Molecular and Integrative Toxicology

George A. Parker *Editor*

Immunopathology in Toxicology and Drug Development

Volume 2, Organ Systems

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George A. Parker
Editor

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Volume 2, Organ Systems

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Preface

This book provides a fundamental understanding of immunopathology and immunopathologic processes, with particular attention to nonclinical toxicology studies. Chapters provide organ system–based summaries of spontaneous pathology and common responses to xenobiotics. A companion volume, *Immunopathology in Toxicology and Drug Development*: *Volume 1*, *Immunobiology*, *Investigative Techniques*, *and Special Studies*, offers an overview of general immunobiology, cells of the immune system, signaling and effector molecules, and immunopathology assays.

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

Durham, NC — George A. Parker

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About the Editor

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Chapter 1
Thymus

Gail Pearse

Abstract The thymus is a primary lymphoid organ where developing T cells (thymocytes) proliferate and differentiate before entering the systemic circulation to populate secondary lymphoid tissues and form the functional T cell repertoire. The thymus tightly controls the antigen specificity of these naive T cells in order to limit reaction with self antigens. In keeping with the importance of T cells to the health of the animal, their development and function are subject to many overlapping pathways and checkpoints. Therefore, many of the key players involved in T lymphocyte development, differentiation and function are potential targets for pharmaceutical intervention in aberrant T cell immune responses resulting in disease. This chapter outlines the complex function of the thymus, the range of changes seen as part of normal physiological and disease processes, and its response to immunotoxicants.

Keywords Thymus • Pathobiology • Immunopathology • Immunotoxicity • Animal models

Abbreviations

AIDz	Autoimmune disease
AIRE	Autoimmune regulator gene
APCs	Antigen-presenting cells
APS	Autoimmune polyendocrinopathy syndrome
CTL	Cytotoxic T cells ($CD8^+$)
CTLA-4	Cytotoxic T lymphocyte antigen

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DOCK8	Dedicator of cytokinesis 8 protein
ETPs	Early thymic progenitor cells
FOXP3	Transcription factor forkhead box P3
iTreg	Induced regulatory T-cells
Lck	Lymphocyte-specific protein tyrosine kinase
MHC	Major histocompatibility complex
nTreg	Natural regulatory T cells ($CD4^+$ $CD25^+$ $FOXP3^+$)
PD-1	Programmed death-1
PSGL1	P-selectin glycoprotein ligand 1
Rag1 and Rag2	Recombinase-activating genes 1 and 2
Runx3	Runt related transcription factor 3
S1P	Sphingosine 1-phosphate
S1P1/S1PR1	S1P receptor 1
Syk	Spleen tyrosine kinase
T-bet	T-box transcription factor
TCR	T cell receptor (CD3)
TEC	Thymic epithelial cells
Th	T helper cells ($CD4^+$)
Th0	Naıve T-cells
ThPOK	Th-inducing BTB/POZ domain-containing Kruppel-like zinc-finger transcription factor
TREG/Treg	Regulatory T cell
ZAP-70	Zeta chain associated protein-70

1.1 Immunobiology of the Thymus

1.1.1 Structure of the Thymus

In mammals the thymus is a bilobed organ located in the anterior mediastinum. Slight species differences in anatomical location have been described by Haley (2013). It is a lymphoepithelial organ composed of a loose meshwork of blood vessels and thymic epithelial cells (TEC) supporting developing T lymphocytes (thymocytes) and antigen-presenting cells (APCs) including macrophages and non-phagocytic, bone marrow derived Dendritic cells (DCs) (Crivellato et al. 2004). Normal histology of the thymus has recently been reported by Pearse (2006). It has a distinct cortex and medulla, separated by a vascular corticomedullary zone. The surrounding connective tissue capsule gives rise to septae dividing the gland into lobules in most species with the exception of the mouse. The cortex is dark staining due to the densely packed, small, immature lymphocytes and sparse epithelial cells and macrophages. Large numbers of these rapidly dividing cortical lymphocytes are short-lived and undergo apoptosis and phagocytosis by macrophages. Apoptotic

bodies and "tingible body" macrophages (so called because of their distinct cytoplasmic apoptotic bodies) are therefore a normal histological feature of the cortex, particularly in young animals. The medulla is pale staining and less densely cellular than the cortex due to the population of more mature T cells which have larger, paler-staining nuclei. The more sparse lymphocyte population also increases the visibility of the pale staining epithelial cells in the medulla. The medulla also contains non-epithelial APCs, such as macrophages and DCs. Ploeman et al. (2003) reported that B lymphocytes are a normal component of the thymic medulla in dogs, which following immunologic activation (e.g., post vaccination) can form prominent lymphoid follicles. Such lymphoid follicles are a normal feature of the thymus in man (Greaves 2012) and lymphoid nodules with well developed germinal centers can be seen in non-human primates (Haley 2013). TEC are divided into four distinct subtypes: subcapsular cortical, inner cortical, medullary and Hassalls corpuscles. Populations differ in antigenic expression, ultrastructural characteristics and their capacity to synthesize the thymic hormones (De Waal et al. 1997). Subcapsular and medullary epithelial cells produce the thymic hormones thymosin, thymopoietin, thymic humoral factor, and thymulin (previously known as serum thymic factor) which are essential for cell growth, differentiation and maturation (Anderson et al. 1996; van Ewijk et al. 1994). Moreover, the thymic epithelium is the main source of the components of the extracellular matrix (ECM), such as laminin, fibronectin and type IV collagen. TECs also secrete cytokines, chemokines and neuropeptides. Hassall's corpuscles are found within the medulla but are rare in rodents when compared with other species. They are composed of concentric layers or clusters of epithelial cells with occasional central cellular debris. Keratinisation in Hassall's corpuscles is seen in man, non human primates (NHP), pigs, dogs and older rats, but is not seen in mice (Haley 2013). The function of Hassall's corpuscles is currently unclear but amongst their proposed functions is the removal of apoptotic thymocytes, T cell signalling including negative T cell selection and driving differentiation of thymic Tregs (Douek and Altmann 2000; Watanabe et al. 2005). Small populations of other cell types can also be seen in the normal thymus. Eosinophils migrate to tissues including the thymus following a short half life in circulation and can be a prominent feature in laboratory animals and man (Rosenberg 2013). In mice, recruitment to the thymus is reportedly greatest in the neonatal period (Throsby et al. 2000). However, Lee et al. (1995) and others have reported eosinophil production from precursors within the thymus of some species including man. The exact role of thymic eosinophils remains unclear. Throsby et al. (2000) reported that they act as sentinal APC associated with class-I restricted selection (positive selection) in normal mice. Preziosi et al. (1995) suggest they have a phagocytic role. Neuroendocrine cells also occur in low numbers. Myoid cells with ultrastructural and immunohistochemical features of striated muscle occur rarely in the thymic medulla of rodents and humans (Suster and Rosai 1992). Their histogenisis is uncertain but some studies have demonstrated shared epitopes with thymic epithelial cells. Both myoid cells and TEC are thought to be involved in an incompletely understood complex process of autoimmunization resulting in Myasthenia Gravis (Willcox et al. 2008).

The corticomedullary junction is characterized by plentiful blood vessels (predominantly arterioles) and a mixture of mature and immature T lymphocytes and dendritic cells. Perivascular B-lymphocytes and plasma cells increase in number with increasing age of the animal. Subcapsular Epithelium-free areas (or holes) have been reported in rats and dogs (Bruijntjes et al. 1993). These lack stromal elements and contain clusters of immature T lymphocytes and occasional tingible body macrophages. They are thought to represent an alternate route for immature T-lymphocytes travelling between the cortex, corticomedullary zone and medulla without coming into contact with the stromal elements concerned with the positive and negative selection process.

1.1.2 Functional Overview and Potential Targets for Therapeutic Intervention

The thymus is a primary lymphoid organ where developing T cells (thymocytes) proliferate and differentiate into the functional T cell repertoire (van Ewijk et al. 1988). It tightly controls the antigen specificity of naive T cells released into the systemic circulation, to limit reaction with self antigens. Following the release into the systemic circulation naive T cells populate T cell areas in secondary lymphoid tissues. On stimulation with antigen, antigen-presenting cells, and cytokines they become activated, undego clonal expansion and differentiate into effector T cells. In keeping with the importance of T cells to the health of the animal, their development and function are subject to many overlapping pathways and checkpoints. Developments in molecular biological techniques and the use of transgenic models lacking specific receptors, transcription factors and cytokines have been used to help dissect these complex immune pathways (Burns-Naas et al. 2001). This provides an opportunity to identify potential targets for pharmaceutical intervention in aberrant T cell immune responses which result in disease. Therefore, many of the outlined key players involved in T lymphocyte development, differentiation and function as well as specific T cell subtypes are the focus of pharmaceutical development programmes.

1.1.3 T Cells and Their Function

The reader is directed to the immunological texts of Janeway (2012) and Kuby (2013) for a comprehensive review of T cell biology. Antigen recognition by T lymphocytes is mediated by the T cell receptor (TCR) linked to the CD3 signal transduction molecule. The TCR is composed of an α and β polypeptide chain each with an antigen binding site. Binding of antigen activates a series of cellular signalling complexes culminating in gene transcription leading to cellular proliferation

and differentiation. Function associated assessory molecules CD8 and CD4 are expressed on mutually exclusive T cell subsets: the cytotoxic T cells ($CD8^+$) and helper T cells ($CD4^+$). Whilst B lymphocytes can recognize intact antigens by virtue of their membrane-bound immunoglobulin, TCRs recognize peptide fragments of protein antigens displayed in conjunction with one of two types of major histocompatability complex (MHC) molecules (Snyder 2012). Class I MHC molecules are expressed on all nucleated cells and platelets and display peptides derived from proteins within the cell such as viral and tumour antigens. Naive $CD8^+$ T cells recognise peptides presented by class I MHC molecules and differentiate into cytotoxic T cells that then kill the infected cells. Class II MHC molecules are expressed on specialist antigen presenting cells (APC) including macrophages, dendritic cells and B lymphocytes. Class II MHC molecules display antigens derived from extracellular microbes and soluble proteins in the extracellular environment. The antigen is internalised into vesicles and proteolytically digested by endosomes or lysosomes and the resulting peptide-MHC II complex is transported to the cell surface. $CD4^+$ T cells bind to class II MHC molecules, and then differentiate into a diverse array of functionally distinct subsets, which usually secrete a set of cytokines directed at the target cell. Signalling through the TCR complex alone is not sufficient to produce lymphocyte activation. This requires a second antigen-independent or 'costimulatory signal'. The second signal is delivered by CD28 on the T cell surface through the specific interaction with one of two ligands B7-1 (CD80) or B7-2 (CD86) on the surface of antigen presenting cells (APCs). It promotes T cell survival, proliferation and cytokine production. If a T cell receives only antigen-specific TCR stimulation in the absence of costimulation, it will be rendered unresponsive (anergic) to antigenic challenge. However, this anergic state induced by insufficient co-stimulation may be reversed later in life (Schwartz 2003). Negative signaling receptors expressed on T cells can counteract the costimulatory signals of CD28.

There are two distinct lineages of T cells: the γδ T cells and αβ T cells. The γδ T cells lack CD4 and CD8 even when mature, and tend to aggregate at skin

Table 1.1 Summary of the main subsets of helper T cells (CD4+)

CD4 T cell lineages	Effector cytokine signature	Mechanism of action	Target/function
Th1	INF ϒ	Activation Mo, NK Expansion CTLs	Intracellular pathogens Tumours
Th2	IL-4, -5, -13	Promotes IgE production and ELS function	Helminths
Th17	IL-17,21	NLS recruitment	Extracellular pathogens (bacteria, fungi)
Treg	IL-10, TGFβ	Regulation of Th cells	Peripheral tolerance

Mo macrophages, *NK* natural killer cells, *CTLs* cytotoxic lymphocytes, *ELS* eosinophils, *NLS* neutrophils

Summary table of the cytokines produced and cells recruited by each of the helper T cell subsets and the main outcomes

and mucosal surfaces as intraepithelial lymphocytes. They are able to recognise peptides, lipids and small molecules without the need for MHC proteins. Although γδ T cells cells predominate early in development, later on more than 90% of thymocytes give rise to αβ T cells. The αβ T cells produced in the thymus give rise to the cytotoxic T cells ($CD8^+$), the helper T cells ($CD4^+$) and natural regulatory (nTreg) T cells ($CD4^+$ $CD25^+$ $FOXP3^+$). Cytotoxic T cells kill infected or otherwise altered cells. Helper T cells stimulate B lymphocytes to make antibodies and activate other leukocytes. The main subsets of helper cells are Th1, Th2 and Th17. A broad overview of the function of these subsets is summarised in Table 1.1. The proinflammatory Th17 subset has been a major focus of research as they are dynamically balanced with Regulatory T cell (TREG/Treg) development. Naturally occurring Treg cells (nTreg) are produced in the thymus, and are distinct from a second group, induced in the periphery (iTreg) from naıve T-cells (Th0). For a comprehensive review of Treg, the reader is directed to Peterson (2012). Treg function is similar in humans and laboratory animal species. Tregs are essential for the dampening of chronic inflammation, maintainance of peripheral tolerance and prevention of AIDz. They also induce tolerance in xenotransplantation, at mucosal sites, and during pregnancy. However, they also limit the beneficial effects of inflammation and immune reactions (e.g., against tumours). Thus the balance between effector T-cells and Treg is critical in determining the outcome of an immune response in the animal. Tregs utilise a number of different mechanisms to inhibit immune responses including: secretion of inhibitory cytokines, induction of apoptosis of effector T cells and inhibition of dendritic cell function (Peterson 2012).

1.1.4 T Cell Development

Early thymic progenitor cells (ETPs) are bone marrow derived stem cells which enter the thymus via the postcapillary venules present in the corticomedullary junction. These early progenitors still have some potential to differentiate into B cells (Takahama 2006). Once they have committed to the T cell lineage, they undergo different stages of maturation in anatomically distinct zones of the thymus as they migrate from the corticomedullary junction to the subcapsular cortex, then through the cortex to the medulla and finally re-enter the circulation at the corticomedullary junction (Fig. 1.1). The time between entry of a T cell progenitor into the thymus and export of its mature progeny is estimated to be around 3 weeks in the mouse. The different anatomical zones are home to distinct populations of thymic stroma with which developing thymocytes must interact appropriately in order to develop (Bunting et al. 2011). In the subcapsular region, specialized epithelial cells known as thymic nurse cells engulf large numbers of developing lymphocytes and play an important role in their differentiation (Brelinska and Warchol 1997). The cortical and medullary epithelial cells (TEC),

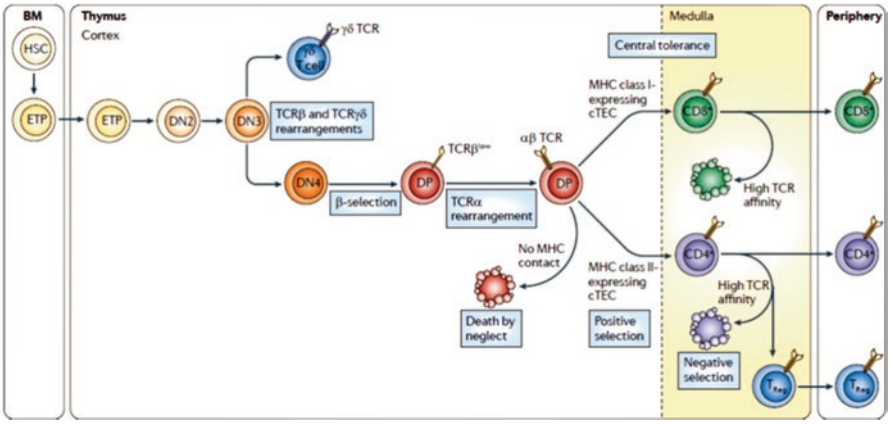

Fig. 1.1 Major events in thymus cell differentiation. Haematopoietic stem cells from the bone marrow give rise to early thymic progenitors (ETPs) then immature double negative (DN) cells, lacking expression of the T cell receptor (TCR) or the co-receptors CD4 and CD8. The DN cells undergo rearrangements of TCR genes. TCRβ gene selection leads to the generation of double positive (DP) cells, which express a properly rearranged TCR β-chain ($TCR\beta_{low}$) and both the CD4 and CD8 co-receptors. This is followed by the rearrangement of the TCR α-chain locus and expression of the αβ TCR. Failure to contact MHC molecules results in death by neglect. Cells with TCRs that bind to MHC class I molecules retain expression of CD8 and lose that of CD4, whereas cells that bind to MHC class II molecules retain CD4 and lose CD8 in the process of positive selection. If the avidity of binding to MHC–peptide ligands exceeds a certain threshold, the cells are deleted by negative selection in the medulla. Undeleted cells eventually migrate out of the thymus to peripheral tissues. A small percentage of self-reactive $CD4^+$ thymic cells with an avidity for MHC class II molecules just below the threshold for negative selection upregulate the transcription factor forkhead box P3 (FOXP3) and exert regulatory T (Treg) cell functions. γδ T cells originate from DN cells that have not yet undergone β-selection. Reproduced with permission from Miller J (2011) The golden anniversary of the thymus. Nat Rev Immunol 11: p 493. doi:10.1038/nri2993

macrophages and reticular/dendritic cells also play a role in development and differentiation of the thymocytes. In the subcapsular cortex the immature cells do not express the T cell receptor (TCR/CD3 complex) or the co-receptors CD4 and CD8 ($CD4^-/CD8^-$) so are said to be double negative (DN). DN cells undergo TCR gene rearrangement giving rise to γδ T cells and αβ T cells. During development of the αβ T cell lineage a critical step is the pre-T cell receptor (pre-TCR) formation. This is formed from the β chain and pre-Tα chain associating with the CD3 group. Notch proteins play a critical role at this point in T cell development and cells that do not express Notch, do not mature beyond this stage. Formation of the pre-T receptor activates signal transduction and the αβ T cells rapidly differentiate into the intermediate double positive (**DP**) phenotype ($CD4^+/CD8^+$) found on the majority of cortical thymocytes. The DP cells are subject to positive and negative selection. Thymocytes finally mature in the medulla for an average of 14 days

during which they become single positive (**SP**) thymocytes. T Cells with TCRs that bind to class I MHC molecules lose expression of CD4 resulting in cytotoxic T cells precursors ($CD4^-/CD8^+$ phenotype). Conversely, cells that bind to class II MHC molecules lose expression of CD8 resulting in helper T cells precursors ($CD4^+/CD8^-$ phenotype).

1.1.5 Self Tolerance

Each T cell is genetically programmed to recognise a specific antigen via its TCR. TCR diversity is generated within the thymus by random somatic rearrangement of the genes encoding the α and β polypeptide chains (TCR gene rearrangement). Because of the random nature of TCR generation, lymphocytes with antigen-binding domains capable of reacting with self molecules can occur. Self tolerance, or the lack of responsiveness of an animal to its own antigens, is therefore a key step in T lymphocyte development. This is achieved by a process of central and peripheral tolerance. The thymus is the site of central tolerance where, by means of a positive and negative selection process, only mature T cells that are self-restricted and non-autoreactive are allowed to enter the circulation. This is reflected in the number of naive T cells leaving the thymus, which is less than 5% of the bone marrow precursors that entered from the circulation. Positive selection takes place in the cortex. Only T cells with an intermediate affinity for major histocompatibility complex (MHC) molecules, in a process called MHC restriction, are allowed to undergo clonal expansion. Failure of positive selection results in apoptosis (Herold et al. 2006). The vast majority of apoptotic cells in the thymus are thought to result from a failure to undergo positive selection because their receptors do not specifically recognise self-MHC molecules (De Waal et al. 1997). The survivors migrate to the thymic medulla where they are subject to negative selection. Cells that react too strongly with self MHC or with self MHC plus self peptide, are again deleted by apoptosis, thereby removing potentially autoreactive cells. A small percentage of self-reactive $CD4^+$ thymic cells with an avidity for class II MHC molecules just below the threshold for negative selection upregulate the transcription factor forkhead box P3 (FOXP3) and exert regulatory T (T Reg) cell functions (Peterson 2012). Presentation of self antigens within the thymus is mediated by several different cell types including epithelial cells, macrophages and dendritic cells. Dendritic cells are non-phagocytic, bone marrow-derived cells, which are especially important in antigen presentation to naive T cells via class I or II MHC molecules. Dendritic cells are more efficient than epithelial cells in mediating negative selection or deletion of self-reactive thymocytes. During negative selection, medullary thymocytes are exposed to a wide variety of autologous protein antigens which are processed and presented by thymic APC in association with self MHC molecules. The thymic medulla contains stable groups of cells displaying the organization, morphology and functional activity of epithelial tissue which is thought to maintain the spectrum of epithelial 'self' (Crivellato et al. 2004). In addition, a small number (1–5%) of

medullary thymic epithelial cells (mTEC) express a mosaic of 'ectopic' tissue-specific molecules, such as parathyroid hormone, thyroglobulin, insulin and C-reactive protein. This'promiscuous' gene expression in mTEC is thought to be under the influence of a transcription factor called autoimmune regulator (AIRE) (Anderson et al. 2005). Thymic stromal cells are themselves supported by the developing thymocytes in a bidirectional signal exchange. This lymphostromal interaction is referred to as thymic crosstalk (van Ewijk et al. 1994). Thus, signals produced by positively selected thymocytes crucially regulate mTEC development and medulla formation. The development of TEC is dependent on many transcription factors including Tbx1, Hoxa3, Pax1, and Foxn1 (Nitta et al. 2008).

1.1.6 Control of T Cell Migration in the Thymus

The colonisation of the thymus by precursor cells, trafficking of thymocytes through the thymus and exit of naive T cells into the circulation, requires coordination between multiple chemokines and chemokine receptors. The current understanding of the role of chemokines in the thymus has been reviewed by Bunting et al. (2011). In order to sustain T cell production in the thymus, bone marrow derived T-cell progenitors are periodically imported from the from circulation. Precursor entry into the thymus occurs in approximately fortnightly waves and is a 'gated' process which is controlled by two feedback mechanisms. The first is dependant on the occupancy status of thymic niches and the second depends on the peripheral lymphocyte pool (Cyster 2009, Bunting et al. 2011). Thymic entry of T cell progenitors requires the interaction of P-selectin glycoprotein ligand 1 (PSGL1) on TPC with P-selectin (on thymic blood vessel endothelium) and the response of CCR9 expressed on TPC to CC-chemokine ligand 25 (CCL25). Space within thymic niches must be available for these cells, and when niche occupancy is low, P-selectin and CCL25 are induced. This increases the rate of progenitor cell recruitment, leading to sustained output of naıve T cells from the thymus. Depletion of the peripheral lymphocyte pool provides positive feedback increasing P selectin expression and increased recruitment of ETPs. Gossens (2009) identified sphingosine 1-phosphate (S1P) as the mediator translating changes in the peripheral lymphocyte pool into altered thymic TPC receptivity. There is evidence that the CCR7/CCL21/CCL19 chemokine axis is also required for precursor entry into the adult thymus, but a limited contribution may be made by other chemokine receptors, including CXCR4 and CCR5 (Bunting et al. 2011). The journey of developing thymocytes through the thymus has yet to be fully understood, since the numerous chemokines involved not only differ according to the developmental stage of the animal, but often have overlapping functions. Chemokine gradients exist between different anatomical compartments and expression of chemokine receptors correspondingly changes in the developing thymocyte populations. A large number of chemokine receptors are expressed by thymocytes, but CCR7, CCR9 and CXCR4 are considered to be the dominant

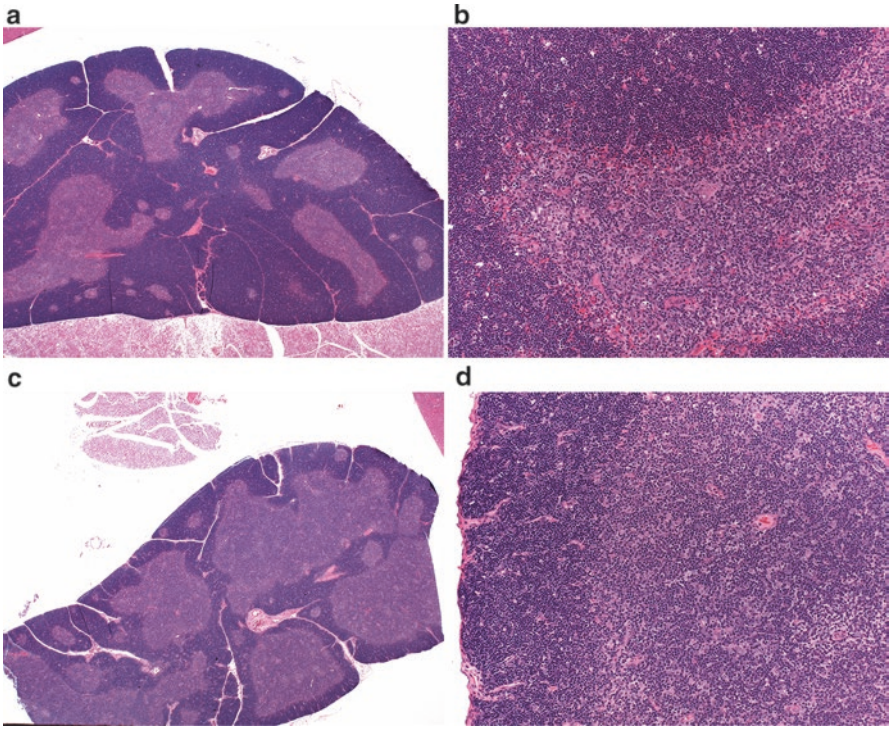

Fig. 1.2 Rat thymus: control (**a, b**) and following treatment with S1P1 agonist (**c, d**). Binding of agonist to S1P1 on lymphocytes within the thymus causes receptor internalization and degradation (functional antagonism). Thymocytes therefore cannot migrate along the chemotactic gradient of S1P1 into the circulation via the vessels at the corticomedullary junction. There is sequestration of unreleased mature thymocytes in the medulla resulting in expansion of the medulla with a decrease in the cortico-medullary ratio (**c**) when compared with the corresponding control (**a**). On higher magnification a greater cellular density can be seen within the medulla (**d**) when compared with the control (**b**). Acknowledgement: Many thanks to Aude Roulois (GSK) for providing these images

receptors controlling migration during fetal development and adult life. The homing and positioning of other cells in the thymus, such as dendritic cells and natural killer T cells is also chemokine-dependent.

Emigration of T cells from the thymus is regulated by sphingosine-1-phosphate (S1P) levels in the blood and S1P receptor 1 (S1P1/S1PR1) on naive T cells (Fig. 1.2). (Matloubian et al. 2004, Venkataraman et al. 2008) S1P is a potent lipid mediator with multiple cell functions, including trafficking of immune cells, which has been reviewed by Spiegel and Milstien (2011). It is produced inside cells by sphingosine kinase which can be activated by numerous stimuli, including pro-inflammatory cytokines. S1P is then exported and activates cell surface receptors in a process called ‘inside-out signalling’. Once formed, S1P is rapidly degraded. However, erythrocytes (and platelets) lack the enzymes required for degradation and are the source of the higher concentration of S1P in the blood when compared

with tissues. Lymph also contains high concentrations associated with the production by lymphatic endothelium. The concentration gradient between the levels of S1P in the circulation and in the tissues including the thymus, and the expression of S1PRs on cells are crucial for the trafficking of immune cells. After surviving negative selection, immature thymocytes upregulate expression of the transcription factor Krüppel-like factor 2 (KLF2) and its target gene S1P1. This enables naive T cells to express S1P1 and exit the thymus via corticomedullary junction blood vessels in response to gradients of S1P produced by perivascular cells and high levels of S1P in the blood. After entering the circulation, lymphocytes internalize S1P1 in response to high S1P levels in the blood, but re-express S1PR1 during transit through non-inflamed secondary lymphoid organs. Pharmacological agents targetting the functions of S1P and its receptors interfere with lymphocyte emigration. They are currently used in treatment of multiple sclerosis (MS) and are of interest in a wide range of inflammatory and autoimmune disorders. FTY720 (fingolimod), when phosphorylated, is a potent sphingosine-1-phosphate receptor 1 (S1P1) agonist used in the treatment of MS. Its effects are consistent with a mechanism of functional antagonism whereby agonist-binding to induced S1P1 on lymphocytes causes receptor internalization and degradation. This in turn prevents lymphocyte migration from lymphoid tissues by chemotaxis along a gradient of endogenous S1P (Wei et al. 2005, Brinkmann et al. 2010). Phosphorylated FTY720 causes sequestration of lymphocytes inside lymphoid organs, rendering them incapable of migrating to sites of inflammation and leading to lymphopenia. As a consequence, there is reduced lymphocyte infiltration into the CNS.

1.1.7 Thymic Commitment and Lineage Decisions

Cellular changes associated with maturation in thymocytes is controlled through numerous transcription factors which act in conjunction with chromatin-modifying enzymes, known as epigenetic regulators. Epigenetic modifications result in reversible, heritable genetic changes that are not associated with changes in DNA sequence. Modifications in the epigenetic code influence the ability of transcription factors to bind to DNA, resulting in changes in gene expression and cell phenotype. Therefore the commitment of a cell to a particular lineage may be flexible depending on environmental cues causing changes in epigenetic marks. Recent advances in epigenetic research have improved our understanding of the events that are required to establish cell lineages (Miller and Weinmann 2010). Targeting the epigenetic code may provide a therapeutic strategy for a variety of immune cell disorders (Allan and Nutt 2014). Key transcription factors in thymocyte development include Notch 1 which switches on genes that drive commitment of thymic progenitor cells to the T cell lineage (Rothenberg and Taghon 2005). It is also involved in determination of αβ versus γδ T cell and CD4 versus CD8 cell production. Commitment of thymocytes to either the CD4 or CD8 lineage is, however, predominantly under the control of the T helper (Th) lineage master regulator ThPOK (Th-inducing BTB/POZ

domain-containing Kruppel-like zinc-finger transcription factor) and Runx3 (Runt related transcription factor 3). ThPOK promotes lineage commitment to $CD4^+$ T cells, whilst Runx3 promotes the lineage commitment of $CD8^+$ cytolytic T lymphocytes (CTL). ThPOK continues to repress the CTL gene program in naïve $CD4^+$ T cells. It also helps the differentiation of $CD4^+$ T cells into effector Th cell subsets in cooperation with other transcription factors. Th1 cell differentiation requires T-bet (T-box transcription factor); Th2 cell differentiation reqires GATA3; Th17 cell differentiation requires RORγt; regulatory T cell differentiation requires FOXP3 and follicular helper T cell development requires Bcl6. However, recent reports suggest Th lineage may be less fixed than previously thought (Cheroutre et al. 2013, Fontenot and Rudensky 2005, Mucida et al. 2013, Naito et al. 2011, Reis et al. 2013).

1.2 Organ-Specific Immunopathological Processes

Due to the dynamic nature of the thymus it varies morphologically according to factors as diverse as age, genetic background, nutritional and immunological status and levels of stress as well as toxic insult. As a result, a number interpretive and descriptive terms have arisen which are often used interchangeably. In order to simplify this potentially confusing situation a standardized descriptive nomenclature has been introduced (Haley et al. 2005) along with principles for enhanced histopathology of immune system organs (Elmore 2006). A complete review of morphological changes seen in the thymus together with differential diagnostic criteria is included in the International Harmonisation of Nomenclature and Diagnostic Criteria (INHAND) document for the lymphoid system, published by the American, British, European and Japanese Societies of Toxicologic Pathology (in press 2015). Selected changes will be discussed here.

1.2.1 Changes in Thymic Cell Populations: Physiological Responses

1.2.1.1 Development

The highest migration of T cells from the thymus to the periphery occurs during embryogenesis and in the early stages of life but with increasing age there is a change in function of the thymus from lymphocyte production to recirculation (Dominguez-gerpe 2003). In the neonatal rat the thymus is moderately well developed with obvious corticomedullary distinction and typical adult microscopic organization by postnatal day (PND) 14 (Parker et al. 2015). In the mouse, thymic development begins at day 12 of gestation developing into an adult-like structure 2–3 days later and reaching maximum size by day 18 of gestation (Ward et al. 1999).

1.2.1.2 Ageing

Progressive thymic involution occurs near the time of sexual maturity in most species and is accompanied by a decreased capacity to export mature T cells to the periphery. The maintainance of circulating T-lymphocyte counts in mature animals is thought to be associated with T-lymphocyte proliferation in the secondary lymphoid organs in a process called homeostatic expansion (Berzins et al. 1998). This may account for the lack of correlation between morphologic changes in the thymus and findings in secondary lymphoid organs or in blood that can occur in routine toxicity studies. The rate of thymic involution varies with species, strain, age, and sex (Haley 2003, 2013). Cytokines, neurotransmitters and increased circulating levels of sex steroids all play a role in this process (Greaves 2012; Ahmed et al. 1985; Sano et al. 2001). Androgens induce a more rapid decrease in thymic size which results in sexual dimorphism between men and women for much of their adult life (Aspinall R, 2000; Boehm and Swann 2013). Similar sexual dimorphism has been reported in cynomologus monkeys (Spoor M et al. 2008). The reduced cellularity seen in involution predominantly involves the cortex, and is considered likely to be associated with decreased proliferation and increased apoptosis of thymocytes (Plecas-Solarovic et al. 2006). The transcription factor STAT-3 derived from TEC, is reported to be a key regulator in T-cell growth and survival, and maintenance of thymic architecture, and likely suppresses pro-apototic genes in thymic atrophy and ageing (Sano et al. 2001; Egwuagu 2009). Evidence suggesting that age-associated involution may be reversed has raised interest in the possibility of therapeutic strategies to enhance thymus function in the elderly (Boehm and Swann 2013; Dominguez-gerpe 2003).

1.2.1.3 Reproductive Status

The reproductive status of an animal can have a profound effect on thymic morphology. 17-estradiol (E2) administration to mice is reported to reduce the size of the cortex but not the medulla, implying an E2-mediated loss of cortical thymocytes. E2 has also been shown to increase the DN1 cells and decrease the DN2 cells, suggestive of a block in T cell development at the earliest stage (Bernardi et al. 2015). In pregnant females, an early increase in thymic weight is followed by a marked reduction in cellularity of the cortex associated with increased progesterone levels. Conversely, there is an increased cellularity of the medulla, considered to be associated with increased Suppressor T cells contributing to foetal antigen tolerance (Kendal and Clarke 2000). Postpartum, thymic cellularity returns to normal (Schuurman et al. 1994), whilst prolactin production during lactation has a stimulatory effect on the thymus.

1.2.2 Effect of Stress

Under conditions of stress, high levels of glucocorticoids causes loss of immature lymphocytes in the thymic cortex (Dominguez-Gerpe and Rey-Mendez 2001). The resulting profound and diffuse thymocyte apoptosis and numerous "tingible-body" macrophages leads to the classical "starry sky" appearance of the cortex. Over time there may be reduction in the size of the cortex as a result of lymphocyte depletion but with limited or no evidence of apoptosis (Haley 2013). Histologically, the splenic white pulp and lymph nodes can be similarly affected, but the predominance of mature lymphoid cells in these organs compared to the thymus make the stress response less dramatic. In the thymus histological changes associated with stress can be morphologically indistinguishable from those caused by a thymotoxic xenobiotic. The impact of stress-induced effects on data interpretation in a toxicology study is therefore considerable and study designs that minimize stress and use appropriate control groups (e.g., cohorts treated with vehicle and subjected to the same procedures as those dosed with test article) are essential. Concurrent changes indicative of stress may be present in the animal such as adrenal cortical hypertrophy with increased relative organ weight and haematological changes (stress leukogram) of increased neutrophils with decreased lymphocytes and eosinophils. For a comprehensive review on the effects of stress in toxicology studies the reader should consult Everds et al. (2013). Concurrent disease will also result in accelerated decrease in thymic cellularity (Smith and Ossa-Gomez 1981). General undernutrition, and specific deficiencies of Vitamin B6, amino acids, fatty acids, and minerals such as zinc cause immunosuppression and a decrease in thymic weight (Robson and Schwarz 1975; Corman 1985; Mittal et al. 1988; Good and Lorenz 1992).

1.3 Immunotoxicity

Immunotoxicity refers to the potentially harmful effects that physical, chemical, or other agents have on the immune system (Koller 1987). Immunotoxicity is a major consideration in the evaluation of drug safety (Spoor et al. 2008). All new human pharmaceuticals, according to the regulatory guidelines ICH S8, require review of their immunotoxic potential, which will include results of the preclinical toxicity studies. The interaction of xenobiotics with various components of the immune system can result in immunostimulation or immunosuppression via decreased lymphoid cellularity or changes in lymphocyte subset populations (Burns-Naas et al. 2001). Immunotoxic reactions commonly manifest themselves as immunosuppression (Gopinath 1996) leading to increased susceptibility to infection or the development of cancer. The latter appears to involve either the failure to control normally repressed oncogenic viruses or the failure of primary tumor surveillance (Weaver 2012).

Immunostimulation (undesired activation of the immune system) can result in acute or chronic inflammatory disease, hypersensitivity reactions or autoimmune disease. The immune-inhibitory pathways, normally ensure immune tolerance and

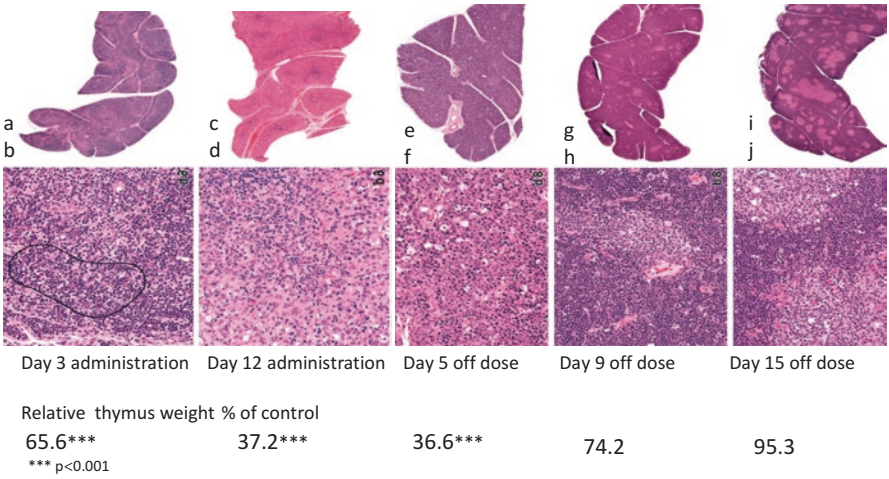

Fig. 1.3 Exposure of the thymus to an immunotoxicant followed by a recovery period, results in a predictable pattern of morphological change. Results from a time course study in the rat with Chlorambucil, show that an initial reduction in the cellularity of the cortex (**a**) is due to apoptosis of thymocytes (circled) (**b**). The progressive depletion of these lymphoid cells, particularly in the cortex, results in a loss of the normal corticomedullary demarcation (**c**). At this stage few thymocytes remain and epithelial stromal cells predominate (**d**). These changes are accompanied by progressive decrease in relative thymic weight. When dosing is stopped, some recovery of overall cellularity can occur as early as Day 1 with the appearance of large lymphoblastic cells in the cortex and medulla. Tingible body macrophages phagocytizing apoptotic debris are also a feature (**not shown**). By Day 5 off dose, the level of cellularity continues to increase. Large lymphoblastic cells and tingible body macrophages remain prominent (**e**, **f**). There is continued restoration of cellularity with a rebound effect, characterized by an increase in the corticomedullary ratio seen at Day 9 (**g, h**). By day 15 off dose, the morphological appearance is indistinguishable from the untreated controls (**i**, **j**). The recovery stages are accompanied by a progressive increase in relative thymic weight reaching 95.3% of control and by Day 15. Reproduced with permission from Pearse G et al. (2009) Time-course study of the immunotoxic effects of the anticancer drug chlorambucil in the rat. Toxicol Pathol 37: 896–898 DOI: 10.1177/0192623309347907

mitigate collateral tissue damage. These regulatory mechanisms include Tregs and the T cell inhibitory receptors such as cytotoxic T lymphocyte antigen (CTLA-4) and programmed death 1 (PD-1). Impairment of these regulatory mechanisms are important contributors to immunostimulation. Microscopic changes in the thymus of animals exposed to immunotoxicants tend to follow a predictable course (Fig. 1.3) However, these changes are not pathognomonic and the distinction between immunotoxic effects from those of normal progressive involution and/or stress-related effects in preclinical safety studies may depend on the weight of evidence available. Initial apoptosis of cortical lymphocytes with increased numbers of apoptotic bodies and tingible body macrophages, results in decreased cortical cellularity. In the absence of recovery, the width of the cortex is reduced resulting in a decreased corticomedullary ratio and loss of the normal corticomedullary demarcation. Eventually the level of cellularity in the cortex may be less

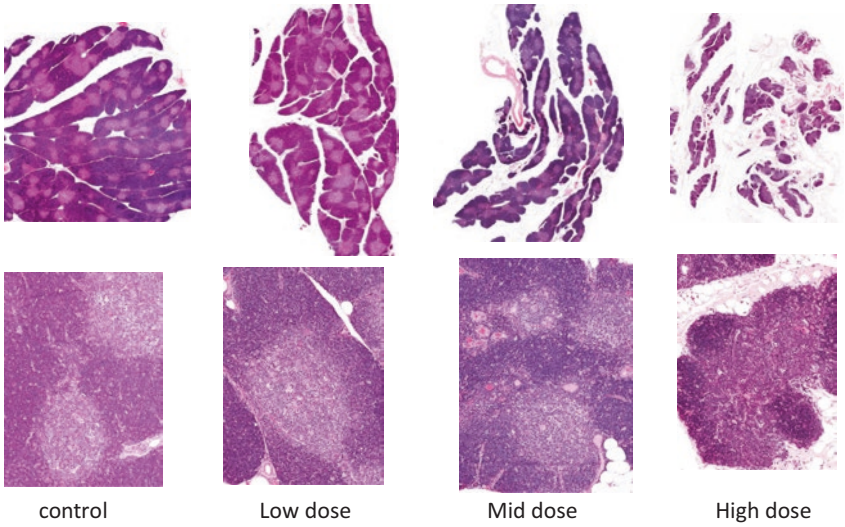

Fig. 1.4 Low and high power view of thymus from dogs given an immunomodulatory drug. All animals were age-matched and the photographs taken at same magnification. There is a dose related decrease in cellularity of the thymus in treated animals when compared with controls. At the low dose there is a minimal decrease in cortical cellularity with a marginal decrease in the size of the thymic lobules. At the mid dose there is a mild decrease in cortical cellularity with a decrease in corticomedullary ratio and increased adipose tissue in between lobules. At the high dose the decrease in cortical cellularity has lead to loss of corticomedullary demarcation and there is a marked increase in adipose tissue deposition between the lobules. Morphologically, these changes are indistinguishable from those of physiological involution or a stress related response, however, the clear dose relationship and lack of other stress associated changes in the animals is indicative of a test article related change

that of the medulla (corticomedullary reversal) or decreased cellularity of both the cortex and medulla may be seen. In some cases degeneration of epithelial cells or epithelial cell proliferation with development of glandular structures containing eosinophilic material can occur. The immune system has a high regenerative capacity, and, depending upon the degree of toxicity, can recover in a relatively short period of time following a toxic insult. Therefore the time between chemical insult and examination of the thymus is an important consideration (Schuurman et al. 1994). Recovery is characterised by a "rebound" hypercellularity of the cortex with an increase in the corticomedullary ratio. The ongoing cellular proliferation, differentiation, lymphocyte trafficking, and gene amplification, taking place in the thymus make it exquisitely sensitive to toxic insult (Koller 1987), and because of this a clear dose-relationship in the histological changes may be present (Figs.1.4 and 1.5). Immunosuppressive effects of a number of different pharmaceutical agents correlate well with changes in histopathology, thymic weight and peripheral lymphocyte counts in the rat and dog. The presence of histopathological changes in other lymphoid tissues may also give weight to thymic changes being a direct effect of immunotoxicity (Greaves 2012; Pearse et al. 2009).

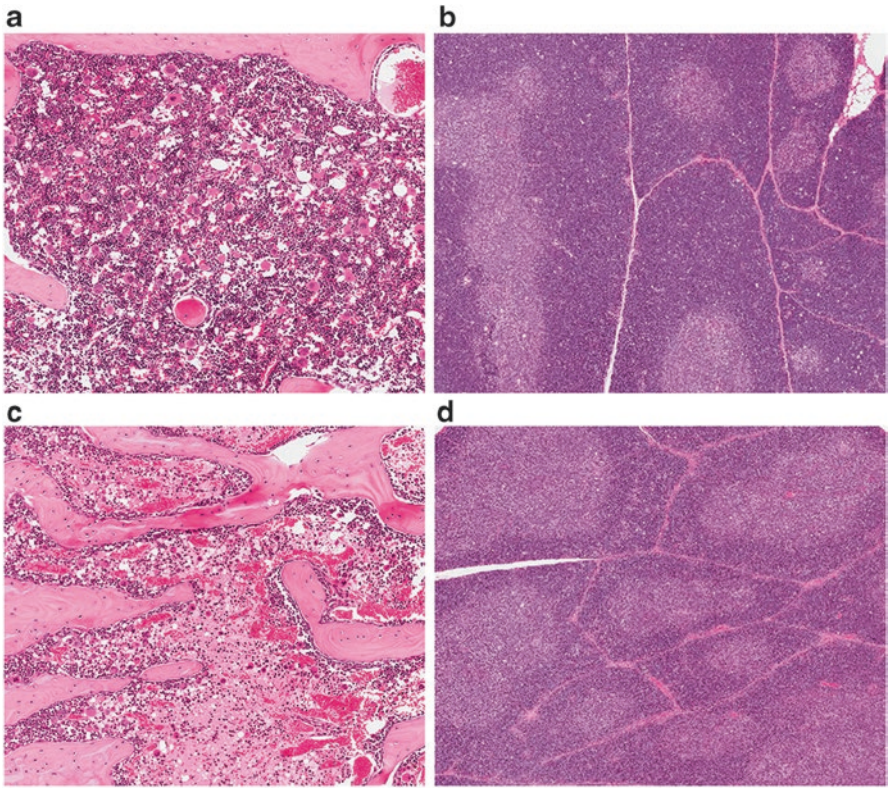

Fig. 1.5 Sections of bone marrow and thymus from control (**a** and **b** respectively) and treated (**c** and **d** respectively) rats from a toxicology study with an immunomodulatory drug. In the bone marrow of the treated animal (**c**) there is extensive necrosis and loss of hematopoietic tissue with hemorrhage into the marrow cavity. This animal also has a decreased cellularity of the thymus (**d**) with a decreased width of cortex when compared with the control. This change is likely to be associated with decreased supply of progenitor cells from the bone marrow

1.3.1 Chemotherapeutics

A number of chemotherapeutic drugs and chemicals including colchicines, cyclosporine A, Dioxin derivatives, nickel and lithium chloride, oxocortisol, maitroxin and etoposide have been shown to induce a transient, reversible decrease in cellularity of the thymic cortex/medulla or both. In the majority of cases, thymic anatomy remains relatively intact and (rebound) thymic hyperplasia may be observed after cessation of treatment (Taub et al. 2005). In the case of Cyclosporine A the effect within the thymus is complex and dose dependant. There is blockage of maturation of

double-positive $CD4^+CD8^+$ thymocytes. Loss of cortical thymocytes is seen only at high doses. The most striking changes are in the medulla, with decreased size and cellularity with small residual islands of medullary tissue, or replacement with thymocytes of cortical phenotype (cortification). Cyclosporine A also causes loss of medullary epithelial cells in rats (Rezzani 2004). Myelotoxic agents such as cyclophosphamide, busulphan, thioacetamide and chlorambucil result in decreased population of the thymus with haematopoietic stem cell (HSC) precursors resulting in a decreased cellularity of the cortex and medulla. In a timecourse study of Chlorambucil administration in the rat (Pearse et al. 2009) rapid reduction in cellularity of the thymus occurred from day 4 of treatment, with reduced corticomedullary ratio, loss of corticomedullary demarcation, and finally generalised decreased cellularity. Recovery was equally rapid (from day 5 of the recovery period) with an initial increased corticomedullary ratio (rebound effect), finally reverting to normal architecture after a 15 day recovery period. The morphological changes in the thymus over time were reflected in changes in organ weight. Many chemotherapeutic agents used to treat malignant diseases cause DNA damage resulting in a profound effect on proliferating lymphocytes as well as the generation and function of antigen-presenting cells derived from hematopoietic stem cells. Thus, many cytotoxic agents suppress cell-mediated immunity, blocking Immune Surveillance (IS) of the malignancy and permitting disease recurrence (Barrett et al. 2009). Further, T cell recovery following lymphopenia is delayed and incompetent compared to other immune cells, contributing to the development of infectious diseases, relapse, and graft-versus-host disease (Williams et al. 2007). The regeneration of T lymphocytes after chemotherapy is derived from two sources. Immediate T cell recovery comes from peripheral clonal expansion of residual mature lymphocytes resulting in an oligoclonal, restricted repertoire of T cells. This results in maximal expansion of anti-tumor clones, but, the loss of T cell repertoire diversity may predispose to auto immunity and infection. Following a return to active thymopoiesis the de novo T cell production decreases peripheral expansion, restores repertoire diversity, and provides new receptors to recognize infectious diseases and tumor antigens. The degree of TCR repertoire diversity (and therefore the degree of thymopoiesis) can be measured in a couple of different ways as described by Williams et al. (2007): In spectratyping there is a PCR-based analysis of length variation in the complementarity-determining region 3 (CDR3) of the TCR β chain. The CDR3 forms the contact site for the binding of peptides and plays a critical role in antigen recognition. The enormous diversity of CDR3 results from the random insertion of nucleotides during the process of VDJ rearrangement leading to a Gaussian distribution of CDR3 lengths. Severe depletion of T cells and antigen driven clonal expansions result in an oligoclonal pattern of limited CDR3 lengths. Renewal of thymopoiesis re-establishes the polyclonal Gaussian patterns in the CDR3 spectratypes, first among naive cells and subsequently among memory T cells.

T cell receptor rearrangement excision circles (TREC) are the episomal DNA circles generated during the rearrangement of the VDJ genes of the TCR α and β chains. These circles are stably retained during cell division but do not replicate, and therefore become diluted among the daughter cells. The signal joint (sj) TREC, formed during rearrangement of the TCRαchain, is readily measurable by PCR assays in circulating peripheral T cells. TREC frequencies are severely reduced by

lymphodepletion, but recover with renewed thymopoiesis. Some of the newer anti-cancer drugs do not act via DNA damage, but by immunomodulation via targeting lymphocyte subsets. For example paclitaxel and cyclophosphamide promote immune reactivity to the tumor through promotion of the T helper 1 cells enhancing NK cell reactivity. The tyrosine kinase inhibitors such as imatinib and dasatinib are immunosuppressive by blocking T cell function but they spare the Tregs (Barrett et al. 2009).

1.3.2 Therapeutic Immunostimulation

As previously noted, immune-inhibitory pathways normally ensure immune tolerance and limit tissue damage resulting from chronic inflammation. In cases where there is immunosuppression mediated by an infectious agent or tumour, antibodies which block inhibitory receptors are used. This therapeutic immunostimulation aims to restore or enhance the immune response but is a delicate balance as the resulting sustained T cell activation can result in immune mediated adverse effects. The potential risks of immunostimulation by biologics is reviewed by Gribble et al. (2007). Antibodies against inhibitory co-receptors on T lymphocytes (CTLA-4 and PD1) are used in cancer immunotherapy to remove the brakes on the immune response (Pardoll 2012). CTLA-4 blockade by monoclonal antibodies (mAbs) promotes anti-tumour T cell activity. The PD-1/PD-L1 pathway exerts inhibitory signals that result in ineffective anti-tumor immunity, mediated at least in part by inducing Treg development and sustaining Treg function. PD-L1 is expressed on a wide variety of tumors, and high levels of PD-L1 expression strongly correlate with unfavorable prognosis in a number of cancers (Francisco et al. 2010). Because the PD-1/PD-L1 pathway functions as immune evasion in some cancers, treatment with mAbs to PD1 or PD-L1 are expected to enhance anti-tumour immunity. Indeed, clinical trials targeting PD-1 and PD-L1 have shown impressive response rates (Ohaegbulam et al. 2015, Nguyen and Ohashi 2015).

1.4 Proliferative Changes

Extensive descriptions of histomorphological patterns and differential diagnosis of reactive or proliferative and neoplastic changes have been carried out by Ward et al. (2006, 2012); Frith et al.(2001) and Greaves (2012) and Haley (2013).

1.4.1 Lymphoid Hyperplasia

Lymphoid hyperplasia occurs in older rodents, especially mice greater than 6 months old. It is more common in females, may be focal or diffuse and involve one or both lobes. It usually occurs in thymuses that have undergone physiological involution/

atrophy. In a retrospective study by Bradley et al. (2012) lymphoid hyperplasia was the most common finding in the thymus in carcinogenicity studies with CD-1 Mice.

1.4.2 Thymic Lymphoma

In mice the onset and magnitude of age-related decline in thymic function has a strong genetic component that may play a part in the development of malignant lymphoma (Hirokawa et al. 1984). T-cell lymphomas of thymic origin are the most common tumour in the thymus of mice, and can occur spontaneously in young animals <3 months of age (Frith et al. 1985). In addition, the specific background strain greatly influences the prevalence and type of lymphoma encountered (Szymanska et al. 2014). T-cell lymphomas, induced by retroviruses, chemicals, irradiation and those found in several strains of GEM, are often of thymic origin, lymphoblastic with widespread metastases (Ward 2006). The **AKR/J** inbred mouse strain displays a high incidence of spontaneous thymic lymphoma which is associated with two classes of endogenous retroviruses: the ecotropic viruses inherited in AKR mice at two chromosomal loci; and the dual tropic recombinant (MCF2 type) virus. In B6C3F1 mice spontaneously occurring thymic lymphomas are rare but can readily be induced by chemicals, viruses, irradiation, and in some types of tumor suppressor gene knockout mice (e.g., p53-deficient mice) (Dunnick et al. 1997; Ward 2006). The most potent chemical carcinogens e.g. *N*-Methyl-*N*-Nitrosourea (MNU) require only a single injection in young mice to induce a high incidence of thymic T-cell lymphoblastic lymphoma (Morton et al. 2008). Induced tumors often have a different cell and tissue of origin and organ distribution to those in control mice. In B6C3F1 and B6129 mice given 1,3-butadiene and DMBA respectively, animals developed thymic T-cell lymphoblastic lymphomas whereas controls had B-cell lymphomas in spleen and mesenteric nodes (Buters et al. 1999; Melnick and Sills 2001). Most spontaneous and induced lymphoblastic lymphomas in the mouse arise within one lobe of the thymus (Frith et al. 1985). The initial lesion is a reduction in thymic size due to loss of cortical lymphocytes. Subsequent to this, a preneoplastic stage of thymic lymphoma called atypical hyperplasia has been described in chemically treated B6C3F1 and p53-deficient mice (Dunnick et al. 1997). Atypical hyperplasia may involve one or both lobes of the thymus. There is a diffuse change with loss of the normal corticomedullary demarcation. Normal architecture is replaced by sheets of large, atypical lymphocytes and fewer admixed small lymphocytes. This preneoplastic lesion can be differentiated from lymphoma by the heterogenous cell population, variable mitotic index, and failure of lymphocytes to extend beyond the capsule of the thymus. Ultimately, there is enlargement of the affected thymus lobe by nodular lymphocytic proliferation which progresses to generalized involvement of the thymus and mediastinum and finally dissemination in the blood with multiorgan involvement (leukemic phase). Thymic lymphoma is rare in F344/N rats. It can be differentiated from mononuclear cell leukemia by the lack of splenic involvement (Stefanski et al. 1990).

1.4.3 Epithelial Hyperplasia

Reindel et al. (2001) reported widespread dose-related epithelial hypertrophy and hyperplasia in cynomologous monkeys given human epidermal growth factor, daily for 2 weeks. Histological changes included hyperplasia of thymic cortical and medullary epithelium and thymic cysts lined by thickened and hypertrophic stratified squamous epithelium.

1.4.4 Thymoma

Thymomas are benign or malignant neoplasms arising from the specialized thymic epithelial cells and have a variable admixed population of lymphoid cells (Rosai and Levine 1976). They are relatively uncommon in mice and rats except for the Wistar and Buffalo rats (Greaves 2012) but a high incidence has been reported in European hamsters (*Cricetus cricetus*) by Brandes et al. (2004). Diverse histological classifications based on epithelial differentiation have been described by Pearse (2006) and others. Thymomas in rats often have a more clearly recognizable epithelial component when compared with mice. Neoplastic epithelial cells partly retain the functional characteristics of their normal counterparts, leading to homing of nonneoplastic immature T cells. However, neoplastic epithelial transformation, associated with abnormalities of the thymic microenvironment, results in failure of positive and negative selection, abnormal T-cell development and paraneoplastic autoimmunity (Brandes et al. 2004). The association between thymoma and autoimmune disease is reviewed by Bernard et al. (2016). A number of autoimmune diseases have been reported in man and animals including myasthenia gravis (MG), systemic lupus erythematosus, inappropriate antidiuretic hormone secretion, pure red cell aplasia, pernicious anemia, pemphigus and autoimmune thyroid diseases (Shahar et al. 2011). MG is the most common autoimmune disease associated with thymoma in man and MG and polymyositis have been recorded in dogs (Brandes et al. 2004). Meriggioli and Sanders (2009) reported that 10–15% of patients with thymoma have MG. In MG the production of autoantibodies predominantly against the acetylcholine receptor (AChR) in the neuromuscular junction of skeletal muscles results in muscle fatigue and weakness. Some MG patients also have antibodies to striated muscle proteins such as titin (a large protein stretching throughout the sarcomere) or rynanodin receptor (RyR) which is the calcium channel of the sarcoplasmic reticulum (SR) (Zisimopoulou et al. 2013).

The role of humeral immunity in the pathogenesis of MG is suggested by the characteristic intrathymic germinal centers, but cellular immunity has also been implicated. A link was found between MG and certain types of thymomas that contain a significant number of CD41/CD81 double-positive T cells, indicating that a malfunction of the thymus medullary selection process observed in these types of thymomas could be the cause of autoreactive T-cell release (Shahar et al. 2011).

1.5 Organ Involvement in Generalized Immunopathological Processes

1.5.1 Autoimmune Disease

In autoimmune disease (AIDz) the immune system mounts an attack on normal self antigens. The development of AIDz is a complex process in which a number of factors work in concert to perturb self tolerance (Smith and Germolec 1999). In a genetically susceptible individual, the immune response to environmental factor(s) in association with defects in immunoregulatory mechanisms can lead to autoimmune disease (Ermann and Fathman 2001). The resulting inflammation and tissue damage can involve a specific cell type (organ specific disease) or a range of cell types and tissues (multisystem disease). In each case enhanced activity of $CD4^+$ T-helper lymphocytes release a broad spectrum of cytokines and cause activation of other immune effector cells. For a comprehensive review see Bolon (2012). The **genetic make** up of an individual is key. In humans, mutations in the autoimmune regulator (AIRE) gene leads to loss of central tolerance and autoimmune polyendocrinopathy syndrome type 1 (APS-1). APS-2 is a more complex disorder associated with genes encoding the major histocompatibility complex (MHC) class II proteins located on APC and targeting of specific tissues by autoreactive T cells (Michels and Gottlieb 2010). Of the many genes predisposing to AIDz that have been identified in humans and animal models the MHC gene complex is the most prevelant (Ermann and Fathman 2001, Agmon-Levin et al. 2011). Genetics play a key role in a number of important role in major diseases such as insulin-dependent diabetes mellitus (IDDM), systemic lupus erythematosis (SLE), rheumatoid arthritis (RA) and multiple sclerosis (MS) and some forms of cancer (Waldron-Lynch and Herold 2011, Bolon 2011). Disease may be be under single gene control but more commonly have multiple genetic and environmental influences (Rogner and Avner 2003). Epigenetic mechanisms do not alter DNA sequence, but change the possibility that gene sequences are transcribed. Alterations in the post-translational modification of histones and DNA methylation are the two major epigenetic mechanisms that may potentially cause a breakdown of immune tolerance and the perpetuation of autoimmune diseases. Specific epigenetic defects have been associated with autoimmune disorders such as SLE, RA and MS (Meda et al. 2011). Many environmental factors such as injury, infection or drug administration can act as triggers of autoimmunity. Exposure of hidden antigens from immune privileged sites (eye, testes, brain) as a result of tissue damage through infection or injury, results in release of antigens into the circulation and an autoimmune response. Any condition causing increased pro-inflammatory signals may be sufficient to overcome anergy in potentially autoreactive T cells in the periphery. Infectious agents have been implicated in the pathogenisis of autoimminity via several mechanisms (Ercolini and Miller 2009, Samarkos and Vaiopoulos 2005, Münz et al. 2009). Activation of APCs in infection can stimulate the activation and proliferation of autoreactive T or B cells in a process known as bystander activation. Tissue damage leads to the uptake of dying cells and enhanced processing and

presentation of self-antigens by APCs to autoreactive cells. Molecular mimicry occurs when a microbial peptide structurally similar to a self peptide leads to cross-reactivity of the immune response. Finally, some microbes are capable of producing super-antigens which nonspecifically cross-link MHC II to TCRs on numerous T-cell lineages primed for myriad epitopes (including autoreactive ones). This leads to polyclonal activation and massive cytokine release (Schiffenbauer et al. 1998). A number of xenobiotics including chemotherapeutics, corticosteroids, polycyclic hydrocarbons, and polyhalogenated hydrocarbons have been linked to AIDz in humans and animals (Holladay 1999; Rao and Richardson 1999). Possible pathogenic mechanisms proposed include loss of immunoregulation due to insufficient generation of Tregs or failure to repress or eliminate autoreactive T cells or both. Alternatively, conjugation of a hapten, such as a small molecule, to an endogenous antigen may cause conformational changes and exposure of new epitopes. The resulting neoantigens are then recognised as foreign by T cells (Griem et al. 1998).

Immune dysregulation can result from disruption of many immune regulatory processes. T cells bearing receptors that encounter self-antigens with high-avidity during thymic development are normally deleted in the process of central tolerance. However, this process is incomplete as not all self-antigens can be sufficiently presented during the thymic selection processes. Autoreactive lymphocytes with pathogenic potential are held in check after entering the circulation (peripheral tolerance) by the processes of clonal ignorance, peripheral deletion, and anergy. Regulation of T cell function via Tregs and inhibitory receptors on T cells is particularly important in maintaining the balance between effective immunity and self-tolerance. The role of Regulatory T-cells is to suppress immune responses including autoimmunity via deletion of autoreactive T-cells. Treg cell function is decreased in many AIDz, which allows survival of autoreactive T-cell clones (Wilde et al. 2010). A rare multisystemic autoimmune disease in man called IPEX (Immunodysregulation, Polyendocrinopathy and Enteropathy, X-linked syndrome) is caused by mutations in the forkhead box P3 (FOXP3) gene in Tregs which results in their absence or dysfunction (Sakaguchi 2005, Michels and Gottlieb 2010). The resulting overwhelming autoimmunity disorder is characterized by enteropathy, food allergy, massive lymphoproliferation, psoriasiform or eczematous dermatitis, nail dystrophy, autoimmune endocrinopathies (primarily insulin-dependent diabetes mellitus and autoimmune thyroid disease), Coombs positive anemia, autoimmune thrombocytopenia, autoimmune neutropenia, tubular nephropathy, and autoimmune skin conditions such as alopecia universalis and bullous pemphigoid (Ochs et al. 2002). Cytotoxic T lymphocyte antigen-4 (CTLA-4) and programmed death-1 (PD-1) are members of the CD28 superfamily of immunoreceptors. Both are involved in the regulation of T cell activation and the induction and maintenance of peripheral tolerance (Fife and Pauken 2011). CTLA-4 is induced on activated T cells and binds to co-stimulatory ligands (B7) on APC with a higher affinity than CD28. Because of this enhanced affinity CTLA-4 may preferentially engage B7 when levels are low, such as when APC present self antigens. Self reactive T lymphocytes receive inhibitory signals from their own CTLA-4 which also inhibits the activation of other T cells. In addition, high levels of CTLA-4 is expressed on the surface Regulatory T cells and is essential for their normal function (Francisco et al. 2010). Reduced expres-

sion in Tregs permits inappropriate activation of naıve T-cells and retention of autoreactive T-cells in organs (Jain et al. 2010). **PD-1** is expressed on thymocytes during development and is induced in peripheral $CD4^+$ and $CD8^+$ T cells, by antigen receptor signaling and cytokines (Francisco et al. 2010). Engagement of PD-1 by either of its ligands (PDL-1 and PDL-2) during TCR signaling limits T cell function. According to Fife and Pauken (2011) PD-1/PDL-1 interactions regulate autoreactive T cells that are specific for tissue-restricted self antigens while PD-1/PDL-2 interactions regulate immune responses to environmental antigens.

1.5.2 Dysfunction of T Cells (T-Cell Activation Defects)

Notarangelo (2014) recently reviewed a growing number of human conditions characterized by phenotypically normal T cells with impared TCR signalling. Defective signalling was associated with mutations in genes for cell surface or signal-transducing molecules including Lck (Lymphocyte-specific protein tyrosine kinase), Syk (Spleen Tyrosine Kinase), ZAP-70 (Zeta chain associated protein-70) and DOCK8 (Dedicator of cytokinesis 8 protein). Lck is important in T cell development and function, is constitutively associated with the cytoplasmic tail of CD4 and CD8, and initiates the TCR signalling process. Mice deficient in Lck demonstrate a substantial reduction in transition from DN to DP thymocytes and a small number of peripheral CD4 and CD8 T cells show only a partial response to TCR activation. The Syk family of protein tyrosine kinases (Syk and ZAP-70) associate with TCRs and undergo tyrosine phosphorylation and activation following receptor engagement. Syk and ZAP-70 have overlapping roles in TCR function and in thymocyte development. Deficiency of DOCK8 results in a range of abnormalities including impared thymic egress with reduced T cells in peripheral lymphoid organs; decreased T cell survival resulting in lymphopenia; defective responses to chemokine stimulation and abnormal T helper cell differentiation with skewing towards Th2 responses. Although the molecular basis underlying these disorders is heterogeneous, defects in the TCR cell signal leads to common clinical and laboratory features. Patients with dysfunctional T cells are reported to suffer from autoimmune disease because of impaired central and peripheral tolerance. Reduced T cell response, with increased susceptibility to viruses and chronic viral infections may trigger continuous stimulation of T cells and lead to exhaustion of $CD8^+$ cells.

1.5.3 Transplant Rejection

In rejection, the recipients immune system recognises the cell surface histocompatibility antigens in the graft or transplant as foreign and attacks it. T cells play a critical role in rejection. Acute rejection results from direct cellular killing by

CD8 T cells or macrophage-associated graft injury. This is mediated by CD4 T cell cytokine secretion resulting in an inflammatory reaction with accumulation of lymphocytes and macrophages. In chronic rejection T cells react against alloantigens in vessel walls. This produces local inflammation and proliferation of vascular endothelium and smooth muscle cells, resulting in local hypoxia/anoxia, and failure of the graft/transplant tissue to survive. For a comprehensive review of transplant rejection and mechanism of drug treatment see Sayeh (1998) and Thomson (2009).

1.5.4 Graft Versus Host Disease (GVHD)

Haematopoietic stem cell transplant (HSCT) is used in the treatment of haematological malignancies. However, GVHD occurs when immunologically competent cells or precursors are transplanted from a donor into an immunologically crippled recipient. The transferred naive and marrow-derived T cells recognise and attack alloantigens in the recipient organs or tissues (such as the skin, liver and gut). Both donor CD4 and donor CD8 T cells have crucial roles in the pathogenesis of GVHD. However, CD4 cells are particularly important as manifestations and severity of GVHD depend on the proportions of naive cells maturing along regulatory T cell, Th1, Th2, or Th17 phenotypes. This maturation is largely influenced by local cytokines (Henden and Hill 2015). A number of therapeutic measures have been taken to curb the activity of donor allogeneic T cells. These include manipulation of cytokine levels, immune-inhibitory pathways (T cell inhibitory receptors and Tregs) TCR signalling and T cell homing pathways (eg S1P1 and selectins). The pathophysiology and treatment of GVHD is reviewed by Blazer et al. (2012).

1.5.5 Severe Combined Immunodeficiency (SCID)

Severe combined immunodeficiency (SCID) is an inherited disorder of humans, mice, horses,and dogs (Perryman 2004). Human SCID is a fatal syndrome of diverse genetic cause characterized by failure of B and T lymphocyte differentiation. It is rapidly fatal, unless treated by hematopoietic cell transplantation (HCT) using bone marrow transplantation from HLA-identical or haploidentical donors. Mutated genes on autosomal chromosomes have been identified in six genetic types of SCID in man: recombinase-activating gene (RAG1 or RAG2) deficiencies, adenosine deaminase (ADA) deficiency, Janus kinase 3 (Jak3) deficiency, IL-7 receptor deficiency, Artemis deficiency, and CD45 deficiency (Buckley 2002). The condition is characterised by lymphopenia and absence of T cell function, which accounts for increased susceptibility to life-threatening viral and opportunistic infections. Concurrent B cell defects and/or lack of helper T cells

also impaires antibody production increasing susceptibility to bacterial infections. Affected infants lack the ability to reject foreign tissue and are therefore at risk for GVHD from maternal T cells that cross into the fetal circulation in utero. The small thymus lacks thymocytes but the epithelium can support normal T-cell development following bone marrow stem-cell transplantation. Secondary lymphoid organs are also depleted of lymphocytes, particularly the T cell areas of the spleen.

1.6 Animal Models of Thymic Immunopathological Processes

1.6.1 Immunocompromised Animal Models

Immunocompromised animal models, such as the SCID mouse or nude rat/mouse, are often utilised for xenograph studies and are an important part of drug discovery in oncology. 'Humanized' mouse strains based on severely immunodeficient mice which support the engraftment of a functional human immune system have been developed. These models permit detailed analyses of human immune biology, development and functions (Shultz et al. 2012). Inherited mutations affecting the murine immune system have historically lead to the identification of novel genes critical to the regulation of the immune system and provided animal models for human immunologic disorders (Clark et al. 1999). However, many diseases under complex genetic control and those with strong interaction between genes and the environment can be more difficult to characterize. The sequencing of the mouse genome and improved methods of genetic analysis have helped in the understanding of these immune driven diseases (Rogner and Avner 2003). Recent genome engineering technologies (reviewed by Hsu et al. 2014) allow highly targeted modification of the genome, epigenetic marks or gene transcripts. This employs CRISPR (clustered regularly interspaced short palindromic repeat)–Cas9 (CRISPR-associated nuclease 9), an RNA-guided nuclease (RGN) which has been used to induce targeted mutations in multiple genes simultaneously, create conditional alleles, and generate endogenously tagged proteins (Harrison et al. 2014). This allows genetic mutations or epigenetic variants associated with biological function or disease to be recreated in an animal or cellular model. Understanding the complex relationships of immune cells and their signalling pathways by use of genetically engineered mouse models, is also an important part of drug discovery by which on and off target effects of potential therapeutic agents for T cell driven diseases can be identified.

1.6.2 SCID Mouse

Two widely used mouse models of SCID are those homozygous for the $Prkdc_{scid}$ mutation and mice with targeted deletion of recombinase-activating genes 1 and 2 Rag1 and Rag2 (Seymour et al. 2006). The function of DNA-dependent protein kinase catalytic subunit (encoded by *PRKDC*) is DNA double strand break (DSB) repair via DNA non-homologous end-joining (NHEJ). NHEJ is also involved in joining V(D)J recombination intermediates during antigen receptor gene assembly in lymphocyte development (Woodbine et al. 2013). More detail of these models including the molecular events leading to SCID are covered in the reviews by Perryman (2004) and Seymour et al. (2006). BALB/c mice homozygous for the spontaneous $Prkdc_{scid}$ mutation, lack functional T and B cells. The thymus and secondary lymphoid organs are small and effectively lack lymphocytes (Fig. 1.6). Some $Prkdc_{scid}$ mice can develop partial immunoreactivity. In these mice, known as "leaky SCIDS", T cell differentiation may be partially restored over time. However, leakiness is effected by strain and is far less prevalent when this mutation is bred into the C3H strain (C3H SCID) mice (Nonoyama et al. 1993).

Mice with targeted mutations of Rag1 or Rag 2 have a phenotype similar to that of the $Prkdc_{scid}$ mouse and develop non-T, non-B SCID similar to that observed in children with RAG-1 or RAG-2 deficiencies. Rag1 and Rag2 null mice have B- and T-cell development arrested at an early stage due to failure to initiate V(D)J recombination. The thymus is small to undetectable. Peyer's patches and lymph nodes are small and lymphopenic, and the spleen has low cellularity. They do not tend to become leaky and therefore can have a more severe combined immunodeficiency than do most scid mice.

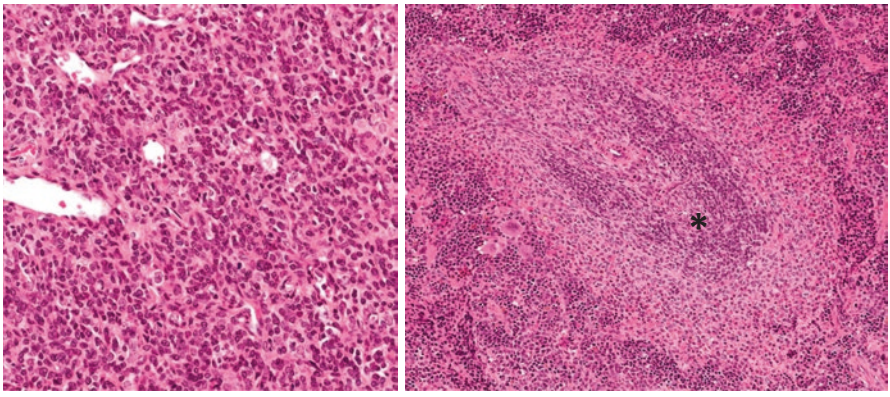

Fig. 1.6 SCID mouse. (**a**) High magnification photomicrograph of thymic area from a SCID mouse showing epithelium and lack of thymocytes. (**b**) Spleen from corresponding animal showing reduction in cellularity of T cell areas (PALS)*

1.6.3 Athymic/Nude Mouse

The nude gene Foxn1 encodes the transcription factor FOXN1, which is exclusively expressed in epithelial cells, mainly in the thymus and skin. Development of the thymus is halted at an early stage, prior to the differentiation of cortical and medullary epithelial cells, in nude mice and mice with targeted disruption of the Foxn1 gene. In the homozygous nude (nu) mouse, there is failure of haircoat development and the thymus remains at a rudimentary stage of epithelial lined canaliculi, lacking in lymphoid cells (Hansen 1978).

1.6.4 NSG (NOD.Cg-Prkdcscid I12rgtm1Wjl/SzJ) Mouse

Immunodeficient mouse models are essential in understanding the function and therapeutic potential of human stem cells, which can be engrafted in vivo into these animals. NOD-scid mice are the standard model in the stem cell research community for evaluation of in vivo human haematopoietic stem cell (HSC) function. However, HSC function in NOD-scid mice is limited by the remaining NK cell activity. In addition, these animals are short lived due to the early occurrence of thymic lymphomas, proposed to be dependent on cytokine signaling mediated through the IL2R chain. Recently, NSG (NOD.Cg-Prkdcscid I12rgtm1Wjl/SzJ) mice have been developed (Fig. 1.7). These mice are severely immunodefficient as they combine the features of the NOD/ShiLtJ background, the severe combined immune deficiency mutation (scid), and an interleukin-2 (IL-2) receptor ϒ chain deficiency. NOD mice lack haemolytic complement and have a unique MHC haplotype, which leads to defects in NK cell and APC function. Homozygosity for the scid mutation results in a double-stranded DNA repair defect and a defect in the rearrangement of genes that code for antigen-specific receptors on lymphocytes. The null mutation in the ϒ chain of the IL-2 receptor leads to deficiencies in cytokine signaling and failure of clonal lymphocyte expansion. As a result, NSG mice lack mature T cells and B cells, NK cells, and hemolytic complement, and are deficient in cytokine signaling. In addition, they are “nonleaky” at more than 1 year of age, do not develop thymic lymphomas, and are relatively long-lived :surviving beyond 16 months old (Shultz et al. 2005). Because they are unable to mount an effective adaptive immune response to foreign organisms or cells they have become a popular tool for xenotransplantation studies. The engrafted human cells retain their tissue function, resulting in “humanized mice”. They have been engrafted with many normal and malignant human cell populations and tissues including human $CD34^+$ hematopoietic stem cells, peripheral blood mononuclear cells, and human embryonic stem cells (Shultz et al. 2005). However, such severely immunocompromised strains are susceptible to infections by bacteria considered to be nonpathogenic in immunocompetent mice. Foreman et al. (2011) reported that nephritis associated with ascending infection with Klebsiella oxytoca and Enterococcus sp. was a major contributor to morbidity in the NSG strain.

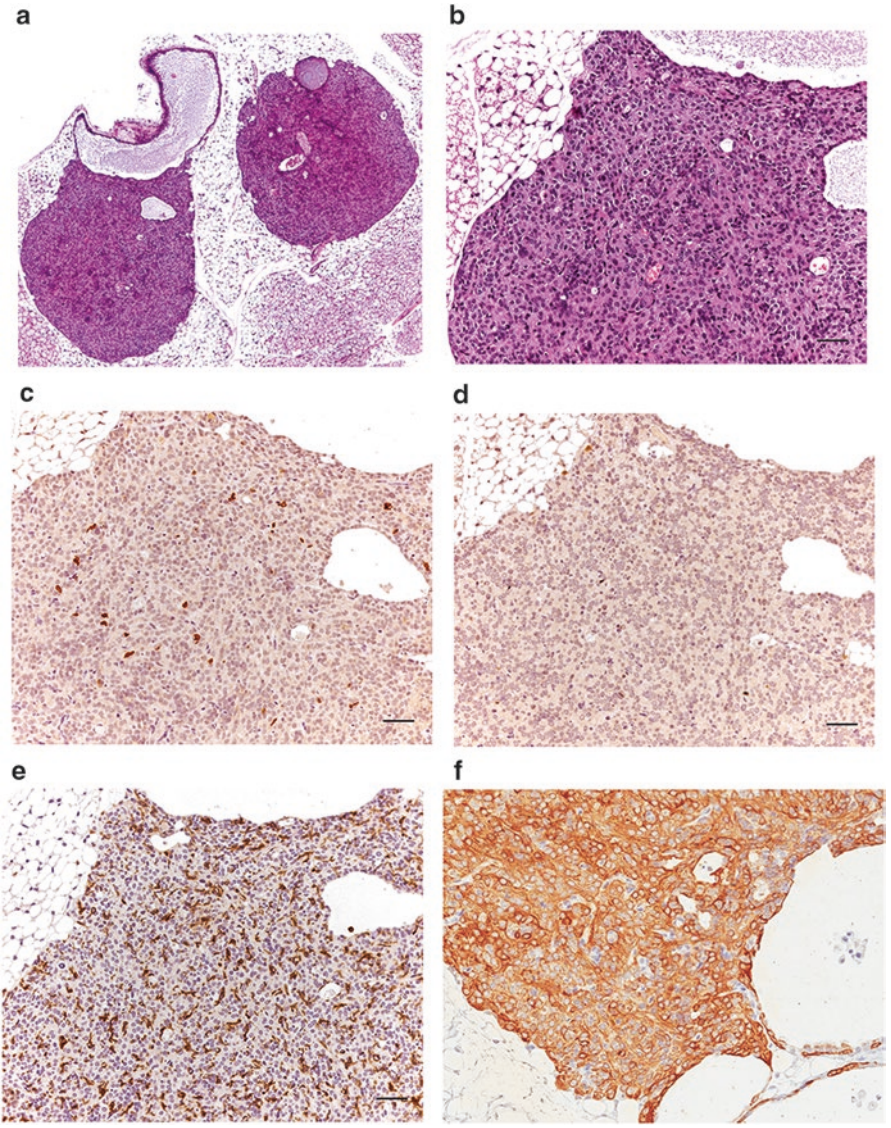

Fig. 1.7 NSG mouse. Morphological appearance of the thymus from a 8–9 month old NSG mouse. (**a**, **b**) H&E stain shows small islands of tissue in the thymic area consisting mostly of stromal cells and cystic structures. (**c**, **d**) IHC staining on the thymus is negative for B and T cells (B220 and CD3 markers, respectively). (**e**) Positively staining macrophages (F4/80 marker) are loosely scattered between epithelial cells. (**f**) Epithelial cell stain positively with cytokeratin marker and form the bulk of the tissue. Acknowledgement: Many thanks to Patrizia Cristofori (GSK) and Francesca Sanvito (Tiget) for providing these images

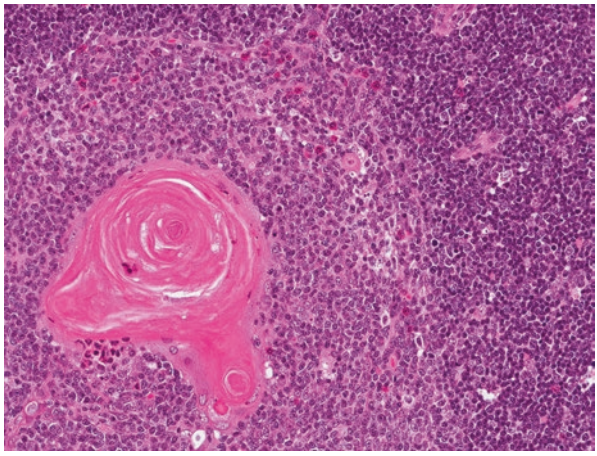

Fig. 1.8 Thymus of minipig showing Hassall's corpuscle (HC). Prominent numbers of eosinophils (*arrows*) are also scattered within the medulla. H&E stain

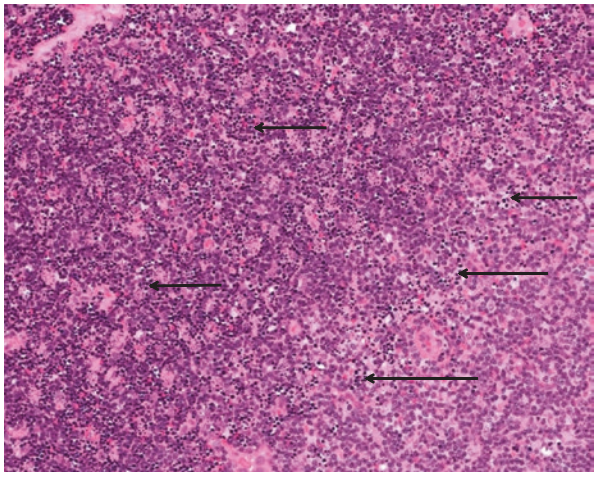

Fig. 1.9 Cortical lymphocyte apoptosis seen as an early test article related change in rat thymus. Note the large number of apoptotic bodies throughout the cortex, many of which are within the cytoplasm of macrophages. The medulla is largely unaffected

1.6.5 The "Scurfy" Mouse

The "Scurfy" mouse, which has a naturally occurring mutation in a gene homologous to the human FOXP3 gene, is an animal model for multisystemic autoimmune disease that resembles IPEX in humans (Ochs et al. 2002). The lack of functional nTreg cells results in uncontrolled cytokine secretion and an overproliferation of $CD4^+$ T-lymphocytes with extensive multiorgan inflammatory cell infiltration including lymph nodes, parenchymal organs and subcutaneous tissues. The phenotype of the Scurfy mouse is similar to other mouse models that lack cytotoxic T-lymphocyte antigen (CTLA)-4. Polymorphisms in the CTLA4 gene also result in AI endocrine disease in man (Ochs et al. 2002). IL-2 knockout (KO) mice also have severe multiorgan autoimmunity as IL-2 is essential for maintainance of T regs (Sakaguchi 2005).

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Chapter 2
Spleen

Tracey L. Papenfuss and Mark F. Cesta

Abstract The spleen, the largest secondary lymphoid organ in the body, functions both as a blood filter and part of the immune system. Histologically, the spleen is comprised of three main components; the red pulp, the white pulp and the marginal zone. The primary functions of the spleen are largely localized to specific anatomic compartments. The splenic red pulp serves as a blood filter to remove effete erythrocytes and platelets from the blood. Red pulp macrophages also have a role in combating blood-borne infection. The white pulp and marginal zone are the primary sites of innate and adaptive immune responses. The marginal zone is at the interface of red and white pulp, and has a predominance of macrophages, dendritic cells, and B cells that play an important role in innate immunity as well as the capture and presentation of antigens to initiate the adaptive immune response. Abundant lymphocytes in the white pulp are distributed into T cell-rich peri-arteriolar lymphoid sheaths and B cell-rich follicles, which work cooperatively to develop adaptive immune responses. A complex interplay between innate and adaptive immune cells and mediators makes the spleen important in the development of effective immune responses, particularly against circulating pathogens. In performing histological and functional evaluations, it is important to consider the wide range of responses in the spleen as well as differences in responses and background findings that can occur in animals of different species, strains, ages, or physiological states.

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2.1 Introduction

The spleen, an organ unique to vertebrate animals, is the largest secondary lymphoid organ in the body (Cesta 2006; Mebius and Kraal 2005). It is also the body's largest blood filter (Mebius and Kraal 2005). As part of the hematopoietic system, the spleen is a site of hematopoiesis (particularly in mice and rats). As a blood filter, it removes abnormal and effete erythrocytes and platelets, bacteria, and particulates from the circulation. As part of the immune system, the spleen is involved in innate and adaptive immune responses (Mebius and Kraal 2005). In some species, such as the dog, the spleen also acts as a reservoir for erythrocytes and platelets.

2.1.1 Development of the Spleen

Spleen embryogenesis requires a complex coordination of cell proliferation, differentiation and cellular specification (Brendolan et al. 2007; Seymour et al. 2006). In mice, embryonic development of the spleen begins at about embryonic day (E) 10.5 with the formation of the spleen anlage from mesenchyme within the dorsal mesogastrium (Brendolan et al. 2007; Golub and Cumano 2013). This is closely associated with the dorsal pancreatic mesenchyme, which begins to separate around E11.5, and the two cannot be reliably distinguished morphologically before E12.5-13 (Brendolan et al. 2007; Golub and Cumano 2013). By around E12, erythroblasts and F4/80-poitive monocytes can be detected in the fetal spleen, and by E12.5-13.5 lymphoid progenitor cells can identified (Brendolan et al. 2007; Golub and Cumano 2013). As early as E14.5, before there is hematopoietic activity in the bone marrow, hematopoietic stem cells can be isolated from the spleen (Mebius et al. 2004). The specific compartments of the spleen develop after birth, however (Mebius et al. 2004). Although recognizable arterioles have been shown to be present at post-natal day (PND) 0, the periarteriolar lymphatic sheaths (PALS) only start to become recognizable by PND7 with PALS and marginal zones being fully developed by PND 28 and mature follicles with germinal centers forming around PND 35 (Parker et al. 2015). Increases in white pulp development have been reported to plateau around 9 weeks after birth (Kodama et al. 2012). Splenic red pulp and associated extramedullary hematopoiesis (EMH) is marked in the first 2 weeks of life but gradually decreases to moderate levels by PND 42 (Parker et al. 2015). Figure 2.1 is a photomicrograph of the spleen of a juvenile rat at PND 42 demonstrating mature follicles with germinal centers and extramedullary hematopoiesis in the interfollicular red pulp. Spleens can be variable in size due to pathologic processes or depending upon the physiologic state.

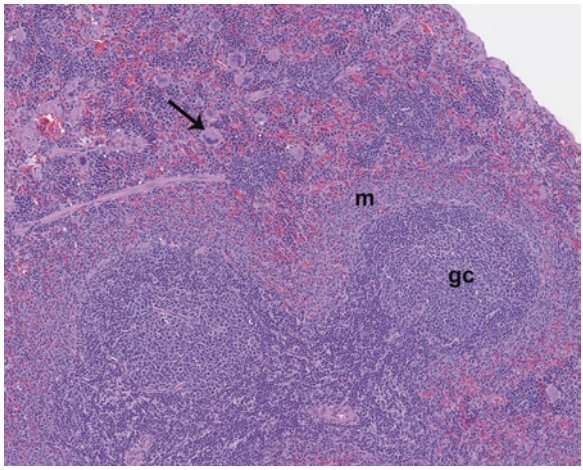

Fig. 2.1 Two splenic follicles in a juvenile (postnatal day 42) rat. Note the presence of germinal centers (*gc*) and prominent marginal zone (*m*). Hematopoiesis is present in the splenic red pulp and contains prominent megakaryocytes (*arrow*) admixed with myeloid and erythroid constituuents. H&E stain, 10× objective magnification

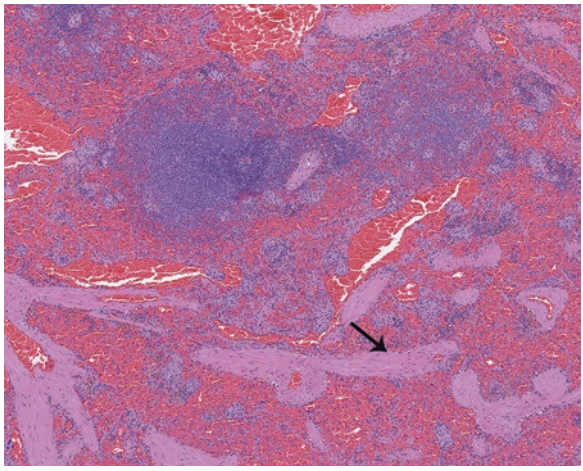

Fig. 2.2 Photomicrograph of the spleen of a dog showing multiple smooth muscle trabeculae (*arrow*). Note the prominent erythrocytic population in the red pulp. Contraction of the muscular trabeculae facilitates expulsion of blood from the spleen in the event of peripheral hemorrhage that results in a reduction in circulating blood volume. H&E stain, 5× objective magnification

2.1.2 Structure and Function of the Spleen

The spleen is located on the left side of the abdomen adjacent to the greater curvature of the stomach. Its blood supply is via the splenic artery, which is a branch of the celiac artery. Grossly, in rodents, it is somewhat dumbbell shaped and roughly triangular in cross-section. Histologically, the spleen has three main compartments: the red pulp, the white pulp, and the marginal zone. It is bound by a capsule that is covered by a layer of mesothelial cells. The capsule is composed of fibrous connective tissue, elastic fibers, and smooth muscle. Trabeculae of smooth muscle arise from the capsule and extend into the red pulp (Fig. 2.2). These smooth muscle trabeculae support the three dimensional network of the red pulp (Blue and Weiss 1981). The spleen has neural input by the sympathetic nervous system with

norepinephrine being the primary regulator of neuro-immune interactions (Nance and Sanders 2007). The spleen lacks afferent lymphatic vessels (Cesta 2006).

The spleen's primary roles are to survey the blood for and respond to infectious agents and foreign material and to remove defective or senescent erythrocytes from the circulation. It can initiate an immune response to infectious agents detected in the blood. The spleen also acts as a reservoir storage site for platelets and erythrocytes and in some species, surveys the health of the platelet population, and is involved in iron storage and turnover (Brendolan et al. 2007; Mebius and Kraal 2005). Splenic red pulp macrophages are considered the primary scavengars for senescent erythrocytes and the balance of different macrophage populations (e.g. M1 versus M2 macrophages) is thought to influence the accumulation or release iron (Borges da Silva et al. 2015). Marginal zone macrophages (MZM) in the spleen can also phagocytose apoptotic cells in the blood, and the clearance of apoptotic cells by these macrophages is thought to contribute to immune modulation and maintenance of peripheral tolerance (Brendolan et al. 2007; Mahnke et al. 2003; Morelli et al. 2003). Besides its important defined role in innate and adaptive immune responses, the spleen also produces factors (e.g., opsonins, properdin and tuftsin) which support opsonization, complement activation and stimulates macrophages and polymorphonuclear cells, respectively (Mebius and Kraal 2005).

2.1.2.1 Red Pulp

The red pulp functions as a filter to remove old or damaged erythrocytes and platelets, apoptotic cells, and infectious agents from the blood (Brendolan et al. 2007). Because of its role in erythrocyte removal, the red pulp is also associated with iron recycling. These functions are accomplished largely by macrophages located in the splenic cords. In rodents, the red pulp is also a significant site of hematopoiesis.

The red pulp has a unique three dimensional structure composed of cords and sinuses. The cords are composed of reticular fibers, fibroblasts and myofibroblasts, basement membranes, and unmyelinated adrenergic nerve fibers (Blue and Weiss 1981; Chadburn 2000; Mebius and Kraal 2005; Saito et al. 1988). The reticular fibers are small (30–50 nm) and lack the collagen core found in reticular fibers of the white pulp (Lockmic et al. 2008). The cords form an open circulatory system with no endothelial-lined blood spaces (Mebius and Kraal 2005; Satodate et al. 1986; Schmidt et al. 1985; den Haan and Kraal 2012). The circulating blood cells (erythrocytes, granulocytes, mononuclear inflammatory cells, and other cells) percolate through the spaces between the splenic cords. There is evidence that, in some species, such as dogs, there are direct connections between the splenic arterioles and the venous sinuses, creating a closed system that coexists with the open circulatory system (Schmidt et al. 1982, 1983). The splenic vein drains into the hepatic portal vein, so the blood leaving the spleen is filtered by the liver before returning to the general circulation (Kraal and Mebius 2006).

Amid the cords and venous sinuses of the red pulp, there are penicillar arteries and their arteriolar and capillary branches, and, particularly in rats and mice,

scattered hematopoietic cells. Within the meshwork formed by the splenic cords, there are plasma cells and plasmablasts that migrate from the follicles and outer PALS after antigenic stimulation, and numerous F4/80-positive red pulp macrophages that monitor the blood for bacteria and other foreign material, and phagocytose old and damaged erythrocytes and platelets (Brendolan et al. 2007; den Haan and Kraal 2012; Jones 1983; Mebius and Kraal 2005; Matsuno et al. 1989). Many macrophages in the red pulp contain hemosiderin pigment from the breakdown of hemoglobin subsequent to phagocytosis of effete erythrocytes. The blood flows from the splenic cords into the venous sinuses. The venous sinuses are lined by discontinuous endothelial cells that are subtended by stress fibers that connect the endothelial cells to the extracellular matrix (den Haan and Kraal 2012; Mebius and Kraal 2005). The arrangement of the endothelial cells and associated stress fibers creates small, slit-like openings which the erythrocytes must pass through to enter the venous sinuses (den Haan and Kraal 2012; Mebius and Kraal 2005). Old and damaged erythrocytes with stiffer membranes do not easily pass through these openings and are phagocytosed by the red pulp macrophages (den Haan and Kraal 2012; Mebius and Kraal 2005). These macrophages also express CD163, a cell surface receptor specific for hemoglobin, which mediates uptake of hemoglobin in the blood (usually bound to haptoglobin) from intravascular erythrocyte destruction (Mebius and Kraal 2005).

Once an erythrocyte is phagocytosed by a red pulp macrophage, the phagosome containing the erythrocyte fuses with a lysosome forming an erythrophagolysosome where the erythrocyte is degraded by proteases (Korolnek and Hamza 2015). The heme catabolism enzyme, heme oxygenase 1, catabolizes the heme into biliverdin and ferrous iron (Fe^{2+}), and the heme transporter, HRG1, has been shown to be important for the transport of heme into the cytoplasm (Korolnek and Hamza 2015; Mebius and Kraal 2005; White et al. 2013). Once degraded, the erythrocyte components are transported into the macrophage cytoplasm. The iron transporter Nramp1, located on the phagolysosomal membrane, may be an important mediator in this step (Korolnek and Hamza 2015; Mebius and Kraal 2005). The iron is either stored as ferritin, which can aggregate to form hemosiderin, or is released as ferritin or as low molecular weight forms that bind to transferrin in the blood (Mebius and Kraal 2005).

Fighting blood-borne infections is another important function of the red pulp. This is accomplished in several ways. Most obviously, the red pulp macrophages phagocytose bacteria and other pathogens in the blood. Also, the uptake of iron by the red pulp macrophages may be important in limiting the growth of pathogens by limiting their supply of iron (Mebius and Kraal 2005). Furthermore, red pulp macrophages produce lipocalin-2, which binds to siderophores, produced by some pathogens, and interferes with bacterial iron uptake (Mebius and Kraal 2005). Lastly, plasmablasts and plasma cells that migrate from the follicles to the red pulp after clonal expansion in the follicles produce antibodies that rapidly enter the bloodstream (Mebius and Kraal 2005).

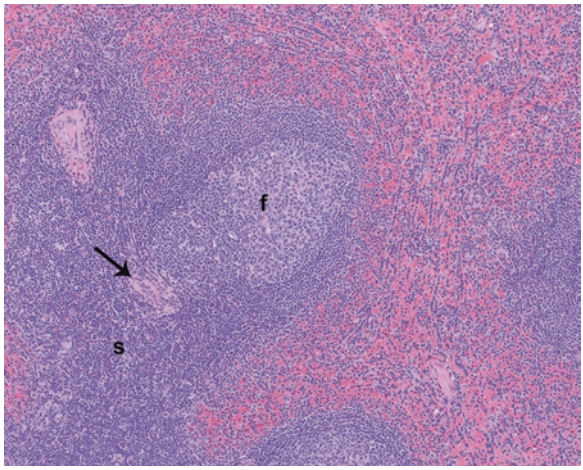

Fig. 2.3 Spleen of a cynomolgus macaque showing a follicle (*f*) and peri-arteriolar lymphoid sheath (PALS) (*s*) surrounding an arteriole (*arrow*). H&E stain, 10× objective magnification

2.1.2.2 White Pulp

The white pulp plays an important part in innate and adaptive immune responses and approximately 25% of the body's lymphocytes are in the spleen (Kuper et al. 2013). The organization of the white pulp bears some resemblance to that of a lymph node, however, it lacks high endothelial venules (Kraal and Mebius 2006). It is composed of the T-cell rich periarteriolar lymphoid sheaths (PALS) and the B cell-rich follicles (Chadburn 2000; Mebius and Kraal 2005). Figure 2.3 demonstrates the close apposition of the T cell-rich PALs region and B-cell rich follicles. Approximately 25% of the spleen is occupied by white pulp (Chadburn 2000). The PALS, which surround the central arterioles, comprise concentric layers of CD3+ lymphocytes with fewer plasma cells, macrophages, and dendritic cells (DCs) within a supporting network of reticular fibers and flattened reticular cells (Cesta 2006; Chadburn 2000). The PALS is divided into two layers, the inner PALS and the outer PALS. The inner zone of the PALS is a T-cell dependent region, containing mainly CD4+ T-cells with fewer CD8+ T-cells, interdigitating DCs, and migrating B cells (Van Rees et al. 1996). The outer PALS zone, which generally stains slightly less intensely in hematoxylin and eosin-stained tissue sections, is populated by small and medium B- and T-cells, macrophages, and, with antigenic stimulation, plasma cells (Matsuno et al. 1989; Kuper et al. 2013; Van Rees et al. 1996). In these T cell zones, T cells interact with DCs and B cells. The chemokines CCL19 and CCL21 are involved in attracting and retaining T cells in the T cell regions (Mebius and Kraal 2005).

The follicles lie adjacent to the PALS, typically at bifurcations of the central arterioles (Ward et al. 1999). The follicles contain numerous B cells with fewer CD4+ T cells and follicular DCs, but typically do not contain CD8+ T cells (Van Rees et al. 1996). The follicles, as in other tissues, are sites of B cell clonal expansion, which is followed by isotype switching, and somatic hypermutation (Mebius and Kraal 2005). They have a central region that contains larger lymphocytes and an

outer region, the corona or mantle zone, which stains more intensely in hematoxylin and eosin-stained tissue sections. The corona contains smaller lymphocytes. Upon antigenic stimulation, follicles develop germinal centers that contain apoptotic cells and tingible body macrophages. Chemokines important for B cell follicle integrity and attraction of B cells to the follicles include CXCL13 and CXCR5 (Mebius and Kraal 2005).

2.1.2.3 Marginal Zone

The marginal zone is a unique region at the interface of the red and white pulp and is fully developed by 10 days of age (Mebius et al. 2004). Macrophages are an important cellular component of the marginal zone and as part of the mononuclear phagocyte system (also known as the reticulendothelial system) serve a critical role for surveying the blood. The marginal zone contains two specific populations of macrophages, the marginal zone macrophages (MZM) and the marginal zone metallophilic macrophages (marginal zone metallophilic macrophages; MM), marginal zone B cells, and DCs amid a framework of reticular fibroblasts and sinus-lining endothelial cells (Kraal and Mebius 2006; Mebius and Kraal 2005). In laboratory rodents, the marginal sinus separates the majority of the marginal zone from the white pulp, but humans lack the marginal sinus (Brendolan et al. 2007). The MZM and the marginal zone B cells are unique to the spleen, but the MM are also present in lymph nodes, surrounding T-cell zones beneath the subcapsular sinus (Kraal and Mebius 2006; Mebius et al. 2004). The marginal sinus and the MM separate the marginal zone from the PALS and follicles. Marginal zone macrophages are highly phagocytic and form a ring at the outer border of the marginal sinus where they extend long cellular processes to help facilitate their important role in trapping particulate antigen (Aichele et al. 2003). The MM are located at the inner border of the marginal zones where they form a thin rim and, although less phagocytic than MZMs, also contribute to the trapping of particulate antigen. However, both the MZM and MM play important roles in trapping antigen, they appear to be less important for antigen presentation (Aichele et al. 2003). The marginal sinus is continuous with the capillaries of the PALS and follicles and the lining endothelial cells express MAdCAM1 (Mebius and Kraal 2005). The MM are found on the PALS side of the marginal sinus, forming a line immediately adjacent to the marginal sinus endothelial cells. On the other side of the marginal sinus are the MZM, B cells, and the DCs, which are scattered throughout the marginal zone. Arterial blood enters the marginal sinus and percolates through the marginal zone into the red pulp. Thus, the marginal zone is ideally situated to survey the blood in search of foreign antigens. Cells entering the white pulp also go through the marginal zone, however, this process requires energy and involves G-protein-coupled receptors (Mebius and Kraal 2005).

The macrophages in the marginal zone express receptors necessary for phagocytosis of opsonized particles, but they also express receptors that allow them to phagocytize nonopsonized particles (Kraal and Mebius 2006). They have numerous

pattern-recognition receptors that recognized damage- and pathogen-associated molecular patterns (DAMPs, and PAMPs, respectively) which often trigger through TLRs, including TLR2, 4 and 9 (Borges da Silva et al. 2015). They are critical in controlling blood-borne viral (e.g., adenovirus and lymphocytic choriomeningitis virus) and bacterial (e.g., *Listeria monocytogenes* and *Neisseria meingitidis*) infections (Borges da Silva et al. 2015). The MM contain high levels of esterase and express Siglec1 (sialic-acid-binding immunoglobulin-like lectin-1, or sialoadhesin), which recognizes sialic acids (Kraal and Mebius 2006; Mebius and Kraal 2005; Mebius et al. 2004). This suggests a role in removal of pathogens and apoptotic cells from the circulation (Kraal and Mebius 2006). Marginal zone metallophilic macrophages also produce the type I interferons (IFN-α and IFN-β), which serve an important role in anti-viral immune responses (Borges da Silva et al. 2015; Mebius and Kraal 2005). It has also been postulated that the MM play a role in adhesion and retention of lymphocytes (especially B cells) in the marginal zone and their migration into the white pulp, and removal of tumor cells from circulation (Mebius et al. 2004).

The MZM express the C-type lectin SIGNR1 (the mouse homologue of DC-SIGN) and MARCO (macrophage receptor with collagenous structure), a type I scavenger receptor (Mebius and Kraal 2005; Mebius et al. 2004). This combination of pattern recognition receptors, and their location relative to the blood flow in the spleen, makes these cells ideally suited to scavenge the blood for pathogens, particularly bacterial pathogens (Kraal and Mebius 2006). SIGNR1 is important in the recognition of polysaccharide antigens (e.g., mannosylated lipoarabinomamman on the surface of Mycobacteirum tuberculosis) and is crucial for uptake and clearance of *Streptococcus pneumoniae* and some viruses (Kraal and Mebius 2006). SIGNR1 also plays an important role in binding yeasts, *E. coli*, HIV, *S. penumoniae*, S. typhimurium (Borges da Silva et al. 2015). It has also been shown that SIGNR1 can interact with Toll-like receptors resulting in increased production of NF-κB (Kraal and Mebius 2006). The SIGNR1 molecule contains a triacid cluster that is thought to be responsible for internalization of bound particles and targeting the SIGNR1-particle complex to lysosomes for processing (Kraal and Mebius 2006; Mebius et al. 2004). Marginal zone macrophages, however, do not express MHC class II; it has been suggested that the degredation products are released from the macrophages and are opsonized by complement (Kraal and Mebius 2006; Mebius and Kraal 2005). It has been demonstrated that SIGNR1 on MZM interacts with marginal zone B cells and is important in IgM antibody production in models of infection with *Streptococcus pneumonia* (Koppel et al. 2008). The marginal zones of SIGNR1 mice have been shown to contain fewer marginal zone B cells, so SIGNR1 seems to be important for marginal zone B-cell survival or trafficking (Koppel et al. 2008). MARCO is class A scavenger receptor for which numerous pathogenic ligands have been identified, including *E. coli* and *S. aureus* (Borges da Silva et al. 2015; Kraal and Mebius 2006). MARCO has also been shown to be important in marginal zone B cell retention in the marginal zone since disruption of the interaction of marginal zone B cells with MARCO results in the migration of marginal zone B cells to the splenic follicles (Koppel et al. 2008).

The marginal zone B cells are another important population of cells in the spleen. They are responsible for early antibody responses. Marginal zone B cells are particularly well-equipped to detect blood-borne antigens, can function as APCs, and play roles in both T cell-dependent and T cell-independent responses (Lopes-Carvalho et al. 2005). They encounter antigens via their presence in the low-flow region where arterioles empty into blood sinuses allowing them to efficiently trap blood-borne antigen-immune complexes and by directly interacting with blood-derived DCs that carry and actively transport antigens to marginal zone B cells (Lopes-Carvalho et al. 2005). They are in a pre-activated state, which allows them to respond rapidly to pathogens. They express high levels of IgM and have a distinct receptor expression pattern in comparison to follicular B cells.

Antigens and pathogens are taken up by APCs in the marginal zone, as well as adjacent regions of the white pulp. APCs that have taken up antigen elsewhere in the body may enter the marginal zone via the blood. Following activation, DCs then migrate into the white pulp to initiate adaptive immune responses in a CCR7 expression-dependent process. Within the white pulp, DCs mediate the initiation of adaptive immune responses. Specifically, DCs can cause helper T cell differentiation to promote humoral or cell-mediated immune responses (see Chapter 1 for description of humoral and cell-mediated immune responses). Follicular DCs produce CXCL13, which facilitates the migration of marginal zone B cells into B cell follicles within the marginal zone (Mebius and Kraal 2005). Follicular helper T cells are CD4+ T cells that play an important role in inducing the differentiation of B cells into plasma cells and memory cells (Ueno et al. 2015). Dendritic cells also play an important role in the differentiation and survival of B cells to become antibody-producing cells and antigen-loaded follicular DCs are necessary for the optimal generation of immunological memory in B cells (Lopes-Carvalho et al. 2005).

Entry of APCs into the white pulp is critical. In the spleen, all cells enter via the marginal zone, unlike lymphoid organs where high endothelial venules (HEVs) are the site of lymphocyte entry (Mebius and Kraal 2005). These activated DCs induce T cell activation which downregulate CCR7 and upregulate CXCR5 allowing them to migrate to the edge of B cell follicles (Mebius and Kraal 2005). Relatedly, CCR7 upregulation in B cells at the edge of B cell follicles promotes their movement to the edge of the follicles where they can interact with T cells (Mebius and Kraal 2005). Following this interaction with activated T cells, isotype switching can occur within the B cells follicles and these B cells can remain in the germinal centers or migrate to other areas of the spleen (e.g., marginal zone or red pulp). Lymphocytes leave the splenic white pulp by downregulating CCR7 expression (particularly in CD8+ T cells) and although the exact anatomical route used is unknown, it is thought that lymphocytes leave white pulp through the marginal zone and then re-enter the bloodstream (Mebius and Kraal 2005). Marginal zone B cells are thought to play an important role in the organization and integrity of the marginal zone and DCs can help promote the differentiation and/or survival of B cells (Mebius and Kraal 2005). The impact of marginal zone B cells on CD8+ T cell responses is not entirely clear but they may serve as targets for viral infection which then result in targeted killing of marginal zone B cells by CD8+ T cells (Lopes-Carvalho et al. 2005). Marginal

zone B cells can also prime naïve CD4+ T cells and drive their differentiation to helper T cell subsets (e.g., Th1, Th2). Conversely, differentiated Th1 and Th2 cells can promote the differentiation of effector B cells (i.e., BE1 and BE2, respectively; reviewed in Lopes-Carvalho et al. 2005). Relatedly, marginal zone B cell interaction with NKT cells has been suggested to be required for antigen-specific regulatory T cell generation (Lopes-Carvalho et al. 2005). Marginal zone and follicular B cells likely arise from a common progenitor but differentiate into cells with distinct roles within the spleen (Mebius and Kraal 2005).

2.1.3 Species Differences in the Histology of the Spleen

Although spleens are relatively similar across species, there are species-specific differences. Spleens can be divided into defense (i.e. few trabeculae and smooth muscle fibers) versus storage-type spleens (i.e. many trabeculae and smooth muscle fibers) or sinusal versus non-sinusal (Banks 1993; Press and Landsverk 2006). The species-differences are apparent both in form and function of the spleen. Sinusal type spleens can store large amounts of blood, while non-sinusal types do not store blood to a large extent (Bacha and Bacha 2000). As such, the size and color of the spleen varies with species and degree of distention. In rodents, it is typically reddish and its size does not vary greatly, while in dogs and other species with a sinusal spleen, its color (red to blue–black) and size vary with the amount of blood is stored. Periarterial macrophage sheaths (PAMs or ellipsoids), which are capillaries surrounded by a sheath of macrophages, can be present in some species. These ellipsoids can be prominent, but vary in appearance between species (Brendolan et al. 2007). In dogs and minipigs, ellipsoids within the red pulp and marginal zone are often prominent, while in humans, they are poorly defined (Brown and Dellmann 1981; Onkar and Govardhan 2013). Figure 2.4 demonstrates prominent ellipsoids

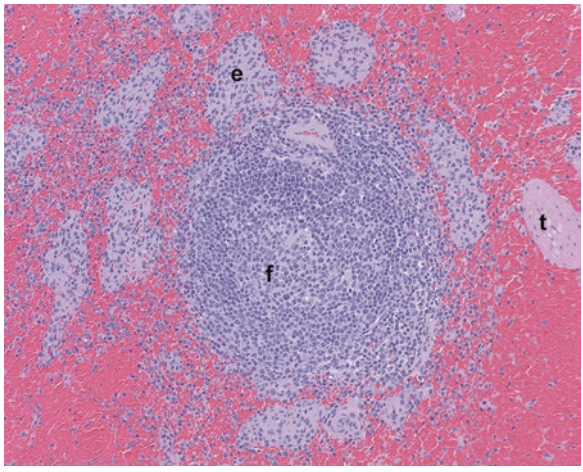

Fig. 2.4 Splenic follicle (*f*) with prominent ellipsoids (*e*) (macrophage-sheathed capillaries) in the spleen of a Gottingen minipig. Note the smooth muscle trabecula (*t*) and prominent erythrocytic population throughout the red pulp. H&E stain, 15.1 objective magnification

present in a Gottingen minipig. Rodents (e.g., mouse, rat, guinea pig) and rabbits lack ellipsoids, though the proposed function for ellipsoids of trapping blood-borne particles still occurs in these species (Brendolan et al. 2007). The amount of white pulp can vary between species with dogs having less white pulp overall compared to humans (Brown and Dellmann 1981; Onkar and Govardhan 2013). Rats, in contrast to other rodents, have prominent marginal zones (Cesta 2006).

2.1.4 Splenectomy Effects on General Immune Functioning

Given the important role of the spleen in both clearing antibody- and complement-coated pathogens, splenectomy can contribute to an increased susceptibility to infections by pathogens whose removal is dependent upon these mechanisms. *Streptococcus peumoniae* is the most common cause of post-splenectomy sepsis although other organisms, such as, *E. coli*, *N. meningitidis and H. influenza type* B can be problematic for splenectomized individuals (Altamura et al. 2001; Mebius and Kraal 2005; Ram et al. 2010). Additionally, since the spleen plays an important role in removing erythrocytes that have reduced deformability, splenectomized individuals have an increased susceptibility to malaria (*P. falciparum*) and babesiosis (*B. microti*) (Ram et al. 2010). Glycoconjugated vaccine approaches may be the most appropriate to facilite IgG-mediated protection in splenectomized individuals (Rosado et al. 2013). The spleen is also an important contributor to natural antibodies since asplenic mice have been shown to lack B-1a B cells (Brendolan et al. 2007; Wardemann et al. 2002). B-1aB cells differentiate into IgA plasma cells in the intestinal mucosa, produce natural antibodies during the initial onset of bacterial or viral infections, and are responsible for T-cell independent immune responses (Brendolan et al. 2007; Kroese et al. 1989).

2.1.5 Stress Effects on General Immune Functioning

Functional changes can be seen with both acute and chronic stress. In the realm of immunotoxicity and immunopathology, many of these functional changes are not assessed with routine histology although some indication of stress can be reflected in a stress leukogram in hematology parameters. Stress can either be acute or chronic. Most references on stress-related responses focus on the effects of chronic stress, since acute stress typically refers to the initial period (minutes to hours) following exposure to a stressor.

During acute stress, leukocytes are commonly depleted, particularly in the spleen and peripheral blood, though they may be increased in other organs such as the skin (Dhabhar 2009; Saint-Mezard et al. 2003). Functional changes during acute stress are often considered immunoenhancing with immune cells (particularly NK cells and granulocytes) leaving the spleen, lung, and marginated pool to enter blood ves-

sels and lymphatics (Dhabhar 2009). There is also enhanced NK cell activitiy, antibody-dependnet cell-mediated cytotoxicity, increased mitogen-induced proliferation of T and B cells, and increased cytotoxic T cells numbers and responses (Dhabhar 2009). In chronic stress, changes in leukocyte number and distribution can be seen both in the circulation and immune organs and have reflected alterations in immune system function. During chronic stress, immune organs are often depleted of various cellular populations and suppression of cell-mediated immune responses, antibody production, NK activity, leukocyte proliferation, skin homograft rejection, virus-specific T cell and NK cell activity, and anti-mycobacterial activity can all be seen (reviewed in Dhabhar 2009).

Stress responses can have a dramatic impact on pathologic endpoints during a toxicologic pathology study (Everds et al. 2013). Often, these changes can be difficult to discern from normal aging changes (e.g., thymic involution) or from test article-related effects. An appropriate assessment of the involvement of stress responses on immunological alterations is critical when assessing the efficacy and safety of a potential therapeutic. The effects of stress on immune organs can cause decreases in total body weights (or weight gain), food consumption and activity, altered organ weights, lymphocyte depletion and altered circulating leuckotye counts. Most commonly, organ weights, such as the spleen and the thymus, are lower due to lymphocyte depletion in these organs. Relatedly, decreased numbers of lymphocytes and eosinophils in conjunction with increased numbers of neutrophils can be seen as a response to stress. Spleen weights can be decreased or unchanged in response to severe or mild stressors, respectively. However, additional factors such as the test article-related effects on the erythroid compartment can impact splenic weights such as increased EMH or hepatocellular hypertrophy which would both cause and increase in splenic weight. Lymphocyte depletion can be in specific anatomic areas of the white pulp (e.g., depletion of marginal zone B cells) and is often seen in conjunction with lymphocyte apoptosis in these compartments although redistribution of various immune cell populations can have variable morphology on B-lymphocyte areas of the spleen. Immunophenotyping by flow cytometry or relative enumeration or quantification of immune cells or compartments by immunohistochemistry and other imaging modalities can be of particular value in identifying the potential impact of stress or test article-effects on the spleen. In the rat spleen, stress most commonly impacts the B cell and NK cell compartments (Everds et al. 2013). Both routine and enhanced histopathologic assessment of various splenic immune compartments should be used to determine whether there are effects on immune cells within the spleen.

2.1.6 Evaluation of the Spleen

Evaluation of the rodent spleen is often performed on a single cross-section. Longitudinal sections, however, provide more area for examination, which may be important when evaluating the white pulp (Suttie 2006). It is important to ensure consistency in sectioning the spleen to maintain the validity of interanimal

comparisons. Fixation in 10% neutral buffered formalin and hematoxylin and eosin staining is adequate for routine screening, though other stains, including immunohistochemical stains for T and B cells, may be useful for further characterization of lesions.

There are a number of parameters, including gross and histopathologic changes, splenic weight, clinical pathology findings, and findings in other organs, that should be assessed In toxicologic studies. In general, a "weight of evidence" approach should be used to integrate and assess the impact of a test article on the spleen to determine potential immunotoxicologic changes. Gross changes of splenic size and color are common parameters that are evaluated and are often related to alterations in splenic organ weights. For example, multiple white areas (nodules) within the spleen suggest expansion of splenic white pulp due to an immune or inflammatory response or neoplastic process, while an enlarged dark red spleen suggests expansion of red pulp due to increased EMH or splenic congestion (depending on the species), or incomplete exsanguination at necropsy (Cesta 2006; Elmore 2006). Splenic weight can also be impacted by stress whether directly or indirectly related to test article administration. With a normal spleen, the spleen-to-body weight ratio is fairly consistent and, in Sprague-Dawley rats, ranges from 0.17 to 0.24% (Losco 1992). Decreases or increases in splenic weight can provide some information regarding changes within the spleen but in general, splenic weight is considered a relatively insensitive indicator of stress and/or immunotoxicity (Elmore 2006; Everds et al. 2013; Luster et al. 1992; Michael et al. 2007). For example, splenic organ weight changes of $\geq$20% are necessary before histological findings of lymphoid depletion are consistently recognized (Everds et al. 2013). Although considered less sensitive than thymic weight and microscopic changes, decreased splenic weight and cellularity are commonly seen in stress, but ascribing stress-related changes rather than ruling out any test article-related alterations should be approached with caution (Everds et al. 2013). Splenic changes related to stress are typically milder than those seen in the thymus. These changes are usually manifested as decreases in white pulp due to both decreased proliferation in T cell regions and increased apoptosis or redistribution of B cells (Dhabhar et al. 1995; Everds et al. 2013; Pruett et al. 2007). Specifically, changes in overall food consumption, body weight, clinical pathology parameters, changes in other organ weights (e.g., thymus, lymph nodes, adrenal glands and reproductive organs) and microscopic changes should all be used to come to a conclusion of stress-related changes within the spleen (Everds et al. 2013).

Decreased splenic weight can indicate depletion of specific cellular components and whether these changes are relatedly to test article administration (either directly or indirectly) must be determined (Cesta 2006). Microscopic evaluation is necessary to assess which regions are impacted and whether an underlying defect may be contributing. For example, athymic animals have decreased cellularity in T cell regions and in the development of secondary follicles (Cesta 2006). In contrast, increased splenic weight is most commonly due to hyperplasia/expansion of specific anatomic compartments within the spleen. Depending on the species and strain, additional processes can increase splenic weight. Pathological processes, such as

lymphoma or other neoplastic processes (e.g., strain-related mononuclear cell leukemia in F344 rats) or procedure-related processes, such as, splenic congestion resulting during euthanasia with barbiturate in dogs are two such examples. Again, microscopic confirmation of immunopathologic alterations is necessary.

For routine screening of the spleen, standard nomenclature is acceptable. However, for evaluation of the spleen in immunotoxicity studies, or when a more detailed assessment is necessary, it is currently recommended that the various compartments of the spleen (red pulp, PALS, follicles, marginal zones) be evaluated separately and that descriptive terms be used for the diagnoses (Elmore 2006; Haley et al. 2005). This enhanced histopathologic approach includes a descriptive terminology includes increased or decreased cellularity, increased or decreased area, and for the follicles, increased number and/or size of germinal centers. If there is an increase in a particular cell type, the diagnosis should be increased numbers and the cell type identified. For example, if there is an increase in plasma cells in the marginal zone, the diagnosis would be "Spleen, marginal zone—Increased cellularity, plasma cell." Severity grades should be applied to these diagnoses. Interpretive diagnoses, such as hyperplasia, should be avoided, but should be included in the discussion where the findings are interpreted. Additional characterization of the cellular population comprising the areas containing increased cellularity (e.g., plasma cells, granulocytes, mast cells, macrophages, etc.) can be valuable but additional staining or immunohistochemistry approaches may be necessary (Elmore 2006). Additional qualitative and quantitative parameters are being incorporated into studies to evaluate immunotoxicity with flow cytometry, morphometry, in situ hybridization/PCR and immune functional assays being the more common techniques being applied (Haley et al. 2005).

The diagnosis and interpretation of lesions in the spleen in immunotoxicity studies may be complicated by age-related or background findings. Additionally, some findings may be associated with or secondary to concurrent findings in other parts of the body. Due to the spleen's exposure to the systemic circulation and its role in filtering the blood, a holistic approach to evaluation of the spleen, taking into account such things as the animal's age, health status, and lesions in other organs, is recommended to properly diagnose and interpret the findings in the spleen. Comparison to age- and gender-matched controls is also very important.

Microscopic changes that may be present in the spleen include congenital changes, disturbances of growth (hyperplasia, dysplasia, neoplasia), degenerative and miscellaneous changes (fibrosis, pigmentation, lipid accumulation, mineralization, amyloidosis EMH,), cell degeneration/death (e.g., apoptosis, necrosis), vascular changes (hemorrhage, periarteritis, infarction) and acute/chronic inflammatory changes (Frith et al. 2000; Suttie 2006). Alterations in PALS and/or marginal zones and changes in the number of germinal centers within follicles are typical cellular changes seen with an immunomodulatory compound (Elmore 2006; Gopinath 1996; Harleman 2000; Kuper et al. 2000). Measures of follicle cellularity and germinal center development are reported to be the most sensitive predictors for potential immunotoxicity while red pulp changes are often more difficult to detect (Elmore 2006; Germolec et al. 2004). Additionally, functional assessment of splenic immune responses can be an important means of assessing immune alterations (Descotes 2006; De Jong and Van Loveren 2007).

2.1.7 Species-Specific Background Findings in the Spleen

There are species-specific differences in the anatomic compartments in rodents. Mice tend to have greater proportion of white pulp than rats but the follicles and marginal zones of mice are less distinct while in rats, the marginal zone comprises up to 28% of the splenic volume and is particularly prominent microscopically (Cesta 2006; McInnes 2012b). In older mice and rats, enlarged spleens can commonly be seen, particularly in the Sprague-Dawley (Elmore 2006). Histologic findings in these aged spleens include increased cellularity in the B cell-rich regions (e.g., follicles or marginal zone), increased myelopoiesis, and red pulp hyperplasia. In general, EMH can be particularly prevalent in rodents, especially younger rodents, compared to other species, such as the dog, where EMH is mostly seen during pathologic conditions such as neoplasia & anemia (Cesta 2006; HogenEsch and Hahn 2001). Additional background lesions that have been described in rats include increased hemosiderin (particularly in females) and lipofuscin in the red pulp of older rats (Elmore 2006; McInnes 2012b; Suttie 2006). Melanin can be seen in the spleen of pigmented mice as well (Taylor 2012).

There are numerous neoplastic and non-neoplastic lesions that may be noted in a safety assessment study. Some of these include accessory splenic tissue, cysts, lipid accumulation, pigment accumulation, congestion, mineralization, angiectasis, hematoma, hemorrhage, atrophy, hyperplasia (lymphoid or stromal), EMH, reactive/inflammatory changes, and lymphoid or hematopoietic neoplasms (Frith et al. 2000). Many of these lesions are common background findings that are associated with aging. Others are congenital or secondary to lesions in other organs. Although many of these findings can be spontaneous, incidental, age-related or background lesions in various species, the impact of test article-related effects on incidence and/or severity should always be evaluated to determine adversity (Kerlin et al. 2016).

Congenital lesions of the spleen are generally uncommon in mice and rats, but may be seen in up to 5% of F344 rats (Losco 1992). As congenital lesions, they are typically 1–4 mm in diameter, and are generally located in the gastrosplenic ligament, tail of the pancreas, or the splenic hilus (Losco 1992). An accessory spleen can also be caused by trauma or injury, in which case, they can occur anywhere in the abdominal cavity (Hobbie et al. 2015; Losco 1992). Accessory spleen is characterized as one or more nodules of splenic tissue in the mesentery or other parts of the abdominal cavity. Though histologically normal splenic tissue, one or more of the splenic compartments may be absent (Hobbie et al. 2015). Accessory spleen, also known as heterotopic spleen or splenosis, is common in some colonies of laboratory rabbits.

The development of T cell-dependent regions of the lymphoid system (i.e. the PALS in the spleen) peaks at puberty, so these areas tend to be larger and more cellular in younger animals. The size of these areas declines beginning around 11 months of age in rats (Losco 1992). Thus, atrophy of the PALS and follicles (lymphoid atrophy) is a normal aging change, so comparison to age- and gender-matched controls is important to identify atrophic changes that are related to test article exposure. Atrophy of these regions can cause the marginal zones to appear larger, though

in aged rats, there may be atrophy of the marginal zones as well (Stefanski et al. 1990). White pulp atrophy has many causes, including irradiation, immunotoxic drugs or chemicals, and viral infections, which may cause apoptosis of lymphocytes (Elmore 2006; Elmore 2007). Apoptotic lymphocytes have small or fragmented, hyperchromatic nuclei, and there is typically a concurrent increase in the number of tingible body macrophages. Lymphocyte apoptosis can be a normal event found in control animals, but if the incidence is higher in animals receiving a test article, an attempt must be made to determine whether apoptosis represents a potential direct effect affecting impacting lymphocyte viability or study-related stress resulting in increased lymphocyte apoptosis (Everds et al. 2013). The red pulp may also be atrophic, though this is not as common as lymphoid atrophy (Stefanski et al. 1990). Red pulp atrophy is often the result of decreased extramedulary hematopoiesis (EMH) or blood in the sinusoids, and is often seen in animals with decreased body weight gain (Stefanski et al. 1990).

Hyperplasia of splenic white pulp can increase in incidence with age and coalescing of enlarged follicles may be seen (Taylor 2012). Hyperplasia of the white pulp may also occur as a treatment effect. In aged animals, this may be associated with early T cell lymphoma (Losco 1992). Hyperplasia of the B cell regions may be associated with an immune response, such as that seen with bacterial or viral infections. Rats with mononuclear cell leukemia may have increased numbers of cells in the marginal zone due to due to infiltration of this region by neoplastic cells. There can also be increased numbers of cells in the follicles or marginal zones of aging rats and mice with no apparent cause (Elmore 2006). Marginal zones may appear larger and more cellular due to increases in the number of cells such as plasma cells, histiocytes, or stromal cells (Losco 1992).

There are also age-related changes in the red pulp. EMH is normally seen in young mice and rats, especially females (Suttie 2006). There may be production of erythroid cells, myeloid cells, megakaryocytes, or any combination of these in the red pulp sinuses. With age, the number of hematopoietic cells in the red pulp decreases, particularly in rats, but some EMH is typically present. EMH can increase when there is a physiologic need for red or white blood cells. Splenic erythropoiesis can increase with anemia, which may be caused by blood loss (e.g., hemorrhage or excessive blood collection) or intra- or extravascular hemolysis, for example. Likewise, myelopoiesis can increase with bacterial or viral infections. In fact, with severe bacterial infections, such as pyometra, there can be such a marked increase in myelopoiesis that it may be difficult to distinguish from myeloid neoplasia.

Immunocompromised rodent models are commonly used in drug discovery and less commonly in later phases of drug development. Depending on the immune alterations in these animals, functional alterations in splenic immune function can readily be determined. However, in routine pathological evaluation, alterations are predominantly noted only in genetically engineered rodent models that specifically lack large components of the immune system (e.g., SCID; severe combined immunodeficiency and Nude rodents) that comprise the spleen. In these animals, there are specific alterations in splenic architecture that can be noted. Specifically, since

SCID mice lack mature B and T cells due to defects in V(D)J recombination, spleens of SCID mice are smaller than wild-type mice and all three regions of the white pulp contain fewer lymphocytes but do have macrophages (Cesta 2006; Perryman 2004). Nude rats are congenitally athymic and are deficient in T cells which results in sparsely populated PALS regions (Cesta 2006). Additionally, since T cell activity is required for the formation of germinal centers, these animals also lack secondary follicles (Cesta 2006).

In non-human primates, the most common background or spontaneous lesions are focal (nodular) lymphoid follicular hyperplasia, capsular fibrosis and germinal centers with accumulations of brightly eosinophilic amorphous material (hyalinization) with or without Russell body formation (Chamanza et al. 2010; Sato et al. 2012). Granulocyte infiltration, pigment deposition and capsular hemorrhage/thrombi are less common (Chamanza et al. 2010; Sato et al. 2012). In dogs, accessory spleens and hemosiderotic plaques along the splenic margin may be present and, like non-human primates, hyalinized material may be seen in the follicles of the spleen (Scudamore 2012). Minipigs commonly have elliposoids which are concentrically arranged around capillaries or small arterioles and consist of phagocytic cells and reticular fibers, referred to as Schweigger-Seidel sheaths (McInnes 2012a). In rabbits, a low incidence of increased EMH in accessory spleens has been reported (Bradley 2012).

Neoplastic changes always warrant careful evaluation. They may be spontaneous, especially in chronic studies, but can complicate histopathologic evaluation of the spleen and when present, are often the cause of increased splenic weight. Types of neoplasms found in the spleen include mononuclear cell leukemia (MCL), malignant lymphoma (multiple T-cell and B-cell types), hemangioma or hemangiosarcoma, histiocytic sarcoma, mast cell tumor and mesenchymal neoplasms (e.g., fibromas/fibrosarcomas, leiomomas, leiomyosarcomas), and others. Some neoplasms can be age or strain-related such as MCL in the Fischer 344 rat (Thomas et al. 2007).

Most splenic neoplasms are relatively uncommon. However, MCL is common in F344 and Wistar-Furth rats (Losco 1992). Though uncommon, it has also been reported in the Sprague-Dawley and conventional Wistar strains (Losco 1992). MCL is also known as large granular lymphocytic (LGL) leukemia because the large granular lymphocyte is thought to be the cell of origin of this neoplasm. MCL is rare in control rats less than 20 months of age, and is more common in males than females (Losco 1992; Stefanski et al. 1990). It is thought to arise in the spleen because the spleen is involved in all cases, but neoplastic cells are commonly found in the liver, lung, lymph nodes, adrenal glands, and kidneys (Stefanski et al. 1990). The spleen is almost always enlarged in advanced stages of the disease, but in early stages, the spleen may not be enlarged. The neoplastic cells are most commonly found in the red pulp and marginal zones (Losco 1992). They are generally pleomorphic cells with round, pale to densely basophilic nuclei, and small amounts of cytoplasm that may have an eosinophilic granular appearance (Losco 1992; Stefanski et al. 1990). Decreased numbers of lymphocytes in the PALS, splenic congestion, decreased EMH and hemosiderin deposits, and erythrophagocytosis by the neoplastic cells are often seen with MCL (Losco 1992; Stefanski et al. 1990).

2.2 Summary

The spleen is a critical immune organ that is considered an essential component to evaluating immune responses and immunotoxicity in drug development. A thorough evaluation of splenic changes including splenic weight or size, color, microscopic changes, and clinical pathologic abnormalities is essential to understand alterations that occur to the spleen during drug development. However, changes noted within the spleen should not be evaluated within a vacuum. Rather, an integrated picture should be developed where the changes within the spleen are considered in conjunction with gross and microscopic changes in other immune organs (e.g., thymus, bone marrow, lymph nodes), pathologic parameters (e.g., clinical pathology parameters) and immunological parameters from flow cytometry of immune functional assays. It is through the integration of these parameters that an overall picture of the immune status and immune effects of a potential new therapy can be fully understood.

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Chapter 3
Lymph Node

Susan A. Elmore and Schantel A. Bouknight

Abstract Lymph nodes are essential for the initiation of the adaptive immune response as they create an environment that filters antigens and pathogens and provide a site for antigen presentation to lymphocytes. Immunologically, responses to the various antigens can be reflected in lymph node histology. These responses can be proliferative or nonproliferative and can be incidental. Species, strain, age of animal, health status, and the type and route of administration of a test article should always be a consideration when evaluating the lymph node. Understanding the physiology and histomorphologic features of the lymph node and alterations of normal lymph node histology are critical to differentiate lesions that occur naturally during development and aging from those that may be treatment related.

Keywords Lymph node structure • Lymph node lobule • Lymph node stromal cells • Lymph node histopathology • Lymph node development

3.1 Lymph Node Development

Lymph node formation is tightly linked to the development of the lymphatic system and in particular the lymph sacs. Lymph sacs are the earliest developments of the lymphatic vasculature that appear around embryonic day (E) 10.5 by budding of endothelial cells that originate from the cardinal vein in mice (Kulkarni et al. 2009, Cupedo and Mebius 2005). Through lymphatic endothelial sprouting these lymph sacs give rise to the lymphatic vasculature, which is complete by E15.5 in mice (Wigle and Oliver 1999, Van de Pavert and Mebius 2014). It is within this timeframe that lymph node formation is initiated. It has been generally accepted

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that lymph nodes originate from primitive lymphatic sacs (Willard-Mack 2006, Cupedo and Mebius 2005, Bailey and Weiss 1975). However, recent studies in mouse embryos that lacked lymphatic vasculature by eliminating Prox-1, demonstrated that lymph sacs are not necessary for the initial formation of the mammalian lymph node anlagen (Vondenhoff et al. 2009). Many insights into the mechanism underlying lymph node formation have come from the analysis of various gene-deficient mice that lack some or all lymph nodes. From these studies, we now understand that lymph node formation requires a coordinated interaction between mesenchymal lymphoid tissue organizer cells (LTo) and hematopoietic lymphoid tissue inducer cells (LTi)(Van De Pavert and Mebius 2010). The interaction of these two cell subsets results in the upregulation of adhesion molecules (vascular adhesion molecule 1 (VCAM1), intercellular adhesion molecule 1 (ICAM1), mucosal vascular adressin cell adhesion molecule 1 (MADCAM1) and homeostatic chemokines [CC-chemokine ligand 19 (CCL19, CCL21 and CXC-chemokine ligand 13 (CXCL13)] which are needed for the attraction and retention of additional hematopoietic cells at the site of developing lymph nodes (Van De Pavert and Mebius 2010).

3.2 Lymph Node Structure and Function

The structure of the lymph node (LN) is critical to its function, as it funnels antigens and antigen-presenting cells towards antigen specific lymphocytes to initiate an adaptive response. Lymph nodes are encapsulated bean-shaped secondary lymphoid organs along the course of lymphatic vessels. Each lymph node is covered by dense connective tissue, the capsule, which extends into the parenchyma to form a number of trabeculae. The lymph node is divided into lobules which are the basic anatomical and functional units of the node (Willard-Mack 2006). Lymphoid nodules are arranged side by side and radiate toward the hilus. The smallest lymph node may contain a few lobules while larger nodes may contain many. Each lobule is subdivided into three main regions: the cortex which contains a more superficial B cell area that is composed of primary follicles and (after antigen stimulation) germinal centers, the deep cortical unit or paracortex (the T-cell rich area) and the medulla. B cell follicles are the primary site of humoral responses whereas the paracortex is the site where circulating lymphocytes enter the LNs and where T cells interact with dendritic cells (DCs). The medulla is a labyrinth of lymph-draining sinuses that are separated by medullary cords, which contain plasma cells, macrophages and T memory cells. Each lobule is surrounded by a system of lymphatic sinuses that are divided into subcapsular, intermediate and medullary sinuses (Ohtani and Ohtani 2008). Afferent lymphatic vessels deliver lymph, which contains tissue-derived antigens and immune cells (including DCs), to the subcapsular sinus (SCS)—a space below the collagen rich fibrous capsule that covers the lymph node. From here, lymph is channeled through the medullary sinuses and blind-ended cortical

sinuses, before leaving the lymph node via the efferent vessels located in the hilum (Girard et al. 2012, Von Andrian and Mempel 2003).

3.2.1 The Parenchyma (Lymphocytes)

Lymphocytes, the parenchymal cells of the lobules, dominate the histological appearance of the lymph node (Willard-Mack 2006). Subsets of lymphocytes include T cells, which participate in cytotoxic adaptive immunity, B cells, which participate in humoral or antibody-driven adaptive immunity, and natural killer (NK) cells that are involved in cell-mediated cytotoxic innate immunity. They are all derived from the same multipotent hematopoietic stem cell and are morphologically indistinguishable from each other until activated. T cells mature and are derived from the thymus whereas B cells are derived from the bone marrow. NK cells differentiate and mature in the bone marrow, lymph nodes, spleen, tonsils and thymus.

Both T and B cells are involved in the adaptive immune response, designed to eliminate or prevent pathogen growth. Immunological memory occurs after an initial response to a specific pathogen, leading to an enhanced response after subsequent encounters with the same pathogen. The adaptive immune response can provide long-lasting protection. The innate immune system is also called the nonspecific immune system because, unlike adaptive immunity, it recognizes and responds to foreign antigens in a generic way. Also, it does not confer long-lasting or protective immunity to the host. Natural killer cells are the effectors in this type of immune response. Also unlike adaptive immunity, NK cells can recognize stressed cells in the absence of antibodies and major histocompatibility complex (MHC). This allows for a much faster immune response.

Although the innate immune response is a good first line of defense, the adaptive immune response is needed in order to mount a much more sophisticated and long-lasting response. Once a foreign antigen is encountered, B cells are activated to secrete antibodies that travel through the blood to bind and inactivate the foreign antigen. Somatic, or V(D)J, recombination occurs in the developing B and T lymphocytes and thus allows for a highly diverse repertoire of antibodies and T cell receptors. This process allows for a very small number of genes to generate a vast number of different antigen receptors that are uniquely expressed on each individual lymphocyte. Thus the process is highly adaptable and efficient.

There are several subsets of T cells. The CD8+ lymphocytes, also called cytotoxic T cells, induce the death of cells that are infected, damaged or dysfunctional. Naïve cytotoxic T cells are activated when their T cell receptor has a strong interaction with a peptide-bound MHC class I molecule. Once activated, clonal selection occurs, allowing the T cells to gain functions and rapidly divide to produce many effector cells. The CD4+ cells, also called helper T cells, are immune response mediators. They have no cytotoxic or phagocytic activity but they direct other cells to perform these tasks. The T cell receptors on

helper T cells recognize antigen bound to Class II MHC molecules. Once activated, cytokines are released that influence other cell types, including antigen-presenting cells. Memory T cells are a subset of antigen-specific T cells that can, upon re-exposure to a cognate antigen, quickly produce large numbers of effector T cells. Suppressor T cells ($CD4^+$Treg) are involved in the maintenance of immunological tolerance. The two main types are $FOXP3^+T_{reg}$ and $FOXP3^-T_{reg}$. The main function of these cells is to suppress autoreactive T cells that have escaped the process of thymic negative selection. Natural killer T cells (NKT) are a heterogeneous group of T cells that have properties of both T cells and natural killer cells. These cells recognize glycolipid antigen presented by the CD1d molecule and, once activated, can perform functions similar to both cytotoxic T cells and T helper cells. Gamma delta T cells are a third type of T cell that shares characteristics of cytotoxic T cells, T helper cells and NK cells. As in adaptive immunity, they can rearrange their TCR genes via V(D)J recombination and thus develop a memory phenotype. They also have functions similar to innate immunity where a restricted TCR or NK receptor may be used as a pattern recognition receptor.

3.2.2 Dendritic Cells

Dendritic cells (DCs) are potent antigen presenting cells (APCs) that are derived from hematopoietic stem cells in the bone marrow. Constitutively distributed throughout most tissues of the body in small numbers, DCs are particularly localized to sites that comprise the body's external barrier, for example the skin and mucosal surfaces (Cavanagh and Von Andrian 2002, Willard-Mack 2006). Within the peripheral tissues, DCs exist in an immature form where they sample their environment to collect and process antigens to present to T cells within lymph nodes (Banchereau and Steinman 1998, Cavanagh and Von Andrian 2002, Willard-Mack 2006). Once activated, antigen-laden DCs undergo maturation into competent APCs, bearing high levels of proteins, MHC and costimulatory molecules that drive T cell activation (Randolph et al. 2005, Banchereau and Steinman 1998). DCs are then mobilized from peripheral tissues to the LNs via the afferent lymph, translocate through the subcapsular sinus and home to the LN paracortex where they present antigen to T cells (Willard-Mack 2006, Miyasaka and Tanaka 2004).

3.2.3 Stromal Cells

Lymph node stromal cells (LNSC) constitute a heterogeneous population of non-hematopoietic cells of mesenchymal and endothelial origins and have long been appreciated to provide a scaffold on which immune cells encounter antigen (Willard-Mack 2006, Hirosue and Dubrot 2015, Fletcher et al. 2015, Chang and Turley 2015, Bajenoff et al. 2006). Recent studies have elucidated that these cells are active in the immune response and are essential regulators of immune cell trafficking, fluid flow, and LN homeostasis (Chang and Turley 2015, Hirosue and Dubrot 2015, Fletcher

et al. 2015, Bajenoff et al. 2006). These lymph node stromal cells can be divided into six sub-populations which are known by their expression of surface markers. These include fibroblastic reticular cells (FRCs: $CD31^-$, $gp38^+$), blood endothelial cells (BECs: $CD31^+$, $gp38^-$) that form high endothelial venules (HEVs), lymphatic endothelial cells (LECs: $CD31^+$, $gp38^+$) that form the lymphatic vascular network, follicular dendritic cells (FDCs: $CD31^-$, $CD21^+$, $CD35^+$, FDC-$M1^+$), integrin α7 pericytes (IAP: $CD31^-$, $GP38^-$, $ITGA7^+$) and a less studied double negative population (DN: $CD31^-$, $gp38^-$) (Hirosue and Dubrot 2015). FRCs comprise 20–50% of the non-hematopoietic compartment of lymph nodes (Fletcher et al. 2015). These stromal cells are immunologically specialized myofibroblasts of mesenchymal origin that can be distinguished from other lymph node resident cells by their expression of podoplanin (PDPN), smooth muscle actin (SMA) and platelet-derived growth factor receptor-α (PDGFRα), and their lack of expression of CD1 and CD45 (Willard-Mack 2006, Junt et al. 2008, Fletcher et al. 2015). They form cell to cell contacts to create a three dimensional open network on which leukocytes migrate. FRCs produce and wrap around parallel reticular fibers that extend from the SCS floor throughout the entire T cell-rich area to form a web of tubes or a conduit network which rapidly transports lymph and associated low molecular weight molecules, such as chemokines, from the SCS towards the HEVs (Junt et al. 2008, Fletcher et al. 2015). In addition to forming the conduit system for fluid transport, FRCs also regulate T cell migration and survival by the production of CCL19 and CCL21-ser, and can directly present antigens that are potentially acquired through FRC conduits (Junt et al. 2008).

The majority of naïve lymphocytes enter LNs from the blood and reach the specific T cell zones through specialized HEVs. HEVs are anatomically distinct postcapillary venules found in lymph nodes and other secondary lymphoid organs (with the exception of the spleen) (Girard et al. 2012, Willard-Mack 2006, Miyasaka and Tanaka 2004). Morphologically, HEV's can be distinguished readily from normal venules by the presence of tall and plump endothelial cells that bulge into the vascular lumen, a thick basal lamina and a prominent perivascular sheath or perivenular channel (Kraal and Mebius 1997, Miyasaka and Tanaka 2004, Girard et al. 2012). HEVs are located mainly in the T-cell zones, such as the paracortical areas of the lymph nodes and the intrafollicular areas of the Peyer's patches, but some are also located in the B-cell zones, particularly in the periphery of the B-cell follicles (Miyasaka and Tanaka 2004). Naïve B and T cells migrate through lymph node HEVs via a multistep adhesion cascade of molecularly distinct signaling events which involves rolling and tethering, sticking, crawling and transmigration (Girard et al. 2012, Von Andrian and Mempel 2003). These steps have been extensively reviewed (Girard et al. 2012, Miyasaka and Tanaka 2004, Von Andrian and Mempel 2003) and are briefly described here. The interaction of lymphocytes with the endothelium of HEV's is initiated by lymphocyte homing receptor L-selectin (CD62L) which mediates the tethering and rolling of lymphocytes along HEV walls (Miyasaka and Tanaka 2004, Von Andrian and Mempel 2003, Girard et al. 2012). L-lectins recognize a family of sulphated, fucosylated and sialylated glycoproteins (6 sulpho sialyl Lewis X motifs decorating O-glycans and N-glycans from HEV sialomcins) (Girard et al. 2012, Miyasaka and Tanaka 2004, Von Andrian and Mempel 2003).

Subsequently, rolling T lymphocytes are induced to arrest (integrin-dependent sticking) by chemokine activation that is mediated by CC-chemokine ligand 21 (CCL21; also known as SLC, TCA4, exodus 2 or 6Ckine). CCL21 is constitutively expressed by high endothelial cells or by chemokines produced by lymph node stromal cells (FRCs and FDCs) and transcytosed to the luminal surface of the HEV endothelial cells (Girard et al. 2012). Signaling through the G protein-coupled receptor CC-chemokine receptor 7 (CCR7), collectively with the shear force of blood flow, induces conformational changes in the lymphocyte integrin known as lymphocyte function-associated antigen 1 (LFA), which mediates firm binding with intercellular adhesion molecule 1 (ICAM1) and ICAM2 on endothelium (Girard et al. 2012). Lastly, lymphocytes rapidly transmigrate between adjacent endothelial cells and penetrate the underlying basement membrane to gain access to the extravascular tissue (Girard et al. 2012, Miyasaka and Tanaka 2004). PECAM is required for this final emigration of leukocytes from vessels (Muller 1993).

The lymphatic vascular network ensures the transport of antigens from peripheral tissues to LNs. Within LNs, LECs line subcapsular, medullary and cortical sinuses which make them well situated to interact directly with lymph borne antigens as well as recirculating T cells and LN-resident DCs. As mentioned above, naïve T cells traffic through the lymph node via a cascade of molecular events. These signals also provide cues for T cell retention but are countered by binding of sphingosine-1 phosphate (S1P) to S1P1 receptor 1, a receptor on LECs, which triggers T cell egress and migration of lymphocytes into cortical sinuses. Within cortical sinuses, fluid flow also promotes egress of T cells into efferent lymphatic vessels(Card et al. 2014). However, if naïve T cells become activated while in the LN, S1PR1 is down-regulated, promoting retention of differentiating T cells in the lymph node.

Follicular dendritic cells (FDCs) are a unique population of cells that are centrally located within B cell follicles of secondary lymphoid tissues (Willard-Mack 2006, Szakal and Tew 1992, Heesters et al. 2014). Unlike DCs, FDCs are not derived from bone marrow hematopoietic cells but develop from perivascular precursors of stromal cell origin that are seeded throughout the body (Heesters et al. 2014). FDCs have a unique ability to retain antigen for an extended period of time and are crucial for the selection of B cells that produce high-affinity antibodies (Heesters et al. 2014, Willard-Mack 2006).

3.3 Factors Affecting Lymph Node Morphology

Although each lymph node has the same basic architecture, histomorphology may vary among species, strain, location, age and health status of animal from which the nodes are collected. In comparison to other species, mice have relatively few identifiable lymph nodes (approximately 22) organized into low numbers of chains while larger animals have more numerous lymph nodes (humans have approximately 450) organized into more complex chains that drain proportionally smaller areas of tissue (Haley 2003, Willard-Mack 2006). In swine, a species increasingly used in toxicology studies, the cortex and medullary components are reversed. The lymphoid follicles and

the cortical areas are located centrally within the lymph node and the medullary sinuses and cords are located peripherally but may penetrate irregularly into the center or occupy the majority of one pole of the node. In pigs, lymph usually flows into the node centrally and leaves the node through efferent vessels located on the capsular surface (Haley 2003). The vascular supply in pigs also differs from that of other animals. Major nodal arteries subdivide into branches which envelop the capsular network which then further branch to penetrate the lymph node parenchyma (Mcinnes 2012).

Anastomosis of afferent lymphatic vessels within lymph node chains differs in large species when compared to smaller species. The lack of lymphatic anastomoses in the rat results in translocation of small amounts of material to portions of the node rather than the entire lymph node. Because extensive anastomosis of lymphatic channels is present in larger species, their lymph nodes demonstrate a more uniform drainage pattern that results in simultaneous antigen exposure throughout the node. Thus, it is important to note that the degree and distribution of xenobiotic-induced change may be the result of differences in lymphatic anastomoses (Haley 2003).

Important lymph node variations exist among different regions of the body. For example, the mesenteric and mandibular lymph nodes drain areas that are continuously exposed to antigens from the environment and therefore are generally in an active state and contain well-developed secondary follicles with large reactive germinal centers. While lymph nodes such as the popliteal and axillary lymph nodes drain areas that are not normally exposed to antigen on a frequent basis and therefore display the classic architecture of a resting node with small numbers of primary follicles and few to no secondary follicles. Aging effects, such as lymphoid atrophy, lipid accumulation, sinus histiocytosis and fibrosis may be profound in the lymph nodes in some species. A decrease in the number of germinal centers has been described in aged Brown Norway and Wistar rats as well as an increase in plasma cells in the medullary sinuses (Greaves 2007). Atrophy of the paracortex, germinal centers and medullary cords have been described in aging CD-1, C3H, B10A and (B10AX A/J) F1 mice (Greaves 2007). In addition to a decreased cellularity of germinal centers, dog and primate lymph nodes may also have mild thickening of the capsule and medullary trabeculae with aging (Maxie et al. 2007).

Genetic mutations in mice and rats, spontaneous or engineered, resulting in immunodeficiency markedly affects the morphology of the lymph node. Most notably among immunodeficient strains is the nude mouse, SCID (severe combined immunodeficiency disease) and RAG (RAG-1 and RAG-2) (recombination activating gene)) mouse models. Nude mice are congenitally athymic therefore, deficient in T-lymphocytes. The lymph nodes are smaller than their wild type cohorts. They have sparsely populated cortical lymphocytes. SCID mice are homozygous for the *Prkdc*^{scid} (protein kinase, DNA activated, catalytic polypeptide) mutation, a protein necessary for joining non-homologous ends of double-stranded DNA. This results in defect in V(D)J recombination of T-cell and B-cell immunoglobulin receptors and a lack of mature B-and T- cells (Perryman 2004). The lymph nodes of SCID mice are smaller than those of wild-type mice. Lymphocytes are generally sparse while macrophages are prominent in sinuses and medullary cords (Custer et al. 1985). Mice with targeted mutations of *Rag1* or *Rag2* have a phenotype similar to

that of the *Prkdc*^{scid} mouse; *Rag1* and *Rag2* null mice have B- and T-cell development arrested at an early stage (Seymour et al. 2006, Shinkai et al. 1992).

The health status of laboratory animals may affect lymph node morphology. It is imperative that the toxicologic pathologist is aware of the numerous pathogens, their biology and how they are expressed in species used in toxicity studies. For example, demodex mites, which are commonly seen on canine hair follicles, may rapidly proliferate under immunosuppressive conditions. Demodectic mite granulomas may be found in lymph nodes (particularly the mandibular nodes) of normal dogs, attesting to their background presence and large numbers may translocate to regional draining lymph nodes under conditions of uncontrollable mange (Haley 2003, Haley 2012).

3.4 Histopathology of the Lymph Node

3.4.1 Lymphoid Necrosis

Lymphoid necrosis can be multifocal or diffuse and is characterized by cell swelling with disruption of cellular membranes, chromatin clumping, karyorrhexis or the nucleus, karyolysis and occasionally eosinophilic cellular debris (Fig. 3.1). Necrosis is often accompanied by inflammatory cells including neutrophils and macrophages. Necrosis should be distinguished from apoptosis which plays a critical role in lymphocyte development and homeostasis. Apoptosis generally involves single cells or small clusters of cells, cell shrinkage, nuclear pyknosis, and fragmentation with apoptotic bodies and tangible body macrophages. With apoptosis, the cell membranes remain intact; the cytoplasm is retained within apoptotic bodies so proinflammatory cells are not released into the surrounding tissue. Therefore, there is generally no inflammation.

Lymph node atrophy, or depletion, is observed as sequelae to chronic necrosis or apoptosis. A number of immuosuppressive drugs, cytokines and biological immune

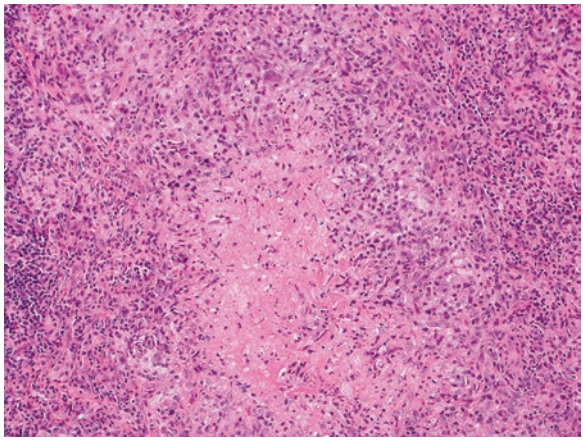

Fig. 3.1 Focal necrosis in the mediastinal lymph node from a Harlan Sprague Dawley rat on a subchronic study treated with abrasive blasting agents (blasting sand). There is a central area of necrosis in the paracortex surrounded by histiocytes and fibrosis. H&E stain, objective magnification 25×

modulators have been shown to produce lymphoid depletion or atrophy in different compartments in preclinical studies (Greaves 2007). The particular compartment of the lymph node affected and the nature of the changes are dependent on the type of agent, dose, timing and duration of administration. Microscopically, lymphocyte depletion is characterized by a decrease in number and size of follicles with few to no germinal centers and/or depletion of paracortical lymphocytes. With depletion of paracortical lymphocytes, the stromal cells of the lymph node are more prominent. Atrophy, particularly in aged rodents, may be accompanied with an increase in adipocytes (lipomatosis) in the hilus region or medulla (Ward et al. 1999, Mcinnes 2012).

3.4.2 Vascular Lesions

In the lymph node, angiectasis is characterized by the dilation and congestion of thin veins within the cortex, medulla, capsule, hilus or surrounding connective tissue (Elmore 2006). Angiectasis is most commonly observed in the mesenteric lymph nodes of rats and mice, including the B6C3F1 mouse and may and may not be accompanied by hemorrhage (Ward et al. 1999). This lesion can be distinguished from a hematoma by the presence of endothelial lining cells, early hemangioma by the absence of large neoplastic endothelial cells and angiomatous hyperplasia by the absence of supporting vascular stroma.

Sinus erythrocytosis, characterized by the presence of free erythrocytes within lymphatic sinuses, can result from a lymph node draining an area of hemorrhage. This finding could also be artefactual and related to euthanasia or handling at procedures at necropsy. Depending on the duration of the inciting lesion, hemosiderin-laden macrophages, erythrophagocytosis and inflammatory cells may be present.

Angiomatous hyperplasia is a non-neoplastic proliferation of capillaries, other vascular structures and varying quantities of fibrovascular stroma (Fig. 3.2). Angiomatous hyperplasia is most often seen in the mesenteric lymph nodes of rats and mice including the CD-1 (Crl: CD-1(ICR) BR) and Crl:WI[Han] rat strains (Bradley et al. 2012, Elmore et al. 2015). The uniform vascular spaces are generally blood filled and endothelial cells are normal-appearing with no mitotic figures. Controversy has existed over how to morphologically classify these angiomatous proliferations as high grade angiomatous hyperplasia can be difficult to differentiate from hemangiomas and hemangiosarcomas (Elmore et al. 2015).

3.4.3 Lymphatic Sinus Ectasia

Lymphatic sinus ectasia (lymphangiectasia, lymphatic cysts, sinus dilation) can involve the medullary and subcapsular sinuses and is typically associated with lymphoid atrophy. It is commonly found in the mesenteric lymph nodes of aged rats and mice but can occur spontaneously in immunodeficient mouse and rat strains with no

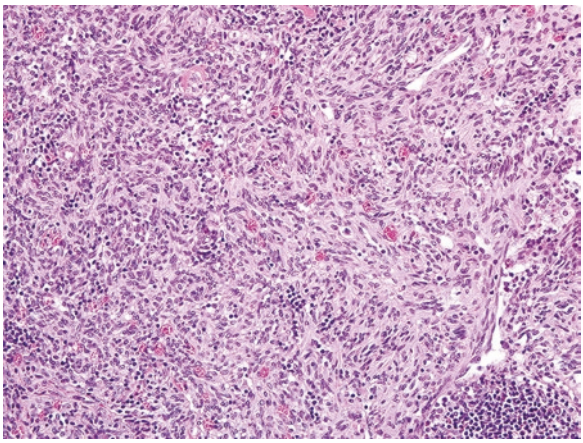

Fig. 3.2 Angiomatous hyperplasia in the mesenteric lymph node of a Wistar Han rat from a 2-year toxicity/carcinogenicity bioassay. Angiomatous hyperplasia is characterized by a focally extensive nonneoplastic change composed of increased number of proliferating endothelial cells and blood-filled vessels and spaces supported by varying quantities of fibrovascular stroma. The endothelial cells lack cellular atypia. H&E stain, objective magnification 20×

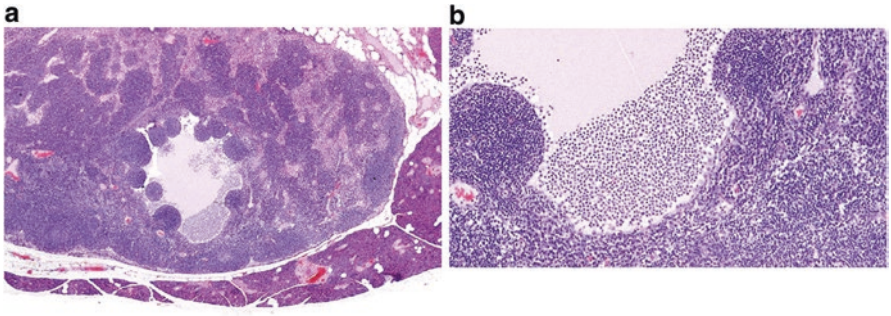

Fig. 3.3 Sinus ectasia from a control 2 year old male F344/N. The low magnification shows a large ectatic medullary sinus (**a**). At higher magnification, the ectatic sinus is lined by plump endothelial cells and contains pale, slightly eosinophilic lacy material admixed with lymphocytes and plasma cells (**b**). H&E stain, objective magnification 8× (**a**) and 40× (**b**)

node predilection (Frith et al. 2000, Sainte-Marie et al. 1997). Dilated sinuses are lined by endothelium and can contain pale eosinophilic/amphophilic material (presumably lymph) (Fig. 3.3a, b). Low numbers of lymphocytes, plasma cells and macrophages can be found admixed with the lymph. Ectasia is probably related to the obstruction of efferent lymph vessels (Mcinnes 2012).

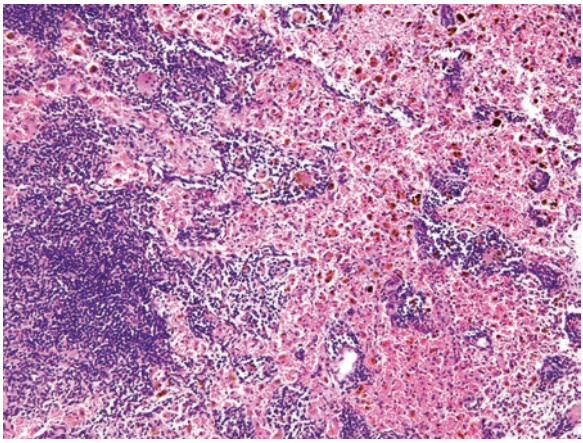

Fig. 3.4 Hemosiderin pigment in the mesenteric lymph node from a Sprague Dawley rat treated in a subchronic study with 3,3,4,4-tetrachloroazobenzene (TCAB). The expanded medullary sinuses contain erythrocytes and macrophages with intracytoplasmic hemosiderin and there is atrophy of the medullary cords. H&E stain, objective magnification 16×

3.4.4 Pigment

Pigment is a common finding in the cytoplasm of control and treated animals. Pigment is most commonly seen in mesenteric lymph nodes, however, can be present in the bronchial and mandibular lymph nodes to a lesser extent. The most common pigments are hemosiderin and ceroid/lipofuscin. Hemosiderin is an iron-containing golden brown, granular material that is most commonly found within medullary cords and lymphatic sinuses of nodes with sinus erythrocytosis (Fig. 3.4). Hemosiderin deposits are often visible within macrophages in the sinuses of local lymph nodes in minipigs. Widespread deposition of hemosiderin is thought to be due to the intramuscular injections of iron administered to neonatal piglets to prevent anemia (Mcinnes 2012). Lipofuscin is also characterized by a golden brown granular appearance but is primarily derived from lipid-containing residues of lysosomal digestion. Ceroid has many of the same histochemical features as lipofuscin. These pigments are difficult to distinguish from one another on routine hematoxylin and eosin stain. Lipofuscin/Ceroid can be identified by several histochemical features such as autofluorescence and staining with stains for fat such as Sudan black, oil-red-O, Periodic acid-Schiff, Ziehl-Neelsen acid fast stain and the Schmorl's reaction. Perl's iron stain or the Prussian blue reaction can be used to differentiate hemosiderin from lipofuscin/ceroid. Melanin is another pigment that can be found within lymph nodes. This pigment is a normal finding in black skinned animals such as mice and is not considered pathological. Histochemical staining of melanin is accomplished by exploiting the chemical reducing properties of this pigment. Two methods are commonly used: The Masson-Fontana silver method and Schmorl's

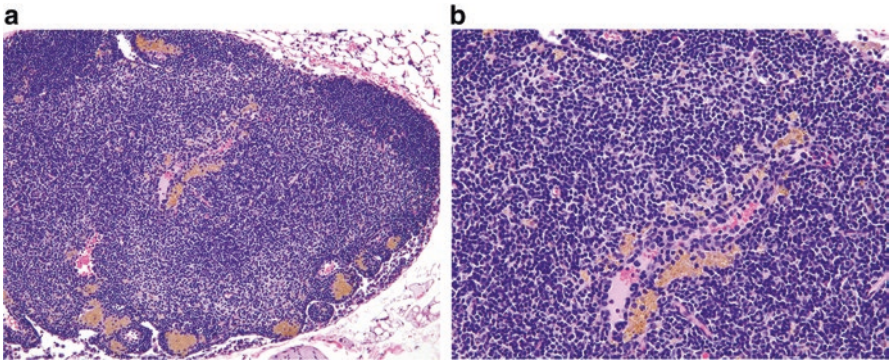

Fig. 3.5 Pigment accumulation in the bronchial lymph node of a B6C3F1 mouse treated in a subchronic study with nanoscale material (Fullerene-C60). At low magnification, there are clusters of pigment in the cortex and peripheral to the deep cortical unit (**a**). Higher magnification illustrated that the golden brown granular pigment is contained with macrophage cytoplasm. The macrophages are either scattered or in clusters (**b**). H&E stain, objective magnifications 20× (**a**) and 40× (**b**)

reaction. DOPA (3,4-dihydroxyphenylalanine)-oxidase is another enzyme histochemical method used to identify melanin.

A number of exogenous pigments may be present within macrophages of regionally draining lymph nodes due to inhalation, ingestion, injection of pigmented substances (Fig. 3.5a, b). Inhaled carbon particles may be found within macrophages of bronchial and/or mediastinal lymph nodes of laboratory non-human primates housed near or within urban areas. Microscopically, carbon presents as fine black granules and may be intra- or extracellular (within macrophages). Due to the non-reactiveness of carbon, there are no histochemical tests to differentiate it from other pigments with similar microscopic characteristics. Tattoo pigment can sometimes be observed as aggregates of scattered brown/black or green material in lymph nodes adjacent to the tattoo. An increase in pigmented macrophages can also be due to the administration of a test article. As such, regional draining lymph nodes to the administration site should be routinely examined for the presence of exogenous material (Elmore 2006). Proteinic test articles, such as biopharmaceuticals, may be present as eosinophilic granules or spicules in the soft tissue near injection sites. These tend to accumulate in macrophages within draining lymph nodes and may also be present in hepatic Kupffer cells and glomerular mesangial cells.

3.4.5 Amyloidosis

With hematoxylin and eosin stain and light microscopy, amyloid appears as an amorphous, eosinophilic, hyalinized extracellular material (Fig. 3.6). Large accumulations of amyloid can cause atrophy of adjacent tissues. Congo red is the most

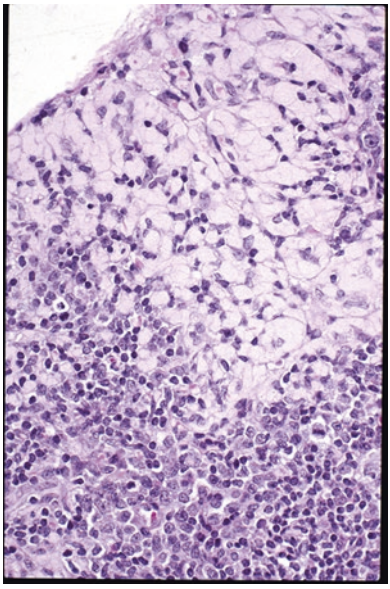

Fig. 3.6 Amyloid accumulation in the cortex of a mesenteric lymph node from a B6C3F1 mouse. The amyloid occurs as extracellular insoluble fibrous protein aggregates that appear as pale eosinophilic or amphophilic material with H&E. Image courtesy of Dr. Jerry Ward. Objective magnification 40×

common stain used to identify amyloid and examination under a polarizing microscope results in green birefringence. Amyloidosis occurs in in most laboratory animal species at a low incidence but in CD-1 mice and Syrian Hamsters, there appears to be a genetic predisposition (Mcinnes et al. 2015, Pour et al. 1979, Frith and Chandra 1991). In both species, amyloidosis is more prevalent in females and the degree of amyloidosis increases with age and is a major cause of death. In mice and hamsters, amyloid deposition occurs in a variety of tissue including the node. In CD-1 mice, the mesenteric lymph node is commonly affected and amyloid deposits primarily accumulate within the periphery of the node in the subcapsular sinuses (Frith and Chandra 1991).

3.4.6 Lymphadenitis

Inflammatory cells can be found in lymph nodes draining sites of inflammation, necrosis, neoplasia, etc. or can be the result of test article administration (irritating test compound or administration procedures). The type of inflammatory cells present depends on the inciting factor and duration (Fig. 3.7). The response can vary from acute (primarily neutrophils) to chronic (macrophages, lymphocytes, and plasma cells) and may be exacerbated in immunocompromised animals. Lymphoid hyperplasia or atrophy may also occur in association with inflammation. Inflammatory cells should be differentiated from extramedullary hematopoiesis (EMH) and granulocytic leukemia. EMH is usually characterized by a mixture of

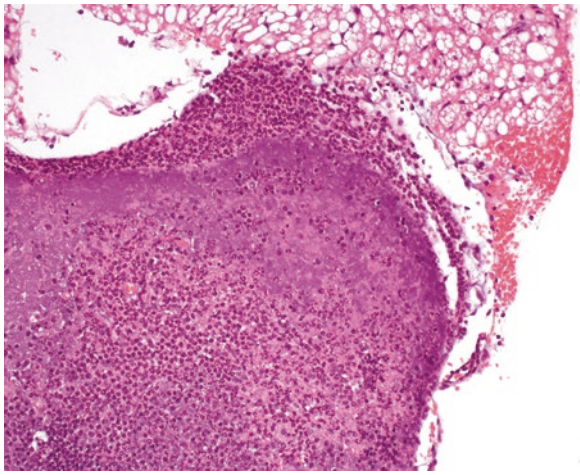

Fig. 3.7 Neutrophilic inflammation in the mandibular lymph node from Swiss Webster mouse 72 h after inhalation of *Yersinia Pestis*. The cortex and medullary sinus contain neutrophils accompanied by large numbers of coccobacilli. Image courtesy of IIT Research Institute. H&E stain, objective magnification 20×

megakaryocytes and other hematopoietic elements. Granulocytic leukemia has a high proportion of immature myeloid cells as well as multiple organ involvement. Granulomatous lymphadenitis should be reserved for lymph nodes with chronic abscesses or granulomatous inflammation as opposed to increased numbers of histiocytes within the subcapsular and medullary sinuses (sinus histiocytosis). Acute abscesses are composed of a central region of necrosis surrounded by neutrophils while chronic abscesses may be surrounded by variable amounts of granulation tissue that can progress to fibrosis. Small abscess are common in the mandibular lymph nodes of minipigs and are thought to be secondary to local irritation or inflammation (Mcinnes 2012). Translocation of microbes or parasites such as demodectic mites in dogs can result in severe destructive changes to the regional draining lymph node (particularly the mandibular nodes) including abscesses, suppurative inflammation and mite granulomas (Haley 2003).

3.4.7 Lymphocyte Hyperplasia

Lymph nodes undergo hyperplasia following a variety of stimuli and can involve different compartments including the cortex, germinal centers and medullary cords (Fig. 3.8a–c). Lymphoid hyperplasia is generally a reactive or immune response and is not considered to be a preneoplastic lesion however, diffuse lymphocyte hyperplasia may result in loss of clear delineation of node architecture and must be differentiated for lymphoma. In normal rodents, the degree of lymphocyte hyperplasia depends on the location of the lymph node, health status and age of the animal and plane of section of the node. As stated in the previous section, the mesenteric and mandibular lymph nodes drain areas that are continuously exposed to antigens from the environment and therefore are generally in an active state and contain well developed secondary follicles with large reactive germinal centers. In nonhuman primates, it is not

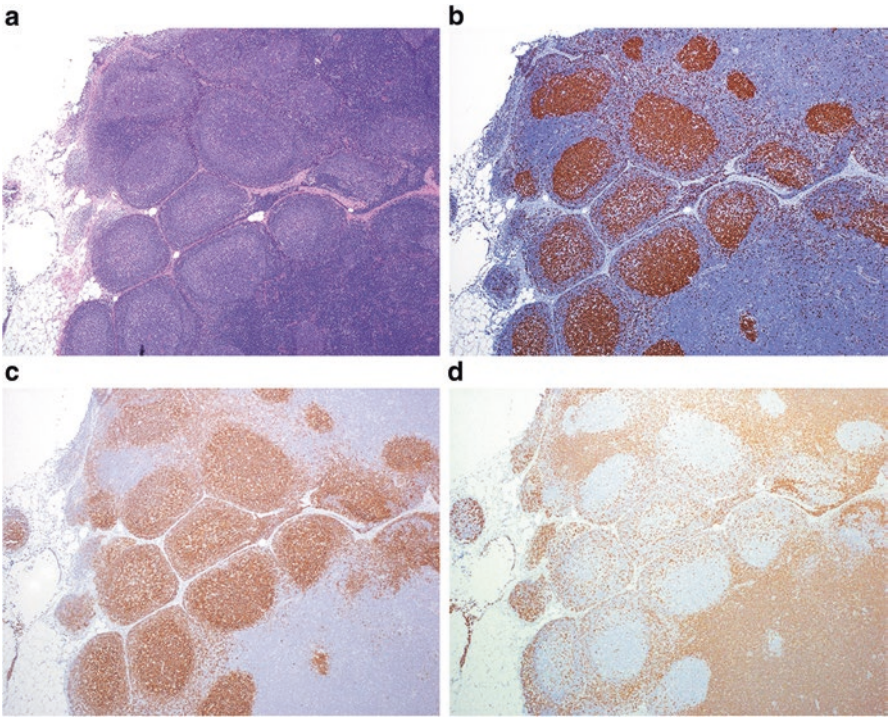

Fig. 3.8 Lymphoid hyperplasia in a mesenteric lymph node from a control Cynomolgus monkey. At low magnification, there is an increase in number and size of lymphoid follicles (**a**). Ki67, a cellular marker for proliferation, shows proliferation of lymphocytes in the germinal centers (**b**) CD20, a marker for B cells, also shows expression of B cells in the germinal centers (**c**) and CD3, a marker for T cells, shows positive cells primarily in the deep cortical unit. Images courtesy of Jennifer Chilton. H&E and Immunohistochemistry stains, objective magnification 4×

uncommon to observe lymphoid hyperplasia in various organs including the lymph node. These findings have been associated with subclinical type D retroviral infections however, since most non-human primates (NHPs) used in nonclinical research are reared in relatively disease free environments and known to be negative for type D retroviruses, the hyperplasia is considered to most likely represent a heightened nonspecific immunosurveillance (Patrick and Rebelatto 2015).

3.4.8 Plasma Cell Hyperplasia

Plasma cell hyperplasia (plasmacytosis) is a common finding in rodents particularly in the mandibular lymph nodes and is usually a response to antigenic stimulation. The primary site for plasma cell hyperplasia is the medullary cords as this is the normal site for resident plasma cells and their precursors (terminally differentiated B lymphocytes). The plasma cells that remain in the medulla tend to be short lived and only secrete antibody, such as IgA or IgG, for a few weeks. This is in contrast

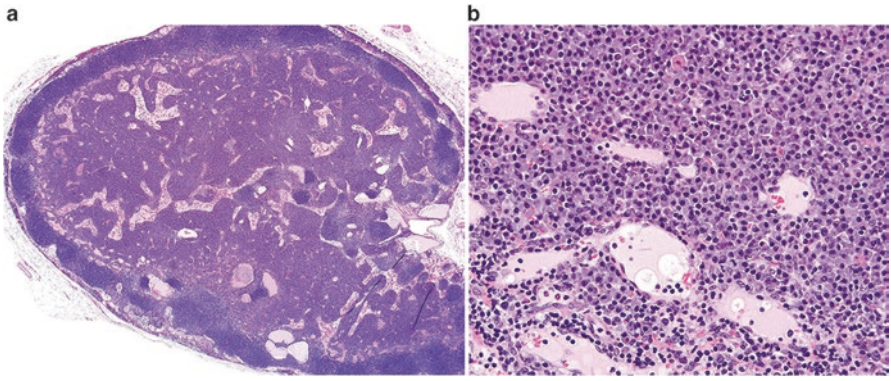

Fig. 3.9 This a mediastinal lymph node from a Wistar Han rat with marked plasma cell hyperplasia involving the medullary region. The node is greatly expanded and the normal architecture is obscured however, the cortex remains intact, there is no evidence of capsular infiltration (**a**) and plasma cells are well differentiated (**b**). Some plasma cells contain Russel's bodies (*arrow*). H&E stain, objective magnification 4× (**a**) and 40× (**b**)

to those plasma cells that migrate to the bone marrow and receive survival signals (Auner et al. 2010). In cases of marked plasma cell hyperplasia, the node may be greatly enlarged; the normal node architecture may be obscured and may be difficult to differentiate from neoplasia (Fig. 3.9a, b). Hyperplasia can be differentiated from neoplasia by the lack of cortical and capsular infiltration, metastasis and atypical plasma cells. Some plasma cells may contain Russell's bodies depending on the duration and intensity of antigenic stimulation.

3.4.9 Sinus Histiocytosis

Sinus histiocytosis is the common term used when histiocytes occur as aggregates within the subcapsular or medullary sinuses. Histiocytes have a characteristic eosinophilic cytoplasm and may contain pigment or other phagocytized material. When aggregates of histiocytes occur within the lymph node parenchyma and obscure the parenchyma, the terms granulomatous inflammation or granulomatous lymphadenitis are appropriate (Elmore 2006). When sheets or aggregates of histocytes occur sporadically in the cortical, paracortical or medullary regions, then the term "histiocytosis" is used (Fig. 3.10a, b). Sinus histiocytosis is commonly observed in rodents and the minipig lymph node. Large eosinophilic histiocytes can be seen in the lymph nodes of New Zealand White rabbits. These histiocytes may have debris within cytoplasmic vacuoles (Mcinnes 2012).

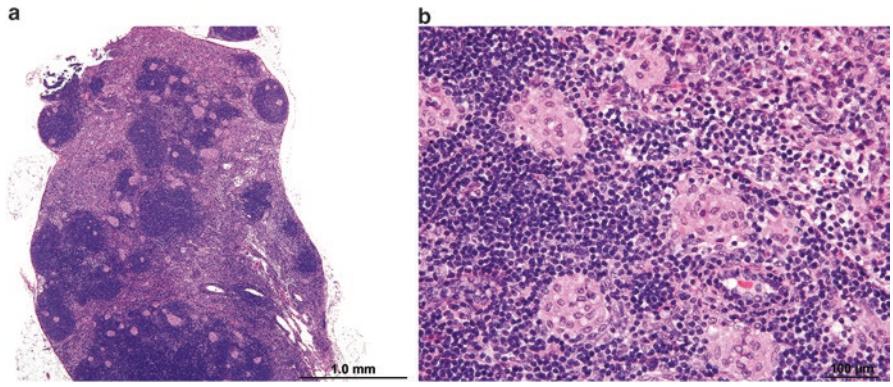

Fig. 3.10 Histiocytosis in a mesenteric lymph node from a F344/NTAC rat treated in a subchronic study with Green Tea Extract. Sheets and aggregates of macrophages are present within the cortical, paracortical and medullary regions (**a**). A higher magnification illustrates the aggregates of macrophages with abundant pale eosinophilic cytoplasm and vesicular nuclei. H&E stain, objective magnifications 4× (**a**) and 40× (**b**)

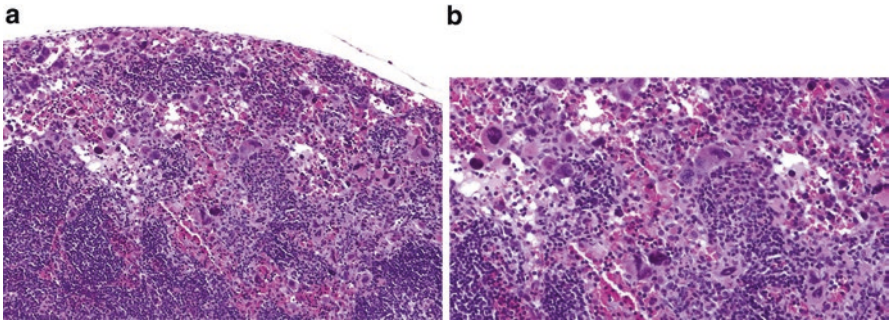

Fig. 3.11 Extramedullary hematopoiesis of a mesenteric lymph node from a control male B6C3F1 mouse. The subscapular and parcortical sinuses contain myeloid, erythroid, and megakaryocytic precursor cells (**a**, **b**). H&E stain, objective magnifications 20× (**a**) and 40× (**b**)

3.4.10 Extramedullary Hematopoiesis

Extramedullary hematopoiesis (EMH) is the production of myeloid, erythroid, and megakaryocytic precursor cells at ectopic sites. Although this process most often occurs in the spleen, it can sometimes be present in the lymph nodes of laboratory animals (Elmore 2006, Chamanza et al. 2010) (Fig. 3.11a, b). EMH is typically a physiological response to a hematoxic insult, anemia or an infection elsewhere in the body. One cell type may predominate depending on the inciting cause. For example, erythroid precursors predominate secondary to hemorrhage or erythrocyte destruction and myeloid precursors predominate secondary to inflammatory or

immunoproliferative conditions. EMH occurs frequently in marmosets in a variety of organs including lymph nodes and usually correlates with the amount and frequency of blood sampling. This spontaneous finding must be differentiated from inflammatory cell infiltrates.

3.4.11 Neoplasia

Lymphoma is the most common primary neoplasm arising in the lymph nodes in many strains/stocks of mice used in safety assessment. Mouse strains such as CD-1, C57BL/6, B6C3F1 and B6;129 have 10–15% incidences of lymphomas in advanced age. There are a variety of sub-classifications of lymphoma however, the majority of these tumors are B cell tumors of the follicular type and arise in the spleen, mesenteric lymph node and/or Peyer's patches (Ward 2006). Malignant lymphoma in the rat occurs at a low incidence with the exception of large granular lymphoma (LGL), formerly mononuclear cell leukemia (MCL), in the Fischer 344 (F344) rats and lymphoblastic leukemia in inbred SD/Cub rats (Stefanski et al. 1990, Matsushima et al. 2010). Spontaneous malignant T-cell lymphoma has been reported in young Long Evans, Wistar and Sprague Dawley rats (Matsushima et al. 2010). In the F344 rat, lymphoma must be distinguished from LGL. Lymphomas are generally characterized by monomorphic sheets of neoplastic lymphocytes. Diagnostic features of lymphoid neoplasia include an increased size of the lymph node, the loss of normal architecture, the presence of a monomorphic population of lymphocytes, and capsular and perinodal fat invasion (Elmore 2006).

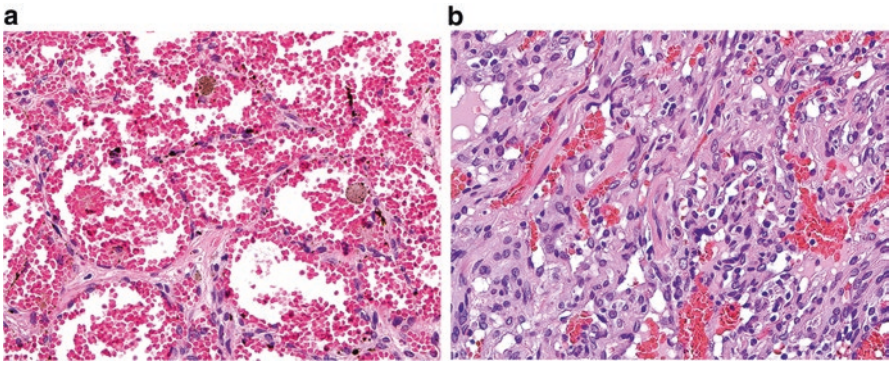

Fig. 3.12 Angiomatous tumors in the mesenteric lymph nodes of Wistar Han rats from a 2-year toxicity/carcinogenicity bioassay. High magnification of hemangioma. This lesion is characterized by a proliferation of variably sized cavernous spaces lined by a single layer of well-differentiated neoplastic cells (**a**). High magnification of hemangiosarcoma. Neoplastic cells are arranged in variably sized vascular spaces that are small, round to anastomosing, and supported by fibrovascular stroma (**b**). H&E stain, objective magnifications 40×

Other types of tumors are relatively uncommon but soft tissue neoplasms (angiomas and angiosarcomas) are occasionally found in the lymph nodes of control rats and mice. Mesenteric lymph node angiomatous tumors (hemangioma and hemangiosarcoma) are a common spontaneous lesion in aged CRL:WI[Han] rats (Deerberg et al. 1980, Reindel et al. 1992, Tucker 1997, Weber et al. 2011). General diagnostic features of hemangiomas are focal non-invasive, circumscribed nodular masses that are composed of variably sized cavernous spaces lined by a single layer of well-differentiated endothelial cells (Fig. 3.12a). Hemangiosarcomas are generally composed of increased number of endothelial cells that form haphazardly arranged anastomosing vascular channels. Endothelial cells can be multi-layered and are often pleomorphic (Fig. 3.12b). These tumors occur at a higher incidence in males and occur more frequently in outbred strains such as the Sprague Dawley and other Wistar strains when compared to inbred F334 rats (Deerberg et al. 1980, Haseman et al. 1998, Creasy 2012). These lesions often provide a diagnostic challenge to pathologists as the biologic behavior is unknown and may represent a progression of a non-neoplastic change such as angiomatous hyperplasia to hemangioma or hemangiosarcoma. Histiocytic sarcoma is a relatively common neoplasm in rodents. It often involves abdominal organs of the mouse, particularly the liver and uterus, however, in BALB/c mice, the bone marrow and lymph nodes are commonly involved (Greaves 2007). Neoplastic cells are often pleomorphic but they usually possess a fairly irregular nucleus with marginated chromatin pattern and occasionally prominent nucleoli. Abundant eosinophilic cytoplasm may show erythrophagocytosis (Frith et al. 1980). Some authors have also noted an association between histiocytic sarcoma in mice and rats with the presence of hyaline droplets containing lysozyme in the cytoplasm of proximal renal tubular cells (Hard and Snowden 1991).

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Chapter 4
Mucosa-Associated Lymphoid Tissues

Christine Frieke Kuper, Marcel V.W. Wijnands, and Serge A.L. Zander

Abstract The mucosal immune system is organized as a complex of two large pools of single immune-competent cells within the epithelium (lymphocytes: IEL) and lamina propria (lymphocytes: LPL) of the mucosa; the lymph nodes draining the mucosa; and the more or less organized lymphoid tissues associated with the mucosal epithelium. *Mucosa-associated lymphoid tissue* (*MALT*) *is defined here as the organized lymphoid tissues associated with the mucosal epithelium*.

The body of humans and animals functions as a symbiotic ecosystem of cells and about a 10-fold higher number of microorganisms, which meet at mucosal barriers. The mucosal immune system plays an important role in this symbiotic relationship, maintaining a delicate balance of tolerating a health-promoting microbiome and allowing uptake of nutrients, while excluding potentially harmful pathogens. In turn, the microbiome is needed to ensure proper functioning of the immune system. Still, many aspects of mucosal immunity and their interrelationships are poorly understood, including the interplay with some non-lymphoid organs like the liver in gut immune functioning.

MALT is included in most guidelines on safety evaluation of drugs, chemicals and food constituents. In view of the importance of the body's mucosal immunity, it is advisable to examine not only MALT at the site of application or exposure like today's common practice, but to include distant MALT as well. There is a need for best practices to select, sample and embed MALT for histopathologic evaluation, because these aspects can profoundly influence the evaluation of MALT pathology and the interpretation of responses against xenobiotics.

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Pathology reports on MALT and single mucosal immune cells are scarce, most likely due to the difficulty to dissect MALT or to properly evaluate MALT in situ, and the difficulty to sample LPL and IEL populations as FACS sorted suspensions. In addition, there may be high resilience in MALT against xenobiotic insults, exactly because of its importance as primary contact site to the outside world. Nevertheless MALT deserves specific attention. This chapter aims to present information on morphology and functioning of MALT to add in the assessment of changes in this important segment of our immune defense and homeostasis.

Keywords BALT • Cryptopatches • IEL • LPL • Lymphocyte-filled villi • GALT • MALT • Mucosal mast cells • NALT • Peyer's patches • Secretory immunoglobulins • Tonsil

Abbreviations

B cell	'Bone marrow- or bursa-derived' lymphocyte mediating humoral immunity
BALT	Bronchus-associated lymphoid tissue
CALT	Conjunctiva-associated lymphoid tissue synonym of EALT
CLP	Common lymphoid progenitor
CMC	Connective tissue mast cell
CP	Cryptopatch
DALT	(Salivary) duct-associated lymphoid tissue
DC	Dendritic cell
EALT	Eye-associated lymphoid tissue synonym of CALT
ETALT	Eustachian tube-associated lymphoid tissue
GALT	Gut-associated lymphoid tissue
GIALT	Gill-associated lymphoid tissue
GIEL	Granulated intraepithelial lymphocyte
GL	Globule/globular leukocyte
GVHD	Graft versus host disease
HEV	High endothelial venule
HSC	Hematopoietic stem cell
iBALT	Inducible BALT
IEL	Intraepithelial lymphocyte
Ig	Immunoglobulin
ILC	Innate lymphoid cell
LALT	Laryngopharynx-associated lymphoid tissue
LDALT	Lacrimal duct-associated lymphoid tissue
LFV	Lymphocyte-filled villus
LPL	Lamina propria lymphocyte
LTi	Lymphoid tissue inducer (cell)
MALT	Mucosa-associated lymphoid tissue

MC	Mast cell
M-cell	Microfold (epithelial) cell
MC_T	Human tryptase-positive chymase-negative mast cell (resembles MMC)
MC_{TC}	Human tryptase- and chymase-positive mast cell (resembles CMC)
MHC	Major histocompatibility complex (HLA in humans)
MMC	Mucosal mast cell
NALT	Nasopharynx/nasal-associated lymphoid tissue
NK	Natural killer lymphocyte
PP	Peyer's patch
SALT	Skin-associated lymphoid tissue
SILT	Solitary intestinal lymphoid tissue
T cell	'Thymus-derived' lymphocyte mediating cellular immunity
TALT	Trachea-associated lymphoid tissue
Th2	T helper 2 lymphocyte

4.1 Introduction

The extensive mucous membranes are the most important contact sites for exogenous substances and infectious agents, besides the skin. Mucous membranes cover the gastrointestinal, urogenital and respiratory tracts, including the oral and nasal cavities, the pharynx and the conjunctiva of the eyes. The mucous membranes are protected by several defense mechanisms, which include mechanical defenses like the mucociliary escalator of the respiratory tract, mucous constituents with antibacterial properties like sialidases (Lewis and Lewis 2012), and extensive adaptive and specific immune defenses mediated mainly by macrophage activity and secretory immunoglobulins, respectively. The mucosal immune system of the gut functions in a delicate balance between excluding antigens and keeping potentially harmful pathogens out, while at the same time allowing for efficient uptake of nutrients and maintaining a healthy microflora. The mucus layer itself is an important determinant in the microbial colonization of the mucosae and vice versa the mucin composition is influenced by the microbiome (Vaishnava 2016). The term mucosal or 'oral' tolerance is generally used to describe the uptake of nutrients and the relationship with the mucosal microflora, which is effectuated by immunological downregulation or suppression of systemic immune responses. The immune system of the respiratory and urogenital mucosae operate in concert with the intestinal immune system. The immune system of the respiratory tract needs to deal with exposure to (inhaled) antigens, and except for the alveolar region, it must also discriminate between commensal and potentially harmful bacteria (Vaughan et al. 2009; Jensen et al. 2013). The respiratory and intestinal immune system are therefore expected to have comparable potential. The immune system of the urogenital mucosa is less examined in laboratory animals, it may be less complex because of seemingly less demanding tasks.

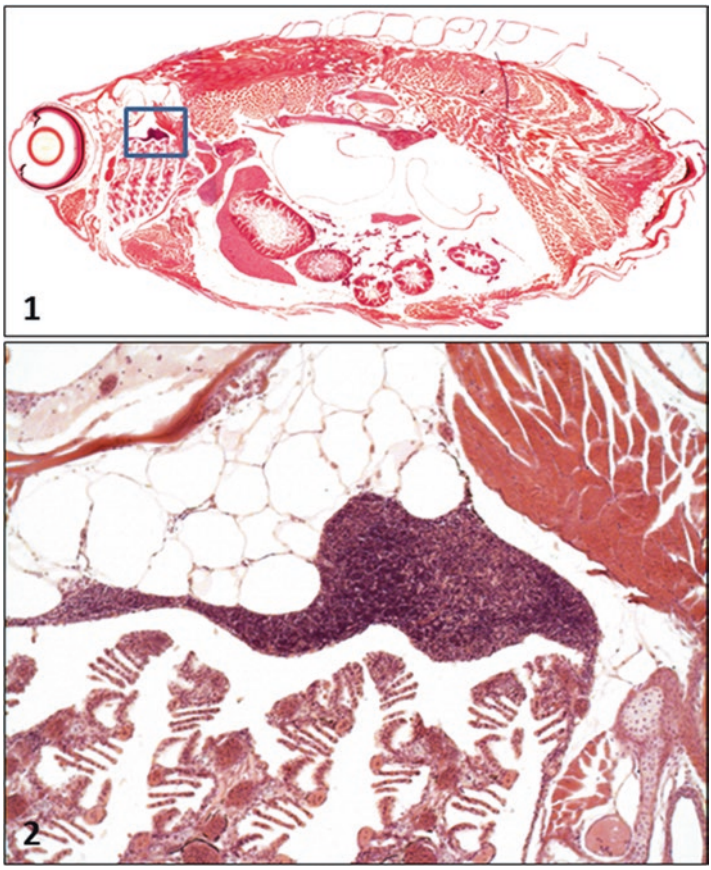

Fig. 4.1 Thymus of zebrafish. (**1**) Longitudinal section through zebrafish with thymus in rectangle; (**2**) Detail of thymus in adipose tissue and associated with the epithelium lining the brachial cavity (courtesy of Dr. Aswin Menke, Triskelion)

The mucosal immune defense is organized as a complex of single immune cells, lymphoid tissues and organs (exemplified by gut mucosal cells and tissues, Fig. 4.1). Based on anatomy and location, the different components can be categorized as (a) lymph nodes, (b) epithelium-associated lymphoid aggregates in the mucosa, and (c) single immune-competent cells scattered in the lamina propria and within the mucosal epithelium. The different compartments can be divided also according to their *main* function, namely inductive sites and effector sites (Brandtzaeg and Pabst 2004; Brandtzaeg et al. 2008). However, the distinction between these functional sites is not absolute. Moreover, the function of some epithelium-associated lymphoid aggregates, like cryptopatches and lymphocyte-filled villi, is not fully understood, so it is not certain whether these particular aggregates have inductive or effector properties or both. For the sake of simplicity, we therefore apply the general term 'mucosa-associated lymphoid tissues' or MALT to all epithelium-associated,

more or less organized lymphoid aggregates in the mucosa. MALT are lymphoid structures, (most of) which are inductive sites where antigen-primed B and T cells are activated and memory and effector cells are generated for mucosal immunity (Brandtzaeg and Pabst 2004; Brandtzaeg et al. 2008). The effector sites are much more diverse, ranging from mucosal epithelium and lamina propria to salivary and mammary glands.

MALT is situated beneath and in association with the mucosal epithelium, and samples antigens from the lumen via this epithelium with its specialized microfold (M) cells (Owen 1977; Van der Brugge-Gamelkoorn et al. 1985; Spit et al. 1989). MALT thus lacks afferent lymph vessels in contrast to lymph nodes. Well-known examples of MALT are the Peyer's patches (gut-associated lymphoid tissue or GALT) and the tonsils and adenoid in the nasal and oronasopharyngeal area (NALT; in man collectively named the Waldeyer's-ring), and bronchus-associated lymphoid tissue (BALT).

The procedures of selection and embedding of MALT structures for histopathological examination and evaluation can influence the results. For example, not all Peyer's patches along the small intestines may react similarly upon oral uptake of xenobiotics, thus standardizing the selection of these structures is needed (Bruder et al. 1999). A cross section of the nasal passages with NALT in situ reveals mostly distinct effects, whereas detection of more subtle effects can only be achieved with dissected NALT, embedded longitudinally (Kuper et al. 1992) and evaluated according to enhanced histopathology principles (Kuper et al. 2000; Elmore 2006, 2012).

MALT is regarded as secondary lymphoid tissue, in contrast to thymus and bone marrow, which are considered predominantly as primary, i.e., antigen-independent, lymphoid organs. Interestingly, MALT and thymus may be evolutionary related (Matsunga and Rahman 2001), and share developmental and structural aspects (Fig. 4.1); Bykova et al. 2003; Bowden et al. 2005). The mucosal epithelium and the epithelium that forms part of the stroma of the thymus have in common that they educate T lymphocytes to mature into cells capable to distinguish self from non-self and harmful from non-harmful, respectively. This illustrates that lymphoid organs have a great plasticity and that compartmentalization in primary and secondary lymphoid organs cannot be strict (Pabst 2007).

The single lymphocytes in the mucosae (lamina propria lymphocytes or LPLs and intraepithelial lymphocytes or IELs) are considered part of the effector sites, as stated above. Their pathology is barely reported despite the fact that the mucosal single T lymphocytes form the largest bulk of T lymphocytes in the body. The lack of published pathology data is most likely related to the difficulty of studying LPLs and IELs in conventionally-stained tissue sections or inability of sampling them as cell suspensions; or maybe this population of cells is just very sturdy. Local lymph nodes are important in the local immune defense and in the connection between the local and systemic immune system (Fig. 4.2). The single lymphocyte compartments and the local lymph nodes play significant roles in mucosal immune defense, but the focus in this chapter is on MALT.

Perspective: Pathology reports on MALT are scarce, despite its key role in maintenance of our microbiome and in the contact with the outside world. This applies also

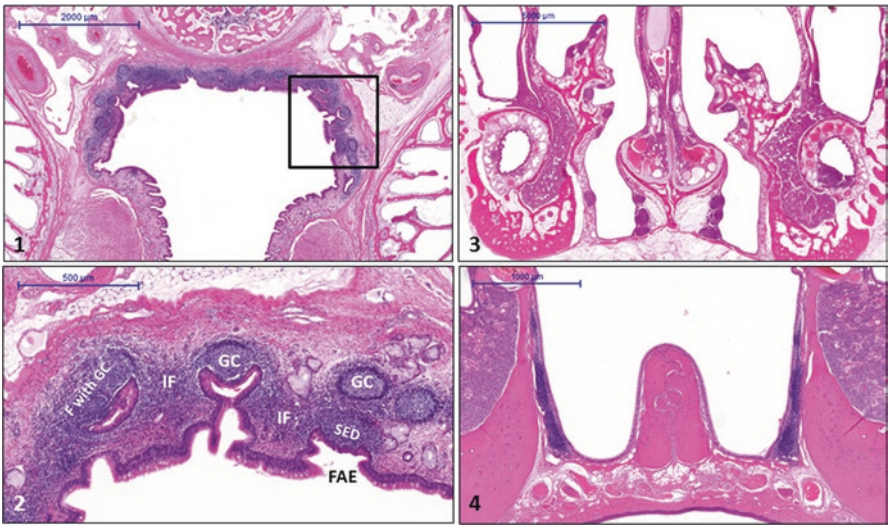

Fig. 4.2 NALT. (**1**) In minipig it is a single organ, like in man (adenoid) and located at the roof of the nasopharyngeal duct; (**2**) Detail of NALT in minipig, see square in (**1**); (**3**) NALT in rabbits consist of multiple lymphoid aggregates dispersed along a relatively large mucosal area in the nasal passages; (**4**) In rodents NALT is paired and located at the ventrolateral wall of the nasopharyngeal duct opening. H&E staining. *FAE* follicle-associated epithelium, *SED* subepithelial dome area, *IF* interfollicular area, *F* follicle, *GC* germinal center

to the single lymphocyte populations in and below the mucosal epithelium, the IELs and LPLs. There are several plausible explanations for this paucity of data: there may have been considerable evolutionary pressure for high resilience in MALT against xenobiotic insults, exactly because of its importance as primary contact site to the outside world. Also MALT pathology may easily go unnoticed, because these tissues are difficult to examine or are affected more in a functional than morphological way, as detectable on H&E-stained sections. Nevertheless, MALT certainly deserves more attention in safety and efficacy than at present and it is a challenge to explore proper examination. It may be advisable to examine not only MALT at the site of application/exposure like today's common practice, but to include distant MALT as well. In this chapter we have tried to present the information on morphology and functioning/histophysiology of MALT to help assess induced changes in this important segment of our immune defense and homeostasis.

4.2 Immunobiology

4.2.1 Development and Morphology of MALT

Anatomically, MALT encompasses a number of organized lymphoid tissues, located predominantly in the mucosal membranes of the gastrointestinal (GALT) and respiratory (NALT and BALT) tracts. A number of less well-organized mucosal

lymphoid aggregates are found in the head region and elsewhere; still others may have gone unnoticed up till now. To our knowledge MALT has not been identified in the urogenital tract. This is surprising, because the urogenital tract has a specific microbiome. Colonic and rectal lymphoid aggregates (patches) and NALT/Waldeyer's ring may function as inductive sites for genital tract immunity (Brandtzaeg 1997; Mestecky and Fulz 1999).

The overall histology of MALT is very similar to that of lymph nodes with B cell-dominated follicles and T cell-dominated interfollicular zones (Figs. 4.2–4.4); less organized MALT have indistinct or no B- and T-cell dominant zones (Fig. 4.3; Tables 4.1 and 4.2). The interfollicular zone of PP and NALT has a dense lymphatic network and high endothelial venules (HEVs) (Sminia et al. 1989; Cesta 2006), and resembles the interfollicular areas and peripheral deep cortex in the lymph nodes. Vulchanova et al. (2007) studied the innervation of porcine PP and found an extensive ganglionated neuronal network with nerve projections into the domes and the interfollicular spaces. The close connection between the nerve projections and the lymphoid cells was highly suggestive for communication and interaction between the mucosal immune system and the central nervous system. BALT is less organized; it is always flanked by a bronchus and an artery and a nerve runs through each of them.

In contrast to lymph nodes, MALT are mostly aggregates of lymphoid and supporting cells, which are incompletely encapsulated or lacking a capsule altogether

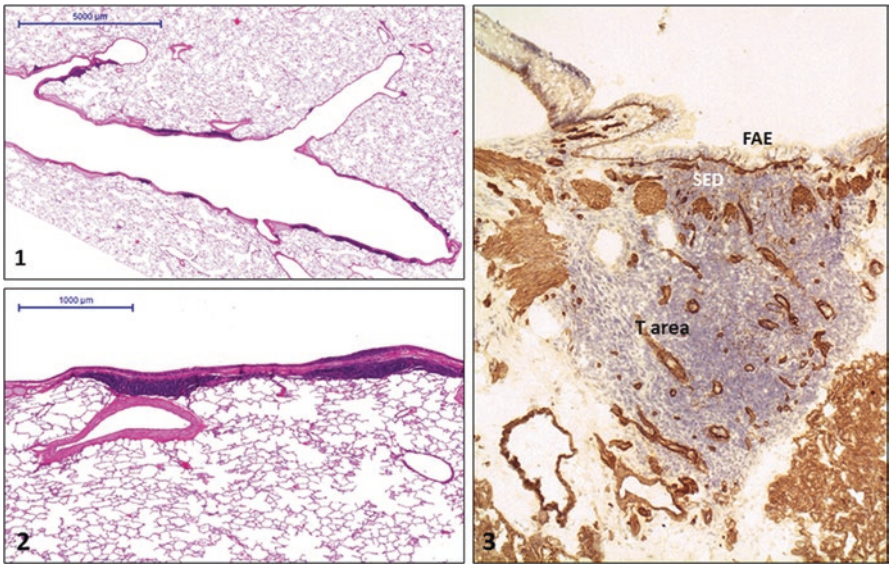

Fig. 4.3 BALT. (**1**) Overview of BALT in lung of rabbit; H&E staining; (**2**) Detail of rabbit BALT; (**3**) BALT in rat lung; laminin staining. *FAE* follicle-associated epithelium, *SED* the subepithelial dome area, located between the epithelium and the interrupted bronchial muscle layer; the T-lymphocyte dominant area contains HEVs, the B-lymphocyte dominant follicle is not clearly distinguishable in this inactive BALT

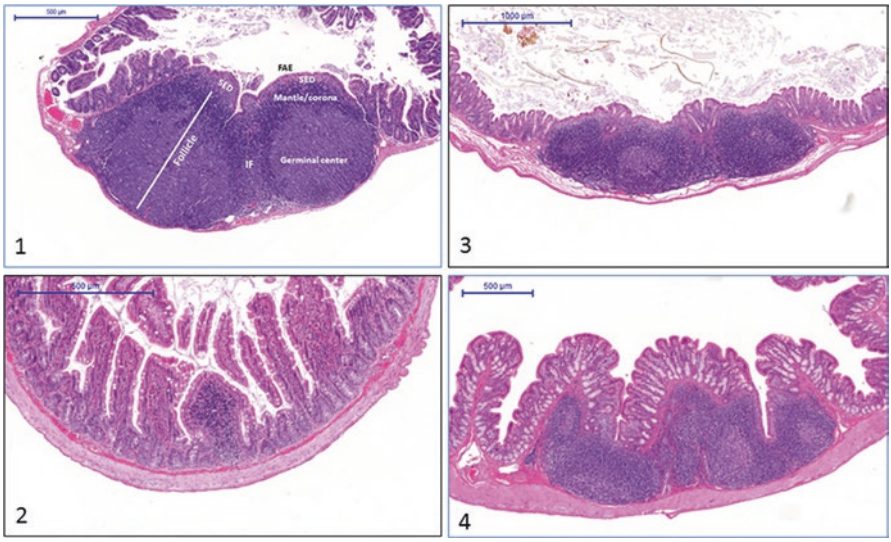

Fig. 4.4 GALT. (**1**) Peyer's patch in small intestine of mouse, well-organized MALT structure. (**2**) Cluster of lymphocyte-like cells in villus of small intestines of rat, not further organized. These clusters are named lymphocyte-filled villi (LFVs); in H&E-stained sections LFVs cannot be distinguished from isolated lymphoid follicles (ILFs). (**3**) Cecal patch rat, with relatively small germinal centers. (**4**) Colonic patch rat with relatively small germinal centers and indistinct T cell-dominated areas. *FAE* follicle associated epithelium, *SED* subepithelial dome area, *IF* interfollicular, *T* lymphocyte-dominated area

(Fig. 4.3c). A second difference with lymph nodes is the lack of afferent lymphatics in MALT as intraluminal antigens are directly sampled from the mucosal surface by specialized M-cells, located in follicle-associated epithelium (FAE). M-cells transfer particulates and microorganisms from the lumen more or less intact to antigen-presenting cells like dendritic cells (DCs), macrophages and B cells, enclosed in their pocket (Corr et al. 2008). M-cells have lysosomes with much less enzymatic activity than the neighboring enterocytes. Nonadherent soluble material can also be taken up by pinocytosis, although transport efficiency across the cell is less than for membrane-bound material. Alternatively, intra- and subepithelial DCs in the nasal mucosa may directly capture antigens and relocate via the efferent lymphatics to draining lymph nodes to become further activated. Chemokines and local vascular adhesion molecules direct the homing of naïve T and B cells via HEVs from the blood towards a particular MALT. Here, B cells with the appropriate antigen specificity become activated inside follicles through stimulatory or modulatory interactions with follicular DCs and T cells, respectively. Primed B cells mature subsequently into plasma or memory cells inside the follicle and on their way towards and inside local draining lymph node sinuses. The third difference with lymph nodes is the lack of medullary cords in MALT, where in lymph nodes the primed B cells and plasma cells go through their final maturation stage.

Table 4.1 Morphologic aspects of MALT, predominantly based on rat data

MALT	Compartments	Cell types	Comments
Peyer's patches (PP)[a]	Specialized epithelium, so-called follicle-associated epithelium	Numerous M cells	Wistar rats[b]: – PP in first two-thirds of small intestines: low number of follicles with a germinal center (approx. 15%). – PP in last one-third of small intestines: high number of follicles with germinal center (approx. 45%). Prenatal development – No. of PP in adult Wistar rats 18 (11–25)[b]; adult BN rats 12 (9–15)[b]; Mice 8–12[c]; Rabbits 5–9[d]; humans 100–300[d] – Interfollicular areas in PP of some species, for example human and minipig, are often quite small
	Dome between epithelium and follicle	Dendritic cells	
	Follicle [with germinal center]	Mainly B cells. In germinal centers: follicular dendritic cells; different stages of maturing B cells; $CD4^+$ T cells; tingible body macrophages	
	Interfollicular area, alternating with follicles	Mainly T cells ($CD4^+$ and $CD8^+$), some B cells, dendritic cells, tissue macrophages, high endothelial venules (HEVs)	
Lymphocyte-filled villi (LFV)	Specialized epithelium?		LFV might be analogous to CP in function and cell constituents, but different in localization (not near crypt but in villus)[e]. Postnatal development
	Lymphoid aggregate; no specific microanatomy	Predominant T cells	
Isolated lymphoid follicles (ILF)[a]	Mature ILF: Specialized epithelium	Numerous M cells	Considered to be developed from CP (in rat: from LFV?) and may be able to mature into PP. Postnatal development
	Microanatomy in between CP and PP. More mature forms of ILF can contain germinal center with dome area between germinal center and specialized epithelium. No distinct T cell areas	Dendritic cells, B cells, T cells, LTi (lymphoid tissue inducer) cells. In germinal centers: follicular dendritic cells; different stages of maturing B cells; CD4+ T cells; tingible body macrophages	
Cryptopatches (CP)[a]	Lymphoid cluster in proximity of intestinal crypts; less specific association with epithelium than ILF and PP and no specialized epithelium. Some structure in that clusters of lymphocytes are surrounded by dendritic cells	Dendritic cells, LTi cells	Present in mouse (>1000) and man; not found in rats

(continued)

Table 4.1 (continued)

MALT	Compartments	Cell types	Comments
Colonic and rectal lymphoid aggregate	Specialized epithelium[f]	Numerous M cells	Often lymphocyte-filled lymph vessels surrounding aggregate
	Lymphoid aggregate; no distinct B- and T-dominant areas. Germinal centers rare/absent		
Lacrimal duct-associated lymphoid tissue (LDALT)	Specialized epithelium	Numerous M cells	– Lymphangiectasis is common – Wistar: low number of germinal centers
	Distinct B- and T-cell areas only upon activation		
Nasal/Nasopharynx-associated lymphoid tissue (NALT)	Specialized epithelium	Numerous M cells	– Wistar: low number of germinal centers – BN: high numbers of germinal centers – Rabbit: NALT at multiple sites throughout nasal passages and nasopharynx – Timeframe of development questionable: may differ between species; rodents may have NALT already at birth[g] or postnatal development[h]; minipig may have NALT just before birth
	Follicle [with germinal center]	Mainly B cells. In germinal centers: follicular dendritic cells; different stages of maturing B cells; $CD4^+$ T cells; tingible body macrophages	
	Interfollicular area, alternating with and underneath follicles	Mainly T cells ($CD4^+$ and $CD8^+$), some B cells, dendritic cells, tissue macrophages, high endothelial venules (HEVs)	
Bronchus-associated lymphoid tissue (BALT)	Specialized epithelium	Numerous M cells	– In mice and man BALT is present only upon activation – Wistar: no to low number of germinal centers – BN: high numbers of germinal centers – Postnatal development in rats
	Rat: distinct B- and T-cell areas only upon activation		

[a]Herbrand and Pabst 2011
[b]Kuper et al. 2007
[c]Taylor and Williams 2005
[d]Makala et al. 2002
[e]Hitotsumatsu et al. 2005
[f]Jacob et al. 1987
[g]Kuper et al. 1992
[h]Lamichhane et al. 2014

Table 4.2 MALT compartments/Peyer's patches and NALT compartments and their constituents

Compartment	Constituents
Follicle-associated epithelium (FAE)	– Microfold (M) epithelial cells in-between enterocytes or ciliated respiratory epithelial cells; only few if any goblet cells – (dendrites of) Dendritic cells (DCs). DCs can be within the epithelium, but more probably they are underneath the basal lamina and project with their dendrites through the epithelium and reach into the mucus layer/lumen – Macrophages, probably incidental. Most macrophages just beneath the basal lamina – Lymphocytes, including IgA^+ cells, in basolateral pocket of M cells (Hernandez & Mantis 2015)[a], and between enterocytes
Subepithelial dome (SED) or Dome	– Dendritic cells, with dendrites extending through epithelium – Macrophages – T lymphocytes – Marginal reticular cells (MRCs; Katakai 2012)[b], extending into follicle – Capillaries
Follicle	Dominant B lymphocyte area – Primary follicles contain small resting virgin B cells – Follicular dendritic cells (FDCs) – Capillaries; Follicles are surrounded by a 'basket' of lymphatics and blood vessels
Mantle	– Small resting virgin B cells like in primary follicle
Germinal centre	– B lymphocytes: development from primary B cell (naive lymphoblast) - in the basal, dark zone - via centroblast and centrocyte to secondary B lymphoblast into B memory cell or plasma (precursor) cell - in the apical light zone – T lymphocytes – Follicular dendritic cells (FDCs) – Tingible body macrophages
Interfollicular area	Dominant T lymphocyte area. *Syn*. parafollicular area – T lymphocytes – B lymphocytes – Fibroblastic reticular cells (FRCs) – Dendritic cells – High endothelial venules (HEV) – Lymphatic plexuses and lacteals

[a]Hernandez and Mantis (2015)
[b]Katakai T (2012)

4.2.1.1 Head Region and the Respiratory Tract

Laryngopharynx-associated (LALT), trachea-associated (TALT), conjunctiva-associated (CALT or eye-associated: EALT), Eustachian tube-associated (ETALT), (salivary) duct-associated (DALT) and lacrimal duct-associated (LDALT; Fig. 4.11a) lymphoid tissues can be distinguished additionally besides NALT (Casteleyn et al. 2011; Cesta 2006).

Knop and Knop (2001) studied LDALT and its relation to the lacrimal drainage system in humans, noting the importance of LDALT for the protection of the ocular surface and proposing to name it the lacrimal *drainage*-associated lymphoid tissue. Tonsils are lymphoid aggregations with or without crypts in the naso-, oro- and laryngopharynx. The lingual, palatine, para-epiglottic, soft palate-associated, tubal and pharyngeal tonsils together form the Waldeyer's ring, which compared to man shows some degree of developmental variability in the domestic species (Casteleyn et al. 2011). In rodents, this typical arrangement of tonsils is lacking, but functionally equivalent pairs of NALT structures can be found in the ventrolateral wall of the nasopharyngeal duct opening (Fig. 4.2d; Koornstra et al. 1991; Kuper et al. 1992; Van der Ven and Sminia 1993). NALT, TALT and CALT form a craniofacial mucosal immune system which appears to be unique because the lymphocytes inhabiting this area exhibit specific characteristics with respect to their dependency on chemokines and adhesion molecules, which differs from that of other MALT and peripheral lymphoid tissues (Okada et al. 2011; Siebelman et al. 2013). NALT in rabbits consist of multiple lymphoid aggregates dispersed along a relatively large mucosal area (Fig. 4.2c; Casteleyn et al. 2010a).

In the lower respiratory tract, BALT is described in a variety of vertebrates, such as mammals, birds and reptiles. According to Haley (2003), rabbits and rats usually have the most BALT, man having the least, while mice and guinea pigs are intermediate. An adult rat has 30–50 BALTs (Reviewed by Sminia et al. 1989). In pigs, BALT can already be found prenatally, but in rodents, rabbits and chickens (Fig. 4.3; Kang et al. 2013) the development starts after birth. In the first weeks they gradually get populated by lymphocytes and other constituents (Reviewed by Sminia et al. 1989). This basic pattern occurs in animals growing up under natural conditions, but also when deprived from antigen stimulation as well as in germ free rats. With increasing age the cell population in BALT shows variation, since further development is dependent on exposure to antigens. The presence of BALT in humans and mice seems to be dependent on postnatal antigenic stimulation (induced or iBALT, Pabst 2007; Foo and Phipps 2010).

4.2.1.2 Gastrointestinal Tract

The oral cavity is the entrance to the gastrointestinal tract and its MALT (the tonsils) has been described above. MALT can be found in the stomach as well (Fig. 4.5a). MALT in the small intestines is the most extensively studied with a number of particular components that show interspecies variability (Fig. 4.6). The large intestines also contain organized lymphoid nodules or clusters, either appearing singularly or grouped into aggregates. Humans and rabbits have a large aggregation known as the appendix (Butler and Sinkora 2013); an appendix is absent in mice and rats, but they have several variably developed aggregates in the wall of the cecum (Haley 2003). The microscopic morphology of the rabbit appendix is shown in Figs. 4.5b and 4.5c. MALT in the intestines is collectively denoted as GALT.

GALT in the small intestines consist of Peyer's patches (PP), isolated lymphoid follicles (ILFs) and cryptopatches (CP). In the mucosa and extending into the

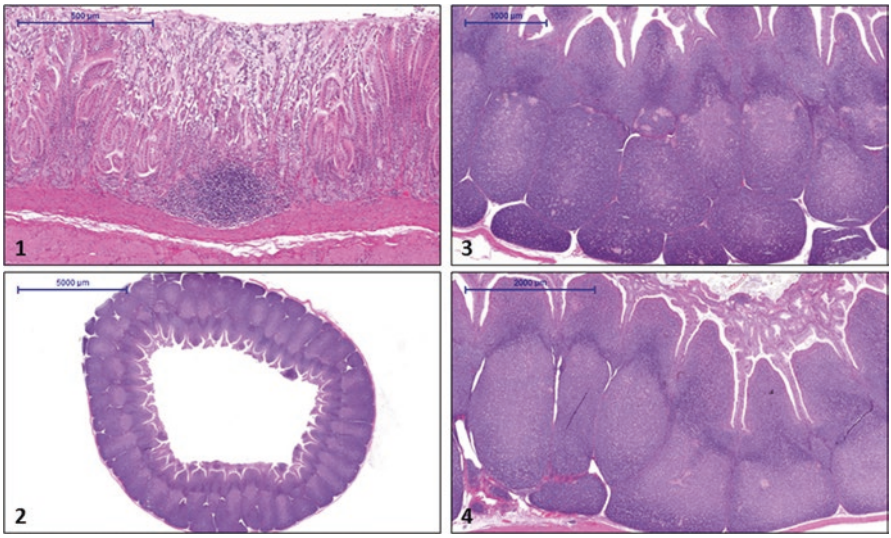

Fig. 4.5 MALT in the rabbit gastrointestinal tract. (**1**) Isolated follicle in stomach; (**2**) Overview of cross section through appendix; (**3**) detail of the appendix; (**4**) detail of sacculus rotundus

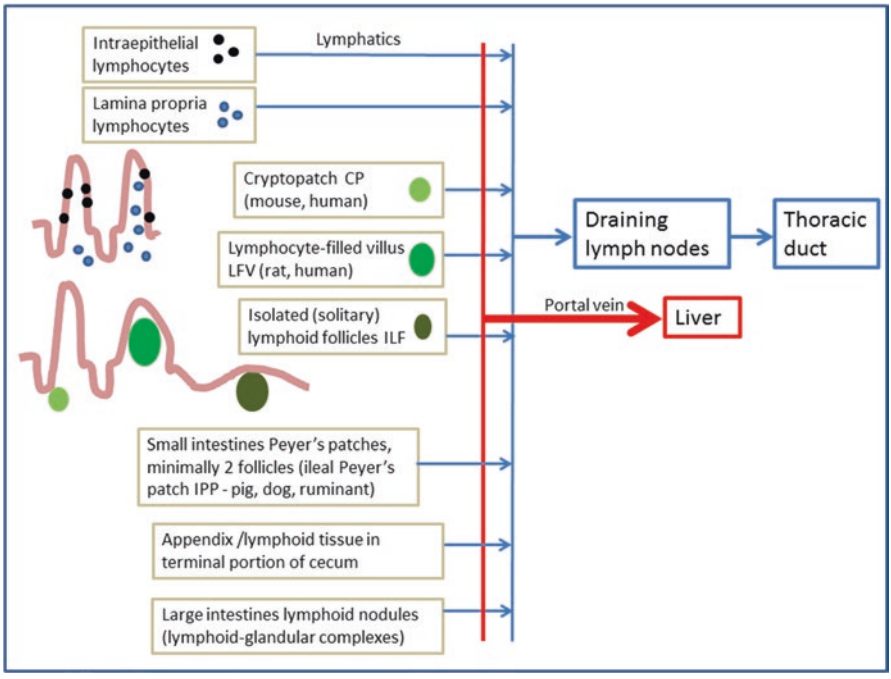

Fig. 4.6 The mucosal immune defense in the gut is organized into different components of single immune cells, lymphoid tissues and organs (modified after Kuper et al. 2013)

submucosa, PPs of most mammals and birds are typical sites of antigen-dependent priming of B and T cells. In PP three regions can be distinguished: the dome (subepithelial dome or SED, a densely innervated region, Vulchanova et al. 2007), which is closest to the epithelium with its M-cells, the follicles with germinal centers (B-cell dominated areas, which are surrounded by basket-like networks of arterioles and venules and lymph vessels, Azzali 2003; Bhalla et al. 1981; Pearson et al. 2012) and the interfollicular areas dominated by T-cells (Table 4.2). The number of PP varies between species (Table 4.1). In addition to the discrete patches in the jejunum and upper ileum found in most species, ruminants, pigs and dogs have an additional, long and continuous PP in the terminal ileum (Haley 2003). This continuous ileal PP acts as a primary lymphoid organ like the bone marrow (Landsverk et al. 1991), where antigen-independent lymphocyte development occurs in a similar fashion as in the bursa of Fabricius in chicken (extrathymic lymphocyte maturation; Casteleyn et al. 2010b). Despite showing a high rate of lymphopoiesis initially, only relatively few B-cells of this ileal PP in lambs (up to 5%) differentiate and emigrate, while the vast majority is cleared by apoptosis and tingible body macrophages in the follicles (Yasuda et al. 2006). After this intense burst of initial mitotic activity, which in sheep starts shortly after birth, involution of the ileal PP begins at about 12 weeks of age and is almost completed by 3–4 months of age with few PP follicles remaining in this location at 18 months of age (Yasuda et al. 2006). In addition to this special ileal PP, 'jejunal-type' PPs can develop in the ileum in response to postnatal antigen stimulation and persist throughout life: these PPs are equivalent to ileal patches of species lacking this special type of PP. Rabbits have a distinctive structure called the sacculus rotundus, which encircles the terminal ileum and can be interpreted as a hypertrophied PP (Fig. 4.5d; Haley 2003); it might be homologous to patches at the ileocecal entrance found in ruminants, described below.

PPs develop already before birth in the mammalian fetus (Fig. 4.7). The cellular developmental and molecular signaling pathways leading up to these structures are complex and still being sorted out (Cherrier et al. 2012). Briefly, IL-7Rα+common lymphoid progenitors (CLPs), derived from the hematopoietic stem cells (HSCs) residing in the fetal liver and bone marrow, give rise to innate lymphoid cells (ILCs, Cording et al. 2014). Three categories of ILCs have been distinguished based on cytokine expression patterns. Of particular interest here are the type 3 ILCs, expressing the nuclear hormone receptor RORγt. Knockout mice, lacking this receptor, fail to develop PPs and lymph nodes (before birth) or isolated lymphoid follicles (ILFs, after birth), because the crucial lymphoid tissue inducer (LTi) cells are not formed. The role of LTi cells is to deliver lymphotoxin (LT)-dependent signals to so-called stromal 'organizer' cells. The LTi cell-organizer cell LT-LTβR interaction results in specific chemokine production and expression of adhesion molecules that sustain this initial cell cluster and drive further recruitment of hematopoietic cell types, forming the ultimate secondary lymphoid tissues (Newberry 2008).

Whereas PP development is programmed prenatally (explaining its designation as a secondary lymphoid tissue, Pabst 2007), CPs and ILFs form postnatally in response to the inevitable colonization of the gut by environmental microbiota (Cherrier et al. 2012). In fact, CPs have been identified in mice and man, and experi-

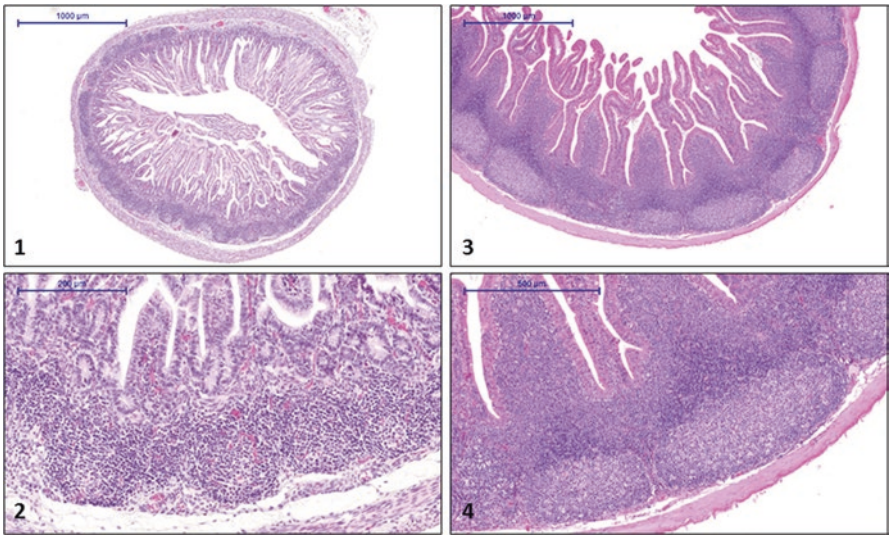

Fig. 4.7 Peyer's patch (PP). (**1**) Overview of (PP) in small intestines of minipig, 1 day before birth; (**2**) Detail of PP in minipig; (**3**) Overview of PP in adult rabbit; (**4**) Detail of PP in rabbit. H&E staining

mental evidence in mice suggests that these clusters of closely packed lymphocytes at crypt bases are actually the precursor structures of ILFs (Nochi et al. 2013; Eberl and Sawa 2010). The concept of solitary intestinal lymphoid tissue (SILT) describes the developmental spectrum from CPs, which mainly consist of Lin-c-Kit+IL-7Rα+cells surrounded by DCs, up to fully matured ILFs that more closely resemble PPs. Analogous to PP development, Lin-c-Kit+IL-7Rα+cells in the CPs act like LTi cells orchestrating the recruitment of additional lymphocytes and DCs via stromal organizer cells by LTβR signaling, and hence driving the formation of mature ILFs from CPs (Lügering et al. 2010). Interestingly, chronic inflammatory processes like inflammatory bowel disease (IBD) in humans are associated with LTi cell-independent tertiary lymphoid tissue. In this case, inflammation-derived chemokines recruit activated LT-expressing lymphocytes that mediate the interaction with LTβR+ stromal cells, forming even less organized lymphoid tissue with or without germinal centers (also termed lymphoid neogenesis; Newberry 2008). Following the discovery of CPs in mice, researchers began to look for similar structures in other species, including man (Moghaddami et al. 1998; Hitotsumatsu et al. 2005). This lead to the identification of lymphocyte-filled villi (LFVs) in the rat and humans, comprising not crypts but villi packed with Lin-c-Kit+IL-7Rα+cells in the lamina propria and increased numbers of IELs in the overlying epithelium. However, it is still unclear what functional role these lymphoid structures play exactly and whether rat and human LFVs are equivalent or not (Lügering and Kucharzik 2006).

GALT of the large intestines in most species consist of ILFs, CPs and larger, more organized lymphoglandular complexes (LGCs) or patches. ILFs and CPs are located in the lamina propria, separated from the lumen only by the follicular epithelium. ILFs are scattered along the colon and rectum in humans (Mowat and Agace 2014), mice (Owen et al. 1991), ruminants and pigs (Lügering and Kucharzik 2006). The more developed LGCs show crypts that communicate with the lumen and extend down through the muscularis mucosae into the submucosa, branching into smaller diverticula where the lymphoid tissue is located (Owen et al. 1991). The epithelial lining of both contains M cells like those of the small intestinal PPs and reduced numbers of goblet cells compared to the surrounding non-lymphoid intestinal epithelium. The large intestinal lymphoid patches or LGCs of ruminants and pigs are concentrated at the ileocaecal entrance, the proximal colon and the rectum around the anal ring; horses show a more diffuse distribution of mostly ILFs (Liebler-Tenorio and Pabst 2006). The LGCs in rodents can be oriented towards the antimesenterial side of the intestine and a rectal patch is consistently located in the mouse within 10 mm from the anus (Cesta 2006; Owen et al. 1991).

Large intestinal lymphoid patches resemble mostly jejunal PPs in their structure and function (Cesta 2006), but colonic patches in rodents may be smaller in size and have smaller-sized germinal centers than small intestinal patches (mouse: Owen et al. 1991; rat: own observations, Fig. 4.2); rectal patches are often completely devoid of germinal centers and T and B cell compartments are difficult to distinguish in H&E-stained sections. Paucity of germinal centers and small size of colonic patches may seem counterintuitive, given that the large intestines function as a reservoir for an extensive number of microbiota, orders of magnitude larger in size than its small intestinal counterpart (Mowat and Agace 2014). However, antigenic stimulation may actually be limited here as microorganisms are mostly aggregated inside fecal boluses and the outer mucus layer, separated from the epithelial lining by a relatively thick inner layer of mucus explaining the regional discrepancy in size (Owen et al. 1991; Mowat and Agace 2014). Hence the mucus barrier, produced by the higher goblet cell density of the large intestine, may modulate germinal center stimulation and limit colonic lymphoid tissue development. This also makes sense given the regional difference in physiological function: the fluidity of the small intestinal content facilitates digestion and uptake of nutrients, while immunity mostly has to deal with tolerance to food proteins; in contrast, the large intestine needs to resorb mostly water, while the immune system just has to keep the commensal microbiota at bay, preventing a detrimental immune response that would otherwise eliminate these useful inhabitants (Mowat and Agace 2014).

4.2.1.3 Non-mammalian Species

NALT in chickens consist of multiple lymphoid aggregates dispersed along a relatively large mucosal area (Kang et al. 2013); NALT develops after hatching. As in mammals, GALT is formed by PP and cecal lymphoid aggregates, but some components are typical for birds: namely the bursa of Fabricius and Meckel's diverticulum (reviewed

by Lillehoj and Trout 1996). The cecal 'tonsils' are situated near the entrance of the ceca and show many structural similarities to PP. Their germinal centers contain both T- and B cells and plasma cells producing IgA, IgG and IgM. The bursa of Fabricius lies near the cloaca and is the central lymphoid organ for proliferation and maturation of B-lymphocytes (hence their name). Meckel's diverticulum is a unique structure. It has been identified in chickens, some other birds and in humans. In all species it is defined as an embryonic vitello-intestinal duct remnant, which in early life connects the yolk sac to the small intestines. In humans, the Meckel's diverticulum has the basic architecture of the small intestines. In contrast to chickens, it does not contain lymphoid tissue, but ectopic gastric fundic or pancreatic tissue in many cases and hence does not play a role in mucosal immunity. On the contrary, being the most common congenital malformation of the gastrointestinal tract it may cause complication ranging from hemorrhages to neoplasms (Sagar et al 2006). In chickens the diverticulum is located about halfway through the small intestinal tract (Branton et al 1987), its wall having the same layered structure as the rest of the small intestines. Its lamina propria contains organized lymphoid tissue, which starts to develop a few weeks post hatching and has germinal centers, lymphocytes and plasma cells (Igbokwe and Abah 2009; Olah et al. 1984).

Like mammals and birds, fish have an immune system including MALT, but there are distinct differences (reviewed by Salinas et al. 2011; Sepahi and Salinas 2016): teleost fish only have single intraepithelial and lamina propria lymphocytes, without organized tissues such as PP. As an equivalent to BALT in animals with lungs, fish have gill-associated lymphoid tissue (GIALT) in gill epithelium and underlying intrabranchial connective tissue. Typical for fish (and amphibians) is the presence of skin-associated lymphoid tissue (SALT). Fish skin is regarded as mucosa, because of its specific anatomical and physiological properties, and MALT here is therefore a cutaneous immune system. In fish MALT macrophages, T and B lymphocytes, plasma cells and granulocytes can be distinguished. In contrast to other animal groups, where IgA is considered the signature antibody of MALT, in fish the prevalent immunoglobulin class is IgT/IgZ in GALT, and possibly also in GIALT and SALT.

4.2.2 Single Mucosal Lymphocytes (IELs and LPLs)

The single lymphocyte pools, diffusely distributed throughout the mucosal effector sites are briefly addressed here, based mostly on information of the single lymphocyte pools in the small intestines. Dependent on the compartment where they reside, intraepithelial lymphocytes (IEL) and lamina propria lymphocytes (LPL) are distinguished.

IELs are abundant (Fig. 4.8), in mice 1 IEL per 5-10 epithelial cells (small intestines) or 1 IEL per 30–50 epithelial cells (large intestines; Beagley et al. 1995), and their phenotypes differ considerably from the LPLs. The IELs express markers that are rare in secondary lymphoid structures, namely CD4/CD8 double positive or CD4/CD8

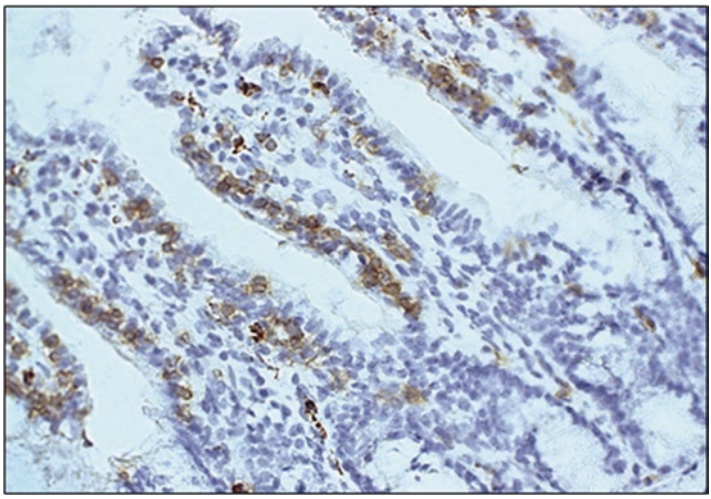

Fig. 4.8 Small intestine of Wistar rat, stained for CD8. Many interepithelial $CD8^+$ lymphocytes, so-called IELs. The lamina propria is almost devoid of $CD8^+$ cells

double negative, and several IELs are NKR-$P1A^+CD3^-CD2^-$ natural killer (NK) cells (Perez-Cano et al. 2005). NK cells are classified as lymphoid cells, which do not respond in an antigen-specific manner. They are therefore grouped under the category 'innate lymphoid cells' (ILCs), together with other lymphoid cells like T helper-like cells (Kim et al. 2016). The ILCs are important players in regulation of homeostasis and inflammation, and dysregulation of the ILCs may lead to allergy and autoimmune diseases (see Sect. 4.2 for MALT in relation to allergy and autoimmunity). The presence of CD8αα instead of the usual CD8αβ T cells contributes to the debate that these T lymphocytes could have matured outside the thymus. More information on the specific character of IELs is given by Vermijlen and Prinz (2014) and Cowley (2014). In the small intestines of Wistar and Fischer rats a significant proportion of the IELs was $CD8\alpha^+\beta^-$. $CD8^+$ cells were situated predominantly close to the basal lamina of the epithelial cells covering the villus. They were not observed in the crypts. $CD4^+$ cells were found mainly close to the apical side of the epithelium, including the crypt epithelium (Bruder et al.. 1999). IELs can express the γδ (TCR γδ; in the gut of mice about 60%, in man about 10%) or αβ T cell receptor (TCR αβ) (reviewed by Qiu et al. 2014). B lymphocytes and (precursor) plasma cells are not present in the epithelium under normal conditions, but they may enter the epithelium upon stimulation by pathogens or cytokines.

The 'conventional' $CD8\alpha\beta^+$ TCR $\alpha\beta^+$ cells probably arrive in the epithelium after being activated in the lymph nodes and spleen (Agace 2008). The trafficking and regulation of the remarkable $CD8\alpha\alpha^+$/TCR $\alpha\beta^+$ and $CD8\alpha\alpha^+$/TCR $\gamma\delta^+$ cells (remarkable because of their absence in secondary lymphoid organs) are still not clear. The number of IELs increases with age, in Lewis rats from below 0.5×10^6 at birth to 1.3×10^6 at PN day 7 and to 3.5×10^6 at reaching adult age (Perez-Cano et al. 2005).

LPLs are primarily conventional $CD4^+$ or $CD8^+$ T cells, positive for TCR αβ. They enter the lamina propria after being activated in lymph nodes or spleen. B cell populations are also present. A distinct B cell population of IgM/IgD double positive cells is situated in the villi of the small intestines and superficial lamina propria of the large intestine (Velazquez et al. 2008). They are present in germ-free mice, thus independent of microbial exposure apparently. Their function seems closely linked to that of NK T cells.

Other single immune cells in the mucosa are monocytes, macrophages and dendritic antigen-presenting cells. Monocytes and macrophages are present in the lamina propria; DCs in the lamina propria and within the epithelium with their processes reaching out into the lumen. An excellent overview of these cell populations and their markers is given by Persson et al (2013).

The IEL and LPL constituents differ between different sites, both quantitatively and qualitatively: between the small and the large intestines (Suzuki 2009; Bowcutt et al. 2014), between the gut and the respiratory tract (Jahnsen et al. 1998), and between the nasal passages, lower airways and within the lungs (Pabst et al. 2008; Wands et al. 2005). The most important differences are related to the distribution of 'unconventional' cells like TCR $\gamma\delta^+$ cells and the diversity of dendritic cell subpopulations, probably reflecting different tasks, fulfilled by the local immune system at different locations. These tasks appear most demanding in the small intestines and indeed the number of IEL and LPL per number of epithelial cells is overwhelmingly large at that site.

The unconventional character of a major part of the single lymphocytes in the epithelium and lamina propria of the mucosae led to the concept that the gut epithelium offers a route for T cell education outside the thymus, thus to be regarded as a primary lymphoid organ (extrathymic maturation; Rocha et al. 1991; Kurd and Robey 2014).

4.2.3 Mucosal Mast Cells (MMC)

Mast cells (MCs) are hematopoietic effector cells of the innate immune system, located strategically close to veins in all vascularized tissues, especially in the dermis of the skin and the lamina propria of mucosal tissues. Their cytoplasm is rich in basophilic granules; upon activation the cells degranulate and release mediators, such as histamine, heparin, leukotrienes, proteases, and various cytokines and chemokines. MCs are major effector cells in inflammatory diseases like asthma, atherosclerosis and arthritis, but have also a protective role as in wound healing, angiogenesis, immune tolerance and defense against pathogens (Kurashima and Kiyono 2014).

MCs develop in the bone marrow via common myeloid and granulocyte/monocyte progenitors; and they share a progenitor cell with basophils (Arinobu et al. 2005). Immature MCs (mast cell progenitors) leave the bone marrow and mature in the peripheral tissues as connective tissue mast cells (CMCs; in rat small intestine

located in the serosa), or mucosal mast cells (MMCs; present in the lamina propria and intraepithelial space of mucosae). This MC heterogeneity is particularly evident in rodents. CMCs maintain granule affinity for metachromatic dyes like toluidine blue after formalin fixation, contain heparin, only express surface IgE and release histamine upon exposure to polyamines like 48/80. In contrast, MMCs lose metachromatic dye affinity of their granules after formalin fixation, contain a non-heparinic proteoglycan, express surface and cytoplasmic IgE, and do not release histamine after 48/80 exposure. Humans have two major subtypes of MCs, based on mast cell-specific protease content: a) MC_T (~ MMCs) contain tryptase but little or no chymase, carboxy peptidase or cathepsin G, and prevail in mucosae; b) MC_{TC} (~ CMCs) contain tryptase, chymase, carboxypeptidase and cathepsin G, and prevail in skin, lymph nodes, submucosae and conjunctiva. MCs also show heterogeneity in other features like heparin content, secreted cytokine spectrum and expression of the receptor for complement factor C5a (Krishnaswamy et al. 2006).

MCs in tissues are long-lived and after de-granulation they re-granulate and live on. The gut of naïve mice appears to have a high number of mast cell progenitors, even in athymic mice and mice lacking T, B, NK and NKT-cells (reviewed by Dahlin and Hallgren 2015). CTMCs are constitutively present in most tissues. MMCs are mainly recruited and activated by a TH2-mediated inflammatory reaction such as in intestinal helminth infections or allergic reactions of the lungs with little or no involvement of resident CTMCs. MMC numbers return to pre-infection values within a few weeks after an inflammatory insult.

MCs have been identified by metachromatic stains such as Giemsa or toluidine blue. Metachromasia is a characteristic change in the color of staining, when the stains bind to particular substances present in tissues or cells. Heparin is strongly metachromatic. Therefore, toluidine blue turns purple to red when it stains heparin. The metachromatic stains have been replaced by immunohistochemistry because it is more sensitive and specific. MCTs in human are mostly identified by tryptase and MMCs in rodents can be characterized by chondroitin sulfate.

Other granulated cells have been described as well in the intraepithelial space, namely granulated intraepithelial lymphocytes (GIELs; containing a cluster of small granules close to the nucleus), globule/globular leukocytes (GLs) and natural killer cells (NKs) or large granulated lymphocytes (LGLs) (Huntley et al. 1984). The terms GIELs and GLs are not common anymore, and whether or not these cell types relate to MMCs or LGLs/NKs is questionable (reviewed by Mowat 1990; Ikeda and Yamashina 1993; Fan and Iseki 1999). Glycosaminoglycan and serine esterase were found in granules of MMCs, GIELs and GLs, but MMC-specific protease was detected in MMC and GL granules, but not in GIEL.

4.2.4 Induction and Regulation of the Immune Response

Fundamental to the immune system of each organism is to discriminate between antigen exposure associated with a threat and antigens that must be tolerated to avoid damaging effector responses (Vaughan et al. 2009). In order to achieve this,

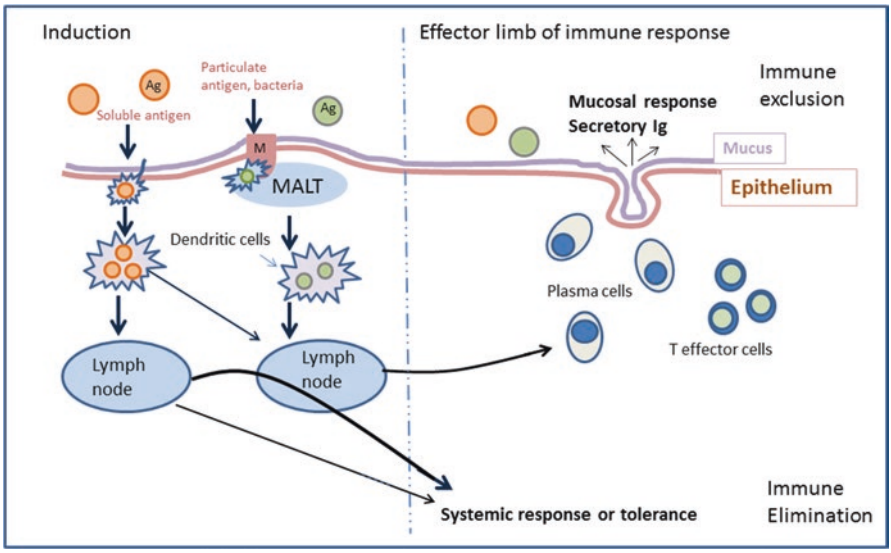

Fig. 4.9 Schematic presentation of induction and effectuation of immune responses via the mucosa (modified after Kuper et al. 2013)

the systemic immune system has to distinguish between self and non-self, whereas the mucosal immune system has to sort out harmless (or even needed) environmental antigens from potentially harmful ones. Several mechanisms enable the immune system to make such a distinction, including the lack of self-reactive B and T cell clones (systemic immunity) and peripheral tolerance (mucosal immunity).

To induce an immune response, antigens must surpass the local defenses, like the mucus layer, and contact the epithelial cells and the antigen-presenting DCs (Fig. 4.9). Upon contact, an immune response is generated in MALT, which is passed on to the draining lymph nodes, such as the mesenteric lymph nodes of part of the intestines and the cervical/submandibular lymph nodes of the upper respiratory tract (Tilney 1971; Kuper et al. 1995). The resulting type of immune response depends on a complex set of factors not only limited to the type of antigen, but also on several host factors (Van den Abbeele et al. 2011). As depicted in Fig. 4.2, the response can be categorized into: (1). elimination of antigen by systemic immunity; (2). exclusion of the antigen at the mucosal surface by secretory immunoglobulins, especially IgA; (3). tolerance to the antigen, which can be regulated via the systemic immune system and locally via molecular events in epithelial cells.

In MALT and local lymph nodes, DCs present antigens to naïve T cells in the T cell region (interfollicular region and paracortex, respectively). The T cells mature into several subtypes like regulatory T cells (Treg), Th1, Th2, Th17 and cytotoxic T cells. Antigen presented to B cells leads to germinal center formation in the B cell area (follicle), where antibody switching occurs and B cells mature into memory cells and precursor plasma cells. The activated, matured T and B cells enter the circulatory system via afferent lymphatics and the thoracic duct and recirculating

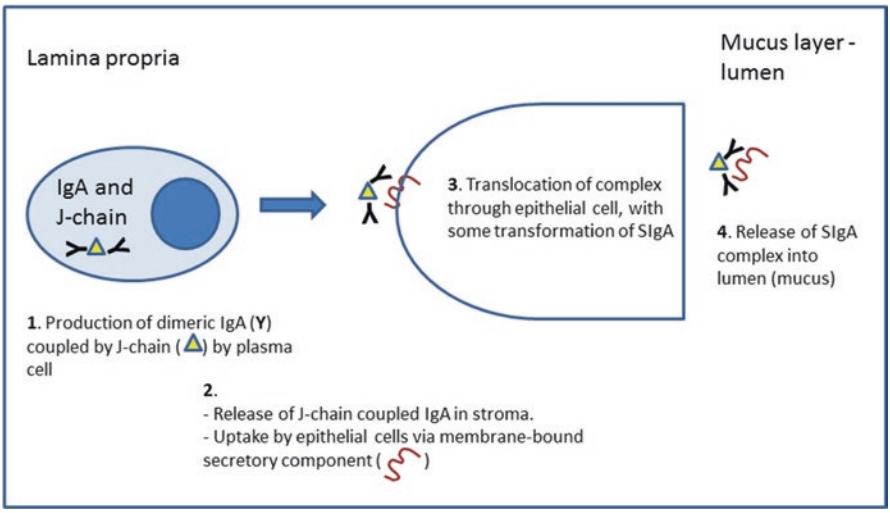

Fig. 4.10 Schematic presentation of production and release of secretory IgA (SIgA) presented in four steps

cells in the blood migrate to the mucosal effector sites. Mucosal immune cells can migrate to antigen-exposed cells as well as to distant mucosal sites. Thus immunization via the nasal mucosa can protect the respiratory as well as the urogenital tract (Mestecky 1987; Brandtzaeg 1997; Wu and Russell 1997; Sato and Kiyono 2012). This protection does not include, however, the entire mucosal system. The original concept of a 'common' mucosal immunity, pioneered by John Bienenstock, no longer applies since more recent research showed particular compartmentalization of effector/memory cell homing towards different mucosal sites, dependent on site-specific endothelial cell adhesion molecule profiles of the HEVs.

The immune response in MALT is especially devoted to the generation of an IgA-antibody response to exclude pathogens, toxins and food antigens, and to regulate host commensal homeostasis (Gommerman et al. 2014). Lamina propria plasma cells produce polymeric immunoglobulins, a process regulated by mucosal T cells. At effector sites, polymeric immunoglobulins (mainly dimeric IgA) are coupled to the J chain within the plasma cells and actively transported by a specific receptor (polymeric Ig receptor or pIgR; Fig. 4.10). This receptor is also called secretory component (SC, a polypeptide of about 70 kDa) and mediates transport of the immunoglobulins across the epithelial barrier into the mucus layer and lumen where bacteria and soluble antigens are bound. By doing so, the secretory immunoglobulins prevent that these bacteria adhere to, and penetrate the mucosal barrier (reviewed in more detail by Brandtzaeg 2013). The mucosal epithelium is thus coated with an 'antiseptic paint'. Secretory immunoglobulins are also secreted via glandular epithelium into saliva, tears and milk. In rodents, hepatocytes produce secretory component as well and thus bile is another source of secretory

IgA into the gut lumen (reviewed by Cesta 2006). SIgM is produced to a lesser extent in addition to SIgA, but the presence of IgG in mucosal secretions is mostly due to passive leakage from the blood. The secretory immunoglobulin system is quantitatively the most significant part of the antibody defense system, 80% of the antibody production by activated B cells in man is taking place in the gut lamina propria (Brandtzaeg 2013).

PP and NALT are the major inductive sites of mucosal immunity, and very significant for systemic immunity. Although most studies are on PP, NALT plays not only the major role in defense of the nasal passages and nearby tissues of ear, eye and oral cavity, but is also the major induction site for protection of the reproductive tissues and adds in protective immunity in the gut (Kiyono and Fukuyama 2004; Ruane et al. 2015). Intranasal immunization against an otitis media pathogen, like *Haemophilus influenzae*, induced specific antibodies in ear wash and specific T memory cells in middle ear mucosa (Kodama et al. 2000). Protection against infection with *Toxoplasmosis gondii* could be achieved by intranasal immunization. The protection was manifested at local sites (nasal, vaginal and intestinal, as well as systemically (Wang et al. 2014). NALT in rodents may function as inductive site for antibodies in tear fluid, and interestingly more efficiently than induction via ocular tissues (Ridley Lathers et al. 1998; Saitog-Inagawa et al. 2000). To overrule tolerance induction, adjuvants may be needed in nasal vaccines, or alternatively sublingual immunization may be applied (Kurono et al. 2012). Induction of tolerance is less explored but is promising in down-regulation of pathogenic reactivity in autoimmune diseases (Xiao and Link 1997). There are indications that PP and NALT may even be involved in brain immunity. Leukocytes from the cerebrospinal fluid of mice with experimental autoimmune encephalitis traffic to PP and orally administered autoantigen induced activation and deletion of autoreactive T cells in PP (Song et al. 2008). NALT might also be involved in draining of brain fluid from the nasal mucosa in small animals like rodents (Kuper et al. 2003).

The scheme in Fig. 4.9 is an oversimplification of the processes taking place at the mucosal surfaces, especially with respect to (a) the functioning of the inflammasomes in epithelial cells (Sellin et al. 2015), (b) immune regulation by neural signaling (Savidge 2016) and (c) with respect to the role of the innate immunity. The innate immune system lacks specificity but complements the adaptive immune system, for example in recognition of true pathogens through pathogen recognition receptors (PRRs; Van den Abbeele et al. 2011; Fig. 4.3). Lymphocyte-like killer cells, macrophages and granulocytes in the mucosa are non-specific effector cells and can kill pathogens, without prior sensitization. In mice, lymphocyte-like killer cells have been characterized as T cells with the specific receptor TCRγδ, which, in contrast to TCRαβ Tc cells, kill targets in an MHC-non restricted manner. They may serve also as initiators of TCRαβ T-mediated responses. While the lamina propria macrophages are of the inflammatory phenotype, and initiate adaptive immune responses against bacteria and tolerance to dietary antigens, the macrophages in the muscularis are more inclined towards tissue protection against environmental disturbances through communication with enteric neurons (Gabyani et al. 2016).

4.3 MALT Pathology

Toxicity to mucosal immune tissues may have consequences that differ from toxicity to systemic lymphoid organs. Also, effects on one component of MALT may influence other MALT components as well. Changes can be part of background pathology or induced. In studies aiming at disclosure of efficacy or toxicity of vaccines or immunosuppressive drugs it can be subject of debate whether the nature of changes observed should be considered as physiologic and intended or pathologic and unintended.

4.3.1 Background Pathology

Dilated lymph vessels are seen regularly in LDALT (Fig. 4.11). LDALT in untreated animals can vary from almost no lymphocyte accumulation to densely populated lymphocyte clusters with some germinal centers; this can depend on animal strain. For example, Brown Norway rats have a more activated appearance of NALT and BALT than Wistar rats (Fig. 4.12). Background findings in NALT and BALT, like a thrombus in NALT area (Fig. 4.11b), are found only incidentally. An interesting incidental finding was the presence of paired lymphocyte clusters on the nasal septum of a rat. Their morphology suggested that they were isolated lymphoid follicles, either belonging to MALT or induced tertiary lymphoid structures. PP may show small foci of mineralisation. This is a common finding in rats and rabbits (Figs. 4.13 and 4.14). Pigment accumulation and clusters of macrophages are also observed regularly (Figs. 4.13 and 4.14). These changes are almost always found in the follicles, especially in the mantle zone of the follicle; the subepithelial dome may be affected as well. Systemic amyloidosis can occur in many species, except most probably rats. This disorder is characterized by progressive depositions of proteinaceous amyloid fibrils in a variety of organs and is usually associated with advanced age. The depositions show a typical green birefringence in Congo-Red stained slides. In systemic amyloidosis, any part of MALT may be affected.

Neoplastic changes of MALT may occur spontaneously, especially in older animals, but they can also be induced by radiation, chemicals or viruses (Fig. 4.14d). Of the primary tumors, follicular cell lymphomas in mice are commonly found in the PP of the ileum. Tumors of other blood cell lines in MALT are infrequent (reviewed by Frith et al. 2001). In general terms, tumors of any organ or tissue that exhibit metastases or an infiltrating growth pattern may affect MALT. Tumor emboli of a neuroepithelial carcinoma originating from the olfactory epithelium have been found in lymph vessels at the base of NALT of a rat (Kuper 2006).

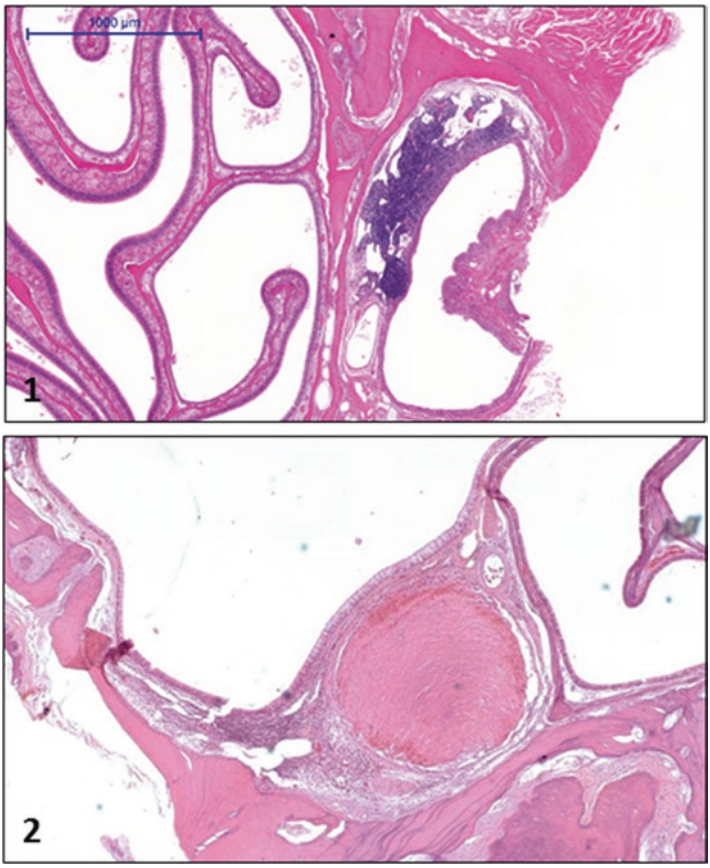

Fig. 4.11 Spontaneous findings in rodent NALT and LDALT. (**1**) Lymphangiectasia, a common observation in LDALT of adult rat; (**2**) Thrombus at NALT site. Accidental finding in nasal section of aged rat. H&E staining

4.3.2 Induced Pathology

Ishikawa et al (2011) studied the histopathological changes of LDALT in rabbits with dacryocystitis induced by inoculation of *Staphylococcus aureus*. The local immune reaction was characterized by an invasion of CD20-positive B lymphocytes, CD3-positive T lymphocytes, IgA positive plasma cells and polymorphonuclear leukocytes and a marked increase of secretory granules in the epithelium covering the lymphoid tissue. Inhalation exposure of mice to a high concentration of glutaraldehyde induced degeneration of the epithelium of NALT (Fig. 4.15). In a few animals, NALT was also less densely populated than NALT of the concurrent untreated controls, which may be a consequence of altered antigen-uptake and antigen

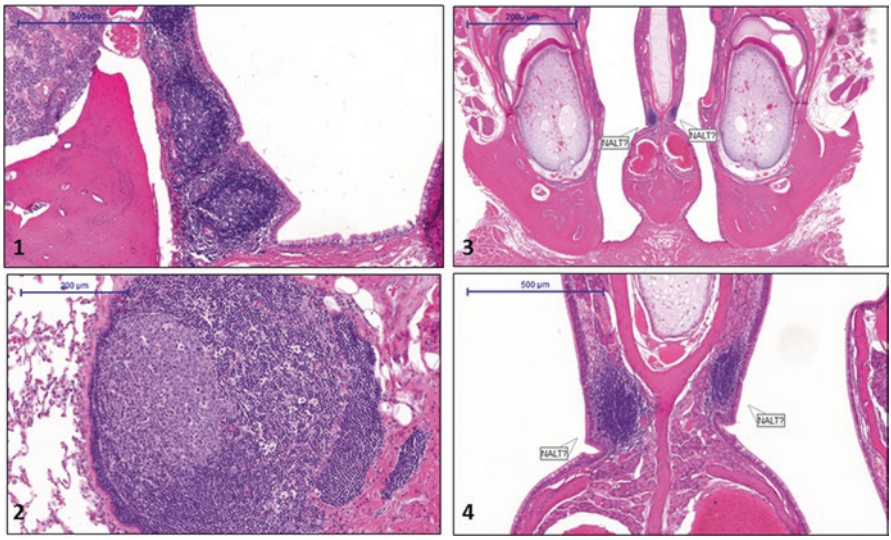

Fig. 4.12 Spontaneous findings in rodent NALT and BALT. (**1**) NALT of Brown Norway rat with several germinal centers; (**2**) BALT of Brown Norway rat with a large germinal center and lymphocyte-filled lymph vessels; (**3**) Rare finding in nasal passages of a Wistar rat: Isolated lymphocyte clusters outside NALT location, (other) features of inflammation were absent. Ectopic NALT, tertiary (induced) lymphocyte cluster? (**4**) Detail of isolated clusters outside NALT location, H&E staining

presentation by the degenerated follicle-associated epithelium in the glutaraldehyde-exposed mice. Changes in the follicle-associated epithelium of NALT has also been observed with high concentrations of formaldehyde (hyperplasia; Kuper et al. 2011) and acetaldehyde (squamous metaplasia). Particles, clusters of (pigmented) macrophages and granulomata are occasionally observed in BALT of animals exposed by inhalation to particles (Fig. 4.16).

Oral treatment of Wistar rats with the immunosuppressant cyclophosphamide resulted in reduced numbers of lymphocytes in all compartments of the PP but also in a reduced number and size of PPs (Fig. 4.17; Kuper et al. 2007). Another immunosuppressant, glucocorticoid, caused a marked reduction of MHC Class II^+ macrophages and T cells in the parafollicular area of BALT (Gemma and Sato 1989).

4.4 MALT Involvement in Generalized Immunopathological Processes

4.4.1 Autoimmune Disease and Allergy

Autoimmunity is an integral part of normal functioning of the immune system, for example in limiting an inflammatory response. However, the regulation of autoimmunity may go wrong and the uncontrolled production of autoantibodies or

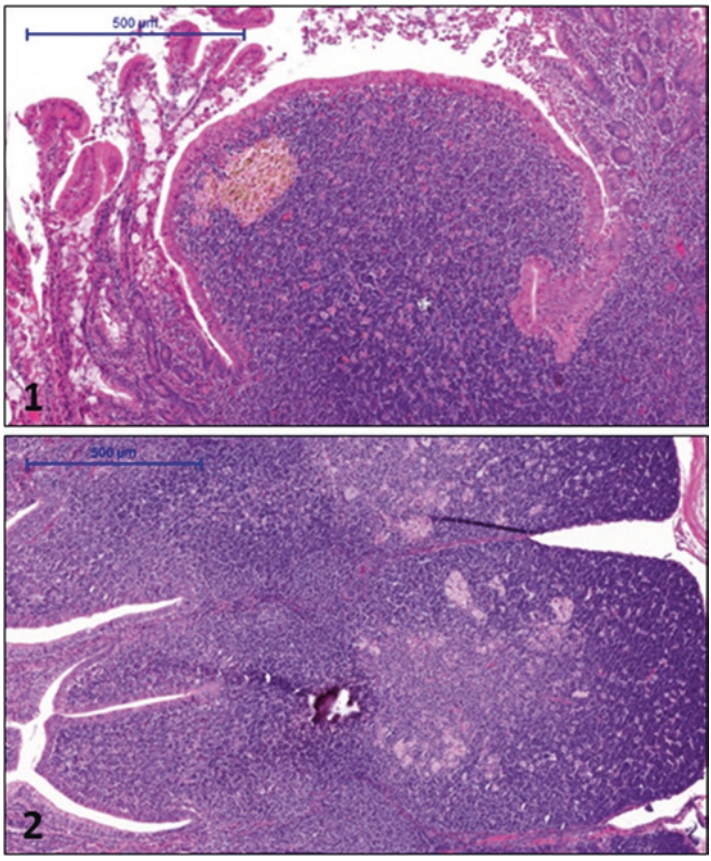

Fig. 4.13 Spontaneous findings in GALT of rabbit. (**1**) Pigment in PP; (**2**) Mineralization and clusters of macrophages in appendix. H&E staining

autoreactive T cell activation will cause serious disease. Central tolerance induction in thymus and bone marrow is key to controlling autoimmunity. Peripheral anergy/tolerance processes, proper functioning of T regulatory cells, cytokine regulation and regulation of apoptosis by clonal deletion also play a role and these processes take place in secondary lymphoid tissues including MALT. These regulations need also to occur at sites of inflammation, to keep inflammation in check. Much attention has been paid recently to the role that mucosal microbiota play in the development of autoimmune and autoimmune-like inflammatory conditions (Tlaskalova-Hogenova et al. 2011). A link between airway mucosal immunity and rheumatoid arthritis has been suggested by Demoruelle et al. (2014), for instance. Involvement of MALT has not yet been addressed in either the induction of exaggerated and uncontrolled inflammation nor as a consequence of it. The exception is PP, which have been recognized as a site of deletion of autoreactive T cells (Song et al. 2008). The consequences of disturbed mucosal regulation is best illustrated by autoimmune liver diseases (reviewed by Trivedi and Adams 2013).

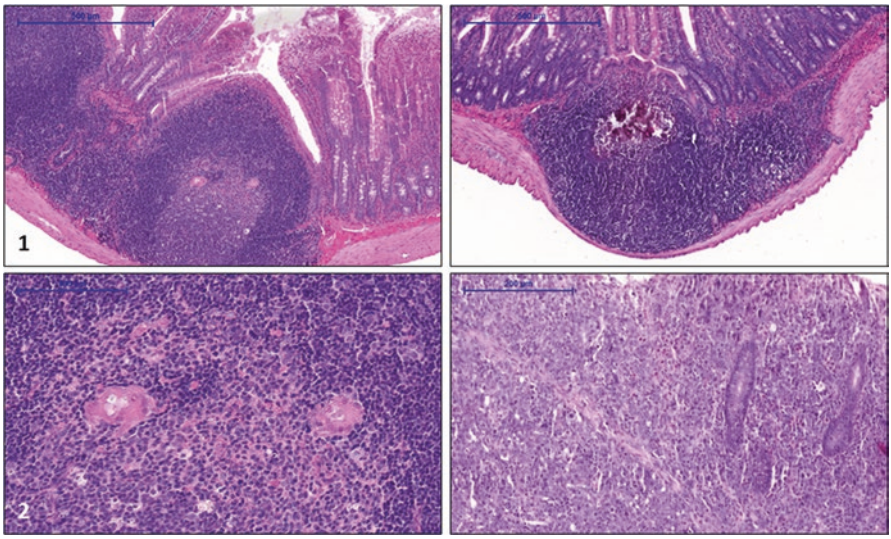

Fig. 4.14 Spontaneous findings in GALT of rat. (**1**) Overview of macrophages clustered around foreign bodies; (**2**) Detail of macrophage clusters; (*upper right image*) Mineralization in Peyer's patches, the subepithelial dome and/or apical zone of follicle appear predilection sites of spontaneous mineralization. In case of induced atrophy (germinal center collaps) mineralization can be observed centrally in the germinal center. (*lower right image*) Lymphoma in PP of Wistar rat. H&E staining

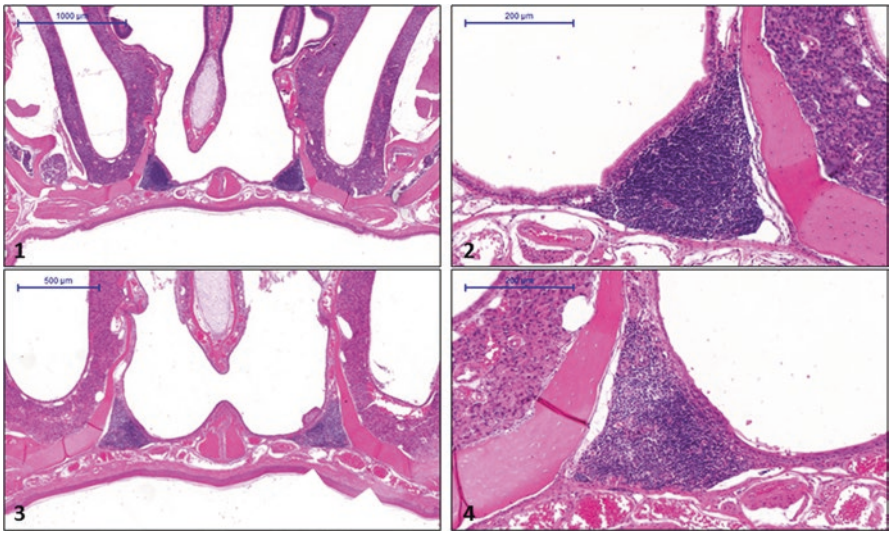

Fig. 4.15 Induced pathology of NALT. (**1**) Overview and (**2**) detail of NALT of untreated concurrent control rat; (**3**) Overview of NALT of rat upon inhalation of a high concentration of glutaraldehyde. The epithelium covering NALT shows squamous metaplasia; (**4**) Detail of NALT, depicted at the left side in the overview. Damage to the epithelium often is accompanied by or leads to a decrease in cellularity and loss of germinal centers of the lymphocyte compartments of NALT. H&E staining

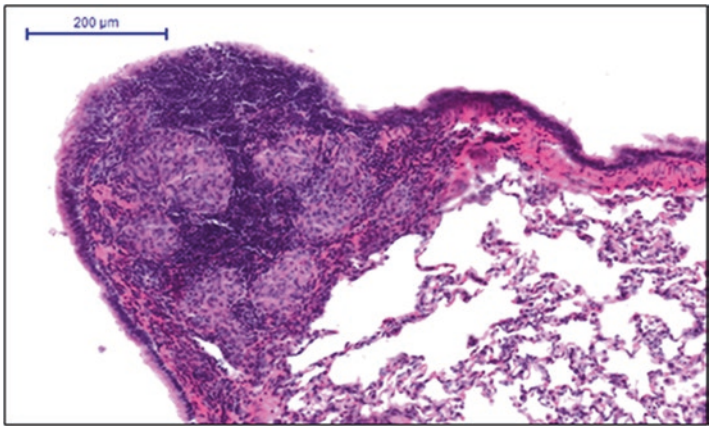

Fig. 4.16 Induced pathology of BALT. Granulomata in BALT of rat upon particle inhalation

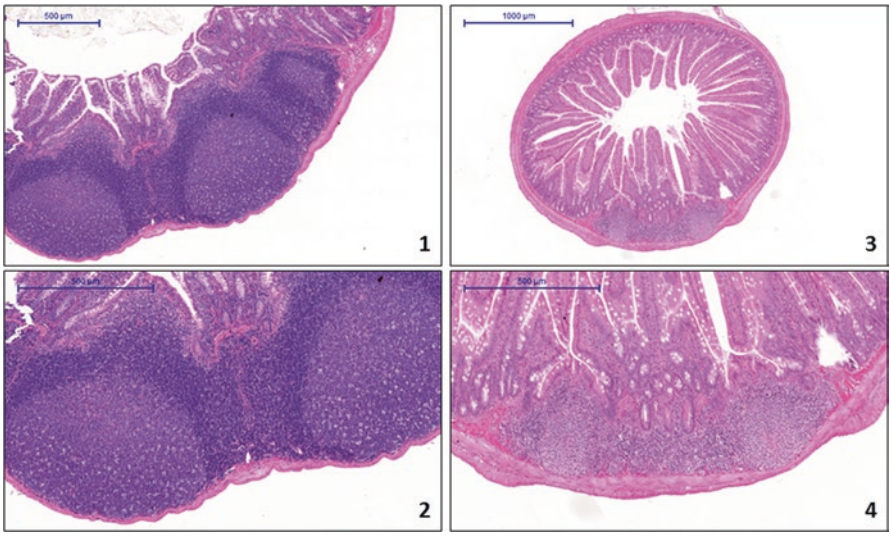

Fig. 4.17 Induced pathology of Peyer's patches (PP) of rat. (**1**) Overview and (**2**) detail of PP of unexposed concurrent control rat; (**3**) Overview and (**4**) detail of PP of rat exposed orally to cyclophosphamide. Marked decreased cellularity, interfollicular areas and follicles, compared to control

Autoimmune hepatitis, primary biliary cirrhosis and primary sclerosing cholangitis are associated with inflammatory bowel disease and coeliac disease. It is hypothesized that aberrant expression of adhesion molecules and chemokines misdirect intestinal lymphocytes towards the liver and to trigger hepatic inflammation.

Allergy is an exaggerated immune-mediated response to essentially innocuous substances manifested itself locally with inflammation at the particular mucosal

sites (or skin), where exposure to the eliciting antigen took place. It is in fact a local manifestation of a systemically altered immune system. Allergy is a two-phased process, with an induction phase upon first contact with the allergen/sensitizer, and a provocation or elicitation phase upon re-exposure. The induction phase is not clinically manifest, but allergen-specific memory and effector T cells and/or allergen-specific antibodies are present in the circulation and at the mucosal sites when an individual is sensitized. The provocation or elicitation phase is clinically manifest, most of the times restricted to the application or contact site, but sometimes also at distant mucosal sites and the skin. Indeed food allergy can lead to intestinal complaints and to shortness of breath and skin rash (Turnbull et al. 2015).

Is MALT involved in induction or provocation of allergy? MALT may not be the primary site of induction. Soluble allergens/sensitizers can be taken up by non-specialized mucosal epithelial cells without the need for M cell involvement, which are clustered above MALT and only scattered in the epithelial lining of the mucosae. Particles are primarily processed by M cells. Examples of particulate allergens are pollen (because of their size deposited predominantly in the upper respiratory tract), and these may be taken up primarily via M cells. Whether processed via ordinary epithelium or M cells, allergens like pollen, to which we are exposed regularly, should normally evoke tolerance (Scheme, Fig. 4.9), otherwise our intestines, respiratory tract and skin would be chronically inflamed. A short-term inhalation study with chemical respiratory allergens in mice did not reveal stimulation or atrophy of NALT, thus morphological signs of involvement of NALT in the induction phase were absent (Arts et al. 2008). In addition, there are to our knowledge no reports describing a link between adenoidectomy (tonsillectomy) in infants and a changed risk of allergic rhinitis development. MALT may play a role in the provocation or elicitation phase, based on the following observations. NALT was stimulated (i.e., showed increased germinal center development and enlarged interfollicular areas) in dermally sensitized mice by inhalation of an aerosol of toluene diisocyanate (TDI; a chemical respiratory allergen; JHE Arts and CF Kuper, unpublished observations). In allergic children, enlarged adenoids responded to sublingual immunotherapy with specific allergens, which suggest that tonsillar tissue may be involved in allergic rhinitis (Masieri et al. 2014). Increased numbers of antigen-presenting cells and eosinophils were observed in the adenoids of allergic children (Vinke and Fokkens 1999). In OVA-specific T cell receptor transgenic mice, the PPs and mesenteric lymph nodes appeared to co-operated in enteropathy development, caused by the food allergen OVA, although the precise role of the PP remained unclear (Nakajima-Adachi et al. 2014). Finally, it has been suggested that the risk of allergy is increased in individuals with SIgA deficiency, lacking compensation in SIgM, but the evidence is weak. Alternatively an increased incidence of microbial infections in the absence of an appropriately working secretory immunoglobulin system promotes allergy development (reviewed by Brandtzaeg 2013).

4.4.2 Immunosuppression and Immunostimulation

MALT can be affected by several types of exposure, ranging from immune modulating drugs to types of diet and exercise. It is not always clear if the effects result from directly targeting the mucosal lymphoid tissues or as a consequence of systemic immune modulation. Also, it is unknown if changes in local mucosal lymphoid tissues automatically affect distant mucosal lymphoid tissues.

Experimental studies in rodents showed that PP were affected along with the thymus, spleen and lymph nodes (Kemmerling et al. 2015; Kuper et al. 2007). The immunosuppressive drugs azathioprine and cyclosporine induced histopathology in several lymphoid organs of rats after oral exposure for 28 days. PP were affected at the higher doses of azathioprine and cyclosporine. Both drugs need to be metabolized to become effective, but the way in which MALT is affected by these drugs is not known precisely. The relatively high doses needed to induce decreased cellularity in PP point to several options, such as (a) the drugs did not reach the PP in sufficient quantities; (b) PP lymphocytes are less sensitive to the drugs; (c) the effects are indirectly induced via the primary lymphoid organs thymus and bone marrow, taking more time (>28 days) to influence PP; (d) examination of PP was inadequate (see Sect. 4.3). Oral exposure of pregnant rats to the immunosuppressive drug cyclophosphamide induced an increase in the number of grossly visible PPs in their offspring, examined at 10 weeks of age (Kuper et al. 2007). The increased numbers of PPs occurred in the absence of a change in weight and morphology of the target organs thymus and spleen. This indicates that PPs can be a rather sensitive lymphoid organ, at least when exposure occurs perinatal. Studies in which another route of exposure was used are scarce. Histopathology was not performed. MALT at different locations may react differently to immunosuppressive drugs. The immunosuppressive drug FTY720 for example induced within hours after administration decreases in the number of peripheral blood, thoracic duct and splenic lymphocytes, but increases in mesenteric lymph nodes and PP lymphocytes (Chiba 2005). These location-dependent effects have been ascribed to sequestration of mature lymphocytes from the circulation into peripheral lymphoid organs including PPs. The environmental chemical hexachlorobenzene has immune-modulating properties and induces increased cellularity in a number of lymphoid organs like bone marrow, spleen, lymph nodes and PPs. In addition, HEVs in PPs are much more prominent in exposed animals (Fig. 4.18). PPs were affected at the same doses as the other lymphoid organs (Schulte et al. 2002).

MALT may be involved in acute graft-versus-host disease (GVHD). GVHD can complicate allogeneic tissue transplantation, when immune cells in the transplanted tissue (the graft) act against the immune-depleted ‘foreign’ host. GVDH target organs are skin and mucosal surfaces (gastrointestinal tract, lungs), and the biliary system. PP may be key lymphoid tissues as differentiation sites of donor-derived anti-host cytotoxic T lymphocytes (reviewed by Zeiser et al. 2004).

Diet can have an effect on immune cells of PPs, and consequently may influence the intestinal immune system. Feeding mice nine weeks with diets high in carbohydrate or fat, decreased the number of $CD19^+$ cells and increased the number of IgA^+ B cells

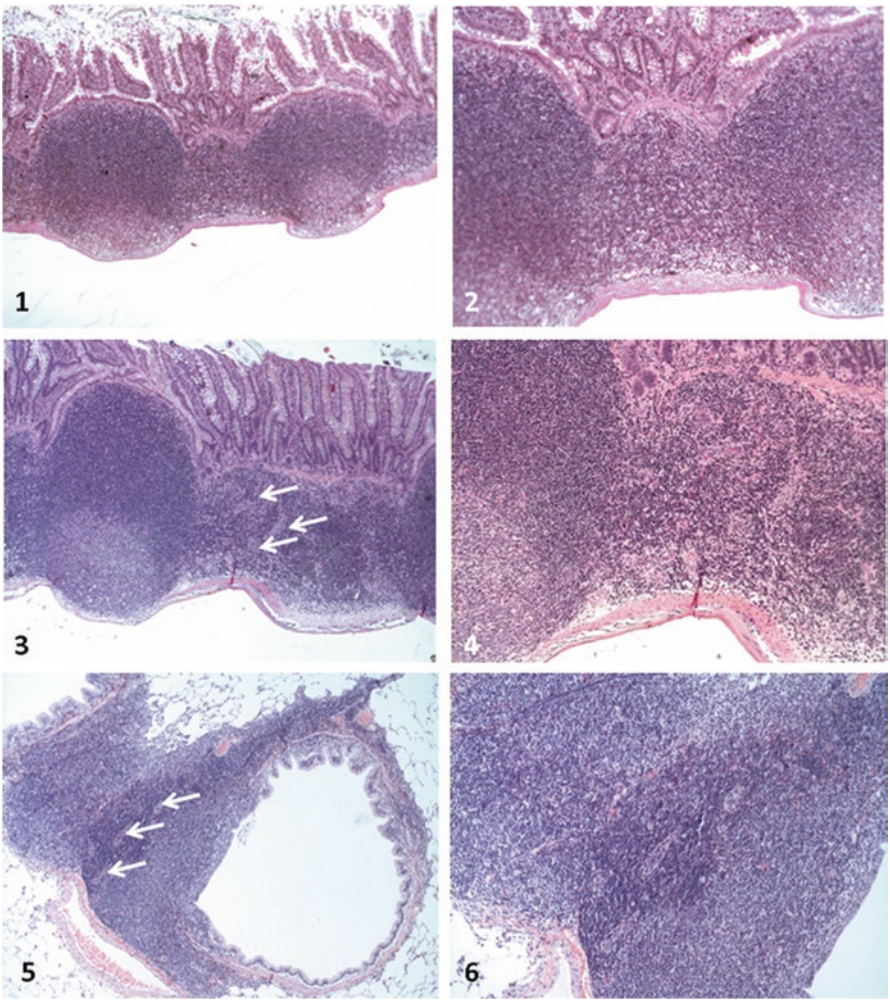

Fig. 4.18 Induced pathology of Peyer's patches (PP) of rat. (**1**) Overview and (**2**) detail of PP of unexposed concurrent control rat; (**3**) Overview and (**4**) detail of PP of rat exposed to hexachlorobenzene (HCB), same magnifications as in Figures of control. The PP is increased in size and the interfollicular area contains many high endothelial venules (HEVs; arrows). (**5**) Overview and (**6**) detail of a single animal exposed to hexachlorobenzene, with a large BALT with large-sized germinal centers and high endothelial venules in the T-cell dominant area; the relationship with the exposure was difficult to verify, because BALT was not sampled in a standardized manner (this particular BALT could be two fused BALTs; also BALT was absent in lung sections of several animals, not being the scope of the study). H&E staining

in PPs (Valdes-Ramos et al. 2010). LPLs and lymphocytes in peripheral blood were similarly changed. Mucosal immunity can also be altered by physical activity. Acute and/or intensive exercise may cause suppression of SIgA in saliva and transient diarrhea, but how this relates mechanistically to mucosal immune functioning is far from clear. Exercise in mice increased T cell proliferation and cytokine production

in PPs. Again, such changes should be interpreted with caution with respect to consequences for overall immune functioning: exercise was found to significantly change lymphocyte numbers but cell function was not changed much.

4.4.3 Sampling and Selection of MALT and IELs/LPLs

MALT histology is often regarded as lacking sensitivity for evaluating effects upon exposure to immune modulating substances. This may relate to the way in which MALT is sampled at necropsy (Kuper et al. 1995). Azathioprine for example decreased the number of grossly visible PPs, but the patches selected for and examined by microscopy appeared normal (Bruder et al. 1999). This may be due to a selection bias of those PPs that remained grossly visible. It may be too laborious to count the number of all visible PPs in the small intestines at routine necropsies. The PPs in the distal part of the small intestines of rats appeared most sensitive to cyclosporine A and cyclophosphamide, with the exception of the large patch in the ileum (Kuper et al. 2007). Although it remains to be investigated if this relatively high sensitivity is a general property of the distal PPs, counting the number of grossly visible PPs within 40 cm from the ileocaecal junction may be sufficient. In addition, standardized selection of a few PPs for histopathologic examination may be a method with sufficient sensitivity to detect potential immunotoxicity. An alternative method is the preparation of so-called Swiss rolls, allowing examination of a considerable larger portion of the small intestines (Moolenbeek and Ruitenberg 1981). An advantage of this method is that cryptopatches and lymphocyte-filled villi can be examined as well.

NALT is usually examined microscopically in cross sections through the nasal passages. Unfortunately, this approach results in transections of NALT at its smallest diameter. Moreover, T- and B-cell areas in NALT are oriented more or less from rostral to caudal. Thus, one or two cross-sections through NALT enable detection of only severe effects. Alternatively NALT can be dissected from the nasal tissues and embedded in toto, but this implies that (part of) the nasal tissues are lost for histopathological evaluation. Methods to isolate NALT in mice have been described by Asanuma et al. (1997). These authors also described a method to isolate nasal IELs. In toto sampling of NALT of rats has been described by Koornstra et al. (1991) and Kuper et al. (2011).

It is common guideline practice to recommend examination of NALT in inhalation and intranasal application studies and to examine PPs in oral studies. However it is very informative to observe if a compound has affected only a specific compartment of the mucosal immune system, at the site of application or exposure, or if the mucosal immune defense in general is changed. For example deoxynivalenol (DON or vomitoxin, a mycotoxin in food contaminated with *Fusarium* spp. fungi) targets the intestinal epithelium and mucosal immune system including PPs (Pinton and Oswald 2014). Importantly, this may be a contributing factor to inflammatory disease. Despite intensive research it is still unclear how DON exerts its immune modulatory effect. To assess the risk of this common food contaminant to humans and evaluate whether PPs are targeted directly or indirectly, it could be helpful to sample NALT as well, when having to decide whether effects are local, restricted to the intestinal immune system, or also affect MALT elsewhere (Escrivá et al. 2015).

As stated in the introduction, single lymphocyte pools are more difficult to study than the more organized MALT. This applies to morphologic assessment in conventionally-stained tissue sections, as well as to isolation by flow cytometry. Several isolation methods have been reported, both for IELs and LPLs, but these are laborious and have their weaknesses (Sheridan and Lefrançois 2012). Importantly, contamination of IELs with LPLs and vice versa as well as contamination with smaller MALT (like cryptopatches and lymphocyte-filled villi LFVs) in the gut occurs easily. Immunohistochemical or immunofluorescent staining can help identify these single cells populations in tissue sections, but with low sensitivity.

4.5 Animal Models: MALT in Immunodeficient Animals

Knock-outs and immunodeficient animals have contributed much to our understanding of MALT development, in particular of PPs (Debard et al. 1999; Paxian et al. 2002; Ferreira et al. 2012), and of the role of dysregulated T cell responses in inflammatory diseases at the different mucosal sites (Hyland et al. 2009; Davis et al. 1998). Severe combined immunodeficiency (SCID) animals lack both functional T and B cells. IgA^+, IgG^+ and IgM^+ B cells and T cells were absent in NALT of C.B-17/lcr-scid Jcl SCID mice, but the epithelium still contained M cells (Karchev et al. 2003). In the remaining section we briefly discuss salient MALT changes in two immunodeficient animal models, the athymic or nude rat and mouse, which are from originally spontaneous mutants.

Nude rats and mice lack a normal thymus and functionally mature T lymphocytes, although the thymus anlage is present as clusters of epithelial cells. The peripheral lymphoid organs including PPs and NALT are small, with reduced T-cell dominated interfollicular areas. CP are present in nude mice and LFV are present in athymic nude rats (Taylor and Williams 2005). The B-cell follicles have often no or small germinal centers because T cell-dependent antibody responses cannot take place. B cell function itself appears unaffected (rat: Hougen 1991). The T-cell areas in PPs and NALT of the nude rat are not devoid of cells, but house many $CD68^+$ (a pan macrophage marker) cells in addition to some B cells (Fig. 4.19a). The high number of macrophages here may reflect a way to compensate the lack of functionally mature T cells and thus an insufficient acquired immune defense (like observed in the *Foxn1* $^{-/-}$ or nude mouse: Cheers and Waller 1975). Other cells of the innate immune system like NK and dendritic cells can compensate in some ways as well (Rolstad 2001). The small intestines of the nude rat house an increased number of lymphocyte clusters, compared to the wild type (unpublished observations; Fig. 4.19b). These clusters may be LFV or isolated lymphoid follicles ILF. The clusters consist of cells negative for immunoglobulin light chains (kappa), CD45 RA (common leukocyte antigen; B cells) and IgA. Many MHC Class II^+ cells were present, several $CD68^+$ cells and some $CD8^+$ and $CD4^{low+}$ cells. With age, the nude rat develops T-like cells (lymphocytes with T-cell associated membrane markers), although it is questionable whether these cells are functionally equivalent to wild-type T cells.

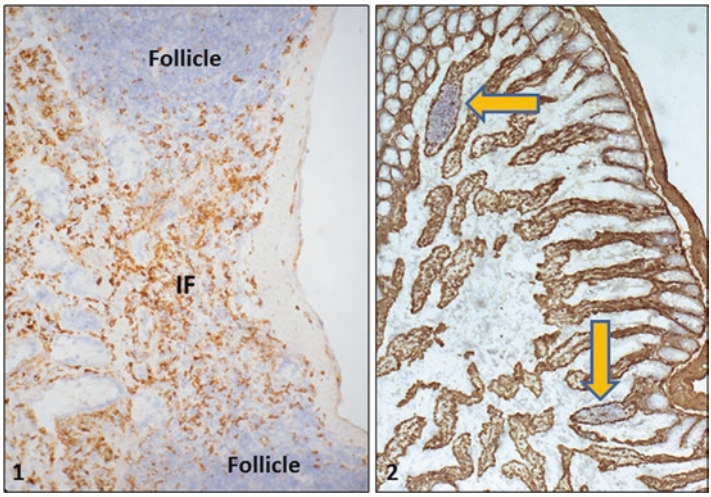

Fig. 4.19 MALT of Nude (athymic rat). (**1**) Peyer's patch, stained for CD68. The interfollicular area houses a high number of $CD68^+$ tissue macrophages instead of T lymphocytes. (**2**) Small intestine, laminin staining. Two lymphocyte clusters, so-called lymphocyte-filled villi or LFV (*arrows*) are located close to each other

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Chapter 5
Immunopathology of the Nervous System

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Abstract The central and peripheral components of the nervous system exhibit extensive integration and interaction with the immune system in both health and disease. Components of the adaptive immune system touch all systems in the body through the blood and lymphatic vascular system, as was described in the chapter on immunobiology in Part I of this book. The nervous system is no exception to adaptive immune system interface. The immunobiology described in this chapter focuses on features of the innate and adaptive immune systems which are specific to the nervous system, and which are especially critical to health and diseases of the central nervous system. Selected immune conditions of the nervous system are explored in more detail to examine the complex interactions of the immune system with the nervous system and the consequences of imbalance between these systems on human health.

Keywords Alzheimer disease • Attention deficit disorder with hyperactivity • Autistic disorder • Blood-brain barrier • Brain injuries • Mental disorders • Multiple sclerosis • Nervous system • Neuroimmunomodulation • Parkinson disease

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5.1 Immunobiology of the Nervous System

5.1.1 Blood-Brain Barrier and Circumventricular Organs

The blood-brain barrier (BBB) is a key component of the innate immune system of the central nervous system (CNS). The BBB acts to limit exposure of the CNS system to various toxicants and infectious agents, as well as to maintain the appropriate environment for neural function. While the BBB limits exposure of the CNS to exogenous insults, this does not imply that the CNS is an immune-privileged site (Lampron et al. 2013; Wraith and Nicholson 2012). There is a high level of immunological activity in the CNS, as will be demonstrated throughout this chapter (Ransohoff and Brown 2012; Ransohoff and Engelhardt 2012).

The BBB is composed of capillary endothelial cells with tight intercellular junctions, pericytes surrounding the capillaries, vascular basement membrane, pericapillary astrocytes, and a collection of receptors, channels, and transporters for selective entry and exit of substances. This is a simplified view of a very complex and selective barrier system, the detailed description of which is beyond the scope of this chapter. The BBB is not a static barrier, but plays an active role in immune surveillance, immune cell trafficking, and modulation of immune responses (Miller 1999). The reader is referred to several excellent publications on the blood brain barrier (Banks 1999; Carvey et al. 2009; Daneman and Prat 2015).

Circumventricular organs (CVOs) are specific areas of the brain which do not have the complete array of traditional BBB components. CVOs have features such as fenestrated capillaries, large perivascular spaces, and specialized ependymal cells which caused them to be thought of as leaky areas of the brain (Miyata 2015). Eight different regions of the brain have been proposed as CVOs. The sensory CVOs, thus named due to their function in sensing blood and cerebrospinal fluid components and homeostasis, include the organum vasculosum of the lamina terminalis, subfornical organ, and area postrema (Miyata 2015). The secretory CVOs, thus named due to their neurosecretory functions, include the neurohypophysis (pars nervosa), median eminence, and pineal gland (Miyata 2015). According to some researchers, the intermediate lobe of the pituitary gland (pars intermedia) is also considered to be a secretory CVO (Cottrell and Ferguson 2004). Additional uncategorized CVOs include the subcommissural organ which lacks fenestrated capillaries, and the choroid plexus which has fenestrated capillaries but lacks neurons (Miyata 2015).

The functions of the CVOs are complex and prohibit blanket categorization of these areas as merely leaky points in the BBB. The CVOs play roles in sensing solute composition in fluids, regulating body temperature and energy balance, sensing blood-derived substances and inflammatory responses, and secretion of critical neuropeptides and hormone-releasing factors, among other specialized functions (Cottrell and Ferguson 2004; Miyata 2015). In addition, due to delimiting barriers around the CVOs, the CVOs allow for communication between the blood and cerebrospinal fluid and local neuroparenchyma without liberal access to the remainder of the brain (Banks 1999).

5.1.2 Glial Cells and Other Inflammatory Cells

Among glial cells, microglia are the resident immune cells of the CNS. While microglia are the primary players, astrocytes and oligodendrocytes also play roles in immune responses in the CNS (Amor and Woodroofe 2014). Microglia are surveillance cells which can become activated and intimately involved in innate immune responses (Kofler and Wiley 2011). Microglial activation is a component of the innate immune response in multiple CNS diseases including epilepsy, amyotrophic lateral sclerosis, multiple sclerosis, Parkinson's disease, heavy metal toxicity, infections, and neoplasia (Amor and Woodroofe 2014).

Astrocytes play an essential role in maintaining normal brain function. Astrocyte functions include synthesis and release of growth factors, regulation of extracellular environment, neuronal support, and removal of toxicants and debris from the cerebrospinal fluid (Sidoryk-Wegrzynowicz et al. 2011). Disruption of normal astrocytic functions in the brain may play a role in neurodegenerative conditions such as Parkinson's and Alzheimer's disease (Sidoryk-Wegrzynowicz et al. 2011).

Some inflammatory cells, such as mast cells, also normally reside in the CNS. Mast cells are often among the first responders in an immune response, recruiting other cells and amplifying the immune response (Dong et al. 2014). Mast cells are capable of synthesis and rapid release of numerous mediators including histamine, serotonin, cytokines, enzymes, neuropeptides, and nerve growth factor (Dong et al. 2014). Their recruitment of immune cells includes monocytes and eosinophils as well as astrocytes and microglia (Dong et al. 2014). There is evidence to support the role of mast cells in inflammatory CNS conditions such as stroke, multiple sclerosis, and Alzheimer's disease (Dong et al. 2014).

Monocytes play a key role in inflammatory conditions in the CNS. Through complex signaling events, recruited monocytes migrate to the CNS where they differentiate into macrophages and dendritic cells and play a role in the modulation of inflammatory responses (Ashhurst et al. 2014). Monocytes are often selectively recruited into the brain during inflammatory responses, in preference over other inflammatory cell types (Persidsky 1999).

T lymphocytes are known to normally circulate in the cerebrospinal fluid even in the absence of an inflammatory response (Wraith and Nicholson 2012). Even when these lymphocytes are activated and come into contact with the BBB, lymphocyte migration and entry into the CNS remains dependent on appropriate antigen encounter and regulation by specific chemokines and receptors (Wraith and Nicholson 2012).

The presence of immune cells in the CNS has its benefits for host defense. However, when the delicate balance between immune system and nervous system function and communication is disrupted, a proinflammatory environment can lead to serious implications in regards to nervous system function (Filiano et al. 2015). Some of these resulting neuroinflammatory conditions are described in more detail in this chapter.

5.1.3 *Inflammatory Reflex*

The interactions between the immune system and the nervous system are bidirectional. While the immune or inflammatory responses can affect the structure and/or function of the nervous system, the nervous system can also directly affect immune system responses.

Activation of the sympathetic nervous system results in release of catecholamines and neuropeptide Y from sympathetic nerve terminals; both act on leukocytes to induce mobilization and increase adhesion (Bedoui et al. 2001).

Activation of nociceptors in the peripheral nervous system causes the release of numerous mediators from the peripheral sensory neurons (Chiu et al. 2012). These sensory mediators of the peripheral nervous system not only communicate with local vasculature, but also activate immune responses (Chiu et al. 2012). This neurogenic inflammation plays a role in host defense and immunopathology (Chiu et al. 2012).

5.2 Selected Immune Conditions of the Nervous System

5.2.1 *Traumatic Brain Injury*

Traumatic brain injury (TBI) is associated with cerebral edema, blood brain barrier (BBB) disruption and inflammation, which contribute in varying degrees to the severity of the injury and subsequent recovery. Primary injury to the head, and thereby brain, can be the result of a variety of circumstances that include direct injury of the brain by the skull (from the skull itself or via abrasion against the interior skull), shearing of the brain via internal motion, cerebral hematomas produced by disruption of the cerebral vasculature with dura mater, and dysregulation of the BBB (Lighthall and Anderson 1994). Traumatic brain injury is the most important cause of disability in individuals under the age of 45 years (Fernandez-Gajardo et al. 2014) and can even lead to the development of delusional disorders and schizophrenia-like psychoses (Fujii and Ahmed 2014). Classically, TBI can occur when the head suddenly and violently hits an object, or when an object pierces the skull and enters brain tissue. Common causes include automobile accidents, physical/blunt trauma (e.g., sports, abuse, accidents), and falls (e.g., bikes, skateboards, ATVs, walkers, windows). In terms of transportation-related deaths, the Center for Disease Control and Prevention (CDC) stated that those insults causing traumatic brain injuries were 6.9 per 100,000 fatalities in 1994 (NHDS et al. 2010). However, more recent data has shown that as of 2010, there were an estimated 1.4–2.5 million cases of TBI registered annually in the United States; the vast majority were classified as mild to moderate with only a small fraction requiring subsequent hospitalization (Twamley et al. 2014; NHDS et al. 2010).

Although there are different types of TBI, such as repeated subthreshold trauma, the following discussion will be limited to a single traumatic event (even though this

information can also be applied to other examples). In many cases, TBI, or associated damage, is classically considered to occur in two distinct stages. The initial or primary injury occurs when the initial damage takes place via external trauma such as physical puncture, hemorrhaging, and cellular damage. The second stage involves a delayed response resulting in damage via acidosis, intracranial hypertension, seizures of unknown origin, hypotension, and other metabolic/physiologic dysregulations that can occur hours to weeks after the initial insult. In all cases, one of the factors that directly affects the outcome following severe TBI is the development and progression of cerebral edema with associated increase in intracranial pressure (ICP), which has been actively debated over the last nearly 100 years (Timofeev and Hutchinson 2006). Unfortunately, little can be done to prevent and reverse the injury itself, so the focus of treatment has been to reduce further injury to the trauma site. This is done by ensuring proper blood supply to the brain and the rest of the body, maintaining adequate blood flow, controlling blood pressure, and controlling inflammation, along with other immune system responses. This indicates that the immune system (and corresponding inflammation) is a key player in the second stage of injury.

5.2.1.1 Neuropathology

The volumetric loss, cellular damage, and associated histopathological changes are generally unique to the specific type of TBI including the location of insult, force of the damage, concurrent non-head trauma, onset of treatment, and preexisting conditions. However, there are commonly identified neuropathological findings of parenchymal hemorrhage, widespread axonal damage (at and away from the site of injury), subarachnoid hemorrhage, gross tissue changes (along with partially/destroyed vasculature), and necrosis at/around the site of insult. The below examples serve to provide some insight into a broad range of TBI; more specific examples are included as part of the animal model section in which additional invasive assessment techniques and research controls can be used to help reduce variability and maximize data collection.

Leijdesdorff et al. (2014) reported that from over 1250 roadside accidents (pedestrians, cyclists, and motorized vehicles) subdural and subarachnoid bleedings were most frequent, with damage to the cerebrum, cerebellum, and brainstem along with skull fractures being the most common sites of injury. Hemorrhage, specifically intraventricular hemorrhage, is one of the leading factors resulting in poor outcomes and long lasting disability in TBI patients. Through Computed Tomography (CT) scan analysis of over 370 patients, intraventricular hemorrhage was found to correspond to corpus callosum damage (Matsukawa et al. 2012). The associated damage to the white and gray matter can vary, depending on the injury, immune response, and post-TBI treatment. In a case study conducted by Shin et al. (2012) over several months, high-definition fiber tracking was used to assess white matter damage following TBI, in part, due to the noted behavioral and motor deficits. This study reported that axonal damage was present at descending fibers at the level of the cortex, internal capsule, and midbrain. Specifically, the authors reported a localized decrease in fibers of the

motor areas (corona radiata) with substantial loss of axon volume in the motor and premotor areas, corresponding to midbrain volume losses and focal fiber breaks in the corticospinal pathway. A longer-term study that evaluated more than 30 patients with Magnetic Resonance Imaging (MRI) for approximately 3 years following the initial TBI report indicated that there was gray matter cortical thinning (frontal and temporal cortex) along with a related decrease in cortical thickness, which corresponded to reported deficits in working memory (Merkley et al. 2008).

5.2.1.2 Pathogenesis of Immune Response

Symptoms of a brain injury often show similarities across classifications, such as headaches, cognitive status, and sensitivity to external stimuli, followed by a dysregulated brain metabolic profile up to a month following the initial insult (Shrey et al. 2011). The key factor from a treatment standpoint often involves focusing on the disruption of metabolic activities within the affected population of cells. Specifically, the inflammatory response and oxidative stress following injury, along with the directly associated physical insult causing neuronal damage, and cellular damage/death can be similar in some cases to what is noted with the other types of severe TBI.

In a review by Hinson et al. (2015), analysis of nearly 20 years of clinically published data indicated that trauma initiates local CNS as well as systemic immune activation. Numerous observational studies describe elevation of proinflammatory cytokines that are associated with important clinical variables including neurologic outcome and mortality. Injury to the brain results in activation of signaling molecules, cytokines, chemokines, and other inflammatory markers. The purpose of the immune system activation is for repair and removal of damaged, dying, or dead cells (including neurons). A small number of clinical trials have included immunomodulating strategies, but no intervention to date has proven effective in improving outcomes after TBI. The role of the immune system in various forms of TBI has been reviewed by several authors (Balu 2014; Barrett et al. 2014; Bordt and Polster 2014).

Neuroinflammation, dysregulated immune responses, and autoantibodies have been investigated in multiple studies (Orsini et al. 2014; de Rivero Vaccari et al. 2014; Raad et al. 2014; Zhang et al. 2014).

Although the brain parenchyma lacks the traditional sentinel cells of the innate immune system found in the periphery, a unique myeloid-derived immune cell type called microglia is present. These microglia function to provide and support immune surveillance and are also able to initiate inflammatory responses within the brain that result in a complicated interaction of altered BBB, activation of microglial and leukocytes, and a more general immune system dysfunction. Inflammation is an indicator of TBI-associated damage, via animal models used in studying treatment efficacy (Mencl et al. 2014). Myeloid-derived cells called microglia provide immune system response and allow for the brain-specific inflammatory responses (Ransohoff and Cardona 2010; Benarroch 2013). Microglia are macrophage-like cells of the CNS that play an important role in the immune system activation following brain injury whereby they become phagocytic (Lull and Block 2010). Although initially constituting a

potentially beneficial response to remove damaged neurons following physical insult, prolonged activation of growth factors, cytokines, phagocytic activity, and proinflammatory molecules becomes detrimental and destructive in nature (Block and Hong 2005). These molecules, including IL-1β, IL-1RA, IL-6, IL-8, IL-10, and TNF-α (Hinson et al. 2015), are immune regulating factors released following TBI.

Reactive oxygen and nitrogen species are "highly reactive species" formed either enzymatically or nonenzymatically as part of the normal metabolic processes in mammalian cells. The factors of importance commonly include superoxide, hydrogen peroxide, hydroxyl radical, nitric oxide, and peroxynitrite. These species can directly negatively impact the cell or cause indirect damage by altering DNA, destroying proteins, and causing general oxidative damage (Zimmerman 1995). Although oxidative stress has been implicated from an enzyme standpoint to result in cellular damage, the time course and related fluctuations in both reactive oxygen and nitrogen species following TBI has not been fully developed. This lack of establishment of a time course pattern has led to seemingly conflicting findings around the importance of oxidative stress in TBI damage (Rodriguez-Rodriguez et al. 2014).

5.2.1.3 Models of Traumatic Brain Injury

There are a variety of TBI models used in animals that allow for the direct manipulation of environmental factors, treatment options, and controls. Clearly, the use of these models is to gain a better understanding of the mechanisms underlying the progression of the damage, biological markers, and treatment options. Each model allows for the evaluation of the injury on the brain from a neurological, cardiovascular, and histopathological standpoint and has been reviewed previously (Cernak 2005). In the laboratory research setting, the conditions of type of insult, force, magnitude, and recovery related variables can be precisely controlled. Although this level of control is of benefit from a research standpoint, the type of damage does not necessarily directly correspond to what is found in "typical" accidents examined in a clinical setting. The fluid percussion, controlled cortical impact, weight drop, and acceleration models produce physical damage that can in many cases be clearly visible and related to behavioral endpoints that are in some cases unique to the model utilized (Lindgren and Rinder 1967; Lighthall 1988; Lighthall and Anderson 1994; Marmarou et al. 1994; Goldman et al. 1991).

The fluid percussion model is a method of inducing localized brain injury (i.e., physical trauma) to an animal model that was first developed by Lindgren, and later modified (Lindgren and Rinder 1967; Lighthall and Anderson 1994). Specifically, this model involves the application of a low pressure pulse of fluid to a surgically exposed region of the brain. As part of the surgical procedure, an appropriately sized section of skull is removed with the injury tube placed over and sealed around the exposed dura. The location, duration, and volume of fluid delivered, in part, determine the degree of damage administered. This particular model has been used in mice, rats, cats, pigs, dogs, and rabbits (Fenn et al. 2015; Hartl et al. 1997; Pfenninger et al. 1989; Hilton et al. 1993). From a histopathological standpoint, the site of

physical damage shows a loss of tissue in and immediately surrounding the area of insult with damage consisting of an increased incidence of gliosis in surrounding neuronal cells. Lin et al. (2015) examined the histopathological changes across three impact forces in the rodent model in which the hematoxylin-eosin staining showed a relationship between the severity of the impact and the degree of peripheral edema and hemorrhage. For example, the intermediate impact group had hippocampal degeneration, necrotic pyramidal cells, subarachnoid hemorrhage which was visible in the ipsilateral ventricle, and edema and inflammatory cell infiltration which was present in the sensorimotor area of the frontal and parietal lobes (Lin et al. 2015). As expected, the most severely damaged group exhibited a higher rate of mortality along with clear and widespread cerebral necrosis.

The controlled cortical impact model is similar to the fluid percussion approach, but involves the direct application of a pneumatic cylinder. The pneumatic cylinder thereby allows for a more controlled and quantifiable measurement of parameters (e.g., force, cylinder impact size, and magnitude of impact). Similarly, this model has been used to study TBI in mice, rats, and even ferrets (Schaible et al. 2014; Lighthall 1988; Zweckberger et al. 2014). The delivery of the fluid to cause a mild to moderate insult to the brain is known to produce a focal site of injury, with a more severe insult resulting in general axonal and brain-stem damage (Marmarou et al. 1994; Lighthall 1988). The controlled cortical impact characterization by Goldman et al. (1991) identified cortical (including subcortical, frontal, entorhinal) histological changes consisting of small spongy like cells that were swollen and/or vacuolated in nature. In general, the pathway of cellular degeneration across brain regions was part of a complicated mechanism in which disturbances in cerebral blood flow, permeability, and cerebral pressure resulted in widespread necrosis and dysfunction.

A third model focuses on the role that acceleration (weight drop) plays in TBI through the use of a weight drop apparatus, which was developed by Marmarou et al. (1994). This graded model allows for a weight to be dropped onto the skull resulting in a higher degree of injury by avoiding direct dural impact, as noted with other models, along with preventing the skull from fracturing. The acceleration model was shown by Foda and Marmarou (1994) to produce a graded pattern of microscopic injury in neurons, axons, and microvasculature primarily in the cerebral cortex. In addition, edema was localized within the cerebral cortex, in the form of pericapillary astrocytic swelling and also in some cases within the brainstem. Outside of the general cerebral cortex, the corpus callosum, internal capsule, optic tracts, cerebral peduncle, cerebellar peduncle, and long tracts in the brain stem also showed axonal injury (Foda and Marmarou 1994).

In a fourth model by Goldman et al. (1991), called the controlled concussion model, a pendulum is utilized to deliver a moderate concussive blow to the head. The concussion is produced by controlled and repeatable mechanical fixed, closed-head injury. This was produced by a pendulum striker with a fixed-head, thus allowing the pendulum to fall on the midline of the skull. Goldman et al. (1991), reported that the most severely impacted regions included the frontal to entorhinal cortex, with an increase of vacuolization, spongy like cells, swollen interspersed cells, and degenerating cells.

In each of the above examples routine behavioral evaluations included the use of rotarod, tail suspension, righting reflex, and functional observational batteries (FOBs) (Fenn et al. 2015). Impairments in each of these endpoints, including decreased rotarod latency times, absent righting reflex, hypoactivity, tonic/clonic movements, seizures, piloerection, salivation, decreased grip strength, and impaired pupillary responses all correspond to brain injury. Quantitative startle response (prepulse inhibition), motor activity, and learning/memory paradigms can also be used to further evaluate exploratory behavior, learning/memory pathways, and motor activity and startle response neural pathways.

5.2.1.4 Current State of Drug Development

The Brain Trauma Foundation is a national organization of experts in the field that publishes guidelines for care, which include recommendations for initial treatment with anti-epileptic drugs, such as phenytoin. Following these guidelines after brain trauma has resulted in a reduction in the incidence of post-traumatic seizures (Brain Trauma et al. 2007). Although TBI directly affects a large number of people and has continued to undergo focused scientific research resulting in some improvements with diagnosis and care, the overall outcome following injury is still poor. Even with the current advances in treatment, there are still elevated incidences of epilepsy, dementia, neurocognitive decline, depression, and direct functional impairments.

In a review of TBI and treatment by Iaccarino et al. (2015), pharmaceutical based post-injury treatment options consist of beta blockers, neuroleptics (haloperidol, quetiapine, droperidol, methotrimeprazine), antiepileptics (valproic acid, phenytoin, and carbamazepine), neurostimulants (methylphenidate, amantadine), benzodiazepines, selective serotonin reuptake inhibitors (SSRIs), and antidepressants. Lithium has neuroprotective effects, stimulates neurogenesis, modulates neuroinflammation, and generally reduces neuronal death. As a result, the use of lithium for treating TBI has increased (Leeds et al. 2014). Less common novel methods being explored involve the prevention of associated oxidative damage following TBI with melatonin, reduced nicotinamide adenine dinucleotide phosphate (NADPH) inhibitors, apocynin, sulforaphane, U-83836E, some polyphenols, methylene blue and general antioxidants treatment (Fernandez-Gajardo et al. 2014; Fenn et al. 2015).

5.2.2 Infectious Diseases

The broad topic of infectious diseases of the nervous system encompasses a range of infectious agents from multicellular trematode parasites to acellular viral particles and prion proteins. Common to all of these diseases are entry of the infectious agent into the nervous system, subsequent host response, and challenges faced by physicians to appropriately treat these agents without further exacerbating the immune response. In an enclosed space such as the cranial vault, the immune response can be as deleterious as the infection itself.

This section will address the major groups of infectious diseases in the nervous system, including parasitic, bacterial, viral and prion-associated diseases with special emphasis on enteroviral meningitis and cerebral malaria.

5.2.2.1 Pathogenesis of Immune Response

There are multiple classes of parasitic diseases which can infect the CNS. Table 5.1 provides a list of common parasitic diseases in humans and their etiologies (Pittella 2013). Many of these diseases are the result of a systemic infection extending to the nervous system; this is particularly true of protozoal infections. Malaria occurs with a high prevalence, particularly in Africa. Although many people recover from the infection without complication, a small percentage will develop life-threatening disease. Children in sub-Saharan Africa as well as adults and teens in Southeast Asia are most likely to develop cerebral malaria (CM). According to the World Health Organization World Malaria Report, approximately 528,000 people died in 2013 (range 315,000–689,000) from complications of malaria in Africa alone;

Table 5.1 Common parasitic diseases and their etiologic agents in humans

Protozoan		
	Cerebral malaria	*Plasmodium falciparum* (other species less commonly)
	Chagas disease	*Trypanosoma cruzi*
	African sleeping sickness	*Trypanosoma rhodesiense* and *Trypanosoma brucei gambiense*
	Toxoplasmosis	*Toxoplasma gondii*
	Cerebral amebiasis	*Entamoeba histolytica*
	Primary amebic meningoencephalitis	*Naegleria fowleri*
	Granulomatous amebic encephalitis	*Acanthamoeba* spp. and *Balamuthia mandrillaris*
	Microsporidiosis	*Encephalitozoon cuniculi* and *Trachipleistophora anthropophthera*
Metazoan		
Trematode	Schistosomiasis	*Schistosoma japonica* (brain) *S. haematobium* and *S. mansoni* (spinal cord)
	Paragonimiasis	*Paragonimus westermani*
Cestode	Cysticercosis	*Cysticercus cellulosae*
	Coenurosis	*Taenia multiceps*
	Hydatidosis	*Echinococcus granulosus* and *E. multilocularis*
Nematode	Gnathostomiasis	*Gnathostoma spinigerum*
	Angiostrongyliasis	*Angiostrongylus cantonensis*
	Toxocariasis	*Toxocara canis*
	Strongyloidiasis	*Strongyloides stercoralis*
	Neural larval migrans	*Baylisascaris procyonis*

many of these are the result of neurologic manifestations of the disease (World Health Organization 2013). Even with antimalarial treatment there is a mortality rate of 15–20% with cerebral malaria (Gordon et al. 2015). This is typically because treatment is initiated too late in the course of disease and damage to the nervous system has already been done. The exact immunopathogenesis for development of CM is not known, however current research suggests that sequestration of parasitized erythrocytes in the microvasculature, endothelial activation, and aberrant host immune-mediated processes all contribute to the development of CM. An imbalance or overwhelming immune response is considered an important contributor to the development of CM.

Bacterial infections in the CNS can manifest as inflammation in the spinal cord (myelitis), brain (encephalitis), membranes surrounding the brain and/or spinal cord (meningitis), or a combination (e.g., meningoencephalitis). Bacteria gain entry into the nervous system through multiple routes including hematogenous spread, direct spread from sinusitis or otitis and less commonly inoculation from trauma or surgery. Additionally, retrograde transport along cranial nerves occurs with *Listeria monocytogenes*. The most frequent pathogens for neonates are Group B streptococci (*Streptococcus agalactiae*), gram-negative bacilli (*Escherichia coli*, *Enterobacter* sp., *Klebsiella pneumonia*, *Citrobacter diversus*), and *Listeria monocytogenes* (Tan et al. 2015).

In children and adults, the most frequent pathogens are: *Streptococcus pneumoniae*, *Neisseria meningitidis* and *Haemophilus influenzae*. *Listeria monocytogenes* is also the most common cause of bacterial meningitis in patients with defective cell-mediated immunity due to hematological malignancy, pregnancy, organ transplantation, human immunodeficiency virus (HIV) infection and chronic corticosteroid therapy (Brouwer et al. 2006).

Viral infections of the CNS system utilize both hematogenous routes of entry and transport through axons in the PNS into the brain and spinal cord. Viral meningitis is caused by a number of neurotropic viruses with human enterovirus including Coxsackie A, Coxsackie B, echoviruses and polioviruses being the most common. Additional agents include herpesviruses, bunyaviruses, arboviruses (viruses transmitted by insect vectors), HIV, and rabies virus. Enteroviruses are small, non-enveloped spherical particles around 30 nm in diameter. Enteroviruses have a worldwide distribution with the EV71 strain causing the most recent outbreaks of viral meningitis in areas of Asia. Infection with enteroviruses is typically confined to children with 2–10% of infections resulting in CNS complications (Denizot et al. 2012). As the name implies, infection with enterovirus occurs first in the gastrointestinal system. Following infection, the virus replicates in the lymph nodes then disseminates throughout the body. In uncomplicated enterovirus infection, the disease manifests as hand foot and mouth disease which clinically presents as ulcers, cold-like symptoms and gastroenteritis. When CNS complications occur, the disease can progress to myocarditis, meningitis, flaccid paralysis, neurogenic pulmonary edema and shock induced sudden death.

Although various infectious agents have different routes of entry, and the pathogenesis of disease is not the same in all cases, the nervous system has a limited

repertoire when it comes to response to infection and histologic changes secondary to infectious agents. Both innate and adaptive immunity play key roles in the response to entry by infectious agents into the nervous system. The innate immune system begins with the BBB which limits entry of larger particles, including organisms, through the capillary endothelium. A second component of the innate immune response is the recognition of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs) such as toll-like receptors, RIG-1-like receptors and NOD-like receptors. First responder cells such as monocytes, macrophages and dendritic cells use PRRs to identify PAMPs. Activation of first responder cells results in direct effects such as phagocytosis of the organism as well as activation of humoral immunity following antigen presentation, primarily by dendritic cells and subsequent acquired immune response. In order for an immune response to an infectious agent to be successful, it must meet three requirements. The response must be rapid, controlled and neuroprotective. If any of these requirements are not met, the disease progression is more severe (Carrithers 2014; Libbey and Fujinami 2014).

5.2.2.2 Models of Infectious Disease

In order to study the pathogenesis and effectiveness of therapeutics for infectious diseases in the nervous system, *in vitro* and *in vivo* models are frequently utilized. *In vitro* studies typically utilize cellular targets of the infectious agent or cells lines which make up the physical barriers to infection in order to examine various aspects of infection and disease pathogenesis. Specifically, cell culture of brain microvascular endothelial cells and intestinal epithelial cells have been used to study the entry of West Nile virus through the BBB and EV71 through the intestinal wall, respectively (Verma et al. 2009; Yang et al. 2009).

Animal models often rely on the natural susceptibility of certain species to the infectious agent of interest or a closely related pathogen. Ideal animal models are readily infected and show a similar disease course with clinical and pathological changes similar to that which is seen in the human disease. For example, infection of (BALB/c × C57BL/6)F1 mice with *Plasmodium berghei* closely recapitulates CM seen in people infected with *Plasmodium falciparum* (Hearn et al. 2000). Similarly, BALB/c mice have been utilized in studies examining potential therapeutics following EV71 infection (Li et al. 2014).

5.2.2.3 Current State of Drug Development

Current therapeutics for bacterial and parasitic infections are directed at eliminating the etiologic agent. In the case of parasitic infections, anthelmentics, antimalarial drugs and antibiotics that can kill protozoal agents are utilized. Antibiotics are used to treat bacterial infections with broad spectrum empirical treatment to initiate therapy followed by specific therapies once cerebrospinal fluid culture and susceptibility have been performed. Unfortunately, these treatments alone frequently fail to cure patients who have already sustained substantial neurologic damage and a rapid

immune response to the death of these agents can result in exacerbation of the disease, particularly metazoan parasites. One retrospective study demonstrated that delaying initiation of intravenous antimicrobials longer than 3 h following hospital admission was a strong predictor of mortality in patients with pneumococcal meningitis (Auburtin et al. 2006).

In the case of viral infections of the nervous system, much of the treatment is supportive and symptomatic. Antiviral medications such as acyclovir, vancyclovir and ganciclovir are typically given to treat specific herpes virus infections and combination antiretroviral treatment may be given for HIV infection. Intravenous immunoglobulin (IVIG) is currently used to treat patients with severe viral infections, particularly with nervous system complications. Additional therapeutics are in development for other viral infections. Specifically, monoclonal antibodies have been considered a potential candidate for the treatment of viral infections (Cho and McKendall 2014).

As previously discussed, even when treatment is successful at eliminating the causative agent in CNS infections, patients frequently suffer long-term debilitation from damage caused during active infection. Therefore, much effort has been spent in developing prevention strategies for these infections. Specifically, the utilization of vaccines in susceptible populations and the development of new vaccines for emerging infectious diseases of the CNS is a major focus of disease management. There has been marked reduction in the incidence of *Streptococcus pneumoniae*, *Neisseria meningitidis* and *Haemophilus influenzae* as a result of successful vaccination programs and development of herd immunity in disease-endemic areas (Tan et al. 2015). Current research is focused on the development of vaccines to viral infections that affect the CNS including West Nile Virus, EV71 and HIV.

5.2.3 Autoimmune Diseases

By definition, autoimmune responses are inappropriate responses of the immune system to self. In the nervous system, self-reactive immune cells attack normal constituents of the nervous system, most commonly components and self-antigens of the myelin sheath. Normally, such self-reactive cells are deleted, regulated or fail to encounter self-antigens but exposure of self-reactive cells to their cognate self-antigens often results in demyelinating diseases and resultant pathology and functional deficits. The most commonly recognized autoimmune disease affecting humans is multiple sclerosis (MS), an inflammatory demyelinating autoimmune disease of the CNS which will be the focus of this section.

5.2.3.1 Multiple Sclerosis

MS is the leading cause of neurological disability in young adults in Western countries (Ellwardt and Zipp 2014). Patients present with one of four clinical presentations (i.e., relapsing remissing [RRMS], secondary progressive [SPMS], primary progressive [PPMS] and progressive relapsing [PRMS]) that likely represent

varying and overlapping contributions of genetic, environmental, hormonal, and immunological effects (Loma and Heyman 2011; Noseworthy et al. 2000). Similar to other autoimmune diseases, there is sexual dimorphism (i.e., women are more commonly affected than men) and an influence of hormones on disease progression (e.g., remission during pregnancy) (Voskuhl and Gold 2012; Whitacre et al. 1999). The pathogenesis of MS is complex and although the definitive etiology (and cognate self-antigen) of MS is unknown, inflammatory injury, demyelination and neurodegeneration have been implicated in disease progression (Ellwardt and Zipp 2014; Loma and Heyman 2011). Numerous immunological and neurological mechanisms contribute to MS pathogenesis, including CD8+ T cells, CD4+ T cells, B cells, macrophages/microglia, natural killer cells, as well as release of soluble inflammatory mediators (e.g., TNF-alpha, IFN-gamma, IL-17, etc.), axonal severing, mitochondrial dysfunction, matrix metalloprotease (MMP) activation, redistribution of various ion (i.e., sodium, potassium, acid-sensing) channels, alterations of neurotrophins (e.g., brain-derived neurotrophic factor), neurite growth factors and oligodendrocyte growth and function, glutamate excitotoxicity and numerous others (Naegele and Martin 2014; Robinson et al. 2014; Ellwardt and Zipp 2014; Frohman et al. 2006; Trapp et al. 1998). Given the complexity of MS pathogenesis, no single model represents the complexity of human disease; however, animal models have been particularly useful to understand and model the various disease manifestations and design appropriate therapeutics.

Models of Multiple Sclerosis: Experimental Allergic/Autoimmune Encephalomyelitis (EAE)

Experimental autoimmune encephalomyelitis (EAE) is the first identified and best characterized animal model for MS and is considered to be the first animal model of human disease (Baxter 2007; Rivers et al. 1933). EAE can be induced in numerous species including the guinea pig, rat, rabbit, macaque, marmoset, hamster, dog, sheep and others. These models mimic clinical and immunological manifestations to varying degrees; however, the mouse is by far the most common species currently used in EAE studies (Handel et al. 2011; Baker et al. 2011; Baxter 2007). The majority of EAE models evaluate the disease utilizing neuroantigen challenge (commonly with an immune stimulus/adjuvant); however, additional models primarily evaluate the demyelination aspect of the disease utilizing either the toxin cuprizone or the infectious agent Theiler's murine encephalomyelitis virus (TMEV) (Simmons et al. 2013; Denic et al. 2011). Within mouse models, different strains have provided important insight into immunopathogenesis, different disease manifestations or pathological patterns of disease (Simmons et al. 2013; Ben-Nun et al. 2014). Specifically, genetically engineered mice such as those transgenic for the T cell receptor specific for a myelin-specific neuropeptide (MOG; myelin oligodendrocyte glycoprotein) or specific combinations of mouse strains (e.g., SJL, B10.PL, etc.) and varying myelin antigens (e.g., PLP; proteolipid protein or MBP; myelin

basic protein, etc.) are used to model RRMS and SPMS (Robinson et al. 2014; Rangachari and Kuchroo 2013; Simmons et al. 2013; Ben-Nun et al. 2014; Papenfuss et al. 2004). Given the availability of genetically engineered mouse strains on the C57Bl/6 background, the MOG-EAE model in C57Bl/6 mice has become the most prevalent (and with MOG antibodies being prominent in MS patients, perhaps the most relevant) model for human MS (Ben-Nun et al. 2014). The applications and utility of EAE as a model to study the immunopathogenesis of MS and to evaluate potential therapeutics are numerous and beyond the scope of this chapter; the reader is directed to numerous excellent reviews (Ben-Nun et al. 2014; Baker et al. 2011; Constantinescu et al. 2011; Denic et al. 2011; Friese et al. 2006; Gold et al. 2006; Handel et al. 2011; Kipp et al. 2012; Mix et al. 2008; Rangachari and Kuchroo 2013; Robinson et al. 2014; Naegele and Martin 2014; Kutzelnigg and Lassmann 2014; Hartung et al. 2014).

The neuroantigen challenge method of inducing EAE is accomplished in two primary ways: active and passive induction (Wekerle 2008; Robinson et al. 2014). In active induction, tolerance is broken and autoreactive cells are generated (or stimulated) to target myelin antigens, induce inflammation, cause demyelination and contribute to neurodegeneration. Typically, active EAE is induced by actively immunizing an animal with a combination of neuroantigen (e.g., emulsified myelin or myelin-specific peptides, such as, MOG, PLP, etc.) and adjuvants which promote an appropriate inflammatory response. Autoreactive T cells present within the circulation are activated and subsequently target self-antigen within the myelin sheath and migrate across the blood-brain barrier (Ben-Nun et al. 2014; Constantinescu et al. 2011; Noseworthy et al. 2000). The combination of inducing neuroantigen and adjuvant required to effectively induce EAE is often very species- or strain-specific (Ben-Nun et al. 2014; Baxter 2007; Gold et al. 2006; Papenfuss et al. 2004). Complete Freund's adjuvant (CFA) is the most commonly employed adjuvant used to successfully induce EAE and it strongly promotes the inflammatory environment and necessary differentiation of naive CD4+ helper T cells into encephalitogenic CD4+ Th1 cells capable of inducing the clinical disease EAE (Ben-Nun et al. 2014; Smith et al. 2011; Hartung et al. 2014; Naegele and Martin 2014). For many years, the induction of Th1 responses (and the prototypical Th1-cytokine IFN-gamma) was considered to be the primary immune response necessary for induction of EAE. However, in 2006, several studies demonstrated the importance of a newly described population of Th17 cells (and associated IL-12, IL-23 and IL-17 production) and there is continued discussion and debate as to the relative importance of Th1 versus Th17 and their requirements for EAE induction (Robinson et al. 2014; Langrish et al. 2005; Adorini et al. 1996a, b). Likely, it is a combination of Th1/Th17 in combination with other contributing factors which result in the clinical manifestations in EAE and in MS patients (Lovett-Racke et al. 2011; Mix et al. 2010). In the active induction process, adjuvants can be used to induce a combination of Th1 and Th17 responses and encephalitogenic cells capable of mediating disease (Smith et al. 2011; Gold et al. 2006; Ben-Nun et al. 2014).

Passive induction of EAE is accomplished by adoptively transferring the encephalitogenic CD4+ T cells induced either *in vitro* from stimulated T cells or T cells obtained from actively immunized mice (Wekerle 2008). The transfer experiments showing that transfer of the encephalitogenic CD4+ T cells alone into a naïve animals could induce EAE clearly demonstrated the important and critical role that CD4+ T cells played in disease and the focus of decades of study have primarily focused on the induction and regulation of the encephalitogenic CD4+ T cells (Lider et al. 1988). Regulation of such pathogenic CD4+ T cells has been a primary focus and various regulatory immune cells have been identified (e.g., regulatory T cells; Tregs, Th2, CD8+ Tc1, CD8+ Tc2, B cells and γδ T cells) which can diminish disease severity and/or the pathogenicity of these pathogenic CD4+ T cells (Mix et al. 2010). However, recent interest in understanding disease pathogenesis and developing novel therapies has focused on cells and pathways beyond the CD4+ T cells implicated in pathogenesis and resolution. Cells including CD8+ T cells, NK-T cells, macrophages, microglia and B cells are just some of the cells known to play important roles in EAE pathogenesis and resolution (Robinson et al. 2014; Mix et al. 2010).

As with any model, there are limitations in the ability of EAE to model MS both for understanding immunopathogenesis, as well as for the development of treatments. There has been criticism that while numerous therapies stop EAE, these rarely translate into cures for MS (Baker et al. 2011; Sriram and Steiner 2005; Weiner 2004). It could be argued that the most utility for EAE comes in understanding the generation of a CNS autoimmune response while drug delivery/dosages have had much more limited success using EAE (Baker et al. 2011). However, several drugs have been developed specifically and extended from EAE studies (e.g., glatiramer acetate, mitoxantrone and natalizumab approved in 1996, 2000 and 2004, respectively) while numerous others are evaluated in and vetted through EAE studies (Steinman and Zamvil 2006; Robinson et al. 2014).

Current State of Drug Development

According to the National Multiple Sclerosis Society (NMSS; of January 2015, there are currently 12 FDA-approved disease-modifying therapies for treating RRMS and one approved for SPMS. Beta interferon treatments were the first approved treatments approved as early as 1993; there have been four drugs approved for use since 2012 and numerous therapies are currently in various stages of clinical trials in human MS patients (http://www.nationalmssociety.org/NationalMSSociety/media/MSNationalFiles/Brochures/Brochure-The-MS-Disease-Modifying--Medications.pdf).

Minimizing the pathology and disease course of a complex disease has required utilization of therapies with various mechanisms of action. Current FDA-approved drugs can be grouped according to these relative mechanisms of action and subsequent therapeutic effects. Specifically, type I IFNs (trade names: Avonex, Betaseron, Extavia, Plegridy and Rebif) can have anti-inflammatory effects, reduce inflammatory cells and cells crossing the BBB, and enhance production of neurotropic factors.

Glatiramer acetate (Copaxone), an amino acid copolymer, has been shown to reduce relapse rates and modulate immune responses via a shift to a Th2 response, reduction of inflammatory responses, potential neurotropic effects, and outcompetition of MHC class II binding of self-antigens (Dhib-Jalbut 2003; Teitelbaum et al. 1996; Aharoni et al. 2005). Approved drugs which modulate lymphocyte trafficking (and limit leukocyte trafficking into the CNS) include natalizumab, alemtuzumab and finolamid (Robinson et al. 2014; Investigators et al. 2008). Natalizumab (Tysabri) is a monoclonal antibody against α4β7 integrin (a.k.a anti-VLA-4) which inhibits adhesion of activated lymphocytes to blood vessels (Robinson et al. 2014). Alemtuzumab (Emtrada), a monoclonal antibody against the CD52 antigen present on many circulating cells, causes profound lymphopenia while fingolamid (Gilenya) is a structural analog of sphingosine which sequesters lymphocytes in lymph nodes and may have neurotropic effects (Robinson et al. 2014; Investigators et al. 2008; English and Aloi 2015). Approved drugs that target rapidly dividing cells and/or result in reduced lymphocyte numbers or function include mitoxantrone (Novatrone), which has numerous anti-inflammatory effects, and teriflunomide (Aubagio), which inhibits rapidly dividing cells and blocks proinflammatory (e.g., NF-kB) signaling pathways (Robinson et al. 2014). Dimethyl fumarate (DMF; trade name Tecfidera) reduces relapses and lesions through a proposed mechanism of reducing IFN-gamma and increasing IL-10 production by helper T cells, inhibiting proinflammatory TNF-alpha, IL-12 and IFN production by peripheral blood mononuclear cells (PBMCs) in humans, and limiting macrophage infiltration into the CNS (English and Aloi 2015; Gold et al. 2012; Ockenfels et al. 1998; Lehmann et al. 2007; Schilling et al. 2006).

In addition to current FDA-approved therapies, numerous other therapies to treat MS are being evaluated, often utilizing EAE for the development and evaluation of such therapeutics (Friese et al. 2006; Ben-Nun et al. 2014; Constantinescu et al. 2011; Robinson et al. 2014). A full description of such potential therapeutic candidates is beyond the scope of this chapter but some of the broad therapeutic focus areas include (1) the induction of regulatory cells, (2) depletion of immune cells and (3) anti-inflammatory therapy. Induction of regulatory T cells has been an area of interest for a while but newer studies have identified other potential regulatory cells including stem cells, immunoregulatory NK cells, type II monocytes, and CD+ T cells (Mix et al. 2008; Frohman et al. 2006; Zhang et al. 2010; Bielekova et al. 2006; Mishra et al. 2012). Lymphocyte depleting and/or immunosuppressive therapies, such as steroids, mitoxantrone, alemuzumab, rituximab, ocrelizumab, and immunoablative treatments, can be useful in treating significant inflammatory flares in MS; however, non-specific depletion may have untoward and potentially severe side effects that limit their application (Robinson et al. 2014; Investigators et al. 2008; Hauser et al. 2008; Hawker et al. 2009; Kappos et al. 2011; Mancardi and Saccardi 2008). Other therapies under development may aim to limit inflammatory responses. Laquinimod, an orally active quinoline carboxamine may interfere with early proinflammatory signaling by S100 and receptor of advanced glycation end product (RAGE) and may downregulate VLA-4 adhesiveness and IL-17 secretion and/or prevent monocytes from entering the CNS (Bjork et al. 2009; Wegner et al. 2010; Schulze-Topphoff et al. 2012; Thone et al. 2012). Reduction of proinflammatory

cytokines is a potential therapeutic target and secukimumab, a human anti-IL-17 monoclonal antibody, may hold promise where previous anti-inflammatory cytokine therapy has failed to show efficacy in the treatment of MS (Robinson et al. 2014; Miossec and Kolls 2012). Although the induction of tolerance and other antigen-specific immunoregulation has theoretical applications, antigen-specific immunomodulation has largely been ineffective with a significant reduction of interest in such therapies following the failure of the large multi-center clinical trial for oral tolerance (Weiner 2004). Such antigen-specific immunotherapy may be largely limited by the fact that MS is a multi-factorial disease and a definitive antigen responsible for inducing MS hasn't been clearly identified. Additionally, neuroprotective therapies addressing some of the neuropathological changes are of interest and include riluzole, phytoid, flecainide, anti-apoptotic molecules, growth factors, activators of oligodendrite transcription factor-1 and blockers of neurite outgrowth inhibitor and other neural growth factors (Frohman et al. 2006). The development and evaluation of such techniques typically employ EAE which demonstrates the utility of EAE as an animal model for MS.

For a complex and multifactorial disease such as MS, no one therapy will likely be able to treat all aspects of inflammation and neurodegeneration. However, thoughtful consideration and selection of different EAE models where appropriate can shed significant light on not only immunopathogenic mechanisms involved in MS development and progression, but also on the potential efficacy of possible MS therapies.

5.2.4 Neurodegenerative Disorders

Neurodegenerative disorders fall into a general class which involves the chronic and progressive loss of function, deficiency in growth, and death of neurons within the central and peripheral nervous systems. In terms of age of onset, these disorders can affect brain function either early in childhood or later in adulthood (with aging as a significant risk factor). This neurodegeneration results in impaired emotion processing, intellectual disabilities, and deficient interpersonal communication, as well as difficulty with social cognition, self-control, and controlled motor movement. Neurodegenerative disorders include Parkinson's disease, Alzheimer's disease, Huntington's disease, amyotrophic lateral sclerosis, and even more broadly dementia.

Of these disorders, two of the more prevalent from an occurrence and diagnosis standpoint that will be discussed in this section are Parkinson's (PD) and Alzheimer's (AD) disease. These are the most common neurodegenerative diseases in the elderly, with AD as primarily dementia or cognitive based and PD as a widespread movement disorder. Both PD and AD share many clinical and pathological features, suggesting that they are possibly part of a larger disease spectrum (Jellinger 2008; Neef and Walling 2006). For example, dementia is also commonly diagnosed in PD patients, with an average prevalence of 30% and is associated with both rapid motor and functional decline (Aarsland et al. 2003; Marras et al. 2002; Levy et al. 2002). In addition, patients with PD and AD also have movement related deficiencies, dopamine dysregulation, and beta amyloid plaques (Jellinger 2008; Neef and Walling 2006).

Although a variety of mechanisms have been investigated concerning neurodegeneration, pathology development and progression, there is information linking the inflammatory response (via microglial activity) to neurodegeneration (Gonzalez et al. 2014; Ringheim and Conant 2004; Perry and Teeling 2013). In addition, immune phenotypes have been associated with neurodegenerative disease as well as chronic immune system activation in the central and peripheral nervous systems (Walker and Lue 2015; Wakabayashi et al. 2010). The following sections will focus on the role of the immune system in relationship to Parkinson's and Alzheimer's disease.

5.2.4.1 Parkinson's Disease

Parkinson's disease (PD) is considered to be the most common neurodegenerative movement disorder, affecting 0.5–1% of the elderly population above the age of 60 and up to 3% over the age of 80 (de Lau and Breteler 2006; Nussbaum and Ellis 2003; Obeso et al. 2010; Coelho and Ferreira 2012; Lesage and Brice 2009). Although a relatively small percentage of PD cases have been related to genetic defects, the root cause for PD largely remains unknown. However, PD is linked to environmental factors such as pesticides (Wirdefeldt et al. 2011), and other players such as medication, stroke, poisoning, or brain tumors.

From a clinical standpoint, PD is characterized by bradykinesia, muscular rigidity, resting tremor, slowed movement, and postural instability (Israel and Hassin-Baer 2005). Although the motor symptoms are treated with the classically defined dopaminergic drugs, as well as other novel drug classes, their effectiveness in treating these motor symptoms typically diminishes as the neurodegeneration worsens resulting in a more pronounced presentation of PD symptoms (Schapira et al. 2009). In a longitudinal study, subclinical motor deficits were noted prior to the clinical onset of PD (Gustafsson et al. 2015). In addition, the clinical severity of these PD deficits is related to the progression of the disease (Fereshtehnejad et al. 2015).

From a neuropathological perspective, the most prominent pathological findings are the classical loss of dopaminergic neurons in the substantia nigra (SN) and presence of Lewy bodies (Lees et al. 2009). When patients suffer motor symptoms, over 60% of all dopaminergic neurons within specific regions of the basal ganglia may have been lost (Bernheimer et al. 1973). As a result, this pathology is considered to be the reason for many of the common non-movement symptoms of PD, including autonomic, cognitive, and psychiatric symptoms (specifically depression) in conjunction with sensory abnormalities ranging from akathisia (inability to sit) to anosmia (inability to smell) (Ding et al. 2015; Marras and Lang 2008). Cognitive deficits, particularly involving memory and higher executive function, can present clinically or more subtly at any of the stages of PD (Muslimovic et al. 2005). In addition, a recent clinical study reported that over half of 120 PD patients suffered from anxiety, sleep problems, and apathy (Lee et al. 2013a). This finding is consistent with other published research indicating that neuropsychiatric symptoms such as depression, hallucinations, anxiety, apathy, sleep disturbances and, to a smaller degree, euphoria and disinhibition are present in patients with PD (Aarsland et al. 1999,

2009). As noted for other disorders/diseases within this chapter, PD is not isolated and is often comorbid with other disorders that have been reviewed in the literature (Alzahrani and Venneri 2015).

Parkinson's disease can be classified into early, moderate, or advanced stages. The placement of a PD patient into the early stage is based on the presentation of mild tremor and/or slight stiffness while being able to carry out normal daily activities. As the disease worsens (moderate), the patient's movement becomes more limited, with worsening tremors and reduced movement. Finally, the advanced stage of the disease corresponds to significantly increased tremors and impaired movement, with corresponding postural imbalance, gait disturbances, and difficulty communicating via speech (Lesage and Brice 2009; Obeso et al. 2010; Coelho and Ferreira 2012). Commonly used diagnostic tools for classifying PD include the Mattis Dementia Rating Scale-2, Hopkins Verbal Learning Test-Revised, Judgment of Line Orientation, Movement Disorder Society Unified Parkinson's Disease Rating Scale Part III, general motor examination, and Montreal Cognitive Assessment, Hoehn and Yahr (H&Y) Scale (Goetz et al. 2004; Movement Disorder Society Task Force on Rating Scales for Parkinson's D 2003; Milenkovic and Dragovic 2013). The cognitive profile in PD differs significantly from that in AD. Performance on tests of orientation and attention are best in differentiating the groups (Bronnick et al. 2007). Other assessment tools such as metabolic profiling have identified over 40 metabolites that could be used to aid in a PD clinical diagnosis (Luan et al. 2015) and help distinguish from Parkinson's-plus syndromes (i.e., progressive supranuclear palsy, corticobasal degeneration, and multiple system atrophy).

Pathogenesis of Immune Response

Although most of the investigation concerning the development, progression, and treatment of PD has been in regards to the dopaminergic system within the SN, the role of the immune system as an active player in PD etiology is becoming clearer. Immune dysfunction has been identified in a large subset of persons with neurodegenerative disorders, such as PD, with inflammatory cytokines present at a level suggesting chronic inflammation, which is localized within the substantia nigra and striatum (Dobbs et al. 1999). Epidemiological evidence suggests an association between neuroinflammation and PD (Whitton 2007). General inflammation dysregulation or overactivation is also believed to be at play in the development of PD (Mogi et al. 1994; Blum-Degen et al. 1995; Ross et al. 2004). The role of the immune system in PD has been reviewed elsewhere, suggesting a strong association of PD to not only autoimmunity, but also inflammation, specifically as it relates to various cytokines, interleukins, and microglia (Tansey and Goldberg 2010; Reale et al. 2009; Kwilasz et al. 2015; Spooren et al. 2011).

Histopathological findings in the brains of postmortem PD patients indicate that chronic neuroinflammation is present specifically within the SN and striatum; this inflammation has been suggested to strongly contribute to the overall pathology (McGeer and McGeer 2001; Brosseron et al. 2014). This chronic inflammation appears to be due to activated microglia, which are the predominant immune cell type

located in the brain and basically function to regulate the innate immune response, as well as being involved with growth and neuron maintenance (Perry and Teeling 2013). However, the complicated relationship between levels of interleukins, brain derived neurotrophic factor (BDNF), biomarkers and the inflammatory response to the progression of PD is not completely understood (Dursun et al. 2015).

Other studies have identified increased microglial activity not only in the SN and putamen, but also in the hippocampus, entorhinal cortex, cingulate cortex and temporal cortex in PD patients (Imamura et al. 2003). Neuroinflammation likely acts via a mechanism involving proinflammatory cytokines, which are secreted by both glial cells and neurons and distributed throughout the CNS (Czlonkowska et al. 2002). Although an in-depth review of each cytokine involved with inflammation and PD neuronal death is beyond the scope of this discussion, a number of cytokines have been implicated, including TNF-α, IL-1 β , -2, -4, -6, -10, -12, TGF-α, - β 1, and - β 2 (Muller et al. 1998; Mogi et al. 1996; Leal et al. 2013; Reale et al. 2009; Brodacki et al. 2008). Immune system studies have identified IL-1 through 11 as cytokines which are key factors in regulating inflammatory response (Dinarello 2011).

For example, TNF-α, IL-1 β , -2, and -6, are increased in the brain and cerebrospinal fluid (CSF) of PD patients (McGeer and McGeer 1997; Mogi et al. 1996; Muller et al. 1998). Interestingly, levels of both IL-1β and TNF-α have been shown to correspond to the degree of inflammation and neurodegeneration in PD patients (Leal et al. 2013). The increased production of these previously mentioned inflammatory cytokines has been associated with various aspects of PD, ranging from cognitive impairment to dopaminergic neuron apoptosis to mortality (Dufek et al. 2015; Magaki et al. 2007; Wahner et al. 2007; Hofmann et al. 2009). For instance, increased levels of IL-6 correspond to elevated PD mortality (Dufek et al. 2015). IL-6 has also been linked to an acceleration of muscle catabolism leading to overall functional disability (i.e., sarcopenia, weakness, and fatigue), as well as depression in PD patients (Ferrucci et al. 2002; Cesari et al. 2004; Pereira et al. 2009; Selikhova et al. 2002). IL-12 acts to increase the production of other proinflammatory cytokines, and is correlated with IL-10, an immunosuppressive and neuroprotective cytokine, in PD patients, further supporting the dysregulated immune system response mechanism (Rentzos et al. 2009).

The interest in inflammation, and the suspected dysregulation of the inflammatory response, has led to the investigation of cytokine genetic polymorphisms as risk factors for PD (Bialecka et al. 2007). Interestingly, investigations on the role of polymorphisms within the interleukin family of cytokines have produced conflicting results, suggesting that the mechanism is not fully understood and additional studies are necessary to clarify their role (Mattila et al. 2002; Moller et al. 2004; Nishimura et al. 2000; Bialecka et al. 2007, 2008; Ross et al. 2004).

The role of autoimmunity in the development and pathology of PD has been reviewed and discussed elsewhere, and has continued to gain interest as a mechanism associated with PD (Benkler et al. 2009; Koutsilieri et al. 2013). Early PD animal research in rodents reported that the transfer of plasma antibodies isolated from PD patients to the SN of rats induced a marked loss of dopaminergic neurons (Chen et al. 1998). These findings have been supported by other studies in which autoantibodies were identified against melanin, α-synuclein, dopaminergic neurons,

and GM1 Ganglioside (Double et al. 2009; Yanamandra et al. 2011; Orr et al. 2005). In addition, the clinical diagnosis of PD-related behaviors (i.e., dyskinesia and depression), has been associated with the presence of anti-neuronal cells, anti-brain lysate, anti-dsDNA, anti-phosphatidylserine, anti-cardiolipin, anti-serotonin, and anti-melanocyte autoantibodies (Benkler et al. 2012).

Neuropathology

Normal motor movement is dependent upon a number of brain pathways, including the motor regions of the cerebral cortex (i.e., premotor and supplementary motor regions of the frontal lobe), SN, caudate nucleus, putamen, globus pallidus, and thalamus, thereby being a major focus for PD-related neuropathology and neuroimaging research (Mak et al. 2015). As discussed previously, selective dopaminergic neuronal loss via apoptosis, along with the presence of Lewy bodies and microglia within the substantia nigra (as well as the brainstem, hippocampus, and amygdala) are characteristic hallmarks of PD neuropathology (Gai et al. 1995; Dickson et al. 1991; Braak et al. 1994).

These findings indicate that there is also abnormal activity within the basal ganglia, cerebellum, and cerebrum along with extensive volume loss throughout the brain (Planetta et al. 2015). Structural MRI studies have revealed prominent grey matter atrophy (frontal, temporal, and parietal cortices) and disruptions of white matter tracts in PD, although PD findings classified as non-dementia are more variable (Burton et al. 2004; Sohn and Kim 1998; Beyer et al. 2007). Although there are a number of brain regions involved with PD pathology, the substantia nigra is the primarily impacted region with elevated levels of glial cells (Hofmann et al. 2009). This fact underscores why the SN is the PD target region of interest as well as explaining the functional motor/non-motor deficits (Benkler et al. 2009; Alexander 2004; Jankovic 2008). In addition, the disruption of normal activity within the motor circuitry, notably the lateralized brain activity pattern is likely an underlying aspect of bilateral motor coordination deficits in PD (Wu et al. 2015). Deficits in motor activity and normal neuronal activity extended to the UAT (tongue–pharynx–larynx–upper esophagus), in which there were PAS-positive axons in all the PD subjects with dysphagia and in none of the controls, and suggest that Lewy pathology affects mucosal sensory UAT axons (Wu et al. 2015).

Although the volumetric findings are somewhat complex in terms of relationship to the state of PD, resting-state functional brain images showed that although there is no significant volume change in the left amygdala, there was an increased amygdalar activity in the PD group compared with the normal control group which correlated with depression scores (Huang et al. 2015). Huang et al. (2015) also found that this was in direct contrast with studies of the right amygdala and fronto-parietal cortex, which found decreased activity correlated to depression scores. These findings clearly indicate that the interactions are not complex, but that abnormal brain function (i.e., amygdaloid complex) is related to the pathologies associated with PD.

Interestingly, another brain region impacted (to varying degrees) in PD patients is the olfactory bulb (OB). Asymmetric deficits in olfactory performance are present in PD patients, similar to deficits noted in other sensory domains (Zucco et al. 2015). There were no significant correlations between OB volume and disease characteristics, including residual smell (Paschen et al. 2015). Pathological examination of a PD brain identified atrophy in the brain regions previously mentioned, as well as Lewy bodies within the anterior olfactory nuclei (Lerner and Bagic 2008).

Models of Parkinson's Disease

The ability of the animal model to be used to predict, in part, clinical data from drug development, disorder etiology, or risk factors is a key component in data interpretation. Assessment of PD in animals has traditionally relied on not only knockout mice/rats, but also a variety of observations and behavioral-based tests, including social interaction, motor activity, startle responsiveness, functional observational battery, and continuous performance. Due to the use of new genetic knockout models and techniques, overall size, historical data available within the literature, and large number of markers and assessment techniques, the rodent (i.e., mouse and rat) has been the animal model of choice. The characterization, sharing of data, and funding of research with these models (and others) has been of particular interest of, and greatly advanced by, the Michael J. Fox Foundation (Baptista et al. 2013).

A variety of PD-related genes have been identified from expression profiles in the lateral and medial SN and frontal cerebral cortex as being differentially regulated Nurr1, NFASC, AMH, CHGB, FGF13, NEFL, SV2B, SYT1, RGS4, Park1 (Park 4, SNCA), Park 8 (LRRK2), Park7 (DJ-1), Park6 (PINK1), VPS35, EiF4G1, and GBA (Parkinson et al. 2015; Baptista et al. 2013; Moran et al. 2006; McGeer et al. 2002). This has led to a number of genetic knockout models (e.g., mouse and rat) being used and characterized in a range of behavioral paradigms examining neuropathology, neurotransmitters, muscle coordination, movement, and strength (Baptista et al. 2013). Knockout mouse models include Parkin (PARK2), DJ-1 (PARK7), PINK1 (PARK6), and MitoPark (mitochondrial dysfunction), as well as Parkin (PARK2), DJ-1 (PARK7), and PINK1 (PARK6) in rats (Harvey et al. 2008; Dave et al. 2014).

Drug- and chemical-induced models of PD are also used to aid in the research concerning disease development and progression in mouse, rat, and non-human primate animal models. For example, MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), a neurotoxin precursor to MPP+ (active form), and 6-OHDA (6-hydroxydopamine), a synthetic neurotoxic compound, are used to induce the destruction of dopaminergic and/or noradrenergic neurons within the CNS. Reserpine is a reversible drug that acts to deplete dopamine, rotenone inhibits mitochondrial complex 1, and paraquat is a pesticide that causes oxidative stress/damage; all have been studied (Leal et al. 2013; Klintworth et al. 2009; Bagga et al. 2015) and reviewed extensively (Simola et al. 2007; Blesa et al. 2012). Finally, lipopolysaccharide (LPS) is an endotoxin generated from gram-negative bacteria which acts to stimulate the neuroinflammation pathway via activating microglia. For a review, see Liu and Bing (2011).

Current State of Drug Development

Unfortunately, the treatment for PD consists of addressing the debilitating symptoms including bradykinesia, muscular rigidity, resting tremor, slowed movement, and postural instability (Gelb et al. 1999; Israel and Hassin-Baer 2005). In addition to standard pharmacological treatment, a variety of other approaches are sometimes used involving physical therapy and deep brain stimulation. However, no current therapy can slow or halt the progression of PD or regenerate the affected brain regions. The relatively slow development of therapies and successful treatments is due to not only the delayed clinical diagnosis, but also the lack of clear mechanisms of PD initiation and progression. As is the case with many neurodegenerative disorders, this should be considered a lifelong disability that worsens over time where treatment is often needed, without a known cure.

Brain stimulation is a therapeutic approach which involves the implantation of electrodes to reduce the overexcitation of targeted brain regions, such as the subthalamic nucleus (Okun 2012; Okun et al. 2012). However, since this treatment doesn't address non-motor deficits (i.e., mania, impulsivity, depression, and apathy), brain stimulation is often used in conjunction with pharmacological treatment (Mosley and Marsh 2015).

As a result of the PD-related pathology, current drug therapies generally act to increase dopamine levels in the striatum via administration of dopamine precursors, dopamine agonists, or selective dopamine reuptake inhibitors (Obeso et al. 2010). For example, several drug treatments include: rotigotine (dopamine agonist and antidepressant), pramipexole (dopamine agonist and antidepressant), ropinirole (dopamine agonist and reduces side effects of SSRIs), levodopa (dopamine precursor), carbidopa (inhibits metabolism of levodopa, can be used with levodopa), levodopa/carbidopa (combination drug; e.g., Sinemet), amantadine (antiviral, NMDA receptor antagonist, dopamine agonist, and dopamine reuptake inhibitor), selegiline (MAO inhibitor; e.g., Eldepryl), and entacapone (cathechol-O-methyltransferase [COMT] inhibitor, sometimes used with levodopa; e.g., Comtan). Interestingly, a combination study by Kim et al. (2015b) investigated the safety and efficacy of rotigotine transdermal system as an add-on to existing therapies using either low-dose pramipexole or ropinirole. This study showed that PD patients responded well to a combination therapy including the rotigotine transdermal system and demonstrated improved clinical outcomes (Kim et al. 2015b).

5.2.4.2 Alzheimer's Disease

Alzheimer's disease (AD) is a neurodegenerative disorder progressing to dementia, most notably in the elderly and affecting an estimated 26–35 million people worldwide (Querfurth and LaFerla 2010; Brookmeyer et al. 2007). A gender difference for AD has been proposed, with women having a slightly higher risk of developing AD than men at 15.5 vs. 13.1%, respectively (Yang and Levey 2015). This is further supported by Rasmuson et al. (2011) who showed sex differences in

hypothalamic-pituitary-adrenal (HPA) axis regulation and steroid hormone clearance in patients with AD, potentially related to IL-6. The age of onset may be attributable to the pathogenesis and virulence of AD, with significant worsening in all age groups on all outcomes over time. However, when the age of onset was later in life, the older groups showed slower rates of AD-related decline than the groups with an earlier age of AD onset (Schneider et al. 2015).

Alzheimer's disease is sometimes misdiagnosed with other similar disorders, such as subcortical vascular dementia, due to overlapping neuropsychological deficits (Graham et al. 2004). Revisions aiding in the neuropathologic assessment of AD (National Institute on Aging–Alzheimer's Association) include the following: awareness of neuropathology with or without clinical manifestations, staging of neurofibrillary tangles, and scoring of neuritic plaques (Montine 2011). This approach to classification and diagnosis has also led to detailed approaches for assessing commonly co-morbid conditions, such as Lewy body disease, vascular brain injury, and hippocampal sclerosis (Montine 2011). In addition to continual revisions and review of diagnostic criteria, metabolic screening tools can aid in proper diagnosis. For example, metabolic profiling reveals inflammatory (increased hs-CRP and globulin:albumin ratio), non-inflammatory, and unique (younger individuals, dyscalculia, aphasia, ApoE4-negative, zinc deficient) subtypes (Bredesen 2015). The degree of severity of these neuropathological findings has been shown to directly correlate to the progression and level of impairment of AD (Sheng et al. 1997), thereby allowing noninvasive markers and profiling to become more useful in the clinical setting. Additional metabolic profiles for AD are being developed concerning impaired homeostasis of histamine, altered metabolism of amino acids (threonine, aspartate and tyrosine), deregulated urea cycle and increased production of eicosanoids (Gonzalez-Dominguez et al. 2015). Novel work in rodent models has suggested that CSF analysis of neurogranin as an AD biomarker could potentially be used to monitor synaptic degeneration, due to the correlation of neurogranin with the rate of cognitive decline (Kvartsberg et al. 2015).

A number of hypotheses have been proposed for AD including genetic, epigenetic, viral, and/or environmental mechanisms. Recently, autoimmune mechanisms were considered to play a role in the pathogenesis of AD (Moscavitch et al. 2009). Although the etiology of AD is not completely understood, the overproduction and accumulation of amyloid-β plaques, apoptosis, and neurofibrillary tangles (aggregates of hyperphosphorylated tau protein) development are key players in etiology and progression (Braak and Braak 1991; Hardy and Selkoe 2002; Ringheim and Conant 2004; Querfurth and LaFerla 2010; Carmichael et al. 2012). Interestingly, the pathology is selective and affects brain regions primarily involved with cognition and higher-level processing (e.g., amygdala, hippocampus and cingulate cortex) (Loring et al. 2001). A three-stage approach in the development of AD has been proposed based on amyloid-β plaque formation, consisting of the presence of plaque formation, neurodegeneration, and then finally subtle cognitive decline (Besson et al. 2015; Sperling et al. 2011).

Pathogenesis of Immune Response

Although most of the investigation concerning the development, progression, and treatment of AD has been in regards to the amyloid-β plaque formation, neurofibrillary tangle development, and apoptosis in the amygdala, hippocampus and cingulate cortex (cognition/higher-level processing centers), there is increasing interest in the role of the immune system as an active player in AD etiology. The role of immune system pathology and AD has been reviewed extensively elsewhere, but the general discussion presented here will include immune system dysfunction, inflammation, and autoimmunity (Aiyaz et al. 2012; Sardi et al. 2011; Ruszkiewicz and Albrecht 2015; Swomley and Butterfield 2015; Bishnoi et al. 2015).

General immune system dysfunction has been identified in AD patients, where neutrophils adhered to and spread inside brain venules and were present in the parenchyma along with neurofibrillary tangles (Zenaro et al. 2015). Further dysfunction has been noted in AD patients, who displayed lower levels of B and T cells with normal NK cell counts (Speciale et al. 2007; Richartz-Salzburger et al. 2007). Although no differences were noted with CD8 compartmentalization, other cluster of differentiation (CD) differences have been identified in AD patients (Pellicano et al. 2012). These differences involve relative decreases in $CD4^+$ cells and T regulatory cells, with increases in effector memory and effector memory RA (TEMRA) cells (Larbi et al. 2009). In addition, AD patients in a study by Pellicano et al. (2012) have also been shown to have altered numbers of naive $CD4^+$, more late-differentiated cells and higher percentages of activated $CD4^+CD25^+$ T cells without a Treg phenotype. This suggests that $CD4^+$ cells might be the result of chronic stimulation by amyloid-β present in the blood (Pellicano et al. 2012). Finally, immune system dysfunction as a result of oxidative stress has also been reviewed, and is closely associated with the cognitive impairment present in AD (Ruszkiewicz and Albrecht 2015; Swomley and Butterfield 2015).

Just as noted for PD, neuroinflammation has been associated with the development of AD (Rogers et al. 1996; Heneka and O'Banion 2007; Wyss-Coray 2006; Akiyama et al. 2000), albeit in response to amyloid-β plaque formation (Emmerling et al. 2000), neurofibrillary tangle formation, and apoptosis (Perry et al. 2007; Holmes et al. 2009; Akiyama et al. 2000). This chronic inflammation appears to be due to activated microglia, which are the primary immune cell type located in the brain. Interestingly, the AD brain pathology does not appear to be due to a deficit in key inflammatory regulating proteins, SOCS-1 through SOCS-7 and CIS (Walker et al. 2015). Although this mechanism of chronic inflammation is not completely understood, the continued apoptosis, amyloid-β plaque formation, and neurofibrillary tangle formation contribute to the continued neurodegeneration and cognitive decline in AD patients (Ryu et al. 2015). In addition to being involved with stimulating the inflammatory response in AD patients, amyloid-β plaques induce the production of chemokines such as MIP-1α, RANTES, and MCP-1 (Li et al. 2009a). Accumulation of amyloid-β in synaptic mitochondria is another pathway associated with neural damage via mitochondrial injury, potentially due to a lack of mitochondrial peptidasomes, which can degrade amyloid-β enzyme (Fang et al. 2015).

Microglia function, in part, to regulate the innate immune response, as well as being involved with growth and neuron maintenance (Perry and Teeling 2013). Other studies have identified increased microglial function to stimulate amyloid-β production through a series of cell surface receptor (e.g., TLR2, TLR4, TLR6, CD14 and CD36) pathways (Bamberger et al. 2003; El Khoury et al. 2003; Reed-Geaghan et al. 2009). In addition, stimulated astrocytes can also produce proinflammatory cytokines and activate microglia, as demonstrated in both in vivo and in vitro models of AD (Lee et al. 2010, 2013b), to further exacerbate this system.

As part of the inflammation mechanism, a number of interleukins, such as IL-1α, -1β, -6, -8, -12, -18, TNF-α, and TGF-β, were reported to be elevated in the peripheral blood of individuals with AD compared with controls (Swardfager et al. 2010; Walker et al. 2001; McGeer and McGeer 2001; Bishnoi et al. 2015; Sutinen et al. 2012). IL-1α, -1β, and -6, are produced by glial cells, and show general elevations within the CNS of patients diagnosed with AD (McGeer and McGeer 2001).

For example, IL-1 positive activated microglia are closely associated with amyloid-β plaque formation in AD patients (Griffin et al. 1989). Interestingly, IL-4 may act as an anti-inflammatory cytokine involved with memory/learning, produced by a number of immune cells, and dysregulated in AD patients, further implicating the abnormal inflammation response (Gadani et al. 2012). Although the comparison of a large number of studies indicate that IL-6 polymorphism is likely not an independent risk factor for AD (Han et al. 2011), there is a positive correlation between IL-6 serum levels and severity of AD (Kalman et al. 1997). Increased levels of IL-8 in the CSF have also been documented in AD and are believed to stimulate plaque formation via other cytokines, which act to further stimulate the inflammation response (Galimberti et al. 2006; Franciosi et al. 2005). Bossu et al. (2008) showed that although circulating IL-18 levels were similar between AD patients and controls, there was a significant increased production of IL-18 from stimulated blood mononuclear cells of AD patients. This also corresponded to a polymorphism in the IL-18 gene promoter region and the level of cognitive impairment (Bossu et al. 2008). Although a number of polymorphisms have been identified for IL-18, only single nucleotide polymorphism has been identified to impact IL-18 gene activity (Kalina et al. 2000; Giedraitis et al. 2001). Increased expression of IL-18 receptor in blood cells has been identified in AD patients with Mild Cognitive Impairment (Salani et al. 2013).

The interest in inflammation and the suspected dysregulation of the inflammation response has led to the investigation of cytokine genetic polymorphisms (as previously mentioned) as risk factors for AD. Specifically, neuronal DNA damage is detected at the earliest stages of AD neuropathology, which corresponds to the level of cognitive impairment (Simpson et al. 2015). Although AD is accompanied by an increase in expression of IL-6 and -18 within the CNS, this has been attributed, in part, to the normal aging process (Ershler and Keller 2000; Tha et al. 2000; Sutinen et al. 2012) along with an overexpression of proinflammatory markers including IL-1, -1β, -6, and -18 (Cacabelos et al. 1994; Vandenabeele and Fiers 1991; Ojala et al. 2009). Furthermore, polymorphisms in the gene for apolipoprotein E (APOE gene ε2, ε3, and ε4) have been shown to be directly related to the development of

AD, potentially through a dysregulated mechanism involving lipid transport, delivery, and distribution (Verghese et al. 2011). Over 100 genes were identified in the amygdala, cingulate cortex, striatum, and cerebellum as being potentially associated with AD pathology involving inflammation, proliferation, protein synthesis, signal transduction, and metabolism (Loring et al. 2001). In addition, genetic assessments of polymorphisms show differences in a large number of genes, including some related to inflammation (i.e., RNA encoding transcription factors, neurotrophic factors, synaptophysin, metallothionein III, IL precursor, dPP1, βAPP, NFIL6) within AD target brain regions, such as the hippocampus (Colangelo et al. 2002). In more general terms, missense mutations in the genes of APP (amyloid precursor protein), PS (presenilin)-1, and PS-2 share the common feature of altering the production of amyloid plaques in both familial and sporadic AD (Wang et al. 2006; Rao et al. 2008). Additional studies generating CSF-based proteomic profiles, have identified BDNF, IL-8, amyloid-β, β2-microglobulin, vitamin D binding protein, apo AII, and apoE as being associated with AD (Zhang et al. 2008).

AD individuals exhibited significantly increased expression of monoclonal antibodies directed against amyloid-β, amyloid-β precursor, and the receptor for advanced glycation, all of which are implicated in immunological processes linked to AD (Mruthinti et al. 2004). Although the microglia within the brain appear to be a major player in AD pathology, these changes are also associated with dysfunction of the peripheral immune system (Perry et al. 2007; Holmes et al. 2009). For example, a significantly increased level of immunoglobulins within the AD brain exhibiting apoptotic features that were observed along with a potentially faulty BBB, also implicates an autoimmune-related mechanism (D'Andrea 2003).

Neuropathology

The brain areas most closely associated with memory loss, cognitive decline, amyloid-β plaque formation, neurofibrillary tangles, and apoptosis include the amygdala, hippocampus, and cingulate cortex (cognition/higher-level processing centers), which has been reviewed elsewhere (Rojo et al. 2008; Vinters 2015). As a result, these regions are of particular interest in neuroimaging and histomorphological investigations. AD neuropathology and biochemistry has been reviewed by Thal et al. (2015).

The National Institute of Aging and the Alzheimer Association, has published general guidelines and criteria in which cases with amyloid-β plaques located in the brain are to be classified as AD pathology regardless of their clinical status (Montine et al. 2012), thereby indicating that this neuropathological finding is the hallmark of AD. In addition, AD is routinely defined pathologically by the presence of amyloid-β plaques, hyperphosphorylated protein tau (neurofibrillary tangles), and apoptosis (Selkoe 2000; Rojo et al. 2008; LaFerla and Oddo 2005). Walker et al. (2015) quantitatively assessed microtubule associated tau (HP-τ), amyloid-β protein, and α-synuclein AD patients, and demonstrated that the topographical distribution of these pathological protein aggregates measured across the cortex (frontal, temporal, occipital), cingulate cortex, hippocampus, caudate nucleus, putamen, striatum,

amygdala, substantia nigra, and locus coeruleus allowed for a neuropathological distinction between the various clinical phenotypes related to AD.

In a study by Barnes et al. (2015), AD- and dementia-related neuropathology was characterized in patients of Caucasian or African American backgrounds. The results from this study showed that patients with African American backgrounds were less likely to have AD pathology as a single dementia pathology than patients with a Caucasian background (19.5 vs 42.0%), and were more likely to have AD mixed with an additional pathology (70.7 vs 50.6%), particularly AD pathology and Lewy bodies, and AD pathology, Lewy bodies, and infarcts. Patients with African American backgrounds with AD dementia were more likely to have mixed brain pathologies as compared to age, sex, education, and cognition matched patients with a Caucasian background with AD dementia (Barnes et al. 2015).

One of several phenotypes associated with AD includes olfactory deficits. These AD-related olfactory deficits involve various brain regions (piriform cortex, transentorhinal cortex, anterior olfactory nucleus, and olfactory bulb) and show preferential vulnerability of somatostatin and calretinin-positive cells colocalized with amyloid-β and an increase in prevalence of parvalbumin positive cells (Saiz-Sanchez et al. 2015).

Another consistent pathological finding is widespread white matter hyperintensity on imaging which is linked to cognitive deficits in elderly AD patients (Capizzano et al. 2004; Provenzano et al. 2013). Over the course of a multiyear longitudinal study using high dimensional imaging (of gray and white matter) brain atrophy occurs with the progression of AD (Farzan et al. 2015; Li and Zhang 2015).

Another phenotype involving cognition corresponds to memory/learning deficits associated with AD. In general, chronic inflammation within the hippocampus is associated with AD, which is related to the noted impaired memory (McGeer et al. 2001). As well as inflammation within the hippocampus, neuronal injury and atrophy within the hippocampus occur in AD (McGeer et al. 2002; Carmichael et al. 2012). Although aging-related changes can also occur within the hippocampus, and other brain regions, neuropathological review with MRI (by multidimensional analysis) allows for classification of hippocampal shape features to discriminate between AD with mild cognitive impairment and non-AD patients (Gerardin et al. 2009).

Models of Alzheimer's Disease

The ability of the animal model to be used to predict, in part, clinical data from a drug development, disorder etiology, or risk factors is a key component in data interpretation. Assessment of AD in animals has traditionally relied on not only knockout mice/rats, but also a variety of metabolic profiling, neuropathological assessments, observations and behavioral based tests. The behavioral assessments include the following standard tests: social interactions, motor activity, functional observational battery, as well as T-maze, Y-maze testing, and contextual fear conditioning, voiding behavior, conditioning procedures, burrowing, and nesting (Biallosterski et al. 2015; Janus et al. 2015; Zenaro et al. 2015; Rojanathammanee et al. 2015).

There are more than ten different murine TLRs involved with the neuroinflammation pathway, which has led to apparent conflicting results across the various AD mouse models (Cameron et al. 2012). Several routinely used transgenic and inducible mouse models include the APPSwe/PS1dE9 for amyloidosis, APP/PS1 involving KM670/671NL and L166P mutations and anxiety behaviors with neuropathology, and Igμ knockout for B-cell and immune system deficiencies, and the APPswe/PS1dE9 derived IL-1β overexpression model with chronic neuroinflammation (Janus et al. 2015; Biallosterski et al. 2015; Shaftel et al. 2007; Radde et al. 2006; Weiner and Frenkel 2006). Interestingly, there has been an increased interest in the use of Ts65Dn (Down syndrome mouse model) in studying age-related cognitive dysfunction and basal forebrain cholinergic neuron degeneration in relationship to AD (Alldred et al. 2015).

Numerous transgenic mouse models are present and used in AD research to investigate the development, progression, and treatment for AD. However, one criticism is that many models used to do not exhibit the severity of neuronal loss and atrophy to adequately investigate and assess AD therapies (Birch et al. 2014; Hall and Roberson 2012) thereby supporting the use of other models. One such novel model is the 3×TgAD triple transgenic AD mouse model for APP, PS1, and TAU, which develops plaques and tangles later in life (Roy et al. 2013).

In addition to the genetic animal models used to study AD, there are also drug/chemical induced models such as administration of lipopolysaccharide into the ventricular system which increases microglial reactivity and cell loss associated with AD (Hauss-Wegrzyniak et al. 1998; Wenk et al. 2000). Similarly, administration of full-length amyloid-β 1–42 into rat hippocampus has also provided similar neuropathological results and behavioral phenotypes with AD (Ryu et al. 2015).

Current State of Drug Development

Current drug therapies are primarily developed to target the previously mentioned neuropathology and/or associated mechanisms of AD in order to slow the progression of the disease (Latta et al. 2015). With the increase in awareness of risk factors, metabolic profiles, and genetic markers, the earlier identification of AD should help with the pharmacological intervention and associated treatment strategy programs to more effectively reduce cognitive decline and AD neuropathology (Mar et al. 2015) and include neurofibrillary tangles, NSAIDs, glucocorticoids, amyloid-β plaque formation, immunization, and miscellaneous targets. Alternative therapies include preventative measures, exercise intervention, cognitive intervention, dietary management, and nutritional supplementation review (Nelson and Tabet 2015).

Anand and Sabbagh (2015) discuss newly emerging neurofibrillary targeting strategies covering a variety of pathways, such as immune-based therapy (AADvac-1, Acl-35, BMS 986168, RG7345), microtubule stabilizers (davunetide, epothilone D, TPI 287), tau aggregation inhibitors (LMTX), and specific protein kinase inhibitors (tideglusib, lithium, valproic acid, L-NBP).

Other approaches include NSAIDs, investigated in various epidemiologic studies and clinical trials (Breitner et al. 1995; Stewart et al. 1997; Imbimbo et al. 2010), which potentially interfere with amyloid-β plaque formation and protect against

apolipoprotein E. Treatment of AD with other anti-inflammatories such as glucocorticoids are believed to reduce proinflammatory cytokines (IL-1β and TNF-α), but have been somewhat controversial in nature (Landfield et al. 2007; Masferrer et al. 1994; Buttini et al. 1997). Rivastigmine (acetyl and butyrylcholinesterase inhibitor) and donepezil (acetylcholinesterase inhibitor) and even nicotine (Xue et al. 2014; Shah et al. 2015) have been shown to attenuate the progression of AD pathology and are often included as part of the treatment program.

Molecules that inhibit amyloid-β such as antioxidants (pinocembrin), peptide-based modulators (JPT1, L2P1, IAM2), small molecule-based modulators (curcumin, resveratrol, orcein), metal chelators (Clioquinol), and enzyme inhibitor to β and/or γ secretase act to prevent plaque formation, as well as potentially aiding in dissolving and destabilization of those plaques (Liu et al. 2014; Rajasekhar et al. 2015). Other amyloid-β plaque formation targeting strategies include nuclear activated T-cell inhibition (key player in amyloid-B plaque formation) such as FK506 and tat-VIVIT, both of which attenuated microgliosis and plaques in treated mice (Adessi et al. 2003; Rojanathammanee et al. 2015).

Amyloid β peptide immunization is another treatment that has provided promising results. For example, a study by Paquet et al. (2015) with AN1792 compared AD patients that were immunized to non-immunized in which the immunized AD patients exhibited an accelerated loss of damaged degenerating neurons (Paquet et al. 2015).

5.2.5 Neurodevelopmental Disorders

Neurodevelopmental disorders are a general class of disorders that involve the impairment, dysregulation, or deficiency of growth and development within the central nervous system (CNS). These disorders can affect brain function either early or late in childhood resulting in problems with emotion, learning/memory, general intellectual disabilities, interpersonal communication, social cognition, self-control, and controlled motor movement. According to the Diagnostic and Statistical Manual of Mental Disorders, neurodevelopmental and common mental disorders include, in part: autism spectrum disorder, fetal alcohol disorder, Tourette syndrome, cerebral palsy, fragile-X syndrome, Down syndrome, attention deficit hyperactivity disorder, Mendelsohn's syndrome, and schizophrenia (American Psychiatric Association 2013). Of these disorders, two of the more prevalent from an occurrence and diagnosis standpoint are autism spectrum disorder (ASD) and attention deficit hyperactivity disorder (ADHD), which will be discussed in further detail within this section. Schizophrenia will be discussed in Sect. 5.3.6, as it is more appropriately classified.

5.2.5.1 Attention Deficit Hyperactivity Disorder

Attention deficit hyperactivity disorder (ADHD), as the name implies, involves a difficulty or deficiency in the ability to maintain attention to a specific task along with associated behavioral symptoms including aggression. As such, symptoms of

ADHD include inattention, hyperactivity, and impulsivity across environmental and social settings. There are a variety of ADHD classifications that depend on the cognitive control, level of hyperactivity, and other associated endpoints. Briefly, there are three subtypes of ADHD, which include a prevalence of inattentive symptoms, predominant hyperactive-impulsiveness, and a combination of both inattention and hyperactivity-impulsivity. Several of the key behaviors, which act to help distinguish ADHD from other neuropsychiatric disorders include fidgeting, disorganization, and impulsivity to help with a differential diagnosis (American Psychiatric Association 2013). This syndrome typically first manifests itself in childhood prior to 7 years of age and is also associated with significant functional impairments (often with other disorders) throughout childhood and even later in adulthood. Although ADHD is typically diagnosed in early childhood, the etiology is unclear and the majority of children (approximately 60–80%) will continue to exhibit symptoms through adolescence and/or adulthood (Wittchen et al. 2011; Dopheide and Pliszka 2009).

From a diagnosis standpoint, ADHD is currently considered to be the most frequently diagnosed childhood neuropsychiatric disorder, occurring in approximately 5–12% of school-aged children during 2010–2012 (Centers for Disease Control and Prevention 2013), many of whom also receive a variety of pharmacological interventions. Other sources have indicated that as of 2010, 3.2 million children under 17 years of age had a medical report of ADHD diagnosed by a health care provider; diagnoses have continued to increase since the late 1990s to mid-2000s (US Department of Health and Human Services HRaSA, Maternal and Child Health Bureau 2013). On a global scale, ADHD is among the most common psychiatric diseases at up to 12% worldwide (Faraone et al. 2003), along with one of the highest degrees of suspected heritability at nearly 75% for twins (Faraone et al. 2005).

Other disorders that commonly coexist with ADHD are among the many factors that make ADHD difficult to accurately diagnose. Therefore, ADHD is considered to be a syndrome that includes a range of neural/neurobehavioral abnormalities. In a review by Lo-Castro et al. (2011), ADHD characteristics were associated with a wide variety of disorders from over 30 publications with prevalence rates ranging from 40 to 65% including fragile-X syndrome, neurofibromatosis 1, DiGeorge syndrome, tuberous sclerosis complex, Turner syndrome, Williams syndrome, and Klinefelter syndrome. Interestingly, Rommelse et al. (2008) among other researchers, reported that there is a genetic and neuropsychological gender difference of male and females, with up to a 9:1 gender difference of males to females (Gaub and Carlson 1997). These gender differences which are present in children tend to normalize into adulthood, with a greater number of females being diagnosed later in life (Biederman et al. 1994). Although ADHD arises from partially unknown genetic and environmental pathways with a range of severities, there are certain behavioral manifestations and treatment modalities that commonly present in patients with ADHD.

Although the etiology for many of the neurodevelopmental disorders remains unknown, the development and subsequent progression of the disorders do involve a complex interplay of related genetic and environmental risk factors. ADHD (and other neurodevelopmental disorders) have been reviewed relative to exposure to

organic pollutants, tobacco, ethanol, lead, heroin, and other neurotoxicants (Liew et al. 2015; Yolton et al. 2014). In addition, a comprehensive list of candidate genes for ADHD, including CNTN6, COMT, MAOA, STS, TPH2, and IL6 (Hu et al. 2015; Elia et al. 2012; Drtilkova et al. 2008), have been reviewed; each gene contributes to a relatively small degree to the heritability of ADHD (Faraone et al. 2008). Interestingly, the immune system is one factor that has been reviewed and shown to play an increasingly important role in the developing nervous system, and that early immune system activation may contribute to the general class of neurodevelopmental disorders (Marques et al. 2015; Stigler et al. 2009; Easson and Woodbury-Smith 2014). For example, early immune activation may result in chronic inflammation and the release of immunomodulatory molecules, cytokines, and reactive oxygen/nitrogen species which could profoundly influence various developmental processes depending upon the temporal spatial patterns of activity.

Neuropathology

The brain areas most closely associated with attention, motivation, cognition, and motor movement are the parietal cortex, prefrontal/frontal cortex, ventral striatum, sensorimotor cortex, and cerebellum. As a result, these regions are of particular interest in neuroimaging and histomorphological investigations. For example, progressive atypical contraction of the ventral striatal surfaces and non-progressive contraction of the dorsal striatal surfaces of the basal ganglia were associated with patients with ADHD (Shaw et al. 2014). Additional neuroimaging studies in children diagnosed with ADHD have also identified alterations such as cortical thinning and white matter microstructure abnormalities in the dorsolateral region of the prefrontal cortex, as well as the cerebellum and corpus callosum (Seidman et al. 2005; Witt and Stevens 2015). These findings are consistent with other studies conducted in preterm infants and children that have noted atypical brain volumes, alterations in cortical thickness, and a corresponding decrease in connectivity. Likewise, MRIs from preterm infants have shown the cerebral tissue within the dorsal prefrontal region to exhibit the largest volume reduction (Bora et al. 2014). In addition, ADHD is associated with smaller overall brain volumes (Hoogman et al. 2015) and that when developmental impairments of cortical thinning occur, they are related to the degree of severity of ADHD (Shaw et al. 2013). A general trend noted by Gurevitz et al. (2014) was that smaller neonatal head circumference out to almost 2 years of age, as well as maternal age and familial history of ADHD, correlated with increased incidence in the development of ADHD. This supports the potential link with premature infant birth, lower birth weights, and other maternal risk factors which can directly impact fetal growth and CNS development. Similarly, reduced volumes of the cortical gray matter, associated white matter, prefrontal cortex, and anterior cingulate cortex were identified in adults with ADHD (Seidman et al. 2006; Makris et al. 2007; Shaw et al. 2007).

Pathogenesis of Immune Response

Although most of the investigation concerning the development of ADHD has been in regards to CNS neurotransmitter pathways including dopamine, serotonin, GABA, and glutamine, the role of the immune system as an active player in ADHD etiology is becoming more important. Immune dysfunction has been identified in a large subset of persons with neurodevelopmental disorders, such as ADHD and ASD (Marques et al. 2015; Easson and Woodbury-Smith 2014; Stigler et al. 2009; Mitchell and Goldstein 2014), particularly concerning inflammation, stress response via the HPA axis, autoimmunity, and the gut-brain axis immune system components.

Neuroinflammation likely acts via a mechanism where proinflammatory cytokines, which are distributed throughout the CNS, stimulate inflammation within the developing brain thereby impacting neuronal development and synaptic transmission (Tohmi et al. 2004; Goshen et al. 2007; Aaltonen et al. 2005; Rogers et al. 2011). Indeed, induction of cytokines via an abnormal or normal immune system response can result in inflammation which will impact brain development, and can be linked to perceptual/cognitive alterations (Nawa et al. 2000). Two proinflammatory cytokines that link neuropsychiatric disorders and inflammation include IL-1 and IL-6, among others (Henje Blom et al. 2012; Pandey et al. 2012; Cheng et al. 2012). Specifically, IL-1 is not only expressed in the brain, but also likely involved with CNS plasticity, dopaminergic differentiation, and neural potentiation in addition to immune system function (Merrill 1992; Potter et al. 1999; Vitkovic et al. 2000). In a study of over 80 children diagnosed with ADHD (and their parents) that investigated the role of the IL-1R antagonist gene, an increased transmission of an IL-1Rα repeat was identified, thereby suggesting a role for cytokine activity in the development of ADHD (Segman et al. 2002). Although this finding was not supported by Misener et al. (2004) in which IL-1Rα and IL-1R was examined in almost 180 families with ADHD (involving 220 children with/without ADHD), these findings suggest a more complicated role of the cytokine pathway, brain activity, and ADHD. The role of IL-1α was also investigated in a juvenile rat study by Tohmi et al. (2004)) which found through the use of a variety of behavioral tests (e.g., startle response prepulse inhibition and social interactions) that only clozapine improved prepulse inhibition performance, although treatment with IL-1α, IL-2, or IL-6 resulted in impaired behavioral outcomes. Other studies have supported the role of IL-6 as a candidate gene for neuropsychiatric disorders; in these studies NSAID decreased the incidence of IL-6 expression, which then decreased the expected ADHD outcome via motor activity and HPA axis through glucocorticoids (Bronson and Bale 2014).

GABA is a well-known inhibitory neurotransmitter that is also targeted as part of the pharmacological treatment for patients suffering from ADHD. Of particular interest in the synthesis of GABA, is the enzyme GAD which catalyzes the conversion of glutamic acid into the GABA and exists as two isoforms, GAD65 and GAD67. Although additional isoforms are present during CNS development, GAD65 is responsible for GABA production in the nerve terminals (Pinal and Tobin 1998). Rout et al. (2012), reported that GAD65 antibodies were detected in over a

quarter of the children with ADHD compared to none of the control children that were not diagnosed with ADHD thereby suggesting serum antibodies as a possible biomarker for patients with ADHD.

In a review by Petra et al. (2015), there is increased recognition and awareness of the gut-brain axis in relationship to the both the immune and nervous systems. The gastrointestinal tract has been well documented in terms of the immune system, containing up to approximately 80% of all immune cells in the body, further supporting the link between the immune and gastrointestinal systems (Stigler et al. 2009). Other than seemingly anecdotal cases in which ADHD symptoms were ameliorated with supplementation of iron, zinc, polyunsaturated fatty acids, and even removal of artificial products (Tsai et al. 2013; Schab and Trinh 2004), individuals with ADHD may have an increased prevalence of gastrointestinal difficulties. For example, in the case of celiac disease, which is a multisystem disorder triggered by gluten in genetically susceptible individuals, Gungor et al. (2013) reported that approximately 10% of patients with celiac disease and 28% of patients with inflammatory bowel disease also have ADHD. Likewise, Niederhofer (2011) has reported that celiac disease is markedly overrepresented among patients presenting with ADHD. Although the significance of these findings, as well as whether or not there is a direct relationship, is not clear, these observations suggest a larger population should be evaluated.

Models of Attention Deficit Hyperactivity Disorder

The ability of the animal model to be used to predict, in part, clinical data from drug development, disorder etiology, or risk factors is a key component in data interpretation. Assessment of ADHD in animals has traditionally relied on a variety of observations and behavioral based tests, including social interaction, motor activity, startle responsiveness, functional observational battery, and continuous performance. Due to the use of genetic knockout models, overall size, historical data available within the literature, and large number of markers and assessment techniques, the rodent (i.e., mouse and rat) have been the animal models of choice.

Paradigms of Study

Behavioral assessments of ADHD in animal models typically involve scoring the findings based on predetermined criteria in order to evaluate a pharmacological treatment or gene knockout. For example, the social interaction test evaluates how a rodent behaves (e.g., anogenital investigation, grooming, aggression, and vocalization) when exposed to a conspecific in a neutral setting. Elevated scores in grooming, vocalization, and investigation could be an indication of ADHD related behaviors. The functional observational battery measures a variety of functional domains including autonomic, neuromuscular, sensorimotor, physiological, and

CNS excitability/activity. Elevated scores in defecation, urination, mobility, tail pinch response, touch response, vocalization, rearing, and tonic/clinic movements could be an indication of ADHD related behaviors. More quantifiable evaluations include the prepulse inhibition and motor activity evaluations. Specifically, the prepulse inhibition involves the pairing of a startle-inducing noise burst with a non-startle-inducing stimulus, with the response to that stimulus being attenuated. Disruption in this attenuation is indicative of ADHD related symptomology (Aga-Mizrachi et al. 2014). By comparison, the motor activity test evaluates exploratory behavior in a novel environment though measuring a quantifiable endpoint of beam breaks to score activity. Increases in overall activity or activity scores identifying repetitive movement is often associated with ADHD related behaviors.

Animal models and associated study paradigms can raise questions concerning how the results apply to the clinical population. The continuous performance task is often used in people and involves a specific predefined task to identify a target of interest (Tomlinson et al. 2015; Manor et al. 2008). As part of this evaluation, the subject presses a button for a target and does not press a button for an off target, all in an attempt to measure inattention. Typical endpoints commonly include button latency, incorrect response count, and omission errors. For example, an increase in target button latency and incorrect responses would be associated with inattention, and thereby correspond to ADHD related behavior.

Genetic/Selective Breeding Models

In addition to the 0.75° of heritability of ADHD, as noted in twin studies (Faraone et al. 2005), there are a large number of neurotransmitter and signaling related candidate genes (i.e., SLC9A, COMT, SLC6A2, MAOA, SLC6A4, TPH2, COMT, 5HTT) some of which have been identified with varying success through linkage analysis and genome wide scans (Elia et al. 2012; Lasky-Su et al. 2008; Muller et al. 2008; Drtilkova et al. 2008; Fisher et al. 2002). This has led to the development of a number of knockout and selective breeding animal models to study ADHD.

In terms of knockout mice, the D4R (dopamine receptor 4) knockout shows that treatment with a dopamine receptor agonist along with COMT inhibition acts to improve attention and response (Tomlinson et al. 2015). A commonly used rat strain to investigate ADHD related behavioral and genetic mechanisms include the spontaneously hypertensive rat (SHR) and control Wistar Kyoto (WKY) counterpart (DasBanerjee et al. 2008; Watterson et al. 2015; Sagvolden et al. 1992). The use of the SHR and WKY control rat strains corresponds to the hypofunctional catecholaminergic system associated in the striatum, nucleus accumbens, and prefrontal cortex along with possible glutamate dysfunction, which is also a key player in the pathophysiology of ADHD (DasBanerjee et al. 2008; Miller et al. 2014).

Current State of Drug Development

Overall, the drugs that treat ADHD are highly efficacious (Shier et al. 2013; Faraone et al. 2006), which makes ADHD as one of the most treatable psychiatric disorders via pharmaceutical manipulation. In many cases (upwards of 70%), pharmacological treatment has been shown to improve ADHD. However, it is worth noting that treatment is palliative in nature, meant to be continued over an entire lifespan, and not meant to be a cure. As of 2010, approximately three quarters of children diagnosed with ADHD were undergoing pharmacological treatment, with less than half undergoing behavioral therapy, and even fewer utilizing dietary supplementation (US Department of Health and Human Services 2013; Visser et al. 2015).

As part of the treatment process, several neurotransmitter systems are implicated in ADHD including GABA, serotonin, and catecholamines. Therefore, major drug class treatments and targets include the following stimulants (methylphenidate, amphetamine, dextroamphetamine, dexmethylphendiate, and clonidine) and antidepressants (nortriptyline, imipramine, desipramine, bupriopion, escitalopram, sertraline, venlafaxine, and atomoxetine which is an inhibitor of the presynaptic norepinephrine transporter acting to enhance the availability of dopamine to increase attention) (Manos 2008; Southammakosane and Schmitz 2015). Typically, MAO inhibitors are not used due to potential deleterious interactions with other prescribed medications. Although these drugs are designed to target neurotransmitters within the brain, the exact method of how they ameliorate ADHD symptoms is not completely known. For example, treatment with a stimulant is generally considered to be the most efficacious and believed to act by increasing dopamine levels in the brain's attentional regions, and increasing the presynaptic dopamine and norepinephrine levels.

5.2.5.2 Autism Spectrum Disorder

Autism spectrum disorder (ASD) encompasses a variety of neurodevelopmental disorders, including autistic disorder, Asperger syndrome, and pervasive developmental disorder not otherwise specified (American Psychiatric Association 2013). Typically, ASD is diagnosed within the first 36 months of life, with diagnosis based upon a child's behavior and development. Even if diagnosis occurs later in life, symptoms would have become present early in childhood. The symptoms exhibited by individuals with ASD fall on a continuum, varying from very mild to severe, but often relate to communication/verbal deficits, social interaction impairments, deficits in social communication, reliance on routines, and sensitivity to environmental changes/stimuli (American Psychiatric Association 2013; Baranek 2002; Smith et al. 2004). In 2010, ASD was diagnosed in approximately one in 68 children, with males being significantly more affected than females indicating a gender bias (Mezzelani et al. 2015; Centers for Disease Control and Prevention C 2015). Other medical conditions commonly found in people with ASD are anxiety, immune

system dysfunction, mitochondrial disease, seizures/epilepsy, and attention deficit disorder. The severity and degree of these conditions may or may not be similar to the general population; however, it remains in the field of study.

Although there is a high concordance rate in identical twin studies, the lack of 100% concordance along with an increasing prevalence rate suggests non-genetic causes. Recent studies have shown up to an 18% recurrence rate within a family, and up to 35% in twin studies (Constantino et al. 2010). In a twin study by Hallmayer et al. (2011), the environmental role for variance in autism risk among twins was found to be at approximately 55%. Of course, the term environment is rather broad and can include anything from pollutant exposure (e.g., pesticides) to maternal nutrition (e.g., folic acid deficiency) during pregnancy, both of which are known to impact neurodevelopment through a variety of mechanisms. Due to phenotypic variability of ASD, earlier screening tools via genetic testing can be applied to children. In a study by Pramparo et al. (2015), leukocyte RNA expression from over 140 children was measured and showed that both the general immune response and inflammation function was ~75% accurate as compared to control children. As is the case for any complex disorder, genetic evaluations (even with heritability and twin studies) for ASD candidate genes have led to not only NLGN3, NLGN4, NEUREXIN1, SHANK3, and CNTNAP2, but also many more involved with neuronal synapse development and behavioral expression (Hu et al. 2009; Hu and Steinberg 2009).

An interesting genetic component of neurodevelopment disorders, which has also gained momentum in ASD research, is the role of disrupted immune system genes. Dysregulated immune function during one or more of the critical periods of development for different organ systems (e.g., gastrointestinal, CNS, and cardiovascular) which persists into adulthood may, in part, be responsible for the behavioral changes and range of symptoms observed in ASD patients. For example, it has been shown that there is an increased frequency of a mutation associated with complement and the major histocompatibility complex (Warren et al. 1996). In a separate review by Ashwood et al. (2006), there is a potential that a dysregulated immune system during neurodevelopment leaves the brain susceptible to insult and generation of characteristic ASD symptoms. Other potentially indirect players impacting the maternal to fetal immune system activation during pregnancy as a result of "environmental" factors could be in the form of fever, rubella, or even general bacterial infection.

Neuropathology

The brain areas most closely associated with and examined in neurodevelopmental disorders involving cognition, motor movement, social interactions, auditory processing, and emotional processing include, in part, the cerebellum, thalamus, hypothalamus, amygdala, and sensorimotor cortex (Ball et al. 2012; Abernethy et al. 2002; Allin et al. 2001). As a result, these regions (as well as others) are of particular interest in neuroimaging and histomorphological investigations. Although a detailed

discussion of all of the many anatomical, developmental, and neurotransmitter related changes is beyond the scope of this discussion, these interactions have been reviewed previously (Shriber 2010).

Libero et al. (2015) conducted a neuroimaging study in 29 males and eight females (half of which were adults with ASD and other half served as controls, all in their mid to late 20s) in which analysis revealed increased cortical thickness across the left cingulate, left pars opercularis of the inferior frontal gyrus, left inferior temporal cortex, and right precuneus along with reduced cortical thickens in the right cuneus and right precentral gyrus. In addition, ASD is known to have cortical abnormalities, such as increased microglial density (Nickl-Jockschat et al. 2012; Morgan et al. 2010). Although there do appear to be volumetric differences in children with ASD showing a larger brain size by typically one year of age, there are some noted reductions in cerebellar and corpus callosum volume, with increases in the volume of the amygdala (Courchesne et al. 2003; Hashimoto et al. 1995; Howard et al. 2000; Nosarti et al. 2004) and frontal cortex (Shen et al. 2013).

Since there are such widespread differences from an anatomical standpoint involving a larger brain volume to altered white and gray matter organization, a number of mechanisms are expected to be involved. To a certain degree, this is likely due to decreased cortical column structures and dendritic growth thereby altering neuronal connections within the brain (Casanova et al. 2006; Stoner et al. 2014). As a result, brain function across multiple regions would be impacted, with the possibility of impacting many of the functional characteristics associated with ASD (Wicker et al. 2008; Deshpande et al. 2013; Muller et al. 2001). The actual anatomical anomalies, white to gray matter organization, and changes in neuronal health suggest multiple mechanisms at play.

As a result of the auditory processing, language, inability to recognize facial cues, and social interaction deficiencies, the superior temporal gyrus has been implicated in autism, where supragranular and infragranular layers of the cortex are located with various abnormal projections (Bigler et al. 2007). However, in a study of eighteen individuals (half with autism and half without) which involved a postmortem examination in order to investigate abnormalities in number/morphology of the cortex, amygdala, and cerebellum showed that there were no alterations of the supragranular to infragranular neurons associated with autism (Kim et al. 2015a). Although these results are potentially conflicting, there is no clear indication concerning the neural circuitry, specific cell populations, or other receptor density, suggesting the need for additional examination of specific ASD subtypes across target brain regions.

Pathogenesis of Immune Response

Much of the investigation concerning the development of ASD has been in regards to the various genetic, morphometric, and CNS neurotransmitter pathways. With the large number of interactions between the immune and nervous systems, the degree of interplay between cytokines, astrocytes, interleukins, and phagocytes in terms of their

direct impact on ASD development is not well understood. In a review of over thirty in utero and neonatal studies with immune-related endpoints, ASD could be due to any number of altered lymphocyte responses and neural autoantibodies, and to differentially regulated cytotoxicity pathways driven by environmental factors (Mead and Ashwood 2015; Ashwood et al. 2006). Even with such a large degree of ambiguity, the importance of the immune system in this mechanism is evident. As a result, the research concerning the role of the immune system as an active player in the development of ASD is bringing about interesting new pathways and mechanisms of development dealing with inflammation, immunodeficiency, oxidation dysregulation, and autoimmunity. The role of immune system dysregulation in ASD has been reviewed in more detail elsewhere (Theoharides et al. 2015; Pervanidou et al. 2007; Wei et al. 2011; Estes and McAllister 2015; Rossignol and Frye 2012).

Immune system dysfunction involving cytokine signaling and immunomodulating factors has been reviewed to varying levels of detail (Rusu et al. 2015; Theoharides et al. 2013; Ashwood et al. 2011). Children diagnosed with ASD have been shown to have altered cytokine profiles, inflammatory profiles, and response patterns (Ashwood et al. 2003; Li et al. 2009b; Morgan et al. 2010). This results in inflammation and dysregulated neuronal connections, growth, and development within specific brain regions. These findings of elevated levels of inflammation have been supported by Singh et al. (1998), in which children diagnosed with ASD exhibit chronic CNS inflammation. From a developmental standpoint, signaling by cytokines, such as IL-1β, -2, -3, -4, -5, -7, -9 and -11 allow for neural-tube development with any pathway dysregulation potentially interfering with CNS development (Mehler and Kessler 1997).

Mutations in the gene for the Methyl-CpG-binding protein (MeCP2) or a lack of the functioning protein have been linked to Rett Syndrome, which is a rare form of autism, with mutations present in a majority of Rett Syndrome children (Amir et al. 1999; Huppke et al. 2000). The protein, MECP2, is a general transcriptional repressor that has been linked to silencing genes within the CNS, often leading to abnormal neuronal connections (Na et al. 2013; Nguyen et al. 2012). Interestingly, children with additional copies of the MECP2 gene (MECP2 duplication syndrome) exhibit a dysfunctional immune system, along with intellectual impairments (Bauer et al. 2015).

Due to the relatively low numbers of antioxidants along with elevated periods of metabolic demand, the brain can be considered somewhat vulnerable to oxidative stressors. Specifically, chronic oxidative stress is commonly reported in children with autism (Chauhan and Chauhan 2006), as well as other classically defined neurodevelopmental disorders (Prabakaran et al. 2004). In addition, children diagnosed with autism also show metabolic profiles from a genotype and phenotype standpoint that is associated with indications of oxidative stress (James et al. 2006). The question that arises is the degree of this immune dysregulation which arises from a whole system or a particular problem within the cell. Mitochondrial dysfunction is implicated in the pathogenesis of neuroimmune disorders (Guo et al. 2012).

From a neuroinflammation and neuroimmune mechanism stance, there appears to be strong activation of microglia and astroglia by TNFα and MCP-1 (Vargas et al. 2005), which may be involved in the development of ASD through a dysregulated

immune system. The cytokines tumor necrosis factor (TNF) and interleukin-6 (IL-6) have been shown to be increased in ASD patients (Zimmerman et al. 2005; Li et al. 2009b). In addition, elevated levels of IL-6 have also been identified in other neuropsychiatric disorders, including depression, schizophrenia, and post-traumatic stress disorder (PTSD) (Al-Hakeim et al. 2015; Wei et al. 2012). Along with nervous system dysfunction concerning immune activation, there is also evidence of autoimmunity issues in children with ASD. Specifically, CNS antibodies have been detected under general *in vivo* and *in vitro* conditions (Rossi et al. 2011; Braunschweig and Van de Water 2012), as well as to specific GABAergic neuron proteins (Rossi et al. 2011). Autoantibodies against the CNS have been reported in the serum of Rett syndrome patients (Klushnik et al. 2001), and also to nuclear brain proteins (Goines P, Van de Water J 2010; Rusu et al. 2015) . In the case of ASD, 30% of children evaluated exhibited elevated antibody levels directed against the cerebellum (Braunschweig and Van de Water 2012). Subsequently, additional studies in patients with ASD have identified the presence of autoantibodies against human neuronal progenitor cells, suggesting that there is an impaired ability of the immune system to recognize brain antigens (Mazur-Kolecka et al. 2014).

Paradigms of Study

Although animal models are useful in the study and understanding of the developmental disorders in people, they do not show a direct relationship to the clinical setting and require a certain level of data interpretation and extrapolation. As such, multiple rodent models (i.e., rat and mouse), pharmaceutical manipulation (minocycline, D-cycloserine, risperidone, clonazepam, or desipramine), and behavioral paradigms are commonly used to assess ASD parameters.

Paradigms of interest involve Y maze, elevated plus maze, motor activity, open field tests, inhibitory avoidance, startle response, sociability compartment, seizure tests, spatial learning/memory, fear conditioning (Lazaro and Golshani 2015; Donaldson and Hen 2015). As part of a rather large panel of neurobehavioral evaluations, many of these rodent paradigms have already been discussed in relationship to other CNS disorders, with the exception of the sociability compartment model. The sociability models utilizing a three compartment model and measuring interactions over multiple sessions between two mice, such as self-grooming, harrowing, anogenital investigation, jumping, and aggression for example, in terms of counts, latency, and duration (Burket et al. 2015; Crawley 2004; Jacome et al. 2011). In each of these paradigms, ASD-related behaviors include general decreased sociability, increased vocalization in pups, decreased vocalization in adults, hyperactivity, stereotypies, increased grooming, and increased anxiety scores.

There are a number of knockout mouse models, two of which include the FMRP (fragile X mental retardation protein) and Mecp2 (methyl-CpG-binding protein) strains. For example, the loss of FMRP leads to dysregulated connections of dendrites and neurons within the CNS. The FMR knockout mouse exhibits mild deficits in behavior with mild to moderate hyperactivity, and some learning deficits; however,

the neuropathological findings are robust and consistent with fragile X-syndrome with an increased amount and length of dendritic spines (Heulens and Kooy 2011; Darnell et al. 2011). The gene MeCP2 plays an important in neuronal connections (Na et al. 2013), with the MeCP2 knockout exhibiting lower brain weight, smaller neurons, reduced synaptic plasticity along with cognitive deficits and altered exploratory activity (Chen et al. 2001), indicative of ASD.

Current State of Drug Development

The treatment of ASD is not to cure the disorder itself, but to ameliorate the often debilitating symptoms including hyperactivity, hostility, irritability, depression, and aggression. In addition to standard pharmacological treatment, a multifaceted approach involving behavioral intervention, counseling, and education is often the most effective. As is the case with many neuropsychiatric disorders, this should be considered a lifelong disability where treatment is often needed, without a known cure.

Pharmacological treatment options of patients diagnosed with ASD include a wide range of drugs, often based on the other disorders which are present. These drugs routinely consist of antipsychotics (haloperidol, risperidone, aripiprazole), antidepressants (fluvoxamine, clomipramine, fluoxetine, citalopram), stimulants (methylphenidate, atomoxetine, clonidine), and anticonvulsants (valproate, divalproex sodium) (Mohiuddin and Ghaziuddin 2013; Shier et al. 2013). Specifically, treatment with antipsychotics, such as haloperidol or risperidone, helps to reduce irritability, aggression, and general behavioral disturbances by acting on dopamine (dopamine type 2 receptor antagonists), serotonin (serotonin type 2A antagonist), or a combination of the two pathways (Aman et al. 2005a, b). Although there is some debate as to the effectiveness of antidepressants in treating ASD, this class of drugs includes selective serotonin reuptake inhibitor and serotonin/norepinephrine reuptake (tricyclics) through the serotonergic and/or noradrenergic pathways to increase the extracellular concentrations of serotonin and/or norepinephrine (Hurwitz et al. 2012; Williams et al. 2013). Through treatment, these antidepressants relieve the ASD related behaviors of irritability, anxiety, aggression, stereotypies, and agitation. The use of a stimulant as a treatment option acts to relieve symptoms of hyperactivity, inattention, aggression, and impulsivity as noted with ADHD which is often a comorbid condition that was discussed previously (Santosh et al. 2006). Anticonvulsant or antiepileptic drug administration acts to not only treat a comorbid condition of epilepsy, but also to aid in social communication and alleviate behavioral disturbances (Hirota et al. 2014; Reilly et al. 2014). Drugs within this class commonly act on the GABAergic system (receptor, transporter, or regulatory enzymes), as well as a number of voltage-gated channels to alleviate related activity.

5.2.6 Common Mental Disorders

5.2.6.1 Anxiety

Anxiety is a normal biological reaction to stressful situations, which can be beneficial, for instance, by increasing alertness in a new environment or learning to predict and avoid harm. However, these responses may become pathological in some individuals, who exhibit irrational fear and dread when there is no danger. As a whole, anxiety disorders affect approximately 42 million adults in the United States (Kessler et al. 2005), which makes anxiety prevalent and a leading cause of days of work lost due to disability (Marciniak et al. 2004). There are numerous specific diagnoses under anxiety disorders, based primarily upon symptom presentation and duration. An exhaustive review of each subtype of anxiety disorder is beyond the scope of this chapter; the reader is referred to Craske et al. (2009). Rather, we will discuss the role of the immune system in the neuropathology of anxiety disorders as a whole, which at their root have commonalities of irrational fear and dread, and we will utilize specific examples from the literature.

It is unknown why some individuals progress to a pathological anxiety condition; however, anxiety symptoms and resulting pathologies are accompanied by heightened sympathetic arousal and emotional processing. Several neurotransmitter systems are implicated, although serotonin (underactivation) and norepinephrine (overactivation) may explain hyperresponsiveness and increased emotionality in anxiety disorders. Given that both the normal and pathological conditions stem from reaction to stressful events, the majority of anxiety research attempts to elucidate the effects of stress, such as psychosocial stress in the case of social anxiety or a traumatic stress in the case of Post-Traumatic Stress Disorder (PTSD), on subsequent emergence of pathologies. Stress activates a complex set of behavioral and neurophysiological changes involving the endocrine, nervous, and immune systems. When a threat is immediate (or perceived), several key brain and endocrine glands known as the hypothalamic pituitary axis (HPA) are activated, namely the paraventricular nucleus (PVN) of the hypothalamus, the anterior lobe of the pituitary gland, and the adrenal gland. Briefly, in response to a stressor, corticotrophin releasing factor (CRF) is released from the hypothalamus to the pituitary gland; and the pituitary releases adrenocorticotropic hormone (ACTH) into systemic circulation. ACTH binds to glucocorticoid receptors on the adrenal glands, resulting in the synthesis and release of glucocorticoids (primarily cortisol in humans and corticosterone in animal models, as discussed below). CRF also activates noradrenergic pathways via the locus coeruleus, resulting in increased norepinephrine and epinephrine. These changes, in a normal threat condition, allow for the "flight-or-fight response" and are short-acting. That is, when the threat has passed, the HPA axis returns to normal via a negative feedback loop whereby the increased cortisol acts upon the hypothalamus to decrease CRF and ACTH. Dysfunctional HPA axis activation, such as persistent activation, may underlie some mood and anxiety disorders. For instance, increased plasma cortisol occurs in patients with social phobia

when exposed to a psychosocial stressor, compared to control subjects (Condren et al. 2002). Psychological stress has also been shown to result in elevated cortisol in PTSD; however, there have been variable reports of altered (lowered) baseline cortisol in the population as well (de Kloet et al. 2006).

Neuropathology

The progression from a normal biological response to pathological anxiety and inappropriate response is accompanied by stress-induced morphologic changes in the brain, which may be due to or exacerbated by chronic HPA dysfunction. For instance, it is known that the hippocampus is particularly sensitive to increased cortisol, and indeed, reduced hippocampal volumes are noted in PTSD and other anxiety disorders (Woon and Hedges 2008). Neuroimaging studies suggest that brain activity is altered across an extensive cortico-striato-limbic network, regions responsible, in part, for interpreting social cues/threat (Martin et al. 2009). Exposing subjects with anxiety disorders to harsh/fearful faces results in activity changes (as measured by functional Magnetic Resonance Imaging) that correspond to symptom severity and are suggestive of higher emotionality and decreased ability for cognitive control. Amygdala activation, a key feature across anxiety disorders, may mediate these network changes (Holzschneider and Mulert 2011), as the amygdala can activate the HPA via serotonergic projection neurons. In addition to amygdala activation, there has been evidence of over-activation of brain regions responsible for sensory function, motor response, facial recognition, and risk (left postcentral gyrus, putamen, and right inferior frontal and middle temporal gyri). Importantly, activation of these brain regions often occurs in conjunction with decreased activation of higher cortical regions involved in self-awareness, memory, and cognitive control (left precuneus, posterior cingulate gyrus, and medial prefrontal cortex) (Martin et al. 2009). The underlying causes of these network dysfunctions are unknown, but likely have both genetic and environmental causes.

Pathogenesis of Immune Response

Thus far, we have considered that pathological anxiety may emerge from dysfunctional HPA axis activation, as the role of stress in anxiety is generally well accepted. Such dysfunction results in increased glucocorticoid (cortisol) secretion and subsequent desensitization of central glucocorticoid receptors, which causes disinhibition and continued HPA activity. However, the mechanisms that drive this continued dysfunction, in comparison to a subject who does not progress to a pathological state remain unclear. One area of focus is the immune system, because proinflammatory cytokines are induced by stress and activation of the HPA via cytokine-mediated neuroendocrine signaling through the adrenal glands (Goshen and Yirmiya 2009). This rise in proinflammatory cytokines

results in further stimulation of the HPA axis. As such, stress-induced activation of the immune response may be one explanation for the progression of anxiety disorders in general. For instance, psychological stress has been shown to transiently increase multiple proinflammatory cytokines (TNFα, IL-6, IL-1Ra, IFNγ) in control subjects, and individuals with high perception of stress also have increased inflammatory markers (Maes et al. 1998). Traumatic events, a precipitating factor in the development of PTSD, also result in increased levels of IL-6 and TNFα; however, these proinflammatory cytokines remain elevated only in patients that subsequently exhibit post-traumatic psychopathology (Sutherland et al. 2003; Tucker et al. 2004). Furthermore, increases in TNF receptors, specifically sTNF-RII, are associated with reduced hippocampal volume, a key feature that correlates with PTSD severity (O'Donovan et al. 2015).

Models of Anxiety Disorders

In order to extrapolate animal data to the clinical population, an animal model should, through use of behavioral paradigms or pharmacologic manipulation, predict performance in human subjects (predictive validity) and be designed to measure the human condition of interest (face validity); further, interpretations from the model should be consistent with/based upon the human condition (construct validity). Assessment of anxiety in animals has traditionally relied on ethologically based tests, relying on the natural exploratory or freezing behavior of rodents. However, pharmacological modeling (e.g., screening for anxiolytics/antidepressants), often used in conjunction with behavioral paradigms, is subject to a number of limitations with extrapolating the behavioral data to the target/clinical population, as discussed below. As such, an increasing focus has been placed on the development of animal models for relevance to specific human pathological conditions For an in-depth review of anxiety models, the reader is referred to Bailey and Crawley (2009) and Steimer (2011).

Stress and trauma are primary risk factors in anxiety disorders, and humans and individuals with anxiety disorders show several indicators of increased stress response, including HPA axis activation, sensitization of limbic circuits, and immune system dysfunction. Given the importance of stress as a precipitating factor in the development of anxiety disorders, a variety of animal models have been developed to evaluate behavioral responses of rodents to acute and chronic stressors, and further to examine the role of the immune system in mediating the behavioral responses to stressors. The type of stressor (psychosocial vs. physical) has important implications for translational validity. For instance, social phobia/anxiety and PTSD have a particularly high prevalence (12.1 and 6.8%, respectively) and are a continuing focus for animal model development and pharmacotherapy; the reader is referred to Campos et al. (2013) for in-depth review.

Psychosocial Stress

Social hierarchies/social interactions have a profound effect on the HPA axis and the immune system in both humans and rodents (DeVries et al. 2003; Bartolomucci 2007; Lukkes et al. 2009). Manipulation of the social environment of a rodent, at varying points in the lifespan, can therefore be used to approximate the effects of social isolation, bullying, and other social situations on physiological and behavioral responses by assessing neurochemical and behavioral responses. Most rodents are naturally social and introduction to a new member (assuming neutral territory, similar weights, and other factors that reduce issues of dominance), will result in social interactions. However, in response to juvenile social isolation or chronic social defeat, a rat will exhibit multiple somatic and behavioral signs, including HPA activation, changes in immune function, and social withdrawal and hiding (Bartolomucci et al. 2001). Wohleb et al. (2013) suggest that immune activation, as evidenced by activated microglia and increased movement of monocytes to the CNS from the periphery, underlie anxiety-like behaviors, as knockout mice deficient in receptors associated with monocyte trafficking did not exhibit symptoms of anxiety or stress.

Acute/Traumatic Stress

PTSD, which affects nearly 7% of the population, can be mimicked by exposing rats to a traumatic event such as a shock or predator. The effects of even single exposures are long-lasting and represent sensitization (non-associative) of fear response that is similar to that of PTSD patients (Campos et al. 2013). Because brain regions underlying fear in rodents are analogous to that in humans, these paradigms can be used to assess the neurobiology, biochemistry, and gene expression in normal versus pathological fear memories. Namely, the behavioral and neurobiological changes during fear learning and memory can be evaluated in response to changing salience of the trauma and in response to pharmacologic intervention. For example, rats exposed to increasing numbers of shocks exhibit increased and longer-lasting plasma corticosterone levels in conjunction with long-lasting exaggerated startle response (Servatius et al. 1995), sensitization to novel stressful stimuli and altered immune function such as increased IL-1β synthesis (Stam 2007). The importance of these models is that behavioral sensitization and immune system changes are long-lasting, providing translational validity to human conditions and opportunities for drug development.

Genetic/Selective Breeding Models

A variety of rat selection models, whereby rats are selected based upon high or low anxiety traits, have been proposed to model anxiety behaviors. These include selective breeding for high or low anxiety behavior (HAB/LAB) or high/low avoidance behaviors (Syracuse rats and Roman Low-Avoidance Rats). These rats, in addition to exhibiting trait and/or state-like anxiety behaviors have varying degrees

of validity such as adrenal gland hypertrophy, altered corticosterone synthesis, and HPA axis activation (Haller and Alicki 2012; Wegener et al. 2012). However, these models allow for the assessment of gene-environment interactions, critically important in studying complex neuropsychiatric disorders such as anxiety, which involves the progression of a normal response to a pathological condition.

Paradigms of Study

Classical tests of anxiety measure the behavior of rodents in a variety of environments, which most commonly include the elevated plus maze, the open field, and the light-dark box. In these tests, the approach/avoidance of the rat or mouse to an aversive stimulus, such as the open arms of an elevated plus maze, the center of an open field, and the bright side of a light-dark box are used to assess the natural anxiety-like behavior of a model relative to a control and/or the anxiogenic/anxiolytic features of pharmacologic interventions. For example, a model developed to mimic an anxiety disorder should show reduced exploratory behavior compared to a control; if a drug increases its exploratory behavior in an open field, time in the open arms of an elevated plus maze, or crossings into the bright side of a box it may show translational promise as an anxiolytic medication for humans. However, this premise depends upon the pharmacologic intervention decreasing natural fear behaviors in rodents, where the response is appropriate for the current condition, and pathological fear/anxiety in humans, where the response is inappropriate for the current conditions (Bailey and Crawley 2009; Haller and Alicki 2012).

Current State of Drug Development

Despite the high prevalence of anxiety disorders, there has been relatively little progress in drug development. In part, this is due to the heterogeneous symptoms, unknown etiology, and the high co-morbidity and symptomatic overlap between anxiety disorders and other neuropsychiatric disorders. Prescriptions generally fall into four classes: anticonvulsants, antipsychotics, benzodiazepines, and antidepressants. The first two medication types are rarely used in current therapy, due to side effects and the relatively high success rate of current antidepressants across anxiety disorders. The mechanism of action of anticonvulsants is not fully understood; however, they may reduce anxiety by modulating inhibitory neurotransmission via gamma alpha butyric acid (GABA) or voltage-gated calcium channels in fear circuits. First- and second-generation antipsychotic medications are approved for use in anxiety disorders; however, there are few long-term studies and they are not recommended as first-line therapies (Pies 2009). Benzodiazepines, among the earliest medications prescribed for anxiety, enhance GABA activity, the major inhibitory neurotransmitter in the brain. Benzodiazepines are extremely effective for reducing acute anxiety, but are associated with numerous risks (dependence, memory impairment, death) and are therefore used only short-term or as a transition while patients adjust to a

longer-acting medication. Selective serotonin reuptake inhibitors (SSRIs) are the first-line pharmacotherapeutics for anxiety disorders as a whole, which is perhaps not surprising given the high co-morbidity (50%) and overlapping neurobiology with depression (Gulley and Nemeroff 1993; Nemeroff 2002; Smeraldi 2012). SSRIs, such as fluoxetine, sertraline, citalopram, escitalopram, and paroxetine, increase the concentration of serotonin in the synapse, which is implicated in mood and arousal. However, the mechanism by which SSRIs improve anxiety is not fully understood, as few drugs that specifically target serotonin receptors, such as buspirone, a serotonin receptor (5-HT1A) partial agonist, have been approved for use as anxiolytic medications (Li et al. 2012). Because it takes several weeks for SSRIs to show anxiolytic effects in humans, alternative mechanisms of action have been proposed. One such mechanism may be through immune system modulation, as antidepressants have been shown to reduce inflammatory markers such as IL-6 and IL-1β in humans (Tucker et al. 2004; Hannestad et al. 2011) and rodents (Song and Wang 2011). As such, it is possible that antidepressants are effective in treating anxiety disorders through a number of mechanisms. Dewall et al. (2010) determined that moderate doses of acetaminophen reduce participants' responses to social rejection via CNS-mediated effects on brain regions that underlie social distress, providing some evidence of potential benefit of immune system modulation on emotionality and social anxiety. However, to the best of our knowledge, there have been no empirical studies to determine whether manipulation of immune system pathways results in changes in anxiety symptoms or related neurochemistry.

5.2.6.2 Depression

Depression occurs in approximately 15 million Americans (nearly 7% of adults) each year (National Institute of Mental Health N 2014) and is characterized by persistent or recurring changes in mood, particularly depressed or sad mood, social isolation, changes in sleeping patterns, and/or physical symptoms such as weight loss or weight gain. Like most neuropsychiatric disorders, the etiology of depression is complex and not well understood. Significant risk factors include biological factors such as gender and genetic vulnerabilities, as women are twice as likely to experience depression (Nolen-Hoeksema 2001) and there is a high familial transmission rate (up to 50% or more) of depression (Wong and Licinio 2001; Lohoff 2010). However, despite the high heritability of major depression, there are no specific genetic abnormalities that predict depression. Rather, a number of genes are suspected to interact with environmental factors, leading to the onset and subsequent relapse in depressive episodes (Lohoff 2010). Imbalances in central neurotransmitters, primarily serotonin, norepinephrine, and dopamine, were considered the primary cause of depression. However, the monoamine hypothesis is limited by a number of considerations, notably the long-term and complex interactions with environmental factors and the delayed therapeutic response to antidepressant medications. These medications produce a pharmacologic effect (increased levels of central monoamines) but take several weeks to show therapeutic efficacy. Of particular relevance are psychosocial factors, particularly negative life events, such as stress and trauma/medical illness.

As discussed for anxiety disorders (see Sect. 5.3.6.1), stress or trauma results in activation of the hypothalamus-pituitary-adrenal (HPA) axis. Depressed patients consistently exhibit markers of increased HPA activation, including increased cortisol production and increased endocrine (pituitary and adrenal gland) activity (Pariante and Lightman 2008). These changes are similar to those noted in anxiety disorders, and indeed anxiety either precedes or occurs in conjunction with depression for the majority of patients diagnosed with a depressive disorder. This may be due to the long-term effects of HPA axis activation on glucocorticoid receptors and neuroplasticity in the hippocampus and prefrontal cortex, as these brain regions are sensitive to elevated cortisol.

Neuropathology

Reduced hippocampal volume is one of the most consistent neuropathological findings in patients with depression (Koolschijn et al. 2009), and is associated with an increase in lifetime duration of depression (MacQueen et al. 2003; Sheline et al. 2003). The hippocampus contains a high concentration of glucocorticoid receptors and is therefore particularly sensitive to cortisol produced as a result of persistent HPA activation. Despite the apparent relationship between hippocampal volumes and depression, studies have failed to find a correlation between hippocampal neuropathology and symptom severity. Furthermore, reduced hippocampus volume has been noted in first episode patients, raising the possibility that morphological abnormalities confer a predisposing risk factor to depression. Animal data have shown that chronic stress or exposure to glucocorticoids results in decreased neurogenesis as well as dendritic spine anomalies (reduced density and/or retraction). Because adult neurogenesis, which occurs in the subventricular zone of the hippocampus, is required for normal stress response, failed neurogenesis may provide an underlying neuropathological mechanism for depressive episodes (Snyder et al. 2011).

Pathogenesis of Immune Response

While diagnosis of major depression/clinical depression is excluded in patients for which the depression can be attributed to a specific medical condition (American Psychiatric Association 2013), initial investigations linking the immune system to depression stemmed, in part, from observations that patients with acute illnesses or infection, such as cancer, cardiac events, and hepatitis, are at risk for depression. Patients treated with cytokines or immunosuppressive compounds for these conditions have a significant chance of developing depression within 12 months of starting treatment (Udina et al. 2012). Depressive symptoms can also be induced by bacterial endotoxins at doses lower than those causing sickness behavior, suggesting that the depressive symptoms are due to immune response rather than acute illness (Reichenberg et al. 2001). In fact, minor increases in inflammation may predict the

development of *de novo* depression (Pasco et al. 2010; Raison and Miller 2011). Specifically, slight elevations in the proinflammatory cytokines IL-6 and C-reactive protein (CRP) are associated with the subsequent development of depression (Valkanova et al. 2013). This suggests that immune system dysregulation is neither necessary nor sufficient for depression but that in a subset of patients, markers of immune function may be predictive of risk for depression. Psychosocial stressors have been shown to increase proinflammatory cytokines and activate the HPA axis, which is dysregulated in depression (Berk et al. 2013). For example, IL-1 stimulates the release of catecholamines such as norepinephrine (Dunn and Chuluyan 1992); these increases correlate with HPA axis activation (Dunn 1992).

Models of Depression

Core symptoms of depression are variable but include primarily subjective measures such as low mood, feelings of worthlessness, hopelessness, and guilt. These distinctly human constructs are challenging to model in animals. However, tasks that evaluate sensitivity to reward or behavioral despair (sucrose consumption and forced swim test) can be utilized in conjunction with adult or developmental models to examine a variety of endophenotypes; the reader is referred to Deussing (2006) and Krishnan and Nestler (2011) for in-depth review.

Stress is often a precipitating factor in the emergence of depressive episodes, which can be modeled in adult rodents by the application of chronic (1–7 weeks) mild or unpredictable physical (restraint, lighting changes) or psychosocial (social defeat) stressors. Stress paradigms result in variable changes depending upon species/strain and precise methodology, but rodents exposed to multiple stressors have exhibited multiple signs of depression including anhedonia, changes in HPA function, and changes in immune markers such as increased IL-1β (Krishnan and Nestler 2011).

Stress during the prepubertal period is also associated with the emergence of depression in adulthood; developmental rodent models provide the ability to assess mechanisms that underlie vulnerability. In early maternal separation, rodent pups are separated (~3 h/day or more) from their dam during the first 2 postnatal weeks. This paradigm can be modified in a number of important ways, such as removing the dam from the pups, which are left in the home cage or removing the pups to a new cage/bedding (either singly or as a litter). These differences can have a significant impact on the degree of functional impairment of the offspring (Vetulani 2013).

Paradigms of Study

Anhedonia, a decreased ability to experience pleasures, is, along with low mood, one of the two essential features of depression (Kennedy 2008). Anhedonia can be measured in rodents by sucrose consumption, in which a 1–2% sucrose solution is offered, usually over a 24-h period. A decrease in the preference for the sucrose solution measured either as total sucrose consumption or in relationship to

preference to control water is interpreted as anhedonia. In the forced swim test, rodents are placed in a cylinder of water, which is inescapable. The time which the rodent spends trying to escape, or conversely the time which the rodent spends immobile, can be correlated to behavioral despair/hopelessness. There are numerous studies which show that administration of antidepressants reduces passive behavior in rodents, which correlates to antidepressant activity in humans. These tests are used to rapidly screen drugs for potential antidepressant activity, although the task has been criticized since acute administration of antidepressants reverses behavioral despair in animal models despite taking several weeks to show clinical efficacy (Krishnan and Nestler 2011).

Current State of Drug Development

In line with the monoamine hypothesis of depression, the majority of depression medications increase the levels of central neurotransmitters implicated in mood and arousal. These primarily include selective serotonin or norepinephrine reuptake inhibitors (SSRIs and SNRIs, respectively) and less frequently, tricyclic antidepressants (TCAs), all of which increase serotonin and/or norepinephrine in the brain. However, as discussed previously the monoamine hypothesis is insufficient to fully explain depression and a number of patients are either treatment-resistant or suffer relapses. Because antidepressants also reduce inflammatory markers and there is evidence of a normalization of inflammatory markers during depression remission (Dahl et al. 2014), there is increased interest in anti-inflammatory agents for the treatment of depression. Trials utilizing anti-inflammatory agents have been conducted with non-steroidal anti-inflammatories (NSAIDs), such as celecoxib, both alone and in conjunction with SSRIs, and have shown to reduce clinical depression scores (Muller et al. 2006; Akhondzadeh et al. 2009). Direct intervention in cytokine synthesis may be effective in some patients with depression, since baseline levels of proinflammatory cytokines have been shown to predict subsequent depressive symptoms. Infliximab, a monoclonal antibody against TNFα, has been shown to improve depressive symptoms in patients with high baseline inflammatory markers (Raison et al. 2013). Specifically, patients receiving infliximab infusions versus placebo exhibited the greatest reductions in CRP (evidence of immune activation) over 12 weeks and had the largest improvement in Hamilton depression rating scales. There are a number of medications approved for immune-modulated diseases such as rheumatoid arthritis which may be used to determine whether drugs that treat inflammation could be beneficial in depression.

5.2.6.3 Schizophrenia

Schizophrenia (SZ) is a chronic and often disabling neuropsychiatric disorder that emerges following adolescence with positive symptoms such as hallucinations and delusions; negative symptoms such as blunted emotion and social withdrawal; and

cognitive deficits, particularly in executive functioning (reasoning, problem solving). SZ occurs with a prevalence of approximately 1% and patient outcome is often dependent upon the degree of cognitive impairment rather than psychosis (Green 1996). Meta-analyses clearly show heritability of SZ with 50–80% concordance in monozygotic twins; cognitive and neurobiological changes are also observed in unaffected first-degree relatives, suggesting that the majority of variance/risk can be explained by genetic factors (Farmer et al. 1987; Tsuang et al. 2001). However, despite decades of research into such genetic factors, there has been no clear "smoking gun" to explain a genetic cause. Rather there is a range of susceptibility genes, including those involved in key cognitive/developmental processes that are proposed to interact with a multitude of environmental risk factors, including psychosocial, biological, and physical factors (Tsuang et al. 2001; Weinberger 2005). Several biological and physical factors, both pre- and postnatally, are implicated in SZ. For instance, obstetric complications (hypoxia, trauma, pre-eclampsia), gestational stress, and illness are linked to increased risk of psychopathologies, including SZ (Davis and Sandman 2012).

Neuropathology

Postmortem analyses show morphometric evidence of developmental abnormalities across several brain regions, including cellular disorganization/altered migration (Kuroki and Matsushita 1998; Benes et al. 1991; Akbarian et al. 1993) and volume reductions (Bogerts et al. 1990) in the hippocampus and prefrontal cortex. These developmental changes are suggestive of decreased numbers of synapses and changes in dendritic/axonal organization (Harrison and Weinberger 2005). In particular, evidence of altered synaptic connectivity has been observed in the hippocampus, nucleus accumbens (NAC), and mediodorsal thalamus (MdThalm) (Harrison and Weinberger 2005). These changes in synaptic activity may underlie deficits in goal-directed activity and poor executive functioning that result in particularly poor functional outcome.

Pathogenesis of Immune Response

Prenatal infections are considered a primary non-genetic cause of SZ (Patterson 2009), with a two- to sevenfold increased risk of SZ following an infection during pregnancy (Brown et al. 2004; Khandaker et al. 2013). The importance of timing of infection has not been established; increased risk has been noted following infection in all three trimesters, but there are insufficient well-designed human studies to definitively state whether subsequent risk or severity and nature of symptoms changes with period of exposure. Interestingly, the type of infection appears to be rather unimportant, with association to multiple viruses, particularly influenza, herpes simplex (Brown and Derkits 2010) and the parasite *Toxoplasma gondii*

(Schwarcz and Hunter 2007). A common factor, then, is immune system activation, either for the mother during pregnancy or for the child during later stages of CNS development. Markers of immune activation (proinflammatory cytokines) are elevated in both mothers (Brown et al. 2004; Fineberg and Ellman 2013; Howard 2013) and offspring who subsequently develop SZ (Potvin et al. 2008; Soderlund et al. 2009). The pathogenesis of immune system changes is unknown, but it is hypothesized that maternal immune activation disrupts fetal neurodevelopment and results in a life-long immune reactivity (Müller 2014). Such immune dysfunction may interact with genetic abnormalities in cytokine pathways, resulting in increased risk of psychosis in the offspring.

The nature of immune dysfunction in SZ is highly variable, perhaps due to the variable nature of the disorder itself or perhaps due to confounds in testing methodologies (inpatient vs. outpatient; medicated vs. non-medicated, etc.). This is an important consideration, as two key meta-analyses (Potvin et al. 2008; Miller et al. 2013) show differences in cytokine dysregulation based upon patient status; notably, a distinct set of proinflammatory cytokines (IL-1β, IL-6, and TGF-β) are presumed state-dependent, as they are increased in first-episode and acutely relapsed patients, but not in stably-medicated patients. In contrast, several other cytokines (IL-12, IFN-γ, and TNF-α) were consistently elevated above control levels, regardless of patient disease or medication status, a potential indication of trait markers for this disorder. The increase in proinflammatory cytokines may provide important new information on this disorder as well as provide new treatment opportunities. For instance, cytokines have been shown to alter tryptophan degradation through the kynurenine pathway (KP) resulting in increased brain levels of kynurenic acid (KYNA). KYNA is an endogenous, astrocyte-derived *N*-methyl-D-aspartate (NMDA) and α7 nicotinic receptor antagonist which is elevated in the brain and cerebrospinal fluid of SZ patients (Erhardt et al. 2001; Schwarcz et al. 2001) and which may underlie the pathophysiology of SZ (Wonodi and Schwarcz 2010; Schwarcz et al. 2012). The KP (Fig. 5.1) is directly modulated by both immune system activation and stress; specifically, indoleamine 2,3-dioxygenase (IDO) and TDO (tryptophan 2,3-dioxygenase), enzymes responsible for the rate-limiting conversion of dietary tryptophan to L-kynurenine (the bioprecursor of KYNA), are up-regulated during immune activation or stress (Schwarcz et al. 2012; Müller 2014). A link to immune dysfunction and KYNA production is evidenced by several clinical reports, including the finding that IL-6, IL-1β, and/or IFNα, proinflammatory cytokines associated with immune activation, result in robust increases in peripheral kynurenine (Raison et al. 2010) and cerebrospinal fluid levels of KYNA (Schwieler et al. 2015). Johansson et al. (2013) similarly found that baseline production of kynurenine and KYNA were increased in fibroblasts from patients with SZ; following treatment with proinflammatory cytokines, KYNA remained elevated in conjunction with increased expression of the enzyme that converts kynurenine to KYNA.

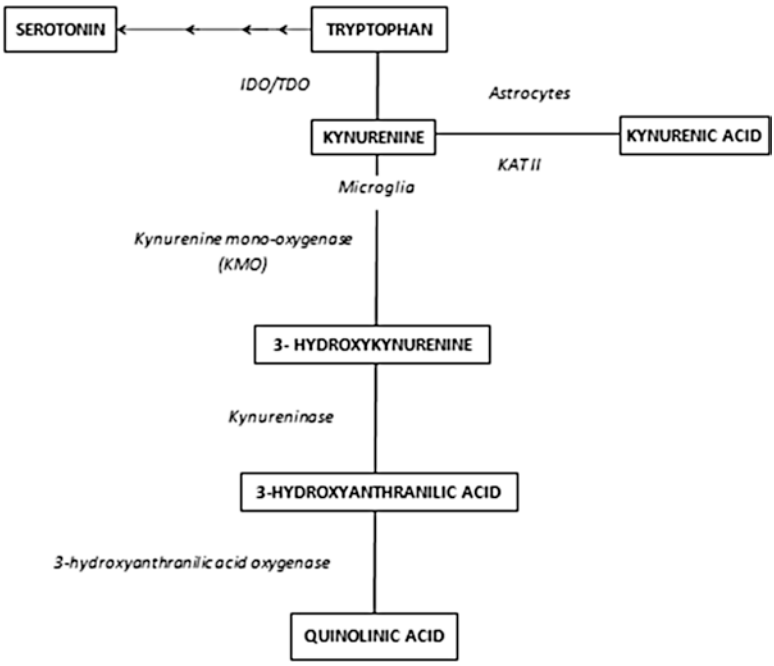

Fig. 5.1 Kynurenine pathway of tryptophan degradation. The majority (99%) of dietary obtained tryptophan is degraded in the kynurenine pathway (KP), although tryptophan also serves as a bio-precursor to serotonin. The KP is a functionally segregated two-arm pathway, in which quinolinic acid is produced in microglia and kynurenic acid is produced in astrocytes. Kynurenic acid is an α7 nicotinic receptor and NMDA receptor antagonist which is elevated in the brain and cerebrospinal fluid of schizophrenia patients

Models of Schizophrenia

There are numerous rodent and non-human primate models of SZ, which vary with degree of face and predictive validity as well as overall cost and complexity. These models include pharmacological manipulations of neurotransmitter systems known to be altered in SZ (dopamine, glutamate), lesions, developmental (tetrodotoxin inactivation of ventral hippocampus, kynurenine administration), and genetic models. An important consideration in selection of a model for this disease is symptom ontogeny. Specifically, symptoms often emerge in the clinical population following puberty; although there are vulnerabilities and risk factors during childhood and adolescence, such as childhood trauma, abuse, and social stress (Varese et al. 2012; Selten et al. 2013). While animal models cannot describe all aspects of complex human psychiatric diseases, particularly when the etiology is unknown, there are animal models of SZ that exhibit the same post-pubertal emergence of schizophrenia-like symptoms when assessed in validated paradigms (discussed below). A full review of animal models is beyond the scope of this book chapter; the reader is

referred to Lipska and Gogos (2010) and Jones et al. (2011) for a comprehensive review. We have selected two particular animal models (Maternal Immune Activation and subchronic L-kynurenine administration) for further discussion, as these models are of particular interest from an immunopathological and neurodevelopmental perspective.

Maternal Immune Activation

As noted above, maternal infection has been established as a significant risk factor for subsequent neurochemical and behavioral abnormalities in the offspring. As such, there are a number of animal models designed to examine the myriad changes both cellular/molecular and behavioral that occur in offspring following maternal immune activation (MIA). Most common MIA models involve rodents, although there are some instances of non-human primates, whereby immunogens are injected in order to induce inflammation. The most common immunogens are lipopolysaccharide (LPS), which is endotoxin derived from gram-negative bacteria or polyinosinic:polycytidylic acid (poly:IC), which is a synthetic analog of viral RNA. While both LPS and poly:IC induce a range of proinflammatory cytokines via activation of toll-like receptors, LPS mimics bacterial infections while poly:IC mimics viral infections (Boksa 2010). While MIA models often utilize either LPS or poly:IC, the route of administration, dose, and timing are considerably variable, ranging from single to multiple ultra-low (nanogram/kg) to mg/kg doses from early to late gestation. An exhaustive review of MIA models is beyond the scope of this chapter. Instead, we focus on a few key findings from studies in rodents utilizing low doses of immunogens, which induce inflammatory changes in the absence of overt sickness response. Sickness behavior can be quite severe, resulting in substantial body weight losses that necessitate supportive dietary care, and may be reasonably expected to affect fetal outcome. In these studies, LPS or poly:IC is administered by intraperitoneal (i.p.) injection to pregnant dams during mid- to late gestation (equivalent to second trimester in humans) and females are allowed to deliver. MIA results in numerous histological/structural abnormalities including abnormal pyramidal cell migration/cell packing; abnormal myelination; ventricular enlargement; and cognitive/behavioral deficits (working memory, pre-pulse inhibition, open-field, social interaction, latent inhibition) (Brown and Derkits 2010; Knuesel et al. 2014). Recently, offspring of MIA models have also been shown to exhibit a variety of immune system changes, including elevations in proinflammatory cytokines as juveniles and adults (Basta-Kaim et al. 2012) that are similar to those observed in the clinical population. Thus, disruption of the maternal immune system during a discrete window during gestation results in long-term immune disruption and corresponding CNS dysfunction in the offspring, which mimic those observed in a variety of neurodevelopmental disorders. While these findings do not show causation, they support further research; for instance direct administration of proinflammatory cytokines such as IL-1β induces schizophrenia-like symptoms that can be reversed by antipsychotics (Nawa and Takei 2006).

L-Kynurenine Administration

Providing L-kynurenine to rodents mimics the observed increases in substrate (kynurenine) and its astrocyte-derived end-product (KYNA) without elucidation of upstream causal factors. KYNA passes the blood-brain-barrier (BBB) poorly; rather, its bioprecursor (L-kynurenine) is transported across the BBB by the large neutral amino acid transporter, System-L (Fukui et al. 1991). L-Kynurenine is then converted to KYNA in astrocytes by the enzyme kynurenine aminotransferase (KAT). There are multiple methods of L-kynurenine administration (acute, via intraperitoneal injection or subchronically in the diet). When administered acutely, L-kynurenine can be administered by i.p. injection, generally at a dosage level of 50–200 mg/kg/day to adults (Alexander et al. 2012; Olsson et al. 2012) or offspring during discrete periods of development (Iaccarino et al. 2013). Alternatively, in order to assess the neurodevelopmental implications of KP changes, L-kynurenine can be provided in the diet to female rats during gestation and/or lactation, which allows for assessment of critical periods of vulnerability in the developing offspring. Specifically, L-kynurenine is provided to females daily in the diet starting at mid-gestation and continuing through gestation day 22 or through lactation. Consumption of the bioprecursor (L-kynurenine) by the dams results in increased plasma and brain KYNA levels in the fetuses and offspring; age-dependent changes in prefrontal- and hippocampal-dependent behavior and brain morphology are also observed in the offspring (Alexander et al. 2013; Pocivavsek et al. 2014; Pershing et al. 2015). Brain KYNA levels in rodents can be modulated through a prostaglandin (E1/E2)-mediated pathway, namely administration of a cyclooxygenase 1 (COX-1) selective inhibitor increases brain KYNA while administration of a COX-2 selective inhibitor decreases brain KYNA (Schwieler et al. 2005). These results provide further evidence to link immune system activation and the kynurenine pathway, as well as provide research opportunities for drug development.

Paradigms of Study

Schizophrenia is a heterogeneous disorder, with a number of uniquely human symptoms such as hallucinations and delusions, which makes task selection challenging. As summarized in Table 5.2, the Measurement and Treatment Research to Improve Cognition in Schizophrenia (MATRICS) and Cognitive Neuroscience Treatment to Improve Cognition in Schizophrenia (CNTRICS) initiatives identified cognitive and molecular targets, and valid clinical tests to assess cognitive impairments in a high throughput manner (Kern et al. 2004), with several points of emphasis including identification of cognitive domains most affected and amenable to treatment utilizing a standard set of criteria for academia, the pharmaceutical industry, and the Food and Drug Administration (Carter and Barch 2007; Carter et al. 2008; Barch et al. 2009). Selected tasks are provided in Table 5.2; the reader is referred to Barch et al. (2009) for full list of proposed tasks.

Table 5.2 Summary of cognitive domains and selected proposed tasks from CNTRICS

Cognitive domains	Proposed tasks
Perception	
Gain control	Contrast-contrast effect task
Integration	Prepulse inhibition of startle
Working memory	
Goal maintenance	Babble task; coherent motion detection
Interference control	Probabilistic reversal learning
Attention	McGaughy and Sarter sustained attention task
Executive control	
Rule generation/selection	Intradimensional/extradimensional shift task
Dynamic adjustment of control	Stroop task
Long-term memory	
Relational encoding/retrieval	Associative inference
Item encoding/retrieval	Inhibition of currently irrelevant memories
Reinforcement learning, including Pavlovian conditioning	Weather prediction task
Affective recognition/evaluation	Penn emotion recognition task

Current State of Drug Development

The majority of FDA-approved medications for schizophrenia are atypical antipsychotics, which have primary efficacy through dopamine (D2-like) receptor antagonism (Correll and Kane 2014). These drugs may be effective for positive symptoms such as hallucinations or delusions, but have adverse effects on the cardiovascular, digestive, and nervous systems (Uçok and Gaebel 2008) and subsequently result in compliance issues for many patients (Freedman 2005). These drugs are also often poor at addressing cognitive dysfunction, which is a critical unmet need in pharmacotherapy because cognitive deficits are the primary predictor of functional outcome. As a result, several new classes of drugs have been explored based upon recommendations by the National Institute of Mental Health-funded programs MATRICS and CNTRICS. These cognitive neuroscience-based research initiatives provide a platform for preclinical translational research across academia and the pharmaceutical industry in disorders for which there are no clear biomarkers (Carter and Barch 2007). These drugs include dopaminergic (D1) agonists, cholinergic (muscarinic and nicotinic) agonists, GABA modulators, and NMDA receptor modulators (glycineB site agonists/antagonists). As described above, the importance of the immune system and the kynurenine pathway have become increasingly well accepted and anti-inflammatory medications, such as COX-2 inhibitors, and KATII inhibitors, which inhibit the formation of KYNA, are being explored.

A variety of anti-inflammatory drugs are under exploration in SZ, with mixed results. The majority of the current research involves off-label use of drugs approved for other indications, including non-steroidal anti-inflammatories (NSAIDs), statins,

neurosteroids, omega-3 fatty acids, and antibiotics. NSAIDs evaluated in SZ have primarily been COX-2 inhibitors, namely celecoxib (Celebrex), which reduces pain and inflammation through the modulation of prostaglandin levels (Aid and Bosetti 2011). Celecoxib administered at 400 mg/day for 6 weeks to 11 months as an adjunctive to the patient's atypical antipsychotic (amisulpride or risperidone) has been shown to significantly improve positive and negative symptoms compared to patients that received anti-psychotic medication plus placebo (Müller et al. 2010; Akhondzadeh et al. 2007); however, some studies have failed to replicate this finding (Rapaport et al. 2005). COX-2 selective inhibitors (parecoxib and meloxicam) have been shown to decrease brain levels of KYNA in rodents through a prostaglandin (E1/E2)-mediated pathway (Schwieler et al. 2005) providing further evidence to link immune system activation and the kynurenine pathway. While this finding has not been replicated in humans, the previously referenced studies indicate that anti-inflammatory medications may be a viable adjunctive medication for some patients with SZ, particularly with enhanced screening or early/targeted intervention. Further, given the interaction of the immune system with the kynurenine pathway and the observations that patients have significantly increased brain and CSF levels of KYNA, targeted manipulation of the kynurenine pathway may be a promising novel treatment option for SZ. Inhibition of the final metabolic step in the kynurenine pathway (synthesis of KYNA from kynurenine) may provide a novel platform with reduced side effects. Only recently have systemically available KATII inhibitors become available (Koshy Cherian et al. 2014). KATII is the primary enzyme responsible for the conversion of L-kynurenine to KYNA, which functions as an NMDA receptor and α7 nicotinic receptor antagonist. Inhibiting this enzyme significantly lowers the amount of KYNA produced in astrocytes, and rodent and non-human primate models of SZ show significant improvements in several tests of cognition that correspond to improved functional outcome in humans following KATII inhibition (Pocivavsek et al. 2011).

Future Directions

The majority of psychiatric conditions do not have known etiologies or readily identifiable, distinct biomarkers or morphological changes. Further, these conditions often have heterogeneous and subjective symptoms that (1) are uniquely human and (2) occur across conditions. A differential diagnosis process is utilized in humans; the overall length of time and type of symptoms with myriad other factors determine diagnosis and eventual treatment, which is often pharmacologic in a rather crude attempt to normalize central neurotransmitter dysfunction (e.g., serotonergic, noradrenergic, or dopaminergic). Further, all common mental disorders discussed in this chapter (anxiety, depression, and schizophrenia) have genetic and environmental influences, with particular risk associated with early developmental insults. This presents challenges in the creation of valid animal models and avenues for future drug development in a number of ways.

Lifetime stress/trauma may contribute to emergence of several neuropsychiatric disorders, including those discussed in this chapter. Immune system involvement is evidenced across these common mental disorders by increased inflammatory markers, although it is unclear whether these changes are a cause or consequence of other pathological changes such as HPA activation. While the current literature for anti-inflammatory drugs shows some promise, particularly when considered as adjunctive to a traditional medication, there are several important limitations. Drug safety and efficacy is considered in clinical trials for rather short periods of time when considering conditions like anxiety, depression, and schizophrenia, which are chronic and recurring. NSAIDs, including celecoxib, have cardiovascular and gastrointestinal risks that increase with duration of use. For instance, long-term use of NSAIDs in SZ patients, who are likely taking antipsychotic medications that have a number of cardiovascular and metabolic risks should be carefully considered. Furthermore, the majority of studies do not consider baseline immune reactivity as a randomization factor, which may explain some of the heterogeneity of the findings. Namely, it is possible that an individualized approach will be more effective when considering future treatment options. IL-1β, IL-6, TNFα and IFNα are cytokines that play a primary role in the CNS and which show abnormalities across mental disorders. There are currently few small molecule inhibitors for these receptors; however, there are antibodies and fusion proteins for IL-1β and TNFα and localized administration of these compounds is under development (Maes et al. 2012). Generation of IL-1β occurs via several pathways, and an additional pathway, the P2X7 receptor is also being explored. The P2X7 receptor is a purinergic, ligand-gated ion channel, and P2X7 antagonists block IL-1β release (Abbracchio et al. 2009; Bartlett et al. 2014). As such, this pathway is under exploration for a number of mood and other neuropsychiatric disorders (Bartlett et al. 2014). Finally, given the current promising data for kynurenic acid for SZ and other disorders as well as the recent availability of systemically available KATII inhibitors, these compounds will likely play a role as new therapies are developed for this chronic disorder.

5.2.7 Neoplasia

Central nervous system neoplasia encompasses a number of diseases from primary neoplasia, including meningioma, glioma and choroid plexus tumors to secondary neoplasms which originate elsewhere and subsequently enter the nervous system such as lymphoma, breast cancer, and melanoma. Although there are significant variations in the molecular mechanisms that drive tumorigenesis in the various nervous system neoplasms, there are marked similarities in the therapeutic challenges faced by practitioners attempting to treat them, most notably the inability for drugs to cross the blood-brain barrier (BBB) and reach therapeutic levels before reaching toxic levels in systemic circulation. This section will focus on the most common and extensively researched primary CNS tumor type, glioma. Nearly half of all primary brain tumors are glial in origin and of those, more than 75% are astrocytomas.

5.2.7.1 Pathogenesis of Immune Response

Glioblastoma multiforme (GBM) is the most common type of malignant astrocytoma. GBMs are typically viewed as either primary, arising in patients de novo, or secondary, deriving from preexisting low-grade astrocytomas. Primary glioblastoma is generally believed to arise from a single glioma precursor cell following multiple concurrent mutations in genes such as EGFR, p16INK4A, PTEN, and VEGF. Secondary glioblastoma is thought to progress in a stepwise fashion with initial mutations in genes such as p53 and PDGFa followed by mutations in genes such as CDK4 and pRB with final progression following mutations in PTEN and PI3K (Kamnasaran 2009). Primary GBM is more frequently seen in elderly patients. They are rapidly growing and have a poorer prognosis than secondary GBMs which are more commonly located in the frontal lobe and have a substantially better prognosis. Aside from a mild reduction in necrosis seen in secondary GBMs, these tumors are largely indistinguishable histologically. Because these tumors have such a different prognosis, it is important to distinguish them clinically. Current studies have focused on identifying unique genetic signatures which can differentiate these two tumor types. While a number of genes may show differences in expression between the tumor types, identification of a mutation in IDH1 is a strong indicator that a GBM tumor is secondary in nature (Ohgaki and Kleihues 2013).

In addition to rapid growth driven by a number of genetic mutations, immunosuppression is a feature of many primary brain tumors, most notably in GBM. The nature and effect of this alteration in immune system function is reviewed in the Handbook of Clinical Neurology (Roth et al. 2012). Briefly, reduced MHC class I expression by glioma cells modulates the immune response via a reduction in antigen presentation (Facoetti et al. 2005). It appears that the level of MHC class I expression is low enough to prevent recognition and killing by T-cells yet remains sufficient to escape NK cell targeting and tumor lysis. Increased secretion of transforming growth factor (TGF)-β by glioblastoma cells is also a major factor in the immune modulation seen with GBM. Not only does TGF-β modulate the innate and adaptive immune response, but also promotes local tumor spread through increased angiogenesis. Immune modulation driven by TGF-β expression includes negative regulation of the differentiation and proliferation of cytotoxic T-lymphocytes (CTLs) (Ludviksson et al. 2000), reduced NK cell activation and cytolytic function (Eisele et al. 2006), and inhibition of dendritic cell maturation (Yamaguchi et al. 1997). It has also been suggested that glioma cells aberrantly express human leukocyte antigen (HLA)-E, a non-classical MHC class I molecule commonly expressed in the placenta, which can suppress NK cell killing (Wischhusen et al. 2005).

5.2.7.2 Models of Neoplasia

There are numerous cell culture based preclinical models for astrocytomas. These models generally fall into one of two categories, somatic astrocyte cell cultures and glioma cell lines. Somatic astrocyte cell cultures typically rely on pre-immortalization via constitutive expression of genes which bypass cellular senescence, such as

telomerase, RAS, AKT, or GATA6. Through this immortalization process, these cells will de-differentiate into high grade astrocytomas. While use of somatic astrocytes allows for preservation of cellular and genetic architecture, these cell lines are difficult to establish and, as mature astrocytes, eventual senescence is inevitable (Kamnasaran 2009). Glioma cell lines include both immortal cultures and primary cell lines. While examination of primary cells is vital to tailoring treatment to individual patients, there is marked heterogeneity in the sample which can interfere with complex mechanistic studies designed to examine only neoplastic cells. Additionally, propagation of these cells may not be possible or may only occur through, *in vivo*, xenograft passages. Immortalized cell lines, however, provide examination *in vitro* through easy propagation. Frequently utilized immortalized glioma cell lines include A172, U118, U251, and U87. While these cell lines have the benefit of immortality without the cellular senescence seen in astrocyte cell cultures, they may have complex genetic signatures that, while useful in understanding complexity of this tumor type, can complicate mechanistic studies targeting single gene mutations.

The use of *in vivo* models involves a diverse group of animals and modalities for recapitulating both the genetic and clinical mosaic of GBM seen in humans. Both human and mouse cell lines have been used in xenograft mouse models. The most widely used xenograft models include the U251 glioma model developed by Pontén (1975) which has been implanted both subcutaneously and orthotopically (intracranial) as well as the U1242 MG intracranial xenograft model (Zhao et al. 2010). These xenograft models display many similarities in growth pattern and gene expression (Camphausen et al. 2005; Radaelli et al. 2009); however, xenografts require the use of an immunocompromised recipient which does not allow for exploration of interaction between the tumor cells and the host microenvironment as seen in spontaneous disease. Another mechanism for modeling intracranial neoplasms is the use of genetically engineered mice (GEMs). Several different GEMs have been developed to explore the growth and pathogenesis of brain tumors. One of the most successful is a GEM that expresses an oncogenic mutant form of H-Ras (V12H) controlled by the GFAP promoter; the mice develop astrocytomas with high mitotic activity, infiltration, necrosis and increased vascularity (Ding et al. 2001). Xenograft models allow for rapid and predictable disease progression which is highly valuable for drug screening and mechanistic studies whereas GEMs allow for disease manifestations which are more realistic in regards to microenvironment and spatial and temporal progression.

Other, less conventional models for intracranial neoplasia include canine, zebrafish, and *Drosophila melanogaster* (fruit fly) models. The growth pattern and progression of spontaneous GBM in dogs as well as their larger size makes them an attractive model for testing of therapeutics. Spontaneous brain tumors occur at a rate of 20 per 100,000 (Dobson et al. 2002) with meningiomas and gliomas being most common and increasing in incidence with age. Viral-induced, non-spontaneous brain tumors (Warnke et al. 1995; Whelan et al. 1988) have also been utilized for modeling human disease. Both zebrafish and fruit flies are advantageous for their rapid growth, large brood size and short gestation. Genetic modifications resulting in defective mismatch repair in zebrafish and activation of EGFR-Ras and PI3K in

a glial-specific manner in fruit flies have resulted in neoplasms resembling gliomas in humans (Feitsma et al. 2008; Read et al. 2009).

5.2.7.3 Current State of Drug Development

GBM presents a formidable challenge to neurooncologists. Current therapies include surgery, radiation therapy, and chemotherapy. Despite aggressive, multimodal therapy, the median survival for GBM is only 13–15 months (Stupp et al. 2009). In order for a therapy to be effective in treating brain tumors, the drug must be able to bypass the BBB and reach adequate therapeutic levels within the tumor prior to reaching systemic toxicity. At present, alkylating agents such as temozolomide and nitrosoureas (carmustine, lomustine, and semustine) are the mainstay of chemotherapeutics. Tumor resistance against alkylating agents is frequently encountered and is a common cause of tumor recurrence. Because drugs must be small, electrically neural, and lipid-soluble to cross the BBB into the CNS, drugs should meet these requirements or delivery methods which bypass the BBB must be used. Methods of delivery which bypass the BBB include disruption of the BBB, convection-enhanced delivery, and direct delivery of drugs into the tumor via polymers or microchips. At present, disruption of the BBB by concurrent treatment with endothelial-shrinking compounds such as mannitol have not proven efficacious. The use of catheters to directly infuse chemotherapeutics and biological agents to brain tumors has shown some increase in survival on an individual basis, but large-scale studies have yet to be done. Convection-enhanced delivery utilizes a small pressure gradient that can deliver high concentrations of drugs into large regions of the brain without causing structural damage. Finally, polymer systems function to release small doses of a drug locally over time. This delivery method has been used with carmustine (BCNU) which showed that sustained and prolonged levels of BCNU could be achieved (Lesniak and Brem 2004).

Current research is focused on precision targeting of chemotherapeutics based on molecular profiles of individual tumors. Molecular targets include EGFR, PDGFRA, PTEN, PIK3CA, NF1, and BRAF. Alteration in expression of each of these genes has been targeted by a pre-existing FDA-approved therapy with modest results. As discussed earlier, a major challenge to utilizing these targeted agents is pharmacokinetics. These molecules are frequently large, hydrophilic, and/or positively charged resulting in poor concentrations within the tumor. Additionally, heterogeneity of the tumor results in variation in vascular permeability as well as variable gene expression throughout (Prados et al. 2015).

Immunotherapy has received a lot of attention in recent years for treating a number of cancers and gliomas are no exception. Unfortunately, as previously described, glioblastoma frequently drives immunosuppression. Additionally, recent studies have shown that traditional treatment with radiation and chemotherapy can actually increase the immunosuppressive nature of remaining GBM cells (Authier et al. 2015). The marked suppression of the immune response is a major challenge for

glioma immunotherapy. In order to overcome this obstacle, new combination therapies are being designed that both block the immunosuppressive microenvironment around gliomas and stimulate a peripheral antitumor immune response via vaccination (Platten et al. 2014).

Brain tumors, GBM in particular, present a unique set of challenges to therapy. Incomplete resection, development of chemotherapy resistance, immunosuppression and the pharmacokinetics within the brain parenchyma all serve as major obstacles in the successful treatment of this disease. In order to overcome all of these issues, multimodal approaches must be utilized.

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Chapter 6
Immunopathology of the Urinary System

Catherine A. Picut

Abstract The pathogenesis of many immune-mediated diseases of the urinary tract has been well elucidated in humans and in their respective animal models. While laboratory animals develop spontaneous immune-mediated renal disease, far less is known about their etiology or pathogenesis. By examining the five types of hypersensitivity reactions that lead to immune-mediated glomerular disease in humans, and understanding the molecular pathogenesis of lesion development, toxicologic pathologists can interpret test article-related lesions in the urinary tract, and help distinguish these lesions from spontaneous disease. Resident macrophages and dendritic cells of the kidney maintain continual tolerance, and any disruption of this homeostasis will result in glomerular and/or tubulo-interstitial inflammation which often self-perpetuates leading to end stage renal disease. Although immune dysfunction leads to renal disease, the reverse is true as well. Uremia leads to immunodysfunction, hypercytokinemia, and multi-organ damage especially to the cardiovascular system.

Keywords Glomerulonephritis • Tubulo-interstitial nephritis • Mesangial cells • Podocytes • Immune-complex • Hypersensitivity nephritis • Nephropathy • Glomerulus • Kidney • Urinary bladder

6.1 Introduction

Immunopathology of the urinary tract includes immune-mediated diseases of the kidney that result from overzealous immune response to *foreign* antigen, or from an inappropriate activation of immune response against *self*-antigen. The unique anatomical structure of the glomerulus makes it a target for immune-complex deposition followed by activation of innate and adaptive immune responses. While the

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immune defenses, in general, are designed to protect the kidney from invasion by microbes, activation of these defense mechanisms by an overzealous or inappropriate response leads to destructive and often self-perpetuating inflammation that causes permanent glomerular and tubular damage. There are strategies in place to suppress unnecessary immune activation, but these strategies are commonly overwhelmed. The immunosuppressive strategies in the urinary system are not as adept as those in the reproductive tract where immune-privilege is important to propagation of the species. Any disruption of immune homeostasis in the kidney, or any insult that causes expressions of damage-associated membrane proteins (DAMPs) in tubular epithelial cells, can set the inflammatory cascade in motion. To be sure, kidney inflammation, once it starts, generally has a depressing fate. By understanding the initial events that lead to immune activation, and those events that perpetuate the inflammation, toxicologic pathologists can better interpret microscopic changes in the kidney, and researchers can develop new therapeutic modalities.

Most of what is known about pathogenesis of immune-mediated kidney disease comes from the use of animal models. These models include "crude" models involving animals injected with crude extracts of renal cortex (i.e., nephrotoxic serum nephritis), on one hand, and highly sophisticated models involving genetically-manipulated mice with deficiencies in specific cell types or mediators, on the other hand. By using both crude and highly sophisticated animal models, researchers have been able to unravel the complex pathophysiology of immune-mediated renal disease.

There are two broad clinical renal conditions in man that are considered immune-mediated and will be used as a scaffold to discuss immunopathology in this chapter: (1) Autoimmune renal disease (glomerulonephritis or tubulo-interstitial disease); and (2) Acute Kidney Injury (AKI). Autoimmune renal disease represents conditions that result from activation of the adaptive immune response to self-antigens. Triggers for activation of the immune system against self are being defined, but the specific trigger in any one individual patient often remains unknown. Acute Kidney Injury, on the other hand represents conditions that result from an overzealous (or self-perpetuating) immune response to a primary exogenous insult. That primary insult can be septic, toxic or ischemic in nature. In humans, there is a third immune-mediated renal condition associated with transplant rejection. This third condition will not be covered in this chapter.

Any one of these immune-mediated kidney diseases (autoimmune or AKI) should be differentiated from non-specific inflammatory kidney disease, such as bacterial-induced pyelonephritis or pyelitis, in which the immune response is *appropriate* and against pathogenic foreign antigens. As long as the inflammatory response is appropriate in severity and duration, the kidney disease should not be considered immune-mediated *per se*, even though immune cells are certainly playing a large role in lesion development.

Another point to clarify is that not all immune-mediated diseases are necessarily inflammatory. Many conditions are not characterized by the conventional infiltration of inflammatory cells, or by the typical edema and/or hyperemia of inflammation. In fact, many of the immune-mediated kidney diseases are generally

"degenerative" in nature with little inflammatory cell response noticed at the light microscopic level (e.g., minimal change disease or membranous glomerulonephritis).

Pathologists will be disappointed to learn that there are very few (if any) pathognomonic light microscopic changes in the kidney that point to a specific pathogenesis. Most renal diseases, regardless of their inciting event, develop a similar presentation by routine light microscopy that includes a mixture of proliferative, degenerative, and/or inflammatory changes. Transmission electron microscopy (TEM) and special immunohistochemical and chemical stains may help elucidate the disease process in question, but these tools (especially EM) are not cost-effective measures available to toxicologic pathologists evaluating tissues from routine preclinical safety toxicology study. However, hopefully this chapter will shed important light on how and why kidney lesions progress as they do; the vast array of possible causes for a kidney lesion far beyond the typical immune complex deposition; and the likely possibility that kidney lesions are a reflection of systemic disease and *visa versa.* While primary immune-mediated renal diseases are rare in laboratory animals, immune-mediated renal diseases are being recognized with increasing frequency as a toxic manifestation of long-term administration of bio therapeutics, especially to non-human primates.

This chapter will start with a discussion of the anatomy of the glomerulus and the immune cell types that play a role in mounting or suppressing immune responses in the kidney, followed by a description of the pathogenesis and morphology of immune-mediated renal diseases in humans and animals that arise by the five hypersensitivity responses. This section will include the more well-known human glomerulonephritides with some discussion of tubulo-interstitial disease. In covering these diseases, the early stages of pathogenesis (i.e., the events that occur *before* immune activation), will be emphasized since each disease has its own set of unique events. When applicable, the animal models of these diseases that have been and are continually utilized to further elucidate these human diseases will be introduced. The tissue changes that occur *after* initial immune activation, will be discussed collectively in Part VI.

A toxicologic pathologist at this point may wonder why so much emphasis would be placed on specific human glomerulonephritides or on experimental animal models, when neither appear to have much bearing on safety toxicologic pathology studies. However, by closely examining the pathogenesis of these human diseases, comes an appreciation for the wide variety of mechanisms that cause derangements in immuno-homeostasis that lead to immunologic attack of the kidney; how glomerular lesions lead to tubular injury and *visa versa*; and how primary renal disease can cause immune-deficiency and multi-organ disease.

Immune-mediated renal disease (i.e., glomerulonephritis and interstitial nephritis) will be followed by a discussion of acute kidney injury (AKI), which is an immune-mediated tubulo-interstitial disease *secondary* to a septic, toxic or ischemic insult. Some of the mechanisms used to activate the immune system are similar to those in primary immune-mediated renal diseases, but there are some unique mechanisms employed that deserve special mention. Because many test articles are

potentially toxic to the filtering kidney, or they disrupt blood flow resulting in degrees of hypoxia, this section will be especially applicable to toxicologic pathologists.

Immunopathology of the urinary tract does not end with a discussion of renal tissues. Immune-mediated renal disease, from whatever cause, results in uremia and a hypercytokinemic state, causing immunodeficiency, multi-organ disease, and in particular cardiovascular disease (Betjes 2013; Kato et al. 2008). Next this chapter will describe the spontaneous kidney diseases in laboratory animals, for which far less is known about pathogenesis than the human counterpart diseases. For a toxicologic pathologist, the practical importance in recognizing commonly encountered, but less well understood, spontaneous immune-mediated urinary tract diseases, is to (1) recognize these as spontaneous; (2) recognize test article-related exacerbations of such diseases; and (2) differentiate them from other test article-related lesions.

The chapter will close with a discussion of the unique immune defenses of the lower urinary tract. Primary immunopathology of the lower urinary tract does not occur to any degree that it occurs in the kidney. Inflammation of the urinary bladder (i.e., cystitis) is usually "appropriate" and therefore not considered immune-mediated. However, immunopathology of the lower urinary tract (i.e., especially when there is *inappropriate* activation of the immune system) could signify there is test article-related breakdown in the normal immune defenses and homeostasis. By understanding the types of defense measures in the lower urinary tract, a pathologist may be able to offer explanation for a test article-related higher incidence and/or severity of inflammation.

6.2 Anatomy of the Glomerulus

The rat has a unipapillary kidney with a cortex and medulla. The cortex is divided into: (1) renal corpuscles (i.e., glomeruli and Bowman's capsule); (2) the proximal and distal convoluted tubular system; and (3) the medullary rays which represent the straight tubular segments. The medulla is divided into: (1) an outer medulla, which contains the outer and inner stripe; and (2) the inner medulla. Since a large portion of immune-mediate pathology involves the glomerulus, the histologic and ultrastructural anatomy of the glomerulus should be emphasized.

A schematic of the renal corpuscle is provided in Fig. 6.1. The corpuscle is a two-part system where the filtration of blood occurs to form filtrate. The glomerulus is a specialized globous extension of the renal interstitium and is composed of capillaries, mesangial cells and mesangial matrix (or mesangium). This globous extension is lined by podocytes which are an extension of specialized proximal convoluted tubular epithelial cells. These podocytes (also termed visceral epithelial cells) lie close to the capillary in the glomerulus, produce the intervening glomerular basement membrane (GBM), and together with the GBM form the filtration apparatus. The epithelial cells *not* in contact with the glomerulus line Bowman's capsule and

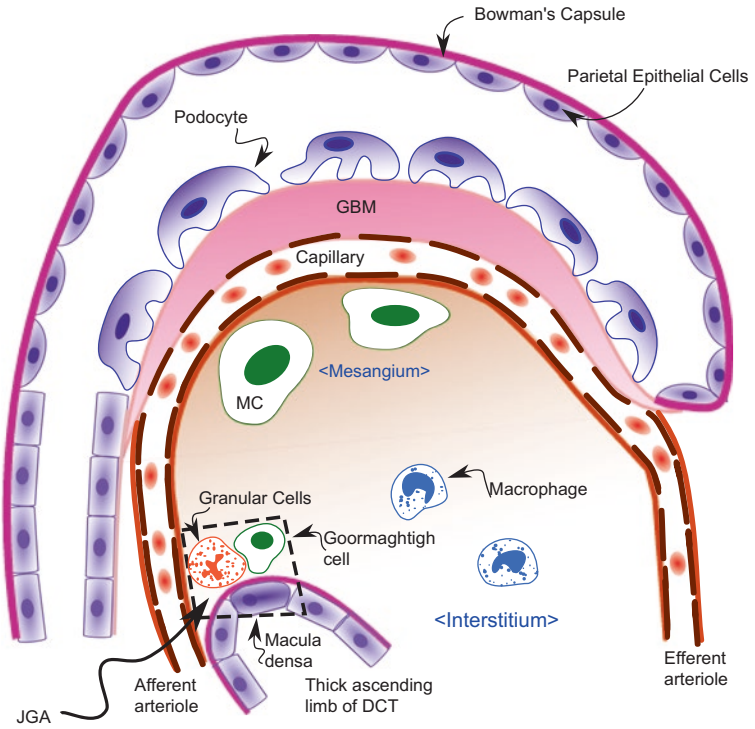

Fig. 6.1 Anatomy of the renal corpuscle. This schematic of the renal corpuscle (glomerulus and Bowman's capsule) emphasizes the cellular relationships between the mesangial cells, renal macrophages, endothelial cells, glomerular epithelial cells and tubular epithelium of the proximal convoluted tubule (PCT). The mesangial cells are in close approximation with the rest of the interstitium and resident interstitial inflammatory cells. Interstitial macrophages have no physical barriers and can readily migrate up into the mesangium. Circulating inflammatory cells, such as neutrophils or monocytes, need only traverse the fenestrated endothelium and thin basement membrane of the glomerular capillary to gain access to the mesangium; however, these cells need to traverse the thick trilaminar anionic glomerular basement membrane to gain access to the podocytes. Note how the podocytes and the parietal glomerular epithelium are modified extensions of the tubular epithelium. Modified mesangial cells (also known as Goormaghtigh or Lacis cells) are intimately associated with (and part of) the juxtaglomerular apparatus (JGA). The JGA is also composed of granular cells which are modified smooth muscle cells that produce renin, and the macula densa which is a group of specialized distal tubular epithelial cells. The JGA is strategically positioned at the end of the afferent arteriole. The macula densa detects changes in NaCl concentration and blood pressure, and responds by having the granular cells increase or decrease activation of the renin-angiotensin-aldosterone system (RAAS) in order to regulate blood pressure. The Goormaghtigh cells help regulate blood flow through the glomerulus, likely through their contractile function and their ability to relay information to the "contractile" mesangial cells nearby in the glomerular tuft. (Schematic by Cynthia Swanson)

are termed parietal epithelial cells. Bowman's capsule and the glomerulus make up the complete corpuscle. Bowman's space is the space between the podocytes and the parietal epithelial cells and is an extension of the tubular lumen.

Figure 6.1 shows the relative relationship between cells of the corpuscle. The epithelium lining the proximal convoluted tubules is continuous with the epithelium lining the visceral and parietal epithelial cells of the up-stream corpuscle. Parietal epithelial cells lining Bowman's capsule are flattened squamous epithelial cells, with the exception of those in the adult male mouse in which these parietal epithelial cells are cuboidal. The visceral epithelial cells (or the podocytes) appear microscopically as plump cells with a round nucleus, and indistinct cytoplasmic margins, and attach to the GBM by a series of foot processes which cannot be appreciated at the light microscopic level. The foot processes contain an intracytoplasmic actin-based cytoskeleton that is linked to the GBM, and the orderly arrangement of these contractile actin filament bundles is critical for the physical maintenance of the foot processes (Faul et al. 2007). The foot processes are laterally connected to each other by a thin zone called a filtration slit. This slit is composed of proteins and likely represent a modified tight junction or adherens junction between the adjacent podocytes. Two important protein components of this slit diaphragm are nephrin and podocin. There is also a thick sialic acid coat on the surface of the podocyte foot processes which coats the slit diaphragm and further helps restrict movement of molecules through this "pore" (Charest and Roth 1985; Miner 2002). The combination of the GBM (discussed below) and the sialic acid-coated protein slits create the physical and electro-chemical filter. This filter restricts molecules based on charge, size and configuration when turning plasma into urine filtrate.

The podocyte foot processes rest on the GBM, which is a thick and specialized extension of the tubular basement membrane. The GBM is a convoluted membrane that serves as the boundary between the "inner" capillaries and the "outer" podocytes. The terms inner and outer would refer to the position of the capillaries and podocytes, respectively, if the glomerulus was artificially expanded. However, because of the convoluted folds of the GBM, the inner and outer positions are not appreciated by light microscopy. The GBM is a trilaminar anionic membrane (110–160 nm in diameter in the rat) and its three layers include luminal rara interna (endothelial side), a middle lamina densa, and a lamina rara externa (podocyte side). The GBM is composed of type IV collagen, proteoglycans (e.g., heparin sulfate, agrin, and perlecan), glycoproteins, laminin and fibronectin.

The glomerulus begins as a hilus and is a stalk-like extension of the interstitium. Besides the podocytes and the GBM, the glomerulus is composed of capillaries, mesangial cells, and mesangial matrix. Capillaries lined by highly fenestrated endothelia extend from the afferent arteriole from the interstitium through the stalk to reside along the inside of the GBM. The capillaries terminate into the efferent arteriole of the interstitium. The unusually high density of fenestrae of these capillaries (constituting 20–50% of the entire endothelial surface) allows high permeability to water and small solutes (Bulger et al. 1983). Deep to the capillaries is mesangial matrix and mesangial cells, which represent a specialized and direct extension of the interstitium. Mesangial cells and the mesangial matrix support the globular structure of the glomerulus. Mesangial cells produce the mesangial matrix which consists of collagens type I, III, IV and V, laminin, fibronectin and proteoglycans.

There is another important structure worth noting and that is the juxtaglomerular apparatus (JGA). This apparatus lies adjacent to the hilus of the glomerulus at the terminal portion of the afferent arteriole as this vessel transitions into the capillaries of the glomerulus. The JGA is a multicellular apparatus composed of the (1) macula dense (specialized cells of the straight portion of the distal nephron); (2) renin-producing granular cells (which are specialized smooth muscle cells of the afferent arteriole); and (3) extraglomerular mesangial cells (also known as Goormaghtigh or Lacis cells) (Goormaghtigh 1939; Bachmann et al. 1986). These cells have extensive (lace-like) cytoplasmic processes and are believed to transmit signals from the tubule back to mesangial cells of the glomerulus to help regulate blood flow.

The physical relationships between the endothelial cells, mesangial cells, visceral and parietal epithelial cells, and cells of the juxtaglomerular apparatus are important in the pathogenesis of immune-mediated renal disease. Several physical relationships important in the pathogenesis of immune mediated renal disease can be noted: (1) The mesangial cells of the glomerulus are separated from blood by nothing other than a conventional capillary basement membrane, and are intimately associated with the juxtaglomerular apparatus and its renin-producing granular cells; (2) The mesangial matrix is an extension of the interstitial mesenchyme of the renal cortex, through which tissue macrophages and immune cells may traverse back and forth; (3) The podocytes are specialized tubular epithelial cells and may share common antigens with tubular epithelium and parietal epithelial cells, such as Fx1A antigen in Heymann nephritis; and (4) Bowman's space is in direct contact with the tubular lumen; therefore, antigens that may leak into the Bowman's space will quickly come into contact with pattern recognition receptors (PRRs) on tubular epithelial cells.

For a more detailed review of the ultrastructural components of the various segments of the glomerulus and the anatomy of different regions of the tubules, the reader is referred to Bachmann et al. (1986), Jones et al. (1986), and Liebelt (1986).

6.3 Immune Cells of the Kidney in Health and Disease

Histology textbooks routinely list and describe the cellular components of the kidney as including the podocytes, the mesangial cells, the tubular epithelial cells, and the interstitial and glomerular capillaries. However, rarely is there any photo microscopic image or schematic depicting normal immune cells of the kidney. There is good reason for this. By light microscopy, immune cells are not normally visualized in the kidney, except rarely as a few perivascular infiltrates of lymphocytes around cortico-medullary vessels. Contrary to what is visualized by light microscopy and standard hematoxylin and eosin stained tissues, there are normal immune cells percolating continuously throughout the kidney parenchyma, including medulla, cortical interstitium, mesangium, and capsule. These immune cells play an important role in maintaining homeostasis such that the kidney is protected from infection and autoimmune attack (Fig. 6.2). When discussing immune cells of the kidney, the

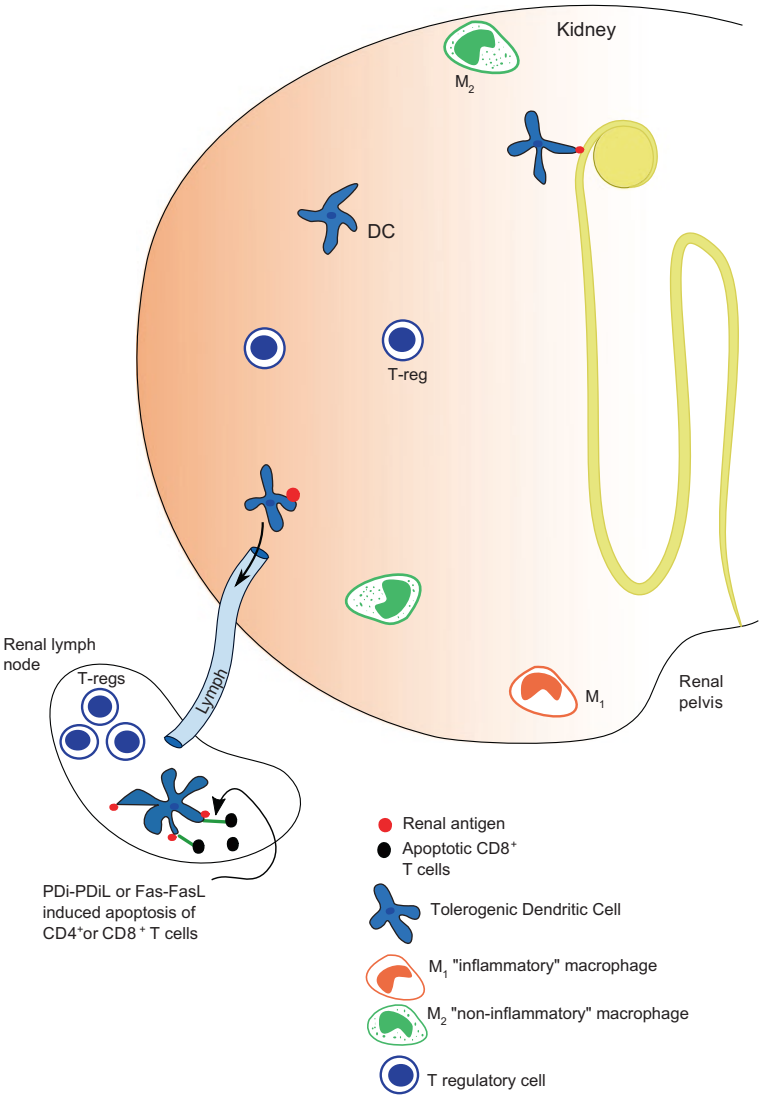

Fig. 6.2 Immune cells of kidney in health. This schematic depicts the typical surveillant immune cells in the kidney and its local (renal) lymph node. In the renal interstitium there are immunosuppressive or tolerogenic regulatory T cells (both $CD4^{+}FoxP3^{+}$ and double-negative T-regs), tolerogenic dendritic cells (DC), macrophages, and rare mast cells (not shown). The majority of the macrophages in the parenchyma are of the wound healing immunosuppressive phenotype (M2), while the M1 pro-inflammatory phenotype macrophages are more prevalent in the medulla. The interstitial DC and macrophages constantly probe for self-antigen or Tamm-Horsfall protein and bring this antigen back to the renal lymph node, where there is clonal deletion of $CD8^{+}$ cytotoxic T cells and effector $CD4^{+}$ T cells (by protein disulphide isomerases (PDi-PdiL) and Fas-FasL (CD95-CD95-L) mechanisms) and proliferation of T-regs. (Schematic by Cynthia Swanson)

discussion should not be limited to the conventional bone-marrow derived immune cells. Rather, somatic cells of the kidney, such as the mesangial cells and the podocytes, are now regarded as having significant immunologic function, especially in perpetuating immune-mediated kidney disease when homeostasis breaks down.

The major bone marrow-derived immune cells of the kidney include macrophages, dendritic cells, and T cells and these cells percolate continuously as resident cells throughout the interstitium (Pindjakova and Griffin 2013). B cells and neutrophils generally only appear when they are responding to immune activation, and are not considered resident cell types in rat or man. The immune cells are not evenly distributed in the kidney. Resident macrophages are more commonly found in the medulla and capsule, while resident dendritic and T cells percolate predominantly in the interstitium of the cortex. There is also some species differences in the populations of immune cells within the kidney under normal conditions. Mice have more resident lymphocytes than rats, and in mice, resident B cells may be found normally in low numbers in the cortex. Setting aside these minor differences in resident immune cells, the major role of the immune cells in the healthy kidney in all species is surveillance for foreign microbial antigens, and to maintain immune tolerance to self-antigens (Timoshanko et al. 2006; Scandiuzzi et al. 2010; Gan et al. 2012).

There are phenotypic and functional subtypes of resident immune cells (i.e., macrophages, dendritic cells and T cells), and these various subtypes can either accelerate or subdue inflammation. The ability of resident immune cells to change phenotype and function is termed plasticity, and the immune cells of the kidney have high plasticity. For example, in Acute Kidney Injury (AKI), specific paracrine factors released from the injured or insulted renal tubular epithelial cells (such as IL-10, damage-associated membrane proteins (DAMPs) or apoptotic bodies) signal the dendritic cells to initiate phenotypic reprogramming of the resident regulatory T cells and immunosuppressive M2 macrophages to effector T cells and pro-inflammatory M1 macrophages, respectively. While this pro-inflammatory phenotype may be necessary to prevent infection and to eliminate an exogenous insult, inflammation will also destroy parenchyma and potentially cause irreversible kidney damage while doing so. Uncontrollable release of inflammatory mediators creates a self-perpetuating condition that can snowball out of hand unless there are mechanisms to halt the inflammation. Therefore, inflammation and immune reactions are a double edged sword. By determining what particular phenotype subdues damaging inflammation, and what triggers the switch from non-inflammatory to inflammatory phenotype, researchers are making significant headway and proposing possible therapies for immune-mediated renal diseases.

Lymphocytes. T cells are occasionally present and B cells are non-existent in the tubulo-interstitium of rat and man under normal circumstances. This is in contrast to the mouse, as mentioned above, in which species B cells are occasionally present in the kidney. Intrarenal resident T cells have a different phenotype from T cells in spleen and blood. The T cells in the kidney are primarily regulatory T cells (T-regs), and in particular, either double-negative T cells (CD3+ CD4−CD8−; DN-T-regs) or CD4+ FoxP3+ T cells (FoxP3+ T-regs). The important point to remember is that the lymphocytes in the kidney under normal circumstances *prevent* activation of the

innate or antigen-specific immune responses. There are some differences how each of the types of T-regs accomplish this task. The DN-T-regs play a role only in suppressing antigen-specific immune responses, and therefore help prevent autoimmune disease and protect against graft rejection. The FoxP3+ T-regs on the other hand are more general players, and dampen *both* antigen-specific and innate immune responses. Immunosuppressive mechanisms common to both the DN-T-regs and the FoxP3+ T-regs are that they both express CTLA4 that downregulates costimulatory molecules CD80 and CD86 on dendritic cells (Gao et al. 2011); they both kill DCs that present antigen through the Fas/FasL pathway; and they both suppress T cell proliferation (Ford McIntyre et al. 2008; Juvet and Zhang 2012).

One mechanism utilized by the DN-T-regs (and not by FoxP3+ T-regs) is trogocytosis, which is a process whereby cells acquire membrane components of other cells. The DN-T-regs acquire antigen (whether it is allo-, foreign-, or auto-antigen) from antigen-presenting cells (APCs) by trogocytosis and these DN-T-regs express this antigen on their own surface. When they do this, these DN-T-regs have the ability to kill antigen-specific CD8+ T cells which may be primed to destroy parenchymal renal cells (Ford McIntyre et al. 2008). The end result is that the DN-T-regs will reduce the population of cytotoxic T cells, and curtail at least one important arm of the antigen-specific immune response (Ford McIntyre et al. 2008).

The phenotype of T cells changes in the diseased kidney. The predominant lymphocyte in disease states of the kidney is the CD4+ T helper cells. CD4+ T helper cells are *not* normal constituents of the kidney in health, but generally indicate activation of the adaptive immune response. Intra- and peri-glomerular infiltrates containing CD4+ T helper cells are regularly observed in immune-mediated kidney disease, such as lupus glomerulonephritis (Couzi et al. 2007; Foster 2007; Masutani et al. 2001) or in IgA nephropathy (Falk et al. 1995). They play a role in mediating these diseases that fall into the class IV delayed-type hypersensitivity reactions (Kurts et al. 2007). They also function backstage in the local or renal lymph nodes (a site separate from the kidney) to induce antibody production by B cells which can then lead to class II or class III hypersensitivity reactions.

There are three types of CD4+ T helper cells: Th1 cells, Th2 cells and Th17 cells. Depending on the functional and surface molecule phenotype, these different T cells have different effector functions, and they exercise phenotypic plasticity depending on the environment. T cells become Th1 cells when they are stimulated by IL-12, IFNϒ and/or when their Toll-like receptors (TLRs) are activated (Kurts et al. 2007). These Th1 cells are pro-inflammatory and are the basis for stimulating macrophages and creating a cell-mediated immune response. T cells will phenotypically become Th2 cells when stimulated by IL-4. Th2 cells will induce immunoglobulin (antibody) production by B cells and stimulate eosinophilic granulocytes for defense against parasites. T cells becomes Th17 cells when induced by TGFβ and IL6 (Tato and O'Shea 2006). When T cells are exposed to IL-23 or IL-1β, they also become Th17 cells that are pathogenic and pro-inflammatory. Pro-inflammatory Th17 cells secrete IL-17 which induces the production of IL-1 and TNFα from endothelial cells, stromal cells, epithelial cells and fibroblasts; and which recruits neutrophils (Iwakura and Ishigame 2006).

Dendritic cells. Dendritic cells (DCs) are constituents of the mononuclear phagocytic system of the kidney (rMoPh). The rMoPh system is the group of cells within the kidney that represent the macrophages and dendritic cells (Nelson et al. 2012). Macrophages and dendritic cells are no longer considered two discrete cell types, but rather one cell type with high plasticity and ability to change function, morphology and phenotype depending on the need or environmental condition. For the purpose of this chapter, a distinction will be made between the renal dendritic cells and the renal macrophages.

The kidney dendritic cells (DCs), as all tissue DCs, are cells derived from bone-marrow precursors, and are specialized for uptake, transport, processing, and presenting antigen to T cells in local lymph nodes. In the kidney, they form an extensive parenchymal network through the entire tubulo-interstitium, and are present within the local renal lymph node; but they are not routinely present in the glomerulus (Kurts et al. 2007; Teteris et al. 2011; Kruger et al. 2004). They are critical to maintain tolerance and prevent activation of the innate or adaptive immune system, but they have been incriminated in perpetuating progression of common renal diseases as well (Segerer et al. 2008; Teteris et al. 2011; Wilde et al. 2009). Several DC subsets have been identified that differ in surface phenotype, activation state and functionality. Depending on the subtype of DC, these cells either encourage peripheral immune tolerance (in the normal homeostatic condition) or they can activate antigen-specific clones of T cells (CD4+ or CD8+ cytotoxic cells). When they activate antigen-specific clones of T cells, inflammation and immune-mediated renal disease ensues.

In health, DCs in the kidney or its lymph node maintain peripheral tolerance to self-antigen. The DC can do this by causing destruction of potentially harmful cytotoxic T cells (i.e., clonal deletion) or by inducing the production of regulatory T cells which suppress the action of harmful T cells (i.e., anergy) (Steinman et al. 2003). DCs from the local renal lymph nodes may be recruited to the tubulo-interstitium by a CX3C-chemokine, known as fractalkine (Segerer et al. 2002). During their brief stay in the kidney, which has been determined to be about 14 days (Dong et al. 2005), the DCs probe the environment for self-antigen. Typically, the DCs peruse the entire tubulo-interstitium and pick up self-antigen, including those antigens from tubular epithelium, self-antigens released by glomeruli, Tamm-Horsfall protein antigens, and systemic antigens that may happen to filter through the glomeruli into the tubular lumen. The renal DCs then take these antigens back to the local lymph nodes where tolerance mechanisms occur (Kurts et al. 1996; Dong et al. 2005). Antigen-bearing DCs in the renal lymph node either cause apoptosis of antigen-specific CD8+ or CD4+ T cells or induce tolerogenic regulatory T cells. There is some suggestion that the tolerance mounted to antigens filtered through the glomerulus might be one way that the body mounts tolerance to food antigens during life (Teteris et al. 2011).

There are several mechanisms used by the tolerogenic DCs to achieve clonal deletion or anergy. These DCs have PDI-L (protein disulfide isomerase ligand) on their surface, which can bind to PDI on lymphocytes mediating either pro-apoptotic or pro-anergic intracellular signaling within the T cells (Gottschalk et al. 2013).

When DCs present autoantigens to CD8+ T cells they can induce Fas and Bim-dependent and Bcl-2-inhibitable apoptosis (Davey et al. 2002; Kurts et al. 1997, 2001). When tolerogenic DCs present autoantigens to CD4+ T cells, they can turn these CD4+ cells functionally and phenotypically into T-regs which will then suppress T cell activation. Tolerogenic DCs also produce anti-inflammatory mediators IL-10 and TGFβ, contributing to the overall anti-inflammatory effect. The unique environment of the local renal lymph node may play a role in tolerance, more so than other lymph nodes draining other tissues. It has been shown that those CD8+ T cells that become localized to the renal lymph node have an increased rate of apoptosis (i.e., clonal deletion) and a decreased production of IFNγ (i.e., they become hypo responsiveness), when compared to T cells in the subcutaneous lymph nodes exposed to the same antigen (Gottschalk et al. 2013).

There is no simple phenotype classification scheme for the dendritic cells in rodents and humans. DCs in the kidney, as in most non-lymphoid tissue, generally express CD11b, and are known as classical CD8-negative DCs. These DCs also express CD11c, CX3CR1, F4/80 and MHC II (Kruger et al. 2004; Heymann et al. 2009). CX3CR1 is the receptor for the chemokine fractalkine that is largely responsible for recruiting DCs into the kidney (Soos et al. 2006). Human renal dendritic cells have a different set of phenotypic markers from mice. Human DCs have CX3CR1 and CD11b and CD11c markers, like mice, but also can express CD103, CD205, CD207 (langerin), CD209 (DC-SIGN), and CD68 (Teteris et al. 2011). In healthy homeostasis, the prevalent phenotype for tolerogenic renal DCs in man is CD205−CD11c+ DCs (Guilliams et al. 2010) or CD103+ DCs (Degauque et al. 2006). Many of these identifying markers in mice and human rDCs are also markers for macrophages (i.e., CD11c, CD11b, F4/80, CD68) which underscores the overlap between DCs and macrophages and the idea that these cells all belong to the highly plastic rMoPh system (Soos et al. 2006). Renal DCs can also be differentiated from each other based on their expression of chemokine receptors other than CX3CR1, such as CCR1, CCR2, CCR5, and CCR7. Variations in these chemokine receptors will dictate whether these DCs can be recruited and invited into healthy or into inflamed/injured kidney (Teteris et al. 2011), and are the focus of possible therapeutic interventions to control inflammation. Correlation between phenotype and function, and particularly determining which subset of rDCs play a role in protecting or potentiating renal disease, are areas ripe for therapeutic research.

The function of DC have been worked out by the creative development of transgenic mouse models. It has been possible for investigators to deplete DCs from a transgenic mouse system expressing human diphtheria toxin receptor. When this mouse model is injected with diphtheria toxin, the DCs are eliminated (Jung et al. 2002). Using this DC-depleted mouse model, and showing that early stage immune-mediated kidney disease (i.e., nephrotoxic serum nephritis) was aggravated in the absence of DC (Scholz et al. 2008), it became clear that DC normally play a role in suppressing immune activation. The DCs immunosuppressive role is important in controlling cisplatin-induced acute kidney injury (Hochheiser et al. 2011a, b), since mice depleted of DCs had more severe renal dysfunction and injury compared to

non-depleted mice (Tadagavadi and Reeves 2010). For a more thorough review of renal DCs, the reader is referred to Shortman and Naik (2007).

While DCs in the kidney are generally regarded as immune cells that attenuate disease, subsets of rDCs can aggravate disease, especially the CD205+ human rDCs. These pro-inflammatory DCs are required to help protect the host from invasion by bacteria and viruses. When the CD205+ DCs capture antigens, they activate CD8+ cytotoxic T cells in secondary lymphoid organs such as the renal lymph node or the spleen. The CD205+ DC are "immature" and are generally triggered by the microbial molecular patterns such as LPS (Mellman and Steinman 2001; Kurts et al. 2007). These DC capture glomerular antigens in the tubulo-interstitium and present it to CD4+ cells causing progression of Th1 cell-mediated disease providing an effective adaptive immune response against microbials. These DCs have costimulatory molecules and produce pro-inflammatory cytokines (such as IL-12, IFNγ, and TNFα), and neutrophilic chemokines that collectively cause inflammation. Although the CD205+ DCs are important for adaptive immune protection against invading microbials, they unfortunately "misbehave" and are the more prevalent phenotype for DCs in immune-mediated glomerular diseases, such as IgA nephropathy and the late stage of nephrotoxic serum nephritis (NSN) (Bagavant and Tung 2005; Markovic-Lipkovski et al. 1990; Hochheiser et al. 2011b). Dendritic cells also potentiate inflammation in lupus nephritis, T cell mediated glomerular injury (NOH model), acute kidney injury and tubulo-interstitial disease (Hochheiser et al. 2011a, b). As will become clearer later, a few misbehaving DCs is all that is needed to start a self-perpetuating disease. Once a few DCs start the inflammatory cascade leading to proteinuria, the protein leaking into the tubular filtrate provides fodder that activates even the well-behaved DCs which then stimulate pathogenic Th1 and Th17 T cells (Hochheiser et al. 2011b).

In some diseases, it is less clear whether DCs prevent disease or potentiate disease, and the difference might depend on the stage of disease. In ischemic reperfusion injury, DCs aggravate injury early on in the disease by producing TNFα, IL-6, MCP-1 and RANTES (Dong et al. 2007), but potentiate repair from such injury by producing IL-10 (Kim et al. 2010). In the early phase of experimental non-accelerated phase of nephrotoxic serum nephritis, the DCs subdue inflammation (Scholz et al. 2008; Hochheiser et al. 2011b; Tadagavadi and Reeves 2010), but they aggravate disease in the later stages (Hochheiser et al. 2011b). What makes the DCs switch from an immunosuppressive cell type (inducing tolerance and anergy) to a pro-inflammatory DC, and *visa versa,* is the important and yet unanswered question, and the answer to which has huge implications for possible therapies in chronic kidney disease.

Macrophages. The kidney macrophages, like the renal dendritic cells discussed above, are part of the renal mononuclear phagocytic (rMoPh) system. While this chapter will make a distinction between the two, the reader is advised that studies are now showing that these two cell types are not distinct. As mentioned above, and which will be emphasized again, there is considerable overlap between the function, phenotype and morphology of the macrophage and the dendritic cell (Nelson et al. 2012).

Renal macrophages are dendritiform cells that form a contiguous network throughout the renal interstitium of rodents and man, where they line the microvascular, peritubular and periglomerular spaces (Nelson et al. 2012). In rats, renal macrophages are more plentiful in the medulla and the capsule. Macrophages may occasionally percolate into the mesangial matrix of the normal glomerulus. Like the rDC, the renal macrophages have phenotypes that make them pro-inflammatory or immunosuppressive, yet the majority of them in healthy homeostasis are of the immunosuppressive phenotype to prevent unnecessary activation of the immune system. The surface markers are generally indicative of their function. The CD11b+/MHC class II+/CX2CR1+/Fr/80+/CD103− (also known as, CD103− or M1) are generally the pro-inflammatory subtype (Nelson et al. 2012) and are found preferentially in the medulla. This makes sense since they are poised to respond to ascending microbials. The non-inflammatory or immunosuppressive phenotypes include the CD103+ and/or the CD11c+ subtypes, and are referred to as M2. These M2 produce IL-10 and induce regulatory T cells. Recall from the discussion above that CD11c+ and CD103+ are also markers for immunosuppressive DCs that induce regulatory T cells (Nelson et al. 2012; Kitching and Holdsworth 2011), once again underscoring the overlap between DC and macrophages. In particular, these immunosuppressive macrophages function like the tolerogenic DCs and sample antigens in the kidney and presents those antigens to T cells in a manner that expands the regulatory T cell population and induces tolerance rather than T cell activation (Nelson et al. 2012).

Macrophages are plentiful in immune-mediated diseases and even with best intentions, and whether they are M1 or M2 phenotype, their presence and function generally harm the kidney and reduce renal function. Even the non-inflammatory/wound healing M2 macrophages contribute to glomerulosclerosis which perpetuates reduced GFR and declining renal function. Macrophages in inflammatory disease come from both the resident and the circulating populations. In acute kidney injury models induced by endotoxemia, ischemia reperfusion, acute urinary obstruction or pyelonephritis, macrophages are increased in size, have upregulated costimulatory molecule expression, and secrete pro-inflammatory mediators. They secrete inflammatory cytokines such as TNFα, IL-6 and IL-1 and secrete chemokines CCL2, CCL5, CXCL10 and CxCL2 that recruit additional inflammatory cells poised to produce inflammatory mediators. Macrophages will also influence the phenotype of the T cell subpopulations in the environment (Kitamoto et al. 2009). In more chronic models of inflammatory kidney disease, the M1 macrophages become M2 macrophages and promote damaging sclerosis and reduced GFR. Regardless of the M1 or M2 phenotype, most tissue damage in immune-mediated kidney disease can be tracked back to the pro- or anti-inflammatory macrophage.

Podocytes and Mesangial Cells. Podocytes and mesangial cells play a large role in perpetuating the immune reaction in the kidney. Normally the mesangial cells are quiescent and inactive, and the podocyte is busy maintaining the GBM and the filtering apparatus. However, both of these non-immune cell types can be induced to produce a number of products that promote inflammation and tissue damage, including IL-1, reactive oxygen species (ROS), platelet activating factor, and thromboxane

B2. In addition they have toll-like receptors (predominantly TLR3 and TLR4 on podocytes; TLR3 on mesangial cells); are equipped with inflammasomes; and can be induced to express class II MHC molecules and present antigens (Schreiner et al. 1981; Shahzad et al. 2015; Lorenz et al. 2014; Banas et al. 2008; Goldwich et al. 2013; Alexander et al. 2007). The mesangial cells also produce growth factors, such as platelet-derived growth factor (PDGF), insulin-like growth factor (IGF), and transforming growth factor (TGF)-β. PDGF and IGF generally cause proliferation of fibroblasts and mesangial cells, while TGF-β stimulates matrix formation by the mesangium and podocytes (Haraldsson et al. 2008).

6.4 Immune-Mediated Glomerulonephritis

Immunopathology of the glomerulus is a complex subject that can be presented in many ways. Some publications divide the disease entities by the source of the antigen (renal, non-renal, foreign) and the staining pattern on immunohistochemistry (linear vs. granular) (Jones et al. 1986). Others describe the various diseases based on the primary morphologic appearance of the glomerulus, such as membranous, membranoproliferative, mesangioproliferative, or crescentic, to name a few. Still others separate renal disease by their clinical presentation (i.e., nephrotic syndrome, hematuria and proteinuria; acute glomerulonephritis, and rapidly progressive glomerulonephritis) (Stephany 2010).

This section will describe specific immune-mediated diseases of the glomerulus based on the type of hypersensitivity response that is primarily operative in that disease. This is commonly the way pathologists subclassify renal disease because it is based on differences in pathogenesis. According to the Gell Coombs classification for hypersensitivity reactions, there are four types of reactions in the glomerulus (Gell and Coombs 1963). These include type I (acute, or Th2-mediated hypersensitivity); type II (anti-glomerular basement membrane disease); type III (immune complex deposition); and type IV (delayed-type hypersensitivity). The types II and III reactions are best known to general pathologists, because these reactions involve immune complex deposition with complement activation within the glomerulus resulting in various morphologic and clinical presentations of glomerulonephritis. However, these two classical well-known reactions no longer complete the story. Now there are immune-mediated glomerulonephritides that correlate to Type I and Type IV hypersensitivity reactions, and there is also a fifth type referred to as “loss of regulatory T cells”. Moreover, the pure type II and type III reactions that typify anti-GBM disease or lupus nephritis, respectively no longer tell the full story either. Rather, multiple types of hypersensitivity reactions are involved in these characteristic immune complex renal diseases.

When toxicologic pathologists are evaluating kidney tissue in a preclinical safety study and recording a diagnosis, they generally subclassify the glomerulonephritis by morphology under the light microscope. Mesangioproliferative (increased number of mesangial cells), membranous (increased thickness of GBM with thickened

capillary loops), membranoproliferative (having features of both mesangioproliferative and membranous), exudative (having intracapillary and intraglomerular inflammatory cells), and crescentic (having the dominant feature as crescents) are commonly used morphologic terms. Since morphologic subtypes are what we see as toxicologic pathologists, and what we record as our diagnosis, one would think this chapter should be organized by morphologic presentation of disease. With few exceptions, the morphologic appearance of the glomerulus can vary considerably with severity and stage of disease, and genetic variants of the patient; morphology is not specific for any one pathogenesis; and morphology alone reveals limited information about the pathogenesis. These revelations can be quite depressing to pathologists who have to interpret renal lesions and shed light on possible pathogenic scenarios. But a pathologist who understands the limits of the light microscope, the various immunologic mechanisms that collectively lead to the same morphologic changes, and how glomerular diseases lead to tubulo-interstitial and multi-organ disease, and *visa versa*, will be able to correlate renal lesions with possible test article-related immunologic mechanisms operating within the kidney itself or within the host as a whole.

In order for the pathogeneses of immune-mediated renal disease to be worked out, scientists had to develop unique animal models through gain and loss of function experiments, where a molecule is either amplified or removed from an *in vivo* system. These animal models have been accomplished by transgenic overexpression or by genetic knock-out, respectively (Anders and Schlondorff 2000). From these knock-out and genetically modified mice, researchers have not only shed light on immune-mediated kidney diseases, but have clarified the pathogeneses of other immune-mediates conditions such as inflammatory colitis, type I diabetes, asthma and multiple sclerosis (Hanninen et al. 2003; Steinman and Zamvil 2006; Groux et al. 1997). A list of the immune-mediated renal diseases in humans that will be discussed in this chapter, and their corresponding animal models and pathogenesis, is provided in Table 6.1.

In this section, only the initial pathogenesis of the diseases (i.e., the events that *initiate* immune activation) will be discussed. The mechanisms that operate *after* immune activation occur will be discussed in the following section (Part VI), when the pathophysiological processes of the various diseases generally converge to provide a similar pattern of histologic lesions (Le Hir 2004).

6.4.1 Type I Hypersensitivity-Acute or Th2-Mediated Hypersensitivity

Type I hypersensitivity is the classical acute allergic response that arises from IgE-mediated mast cell degranulation, histamine production, and infiltration of eosinophils. The reaction is initially mediated by Th2 helper T cells that produce IL-4, IL-5 and IL-13. The IL-4 and IL-13 induce B cells to produce IgE antibody that

Table 6.1 Animal models and pathogenesis of selected human immune-mediated renal diseases

Animal model	Human disease	Pathogenesis
Auto-immune glomerulonephritis		
IL-13 transfected rats	Minimal change nephropathy	Type I - *Mediated by Th2 helper cells producing IL13* Type V - *Deficiency of Tregs to produce CTLA-4*
Mercury chloride-induced nephropathy *Brown Norway rats*	Anti-GBM disease Non-specific glomerulonephritis	Type I - *Th2 helper cells and elevated IgE* Type II - *Anti-GBM deposits*
Puromycin nephropathy *Rats/mice*	Minimal change nephropathy	Type II - *C5-9 MAC damage to podocytes* Type IV Interstitial nephritis
Experimental autoimmune Glomerulonephritis *Wystar-Kyota rats, and Zeno II transgenic mice* Spontaneous GN in NZ White rabbits	Anti-GBM disease	Type II- *Antigen is megalin in mice* Type III - *Formation of CICs from leakage of antigen* Type IV - *CD8+; CD4+ Th1 and Th17 effector cells when crescentic* Type V
Passive or active Heymann *Using Fx1a antigen in Sprague-Dawley rats and mice* Spontaneous Heymann in rats	Membranous GN *Autoimmune to phospholipase A2 receptor (PLA2R) on podocytes* Antenatal alloimmune reaction *to neutral endopeptidase (NEP) on fetal podocytes*	Type II - *In autologous phase* Type III - *In heterologous* phase Type IV - *CD8+ T cells*
Nephrotoxic serum nephritis *Mouse, rats, rabbits* BSA-injections *rabbits, dogs, mice, rats*	Human crescentic nephritis Nonspecific immune complex nephritis	Type II Type V - *CD4+ Th1/Th17, macrophages, CD8+*
Lupus nephritis *NZB/NZW F1 rats* *MRL/lpr mice*	Lupus nephritis	Type III Type IV Type V
IgA nephropathy *ddY mouse strain* Dietary administration of mycotoxin in mice	IgA nephropathy	Type III - *IgA deposits* Type IV - *CD4+ and CD8+*

(continued)

Table 6.1 (continued)

Animal model	Human disease	Pathogenesis
Anti Thy 1 *Mouse or Lewis rat* *Antibody to mesangial cells*	Non-specific glomerulonephritis	Type IV - *CD4+ and CD8+*
Adriamycin-induced glomerulonephritis	Focal segmental glomerulosclerosis	Type IV - *CD8+* Type V
OVA/HEL (NOH) model *Mouse*	Non- specific glomerulonephritis	Type IV - *CD4+ and CD8+*
Immune-mediated tubulo-interstitial nephritis		
Kdkd mouse Lewis rats *Inject with syngeneic kidney*	Immune-mediated tubulo-interstitial nephritis	Type IV Type V
Anti-TBM disease *Rats/mice*	Anti-TBM disease Immune-mediated tubulo-interstitial nephritis	Type IV Type V
Acute kidney injury		
Nephrectomy (5/6) *Rat*	Acute kidney injury	Innate ≫ Adaptive
Septic acute kidney injury *Inject LPS*	Acute kidney injury	Innate ≫ Adaptive
Aseptic acute kidney injury *Inject cisplatin in rats/mice* *Treat with N-(3,5-dichlorophenyl)-succinimide (rats)* Ischemic reperfusion injury	Acute kidney injury	Innate ≫ Adaptive

binds to mast cells and cause degranulation and histamine release. IL-5 recruits eosinophils. Conventionally, acute allergic or acute hypersensitivity reactions in tissues such as skin are characterized by eosinophils, mast cells, and edema, with lymph nodes containing Th2 cells and IgE-producing B cells. This is not a common typical tissue reaction seen in the kidney. It is true that mast cells and eosinophils may increase in various renal diseases (Blank et al. 2007), and eosinophil activation has been reported in some forms of drug-induced nephritis, but these cells are never the predominate immune cell in the kidney (Koren 1989) (Fig. 6.3). More typically, type I allergic reactions in the kidney are due to a "hypercytokine" effect, in the absence of the typical tissue infiltrates of mast cells and eosinophils ordinarily associated with allergic reactions in other tissues. Minimal change disease (also known as Nil Disease or lipoid nephrosis) is a disorder that is now believed to represent a type I hypersensitivity reaction in the kidney due to the cytokine IL-13.

Minimal Change Disease: Minimal change disease (MCD) is a common glomerular disease in children and affected individuals present with hypoalbuminemia, proteinuria, and the nephrotic syndrome. The nephrotic syndrome is characterized by hypercholesterolemia and thromboembolic events. MCD is the major histologic

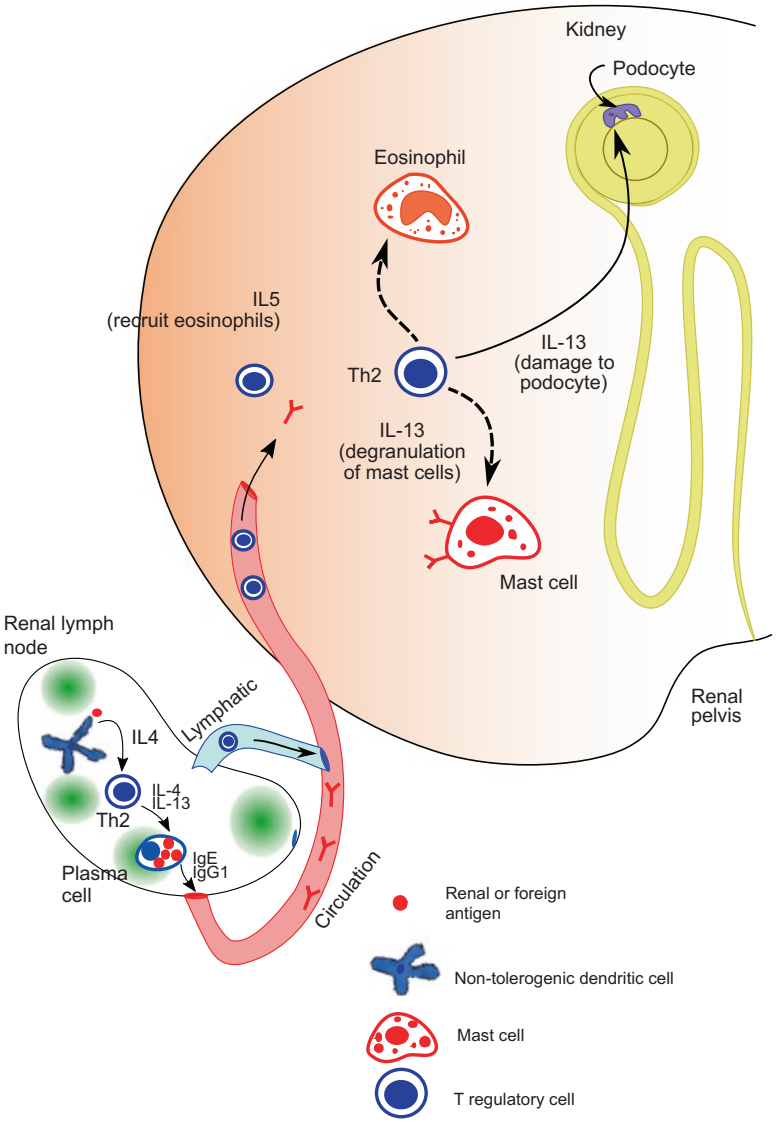

Fig. 6.3 Type I Hypersensitivity. Th2-mediated disease. This schematic shows how a Th2 response can cause interstitial inflammation, and also glomerular disease. Non-tolerogenic DCs (with allergen) travel to the renal lymph node. The DCs produce IL-4 which activates T cells to switch to Th2 phenotype. The Th2 cells, in turn, secrete IL-4 and IL-13, resulting in class switching of immunoglobulin production by B cells to that of IgE (as well as IgG1). The Th2 cells and the IgE travel by lymphatic and/or blood circulation to the renal interstitium. Once in the interstitium, IgE can bind to the surface of mast cells, leading to degranulation. The Th2 cells secrete IL-5 and IL-13. IL-5 attracts eosinophils, and IL-13 assists in mast cell degranulation. More typically, however, the IL-13 damages podocytes, causing upregulation of CD80 (B7-1) and leading to proteinuria. (Schematic by Cynthia Swanson)

variant of idiopathic nephrotic syndrome and accounts for the vast majority (approx. 90%) of cases of childhood nephrotic syndrome (Kaneko et al. 2015). As an aside, focal segmental glomerulosclerosis (FSGS) is the other histologic variant for nephrotic syndrome and accounts for 10% of cases (Kaneko et al. 2015). MCD and FSGS may represent different ends of the same disease spectrum with same underlying pathophysiologic process. In either event, MCD represents a type I hypersensitivity reaction.

The cause for MCD is largely unknown, but 10–20% of patients have an identifiable triggering event. Nonsteroidal anti-inflammatory drugs, rifampin, mercury, lithium, bee stings, Hodgkins lymphoma, hematopoietic stem cell transplantation, and drug-induced interstitial nephritis are a few known triggers for MCD. The pathology is non-specific, and it is a diagnosis based more on exclusion than on any specific finding. The glomerulus is normal by light microscopy, hence the term "minimal change disease". While microscopically normal, the GBM has lost its charge barrier, and therefore protein leaks into the filtrate. There may be intratubular protein and intraepithelial resorption of protein and lipid by tubular epithelial cells. Ultrastructurally there is fusion and/or effacement (or retraction) of foot processes, with intracytoplasmic vacuolation and formation of microvilli on podocytes. If MCD is secondary to drug-induced interstitial nephritis, then obviously interstitial inflammation may co-exist. In toxicologic pathology, a diagnosis of MCD or "allergic glomerulitis" would not be common diagnosis, since the tools needed to make this diagnosis (i.e., electron microscopy, or B7-1 urinary excretion levels) (Ling et al. 2015; Garin et al. 2009) are not routinely done in laboratory animals. However, when confronted with "normal appearing" glomeruli with intratubular and endocytosed protein and lipid, proteinuria, and no etiology or explanation, a type I hypersensitivity should be considered.

The pathogenesis of MCD is not clear, and much of the advances in our knowledge are due to the use of animal models. One animal model is to induce proteinuria by injection of lipopolysaccharide (LPS) in severe combined immunodeficiency (SCID) mice devoid of T and B cells (Reiser et al. 2004). Other models include mice transfected with IL-13 (Lai et al. 2007) and administration of puromycin aminonucleoside to rats (Grond et al. 1988).

The working hypothesis that brings together most of the research findings is that MCD is caused by a circulating factor that causes podocyte damage, which leads to sustained upregulation of B7-1 on podocytes due to T-reg dysfunction. Both the initial podocyte damage (type I hypersensitivity) and the T-reg dysfunction (type 5 hypersensitivity) are required for the disease to manifest (Shimada et al. 2011). The initial damage to the podocyte is due to some circulating factor (e.g., IL-13, allergen, microbial product), and this damage results in upregulation of B7-1 expression by podocytes. This increased B7-1 expression results in disruption of the actin cytoskeleton, resulting in shape changes to the foot processes and proteinuria through a mechanism that awaits to be clarified. B7-1 over-expression fails to resolve because of a dysfunction of a regulatory mechanisms that normal keeps B7-1 expression under control. This continual B7-1 expression perpetuates the proteinuria and may

lead to more advanced progression of the disease that is microscopically apparent, namely focal segmental glomerulosclerosis (FSGS).

Several circulating factors can cause the initial podocyte damage that leads to upregulation of B7-1. One such factor is IL-13, although LPS binding to Toll-like receptor 4 on the surface of podocytes, can also lead to upregulation of B7-1 expression (Lai et al. 2007; Garin et al. 2010; Reiser et al. 2004). Therefore, over-expression of B7-1 on podocytes is not specific for MCD or type I hypersensitivity or IL-13-induced podocytopathy, and has been reported with nephrotic syndrome, puromycin nephropathy, and lupus nephritis (Reiser and Mundel 2004). Since this section of the chapter is focused on type I hypersensitivity reactions in the glomerulus, the discussion will focus on IL-13-induced podocyte damage and IL-13-induced B7-1 expression. IL-13 is a cytokine released by Th2 cells and this cytokine is responsible for immunoglobulin class switching of B cells to produce IgE. It is proposed that Th2 cells releases excess IL-13 circulates to the glomerulus and causes podocyte damage and protein leakage (Kurts et al. 2007; Lai et al. 2007) (Fig. 6.3). Minimal change disease therefore can be thought of as a hypercytokine event (Mathieson 2003). The direct morphologic effects of IL-13 on podocytes was demonstrated using an IL-13 overexpression transfected Wistar rat, in which the podocytes had foot process fusion (Li et al. 2007). Further support for the IL-13-induced damage to podocytes comes from the fact that MCD can be associated with Hodgkins lymphoma, a disease involving proliferation of the Reed-Sternberg cell that in turn relies on IL-13 as its autocrine growth factor (Kapp et al. 1999). It is speculated that IL-13 induces the podocyte to over-express B7-1, and B7-1 expression results in fusion of foot processes. How B7-1 leads to foot process fusion is not clear, but B7-1 over-expression is still considered critical to the pathogenesis of MCD.

B7-1 (also referred to as CD80) is a co-stimulatory molecule normally expressed on antigen presenting cells (macrophages and dendritic cells) that binds to CD28 on T cells and helps modulate the T cell receptor signal (Greenwald et al. 2005). Podocytes however also express B7-1, and its increased expression or availability is directly correlated with disease and glomerular leakage: Mice lacking B7-1 are protected from LPS-induced nephrotic syndrome (Reiser et al. 2004). Normally, increased B7-1 expression by podocytes is transient and the degree of expression of B7-1 and its function on T cells is controlled by the T regs (Fig. 6.4). T regs produce CTLA-4 which binds to B7-1 and blocks its ability to bind to CD28 on T helper cells. T regs also produce IL-10 that (1) suppresses activation of T helper cells; (2) limits the production of IL-13 by Th2 cells which would otherwise cause additional damage to podocytes and continued upregulation of B7-1; and (3) limits expression of B7-1 by podocytes (Shimada et al. 2012; Wing et al. 2008). Therefore, if B7-1 expression is increased by IL-13, (or an allergen, or LPS, or some other cytokine), and proteinuria occurs, the T-regs should and will quickly bring B7-1 expression under control to resolve the proteinuria. In MCD, there is believed to be an additional dysfunction of Tregs characterized by reduction in CTLA-4 (which would normally bind to and block B7-1), and a reduction of IL-10 (which would normally reduce B7-1 expression). Therefore patients with this T reg dysfunction have sus-

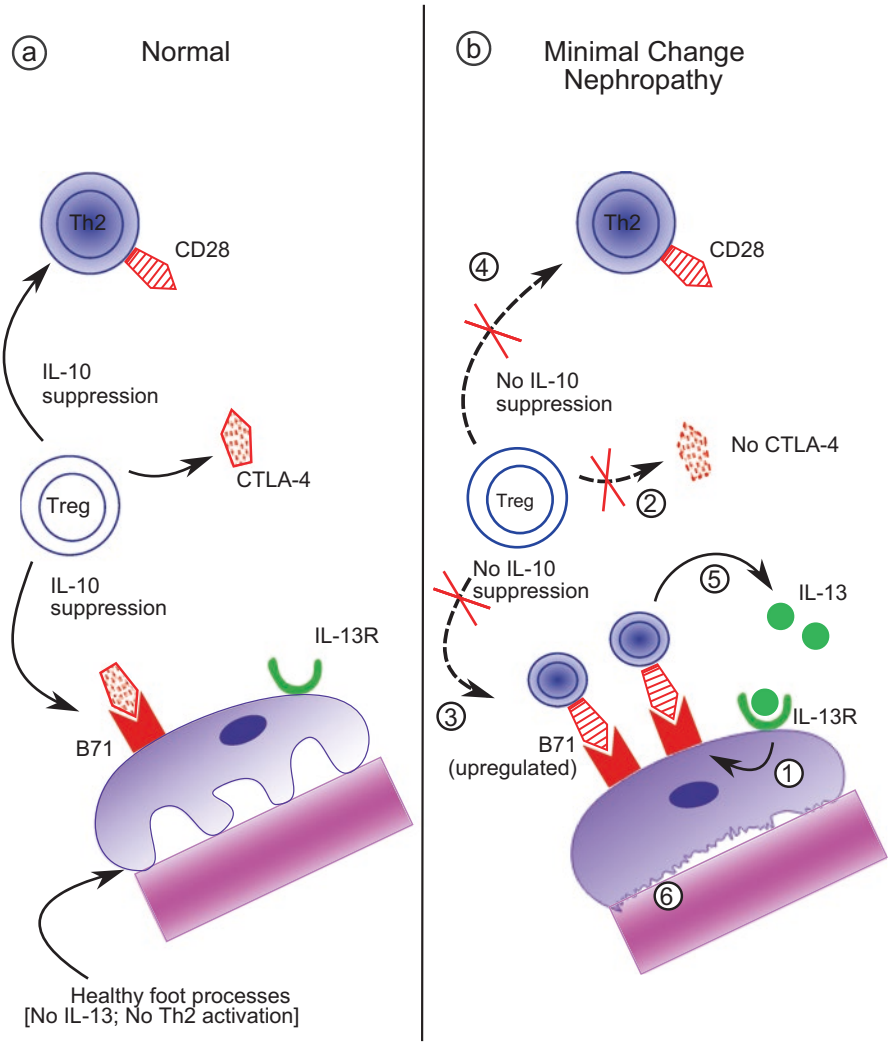

Fig. 6.4 Type I hypersensitivity. IL-13 and B7-1 mediated podocyte injury. Panel (**a**) represents the healthy normal glomerulus with podocytes having foot processes attached to the basement membrane. The podocytes normally express a low number of CD80 (B7-1) co-stimulatory molecules on their surface. Many of these CD80 molecules are bound to circulating CTLA-4 produced by regulatory T cells. Therefore, little CD80 is available to bind CD28 co-stimulatory molecules on Th2 cells. Th2 cells are therefore not activated and cytokine production is subdued. Production of CTLA-4 is one of the ways T-regs maintain immunosuppression. Another way T-regs maintain immunosuppression is by production of IL-10, which has an inhibitory effect on production and proliferation Th2 cells and on CD80 expression by podocytes. Panel (**b**) represents the kidney in Minimal Change Disease (MCD) which is considered a Th2-mediated disease. In MCD, there is increased availability of CD80 molecules on the surface of podocytes. This increased availability is due in part to IL-13 induced—damage to the podocyte, which responds with over-expression of CD80 on its surface (*1*). Increased availability of CD80 binding sites is also due to a deficiency of T-regs to produce secretory CTLA-4 (*2*). With a deficiency of CTLA-4, there is increased available

tained high level of B7-1 expression and a high number of B7-1 available binding sites to further activate T cells (Araya et al. 2009). Therefore, there is (1) uncontrolled Th2 activation; (2) increased secretion of IL-13; and (3) persistent proteinuria.

Th2-cell activity is classified as a Type I hypersensitivity. This is because IL-13 produced by Th2 cells is the cytokine that is involved in type switching B cells to produce IgE, the immunoglobulin involved in triggering acute hypersensitivity. However other than overexpression of IgE receptors (CD23) on circulating lymphocytes in man with MCD (Shao et al. 2009), IgE is not believed to be directly involved in the pathogenesis of the glomerular injury in MCD. Classifying MCD as a Type I hypersensitivity disease is also appropriate given the clinical history of the disease. MCD is secondary to events considered to trigger classical hypersensitivity reactions, such as bee stings and drug-induced interstitial nephritis (Koren 1989; Kurts et al. 2007). The take home message is that a type I allergic hypersensitivity reaction should be entertained as a possible explanation for proteinuria even though eosinophils, mast cells, edema, and hyperemia are not features of the disease.

Mercury Chloride Induced Nephropathy is an example of a Th2 mediated (or Type I hypersensitivity) disease, that also involves a type II hypersensitivity reaction. With repeated low dose subcutaneous injections of mercury chloride to Brown Norway (BN) rats or rabbit, there is Th2 cell proliferation, polyclonal B cell activation, production of autoantibodies (IgG) and excess IgE levels (van Alderwegen et al. 1997; Fournie et al. 2002; Savignac et al. 2004; Sapin et al. 1984). The Th2 proliferation with excess IgE production characterizes this experimentally- induced disease as a type I hypersensitivity reaction, even though the glomerular disease is more typical of a type II reaction.

Many of the autoantibodies produced following injection of mercury chloride are specifically directed to the GBM (Goldman et al. 1991; Nieto et al. 2002), which means this nephropathy is also an animal model of anti-GBM disease (Type II hypersensitivity, Part IV, B). In fact, the majority of the lesions visible by light microscopy are due to the Type II hypersensitivity reaction. The presentation in rats is that of a membranous glomerulonephritis with thickened GBM and capillary basement membrane with possible increase in mesangial matrix and immune complex deposits in the basement membrane. The disease is characterized by linear IgG deposits in the glomerulus (typical of anti-GBM disease discussed Part IV, subpart B) and tubulo-interstitial nephritis characterized by lymphocytes and monocytes and a barrage of pro-inflammatory cytokines such as TNFα (Nieto et al. 2002;

Fig. 6.4 (continued) CD80 binding sites on podocytes to bind to the costimulatory CD28 molecules on Th2 cells. T-regs also are deficient in production of IL-10 which normally controls CD80 expression (*3*) and Th2 proliferation (*4*). When CD28 on Th2 cells bind to CD80 on podocytes, the Th2 cells become activated, produce more IL-13 which binds to IL-13 receptors on podocytes (*5*), and the process self-perpetuates. IL-13-injured podocytes have reduced synthesis of polyanions leading to loss of charge at the filtration barrier in the glomerular basement membrane. The injured podocytes has ultrastructural changes characterized by fusion or retraction of foot processes from the glomerular basement membrane (*6*). There are no ultrastructural changes in the glomerular basement membrane itself. (Schematic by Cynthia Swanson)

Mampaso et al. 1989). While the interstitial inflammation strongly suggests a T cell-mediated delayed-type hypersensitivity (DTH) response, DTH has not been documented (Savignac et al. 2004; Ghielli et al. 1997). While low doses of mercuric chloride induces a glomerulonephritis (Type I and Type II), high doses of mercuric chloride results in acute tubular necrosis (see AKI, Chapter 6, Part 7) (Stacchiotti et al. 2003).

Only the BN rat has a Th2-mediated glomerulopathy in response to mercuric chloride. The Lewis rat does not respond in the same way as the Brown Norway rat, but rather responds to mercury chloride by a proliferation of TGF-β and IL-10 producing T-regs (not by a proliferation of Th2 cells) (Fournie et al. 2002).

6.4.2 Type II Hypersensitivity: Anti-glomerular Basement Membrane Nephritis; Membranous Nephropathy (MN); Antenatal Membranous Nephropathy

Type II hypersensitivity reactions are those in which antibodies are produced against renal autoantigens and a reaction is instigated when autoantibody comes out of circulation and binds to the antigen resulting in immune complex deposits. Typically, the autoantigen is on the surface of cells, or embedded in the glomerular basement membrane. These autoantigens are inappropriately recognized by non-tolerogenic macrophages or non-tolerogenic dendritic cells which present the autoantigen to CD4+ T helper cells, which then causes a B cell response with production of autoantibody subclass that is adept at fixing complement (e.g., IgG2 subclass in the mouse). Autoantibody produced by B cells in the renal lymph node travels by the blood to the kidney and binds to the antigen forming an immune complex in situ. The complex activates complement, and the host cell is destroyed or injured. In addition to complement-mediated destruction, the cells bearing the autoantigen can also be destroyed by antibody-dependent cell mediated cytotoxicity (ADCC). In essence, the antibody forms a bridge between the autoantigen and NK cells or macrophages.

The classic type II hypersensitivity condition recognized in the kidney is anti-GBM nephritis, and the inciting autoantigen is the NC1 domain of the alpha-3 chain of type IV collagen (hereinafter, referred to generally as type IV collagen) in the GBM. Other examples of type II hypersensitivity diseases are membranous glomerulonephropathy in which the autoantigen is phospholipase A2 receptor on podocytes; Myasthenia Gravis in which the autoantigen is the acetylcholine receptor on myofibers; and Grave's disease in which the autoantigen is the thyroid stimulating hormone (TSH) receptor on thyroid follicular cells.

Anti-Glomerular Basement Membrane (anti-GBM) Disease. Anti-GBM disease is a rare autoimmune disease in man where antibodies form against intrinsic antigen in the GBM, and attack the basement membranes of the kidney. In the kidney, auto-antibodies can attack both the glomerular and the tubular basement mem-

branes (GBM and TBM, respectively). In 50% of the cases, anti-GBM disease is often associated with autoantibodies directed against the basement membrane of the lungs, in which case, the condition is referred to as Goodpasture's syndrome first described in 1919 (Goodpasture 2009). *In vivo* studies indicate that the alpha-3 epitopes are normally sequestered (i.e., hidden) and become exposed due to some disruption of the GBM. Some triggers that might lead to exposure of these sequestered antigens might be smoking (exposure of alveolar basement membrane), or damage to the glomerular or capillary basement membrane by ROS associated with underlying infection/inflammation. In humans, certain HLA-DR subtypes are genetically predisposed to this disease. Since autoantigen is present throughout the GBM, immunohistochemistry reveals antibody (IgG and occasionally IgA and IgM) binding in a linear manner on the GBM and possibly the TBM of distal tubules, with *in situ* formation of immune complexes (Salama et al. 2001). In addition to *in situ* formation of immune complexes, there can also be formation of circulating immune complexes (CIC), presumably due to release of antigen into the circulation, and these CIC deposit in the subendothelial space. Much of the pathology of tissue damage is mediated in large part by the deposition of these CICs, which is represents a type III hypersensitivity reaction (see Chapter 6, Part 4.3). As part of the pathogenesis, there is also proliferation CD4+ T helper cells. So one can see that a pure type II hypersensitivity response is not typically the case in anti-GBM disease.

Histopathology reveals crescentic and/or rapidly progressive glomerulonephritis, which has a fatal outcome in about half of the patients (Lahmer and Heemann 2012) The pathogenesis for lesion development in anti-GBM disease is discussed in more detail later, but it begins by immune complexes activating complement. Briefly, complement activation spawns C3a and C5a anaphylatoxins that lead to recruitment and adhesion of neutrophils and macrophages. Release of cytokines and lysosomal enzymes from these granulocytes causes endothelial damage, and breakdown of GBM leading to leakage of plasma proteins into Bowman's space, and crescent formation (Olson et al. 2011). There is mesangial cell proliferation, and infiltration of T cells, macrophages and neutrophils into the glomerulus. Some novel therapies being investigated is suppression of T cell proliferation via blockade of CD28-B7 (the co-stimulatory pathway for T cell activation).

In addition to Mercury Chloride induced nephropathy (discussed above under Chapter 6, Part 4.1), the experimental model for anti GBM disease is **experimental allergic glomerulonephritis (EAG)**. In the most simple and pure sense of the model, it is induced by immunizing mice or rats with non-collagenous α3 chain of type IV collagen from rats (Hopfer et al. 2003; Meyers et al. 2002; Kalluri et al. 1997). This is also known as Goodpasture's antigen (Saus et al. 1988). The Wystar-Kyoto strain of rat is used almost exclusively for the animal model, because in this strain, the disease is characterized by circulating anti-GBM antibodies, the same renal pathology (a rapidly progressive glomerulonephritis with crescents), and pulmonary involvement (Sado et al. 1998). The injection of the homologous Goodpasture's antigen "immunizes" the animal, and the animal responds with a proliferation of antigen-specific T cells and B cells in lymph nodes, with production

of antibodies against the injected antigen. This antibody reacts with the endogenous collagen in the GBM, and this deposition helps diagnose the disease. When there is formation of *in situ* immune complexes or CICs, then complement gets fixed and the glomerular lesions develops. There is proliferative glomerulonephritis with necrosis and crescent formation (Reynolds et al. 1998). Evidently, the injection of the auto-antigen overpowers the normal tolerogenic DC and tolerogenic T regs, and homeostasis breaks down.

Other animal models of anti-GBM disease are a bit more complicated than simply injecting the Goodpasture's antigen into mice or rats. The more complicated models start with first inducing autoantibody production to the antigen in a heterologous rabbit, then injecting that heterologous rabbit anti-GBM antibody into a rat or mouse. The rabbit anti-GBM antibody immediately binds to the rat or mouse GBM or TBM during this initial "autologous" phase. Then the rat or mouse starts to mount an antibody response to the injected heterologous rabbit immunoglobulin, and in about 5–7 days, the anti-rabbit antibody either binds to the rabbit anti-GBM antibody already deposited in the basement membranes or forms immune complexes with any circulating rabbit antibody. Circulating immune complexes then may deposit in the GBM or in the mesangium. This second delayed phase of the disease is called the heterologous phase and since it involves deposition of CIC, it can be associated with more advanced and rapidly progressive disease. This is not unlike the fact that deposition of CICs in anti-GBM disease in people instigates the more advancing and progressive lesions.

Since the anti-GBM disease is profound and aggressive; since antibodies alone cannot induce the experimental disease; and since T cells and macrophages are present in the interstitium, Type II hypersensitivity or type III hypersensitivity (due to CIC deposition) might not be the only pathogenesis involved in lesion development (Kalluri et al. 1997; Tipping et al. 1985). There is suggestion that type IV hypersensitivity, with activity by CD8+ cytotoxic T cells, is also involved (Reynolds et al. 1993, 2002; Tipping and Holdsworth 2006). A role for Th17 helper cells has been shown to play a role in producing IL-17 and recruiting damaging neutrophils to the site of injury in the glomerulus in crescentic glomerulonephritis (which is often the presentation of anti-GBM disease) (Turner et al. 2012). Type V hypersensitivity, with insufficient function of T regs and loss of tolerance to GBM antigens, might be important in disease progression as well. It has been shown that nasal application of antigen or oral feeding of GBM antigen to animals with EAG, there is regression of kidney lesions. This is likely because these applications boosted immune tolerance by T-regs (Reynolds and Pusey 2001; Reynolds et al. 2005).

Antenatal Membranous Nephropathy. In humans, there is another renal disease that results from a type II hypersensitivity reaction to endogenous antigen in the glomerular basement membrane. However, in this disease the antibody response is maternal and the endogenous antigens are on the fetal podocytes *in utero.* The inciting antigen is neutral endopeptidase (NEP). This entity called antenatal membranous nephropathy results from maternal antibodies that cross the placenta and bind to fetal glomerular podocytes mediating renal disease in the fetus (Debiec et al. 2002; Ronco et al. 2006). NEP first appears in S-shaped fetal nephrons and persists

on podocytes of the mature kidney. NEP can also be present on syncytiotrophoblasts (Debiec et al. 2002). The NEP immunizes the mother who builds up antibodies against it. The anti-NEP antibodies produced by the mother get transferred to the fetus in the last trimester of pregnancy and if the antibody is IgG1, that antibody deposition results in membranous nephropathy in the fetus (Debiec et al. 2002; Ronco et al. 2006). From about 18 weeks of gestation onward, maternal antibodies are actively transported across the placental to the fetus normally. The mothers that form NEP antibodies are those individuals who have a mutation of the membrane metallo-endopeptidase (MME) gene (Debiec et al. 2002), and therefore do not express NEP themselves. This alloimmune disease is assuredly underreported, since maternal IgG in the neonate has a short half-life and by the time a neonate presents with renal disease, the maternal IgG is no longer present. It is suspected that antenatal and perinatal nephron loss that results from this form of membranous nephropathy may be one of the causes of chronic renal failure later in life (Nortier et al. 2006).

Membranous Nephropathy (MN). Another endogenous autoantigen has been implicated in type II hypersensitivity renal disease. This condition is called autoimmune membranous nephropathy, and the target antigen is phospholipase A2 receptor (PLA2R), which is an integral membrane glycoprotein of podocytes (Glassock 2012). The disease is caused by autoantibody to PLA2R binding to PLA2R and forming subepithelial deposits that activate complement (Fig. 6.5). There are genetic variants in people to explain why they suddenly mount autoantibody to PLA2R on the surface of podocytes in membranous nephropathy. A combination of triggering gene variants determine not only whether disease is initiated but also the rate of progression. For example, preferential production of immunoglobulin subtype IgG4 is associated with increased rate of progression of disease. This correlation results from the fact that IgG4 is associated with higher levels of plasminogen activator inhibitor type 1 (PAI1) and higher PAI1 levels results in more extensive fibrosis. In addition to PLA2R, other antigens have been implicated in autoimmune membranous nephropathy in humans including lecithin cholesterol acetyltransferase (LCAT; an enzyme involved in maintaining cholesterol homeostasis) (Takahashi et al. 2013), and gp330 found in the coated pits on proximal tubular epithelial cells and podocytes (Debiec et al. 2002; Salant et al. 1980; Cybulsky et al. 1988; Andres et al. 1986).

Membranous nephropathy is histologically characterized by distortion and thickening of the GBM. Ultrastructurally, there are subepithelial immune deposits, effacement and displacement of foot processes, and microvillus transformation of the podocyte (Cybulsky et al. 1988).

Experimental Heymann nephritis. Experimental Heymann nephritis is a way to produce a membranous glomerulonephritis in rats or mice. There are two forms of the experimental model: Active and Passive. Active Heymann is induced by immunizing rats or mice with autologous renal cortical homogenate (Fx1A, which is megalin and glycolipids) resulting in autoantibodies that bind to antigen on the surface of podocytes resulting in immune complexes in the subepithelial locations (Cavallo 1994; Couser and Nangaku 2006). Creating a Passive Heymann model is a

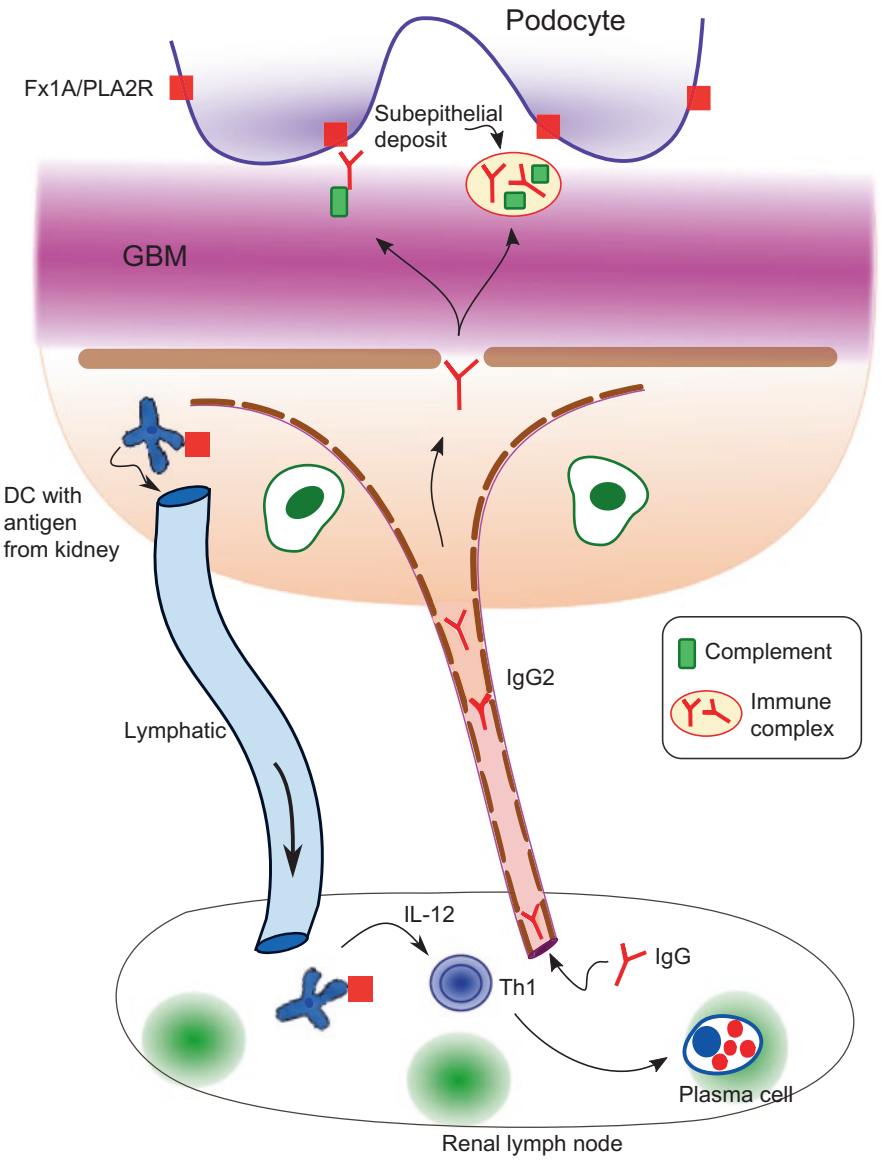

Fig. 6.5 Type II hypersensitivity. Dendritic cells (DC) pick up a kidney autoantigen on the surface of podocytes, such as phospholipase A2 receptor (PLA2R) in man or Fx1A in rodents. DCs take this antigen to the renal lymph node, where IL-12 is produced. The IL-12 stimulates the Th1 phenotype of T cells. The Th1 cells (and the associated IFN-γ-rich environment) encourage production of IgG by B cells or plasma cells. The IgG circulates to the kidney and binds to the autoantigen on the surface of podocytes and/or forms electron dense immune deposits in the sub-epithelial region. Complement fixes to the Fc portion of bound antibody, and results in leukocyte-independent complement-mediated destruction. (Schematic by Cynthia Swanson)

bit more complicated and entails first producing antibodies in sheep against rat Fx1A antigens. Then these sheep antibodies are injected into the rat, and the antibodies plant in the subepithelial location (Susani et al. 1994). During the first autologous phase, when the injected antibody binds to megalin on the podocyte, the reaction could be classified as a type II mediated hypersensitivity. There is a second phase, i.e., the heterologous phase, where the rat mounts an antibody response to the injected sheep antibody. This results in formation of circulating immune complexes and/or the *in situ* formation of immune complexes. The heterologous phase is classified as a type III hypersensitivity reaction (Couser and Nangaku 2006). It is important to point out that the initial autologous phase of passive Heymann nephritis does not mimic human spontaneous membranous nephropathy, because sheep IgG cannot activate rodent complement. As with membranous nephropathy in man, there is some indication that delayed type hypersensitivity, and in particular activation of intraglomerular cytotoxic T cells, are involved in the pathogenesis of experimental Heymann nephritis (Spicer et al. 2001; Walters et al. 2001).

Heymann nephritis may occur spontaneously in rats (Heymann et al. 2009; Van Damme et al. 1978; Couser and Nangaku 2006). It is characterized by subepithelial formation of immune complexes directed against a podocyte membrane protein megalin (Kerjaschki and Farquhar 1982, 1983). Megalin is expressed in human podocytes, but has not been associated with human membranous nephropathy (Debiec et al. 2002).

Membranous nephropathy has been associated with repeated chronic injections of soluble antigens or biopharmaceutical products in monkeys, with subepithelial immune-complexes (IgG and C3), spikes on silver stains (Kahn et al. 2002).

6.4.3 Type III Hypersensitivity: Immune Complex Glomerulonephritis; Lupus Nephritis; IgA Nephropathy

Type III immune complex glomerulonephritis is the deposition of circulating immune complexes in the GBM, or it may be due to *in situ* formation of immune complexes when foreign antigen is first planted in the GBM. The pathogenesis is as follows: CD4+ T cells stimulate B cells to produce antibodies against circulating antigens; large immune complexes form within the circulation; these large immune complexes precipitate in glomerular capillary walls (subendothelial or mesangial, in general); complement binds to the Fc receptors, and triggers the damaging complement cascade. The main disease conditions in man that represent type III immune complex glomerulonephritis are systemic lupus erythematosus (SLE or lupus nephritis), and the heterologous phase of experimental Heymann nephritis (discussed in Chapter 6, Part 4.2).

Lupus Nephritis. The immunopathology of lupus nephritis is based on an aberrant innate and adaptive immune response against many nuclear autoantigens, including double stranded DNA (dsDNA) (Fig. 6.6). A complex combination of

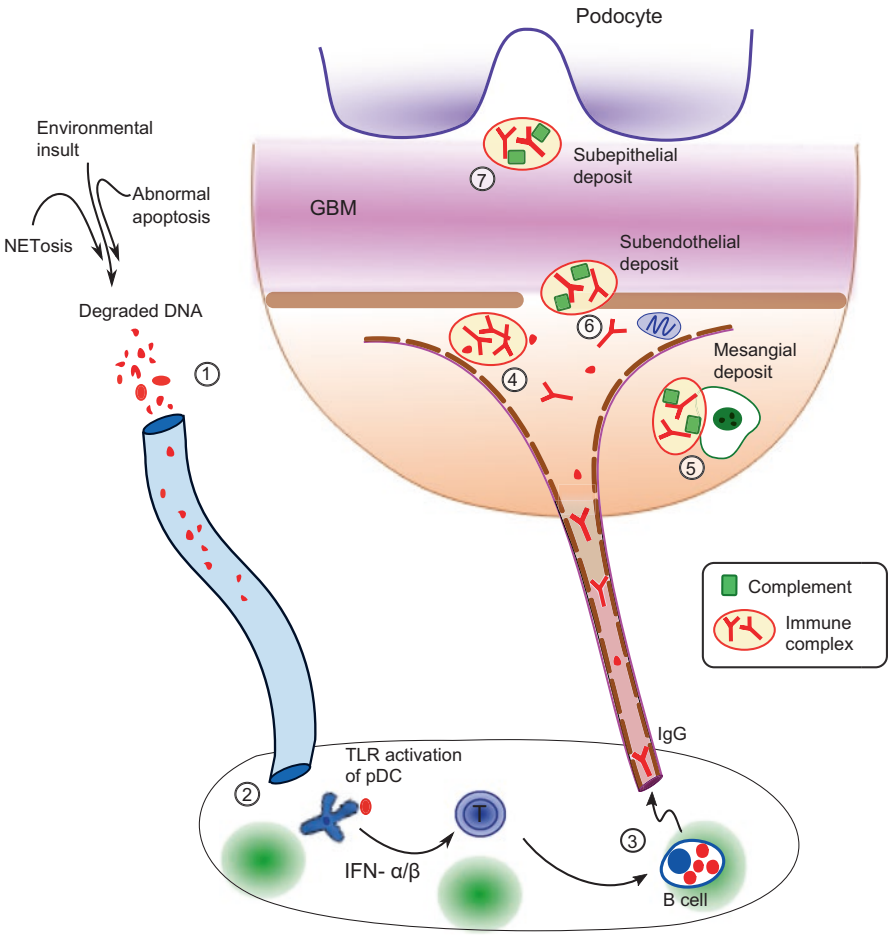

Fig. 6.6 Type III hypersensitivity. This schematic shows the pathogenesis of systemic lupus erythematosus (SLE), as an example of a Type III hypersensitivity disease resulting from deposition of immune complexes in the glomerulus. In SLE, there is excess cell destruction with release of DNA (*1*). This occurs either because of genetic variation (that may result in abnormal apoptosis or excess NETosis of neutrophils) or by injury from environmental factors. The DNA degrades and loses methylation (which otherwise prevents it from activating toll-like receptors (TLRs) on dendritic cells). Degraded un-methylated DNA activates TLRs 7 and 9 on DCs (*2*). When plasmacytoid DCs (which are a subtype of DCs rich with TLR 7 and 9) are activated and the negative feedback loop on IFN production is short-circuited, excess IFNα and IFNβ is produced. This IFN-rich environment stimulates T helper and B cells, leading eventually to autoantibody production (*3*). The antibody enters the circulation and binds to the circulating dsDNA, forming circulating immune complexes (*4*). These large immune complexes deposit in the mesangium first (*5*), and then in the subendothelial (*6*) and subepithelial (*7*) locations, in that order. The site of immune complex deposition will dictate the nature of the injury and light microscopic presentation. These complexes trigger the complement cascade, which damages podocytes and causes an influx of inflammatory cells. (Schematic by Cynthia Swanson)

genetic variants ultimately promote loss of tolerance for nuclear autoantigens. Just like there are genetic variants in people to explain why they suddenly mount autoantibody to PLA2R or gp330 on the surface of podocytes in membranous nephropathy, so too there is a combination of unfortunate genetic variations that cause people to initially mount an immune response to dsDNA in systemic lupus erythematosus (SLE). Other genetic variants that facilitate production of autoreactive T and B cell clones control the rate of disease progression. The unique combination of genetic risk factors will determine onset of disease and pattern of disease progression (Anders and Schlondorff 2000).

One genetic aberration that incites SLE is an aberration in apoptosis. Normally, nuclear particles are confined by cell membrane following apoptotic cell death and this cell membrane prevents nuclear material from being exposed to the immune system. However, in SLE, there are some genetic variations in apoptotic mechanisms that allow such exposure of nuclear material to the immune system (Saxena et al. 2011; Migliorini and Anders 2012; Hakkim et al. 2010; Garcia-Romo et al. 2011). Also, some people with SLE have a complement deficiency that prevents opsonization of apoptotic cells thus allowing an abundance of apoptotic bodies to loiter increasing the likelihood of exposing dsDNA to the immune system (Munoz et al. 2010). Abnormal exposure of nuclear material to the immune system can also be due to a dysregulation of NETosis. As part of the innate immune system, NETosis is a normal process involving massive suicide by neutrophils, whereby microbials are killed in its wake. NET stands for a "neutrophil extracellular trap" (Yu and Su 2013). When the neutrophil commits suicide, it forms an extracellular "net" of chromatin, ROS, histones, and other proteins that ensnares and kills microbes. Abnormal or excessive NETosis has been found in mice and people with SLE. As with most immunologic mechanisms, too much of it can be damaging, because the nets are composed of nuclear material. Excess NETosis not only provides antigenic fodder for SLE, but it triggers thrombosis, causing vascular endothelial damage. Damaged endothelial cells provide yet additional source of nuclear autoantigen.

Aside from the genetic risk factors that expose DNA to the immune system (Cui et al. 2013), there are environmental risk factors that affect onset and progression of SLE. Infections, hormones, UV light, and drugs, can result in massive cell death, exposure of the immune system to large quantities of dsDNA, and an expansion of autoreactive lymphocyte clones (Anders and Schlondorff 2000). Regardless of the genetic or environmental cause, these changes collectively result in the production and persistence of nuclear material in the extracellular space and antibody response thereto.

Normally, there is self-tolerance to one's DNA. However, the persistence of exposed nuclear antigens lead to loss of self-tolerance. When there is a delay in clearance of dead cells or their apoptotic bodies, nucleic acid in these cells degrades and these degraded particles (often with lost methylation) can activate toll-like receptors (TLRs) on dendritic cells or macrophages (i.e., the innate immune system) (Bosch 2011; Munoz et al. 2010; Guiducci et al. 2010; Patole et al. 2005; Pawar et al. 2007), thus stimulating the innate immune system. Under normal circumstances, methylation of RNA and DNA prevents the nuclei acid from being

recognized by toll-like receptors (TLRs) 3, 7, and 9. But when nuclear particles degrade, their methylation status changes, and they can turn into endogenous immune adjuvants (Kariko et al. 2004). The binding of these degraded DNA/RNA to TLRs induce expression of costimulatory molecules which shifts the process of presented autoantigens from tolerance to immune activation of T and B effector cells (Zikherman et al. 2012). Activation of the TLR 7 and TLR 9 helps perpetuate the disease since signaling of these toll-like receptors prolongs the life span of the activated dendritic cells by making them resistant to glucocorticoid-induced death (Guiducci et al. 2010).

One of the reasons why TLR activation causes a switch from tolerance to immune activation may be related to abnormally high levels of IFNα and IFNβ. Under healthy conditions, DCs (without costimulatory molecules) normally capture apoptotic bodies and present autoantigens to autoreactive lymphocytes, leading to apoptosis of B cells, CD4+ T cells and CD8+ T cells in a way to maintain tolerance. In SLE, however, activation of TLR 7 and 9 receptors on the surface of plasmacytoid DCs or endothelial cells result in abnormally high production of IFNα and IFNβ. Viral infections in particular activate TLR 7 and 9 on the surface of plasmacytoid DCS (pDC). Regardless of how TLR 7 or 9 is activated, the resulting interferon upregulates costimulatory molecules that trigger expansion of autoreactive B and T cells (rather than clonal deletion of these effector cells) (Banchereau and Pascual 2006). Autoreactive B cells generate autoantibody to nucleic acids and form immune complexes. Immune complexes perpetuate the disease since immune complexes provide sustained activation of TLR 7 and 9 (Barrat et al. 2005; Vollmer et al. 2005) and trigger production of more IFNα and IFNβ from endothelial cells.

While viral infections or excess NETosis may create non-tolerogenic dendritic cells, there are many additional genetic alterations that might lead to sustained overproduction of IFNα and IFNβ, such as a deficiency in the normal feedback between IFN levels and pDC production. Normally healthy pDCs only secrete IFN for a few hours, and then they produce TNF which shuts down IFN production by an autocrine feedback mechanism (Hanada et al. 2003). In SLE, this autocrine feedback might not work because of increased amounts of soluble TNF receptors that bind up all the circulating TNF, thus short-circuiting the feedback loop (Gill et al. 2002; Palucka et al. 2005).

The importance of knowing the scope of possible triggers or potentiators for SLE has implications for toxicologic pathologist. While SLE is not a spontaneous disease commonly encountered in laboratory animals, test articles could possibly cause increased apoptosis of cell types, deficiency of opsonization, massive cell death, excess NETosis, activation of TLR 7 and TLR 9, increased production of IFN, and/or loss of self-tolerance to dsDNA, leading to type III hypersensitivity reactions. After all drugs are one of the triggering events for SLE in man.

Autoantibodies that result from B cell proliferation can form circulating immune complexes, or the autoantibodies may bind to intrarenal nuclear autoantigens and form complexes *in situ*. The immune complexes predominantly locate in the mesangium (classes I and II lupus nephritis), but can lodge in the subendothelial region (classes III and IV lupus nephritis), and also in the subepithelial locations (class V

lupus nephritis). Regardless where they lodge, the complexes activate complement. As will be described in more detail later in the chapter, complement activation results in podocyte loss, proliferation of mesangial cells, proteinuria and persistent interstitial inflammation, glomerulosclerosis and interstitial fibrosis, and ultimately progressive chronic kidney disease (CKD) and end stage renal disease.

There are many histologic presentations of lupus nephritis, including glomerulosclerosis, or a proliferative, mesangioproliferative, membranous or crescentic glomerulonephritis (Weening et al. 2004). The histologic lesion can be focal or diffuse (<50% or > 50% of glomeruli in the kidney section, respectively), and segmental or global (<50% or >50% of each individual glomerular tuft, respectively). Light microscopic changes include increased matrix, mesangial cell proliferation, capillary endothelial cell proliferation, thickening of capillary walls (wire loop effect due to rigid thickening of capillary loops due to subendothelial deposits), glomerular tuft necrosis, fibro cellular crescents, karyorrhexis, hyaline thrombi (which are actually intravascular immune complexes), and glomerular sclerosis. While hyaline thrombi and wire loop lesions are typical of SLE, the only change considered pathognomonic of lupus is the hematoxylin body. The hematoxylin body is a round, smudgy lilac-staining structure within the tuft that represents degenerated and engulfed nuclear material. This smudge cell corresponds to the LE cells described in the blood of patients with lupus.

There has been considerable increase in understanding of how SLE triggers the different histologic presentations or classes of lupus nephritis, and how autoimmune and non-autoimmune factors promote the progression to end stage kidney disease (Anders and Schlondorff 2000). This understanding has come from the use of animal models of lupus nephritis. The lesion of lupus nephritis is dependent on T cells, and this is a common feature of all lupus models in MPR/lpr and NZB and NZB mice. MRL/lpr mice with B cells unable to secrete antibodies still develop lupus (Chan et al. 1999), and those mice with a genetic deficiency of the B cell activating factor BAFF do not have attenuated disease (Jacob et al. 2006). Therefore, SLE is not only a type III hypersensitivitiy reaction, but also represents a type IV delayed hypersensitivity and perhaps a deficiency of regulatory T cells. The disease typically involves all three classes of immunoglobulins and activation of both the classic and alternate complement pathways in its pathogenesis.

There are six classes of glomerular lesions as follows, and the different light microscopic presentations depend on whether the complexes deposit in the mesangium, the subendothelium or the subepithelium (Weening et al. 2004).

Class I and Class II SLE is referred to as “Minimal Mesangial” and “Mesangial” lupus nephritis, respectively. In this early stage of disease, the immune complexes are limited to the mesangium. Microscopically, mesangial lupus nephritis may or may not have mesangial cell proliferation. All immunoglobulins isotypes have access to mesangium including IgA and IgM an IgG. This is because the mesangium is not separated by a GBM from the intravascular compartment. The immunoglobulin merely has to pass through the fenestrated glomerular capillary endothelial cells to reach the mesangium (Nowling and Gilkeson 2011; Yung et al. 2010). Immune complexes generally form *in situ*. Cross reactivity of the antibody to α –

actinin or annexin II on mesangial cells might facilitate the location of immune complexes in the mesangium (Yung et al. 2010; Zhao et al. 2006). Once formed, both the FcR and the nuclei acid component of the deposit trigger injury and signal the intraglomerular macrophage and dendritic cells to produce IFNα and other pro-inflammatory cytokines (Anders and Schlondorff 2000). Depending on the capacity of the deposits to trigger cell injury and inflammation, the deposition will cause no (class I), mild or massive (class II) mesangial proliferation (see Fig. 6.12).

Class III SLE is called Focal SLE and Class IV SLE is called Diffuse SLE. In these classes III and IV, immune complexes are located in the mesangium but also in the subendothelial space. In addition to the mesangial proliferation (as seen in Class II SLE), there may be necrotizing or sclerosing lesions. The close proximity of the immune deposit to the endothelial cell causes endothelial cell activation by the same complement, Fc-receptor and nuclei acid sensor-related mechanisms as in Types I and II. This triggers release of IFNα by endothelial cells, and results in formation of characteristic tubuloreticular inclusions in the glomerular endothelial cells. TRIs are characteristic ultrastructural findings that indicate a reaction to excess IFNα or IFNβ signaling (Fairhurst et al. 2009; Venkataseshan et al. 1991; Klippel et al. 1985; Rich 1981). Tubuloreticular inclusions are not pathognomonic for SLE, and can be seen in idiopathic glomerulonephritis (Yang et al. 2009). TRIs are anastomosing tubule like structures within endoplasmic reticulum and provide evidence that the cell has been acted upon by alpha or beta interferon. With large amounts of subendothelial deposits, the capillary walls are thickened and form a thick wire-loop at the edge of the glomerular tufts.

Class V SLE is called Membranous SLE, and immune complexes are subepithelial, mesangial and subendothelial. This is the most common and severe form of lupus and can be global or segmental (affecting <50% of the cortex). The segmental presentation of class V surprisingly carries a worse prognosis, yet has fewer immune complex deposits than the global lesion. This seems counterintuitive. However, the segmental distribution of the lesion indicates that the lesion is driven mostly by a vasculitis rather than by the bulk deposition of intraglomerular immune complexes. Since vasculitis and leaky capillaries are at the root of its pathogenesis, the segmental SLE is associated with more necrosis and more crescent formation. This segmental and most severe form of lupus nephritis (Class V) is more similar to ANCA (anti-neutrophil cytoplasmic autoantibodies) -associated glomerulonephritis or polyangiitis, which is an autoimmune disease that affects small and medium-sized blood vessels. With subepithelial immune deposits, there is proliferation of GBM around the deposits and formation of spikes of basement membrane. These spikes are visualized with silver stains.

Class VI SLE is called “Advanced Sclerosing” lupus nephritis. The glomerulosclerosis within the tuft in this form of disease occurs because there is podocyte loss with insufficient epithelial regeneration. In the tuft, scarring occurs upon the loss of 30% or more of the podocytes (Wharram et al. 2005). The advanced sclerosing lupus nephritis, while having started as a type III hypersensitivity, also involves the Th1 cells, making it also a form of DTH.

The pathogenesis of the damage/lesions in the glomerular tuft attributable to immune complexes are described in more detail below, since the proliferative and degenerative changes are non-specific once immune activation occurs.

Interstitial nephritis is additionally noted in many forms of SLE, and the inflammatory infiltrate is generally composed of lymphocytes, macrophages and plasma cells. Interstitial inflammation commonly adjacent to crescents, especially when the lesion involves Bowman's capsule. The reason for the tubulo-interstitial inflammation can be attributed in part to the leakage of protein/albumin through the GBM. Albumin activates and upregulates inflammasomes (such as NLRP3) in renal tubular epithelium; and stimulates the proximal tubular cells to synthesize chemokines (MCP-1 and RANTES) that recruit macrophages and T cells into the interstitium; which in turn produce chemokines that attract neutrophils. Macrophages and neutrophils produce reactive oxygen species that further damage tubular epithelial cells. Tubular atrophy and dilatation results. The pathogenesis of immune mediated tubulo-interstitial disease is covered in a later section (see Chapter 6, Part 6.).

NZB/NZW F1 and MRL/lpr mice: Several murine models are employed to study lupus nephritis, such as MRL/lpr mice or NZM mice (Foster et al. 2007). NZB NZW F1 hybrids develop a spontaneous acute phase of SLE, when the kidneys are café au lait or dusky pink and enlarged and the capsule is non-adherent. Later in the chronic course of disease the kidneys are shrunken, the capsule is adherent, and a granular pitted surface with small cysts and petechiae are present in the cortex on cut surface. As in man, the earliest lesion are mild endothelial and mesangial cell proliferation. With more advanced disease there is subepithelial deposition of PAS positive immune complexes. Eventually, there is fibrosis and collagen formation. Active lesions have fibrinoid necrosis of small vessels. In chronic, cases, thickened glomerular basement membrane with occlusion of capillary loops is extensive. Epithelial crescents, fusion of epithelial foot processes, and accumulation of lymphocyte and plasma cells in the interstitium occur. The antigen in the NZB/W mice is DNA, and the immunoglobulin subtype that forms complexes in the endothelium and mesangium is generally IgG2a. In the NZB and in the NZB and NZB/W F1 hybrids, the antibody is also against antigens of the Gross Leukemia Virus (Andrews et al. 1979).

Non-specific Type III Immune Complex Glomerulonephritis. Immune complexes to antigens other than dsDNA can occur. This non-specific deposition of immune complexes in the GBM is referred descriptively as crescentic glomerulonephritis in man, and the lesion is proliferative, sclerosing and crescentic when there are mesangial, subendothelial and subepithelial deposits. The lesion is not unlike class V SLE.

The experimental animal model for non-specific immune complex glomerulonephritis is referred to as **Nephrotoxic Serum Nephritis (NTN)** or Masugi nephritis (Tipping and Holdsworth 2006). This model is produced by first vaccinating sheep or rabbits with antigen (such as homogenized murine kidney cortex), and then injecting the antibody-laden ("toxic") serum into mice or rats. In the mice or rats, there are two stages of the disease. In the initial phase (i.e., the autologous phase),

the sheep or rabbit heterologous antibody hones in on the antigens in the kidney cortex and form *in situ* immune complexes. During this autologous phase, there are immune complexes that deposit, along with proteinuria, but there is little to no histopathology. The second phase (i.e., the heterologous phase) occurs later because it takes some time for the mouse or rat to mount antibodies to the injected heterologous immunoglobulin. Once an antibody response is mounted, the mouse antibodies bind to the sheep immunoglobulin and forms immune complexes with exposed Fc-receptors. These complexes lodge in the glomeruli. Rather than have two discrete phases to a disease process, some investigators have come up with an "accelerated" NTN model. In the accelerated model, the mouse is initially vaccinated with sheep IgG. In this way, when the nephrotoxic serum (containing sheep IgG specific for murine kidney cortex) is first injected into the mouse, both the autologous and the heterologous phases occur simultaneously. No delay is required for the mouse to mount any response to the injected sheep IgG. The accelerated NTN model produces a more pronounced rapidly progressive disease state.

The terminology can be confusing when discussing the names for the animal models of renal disease. It should be noted that in the NTN model (non-accelerated), the autologous phase is nothing more than anti-GBM disease if GBM antigens are used to immunize sheep, or Heymann nephritis if Fx1A antigens are used to immunize sheep. In some cases, publications on anti-GBM disease or Heymann nephritis will refer to the animal model in the broad sense as NTN, and this should not confuse the reader.

In either event, and regardless of the antigen used to produce the model, the Fc receptor of the murine IgG binds to Fc receptors on immune effector cells, including macrophages, DC, T cells, B cells, NK cells and mast cells, which promote inflammation. There is cross linking of receptors, degranulation, superoxide generation, cytokine release, phagocytosis and antibody-dependent cellular cytotoxicity by NK cells. Neutrophil aggregation, thrombi formation, and endocapillary and mesangial cell proliferation are due to the activation of complement and coagulation cascades. An influx of CD8+ T cells and macrophages into the glomerulus perpetuate the disease (Tsuchiyama et al. 2000; Chen et al. 2002). Both Type III immune complex hypersensitivity and delayed type hypersensitivity (involving Th1 and CD8+ T cells) are involved (Fujii et al. 2003; Groggel and Terreros 1990). Glomerulosclerosis and crescents occur.

There are a host of publications regarding differences in disease progression and these differences are due to the type of IgG class, whether there is skewing of the IgG subclass, the type of Fc receptors, the affinity of the IgG class for both inhibitory and activating Fc Receptor, the amount of injected heterologous immunoglobulin that affects the induction of activating or inhibitory Fc receptors on effector cells, and the cytokine milieu. It is well known that IFNγ, TGFβ, and C5a upregulate activating Fc receptors (Kaneko et al. 2006; Shushakova et al. 2002; Ravetch 2002; Samuelsson et al. 2001), thereby creating a self-perpetuating inflammatory and destructive disease For a review of the immunoglobulin subclass, and its Fc receptors, the reader is referred to Delves et al. (2011).

In rabbits, type III immune complex nephritis can also be induced by injection of cationized **bovine serum albumin (BSA)** which embeds itself in the subepithelial region of the GBM and serves as a planted antigen (Border et al. 1982). The cationic BSA binds to anionic heparin sulfate proteoglycans. The IgG produced in response to this injected antigen forms subepithelial immune complexes triggering complement activation. This model has the advantage of varying the location of the immune complexes. If anionic or neutral BSA is injected, mesangial deposits result (Koyama et al. 1986a). This model is also established in dogs, mice and rats (Koyama et al. 1986b; Kobayashi et al. 1998).

IGA Nephropathy. Human IgA Nephropathy (IgAN) is the most common primary chronic glomerulonephritis in Japanese people, and 40–50% of cases result in end stage renal dysfunction within 20 years of onset (Floege et al. 2014). Most persons with IgAN do not show clinical signs, and most run a benign course that spontaneously resolves. IgA nephropathy (IgAN) is not like other immune-mediated diseases. While its pathogenesis involves antibody deposition within the glomerular basement membrane, these deposits are not conventional antigen-antibody complexes. However, these deposits nonetheless instigate a type III hypersensitivity disease response. The deposits are a four-way complex involving abnormal polymeric IgA and the transferrin receptor on the surface of mesangial cells (Fig. 6.7). First of all the polymeric IgA is abnormally glycosylated at the IgA1 sub fraction, and because of this abnormal "under" glycosylation (that is, the IgA1 sub fraction is galactose-deficient), it forms immune complexes with soluble CD89 (sCD89). CD89 is the Fc- receptor for IgA. This complex then binds avidly to the transferrin receptor (TfR1) on the surface of mesangial cells (Gharavi et al. 2008; Monteiro 2005; Novak et al. 2008; Daha and van Kooten 2013). The TfR1-CD89-IgA1 complexes then induces transglutaminase 2 (TGase2) expression on the surface of mesangial cells and forms a four-way complex. The four-way complex of sIgA-CD89-TfR1-TGase2 on the surface of mesangial cells are necessary for the full blown clinical disease, as this four way complex induces mesangial cell proliferation, expansion and remodeling of the extracellular matrix, activation of complement, secretion of pro-inflammatory cytokines, crescent formation, glomerulosclerosis, tubulo-interstitial fibrosis, and proteinuria (Working Group of the International Ig et al. 2009). Histologically, IgA nephropathy is a mesangioproliferative glomerulonephritis. The disease can snow-ball since there is self-perpetuation or auto-amplification of the complex deposition: the TfR1-CD89-IgA induces TGase2 expression, and TGase2 increases the affinity of IgA1-sCD89 to TfR1 (Daha and van Kooten 2013; Berthelot et al. 2012). If fewer than these four components are present, the complexes may still deposit in the mesangium, but the progression of disease will be attenuated (Gharavi et al. 2008).

Complement activation is quite important in lesion development in IgAN (Roos et al. 2001). While normal polymeric IgA can readily activate complement by either the alternate or lectin pathways (Hiemstra et al. 1987; Rits et al. 1988), the under-glycosylated O-linked sugar chain of the abnormal IgA activates complement by the

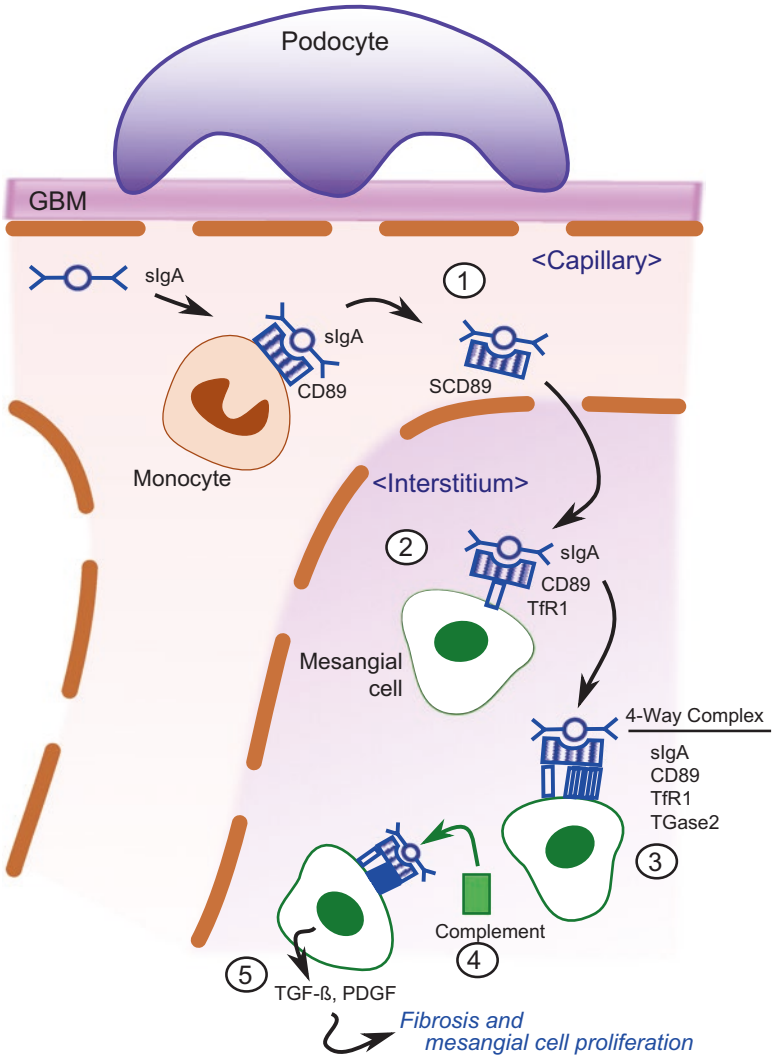

Fig. 6.7 IgA nephropathy. Schematic model of molecular interactions involved in the deposition of IgA1 in IgA nephropathy. IgA1 produced by B cells is under-glycosylated and can interact with CD89 on monocytes. This interaction results in the release of soluble CD89 molecules and sCD89-IgA1 complexes form in the circulation (*1*). These two-way complexes travel from circulation to the kidney and can interact with transferrin receptor (TfR1) on the surface of mesangial cells (*2*). This interaction induces TGase2 expression which binds sCD89-IgA1-TfR1 (*3*). The combined action of these four components (sCD89, IgA1, TfR1 and TGase2) result in an amplification of binding as well as an increased production of inflammatory mediators and platelet-derived growth factor, resulting in mesangial cell proliferation. The hinge region of the IgA1 activates the lectin complement pathway (*4*), which results in production of inflammatory mediators and growth factors, such as PDGF and TGFβ. TGFβ generally promotes fibrosis, while PDGF causes mesangial cell proliferation. (Schematic by Cynthia Swanson)

lectin pathway (Malhotra et al. 1995; Matsuda et al. 1998; Roos et al. 2001; Endo et al. 1998). Complement can also be activated by the classical complement pathway if co-deposited IgG happens to bind to the under-glycosylated hinge region of IgA1 in the complex (Tomana et al. 1997; Suzuki et al. 2009).

As with lupus nephritis and membranous nephropathy, IgA nephropathy has a variety of clinical presentations and rates of progression due to genetic variants (Orth et al. 1998; Berthoux et al. 2011; Bonnet et al. 2001). There might be genetically-based variable expression of TfR1 on mesangial cells (Haddad et al. 2003; Moura et al. 2004) to explain the different clinical presentations and accentuations of IgAN. There also may be genetic variation that controls the rate of shedding of CD89 from circulating mononuclear cells. In patients with IgAN, there is increased shedding of CD89 from the surface of mononuclear cells into the circulation, and increased shedding will allow more IgA1-sCD89 complexes to form. The ratio of polymeric/monomeric IgA in the circulation is also genetically based, and those patients with a higher polymeric/monomeric ratio are at greater risk for clinically severe disease.

The pathogenesis of IgAN involves type III hypersensitivity reaction, but also involves cell mediated CD4+-Th1 and CD8+ cytotoxic T cell delayed type hypersensitivity reactions. The ddY mouse strain is an animal model for spontaneous IgA nephropathy (Kurts et al. 2007). Using this model, it was shown that depletion of CD4+ helper cells in these mice reduced glomerular IgA deposits without affecting circulating IgA levels, suggesting the CD4+ T cells controls, in part, the deposition of IgA (Ohmuro et al. 1994; Tomino et al. 1993). Others have shown that CD8+ cytotoxic T cells perpetuate disease, since depletion of CD8+ reduced renal disease (Shimamine et al. 1998). Clearly, the roles for the CD4+ and CD8+ cells need further investigation, but IgAN, at least in a couple of animal models, involves DTH hypersensitivity or cell-mediated immunity (Suzuki et al. 2007).

Animal Model of IgA nephropathy. Although most of the pathogenesis of IgAN has been worked out in animal models, finding the perfect animal model that has all the pathogenic factors similar to humans is difficult. The ddY strain of mouse develops a spontaneous early onset IGA nephropathy, where these is deposition of IgA and co-deposition of IgG, IgM and C3 (Imai et al. 1985). This strain is classified into three groups: (1) early onset at 20 weeks; (2) late onset at 40 weeks; and (3) quiescent. Selective intercrossing has established a novel strain in which 100% of animals develop severe early onset kidney disease resembling severe IgAN in humans (Okazaki et al. 2012). In addition there is a High IgA mouse exhibiting high serum level of IgA, and this strain was established by interbreeding ddY (Muso et al. 1996).

Rifai et al. (1979) published an animal model using mouse IgA hybrinoma model actively immunized with dinitrophenol conjugated to BSA. It resulted in mesangial IgA deposition and hematuria (Rifai et al. 1979). IgA nephropathy with deposits of IgA in the mesangium has also been induced in mice by feeding the mycotoxins

nivalenol or trichothecene (Hinoshita et al. 1997; Pestka et al. 1989). Evidently, the mycotoxins induce helper Th2 cells to produce cytokines that lead to overproduction of IgA. Recently the transgenic expression of LIGHT (a ligand for lymphotoxin beta receptor) in mice was shown to stimulate polymeric IgA overproduction which resulted in glomerular IgA deposits, interstitial inflammation and hematuria (Wang et al. 2003). These early experimental models (i.e., DNP-BSA, mycotoxins, LIGHT transgenic) were not very indicative of the human condition because mouse IgA differs from human IgA. Mice do not have a hinge region and it is this hinge region where the O-glycan abnormality occurs in man; mice do not have circulating IgA Fc receptor CD89; and mice have lower polymeric/monomeric IgA ratios than humans. Therefore a replica of the Gal-deficient polymeric IgA1 that bound to sCD89 was not feasible in the hybridoma model. However, a lot has transpired in IgAN model studies. Increase in aberrantly glycosylated polymeric IgA levels together with mesangial IgA deposits, hematuria, and proteinuria were observed in mice transgenic for the B cell activation factor of the TNF family (BAFF) (McCarthy et al. 2011). There is also mouse hybridomas producing an IgA rheumatoid factor that produce IgA allotypes bearing the O-glycan (Otani et al. 2012); A CD89 transgenic animal model that produced CD89 (Monteiro 2005; Kanamaru et al. 2007); and a transgenic mouse models that have a high polymeric/monomeric IgA ratio similar to humans has been developed (Coulon et al. 2011; Kurts et al. 2007). The point is that any comparison between IgA nephropathy in mice and in man must be careful to take into consideration differences in many of the factors that play a role in the pathogenesis of IgAN.

6.4.4 Type IV Hypersensitivity: Delayed-Type Hypersensitivity

There is no spontaneous or experimentally-induced glomerular disease that is recognized as being caused solely by a delayed type hypersensitivity reaction in man or animals. However DTH plays a role in lesion progression in many of the glomerular diseases mentioned above, such as IgA nephropathy, lupus nephritis, and membranous nephropathy (Clarke et al. 1983; Schatzmann et al. 1999; Tipping and Holdsworth 2006). Delayed type hypersensitivity reactions are typically those mediated by cells, not antibody. They are inflammatory conditions triggered by the activation of macrophages, T cells, and NK cells. The T cells are generally Th1 cells which produce pro-inflammatory cytokines such as IFNγ and TNFα. This is as opposed to Th2 cells that produce IL-4 and IL-10 and IL-13, and stimulate B cells to produce antibody (Schatzmann et al. 1999). In DTH, antigen planted in the kidney is presented to CD4+ T cells in the local renal lymph node by macrophages or dendritic cells (Kelley and Singer 1993). There is clonal proliferation of antigen-specific CD4+ Th1 cells and cytotoxic T (Tc) cells. Th1 cells enter the kidney interstitium and stimulate cellular immune effector cells such as inducing glomerular and/or interstitial macrophages to release reactive oxygen species and enzymes that injure glomerular tissue and the tissue of Bowman's capsule. This Th1- mediated

injury stimulates epithelial cell proliferation and fibrosis and the formation of crescents (Panzer et al. 2007; Radeke et al. 1994) (Fig. 6.8).

While DTH reactions classically involve Th1 cells and macrophages, DTH reactions can also involve CD8+ T cells. Clonal proliferation of autoreactive CD8+ cells cause cytotoxic damage (Bevan 2004). CD8+ cytotoxicity plays some role in lupus, since there is poorer prognosis of lupus nephritis when CD8+ T cells are detected in periglomerular infiltrates (Couzi et al. 2007; Kurts et al. 2007). Also when CD8+ cell are experimentally depleted from lupus animal models, there is lessening of disease progression (Kawasaki et al. 1992; Reynolds et al. 2002).

There are a few animal models of glomerular disease that represent *primarily* a DTH hypersensitivity reaction. One model is **anti-Thy 1.1** glomerulonephritis in mice or rats. This is a mesangioproliferative glomerulonephritis induced by injecting antibody specific for Thy 1.1 antigen which is an antigen present on the surface of normal mesangial cells (Harendza et al. 1999). The antibody binds to mesangial cells resulting in mesangiolysis in the initial stage of disease. At 3 days, there is temporary microvascular aneurysms or capillary ballooning (Oyanagi-Tanaka et al. 2001). After 3 days, there is compensatory proliferation of mesangial cells and sclerosis. Both CD4+ and CD8+ cells are present, and both appear to play a role in disease progression. The leukocytes secrete mediators (such as platelet- derived growth factor) that induce mesangial cell proliferation (Ikezumi et al. 2000, 2004; Yamamoto and Wilson 1987), and eventually TGFβ which halts mesangial cell proliferation but promotes deposition of extracellular matrix. Another model for DTH or cell-mediated immunity may be **focal segmental glomerulosclerosis (FSGS) following 5/6 nephrectomy** in the rat. A role of T cells (and therefore DTH) exists in this model based on limited experimentation (Hamar et al. 1999).

There exists a T cell mediated model of glomerular injury in transgenic mice, known as the **nephrin-OVA/HEL (NOH) model** (Heymann et al. 2009). This mouse expresses antigens ovalbumin and hen egg lysozyme in glomerular podocytes. The injury is produced by injecting CD8+ cytotoxic T lymphocytes and CD4+ T cells into the mice, resulting in periglomerular mononuclear infiltrates and inflammation of parietal epithelial cells. Eventually there is pronounced tubulointerstitial infiltration by mononuclear cells, focal tubular atrophy, intratubular protein casts and glomerulosclerosis. Much of the progression of the disease is due to DCs within the renal lymph nodes. Before the CD4+ T cells or the CD8+ T lymphocytes are injected into the model, the rDCs already took the glomerular antigen (OVA/HEL) to the renal lymph node and established a state of tolerance. These rDCs functioned in the expected manner. However, in addition to the tolerogenic rDCs, there is also a population of non-tolerogenic DC in the renal lymph node that politely remains quiescent yet perched for action. Therefore, when CD8+ cytotoxic and CD4+ helper T cells are injected, these non-tolerogenic DCs are suddenly stimulated into action and present this OVA/HEL antigen to the injected effector lymphocytes. These lymphocytes travel to the tubulo-interstitium where these cytotoxic lymphocytes and T helper cells incite inflammation (Heymann et al. 2009). This model has elucidated the important fact that under healthy and normal circum-

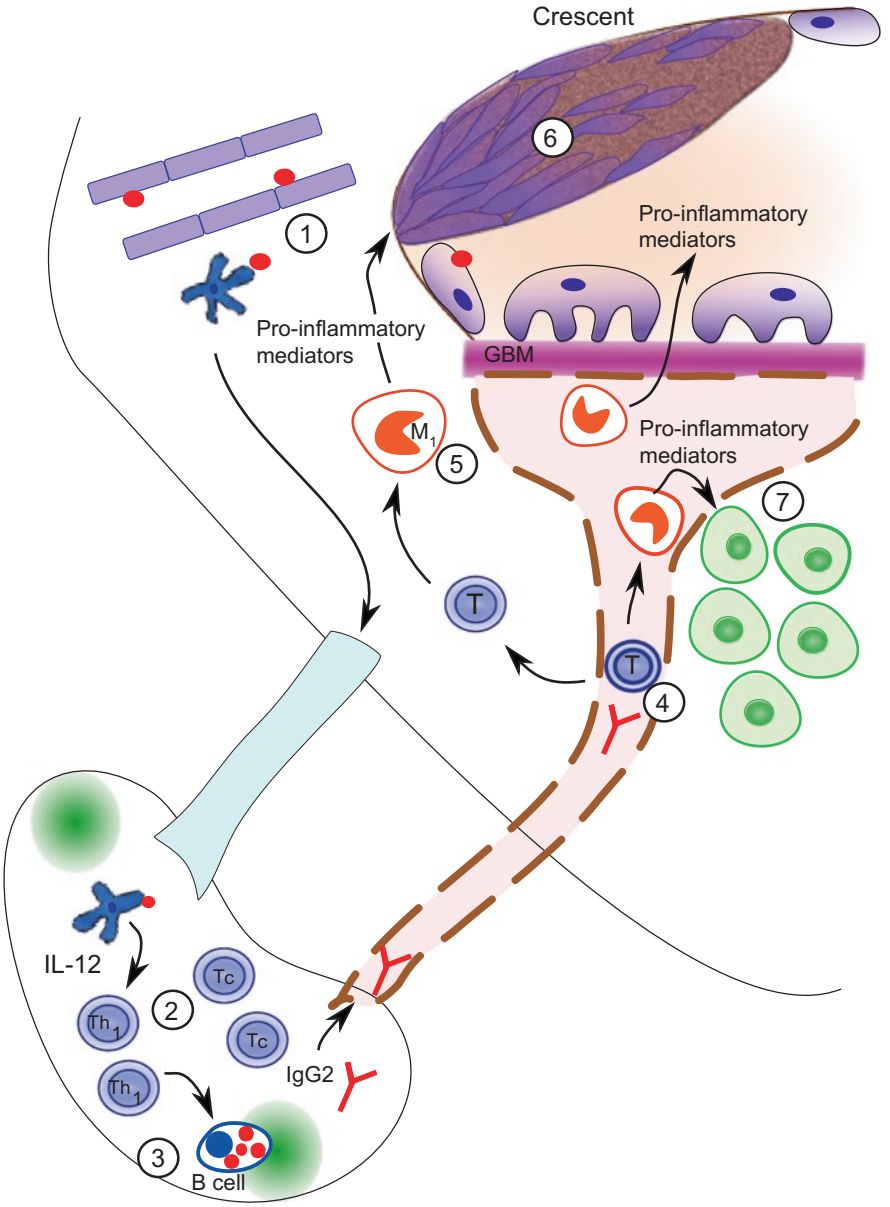

Fig. 6.8 Type IV hypersensitivity This schematic shows the pathogenesis of delayed-type hypersensitivity reactions in the glomerulus. Non-tolerogenic DCs pick up autoantigen (or foreign antigen) in the kidney (*1*) and take it to the local renal lymph node. DC present antigen to $CD4^+$ Th1 cells and/or $CD8^+$ cytotoxic T cells (Tc) resulting in proliferation of antigen-specific Th1 and Tc cell populations (*2*). In the meantime, some T cells stimulate B cells (or plasma cells) in that lymph node to produce antibody (*3*). Antigen-specific Th1 and Tc cells, and autoantibody, travels back to the kidney interstitium and glomerulus (*4*). When these effector Th1 cells meet antigen back in the kidney, they induce macrophages (M1) to produce pro-inflammatory cytokines such as ROS, IL-1

stances there are various phenotypes of renal lymph node dendritic cells that can cross-present renal autoantigen and either induce tolerance or inflammation.

6.4.5 *Type V Hypersensitivity: Loss of Regulatory T Cells*

Just like there was no single glomerular disease that is causes exclusively by a type IV hypersensitivity (i.e., DTH) response, there is no known renal disease that is caused exclusively by a deficiency in regulatory T cells. Regulatory T cells represent a subset of CD4+ cells that express CD25 and FoxP3, and their role is to suppress effector T cells (Bacchetta et al. 2005). They produce IL10 (which is immunosuppressive in general), arise in the thymus, and are induced in the periphery under influence of TGFβ. IL-10 producing T-regs prevent autoimmunity in the gut, lung and skin, and lessen disease in the kidney (Kurts et al. 2007; Scholz et al. 2008; Mahajan et al. 2006). In most glomerular diseases, T-regs are decreased in number and this loss of regulation leads to increase in effector T cells and increased disease (Mahajan et al. 2006; Scholz et al. 2008; Wolf et al. 2005).

A deficiency in regulatory T cells has been suspected as *part* of the pathogenesis in IL-13 transfected rats causing minimal change nephropathy (see above); in experimental allergic glomerulonephritis; and in nephrotoxic serum nephritis (Araya et al. 2009; Scholz et al. 2008; Mahajan et al. 2006). $HgCl_2$ induced glomerulopathy may also be related to immunodysregulation of the T effectors and T regulatory cells (Weening et al. 1981).

Adriamycin nephropathy may be considered an animal model of glomerulonephritis caused by the loss of regulatory T cells. Adriamycin (trade name for doxorubicin) is a DNA intercalating anthracycline antibiotic. Injection leads to non-immune glomerulopathy resembling human focal glomerulosclerosis. This is followed by tubulo-interstitial changes with interstitial infiltration of CD4+ and CD8+ T cells and macrophages. The inflammation is mediated primarily by CD8+ cytotoxic T cells, because experimental removal of CD8+ T cell attenuates inflammation (Wang et al. 2001b). Based on a series of experiments, it has been suggested that Adriamycin is toxic to regulatory T cells, which normally keeps immune activation in check (Wang et al. 2006; Kurts et al. 2007). When T-regs are reduced, immune

Fig. 6.8 (continued) and TNFα, and growth factors/mediators such as PDGF (*5*). These cytokines and mediators collectively injure Bowman's capsule and the glomerular basement membrane, leading to crescent formation (*6*) and proliferation of mesangial cells (*7*). Tc will bind to antigen on renal cells and directly induce cytolysis of mesangial cells or podocytes (not shown). The normal tolerogenic dendritic cells, Th2 lymphocytes and M2 macrophages are overwhelmed, and the balance is tipped in favor of a pro-inflammatory cascade of events. In addition to the cell-mediated Type IV reaction, autoantibody produced by B cells contribute to the disease progression. Autoantibody can bind to circulating or planted antigen, form complement-fixing immune complexes, and contribute to the renal lesion through a more classical type III hypersensitivity reaction (not shown). (Schematic by Cynthia Swanson)

homeostasis is upset and inflammatory cytokines cause glomerulonephritis and tubulo-interstitial inflammation. Deficiency of T regs is also implicated in the pathogenesis since adoptively transferred CD4+CD25+ T regs protects against renal disease (Mahajan et al. 2006), and replenishment of regulatory CD 4+ T cells following Adriamycin treatment resulted in increased secretion of TGFβ and reduction of disease (Wang et al. 2006) (Wang et al. 2001a).

The immune-mediated disease caused by T reg deficiency with Adriamycin must be distinguished from the more direct toxic effects of Adriamycin. Adriamycin causes direct podocyte damage by altering nephrin, podocin, and NEPH1 in a non-immune-mediated fashion, and can be seen in SCID mice (Lee et al. 2006).

6.5 Immune-Mediated Tubulo-Interstitial Nephritis

Immune-mediated tubulo-interstitial nephritis can be either primary resulting from immune activation against renal tubular antigens that ordinarily should be innocuous; or secondary resulting from a spill-over effect from immune-mediated glomerulopathy. Renal tubular antigens that cause a primary immune-mediated tubulo-interstitial nephritis include collagen IV (Goodpasture's antigen) in the tubular basement membrane (resulting in anti-TBM disease), and gp330 in the coated pits of both podocytes and of proximal tubular epithelium. Secondary immune-mediated tubulo-interstitial nephritis is most commonly the sequel or byproduct of immune-mediated glomerular disease. Glomerular changes associated with glomerulonephritis, such as activation and proliferation of mesangial cells and macrophages with release of pro-inflammatory mediators, results in recruitment of T cells, macrophages and dendritic cells not only to the glomerulus, but also to the kidney interstitium. Albuminuria as a result of the damage to the GBM stimulates the tubular epithelial cells to produce inflammatory cytokines, thereby engaging the tubulo-interstitium in the inflammatory process. Therefore, interstitial inflammation often is found secondary to glomerular injury.

Like glomerulonephritis, tubulo-interstitial nephritis (primary or secondary) is an inflammatory disease condition that self-perpetuates, leading to fibrosis and scarring and decreased kidney function. A more detailed description of the pathogenesis of immune-mediated tubul0-interstitial disease will be covered with Acute Kidney Injury (AKI, Chapter 6, Part 7).

Immune-mediated tubulo-interstitial nephritis should be differentiated from inflammatory kidney disease, such as bacterial pyelonephritis, that represents an appropriate immune response to infection and from Acute Kidney Injury (AKI) which is tubulo-interstitial nephritis *secondary* to a non-immunologic insult (ischemic, toxic or septic insult). Oftentimes, a primary infection or the primary insult cannot be identified, especially in laboratory animals, and the pathologist is confronted with a non-specific chronic tubulo-interstitial inflammatory disease process.

There are murine models for primary immune-mediated tubulo-interstitial nephritis (Meyers and Kelly 1991; Neilson 1993; Zakheim et al. 1984). One such model is the **kdkd mouse**, which is a mutant subline of the CBA strain. At 8-10 weeks of age, these mice get spontaneous interstitial nephritis, that progresses to renal damage by 4–6 months (Neilson et al. 1984). The disease is mediated by T cells and is a form of DTH hypersensitivity against renal tubular antigens. Research has shown that disease correlates with a functional inactivation of the suppressor T cells (which are now known as regulatory T cells) (Kelly and Neilson 1987). Under normal conditions, the regulatory T cells would maintain self-tolerance. The specific cause for the deficiency in T-regs in this model has not been worked out.

Another animal model for primary immune-mediated tubulo-interstitial disease is **anti-TBM disease** (Kelly et al. 1985). It can be experimentally produced in rodents (e.g., rats or mice) immunized with heterologous (i.e., rabbit) renal tubular antigens such as collagen IV of the TBM. The recipient get interstitial mononuclear infiltrates following injection of the rabbit toxic serum (i.e., rabbit anti rodent-TBM antibody.) This condition is a T cell-mediated form of DTH (Type IV hypersensitivity) and one that might also be caused by a deficiency in regulatory T cells (Type V hypersensitivity). The disease can be abrogated when T cells are removed with cyclophosphamide. The renal disease can also be ameliorated by a low protein diet (from 27 to 3%). Low protein has a beneficial effect because it blunts the T cell effector cell functions (Agus et al. 1985). A deficiency in antigen-specific T cell activity and a blunting of a DTH reaction has been noted in several disease conditions associate with low protein diets (Mainali and McMurray 1998), including chronic progressive nephropathy in rats, the pathogenesis of which is unknown. Using this experimental anti-TBM disease, it has also been discovered that prostaglandin E result in suppression of autoimmunity, and diets high in polyunsaturated fatty acid precursors of this favorable arachidonic acid may be beneficial and help curtail disease progression.

Another model of tubulo-interstitial nephritis that may involve T cells is Puromycin Aminonucleoside Nephropathy **(PAN)**. In rat PAN, while T cells may be absent from glomeruli, they infiltrate the interstitium and are associated with progression of the disease (Saito and Atkins 1990). Interstitial nephritis has also been experimentally induced in rats with N-(3,-dichlorophenyl) succinimide (Barrett et al. 1983).

6.6 Pathogenesis of Glomerular Injury in Immune Mediated Glomerulonephritis

So far in this chapter, we have reviewed the specific immune cell types that play a role in renal disease. In addition, we have reviewed the pathogenesis of how renal disease is initiated by covering the five major hypersensitivity reactions. Some of these reactions involve immune complex formation (i.e., type II and type III reactions), and others do not (i.e., type I, type IV and type V). Most of the diseases

involve participation of more than one of these hypersensitivity reactions. Most of the diseases result in a similar tissue response in the kidney that involves a mixture of proliferative, degenerative, inflammatory, and atrophic changes. Since the kidney, and glomerulus in particular, have a limited repertoire in their response, the morphologic presentation of an immune-mediated lesion is often not pathognomonic or specific for any one pathogenesis (Le Hir 2004). The pathognomonic smudge cells of lupus nephritis may be an exception, but they are present in only 2% of renal biopsies of patients. Once the immune system is activated, the changes to the glomerulus under light microscopy may be similar and the morphologic presentations for the diseases converge. This section of the chapter will focus on the pathogenesis of the tissue response in the glomerulus seen at the light microscopic level. The molecular basis for the different changes will be provided, and hopefully this will give the pathologist an understanding of how and why certain changes develop.

After immune complexes deposit in the glomerulus (mesangial, subendothelial, or subepithelial), and complement is activated, a cascade of inflammatory events occurs. Virtually all of the cell types of the glomerulus are involved in perpetuating the inflammation, including mesangial cells, endothelial cells, podocytes, and resident macrophages. Depending on the nature of the initial reaction, cytokines and chemokines released from these cells recruit neutrophils, macrophages, dendritic cells and T cells to perpetuate the damage. The location of the immune complex, the involvement of either Th2 or Th1 cells, the degree of activation of complement, and the phenotype of the macrophages (inflammatory or non-inflammatory) and dendritic cells (tolerogenic vs non-tolerogenic), will dictate the nature of the glomerular injury. The mechanics of immune complex deposition are reviewed by (Wilson and Dixon 1986). Briefly factors that determine the location and deposition of immune complexes includes factors that affect permeability such as platelet activating factor, immune complex size, antigen:antibody ratio of the immune complex, antigen size, antibody subclass, antibody avidity and blood flow. In the human and experimental animal diseases described in the first part of this chapter, the location of these immune complexes have been worked out. Even *without* formation of immune complexes or with conventional immune cells recruited into the tissue, the parenchymal cells (including mesangial cells, podocytes, endothelial cells, and renal tubular epithelial cells) collectively contribute to the self-perpetuation of inflammatory and/or degenerative changes in the renal parenchyma.

The types of tissue reactions that occur in the glomerulus at the light microscopic level, include necrosis of mesangial cells, mesangial cell proliferation, intraglomerular inflammatory cells, endothelial cell proliferation, thickening/spiking of the GBM, deposition of mesangial matrix, podocyte loss, fusion of podocyte foot processes, capillary thrombi, fibrosis, crescent formation (cellular, fibro cellular or fibrous), and podocyte/parietal cell transformation. Some of these changes indicate acute damage (infiltrate of inflammatory cells; hyaline thrombi; necrosis of mesangial cells), others suggest subacute changes (spike formation, podocyte loss, fusion of podocyte foot processes, mesangial cell proliferation), and others characterize chronic changes (deposition of mesangial matrix, fibrosis, fibrous crescent forma-

tion, and epithelial cell transformation). The pathophysiology of acute to subacute glomerular injury is schematically portrayed in a series of three figures: Figs. 6.9, 6.10, and Fig. 6.12. Several features of glomerular lesions are depicted in images of glomerulopathies of dogs at the end of this chapter. (see below, Figs. 6.25, 6.26, 6.27, 6.28, 6.29, 6.30, 6.31, 6.32, 6.33, and 6.34).

As a brief overview, most of the injury begins with complement fixation, leading to release of inflammatory mediators produced from the various populations of parenchymal cells. The mediators cause direct or indirect damage to the GBM, which leads to albuminuria. During the acute to subacute stages of glomerular disease, there can be cellular proliferation (endothelial and mesangial), changes in the GBM and capillary basement membrane, platelet aggregation and micro thrombi within capillaries, podocyte foot process fusion and podocyte loss, and an influx of neutrophils, macrophages, and possibly Th1/Th17 cells. During the more chronic stages of the disease, any attempts at tissue repair by fibrosis does little to heal the kidney, but rather promotes hypoxia, more fibrosis, and decreased glomerular filtration rate (GFR).

Complement Activation. Complement activation is a major cause of glomerular injury and is often the initial inciting cause for injury associated with Type II and Type III hypersensitivity reactions. Activation of a complement results in direct cell damage and in the release of products that have pro-inflammatory and/or immunoregulatory properties (Fig. 6.9). Complement can be activated by classical, lectin and/or alternative pathways. Typically, C1q, the recognition molecule, binds to the Fc portion of the immune complex to activate the classical pathway. Complement fixation is more prominent with IgG1 and IgG3 subclasses of immunoglobulin, and less frequent with the IgG2 subclass. In the case of IgA nephropathy and bacterial infections, the alternative pathway is activated. In addition, mannose-binding lectin structures on a variety of cell types can bind to the carbohydrate moieties on microorganisms and activate the lectin pathway as well. Regardless of how complement is activated, all pathways lead to formation of C3 component which is acted upon by C5 convertases to become C5. C5 is cleaved to generate C5b, the first step in assembly of C5b-9 (the membrane attack complex, MAC).

The glomerulus has some defensive mechanisms by which to prevent complement activation. Complement activation is normally strictly regulated to prevent aggressive damage to host cells. Podocytes have surface bound regulatory proteins (such as CR1 which is the C3b receptor), membrane cofactor proteins (such as MCP and CD46), and decay accelerating factor (DAF or CD55), that work together to shorten the half-life of assembled C3 and C5 convertases. Other proteins such as membrane-bound CD59, and plasma-derived clusterin and vitronectin, prevent assembly and insertion of C5b-9 complexes (Fig. 6.9). For a review of the complement regulatory protein in glomerular disease, see Nangaku (1998).

Another protective mechanism of the podocyte is its ability to endocytose the C5b-9 complex and then exocytose it into the filtrate. Urinary excretion of C5b-9 may be a biomarker for ongoing immunologic injury in people (Kerjaschki et al. 1989; Kon et al. 1995).

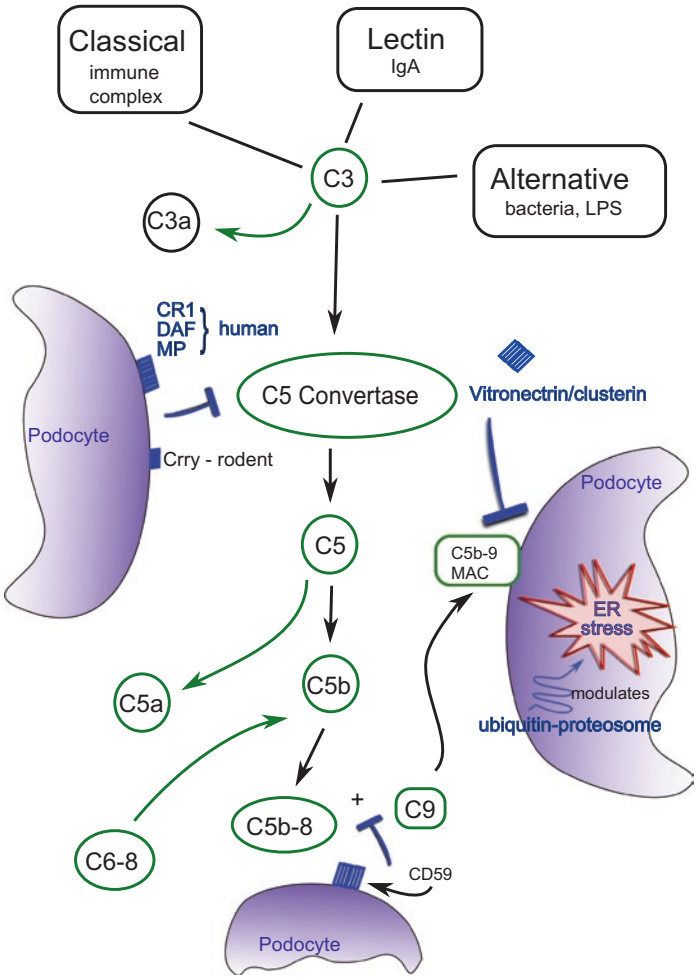

Fig. 6.9 Complement and its regulation. This schematic depicts complement activation and the regulation of its activation in glomerulonephritis. The complement cascade becomes activated by classical, lectin or alternative pathways. All pathways converge to cell-surface assembled C5 convertase, which generates C5. C5 then is cleaved to generate C5b, which forms the C5b-9 complex. This C5b-9 complex is a lipophilic complex that forms a pore within cell membranes, and lysis/damages the cell. In nucleated cells, the C5b-9 MAC does sub-lytic damage resulting in accumulation of misfolded proteins and endoplasmic reticulum (ER) stress. There are a number of factors that inhibit complement activation or its damage, and they are color-coded royal blue. First, surface bound regulatory proteins, such as the complement receptor-1 (CR1), decay-accelerating factor (DAF), and membrane cofactor protein (MCP), work together to shorten the half-life of cell surface assembled C5 convertase in humans. These complement regulatory proteins are referred to more generally as Crry in rodents. Final formation of the membrane attack complex is regulated by podocyte expressed CD59. Plasma-derived complement regulatory proteins (clusterin and vitronectin) prevent insertion of C5b-9 terminal complex into the cell membrane. If the C5b-9 complex does insert into cell membranes, intracellular ubiquitin-proteasome system is designed to help protect against ER stress. (Schematic by Cynthia Swanson)

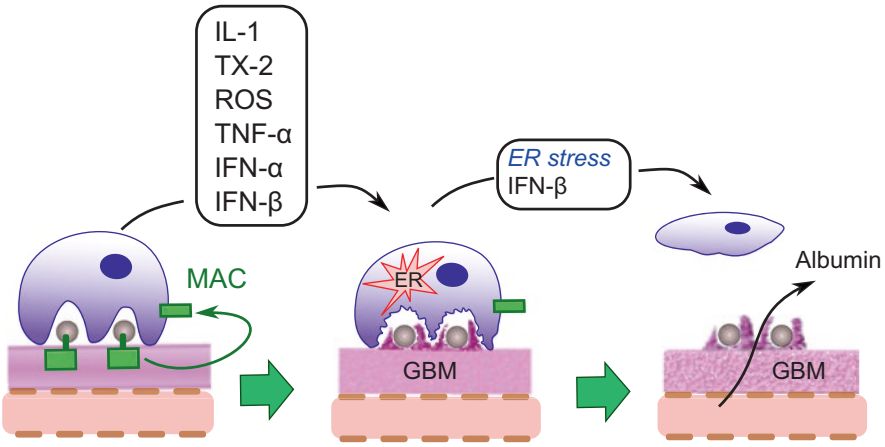

Fig. 6.10 Acute/subacute glomerular injury (leukocyte independent). The schematic shows the pathogenesis of glomerular injury during the acute and subacute stages, when the immune complex deposits are in the subepithelial location. (*Left*) Subepithelial immune complexes fix complement, especially those involving IgG1 and IgG3 subclasses of immunoglobulin. After complement is activated, the complement components such as C3a and C5a cannot readily enter the circulation. Therefore they are ineffective at recruiting neutrophils or monocytes, and the damage that results is leukocyte-independent. The C5b-9 membrane attack complex however is capable of attacking the podocyte and causing sublytic damage. Damaged podocytes produce IL-1, TX2, ROS, TNFα, and IFNα and IFNβ. (*Middle*) The damaged podocyte has endoplasmic reticulum (ER) stress and is also bombarded by inflammatory cytokines. In response, the podocyte instigates deposition of matrix material around the immune complex, resulting in spike formation that is visible on silver-stained histologic sections. There is restructuring of the actin cytoskeleton and retraction of foot processes. (*Right*) IFNβ and ER Stress can eventually cause apoptosis of the podocyte. The damaged podocytes fail to maintain the polyanion composition of the glomerular basement membrane, or the nephrin and α-actinin-4 composition of the slit diaphragms. With continued retraction of foot processes, there is loss of the filtration capability, resulting in proteinuria. (Schematic by Cynthia Swanson)

Acute/Subacute Glomerular Injury. Regardless of where the immune complex is located in the subepithelial or subendothelial region, or in the mesangium, the C5b-9 membrane attack complex (MAC) is responsible for direct cellular damage to podocytes, endothelial cells and/or mesangial cells, respectively. Since these three cell types are nucleated cells, and nucleated cells are relatively resistant to lysis by the C5b-9 MAC, there is no abrupt lysis of the cell, like one would otherwise see when complement attacks red blood cells. Rather the damage by C5b-9 to nucleated cells is sub-lytic.

The following discussion will focus on the podocyte damage caused by the C5b-9 MAC (Fig. 6.10). The "sub-lytic" damage to the podocyte results in an increased synthesis of prostaglandins and thromboxane (Lovett et al. 1987), IFN (Gurkan et al. 2013), reactive oxygen species (Adler et al. 1986), and IL-1 and TNFα (Lovett et al. 1987) from that podocyte. Aside from inducing release of pro-

inflammatory mediators, the C5b-9 MAC also sets into motion a cascade of events including calcium influx, oxidative injury, cell cycle dysregulation, disruption of the actin cytoskeleton, and internalization of podocyte specific proteins (Beck and Salant 2014; Meyer-Schwesinger et al. 2009). The disruption of the actin cytoskeleton and the internalization of podocyte specific proteins at the slit diaphragm (namely nephrin and α-actinin 4) results in fusion and retraction of foot processes and loss of filtering ability of the slit diaphragm (Meyer-Schwesinger et al. 2009). These collective injurious actions of C5b-9 results in an accumulation of misfolded protein in the endoplasmic reticulum leading to endoplasmic reticulum (ER) stress (Cybulsky 2010). Sustained ER stress may by cytotoxic by causing activation of pro-apoptotic pathways leading to destruction of the podocytes (Rong et al. 2015; Hetz 2012).

ER stress-induced apoptosis occurs by two pathways: (1) the activation of CCAAT/enhancer-binding protein (C/EBP) homologous protein (CHOP/GADD153); and (2) caspase-12 (Cao et al. 2014). The podocyte has a ubiquitin-proteasome system and a defensive mechanism of autophagy that are both capable of reducing the ER stress of the podocyte following injury by C5b-9 MAC (Hartleben et al. 2010; Meyer-Schwesinger et al. 2009; Wang et al. 2012; Beck and Salant 2014).

The pro-inflammatory mediators (IFNα, IFNβ, prostaglandins, thromboxane, ROS, and TNFα) produced by the damaged podocyte itself potentiates its own destruction and loss (Ryu et al. 2012). IFNβ directly induces podocyte death *in vitro* and *in vivo* while IFNα inhibits podocyte progenitor migration and proliferation and thereby prevents any repair (Migliorini et al. 2013). More specifically, IFNβ causes mitotic podocyte death (mitotic catastrophe). It provides a stimulus for this post-mitotic cell to pass through the M phase of nuclear division, but the highly differentiated post-mitotic podocyte has insufficient tools to complete this division, and the podocyte dies (Castedo et al. 2004). In summary, at this early point in the stage of complement fixation and activation, self-induced structural damage occurs to the podocyte without any leukocyte involvement at all (Takano et al. 2013).

Leukocyte Independent Events: After the initial insult by the C5b-9 MAC with release of ROS and pro-inflammatory cytokines, the eventual extent of cell damage depends on the numbers and types of leukocytes that are recruited to the area. As mentioned above for the podocyte, there can be much destruction without any leukocyte involvement at all. In general, those immune complexes that fix complement in the subepithelial location are leukocyte independent, and those in the subendothelial area or mesangial area are those that are leukocyte dependent. This makes sense because immune complexes in the subepithelial region are relatively far from the circulation, and C5a and C3a, cannot readily diffuse into the circulation against the pressure gradient. With leukocyte independent events, there is thickening of the GBM with spike formation (Fig. 6.10). The podocyte injured by complement stimulates the production of extracellular matrix that thickens the GBM and creates spikes of material between the subepithelial deposits. These spikes appear on silver stain since the intervening translucent complexes do not stain with silver (Fig. 6.11).

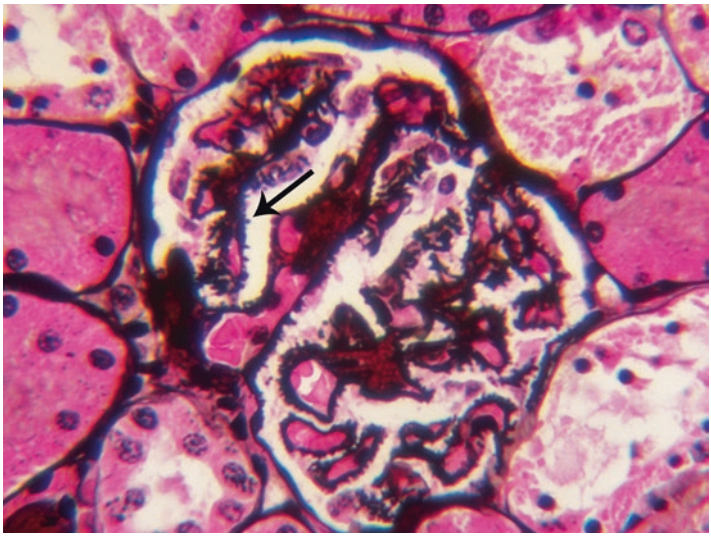

Fig. 6.11 Spike formation of the glomerular basement membrane (GBM). This section is from the kidney of a *Mastomys natalensis* (multi-mammate mouse) with spontaneous glomerulonephritis. The section was stained with periodic acid—Schiff-methenamine silver, and demonstrates the spikes of the GBM commonly associated with subepithelial immune deposits. Thin section periodic acid-Schiff/methenamine silver (PAMS) stain, 40x objective magnification. (Photo courtesy of Dr. George A. Parker)

Leukocyte Dependent Events: Those immune complexes in the subendothelial area are generally leukocyte dependent lesions, and additional damage is attributed to the influx of neutrophils and macrophages (Salant et al. 1985) (Figs. 6.12, 6.13, and 6.14). The leukocyte-dependent glomerular injury can be visually more dramatic and progressive, because it involves conventional inflammation, capillary leakage, and proliferation. Neutrophils (and monocytes) are most prominent in the glomerulus following post-infectious GN (post streptococcal) and in the crescentic glomerulonephritides in humans (Hooke et al. 1987) which are two immune-mediated glomerulopathies associated with mesangial and subendothelial deposits. The glomerular lesion when it involved neutrophils is often termed exudative glomerulonephritis or crescentic glomerulonephritis. C5a and C3a cleavage products from complement activation enter the circulation (from the subendothelial or mesangial deposits) and lead to neutrophil chemotaxis and neutrophil adherence, respectively. These anaphylatoxin products also stimulate leukocytes that enter the mesangium to release pro-inflammatory mediators such as IL-1, procoagulants, and arachidonic acid metabolites. The migration of leukocytes into the mesangium occurs more readily after complement activation because vasoactive C5a also results in increased transcapillary pressure (Cybulsky et al. 1988).

Those immune complexes in the mesangium activate complement that causes sublytic damage to the mesangial cell, in the same way complement induces sub-

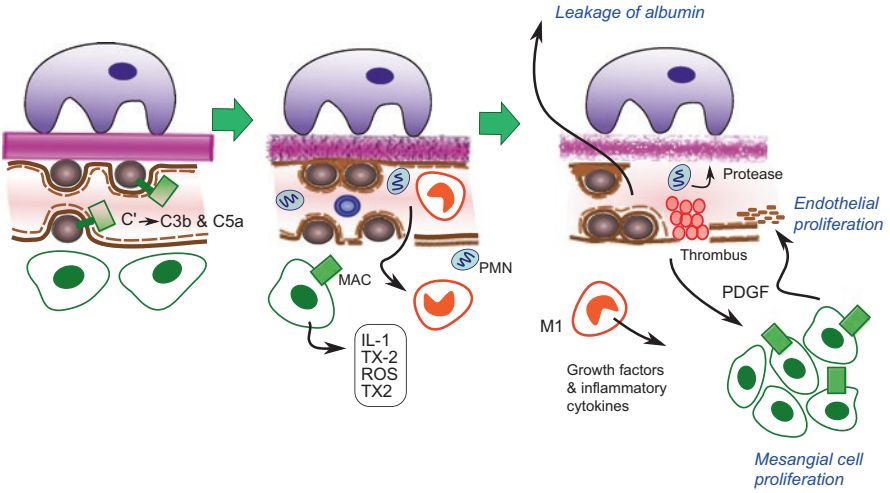

Fig. 6.12 Acute/subacute glomerular injury (leukocyte dependent). The schematic shows the pathogenesis of glomerular injury during the acute and subacute stages, when the immune complex deposits are in the mesangial and/or subendothelial location, and stimulate leukocyte involvement. (*Left*) Immune complexes in the subendothelial space fix complement. C3a and C5a components have access to the circulation and recruit neutrophils and monocytes. (*Middle*) The recruited macrophages produce pro-inflammatory mediators such as IL-1, TXB2, and ROS, which in turn stimulate the mesangial cells to secrete its own battery of mediators and chemokines. C5a also results in increased transcapillary pressure with dilatation of the capillary promoting transcapillary migration of inflammatory cells. (*Right*). The circulating neutrophils release damaging enzymes, especially neural serine proteases which cause damage to the glomerular basement membrane. The neutrophilic enzymes and inflammatory cytokines also cause endothelial cells to retract from the basement membrane, leaving room for platelet aggregation (thrombi) and activation. The activated platelets produce PDGF (as does the mesangial cells) and PDGF along with TXB2 promotes endothelial and mesangial cell proliferation. A thick basement membrane forms around the immune deposits resulting in a wire loop appearance. Often there also is a response of duplication of the basement membrane. Often there can be interposition of mesangial cells between the two membranes (not shown). These changes to the basement membranes (duplication and wire-loop) can be detected by light microscopy with silver and possibly PAS stains. (Schematic by Cynthia Swanson)

lytic damage to the podocyte. IL-1 produced by infiltrating macrophages also establish a cross-talk with the mesangial cells. Mesangial cells respond to insertion of C5b-5 MAC or to the mediators released by macrophages by releasing their own battery of pro-inflammatory mediators, such as IL-1, TXB2, TNFα, and reactive oxygen species (Satriano et al. 1993; Gomez-Guerrero et al. 1994; Sedor et al. 1987). Mesangial cells also secrete chemokines such as MCP-1 leading to the recruitment of macrophages (Hora et al. 1992) and by the expression of fractalkine (CX3CL1), a potent leukocyte chemoattractant on mesangial cells (and endothelial cells) (Cockwell et al. 2002; Furuichi et al. 2001).

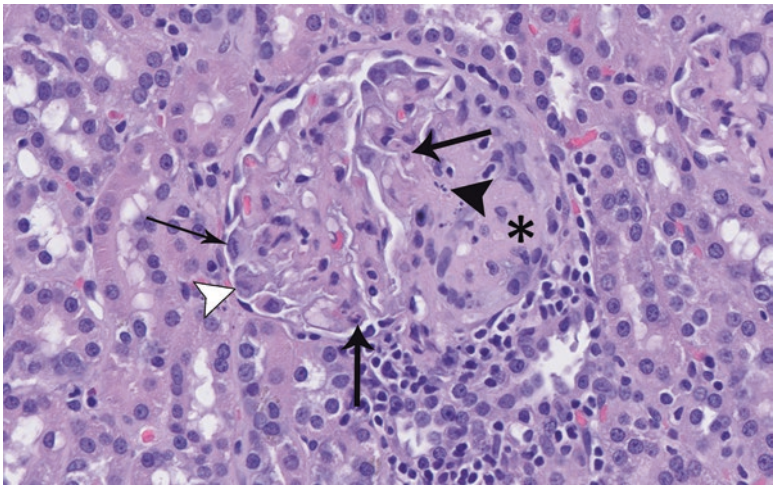

Fig. 6.13 Exudative glomerulonephritis in rat. This section of kidney is from a rat that has changes within the glomerulus consistent with exudative glomerulonephritis. The cause of this lesion is unknown, yet the lesion provides opportunity to point out various changes commonly associated with immune-mediated glomerulopathies. There are circulating inflammatory cells in the capillary lumen (*thick arrows*), mesangiolysis with necrosis of cells in the mesangium (*black arrowhead*), deposition of mesangial matrix (*asterisk*), and podocyte hypertrophy (*white arrowhead*). One podocyte (*thin arrow*) may be in the early stage of mitosis, which, for the podocyte, results in mitotic catastrophe and podocyte death. The attraction of lymphocytes to the periglomerular interstitium (below the glomerulus) is not uncommon in leukocyte-dependent glomerulopathies. H&E stain. 54× objective magnification

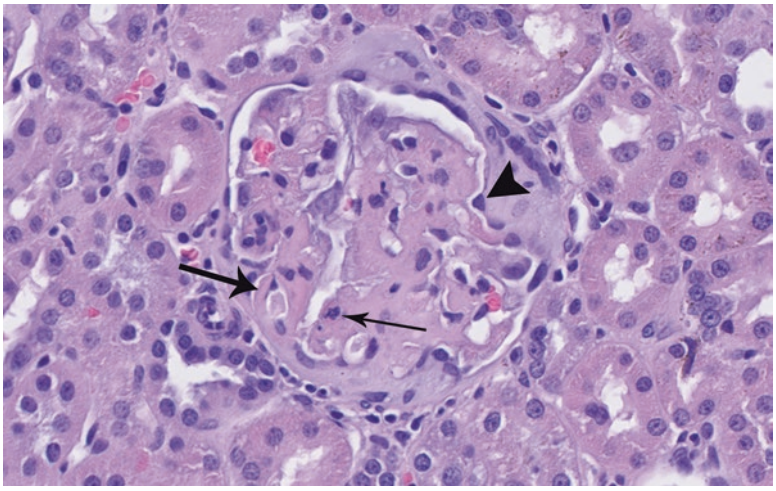

Fig. 6.14 Exudative glomerulonephritis in rat. In this tuft from the same animal as in Fig. 6.13, there is one thickened capillary forming a wire-loop (*thick arrow*) containing an intravascular hyaline thrombus. This thrombus could represent either clotted blood or immune complexes. Note the parietal epithelial cells extending to the tuft and forming a synechia, in an attempt cover denuded GBM (*arrowhead*). An intracapillary neutrophil is present (*thin arrow*). H&E stain. 61× objective magnification

Neutrophils contribute to the destruction of the glomerular structure by releasing numerous enzymes. Neutrophils release proteases, prostaglandins, leukotrienes, platelet activating factor, collagenases, lysozymes, and lactoferrin. Perhaps the most damaging of these is the neutral serine proteases (e.g., elastase and cathepsin G) released from the primary granules of neutrophils. Neutral serine proteases are those enzymes primarily responsible for degradation of the GBM (Davies et al. 1984).

Thromboxane B2 (TXB2) released from mesangial, cells, podocytes, or endothelial cells promotes platelet aggregation, hyaline thrombi formation, hypoxia, and eventual decreased circulation and GFR (Hassid et al. 1979). It should be cautioned at this point that hyaline thrombi caused by this hypercoagulable state is hard to differentiate by light microscopy from the hyaline thrombi that represent intravascular immune complexes (commonly seen in lupus nephritis). The aggregation and activation of platelets give rise to the release of another bout of mediators and growth factors. One of these is platelet derived growth factor (PDGF) that causes proliferation of endothelial cells and mesangial cells. Other mediators present during this intraglomerular inflammatory lesion that cause mesangial cell proliferation include angiotensin converting enzyme, leukotriene D3, and matrix metalloproteinases, since studies show that inhibitors of these substances curtail mesangial cell proliferation in glomerular disease models (Kurogi 2003). TXB2 released from podocytes result in vasoactive effects leading to increased transcapillary pressure and increased permeability of the GBM and proteinuria (Stahl et al. 1987; Zoja et al. 1987; Cybulsky et al. 1988; Macconi et al. 1989). The direct damaging effects of TXB2 have been shown in experimental studies (Prickett et al. 1983; Kelley et al. 1985; Coffman et al. 1985).

Monocytes and neutrophils also produce myeloperoxidase (MPO), which is a cationic enzyme and reacts with H_2O_2 to form hypohalus acid that injures cells such as endothelial cells leading to denuded GBM. MPO, since it is cationic, binds to the anionic sites in the capillary walls. These damaged capillary walls become a nidus to which any circulating immune complexes and platelets can further attach.

T lymphocytes (and DTH in general) play a minor role in glomerular injury, although they play a significant role in tubulo-interstitial disease. For example, in lupus nephritis, both CD4+ and CD8+ cells caused a DTH disease which effect primarily the interstitium. Th1 cells produce pro-inflammatory cytokines IL-1, IFNϒ, and TNFα which affect the interstitium and by extension, can affect the mesangium (Kamoun et al. 1981; Hooke et al. 1987). Th1 cells are not the only T cells that produce damaging cytokines. Th17 cells participate and often cause more rapidly progressive damage than if only Th1 cells are present (Kitching and Holdsworth 2011). Cytokines from macrophages and mesangial cells, such as IL-6 and TGFβ, direct T cells to become a Th17 phenotype. Th17 cells mobilize and activate neutrophils, and induce macrophage production of additional pro-inflammatory cytokines, such as IL1β and TNF. IL17 also stimulates mesangial cells (and tubular epithelial cells) to produce chemoattractant for T cell, macrophage and neutrophils (Van Kooten et al. 1998).

Above, we discussed how complement initiates the entire cascade of inflammatory and destructive events in the glomerulus. In particular, complement activation is followed by self-destructive actions of the podocytes and the production of damaging cytokines from recruited leukocytes and from resident endothelial and mesangial cells. However, complement continues to play a role even after the initial event. Complement components are synthesized within the podocytes, mesangial cells, and endothelial cells (as well as tubular epithelial cells) when induced by pro-inflammatory cytokines (Zhou et al. 2001; Sacks et al. 1993). Therefore, complement activation can continue to occur, even in the absence of initial activation of complement by immune complexes. The C3a and C5a components that result from *intrarenal* synthesis of complement induces the production of TGFβ by mesangial cells and podocytes which plays a role in the fibrotic (chronic) stage of disease (Fearn and Sheerin 2015).

Glomerular Disease spills over into the tubulo-interstitium. The damaging effects of pro-inflammatory cytokines is not limited to the glomerulus. The events occurring in the glomerulus during acute/subacute injury spill-over into the interstitium leading to interstitial inflammation and progressive chronic renal disease. The involvement of the interstitium occurs by several mechanisms, including a direct encroachment into the interstitium at the glomerulo-tubular junction or via the tubular filtrate (Kriz and LeHir 2005). The glomerular tubular cross-talk is due to a number of factors that leak into the filtrate, including TNFα, angiotensin II, albumin, and complement components. For example, TNFα produced by mesangial cells is one pro-inflammatory cytokine that activates tubular cells by a paracrine mechanism to induce its own pro-inflammatory mediators (Chan et al. 2005b). Angiotensin II leaking into the filtrate is another protein that can incite interstitial inflammation. With hemodynamic changes associated with glomerular disease, there is activation of the renin-angiotensin-aldosterone system, upregulation of angiotensinogen by the liver, and increased circulating levels of angiotensin II. Angiotensin II directly triggers apoptosis not only of podocytes but also of downstream tubular epithelial cells after it leaks through the GBM (Chan et al. 2005a; Sanz et al. 2008; Nijenhuis et al. 2011; Durvasula et al. 2004; Bhaskaran et al. 2003). Apoptotic bodies or DAMPS derived from the angiotensin-II damaged RTE bind to pattern recognition receptors (PRRs) on RTE which activates inflammasomes within tubular epithelial cells. Albumin also leaking through a damaged GBM can activate this inflammasome system within RTE. The activated inflammasome system permit the RTE to produce IL-1 and IL-18, which recruits inflammatory cells to the kidney interstitium. Angiotensin II also directly activates TLR4 (on tubular epithelial cells and mesangial cells) that results in the production of pro-inflammatory molecules and chemokines. Therefore, as a result of angiotensin II and albumin, monocytes and macrophages and T cells are recruited from the vasculature into the interstitium (Pearse et al. 2008; Gajjala et al. 2015). In the same manner that complement components induced production of TGFβ by mesangial cells to cause fibrosis of the glomerulus, complement components (e.g., C3a and C5a) leaking through the GBM or produced by podocytes directly can induce production of

TGFβ by RTE. Thus, interstitial fibrosis spills over from primary glomerular disease via the tubular filtrate.

Encroachment of glomerular injury into the glomerulo-tubular junction is also a very important mechanism by which glomerular injury spreads to the tubulointerstitium. In Fig. 6.1, one can see where the highly specialized GBM and its podocytes transitions into the thin and ordinary basement membrane of the proximal tubule and its overlying tubular epithelial cells. It is at this site (i.e., the glomerulo-tubular junction) where misdirected filtration through the GBM in the damaged glomerulus extends between tubular epithelial cells and tubular basement membrane (Kriz and LeHir 2005). This separation between epithelial cells and basement membrane may spread along the entire proximal convolution, and occurs with inflammatory and/or degenerative glomerulopathies. Another way that glomerular disease spreads to the interstitium is through a growing crescent that encroaches on the glomerulo-tubular junction, whereby the initial segment of the proximal tubule gets incorporated into the crescent (Kriz et al. 1998). The formation of crescents is further described in the following section. Cellular overgrowth of the glomerulo-tubular junction and the encroachment of the filtrate between the epithelium and basement membrane directly leads to tubular degeneration with early obstruction, resulting in atrophy and loss of tubules and eventually complete nephron degeneration. The final decomposition of the glomerular remnant usually occurs after final decomposition of the tubule (Kriz and LeHir 2005). The histopathologic changes associated with tubular atrophy, tubular degeneration, and decomposition of the atubular glomerulus are detailed in Kriz and LeHir (2005) and are beyond the scope of this chapter.

Chronic Injury and Crescent Formation. In the chronic stage of glomerular disease, glomerulosclerosis and fibrosis occurs. The growth factors, PDGF and IGF, released by mesangial cells and endothelial cells have a proliferative effect on mesangial cells. TGFβ induces the formation of increased extracellular matrix by these mesangial cells and increased GBM matrix by the podocytes. Increased matrix (or sclerosis) results in decreased capillary blood flow and decreased GFR. Damaged podocytes and podocytopenia is the result of major injury, and this "heals" as areas of sclerosis within the tuft but also with formation of crescents (Fig. 6.15). Such sclerosis and crescents are a poor prognostic sign, because both lead to less blood flow that only accentuates the loss of GFR (Lemley et al. 2002; El Karoui et al. 2011). Therefore, the "wound healing" response of the glomerulus results in continued loss of function and is not beneficial.

The formation of crescents can be considered as a failed attempt by the glomerulus to heal itself, and crescents arise from the mesenchymal transformation of both the parietal and the visceral epithelial cells. Normally, when podocyte loss is minimal, the podocytes may regenerate: the parietal epithelial cell layer contains podocyte progenitors which have limited capability of differentiating into podocytes. This limited ability at regeneration is rapidly curtailed by the albuminuria associated with glomerular disease. The albuminuria associated with glomerulonephritis (because of damage to the GBM by serine proteases and TXB2) binds up all the reti-

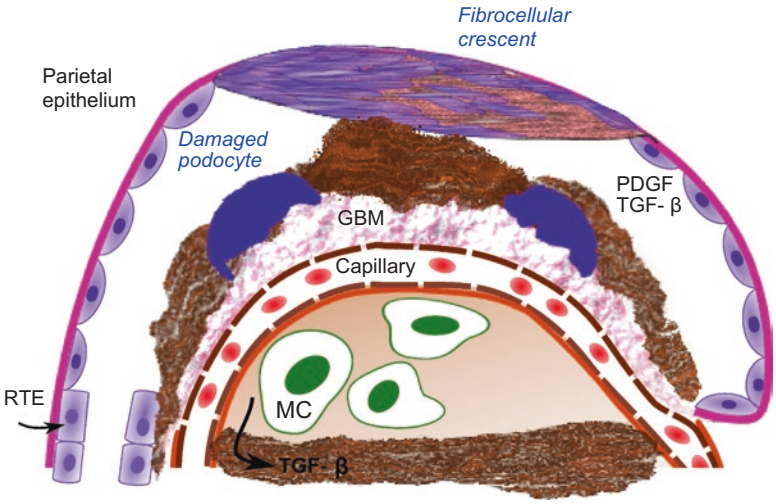

Fig. 6.15 Chronic glomerular injury and crescent formation. The schematic has a renal glomerulus with Bowman's capsule lined by parietal epithelium and the glomerular tuft. Podocytopenia is one reason for crescent formation. This particular tuft has a loss of podocytes and there is damage to the glomerular basement membrane (GBM). The epithelial cells transform into matrix producing mesenchymal cells that layer down matrix. Epithelial cells produce TGFβ and PDGF which promote proliferation of parietal epithelial cells and increased production of extracellular matrix that covers denuded GBM. The end result is the formation of a fibro cellular or fibrous crescent. The scar tissue encroaches into the glomerulo-tubulo junction, leading to degeneration of RTE (*left lower corner*). Mesangial cells, which have proliferated under the influence of PDGF, angiotensin converting enzyme, and other mediators, produce TGFβ which causes increased production of mesangial matrix, leading to glomerulosclerosis. (Schematic by Cynthia Swanson)

noic acid in Bowman's space. Since retinoic acid is a mandatory differentiation factor for podocyte progenitors, there is loss of even the limited regenerative capability of the podocyte (Peired et al. 2013).

When this limited ability of regeneration is no longer possible, parietal epithelial cells and podocytes (the visceral epithelial cells) both revert to a mesenchymal-type cell and form a fibro cellular crescent in an attempt to patch over the denuded GBM. This transformation is called epithelial-mesenchymal transformation (EMT). The contribution of podocytes to crescent formation is a relatively new concept. Originally it was believed that podocytes were not involved in the pathogenesis of crescent formation because podocytes are terminally differentiated (Henique et al. 2014).

The process of epithelial mesenchymal transition (EMT) is a reverse form of embryogenesis. EMT is mediated by both platelet-derived growth factor (PDGF) and TGFβ, which are both produced by the glomerular epithelial cells themselves (van Roeyen et al. 2011). The PDGF stimulates proliferation and transformation of the glomerular epithelial cells and the TGFβ induces these cells to produce extracellular matrix. The transitioning podocytes lose polarity, have reduced expression of nephrin and ZO-1 (zonula occludins protein), lose their phenotypic and structural

integrity (Li et al. 2008; Loeffler and Wolf 2014), become migratory, and produce extracellular matrix. Some of the parietal epithelial cells migrate on to the top of the existing podocytes, form a layer of extracellular matrix, thereby producing a synechia (Besse-Eschmann et al. 2004; Moeller et al. 2004). With more than 40% loss of podocytes, these synechia can be seen microscopically (Barisoni et al. 1999).

The stimulus for transitioned epithelial cells to produce extracellular matrix (EMT) is TGFβ (Loeffler and Wolf 2014). Increased levels of angiotensin II and renin (produced by the JGA apparatus in instances of reduced GFR) induce TGFβ production by renal cells thereby encouraging EMT and fibrosis. Virtually all cells of the glomerulus produce TGFβ, including mesangial cells, visceral and parietal epithelial cells, endothelial cells, and macrophages. The scarring process leads to irreversible glomerulosclerosis and nephron loss (Kriz and LeHir 2005).

TGFβ is essential for normal development, tissue repair and maintenance of organ function, but excess production is linked to kidney diseases, such as FSGS, IgA nephropathy, and lupus nephritis (Kitamura and Suto 1997). TGFβ not only assists in the increased extracellular matrix of the mesangium, but of the thickening and spike formation of the GBM, of the thickening and reduplication of the capillary basement membrane, and in crescent formation. TGFβ stimulates the production of matrix, by increasing production of its proteins (collagen, laminin, fibronectin), decreasing the activity of matrix proteinases, and upregulating the synthesis of proteinase inhibitors like PA1-I (Kitamura and Suto 1997; Lopez-Hernandez and Lopez-Novoa 2012). TGFβ exerts this pro-fibrinogenic effect on the epithelial cells and the mesangial cells of the glomerulus (Wolf and Ziyadeh 2007).

Although crescents develop after podocyte loss and by EMT, crescents can also occur following rupture of the capillary wall or vasculitis. When capillary walls and GBM are damaged, there is release of mitogens into Bowman's space, proliferation of parietal epithelial cells and fibroblasts of Bowman's capsule, and infiltration of macrophages and T cells into Bowman's capsule. Regardless of whether they arise following EMT or from capillary wall destruction, the cellular and fibrous components of a crescent obstruct Bowman's space further promoting the loss of GFR (Smeets et al. 2009). Depending on the amount of cellular or fibrous components, crescents can be morphologically referred to a cellular, fibro cellular or fibrous. Cellular crescents contain about 35% macrophages, 12% neutrophils and 12% podocytes (Johnson et al. 1988). Macrophages enter the crescents usually from the periphery outside of, and through breaks in, Bowman's capsule. For an excellent discussion of morphologic development and progression of crescents, see (Kriz and LeHir 2005).

Role of Renin-Angiotensin-Aldosterone System in Renal Lesions. Activation of the renin-angiotensin-aldosterone (RAA) system in chronic kidney disease (whether that disease is glomerular or tubular) can perpetuate the kidney disease and also impact other organ systems. In response to decrease in renal perfusion pressure, renin is produced by the JGA and along with angiotensin converting enzyme (ACE) converts angiotensin I to angiotensin II. Angiotensin II constricts the efferent arteriole, resulting in decreased blood flow and increased glomerular capillary pressure. Angiotensin II also causes increased aldosterone release in the adrenal

cortex and increased sodium retention. Aside from its effects on the vascular system, the components of the RAA system play a role in lesion development in the glomerulus and interstitium. Recall in acute/subacute injury, angiotensin II can cause podocyte apoptosis and after these apoptotic bodies bind to TLRs, can activate macrophages and dendritic cells to release pro-inflammatory cytokines. ACE, which is also elevated with activation of the RAA system plays a role in inducing mesangial cell proliferation. In chronic injury, angiotensin II also participates in lesion development. Angiotensin II induces production of TGFβ by podocytes, mesangial cells and macrophages; and aldosterone induces the production of TGF-β distal tubular epithelial cells and collecting ducts, resulting in glomerular and peritubular fibrosis, respectively (Ketteler et al. 1995; Wolf and Neilson 1993).

6.7 Acute Kidney Injury (AKI)

Under many of the hypersensitivity reactions discussed above that initially affect the glomerulus, most of the initial immune damage is due to inflammatory cytokines released by resident mesangial cells, podocytes, or resident macrophages and dendritic cells. Only after these inflammatory cytokines stimulate cell damage, do T cells or additional macrophages infiltrate and perpetuate the tissue destruction and spill over into the tubulo-interstitium. More often than not in the glomerulus, a typical inflammatory condition never exists, and instead there is light microscopic findings of "non-inflammatory" fibrosis, glomerulosclerosis and crescents.

Acute Kidney Injury (AKI) is different. Acute Kidney Injury presents as a conventional inflammatory response associated with *appropriate* activation of the innate arm of the immune system, followed in many instances by activation of the adaptive immune response and self-perpetuation of disease. The triggering event may be septic (i.e., microbial infection) or aseptic (i.e., toxicity such as cisplatin or ischemia). Unlike immune-mediated tubulo-interstitial disease, AKI involves a primary and direct non-immune-mediated insult to the tubules, and is not merely a "spill-over" from glomerular injury or an autoimmune reaction to some tubular antigen. In AKI, the immune system's activation is readily apparent by the marked infiltrate of inflammatory cells. Depending on the inciting insult, the infiltration is composed of neutrophils, monocytes, macrophages and lymphocytes, as well as dendritic cells. The pathogenesis of the inflammatory disease is not unlike that described for immune mediated interstitial disease. The renal tubular epithelium (RTE) is increasingly identified as an accomplice in acute kidney injury, since RTE express toll-like receptors, possess inflammasomes, and produce inflammatory cytokines (Anders and Muruve 2011). The immune responses are implicated not only in the pathogenesis of the disease but also in the repair of the AKI. Since the inflammatory reaction and the tissue destruction in AKI tends to snowball out of control even when the inciting event is eliminated, AKI will be covered in this chapter as an immune-mediated disease.

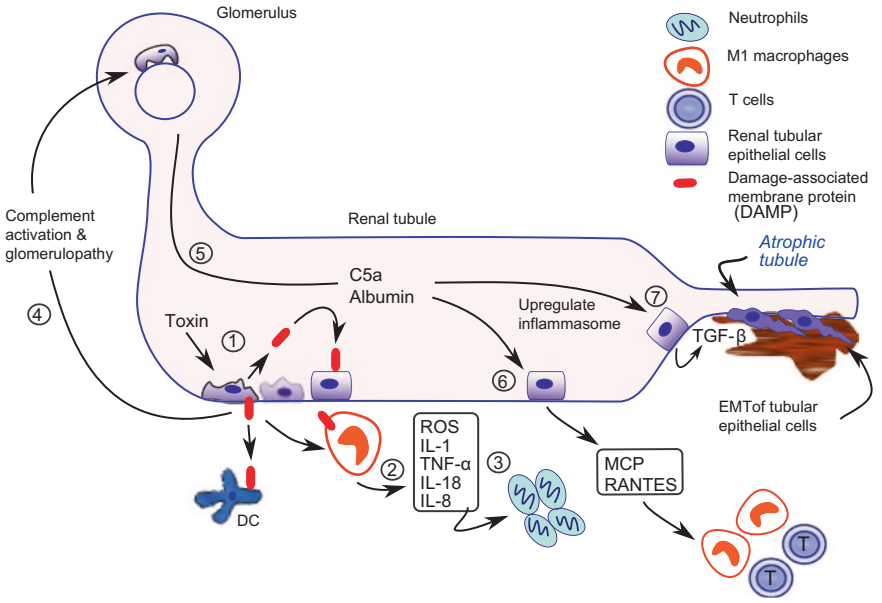

Fig. 6.16 Pathogenesis of non-septic acute kidney injury (AKI). One cause of AKI is toxicity that damages renal tubular epithelial (RTE) cells, resulting in exposure of damage markers such as damage associated membrane protein (DAMPS) (*1*). DAMPS bind to pattern-recognition receptors (PRR) on macrophages, dendritic cells, and RTE, followed by release of pro-inflammatory mediators ROS, IL-1, IL-8, IL-18 and TNFα (*2*). These mediators lead to further destruction of the tubular epithelial cells and capillary walls, and recruit neutrophils with possible formation of abscesses (*3*). Back at the site of initial insult, DAMPs also activate complement (*4*). Complement causes a glomerulopathy resulting in albuminuria and leakage of complement components (*5*). Intratubular albumin causes RTE cells to produce MCP1 and RANTES resulting in additional influx of macrophages and T cells (*6*). Complement components upregulate inflammasomes inducing RTE to produce TGF-β (*7*). TGF-β promotes fibrosis by two pathways. First, TGF-β causes transition of pro-inflammatory M1 macrophages into "wound healing macrophages" (M2 macrophages) that stimulate fibrosis. Secondly, TGF-β also encourages the RTE to undergo epithelial mesenchymal transition, and migrate to the interstitium to produce extracellular matrix. Eventually the tubule becomes atrophic. (Schematic by Cynthia Swanson)

An animal model for septic AKI is the administration of LPS. The best known model for aseptic AKI is the ischemia reperfusion injury (IRI) model, induced by surgically clamping *in vivo* the renal pedicles under normothermic condition, or under 4 °C conditions. The IRI model has been used to work out much of the pathogenesis of AKI. Another model of aseptic AKI is the nephrotoxic AKI model induced by cisplatin.

The pathogenesis of non-septic AKI is found in the attached schematic (Fig. 6.16). AKI is triggered by the binding of danger signals to renal tubular epithelial cells or interstitial dendritic cells or macrophages. These danger signals may be either PAMPs (pattern associated molecular patterns) from the surface of microbes (as in septic AKI), or they may be DAMPs (damage associated molecular

patterns, such as heat shock protein (HSP)), HIFs (hypoxia-induced factors), or other stress/injury factors such as reactive oxygen species, extracellular ATP, uric acid, nuclei acid, or extracellular matrix components such as hyaluronan and biglycan (Anders and Schlondorff 2000; Anders and Muruve 2011). In cases of septic injury, the microbe itself or part of the microbe is the danger signal. But in aseptic conditions, an initial insult such as toxicity or ischemia will produce these danger signals from renal tubular epithelial cells.

Once formed, the danger signals bind to pattern recognition receptors (PRRs) on the surface of adjacent or downstream renal tubular epithelial (RTE) cells. These PRRs include toll-like receptors (TLRs), retinoic acid- induced gene- like (RIG)-like receptors, nucleotide-binding domain-leucine-rich repeat (NLR) receptors (of which the nucleotide-binding oligomerization domain (NOD) is one), and C-type lectins. When bound, the pro-inflammatory NF-κB pathway is engaged, leading to production of pro-inflammatory cytokines and chemokines (especially IL-1β and IL-18) (Allam et al. 2012; Zhang et al. 2008; Lee et al. 2006; Hochheiser et al. 2011b). It has recently been discovered that in order to produce mature IL-1β and IL-18, another second pathway must be engaged, and that is the inflammasome-induced caspase-1 pathway. To form an inflammasome and activate the caspase pathway, a subfamily of NLR receptors, called NLRP (nucleotide-binding oligomerization domain, leucine-rich repeat and pyrin-domain containing) receptors must be involved. When NLRP is activated, not only is NFκB engaged, but there is formation of an inflammasome, which is a multi-protein complex that activates caspase 1. Caspase 1 allows for transformation of procytokines to mature cytokines (i.e., mature IL-1β and IL-18).

Once the PRRs on RTEs or the interstitial dendritic cells or macrophages are activated, there is production of many pro-inflammatory mediators, including IL-1β, IL-8, IL-18, TNFα, and ROS, and chemokines MCP-1 and CX3CL1. IL-8 recruits neutrophils. MCP-1 recruits macrophages and CX3CL1 (also known as fractalkine) recruits dendritic cells, macrophages, NK cells, effector T cells, and cytotoxic T cells (Furuichi et al. 2001; Wu et al. 2007; Allam et al. 2012; Leemans et al. 2005). Fractalkine can be either a soluble circulating or membrane-bound chemokine, and in the kidney it is positioned on apical surface of RTEs. This position facilitates recruitment and retention of leukocytes to the site of injury (Durkan et al. 2007). IL 18 induces INFϒ production, Th1 cell polarization, and Fas ligand-mediated cell death of renal tubular epithelial cells (Turner et al. 2014). Neutrophil recruitment may lead to abscess formation, and neutrophils perpetuate inflammation and tissue destruction by releasing proteases (e.g. elastases), oxygen radicals (Anders and Muruve 2011), and platelet activating factor (Nemoto et al. 2001; Rabb et al. 1994; Rouschop et al. 2005). For a complete review of the role of immune cells and the mediators they produce in experimental AKI, see Jang and Rabb (2015).

Role of Complement and C3 Glomerulopathy secondary to tubular injury. The initial insult to RTE cells will not only activate PRRs leading to inflammatory cytokine production, but the insult will also trigger complement activation and drive a C3-mediated glomerulopathy. Complement can be activated by either the alternative or lectin pathway. The high ammonia load within the tubules of diseases kidneys

activates C3 components in the tubular brush border resulting in activation of the alternate pathway (Nath et al. 1985). It should be noted that while there is a reduction in *overall* ammonia production by diseased kidney, there can be a doubling of the ammonia level *per nephron* (Nath et al. 1985; MacClean and Hayslett 1980). The lectin pathway is also activated because complement binds to C-lectin carbohydrate moieties (one of the PRRs) on the surface of injured tubular epithelial cells. Another cause for complement activation following injury to renal tubular epithelial cells is that damaged tubular epithelial cells (and in particular ischemic cells) have a reduced number of complement regulatory proteins. It should also be noted that proximal tubular epithelial cells can activate complement due to intrinsic convertase activity (Camussi et al. 1983). Eventually massive activation of complement leads to anaphylatoxins C3a and C5a that causes systemic (and glomerular) increased capillary permeability (McCullough et al. 2013). In the leaky capillaries of the glomerulus, circulating complement factors are able to leak into the mesangium and to the podocytes where they can cause sublytic damage by C5b-9 MACs (see Part VII). Albuminuria results. The C3a and C5a will also recruit neutrophils to the glomerulus and lead to further destruction of the GBM. Hence upstream glomerular injury is secondary to the downstream tubulo-interstitial inflammation in AKI.

C3 glomerulopathy secondary to tubular injury has been reported in monkeys associated with chronic administration of second generation antisense oligonucleotides (Frazier et al. 2014). In that study, test article deposited as granular material in proximal tubular epithelial cells within lysosomes. It was concluded that this tubular lesion led to a glomerulopathy in monkeys similar to C3 glomerulopathy.

Leakage of albumin and complement components into the glomerular filtrate will promote the tubulo-interstitial inflammation of AKI as described in preceding paragraphs. Albumin stimulates the proximal tubular cells to synthesize chemokines (MCP-1 and RANTES) that recruit monocytes and Th1 cells, and albumin upregulates inflammasomes in RTE cells, resulting in continual production of pro-inflammatory IL-1β and IL-18 (Chang et al. 2014). Albumin also causes the renal tubular epithelial cells to produce C3 that can then generate C3a and C5a (complement components). C3a and C5a (either generated following local production of C3 by the RTE or that leak from the glomerulus) promote increased extracellular matrix deposition, fibrosis, and scarring (Fearn and Sheerin 2015). This is because C5a can induce the production of TGFβ by RTE, and this TGFβ then signals to the interstitial fibroblasts to produce PDGF that promotes fibrosis (Boor et al. 2007). C5a may also shift the T cells to Th2 cells, with their cytokine response pattern (i.e., IL-13 and TGFβ) which has a pro-fibrotic action (Wynn 2004). To be sure, expanding evidence now suggests that complement derived by local synthesis (secondary to tubular protein) has a substantial effect on the progression to end stage renal disease.

The inflammatory reaction soon transforms into one that promotes tissue remodeling. For reasons that are not entirely clear, the pro-inflammatory environment of neutrophils, M1 macrophages, and Th1 lymphocytes that have been causing much of tissue destruction dissipates and the M1 macrophage/Th1 lymphocyte populations give way to a predominant M2 macrophages/T-reg environment. Production of complement components by the renal tubular epithelial cells may be one reason for

this phenotype switch (Wynn 2004). The forces that facilitate the change from pro-inflammatory cells to M2/T-reg repair are though largely unknown, but it is due to changes in the intrarenal microenvironment and involves adenosine signaling (Kinsey et al. 2012), CSF-1 signaling, and IL-1 receptor associated kinases (IRAKs) (Zhang et al. 2008; Lech and Anders 2013). Understanding the pathophysiology of AKI, and what tips the homeostasis in favor of T-regs and M2 phenotype, is a cornerstone of exploration of novel diagnostic and therapeutic strategies for AKI.

When T-regs and M2 phenotypes are favored, inflammation subsides and tubular regeneration occurs (Gandolfo et al. 2009). TGFβ is probably the primary growth factor that brings about these beneficial effects of reducing inflammation. However, with healing comes tubular atrophy and interstitial fibrosis, which may be beneficially to curtailing inflammation, but are certainly not beneficial to renal function. As in the glomerulus, the TGFβ instigates much of the tubulo-interstitial fibrosis and is produced by renal tubular epithelial cells. Angiotensin II, renin and aldosterone upregulate its production (Loeffler and Wolf 2014).

The parallels between the events happening in the glomerular podocyte and in the renal tubular epithelial cells is striking. Similar to what happens with crescent formation during chronic injury in the glomerulus, there is endothelial- mesenchymal and epithelial-mesenchymal transition (EndMT, EMT, respectively). Both endothelial cells and tubular epithelial cells become relatively undifferentiated mesenchymal cells similar to myofibroblasts. TGFβ and PDGF orchestrates this transition as they do in the glomerulus. The transitioned cells produce extracellular matrix resulting in interstitial fibrosis and thickened tubular basement membranes. As tubular epithelial cells transition to interstitial myofibroblasts, they lose their cell to cell contacts (i.e., loss of E-cadherin and ZO-1), express mesenchymal markers, and migrate into the interstitium, resulting eventually in tubular loss (Loeffler and Wolf 2014; Hills and Squires 2011; Liu 2004; Yang and Liu 2001; Bottinger and Bitzer 2002; Lopez-Hernandez and Lopez-Novoa 2012).

TGFβ aids in their migration through the tubular basement membrane since TGFβ induces proteases matrix metalloproteinase-2 and matrix metalloproteinase-9 that disrupts intact TBM to allow for this migration (Loeffler and Wolf 2014). The contribution of EMT to renal fibrosis differs among kidney diseases, and occurs more in unilateral ureteral obstruction and ischemic nephropathy in SJL mice, rather than Adriamycin nephrosis or nephrotoxic serum nephritis (Inoue et al. 2015). TGFβ also induces fibrosis through means other than EMT and EndMT. TGFβ induces fibroblast activation through mammalian TOR (mTOR) kinase signaling (Li et al. 2015).

In general, the repair process is maladaptive leading only to further functional deficiencies of the kidney, reduced vascular perfusion (especially as endothelial cells are also transitioning into mesenchymal cells), and scarring. The compensatory mechanism of fibrosis results in a vicious progression to end stage disease (Liu 2011).

Tubular hypertrophy is sometimes associated with interstitial fibrosis. The same growth factors that cause fibrogenesis (e.g. TGFβ and PDGF) are the same growth factors that induce cellular hypertrophy (Wolf and Ziyadeh 1999). The hypertrophy

however is temporary. Eventually, fibrosis and tubular atrophy represent the final common pathway of all kidney disease leading to chronic renal failure (Hills and Squires 2011; Zeisberg and Neilson 2010).

Protective Strategies. The kidney has some protective mechanisms to control against run-away inflammation associated with tubulo-interstitial disease. Progranulin (PGRN) is a glycoprotein produced by RTE that limits inflammation associated with AKI, and it is believed to work by several mechanisms. First, it interferes with the TGF-mediated induction of NOD2 expression. Recall NOD2 is one of the pattern recognition receptors for DAMPs. Next, PGRN blocks TNF receptors thus interfering with the pro-inflammatory actions of this mediator. Next, PGRN promotes the expansion of regulatory T cells (Hu et al. 2014). The end result is that PGRN attenuates production of inflammatory cytokines and chemokines and infiltration of leukocytes (Tadagavadi and Reeves 2015; Zhou et al. 2015).

There are numerous antimicrobial and immuno-suppressive peptides and proteins in the kidney that help modulate the immune responses in the tubulo-interstitium, such as Tamm Horsfall protein, defensins and calgranulins. Since these defense mechanisms are present also in the lower urinary tract, these immune-modulators will be discussed under that section.

Autophagy may also be considered a protective mechanism in AKI or in any form of renal disease for that matter. Autophagy is an intracellular catabolic process that contributes to homeostasis, and there is emerging evidence that renal autophagy plays a protective role in kidney diseases, and in particular acute and chronic kidney injury (Leventhal et al. 2014; Kaushal 2012). Autophagy controls inflammasome function and can modify the intrarenal inflammatory milieu, and prevent immune cell infiltration. It does this by mediating the clearance of altered mitochondria and removal of protein aggregates such as DAMPS, which otherwise activate inflammasomes and apoptotic pathways (Fougeray and Pallet 2015). Autophagy however may also promote inflammation, since autophagy can promote the ability of renal epithelial cells to act as antigen presenting cells (Leventhal et al. 2014). Because autophagy may either promote or protect against kidney injury, further research is needed to understand the role of this system in different renal diseases. Developing novel agents that promote or inhibit various steps in the autophagy pathway, and determining their use in controlling the progression of various kidney diseases, is a ripe area research (Leventhal et al. 2014).

Multi-organ involvement following AKI. AKI, and especially ischemic AKI, has grave implications and commonly results in remote organ failure through multi-organ cross-talk. This cross-talk is due to cytokines, and results in such far-reaching injury as apoptosis of lung epithelium; apoptosis and oxidative stress of hepatocytes; inflammation in the liver; activation of microglia in the brain; increased vascular permeability in the brain; and apoptosis and neutrophil trafficking in the heart (White and Hassoun 2012).

Animal Models of AKI: Cisplatin-Induced Acute Kidney Injury in mice or rats is an animal model of aseptic AKI in humans. The pathogenesis of the toxicity has been well elucidated. It involves direct injury to proximal tubular epithelial cells causing oxidative stress, followed by apoptosis or necrosis, and inflammation

(Ozkok and Edelstein 2014). In addition to the toxic injury, there is also a direct endothelial damage resulting in ischemia of the renal medulla. Within the RTE, cisplatin depletes glutathione rapidly, resulting in oxidative stress. Oxidative stress or ER stress leads to activation of proapoptotic Bax and Bak proteins. Apoptosis can also be induced by activation of Fas and TNFα receptors on the surface of RTE followed by downstream activation of caspases. In any event the DAMPs on the apoptotic cells bind to TLR4 stimulating production of pro-inflammatory cytokines, including IL-1β, IL6, IL-18, IL-33, TNFα, MCP1, fractalkine (CXC3), RANTES, and TGFβ. By a series of experiments using animal models deficient in one or more cell types or in one or more cytokines, it has been determined that the IL-33 and TNFα are responsible for most of the injury however (Akcay et al. 2011). IL-33 is a IL-1-like pro-inflammatory cytokine that is proposed to be released from necrotic (but not apoptotic) cells, bind to immune cells, and increase secretion of pro-inflammatory cytokines (Lamkanfi and Dixit 2009). IL-33 also is a chemoattractant for CD4+ T cells, and these activated T lymphocytes express Fas-L and cause apoptosis of Fas-bearing RTE. Interestingly enough, RTE cells also express Fas-L and participate in the fratricide killing of its own cell type (Linkermann et al. 2011). Mast cells containing preformed TNFα potentiate the injury. Cisplatin is not a model of pure toxic AKI. Cisplatin also induces an ischemia to the renal medulla, due to its vasoconstrictive effects associated with endothelial dysfunction.

Treatment of WAG-strain rats with N-(3,5-dichlorophenyl)-succinimide induces a tubulo-interstitial nephritis with prominent interstitial renal fibrosis, and could be considered an animal model for AKI. The chemical causes initial areas of focal necrosis of proximal tubules (Barrett et al. 1983).

6.8 Immune Deficiency Associated with Chronic Renal Disease

In the first parts of this chapter, the role of the immune system is causing and perpetuating renal disease was described. However, renal disease also has an effect on the immune system. Chronic renal disease causes a condition known as uremia, which results in immunodysfunction. Uremia is a systemic condition secondary to chronic renal disease that is due to high blood urea nitrogen and high serum creatinine. The uremic state causes simultaneous immunosuppression, immune activation, and hypercytokinemia (Fig. 6.17). The immunosuppression contributes to high prevalence of infection, and infection is one of the underlying causes for the high mortality in patients with end stage kidney disease. Uremia causes a suppression of the innate and the adaptive immune response. In particular uremia is associated with lower antigen presenting capabilities of DC and macrophages due to altered costimulatory molecules CD 80/86; depletion and impaired inhibitory activity of T-regs; B cell lymphopenia and impaired humoral immunity; and depletion of dendritic cells and memory T cells (Vaziri et al. 2012).

Uremia is also associated with immune activation, characterized by increased expression of TLR2 and TLR4; increased cytokine production; upregulation of pattern recognition receptors (DAMP, HIF); spontaneous activation, degranulation and increased reactive oxygen species production by neutrophils; complement activation (due to increased levels of mannose binding lectin); loss of regulatory T cells; and increased proportions of pro-inflammatory subsets of T cells and monocytes (Vaziri et al. 2012; Satomura et al. 2002; Betjes 2013; Kato et al. 2008). The pro-inflammatory subsets of leukocytes is similar to that of healthy elderly individual, which has led some people to suggests that uremia causes premature ageing of the immune system (Betjes 2013).

Hypercytokinemia is also a typical feature of uremia. In chronic kidney disease, cytokines IL-6, IL-1 and TNFα are elevated. Increased levels of circulating cytokines is due to (1) decreased renal elimination of cytokines in the circulation associated with lower GFR; and (2) increased generation of these cytokines due to uremic toxins; and (3) the loss of T-reg function that would otherwise suppress inflammation (Vaziri et al. 2012). Hypercytokinemia is associated with high prevalence of cardiovascular disease, and contributes to the high mortality in patients with end stage kidney disease. Cytokines promote atherosclerosis since they enhance production of adhesion molecules and increase Th1/Th2 ratio of T cell subsets.

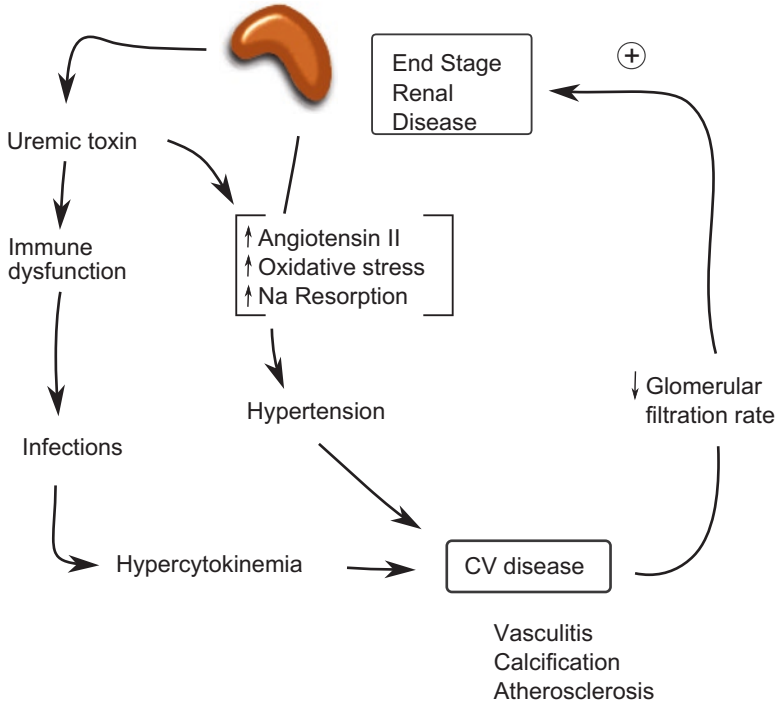

Fig. 6.17 Kidney disease and systemic effects

Increased amounts of circulating pro-inflammatory cytokines on one hand, yet functional immunosuppression on the other seems counterintuitive. In a nutshell, functional immunosuppression leads to increased infection and infection leads to increased inflammation and hypercytokinemia. This relationship is another example of how any inflammation in the kidney can snowball: Kidney inflammation causes renal disease/uremia which causes immunodeficiency, then infections, and then more inflammation (Fig. 6.17).

Hypercytokinemia leads to atherosclerosis and cardiovascular disease. In particular hypercytokinemia promotes adhesion of leukocytes to vascular endothelium and early plaque formation. The general pro-inflammatory state also is associated with upregulation of fibrinogen, lipotriene-α, and C reactive protein, which are associated with cardiovascular disease (Kato et al. 2008). The uremia associated with chronic renal disease is not the only cause for cardiovascular disease. Chronic renal disease also causes hypertension. The animal model for hypertension (Dahl salt-sensitive hypertensive rats) has allowed pathologist to determine the causative connection between renal disease and hypertension. Lymphocytes and macrophages result in increased renal oxidative stress that causes increased production of angiotensin II, and increased reabsorption of sodium (Rodriguez-Iturbe et al. 2002; Mattson et al. 2006). Uremic toxins can also cause the damaging oxidative stress that contributes to this hypertension. Natural uremic toxins such as paracresol sulfate and indoxylsulfate stimulate NADPH oxidase in endothelial cells with increase ROS formation. With widespread endothelial damage, the heart, kidney, and vascular cardio renal equilibrium is disrupted (Gajjala et al. 2015).

With immunodeficiency for whatever reason, be it secondary to uremia or due to a primary effect of test article, the pathologist should be on the lookout for infectious diseases in the kidney that are associated with immunodeficiency. Examples include Polyoma virus infection in Cynomolgus monkeys (Fig. 6.18) or *Candida albicans* infection (van Gorder et al. 1999). Polyoma viruses are ubiquitous but can cause overt disease in immunocompromised individual. The Simian Polyoma virus affects multiple organs, but can cause a tubulo-interstitial nephritis. Oftentimes, the infection associated with the immunodeficiency will instigate an innate immune response leading to tubulo-interstitial nephritis. Such is the case with Polyoma virus infection in monkeys.

6.9 Spontaneous Disease in Rats

Chronic progressive renal disease is a spontaneous tubulo-interstitial disease of rats. It is of unknown etiology that is affected by protein and caloric intake and male sex hormones. CPN is characterized by primarily a tubulo-interstitial disease with glomerular involvement (Fig. 6.19). There is kidney enlargement, with dilated tubules filled with casts, notable absence of vascular changes, variable degrees of inflammation, and no known primary immunologic or autoimmune basis. The kidney has increased weight and can exceed twice of more that of the mature breeder

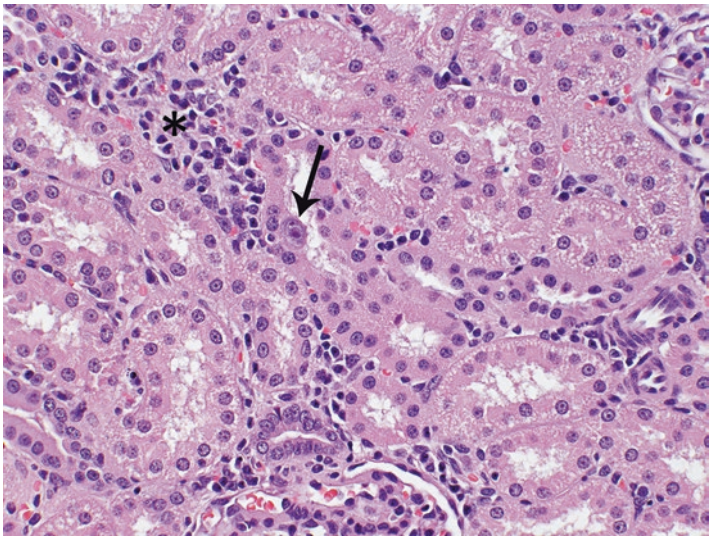

Fig. 6.18 Viral nephritis in cynomolgus monkey. This section is the interstitium of the kidney of a cynomolgus monkey that has a test article-related immunomodulation. The immunomodulation results in expression of a cryptic viral infection, probably cytomegalovirus or polyoma virus, with resultant tubulo-interstitial nephritis that includes infiltrations of mononuclear inflammatory cells (*asterisk*). Note the enlarged tubular epithelial cell with the intranuclear inclusion body (*arrow*). H&E stain. 20× objective magnification (Image courtesy of Dr. George A. Parker)

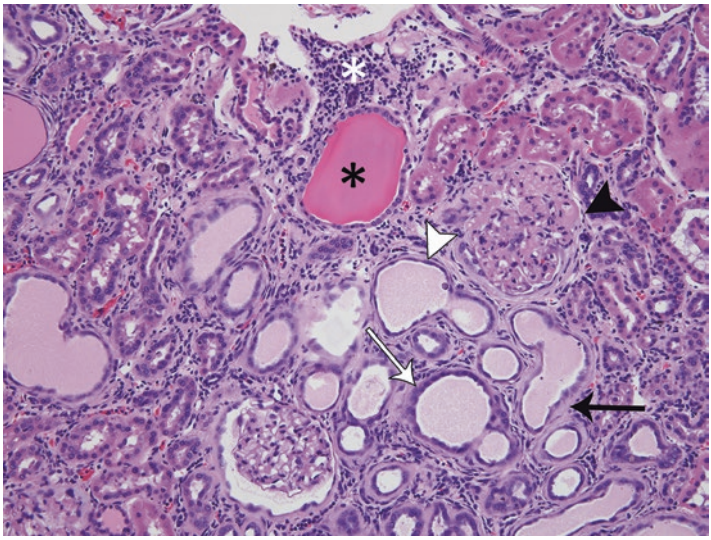

Fig. 6.19 Chronic progressive nephropathy in rat. This section of kidney from an adult rat shows the typical appearance of spontaneous chronic progressive nephropathy. The lesion is typified by peritubular fibrosis or thickened tubular basement membranes (*black arrow*) and basophilic tubular epithelial cells (*white arrow*). Commonly there is interstitial inflammation (*white asterisk*), tubular protein and protein casts (*black asterisk*), atrophic tubules lined by flattened epithelium (*white arrowhead*) and commonly a focal membranoproliferative glomerular lesion. Note the thickened capillary loop in this affected glomerulus (*black arrowhead*). From this appearance of CPN, it is easy to see why toxic changes or a primary immune-mediated glomerulopathy would be difficult to detect in kidneys affected by underlying spontaneous CPN disease. H&E stain. 20× objective magnification

rat. Protein casts, basophilic tubules and thickened basement membranes are widely scattered early in the disease. The triad of findings (protein cases, basophilic tubules, and thickened tubular basement membranes) has no human counterpart. CPN has features of not only degenerative disease, but there are regenerative changes as well, including tubules with a high proliferative rate (Hard and Khan 2004; Hard et al. 2009, 2013). As disease progresses there are secondary and adaptive changes including inflammation, fibrosis and glomerulosclerosis. While CPN is considered a tubulo-interstitial disease primarily, there are early ultrastructural changes in the glomerulus such as swollen endothelial cells and hypertrophied podocytes with swollen foot processes.

The cause of CPN is unknown. The disease has a genetic component and is more prevalent in the Sprague-Dawley and Fischer 344 strains than in the Wistar, Lewis or Brown-Norway strains. Its importance arises because the toxicologic pathologist must recognize its spontaneous occurrence and not confuse the lesion with test article-related kidney disease. Some chemicals exacerbate CPN, but these equivalent baseline or exacerbations are not seen in humans, and therefore exacerbation of CPN has little human relevance in the preclinical safety assessment. While the cause of CPN has not been established, lesion development has some features of delayed type hypersensitivitiy reaction, resulting in combined degenerative and regenerative changes. That low protein diets help minimize CPN is consistent with the fact that low protein diets help abrogate anti-TBM disease in rodents (Kelly et al. 1985).

CPN also exists in mice, where there is questionable sex predilection (Ettlin et al. 1994), and the lesion generally has a more prominent glomerular involvement than in rats. CPN in mice is discussed below under spontaneous glomerulonephritis in mice (below).

Spontaneous Tubulo-interstitial Nephritis. Spontaneous tubulo-interstitial nephritis can occur in rats, but is difficult if not impossible to distinguish from the lesion known as spontaneous CPN in rats. In safety toxicology studies, any tubular toxic renal injury generally results in a condition referred to as exacerbated CPN. Straight forward tubulo-interstitial nephritis is easier to identify in the dog, where baseline CPN does not complicate the lesion. Tubulo-interstitial nephritis may be focal or generalized and commonly has a mixed mononuclear inflammatory cell infiltrate composed of lymphocytes, plasma cells and macrophages with fibrosis. Neutrophils are rare (Hard et al. 1999).

Spontaneous tubulo-interstitial disease in mice is infrequent. It can be infectious and caused by bacteria (Proteus, Pseudomonas, Leptospirosis), or it can be due to an immune reaction to virus such as Chronic Lymphocytic Choriomeningitis. Polyoma virus in Cynomolgus monkeys is another cause for interstitial nephritis. Leptospirosis can cause tubulo-interstitial disease in all species; Encephalitozoon can be the cause of tubulo-interstitial disease in dogs and rabbits; and Ehrlichia and adenovirus can cause tubulo-interstitial disease in dogs.

A unique tubulo-interstitial disease exists in CBA/J mice. It is characterized by interstitial infiltrate of lymphocytes and macrophages at the corticomedullary junction with multinuclear giant cells and destruction of tubules (Rudofsky 1978).

Spontaneous Glomerulonephritis in Laboratory Animals. When confronted with spontaneous glomerulonephritis in pre-clinical safety toxicity studies, it is recommended to take 2 μm paraffin sections stains with PAS and/or appropriate silver techniques to define the morphologic pattern of the different types of glomerulonephritis. In preclinical safety studies, it is most important to distinguish any spontaneous disease from test article-related glomerulonephritis. The morphologic diagnosis of either mesangioproliferative GN (MesPGN), membranoproliferative GN (MPGN), membranous GN (MGN), or crescentic GN can and should be distinguished. The morphologic diagnosis of **mesangioproliferative GN** is made when there is axial accumulation of PAS positive mesangial matrix with mesangial cell proliferation. There may also thickening of Bowman's capsule with occasional synechia. If intracapillary neutrophils are present within these proliferative tufts, then the term exudative glomerulonephritis or intracapillary proliferative glomerulonephritis may be used. The morphologic diagnosis of **membranous GN** is made when there is a glomerulus with normal cellularity, but with thickened GBM and capillary basement membranes. The morphologic diagnosis of **membranoproliferative glomerulonephritis** is made when there is a mixture of both mesangial cell proliferation and thickened GBM and capillary loops, and increased mesangial matrix. There may be an increase in mesangial matrix, as well as spikes (indicating subepithelial complexes) or wire loops (indicating subendothelial complexes). The morphologic diagnosis of **crescentic GN** should be made when the glomerulus has crescents, adhesions between the capsule and the tuft, increased matrix, and proliferation of parietal and visceral epithelial cells (Hard et al. 1999).

Despite the large number of experimental animal models of glomerulonephritis in rats, spontaneous glomerulonephritis is not common in rats. It is however common in mice. **Spontaneous glomerulonephritis in mice** is likely the same condition as that described as CPN in mice. In one report, this primary glomerulonephritis was associated with deposition of IgG and IgM in older mice (Yumura et al. 1989) and characterized by initial mesangial proliferation, glomerulosclerosis and albuminuria. The kidneys of 50 1-year old C57BL/6 mice were examined and 48 of them had histologic evidence of mild to moderate glomerulosclerosis. All 50 animals had deposits of IgG and 41 of the 50 had C3 in the mesangium. When eluted, 57% of the Ig reacted with murine leukemia virus cell surface antigen. Therefore, the spontaneous glomerulonephritis in mice appears to be an immune complex glomerulonephritis triggered by murine leukemia virus antigen (Andrews et al. 1979). The glomerular change is characterized by a mesangial proliferation, glomerulosclerosis, and thickening of Bowman's capsule. The interstitial change is characterized by thickened tubular basement membranes, intratubular eosinophilic casts, and an interstitial mononuclear cell infiltrate (Wolf and Hard 1996). Terms such as CPN, glomerulosclerosis, and mesangioproliferative glomerulopathy have been used to refer to this spontaneous glomerulonephritis in mice.

There is a **mesangioproliferative glomerulopathy in rats** characterized by hypertrophic mesangial cells and increased mesangial matrix along the axial distributions. It has been reported following morphine administration (Weber et al. 2008) and in genetically manipulated mice with thrombopoietin overexpression (Shimoda et al. 2007).

A **hyaline glomerulopathy** in B6C3F1 mice has been reported by Wojcinski et al. (1991). The PAS-positive, Congo-red negative, hyaline deposits were reported to be immunoglobulin complexes that localized along glomerular capillary walls and are composed of granular amorphous material suggestive of immune complex deposition, and may be composed of fibrils in a finger-like pattern on electron microscopy (Frazier et al. 2012). Hyaline glomerulopathy is considered a rodent specific syndrome (Frazier et al. 2012). Hyaline glomerulopathy is characterized by loss of cellularity of the glomerular tufts, unlike glomerulonephritis which generally is membranoproliferative and may have mononuclear cell infiltration. It has been reported following chronic administration of antisense oligonucleotides (Frazier 2015). Ultrastructurally, the hyaline glomerulopathy induced by antisense oligonucleotides were characterized by thickened irregular endothelial lining, hypertrophic endothelial cells, thickened reduplicated capillary basement membranes, and electron dense and electron lucent deposits in the membrane. The endothelial cell was the apparently the cell of target, and these cells are sensitive to cytokine mediated injury. There was upregulation of CD68 gene, which have been associated with TLR activity and amyloid formation, suggesting that immune stimulation has a role in pathogenesis of this glomerular injury.

Spontaneous glomerulonephritis in nonhuman primates is occasionally noted in the literature, yet it is sporadic (Andrews et al. 1979). Histologically, the lesion is a mesangioproliferative (or mesangiocapillary) glomerulonephritis. The glomeruli are enlarged and have lobular proliferation of mesangial cells and increased mesangial matrix. By light microscopy using silver stain, there is mesangial interposition between the GBM causing a double-contoured membrane {Sato, 2012 #7759}. One study reported that the GBM is thickened in approximately 10% of control Cynomolgus monkeys and electron dense deposits may be found in the subendothelial, subepithelial or mesangial regions with podocyte foot process fusion in 20% of control cynomolgus monkeys (especially those of Chinese origin as opposed to those from Mauritius) (Khan et al. 2013). Therefore, subclinical glomerulonephritis may complicate the evaluation of kidney tissue in monkeys on preclinical safety studies.

Membranoproliferative glomerulonephritis in Owl Monkeys. A spontaneous membranoproliferative GN has been described in owl monkeys (*Aotus trivirgatus*), which is characterized by electron dense deposits in the GBM to the subepithelial area, with segmental thickening of the GBM, thickening and reduplication of the capillary basement membrane, mesangial cell proliferation and increased mesangial matrix. Adhesions to a thickened Bowman's capsule with periglomerular fibrosis and an interstitial infiltrate of lymphocytes, eosinophils and plasma cells further characterize the spontaneous lesion (King et al. 1976) (Figs. 6.20 and 6.21). It has been associated with hemolytic anemia, and these two diseases are likely connected, but that connection has not been determined (Chalifoux et al. 1981).

Spontaneous Glomerulonephropathies in Dogs. Glomerulopathies occur spontaneously in dogs with a median age of 6–8 years (Schneider et al. 2013). Spontaneous glomerulonephritis is rare in young dogs, but immune complex glomerulonephritis has been reported in a dog as young as 4 months of age (Schneider

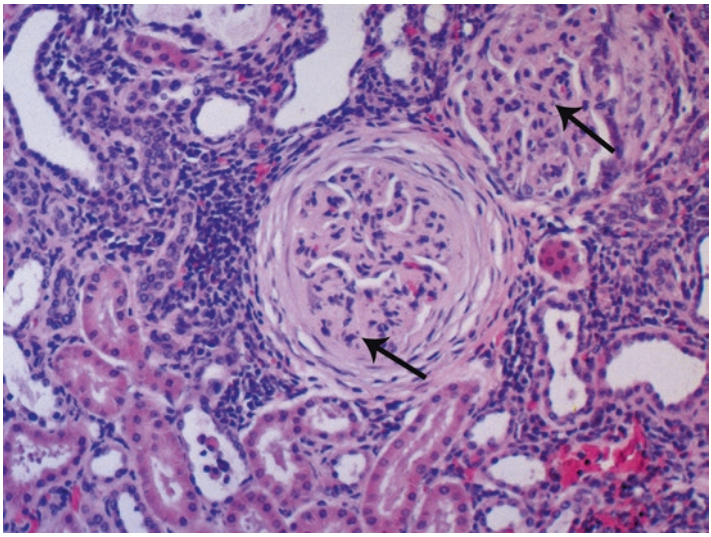

Fig. 6.20 Glomerulonephritis in owl monkey (*Aotus trivirgatus*). This section is from an owl monkey with spontaneous glomerulonephritis. There is proliferation of cells and matrix within the tuft that is adherent to Bowman's capsule. Note the pronounced periglomerular fibrosis and the infiltrate of lymphocytes and plasma cells in the periglomerular interstitium. The glomerulus in the upper right corner of the image has less sclerosis, but shows evidence of cellular proliferation and increased mesangial matrix. Necrotic cell debris is occasionally noted within the affected tufts (*arrows*). These changes in the kidney are nonspecific yet compatible with subepithelial and intramembranous deposition of immune complexes. H&E stain. 20× objective magnification (Image courtesy of Dr. George A. Parker)

et al. 2013; Cianciolo et al. 2016). Therefore, for the toxicologic pathologist evaluating tissues from dogs less than 2 years of age on pre-clinical safety studies, spontaneous immune-mediated renal disease is rare. Spontaneous glomerulopathies in dogs have received considerable attention recently as there is effort to classify the various etiologic, clinical and morphologic presentations of the disease (Cianciolo et al. 2016; Schneider et al. 2013). Of the causes for spontaneous glomerulonephritis in dogs, 48% represent immune complex glomerulonephritis (ICGN), and as the name implies, is due to immune complex deposition (Schneider et al. 2013). Other leading causes of glomerulopathies include primary glomerulosclerosis (20%), amyloidosis (15%) and nonimmune complex glomerulopathy (9%) (Schneider et al. 2013).

Since ICGN is the most common immune-mediated condition in dogs, it will be further discussed and illustrated herein. ICGN in dogs presents in a membranous or membranoproliferative pattern (Figs. 6.25, 6.26, 6.27, 6.28, 6.29, 6.30, 6.31, and 6.32). The high volume of spontaneous cases of canine ICGN in veterinary medicine and the opportunity to obtain renal biopsies of clinical cases offer an excellent opportunity to showcase some of the pertinent features of glomerulonephritis in mammals.

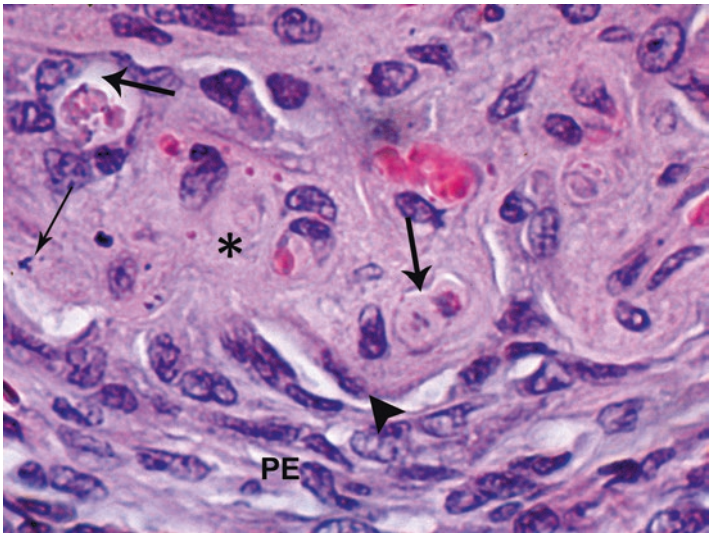

Fig. 6.21 Glomerulonephritis in owl monkey (*Aotus trivirgatus*). This high power section of an affected glomerulus is taken at a point where the glomerular tuft adheres to the parietal epithelium (PE) lining Bowman's capsule. The proliferating spindle shaped cells making up the parietal epithelial layer likely represent a proliferation of parietal epithelial cells as a result of epithelial-mesenchymal transition. The capillaries in the tuft have intracapillary hyaline thrombi (*thick arrows*) which may represent coagulated blood or immune complexes. There is abundant mesangial matrix (*asterisk*). An elongated spindle-shaped cell in the location of a glomerular podocyte (*arrowhead*) represents epithelial mesenchymal transition of either the visceral or parietal epithelial cells. Note the necrotic cell debris (*thin arrow*) suggesting a necrotizing component to this particular lesion. H&E stain. 40× objective magnification. (Photo courtesy of Dr. George A. Parker)

Primary glomerulosclerosis in dogs is a diagnosis of exclusion made when there are light microscopic features of sclerosis of the glomerular tuft, and when electron microscopy rules out the presence of immune complexes. Primary glomerulosclerosis in dogs is generally limited to dogs over 2 years of age, and is focal and segmental (similar to FSGS in humans). As in man, primary glomerulosclerosis represents an abnormality in the podocyte, which can be primary or secondary after insults due to hypertension, obesity, diabetes (Schneider et al. 2013). Histologically there is focal and segmental solidification of the capillary tuft of at least one glomerulus (Fig. 6.33), and this solidification is due to mesangial matrix expansion, effacement of the capillary lumen, and mesangial hypercellularity (Cianciolo, et al. 2016). Ultrastructurally, there is non-specific foot process effacement which typifies the pathogenesis of podocyte injury (Fig. 6.34) but there may also be GBM rarefaction, mesangial cell interpositioning, microvillus transformation of podocytes, and synechia. While not a classic and obvious immune-mediated disease like that of ICGN, the immune system is likely involved in the etiology and/or progression of disease. After all, the pathogenesis of human FSGS and minimal change nephropathy involves immune dysregulation, immune mediators, and/or type I hypersensitivities and it is reasonable to assume that there is similar involvement of the immune system in the canine counterparts.

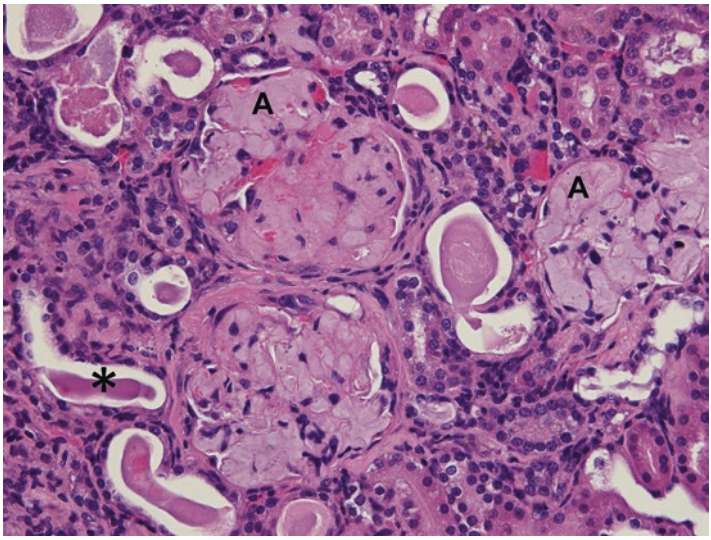

Fig. 6.22 Amyloid in mouse kidney. This section of a kidney from a mouse in a chronic 2-year study shows the commonly-encountered spontaneous amyloid deposition in the glomerular tufts. Amyloid (A) is a pale pink homogenous material on H&E stain stained sections, and can be definitively differentiated from mesangial matrix by Congo Red stain (not shown). The protein casts within adjacent tubules (*asterisk*) and the associated tubule-interstitial nephritis characterized by a diffuse infiltration of mononuclear inflammatory cells in the interstitium are common sequel. Amyloid destroys the filtration integrity of the glomerular basement membrane. Intra-tubular albumin activates inflammasomes. H&E stain. 40× objective magnification. (Photo courtesy of Dr. George A. Parker)

Amyloidosis in laboratory animals. Although relative common in Syrian hamsters and specific strains of mice especially CD-1, renal amyloidosis is rare in rats. In mice and rats, the glomerular amyloid may involve the tubular interstitium. In rats, there is glomerular, peritubular and/or interstitial deposits of eosinophilic amyloid that stains positive with Congo red (Hard et al. 1999). The most prominent amyloid deposits will be in the glomerulus (Seely 1999) (Fig. 6.22). In mice, renal amyloid is seen in the CD-1, SJL, C57Black and less commonly in the B6C3F1 mouse, where its incidence is less than 1% (Frith and Chandra 1991). The incidence and severity of amyloidosis increases with age, and may be variable with stress, caging, and pathogen status (Lipman et al. 1993).

Amyloidosis is not a spontaneous disease in dogs less than 2 years of age on preclinical toxicity studies. It can be seen in about one-sixth of dogs with spontaneous glomerulopathies, is most commonly seen in Chinese Shar Peis and English Bulldogs, and the earliest age it presents clinically is 3.6 years in the Shar Pei (Cianciolo et al. 2016; Segev et al. 2012). Though not directly related to toxicologic pathology and immunotoxicology, scientists involved in these fields should be aware of the animal model known as systemic amyloidosis in Chinese Shar Pei dogs (DiBartola et al. 1990). Affected dogs have recurrent fever of unknown origin (known clinically as 'Shar Pei fever'), which is associated with elevated circulating levels of IL-6 (Rivas et al. 1992). The disease appears to have a genetic basis (DiBartola et al. 1990), and is proposed as a model for a somewhat similar disease in humans (Familial Mediterranean Fever) (Rivas et al. 1992).

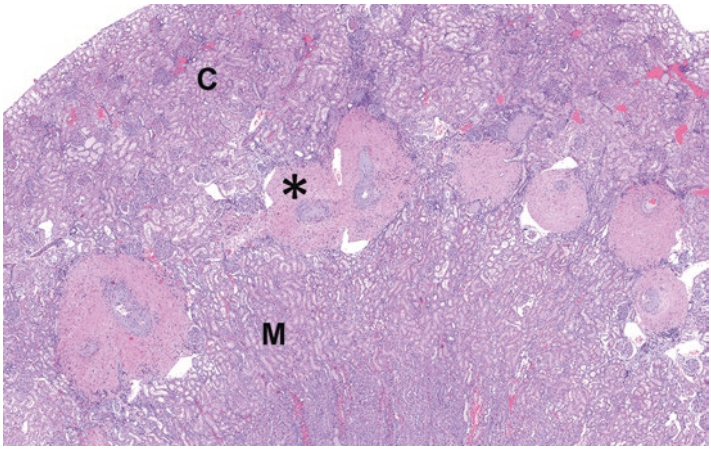

Fig. 6.23 Polyarteritis nodosa in kidney of rat. This section of kidney from an adult rat with polyarteritis nodosa shows the marked thickening of the walls of the medium sized arteries at the cortico-medullary junction. Each of the affected arteries has marked perivascular fibrosis (*asterisk*) forming prominent nodules that can often be visualized grossly. Cortex (C); Medulla (M). H&E stain. 27× objective magnification

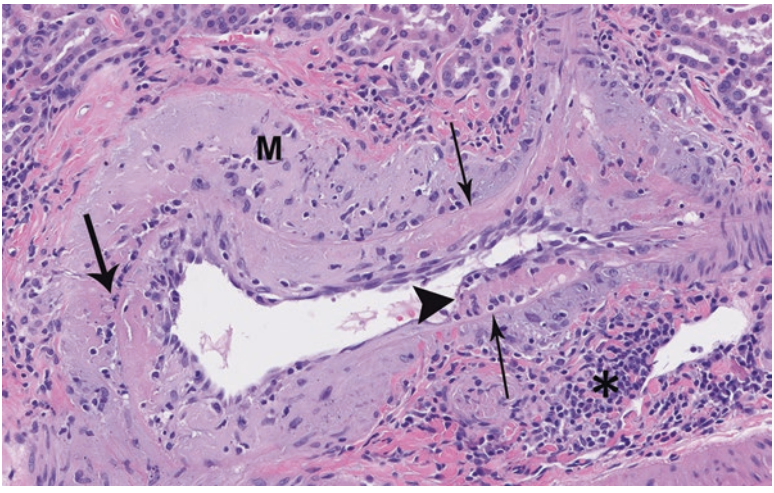

Fig. 6.24 Polyarteritis nodosa in kidney of rat. This section is a high power of a medium sized artery in the kidney affected by polyarteritis nodosa. This lesion is a necrotizing vasculitis, with intimal thickening (*arrowhead*) on the luminal side of the elastic lamina (*thin arrows*), fibrinoid necrosis of the vessel wall (*arrow*), thickening or medial hypertrophy of smooth muscle wall (M), and a perivascular infiltration of lymphocytes and macrophages (*asterisk*). This vessel does not have the prominent perivascular fibrosis that is typical of the more chronically affected vessels shown in Fig. 6.23. H&E stain. 40× objective magnification

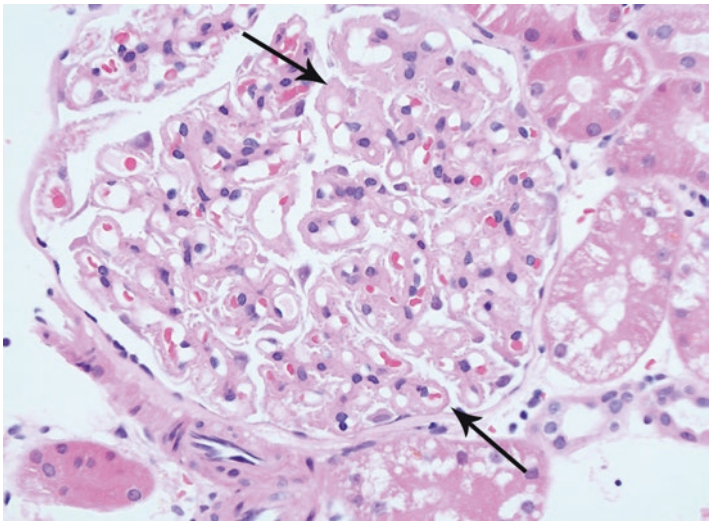

Fig. 6.25 Membranous glomerulonephritis in dog (H&E). This image illustrates membranous glomerulonephritis in a Doberman Pinscher dog. The glomerular tuft has thickened capillary walls (*arrows*) without an appreciable increase in mesangial cell number. H&E stain, 40× objective magnification (Image courtesy of Dr. Rachel E. Cianciolo)

Periarteritis/polyarteritis. Periarteritis (or polyarteritis nodosa) is a chronic immune mediated lesion in rats and mice, Beagles, and has been reported in the cat, pig and non-human primates (Porter et al. 2003; Snyder et al. 1995; Hamir 1980; Altera and Bonasch 1966) (Fig. 6.23 and 6.24). It affects medium-sized muscular arteries and can affect the kidney. There is fibrinoid necrosis of the arterial wall, disruption of the internal elastic lamina, and a neutrophilic reaction with eosinophils. Chronic lesions are characterized by a perivascular mixed chronic inflammatory infiltrate, thickened muscular walls with medial and adventitial fibrosis, and luminal narrowing or obliteration. The lesion has an immune component and can be associated with type III immune complex deposition. Most speculation is that immune complexes deposit with subsequent activation of the complement cascade, neutrophil and monocyte chemotaxis, and release of lysosomal enzymes, oxygen radicals and pro-inflammatory mediators. The release of vasoactive amines from platelets increase the vascular permeability allowing additional intramural deposition of immune complexes. Elevated numbers of CD4+ T cells and macrophages point to a delayed type hypersensitivity (cell-mediated) immune response in addition to a type III hypersensitivity. The lesion can also develop as a result of hypertension, and particularly angiotensin II, in rats. The ACE inhibitor captopril prevents production of formation of angiotensin II and development of PAN in rats (Peters et al. 2010). This disease entity underscores the intimate relationship between the cardiac, vascular, and renal systems.

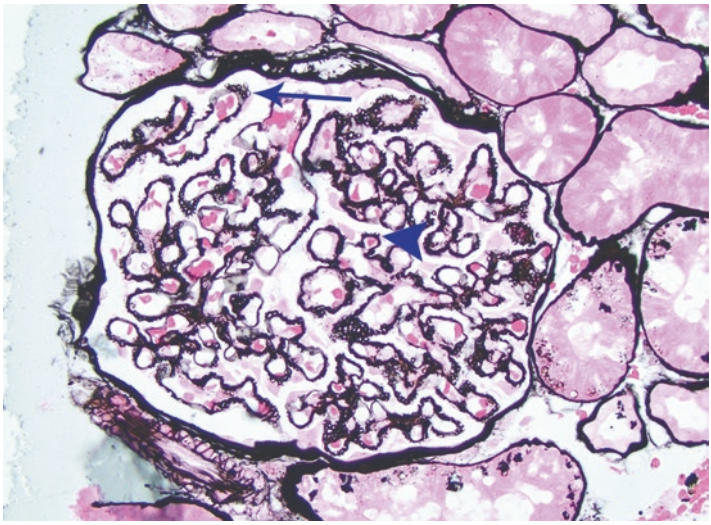

Fig. 6.26 Membranous glomerulonephritis in dog (Silver Stain). This image represents membranous glomerulonephritis in a Doberman Pinscher dog. Spikes (*arrowhead*) protruding from the glomerular basement membrane (GBM), and holes (*arrow*) within the GBM are visible with silver stains. The spikes represent GBM matrix forming on the sides of non-stained immune complex deposits. The clear holes represent unstained immune complex deposits. Jones methenamine silver stain. 40× objective magnification (Image courtesy of Dr. Rachel E. Cianciolo)

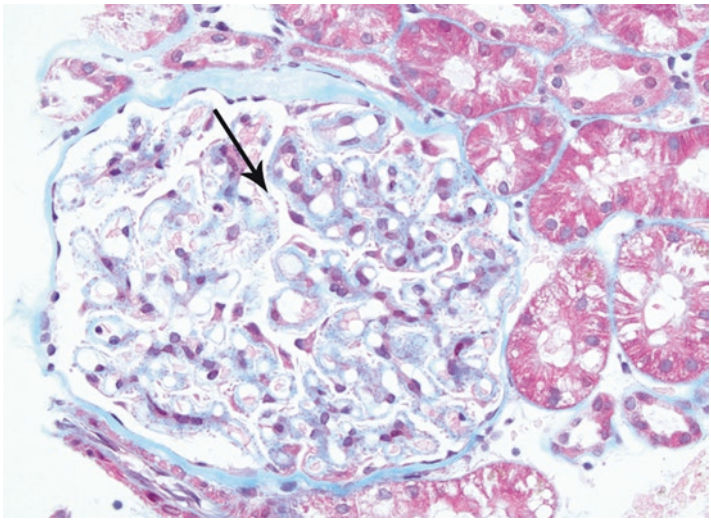

Fig. 6.27 Membranous glomerulonephritis in dog (trichrome). This image illustrates membranous glomerulonephritis in a Doberman Pinscher dog. Red to orange nodular deposits (*arrow*) throughout the glomerulus represent immune complexes. Masson's trichrome stain. 40 × objective magnification. (Image courtesy of Dr. Rachel E. Cianciolo)

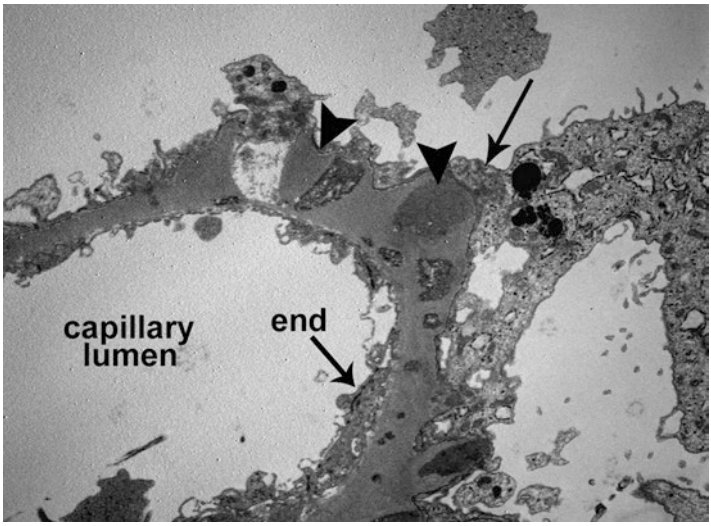

Fig. 6.28 Membranous glomerulonephritis in dog (TEM). This image illustrates membranous glomerulonephritis in a Doberman Pinscher dog. In this transmission electron microscopic image there are sub-epithelial immune deposits (*arrowheads*) and irregular thickening of the glomerular basement membrane. The podocyte has fused foot processes (*arrow*). An endothelial cell (end) is present in the image. (Image courtesy of Dr. Rachel E. Cianciolo)

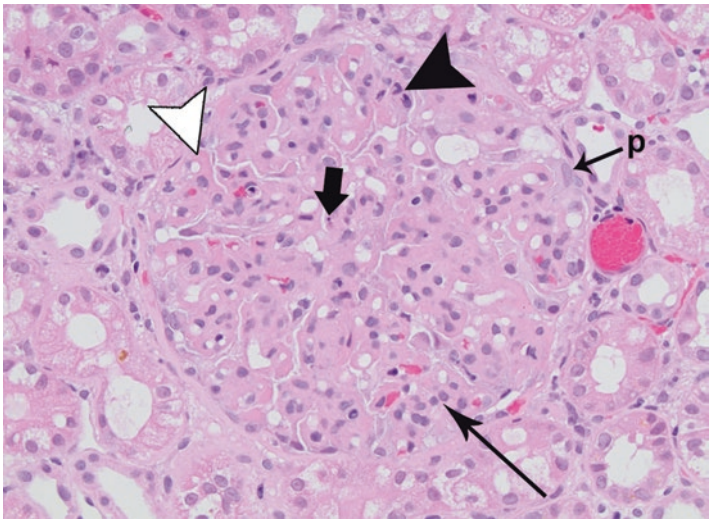

Fig. 6.29 Membranoproliferative glomerulonephritis in dog (H&E). This image illustrates membranoproliferative glomerulonephritis in a 6-year-old Border Collie dog with Lyme borreliosis. The glomerular tuft has an increased number of mesangial cells (*thin arrow*), increased amount of mesangial matrix, thickened capillary loops (*white arrowhead*), occasional intravascular inflammatory cells (*black arrowhead*), and necrotic debris (*thick arrow*). Capillary lumens are constricted. There is at least one hypertrophied parietal cell or podocyte (P). H&E stain. 40× objective magnification (Image courtesy of Dr. Rachel E. Cianciolo)

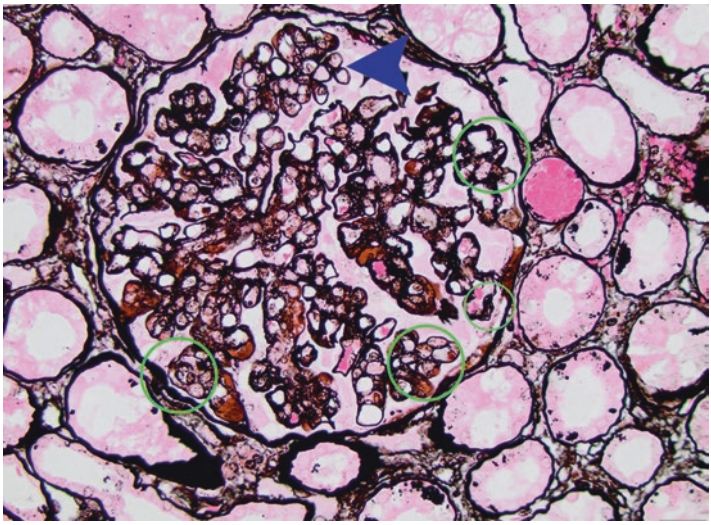

Fig. 6.30 Membranoproliferative glomerulonephritis in dog (Silver Stain). This image represents membranoproliferative glomerulonephritis in a 6-year-old Border Collie dog with Lyme borreliosis. Thickened basement membranes result in a wire-loop appearance (*arrowhead*) when stained with silver. Reduplication of basement membrane (*encircled*) due to interposition of the mesangial cells is apparent. Jones methenamine silver stain. 40× objective magnification (Image courtesy of Dr. Rachel E. Cianciolo)

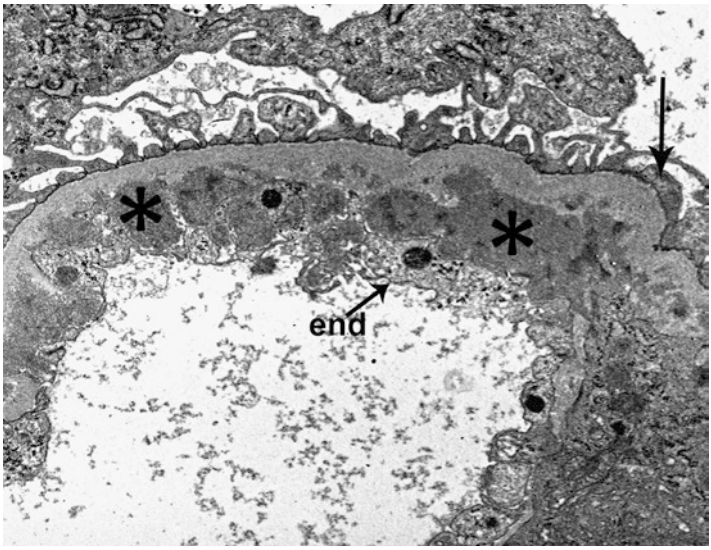

Fig. 6.31 Membranoproliferative glomerulonephritis in dog (TEM). This electron microscopic image represents membranoproliferative glomerulonephritis in a 6-year-old Border Collie dog with Lyme borreliosis. Sub-endothelial immune deposits (*asterisks*) can clearly be seen, as well as fusion of foot processes (*arrow*). An endothelial cell (end) is present. (Image courtesy of Dr. Rachel E. Cianciolo)

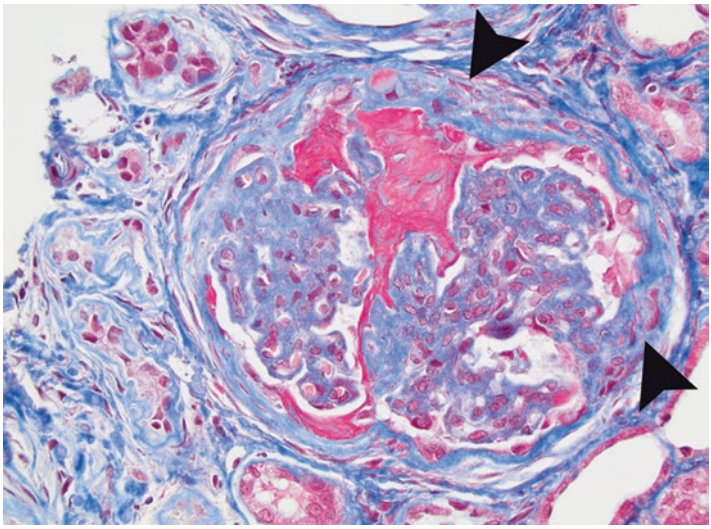

Fig. 6.32 Membranoproliferative glomerulonephritis with crescent formation in dog (trichrome). These images are from the kidney of an 8-year-old American Pit Bull dog with membranoproliferative glomerulonephritis (MPGN) that has crescent formation. The crescents consists of proliferation of elongated parietal epithelial cells admixed with fibrosis (*arrowheads*). Masson's trichrome stain. 40× objective magnification (Image courtesy of Dr. Rachel E. Cianciolo)

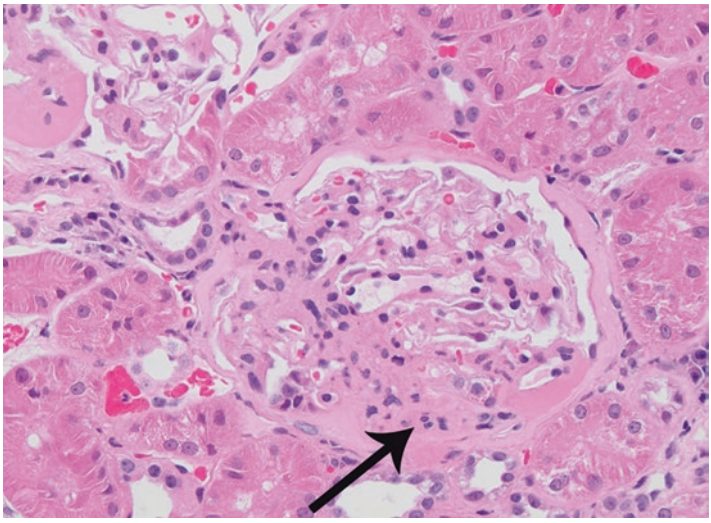

Fig. 6.33 Focal segmental glomerulosclerosis (FSGS) in dog (H&E). The images are from a 7-year-old Cavalier King Charles Spaniel dog. There is segmental localized obliteration of the tuft by sclerosis (*arrow*). H&E stain. 40× objective magnification (Image courtesy of Dr. Rachel E. Cianciolo)

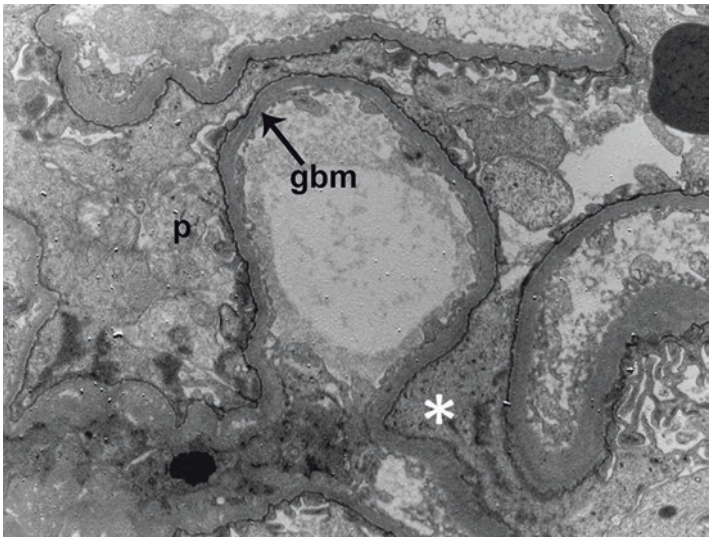

Fig. 6.34 Focal segmental glomerulosclerosis (FSGS) in dog (TEM). The transmission electronic microscopic image is from a 7-year-old Cavalier King Charles Spaniel dog. Electron microscopy reveals a normal glomerular basement membrane. The podocytes (P) have swollen cytoplasm with extensive fusion of foot processes (*asterisk*). (Image courtesy of Dr. Rachel E. Cianciolo)

6.10 Immune Defense of the Lower Urinary Tract

The lower urinary tract includes the ureter, urinary bladder and urethra. There are no specific autoimmune or immune-mediated diseases of the lower urinary tract. There is however a high incidence of septic inflammatory conditions that cause considerable problem in women. These septic inflammatory conditions are a direct result of the innate immune system to rid the infection. For that reason, they are not considered immune-mediated and will not be covered in this chapter. What will be covered is the laundry list of mechanisms and strategies the lower urinary tract has to avoid infection. When there is any breakdown in these defense mechanisms, inflammatory cystitis and urethritis occurs.

The bladder mucosa is widely accepted as a sterile environment, and physical and non-physical features make it a good defense against invading microbes. The alkaline pH, high urea content and absence of glucose make urine inhospitable to bacteria. A pH below 6 or higher than 7 is inhospitable to bacterial growth. Urine osmolality can affect bacterial growth. In general bacterial growth is inhibited when osmolality is less than 200 mosm/L or greater than 1200 mosm/L (Spencer et al. 2014). The luminal surface of the bladder also contains highly sulfated and anionic glycosaminoglycans that prevents adherence of microbes to the urothelium, and contributes to the impermeability of the bladder wall (Theodoros 2011).

There are mechanical forces that minimize UTI in addition to the urine itself. Ureteric peristalsis, urine flow and voiding, mucus shedding and epithelial cell

sloughing are some of the physical barriers to infection. The ureter is not a quiescent tube, but rather there is constant peristalsis of this muscular tube that propels urine and dynamically prevents ascending bacteria (Floyd et al. 2012; Spencer et al. 2014; Osman et al. 2009). Umbrella cell sloughing is triggered by an interesting pathophysiologic event. If *E.coli* can attach to the uroplakin receptors on urothelium, there is a rapid response to exfoliate the adulterated epithelial cells. This exfoliation is an innate immune response designed to prevent bacterial invasion into the wall of the bladder. The binding causes the uroplakin receptor to be phosphorylated on its cytoplasmic tail, which signals a cascade of events, starting with increased intracellular calcium and apoptosis (Thumbikat et al. 2009; Sivick and Mobley 2010). Some immunomodulating therapy takes advantage of this exfoliation mechanism. Exposing bladder to protamine sulfate, a highly cationic protein, removes surface-bound and intracellular *E. coli* by causing the umbrella cells to exfoliate more readily.

In addition there is a highly conserved innate immune response to provide front line defense against microbial insult and leads to subsequent activation of an adaptive immune response. The innate provides immediate defense, and the adaptive confers long lasting immunity. There are toll-like receptors on urothelium that are activated when microbials invade, such as TLR4, TLR5 and TLR11. There are antimicrobial peptides and Tamm Horsfall protein that help prevent bacterial infection. The cytokines IL-6, TNFα, IL-1B, G-CSF, and IL-17 and chemokines CXCL1, CXCL2, CXCL3, CXCL8 and CCL4 are detected in the infected mammalian bladder. Neutrophils are the most abundant early responders to infected bladders. Antigen presenting cells (APCs), such as macrophages and dendritic cells and ϒδ-T cell are also part of the host defense. IL-17 works as both an innate and adaptive immunomodulatory cytokine. It is upregulated in the bladder in response to acute infections, and is secreted by CD4+ Th17 T lymphocytes, cytotoxic T cells, ϒδ-T cells, NK-T cells, neutrophils, eosinophils and monocytes. ϒδ T cells are a key source of IL-17A production. IL-17 enhances neutrophil migration to infected tissue; and enhances chemokines CXCL2 and CCL20 (important for infiltration of neutrophils); CXCL10 and CCL5 (important for infiltration of T cells) and CXCL10and CCL20 (important for infiltration of dendritic cells). Its presence is undoubtedly useful for proper clearance of *E. coli* (Sivick and Mobley 2010). IL-17 also increases other pro-inflammatory cytokines such as IL-6, IFNϒ, and IL-4. IL-4 is a Th2 "immunosuppressive" cytokine by also is important to stimulate B cell in an adaptive immune response. Therefore IL-17 is a liaison between the innate and adaptive immune responses.

The most common TLR in the urinary tract are TLR2 (recognizing bacterial lipoteichoic acid or lipoprotein), TLR3 (ds RNA), TLR4 (LPS), TLR5 (flagellin), TLR9 (unmethylated CG DNA of bacteria), and TLR11 (recognizes parasite). The best studied is TLR4 which is expressed kidney and bladder TLR4 epithelium and contribute to increase mucosal immune responses. As soon as TLR4 is bound, there is a release of IL-8 to recruit neutrophils (Samuelsson et al. 2004). Genetic variation in the TLR4 expression and IL-8 receptor expression (CXCR1) on neutrophils renders some people more susceptible to bladder infections. People with low IL-8

receptor expression have a high incidence of acute pyelonephritis, recurrent cystitis and bacteriuria (Theodoros 2011; Lundstedt et al. 2007). The genetic variation of TLR4 expression predisposes humans to infection. Employing TLR 4 ligands to boost immunity in patients with defective toll-like receptor genes is one avenue of drug development to combat lower urinary tract infections. Activating TLR4 with astragalus, a Chinese herbal medicine, can activate pro-inflammatory factor secretion.

Uropathogenic *E. coli* commonly hides in intraepithelial fusiform vesicles, and is one of the ways uropathogenic *E. coli* can escape antibiotic therapy and cause chronic bouts of urinary tract infections. However, this hiding place has recently been a target for therapeutic intervention. Uropathogenic *E. coli*, after attachment to the urothelium, can form intracellular bacterial colonies where they remain quiescent in the bladder, hidden from the innate immune system, and are responsible for chronic UTI (Anderson et al. 2003; Mysorekar and Hultgren 2006). These colonies are hidden from surface Toll-like receptors. When the bladder expands under normal circumstances, the vesicles fuse with apical surface in order to increase membrane surface area, and this fusion exposes the hiding place for *E. coli.* Increased cAMP is required for this normal fusion process (Apodaca 2001). Researchers have shown that this fusion can be brought about by ways other than stretching the bladder wall. Rather the fusion can be instigated by increased cAMP in the urothelial cells. This can be done by activating TLR4 or treating with a drug such as forskolin that increases cAMP. With increased intracellular cAMP, there is increased fusion of these vesicles with the membrane, and increased exocytosis and exposure of the hidden *E. coli* (Song et al. 2007; Bishop et al. 2007).

The urinary bladder also produces antimicrobial peptides as part of its defense. The most well studied AMP peptides are defensins and cathelicidin (LL-37). Cathelicidin is expressed on all epithelial surfaces and on circulating white cells, including neutrophils, monocyte, NK cells. The alpha defensins, NHP1 and HNP4, are present in neutrophils. The beta defensins are found throughout the epithelial cells, and HBD1 through HBD4 (four of them) have been studies in detail. There are produced throughout the urinary tract. In particular, HBD1 is produced by collecting ducts, distal tubules and loop of Henle, and is positioned to defend the kidney from ascending infectious microbes (Valore et al. 1998; Lehmann et al. 2002; Morrison et al. 2002). HBD1 can also disrupt bacterial membranes and can be chemotactic for immune cells (Zasloff 2007). It has been shown that mice deficient in B defensin 1 have a higher incidence of bacteriuria (Morrison et al. 2002). Some HBDs are inducible and not normally produced, such as HBD2. Cytokines IL-1 or TNFα produced by inflammatory cells induce these AMPs.

Other antimicrobial proteins include Tamm Horsfall protein (THP), lactoferrin and lipocalin. THP, also known as uromodulin, is a glycoprotein produced exclusively by the epithelial cells lining the thick ascending limb of the loop of Henle (Devuyst and Bochud 2015). It serves an anti-infective role by stopping the microbes, especially fimbriated *Escherichia coli*, from adhering to epithelial cells (Bates et al. 2004; Devuyst and Bochud 2015). THP binds to type I fimbriae on *E. coli* to prevent adherence to uroplakin receptors. THF acts as a chemoattractant and

activates TLR4 on DCs (Theodoros 2011). Lactoferrin and lipocalin restrict the availability of iron, an essential microbial nutrient (Goetz et al. 2002; Abrink et al. 2000). Lactoferrin is in collecting tubules, and lactoferrin and lipocalin is found in the fluid layer immediately adjacent to the urothelial cells.

Hepcidin is an AMP that also restricts the use of iron for bacterial growth. Hepcidin is synthesized in the liver and excreted by the kidneys. It is induced by IL6, and inhibits the availability of iron to bacteria. Hepcidin binds to and degrades the iron exporter ferroportin. This prevents iron-containing cells, such as macrophages from releasing this valuable iron to the bacteria (Reygaert 2014).

Calgranulins A, B, and C are involved in innate immunity and inflammation. As secreted proteins, they inhibit microbial growth in the extracellular milieu presumably though their ability to chelate zinc. Cells that express calgranulins are resistant to bacterial adherence and invasion (Reyes et al. 2011; Hsu et al. 2009). Calgranulins A and B are in granulocytes, monocytes, dendritic cells, epithelial cells, and keratinocytes (Foell et al. 2007), and Calgranulin C resides in granulocytes (Vogl et al. 1999). The direct antimicrobial functions of calgranulins are probably not the major biologically effective functions of calgranulins in the urinary tract. Instead calgranulins have a multi-functional role in regulating the degree of inflammation, and helping to assure an optimal immune response that eliminates infection without causing unnecessary tissue damage from protease and reactive oxygen species. Calgranulins inhibit immunoglobulin G production in lymphocytes (Brun et al. 1994); they inhibit neutrophils recruitment (Sroussi et al. 2006, 2007); induce apoptosis in immune cells (e.g. T cells); and promote the regulatory macrophage phenotype (M2). Their function to protect against too much inflammation and innate immune activation needs to be calibrated carefully. Calgranulins in excess contribute to the complicated inflammatory disease such as struvite urolithiasis, pyelonephritis and interstitial cystitis. Calgranulins are used as a biomarker for chronic active inflammation, but also they may play a role in the pathogenesis of rheumatoid arthritis, systemic lupus, Sjogrens, and atherosclerosis (Foell et al. 2007; Johne et al. 1997; Pereira et al. 2011).

When infection occurs and TLR are activated, the neutrophils and macrophages are called into the tissue. The neutrophils are a powerful defense but their enthusiasm to fight infection must be controlled or else there will be collateral tissue damage. The macrophages play a role in regulating the neutrophilic response in the urinary bladder. The sentinel macrophages trigger an alarm to release chemokines that in turn recruit neutrophils. At the same time however, these macrophage also lure in helper macrophages that provide a safety mechanism. It is these helper macrophages that arrive later to the scene that decide how many neutrophils will be recruited (Schiwon et al. 2014). Only with a thumbs-up positive signal from the helper macrophage, and with TNF-mediated intimate communication between the two sets of macrophages, will the sentinel tissue macrophage produce CXCL2 to allow neutrophil migration through the basal membrane of the urothelium (Schiwon et al. 2014). This model of an initial resident sentinel macrophage interacting with helper macrophages is tantamount to a macrophage asking for a second opinion from the helper macrophage (Schiwon et al. 2014).

While the innate immune system is probably the initial and the strongest defense for the bladder to avoid infection, the bladder also utilized adaptive cell-mediated and humoral immunity. Recruitment of activated T cell in the bladder and the development of specific IgG in serum and urine were observed (Thumbikat et al. 2006). This adaptive immunity with urine IgG suggests that vaccines can be made that are effective against chronic urinary tract infections.

6.11 Summarized Points

1. In the kidney, peripheral tolerance is maintained by the continual surveillance of dendritic cells taking autoantigen back to the renal lymph node, and maintaining a population of T-regs and M2 macrophages in the kidney.
2. Glomerular disease may occur by one of five hypersensitivity reactions, and these disease processes generally converge to produce a limited repertoire of tissue lesions.
3. Immune-mediated renal disease is not necessarily inflammatory. There can be sublytic damage of podocytes by complement, which sets into motion a chain of events leading to production of TGFβ and PDGF by podocytes and mesangial cells leading to proliferation of mesangial cells and crescent formation.
4. The mesangial cells, podocytes, and renal tubular epithelial cells have immune function: They are capable of producing inflammatory mediators, such as IL-1 and TNFα that potentiate inflammation, and growth factors that cause cellular proliferation, and production of extracellular matrix.
5. Glomerular injury leads to tubulo-interstitial injury, and this is mediated by albumin, C3a, C5a, and angiotensin II in the tubular filtrate activating TLR and upregulating inflammasomes in renal tubular epithelial cells.
6. Tubulo-interstitial nephritis leads to glomerular injury by activating complement, and causing a complement-mediated glomerulopathy.
7. Renal tubular, visceral, and parietal epithelial cells undergo epithelial-mesenchymal transition mediated by TGF-β, leading to crescent formation, glomerulosclerosis, interstitial fibrosis and eventual tubular atrophy.
8. Once inflammatory glomerular or tubulo-interstitial disease starts, there is self-perpetuation of the disease process and even the repair stage can further reduce renal function.
9. Uremia results in production of uremic toxins that cause widespread endothelial damage and vascular disease, and hypertension which aggravate renal disease. Uremia also causes immunodysfunction, leading to infection and a hypercytokinemic state, which perpetuates the vascular disease and disrupts the cardio-renal equilibrium.
10. The lower urinary tract is armed with powerful innate immune defenses including ureteric peristalsis, numerous antimicrobial proteins, and a large number of toll like receptors, and calgranulins that keep the inflammation under control.

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Chapter 7
Immunopathology of the Hepatobiliary System

Danielle L. Brown

Abstract The liver is the largest internal organ in the body and contains many cell types that are involved in local and systemic immunity, including those of both the innate and adaptive immune systems. Approximately 10% of the cells of the liver belong to the immune system, including the largest supply of tissue-specific macrophages and natural killer cells in the body. One of the main roles of immune cells in the liver is to establish an immunotolerant microenvironment, as the liver is the first tissue to receive portal blood flow from the gastrointestinal tract, which includes endotoxin, self-antigens, and ingested antigens. This prevents unwanted systemic immune responses towards these everyday encountered antigens. Kupffer cells and sinusoidal endothelial cells in the liver play important roles in removing pathogens and other possible antigens from the portal blood prior to them reaching the systemic circulation, also preventing unwanted reactions. Autoimmune disease can result if this immunotolerant microenvironment is disrupted. The liver also plays a role in local and systemic inflammation through the production of acute phase proteins, cytokines, and other pro-inflammatory mediators. Immune cells play an important role in many liver diseases of humans and animals, including drug-induced liver injury, viral infection, and neoplasia. In addition, the liver plays an important role in systemic disorders, particularly septicemia. This chapter outlines the normal immunobiology of the liver, including the cell types involved in the immune response, and their roles in various diseases of the liver as well as systemic disorders. Animal models of liver diseases involving the immune system are also discussed.

Keywords Immunotolerance • Kupffer cells • Dendritic cells • Sinusoidal endothelial cells • Lymphocytes • Stellate cells • Hepatocytes • Biliary epithelial cells

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7.1 Immunobiology of the Hepatobiliary System

7.1.1 Macroscopic and Microscopic Structure

The liver is the largest internal organ in the body, making up 2–4% of the body weight in most species. It is situated in the cranial abdominal cavity, abutting the diaphragm cranially and the gastrointestinal tract caudally.

The liver has a dual blood supply. Approximately 80% of its afferent blood supply comes from the portal vein, which drains the gastrointestinal tract, and the remainder is from the hepatic artery (McCuskey 2008). Approximately 30% of the total blood in the body passes through the liver every minute (Ahlensteil and Rehermann 2007), and the entire blood volume passes through the liver approximately 360 times per day (Knolle and Gerken 2000). Because of this, the liver is continuously exposed to a large load of antigenic stimuli, including exogenous pathogens, dietary components, and xenobiotics (Bogdanos et al. 2013). The blood from these two sources mixes within the hepatic sinusoids and exits via the hepatic vein, emptying into the caudal vena cava.The liver is also the largest source of lymph in the body, contributing 25–50% of the thoracic duct flow (Bertolino et al. 2002).

Microscopically, the primary parenchymal cells of the liver are referred to as hepatocytes. The hepatocytes comprise approximately 80% of the liver tissue, the remainder of the organ being composed of non-parenchymal cells (Kupffer cells, dendritic cells, lymphocytes, sinusoidal endothelial cells, hepatic stellate cells, and biliary epithelial cells) and the extracellular space. The hepatocytes are arranged in one-cell thick cords or plates separated by vascular spaces called sinusoids (Fig. 7.1). The classical functional subunit of the liver is the lobule. The hepatic lobule is hexagonal and contains a central vein in the middle of the hexagon and portal tracts at each angle (Fig. 7.2a). Another way to view the subunit of the liver is the acinus,

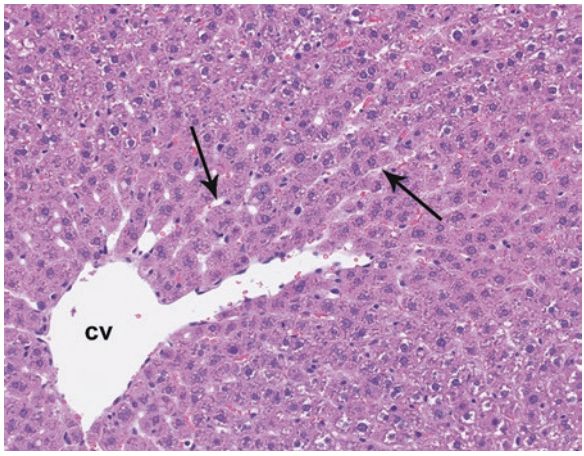

Fig. 7.1 Photomicrograph of a rat liver at high magnification. Hepatocytes are arranged in one-cell thick cords, separated by vascular sinusoids (*arrows*). *CV* central vein. Hematoxylin and eosin (H&E) stain, 20× magnification

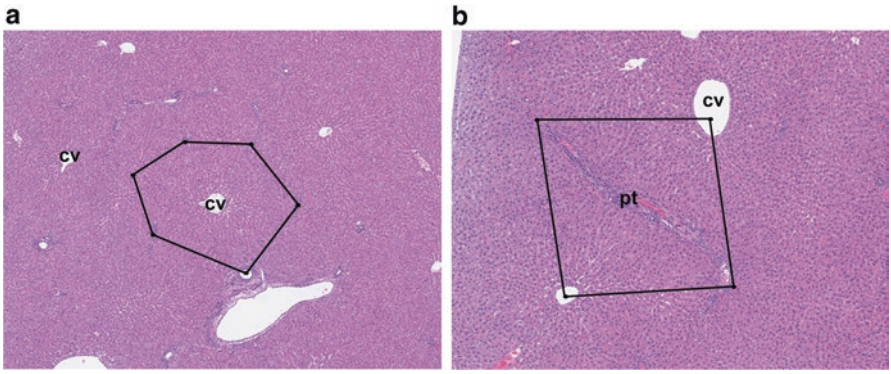

Fig. 7.2 (**a**) Photomicrograph of a rat liver demonstrating the classical functional unit, the hepatic lobule. The lobule contains a central vein at the center (*cv*) and portal tracts at each point of the hexagonal structure. H&E stain, 5× magnification, (**b**) Photomicrograph of a rat liver demonstrating another way to view subunits of the liver, the hepatic acinus. The acinus is diamond-shaped and contains a portal tract at the center (*pt*) and central veins (*cv*) at the points. H&E stain, 7× magnification

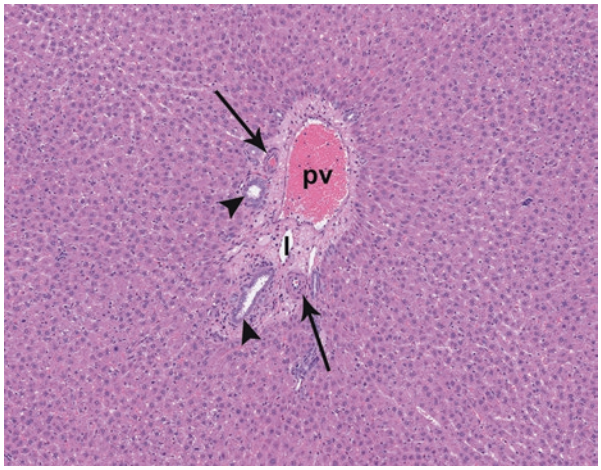

Fig. 7.3 Photomicrograph of a rat liver demonstrating the microanatomy of the portal tract. The portal tract is composed of a large portal vein (*pv*) and adjacent smaller bile ducts (*arrowheads*), hepatic arteries (*arrows*), and lymphatic vessels (*l*). H&E stain, 10× magnification

which is a diamond-shaped structure with a portal tract in the center (also termed zone 1) and central veins at the tips of the diamond (zone 3) (Fig. 7.2b). The portal tract is composed of a portal vein, bile duct, hepatic artery, and lymphatic vessels (Fig. 7.3). Blood enters the liver via the portal vein and hepatic artery, mixes in the sinusoids, and exits via the central veins. In contrast, bile flows in the opposite direction. It begins within the biliary canaliculi (interdigitations in the cell

membranes of adjacent hepatocytes) in the centrilobular areas (adjacent to the central veins) and flows through canaliculi towards the portal areas, emptying into the bile ducts.

Hepatic sinusoids are unique vascular structures that are lined by discontinuous endothelial cells that lack a basement membrane (sinusoidal endothelial cells). The sinusoids contain several non-parenchymal cells, including Kupffer cells, which are resident macrophages within the liver (Fig. 7.4). In addition, between the sinusoidal endothelial cells and the hepatocytes there is a gap termed the space of Disse. Hepatic stellate cells are located within that space.

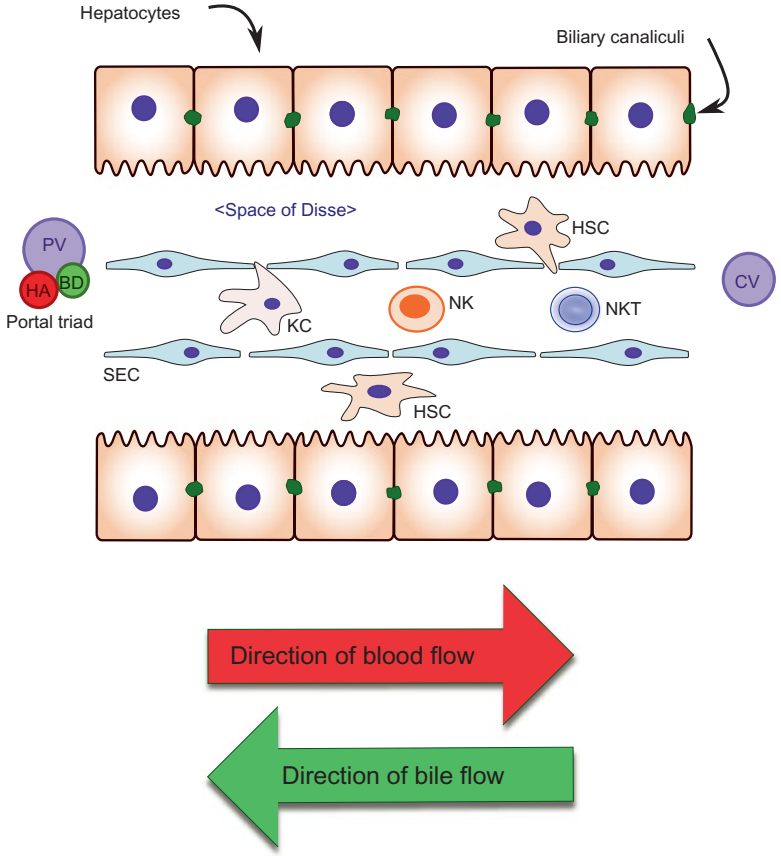

Fig. 7.4 Anatomy of the hepatic sinusoid. Cords of hepatocytes are separated by sinusoids, which are modified vascular spaces lined by sinusoidal endothelial cells (*SEC*). Within the sinusoids reside several immune system cells, including resident hepatic macrophages termed Kupffer cells (*KC*) and lymphocyte subsets including natural killer cells (*NK*) and natural killer T cells (*NKT*). In addition, a space exists between the sinusoidal endothelial cells and hepatocytes, termed the *space of Disse*. Within this space reside hepatic stellate cells (*HSC*). Sinusoids carry blood from the portal veins (*PV*) and hepatic arteries (*HA*) within the *portal triads* towards the central veins (*CV*). Conversely, bile flow travels in the opposite direction and flows between hepatocytes through *biliary canaliculi*

7.1.2 Cell Types Involved in Immunobiology

The liver contains many cell types that are involved in local and systemic immunity. Approximately 10% of the cells in the liver belong to the innate or adaptive immune system, and the liver contains the largest pool of mononuclear phagocytes and natural killer cells in the body (Cullen and Brown 2012).

7.1.2.1 Kupffer Cells

Kupffer cells are the resident macrophages within the liver. They constitute approximately 80% of all macrophages within the body (Knolle and Gerken 2000), making them the largest population of tissue macrophages. They reside within the hepatic sinusoids and make up approximately 30–35% of all non-parenchymal cells within the liver (Parker and Picut 2005; Jenne and Kubes 2013). The Kupffer cell population is established prior to birth and is maintained in adult life through self-renewal and longevity, with virtually no contribution from the circulating monocyte pool (Yona et al. 2013). Kupffer cells have a long lifespan and, during health, have long residence times and slow rates of self-replication (McCuskey 2008).

Kupffer cells are most numerous in the periportal areas of the liver and least numerous in the centrilobular areas (Parker and Picut 2005). Kupffer cells within different zones of the liver are also different phenotypically. Periportal Kupffer cells are larger, have higher lysosomal enzyme activity, and have greater phagocytic activity, whereas those closest to the central veins are smaller and produce larger amounts of cytokines (Sleyster and Knook 1982; Liaskou et al. 2012).

Kupffer cells have an important role in nonspecific phagocytosis, comprising the first line of defense against pathogenic organisms, endotoxin, and foreign material absorbed from the intestine into the portal circulation. Through this strategic location, Kupffer cells act as an immune sentinel, alerting other components of the immune system to the presence of harmful pathogens (Bilzer et al. 2006). They express several toll-like receptors (TLRs) that respond to these pathogens, such as TLR-4, the ligand for lipopolysaccharide (LPS). A major differentiating ability of Kupffer cells when compared with other cells of the mononuclear phagocyte system is their ability to phagocytose bacteria under flow conditions versus only under static conditions (Jenne and Kubes 2013). Kupffer cells can also phagocytose activated and apoptotic neutrophils, which contributes to resolution of inflammation (Shi et al. 2001). Interestingly, in pigs, most blood-borne material is cleared by pulmonary intravascular macrophages rather than Kupffer cells (Cullen and Brown 2012).

Kupffer cells also serve as conventional antigen-presenting cells. They express major histocompatibility complex (MHC) class I and II molecules, costimulatory molecules CD80 and CD86, and adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) (Li and Tian 2013). However, in contrast to antigen presenta-

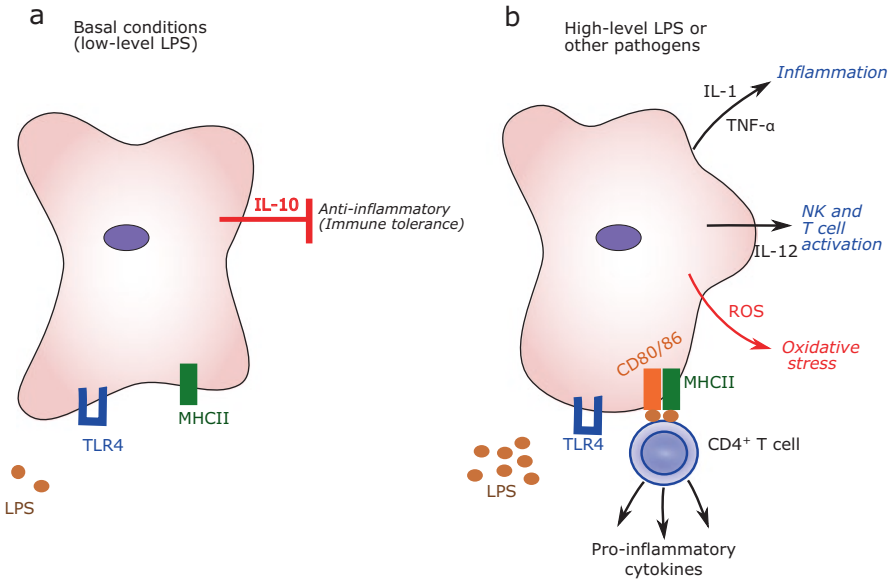

Fig. 7.5 (**a**) Under basal conditions (low levels of lipopolysaccharide (*LPS*) as commonly encountered from the portal blood), Kupffer cells (*KC*) secrete *IL-10*, which has anti-inflammatory effects and induces immune tolerance. (**b**) Under high levels of LPS or other pathogens, *KC* become activated and transform into immunogenic antigen-presenting cells. Activated *KC* secrete inflammatory cytokines such as *IL-1* and *TNF-α*, activate natural killer (*NK*) and T cells through secretion of *IL-12*, and produce reactive oxygen species (*ROS*). They can also present antigen to CD4+ T cells through *MHCII* with the help of co-stimulatory molecules *CD80/CD86* and induce additional production of pro-inflammatory cytokines by the activated T cells. *TLR4* Toll-like receptor 4

tion in other tissues, antigen presentation by Kupffer cells in the liver often results in immune tolerance rather than activation (Thomson and Knolle 2010). Under basal conditions, Kupffer cells are poor activators of the adaptive immune response due to continual exposure to lipopolysaccharide from the intestines (You et al. 2008). They can stimulate regulatory T cells (Tregs) to secrete the immunosuppressive cytokine IL-10 or even produce this cytokine themselves in response to the continuous low-grade LPS exposure, a phenomenon called "LPS tolerance" (Thomson and Knolle 2010; Nakamoto and Kanai 2014; Knolle et al. 1995). This tolerance induction has an important role in preventing harmful immune reactions to soluble antigens from the intestine as well as in the prevention of liver transplant rejection. However, under inflammatory conditions or in the presence of hepatocellular damage, Kupffer cells can become activated and switch their role from tolerance-inducing to immunogenic antigen-presenting cells (Crispe 2011) (Fig. 7.5). Furthermore, when activated by large quantities of LPS, Kupffer cells actually play a prominent role in promoting liver injury rather than in protecting the liver, and experimental inactivation of Kupffer cells has been shown to abrogate liver injury (Adachi et al. 1994).

Upon activation Kupffer cells have the ability to produce and secrete several pro-inflammatory cytokines, including interleukin (IL)-1, IL-6, and tumor necrosis factor (TNF)-α (Liaskou et al. 2012). They can also secrete chemokines such as macrophage inflammatory protein (MIP)-1α and regulated on activation normal T cell expressed and secreted (RANTES) and express several cell surface receptors involved in immunity, including complement, Fc, mannose, and scavenger receptors (Liaskou et al. 2012). In response to bacterial infection or tumor antigens, Kupffer cells can activate natural killer (NK) cells and T cells through secretion of IL-12 (Seki et al. 2000). Activated Kupffer cells can contribute to hepatic injury indirectly through the production of these cytokines and chemokines and amplification of the inflammatory process. They can also contribute directly through the production of reactive oxygen species (ROS).

Overall, Kupffer cells have a dual role within the context of hepatic immunity. In the steady state, they support tolerance induction towards circulating and hepatocyte-derived antigens, preventing an immune response towards self and orally ingested antigens. However, in the context of microbial infection, Kupffer cells become activated and induce a strong immune response.

7.1.2.2 Dendritic Cells

Dendritic cells are also members of the mononuclear phagocyte system. The normal liver contains resident dendritic cells that mature as they migrate from the portal vein toward the central vein, eventually migrating to and concentrating in the regional lymph nodes (Sato et al. 1998). The liver contains more dendritic cells than any other parenchymal organ (Steptoe et al. 2000), which may result from the large number of pathogen-associated molecular patterns (PAMPs) present in the portal circulation (Thomson and Knolle 2010). However, they still make up less than 1% of the non-parenchymal cells in the liver (Bertolino et al. 2002; Nakamoto and Kanai 2014). Dendritic cells in the liver are most concentrated in the periportal and centrilobular areas (Prickett et al. 1988).

There are five subpopulations of dendritic cells with the liver based on their phenotypic characteristics: myeloid, plasmacytoid, mixed myeloid and plasmacytoid, lymphoid, and natural killer dendritic cells (Bogdanos et al. 2013; Pillarisetty et al. 2004; Hsu et al. 2007). Myeloid and plasmacytoid dendritic cells are the classical types and are the main subpopulations in the liver, the liver containing more plasmacytoid dendritic cells than even lymphoid organs (Crispe 2009). They are biased toward tolerance induction rather than activation of immunity. For example, plasmacytoid dendritic cells can induce anergy or deletion of circulating T cells (Thomson and Knolle 2010).

Dendritic cells in the liver differ from those in the spleen and other tissues. In particular, they are less immunogenic, likely due to a lack of expression of costimulatory molecules (Pillarisetty et al. 2004) or partial tolerance induced by constant exposure to endotoxin (LPS) from the portal circulation (De Creus et al. 2005). Hepatic dendritic cells also exhibit a more immature phenotype when compared

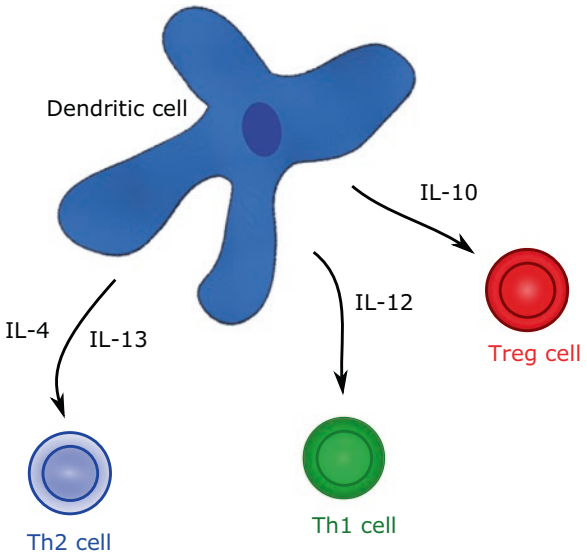

Fig. 7.6 Hepatic dendritic cells play diverse roles in local and systemic immunity. They have the ability to activate regulatory T cells (*Tregs*) through secretion of *IL-10*, *Th1* cells through secretion of *IL-12*, and *Th2* cells through secretion of *IL-4* and *IL-13*

with those in other tissues. For example, they have lower expression of CD11c (Hsu et al. 2007) and lower levels of MHC class II molecules and costimulatory molecules, such as CD80 and CD86, making them weak T cell stimulators (Kingham et al. 2007).

In the normal liver, dendritic cells reside as immature antigen-presenting cells. If they capture self-antigen, endotoxin, or dietary antigen they induce antigen-specific T cell tolerance (Abe and Thomson 2007). However, upon capture of exogenous antigen or in the presence of inflammation or hepatic damage, they can migrate to the regional lymph nodes where they become activated and prime naïve antigen-specific T cells (Abe and Thomson 2007). Once they migrate to the lymph nodes, they can also activate NK cells and NK T cells (Hermans et al. 2003). Therefore, hepatic dendritic cells can play diverse roles in local and systemic immunity. They can promote tolerance and immunosuppression through activation of Tregs (Steinbrink et al. 1997), they can induce a Th1 response through upregulation of IL-12 (Martin-Fontecha et al. 2004), or can induce a Th2 response via IL-4 and IL-13 secretion (Soumelis et al. 2002) (Fig. 7.6).

7.1.2.3 Sinusoidal Endothelial Cells

Sinusoidal endothelial cells (SECs) comprise approximately 50% of the non-parenchymal cells in the liver (Blouin et al. 1977; Racanelli and Rehermann 2006), and are more than twice as abundant as Kupffer cells. They are specialized endothelial cells that are fenestrated (contain true discontinuities) and lack a basal lamina. This allows for solutes and small particles to pass freely from the sinusoidal lumen into the space of Disse, which contains hepatic stellate cells and microvilli of

hepatocytes. Approximately 5–10% of the surface of SECs are perforated by these fenestrae (Le Couteur et al. 2008), which are grouped into clusters called "sieve plates" (Wisse et al. 1985). In general, periportal sinusoids have a smaller diameter and SECs in that zone of the lobule contain larger but fewer fenestrae when compared with centrilobular areas (Wisse et al. 1985). However, fenestrae are dynamic structures and their diameter can be affected by changes in sinusoidal luminal blood pressure, vasoactive substances, hormones, drugs, toxins, disease, and aging (Wisse et al. 1985; Braet and Wisse 2002). The fenestrations play a role in immunity by mediating interactions between hepatocytes and circulating lymphocytes, both of which can project cell membrane extensions through the fenestrae to interact with one another (Le Couteur et al. 2008).

SECs have important roles in regulation of sinusoidal blood flow as well as in host defense mechanisms. They have a unique role in the disposal of waste molecules generated through inflammatory, immunologic, or general homeostatic processes, via the action of specific endocytic receptors on their cell membrane (Parker and Picut 2012). In fact, SECs may represent the most active endocytic cells in the body (Smedsrod et al. 1990). They have the ability to endocytose a variety of macromolecules, including proteins, glycoproteins, lipoproteins, and glycosaminoglycans (Smedsrod et al. 1985a; Wisse et al. 1996). SECs appear to be the main cell type to endocytose circulating connective tissue molecules, which is mediated by mannose, collagen, or scavenger receptors (Smedsrod et al. 1990; Malovic et al. 2007). In the absence of functional Kupffer cells, SECs can also take up larger particulates (Steffan et al. 1986).

SECs have a unique phenotype, which more closely represents immature dendritic cells than endothelial cells from other organs (Knolle 2007). This unique phenotype allows them to function as antigen-presenting cells. They constitutively express MHC class I and II, costimulatory molecules CD80 and CD86, and adhesion molecules such as ICAM-1 (Knolle and Limmer 2003) (Fig. 7.7). However, they appear to be poor stimulators of naïve T cells (Katz et al. 2004). They are particularly efficient at cross-presentation of extracellular antigens in the context of MHC I, allowing for both CD4+ and CD8+ T cells to be exposed to circulating antigens from the intestinal tract (Jenne and Kubes 2013). CD4+ T cells that are activated by SECs often differentiate into Treg cells (Knolle et al. 1999), which is critical for the development and maintenance of immune tolerance. However, it can present a problem in developing an efficient immune reaction against viral infections or neoplasia in the liver.

SECs express several cell surface receptors (Fig. 7.7). Many of these receptors allow for endocytosis of molecules as outlined above, including scavenger, mannose, collagen, hyaluronan, apo E, and galactose receptors (McCuskey 2008). They also express Fc receptors for uptake of immune complexes and several pattern recognition receptors, such as TLRs. Engagement of TLR4 on the surface of SECs by a ligand, such as LPS, results in secretion of IL-10 and immune tolerance (Crispe 2009).

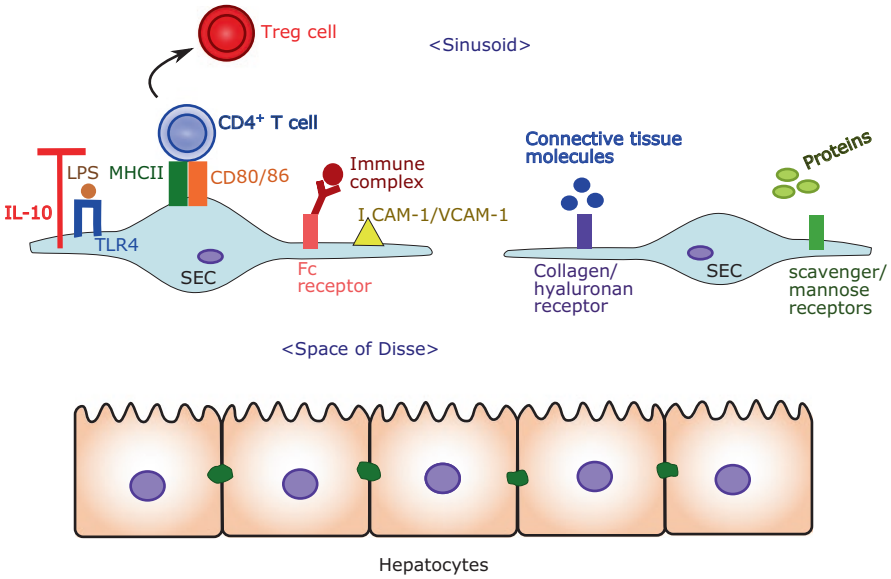

Fig. 7.7 Sinusoidal endothelial cells (*SEC*) have a unique phenotype and express several receptors. *TLR4* on the surface of SEC can interact with *LPS* and induce secretion of *IL-10*, leading to immune tolerance. Although SEC express *MHCII* and *CD80/CD86* and can act as antigen-presenting cells, they usually induce *CD4+* T cells to differentiate into immunosuppressive *Treg* cells. Other important receptors on SEC include *Fc receptors*, which bind circulating *immune complexes*, *ICAM-1/VCAM-1*, which bind inflammatory cells, *collagen/hyaluronan receptors*, which bind *connective tissue molecules*, and *scavenger/mannose receptors*, which bind *proteins*

7.1.2.4 Lymphocytes

The liver contains a significant number of lymphocytes, including T cells, B cells, NK cells, and NK T cells. Lymphocytes comprise approximately 25% of all non-parenchymal liver cells, and are composed of over 60% T cells (including NK T cells), approximately 30% NK cells, and less than 10% B cells (Blouin et al. 1977; Ahlensteil and Rehermann 2007; Racanelli and Rehermann 2006). Within the normal liver, lymphocytes are mainly located within portal tracts but can also be scattered throughout the parenchyma (Doherty et al. 1999; Norris et al. 1998; Hata et al. 1990). However, the composition and localization of lymphocytes within the liver changes drastically when the hepatic architecture is disrupted by inflammation. The composition of hepatic lymphocytes also depends on the species. For example, the mouse liver contains more NK T cells versus the rat and human liver contain more NK cells (Gao et al. 2009).

T Lymphocytes Hepatic T cells are different from those in the blood, lymph nodes and spleen. For example, the CD4:CD8 ratio of hepatic T cells is reversed, being 1:3.5 in the liver as compared to 2:1 in the blood (Norris et al. 1998). The liver also has a higher percentage of double-positive (CD4+CD8+) and double-negative (CD4−CD8−) T cells when compared with other tissues or the blood (Norris et al.

1998). Furthermore, over 15% of hepatic T cells express the gamma/delta form of the T cell receptor (γδTCR), which is up to five times higher than the percentage in the bloodstream (Carding and Egan 2002; Brandes et al. 2005). All of these differences suggest that there is local control of function and/or differentiation of lymphocyte populations in the liver. In fact, CD4−CD8− T cells are known to develop extrathymically in the liver. The liver also functions as a "sink" for activated T cells. These T cells are sequestered in the liver and then undergo apoptosis due to a lack of costimulatory molecules and insufficient antigen presentation (Mehal et al. 1999).

B Lymphocytes B lymphocytes comprise only 5% of the total lymphocytes in the liver (Doherty et al. 1999; Norris et al. 1998; Hata et al. 1990).

Natural Killer Lymphocytes (NK cells) The liver contains a large number of resident NK cells, which used to be referred to as pit cells. These cells comprise 20–40% of all hepatic lymphocytes in the human and 10–20% of those in the mouse, compared with less than 5% seen in peripheral blood (Doherty et al. 1999; Tian et al. 2013). Hepatic NK cells are intrasinusoidal and adhere to SECs and Kupffer cells. They are bone marrow-derived mononuclear cells that have markers of both T cells and macrophages, and are morphologically defined as large granular leukocytes (LGLs) because they contain distinct azurophilic cytoplasmic granules. Circulating NK cells migrate to the liver and attached to the sinusoidal wall, where they then mature into liver-specific NK cells (McCuskey 2008; Nakatani et al. 2004). Once in the liver, they remain there for 2 weeks and are dependent on Kupffer cells, proliferating locally when stimulated by IL-2 (Parker and Picut 2005).

The phenotype of NK cells is species-dependent. For example, human NK cells are CD56+ and CD3- (Tian et al. 2013) versus mouse NK cells are NK1.1+ and CD3− (C57CL/6 mice) or CX5+ and CD3− (other strains) (Koo et al. 1986) and rats are NKR-P1A+ and CD3− (Gao et al. 2009). Hepatic NK cells also show different immunophenotypical, morphological, and functional characteristics when compared with peripheral NK cells. For example, they have higher expression of CD11a/CD18 (LFA-1) adhesion molecule, which plays an important role in the overall effectiveness of hepatic NK cells in tumor cell killing (Luo et al. 1999). They also have higher expression of the inhibitory receptor NKG2A, which may contribute to immune tolerance in the liver, and higher perforin/granzyme activity and TNF-related apoptosis-inducing ligand (TRAIL) expression, which contribute to increased cytotoxicity (Tian et al. 2013; Gao et al. 2009). Hepatic NK cell activity also appears to be influenced by species and strain, and rats have higher activity than mice (Wright and Stacey 1991).

Hepatic NK cells have important roles in surveillance for infection (particularly viral infection), non-specific cell killing, and resistance to tumor invasion (Parker and Picut 2012), and their functions are strongly influenced by the tissue microenvironment (Fig. 7.8). They are considered the first line of defense against viral infections (Biron et al. 1999). Upon activation, they release their cytoplasmic granules containing perforin and granzyme, which can attack cell membranes and cause apoptosis in target cells (Parker and Picut 2005). Activated NK cells can also produce high levels of cytokines such as interferon (IFN)-γ that can shape the immune

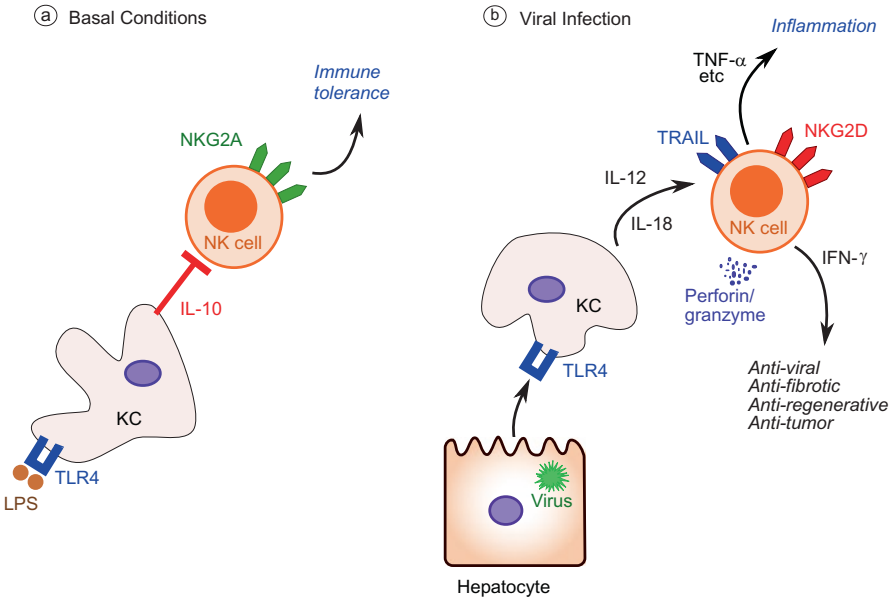

Fig. 7.8 (**a**) Under basal conditions, Kupffer cells (*KC*) secrete *IL-10* and natural killer (*NK*) cells express high numbers of inhibitory *NKG2A* receptors, leading to immune tolerance. (**b**) During viral infection, *KC* secrete *IL-12* and *IL-18*, which activate *NK* cells. In addition, expression of stimulatory *NKG2D* and *TRAIL* receptors by *NK* cells is upregulated. In turn, activated *NK* cells secrete pro-inflammatory cytokines such as *TNF-α*, release *perforin* and *granzyme*, which are involved in killing of infected cells, and secrete *IFN-γ*, which has anti-viral, anti-fibrotic, anti-regenerative, and anti-tumor effects

response (Jenne and Kubes 2013). IFN-γ is the most important cytokine produced by NK cells and has anti-viral, anti-fibrotic, anti-regenerative, and anti-tumor effects (Wang et al. 2008; Sun and Gao 2004; Subleski et al. 2006; Radaeva et al. 2006).

The ability of NK cells to kill target cells is determined by opposing signals from inhibitory (e.g. NKG2A) and stimulatory (e.g. NKG2D) receptors on the NK cells and their interactions with corresponding ligands on target cells (Raulet 2003; Lanier 2005) (Fig. 7.8). For example, inhibitory receptors recognize MHC Class I molecules expressed on target cells and subsequently inactivate NK cell functions (Gao et al. 2009). Expression of these receptors is significantly altered during liver disease, and NKG2D is upregulated after viral infection or transformation of hepatocytes (Crispe 2009).

NK cell activity is also highly influenced by cytokines. IL-12 production by activated dendritic cells and monocytes, as well as IL-18, lead to NK cell activation manifested by production of IFN-γ and enhanced perforin-dependent cytotoxicity, respectively (Tian et al. 2013; Dao et al. 1998; Biron and Brossay 2001). In addition, type I interferons (IFN-α and IFN-β) are potent activators of NK cell cytotoxicity and IL-15 promotes NK cell proliferation (Tian et al. 2013). NK cells can also

become activated by cell-cell contact with other immune cells, such as TLR ligand-activated Kupffer cells (Tu et al. 2008), or through stimulation of TLRs on NK cells themselves (Katsargyris et al. 2009; Moretta et al. 2006). However, cytokines and immune cells can also suppress NK cell activity, creating a system of checks and balances. For example, ligation of TLR4 on Kupffer cells leads to upregulation of inhibitory NKG2A receptor ligands (Eissmann et al. 2010). Also, cytokines such as IL-10 and transforming growth factor (TGF)-β are potent inhibitors of NK cell function (Tian et al. 2013; Miethke et al. 2010).

Although NK cells are most known for their roles in innate immunity, they also play critical roles in shaping the adaptive immune response through influencing other cells of the immune system including dendritic cells, Kupffer cells, T cells, B cells, and SECs. This can occur through direct cell-cell interactions, production of cytokines, chemokines, and growth factors, or through innate immune recognition (Tian et al. 2013). On the other hand, NK cells can also dampen the adaptive immune response through selective killing of macrophages, dendritic cells, or T cells (Lu et al. 2007; Moretta 2002).

NK cells expressing T cell receptor (NKT cells) NKT cells are lymphocytes that express both NK cell and T cell markers on their cell surface. NKT cells are important in non-specific cell killing and play a significant role in resistance to tumor invasion (Parker and Picut 2005; 2012). They also play diverse roles in liver injury, inflammation, fibrosis, and regeneration (Gao et al. 2009; Park et al. 2009).

NKT cells are abundant in the liver, accounting for 20–35% of all lymphocytes in the mouse liver and 10–15% of all lymphocytes in the rat or human liver (Gao et al. 2008, 2009; Racanelli and Rehermann 2006). In fact, the liver has the highest ratio of NKT cells to conventional T cells of any organ in the body (Eberl et al. 1999) and a higher number of NKT cells than the blood in humans and the lymphoid organs in mice (Crispe 2011). NKT cells can arise in the thymus or develop extrathymically from liver precursors (Shimamura et al. 1997). Liver NKT cells can be divided into two groups: "classical" and "non-classical", the former being CD1d-restricted (Mocchegiani et al. 2004).

The T cell receptor on NKT cells interacts with CD1 on the surface of antigen-presenting cells such as Kupffer cells, dendritic cells, and SECs (Crispe 2009). NKT cells are activated by ligation of their T cell receptor or through IL-12, derived from Kupffer cells and dendritic cells (Brigl et al. 2003). Upon activation, they can incite both inflammatory and anti-inflammatory responses through the production of a wide array of cytokines, including IFN-γ, IL-1, IL-17, IL-4, and IL-10 (Brennan et al. 2013; Matsuda et al. 2008; Lalazar et al. 2006; Bendelac et al. 1995) (Fig. 7.9). NKT cells also have the ability to detect distant tissue insults and, in turn, modulate the global immune status of the animal (Jenne and Kubes 2013).

NKT cells actively patrol the liver vasculature in search of invading pathogens or tumor cells, and are the only liver-resident lymphocyte to do so (Jenne and Kubes 2013). Once they detect tumor cells or pathogens, the can directly kill the target cells via the perforin-granzyme or Fas/Fas ligand pathways (Matsuda et al. 2008).

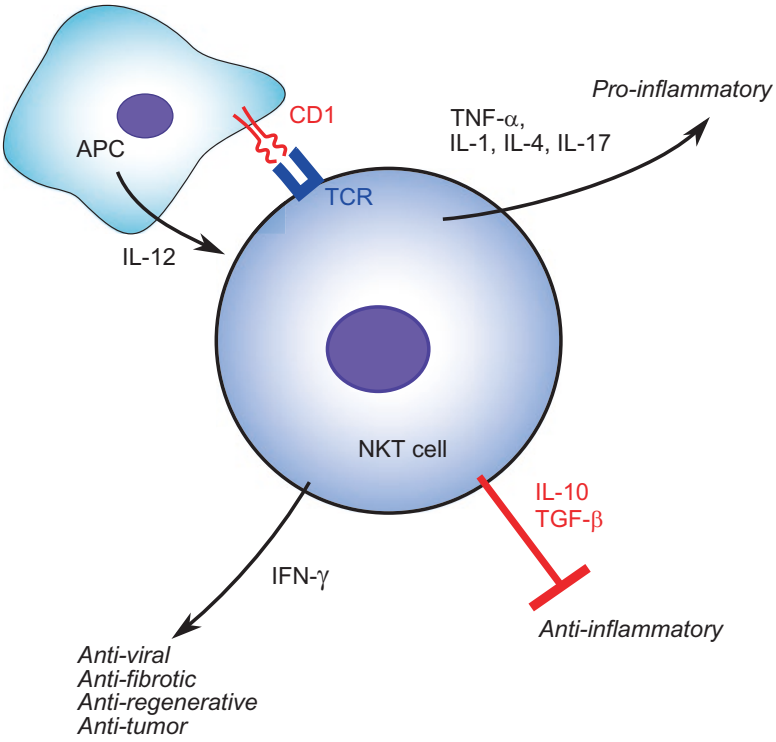

Fig. 7.9 Activation of *NKT* cells occurs through secretion of *IL-12* by antigen-presenting cells (*APC*), or by interaction of the T cell receptor (*TCR*) on the surface of the *NKT* cell with *CD1* on the surface of the *APC*. In turn, *NKT* cells secrete pro-inflammatory cytokines such as *TNF-α*, *IL-1*, *IL-4*, and *IL-17*, anti-inflammatory cytokines such as *IL-10* and *TGF-β*, and *IFN-γ*, which has anti-viral, anti-fibrotic, anti-regenerative, and anti-tumor properties

7.1.2.5 Stellate Cells

Stellate cells (previously referred to as Ito cells), comprise approximately one-third of the nonparenchymal cell population in the liver (Jezequel et al. 1984). They reside in the space of Disse between the SECs and hepatocytes and can be found at a higher frequency in periportal areas when compared with centrilobular areas (Wake 1980).

The main functions of quiescent stellate cells are to store vitamin A, regulate sinusoidal blood flow, mediate intercellular communications, synthesize extracellular matrix components, and maintain sinusoidal homeostasis (Weiskirchen and Tacke 2014).

Under physiological conditions, 50–80% of the total retinoids in the body are stored in the liver, of which 80–90% is stored in stellate cells (Blomhoff et al. 1990; Hendriks et al. 1988). Vitamin A regulates innate immunity through modulating leukocyte function, impeding regeneration of mucosal barriers, and modulating

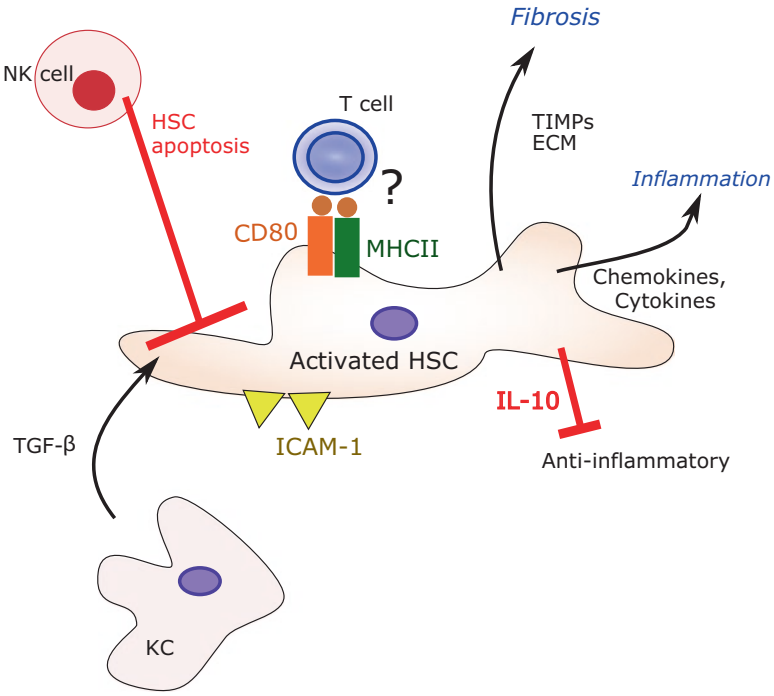

Fig. 7.10 Upon activation, hepatic stellate cells (*HSC*) induce fibrosis through production of tissue inhibitors of matrix metalloproteinases (*TIMPs*) and secretion of extracellular matrix (*ECM*) components. They also secrete pro-inflammatory cytokines such as *IL-6*, *chemokines*, and anti-inflammatory cytokines such as *IL-10.* Further activation of *HSC* can occur through secretion of *TGF-β* by Kupffer cells (*KC*). In contrast, *NK* cells can induce apoptosis of *HSC*. The role of activated *HSC* as antigen-presenting cells to *T cells* through *MHCII* is under debate

myeloid cell differentiation (Stephensen 2001). It is particularly important for differentiation of mucosal and splenic dendritic cell subsets involved in mucosal immunity (Beijer et al. 2014). Vitamin A is also involved in adaptive immunity through modulation of T and B lymphocyte development (Kluwe et al. 2011; Chen et al. 2013).

Stellate cells play important roles in mediating intercellular communications through their subendothelial processes, which wrap around the sinusoids between endothelial cells and hepatocytes (Wake 1995). This may facilitate intercellular transport of soluble mediators and cytokines.

During hepatic injury, stellate cells become activated (Fig. 7.10). They lose their characteristic cytoplasmic retinoid droplets and evolve into myofibroblast-like cells, including acquiring expression of alpha-smooth muscle actin (α-SMA), which is the single most reliable marker of stellate cell activation (Friedman 2008a). They contribute to the formation of hepatic fibrosis through secretion of tissue inhibitors of matrix metalloproteinases (TIMPs) and deposition of collagen (Friedman 2008b). They also become an important source of cytokines and can amplify the acute-

phase response through secretion of IL-6 (Tiggelman et al. 1995) as well as the general inflammatory response through production of chemokines that recruit neutrophils and mononuclear cells (Friedman 2008a). Activated stellate cells also have increased expression of adhesion molecules such as ICAM-1 (Weiskirchen and Tacke 2014). In addition to amplifying the inflammatory and immune response, activated stellate cells can also suppress the immune response through production of the anti-inflammatory cytokine IL-10 (Friedman 2008a).

There is extensive interaction between stellate cells and other immune cells within the liver (Fig. 7.10). As previously stated, activated stellate cells produce chemokines which recruit monocytes into the liver from the peripheral blood. In turn, these cells produce TGF-β, which further activates stellate cells (Karlmark et al. 2009). Resident Kupffer cells also produce several cytokines that promote survival of activated stellate cells (Pradere et al. 2013). In contrast, NK cells can selectively kill early activated or senescent stellate cells (Gao and Radaeva 2013), and stellate cells can induce T cell apoptosis through expression of the molecule B7-H1 (Yu et al. 2004).

Stellate cells express several TLRs, including TLR2, TLR3, TLR4, TLR7, and TLR9 (Weiskirchen and Tacke 2014). TLR2 responds to lipid-containing PAMPs and contributes to hepatic inflammation and fibrosis (Miura et al. 2013). TLR4 recognizes LPS, and activation of TLR4 results in increased expression of adhesion molecules and chemokines by stellate cells (Paik et al. 2003).

Some studies have claimed that stellate cells are potential antigen-presenting cells but this is controversial (Crispe 2011). They have been shown to cross-prime CD8+ T cells and present lipid antigens to NKT cells (Winau et al. 2007), and they express MHC molecules and costimulatory molecules (Vinas et al. 2003). However, these are expressed in relatively low amounts (Winau et al. 2007); therefore, the true antigen-presenting ability of stellate cells is uncertain.

7.1.2.6 Hepatocytes

Hepatocytes can also aid in the host immune response. They express innate immune receptors and can detect pathogens (Jenne and Kubes 2013). They also express MHC Class I and CD1, which allows them to act as unconventional antigen-presenting cells (Li and Tian 2013). However, they lack costimulatory molecules and therefore lead to immune tolerance rather than activation (Li and Tian 2013). The lack of costimulatory molecules leads to premature T cell death, a process termed “death by neglect” (Thomson and Knolle 2010). Under inflammatory conditions, some hepatocytes can be induced to express MHC Class II molecules, allowing them to function as antigen-presenting cells for CD4+ T cells, leading to a preferential induction of the Th2 response and defective immunity (Herkel et al. 2003; Wiegard et al. 2007). They also express TLRs and are able to respond to stimulation by TLR ligands (Broering et al. 2011).

Hepatocytes can interact with other cells of the immune system and contribute to the immune tolerant state of the liver. Interaction between hepatocytes and NK cells

can result in increased IL-10 and decreased IFN-γ production by NK cells (Jinushi et al. 2007). When encountering and activating NKT cells, hepatocytes can also induce IL-10-producing CD8+ T cells (Thomson and Knolle 2010; Wahl et al. 2007). Hepatocytes can also induce antigen-specific Treg cells, which can prevent the development of autoimmune disease (Luth et al. 2008).

7.1.2.7 Biliary Epithelial Cells

Biliary epithelial cells (also called cholangiocytes) play an important role in mucosal immunity through secretion of IgA into the bile (Woof and Mestecky 2005). This provides a barrier of defense against infective pathogens in the biliary tract by aggregating bacteria and preventing binding of pathogens to the mucosal surface (Sung et al. 1992).

Biliary epithelial cells express TLRs that recognize PAMPs (Syal et al. 2012; Harada and Nakanuma 2010). However, they possess "endotoxin tolerance", meaning that they are constantly exposed to PAMPs through the bile and/or portal circulation but fail to elicit an inflammatory response to these antigens (Harada and Nakanuma 2010). This is important for maintenance of immune tolerance in the liver. They also express several adhesion molecules, including ICAM-1 (Syal et al. 2012).

Cholangiocytes can release inflammatory cytokines and chemokines during times of hepatic injury, particularly in inflammatory processes involving the bile ducts (Syal et al. 2012; Harada and Nakanuma 2010). They also have the ability to induce apoptosis of infected cells (Syal et al. 2012).

Although controversial, some studies have shown that cholangiocytes have the ability to act as non-conventional antigen-presenting cells during persistent liver inflammation through expression of MHC Class II molecules, similar to hepatocytes (Bogdanos et al. 2013). These unconventional antigen-presenting cells may account for the liver's failure to mount an effective immune response during certain disease processes (Crispe 2011).

7.1.3 Innate (Nonspecific) Immunity

The liver plays a central role in local and systemic innate immunity (Fig. 7.11). Many factors that are required for mounting a systemic inflammatory response are synthesized in the liver by hepatocytes, Kupffer cells, and hepatic dendritic cells (van Oosten et al. 2001; Rowell et al. 1997). Furthermore, upregulated expression of receptors for cytokines and bacterial constituents on cells in the liver amplifies this response, leading to enhanced synthesis and secretion of cytokines, type I interferons, acute-phase proteins, and antimicrobial peptides. This upregulated expression is often triggered through microbial products such as endotoxin, which has been shown to stimulate Kupffer cells to produce inflammatory mediators, oxygen

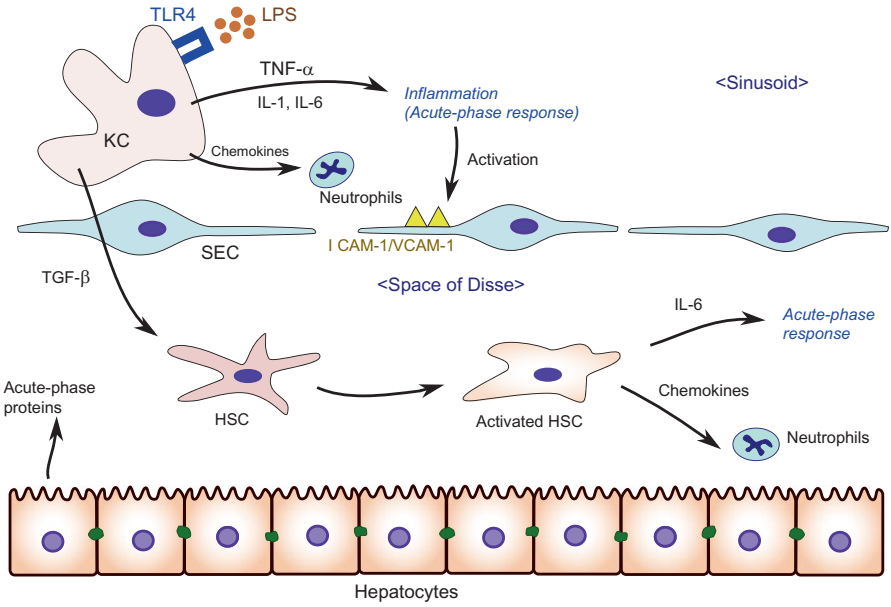

Fig. 7.11 The liver plays a central role in innate immunity. Upon binding of *LPS* to Kupffer cells (*KC*), they secrete cytokines involved in the acute-phase response, including *TNF-α*, *IL-1*, and *IL-6*. They also produce several *chemokines*, which attract neutrophils and other inflammatory cells. Pro-inflammatory cytokines secreted by *KC* activate sinusoidal endothelial cells (*SEC*), which in turn upregulate expression of adhesion molecules like *ICAM-1* and *VCAM-1*. In addition, secretion of *TGF-β* activates hepatic stellate cells (*HSC*). These activated *HSC* in turn produce additional *chemokines* and pro-inflammatory cytokines such as *IL-6*. *Hepatocytes* are also central to innate immunity in that they produce many of the *acute-phase proteins* involved in the systemic inflammatory response

free radicals, and proteases upon its binding (Abe and Thomson 2007) and to stimulate hepatic stellate cells to enhance expression of chemokines and adhesion molecules (Paik et al. 2003). TNF-α, produced primarily by Kupffer cells but also by hepatocytes, SECs, and activated hepatic stellate cells, is a key modulator of the hepatic innate immune response (Kowalewska et al. 2011). Hepatocytes also produce serum mannose-binding lectin, which recognizes microbial-specific sugar motifs and in turn activates innate immunity and microbial clearance through opsonization (Wagner et al. 2003). Activated stellate cells can also amplify innate immunity by enhanced acute-phase protein production through secretion of IL-6 (Tiggelman et al. 1995) and enhanced infiltration of inflammatory cells through chemokine production (Friedman 2008a).

Several roles of the liver in the innate immune response are outlined in more detail below.

7.1.3.1 Production of Acute-Phase Proteins

The liver is the major site of production of acute-phase proteins associated with acute inflammation (Parker and Picut 2005; 2012). These proteins are produced in response to cytokines, mainly released by macrophages at the site of injury (Evans 2011). A focal, extrahepatic injury prompts local tissue macrophages to release a first wave of cytokines, including IL-1, TNF-α, and small amounts of IL-6 (Fey et al. 1994). In addition, these cytokines can also be released by macrophages upon phagocytosis of pathogenic material (Liaskou et al. 2012). This is followed by a second wave of cytokines composed of large amounts of IL-6, which promotes a massive production of acute-phase proteins by hepatocytes (Fey et al. 1994). Hepatic production of acute-phase proteins begins within 24 h after acute tissue injury (Evans 2011).

Some acute-phase proteins are increased following an inflammatory response (referred to as positive acute-phase proteins), while others are decreased (referred to as negative acute-phase proteins). The systemic concentration of these proteins usually changes by at least 50% following an inflammatory injury (Gabay and Kushner 1999). Acute-phase proteins play a role in the immune response, provide protection against oxidative stress produced during inflammation, and have various anti-infective properties.

The liver produces several acute-phase proteins, including serum amyloid A (SAA), fibrinogen, C-reactive protein (CRP), complement factors C3 and C9, haptoglobin, hemopexin, ceruloplasmin, proteinase inhibitors, CD14, liposaccharide-binding protein (LBP), and serum mannose-binding lectin.

SAA promotes recruitment of inflammatory cells to the site of inflammation (Stockham and Scott 2008) and is a useful indicator of inflammation in several species, such as dogs (Evans 2011).

Fibrinogen is a precursor of fibrin and is a reliable indicator of inflammation in rats (Dasu et al. 2004). The liver also produces other coagulation factors, such as prothrombin, factor VIII (von Willebrand's factor), and plasminogen (Liaskou et al. 2012).

CRP is a pentraxin that acts as an opsonizing agent, activates complement, binds IgG receptors on mammalian cells and phosphocholine in bacterial membranes, recognizes nuclear constituents in damaged cells, and induces cytokine production (Stockham and Scott 2008). CRP production increases rapidly up to 1000-fold within 24–48 h in response to infection or trauma, and also reduces rapidly upon resolution of the inflammatory process (Liaskou et al. 2012). CRP is mainly produced by hepatocytes but can also be produced by Kupffer cells, monocytes, and subsets of lymphocytes (Park et al. 2005). CRP is a reliable indicator of inflammation in humans (Dupuy et al. 2003) and is also a major responder in dogs and pigs (Evans 2011).

Haptoglobin binds the globin portion of hemoglobin that is released by erythrocytes upon erythrolysis. It is considered to be a reliable indicator of inflammation in pigs (Evans 2011; Chen et al. 2003) and rats (Dasu et al. 2004).

Several proteinase inhibitors produced by the liver act as acute-phase proteins. These include α-2 macroglobulin, α-1 antitrypsin, α-1 antichymotrypsin, and α-1 cysteine proteinase inhibitor. These proteins act to reduce the tissue damage caused by proteinases that are released by dead or dying cells in sites of inflammation. Of these, α-2 macroglobulin is a reliable indicator of inflammation in rats (Dasu et al. 2004).

7.1.3.2 Nonspecific Phagocytosis of Particles and Nonspecific Pinocytosis of Molecules

The liver plays an important role in phagocytosis of particulate matter and pinocytosis of soluble materials by Kupffer cells and SECs, respectively.

Kupffer cells have the ability to clear bacteria, viruses, fungi, parasites, immune complexes, tumor cells, liposomes, lipid microspheres, iron, and other microparticles (Abe and Thomson 2007). One of the main roles of Kupffer cells in innate immunity is the uptake of endotoxin. Bacterial endotoxin (LPS) derived from the gastrointestinal (GI) tract is cleared principally by the liver through uptake by Kupffer cells (Naito et al. 2004), and periportal Kupffer cells (those closest to the portal blood from the GI tract) have the greatest phagocytic ability (Sleyster and Knook 1982). This occurs through LPS binding of LBP (LPS-binding protein, an acute-phase protein produced by the liver), which facilitates the transfer of LPS to CD14 on the surface of Kupffer cells. This activates TLR4 signaling, which leads to activation of Kupffer cells and their direct involvement in innate immunity (Su 2002). Kupffer cells also have Fc receptors for phagocytosis of immunoglobulin-coated cells or antigen-antibody immune complexes, complement receptors for binding and phagocytosis of erythrocytes coated with complement fragments (particularly C3b), and several scavenger receptors (Smedsrod et al. 1985b).

Pinocytosis of soluble materials occurs via specific receptors on SECs. These cells have several types of surface receptors, including collagen, scavenger and mannose receptors, that allow them to scavenge macromolecules and pathogenic agents from the sinusoidal blood, and can even engage in phagocytosis of particles less than 200 μm (Steffan et al. 1986). SECs can also engage in receptor-mediated uptake and removal of apoptotic bodies (Dini et al. 1995). Mannose receptors bind collagen α-chains, lysosomal enzymes, myeloperoxidase, and tissue plasminogen-activating factor (Sorensen et al. 2012; Malovic et al. 2007). SECs also contain Fc receptors for endocytosis of small soluble IgG immune complexes, particularly those which are too small for phagocytosis by Kupffer cells (Lovdal et al. 2000).

Hepatic stellate cells have also been shown engage in phagocytosis. Studies have demonstrated phagocytosis of hepatocyte-derived apoptotic bodies by stellate cells, resulting in their activation (Canbay et al. 2003).

7.1.3.3 Nonspecific Cell Killing

Effector cells in the liver that are involved in nonspecific cell killing include NK cells and NKT cells (Parker and Picut 2005). The constitutive activation of NK and NKT cells in the sinusoids of the normal liver likely plays a key role in immune surveillance and removal of circulating tumor cells from the body (Gao et al. 2009). Unlike cytotoxic (CD8+) T lymphocytes, recognition of target cells by NK and NKT cells is not restricted to MHC antigen presentation and does not involve immunologic memory, meaning that cell killing by NK cells is truly part of the innate immune system.

NK and NKT cells can exert antitumor effects through several pathways, including exocytosis of perforin/granzyme-containing granules, induction of apoptosis, or production of cytokines that augment function of other immune cells (Nakatani et al. 2004). Therefore, they can directly or indirectly kill pathogens, tumor cells, and stressed hepatocytes or hepatic stellate cells (Tian et al. 2013). NK and NKT cells cause apoptosis of target cells through the Fas/Fas ligand (FasL) pathway. In this pathway, FasL binds to its receptor (Fas) and activates "death domain" signaling elements in the target cell, leading to activation of the caspase cascade and apoptosis. Exocytosis of perforin/granzyme-containing granules leads to perforin-mediated introduction of pores into the cell membrane of the target cell and introduction of granzymes through this pore into the cytoplasm. This bypasses signaling molecules and proceeds directly to the caspase cascade through direct activation of caspase-3 by granzyme B. This pathway is thought to be a backup defense against those cells resistant to Fas/FasL-mediated apoptosis. However, some studies have shown that human and rat hepatic NK cells utilize the perforin/granzyme pathway exclusively in induction of apoptosis in tumor cells that are resistant to killing by splenic blood NK cells (Vermijlen et al. 2002).

Upregulation of NK and NKT cells occurs through cellular cross-talk with other cells in the liver. For example, activated Kupffer cells produce IL-18, which promotes Fas/FasL-mediated apoptosis (Tsutsui et al. 1996) as well as perforin/granzyme-mediated cell killing (Dao et al. 1998) by NK and NKT cells. Activated Kupffer cells also produce IL-22, which has been shown to stimulate NK cells (Tsutsui et al. 1996). In addition, the liver has high levels of IL-12 and other inflammatory cytokines. IL-12 promotes the maturation of NKT cells, as well as other cytotoxic cells (O'Farrelly 2004).

7.1.3.4 Disposal of Waste Molecules of Inflammation and Nonspecific Immunity

Hepatic SECs play an important role in disposal of waste molecules. They are professional pinocytes and engage in receptor-mediated pinocytosis through four categories of surface receptors: collagen receptor, mannose receptor, scavenger/hyaluronan receptor, and Fc receptor (Parker and Picut 2005).

SECs of rat liver express a receptor that specifically recognizes and mediates endocytosis of collagen α-1 monomers and denatured collagen (Smedsrod et al. 1985a), which has recently been determined to be the mannose receptor (Malovic et al. 2007). The mannose receptor can also clear the C-terminal propeptide of type I procollagen and the scavenger receptor can clear the NH_2-terminal propeptides of types I and III procollagen (Smedsrod et al. 1985a; Melkko et al. 1994).

The mannose receptor on SECs is also essential in the regulation of serum glycoprotein homeostasis and in control and resolution of inflammation (Lee et al. 2002). The scavenger receptor can clear polysaccharides and proteins released by extracellular matrix turnover, intracellular macromolecules, modified serum proteins and bacterial and fungal proteins (Smedsrod et al. 1990). In addition, the hyaluronan receptor on SECs is the main route of clearance of hyaluronan from the blood, which increases with immune-mediated and liver diseases as well as with certain malignancies (McCourt et al. 1999). This receptor shares many functional properties with the scavenger receptor family.

7.1.4 Adaptive (Acquired or Specific) Immunity

The liver contains several effector cells of adaptive immunity, and is involved in both cell-mediated and antibody-mediated responses. There is considerable cross-talk in the liver between cells involved in innate and adaptive immunity (Fig. 7.12). For example, activated NK and NKT cells and Kupffer cells rapidly produce copious amounts of cytokines which modulate adaptive immune responses (Gao et al. 2009). Important roles of the liver in the adaptive immune response are outlined below.

7.1.4.1 Deletion of Activated T Cells

The liver is a preferential site of T cell apoptosis (Bertolino et al. 2002). This allows for clearance of activated T cells following local inflammatory reactions as well as removal of T cells that were activated by inflammation at distant sites.

Leukocyte emigration in the liver occurs in the sinusoids and is unique in that it does not require the initial selectin-mediated rolling step that occurs in the postcapillary venules (Wong et al. 1997). Instead, SECs constitutively express high levels of the adhesion molecules ICAM-1 and VCAM, which facilitates integrin-mediated adhesion of activated T cells in the absence of local inflammation (Mehal et al. 1999). Antigens expressed by antigen-presenting cells in the liver actively cause CD8+ T cell accumulation through T cell receptor-activated ICAM-1 adhesion, and can also passively sequester CD8+ T cells that do not recognize intrahepatic antigen through VCAM adhesion (John and Crispe 2004).

Once T cells are sequestered in the liver, the absence of costimulatory molecules and helper molecules and insufficient antigen presentaion in the liver microenvironment

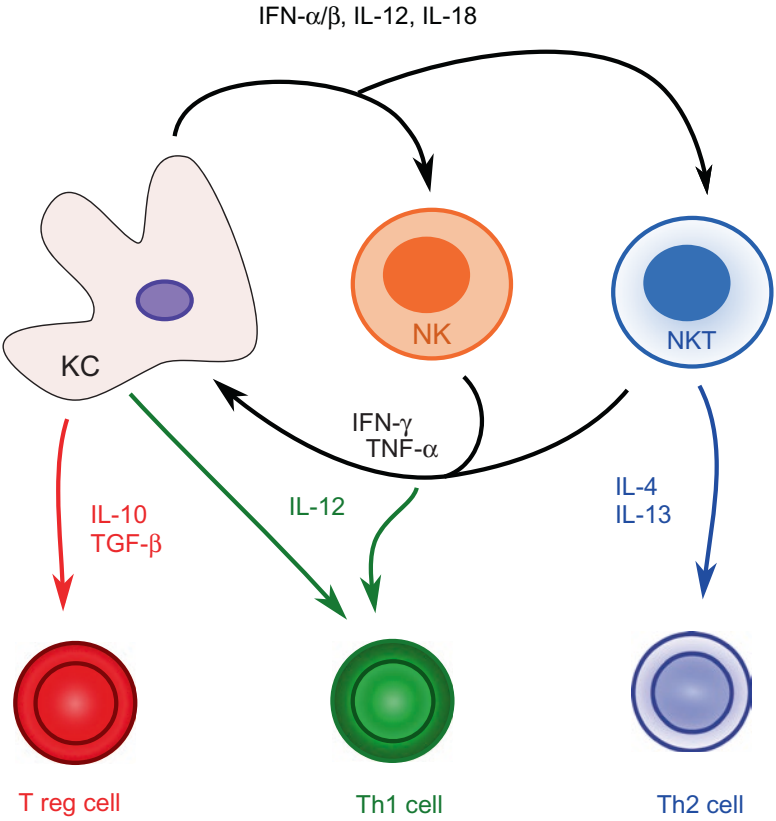

Fig. 7.12 Considerable cross-talk exists in the liver between cells of the innate immune system (*KC*, *NK*, *NKT*) and cells of the adaptive immune system (T cells). For example, Kupffer cells (*KC*) can activate regulatory T (*Treg*) cells through secretion of *IL-10* and *TGF-β* or *Th1 cells* through secretion of *IL-12*. They can also activate other innate immune cells (*NK* and *NKT* cells) through secretion of type I interferons (*IFN-α* and *IFN-β*), *IL-12*, and *IL-18*. *NK* and *NKT* cells can activate *KC* and *Th1* cells through secretion of *IFN-γ* and *TNF-α*. In addition, *NKT* cells can activate *Th2* cells through secretion of *IL-4* and *IL-13*

results in apoptosis of the T cells, a process referred to as "death by neglect" (Mehal et al. 1999). Therefore, the liver is regarded as a "sink" for activated T cells.

Several cell types in the liver can facilitate T cell apoptosis. Hepatic dendritic cells (particularly the lymphoid subset) promote apoptosis of T cells and resulting immune suppression (O'Connell et al. 2001). Activated T cells that are bound to SECs have been shown to be actively killed by the beta-galactoside binding protein galectin-1 (Lotan et al. 1994; Perillo et al. 1995; Rabinovich et al. 1998). Activated hepatic stellate cells have a mechanism for inducing T cell apoptosis through enhanced expression of B7-H1, also known as programmed death ligand 1 (PD-L1), which plays a major role in suppressing the adaptive immune response (Yu et al. 2004). This molecule, which is not expressed by quiescent stellate cells, is upregulated either by IFN-γ or by contact with activated T cells (Weiskirchen and Tacke 2014).

Table 7.1 Cell types involved in immunotolerance in the liver and their effects

Cell types	Mechanism	Effects
KC DC HSC SEC NKT HEP	IL-10 production	Proliferation of Treg cells, suppression of inflammatory cytokine production
KC DC HSC	TGF-β production	Proliferation of Treg cells, suppression of inflammatory cytokine production
KC DC	Prostaglandin production	Inhibition of antigen-specific T cell activation, apoptosis of activated T cells
DC HSC SEC HEP	B7-H1 expression	T cell tolerance, T cell apoptosis
KC SEC	FasL expression	Apoptosis of activated T cells
DC	Expression of PD-1 and CTLA-4	Induction of CD8+ T cell tolerance
SEC	C-type lectin expression	Inhibition of T cell proliferation, apoptosis of T cells
HEP	Lack of co-stimulation	T cell "death by neglect"

DC dendritic cells, *HEP* hepatocytes, *HSC* hepatic stellate cells, *KC* Kupffer cells, *NKT* natural killer T cells, *SEC* sinusoidal endothelial cells

7.1.4.2 Induction of Tolerance to Ingested and Self-Antigens

The liver is constantly exposed to ingested antigens through the portal bloodstream, and an important job of the liver is to promote immunologic tolerance (prevent reaction) toward potentially antigenic molecules absorbed from the intestinal tract. The liver also plays a role in tolerance to self-antigens through selective apoptosis of self-reactive T cells, as described above.

Several cell types are involved in creating an immunotolerant microenvironment in the liver (Table 7.1). In response to continuous low levels of bacterial endotoxin (LPS), Kupffer cells and SECs down-regulate immune reactivity through secretion of immune-suppressing cytokines, such as IL-10, TGF-β, and prostanoids, creating a tolerogenic cytokine milieu (Knolle and Gerken 2000; Crispe 2009; Thomson and Knolle 2010). This has several downstream effects. Under TGF-β stimulation, hepatocytes secrete IL-10, leading to amplification of immune-suppression (Bissell et al. 1995). IL-10 and prostanoids downregulate leukocyte adhesion molecules on the surface of SECs (Knolle and Gerken 2000) and inhibit antigen-specific T cell activation by Kupffer cells, dendritic cells, and SECs (You et al. 2008; Thomson

et al. 1995; Knolle et al. 1998), often through downregulation of MHC Class II and costimulatory molecules. IL-10 production also leads to decreased NK cell activation (Tu et al. 2008). TNF-α is also released by Kupffer cells in response to physiologic concentrations of LPS and it downregulates CD4+ T cell activation by SECs (Knolle and Gerken 2000).

As outlined previously, several cells in the liver can act as antigen-presenting cells. However, the result of this antigen presentation is often tolerance rather than immunity.

Liver myeloid, plasmacytoid, and other subsets of dendritic cells are, according to some references, the main initiators of immune tolerance in the liver (Crispe 2011; Hsu et al. 2007). Hepatic dendritic cells tend to prime naïve T cells into Th2, Th0 or Treg cells and only with further activation signals can they acquire the co-stimulatory signals needed to generate a Th1 cell response (Hsu et al. 2007). They also express the inhibitory molecules PD-1 and CTLA-4, which induce circulating CD8+ T cell tolerance (Probst et al. 2005).

SECs express MHC Class II molecules and can take up antigen and present it to CD8+ T cells. However, this results in antigen-specific tolerance rather than immunity (including tolerance towards oral antigens), likely because there is a lack of input from helper T cells (Rubinstein et al. 1986, 1987; Limmer et al. 2000, 2005). Upon recognition of antigen presented by SECs, CD8+ T cells proliferate rapidly but do not produce effector cell cytokines and demonstrate a marked reduction in antigen-specific cellular cytotoxicity (Berg et al. 2006). This appears to mainly depend on expression of inhibitory molecules B7-H1 on SECs and PD-1 on CD8+ T cells (Diehl et al. 2008). In addition, SECs express various C-type lectins which inhibit T cell proliferation and induce T cell apoptosis (Liu et al. 2004) and they can also induce T cell apoptosis through the Fas/FasL pathway (Onoe et al. 2005). A unique feature of SECs is the ability to efficiently cross-present extracellular antigens in the context of MHC Class I, allowing for both CD4+ and CD8+ T cells to be exposed to blood-derived antigen, leading to development and maintenance of immune tolerance (Jenne and Kubes 2013). SECs can also affect the activity of other liver cells. For example, contact of SECs with neighboring antigen-presenting dendritic cells leads to suppression of their ability to fully activate naïve CD8+ T cells through reduced expression of co-stimulatory molecules (Schildberg et al. 2008).

Hepatocytes have been shown to act as non-conventional antigen-presenting cells. However, this leads to premature T cell death or tolerance rather than activation, likely representing “death by neglect” due to lack of costimulatory signals (Li and Tian 2013). T cells that do become activated by hepatocytes express lower levels of survival gene products and have less IL-2 mRNA compared with those activated by professional antigen-presenting cells (Parker and Picut 2005).

Hepatic stellate cells also have tolerogenic capability. Immunomodulation by stellate cells is regulated by the inducible expression of the inhibitory molecule B7-H1 (Chen et al. 2006). Stellate cells can also produce vitamin A-derived retinoic acid and TGF-β, both of which contribute to a tolerogenic environment (Bogdanos et al. 2013).

Regulatory T cells (Tregs) have an important role in restricting the immune response towards self and foreign antigens. The liver has relatively few of these cells but they nonetheless play an important role in regulating hepatic immunity (Li and Tian 2013). Most antigen-presenting cells in the liver can induce Treg development and/or recruit circulating Tregs. Tregs can suppress other cells in both direct and indirect manners. They can directly interact with effector T cells and antigen-presenting cells and they can secrete regulatory cytokines such as IL-10 and TGF-β (Bopp et al. 2007; Sakaguchi et al. 2008). CD8+ T cell responses to non-self antigens are controlled by Treg cells that secrete IL-10 in response to antigen (Breous et al. 2009).

Hepatic NK cells contribute to immune tolerance through production of chemokines, including macrophage inflammatory protein-1α (MIP-1α) and MIP-1β, which induce hepatocytes and SECs to secrete CXCL9. This in turn recruits T cells into the liver, which are then deleted or induced to become tolerogenic (Crispe 2003).

Interestingly, tolerance induction by the liver is not restricted to the local hepatic microenvironment. Significant crosstalk occurs between the liver and other organs, often leading to systemic tolerance (Li and Tian 2013). Induction of systemic tolerance by liver antigen-presenting cells has been attributed to peripheral deletion and induction of antigen-specific Tregs (Crispe 2009). One quite interesting characteristic of peripheral tolerance induced by the liver is its ability to transfer tolerance from a tolerized animal to a naïve animal by adoptive transfer of leukocytes (Gorczynski 1994).

7.1.4.3 Extrathymic Proliferation of T Cells

Extrathymic pathways of T cell differentiation have been shown to occur in the liver, where T cell populations may arise from their own preexisting precursor cells rather than from the thymus (Sugahara et al. 1999). These cells are generated in situ in the hepatic parenchyma and migrate to the sinusoidal lumen, which is accompanied by reverse migration of normal thymus-derived T cells from the sinusoids into the hepatic parenchyma (Yamamoto et al. 1999). The livers of adult mice have been found to contain c-kit-positive stem cells that can reconstitute thymocytes, multiple lineage cells, and bone marrow stem cells (Taniguchi et al. 1996; Watanabe et al. 1996), and these cells may contribute to this process.

This process has increased significance with aging, and has also been shown to be predominant in mice with autoimmune diseases or athymic mice (Parker and Picut 2005). It also plays a pivotal role in immune reactions that occur with bacterial infections, malignancies, autoimmune diseases, and pregnancy (Abo 1993).

Extrathymically-derived T cells exhibit several phenotypic differences when compared with T cells derived from the thymus. These include: constitutive expression of the IL-2Rβ chain, expression of the αα homodimer of CD8 in CD8+ cells, intermediate expression of the T cell receptor (TCRint), and the presence of self-reactive clones (Abo 1993; Ohteki et al. 1992). In addition, a large proportion

of extrathymically-derived T cells contain the γδ form of the T cell receptor and are double-negative (CD4 and CD8 negative). There is also a gender difference in the number of these cells. Studies have found that females contain more TCRint cells in the liver and other organs when compared to males (Kimura et al. 1994) and that they are even higher in number during gestation (Kimura et al. 1995) or after estrogen injection (Yahata et al. 1996).

7.1.4.4 Disposal of Waste Molecules of Specific Immunity

The liver is the primary site for removal of experimentally administered antigen and immune complexes (Parker and Picut 2005), which can occur via phagocytosis by Kupffer cells or pinocytosis by SECs.

Kupffer cells contain Fc receptors which recognize the Fc domain of antibodies. This results in nonspecific phagocytosis of immune complexes as well as antibody-coated particles such as microorganisms and eukaryotic cells (Parker and Picut 2005). Kupffer cells and SECs produce mRNA for FcγRIIB2 and FcγRIII, which mediate the uptake of immune complexes (Parker and Picut 2005; Lovdal et al. 2000). This is in contrast to hepatocytes, which have no demonstrable production of Fc receptors (Lovdal and Berg 2001). There is conflicting data as to whether Fc receptors on Kupffer cells are recycled following binding to immune complexes or whether they are destroyed along with the immune complexes in lysosomes.

The liver is also a major site of deletion of signaling and effector molecules, allowing clearance of those molecules following inflammatory reactions. This includes assimilation of cytokines, and several cell types in the liver have a high density of cytokine receptors (Fey et al. 1994).

7.2 Organ-Specific Immunopathological Processes

7.2.1 Hepatic Inflammation

The general process of hepatic inflammation can have many etiologies, some of which are described in further detail below (viral hepatitis, autoimmune disease, drug-induced liver injury, etc.). However, in order to understand the pathophysiology of each of these diseases and how the immune system affects or is affected by them, a general description of the process of hepatic inflammation is needed.

Inflammation in the liver can be sterile or septic. During sterile inflammation (e.g. drug-induced liver injury, ischemia-reperfusion injury), necrotic cells release their contents, some of which act as alarmins or danger-associated molecular patterns (DAMPs) to activate the immune system (Adams et al. 2010). An important alarmin is the nuclear protein high mobility group box protein 1 (HMGB-1) (Scaffidi et al. 2002). HMGB-1 is detected by TLR4 or the receptor for advanced glycation endproducts (RAGE) on the surface of dendritic or Kupffer cells or TLR4 on SECs

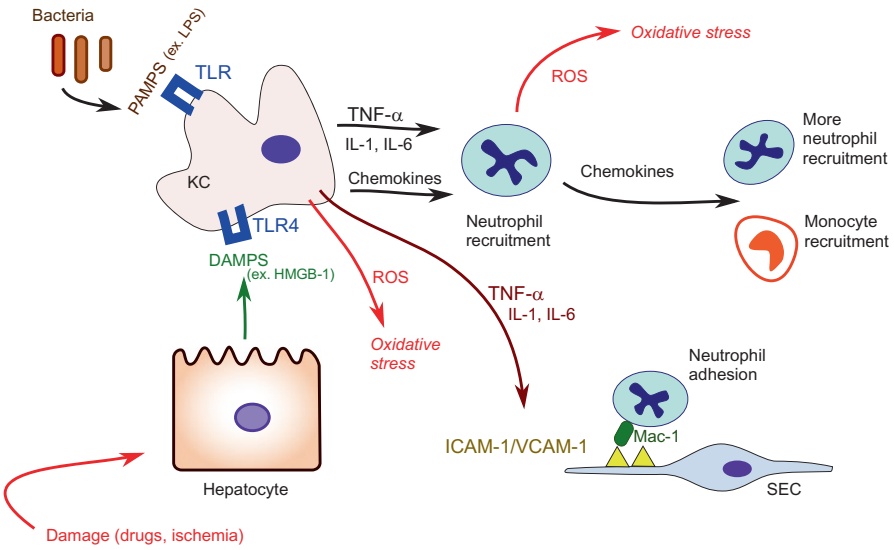

Fig. 7.13 Hepatic inflammation can be sterile (e.g. ischemic or drug-induced) or septic. In sterile inflammation, hepatocyte damage leads to release of damage-associated membrane proteins (*DAMPs*) such as *HMGB-1* and in septic inflammation, the bacteria contain pathogen-associated membrane proteins (*PAMPs*) such as *LPS*. In both cases, the proteins bind to *TLR*s on Kupffer cells (*KC*), leading to *KC* activation. Activated *KC* produce pro-inflammatory mediators (e.g.*TNF-α*, *IL-1*, *IL-6*) and *chemokines* which act to recruit *neutrophils* to the liver. Neutrophils also produce *chemokines*, leading to *monocyte* and additional *neutrophil* recruitment. Pro-inflammatory cytokines also upregulate expression of adhesion molecules (*ICAM-1*, *VCAM-1*) on sinusoidal endothelial cells (*SEC*) and *Mac-1* expression on neutrophils, leading to neutrophil adhesion within the sinusoids. Activated *KC* and *neutrophils* can also produce reactive oxygen species (*ROS*)

(van Golen et al. 2012). During septic inflammation, Kupffer cells and resident dendritic cells detect the presence of invading pathogens via pathogen-associated molecular patterns (PAMPs). In either case, Kupffer cells then become activated and recruit other immune cells to the liver (Jaeschke 2011) (Fig. 7.13).

During the initial event of hepatic inflammation, innate immune cells such as neutrophils, monocytes, and NK cells are recruited to the liver. Important inflammatory mediators that have been shown to recruit neutrophils to the liver microvasculature include TNF-α, CXC chemokines (e.g. IL-8/CXCL8, MIP-2/CXCL2, and CXCL1), platelet-activating factor (PAF), and IL-1 (Kowalewska et al. 2011). Unlike that which occurs in capillaries elsewhere, neutrophil recruitment to the hepatic sinusoids is believed to be an active process rather than a simple mechanical trapping (Kowalewska et al. 2011). In contrast to sepsis, neutrophils responding to sterile inflammation are highly motile within the sinusoids, and can migrate intravascularly towards the site of injury, ultimately infiltrating directly into the area of damage (McDonald et al. 2010).

Recruitment of neutrophils and other cells of innate immunity is followed by recruitment of adaptive immune cells. Liver-resident dendritic cells sample foreign

antigen and carry it to local draining portal lymph nodes, where the antigens are presented to adaptive naïve T cells. Following antigen presentation, different types of antigen-specific effector T cells leave the lymph nodes, drain back into the systemic circulation, and are recruited via the hepatic sinusoids towards the site of injury or inflammation (Liaskou et al. 2012). Lymphocyte recruitment increases in response to inflammation and intrahepatic localization of lymphocytes determines the ultimate pattern of disease (Adams et al. 2010). Chemokine ligands (i.e. CCL-3 through CCL-5) are also strongly expressed by portal vascular endothelium, where they mediate lymphocyte recruitment in a range of inflammatory diseases (Murai et al. 1999). Additionally, lymphocytes can amplify the immune response. For example, Th17 cells secrete IL-17, which attracts neutrophils and helps to link innate and adaptive immunity (Liaskou et al. 2012).

As said previously, leukocyte emigration in the liver occurs in the sinusoids and is unique in that it does not require the initial selectin-mediated rolling step that occurs in post-capillary venules (Wong et al. 1997). This is because hemodynamic factors, Kupffer cells, and leukocyte interactions with vessels walls serve to slow the rate of blood flow through the sinusoids. Sinusoidal endothelial cells constitutively express high levels of the adhesion molecules ICAM-1 and VCAM, facilitating integrin-mediated adhesion of activated T cells. Sinusoidal endothelial cells also constitutively express vascular adhesion protein-1 (VAP-1), which mediates lymphocyte recruitment and adhesion within the liver (Lalor et al. 2002). CXCR6 (receptor for chemokine CXCL16) is expressed on liver-infiltrating T cells, permitting their localization to hepatocytes and biliary epithelial cells, both of which co-express CXCL16 and VCAM-1 in areas of liver injury (Boisvert et al. 2003; Heydtmann et al. 2006). This allows recruited lymphocytes to hone in on areas of injury. CXCL16 supports the adhesion of lymphocytes directly and also promotes activation of β_1-integrins, thus enhancing lymphocyte binding to VCAM-1 (Heydtmann et al. 2006).

Neutrophil adhesion involves an interaction between Mac-1 on neutrophils and ICAM-1 on sinusoidal endothelial cells (McDonald et al. 2010). Kupffer cells also play a direct role in leukocyte adhesion by trapping leukocytes within sinusoids and providing adhesive ligands (Matsuno et al. 2002), and activated hepatic stellate cells secrete chemokines and express adhesion molecules (e.g. ICAM-1 and VCAM-1) that are important for lymphocyte adhesion (Adams et al. 2010). Additionally, stellate cells have a critical role as regulators of post-endothelial migration and positioning of cells within the liver parenchyma (Holt et al. 2009).

Neutrophils responding to sterile inflammation function to clear debris and initiate wound-healing (Nathan 2006). They are emerging as the central orchestrators of resolution and restitution following tissue injury (Soehnlein and Lindbom 2010). However, over-exuberant or prolonged neutrophil infiltration can instead exacerbate tissue injury and lead to disease (McDonald and Kubes 2012). This tissue injury occurs through the production of reactive oxygen species (ROS), reactive nitrogen species (RNS), and proteases (Jaeschke and Bajt 2006). Neutrophil adhesion triggers adherence-dependent ROS formation and degranulation, leading to

tissue damage. Proteases are important in extravasation and migration, but can also lead to cytokine production.

Inflammatory monocytes recruited to the inflamed liver can differentiate into macrophages and play a role in tissue homeostasis through clearance of senescent cells and remodeling and repair of tissue (Gordon 1998). This includes removal of cellular debris generated during tissue remodeling and phagocytosis of necrotic debris.

If hepatic inflammation becomes more chronic, tertiary lymphoid structures may develop, referred to as "portal tract-associated lymphoid tissue" (Grant et al. 2002; Yoneyama et al. 2001). These areas contain discrete T and B cell regions as well as dendritic cells and represent an environment of ongoing recruitment and retention of lymphocytes within the liver. Chronic inflammation can also lead to fibrosis, which is outlined in detail below.

7.2.2 Hepatic Fibrosis

Normally, the hepatic extracellular matrix (ECM) comprises less than 3% of the relative area of the liver and the matrix in the space of Disse is mainly composed of collagen IV and collagen VI. However, following persistent liver injury of virtually any etiology, it is replaced by fibrillary collagens I and III and fibronectin (Hernandez-Gea and Friedman 2011).

Fibrosis and wound healing are part of the normal innate immune response to tissue damage and self-limiting fibrosis is also observed in acute liver disease. The problem occurs when the fibrosis becomes dysregulated and excessive scarring occurs in response to persistent injury, leading to altered tissue function (Pellicoro et al. 2014). This chronic activation of the wound-healing reaction is characterized by persistent hepatocellular and/or cholangiocellular damage, complex inflammatory infiltrate, activation of ECM-producing cells, and marked changes in the quality and quantity of the ECM (Pinzani and Macias-Barragan 2010).

Both adaptive and innate immune systems play important roles in hepatic fibrosis (Table 7.2). Studies using animal models have shown that animals deficient in macrophages, T cells, B cells, and NKT cells show reduced fibrosis, meaning that these cells all contribute to fibrosis; however, neutrophils do not appear to contribute (Holt et al. 2008a). In response to liver injury, hepatic macrophage populations markedly change, with a drop in the number of Kupffer cells and a marked increase in the number of monocyte-derived macrophages, suggesting that pro-fibrogenic macrophages are derived from this population (Ramachandran et al. 2012).

An important component of hepatic fibrosis is sinusoidal remodeling (Fig. 7.14). Deposition of ECM in the space of Disse leads to loss of fenestrations in sinusoidal endothelial cells (Hernandez-Gea and Friedman 2011). Defenestration and "capillarization" of the sinusoids initiate perisinusoidal fibrosis through altered retinol metabolism within hepatic stellate cells, leading to the transformation of the stellate cells into myofibroblasts (Fraser et al. 1991; Braet and Wisse 2002). These

Table 7.2 Role of innate and adaptive immune cells in the development of hepatic fibrosis

Cell type	Role in hepatic fibrosis	Cytokines involved
Kupffer cells	Secrete pro-inflammatory and fibrogenic mediators, ROS	IL-1, IL-6, TNF-α, TGF-β
Dendritic cells	Enhanced activity, stimulation of NKT cells and HSCs	IL-6, IL-12
Monocyte-derived macrophages	Perpetuation of fibrosis, secrete fibrogenic mediators	IL-1, IL-6, TNF-α, PDGF, TGF-β
NK cells	Anti-fibrotic through apoptosis of HSCs	TNF-α (TRAIL), IFN-γ
NKT cells	Drive Th2 response	IL-4, IL-13
Th1 cells	Anti-fibrotic response, stimulate NK cells	IL-2, IL-12, IFN-γ
Th2 cells	Pro-fibrotic response	IL-4, IL-5, IL-6, IL-13
B cells	Pro-inflammatory and pro-fibrotic response	IL-1, IL-8, IL-12, TNF-α

HSC hepatic stellate cells, *IFN* interferon, *IL* interleukin, *NK* natural killer, *NKT* natural killer T, *ROS* reactive oxygen species, *TGF* transforming growth factor, *Th1* T helper type 1, *Th2* T helper type 2, *TNF* tumor necrosis factor, *TRAIL* tumor necrosis factor-related apoptosis ligand

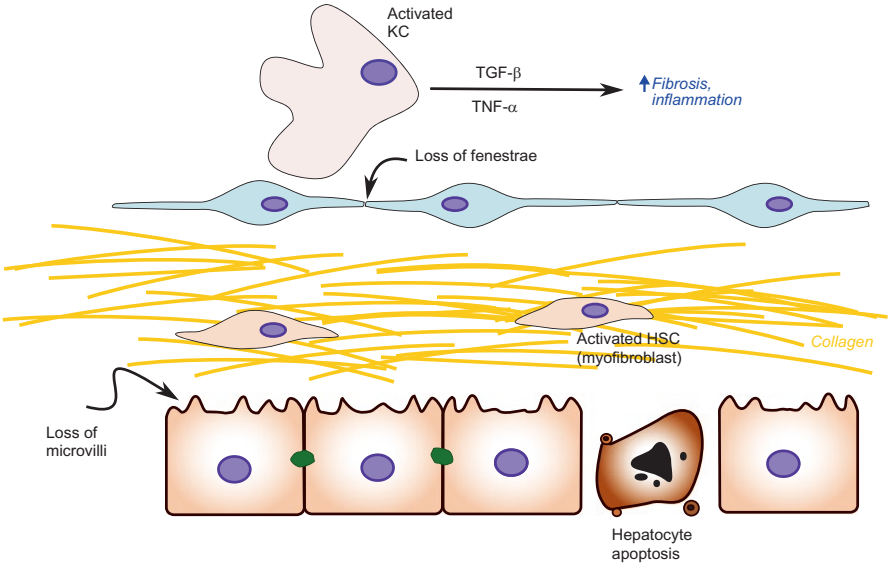

Fig. 7.14 Sinusoidal remodeling is an important component of hepatic fibrosis. Extracellular matrix deposition in the space of Disse leads to *loss of fenestrae* between sinusoidal endothelial cells, also termed "capillarization". Loss of fenestration as well as *TGF-β* production by activated Kupffer cells (*KC*) leads to activation of hepatic stellate cells (*HSC*), which assume a *myofibroblast* phenotype. These cells produce additional extracellular matrix components, leading to accumulation of abundant *collagen* within the space of Disse. This sinusoidal remodeling leads to *loss of microvilli* on hepatocytes and eventually *hepatocyte apoptosis*

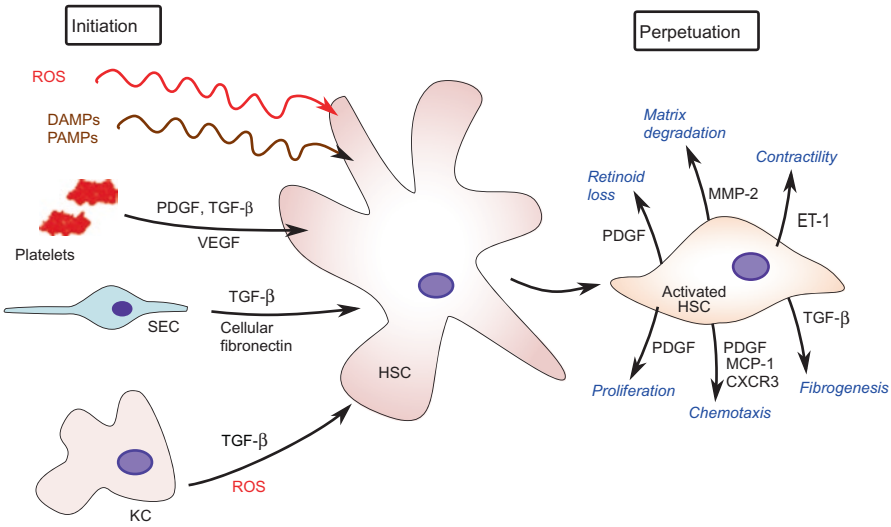

Fig. 7.15 The two major phases of hepatic stellate cell (*HSC*) activation are *initiation* and *perpetuation*. Initiation can occur with oxidative damage (*ROS*), sterile or septic inflammation (*DAMPs*, *PAMPs*), or from cytokines produced by other cells. *Platelets* can initiate *HSC* activation through secretion of several growth factors, including *PDGF*, *TGF-β*, and *VEGF*. Sinusoidal endothelial cells (*SEC*) can activate HSC through secretion of *TGF-β* and *cellular fibronectin*. Kupffer cells (*KC*) can initiate *HSC* activation through production of *TGF-β* and *ROS*. Perpetuation involves cytokines and other molecules secreted by the activated *HSC* themselves. This includes *PDGF*, which leads to HSC proliferation, chemotaxis, and retinoid loss; endothelin-1 (*ET-1*), which is important for *HSC* contractility; *TGF-β*, which is important for fibrogenesis; *MMP-2* which leads to matrix degradation; and several other chemokines, including *MCP-1* and *CXCR3*

activated stellate cells are proliferative, fibrogenic, and contractile, and have a central role in the development of hepatic fibrosis through secretion of tissue inhibitors of matrix metalloproteinases (TIMPs) and deposition of collagen.

The activation of hepatic stellate cells consists of two major phases: initiation and perpetuation (Fig. 7.15). This may be followed by resolution of fibrosis if the injury subsides.

Initiation consists of early changes in gene expression and phenotype that render stellate cells responsive to cytokines and stimuli, and results mainly from paracrine stimulation by neighboring sinusoidal endothelial cells, Kupffer cells, hepatocytes, and platelets (Friedman 2008a). Initiation can also be stimulated by soluble factors such as reactive oxygen species (ROS), DAMPs such as apoptotic hepatocyte DNA, or PAMPs such as LPS (Xu et al. 2012). Platelets produce platelet-derived growth factor (PDGF), TGF-β, and vascular endothelial growth factor (VEGF), all of which stimulate fibroblastic activity. Sinusoidal endothelial cells stimulate stellate cells through the production of cellular fibronectin and conversion of TGF-β to its active profibrogenic form (Jarnagin et al. 1994). Kupffer cells stimulate matrix synthesis, cell proliferation, and the release of retinoids through production of cytokines (par-

ticularly TGF-β) and ROS (Bilzer et al. 2006). Hepatocytes are a source of fibrogenic lipid peroxides (Novo et al. 2006).

The initiation stage is followed by perpetuation, which results from the effects of the above stimuli on maintaining an activated phenotype and generating fibrosis. It involves both paracrine and autocrine loops (Friedman 2010). Perpetuation of activated stellate cells involves chemotaxis, fibrogenesis, contractility, matrix degradation, and retinoid loss. PDGF is a potent mitogen for stellate cells and is produced by platelets, macrophages, and even other activated stellate cells (Pinzani 2002; Pellicoro et al. 2014; Mehal and Friedman 2007). Other compounds with mitogenic activity towards stellate cells include VEGF, thrombin, epidermal growth factor (EGF), keratinocyte growth factor (KGF), and basic fibroblast growth factor (bFGF) (Xu et al. 2012). Recruitment of activated stellate cells to the site of injury occurs through the production of chemokines, including PDGF, MCP-1/CCL2, and CXCR3 (Kinnman et al. 2000; Marra et al. 1998; Bonacchi et al. 2001). Once stimulated, stellate cells can generate fibrosis through increased cell number and increased matrix production per cell (Friedman 2008a). Activated stellate cells produce collagen types I and III, and the most potent stimulus of collagen production is TGF-β (Gressner et al. 2002). TGF-β can also stimulate its own production by myofibroblasts, creating an autocrine loop (Xu et al. 2012). Activated hepatic stellate cells also exhibit contractility (due to secretion of endothelin-1), and can impede portal blood flow by constricting individual sinusoids (Friedman 2008a; Mehal and Friedman 2007). This contractile potential is mediated by expression of the cytoskeletal protein α-smooth muscle actin (α-SMA) by activated stellate cells (Rockey et al. 1992). In addition to fibrogenesis, activated stellate cells are also involved in matrix degradation through production of matrix metalloproteinases (MMPs), particularly MMP-2 (Mehal and Friedman 2007). The early disruption of normal hepatic matrix by MMPs hastens its replacement by scar matrix (Friedman 2008a).

Resolution of fibrosis can occur through pathways that drive stellate cell apoptosis or contribute to their reversion to a quiescent phenotype. Activated stellate cells express death receptors such as Fas and TRAIL, and withdrawal of fibrogenic and anti-apoptotic signals and/or stimulation of death receptors by ligands may stimulate apoptosis (Pellicoro et al. 2014). NK cells have been shown to induce apoptosis of early activated or senescent activated stellate cells by a TRAIL-mediated mechanism (Radaeva et al. 2006). In contrast, quiescent stellate cells and fully activated stellate cells are relatively resistant to killing (Gao et al. 2009). IL-22 induces senescence of stellate cells, limiting fibrosis and accelerating its resolution (Kong et al. 2012). These senescent stellate cells have decreased matrix and cytokine synthesis, decreased MMP secretion, and a gene expression profile that is consistent with exit from the cell cycle (Krizhanovsky et al. 2008). Macrophages are required for recovery of fibrosis following withdrawal of the inciting agent (Pinzani and Macias-Barragan 2010). Macrophages secrete proteases which act to degrade cross-linked collagen (Fallowfield et al. 2007). In addition to the effects of pro-resolution macrophages, disappearance of pro-inflammatory and pro-fibrotic macrophages may also modulate the local microenvironment to favor resolution (Pellicoro et al. 2014).

The outcome of fibrosis may depend on the type of T cell reaction occurring in the underlying disease process. In general, Th1 reactions will inhibit the development of fibrosis versus Th2 reactions will stimulate it. Th1 cytokines (especially IFN-γ and IL-12) stimulate NK cell function, stimulate enzymes active in collagen degradation, and increase apoptosis of stellate cells (Mehal and Friedman 2007; Muhanna et al. 2008). In contrast, Th2 cytokines IL-4 and IL-13 increase TGF-β activity and collagen synthesis by stellate cells (Mehal and Friedman 2007; Pellicoro et al. 2014). CD8+ T cells can also play a pro-fibrogenic role in the liver (Safadi et al. 2004). Other immune cells involved in the development of fibrosis include dendritic cells and NKT cells. During chronic hepatic injury, dendritic cells in the liver can transform from a tolerogenic to an immunogenic phenotype, leading to pro-fibrogenic effects (Xu et al. 2012). A subset of innate-like invariant NKT (iNKT) cells are activated to a pro-fibrogenic state following acute or chronic liver injury (Pellicoro et al. 2014).

In addition to hepatic stellate cells, other cells have also been found to be sources of myofibroblasts in the liver (Xu et al. 2012). The most researched of these cell types are the portal fibroblasts. Portal fibroblasts can be recruited and activated into myofibroblasts, particularly during biliary diseases (i.e. cholestasis) and ischemic injury (Xu et al. 2012; Dranoff and Wells 2010). Their proliferation and differentiation is stimulated by fibroblast growth factor 2 (FGF-2) and, in opposition to stellate cells, is actually inhibited by PDGF (Penz-Osterreicher et al. 2011) and TGF-β (Wells et al. 2004). Extensive cross-talk exists between biliary epithelial cells and portal fibroblasts in cholestatic liver disease. Proliferating small bile ducts (referred to as the "ductular reaction") express numerous adhesion molecules, cytokines, chemokines, growth factors, and pro-fibrogenic stimuli that have autocrine and paracrine effects on myofibroblasts (Penz-Osterreicher et al. 2011). For example, cholangiocytes upregulate MCP-1/CCL2 during biliary fibrosis, which increases smooth muscle actin expression and pro-collagen production by portal myofibroblasts (Kruglov et al. 2006). In contrast, quiescent cholangiocytes do not produce these peptides. Other cell types that have been proposed to contribute to hepatic fibrogenesis but are less substantiated include bone marrow-derived cells and hepatic epithelial cells (hepatocytes and/or biliary epithelial cells), the latter thought to contribute through epithelial-mesenchymal transition.

7.2.3 Chronic Liver Failure (Cirrhosis)

Cirrhosis is the term used to describe an end-stage liver secondary to any cause of chronic liver failure. The switch from acute resolving hepatic disease to chronic persistent inflammation is associated with the development of a specialized microenvironment that maintains leukocytes, particularly lymphocytes, in close proximity with activated hepatic stellate cells (myofibroblasts) at sites of tissue injury (Muhanna et al. 2007). This close proximity is important, as cycles of tissue injury

and repair during chronic liver disease lead to local secretion of cytokines, including IL-6, IL-12, and TNF-α, by activated stellate cells which promote additional inflammation and antigen-independent bystander T cell activation (Holt et al. 2008a). This cytokine milieu favors lymphocyte survival and promotes effector T cell function even in the absence of the original inciting agent (Abrignani 1997).

Cirrhosis almost always involves a progression of hepatic fibrosis, and two main features are responsible for this. The number of hepatic myofibroblasts reaches its peak level and there is progressive reduction of the ability to degrade and remodel fibrillary collagen (Friedman 2008b; Bataller and Brenner 2005; Pinzani and Macias-Barragan 2010).

Aside from loss of liver function, hepatic cirrhosis can also lead to the development of endotoxemia and increased risk of systemic infection due to shunting of portal blood through numerous acquired portosystemic shunt vessels, bypassing Kupffer cells (Cullen and Brown 2012).

7.2.4 Drug-Induced Liver Injury

Drug-induced liver injury (DILI) accounts for approximately 13% of acute liver failure cases in the United States and is a leading cause of drug withdrawal from the market (Schiodt et al. 1999; Ostapowicz et al. 2002; Lumley 1990). Most are idiosyncratic, and in the majority of cases in which toxicity during clinical trials led to termination of drug development, the incidences were not predicted by animal studies (Lumley 1990). The main culprits of DILI are anti-infective, central nervous system, musculoskeletal, and gastrointestinal drugs, which are broad categories (Andrade et al. 2005). Histologically, DILI is characterized by acute hepatocellular necrosis, biliary injury, or a combination of both (Zimmerman 2000).

DILI can be categorized as predictable (type A reactions) or idiosyncratic (type B reactions) (Deng et al. 2009). Hepatic injury in predictable reactions can occur due to the parent compound or one of its metabolites (Njoku 2014). There are three mechanisms by which toxic parent compounds or their metabolites induce hepatotoxicity. This includes direct injury to hepatocytes by interfering with critical cellular functions, sensitization of hepatocytes to cytokine-induced damage, or irreversible covalent modification of native proteins by reactive metabolites, leading to an immune reaction against the altered proteins (Njoku 2014)

As previously stated, the majority of DILI is idiosyncratic. With idiosyncratic DILI (IDILI), the mechanism of toxicity is unknown but likely does not involve the pharmacological action of the drug (Shaw et al. 2010). In addition, toxicity typically occurs at doses that are considered generally safe for the majority of patients, a wide range of reactions can be seen, and there is an inconsistent temporal relationship to drug exposure (Shaw et al. 2010). IDILI can be further divided into metabolic idiosyncrasy and immune idiosyncrasy (Zimmerman 1999). Metabolic idiosyncrasy is based on differences in drug metabolism between individuals, most often due to genetic polymorphisms (Adams et al. 2010). As a result, toxic metabolites accumulate in some individuals and lead to hepatotoxicity. This may be the

case for hepatotoxicity caused by isoniazid, ketoconazole, troglitazone, and pyrazinamide (Adams et al. 2010). In contrast, immune idiosyncrasy involves the development of auto-antibodies against drug-modified liver components (haptenization) or its metabolites (Ju and Uetrecht 2002; Liu and Kaplowitz 2007). Drugs that may cause IDILI by this mechanism include halothane and tienilic acid (Adams et al. 2010). However, the causal link between reactive metabolite accumulation and hepatotoxicity or between auto-antibody production and hepatotoxicity has not been established for many compounds (Shaw et al. 2010; Deng et al. 2009) and in many cases, the underlying pathogenesis remains unknown.

A more recent hypothesis for the underlying pathogenesis of IDILI is the "danger hypothesis". This suggests that, in addition to immunization and challenge, a second "danger signal" is required in order to precipitate the adaptive immune response and lead to hepatotoxicity (Seguin and Uetrecht 2003; Pirmohamed et al. 2002). This signal could be cell death, cytokine release, intestinal microbial disturbance, infection, or even a signal caused by the drug metabolite itself (Shaw et al. 2010; Seguin and Uetrecht 2003). For example, the drug may cause cellular injury and necrosis, which results in release of cellular contents (e.g. DNA, RNA, heat shock proteins) that act as DAMPs (Adams et al. 2010). These DAMPs induce complement activation or bind TLRs on neutrophils and macrophages and recruit them to the liver (Fig. 7.16). The inflammatory cells can then cause further cell death.

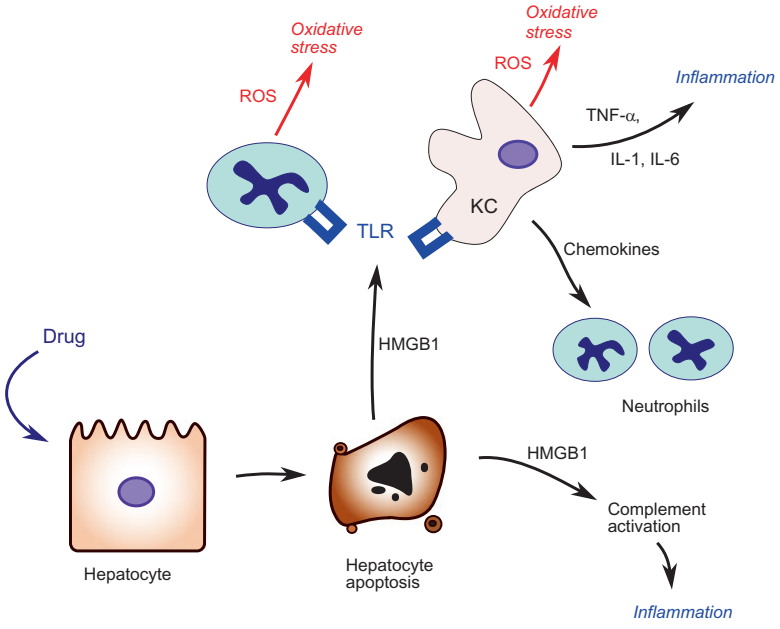

Fig. 7.16 The "danger hypothesis" of idiopathic drug-induced liver injury (IDILI). An administered drug causes *hepatocyte apoptosis*. This leads to the production of the DAMP *HMGB1* by injured and dying hepatocytes. *HMGB1* is recognized by *TLR*s on *neutrophils* and Kupffer cells (*KC*), leading to production of *ROS* and oxidative stress. *KC* also produce pro-inflammatory cytokines (*TNF-α*, *IL-1*, *IL-6*) and *chemokines. HMGB1* can also induce inflammation through *complement activation*

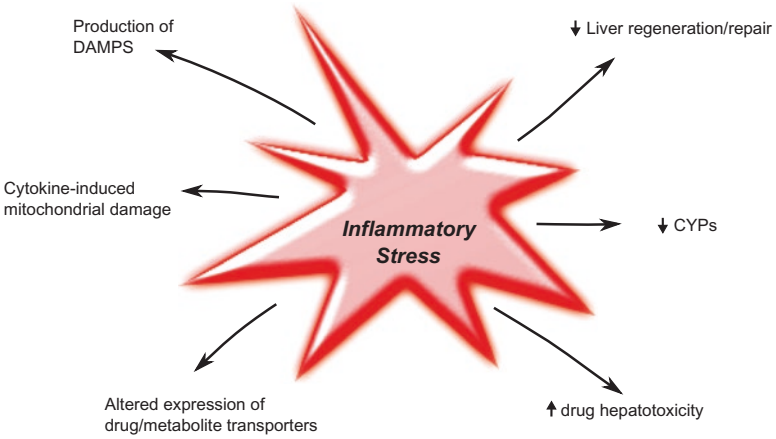

Fig. 7.17 Hypothesis of *inflammatory stress* leading to drug-induced liver injury (IDILI). Inflammatory stress can lead to production of *DAMPs* (e.g. HMGB1), leading to the path of injury outlined by the "danger hypothesis". It can also decrease the activity of cytochrome P450 enzymes (*CYPs*) or alter the expression of drug or metabolite transporters, which can lead to build up of potentially toxic drug metabolites or the parent compound. Inflammatory stress can heighten the toxicity of already hepatotoxic drugs and can lower the ability of the liver to regenerate and repair itself, perpetuating drug-induced hepatic damage. Pro-inflammatory cytokines released during inflammatory stress can also cause mitochrondrial damage

There are also several studies suggesting that inflammatory stress during drug therapy can contribute to IDILI (Hussaini and Farrington 2007) (Fig. 7.17). For example, the presence of viral hepatitis is a risk factor for HIV patients developing IDILI from anti-retroviral drugs. In addition, antibiotics and non-stereoidal anti-inflammatory drugs (NSAIDs) that are used in inflammatory conditions are also some of the most common culprits of IDILI. There are several mechanisms in which inflammation may contribute to IDILI (Deng et al. 2009). For example, minor hepatic injury elicited by some drugs may progress to more serious injury as damaging inflammatory mediators are released. On the other hand, inflammatory stress itself may initiate hepatotoxicity that is exaggerated by the drug. Inflammation can also inhibit or delay liver regeneration and repair and can inhibit cytochrome P450 enzymes. Lastly, concurrent inflammation may modify the intrahepatic distribution of drugs by altering expression of drug transporters including ATP-binding-cassette (ABC) transporters in hepatocytes (Petrovic et al. 2007). Several animal models have demonstrated that mild inflammation from small doses of LPS interacts with drugs known to cause IDILI in humans, leading to the development of similar liver injury in animals (Deng et al. 2009). Inflammatory stress can also be linked with other hypotheses for IDILI pathogenesis (Shaw et al. 2010). For instance, genetic polymorphisms in cytokines or cytokine receptors (rather than biotransformation enzymes) may make individuals more susceptible to hepatotoxicity. Inflammatory stress may also regulate the expression of biotransformation enzymes or transporters for reactive drug metabolites. In addition, inflammation may lead to the production of DAMPs, linking it to the danger hypothesis.

Immune system involvement in DILI is extensive. Tissue damage in DILI may involve auto-antibodies reacting with liver-specific antigens (B cell involvement), cell-mediated immunity against hepatocytes (T cell involvement), and the activation of innate immunity to both initiate and induce drug-specific adaptive immune responses (Liu and Kaplowitz 2007). Innate immune cells involved in the development of DILI include NK cells and Kupffer cells. In most cases, involvement of neutrophils is controversial. Activation of NK cells by other factors (e.g. viral infection) has been shown to accelerate drug-induced hepatotoxicity (Gao et al. 2009). Kupffer cells are thought to be protective against DILI through secretion of IL-10 (Kumagai et al. 2007). Loss of IL-10 has been shown to result in increased pro-inflammatory cytokines such as TNF-α and IL-6 and more severe hepatocellular necrosis (Kumagai et al. 2009). T cells also play an important role, and the balance between recruitment and survival of regulatory and effector T cells may determine the outcome of DILI. Th2 cell activation by IL-4 has been shown to initiate drug-induced hepatic inflammation and hepatotoxicity in animal models (Njoku 2014). DILI can also affect the systemic immune response through disruption of normal hepatic immune function (Parker and Picut 2012).

Acetaminophen (APAP) is the most common cause of DILI in humans. It is an example of predictable DILI, or type A reaction. APAP is metabolized in the liver by CYP2E1 to the highly electrophilic intermediate N-acetyl-p-benzoquinone-imine (NAPQI). NAPQI is usually detoxified through glutathione conjugation, but after a toxic dose glutathione is depleted and NAPQI accumulates. Excess NAPQI covalently binds to cellular proteins to form APAP-protein adducts, resulting in oxidative stress, mitochondrial dysfunction, and DNA damage, ultimately causing hepatocellular death. Liberation of free DNA from dying cells triggers an inflammatory response via activation of innate signal receptors (Tsutsui and Nishiguchi 2014). Activation of the innate immune system (Kupffer cells, NK cells, and NKT cells) results in the release of inflammatory mediators and recruitment of leukocytes into the liver. These pro-inflammatory mediators include TNF-α, IL-1, and nitric oxide (Laskin et al. 1995). A study in mice found that decreasing serum concentration of these inflammatory mediators reduced the pathophysiological responses associated with APAP toxicity (Blazka et al. 1997). In addition to resident macrophages (Kupffer cells), the liver of mice with APAP-induced hepatotoxicity also contains a population of infiltrating macrophages, which have an anti-inflammatory role (Holt et al. 2008b). This population of macrophages appears to be the most critical phagocytes for clearing necrotic cell debris and shutting down inflammation (Jaeschke et al. 2012).

A prototypical example of IDILI is halothane hepatitis. Halothane is oxidized in the liver by CYP2E1 to the reactive intermediate trifluoroacetic acid (TFA), which covalently binds liver proteins and forms adducts. The adducts can then be presented on the plasma membrane of hepatocytes (Kharasch et al. 1996). Most patients that develop halothane hepatitis were exposed previously, and many have serum auto-antibodies that react with specific hepatic proteins such as CYP2E1 (Bourdi et al. 1996). These auto-antibodies cannot be cleared by the complement system and form immune complexes, which precipitate and cause liver damage (Njoku et al.

2006). An inflammatory response is triggered, with increased production of proinflammatory cytokines and leukocyte infiltration. Neutrophils appear to have a critical role in the pathogenesis of halothane-induced hepatitis (You et al. 2006), and are recruited by many cells, including NKT cells (Cheng et al. 2010). In summary, the formation of TFA adducts leads to initial hepatocyte damage, which is proceeded by immune complex formation and an inflammatory reaction. This inflammatory response appears to be a critical factor in determining the degree of hepatic injury and may contribute to variability in susceptibility among patients (Ju and Reilly 2012).

7.2.5 Autoimmune Liver Disease

There are several types of autoimmune liver diseases in humans, including autoimmune hepatitis, primary biliary cirrhosis, and primary sclerosing cholangitis. A type of helper T cell, referred to as the Th17 cell, appears to be a major component of the immune response in autoimmune reactions (Romagnani et al. 2009). The intestine is considered to be a key regulator of Th17 responses, and patients with autoimmune liver diseases often have concomitant intestinal inflammation; therefore mucosal immunity in the gastrointestinal tract may be involved in the pathogenesis of these disorders (Trivedi and Adams 2013). In the liver, γδT cells also appear to be involved in induction and maintenance of autoimmunity, as they are shown to be increased in both liver and peripheral blood of patients with active autoimmune liver diseases (Martins et al. 1996; Wen et al. 1992; Ferri et al. 2010). In addition, double-negative (CD4−CD8−) T cells in the liver may be critical to induction of autoimmunity (Thomson et al. 2006; Masuda et al. 1991). This population is extrathymically derived and the liver has a higher population of these cells compared with the peripheral circulation. Dendritic cells may also play essential roles in initiation and perpetuation of autoimmune diseases, as they are frequently observed in patients with primary biliary sclerosis (Demetris et al. 1989), and decreased MHC Class II expression is observed on dendritic cells from patients with autoimmune hepatitis (Hiasa et al. 2002). Environmental factors may also play a role. For example, it has been shown that gestational exposure to dioxin exacerbates autoimmune disease in mice (Mustafa et al. 2009). Overall, the pathogenesis of these disorders is extremely complex and they arise from an interaction of both environmental and genetic factors.

Autoimmune hepatitis is characterized by infiltration of mononuclear cells within portal tracts and into the adjacent parenchyma, with disruption of the limiting plate (interface hepatitis). Like many other autoimmune diseases, it has a strong female bias, the cause of which remains uncertain (McFarlane and Heneghan 2004). Patients with autoimmune hepatitis have self-reactive B and T cells and autoantigens that are presented in conjunction with MHC Class II molecules by antigen-presenting cells. Initiation of the disease process is believed to occur through recognition of liver self-antigens by naïve T cells (Liberal et al. 2013), and hepatic

damage is most likely mediated by antibody-dependent cell-mediated cytotoxicity (Bogdanos et al. 2013). This is believed to mainly occur through CD4+ T cells; however, recent studies support involvement of more diverse cell populations, including Th17 cells (Longhi et al. 2012). Genetic susceptibility, molecular mimicry, and impaired immunoregulation, particularly involving Treg cells, also contribute to initiation and perpetuation of the autoimmune attack (Pellicoro et al. 2014). Molecular mimicry can occur through repeated exposure to a triggering sequence that is common in diverse antigens, leading to eventual loss of self-tolerance (Bogdanos et al. 2001).

Autoimmune hepatitis is a polygenic disorder, and aside from disease susceptibility, genetic factors also influence clinical manifestations and disease outcomes (Czaja 2008; Czaja et al. 2002). It can be difficult to diagnose, as disease-specific autoantibodies are only detectable in approximately 30% of people with the disease (Baeres et al. 2002). In addition, a long lag time between sensitization to the triggering antigen and actual onset of disease can complicate the ability to identify etiological triggers (Czaja 2010).

Primary biliary cirrhosis (PBC) is an autoimmune disease causing lymphocytic inflammatory destruction of small and medium intrahepatic bile ducts, leading to fibrosis, cholestasis, and cirrhosis (Kaplan and Gershwin 2005; Hirschfield and Gershwin 2013). The disease is characterized by a clinical homogeneity among patients, an overwhelming female predominance, and the production of a multilineage immune response against mitochondrial autoantigens (Hirschfield and Gershwin 2013). Autoantibodies are mainly targeted against the E_2 subunit of the pyruvate dehydrogenase complex (Gershwin et al. 1987), and anti-mitochondrial antibodies can be detected in over 90% of patients (Walker et al. 1965). Antigens are released from apoptotic blebs of biliary epithelial cells or from molecular mimicry of infectious agents or altered xenobiotics (Selmi et al. 2010). Innate immunity is also thought to be involved, as cholangiocytes express a variety of TLRs and patients with PBC have upregulation of TLR4 and TLR9 in cholangiocytes (Selmi et al. 2009). Monocytes in patients with PBC are also more susceptible to activation of TLRs, resulting in secretion of proinflammatory cytokines (Broering et al. 2011). NK cells appear to play a dual role in the pathogenesis of PBC. On one hand, NK cells may promote progression of the disease by causing apoptotis of cholangiocytes via a TRAIL-dependent mechanism and by producing pro-inflammatory cytokines (Shimoda et al. 2011). On the other hand, NK cells can diminish the progression of PBC by inhibiting adaptive immune responses through IL-10 production and induction of apoptosis in immune cells (Schleinitz et al. 2010).

The portal lymphocytic infiltrate in PBC is composed of both αβ CD4+ T cells and CD8+ T cells (Hirschfield and Gershwin 2013). CD8 cells may play a more significant role in mediating destruction of the bile duct, particularly under a background of loss of Treg cell function (Shi and Zhang 2012; Hsu et al. 2009). Patients with PBC also have an enhanced ratio of Th1 to Th2 cells (Harada et al. 2002). As with autoimmune hepatitis, Th17 cells also may play an important role (Lafdil et al. 2010). The innate immune response stimulates cholangiocytes to produce Th17-inducible and maintaining cytokines. In addition, Th17 cells produce

IL-17, which stimulates cholangiocytes to produce chemokines leading to migration of inflammatory cells.

Primary sclerosing cholangitis (PSC) is characterized by chronic inflammation and concentric periductal fibrosis ("onion-skinning") leading to obliteration of intra and/or extrahepatic bile ducts, particularly medium and large-sized ducts (Penz-Osterreicher et al. 2011). The underlying pathogenesis involves misdirected homing of intestinal mucosal effector T cells to the liver secondary to hepatic inflammation and activation of vascular adhesion protein 1 (VAP1), which results in aberrant expression of mucosal vascular addressin cell adhesion molecule 1 (MADCAM1) and CC-chemokine ligand 25 (CCL25) (Liaskou et al. 2011). These gut-primed adaptive and innate immune responses contribute to chronic progressive biliary inflammation (Pellicoro et al. 2014). Fifty percent of patients with PSC have antinuclear antibodies and 80% have perinuclear antineutrophil cytoplasmic antibodies (pANCA) (Mulder et al. 1993).

7.2.6 Viral Hepatitis

Hepatitis B and C viruses are main causes of chronic liver disease worldwide, and the host immune response to the virus is critical in determining whether the infection will resolve or progress to chronic disease (Bertoletti and Ferrari 2003). An immune response that is too weak to clear the virus but sufficient enough to perpetuate destruction of infected hepatocytes can induce a chronic inflammatory disease, eventually leading to cirrhosis.

Natural immune responses control hepatitis B virus (HBV) infection in over 90% of adult patients. Defective innate immunity and exhausted adaptive immunity characterize chronic infection (Pellicoro et al. 2014). Most chronic HBV infections are caused by vertical transmission from mother to child in utero or during early infancy; therefore, viral persistence is due to immaturity of the immune system or tolerance to the antigen in utero (Rehermann 2013). In contrast, most persistent HCV infections are due to horizontal transmission between immunocompetent adults and viral escape mutations are more important in establishing persistence in immunocompetent adults (Rehermann 2013). It is more difficult to achieve spontaneous clearance of hepatitis C virus (HCV), as it attenuates both innate and adaptive immune responses. Spontaneous clearance of HCV is associated with vigorous and multispecific T cell responses. If clearance cannot be achieved and a chronic infection is established, the cellular immune response to the virus triggers chronic liver inflammation and progressive disease (Pellicoro et al. 2014).

A summarized hepatic immune response to viral infection is presented in Fig. 7.18. Expression of certain pattern recognition receptors by sinusoidal endothelial cells is targeted by hepatotropic viruses in order to leave the vascular compartment and infect hepatocytes (Knolle 2007). Once hepatocytes are infected by virus, they present antigen to antigen-presenting cells, which then activate naïve CD4+ T cells, CD8+ T cells, NK cells and NKT cells (Crawford and Burt 2012). CD4+ T cells

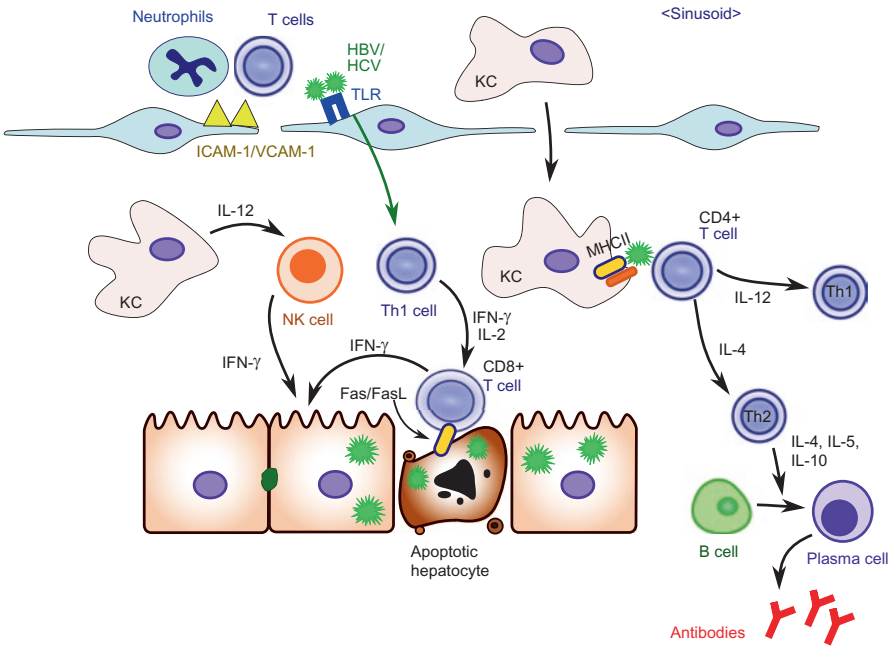

Fig. 7.18 The hepatic immune response to viral infection. Viral particles (*HBV/HCV*) target pattern recognition receptors (*TLRs*) on sinusoidal endothelial cells to gain entrance to the space of Disse and infect hepatocytes. Infection leads to activation of antigen-presenting cells like Kupffer cells (*KC*), which in turn activate *NK* cells through secretion of *IL-12* and *CD4+ T* cells through presentation of antigen with *MHCII*. Activated *NK* cells produce *IFN-γ*, which has strong antiviral properties. Activated CD4+ T cells differentiate into *Th1* cells under the influence of *IL-12*, or *Th2* cells under the influence of *IL-4*. *Th1* cells activate *CD8+ T* cells through secretion of *IFN-γ* and *IL-2*, which themselves secrete *IFN-γ* and induce apoptosis of infected hepatocytes through *Fas/FasL* interactions. *Th2* cells secrete cytokines (*IL-4*, *IL-5*, and *IL-10*) that stimulate *B cells* to become *antibody*-secreting *plasma cells*. Viral infection also upregulates the expression of adhesion molecules *ICAM-1* and *VCAM-1* on the surface of sinusoidal endothelial cells, leading to increased adhesion of neutrophils and lymphocytes

differentiate into Th2 cells via IL-4 stimulation and secrete IL-4, IL-5, and IL-10, which stimulate B cell maturation into antibody-secreting plasma cells. IL-12 production by activated hepatocytes also leads Th1 cell differentiation and stimulation of NK cells (Bertoletti and Ferrari 2003; Koziel 1999). Resulting Th1 cells secrete IFN-γ and IL-2 to stimulate CD8+ T cells to become cytotoxic lymphocytes (Crawford and Burt 2012). Cytotoxic lymphocytes, NK cells, and NKT cells also secrete IFN-γ, which has an antiviral effect on hepatocytes, modulates production of pro-inflammatory cytokines IL-1 and TNF-α, and increases MHC expression (Bertoletti and Ferrari 2003; Foster 1997).

In HCV infection, type I interferons (IFN-α and IFN-β) serve as the first line of defense and have antiviral and anti-proliferative effects (Invernizzi et al. 2007). These type I interferons are produced by macrophages through activation of TLR3

and retinoic acid-inducible gene-1 (RIG-1), and they bind to receptors on neighboring cells (Terilli and Cox 2013). This activates the Jak/Stat pathway, leading to induction of interferon stimulated genes which degrade viral RNA and amplify the interferon response (Terilli and Cox 2013). Interferons cause an antiviral state in uninfected cells and inhibit viral replication in infected cells (Terilli and Cox 2013). They also activate effector cells of the immune system, serving as a link between innate and adaptive immunity (Li and Lemon 2013). However, HCV has evolved mechanisms to prevent this signaling from occurring (Gale and Foy 2005). For example, HCV serine protease NS3/4A cleaves IFN-β promoter stimulator 1 (IPS-1), which is an adaptor in RIG-1 signaling. It also cleaves Toll/IL-1 receptor/resistance domain-containing adaptor-inducing IFN (TRIF) protein, which is an adaptor molecule for TLR3. In contrast to HCV, HBV infection does not induce a strong initial type I interferon response, garnering it the term "stealth" virus (Wieland et al. 2004). However, it still may be recognized by resident cells in the liver and may therefore activate innate immunity without type I interferon induction. It also stimulates the release of other cytokines from Kupffer cells and sinusoidal endothelial cells (Hosel et al. 2009).

Accumulating evidence suggests that NK cell activity is actually a major determinant of the clinical outcome following viral infection of the liver (Busca and Kumar 2014). Mice with viral disease have significant accumulation of NK cells in the liver, and it has been shown that these play a key role in innate immune defenses against viral infection (Gao et al. 2009). In the steady-state, hepatic NK cells promote liver tolerance. However, following viral infection, chemokines produced by resident cells in the liver recruit more NK cells and these cells acquire an activated phenotype to control viral infection (Shi et al. 2011). NK cell expression of TRAIL is upregulated, leading to apoptosis of infected cells, hepatocellular damage and inflammation, viral clearance, and inhibition of fibrosis (Ahlenstiel et al. 2011; Stegmann et al. 2010; Dunn et al. 2007; Glassner et al. 2012). Non-infected cells are not killed by NK cells because inhibitory signals from MHC Class I molecules prevail over activating signals. In contrast, virus-infected cells have altered expression of MHC molecules which disrupts the inhibitory signals and allows for NK-mediated lysis of infected hepatocytes (Bogdanos et al. 2013). However, HCV in particular can evade NK cell surveillance via multiple mechanisms, resulting in persistent infection in most patients (Golden-Mason and Rosen 2006). With disease persistence and chronicity, the phenotype of NK cells in the liver changes. Cytotoxicity remains increased, but production of IFN-γ and TNF-α are suppressed, due to chronic exposure to cytokines (Rehermann 2013). NK cells become less capable at activating dendritic cells (Golden-Mason and Rosen 2006), and expression of NK cell receptors is significantly altered with increased inhibitor receptor NG2A expression (Jinushi et al. 2004; Nattermann et al. 2006). NK cells begin to promote persistence of pathogen and liver injury while inhibiting fibrosis and tissue regeneration (Shi et al. 2011). With time, NK and NKT cells become significantly depleted as viral infection progresses to cirrhosis (Yamagiwa et al. 2008; Seki et al. 2011). This depletion in NK and NKT cells, and their decreased antitumor activi-

ties, may explain the risk of hepatocellular carcinoma development in chronic HCV patients (Seki et al. 2011).

The Th1 response has also been shown to be necessary to counteract viral infections in the liver (Invernizzi et al. 2007). Th1 cells stimulate the influx of viral-specific cytotoxic (CD8+) T cells into the liver (Chisari et al. 2010). CD8+ T cells play a fundamental role in viral clearance during acute, self-limiting HBV and HCV infection and it has been shown that an efficient HBV-specific CD8 response can promote control of viral infection without persistent liver disease (Invernizzi et al. 2007; Guidotti and Chisari 2006). In order to achieve this, they require simultaneous CD4+ T cell help (Klenerman and Thimme 2012; Terilli and Cox 2013). CD8+ T cells control viral replication through IFN-γ production and destroy infected hepatocytes through direct cytotoxicity (Guidotti et al. 1999). However, chronic infection is thought to involve functionally inefficient CD8+ T cells that don't eradicate infection but instead sustain repetitive cycles of immune-mediated necrosis, regeneration, and inflammation (Guidotti and Chisari 2006). Prolonged exposure to viral antigens is thought to be the main cause for reduced frequency and impaired effector function of virus-specific CD8+ T cells (Bucks et al. 2009; Larrubia et al. 2013). This is also referred to as "T cell exhaustion", occurring due to overexpression of inhibitory co-receptors, altered development and maintenance of memory, modified cytokine production, and metabolic and bioenergetics deficiencies (Loggi et al. 2014).

Neutralizing anti-envelope antibodies are also an important component to protective immunity against HBV (Rehermann et al. 1996). They mediate immunity by both complexing with free viral particles and by preventing their uptake by uninfected hepatocytes (Alberti et al. 1978). In contrast, neutralizing antibodies are not important in the clearance of HCV infection (Rehermann et al. 1996). An antibody response appears only after chronic HCV infection is established, and antibodies are unable to clear infection at that stage (von Hahn et al. 2007; Chen et al. 1999).

In summary, a combination of immune escape strategies, depletion or exhaustion of cytotoxic T cells, and a tolerogenic hepatic microenvironment that suppresses virus-specific T cell effector functions contributes to the persistence of viral infections in the liver (Table 7.3). Common features of chronic HBV and HCV infections include downregulation of virus-specific T cell responses, an inflammation-induced increase in Treg cells, and a shift in the ratio between T cell-sustaining cytokines and T cell-suppressive cytokines (Penna et al. 2007; Wedemeyer et al. 2002; Stoop et al. 2005; Rehermann 2013)

7.2.7 Alcoholic Liver Disease

Alcoholic liver disease is characterized by infiltration of the liver by inflammatory cells, subsequent hepatocellular injury, and fat accumulation (Bogdanos et al. 2013; Adams et al. 2010). Activation of innate immunity through TLR4 and complement factors C3 and C5 plays an important role in disease initiation through activation of

Table 7.3 Selected immune escape strategies utilized by hepatitis viruses

Virus	Mechanism of immune escape
HBV and HCV	Down-regulation of virus-specific T cell responses
HBV and HCV	Inflammation-induced increase in Treg cells
HBV and HCV	Increased T cell-suppressive cytokines
HBV and HCV	Prolonged exposure to viral antigen causing impaired effector function of virus-specific CD8+ T cells ("T cell exhaustion")
HCV	Serine protease NS3/4A cleaves adaptor molecules involved in RIG-1 and TLR3/IRF3 signaling
HCV	Evasion of NK cell surveillance
HCV	Viral mutations allowing escape from virus-specific CD8+ T cells and antibodies
HBV	Lack of strong initial type I IFN response ("stealth" strategy)

Kupffer cells, followed by production of pro-inflammatory cytokines such as TNF-α, IL-1, and IL-6 (Pellicoro et al. 2014; Pritchard et al. 2007; Bode and Bode 2005), leading to the development of steatohepatitis and fibrosis. By contrast, the role of adaptive immunity in the process is largely unknown (Gao and Bataller 2011).

Neutrophil accumulation is a prominent feature of alcoholic liver disease, and neutrophils appear to mediate tissue injury (Jaeschke 2002). Neutrophil infiltration in alcoholic liver disease is primarily mediated by IL-8, CXCL1, and IL-17 (Gao and Bataller 2011). In addition to directly inducing neutrophil recruitment, IL-17 also stimulates hepatic stellate cells to produce IL-8 and CXCL1, further amplifying the process (Lemmers et al. 2009).

The development of steatohepatitis in alcoholic liver disease may be related to alcohol's effects on lipid metabolism. Alcohol increases fatty acid synthesis in hepatocytes via upregulation of the transcription factor sterol regulatory element-binding protein 1c (SREBP-1c) (Gao and Bataller 2011). It also inhibits fatty acid oxidation via inactivation of peroxisome proliferator-activated receptor α (PPAR-α) (Yu et al. 2003; Galli et al. 2001). In addition, ethanol can inhibit the serine-threonine kinase that inactivates acetyl-coA carboxylase, the rate-limiting enzyme for fatty acid synthesis (Gao and Bataller 2011).

Cross-talk between the liver and gut may also be involved in the pathogenesis of alcoholic liver disease (Fig. 7.19). Excessive alcohol intake induces changes in the intestinal microflora that can lead to bacterial overgrowth and disruption of tight junctions, leading to increased intestinal permeability (Rao 2009; Bode and Bode 2003; Ma et al. 1999; Edwards and Wanless 2013). Endotoxin can then translocate across intestinal epithelial cells and travel to the liver through the portal vein, stimulating TLR4 on Kupffer cells and stellate cells and ultimately lead to inflammation, steatosis, and fibrosis (Seki and Schnabl 2012). Alcohol further enhances endotoxin-related inflammation through attenuation of STAT3 activation in monocytes and sinusoidal endothelial cells (Mandrekar and Szabo 2009).

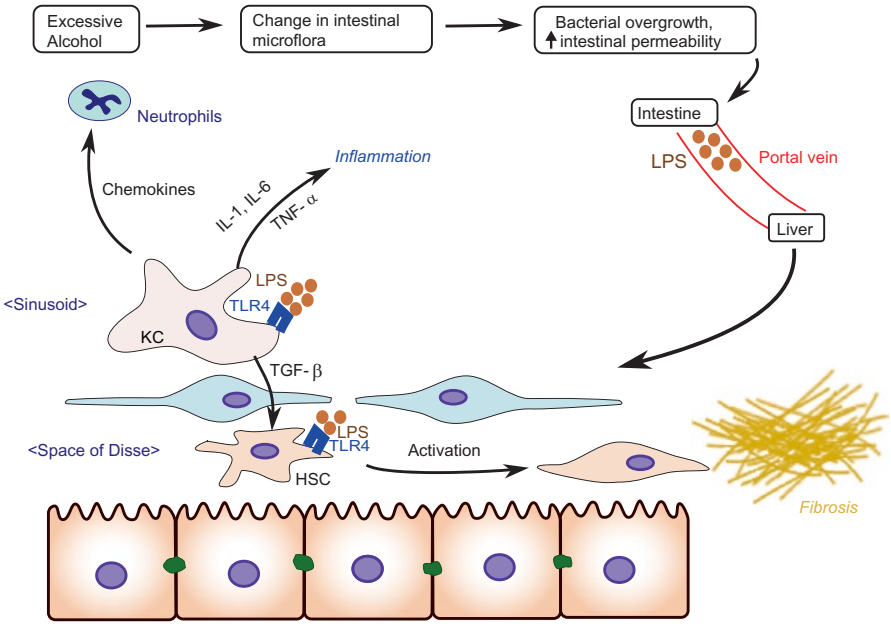

Fig. 7.19 Intestinal involvement in the pathogenesis of alcoholic liver disease. Excessive intake of alcohol can lead to changes in intestinal microflora, resulting in bacterial overgrowth and increased intestinal permeability. This leads to higher amounts of *LPS* entering the portal circulation to the liver. Once in the liver, the excess LPS is recognized by *TLR4* on Kupffer cells (*KC*), leading to *KC* activation. Activated *KC* produce pro-inflammatory cytokines (*IL-1*, *IL-6* and *TNF-α*), *chemokines*, and *TGF-β*, the latter of which activates hepatic stellate cells (*HSC*). *LPS* can also activate *HSC* directly through *TLR4*. Activated *HSC* assume a myofibroblast phenotype and induce fibrosis

Alcohol intake can exacerbate other liver conditions. For example, ethanol attenuates the function of NK cells, abolishing their antiviral and antifibrotic effects. This can accelerate the progression of co-existing viral hepatitis or accelerate the development of fibrosis in chronic hepatitis due to other causes (Pellicoro et al. 2014; Jeong et al. 2008).

7.2.8 *Nonalcoholic Fatty Liver Disease and Nonalcoholic Steatohepatitis*

Non-alcoholic fatty liver disease (NAFLD) is the leading cause of liver disease in Western countries, with a prevalence of 30% in the general population and 75–100% in obese individuals (Jin et al. 2013). It is considered to be the hepatic manifestation of metabolic syndrome, and is increasing in prevalence with the obesity epidemic. There is a close link between insulin resistance and hepatic inflammation, and Kupffer cell depletion in experimental models has shown to prevent the

development of diet-induced steatosis and insulin resistance (Pellicoro et al. 2014). Certain cytokines have been shown to exacerbate both NAFLD and insulin resistance, such as TNF-α and IL-6, whereas others have been shown to be protective against both disorders, such as IL-10 and adiponectin (Pellicoro et al. 2014).

The danger with NAFLD is that approximately 20% of cases will progress to non-alcoholic steatohepatitis (NASH) (Jin et al. 2013). NASH is a metabolic liver disease in which steatosis is associated with hepatic infiltration of immune cells that leads to inflammation and fibrosis. Insulin resistance is also of central importance in the development and progression of NASH through the production of oxidative stress (Peverill et al. 2014).

The exact molecular mechanisms underlying the pathogenesis of NASH are unknown; several hypotheses exist. The "two-hit hypothesis" speculates that the first hit causes fat accumulation in hepatocytes (due to obesity and/or insulin resistance) and the second hit causes inflammation and fibrosis (Day and James 1998). Fat accumulation in hepatocytes is initiated by insulin resistance, which inhibits regulation of lipase in adipose tissue, resulting in the release of large quantities of free fatty acids (Fabbrini et al. 2008). In addition, newly-generated free fatty acids are synthesized by de novo lipogenesis and excess dietary fatty acids. These factors together overwhelm the capacity of protective oxidative metabolic pathways, and the oversupply of free fatty acids leads to steatosis (Peverill et al. 2014; Hijona et al. 2010). The second hit is thought to be caused by oxidative stress, pro-inflammatory cytokines, altered adipokines (low adiponectin and high leptin), and mitochondrial dysfunction (Hijona et al. 2010; Day and James 1998). This step is also related to insulin resistance and obesity, which leads to pro-inflammatory signaling via TLR4/NF-κB pathways (Kim et al. 2007). In addition, beta-oxidation of free fatty acids produces reactive oxygen species, leading to oxidative stress and lipid peroxidation (Weltman et al. 1998; Garcia-Monzon et al. 2000).

The "two-hit hypothesis" has recently been modified to the "multi-hit hypothesis" due to the involvement of complex factors and interactions between lipid dysregulation, adipokine imbalance, adipose inflammation, oxidative stress, and insulin resistance (Tilg and Moschen 2010) (Fig. 7.20). Imbalanced lipid metabolism and insulin resistance is considered the "first hit", with hyperinsulinemia resulting in steatosis via increased hepatic lipogenesis, decreased free fatty acid oxidation, decreased VLDL secretion, and increased lipolysis of adipose tissue (Jung and Choi 2014). After development of steatosis, the liver becomes vulnerable to "multi-hits" (often multiple parallel hits) including gut-derived bacterial toxins, adipokine/cytokine imbalance, mitochondrial dysfunction, oxidative damage, dysregulated apoptosis, release of pro-fibrogenic factors and pro-inflammatory mediators, and activation of Kupffer cells and stellate cells (Jung and Choi 2014). Lipotoxicity leads to cell death, mainly by apoptosis, which drives inflammation and fibrosis (Machado and Cortez-Pinto 2011). Excess free fatty acids can sensitize hepatocytes to the cytotoxic effects of death ligands such as TRAIL and Fas, further amplifying the process (Malhi et al. 2007). Intestinal derived factors that may contribute are similar to those observed with alcoholic liver disease and include small intestinal bacterial overgrowth, tight junction disruption, and increased intestinal permeability (Miele et al. 2009).

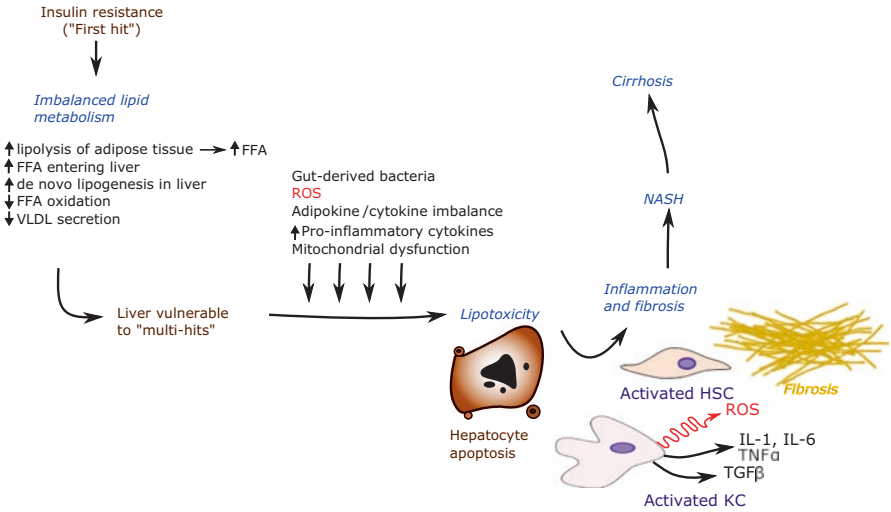

Fig. 7.20 The "multi-hit" hypothesis of non-alcoholic fatty liver disease (NAFLD). Insulin resistance is considered the "first hit" and leads to an imbalance in lipid metabolism. This makes the liver vulnerable to several "multi-hits", any of which can culminate in lipotoxicity. Lipotoxicity causes *hepatocyte apoptosis* and activation of hepatic stellate cells (*HSC*), leading to fibrosis, as well as activated Kupffer cells (*KC*) leading to inflammation through the production of cytokines. This fibrosis and inflammation can progress to non-alcoholic steatohepatitis (*NASH*) and eventually *cirrhosis*

7.2.9 Ischemia-Reperfusion Injury

Ischemia-reperfusion injury occurs after a prolonged ischemic insult followed by restoration of blood perfusion (Abu-Amara et al. 2010). It is a major limitation to liver transplantation and can also occur during surgical resection (Abu-Amara et al. 2010; Fondevila et al. 2003).

The pathogenesis of ischemia-reperfusion injury is illustrated in Fig. 7.21. During ischemia, there is a lack of ATP production by Kupffer cells and sinusoidal endothelial cells, leading to cell swelling (Selzner et al. 2007). That, along with increased vasoconstriction, leads to narrowing of sinusoids (Abu-Amara et al. 2010). Cells undergoing oxidative stress or damage during ischemia are programmed to warn the host of imminent cell death by liberating cellular constituents, which serve as DAMPs (van Golen et al. 2012). A key DAMP is HMGB1, which serves as a critical inflammatory mediator during reperfusion through activation of the TLR4 receptor (Tsung et al. 2005). Upon reperfusion, there is increased adhesion and aggregation of neutrophils and platelets in the sinusoids, resulting in reduction of microcirculatory blood flow (Abu-Amara et al. 2010). In addition,

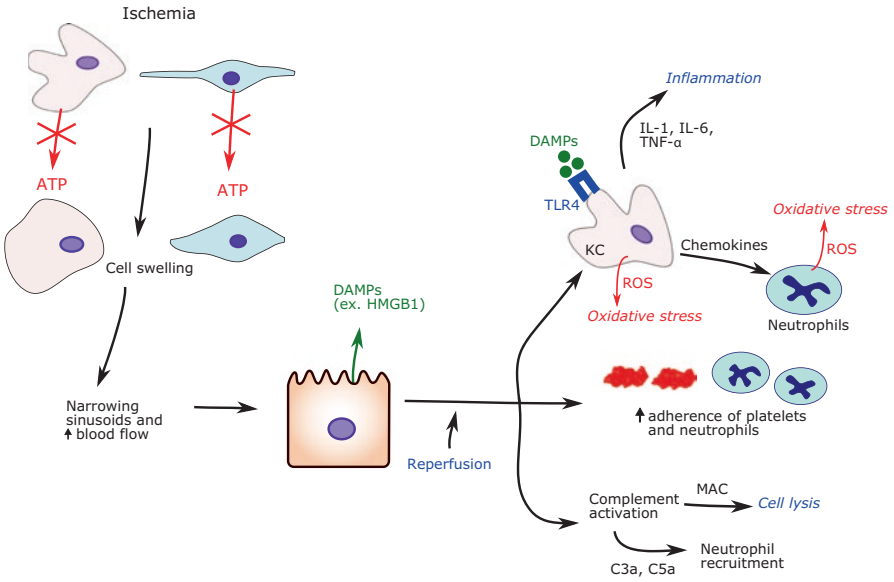

Fig. 7.21 Pathogenesis of ischemia-reperfusion injury. *Ischemia* causes a lack of *ATP* production by Kupffer cells and sinusoidal endothelial cells. This leads to cell swelling, which narrows sinusoids and restricts blood flow. Restricted blood flow causes hepatocytes to release *DAMPs*. Upon *reperfusion*, released *DAMPs* bind to *TLR4* on Kupffer cells (*KC*), leading to release of pro-inflammatory cytokines (*IL-1*, *IL-6*, *TNF-α*), *chemokines*, and reactive oxygen species (*ROS*). In addition, there is increased adherence of platelets and neutrophils to sinusoidal endothelial cells and activation of complement, which leads to cell lysis by the membrane attack complex (*MAC*) and neutrophil recruitment by *C3a* and *C5a*

released DAMPs are brought into contact with innate immune system cells such as liver-resident dendritic cells and Kupffer cells, triggering an inflammatory response (van Golen et al. 2012; Zhai et al. 2011). This leads to homing, adhesion, and activation of more inflammatory cells, including T cells, monocytes, and neutrophils, which sustains the immune response and further amplifies local tissue destruction (McDonald and Kubes 2012; Zhai et al. 2011). The complement system is activated, which leads to liver damage both directly, through cell lysis by the membrane-attack complex, and indirectly, through recruitment and activation of neutrophils and Kupffer cells (Fondevila et al. 2008; Montalvo-Jave et al. 2008). Reintroduction of oxygen also exacerbates oxidative stress by amplifying mitochondrial reactive oxygen species generation to cytotoxic levels (Gujral et al. 2001; Bhogal et al. 2010). Overall, the process culminates in direct and indirect cytotoxicity of hepatocytes (Zhai et al. 2011).

Neutrophils constitute the most cytotoxic leukocyte subset and mediate large scale tissue destruction through the release of reactive oxygen and nitrogen species and proteolytic enzymes (Jaeschke 2006). Kupffer cells also play an important role in initiating and propagating cellular damage and death in ischemia-reperfusion

injury. They are activated during both ischemic and reperfusion phases and produce abundant reactive oxygen species and pro-inflammatory cytokines, which propagate injury (Caban et al. 2002). Activation of Kupffer cells occurs via the complement system (particularly C5a) and IFN-γ production by CD4+ T cells and NK cells (Brock et al. 2001; Caldwell et al. 2007; Jaeschke 2011). The main role of Kupffer cells during ischemia-reperfusion injury is to recruit and activate circulating neutrophils (Abu-Amara et al. 2010). However, they also recruit and activate CD4+ T cells and activate sinusoidal endothelial cells, eventually contributing to their damage (Hanschen et al. 2008). They stimulate hepatocytes to produce reactive oxygen species, stimulate platelets to adhere to sinusoidal endothelial cells, and amplify inflammation through production of TNF-α (Taniai et al. 2004; Nakano et al. 2008; Zhai et al. 2011). CD 4+ T cells serve as inflammatory signal amplifiers that activate Kupffer cells and promote further influx of neutrophils via release of chemotactic factors (Shen et al. 2009; Kuboki et al. 2009).

Sinusoidal endothelial cells are the most sensitive cell type to ischemia-reperfusion injury (Caldwell-Kenkel et al. 1989). They interact extensively with other immune cells during ischemia-reperfusion injury, including facilitating platelet and neutrophil migration and adherence and enhancing T cell transmigration through transcytosis and surface presentation of chemokines (Khandoga et al. 2006; Schrage et al. 2008).

Hepatic ischemia-reperfusion injury can have systemic consequences. It increases the risk of systemic and portal endotoxemia (Thomson and Knolle 2010) through damage to sinusoidal endothelial cells and loss of the tolerogenic microenvironment.

7.2.10 Hepatic Neoplasia

Although several mechanisms of non-specific cell killing exist in the liver (see Sect. 7.1.3.3), a high mortality rate from hepatic neoplasia (primary and metastatic) still exists. Therefore, these defense mechanisms must fail at some point.

NK cells play a central role in the innate immune response to tumors. NK cells are mainly activated by IL-12 produced by Kupffer cells, and the most prominent NK cell-produced cytokine with anti-tumor effects is IFN-γ (Seki et al. 2011; Subleski et al. 2006). NKT cells also may play an important role depending on the stimulus involved (Seki et al. 2011). Central to tumor cell killing by NK cells is reduced or abolished expression of MHC Class I molecules by the tumor cells (Diefenbach and Raulet 2002). NK cell killing of tumor calls has been shown to occur primarily by the perforin/granzyme pathway rather than the Fas/Fas ligand pathway (Vermijlen et al. 1999).

CD8+ T cells are the main effector cells in the antitumor immune response through presentation of tumor antigens by MHC Class I molecules (Bowlus 2007). Tumor antigens can also be taken up by dendritic cells, which present them on MCH Class II molecules to CD4+ T cells (Bowlus 2007). This can further enhance

the proliferation and effector function of CD8+ T cells via Th1 cytokines and can also activate B cells via Th2 cytokines, promoting antibody-dependent cellular cytotoxicity.

Unfortunately, tumor cells have developed several immune escape mechanisms, including induction of Treg cells and myeloid-derived suppressor cells (Bogdanos et al. 2013). Tumor surveillance functions of NK cells have been shown to be suppressed in both precancerous fibrotic and cirrhotic livers as well as tumor-containing livers (Tian et al. 2013). In hepatocellular carcinoma, dendritic cells are reduced in number and function, with suppressed T cell stimulatory capacity (Chen et al. 2000; Lee et al. 2004). Metastatic colon carcinoma cells have been shown to express Fas ligand, which permits the deletion of activated T cells through "Fas counterattack" as well as apoptosis of Fas-containing hepatocytes (O'Connell et al. 2000; Yoong et al. 1999).

Toll-like receptor stimulation and subsequent activation of NF-κB and JNK pathways appear to be critical for production of cytokines associated with tumor progression in the liver (Broering et al. 2011). It is even proposed that NF-κB activation is directly associated with tumor cell proliferation. In addition, Th17 cells are thought to have a role in tumor progression through promotion of angiogenesis and production of IL-22, which may stimulate proliferation of tumor cells (Lafdil et al. 2010).

7.3 Organ Involvement in Generalized Immunopathological Processes

7.3.1 Widespread Acute Inflammation (Septicemia/ Endotoxemia)

The liver plays an important role in modulating the systemic inflammatory response. Activated hepatocytes, sinusoidal endothelial cells, and Kupffer cells participate in concert to activate and recruit other innate immune cells such as monocytes and dendritic cells, which act to engulf and destroy pathogens.

The innate immune system in the liver can be triggered by bacterial toxins, bile, drugs, alcohol, other toxins, or viruses (Edwards and Wanless 2013). Molecules bearing PAMPs or DAMPs interact with PRRs expressed on Kupffer cells, sinusoidal endothelial cells, neutrophils, and hepatocytes. This results in the elaboration of pro-inflammatory mediators that recruit additional inflammatory cells, particularly neutrophils, which amplify the response. Eventually, this leads to phagocytosis, cell killing, and activation of hepatic stellate cells to synthesize collagen, producing fibrosis.

Endotoxin (LPS) is the most frequent activator of the innate immune system (Edwards and Wanless 2013). It binds to TLR4 on macrophages (particularly Kupffer cells in the liver) and other cells and initiates activation of MAP-kinase and

NF-κB, leading to transcription of the pro-inflammatory mediator TNF-α. TNF-α induces the production of other cytokines and chemokines, leads to reactive oxygen species generation, induces nitric oxide synthase (NOS) in sinusoidal endothelial cells, and increases expression of integrins and other cellular adhesion molecules (Szabo et al. 2002; Nolan 2010). TNF-α and other pro-inflammatory mediators promote recruitment and activation of neutrophils (Szabo et al. 2002; Bajt et al. 2001). In addition, endotoxin and other bacterial products can activate complement, which primes neutrophils for reactive oxygen formation (Jaeschke et al. 1993).

Regardless of the initial site of infection, the development of septicemia leads to sequestration of activated neutrophils in internal organs containing an extensive capillary network, such as the liver and lungs (Brown et al. 2006). This sequestration is mediated by CD44, the receptor for hyaluronan, due to increased deposition of serum-derived hyaluronan-associated protein on sinusoidal endothelial cells (McDonald et al. 2008). This adhesion process is different than that observed in sterile inflammation (see Sect. 7.2.1). Neutrophilic infiltration can significantly contribute to hepatocellular and sinusoidal endothelial cell damage, hepatocellular production of coagulation factors, complement, and acute phase proteins, vascular hypoperfusion, and multi-organ dysfunction (Dhainaut et al. 2001). Therefore, hepatic inflammation and necrosis are very common changes occurring in severe septicemia, regardless of the inciting cause.

The clearance of bacteria by neutrophils during septicemia may be mediated by a novel effector mechanism termed "neutrophil extracellular traps" (NETs), which enable the cells to capture and kill microbes in a much more efficient manner than by phagocytosis of individual bacteria (McDonald and Kubes 2012). NETs are webs of decondensed chromatin decorated with antimicrobial granule proteins expelled from the nucleus of activated neutrophils (Brinkmann et al. 2004). It is hypothesized that neutrophils recruited to sinusoids during septicemia may cast NETs into the bloodstream to capture circulating bacteria (McDonald and Kubes 2012). In turn, these NETs may contribute to hepatic damage and vascular dysfunction.

The liver can impact other organs as well, through production of acute-phase proteins and numerous pro-inflammatory mediators (McDonald and Kubes 2012). On the other hand, the liver can help to control the systemic inflammatory response through the production of anti-inflammatory mediators (Kowalewska et al. 2011).

7.3.2 Age-Related Changes

The liver is a site of immune maturation, differentiation, and functions that, during the aging process, may partially compensate for thymic atrophy (Mocchegiani et al. 2004). However, aging of the liver is also associated with many significant alterations including impaired drug metabolism, adverse drug reactions, and increased susceptibility to toxins.

There is a general age-related decline in T cell-mediated immunity, as illustrated by studies in aged mice that show an inability to mount a cell-mediated immune response against tuberculosis (Orme 1987). The T cell population also appears to shift from those with the αβ T cell receptor to those with the γδ T cell receptor (Tsukahara et al. 1997). In addition, the number of activated CD8+CD122+ T cells increases with age, leading to an increased susceptibility to endotoxin (Sato et al. 2005). These T cells may also have positive effects on the host in that they exhibit high IFN-γ production and thus have anti-tumor function (Motegi et al. 2008). This has also been demonstrated in rats, along with increased neutrophils in sinusoids, phagocytosis of platelets and neutrophils by Kupffer cells, more pronounced hepatocellular injury, and the presence of serum biomarkers associated with hepatic damage (Durham et al. 1990).

Kupffer cell number and activity level also changes with age, and may vary between species. Studies in aged F344 rats have demonstrated increased Kupffer cell numbers and greater phagocytic activity (Hilmer et al. 2007). Studies in aged mice have also shown greater numbers of Kupffer cells; however, lower phagocytic activity was seen (Videla et al. 2001). Although increased phagocytic activity is seen in aged rats, decreased Kupffer cell activation has also been demonstrated. In a study of aged F344 rats, resistance to cadmium-induced hepatotoxicity was observed and attributed to impaired Kupffer cell activation (Yamano et al. 2000).

Changes in the NK and NKT cell populations have also been demonstrated in aging livers. The NK cell phenotype that is associated with NK cell activity in the liver (NK1.1+TCR(int)) increases until middle age and then declines (Parker and Picut 2005). This results in an age-related decline in first-line defense against invading tumor cells. In contrast, NKT cell-mediated apoptosis has been demonstrated to increase with age. Injection of CpG oligodeoxynucleotides (CpG-ODN) to aged mice results in increased hepatic injury and multi-organ dysfunction syndrome (MODS), attributed to NKT cell activity via TNF and Fas/FasL-mediated pathways (Kawabata et al. 2008).

Aging has several effects on the sinusoidal endothelial cells and hepatic microcirculation (Fig. 7.22). The most prominent effect is termed “pseudocapillarization”. Pseudocapillarization is characterized by thickening (up to 50%) and defenestration of sinusoidal endothelial cells as well as a 6–10% reduction in sinusoidal diameter (Ito et al. 2007). Sinusoidal endothelial cells also possess lower endocytic ability and show increased expression of von Willebrand’s factor (vWF) and ICAM-1 and reduced expression of caveolin-1 (Ito et al. 2007; Le Couteur et al. 2008). Upregulation of vWF and ICAM-1 lead to increased adhesion of platelets and leukocytes, respectively. These changes are accompanied by deposition of collagen in the space of Disse and increased numbers of fat-engorged, inactivated stellate cells (Le Couteur et al. 2008; Warren et al. 2005). Overall, these changes lead to a 35% reduction in sinusoidal blood flow in aged mice (Ito et al. 2007). Defenestration of sinusoidal endothelial cells has several downstream effects, including impaired lipoprotein clearance, impaired drug clearance leading to adverse drug reactions, and possible contribution to autoimmune disease by impeding interactions between naïve T cells and hepatocytes (Le Couteur et al. 2002,

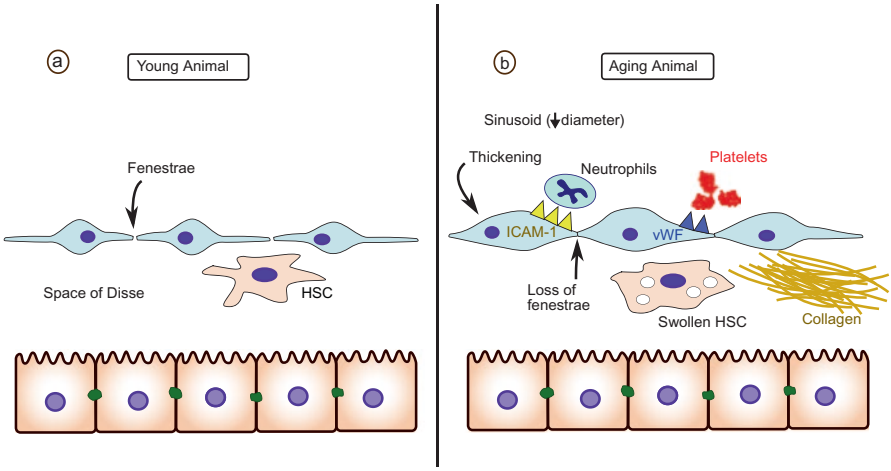

Fig. 7.22 (**a**) Sinusoids of a young animal are lined by endothelial cells that are separated by *fenestrae*. The space between the endothelial cells and hepatocytes (space of Disse) is devoid of collagen and contains inactive hepatic stellate cells (*HSC*). (**b**) In aging animals, sinusoids exhibit decreased diameter due to swelling of endothelial cells, and there is a loss of fenestrae. Endothelial cells have increased expression of *ICAM-1* and von Willebrand's factor (*vWF*), resulting in increased adhesion of *neutrophils* and *platelets*, respectively. *HSC* become swollen with retinoid droplets, and there is deposition of *collagen* in the space of Disse

2005; Warren et al. 2006). Interestingly, caloric restriction has been shown to reduce the level of pseudocapillarization in rats (Jamieson et al. 2007).

Overall, the liver of aged mice shows an increase in inflammatory cytokines and chemokines (Singh et al. 2008). This corresponds to an enhanced inflammatory infiltrate, forming clusters of immune cells near perivascular regions which, over time, can form ectopic lymphoid structures (Singh et al. 2008).

Autoreactivity has also been demonstrated to increase with age. T cells in aged mice have been shown to be resistant to activation-induced cell death in vitro and increased autoreactive T cells have been found in aged mice in vivo (Parker and Picut 2012). Altered self-antigen presentation with age is suspected, and extrathymic T cells increase with age, apparently in synchrony with thymic involution (Parker and Picut 2012).

Age-related effects on acute-phase protein production by the aging liver are numerous. The production of haptoglobin, α-1 acid, and T kininogen glycoprotein is reduced with aging (Gomez et al. 2008). Overall, studies have shown an increased time required for acute phase protein mRNA to be produced and a decreased maximum level of mRNA produced (Carter et al. 1991). In studies examining the liver of aged rats, initial production of IL-6 appears to be normal; however, the subsequent IL-6 response was increased relative to that seen in young rats (Parker and Picut 2012). This lower level of acute phase protein production in the face of increased IL-6 suggests an underlying hepatocellular deficit in acute phase protein production rather than insufficient signals for production.

An age-related decline in autophagy has also been demonstrated in the liver. Autophagy is the mechanism of cell degradation of unnecessary or damaged organelles or other material by lysosomes, and aids in cell survival. A marked decline in expression of microtubule-associated light chain 3 (LC3), a marker for autophagy, is observed in the liver of aged mice (Uddin et al. 2012). This reduction in autophagy may result in reduced presentation of self and ingested antigens on the surface of hepatocytes, culminating in a reduced ability to kill potentially self-reactive T cells.

7.3.3 Systemic Autoimmune Disease

The liver's important role in inducing and maintaining systemic tolerance has been discussed previously. Thus, it would make sense that the liver would be involved in systemic autoimmune diseases. In fact, patients with systemic lupus erythematosus (SLE) often have liver disease as a component of their disorder (Cojocaru et al. 2013). Hepatic inflammation with SLE is more lobular than that seen with autoimmune hepatitis, which has a more periportal or interface pattern. Nodular regeneration of the liver can also be seen in SLE patients secondary to immune complex deposition in small vessels, resulting in obliterative venopathy (Cojocaru et al. 2013). Patients with autoimmune disorders have also been shown to have increased extrathymic T cells, which are mainly produced in the liver and intestine (Abo 2001).

7.4 Animal Models of Organ-Specific Immunopathological Processes

7.4.1 Mouse Models of Acute Hepatitis Using Concanavalin A

Concanavalin A (Con A) is a jack bean-derived lectin with powerful T cell mitogenic activity. Intravenous injection of Con A induces acute liver failure in mice that resembles human autoimmune hepatitis (Tiegs et al. 1992). It rapidly induces hepatic necrosis and inflammation, with elevation of a wide variety of cytokines including Th1 (IFN-γ), Th2 (IL-4), and Th17 (IL-17 and IL-22) cytokines (Lafdil et al. 2010).

Massive liver injury is accompanied by abundant intrasinusoidal fibrin deposition and thrombosis (Kato et al. 2013). Cytokines such as IFN-γ, TNF-α, and IL-4 appear to be involved in this process. IFN-γ-mediated signal transduced and activator of transcription-1 (STAT1) signaling in both Kupffer cells and sinusoidal endothelial cells induces tissue factor expression, resulting in massive prothrombotic liver injury (Tsutsui and Nishiguchi 2014).

TNF receptor 2 and neutrophils have also been shown to be important in mediating tissue damage (Kusters et al. 1997; Bonder et al. 2004). In addition, NKT cell activity has shown to be crucial to the development of the lesions, as mice lacking NKT cells are resistant to Con A-induced hepatitis (Takeda et al. 2000).

7.4.2 Mouse Model of Endotoxin-Induced Liver Injury

Although mice are relatively resistant to endotoxin (LPS), systemic administration of heat-killed *Propionibacterium acnes* (an indigenous bacterium in the skin) renders them highly susceptible (Mizoguchi et al. 1987). Kupffer cells appear to be primarily responsible for *P. acnes*-induced endotoxin sensitization, both systemically and locally, through induction of hepatic granuloma formation (Tsutsui and Nishiguchi 2014). Other experiments have demonstrated that inactivation of Kupffer cells abrogates liver injury associated with LPS, further confirming the importance of Kupffer cells in this process (Adachi et al. 1994).

Kupffer cells ingest the bacteria, which activates the TLR-MyD88-NF-κB pathway, leading to production of IL-12 (Tsutsui and Nishiguchi 2014). In turn, IL-12 induces lymphocytes to produce IFN-γ, which leads to granuloma formation.

IL-18 is also involved in the pathogenesis of endotoxin-induced hepatic injury. After LPS challenge, the TLR4-TRIF-mediated signal is induced in *P. acnes*-activated Kupffer cells, which leads to activation of the NLRP3 inflammasome (Tsutsui and Nishiguchi 2014). The inflammasome activates caspase-1, which cleaves precursor IL-18 to mature IL-18. IL-18 then activates lymphocytes such as NK cells to express FasL and produce TNF-α. This culminates in hepatocyte apoptosis through the Fas/FasL and TNF-α/TRAIL pathway. It is important to note that only Kupffer cells from *P. acnes*-primed mice express Fas (Tsutsui and Nishiguchi 2014). IL-18 is also involved in *P. acnes* induction of LPS sensitization by inducing IFN-γ expression.

7.4.3 Mice Injected with α-Galactosylceramide

α-Galactosylceramide (α-GalCer) was originally identified and extracted from a marine sponge. Mice injected with α-GalCer develop findings similar to viral hepatitis, such as piecemeal necrosis and Councilman bodies in and around portal areas. Thus, it has been developed as an animal model of hepatitis.

α-GalCer activates NKT cells, which induce hepatotoxicity as a result of increased FasL expression (Nakagawa et al. 2001). Activated NKT cells can indirectly recruit Treg cells into the liver through secretion of IFN-γ, which increases CXCL10 chemokine expression by Kupffer cells, hepatocytes, and biliary epithelium (Santodomingo-Garzon et al. 2009). IFN-γ produced by activated NKT cells can also stimulate NK cells, which are antitumor effectors (Nakagawa et al. 2001).

In addition, IL-17 derived from NKT cells can prevent monocyte infiltration in the liver (Wondimu et al. 2010). Thus, NKT cells can play a dual role, having both a damaging and a protective effect.

7.4.4 Animal Models of Idiosyncratic Drug-Induced Liver Injury (IDILI)

7.4.4.1 LPS-RAN Model

IDILI is observed in a small percentage of humans taking the drug ranitidine (RAN) (Vial et al. 1991). It does not appear to be related to the pharmacological action of the compound and is not observed with similar drugs like famotidine. The reactions are typical of IDILI in that the time of onset is highly variable and re-challenge does not always result in reoccurrence of toxicity; therefore, it does not seem consistent with an adaptive immune response (Graham et al. 1985). It also does not appear to be caused by metabolic bioactivation, as no known metabolite of RAN has been reported to be hepatotoxic (Deng et al. 2009).

A rat model has been developed that combines the administration of RAN with LPS. Ranitidine alone is not hepatotoxic to rats; however, when modest underlying inflammation induced by small nontoxic doses of LPS is present, IDILI-like injury is observed after RAN administration (Luyendyk et al. 2003). This reaction is not observed in rats after famotidine administration, which correlates with that seen in humans.

The main infiltrating cells in RAN-induced hepatic injury are neutrophils. Ranitidine itself does not activate neutrophils, but it can when given in combination with LPS (Deng et al. 2009). Ranitidine given together with LPS also causes increased TNF-α production to last longer than in rats receiving LPS alone (Deng et al. 2009). This potentiation also cannot be reproduced with famotidine.

7.4.4.2 LPS-DCLF Model

Diclofenac (DCLF) is a non-steroidal anti-inflammatory drug (NSAID) that causes rare IDILI in humans with a variable latency period (Banks et al. 1995). The lesions are mainly hepatocellular, are observed most often in women with osteoarthritis, and are not accompanied by signs associated with an adaptive immune response (Banks et al. 1995).

As with RAN, a small non-toxic dose of LPS given to rats makes them susceptible to liver injury when also given a non-toxic dose of DCLF (Deng et al. 2006). This suggests that inflammatory stress may be a susceptibility factor for RAN- and DCLF-induced IDILI. The mechanism of DCLF-induced IDILI in rats appears to be dependent on neutrophils (Deng et al. 2009).

In contrast, large toxic doses of DCLF in animal models cause hepatotoxicity through a mechanism that involves gut-derived inflammatory stimuli (Deng et al. 2009). These stimuli likely induce oxidative stress and apoptotic signaling or altered lipid metabolism.

7.4.4.3 Halothane Injection

Intraperitoneal injection of halothane in mice leads to the development of liver inflammation, similar to that observed in humans. However, this is strain specific, with female Balb/c mice being most susceptible and C57BL/6 mice being resistant (You et al. 2006). There is also a guinea pig model of halothane-induced hepatotoxicity (Lunam et al. 1985).

7.4.5 Animal Models of Autoimmune Liver Disease

Unfortunately, a universal model of autoimmune hepatitis does not exist. Loss of immune tolerance is difficult to achieve because of the normal micro-environment of the liver and is not naturally occurring in animals. Therefore, it must be artificially created and thus is never fully comparable to the human disease (Czaja 2010). There are several animal models of autoimmune liver disease, and they are categorized below and summarized in Table 7.4.

7.4.5.1 Immunization/Injection Models

Autoimmune hepatitis can be induced in mice and rabbits by immunization with liver homogenates plus adjuvants, DNA encoding human antigens, or autoimmune hepatitis-associated autoantibodies (Biburger and Tiegs 2007).

Early models of autoimmune hepatitis were based on administration of crude liver homogenates combined with adjuvants (typically complete Freund's adjuvant), with no knowledge of the target antigens responsible for the disease (Christen et al. 2007). Unfortunately, the resulting hepatitis in these models was self-limiting. Immunogenic components were subsequently isolated by using fractions of hepatocyte isolates. In these models, autoantibodies were produced, liver-infiltrating T cells could be identified, and mononuclear portal infiltrates were observed (Buschenfelde et al. 1972; Kuriki et al. 1983). These models were valuable in that they demonstrated that autoantibodies were not pathogenic and that the process was mediated by activated immune cells. However, they still did not produce self-perpetuating aggressive inflammation or autoantibodies that were identical to humans and were limited by poor characterization of the triggering antigens and no control over genetic factors (Czaja 2010). Subsequent models utilized LPS as an adjuvant. In one model, inbred SMA mice were administered injections of liver

Table 7.4 Animal models of autoimmune hepatitis

Animal model	Contributions/advantages	Disadvantages
Immunization with liver homogenates and adjuvants	Demonstrated association between genetics and susceptibility, showed importance of T cells	Hepatitis is self-limiting, can induce immune response to unspecified antigens
Hepatic lysate fractions	Production of autoantibodies, identification of liver-infiltrating T cells, observation of mononuclear portal infiltrates	Hepatitis is self-limiting, autoantibodies are not identical to humans, poor characterization of triggering antigens, no control over genetics
Inbred models injected with liver homogenates plus LPS	Moderate to severe periportal infiltrate, autoantibodies to liver-specific membrane proteins, genetic component demonstrated	Non-specific antigens can cause polyclonal immune response
Concanavalin A injection	Strong inflammatory response, demonstrated importance of cytokines and T cells in hepatic damage	Lacks liver autoantigen-specific effector T cells, transient hepatitis
α-GalCer injection	Lymphocytic infiltrates observed, autoantibodies against nuclear factors, demonstrated importance of NKT cells in disease	Lack of control over genetics, transient hepatitis
Neonatal thymectomy	Provided understanding of role of thymus in maintaining self-tolerance	Transient hepatitis
Transgenic antigen or TCR expression	Well-defined antigens, control over genetics, lobular and portal infiltrates	Autoimmune disease mainly induced by CD8+ T cells rather than CD4+ T cells, mainly acute transient hepatitis
Knockout models	Identified role of single genes in immune response, links between genetic background and specific genes, develop periportal inflammation and autoantibodies	Clinical autoimmune hepatitis is not single gene-mediated, models can induce multi-organ disease
Vaccination or infection with viral vector and human antigens	Compatible histology, antibodies to human liver-specific antigens, severe persistent hepatitis	No strong association between autoimmune hepatitis and viral infection

homogenates plus LPS, and these mice developed moderate to severe periportal lymphoplasmacytic infiltrates plus autoantibodies to liver-specific membrane proteins (Kuriki et al. 1983). Other mouse strains were subsequently used and it was noted that different strains varied in their susceptibility to inflammation, thus showing a genetic component to the disease (Yuksel et al. 2014). Overall, a major drawback to these models using liver homogenates was that the homogenates may contain unspecified antigens that cause a polyclonal immune response. Thus, more specific models were needed.

As discussed previously, hepatitis can also be induced by injection of T cell mitogens like Con A into mice or rats, leading to acute or chronic inflammation

caused by activation of T cells and macrophages (Biburger and Tiegs 2007). However, this model lacks the liver autoantigen-specific effector T cells that are observed with autoimmune hepatitis (Yuksel et al. 2014). Also discussed above, injection of NKT cell-activating α-GalCer in mice can lead to the development of hepatitis secondary to increased expression of Th1 and Th2 cytokines (Biburger and Tiegs 2007). In this model, lymphocytic infiltration is observed and autoantibodies against nuclear factors such as dsDNA are produced, illustrating an important role for NKT cells in autoimmune hepatitis development (Yuksel et al. 2014). The environmental toxicant trichloroethylene can induce autoimmune hepatitis through acceleration of innate immune responses in already autoimmune-prone mouse strains (such as MRL mice). In this model, activated CD4+ T cells cause a Th1 cytokine response, periportal mononuclear infiltrates are observed, and serum antinuclear antibodies develop (Griffin et al. 2000).

Primary biliary cirrhosis (PBC), another autoimmune disease of the liver, can be induced in mice, guinea pigs, and rabbits following chemical xenobiotic immunization (Wakabayashi et al. 2008, 2009; Amano et al. 2004; Leung et al. 2007). For example, C57BL/6 mice immunized with 2-octynoic acid coupled to bovine serum albumin develop antibodies to PDC-E2 and histologic changes similar to PBC (Wakabayashi et al. 2008). Additional animal models of PBC have been developed based on immunization with carbonic anhydrase-II or PDC-E2, as well as immunization with biliary epithelial cells (Biburger and Tiegs 2007).

Primary sclerosing cholangitis (PSC) can be induced by feeding 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DCC) or lipotocholic acid to mice (Fickert et al. 2006, 2007). DCC leads to biliary secretion of porphyrin with subsequent inflammation and lipotocholic acid leads to mechanical injury and obstruction of small bile ducts. In addition, oral administration of alpha-naphthylisothiocyanate (ANIT) to rats results in portal inflammation and extensive fibrosis (Mclean and Rees 1958).

7.4.5.2 Inbred Models

Inbred animal models provide the next level of improvement over models induced by administration of liver homogenates. These models ensure consistency of genetic components that may affect the immune response.

Supernatants for injection were further refined using syngeneic liver homogenates and the resulting disease is characterized by perivascular infiltrates, mononuclear portal infiltrates, and lobular hepatitis, as well as the development of antibodies to liver-specific proteins (Lohse et al. 1990).

Neonatal thymectomy has been shown to enhance the inbred mouse model, providing understanding of the role of the thymus in maintaining self-tolerance (Watanabe et al. 1987).

7.4.5.3 Transgenic and Knockout Mice

Transgenic mice expressing specific antigens provide a good alternative to immunization studies with liver homogenates containing undefined antigens. However, most of these models induce autoimmune hepatitis with CD8+ T cells, whereas CD4+ T cells and plasma cells are the main lymphocytic populations in humans with autoimmune hepatitis (Yuksel et al. 2014). Adoptive transfer of naïve transgenic ovalbumin-specific CD8+ T cells into transgenic mice expressing the neoantigen ovalbumin in hepatocytes leads to acute hepatitis with lobular and portal infiltrates and increased serum alanine aminotransferase (ALT) (Derkow et al. 2007; Buxbaum et al. 2008). However, hepatitis is still short-lived in this model. It did show that antigen-specific naïve CD8+ T cells can be activated in the liver even in a non-inflammatory microenvironment. Transgenic BALB/c mice expressing influenza virus hemagluttinin (HA) under the control of mouse albumin promoter were shown to develop spontaneous chronic liver inflammation (Zierden et al. 2010). Transgenic expression of IFN-γ produces histological features of autoimmune hepatitis but does not induce fibrosis (Toyonaga et al. 1994).

Several TGF-β and TGF-β receptor knockout models have been developed as animal models of autoimmune liver diseases (Oertelt et al. 2006; Aoki et al. 2005). TGF-β1 is one of the key negative regulators of immune homeostasis and its knockout causes autoimmune disease in several organs (Penz-Osterreicher et al. 2011). TGF-β1 knockout mice develop lymphocytic periportal inflammation and antimitochondrial autoantibodies and are considered to be an animal model for PBC (Oertelt et al. 2006). In addition, breeding TGF-β1 knockout mice onto a BALB/c background dramatically modifies the phenotype, resulting in the development of spontaneous hepatitis resembling autoimmune hepatitis (Gorham et al. 2001). These mice show extensive inflammation and hepatocyte necrosis versus those on a 129/CF background are normal, again illustrating the importance of genetics in development of autoimmune disease (Yuksel et al. 2014). Double TGF-βRII knockout mice also develop histological and serological characteristics of PBC, with antimitochondrial autoantibody production and lymphocytic cholangitis, bile duct destruction, and portal granulomas (Oertelt et al. 2006).

Other specific mouse strains have been developed as animal models for autoimmune diseases in the liver. NOD.c3c4 mice were the first spontaneous mouse model of PBC, developing PDC-E2 antibodies like humans (Irie et al. 2006). IL-2Rα knockout mice have loss of Treg cell activity leading to autoimmunity. They have similar features to PBC in humans but also have ulcerative colitis, which can be seen with primary sclerosing cholangitis (PSC) but not usually PBC (Wakabayashi et al. 2006). In this model, anti-PDC-E2 antibodies are usually present in sera and there are profound lymphocytic infiltrates in the portal tracts, with CD8+ T cells predominating (Hirschfield and Gershwin 2013). The Scurfy mouse is characterized by loss of functional Treg cells due to a Foxp3 mutation and develops antimitochondrial autoantibodies, lymphocytic portal infiltrates, and severe interlobular bile duct destruction similar to PBC (Zhang et al. 2009). However, these mice have an extremely short life span. Multidrug resistance gene (Mdr2) knockout mice lack

phosphatidylcholine excretion in bile which renders it toxic and leads to nonsuppurative cholangitis, periductal fibrosis, and obliterative cholangitis reminiscent of PSC (Fickert et al. 2004).

Conditional knockout mice with ablation of the ubiquitin ligase Traf6 (TNFR-associated factor 6) in the thymic medullary epithelial cells have a dramatically reduced number of these cells and develop spontaneous and chronic liver inflammation with pathological features of autoimmune hepatitis, including interface hepatitis, anti-nuclear antibodies, and chronicity (Alexandropoulos et al. 2015). However, they also develop inflammation and autoantibodies against other organs such as the lung, kidney, and intestine, therefore showing a more systemic autoimmune phenotype (Yuksel et al. 2014).

As with previous models, the frequent tolerance of liver-specific antigens proves to be a major limitation in the development of transgenic models of autoimmune liver disease (Czaja 2010). Generating a preconditioned state has been recognized as an important requisite to overcoming this natural immune tolerant state and has improved transgenic models of autoimmune hepatitis (Czaja 2010). Examples of preconditioning include adoptive transfer of activated T cells from antigen-primed donors (Moriyama et al. 1990), adoptive transfer of antigen-specific T cells in combination with infection (Voehringer et al. 2000), and vaccination with dendritic cells combined with direct administration of IL-12 (Tamaki et al. 2005). For example, transfer of lymphocytes from the peripheral blood of humans with PBC into SCID mice causes them to develop anti-mitochondrial and anti-PDC-E2 antibodies and marked lymphocytic cholangitis (Krams et al. 1989). However, even with preconditioning, many models still fail to develop chronic aggressive hepatitis.

Current promising animal models are based on transgenic engineering, viral vectors, and human antigenic targets of type 2 autoimmune hepatitis (CYP2D6 and formiminotransferase cyclodeaminase) in contrast to the more common type 1 autoimmune hepatitis, which lacks a distinctive target antigen (Czaja and Manns 1995). In mice, the vaccination regimen uses plasmids coding for lymphocytic choriomeningitis virus (LCMV) nucleoprotein and IL-12, and generates a type I cytokine response, migration of CD8+ T cells, and cytolysis of hepatocytes. It produces antigen-specific, liver-infiltrating T cells with cross-reactivity between viral and self-antigens (Djilali-Saiah et al. 2002).

7.4.6 Animal Models of Viral Hepatitis

There are several natural animal models of viral hepatitis, including woodchuck hepatitis virus, chimpanzee hepatitis B virus infection, duck hepatitis virus, ground squirrel hepatitis virus, and lymphochoriomeningitis virus (LCMV) in mice. Woodchuck hepatitis virus is a hepadnavirus that involves activation of NK and NKT cells in the early stages of infection, similar to HBV and HCV (Guy et al. 2008). Chimpanzees can be infected with HBV, similar to man. However, this is not

a highly researched model due to monetary and ethical concerns and it differs somewhat from human infection in that an early innate immune response is not observed and NK and NKT cell activation is linked to liver injury (Gao et al. 2009; Busca and Kumar 2014). LCMV infection in mice has been used to study the pathogenesis of viral hepatitis, but also lacks several characteristics of human HBV and HCV infection (Rehermann 2013).

Transgenic mouse models have also contributed greatly to the understanding of immunopathological processes involved in chronic viral hepatitis. A transgenic mouse model of HCV was produced using a *Cre/loxP* switching system (Kimura and Kohara 2011). Other transgenic mouse lines have been developed by injecting portions of the viral genome into fertilized eggs of inbred mice. HCV transgenic mice have significantly reduced apoptotic cell death compared with humans, which has been attributed to resistance to Fas-mediated apoptosis (Machida et al. 2001). HBV transgenic mice harbor the viral genes in their germline DNA and do not develop significant liver injury despite high levels of replication (Guidotti et al. 1995). However, several characteristics of disease pathogenesis have been discovered through this model. For example, studies have shown that CD8+ T cells are important in controlling viral replication and that liver disease has an immunological basis rather than being mediated by the virus itself. Furthermore, HBV transgenic mouse models have shown that functional impairment of dendritic cells (lower MHC Class II expression) contributes to defective immune responses against the virus (Akbar et al. 1993).

Human hepatocyte chimeric mice have also been developed through introduction of human hepatocytes into the liver of uPA/SCID mice, making them susceptible to HBV and HCV infection (Tsuge et al. 2005; Mercer et al. 2001). However, SCID mice are immunodeficient; therefore, the study of the underlying role of immunity and inflammation are limited in these models. Recently, a transgenic immunocompetent mouse expressing essential HCV entry factors has been developed, allowing viral entry into the host system. This model has been used to study inhibition of viral entry by various virus- and receptor-specific antibodies (Dorner et al. 2011).

7.4.7 Animal Models of NAFLD

Several animal models of NAFLD have been created, including nutritional models and genetically-engineered models. NAFLD has been induced in rodents by feeding diets deficient in leptin or high in fat and sucrose, and these diets have shown the importance of NKT cells in playing a protective role against the disease (Li et al. 2002, 2004, 2005). Methionine and choline-deficient diets also lead to hepatic steatosis with inflammation and fibrosis, although these animals lack obesity and insulin resistance (Rinella and Green 2004). Studies have shown that feeding a high-fat diet has variable results depending on the rodent strain, fat content of the diet,

composition of the dietary fat, and duration of treatment (Nakamura and Terauchi 2013; Kanuri and Bergheim 2013). For example, long-term high fat diet can induce obesity and insulin resistance in C57BL/6 mice, leading to NASH and liver tumorigenesis (Hill-Baskin et al. 2009). Additionally, a diet high in fructose and cholesterol in addition to fat induces NASH in the same mouse strain (Clapper et al. 2013).

Genetically-engineered models of NAFLD include sterol regulatory element binding protein (SREBP)-1c-transgenic mice (Nakayama et al. 2007) and phosphate and tensin homologue deleted on chromosome 10 (PTEN)-null mice (Horie et al. 2004).

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Chapter 8
Immunopathology of the Respiratory System

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Abstract Respiratory immunity is accomplished using multiple mechanisms including structure/anatomy of the respiratory tract, mucosal defense in the form of the mucociliary apparatus, innate immunity using cells and molecules and acquired immunity. There are species differences of the respiratory immune system that influence the response to environmental challenges and pharmaceutical, industrial and agricultural compounds assessed in nonclinical safety testing and hazard identification. These differences influence the interpretation of respiratory system changes after exposure to these challenges and compounds in nonclinical safety assessment and hazard identification and their relevance to humans.

Keywords Respiratory • Nasal • Larynx • Trachea • Lung • Cilia • Mucin • Alveoli • Macrophage • Inhalation • Mucus • Ciliated epithelium • Alveolar macrophage • Dendritic cell • Asthma

8.1 Introduction

Immunity in the respiratory system is similar to that of other organ systems with innate and acquired systems working in concert to recognize and eliminate foreign substances from the respiratory tract while maintaining homeostasis and normal function. The majority of the respiratory tract is exposed to the external environment with its myriad of pathogens, particulates, gases and toxins. This constant exposure to the environment requires initial protection of mucosal surfaces of the nose, larynx and tracheobronchial tree to allow innate and acquired immunity to eliminate challenges and maintain oxygen exchange in the lung.

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The anatomical structure of the upper respiratory tract along with mucociliary apparatus and components of the innate immune system are the initial protection against environmental threats which in turn signal and prime the acquired immune system for final elimination of foreign substances and protection against future threats. These protective mechanisms have a hierarchy with robust structural and mechanical mechanisms in the nose followed by the larynx and tracheobronchial tree that are designed to protect the lower respiratory tract (bronchioles and alveoli) from injury

Even though the goal of respiratory immunity is to eliminate threats and maintain homeostasis, pathogens, environmental toxins and pharmaceutical and industrial compounds can perturb the innate and acquired immune systems causing injury to the respiratory tract. Species differences in mucosal defense and innate and acquired immunity also play a role in the response of the respiratory immune system and these differences play a role during nonclinical safety assessment of pharmaceutic, agricultural and industrial compounds.

8.2 Anatomical Structures

8.2.1 Nose

The primary purpose of the nose is to warm, humidify and filter the air prior to its arrival in the lung. Filtration takes place via structural, mechanical and immunologic means.

Nasal cavity complexity results from the turbinate shape and varies between species with regards to the complexity and location of turbinates. Humans and monkeys are similar with the least complex turbinate structure. The human has three turbinates (superior, middle, inferior) and the nonhuman primate has two turbinates (maxilloturbinates, ethmoid turbinates) that extend throughout the nasal cavity and have a simple shape. Dogs and rodents (rats and mice) have more complex turbinate structure with dorsal nasal and maxilloturbinates in the rostral portion of the nasal cavity and multiscrolled ethmoid turbinates in the caudal portion of the nasal cavity (Fig. 8.1) (Harkema et al. 2006).

Turbinate complexity influences how air flows through the nasal cavity. Air flow through the nose influences the exposure of turbinate epithelium to inhaled particles and gases and subsequent innate and acquired immune responses in the nasal cavity as well as potential exposure of the larynx, trachea and lung to inhaled gases and particulates. Maxilloturbinates in rodents and dogs (Figs. 8.2 and 8.3) are more complex than in nonhuman primates and are better suited, especially in the rodent, for filtration, absorption and clearance of particulate matter and gases (Harkema et al. 2006). Ethmoid turbinates, observed in rodents, dogs and nonhuman primates are associated with the more extensive olfactory capabilities of these species. These turbinates are not involved in filtration but are susceptible to particulate and gas exposure.

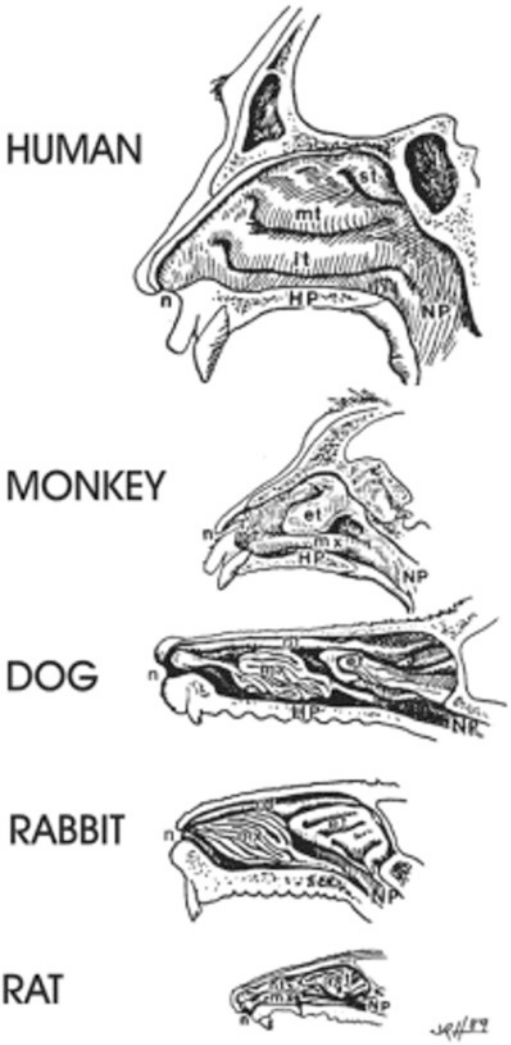

Fig. 8.1 Diagrammatic representation of the exposed mucosal surface of the lateral wall and turbinates in the nasal airway of the human, monkey, dog, rabbit and rat. *HP* hard palate, *n* naris, *NP* nasopharynx, *et* ethmoturbinates, *nt* nasoturbinates, *mx* maxilloturbinates, *mt* middle turbinate, *it* inferior turbinate, *st* superior turbinate. Figure and caption from Harkema J (2006) The nose revisited: a brief review of the comparative structure, function and toxicologic pathology of the nasal epithelium, Toxicol Pathol 34 (3) (Harkema et al. 2006)

8.2.2 Larynx

The larynx is a conduit for airflow from the nose to trachea; it diverts ingested material from the airways using the epiglottis; it is part of the mucociliary apparatus for expelling of mucus into the pharynx and gastrointestinal tract and is used for vocalization. The lining epithelium varies by region in the larynx along with the various folds, pouches and cartilages (Renne and Gideon 2006). Stratified squamous epithelium of the epiglottis is thicker in dogs and nonhuman primates, extends more caudally and potentially allows more protection against inhaled materials. Lateral

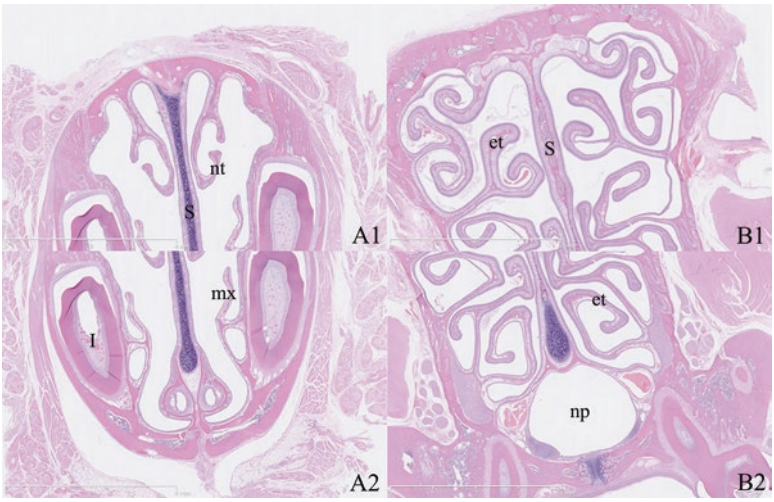

Fig. 8.2 Nasal cavity from a Sprague–Dawley rat showing the turbinate pattern.. Nasoturbinate (nt) and maxilloturbinates (mx) in the rostral section (A1, A2) are lined by transitional epithelium. Ethmoid turbinates (et) with more complex scrolls in the caudal nasal cavity (B1, B2) are lined by olfactory epithelium. *I* incisor, *S* nasal septum, *np* nasopharynx with nasal associated lymphoid tissue (H&E, 1.25× objective magnification)

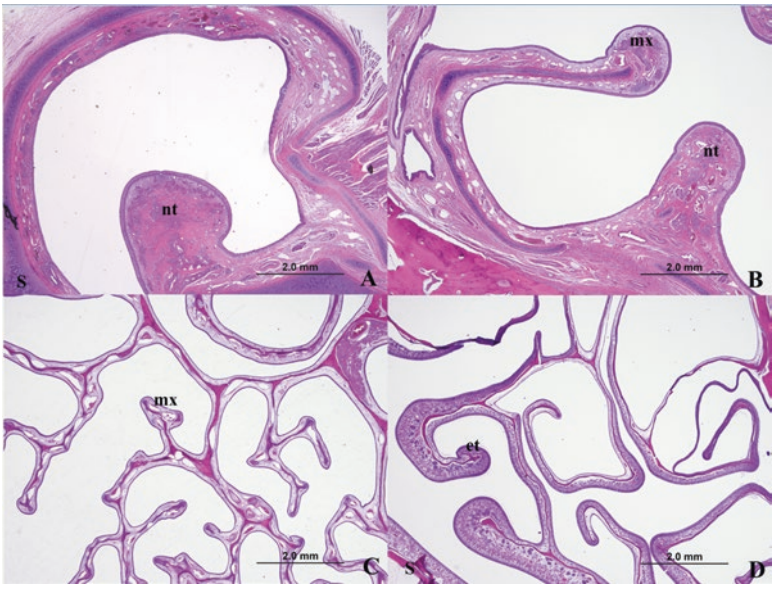

Fig. 8.3 Nasal cavity from a beagle dog highlighting the turbinate pattern. Nasal levels taken at level of incisors (**a**), at level of canine teeth (**b**), at level of second premolar tooth (**c**) and at level of the molar teeth (**d**). *S* nasal septum, *nt* nasoturbinate, *mx* maxilloturbinate, *et* ethmoid turbinate (H&E, 2× objective magnification)

ventricles are more lateral in dogs and monkeys but extend ventrally in rodents. Transitions from squamous to respiratory epithelium are at the arytenoid cartilages in dogs and monkeys (Renne and Gideon 2006).

8.2.3 Trachea

The main function of the trachea is to move air from the upper respiratory system (nose, larynx) to the lung. It is relatively uncomplicated with respect to structure (straight tube) and microscopic appearance (respiratory epithelium and submucosal glands). The main species differences in tracheal morphology involve submucosal glands: their number and their location along the trachea. The histology of the rat, dog and monkey is shown in Fig. 8.4 (Choi et al. 2000). Tracheal submucosal glands are typically more numerous in the ventral trachea (except for the pig in which the dorsal trachea has more glands). Submucosal glands are found throughout the trachea in all species but are in higher numbers in association with the first three cartilage rings in the rat and are only found at the junction of the larynx and trachea in the mouse. Submucosal gland position in relationship to cartilage rings varies between species. Glands are more often between tracheal rings in rats and are found between and about cartilage rings in the nonhuman primate and dog (Choi et al. 2000). Tracheal length influences particle deposition in the respiratory tract. Tracheal length in relationship to its diameter is longer in the dog, rat and mouse when compared to the human and results in less particle deposition in the large airways of these animals (Lippmann and Schlesinger 1984).

8.2.4 Lung

The main function of the lung is oxygen exchange at the level of the alveolus. All structures in the respiratory tract that eventually lead to the alveolus are designed to deliver air that is warm, humidified and free of particulate matter, microorganisms (viruses, bacteria, fungi, etc.) and toxins. Structural protection in the lung consists of dichotomously branching airways which trap materials to allow clearance by the mucociliary apparatus. Airway branching can be symmetric (daughter airways of similar diameter) or asymmetric (major and minor daughter airways). Airway branching varies in species being symmetric in primate species (human, monkey) and asymmetric in dogs and rodents (Fig. 8.5). Airway branching affects distribution of inhaled material with humans and monkeys having greater upper bronchial particle deposition and deposition at bifurcations (which is also influence by oral breathing in humans) (Lippmann and Schlesinger 1984).

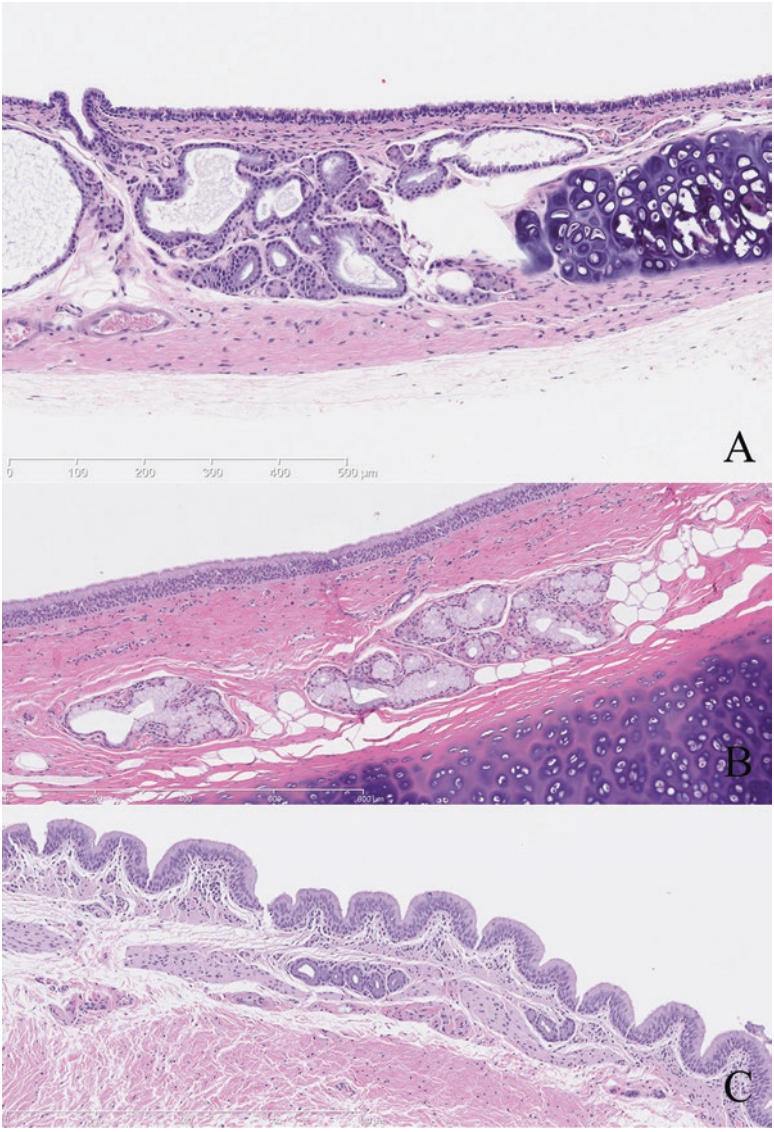

Fig. 8.4 Trachea histology in the Sprague-Dawley rat (**a**), Beagle dog (**b**) and cynomolgus macaque (**c**). (H&E, 10× objective magnification)

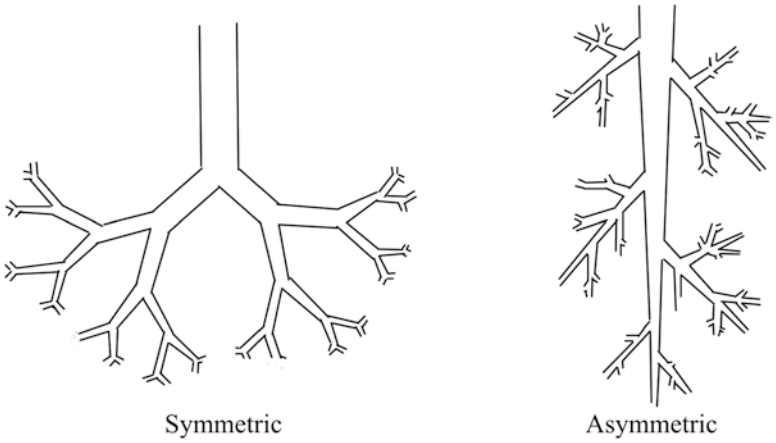

Fig. 8.5 Detail of airway branching found in the lungs of humans and nonhuman primates (symmetric) and dogs and rodents (asymmetric). Figure adapted from Treatise on Pulmonary Toxicology, Volume 1: Comparative Biology of the Normal Lung (McBride 1992)

8.3 Mucociliary Apparatus

8.3.1 Normal Mucociliary Apparatus

The first line of defense in the respiratory tract is the mucociliary apparatus which is composed of pseudostratified columnar respiratory epithelium (which includes ciliated cells, mucus (goblet) cells and club cells) and mucus (produced by mucus (goblet) cells and submucosal glands). Components of the mucociliary apparatus vary along the respiratory tract (Figs. 8.6 and 8.7). Mucus (goblet) cells are observed in the respiratory epithelium of the nose and larger airways of the lungs and in lower numbers in the trachea. Respiratory epithelium in the rat larynx does not contain mucus (goblet) cells.

Respiratory epithelium is more extensive in the rostral portion of the nose and the nasopharynx (Harkema et al. 2006). When examining the six levels of the rat nasal cavity (Mery et al. 1994), respiratory epithelium lines the nasal septum (Fig. 8.6) and the dorsal meatus in the most rostral section caudal to the nares (section between the upper incisor and incisive papilla); it lines the nasoturbinates and dorsal septum in the section at the level of the incisive papilla; it then lines the most ventral aspect of the nasal cavity (ventral aspect of most ventral ethmoturbinates, nasopharyngeal duct and nasopharynx) in the sections of the caudal nose (Fig. 8.6) at the level of the second palatal ridge and molars (Mery et al. 1994). Respiratory epithelium is present in the larynx (base of epiglottis in rodents, diverticula of monkeys and caudal aspect of larynx surrounded by cricoid cartilage in rodents, dogs and monkeys) but generally does not contain mucus (goblet) cells. (Renne and Gideon 2006). Ciliated cells are abundant in the respiratory epithelium but with decreasing airway size in the lung, the proportion of ciliated cells decreases

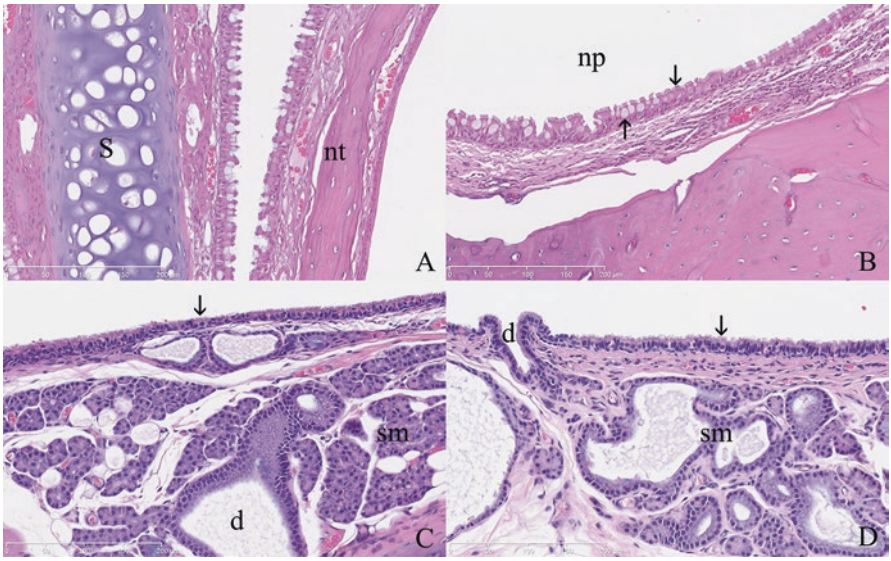

Fig. 8.6 Respiratory epithelium in the nose (**a**, **b**), larynx (**c**), and trachea (**d**). Ciliated cells (↓) are observed throughout the respiratory tract. Mucus (goblet) cells (↑) in the upper respiratory tract are most prevalent in the nose. *S* nasal septum, *nt* nasoturbinate, *np* nasopharynx, *sm* submucosal glands, *d* submucosal gland duct (Sprague-Dawley rat, H&E, 20× objective magnification)

along with the cilia height (Stannard and O'Callaghan 2006). The most abundant type of cilia in respiratory tract epithelium are motile cilia which are found in clusters of 100 to 300 on the apical cell surface (Jain et al. 2010). Motile cilia have a basal foot process which results in cilia pointing in the same direction and allows them to have a coordinated beat to clear mucus from the respiratory tract (Stannard and O'Callaghan 2006). Motile cilia have a 9+2 microtubular structure composed of 9 doublets on the periphery with dynein arms and a central microtubular pair (Fig. 8.8). The central pair is attached to the peripheral doublets via radial spokes and the tubular structures are linked by nexin (A and B tubule doublets) or surrounded by an inner sheath (central microtubules) (Fig. 8.8). In addition to motile cilia, primary cilia have been identified in the respiratory tract and have a 9+0 tubule arrangement devoid of a central microtubule pair, dynein arms or radial spokes. Primary cilia have a sensory role and are observed in the retina, nasal cavity, biliary ducts and endothelium (Jain et al. 2010).

The mucociliary apparatus in the nose of the rat has two regions—anterior and posterior. Nasoturbinates and maxilloturbinates are in the anterior region; the mucus is transparent and drains into the ventral meatus. The maxillary recess and ethmoturbinates are located in the posterior region, the mucus is more opaque and drains just anterior to the entrance of the nasopharynx (Morgan et al. 1984). Mucus turnover time in the rostral nose of the rat is much faster (10 min) than the caudal nose (several days) (Harkema et al. 2006). The mucociliary apparatus in the larynx and trachea of the dog shows a velocity gradient with greater flow in the upper trachea and cricoid regions which extends into the interarytenoid region and eventually slows in the epiglottis

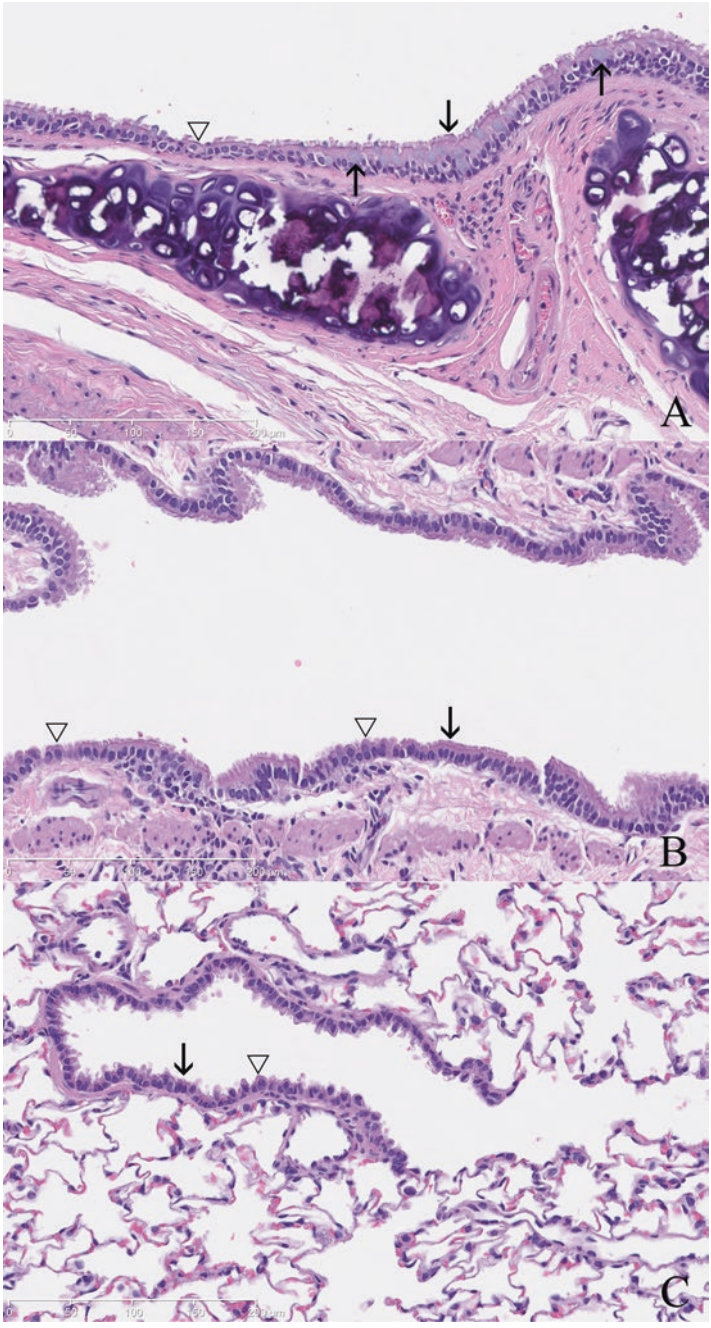

Fig. 8.7 Respiratory epithelium in the lung of a Sprague-Dawley rat. Bronchi (**a**), large diameter bronchiole (**b**) and terminal bronchiole (**c**) show that mucus (goblet) cells (↑) are most numerous in the bronchus. As the airways branch into bronchioles, the number of mucus (goblet) cells and ciliated cells (↓) decrease and the number of nonciliated cells (club cells, ∇) increase (H&E, 20× objective magnification)

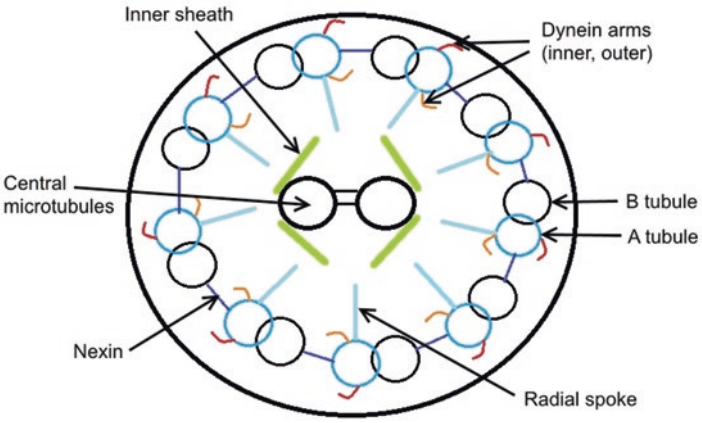

Fig. 8.8 Cross section of motile ciliiary axoneme detailing the 9+2 arrangement with dynein arms and radial spokes. Figure adapted from (Stannard and O'Callaghan 2006)

regions where the mucus can be swallowed (Bridger and Proctor 1972). The transition from squamous to respiratory epithelium in the larynx occurs at the base of the epiglottis in rats but squamous epithelium extends more caudally in the dog and monkey (Renne et al. 2007, Renne and Gideon 2006). Ciliated epithelium in the larynx is more abundant in the dorsal larynx (Renne and Gideon 2006). In the lung, decrease in mucus transport velocity occurs as airways branch most likely due to lower numbers of ciliated cells as well as the smaller airway diameter.

Mucus is a viscoelastic gel composed of mucin glycoproteins, water, ions, proteins (e.g., lysozymes and immunoglobulins) and lipids. Mucus lining the respiratory tract is produced by mucus (goblet) cells and/or submucosal glands in the nose, larynx, trachea and bronchi (Fig. 8.9) (Spicer and Martinez 1984, Kim et al. 1997). Mucus has thixotropic properties, acting as a fluid when stirred and semisolid when standing (Lai et al. 2009) which is accomplished through the varied properties of the mucus associated with the epithelium. Periciliary mucus is liquid while that overlying cilia is a gel allowing cilia to beat rhythmically and propel mucus rostrally. Mucus act as a first responder to inhaled particulate matter (inorganic, bacteria, viruses), gases and toxins through a variety of mechanisms (Lai et al. 2009). Mucus not only traps particulate matter and gases but also has antimicrobial and clearance properties. Stored mucus (intraepithelial mucus) is more abundant in the rostral nasal cavity and this is consistent with abundant mucus (goblet) cells in nasal respiratory epithelium (Fig. 8.6).

Mucin glycoproteins are a major constituent of mucus with mucin gene and protein expressed in the upper and lower upper respiratory tract of the human, nonhuman primate, rat, mouse and dog. Mucins are glycosylated macromolecules with a protein core and sugar chains that vary in size and composition that give mucus its viscous and elastic nature and the ability to trap foreign substances in the respiratory tract (Thornton et al. 2008). Mucins in the respiratory tract are divided into two categories, secreted (Table 8.1) and tethered (Table 8.2) which form a raft overlying the epithelium (secreted and tethered) or are associated with the cell membrane (tethered) (Fig. 8.10). Secreted (gel-forming) mucins are the most abundant and make up

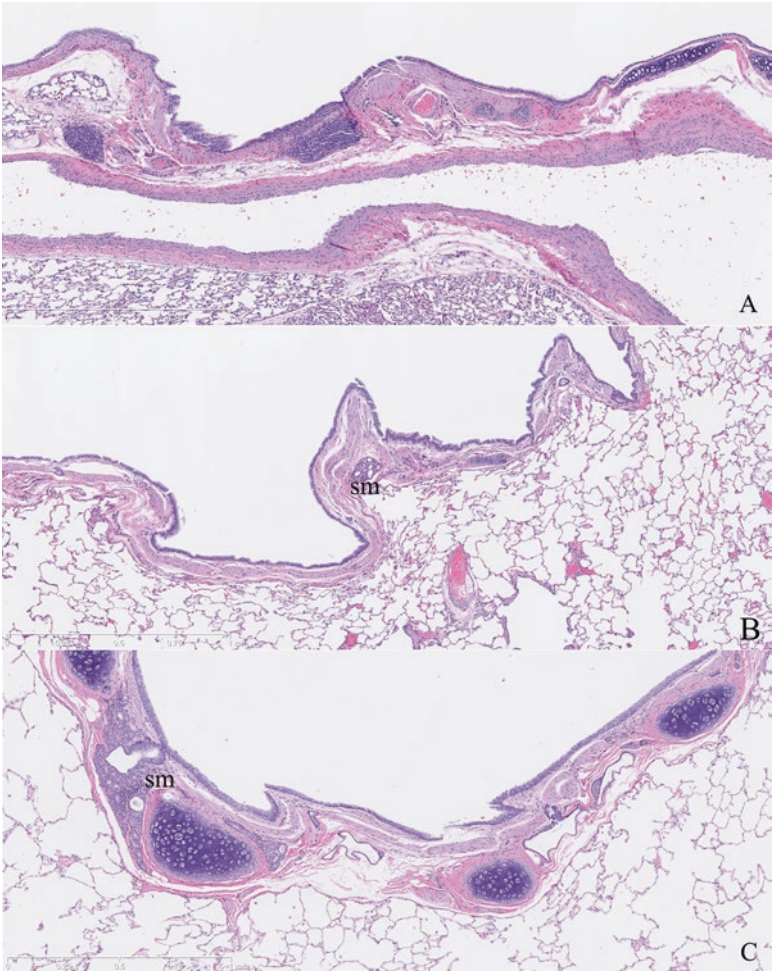

Fig. 8.9 Bronchi in the lung of Sprague-Dawley rat (**a**), dog (**b**) and cynomolgus macaque (**c**) showing the respiratory epithelium lining the airway of all three species and submucosal glands (sm) in the dog and cynomolgus macaque (H&E, 5× objective magnification)

about 90% of the respiratory mucin content in the human, are the gel portion of mucus and are stored in mucus (goblet) cells of the respiratory epithelium and the mucus cells or serous cells of the submucosal glands. Secreted mucins are released in response to secretagogues (e.g., eicosanoids, inflammatory mediators, bacterial products, inhaled toxins) (Rose and Voynow 2006, Hattrup and Gendler 2008, Thornton et al. 2008, Adler and Li 2001). Tethered mucins make up approximately 10% of the total respiratory mucin content in humans and are produced by ciliated cells. Tethered mucins are on the cell surface, associated with cilia and microvilli with a membrane-spanning domain and a cytoplasmic tail and make up the periciliary layer of mucus. Tethered mucins participate in the barrier funciton of mucus with secreted mucins but may also trigger signaling pathways (Williams et al. 2001,

Table 8.1 Expression of secreted mucin genes/protein in the normal respiratory tract

	Human	Non-human primate	Rat	Mouse
MUC2	Lung (mucus (goblet) cells)., Nose (mRNA in serous and mucous cells of submucosal glands, ciliated and basal cells, occasional mononuclear inflammatory cells) (Jeffery and Li 1997) Inferior turbinates of nose (mucus (goblet) cells) (Aust et al. 1997)		Trachea (after infection) (Sharma et al. 1998) Lung airway epithelium (mRNA) (Rose and Voynow 2006)	
MUC5AC	Lung (mucus (goblet) cells) (Jeffery and Li 1997); Inferior turbinates of nose (mucus (goblet) cells and occasional submucosal glands) (Aust et al. 1997)	Mucus (goblet) cells (trachea and lung) (Wiede et al. 1999) Nasal mucus (goblet) cells	Trachea (Borchers et al. 1998) Nasal mucus (goblet) cells (Harkema et al. 1994); metaplastic mucus (goblet) cells after ozone exposure (Cho et al. 2000) Lung mucus (goblet) cells (Rose and Voynow 2006)	Rare in lungs in naïve animals (increases with allergen sensitization) (Zuhdi Alimam et al. 2000)
MUC5B	Mucous cells in submucosal glands (bronchus) (Voynow et al. 1998, Sharma et al. 1998) Inferior turbinates of nose (submucosal glands) (Aust et al. 1997)	Submucosal glands (trachea and lung) (Wiede et al. 1999)		Proximal trachea (mRNA) (Rose and Voynow 2006)
MUC7	Serous cells in submucosal glands (bronchus) (Sharma et al. 1998) Inferior turbinates of nose (submucosal glands) (Aust et al. 1997)			
MUC8	Mucous cells of submucosal glands (lung, trachea) (Voynow et al. 1998)			
MUC19	Mucous cells of submucosal glands (lung, trachea) (Voynow et al. 1998) Trachea (ISH) (Chen et al. 2004)			Trachea (submucosal glands via ISH) (Chen et al. 2001) Proximal trachea (mRNA) (Rose and Voynow 2006)

Table 8.2 Expression of tethered mucin genes/protein in the normal respiratory tract

	Human	Rat	Mouse	Dog
MUC1	Lung (apical respiratory epithelium) (Voynow et al. 1998) Inferior turbinates of nose (respiratory epithelium, submucosal glands) (Aust et al. 1997)	Lung airway epithelium (protein) (Rose and Voynow 2006)	Lungs (Zuhdi Alimam et al. 2000)	Trachea (apical epithelium and soluble) (Mcneer et al. 1998)
MUC4	Lung (respiratory epithelium, on surface of cilia) (Voynow et al. 1998, Hattrup and Gendler 2008) Inferior turbinates of nose (mucosal surface and occasional submucosal gland) (Aust et al. 1997)	Lung airway epithelium (protein, rose)		
MUC13	Trachea (ISH) (Williams et al. 2001)		Lung (pneumocytes), Trachea and Bronchial epithelium (low levels) (Williams et al. 2001)	
MUC16	Respiratory epithelium (ciliated cells) (Hattrup and Gendler 2008)			
Others	mRNA expression of MUC11*, MUC15*, MUC20* (sublocation unknown) (Rose and Voynow 2006)			CTM-A, CTM-B (from canine tracheal mucus secretions) (Shankar et al. 1991)

Hattrup and Gendler 2008). MUC1 and MUC16 have epitopes to allow adhesion of various molecules while MUC4 does not and may allow cells to evade immune recognition (Hattrup and Gendler 2008, Kesimer et al. 2013). There are species differences in types of mucin expression in the respiratory tract (Tables 8.1 and 8.2), with the more thorough investigations of mucins reported in the trachea and lung, especially of the human and to a lesser degree in the nonhuman primate, rat and mouse. Little has been published on mucin types in the dog and in the nasal cavity of all species but there has been limited examination in the human nose (Aust et al. 1997). Mucins from respiratory mucus (goblet) cells are typically nonsulfated in the rat (sialomucin) and sulfated in the dog (sulfomucin) (Spicer and Martinez 1984).

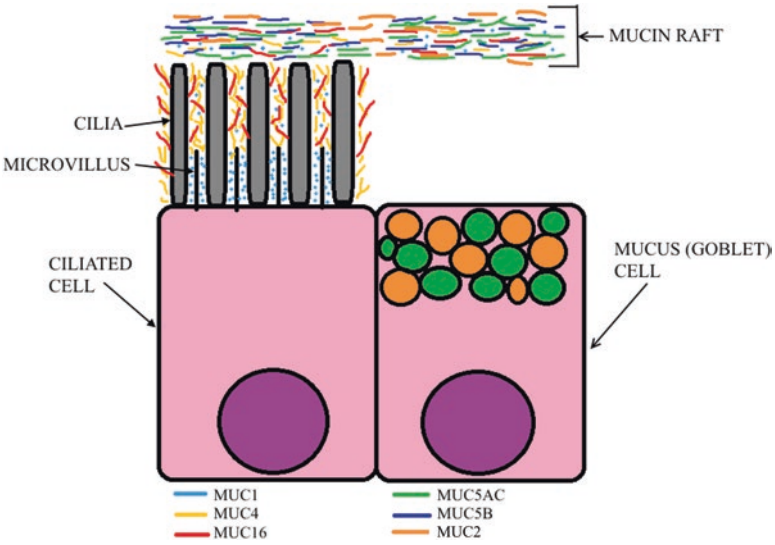

Fig. 8.10 Mucin types in the respiratory tract. Tethered mucins (e.g., MUC1, MUC4 and MUC16) are produced by ciliated cells, observed in close associated with the cilia (MUC4, MUC16) and microvilli (MUC1) and to a lesser extent in the mucin raft overlying the epithelium. Secreted mucins (MUC2, MUC5AC and MUC5B) are found primarlily in the mucin raft and originate from mucus (goblet) cells (MUC2, MUC5AC) or submucosal glands (MUC5B). Figure adapted from (Hattrup and Gendler 2008)

8.3.2 Mucociliary Apparatus and Toxicity

Impairment of the mucociliary apparatus can result from changes to the mucus layer (amount, viscosity, and pH) and/or changes to the ciliated cells (loss of ciliated cells, loss of cilia, impairment of ciliary function). Changes to the apparatus decrease its ability to function and increase the susceptibility to bacterial/viral infections and other environmental toxins, and/or allow particulate matter into the pulmonary alveoli with subsequent pulmonary pathology. For example, changes to the mucociliary apparatus can lead to chronic respiratory disease in rats caused by *Mycoplasma pulmonis*, once a significant colony health problem in laboratory rodents. *M. pulmonis* is ubiquitous in the nasal passages and opportunistic infection of the respiratory tract occured after exposure to increased ammonia in the bedding (urease in intestinal flora converts urine 2 μ globulin into ammonia). Ammonia exposure allows *M. pulmonis* to multiply in the nose, larynx, trachea and lung with subsequent chronic inflammation/infection (Lindsey et al. 1971, Schoeb et al. 1982).

Increased mucus production is a common lesion associated with respiratory tract toxicity and results from increased production of mucus and/or increased numbers of mucus (goblet) cells. Increased mucus production due to hypersecretion by mucus (goblet) cells and/or submucosal glands can be induced by ozone, cigarette smoke, cytokines [TNFα, IL-1β, IL-13, IL-17], neutrophil elastase, allergens and

microbial pathogens (Ganesan et al. 2013, Adler and Li 2001). Increased mucus will also occur due to increased numbers of mucus (goblet) cells as a result of hyperplasia or metaplasia (Samet and Cheng 1994). Mucus (goblet) cell metaplasia and hyperplasia are induced by EGFR and IL-13 (Ganesan et al. 2013). Increased EGFR expression can result from oxidants derived from neutrophil elastase, cigarette smoke and diesel emissions (Casalino-Matsuda et al. 2006). In addition to greater amounts of mucus, increased expression of specific mucins is observed with toxin exposure. Acrolein, an aldehyde found in smog and tobacco smoke, will induce mucus hypersecretion and increased expression of Muc5ac (trachea and lung) (Borchers et al. 1998). Muc5ac expression is also elevated after ozone exposure in the nose and lung (Samet and Cheng 1994, Fanucchi et al. 1998). Occasionally, mucin production is decreased, as was noted in a murine asbestos inhalation model using osteopontin null mice in which the amount of mucin observed on Periodic Acid Schiff stained lungs was decreased in distal bronchioles (Sabo-Attwood et al. 2011). Mucus composition can be altered after exposure to air pollutants. For example, pH alteration after sulfur dioxide exposure reduces mucus viscosity while increased mucus viscosity can result from protein cross-linking after formaldehyde exposure (Samet and Cheng 1994) and mucus acidification.

Loss of ciliated cells/cilia is observed with direct cell injury, which may be noted with exposure to ozone, sulfur dioxide and cigarette smoke. This loss may be temporary, followed by regeneration of ciliated cells, but with sustained injury and repair, hyperplasia and/or metaplasia may occur. The latter changes may or may not be reversible and there may be permanent mucociliary impairment. Exposure to aged and diluted sidestream cigarette smoke followed by naphthalene exposure in mice results in impaired respiratory epithelial repair in terminal bronchioles resulting in loss of normal club and ciliated epithelium (Van Winkle et al. 2001). Impairment of ciliary function can be noted with exposure to sulfuric acid, sulfur dioxide, ammonia, cigarette smoke and formaldehyde. However, other respiratory pathology with these compounds has a more pronounced effect and the ciliary impairment may not be a major factor in the overall pathologic process (Samet and Cheng 1994). Ciliary dysfunction can also occur in association with increased amounts and viscosity of mucus (Ganesan et al. 2013). Impairment of ciliary function without loss of numbers of ciliated cells or cilia can occur during anesthesia or with cold temparatures (Christopher et al. 2014).

8.4 Inflammatory Cells and Molecules of Innate Immunity

The main inflammatory cell populations associated with innate immunity in the respiratory tract are granulocytes (neutrophils and eosinophils), mast cells, macrophages, dendritic cells and natural killer cells. These cells and their associated inflammatory mediators (cytokines, chemokines, complement) are involved in phagocytosis, neutralization and killing of infectious agents and interact with the acquired immune system. Innate inflammatory cells are also recruited/induced after exposure to certain chemicals, particulate matter and pharmaceutical compounds.

Neutrophils, the main immune cell in the peripheral blood, are vital in control of bacterial and fungal infections with proteases, reactive oxygen species and antimicrobial peptides (Kruger et al. 2015). Neutrophils are primed after exposure to pro-inflammtory cytokines during acute inflammation and these primed neutrophils can be retained in the lung as a protective mechanism (Kruger et al. 2015, Summers et al. 2014). Retained, primed neutrophils are eventually deprimed and sent back into circulation but this depriming may be impaired in patients with Acute Respiratory Distress Syndrome (ARDS) (Summers et al. 2014). Neutrophil recruitment occurs after chemokines such as IL-8, MIP-2 (macrophage inflammatory protein-2) and KC are released. MIP-2 and KC are produced by tissue macrophages after exposure to bacterial lipopolysaccharide (LPS) (DE Filippo et al. 2008). LPS binds to lipopolysaccharide binding protein (LBP) which is an acute phase protein produced in the liver as well as the lung. LBP is important for bacterial clearance in the lung (Fan et al. 2002). IL-8 is produced by monkey airway epithelium (ciliated cells) very soon after ozone exposure and has been associated with neutrophil influx in the lung. IL-8 production declines 24 h after ozone exposure (Chang et al. 1998). Neutrophil influx in the nose after ozone exposure upregulates mucin (MUC5AC) mRNA and mucus cell metaplasia in nasal transitional epithelium in the rat (Cho et al. 2000). Cytokine production (TNF and IL-8) after zinc oxide (welders) or lead oxide (experimental) exposure also results in neutrophil recruitment in the lung. Neutrophil recruitment with subsequent degranulation leads to elevated concentrations of free radicals and hydrolytic enzymes with associated tissue damage (Albright and Goldstein 1996). Neutrophils in the trachea of mice following influenza infection attracts CD8+ T cells via the CXCL12 chemokine that is primarily produced by neutrophils and forms a trail for the T cells to follow (Lim et al. 2015).

Eosinophils, peripheral blood granulocytes that contain granules with major basic protein, eosinophil cationic protein and peroxidase among others, are the primary granulocyte response to parasites. Eosinophil development, differentiation and maturation require GM-CSF, IL-3 and IL-5. Eosinophils produce cytokines during rest but these are upregulated during inflammation and include many pro-inflammatory cytokines, chemokines and lipid mediators (Blanchard and Rothenberg 2009). Eosinophils can be found normally in many tissues but not the lung. Eosinophil recruitment occurs with chemokines such as MIP-1α and -1β, RANTES, eotaxin, and MCP (monocyte chemoattractant protein) among others (Oliveira and Lukacs 2003).

Mast cells originate from the bone marrow prior to terminal differentiation and migrate to tissues with their final differentiation relying on the local environment. Mast cells (mucosal and connective tissue types) reside in mucosal and epithelial tissues in many species and the thoracic and peritoneal cavities of rodents (Amin 2012, Krystel-Whittemore et al. 2015). Mucosal mast cells are found in the respiratory tract of rats and mast cell progenitors here are typically found in low numbers but will increase in number after antigen-induced inflammation and express chymase and tryptase (Amin 2012, Krystel-Whittemore et al. 2015). Mucosal mast cells are T-cell dependent and will increase in number during T cell immune responses in contrast to connective tissue mast cells (Metcalfe et al. 1997). Mouse lung mast cells are rare and noted primarily around the mainstem bronchi while primate lung mast

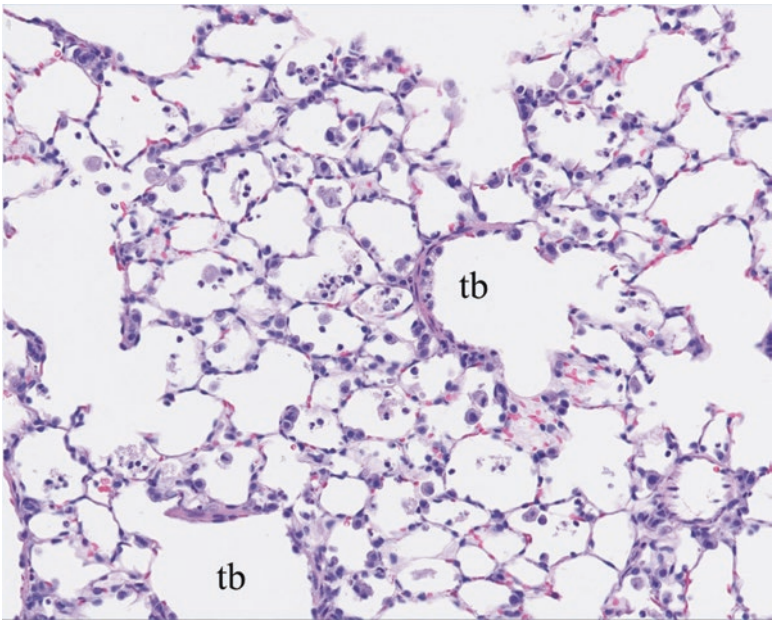

Fig. 8.11 Sprague-Dawley rat lung with alveolar macrophages and fewer neutrophils in alveolar spaces. *tb* terminal bronchiole (H&E, 20× objective magnification)

cells are more numerous around bronchioles (Miller and Pemberton 2002). In contrast to this observation, tryptase and chymase positive mast cells in infant rhesus macaques are most numerous in the trachea and least numerous in terminal bronchioles (Van Winkle et al. 2010).. Canine lung mast cells are most numerous in bronchial lamina propria with tryptase the predominate granule protease (Kube et al. 1998). Mast cell granules contain tryptase, chymase, histamine (more abundant in connective tissue mast cells) and serotonin with tryptase predominating in human mast cells (Beil et al. 2000, Welle 1997). Mast cell degranulation in the respiratory tract results in smooth muscle contraction with airway constriction, increased mucus production, increased vascular permeability with edema and coughing (Krystel-Whittemore et al. 2015). Mast cells are involved in innate and adaptive immunity by recognizing pathogens via toll like receptors with recruitment of other inflammatory cells such as neutrophils, natural killer cells and eosinophils; processing antigens via MHCI and MHCII and activating dendritic cells (Krystel-Whittemore et al. 2015). Eosinophils and mast cells both increase in number in allergic airway disease and influence airway remodeling through changes in airway collagen and smooth muscle (Van Winkle et al. 2010, Amin 2012, Humbles et al. 2004).

Macrophages are the first and main line of defense for the lower respiratory tract (Fig. 8.11). Macrophages are found in the bronchi, interstitium and alveolar spaces. Macrophages are also found in the capillaries (pulmonary intravascular macrophages, PIMs) of humans, cats, dog and sheep but not rodents or macaques (Kopf et al. 2015, Balhara and Gounni 2012). PIMs are attached to the capillary endothe-

lium on the thicker side of the alveolar septum and are found constituatively in the lung and can be induced in the species mentioned. While not normally found in the lung, PIMs can be induced in rats and mice after LPS exposure (Schneberger et al. 2012). PIMs are phagocytic and can influence lung inflammation (Schneberger et al. 2012). Alveolar macrophages are derived from blood monocytes, reside in the interstitium and migrate into alveolar spaces when needed where they mediate pulmonary responses after toxin exposure (Landsman and Jung 2007, Harkema et al. 2013). In addition, the lung is populated by alveolar macrophage progenitors which mature locally and are able to proliferate and self-renew (Kopf et al. 2015). Alveolar macrophages, which do not stimulate T-cells, can divide and are distinct from dendritic cells in the interstitium (which can stimulate T-cells) (Landsman and Jung 2007). There are two subpopulations of lung macrophages, M1 and M2. M1 macrophages are important in resistance to intracellular pathogens and are driven by interferon-γ and lipopolysaccharide. M2 macrophages deal with foreign material and apoptotic debris and are driven by IL-4 and IL-13 (Balhara and Gounni 2012). Macrophages are recruited from the interstitium of the lung via signals from pneumocytes (e.g., MCP-1) (O'Brien et al. 1998, Kannan et al. 2009).

Alveolar macrophages respond to particulate matter by phagocytosing and digesting material (if possible) in the alveolar space, releasing cytokines (TNFα) which then cause increases in chemokines (MIP-2 and IL-8) followed by recruitument of inflammatory cells (Driscoll et al. 1997). Alveolar macrophage response to particulate matter can vary based on paritcle type. Chrysotile, crocidolite asbestos, silica and coal mine dust are associated with increased alveolar macrophage TNFα while short asbestos fibers and diesel dust do not result in increased TNFα production (Dorger and Krombach 2002). Alveolar macrophage function can also be affected by toxin exposure. For example, phagocytic activity of alveolar macrophages can be impaired by ozone (Van Loveren et al. 1990, Oosting et al. 1991) and phagocytosis of ultrafine particles (Lundborg et al. 2001, Renwick et al. 2001). Alveolar macrophages contribute to pulmonary fibrosis and progressive lung lesions through production of platelet derived growth factor, fibroblast growth factor and fibronectin after particle exposure (Dorger and Krombach 2002). Alveolar macrophages respond to reactive oxygen species resulting from neutrophil degranulation (e.g., observed with ozone) (Chang et al. 1998, Albright and Goldstein 1996). Activated alveolar macrophages have increased inducible nitric oxide synthase (iNOS) expression and produce nitric oxide. This has been reported in rat alveolar macrophages in vitro after stimulation with IFN-γ., but was not observed in nonhuman primate and human alveolar macrophages (Jesch et al. 1997). Nitric oxide has antimicrobial, anti-inflammatory and antioxidant properties, but may also be involved in promoting further lung injury through production of reactive oxygen species such as peroxynitrite and nitrogen dioxide (Van Der Vliet et al. 2000). Alveolar macrophages play a role in asthma, having both pro-inflammatory and immunosuppressive roles. The pro-inflammatory role of alveolar macrophages in asthma results from release of IL-17, reactive oxygen species, TNF, IL-1β, IL-8, etc. The immunosuppressive action of macrophages in asthma is due to IL-10, IL-12 and nitric oxide release (Balhara and Gounni 2012. Nikander 1991). Alveolar macrophages produce TGF-β which in turn induces T_{reg} cells resulting in tolerance to

inhaled antigen and inhibition of T_H2 inflammation (Kopf et al. 2015). This tolerance mechanism is overrided when inhaled antigens bind to Toll-like receptor 4 and tolerogenic alveolar macrophages become inflammatory (Kopf et al. 2015).

Lung dendritic cells (DC) are found along the length of the respiratory tract (nose to pulmonary alveolus) and decrease in density moving from the mucosa of the nose to the lung (Condon et al. 2011). Dendritic cells are found in the mucosa of the nose, larynx, trachea and conducting airways and in the alveolar wall (Lambrecht et al. 2001). Dendritic cells residing in the mucosa migrate to regional lymph nodes for routine antigen sampling. Mucosal and alveolar dendritic cells extend cytoplasmic projections into airway or alveolar lumens to sample inhaled antigens (Lambrecht et al. 2001, Condon et al. 2011, Kopf et al. 2015). Alveolar dendritic cells have a low turnover time and do not appear to migrate to lymph nodes unlike those found in the respiratory mucosa (Lambrecht et al. 2001). Dendritic cells of the respiratory tract originate in the bone marrow, first as a monocyte and DC precursor (MDP) which gives rise to peripheral blood monocytes and the common dendritic cell precursor. As with other inflammatory cells, differentiation is partial in the bone marrow with full maturation occuring in the final tissue destination (Kopf et al. 2015). Peripheral blood monocytes also replenish resident lung dendritic cells (Condon et al. 2011, Kopf et al. 2015). Dendritic cells residing in the respiratory stroma are immature and unable to activate naïve T-cells and this immaturity is maintained by alveolar macrophages (Lambrecht et al. 2001, Kopf et al. 2015). The dampening or suppression of inappropriate inflammation by alveolar macrophages and epithelium in alveoli and terminal bronchioles by suppression of alveolar dendritic cells is vital in maintaining adequate gas exchange (Kopf et al. 2015, Lambrecht et al. 2001).

Natural killer (NK) cells are lymphocytes of the innate immune system that identify resident cells affected by pathogens or those that have become neoplastic. NK cells originate in the bone marrow from a common lymphoid progenitor but do not have active recombination activation genes unlike T and B lymphocytes. Natural killer cells produce interferon-γ, cytokines (e.g., IL-5 and IL-13), are cytolytic (cytoplasmic granules contain perforins and granzymes) and regulate immunity (Ivanova et al. 2014, Okada et al. 2015, Culley 2009, Campbell and Hasegawa 2013). Up to 10% of lymphocytes in the lung are NK cells and they are found as mature and immature phenotypes which can be influenced by the lung environment (Ivanova et al. 2014). Bronchial epithelium produces IL-15, which promotes NK cell survival (Culley 2009). NK cells are more numerous in the mouse lung when compared to the human lung (Haley 2003). NK cells in the mouse nose are similar to those in the lung with mature and immature phenotypes and are more numerous in the lamina propria of the nasal septum (Okada et al. 2015). More NK cells in the mouse nose have Ly49 receptors, which recognize MHC class Ia molecules and regulate cell mediated cytotoxicity, than those in the mouse lung (Okada et al. 2015). Additionally, NK cells in the mouse lung and nose have less upregulation of CD107a (LAMP-1) than those in the spleen after stimulation, indicating weaker degranulation (Okada et al. 2015). NK cells at mucosal surfaces have little CD56 expression ($CD56^{dim}$) in contrast to those in the lymph node which have high levels of CD56 ($CD56^{bright}$). CD56 expression is associated with greater cytokine production while cells with less expression have greater cytotoxic capabilities which is

more important at mucosal surfaces that are more likely to be exposed to pathogens (Culley 2009). NK cells are important in defense against respiratory viruses such respiratory vaccinia virus, respiratory syncytial virus and influenza virus and their activation and eventual regulation are controlled by interferons and IL-12 (Biron 1997). NK cells are activated by IL-1, IL-6, TNF-α and IL-12 (produced by macrophages), produce IFN-γ which primes T helper 1 cells and lyse cells with virus (Tamura and Kurata 2004, Waldhauer and Steinle 2008). NK cells eliminate tumor cells directly through cytolysis but also stimulates dendritic cells (through IFN-γ) and eventually an antitumor CD8 T-cell response (Waldhauer and Steinle 2008). Circulating NK cell numbers and activity have been shown to be suppressed in cigarette smokers and this suppression can last even after cessation of smoking which may increase the risk of lung cancer (Tollerud et al. 1989). Ozone exposure was shown to either increase or decrease NK activity in the rat lung, depending on exposure concentration. Lower exposure increased NK activity; higher doses decreased NK activity (Van Loveren et al. 1990). Short term exposure of mice to JP-8 jet fuel for 1 hour a day for 7 days resulted in loss of NK cell and helper T cell funciton as well as altering cytotoxic T cell function (Harris et al. 2000).

8.4.1 Molecules

Many secretions found in the respiratory tract are produced by a variety of cells including mucus (goblet) cells, secretory cells (non-ciliated epithelium), phagocytic cells or lymphocytes that have protective qualities. These include trefoil factors, defensins, inflammatory mediators (cytokines, chemokines, complement), immunoglobulins, lactoferrin, lysozyme and transferrin, some of which have already been discussed (Nelson and Summer 1998).

Trefoil factors are 7–12 kDa, protease resistant proteins and include trefoil factor 1 (TFF1), trefoil factor 2 (TFF2) and trefoil factor 3 (TFF3). Trefoil factors are highly expressed at mucosal surfaces where they are intimately associated with mucins and participate in the health and maintenance of the mucosa (Goke and Podolsky 1996). The function of these molecules has been most extensively studied in the gastrointestinal tract. Trefoil factors are involved in mucosal restitution after epithelial injury; they maintain viscosity of the mucus layer and can modulate the immune system by controlling lymphocyte migration. Trefoil factors are most often found in conjunction with mucins in mucus (goblet) cells and in submucosal glands. In the respiratory system, they have been found in mucus (goblet) cells, submucosal glands and club cells in the trachea and lung. Species differences in trefoil expression are presented in Table 8.3. TFF2 has the highest expression in the mouse lung but is undetectable in humans. In contrast, TFF3 has the highest expression in human lungs. TFF1 and TFF3, with normal low expression in mice, show elevations after epithelial injury using a naphthalene model of club cell injury and repair (Greeley et al. 2010). After experimental allergen exposure using OVA and albumin, increased TFF2 expression in mice is regulated by a Th2 response (Nikolaidis et al.

Table 8.3 Expression of trefoil factors in lungs of different species

	TFF1	TFF2	TFF3
Human (adult)	Hardly detectable (Wiede et al. 1999) In Club and ciliated cells (Dos Santos Silva et al. 2000)	Not detectable (Dos Santos Silva et al. 2000)	In SM glands (w/ MUC5B) and mucous cells (w/ MUC5AC) (Wiede et al. 1999) In airway epithelium (Club, ciliated cells) & submucosal glands (Wiede et al. 1999, Dos Santos Silva et al. 2000)
Rhesus macaque (adult)	Low to moderate expression in mucous cells (cytoplasm and nuclei) that increases with age during postnatal development and ciliated cell/Club cell nuclei (Greeley 2008)	Not detectable (Greeley 2008)	Highest expression and is expressed throughout postnatal development – mucous cells and submucosal glands (Greeley 2008)
Balb/c mice (adult)	Not examined	Induced by allergen exposure (OVA and A. fumigatus) and regulated by Th2 and STAT6 (Nikolaidis et al. 2003)	Not examined
C57Bl/6 mice (adult)	Low expression normal (club cells) (Kouznetsova et al. 2007, (Hertel et al. 2004) Increased expression after allergen exposure (Kouznetsova et al. 2007)	Highest expression (Kouznetsova et al. 2007, Hertel et al. 2004)	Not detectable (Kouznetsova et al. 2007, Hertel et al. 2004)
NIH Swiss mice (adult)	Low to moderate expression – Club and ciliated cells, attenuated fibroblasts (Greeley 2008)	Highest expression (sublocation could not be determined) (Greeley 2008)	Lowest expression—club cells, ciliated cells (Greeley 2008)

2003). TFF2 may be involved in neutrophil and eosinophil recruitment after house dust mite antigen exposure and in goblet cell metaplasia and eosinophil recruitment after IL-13 administration in the mouse lung (Wills-Karp et al. 2012). TFF1, which normally has low expression in club cells, is induced in the mouse lung after experimental allergen exposure using *Aspergillus fumigatus* antigen, and this expression is noted largely in transdifferentiating club cells (Kouznetsova et al. 2007). Rhesus macaques (6 and 12 months old) exposed to ozone for 11 cycles (cycle = 0.5 ppm for 8 h for 5 days) had no change in TFF1 expression, but TFF3 expression increased and was noted in both mucus cells and ciliated cells in the proximal airways of the lung (Greeley 2008).

Defensins have been observed in many mammalian species as well as birds and even fish. There are two types of defensins, α and β, which have a similar β sheet structure but differing cysteine disulfide bonds (Ganz 2002). Defensins

are constituitively expressed in neutrophils and epithelium but can also be induced by cytokines and endotoxin in monocytes and CD8 lymphocytes (Oppenheim et al. 2003). α-defensins are primarily found in leukocyte (neutrophil) granules in humans, rats and monkeys, in alveolar macrophages of rabbits and are also noted in epithelia (rabbit kidney, human female reproductive tract and Paneth cells) (Selsted and Ouellette 2005). Only the mouse lacks neutrophil α-defensins (Ganz 2002). α-defensins have antimicrobial and antiviral activity, can regulate complement activation, degranulate mast cells, are chemotactic for T-cells and immature dendritic cells, block endotoxin binding and can block ACTH receptors (Oppenheim et al. 2003). Rhesus macaques, baboons and orangutans also have Θ-defensin in their neutrophil granules. Θ-defensin mRNA has been found in human bone marrow but this mRNA is not translated into protein. β-defensins are observed in epithelia of humans, monkeys, rats and mice, including the respiratory tract (Ganz 2002). β-defensins also have antimicrobial activity, can induce prostaglandin D_2 production, degranulate mast cells and are chemotactic for CCR6 dendritic cells (Oppenheim et al. 2003). Three human defensins (HBD-1, -2 and -3) are found in the respiratory tract (Ganz 2002). HBD-1 and -2 RNA and protein were expressed in cell cultures of human trachea and bronchi (localized to surface epithelium and submucosal glands). HBD-2 expression increased with IL-1β exposure (Singh et al. 1998) whereas HBD-1 expression did not change, even with exposure to lipopolysaccharide or other cytokines (Singh et al. 1998). Mouse β-defensins 1 and 3 are found in respiratory epithelium of the nose, trachea, large proximal airways and distal airways. The expression of β-defensin 1 is greater than β-defensin 3 in the mouse respiratory tract. β-defensin 3 is upregulated after infection with *Pseudomonas aeruginosa* (Bals et al. 1998, Bals et al. 1999). A novel β-defensin (β-defensin 4) has also been found in the trachea of mice (Jia et al. 2000). Rat β-defensins are found in the respiratory epithelium of the trachea (β-defensin 1) and in type II pneumocytes of the lung (β-defensin 2) (Jia et al. 1999).

Inflammatory mediators such as cytokines, chemokines and complement, some of which have already been discussed, are typically produced by macrophages, monocytes, epithelial cells and fibroblasts (Thacker 2006). After exposure to bacterial LPS, TNFα and IL-1 levels are elevated in bronchoalveolar lavage fluid 30–90 min after exposure (Thacker 2006). These elevations are observed even when alveolar macrophages are depleted, suggesting other cells in the lung, such as interstitial macrophages or type II pneumoncytes, can produce these inflammtory mediators (Elder et al. 2005). IL-22 is produced by Th22, Th1, CD8+ T cells, NK cells and γδ T cells with pro-inflammatory and protective effects in the lung and these effects are mediated by IL-17A (Besnard et al. 2011, Sonnenberg et al. 2010). IL-22 transcript is elevated in the lung of mice after experimental asthma (ovalbumin exposure) and is needed for allergic airway inflammation through increased Th2 cytokines during the antigen sensitization phase of asthma. IL-22 can upregulate MUC1, β-defensins and neutrophil chemokines in respiratory epithelium (Besnard et al. 2011). Inflammation and associated epithelial injury with production of IL-22 and IL-17A by CD4+ TH17 cells is observed in mice after bleomycin administration. IL17 null mice have higher IL-22 expression but less bleomycin-induced inflammation and reduced apoptosis of airway epithelium (Sonnenberg et al. 2010).

8.5 Acquired Immunity

8.5.1 Normal Respiratory Tract

The acquired immune system in the respiratory tract is composed of mucosal associated lymphoid tissue in the nose (NALT, Fig. 8.12), larynx (LALT) and the lung (BALT, Fig. 8.13) and associated draining lymph nodes. Rats have the most BALT and humans have the least, where it is typically found in healthy children and adolescents but is induced in adult humans (Haley 2003, Tschernig and Pabst 2000). NALT is only observed on the ventral aspect of the lateral walls at the opening of the nasopharyngeal duct of rats but NALT is observed on the lateral and septal walls in nonhuman primates (Haley 2003). NALT in humans is composed of the lymphoid tissue of Waldeyer's pharyngeal ring (adenoids and palatine tonsils) (Brandtzaeg et al. 2008). Mucosal associated lymphoid tissue in the respiratory system is also discussed in Chap. 16.

Lymphocyte populations in the respiratory tract and draining lymph nodes include T cells (CD4, CD8, and T_{reg} cells) and B cells which rely on the innate immune system for their activation and regulation. Antigen-specific B- and T-cells are produced after dendritic cells in the respiratory tract interact with inhaled antigen and are transported to draining lymph nodes. Memory CD8 and CD4 T cells that are specific for the pathogens like influenza virus can reside in lung long after infection, are found near the airways and bronchovascular bundles, and can migrate throughout the lung and distant tissues after reexposure to influenza virus for protection (Turner et al. 2014, Cauley and Lefrancois 2013).

The upper respiratory tract (nose, larynx, trachea) is drained by cervical lymph nodes. The lung in the rat and mouse is drained by two mediastinal lymph nodes while the dog has three to five tracheobronchial lymph nodes and the human has 35 or more tracheobronchial lymph nodes divided into tracheal, bronchial, bronchopulmonary and pulmonary nodes (Haley 2003). Bronchial lymph nodes in the rodent also drain the lung and pleural space. Rodent lymph nodes are organized in simple chains while human lymph nodes form complex chains with anastamoses of lymphatic vessels (Haley 2013). The lack of extensive lymphatic anastamoses in the rat results in translocation of particulate matter or antigen from the lung to portions of the lymph node instead of the entire node as in the human (Haley 2013).

8.5.2 Acquired Immunity and Toxicology

Examples of acquired immune responses in the lung include hypersensitivity (asthma and beryllium) and changes to acquired immunity after exposure to environmental and occupational metal. Asthma is an ongoing type I hypersensitivity in the lung that begins with initial exposure to antigen and involvement of the innate immune system (neutrophils, lung macrophages) and subsequent involvement of the acquired immune system (T-helper 2 cells and IgE), which results in airway

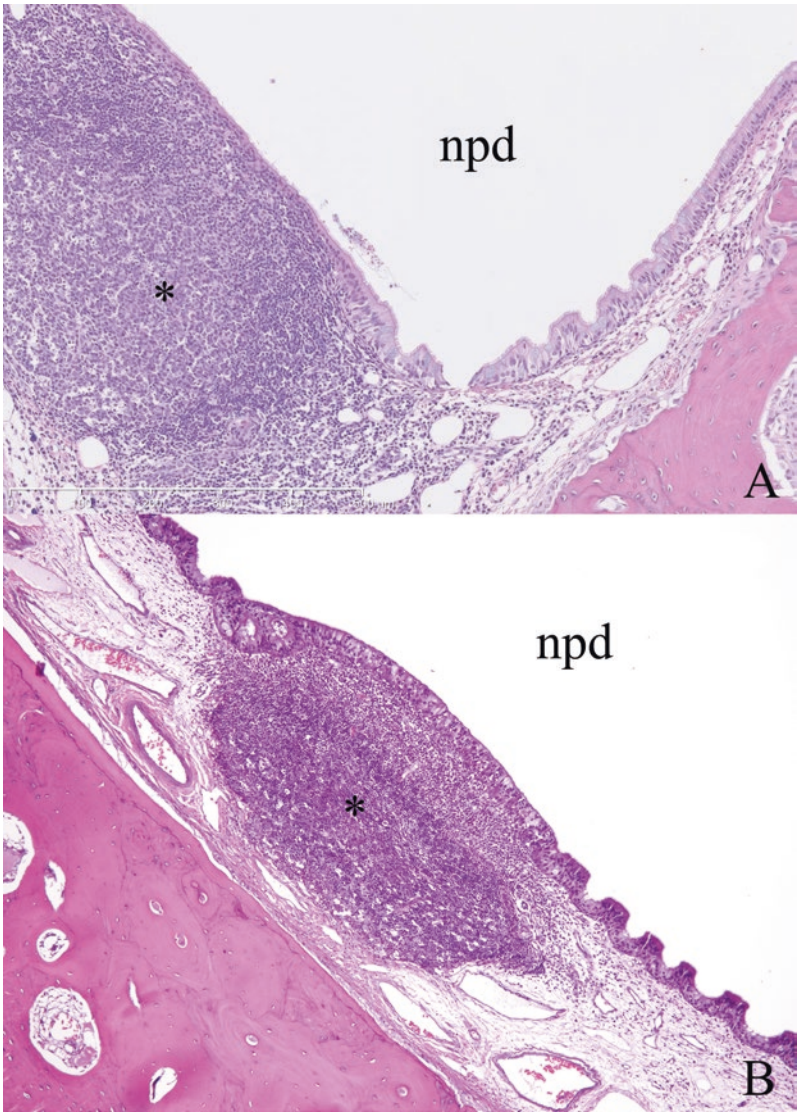

Fig. 8.12 Nasal associated lymphoid tissue (*, NALT) with germinal centers in the nasal cavity of the Sprague-Dawley rat (**a**) and beagle dog (**b**). *npd* nasopharyngeal duct (H&E, 10× objective magnification)

hyperreactivity, chronic eosinophilic airway inflammation, mucus cell hyperplasia and airway muscle changes. Asthma in juveniles can be induced by exposure to oxidant air pollutants (including ozone) in early childhood during postnatal lung development, resulting in alterations in alveolar morphogenesis, airway branching and development, and airway innervation (Tran et al. 2004, Plopper et al. 2007,

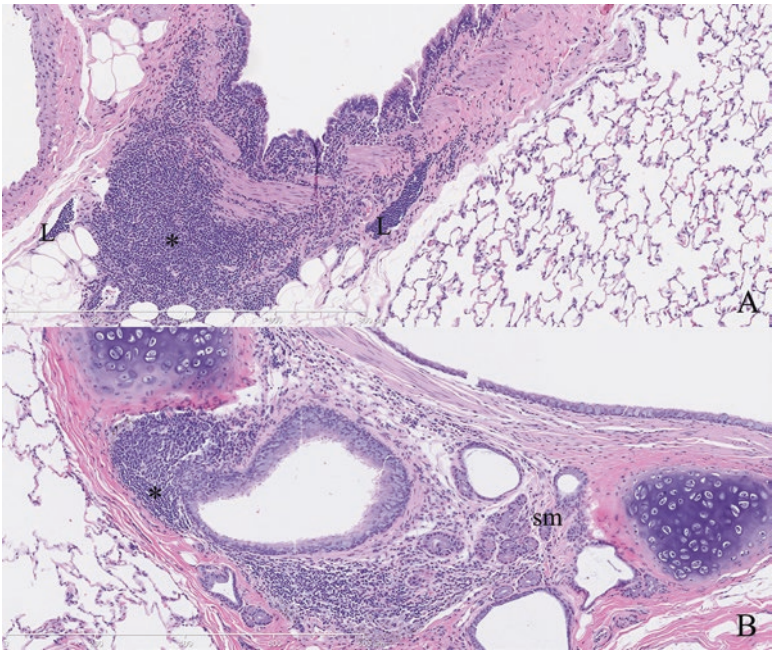

Fig 8.13 Bronchus associated lymphoid tissue (*, BALT) in the bronchus propria-submucosa of Sprague-Dawley rat (**a**) and cynomolgus macque (**b**). *L* lymphatic, *sm* submucosal glands (H&E, 10× objective magnification)

Plopper and Fanucchi 2000, Miller et al. 2003). Conversely, asthma may be prevented by exposure to endotoxin and antigens during childhood and this has been noted in children growing up on farms (Kaiser 2015). Exposing mice to endotoxin prior to exposure to house dust mite antigen blunts the initial innate immune response to that antigen (Schuijs et al. 2015). A potential similar benefit has been noted with early exposure to household pets (cats and dogs) but the results have been contradictory in the multiple cohort studies conducted (Lau et al. 2000, Remes et al. 2001, Chen et al. 2010).

Exposure to metal cations in the lung can result in hypersensitivity. Exposure to beryllium itself does not induce hypersensitivity in the lung. Initially, it was thought that beryllium bound to an unspecifed protein in the lung resulted in antigenicity of beryllium and $CD4^+$ T cells reactive to the beryllium hapten with eventual granuloma formation in the lung (Albright and Goldstein 1996). More recent research has shown that reaction to inhaled beryllium shows both hypersensitivity and autoimmunity (Clayton et al. 2014). Beryllium is buried in a complex composed of HLA-DP2 (MHC II allele) and a self peptide, thus altering the MHC II molecule's recognition by the T cell receptor and subsequent hypersensitivity. Exposure to other metals such as aluminum (smelters, miners), arsenic and cadmium (tobacco smoke and/or fungicides), chromium (manufacturing), nickel (fossil fuel combus-

tion, mining) and vanadium (coal burning) may result in lung pathology but the extent of the pathology is dependent on metal solubility (Cohen 2004). Exposure to aluminum may result in alveolar macrophage activation and neutrophil influx. Arsenic inhalation may cause an initial influx of neutrophils and alveolar macrophages and eventual suppression of lymphocyte IL-2 secretion, decreased complement levels and production of acute phase proteins (Cohen 2004). Cadmium exposure also results in suppression of cell-mediated immunity. Chromium can enhance T-lymphocyte proliferation at low concentrations but suppress it at higher concentrations given that it may be clastogenic (Cohen 2004). Nickel exposure suppresses humoral immune responses in mice (inhalation exposure) and rats (drinking water exposure), but immune suppression has not been readily documented in human exposure. Human exposure to nickel can result in asthma and may result from Ni-albumin conjugates (Cohen 2004). Inhalation of vanadium is followed by systemic exposure and formation of pentavalent vanadates and oxides with alterations in pulmonary immunity (e.g., decreased alveolar macrophage phagocytosis, reduced cytokine levels) and subsequent bacterial pneumonia, bronchitis, asthma, rhinitis and pharyngitis (Cohen et al. 2007, Cohen 2004).

8.6 Pharmaceuticals and Immunopathology

Many pharmaceutical compounds and components of those compounds can induce lung immunopathology. Noteworthy examples include nanoparticles, drugs that induce phospholipidosis and drugs that induce lung eosinophilia.

Nanotubes are being designed for many applications including delivery of pharmaceutical compounds and as antimicrobials. Nanotubes typically range in size from 1 to 100 μm and can be composed of carbon, gold, boron, zinc oxide, titanium dioxide, cerium oxide and silver (Hubbs et al. 2011, Thompson et al. 2014). Composition and deposition location of nanotubes will affect the immune response (Thompson et al. 2014). As smaller nanotubes are constructed, their toxicity increases because they can penetrate many organs, including the lung (Thompson et al. 2014). Nanotubes can have immunostimulatory or immunosuppressive effects (Thompson et al. 2014). After inhalation of single-walled carbon nanotubes, pulmonary granulomas were noted in rats that had either inhaled or aspirated the nanotubes (Hubbs et al. 2011). Granuloma formation with nanotubes occurs after aggregation of nanotubes, which is more likely with intratracheal instillation or oropharyngeal aspiration. Interstitial fibrosis occurs when nanotubes are more dispersed in the lung, which may occur after inhalation of dry aerosolized or nebulized suspensions (Thompson et al. 2014). Inhalation of single-walled carbon nanotubes by mice or cerium dioxide nanoparticles in rats was associated with neutrophil influx in the lung (Hubbs et al. 2011, Morimoto et al. 2015). In another study with highly dispersed single-walled carbon nanotubes, neutrophil inflammation did not persist but interstitial fibrosis was observed after only 4 days of exposure (Hubbs et al. 2011). Exposure of mice to low concentrations of multi-walled carbon nano-

tubes for 14 days resulted in suppression of T-cell dependent immune functions but exposing mice at higher concentrations for up to 13 weeks resulted in granulomatous inflammation and pleural thickening (Hubbs et al. 2011). Inhalation of silver nanoparticles by rats for 28 days resulted in increased size and number of goblet cells that contained neutral mucins with no change in sulfo- or sialomucins (Hyun et al. 2008) Inhalation of silver nanoparticles by rats for 90 days resulted in alveolar inflammation, perivascular infiltrate and alveolar macrophage accumulation in the lungs (Sung et al. 2009). . Inhalation of carbon black nanoparticles followed by ovalbumin challenge will result in enhanced inflammatory response to the ovalbumin due to enhancement of IL-4, IL-5 and IL-13 by carbon black nanoparticles (Hubbs et al. 2011).

Many drugs (e.g., amiodarone, antidepressants, antibacterials) have been shown to cause accumulation of phospholipids in cells of the monocyte/macrophage family, including alveolar macrophages forming lamellar bodies. Cationic amphophilic drugs cause accumulation/retention of the cell membrane component, phospholipid, in cell lysosomes in many tissues. This accumulation results from the affinity of the basic cationic amphophilic drugs to the acidic environment of the lysozome, with ionization of the drug and subsequent inability to leave the lysozome, as well as the direct interaction with the drug and phospholipid of the membrane (Anderson and Borlak 2006) . In the lung, the alveolar macrophage and type II pneumocyte are susceptible to phospholipid accumulation. There is accumulation of large, foamy alveolar macrophages and amorphous material in alveolar spaces. This accumulation is reversible after cessation of the compound (Halliwell 1997). Macrophage accumulation of lamellar bodies may inhibit cellular phospholipases and prevent cytotoxicity of phagocytosed silica (Reasor and Kacew 2001, Anderson and Borlak 2006) and may also enhance phagocytic activity of macrophages (Anderson and Borlak 2006).

Lung eosinophilia with associated clinical signs of dyspnea and radiographic infiltrates have been observed with anti-convulsants such as phenytoin sodium (Michael and Rudin 1981, Dixit et al. 2009) and carbamazepine (Lewis and Rosenbloom 1982) which are both members of the arene oxide-producing anticonvulsants. These hypersensitivity reactions are relatively uncommon and likely have a genetic determination (Vittorio and Muglia 1995). Other pharmaceutical and over-the-counter compounds that have been associated with hypersensitivity reactions and eosinophils in the lung include acetylsalicylic acid (i.e., aspirin), bleomycin, captopril, hydrochlorothiazide, mesalamine, minocycline, nitrofurantoin, penicillamine, sulfasalazine and sulfonamides (Campos and Pereira 2009). Information on these compounds and many others can be found on www.pneumotox.com.

Alveolar macrophages are also commonly induced during nonclinical safety assessment of pharmaceutical compounds. Increases in numbers of alveolar macrophages were a common finding in rats when pharmaceutical compounds, typically for asthma and allergic rhinitis therapy or other pulmonary inflammatory conditions, were given via inhalation (Nikula et al. 2014). Some of these compounds include Budesonide, Ciclesonide, Mometasone, Tilade and Advair amongst others (Nikula et al. 2014). The assessment of alveolar macrophage increases for adversity during nonclinical safety assessment should take into consideration the presence or

absence of other adverse lung findings, the progression of macrophage accumulation in longer duration studies with the same compound and resolution of alveolar macrophage accumulation with recovery (Nikula et al. 2014).

8.7 Development and Immunopathology

The development of innate and acquired immunity during post-natal development involves the presence of alveolar macrophages in airway and alveolar lumens and the maturation and increased number of T and B lymphocytes in BALT and regional lymph nodes (bronchial, mediastinal) (Miller 2004). Neonatal rodents have a lower level of lymphocyte trafficking from regional lymph nodes and have fewer antigen presenting cells (MHC II) that function poorly when compared to adults (Miller 2004). Alveolar macrophage numbers are greater in human infants when compared to teenagers and adults, and a similar trend is noted in nonhuman primates (Miller 2004). However, alveolar macrophages in newborn monkeys have poor phagocytic and killing capabilities. In contrast, alveolar macrophages of neonatal rats have greater phagocytic and killing ability for certain pathogens such as gram negative and positive bacteria (Miller 2004). Dendritic cells in the respiratory tract are first observed at the base of the nasal turbinates in newborn rats and then increase in number with age along the respiratory tract (Condon et al. 2011). Mucosal and connective tissue mast cells are in very low numbers in the rat at birth but increase with age (Wilkes et al. 1992).

Mucin-containing cells in rats are first noted in the most proximal portion of the trachea adjacent to the larynx after the third postnatal week (Smolich et al. 1967). Mucous cells containing PAS-positive material were noted in the bronchi in developing mouse lungs starting at embryonic day 15.5 and postnatal day 28 (Roy et al. 2011). Mucous cell morphology in developing mouse lungs was further confirmed by the presence of Muc5b, Muc1 and Muc4 transcripts in the lung starting at embryonic day 14.5 as well as protein expression (confirmed by immunohistochemistry) of Muc5b in tracheobronchial epithelium from embryonic day 15.5 to postnatal day 28. Muc5ac and Muc16 are expressed at much lower levels during mouse lung development (pre- and postnatal development). Muc5ac, which has increased expression in asthma models, is not detectable in the developing mouse lung until postnatal day 14 (Roy et al. 2011). In the human fetus, MUC4 is the earliest expressed gene in the foregut (gestation week 6.5) with MUC1 and MUC2 following at gestation week 9.5 in the trachea, bronchi and developing lung (Buisine et al. 1999). MUC5AC and MUC5B and are expressed starting at gestation week 13 while MUC7 starts to be expressed at gestation week 23 (Buisine et al. 1999).

The development of innate and acquired immune protection in the developing lung can influence the response of the developing lung to toxic insults. As compared to adult rodents, neonatal rats have higher expression of CINC-1 and MIP-2 (correlates of IL-8 and GRO-β) and neonatal mice have increased expression of TNFα, IL-1β and IL-6 when exposed to hyperoxic conditions. Higher expression of these factors occur prior to airway inflammation during hyperoxic conditions which is thought to be protective against the toxic effects of high oxygen concentrations in the lung (Miller 2004).

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Chapter 9
Immunopathology of the Cardiovascular System

Molly H. Boyle

Abstract Both innate and adaptive immune responses are activated in the heart in response to tissue injury. The purpose of a cardiac inflammatory response is to resolve the trigger for it, thereby allowing the heart to adapt to substandard conditions acutely, and ultimately restore homeostasis and cardiovascular function. Infectious and non-infectious myocardial insults may induce chronic inflammatory myocarditis with an autoimmune component directed to antigens such as myosin and troponin. Immune responses and inflammation in the cardiovascular system are discussed in detail. An understanding of Chagas cardiomyopathy and streptococcal infections has shed insight into general immunopathologic processes relating to the heart. Considerable knowledge pertaining to cholesterol metabolism and the immune system has developed as a result of a combined approach in which patient data are used to formulate hypotheses that are investigated in genetically modified murine models. Hypercholesterolemia decreases immune responsiveness through various processes. Studies in the cardiovascular arena and resultant therapeutic options have inspired rheumatic disease investigations, and similarly, rheumatic disease data have informed on cardiovascular research. Efforts are underway to move immunomodulatory treatments into clinical trials for atherosclerosis and vascular disease. The interplay between cardiovascular and immune therapeutics is reviewed. Animal models do not consistently recapitulate the human pathophysiology of cardiovascular disease, whereas human studies lack access to patient arteries for histopathologic analysis, and the human condition exhibits slower disease progression. As a result, there is a need for better models to study respective contributions of immune factors in cardiovascular pathology. Background cardiovascular pathology with immune-mediated associations in nonclinical species and routine animal models are also discussed.

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Abbreviations

ADA	Anti-drug antibodies
ADCC	Antibody-dependent cell-mediated cytotoxicity
ANCA	Anti-neutrophil cytoplasmic antibodies
ApoE	Apolipoprotein E
CCC	Chronic Chagas disease cardiomyopathy
CCR	Chemokine receptor
CD	Cluster of differentiation
CDC	Complement-dependent cytotoxicity
CLR	C-type lectin receptors
CMV	Cytomegalovirus
COL14A1	Collagen type XIV alpha 1
COX-2	Cyclooxygenase-2
CRP	C-reactive protein
DAMP	Damage-associated molecular patterns
DIVI	Drug-induced vascular injury
EC	Endothelial cell
FcR	Fc-receptor
FcγR	Fc region of IgG
GlcNAc	*N*-acetyl-beta-D-glucosamine
HLA	Human leukocyte antigen
HSP	Heat shock protein
HSPB8	Heat shock protein family B (small) member 8
IC	Immune complex
ICAM	Intercellular adhesion molecule
IFN-γ	Interferon gamma
IgG	Immunoglobulin G
IL	Interleukin
LDL	Low density lipoprotein
LDL-C	Low density lipoprotein cholesterol
LDLR	Low-density lipoprotein receptor
LPS-RS	Lipopolysaccharide from the photosynthetic bacterium *Rhodobacter sphaeroides*
mAb	Monoclonal antibody

MCP -1	Monocyte chemoattractant protein-1
MEF2A	Myocyte enhancer factor 2A
MMP	Matrix metalloproteinase
MPO	Myeloperoxidase
NF-κB	Nuclear factor-kappa B
NLR	Nucleotide-binding oligomerization domain-like receptors in short NOD-like receptor
NLRP3	NLR family pyrin domain containing 3
NO	Nitrous oxide
oxLDL	Oxidized low-density lipoprotein
PAMP	Pathogen-associated molecular patterns
PAN	Polyarteritis nodosa
PCR	Polymerase chain reaction
PD-L	Programmed death-ligand
PLA	Phospholipid antibody
PPAR	Peroxisome proliferator-activated receptor
PRR	Pattern recognition receptors
RAAS	Renin-angiotensin-aldosterone system
SAP	Serum amyloid P-component
SLE	Systemic lupus erythematosus
SNP	Single nucleotide polymorphism
Th1	(Type 1) T helper cell
TLR	Toll-like receptor
TNFα	Tumor necrosis factor alpha
TNFα	Cachexin or cachectin
Tregs	Regulatory T cells
VCAM-1	Vascular cell adhesion molecule 1
VSMC	Vascular smooth muscle cells
β2GP1	Plasma protein β-2-glycoprotein 1
γδ T-cells	Gamma delta T cells

9.1 Introduction: Immune Responses and Inflammation in the Cardiovascular System

Both innate and adaptive immune responses are activated in the heart in response to tissue injury. The innate immune system provides global, nonspecific defense, and inflammatory responses induced by it can be physiological and result in the upregulation of a portfolio of cytoprotective responses that characterize a short-term adaptation to the stressor. Alternatively, the inflammatory response can become pathophysiologic and dysregulated, resulting in collateral myocardial damage that leads to progressive left ventricular dysfunction and subsequent adverse left ventricular remodeling (Mann 2015). Although multiple risk factors and comorbidities

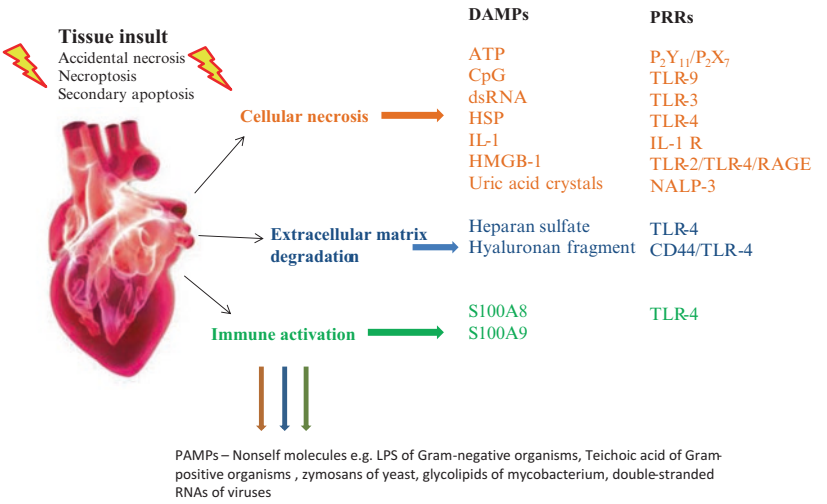

Fig. 9.1 Cardiac innate immune responses. Innate immune responses in the heart originate with nonself PAMPs or hidden-self DAMPs by PRRs, which are germ-line encoded. Cells that undergo overt necrosis, planned necrosis (necroptosis), or apoptosis release their contents into the extracellular space and commence inflammation through PRRs. Cardiac PRRs distinguish the molecular patterns of endogenous host material that is released by these injured cells. The immune response that is initiated by DAMPs has a consistent timeline, characterized by neutrophilic infiltration followed by monocytes at the site of injury and has been called sterile inflammation [adapted from Mann DL (2015) Circ Res 116:1254–1268].

must be considered, elevation in inflammatory biomarkers including the pentraxins C-reactive protein (CRP) (and its rodent paralog SAP), interleukin (IL)-1β, IL-6, TNF-α, and TNF receptor 1 and 2, have been causatively implicated in heart failure. Indeed, non-hemodynamic or contractile targeted therapies have been investigated to these ends and are discussed later (Tanigaki et al. 2015; Shalhoub et al. 2011; Mann 2015; Dick and Epelman 2016).

Cardiac innate immune responses are initiated by the detection of pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) by an established number of germ-line encoded pattern recognition receptors (PRRs) (Fig. 9.1). The innate immune system has evolved to detect molecules that are nonself (PAMPs) as well as intracellular molecules (DAMPs) that are concealed by the plasma membrane and not typically present in extracellular fluids without cell leakage, blebbing, or death. This latter revelation has provided more insight into the link between tissue injury, activation of proinflammatory mediators, and the resulting myocardial response to insult. PRRs encountering PAMPs and DAMPs trigger signaling cascades that activate nuclear factor-κB, activator protein 1, and interferon regulatory transcription factors that control target genes encoding proinflammatory cytokines and interferons in the heart. Another subset of PRRs in the heart trigger a proinflammatory mechanism that requires assembly of cytosolic

protein complexes called inflammasomes. Canonical inflammasomes convert procaspase-1 into the catalytically active protease which produces IL-1β and IL-18, thereby initiating inflammatory responses in the heart (Mann 2015).

PRRs can be subdivided into two major classes based on their subcellular localization: Toll-like receptors (TLRs) and C-type lectin receptors (CLRs). TLRs and CLRs are located on plasma membranes or endosomes where they sense the presence of PAMPs or DAMPs. The relative expression levels for TLR mRNAs in the human heart is TLR4 > TLR2 > TLR3 > TLR5 > TLR1 > TLR6 > TLR 7 > TLR8 > TLR9 > TLR10, with TLR4 being preferentially upregulated in the failing human heart. Additionally, TLR4 and TLR2 have a substantial effect on cardiac remodeling in ischemia-reperfusion injury and myocardial infarction. Blocking TLR2 signaling reduces pro-inflammatory pathways in an in vitro model of human atherosclerosis. For example, administration of anti-TLR2 therapy with a specific monoclonal antibody minutes before reperfusion decreases infarct size and preserves cardiac function by reducing leukocyte influx and cytokine production in mice and pigs. Anti-TLR4 therapies are also being investigated. CLRs can signal independently or control signaling through TLRs. The relative expression levels of CLR mRNAs in the human heart are similar to the murine heart: Bcl-10 > Galectin-1 > mannose receptor 2 > DC-SIGN (CD209) > Src > mannose receptor 1, >Dectin-1, triggering receptor expressed on myeloid cells 1 and Card-9 (Mann 2015).

(NOD)–like receptors (NLRs) act as cytosolic sensors to intracellular DAMPs and PAMPs. Analysis of human heart tissues has demonstrated that NOD (NOD2 [NLRC2]), NOD1 (NLRC1), and NLR family, pyrin domain–containing protein 2 (NLRP2 [NALP2]), NLRP3 (NALP3), also known as cyropyrin, are expressed. Both NOD1 and NLRP3 activate canonical inflammasomes in the heart, and are important in adverse cardiac remodeling after ischemia-reperfusion injury and myocardial infarction (Mann 2015). Reparative inflammasomes are enriched in cardiac macrophages, which are critical for TGF-β-induced expansion of cardiac myofibroblasts in an attempt to restore homeostasis post-infarction (Dick and Epelman 2016).

Cardiac macrophages can be stimulated or inhibited by a number of mechanisms irrespective of T-cell activation and adaptive immunity. The renin-angiotensin-aldosterone system (RAAS) exhibits well known physiologic effects on blood pressure through cardiac hypertrophy, remodeling, and modulation of renal perfusion, but also through immune mechanisms. Macrophage phenotypic heterogeneity is an important phenomenon in cardiac, and other pathology, and macrophage differentiation to the classical proinflammatory phenotype predominates with activation by mineralocorticoids of the RAAS. Agonism or genetic manipulation of RAAS components results in skewing of macrophage differentiation to an alternative or wound-healing and protective phenotype. Heat shock proteins (HSP) and oxidized low-density lipoprotein (LDL), discussed in greater detail later, can also independently activate macrophages (Frieler and Mortensen 2015; Shalhoub et al. 2011; Virella 2010). Investigations into the genesis and perturbation of cardiac and vascular macrophages should note the ability of vascular smooth muscle cells (VSMCs) to undergo macrophage-like transdifferentiation, and internalized low-density

lipoprotein cholesterol (LDL-C), as described for cardiac or bone marrow-derived macrophages (Zeller and Srivastava 2014).

The adaptive immune system imparts a specialized response mediated by B and T cells which can be modulated by various components of the cardiovascular system, including cardiomyocytes, endothelial cells (ECs), VSMC, and cardiac fibroblasts. The T-cell costimulatory receptors CD80 and CD86 are expressed on the surface of antigen-presenting cells, not on ECs or VSMCs. However, ECs and VSMCs express other proteins including PD-L1, PD-L2, and ICAM-1. PD-L1 and PD-L2 expression on VSMCs contributes to the relative immunoprivilege of the vascular medial compartment, as does low expression of MHC II and other physical and innate barriers, including the production of TGF-β (Tellides and Pober 2015). $CD4^+$ T-cells have a role in modulating ischemia–reperfusion injury, but are also required for proper healing and may attenuate chronic remodeling after myocardial infarction. Regulatory T-cells (Tregs) have been demonstrated to prevent cardiac hypertrophy and diminish infarct size clinically and nonclinically, respectively. IL-17, produced by γδ T-cells and Th17 cells, is increased in hypertension and Tregs have been shown to suppress Th17 differentiation (Frieler and Mortensen 2015; Hofmann and Frantz 2015). B cells can have detrimental effects in that they impair healing and potentially contribute to autoantibody formation in chronic ischemic cardiomyopathy, although they may play a critical role in the homing of splenic macrophages to infarct zones (Hofmann and Frantz 2015).

The purpose of a cardiac inflammatory response is to resolve the trigger for it, thereby allowing the heart to adapt to injurious conditions acutely, and ultimately restore homeostasis and cardiovascular function, despite the inability to fully replace or repair damaged myocardium. If substandard conditions are sustained, chronic inflammation endures and furthers disease progression. Lasting expression of proinflammatory cytokines in the failing heart signify malfunction of the myocardium to restore homeostasis, leading to a state of ongoing inflammation that is intermediate between the baseline state and acute inflammation. This intermediate state has been termed “parainflammation” and represents an active prolonged inflammatory response in the dysfunctional state that continuously attempts repair. Additionally, neurohormonal system activation in heart failure through the RAAS and the adrenergic nervous system can elicit inflammation in the heart resulting in parainflammation. Parainflammation can persist without apparent tissue injury or infection. Down-regulating parainflammation in the failing heart to prevent collateral damage may disrupt homeostatic responses at critical timepoints. Because parainflammatory responses range from physiological minimal levels to robust inflammation depending on the disease or insult, it is critical to know when, on this spectrum, it is appropriate to target inflammation in the failing heart (Mann 2015).

The understanding of atherogenesis has developed as a result of a combined approach in which patient data are used to formulate hypotheses that are investi-

gated in genetically modified models, typically mice. These animal models show that vascular beds in various tissues have different endothelial phenotypes and regulation pathways. The local endothelium integrates innate immune system signals from the blood, blood vessel walls, and surrounding parenchymal tissue in a site-specific manner and congruently alters the properties of its luminal surface. The local hemostatic potential within these individual vessel segments varies according to resident conditions, as dictated by endothelial surface expression of anti-inflammatory/antithrombotic and proinflammatory/prothrombotic factors. Vascular hemostasis is regulated by organ-specific endothelial pathways that include tissue-type plasminogen activator, with the addition of thrombomodulin in the heart and brain vasculature. Shifts in such pathways can contribute to age-related increases in vascular pathophysiology (Hallenbeck et al. 2006).

Some transcription factors exhibit inflammatory properties and can modulate the initial cascade of transcriptional activation in response to inflammatory stimuli. The prototypic transcription factor for mediating these responses is NF-κB. In addition to NF-κB, the purported "immediate-early genes", including c-jun and c-fos, contribute to initial inflammatory responses. Activation of PPAR-α and PPAR-γ is associated with favorable effects in lipid metabolism, insulin sensitivity, inflammation, and relatedly, limiting the development of atherosclerosis. Mutations in the gene for the transcription factor MEF2A have been linked to a subset of patients with premature coronary heart disease (Hallenbeck et al. 2006).

The amount of circulating anti-HLA (human leukocyte antigen) antibodies and their complement-binding capacity are strongly correlated with arteriosclerosis severity. Antibody-associated severe arteriosclerosis has a distinct phenotype that is characterized by endothelial activation, endarteritis, and local complement deposition. Circulating anti-HLA antibodies are major determinants, independent of traditional cardiovascular risk factors, and exert long-term detrimental effects that are characterized by an increased risk for major cardiovascular events. It is therefore prudent to identify patients with circulating anti-HLA antibodies to screen them for cardiovascular diseases and aggressively treat traditional risk factors (Loupy et al. 2015).

Vascular disease of the heart is characterized by neurovascular remodeling, an adaptive response to chronic (hypertension) or acute (ischemic) insults. Neurovascular remodeling is established by alterations to the anatomic organization of vessels, characterized by inward hypertrophic remodeling, intimal hyperplasia, formation of new vessels (angiogenesis), and functional changes (Fig. 9.2). The arterial media is often spared from the consequences of inflammatory processes affecting the vessel wall. In the absence of medial immunoprivilege, coronary atherosclerosis and other vasculopathies would likely result in earlier complications and enhanced disease progression. Pharmacological enhancement of these protective responses may pose a novel therapeutic strategy in advanced arteriosclerosis or other types of arteritis in which medial immunoprivilege is lost (Tellides and Pober 2015).

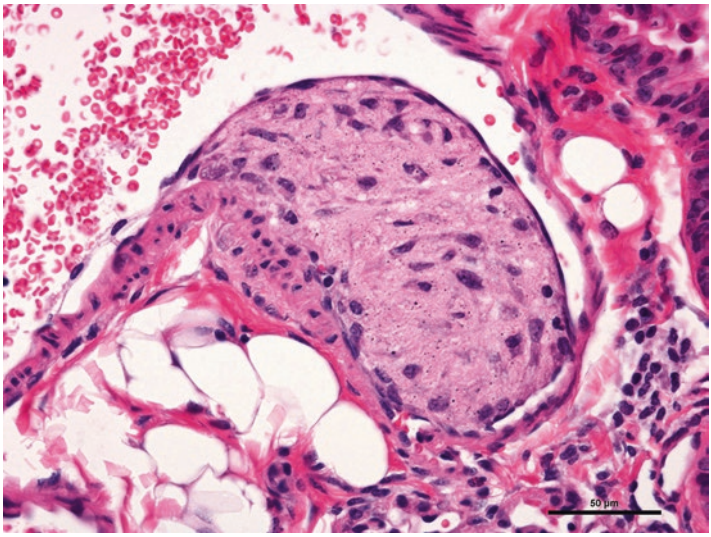

Fig. 9.2 Arterial layers and the immune response. Using an atherosclerotic plaque as a classic example of vascular inflammation, this photomicrograph illustrates some of the involved structures. The intima is the site of the plaque, composed of lipid droplets, macrophage foam cells, T lymphocytes, and depending on age of the lesion, a necrotic core with an overlying fibrotic cap. The intima has a microvasculature (not visible through traditional light microscopy) that serves as the portal for entry and exit of leukocytes involved in the immune response. The internal elastic lamina separates the intima from the media and may undergo degeneration and fragmentation in areas subjacent to the intimal plaque. The tunica media is mostly composed of extracellular matrix and smooth muscle cells, the latter containing indoleamine dioxyenase which contributes to the media's immunoprivileged state. The media often becomes attenuated in the presence of an atherosclerotic plaque and will grow outward in a process called geometic remodeling. The external elastic lamina separates the media from the adventitia. The adventitia serves as a site for much immunologic activity. There are mast cells, T cells, B cells, and plasma cells within this layer and nerve endings secrete mediators that regulate some vascular function. The vasa vasorum, located within the adventitia, intitiate plaque vasculature. There are also lympatic vessels that allow for travel of immune cells to regional lymph nodes and tertiary lymphoid structures in the adventitia. Dendritic cells circuit the adventitia and plaque, appraising antigens and presenting them to T lymphocytes [Adapted from (Libby and Hansson 2015)]

9.1.1 Cholesterol Metabolism and the Immune System

Cellular and molecular mechanisms of atherogenesis are based on the conventional lipid theory of atherosclerosis, which infers a key role of cholesterol in the initiation of atherosclerotic lesions. Contribution of shear stress and other rheologic factors are critical to the predilection of certain sites of cholesterol and LDL-mediated endothelial and vascular injury (Shalhoub et al. 2011; Ward et al. 2009). The source of accumulating cholesterol is LDL-C. Native (intact) LDL-C does not cause cholesterol accumulation in arterial cells, but chemically modified LDL-C is atherogenic, through pathways resulting in intracellular cholesterol accumulation.

Modified LDL-C possesses antigenic properties which induce the production of autoantibodies, leading to the formation of LDL-C-containing circulating immune complexes (ICs). Large LDL-C-containing aggregates result, the metabolism of which at the cellular level is different from the classical receptor-dependent pathway. Internalization of such particles is predominantly via uncontrolled phagocytosis, leading to massive intracellular accumulation of cholesterol, mainly in the form of lipid droplets. Such cholesterol-laden cells represent foam cells typical of atherosclerotic lesions (Patel et al. 2015).

Cholesterol metabolism may have an impact on the immune system by increasing macrophage chemotaxis and predisposing the microvasculature to enhanced leukocyte-endothelial adhesion in response to inflammatory stimuli. Additionally, increased plasma lipoprotein levels reduce systemic cytokine responses. Weakened antibacterial immune responses have been observed in hypercholesterolemic ApoE-deficient mice. Similarly, hypercholesterolemic mice lacking the LDL receptor are highly susceptible to disseminated *Candida albicans* infection. Furthermore, ApoE- and LDLR-deficient mice demonstrate imparied antiviral cellular immune responses leading to delayed viral clearance. These findings indicate that hypercholesterolemia decreases general immune responsiveness (Laman and Ludewig 2007).

The detrimental consequences of chronic T-cell mediated inflammatory responses in the vascular wall have been demonstrated in transgenic mouse models of inducible cardiovascular immunopathology. In SM-LacZ mice, the microbial β-galactosidase antigen is expressed exclusively in cardiomyocytes of the right heart and in arterial smooth muscle cells. The transgene functions as a self-antigen mimicking a bacterial antigen that resides in the cardiovascular system. T-cells ignore the peripherally expressed antigen until the antigen is efficiently presented in secondary lymphoid organs. Immunization with dendritic cells presenting α-galactosidase peptide eventually elicits arteritis and myocarditis. When SM-LacZ mice are crossed onto the hypercholesterolemic ApoE$^{-/-}$ background, hypercholesterolemia enhances and perpetuates T-cell mediated arterial inflammation, and arterial inflammation significantly increases the susceptibility of the arterial wall to cholesterol-induced atherosclerosis. Similar examples of vascular immunopathology and hypercholesterolemia have been observed in other experimental systems. For example, the number of IFN-γ producing T-helper 1 cells infiltrating atherosclerotic lesions decreases under severe hypercholesterolemic conditions and the subsequent T-helper 1 to T-helper 2 switch results in the formation of IgG autoantibodies directed against oxidized LDL (Laman and Ludewig 2007).

Mice with impaired IFN-γ responses are more susceptible to infection with murine cytomegalovirus (CMV) or γ-herpesvirus 68, and develop progressive chronic arterial inflammation. Also, *Chlamydia pneumoniae* preferentially infects predamaged atheromatous areas of the aorta in severely hypercholesterolemic ApoE- or LDLR-deficient mice, but only very rarely affects healthy aortas of normocholesterolemic C57BL/6 mice or aortas of hypercholesteromic mice before the development of cholesterol-induced lesions. *C. pneumoniae* infection also aggravates diet-induced atherosclerosis in normal C57BL/6 mice. Altered immune responses favor virus-induced vascular immunopathology and similarly, in viremic

states, there is anatomic predilection for impaired arteries. It is therefore likely that the association of *C. pneumoniae* and human CMV infection with atherosclerotic disease is due, at least in part, to altered immune states and resulting compromised pathogen control. In humans, herpesvirus infection can alter cholesterol metabolism. Thus, self-maintaining immunopathological disease states may develop when chronic hypercholesterolemia-mediated immunosuppression impairs the usually well-balanced host-pathogen equilibrium (Laman and Ludewig 2007).

The TLR4 antagonist lipopolysaccharide from the photosynthetic bacterium *Rhodobacter sphaeroides* (LPS-RS) has been shown to reduce atherogenesis in diabetic ApoE$^{-/-}$ mice; and, treatment with an anti-TLR4 antibody leads to lower blood pressure in spontaneously hypertensive rats. A protective function of TLRs 7 and 9 during atherogenesis has also been shown (Zimmer et al. 2015). Heat shock proteins (HSPs) have been proposed to play a role in the pathogenesis of atherosclerosis. Human HSP65 was the first candidate HSP-related antigen for a pro-atherosclerotic immune response. The link between the immune response to HSPs and the role of infectious agents in atherosclerosis was realized by the association between IgG antibodies to chlamydial HSP60. In addition to eliciting a humoral response, HSP60- and HSP65-derived peptides are recognized by T cells from human atherosclerotic lesions, a role that has also been proposed for peptides derived from modified LDL-C. The presence of T regulatory cells, dendritic cells, and a variety of pro- and anti-inflammatory cytokines in the plaque suggests that a complex regulatory network exists therein. Dendritic cells located in atherosclerotic regions likely play a significant pro-inflammatory role, as a consequence of direct activation by oxidized low-density lipoprotein (oxLDL) and HSP60 (human and chlamydial) primarily through the TLRs 2 and 4 (Virella 2010).

Fcγ receptors (FcγRs) have varying roles in ECs, VSMCs, and monocytes/macrophages, and classically modulate intracellular signaling on binding of the Fc region of IgG in immune response cells. FcγRI, IIA, and IIIA activation cause a variety of cellular responses, including increased reactive oxygen species and apoptosis, decreased nitrous oxide (NO) production and vasodilation, and increased tissue factor expression that may contribute to vascular disease pathogenesis. Isolated stimulation of the lone inhibitory FγcR, FcγRIIB, has adverse consequences in endothelial cells by antagonizing NO production and reparative mechanisms. In preclinical disease models, activating FcγRs promote atherosclerosis and enhance thrombotic vascular occlusion, whereas FcγRIIB is protective, by decreasing atherosclerotic burden and peripheral insulin resistance. The FcγR ligand C-reactive protein (CRP) has undergone intense study. Although in rodents CRP does not affect atherosclerosis, it causes hypertension, insulin resistance, and worsens myocardial infarction. While an association between increases in circulating CRP and coronary heart disease in humans has been realized, Mendelian randomization studies have suggested that CRP is not likely a disease mediator (Tanigaki et al. 2015).

Genetic variants at the SORT1 gene locus are associated with plasma LDL-C levels and subsequent myocardial infarction and coronary artery disease. SORT1 encodes the protein sortilin, which can mediate the uptake and degradation of LDL-C in hepatocytes. Deletion of sortilin in macrophages reduces foam cell formation

and atherosclerosis without influencing plasma LDL-C levels. Macrophage sortilin is a receptor-mediated pathway promoting uptake of native LDL by macrophages, and advancing foam cell formation and atherosclerosis. SORT1 deficiency in macrophages reduces LDL-C uptake and macrophage cholesterol loading, independent of the LDLR or macropinocytosis, and subsequently protects against the development of atherosclerosis. The macrophage sortilin pathway is therefore a pathway of macrophage cholesterol loading that quantitatively contributes to atherosclerosis (Patel et al. 2015).

9.1.2 Interplay Between Cardiovascular Pathology and Immune Dysfunction

There are mechanistic connections between cardiovascular events and rheumatic diseases. Studies in the cardiovascular arena and resultant therapeutic options have inspired rheumatic disease investigations, and similarly, rheumatic disease data have informed on cardiovascular research. For example, the role of matrix metalloproteinases (MMPs) in connective tissue breakdown in atherosclerotic plaques and in the remodeling of the left ventricle after myocardial infarction has similarities with joint destruction in rheumatoid arthritis (Libby et al. 2002). MMPs have also been associated with vascular remodeling and alteration severe enough to potentiate aortic aneurysm formation. MMP2 and MMP9 have been implicated in the degradation of extracellular matrix proteins that occurs during the development of aneurysms (Sahota 2013).

Phospholipid antibodies (PLAs) play a pathogenic role in accelerated atherosclerosis that afflicts patients with systemic lupus erythematosus (SLE). Most PLAs that exist in patients with SLE and phospholipid antibody syndrome react with beta-2-glycoprotein 1 (β2GP1) and are predominantly of the IgG isotype. The atherogenic mechanism of these antibodies involves association of β2GP1 with oxLDL, followed by the formation of pro-inflammatory IgG–**β**2GP1–oxLDL complexes (Virella 2010).

Infectious and non-infectious myocardial insults may induce chronic inflammatory myocarditis with an autoimmune component directed to antigens such as myosin and troponin. This postulate is grounded in animal model experiments with coxsackie B3 virus, murine cytomegalovirus, and others. Autoantibodies to myosin result, and can be reproduced by immunization with myosin or myosin-derived peptides. Cardiac troponin I is also involved in autoimmune reactions of patients with myocardial damage. Myocarditis can be induced by passive transfer of troponin I specific T cells; and troponin I autoantibodies are detectable in 7–9% of human patients with dilated cardiomyopathy or ischemic cardiomyopathy (Virella 2010). Evaluation of an antibody response to disease-related self-determinants may help to assess both the susceptibility to, and the progression rate of, autoimmune cardiac damage.

Viral infection triggers an inflammatory process by expression of cytokines, chemokines, and abundant infiltration of T cells and macrophages. This inflammation outlasts the initial replicative phase and may lead to post-viral autoimmunity. Chronic inflammation then leads to tissue injury, endothelial dysfunction, and cardiac remodeling, resulting in deterioration of myocardial contractility and left ventricular ejection fraction. Disarrangement of the immune response or molecular mimicry often triggers exposition of autoantigens, followed by a chronic autoimmune disorder called inflammatory cardiomyopathy (Skurk and Schultheiss 2015).

In the absence of a virus and presence of symptomatic heart failure, inflammatory cardiomyopathy is currently treated with immunosuppressive therapy. A potential therapeutic option for virus-negative autoimmune cardiomyopathy has been reported using an approach originally employed in stem cell therapy: injecting the secretome (i.e., secreted proteins) of mononuclear cells into mice with experimental autoimmune myocarditis (EAM). In a T cell-dependent model of autoimmune myocarditis, cardiac inflammation was limited as a result of $CD11^+$ monocytes suppressing the $CD4^+$-dependent autoimmune response. Supporting evidence that secretome injections may prove beneficial is suggested by a similar cytokine profile of mononuclear preparations in human subjects (Skurk and Schultheiss 2015).

9.1.2.1 Chagas Disease Cardiomyopathy: Immunopathology and Genetics

Trypanosoma cruzi, the etiological agent of Chagas disease (CD), initiates the polyclonal activation of B cells and production of antibodies. These antibodies have multiple targets including heart muscle, DNA, and collagen. Antibodies directed toward cardiomyocytes recognize the C-terminus of the trypanosome ribosomal P0 protein, the second extracellular loop of the human adrenergic receptors (β1 and β2), and the M2 muscarinic receptor. Sequence comparisons between the β1 receptor and the ribosomal protein show a five-amino-acid sequence homology (AESEE in the ribosomal protein and AESDS in the receptor). Interactions between antibodies and cardiomyocyte adrenergic receptors modulate CAMP-activated calcium channels (Flaherty 1999).

Chronic Chagas disease cardiomyopathy (CCC) is characterized by a Th1 T cell-rich myocarditis, cardiomyocyte hypertrophy, and prominent fibrosis. CCC presents an excellent example of the complex processes of hypertrophy and fibrosis involving all cell types present in the heart and interactions with circulating cells. There are familial trends in CCC cases and a 30% incidence of CCC in Chagas Disease. Trends of association have been detected for markers located around the SLCO1B1 gene. SLCO1B1 is a membrane transporter that belongs to a solute carrier family and plays a role in drug metabolism. It is expressed in the liver, brain, heart, and kidney and transports organic anions, such as digoxin, bilirubin, methotrexate, and statins. In addition, loss-of-function mutations may be associated with impaired drug action in target tissues. A cluster of 12 SNPs within introns of Collagen type XIV alpha 1 (COL14A1) is associated with PCR positivity.

COL14A1 is a fibril-associated collagen which interacts with the fibril surface and regulates fibrillogenesis. Furthermore, HSPB8 is a small heat shock protein whose heart-specific overexpression induces myocardial hypertrophy. HSPB8-transgenic mice bearing the K141N mutation express myocardial hypertrophy, ventricular dysfunction, and apical fibrosis. Expression of HSPB8 is selectively increased in myocardial tissue from CCC patients compared to idiopathic dilated cardiomyopathy patients. No polymorphisms in immune-related genes are known (Cunha-Neto and Chevillard 2014).

9.1.2.2 Cardiac Pathology Associated with *Streptococcus* spp. Infections

Group A β hemolytic streptococci are a classic example of molecular mimicry in that they very basically have antigens that are similar to those found on cardiac muscle. During a streptococcal infection, antibodies directed at the bacteria also react with cardiac tissue, thereby causing an autoimmune reaction which can cause heart valve pathology associated with rheumatic fever (Flaherty 1999). The cross-reactive autoantibodies target the dominant group A streptococcal epitope of the group A carbohydrate, N-acetyl-beta-D-glucosamine (GlcNAc), and heart valve endothelium, laminin, and laminar basement membrane. Disease is initiated by a two-hit hypothesis typified by antibody attack on the valve endothelium, followed by extravasation of T cells cross-reactive with streptococcal M protein and cardiac myosin. Cardiac myosin epitopes target the S2 region of cardiac myosin and are similar among populations with rheumatic carditis worldwide, irrespective of the offending group A streptococcal M serotype. Immune responses to collagen I also take place due to likely release of it from the damaged valve. Neovascularization potentiates further injury from antibodies to the once immunoprivileged valve (Cunningham 2012).

Histopathologically, the lesion of rheumatic fever is characterized by mononuclear to granulomatous inflammation, Aschoff bodies, edema, and fibrinous vegetations, depending on chronicity. T cell infiltration, predominantly $CD4^+$ cells, is the result of vascular cell adhesion molecule-1 (VCAM-1) on the valvular endothelium, while granulomatous inflammation is due to interferon gamma (IFNγ). As the lesion progresses, autoantibodies against the group A streptococcal carbohydrate epitope GlcNAc, cardiac myosin, and its peptides accumulate. Interestingly, these same pathogenic mechanisms of cross-reactive autoantibodies targeting the valve in classic rheumatic heart disease, also target neurons in Sydenham chorea. The streptococcal epitope GlcNAc is shared in both these clinical conditions associated with *Streptococcus* spp. (Cunningham 2012).

The use of animal models has led to a better understanding of the human disease through defining mimicry in heart disease and elucidating pathogenic epitopes of the autoantigens and microbial antigens involved. An animal model with similar physiologic and morphologic valvular disease was established in Lewis rats immunized with streptococcal M6 protein (Cunningham 2012).

9.1.3 Therapeutics

Tables 9.1 and 9.2 summarize much of the data presented in this section.

9.1.3.1 Monoclonal Antibodies and Potential Immunotoxicity

Monoclonal antibodies (mAbs) can have potential for immunotoxicity via mechanisms related to immunosuppression, immunostimulation, and immunogenicity. Cytokine release syndrome may occur with some T cell-engaging therapies, and can be dose- or treatment-limiting. Cytokine release syndrome is characterized by

Table 9.1 Marketed therapies with immune and cardiovascular implications

Class	Indication(s)	Potential Toxicity(ies)
Monoclonal antibodies	Allergy, autoimmune, cancer, cardiovascular, hematologic, infectious disease, neurologic, ophthalmologic, orthopedic	Cytokine release syndrome; Off target toxicities; ↑LDL-C
Beta antagonists	Angina, arrhythmias, bleeding esophageal varices, coronary artery disease, asymptomatic and symptomatic heart failure, hypertension, migraine, secondary prevention post-myocardial infarction	General ↓immune response, Lupus syndromes
Calcium antagonists	Angina, paroxysmal supraventricular tachycardias, hypertrophic cardiomyopathy, Raynaud phenomenon, pulmonary hypertension, esophageal spasms, migraine	General ↓immune response
Vasodilator (hydralazine)	Hypertension	Lupus syndromes
Diuretics	Hypertension, glaucoma, edema	Lupus syndromes
Angiotensin-converting enzyme inhibitor (captopril)	Hypertension	Pemphigus
Methyldopa	Hypertension	Autoimmune-mediated hemolytic anemia
Statins	Hypercholesterolemia	T cell deviation
AN1792, beta-amyloid immunotherapeutic agent	Alzheimer's disease	Cerebral inflammation with microhemorrhage
COX-2 inhibitors	Inflammation	Thrombosis
Biphosphonates	Osteoporosis	Atrial fibrillation
TNFα inhibitors	Autoimmune/immune-mediated inflammatory diseases	Cardiomyopathy, heart failure
Protease inhibitors (e.g. ritonavir, indinavir, amprenavir)	Antiviral	Atherosclerosis

Table 9.2 Marketed immune therapies with potential cardiovascular treatment indications

Drug, action	Immunologic indication(s)	Potential cardiovascular indication(s)
Canakinumab, human monoclonal antibody that inhibits IL-1β	IL-1β–driven rare hereditary illnesses (e.g. cryopyrin-associated autoinflammatory syndromes); Drug-resistant acute gouty arthritis	↓Recurrent vascular events (CANTOS)
Anakinra, IL-1RA agonist	Rheumatoid arthritis	Left ventricular function improvement; ↓ IL-6 and CRP levels; beneficial plaque remodeling
Colchicine, NLRP3 inflammasome suppressor	Gouty arthritis	↓Cardiovascular events
Methotrexate, antimetabolite	Cancer; psoriasis, rheumatoid arthritis	↓IL-6 and TNF-α, ↓atherosclerosis

hyperpyrexia (>40 °C) associated with cardiovascular disturbances, including a drop in blood pressure, or even cardiovascular collapse potentially leading to cardiac ischemia, and various neurological disorders (Descotes 2009). Electrocardiogram assessment can be timed to coincide with cytokine release sampling to assess whether any observed increased cytokine levels correlate with cardiovascular effects (Brennan et al. 2010).

Toxicity can result from binding to target antigen in tissues other than those necessary for therapeutic effect. For example, cardiotoxicity observed with trastuzumab (anti-HER2; Herceptin) has been attributed to the expression of the targeted antigens in cardiac muscle. If the biology and tissue distribution of the target are well-defined, potential target organs of toxicity can often be predicted. Therefore, the choice of IgG isotype (1, 2 or 4) and the design of the Fc portion of the antibody to minimize or enhance Fc-mediated antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) activity impacts the toxicity to target and non-target tissues (Brennan et al. 2010).

Tocilizumab (anti–IL-6R antibody) as well as infliximab and adalimumab (both anti–TNF-α antibodies) are used in patients with arthritis. However, their major disadvantage is an adverse increase in LDL-C, which could have harmful effects in patients with preexisting coronary artery disease (Zimmer et al. 2015).

9.1.3.2 Marketed Cardiovascular Therapies and Potential Immunotoxicity

Beta-adrenergic inhibitors (β-blockers) and calcium antagonists (verapamil, nifedipine, and dilthiazem) have been inconsistently shown to decrease immune responses. Autoimmune reactions are the most common immunotoxic adverse effects of heart disease treatments. Specifically, lupus syndromes have been

described in association with hydralazine, diuretics, and β-blockers, with acebutolol and practolol as the most commonly incriminated derivatives. Other autoimmune reactions include pemphigus associated with captopril; and autoimmune hemolytic anemia related to methyldopa treatment (Laman and Ludewig 2007). Small molecules such as statins can induce immune system deviation of T cells, such that their cytokine profiles shift from proinflammatory to immunomodulatory (Hallenbeck et al. 2006).

9.1.3.3 Immunomodulatory Therapies with Cardiovascular Implications

Efforts are underway to move immunomodulatory therapies into clinical trials for atherosclerosis and vascular disease (Hallenbeck et al. 2006).

Canakinumab, a human monoclonal antibody that inhibits IL-1β, is approved for the treatment of rare IL-1β–driven hereditary illnesses, such as cryopyrin-associated autoinflammatory syndromes, and for the treatment of drug-resistant acute gouty arthritis. The Canakinumab Anti-inflammatory Thrombosis Outcomes Study (CANTOS) examines if IL-1β antagonism can reduce recurrent vascular events. Anakinra, an IL-1RA agonist, blocks the biological activity of naturally occurring IL-1 and is approved for the treatment of rheumatoid arthritis. Anakinra has been shown to improve left ventricular function in patients with rheumatoid arthritis and to decrease IL-6 and CRP levels in patients with type 2 diabetes mellitus. Interestingly, analysis of IL-1 receptor type I-deficient ApoE$^{-/-}$ mice with total functional inactivation of IL-1 signaling exhibit multiple features of increased plaque instability through inhibition of outward vessel remodeling. IL-1 signaling may partake in a process that facilitates seemingly advantageous plaque remodeling (Zimmer et al. 2015).

Drugs that target the NLRP3 inflammasome have been evaluated in clinical trials. Colchicine is generally effective for the treatment of gout, and colchicine decreases cardiovascular events in patients with stable coronary artery disease. Methotrexate not only inhibits proinflammatory cytokines of the IL-1 family, but also reduces other proinflammatory cytokines, including TNF-α and IL-6. Methotrexate has been shown to reduce atherogenesis in cholesterol-fed rabbits. Direct inhibition of IL-6 and TNF-α therefore has therapeutic potential for the treatment of atherosclerosis (Hallenbeck et al. 2006; Mason and Libby 2015).

AN1792, a beta-amyloid immunotherapeutic agent, did not produce adverse pathology nonclinically. However, in the Phase IIa study, about 6% of the volunteers demonstrated adverse inflammatory episodes accompanied by microhemorrhage in the brain. Transgenic mice had a similar lesion consisting of cerebral microhemorrhages that were Prussian Blue positive. Cerebrovascular lesions were thought to have developed as a result of one or a combination of vascular NO dysfunction, MMP extracellular matrix degradation, modified ApoE lipid metabolism, and/or decreased telomerase activity with mitochondrial membrane potential damage (Sahota 2013).

Small vessel pulmonary vasculitis has been observed in mice with administration of high doses of a human recombinant IL-2. In rats, the lesions consists of infiltration of lymphocytes and eosinophils in the interstitium and vessel (venular, arteriolar) walls. Perivascular eosinophils have been previously reported with administration of the immunosuppressive macrolide FK-506, tacrolimus. It has been hypothesized that lymphocytes release an eosinopoietic cytokine with IL-2 stimulation (Sahota 2013).

9.1.3.4 Immune-Based Cardiotoxicity of Noncardioactive Agents

There are some pharmacologic agents that are not designed to treat cardiovascular disease, but may be associated with cardiotoxicity through immune activation. Selective cyclooxygenase-2 (COX2)-induced cardiotoxicity is an interesting example. During inflammation and angiogenesis, COX2 expression is promoted by growth factors and cytokines in small vessel endothelium. Metabolism of endocannabinoids by endothelial COX2 coupled to prostacyclin synthase activates PPARδ. EC activation downregulates expression of tissue factor, the principal coagulation cascade initiator. PPARδ activity is therefore diminished by selective COX2 inhibitors, which results in regulation loss of tissue factor expression in ECs, elevated circulating levels of tissue factor, and heightened susceptibility to thrombosis. A thromboxane imbalance caused by prostacyclin reduction further potentiates platelet aggregation and thrombosis (Sahota 2013).

Atrial fibrillation has been observed with administration of bisphosphonates and is thought to result from an increase in inflammatory cytokines and dysregulation of calcium. Additionally, cardiomyopathy and heart failure has been associated with TNFα inhibitors due to coagulation cascade activation. Finally, atherosclerosis is a cardiotoxic effect observed with administration of protease inhibitors (e.g., ritonavir, indinavir, amprenavir), subsequent to upregulation of the cholesterol uptake receptor CD36 on macrophages, impaired endothelial relaxation, and increased carotid intima-media thickness (Sahota 2013).

9.1.4 Nonclinical Species Background Cardiovascular Pathology with Immune-Mediated Associations and Routine Animal Models

9.1.4.1 Spontaneous Polyarteritis and Other Vascular Lesions

Polyarteritis nodosa (PAN) (polyangitis, panarteritis)-like diseases have been described in several species including primates, dogs, cats, pigs and rodents. Although the pathogenesis of PAN is not well understood, it is generally thought to involve an immune-mediated disease process. IC deposition with complement activation may play a role in initiating the disease, while cell-mediated immune

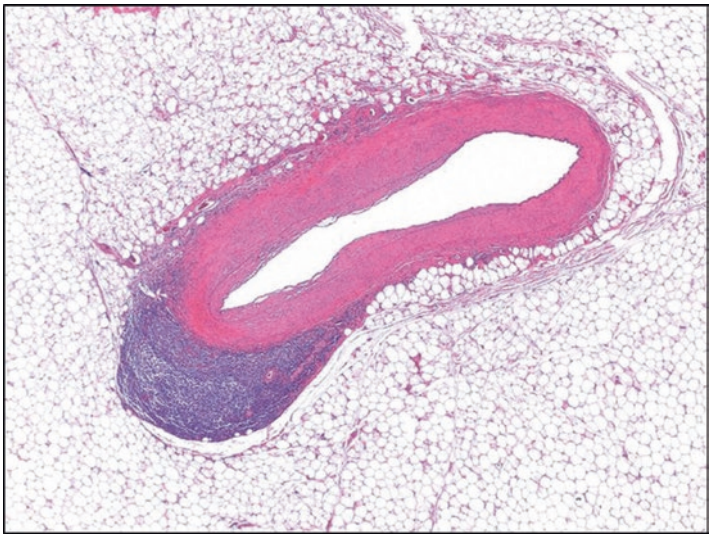

Fig. 9.3 Canine idiopathic extramural coronary arteritis. Photo courtesy of Klaus Weber

interactions are likely to contribute to the progression of lesions. While clinical signs are not always apparent, the acute febrile phase in dogs is characterized by elevation of acute phase proteins, especially IL-6. It's important to differentiate the spontaneous disease from drug-induced vascular injury (DIVI), but realize that spontaneous polyarteritis may be potentiated by immunomodulating drugs (Sahota 2013).

Polyarteritis in humans is multisystemic and characterized by necrotizing lesions in small and medium-sized arteries. In monkeys, spontaneous polyarteritis may occur systemically (kidney, lung, meninges, sciatic vessels) or be localized to the coronary arteries. Histopathologically, left and right ventricular vessel walls contain focal hemorrhage, cellular debris, smooth muscle cell degeneration/regeneration, and/or mononuclear cell infiltrates (Sahota 2013).

In rats, PAN is a well-described entity and the incidence varies among different strains. Macroscopically, nodular lesions affecting predominantly the pancreatic, mesenteric, and/or spermatic arteries may be observed. Depending on the chronicity, microscopic features include fibrinoid necrosis of muscular arteries, mixed inflammatory cell infiltrate of vessels and surrounding tissue, and fibrosis. Spontaneous arterial diseases described in dogs include idiopathic necrotizing polyarteritis in beagle dogs (beagle pain syndrome) and idiopathic extramural coronary arteritis (Figs. 9.3 and 9.4), which may be a component of beagle pain syndrome. Drug administration can be associated with both non-necrotizing and necrotizing inflammatory vascular lesions, which may be induced by hypersensitivity reactions. Examples of drug-related vascular injury include mesenteric vasculitis in rats

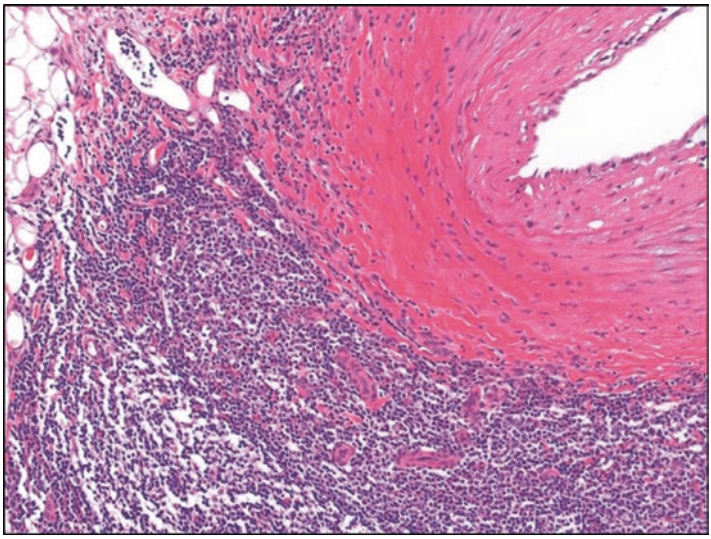

Fig. 9.4 Canine idiopathic extramural coronary arteritis. Photo courtesy of Klaus Weber

treated with phosphodiesterase III inhibitors, and coronary and systemic arteritis associated with endothelin A receptor antagonists in dogs and monkeys (Porter et al. 2003; Albassam et al. 1999; Percy 2007; Schoen and Cotran 1999).

Spontaneous lesions resembling atherosclerosis histopathologically have been reported in monkeys. Irregular aortic, coronary artery, and general large vessel intimal thickening occurs in pigs (Sahota 2013).

9.1.4.2 Spontaneous Murine Lupus-Like Syndromes

New Zealand mice, particularly the (NZB X NZW)F_1 and NZB X W mice, have been historically used as an experimental model of human SLE due to development of spontaneous lupus-like syndromes. Other strains which also develop a more acute SLE-like disease include MRL/1, MRL/n, and BXSB. Retroviruses, thymic atrophy or failure, anti-thymocyte antibodies, immunologic hyperreactivity, suppressor T cell deficiency, abnormal T cell cytotoxicity, and phagocytic cell aberrations have all been postulated. Genetic analysis has demonstrated that multiple genes are involved. Moreover, the SLE-like syndromes in all strains are clinically and immunopathologically similar, and the variations expressed between the strains are no different than those found in an unselected series of humans with SLE. All affected mice share B cell hyperreactivity, autoantibodies, circulating ICs, abnormalities of Ig and complement, thymic cortical atrophy, and IC-type glomerulonephritis with retroviral glycoprotein subunit, gp70, glomerular deposits. Differences

among the strains relate to the amounts and specificities of autoantibodies, age of onset, rapidity of disease progression, sex differences, presence of arteritis, and extent and nature of lymphoid hyperplasia (Andrews et al. 1978).

NZB/W mice are a well-characterized autoimmune strain of mice that develop pulmonary vasculitis with plexiform morphology in an age-related fashion. Mild perivascular and peribronchiolar lymphoid hyperplasia first seen around 4 months of age progresses into severe hyperplasia by 8 months. This precedes the development of angio-destructive lesions of arteries and veins, first appearing at approximately 10 months. By 12 months of age, 100% of mice have multilobular transmural plasma cell, histiocyte, and mature lymphocyte infiltration of the vascular walls. The inflammatory cell infiltrate often extends into the interstitium. Vessel lumens may be narrowed and even obliterated, with focal disruption of the elastic membranes (Staszak and Harbeck 1985). Most lymphocytic cells within the lesions are T cells that express the Lyt-1 phenotype, whereas cells expressing Lyt-2 are rarely observed. Cells reacting with a mAb recognizing cells of the B-lineage and cells expressing Ia antigens have also been observed. Before the development of vasculitis, B and T cells are randomly distributed throughout lymphoid hyperplasia. In older animals with vasculitis, T cells expressing Lyt-1 are associated with vessel lumens and therefore primarily responsible for the vascular infiltration, to the apparent exclusion of other lymphoid cell types. B cells and Ia^+ cells may also be localized at the periphery of the lesions (Harbeck et al. 1986).

9.1.4.3 Spontaneous "Cardiomyopathy" in the Mouse

Age-related myocardial degeneration, inflammation, and fibrosis can be seen spontaneously in the mouse. This lesion often occurs in the ventricular myocardium, subjacent to the mitral valve insertion ring. It is mentioned here because it should be differentiated from viral myocarditis, particularly caused by Coxsackie virus or murine CMV. The viral lesion is characterized by lymphoplasmacytic inflammation that may be accompanied by necrosis (Sahota 2013).

9.1.5 Translational Challenges

Risk and exacerbation factors to arteriosclerosis include hypertension, tobacco use, and aging. However, immune and inflammatory processes play a role in arteriosclerosis and more generally in atherosclerosis, as supported by animal experimental data and clinical observations in autoimmune diseases. Animal models are limited by the fact that they do not recapitulate human pathophysiology, whereas human studies lack access to patient arteries for histopathologic analysis, and the human condition exhibits slower disease progression. As a result, there is an incomplete understanding of the respective contributions of nonimmune and immune factors in arteriosclerosis (Loupy et al. 2015).

Solid organ transplant offers a unique opportunity to address the general pathogenesis of arteriosclerosis by using kidney transplantation as a human prototype for the antibody-dependent immune response that coexists with traditional risk factors. Knowledge of mechanisms that provoke accelerated arteriosclerosis in solid organ allografts has illuminated the role of the adaptive immunity in usual atherosclerosis (Loupy et al. 2015).

For screening of anti-atherosclerotic substances, a cell-based model of a primary culture of human monocyte-derived macrophages has been used to analyze blood serum from patients after drug administration. The test system is designed for complex analysis of monocyte activity in individuals and to also diagnose immunopathology and monitor treatment efficacy (Orekhov et al. 2015). It is important to note the similarities and differences in clinical and nonclinical patient-derived macrophages. Classical or "inflammatory" human $CD16^-$ and paralogous mouse $Ly6C^+$ monocytes highly express the chemokine receptors CCR1 and CCR2 that bind MCP-1; however, PPARγ is highly expressed in $Ly6C^+$ mouse monocytes but not human $CD16^-$ monocytes (Frieler and Mortensen 2015; Zeller and Srivastava 2014).

In nonclinical species, most DIVI is caused by excessive hemodynamic activity resulting in functional damage manifesting as medial necrosis and hemorrhage. In humans, DIVI is more commonly immune-mediated. Because of these differences and translational challenges, the term 'vasculitis' may be appropriate in nonclinical species when acute arterial lesions are induced by vasoactive agents and inflammation is a predominant histopathologic feature (Sahota 2013).

Nonclinical DIVI may transpire as the result of IC deposition secondary to an immune response to therapeutic proteins, antibodies, or small molecules. It most often occurs in small cutaneous arteries and venules in humans, monkeys, and less commonly, dogs. Macroscopically, it sometimes manifests as a skin rash. In general, compounds causing IC-mediated DIVI have a predilection for vessels with slow blood flow (e.g., renal glomerulus, choroid plexus, epididymis, and/or the pampiniform plexus). Furthermore, three mechanisms have been implicated in nonclinical species IC-mediated vasculitis: (1) therapeutic antibody recognition of an epitope expressed by ECs in which anti-drug antibodies (ADA) bind to the therapeutic antibody immobilized on ECs, resulting in complement activation and subsequent vascular damage (2) therapeutic antibody binding to a soluble target, the formation of ICs by ADA, followed by complement activation (3) binding of aggregates of therapeutic antibody by ADA, activation of complement, and deposition of the ICs. In humans, vasculitis originating from the induction of antineutrophil cytoplasm antibody (ANCA) antibodies suggests direct activation of cytokine-primed neutrophils and monocytes by ANCAs, which results in degranulation and subsequent EC damage by both cell types (Mikaelian et al. 2014). Cytoplasmic-ANCA is usually induced by protease-3 and perinuclear-ANCA is associated with anti-MPO, elastase, lysozyme, and lactoferrin antibodies (Sahota 2013).

DIVI in humans usually reflects a hypersensitivity reaction characterized by a mixed inflammatory cell infiltrate within the walls of capillaries, arterioles, venules,

and veins. Necrosis is not a feature, and larger muscular arteries are generally spared. Hypersensitivity vasculitis in humans has been associated with numerous and diverse pharmacologic therapies including penicillins, sulfonamides, minocycline, allopurinol, thiazides, pyrazolones, retinoids, quinolones, hydantoins, propylthiouracil, hydralazine, colony-stimulating factor, and methotrexate. (Sahota 2013)

Validated in vitro cellular models of endothelial responses to relevant immunological and proinflammatory mediators should be comprehensively investigated by cellular biological, molecular genetic, biochemical, pharmacological, and immunological imaging (e.g., bioluminescence for T-cell trafficking, annexin V for apoptosis) approaches. Ideally, culture systems examine endothelial function and the interaction between endothelial cells and organ-specific parenchymal cells. Establishing good in vitro models to study a complexity of cellular interactions of the neurovascular unit is important, but translation will be difficult without good in vivo models assessed by multiple outcome measures, including imaging, neurological/behavioral, histological, and molecular endpoints as appropriate (Hallenbeck et al. 2006).

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Chapter 10
Immunopathology of the Male Reproductive Tract

Catherine A. Picut, Eveline P.C.T. de Rijk, and Darlene Dixon

Abstract This chapter reviews the immunophysiology and immunopathology of the male reproductive tract organs, and explains the strategies and operable mechanisms by which the male reproductive tract maintains immune privilege to protect germ cells. Immunologic mechanisms involving the testes and the excurrent duct system are similar because they both involve a consortium of physical and functional barriers to maintain immune privilege for the germ cells. In both, there are complex interactive relationships between the endocrine and the immune systems that provide for a strong innate, but a suppressed adaptive, immune response. In order to tease apart these complex interactions, the roles of each conventional immune cell (macrophages, dendritic cells, T cells, and NK cells) are first described, and their dual immune and endocrine functions are emphasized. The somatic Leydig cells and Sertoli cells also have dual immunologic and endocrinologic functions. The Sertoli cells and Leydig cells promote spermatogenesis on one hand, yet defend against immune activation and inflammation on the other. Understanding how the endocrine and immune systems interact will aid the pathologist in identifying or explaining the pathogenesis of histologic findings for either spontaneous or xenobiotic-related lesions.

Keywords Testes • Immunology • Macrophage • Cytokine • Spermatogenesis

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10.1 Introduction

The male reproductive tract of mammals has evolved into an organ system with an adept innate non-specific arm of the immune system, yet with deliberate suppression of the adaptive (antigen-specific) immune system. Suppression of the adaptive immune system protects germ cells expressing "foreign" antigens from being immunologically destroyed by the host. The balance in favor of innate immunity is necessary to meet the physiologically challenging demands for insuring successful spermatogenesis, yet maintain full protection of the testis against microbial invasion. The suppression of the adaptive immune system is achieved by different mechanisms: (1) production of immunosuppressive factors by Sertoli and Leydig cells; (2) maintenance of populations of immunosuppressed macrophages, tolerant dendritic cells, and tolerant regulatory T cells; and (3) implementation of several immunoregulatory systems (such as [1]Fas-FasL and the [2]Gas6/Pro-S-TAM system). To achieve the enhanced innate immunity, special features, such as the production of antiviral proteins and expression of Toll-like receptors by Sertoli cells and Leydig cells are in place.

The unique balance between innate and adaptive immunity is complex and difficult to tease apart. It involves a highly complex interactive relationship between the conventional adaptive immune cells (macrophages, dendritic cells, T cells, and NK cells) and somatic cells (Leydig cells and Sertoli cells), the latter normally regarded as having purely endocrine function. Contrary to popular understanding, these "endocrine" somatic cells are key to the immunologic protection of the germ cells. They actually perform much of their "endocrine" function of supporting spermatogenesis by activating mini-inflammatory reactions. Therefore, these somatic cells can be considered as having dual endocrine and immunologic functions. Recruiting these somatic cells to be engaged in immunologic activity, in an evolutionary sense, has been necessary to help achieve the unique and necessary homeostatic balance in the male reproductive tract. Not only do the conventional endocrine cells of the testis take on immunologic roles, but the reverse is true as well: conventional immune cells take on important endocrine roles. For example, testicular macrophages are critical for the development of Leydig cells.

Cross function between the endocrine and immune cells of the testis underscores the close interrelationship between the immune and reproductive systems in general. Toxicologic pathologists should be fully aware of this exceptionally close interrelationship since xenobiotic targeting of one cell type in the testis can have both endocrinologic and immunologic consequences. Furthermore, xenobiotics that broadly target one system (i.e., the immune system) can have broad sweeping effects on the endocrine system.

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[1] **Fas ligand** (**FasL** or CD95L) is a type-II transmembrane protein that belongs to the tumor necrosis factor (TNF) family. Its binding with its receptor induces apoptosis.

[2] Activation of **Toll-like receptors (TLRs)** triggers rapid inflammatory cytokine production in various cell types. The exogenous product of growth-arrest-specific gene 6 (**Gas6**) and Protein S (**ProS**) inhibit the TLR-triggered inflammatory responses through the activation of Tyro3, Axl and Mer (**TAM**) receptors.

Lastly, the complex immunophysiology of the testis is affected by both local and systemic endocrine regulation involving the central nervous system (CNS), hypothalamus, pituitary, thyroid gland, and adrenal gland.

This chapter is entitled immunopathology of the male reproductive tract even though a large portion of the chapter is devoted to normal immunophysiology. Under normal circumstances, the male reproductive tract has a redundancy of mechanisms in place to *avoid* immunopathology and immune activation. Immunopathology results when there is a breakdown of immune homeostasis, and spontaneous breakdown in the testis happens rarely in laboratory animals. Despite some differences between human and animal testicular anatomy and structures (e.g. intratesticular lymphatics), the immunophysiology is similar in both humans and rodent species. The rodent species therefore have provided appropriate models for pathology of the human testis.

The goal of this chapter is to give the toxicologic pathologist and toxicologist a substantive yet concise understanding of the complexities of immune homeostasis of the male reproductive tract. The chapter will give sufficient detail so that a toxicologic pathologist or toxicologist, when confronted with a test article causing microscopic evidence of injury to the male reproductive tract, could appreciate the ramifications of that injury on both the endocrine and immunologic systems. By the same token, if confronted with an immunologic toxicant, the pathologist or toxicologist will be able to predict and interpret the changes in the reproductive tract. In the interest of clarity, this chapter is not an exhaustive review of the literature. Sufficient references are provided so that readers interested in pursuing a particular topic may do so.

The chapter starts with a brief review of the histologic anatomy of the male reproductive tract, followed by the features of immunologic privilege and the role that each cell type plays in maintaining this status. Then the complex interactions between the endocrine and immunologic systems will be summarized, and the pathogenesis of inflammatory disease in the testis will be discussed. The last part of the chapter will include an overview of experimental models used in the field of immunobiological research as it relates to the male reproductive system, and of spontaneous diseases affecting the male reproductive organs in laboratory animals.

10.2 Histologic Anatomy

10.2.1 Overview

The male reproductive tract is composed of the testes, excurrent duct system and accessory sex glands. This first section will provide an overview of the cell types and the microscopic anatomy of the male reproductive tract tissues, but will concentrate on structures relevant to understanding the interface between the immune system and the male reproductive tract.

The testis is composed of convoluted loops of seminiferous tubules separated by interstitium containing Leydig cells, mononuclear inflammatory cells (predominantly macrophages), a testosterone-rich ultrafiltrate, blood and lymphatic vessels, and supporting loose fibrous connective tissue. The seminiferous epithelium lining the indi-

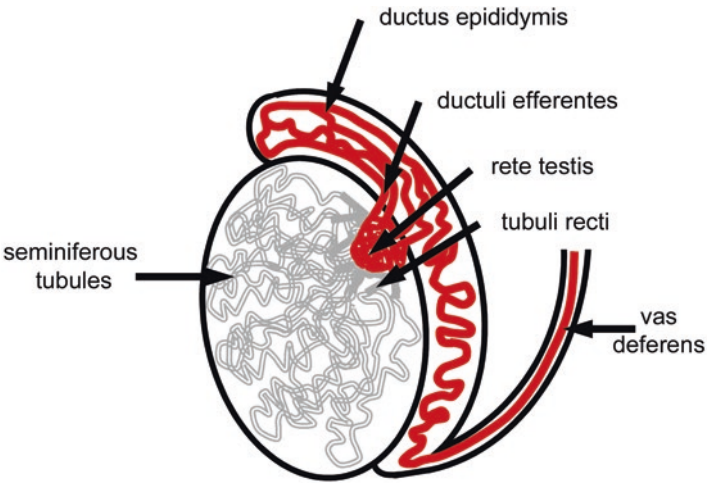

Fig. 10.1 Schematic of excurrent duct system. The excurrent duct system of the male reproductive tract begins at the end of the tubuli recti, and consists of rete testis, ductuli efferentes (efferent ducts), ductus epididymis (epididymal tubules), and vas deferens

vidual tubules is complex. It is composed of basally-located Sertoli cells supporting successive synchronized populations of maturing germ cells, namely spermatocytes, round spermatids and elongating spermatids. Both ends of the blind seminiferous tubules, ensheathed by contractile myoid cells, terminate into the tubuli recti. The tubuli recti (i.e., the distal ends of blind seminiferous tubules) are the first component of the excurrent duct system. The excurrent duct system consists of a continuum of channels and begins with the rete testis (within the testis), which transition into the efferent ductules (ductuli efferentes), epididymis (ductus epididymis), and finally the vas deferens (Figs. 10.1 and 10.2).

10.2.2 Testis

The testis is functionally and anatomically separated into an endocrine compartment (vascularized interstitial tissue) and the gametogenic compartment (avascular seminiferous tubules).

Vasculature. The vascular supply to the testis consists of a long, highly coiled spermatic artery that arises from the abdominal aorta. Non-fenestrated arterioles, capillaries and venules permeate the interstitial tissue of the testis. Even though fenestrations are absent, there is essentially no restriction on the exchange of molecules between the blood and the testicular interstitium, with the result that interstitial fluid is very similar to circulating blood in overall composition (Hedger 2012).

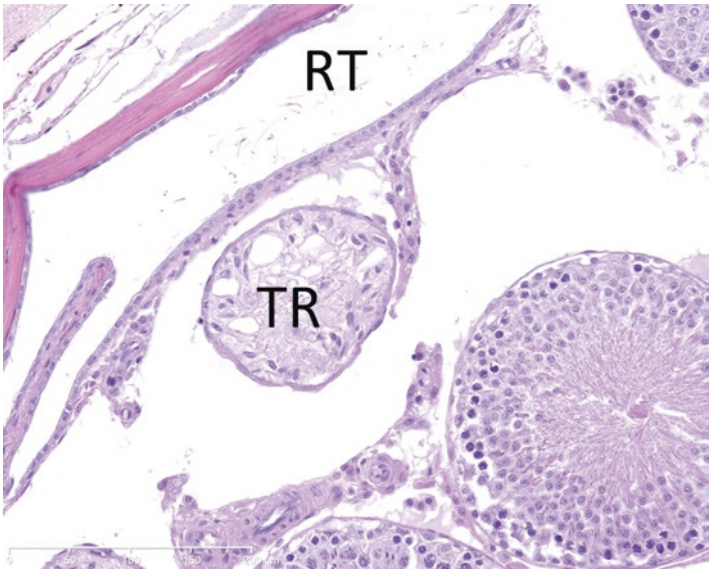

Fig. 10.2 Tubuli recti and rete testis of rat testis. This histologic section of rat testis shows the tubuli recti (TR) which are the termination of the seminiferous tubule. The TR are lined by Sertoli-like cells, and transition into the rete testis (RT) which is the beginning of the excurrent duct system. Periodic acid-Schiff/hematoxylin (PASH) stain. 20× objective magnification

The lymphatics of the testis are variable in structure between species (Fig. 10.3). In rodents, the lymphatics are irregular channels or sinusoids incompletely bounded by visceral endothelial cells, thus exposing the Leydig cells and interstitial macrophages directly to lymph (Fawcett et al. 1973; Fawcett 1973). In humans, the interstitium has large discrete lymphatic vessels, and in the pig the interstitium has many small discrete lymphatic vessels (Hedger 2012; Fawcett et al. 1973; Fawcett 1973). Regardless of the architectural arrangement, there are no physical barriers to lymphatic drainage of the testis and epididymis in any species, and the lymphatics have full access to immune cells of the interstitium of the testis and epididymis. In man, the lymphatics drain into the lumbar or para-aortic lymph nodes. In rats, the lymphatics primarily drain into the iliac and renal nodes (Robaire et al. 2006).

Leydig Cells. Leydig cells (LC), also commonly called interstitial cells, have a role in both the endocrine and immunologic systems. Leydig cells are best known for their endocrine function because they produce androgen, and are responsible for the high levels of testosterone in the interstitium. Testosterone diffuses across the basement membrane of seminiferous tubules, binds to the androgen receptor (AR) on Sertoli cells, and facilitates the Sertoli cell as it supports spermatogenesis. Leydig cells have an important immune function as well, as they recruit macrophages into the testis. This recruitment is regulated indirectly by luteinizing hormone (LH), one of the gonadotrophins produced by the pituitary gland. Once recruited to the testes, the *function* of the macrophages is then under control of the Sertoli cells in an FSH-

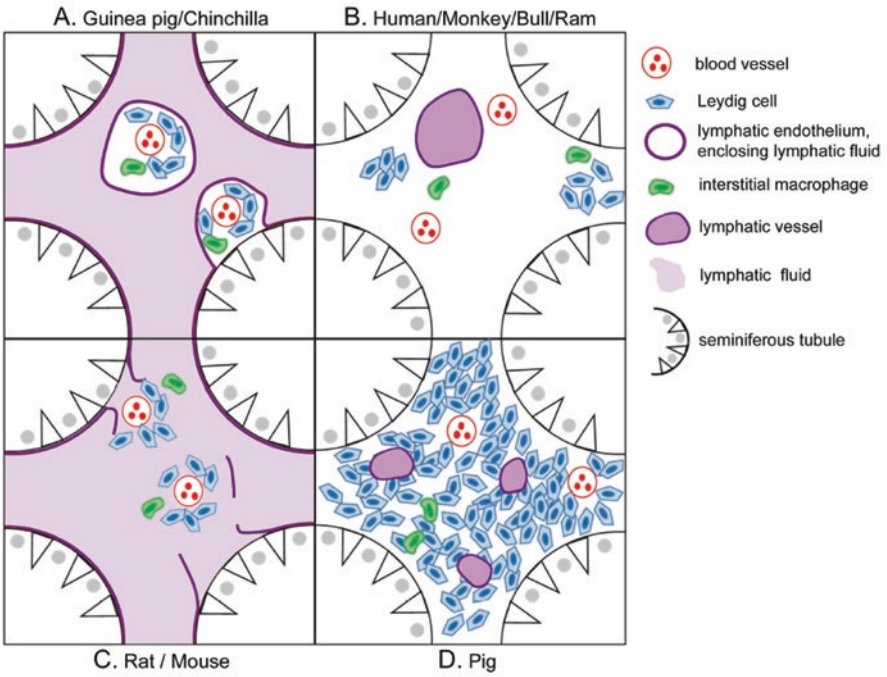

Fig. 10.3 Anatomy of the interstitium of the testis in mammalian species. This diagram illustrates the anatomy of the interstitial tissue of the testis of various species of mammals. (**a**) **Guinea pig and chinchilla**. The lymphatic channel is a a sinusoidal structure that is present throughout the interstitium. Lymphatic endothelium (shown in purple) forms a solid barrier between lymph and the remaining interstitial components such as Leydig cells, macrophages, and interstitial blood vessels. The lymphatic endothelium also forms a solid barrier between lymph and the basement membrane of seminiferous tubules. (**b**) **Human, monkey, bull, and ram**. There are discrete endothelium-lined lymphatic vessels within the interstitium. Leydig cells and macrophages form clusters within an abundant amount of loose fibrovascular stroma. (**c**) **Rat and mouse**. The lymphatic endothelium is a sinusoidal channel, somewhat similar to (**a**). However, the lymphatic endothelium is discontinuous, thereby allowing Leydig cells, macrophages and blood vessels to directly contact, and be bathed in, lymphatic fluid. There are approximately 4–5 Leydig cells per macrophage, and the Leydig cells tend to cluster close to the blood vessels. (**d**) **Pig**. In the pig, there are multiple small discrete endothelium-lined lymphatic vessels in each interstitial space. The interstitium also contains numerous Leydig cells. Macrophages are rarely noted due to the large numbers of Leydig cells. [modification from Fawcett et al (1973)]

dependent manner (Duckett et al. 1997). The distribution of Leydig cells varies between species. In the rodent, Leydig cells cluster around blood vessels. In man, small clusters of Leydig cells are distributed more randomly in the loose connective tissue of the testicular interstitium. In pigs, abundant densely packed Leydig cells throughout the interstitium obscure the interstitial stroma (Fig. 10.3).

Macrophages. The predominant mononuclear inflammatory cell type in the interstitium is the resident testicular macrophage. These cells police the interstitium and are phagocytic antigen-presenting cells (APCs). Their primary role is to maintain security

against invading microbial organisms. Macrophages typically have a dual role in both innate and adaptive immunity but, as will be discussed later in more detail, the macrophage of the testis is generally of an immunosuppressed subtype. Being stripped of its immunologic role, the testicular macrophage arguably has a more important endocrine function than an immunologic function in the normal testis. However, being part of the immunologic reserve, it is poised to respond aggressively to invasion by microbial organs. There are substantial numbers of macrophages in the non-inflamed testis of the rat, mouse and human, but macrophages are rarely apparent microscopically in the non-inflamed boar testis where densely packed Leydig cells overwhelm the interstitium (Figs. 10.3, 10.4, and 10.5).

Germ cells. The germ cells, depending on their stage of development, vary in chromosome number, morphology and susceptibility to harmful influence. Spermatogonia represent the major proliferative germ cell population residing outside the blood-testis barrier (BTB, as discussed in this chapter, Part 3.2). The diploid spermatogonia are vulnerable to cytotoxic agents because they have a high mitotic activity and reside outside the BTB, thus are directly exposed to the interstitial milieu. Spermatocytes, which are the next phase of spermatogenesis, are mostly tetraploid and represent the meiotic phase of differentiation. The earliest stage of the spermatocyte development is the pre-leptotene spermatocyte. Along with spermatogonia, these pre-leptotene spermatocytes, also reside *outside* of the BTB. The remainder of the spermatocytes (i.e., leptotene, zygotene, and pachytene), the secondary spermatocytes (i.e.,

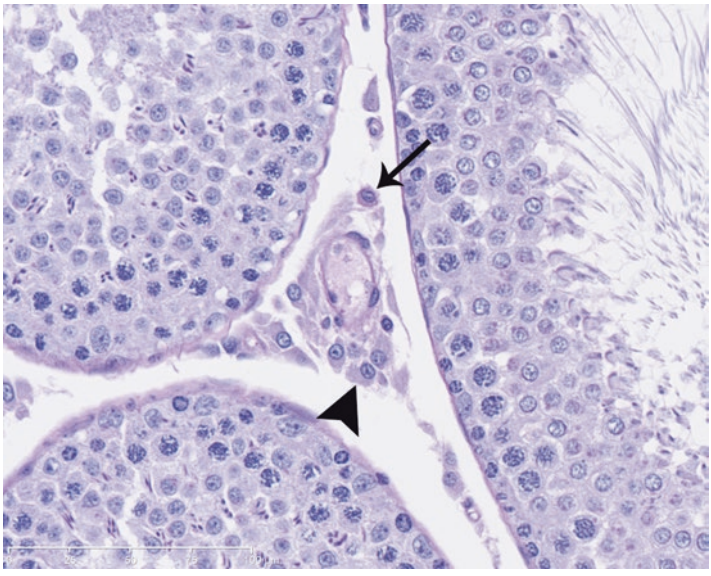

Fig. 10.4 Interstitial macrophages in the rat testis. Interstitial macrophages and Leydig cells are in a perivascular position. The interstitial macrophages are difficult to distinquish from Leydig cells in H&E-stained sections. The cytoplasm of interstitial macrophages is PAS-positive (arrow), which may help distinguish them from PAS-negative Leydig cells (arrowhead). Periodic acid-Schiff/hematoxylin (PASH) stain. 40× objective magnification

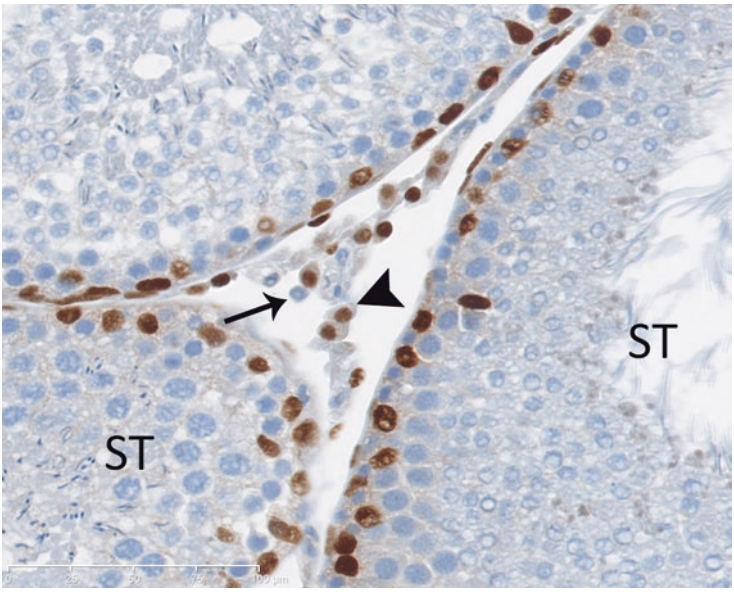

Fig. 10.5 Interstitial macrophages in the rat testis. Immunohistochemical stains can also be used to distinguish interstitial macrophages from Leydig cells. This section of testis of an adult rat was stained with antibody to GATA-4. Leydig cells are GATA-4 positive (arrowhead). A few unstained interstitial macrophages are present (arrow). The approximate ratio of 4–5 Leydig cells: 1 macrophage can be appreciated. The Sertoli cell nuclei along the basement membrane of the seminiferous tubule (ST) also stain with GATA-4. GATA-4 immunohistochemical stain with diaminobenzidine chromagen and hematoxylin counterstain. 40× objective magnification

the spermatocytes that result from the first meiotic division of primary spermatocytes), and the haploid spermatids are protected *within* the BTB. Due to DNA exchange during the process of meiosis, spermatocytes, spermatids and spermatozoa can become antigenically foreign. Were it not for immunologic privilege by the physical and physiologic barriers afforded to these cells, these "foreign" germ cells would incite an inflammatory response.

Sertoli cells. Sertoli cells are large non-proliferative cells that reside on the basement membrane of the seminiferous tubules. They serve multiple complex roles in the endocrinologic and immunologic arenas, including support of synchronous differentiation of cohorts of germ cells, maintenance of BTB, secretion of seminiferous fluid, release of mature spermatids, phagocytosis of residual bodies and apoptotic germ cell remnants, and functional maturation of resident macrophages. Like spermatogonia and pre-leptotene spermatocytes, the Sertoli cells reside in the vulnerable position outside of the BTB where they are readily exposed to xenobiotics along their basolateral membranes. Since Sertoli cells have so many endocrinologic and immunologic functions and reside partially outside the BTB, it is quite common for Sertoli cells to become functionally inactive in one or more of its functions when exposed to xenobiotic insult. Any functional deficiency that occurs as a result of such insult has profound downstream consequences, often leading to germ cell degeneration.

The Sertoli cells are sometimes regarded as exceptionally hearty and stoic cells, because they commonly are the sole survivors within seminiferous epithelium that has been largely wasted by degenerative processes. However, nothing could be further from the truth. While the Sertoli cell might *phenotypically* appear as the only cell type that remains alive in the seminiferous tubule (the so-called 'Sertoli-only tubule'), the Sertoli cell is more commonly *functionally* injured, and this injury is the proximate cause for germ cell demise or immunologic imbalance.

Myoid Peritubular cells. Myoid peritubular cells (MPCs) surrounding the seminiferous tubules contain contractile elements that help transport immotile spermatozoa into the epididymis. There are several layers of MPCs in the walls of human seminiferous tubules, but only one layer in rodents. MPCs express androgen receptors; thus, they are under the influence of the Leydig cells and their upstream endocrinological signaling pathways.

10.2.3 Excurrent Duct System

The excurrent duct system is the system of ducts that is contiguous with the seminiferous tubules and permit spermatozoa to be channeled from the testis to the ejaculate, and includes the rete testis, efferent ducts, the epididymis and the vas deferens (see Fig. 10.1).

Rete Testes. The rete testis is the continuation of the tubuli recti, constitutes the first segment of the excurrent duct system, and resides within the testis proper (see Fig. 10.2). The rete testis is lined by low cuboidal epithelium. The rete of rat testes is be found in the subcapsular areas, and must not be confused with the tubuli recti, which are the distal-most aspect of the seminiferous tubules and are lined by Sertoli-like cells. The rete has a deficient, if not absent, BTB and is not afforded the unique population of immunosuppressed interstitial macrophages provided to the rest of the testis (Tung et al. 1987a). Aside from transport of spermatozoa, the rete testes resorbs intratubular fluid secreted by the Sertoli cells.

Efferent Ducts. The efferent ducts connect the rete testis to the epididymis. The pattern, number and location of efferent ducts varies between species. The efferent ducts in rats and mice form a funnel pattern of anastomosing tubules that end into a single tubule called the common ductulus efferens (Foley 2001; Hess et al. 2002; Ilio and Hess 1994). Since the efferent ducts in rats and mice are embedded in the epididymal fat pad, these ducts are typically not examined histologically in routine toxicity studies. In dogs, the efferent ducts are embedded within the initial segment of epididymis and are readily available for examination in toxicity studies. In monkey, the efferent ducts are enclosed within connective tissue capsule at the proximal pole of the epididymis but at this proximal location, are generally not routinely sectioned. The efferent ducts, together with the rete testes, resorb 90% of intratubular fluid secreted by Sertoli cells (Hess et al. 2002). The efferent ducts empty into the initial segment of the epididymis.

Epididymis. The epididymis is composed of a highly coiled duct linking efferent ducts to the vas deferens. It consists of an initial and an intermediate segment which then enters the main portion of the epididymis, which is divided into caput, corpus and cauda. As the spermatozoa transit the epididymis, they mature and gain capability for forward mobility. While the proximal region (caput) of the epididymis helps the rete testis and the efferent ducts to reabsorb intratubular fluid secreted by the Sertoli cells, the major functions of the epididymal epithelium is protein secretion and endocytosis.

Cell types of the epididymis include principal, basal, apical, narrow, clear and halo cells, which are morphologically distinct and show region specificity. Virtually all of the cell types are present in the initial segment (Figs. 10.6, 10.7, 10.8, and 10.9). **Principal cells** comprise the majority 65–80% of the epididymal epithelium, and have major functions in protein secretion and endocytosis. **Basal cells** adhere to the basement membrane, often are dome-shaped with a basally-located nucleus, and have attenuated cytoplasmic processes that extend parallel to, and along, the basement membrane. The basal cells are a source of prostaglandins (Seiler et al. 2000; Leung et al. 2004). They are purported to protect the epididymal sperm from damaging levels of reactive oxygen species and oxygen radicals by producing superoxide dismutase (SOD) and glutathione-S transferase, and to scavenge metal ions by producing metallothioneines (Nonogaki et al. 1992);(Gregory and Cyr 2014). As will be discussed in the next paragraph, these basal cells may be intra-epithelial macrophages. **Apical cells** are found in the initial and intermediate segments of the epididymis, and play a role in endocytosis. **Narrow (or pencil) cells**, which are highly attenuated cells restricted to the initial and intermediate zones, are involved in endocytosis and H^+ ion secretion. **Halo cells** are round cells with a perinuclear rim of clear cytoplasm that reside in the basal zone throughout the epididymis. Halo cells have intracytoplasmic dense core granules. They are the primary immune cells of the epididymis and represent either cytotoxic T lymphocytes (CD8+) or CD4+ T helper cells, but are not B lymphocytes (Robaire et al. 2006; Serre and Robaire 1999). The literature does not mention NK cells as a normal resident of the epididymis. The majority of the intraepithelial lymphocytes are CD8+ T cells, which is a common feature of mucosal epithelia in general. There is an increase in halo cells throughout the epididymis with age in rats (Serre and Robaire 1999; Levy and Robaire 1999; Robaire et al. 2006). Accumulation of damaged epithelial cells and antigens of germ cells leaking through senescent/dysfunctional blood-epithelial barrier may contribute to the active recruitment of halo cells, resulting in an increased number of these T cells with age (Levy and Robaire 1999). **Clear cells** are generally limited to the caput, corpus, and cauda and have abundant clear cytoplasm that contains lipid droplets and lysosomes. They are primarily endocytic (not phagocytic) cells that clean up cytoplasmic droplets released by sperm traversing the epididymal duct.

There may be scattered **mast cells** in the interstitium of the epididymis of the adult rat, and the population density peaks at approximately 90 days of age (Jimenez-Trejo et al. 2007).

It is clear that the epididymis, unlike the testis, has an **intra-epithelial macrophage** population. However, the phenotype, appearance, and function of this macrophage has yet to be elucidated. These intra-epithelial macrophages provide stark distinction of the epididymal immunologic condition from that of the seminiferous tubules, which are

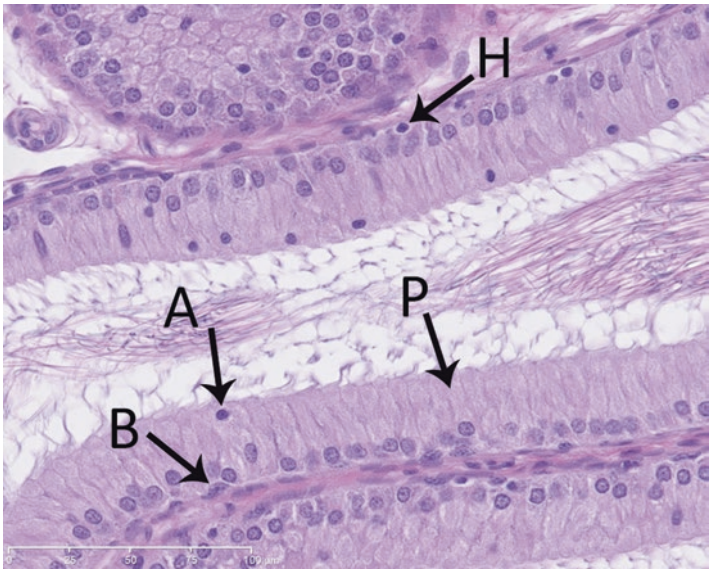

Fig. 10.6 Epididymis-initial segment. The initial segment of the epididymis is lined by tall columnar epithelial cells, called principal (P) or narrow cells, that have stereocilia on the apical (luminal) surface. In addition, the epididymal epithelium contains apical cells (**A**), halo cells (H) representing intra-epithelial $CD8^+$ T cells, and basal cells (**B**). H&E stain. 40× objective magnification

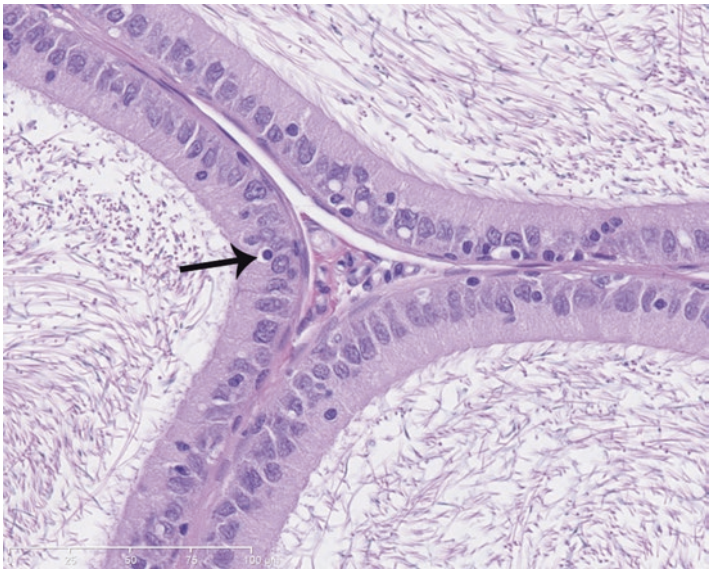

Fig. 10.7 Epididymis-caput. Caput epididymis showing several halo cells (arrow). H&E stain. 40× objective magnification

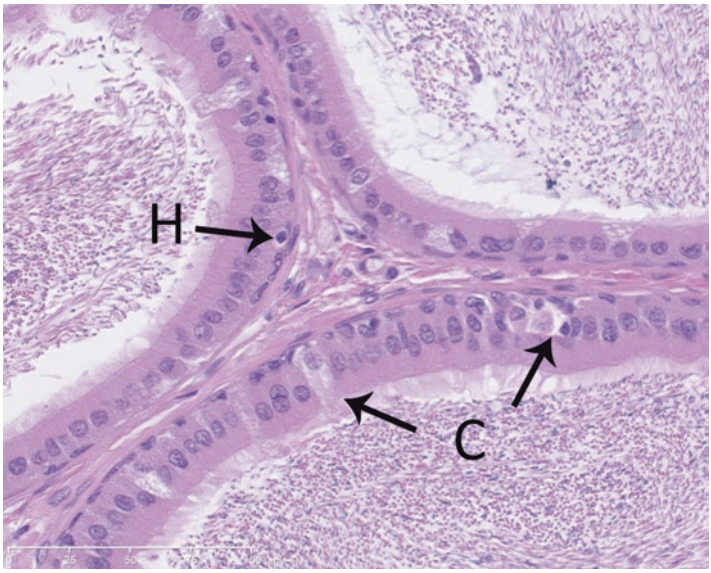

Fig. 10.8 Epididymis-cauda. Cauda epididymis showing clear cells (C) and halo cells (H). H&E stain. 40× objective magnification

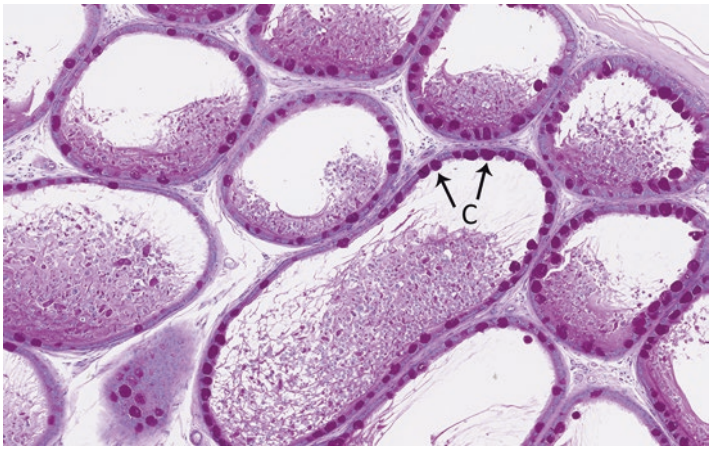

Fig. 10.9 Epididymis-cauda. This section shows hypertrophy of clear cells (C) associated with degeneration of spermatogenic tubules in the ipsilateral testis (not shown) of a rat. The PAS-positive clear cells are responsible for endocytosis of debris, and commonly contain the PAS positive cellular debris that originates from degenerating spermatids. Periodic acid-Schiff/hematoxylin (PASH) stain. 10× objective magnification

entirely devoid of any such intraepithelial immune cell type. The intra-epithelial macrophages are generally MHC class II *negative* (unlike most other circulating or tissue macrophages) and were thought to appear microscopically as "halo" cells (Nashan et al. 1989, 1990). Now it is not clear as to whether the halo cells represent lymphocytes *and* macrophages, or just lymphocytes. There has been suggestion that the basal cells

of the epididymis represent intra-epithelial macrophage in the epididymis, because of their antigenic properties and ultrastructural similarities to macrophages (Yeung et al. 1994; Seiler et al. 1999). Most recently, clear distinction has been made between the F4/80 positive intraepithelial macrophages and the basal cells of the mouse epididymis (Shum et al. 2014). Therefore, whether the intra-epithelial macrophages appear as basal cells, halo cells, or another distinct cell type is unclear at this time.

Urethra: The urethra is the distal end of the male reproductive tract. The opening of the penile urethra transitions from keratinized stratified squamous epithelium to non-keratinized stratified squamous epithelium in the fossa navicularis. As the non-keratinized stratified squamous epithelium enters the shaft of the penis, it transitions into the pseudostratified glandular columnar epithelium, which lines the length of the penile urethra. In this epithelium are deep invaginations, known as Littre glands, which provide lubrication and contribute to innate immunity by producing antimicrobial proteins. The pseudostratified columnar epithelium contains intraepithelial CD4+ and CD8+ T cells, NK cells, dendritic cells and resident macrophages. The epithelium allows transepithelial passage of immunoglobulins A, M and G (IgA, IgM and IgG) as well as interferon (IFN), and the epithelium secretes mucins, and antimicrobial proteins, and expresses Toll-like receptors (Nguyen et al. 2014).

10.2.4 Accessory Sex Glands

There is marked species-related variability in the anatomy, biology, and number of the male accessory sex glands. The accessory glands include the prostate, the coagulating gland, the seminal vesicles, the ampullary glands, the bulbourethral gland, the urethral gland and the preputial gland. All these glands are present in rats and mice, but only the prostate, seminal vesicle, coagulating gland, and bulbourethral gland are routinely examined in preclinical toxicity studies in rodents. The secretions from these glands are critical to the maturation of sperm in preparation for conception, but also for innate immune responses. The prostate gland is responsible for producing the secretory component of IgA which is required for transport of IgA across the mucosa (Stern et al. 1992) and for producing non-specific anti-bactericidal proteins, such as prostasomes (see this chapter, Part 4.4).

10.3 Immunologic Privilege and Immunosuppression of the Testis

10.3.1 Immune Privilege in General

The human testis produces approximately 100 million highly differentiated spermatozoa each day from a few hundred thousand spermatogonial stem cells. These highly differentiated spermatozoa appear late in developmental life, long after maturation of the immune system and after the establishment of systemic immune

tolerance. In man, the first appearance of the earliest meiotic germ cells (spermatocytes) is about 10 years of age. In rats, meiotic spermatocytes that could be recognized as foreign first appear in the early juvenile period at postnatal day (PND) 15–20 (Picut 2014). These post-meiotic cells express a broad range of novel structural proteins, surface receptors, signaling molecules and enzymes that have the potential to be seen as foreign by the mature immune system. It is essential that these "foreign" germ cells be protected from the host's immune system.

The testis is a privileged site where germ cells are protected from attack by the host's immune system. Immune privilege is not unique to the testis. Besides the testis, immune privilege has been recognized also in the brain, the anterior chamber of the eye and the placenta in mammals. Immune privilege is a condition in which tissues and associated antigens that might be recognized as foreign to the host are inaccessible to the host's immune system. The immune privilege in the testis was initially believed to be solely based on the physical sequestration of antigens from antibodies by the blood testes barrier (BTB). However, this view of immune privilege has expanded considerably, thus not only the physical barrier of the BTB but also functional barriers in the interstitial space are now included. This expansion of our understanding of immune privilege was mainly based on the findings that autoantigens were present on pre-leptotene spermatocytes and spermatogonia, cell types that reside *outside* the BTB (Chen et al. 2009; Wong et al. 2008; Yule et al. 1988), so clearly additional mechanisms are needed to fully explain the immune privilege. In the interstitial space, immune privilege is afforded by: (1) a selection of a unique population of anti-inflammatory macrophages that have reduced antigen presenting capabilities; (2) a preponderance of suppressor and regulatory T cells as opposed to effector and cytotoxic T cells; and (3) a microenvironment rich in anti-inflammatory factors, hormones (e.g., androgens) and operational immunoregulatory systems (such as the Gas6/ProS-TAM system) that dampen the immunologic response (Table 10.1).

There are two misconceptions about immune privilege of the testis. The first is that the testis has a deficient or abnormal lymphatic system and that restricted movement of immune cells and other effectors contribute to an absence of immune responses within the testis. It has been clearly established in several species that the testis possesses effective lymphatics draining directly to local lymph nodes (Hedger 2012; Itoh et al. 1998). The second misconception is that the lower temperature of the testis in most mammalian species contributes to immune privilege. However, no studies show that lower temperature contributes to immune privilege (Head and Billingham 1985; Hedger 2012; Selawry and Whittington 1984).

Reviews on the topic of immunoprivilege in the testes can be found in (Hedger 2011a; Li et al. 2012; Zhao et al. 2014).

10.3.2 Blood Testis Barrier

The major physical barrier that results in immune privilege of the testis is the blood-testis barrier (BTB) located within the seminiferous tubules. With rare exception of the pre-leptotene spermatocyte, the BTB separates the spermatocytes and spermatids (both

Table 10.1 Cytokines and factors that suppress/modulate inflammation in the testis

Factor	Source	Main role
TGF-β	Sertoli cells Macrophages Peritubular myoid cell	Anti-inflammatory Inhibits T and B cell function Controls dynamic of BTB
Activin A	Sertoli cells Peritubular myoid cell	Anti-inflammatory Inhibits T and B cell function
IL-1α	Sertoli cells Germ cells	Stimulates spermatogonia
Alpha-melanocyte stimulating hormone (α-MSH)	Macrophages Leydig cells	Stimulates IL-10 production Inhibits IL-2 response Inhibits T and NK cytotoxicity
IL-6	Sertoli cells Leydig cells	Anti-inflammatory Stimulates meiosis of spermatocytes
IL-10	Macrophages T cells	Anti-inflammatory
iNOS/NO	Leydig cells Sertoli cells Spermatocytes	Controls dynamic of BTB
Macrophage inhibiting factor (MIF)	Leydig cells	Inhibits T cell and NK cytotoxicity
Fas ligand	Germ cells	Apoptosis of Fas-bearing T cells
PGE2	Macrophages Leydig cells	Modulates inflammatory function of macrophage Inhibits T cell proliferation Inhibits NK cytotoxicity
Clusterin	Sertoli cells	Inhibits T cell activation
Androgen	Leydig cells Sertoli cells	Induces IL-10 production by T cells Shifts T cell population to T-regs Decreases IFNα and IL-2 from T cells Decreases TNFα and IL-6 from macrophages
MCP-1 (monocyte chemotactic protein-1)	Mast cells Peritubular myoid cells	Attracts macrophages
TNF-α (low levels)	Germ cells Sertoli cells	Protects germ cells from apoptosis Controls dynamics of BTB
TAM/Gas6-ProS	Sertoli cells Leydig cells	Suppresses immune activation
Prostasomes, polyamines	Prostatic epithelium	Inhibits T cell activation Inhibits macrophage phagocytosis
B defensin	Germ cells Epididymal epithelium Sertoli cells	Antimicrobial (inhibits activation of the innate immune system)
Toll-like receptors (TLR)	Germ cells Sertoli cells Leydig cells	Induce apoptosis of germ cells and T cells, and bolsters innate immunity

of which are in the meiotic phases of division) from the mitotic cells (i.e., the spermatogonia). Inside (or above) this BTB (also known as the adlumenal compartment) reside the spermatocytes and spermatids; and outside (or below) this BTB reside spermatogonia and pre-leptotene spermatocytes. As a consequence, all germ cells within the BTB are entirely dependent on the Sertoli cells for their support, regulation, and protection, while cellular elements outside the BTB are nourished by the general vascular supply.

The BTB consists of a complex of various junctions including tight junctions, basal ectoplasmic specializations (or occluding junction), gap junction and desmosome-like junctions between adjacent Sertoli cells (Su et al. 2011; Cheng et al. 2011b). The basal ectoplasmic specialization (ES) is found on both lateral sides of Sertoli cells and is composed of tightly packed actin filament bundles oriented perpendicular to the plasma membrane (Cheng et al. 2011a). Due especially to the basal ectoplasmic specialization, the BTB is one of the tightest physical seals in the body. It should be noted here that the ES is not restricted to the BTB in the seminiferous tubule, but may also be found at the Sertoli cell-spermatid interface (from spermatid steps 9-19 in the rat) where it is known as the apicale (Russell 1977). In the apicale, the actin bundles are only present on the Sertoli side The BTB is completely impermeable and restricts intercellular flow of water, electrolytes, ions, nutrients, hormones, paracrine factors and biological molecules that would otherwise occur between Sertoli cells into the tubular lumen. It also sequesters "foreign" antigen on germs cells to prevent the production of antisperm antibodies, and keeps white blood cells and those few anti-sperm antibodies that do develop away from the "foreign" gametogenic cells (Fijak et al. 2011). The BTB is anatomically more complex than other blood tissue barriers such as the blood-brain barrier or the blood-retinal barriers which are constructed exclusively of tight junctions between endothelial cells, or the blood-CSF barrier or the blood-epididymal barrier which are constructed of tight junctions at the apical edge of epithelial cells. See Su et al for a detailed review of the anatomy and composition of the BTB (Su et al. 2011).

Although the ES is one of the tightest blood tissue barriers, the BTB undergoes dynamic and consistent restructuring to facilitate the transit of pre-leptotene spermatocytes to the leptotene stage (Russell 1977). It is the dynamic process of dissolution and reconstruction of the BTB by *physiologic* amounts of "inflammatory" cytokines produced by the Sertoli cell that renders the BTB especially vulnerable when *pathologic* inflammation occurs.

Many toxicants, especially kinase inhibitors, have adverse impact on the BTB. Compounds such as Bisphenol A and cadmium target a number of protein kinases that are important for dissolution and restructuring the BTB. Cadmium in particular effects the occludin/ZO-1/focal adhesion kinase complex at the BTB (Cheng et al. 2011a). For a more complete discussion of the role of protein kinases on spermatogenesis in general, and the BTB in particular, the reader is referred to Cheng et al. (2011c) and Jenardhanan and Mathur (2015).

The BTB exists only in the seminiferous tubules. The rete testis epithelium lacks Sertoli cells and their highly specialized ectoplasmic specializations. The epithelial barrier restricting movement from the blood into the rete testis is much less effective than that of the seminiferous epithelium, thus immunoglobulin and immune cells

are able to cross the epithelium of the rete testis (Dym and Romrell 1975; Koskimies et al. 1971; Koskimies and Kormano 1973; Tung et al. 1970).

The BTB develops postnatally in advance of spermatogenesis. In rat, the BTB begins to assemble by PND 15-16 and it is completely developed by PND 18-21 (Vitale et al. 1973; Bergmann and Dierichs 1983; Russell et al. 1989; Toyama et al. 2001). In other species, these timelines of BTB formation are 13–17 weeks of age for dogs and 8 years for man. The BTB is completed by postnatal week 20 in dogs and at puberty in man.

10.3.3 Immune Cells of the Testis

There are a number of conventional immune cells in the testis, including T cells, macrophages, and NK cells. In addition, Sertoli cells and Leydig cells play important roles in regulating the immune responsiveness of these conventional immune cells. Taken together, both the conventional immune cells and the unconventional immune cells (Leydig cells and Sertoli cells), dampen the adaptive immune response in the testis, while preserving the innate immune responsiveness. This next section will explain how these immune and somatic cells work together to achieve this selectively immunosuppressed organ.

Stereologic analysis of testes from Sprague-Dawley rats and humans have been performed to indicate the composition of immunologic cells in normal teste (Hedger and Hales 2006) (Table 10.2). The majority of the immunocytes in the testicular interstitium of rats are macrophages (approximately 75%). The next most common immune cell is the T cell (approximately 15%) and then the NK cell (approximately 8%), followed by the rare dendritic cell (approximately 2%). Of the T cells, the vast majority are CD8+ cells (80%) and a minority are CD4+ cells (20%) (Hedger and Hales 2006) (Fig. 10.10) As will be clear in the following discussion of each immune cell type in the testes, immune cells that enter the male reproductive tract environment become functionally modified to restrict their pro-inflammatory activity and provide an immunologically restrained environment where adaptive immune responses are closely controlled (Hedger 2015b).

Macrophages. Macrophages in the rat and mouse testis have reduced inflammatory and co-stimulatory activities and represent an immunosuppressed cell type that

Table 10.2 White cell populations in the rat testis

Cell type	Rat (% of total leukocytes)
Macrophages	75.0%
T-cells	15.0%
CD8+(80%) CD4+(20%)	
NK	8.0%
Dendritic cells	2.0%

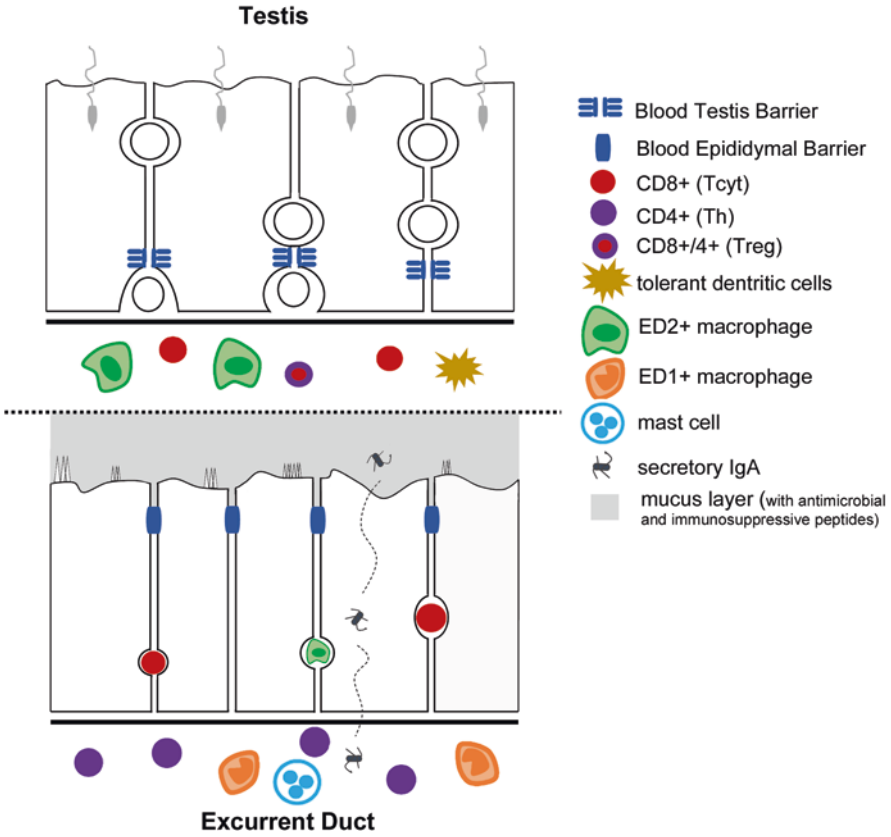

Fig. 10.10 Predominant inflammatory cells of the testis (top panel) and excurrent duct system (bottom panel). **Top panel**. In the testis, immunoregulation is afforded by the occluding junctional complex of the blood-testis barrier, interstitial macrophages of the non-inflammatory ED2$^+$ subtype, tolerant dendritic cells, CD8$^+$ T cells with cytotoxic activity, and CD4$^+$CD8$^+$ T-reg cells that are important in maintaining tolerance. There is a paucity of immune cells such as CD4$^+$ T helper cells that are capable of mounting antigen-specific adaptive immunity. **Lower panel**. In the excurrent duct system, such as the epididymis, the epithelium is lined by stereo-ciliated epithelial cells, and immunoregulation is provided by the normal mucosal immunity, including mucoid secretions, secretory IgG, intraepithelial CD8$^+$ T cells, and intra-epithelial macrophages (most likely of the ED2$^+$ phenotype). In addition, antigen-specific adaptive immunity is provided by CD4$^+$ T cells and resident macrophages of the ED1$^+$ pro-inflammatory subtype. Mast cells may be normally present in the interstitium. The apically positioned tight junctions between lining epithelial cells establish the blood-epididymis barrier, which helps prevent antigenic stimulation by luminal sperm. A host of immunosuppressive (e.g., spermine, prostasome) and antimicrobial (e.g., defensins) peptides in the luminal mucus layer also dampen any immune response (adapted from Hedger et al. 2006)

restrains cell-mediated immune reactions. Macrophages present in the interstitium of the testis are referred to as resident macrophages. There are substantial populations of testicular resident macrophages in most species, including rats, mice, guinea pigs, hamsters, horses, bulls and humans. Humans have about twice as many interstitial testicular macrophages per gram of tissue ($10–25 \times 10^6$ per g) as rats

($5–10 \times 10^6$ per g) (Hedger and Hales 2006). There are few testicular macrophages in the boar and ram. The ratio of macrophage to Leydig cells in rats and mice is approximately 1 macrophage per 4–5 Leydig cells (Niemi et al. 1986).

The testicular macrophages retain their potential to carry out conventional functions of macrophages in other organs. They retain machinery to phagocytize, have surface MHC-class II molecules and possess cytokine receptors. Most of the testicular macrophages, however, are predominantly of the immunosuppressed phenotype. They have been placed in immunologic "reserve" and have been recruited for an additional tissue-specific role in endocrine function.

Testicular macrophages are not a homogeneous population of cells and can be separated into distinct subsets as defined by the expression of molecules recognized by the monoclonal antibodies, ED1 and ED2 (Wang et al. 1994). Most interstitial macrophages in the testis express CD163 (a cell surface glycoprotein), which is the antigen that recognizes monoclonal antibody ED2 in immunohistochemistry (IHC) staining. The $ED2^+$ macrophages, which are the main subpopulation in the normal rat testis, secrete the anti-inflammatory cytokine IL-10, thus contributing to the dampened immune response in the testis (Winnall et al. 2011). A minor subset of testicular macrophages (15–20%) expresses the lysosomal glycoprotein CD68, recognized by ED1, which is important in phagocytosis and innate immunity. These CD68 or $ED1^+$ macrophages have a smaller nuclear diameter than the $ED2^+$ macrophages, produce and secrete pro-inflammatory cytokines such as IL1, TNFα and IFNϒ (Perez et al. 2013), and produce nitric oxide. It has been suggested that these $ED1^+$ macrophages represent an immature population of testicular macrophages that are newly arrived from the circulation, because these pro-inflammatory features are more typical of circulating monocytes (Gerdprasert et al. 2002). The testis also has an "intermediate" macrophage population that possesses both ED1 and ED2. Taken together, the conventional wisdom is that there are several populations of macrophages in the rat testis, putatively representing different stages of development or functional states. Figure 10.11 shows the functional and phenotypic development of the pro-inflammatory circulating monocyte into the non-inflammatory interstitial macrophage.

The $ED2^+$ macrophages of the rat are non-inflammatory because they produce TGFβ, and IL-10; and IL-10 is a powerful inhibitor of helper T cells. In addition to anti-inflammatory cytokine/chemokine production, there are other ways that $ED2^+$ macrophages contribute to the immunosuppressive environment. The $ED2^+$ macrophages in the mouse have deficient expression of MHC class II molecules that are required for antigen presentation and therefore cannot present antigen to T cells (Hedger 2002). While the $ED2^+$ cells in the testis do not express MHC II in the mouse, the $ED2^+$ cells located specifically around the rete testis do express MHC II (Mahi-Brown et al. 1987). This may explain why the rete testis is so susceptible to sperm granulomas in this species. Testicular macrophages in the mouse also lack co-stimulatory B7-1 and B7-2 molecules. The lack of these B7-1 (CD80) and B7-2 (CD86) molecules render the macrophage incapable of activating T helper cells to mount an antigen-specific response. These specific immunosuppressive mechanisms (lack of MHC class II and co-stimulatory B7 molecules) in the mouse have not been demonstrated in the rat (Kern and Maddocks 1995; Kern et al. 1995; Hayes et al. 1996;

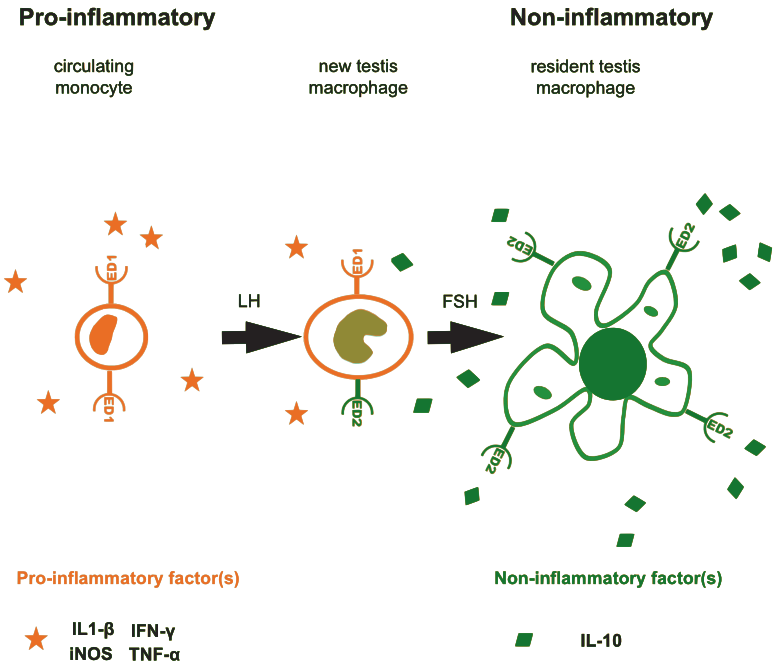

Fig. 10.11 Phenotypic maturation of resident testicular macrophages. Conventional wisdom suggests that pro-inflammatory circulating monocytes mature into non-inflammatory resident testicular macrophages by the action of LH and FSH. LH, acting through the Leydig cell, recruits circulating monocytes to the interstitium. Once in the interstitium, FSH working in concert with Sertoli cells enables the monocyte to mature into a non-inflammatory phenotype. This non-inflammatory phenotype is characterized by an increase in nuclear and cytoplasmic volume, loss of markers that recognize ED1$^+$ antibody, upregulation of IL-10 non-inflammatory cytokine, and loss of ability to produce pro-inflammatory molecules such as IFN-γ, IL-1β, TNF-α, or iNOS. During this development and maturation, there is an intermediate phenotype of the "new testis macrophage" that has phenotypic and functional abilities of both the pro-inflammatory monocyte and the non-inflammatory resident testis macrophage

Meinhardt et al. 1996). Nevertheless the ED2$^+$ macrophages in the interstitium of the rat testis produce IL-10 and play a major role in suppressing the immune response.

Lymphocytes (T cells), NK cells and Dendritic cells. The absence of significant subpopulations of immunocytes (no B cells and rare T helper cells) demonstrate that adaptive immunity in the testis is deficient (Perez et al. 2013). Lymphocytes of any subtype are relatively sparse in the testis of all species compared to the number of macrophages. In rat and man, lymphocytes represent about 10–20% of the total leukocyte population (Hedger 2015b). When present, lymphocytes are more commonly adjacent to the epithelium of the rete testis. With advancing age in the rat, interstitial lymphocyte populations increase, and this might be a reflection of the normal breakdown of immune privilege with aging.

T cells are generally separated into regulatory and effector subsets. T cells that express CD4 are mostly helper or regulatory cells that are activated by antigen-presenting cells bearing MHC Class II. T cells that express CD8 markers are generally

cytotoxic and are activated by antigen presented in context of MHC class I molecules. The $CD8^+$ T cells are therefore important in protection against viral infections and proliferation of transformed tumorigenic cells. Regulatory T cells (Tregs) are involved in maintaining immune tolerance to germ cell antigens (Hedger and Hales 2006). Tregs express CD4 but also CD8 and Foxp3.[3] Tregs encounter tissue autoantigens, maintain peripheral tolerance to these antigens, and prevent autoimmune reactions (Wheeler et al. 2011). The preponderance of Tregs over traditional $CD4^+$ T helper cells helps dampen the autoimmune response to sperm following vasectomy (Wheeler et al. 2011). By the same token, the relative deficiency of $CD4^+$ T helper cells limits clonal expansion of antigen-specific T cells and subsequent antibody production by B cells, thus achieving the end result of a suppressed or deficient antigen-specific arm of the immune response. Less is known about the various subpopulations of T cells in man (Ritchie et al. 1984; Hedger 1997; el-Demiry et al. 1987).

NK cells are a major immunocyte subpopulation within the rat testis, and are responsible for innate non-specific cell killing and phagocytosis along with macrophages and $CD8^+$ T cells. NK cells can recognize and destroy tumor cells and virally-infected cells via specific receptor mechanism that does not involve MHC class II (Lanier et al. 1992). The NK cells bolster the testes' innate immune protection (Hedger 1997).

The testis houses a unique population of **dendritic cells (DC)** that results in overall immunosuppression of adaptive immunity. Dendritic cells represent a minor population of interstitial cells in the normal rat testes (Li et al. 2012) and there are 10x as many macrophages as there are dendritic cells (Meinhardt et al. 1998). Dendritic cells in general act like macrophages by activating naïve T cells and playing a role in T cell-dependent immunity. They are the most powerful antigen presenting cells, express MHC class II and costimulatory molecules CD80 and CD86, and they can either (1) induce activation of lymphocytes in response to allo-antigen, or (2) minimize autoimmune response by tolerizing T cells to autoantigens. The DCs in the testis are of the tolerogenic subtype described as "immature", and are unable to induce T cell proliferation (Rival et al. 2007; Guazzone et al. 2009, 2011). This is one of the mechanisms by which adaptive immune reactions are suppressed in the testis.

Mast Cells and Eosinophils are also occasionally present in the testes of multiple species, but to a very limited degree. The number of mast cells in the testis appears to be under control of the Leydig cells (Hedger 2015b). In the rat, mouse and dog, mast cells and eosinophils may be present around blood vessels in the testicular capsule, and are confined to this subcapsular region (Gaytan et al. 1989). In the pig, horse and human, mast cells may be found normally throughout the testis. These cells play a role in innate immunity but also are involved in the control testicular blood flow. The mast cell population in human testes is known to increase with male infertility and decline with advancing age, but the biologic importance of these changes are not clear (Apa et al. 2002). It has been suggested that mast cells are essential intermediaries in regulatory T cell tolerance (Lu et al. 2006). They produce

[3] Foxp3 appears to function as a master regulator (transcription factor) in the development and function of regulatory T cells.

monocyte chemoattractant protein -1 (MCP-1), indicating they may play a role in regulating the influx or recruitment of macrophages during inflammation (Li et al. 2012). At this time there are no specific roles assigned to mast cells or to eosinophils in maintaining immune privilege in the normal testis.

Sertoli Cells. The Sertoli cell is critical to immune privilege in the testis. It is the cell that gives rise to and maintains the BTB. However, Sertoli cells also have immunosuppressive functions. One important role for Sertoli cells in preventing damaging inflammation in the testis is phagocytosis of apoptotic germ cells. In this way any foreign antigens associated with the dead germ cells are quickly removed before the antigens are exposed to the immune system. Not only do the dead germ cells have foreign antigens, but there is also evidence that they express endogenous Toll-like receptors (TLRs) (Wang et al. 2012; Chen et al. 2014). Unnecessary activation of these TLR ligands from necrotic germ cells can upregulate pro-inflammatory cytokines and induce inflammation (Piccinini and Midwood 2010; Zhang et al. 2013; Wang et al. 2012; Chen et al. 2014). Therefore it is important that necrotic germ cells be rapidly removed before they incite damaging non-specific inflammation and tissue damage (Pelletier et al. 1998; Schuppe et al. 2008). Sertoli cells have enhanced ability to phagocytize necrotic germ cells which is facilitated by TAM receptors on their surface (Xiong et al. 2008). TAM receptors—Tyro3, Axl, and Mer—comprise a family of receptor tyrosine kinases that, together with Gas6 and Protein S, promote efficient phagocytosis of apoptotic cells, and act as inhibitors of the innate inflammatory response to pathogens.

Sertoli cells play an active role in suppressing adaptive immunity. Sertoli cells are responsible for producing anti-inflammatory mediators, which includes TGFβ, activin, and clusterin, all of which inhibit T cell activation. Activin and TGFβ inhibit activated macrophages and dendritic cells from producing proinflammatory IL6 or TNFα. TGFβ inhibits activated T cells from differentiating into the Th1 phenotype. Sertoli cells also produce IDO (indoleamine 2,3-dioxygenase) that depletes tryptophan and inhibits protein synthesis in activated T cells (Fig. 10.12).

Sertoli cells also express FasL and PD-L1 (programmed death-ligand or B7-H1) that promotes apoptosis of activated T cells following engagement with FAS and PD-1 receptors, respectively (Francisco et al. 2009; Butte et al. 2007) (Fig. 10.12). The importance of the FasL-Fas system in causing apoptosis of invading T cells has been considered a major component of this apoptotic system, but recent evidence suggests the FasL-Fas system is more important in causing apoptosis of germ cells when the testis gets insulted in any way (Lee and Huang 1997; Hedger 2015a). FasL expression is upregulated on Sertoli cells by TNF and IFNγ, and this upregulation then causes increased apoptosis of the Fas-bearing germ cells. This mechanism explains high numbers of apoptotic germ cells after physical or toxicologic insult (Ogi et al. 1998; Hedger 2015b; Riccioli et al. 2000). Rather than being purely destructive, the increased apoptosis may be a defensive strategy. This admittedly speculative "Sophie's choice'-type process proposes that elimination of a subpopulation of germ cells that would utilize too many resources of the Sertoli cell allows the Sertoli cell to conserve its resources and preserve at least some germ cells (Lee and Huang 1997). The FasL-Fas system is a normal physiologic mechanisms that occurs

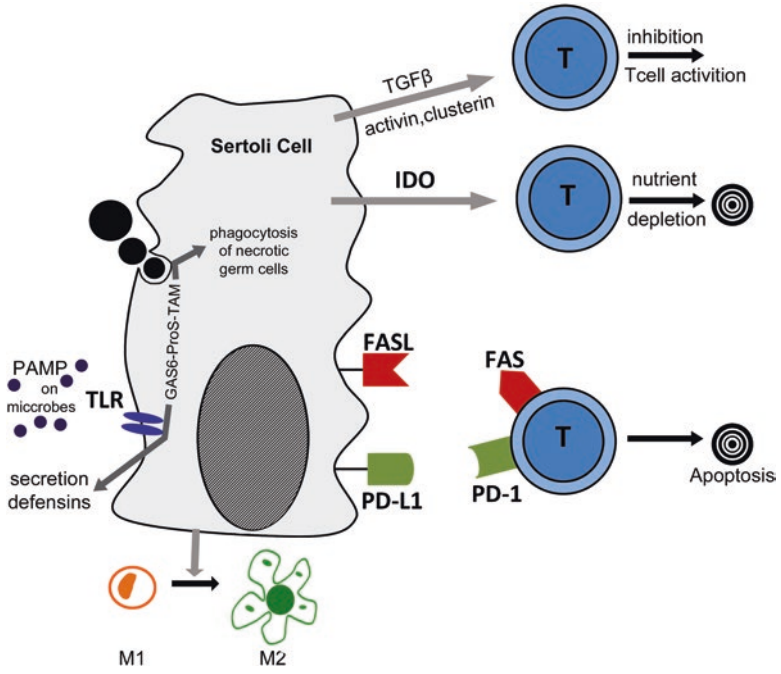

Fig. 10.12 Immune functions of the Sertoli cell. The Sertoli cell suppresses activation of adaptive immunity while maintaining an active innate immune response. The Sertoli cells play a significant role in preventing antigen-specific immunologic destruction of germ cells by T cells in three ways. First, Sertoli cells produce IDO (indoleamine-2,3 dioxygenase), which depletes tryptophan, an essential T cell nutrient, leading to diminished T cell proliferation and eventual destruction. Second, Sertoli cells express FasL and PD-L1 ligands, which bind to Fas and PD-1 on T cells in the vicinity. Since no second signal is received by the T cells, the binding results in apoptosis of T cells, or at least an inhibition of proliferation. Third, Sertoli cells produce anti-inflammatory mediators, such as TGFβ, activin and clusterin, which inhibit T cell activation. On the other hand, Sertoli cells are important in maintaining the innate immune response. Sertoli cells express TLRs that are activated by PAMPS on microbes; secrete antimicrobial defensins; phagocytize germ cells to prevent unnecessary exposure of foreign antigens; and, with the help of IL-1 and FSH, mold the interstitial macrophages into the non-inflammatory M2 phenotype. The Gas6-ProS-TAM receptors control the damaging inflammation that could otherwise result from TLR activation

at all times: Sertoli cells use FasL-Fas to continually induce apoptosis of germ cells at low levels (Print and Loveland 2000). In fact, in the rat every germ cell type can undergo normal physiologic apoptosis (Blanco-Rodriguez 1998), but the apoptosis is held in check by a delicate balance between pro- and anti-apoptotic proteins (Bax and Bcl), and a close interrelationship between the Sertoli cell and germ cells. For an excellent review of the intrinsic and extrinsic apoptotic pathways operable in normal spermatogenesis and toxicant injury, refer to Murphy and Richburg (2014).

Sertoli cells also produce antiviral and antibacterial molecules such as defensins which will function within the excurrent duct system to reduce pathogen invasion. Defensin is known as a host-defense peptide involved in killing phagocytized bacteria, fungi and viruses, and is important in the innate immune system of the testis. Like

germ cells, Sertoli cells express Toll-like receptors (TLRs) which are poised to aggressively activate the innate immune system upon intrusion by microbes. This activation is kept under control by the down-regulatory influences of Gas6/ProS-TAM signaling (Lemke and Rothlin 2008; Rothlin et al. 2007). In summary the TLRs on Sertoli cells have a positive influence on the innate immune system, but the Gas6/ProS-TAM keeps its activation under control. As important as the innate system may be, inflammatory conditions that result from its activation are always damaging to testicular function (Sun et al. 2010).Therefore the Gas6/ProS-TAM dampening mechanism is probably more crucial to continued normal function of the testis.

The Sertoli cell has a central role in establishing the suppressive subpopulation of ED2$^+$ macrophages. After the macrophages are recruited by the Leydig Cells, FSH through its action on Sertoli cells (and along with growth hormone and cytokines such as IL-1) mold the macrophages into the immunosuppressive phenotype (Yee and Hutson 1983). In particular, FSH simulates the increase in mean nuclear diameter of the testicular macrophage in rats as they transforms from ED1$^+$ to ED2$^+$ phenotype (Fig. 10.11) (Duckett et al. 1997; Hedger and Hales 2006).

Leydig Cells. Leydig cells are known as a major steroidogenic cell of the testis, but this endocrine cell has also functions as an "immunologic" cell that plays a huge role in maintaining and suppressing the immune system. Leydig cells control the trafficking of lymphocytes in the testis and in particular maintain the high CD8$^+$/CD4$^+$ ratio. As a demonstration of this function, when Leydig cells are removed from the testes by ethylene dimethanesulphonate (EDS) treatment there is a decrease in the ratio of CD8$^+$ to CD4$^+$ cells (Hedger and Hales 2006).

Leydig cells recruit and modify the function of resident macrophages. Luteinizing hormone acting through Leydig cells controls the recruitment of macrophages to the testis and FSH (working in concert with the Sertoli cells) controls the maturation of the testicular macrophage phenotype (Meinhardt et al. 1998; Raburn et al. 1993). This close association between Leydig cells and macrophages explains why the macrophage population expands dramatically during development, coinciding with the proliferation of adult Leydig cells (Hardy et al. 1989; Raburn et al. 1993). Leydig cell produce the chemoattractant macrophage inhibitory factor (MIF), and this may be the non-androgenic product responsible for recruitment of circulating monocytes (Raburn et al. 1993). Another role for MIF is to act as an inhibitory factor of T cell activation and NK cell cytotoxicity, thus adding to the anti-inflammatory cytokine milieu of the testis.

In addition to Leydig cell-macrophage interaction, there is Leydig cell-mast cell interaction in the testis. Leydig cells control and inhibit the population of mast cells, and in so doing dampen the tissue damage that could be caused by these pro-inflammatory cells. This inhibitory function is mediated by androgens. When Leydig cells are experimentally ablated by EDS, the mast cell population increases: When neonatal rats are treated with estrogen, testicular mast cell numbers increase (Gaytan et al. 1989). Leydig cells not only control mast cell populations, but mast cells also control Leydig cell function. The reciprocal relationship also exists, as mast cells regulate, and generally inhibit, steroidogenesis by Leydig cells (Aguilar et al. 1995). This close association between mast cells and Leydig cells might explain why infertility in humans is often associated with increased numbers of mast cells (Hussein et al. 2005).

Leydig cells also are capable of producing PGE2, which modulates inflammatory functions of macrophages and produces immunosuppressive androgens and other protein hormones. The immunosuppressive properties of androgens and other protein hormones will be discussed in greater detail later in this chapter.

To this point we have focused on mechanisms by which Leydig cells suppress the immune system. However, Leydig cells have an important positive immunologic role, which is serving as a first line of defense against invading microorganisms. Leydig cells positioned in the interstitium encounter invading bacteria first and need some ability to activate the innate immune response. Fortunately, Leydig cells express TLR (i.e., TLR3 and TLR4 in mice) which are important in initiating innate immune responses. Since inflammation can be damaging, the Leydig cells also have the Gas6-ProS-TAM system to help regulate the degree of TLR activation, as it does in the Sertoli cells (Wang et al. 2005).

When TLR are activated on Leydig cells, there is reduced production of testosterone. Since testosterone is immunosuppressive (see this chapter, Part 5.1), the reduced synthesis of testosterone is but one way by which the innate immune response is bolstered. TLR activation causes increased inflammatory cytokine production (such as TNFα and IL6) and these cytokines inhibit testosterone biosynthesis in Leydig cells (Diemer et al. 2003). The TAM mechanism also works directly on the Leydig cell to inhibit biosynthesis of testosterone. Regardless of whether lower testosterone is caused by inflammatory cytokines or the TAM mechanism, the resulting lower level of otherwise immunosuppressive testosterone results in "enhanced" innate immunity when TLR is activated (Rettew et al. 2008; Page et al. 2006).

Germ Cells. There is emerging evidence that germ cells also may participate in protecting themselves from their own immunologic destruction and development of autoimmunity. Germ cells produce and secrete low physiologic amounts of Il-1α and TNFα (De et al. 1993; Haugen et al. 1994), and it has been shown that the *low* physiological level of TNFα protects the germ cell from apoptosis in *non-inflammatory* conditions (Theas et al. 2008). It was discussed above that germ cells express Fas, which is one way the Sertoli cells can eliminate germ cells by apoptosis. Germ cells also express FasL, and have the power to cause apoptosis of lymphocytes that sneak through the basement membrane of the seminiferous epithelium (D'Alessio et al. 2001; Suda et al. 1993). Germ cells also produce antiviral and antibacterial molecules such as defensins which function within the excurrent duct system to reduce pathogen invasion and the need for immune response (Cheng and Mruk 2012).

Part of the theme of this overall chapter is that the testis has developed mechanisms to suppress adaptive immunity leaving intact the innate immune system that includes NK cells. If this were the scenario case, the NK cells would kill the germ cells that lack classical MHC class I antigens. Germ cells have developed a mechanism whereby NK cells (of the innate arm) and cytotoxic T cells (of the adaptive arm) can exist in the testis in their fully functional capacity against invading pathogenic microbes, but do not kill the germ cells that express foreign antigen. Recall that the meiotic divisions of germ cells result in expression of foreign antigen at a time well after tolerance has been established in the animals. These germ cells with foreign antigen avoid attack from cytotoxic T cells or NK cells by not expressing

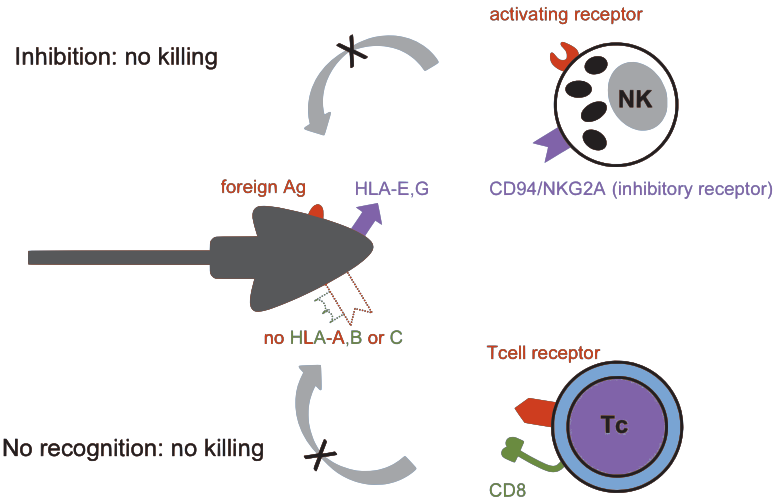

Fig. 10.13 Role of germ cells in avoiding immune attack. Germ cells modify their expression of MHC class I molecules to avoid immunologic attack by either cytotoxic T cells or NK cells in the vicinity. The germ cells fail to express classical MHC class I molecules (HLA-A, HLA-B, or HLA-C), and instead express non-classical HLA E and/or HLA-G. Cytotoxic T cells do not recognize these "non-classical" germ cells as "self" and fail to kill them. Cytotoxic T cells recognize only those antigens which are presented in context of classical MHC class I (HLA-A, -B and -C in humans), therefore absence of the classical MHC class I prevents Tc-mediated killing of germ cells. In contrast to the situation with Tc cells, the HLA-E and HLA-G molecules are capable of binding to inhibitory receptors on NK cells (e.g. CD94/NKG2A and ILT2, respectively), in much the same way that the classical MHC I molecules bind to the inhibitory KIR receptors on NK cells. NK cells typically kill cells that do not express MHC class I, but in this special situation the NK cells are prevented from killing germ cells even though the germ cells do not express the classic MHC class I molecules (HLA-A, B or C). Any foreign antigens on the germ cells might bind to activation receptors on NK cells, but likely will not overpower the inhibitory signal when HLA-E or HLA-G binds to CD94/NKG2A

the classical MHC Class I molecules (HLA-A or HLA-B or HLA-C), and instead expressing the non-classical class I molecules HLA-E and G (Fiszer et al. 1997) (Fig. 10.13). Because cytotoxic T cells require target cells to express classical MHC-I molecules in order to recognize "self" along with antigen recognition, the germ cell avoids detection by cytotoxic T cells. However, the germ cells, if devoid of HLA-A, HLA-B, or HLA-C, run the risk of getting killed by NK cells that are programmed to kill any cells which do not express MHC I. These classical MHC I molecules, which are necessary to bind to the KIR (killing immunoglobulin receptors) on NK cells to turn off the killing machinery, are not present on germ cells. If this KIR inhibiting receptor is not bound by classical MHC I, then the NK cell will attack. As a solution, the germ cells express HLA-E and G (i.e., the non-classical forms of MHC I), which also binds to a class of inhibiting receptors (e.g., CD94/NKG2A) on NK cells to inhibit NK cell killing. The ability of HLA-E and G to turn off NK cell killing by these additional inhibitory receptors is not absolute. These inhibiting receptors CD94/NKG2A can be overpowered by foreign antigens on the

germ cells, which bind to activating receptors on the NK cells. Therefore, as long as any foreign antigens don't *strongly* bind to *many* activating ligands on the NK cell in a manner that over powers the inhibitory signal, the NK cell stands down in the face of foreign antigen on the germ cells. In short, the "foreign" germ cells, due to modification of their MHC class I molecule expression, has been able to circumvent killing by either the cytotoxic T cells and the NK cells.

Neutrophils. Neutrophils are not present in the normal testis, and are present only in conditions of testicular inflammation or damage. Therefore, they play no role in maintaining immunological homeostasis.

Myoid Peritubular Cells (MPC's). The role of MPC's in inflammation or controlling the immune response is unknown. They participate in the testicular immune environment because they have been shown to produce the immunosuppressive factors TGFβ and activin A that inhibit T cell activation, and also produce monocyte-chemoattractant protein-1 (MCP-1) that may be responsible for recruitment of macrophages into the testis (Li et al. 2012).

10.4 Immune Privilege and Immunosuppression of the Excurrent Duct System

From the rete testis to the urethra, immunoregulation is provided by the common mucosal immune system, the blood-epithelial barrier, and a host of immunosuppressive and antimicrobial proteins (Fig. 10.10) In the section on the testis above, it became clear that while the adaptive immune system is suppressed, the innate immune system is fully functional and actually quite powerful. The TLRs on Leydig cells and Sertoli cells, and the NK cells, are ready to be called into action. If the innate system in the testis were activated however, inflammation in the testis would ensue, and this inflammation would have damaging effects on spermatogenesis. Therefore, the excurrent duct system becomes a very important structure to prevent upstream migration of microbes into the testis; to be the first line of defense against microbial; and to minimize any possibility of activating the testicular innate immune system which would effect spermatogenesis. In the excurrent duct system as in the testis, there is suppressed antigen specific immunity, but enhanced innate immunity.

10.4.1 Common Mucosal Immunity

Similar to the common mucosal immune system in other tissues, immune protection in the excurrent duct system is provided by the common mucosal immune system, which is predominantly composed of mucoid secretions, secretory IgA and intraepithelial $CD8^+$ T cells possessing cytotoxic activities. These defenses along with interstitial $CD4^+$ T cells, NK cells, and macrophages (both interstitial and intra-epithelial) contribute to normal innate and adaptive immune responses. Since there is no

mucosa-associated lymphoid tissue (MALT) in the male genital tract, the epithelial cells lining the genital tract are believed to act as antigen presenting cells (APC's) and present antigen to the intra-epithelial lymphocytes.

10.4.2 Immune cells of the Excurrent Duct System

Compared to the testis, macrophages and lymphocytes are more frequently observed within the epithelium and within the interstitium throughout the excurrent duct system and accessory sex glands. The interstitial lymphocytes are predominantly $CD4^+$, while the intraepithelial lymphocytes are predominantly $CD8^+$ (Ritchie et al. 1984; Hedger 2015a) (Fig. 10.10). In contrast to the seminiferous tubules, lymphocytes are readily able to cross the epithelium lining the excurrent duct system (Ritchie et al. 1984; Hedger 2015a).

The striking difference between the distribution and types of immune cells of the testis from that of the epididymis, is that in the epididymis, immune cells are present *both* within the interstitium and within the epithelium, while in the testis there are no lymphocytes within the epithelium. There are $CD4^+$ and $CD8^+$ T cells ('halo cells') as well as macrophages that reside in the epithelium of the epididymis. Basal cells of the epididymis may be intraepithelial macrophages (see discussion above). The interstitial macrophages bear MHC class II molecules and are effective antigen presenting cells, but the intraepithelial macrophages are MHC class II negative in rats and man (Ritchie et al. 1984) (Ritchie et al. 1984). These intra-epithelial MHC class II negative macrophages are primarily responsible for phagocytosis of senescent and excess sperm, and their deficient APC capabilities help keep the adaptive immune system somewhat suppressed even in the epididymis.

10.4.3 Blood-Epididymis Barrier (BEB)

There is evidence for a blood-epididymis barrier (BEB) that restricts movement of molecules across the epididymal epithelium, similar to that of the BTB. The BEB is the major way that adaptive immunity is suppressed in the excurrent duct system. The BEB is composed of tight junctions between adjacent principal cells, similar to the organization of other epithelial barriers. Sperm transport occurs in the epididymis of the rat in one to two weeks, and thereafter sperm is stored in the corpus for an indeterminate time. A BEB is important to keep the adaptive immune system from contacting the "foreign" antigens on the sperm, which can arise following the meiosis of germ cells throughout life after the host's immune tolerance is established. Even though the BEB may not be as tight as the BTB (Levy and Robaire 1999), circulating immunoglobulin cannot readily pass the BEB into the epididymal fluid. However, it appears that circulating immunoglobulin is not entirely excluded from getting into the epididymal fluid, because 2% of the circulating level of IgG can be found in the caudal epididymal fluid (Mital et al. 2011). The presence of IgG

in the caudal fluid does not necessarily indicate there is transepithelial passage of immunoglobulin, because this amount of IgG in the epididymal fluid could originate from 'leakiness' of the rete testis (Knee et al. 2005).

10.4.4 Immunosuppressive Proteins of the Excurrent Duct System

Throughout the excurrent duct system there are immunosuppressive proteins that actively suppress the need for activation of either the innate or adaptive immune systems. Seminal fluid contains immunosuppressive substances that inhibit T cell and NK cell activities. These substances include prostasomes, oxidized polyamines, prostaglandins of the E series, nonspecific lymphocyte suppressing proteins and immunoregulatory cytokines TGFβ1 and TGFβ2, activin and IL-10. Prostasomes and polyamines are responsible for much of the immunosuppressive activity of the ejaculate. Prostasomes are multilaminar vesicles secreted by the normal prostate and are a major component of human semen. They inhibit mitogen-induced T cell proliferation and macrophage phagocytic activity. Oxidized polyamines such as spermine and spermidine inhibit cell growth. Prostaglandins of the E series inhibit T cell proliferation and NK cytototoxicity (Quayle et al. 1989; Tarter et al. 1986). Other immunosuppressive factors are TGFβ1 and TGFβ2 derived from the seminal vesicles and prostate gland; IL-10 derived from macrophages within these accessory glands; and activin A derived from the prostate, testes and epididymis. As with Sertoli cells and germ cells, epididymal epithelium produces β-defensin and defensin-like proteins with antimicrobial properties. Some of these proteins are epididymis-specific (e.g. bin1b, EP2, lactoferrin, cystatins, Eppin and certain cathelicidins) (Li et al. 2012). Most of these peptides have other functions in addition to their antimicrobial function such as involvement in initiation of sperm motility (i.e., Bin1b) (Zhou et al. 2004) or involvement in the ability of sperm to fertilize and egg (i.e., eppin) (O'Rand et al. 2011).

10.5 Endocrine-Immune System Interaction

There is a close relationship between the development and maintenance of immune and reproductive systems in mammals. Normal development of the male reproductive system is linked to the normal development of the local immune environment and *vice versa*. The close association of development of the testes and the immune system was reported by Calzolari (1989) after he found that castration of rabbits before puberty led to an increase in the size of the thymus. His work established that products of the testis, the sex steroids in particular, regulate the immune system directly or indirectly (Calzolari 1989; Sasson and Mayer 1981; Kimura et al. 1995). Even after postnatal development of the reproductive system is complete and immunologic tolerance is established, the endocrine system still maintains asuppressive effect on the immune system in males.

10.5.1 Hormones That Suppress the Immune Response

Androgens exert an inhibitory effect on innate and adaptive immune activation and the presence of androgen within the interstitium contributes to the immune privilege of the testis. Compared to females, males have lower serum immunoglobulin levels, reduced cellular immune responses to antigenic challenge, and a lower incidence of autoimmune diseases (Ahmed and Talal 1990; Castro 1974; Grossman 1984; Roubinian et al. 1978). The absence of androgen receptors on circulating white blood cells suggests the inhibitory effects of androgens on immunity are exerted indirectly. However, even though the classic androgen receptors (AR receptors) are not present on circulating lymphocytes, androgens might still be acting directly on these cells. Androgens may alter Ca^{++} fluxes in lymphocytes and macrophages by interacting with membrane-bound G protein-coupled receptors and thereafter modulating transcription factors (Benten et al. 2004). In addition, androgens act on the immune cells by inhibiting NFκB and the subsequent expression of inflammatory genes such as TLR3, IL-1, IL-6 and TNF (Rettew et al. 2008; Norata et al. 2006). The immunosuppressive role of testosterone in the immune privileged testis is mainly derived from experiments with "experimental autoimmune orchitis" (EAO) models and is discussed further below (Fijak et al. 2011).

The specific actions of androgens on maintenance of immune privilege in the testis are complex, but in general testosterone and dihydrotestosterone (DHT) dampen both non-specific and antigen-specific immunity by several mechanisms. Testosterone dampens inflammation by directly binding to the AR receptor on $CD4^+$ T cells to produce the anti-inflammatory cytokine IL-10 (Liva and Voskuhl 2001). Il-10 production would help shift the balance in favor of Th2 or Th3 cells for antibody production and/or tolerance rather than the Th1 cells that favor cell-mediated immunity (Hedger and Hales 2006). Androgens also are associated with a shift in favor of increased regulatory T cells ($CD4^+$ $CD25^+$ $Foxp3^+$) with decreased numbers of $CD4^+$ helper T cells in the interstitium.

Androgens are also involved in maintaining the integrity of the BTB, which is a major component of the immune privilege in the testis (Janecki et al. 1992; Chung and Cheng 2001; McCabe et al. 2010; Yan et al. 2008). Without androgen, mice have a defective BTB with reduced proteins in the ectoplasmic specializations, likely a result of perturbed Sertoli cell maturation and polarization (Willems et al. 2010a, b). Androgens are also important in the on-going dis-assembly and re-assembly of the BTB during spermatogenesis, and in particular the relocation of integral membrane proteins from the old BTB site above the pre-leptotene cells to the new BTB site below these cells via transcytosis (Cheng and Mruk 2010; Su et al. 2010).

In addition to androgen, other protein hormones and neuropeptides produced by the Leydig cells can affect the immune response. The pro-opiomelanocortin peptide, α-melanocyte-stimulating hormone and a macrophage inhibiting factor (MIF) produced by Leydig cells have inhibitory effects on lymphocyte and macrophage activity and specifically inhibit the cell killing activity of cytotoxic T cells and NK cells (Meinhardt et al. 1996; Hedger and Hales 2006).

10.5.2 Role of Inflammatory Mediators in Spermatogenesis

Above, we discussed how hormones produced by the "endocrine" cells of the testis are involved in maintaining immune balance. In particular the Sertoli cell, through its production of immunosuppressive substances (such as clusterin, actin and TGFβ) and its mechanisms to prevent immune activation (such as IDO production) plays a role in keeping damaging inflammation in abeyance. However, there is some irony in this system, because Sertoli cells actually produce a large number of inflammatory cytokines and do so continually during normal homeostasis. Sertoli cells actually incite a mini-inflammatory reaction in order to support normal spermatogenesis. As long as these inflammatory mediators are controlled to very low physiologic levels, normal spermatogenesis ensues, and tissue inflammation is prevented. The following section will discuss the pro-inflammatory mediators produced by the somatic cells. It will emphasize how low levels are required for endocrine function, but pathologic levels are destructive.

Inflammatory mediators produced by Sertoli cells and germ cells play a significant role in spermatogenesis under normal circumstances. During spermatogenesis, the Sertoli-Sertoli and Sertoli-spermatid ectoplasmic specializations (i.e., the BTB) must be constantly dis-assembled and re-assembled as spermatocytes proceed through their maturation. Hormones such as testosterone, estrogen, and FSH play a role in this process (Yan et al. 2008); however, "inflammatory" cytokines participate as well. Cytokines and inflammatory mediators, such as IL-1α, TGF-β, TNFα (Xia et al. 2009) and nitric oxide (Yan et al. 2008; O'Bryan and Hedger 2008) are produced by the Sertoli cells and germ cells under normal circumstances to break down and rebuild the BTB (Fig. 10.14). Residual bodies left over from normal spermiation drive this localized inflammatory process. Residual bodies are the redundant organelles and excess cytoplasm shed from the elongating spermatid in its final steps of maturation. These residual bodies activate a localized mini-inflammatory reaction by stimulating production of the pro-inflammatory mediator IL-1α by Sertoli cells. IL-1α in turn activates IL-6 and activin (Syed et al. 1995; Perez et al. 2013; Okuma et al. 2005). All three of these cytokines play a role in spermatogenesis. IL-1 has stimulatory effects on spermatogonial proliferation and spermatocyte survival and stimulates testosterone production by Leydig Cells. IL-6 produced by Sertoli cells generally down-regulates meiotic DNA synthesis in the pre-leptotene spermatocytes and is generally regarded as having inhibitory effects on spermatogenesis (Hakovirta et al. 1995; Pollanen et al. 1989; Parvinen et al. 1991). Activin that is produced by Sertoli-cells modulates the effects of both IL-1 and IL-6 in the performance of their spermatogenic function (Pollanen et al. 1989). While the Sertoli cell is regulating proliferation and maturation of spermatocytes, the germ cells themselves are producing TNFα and nitric oxide important for the reorganization of the tight junctions. This localized mini-inflammatory response is easily thrown out of balance by an exogenous or toxicologic insult.

There is additional work underway to identify the role, if any, of TLRs on the Sertoli cells during normal spermatogenesis. Above, we learned that TLRs are

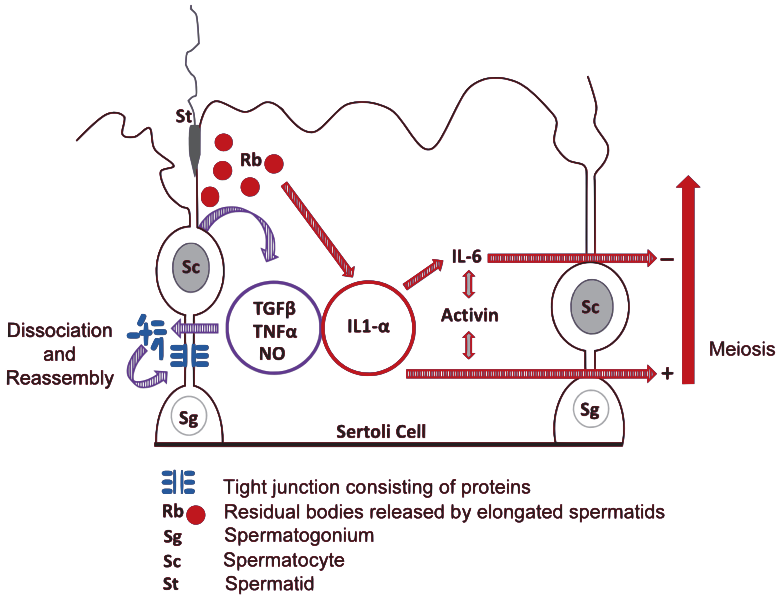

Fig. 10.14 Inflammatory mediators in normal spermatogenesis. Inflammatory mediators are a normal part of spermatognesis. Residual bodies (Rb) shed from the elongating spermatid (St) in the final steps of maturation stimulate production of IL-1α by Sertoli cells (Sc), which in turn activates IL-6 and activin. IL-1 stimulates, and IL-6 inhibits, meiotic activity of germ cells. Activin modulates these competing effects. Sertoli cells and germ cells also produce the pro-inflammatory mediators TGF-β, TNF-α, and nitric oxide (NO), which are critical for the repeated dissociation and re-assembly of the BTB

perched and ready to bind to pathogen-associated molecular patterns (PAMPs) on invading microorganisms, and represent part of the innate immune system to prevent initial infection. However, these TLRs on Sertoli cells might play a role during normal spermatogenesis as well. When TLRs are activated, the Sertoli cells produce IL-1α and IL-6, and this observation has prompted investigators to search for a role of TLRs during the normal localized mini-inflammatory reaction associated with normal spermatogenesis (Hedger 2015a; Winnall et al. 2011).

10.5.3 Endocrine Function of Immune Cells

In the section above, we discussed how the Sertoli cell uses "immunologic mechanisms" to perform its role to support spermatogenesis. The conventional immune cells, which are ironically subdued from using their immunologic mechanisms, take on an unconventional endocrine role. One such immune cell is the ED2 macrophage. Although they have an important immunological role in production of immunosuppressive IL10 and phagocytosis of exogenous material in the interstitium, they

also facilitate development and function of the Leydig cell (Hedger and Hales 2006). In fact, the trophic effect of ED2$^+$ interstitial macrophages on Leydig cell development might be the macrophage's most important role in the testis. Macrophages also produce IL-1β that plays a role in the proliferation of Leydig cells during prepubertal development (Raburn et al. 1993). Moreover, it has been demonstrated that Leydig cells will not develop in immature rats in the absence of functional macrophages (Gaytan et al. 1994a, b). In the normal adult, testicular macrophages provide substrate for the Leydig cells to produce steroid. The macrophages express cholesterol 25 hydroxylase enzyme and produce 25 hydroxycholesterol from cholesterol. The 25-hydroxycholesterol serves as a substrate for testosterone biosynthesis that bypasses the need for steroidogenic acute regulatory protein (STAR) (Lukyanenko et al. 2002). As an interesting side note, the 25 hydroxylase enzyme is negatively regulated by testosterone, suggesting that there may be a small internal feedback loop between the testicular macrophages and the Leydig cells) (Lukyanenko et al. 2002).

The cross talk between Leydig cells and macrophages is achieved by specialized interdigitation linking the Leydig cells and macrophages (Hutson 1992). With aging these interdigitations are lost (Giannessi et al. 2005). This lack of interdigitation and cross-talk between macrophages and Leydig cells might play a role in reduced fertility in aged animals.

The interstitial macrophages always have potential to be activated into a pro-inflammatory state, should there be physical tissue damage or breakdown of the BTB. If macrophages are activated, they secrete pro-inflammatory cytokines that overwhelm the trophic effects that ED2$^+$ macrophages have on steroidogenesis, and these overwhelming pro-inflammatory cytokines restricts the ability of the Leydig cells to produce androgen (Hales 2002). Under normal homeostatic conditions, the macrophage supports the Leydig cell, but under adverse conditions of inflammation or disruption of homeostasis, the macrophage becomes the enemy of the Leydig cell.

10.6 Inflammatory Disease

There is a delicate balance of endocrine and immunologic systems operating in the testis. On one hand there is positive inter-relationship between androgen levels and maintenance of an anti-inflammatory microenvironment. On the other hand, spermatogenesis requires "inflammatory cytokines". The homeostasis of the testis is highly vulnerable to imbalances by any upset of the endocrine or the immune system. As will be discussed in the following sections, activation of the immune system and inflammation leads to androgen insufficiency and/or infertility. Androgen insufficiency, in turn, results in immune activation. Regardless of the cause, immune activation and androgen deficiency enter a death spiral where one process accentuates the other with the end result of aspermatogenesis. Therefore it is imperative that normal homeostasis be maintained, because anything other than homeostasis is damaging to the testis and to the germ cells.

10.6.1 How Inflammation Affects Spermatogenesis

Non-specific inflammation of the testis (orchitis) due to trauma, heat, or infectious has devastating effects on reproductive function. Fever and a raised body temperature associated with inflammation is but one cause for testicular failure associated with inflammation. Inflammation, regardless of the inciting cause, leads to reduced steroidogenesis, androgen deficiency, and reduced spermatogenesis.

Many of the pathogenic mechanisms leading from inflammation (orchitis) to infertility have been elucidated using animal models for inflammation and autoimmune orchitis. As discussed above, numerous inflammatory mediators and immunoregulatory molecules are produced normally by Sertoli cells, Leydig cells and even spermatogenic cells during homeostasis. These molecules include IL-1, IL-6, TNFα, TGFβ, and nitric oxide. In inflammation, the population of testicular macrophages changes, with an influx of pro-inflammatory ED1$^+$ (immature) macrophages that produce high levels of IL1, TNFα and nitric oxide (Rival et al. 2008) (Fig. 10.11). An influx of T cells and neutrophils accompanies the increase in macrophages in the inflamed testis (Tung et al. 1987c; Wang et al. 1994; Widmark et al. 1987; Bergh et al. 1993; Hedger 1997) and these T cells and neutrophils produce additional pro-inflammatory cytokines like IL-2 and IFNγ. The high cytokine level leads to disruption of the BTB, sloughing of spermatocyte and spermatids, and increased apoptosis of germ cells. In particular, excess IL-1α released by invading macrophages facilitates BTB opening by affecting the actin cytoskeleton (Sarkar et al. 2008), and disrupts the co-localization of proteins like cadherin, catenin, claudin and ZO-1 (Perez et al. 2012). Other specific molecular events associated with breakdown of the BTB following Sertoli cell toxicity and excess pro-inflammatory cytokines can be found in literature describing the pathogenesis of cadmium and bisphenol-A (BPA) toxicities (Cheng et al. 2011b).

Mast cells participate in the inflammatory process and can be present in granulomas (Apa et al. 2002). They can increase more than tenfold in number in EAO models (Iosub et al. 2006). They secrete the serine protease tryptase, which induces fibroblast proliferation and collagen synthesis, resulting in tissue fibrosis and sclerosis (Abe et al. 1998).

The inflammatory mediators induced by inflammation serve to reduce androgen production locally at the level of the Leydig cell, but also centrally at the level of the pituitary gland (Fig. 10.15). As mentioned above, macrophages assist in steroidogenesis by Leydig cells under normal condition but will inhibit steroidogenesis in inflammatory conditions (Hedger 2012). IL-1 and TNFα produced by activated ED1$^+$ macrophages repress steroidogenic enzyme gene expression in Leydig cells leading to lower androgen production. Reactive oxygen species produced by ED1$^+$ macrophages result in perturbation of mitochondria and inhibition of steroidogenic acute regulatory protein (STAR) expression. Activated T-cells in areas of inflammation produce cytokines IFNγ and IL-2, and both of these cytokines inhibit steroidogenesis in Leydig cells. IFNγ inhibits cholesterol transport into the mitochondria at level of STAR, and IL-2 inhibits the Leydig cell's ability to respond to luteinizing hormone, all contributing to lower androgen levels. However, the lower androgen

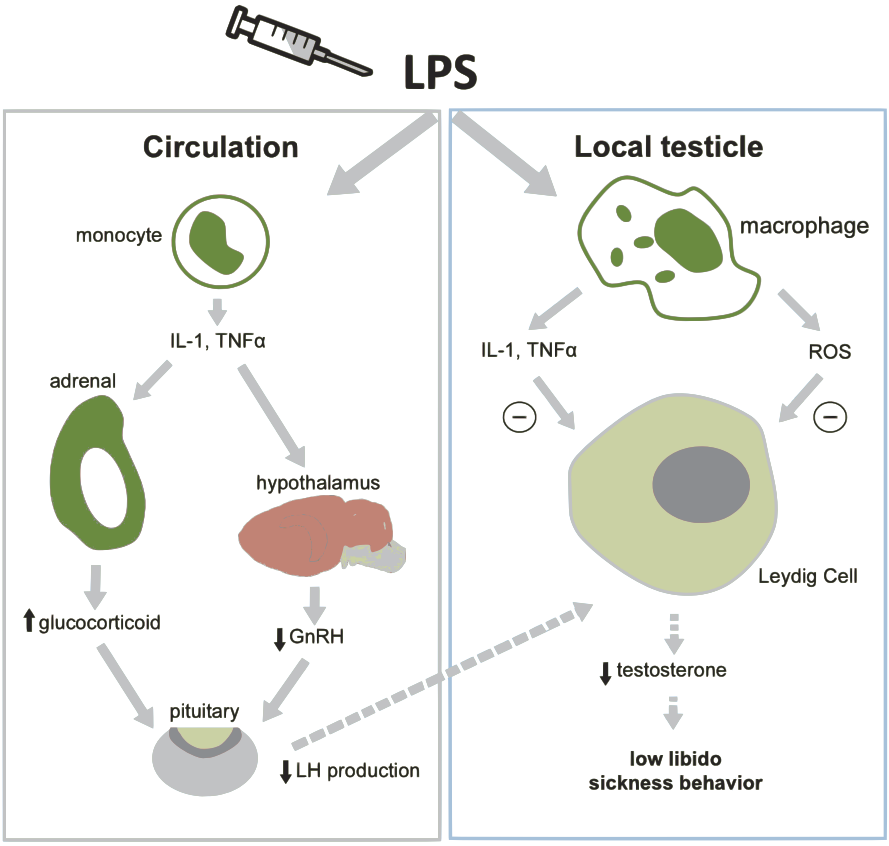

Fig. 10.15 Sickness behavior. Activation of the innate immune system with LPS has both local and systemic effects that result in low testosterone. LPS effects local resident testicular macrophages to increase production of ROS, IL-1 and TNF-α. These pro-inflammatory mediators turn off steroidogenesis at the level of the Leydig cell. LPS also stimulates circulating monocytes to produce IL-1 and TNF-α. These systemic pro-inflammatory mediators result in increased glucocorticoid production by the adrenal gland, which reduces gonadotropin release from the pituitary. IL-1 and TNF-α may also directly activate neural pathways to turn off LH production at the level of the pituitary gland. Lower testosterone level is the end result of local and systemic increases in pro-inflammatory mediators

level that results from pro-inflammatory cytokines and reactive oxygen species does not result in diminished spermatogenesis, because there normally is a surplus of intratesticular androgen. Even relatively large declines in testicular androgen would result in only minor losses of spermatogenic efficiency (Zirkin et al. 1989, 1993). However, any reduction in circulating androgen could have profound effects on the androgen-dependent functions, such as accessory gland function, secondary sex characteristics and libido. It follows that loss of secretions of the accessory sex glands (e.g., decreased defensins, prostasomes, etc.) would reduce the innate defense mechanisms of the excurrent duct system.

In addition to local decrease in androgen synthesis, the pro-inflammatory cytokines produced by macrophages in the testis can indirectly lead to lower androgen production indirectly through the pituitary. Pro-inflammatory cytokines activate the hypothalamic-pituitary-adrenal gland (HPA) axis, leading to increased secretion of ACTH and increased production of glucocorticoid by the adrenal cortex. The glucocorticoids result in an inflammation-induced "stress response" which down-regulates pituitary gonadotropins with eventual decline production of testosterone by Leydig cells (Whirledge and Cidlowski 2010).

Within this understanding that inflammation leads to lower androgen levels, it is no wonder that male sexual function and general well-being operate in a reciprocal relationship. The yin/yang relationship between the male reproductive endocrine system and immune system has been coined as "sickness behavior" (when the immune system is activated during sickeness) and "testosterone behavior" (under normal reproductive conditions when libido and feelings of well-being dominate and the immune system is not activated). Sickness behavior represents an organized behavioral strategy orchestrated through the macrophage and Leydig cell interaction along with the systemic endocrine feedback loops (Fig. 10.15). It is characterized by depressed male aggression, and loss of libido. In general, sickness behavior does not cause deleterious effects on spermatogenesis. This is because the interstitial microenvironment has a surplus of testosterone that provides sufficient support for the Sertoli cell and the BTB to support spermatogenesis.

10.6.2 Autoimmune Infertility

Autoimmune infertility is a condition in humans in which there is infertility and a circulating titer of sperm autoantibodies. Sperm autoantibodies can be identified in 5–10% of all cases of human male infertility (Hedger 2015a; Lenzi et al. 1997; Baker et al. 1983). Sperm autoantibodies can be induced by damage to the testis (by trauma, vasectomy, heat or inflammation) or it may be idiopathic. After vasectomy, seventy percent of human patients develop sperm autoantibodies (Ansbacher 1973; Hellema et al. 1979; Bigazzi 1981; McDonald 2000). Sperm autoantibodies may even occur in the absence of any tissue damage, and the cause is unknown. Whether the autoantibodies are induced by damage or arise spontaneously, a genetic predisposition for development of sperm autoantibodies is suspected. In autoimmune infertility, it is difficult if not impossible to determine if the autoantibodies are responsible for the infertility or if the infertility and sperm autoantibodies are coincidental consequences of the same genetic trait. However, sperm autoantibodies can cause or magnify the infertility by their effect on sperm transport, sperm capacitation, acrosome reaction, and sperm-egg interaction (Gleicher 1998). Autoimmune infertility in man involves multiple antigens, which differ from individual to individual, and there is no single dominant antigen (Hedger and Hales 2006). The autoimmune reaction to multiple antigens as opposed to a single antigen makes sense

when one considers the pathogenesis. In certain autoimmune diseases, such as type I diabetes, pemphigus or autoimmune gastritis, the development of autoimmunity is due to disruption of the normal regulatory controls of the immune system, and immunologic tolerance to a specific antigen breaks down (Tsunoda et al. 2002; van Driel et al. 2002). With breakdown of tolerance, the immune system attacks antigens that are normally ignored. In these disease states, a dominant autoantigen is usually identified (Toh et al. 2000). In the case of male infertility (or autoimmune orchitis) on the other hand, tolerance to sperm foreign antigens has never been established in the first place. So the pathogenesis is not due to loss of tolerance, but rather to inappropriate exposure of foreign antigens to the immune system, or loss of suppression of the immune system in general. Therefore, multiple antigens are involved in autoimmune infertility (or autoimmune orchitis) (Hedger 2015a).

10.6.3 Autoimmune Orchitis

Inflammation of the testis is called orchitis. When orchitis is also associated with circulating sperm autoantibodies, the term autoimmune orchitis is used to subclassify the inflammatory condition. Autoimmune orchitis can develop secondary to a primary inflammatory condition (orchitis), or the autoimmune condition can be the inciting cause for the inflammation. Any non-specific inflammation, incited by any means, can result in breakdown of the BTB by pro-inflammatory cytokines (TNF, IFNγ, and nitric oxide), and exposure of the immune system to germ cell "foreign" antigens with production of sperm autoantibodies. The bottom line is that when the normal mechanisms of immune privilege breaks down, and there is disruption of the local immunoregulatory mechanisms, orchitis occurs. It should be noted that sperm autoantibody production, by itself, does little to perpetuate the inflammation, and there is little correlation between the presence or severity of testicular inflammation and the level of sperm autoantibodies. Only a very low percentage of these autoantibody-positive patients ever develop orchitis. Furthermore, many sperm autoantibody-positive males have normal fertility. This is because sperm autoantibodies are not considered a significant contributing factor to testicular inflammation, and antigen-antibody immune complexes are not important to the pathogenesis of orchitis. Instead, T cell-mediated immunity is what fuels the inflammation in the testis (Gleicher and el-Roeiy 1988). This is not to say sperm autoantibodies are useless or meaningless. Sperm autoantibodies are important *diagnostic* tool in humans because they provide a clue as to the autoimmune pathogenesis. Sperm autoantibodies also have clinical relevance because the antibodies can lead to infertility by their action on the ejaculate.

Autoimmune orchitis can also occur in instances where there is cross-reactivity of sperm antigens to salivary gland antigens. This cross-reactivity is not dissimilar to the proposed cross-reactivity between thyroid and ovarian antigens in females with autoimmune thyroiditis and premature ovarian failure (Cohen and Speroff 1991).

10.6.4 Experimental Models of Testicular Inflammation and Autoimmune Orchitis

Experimental models of testicular inflammation in rats and mice are used by researchers in the field of reproductive physiology to elucidate the pathogenesis of inflammation and sperm autoantibody production, whether that inflammation is caused by innate immunity (LPS, HCG) or adaptive immunity (vasectomy, EAO). Regardless of the pathogenesis of the inflammation, whether it is primary inflammation or secondary to autoimmune disease, the histologic effects on spermatogenesis are similarly devastating. This section will highlight those experimental models used in immunophysiological research.

LPS induced inflammation is one such model for studying the effects of activation of the innate immune system on testicular steroidogenesis. Acute systemic administration of LPS causes an endotoxic shock-like condition with vasodilatation leading to increased numbers of intratesticular macrophages in the rat (Gerdprasert et al. 2002) and neutrophils in the boar (Wallgren et al. 1993, 1995). It eventually leads to apoptosis of spermatogonia and spermatocytes with sloughing of spermatocytes and early round spermatids from the epithelium *after* the endotoxic condition is resolved (O'Bryan et al. 2000). The damage is due to disruption of the BTB by overwhelming amounts of pro-inflammatory cytokines.

HCG Hyperstimulation. This inflammatory model is induced by injection of high doses of HCG. HCG causes a hyperstimulation syndrome consisting of a transient decrease in testicular blood flow, followed by increased blood flow, opening of vascular endothelial cell junctions and increased interstitial fluid 16-24 hours later. Other histologic changes include accumulations of intravascular and interstitial neutrophils, failure of mature sperm release, vacuolation of seminiferous epithelium, and apoptosis and loss of spermatogonia and primary spermatocytes. The HCG is speculated to cause dramatic production and secretion of the pro-inflammatory mediator IL-1β by Leydig cells (Lin et al. 1993; Bergh et al. 1996; Veijola and Rajaniemi 1986) and can be eliminated by depletion of the Leydig cells with ethane dimethane sulfonate (EDS) (Sowerbutts et al. 1986; Setchell and Rommerts 1985; Veijola and Rajaniemi 1985).

Ischemia (transient testicular ischemia or torsion of spermatic cord.) Transient testicular ischemia **i**n mice results in apoptosis of spermatogonia, an influx of neutrophils in the testicular subcapsular venules, and an increase in reactive oxygen species (ROS) in the interstitium (Lysiak et al. 2001, 2003). Ischemia causes a similar accumulation of neutrophils in the rat. Locally produced IL-1β and TNFα are implicated as mediators of this response. This ischemia model is similar to HCG induced inflammation because both are associated with recruitment of neutrophils and both involve IL-1β. Ischemia causes rapid apoptosis of spermatogonia and early spermatocytes (Tjioe and Steinberger 1970; Turner et al. 1997; Lysiak et al. 2001).

Following ischemia by spermatic cord torsion, the contralateral testis is affected by an influx of macrophages and mast cells, with apoptosis and sloughing of germ

cells. The effect on the contralateral testis is due to humoral and cellular immune-mediated testicular cell damage (Rodriguez et al. 2006).

Experimental Vasectomy. Vasectomy leads to inflammation followed by development of sperm antibodies and inflammation of the testis and epididymis, and is an animal model for autoimmune orchitis. In the Lewis rat, certain strains of mice, and in guinea pig and rhesus monkey, vasectomy results in rapid progression to full blown orchitis (Bigazzi et al. 1977; Herr et al. 1987; Kojima and Spencer 1983; Taguchi and Nishizuka 1981). Often the contralateral testis is involved, by a condition called sympathetic orchiopathia, which results from transfer of $CD4^+$ cells and autoreactive lymphocytes to the opposite testis. However, vasectomy in most strains of rats and man leads only to formation of sperm antibody without significant inflammation, and at most, sperm granulomas in the epididymis in mice and rats (Tait et al. 2000). In the B6AF1 mouse, vasectomy leads to tolerance and resistance to EAO, presumably due to a shift toward $CD4^+CD25^+Foxp3^+$ regulatory T cells. Therefore, whether there is resistance to EAO or full blown orchitis following vasectomy is dependent on the strength of the Treg response, which is apparently under genetic control (Wheeler et al. 2011).

Animal Model of PGA syndrome. Type A polyglandular autoimmune syndrome (PGA) is a generalized autoimmune disorder where the body develops antibodies against steroidogenic specific antigens expressed by steroidogenic cells, including the Leydig cells and adrenal cortical cells. Autoimmune orchitis and Addison's disease are the typical presentation. The autoimmune orchitis that ensues then leads to breakdown of the BTB and eventual production of sperm autoantibodies. The animal model for PGA syndrome involves thymectomizing mice at post-natal day (PND) 3. Removal of the thymus causes a polyglandular autoimmunity similar to that found in human PGA syndrome (Sakaguchi and Sakaguchi 1990; Tung et al. 1987a, b). The thymus is critical to the development of tolerance to self-antigen, and the absence of a thymus would result in lack of this tolerance and susceptibility to autoimmune disease. Thymectomy at 3 days of age causes a reduction in regulatory T cell subsets in the adult, results in spontaneous epididymo-vasitis and eventually orchitis (Lipscomb et al. 1979; Taguchi and Nishizuka 1981; Tung et al. 1987a, b; Hedger 2012).

The Aire-deficient mouse has a deficiency in the autoimmune regulator gene (Aire gene) that regulates thymic expression of various tissue specific autoantigens, particularly autoantigens of the endocrine system (Ramsey et al. 2002; Kriegel et al. 2004; Hedger 2012). The hypogonadism in this Aire-deficient mouse is due to autoimmunity against the Leydig cells (steroid producing cells), thus the damage to spermatogenesis and sperm production is considered secondary (Maclaren et al. 2001). Sperm antibodies develop in the Aire-deficient mouse and the epididymis contains numerous inflammatory cell infiltrates (Hubert et al. 2009).

Experimental Autoimmune Orchitis (EAO). EAO is an animal model for human autoimmune orchitis. In this model, rats or mice are immunized with spermatogenic cells, whole sperm, or with testicular or epididymal extracts with adjuvants. EAO

can also be induced by the adoptive transfer of $CD4^+$ T cells to mice (Tung et al. 1971, 1987a, b, c; Hedger 2012). Following immunization or adoptive transfer of T cells, there is sperm autoantibody production, invasion of mixed inflammatory cells (monocyte, lymphocytes, neutrophils, mast cells and eosinophils) into the seminiferous tubules, focal necrosis of spermatogenic epithelium, sloughing of germ cells, and finally aspermatogenesis (Kohno et al. 1983; Doncel et al. 1989; Zhou et al. 1989). The epididymis and vas deferens are involved as well. At first, the inflammatory lesions are confined to several foci of a few seminiferous tubules, but then extend to the whole organ in the chronic severe stage in which formation of granulomas is frequent. In mice and rats, EAO invariably involves the rete testis, since this area has greatest access to the antigens of spermatogenesis (due to deficient BTB), and the interstitial tissue around the rete is where there is a large concentration of MHC class II macrophages.

The tissue damage and inflammation of EAO is due primarily to the testicular macrophage and the interstitial dendritic cells (Perez et al. 2012; Rival et al. 2008). IL-1α is responsible for disruption of the BTB in a manner similar to LPS induced inflammation model (described above). The dendritic cells in EAO undergo a process of maturation with higher expression of CCR7 (the chemokine receptor involved in cell migration to lymph nodes). This result is associated with an increased number of dendritic cells in the draining lymph nodes. The activated dendritic cells in the draining nodes express high levels of MHC class II and IL-12p35 mRNA and significantly enhance the proliferation of naïve T cells to become Th1 cells active in cell-mediated immunity (Guazzone et al. 2011).

Apoptosis of germ cells is a feature of EAO. It is most likely that apoptosis is a defense mechanism of the Sertoli cell, causing apoptosis of germ cells by the FasL-Fas mechanism to preserve its reserves. In EAO, it has been shown that the number of Fas + germ cells correlated with increased number of apoptotic germ cells. In the case of any type of insult, up-regulation of Fas on germ cells, and up-regulation of FasL on Sertoli cells might be a way to enable the Sertoli cells to eliminate germ cells which cannot be supported adequately, and for the Sertoli cell to save its resources to support the cells that it can save (Lee and Huang 1997).

10.7 Spontaneous Disease Associated with Immune Disruption

Inflammation of the testis is uncommon in laboratory animals (Creasy et al. 2012; Boorman et al. 1990). Inflammation of the testis may be caused by trauma, bacterial infections or by spontaneous autoimmunity. Inflammation, regardless of its origin, will result in an influx of neutrophils, macrophages, and lymphocytes (Figs. 10.16 and 10.17). In general, however, a neutrophilic infiltrate is usually associated with tubular necrosis, which generally is associated with vascular compromise or ischemia. A macrophage or granulomatous infiltrate is usually associated with foreign body reactions to leakage of sperm, and a lymphocytic

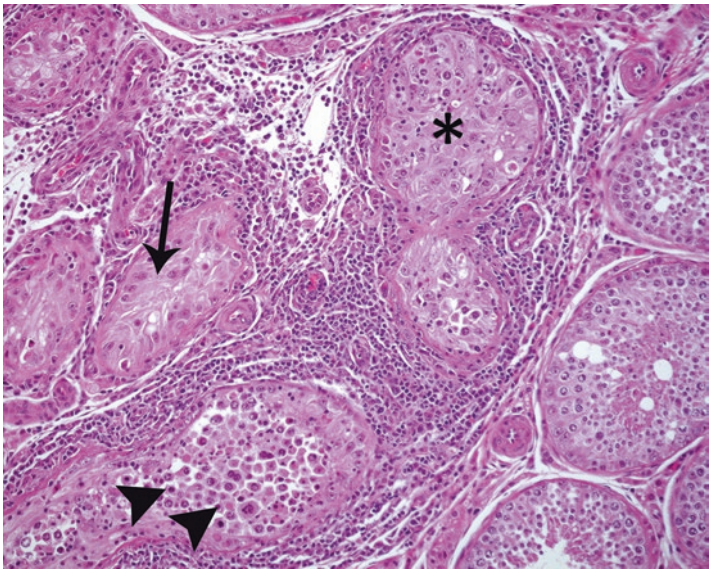

Fig. 10.16 Orchitis in dog. This is a case of spontaneous orchitis in a dog with non-specific lymphocytic inflammation. There is a diffuse infiltration of lymphocytes and fewer plasma cells throughout the interstitium with some breach of these inflammatory cells into the seminiferous tubules (asterisk). There is loss of orderly spermatogenesis in most tubules, with apoptosis/degeneration of germ cells (arrowhead). While the inciting event for this inflammation is unknown, the lymphocytic infiltrate suggests a major component of the inflammatory reaction is an immunologic response, most likely to germ cell antigens. Excess inflammatory cytokines released from the inflammatory cells will overpower the normal level of cytokines produced by the Sertoli cells to support spermatogenesis. The high level of inflammatory cytokines leads to loss of spermatogenesis (arrow) and breakdown of the BTB. This in turn leads to leakage of antigenic protein into the interstitium which incites more inflammation, thus the inflammatory process self-perpetuates. The apoptotic germ cells result from inflammatory cytokine-induced upregulaton of Fas-FasL on Sertoli and/or germ cells. H&E stain. 10× objective magnification (image from personal files of Dr. Dianne Creasy)

infiltrate generally indicates an autoimmune cell-mediated reaction. The infiltration is generally accompanied by edema and or hemorrhage, and it may destroy and replace tubules (Creasy et al. 2012). Inflammation, regardless of the type, generally indicates that the tight junctions between Sertoli cells are breached or the Sertoli cells have been damaged.

A strain of mink (Tung et al. 1981a, b), the Brown Norway rat and a certain breed of dog (Fritz et al. 1976) have naturally arising condition of **spontaneous autoimmune orchitis** (Furbeth et al. 1989). Lymphocytic orchitis associated with thyroiditis has been identified in a colony of beagle dogs (Fritz et al. 1976). The lesion in the testis consisted of variable degrees of lymphocytic infiltrates with nodular, diffuse or aggregate distribution, associated with varying degrees of tubular degeneration and atrophy. Plasma cells and histiocytes were occasionally noted. However, an appreciable number of dogs had tubular atrophy and degeneration in the absence of

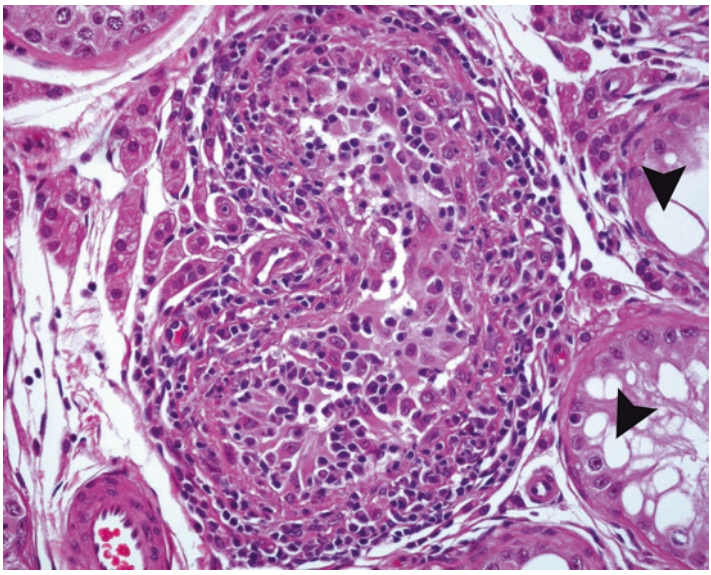

Fig. 10.17 Orchitis in dog. In this image the lymphocytic inflammatory infiltrate is obscuring a seminiferous tubule. Other seminiferous tubules to the right are lined only by Sertoli cells, indicating the inflammation has a general damaging effect on spermatogenesis. Note the vacuolated Sertoli cells (arrowhead), which is a non-specific indication of damaged, yet viable, Sertoli cells. Based on histopathology alone, the inciting cause for the inflammation cannot be determined in this spontaneous case of orchitis. H&E stain. 20× objective magnification (image from personal files of Dr. Dianne Creasy)

a lymphocytic reaction In humans, orchitis **c**an be caused by viral infections (such as mumps, Coxsackievirus B, varicella, human immunodeficiency virus, Dengue fever and Epstein Barr virus), bacterial infections (E coli, Chlamydia, and Neisseria syphilis) and mycobacterial infections (M. tuberculosis). In dogs, *Brucella* spp., *Esherichia coli*, *Leishmania* spp. (Diniz et al. 2005) and blastomycoses (Totten et al. 2011) can cause orchitis. Brucellosis of dogs deserves special mention. *Brucella canis* is an intracellular bacterium that targets the reproductive steroid-dependent tissue, such as the prostate, testis and epididymis in males, and the fetus, gravid uterus and placenta in females. The cellular damage caused by Brucella infection results in orchitis and epididymitis followed by leakage of sperm antigen, sperm granuloma formation, and immune-mediated orchitis and epididymitis. Brucella is taken up by macrophages. By yet unknown ways, the organism avoids the adaptive immune response by down regulating MHC II and is able to survive in macrophages without its antigens being processed (Forestier et al. 2000; Barrionuevo et al. 2008).

Inflammation of the epididymis is uncommon for the same reason it is uncommon in the testis. However, inflammation of the epididymis and vas deference has been reported in adult rats secondary to neonatal administration of diethyl-stilbestrol

(Atanassova et al. 2005). In rodents, the presence of prominent mast cells in the interstitium of the epididymis is a common background finding that should not be confused with inflammation. As mentioned above, mast cell populations vary with sexual maturation, with a peak in mast cell numbers at approximately 90 days of age (Jimenez-Trejo et al. 2007).

Sperm Granulomas. Spontaneous sperm granulomas in epididymis (Figs. 10.18 and 10.19) and of the rete (Figs. 10.20 and 10.21) are occasionally encountered in mice and rats, because this is the site where the immune privilege breaks down. Dogs also develop spontaneous sperm granulomas, and these might be attributed to the blind-ended structures within the efferent ducts in this species (Foley et al. 1995). Epididymal sperm granulomas can be intratubular or extend into the surrounding interstitium. The typical inflammatory cell population of a sperm granuloma includes macrophages, polymorphonuclear leukocytes, fibroblasts and sperm (Fig. 10.22 and 10.23). There may be hypertrophy/hyperplasia of the epididymal epithelium, leading to intraductal granulomas surrounding impacted sperms (Creasy et al. 2012).

The most common pathogenesis for the formation of granuloma is increased intraluminal pressure. The increased pressure may be the result of too much fluid due to inadequate resorption, or constriction of the outflow tubules with increased "up-stream" pressure. In both cases, the increased pressure leads to atrophy and degeneration of tubules, followed by rupture of the tubule and eruption of the sperm

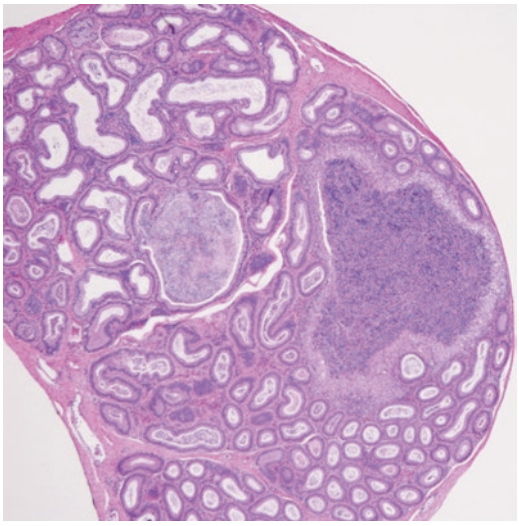

Fig. 10.18 Sperm granuloma in epididymis of mouse. This spontaneous sperm granuloma is located in the caput of the epididymis in a mouse, which is a site of lowered immune privilege compared to the testis. H&E stain. 5× objective magnification

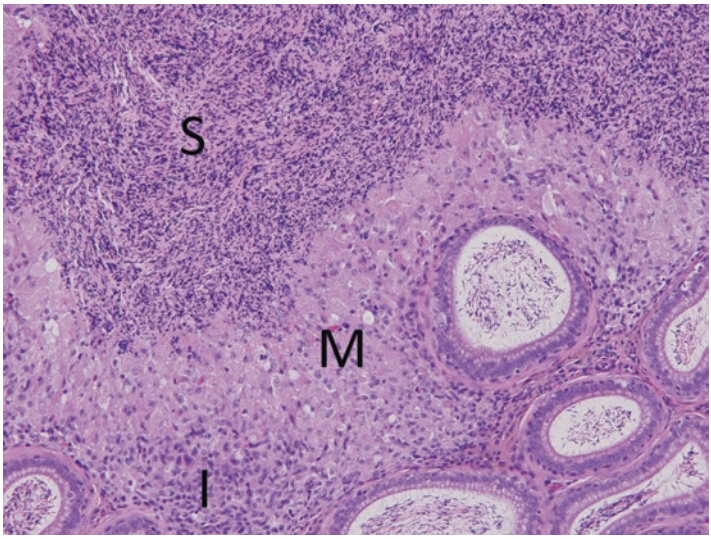

Fig. 10.19 Sperm granuloma in epididymis of mouse. At higher magnification the sperm granuloma has macrophages (M), mixed inflammatory cells (I), and accumulation of degenerating spermatozoa (S). This sperm granuloma is extra-tubular. Compare to the intratubular sperm granuloma (Figs. 10.22 and 10.23). H&E. 20× objective magnification

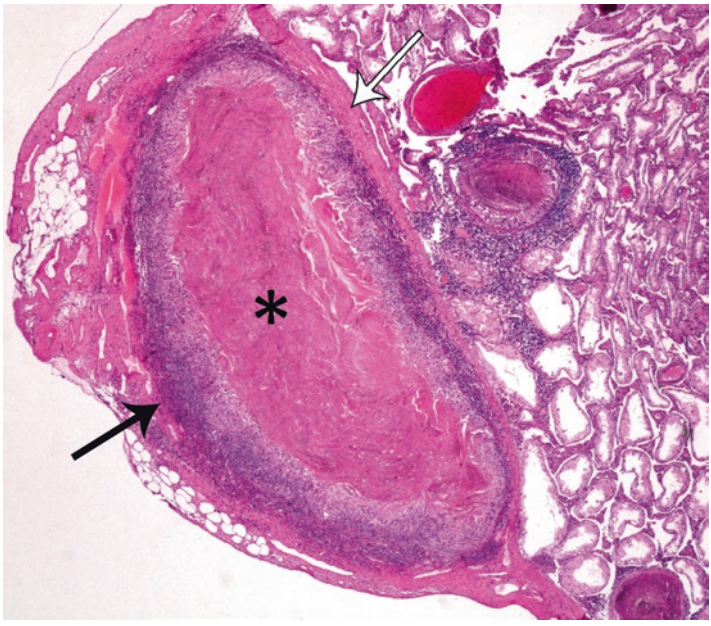

Fig. 10.20 Sperm granuloma in rete testis of rat. Sperm granulomas also occur in the rete testis (in the body of the testis), where the BTB transitions into the less-protected status of the excurrent duct system. The lumen of the granuloma consists of compacted sperm (asterisk), the basophilic rim (black arrow) consists of an inner layer of pale pink macrophages and an outer deeply basophilic layer of lymphocytes.The dense eosinophilic capsule (white arrow) is a reactive fibroproliferative reaction that attempts to contain the area of inflammation. H&E stain. 10× objective magnification

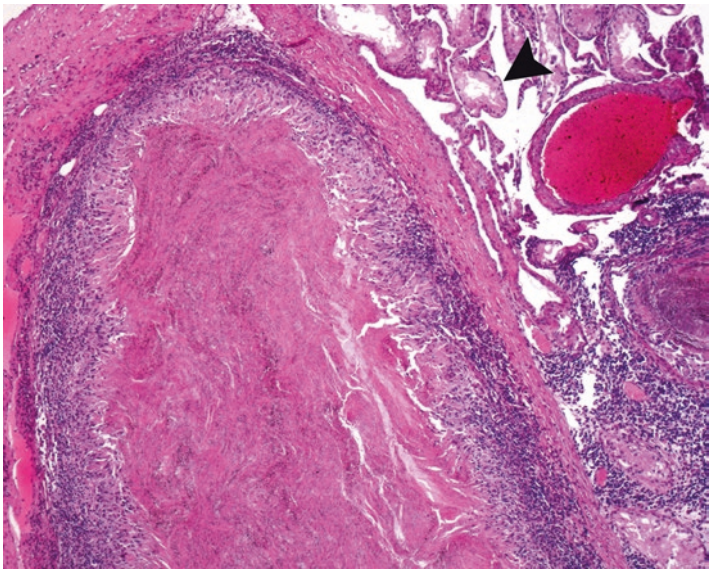

Fig. 10.21 Sperm granuloma in rete testis of rat. At higher magnification the well delineated sperm granuloma has a central layer of macrophages and lymphocytes and an encapsulating layer of collagenous fibrous tissue. There is a lymphocytic inflammatory cell infiltrate into the surrounding testicular interstitium. The seminiferous tubules (arrowhead) in this image are lined only by Sertoli cells, with no spermatogenesis. This might be the result of increased intraductular pressure caused by the physical mass of the granuloma, leading to secondary "upstream" atrophy. Alternatively, it could be due to inflammatory cytokines overwhelming the normal Sertoli cell regulation of spermatogenesis. H&E stain. 20× objective magnification

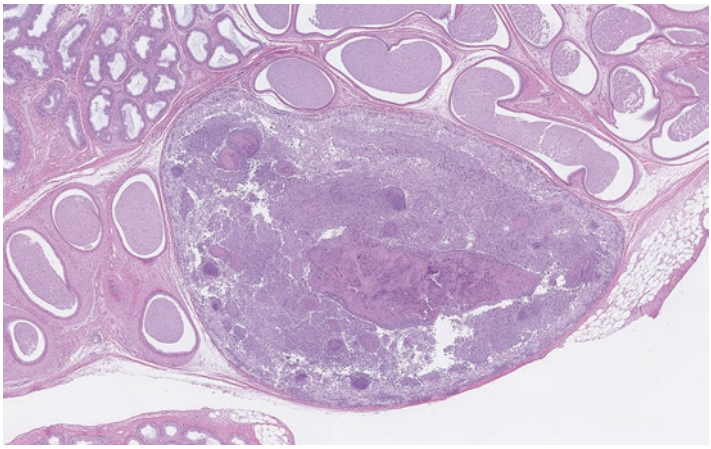

Fig. 10.22 Intraductular sperm granuloma in epididymis of rat. This sperm granuloma is confined to one epididymal tubule. There is a thin wall surrounding the accumulation of sperm within the duct. At this low magnification, one could mistake this finding for a spermatoceole (a non-inflammatory expansion of a tubule by spermatozoa). H&E stain. 10× objective magnification

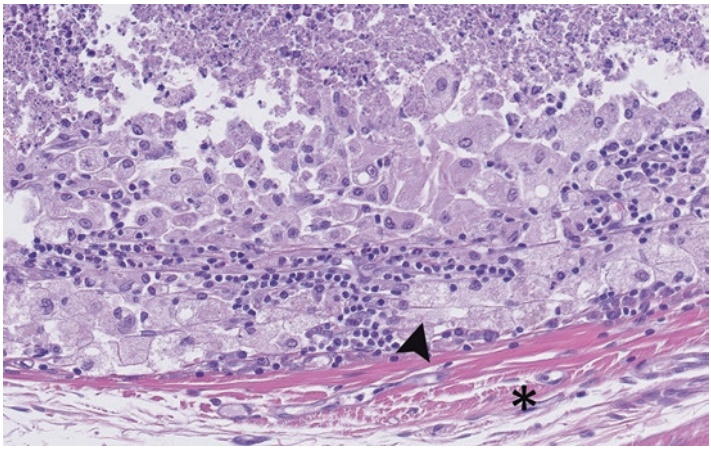

Fig. 10.23 Intraductular sperm granuloma in epididymis of rat. This is a higher magnification of the previous image showing the layer of large foamy macrophages (arrowhead) lining the inside of the tubule. Lymphocytes are present in lower numbers. H&E stain. 40× objective magnification

into the interstitium, and then by a responsive inflammation which forms a nodule encapsulating the sperm (Hibi et al. 1995; Chapin et al. 2014; Kempinas and Klinefelter 2014). A good example of the sperm granulomas resulting from excessive fluid are those which develop in the epididymis or rete testis as a result of hormonally-induced fluid imbalance (Sasaki et al. 2011). The sperm granulomas induced by the α-adrenergic antagonist guanethidine are located at the vas deferens-epididymal junction, presumably due to constriction of outflow associated with smooth muscle contraction (Kempinas et al. 1998a, b).

In addition to increased intraductal pressure, other causes for leakage of sperm through the epithelium includes compromise of the wall due to direct damage. Sperm granulomas may be due to experimental vasectomy (McGinn et al. 2000), ischemia, administration of toxic substances or hormones, or as secondary to autoimmune or infectious orchitis or epididymitis. At least with experimental vasectomy, the pathogenesis is clear. Physical obliteration or damage to the outflow tract leads to increased intraluminal pressure upstream, leading to ruptured ducts and sperm granulomas. Damage to the epithelium might also occur with ischemia associated with polyarteritis nodosa. In fact polyarteritis is one of the more common causes for sperm granulomas of the testis and epididymides of rats (Boorman et al. 1990).

Toxicant-induced sperm granulomas are caused by direct damage to the epididymal wall. Methylchloride administration to Fischer rats results in destruction of ductule epithelium and inflammation, followed by formation of sperm granulomas primarily in the cauda (Chapin et al. 1984). Dibromochloropropane and alpha chlorohydrin administration to rats (Boorman et al. 1990) or 2-methylimidazole admin-

istration to mice (Tani et al. 2005), are associated with efferent duct and epididymal caput granulomas; and at least with dibromochloroprane and alpha-chlorohydrin, the inciting event was primary epithelial damage. L-Cysteine administration to rats is associated with epididymal corpus and cauda granulomas, but the pathogenesis is less clear (Sawamoto et al. 2003).

Other causes for sperm granulomas might not be as physical as obstruction, intraluminal pressure or direct destruction of the wall. Some toxicants cause sperm granulomas by degrading the molecular components of the BEB with increased permeability. Polychlorinated biphenyls cause sperm granulomas in mice because of a reduction in the tight junction proteins such as ZO-1 (Cai et al. 2013). Another inciting event might be the imbalance between immune tolerance and immune activation, with tipping of the scale toward immune activation. This might be due to toxicant-induced stimulation of pro-inflammatory gene expression in the epididymal epithelium (Gregory and Cyr 2014).

Hormonal imbalance can cause sperm granulomas in the epididymis. Anti-androgens can disrupt the basal cell's ability to scavenge ROS, and ROS can overwhelm the basal cells and cause tissue damage to the epididymal wall (Shum et al. 2008; Gregory and Cyr 2014). Once started, sperm granulomas perpetuate the inflammation. The physical presence of the fibroblast- and macrophage-rich nodule leads to higher intraductal pressure, which further accentuates damage to the ductal wall, breakdown of the BEB and further leakage of sperm antigen. The inflammation that results perpetuates the granuloma: Reactive oxygen species (ROS) released by interstitial inflammatory cells can activate matrix metalloproteinases (MMPS) which leads to further degradation of tight junction proteins and increased leakage (Gregory and Cyr 2014).

Polyarteritis Nodosa. The testis and epididymis are a common site for expression of polyarteritis nodosa, a systemic vascular disease. It is characterized by hyaline eosinophilic amorphous material (fibrinoid change) in the tunica media of medium sized blood vessels, fragmentation of smooth muscle of the tunica media, and hemorrhage and inflammation (Creasy et al. 2012). The inflammation usually consists of lymphoplasmacytic infiltration around small arterioles, with associated medial hypertrophy and fibrinoid necrosis. Polyarteritis nodosa is much more common in the rat than in the mouse, and more common in the testis than the epididymis (Creasy et al. 2012). Polyarteritis is one of the more frequent causes of sperm granulomas in the testis and epididymides (Boorman et al. 1990). Polyarteritis results in tissue ischemia, and the ischemia results in damaged epididymal or seminiferous tubular walls, loss of BEB or BTB, respectively, and leakage of germ cells or spermatozoa. Even without frank ischemia, the locally extensive inflammation around the affected artery produces an elevated inflammatory cytokine milieu that overwhelms the normal physiologic "inflammatory" cytokine balance that is required for spermatogenesis. The elevated inflammatory cytokine milieu then results in decreased spermatogenesis in the area (Fig. 10.24).

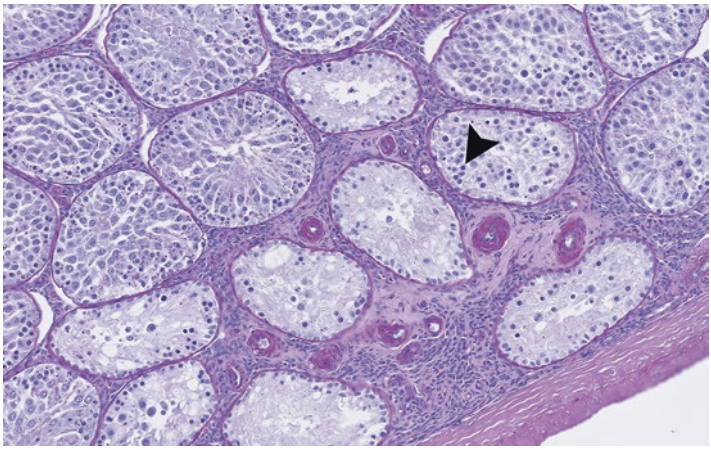

Fig. 10.24 Polyarteritis nodosa in a rat testis. Small arterioles of the testis have fibrinoid necrosis of the wall with deposition of PAS-positive material. A locally extensive lymphocytic infiltrate around the blood vessels extends between individual seminiferous tubules. Nearby tubules have depletion or paucity of germ cells with apoptotic cells (arrowheads). Most of these depleted tubules have intact live Sertoli cells (Sertoli-only tubules). The most likely explanation for the regional loss of germ cells is that inflammatory cytokines associated with the inflammatory reaction overpowered the normal physiologic cytokine production of Sertoli cells. Too many pro-inflammatory cytokines will disrupt the BTB and lead to loss of germ cells. As described in the text, excess IL-1 released by invading macrophages affects the actin cytoskeleton and disrupts the co-localization of proteins (cadherin, catenin, claudin and ZO-1) essential to the BTB. The apoptotic germ cells are likely the result of upregulation of FasL-Fas by the germ cells. Periodic acid-Schiff/hematoxylin (PASH) stain. 16× objective magnification

Prostatic inflammation. Inflammation of the prostate is a common background incidental finding in rats and mice, and the incidence increases with age. Ascending prostatitis has been associated with ascending bacterial infections such as *Pasteurella pneumotropica* in rats (Sebesteny and Lee 1973) and *Staphlococcus aureus* and *Mycoplasma* spp. infection in mice. Prostatitis has also been attributed to hormonal imbalances like hyperprolactinemia and estradiol (Tangbanluekal and Robinette 1993; Van Coppenolle et al. 2001). In the rat (but not the mouse), there is a positive association between prostatitis and pituitary gland tumors (presumably prolactin secreting tumors) (Suwa et al. 2001, 2002). The frequency of inflammation is highest in the dorsolateral lobe of the prostate, less in the ventral lobe, even less in the coagulating gland, and has the lowest incidence in the seminal vesicle (Suwa et al. 2001, 2002).

”Background’ prostatitis is characterized primarily by lymphocytic infiltrates, though suppurative lesions or large abscesses are possible. Focal mineralized concretions are often present in these inflammatory foci.

Prostatic inflammation in genetically engineered mice has been attributed to urinary stasis and/or alteration in immune function or inflammatory responses in the dorsal lobe of the prostate in the senescence-accelerated mouse (Suwa et al. 2002; Shappell et al. 2004; Stolte 1993; Boorman et al. 1996; Barthelemy et al. 2004).

10.8 Implications for Toxicologic Pathology

There are a number of ingenious strategies that have evolved to protect the germ cells from immunologic demise. Virtually every cell type in the testes has some unique mechanism to suppress immune system activation. In general, there is a suppression of the adaptive immune system yet strong innate immunity, which serve to protect germ cells from being recognized as "foreign" by the host.

Teasing apart the complex working of the immune and endocrine system in the various cell types of the male reproductive tract is a challenge, and one that is ordinarily reserved for reproductive physiologists elucidating the molecular basis for each cell's function.

The toxicologic pathologist generally is at a disadvantage and has to work back from a non-specific lesion to the specific cause, and this is generally not possible or requires much speculation. The toxicologic pathologist generally is presented with sperm granulomas, orchitis or epididymitis, and varying degrees of tubular degeneration and hypospermatogenesis. These changes are often non-specific and don't point to any one specific mechanism of action.

By understanding how the endocrine and immune system interact, and appreciating the delicate balance required for spermatogenesis to proceed, the pathologist will be able to formulate possible mechanisms of action for a given lesion. One of these possible mechanisms to consider could be immune system dysregulation. In particular, when confronted with non-inflammatory degeneration or hypospermatogeneis, it is possible that the Sertoli cell functions were disturbed, leading to an imbalance in the normal localized micro-inflammatory reaction that the Sertoli cell normally orchestrates, leading to deficient spermatogenesis (Hedger 2011b).

When confronted with nonspecific inflammation in the tissues, the pathologist can evaluate the infiltrate to help decide if it is autoimmune or cell-mediated (lymphocytic), neutrophilic with necrosis (bacterial or ischemia-related), or granulomatous (foreign body reaction to leakage of sperm). Any evidence of reduced spermatogeneis in the region of the inflammation could be explained by the overwhelming inflammatory cytokines affecting normal spermatogenesis. Loss of spermatogenic epithelium in areas adjacent to the inflammation is likely caused by increased production of inflammatory cytokines overwhelming normal homeostasis. Apoptosis of the germ cells following physical or chemical insult are likely due to upregulation of the Fas-FasL expression by germ cells and Sertoli cells, respectively (Kajihara et al. 2006; Lee and Huang 1997).

Stress is often suspected in toxicity studies, and the effects of stress must be teased apart from the direct effects of test article on the testis or epididymis. In general, stress will cause a decrease in circulating androgen levels, but have very little intra-testicular effects, due to the surplus of androgen in the interstitial testicular tissue. If there is test article-related endocrine disruption with substantially decreased androgen production, there may be sufficiently reduced intra-testicular androgen levels to see the characteristic findings such as degeneration of pachytene spermatocytes in stage VII and depletion of elongating spermatids, but generally this does not

occur as part of a stress response. With stress, however, there can be sufficiently low circulating androgen levels to see an effect on the accessory glands, resulting in atrophy and decreased secretions. The lower circulating androgen levels might also explain "sickness behavior" in animals.

For a more detailed account of pathogenesis of male reproductive toxicity, the reader is referred to (Creasy 2001).

10.9 Summarized Points

- In the testis, there is suppression of adaptive immune system to protect attack against foreign antigens that develop on germ cells during meiosis. This immunosuppression is achieved by.
  1. Production of immunosuppressive androgen (by Leydig cells).
  2. Maintenance of the blood-testis barrier to prevent immune cells from entering spermatogenic epithelium.
  3. Production of indoleamine 2,3,dioxygenase (IDO) by Sertoli cells to inhibit proliferation of T cells.
  4. Maintenance of $ED2^+$ testicular macrophages (that produce IL-10 a powerful inhibit of helper T cells).
  5. Use of Fas-FasL and Gas6/PRoS-TAM by Sertoli cells to cause rapid apoptosis of germ cells to minimize exposure of foreign antigen.
  6. Aberrant expression of MHC class I molecules by germ cells to prevent their attack by either NK cells or cytotoxic T cells.
- Testicular $ED2^+$ macrophages have not only an immunosuppressive role, but also have an endocrine role. They provide substrate for androgen production to the Leydig cell and are responsible for maturation of Leydig cells during development.
- Sertoli cells produce inflammatory cytokines that cause mini-inflammatory reactions repeatedly at the site of the blood-testis barrier (BTB), and close titration of these inflammatory reactions are critical for normal spermatogenesis. Any excess inflammatory cytokines due to inflammation will disrupt the BTB, and cause germ cell depletion and/or apoptosis.
- Inflammation can also effect spermatogenesis by the systemic route. Inflammatory cytokines turn off gonadotropin production at the level of the pituitary leading to lower androgen levels and male sickness behavior.
- In the excurrent duct system, the adaptive immune responses are far less suppressed than in the testis, as there are intraepithelial $CD8^+$ T cells and macrophages; interstitial $CD4^+$ T helper cells and pro-inflammatory $ED1^+$ macrophages; and a relatively weak blood-epididymis barrier. Therefore, sperm granulomas occur more frequently in the excurrent duct system.

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Chapter 11
Immunopathology of the Female Reproductive Tract and Mammary Gland

Catherine A. Picut, Darlene Dixon, and Eveline P.C.T. de Rijk

Abstract The female reproductive tract (FRT) has unique requirements for regulation of immune protection in pregnant and non-pregnant states. During the non-pregnant state, the ovary creates a protective environment for the ova by controlling the normal inflammatory reactions that occur during cycling of the ovary, and a modified mucosal immunity protects the remainder of the FRT from invading microorganisms. During pregnancy, to ensure that the fetus or placental tissues are not rejected as being "foreign", the placenta has creative mechanisms to down-regulate antigen specific immunity and selective aspects of innate immunity. Within the metrial gland, the uterine natural killer cell (uNK) works cooperatively with the trophoblast to establish tolerance to the fetal antigens. This chapter will cover the animal models used to study the pathogenesis of diseases of women in which immune dysregulation of the FRT is implicated, including premature ovarian failure, polycystic ovary syndrome, endometriosis, preeclampsia, and recurrent miscarriages.

Keywords Ovary • Immunology • Macrophage • Natural killer cell • Pregnancy • Placenta • Trophoblast

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11.1 Introduction

The female reproductive tract (FRT), consisting of the ovaries, oviducts, uterus, cervix, vagina, and placenta, has unique requirements for regulation of immune functions in both the pregnant and non-pregnant states. The FRT must be capable of resisting bacterial and viral pathogens, yet sufficiently tolerant to disregard allogeneic spermatozoa, and not reject the immunologically distinct fetus.

During the **non-pregnant state**, a modified mucosal immunity protects the majority of the FRT (oviduct, uterus, cervix and vagina) from invading microorganisms and infection. As part of this modification, the epithelial cells of the oviduct, uterus, cervix and vagina take on important immunologic functions to facilitate effective mucosal immunity. The innate and adaptive immune responses are both accentuated in some regards to protect the FRT from microbial infection, yet those immune responses are carefully modulated through fluctuating hormone levels in order to protect the tissue from damage due to inflammation and to maintain an environment that is conducive to pregnancy. The ovary, on the other hand, is a part of the FRT that does not rely on conventional mucosal immunity. Instead, the ovary creates an environment with limited innate and adaptive immunity, where the macrophage is the primary player. The ovarian macrophage utilizes all of its versatile "immunologic" functions to help orchestrate phase cycling of the ovary. The macrophage instigates repeated mini-inflammatory reactions that (1) cause ovulation and luteolysis; (2) produce growth factors that allow for angiogenesis and cell proliferation associated with follicular and luteal growth; and (3) phagocytizes apoptotic granulosa cells and luteal cells following atresia. The macrophage is available to fill conventional immunologic roles involved in resisting microbial invaders. The central role of the macrophage in ovarian immunity is reflected in evidence that dysregulation of the macrophage is involved in premature ovarian failure (POF), polycystic ovary disease (POS), and endometriosis.

During **pregnancy**, the adaptive and innate immune systems are modified to ensure that the fetus or placental tissues are not rejected as being "foreign". Central to this modification is the uterine natural killer cell (uNK), which is a unique natural killer cell in the placenta. The uNK cell works cooperatively with trophoblast cells to down-regulate its own cytotoxic activity and to create an immunosuppressive environment for the placenta and fetus. In addition to down-regulating the killing function of uNK cells, there are many alternate mechanisms in place to ensure that the adaptive immune system is suppressed sufficiently to protect the fetus, yet remains a level of activity that is necessary to prevent invasion of microorganisms. Interference with this delicate balance of the immune system during pregnancy is regarded as a major contributing factor for the development of pregnancy complications such as preeclampsia or recurrent miscarriages.

Immunopathology of the FRT occurs when there is breakdown of the immune-protective environment of the FRT in either its non-pregnant or pregnant state. With new pharmaceuticals or environmental chemicals, especially those disrupting either the immune or endocrine system, there is potential for this disruption to be manifested as non-specific reproductive endpoints such as reduced fertility in labo-

ratory animals. In rare instances, a pathologist might identify specific microscopic changes that could explain the reproductive or immunologic effect, but more often than not, the cause of the reproductive failure is not apparent microscopically. Instead, it may be necessary to correlate any changes in reproductive endpoints back to some disturbance of the immune system. This would be possible only if the pathologist is knowledgeable about the interaction of the immune and reproductive systems. Therefore, pathologists and toxicologists must understand how the endocrine and immune systems interact to explain non-specific changes in endpoints or findings that appear unrelated to the known action of the test article.

This chapter will begin by a discussion of the normal immunologic regulation of the FRT in the non-pregnant state, followed by a discussion of the immunomodulation required for a successful pregnancy. In the section on the pregnant state, the uNK cell will be introduced, as well as many of the creative mechanisms that are utilized by fetal tissues to avoid or actively suppress the adaptive immune system and selective parts of the innate immune system. The chapter covers some common diseases in which immune dysregulation of the FRT is implicated, including premature ovarian failure, polycystic ovary syndrome, endometriosis, preeclampsia, and recurrent miscarriages. Appropriate animal models will be mentioned.

11.2 Non-Pregnant State

11.2.1 Immunological Regulation in the Ovary

11.2.1.1 Immunological Protection of the Oocyte

The ovary is an immunologically privileged tissue because it houses the oocyte, which can have surface antigens that are recognized as foreign by the host even though the oocyte developed within the host. During follicular development the primary oocyte within the primordial follicle begins to undergo meiosis with duplication of chromosomes to the tetraploid state. The oocyte is held in prophase, until it begins its first meiotic division at about the time of ovulation. This first division can involve cross-overs of DNA leading to expression of new "foreign" surface antigens in much the same way as primary spermatocytes and spermatids become "foreign" to their host. The second meiotic division begins in the fallopian tubes (or oviducts, in rats) when the oocyte is arrested in metaphase, and this division is complete only if fertilization occurs.

The primary oocyte must be secluded from the immune system in order that its "foreign" antigens, which are expressed following the first meiotic division, do not incite an immunologic response, yet the immune system must respond to any invading microorganisms. The oocyte is protected by both structural and immunological mechanisms (Fig. 11.1). The structural barriers around the oocyte include the zona pellucida, several layers of granulosa cells with tight junctions, a thick basement membrane and a peripheral rim of hormone-producing theca cells. These layers

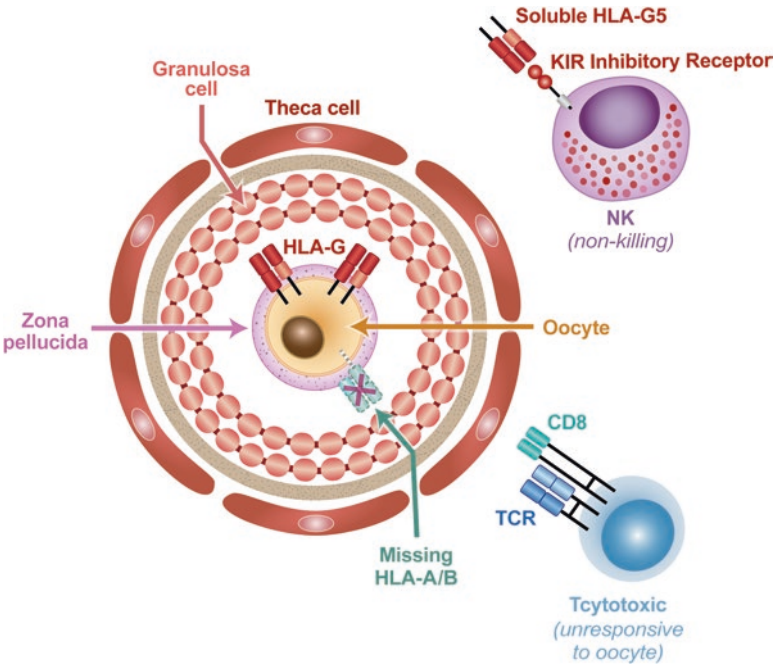

Fig. 11.1 Immunological protection of oocyte in ovary. This is a schematic of an oocyte within a follicle. The oocyte that may express "foreign" antigens is protected by both structural and immunological barriers. The oocyte is enshrouded by a zona pellucida, granulosa cells, a basement membrane, and thecal cells, all of which pose a structural barrier to trafficking of immune cells. A number of immunological mechanisms that serve to protect the oocyte. The thecal cells produce progesterone that has an immunosuppressive effect on macrophages within the interstitium of the ovary. Oocytes also express HLA-G and secrete soluble HLA-G5, both of which bind to KIR (killing-immunoglobulin-like receptors) on NK cells thus disabling the killing mechanism of NK cells. Any $CD8^+$ cytotoxic T cells (Tcytotoxic) in the interstitium fail to recognize "foreign" antigens on the oocyte because cytotoxic T cells recognize antigen only when it is presented in the context of classical MHC Class I molecules (HLA-A or HLA-B), and the oocyte does not express these MHC Class I molecules. In this way, oocytes utilize differences in MHC Class I expression to avoid killing by either NK cells or $CD8^+$ cytotoxic T cells

collectively control trafficking of macrophages or neutrophils into the antrum of the maturing follicle. Furthermore, macrophages in the interstitium of the ovary are maintained in an immunosuppressive phenotype, largely due to the high local level of progesterone produced by the thecal cells.

The oocyte is also protected because the ovary permits only a limited number of immune cells in its parenchyma. Conventional immune cells present in the normal ovary are the macrophages, occasionally neutrophils, and fewer eosinophils, cytotoxic T cells and NK cells. The populations of these cells vary depending on the cycle, and serve to orchestrate normal functions of follicular growth and atresia, ovulation, and luteal growth and regression. B cells and T helper cells are generally absent from the ovary (Bukovsky et al. 1995).

Even though there are few cells of the adaptive immune system in the ovary (except for the macrophages), there is a protective population of regulatory T cells (Tregs) that is maintained in the local lymph nodes. The Tregs maintain peripheral tolerance to certain ovarian auto-antigens to which central tolerance was never established, i.e., zona pellucida antigens (Samy et al. 2005, 2006).

In addition to the structural barriers and populations of immunosuppressed M2 cells and Tregs, an immunologic strategy involving class I MHC molecules also helps protect the oocyte against killing by cytotoxic T cells or NK cells. Oocytes of humans lack MHC Class I (HLA-A and HLA-B), and MHC Class II (HLA-D) molecules (Comiskey et al. 2013). Missing the MHC Class I molecules A and B protects the oocyte (that might express "foreign" antigens on its surface) from being recognized and attacked by surveillant $CD8^+$ T cells. However, the absence of MHC I (HLA-A and HLA-B) is a double-edged sword, because the absence of classical MHC 1 molecules also makes the oocyte vulnerable to attack by NK cells that would identify the oocyte as "non-self. However, even though *classical* MHC Class I A and B molecules are missing from the oocytes of humans, the oocyte expresses the polymorphic HLA-G molecule which is a *non-classical* MHC I molecule. HLA-G can bind to inhibitory receptors (KIR, killing immunoglobulin-like receptors) on NK cells and suppress NK cells that come into contact with the oocyte (Hunt et al. 2005). This mechanism of non-standard MHC I expression is discussed in more detail below in the second part of this chapter.

As opposed to human or rat oocytes, conventional Class I MHC molecules are present on mouse oocytes (Ewoldsen et al. 1987). Therefore in the mouse, the role of the zona pellucida in physically protecting the oocyte from cytotoxic T cell attack becomes particularly important.

11.2.1.2 Ovarian Macrophages

The ovarian interstitial stroma contains an abundance of macrophages, which are the most prevalent immune cells of the ovary. Macrophages are present in the immature pre-ovulatory ovarian interstitium, and expand in numbers as the ovary matures (Brannstrom and Enskog 2002). In the immature ovary, macrophages are mainly confined to the interstitium; however, macrophages also infiltrate the thecal and luteal cell layers and occasionally can be found in the granulosa cell layers (Brannstrom and Enskog 2002; Wu et al. 2004; Bowen and Keyes 2000; Li et al. 1998; Van der Hoek et al. 2000). Macrophages in the *human* corpora lutea are present primarily in the more peripheral "theca-lutein" areas of the corpus luteum, although some are found in the more central "granulosa-lutein" cell layer (Gaytan et al. 1998; Weissenbacher et al. 2014). In contrast, macrophages in the corpora lutea of the rat are more plentiful in the center of the corpora lutea (Brannstrom and Enskog 2002). Since the regional distribution pattern of small and large luteal cells seen in humans does not exist in the rat, (Tomac 2011; Leung and Adashi 2003), it is not surprising that rats and humans do not share the same regional distribution pattern for luteal macrophages. Any differences in distribution patterns of macrophages in any species described in the literature are likely due to variation in the different surface markers

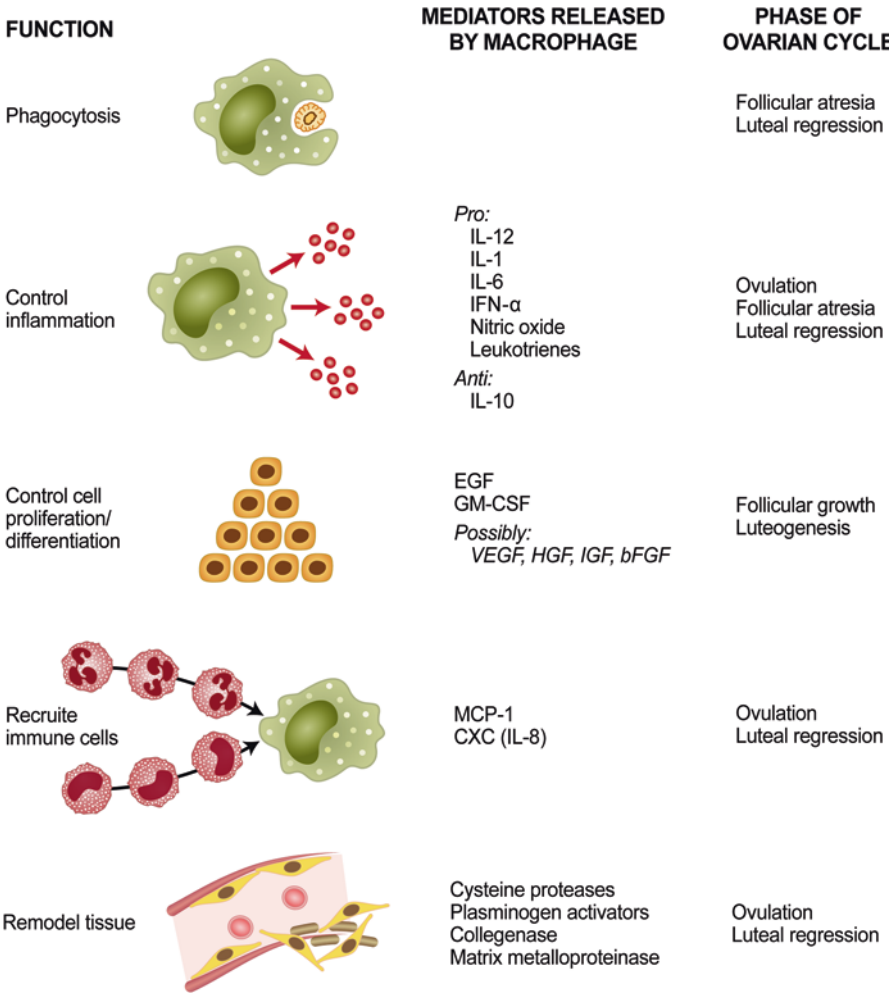

Fig. 11.2 Functions of ovarian macrophages

used to identify the macrophages, such as CD68 (i.e., the ligand for ED1), MHC class II, Mac-1/Cd-11b, and F4/80 (Wu et al. 2004).

Ovarian macrophages, like macrophages elsewhere, are multifunctional cells with diverse functions, which include phagocytosis, degradation and presentation of foreign antigens, matrix dissolution, tissue remodeling, and production and secretion of cytokines, chemokines and growth factors. In the ovary, these conventional functions of macrophages incite localized "inflammatory reactions" that facilitate normal cycling events, including folliculogenesis, follicular atresia, ovulation, and corpora luteal formation and regression (Fig. 11.2) (Wu et al. 2004). Ovulation and corpora lutea regression share features of conventional tissue inflammation, such as infiltration of $ED1^+$ and $ED2^+$ macrophages, increased staining for monocyte chemoattractant protein 1 (MCP1), up-regulation of TNF-α, apoptosis mediated in part

Table 11.1 Functions of ovarian macrophages

Function	Mediators	Consequence in tissue	Phase
Phagocytosis		Remove apoptotic granulosa cells and luteal cells	Follicular atresia Luteal regression
Growth Factors	EGF GM-CSF TGFα TGFβ (VEGF, HGF, IGF, bFGF)[a]	Proliferation Differentiation Steroidogenesis	Follicular growth Luteogenesis
Cytokines	IL-1 IL-6 IFNα TNFα Nitric oxide Leukotrienes Prostaglandins	Controlled Inflammation Vasodilation	Ovulation Follicular atresia
Chemokines	MCP-1 CXC (IL-8)	Recruit and activate macrophages, neutrophils, T cells	Ovulation Luteal regression
Proteases	Cystine proteases Plasminogen activators Collagenase	Matrix remodeling Matrix dissolution	Ovulation Luteal regression

[a]Not necessarily from macrophages

by Fas/FasL (Kuranaga et al. 1999), and increased numbers of $CD8^+$ T cells (Sander et al. 2008; Davis and Rueda 2002).

The cytokines, chemokines and growth factors released by ovarian macrophages collectively orchestrate normal ovarian cycling. These factors are summarized in Table 11.1, and have been reviewed extensively (Brannstrom et al. 1993; Katabuchi et al. 1996; Terranova and Rice 1997; Bukulmez and Arici 2000; Wong et al. 2002; Tamura et al. 1998; Hagglund et al. 1999; Curry and Osteen 2001). Determining which factors are critical to the different phase of ovarian function is an area of active investigation. The extensive and intimate involvement of macrophages in all phases of follicular and corpora luteal development is the basis for suspecting malfunction of macrophages in ovarian diseases such as polycystic ovary syndrome (POS), endometriosis and premature ovarian failure (POF).

Role of Macrophages in Life Cycle of Follicle

The earliest stage of follicular growth (primordial to primary follicle) is independent of macrophages, but during continued growth ovarian macrophages localize to the theca cell layer of healthy follicles. A number of macrophage factors allow for granulosa cell proliferation, including granulocyte macrophage colony-stimulating factor (GM-CSF) , hepatocyte growth factor (HGF), basic fibroblast growth factor (bFGF), transforming growth factor (TGF-α), epidermal growth factor (EGF), and insulin-like growth factor (IGF), to name a few. Inflammatory cytokines released from macrophages during follicular growth can also help stimulate cell proliferation of granulosa cells. TNF-α,

produced at low levels in the developing follicle, helps stimulate cell proliferation, even though high levels of TNF-α cause apoptosis of follicular or luteal cells at another phase (Davis and Rueda 2002; Henkes et al. 2008; Gadsby et al. 2006).

One interesting rodent model helps implicate the ovarian macrophage in follicular development. The osteopetrotic (op/op) mouse has severely reduced numbers of mature macrophages due to a natural mutation in the macrophage colony stimulating factor (M-CSF-1) gene (Mikkelsen and Thuneberg 1999). This model also has reduced ovarian follicle growth and impaired fertility, thereby implicating the macrophage as critical to follicular development (Cohen et al. 2002).

Ovulation and acute inflammation share many of the same features such as edema, vasodilation, heat, and inflammatory mediators. During ovulation, the wall of the follicle is weakened by proteolytic enzymes and the follicle ruptures when granulosa cells along the apical surface are lysed and sloughed away. The follicle becomes hyperemic, is infiltrated by neutrophils, eosinophils and macrophages. There is evidence of elevated levels of circulating auto-antibodies to ovarian surface antigens, as well as increased complement activity. Furthermore, there is increased vascular permeability and edema associated with dissolution of the thecal collagen and extra-cellular matrix; and upregulation of IFNγ, TNF-α, and IL-1 in macrophages and granulosa cells.

The controlled inflammation of ovulation is mediated, in large part, by macrophages. Immediately before ovulation, macrophages migrate into the thecal layer in humans and rats (Brannstrom et al. 1994a, b). This is likely a response to local upregulation of the chemokine MCP-1 (monocyte chemoattractant protein-1) produced by macrophages themselves (Wong et al. 2002). Macrophages bring about ovulation by virtue of their pro-inflammatory cytokines and protease enzymes that help dissolve the follicular wall. However, macrophages are not solely responsible for initiating ovulation, as they recruit neutrophils to the site that in turn contribute matrix metalloproteinases to help dissolve the follicular wall. Experimental depletion of ovarian macrophages decreases ovulation (Van der Hoek et al. 2000), and experimental enhancement of the number of macrophages increases the number of oocytes that ovulate in the presence of luteinizing hormone (LH) (Hellberg et al. 1991). When macrophage numbers are decreased following feed restriction, there is decreased ovulation rate (Wu et al. 2004).

Follicular atresia is also a controlled inflammatory reaction. During normal atresia, the complement system is active, there is pyknosis and fragmentation of oocyte and granulosa cells, and white blood cells (macrophages and fewer neutrophils) invade the atretic follicles. It is clear that macrophages play an important role in removing atretic granulosa cells in multiple species such as mice, humans, and guinea pigs (Paavola 1979; Kuryszko and Adamski 1987; Kasuya 1997).

Role of Macrophages in Life Cycle of Corpus Luteum

There are multiple aspects of luteal function that are influenced by ovarian macrophage. The macrophages secrete growth factors that stimulate corpora luteal formation (in much the same way they stimulate follicular growth), and then facilitate its destruction (in much the same way they facilitate follicular atresia).

Luteal formation occurs immediately after ovulation, when there is neovascularization, differentiation of granulosa cells into lutein cells and migration of macrophages into the corpus luteum (Figs. 11.3 and 11.4). Macrophages are by far the most prominent immune cell in human and rat corpora lutea, and will remain the most prominent immune cell in the corpus luteum throughout its lifespan. The macrophages secrete growth factors, such as VEGF, EGF, and bFGF, which stimulate progesterone production by luteal cells.

Although macrophages are important in luteal formation, macrophage numbers are highest during luteal regression. Prior to luteal regression, there is an influx of macrophages into the corpus luteum, with fewer numbers of eosinophils and $CD8^+$ T cells (Niswender et al. 2000; Sander et al. 2008). The process of luteolysis or regression is known to involve increased synthesis of PGF2α, decreased progesterone production, and luteal cell apoptosis. Ovarian macrophages affect all three of these events. First, macrophages may be a primary source of PGF2α, but more likely they produce, IL-1 and TNFα that in turn stimulate luteal cells to produce PGF2α (Brannstrom et al. 1993). The TNFα produced by macrophages has a dual role during regression, because it directly inhibits basal progesterone secretion from luteal cells and promotes luteolysis (Okuda and Skumoto 2003). Macrophages are also a source of reactive oxygen species (ROS), which together with TNFα and PGF2α induces an apoptosis cascade (Brannstrom et al. 1993; Niswender et al. 2000). The role of eosinophils and $CD8^+$ T cells in luteal regression is not clear, but to be sure, the role is minor compared to the macrophages.

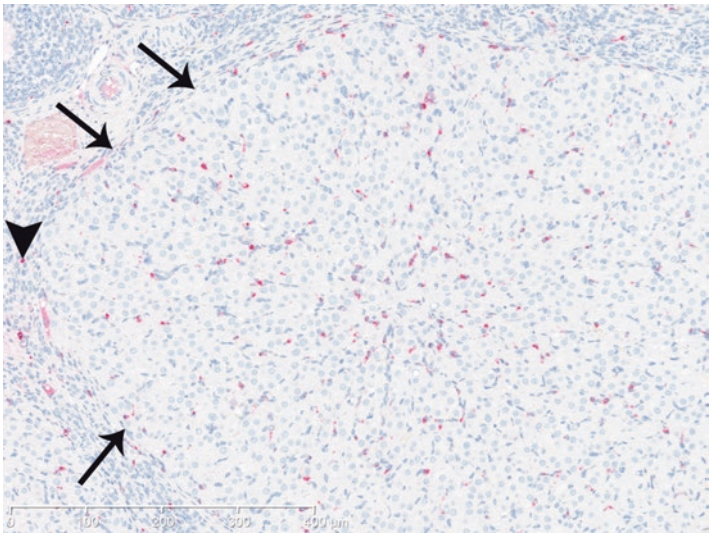

Fig. 11.3 $ED1^+$ macrophages in corpus luteum of rat. Note the positively stained $ED1^+$ pro-inflammatory macrophages dispersed evenly throughout a corpus luteum of the current estrous cycle. A few macrophages are present in the interstitium. Outer edge of corpus luteum delineated with *arrows*. Immunohistochemistry using ED1 antibody, 3-amino-9-ethylcarbazole (AEC) chromagen, and hematoxylin counterstain. 10× objective magnification

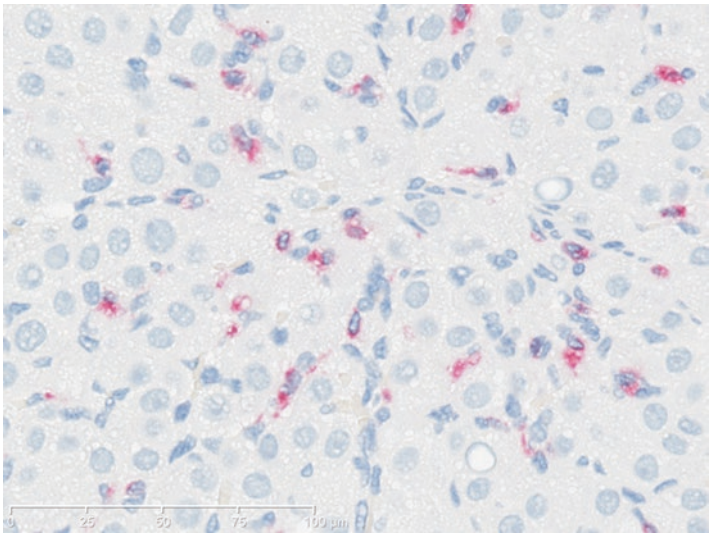

Fig. 11.4 ED1^{+} macrophages in corpus luteum of rat. Figure 11.3 taken at higher magnification to show the ED1+ macrophages dispersed among luteal cells. Immunohistochemistry using ED1 antibody, 3-amino-9-ethylcarbazole (AEC) chromagen, and hematoxylin counterstain. 40× objective magnification

11.2.2 Modified Mucosal Immunity of FRT in the Non-pregnant State

The female reproductive tract (FRT), including the oviduct, uterus, cervix and vagina, is considered part of the common mucosal immune system. The FRT mucosal system, however, has some distinct differences from other mucosal surfaces. These differences are important since the FRT has unique anatomical characteristics and performs unique functions when compared to other mucosal immune systems, such as the gastrointestinal tract. One major difference between the genital tract and the intestinal tract is the compartmentalization of the genital mucosa into sterile and non-sterile compartments. The vagina and ectocervix have commensal flora (predominantly *Lactobacillus* spp., in humans), whereas the endocervix, uterus, and oviducts (or fallopian tubes) are sterile and lack microbial flora (Johansson and Lycke 2003; Reis Machado et al. 2014; Quayle 2002; Wira et al. 2005; Fahey et al. 2006). To help maintain sterility, the ectocervix and vagina must provide a strong barrier with cervical mucus to help prevent bacteria from ascending into the sterile compartments (Johansson and Lycke 2003). The mucins form a thick gel that functions as a physical barrier to pathogens. Its aqueous component is rich in immunoglobulins and in antimicrobial peptides, which provides additional protection (Reis Machado et al. 2014). One feature of the mucus is its antibacterial acidic pH, which is due to the production of lactic acid and hydrogen peroxide by the commensal *Lactobacillus* bacteria. The sterile compartments of the upper FRT do not have the benefits of mucus protection, but have additional features to help maintain sterility. The uterine epithelial cells assume an active immunologic role that enhances innate immunity, as discussed below.

Another major difference between the FRT and other mucosal surfaces is that the FRT is under control of hormones, which can have profound impact on the function of immune and non-immune cells. While it is clear that estrogen and progesterone exert profound effects on cytokine and chemokine production, transport of immunoglobulins, fluctuations in immune cell populations, antigen presenting capabilities, and secretion of antimicrobial proteins (Wira et al. 2005; Fahey et al. 2006), the literature is riddled with isolated facts and seemingly conflicting information, with few consistent patterns of effects that can be summarized. Examples of these inconsistencies are as follows: i) estrogen causes increased IgA levels in the uterus, but decreased IgA levels in the vagina (Ahmed et al. 1990); and ii) estrogen causes increased antigen presenting cell (APC) capability of uterine epithelial cells, but decreased APC function of stromal macrophages (Wira and Rossoll 2003). Indeed, the effects of estrogen or progesterone on the immune parameters varies with specific anatomic site with the FRT, cell type, stage of the estrous or menstrual cycle, state of pregnancy, and species.

Even given apparent inconsistencies and complexities, there are a few generalities to be made with regard to hormonal effects on the immune system of the non-pregnant female reproductive tract. One generalization is that hormonal changes during the estrous (or menstrual) cycle regulate the immune system throughout the FRT in a way that favors conditions for sperm migration, fertilization, implantation and pregnancy (Reis Machado et al. 2014). The FRT is less protective against infection (i.e., the immune system is less active) in the progesterone-dominated phase, and most protective against infection (i.e., the immune system is more active) in the estrogen-dominated phase. Because immune protection is dampened during the progesterone phase (secretory phase in man; diestrus phase in rodents) of the cycle, there is a "window of vulnerability" during which potential pathogens can infect the FRT (Wira et al. 2015). In fact, this vulnerability renders humans more likely to be infected with HIV and other sexually transmissible diseases during the secretory (progesterone-dominated) phase of the menstrual cycle (Wira et al. 2015).

The complexity of hormonal effects on immunologic function is understandable when one considers that the reproductive tract must constantly trade-off between an immunosuppressed condition that favors reproductive success versus a status that maximizes protection against microbial infection. Recognition that immune status varies with changing hormonal profiles, and that the effects on hormone levels could alter the immune system of the female reproductive tract, are the most practical bits of knowledge for a pathologist or toxicologist. Teasing apart sex hormone effects on the different players of the immune system and then correlating those results with microscopic findings in the animal is an academic exercise that has minimal practical application in standard toxicology studies.

11.2.2.1 Role of Epithelial Cells in Mucosal Immunity

The epithelial cells (EC) lining the FRT are adapted to be both a structural and immunological barrier against invading microorganisms. Unlike epithelial cells lining other mucosal surfaces, the EC lining the FRT have been adapted to take on certain roles ordinarily reserved for conventional macrophages (Fig. 11.5). All of

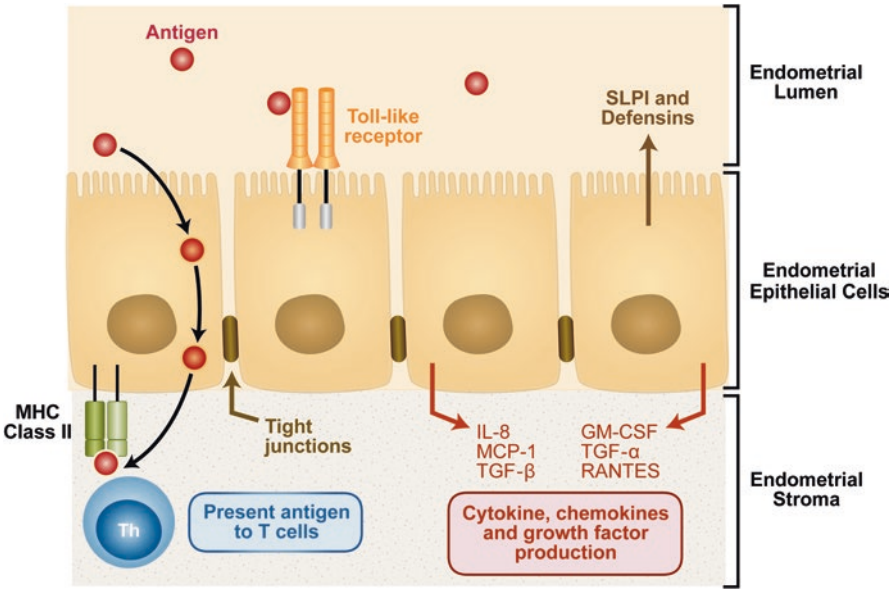

Fig. 11.5 Immunologic role of epithelial cells of the female reproductive tract. The epithelial cell (EC) of the female reproductive tract (FRT) is modified to assume an immunological roles. The uterine EC expresses MHC Class II molecules and is capable of processing and presenting antigen. Many antigens arrive from the lumen after passing through endocytosis, passage through phagosomes, and are expressed in the groove on MHC Class II. Tight junctions between neighboring ECs prevent paracellular flow of pathogens or antigens. The EC also communicates with underlying stromal and inflammatory cells by producing a variety of chemokines, cytokines, and growth factors that help control the immunological reaction in the endometrial stroma. The epithelial cells also produce and secrete antimicrobial defensins as well as secretory leukocyte proteinase inhibitor (SLPI) into the lumen. These substances have immunosuppressive effects on inflammation. Toll-like receptor (TLR) populations vary throughout regions of the FRT, but are utilized to provide additional defense against invading microorganisms

the functions of the EC, be they structural or immunological, are affected by changing hormone levels.

As a first line of defense against ascending microorganisms, the ECs first provide a structural barrier with intact tight junctions that help keep microorganisms from invading into tissue. This barrier of desmosomes, tight junctions and adherens junctions exists in the upper, more fragile, columnar epithelium of the uterus (i.e., upper FRT) but does not exist between the loose connections associated with the thicker squamous epithelium of the vagina and ectocervix (i.e., lower FRT) (Ochiel et al. 2008). The integrity of the barrier is diminished by estradiol (Weissenbacher et al. 2014). This could be considered counterintuitive and one of the "inconsistencies" between hormone levels and immune status. If high estradiol levels are generally associated with heightened innate immunity, they why is it also associated with degraded tight junctions that allow for greater access to microbes. Degraded tight junctions have a reproductive importance for allowing greater

movement of proteins during the time of ovulation in preparation for implantation. That degraded tight junctions also permits pathogens to gain access to the tissue underscores the point that immune competence and reproductive success are often in conflict with each other.

To compensate for the estradiol-associated decrease in the integrity of the physical barrier, plus absence of the antimicrobial mucus in the upper FRT, the upper FRT is modified in other ways to prevent possible infection. The ECs of the uterus, cervix and vagina function as immune cells by expressing MHC class II molecules and by processing and presenting antigen (Fahey et al. 1999, 2006; Wallace et al. 2001). Assuming this APC function by the EC is important since the FRT does not have the equivalent of the intestinal M cells in the intestinal mucosal immune system (Kucharzik et al. 2000). The APC function of the FRT epithelial cells is bolstered by additional APC function of the underlying stromal macrophages and dendritic cells (DC) (Quayle 2002; Johansson and Lycke 2003; Wira et al. 2005). The APC function of the EC is under hormonal control like virtually every other functional parameter and cell type of the FRT. Estradiol increases APC function of uterine EC, but inhibits the APC function of other antigen-presenting cells (i.e., macrophages and dendritic cells) in the underlying stroma. Interestingly, it is the EC cell itself that inhibits the APC function of these other underlying cells. When estradiol acts on the EC, the EC produces TGF-β, which then suppresses the antigen presenting ability of the underlying stromal macrophages and dendritic cells (Wira and Rossoll 2003). The strategy for estrogen increasing APC function in the EC, yet at the same time blunting the APC function of dendritic cells or stromal macrophages is not apparent, but the differential effect of estrogen on the APC function of different cell types in the same anatomical location underscores how difficult it is to generalize about hormonal effects on the immune system of the FRT.

Epithelial cells in the oviduct, uterine endometrium, cervix and ectocervix also express pattern recognition receptors (PRRs) (Reis Machado et al. 2014). Activation or binding to these PRRs, including TLRs, RIG-1-like receptors (RLR) and NOD-like receptors (NLR) trigger the production and secretion of immunologically active cytokines and chemokines from the uterine EC (McClure and Massari 2014; Hart et al. 2009). Expression of PRR is a major part of the innate immune response of the FRT. The various TLRs are site specific. TLR2 and TLR4 expression is low in the lower FRT since the lower FRT has a population of commensal bacteria, and is adapted for tolerance to these organisms (Fazeli et al. 2005). By contrast, TLR7, TLR8 and TLR9, which are activated by viral PAMPS, are evenly expressed throughout the FRT (Hart et al. 2009). There is no known consistent hormonal effect on TLR expression in the FRT; and there is no direct link between PRR (or TLR) expression and protection against or susceptibility to specific pathogens in the FRT (Wira et al. 2015). For a review of TLR expression by epithelial cells in general, see McClure and Massari (2014).

EC of the FRT also act as immunologically active cells by secreting cytokines and chemokines to communicate with underlying immune cells (Parsons et al. 2002; Quayle 2002; Wira et al. 2015). ECs produce factors such as GM-CSF, G-CSF, TNF-α, IL-1, IL-6, LIF, TGF-β, MCP-1, RANTES, IFN-ε, and IL-8

(Weissenbacher et al. 2014; Fung et al. 2013). These immune mediators are not for the sole purpose of combatting infection, but are useful in the normal tissue reconstruction (proliferation and sloughing) of the endometrial stroma that occurs during the menstrual cycle, and in preparation for implantation. In this regard the inflammatory mediators serve an endocrine/reproductive role. The production of these factors varies with fluctuations in estrogen and progesterone throughout the ovarian cycle. In general, estrogens drive the synthesis of certain cytokines in EC, including GM-CSF, IFN-ε, CSF-1, IL-8, and TNF-α (Robertson et al. 1994), and at implantation, these pro-inflammatory cytokines cause normal controlled inflammatory "injury" to the uterus. This inflammatory "injury" to the endometrium during implantation favors success of implantation (Mor et al. 2011). The inflammatory reaction that serves an important endocrine function in implantation is not unlike the inflammatory reaction that causes ovulation in the ovary.

Not only estrogen, but progesterone also effects the production of the EC cytokines, and in general has an anti-inflammatory and immunosuppressive effect. Progesterone directly suppresses production of GM-CSF, IL-1, and at high levels it may act through the glucocorticoid receptor to down-regulate production of these inflammatory cytokines (Jones et al. 2008). Progesterone also controls the chemokine expression profiles from the cell types of the endometrium, and it is these chemokines that regulate the endometrial leukocyte lineage. In humans, certain chemokines will cause the uNK cell recruitment required for pregnancy, while at other times, there is macrophage, eosinophil and neutrophil recruitment for menstrual shedding (Jones et al. 2004).

There is considerable crosstalk between uterine ECs and underlying stromal immune cells. In the previous paragraphs, it was explained how ECs of the uterus communicate with the underlying stromal immune cells by secreting cytokines and chemokines. The reverse is true as well: stromal immune cells also communicate with the overlying uterine ECs. The stromal inflammatory cells communicate with ECs by soluble factors to maintain epithelial barrier function. These soluble factors can increase or decrease the transepithelial resistance of the EC tight junction (Weissenbacher et al. 2014). The proliferation of ECs due to estrogen is also dependent on the release of mediators (e.g., HGF, IGF, KGF) by the endometrial macrophages and stromal cells (Weissenbacher et al. 2014).

ECs are very important in the innate immune response, because they produce defensins, which are natural antimicrobial peptides. These defensins are regulated, in part, by the hormonal environment (King et al. 2003; Chen and Fang 2004). Defensins β-1 and β-2 are the best-known defensins of the FRT. Additional antimicrobial functions of uterine ECs include their ability to produce a neutrophil elastase inhibitor (secretory leukocyte proteinase inhibitor, SLPI) which is produced by the glandular epithelial cells, and which is upregulated by estrogen in the rat. Aside from its antimicrobial actions, SLPI also down-modulates inflammatory reactions in the uterine endometrium during critical phases of implantation and early pregnancy. SLPI does this by inhibiting the pro-inflammatory NFκB pathway (Weissenbacher et al. 2014). How the hormones effect the production of the various defensins and SLP1 is complicated, as there is site specificity to their effect. For example, estrogen

stimulates epithelial cells to produce SLP1 and HBD2 (human β defensing-2) in the uterus but decreases the secretion of HBD2 in the vagina. The concentrations of antimicrobial proteins such as SLPI and HBD2 generally remain low during the progesterone dominated secretory phases in rats, but the opposite is true in man, in which SLPI surges during the progesterone dominated phase of the menstrual cycle (Weissenbacher et al. 2014). The point to be made is that hormonal differences in the production of antimicrobial or immunomodulating substances may lead to anatomical differences in antiviral and antibacterial activity between upper and lower FRT and between different times of the cycle. For a review of the regional, cell specific and hormonal variation in the secretion of cytokines, chemokine and antimicrobial proteins, the reader is referred to reviews (Wira et al. 2015; Fahey and Wira 2002; Weissenbacher et al. 2014).

11.2.2.2 Leukocytes of the Non-pregnant FRT

The uterus, cervix and vagina contain both T and B lymphocytes as well as macrophages, dendritic cells, NK cells, and neutrophils in the stroma (Figs. 11.6 and 11.7). In humans, the majority of the leukocytes in the uterus, endocervix and ectocervix are T cells (40–50%). The next most prominent cell type in humans is the NK cell (30%), followed by approximately equal proportions of macrophages, dendritic cells and neutrophils (10–20% of each). B cells are relatively rare (Wira et al. 2015). In rodents, uterine NK cells are rare except in the pregnant state (see below). The

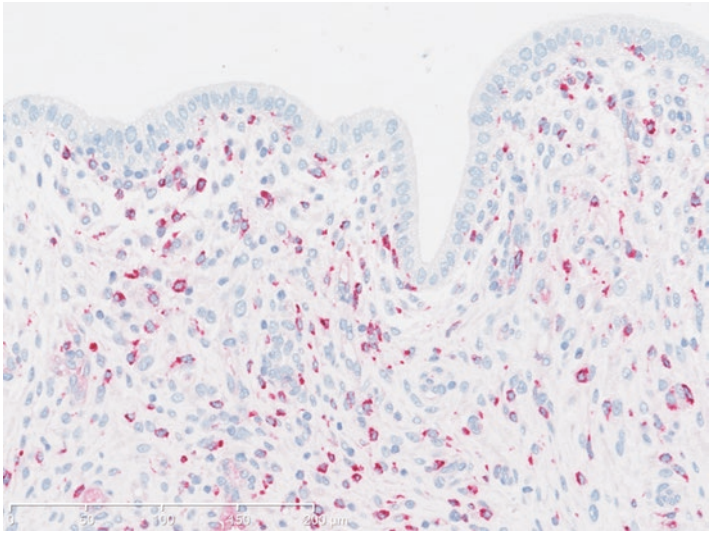

Fig. 11.6 ED1^{+} macrophages in uterus of diestrus rat. There is a high density of ED1^{+} macrophages throughout the endometrial stroma in the uterus of a normal adult rat in diestrus. Uterine epithelial cells (EC). Immunohistochemistry using ED1 antibody, 3-amino-9-ethylcarbazole (AEC) chromagen, and hematoxylin counterstain. 20× objective magnification

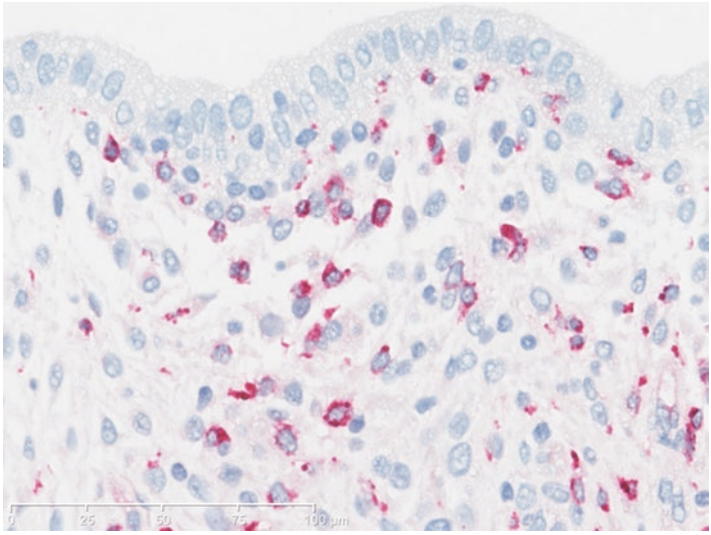

Fig. 11.7 ED1$^+$ macrophages in uterus of diestrus rat. Note the ED1$^+$ pro-inflammatory macrophages in the endometrial stroma, often extending into the overlying endometrial epithelial cells (EC). Immunohistochemistry using ED1 antibody, 3-amino-9-ethylcarbazole (AEC) chromagen, and hematoxylin counterstain. 40× objective magnification

leukocytes, and their mediators, mount innate and adaptive immune responses throughout the FRT during non-pregnancy, even though there is fluctuation of cells with hormones and with anatomical location within the FRT.

These cell populations vary with hormonal levels. As a broad generality, total leukocyte numbers increase under the influence of estrogen (Weissenbacher et al. 2014). Estrogen tends to increase the innate and adaptive immune responses, and maintains a generally heightened level of immune readiness in the FRT, because estrogen increases the APC function of the EC, increases entry of leukocytes into the uterus, increases the ability for T helper cell activation, and increases transport of IgG and IgA into the uterine lumen. Cells of the lower FRT are far less effected by fluctuations in sex hormones, as the cell populations in the lower FRT remain constant throughout the cycle (Wira et al. 2015; Pudney et al. 2005).

T lymphocytes are by far the most abundant leukocyte subset in the FRT, and they are in higher proportion in the lower, rather than the upper, areas of the tract (Wira et al. 2015). In the lower FRT, CD4$^+$ and CD8$^+$ T cells are equally abundant, but in the upper FRT (i.e., endometrium) CD8$^+$ T cells predominant. Although more abundant in the upper FRT, the CD8$^+$ T cell activity is suppressed during the progesterone-dominated (secretory) phase presumably to minimize cytotoxicity to allogeneic sperm or fetal tissue. The CD8$^+$ T cells in the lower FRT are active, are not suppressed by progesterone, and offer constant protection against potential pathogens.

Many of the T cells dispersed throughout the FRT are regulatory T cells, and regulatory T cells are more common in the pregnant uterus where they play a key

role in maintaining tolerance. T regs in the pregnant state will be discussed under Part II. Progesterone has an effect on Treg populations in the non-pregnant uterus. Rising progesterone levels in the luteal phase of the estrous cycle are important to promote differentiation of T cells into the Treg phenotype, and to prevent their differentiation into pro-inflammatory Th17 cells. Progesterone is associated with increased systemic and local uterine proportions of Tregs in mice (Mao et al. 2010; Lee et al. 2011). Th17 T cells are also present in the uterus, but are generally lower in number in the endometrium compared to the cervix and lower FRT. Th17 cells are involved in host defense against extracellular bacteria and fungi, and are generally pro-inflammatory type cells that participate in autoimmune disease and chronic inflammatory diseases. Their numbers also fluctuate somewhat with hormonal cycling, and estrogen generally is associated with lower Th17 cells. The higher proportion of Th17 cells in the lower FRT makes sense because this is the site where the FRT provides initial assault against bacterial and fungal infections. The role of Th17 cells in reproductive success is unclear, but Th17 cells have been implicated in pregnancy loss (Fu et al. 2014).

B lymphocytes are present throughout the FRT, but are rare compared to T lymphocytes. B cells produce immunoglobulin to mount humoral responses as part of adaptive immunity. Instead of discussing B cell numbers, we should discuss immunoglobulin levels. The immunoglobulins found in the mucosa and lumen of the genital tract are IgG and IgA, with IgG levels being greater than IgA levels. The IgA is in its polymeric form, similar to that in the intestinal tract. The reason why IgG is more plentiful than IgA is unclear, but is unique to the FRT, since in most other tissue sites IgA is the major immunoglobulin found in secretions (Woof and Mestecky 2005). The amount of immunoglobulin (IgA or IgG) varies with hormone levels. In rats, estrogen increases the amount of IgA and IgG in the uterus, but is associated with less immunoglobulin in the cervix (Weissenbacher et al. 2014). In humans, the effect appears reversed. Estrogen is associated with decreasing IgA and IgG levels in humans during the follicular phase, reaching a minimum during ovulation, and immunoglobulin levels increase during the luteal phase (Shrier et al. 2003). In humans, this feature of lower immunoglobulin during rising estrogen levels may facilitate the survival of sperm in the mucosa and ensure the efficiency of fertilization. The lower immunoglobulin levels, however, may render the FRT more susceptible to infection, because minimal immunoglobulin levels in secretions correlate to the same time when the tight junction in the epithelial cells is “degraded” under high estradiol influence (Wira et al. 2015).

Macrophages in the FRT are concentrated in the endometrium and in the myometrial connective tissue. While in the endometrium, they are fluctuate in number with estradiol and progesterone variations (Starkey et al. 1991), but in the vagina, the number of macrophages remains stable throughout hormonal cycling (Wira et al. 2005). Estrogens stimulate the influx of macrophages into the mouse uterus by enhancing the production of MCP-1 by dendritic cells (Moffett-King 2002; Trundley and Moffett 2004). Others have reported that estrogens inhibit the production of the macrophage chemotactic protein (MCP-1) by uterine epithelial cells (Weissenbacher

et al. 2014) Therefore, hormonal control over macrophage numbers is not entirely straight-forward.

Dendritic cells are present in the endometrial stroma and myometrial connective tissue throughout the FRT. In the uterus, the DC do not extend into the epithelium, but intra-epithelial DC (i.e., Langherhans cells) are present in the vagina (Iijima et al. 2008). The dendritic cells of the endometrial stroma are generally tolerogenic, in that they have decreased expression of CD83 and CD86 costimulatory molecules and have decreased TLR expression. The EC secrete cytokines that encourage this tolerogenic phenotype in the stromal DC of the uterus (Reis Machado et al. 2014; Schulke et al. 2008).

The NK cells of the uterus have a phenotype unique to the uterus, and will be discussed in more detail below (Section II, C).As a general comment, the uterine NK cells and dendritic cells are especially useful in facilitating the decidual response at implantation. NK cells are more prominent in the upper rather than the lower tract (Wira et al. 2015). As will be discussed later, progesterone is important for the adequate function of the uterine NK populations (Moffett-King 2002; Trundley and Moffett 2004).

Neutrophils are present throughout the FRT, but are found in largest quantities in the oviduct (fallopian tubes in humans) and progressively decrease from the upper FRT to the lower FRT (Weissenbacher et al. 2014; Wira and Rossoll 2003). Their numbers fluctuate widely in the endometrial stroma since neutrophils assist in normal reconstruction of endometrial tissue, and also have a major function in innate immune defense (Selsted and Ouellette 1995). Estrogen increases the production of IL-8, which is a potent chemotactic factor for neutrophils (Weissenbacher et al. 2014). Not surprising, the highest numbers of neutrophils are recovered from oviducts/fallopian tubes, where IL-8 levels are the highest (Weissenbacher et al. 2014).

Chlamydia, *Neisseria gonorrheoae*, and HSV type 2 are the causative agents of three of the most common sexually transmitted infections in humans. Significant progress has been made to advance understanding of the pathogenic mechanisms and the immune responses to these agents by utilizing animal models. An overview of the animal models of these infections, as representative of FRT infections, are summarized in Kaushic (2015). For specific pathogenesis on the mucosal immune response to HIV and HPV infections in man, the reader is referred to Reis Machado et al. (2014).

11.2.2.3 Immune Function of Seminal Fluid

Above we learned that estrogen primes uterine epithelial cells to produce cytokines and chemokines that result in leukocyte recruitment and activation of innate and adaptive immune events. This activation results in minor, locally controlled inflammation that facilitates implantation. Seminal fluid from the male is thought to have a fundamental role in triggering this beneficial inflammatory cascade. In mice, within hours of mating, there is an infiltration of macrophages, DCs, and granulocytes into the stroma and the lumen of the uterus. Components of the seminal fluid elicit

pro-inflammatory cytokines (IL-6, IL-8 and IL-1) and a robust recruitment of macrophages, tolerogenic dendritic cells and T cells. The high level of TGF-β in seminal fluid allows for presentation of "foreign" sperm antigen by these tolerogenic dendritic cells to expand a population of inducible Tregs (Robertson et al. 2009). Tregs circulate through blood and migrate into the endometrium to generate and maintain immune tolerance to the future conceptus (Guerin et al. 2011; McMaster et al. 1992). A similar inflammatory reaction is seen in women, where seminal fluid induces inflammation in the cervical tissue (Sharkey et al. 2012). The inflammatory response to fluid depends on the high level of TGF-β in the seminal fluid. The TGF-β inseminal fluid is comparable to colostrum, and is five-fold higher than that of serum. In fact, seminal fluid is considered the most potent biological source of TGF-β known (Robertson et al. 2002). High levels of prostaglandins of the E series in seminal plasma also mediate immune-regulatory functions (Kelly 1997). Though seminal fluid facilitates a successful implantation and pregnancy, it is apparently not required, since artificial insemination is effective.

11.2.3 Reproductive Disease of the Non-pregnant State

Because macrophages mediate so many complex and seemingly disparate functions, macrophage dysregulation has been implicated in the pathogenesis of ovarian dysfunctions that involve excessive inflammation.

11.2.3.1 Premature Ovarian Failure (POF)

POF in women is defined by cessation of menses due to failing ovaries before the age of 40 and by increased circulating gonadotropin levels (Blumenfeld et al. 1993). It is clear that autoimmune oophoritis and autoimmune polyglandular syndrome are precursors of POF (Biscotti et al. 1989). Autoimmune abnormalities are detectable in up to 66% of the cases (Cohen and Speroff 1991). In particular, autoantibodies to ovarian antigens such as the zona pellucida, inhibin-α, and/or the LH receptor have been identified. Polyglandular syndrome includes not only autoimmune oophoritis, but hypothyroidism, insulin-dependent diabetes mellitus, and/or Addison's disease. In polyglandular syndrome, autoimmune oophoritis may include antibodies not only against ovarian proteins, but also autoantibodies to the P450-17α hydroxylase of steroid-producing cells of the adrenal cortex and other endocrine tissues. Seventy-eight percent (78%) of patients with POF are positive for antibodies to steroid-producing cells (Tuohy and Altuntas 2007).

While autoimmune conditions are a precursor of POF, lymphocytic or inflammatory changes in the ovary are not characteristic for POF. In women with POF, there is lymphocytic infiltration of ovaries in only 11% of cases (Tuohy and Altuntas 2007). However, even without microscopic evidence of inflammation, a cell mediated immune response is operative in POF. POF patients also have higher circulating

$CD4^+$ T cells and higher $CD4^+/CD8^+$ ratio of circulating leukocytes compared to non-affected individuals.

It has been suggested that a deficiency of Tregs and production of antibodies to inhibin may both be involved in the pathogenesis of POF in women. Lack of Tregs would decrease any tolerance to self-antigens, allowing for antibody production to inhibin-α. This antibody would form a complex with inhibin-α, rendering it useless, and leading to higher levels of FSH (due to the loss of inhibin's inhibitory effect on the pituitary). Excess FSH would lead to initial overstimulation of the ovary followed by depletion of follicles.

The experimental model that most closely mimics this scenario of POF in humans is the immunization of SWXJ female mice with $CD4^+$ T cells activated against inhibin-α (Altuntas et al. 2006). This induces a biphasic disease. The early stage is that of *enhanced* fertility with high levels of inhibin-α neutralizing antibody that prevents inhibin-mediated down regulation of pituitary FSH release. Therefore, there are high levels of FSH and enhanced fertility with superovulation and accelerated rate of follicle maturation during the first phase. The persistence of this dysregulation ultimately leads to the second phase, which is characterized by accelerated depletion of the primordial follicle pool and premature infertility. The hallmark features of human POF also exist in the late stage of this animal model: high titers of antibodies to an ovarian protein, elevated serum levels of FSH, and depletion of follicles. The SWXJ mouse model is particularly appealing to investigators because there are indications that a heritable specific promoter haplotype of the inhibin α gene is a risk factor for POF (Altuntas et al. 2006).

Other animal models of POF are those designed to produce experimental autoimmune oophoritis (EAO), since autoimmune oophoritis is a precursor event of POF in women. One such mouse model for EAO is the thymectomized mouse (Miyake et al. 1988; Smith et al. 1991). In this model, tolerance to self is lost and the immune system attacks multiple self-antigens, including multiple ovarian antigens. The effector cell is the $CD4^+$ T cell, and mice have circulating antibodies to the oocyte, zona pellucida (with destruction of the oocyte), non-germ line ovarian specific antigens like inhibin-α, and/or steroid-producing (granulosa and theca) cells within the ovary. Antibodies are generally produced within 4 weeks of thymectomy. These thymectomized mice also develop autoimmune gastritis (Smith et al. 1992), thyroiditis, and inflammation of the parotid and lacrimal glands 3–14 weeks after thymectomy. The inflammation in the ovary generally subsides within 14 weeks, and eventually, there is loss of oocytes, elimination of follicles, and ovarian atrophy. These end stage features resemble POF in women.

Another model of EAO (as a precursor to POF) is immunization of mice with a 15 amino acid ZP3 peptide, which is the sperm binding site of the zona pellucida (Rhim et al. 1992). As in the thymectomized model, there is induction of T cells and autoantibodies, with the T cell response being the cause of disease in the ovary. EAO may also be induced in rats and BALB/c mice by immunization with rat or bovine ovarian homogenate in Freund's adjuvant. Inflammation occurs in the ovaries within 14 days following these induction procedures. The expansion of germinal centers and increased splenic activity indicate both B and T cells, i.e., both humoral

and cellular immunity, are involved. Serum antibodies appear by day 28 following induction procedures, and antibodies may passively transfer decreased fertility to recipients. Yet another experimental model of EAO is immunization of New Zealand white rabbits with heterologous porcine zona pellucida antigens. This results in a form of EAO with eventual follicle depletion and ovarian failure. It is associated with a marked atretic appearance and reduction and eventual disappearance of primary growing follicles. An inflammatory oophoritis does not occur.

11.2.3.2 Endometriosis

Endometriosis is a chronic inflammatory disease that is defined as the presence of endometrial tissue outside the uterus, causing pelvic pain and subfertility. While the lesions of endometriosis are generally the growth of endometrial tissue outside the uterus, it is also characterized by retarded follicular growth in the ovary. Endometriosis is discussed in this chapter because the pathogenesis of endometriosis may involve dysregulation of the immune system. Patients with endometriosis have increased numbers of Tregs in regional lymph nodes (Budiu et al. 2009; Bergqvist et al. 2001; Santanam et al. 2002), a Th2 dominant phenotype, and autoantibody production (Gleicher et al. 1987). In short, there is circumstantial evidence for defective adaptive immunity in endometriosis, but the details are not yet clarified.

Endometriosis occurs as a spontaneous disease in humans and some non-human primates. Rodent animal models involve surgical transfer of autologous uterine tissue to the intestinal mesentery (Pelch et al. 2012) or surgical transfer of human endometrial tissue into the peritoneum of immunocompromised mice (Awwad et al. 1999; Grummer et al. 2001; Ozawa et al. 2006). Additional rodent models include genetically modified Kras mice form benign ovarian endometriosis-like lesions following injection of a Cre-encoding adenovirus into the ovarian bursa (Dinulescu et al. 2005) or genetically modified Kras mice expressing the human MUC1 tumor-associated antigen (Budiu et al. 2009). Using these animal models, investigators are honing in on a possible immunologic basis for the disease.

11.2.3.3 Polycystic Ovary Syndrome (PCOS)

PCOS is associated with chronic low-grade inflammation evidenced by elevated levels of C-reactive protein, chronic inflammation, and a hypercytokinemic state of pro-inflammatory mediators such as TNF-α and IL-6 (Samy et al. 2009). The cysts might be the result of alteration in the normal inflammation-like ovulatory process (Schmidt et al. 2014). The animal model of PCOS is estradiol-induced cystic ovaries in mice, which is also associated with higher levels of the pro-inflammatory mediators TNF-α and IL-6. Therefore, the pathogenesis of PCOS may involve, or be perpetuated by, abnormalities in the immune system, but the pathogenesis is far from being fully elucidated.

11.3 Pregnant State

11.3.1 Anatomy of the Placenta

The placenta is a mixture of fetal (trophoblastic) cells and maternal (decidual) cells. The gross and the microscopic anatomy differs between species, but there is not much difference in the immunology of the placenta between species. The placenta serves as the site where allogeneic "foreign" antigen of the fetus comes in contact with the maternal host for an extended period of time (from implantation through gestation). Before discussing the immunologic mechanisms operable in the placenta, a brief review of anatomy would be useful.

There are defined tissue layers that make up the placenta and separate the fetus from the endometrium. Starting closest to the fetus, the layers between fetus and dam in the rat are amnion, yolk sac, Reichert's membrane, placental labyrinth, basal zone (trophospongium), and decidua basalis with its embedded metrial gland (Fig. 11.8).

The decidua basalis and metrial gland are composed of "maternally derived" tissues, and it is the embedded metrial gland where immunologic "tolerance" to the fetus occurs. Immediately after implantation, the maternal endometrium undergoes morphologic change into decidual tissue, where the endometrial fibroblasts physically transform into ovoid plump decidual cells. Coursing into and through the decidua are the maternally-derived spiral arteries lined by maternally-derived endothelium. As pregnancy progresses, fetal trophoblasts invade into the decidua (invasive trophoblasts) and into the lumen of the spiral artery (endovascular trophoblasts), where they physically replace maternal endothelial cells. This highly intimate relationship between the invasive and endovascular trophoblasts and the decidua is important for a successful pregnancy, and would not be possible without special immunologic tolerance for these trophoblasts that have surface "foreign" fetal antigens. This tolerance is provided by the metrial gland, which is a cluster of maternally-derived immune cells in the decidua adjacent to the spiral artery. These immune cells include uterine natural killer (uNK) cells, macrophages, dendritic cells and regulatory T cells. The immune cells arrive to the site by the spiral artery and migrate into the supporting stroma of the decidua to form a dense collection of immune cells, termed the metrial gland. This is the site where fetal alloantigens meet maternal immune cells and tolerance is established (Figs. 11.9, 11.10, 11.12, and 11.13). The metrial gland does more than establish immunologic tolerance. The immune cells of the gland control the degree of fetal trophoblast invasion into the decidua and spiral arteries. Trophoblast invasion is required for adequate blood supply to the fetus. When trophoblasts invade the spiral artery, they also replace the maternal endothelium and any smooth muscle cells of the original artery. By so doing, a non-contractile, non-elastic, vessel that is not controlled by maternal vasoconstrictors, forms and this will ensure abundant blood supply to the fetus for the remainder of gestation. Hence the immune cells of the metrial gland have dual immunologic and reproductive roles. The immunologic mechanisms by which the metrial gland accomplishes tolerance will be the focus of this section.

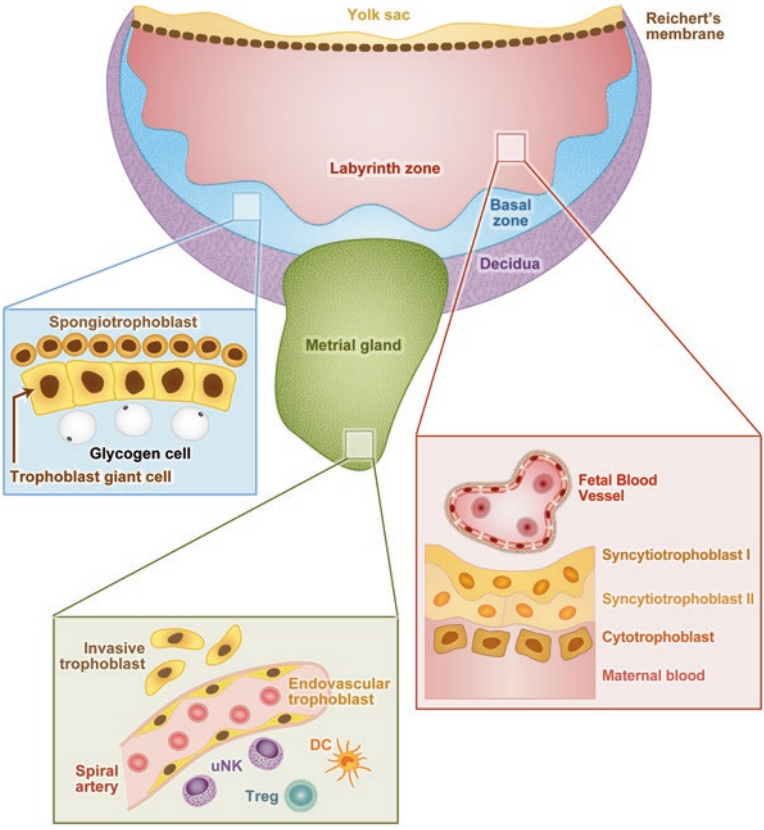

Fig. 11.8 The hemotrichorial rat placenta. The first structure separating the fetus from the uterus is the yolk sac, then Reichert's membrane, followed by the placenta proper. The placenta is comprised of a labyrinth zone (LZ), a basal zone (BZ or trophospongium), decidual zone (DC), and a metrial gland (MG). The **labyrinth** is the zone where fetal and maternal blood are most closely approximated, and in the hemotrichorial placenta of the rat are separated by three layers of trophoblasts. Fetal nucleated red cells are encased by fetal endothelial cells and a basement membrane, followed by two layers of syncytiotrophoblasts and one layer of cytotrophoblasts. The cytotrophoblasts are exposed directly to the maternal blood. The **basal zone** is comprised of three cell types:spongiotrophoblasts, giant trophoblasts, and glycogen cells. The glycogen cells give rise to the invasive and endovascular trophoblasts in the metrial gland. The **metrial gland** is embedded in the decidual zone and extends into the mesometrial triangle. It is comprised of decidualized endometrial stromal cells (as in the decidua) and spiral arteries. In the supporting decidualized tissue of the metrial gland there are large uterine NK cells and Tregs from the maternal blood. Trophoblasts invade from the basal zone and replace endothelium of the spiral artery. The endovascular trophoblasts are critical to spiral artery remodeling and success of the pregnancy. The endovascular trophoblasts replace maternal endothelium and maternal smooth muscle of the spiral artery, thus allowing for wide, non-elastic, incontractile tubes that are free of vasomotor control, and will increase uteroplacental perfusion to meet the requirement of the fetus. Most of the immunoregulation of pregnancy occurs in the metrial gland. The **decidua** (not enlarged) is comprised of decidualized and plump endometrial stromal cells

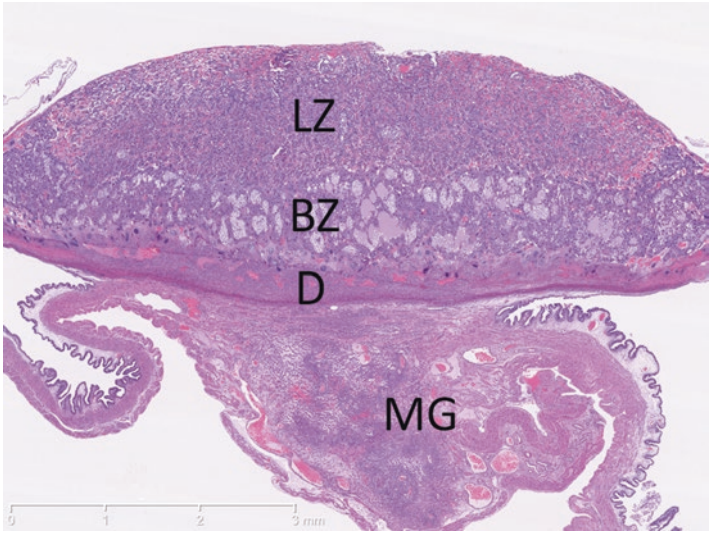

Fig. 11.9 Rat placenta at GD 11. At gestational day 11 (GD 11), the placenta consists of a labyrinth zone (LZ), basal zone (BZ), decidua (D) and a small metrial gland (MG). In the metrial gland, angiocentric uterine NK cells are present. H&E stain. 2.5× objective magnification

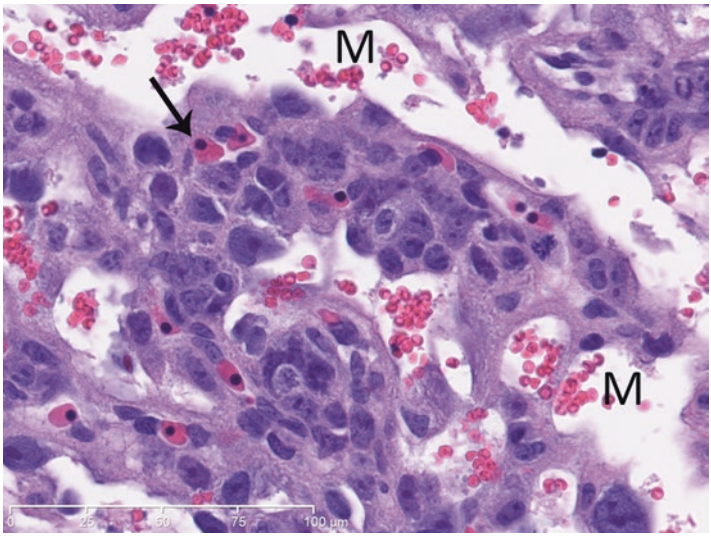

Fig. 11.10 Labyrinth zone of rat placenta at GD 14. In the labyrinth zone, the nucleated fetal blood cells (*arrow*) are separated from maternal blood cells (M) by multiple layers of trophoblasts. H&E stain. 40× objective magnification

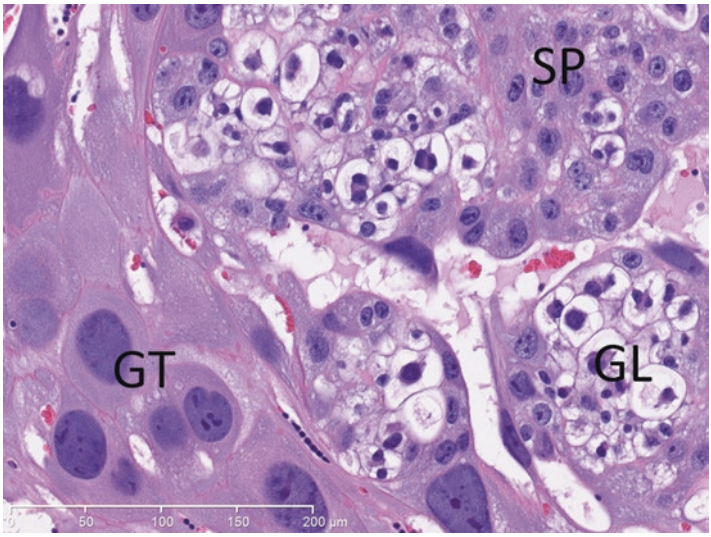

Fig. 11.11 Basal zone of rat placenta at GD 14. In the basal zone are three cell types of fetal origin, including spontiotrophoblasts (SP), glycogen cells (GL), and the giant trophoblasts (GT). H&E stain. 20× objective magnification

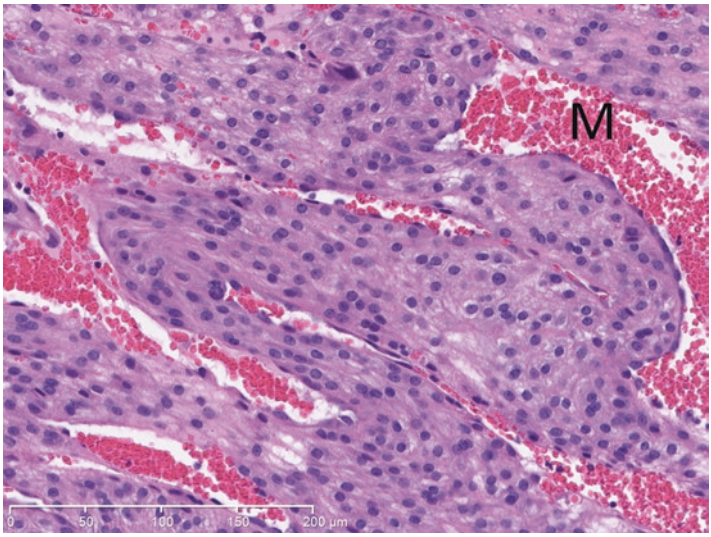

Fig. 11.12 Decidua of rat placenta at GD 14. The decidual zone is comprised of modified thickened "decidualized" endometrial stromal cells. Spiral arteries contain maternal blood cells (M). H&E stain. 20× objective magnification

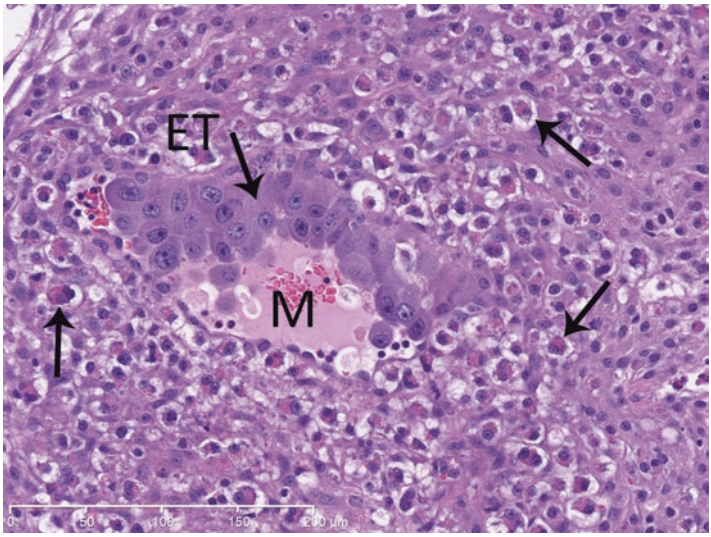

Fig. 11.13 Metrial gland of rat placenta at GD 16. The spiral arteries containing maternal blood (M) are intimately in contract with the endovascular trophoblasts (ET) of fetal origin. The metrial gland stroma is comprised of decidualized stromal cells, invading trophoblasts, and maternally derived immune cells, including uterine NK cells (uNK), macrophages, dendritic cells, and T cells. The uNK cells (*arrows*) can be readily distinguished from other cells by presence of eosinophilic intracytoplasmic granules. H&E stain. 20× objective magnification

The trophospongium or basal zone is of fetal origin. This layer consists of spongiotrophoblasts, a deeper layer of giant cell trophoblasts, and islands of glycogen-rich cells admixed therewith. This layer is the source of the trophoblasts that invade into the decidua and spiral arteries. The labyrinth layer is where maternal blood comes in close contact with fetal blood for exchange of nutrients, waste products, and oxygen. Aside from allowing maternal blood to enter, this layer is of fetal origin, and composed mostly of fetal trophoblasts that form the boundary layer between fetal blood and maternal blood. In the rat, the fetal blood is separated from maternal blood by fetal endothelium, and then three cell layers of trophoblastic cells (one layer of cytotrophoblasts and two layers of syncytiotrophoblasts), with the cytotrophoblast layer being adjacent to maternal blood. These three chorion layers are the basis for subclassifying the rodent placenta as hemo*tri*chorial. Because of the immunologic tolerance established at the metrial gland, close contact between the maternal and fetal tissues is permitted in this labyrinth zone.

The reader is referred to Furukawa et al. (2014) for a concise review of the anatomical comparisons of the placenta in other species such as the rabbit and human. A few differences between species will be mentioned here. The rabbit is similarly a hemochorial placenta, similar to the rat, but in the rabbit there are only two chorion layers separating maternal and fetal blood in the labyrinth zone: one layer of syncytiotrophoblasts and one layer of cytotrophoblasts. The syncytiotrophoblast layer is adjacent to the maternal blood in the rabbit (unlike the rat in which the cytotrophoblasts borders maternal blood). The zones of placentation are somewhat different in

the rabbit than in the rat. There is a labryrinth zone (similar to the rat) which is the zone close to the fetus where there is nutrient and gaseous exchange between maternal and fetal blood, then an intervening junctional zone, and then the maternal decidua. There is no defined metrial gland within the decidua of the rabbit as there is in the rat. Rather, the junctional zone is the site where PAS positive cells (i.e., uterine NK cells) cluster, and the junctional zone is therefore the likely the equivalent of the metrial gland of the rat and the site where immune tolerance to fetal antigens is established.

Human placental anatomy and histology is similar to that of rodents, except that in humans, (1) there is no yolk sac; (2) the placenta is villous rather than labyrinthine; (3) there is only one complete trophoblast layer (i.e., the syncytiotrophoblasts) separating the fetal blood from maternal red blood cells; and (4) there is no physically defined metrial gland. In humans uterine natural killer cells percolate throughout the decidua and do not cluster into defined glands.

11.3.2 General Features of Immunology of Pregnancy

The immune system is designed to protect against disease and contamination by microbes. Mammalian pregnancy, which features gametes and embryos expressing unique antigens, presents a serious biological challenge to the maternal immune system. The fetus and placenta, taken together, constitute a graft of foreign material. Allograft is the term used when tissue is transferred between genetically different members of the same species; and isograft is the transfer of tissue between genetically identical individuals. Pregnancy is therefore best classified as a semi-allograft (Hill et al. 1995), because it is partly an isograft, and partly an allograft. The maternal component of the fetal DNA is from the mother (i.e., an isograft), yet the paternal component of the fetal DNA is from the father, which is a different member of the same species (i.e., an allograft).

Graft rejection is generally mounted by $CD8^+$ T cells and/or NK cells. Cells from an isograft would not express any "foreign" antigen, and cytotoxic T cells would recognize the MHC class I molecules on the surface of the isograft as "self". Cells from the same isograft would also be ignored by NK cells because MHC Class I (HLA-A or HLA-B) molecules on the surface of the isograft cells would bind to inhibitory receptors on the NK cell and avoid being killed.

The situation is different with a semi-allograft, e.g., tissue graft from relative. Somatic cells in a semi-allograft may or may not express HLA-A or HLA-B molecules of the host, and may or may not have foreign antigens. If the semi-allograft cells express HLA-A or HLA-B and express foreign antigens, they are attacked by cytotoxic T cells. If the semi-allograft cells do not contain the HLA-A or HLA-B, yet are cloaked with foreign antigens, they will be killed by NK cells (Fig. 11.14). This is because NK cells have two types of receptors: activating receptors that trigger killing when recognizing ligands that should not be present (i.e., foreign antigens on the semi-allograft cells), and inhibitory receptors that restrain from killing when recognizing classical MHC I molecules. Therefore, in the semi-allogeneic "graft", the fetal

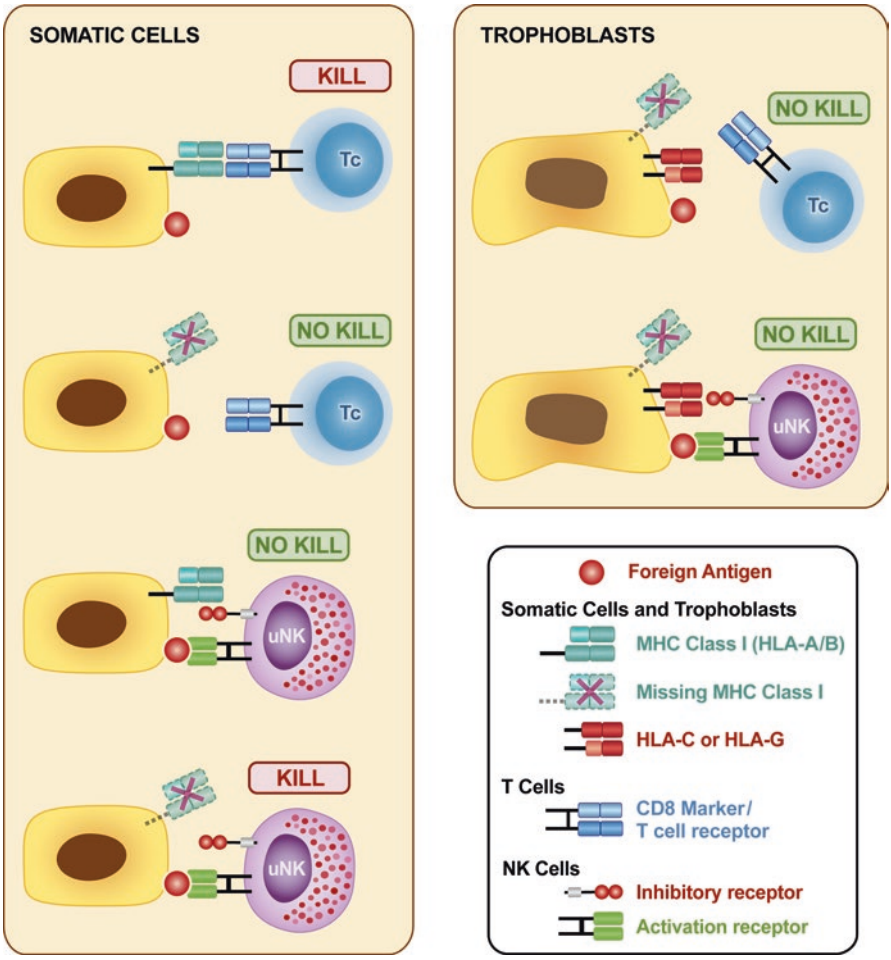

Fig. 11.14 Natural killer cell and cytotoxic T cell killing activity against semi-allogeneic somatic cells and trophoblasts. The *left hand panel* represents the type of somatic cells that could be present in a semi-allogeneic graft of donor cells, and shows how these donor cells can be killed by either cytotoxic T cells or NK cells. In the **first** (*top*) scenario, donor somatic cells express classical MHC class I of the host and foreign antigens. While antigen normally resides in the cleft of the MHC class I, the antigen and MHC molecule are physically separated for clarity in this schematic. Cytotoxic T cells of the host will kill this cell since it recognizes the HLA-A/B. In the **second** from the top scenario, donor somatic cells do not express MHC class I of the host, but has surface foreign antigens. In this instance, cytotoxic T cells will not recognize the donor cell and will not kill it, even though it expresses foreign antigen. In the **third** scenario from the top, donor somatic cells express MHC class I of the host and foreign antigens (as in the top scenario). The HLA-A or HLA-B will activate the inhibitory receptor on normal tissue NK cells and the NK cells will not kill the donor cells. However, this assumes that any foreign ligands on the donor cell that bind to activating receptors do not overpower or outnumber the inhibitory receptors. In the **fourth** (*bottom*) scenario, donor somatic cells do not express MHC class I of the host, but do express foreign antigens. As a result, the inhibitory receptors on NK cells are not activated by MHC, but the activating receptors on the NK cells are activated by foreign antigen. The NK cell will proceed to kill the

cells could be vulnerable to both cytotoxic T cells and NK cells, depending on whether the fetal cells express the same MHC class I molecules as the mother.

The semi-allograft of pregnancy is unique however. The placenta has taken advantage of the various classical and non-classical MHC class I molecules, and a number of other strategic maneuvers to avoid recognition by either NK cells or cytotoxic T cells. The placenta therefore not only provides the hormonal, nutritional and oxygen support to the fetus, but modulates the maternal immune response during pregnancy to prevent semi-allogeneic graft rejection (Mincheva-Nilsson and Baranov 2014).

Before the mechanisms of immunologic avoidance, including modified MHC class I expression, can be properly discussed, the constituent cell types of the immune system operative in the placenta must be introduced. These cell types include uterine natural killer (uNK) cells, placental macrophages, dendritic cells, and subpopulations of T cells. Most of these cells are clustered into the metrial gland of the decidua (at least in the rat), where most of the immune regulation of pregnancy occurs.

11.3.3 Immune Cells of the Placenta

The immune cells of the placenta include uterine natural killer (uNK) cells (approximately 70%), placental macrophages (approximately 15%), T cells (approximately 15%), and dendritic cells (approximately 1%) (Fig. 11.15). Granulocytes (i.e., neutrophils, eosinophils, and basophils) and B cells are essentially absent from normal placentas (Bulmer et al. 1998; Vassiliadou and Bulmer 1996).

11.3.3.1 Uterine Natural Killer (uNK) Cells

Uterine natural killer (uNK) cells are a unique population of NK cells that exist in the pregnant uterus of mammals. Uterine NK cells can also be found in the non-pregnant state in the human uterus, but are generally limited to the pregnant uterus in rats and mice. They represent 70% of the inflammatory cells in the placenta, and have three extremely important functions therein: (1) recruiting or promoting trophoblast invasion of the decidua and spiral arteries; (2) releasing cytokines that promote vascular remodeling and angiogenesis, and (3) producing cytokines that immunosuppress

Fig. 11.14 (continued) donor cell. This is referred to as the "missing-self" scenario. The *right hand panel* represents how trophoblasts of semi-allogeneic pregnancies avoid killing by both cytotoxic T cells and uterine NK (uNK) cells. In the **first** scenario, which presents the T cell situation, trophoblasts express only the non-classical HLA-C or HLA-G MHC Class I molecules, which present foreign antigens. The cytotoxic T cell cannot recognize the fetal cell as self (because it recognizes only the classical HLA-A and B molecules), and therefore does not kill the fetal trophoblast. In the **second** scenario, which presents the uNK situtation, the HLA-C or HLA-G expressed by the fetal cells are capable of binding to the inhibitory receptors on the uNK cells. The uNK cell will not kill the donor cells, despite presentation of foreign antigen in HLA-C or HLA-G context. As above, this assumes the foreign antigens that bind to any activating receptors on uNK cells do not overpower or outnumber the inhibitory receptors

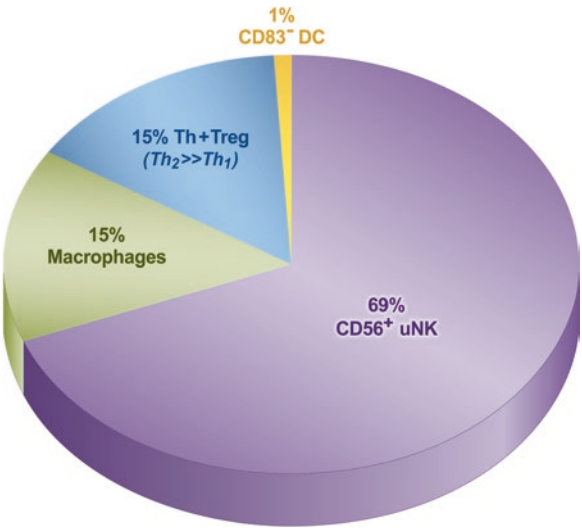

Fig. 11.15 Leukocyte populations in the placenta. This pie-chart depicts the relative numbers of various leukocytes in the placenta. The majority (69%) of leukocytes are the CD56⁺ uterine NK (uNK) cells . Placental macrophages, predominantly of the M2 non-inflammatory type, represent 15%. T cells, predominantly of Th2 or Treg phenotype, account for 15%. Dendritic cells, many of which are immature CD83⁻, DC-SIGN⁺ cells with suppressed antigen presenting function, account for 1% of the placental leukocytes

macrophages and T cells. Uterine NK cells are concentrated in the metrial gland of the rat during pregnancy (and in the junctional zone of rabbits during pregnancy). The uNK cell of the metrial gland is as central to placental growth as the ovarian macrophage is to follicular and luteal growth of the ovary.

Uterine NK cells are derived from circulating CD56⁺ NK cells and are recruited to the pregnant endometrium by chemokines, especially macrophage chemotactic protein-1 (MCP-1) produced by cytotrophoblasts. In addition to recruitment, local proliferation of uNK cells may also be possible (King et al. 1999), thus mitotic activity in the uNK cell population is not uncommon (Picut et al. 2009). Other names for uNK cells in the literature include LGL (large granulated lymphocytes), endometrial granulocytic leukocytes, decidual granulated lymphocytes, and granulated metrial gland cells (in rats and mice) (Picut et al. 2009). CD56⁺ NK cells (i.e., uNK cells) can be found in the circulation, where they represent 10% of the circulating NK cells and are referred to as regulatory NK cells. The majority of *circulating* NK cells are CD16⁺ conventional type and are more highly cytotoxic than the uNK cell.

In humans, uNK cells exist in low numbers in the non-pregnant uterus during the proliferative stage of menstrual cycle, and increase during the secretory phase. If pregnancy occurs, uNK cell numbers remain high (Bulmer et al. 1991), reaching a peak during weeks 8–13 of human pregnancy (Moffett-King 2002) when they account for 70% of the decidual leukocytes. After 20 weeks of gestation, uNK cells

are absent in the human decidua (King et al. 1998). In the placenta, uNK cells encircle vessels and concentrate adjacent to the fetal-derived extravillous trophoblast cells that tap into the spiral arteries (Kam et al. 1999; Bulmer et al. 1987).

In mice and rats, uNK cells are completely absent from the non-pregnant uterus, and limited to the pregnant uterus where they are concentrated in the metrial gland (Picut et al. 2009). The metrial gland is a nodular aggregate of heterogenous tissue in the mesometrial triangle in the uterine wall, in the deep aspect of the decidual basalis (see Figs. 11.9 and 11.16). It is comprised of uNK cells, trophoblasts and fibroblasts and is critical for vascular remodeling and immunoregulation. Rare and scattered uNK may be found in the endometrial stroma of the pregnant uterus at GD 4 (de Rijk et al. 2002). Uterine NK cells start to concentrate in the mesometrial triangle as the metrial gland develops by GD 8 (Picut et al. 2009). By GD 11, uNK cells are clearly organized into angiocentric aggregates (Figs. 11.6 and 11.17) and increase in number through GD 15 in the rat. There is spatial and temporal associations between the uNK cells and the endovascular proliferation of trophoblasts at this time, and this is the focus where immunoregulation for a successful pregnancy will occur. After GD 15, the uNK cells retreat to a peripheral location in the gland (Picut et al. 2009) and begin to undergo apoptosis. The uNK and metrial gland disappear by the end of gestation(de Rijk et al. 2002). The uNK cells are readily recognized in histologic sections, but there are some species-related differences in histological appearance. Rat uNK cells are 25–30 μm in diameter with 1- to 3-μm

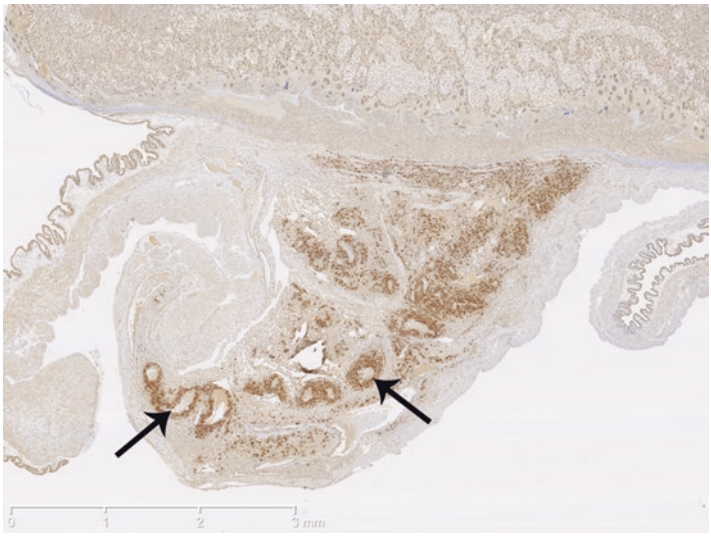

Fig. 11.16 Metrial gland at GD 16. The metrial gland can be identified by the collection of perivascular perforin-positive uterine NK cells in the mesometrial triangle. Note the concentration of brown stain around the spiral arteries (*arrow*). Immunohistochemistry using anti-perforin antibody, diaminobenzidine (DAB) chromagen, and hematoxylin counterstain. 1.25× objective magnification

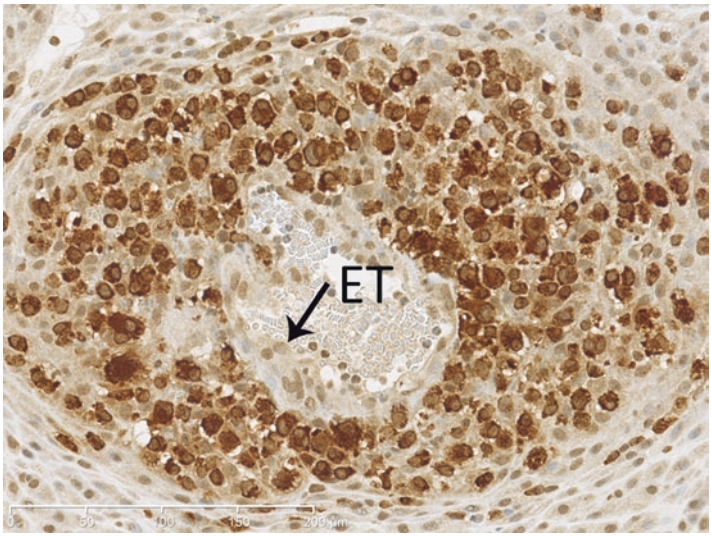

Fig. 11.17 Metrial gland of rat placenta at GD 16. The perforin-positive uterine NK (uNK) cells concentrate around a spiral artery filled with maternal blood cells (*center of figure*). The endothelial cells of this artery have been replaced by perforin-negative (unstained) endovascular trophoblasts (ET) of fetal origin, as determined by H&E stained sections (not shown). Immunohistochemistry using anti-perforin antibody, diaminobenzidine (DAB) chromagen, and hematoxylin counterstain. 20× objective magnification

granules, and are slightly smaller than mouse uNK cells that are 30–35 μm in diameter with 2–4 μm granules (Stewart 1998).

The role of the uNK cell is to suppress the local immune responses and to work in concert with the trophoblast to build the blood supply of the placenta. The uNK cell is not a cell of *destruction*, but rather a cell of *construction*. The suppressed cytotoxic ability of the uNK cell is due, in large part, to trophoblast production of HLA-G (i.e., non-classical MHC-class I) that binds to the inhibitory receptors of uNK cells (discussed later under mechanisms to prevent graft rejection). The binding of uNK to HLA-G on trophoblasts not only turns off the killing function of uNK cells, but the binding also promotes production of factors, such as VEGF, placental growth factor, angiopoietins and proteases, necessary for vascular remodeling of spiral arteries and angiogenesis (Greenwood et al. 2000; Kaloglu and Bulut 2007; Wang et al. 2003). The uNK cell also secretes chemokines CXCL8 (IL-8) and CXCLK10 that recruit trophoblasts to the area to do most of the vascular remodeling work. These trophoblasts are capable of invading the walls of spiral arteries, where they replace endothelial and smooth muscle cells to create a low resistance vascular channel. The uNK cell also takes an active role in suppressing immune responses in the vicinity of the metrial gland. The uNK cells ensure there is a T_{H2}-type cytokine environment of IL-10 and TGF-β that subdues any immune activation against fetal tissue (Eriksson et al. 2004).

While uNK cells recruit trophoblasts to the area, the reverse is also true. Trophoblasts produce MCP-1 which recruits uNK cells into the decidua. Trophoblasts

also produce adrenomedullin, which has also been shown to recruit uNK cells. Adrenomedullin is a vasodilatory, angiogenic and anti-inflammatory protein that binds to G-coupled protein receptors. It is produced not only by trophoblasts, but also by uterine epithelial cells. Estrogen, progesterone and hypoxia (all elevated in placenta) dramatically upregulate adrenomedullin in human and rodent uterus, ovary, and placenta (Matson and Caron 2014). The recognition of estrogen-induced adrenomedullin-recruiting uNK cells is consistent with previous reports that prolactin and estrogen are both important for uNK cells to proliferate and aggregate in the metrial gland (Ain et al. 2003; Stewart 1983, 1998).

11.3.3.2 Placental (or Decidual) Macrophages

Macrophages represent 15% of the leukocytes of the placenta and are the most frequent APC throughout pregnancy (Hunt et al. 2000). In the placenta, there are two populations of macrophages: one is the decidual macrophage of maternal origin. This decidual macrophage originates from hematopoietic stem cell origin and resides in the maternal endometrial stroma or decidua basalis portion of the placenta. The other placental macrophage is the Hofbauer cell, which is of fetal yolk sac origin and comes to reside in the chorionic villus (Pinhal-Enfield and Leibovich 2012). For the purposes of this chapter, the term placental macrophage will be used to refer to both types of macrophages, unless otherwise specified. Placental macrophages are classified as M2 (immune modulation and tissue remodeling, similar to $ED2^{+}$), or M1 (pro-inflammatory type, similar to $ED1^{+}$). The majority of the placental macrophages are of the M2 subtype, express CD163, produce IL-10, and express low levels of CD86 costimulatory molecule. By virtue of their low level expression of CD86, they have a reduced ability to activate T cells to initiate an adaptive immune response (Pinhal-Enfield and Leibovich 2012). The M2 macrophages are designed to resolve inflammation through production of anti-inflammatory Th2 cytokines; perform enhanced phagocytosis; and secrete growth and angiogenic factors (Pinhal-Enfield and Leibovich 2012). The placental macrophages are also immunosuppressed because they have receptors for HLA-G. Soluble HLA-G5 produced by trophoblasts has immunosuppressive effects on macrophages.

It is important to understand that the M1 and M2 phenotypes are on opposite sides of the spectrum, yet macrophage phenotypes in the placenta represent a series of gradations and nuances within this broad spectrum between the pro-inflammatory M1 on one end of the spectrum and the anti-inflammatory M2 macrophage on the other end (Pinhal-Enfield and Leibovich 2012).

While the M2 macrophages are immunosuppressed, they can be called into action at any time. The M1-associated genes for pro-inflammatory IL-1 are hypermethylated and thereby inactivated, but they can protect the fetus against invading pathogens if necessary. Transformation of M2 macrophages into the pro-inflammatory M1 macrophages is suggested to occur pathologically in preeclampsia and physiologically in gram negative infections (Hunt 1989).

Both the Hofbauer cell (the placental macrophage of fetal origin) and the decidual macrophages (of maternal origin) contribute to angiogenesis in the placenta in different ways. Placental Hofbauer cells are programmed to secrete angiogenic factors that promote angiogenesis. Hofbauer cells secrete VEGF, which binds the fms-like tyrosine kinase 1 receptor (Flt-1) expressed on trophoblasts and endothelial cells (Pinhal-Enfield and Leibovich 2012). Decidual macrophages on the other hand encourage this VEGF-induced angiogenesis by controlling the amount of secretory Flt-1 receptor (sFlt-1) in the area. They do this by phagocytosing excess sFlt-1 receptor that would otherwise render the VEGF unavailable. Decidual macrophages (of maternal origin) also control angiogenesis by regulating the amount of placental lactogen in the area. Placental lactogen is a prolactin hormone produced by trophoblasts that promotes placental angiogenesis. Decidual macrophages express Stabilin-1, a receptor for placental lactogen. The decidual macrophage, through its Stabilin-1 receptor, is able to bind, engulf, and digest placental lactogen and thereby control the amount of extracellular lactogen available to promote angiogenesis (Pinhal-Enfield and Leibovich 2012). By phagocytosing sFlt-1 receptor on one hand, and engulfing lactogen on the other hand, the decidual macrophage can titrate closely the rate of angiogenesis in the placenta.

Perhaps one of the decidual macrophage's most vital roles is the swift phagocytosis of apoptotic trophoblasts (Abrahams et al. 2004a). Apoptotic trophoblasts are a normal occurrence in the growing placenta, yet these apoptotic cells could be identified as foreign, inciting a specific immune response by the mother. The M2 placental macrophages engulf apoptotic cells swiftly in order to prevent release of pro-immunogenic and pro-inflammatory intracellular contents.

11.3.3.3 Dendritic Cells

Dendritic cells (DC) comprise 1% of all decidual stromal cells in the uterus at times of pregnancy. There are three types of dendritic cells. Classical mature $CD83^+$ DC, immature DC-SIGN-expressing DC, and a small group of DEC 205^+ immature DC (Kammerer et al. 2007). The mature $CD83^+$ (also expressing CD25, CD40, CD45, CD80/86)) are of the immunostimulatory type and are professional APCs that present antigen to T cells and activate Th1 and Th17 cells. Immature $CD83^-$ cells on the other hand induce T cell tolerance and activate Tregs. These $CD83^-$ DC express CD14 and DC-SIGN (dendritic cell-specific ICAM-grabbing nonintegrin, also known as CD209) (Kammerer et al. 2007). These immature dendritic cells are relatively weak at presenting antigen compared to mature DCs. This is partly because DC-SIGN alters both cytokine production and antigen presentation in ways that benefit foreign antigens or pathogens (van Kooyk and Geijtenbeek 2003). These immature dendritic cells could be central players in the maintenance of immune tolerance in the pregnant uterus by creating an immune privileged site benefitting the "foreign" fetus. The majority of the population of decidual DCs regulate the Th1/Th2 balance of T cells and maintain an immunosuppressive Th2 dominant state.

11.3.3.4 T Cell Subpopulations

Although the non-pregnant uterus is home to several subpopulation of leukocytes with ability to initiate adaptive immune responses, major modifications are made in the uterine leukocyte population at the time of implantation to reduce the likelihood of anti-fetal immunity. The pregnant uterus becomes reprogrammed from a mucosal site with Th1 and B lymphocytes (albeit small numbers of B lymphocytes) into a site where innate immune cells predominate and T cell adaptive immunity is suppressed. After all, adaptive immunity directed toward the conceptus antigens would pose significant danger to ongoing fetal development.

The placenta is richly populated by maternal leukocytes, especially where the extravillous trophoblasts invade the spiral arteries in humans and in the metrial gland in rats. In humans, T lymphocytes account for 15% of the total number of immune cells in the pregnant uterus (Du et al. 2014). The most prominent leukocyte subpopulations in the preimplantation uterus are NK cells and APCs that include macrophages and dendritic cells. Any scarce B cells and many of the T cells that were percolating throughout the FRT disappear at implantation, leaving immune suppressive, anti-inflammatory regulatory T cells as the major remaining T cell subset (Heikkinen et al. 2004). In general, T cells can be classified as either cytotoxic T cells ($CD8^+$), helper T cells ($CD4^+$), or regulatory T cells ($CD4^+$, $CD25^+$, FoxP3+). The $CD4^+$ T helpers can be further divided into Th1 cells that produce inflammatory cytokines (IL-1β, IFNϒ, TNFα), the Th2 cells that produce anti-inflammatory cytokines (IL10, IL4, TGFβ), and the more recently described Th17 cells that produce pro-inflammatory cytokines (IL-17 and IL-23). T regs are T cells that express $CD4^+CD25^+$ FoxP3+, and are responsible for maintaining tolerance and suppressing immune activation. In the pregnant uterus, there is an increase in the ratio of Th2/Th1 cells, a decrease in the number of Th17 cells, and an increased percentage of Tregs, compared to the circulation. This lymphocyte profile creates an immunosuppressive environment in the pregnant uterus (Lin et al. 1993). Pregnancy has been termed a Th2 type phenomenon (Wegmann et al. 1993). During normal non-pregnant states, the Th1/Th2 ratio in the endometrium is 147.5 during the proliferative phase; 37.4 during the secretory phase; and merely 1.3 in the early pregnancy decidua (Saito et al. 1999; Mjosberg et al. 2010).

Cytotoxic T cells were present in the non-pregnant uterus, and in fact there were more cytotoxic T cells in the uterus, than in the lower FRT. During pregnancy, however, there is a banishment of cytotoxic T cells from the uterus and the placenta. The banishment of T cytotoxic cells from the placenta has been shown recently to be due to a T cell silencing chemokine. Normally decidual stromal cells produce T cell chemoattractants (e.g., CXCL9) that attracts cytotoxic T cells when induced with endotoxin. This silencing cytokine prevents the cytotoxic T cell from responding even in the presence of strong systemic inflammatory signals (Nancy et al. 2012). Therefore, the pregnant uterus has virtually no cytotoxic T cells that could kill the conceptus.

The immunosuppressive Th2 dominant state is created by numerous cytokines, chemokines and hormones. Progesterone has been implicated as the cause for the

Th2/Th1 shift as well as the cause for the increased numbers of Tregs (Porter and Scott 2000; Mao et al. 2010). Moreover, at least 18 chemokine receptors are present at the maternal-fetal interface (Du et al. 2014), and the decidual stromal cells and trophoblasts secrete an array of chemokines, many of which are involved in the recruitment and trafficking of leukocytes. A review of these chemokines is available (Du et al. 2014). Substances produced by immature dendritic cells also might affect the Th2 preponderance (Saito et al. 2010). High estrogen, PGE2 and IL10 all favor the Th2 phenotype, and they do so by inhibiting IL-12 production by decidual DCs (Saito et al. 2010).

To further support the immunosuppressive phenotype of a Th2 dominant environment, regulatory T cells (Tregs) are increased locally and promote immune tolerance to the environment. T regs are present in increased numbers both in the circulation and in the decidua during the first and second trimesters of human pregnancy (Somerset et al. 2004; Heikkinen et al. 2004). In mice, a substantial increase in $CD4^+$ $CD25^+$ T cells are noted in spleen and lymph nodes draining urogenital tract as early as day 2 of pregnancy (Aluvihare et al. 2004). These Tregs represent approximately one third of all $CD4^+$ T cells in the pregnant uterus. Tregs suppress activation of $CD4^+$ T cells (Th1 and Th17 cells) and inhibit generation of cytotoxic $CD8^+$ T cells and cytotoxic natural killer cells. T_{regs} also interact with macrophages to support maintenance of the M2 phenotype and with DCs to support maintenance of the tolerogenic DC phenotype. The Tregs secrete TGF-β and IL-10 to condition the dendritic cells to become tolerogenic. Unlike the Th1 or Th2 subpopulations, the increased formation of T_{regs} is not a response to hormonal changes during pregnancy, but rather dependent on exposure of T cells to paternal/fetal alloantigens (Schumacher et al. 2007). As mentioned above, the seminal fluid with its high TGF-β is the first step in causing the induction of Tregs. Tregs and tolerogenic DCs self-perpetuate one another.

11.3.4 Suppression of Fetus Rejection during Pregnancy

In the semi-allogeneic pregnant state, the immune system must be circumvented to prevent graft rejection. Fortunately, there are mechanisms in place to prevent intimate associations between maternal and fetal cells. These mechanisms are especially important in species with a hemochorial placenta (human, monkey, rodents), where there is most invasion of the maternal tissue and most intimate contact between the maternal immune system and fetal tissue. The immune protective mechanisms developed to facilitate semi-allogeneic pregnancy in mammals with hemochorial placentation are contributed by both the mother and the fetus. The mechanisms to circumvent the immune activation of graft rejection involves changes in the distribution of leukocyte subpopulations in the uterus (Th2 dominance; increased Tregs); selection of the M2 immunosuppressive and non-inflammatory phenotypes of placental macrophages (described above); production of immune suppressive molecules by both maternal and fetal cells

(progesterone, prostaglandin, cytokine, growth factors, and indoleamine-2,3-dioxygenase); restriction on fetal trophoblast cell expression of the HLA class I molecules; expression of inhibitory B7 molecules on trophoblast cells and macrophages; high expression of complement-activating regulatory proteins on trophoblasts; engagement of pro-apoptotic FasL-Fas system; and use of morphologically defined microvesicles called exosomes to act as decoys (Trundley and Moffett 2004) (Figs. 11.18, 11.19, 11.20, and 11.21). Collectively, these strategies provide successful avoidance of adaptive immune activation, and selective suppression of the uNK cells as part of the innate immune system. When these mechanisms are working, the fetal/placental semiallograft is not rejected, which the laws of transplantation biology would otherwise dictate.

11.3.4.1 Production of Immunosuppressive Molecules

Virtually all cells of the placenta, including trophoblasts, macrophages, Th cells, Tregs, and decidual stromal cells produce immunosuppressive and/or anti-inflammatory factors. This is the overall strategy of the placenta to limit the chance that the mother will reject the foreign antigens on fetal tissues. All subpopulations of trophoblasts cells (i.e., syncytiotrophoblasts, cytotrophoblasts, and extravillous cytotrophoblasts (in humans)) act as an efficient structural barrier to trafficking of lymphocytes or other immune cells into the fetal tissue. The trophoblasts express cell surface molecules (e.g., HLA-G) and secrete substances (e.g., sHLA-G5, TGF-β1, IL-10, progesterone) that down-regulate cell-mediated immune responses.

Progesterone might be considered the instigator of the immunosuppressive cascade (Fig. 11.18). Progesterone produced by trophoblasts interferes with the NFκB pathway (a pro-inflammatory pathway) and drives cells (macrophages, T cells, NK cells) into an immunosuppressive profile in humans and mice. More specifically, progesterone programs T helper cells to produce IL-10 and IL-4, and macrophages and Tregs to produce IL-10 and TGF-β (Lee et al. 2011; Piccinni et al. 1998). Progesterone creates its own negative feedback loop within the placenta. It stimulates T cells to produce Progesterone-Induced Blocking Factor (PIBF), which down-regulates the effects of progesterone. TGF-β and IL-10 potentiate the immunosuppression of macrophages by inhibiting production of reactive oxygen species, down-regulating expression of MHC class II (i.e., limiting the APC function), and quelling the cytotoxic enthusiasm of both macrophages and uNK cells. Collectively, an anti-inflammatory immunosuppressive cytokine environment is created where antigen presentation and helper T cell activation is minimized.

Additional immunosuppression might be afforded during certain times of pregnancy by secretory leukocyte proteinase inhibitor (SLPI), which is a neutrophil elastase inhibitor produced by maternal uterine glandular epithelial cells. SLPI is not only an antimicrobial, but also inhibits production of NFkB. This inhibition of NFkB would further squelch inflammatory cytokine production. SLPI helps keep inflammation to a minimal during the critical phases of implantation and early pregnancy (Weissenbacher et al. 2014).

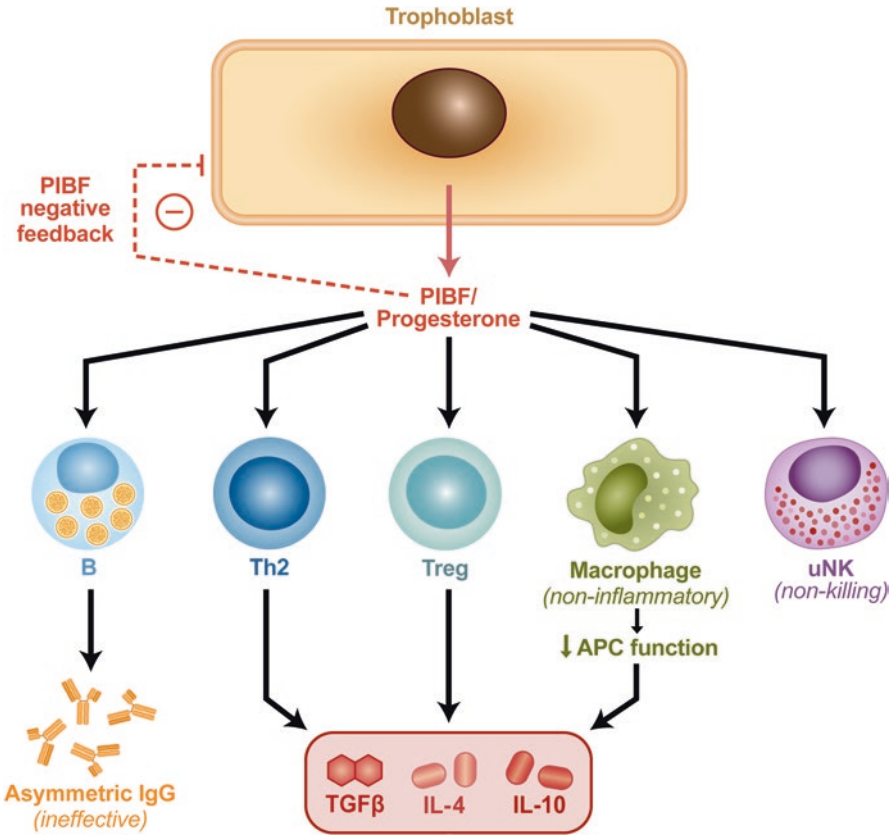

Fig. 11.18 Mechanism of immunosuppression—progesterone cascade. Progesterone produced from trophoblasts instigates the immunosuppressive cascade, though IL-10 and TGF-β potentiate the effect. Progesterone induces a Th2 cytokine profile for helper T cells, increases the number of Tregs, and suppresses the antigen presenting abilities of macrophages. The end result is an environment rich in anti-inflammatory cytokines such as IL-4, IL-10 and TGF-β at the maternal-fetal interface. Progesterone also induces the production of Progesterone-Induced Blocking Factor (PIBF), which can be produced by Th2 cells. PIBF in turn functions as negative feedback on the trophoblast to decrease production of progesterone, and this negative feedback loop helps maintain homeostasis. PIBF also induces B cells (or plasma cells) to produce asymmetric (or ineffective) IgG. This helps prevent complement-fixing antibody reactivity against paternal antigen

After progesterone initiates the immunosuppressive cascade, IL-10 cytokine from T helper cells takes over (Fig. 11.19). IL-10 not only directs other T lymphocytes away from killer activities, it promotes expression of HLA-G by trophoblasts and inhibits expression of classical MHC I molecules. HLA G (the membrane bound form) and HLA-G5 (its soluble isoform) on trophoblasts have profound effects on uNK cells. The uNK cells have KIR inhibiting receptors for soluble HLA-G. When KIR receptors are bound to HLA-G, the uNK cells have reduced capacity to synthesize certain pro-inflammatory mediators and the uNK killing function is dismantled (Moretta et al. 2001). The binding results in production of IL-10 and

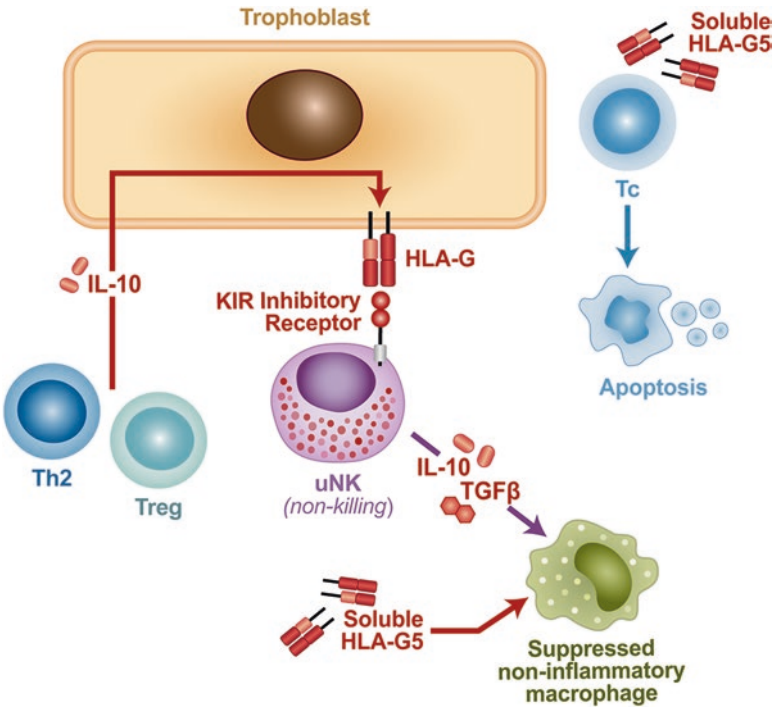

Fig. 11.19 Mechanism of immunosuppression—upregulation of HLA-G. Th and Treg cells both produce IL-10 (see Fig. 11.18). The IL-10 upregulates expression of HLA-G on trophoblasts. HLA-G binds to KIR (killing immunoglobulin-like receptors) on uterine NK (uNK) cells, dismantling their killing action. Binding of HLA-G to uNK cells further upregulates production of anti-inflammatory mediators IL-10 and TGF-β from uNK. The soluble form of HLA-G (HLA-G5) has additional immunosuppressive roles. HLA-G5 binds to cytotoxic T cells, resulting in T cell apoptosis; and binds to macrophages to further subdue any pro-inflammatory tendencies

TGF-β, which drives macrophages into an anti-inflammatory phenotype. Soluble HLA-G5 is not only immunosuppressive to uNK cells, but it has immunosuppressive effects on T lymphocytes and macrophages directly. HLA-G5 binds to T lymphocytes and stimulates death of activated lymphocytes ($CD8^+$ or $CD4^+$) (Solier et al. 2002), reduces expression of $CD8^+$ on cytotoxic lymphocytes, and directly induces immunosuppressive profiles in mononuclear phagocytes and T cells (van der Meer et al. 2004).

Trophoblasts also express HLA-E (non-classical MHC I molecule) and certain haplotypes of HLA-C (classical MHC I molecule), which can bind to inhibitory receptors referred to as CD94/NKG2 (a C-type lectin receptor) and ILT2 (Ig-like transcript 2), respectively, on uNK cells, further ensuring that the killing function of these cells are turned off (Table 11.2).

In addition to expressing only certain MHC class I molecules that turn off uNK cell cytotoxicity, there is another mechanism by which trophoblasts suppress the

Table 11.2 NK receptors and ligands in humans and rats/mice

NK receptor	Ligand on trophoblast	Primary function
K I R (LY49)	HLA-C HLA-G (QA-2)	Inhibitory Inhibitory
CD94/NKGs	HLA-E (QA-1) MIC-A, MIC-B	Inhibitory Activating
ILT-2	HLA-C HLA-G	Inhibitory Inhibitory

QA-2 is the functional homologue of HLA-G in man
QA-1 is the functional homologue of HLA-E in man
KIR killing immunoglobulin-like receptor, *ILT-2* immunoglobulin-like transcript 2

immune system. Trophoblasts, and to a lesser degree placental macrophages, generate toxic tryptophan metabolites by the enzyme indoleamine 2,3,dioxygenase (IDO) (Fig. 11.20). IDO suppresses virtually every type of immune cell, especially T cells (Munn et al. 1998; Trowsdale and Betz 2006; Hwu et al. 2000; Mellor et al. 2002), because it degrades the essential amino acid L-tryptophan. T cells are uniquely sensitive to fluctuations in the local concentrations of the amino acid tryptophan. A drop in tryptophan concentration results in decreased T cell proliferation (Sedlmayr et al. 2002) and induces a tolerogenic phenotype in dendritic cells (Bansal et al. 2012). There are negative feedback mechanisms to keep IDO in check. IDO production increases when there are proinflammatory molecules in the vicinity, such as IFN-γ, TNF-α, and IL-1, but IDO production decreases in the presence of anti-inflammatory cytokines IL-4, IL-10 and TGF-β (MacKenzie et al. 1999). Therefore, pro-inflammatory cytokines will activate the IDO suppressive mechanism, and *vice versa*, helping to keep inflammation in check and well-regulated.

Vasoactive intestinal peptide (VIP) is a neuropeptide expressed in trophoblast and contributed to smooth muscle relaxation and vasodilation in the uterus (Marzioni et al. 2005). VIP also has anti-inflammatory effects and, like progesterone, promotes a Th2 shift through the stimulation of IL-10. DCs differentiated in the presence of VIP induce T_{regs}, inhibit allogeneic $CD4^+$ responses and increase TGF-β production (Fraccaroli et al. 2009).

11.3.4.2 Inhibitory co-stimulatory Proteins on Trophoblasts and Macrophages

There are many members of the B7 family of immunodulators that play a role in immune tolerance during pregnancy. The B7 family of molecules mediate cell to cell interactions with receptors such as CD28 or CTLA-4 on T lymphocytes. These ligand-receptor interactions can have stimulatory (CD28) or inhibitory (CTLA-4) effects on T cells. Trophoblasts express B7H1 and B7H2, both of which down-modulate T cells through several ligands of the CD28 family, such as PD-1, ICOS (inducible CO-Stimulator of T cells), or CTLA-4, that conduct the inhibitory signal (Petroff 2006). The end result is to shut off T cell activation and

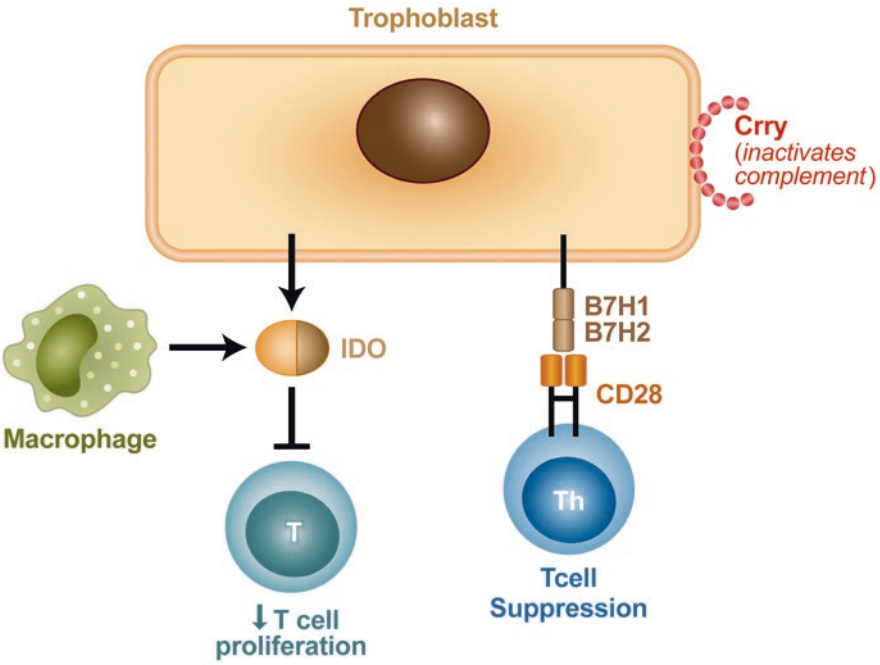

Fig. 11.20 Mechanism of immunosuppression—indoleamine-2,3,dioxygenase (IDO) , B7 expression, and Crry proteins. Additional mechanisms by which trophoblasts suppress the local immune system are depicted in this figure. Trophoblasts and placental macrophages produce indoleamine-2,3, dioxygenase (IDO) . IDO inhibits proliferation of T cells by starving the cell of the essential nutrient tryptophan. Trophoblasts also express B7 co-stimulatory molecules of the inhibitory type (B7-H1 and B7-H2). These B7 inhibitory molecules bind to ligands of the CD28 family (such as CD28, PD-1, ICOS, and CTLA-4) on T cells, causing suppression of T cell function and proliferation. Trophoblasts protect themselves from complement-mediated destruction by expressing Crry, a complement-activating regulatory protein, on their surface. Crry will hinder complement activation by inhibiting the enzyme C3-convertase which is critical to both the alternative and classical complement cascade

antigen specific immune responses (Fig. 11.20). These inhibitory members of the B7 family are expressed not only by trophoblasts, but also by placental macrophages (Scodras et al. 1990).

11.3.4.3 Complement-Activating Regulatory Proteins on Trophoblasts

The placenta has an effective protective mechanism to prevent destruction of the placental tissue by complement, which can be activated by either cytotoxic antibodies or by the large of amount of cell debris that accumulates as a result of tissue remodeling. Trophoblasts have a number of membrane-bound complement regulatory proteins that interfere with complement deposition or activation. This

effectively protects the placenta from complement-mediated cytotoxicity in the event IgG binds to the trophoblast (Fig. 11.20). Complement-control or complement-activating regulatory proteins are better characterized in humans than in rodents. In humans, they include DAF (decay accelerating factor) and membrane cofactor protein (MCP), and in rodents, they are referred to generally as Crry (or complement regulatory proteins). Crry in mice inactivates C3-convertase on the surface of trophoblast cells, which halts both the classical and alternative complement cascades. Mice with a knock-out gene for the Crry complement regulatory protein have a high incidence of placental inflammation and fetal loss (Trowsdale and Betz 2006).

Considering that the majority of the leukocytes in the placenta are T cells, one would expect that immunoglobulin production or antibodies in the placenta would be minimal. However, there is significant production of immunoglobulin by B lymphocytes at the maternal-fetal interface, and some of the immunoglobulinis directed against the fetal trophoblast. Anti-HLA antibodies are relatively common in women, but they do not appear to cause any effect on pregnancy. Anti-HLA antibodies are found in 20% of first pregnancies and this rises to 75–80% of multiparous women. Part of the reason the anti-HLA antibodies fail to pose a problem is because of complement regulatory proteins on the trophoblasts.

The maternal fetal interface is also an important site where immunoglobulin is normally transferred to the fetus. Antibodies of IgG subtype are normally transported across the placenta from maternal blood to fetal blood via endosomes or transcytosis. Synctiotrophoblasts bathed in maternal blood internalize maternal IgG in endosomes. FcRn (the neonatal receptor for Fc portion of immunoglobulin) is expressed on the internal surface of these endosomes and bind to the IgG in this acidic environment ($pH < 6.5$). While bound to FcRn, the IgG is protected from degradation, is transported to the fetal side of the synctiotrophoblasts and released into the fetal blood. The release of IgG is due to physiological disassociation from the FcRn due to higher pH of fetal blood (pH 7.4). The same FcRn may be important in transporting maternal IgG from mother's milk across intestinal cells of the young infant. The FcRn exhibits pH dependency for IgG binding, and has high affinity for IgG at relatively low pH of 6, but very low affinity at physiological pH 7.4 (Palmeira et al. 2012). Thus FcRn is able to bind to, protect, and carry IgG in the protected acidic endosomes, but is unable to bind IgG at the apical side of the synctiotrophoblasts facing maternal blood.

11.3.4.4 Modified MHC-Class I Expression

Above, it was mentioned that trophoblasts express HLA-G and can produce soluble HLA-G5 as a means to immunosuppress T cells and macrophages in the vicinity of the placenta. Expression of HLA-G, however, is part of the overall plan to modify the regular expression of MHC Class I molecules. Normally, all nucleated cells express MHC Class I (HLA-A and HLA-B; and the mouse equivalent H-2 K and H-2D) in order to be recognized as "self". In the placenta, however, this strategy

does not work, because the placenta is not "self". The placental trophoblasts therefore express a modified unconventional profile of MHC Class I molecules. Instead of expressing HLA-A and HLA-B, they express non-classical HLA-G, HLA-E, and only certain haplotypes of HLA-C (classical). When cloaked in this unconventional ensemble of MHC class I molecules, the relatively non-discriminating uNK cell sees the trophoblast as "self" (Fig. 11.21). Similar to the binding of classical HLA-A and HLA-B to inhibitory receptors on NK cells, HLA-C, HLA-G and HLA-E bind to inhibitory KIR, ILT2 and CD94/NKG2 receptors on uNK cells (Collins and Baltimore 1999; Trowsdale and Betz 2006). Based on this inhibitory signaling that interrupts uNK-mediated killing, the uNK cells allow the trophoblasts to survive. Simultaneously, the highly discriminating cytotoxic T cells that require antigen presentation in the context of classical MHC class I molecules (HLA-A and HLA-B) to initiate cell killing overlook the trophoblast cells because antigen is not presented in classical MHC I context. By utilizing atypical MHC class I expression the trophoblast has successfully protected itself and the fetus from the two primary mechanisms for graft rejection, which are based on cell killing mediated by NK cells and cytotoxic T cells. .

One reason why HLA-C markers are not recognized as self by cytotoxic T cells is the relatively short half-life of HLA-C. In addition, the process whereby HLA-C on trophoblasts binds to inhibitory receptors on uNK cells is complex, and the focus of current research. At this point it is clear there are multiple variants of fetal HLA-C gene haplotypes and multiple subtypes of killer inhibitory receptors (KIRs) on uNK cells that bind to the HLA-C ligands. Research is focused on identifying which combinations of KIR receptors and HLA-C ligands inhibit (i.e., activate the inhibitory receptor) and which combinations fail to inhibit (i.e., fail to activate the inhibitory receptor) uNK cells. Those that fail to inhibit uNK cell killing may be the ones associated with pregnancy complications such as preeclampsia.

11.3.4.5 Pro-apoptotic Mechanisms (Fas–Fas L) and Microvesicles as Decoys

Fas–FasL at the maternal fetal interface contributes to the lack of immune attack of mother against fetus. Fas L (or CD95L) present on trophoblasts and uterine glandular epithelium will bind to Fas on maternal NK and T cells, resulting in apoptosis of the NK or T cells, thus preventing them from launching an offensive cytotoxic attack (Hunt et al. 1997). The role of FasL has been studied in the *gld* mutant mouse that lack Fas-L. Pregnancy in gld mice is characterized by extensive infiltration of lymphocytes and necrosis at the interface of the decidua (Hunt et al. 1997). Other studies point to the importance of Fas-FasL in preventing immunologic rejection in humans. Ohshima and colleagues studied the relationship between FasL expression and NK cell infiltration in human placenta during early pregnancy (Ohshima et al. 2001). They showed that a reduction in FasL expression by trophoblasts was closely associated with activation and infiltration of maternal NK cells, and destruction of uterine glands, resulting in rejection of the fetus. Thus, expression of FasL in the

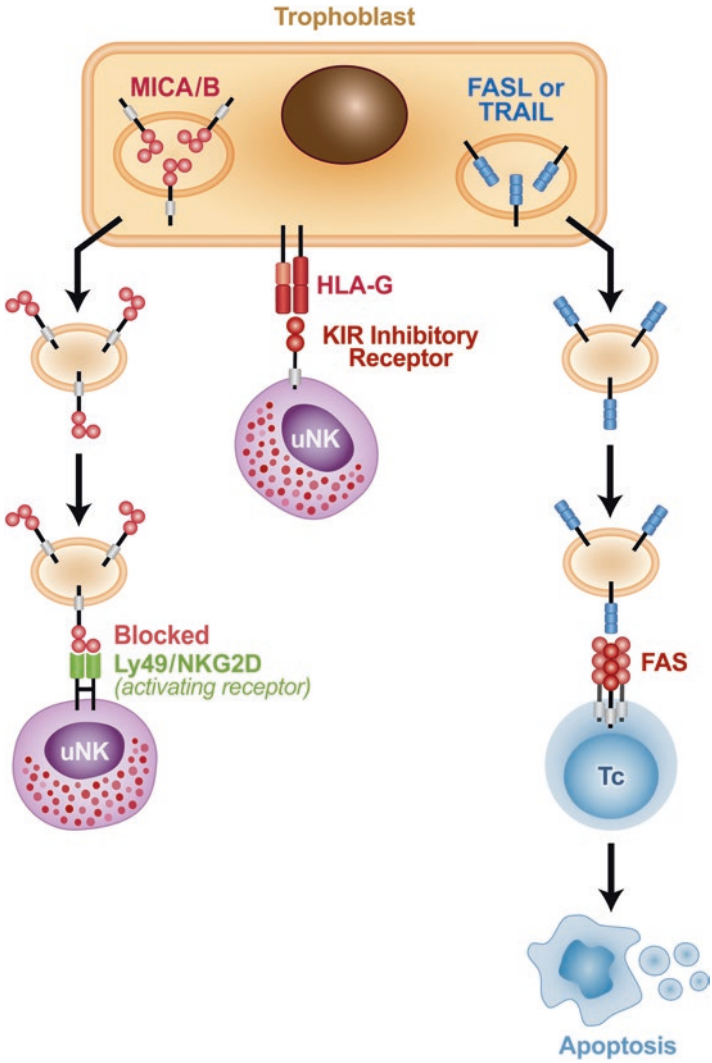

Fig. 11.21 Mechanism of immunosuppression—use of exosomes, MIC-A and -B, and Fas-FasL. The use of MIC-A, MIC-B and Fas-FasL mechanisms with exosomes is an ingenious way for the trophoblast to control immunologic attack against itself. Stress HLA molecules (MHC class I chain-related proteins A and B, MIC-A and MIC-B), are produced by trophoblasts. These molecules exist only on the cytoplasmic membranes of exosomes, and not on the trophoblast cell membrane. MIC-A and MIC-B are MHC class I glycoprotein molecules encoded by the *MICA* or *MICB* gene and do not have associated β2 microglobulin. MIC-A and MIC-B are incapable of binding to the KIR inhibitory receptors on uNK cells. Instead they bind to the CD94/NKG2D *activating* receptors. When MIC-A and MIC-B are exported a distance from the trophoblast on the surface of exosomes, they essentially block all the CD94/ NKG2D activating receptors on uNK cells through a form of competitive inhibition. The uNK cells are left impotent. This mechanism assures that uNK cells (even those some distance from the trophoblast) become inactive killers. Those uNK cells in close contact with the trophoblasts utilize the convential HLA-G/KIR binding to be rendered inactive. Fas-L is also expressed on exosomes within the trophoblasts. When FasL is released by exosomes, it binds to Fas on the surface of uNK or cytotoxic T cells, resulting in apoptosis of the uNK or cytotoxic T cell

uterine glandular epithelium and cytotrophoblasts may play a role in downregulating the maternal immune response, thereby helping to maintain pregnancy.

It is now understood that FAS-L is exported away from the trophoblast by exosomes, in much the same way that exosomes transport TGF-β1 and MIC-A and MIC-B (discussed below) to sites removed from the trophoblast (Fig. 11.21). The FAS-L molecule is actually not on the surface of syncytiotrophoblasts, but concentrates only on the membrane of its exosomes (Mincheva-Nilsson and Baranov 2014). The exosomes are <100 nm microvescular bodies that are actively exteriorized after fusion with the plasma membrane (Mincheva-Nilsson and Baranov 2014). Removing the Fas-FasL apoptosis activity away from the fetal-maternal interface would be beneficial because it reduces the potential for accumulations of cell debris that could activate complement. Exosomes have an added benefit of protecting the FAS-L from degradation by proteases (Vargo-Gogola et al. 2002; Stenqvist et al. 2013). The exosomes can also contain MHC class II antigens that can influence expression of T cell activation markers (Taylor et al. 2006), and immunosuppressive proteins such as B7 family proteins that can influence macrophage function and differentiation (Atay et al. 2011; Hedlund et al. 2009).

TRAIL (TNF-related apoptosis inducing ligand) is another pro-apoptotic TNF-related molecule which, like FAS-L, is expressed in exosomes in trophoblasts and transported to cause apoptosis of inflammatory cells (Stenqvist et al. 2013). TRAIL is highly expressed by trophoblasts cells, is released into serum, and is toxic to leukocytes bearing its receptors (Phillips et al. 1999).

Recently, it has been shown that MHC class I chain-related proteins A and B (MIC-A and MIC-B) are expressed in exosomes within the cytoplasm of the trophoblast (Mincheva-Nilsson et al. 2006; Mincheva-Nilsson and Baranov 2014). These molecules are not the complete MHC Class I molecule because they do not bind to β2 microglobulin. MIC-A and MIC-B containing exosomes are released from the trophoblasts and the MIC-A and MIC-B bind to the activating NK receptor CD94/NKG2D (Gonzalez et al. 2008). At first glance, one would think that activating the NK cells with MIC-A or MIC-B-containing exosomes would be detrimental, leading to cell killing by the uNK cell. However, there is evidence that the exosomal release of MIC-A or MIC-B acts as a decoy and blocks the CD94/NKG2D "activation" receptor ligands on NK cells, preventing activation of killing against the trophoblast itself (Fig. 11.21). This "decoy" maneuver has been revealed when exosomes containing MIC-A and MIC-B downregulate NK cells in a dose dependent manner (Hedlund et al. 2009).

Fig. 11.21 (continued) (Tc is shown in schematic). This FasL-Fas mechanism helps to maintain immunologic homeostasis by causing destruction of "killing" cells. Because the Fas-L exosome can travel a distance, it also ensures that the apoptosis occurs far from the maternal-fetal interface. In this way, apoptotic debris does not stimulate unnecessary inflammation right at the maternal-fetal interface. The TRAIL (TNF-related apoptosis-inducing ligand) and PD-L1 (not shown) are other pro-apoptotic ligands that can be found on exosomes and transported far from the trophoblast to cause death of activated immune cells

11.3.4.6 Multinucleated Cell Transformation

Multinucleated and karyomegalic trophoblasts in the trophospongium (see Fig. 11.8) are the result of evolution of the immunosuppressive mechanisms, and serve as a visual reminder of the unique immunologic environment in the placenta. The syncytial trophoblasts (in the trophospongium) form a true syncytium with no extracellular spaces between cells. This structural barrier helps limit exchange of migratory immune cells between the embryo and mother. However, the syncytial trophoblasts are not in the metrial gland of the rat, where the majority of active immunosuppression takes place. Therefore, this structural barrier afforded by the syncytial trophoblasts is interesting, but probably not critical for successful pregnancy. The fusion of trophoblasts is caused by viral fusion proteins from endosymbiotic endogenous retroviruses (ERVs) (Mi et al. 2000). The genome of mammals is littered with ERVs. Most have been inactivated in the course of evolution, but interestingly, the ERVs in the placenta have not been inactivated. The endogenous retroviruses have a gene that codes for a protein called syncytin, which is derived from the genes that encode env proteins (Mi et al. 2000). An immunoevasive action was the initial purpose for this viral protein; however, this viral protein also enables the fetus to better resist the immune system of the mother (Carter 2011). Thus far, six syncytin genes have been discovered, including two in the mouse and two in higher primates. Each gene represents an independent capture from a retrovirus, and an independent evolutionary occurrance. Taken together, the viruses represent a prime example of convergent evolution, which occurs when separate evolutionary lineages (i.e., the incorporation of each syncytin gene) reach a similar end result (i.e., immunoprivilege) (Carter 2011).

11.3.5 Enhancement of Systemic Innate Immune Response During Pregnancy

As we saw above, there are a number of sophisticated mechanisms in place to selectively suppress the innate (uNK) and generally suppress the adaptive (cytotoxic T cells; Tregs, Th2/T1 ratio) immune systems within the uterus so that the fetus is not rejected by the mother. While the *local* innate system is depressed, this is not the case for the *systemic* innate immune system. In fact, the systemic innate immune system in the host is highly activated during pregnancy and ready to protect the mother against invasion by microbials (Wilczynski 2006).

During pregnancy there are higher numbers of circulating monocytes and granulocytes, resulting in increased numbers of total leukocytes during pregnancy (Svensson-Arvelund et al. 2014). In a review of hematology changes during pregnancy in the rat, it was found there is a linear increase in absolute numbers of total white blood cells, and of monocytes and granulocytes (de Rijk et al. 2002). Histologic evidence of the circulating leukocytosis is seen in the placenta where neutrophils plug capillaries in the basal zone of the rat placenta from GD 6 to GD

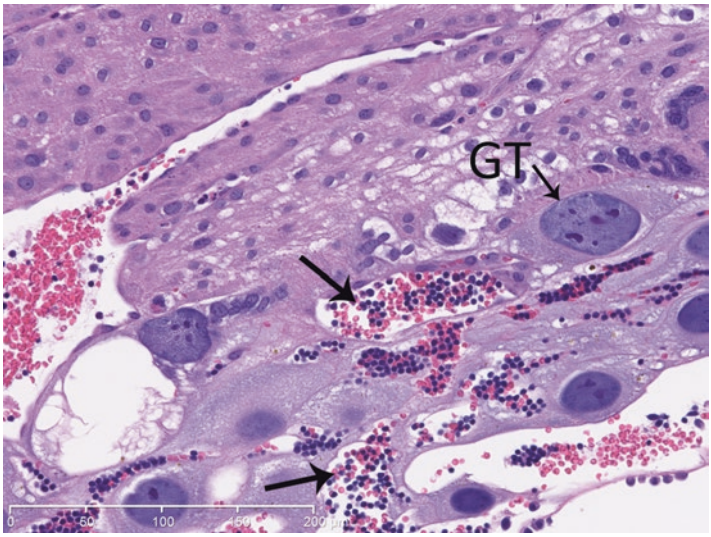

Fig. 11.22 Rat placenta with circulating leukocytosis GD 12. At mid-gestation, there is circulating leukocytosis in blood vessel of the basal zone (*arrow*). Here the blood vessels are in close approximation to giant trophoblasts (GT). H&C. 20×

13 (de Rijk et al. 2002) (Fig. 11.22). These neutrophils are necessary to phagocytose debris from dead decidua following invasion of trophoblasts. In humans, circulating leukocytes are phenotypically and functionally activated. with upregulation of activation markers CD11b or CD64 on circulating monocytes and granulocytes (Svensson-Arvelund et al. 2014). In the third trimester of human pregnancy, there are increased numbers of circulating pro-inflammatory monocytes that produce IFNγ and TNF-α. Circulating granulocytes show increased production of oxidation radicals following activation and have increased phagocytic activity during pregnancy(Weissenbacher et al. 2014). Increased complement parameters and acute phase proteins (i.e., ceruloplasmin, fibrinogen, globulin, alpha 1 antirypsin, clotting factors), in addition to decreased albumin are all characteristic of the blood during pregnancy (Weissenbacher et al. 2014). Many of the leukocyte and serum chemistry profiles during pregnancy are similar to those seen in sepsis (Sacks et al. 1998; Luppi et al. 2002).

The Th1/Th2 balance in *circulating* T cells in normal pregnancy is not well characterized. What is certain is that the high Th2/Th1 ratio in the placenta is not duplicated in the peripheral blood (Saito et al. 2010). There is, however, an increase in circulating Tregs, and a decrease in the numbers of circulating dendritic cells.

The thymus is also undergoing changes during pregnancy (Figs. 11.23a, b). There is thymic cortical involution during pregnancy, yet the thymic medulla enlarges and rearranges to create a microenvironment containing increased numbers of mature thymocytes. Estrogen and progesterone are the agents that facilitate this cortical thymocyte loss (Kendall and Clarke 2000; Mincheva-Nilsson et al. 2006),

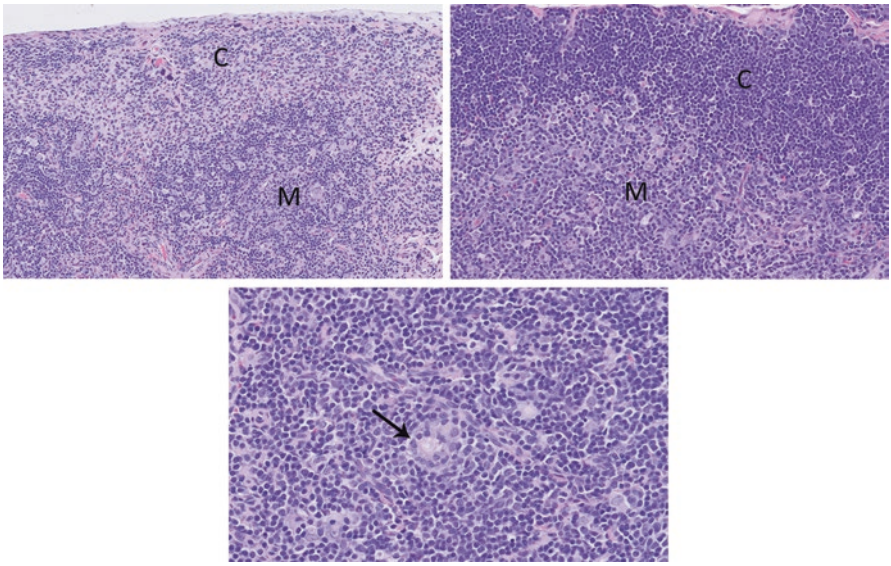

Fig. 11.23 (**a**, upper left figure) Thymus from pregnant rat. This thymus was taken from a pregnant rat at gestational day 10. The cortex (C) of the thymus has a paucity of lymphocytes while the medulla (M) has a dense population of lymphocytes, which is the reverse of the normal situation. The medulla (M) generally contains the tolerant lymphocytes. H&E stain. 30× objective magnification. (**b**, upper right figure) Thymus from non-pregnant rat. This thymus from a non-pregnant rat shows the typical cortico-medullary architecture with densely cellular cortex (C) and a relative paucity of lymphocytes in the medulla (M). H&E. 30×. (**c**, lower figure) Thymus from pregnant rat. The medulla of the thymus of a pregnant rat at GD 10 is densely cellular and has a characteristic epithelial ring (*arrow*). H&E. 50×

which is at its greatest in mid-pregnancy and maintained until lactation ceases. The expanded population of T cells in the medulla are suggested to be suppressive, contributing to the immune suppression to the semi-allogeneic fetus (Clarke and Kendall 1994; Mincheva-Nilsson et al. 2006). In the mouse, the medullary epithelial cells undergo mitosis and surround collection of lymphocytes to form characteristic medullary epithelial rings (Kendall and Clarke 2000). The thymus of pregnancy can be differentiated from the thymic involution of glucocorticoids and stress, because the thymus of pregnancy has a very active medulla (Mincheva-Nilsson et al. 2006). The loss of cortex does not correlate to changes in the peripheral population of T cells, presumably due to the long life of peripheral lymphocyte.

Although the innate and adaptive immune systems are dampened during pregnancy, there are some features of the innate immune system that are still quite active. Despite all the dampening of the immune responses (i.e., Th2 dominance; high numbers of Tregs, MHC-class I modification, B7 and Crry expression), expression of TLRs on the trophoblasts is not dampened. TLR2 and TLR4 are expressed on trophoblasts (Holmlund et al. 2002), especially the villous cytotrophoblasts and extravillous trophoblast cells. The syncytiotrophoblast cells in humans do *not*

express TLRs. There is a sound reason for this variable expression of TLR on different types of trophoblasts. In humans, the syncytiotrophoblast is adjacent to the maternal blood (unlike the situation in rats, where the cytotrophoblast is closest to maternal red cells). Keeping TLR expression away from maternal blood is a mechanism to prevent overstimulation of the innate system by commensal organisms in the blood stream. In this way, TLRs are activated only if the syncytiotrophoblast cell layer is breached by a pathogen (Abrahams and Mor 2005). Following activation of TLR 4, trophoblasts produce IL-6 and IL-8, and trigger the NFκB inflammatory cascade of events, which may result in destruction of trophoblasts. Activation of TLR2 causes direct caspase-mediated apoptosis of the trophoblast itself (Takeda and Akira 2003).

11.3.6 Increased Susceptibility to Infection During Pregnancy

The increased immune tolerance of pregnancy under the Th2dominated placenta may decrease susceptibility to some diseases, but may increase susceptibility to certain infections during pregnancy. Pregnant women are more severely affected by influenza, hepatitis E, cytomegalovirus, poliovirus, herpes simplex, and malaria, and possibly coccidioidomycosis, measles, smallpox and varicella (Kourtis et al. 2014). The success of these infections from the pathogen's point of view depends on Th1 suppression in the Th2 dominated environment. On the other hand, rheumatoid arthritis and multiple sclerosis, which are associated with Th1/Th17 type (cell-mediated) responses, often are ameliorated during the Th2-dominated environment provided by pregnancy. However, even though these disease processes subside during pregnancy, they can worsen or rebound during the postpartum period, when the Th2 dominance is lost.

Ahmed and Talal (1990) has reviewed the effects and influence of sex hormones on the immune system. In general, estrogen and progesterone increase immune responsiveness (at low doses). On the other hand, androgens (dihydrotestosterone or testosterone) generally inhibit cell mediated or humoral immunity. Therefore, females have higher IgG and IgM production and better cell mediated immune responses than their male counterparts when challenged by pathogens. This is not always an advantage, because this also explains why females are more susceptible to autoimmune diseases, whether those diseases are cell-mediated or antibody-mediated. Examples of these autoimmune diseases include systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), Sjorgens syndrome, and Hashimoto's thyroiditis. The female susceptibility to these autoimmune diseases is also observed in laboratory animal models, such as the NZBxNZW mouse for SLE, polyarthritis in LEW/N rats, thyroiditis in BUF and PVG/c rats, and Sjorgen's syndrome in B/W mice. The reason why estrogen upregulates the immune response is uncertain, but may be related to intracellular receptors for estrogen in lymphocytes.

11.3.7 Pregnancy Problems Associated with Immune Dysregulation

There is delicate crosstalk and collaboration between fetus-derived trophoblasts and maternally derived immune cells that establish a unique maternal fetal immune milieu that contributes to embryo survival. Dysregulation or breakdown of this immunologic crosstalk is linked to preeclampsia, intrauterine growth retardation and recurrent miscarriages.

11.3.7.1 Pre-eclampsia

Preeclampsia is associated with shallow trophoblast invasion and less extensive remodeling of the spiral arteries, leading to hypertension, proteinuria and edema. It is primarily a disease of humans, although a few spontaneous cases have been reported in non-human primates (Baird 1981; Thornton and Onwude 1992; Stout and Lemmon 1969). Preeclampsia is known as a new-onset hypertension with proteinuria and increased levels of inflammatory cytokines (Kupferminc et al. 1994). Preeclampsia is characterized by insufficient invasion of trophoblasts and insufficient remodeling of spiral arteries, leading to a reduction in uteroplacental perfusion, followed by fetoplacental ischemia (Hladunewich et al. 2007). The ischemic placenta releases cytokines and reactive oxygen species. Decreased levels of IL-10 (Hennessy et al. 1999; Orange et al. 2003; Wilczynski et al. 2002; Murphy et al. 2005; Kalkunte et al. 2011) and increased levels of IFNγ and TNF-α (Serin et al. 2002; Kupferminc et al. 1994; Kumar et al. 2013) further characterize pre-eclampsia. Angiotensin II type I receptor autoantibodies (AT1-AA) also develop (Wallukat et al. 1999).

The underlying cause for the failure of trophoblast invasion and spiral artery remodeling has received considerable attention, and there are as many proposed mechanisms as there are cell types and cytokines in the placental environment. All of the proposed mechanisms feed into each other, creating a complex scenario of a pro-inflammatory state.

There is some conventional understanding that preeclampsia is associated with a high Th1/Th2 ratio of lymphocytes, suggesting the normal Th2 immunosuppressive phenotype of normal pregnancy is not present. Some evidence indicates that preeclampsia might be due to activation of uNK cells, and associated with a certain combination of HLA-C haplotypes on the trophoblast and a certain phenotype of KIR receptors on the uNK cells (Hiby et al. 2004, 2010; Svensson-Arvelund et al. 2014). Regardless of the specific pathogenesis, autoimmune destruction of the trophoblast would prevent its proliferation and invasion, thus reducing its ability to support the fetus through gestation.

Loss of Tregs and loss of immune tolerance may also play a roll in preeclampsia. In preeclampsia, T_{regs} are significantly reduced in the peripheral blood and decidual tissue compared to women with healthy pregnancies (Sasaki et al. 2007). Women who have not had prior intercourse with a conceiving partner over several months

prior to conception have increased likelihood of preeclampsia. This might be due to a reduction in the Treg cell population which specifies the partner's HLA antigens. The seminal fluid of the conceiving partner induces Tregs upon repeated coitus during weeks or months prior to conception (Kho et al. 2009). If the female had no prior exposure to the seminal fluid, there might be insufficient Tregs available, and the uterus would be disposed towards inflammation and development of Th1 and Th17 cell subsets.

Placental macrophages have also been implicated as a cause of preeclampsia. Normally, placental macrophages are present around the uNK cells and the endovascular trophoblasts in the spiral arteries. In preeclampsia, there is an increased incidence of macrophages producing TNF-α, and these clusters are present between the spiral artery and the invading trophoblasts. The placental macrophages are in essence inhibiting the normal interaction of uNK cells and trophoblasts, preventing trophoblast invasion (Fig. 11.24).

Another way placental macrophages could be at the root of preeclampsia is by failure of the macrophages to sequester soluble Flt-1 receptor. Recall that Flt-1 is

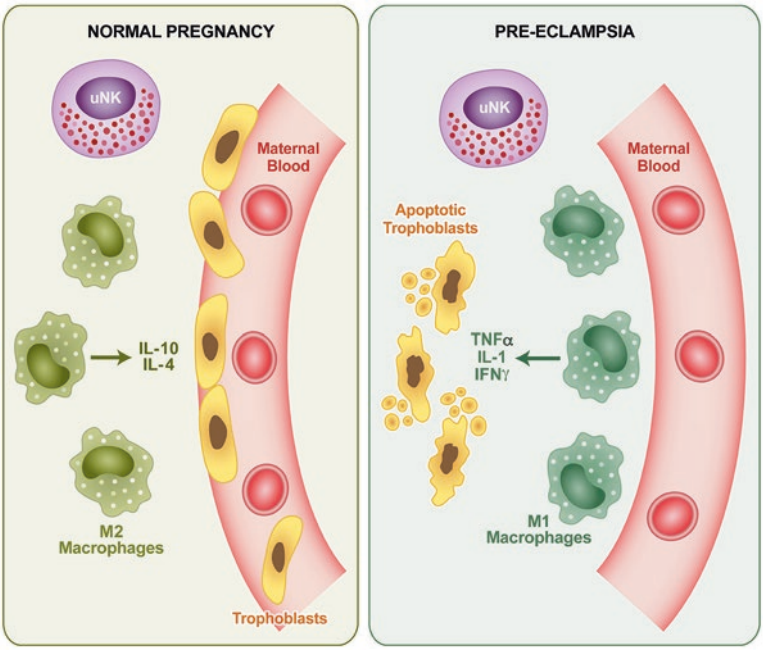

Fig. 11.24 Proposed mechanism in pre-eclampsia—activated M1 macrophages. Normal M2 placental macrophages reside in the stroma outside of the spiral arteries and endovascular trophoblasts. These macrophages support trophoblast invasion by secreting IL-10 and IL-4. In pre-eclampsia, placental macrophages are of the M1 subtype and are intimately associated with the wall of the spiral artery. They physically impede the necessary invasion of trophoblasts into the artery. These M1 macrophages produce pro-inflammatory cytokines TNF-α and IFNγ that lead to apoptosis of the trophoblasts

the fms-like tyrosine kinase 1 receptor for VEGF on trophoblasts and endothelial cells (see Section II, Part C, 2). Phagocytosis of excess sFlt-1 receptor is a mechanism whereby decidual macrophages control the amount of VEGF available for angiogenesis. In preeclampsia the placenta produces either elevated levels of soluble Flt-1 receptor (sFlt-1), or the placental macrophage is deficient in phagocytizing the excess receptor. In any event, excess sFlt-1 results and this excess of sFLT-1 sequesters the available VEGF ligand (produced in large part by the Hofbauer fetal macrophage) rendering VEGF unavailable for angiogenesis or spiral artery remodeling (Pinhal-Enfield and Leibovich 2012).

A subclinical infection might also be at the root of preeclampsia. Clinical studies show a strong association between preeclampsia/intrauterine growth restriction and infection (Conde-Agudelo et al. 2008; Resnik 2002). It is the trophoblast with its TLRs that might be responding to pathogens, leading to the demise of the pregnancy. Activation of TLR2 on trophoblasts by bacterial pathogens results in trophoblast necrosis. Certain intrauterine infections during pregnancy may have either a direct or indirect effect on trophoblast survival, depending on whether TLR2 or TLR4 is activated (Fig. 11.25). Gram-positive bacteria expressing peptidoglycan or lipotechoic acid may activate TLR2 and cause direct necrosis of the trophoblast. A gram negative infection activates TLR4 and only indirectly results in apoptosis of the trophoblast (Deng et al. 2009; Schatz et al. 2012). Activation through TLR4 triggers trophoblast cells to produce high levels of TNF-α and IFNγ, which in turn are toxic to other trophoblasts. While pre-eclampsia is a human disease, the notion that subclinical infections lead to pregnancy complications has implications for toxicology studies in animals. There is an association between increased incidence of infections and lower pregnancy rates in breeding rodent colonies (Trowsdale and Betz 2006).

Regardless of how the necrosis of trophoblasts begins (subclinical infection or not), necrosis of trophoblasts sets in motion a cascade of detrimental effects. With increased necrosis, there is increased production of anti-phospholipid antibodies (Chen et al. 2009). These antibodies could bind to surface expressed Fc receptors on placental macrophages, resulting in secretion of more pro-inflammatory cytokines such as TNF-α. Pro-inflammatory macrophages could then induce Fas expression by trophoblasts and intensify trophoblast sensitivity to apoptosis/necrosis (Abrahams et al. 2004b).

The hypoxia caused by improper spiral artery remodeling and improper trophoblast invasion (regardless of inciting mechanism) is a stressful condition, and this stress perpetuates further hypoxic damage by causing glucocorticoid release (Fig. 11.26). It is proposed that reduced uterine placental perfusion (hypoxia) initially activates stress pathways in the fetus leading to elevated cortisol. Elevated cortisol leads to upregulation of plasminogen activator inhibitor 1 (PAI-1) levels in trophoblasts, which leads to excess levels of fibrin and extracellular matrix proteins (ECM). The excess fibrin and ECM leads to further reduction of flow of nutrients and infarction.

Another possible cause for preeclampsia might be enhanced shedding of syncytiotrophoblast microparticles (STMP, also known as syncytial bodies or syncytial

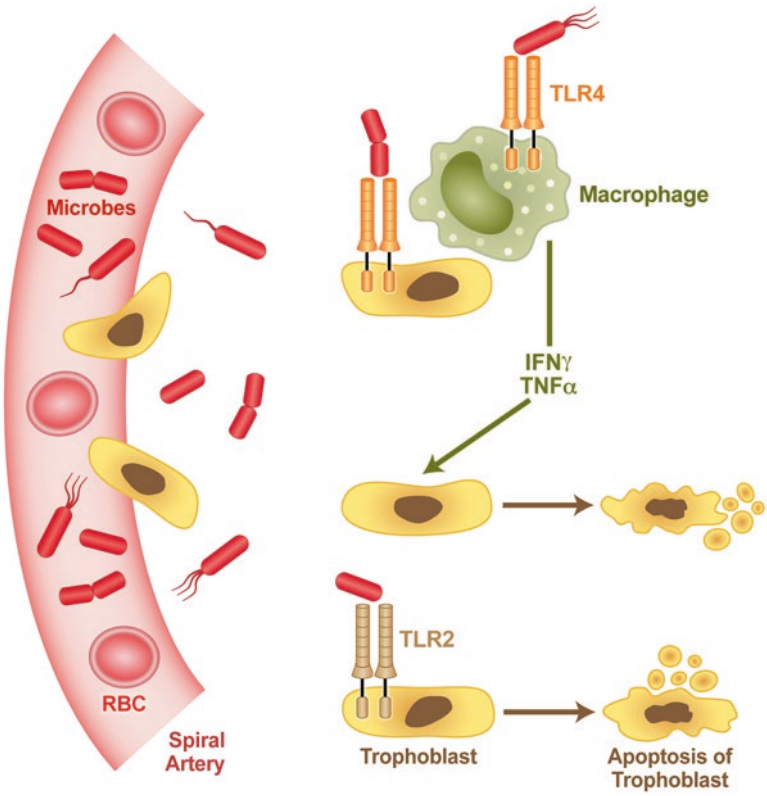

Fig. 11.25 Proposed mechanism in pre-eclampsia—infection-induced activation of TLR. Trophoblasts express a number of TLR receptors, most notably TLR2 and TLR4. Activation of TLR2 or TLR4 by microbial agents can result in direct apoptosis of trophoblasts, or indirect apoptosis of trophoblasts, respectively. Gram positive organisms generally activate the TLR2 receptors directly causing apoptosis of the trophoblast. Gram negative infections activate the TLR4 receptors on macrophages or trophoblasts. When TLR4 receptors are activated, there is increased production of pro-inflammatory cytokines (e.g. TNF-α and IFNγ) that are toxic to neighboring trophoblasts. The end result is the same: increased apoptosis of trophoblasts, followed by deficient spiral artery remodeling and fetal hypoxia

knots) that bleb and shed from the plasma membrane. These particles are present in low concentration in normal pregnancy, but enhanced shedding of STBM may promote immunologic attack of antigens on the microparticles, with inflammation and necrosis/apoptosis and preeclampsia (Guller 2009; Redman and Sargent 2010). There are two types of structures that can form from the trophoblast and circulate in the maternal blood, causing immunologic responses by virtue of the exchange of material from fetal to maternal systems (Redman and Sargent 2007). These are syncytial knots (100–200 μm) or exosomes (20–100 nm). Exosomes were discussed previously as a decoy tool and to prevent immune activity at the site of the tropho-

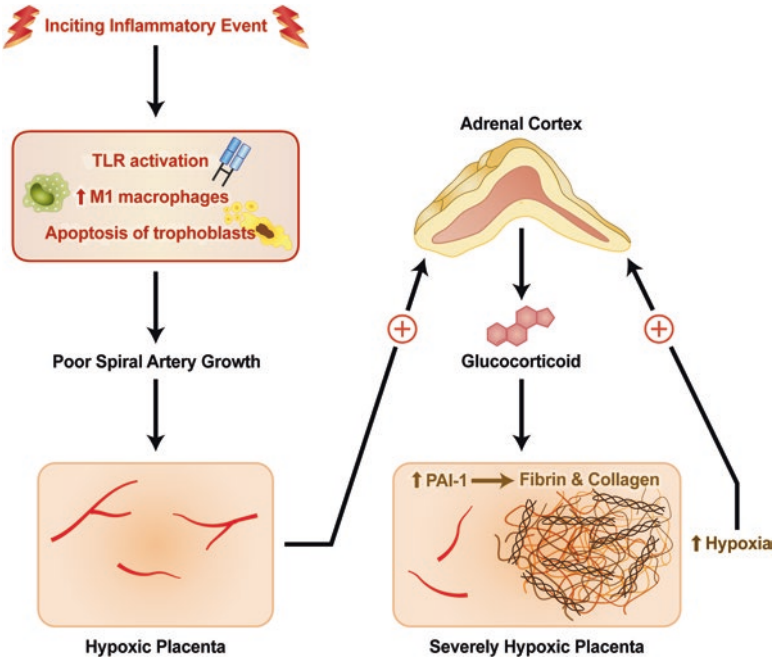

Fig. 11.26 Glucocorticoid (stress) exacerbates hypoxia associated with pre-eclampsia. Glucocorticoids (GC) accentuate the hypoxia in pre-eclampsia. As seen in Figs. 11.25 or 11.26, activation of TLR or pro-inflammatory macrophages result in apoptosis of trophoblasts and poor spiral artery remodeling. This creates a hypoxic environment with few blood vessels. The hypoxia of this pre-eclampsia condition (regardless of its inciting event) leads to glucocorticoid release from the adrenal cortex. GCs contribute to the hypoxic condition, because they upregulate plasminogen activator inhibitor 1 (PAI-1) on trophoblasts. Excess PAI-1 leads to decreased plasminogen activator and then excess fibrin and extracellular matrix protein (ECM) in the placenta. The fibrin and ECM impede oxygenation of the tissues, leading to more stress and more GC release, and eventually to infarction of placenta and pregnancy loss

blast. Syncytial knots are larger than exosomes (about 100–200 μm in diameter) and form from the normal expected apoptotic parts of trophoblast nuclei following syncytitial formation. Following trophoblast syncytialization, excess nuclei die by apoptosis and cluster into syncytial knots. These knots can be extruded from the cell and occasionally clog a capillary, constituting the "fetal" cells that are sometimes observed histologically in maternal lung tissue. Excess syncytial knots have been noted in women with pre-eclampsia. Depending on how they are formed (i.e., by apoptosis or necrosis) the knots may have an impact on inflammation and disease. Those that normally form by apoptosis in low numbers do not activate endothelial cells to incite inflammation. However, those that form after the death of the trophoblast (i.e., by necrosis) do excite the endothelial cell and are the ones believed to play a role in pre-eclampsia (Redman and Sargent 2007). Anti-phospholipid autoantibodies have been shown to increase the syncytial knots that form by non-

apoptotic (i.e., the aberrant necrotic) trophoblast shedding. Since it is only the "necrotic" knots that activate endothelial cells and contributes to pre-eclampsia, there is a connection between anti-phospholipid antibody and pre-eclampsia (Chen et al. 2006, 2009). Other factors besides anti-phospholipid antibody can result in knot formation following necrosis rather than apoptosis. Variations in the numbers of complement regulatory proteins or excess activation of the alternate pathway can also shift the trophoblast death from apoptosis to necrosis (Huppertz et al. 2003).

Many animal models for preeclampsia exist. There are several genetically manipulated mouse models that affect a single gene, such as sFlt-1, the renin-angiotensin system, VEGF, endothelin, and endothelial nitric oxide-synthetase, in addition to others (Pennington et al. 2012). Readers are referred to Pennington et al. (2012) for more information on mouse models of preeclampsia. One animal model deserves special attention, because it mimics the changes in maternal circulation or immune function associated with preeclampsia. This model of reduced uterine perfusion pressure (RUPP) in pregnant rats closely mimics preeclampsia, because it involves hypertension, immune system abnormalities, renal vasoconstriction, increased levels of TNF-α and IL-6, and oxidative stress in the mother and intrauterine growth restriction of the fetus (Li et al. 2012). For a review of the model, readers are referred to Li et al. (2012). While the RUPP model does not provide a model to study the mechanisms responsible for abnormal placentation that ultimately leads to vascular ischemia, it is useful for studies of the consequences of reduced uterine blood flow.

11.3.7.2 Recurrent Miscarriages

Human reproductive failure may be a consequence of aberrant expression of immunological factors during pregnancy. Fifteen percent (15%) of clinically recognized human pregnancies are lost prior to the 20th week of gestation (Alberman 1988). Of these, 40% are unexplained, 20% are due to autoimmunity, and 40% are due to endocrinological (17–20%), infectious (0.5–5%), anatomical (10–15%) or chromosomal (2–5%) problems. Of the potential immunologic causes for pregnancy loss, evidence suggests that aberrant HLA expression, anti-sperm or antiphospholipid antibodies, alteration in cytokine production, and abnormal uNK cells are responsible. Up to this point, this chapter has addressed the immunologic principles governing the pregnant state. Any disruption of any one of those important mechanisms could result in spontaneous abortion or lower pregnancy rates. A few areas of research regarding potential causes of recurrent miscarriages are discussed briefly below, but this discussion only scratches the surface. There is considerable overlap between the causes of recurrent miscarriages and the causes of preeclampsia.

Aberrant HLA expression has been shown to influence gamete development, embryo cleavage rates, implantation and fetal development. As stated in the chapter, HLA-G expression and binding to T cells and uNK cells are necessary for the tissue to establish the immunosuppressive phenotype. Aberrant HLA-G expression might

also cause pregnancy loss by its effect on cytokine production by T cell and uNK cells. When HLA-G expression is down-regulated, Th1 cells are activated, there is a loss of the Th2/Th1 dominance, and Th1 cells release relatively high levels of pro-inflammatory cytokines IFNγ, TNF-α and IL-1. These pro-inflammatory cytokines inhibit human placental trophoblast cell growth and/or destroy the trophoblast (Raghupathy 1997). A perturbation in the balance of pro- and anti-inflammatory cytokines and, in particular, higher Th1 cytokines and lower Th2 cytokines have been associated with recurrent miscarriages (Raghupathy 1997; Lim et al. 2000). Therefore, aberrant HLA expression might affect pregnancy via immunologic (loss of immunosuppressive Th2 phenotype), or physiologic (loss of angiogenesis, tissue remodeling) mechanisms.

Alterations in the population or phenotype of uNK cells might affect pregnancy (Moffett et al. 2004). $CD56^+$ NK cells (or the uterine NK cells) secrete cytokines to promote placental growth, but if the NK cells express $CD16^+$, they may inhibit conceptus growth and trigger killing. So reproductive failure may result from alteration of cytokine secretion from $CD16^+$ NK cells.

Anti-phospholipid antibodies (APAs) can be found in 5–15% of patients with recurrent pregnancy loss. APAs targeted toward phosphatidylserine, phosphatidylcholine, phosphatidylglycerol, phosphatydlinositol, and phosphatidyethanolamine will effect the embryo, and APAs against phosphatidylserine and phophatidylethanolamine will effect the trophoblast and interfere with invasion and attachment to the uterus (Peaceman and Rehnberg 1993). APA binding to trophoblasts activate complement, which promotes platelet and endothelial cell activation, and inflammation and thrombosis (Salafia and Parke 1997; Holers et al. 2002).

Not all antibodies are damaging to the pregnancy. Anti-HLA antibodies are now regarded as necessary for normal pregnancy and their *absence* could be a marker of inappropriate maternal immune response to the fetus (Nielsen et al. 2010; Weissenbacher et al. 2014). Some women with recurrent miscarriages have *lower* anti-HLA antibodies, yet now it is believed that lower levels of antibody could be due to a pregnancy loss before maternal antibodies are normally produced. Currently, anti-HLA antibodies are not considered a significant prognostic factor, one way or the other, in human pregnancy.

11.4 Mammary Gland

The mammary gland is a specialized integumentary gland. In the rodent, it is comprised of six ventrolateral pairs of glands, each comprised of a series of branching ducts. Starting at the nipple, there is a collecting duct lined by keratinized stratified squamous epithelium, then lactiferous ducts and ductules lined by a double layer of cuboidal epithelium, and finally alveoli lined by a single layer of secretory cells. This epithelial branching structure is associated with smooth muscle, adipose tissue, connective tissue stroma, lymph nodes, lymphatics, and nerves. During puberty, which generally is associated with vaginal opening between post-natal days 35–40,

the mammary glands in the rat undergo initial expansion under the control of growth hormone and prolactin. Throughout life, the mammary gland fluctuates in size and morphology with estrous cycling, pregnancy, lactation, and involution.

The mammary gland has a sophisticated innate immune response similar to other integumentary structures. The process of milk production and the innate immune response are related on an evolutionary scale. There is thought that mammary gland first evolved as an integumentary gland to provide innate immune response to tissue damage and infection, and only thereafter evolved its nutritional role for newborns (Vorbach et al. 2006). These dual immune and nutritional roles persist into the present day mammalian mammary gland, since the milk's purpose is to provide immune protection and nutrition of the newborn in many species. The mammary gland presumably first evolved as a mucous-secreting skin gland containing a variety of antimicrobial molecules to protect maternal or newborn skin. Over time, antimicrobial substances (e.g., xanthine oxidase reductase and lysosyme), genetically evolved to allow for the production and secretion of fat droplets, the production of whey protein and sugar, and the accumulation of water into milk (Vorbach et al. 2006). Other evidence that the nutritional role of the mammary gland is evolutionarily tied to the innate immune system is based on the fact the NF-κB and Jak/Stat signaling pathways play a central role in both lactation and inflammation. NF-κB controls expression of various antimicrobial molecules, cytokines and costimulatory molecules (Baeuerle and Henkel 1994), yet it also controls mammary gland differentiation and milk production (Cao et al. 2001). Prolactin also has a dual role. Prolactin acts as an anti-inflammatory or proinflamamtory "cytokine", helps regulate the secretion of IgA and the homing of T cells in the gland (Barber et al. 1992; Mackern-Oberti et al. 2013), yet it also stimulates the uptake of amino acids and glucose as well as the synthesis of nutritional components (casein, lactose, and α-lactalbumin, to name a few) of milk (Barber et al. 1992).

11.4.1 General Immune Components of Mammary Gland Tissue

Most of the work done with general immunity of the mammary gland in the veterinary literature has been done in cows (Ezzat Alnakip et al. 2014; Rainard and Riollet 2006). The primary defense mechanism of the mammary gland is the teat canal which is both a physical barrier provided by the smooth muscle sphincter and a source of anti-microbial substances. The teat duct keratin contains fatty acids that slow the growth of bacterial pathogens. The fibrous proteins in the keratin bind electrostatically to microorganisms and hinder their migration.

The mammary gland stroma contains resident leukocytes (macrophages, B lymphocytes and T lymphocytes) that provide surveillance and aid in the restructuring the gland during involution (Barber et al. 1999). Macrophages constitute the largest population of conventional immune cells of the normal virgin and primiparous mammary gland (ten Napel et al. 2009). Macrophages participate in the process of

involution, thus are present in increased numbers in primiparous rats when compared to virgin rats (Zhao et al. 2010). B cells/plasma cells and T cells are also present in the mammary gland, and are present in higher numbers in the involuted gland compared the gland of virgin animals. Prolactin regulates T cell homing to the mammary gland (Roux et al. 1977; Weisz-Carrington et al. 1977). While the prolactin-mediated mechanism for T cell homing is not completely known, there is evidence that prolactin induces expression of T cell chemokines (CCL20, CxCL9, CXCL10 and CXCL11) in epithelial cells (Mackern-Oberti et al. 2013)

There is a higher number of IgA-secreting plasma cells in the involuted gland compared to the virgin gland, and this is due to the fact that the plasma cells in the involuting gland have been exposed to antigen and the plasma cells are primed to produce IgA. In the virgin gland, this priming of the plasma cells to produce IgA has not yet occurred. This difference in ability of the plasma cells to produce IgA has implications for adaptive immunity. Antigen infusion into the virgin mammary gland fails to elicit local production of IgA but does elicit a response in the primiparous gland, resulting in local production of IgA. This IgA response can be enhanced by simultaneous stimulation of the intestine, providing evidence that the IgA-producing plasma cells of the mammary gland are derived in part from the GALT (Larson et al. 1980).

The mammary gland also has an acitve innate immune system. The mammary gland epithelium is prepared to respond to invading microorganisms via expression of pattern recognition receptors such as toll-like receptors (TLRs) (Vorbach et al. 2006).

11.4.2 Immune Components of Colostrum/Milk

Milk serves to confer passive immune protection to the neonate (especially in ruminants and pigs), provide nutrition to the newborn, and provide anti-microbial substances to prevent gastrointestinal infections in the newborn. However, it is also important that the milk within the mammary gland has protection against overgrowth of bacteria. To meet this requirements, colostrum/milk contains most components of the innate and adaptive immune systems, including immunoglobulin, leukocytes, antimicrobial proteins, and complement. Each of these components will be discussed in this section.

Immunoglobulin component of colostrum. There is a marked species difference in the nature and composition of the immunoglobulin component of colostrum. The colostrum of ruminants and pigs contains abundant IgG, which is assimilated by the neonate for 24–48 h after birth. The colostrum of rats, on the other hand, contains predominantly IgA and has less IgM and IgG (Larson et al. 1980).

The reason for the species differences is based on whether the passive transfer of immunity occurs through the colostrum, or if it occurred prenatally through the placenta. In rodents, humans and rabbits, passage of maternal immunity is mediated

both prenatally by the yolk sac/placenta, and postnatally by the colostrum. By contrast, in ruminants and pigs, maternal IgG is transferred almost exclusively via colostrum during the postnatal period. It follows that the ruminant and pig intestinal tract is highly adept at absorbing IgG.

The transport of immunoglobulins of any subtype into milk occurs by two means—by transfer from the blood (generally IgG) and by local production (IgA and IgM). The blood transfer of immunoglobulin occurs primarily in ruminants and pigs. IgG1 is the predominant immunoglobulin subtype that is transferred from blood to milk in the cow (Watson 1980; Larson et al. 1980). On the other hand, the local production of immunoglobulins in a healthy mammary gland is performed by plasma cells located adjacent to the secretory epithelium in the mammary gland. IgA and IgM are the predominant subtypes produced by those plasma cells in all species, including man. Except for ruminants and pigs (where IgG is the principal immunoglobulin in colostrum and milk), the principal immunoglobulin class in most mammalian milk (and most mammalian exosecretions from any orifice) is IgA (Larson et al. 1980): IgG and IgM is present in relatively smaller quantities, and immunoglobulins IgD or IgE are considered absent in mammary secretions. Human colostrum is 95% IgA, 4% IgM, 1% IgG (Larson et al. 1980).

Transport of IgG across epithelium. The transfer of IgG from blood to milk is highly important in ruminants and pigs, and occurs by receptor-mediated endocytosis. The tight junctions between ductular and alveolar epithelium, located at the apical surfaces, make it impossible for IgG to pass between epithelial cells and the macromolecular size of immunogloblin molecules makes it impossible for these molecules to transit by passive diffuse. The receptor for IgG is termed the neonatal Fc receptor (FcRn). These receptors is present in large numbers in those animals with abundant IgG in the milk (i.e., ruminants and pigs), and is far less common in the mammary gland of species with low transfer of IgG from blood to milk (i.e., rat and man) (Cui et al. 2014). The FcRn has unique pH dependent properties which enable the binding to IgG at acidic pH (<6.5) and release of IgG at neutral pH (7.0–7.4) (Vaughn and Bjorkman 1998; Rodewald and Kraehenbuhl 1984; Andersen et al. 2006).

Mammary gland cells, like most cells, are neutral pH, which means those FcRn at the cytoplasmic surface of mammary gland epithelial cells will not bind avidly to IgG. However, the cell accommodates for this and first takes up the IgG by nonspecific pinocytosis. Once the pinocytosed immunoglobulin is transferred from pinocytotic vesicles to acidic endosomes, the IgG then binds to FcRn receptors lining the membrane of these endosomes that have an internal environment at pH 6.5. The receptors carry the IgG across the cell by transocytosis and release the IgG into the neutral pH of milk. The direction of IgG travel in ruminant milk is primarily from the basolateral surface of epithelial cells to the apical side (i.e., from blood to milk) and the FcRn receptors are accordingly found predominantly on the basolateral surface of the epithelial cells. In humans and rats, the IgG can travel from blood to milk or milk to blood depending on which side of the epithelial cell the FcRn receptors predominate. In rats, the predominant FcRn receptors are at the apical side of the epithelial cell, and therefore transfer of IgG, if it occurs at all, is from milk to blood

(Cianga et al. 1999). The purpose of IgG transport in the rat mammary gland (since it generally is in the direction of milk to blood) is apparently for alternative purposes to recycle IgG and prolong its half-life, rather than to enrich IgG in the milk for passive transfer (Cianga et al. 1999).

This initial uptake of IgG into acidic endosomes that favors binding of IgG to FcRn is the same process used by the syncytiotrophoblast in man (Simister et al. 1996) and rat (Roberts et al. 1990). Therefore an extracellular acidic pH is not a prerequisite for receptor-mediated IgG uptake. However, in the neonatal intestine, the acidic pH is extracellular and the initial pinocytosis of IgG into endosomes is therefore not required. IgG will avidly bind to FcRn on the apical surface of intestinal epithelial cells.

Local Production of IgA. Plasma cells within the mammary gland stroma are responsible for local production of IgA and to a lesser degree IgM. These plasma cells originate and are recruited from gut-associated lymphoid tissue (Weisz-Carrington et al. 1977). The recruitment of IgA secreting plasma cells from the GALT is due to the "entero-mammary link" that is a system of homing receptors and adhesion molecules. The IgA that is produced and ultimately secreted into milk has specificity for antigens in the maternal digestive tract (and therefore for antigens in the neonatal digestive tract) (Salmon 2000).

The number of plasma cells in the gland increases in the mammary gland during pregnancy (Tanneau et al. 1999; van der Feltz et al. 2001) and their numbers are regulated by chemokines and cytokines (Mackern-Oberti et al. 2013).

Antimicrobial components of milk. The milk contains peptides, small proteins and enzymes that provide innate immune protection against invading microorganisms. The epithelial cells produce and secrete these anti-microbial proteins and peptides, such as defensins and cathelicidins, into milk (Singh et al. 2008). Other anti-microbial components of milk include lactoferrin, transferrin, lysozyme, and lactoperoxidase. Lactoferrin and transferrin bind iron and essentially starve those bacteria having high iron requirments. Lysozymes lyse bacterial cell walls. Lactoperoxidase acts on hydrogen peroxide elaborated by lactobacilli (i.e., the normal milk flora) and catalizes the release of oxygen, which is harmful to certain bacteria.

Leukocytes in milk. Functional macrophages, B cells, T cells and neutrophils migrate into the milk (Kumar et al. 1985). The macrophage is the predominant milk leukocyte in the rat (Larson et al. 1980). The B and T lymphocytes of colostrum and milk have been shown to be capable of eliciting humoral and cellular immune responses (Larson et al. 1980). Lymphocytes from the dam cross the intestinal barrier of the neonate and constitute a significant component of the circulating leukocytes of the neonate

While there are functional leukocytes in the mammary gland stroma and the milk, the mammary gland and milk are thought of as being immune-compromised compared to other organs of the host (Ezzat Alnakip et al. 2014): Lymphocytes in the milk exhibit hypo-responsiveness to mitogenic, antigen, or allogenic stimuli

compared to blood lymphocytes (Taylor et al. 1994; Weber et al. 1983); bovine milk macrophages secrete less IL-1 compared to blood monocytes and are less efficient at promoting lymphocyte activation (Politis et al. 1991); and CD8+ suppressor or cytotoxic T cells predominate over CD4+ effector cells in milk compared to blood. CD8+ cells are mostly cytotoxic during mid-lactation, but are suppressor $CD8^+$ cells during the early postpartum period (Ezzat Alnakip et al. 2014).

Complement in milk. Complement components are another part of the immune system present in milk. The components are in lowest concentration in healthy mammary gland during lactation, and higher levels in colostrum and in milk during late lactation and during involution. The alternate pathway is the sole complement pathway operative in the healthy mammary gland. The classical pathway is generally non functional in the mammary gland due to the low to absent amount of C1q factor in milk compared to blood (Rainard 2003).

11.4.3 Process of Mammary Gland Involution

The process of mammary gland involution can be thought of as a form of sterile inflammation. Mammary gland involution is a process where epithelium undergoes programmed cell death and removal by phagocytosis; milk fat globules are removed; and the stroma is repopulated with differentiated adipocytes. In the mouse, involution takes approximately 5 days and starts when the pups stop suckling. The gland reaches maximal weight within the first 24 h after the pups stop suckling, as the milk distends the gland. Then the epithelium becomes apoptotic. At the 48 h mark after the onset of involution, there is a highest proportion of apoptotic cells within the gland (Atabai et al. 2007). Removal of these apoptotic cells and milk fat globules are performed by mammary gland epithelial cells, endothelial cells, fibroblasts and macrophages. The epithelial cell plays the dominant role early in the involution process in rodents (during the first 24–48 h). During the later stage of involution, the macrophage predominates.(Atabai et al. 2007). That the epithelial cell is the dominant phagocytic cell early in involution is evidence by the fact that macrophages are rare prior to day 4 of involution ((Stein et al. 2004) and are not detectable before day 2 (Hanayama and Nagata 2005) (Atabai et al. 2007).

During phagocytosis of apoptotic cells, both the epithelial cells and the macrophages produce cytokines and growth factors, such as TGF-β and VEGF which assist in the remodeling of the gland (Monks et al. 2002; Golpon et al. 2004).

Uterocalin (also known as lipocalin-2) is an acute-phase protein produced by the liver and promotes apoptosis specifically of neutrophils in the mammary gland. This apoptosis of neutrophils prevent the involvement of this inflammatory cell type in the early phase of involution of the mammary gland and helps subdue tissue inflammation during this process of tissue destruction (Nilsen-Hamilton et al. 2003; Sultan et al. 2012; Ryon et al. 2002).

11.5 Summarized Points

- The ovarian macrophage has a dual endocrine and immunologic role. Using its conventional immune functions of cytokine and growth factor production, phagocytosis and tissue remodeling, the ovarian macrophage orchestrates normal cycling of the ovarian structures.
- The non-pregnant uterus has modified mucosal immunity that is generally enhanced under estrogen influence and dampened under progesterone influence. Key features of this mucosal immunity include uterine epithelial cells that assume immunologic functions, such as antigen presentation; production of cytokines, growth factors, and defensins; and expression of toll-like receptors.
- The non-pregnant uterus has a suppressed adaptive immunity. Seminal fluid and uterine epithelial cells produce high levels of TGF-β that leads to a relatively high population of tolerogenic dendritic cells and T regs in the uterus in preparation for pregnancy.
- During pregnancy, the metrial gland (and in particular the interaction between uterine NK cells and trophoblasts) establish immunologic tolerance to the fetus and control vascular remodeling of the placenta. Disruption of this intimate interaction may be the basis for preeclampsia and recurrent miscarriages.
- During pregnancy, a suppressive adaptive immunity and modified innate immunity in the placenta is due to trophoblasts producing progesterone that creates a tolerogenic environment of T regs and anti-inflammatory cytokines (IL-4, IL-10, and TGFβ). Specific immunosuppressive mechanisms include (i) trophoblast expression of modified MHC Class I molecules (HLA-C and HLA-G) that prevent killing of fetal tissues by either the uNK cells or the cytotoxic T cells; (ii) trophoblast production of indoleamine 2,3,dioxygenase (IDO) and expression of B7 costimulatory molecules to limit T cell proliferation; (iii) trophoblast expression of complement regulatory proteins (Crry) to prevent complement activation; (iv) trophoblast use of exosomes to regulate the location and degree of apopotosis; and (v) B cell production of asymmetric (ineffective) immunoglobulin.

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Chapter 12
Immunopathology of the Musculoskeletal System

Brad Bolon and Jairo Nunes

Abstract The musculoskeletal system is composed chiefly of bone, cartilage, skeletal muscle, tendons, and ligaments, and as such does not serve as a prominent location for immune response elements under normal conditions. However, bone marrow and regional lymph nodes within or near large muscles as well as the existing vascular supply do provide ready access for many immune cell lineages to musculoskeletal components, which may help drive inflammation of these tissues during autoimmune or infectious diseases. Immune-mediated skeletal (bone and joint) diseases are driven by multiple bone-regulatory molecules (including the canonical RANK/RANKL/OPG pathway as well as non-canonical [RANKL-independent] pathways) and also many immune cell-derived pro-inflammatory molecules (especially cytokines and chemokines released by activated lymphocytes, macrophages, and fibroblast-like synoviocytes). Immune-mediated conditions affecting soft tissues (i.e., skeletal muscle and tendons) also are propelled by lymphocyte- and macrophage-derived pro-inflammatory cytokines. Immune system assaults on musculoskeletal tissues often are driven by a loss of self-tolerance to endogenous antigens (i.e., autoimmunity) or to mistaken identity (i.e., molecular mimicry between a pathogen-derived epitope and an endogenous molecule). Improved understanding of immunopathologic diseases affecting the musculoskeletal system has been achieved by evaluating animal models of disease and human patients. The current chapter describes the cellular and molecular components of musculoskeletal immunology and explores common immunopathologic conditions affecting musculoskeletal tissues, including the animal models used to assess potential etiologies, disease mechanisms, and disease-modifying therapies.

Keywords Arthritis • Myositis • Osteoarthritis • Osteoimmunology • RANKL • Rheumatoid arthritis

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12.1 Introduction

The musculoskeletal system is composed of bone, cartilage, skeletal muscle, tendons (which connect bones and skeletal muscles), and ligaments (which bridge joints and connect adjacent bones). Support elements such as blood vessels, lymphatic vessels, and nerves are encountered commonly in bone and skeletal muscle but are essentially absent in cartilage, tendons, and ligaments. Various connective tissues (e.g., dense fibrous capsules, white fat) also are found within many muscles and sometimes inside the medullary cavities of long bones. In general, however, organs of the musculoskeletal system harbor few immune cells within their parenchyma under normal circumstances.

Nonetheless, the musculoskeletal and immune systems maintain a close relationship during both health and disease. Bone marrow is the key site for hematopoiesis, including many immune effector cells (mainly those participating in innate immune reactions), in both adult animals and humans (see Volume 1, Chapter 2 for further details). Certain regional lymph nodes (e.g., axillary and popliteal) are found near large, very active skeletal muscles, thus providing a local niche to mount an adaptive immune response. Some lineages of musculoskeletal cells (e.g., synoviocytes) may be recruited to serve as facultative immune cells in launching or sustaining immune responses directed against other musculoskeletal tissues. Therefore, one or multiple elements of the musculoskeletal system may be targeted easily by nearby populations of immune cells during immunopathologic diseases.

Immune-mediated diseases affecting the musculoskeletal system may exhibit a limited number of macroscopic and microscopic appearances using conventional clinical diagnostic and anatomic pathology techniques. Nonetheless, the inflammatory components of various skeletal diseases are distinct in terms of both immune cell composition and cytokine signature (Stolina et al. 2008; Stolina et al. 2009; de Lange-Brokaar et al. 2012; van de Sande and Baeten 2016; Malik et al. 2016). These differences also may exist for discrete patient subpopulations for a given disease, or even between distinct disease stages in a single patient (van de Sande and Baeten 2016). Accordingly, individuals seeking to understand the immunopathology of musculoskeletal pathology will need to expend considerable effort to integrate clinical and experimental data from many sources in constructing a model for any given disease.

The current chapter briefly reviews major features of musculoskeletal immunobiology and immunopathology. Key cell types, their functions, and critical signaling pathways are described. Common immune-mediated lesions of the musculoskeletal system and their pathogeneses are defined. Where possible, emphasis will be placed on cross-species similarities to facilitate the growing translational medicine effort to find innovative therapeutic agents to reduce, prevent, or reverse immune-mediated damage to musculoskeletal tissues.

12.1.1 Immune Cells and Signals in Musculoskeletal Tissues During Health and Disease

The relationship of musculoskeletal tissues to immune response elements may be understood most easily by considering each entity in isolation. Bones and joints (primarily synovium rather than tendons and ligaments) may be reached readily by immune cells in health based on their close proximity to bone marrow and their rich vascular supplies. By comparison, normal skeletal muscle has no immune elements except a few sparsely scattered resident histiocytes for immune surveillance.

12.1.1.1 Bones and Joints (including Tendons and Ligaments)

Investigations of bone metabolism and bone marrow immunology traditionally have been treated as separate entities, but increasing evidence in the last decade indicates that cells of bone marrow and lymphoid origin work together with those of the skeletal system, compromising a reciprocal "osteoimmune" signaling system (Danks and Takayanagi 2013; Takayanagi 2012; Guerrini and Takayanagi 2014). These interactions are orchestrated by the release of chemical mediators and direct cell-cell contact that are actively engaged in the maintenance of bone homeostasis (Caetano-Lopes et al. 2009). On the other hand, deregulation of immune cells and their mediators at sites of inflammation can activate excessive bone resorption, which contributes to the pathogenesis of immune-mediated skeletal diseases like rheumatoid arthritis. Importantly, several molecules identified initially as immunoregulatory factors now have been shown to have essential functions in bone metabolism (David 2007; Takayanagi 2007). In like manner, altered osteoblast signaling contributes to oncogenic transformation of hematopoietic cells (Kode et al. 2014). Future work in osteoimmunology undoubtedly will discover many new examples of collaboration among bone, synovial, and immune cells.

Effector Cells in Bone Remodeling. Growth, maintenance, repair, and degradation during normal and pathologic skeletal remodeling is modulated by the interplay between bone cells with counteracting functions. In particular, the plasticity of bone structure is controlled by the balance between osteoblastic (bone-forming) and osteoclastic (bone-eroding) activities. These activities are regulated in health chiefly by reciprocal cross-talk between osteoclasts and osteoblasts. In immune-mediated musculoskeletal diseases, however, soluble signaling molecules produced by leukocytes can alter the balance in favor of osteoclastic activity (Braun and Zwerina 2011; Sato and Takayanagi 2006).

Osteoblasts arise from mesenchymal stem cells in bone marrow. Their primary function is to generate a proteinaceous bone matrix that serves as a scaffold on which mineral (hydroxyapatite) crystals are deposited. Osteoblasts express receptors for most major molecules that regulate bone metabolism, including factors that impel bone formation as well as those that drive its resorption. Importantly, osteoblasts secrete several cytokines that regulate osteoclast differentiation (Baron 2008).

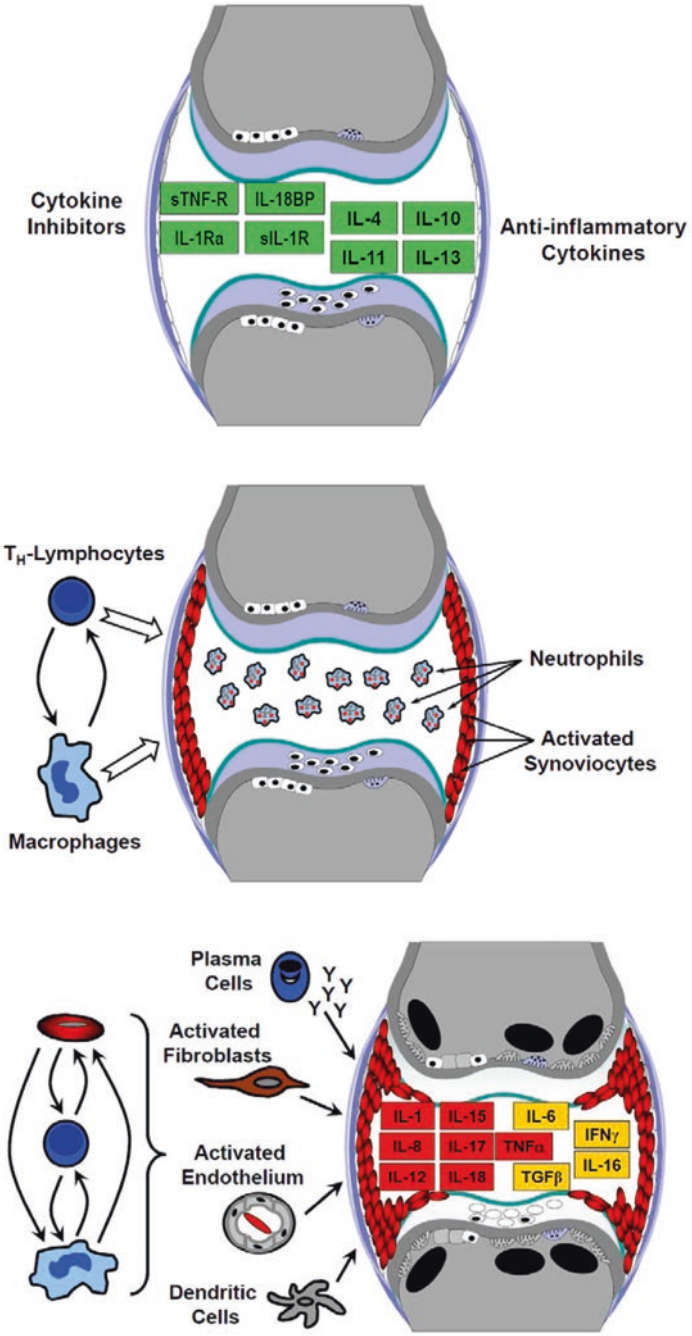

Fig. 12.1 Schematic diagram of a diarthrodial joint (a common target site for autoimmune damage in many species, including humans). Induction of autoimmune disease depends on the balance between pro- and anti-inflammatory stimuli in a target organ. In health (*top panel*), tissues express a diverse population of immune-dampening molecules (denoted by *green boxes*), including

In addition, osteoblasts release multiple factors that provide a favorable peri-endosteal marrow microenvironment for hematopoietic stem cell (HSC) maintenance and differentiation (Calvi et al. 2003), termed the 'HSC niche' (Takayanagi 2007; Levesque et al. 2010). Thus, osteoblasts act not only directly to build bone but also indirectly via soluble signaling molecules to control bone erosion and the production of multiple immune cell lineages.

Osteoclasts originate from phagocytic precursors of the monocyte/macrophage lineage and digest bone matrix. In health, their main function is removal of bone matrix during physiologic bone remodeling. However, during skeletal disease (including immune-mediated conditions), excessive osteoclast production results in increased resorption activity and pathologic bone loss (Boyle et al. 2003); indeed, osteoclasts are the major effector cells that cause bone erosion in autoimmune arthritis (Sato and Takayanagi 2006). Conversely, reduced osteoclast function results in increased bone mass (osteopetrosis) and abnormal bone marrow formation (Teitelbaum and Ross 2003).

Regulatory Cells in Bone Remodeling. As noted above, osteoblasts are a primary influence in controlling the activation state of osteoclasts. This level of control is active chiefly in health. During immune-mediated skeletal diseases, other cell types provide a much greater impetus for enhancing osteoclast numbers and function (Fig. 12.1). In particular, immune cells participating in immune-mediated musculoskeletal disease typically are located in the bone marrow (especially beneath joint surfaces) and synovium (Bugatti et al. 2012). Immune reactions directed against joint tissues may spread into adjacent soft tissues, and thus impact tendons passing near joints as a bystander effect.

Lymphocytes are a major regulatory cell in immune-mediated skeletal disease. Helper ($CD4^+$) T-cells are essential activators of osteoclasts (Kong et al. 1999;

Fig. 12.1 (continued) anti-inflammatory cytokines such as interleukins (IL)-4, -10, -11, and -13 as well as inhibitors like receptor antagonists (IL-1Ra), soluble binding proteins (IL-18BP), and soluble receptors (sIL-1R and sTNF-R for removing IL-1 and tumor necrosis factor-α [TNF-α], respectively). Joint architecture is within normal limits: cartilage lacunae (*white ovals*) contain chondrocytes (*off-center black ovals*); cartilage matrix (*pale lavender*) is intact; synovium (*flat white ellipses*) lining the joint capsule (*curved, lavendar, vertical lines*) are in a resting state; and subchondral bone contains numerous osteoblasts (*white rectangles with black nuclei*) but few osteoclasts (*pale lavendar cells with one flat margin defined by multiple ridges*). In early inflammation (*middle panel*), T-helper (T_H)-lymphocytes and macrophages secrete large batteries of cytokines to communicate to each other, recruit other leukocytes (e.g., neutrophils), and upregulate the function of local facultative immune cells (e.g., synoviocytes). Activated synoviocytes (*bright red ovals*) become more plump (hypertrophic) and increase in number (hyperplasia), but other joint structures are relatively unaffected. As the inflammatory response progresses (*bottom panel*), dendritic cells and B-lymphocytes/plasma cells as well as many non-immune but activated cells (e.g., endothelium, fibroblasts, synoviocytes) also release a plethora of pro-inflammatory cytokines. Activated synovium proliferates across the face of the articular cartilage, resulting in degradation of the matrix (*white*) and chondrocyte loss within most lacunae (*empty white ovals*). The subchondral bone is eroded (*solid black ovals*) due to extensive expansion of osteoclasts and osteoblast death (*pale gray rectangles*). Most of these mediators are purely pro-inflammatory signals (shown as *red boxes*), including IL-1, -8, -12, -15, -17, and -18 as well as TNF-α; a few ligands (*yellow boxes*) like IL-6 and -16, interferon (INF)-γ, and transforming growth factor (TGF)-β exhibit pro-inflammatory properties during autoimmunity but help modulate inflammation in other circumstances. Figure reprinted from (Bolon 2012)

Takayanagi et al. 2000b) and also control the functions of many other leukocyte classes. These tasks are undertaken not only by T-helper (T_H) cells at the site of the inflammatory process (Wechalekar and Smith 2014) but also by follicular T-helper (T_{FH}) cells in regional lymph nodes (Chen et al. 2012; Yu et al. 2015). Certain T-helper cell classes, especially those producing substantial quantities of interleukin (IL)-1 (T_H1) or IL-17 (T_H17), have been shown to be especially prone to driving immune-mediated skeletal destruction (Dong and Flavell 2000; Gaston 2008; Takatori et al. 2008). Many additional pro-inflammatory, lymphocyte-derived cytokines (Burmester et al. 2014; Mateen et al. 2016) and chemokines (Thieblemont et al. 2016; Szekanecz and Koch 2016) also are being investigated for their roles in driving immune-mediated skeletal diseases. The pro-inflammatory effects of T_H1 and T_H17 T-cells are countered in health by the actions of regulatory T-lymphocytes (Treg) to suppress autoreactive T-cells and B-cells (Cooles et al. 2013; Alunno et al. 2015), but in disease a decline in Treg cells permits T_H1 and T_H17 T-cells to carry out their pro-inflammatory missions (Mateen et al. 2016; Cooles et al. 2013). Mature B-cells expressing antibodies against locally expressed autoantigens also are recruited to sites of immune-mediated skeletal disease (Bugatti et al. 2014).

Macrophages and ***dendritic cells*** also impact skeletal integrity during skeletal diseases. In immune-mediated arthritis, macrophages are numerous within the synovium, and the extent of joint erosion is well correlated with the degree of macrophage infiltration (Udalova et al. 2016). As with lymphocytes, macrophages entering the synovium are heterogeneous with respect to their molecular signatures; in disease, the number of pro-inflammatory cells exceeds those acting to suppress inflammation (Li et al. 2012). Macrophages also represent an important element of the inflammatory reaction in subchondral bone marrow (Bugatti et al. 2012). Dendritic cells are bone marrow-derived cells that regulate both innate and adaptive immune responses against a specific epitope. Some dendritic cells serve as antigen-presenting cells (APCs) to activate lymphocytes and other leukocytes which carry out immune-mediated attacks, while others serve as regulatory (or "tolerogenic") dendritic cells to maintain self-tolerance (Raïch-Regué et al. 2014). In recent years, interest has surged in treating immune-mediated musculoskeletal diseases by seeking to increase numbers of tolerogenic dendritic cells (Schinnerling et al. 2015).

Synoviocytes serve as facultative (i.e., inducible, "as needed") regulators of immune-mediated skeletal disease (Lowin and Straub 2015). Synoviocytes are the principal components of the intimal (inner) layer of synovium, the soft tissue that lines diarthrodial joints, tendon sheaths and bursae. In health, resident ('resting') synoviocytes exhibit one of two phenotypes: macrophage-like (type A) and fibroblast-like (type B cells). The microenvironment in inflamed joints activates type B cells so that they can produce and release pro-inflammatory and pro-erosive cytokines, chemokines and matrix metalloproteinases. Activated type B cells also express receptors for several neurotransmitters (e.g., dopamine, norepinephrine, glutamate), thus providing a means whereby immunomodulatory activities of the osteoimmunology and neuroimmunology systems in the joint may be integrated. Interestingly, activated synovial fibroblasts may harbor an altered DNA methylation pattern that allows them to retain an activated phenotype at distant sites (i.e., those

without a pre-existing pro-inflammatory milieu), which permits any circulating synovial fibroblasts to colonize previously unaffected joints and initiate arthritis (Lowin and Straub 2015).

Molecules that Regulate Bone Remodeling. Skeletal remodeling is a closely regulated process that depends on reciprocal communication between osteoclasts and osteoblasts, with osteoblasts both supporting and regulating osteoclast activity (Jimi et al. 1996). The main intercellular signaling pathway that regulates bone remodeling and underpins osteoimmunologic activities consists of three members of the tumor necrosis factor (TNF) and TNF receptor (TNFR) superfamily: receptor activator of nuclear factor-κB (RANK, also termed TNFR superfamily member 11A, TNFRSF11A); RANK ligand (RANKL); and osteoprotegerin (OPG, also termed TNFRSF11B) (Bolon et al. 2002; Walsh and Choi 2014). In health, the extent and severity of bone loss is controlled by the balance between OPG and RANKL in the bone microenvironment. In immune-mediated skeletal diseases, the bone- and joint-destructive effects of RANKL, which is present in relative excess to OPG, are augmented by those of many pro-inflammatory cytokines released by activated leukocytes and fibroblast-like (type B) synoviocytes (Keyszer et al. 1994; Lerner 1994; Taubman and Kawai 2001; Okamoto and Takayanagi 2011). Activities of pro-inflammatory and pro-erosive factors are countered by a large constellation of molecules that down-regulate, physically bind, or functionally antagonize these skeletal-damaging influences.

RANK is a membrane-bound receptor found on osteoclasts. It is the only known receptor for RANKL. Expression of RANK on osteoclasts depends on stimulation by macrophage colony-stimulating factor (M-CSF) (Suda et al. 1999), which is produced locally by parathyroid hormone (PTH)-activated osteoblasts. Expression of RANK on mature T-cells and dendritic cells provides an avenue whereby RANKL can influence immune system activities (Kong et al. 2000).

OPG is a soluble decoy (non-signaling) receptor for RANKL, the presence of which prevents RANKL from binding to RANK and launching the pro-erosive intracellular machinery. OPG is produced by several cell types, chiefly osteoblasts in bone (Udagawa et al. 2000) as well as B-cells and dendritic cells (Mizuno et al. 1998). Secretion of OPG by B-cells and dendritic cells is controlled by the costimulatory molecule CD40 (Bengtsson and Ryan 2002). In general, mature osteoblasts appear to generate more OPG than RANKL, while the less mature osteoblasts that are characteristic of remodeling bone (whether during development or in a pathologic state) release more RANKL than OPG (Nagai and Sato 1999). Via its ability to sequester RANKL (see below), OPG may function as an immunomodulatory molecule (Bolon et al. 2002). In practice, however, in the face of severe immune-mediated musculoskeletal disease OPG is much more effective at mitigating skeletal erosion than reducing inflammation (Stolina et al. 2009; Campagnuolo et al. 2002).

RANKL is the main factor that controls osteoclast activity. Importantly, it appears to be the final common mediator for the canonical (i.e., RANKL-dependent) pathway used by many pro-resorptive ligands under both physiologic and pathologic conditions (Bolon et al. 2002). The soluble homotrimer form of RANKL is released by proteolytic cleavage of a membrane-bound protein; both soluble and membrane-bound forms are

potent RANK agonists (Wong et al. 1997; Kong et al. 1999). RANKL is produced by osteoblasts, especially near regions with intense bone remodeling (Lacey et al. 1998), as well as $CD4^+$ and $CD8^+$ T-cells (Wong et al. 1997; Kong et al. 1999) and activated fibroblast-like (type B) synoviocytes (Takayanagi et al. 2000a); in immune-mediated skeletal disease, the key sources of RANKL are T-cells and synoviocytes. In adults, RANKL also is highly expressed in intestine and lymph nodes (Lacey et al. 1998). When bound to RANK, secreted RANKL is the primary driver of bone erosion in immune-mediated musculoskeletal diseases (Danks et al. 2016). The 'classic' pathway for initiating osteoclast differentiation requires RANKL binding followed by recruitment of TNF receptor-associated factor 6 (TRAF6). Functional TRAF6 is essential for osteoclast-mediated bone resorption (Lomaga et al. 1999), likely because TRAF6 activates a cascade of mitogen-activated protein kinases (MAPKs) that stimulates the NF-κB pathway (Takayanagi 2007). When activated, NF-κB works in collaboration with nuclear factor of activated T-cells 2 (NFATc2) to trigger the transcription factor NFATc1, the master regulator of osteoclastogenesis, ultimately resulting in terminal differentiation of osteoclasts from their precursors (Takayanagi et al. 2002). NFATc1 is also important for multi-nucleation and activation of osteoclasts (Ishida et al. 2002).

Non-canonical pathways mediate osteoclast activation in a RANKL-independent manner (Sabokbar et al. 2016). One alternative is mediated by binding of M-CSF to colony-stimulating factor-1 receptor (c-fms) on osteoclast precursors, after which activation of extracellular signal-regulated kinases (ERK) and the PI3K/Akt pathway leads to enhanced osteoclast proliferation and survival (Kim and Kim 2016). M-CSF signaling also has been shown to boost the resorptive activity of mature osteoclasts (Hodge et al. 2011). Another non-canonical alternative involves calcium-mediated signaling as a co-stimulatory pathway for RANK, where long-lasting oscillations in intracellular Ca^{2+} reserves due to activation of immunoreceptor tyrosine-based activation motif (ITAM)-harboring adapters allow persistent auto-amplification of NFATc1 (Negishi-Koga and Takayanagi 2009). Finally, dysregulation of endogenous inhibitors of the Wnt signaling pathway, such as Dickkopf1 (DKK1) and sclerostin, leads to an imbalance in bone formation (since Wnt signaling is critical for osteoblast differentiation) relative to bone erosion in immune-mediated arthritis (Choi et al. 2009).

Pro-inflammatory molecules can influence the degree of bone erosion in immune-mediated skeletal diseases by modulating the relative levels of RANKL and OPG (i.e., the "RANKL:OPG ratio") so that the skeletal microenvironment favors enhanced osteoclast production and activity (Fig. 12.1). Pro-inflammatory cytokines typically upregulate RANKL production leading to pro-erosive osteoclast activity, including IL-1ß (Hofbauer et al. 1998; Hofbauer et al. 1999c; Braun and Zwerina 2011), IL-11 (Yasuda et al. 1998b; Horwood et al. 1998), IL-17 (Kotake et al. 1999; Sato et al. 2006; Braun and Zwerina 2011), IL-18 (Dai et al. 2004), IL-20 (Hsu and Chang 2010), IL-22 (Kim et al. 2012), IL-23 (Yago et al. 2007), TNF-α (Brändström et al. 1998; Hofbauer et al. 1998; Hofbauer et al. 1999c; Braun and Zwerina 2011), and TNF-β (Brändström et al. 1998). Importantly, expression of both RANKL and OPG have been shown to be enhanced by most of these cytokines, particularly IL-1β (Hofbauer et al. 1998; Hofbauer et al. 1999c; Braun and Zwerina 2011), IL-17

(Kotake et al. 1999; Sato et al. 2006; Braun and Zwerina 2011), and TNF-α (Brändström et al. 1998; Hofbauer et al. 1998; Hofbauer et al. 1999c; Braun and Zwerina 2011), as well as TGF-ß (Murakami et al. 1998). These cytokines are commonly found in the synovium and synovial fluids of patients with immune-mediated skeletal disease (Danks and Takayanagi 2013) and likely work by inducing RANKL expression (Braun and Zwerina 2011). Addition of OPG to cultures of RANKL-expressing cells harvested from joints of rheumatoid arthritis (RA) patients blocks osteoclastogenesis and bone resorption, with the extent of the inhibition depending on the RANKL:OPG ratio (Haynes et al. 2001). Numerous chemokines of both the CC class (e.g., CCL2, CCL3, CCL4, and CCL5) and the CXC class (e.g., CXCL5, CXCL8, CXCL9, and CXCL10) that control cell trafficking and neovascularization have been identified in inflamed tissues of RA patients (Godessart and Kunkel 2001). Other pro-inflammatory molecules such as adenosine (Teramachi et al. 2011) and histamine (Marzaioli et al. 2012) can heighten imbalances in the RANKL:OPG ratio, thereby exacerbating immune-mediated skeletal disease. Finally, various acute phase reactants and coagulation cascade components that are found at sites of inflammation can increase bone resorption in in vitro systems (Lerner 1994).

Anti-inflammatory molecules function in health to quench immune reactions in bones and joints (Fig. 12.1). Anti-inflammatory cytokines like IL-4, IL-10, IL-11, and IL-13 actively counter the effects of pro-inflammatory cytokines. Decoy receptors like soluble IL-1 and TNF receptors (sIL-1R and sTNF-R, respectively) and IL-18 binding protein (IL-18BP) serve as sinks to keep ligands from interacting with their receptors, thereby preventing the launch of pro-inflammatory signaling cascades; this strategy mirrors that employed by OPG in preventing RANKL-mediated osteoclast activation. Finally, competing molecules like IL-1 receptor antagonist (IL-1Ra) thwart docking of a pro-inflammatory cytokine with its cellular receptor. These damping mechanisms are overwhelmed by the excessive production of pro-inflammatory stimuli in immune-mediated joint diseases.

Other signal molecules also may impact the RANKL:OPG balance in skeletal tissues. In health, bone resorption is inhibited by signals that boost OPG but lessen RANKL expression, including elevated calcium levels (Yasuda et al. 1998b). Many molecules that drive osteoclastic bone dissolution increase RANKL but reduce OPG expression (Bolon et al. 2002). Agents that elicit this pattern in vitro include glucocorticoids (Hofbauer et al. 1999a), immunosuppressants (Hofbauer et al. 2001), and many hormones such as androgens (Hofbauer et al. 2002), estrogens (Hofbauer et al. 1999b), parathyroid hormone (PTH) (Horwood et al. 1998), and vitamin D (Yasuda et al. 1998a; Yasuda et al. 1998b). Autonomic nervous system activity can influence the severity of inflammatory skeletal diseases, indicating the strength of the neuroimmunologic axis. Increased sympathetic (ß2-adrenergic) signaling increases the severity of osteoarthritis (Jiao et al. 2015). In contrast, stimulation of parasympathetic (cholinergic) nerves reduces the extent of collagen-induced arthritis (Levine et al. 2014). Many neuropeptides (which are found in nerve fibers within bone) and also leptin (by acting on sympathetic neurons in the hypothalamus) can modulate bone formation and resorption (Lerner and Persson 2008).

Clearly, control of bone erosion in immune-mediated skeletal diseases is regulated via the concerted action of many local or systemic factors.

Bone-Regulating Molecules that Modulate Immune Cell Function. In addition to its functions in controlling bone metabolism, RANKL also plays an important regulatory role in the immune system. RANKL is needed for lymph node formation during development (Dougall et al. 1999). RANKL also directs such essential lymphocyte processes as differentiation, activation (especially for T-cells), and tolerance induction (Yasuda et al. 1998a; Cheng and Fong 2014). These roles are made possible by RANK expression on mature T-cells and dendritic cells (Kong et al. 2000). OPG, by preventing RANKL from interacting with RANK expressed on dendritic cells, may control immune responsiveness (Bengtsson and Ryan 2002), though it cannot quench the entire immune response (Stolina et al. 2009; Campagnuolo et al. 2002).

Bone-regulating molecules do play a key role in governing the hematopoietic stem cell (HSC) niche. Osteoblasts lining the endosteal surface support HSCs under the influence of bone morphometric protein (BMP) (Zhang et al. 2003) and the Notch signaling pathway (Calvi et al. 2003). Osteoclasts are not required for HSC maintenance or mobilization (Miyamoto et al. 2011), although PTH and RANKL help drive HSC mobilization (Li et al. 2015). In addition, PTH enhances HSC colonization in this niche (Song et al. 2010). To our knowledge, no reports exist defining the degree to which bone-regulating molecules dictate conversion of HSCs to the several leukocyte classes, especially in the context of initiating and sustaining musculoskeletal disease.

12.1.1.2 Skeletal Muscle

In health, skeletal muscle contains few resident and trans-migrating leukocytes. Immune-mediated muscle diseases are driven primarily by infiltrating lymphocytes (chiefly $CD4^+$ and $CD8^+$ T-cells as well as plasma cells), macrophages, and dendritic cells responding to the release of muscle fiber antigens; the proportion of each cell type depends on the pathogenesis of the myopathy (Simon et al. 2016; Gherardi 2011). However, muscle cells may produce cytokines (Nagaraju et al. 1998; Sugiura et al. 2000) and can act as facultative antigen-presenting cells (Wiendl et al. 2005a), and therefore are active participants in the immune response in muscle tissue. The activated endothelial cells in capillaries within inflamed muscle tissue also release cytokines and express adhesion molecules that help leukocytes exit the vasculature.

The molecular pathogeneses of various immune-mediated muscular diseases are poorly understood due to the lack of animal models and the rarity of the human conditions. However, existing reports indicate that immune system attacks on skeletal muscle are driven by a witch's brew of pro-inflammatory molecules (De Paepe and Zschüntzsch 2015; Gherardi 2011). The innate immune response is dependent chiefly on leukocyte-derived molecules like interferon (IFN)-α/β/γ, many interleukins, and TNF-α (Rayavarapu et al. 2013). The adaptive response in muscle typically is of the T_H1 type, which is driven by IFN-γ, IL-2, IL-12, IL-15, and TNF-α

(De Paepe and Zschüntzsch 2015). An additional effect of many pro-inflammatory cytokines is that they trigger myofiber catabolism and thus promote muscle atrophy (Costamagna et al. 2015). High-mobility group box-1 (HMGB1)—a mediator of innate immunity in skeletal myofibers—is upregulated in muscle inflammation, is modulated by certain combinations of pro-inflammatory cytokines (e.g., IL-1β and IFN-γ), and can promote myofiber degeneration (Muth et al. 2015). Costimulatory molecules of importance in controlling cytokine release in muscle tissue include CD40 (Sugiura et al. 2000) and inducible costimulator (ICOS) ligand (Wiendl et al. 2005b). In summary, many mediators are responsible for launching and sustaining immune-mediated muscle disease.

12.1.2 Immune-Mediated Musculoskeletal Diseases

Humans suffer from a spectrum of immune-mediated musculoskeletal diseases. In terms of numbers, the most common conditions are arthritides, which affect joints (cartilage, synovium, and bone) as well as the contiguous ligaments, tendons, and tendon sheaths. Multiple animal models, exhibiting variable pathologic features and presentations, have been produced to examine the cellular and molecular elements that drive immune-mediated joint diseases. These models recapitulate the clinical and pathologic features of their human counterparts to a variable degree, and as such can be used in defining possible new cellular/molecular mechanisms and developing novel anti-arthritic agents. Humans also develop several forms of immune-mediated myositis, but few animal models have been described that repeat the specific characteristics of the distinct human diseases. Immune-mediated osteitis (or more commonly osteomyelitis, as bone marrow also is affected) is uncommon relative to inflammatory bone disease associated with traumatic or vascular introduction of an infectious agent. However, bacterial and viral infections localized to the epiphyses (usually of long bones) can lead to subsequent recurrent infections that have been linked to a greater proclivity to develop immune-mediated joint disease (Berthelot and Sibilia 2016).

12.1.2.1 Arthritis

In humans, the joints are susceptible to multiple variants of immune-mediated attack, which differ in their lesion patterns and severity (http://www.rheumatology.org/I-Am-A/Patient-Caregiver/Diseases-Conditions). Most variants in humans may be classed as “inflammatory” polyarthritides due to the large numbers of mixed leukocytes and myriad pro-inflammatory signal molecules present within the many affected joints. Rheumatoid arthritis (RA) is the prototype for this category. In contrast, “non-inflammatory” arthritides exhibit a much less florid leukocyte influx and may be limited to one or a few joints. Osteoarthritis (OA) is the classic example of this class.

"Inflammatory" Arthritis

Rheumatoid arthritis (RA), with an estimated global prevalence of 1–2%, is the most common type of severe immune-mediated arthritis affecting humans. Factors such as age, gender (hormonal status), genetic background, environmental conditions, and intestinal microbiota influence the molecular events that control the initiation and persistence of RA (Klippel et al. 2008; Forbes et al. 2016). The disease affects young women approximately three times as often as men, often presenting first as pain, stiffness, swelling, and warmth in multiple small joints (e.g., intercarpal, intertarsal, metacarpophalangeal, metatarsophalangeal, and proximal interphalangeal) of the distal limbs. More and larger joints may become involved over time. Rheumatoid factors (i.e., auto-antibodies directed against such entities as citrullinated proteins) circulating in the blood can be used as biomarkers to follow disease progression and "flares" (i.e., periods of exacerbation) in many patients (McInnes and Schett 2011).

Two other forms of immune-mediated joint disease in humans must be differentiated from RA. ***Psoriatic arthritis*** is a RA-like disease that occurs in approximately 15% of patients who have psoriasis. Common sites tend to be large joints of the lower limbs, distal interphalangeal joints of all limbs, and sometimes the sacroiliac joints and intervertebral discs. The disease tends to strike individuals in mid-life, and in about 40% of cases affects multiple family members. The disorder has been linked to streptococcal throat (particularly tonsil) infections (Muto et al. 1996; Rantakokko et al. 1997; Wang et al. 1999). ***Ankylosing spondylitis***, a form of spondyloarthritis (or spondyloarthropathy), affects the entheses (i.e., the fibrocartilaginous or fibrous attachments between a bone and a ligament or tendon) associated with the sacroiliac joints and intervertebral discs, and to a lesser extent joints in the limbs. Young males are affected more commonly than other members of the population, and most patients are Caucasians with the genotype HLA-B27 (a major histocompatibility complex [MHC] class I surface antigen needed to present antigens to T-cells).

Several autoimmune diseases of the musculoskeletal system primarily attack connective tissue (https://www.merckmanuals.com/home/bone,-joint,-and-muscle-disorders/autoimmune-disorders-of-connective-tissue/overview-of-autoimmune-disorders-of-connective-tissue). ***Systemic lupus erythematosus*** (SLE) is a pantropic autoimmune condition in which immune reactions are mounted not only in joints but also in numerous other organs (especially the kidneys, lungs, and skin). The disease commonly affects women of child-bearing age, often those having a non-European (African, Asian) ethnic background. ***Scleroderma*** (or systemic sclerosis) also is a pantropic autoimmune disease. It affects women, usually of child-bearing age, approximately four times as often as men. Over time, inflamed joints may become fixed in a flexed position (i.e., contracture) due to the extensive formation of new fibrous beds within the adjacent soft tissues. ***Secondary Sjögren's syndrome*** presents as inflammation of the salivary glands and tear ducts in patients with another rheumatologic disease (primarily RA or SLE). The condition occurs most often in older women. ***Mixed connective tissue disease*** (MCTD) is a rare condition in which the victim simultaneously exhibits features of multiple autoimmune diseases (so-called "overlap syndrome"). Comorbid diseases typically include SLE, scleroderma, and

often some variant of polymyositis. This progressive disease affects primarily women (especially in late adolescence), though vulnerability extends from childhood throughout life. Arthritis often is a prominent component of MCTD.

The immune-mediated arthritides, while severely impacting joints, may be associated with comorbidities (i.e., autoimmune conditions of other organs and systems). For example, RA may occur together with autoimmune thyroiditis (Punzi and Betterle 2004) and inflammatory bowel disease (Forbes et al. 2016). The variants of immune-mediated arthritis are distinguished more by their clinical presentations and lesion patterns than by differences in the molecular mechanisms that drive inflammation. Many disease-modifying anti-rheumatic drugs (DMARDs) may be used to treat these conditions.

In RA patients, affected joints initially develop a synovitis characterized by synovial cell activation, hypertrophy, and hyperplasia along with infiltration of leukocytes (neutrophils, $CD4^+$ T-cells, macrophages, and dendritic cells) into the peri-synovial connective tissue. Chronic RA usually is characterized by intense inflammation in the synovium and periarticular soft tissues (including the formation of lymphoid nodules with germinal centers and many B-cells), and to some extent the nearby bone marrow, as well as production of pannus (an aggressive neotissue bed comprised of activated fibroblasts, immune cells, and blood vessels that will migrate over the surfaces of articular cartilages). The plethora of pro-inflammatory cytokines and chemokines released in this environment promote erosion of the cartilage and subchondral bone. Without treatment, loss of cartilage will lead to joint narrowing, osteophyte production, and eventually fusion (ankylosis) in an attempt to stabilize the collapsing bones. Inflammation recedes if the autoantigen is removed, but any damage to bone or cartilage remains. Despite the common pattern of structural damage, the pathogenesis of RA differs among individuals; this fact is proven because inhibitors directed against master pro-inflammatoy cytokines like IL-1β and TNFα are effective treatments in subsets of human RA patients (Kulmatycki and Jamali 2005; Koller 2006). Therefore, RA in humans actually represents a syndrome in which a common set of morphologic lesions is elicited by one or several potential cellular/molecular abnormalities. In contrast to RA, patients with autoimmune diseases of connective tissue tend to have intermittent episodes of arthritis that tend to produce modest joint erosion and little (if any) joint deformity.

Immune-mediated polyarthritis in animal models is similar to that found in RA patients, although it differs from that of RA in humans in several key respects (Bendele et al. 1999). Destruction of articular cartilage and associated bone is more extensive in animals with induced arthritis, and occurs much more quickly (days to a few weeks) relative to disease progression in humans with spontaneous RA (months to years). In animals, the extent of joint swelling (Fig. 12.2), the spectrum of affected joints, and the time course during which disease progresses vary with the arthritogenic agent (Bolon et al. 2011). Some models develop mainly joint lesions (e.g., collagen-induced arthritis [CIA] and streptococcal cell wall [SCW]-induced arthritis), sometimes with extra-articular lesions at a few sites (e.g., adjuvant-induced arthritis), and thus more closely mirror classic RA (Table 12.1). Other animal arthritis models exhibit systemic inflammation (e.g., homozygous MRL-*lpr/lpr* mice), and thus resemble SLE (Table 12.1). Profiles of pro-inflammatory molecules associated with active disease are distinct for different animal RA models (Stolina et al. 2008; Stolina et al. 2009). Multiple cytokines participate in the induction and maintenance of disease in such

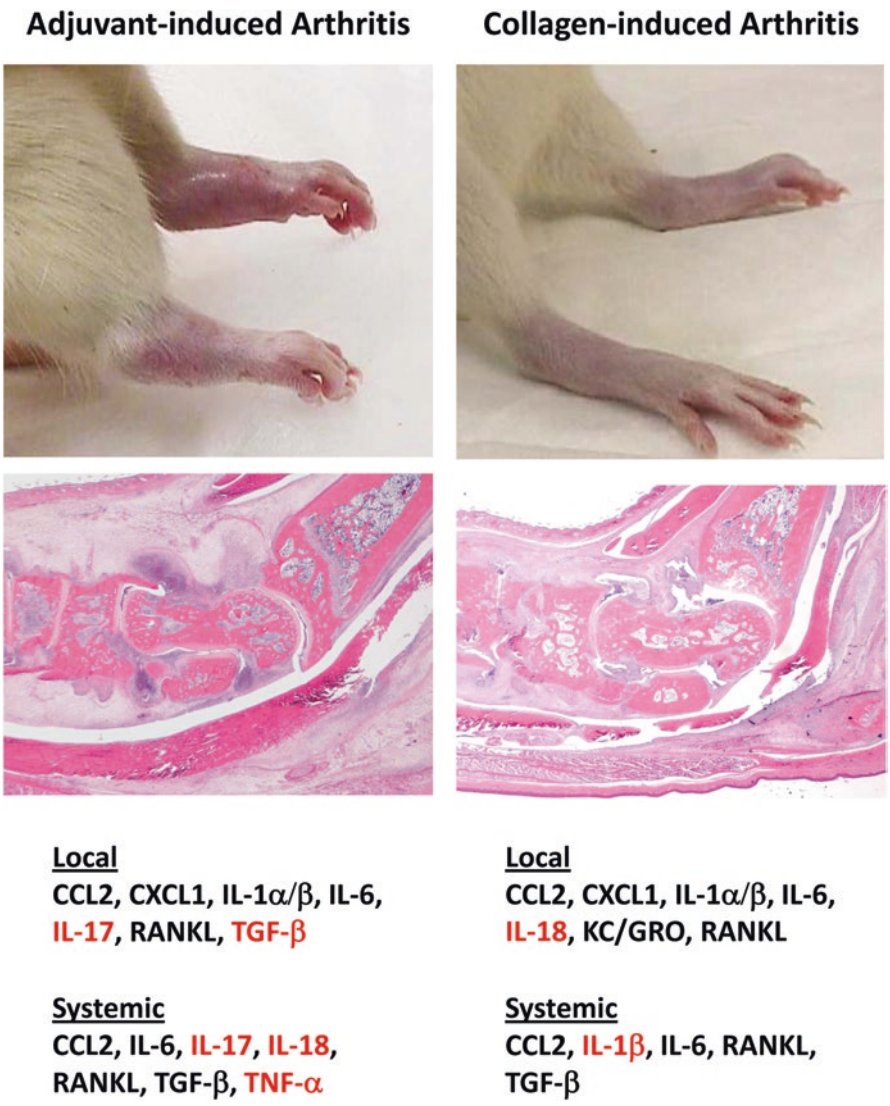

Fig. 12.2 A given target organ may be affected by multiple autoimmune conditions, each having some unique clinical, pathological, and biochemical features. For example, adult Lewis rats develop more severe arthritis (indicated here by greater swelling and reddening of the tibiotarsal region [hock or "ankle"]) 7 days following treatment with a single inoculation of heat-killed *Mycobacterium* in adjuvant than occurs after administration of multiple injections of collagen type II. One proposed explanation for this divergence is the variation in cytokine profiles between the models; whether measured locally (in joint extract) or systemically (in serum), some pro-inflammatory molecules (shown in *red*) are produced in only one model. Abbreviations are defined in the legend for Fig. 12.1, with these exceptions: *CCL2* chemokine (C-C motif) ligand 2 (formerly MCP-1); *chemokine* (C-X-C motif) ligand 1 (formerly KC/GRO); *RANKL* receptor activator of nuclear factor-κB ligand. Gross images reprinted from (Bolon et al. 2011), while cytokine signatures were reported in (Stolina et al. 2008; Stolina et al. 2009); figure arrangement is reproduced from (Bolon 2012)

Table 12.1 Selected models of "inflammatory" variants of immune-mediated arthritis

Categories	Induction principle	Examples	Inciting agents/genetic alteration	Species
Genetically engineered	Deliberate manipulation of one or more genes encoding proteins that regulate the immune response	D1CC transgenic	Major histocompatibility complex (MHC) class 2 molecule (over-expression limited to joints)	Mouse
		HLA-B27 transgenic	Human leukocyte antigen (HLA) B27 (a MHC class 1 molecule) and human β2-microglobulin	Rat
		HLA-DR transgenic	Human leukocyte antigen, D-related (a MHC class II molecule)	Mouse
		IL-1Ra knockout	Interleukin-1 receptor antagonist (IL-1Ra) null mutation	Mouse
		K/BxN (bi-transgenic)	Human T-cell receptor (KRN) and a human MHC class II molecule	Mouse
		TNF-α transgenic	Tumor necrosis factor-α	Mouse
Induced	Intra-articular administration of an exogenous material (usually into the femorotibial ["knee"] joint)	Antigen-induced arthritis	Albumin	Mouse, rat
		Cytokine-induced arthritis	Purified cytokines (e.g., IFN-γ, IL-1ß, TNF-α)	Mouse, rat, rabbit
	Systemic (subcutaneous) injection of an exogenous material in slow-desolving depot material (adjuvant)	Adjuvant-induced arthritis (AIA)	Lipoidal amine	Rat
			Mycobacterium tuberculosis (heat-killed)	Rat
			Pristane	Mouse, rat
		Bacterial cell wall-induced arthritis	Bacterial cell wall peptidoglycan (polysaccharide): *Lactobacillus* sp., *Streptococcus* sp. (SCW)	Rat
		Collagen-induced arthritis (CIA)	Type II collagen (bovine, porcine, rodent)	Mouse, rat, monkey
	Passive transfer of exogenous cells or antibodies	Antibody-induced arthritis	Monoclonal auto-antibodies to type II collagen, given to vulnerable sub-strains (e.g., B.10 or DBA/1, which have the H-2^q genotype)	Mouse
		Cell-mediated arthritis	Adoptive transfer of spleen-derived $CD4^+$ T-cells from DBA/1 animals to *scid* animals (which lack functional T-cells and B-cells)	Mouse
Spontaneous	Spontaneous mutations leading to immune-mediated joint disease	MRL/*lpr* (formally: MRL/Mpj-*Fas*^{lpr}/*2J*)	Mutation in *Fas*^{lpr}, a membrane protein in the TNF receptor family that modulates apoptosis	Mouse
		SKG (BALB/c genetic background)	Mutation in zeta (ζ) chain-associated protein kinase 70 kDa (ZAP-70), a membrane element involved in T-cell receptor-mediated signaling	Mouse

models as treatment with inhibitors to simultaneously block two master pro-inflammatory molecules provides synergistic anti-arthritic activity relative to administration of either inhibitor alone (Feige et al. 2000).

Collagen-induced arthritis (CIA), although not identical to RA, is the animal model that seems to best replicate the clinical and structural features of human RA (Myers et al. 1997; Bolon et al. 2011), so this model is a preferred platform for translational medicine efforts to determine mechanisms and find innovative therapies relevant to human immune-mediated joint diseases. CIA may be induced in many animal species (including nonhuman primates) and follows a less aggressive course (in both speed and severity) than many adjuvant-induced arthritis models (Bolon et al. 2011) (Fig. 12.2). Rodents are favored subjects for CIA experiments due to the availability of strains with differing vulnerabilities (Wilder et al. 1999) and immune capabalities (Fox et al. 2007), the availability of key reagents for immunological testing, and the proven track record for predicting efficacious human responses (Bendele et al. 1999; Hegen et al. 2008).

Potential Mechanisms of "Inflammatory" Immune-Mediated Arthritides

The cause(s) and pathogenesis of immune-mediated joint diseases are not fully understood. Indeed, to our knowledge, the molecular pathogenesis of arthritis where the inciting agent lacks inherent immunogenicity (e.g., pristane-induced arthritis (Vingsbo et al. 1996)) is not known. However, the cumulative literature supports the existence of several mechanisms that likely contribute to the onset and maintenance of "inflammatory" immune-mediated joint diseases.

One mechanism is likely to be "molecular mimicry," a situation in which a self-antigen is incorrectly recognized as "non-self" due to its resemblance to a foreign molecule (Bläss et al. 2001). For example, purified type II collagen from other mammalian species reliably induces CIA in mice and rats (Bolon et al. 2011). Similarly, synovial T-cells have been suggested to mediate attacks against nearby cells that express lymphocyte function-associated antigen-1 (LFA-1). The amino acid sequence in the alpha chain of LFA-1 is highly homologous to an epitope of the spirochete outer surface protein A (OspA) carried by *Borrelia burgdorferi*—the bacterium responsible for Lyme disease arthritis (Gross and Huber 2000). Numerous other bacterial and viral pathogens also have been associated with pathologic processes that resemble RA (Arleevskaya et al. 2016). For instance, *Yersinia*-triggered arthritis has been attributed to deposition of bacterial antigens (Nikkari et al. 1992) and the production of *Yersinia*-specific T-cells (Viner et al. 1991) in the synovium. The ability of bacterial products to incite adjuvant-induced arthritis (via heat-killed *Mycobacterium*) or streptococcal cell wall (SCW)-induced arthritis also may represent instances of altered self-tolerance by T-cells within affected tissues (van den Broek et al. 1992).

Importantly, joint-directed immune reactions in psoriatic arthritis have been posited to arise in part from extra-articular bacterial infections. This disease has been associated with streptococcal tonsillitis (Muto et al. 1996; Rantakokko et al. 1997; Wang et al. 1999), a condition which also may trigger psoriasis in the absence of arthritis due to greater numbers of skin-homing $CD4^+$ and $CD8^+$ T-cells (Sigurdardottir

et al. 2013). The link between psoriatic arthritis and streptococcal tonsillitis has been suggested to arise from circulating antibodies against bacterial cell wall constituents (Muto et al. 1996; Rantakokko et al. 1997); microbial ribosomal RNA but not bacteria in the blood, and occasionally the synovial fluid (Wang et al. 1999); and increased activation of innate immune cells mediated by Toll-like receptor (TLR)-2 (Carrasco et al. 2011). However, the connection may instead represent a generic response by infiltrating synovial T-cells to streptococcal superantigens (i.e., molecules that cause non-specific, polyclonal T-cell activation) rather than a disease-specific immune response to conventional streptococcal antigens (Thomssen et al. 2000). Though the exact pathogenesis must be clarified, tonsillectomy of patients with chronic bacterial infections does reduce the severity of psoriasis (Thorleifsdottir et al. 2012) and psoriatic arthritis (Kaneko et al. 2015).

A second and related mechanism is epitope spreading (Arleevskaya et al. 2016). In this situation, the acquired T-cell and/or B-cell response attacks progress beyond the original epitope to include associated epitopes, usually those of an infectious agent but sometimes self-epitopes in the pathogen-damaged tissue. Epitope spreading may result from post-translational modifications in protein structure that lead to production of neoantigens that will be seen as "non-self." For instance, conversion of arginine to citrulline in connective tissue proteins like vimentin (Ménard et al. 2000) will yield a series of molecules that may modulate many processes, including immune cell apoptosis and NETosis (where neutrophils exude chromatin strands as carriers for peptides with antimicrobial and immunoreactive properties—including service as neoantigens that can elicit autoimmunity (Thieblemont et al. 2016)). Citrullinated molecules have been shown to play a role in initiating autoimmune diseases (Dwivedi and Radic 2014; Valesini et al. 2015).

A third mechanism is an altered balance between pro-inflammatory and anti-inflammatory signaling cascades (Fig. 12.1), presumably resulting from constitutive activation of resident immune surveillance cells (histiocytes and synoviocytes) leading to chronic overproduction of myriad pro-inflammatory and pro-erosive cytokines and chemokines (Bläss et al. 2001). Over time, the higher basal levels of signaling molecules produced by these resident cells would serve to attract and activate other osteoimmune cells (including osteoblasts, T-cells, and dendritic cells), which would perpetuate and accelerate the evolution of the pro-inflammatory microenvironment. An important aspect of this control is provided by the "gut-joint axis." In health, the normal microbiome in the digestive tract provides a system-wide immunoregulatory influence on the immune system (Forbes et al. 2016). Dysbiosis of the intestinal microbiota is a known risk factor for many autoimmune diseases, especially RA and ankylosing spondylitis (Forbes et al. 2016). The key alteration during immune-mediated diseases appears to be a shift in T-cell sub-populations in which the inhibitory role of regulatory (Treg) T-cells can no longer compensate for the relative excess of activated T-helper cells (especially of the T_H1 and T_H17 varieties) (Cooles et al. 2013; Mateen et al. 2016). Indeed, some T-cells that express FOXP3 (forkhead box P3, a protein that controls Treg cell differentiation) can actually convert to T_H17-type T-cells (Komatsu and Takayanagi 2015). The importance of the gut microflora is confirmed by studies in which conventionally housed and germ-free rodents with adjuvant-induced arthritis or

CIA exhibit different severities of disease depending on their pathogen status (Dorożyńska et al. 2014; Asquith et al. 2016). Recent studies suggest that commensal segmented filamentous bacteria (SFB) modulate immune-mediated arthritis by driving the differentiation and peripheral migration of follicular T-helper (T_{FH}) cells that are primed to recognize self-antigens (Teng et al. 2016; Chappert 2014; Block et al. 2016)

A fourth mechanism of relevance to arthritis induction is polyclonal lymphocyte activation (Arleevskaya et al. 2016). Both T-cells and B-cells might be affected, but in RA the principal impact seems to be on T-cells. Furthermore, both cells within the synovium and those in more distant lymphoid organs are affected. Importantly, lymphocyte function is deficient when multiple clones are activated at the same time. However, the polyclonal increase in lymphocyte activation typically is accompanied by impaired deletion or deficient anergy of autoreactive clones, which sustains the autoimmune response even though the individual effector cells are less active than their counterparts in a more limited inflammatory response.

A fifth proposed mechanism that might influence immune-mediated arthritis is age-related changes in immune system function. Chronic, low-level inflammation (or "inflammaging") is a common finding in older people that has been linked to persistent elevations in circulating levels of many pro-inflammatory cytokines, acute phase proteins, and pro-coagulating factors (Franceschi and Campisi 2014; Dietert et al. 2012). Deficient immune function (or "immune senescence") in the elderly has been associated with skewed T-cell sub-populations as well as reduced capacity for T-cell activation and differentiation (Ponnappan and Ponnappan 2011). The predisposition for individuals with longstanding immune-mediated musculoskeletal diseases of the "inflammatory" type (i.e., those with aggressive multifocal inflammation due to autoimmune attack on bones or joints) to have shorter lifespans relative to healthy individuals therefore may be a consequence not only of their immune-mediated disease but also a consequence of increased susceptibility to other age-related diseases (most of which have a pathogenesis based partly on dysregulated immune system responsiveness). We posit that inflammaging is unlikely to play a key role in the majority of immune-mediated musculoskeletal diseases of the "inflammatory" type, which develop during the first few decades of life and involve extensive inflammation in their own right. Instead, if an age-related mechanism plays a role in the onset of "inflammatory" arthritides, we believe that the most likely culprit will be premature senescence of immunosuppressive T reg cells.

"Non-inflammatory" Arthritis

Osteoarthritis (OA) is a slowly progressing, erosive joint disease that affects approximately 10% of men and 13% of women over the age of 60 years in the United States (Zhang and Jordan 2010). The number of affected individuals in industrialized countries is anticipated to reach 25% of the elderly by 2030 (Hootman and Helmick 2006). The disease tends to affect weight-bearing joints like the coxofemoral ("hip"), femorotibial ("knee"), intervertebral, intercarpal ("wrist"), and hallux ("big toe") interphalangeal joints. The lifetime risk for developing OA in

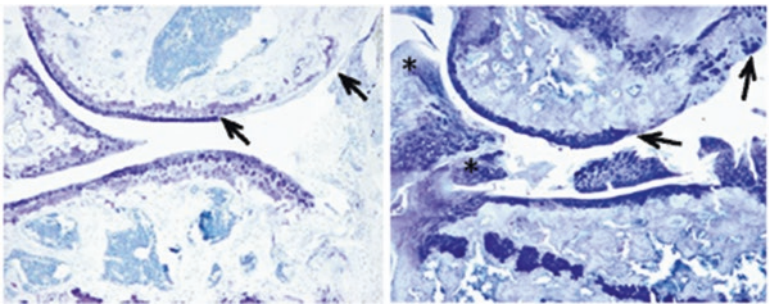

Fig 12.3 Degenerative joint disease is the hallmark feature of osteoarthritis (OA). Mild changes (*left panel*) include focal loss (between the *arrows*, femur only) or narrowing (femur and tibia) of the non-mineralized (deep purple) articular cartilages. In more severe disease (*right panel*), the articular surfaces of both the femur and tibia are rough due to the loss of the superficial (non-mineralized) and sometimes also the deeper (mineralized) cartilage (between the *arrows*, femur only), and large osteophytes (*asterisks*) are found at the joint margins. Femorotibial ("knee") joint, 9-month-old osteoprotegerin (Opg) wildtype ($Opg^{+/+}$ *left panel*) and Opg knockout ($Opg^{-/-}$ *right panel*) mice. Toluidine blue stain (to demonstrate loss of cartilage matrix). Images are reproduced from (Bolon et al. 2015)

the hip and knee is approximately 25% and 46%, respectively (http://www.rheumatology.org/I-Am-A/Patient-Caregiver/Diseases-Conditions/Osteoarthritis#sthash. MqVd2ae1.dpuf). Clinically, patients usually present with joint crackling or grinding, pain, stiffness, and swelling. Both OA and RA can occur together. Recent literature suggests that despite its typical localization to one or a few joints, OA may represent a systemic skeletal disease (Malemud 2015).

All tissues within OA joints exhibit anatomic evidence of disintegration. Cardinal structural changes of the disease include cartilage fibrillation ultimately leading to fragmentation and loss, eburnation (osteosclerosis) of subchondral bone, deterioration of the ligaments and tendons, and clonal expansion of chondrocytes (Fig. 12.3). The degree of synovial inflammation is variable but fairly mild relative to that observed in "inflammatory" forms of immune-mediated joint diseases like RA. Immune cells found within the synovial tissues of OA joints are predominantly T-cells, macrophages, and mast cells. The numbers of infiltrating immune effector cells as well as the kinds and quantities of pro-inflammatory cytokines are lower in OA joints relative to RA joints (de Lange-Brokaar et al. 2012). Interestingly, RANKL expression in joints is reduced in OA relative to RA (Crotti et al. 2002), thus suggesting that cartilage changes in OA joints are consequences of mechanical trauma and/or RANKL-independent biochemical changes in the degenerating articular cartilage. However, altered RANKL metabolism in some osteoblast subsets may be responsible for a portion of the abnormalities in the sclerotic subchondral bone (Tat et al. 2009). Joint destruction in OA is mediated mainly by cytokine-induced stimulation of matrix metalloproteinases (MMPs), which then degrade cartilage matrix (i.e., proteoglycans) and collagen. The extent of cartilage damage in OA depends on the ratio of MMPs to tissue inhibitors of metalloproteinases (TIMPs) (Ishiguro et al. 1999).

Models of OA are an imperfect means of evaluating the pathophysiology of human disease in vivo. Many options are available, and as yet no one model represents a "gold standard." Human tissue harvested for in vitro experimentation usually is obtained during joint replacement surgery, and thus represents end-stage disease. Instead, in vivo animal testing is employed to model a few key aspects of OA (McCoy 2015). Importantly, different OA models exhibit divergent molecular mechanisms and structural lesions (Little and Zaki 2012), so the choice of model will depend on the hypothesis to be tested. Common permutations include investigation of spontaneous disease (Huebner et al. 2002) or OA-like disease induced by genetic engineering (Glasson 2007), surgical intervention to destabilize (Bendele 2001) or induce a sizeable cartilage defect (Ahern et al. 2009) in a weight-bearing joint (typically the femorotibial), or intra-articular chemical administration to produce cartilage degeneration (Table 12.2). Typically, initial discovery and development efforts in OA are done using rat models (Gerwin et al. 2010), but genetically engineered mouse models (Glasson et al. 2010; Culley et al. 2015) and spontaneous or surgically destabilized guinea pigs (Kraus et al. 2010) often are employed to explore specific biochemical alterations while large animals (rabbits, dogs, goats, sheep, and even horses) are utilized to determine whether or not therapeutic modalities are applicable to human-sized joints (Cook et al. 2010; Laverty et al. 2010; Little et al. 2010, McIlwraith et al. 2010). Regardless of the model, consistent histopathological scoring using species-specific scoring criteria is necessary when translating animal findings to predict potential human responses.

Potential Mechanisms of "Non-inflammatory" Immune-Mediated Arthritis

Molecular signals that influence the onset and progression of OA include some factors that also drive the "inflammatory" category of immune-mediated joint diseases. However, the combination of signals as well as their comparative importance in "inflammatory" (RA-like) and "non-inflammatory" (OA-like) conditions vary considerably. For example, molecular mimicry is likely to be of limited relevance to OA since the level of inflammation is variable but often low, suggesting that a substantive acquired immune response is not being mounted against self-antigens within joint tissues. In contrast, "inflammaging" is a clear risk factor for developing OA (Greene and Loeser 2015). The innate immune system seems to be the main driver of OA.

Pro-inflammatory cytokines clearly are key signals in both OA and animal models of OA, although the extent of synovitis varies considerably among individuals. In this regard, IL-1 and TNF-α both are master cytokines in initiating OA, while IL-1 alone drives later stages of the disease (Goldring 1999). Importantly, chondrocytes have receptors for and release multiple cytokines and chemokines, which can alter the metabolic programming of chondrocytes so that they shift from anabolic (cartilage-building) functions in health to catabolic (cartilage-degrading) and abortive repair processes during disease (Houard et al. 2013). Pro-inflammatory cytokines stimulate chondrocytes to release cartilage-degrading enzymes like

Table 12.2 Selected models of ("non-inflammatory" immune-mediated) osteoarthritis

Categories	Induction principle	Inciting agents/genetic alteration	Species
Genetically engineered	Deliberate manipulation of one or more genes	A disintegrin and metalloproteinase with thrombospondin motifs (*Adamts*) null mutations	Mouse
		Matrix metalloproteinase (*Mmp*)-13 null mutation	Mouse
		Osteoprotegerin (*Opg*) null mutation	Mouse
Induced	Intra-articular administration of an exogenous material (usually into the femorotibial ["knee"] joint)	Collagenase injection	Mouse, rat
		Estradiol injection (repeated)	Rabbit
		Monosodium iodoacetate injection	Rat
	Systemic administration of toxicants that damage cartilage, ligament, and/or tendon integrity	Quinolone treatment (oral or parenteral)	Rat, guinea pig, rabbit, dog, monkey
	Surgical destabilization (usually of the femorotibial joint)	Cartilage defect (fracture or hole) creation	Mouse, rat, rabbit, dog, goat, sheep, pig
		Cartilage (or osteochondral) fragment creation	Horse
		Cranial (anterior) cruciate ligament transection	Guinea pig, rat
		Meniscal destabilization (removal or tear—usually medial)	Guinea pig, rat, rabbit
		Partial osteotomy (tibial plateau)	Rat, sheep
Spontaneous	Degenerative joint disease	Age-related, progressive osteoarthritis	Guinea pig (Hartley)
		Age-related, progressive coxofemoral ("hip") dysplasia	Dog (German Shepherds and other large breeds)

MMPs and ADAMTS5 (a disintegrin and metalloproteinase with thrombospondin motifs 5), which are primary agents responsible for cartilage matrix degradation (Ameye and Young 2006; Silverstein et al. 2016). The milieu of pro-inflammatory mediators released in OA is less extensive than that produced in RA (Kuca-Warnawin et al. 2016).

12.1.2.2 Myositis

Autoimmune myopathies are a group of rare, chronic, idiopathic diseases affecting skeletal muscles (Malik et al. 2016). The most common variants are dermatomyositis, polymyositis, necrotizing autoimmune myopathy, and sporadic inclusion body myositis. The most frequently affected sites are the proximal muscles (i.e., large bellies of the neck, shoulder, and hip regions). Patients typically present with difficulty in climbing, sitting, and standing, usually with little or no pain. Diagnostic tests include magnetic resonance imaging (to detect muscle edema), electromyography (to assess myofiber activity), serum chemistry (to measure creatine kinase [CK] activity, an indicator of cytosolic leakage from damaged myofibers), and histopathology of affected muscles (to visualize immune effector cells).

Dermatomyositis (DM) manifests as symmetrical proximal muscle weakness preceded or accompanied by a characteristic skin rash. Children and middle-aged adults, more often females, are the victims. ***Polymyositis*** (PM) is an exclusionary diagnosis in that the muscular signs and symptoms of DM occur in the absence of cutaneous involvement. PM occurs mostly in women, though it usually arises in young adults (but not children). ***Necrotizing autoimmune myopathy*** (NAM) is similar to PM (i.e., muscle weakness without a rash) but is more rapid in onset and severe in presentation. This condition exhibits particularly high serum CK activity, and it may be initiated by treatment with pharmaceutical agents. ***Sporadic inclusion body myositis*** (sIBM) is a unique immune-mediated myositis in that it develops in middle-aged males, usually progressing slowly over several years; involves both proximal and distal muscles; and may be symmetric or asymmetric. Dysphagia and facial weakness are common in this disorder.

Pathology assessment, especially muscle biopsy, remains the gold standard for discriminating the various immune-mediated muscle diseases (Malik et al. 2016; Vattemi et al. 2014; Simon et al. 2016). Serum CK activity is the most sensitive biomarker—elevations of 100-fold in NAM, 50-fold in DM, and at least 5-fold in PM are customary—but this measurement does not correlate well with the severity of the disease symptoms (Malik et al. 2016). Many myositis-specific antibodies have been identified, but they tend to be prognostic indicators of disease progression and therapeutic response in only a small subset of patients. Biopsies for microscopic analysis should be harvested from moderately affected muscles to maximize the likelihood of detecting immune-mediated lesions. Samples from mildly weakened muscles may show little inflammation, while specimens of severely affected muscles may exhibit more end-stage fibrosis than inflammation. In DM, the infiltrate consists of $CD4^+$ T-cells, B-cells, and plasmacytoid dendritic cells that collect in the connective tissue between fibers; myofibers in the region tend to be slightly to profoundly atrophied but not necrotic, and blood vessels retain deposits of C5b-9 membrane attack complexes and may be ringed by leukocytes. In PM, the endomysium contains $CD8^+$ T-cells and macrophages, which invade and destroy nearby myofibers but do not harm regional blood vessels. In NAM, the key immune effector cells are thought to be macrophages, although few infiltrating leukocytes are evident. Blood vessels are associated with C5b-9 membrane attack complexes but not with perivascular leukocyte infiltrates. In sIBM, the primary infiltrating cells are $CD8^+$

T-cells and macrophages. The key myofiber finding is centrally located, rimmed vacuoles. While histopathologic examination is required to provide a definitive diagnosis for these diseases, evidence of myositis may be minimal and non-specific, with features that appear consistent with several diseases (Gherardi 2011).

Several animal models of immune-mediated myositis have been devised to investigate novel molecular mechanisms and potential therapeutic agents. Experimental autoimmune myositis (EAM) usually is induced in rodents by injection of myosin (Matsubara and Okumura 1996; Matsubara and Takamori 1987) or myosin-binding protein C (Kohyama and Matsumoto 1999) derived from skeletal muscle. Infiltrating immune cells include $CD8^+$ T-cells and macrophages with fewer $CD4^+$ T-cells, but not B-cells (Allenbach et al. 2009). Disease may be passed to naïve animals by transfer of $CD4^+$ T-cells (harvested from regional lymph nodes) or anti-myosin auto-antibodies, and ameliorated by transfer of Treg cells (Allenbach et al. 2009; Matsubara and Okumura 1996). The extent of myositis may be increased by administration of myotoxins (Kang et al. 2015; Wen-Jing et al. 2015), thereby permitting the onset and progression of myositis to follow a more reproducible course.

The specific causes and pathogeneses of immune-mediated muscle diseases are not known. Activated T-cells (especially $CD8^+$) and macrophages are thought to be responsible for most of the direct myofiber damage, although ischemia related to microvasculature injury is considered to play a secondary role in initiating myofiber atrophy. Myofiber destruction by $CD8^+$ T-cells uses perforin pore formation to penetrate the sarcolemma (Sugihara et al. 2010). Auto-antibodies against many myofiber constituents have been detected and are proposed as an additional mechanism of muscle damage in these diseases, but it is possible that such molecules instead represent a sequel to rather than a cause of myofiber degeneration. Many pro-inflammatory cytokines may be detected in inflamed muscles, including IFN-α/β/γ, IL-1, IL-6, IL-12, IL-15, IL-17, and TNF-α (De Paepe and Zschüntzsch 2015). Chemokines such as CCL2/3/4/8/19/21 and CXCL9/10/11/13 are also rampant (De Paepe and Zschüntzsch 2015). The rich milieu of pro-inflammatory molecules that sustain these conditions suggests that therapeutic strategies designed to specifically inhibit one or more cytokines will become an increasingly popular means of targeted therapy for immune-mediated myositis.

12.1.2.3 Osteitis

Most cases of osteitis result from hematogenous or traumatic introduction of pathogens (mainly bacterial) into the nutrient-rich marrow cavity. However, sterile osteitis does occur. Such non-microbial conditions appear to represent an auto-inflammatory state (i.e., a disorder initiated and sustained chiefly by the innate immune system) rather than an autoimmune scenario (i.e., a disease in which the acquired immune system is induced to attack the body's own tissues).

In humans, non-bacterial osteitis exhibits one of several presentations (Jansson et al. 2007). The two most common presentations are the SAPHO syndrome (for synovitis, acne, pustulosis, hyperostosis, osteitis) and CRMO (for chronic recurrent multifocal osteomyelitis). Affected individuals may be of any age, although SAPHO

tends to occur in adults and CRMO in juveniles. Patients with both disorders usually exhibit good health (i.e., no cyclic fevers, organomegaly, weight loss, or fatigue, as occurs with bacterial osteomyelitis) except for localized bone pain at one or several sites. Radiographs demonstrate hyperostosis of bones forming the anterior thoracic wall, and to a lesser extent vertebrae and long bones. Lesions (especially in long bones of the legs) typically are located in the metaphyses adjacent to physeal (growth) plates; involvement usually exhibits bilateral symmetry. Some but not all patients develop palmar-plantar pustulosis (i.e., multiple small vesicles of the palms and soles). Biopsies of affected bones demonstrate a sterile osteitis with a non-specific, bland lymphoplasmacytic infiltrate that may include scattered neutrophils and macrophages within intra-medullary beds of fibrotic tissue. In general, autoimmune osteitis is not associated with immune-mediated arthritis. The tendency for several members of a single family to develop aspects of these syndromes suggests that a strong genetic component influences the onset and cyclic progression of immune-mediated osteitis, as is true for many autoimmune diseases (Smith and Germolec 1999; Bolon 2012).

The etiology and pathogenesis of sterile osteitis syndromes are not yet known, but the release of pro-inflammatory molecules that activate the inflammasome is presumed to be the mechanism (Hofmann et al. 2012). In addition, mutations in PSTPIP2 (proline-serine-threonine phosphatase-interacting protein 2) have been implicated in the SAPHO syndrome and CRMO (Hofmann et al. 2012), though they do not occur in all human patients (Jansson et al. 2007). Two mouse models, cmo (chronic multifocal osteomyelitis) (Ferguson et al. 2006) and Lupo (Grosse et al. 2006), have recessive mutations of Pstpip2 that are associated with multifocal osteomyelitis and osteonecrosis. Lesions in both models are more severe than those of human patients. Lupo mice also develop cutaneous manifestations, although the lesions are characterized not by pustulosis but by epithelial inflammation and thickening. The distinct differences between the human and mouse conditions indicate that the murine models only partially replicate the human diseases, indicating that further understanding of the SAPHO syndrome and CRMO must await additional genetic and epigenetic testing in human cases and the discovery of new animal models.

12.1.3 Summarized Points

1. Osteoimmune signals that control bone and joint integrity are mediated chiefly by signal molecules released by osteoblasts and (in disease) infiltrating leukocytes, primarily $CD4^+$ and $CD8^+$ T-cells, macrophages, and activated synoviocytes.
2. Key osteoimmune signaling molecules include RANKL (a pro-erosive factor) and many pro-inflammatory cytokines (especially IL-1β, IL-17, and TNF-α) and chemokines.

3. Immune-mediated arthritis may be divided into "inflammatory" and "non-inflammatory" variants (with rheumatoid arthritis [RA] and osteoarthritis [OA] as the main prototypic manifestations, respectively, of these variants).
4. Inflammation and cytokine production are more extensive in "inflammatory" arthritides due to the greater response by the acquired immune system (especially by $CD4^+$ T-cells).
5. The "non-inflammatory" arthritis OA is characterized more by dysregulated chondrocyte metabolism, leading to defective maintenance and repair, with relatively little inflammation mediated mainly by the innate immune system.
6. Immune-mediated myositis is a group of related conditions characterized by variable $CD8^+$ T-cell-mediated myofiber destruction, sometimes in association with immune complexes within the microvasculature.
7. Immune-mediated osteitis is a sterile auto-inflammatory response (i.e., due to an overactive innate immune reaction) rather than a classic autoimmune response (i.e., an attack by cells of the acquired immune system directed against "self" antigens).
8. Animal models of immune-mediated musculoskeletal diseases reproduce only a portion of the complex pathogeneses of the human diseases, so the proper choice of animal model is critical to obtaining useful information during basic translational medicine research and product development studies.

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Chapter 13
Immunopathology of the Endocrine System

Thomas J. Rosol and Brent E. Walling

Abstract It is well recognized that there is important cross-communication and inter-regulation that occurs between the endocrine, immune, and nervous systems during development and adult life. This chapter will specifically focus on interactions between the immune and endocrine systems related to (1) autoimmune diseases of the endocrine glands due to disruption of tolerance and development of adaptive immunity, (2) the effects of drugs and chemicals on induction of endocrine autoimmune diseases, (3) the newly recognized role of innate immunity in endocrine disorders, such as diabetes mellitus and obesity, (4) the effects of cytokines from immune cells on endocrine cells, and (5) effects of select hormones, such as glucocorticoids, prolactin, and parathyroid hormone on the immune system.

Keywords Immune-mediated • Autoimmunity • Endocrine • Pituitary • Thyroid • Parathyroid • Adrenal • Islets of Langerhans

13.1 Introduction

Immunoendocrinology is the branch of science that covers the interactions between the endocrine and immune systems as they relate to the maintenance of normal physiology as well as contribute to the manifestation of pathophysiologic conditions (Stelzer and Arck 2016). This chapter will focus principally on the endocrine diseases that are due to immune-mediated conditions, innate immunity, and cytokines produced by the immune system. It will also briefly cover a few of the classic endocrine hormones that directly affect the immune system and its cellular

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elements. Some of the earliest evidence of the interactions between the immune and endocrine systems involved the observation that stress was associated with adrenal cortex-mediated immunosuppression, even before the discovery of glucocorticoids (Selye 1955). After the discovery of the adrenal corticosteroids, it became evident that cortisol and the more potent synthetic corticosteroids were very effective at reducing inflammation and could be used to control autoimmune disorders. Synthetic corticosteroids are used widely today to treat various inflammatory and immune-mediated conditions and are an important part of immunosuppressive regimens. Unfortunately, chronic use of corticosteroids is associated with many side effects, and it has been particularly challenging to separate their anti-inflammatory actions from the many other effects they have on normal physiology.

Endocrinology has classically involved the investigation of cell signaling molecules that are secreted into the blood and regulate cells or tissues distant from the site of synthesis and secretion. However, endocrinology has expanded to the study of signaling molecules that have paracrine, autocrine, and even intracrine functions. The immune system and its cells, namely the lineages of lymphocytes and monocytes/macrophages, secrete or release a wide array of biologically active cytokines and chemokines that have both systemic and local (paracrine) effects. In addition, under certain conditions endocrine cells secrete the same cytokines. Therefore, there is a significant overlap of signaling molecules used by both the endocrine and immune systems, which play a role in both normal physiology and disease states. Certain drugs, including the cytokines and anti-cytokines used to treat various diseases, can induce or exacerbate subclinical autoimmune endocrine diseases.

Autoimmune endocrine disease results when there has been loss of self-tolerance due to inadequate suppression of lymphocytes resulting in the production of autoantibodies and cell-mediated immunity (Greenen and Chrousos 2004). The thyroid and adrenal glands are the most common endocrine organs to suffer from spontaneous autoimmunity and the clinical conditions are well characterized. More recently, the genetic susceptibility and pathogenesis of the various autoimmune endocrine disorders has been further clarified. This chapter emphasizes the genetic markers and susceptibility genes and their signaling pathways involved in the immune-mediated endocrine disorders. With the rise in the incidence of diabetes mellitus (DM) in the world, investigations have emphasized the role of the adaptive immune response in Type 1 DM and the unexpected role of the innate immune system in Type 2 DM. Infectious agents, particularly viruses and bacteria, also have a contributory role in the development of autoimmunity due to their ability to stimulate the immune system, interferon production, and, in some cases, cross-reacting antibodies. Therefore, even with co-evolution of the nervous, immune, and endocrine systems from the time of primitive fishes, the endocrine system of humans and laboratory animals remains susceptible to development of specific autoimmune conditions that affect their endocrine glands.

13.2 Autoimmune Diseases of Endocrine Glands

The incidence, onset, and severity of autoimmune diseases of the endocrine glands in an individual are influenced by genetics, sex, infectious agents, and environmental factors. Genetic factors include the genotype of the highly variable major histocompatibility complex (MHC), which encodes the class I and II MHC molecules and participates in the regulation and normal development of immune tolerance. In humans the MHC is called the HLA (human leukocyte antigen), the DLA (dog leukocyte antigen) in canines, and the H-2 in mice. Additional genes that are involved include those that regulate the immune system and tolerance. Some autoimmune diseases predominantly affect single organs and others are less specific and can affect a few or multiple organs.

Genetic susceptibility to developing autoimmune endocrine diseases has been established over 40 years of research. Examination of the familial history of patients with autoimmune endocrine diseases such as type 1 diabetes and autoimmune thyroid disease demonstrated a higher risk of developing disease among siblings compared to the general population. For instance, a genetic predisposition towards developing autoimmune thyroid disease (AITD) has been demonstrated by showing an increased presence of anti-thyroid antibodies in siblings of patients with AITD, clustering of AITD in families, and a higher risk ratio for developing AITD in monozygotic twins compared to dizygotic twins (Tomer et al. 2003). Much effort has been expended to identify genes that increase or decrease susceptibility to autoimmune endocrine diseases. Through genome screening, association studies, and linkage analysis, mutations and single nucleotide polymorphisms (SNPs) have been identified in multiple gene families. Results have demonstrated that most autoimmune endocrine diseases are polygenic and genetic susceptibility for some autoimmune endocrine diseases differs among ethnic groups. Unsurprisingly, the majority of genes associated with the development of autoimmune endocrine diseases are generally immune-regulatory genes including those involved in the development of central and peripheral tolerance. Reviews of genetic factors associated with autoimmune endocrine diseases have been recently published (Anderson 2008; Lee et al. 2015; Eisenbarth 2011; Husebye and Lovas 2009). In this chapter the primary genes associated with autoimmune endocrine diseases will be highlighted (Table 13.1).

13.2.1 Molecules and Signaling Pathways of Endocrine Autoimmunity

13.2.1.1 HLA Family

The primary determinant of genetic susceptibility to type 1 diabetes (T1D) and additional autoimmune endocrine diseases is localized in the HLA cluster of genes within the major histocompatibility complex (MHC) region on chromosome 6p21.

Table 13.1 Important susceptibility genes associated with autoimmune endocrine diseases and their function

Gene	Immune function	Associated autoimmune endocrine disease
HLA	Antigen presentation	T1D, GD, HT, CD, AAD, IAS, APS-2
CTLA-4	Immune checkpoint; downregulation of immune system	T1D, GD, HT, CD, AAD
PTPN22	Immunoregulation; T cell signaling	T1D, GD, HT, AAD
FOXP3	Maintenance of self-tolerance	T1D, GD, HT, IPEX
IL-2Rα (CD25)	Maintenance of self-tolerance	T1D, GD
INS	Blood glucose regulation	T1D
CD40	Co-stimulation	GD
TSHR	Signaling for thyrotropin synthesis	GD
FCRL3	B lymphocyte stimulation	GD, HT
PD-L1	Immune checkpoint; downregulation of immune system	GD, AAD
AIRE	Self-tolerance	APS-1
NALP1	Innate immune system stimulation	T1D, GD, HT, AAD

Table modified from Lee et al. (2015) and Husebye et al. (2009)
T1D type 1 diabetes mellitus; *GD* Graves' disease; *HT* Hashimoto's thyroiditis; *CD* celiac disease; *AAD* autoimmune Addison's disease; *IAS* insulin autoimmune syndrome; *APS-1,2* autoimmune polyendocrine syndrome 1 or 2; IPEX immune dysregulation, polyendocrinopathy, enteropathy, X-linked

Increased susceptibility or resistance to T1D has been observed, with structural alterations in the MHC class II molecules associated with susceptible or resistant haplotypes in humans and mice (Cucca et al. 2001). The HLA class II alleles, DQ, DR, and DP, are the most important haplotypes which can confer susceptibility or protection to T1D (Michels et al. 2011), although the contribution of individual genes has been difficult to identify due to strong linkage disequilibrium between the two loci (Pociot and McDermott 2002). These class II molecules are expressed on antigen-presenting cells (APC) and polymorphisms at these loci alter the structure of the peptide binding sites of the chains which make up the MHC class II molecules. Additional loci in the class II region, as well as loci in the class I and class IV regions, have been studied for their association with T1D susceptibility (Noble et al. 2010).

Polymorphisms in the HLA-DR3 allele have been linked to autoimmune thyroid disease (AITD). It has been hypothesized that polymorphisms of HLA-DR3 enable the presentation of thyroid peptides, thyroglobulin (Tg) in particular, to T cells which trigger AITD (Muixi et al. 2008). Similar to other autoimmune diseases, specific HLA alleles increase susceptibility to the development of Graves' disease (GD) while others are protective (Michels and Eisenbarth 2010). Polymorphisms in the thyroglobulin gene itself have been associated with AITD, including GD and Hashimoto's thyroiditis (HT), in humans and mice (Ban et al. 2003b) and there is strong evidence that interactions between specific polymorphisms of the HLA-DR3 gene and thyroglobulin gene increase the risk for developing AITD (Ban et al.

2003b; Hodge et al. 2006). One proposed mechanism is the aberrant expression of HLA class II molecules and presentation of thyroid autoantigens to T cells by thyroid epithelial cells (Hanafusa et al. 1983; Jacobson and Tomer 2007b). Polymorphisms in class I HLA genes also confer an independent risk in developing GD (Simmonds et al. 2007).

A role linking HLA haplotypes and genetic susceptibility to developing HT in humans has yet to be uncovered. A model of HT, the NOD $H2^{h4}$ mouse, can develop spontaneous thyroiditis similar to HT (Burek and Talor 2009). Transgenic MHC class II knockout mice on a NOD background with the human DR3 transgene were susceptible to developing pronounced experimental autoimmune thyroiditis induced by both mouse and human thyroglobulin but was only minimally effective in NOD $H2^{h4}$ mouse with the human DQ8 transgene (Flynn et al. 2007). This suggests a role of the MHC haplotype and susceptibility to HT.

Two specific HLA haplotypes, DR3-DQ2 and DR4-DQ8, have been positively associated with autoimmune Addison's disease (AAD) (Falorni et al. 2008; Yu et al. 1999; Myhre et al. 2002) while other haplotypes appear protective (Falorni 2011). These polymorphisms alter the amino acids that comprise the HLA molecules which, in turn, may alter peptide binding capabilities (Hammer et al. 1995). Consequently, this may influence the presentation of autoantigens to autoreactive T cells. A study by Bratland et al. (2009) demonstrated preferential binding of 21-hydroxylase (21-OH) peptides to an HLA haplotype associated with increased genetic susceptibility to AAD (Bratland et al. 2009). Additionally, carriers of an HLA class I allele, B8, had an increased risk of developing AAD (Baker et al. 2010) which binds a specific region of the 21-OH peptide and elicits an inflammatory response by CD8 T cells (Rottembourg et al. 2010). Polymorphisms of MHC class 1 chain-related a (MICA) have also been associated with AAD (Gambelunghe et al. 1999). MICA does not bind antigens but acts as a ligand for activating receptor (NKG2D) on gamma-delta T cells, natural killer cells, and activated cytotoxic T lymphocytes (Falorni 2011; Bratland and Husebye 2011). However, its role in AAD remains to be clarified. Polymorphisms of the class II transactivator gene (CIITA), which regulates expression of MHC class II molecules in APC cells including B cells, monocytes, and dendritic cells, is also associated with AAD (Ghaderi et al. 2006; Skinningsrud et al. 2008) independent of other HLA risk factors of AAD.

Lymphocytic hypophysitis (LYH), inflammation of the pituitary gland, is a rare condition and minimal data exists which link any genes with LYH. Recently, Heaney et al. (2015) identified two HLA haplotypes, DQ8 and DR53, present in patients with LYH at a higher frequency than the general population (Heaney et al. 2015). However, a causal role for the identified haplotypes and hypophysitis has not been determined.

Genetic susceptibility plays an important role in celiac disease (CD) and is found in individuals with HLA-DQ2 and HLA-DQ8 haplotypes (Diamanti et al. 2013), although polymorphisms in non-HLA genes involved in control of the immune response have also been linked to CD (Zhernakova et al. 2009). Many of the genetic loci associated with susceptibility for developing CD are also shared with other auto-immune diseases including HLA-DQ2 and HLA-DQ8, IL-2, and CTLA-4 (Smyth et al. 2008; Zhernakova et al. 2009).

13.2.1.2 CTLA-4/PTPN22

Two genes associated with regulation of activated T cells, cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and protein tyrosine phosphatase-22 (PTPN22), have been linked to multiple autoimmune endocrinopathies. PTPN22 encodes for lymphocyte tyrosine phosphatase (LYP) and has a role in regulating T cell signaling. A single mutation in the gene PTPN22 results in a gain of function mutation suppressing T cell receptor signaling (Bottini et al. 2004) and has been associated with autoimmune diseases including Graves' disease (GD), Hashimoto's thyroiditis (HT), and type 1 diabetes (Jacobson and Tomer 2007b). The reason is unclear but the prevailing hypothesis is this mutation permits autoreactive T cells to escape negative selection (Bottini et al. 2004; Vang et al. 2005). CTLA-4 is a transmembrane immunoregulatory protein expressed on the surface of activated T cells and is an important negative regulator of T cell activity. Polymorphisms in CTLA-4 have been associated with GD, HT, and other autoimmune diseases (Ban et al. 2003a; Jacobson and Tomer 2007a; Michels et al. 2011). Polymorphisms in either gene also have been associated with the development of T1D (Vang et al. 2005; Michels et al. 2011; van Belle et al. 2011) and modulate the risk of developing AAD (Blomhoff et al. 2004; Roycroft et al. 2009).

13.2.1.3 FOXP3/IL-2R/CD25

Forkhead box P3 (FOXP3) and interleukin-2 receptor (IL-2R), also known as CD25, are both involved in peripheral tolerance and are highly expressed in CD4+25+ regulatory T cells. Regulatory T cells have a vital role in inhibiting autoimmune disease (Shevach 2001). The growth and survival of regulatory T cells depend on IL-2 signaling through the IL-2 receptor and single nucleotide polymorphisms in the interleukin-2 receptor-α (IL-2Rα) gene have been associated with increased T1D susceptibility (Qu et al. 2007) likely through disruption of the IL-2 signaling pathway (Michels et al. 2011). Single mutations in the FOXP3 gene results in dysfunctional regulatory T cells (Bacchetta et al. 2006) and polymorphisms in either gene have been associated with AITD as well as additional autoimmune diseases, including IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked), which affects multiple endocrine organs (Lee et al. 2015).

13.2.1.4 IDDM2

The second most important genetic predisposition for T1D has been identified at the IDDM2 locus in the insulin gene (INS) region identified as VNTR (variable number of tandem repeats) polymorphisms. Susceptibility to T1D has been associated with proinsulin expression in the thymus and related to the number of tandem repeats in the promoter region of the insulin gene. Individuals homozygous for fewer repeats (VNTR I) were at the highest risk of developing T1D while individuals with high numbers of repeats (VNTR type III) were protected (Vafiadis et al. 1997). Higher number of repeats was associated with higher levels of INS mRNA in the thymus and it was

proposed that greater proinsulin expression in medullary thymic epithelial cells results in increased deletion of autoreactive T cells by negative selection (Pugliese et al. 1997).

13.2.1.5 CD40

Polymorphisms of genes involved in the activation of leukocytes have been associated with GD (Jacobson and Tomer 2007a). CD40 is expressed on antigen-presenting cells as well as different cell types including thyroid epithelial cells. Different CD40 SNPs have been associated with many immune-mediated diseases. One SNP of CD40 was shown to increase translational efficiency in B cells and increase synthesis of CD40 protein, which could lower the threshold of activation for B cells and enhance a proinflammatory response following T cell co-stimulation (Jacobson and Tomer 2007a; Lee et al. 2015). A second hypothetical mechanism involves increased CD40 by thyroid epithelial cells resulting in autoimmunity from bystander mechanisms (Lee et al. 2015).

13.2.1.6 TSHR

The thyrotropin receptor (TSHR) is expressed on the surface of thyroid epithelial cells and binding of thyroid stimulating hormone to TSHR stimulated iodide uptake and thyroid hormone synthesis and secretion. At least five TSHR polymorphisms have been recognized and studies identified polymorphisms associated with GD (Cuddihy et al. 1995). One SNP has been associated with decreased thymic expression of TSHR mRNA in the thymus, which may result in failure of the development of central tolerance for TSHR (Colobran et al. 2011). Furthermore, it has been shown that IFN-α interacts with a particular SNP of TSHR in the thymus and results in decreased TSHR mRNA, suggesting a role for cytokines contributing to decreased central tolerance and the development of GD (Stefan et al. 2014).

13.2.1.7 FCRL3

FCRL3 encodes for Fc receptor-like protein 3 which has a role in the NF-κB signaling pathway and may be involved in the innate and adaptive immune response (Wu et al. 2015). Polymorphisms in FCRL3 have been associated with AITD and Graves' ophthalmology (Kochi et al. 2005; Simmonds et al. 2006; Wu et al. 2015) but the underlying mechanism(s) have not been identified.

13.2.1.8 AIRE

Mutations in AIRE (autoimmune regulator) interferes with the thymic deletion of autoreactive T cells, leading to APS-1 (autoimmune polyendocrine syndrome type 1) (Bussone and Mouthon 2009). In both syndromes, T1D is a common manifestation. Polyendocrine syndromes are discussed separately in this chapter.

13.2.1.9 Others

The role of vitamin D as an immunomodulator has been recently reviewed (Szymczak and Pawliczak 2016) and polymorphisms of the vitamin D receptor (VDR) and CYP27B1; 25-hydroxyvitamin D 1-α hydroxylase) have been associated with AAD and other autoimmune endocrine diseases (Pani et al. 2002; Lopez et al. 2004). Genetic variants of programmed death ligand (PD-L1), a ligand for the programmed death molecule expressed on T cells with a role in downregulating T-cell responses and cytokine production, has been associated with increased risk of both AAD and Graves' disease (Mitchell et al. 2009). Finally, polymorphisms in NACHT leucine-rich-repeat protein 1 (NALP1), have been associated with an increased risk of developing AAD as well as other autoimmune diseases (Magitta et al. 2009), some of which may be associated with vitiligo (Jin et al. 2007). NALP1 is a member of the NOD-like receptors family that sense microbial peptides and stimulate the innate immune system. Therefore, a link between NALP1 and AAD demonstrate a role of the innate immune system in the development of autoimmune diseases.

13.2.2 Organ Involvement in Endocrine Autoimmunity

13.2.2.1 Pituitary Gland

Lymphocytic hypophysitis (LYH) is a rare inflammatory lesion of the pituitary gland with only about 500 cases being reported in the literature as of 2008 (De Bellis et al. 2011b), although the true prevalence of this disease may be underestimated (Bellastella et al. 2003). LYH has been further classified based on morphologic location (adenohypophysitis (LAH), infundibuloneurohypophysitis (LINH), panhypophysitis (LPH) (Caturegli et al. 2005; Tashiro et al. 2002); however, because of limitations on accurate sampling of the pituitary gland, these classifications may represent different disease entities or different facets of the same disease (Caturegli et al. 2008). Symptoms arise in patients related to the deficiency of one or more hormones. Decreased adrenocorticotropic hormone (ACTH) is most often observed in patients with LYH followed by luteinizing hormone (LH), follicle-stimulating hormone (FSH), growth hormone (GH), thyroid stimulating hormone (TSH), and prolactin (PRL) deficiency (De Bellis et al. 2011a). Hyperprolactinemia with increased PRL, suspected to be secondary to inflammation, has also been observed in LYH patients (De Bellis et al. 2011b). LAH is more common in women and has been associated with pregnancy while LINH affects men and women equally (Caturegli et al. 2005). Additionally, hypocortisolemia and hypothyroidism are more often associated with LAH while polydipsia-polyuria is observed most often with LINH (Caturegli et al. 2005).

A review of patients with LYH described the histologic appearance of LYH as a lymphocytic infiltrate composed predominantly of lymphocytes and plasma cells

that disrupts the normal architecture of the pituitary gland with minimal necrosis (Caturegli et al. 2005). Occasionally aggregates formed lymphoid follicles with germinal centers and fibrosis was common. Immunochemistry revealed a mixture of T and B lymphocytes. Mast cells also have been identified in patients with LYH (Vidal et al. 2002). Increased numbers of activated folliculo-stellate cells are present (Horvath and Kovacs 2002), and the folliculo-stellate cells may initiate the primary T cell response in LYH (Caturegli et al. 2008). Additional work has identified both CD4+ and CD8+ T lymphocytes in the pituitaries of LYH patients as well as small numbers of macrophages expressing MHC II molecules (Gutenberg et al. 2005). Additionally, aberrant expression of MHC II molecules and overexpression of MHC I molecules by pituitary cells were observed, suggesting CD8+ T-cell mediated cytotoxicity as an important facet of LYH. Immunohistochemical and immunofluorescence analysis of pituitary biopsies from two patients diagnosed with LYH identified an abundance of IL-17+ cells and absence of regulatory T cells in one patient with diffuse lymphocytic infiltrates and abundant regulatory T cells in the second patient with more organized lymphocyte structures (resembling germinal centers). It was hypothesized that an absence of regulatory T cells was important in developing genuine autoimmune LYH (Mirocha et al. 2009).

Multiple autoantibodies have been identified as potential markers for LYH, including anti-gonadotropin hormones (GH) (Takao et al. 2001), α-enolase (O'Dwyer et al. 2002), pituitary gland-specific factors 1a and 2 (Tanaka et al. 2002), and secretogranin II (Bensing et al. 2007), and more recently, chromosome 14 open reading frame 166 (C14orf166) and chorionic somatomammotrophin (CSH) (Lupi et al. 2008). The sequence identified by the antibody to CSH is also found in placental CSH and pituitary GH1. Given the association of LYH and pregnancy, one potential mechanism of LYH is anti-placental CSH antibodies recognizing the GH1 antigen by molecular mimicry (Lupi et al. 2008).

An anticancer monoclonal antibody (Ipilimumab) that targets CTLA-4 has been associated with inducing hypophysitis (Mahzari et al. 2015). This is one of a variety of immune-related adverse events (IRAEs) associated with checkpoint inhibitors (Michot et al. 2016). The mechanism of anti-CTLA-4 antibody induced hypophysitis is still under investigation but evidence implicates an autoimmune event where CLTA-4-mediated downregulation of T cell activation is inhibited followed by T cell destruction of the pituitary resulting in hypopituitarism (Torino et al. 2013). Patients receiving Ipilimumab and suspected hypophysitis also had abnormal hormone profiles including low cortisol with low ACTH, low free T_4 with low TSH, or low testosterone with low LH and/or low FSH, further implicating the pituitary gland as a target organ (Mahzari et al. 2015).

Iwama et al. (2014) were able to replicate early hypophysitis in SJL/J mice following injections of anti-murine CTLA-4 antibodies (Iwama et al. 2014). The pituitary gland from the treated SJL/J mice had a mononuclear infiltrate composed of both lymphocytes and macrophages. Additionally, the treated mice developed antibodies directed against the anterior pituitary gland, specifically towards PRL-secreting and ACTH-secreting cells. Furthermore, they demonstrated CTLA-4 expression in the pituitary glands of both mice and humans and this expression was

predominantly found in PRL- and TSH-secreting cells. They successfully demonstrated the deposition of complement factors C3 and C3d on PRL-secreting cells and C4d on PRL- and TSH-secreting cells following anti-CTLA-4 antibody injection in mice and proposed one mechanism of anti-CTLA-4 antibody hypophysitis as a type II hypersensitivity reaction mediated by complement activation (Iwama et al. 2014). Hypophysitis also has been diagnosed in patients receiving check-point inhibitors targeting PD-1 (e.g. tremelimumab), but with a lower incidence (Joshi et al. 2016).

A reliable mouse model of experimental LYH has been developed by Tzou et al. (2008) in which female SJL/J mice were immunized with mouse pituitary extracts and developed a severe lymphocytic infiltration of the anterior pituitary which mimics human LYH (Tzou et al. 2008). CD4+ T cells were the most prominent infiltrating cell type followed by CD8+ T cells. The disease followed a similar course to human LYH with inflammation and enlargement of the pituitary gland followed by atrophy and replacement with fibrous connective tissue and clinical hypopituitarism with decreased corticosterone and thyroxine levels. Subsequent work identified prohormone convertase 2 (PC2) as a possible pituitary autoantigen, although immunization with PC2 only produced a mild to moderate pituitary-specific infiltrate indicating that additional antigens contribute to experimental LYH (Tzou et al. 2008). Other animal models of LYH were have been historically inconsistent and have fallen into disuse.

13.2.2.2 Thyroid Gland

Autoimmune thyroid disease (AITD) comprise multiple clinical entities. Two of which, Graves' disease (GD) and Hashimoto's thyroiditis (HT), are some of the most commonly occurring autoimmune endocrine diseases. AITD begins with the development of antibodies against thyroid peroxidase (TPO), thyroglobulin (Tg), and thyroid stimulating hormone receptor (TSHR), which results in lymphoplasmacytic infiltration of the thyroid gland and clinical manifestations of hyperthyroidism with GD and hypothyroidism with HT.

Epidemiologic studies have implicated iodine as a potential environmental trigger in the development of HT and studies have demonstrated that high iodine intake accelerates the development of autoimmune thyroiditis in animal models of AITD (Rasooly et al. 1996; Mooij et al. 1993; Kolypetri et al. 2010) and iodine restriction reverted hypothyroid patients to normal (Kasagi et al. 2003). Several mechanisms have been proposed that implicate iodine itself or the iodination of thyroglobulin as the cause of AITD, which have been reviewed (Wang and Baker 2011). In addition, multiple environmental factors have been associated with the development of Graves' disease including iodine intake, stress, smoking, medications, and viral and bacterial infections (Morshed et al. 2011b). One interesting environmental factor is the hygiene hypothesis and its possible link to GD. The hygiene hypothesis suggests that an immune system exposed to different infections is better able to control autoimmune responses and improved living standards have increased the risk of developing

autoimmune diseases. Infection in a mouse model of GD induced with *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) suppressed GD development and was attributed to an immune response to the TSHR by lymphocytes with a Th1 phenotype (Nagayama et al. 2004). However, this contrasts with reports that implicate infectious agents in the pathogenesis of both GD and HT (Tozzoli et al. 2008).

Graves' disease begins with the development of autoantibodies to the thyroid stimulating hormone receptor (TSHR). TSHR is primarily expressed in the thyroid and undergoes complex post-translational processing, which may contribute to the antigenicity of the receptor (Morshed et al. 2011b). B cells appear to have a central role in GD. The extracellular domain of the TSRH molecule provides multiple epitopes which can bind autoantibodies and three types of antibodies are common in GD. Stimulating antibodies bind to TSRH and can induce thyrocyte activity similar to TSH. Blocking antibodies prevent TSH binding to the TSHR and can induce hypothyroidism but also may act as a week TSH agonist (Morshed et al. 2009). Neutral TSHR antibodies do not block TSH or induce T4 production (Morshed et al. 2011a). With the different biological activities of anti-TSHR antibodies, GD can be associated with hyperthyroidism or hypothyroidism. Antibody titers can fluctuate in Graves' disease and, in rare cases, alternating hyperthyroidism and hypothyroidism, referred to as Thyroid Yo-Yo syndrome, can be observed (Gillis et al. 1998). Multiple epitopes of the TSHR appear to stimulate T cells (Martin et al. 1997). Autoantibodies, in turn, bind to TSHR and can stimulate or inhibit thyroid hormone secretion and hyperthyroidism occurs when the balance of the autoantibodies favors thyroid cell activation (Michels and Eisenbarth 2010).

One syndrome associated with hyperthyroidism is Graves' ophthalmology where there is an increase in orbital fat and muscle volume. Histopathologic changes observed include an accumulation of granular material between muscle fibers, muscle edema, and lymphocytic infiltration of the orbital tissue (Bahn 2010; Michels and Eisenbarth 2010). In vitro cultured orbital fibroblasts express the TSHR mRNA and protein (Starkey et al. 2003; Bahn 2010) and the TSHR or a similar protein expressed by the orbital fibroblasts or adipocytes may be a target for anti-TSHR antibodies. A model of Graves' ophthalmology has been proposed by Bahn (2010) where anti-TSHR antibodies bind to orbital fibroblasts and induce differentiation and proliferation of fibroblasts to adipocytes with increased TSHR expression. The adipocytes secrete IL-6, which enhances B cell maturation and production of anti-TSHR antibodies by plasma cells. Simultaneously, cytokine production by infiltrating macrophages and lymphocytes stimulate production of prostaglandin E_2 and hyaluronan, which accumulates between the muscle fibers.

Hashimoto's thyroiditis (HT) is the most common endocrine autoimmune disease and is characterized by infiltration of the thyroid gland by T cells and plasma cells (Fig 13.1). One model proposes that the initiation of HT occurs in two immune-mediated stages (Wang and Baker 2011). The first stage is the presentation of antigen by antigen-presenting cells (APCs), which may be enhanced by iodine, toxins, or localized infections. The second stage is the interaction of lymphocytes with the antigen presenting APCs, breakdown of immune tolerance, and the activation of both CD4+ and CD8+ T cells (Hutchings et al. 1999) and B cells (Khan et al. 2015).

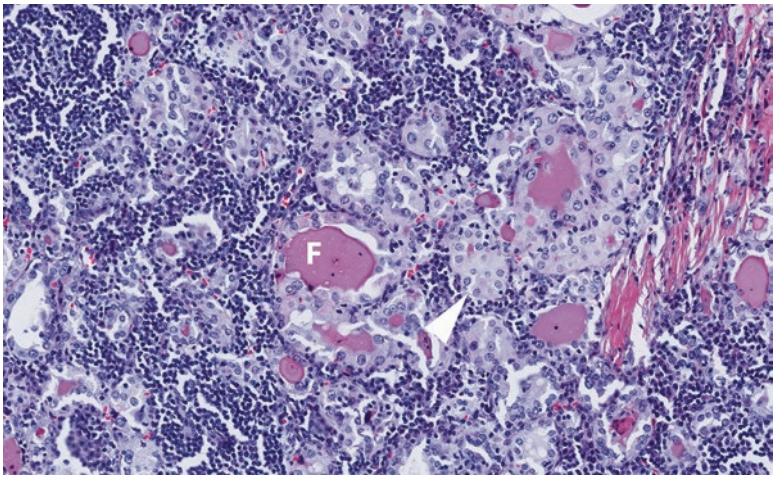

Fig. 13.1 Human thyroid gland with Hashimoto's thyroiditis. Notice the intense interstitial lymphoplasmacytic infiltrate with loss of follicles (F), destruction of follicles, penetration of follicles by the inflammatory cells, and sparing of C cells (*arrowhead*)

The destruction of the thyroid gland is mediated, in part, by cytotoxic T cells (Khan et al. 2015) while the presence of CD4+ T cells are required for development and maintenance of HT (Braley-Mullen et al. 1999; Braley-Mullen and Yu 2000) and have multiple roles including cytokine production and B-cell activation. B cells are required for activation or selection of autoreactive T cells (Wang and Baker 2011) and the absence of B cells inhibits initiation and maintenance of HT (Yu et al. 2008). The absence or dysfunction of CD4+CD25+ regulatory T cells has been associated with the development of many autoimmune diseases including HT (Wei et al. 2005). Wang and Baker (2011) proposed that defective regulatory T cells result in increased B-cell activity and increased expression of proapoptotic molecules in thyroid epithelial cells. Damage to thyroid follicular cells further enhances development of autoantibodies to thyroid peroxidase (TPO), thyroglobulin, and the TSHR (Michels and Eisenbarth 2010) and provides a positive feedback loop in HT.

13.2.2.3 Animal Models for Autoimmune Thyroid Disease

The first model of experimental autoimmune thyroiditis was in rabbits induced by injections of thyroid extracts with complete Freund's adjuvant (Rose and Witebsky 1956). Currently, the murine experimental autoimmune thyroiditis (EAT) model is favored by investigators. EAT can be induced in genetically susceptible mice with autologous or heterologous thyroglobulin (Tg) or thyroid peroxidase (TPO), in conjunction with complete Freund's adjuvant or lipopolysaccharide (Jacobson and Tomer 2007a). Genetic susceptibility varies between mouse strains and is related to MHC haplotypes and the antigen used (Tg vs. TPO) (Nagayama and Abiru 2011).

Spontaneous models of HT have also been established in the Obese Strain (OS) chicken, nonobese diabetic (NOD) mouse, NOD-H2^{h4} mouse, and a transgenic mouse model expressing human TPO-specific T cell receptor (Podolin et al. 1993; Quaratino et al. 2004; Nagayama and Abiru 2011). Additionally, spontaneous thyroiditis occurs in marmosets (Levy et al. 1972; Tucker 1984) and dogs (Rosol and Gröne 2015), and often results in hypothyroidism. In marmosets, immature or mature animals can be affected. Spontaneous thyroiditis occurs in older dogs, which limits its usefulness as an animal model in the research setting.

Spontaneous GD has not been reported in animals. Experimental Graves' disease has been induced in mouse and hamsters by injection with TSHR-expressing cells or vaccination with TSHR-DNA or TSHR-adenovirus (McLachlan et al. 2005). Spontaneous hyperthyroidism is common in cats and has been used as a model for toxic nodular goiter in humans (Peterson 2014). However, this model lacks circulating antibodies to the TSHR and is not useful for research on GD. Recently, an animal model of spontaneous generation of anti-TSHR antibodies was reported where a transgenic NOD-H2^{h4} mouse carrying the human TSHR A subunit (TSHR/NOD.H2^{h4}) developed anti-TSHR antibodies when drinking water was supplemented with sodium iodide (Rapoport et al. 2015). However, only a subset of females developed increased serum T_4 concentrations.

13.2.2.4 Adrenal Gland

Autoimmune Addison's disease (AAD), also known as primary adrenal insufficiency (PAI), is a clinical condition resulting from the destruction or impaired function of cells of the adrenal cortex that results in decreased production of glucocorticoids and in some cases mineralocorticoids and androgens (Falorni 2011). PAI is relatively uncommon compared to other autoimmune diseases of endocrine organs. Although there are multiple etiologies of PAI, autoimmune adrenalitis causes 80–90% of the cases of PAI in developed countries (Bratland and Husebye 2011). The timeline of AAD is similar to type 1 diabetes in which the onset of the development of autoantigens towards the adrenal cortex and cortical destruction occurs gradually, possibly months to years, prior to the development of clinical disease (Mitchell and Pearce 2012). The functional reserve of the adrenal cortex is considerable and the clinical effects of hormonal deficiency do not appear until 90% of functioning cortical cells have been destroyed (Rosenthal et al. 1978). AAD is an important spontaneous endocrine disease in older dogs and the disease may mimic the human pathogenesis (Rosol and Gröne 2015). AAD-associated loci were identified on *Canis familiaris* (CFA), chromosomes CFA12 and CFA 37, counter parts of loci in human genes HLA and CTLA-4, respectively (Husebye and Lovas 2009).

The mechanisms of autoimmune destruction of adrenocortical cells remains largely unknown but T cells likely play a major role (Freeman and Weetman 1992). Histopathologic findings of the adrenal gland from AAD patients demonstrate a diffuse infiltrate composed of lymphocytes, macrophages, and plasma cells (Bratland and Husebye 2011). Steroid 21-hydroxylase has been identified as the

major autoantigen in AAD (Bednarek et al. 1992) and autoantibodies have been present in more than 90% of recent onset patients (Michels and Eisenbarth 2010). The role of antibodies is unclear but most likely are involved in complement- or antibody-dependent cytotoxicity (Bratland and Husebye 2011). Additionally, IFN-γ production by peripheral blood mononuclear cells from AAD patients was enhanced following stimulation by steroid 21-hydroxylase in the presence of steroid 21-hydroxylase autoantibodies, suggesting that they act as a natural adjuvant (Bratland et al. 2009). At the end stage of the disease, the three hormone-producing cell layers of the adrenal cortex have been replaced by fibrous connective tissue.

Individual target tissues may play an active role in autoimmune disease since tissues targeted for autoimmune destruction can modulate inflammation (Hill et al. 2007). Adrenal cortical cells produce cytokines important in immune-endocrine cross-talk and AAD (Bornstein et al. 2004; Rotondi et al. 2005) and also express Toll-like receptors (Bornstein et al. 2004; Kanczkowski et al. 2009). Polyinosine–polycytidylic acid (poly (I:C) induces production of CXCL10 (elevated in ADD patients) by adrenocortical cells mediated through TLR-3 (Bratland et al. 2013). Type 1 and 3 interferon receptors are expressed in the adrenal cortex and interferons increase HLA class I expression (Hellesen et al. 2014). In addition, interferons can increase steroid 21-hydroxylase and with poly (I:C) increase CXCL10 production greater than poly (I:C) or IFN-γ alone. This suggests that viral infections, which induce IFN production, may be linked with the development of ADD, similar to the autoimmune conditions, T1D and AITD. Viral infections of the adrenal gland have been documented (Paolo and Nosanchuk 2006) and it has been suggested that a local IFN response may promote the development of AAD.

An animal model for spontaneous AAD does not currently exist. Experimental AAD can be induced using adrenal extracts in Freund's adjuvant in guinea pigs, rabbits, and rats to study autoimmune adrenalitis (Bratland and Husebye 2011). However, these models are do not effectively replicate human AAD. Spontaneous Addison's disease occurs sporadically in adult dogs, but it is relatively infrequent and may not be useful as a model in a research setting (Rosol and Gröne 2015). However, the genetics of AAD in dogs may be informative when it is elucidated.

13.2.2.5 Pancreas and Type 1 Diabetes Mellitus (T1D)

Type 1 diabetes (T1D) is a form of diabetes mellitus that results from destruction of the insulin-producing β cells in the islets of Langerhans of the pancreas. Onset of clinical T1D occurs when the functional mass of β cells, along with insulin production, has declined to the point where homeostatic mechanisms can no longer maintain euglycemia and symptoms of hyperglycemia appear. Although onset of clinical T1D is relatively rapid, it is a chronic disease with a presymtomatic phase (prediabetes) that may last from months to years (Lamb and Morris 2011). The destruction of β cells is immune-mediated in 70–90% of T1D patients, and is often referred to as Type 1A diabetes. Type 1B diabetes is an uncommon manifestation of T1D where the pathogenesis of β cell destruction is unclear and there is no evidence of β cell autoimmunity (American Diabetes 2014; Atkinson et al. 2014). Historically T1D

was attributed to autoreactive T cells evading negative selection in the thymus and infiltrating the islets ("insulitis"). The pathogenesis of T1D is complex and involves both the innate and adaptive arms of the immune system.

The genetic background of T1D patients and environmental triggers figure prominently in the mechanisms underlying T1D. Disease progression is heterogeneous and has resulted in different models of β cell loss. A most widely referenced model from 1986 describes a constant linear decline of β cell mass in genetically susceptible patients following an encounter with an environmental trigger (Eisenbarth 1986). However, others have proposed that the progression of β cell loss and disease is variable.

Relative genetic risk (HLA haplotype vs subtle mutations) impacts the requirement of an environmental trigger, and islet autoimmunity may be counterbalanced by β cell regeneration, resulting in a relapsing-remitting disease model (van Belle et al. 2011; Oram et al. 2014). In addition, CD8+ T cell exhaustion has been observed, which was associated with a favorable long-term outcome (McKinney et al. 2015). There is some evidence that β cells can regenerate in T1D patients (Willcox et al. 2010), which further supports a waxing-waning model of T1D. Both models are likely valid and depend on genetic background and the nature and frequency of environmental triggers, which influence the rate of β-cell destruction, along with individual health and response to therapy.

Extensive research into the identification of environmental risk factors or triggers for T1D has identified dietary, pathogenic, and other factors, especially in individuals with a genetic predisposition for T1D. Dietary factors include early exposure to cow's milk, timing of breast feeding and the introduction of cereals to infants, and the consumption of gluten or other wheat proteins as having potential roles in the development of T1D (Åkerblom and Knip 1998; Lamb et al. 2015). The role of wheat proteins, particularly gluten, is of interest in part to its role in the development of celiac disease. Some studies cast doubt on the role of cow's milk, but suggest omega-3 fatty acid and vitamin D may be protective in genetically susceptible individuals (Norris et al. 2007; van Belle et al. 2011).

Gastrointestinal microbiota also may have a role in the susceptibility and development of T1D. Roesch et al. (2009) correlated a difference in the relative abundance of gastrointestinal bacteria with the onset of diabetes in Biobreeding diabetes-resistant and Biobreeding diabetes-prone rats, finding a greater abundance of probiotic *Lactobacillus* bacteria present in the diabetes-resistant rats (Roesch et al. 2009). Probiotic administration, which includes the presence of lactobacilli, inhibited T1D in the NOD mouse (Calcinaro et al. 2005; Roesch et al. 2009). Wen et al. (2008) were able to attenuate T1D in MyD88-negative NOD mice by colonizing the gastrointestinal system with a defined human microbiota while germ-free MyD88-negative NOD mice developed T1D. This reinforced studies indicating an important role of the innate immune system and intestinal microbes contributing to developing T1D (Wen et al. 2008).

Viral infections are also suspected of contributing to the pathogenesis of T1D. Enterovirus, mumps, rubella, measles, chicken pox, parvovirus, cytomegalovirus, and rotavirus have been examined for their association with T1D (Devendra and Eisenbarth 2004; Lamb and Morris 2011) with enteroviruses and coxsackie

virus emerging as the strongest candidates (Hober and Sauter 2010). Mechanisms by which viral infections are involved in the pathogenesis have been reviewed and include viral infection of β cells which, in turn, may cause β cell lysis; breakdown of self-tolerance by expression of viral antigens, and altered β cell antigens, and/or MCH-antigens and cytokines; and induction of bystander activation of T cells (van der Werf et al. 2007). Viral protein expression can act as a superantigen resulting in activation and proliferation of autoreactive T cells directed at β cell antigens, viral disruption of the balance of activated and regulatory T cells to favor the presence of activator T cells, molecular mimicry, and repeat infections which may induce autoimmunity by activation and modification of T cells (Christen and von Herrath 2004; Merkler et al. 2006; van der Werf et al. 2007).

Islet autoimmunity (IA) with the presence of autoantibodies can be detected years before clinical T1D. There are four primary autoantibodies reactive to islet cell antigens, which include, insulinoma-associated antigen-2 (IA-2), (pro)insulin, glutamic acid decarboxylase 65 (GAD65), and zinc transporter 8 (ZnT8) (van Belle et al. 2011). The presence of anti-pancreatic antibodies demonstrates an early role for B cells in the pathogenesis of T1D (Pescovitz et al. 2009), which likely involves the development of anti-pancreatic antibodies by plasma cells and/or expression of antigens by B cells to T cells.

Beta cell destruction in T1D is thought to occur predominantly through autoreactive CD8+ T cells (Pinkse et al. 2005) and during early insulitis granzyme B and perforin secretion plays an initial role in β cell necrosis (Wilcox et al. 2016). Interestingly, the majority of CD8+ T cells in islets are not autoreactive to islet antigens, including IA-2, insulin, and GAD65 (Coppieters et al. 2012). Reasons for this include the presence of autoantigens which have yet to be identified, post-translational modification of known antigens (which can be enhanced by endoplasmic reticulum stress or environmental triggers), and the recruitment of bystander leukocytes by chemokines (Magnuson et al. 2015). Macrophages are also present and may contribute to β-cell death by the release of proinflammatory cytokines, which initiate apoptosis through tumor necrosis factor (Wilcox et al. 2016) or Fas-FasL activation by IL-1β (Cnop et al. 2005). During insulitis there is an upregulation of MHC class I molecules and IFN-α secretion by β cells (Bottazzo et al. 1985; Foulis et al. 1987), which supports a possible link to viral infections (Devendra and Eisenbarth 2004). IFN-α may contribute to β cell apoptosis via the JAK-STAT pathways (Wilcox et al. 2016). As insulitis progresses, an increase in B cells occurs in islets, and B cells are thought to be important in maintaining CD8+ T cell survival (Brodie et al. 2008) and low to modest antibody secretion (Willcox et al. 2009). CD4+ T cells were not a major component of the inflammatory infiltrate (Willcox et al. 2009). NK calls were also rare, since overexpression of MHC 1 antigens by β cells (Foulis et al. 1987) inhibits NK function (Vivier et al. 2004). Regulatory T cells were negligible. The immunological phases and β-cell mass over time has been described by van Belle et al. (2011), which integrates the anatomic sites, immune cell populations, key events, and cytokines/chemokines involved in the development of T1D (van Bergen et al. 2015; van Belle et al. 2011).

Evidence of a role of the innate immune system in the development of T1D comes from studies of pattern recognition receptors (PRR), which coordinate the innate inflammatory response to endogenous and exogenous stimuli. A recent review of the role of PRRs in T1D by Tai et al. (2016) showed that the majority of toll-like receptors (TLR), NRL proteins Nod1, Nod2, and NRLP3, and MyD88 were important in the pathogenesis of T1D. Knockout of individual TLRs and NRL proteins in diabetic-prone NOD mice or BioBreeding rats conferred protection from developing T1D (Tai et al. 2016). In particular, TRL7 and TRL9, as well as NRLP3 and MyD88, have critical roles in development of T1D in NOD mice (Wen et al. 2008; Hu et al. 2015; Tai et al. 2016; Zhang et al. 2010). Interference with PRR pathways in macrophages and dendritic cells inhibits activation of proinflammatory transcription factors, including NFκB and IRFs. In turn, production of inflammatory cytokines and type 1 interferon, which may stress β cells, is reduced. Secreted cytokines facilitate activation of naïve T cells. The role of gastrointestinal microbiota in T1D development appears related to the innate immune response and PRRs (Wen et al. 2008), but further research is needed to understand mechanisms underlying the influence of innate immunity and PRRs in T1D.

13.2.2.6 NOD Mouse and Type 1 Diabetes Mellitus

The non-obese diabetic (NOD) mouse is the most extensively used strain for studies of autoimmune disease and has greatly enhanced the understanding of the pathogenic mechanisms underlying T1D. NOD mice spontaneously develop insulitis and T1D characterized by T-cell destruction of β cells. Similar to human T1D, NOD T1D is associated with multiple genetic polymorphisms, including a unique sequence of the MCH class II allele I-A, which is homologous to DQ of humans (Zhang and Eisenbarth 2011). Additional polymorphisms associated with T1D in NOD mice include genes for β-2-microglobulin, IL-2 expression, and CTLA-4 (Zhang and Eisenbarth 2011). CD4+ and CD8+ T lymphocytes as well as B lymphocytes are essential in in the pathogenesis of T1D in NOD mice.

13.2.2.7 BioBreeding (BB) Rat and Type 1 Diabetes Mellitus

The BioBreeding (BB) rat is also commonly used for T1D research and develops spontaneous diabetes with a marked inflammatory infiltrate of β cells and, unlike NOD mice, will succumb rapidly to metabolic alterations secondary to T1D (Dalberg et al. 2011). Inflammatory cell infiltrates follow a predictable pattern with antigen-presenting cells (APCs), macrophages, and dendritic cells (DC) first arriving in islets followed by natural killer (NK) cells, CD4 and CD8 T cells, and then B cells (Hanenberg et al. 1989). The presence of APCs, macrophages, and DCs are essential for attracting T cells during early insulitis. Macrophages from BB rats produce high amounts of nitric oxide, which is thought to contribute to β-cell toxicity (Wu and Flynn 1993). Dendritic cells from BB rats are very responsive to IL-1

and GM-CSF, which increases DC activity and decreases IL-10 production (Dalberg et al. 2011). The functional differences of the macrophages and DCs in BB rats likely contribute to T1D. Additionally, the processes of developing tolerance to self-antigens in the thymus and periphery are impaired in BB rats. FoxP3 expression is reduced in CD4+CD25+ regulatory T cells, which behave as activated T cells (Dalberg et al. 2011). The roles of both NK cells and B cells are unclear in the pathogenesis of T1D in BB rats.

13.2.2.8 Insulin Autoimmune Syndrome

Insulin autoimmune syndrome (IAS), also known as Hirata's disease, is a rare autoimmune disease caused by autoantibodies which bind insulin/proinsulin and can also bind to the insulin receptor. Binding of antibodies to insulin/proinsulin results in spontaneous hypoglycemia and is the third leading cause of hypoglycemia in Japan (Uchigata and Hirata 2011). Similar to other endocrine autoimmune diseases, individuals with HLA-DR4 are genetically predisposed to develop IAS (Uchigata et al. 1995). Two potential major triggers for developing IAS have been identified: infection and medications. Viral infections, including coxsackie B, influenza, mumps, rubella, and hepatitis C, have been associated with an increase in endogenous insulin antibodies (Bodansky et al. 1986; Ruíz-Giardín et al. 2002) and are a risk factor for IAS. Medications include compounds containing sulfur/sulfhydryl groups (-SH) such as methimazole, captopril, D-penicillamine, hydralazine, glutathione, methionine, mercaptans, clopidogrel, aurothioglucose, imipenem, penicillin G and diltiazem as well as α-lipoic acid (Uchigata et al. 2009; Ismail 2016). One proposed mechanism of IAS involves the binding of insulin and proinsulin by antibodies. Persistent postprandial hyperglycemia results because bound insulin and proinsulin are not effective in lowering blood glucose. In turn, the pancreas continues to secrete insulin, proinsulin, and C-peptide until the levels of circulating insulin exceed antibody binding capacity and blood glucose returns to normal. Subsequently, bound insulin disassociates from the antibodies at a constant rate and is not under feedback control (Goldman et al. 1979). Under a high rate of disassociation, free insulin would continue to lower blood glucose and results in hypoglycemia (Goldman et al. 1979; Redmon and Nuttall 1999).

A second form of IAS involves antibodies to the insulin receptor (type-B insulin resistance). This is more common in women and has been associated with both hyperglycemia and/or hypoglycemia (Lupsa et al. 2009; Ismail 2016). Antibodies bound to the insulin receptor prevent degradation of insulin which leads to hyperinsulinemia and hypoglycemia (Lupsa et al. 2009). Hyperinsulinemia has been attributed to increased insulin secretion by the pancreas in response to insulin resistance and reduced insulin clearance (Ismail 2016). IAS has been observed in patients that developed antibodies to insulin, proinsulin, the insulin receptor, or a combination of the three, which results in a variety of clinical presentations and mimics other causes of hypoglycemia (Ismail 2016).

13.2.2.9 Parathyroid Gland

Autoimmune hypoparathyroidism (AH) is the second most common cause of acquired hypoparathyroidism following anterior neck surgery (Bilezikian et al. 2011). AH can occur as part of an autoimmune syndrome (e.g. APS-1) or as an isolated syndrome. Patients with AH have low levels of parathyroid hormone (PTH) along with hypocalcemia and hyperphosphatemia.

Identification of autoimmune antibodies specific for parathyroid tissue has been challenging. Anti-thyroid antibodies in patients with idiopathic hypoparathyroidism (IH) were first reported in 1966 (Blizzard et al. 1966), but subsequent studies indicated that the antibodies were not specific for parathyroid tissue (Kumar et al. 1996). Autoantibodies were identified that specifically targeted the extracellular domain of the calcium-sensing receptor (CaSR) in patients with AH (Li et al. 1996) and subsequent studies demonstrated anti-CaSR antibodies in patients with IH or APS-1 (Goswami et al. 2004; Gavalas et al. 2007). Calcium and phosphorus homeostasis is maintained by the minute-to-minute regulation of PTH secretion from parathyroid chief cells. High serum calcium levels bind to the CaSR and inhibit PTH secretion (Brown 2009). The role of anti-CaSR antibodies in AH is not fully understood. CaSR antibodies were identified in AH patients without destruction of the parathyroid glands (Kifor et al. 2004). It was suggested that the anti-CaSR antibodies inhibited PTH release by activating the receptor. Alternatively, hypoparathyroidism secondary to infiltration of the parathyroid glands by mononuclear cells and parathyroid atrophy has been observed in APS-1 patients (Betterle and Zanchetta 2003).

Autoimmune hypocalciuric hypercalcemia (AHH) is a condition where patients develop PTH-dependent hypercalcemia secondary to inactivating antibodies to CaSR (Kifor et al. 2003; Pallais et al. 2004). The mechanisms of CaSR-mediated inhibition of PTH secretion remain to be elucidated. However, one mechanism may be the inhibition of calcium/G_i-dependent ERK 1/2 phosphorylation which mediates calcium-dependent inhibition of PTH secretion (Makita et al. 2007).

There has been some indirect evidence in support of an autoimmune etiology for primary hyperparathyroidism, a counterpart to hypoparathyroidism. In patients with primary hyperparathyroidism circulating anti-parathyroid antibodies were identified and biopsy specimens showed induced MHC class II expression on parathyroid parenchymal cells in a subset of patients that had parathyroid adenomas or hyperplastic parathyroid glands (Bjerneroth et al. 1998). There was an absence of leukocytes in the biopsy specimens, indicating that MHC expression was not induced by inflammatory cytokines secondary to local inflammation. Antibodies directed towards parathyroid adenomas and CaSR have also been detected in a patient with hyperparathyroidism and autoimmune polyglandular syndrome (Pelletier-Morel et al. 2008), suggesting that in some cases primary hyperparathyroidism may be an autoimmune disease and CaSR could have a role in its pathogenesis.

13.2.2.10 Polyendocrine Syndromes

Autoimmune polyendocrine syndrome type I (APS-I), also referred to as type I polyglandular autoimmune (PGA) syndrome and autoimmune polyglandular syndrome type I, is a rare disease which primarily manifests itself in childhood, although adult cases of clinical APS-I occur. A clinical diagnosis is made when two of three primary components including mucocutaneous candidiasis, hypoparathyroidism, and Addison's disease are present (Huseby and Kämpe 2011; Betterle et al. 1998). Additional conditions are associated with APS-I including endocrine disease (gonadal insufficiency, T1D, autoimmune thyroid disease, hypophysitis), gastrointestinal disease (hepatitis, gastritis, malabsorption), and skin manifestations (alopecia, vitiligo), which have the alternative name APECED (autoimmune polyendocrine-candidiasis-ectodermal dystrophy), as well as ocular diseases (keratoconjunctivitis, cataract, iridocyclitis, retinal detachment, optic atrophy), enamel hypoplasia, anemia, vasculitis, asplenism, Sjögren's syndrome, and Turner's syndrome (Huseby and Kämpe 2011; Betterle et al. 1998; Ahonen et al. 1990).

The genetics of APS-I are relatively straightforward with over 95% of the cases attributed to polymorphisms in the autoimmune regulator (AIRE) gene (Aaltonen et al. 1997) and over 60 different polymorphisms reported (Huseby and Kämpe 2011). The AIRE gene encodes a transcription factor for expression and presentation of self-antigens in the thymus to developing T cells, which are important for developing central tolerance and preventing autoimmunity (Michels and Eisenbarth 2010; Anderson et al. 2002). A full discussion of the AIRE gene is discussed elsewhere in the Immunology chapter.

Autoimmune polyendocrine syndrome type 2 (APS-2), also called Schmidt's syndrome, is the most common polyendocrine syndrome in humans (Michels and Eisenbarth 2010). The definition of APS-2 differs slightly among contemporaries. One definition of APS-2 is Addison's disease with either autoimmune thyroid disease or T1D (Michels and Eisenbarth 2010), while an alternative definition is the occurrence of two or more organ-specific autoimmune diseases and not APS-1 (Barker 2011). APS-2 has been subdivided into APS-2 (Addison's disease and autoimmune thyroid disease or type 1 diabetes), APS-3 (autoimmune thyroid disease and another autoimmune disease – not Addison's diseases or type 1 diabetes), and APS-4 (any combination of two other organ-specific autoimmune diseases but not APS-1, 2, or 3) (Barker 2011; Betterle and Zanchetta 2003). Celiac disease is also relatively common in patients with APS-2 while other diseases including alopecia, vitiligo, pernicious anemia, myasthenia gravis, and stiff man syndrome have been associated with APS-2, but are uncommon (Michels and Eisenbarth 2010).

Unlike APS-I, APS-2 affects predominantly adults and may have a prolonged preclinical phase. Similar to other autoimmune diseases, APS-2 is a polygenic disease and many of the genes associated with increased risk of developing APS-2 also are associated with development of individual autoimmune endocrine diseases including T1D and Addison's disease.

The IPEX syndrome (immune dysfunction, polyendocrinopathy, enteropathy, X-linked) is rare, only occurs in males, usually presents in newborns and children

under 1 year of age, and results in multi-organ autoimmunity and recurrent infections (Bacchetta et al. 2011). IPEX is most often a result of mutations in the gene for transcription factor forkhead box p3 (FOXP3). FOXP3 is expressed in regulatory T cells and is crucial in their function of modulating immune responses to foreign antigens and preventing responses to self-antigens (Bacchetta et al. 2006; Sakaguchi et al. 2006). The scurfy mouse model of IPEX contains a mutation in the scurfin gene, homologous to human FOXP3, and develops a disease similar to IPEX with lymphocytic infiltrates of multiple organs (Brunkow et al. 2001). The mechanism identified was absence of immune regulatory activity by CD4+CD25+ T cells (Khattri et al. 2003).

POEMS (polyneuropathy, organomegaly, endocrinopathy, monoclonal gammopathy, and skin changes syndrome) is a rare multisystem paraneoplastic disorder with an underlying plasma cell dyscrasia that can manifest in a variety of clinical conditions. Endocrine manifestations are often observed in patients with POEMS and include gonadal failure, diabetes mellitus, hypothyroidism, hypoadrenocorticism, hyperprolactinemia, and hypoparathyroidism (Ji et al. 2012; Kumar and Sharma 2015). The mechanism underlying endocrine disorders seen in POEMS remains to be elucidated, but identification of IgG lambda antibodies from serum of a POEMS patient that bound to rat and dog pituitary glands suggested that the pituitary gland was the primary target (Reulecke et al. 1988).

13.2.2.11 Celiac Disease

Celiac disease (CD) is an immune-mediated disorder that results in T cell infiltration of the small intestinal mucosa. Individuals with CD have attenuated mucosal epithelium and flattened villi and suffer from a number of symptoms associated with impaired ability to properly digest and absorb nutrients. Although CD is not directly associated with the endocrine system, it is closely associated with endocrine autoimmune diseases including T1D and autoimmune thyroid disease.

Environmental factors have been associated with the development of CD and other autoimmune diseases. The timing of the introduction of gluten to infants and duration of breastfeeding is thought to influence the risk of developing CD. Patients with CD have an altered intestinal microbiota that may be related to HLA-DQ2 phenotype (Nadal et al. 2007; Olivares et al. 2015), The combined breastfeeding pattern and HLA-DQ phenotype could alter the risk of developing CD (Palma et al. 2012). The association of altered intestinal microbiota with CD (Marasco et al. 2016) is further supported by increased Toll-like receptor-9 expression and decreased Toll-like receptor-2 and Toll-interacting protein in gastrointestinal biopsies from CD patients (Kalliomäki et al. 2012). CD has been linked with certain infectious agents (Kagnoff et al. 1987). The mechanisms underlying the association of infections and autoimmune diseases include polyclonal activation of lymphocytes, increased immunogenicity of self-antigens, and inflammation mediated by antigen molecular mimicry (Diamanti et al. 2013; Bach 2005).

Both the adaptive and innate immune response contribute to the pathogenesis of CD. A potential mechanism which connects the adaptive and immune response has been described by Verdu et al (2015). When genetically susceptible individuals consume gluten, partially digested peptides, including gliadin, translocate across the epithelial barrier and transglutaminase deamidates the amino acid glutamine into glutamic acid, which fits well into pockets of the DQ2 and DQ8 molecules (Mereiles et al. 2011). Deamidated gliaden peptides are taken up by dendritic cells in the lamina propria and expressed by the MHC II molecules, inducing a proinflammatory response by gluten-sensitive CD4+ T lymphocytes with the production of IFN-γ and IL-21. IFN-γ is potentially cytotoxic to intestinal epithelial cells (Przemioslo et al. 1995), which promotes activation of intraepithelial cytotoxic T lymphocytes with subsequent epithelial cell destruction (du Pre and Sollid 2015). CD4+ T lymphocytes also stimulate anti-gliadin and anti-transglutaminase antibody production by B cells (du Pre and Sollid 2015; Verdu et al. 2015). Cooperation between CD4+ T cells and B cells in CD through binding and presentation of deamidated gluten by B cells to T cells results in both B cell differentiation into plasma cells and clonal expansion of T cells has been proposed as a mechanism of CD (du Pre and Sollid 2015). The innate immune response also contributes to CD with induction of IL-15 production by stressed epithelial cells. IFN-γ may induce IL-15 production by epithelial cells (van Bergen et al. 2015). IL-15 plays a role in the proliferation and activation of intraepithelial lymphocytes which, in turn, can kill epithelial cells, and IL-15 can inhibit regulatory T-cell function and induce proinflammatory dendritic cells (Verdu et al. 2015).

13.3 Drug-Induced Endocrine Autoimmunity (DIEA)

Drug-induced autoimmunity (DIA) is due to an immune-related reaction associated with continuous drug treatment (Xiao and Chang 2014). DIA often resolves after discontinuation of administration of the particular drug. Mechanisms include formation of autoantibodies, activation of T cells, inhibition of T suppressor cells, activation of leukocytes, direct organ damage, or changes in the conformation of proteins. Drug-induced endocrine autoimmunity (DIEA) occurs in genetically predisposed individuals or animals. Interferon-α (IFN-α) is the most common therapeutic agent associated with DIEA. Other drugs include interleukin-2 (IL-2), Ipilimumab (anti-cytotoxic T-lymphocyte-associated protein 4, CTLA4), granulocyte-macrophage-colony stimulating factor (GM-CSF), Campath-1H (Alemtuzumab; anti-CD52 monoclonal antibody), amiodarone, antiretroviral therapy (HAART), and sulfhydryl compounds (Prummel et al. 2004; Barker 2011). The thyroid gland is most often affected, but other endocrine organs such as the pituitary gland (hypophysitis with Ipilimumab) or islets of Langerhans (sulfhydryl compounds) can be affected by some drugs (Iwama et al. 2014).

13.3.1 Interferon-α

Interferon-α is often used to treat patients with Hepatitis C virus infection, various cancers, or hematological diseases. IFN-α therapy can result in interferon-induced thyroiditis (IIT) or diabetes mellitus in some genetically predisposed patients (Tomer et al. 2007; Jacobson and Tomer 2007b). IIT is either autoimmune or non-autoimmune. The autoimmune forms of IIT include Hashimoto's thyroiditis and Graves' disease. The nonautoimmune form of IIT is due to direct damage to the thyroid gland by IFN-α. IIT and Hashimoto's thyroiditis is associated with antithyroid antibodies, including antithyroperoxidase and antithyroglobulin. Antithyroid antibodies can remain in circulation even after discontinuation of IFN-α. Graves' disease is less common and is due to functional anti-thyroid stimulating hormone (TSH) receptor antibodies. If patients have antithyroid antibodies prior to the initiation of IFN-α therapy, they would be at increased risk for developing IIT. The non-autoimmune effects of IFN-α can include inhibition of the actions of TSH on thyrocytes, decreased iodine organification of thyroxine, and decreased thyroxine secretion (Mandac et al. 2006). Type I diabetes mellitus is an uncommon complication of IFN-α therapy and the pathogenesis is poorly understood, but may involve anti-islet antibodies (Nakamura et al. 2011).

13.3.2 Interleukin-2 (IL-2) and Autoimmune Thyroiditis

IL-2 is used to treat HIV and some forms of cancer such as malignant melanoma and renal carcinoma (Antony and Dudek 2010). IL-2 is a lymphokine that is involved in T- and B-cell proliferation and function, natural killer cell activity, and monocyte activation. Treatment with IL-2 can disrupt self-tolerance and lead to autoimmune thyroiditis, especially in patients with pre-treatment antithyroid antibodies (Kroemer et al. 1992). It is thought that IL-2 treatment allows for the recovery of previous deleted thyroid-specific T cells.

13.3.3 Anti-CTLA4 Antibody (Ipilimumab)

Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4; CD152) is an inducible receptor on helper T cells that functions as an immune checkpoint and "off" switch when bound to CD80 or CD86 on antigen presenting cells. Ipilimumab is a humanized monoclonal antibody to CTLA-4 used to treat cancer, such as melanoma. Some patients treated with Ipilimumab have a breakdown of tolerance to self-antigens and development of autoimmune diseases, such as colitis, hepatitis and less commonly hypophysitis (Weber 2007). Hypophysitis occurs is about 4% of treated patients. Repeated injection of Ipilimumab to SJL/J or C57BL/6J mice induced lymphocytic

hypophysitis and anti-pituitary antibodies due to expression of CTLA-4 on pituitary endocrine cells, particularly the luteotrophs and thyrotrophs (Iwama et al. 2014).

13.3.4 Amiodarone

Amiodarone is one of the most commonly used antiarrhythmic drugs in the world for treatment of both atrial and ventricular dysrhythmias, such as ventricular tachycardia. Amiodarone has a high iodine content and multiple effects on the thyroid gland including inhibition of thyroid hormone synthesis and iodine potentiation of pre-existing autoimmune thyroiditis (Rabinowe et al. 1986; Narayana et al. 2011).

13.3.5 Insulin Autoimmune Syndrome (IAS, Hirata's Syndrome) and Sulfhydryl Compounds

IAS was initially described in Japan in patients with anti-insulin antibodies, high serum insulin concentrations, hypoglycemia, and no history of prior treatment with insulin (Archambeaud-Mouveroux et al. 1989). The Japanese may have a genetic predisposition to this condition (Barker 2011). Sulfhydryl groups have a reducing effect, which may modify the structure of proteins and stimulate the induction of autoimmunity. Examples of drugs with sulfhydryl groups include methimazole, captopril, penicillamine, among others (Uchigata et al. 2009).

13.4 Innate Immunity in Endocrine Diseases

The innate immune system functions both in acute and chronic diseases and involves neutrophils and macrophages. Macrophages are a predominant cell type in chronic conditions and they are easily underestimated in histopathologic evaluations if immunohistochemical localization is not used (e.g., F4/80 immunostaining in mice). Macrophages have multiple subtypes, which are not easily discerned by their cellular morphology (Lavin and Merad 2013). Initially, macrophages were classified as type M1 and M2. M1 macrophages refer to the typical inflammatory macrophages that are activated by interferon-γ or lipopolysaccharide (LPS) and secrete abundant IL-12. M2 macrophages are involved in repair processes and secrete abundant IL-10, which is anti-inflammatory. It is thought that M2 macrophages play a role in the progression of cancer due to their anti-inflammatory and growth promoting properties. It is now recognized that this is an overly simplistic paradigm (Ostuni et al. 2015). It is likely that further subtypes will be elucidated. Therefore, the humoral and paracrine regulation and cellular function of the different subtypes of

macrophages cannot be evaluated in routine histopathological sections. Investigations on the subtypes and functions of the macrophage subtypes requires functional staining of tissue sections (immunohistochemistry and in situ hybridization for specific markers), flow cytometric or molecular analysis of isolated cells, or in vitro mechanistic investigations.

13.4.1 Type II Diabetes Mellitus

Type 2 diabetes mellitus (T2D) is a multifactorial disease that is associated with obesity and reduced physical activity. Mechanisms of islet β-cell failure include glucotoxicity, lipotoxicity associated with long-chain free fatty acids, oxidative stress, endoplasmic reticulum stress, and possibly amyloid deposition. More recently, it has become apparent that T2D is also an inflammatory disease with contributions by circulating inflammatory factors and inflammation (notably macrophages) in the islets of Langerhans (Donath and Shoelson 2011). Obese individual have chronic inflammation of adipose tissue, which is due to infiltration by lymphocytes and macrophages. Cytokines and chemokines, such as IL-1β and TNF, are released from the adipose tissue. In addition, stressed β-cells secrete IL-1β and CC- and CXC-chemokine ligands (CCL2 and 3 and CXCL8), which are responsible for the recruitment of macrophages to the islets. The activated macrophages also secrete IL-1β in the islets, which bind to IL-1β receptors on β-cells. Therefore, IL-1β originates from the adipose tissue and resident macrophages in the islets. Clinical studies have shown that treatment with anti-inflammatory drugs (nonsteroidal anti-inflammatory drugs, salsalate, IL-1β blockade, or IL-1β receptor inhibitors) have the potential to slow the progression of pre-diabetes or T2D and improve the treatment of T2D (Bellucci et al. 2016; Donath and Shoelson 2011).

13.4.2 Metabolic Syndrome

Metabolic syndrome is a collection of risk factors for cardiovascular disease (CVD), which include abdominal obesity, dyslipidemia, hyperglycemia, insulin resistance, and hypertension (Huang 2009), and most people with metabolic syndrome are at increased risk for developing type 2 diabetes (T2D) (Pop-Busui and Pietropaolo 2011). The National Cholesterol Education Program's Adult Treatment Panel III report (ATP III) identified a proinflammatory state as one of six components of metabolic syndrome related to CVD (Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III) Final Report 2002). Furthermore, there is evidence that systemic inflammation is linked to both T2D and metabolic syndrome and abdominal adipose tissue significantly contributes to chronic inflammation.

Obesity is associated with increased inflammation and oxidative stress. Excessive nutrient consumption results in expansion of adipose tissue primarily through hypertrophy which, in turn, activates endoplasmic reticulum and mitochondrial stress responses (Andersen et al. 2016). Subsequently, adipose tissue secretes a number of bioactive proteins including the proinflammatory cytokines TNFα, IL-6, IL-1β, and MCP-1, which contribute to the development of T2D (Kern et al. 1995; Pop-Busui and Pietropaolo 2011). TNFα has a significant role in T2D and inhibits insulin signaling at multiple points. TNFα inhibition of the tyrosine kinase activity of the insulin receptor promotes insulin resistance (Hotamisligil et al. 1996). In addition, TNFα can impair insulin secretion through nitric oxide (Kwon et al. 1999), block insulin-mediated cell signaling by impairing phosphorylation of the insulin receptor (Pickup 2004), and/or stimulate IL-6 production (Pakala et al. 1999), which promotes insulin resistance (Mohamed-Ali et al. 1997). Importantly, both endoplasmic reticulum stress, through IKKβ, and TNFα can activate NF-κB which results in production of proinflammatory cytokines. IL-1β also can impair insulin secretion by pancreatic β cells (Pop-Busui and Pietropaolo 2011). MCP-1 (CCL2) is expressed by adipocytes in proportion to adiposity (Weisberg et al. 2006) and contributes to insulin resistance (Kanda et al. 2006).

The proinflammatory cytokine, resistin, is produced by adipocytes and immune cells and is upregulated by proinflammtory cytokines. Resistin, in turn, promotes proinflammtory cytokine production through NF-κB (Pop-Busui and Pietropaolo 2011). Resistin, IL-6, and TNFα impair endothelial nitric oxide synthase function by endothelial cells leading to elevated cytotoxic peroxynitrite anion levels and endothelial dysfunction, which precedes atherosclerosis (Huang 2009). MCP-1 also recruits macrophages to infiltrate the adipocytes. Secretion of proinflammatory cytokines by adipocytes and macrophages results in adipocyte dysfunction and release of free fatty acids which, in turn, results in insulin resistance in skeletal muscle by disruption of metabolic functions and/or inhibition of insulin receptor signaling (Guilherme et al. 2008). Free fatty acids and lipids also accumulate in the liver and β cells of the pancreas and disrupt normal function (Andersen et al. 2016) and increase production of the atherogenic small dense low-density lipoproteins (Huang 2009). The aforementioned changes underlie the metabolic alterations observed with metabolic syndrome. Although originally the cause of metabolic syndrome was assumed to be adipose tissue alone, it is apparent that the secretion of cytokines, along with adipokines from adipose tissue and chronic inflammation trigger the alterations in metabolic parameters and cardiovascular function, which define metabolic syndrome.

13.5 Cytokines and Endocrine Organs

The endocrine organs and their parenchymal cells can be influenced by cytokines, which are produced by lymphocytes and monocytes and many other tissues, including endocrine organs. Although cytokines were initially identified in associated with

immune cells, they have a variety of functions depending on their location of production and secretion. The cytokines most often associated with alterations in endocrine function are interleukin-1 (IL-1), IL-2, IL-6, and tumor necrosis factor-α (TNF-α) (Spangelo 1997). The production of cytokines is inductive and usually not constitutive, and regulation of cytokine gene transcription is central to cytokine responsiveness. Circulating concentrations of cytokines are increased in patients with systemic illness or systemic inflammatory response syndrome (SIRS). In addition to cytokines, such as IL-1, IL-6 and TNF-α, procalcitonin is also an excellent diagnostic marker, because procalcitonin levels are very low in normal patients and markedly increased in patients with sepsis or SIRS (Jekarl et al. 2013). Cytokines in the circulation can affect endocrine tissues and cytokines can be produced in endocrine organs to have local (paracrine) effects. Cytokines are also important in the endocrine functions of nonclassic endocrine tissues, such as bone and muscle, but this is beyond the scope of this chapter. Endotoxin can increase IL-1, IL-6, and TNF-α production in the pituitary gland and adrenal cortex. This section also contains a summary of Euthyroid Sick Syndrome, which is a cytokine-induced syndrome of reduced circulating thyroid hormones in sick and critically ill patients.

13.5.1 Interleukin-1 (IL-1)

The interleukin-1 family consists of IL-1α, IL-1β, and IL-1 receptor antagonist (IL-1ra). IL-1α α and β are only 22% homologous at the amino acid level. IL-1 is not processed through the endoplasmic reticulum (ER) and activated macrophages contain IL-1 in the cytosol and cell surface. IL-1ra is processed and secreted through the ER and Golgi apparatus and binds to (but does not activate) IL-1 receptors with equal affinity to IL-1. There are two IL-1 receptors (Type I and II). The Type I receptor is on most cells and the Type II receptor is on neutrophils, B cells, and monocytes/macrophages. Only the type I receptor transduces a signal and the Type II receptor may be a decoy receptor. Signal transduction pathways include phospholipase C, sphingomyelinase, and Jak 1 and 3 pathways. IL-1 is pyrogenic. IL-1 can be produced by endocrine cells, including those in the hypothalamus, pituitary gland, β-cells of the islets of Langerhans, adrenal cortex and medulla, among other endocrine cells.

IL-1 activates the hypothalamic-pituitary-adrenal (HPA) axis (stress response) (Everds et al. 2013). IL-1β is produced by thyrotropes of the adenohypophysis. IL-1ra is constitutively expressed in the rat anterior pituitary gland, and IL-1 and IL-1ra receptors are present in the pituitary gland (Spangelo 1997; Spangelo et al. 1995). IL-1β decreased PTH secretion and mRNA synthesis in parathyroid chief cells (Toribio et al. 2003). There was a concomitant increase in expression of the calcium-sensing membrane receptor. It is possible that IL-1 plays a role in the hypocalcemia that occurs during endotoxemia or septicemia. IL-1 is produced in the zona fasiculata and reticularis of the human adrenal gland. The adrenal medullary chromaffin cells also contain IL-1, but not IL-1 mRNA, so it may originate from the

adrenal cortex and arrive in the medulla through the adrenal portal circulation. See the sections on Type 1 and 2 diabetes mellitus for information on the role of IL-1 in diabetes.

13.5.2 Interleukin-2 (IL-2)

IL-2 is a lymphokine (cytokine) produced by antigen-stimulated helper T cells and is a T-cell mitogen. The IL-2 receptor has α, β, and γ chains, and the α and β chains are shared by other cytokine receptors. Somatotrophs, lactotrophs, and corticotrophs in the anterior pituitary have IL-2 receptors and IL-2 may regulate pituitary cell mitosis in an autocrine or paracrine manner (Spangelo 1997). IL-2 infusion in renal cancer patients increased serum cortisol and beta-endorphin concentrations, decreased melatonin, and disrupted the normal circadian rhythms (Lissoni et al. 1997).

13.5.3 Interleukin-6 (IL-6)

IL-6 activates T cells and stimulates differentiation of B cells, including stimulation of antibody production. It has many other functions in the body, including as an endocrine cytokine (Papanicolaou and Vgontzas 2000). The IL-6 receptor is a trimer with an α-subunit and a homodimer of GP130. Signaling is triggered by formation of GP130 homodimers through the Stat3 and 5, Tyk1, and Jak 1 and 2 pathways. The folliculostellate cells of the rat pituitary gland contain IL-6, which may function as a paracrine hormone in the adenohypophysis. Human ACTH- and growth hormone-producing pituitary adenomas produce IL-6 and have IL-6 receptors (Spangelo 1997).

IL-6 has been immunohistochemically localized to the human adrenal cortex (all three zones), adrenal medulla, thyroid follicular cells, parathyroid chief cells, and islets of Langerhans, but not the C cells (Kontogeorgos et al. 2002). Human thyroid follicular cells produce IL-6 including those from patients with Graves' disease. IL-6 may be important in the pathogenesis of autoimmune thyroid disease. The rat FRTL-5 thyroid follicular cell line constitutively produces IL-6, which was increased by IL-1 (Iwamoto et al. 1991).

13.5.4 Tumor Necrosis Factor-α (TNF-α)

TNF-α was first identified as cachectin and induces fever, an acute-phase response, and weight loss in severe clinical illness and cancer. TNF-α signal transduction involves phospholipase C and sphingomyelinase. TNF-α has been localized to

endocrine cells of the hypothalamus, pituitary, adrenal, and thyroid. Endotoxin can increase the production and secretion of TNF-α by endocrine cells (Spangelo 1997). Systemic administration of human TNF-α in rats increased plasma glucagon, corticosterone, and norepinephrine concentrations (Darling et al. 1989).

13.5.5 Euthyroid Sick Syndrome (ESS) or Nonthyroidal Illness Syndrome (NTIS)

The euthyroid sick syndrome (ESS) is a condition of abnormal thyroid circulating hormone concentrations in a patient with nonthyroidal systemic illness and normal thyroid gland function. The magnitude of the circulating thyroid hormone perturbations are proportional to the severity of the systemic illness. The most striking changes are decreased triiodothyronine (T_3) and increased reverse T_3 concentrations in the blood. Total thyroxine (T_4) and free T_4 concentrations may also be decreased in severe conditions. Thyroid stimulating hormones (TSH) concentrations will be in the high range of normal or minimally increased in severe conditions. Upon recovery the circulating thyroid hormone concentrations return to normal. The condition may result from changes in thyroid hormone synthesis and secretion, regulation of the hypothalamic-pituitary-thyroid axis, or peripheral thyroid hormone metabolism. However, the patients are not considered to have hypothyroidism. Lower concentrations of thyroid hormones are thought to have an energy-sparing role in sick individuals.

Increased circulating cytokines are thought to play a role in the pathogenesis of ESS, particularly IL-1, IL-6, TNF-α, and interferon-γ. Cytokines can reduce the production of thyroid-releasing hormone (TRH) from the hypothalamus, thyroid stimulating hormone (TSH) from the pituitary gland, and thyroid hormone (T_4 and T_3) synthesis by the thyroid gland (Bartalena et al. 1998; de Vries et al. 2015). Cytokines have direct and indirect effects on thyroid follicular cells by inhibiting the uptake of iodide by the sodium iodide symporter, reducing transcription of thyroglobulin, inhibiting thyroperoxidase and iodination of thyroid hormones, and reduced deiodination of T_4 to form the active thyroid hormone, T_3. There is also reduced deiodination of T_4 to T_3 in the peripheral tissues due to inhibition of the Type 1 deiodinase enzyme, particularly in the liver. The Type 2 deiodinase enzyme is responsible for local tissue production of T_3 from T_4, and the Type 2 deiodinase is downregulated by T_3. During illness, the Type 2 deiodinase in the hypothalamus is activated, which increases local T_3 concentrations, and downregulates TRH (and subsequent TSH) production. The Type 3 deiodinase is a major T_4 inactivating enzyme, especially in the liver. Even though the Type 3 deiodinase (D3) is increased during illness, it is not thought to play a role in ESS because D3 knockout mice have a similar reduction in thyroid hormones compared to control mice during systemic inflammation (Boelen et al. 2009).

13.6 Effects of Hormones on the Immune System

13.6.1 Glucocorticoids

Glucocorticoids were discovered in the 1940s and the natural and synthetic glucocorticoids (with increased potency) are commonly used to suppress inflammation and are part of immunosuppressive treatment protocols (Coutinho and Chapman 2011). Unfortunately long term treatment with glucocorticoids leads to significant side effects including osteoporosis, muscle wasting, hypertension, dyslipidemia, Type 2 diabetes mellitus, glaucoma, cataracts, among others. Development of compounds that separate the anti-inflammatory actions of glucocorticoids from the metabolic actions have not been very successful (Quax et al. 2011). Many of the biological effects of glucocorticoids are mediated by the classic mineralocorticoid and glucocorticoid cytoplasmic receptors that translocate to the nucleus. Glucocorticoids can increase or decrease the numbers or activity of these receptors depending on the tissue and physiological condition, but in general chronic stress causes down-regulation (Zhe et al. 2008). Glucocorticoids can also signal through nongenomic processes, and this may represent new targets for therapy or immunosuppression (Schoneveld et al. 2011; Lowenberg et al. 2007).

The effects of glucocorticoids on inflammatory cells include increased peripheral neutrophil counts and bone marrow myeloid cells, decreased peripheral lymphocytes and promotion of lymphocyte apoptosis, and decreased peripheral eosinophil counts (Everds et al. 2013). Glucocorticoids reduce the number and function of both T and B cells. Proliferation is decreased and apoptosis is increased. The production of many cytokines is suppressed, including IL-2 and interferon-γ. Phagocytosis is suppressed in neutrophils and macrophages. Inflammation is reduced because increased annexin-1 (lipocortin-1) production suppresses phospholipase A2 with subsequent decreased eicosanoid production and decreased leukocyte adhesion, chemotaxis and phagocytosis. Thymic atrophy will occur, especially in the young.

The interactions of stress and the hypothalamic-pituitary-adrenal (HPA) axis on the immune and endocrine systems has recently been thoroughly reviewed (Everds et al. 2013). During acute and chronic stress there is activation of the hypothalamic-pituitary-adrenal (HPA) axis, which causes an increase in circulating glucocorticoids (principally cortisol in hamsters, dogs, cats, and primates and corticosterone in birds, rodents, and rabbits (Rosol et al. 2001)). Rabbits are unusual in that they have significant amounts of both cortisol and corticosterone. During stress, rabbits tend to shift synthesis towards cortisol. Glucocorticoid concentrations increase tenfold or more in life-threatening stress and by three- to fivefold in more moderate stress. Mild chronic stress may only increase trough concentrations, while mean peak concentrations may remain in the normal range. Under normal conditions, glucocorticoids are secreted intermittently in a pulsatile manner, approximately once per hour. In addition, glucocorticoids exhibit a circadian pattern with peak concentrations at times of peak activity (e.g., for rodents during the first few hours

of the dark period) and trough concentrations at times of reduced activity (e.g., for rodents about the first 6 h of the light period). Circadian rhythms of glucocorticoids in diurnal (i.e., non-nocturnal) animals are the opposite of those of nocturnal animals. Stress during the dark period induces less ACTH and corticosterone response in rats compared to stress during the light phase (Retana-Marquez et al. 2003). Circadian secretory patterns are reduced or lost in pregnant or lactating rats.

13.6.2 Prolactin

Prolactin (luteotropin) is best known as the hormone produced from the anterior pituitary luteotrophs that regulates mammary gland development and lactation. However, prolactin has over 300 functions and the cell membrane receptors are widespread in the body. Prolactin has important roles in the immune system and is produced as a local cytokine by macrophages, NK cells, and T and B cells (Díaz et al. 2013). Lymphocyte prolactin expression is controlled by an alternate promoter, which is stimulated by cAMP, retinoic acid, and calcitriol, and inhibited by glucocorticoids and some interleukins (such as IL-2 IL-1β, and IL-4). Prolactin increases the weight of the spleen and thymus in rats. Prolactin has an immunomodulatory role, but development of the immune system was unaffected in mice after genetic knockout of prolactin or its receptor (Dorshkind and Horseman 2000). Prolactin receptor knockout mice had reduced T-cell proliferation and IL-2 production in response to PHA. Prolactin can also inhibit T-cell apoptosis.

Prolactin may play an important role in the pathology of autoimmunity (Díaz et al. 2013); however, its role is poorly understood and somewhat controversial. Autoimmune diseases are more common in females, and both estrogen and prolactin modify the immune phenotype. Hyperprolactinemia can break B-cell tolerance (Saha et al. 2009) and has an autostimulatory effect by increasing the production of immunoglobulin, cytokines, and autoantibodies. Prolactin induces monocyte maturation to dendritic cells, T-cell activation, and production of pro-inflammatory cytokines. These mechanisms appear to allow hyperprolactinemia to promote autoimmune endocrine diseases, such as Type 1 diabetes mellitus and Hashimoto's thyroiditis.

13.6.3 Parathyroid Hormone

Parathyroid hormone (PTH) is an 84-amino acid peptide produced by chief cells of the parathyroid gland. Its principle function is to regulate serum calcium concentration through actions on the kidney, bone, and intestines. PTH receptors also are present on neutrophils and B and T cells (Yamamoto et al. 1988; McCauley et al. 1992). PTH has a potential role as an immunomodulator; however, experiments have shown inconsistent effects (Geara et al. 2010). Secondary hyperparathyroidism with high serum PTH concentrations occurs frequently in patients with chronic

renal disease and recombinant PTH 1-34 (teriparatide) is used to treat osteoporosis in the elderly by daily injections. Evidence suggests that PTH functions as one of the uremic toxins (Rodriguez and Lorenzo 2009). Lymphocytes from patients with uremia often have functional defects, which can be reversed by parathyroidectomy, but it is not clear whether the functional defects were due directly to PTH. Parathyroid hormone-related protein (which binds to PTH receptors with equal affinity to PTH) can be produced by normal, neoplastic, or activated lymphocytes and macrophages, which can function as an autocrine or paracrine growth inhibitor for lymphocytes (Adachi et al. 1990).

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Chapter 14
Ocular Immunopathology

Meg Ferrell Ramos, Leandro Teixeira, Curtis R. Brandt, and Diana Auyeung-Kim

Abstract The eye has traditionally been regarded as an immune privileged site because inflammatory responses that would otherwise be detrimental to vision are modulated in the ocular environment. Numerous immunosuppressive mechanisms exist in the eye to accomplish this but it is clear that this status can be "broken" such as during infections and in the development of uveitis. Components of the innate immune system that sense pathogen components are present in ocular cells and activation results in the production of pro-inflammatory cytokines. Dendritic cells, which are critical for presenting antigen to the adaptive immune system are also present in the cornea, iris, and retina and can bind and process antigens and then traffic to the local draining lymph node or spleen resulting in antibody production, the generation of pathologic T-cells, or immunosuppressive regulatory T-cells. Innate and adaptive responses may similarly occur in response to the administration of ocular biologic drugs and viral gene delivery vectors, resulting in pathology that is immune-mediated and confounding drug development. This chapter reviews our current understanding of ocular immunology and disease mechanisms, known immunological pathology associated with drug development, comparative anatomical features of the eye, and regulatory aspects specific to ocular drug development and nonclinical testing strategies.

Keywords PAMP receptors • Dendritic cells • Immunosuppression • ACAID • Uveitis • CD4+ cells • CD8+ cells • Th-17 cells • RPE • Ocular drug delivery • Ocular pathology • Ocular immunopathology • Ocular anatomy • Ocular drug development

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14.1 Introduction

The eye is an exquisitely complex organ comprised of multiple, specialized tissue components arranged in a manner that enables the conversion of light energy into electrical nerve impulses that ultimately translate into visual perception. Despite the preciseness of the anatomical construct and the seeming sensitivity of the neural components—the retina and optic nerve—the eye is remarkably resilient to disease or injury, attributable to a number of factors, i.e., the tough outer structural components that minimize internal injury (sclera and cornea); vascular characteristics that promote ocular homeostasis and immune privilege (blood-aqueous and blood-retinal barriers); and additional features (lack of intraocular lymphatics, the presence of intraocular immune modulators and cells, and the exclusion of others) that modulate inflammation that may otherwise be tissue-destroying. The later is particularly relevant to a specialized organ such as the eye, composed largely of tissues incapable of regeneration, and with the exception of occurring as a pair, lack functional redundancy. Conversely these attributes may contribute to ocular pathology from exposure to molecules (chemical or biologics) irrespective of administration route (ocular or systemic, belying the vulnerability of the blood barriers to systemically administered drugs), and more recently, from ocular-specific drug delivery systems, whether biological, mechanical, or chemical. Sometimes these occur in unpredictable ways that confound drug development strategies.

Ocular assessment in drug development has historically focused on potential toxicity due to chemical injury, generally associated with an inflammatory response that is proximal to drug administration; hypersensitivity, developed over time to drug administration, such as that which can occur with topical administration of some glaucoma drugs; or pharmacology, which may result without an anatomical correlate detectable by in-vivo assessment or routine histological analysis. With pharmacology toxicity, pathologies with an immune component are generally secondary inflammatory reactions to anatomical changes. Ocular toxicity observed during preclinical development of small molecules is often predictive of clinical occurrences, and findings are used to guide clinical development and manage patient risk, although it's also well known that association of adverse events of low frequency to the administration of a specific drug may follow years of post-marketing experience. Selection of a relevant species is important, not only for pharmacological activity, but also for the assessment of potential toxicity in metabolites; non-human primates may not always be the most relevant animal model, and despite anatomical differences, alternate species such as the rabbit or dog may be more suitable for conducting safety studies.

More recently, the development of biopharmaceuticals to treat a spectrum of ocular diseases, and in particular the associated need for intraocular delivery of such treatments, has demonstrated a need for better understanding of the physiology and immune regulatory pathways that are unique to the eye. Both innate and adaptive arms of the immune system can be activated with the administration of biologics into the eye. Needle insertion alone can induce transitory changes consistent with activation of the innate system. Attention to formulation details that minimize tissue injury, and avoiding progression of events arising from innate activation, may be important in avoiding

systemic exposure of 'novel' eye proteins that may subsequently become immunogenic. With respect to the biologic administered, immunogenicity is dependent on a number of factors including structural properties of the molecule, glycosolation, post-translational modification, epitopes, aggregation; to host factors, including species, genetics, and state of health; and to route of administration and administrative interval (Caspi 2014; Ponce et al. 2009). Important considerations to the resolution of an immunological response involving the eye include the compartment and thus the matrix (resolution of cellular infiltrates in the vitreous tends to be prolonged) in which the immune reaction is occurring, the tissues that are targeted as part of the immune response, and the secondary immune response of resident ocular cells, including the elaboration of immune mediators and other cytokines. As biologics have become increasingly more humanized, immunogenicity in patients has diminished. However, immunogenicity persists in animals used for non-clinical development due to cross-species differences in protein sequence, and findings related to immunogenicity are generally not considered predictive of human clinical experience (Ponce et al. 2009). Non-human primates have presumed similarity in proteins, but should not be the default species for safety studies. For intra-ocular delivery, however, they often are the most relevant species, and despite similarities in proteins, immunological pathology does occur and can confound interpretation of pathology findings. Assessment of ocular status is critical during the conduct of a safety study to sorting out the differences between formulation, immunological or pharmacological drug-related findings. Histological evaluation and interpretation should take into account in-vivo findings and timing of occurrence to drug administration, PK, and ADA data for proper risk assessment.

This chapter reviews the anatomical and immunological features of the eye, assessment of the eye in toxicology studies, known immunological pathology associated with drug development, and regulatory aspects specific to ocular drug development and nonclinical testing strategies. Although this chapter is focused on ocular immunopathology, it should be kept in mind that the eye is particularly susceptible to pharmacological toxicity that result in functional change that often lack a histological correlate, and/or to degenerative changes that are devastatingly irreversible that may remarkably lack an obvious immunological component. Although beyond the scope of this chapter, the reader is referred to a number of reviews that detail these findings (Fraunfelder 2015; Penedones et al. 2015; Izazola-Conde et al. 2011); *Drug-Induced Ocular Side Effects* (Fraunfelder 2015) is particularly comprehensive, and includes ocular and systemic drugs.

14.2 Ocular Immunology

In order to function properly the visual axis must remain clear and able to transmit light without distortion. Inflammatory responses that result in protein secretion and infiltration of immune cells pose a significant threat to vision that is not a concern for non-ocular sites such as the skin. As a result the eye has specific mechanisms to

down-regulate inflammation. The eye has been thought to have a specialized immune system for some time. In 1873, van Dooremaals reported the proliferation of heterologous tumor cells after intracameral injection (Van Dooremaal 1873). At that time, very little was known about the immune system including the concept of graft rejection. In 1948, Medawar published similar results and coined the term "immune privilege" to describe the ocular environment (Medawar 1948). This was attributed to a lack of a lymphatic system in the eye (Streilein 1990). Since the initial observations much has been learned about the immune system in the eye, including many of the mechanisms behind immune privilege. However, it is clear that immune privilege can be "broken" as evidenced by severe immunopathological responses to ocular pathogens that lead to blindness and non-infectious uveitis in patients (de Smet and Chan 2001). We now know that many components of innate, antibody-mediated, and cell-mediated immunity are present in the eye and that they can be activated and override the immunosuppressive mechanisms.

14.2.1 Innate Immune Systems

It has been increasingly recognized that several innate mechanisms can prevent infections and signal to the rest of the system that an infection is present. This subsequently results in activation of adaptive immune responses and in fact, innate immune effector functions play a role in modulating the adaptive response (Owen et al. 2013a). The cornea is constantly exposed to potential immunological threats and has several defense mechanisms to prevent infection. These include the secretion of proteins, such as lysozyme, lactoferrin, and lactoferracin B in tears (McDermott 2013; Pearlman et al. 2013). Mucins on the corneal epithelium also prevent infection by interfering with attachment to cells. In addition, small antimicrobial peptides such as α- and β-defensins and LL-37 (cathelecidin) are present in tears and are synthesized by corneal epithelial cells. Some defensins are constituitively expressed while others are up-regulated following infection (McDermott 2013; Kolar and McDermott 2011; Hazlett and Wu 2011). Several peptides have been studies for their potential therapeutic use (Silva et al. 2013; Brandt 2014) although none are approved clinically.

Several innate recognition systems that can sense the presence of pathogens are present in cells including ocular cells (Fig. 14.1). Stimulating substances are referred to as Pathogen Associated Molecular Patterns (PAMPS) and chemically can be proteins, cell wall components of both bacteria and fungi, or several types of nucleic acids. Several receptor systems are present in cells. The best studied are the Toll-Like Receptors (TLRs) for which humans express ten (Pearlman et al. 2008; Mai et al. 2014; Chang et al. 2006; Lambiase et al. 2011; Redfern and McDermott 2010). Mice express additional TLRs but the systems are very analogous in their function. Several TLRs are expressed on the cell surface (Fig. 14.1) and predominantly recognize microbial proteins (eg. TLR2) or cell wall components including bacterial endotoxin (TLR4). Four TLRs, 3, 7, 8, and 9 are found in endocytic vesicles in cells and they recognize various types of nucleic acids including uncapped RNA, various

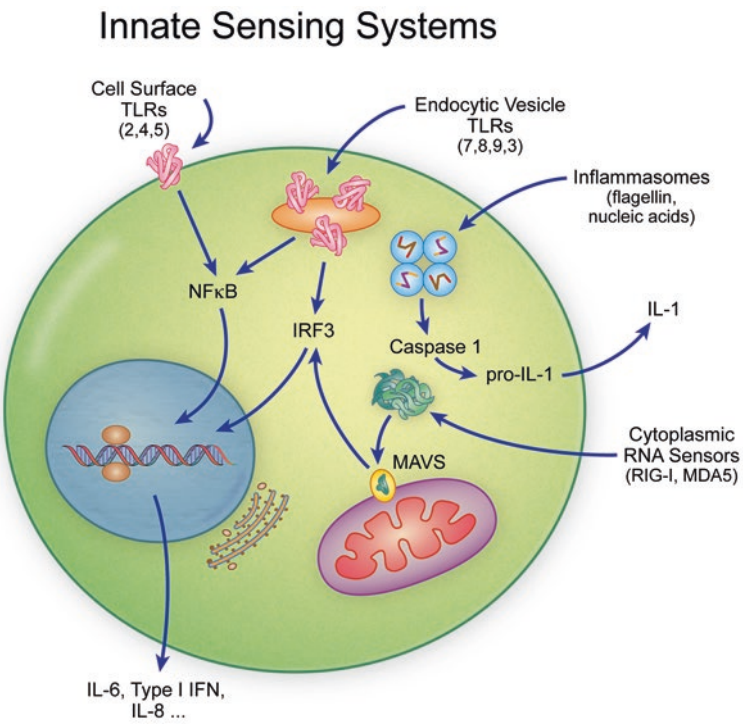

Fig. 14.1 Schematic diagram of the Toll-like receptor (TLR), inflammasome, and cytoplasmic RNA innate sensing systems. TLR proteins are located either at the cell surface or in endocytic vesicles and sense proteins, fungal and bacterial cell wall components, or nucleic acids associated with pathogens. They then signal through the adapter protein MyD88 (all TLRs except TLR3) to activate NFκB. TLR3 signals through Interferon Regulatory Factor 3 (IRF3) to induce synthesis of Type I interferons. Inflammasomes, located in the cytoplasm sense similar pathogen associated molecules resulting in the activation of Caspase 1 with subsequent cleavage and release of IL-1 and IL-18. The cytoplasmic sensors, RIG-I and MDA5, sense various types of pathogen associated RNA and interact with Mitochondrial Antiviral Signaling Protein (MAVS) to activate IRF3 resulting in IFN induction and secretion

single and double stranded RNAs (TLR 3, 7, and 8) or unmethylated CpG motif (TLR9) containing DNA molecules (Desmet and Ishii 2012). It is important to note that the TLRs can recognize these PAMPs whether they are associated with an infection or are present as contaminants in biological drugs. Biological drugs can be manufactured in *E. coli* or yeast and endotoxin or cell wall contaminants can trigger inflammatory responses through the TLRs. Plasmid DNA prepared in *E. coli* can also be contaminated with endotoxin.

Most of the TLRs use a common signaling pathway. Dimerization of the TLR upon ligand binding results in binding of the TLR to a cytoplasmic protein called Myd88. This results in recruitment of IRAK kinases to the TLR/MyD88 complex and activation of TRAF6, which polyubiquitinates itself and TAK1. The polyubiquitinated proteins then bind to IKK-β which is degraded. This releases NFκB which translocates to

the nucleus where it activates expression of inflammatory cytokines as well as other genes (Kawai and Akira 2010). This system is present in several ocular cell types (Kumar and Shamsuddin 2012; Lambiase et al. 2011). TLR3, which recognizes double stranded RNA, uses a different signaling mechanism. The adaptor in this case is TRIF which activates two kinases, TBK1 and RIPK1 which then phosphorylates Interferon regulatory factor 3 (IRF3) resulting in translocation of IRF3 into the nucleus where it primarily activates type I interferon (Kawai and Akira 2010).

Another nucleic acid sensing system consists of two proteins containing "dead box" helicase motifs, RIG-I and MDA5 (Yoneyama et al. 2004; Jensen and Thomsen 2012; Reikine et al. 2014; Desmet and Ishii 2012). These are cytoplasmic proteins that bind long or short double stranded RNAs or RNAs with a 5' triphosphate. After interaction with their ligands, RIG-I and MDA5 bind to a protein, MAVS, located on the surface of mitochondria, which results in the phosphorylation of IRF3 by TBK1. Phosphorylated IRF3 then translocates to the nucleus where it activates type I interferon genes. Activation of a second kinase, IKK, results in NFκB translocation to the nucleus and induction of pro-inflammatory as well as other immune genes (Reikine et al. 2014; Yoneyama et al. 2004; Jensen and Thomsen 2012).

Inflammasomes constitute another innate sensing system located in the cytoplasm (Vanaja et al. 2015; Rathinam et al. 2012; Franchi et al. 2012). Inflammasomes are a complex of several proteins, including caspase 1, PYCARD and NALP (the ligand binding component) and several forms are present in ocular cells each of which recognize different ligands. Upon ligand binding, the complexes form resulting in the activation of caspase 1. Caspase 1 then cleaves pro-IL1 and pro-IL18 resulting in their release from the cells. Both IL-1 and IL-18 are pro-inflammatory and chemotactic for neutrophils. Retinal pigment epithelial cells constituitively express pro-caspase 1 and pro-IL18 and inflammasome responses to drusen products may play a role in Age Related Macular Degeneration (Gao et al. 2015). Mutations in inflammasome components are involved in periodic fever syndromes as well (Henao-Mejia et al. 2012).

Multiple cell types in the eye express various components of these innate sensing systems and in the presence of inflammatory stimuli, will up-regulate expression of these proteins (Kumar and Yu 2006; Kumar et al. 2004; Kleinman and Ambati 2012; Campbell and Doyle 2013; Ueta et al. 2011). However, a complete cataloging of the ocular cells that express these innate receptor systems is not yet available. Glial cells in the retina, both macroglia (Müeller cells) and microglia (astrocytes), are important components of the innate response in the retina, express a number of innate immune receptors, and respond by secreting cytokines that modulate downstream immune functions as well as up-regulate host defenses against microbes. They also appear to have a role in pathological responses and non-infectious disease (Karlsetter et al. 2015; Kumar and Shamsuddin 2012).

14.2.1.1 Effector Functions Resulting from Innate Sensing

The release of pro-inflammatory cytokines and interferon by ocular cells results in several downstream responses (Kijlstra 1994; Torres and Kijlstra 2001). First, cells exposed to these cytokines up-regulate the expression of a number of different genes

including additional cytokines that amplify the signals. These cells also up-regulate matrix metaloproteases that cause tissue damage and can result in the activation of other proteins such as TGF-β. Interferon also up-regulates the antiviral response in non-infected cells. The increased expression of cytokines sets up concentration gradients resulting in chemotaxis of immune cells into ocular tissues which normally don't have large numbers of these cells. In many ocular inflammatory diseases neutrophils are the predominant early infiltrating cell type. Upon infiltration neutrophils begin releasing mediators such as reactive oxygen species and additional cytokines that cause tissue damage and further amplify responses. Many cytokines released by resident cells and infiltrating neutrophils are chemotactic for T-cells that infiltrate tissues.

Another important downstream effect is the role played by innate signaling responses in regulating the adaptive immune response in the eye (Ueta and Kinoshita 2012; Perez et al. 2013). Although we don't completely understand dendritic cell functioning in ocular tissues, we do know that innate effector mechanisms have several effects on dendritic cells and the type of signals seen by the dendritic cells can alter the adaptive immune response (Forrester et al. 2010; Barabino et al. 2012). For example in the cornea, resident dendritic cells have recently been identified (Barabino et al. 2012). In addition, immune stimuli in the cornea result in the infiltration of Langerhans Cells from the corneal limbus (Knickelbein et al. 2014). Exposure of dendritic cells to cytokines present after activation of the innate sensing pathways has several effects. First, the dendritic cells can migrate towards areas where antigens are present. Dendritic cells are activated upon exposure to these cytokines and become capable of antigen processing and presentation. In particular, histocompatibility antigens are up-regulated in the DC as are antigen processing systems as well as co-stimulatory molecules. The activated DC pick up antigens and then traffic to the local draining lymph nodes where they present the antigens to T-cells and B-cells (Kaufman 2013). Depending on the nature of the antigen either the exogenous (Class II pathway) or endogenous (Class I) antigen presenting pathways can be operative. B-cells can also become antigen presenting cells through the endogenous pathway in the presence of the appropriate cytokines. The activated T-cells then chemotax back to the tissue where they exert their effector functions. Depending on the exact signals they receive, dendritic cells can promote different types of adaptive responses.

14.2.2 Adaptive Immune Responses

By definition, adaptive immunity is the generation of an immune response directed towards a specific target, referred to as an epitope. Most epitopes are composed of peptides or proteins although carbohydrates and certain lipid structures can be targeted. Because the majority of "ocular biological drugs" in use are proteins, they have the potential to serve as targets for adaptive immunity. Viral gene delivery vectors also have protein components and these can serve as targets. Adaptive immunity is divided into humoral immunity which is mediated by antibody molecules and cellular immunity, which is mediated by various types of T-cells (Owen et al. 2013c). The innate and adaptive systems are constantly communicating with each other.

Antigen presentation is absolutely dependent on the presence of "MHC" molecules on the surface of antigen presenting cells (APC), which bind the target structures and allow for the adaptive system to "see" the antigen (Davey and Rosenbaum 2000; Hansen and Bouvier 2009). In mice, these proteins are referred to as the Major Histocompatibility Complex (MHC) and in humans it is referred to as the Human Leukocyte Antigen (HLA) system. There are two systems, endogenous and exogenous (see below). In addition, in order to properly activate an adaptive response the cells must see a second signal that is referred to as co-stimulation. These are separate receptor-ligand interactions from the MHC interactions. T-cells that do not receive simultaneous MHC and co-stimulatory signals either undergo apoptosis and die or are "tolerized" and unable to respond to the presence of the target epitope.

To generate an adaptive immune response an antigen must be presented to the system and this is a significant point of interaction between innate and adaptive immunity (Owen et al. 2013b). As noted above, cytokines released from cells that have been stimulated through TLRs, RIG-I, MDA5, and other sensing systems activate resident dendritic cells in ocular tissues. Interferon is one of the most important of these. The dendritic cells respond by up-regulating the expression of several proteins that are needed to present antigens. These include proteins involved in binding, uptake, and proteolytic processing of antigens. Activation also increases the migratory capacity of the APCs. Once an APC has been activated and acquires antigen it traffics to the local draining lymph node or the spleen. In the lymph node and spleen the APCs encounter T-cells or B-cells and they present antigen to these cells resulting in the activation of specific responses.

In the eye, the dendritic cells ($Cd11c^+$) are located in the conjunctiva, the cornea itself, and in the retina. Additional APCs (Langerhans cells) migrate into the central cornea from the limbus in response to a threat such as an infection (Fig. 14.2). There are significantly fewer dendritic cells ($Cd11c^+$) in anterior chamber tissues and in the neural retina but they are present in sufficient numbers to trigger responses (Lehmann et al. 2010; Heuss et al. 2012; McPherson et al. 2014; Streilein 2003). It should be noted that once a response is initiated there is an amplification loop that results in migration of additional APCs into the site and increased inflammation so responses may not require large numbers of cells for initiation. Local APC also play important roles in promoting tolerizing responses depending on the circumstances of their activation (Streilein 2003; Caspi 2006; Biros 2008; McPherson et al. 2014; Heuss et al. 2012). Several cell types in the eye will upregulate MHC expression upon exposure to cytokines such as IFN allowing them to be recognized by cytolytic $Cd8^+$ T-cells. Another potential route for the generation of an adaptive response to a biological or gene delivery vector is leakage from the eye and eventual appearance in a local draining lymph node or the spleen via the circulation. Currently it is not clear whether this is a significant route.

The endogenous mechanism for antigen presentation involves Class I MHC molecules. In this pathway, antigens are expressed in the cell, are then cleaved into peptides that then traffic to the endoplasmic reticulum (ER). In the ER, they bind to the Class I MHC molecule in a peptide binding cleft and then traffic through the secretory pathway to emerge on the cell surface. This process also occurs in target cells such as those infected with a virus. The exogenous pathway does not require

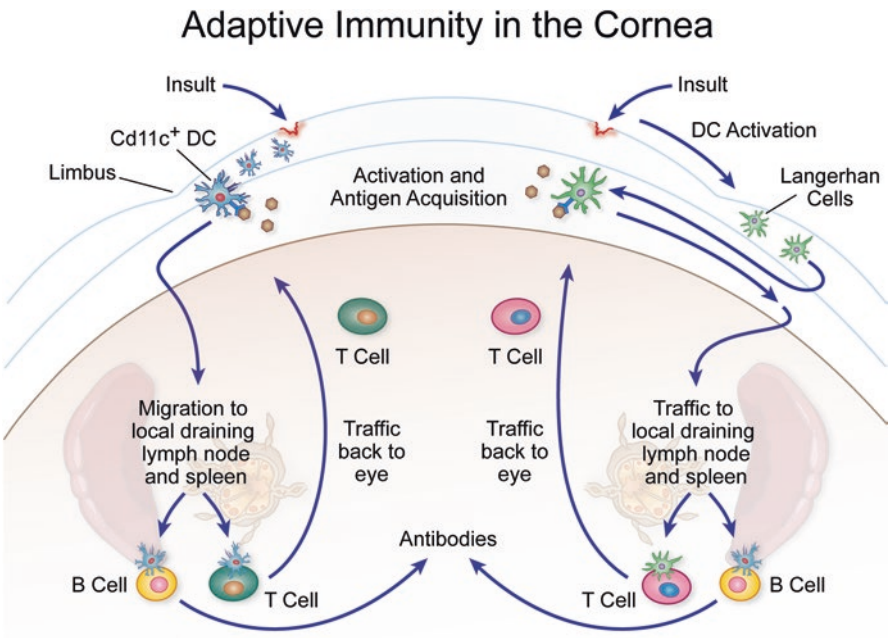

Fig. 14.2 Adaptive immunity in the cornea. Two types of dendritic cells participate in generating adaptive responses in the cornea. Resident Cd11c$^+$ cells are activated and become antigen presenting cells (APC) after exposure to cytokines such as Interferon. These cells acquire and process antigen while trafficking to either the local draining lymph node or spleen where they present antigen to T-cells or B-cells, in the presence of Cd4$^+$ helper T-cells. Langerhans cells reside at the corneal limbus and upon detecting an insult, they begin differentiation into APCs and migrate into the cornea where they acquire antigen. The Langerhans cells then migrate to the local draining lymph node or spleen and present antigen to T and B-cells like the Cd11c$^+$ cells. The T-cells, which can have a CD8$^+$, Th17, or Cd4$^+$ delayed type hypersensitivity phenotype, then traffic back to the eye where they carry out their effector functions. The B-cells differentiate into plasma cells and secrete antibodies in the circulation which can then cross a damaged blood-ocular barrier into the eye. Some B-cells have been shown to migrate into the cornea in certain inflammatory conditions but their significance is not clear

expression of the antigen in the presenting cell. For this pathway, antigens bind to the cell surface, and are endocytosed. The endocytic vesicles then fuse with lysosomes and the antigen is proteolytically cleaved into peptides. The endo-lysosome then fuses with vesicles carrying the Class II MHC molecules, the peptide is loaded onto the Class II protein, and then is exported to the cell surface.

Ocular biologicals, which are usually humanized monoclonal antibodies, are not expressed in cells and thus, the exogenous antigen presentation pathway is how these are recognized. Ocular biologics tend to trigger antibody-mediated responses. There are two potential ways that an ocular biologic protein might be presented. First, these are used in diseased eyes where there may be disruptions in the blood-ocular barrier. In this circumstance the biologic might leak from the eye and travel to the spleen. Second, as discussed below, intravitreal injection of the biologic could result in endogenous APCs in the retina taking up the antigen and then trafficking to

the local draining lymph node or the spleen. Viral gene delivery vectors are similar in that most cells don't encode viral proteins so vector particles are taken up and presented through the exogenous pathway. An exception for viral vectors can result if the transgene expressed in the target cell can be recognized as foreign, in which case, the endogenous pathway would be used.

Optimal adaptive responses also require T-cell help. Helper T-cells, which express the CD4 protein, interact with APCs and the eventual effector cell to form a complex. The APCs and helper T-cells secrete cytokines that bind to the effector cell enhancing the response. In addition, the particular set of cytokines secreted by the APCs and CD4+ T-cells can modulate the response. For example, the response can result in the generation of CD8+ cytolytic T-cells, Th17 T-cells, delayed type hypersensitivity T-cells ($T_{DTH)}$ or regulatory T-cells ($T_{reg;}$; T-cells that actively suppress immune responses—see below). For the generation of antibody responses, depending on the cytokine milieu one can generate IgE or various isotypes of IgG antibodies that have different effector functions. In terms of inflammatory reactions in the eye $CD8^+$ cells, T_{DTH}, and Th17 cells have all been shown to be capable of causing pathology.

For the ocular surface, resident dendritic cells or Langerhans cells that migrate from the corneal limbus, are activated and acquire antigen (Fig. 14.2). These cells then traffic to the local draining lymph nodes or spleen where they interact with T-cells resulting in antigen specific activation. The activated T-cells (both CD4+ and CD8+) can then migrate back to the cornea to carry out their effector functions leading to corneal clouding. The dendritic cells also interact with B-cells and CD4+ helper T-cells resulting in conversion of the B-cells into plasma cells that secrete antigen specific antibodies. Inflammatory processes loosen the blood ocular barrier allowing antibodies access to the eye. Corneal neo-vascularization also helps increase access of T-cells and antibodies to corneal and conjunctival tissues.

In the retina, small numbers of resident APCs reside in the ganglion cell layer and are involved in both activating and suppressive adaptive responses depending on the circumstances (Fig. 14.3). In retinas that are considered quiescent and have not received some insult, they promote immunosuppressive responses (Heuss et al. 2012; Silver et al. 2015). In retinas that have received some injury, they promote immunopathological responses (Fig. 14.3). For example, exudative lipid products in drusen or oxidative stress are pro-inflammatory and their presence can lead APCs to present antigen in a way that generates a pathological response including cytokine secretion and increased expression of MHC antigen (Ambati et al. 2013; Nussenblatt et al. 2014; Gao et al. 2015).

Both humoral and cellular adaptive responses play roles in various ocular pathologies and in suppressing pathological responses. Antibody responses seem to predominate for ocular biologicals and also play a role in infectious disease responses (Meek et al. 2003; de Smet and Chan 2001). For example antibodies to HSV-1 glycoprotein K (gK) may be involved in the pathogenesis of herpetic keratitis in humans (Ghiasi et al. 1997). Regulatory T cells play critical roles in dampening pathological immune responses (Silver et al. 2015; Mochizuki et al. 2013) whereas $CD8^+$ cytolytic and $CD4^+$ delayed type hypersensitivity cells and Th17 T-cells play pathological roles in uveitis and keratitis (Caspi 2010; Rowe et al. 2013).

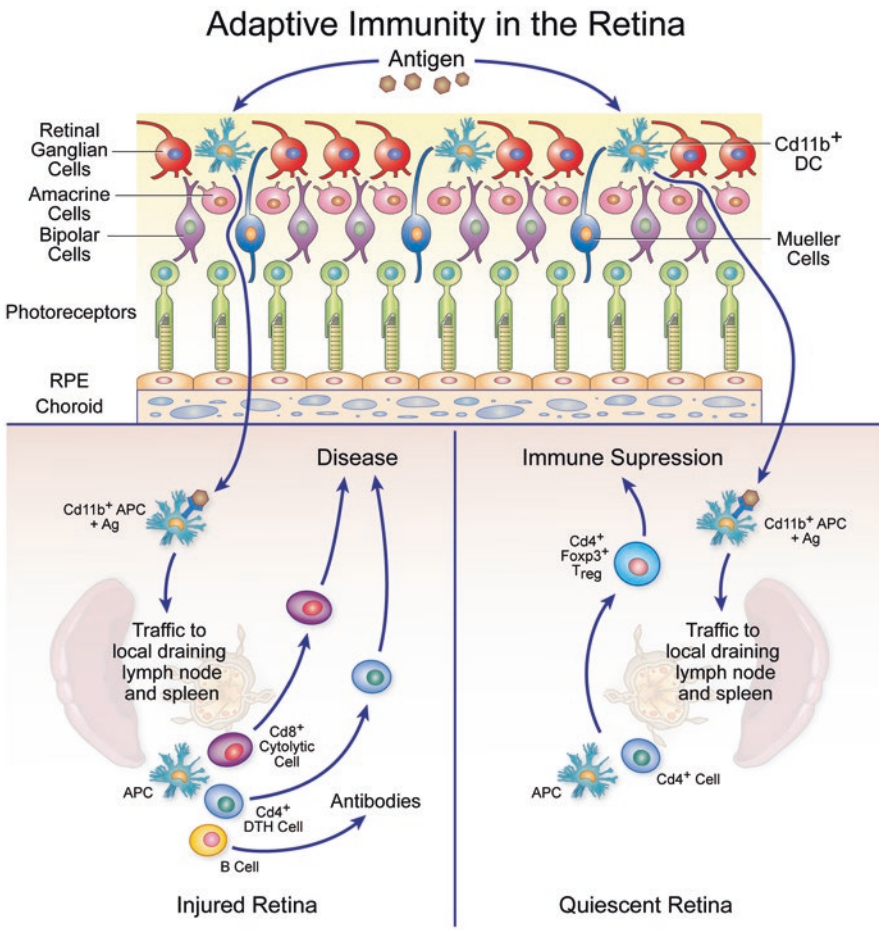

Fig. 14.3 Adaptive immunity in the retina. Small numbers of APCs, present in the ganglion cell layer, acquire antigen and then traffic to the local draining lymph node or spleen where they present antigen to T-cells. For a retina that has not received an inflammatory signal (quiescent) this results in the generation of suppressor T-cells that traffic back to the eye and dampen inflammation. For a retina that has been exposed to an insult (injured) the response results in the activation of $CD8^+$ cytolytic cells or $CD4^+$ DTH type cells that then infiltrate the retina causing inflammatory damage

14.2.3 Immune Privilege in the Eye

Inflammatory responses in the eye are generally not desirable because they result in interference with light paths through the cornea, aqueous, lens, or vitreous or they result in tissue damage directly to the retina. For this reason, the eye has a number of passive and active mechanisms to suppress inflammatory responses, although this suppression or privilege is not absolute and clearly can be broken during infections and in non-infectious uveitis (Streilein 2003; Caspi 2006; Forrester et al. 2008).

The major passive mechanism is the blood-ocular barrier which is present in both the anterior and posterior segments. An additional mechanism is a lack of lymphatic vessels in the eye. Lymphatic vessels are present in the conjunctiva and under inflammatory conditions such as dry eye and infection they grow into the cornea (Barabino et al. 2012; Bryant-Hudson et al. 2014; Park et al. 2015). To date lymphatic vessels have not been found in other tissues such as the iris and retina. For the retina, the barrier is established between retinal pigment epithelium and vascular endothelial cells through tight junctions. In the anterior chamber, vascular endothelial cell tight junctions help establish the barrier. If the barrier is intact, molecules even as small as 376 Da (fluorescein for angiography), can be excluded, so potential protein antigens normally don't cross the barrier. Early events in an inflammatory process involve the release of various cytokines and chemokines from cells and these can have negative effects on barrier function.

Several active immunosuppressive mechanisms exist in the eye (Mochizuki et al. 2013; Mochizuki 2010; Ishida et al. 2003; Chen et al. 2014). First, there is a lack of MHC expression on cells in the eye reducing the possibility that antigen presentation can occur. In the presence of cytokine stimulation, such as Interferon, MHC antigen expression can be upregulated on several cell types in the eye which could make them targets for T-cells (Hamel et al. 1990; Brandt et al. 1990). Ocular cells also express a number of surface proteins that have direct anti-inflammatory activities. These include membrane bound TGF-β, Fas ligand (FasL), galectin-1, thrombospondin, and the B7-CTLA4 co-stimulatory interaction. In addition, a number of factors are secreted into ocular fluids. Large amounts of TGF-β2, which is directly immunosuppressive, are found in ocular fluids. In addition there are immune suppressive peptides such as vasoactive intestinal peptide, calcitonin-gene-related peptide, somatostatin, and α-melanocyte stimulating hormone. Cytokines such as migration inhibitory factor (MIF) and at least the mRNA for an IL-10 related protein are also present. Complement activation inhibitors are also present in the eye.

Pigmented epithelial cells in the eye (RPE, ciliary body, and iris) play an important role in regulating immune responses in the eye. Co-culture of T-cells with pigmented epithelial cells results in suppression of T-cell activation (Ishida et al. 2003; Kaestel et al. 2002). Of these cell types, retinal pigment epithelial cells are the best characterized in terms of immunosuppressive functions. Expression of galectin-1 by RPE cells is at least partially responsible for this activity (Ishida et al. 2003). TGF-β, prostaglandin E2, and nitric oxide production by RPE cells have also been shown to contribute to the suppressive function (Holtkamp et al. 2001; Liversidge et al. 1994; Liversidge et al. 1993). RPE cells have also been shown to induce T-cell anergy during antigen presentation (Gregerson et al. 2007) and they secrete soluble CD54 (ICAM-1) which interferes with T-cell homing functions (Wallace et al. 2013).

14.2.3.1 Anterior Chamber Associated Immune Deviation (ACAID)

In 1975 Kaplan et al. reported that introduction of soluble antigen into the anterior chamber resulted in systemic suppression of Th1-mediated immune responses such as the delayed type hypersensitivity response (Kaplan et al. 1975; Biros 2008).

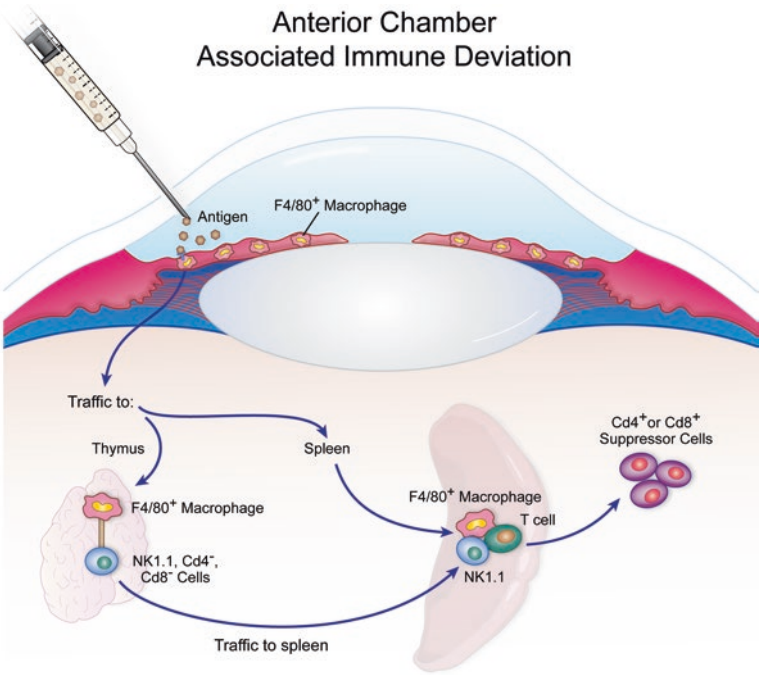

Fig. 14.4 Anterior chamber associated immune deviation (ACAID). Macrophages resident in the anterior chamber acquire antigen and mature in the presence of proteins such as TGF-β and other factors that promote their ability to induce the suppressor phenotype and then traffic to the thymus and spleen. In the thymus the macrophages interact with NK1.1, Cd8-, Cd4- T-cells that then traffic to the spleen. In the spleen the macrophages, which present the soluble antigen, interact with NK1.1 cells and naïve T-cells resulting in the generation of $Cd4^+$ or $Cd8^+$ suppressor cells. The immune suppression is antigen specific and systemic

Subsequently it was shown that this results in the generation of regulatory T-cells (Treg) that actively suppress immune responses. A number of components are needed for the generation of ACAID including an eye with an intact blood ocular barrier, an intact spleen and thymus, IL-10, B cells, efferent suppressor cells, blood-borne APCs, invariant natural killer T cells (iNKT), β2 microglobulin, γδ T cells, and TGF-β in anterior chamber fluid (Fig. 14.4).

The process begins with class II negative $F4/80^+$ macrophages (APCs) acquiring the antigen in the intracameral space. These APCs develop in the presence of high concentrations of TGF-β which results in suppressed IL-12 expression by the cells. Both the complement C3b receptor and CD1d (class I MHC) on the cell surface are required for ACAID. An intact thymus may also be involved in providing $CD4^-/CD8^-/NK1.1$ thymocytes which migrate to the spleen and are the precursors for the Treg cells.

After antigen acquisition, the $F4/80^+$ cells traffic to the spleen where they interact with NKT cells and B cells and the NK1.1 T cells in the presence of IL-10, IL-13, and MIP-2, which act to differentiate the NK1.1 T cells into $CD4^+$ or $CD8^+$ Treg cells that then suppress antigen specific immune responses (e.g., delayed type hypersensitivity responses) throughout the body. It is important to note that anti-

body responses are not affected by ACAID. The purpose of ACAID is not entirely clear but may exist to prevent immune responses to proteins expressed in ocular cells that are released into the aqueous or vitreous compartments. The ability to suppress antigen specific immune responses is being actively pursued as a mechanism to reduce or prevent allograft rejection.

14.3 Immunopathology of the Eye

The eye has a limited repertoire of responses when immunologically provoked, with similarities to those that occur systemically. Despite being a site of immune privilege (immune deviation), immune responses (innate or adaptive inflammation to foreign proteins, including pathogens, exogenous proteins, biological therapeutics, and to endogenous ocular proteins) do occur as described above. However, concentrated within the ocular space, inflammation is slow to resolve and more likely to have long-term consequences on sensitive tissues that do not regenerate. As previously described, the environment of the intra-ocular space is immunosuppressive, and immune privilege is predicated on the presence of intact blood-ocular barriers (BOBs). The presence of inflammation in the eye implies a compromise to the BOBs and to the conditions that create immune deviation. It has not been established if administration of an antigenic biologic to an eye with compromised immune privilege may exacerbate immunological responses, but the possibility should be considered. Primary mediators to immune suppression and to immune deviation previously described include TGFβ cytokines, in particular TGFβ2. Inflammation causes a local up-regulation of TGFβ2 production, which can modulate the immune outcome. However, untargeted soluble TGFβ2 has roles in cellular transdifferentiation, migration, and proliferation, and has been implicated in common spontaneous diseases that result in formation of myofibroblastic membranes in the subretinal space (AMD), on top of the retina (epiretinal membranes), and within the vitreous (vitreous bands and membranes) (see below). Secretion of extracellular matrix and subsequent membrane formation in the eye recapitulates systemic events in wound healing, and may be an undesirable outcome associated with inflammatory events occurring with intraocular administration of biological therapeutics.

14.3.1 Terminology

The eye is a complex organ with numerous tissue types and not infrequently develops unique lesions. As a result, ‘ocular-centric’ terminologies in clinical use are common, many of which are presented parenthetically here. Some terms may seem generic but can convey specific immunological mechanisms in a clinical setting, i.e., ‘uveitis’, which diagnostically implies inflammation within the uvea, but clinically might imply the presence of autoimmune disease directed towards ocular

antigens. The reader is advised to avoid such terms and use diagnostic nomenclature recognized globally by regulatory agencies in compliance with SEND (Standard for the Exchange of Nonclinical Data), found in International Harmonization of Nomenclature and Diagnostic Criteria (INHAND) publications (Keenan et al. 2015; Mann et al. 2012), and specifically, for ocular tissues (in progress).

14.3.2 Innate Immune Responses

Delivery of a drug into the intraocular space breaches BOBs providing the opportunity for activation of the innate immune system. Needle insertion through the cornea immediately anterior to the limbus into the intra-cameral space, or through the pars plana into the vitreous, may result in a minimal amount of protein extravasation into the anterior chamber, which may or may not be accompanied by an infiltrate of white blood cells. Neutrophils, or other polymorphonuclear cells (PMNs; heterophils in rabbits) are first cellular responders, and can be visualized using a slit lamp. In-vivo, protein in the anterior chamber is observed as flare using a using a laser flare photometer. Flare is characterized on histology as a pale eosinophilic matrix (resembling edema in other tissues) of low cellularity within the anterior chamber. Uncomplicated, flare rapidly resolves within hours. Resolution of inflammation presumably occurs concurrently with re-establishment of BOBs, ocular homeostasis, and thus immune deviation.

Administration of solutions that are not physiologically optimal can result in an acute, innate inflammatory response and is associated with an influx of predominantly PMNs and extravasation of plasma of higher protein content into the anterior chamber. When severe, red blood cells denoting hemorrhage are also present. Vascular changes, including vascular inflammation may be observed in the ciliary processes, ciliary body, and iris. Vascular congestion of the iris can be observed on ophthalmic examination, particularly in albino animals. Vascular congestion has been ascribed as 'iritis' clinically, an unfortunate term given that congestion may be observed upon administration of a number of drugs that increase blood flow within the uvea, but are not necessarily associated with inflammation. Diagnoses of inflammation of the iris, or of vessels within the iris, should follow INHAND recommendations. Postmortem vascular pooling may appear as vascular dilation and congestion on histological examination, but generally are poorly correlated with meaningful clinical changes. These terms are best avoided as independent diagnoses lacking in-vivo or other histological correlates.

Acute inflammation into the ocular space is predominantly neutrophilic, although variable numbers of lymphocytes and macrophages may also be present. Cells may be dispersed throughout the anterior chamber, but accumulation at the surface of the cornea endothelium and anterior margins of the iris is common. Cellular aggregates may form, and in severe inflammation, hypopyon may occur, most often observed clinically as a fluctuant, white mass in the ventral margins of the anterior chamber. Protein deposits or cellular debris (keratotic precipitates) may be observed on the cornea endothelium on ophthalmoscopy. These are generally difficult to detect on

histology unless careful sectioning has been planned with advance knowledge of their presence and precise location. Good communication between the pathologist, study ophthalmologist, necropsy team and histologists is critical to ensure proper collection and processing of ocular tissues for postmortem evaluation and correlation with in-life findings. Inflammation can extend throughout the entire uveal tract, resulting in inflammation in the choroid (panuveitis), or extend into the vitreous (ophthalmitis).

Initiation, course, and resolution of ocular inflammation are dependent on the primary cause and individual animal susceptibility. Responses may vary drastically between or within a species. A spectrum of responses can occur within a given dose group during a safety study, indicating the importance of individual host factors such as genetic variation, immune status, and concurrent morbidity. Ocular inflammation occurring during drug development may be associated with the administration of formulations that contain pyrogenic factors, such as endotoxin. Endotoxin is a component of the outer cell wall, lipopolysaccharide (LPS), found in gram-negative bacteria such as *E. coli*, and can be a contaminant of medical solutions, surgical instruments, and devices. As previously mentioned, residual endotoxin and other host cell proteins occur in biologics that are produced in bacterial expression systems, such as *E. coli*, and thus may be present in formulations used in preclinical development that have not been manufactured as stridently required for Good Manufacturing Practice (GMP). Endotoxin activates TLR signaling pathways through PAMP recognition (described above in Ocular Immunology). Some host cell proteins (flagellin, nucleic acids) activate other pathways such as inflammasomes (Franchi et al. 2012). Regardless of pathway, the inflammatory response appears similar.

Several studies have characterized the acute inflammatory process that occurs with intraocular administration of endotoxin, most often in rabbit eyes (Buchen et al. 2012; Nussenblatt et al. 2012). Rabbits may be exquisitely sensitive to endotoxin compared to other species (however, a paucity of published data in non-human primates may be indicative of a lack of adequate studies, rather than actual sensitivity). However, even amongst rabbits there is apparent individual sensitivity, with some individuals exhibiting little reaction, while others exhibit prolonged inflammatory responses.

Inflammation resulting from endotoxin contamination $\geq$0.1 EU/eye can be detected on ophthalmic examination. However, histologically, inflammation can be observed with endotoxin contamination as low as 0.01 EU/eye, suggesting that in some cases histopathological examination may be a more sensitive means of detecting inflammation in the eye (Streit et al. 2015). Endotoxin causes an acute influx of protein and PMNs (heterophils) into the anterior chamber within an hour of administration to either the anterior chamber or vitreous, and is generally dose-dependent in severity. Leukocytes may be observed transiting the uveal tissues at the ciliary body, processes, and the pars plana. Clinical observations show that inflammation tends to peak in the anterior chamber by Day 2 following administration, and, if not complicated by other factors, resolves by Day 4. More recently, it was shown that vitreous inflammation lags that of the anterior chamber but persists beyond Day 7, with an infiltrate of mononuclear cells (macrophages and lymphocytes) replacing the initial, predominantly heterophilic response (Streit et al. 2015).

Histologically, inflammation in the vitreous induced by endotoxin is typified by inflammatory cell infiltrates at the peripheral margins of the vitreous, primarily posterior to the ciliary processes, at the retinal surface, and behind the lens. However, the seemingly restricted location of the inflammatory response to these areas is also likely associated with the structure of the vitreous and fluid dynamics, the distribution of the drug or solution injected into the vitreous, the location of resident hyalocytes (periphery of the vitreous), and generation of subsequent diffusion gradients of elaborated inflammatory mediators that recruit additional cells to the site. The predominantly gel vitreous of most laboratory animals (with the notable exception of non-human primates) (Denlinger and Balaz 2014) may help to restrict the inflammatory response to the anterior and peripheral margins of the vitreous during acute inflammatory phases.

Innate inflammation of increased severity may extend into the superficial retina, and occasionally be associated with cell loss and necrosis of the inner layers. Similar to other forms of retinal injury, cell degeneration, apoptosis, and so-called 'nuclear drop-out' (nuclei from inner or outer layers appearing to migrate out the retina, in the direction of the RPE layer) may be observed. Most often, inflammation from endotoxin uncomplicated by other factors resolves gradually within the vitreous and is generally uneventful, although cells may persist for weeks in the vitreous due to slow fluid dynamics. In preclinical development, persistence of infiltrates—often consisting of degenerating macrophages—demonstrate slow recovery, and may still be present despite a lapse of 2 months or more from time of last drug administration.

The majority of intraocular drugs currently on the market are small molecules, and anti-inflammatory. Trace endotoxin that is not clinically relevant may have a negligible impact when administered concurrently with an anti-inflammatory drug. However, trace impurities administered with an antigenic biologic may confound safety interpretation and have a negative impact to a program. The induction of inflammation on delivery of a biologic—or any drug—may have a role in priming the local environment for type II MHC presentation, facilitating an immunogenic response, and possibly resulting in delayed hypersensitivity (DH). Although as mentioned above, antibody responses are not affected by ocular immune deviation, severity of inflammation may parallel anti-drug antibody (ADA) titers and thus correlating ADA to findings is often used as a risk-mitigation strategy in drug development (see below). In mouse models of uveitis, the innate immune environment that is present at the time of antigen exposure drives the effector response (Caspi 2014). In models of DH, mice co-administered endotoxin or host cell protein systemically with ovalbumin showed increased antigen-specific IgG to ovalbumin over those receiving ovalbumin alone, suggesting impurities may synergistically facilitate an immunogenic response (Verthelyi and Wang 2010). Thus, the severity of inflammation in the eye concurrent with the presence of a biological therapeutic may influence the ensuing adaptive response (predominantly Th2, Th1, or Th17).

Regardless, the presence of active inflammation initiated upon drug administration indicates a breach in BOB, and thus a loss of immune deviation. A break in immune privilege in the eye can potentially result in an immune response to endogenous ocular proteins in addition to that of the administered protein. Ocular-specific T cells do exist in peripheral blood; likely these cells are 'ignorant' rather than 'tolerant' because the BOB inherently hinders establishment of tolerance as occurs

with self-antigens in the periphery (Caspi 2014). Given our current understanding of immunogenicity, it seems prudent to formulate drugs intended for preclinical development in the eye in a manner that minimizes inflammation on administration, minimizing recruitment of systemic APCs and local APC expression of MHCII.

14.3.3 Adaptive Immune Responses

The administration of foreign proteins typically provokes an adaptive immune response with the generation of antibodies, and is a fundamental strategy utilized for immunization. Specific factors known to enhance immunogenicity to therapeutic proteins are beyond the scope of this chapter, but are discussed elsewhere in this volume (see *General Pathology*). Although much can be gleaned from this knowledge, there are numerable gaps in our general understanding of immunogenicity of biological therapeutics and impact to patient safety. Much less is known regarding the potential for immunogenicity when biologics are administered to the eye, an environment where regulatory T-cells normally would predominate when recruited. Immune privilege (deviation) does not imply that an immune response is not generated. Soluble proteins may exit the eye either through Schlemms canal or the posterior vasculature (Lin et al. 2014), and enter the systemic vasculature with subsequent presentation in lymphoid organs, resulting in a humoral response (Rocha et al. 1992). APCs bearing ocular antigens are also directly transported by the vasculature to the spleen (Masli and Vega 2011). Thus, it seems likely if antibodies develop to a systemically administered biologic, antibodies can also develop when the biologic is placed in the eye. However, the origin of the APCs (peripheral lymphoid tissues; resident dendritic cells; recruited APCs) that have a primary role in directing polyclonal B activation towards protein therapeutics administered to the eye has not been fully elucidated. Hypothetically, the first two options could occur under the umbrella of immune deviation; non-complement fixing antibodies and $CD8^+$ T cells may develop, but a pro-inflammatory outcome is thwarted by Tregs (Masli and Vega 2011). If the eye is inflamed, conventional MHCII APCs may be present in the eye and result in generation of antibodies or DH response. There can be marked individual variation in both humoral (i.e., ADA production) and inflammatory responses observed within and across dose groups in a safety study, however the mechanisms and association with loss of immune deviation have not been fully elucidated.

Humoral responses are generally detected 10-14 days following administration of a biologic in the eye, similar to systemic delivery, when they occur. Histological findings (infiltrates of lymphocytes and macrophages) may correlate with antibody production and detection in the eye or serum. There is a certain level of tolerance from regulatory agencies to histological findings for an expected adaptive immune response (Ponce et al. 2009). In the eye, an adaptive response of minor consequence is observed as minimal to mild perivascular infiltrates of lymphocytes around retinal and optic nerve vessels. These generally occur as small cuffs, 2–4 cell layers thick, with minimal impact to the adjacent tissue (Fig. 14.5). Similar cuffs are also observed at the injection site, and infiltrates are commonly present within the ciliary body (Fig. 14.6)

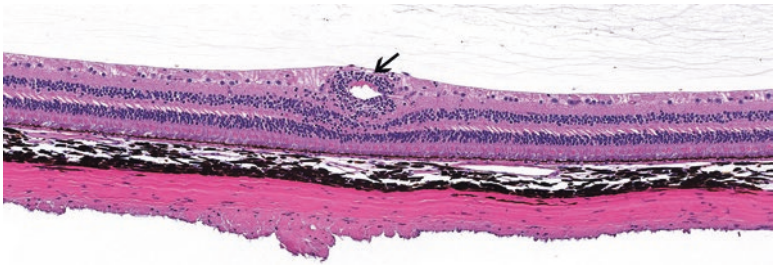

Fig. 14.5 Perivascular infiltrates in the retina associated with an immune response to the IVT administration of a biologic. Small cuffs of lymphocytes are present surrounding a retinal vessel (*arrow*). Note that there is minimal reaction to the surrounding cell layers. Such responses typically recover with no impact to the anatomy or visual function. Cynomolgus monkey, 200×

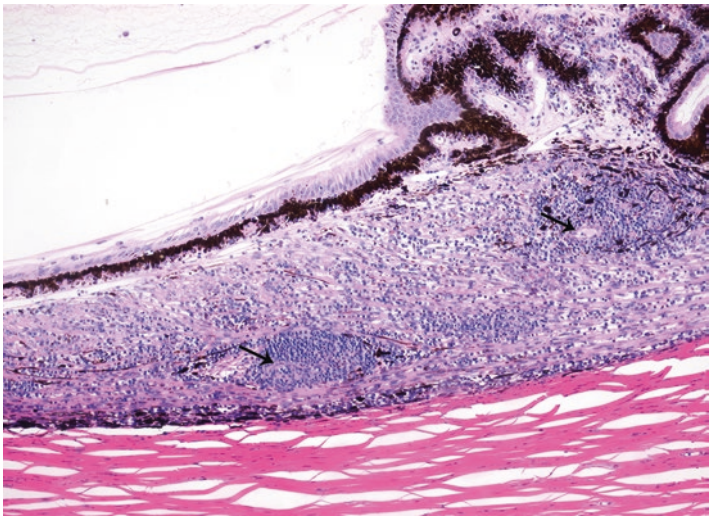

Fig. 14.6 Antigenic immune response in ciliary body of a cynomolgus monkey administered a biologic. Numerous infiltrates of lymphocytes are present within the ciliary body and ciliary processes. Note the perivascular cuffs (*arrows*). Cynomolgus monkey, 100×

and choroid, although these must be differentiated from background changes, particularly in non-human primates. Such infiltrates generally resolve without incidence, have no functional impact, and within the retina, seem to rarely result in cellular loss beyond isolated cells in the inner nuclear layer. Perivascular infiltrates can be observed surrounding retinal vasculature on fundic imaging as white/off-white expansions of the normal vasculature pattern, colloquially termed 'frosted branch angiitis' (Fig. 14.7). Infiltrates of lymphocytes and macrophages are frequently observed within the body of the vitreous, at the peripheral margins of the vitreous body (previously described), and at the surface of the optic disc. On occasion, perivascular lymphocytic infiltrates may only be observed at the optic disc, or within the optic nerve (Fig. 14.8). Drugs injected into the vitreous exit via the trabecular meshwork anteri-

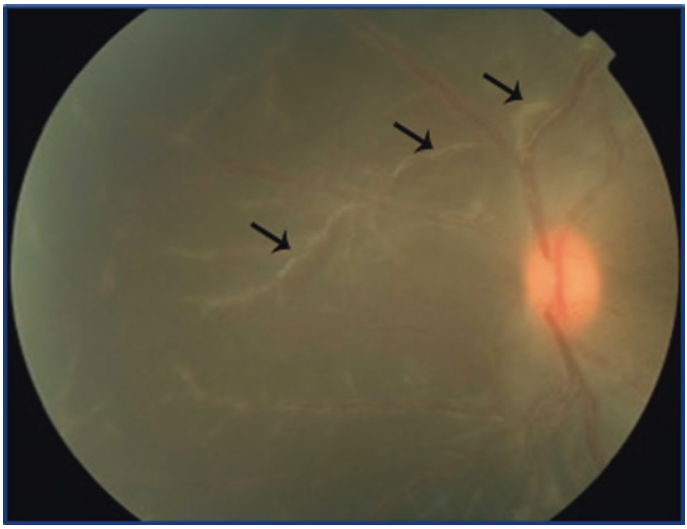

Fig. 14.7 'Frosted branch angiitis'. Fundic image of retinal blood vessels surrounded by cuffs of leukocytes (*arrows*). Antigenic immune response to the IVT administration of a biologic to a cynomolgus monkey

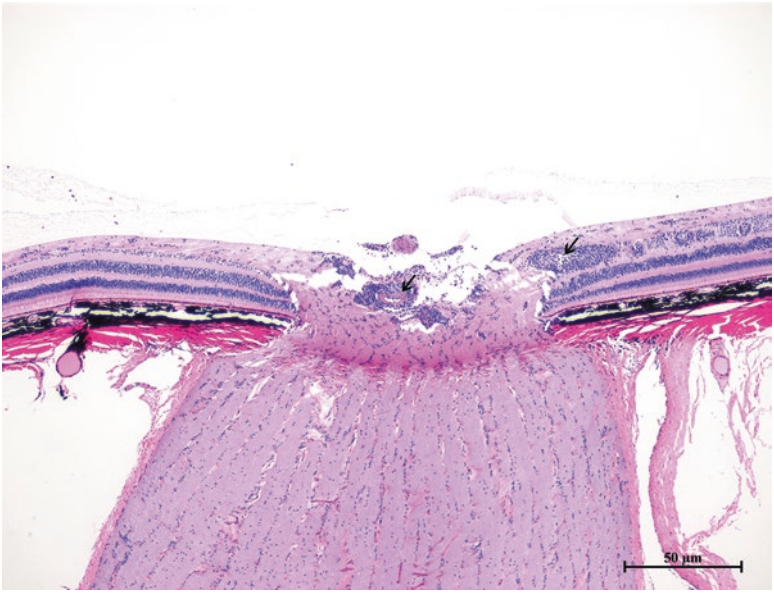

Fig. 14.8 Perivascular inflammation of the optic nerve head associated with the IVT administration of a biologic. Small perivascular cuffs of lymphocytes are present around the blood vessels of the optic nerve and adjacent retina (*arrows*). Cynomolgus monkey, 40×

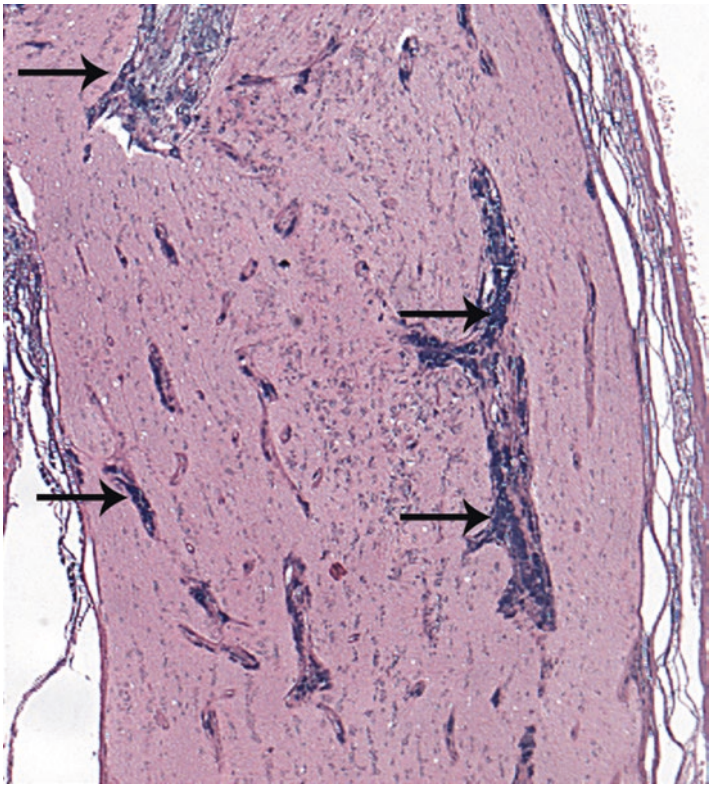

Fig. 14.9 Perivascular macrophage infiltrates of the optic nerve associated with the IVT administration of a pigmented solution. Thick perivascular cuffs of macrophages containing engulfed pigment are present around blood vessels of the optic nerve, suggesting this as a clearance pathway for foreign matter out of the posterior segment of the eye. Rabbit optic nerve, 50× (photo courtesy of Dr. Ken Schafer)

orly, or through the posterior blood-retinal barrier (Lin et al. 2014). Lymphocytes are often observed in the optic nerve following administration of a biological therapeutic. Cytokine honing mechanisms likely have a role, as drugs may exit via the vasculature of the optic nerve. For example, macrophages bearing pigmented particles following IVT administration have been observed egressing via the nerve (Fig. 14.9) (Schafer and Render 2013). Significant inflammation in the optic nerve includes vascular damage, influx of neutrophils, tissue necrosis ('optic neuritis'), and hemorrhage. Optic neuritis can be readily detected on fundic examination.

Vitreous inflammation is slow to resolve and when global, can have a significant impact to visual function, preventing repeat dosing on a safety study. Matrix changes to the vitreous can create generalized opacities that obscure the visual pathway. For these reasons, dosing through hypersensitivity responses with the intent of inducing tolerance is not possible in the eye, although it can be a successful strategy employed systemically. More often, overt inflammation in the eye resolves with physical bystander damage to sensitive neural tissues, which does not recover, but resolves with loss of structure (retinal or optic nerve atrophy), or scarring.

It may be challenging to differentiate the varying types of inflammatory responses in the eye without extensive analysis. Once initiated, unchecked inflammation tends to amplify with recruitment of peripheral leukocytes, leading to increased tissue damage. However, a preponderance of T-cells differentiates hypersensitivities from chronic inflammation. In delayed hypersensitivity (Type IV hypersensitivity), tissue damage is mediated by Th1 or Th17 lymphocytes, regardless if the inciting cause is a pathogen, a self-antigen, or a foreign protein, bound or present within cells or tissue (Gery and Chan 2009). Infiltrates of lymphocytes occur in the retina, choroid, optic nerve, and vitreous, and may be associated with extensive retinal necrosis. Immune complex disease, the formation of antigen-antibody complexes that deposit in tissues and subsequently activate compliment (Type III hypersensitivity), can provoke an inflammatory reaction primarily around blood vessels (vasculitis) within the retina and optic nerve. While mechanisms of immune deviation generally impair production of compliment-fixing antibodies (Rocha et al. 1992), compliment activation has been shown to have a role in spontaneous diseases in the eye, such as age-related macular degeneration (AMD) (Hageman et al. 2001), and may similarly have a role when immune privilege has been lost in the presence of other antigens. The activation of compliment ultimately leads to cell death, and the recruitment of additional inflammatory cells. Vascular cuffs composed of WBC infiltrates are larger than described above, and in addition to lymphocytes, contain neutrophils and macrophages. Cell loss and displacement may be evident in the adjacent tissue, and tissue necrosis may occur (Fig. 14.10). Vascular congestion, accumulation of intravascular WBCs and extravasation, endothelial cell activation and fibrinous changes within the vascular wall may be observed, and the possibility of intravascular thrombi should be considered. Fundic imaging may be obscured due to inflammation and vascular leak. Strategies to minimize the undesirable immune responses by the host include constructing antibodies without the Fc portion of the molecule to reduce effector function (Treacy and Martin 2008).

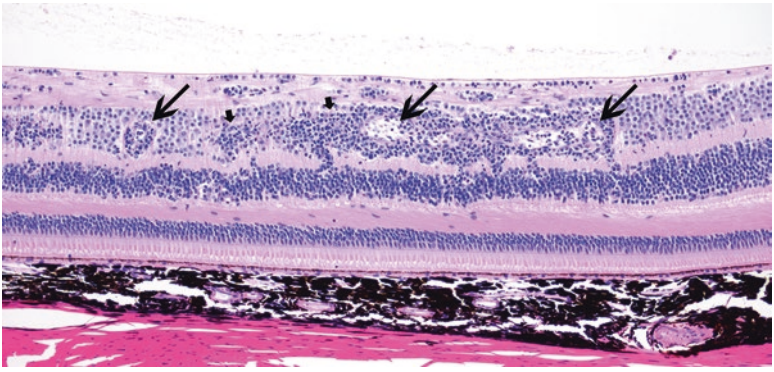

Fig. 14.10 Antigenic immune response in the retina of a cynomolgus monkey administered a biologic. Numerous infiltrates of lymphocytes surround retinal blood vessels (*arrows*) and are associated with necrosis and nuclear loss of the inner nuclear layer (*block arrows*). Cynomolgus monkey, 200×

It's important to distinguish inflammation resulting from immune-mediated causes, from possible pharmacological causes, particularly vascular inflammation. The former may not be clinically relevant in humans, but the later may curtail further drug development. Detection of immune complexes on tissue sections with vascular lesions by immunohistochemistry (IHC) can mitigate otherwise negative findings. Rather than construct an antibody sensitive to the drug, human anti-IgG is often used as a surrogate; antibodies to IgG and IgM derived from the test species are used to detect endogenous Ig. Co-localization of these antibodies to C3 within lesions is taken as evidence of immune complex disease. The reader is referred to recent excellent reviews for further details on the immunohistochemical detection of immune complexes in drug development (Frazier et al. 2015; Leach et al. 2014; Rojko et al. 2014).

Definitive evidence for immunogenicity can be lacking, as for example, antigen-antibody complexes that have been cleared from tissue prior to necropsy. In these cases, a 'weight of evidence' approach must be applied. Correlating exposure data, systemic ADA, and ADA in ocular fluids or tissues may bolster a case for immune complex disease (Leach 2013a; Leach et al. 2014) (see Regulatory Aspects of Ocular Drug Development, below).

14.3.4 Immunopathology Outcomes

Ocular immunology and concepts of ocular immune privilege were previously described (Ocular Immunology). The cellular and cytokine milieu of the eye at homeostasis minimize inflammatory responses that are potentially tissue destructive. From an evolutionary and developmental perspective, this seems prudent, as the tissues of the eye are not redundant and do not regenerate. The evolution of tolerance within the eye is observed with some infectious diseases, such as CMV or Toxoplasmosis, where infected retinal cells may bypass immune surveillance. In other cases, fibrotic-like mechanisms appear to be present to wall off, or isolate offending invaders. This can also occur in drug development in response to administration of chemically or physically irritating agents, including solutions or devices.

Cytokines within the TGFβ family, and particularly TGFβ-2 (Caspi 2010, 2014; Masli and Vega 2011; Mo and Streilein 2001; Streilein 1990) have key roles in modulating inflammation and maintaining an environment that is anti-inflammatory. However, TGFβ is also elaborated under inflammatory conditions, and conversely activation of untargeted soluble TGFβ has been implicated in ocular fibrotic disease (Masli and Vega 2011). TGFβ-2 in concert with other cytokines (PDGF, HGF, MCP-1) are participants in tissue repair processes, and regulate the proliferation, transdifferentiation, and migration of cells (including ocular cells) to assume a fibroblastic, membranous phenotype (Kita et al. 2014; Kohno et al. 2009; Mehta et al. 2014). This is readily observed in spontaneous disease of the eye, such as AMD, which is characterized in part by transdifferentiation and proliferation of RPE within the region of the macula, and is associated with neovascularization and vascular leak. AMD is recapitulated in animal models of choroidal neovascularization (CNV), such as monkey laser CNV (Goody et al. 2011; Grossniklaus et al. 2010) (Fig. 14.11).

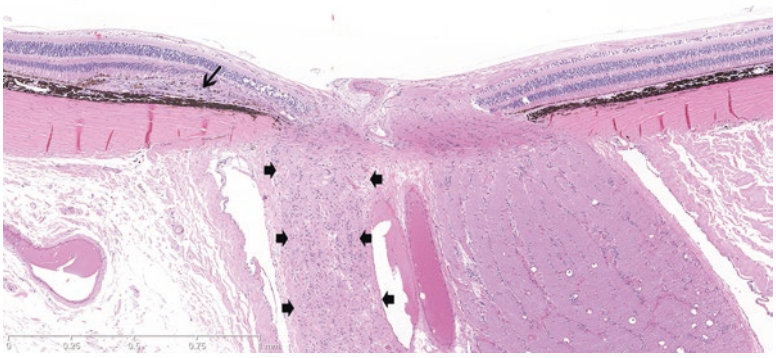

Fig. 14.11 Subretinal membrane and medial optic nerve atrophy of unknown cause. A multilayer mass of fibro-membranous tissue is present in the subretinal space (*arrow*) resulting in cell loss and atrophy of the undermined retina. This lesion is similar to that which occurs in AMD, and laser models of CNV. In this eye, the adjacent, medial portion of the optic nerve demonstrates atrophy (between *block arrowheads*), associated with a paucity of ganglion cells [compare to the retina and optic nerve fibers on the lateral side (*right aspect* of photo)]. Spontaneous optic nerve atrophy has been reported in the literature. Cynomolgus monkey, 50×

The formation of epiretinal membranes (ERM) and vitreous membranes are other manifestations of ocular cell plasticity. Both types of membranes have morphological resemblance to fibrous tissue reactions involved in systemic wound healing, and likely have a similar role in the eye, as a response to retinal trauma, or as attempts to isolate a foreign body or infectious entity. ERMs are composed of linear proliferations of ocular cells on the inner surface of the retina that are primarily derived from RPE, glial, or Müller cell populations. Vitreous membranes are composed of collagenous matrix and fusiform cells oriented in linear arrays within the vitreous. The predominant cell types have been identified using specific cell markers by IHC as hyalocytes, glial cells, Müller cells, RPE, and astrocytes (Joshi et al. 2013; Schumann et al. 2011; Zhao et al. 2013). Many of these cell types secrete, as well as respond to, cytokines that have key roles in the pathogenesis of membranes, including TGFβ-2, PDGF, fibronectin, FGF and IGf-1 (Mehta et al. 2014). Hyalocytes express all four TGFβ isotypes (Lutty et al. 1993), and secrete both TGFβ-2 and connective tissue growth factor (CTGF) under inflammatory conditions, promoting subsequent production of ECM, glycosaminoglycans and fibronectin, important constituents of proliferative membranes (Kita et al. 2014). Regardless of origin, cells in ERMs and vitreous membranes tend to have a myofibroblastic phenotype. Transdifferentiated RPE and hyalocytes have been shown to express α-smooth muscle actin (α-SMA) and myosin light chain, and contract when exposed to TGFβ-2 (Kohno et al. 2009; Mehta et al. 2014; Zhao et al. 2013).

Vitreous membranes often appear to originate in the vicinity of the ciliary process and pars plana, often in proximity to the site of injection. Inwardly protruding plugs of fibrous connective tissue at the injection site often co-mingle with vitreous membranes, which in turn tend to co-mingle with cells that transfix the lens (zonule fibrils), as well as those of the superficial retina (glial, astrocytes, Müller cells) and vitreous (hyalocytes). Transitional

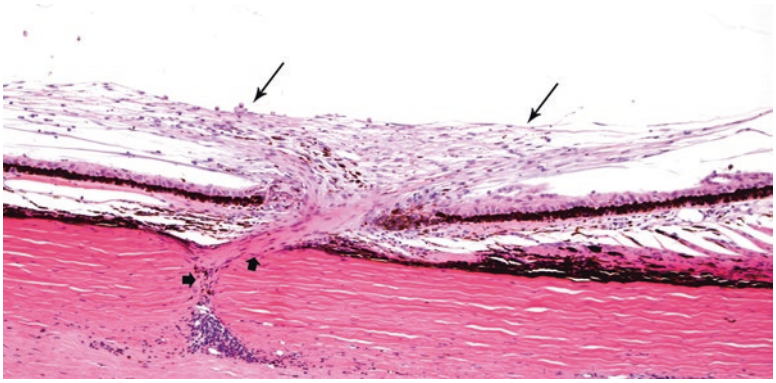

Fig. 14.12 Vitreal membranes at an injection site, associated with the IVT administration of a biologic. Linear layers of fibroblastic membranes are present at the surface of the pars plana (*arrows*). The site of injection contains a plug of dense fibrous tissue (*block arrows*), extending through the sclera and pars plana. The protruding mass comingles with the membrane at the surface. A minor inflammatory infiltrate is present at the injection

cells forming membranes may be difficult to distinguish morphologically by light microscopy from typical resident populations (Fig. 14.12). Vitreous membranes have a tendency to tether to fixed structures such as the retina, lens, and optic nerve, and because they are contractile, place tension on those structures. Contraction can result in lens displacement, retinal detachment, and prolapse of the optic nerve (personal observation). At minimum, the opaque nature of membranes within the vitreous have the potential to interfere with optical light pathways and impair vision. Vitreous membranes are progressive, and while they may mature and become quiescent, do not recover. Vitreous membranes are unpredictable, but their occurrence can be provoked by any noxious stimulus, including pyrogenic factors. Rabbits may be predisposed (personal observation).

Membranes may develop following administration of physical devices. Devices, or solutions that aggregate and form gels on administration, tend to gravitate to the ventral aspect of the vitreous. Prolonged contact with the retina can provoke epiretinal membrane formation, likely in response to superficial physical irritation. Membranes may develop in absence of any significant inflammatory response. Similar to vitreous membranes, ERMs have contractile properties, and tend to place tension on the underlying retina, which can result in retinal displacement or detachment.

Devices with biophysical or chemical properties that are less than optimal can induce an innate inflammatory response. This can be associated with formation of a provisional matrix over the device (viewed as an eosinophilic matrix on histology), derived from the initial leakage of serum and protein in the eye, which then serves as a substrate for attachment of WBCs and compliment factors (Anderson 2012). The release of cytokines from WBCs promotes cellular recruitment and membrane formation, which may serve to isolate the device within the ocular space.

Recruitment of WBCs to these sites can be associated with “frustrated phagocytosis” - characterized by macrophage breakdown and release of lysosomal contents

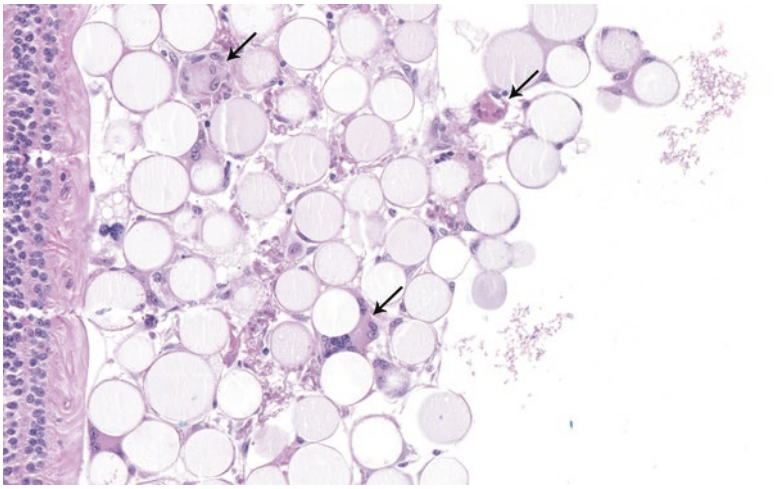

Fig. 14.13 Granulomatous inflammation incited by IVT administration of microspheres containing a biologic. Numerous aggregates of microspheres are present in the anterior vitreous adjacent to the retina. Note that many are surrounded and/or engulfed by macrophages (*arrows*). Rabbit, 100×

(which contain proteolytic enzymes of low pH), in proximity to sensitive ocular tissues and the generation of free radicals causing further necrosis. Frustrated phagocytosis can occur on devices exceeding the size limitation of a macrophage to engulf it (~10 um). Devices ≥ 10 um may also elicit a foreign body or granulomatous reaction, with accumulation and fusion of macrophages (multinucleated giant cells) around the device.

Smaller delivery devices may be innocuous, but can stimulate a granulomotous response (Fig. 14.13. Microspheres have a tendency to aggregate at the periphery of the vitreous gel and cause retinal compression at points of contact. Microspheres sometimes migrate into problematic places, including the stroma of the iris, the trabecular meshwork, retina, sub-retinal space, choroid, and optic nerve.

Delivery devices below 5–10 μm, including nanoparticles, may be susceptible to phagocytosis and elimination through macrophages or hyalocytes. Because of the acidic and enzymatic nature of the internal lysosomal environment, consideration with respect to drug pharmocometrics should account for such conditions.

14.4 Ocular Anatomy

14.4.1 Introduction

The eye is a specialized organ composed of tissues originating from multiple embryological layers with substantial contribution from the neural crest, mesoderm, neuroectoderm and surface ectoderm. This diverse embryological tissue origin is

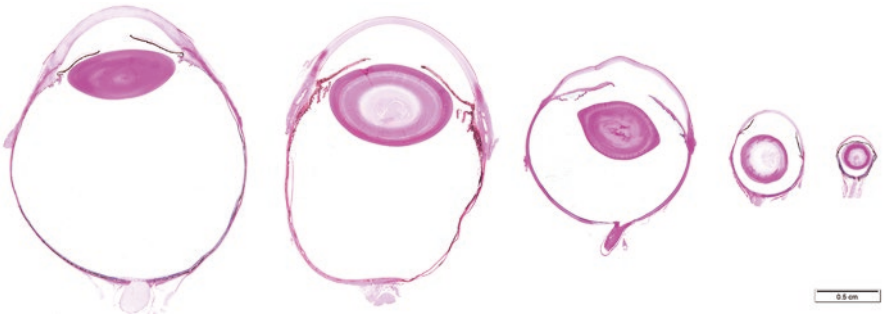

Fig. 14.14 Sub-gross images comparing the ocular shape and dimension of commonly use species in toxicology. From left to right: globes of a cynomolgus monkey, beagle dog, New Zealand white rabbit, rat and mouse. H&E, bar = 0.5 cm

evident in the broad spectrum of cellular differentiation and consequent formation of complex and highly specialized ocular tissues. Its main function is photoreception, a process in which light energy is transformed into electrical impulses by cone and rod photoreceptors located in the retina, and transmitted via the optic nerve to the brain where it's perceived as vision. In order for the eye to effectively focus light and generate a visual response, several structural characteristics need to be preserved. The size and rigidity of the globe have to be precisely matched with the refractive properties of the corneal surface curvature and the location and shape of the lens such that light passing through the various ocular tissues is focused exactly on the retinal layer containing the photoreceptor outer segments. These properties are acquired during development and must be maintained throughout life if the function of the eye is to remain intact.

The eye can be divided in three basic layers or tunics: (1) the fibrous tunic, the external part of the eye composed of the corneal and sclera, (2) the uveal tract, internal to the fibrous tunic composed of the iris, ciliary body, and choroid which houses the vascular supply to the eye and the internal (3) neural layer, composed of the retina and optic nerve head. The eye can be further divided in anterior and posterior segments, the first containing the cornea, anterior and posterior chambers, iris, ciliary body, and lens, and the second, composed of the vitreous and vitreal space, retina, choroid, sclera and optic nerve. The anatomical and histo-morphological features of each specific ocular tissue are described below, with anatomical differences across species noted (Fig. 14.14).

14.4.2 Eyelids

The primary function of the eyelids is protection of the ocular surface from exposure or accidental trauma but they also play roles in tear film formation and distribution (see below). In rodents, rabbits and dogs, the upper and lower lids are fused at

birth and separate only after retinal development is adequate for vision, between 12 and 15 days of age (Gellat et al. 2014; Smith 2002).

The eyelid is composed of a palpebral (inner) surface lined by the conjunctiva, and an outer surface of haired skin that has a specialized lid margin containing meibomian (sebaceous) glands. The lid margin is supported by a firm, layer of dermal collagen which in the upper lids of nonhuman primates, forms a robust rigid structure called the tarsal plate. Carnivores and humans have an almond-shaped eye fissure, while most rodents and nonhuman primates have a round one. Lid closure (blinking) is controlled by the circumferential *orbicularis oculi* muscle, and by the sympathetically innervated superior tarsal and inferior palpebral muscles. Opening of the lid is controlled by contraction of the *levator palpebrae* muscle and relaxation of the *orbicularis oculi* (Gellat et al. 2014).

The process of blinking is of extreme importance for cleaning the cornea and the maintenance and spread of the pre-corneal tear film throughout the ocular surfaces. Cilia are long, wide hairs produced by specialized hair follicles near the mucocutaneous junction. Cilia are present in the eyelid margin of most species but are absent in the cat and only present in the upper eyelid of dogs. Cilia form an outward curving tactical mesh in front of the eye that helps protect the cornea. Histologically, the pilosebaceous units of the eyelids are similar to truncal hairs, but larger.

Of the species used in non-clinical drug development, the dog, cat, and rabbit possess a nictitating membrane (third eyelid), a large fold of conjunctiva that protrudes from the medial canthus over the corneal surface of the globe and is supported by a curved, T-shaped plate of hyaline cartilage. A mixed (seromucinous) gland (gland of the third eyelid) surrounds the base of the cartilage. Small aggregates of lymphoid tissue are typically present in the bulbar (inner) conjunctival epithelium and may be numerous. The third eyelid mechanically protects the cornea, aids in tear distribution over the corneal surface, and contributes 30–50% of the aqueous portion of the tear film via multiple small ductules that enter the bulbar conjunctival surface near the fornix.

14.4.3 Conjunctiva

The conjunctiva is a mucosal membrane that lines the inner aspect of the eyelids (palpebral conjunctiva), the surface of the sclera (bulbar conjunctiva), and the inner and outer aspects of the third eyelid (when present). The conjunctival epithelium is a thin, multilayered, non-keratinized epithelium that contains many unevenly distributed goblet cells. At the limbus (the border between the cornea and the sclera), the conjunctival epithelium forms a specialized, circumferential zone containing the stem cells of the cornea. The cells that migrate and repopulate the corneal epithelium during normal cell turnover, and under pathological cell loss that result in corneal defects are derived from these stem cells. The substantia propria, a connective tissue layer beneath the epithelium, contains numerous glands, and is densely populated with both blood and lymphatic vessels. Lymphatic vessels predominate in the underlying loose fibrous connective tissue.

The conjunctiva provides a loose flexible connection between the globe and the skin, anchoring the globe in the orbit while still facilitating its motion. Goblet cells located in the conjunctiva contribute to the mucinous component of the tear film, which has roles in the maintenance and redistribution of the tear film during blinking. The conjunctiva is both a physical and immunological barrier to harmful microbes, the later through conjunctiva-associated lymphoid tissue (CALT) often observed in the inner marginal rims of the conjunctiva. CALT functions locally in immune surveillance and modulation of the immune response of the anterior segment and ocular surface structures. CALT is thought to protect the ocular surface by performing antigen uptake and processing to initiate local immune responses. Antigen processing occurs in lymphoid tissue located on the bulbar surface of the nictitans in the dog and rabbit, and in the palpebral conjunctiva in rodents and nonhuman primates. As with most related lymphoid tissues in the body, the amount of CALT varies depending on the age and extent of immune stimulation (principally to antigens in the conjunctival sac, but to a lesser degree to those delivered systemically).

14.4.4 Cornea

The cornea is a highly specialized and ordered tissue constructed to attain optical clarity (transparency) and to aid light refraction (focusing). The surface is comprised of several layers of remarkably uniform non-keratinizing, stratified squamous epithelium consistent in thickness and structure across its length. There are three primary layers to the cornea epithelium, (1) basal cells, a deep layer resting on basal lamina, (2) wing cells, an intermediate cell layer, and (3) superficial squamous cells, varying from 3 to 5 layers in the mice, rats, and rabbits, to 5-7 layers in the dogs, cats, and primates (Gellat et al. 2014). A modified acellular region of stroma (Bowman's layer) composed of fine, randomly arranged collagen fibers is present posterior to the epithelial basal lamina in humans and non-human primates. Although Bowman's layer is not visible by light microscopy in the mouse, a thin layer of randomly arranged collagen fibrils can be seen immediately underneath the corneal epithelium by electron microscopy (Smith 2002). The function of Bowman's membrane is not known. Optical transparency of the cornea is dependent on a precise spatial relationship between collagen fibers and non-collagenous matrix, and the functional cells (keratocytes) in the stroma. A monolayer of low cuboidal epithelial cells (corneal endothelium) separates the cornea from the aqueous humor of the anterior chamber. The corneal endothelium secretes Descemet's membrane, a thick basement membrane positioned between the endothelium and the inner surface of the corneal stroma. Descemet's membrane is composed mainly of laminin, fibronectin and type IV collagen, which gives it elastic properties. The morphology of corneal endothelium is similar across mammalian species (Fig. 14.15), but cellular density can vary from 2211 cells/mm^2 in the rat to 4,450 cells/mm^2 in nonhuman primates and humans (Collin and Collin 1998). The endothelium actively moves water and electrolytes between the corneal stroma and anterior chamber, which simultaneously supplies the cornea with nutritional support from the aqueous, and maintains corneal transparency.

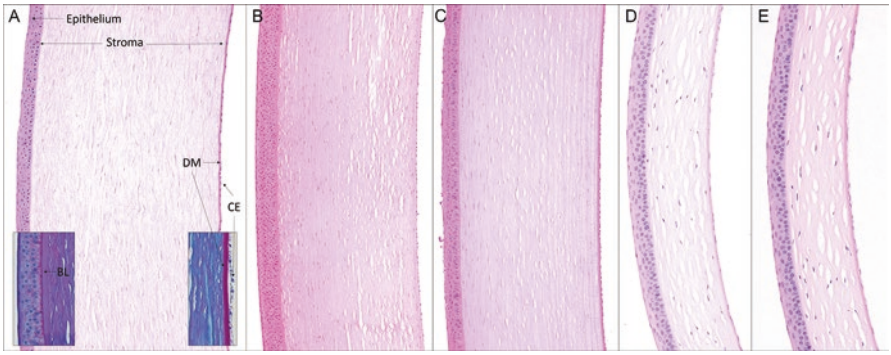

Fig. 14.15 Normal axial cornea microscopic anatomy of commonly use species in toxicology. Note the differences in thickness, especially of the corneal stroma between species. (**a**) Cynomolgus monkey. Humans and non-human primates present a prominent Bowman's layer (BL) subtending the corneal epithelium (*left inset*). Descemet's membrane (DM), the basement membrane of the corneal endothelium (CE) is highlighted on the right inset. (**b**) Beagle dog. (**c**) New Zealand white rabbit. (**d**) Rat. (**e**) Mouse. H&E, *insets*: Alcian Blue – PAS

Blood vessels and lymphatics are not present in the normal cornea, an important feature that contributes to transparency. Avascularity is maintained by cytokines that have been identified in the cornea known to inhibit angiogenesis such as VEGFR 1 and 3, thrombospondins 1 and 2, endostatin, and inhibitors of metalloproteinases (Cursiefen et al. 2006). Angiogenic privilege, an important component of immune privilege (previously described), is critical to maintaining avascularity under inflammatory conditions. The vascular supply to the cornea is derived from an arterial circle that originates from the superficial branch of the anterior ciliary artery (Riordan-Eva 2011). Arteriole branches from this source form an arcade within the limbus surrounding the globe to supply the metabolic needs of the peripheral cornea, while diffusion of nutrients from the aqueous supplies the primary needs of the central cornea. Capillaries extend anteriorly to the level of Bowman's membrane, forming loops that lead back to towards the limbus, or less commonly, terminate abruptly within the peripheral stroma.

14.4.5 Ocular Glands and the Pre-corneal Tear Film

The lacrimal system is comprised of a series of extraocular apocrine glands whose secretions contribute important elements to the tears that lubricate the surface of the eye.

Primates have one lacrimal gland located dorsal and lateral to the globe. Dogs, cats and rabbits have both an extra ocular lacrimal gland and a gland of the third eyelid, although the latter is very small in rabbits. Rabbits and rodents also have an intraorbital gland (harderian gland), located behind the globe in a medial (nasal) location (Payne 1994). In rodents and primates, in which the nictitating membrane is a vestigial remnant, the lacrimal glands are small, and are dispersed through the medial canthus in the

vicinity where the root of the membrane would be. Lacrimal glands are seromucinous glands that mainly produce the aqueous component of the tears. In contrast the harderian gland produces significant quantities of lipid, particularly in rabbits. In all species, these glands secrete immunoglobulins (IgA) and tissue cytokines (IL-1, IL-6 and IL-8) as well as enzymes and anti-microbial substances such as lysozyme, lactoferrin and bactericidal permeability-increasing protein (BPI) and other peptides and proteins that prevent infection of the anterior cornea (Chen et al. 1997). The hardarian and lacrimal glands and the conjunctival goblet cells also secrete mucins and trefoil factor family (TFF) peptides that stabilize the connection between the surfaces of the corneal and conjunctival epithelium and the aqueous component of the tear film, and provide a continuous barrier across the ocular surface that prevent pathogen colonization and penetration (Paulsen and Berry 2006). The pre-corneal tear-film is an acellular fluid layer that is adherent to the most superficial layer of the corneal epithelium and is essential for maintaining both the health of the cornea and the high-quality optics of the corneal surface. It is composed of three main layers: mucinous, aqueous and lipid. The inner mucinous layer is secreted largely by the conjunctival goblet cells with contributions from the lacrimal glands and its main function is to adhere to the cell membrane of the corneal and conjunctival epithelium, holding the aqueous component of the term film in place by adsorption. The middle, aqueous layer is secreted by the glands of the lacrimal system. The aqueous component keeps the corneal surface moist, and serves as a media for nutrient exchange and oxygenation for the anterior corneal tissue, which is avascular. The outer lipid layer is secreted principally by the sebaceous (meibomian) glands of the eyelid margins, and when present by the harderian glands. Lipids form a monolayer on the tear surface that enhances surface tension and integrity of the tear film, reduces tear film evaporation, and provides a glassy smooth interface between the corneal surface and air (Chen et al. 1997). The tears are cleared from the ocular surface by a system of drainage ducts located in the upper and lower conjunctiva near the medial canthus. Ductal openings (puncta) on the conjunctiva communicate with ducts (canaliculus); these converge in a bladder-like sac called the nasolacrimal sac. This sac, in turn, connects to the nasal cavity through the nasolacrimal duct. This system is called the nasolacrimal drainage system because the tear film material is deposited into the nasal cavity (Ding et al. 2010).

14.4.6 Sclera

The sclera forms the main part of the fibrous tunic of the eye and functions to protect the ocular contents, maintain IOP, and maintain the shape of the globe, even during contraction of the extraocular muscles which have tendons inserted on its surface (Forrester 2007). The sclera is relatively avascular and possesses great tensile strength, extensibility and flexibility. In many species, including nonhuman primates, dogs and cats, the sclera is thickest at the limbus, where it originates, and thinnest at the equator where the tendons of the extraocular muscles insert (Walls 1942). The sclera is composed of fibroblasts and dense irregular connective tissue, mainly collagen type I, with scant amounts of collagens type II, IV, V, VI VIII, XII

and XIII (Rada et al. 2006). Anteriorly the sclera blends with the cornea at the limbus. The sclera surrounds the limbus and extends posteriorly where it's penetrated by the optic nerve exiting the eye at, the lamina cribrosa. The sclera merges with dura mater surrounding the optic nerve.

A dense membrane of collagen bundles (Tenon's capsule) courses over the sclera to the optic nerve. Tenon's capsule is attached at the limbus and merges with the perimysium of the ocular muscles, and functions as a type of pulley for the ocular muscles (Rada et al. 2006). The sclera is densely innervated, and while it's penetrated by the ocular supply to the eye, it lacks a specific vascular bed. As a consequence, inflammatory conditions can be particularly prolonged and painful. Inflammation in the sclera often culminates in necrosis as a result of slow removal of fluid and cellular debris.

14.4.7 Uveal Tract and Filtration Angle

The uveal tract (or uvea) forms the continuous, heavily vascularized and often pigmented middle tunic of the globe. Its main function is to provide the globe's blood supply and, through its high melanin pigment content, absorb reflected light and prevent glare when light is focused on the retina. The uveal tract can be divided into iris, ciliary body (anterior uvea) and choroid (posterior uvea).

The iris is a thin contractile circular disk analogous to the diaphragm of the camera. It is cantilevered across the front of the globe where it separates the anterior from the posterior chambers. *The anterior chamber* represents the space between the cornea and the anterior surface of the iris and *the posterior chamber* the space between the posterior surface of the iris, lens and anterior face of the vitreous. The chambers are connected by the pupillary space, which is formed within the inner marginal rim of the iris. Both chambers contain aqueous humor, which is secreted by the ciliary epithelium into the posterior chamber, and flows through the pupil into the anterior chamber. The anterior surface of the iris has no epithelial lining, so aqueous is free to diffuse from the iris stroma to the anterior chamber with no barrier. The iris stroma consists of a loose connective tissue containing fibroblasts, melanocytes and collagen fibers. The posterior surface of the iris is lined by simple cuboidal pigmented epithelium (posterior pigmented epithelium), which is in direct contact with the posterior chamber. Tight junctions between these cells create a barrier between the posterior chamber and iris stroma.

The dilator muscle is a contractile myoepithelial tissue that is present in the posterior iris stroma and originates from the anterior iris epithelium. Contraction of the dilator muscle is controlled by sympathetic innervation, resulting in mydriasis (dilation of the pupillary space). The sphincter muscle is a circumferential ring of smooth muscle fibers that encircles the pupillary margin. Contraction of the sphincter muscle is controlled by parasympathetic innervation, resulting in miosis (contraction of the pupillary space). Pupil shape varies between species and state of contraction. It is generally round in the rodent, dog, and primate, vertically oval in the rabbit, and vertical slit-like in the cat.

The major arterial circle of the iris is supplied by anastomoses of the anterior ciliary arteries and the long posterior arteries (Riordan-Eva 2011). Stromal blood vessels are relatively thick, and allow for constant blood flow regardless of pupil constriction or dilation (Barskey 2006). The blood aqueous barrier of the anterior segment is dependent on the tight junctions present between the endothelial cells of the iridal vessels, and the tight junctions between the pigmented epithelial cells of the posterior iris, ciliary body, and ciliary processes, an important component of ACAID (previously discussed).

Resident stromal macrophages and dendritic cells exhibit attenuated behavior that modulates, rather than promotes, inflammatory reactions (Masli and Vega 2011), and thus have an important role in maintaining immune privilege.

The color of the iris is attributed to type and density of melanin pigment, degree of vascularization, and backscatter of incident light from stromal collagen fibers, of which the latter accounts for the bluish pink iris color of albino rodent species (Wilkerson et al. 1996). Pigmentation of the iris limits light transmission through the pupil and has a protective role to the retina.

The ciliary body is subdivided into (anterior) *pars plicata* and (posterior) *pars plana*. In the pars plicata there are multiple radially arranged folds, known as the ciliary processes. The inner aspect of the ciliary body is lined by a neural tube-derived double epithelium composed of an inner layer of non-pigmented epithelium and an outer layer of pigmented epithelium. The ciliary epithelium secretes aqueous humor and vitreous glycosaminoglycans and collagen (Walls 1942). The zonular ligaments that suspend the lens (see below) are also produced by the ciliary epithelium and are anchored on the epithelial apical basement membrane. The ciliary body stroma is similar to the iris stroma and contains the ciliary muscle, a smooth muscle which functions in visual accommodation by controlling the suspension of the lens and thereby its curvature. The ciliary muscle also plays a role in aqueous filtration in primates by regulating the tension of the trabecular meshwork at the level of Schlemm's canal. The relative tone of smooth muscle within the ciliary body controls the visual accommodation of the lens; thus the relative amount of smooth muscle present in the ciliary body is reflective of visual acuity. The ciliary muscle is robust in primates and accommodation is, by far, more effective in these species than in most other mammals (Fig. 14.16). Accommodation is poor in rodents and dogs. The ciliary muscle also plays a role in aqueous filtration in primates by regulating the tension of the trabecular meshwork at the level of Schlemm's canal. The pigmented ciliary epithelium (along with the iris and retinal pigmented epithelium) plays an important role in regulating the immune response in the eye by suppressing T- cell activation (see previous section).

The aqueous, produced by the ciliary body epithelium, is formed in three stages. First, water, ions, proteins and metabolic fuel are delivered by the ciliary circulation. Next, the differences in oncotic and hydrostatic pressures, and concentration gradients promote ultrafiltration and diffusion from the capillaries into the stroma. Finally, the fluid moves into the posterior chamber guided by the osmotic gradient produced by the ionic transport into the basolateral spaces between the nonpigmented epithelial cells. Because of the blood-aqueous barrier, the relative lack of

protein in aqueous humor establishes a Starling equilibrium that opposes passive fluid movement across the epithelial bilayer, and so aqueous humor production is considered an active process requiring the expenditure of metabolic energy (Kiel et al. 2011). *The filtration apparatus* is made up of several structural features which function to reabsorb aqueous humor back into the blood. Aqueous is secreted into the posterior chamber, passes through the pupil into the anterior chamber, and is drained laterally though the trabecular meshwork (TM) situated within the iridocorneal angle (ICA). The balance between secretion and drainage of the aqueous humor determines IOP. Under normal conditions, IOP is maintained at a relatively constant level of 15–20 mmHg in all mammalian species of interest for ocular toxicology research (Johnson 2005). The ICA resides in the limbus, formed by the base of the iris and the corneal-scleral tunic. The ICA extends into the anterior ciliary body forming a recession, the ciliary cleft or cilioscleral sinus, where the TM is located (Fig. 14.16). The TM is composed of crisscrossing cords or sheets of collagen and elastin that appear to be anterior tendinous extensions of ciliary body musculature. The TM surface is covered by a unique population of endothelial-like trabecular cells that are continuous with the endothelium of the cornea and the downstream collecting duct (Samuelson and Streit 2012). The TM is subdivided into three distinct layers (in order of aqueous flow): uveoscleral, or uveal meshwork (USM); corneoscleral meshwork (CSM); and juxtacanalicular, or cribriform meshwork (JCM). The corresponding intratrabecular spaces become progressively smaller, resulting in increased aqueous outflow resistance as aqueous flows through the TM, and out the JCM. Aqueous humor exits the JCM and flows into collecting ducts (Schlemm's canal, angular aqueous sinus, or angular aqueous plexus), through

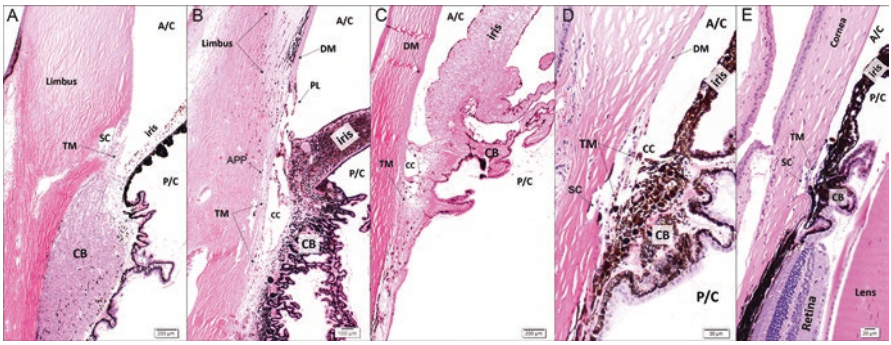

Fig. 14.16 Normal iridocorneal angle microscopic anatomy of commonly used species in toxicology. (**a**) Cynomolgus monkey. Humans and non-human primates present a Schlemm's canal (SC) and a prominent ciliary body muscle (CB). (**b**) Beagle dog. (**c**) New Zealand white rabbit. Dogs, rabbits and also cats do not posses a Schlemm's canal, instead they have intermittent drainage canals called angular aqueous plexus (AAP). (**d**) Rat. (**e**) Mouse. Similar to humans and NHP, rats and mice present a Schlemm's canal (SC). Note however how thin the trabecular meshwork is compared to the Cynomolgus monkey. *A/C* anterior chamber, *P/C* posterior chamber, *DM* descemet's membrane, *PL* pectinate ligament, *TM* trabecular meshwork, *SC* Schlemm's canal, *AAP* angular aqueous plexus, *CC* ciliary cleft, *CB* ciliary body. H&E

interscleral channels, exiting the eye though episcleral and conjunctival veins. This system forms the classic pathway through which aqueous is removed from the eye. In primates, rats and mice, aqueous flows into Schlemm's canal, a circumferential duct lined by endothelium. Schlemm's canal is absent in dogs, cats and rabbits, which instead have intermittent drainage canals called angular aqueous plexus (AAP) that connect the corneoscleral meshwork to the intrascleral venous plexus (McLellan and Teixeira 2015; Samuelson and Streit 2012). The process of aqueous drainage through this route is not well understood; it is energy dependent, and the magnitude of absorption is proportional to IOP. Aqueous can also be drained by an alternate pathway into a potential space posterior to the trabecular meshwork (i.e., into the anterior face of the ciliary muscle and suprachoroidal space) just inside the sclera. This is a passive system not dependent on IOP that permits aqueous reabsorption by vessels located within the choroid or sclera. The relative contributions of these two outflow pathways vary among species, with the alternative pathway accounting for 30–65% in primates, 13% in rabbits, 3% in cats, 15% in dogs and 4–14% in humans (Gellat et al. 2014).

The choroid is the posterior portion of the uvea, a layer of blood vessels and connective tissue situated between the sclera and the retina. The primary function of the choroid is to service the high metabolic demands of the cells located in the outer retina, however, the choroidal space also provides a conduit for vessels traveling to other parts of the eye, and in the absorption of excess light radiation, reducing the amount of light reflection back to the retina. The choroidal vessels originate from the short posterior arteries (Riordan-Eva 2011). The choroid supplies oxygen and nutrients to the retina primarily through the choriocapillaris, an extensive interconnected capillary network posterior to the retinal pigmented epithelial (RPE) cells. The choriocapillaris is separated from the RPE layer by Bruch's membrane, a structure formed by five layers: the RPE basal lamina, an inner collagenous zone, a middle elastic layer, and outer collagenous zone and the basement membrane of the endothelial cells of the choriocapillaris. Bruch's membrane selectively filters the passage of macromolecules between the retina and choriocapillaris, which have a fenestrated endothelium through which macromolecules leak into the extracellular space of the choroid. The choriocapillaris has the highest perfusion rate of any capillary vascular bed in the body, which is reflective of the high metabolic demands of the ocular tissues. In humans, Bruch's membrane is subject to a variety of senescent changes representing a significant cause of age-related visual defects, including age-related macular degeneration (AMD).

The external choroid consists primarily of a venous complex that drains into vortex veins, which drain into the superior and inferior orbital veins. Pigmented reticular tissue residing in the choroidal space supports a network of mid-sized anastomosing vessels that are distributed between the choroid and choriocapillaris. Melanocytes are dispersed in the network and have a role in absorption of excess radiation. Most domestic species, including the dog and cat, have a tapetum lucidum, a layer of reflective tissue interspersed between the choriocapillaris and the mid-sized vessels. The tapetum is composed of a multi-layered complex of polyhedral cells (iridocytes) that contain cytosplasmic light reflective crystals. In the dog, these are composed of zinc cysteine (Ollivier et al. 2004). The tapetum is thought to

enhance vision under scotopic conditions by reflecting light that has passed through the retina, back to the photoreceptors, increasing their activation. The organelles and crystal composition of these cells varies across species, and may have drug-binding characteristics that are also species variable.

14.4.8 Lens

The lens is a transparent tissue composed of highly specialized cells (lens fibers) that is an important part of the ocular optical system. The lens functions to refract light and focus images on the plane of the retina. It is suspended in the aqueous humor of the posterior chamber and held in place by the zonular fibers (zonules) and the anterior face of the vitreous body. Structurally, the lens composed of three parts: the lens capsule, the lens epithelium and the lens fibers.

The lens capsule is a thick basement membrane produced by the lens epithelium that completely envelops the lens. The anterior aspect of the capsule is characteristically thicker than the posterior. It is composed of collagen type IV and sulfated glycosaminoglycans which confers the elastic capacity necessary for the change in shape that drives visual accommodation in the mammalian eye (Peczon et al. 1980).

The lens epithelium is composed of simple cuboidal cells that are only present under the anterior and equatorial lens capsule. These cells become columnar, and migrate towards the lens equator where they elongate, lose their nucleus and transform to lens fibers that comprise the majority of the lens. As the lens fibers elongate, they expand to become hexagonal structures up to 4×7 μm in cross-section, and can be up to 12 mm in length extending from the anterior to the posterior pole of the lens (Forrester 2007; Cain et al. 2006).

14.4.9 Vitreous

The vitreous body, an optically clear viscoelastic gel-like extracellular matrix, fills the vitreous cavity, the space that spans the distance from the posterior pole of the lens to the inner aspect of the retina. The presence of the vitreous maintains the position of both the lens and the globe, and provides shape to the globe. More than 95% of the vitreous gel weight is water, while the remaining balance is comprised of the structural components hyaluronon, heterotypic fibrils of collagens type II, V/XI, and IX, fibronectin, fibrillin, opticin and a small population of resident macrophages (hyalocytes) (Bishop 2000; Crafoord et al. 2014). The transparency of the vitreous allows light to pass through unaltered, and is dependent on the spatial arrangement of these components. Sourcing for vitreous collagen appears controversial, and has been variably ascribed to synthesis within the ciliary body, hyalocytes, and retinal Müller cells (Bishop 2000; Sebag et al. 1992). Collagen molecules assemble into

highly organized, long, thin fibrils of copolymers: a central core of collagen V/XI, surrounded by a cross-linked, staggered array of collagen II (Bishop 2000). Type IX collagen is a chondroitin sulfate proteoglycan that is distributed and cross-linked to the surface of fibril bundles, and has a role in the spacing and distribution of the fibrils within the vitreous. Collagen bundles are interlinked by additional collagen fibrils to form a scaffold inflated by arrays of hyaluronon, a hydrophilic glycoprotein interspersed between the collagen bundles that attracts both water and counter ions (Crafoord et al. 2014). Vitreous structural differences across species are related to differences in hyaluronan concentration, which forms the liquid vitreous. The rabbit, dog, cat, and rodent have a predominantly gel vitreous throughout life; the concentration of hyaluronan relative to collagen is low compared to that of humans and non-human primates (Denlinger and Balazs 2014). Amongst laboratory animals, the vitreous of the rhesus monkey (Macaca mulatta) has the most similarities to that of the human, with respect to collagen and hyaluronan content, structural characteristics, and aging (Denlinger and Balazs 2014). Differential distribution of collagen and proteoglycans within the vitreous results in two basic zones of varying densities, the cortical (peripheral) vitreous and medullary (core) vitreous. The cortical vitreous is relatively more condensed and fibrillar compared to the medullary vitreous, which in some species tends to be more liquid. The highest concentration of collagen fibers occurs in the vitreous base of the cortex, which circumferentially straddles the ora serrata. The fibers of the vitreous base are arranged perpendicular to the surface of the retina. Tight mechanical adhesions are formed anterior and posterior to the ora serrata through interdigitations of these fibers with epithelial cells of the ciliary body, and neuroglia of the peripheral retina, respectively (Tozer et al. 2014). The anterior vitreous cortex extends anterior from the vitreous base to adhere to the posterior lens capsule. The vitreous attaches to the lens capsule in a ring-like manner, forming the ligament of Wieger (Sebag et al. 1992). The posterior vitreous extends posteriorly from the vitreous base to the rim of the optic disc, which is not covered by vitreous (Tozer et al. 2014). Collagen fibers in the posterior cortex run parallel to the retinal surface. Tight adhesions are formed between the vitreous fibers and the internal limiting membrane (ILM) and glia surrounding the optic disc, macula (primates), and medullary ray (rabbit). These regions are particularly vulnerable to retinal pathology such as neovascularization or vascular malformations. Separation of the vitreous can place traction on these attachments, leading to vitreous hemorrhage or retinal tears. Persistent vitreous traction on retinal tears is an important factor in the development of a retinal detachment. Molecular components of the ECM (chondroitin sulfate glycosaminoglycans, fibronectin, opticin, laminin) residing within the vitreoretinal interface are currently thought to provide a biomechanical adhesion between the retina and the remainder of the posterior vitreous (Halfter et al. 2014; Tozer et al. 2014). The bulk of the vitreous is formed by the medullary vitreous, a cell-free mixture of collagens and hyaluronic acid (HA) existing in either a gel or a liquid state depending on the species, age, and condition of the eye. Within the medullary vitreous, the collagen fibrils generally course in an anterior posterior direction. Anteriorly, these fibrils blend with those of the basal vitreous and posteriorly they insert into the vitreous cortex.

Average vitreous volumes are as follows: human, 4.5 mL; dog, 2.9 mL; cynomolgus macaque, 2.2 mL; rabbit, 1.6 mL, rat 0.03 mL (30 μL); mouse 0.01 mL (10 μL) (Attar et al. 2013; Remtulla and Hallett 1985). In the adult eye, the vitreous volume is a relatively permanent and is fixed. However, the vitreous structure is variable in its consistency at different ages due to differences in the proportions of gel to liquid vitreous that naturally occur with aging. In some species (humans, rhesus monkey), aging of the vitreous is associated with a loss of collagen IX, collapse of the network and close apposition of the bundles known as vitreous liquefaction (Bishop 2014). However in many animals, including rodents, the vitreous remains in a predominantly gel state (Denlinger and Balazs 2014).

A small population of hyalocytes, resident phagocytic cells, resides in the cortical reous, mainly in the peripheral cortex abutting the inner surface of the retina, concentrating in the anterior vitreous base, near the ciliary processes, and surrounding the optic disc (Halfter et al. 2014). Hyalocytes express cell surface antigens characteristic of monocyte/macrophage leukocyte lineage including CD45 (leukocyte common antigen), CD64 (Fc receptor I), CD11a (leukocyte-function antigen-1), histocompatibility complex (MHC) class II antigens (Qiao et al. 2005). Hyalocytes also express F4/80, a marker common to tissue macrophages. Experimental evidence suggests that hyalocytes in the vitreous have a role in conferring immune deviation and modulating inflammation that occurs with delayed hypersensitivity to antigens (Kita et al. 2014; Sakamoto and Ishibashi 2011). In non-inflamed eyes, this has been coined vitreous cavity-associated immune deviation (VCAID). Immune privilege has been shown to be lost in mice with inflamed eyes, and in knockout mice deficient in natural killer T cells, a critical component of immune deviation (Sonoda et al. 2005). However, current literature also suggests that hyalocytes play a crucial role in the formation and contraction of proliferative membranes that form in diseased eyes, such as proliferative diabetic retinopathy (PDR) and proliferative vitreoretinopathy (PVR), in response to overexpressed growth factors (Kita et al. 2014).

The vitreous contains a number of molecules that regulate or inhibit angiogenesis, including opticin, pigment epithelium derived growth factor (PEDF), leucine-rich alpha-2 glycoprotein (LRG1), and thrombospondins. Opticin, a glycoprotein distributed throughout the vitreous, but particularly in association with the internal limiting membrane (ILM) of the retina, is an important inhibitor of angiogenesis that prevents endothelial cell adhesion to collagen fibrils (Bishop 2014). Thrombospondins 1 and 2 inhibit the proliferation and migration of endothelial cells (Bishop 2014; Masli and Vega 2011).

14.4.10 Retina

The retina is a highly differentiated and complex multi-layered neural tissue derived from an outward extension of the rostral neural tube. Vertebrate vision is initiated through the transduction of light energy to neural impulses by the retinal

photoreceptors. The retina can be roughly divided into an inner and outer neural retina. The outer neural layer is supported by the retinal pigment epithelium (RPE), which are not physically attached to one another, but are separated by a potential space. The outer neural retina, particularly the photoreceptor cells, consumes oxygen and nutrients at a higher rate than any other tissue in the body. For this reason, the choroidal blood supply to the RPE is designed to deliver oxygenated blood and remove metabolic wastes through high volume, fast flow rates. The metabolic needs of the inner retina are less than that of the outer retina, and supplied by an endogenous retinal vasculature, which has a more conventional flow rate. Mice, rats, dogs, and nonhuman primates have an extensive retinal vascular bed (i.e., a holangiotic pattern). In contrast, rabbits have a limited retinal vascular system (i.e., a merangiotic pattern), and most of the retina lacks an endogenous blood supply. Vessels in the rabbit are located internal to the surface of the medullary rays, and attach to the retinal surface by glial cell processes (Matsumoto et al. 1984). The metabolic needs of the rabbit retina are largely met through diffusion.

The neural retina is a 9-layered neural tissue responsible for phototransduction and the signal processing across a three-neuron network that ultimately ends in the transmission of nerve impulses to the brain by the retinal ganglion cells. The layers of the neural retina are listed below in order, from the inner to the outer layer of the retina.

1. Inner limiting lamina (ILL). A thin, transparent basement membrane, synthesized by the basal foot processes of the Müller cells (retinal glia). The ILL separates the retina and the vitreous body. The ILL ceases at the rim of the optic disc, which is instead covered by a basal lamina, thought to be derived from astrocytes in the disc (Halfter et al. 2014).
2. Nerve fiber layer. Axons from the retinal ganglion cells form the nerve fiber, and course the surface of the retina to converge at the optic disc, forming the optic nerve. Axons are interspersed with astrocytes and other glial cells. The vasculature of holangiotic species resides in the nerve fiber layer.
3. Retinal ganglion cell layer. Ganglion cells are third order neurons that collect integrated signals from interneurons of the inner nuclear layer, and transmit nerve impulses via the optic nerve to the brain. Astrocytes are also present in this layer.
4. Inner plexiform layer (IPL). Synapses between retinal ganglion cells and the interneurons of the inner nuclear layer (bipolar cells, amacrine, and interplexiform cells) are formed in the IPL.
5. Inner nuclear layer (INL). Interneurons (second order neurons; bipolar, amacrine, horizontal, and interplexiform cells) and Müller cells are housed in the INL.
6. Outer plexiform layer (OPL). Synapses between the photoreceptor cells and the interneurons of the inner nuclear layer are formed in the OPL. Horizantal cells form connections between groups of rods and cones through lateral synapses. Rod spherules and cone pedicles also reside in the OPL.
7. Outer nuclear layer (ONL). Photoreceptors are bipolar cells oriented perpendicularly within the retina. The cell bodies and nuclei of the rod and cone photoreceptors reside in the ONL. Rod cell nuclei are distributed throughout the layer, while cone nuclei form a row inner to the external limiting membrane. Photoreceptor axons extend into the OPL, while dendrites form the photoreceptor segments.

8. Outer limiting membrane (OLM). Visualized as a line on H&E, the OLM reflects the presence of an anastomosing network of tight junctions formed between photoreceptor cells and Müller cells.
9. Photoreceptor Segments. Photoreceptor dendrites form the inner and outer segments of the retina. The portion of the dendrite residing in the inner segment has a high content of mitochondria, golgi, and rough endoplasmic reticulum necessary for high-efficiency protein synthesis. Phototransduction occurs in the outer segment, which houses the discs of the rods and cones.

There are marked morphological differences in the retina across species (Fig. 14.17), however the circuitry and function of the retina is remarkably conserved. The outer segments are composed of stacked membranous discs that house the photosensitive pigments used in the visual cycle. Rhodopsin, used in low light (scotopic) conditions and motion detection, present in rods, while the cones, which provide visual acuity, contain one of three opsins with sensitivity to either red, green, or blue light (long, medium, and short wavelength, respectively). Opsins collectively permit visual detection within the spectral range of red through violet. For mammalian species, only primates are known to have all three cone types (Boycott and Wassle 1999).

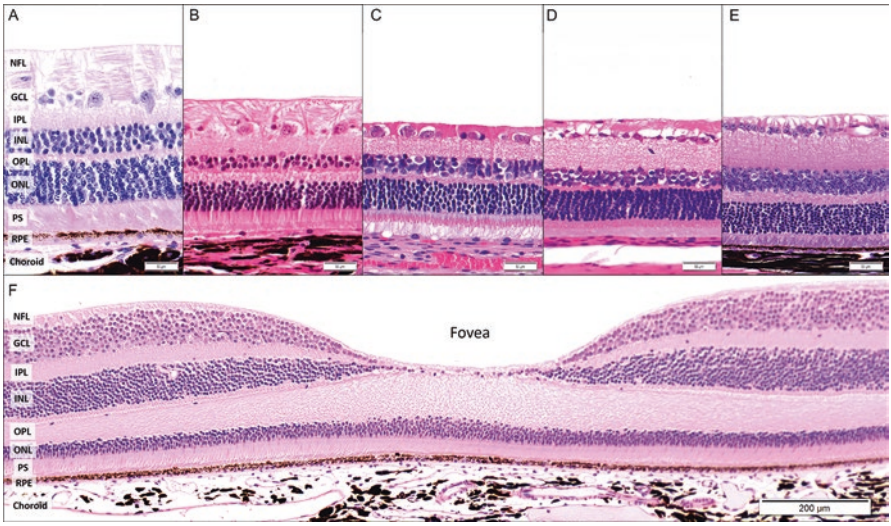

Fig. 14.17 Normal retinal microscopic anatomy of commonly use species in toxicology. Note the differences in thickness and cellular density of the different retinal layers between species. (**a**) Cynomolgus monkey. (**b**) Beagle dog. (**c**) New Zealand white rabbit. (**d**) Rat. (**e**) Mouse. (**f**) Foveal region of the retina of a cynomolgus monkey. The fovea is characterized by a depression within an avascular zone of the central retina (macula) seen in humans and most non-human primates. This region is cone exclusive and is bordered by a high density of ganglion cells. *NFL* nerve fiber layer, *GCL* ganglion cell layer, *IPL* inner plexiform layer, *INL* inner nuclear layer, *OPL* outer plexiform layer, *ONL* outer nuclear layer, *PS* photoreceptor segments, *RPE* retina pigmented epithelium. H&E

The predominant photoreceptor in the mammalian retina is the rod (95% in humans (Massey 2006)). Rod discs are cylindrical and ensheathed by the apical processes of the RPE. Discs are continuously shed and phagocytized by RPE, which recycle the vitamin A analogs crucial to conversion of light into an electrical transduction impulse. Discs are continuously replaced from the inner segment, where the mitochondria and Golgi reside, which is connected by a thin dendritic process to the outer segment. In cones, discs taper in the outer segment to form a cone-like shape; the widest part is continuous with the inner segment of the dendrite. RPE processes form leaf-like sheaths around cone discs. Mechanisms of cone disc shedding are less clear than those known for rods. The outer segments of the photoreceptor cells are amongst the highest metabolically active cells in the body. Photoreceptors are maintained in a depolarized state when the cells are not functioning, and thus they require a continuous energy supply. There is also a continuous production of discs and opsin proteins, as well as a host of enzymes and transport proteins, that are consumed and recycled by RPE phagocytosis. These processes take place in the presence of focused light, and thus are associated with oxidative stress.

Although rods predominate in all species, the respective numbers of cones and rods and the spatial arrangement within the retina have considerable species variation. The fovea, a depression within an avascular zone of the central retina (macula), of humans and most non-human primates is cone exclusive. The fovea is devoid of other cells with the exception of Müller cells. The distribution of red, green, and blue cones in the primate eye appears to be random, with the exception of the fovea, where blue cones are absent (Massey 2006). Cone pedicles are densely packed and interconnected by gap junctions of fine processes that mediate electrical coupling, particularly between red and green sensitive cones. This reduces noise while simultaneously reinforcing the light signal between neighboring cones (Massey 2006). In humans and non-human primates, cell axons are radially arranged around the macula. This design permits maximal visual acuity under bright light (photopic) conditions. Rods, which function under scotopic conditions, progressively dominate in the peripheral regions of the retina. Although rods can respond to a single photon, signals are pooled, which improves sensitivity in dim light (Massey 2006).

The regional concentration of cones in the retinas of other species and relative proportion to rods likely reflects behavioral accommodations for visual needs pertinent to the environmental niche occupied. Most mammals are dichromatic; however, they may possess hybrid cones with divergent spectral sensitivity that likely similarly reflects their behavioral needs (see Peichl for review (Peichl 2005)). Rabbits have a wide horizontal plane of vision (visual streak) suited for assessing predator encroachment. The visual streak is a linear, horizontal band (3–2 mm wide) centered ~3 mm ventral to the optic disc, parallel and below the ventral margin of the medullary ray (Prince and McConnell 1964). The retina is thicker in the ventral streak, which contains a higher density of photoreceptors and inner nuclear cells for sensory processing, and ganglion cells for signal transduction. Cones are sparsely confined to this region and are predominantly a red/green hybrid. Although the

proportion of rods is overall greater, the combination of cones and rods in this region makes the rabbit eye suitable for crepuscular activities. Blue cones may be found in the ventral retina below the visual streak (Juliusson et al. 1994).

The carnivore retina concentrates cones and ganglion cells in an oval zone arranged in a horizontal plane dorsolateral to the optic disc (Mowat et al. 2008). In dogs, cones are predominantly a red/green hybrid, with fewer blue cones (Mowat et al. 2008). Rodents may have blue, blue/green, or green/red hybrid cones and have regional distribution patterns that vary by species (Peichl 2005). Some mice have a preponderance of blue cones in the ventral retina (Juliusson et al. 1994). Retinas of nocturnal rodents contain few cones, however diurnal rodents have a relatively high concentration of cones (30–40%) (Bobu et al. 2008; Saidi et al. 2011) which may make them better models of photoreceptor pathologies.

The nuclei and cell body of the photoreceptors are housed in ONL. Rod nuclei predominate in the inner aspect of the ONL, while cone nuclei are located adjacent to the OLM. Rod degeneration usually occurs in-situ, but cone degeneration sometimes leads to a displacement of the cell into the outer retina (nuclear drop-out), which eventually undergoes RPE phagocytosis.

The INL contains the interneurons responsible for initial processing of a visual stimulus. Spatial distribution is organized by cell type: horizontal cells reside in the outer zone, bipolar cells are located central, and amacrine cells toward inner aspect. Horizontal cells have lateral connections that modify signals through negative feedback between photoreceptors and bipolar cells, modulating image boundaries (which increases resolution) (Massey 2006). Bipolar cells function vertically to modulate brightness and color information, and amacrine cells modify signal input to ganglion cells. Müller cells, which function as retinal glial cells, are also present in the INL. Müller cell processes extend from the innermost retina to the outer limiting membrane and are intimately entangled with the processes of other retinal neurons. Müller cell processes surround blood vessels, and have a role in the distribution of nutrients and metabolic support within the retina. An early indication of metabolic stress is associated with a change in Müller expression. In the healthy retina, Müller cell expression of glial fibrillary acidic protein (GFAP) is limited to the inner aspect near the inner limiting membrane, but when stressed (e.g., detachment, hypoxia or phototoxicity) GFAP expression occurs throughout the length of the cell.

The summation of the visual stimulus is transmitted as a nerve impulse by retinal ganglion cells. Ganglion cell axons pass through the optic disc into the optic nerve. Damage at any level of the nerve axons can interrupt signal transmission, and prevent retrograde delivery of vital nutrients from the brain (i.e., brain-derived neurotrophic growth substances) necessary to maintain the integrity of the ganglion cell. Damage to the axons of the ganglion cells in the nerve fiber layer, optic disc, lamina cribrosa or anywhere in the optic nerve optic (including the chiasm and optic tracts) will result in ganglion cell death. Single cell ganglion cell death can be observed as dislocation of pyknotic nuclei in the vitreous-retinal interface (nuclear drop-out).

14.4.11 Retinal Pigmented Epithelium

The Retinal pigment epithelium (RPE) is derived from neural tube ectoderm and is analogous to ependymal cells in form and function. As the optic vesicle folds on itself to form the optic cup, the RPE remains as a single cell layer in direct contact with the outer cells of the neural retina that eventually become the photoreceptor cells. The photoreceptor cells and the adjacent RPE function as a unit, and together they are responsible for phototransduction, the transformation of light energy into the electric signals that are the basis of vision.

The RPE cells are smooth and hexagonal in shape when viewed from the inner surface. In histologic sections, the RPE cells consist of an outer non-pigmented basal region with an oval nucleus and an inner, pigmented portion, which extends as a series of straight villous processes between the photoreceptor outer segments. The close association between the outer segments and the apical processes enables phagocytosis of senescent discs as they are shed from the photoreceptors (Besharse and Defoe 1998). Shedding is triggered by light stimulus following a period of darkness, a process that results in complete disc turn-over within 11 days. RPE have a critical role in the uptake and cycling of vitamin A analogs (derived from the blood, interphotoreceptor matrix [IPM], and through phagocytosis of shed discs), and subsequent generation of functional 11-*cis* retinal essential to vision. Interphotoreceptor-binding protein, secreted by RPE, manages the transport of retinoid between the photoreceptors and the RPE within the IPM, a specialized extracellular matrix containing hyaluronan, proteoglycans, glycosaminoglycans, matrix metalloproteases, and growth and immunosuppressive factors secreted by RPE (Strauss 2005). The IPM has roles in mediating retinal adhesion and facilitates the supportive function the RPE has to the retina. The IPM also helps prevent vascular proliferation in the surrounding tissues. An anastomosing network of tight junctions near the apex of the RPE cell layer forms the outer blood–retinal barrier, which is similar in structure and function to the blood-brain barrier, and has an important role in maintaining immune privilege within the eye. RPE actively transports nutrients such as amino acids, omega-3 fatty acids, and glucose to the outer retina across the tight junctions. Transcellular movement is regulated by a series of receptors, ion channels and exchangers, and organelles that have a polar distribution in the RPE, creating RPE membrane potential measurable by ERG. The movement of ions by the RPE creates an ionic gradient in the IPM that maintains photoreceptor excitability, permitting the electrical transmission of the light stimulus by the photoreceptor. Ionic flux also stabilizes IPM pH, and contributes to the passive removal of water from the retina, an end-product of metabolic activity.

The basal surface of the RPE cells is characterized by extensive infolding. The basal lamina of the RPE cell forms the innermost layer of the five-layered Bruch's membrane which separates the retina from the choriocapillaris. Melanin pigment in the RPE protects the outer retina from excessive light reflection and glare, reducing oxidative damage.

14.4.12 Optic Nerve

"Optic nerve" is a misnomer since this structure actually is a central nervous system (CNS) tract. The optic nerve is composed primarily of the axons from ganglion cells (GC), which extend from the inner retina, coursing posterior and centripetally within the inner nerve fiber layer to converge into bundles at the optic nerve head (also known as the optic disc) and form the nerve. The position of the optic disc demonstrates some species variation. In dogs and primates, it tends to be positioned equatorial, and in the rabbit, in the dorsal posterior pole. The central area of the optic disc is depressed and is supported by a thickening of the retinal inner limiting membrane (called the supporting meniscus of Kuhnt). Before exiting the eye, the GC axons are arranged into bundles surrounded by retinal astrocytes. As the axon bundles exist the eye posterior to the choroid, they transverse an open meshwork of collagenous beams or plates continuous with the sclera (lamina cribrosa). The lamina cribosa (LC) contains elastin and provides structural support for the nerve. In glaucoma, physical distortion of the globe resulting from elevated IOP causes outward bowing of the LC; the physical distortion and misalignment of the laminar plates results in axon compression, atrophy, and subsequent ganglion cell death. The composition of the LC is similar across species, but there are structural differences. Nonhuman primates, pigs, dogs, cats and rabbits have a robust LC comparable to humans; in rats, single bundles of collagen form the trabeculae resulting in a delicate LC. Mice do not have a distinct LC (May 2008).

The axons of the optic nerve are myelinated by oligodenrocytes, the extent of which varies across species. In dogs, myelination occurs within the optic disc, with variable extension within the peri-papillary nerve fiber layer; these differences account for variability in shape of the optic disc observed on fundic imaging. The rabbit is unique, in that it has a medullary ray, a vascularized region on either side of the optic nerve that appears as a zone of white rays emanating from the optic disc on fundic imaging. The appearance is attributed to the myelination of ganglion cell axons as they course over the posterior retina, prior to converging at the optic disc. In other species, (nonhuman primates, cats, pigs and rodents), myelination commences at the outer margins of the lamina cribosa similar to humans (May 2008). In these species, the optic disc appears comparatively small, round, and dark. Myelination extends to the optic chiasm. There is no sexual difference, or difference between right and left globes, in the number of myelinated fibers in the optic nerve, but the degree of myelination varies significantly among species. As an example, myelination in primates is tenfold that of mice. In mice, myelination diverges substantially among the different strains (Smith 2002).

The optic nerve is surrounded by extensions of the three meningeal sheaths of the central nervous system. The pia mater covers the surface of the nerve and forms interdigitations and septations throughout the neuropil, separating the axons into discrete nerve bundles. The subdural and arachnoid spaces communicate directly with their counterparts inside the cranium.

The optic nerve extends from the back of the globe to the floor of the cranial cavity, where the two nerves intersect at the optic chiasm. The degree of crossover in the chiasm varies among species, from relatively little (e.g., dog) to more than 50% (e.g., nonhuman primates). The axons synapse in the visual relay center within the lateral geniculate body of the midbrain.

The vascular supply to the optic nerve is complex. The optic disc and region anterior to the LC is supplied by collaterals from the choroid and retinal circulation. The LC zone is supplied by branches from the short posterior ciliary arteries and arteries that supply the pia. The nerve posterior to the LC is supplied by the arteries to the pia. Venous drainage occurs through the central retinal vein and pial veins (May 2008).

In dogs, a small dark spot viewed on fundic examination within the center of the optic disc, known as the physiologic pit, is considered a remnant of the hyaloid artery. An exaggeration of the normal thickening of glial cells over the optic nerve (called Bergmeister's papilla) is also considered a remnant of the hyaloid artery (Gellat et al. 2014).

14.4.13 Ocular Blood Supply

The ocular blood supply is derived from the ophthalmic artery (internal, and/or external), the first branch off the internal carotid artery (humans, primates), or from a branch off the internal maxillary artery supplied by the external carotid (most domestic species) (Samuelson 2007).

The central retinal artery (CRA), the short and long posterior ciliary arteries (PCA), and the anterior ciliary arteries (ACA) are branches from the ophthalmic artery.

The rectus muscles are supplied from branches the ACAs, the superficial ciliary arteries. These continue forward to form the episcleral arterial circle, a ring of connected vessels that encircles the globe posterior to the limbus and superficial to the intra-ocular circle. The intra-ocular circle is supplied by the long PCA.

The iris and ciliary body are supplied by the ACA, the long PCA, and anastomotic connections from the anterior choroid vasculature. The major arterial circle of the iris is supplied by the ACAs, which pierce the scleral near the limbus, and the long PCAs, which enter the sclera near the posterior pole, and travel anterior in the choroidal space. The major arterial circle of the iris gives off branches to both the iris and ciliary body.

There are distinct species differences in the inner retinal vascularization that reflect differences in retinal design and functional adaptation to environmental demands. In most mammals, the blood distribution to the retina is holangiotic (the rabbit, a notable exception, is merangiotic).

In the holangiotic retina, blood flow is derived from the CRA, branching off the ophthalmic artery in humans and non-human primates (Harris et al. 2006), species

that have a macula with a fovea, a cone-exclusive region of the retina designed for acute photopic vision. The CRA emerges from the optic nerve head to form superior and inferior branches. The temporal branches supply the macula, arching and creating a 0.4 mm capillary-free zone around the fovea (Hayreh 1974). This design optimizes light transmission while minimizing scatter to the cone-rich photoreceptors of this region that permits vision acuity. The inner 2/3 of the retina is supplied by arteries limited to the nerve fiber layer; branching superficial capillaries supply the ganglion cell and nerve fiber layer, while deeper capillaries penetrate the retina to the inner nuclear layer.

In species that lack a macula, the retinal blood supply is derived from the posterior ciliary arteries branching off the external ophthalmic artery. Although the vascular pattern is divergent across species, in general the vessels form a radial pattern originating from the posterior ciliary artery at the optic disc. In dogs and pigs, the retina contains a region of increased ganglion cell and cone-rich density that is lateral and slightly dorsal to the optic disc, referred to as the area centralis. The area centralis is relatively devoid of blood vessels, and a fovea of sorts is present that is the source of visual acuity. Rodents display weak propensities for centralized cone concentrations in any portion of the retina, reflected in a more uniform distribution of the vessels. In the rabbit, which is merangiotic, the retina is avascular. Arteries supplying the retina are derived from the posterior ciliary arteries and lie internal to the retina in close opposition to the internal limiting laminae through glial cell attachments, and within invaginations of the neural fiber layer (Tripathi and Ashton 1971). Blood vessel loops extend from the optic disc in the ventral and lateral planes over the retinal surface the extent of the medullary rays, (myelinated axons of the ganglion cells which course on top of the retina before exiting the globe through the optic nerve). Branches from these loops form a modest capillary network that extends into the inner retina to the level of the outer plexiform layer. Rabbits possess an extended region within the retina comparable to the area centralis known as the visual streak, a horizontal band in the posterior retina inferior to the optic disc. Ganglion cells and photoreceptors occur in higher density in the visual streak than that of the peripheral or dorsal retina.

Many mammals have no endogenous retinal vessels (i.e., a parangiotic or anangiotic pattern). Among these species, the guinea pig is commonly used in laboratory experiments to evaluate basic visual system biology.

14.4.14 Lymphatics and Draining Lymph Nodes

The presence and distribution of lymphatic vessels throughout the ocular tissues has been a relevant and controversial topic. The eyelids and conjunctiva are rich in lymphatics (Gusev 1964) (Walls 1942). Except for those structures, the eye was thought to lack lymphatic vessels; however studies utilizing lymphatic endothelial markers (e.g., Lyve-1 or podoplanin) and lymphangiogenic factors (e.g., VEGF-C), have revealed the existence of lymphatics in the corneal limbus, lacrimal gland, orbital

meninges, and extraocular muscles (Dickinson and Gausa 2006; Gausas et al. 1999; Krebs and Krebs 1988). Multiple studies attempted to demonstrate the presence of lymphatic channels in the uveal tissues with contradictory results. Some authors described the presence of distinct lymphatic channels in the human ciliary body, and suggested the presence of a novel "uveolymphatic" outflow pathway (Yücel et al. 2009),(Birke et al. 2010). Similarly, the presence of a lymphatic-like system in the choroid of birds and primates has been described by electron microscopy but, recent publications using the previously mentioned lymphatic markers failed to demonstrate these vessels in the human choroid (De Stefano and Mugnaini 1997; Matsumoto et al. 1984; Schroedl et al. 2008).

In humans and most species of interest, the lymphatic drainage from the eye occurs mainly through the peri-auricular, parotid and mandibular lymph nodes (Cook et al. 2002; Evans and de Lahunta 2013). A specific pattern of lymphatic drainage in seen in the eyelids. In cynomolgus monkey lymphoscintigraphy studies reveal discrete lymphatic drainage pathways for the upper and lower eyelids (peri-auricular, parotid lymph nodes) and a dual pathway for the central upper eyelid (peri-auricular and submandibular-anterior cervical lymph nodes) (Cook et al. 2002).

14.5 Regulatory Aspects of Ocular Drug Development

14.5.1 Preclinical Development of Ocular Drugs

Ocular drug development is very active in pharmaceutical and biopharmaceutical companies. Ocular drugs may be delivered through various routes, including topical, intravitreal, periocular (subconjunctival, retrobulbar, peribulbar, and posterior subtenon injections)(Short 2008). Indications may include conjunctival diseases, ocular hypertension, dry eye, retinopathy, and macular degeneration. Table 14.1 lists a few examples of the nonclinical programs of drugs for ophthalmology approved by the U.S. Food and Drug administration (FDA). This section provides an overview of the regulatory aspects of ocular drug development and more specifically as it relates to immunopathology; more comprehensive discussion related to regulatory aspects and nonclinical study parameters and designs can be found in Weir and Collins (2013) (Weir and Collins 2013), Attar et al. (2013) or direct interaction with the regulatory agencies.

The U.S. Food and Drug Administration (FDA) and Organisation for Economic Co-operation and Development (OECD) provide some guidance for ophthalmic drug development (ICH-M3(R2) 2010; ICH-S2(R1) 2011). However, there is currently no distinct regulatory pathway for preclinical development of ocular drugs (Huml et al. 2009; Novack 2009; Short 2008). Within the U.S., the ophthalmic drug products may be regulated by one of the following centers within the FDA:

- Center of Drug Evaluation and Research (CDER), Office of New Drugs, Division of Transplant and Ophthalmology (DTOP);

Table 14.1 Nonclinical programs of FDA approved ocular drugs

	Xalatan	Travatan	Lumigan	Lucentis	Eylea
NDA/BLA (approval year)	NDA 020597 (1996)	NDA 021257 (2001)	NDA 021275 (2001)	BLA 125156 (2006)	BLA 125387 (2011)
Active ingredient	Latanoprost	Travoprost	Bimatoprost	Ranibizumab	Aflibercept
Dosage form/route	Solution/topical ocular (TO)			Solution/intravitreal injection	
Indication	Reduction of elevated intraocular pressure in patients with open angle glaucoma or ocular hypertension			Neovascular (wet) age-related macular degeneration	
Pharmacokinetics[b]	Rats (PO, IV, TO), rabbits (IV, TO), dogs (IV), monkeys (PO, IV, TO)	Rats (IV, SC), rabbits (IV, TO), dogs (IV, TO)	Mice (IV, PO), rats (IV, PO), rabbits (TO), monkeys (IV, PO, TO),	Rabbits (IV, IVT), monkeys (IVT)	Rats (IV, SC), rabbits (IVT), monkeys (IV, IVT, SC)
Repeat-dose ocular toxicity[c]	1 year: rabbits, monkeys (TO)	6 months: rabbits (TO); 1 year: monkeys (TO)	6 months: rabbits (TO); 1 year: monkeys (TO)	26 weeks: monkeys (IVT)	8 months: monkeys (IVT)
Repeat-dose systemic toxicity[c]	13 weeks: mice (PO), rats (PO, IV), dogs (IV)	13 weeks: mice, rats (IV); 6 months: rats (SC)	1 year: rats (PO);17 week: monkeys (IV)	–	13 weeks: rats (SC);6 months: monkeys (IV)
Genotoxicity	√	√	√	–	–
Carcinogenicity	Mice, rats (PO)	Mice, rats (SC)	Mice, rats (PO)	–	–
DART	Rats, rabbits (IV)	Mouse, rats (IV, SC)	Mouse, rats (PO)	–	Rabbits, monkeys (IV)
Safety pharmacology	√ (IV)	√ (IV, SC)	√ (IV)	√ (IVT)[d]	√ (IV)

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[a] *Sources*: www.fda.gov; www.pharmapendium.com; product inserts

[b] Absorption, distribution, metabolism, and/or elimination

[c] Studies with the longest treatment duration; ocular toxicity studies included evaluation of systemic tissues

[d] Safety pharmacology endpoints were incorporated into repeat-dose toxicity studies

- Center for Biological Evaluation and Research (CBER) Office of Cellular, Tissue and Gene Therapy or Office of Blood Research and Review; or
- Center for Devices and Radiological Health (CDRH), Office of Device Evaluation, Division of Ophthalmic, Neurologic and Ear, Nose and Throat Devices.

Table 14.2 provides a comparison of nonclinical studies designed to support an ophthalmic drug versus a drug administered systemically. The extent of studies required for ophthalmic drug development can vary depending on several factors, such as if it is a new molecular/biological entity (NME/NBE), reformulation or new route of administration of currently marketed drug, combination product, sustained release product, or delivered as a device. The guiding principles for chemical entities (small molecules) are included in ICH M3(R2)(ICH-M3(R2) 2010) and the guiding principles for biologics (e.g., cytokines, plasminogen activators, recombinant plasma factors, growth factors, fusion proteins, enzymes, receptors, hormones, and monoclonal antibodies and applicable recombinant DNA protein vaccines, chemically synthesized peptides, plasma derived products, endogenous proteins extracted from human tissue, and oligonucleotide drugs) are included in ICH S6(R1)(ICH-M3(R2) 2010; ICH-S6(R1) 2011). For NCE/NBE, a full development package, including

Table 14.2 Comparison of nonclinical studies designed to support an ophthalmic drug vs. a drug administered systemically

Study type	Needed for ophthalmic drug	Needed for systemically administered drug
Pharmacology	Yes	Yes
In vitro metabolic stability and plasma protein binding for humans and animals	Yes	Yes
Pharmacokinetics	Yes	Yes
Safety pharmacology	Often no	Yes
Genotoxicity	Yes (generally limited to small molecules)	Yes (generally limited to small molecules)
General toxicology	Yes (generally using an ocular and systemic route of administration)	Yes (generally limited to the clinical route of administration)
Reproductive toxicology[a]	Potential for waiver	Yes
Fertility[a]	Generally yes	Yes
Embryo-fetal development[a]	Yes	Yes
Pre-, postnatal development[a]	Potential for waiver	Yes
Photosafety[a]	Yes	Yes
Carcinogenicity[a]	Potential for waiver	Yes (if needed)
Tissue cross-reactivity	Yes (generally limited to monoclonal antibodies)	Yes (generally limited to monoclonal antibodies)

[a] If conducted, generally route of exposure is systemic (e.g., intravenous, subcutaneous, oral). Reproduced and adapted by permission from Weir and Collins (2013) (Weir and Collins 2013)

local and systemic repeat-dose, safety pharmacology, genotoxicity, reproductive and developmental toxicity, and/or carcinogenicity may be required for registration. For reformulation, new route of administration, and some combination products of currently marketed products, an abbreviated drug package (Table 14.2) that includes only local toxicity studies may be necessary for registration if preclinical systemic toxicity studies have already been conducted. For drug delivery systems, the product would most likely be considered a drug if the delivery system is a platform for delivery; however, if the delivery system is itself a device with its own indication, then it would most likely be regulated as a combination product which would then include a mixture of drug and device studies (Novack 2009).

The pivotal toxicology studies, in addition to safety pharmacology studies, are expected to be performed in compliance with Good Laboratory Practice (GLP); whereas the early pharmacodynamics, pharmacokinetics, and dose-range toxicity studies are generally conducted non-GLP. Additionally, some specialized toxicology parameters within a study may not be able to comply fully with GLP (e.g., instrument or method not validated), but may still be used to support regulatory submissions and these items are cited as such on the report compliance section.

14.5.2 New Molecular/Biological Entities

The development of NME/NBE require conduct of the typical systemic drug development package in addition to ocular pharmacology, pharmacokinetics, and toxicity studies (ICH-M3(R2) 2010; ICH-S6(R1) 2011). It is critically important that toxicity findings are correlated to pharmacology, pharmacokinetics, and/or immunogenicity to understand the how the effects may translate to humans.

14.5.3 Ocular Pharmacology

Ocular pharmacology/pharmacodynamics studies are generally performed to test the efficacy of the drug and/or to determine its mode of action. Additionally, pharmacology studies may be used to justify species selection for preclinical studies (e.g., sequence homology, in vitro binding, cross reactivity, binding affinity, functional activity). These studies may be conducted in laboratory animals or in vitro systems and are used to characterize primary pharmacodynamics (related to the therapeutic target), secondary pharmacodynamics (not related to the therapeutic target), and safety pharmacology (effects on physiological functions). Studies that address pharmacodynamic may include sequence homology, in vitro binding, cross reactivity, relative binding affinity, and in vitro and in vivo functional activity. Typically the pharmacodynamics studies are not conducted in accordance with GLP. Safety pharmacology studies may include stand-alone in vivo studies (e.g.,

cardiovascular, CNS, respiratory) in addition to in vitro hERG activity assays. Stand-alone studies in vivo may not be needed for ocular drugs where the pharmacology of the drug substance is well characterized, and where systemic exposure or distribution to other organs or tissues is demonstrated to be low (ICH-S7A 2000). If systemic exposure from ocular dosing is high, an acceptable option may be to include the safety pharmacology parameters (e.g., physical examination, respiration rate, heart rate, and body temperature) in the repeat-dose study if sufficient exposure can be achieved (Authier et al. 2013); otherwise, a stand-alone study with systemic administration may be necessary. Safety pharmacology studies or parameters that are included in pivotal toxicity studies that includes the safety pharmacology parameters, as well as the in vitro hERG activity assay, are typically conducted in accordance with GLP (ICH-S7A 2000) and are conducted prior to any clinical trials.

14.5.4 Ocular Pharmacokinetics

Ocular pharmacokinetic studies are conducted to evaluate the systemic bioavailability (e.g., blood, plasma, or serum) and ocular distribution in relevant tissues (e.g., conjunctival, cornea, iris/ciliary body [ICB], choroid, sclera, lens, lacrimal gland, trabecular meshwork, retina, aqueous and vitreous humor, eyelid) after ocular dosing in animals. There are challenges associated with ocular pharmacology assessments due to the variability associated with non-serial sampling and development of sensitive bioanalytical methods in multiple matrices (Attar et al. 2013). Additionally, an in vitro assay to determine whether the drug binds to melanin may also be performed. The in vivo pharmacokinetic studies are generally performed in one relevant species, typically rabbit, dog, monkey or pig due to the eye size and anatomical considerations. These studies are generally not conducted according to GLPs.

Systemic exposure (Cmax, AUC, and apparent half-life) after ocular dosing should be evaluated prior to clinical trials, and typically is characterized in the pivotal toxicology studies (see below). Systemic exposure is usually assessed after the first and last dose, and for a sufficient time period to demonstrate clearance. Additionally, prior to Phase 3, the absorption, distribution, metabolism, and excretion should be characterized but typically these are conducted prior to any clinical trials (ICH-M3(R2) 2010). In some cases, such as ocular sustained –release products, the systemic exposure may not reflect clearance of the drug from the eye (Weir and Collins 2013). As such, ocular distribution after dosing should be characterized.

Assessment of melanin binding for NME is recommended because the pharmacokinetics and pharmacodynamics of a drug can be affected by melanin retaining the drug and subsequently releasing it (Attar et al. 2013; Guadana et al. 2010). Therefore, it is important to determine whether a drug binds to melanin as it affects the species selection for ocular toxicity studies (e.g., conducted in the standard non-pigmented ocular model [New Zealand White (NZW)] Rabbit) or a pigmented strain [e.g., Dutch-Belted Rabbit]).

14.5.5 Ocular Toxicity Studies

To establish the toxicity profile of a drug after ocular dosing, single- and repeat-dose ocular toxicity studies are conducted. These studies are conducted to identify local ocular effects and if there are systemic target organs. If adverse effects are observed, these studies should assess whether they are reversible. The shorter-term studies (e.g., tolerability studies) are used to establish the dose levels for longer-term pivotal studies (e.g., toxicity studies) and the longer-term studies are used to establish the starting dose levels in the clinic.

The vehicle and intended clinical formulation and same route of exposure should be used in the pivotal nonclinical ocular toxicity studies. The excipients used in the formulations listed in the FDA inactive ingredient database (http://www.accessdata.fda.gov/scripts/cder/iig/index.cfm) in a similar manner may preclude the need for additional studies as the case is for novel excipients. In cases where the drug product is modified during the course of development (e.g., change of excipients), bridging studies to compare bioavailability of the formulations may be required to demonstrate safety.

The dosing frequency in the ocular toxicity studies should be at least as frequent as that intended for humans and the duration should be as long as the clinical duration, similar to systemic toxicity studies (ICH-M3(R2) 2010). Termination of the main study animals should coincide with the potential detection of acute toxicity, typically shortly after the last dose.

The range of doses should enable an assessment of dose-response relationships and a determination of an adequate margin of exposure to the clinical dose. To allow for exaggerated exposure, an enriched formulation (e.g., greater than clinical dose) should be included; however, if it is not feasible, then the dosing frequency may be increased to achieve greater exposure. For small molecules, toxicity should be demonstrated at the high dose (ICH-M3(R2) 2010). For biologics, dose selection should be based on a dose which provides the maximum intended pharmacological effect in the preclinical species; and (2) a dose which provides at least a tenfold exposure multiple over the maximum exposure achieved in the clinic (ICH-S6(R1) 2011; Leach 2013b). The higher of these two doses should be chosen for the high dose group unless there is a justification for using a lower dose (e.g., maximum feasible dose). The low dose should match the human exposure (Leach 2013b). For both small molecule and biologics, toxicity may be not observed with ocular dosing and dosing to toxicity is not feasible. Generally, ocular toxicity studies should be designed to assess a dose response relationship and provide an adequate margin of exposure to the clinical doses. Systemic toxicity studies are also conducted to identify toxicity at higher exposures.

For ocular toxicity studies, typically non-rodent models are used (e.g., rabbit, dog, monkey, pig) because their large eye permits easier dosing and ophthalmic examination. The relevant species may depend on pharmacology, drug metabolism, and species anatomy. In cases where the drug binds to melanin, the relevant model would be a species with pigmented eyes (e.g., Dutch-belted rabbit, dog, monkey). For NME,

typically toxicity is evaluated in two species. For NBE, such as humanized antibodies, there monkey may be the only relevant species to conduct the toxicology studies.

For sustained release products, the interval between dosing in repeat-dose animal studies should be dependent on the expected duration of drug release and should be similar to or shorter than that proposed in the clinical studies (FDA 2015c; ICH-S6(R1) 2011). Drug release typically is estimated by in vivo pharmacodynamic and in vitro release data. Ocular distribution studies should also be performed to determine kinetics and clearance of the drug from the eye.

The minimal standard parameters that are typically evaluated in the ocular toxicity studies are listed in Table 14.3. Collection of systemic parameters (e.g., clinical pathology, gross necropsy, ECGs) should occur at time of maximal exposure (C_{max}) to assess acute toxicities or effect or where minimal, if any, recovery has occurred. Reversibility of effects should also be evaluated.

Ocular examinations evaluate the anterior segment of the eye using slit-lamp biomicroscopy (with and without fluorescein staining of the cornea, if required), in addition to the posterior segment of eye using direct and indirect ophthalmoscopy. For ocular irritancy and inflammation in the anterior segment of the eye, the modifications to the eye may be quantified using McDonald-Shadduck, Draize, Hackett-McDonald, and/or standardization of uveitis nomenclature (SUN) scoring systems (Thomasy et al. 2016). Additional ocular endpoints that may be considered are: optical coherence tomography (OCT), gonioscopy, pachymetry (corneal thickening), corneal sensitivity, pupillary response, Schirmer tear test (STT), and fluorescein angiography. Typically, the endpoints are evaluated at Cmax and at the end of study, and during recovery.

Histopathology examination of the ocular tissues are conducted to identify structural changes in the eye from ocular dosing. Typically, during necropsy, the eye is removed by enucleation and the ocular tissues are carefully processed and sectioned (Attar et al. 2013). The microscopic findings should be correlated to any ocular findings, and in particular timing of observations with respect to timing of administration. For NME, full systemic evaluation in pivotal repeat dose ocular studies is generally recommended even if systemic exposure is expected to be low or below the level of quantitation (BLQ). In certain cases, systemic evaluation in ocular study may substitute for systemic study in a second species if sufficient systemic exposure to evaluate toxicity can be achieved with ocular dosing. Gross and microscopic systemic tissues may be evaluated or, minimally, should be collected from pivotal ocular studies and stored for possible future evaluation should findings occur in systemic studies. Clinical pathology and ECGs may also be considered if sufficient systemic exposure occurs with ocular dosing. However, if the systemic exposure at the NOAEL in the systemic study is considerably higher than that observed in the ocular toxicity studies, then systemic parameters likely will not need to be evaluated in ocular studies.

There is tolerance from the regulatory agencies to histologic findings from an expected adaptive immune response (Ponce et al. 2009), but adverse pharmacological effects may lead to the discontinuation of the development program (see above section, Immunopathology of the Eye). The FDA approved LUCENTIS and EYLEA with transient increase in intraocular pressure (IOP) due to the increased intraocular

volume and inflammation in both the anterior and posterior segments that likely resulted from the intravitreal dosing procedure (as previously described in Immunopathology of the Eye, Innate Responses) in the preclinical studies, as these finding did not represent a major clinical concern (Genentech 2006; Regeneron Pharmaceuticals 2011). In both cases, the effects were dose-responsive, transient and reversed or were reduced during the recovery period and no abnormal IOP, cataract, or fluorescein leakage changes were noted.

Similar to biologics administered systemically, pharmacokinetics and immunogenicity assays in blood and tissues should be conducted in preclinical ocular studies to detect and confirm the presence of anti-drug antibodies (ADAs) if warranted. Within toxicology studies, immunogenicity samples should be collected in conjunction with PK samples at potentially peak concentrations (10-14 days postdose) (Leach 2013b). Immunogenicity may affect the pharmacokinetics or efficacy of biologics by reducing or eliminating the activity of the test article, neutralizing the endogenous molecule, and/or result in hypersensitivity reactions (e.g., either immediate [type I] or immune-complex mediated [type III]) (Leach et al. 2014)·(ICH-S6(R1) 2011). Therefore, any pharmacological and/or toxicological changes in preclinical studies should be correlated with the presence of ADAs. Immunogenicity in nonclinical species may not be predictive of immunogenicity in humans, and should be taken into consideration prior to modifying the study design (e.g., dosing regimen or termination) unless the immune response neutralizes the pharmacological and/or toxicological effects of the biopharmaceutical in a large proportion of the animals (Buelski and Treacy 2004; ICH-S6(R1) 2011; Leach 2013b). Development of ADA may preclude the evaluation of the test article in long-term safety studies or carcinogenicity study due to reduced exposure in certain species, as such, alternative animal models may need to be considered. Additionally, for monoclonal antibodies, the immunological properties of the antibody should be described in detail in regulatory filings, including its antigenic specificity, complement binding, and any unintentional reactivity and/or cytotoxicity towards human tissues distinct from the intended target (ICH-S6(R1) 2011).

Table 14.3 Standard parameters evaluated in nonclinical ocular toxicity studies

Ocular endpoints	Systemic endpoints
Gross observations	Clinical observations
Slit lamp bimicroscopy	Body weight
Funduscopy	Food consumption
Tonometry	Clinical pathology
Electroretinography	Necropsy
Histopathology	Organ weights
	Histopathology
	Toxicokinetics
	Immunogenicity (biologics)

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14.5.6 Systemic Toxicity

Systemic toxicity studies are also conducted to supplement ocular toxicity studies for which systemic concentrations may be too low. If the systemic bioavailability after ocular dosing is low and not associated with adverse effects, some study types may not be required or may be waived by the FDA as presented in Table 14.1. The parameters evaluated in the systemic studies are listed in Table 14.3 for systemic endpoints, but may also include central nervous system (CNS) or electrocardiogram (ECG) evaluation at T_{max}.

For systemic toxicity, the route of administration may depend on the ocular route of delivery. For evaluation of topical ocular drugs, the oral route may be used because the drug may be swallowed (e.g., through drainage of the nasolacrimal duct) and be metabolized through the gastrointestinal tract. For ITV dosing, the more appropriate systemic route of exposure may be parenteral (IV). Systemic toxicity is typically conducted in two relevant species. However, there may be only one relevant species in the case of most biologics or if systemic exposure with systemic dosing is negligible, a single species may be considered adequate. Additionally, in short term pivotal studies, if two species show similar findings, then a single species may be adequate.

Carcinogenicity studies for NME may only need to be conducted for ocular drugs for which there is a cause for concern or significant systemic exposure (ICH-S1A 1995). For biologics, genotoxicity studies are not performed if it is not expected to interact with DNA or chromosomal material (ICH-S2(R1) (2011)). ICH S6(R1) requires a weight of evidence approach to assess the carcinogenicity potential of biologics (if warranted) and feasibility of study conduct as studies in non-relevant species generally not warranted (ICH-S6(R1) 2011). Rather, long-term safety studies with systemic exposure showing lack of preneoplastic or neoplastic lesions histologically may be sufficient or, if feasible, a homologous protein or transgenic model expressing the human receptor may be considered (ICH-S6(R1) 2011). However, use of a homologous protein or transgenic model has not been reported to date for any ocular products. For biologics, if the toxicological findings are similar or the findings are understood from the mechanism of action of the product in short term studies, then evaluation in one species may be sufficient for longer-term general toxicity. For example, ranibizumab (LUCENTIS®), the pivotal ocular study was conducted only in monkeys because earlier ocular intravitreal tolerability studies in rabbits showed low inflammation and high immunogenicity (Genentech 2006).

Similar to safety pharmacology and carcinogenicity studies, if there is limited systemic exposure from ocular dosing or sufficient justification due to indication, the requirements for certain reproductive toxicity studies may be waived. For biologics, ICH S6(R1) indicates that mating studies are not practical for nonhuman primates (ICH-S6(R1) 2011). As such, the effects on male and female fertility in nonhuman primates can be assessed by evaluating the reproductive organs (organ weight and histopathological evaluation) in repeat dose toxicity studies of at least 3 months duration (ICH-S6(R1) 2011).

14.5.7 Reformulation and/or New Route of Exposure

Recently released guidance for reformulated drug products and products intended for administration by an alternate route (FDA 2015c) indicate that if the drug substance has not been previously administered by the proposed ocular route then toxicity studies in two species with complete ocular and systemic evaluation for the appropriate duration should be carried out with the new formulation. A study in a single most appropriate species may be adequate if the drug substance has been previously administered by the proposed ocular route. As with NME/NBE, the studies generally should be conducted with vehicle control and complete clinical formulation and include the evaluation of systemic exposure. Ocular tissue distribution should also be assessed to understand pharmacokinetics.

If the ocular drug product is a reformulation of an existing marketed product and/or was not previously administered ocularly, or is part of a combination drug that the Sponsor does not own nor have the right to reference the data, the Sponsor may use a 505(b)(2) approach (FDA 2015c). The FDA uses the 505(b)(2) abbreviated process to approve drugs that rely on published literature evaluation or Agency findings of safety and effectiveness as detailed in the Summary Basis of Approval (SBA) for an approved drug. This includes using systemic toxicity information (e.g., toxicity or NOAEL) with an appropriate bridge to the new formulation. Examples of ophthalmic products that used this process include Akten (lidocaine hydrochloride) Ophthalmic Gel (Akorn 2008) and Phenylephrine Hydrochloride Ophthalmic Solution USP, 2.5% and 10% as a mydriatic agent (Akorn 2014).

For biologics, the 505(b)(2) approach is limited to biological products that are regulated as drugs under the Food Drug & Cosmetic Act (FD&C), whereas biologics regulated under the Biologics Price Competition and Innovation Act (BPCIA) would use the 351(k) pathway for biosimiliars (FDA 2015b). If the structural and functional data are limited in scope or there are concerns about the proposed product quality, a general toxicology study may be needed that includes full systemic histopathology, pharmacodynamic, pharmacokinetic, and immunogenicity assessments (FDA 2015b). However, if sufficient clinical data for the drug with the same route of administration and formulation (e.g., from studies or marketing experience outside the U.S.) that provides ample evidence for its safe use, then additional animal studies may not be necessary.

14.5.8 Combination Products

The FDA defines a combination product as the following (21 CFR § 3.2(e)):

- A product comprised of two or more regulated components, i.e., drug/device, biologic/device, drug/biologic, or drug/device/biologic, that are physically, chemically, or otherwise combined or mixed and produced as a single entity;

- Two or more separate products packaged together in a single package or as a unit and comprised of drug and device products, device and biological products, or biological and drug products;
- A drug, device, or biological product packaged separately that according to its investigational plan or proposed labeling is intended for use only with an approved individually specified drug, device, or biological product where both are required to achieve the intended use, indication, or effect and where upon approval of the proposed product the labeling of the approved product would need to be changed, e.g., to reflect a change in intended use, dosage form, strength, route of administration, or significant change in dose; or
- Any investigational drug, device, or biological product packaged separately that according to its proposed labeling is for use only with another individually specified investigational drug, device, or biological product where both are required to achieve the intended use, indication, or effect.

The FDA Office of Combination Products (OCP) oversees the regulation of combination products by coordinating the reviews among the different FDA Centers. Generally, DA is required to assign a combination product to a lead Center based on the primary mode of action of the product. Generally, if the device serves as a delivery platform for the drug, it would most like be regulated as a drug (Novack 2009). Regulation of combination drugs are included in ICH M3(R2) (ICH-M3(R2) 2010) and guidance documents on combination products can be found at http://www.fda.gov/RegulatoryInformation/Guidances/ucm122047.htm.

In addition to safety testing, the ocular devices are subjected to biocompatibility studies to ensure its safety with biological systems. The ocular devices, materials and/or their extracts are evaluated in a series of in vitro and/or in vivo cytotoxicity, hemocompatibility, sensitization, and irritation tests that are conducted in accordance with International Organization for Standardization (ISO)-10993(http://www.iso.org/iso/). The devices that will be used in the clinic is also tested in ocular toxicity studies, typically the device alone is tested as a placebo group. The nonerodible implant Retisert was reviewed and approved as drugs and included ISO-10993 testing (Bausch&Lomb 2005).

14.5.9 Other Considerations

14.5.9.1 Impurities

For small molecules, guidance related to impurities in drug substances and drug products have been issued by ICH. Theses guidance documents cover the qualifications and control for impurities in drug substances and drug products for registration (ICH-Q3A(R2) 2006; ICH-Q3B(R2) 2006), in addition to providing a framework for the identification, categorization, qualification of control of mutagenic impurities to limit carcinogenic risk for drugs that have the potential to result in DNA

damage (ICH-M7 2015; Industry 2008). For qualification, the level of impurities used in the preclinical studies should be similar or greater than the level used in the clinical studies to ensure adverse effects, including immune responses, are not due to impurities. If levels are not sufficient, additional genotoxicity studies for the impurity and toxicity studies with the higher levels of the impurity (e.g., present in the drug substance or drug product or spiked in the formulation) in the clinical formulation may be required to qualify the impurity prior to use in clinical trials. This may be challenging as some drug impurities may not be easily manufactured to levels necessary to run stand-alone tests.

Alternatively, the Threshold of Toxicological Concern (TTC) can be used if toxicity data is not available for the impurity (Munro et al. 2008; EMEA 2006). The TTC concept is based on a probabilistic approach that there is a very low probability of an appreciable risk (excess cancer risk of <1 in 100,000 over a lifetime) to human health if the human exposure to a genotoxic impurity is below 1.5 μg/day. Therefore, if human exposure is below the threshold of 1.5 μg/person/day, no toxicity data needs to be generated for the impurity unless there is a cause for concern.

14.5.9.2 Endotoxin in Devices

Endotoxins may be introduced into devices during the manufacturing, sterilization, or packaging process. The previously accepted endotoxin level was 0.5 EU/ml (ISO-15798:2013 2013). FDA recently released a draft guidance, "Endotoxin Testing Recommendations for Single-Use Intraocular Ophthalmic Devices"(FDA 2015a) that recommends an even lower level of endotoxin due to the increased frequency of toxic anterior segment syndrome (TASS) cases. TASS is thought to be associated with endotoxin or other pyrogenic contamination following intraocular surgery and poses a risk to patient vision. The importance of regulating the endotoxin levels is to prevent the occurrence of inflammation, allergic reaction or other immunopathological response. The pathological effects of endotoxin and other pyrogens are described elsewhere in this chapter.

The current draft recommendations are as follows:

- Intraocular fluids: ≤0.2 EU/ml.
- Anterior segment solid intraocular devices: ≤0.2 EU/device. For glaucoma devices, this limit applies to the segment of the device placed in the anterior chamber and the segment(s) contacting the aqueous humor even though the main portion of the device may reside outside the eye.
- Posterior segment solid intraocular devices: ≤0.5 EU/device. This limit applies to the segment of the device placed in the posterior segment and any segment(s) contacting the vitreous even though part of the device may reside outside the eye. For devices that contact the aqueous humor and the vitreous humor or posterior segment, the guidance refers to the Division of Ophthalmic and Ear, Nose and Throat Devices for recommended limits.

14.5.9.3 Ocular Irritation

For topical products administered by other routes of administration (e.g., dermal), ocular irritation studies may be required. In light of the 3R's (reduce, refine, and replace), to evaluate ocular irritation, numerous ex vivo, in vitro, and in silico models have been developed. These include (Wilson et al. 2015; Hutala et al. 2008):

- Ex vivo: e.g., bovine corneal opacity permeability assay (BCOP), porcine corneal opacity permeability assay (PCOP), isolated rabbit eye (IRE), isolated chicken eye (ICE)
- In vitro cytotoxicity tests: e.g., thymidine incorporation, Coomassie brilliant blue protein measurements, crystal violet and Lowry reagent, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays (MTT assays), lactate dehydrogenase leakage (LDH), fluorescein leakage (FL) trypan blue exclusion, florescent staining with propidium iodide, neutral red uptake/release tests, and in vitro corneal epithelium models, and
- In silico

Of these, the most accepted screening assessments for severe irritation is the BCOP, which evaluates the opacity (amount of light transmitted through the cornea) and permeability (amount of fluorescein dye that penetrates through the cornea) (OECD 2013; Wilson et al. 2015). However, a single in vitro study is not sufficient to evaluate ocular toxicity and does not replace in vivo ocular toxicity studies in pharmaceutical regulatory filings for ocular drugs.

14.5.9.4 Safety Margins

For ocular studies, the safety margins are calculated similarly to the systemic studies. For intravitreal dosing, additional calculations may be performed based on dose administered in the vitreous volume.

Exposure ratios for systemic toxicities are typically calculated by comparing the systemic exposure (based on maximal concentration (Cmax) and/or area under the concentration time curve [AUC]) at the NOAEL or LOAEL in the nonclinical toxicity studies with the exposure observed in human clinical trials. Additionally, the comparison can also be made on the basis of weight (mg/kg/day) or body surface area (mg/m^2/day) by extrapolating the animal dose to human equivalent dose (HED)(FDA 2005). Large margins of safety suggest that the drug may not cause adverse effects in humans at the recommended clinical doses. If feasible, at least a tenfold exposure margin between the preclinical model and human is desired.

For intravitreal administration, theoretical exposure margins may be assessed by comparing the intended clinical dose to the dose administered in the preclinical model with corrections for vitreous volume differences between humans and animal models. For EYLEA®, the FDA utilized a vitreous volume of 2 mL for monkeys and 4 mL for humans (twofold) (Regeneron Pharmaceuticals 2011; Short 2008), whereas earlier approval for LUCENTIS®, vitreous volumes of 1.5 mL for monkeys and 4.5 mL for humans (threefold) were used (Genentech 2006).

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Chapter 15
Immunopathology of Drug and Toxin-Related Skin Reactions

Rony Shreberk-Hassidim and Yuval Ramot

Abstract The skin has an important role as a barrier, protecting the organism from its environment. Therefore, it participates significantly in several immunological processes, and this novel role is gradually revealed in recent studies. We overview the main components of the cutaneous immunological response, divided into contact and systemic antigen exposures. The major players of the immunopathological skin reactions are the keratinocytes, Langerhans cells and dermal dendritic cells. In addition, we discuss the use of animal models for exploring the complexity of these reactions.

Keywords Allergic contact dermatitis • Aryl hydrocarbon receptor • Contact hypersensitivity • Dermal dendritic cells • Langerhans cells • Steven–Johnson syndrome • Toxic epidermal necrolysis

15.1 Skin Anatomy and Immunobiology

The skin is the largest organ system of the body, covering the entire surface of the organism. Its main function is to serve as a barrier between the organism and the external environment, preventing penetration of pathogens, toxic chemicals and physical factors. It is composed of two layers, the outer epidermis and the dermis beneath it (Urmacher 1990). The major cell population which composes the epidermis is the keratinocytes (KCs) (>90%), and its minor cell populations are melanocytes, Langerhans cells (LCs) and Merkel cells. The dermis, which lies underneath the epidermis, is a vascular layer containing collagen and elastin fibers, in addition to fibroblasts, macrophages and dermal dendritic cells (dDCs). Under the dermis lies the hypodermis, which is composed mainly of adipose tissue, and is not considered as part of the skin (Vandergriff and Bergstresser 2012, Velazquez Es 2009). The major skin compartments are illustrated in Fig. 15.1.

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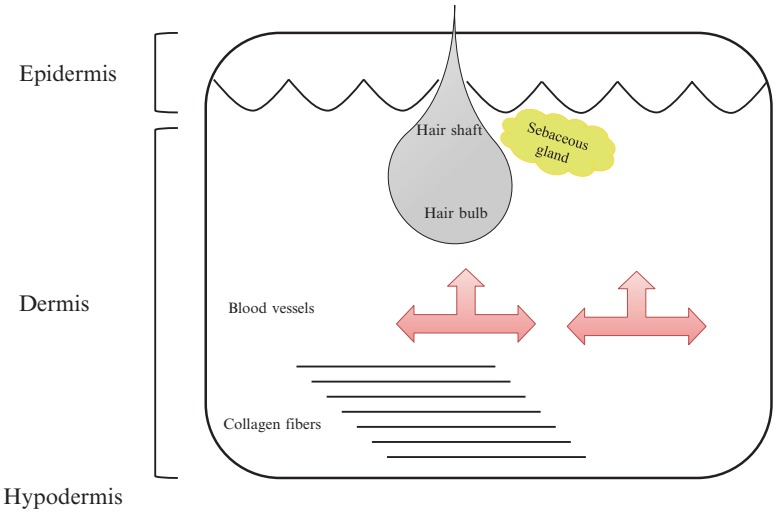

Fig. 15.1 Illustration of the basic skin structure

Table 15.1 Summary of cell populations involved in the dermatological immune response

Type of cell	Location	Main role in the dermatological immune response
Keratinocytes	Epidermis	• Secretion of immunological mediators—IL-1, IL-6, IL-7, IL-8, IL-10, IL-11, IL-12, TNF-alpha • Expression of immunological surface molecules—MHC-I, MHC-II, ICAM-1, CD80
Langerhans cells	Epidermis	Immune surveillance—sampling and ingesting antigens in the epidermis, trafficking them to regional lymph nodes, where they are presented to T cells. LCs may elicit immunological tolerance or inflammatory response, depending on their maturation state
Dermal dendritic cells	Dermis	Immune surveillance—sampling and ingesting antigens in the dermis, trafficking them to regional lymph nodes, where they are presented to T cells. dDCs may elicit immunological tolerance or inflammatory response, depending on their maturation state or subpopulation

dDCs, Dermal dendritic cells; ICAM, Intercellular adhesion molecule; IL, Interleukin; LCs, Langerhans cells; MHC, Major histocompatibility complex; TNF, Tumor necrosis factor

It is now known that in order to provide protection from external insults, the skin harbors complex immunologic processes, and in fact serves as an active immune organ, maintaining active interaction between its different cell populations, the immunologic system and cytokines (Salmon et al. 1994). We will elaborate on the leading cell populations known to be involved in the dermatological immune responses: KCs, LCs, and dDCs (Table 15.1).

15.1.1 Keratinocytes

Development and origin: The KCs are epithelial cells, arranged in four layers from inside to outside of the epidermis: basal layer, spinous layer, granular layer and cornified layer. KCs proliferate in the basal layer and differentiate whilst progressing to the outer layers (Urmacher 1990).

Function, related mediators and expression of co-stimulatory molecules: In the past, KCs were considered only as keratin producers, providing mechanical and physical defense to the skin by forming the compact hydrophobic cornified layer (Vandergriff and Bergstresser 2012) and multiple intercellular tight-junctions (Brandner et al. 2002). However, in 1976, it was first suggested that KCs can influence T-cell migration (Edelson 1976). Since then, more data was revealed on the significant role of KCs in the immune responses of the skin. Two main mechanisms mediate the role of the KCs in the immunologic process: secretion of immunological mediators and expression of immunological surface molecules.

- Secretion of immunological mediators: It was recognized that KCs synthetize and secrete cytokines. The first to be recognized was interleukin (IL)-1 (Luger et al. 1983), and it is now known that KCs secrete mainly IL-1-alpha (Kupper et al. 1986). Other cytokines secreted by the KCs are IL-6, IL-7, IL-8, IL-10, IL-11, IL-12 and tumor necrosis factor (TNF)-alpha. Additionally, they secrete granulocyte-macrophage colony-stimulating factor (GM-CSF) and growth factors such as fibroblast growth factor (FGF), insulin-like growth factor (IGF), platelet-derived growth factor (PDGF) and transforming growth factor (TGF) type alpha and beta (Luger and Schwarz 1990, Feliciani et al. 1996). KCs also secrete a large array of neuropeptides such as substance P, proopiomelanocortin (POMC)-derived peptides, calcitonin gene related peptide (CGRP) and vasointestinal peptide (VIP) (Luger 2002).

  It was shown that IL-1 exists in the cornified layer of the intact epidermis, and after injury it is released and induces local and systemic inflammatory processes (Gahring et al. 1985). Ultraviolet (UV) radiation, chemicals and infections are also stimulants of cytokine synthesis and release (Luger and Schwarz 1990). Over the years, it was discovered that almost all the known cytokines, except mostly IL-2, IL-4 and interferon (IFN)-gamma, and various chemokines and growth factors, are secreted and participate in epidermal immunological processes, where they regulate the cutaneous inflammatory responses (Luger et al. 1996). Imbalance in this delicate complex network of immunological mediators may lead to dermatological disorders (Feliciani et al. 1996).
- Expression of immunological surface molecules: Major histocompatibility complex (MHC)-I molecules are expressed on all nucleated cells, including KCs, and are involved in lysis by cytotoxic or CD8+ T lymphocytes (Symington and Santos 1990). Later, it was found that in specific diseases, KCs also express MHC-II molecules (Volc-Platzer et al. 1984, 1987), known previously to be expressed only by LCs. In vitro studies demonstrated that MHC-II expression was stimulated by pure recombinant exogenous IFN-gamma (Basham et al. 1984), and may be related to inflammatory skin diseases such as lichen planus, allergic contact dermatitis and

cutaneous graft versus host disease (GVHD). More data suggest that the expression of MHC surface molecules during the inflammatory cutaneous response enables KCs to function as primary antigen presenting cells (APCs) (Fan et al. 2003).

In addition, KCs express co-stimulatory molecules, including for example intercellular adhesion molecule-1 (ICAM-1) (Fan et al. 2003) and CD80 (Wakem et al. 2000), which are obligatory signals in the activation of T cells by APCs. In contrast to previous reports, which demonstrated induction of T cell tolerance following presentation of autoantigens by KCs (Bal et al. 1990), further studies showed that KCs serve as APCs with a potential to activate T cells (DE Bueger et al. 1993), for example, priming CD8+ T cells (Kim et al. 2009). This highlights the fact that KCs have a crucial role in the initiation and enhancement of the cutaneous adaptive immune response as well as the primary role in the innate immune system of the skin.

15.1.2 Dendritic Cells

Dendritic cells (DCs), a subtype of APCs, are named for their stellate shape, with "dendritic" meaning "treelike" in Greek. They process and present antigens to T cells, leading to either immunity or tolerance, according to the antigen presented (Steinman 2007). DCs include plasmacytoid DCs (pDCs) and myeloid DCs (mDCs) subpopulations: the first circulate in the blood or lymphoid organs and the latter are found in peripheral tissues, secondary lymphoid organs or the blood. In the skin, mDCs are divided into LCs in the epidermis and dDCs which are localized to the dermis (Banchereau et al. 2009). Following inflammatory or other stressogenic stimuli or even in steady-state, these cells migrate from the skin to lymph nodes, leading to cell maturation and initiation of immune response. While LCs and dDCs have a common pathway of action, they differ in their characteristics, function and kinetics (Table 15.2) (Steinman et al. 1995), discussed in the next section.

15.1.3 Langerhans Cells

Development and origin: In 1868, Paul Langerhans published an article describing for the first time non-pigmentary dendritic epidermal cells, which were later named after him (Jolles 2002, Langerhans 1868). At the beginning, they were thought to be cells of the nervous system, but only after more than a century it was discovered that they are derived from the bone marrow and comprise 3–8% of the epidermal cells (Katz et al. 1979). During embryogenesis, early precursors of LCs originate from primitive macrophages of the yolk-sac and later from myeloid progenitors found in the fetal liver. Precursors of LCs from both origins migrate to the skin before birth, where they differentiate (Hoeffel et al. 2012). Their differentiation is dependent on receptor for colony stimulating factor-1 (CSF-1R) (Ginhoux et al. 2006) and IL-34 as its specific ligand in the skin (Wang et al. 2012). In addition, TGF-beta 1 is also

Table 15.2 Summary of the different characteristics of Langerhans cells versus dermal dendritic cells

Characteristics	Langerhans cells	Dermal dendritic cells
Location	Epidermis	Dermis
Origin	Primitive macrophages of the yolk-sac in early embryogenesis and later from myeloid progenitors found in the fetal liver	Myeloid precursors derived from the bone marrow
Renewal	• In steady state—self-renewal in the epidermis, radioresistant • In inflammation—circulating monocytes, migrating from blood vessels into the dermis	Continuous replacement by blood-borne bone marrow precursors, radiosensitive
Motility	• In steady state—mostly sessile • In inflammation – peak arrival to the regional lymph nodes 4 days after the stressogenic stimuli	More motile, peak arrival to the regional lymph nodes 1–2 days after the stressogenic stimuli
Phenotype markers	MHC-II, Birbeck granules, E-cadherin, CD1a, Langerin (CD207)	MHC-II Subsets: $Langerin^{+}CD103^{+}$ $Langerin^{+}CD103^{-}$ $Langerin^{-}CD11b^{+}$ $Langerin^{-}CD11b^{-}$

MHC, Major histocompatibility complex

important for LCs development (Borkowski et al. 1996). TGF-β1 is known to be secreted in the skin by KCs, but it was shown that its autocrine and paracrine secretions by LCs are required for their development and survival (Kaplan et al. 2007).

Function, related mediators and expression of co-stimulatory molecules: LCs serve as APCs in the epidermis, where they are arranged as a network between KCs, a position that allows them to sample antigens in their adjacent environment (Larregina and Falo 2005). The expression of the adhesive molecule E-cadherin by LCs mediates their conjugation to KCs (Tang et al. 1993). They have typical features distinguishing them from other DCs, including high-level-expression of CD1a, which presents lipid antigens to T cells (Hunger et al. 2004). In addition, they are characterized by unique cytoplasmic organelles resembling a "tennis-racket", known as Birbeck granules (Wolff 1967). The formation of these granules is induced by langerin (CD207), a C-type lectin receptor (Valladeau et al. 2000). Previously, langerin, though its contribution to antigen processing is unclear, was considered to be the only available specific marker for LCs (Hunger et al. 2004). Later, it was discovered that langerin is expressed on a small subpopulation of dermal DCs as well (Bursch et al. 2007).

The explicit contribution of LCs to the skin immune system is still vague, and apparently their function, mediators and dynamics differ in steady state versus inflammatory response, as follows:

- In steady state: After birth, the LC population is maintained by independent proliferation in the epidermis, not requiring replacement by circulating precursor cells (Merad et al. 2008, Kanitakis et al. 2011). This autoreplacement is a distinc-

tive characteristic of LCs when compared to other DCs, and may explain why they are considered "radio-resistant", remaining of recipient rather than donor origin after haematopoietic progenitor cell transplantation following irradiation (Merad et al. 2008). They participate in immune surveillance, sampling antigens in the epidermis and trafficking them to the skin-draining lymph-nodes (Hemmi et al. 2001) in a very low motility rate (Kissenpfennig et al. 2005). Yet, their impact on T cells in steady state is still debated—previous studies support their role as tolerance inducers, while other studies question this role (Lutz et al. 2010). For example, it was shown that in steady state, LCs present self-antigens to T cells in the regional lymph nodes, but this did not result in tolerance of CD8+ T cells (Mayerova et al. 2004). In an additional study, an opposite result was found, demonstrating deletional tolerance of T cells after self-antigen presentation by LCs (Waithman et al. 2007). The migration of LCs in steady state is thought to be mediated by TGF-beta, as mentioned above, and IL-10 (Larregina and Falo 2005), which is believed to contribute to tumor cell tolerance in metastatic melanoma (Enk et al. 1997). An attempt to identify a specific phenotype responsible for inducing tolerance by LCs in the steady state failed, but showed that the involved cells have lower expression of co-stimulatory molecules such as CD40 and CD80, lower production of pro-inflammatory cytokines such as IL-12 and minor induction of proliferation of antigen-specific T cells (Stoitzner et al. 2005). The current hypothesis is that tolerance induction is dependent on the maturity state of LCs: incompletely matured LCs with low to moderate expression of co-stimulatory molecules and cytokines may lead to T cell tolerance in the skin-draining lymph nodes (Mutyambizi et al. 2009).

- In inflammation: During inflammation, the LC population is maintained in a different manner than in steady state. After inflammatory stimuli such as UV light, the LC population is renewed by circulating monocytes, migrating from blood vessels into the dermis (Merad et al. 2002). This process is dependent on the expression of the C–C chemokine receptor (CCR)2 and CCR6 by the circulating cells, attracted in a gradient pattern to their ligands, C–C chemokine ligand (CCL)2 and CCL20, respectively, and other chemokines secreted by the inflamed skin (Vanbervliet et al. 2002, Nagao et al. 2012). In addition to recruiting LC precursors to preserve the epidermal population during inflammation, when activated, LCs separate from adjacent KCs by down regulation of adhesion molecules such as E-cadherin, become motile and migrate towards the lymphoid tissue (Jakob et al. 2001) in a CCR7-dependent manner (Ohl et al. 2004). Surprisingly, the migration of LCs into the lymph nodes is slow, peaking 4 days after the inflammatory stimuli, lagging the arrival of DCs and actually the beginning of immunological response (Kissenpfennig et al. 2005). It may suggest a regulatory effect of LCs on the cutaneous immunological response, rather than a pro-inflammatory role, which is also supported by the observation of amplified contact hypersensitivity reaction in the absence of LCs in a mouse model (Kaplan et al. 2005, Bobr et al. 2010) and activation of regulatory T cell by LCs (Van Der Aar et al. 2013). However, during inflammation, the number of LCs is

increased in the regional lymph nodes, expressing more co-stimulatory molecules such as CD40, CD86, B7-H1, and B7-DC, producing higher amounts of the pro-inflammatory cytokine IL-12 and inducing proliferation of antigen-specific T cells with their secretion of IFN-gamma (Stoitzner et al. 2005). These complex and somewhat contradicting findings emphasize the sophistication of the impact that LCs have on the immunologic process in inflammatory state, allowing them to be stimulators versus regulators according to their maturation state and the antigen presented.

15.1.4 Dermal Dendritic Cells

Development and origin: Only recently it was learned that the cutaneous APC population does not include only LCs, but also other dendritic cells that reside in the dermis, termed dDCs (Allan et al. 2003). The dDCs are composed of functionally and phenotypically heterogeneous subpopulations, which originate from different myeloid precursors of the bone-marrow with varied differentiation and maturation pathways (Sallusto 2001). Unlike LCs, dDCs are replaced continuously by those precursors, arriving to the dermis through the vascular system, explaining their radiosensitivity (Liu and Nussenzweig 2010). In addition, all DCs are replaced by the donor-derived precursors following bone-marrow transplantation (Merad et al. 2002). Like other DCs in the spleen and lymph nodes, the development of dDCs is mostly dependent on FMS-like tyrosine kinase receptor 3 (Flt3) (Waskow et al. 2008, McKenna et al. 2000), in contrast to LCs which are M-CSF dependent.

Function, related mediators and expression of co-stimulatory molecules: In steady state, dDCs are located in perivascular areas of the superficial dermis (Stingl 1990), and sample antigens in order to present them to T cells in the skin-draining lymph nodes (Ginhoux et al. 2007). When examining the cutaneous-draining lymph nodes in steady state, dDCs compose only a minor component of all DCs in the lymph node. However, during inflammation, there is enhanced migration of dDCs to the lymph nodes (Jakubzick et al. 2008). In both conditions, their migration across the dermis towards the lymph nodes is dependent on CCR7 (Ohl et al. 2004). Following inflammatory stimuli, dDCs migrate rapidly, arriving to the regional lymph nodes within a peak of 1-2 days, while LCs follow and peak only after 4 days of those stimuli (Kissenpfennig et al. 2005).

The dDCs are divided into distinct subsets, according to the expression of various markers. At least four subsets of dDCs were identified in murine models, and all of them reside in dermis during steady state. The major subpopulation, composing 80% of the dDCs, is negative for langerin expression, and can be further subdivided according to the expression of CD11b (Henri et al. 2010). The Langerin $(CD207)^+$ dDCs, which were only recently recognized (Bursch et al. 2007), represent a small

proportion of the dDCs and may be further classified based on the expression of CD103 (Henri et al. 2010).

Large efforts are invested in the last few years to explore the connection between the phenotype of specific subpopulations of dDCs and their function in response to antigen presentation. For example, a murine model of skin infection by Candida albicans discovered that Langerin^{+} dDCs promote antigen-specific cytotoxic lymphocytes and T helper (Th) 1 cells, and inhibit differentiation of Th17 cells, in contrast to Langerin^{-} dDCs, which lead to Th17 cell differentiation (Igyártó et al. 2011). In a murine model of contact hypersensitivity, another subset of dDCs was recognized, expressing CD301 (named also macrophage galactose-type C-type lectin 2) and inducing a Th2-mediated immune response (Murakami et al. 2013). These opposite functions may help clarify the previous reports of induction of antigen-specific immune response and tolerance to self-antigens by dDCs (Waithman et al. 2007). Another hypothesis is that the maturation phase of dDCs affects their immunological role. For example, in steady state, dDCs that are not fully mature lead to tolerance, while stressogenic stimuli generate functionally mature dDCs resulting in a T-cell inflammatory response (Jiang et al. 2007).

15.1.5 Other Components Involved in the Dermatological Immune Response

The skin contains few additional structures as sebaceous glands, sweat glands and hairs, which are defined as the skin adnexa. Recent data suggest that these structures participate in the dermatological immune response. We will mention few of them.

For example, sebaceous glands secrete a lipid mixture which some of its products were shown to have inflammatory effects, especially free fatty acids as palmitic acid (Zhou et al. 2013). In addition, protease-activated receptor-2 (PAR-2), which has a pivotal regulatory role on cutaneous barrier, inflammation and wound healing, was expressed by sebocytes and was involved in their differentiation, lipid secretion and immunological response to *Propionibacterium acnes* (Lee et al. 2015). It may add to the understanding of the pathogenesis of inflammatory skin disorders of the sebaceous gland, such as acne.

Adipose tissue, the main component of the hypodermis, may contribute to the cutaneous immune response as well. There is cumulative evidence that adipocytes are immunologically active, secreting different adipokines and cytokines as adiponectin, leptin, resistin, IL-6, and TNF (Schaffler and Scholmerich 2010). In the skin, for instance, infection by *Staphylococcus aureus* was controlled by expansion of subcutaneous adipocytes and by their production of antimicrobial peptides, such as cathelicidin (Zhang et al. 2015). It raises questions regarding the immunological influence of obesity and weight-reduction methods, yet to be answered.

15.2 Specific Immunopathological Processes of the Skin: Contact Exposure to Drugs and Toxins

15.2.1 Epidemiology and Types

The skin, as the outer surface of the body, is exposed to many external stimuli, which can result in some cases in cutaneous inflammation known clinically as contact dermatitis. It is characterized by an itchy eczematous eruption composed of erythema, edema and vesicles in its acute phase or scales and lichenification in its chronic phase (Mowad and Marks 2012). This inflammation may be induced by an irritant or allergic mechanism, dividing contact dermatitis into two types (Vocanson et al. 2009, Mowad and Marks 2012):

- Irritant contact dermatitis (ICD): Caused by a direct cytotoxic effect of a chemical agent activating the innate immune system. This is the more common form of contact dermatitis.
- Allergic contact dermatitis (ACD): A T-cell mediated type IV delayed-type hypersensitivity response, initiated after an exposure to a specific allergen.

Both types are very common, and are considered the leading cause for occupational skin diseases (Rietschel et al. 2002, Diepgen 2003) having a large impact on public-health burden (Chew and Maibach 2003). In this section, we will focus on the pathogenesis of ACD, which represent a more complex immunological pathogenesis.

15.2.2 Pathogenesis of ACD

The allergen is a hapten, a low-molecular weight molecule able to bind a protein, which can convert it into an immunogenic antigen (Pichler et al. 2011). Many haptens may cause ACD, the most common ones include metals such as nickel, topical antibiotics such as bacitracin or neomycin sulfate and preservatives and dyes such as formaldehyde or p-phenylenediamine (Fransway et al. 2013). The small-sized haptens are able to enter the corneal layer effortlessly, where they bind to self-cutaneous proteins (Roberts and Aptula 2008). Most haptens are not electrophilic and need to be modified by reactions in their environment (known as prehaptens) or by skin-enzymatic changes (known as prohaptens), prior to the protein-binding (Basketter et al. 1995, Karlberg et al. 2007).

The understanding of the pathogenesis of ACD is based on animal models of contact hypersensitivity (CHS), where the hapten is painted on the skin of a pre-sensitized animal (Christensen and Haase 2012). The CHS is composed of two key phases—sensitization and elicitation phases:

- Sensitization phase—known also as induction or afferent phase, begins at the first encounter of a hapten with the skin, followed by its ingestion by APCs,

which migrate to the regional lymph nodes, where they present the hapten to naïve T cells. This step lasts several days, up to 7 days in mice and 15 days in humans, and usually does not result in a clinical dermatological response (Vocanson et al. 2009). The hapten binds an amino-acid residue, causing modification of self-proteins, a process called haptenization, resulting in new unfamiliar antigens. These antigens are sampled by the cutaneous APCs—LCs and dDCs. Their maturation and activation state is influenced by interactions with cytokines and other substances, representing "danger signals", released in response to provocation by the hapten itself. For example, fragments of hyaluronic acid, a ligand of Toll-like receptor 4 on APCs (Termeer et al. 2002), extracellular adenosine triphosphate (ATP), a ligand of the receptor $P2X_7$ (Weber et al. 2010) or prostaglandin E2 (Kabashima et al. 2003), were shown to promote APCs activation. Those danger signals lead to secretion of IL-1-beta and TNF-alpha, which are crucial for activation and stimulation of APCs to migrate (Cumberbatch et al. 1997), as explained in detail in the previous section.

Following stimulation, APCs, loaded with haptenated peptides, migrate to the skin-draining lymph nodes, presenting antigens located on MHC I and II surface-molecules to naïve T cells (Weltzien et al. 1996) (Fig. 15.2). This process results in the activation of antigen-specific T cells, including $CD4^+$ and $CD8^+$ cells (Xu et al. 1996), which differentiate into effector and memory T cells, as detailed below and summarized in Fig. 15.3:

- T-cell activation—the co-stimulatory signals required for the activation of T cells by APCs in the local lymph nodes are MHC II molecules interacting with T cell receptors, representing the first and principal signal, CD80 and CD86 interacting with CD28 receptor on T cells and interaction of CD40 on APCs with CD154 on T cells, although its exact role is still debated (Christensen and Haase 2012).
- T-cell differentiation—cytokines secreted during the interaction between APCs and T cells, mediate T cell differentiation into Th1, Th2 or Th17 cells. The differentiation into Th1 cell is dependent on IL-12 and IFN-gamma, which produce the same cytokines after their differentiation. The differentiation into a Th2 cell phenotype is under the effect of IL-4, and these cells secrete IL-4, IL-5 and IL-13 (Nguyen and Casale 2011). $CD8^+$ cells are influenced by the same cytokines, inducing similar subsets of cells—TC1 secreting Th1-like cytokines and TC2 secreting Th2-like cytokines (Sad et al. 1995). The differentiation to Th17 is mediated by TGF-beta, IL-6 and IL-21, and these cells secrete IL-17 and IL-22 (Awasthi and Kuchroo 2009).

Sensitization phase has been shown that the predominant induced effector T cells are $CD8^+$ cells, while $CD4^+$ cells have an effect of down-regulation of the CHS response (Xu et al. 1996). In the presence of a highly pro-inflammatory hapten, a $CD8^+$ induction does not require signals from $CD4^+$ cells, which are usually needed for its activation (Saintmezard et al. 2004).

Sensitization phase, the interaction with APCs results in the formation of antigen-specific memory T cells, divided to subsets based on their expression of

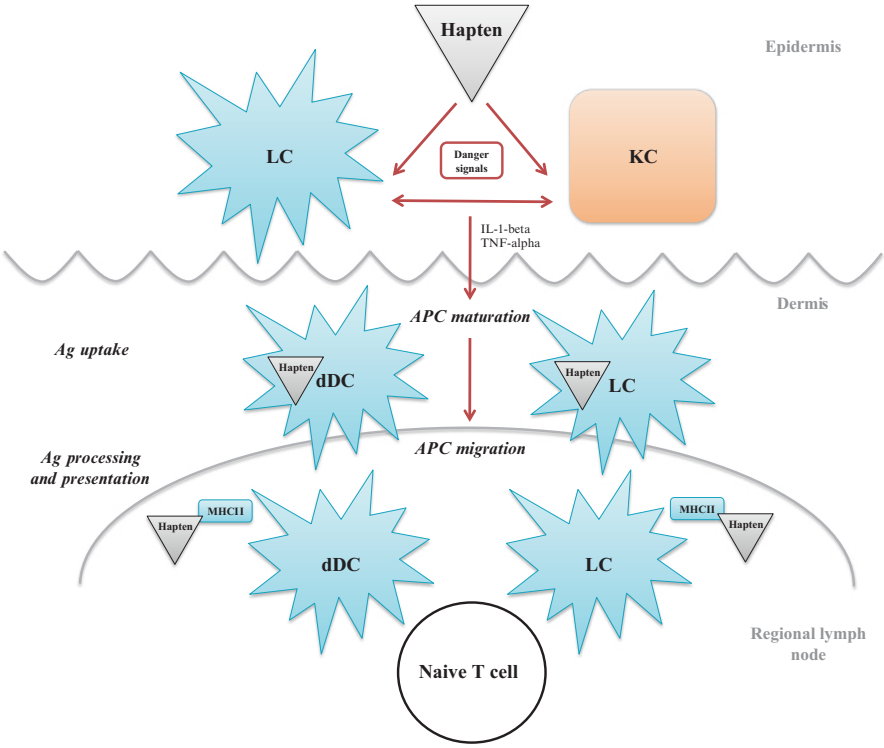

Fig. 15.2 Illustration of the maturation and migration process of antigen-presenting cells during the sensitization phase of contact hypersensitivity. *Ag* antigen, *APC* antigen-presenting cell, *dDC* dermal dendritic cell, *IL* interleukin, *KC* keratinocyte, *LC* Langerhans cell, *MHC* major histocompatibility complex, *TNF* tissue necrotic factor

CCR7. Sensitization phase Effector memory T cells, which are negative for CCR7, can migrate quickly to the irritated tissue, leading to a rapid effector response. In contrast, central memory T cells express CCR7, which enables them to circulate between lymph nodes and blood vessels, stimulating recruitment of DCs and effector cells after re-exposure to the specific antigen (Sallusto et al. 1999).

Effector and memory T cells that have been activated in the skin-draining lymph nodes express new surface molecules, including CD43 and cutaneous lymphocyte antigen, named also P-selectin glycoprotein ligand 1 (PSGL-1), which is essential for the ability of T cells to enter the skin (Santamaria-Babí, 2004, Matsumoto et al. 2007). Migration of activated antigen-specific T cells from the lymph nodes to the circulation is the last part of the sensitization phase.

- Elicitation phase—known also as the efferent or challenge phase, starts after subsequent exposure of the skin to previously-sensitized hapten. The duration of this phase is approximately 72 h in humans and 24–48 h in mice, terminating after a few days due to active down-regulatory mechanisms (Bour et al. 1995, Vocanson et al. 2009). In contrast to the sensitization phase, the relevant hapten

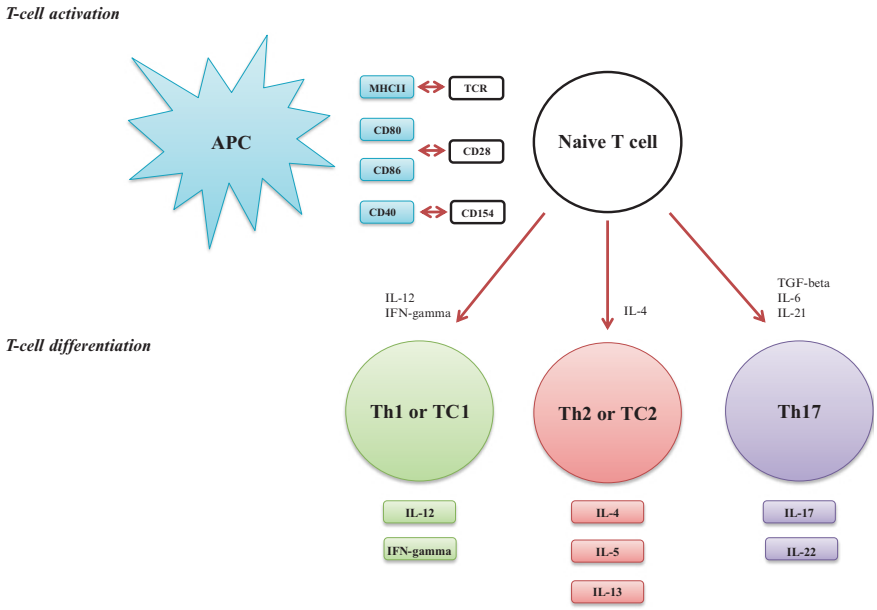

Fig. 15.3 Illustration of selected co-stimulatory molecules and cytokines involved in the T-cell activation and differentiation processes in the skin-draining lymph nodes during the sensitization phase of contact hypersensitivity. *IFN* interferon, *IL* interleukin, *MHC* major histocompatibility complex

may be presented not only by MHC class II-expressing APCs, but also by other cutaneous cells such as KCs, mast cells, endothelial cells and macrophages in an MHC class I fashion (Barker et al. 1991, Grabbe and Schwarz 1998, Grabbe et al. 1995). The activation of hapten-specific T cells, following antigen-presentation, is hypothesized to occur at the skin and not at the skin-draining lymph nodes, resulting in subsequent local inflammatory and clinical eczematous rash. The elicitation phase is further sub-divided into an early response, occurring at the first 2 h following exposure to the hapten, and to a late response occurring 24 h after exposure (Van Loveren et al. 1983).

– Early response: In recent years it was shown that the initial elicitation reaction, which is considered to be T-cell dependent, actually requires the participation of the innate immune system including complement, mast cells and neutrophils, in addition to B cells from the adaptive system (Table 15.3) (Christensen and Haase 2012). C5a, part of the complement immune system, activates platelets and mast cells through their C5a receptors, causing the release of vasoactive mediators such as serotonin (from both platelets and mast cells) and TNF-alpha (only from mast cells) (Meuer et al. 1981, Tsuji et al. 2000). Under the influence of these vasoactive agents, post-capillary venules express ICAM-1 and vascular cell adhesion molecule-1 (VCAM-1)

Table 15.3 Summary of immunological components (not including T cells) participating in the early response of the elicitation phase of contact hypersensitivity

Component of the immune system	Role in early response
Natural killer T cells	Participate in the activation of B1 cells mediated by IL-4
B cells	B1 cells produce hapten-specific IgM antibodies during the sensitization phase. Upon re-exposure, IgM antibodies create immune complexes with the hapten, activating complement system to generate C5a
Complement and mast cells	C5/C5a complement activation of mast cells, which secrete vasoactive mediators such as serotonin and TNF-alpha. These mediators induce the expression of adhesion molecules on endothelial cells attracting effector T cells to the skin
Neutrophils	Infiltrate the challenged skin in a CXCL1-dependent fashion and subsequently recruit antigen-specific T cells by CXCL9 and CXCL10

CXCL, CXC chemokine ligand; IL, Interleukin; TNF, Tumor necrosis factor

on their luminal surface, attracting effector T cells to the skin (Mchale et al. 1999). CXC chemokine ligand (CXCL), produced after exposure to the hapten, directs the infiltration of neutrophils to the affected skin. Subsequently, neutrophils recruit antigen-specific T cells by the secretion of CXCL9 and CXCL10 (Engeman et al. 2004, Kish et al. 2009). The participation of B cells in this phase was confirmed using a model of B-cell-deficient mice, demonstrating a diminished CHS response compared to wild-type mice (Askenase et al. 1998). It was shown that B1 cells produce hapten-specific IgM antibodies one day after their first exposure to a hapten during the sensitization phase. Following secondary exposure, these antibodies form cutaneous immune complexes, activating the complement system to generate C5a, which is the initial step of the elicitation phase (Tsuji et al. 2002). Natural killer T (NKT) cells were shown to be a part of this complex response as well, via involvement in the activation of B1 cells mediated by IL-4 (Askenase et al. 2005).

– Late elicitation response: This response is dependent on the extravasation of effector T cells from the circulation towards the challenged skin (Muller 2013), according to the next steps (Fig. 15.4):

1. *Expression of adhesion molecules on endothelial cells*—under the influence of LCs and KCs-derived IL-1-beta and TNF-alpha, the activated nuclear-factor (NF) kappa-B pathway in endothelial cells induces the transcription of adhesion molecules such as E-selectin, ICAM-1 and VCAM-1 (Epstein et al. 1997). Another key adhesion molecule, P-selectin, exists preformed in endothelial cells, in which it is stored in Weible-Palade bodies and released after specific stimuli (Bonfanti et al. 1989, Lowenstein et al. 2005).
2. *T-cell tethering*—T cells interact with the low affinity endothelial-cells-adhesion molecules E-selectin and P-selectin by their receptors CD34 and

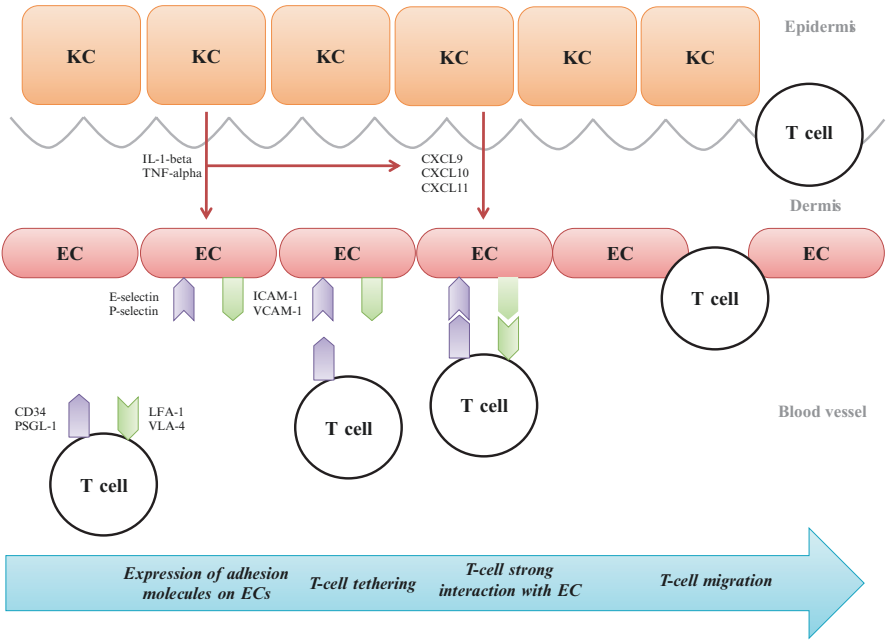

Fig. 15.4 Illustration of the steps composing the extravasation of T cells during the late elicitation phase of contact hypersensitivity. *CXCL* CXC chemokine ligand, *ICAM* intercellular adhesion molecule, *IL* interleukin, *LFA* leukocyte function-associated antigen, *PSGL* P-selectin glycoprotein ligand, *TNF* tumor necrosis factor, *VCAM* vascular cell adhesion molecule, *VLA* very late antigen

PSGL-1, respectively. This interaction forces T cells to slow their motion in the bloodstream (Matsumoto et al. 2007, Epstein et al. 1999), a process known to immunologists as 'rolling' and to histopathologists as 'pavementing' of leukocytes.

3. *Strong interaction with endothelial cells*—a stronger interaction between high affinity integrins as very late antigen-4 (VLA-4) on T cells and VCAM-1 on endothelial cells, in addition to leukocyte function-associated antigen-1 (LFA-1) on T cells and ICAM-1 on endothelial cells, results in the arrest of T-cells rolling in the circulation (Epstein et al. 1999). It is prompted by the release of chemokines from KCs (caused by IL-1-beta and TNF-alpha stimulation), as CXCL9, CXCL10 and CXCL11 binding CXC chemokine receptor (CXCR) 3 on T cells (Sebastiani et al. 2002).
4. *Migration*—After the arrest in their motion, T cells migrate across endothelial cells into the dermis and lastly arrive to the epidermis. Platelet-endothelial cell adhesion molecule (PECAM, CD31) facilitates this transmigration through the wall of the blood vessel (Muller 2013).

Late elicitation response main effector T cells that mediate this response are $CD8^+$ cytotoxic lymphocytes, recruited rapidly to the affected site in the epider-

mis. $CD4^+$ T cells infiltrate the skin later, participating in the regulation of the inflammatory response (Akiba et al. 2002, Bour et al. 1995). Few studies demonstrated that a small fraction of the $CD4^+$ cells, especially $CD4^+$ Th1 cells, serve as effector cells, whereas the majority of the $CD4^+$ cells possess a regulatory role (Christensen and Haase 2012).

Late elicitation response arrival to the epidermis, $CD8^+$ cells have a cytotoxic effect on KCs, causing their apoptosis. This process is mediated by perforin/granzyme and Fas/Fas ligand pathways (Kehren et al. 1999). $CD8^+$ cells produce IFN-gamma, which stimulates the secretion of cytokines, chemokines and reactive oxygen species (ROS) (He et al. 2009) from the adjacent macrophages, KCs and neutrophils, leading to enhancement of the inflammatory response.

Late elicitation response resolution of the elicitation phase occurs 24–48 h after the exposure to the hapten, and is dependent on the activation and arrival of $CD4^+$ regulatory T cells. Few subpopulations of regulatory $CD4^+$ cells have been previously described, including $CD24^+$ $CD25^+$ T cells and IL-10-releasing Tr1 cells (Vocanson et al. 2009).

15.2.3 The Aryl Hydrocarbon Receptor

The aryl hydrocarbon receptor (AhR) is found in all cutaneous cells. It is a transcription factor known to serve as a sensor for chemical signals, to which the skin is frequently exposed. Before activation, the AhR is located in the cytoplasm, and following activation by its ligands—low molecular weight chemicals—it migrates into the nucleus. In the nucleus it stimulates transcription of genes containing xenobiotic response elements (Abel and Haarmann-Stemmann 2010). The subsequent cutaneous response is the result of induction of a range of genes, depending on the timing and the quality of the AhR activation by various ligands (Mitchell and Elferink 2009). The AhR is also expressed in other organs, which are frequently exposed to exogenous chemicals, such as the liver, lungs and gut. Recently, there has been a great interest in the cutaneous function of AhR, demonstrating its involvement in almost every aspect of the skin, including skin physiology, immunology and skin diseases such as vitiligo, chloracne, psoriasis, pityriasis versicolor and skin cancer (Esser et al. 2013, Noakes 2015). We will focus on its role in the immunological response to an exogenous stimulus encountered by the skin.

- Role in toxic skin response: Described for the first time by Herxheimer in 1899 (Herxheimer 1899), chloracne is an acne-like eruption developing after direct contact with aromatic hydrocarbons, including furans of biphenyls and dioxins, especially 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (Passarini et al. 2010). These are well-known industrial pollutants infamous due to previous industrial accidents such as the accident at Ludwigshafen in 1953, Seveso in 1976, and the poisoning of Victor Yushchenko, the former president of Ukraine, in 2004 (Thiess et al. 1982, Sorg et al. 2009, Caputo et al. 1988), in addition to the military use

of Agent Orange, a formulation sprayed during the Vietnam war which unintentionally contained TCDD (Yi et al. 2014). Chloracne is actually a misnomer, representing dermal hamartomas which gradually develop over a few years, with the suggested name—metabolizing acquired dioxin-induced skin hamartoma (MADISH). Histo-pathologically they are characterized by the lack of sebaceous glands, in contrast to acne which is characterized by sebaceous gland hypertrophy (Sorg 2010, Saurat et al. 2011, Ramot et al. 2009). Previously, it was shown that dioxins bind AhR in high-affinity, suggesting its key role in the pathogenesis of chloracne (Van den Berg et al. 1994). However, this association was re-challenged in recent studies, owing to the findings of therapeutic agents such as kinase inhibitors causing AhR-independent chloracne-like symptoms, while other AhR agonists that can be found in food and vegetables do not cause any dermatological symptoms (Sorg 2014).

- Role in the immunological skin response: In a murine model, activation of AhR in KCs resulted in severe skin inflammation and immunological imbalance; emphasizing the role of AhR in the immunological function of KCs (Tauchi et al. 2005). Furthermore, it was shown that intracellular signaling pathways dependent on AhR lead to KC production of cytokines such as IL-1-beta and IL-8 (Henley et al. 2004, Tsuji et al. 2011). In relation to other cytokines, AhR is essential for generating IL-22 by Th17 (Veldhoen et al. 2008). The complexity of its role was enhanced when it was shown to induce production of IL-21 and IL-23, and shift the immune response towards regulatory T cells, while inhibiting Th17 cells, depending on the AhR ligand (Mezrich et al. 2010). In addition, LCs, the epidermal APCs, express high levels of AhR, and LCs deficient of AhR exhibit impaired maturation and function, resulting in diminished CHS reaction (Jux et al. 2009).

15.3 Skin Involvement in Generalized Immunopathological Processes: Systemic Exposure to Drugs and Toxins

15.3.1 Epidemiology and Types

Along with the development and use of a variety of drugs in the modern world, adverse drug reactions (ADRs) are an unavoidable outcome. They are defined as an unintended injury secondary to a medication, which was used in accepted doses and indications (Kuljanac 2008, Edwards and Aronson 2000). ADRs are common, occurring at 5–15% of therapy usage, resulting in substantial cost in relation to morbidity and mortality (Jick 1984). In the 1970s, ADRs were classified to two types of reactions according to their mechanism and dose-dependency (Rawlins and Thompson 1977). Over the years, this classification was expanded, and at present it includes six subtypes, named alphabetically as follows (Edwards and Aronson 2000):

- Type A reaction, "Augmented"—predictable dose-dependent reaction, a consequence of the pharmacologic effect of the drug. Consists the majority (~80%) of ADRs, generally considered as a non-immunological reaction (Thien 2006).
- Type B reaction, "Bizarre"—unexpected, not related to the dose or to the pharmacological effect of the drug. Less common, includes 10–15% of ADRs and is mainly immunological in nature (Gomes and Demoly 2005).
- Type C reaction, "Chronic"—dependent on cumulative dose of the drug over time.
- Type D reaction, "Delayed"—occurs long time after the exposure to the drug.
- Type E reaction, "End of use"—withdrawal effects occurring at cessation of drug.
- Type F reaction, "Failure"—unanticipated failure of the drug.

Further classification is by the severity of ADR, considered as serious or severe if it is lethal or life-threatening, or if it causes significant disability or a need for hospitalization (Edwards and Aronson 2000).

The immunologically-mediated ADRs derive from one of the four pathophysiological types of immunological responses (according to Gell and Coombs classification)—immediate IgE-dependent reaction (type I), cytotoxic antibody reaction (type II), immune complexes-dependent reaction (type III), and delayed-type hypersensitivity reaction (type IV) (Gell and Coombs 1963).

Any organ may be involved, but the skin is the most common target for ADR. The incidence of rashes is about 2–3% among patients receiving an average of 8–9 drugs (Bigby 2001), and in 2% of hospitalized patients the ADR involving the skin is severe (Roujeau and Stern 1994).

Cutaneous ADRs (CADRs) are a group of eruptions developing during drug therapy, with diverse clinical and histo-pathological characteristics and different mechanisms (Kuljanac 2008). For example, exanthematous drug eruption, also known as maculopapular eruption, is the most common, representing 95% of the CADRs (Kuljanac 2008). Its histo-pathological changes are of interface dermatitis with degeneration of the basal-layer-KCss, caused by cytotoxic effects of drug-specific $CD4^+$ T cells, expressing the cytotoxic proteins perforin and granzyme B (Yawalkar 2005). Other CADRs include urticaria, usually activated by an immediate IgE-dependent reaction and other delayed-type hypersensitivity reactions as bullous or pustular drug eruptions, in addition to vasculitis, which is believed to result from an immune-complex reaction (Kuljanac 2008, Pichler 2003). Severe CADRs are uncommon, as mentioned previously, however may lead to a high mortality rate of 10–30% including drug eruption with eosinophilia and systemic symptoms (DRESS) syndrome, Stevens-Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN) (Wolf et al. 2005). We will focus on the pathogenesis of SJS and TEN, which are the most studied adverse drug eruptions.

15.3.2 Pathogenesis of SJS and TEN

SJS and TEN represent different severity grades of the same clinical spectrum: SJS includes involvement of less than 10% of the skin body surface area (BSA), TEN is involvement of above 30% of the BSA and overlapping SJS-TEN is defined as involvement of 10–30% of the BSA (Roujeau 1994). They are characterized by rapid detachment of the epidermis manifesting as bullous and target-like lesions on the skin and erosions in the mucosal membranes (Roujeau and Stern 1994). The most common trigger of these reactions is exposure to drugs, with over 100 chemicals described; the most common ones are trimethoprim-sulfamethoxazole, carbamazepine, aminopenicillins and allopurinol (Roujeau and Stern 1994).

Due to its severity and high mortality-rates, the pathogenesis of SJS and TEN is extensively studied. Over the years, an association between genetic predisposition and immunological mechanisms upon exposure to a specific drug has been found. The main hypothesis is that drug-specific T cells are activated and differentiate to cytotoxic T lymphocytes (CTL) via presentation of the drug by specific human leukocyte antigen (HLA) alleles, however, the pathogenesis of SJS and TEN is still not entirely clear (Chung and Hung 2012). We will discuss the genetic and immunological components that contribute to the pathogenesis of SJS and TEN, summarized in Figure 15.5.

- Genetic susceptibility: The first report demonstrating genetic susceptibility to SJS/TEN revealed an association between HLA-A29, HLA-B12 and HLA-DR7 to sulfonamides-induced TEN and between HLA-A2 and HLA-B12 to oxicam-induced TEN in European populations. It advocated the hypothesis of genetic predisposition to SJS/TEN involving HLA genes, which represent the MCH genes in humans (Roujeau et al. 1987). Further studies showed the following associations—HLA-B58:01 in allopurinol-related SJS/TEN or DRESS syndrome in Han Chinese (Hung et al. 2005), HLA-B15:02 in carbamezapine-related SJS/TEN in Han Chinese population (Chung et al. 2004), with different alleles associated with other types of CADRs in this population (Hung et al. 2006). In the Japanese population, HLA-B15:11 was found to be linked with carbamezapine-induced SJS/TEN (Kaniwa et al. 2010), and lately, HLA-B59:01 was found in the Korean population with methazolamide-induced SJS/TEN (Kim et al. 2010). These observations show that HLA association to SJS/TEN varies between different ethnic groups.
- Immunological response: The interaction between the drug, HLA molecule and T cell response is the main focus of interest in the effort to explore SJS/TEN pathogenesis. In a recent study, using an in-vitro carbamezapine-induced SJS/TEN model, a direct binding of the drug and its derivatives to HLA-B15:02 was detected, resulting in its presentation to CTLs (Wei et al. 2012). CTLs are activated, and further induce a cellular immune response directed towards KCs, mediated by cytotoxic proteins as granulysin, Fas/Fas ligand,

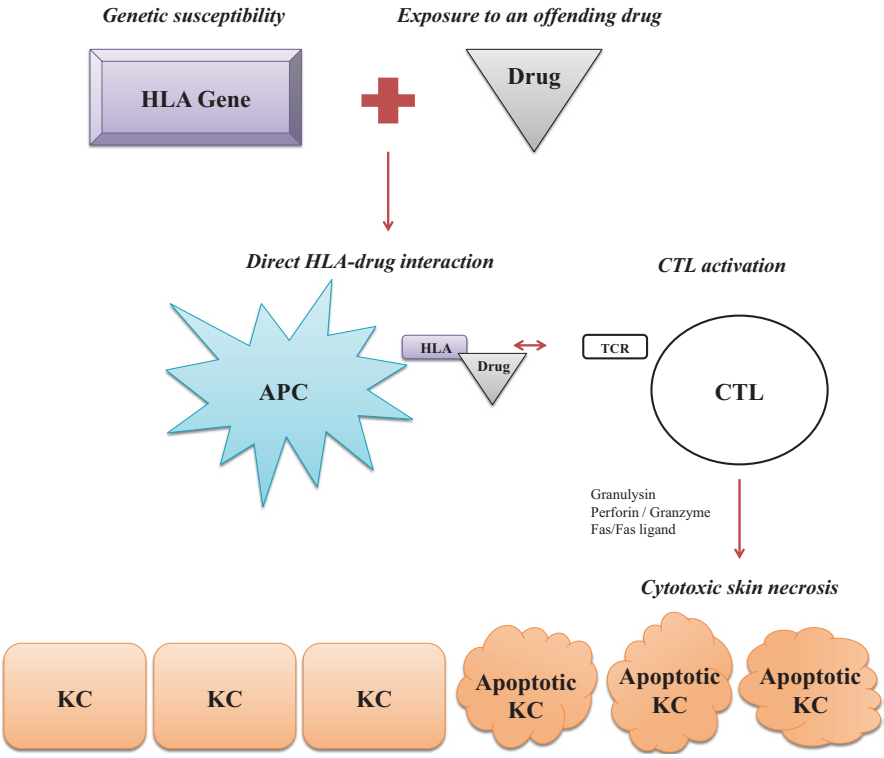

Fig. 15.5 Illustration of the main components involved in the pathogenesis of SJS and TEN. *APC* antigen presenting cells, *CTL* cytotoxic T lymphocyte, *HLA* human leukocyte antigen, *KC* keratinocyte cell, *TCR* T cell receptor

perforin/granzyme B, cytokines and chemokines (Su and Chung 2014) as described below:

– *Granulysin*: A pore-forming cationic protein which resides in the granular compartments of the cytoplasm of CTLs and natural killer (NK) cells (Peña et al. 1997). Upon activation of CTLs or NK cells, granulysin is released extracellularly, resulting in cytotoxic effects on KCs (Chung et al. 2008), induced by its permeabilizing abilities (Okada et al. 2003). High levels of granulysin ribonucleic acids (RNA) and proteins were demonstrated in SJS/TEN blister fluid, in addition to epidermal necrosis and bullous rash developing in murine models following skin injections of granulysin. Furthermore, depletion of granulysin resulted in diminished apoptosis of KCs (Chung et al. 2008). These findings emphasize the pivotal role of granulysin in the pathogenesis of SJS/TEN, especially as an inducer of KC apoptosis, not requiring a direct cellular interaction. Surprisingly, granulysin is not only a cytotoxic

protein, but also a chemoattractant of the immunological response. It was shown to stimulate recruitment and activation of APCs, T cells, monocytes and NK cells and enhance secretion of pro-inflammatory chemokines such as CCL3 and CCL5 and cytokines such as IL-1, IL-6, IL-10 and IFN-gamma (Deng et al. 2005).

- *Perforin/Granzyme B*: Two cytosolic proteins, which are released by CTLs and NK cells following activation. These proteins co-operate for the generation of a cytotoxic effect (Trapani and Smyth 2002), with perforin creating pores in the cell membrane, enabling granzymes B, a serine protease, to enter into the target cell and prompt apoptosis via caspase-dependent and caspase-independent pathways (Bots and Medema 2006). It was shown that perforin and granzyme B are the main contributors to the induction of KCs apoptosis in SJS/TEN, as their inhibition resulted in reduced cytotoxicity. In contrast, the inhibition of Fas/Fas ligand apoptosis pathway did not seem to impact on the cytotoxic response in SJS/TEN (Nassif et al. 2002).
- *Fas/Fas ligand*: Fas is a transmembrane receptor of the death-receptor family, triggered after the binding of Fas ligand on its extracellular domain. It leads to the activation of intracellular cascade of caspases resulting in DNA degradation and cell death (Strasser et al. 2009). The data on its role in SJS/TEN display contradicting results. On the one hand, high levels of Fas ligand on KCs and soluble Fas ligand were found in TEN patients, in addition to inhibition of KCs apoptosis by anti-Fas antibodies (Viard et al. 1998). Later, it was shown that high levels of soluble Fas ligand in the serum of SJS/TEN patients were released not from KCs but from peripheral blood mononuclear cells (PBMCs), and were further stimulated to secrete soluble Fas ligand after exposure to the causal drug (Abe et al. 2003). On the other hand, results of a study performed by Nassif et al. mentioned previously (Nassif et al. 2002) and O'Reilly et al. demonstrated that only membrane-bound Fas ligand, and not soluble Fas ligand, induce cell apoptosis (O'Reilly et al. 2009), therefore questioning the contribution of this pathway in the induction of KCs apoptosis in SJS/TEN.
- *Cytokines and chemokines*: TNF-alpha and IFN-gamma are the main cytokines found to be highly expressed in SJS/TEN lesions, participating in the immunological cytotoxic effects on KCs (Paquet et al. 1994, Viard-Leveugle et al. 2013). Other cytokines and chemokines include mainly IL-2, IL-5, IL-6, IL-10, IL-13, CCR3, CXCR3 and CXCR4 (Correia et al. 2002, Caproni et al. 2006).

15.4 Animal Models

The use of animal models for understanding immunopathology in general, and specifically the immunopathology of the skin, has increased significantly in recent years. Most of these advances are attributed to the discovery of relevant transgenic mice models for inflammatory skin diseases.

In this section, we will review the most prominent mouse models for inflammatory skin disorders, with special emphasize on atopic dermatitis and psoriasis, two skin conditions for which much progress was made lately in our understanding of their pathogenesis. We summarize by providing some general concepts regarding the use of animal models in skin studies, including potential problems and considerations for choosing the most suitable animal models.

15.4.1 Atopic Dermatitis

A large number of compounds have been used to elicit an inflammatory reaction in animal skin, leading to dermatitis. Such compounds have been used to mimic atopic dermatitis or contact dermatitis in different animal species. Some of the compounds which are commonly used are listed here.

2,4,6-trinitrochlorobenzene (TNCB) is a sensitizing agent, which when administered repeatedly to the skin of mice leads to swelling, increase in skin thickness and edema (Harada et al. 2005). Interestingly, it was demonstrated that there is a difference in the skin reaction based on the number of times the compound is applied to the skin. When applied repeatedly to the skin of mice, there is a change from the characteristic delayed type reaction to an immediate type hypersensitivity response. This is followed by a phenotype resembling chronic dermatitis, characterized by epidermal hyperplasia, increase in IgE levels and the accumulation of $CD4^+$ T cells and mast cells in the dermis. Overall, there is a gradual change in this model in the cytokine milieu from Th1 response to Th2 response (Kitagaki et al. 1997), which is reminiscent of the findings in atopic dermatitis in humans, but in a site-specific fashion. Therefore, it is invaluable for the research of the mechanisms involved in the development of chronicity in this disorder.

Another compound which has been shown to cause an atopic dermatitis-like rash in animals is Paraphenylenediamine. When applied to mice (Yokozeki et al. 2001) or guinea pigs (Magnusson 1974), it leads to chronic hypersensitivity reaction, that was shown to be accompanied by increased IgE levels, mast cell recruitment and enhancement of the Th2 cytokine milieu (Yokozeki et al. 2003). The epicutaneous administration to mice of dust mite allergen, which has also been connected to atopic dermatitis in humans (Kimura et al. 1998), caused a severe dermatitis with a Th2-skewed reaction, again mimicking atopic dermatitis (Huang et al. 2003). This model has also been shown to cause concomitant neurogenic inflammation, again similar to atopic dermatitis in humans (Ebertz et al. 1987).

Several transgenic mice models for atopic dermatitis have been developed. The most commonly used mouse model is the NC/Nga mouse, that when kept in an air-uncontrolled conventional room (as opposed to a laminar filter-air flow enclosure in a bioclean room) develops eczematous skin rash accompanied by itching, scaling, erythema, dryness and alopecia (Matsuda et al. 1997). Serum IgE levels increase in parallel to the induction of the skin lesions. While this model has been used extensively, even for the evaluation of potential treatment modalities (Hiroi et al. 1998),

its main drawback is the relatively low incidence of spontaneous development of skin lesions.

Two additional models are based on the activation of IL-18, which can strongly stimulate both IFN-gamma secretion and secretion of Th2 cytokines (Ahn et al. 1997, Hoshino et al. 2000). The IL-18-transgenic (KIL-18 Tg) mice overexpress IL-18 in the skin, leading to the development of atopic dermatitis-like skin lesions after 6 months under specific pathogen-free conditions (Konishi et al. 2002). Another mouse model, KCASP1Tg, overexpresses caspase-1 in its KCs, inducing IL-18 secretion, and develops atopic dermatitis-like lesions much more rapidly, with lesions appearing after 8 weeks (Konishi et al. 2002). Another mouse model, which develops atopic dermatitis-like lesions, is the RelB knockout mouse (Barton et al. 2000). RelB is a transcription factor which belongs to the NF-kappa-B/Rel family, and these mice develop a complex inflammatory phenotype that is not restricted to the skin. In addition to the skin lesions, the mice demonstrate increased number of eosinophils in the blood, IgE overproduction and mast cell degranulation, which are accompanied by pulmonary inflammation, mimicking in part the asthma that is found in association with atopic dermatitis in humans.

15.4.2 Psoriasis

Recently, much progress has been made in elucidating the pathogenesis of psoriasis, allowing the development of new effective drugs. However, until now, a reliable animal model for psoriasis has been unavailable. The most common animal used as a model for psoriasis is the mouse, and a recent review by Wagner et al. provides details on 27 mouse models for psoriasis (Wagner et al. 2010). These models are divided into several groups:

1. Spontaneous mutations: These mouse models are considered to be of limited use, because they lack T cell infiltration by histopathology and don't respond to antipsoriatic treatments (Gijbels et al. 2000).
2. Genetically altered mice:
   (a) Transgenic mouse models: A large number of mice models were developed that over-express cytokines or transcription factors known to be involved in psoriasis pathogenesis. These include for example K5-STAT3C mice,(Sano et al. 2005) K14-VEGF mice (Detmar et al. 1994), overexpression of TGF-beta 1 (Li et al. 2004) and overexpression of TNF (Cheng et al. 1992).
   (b) Gene knock-out mouse models: This is considered a more physiologically relevant mouse model than the transgenic mouse approach. Knock-out mouse models for psoriasis include for example deletion of the IL-1 receptor antagonist gene,(Shepherd et al. 2004) epidermal deletion of the serum response factor (Koegel et al. 2009) and epidermal deletion of the Jun proteins (Guinea-Viniegra et al. 2009, Meixner et al. 2008).

3. Cytokine injections: Subcutaneous injections of IL-12 and IL-21 can lead to inflammatory skin lesions resembling psoriasis (Caruso et al. 2009). Application of imiquimod, which is a strong immune activator, leads to psoriasis-like skin lesions, mediated by activation of the IL-23/IL-17 axis (Van der Fits et al. 2009). A novel promising model for psoriasis is the dermal injection of IL-23 (Hedrick et al. 2009, Jiang et al. 2013). This model showed great resemblance to human disease when compared to human psoriasis transcriptome (Suarez-Farinas et al. 2013).
4. Transplantation mouse models:
   (a) Cell transfer models: Prkdc^{scid} mice which received CD4^{+}CD45RBhi T cells developed psoriasis-like skin lesions (Schon et al. 1997).
   (b) Humanized xenotransplantation models: This is one of the most elegant models for psoriasis, and these models are considered to most closely resemble psoriasis in humans. In these models, human psoriasis-prone skin is transplanted to immunodeficient mice (Gudjonsson et al. 2007). While these models are very representative of the human disease, they have several disadvantages: the systemic effects of psoriasis cannot be examined (Wagner et al. 2010), and the production of such models is expensive and challenging, large skin samples are required, and they should be implanted quickly to avoid ischemia.

15.4.3 Acne

The animal models for acne are highly limited. One of the more common animal models for studying acne is the hairless rhino mouse model. The skin of this mouse contains a large number of horn-filled utriculi, which closely mimic comedones in humans, the non-inflammatory skin lesions of acne (Nakano et al. 2007). These utriculi are superficial cysts that stem from the hair canal and open to the surface. They contain keratinous material and are lined with squamous epithelium (Ashton et al. 1984). Recently, a Kyoto rhino rat model was developed, which also develops non-inflammatory comedones on their backs (Yoshimasu et al. 2014). While these rats develop non-inflammatory lesions spontaneously, it is possible to induce inflammatory acne by injecting *Propionibacterium acnes* intradermally. Such approach was used in rats (Fan et al. 2013) and in mice (Zhang et al. 2013).

15.4.4 Phototoxicity

Phototoxicity reaction is seen in the skin of humans following systemic administration of photodynamic drugs or chemicals followed by exposure to UV radiation. This reaction is characterized by redness, swelling, skin burning and even blisters (Matsumoto et al. 2010). Such photoreactive drugs include antibiotics, psoralens,

nonsteroidal anti-inflammatory drugs and tranquilizers (Marrot et al. 2003, Yoshikawa et al. 2013). Similar reactions can also be seen in herbivores after ingestion of photosensitizing plants, such as St John's wort and buckwheat (i.e., primary photosensitization, type I), in animals with defective porphyrin metabolism (type II) or in animals with severe liver damage (hepatogenous photosensitization, type III) (Galitzer and Oehme 1978). Several animal models have been described to test for phototoxic effects of drugs, and include the use of mice, rabbits, guinea pigs, and swine (Matsumoto et al. 2010, Yoshikawa et al. 2013). Different protocols exist, but in all studies the animal is administered with a specific compound, followed by irradiation with specific UV wavelengths. As control, several sites of the animal are covered during the UV exposure. Phytophotodermatitis, a common skim reaction in humans induced by contact with a photosensitizer substance (furanocoumarins or psoralens) derived from plants followed by sun exposure (Stoner and Rasmussen 1983), has also been reproduced in rats (Goncalves et al. 2005).

15.4.5 General Considerations

The choice of the animal used for the dermatotoxicology experiments is complicated. However, several authors have pointed out the pig as a preferred animal for these studies for several reasons (Mahl et al. 2006):

1. The permeability and metabolic characteristics of the porcine skin are relatively similar to human skin (Ngawhirunpat et al. 2004), leading to comparable penetration of compounds across human and porcine skin (Reifenrath et al. 1984).
2. The skin of pigs shows high morphological and physiological similarity to humans in terms of general morphology, epidermal thickness, cellular composition and turnover and immunological reactivity (Lavker et al. 1991).
3. The pig has a relatively hairless and non-pigmented skin, making it optimal for clinical evaluation of surface changes (Yabuki et al. 2007).

Nevertheless, several differences should be taken into account, including scaling of the skin, vascularization of the dermis and function and prevalence of eccrine and apocrine glands (Mahl et al. 2006). The interpretation of dermal toxicity studies should be done very carefully, as there are many considerations that should be taken into account, This has been recently reviewed in detail by Chandra et al. (2014b), and the main points are summarized below:

1. Hair removal: The specific technique used for hair removal can affect the interpretation of results. Hair clipping can cause epidermal hyperplasia and hyperkeratosis and chemical depilation or tape stripping can lead to reduction of the stratum corneum.
2. Wrapping procedure: The test sites on the skin are commonly occluded, and accompanied with an elastic bandage. This kind of wrapping can lead to sys-

temic effects, such as hepatic necrosis (Ramot et al. 2012), the so called "corset liver" (Nyska et al. 1992, Parker and Gibson 1995).

3. Species differences: Species and gender differences can be observed not only to the active compounds, but also, surprisingly, to the vehicle. Such was the case in the unexpected finding that New Zealand white rabbits are sensitive to white petrolatum for example (Chandra et al. 2014a). Of course, there are also anatomical variations between species that can affect interpretation of results, such as variability in pigmentation or sebaceous gland size.

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