Chapter 2
Spleen

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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.

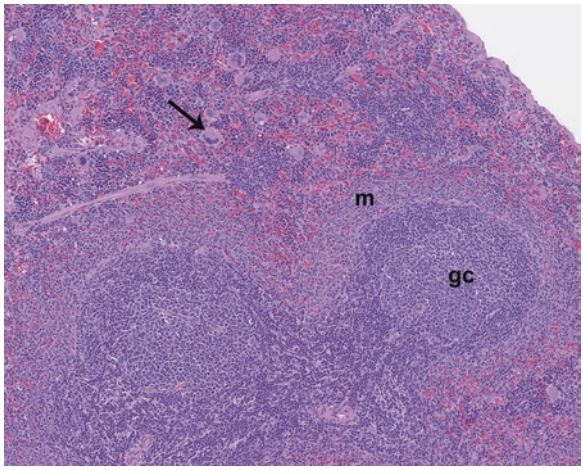

Fig. 2.1 Two splenic follicles in a juvenile (postnatal day 42) rat. Note the presence of germinal centers (*gc*) and prominent marginal zone (*m*). Hematopoiesis is present in the splenic red pulp and contains prominent megakaryocytes (*arrow*) admixed with myeloid and erythroid constituuents. H&E stain,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

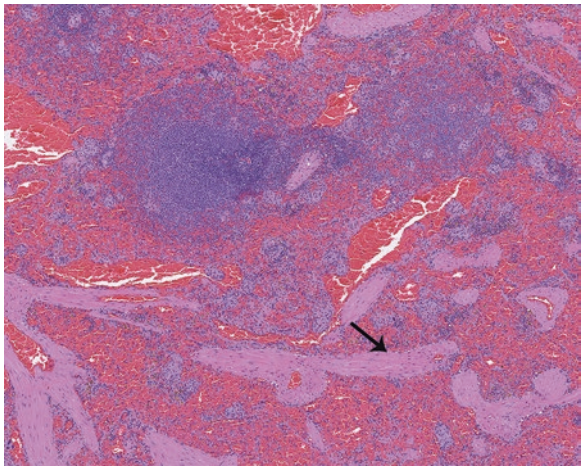

Fig. 2.2 Photomicrograph of the spleen of a dog showing multiple smooth muscle trabeculae (*arrow*). Note the prominent erythrocytic population in the red pulp. Contraction of the muscular trabeculae facilitates expulsion of blood from the spleen in the event of peripheral hemorrhage that results in 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).

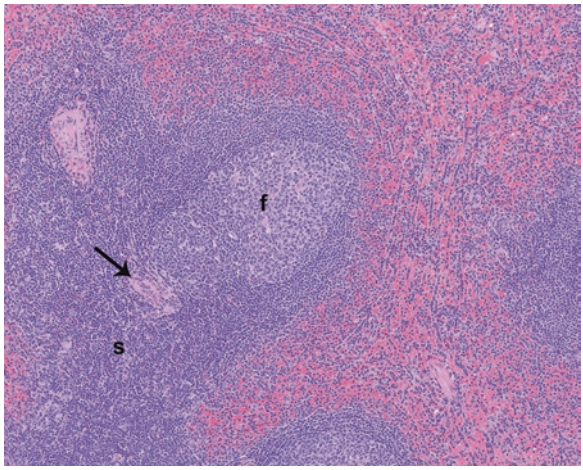

Fig. 2.3 Spleen of a cynomolgus macaque showing a follicle (*f*) and peri-arteriolar lymphoid sheath (PALS) (*s*) surrounding an arteriole (*arrow*). H&E stain, 10× objective magnification

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

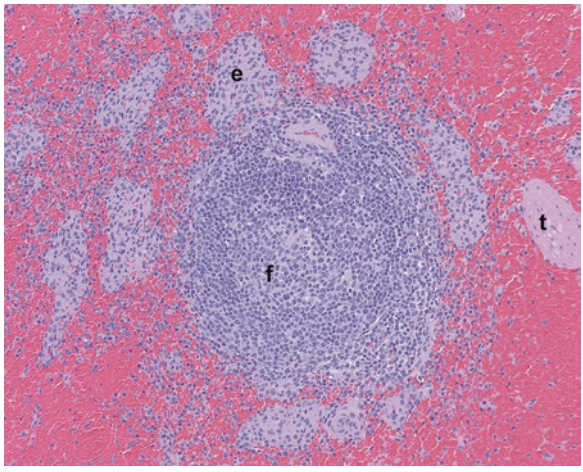

Fig. 2.4 Splenic follicle (*f*) with prominent ellipsoids (*e*) (macrophage-sheathed capillaries) in the spleen of a Gottingen minipig. Note the smooth muscle trabecula (*t*) and prominent erythrocytic population throughout the red pulp. H&E stain, 15.1 objective magnification

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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