
2.1 Introduction

The major functions of the testis are spermatogenesis and steroidogenesis. The histological structure of the adult testis is organized by two distinct regions: the seminiferous tubules and the interstitial spaces between tubules. Spermatogenesis takes place within the convoluted seminiferous tubules, which originate from the peripheral testis, connect the tubuli recti, and then terminate at the rete testis (Fig. 2.1). Testicular germ cells (TGC) leave the testis from the rete testis. The seminiferous tubules, tubuli recti, rete testis, ductuli efferentes, epididymal ducts, and vas deferens form a functional unit and are in direct continuity for the transport of germ cells (Fig. 2.1). Steroidogenesis is fulfilled by Leydig cells in the interstitial spaces. The sex hormones, mainly testosterone, are critical for normal spermatogenesis by their paracrine action on the seminiferous epithelium. These histological structures for spermatogenesis and steroidogenesis are broken by local inflammation. Testicular autoimmunity is caused by immune responses against various testicular autoantigens, resulting in the lymphocytic infiltration and the spermatogenic disturbance. For induction or inhibition of testicular autoimmunity, controls of both systemic immune responses against testicular autoantigens and local immuno-circumstances inside the testis are important.

2.2 Autoimmunogenicity of Spermatids and Spermatozoa

The seminiferous epithelium is subdivided into two compartments. The first (basal) compartment, which is basal to the blood-testis barrier (BTB), contains spermatogonia and young spermatocytes that have free access to substances from the vascular compartment; the second (adluminal) compartment lies above the BTB and represents the microenvironment that is isolated from the vascular system, where meiosis, spermiogenesis, and spermiation occur.

Fig. 2.1 Anatomy of germ cell duct system in males.
D ductuli efferentes, *E* epididymis, *R* rete testis, *S* seminiferous tubule, *T* tubuli recti, *V* vas deferens

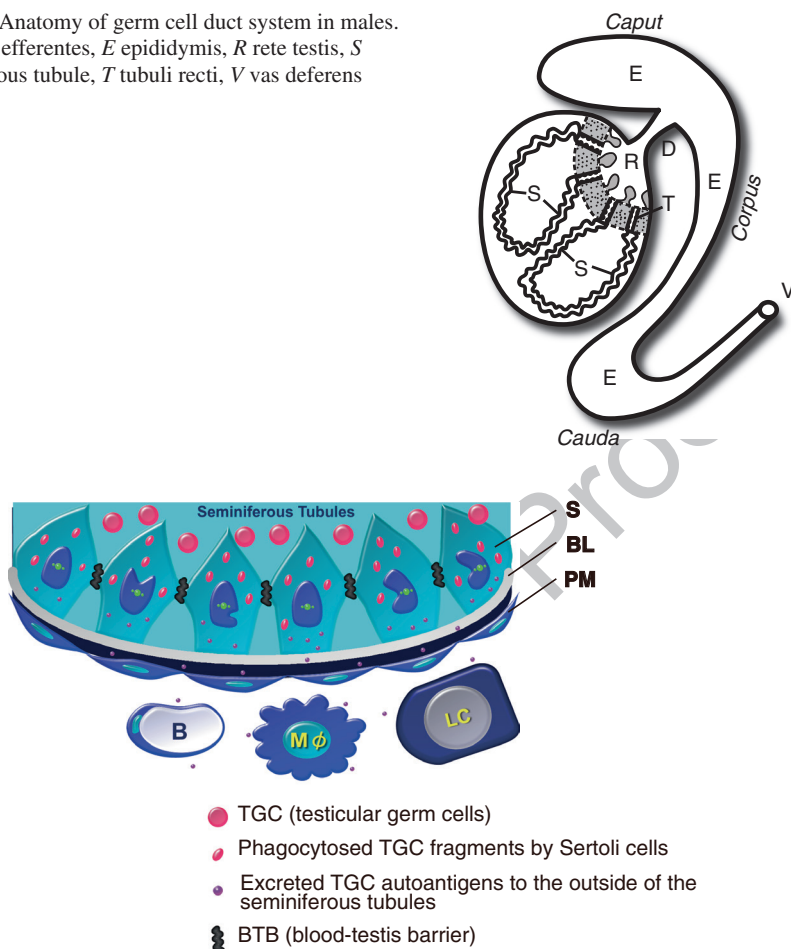


Fig. 2.2 A hypothesis of excretion of TGC autoantigens to the testicular interstitium under physiological condition. *B* blood capillary, *BL* basal lamina, *LC* Leydig cell, *Mφ* macrophage, *PM* peritubular myoid cell, *S* Sertoli cell

Spermatids and spermatozoa emerging from meiosis do not appear in the seminiferous epithelium until puberty, when immune tolerance has already been established. Different from spermatogonia and preleptotene spermatocytes having 46 chromosomes, spermatids and spermatozoa are quite new in that they have only 23 chromosomes and express various new developing antigens (Ike et al. 2007). A large number of novel proteins are expressed in developing TGC during spermatogenesis (Fig. 2.2). Hence, they contain various autoimmunogenic antigens which are recognized as foreign bodies, alien substances, nonself, or neo-self by self-immune system, leading to a challenge to the immune system. However, under normal conditions, the haploid cells are sequestered and preserved from the circulatory system by the BTB.

Localization of autoantigens detected by circulating autoantibodies in mice injected with syngeneic TGC alone was immunohistochemically studied by reaction of the immune sera with frozen sections of the testes from various aged mice (Itoh et al. 1994). The results showed that the reactivity of immune sera was detected in the testis sections of normal mice older than 24 days of age but not in those mice younger than 20 days of age. It is well known that the seminiferous tubules of mice contain spermatogonia at the day of birth, preleptotene spermatocytes at 1 week of age, pachytene spermatocytes at 2 weeks of age, round and oval spermatids at 3 weeks of age, the elongating spermatids at 4 weeks of age, and most mature (elongated) spermatids at 5 weeks of age (Vergouwen et al. 1995). Therefore, the immunostain was detected on spermatids of various stages, and immature TGC, such as spermatogonia, preleptotene spermatocytes, and pachytene spermatocytes, are not reactive with the immune sera. Using microarray analyses of 1315 testicular genes in adult and juvenile mice, 46% exhibited an increase of twofold or more in adults compared to juveniles and 22% decrease of twofold or more (Ike et al. 2007). On the basis of the fundamental difference in expression profiles, and molecular functions of the encoded products, the genes were classified into seven groups: the postmeiotically upregulated genes encoding various enzymes, structural proteins, regulatory proteins, chaperones, downregulated genes encoding hemoglobulins, oxidation-/reduction-related proteins, or machinery associated with protein synthesis, such as ribosomal proteins. Liu et al. (1992) have immunohistochemically demonstrated that mouse sperm antigen (MSA-63) detected by a monoclonal antibody (HS-63), which mainly recognizes proteins on the acrosomal region of the spermatozoa, was not expressed until 25 days after birth. This period is in accord with the time at which TGC initially acquire a delayed-type hypersensitivity (DTH)-eliciting capacity and lymphostimulatory activities for cytokine production in the immunized mice (Soramoto et al. 1993). In addition to the appearance of new autoantigens in mice older than 24 days of age, TNF- α , a potent pro-inflammatory cytokine, is endogenously synthesized by TGC within normal seminiferous tubules (De et al. 1993).

Severe inflammation is easily and locally induced by subcutaneous injection with prepared syngeneic spermatozoa without the use of any adjuvants in mice (Ball 1984). The lesion at the injection site developed in a characteristic sequence which is divided into four stages: acute inflammation, simple chronic inflammation, granuloma formation, and repair. The acute inflammation was seen at 6 and 24 h. Large numbers of neutrophils, many containing phagocytosed sperm heads, were present. The small number of macrophages was noted at 6 h, but they were more conspicuous by 24 h. Between 2 and 4 days, large numbers of spermatozoa persisted and were associated with an infiltrate of many macrophages. Neutrophils were rarely seen in such lesions. From 5 to 9 days, discrete masses of mononuclear inflammatory cells, predominantly macrophages but including some epithelioid cells and a few Langhans-type giant cells, were present. The lesions generally contained few spermatozoa. Some lymphocytes and eosinophils were seen at the periphery of the lesions, and there was a fibrous connective tissue at this site. By 14–21 days, small numbers of extracellular spermatozoa were associated with an infiltrate of macrophages surrounded by a zone of immature fibrous tissue. The acute inflammatory phase of the lesion appeared more

severe and vigorous in the mice previously immunized against syngeneic spermatozoa than in its immunologically innocent counterparts. There is a sex difference in DTH reaction to syngeneic TGC. Both male and female mice were subcutaneously injected with TGC, and the DTH reaction was elicited with TGC 6 days after the immunization. The results showed that the DTH was detected in female mice, but not in male mice. A sex difference in the DTH reaction to sheep red blood cells was not detected. It is noted that the sex differences in the DTH against TGC were attributed to TGC other than spermatozoa because a sex difference was not detected against spermatozoa (Ball 1984; Yoshida et al. 1983).

2.3 Protection of Autoimmunogenic Spermatids and Spermatozoa by the Blood-Testis Barrier (BTB)

The functional meaning of the BTB can be referred to two main aspects: (1) the maintenance of a specific milieu for the differentiation of TGC and (2) their segregation from the immune system of the body. The BTB is created near the basal lamina by various junctions including tight junction, basal ectoplasmic specializations, gap junctions, and desmosome-like junctions between two adjacent Sertoli cells (Mruk and Yan Cheng 2015). The BTB function is to completely exclude all cellular and molecular traffic via the extracellular space between the Sertoli cells (Dym and Fawcett 1970). Basal to the tight junctions, the premeiotic spermatogonia are located near the basal lamina and the bloodstream. After the first step in meiosis, a new tight junction is formed below the developing spermatogonia, and the old tight junction above the new one will be lost (Bart et al. 2002). During spermatogenesis, maturing spermatogonia are transported toward the luminal side of the seminiferous tubules. The BTB must physically disassemble permitting the passage of preleptotene and leptotene spermatocytes. This dynamic process is regulated by transforming growth factor-beta-3 and TNF-alpha (Bronson 2010). It is absolutely essential for creating a highly specialized biochemical environment for the meiotic and postmeiotic cells, but the BTB also limits the access of systemic immunity and sequesters the majority of the autoimmunogenic TGC. The BTB, formed by Sertoli cells, primarily protects postmeiotic spermatids and spermatozoa from attack by the self-immune system. Most recently, transplantation of rat spermatogenesis inside the BTB of immunocompetent mice has succeeded (Qu et al. 2012; Hirayanagi et al. 2015). This indicates that the BTB protects not only autologous but also xenogeneic TGC from attack by self-immune system. However, if the BTB is functionally damaged, autoantigens of these haploid cells leak out beyond the BTB, leading to a continuous supply of the autoantigens to the immune system, with the resultant chronic inflammation in the testis for a prolonged length of time (Itoh et al. 2005; Naito and Itoh 2008). In particular, the BTB at the tubuli recti and the rete testis is not as strong as that in the peripheral testis. In a study of topographical uptake of blood-borne horseradish peroxidase in the testis, no horseradish peroxidase infiltrated into the lumen of the seminiferous tubules; however, some horseradish peroxidase was detected in tubular walls and epithelial cells lining the tubuli recti and the rete testis, indicating that these

regions are permeable to horseradish peroxidase (Itoh et al. 1998a). IL-1, a pro-inflammatory cytokine, is expressed in the testis and regulates testicular functions under physiological conditions. IL-1 facilitates the BTB opening by affecting the actin cytoskeleton (Sarkar et al. 2008).

Under normal condition, the blood-epididymal barrier formed by epididymal epithelial cells also protects autoimmunogenic spermatozoa from attack by the self-immune system. However, the blood-epididymal barrier is less exclusive than the highly specialized tight junctions of the BTB (Hinton and Palladino 1995). Consequently, immune cells are common among the epithelial cells of the epididymal duct (Wang and Holstein 1983; Ritchie et al. 1984).

2.4 Protection of Autoimmunogenic Spermatids and Spermatozoa by Various Local Elements Other Than the BTB

There are certain areas in the body where immune responses are “forbidden.” These sites are referred to as “immunologically privileged” sites. The mechanisms underlying immune privilege result from “immunologic ignorance” or “immunologic tolerance.” Testicular immune privilege was once believed to be mainly based on the sequestration of autoantigens from the immune system by the BTB in the seminiferous tubules. However, recent evidence has suggested that the testicular interstitium represents the first line of testicular defense against pathogens from the bloodstream. The multiplex immunosuppressive mechanisms, involving various local elements of different origins, participate in the formation of an immunologically privileged area within the testis. Indeed, the testicular interstitium outside the BTB, where many resident macrophages and Leydig cells are normally present, is also protected from attack by the self-immune system.

All testicular cells, involving Sertoli cells, Leydig cells, testicular macrophages, peritubular myoid cells, endothelia of blood and lymph capillaries, lymphocytes, and TGC themselves, may modulate local immunity in the testis (Itoh et al. 1995a, 1999a, 2005). To maintain the testicular immune privilege, the testicular cells express and secrete numerous immunoregulatory molecules including androgens, macrophage migration inhibitory factor, activin, Fas ligand, protein S, IL-10, IL-35, transforming growth factor-beta, programmed death-ligand 1, toll-like receptors (TLR), and Tyro-3, Axl, and Mer (TAM) receptors, which play critical roles in regulating immune responses in the testis (Fijak and Meinhardt 2006; Sun et al. 2010; Meinhardt and Hedger 2011; Winnall et al. 2011a, b; Li et al. 2012; Terayama et al. 2014; Deng et al. 2016). Both activin A and activin B are abundantly expressed by Sertoli cells. Activin A inhibits the expression of pro-inflammatory cytokines, including IL-1 and IL-6, thus suppressing the testicular inflammatory responses (Phillips et al. 2009). The Fas/Fas ligand system suppresses immune responses by inducing the apoptosis of Fas-bearing activated lymphocytes. Fas ligand, also called CD95L, is abundantly expressed in the testis. Testicular immune privilege is maintained by inducing lymphocyte apoptosis via Fas ligand expressed mainly in Sertoli cells. Fas ligand is also

expressed in TGC. It remains to be investigated whether Fas ligand that is expressed in the TGC induces lymphocyte apoptosis and contributes to immune privilege within the seminiferous tubules. Programmed death receptor-1/programmed death-ligand 1 is another T cell tolerance system. Programmed death-ligand 1 inhibits T cell activation through programmed death receptor-1. It is constitutively expressed mainly by spermatocytes and spermatids in the seminiferous tubules and involved in the survival of allogeneic islet allografts, suggesting that programmed death receptor-1/programmed death-ligand 1 system is also a mechanism that underlies testicular immune privilege (Cheng et al. 2009). Furthermore, the testis locally generates an efficient innate immune system against pathogens. TLRs are pattern recognition receptors that recognize pathogen-associated molecular patterns. Various TLRs are expressed in various testicular cells of different species (Bhushan et al. 2008, 2011; Fujita et al. 2011; Hedger 2011a, b). Therefore, TLR-mediated innate immune responses by nonimmune cells in the testis also play a critical role in the protection of the testis from infectious diseases. Tyro-3, Axl, and Mer (TAM) receptors are negative regulators of TLR-initiated systemic innate immunity and play critical roles in regulating immune responses (Sun et al. 2010). They are abundantly expressed in Sertoli cells and Leydig cells under normal state and therefore regulate the tissue homeostasis in immunoprivileged testis (Deng et al. 2016).

2.5 Cell Populations Involved in the Testicular Immunoregulation

2.5.1 Sertoli Cells

The testicular cells that appear to be central to maintain testicular immune privilege are the Sertoli cells. These columnar cells extend from the basal lamina to the lumen of the tubules. The Sertoli cells are responsible for physical support of TGC, providing them with essential nutrients and growth factors. Sertoli cells forming the BTB support TGC development and also secrete some proteins for the suppression of lymphocyte proliferation to the outside of the BTB (Wyatt et al. 1988; De Cesaris et al. 1992). Inhibin and activin produced by the Sertoli cells also, respectively, accentuate and reduce thymocyte proliferation in response to mitogen (Lee et al. 1989; Hedger et al. 1989). Sulfated glycoprotein 2, a major product of the Sertoli cell, has been shown to inhibit cell lysis by the complement attack complex, C5b6789 (Jenne and Tschopp 1989; Griswold et al. 1986; Law and Griswold 1994). Sertoli cells secrete inhibitors of granzyme B, which is part of the destructive arsenal of cytotoxic lymphocytes (Sipione et al. 2006). Transforming growth factor-beta secreted by Sertoli cells also contributes to testicular immune privilege through their immunosuppressive activities (Pollanen et al. 1993). It was demonstrated that transforming growth factor-beta facilitates Sertoli cells to support graft survival in co-transplantation experiments (Suarez-Pinzon et al. 2000). In cultured Sertoli cells, inflammatory cytokines such as TNF-alpha, IL-1, IFN-gamma, and lipopolysaccharide strongly enhance the surface expression of integrin ligands, intercellular

adhesion molecules-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1). These molecules are known to be specific for binding of lymphocytes (Riccioli et al. 1995). This suggests that both direct and paracrine mechanisms of interaction between Sertoli cells and lymphocytes are present for the control of immune reactions in the testis (Fillipini et al. 2001).

The Fas/Fas ligand system has been considered to be one of the central mechanisms in homeostasis of the immune response. Fas is a type I membrane protein that belongs to the TNF receptor family and is known to be a receptor for Fas ligand, a cytokine also belonging to the TNF family. Activated lymphocytes that express Fas undergo apoptosis on interaction with cells bearing the Fas ligand. In the testis, the Fas ligand has been mainly detected in the Sertoli cells of the rodent testis (Bellgrau and Selawry 1990; Bellgrau et al. 1995). A developmental study in men demonstrated that Fas ligand expression was found in Sertoli cells of adult testis but not in fetal testis (Francavilla et al. 2000). In rat testes, Fas ligand expression is present from the early postnatal days up to adult, and the Sertoli cells are the main Fas ligand-expressing cell within the seminiferous tubules (D'Abrizio et al. 2004). Importantly, seminiferous tubules of normal mice survived under the kidney capsule of an allogeneic host for much longer periods than seminiferous tubules from gld mutant mice that lack a functional Fas ligand (Bellgrau et al. 1995). Therefore, the constitutive expression of Fas ligand on Sertoli cells may contribute to the testicular immune privilege by inducing the apoptosis of infiltrating T cells that express Fas (Takeda et al. 1998; Koji 2001; Koji et al. 2001). The interaction of Sertoli cells expressing Fas ligand with Fas-bearing autoreactive lymphocytes, leading to the death of the latter by apoptosis, has been proposed as the mechanism responsible for the maintenance of immune tolerance in the testis. The Sertoli cells also express the negative costimulatory ligand CD274 antigen (also called B7-H1), which has the capacity to induce apoptosis in antigen-specific T cells (Dal Secco et al. 2008). That may be why isolated Sertoli cells can survive in both allogeneic and xenogeneic barriers and also provide localized immunoprotective environment for allografts and xenografts (Selawry and Cameron 1993; Sanberg et al. 1996; Korbitt et al. 1997; Suarez-Pinzon et al. 2000; Dufour et al. 2008).

Sertoli cells have a strong phagocytic activity *in vivo*, like macrophages. Cultured Sertoli cells are also active phagocytes *in vitro* but do not express constitutively MHC class II antigens or macrophage-specific markers (Kohn et al. 1983). Phagocytic removal of the apoptotic TGC and residual bodies by Sertoli cells is critical for intact TGC to differentiate. The uptake of residual bodies and apoptotic TGC may endow Sertoli cells with producing factors necessary for spermatogenesis. Phagocytosis of apoptotic TGC by Sertoli cells may be also a process that regulates immunity to TGC autoantigens and supports self-tolerance. Therefore, timely removal of apoptotic TGC and residual bodies by the Sertoli cells is important to avoid autoimmune responses. It is unclear how Sertoli cells phagocytosing apoptotic TGC induce self-tolerance to TGC autoantigens; however, there is a possibility that Sertoli cells excrete some phagocytosed materials to the outside of BTB (Fig. 2.2). The latent leakage of small amount of TGC autoantigens into the testicular interstitium under normal condition may play a role on peripheral tolerance preventing testicular autoimmunity (Naito et al. 2008).

Differing from the study by Kohno et al. (1983), it was later reported that, in response to IFN- γ , mouse Sertoli cells strongly upregulate expression of MHC class II antigens, indicating an antigen-presenting capability of Sertoli cells (Dal Secco et al. 2008). Dal Secco et al. (2008) also demonstrated that Sertoli cells upregulate the negative costimulatory ligand B7-H1 (CD274) in response to IFN- γ . Blockade of B7-H1 on the Sertoli cell surface resulted in enhanced proliferation of CD8 $^{+}$ T cells co-cultured with Sertoli cells. Moreover, co-culturing T cells with Sertoli cells can induce an increase in CD4 $^{+}$ CD25 $^{+}$ T cells and a decrease in CD4 $^{+}$ CD25 $^{-}$ T cells, suggesting Sertoli cell-mediated Treg conversion; this process was found to be B7-H1 independent (Dal Secco et al. 2008). It implies that Sertoli cells are potentially capable of downregulating the local immune response, on the one hand by directly inhibiting CD8 $^{+}$ T cell proliferation through B7-H1 and, on the other hand, by inducing an increase in Treg that might suppress other bystander T cells.

Testosterone produced by Leydig cells is not only one of immunosuppressive steroid hormones but also has an effect on the BTB composed of inter-Sertoli cell junctions. Sertoli cell-specific deletion of the androgen receptor in knockout mice disrupts testicular immune privilege (Meng et al. 2005), possibly because androgens regulate Sertoli cells' tight junctions (McCabe et al. 2010, 2012, 2016; Meng et al. 2011).

Sertoli cells participate in the testicular defense system against viruses and bacteria. Mouse and rat Sertoli cells express functional TLR from TLR1 to TLR6 for innate immunity (Riccioli et al. 2000, 2006; Starace et al. 2008; Wu et al. 2008; Hedger 2011b; Winnall et al. 2011b). Under stimulation of TLR with various invading pathogens, Sertoli cells produce the pro-inflammatory molecules. Therefore, TLR3 activation induces antiviral immune responses in mouse Sertoli cells (Starace et al. 2008). Sertoli cells may create an immune-privileged microenvironment that protects TGC from interstitial and/or ascending pathogens; however, there is a risk that innate immune responses through TLR activation breakdown of a milieu of the spermatogenesis will result in male infertility.

TAM receptors composed of Tyro-3, Axl, and Mer are negative regulators of TLR-initiated systemic innate immunity and play critical roles in regulating immune responses (Sun et al. 2010). Indeed, various TLRs are expressed and functional in Sertoli cells, and TAM receptors inhibit TLR-mediated inflammatory cytokine production by Sertoli cells. Moreover, TAM system facilitates phagocytic clearance of apoptotic TGC by Sertoli cells. The removal of apoptotic TGC by phagocytes facilitates the elimination of the autoantigens, which may reduce endogenous inflammation.

In regard to the modified Sertoli cells at the tubule recti, they normally phagocytose apoptotic TGC for the elimination of unwanted spermatozoa (Naito et al. 2008, 2009). This phenomenon is regarded as a normal mechanism for transport of normal spermatozoa to the ductuli efferentes. It may be that some TGC autoantigens are excreted from the modified Sertoli cells into the area of adjacent multilayered basal lamina and peritubular myoid cells for inducing self-tolerance to TGC antigens (Fig. 2.3). In particular, both the basal lamina and the cytoplasm of modified Sertoli cell intimately interconnect with each other, similar to the sagittal suture in the human skull (Tainosho et al. 2011). This suggests that the modified Sertoli cells

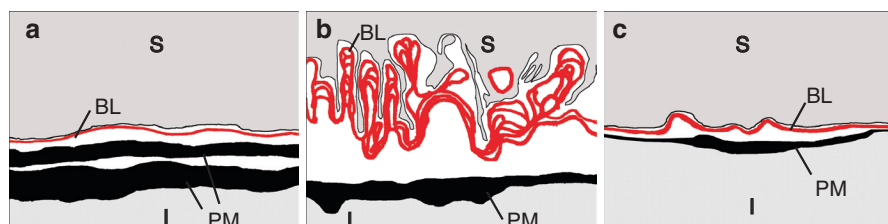


Fig. 2.3 Spaces among Sertoli cells, basal lamina, and peritubular myoid cells at seminiferous tubules (a), tubuli recti (b), and rete testis (c) in the mouse. *BL* basal lamina of the tube (red colored), *I* interstitium, *PM* peritubular myoid cell cytoplasm, *S* Sertoli cell cytoplasm

have a wide surface area at their base and therefore can effectively excrete intratubular materials to the outside of tubuli recti or take various extra-tubular molecules into the tubuli recti. However, the modified Sertoli cells at the tubuli recti and epithelial cells at the rete testis may not directly present the autoantigens to lymphocytes because of a lack of class II MHC antigens, as seen in Sertoli cells in vivo (Itoh et al. 1991).

2.5.2 Leydig Cells

The normal testis is an organ packed compactly with seminiferous tubules, having little interstitium. However, many Leydig cells are compacted in its interstitium. Testosterone, an immunosuppressive steroid hormone, is produced by Leydig cells and suppresses lymphocyte proliferation. Testosterone also reduces TLR4 expression in macrophages (Rettew et al. 2008). Administration of testosterone suppresses autoimmune diseases (Cutolo et al. 2004; Gold and Voskuhl 2009). The intratesticular testosterone concentration is tenfold higher than the serum concentration and is far greater than necessary for the maintenance of normal spermatogenesis (Jarow et al. 2005).

Leydig cells exhibit high antiviral activities in response to viral infections (Dejucq et al. 1995, 1998; Melaine et al. 2003). TLR2, TLR3, and TLR4 are present in Leydig cells, and they trigger innate immunity in response to ligand stimulation (Shang et al. 2011). Although TLR activation of Leydig cells provides defense against invading pathogens, innate immune inflammation in testicular interstitium may also affect the spermatogenesis (Wu et al. 2016). It was also reported that Leydig cells regulate the expansion of testicular macrophages and lymphocyte numbers in the testis (Raburn et al. 1991, 1993; Hedger and Meinhardt 2000). Testosterone production by Leydig cells was stimulated by a conditioned medium from culture of testicular macrophages in a dose-dependent manner in vitro (Yee and Hutson 1985). It is also known that Leydig cells spontaneously adhere to lymphocytes and nonspecifically suppress the proliferation of lymphocytes in vitro without any dependence on testosterone (Born and Wekerle 1981,

1982). In addition to immunosuppressive testosterone, immunosuppressive cytokines such as transforming growth factor-beta and IL-10 are secreted from Leydig cells (Teerds et al. 1990; Avallet et al. 1994; Verajankorva et al. 2001; O’Bryan et al. 2005).

In the brain, astrocytes are known to have an important role in establishing structural properties of blood-brain barrier composed of endothelial cells (Bart et al. 2002). Astrocytic end feet produce basic fibroblastic growth factor, which is also produced by Leydig cells. Desert hedgehog gene is a signaling molecule expressed by Sertoli cells. Its receptor is localized to Leydig cells and also peritubular fibroblastoid cells (Clark et al. 2000). In mice lacking the desert hedgehog gene, adult-type Leydig cells were lacked, and numerous undifferentiated fibroblastic cells were in the testicular interstitium with production of abundant collagen. Moreover, the basal lamina, normally present between the peritubular myoid cells and Sertoli cells, was focally absent (Clark et al. 2000). This indicates that Leydig cells are also involved in maintaining the BTB. Fibroblastic cellular constituents exhibiting fibroblastoid morphology surrounding the seminiferous tubular wall are regarded as precursors of the functional Leydig cells (Hatakeyama 1965; Teerds et al. 1988, 1999). These peritubular precursor cells may support the BTB. Other studies demonstrated that testosterone regulates the permeability of the BTB by regulating the expression of a Sertoli cell tight junction protein, claudin-3 (Meng et al. 2005, 2011).

2.5.3 Testicular Leukocytes

In the normal testis, leukocytes are found almost exclusively within the interstitial tissue between the seminiferous tubules and under the tunica albuginea. These cells chiefly comprise of resident macrophages, dendritic cells, and circulating lymphocytes, although variable numbers of mast cells and eosinophils are also present, depending upon the species. For example, in the macaque, 42.7% of the testicular leukocytes were CD163⁺ macrophages, while 4.5% were CD14⁺CD163⁻ monocyte-like macrophages. 30.8% of testicular leukocytes were CD3⁺ T cells, with CD4⁺ and CD8⁺ cell proportions similar to those in the blood. B cells and granulocytes were 0.24% and 3.3% of testicular leukocytes, respectively. Small populations of dendritic cells, plasma cells, NK cells, and NKT cells were also detected (De Rose et al. 2013) (Table 2.1).

Table 2.1 Resident leukocytes in the testis

Macrophages (CD163 ⁺ /CD68 ⁺)/dendritic cells
T lymphocytes (CD4 ⁺ /CD8 ⁺) (CD25 ⁺ /CD25 ⁻)
B lymphocytes/plasma cells
NK cells/NKT cells
Mast cells
Eosinophils

2.5.3.1 Macrophages

Not only Leydig cells but also macrophages are major components in testicular interstitium. Hume et al. (1984) were the first to identify testicular macrophages in the mouse by the use of the F4/80 marker and showed that approximately 20% of all interstitial cells in the normal testis were macrophages. There are many studies which demonstrate organ-specific features of testicular macrophages. The exposure to the testis-specific environment may give the testicular macrophages its unique functions. In the testicular interstitium, Leydig cells and testicular macrophages are coupled structurally by way of specialized membrane digitations in adult rats (Hutson 1990, 1992). The macrophages start to form these junctions with Leydig cells when the major increase in secretory activity of Leydig cells occurs during puberty. In vitro studies showed that co-culture of rat testicular macrophages and Leydig cells stimulates Leydig cell steroidogenesis when the macrophages are stimulated with lipopolysaccharide, while stimulated peritoneal macrophages had no such effect. Furthermore, conditioned medium from cultures of rat testicular macrophages but not of peritoneal macrophages stimulates testosterone production in a dose-dependent manner when added to Leydig cells in vitro (Yee and Hutson 1985; Lombard-Vignon et al. 1992). Another in vitro study demonstrated that follicle-stimulating hormone-treated testicular macrophages have a more potent effect on Leydig cell steroidogenesis when compared to untreated ones (Yee and Hutson 1983, 1985). Testicular macrophages but not peritoneal macrophages have specific receptors for follicle-stimulating hormone and respond to follicle-stimulating hormone in a dose-dependent manner by an increased secretion of lactate. An in vivo study showed that treatment of neonatal rats with human chorionic gonadotropin increases the numbers of macrophages in the testis but not in the liver (Yee and Hutson 1983, 1985). Therefore, testicular macrophages play endocrine and paracrine roles in the testis. Differences between macrophage populations from different tissues or organs are called “the inter-population heterogeneity” of macrophages. Local stimuli such as specific cell-cell interactions and production of organ-specific factors are likely to contribute to producing such heterogeneity.

Differing from the inter-population heterogeneity, differences between macrophage subpopulations obtained from one particular tissue or organ are called “the intrapopulation heterogeneity” of macrophages (Itoh et al. 1995b; Winnall and Hedger 2013). In the testis, there are resident macrophages and circulating macrophages. Rat testicular macrophages can be divided into two morphologically different types of cells: large round cell and small elongated cells (Pollanen and Maddocks 1988). In mice, macrophages in the testicular interstitium were various in shape, such as irregular-, elongate-, round-, or oval-shaped cytoplasm with short or elongate cytoplasmic protrusions (Itoh et al. 1995b). Much attention has been directed to a possible cell-cell interaction between macrophages and Leydig cells. However, macrophages are also closely associated with the seminiferous tubular walls or blood capillary walls, suggesting a functional coupling between macrophages and seminiferous tubules and blood capillaries (Itoh et al. 1995b). An immunohistochemical study showed that all testicular macrophages in the human fetus can be

labeled by pan-myelomonocytic cell markers, but not all can be labeled by more mature myelomonocytic cell markers (Dechelotte et al. 1989). Examination of the staining pattern of the various antibodies against macrophage/dendritic cell antigens (F4/80, BM8, MP23, MOMA1, MOMA2, M5/114, BMDM1, and NLDC145) demonstrated that these cell antigens differed from each other in amount present and localization, indicating phenotypical heterogeneity of the testicular macrophages in mice. The intrapopulation heterogeneity of testicular macrophages might reflect diversities in their function, which may depend on their localization, differentiation stages, and degree of activation (Itoh et al. 1995b).

There is a speculation that testicular macrophages actively exert immunosuppressive activity. Allografts and xenografts have been shown to survive in the testes of rats for remarkably long times. However, the grafts were rejected from the testes of rams. Histochemical analysis of the testes revealed that the rat testicular interstitium contained approximately eight times as many macrophages/interstitial area as did ram testicular interstitium (Pollanen and Maddocks 1988). Moreover, large and round macrophages are rich in the rat but poor in the ram. Although the rat also contains some small and elongated macrophages, all testicular macrophages in the ram are small and elongated. This implies that round and large macrophages in the rat contribute to the immune privilege of this site by modifying directly or indirectly the activity of Leydig cells. Testicular macrophages are functionally classified into two populations, which are designated as “resident” and “newly arrived or circulating” according to the differential expression of surface scavenger receptor CD163. CD163⁺ (ED2⁺) macrophages, which are considered to be resident cells in the testis, represent the majority (approximately 80%) of testicular macrophages in rodents. They constitutively produce immunosuppressive IL-10 and are poor stimulators of T cell proliferation, indicating that they contribute to the maintenance of the testicular immune privilege. In mice, approximately 40% of testicular macrophages are constitutively IL-10 positive (Verajankorva et al. 2001). In cases of testicular inflammation, testicular macrophages produce more immunosuppressive transforming growth factor-beta and IL-10, which may contribute to the testicular immune privileged status. On the other hand, CD68⁺ (ED1⁺) macrophages, which are presumably derived from circulating monocytes/macrophages that have only recently arrived in the testis, represent a minor (approximately 20%) proportion of all testicular macrophages in rodents. They have a pro-inflammatory phenotype and express IL-1-beta, TNF-alpha, IL-6, activin A, inducible nitric oxide synthase, and other inflammatory factors (Terayama et al. 2014). However, TNF-alpha, which is synthesized by testicular macrophages, protects TGC from apoptosis at a physiologically low concentration in normal testis (Xiong and Hales 1993). What factors regulate the balance of ED1⁺ pro-inflammatory cells and ED2⁺-resident immunosuppressive macrophages in the testis under physiologic condition are still unknown. Systemic inflammation in response to lipopolysaccharide leads to an influx of CD163⁻ monocyte-like “infiltrating” macrophages into the rodent testis *in vivo*. However, “newly arrived” CD163⁻ testicular macrophages in the rat testis show very little response to lipopolysaccharide stimulation *in vitro* under normal homeostatic conditions. It indicates the presence of intrapopulation heterogeneity of CD163⁻ testicular macrophages. “Newly arrived” testicular macrophages of the normal rat testis are derived from a monocyte subset that continuously repopulates the testis and distinct from the monocyte-like “infiltrating”

subset, from which pro-inflammatory testicular macrophages may be derived during systemic inflammation (Winnall and Hedger 2013).

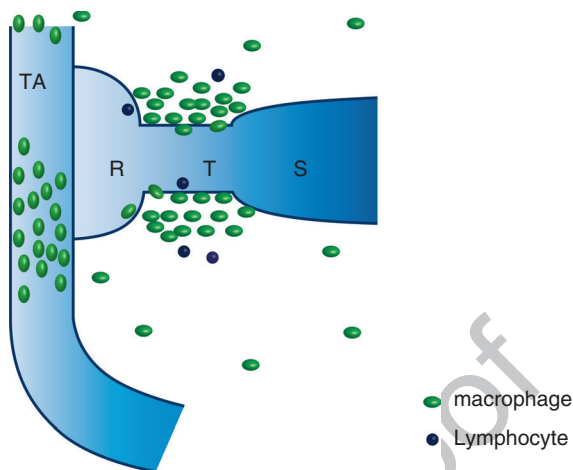
Testicular macrophages were purified and fractionated on discontinuous Percoll gradient in mice, and their antigen-presenting capacity in humoral and cell-mediated responses was tested *in vitro* (Mishell-Dutton cultures, proliferation assay) and *in vivo* (induction of contact sensitivity reaction) (Bryniarski et al. 2004). Heavier Percoll fractions produce little transforming growth factor-beta and are efficient antigen-presenting cells in humoral- and cell-mediated immune responses. Lighter fractions produce high amounts of transforming growth factor-beta and are rather tolerogenic than immunogenic. Their immunosuppressive activity can be prevented by *in vivo* treatment of donor testicular macrophages with cyclophosphamide or *in vitro* treatment with anti-transforming growth factor-beta monoclonal antibodies. In non-separated testicular macrophage population, the immunosuppressive activity prevails. Therefore, subpopulation of testicular macrophages able to trigger specific immune responses is present in the testis but remains under control by other testicular macrophage populations which minimize the risk of development of autoimmune reactions.

IL-35 is another immunosuppressive and anti-inflammatory cytokine, which is produced by Foxp3⁺ Treg, suppresses cell proliferation, and downregulates Th17 cell development. IL-35 is a heterodimeric protein composed of two different subunits, Epstein-Barr virus-induced 3 (EBI3) and the p35 of IL-12. Intriguingly, EBI3- and p35-double-positive resident macrophages were found in the testicular interstitium (Terayama et al. 2014). They may produce IL-35 under physiological conditions as Treg. The lack of EBI3, p35, and IL-12 receptor caused significant infiltration of lymphocytes into the testicular interstitium with increased IFN-gamma expression and autoantibody production, resulting in spermatogenic disturbance (Terayama et al. 2014). Therefore, IL-35 may contribute to maintain the testicular immune privilege, and its lack develops autoimmune-like disorder in the testis.

Treatment of rats with human chorionic gonadotropin induces inflammation-like changes in the testicular microcirculation, and the gonadotropin-induced inflammation-like response is enhanced in macrophage-depleted rat testes (Bergh et al. 1987, 1993). Liposome-entrapped dichloromethylene diphosphonate was injected locally into the unilateral testes in order to deplete testicular macrophages. After the chorionic gonadotropin treatment, there was a large increase in the number of leukocytes in testicular blood vessels, and numerous leukocytes had migrated into the interstitial tissue of the unilateral testes. This response was greater than in the intact contralateral testis. This suggests that testicular macrophages are not the origin of the inflammatory mediator after the gonadotropin treatment. On the contrary, it appears that testicular macrophages secrete some factors inhibiting this type of inflammation.

The BTB at the tubuli recti and the rete testis is known to be incomplete. This implies that the testicular tissue around the tubuli recti is where autoreactive lymphocytes can gain access to autoimmunogenic TGC antigens. Many macrophages accumulate around the tubuli recti, and a few macrophages penetrate into the tubuli recti (Itoh et al. 1995a) (Fig. 2.4). Under normal condition, they may take materials leaked from the tubuli recti for prevention of inflammatory responses (Itoh et al. 1999b; Takahashi et al. 2007). However, when the testicular immune privilege is broken, the tubuli recti should be comprised of immunological-specific region, where lymphocytes are attracted.

Fig. 2.4 Localization of lymphocytes and macrophages under normal condition. *R* rete testis, *S* seminiferous tubule, *T* tubuli recti, *TA* tunica albuginea



In summary, the testicular macrophages are the largest population of leukocytes in the immune-privileged testis, where both innate and acquired immune responses are effectively suppressed, and the testicular macrophages are predicted to be responsible for regulating this immunosuppression.

2.5.3.2 Dendritic Cells

Class II MHC antigen-bearing cells are known to be antigen-presenting cells for CD4⁺T cells. Dendritic cells, the most powerful antigen-presenting cells, induce activation and differentiation of lymphocytes in response to alloantigens but also minimize the autoimmune response by tolerating T cells to autoantigens under physiological conditions (Banchereau and Steinman 1998). In human testis, no class II MHC antigens were identified on any cells within seminiferous tubules, and the antigens were found on dendritic-like cells between the seminiferous tubules and on vessel endothelium, although the expression was extremely limited (Pollanen and Niemi 1987). In a normal mouse testis, both the seminiferous tubules and the surrounding interstitium were negative for class II MHC antigens (Itoh et al. 1991). This indicates that antigen presentation to CD4⁺T cells is not a common event inside the testis. In the rat, dendritic cells also represent a minor population of the interstitial cells, numbering about one-tenth of the macrophages (Rival et al. 2006). The dendritic cells of the normal testis have not yet been well physiologically characterized, but evidence from the rat suggests that the crucial antigen-presenting cells exert immunoregulatory functions in the testis under normal condition. However, in inflammatory condition, the number of class II MHC antigen-bearing cells increases in number (Tung et al. 1987; Itoh et al. 1991). Therefore, stimulated dendritic cells may convert immune privilege into expansion of autoreactive T cells (Rival et al. 2006, 2007; Fijak et al. 2011).

2.5.3.3 Lymphocytes

In the mouse, under normal conditions, it is noteworthy that the testis sections stain neither for T and B cell markers nor for immunoglobulins and complements; however, a small number of lymphocytes are observed in the epididymis (Itoh et al.

1991). Therefore, the presence of extremely limited number of lymphocytes in the testis may be derived from a poor microcircumstance for attraction of lymphocytes. By minute microscopic observation, only a few lymphocytes can be found in and around the tubuli recti and the rete testis of the mouse (Naito et al. 2008) (Fig. 2.4). Because of that, the tubuli recti and the rete testis are permeable to IgG and exogenously administered HRP; in the normal state, the presence of a few lymphocytes and macrophages penetrating the tubuli recti and the rete testis may be important for access to TGC autoantigens (Fig. 2.5). To investigate the function of the penetrating lymphocytes, the changes and distribution of these lymphocytes in some abnormal conditions, such as experimental cryptorchidism and experimental obstruction of TGC transport to the ductuli efferentes and epididymis, should be examined.

In the rat, intratesticular lymphocytes are consistently present under normal condition, and the population is skewed toward class I MHC antigen-restricted CD8⁺ T cells, and CD8⁺ T cell subset predominates over the CD4⁺ T cell subset. Testicular CD8⁺ T cell population also includes significant numbers of cytotoxic T cells (Tompkins et al. 1998; Hedger and Meinhardt 2000, 2003). In contrast to the peripheral blood, in which the CD4⁺ T cell subset was the major lymphocyte subset, not only CD8⁺ T cells but also NK cells were numerous in a normal rat testis (Tompkins et al. 1998). Destruction of the Leydig cells by the treatment with ethane dimethane sulfonate caused a rapid preferential increase in testicular CD4⁺ T cells, which was followed by an increase in both CD8⁺ T cell subset and T cell-inhibiting activity in the Leydig cell-deficient testis (Hedger et al. 1998). After Leydig cell recovery, there was a significant shift toward the CD8⁺ T cell subset.

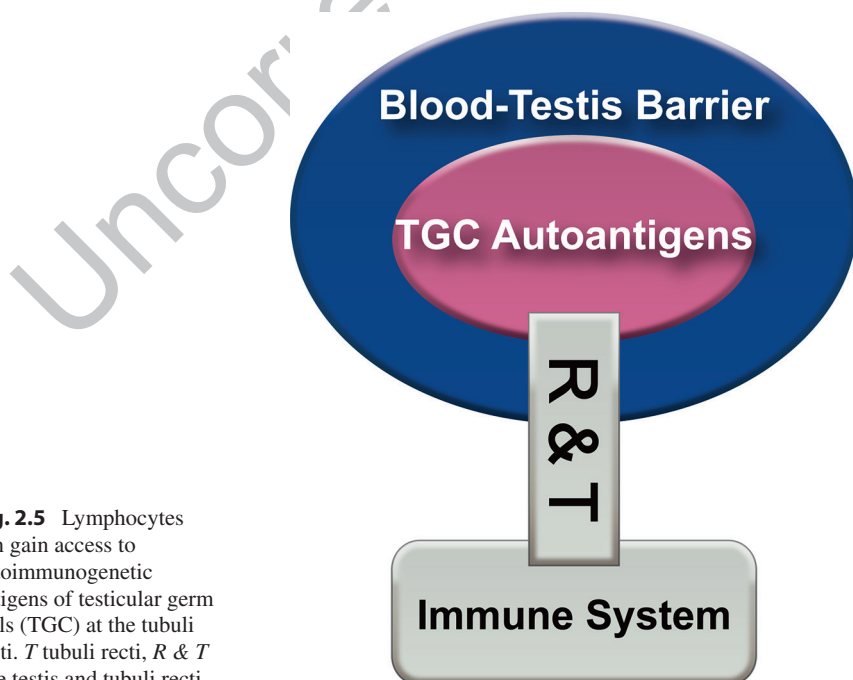


Fig. 2.5 Lymphocytes can gain access to autoimmunogenetic antigens of testicular germ cells (TGC) at the tubuli recti. *T* tubuli recti, *R & T* rete testis and tubuli recti

More recently, some CD4⁺CD25⁺ Treg are found within the testicular interstitium under physiological conditions (Jacobo et al. 2009). Foxp3⁺ Treg are central to the maintenance of immunological homeostasis and tolerance. Recently, it was reported that a conditioned medium from primary mouse Sertoli cells may be able to induce Treg (Campese et al. 2014). Generation of CD4⁺CD25⁺Foxp3⁺ Treg in Leydig cell-conditioned media was determined to investigate the influence of testosterone (Fijak et al. 2015). Leydig cell-conditioned media dose dependently stimulated expression of transcription factor Foxp3 and secretion of IL-10 in splenic CD4⁺ T cells, an effect abolished by addition of the antiandrogen flutamide.

2.5.3.4 Other Leukocyte Populations

Distribution and numbers of mast cells or eosinophils were studied in the testes of 12 mammalian species (Anton et al. 1998). Mast cells were frequently found in horse and human testis, whereas eosinophils were nearly absent. On the contrary, eosinophils were abundant in the rabbit testis, while mast cells were lacking in the pig testis. Otherwise, mast cells and eosinophils were absent from the testicular interstitium proper in rats, dogs, cats, bulls, and deer, although they were present around blood vessels in the tunica albuginea. It remains unknown whether the presence of eosinophils in the testis plays a role in local immunoregulation. On the other hand, it is known that mast cells in the testis regulate steroidogenesis by Leydig cells (Aguilar et al. 1995). Mast cells secrete serine protease tryptase, which promotes the proliferation of fibroblasts and synthesis of collagen (Abe et al. 1998, 2000), leading to fibrosis, sclerosis, and thickening hyalinization of tissues. Mast cells also synthesize TNF-alpha, which protects TGC from apoptosis at a physiologically low concentration in normal testis (Xiong and Hales 1993). In human, mast cells in the interstitium, mediastinum, and tunica albuginea increase in number slightly during infancy, decrease during childhood, and then increase again at puberty, when active spermatogenesis starts. During adulthood, the number of mast cells progressively decreases in the testicular interstitium (Nistal et al. 1984, 1986). In the rat, mast cells in the testis were found almost exclusively around subcapsular blood vessels. In neonatally estrogen-treated rats, a greater number of mast cells were present in the testicular interstitium, whereas no significant increase in the number of mast cells was found for the epididymis, despite the induction of stromal proliferation. On the other hand, androgen-treated rats did not have increased mast cell numbers in any organ. These results indicate that the increase in mast cell numbers was estrogen dependent, specifically related to the testis, and did not seem to be a consequence of the increase in the connective interstitial tissue (Gaytan et al. 1989). By the treatment of rats with ethylene dimethane sulfonate, simultaneous proliferation and differentiation of mast cells and Leydig cells were found in the testis, indicating that Leydig cells and mast cells share some common regulatory factors (Gaytan et al. 1992). Clinically, in men with alcoholic and nonalcoholic cirrhosis, the testicular interstitium, consisting of small and poor collagen fiber bundles, was less developed than in normal testes. The appearance of connective tissue was associated with a decrease in the number of mast cells in the testes of cirrhotic males, suggesting the involvement of mast cells in the synthesis, packing, and organization of collagen fibers. The cause of the decrease in mast cell numbers may be related to hormone alterations, in particular testosterone

deficiency (Nistal et al. 1986). Mast cells can be divided into two subtypes based on differences in their neutral serine protease content. MC_T contains only tryptase, whereas MC_{TC} contains both tryptase and chymase in addition to other proteases, including cathepsin G and carboxypeptidase (Yamanaka et al. 2000). In the normal testes, MCT are the predominant subtype. However, in male infertile patients, total number of mast cells increases, and the predominant subtype of mast cell is shifted to MCTC. Small populations of testicular leukocytes include B cells, plasma cells, NK cells, and NKT cells (De Rose et al. 2013).

2.5.4 Peritubular Myoid Cells and Basal Lamina

The seminiferous tubules are surrounded by peritubular myoid cells, which together with Sertoli cells secrete components of the basal lamina that encloses the seminiferous epithelium. Peritubular myoid cells are smooth muscle-like and surround the seminiferous tubules for autonomic contraction of the tubular walls to excrete immotile TGC to the epididymis. They may also contribute to the BTB. In an experimental study of permeation of administered lanthanum as a tracer on rats, electron microscopy showed that tight junctions between the peritubular myoid cells largely prevented permeation of the tracer to the seminiferous epithelium (Bart et al. 2002). Peritubular myoid cells also express androgen receptors and mediate androgen actions on fetal Sertoli cell proliferation. They support the inter-Sertoli cell junctions and also secrete some cytokines (Schuppe and Meinhardt 2005).

In regard to cytokines, peritubular myoid cells release transforming growth factor-beta-2, monocyte chemotactic protein-1, and leukemia inhibitory factor. Transforming growth factor-beta is an immunosuppressive protein, which is regulated by macrophage migration inhibitory factor secreted from Sertoli cells and Leydig cells (Muller et al. 2005). Monocyte chemotactic protein-1 should account for the recruitment of inflammatory monocytes and/or macrophages. Peritubular myoid cells also express TNF-alpha receptors 1 and 2, which mediate the expression of other inflammatory molecules, including IL-6 and cyclooxygenase-2 (Schell et al. 2008). Peritubular myoid cells are regulated by mast cell and macrophage products and produce factors that can fuel inflammatory changes (Mayerhofer 2013). Peritubular myoid cells were also able to markedly express IFNs and IFN-induced antiviral proteins after viral exposure (Dejucq et al. 1995, 1998). They possess a high degree of plasticity, which results in hypertrophy and loss of contractile abilities. Further studies should provide insights into the repertoire of the secretion products, contractile properties, and plasticity of peritubular myoid cells.

Basal lamina also provides a selective barrier and is an important component of the BTB. In general, the main components of basal lamina are type IV collagens, laminins, entactin/nidogen, and heparin sulfate proteoglycan, which are present on the basal surface of all epithelia. Morphological alterations of the basal lamina at the seminiferous tubules in various pathological conditions including a varicocele, cryptorchid testes, hypogonadotropic hypogonadism, and irradiated testes have been reported, suggesting the possibility that the basal lamina of the seminiferous tubules influences spermatogenic activity (Tainosho et al. 2011). The basal lamina

of the modified Sertoli cells at the tubuli recti exhibited a wavy and multilayered structure, but the Sertoli cells of the seminiferous tubules and the epithelium of the rete testis had an almost flat and single-layered basal lamina (Tainosho et al. 2011) (Fig. 2.3). The wavy and multilayered basal lamina may function as a flexible and movable junction between the seminiferous tubules and the rete testis. Its structure may facilitate opening or closing of the valve-like structure formed by the modified Sertoli cells at the tubuli recti. It was also noted that wide gaps existed between the modified Sertoli cells, the basal lamina of the epithelial layer, and the peritubular myoid cell layer at the tubuli recti. The presence of the wide gaps might provide the microcircumstance in which lymphocytes can easily penetrate into the tubuli recti. Therefore, this characteristic structure of the basal lamina of the tubuli recti may be one of the factors for its incomplete BTB (Tainosho et al. 2011).

2.5.5 Blood Capillary Endothelium

Distribution of blood capillaries in the testis and the epididymis is quite regional (Hirai et al. 2010, 2012). In the epididymis, blood capillaries are dense in both the initial segment and cauda but not abundant in the caput and the corpus. In contrast, blood capillaries are relatively sparse throughout the testis.

Blood capillaries in the testis exist in its interstitium but never extend into the tubular walls of the seminiferous tubules, the tubuli recti, and the rete testis. In sharp contrast, some blood capillaries of the epididymis extend beyond the peritubular myoid cell layer and reach beneath the basal lamina of the epididymal ducts. Distinct from the epididymis and prostate, the testis is not vulnerable to nonspecific extravasation of polymorphonuclear leukocytes (Itoh et al. 1995a). Therefore, there is a possibility that a permeability of capillary blood vessels against leukocytes in the testis may first contribute to the immune-privileged status of the organ. Before the immunosuppressive actions by Leydig cells, testicular macrophages, peritubular myoid cells, and Sertoli cells, the testicular capillary vessels might primarily function as first BTB to protect the testis from inflammatory cell responses. Ultrastructural studies in rat testes showed that testicular capillaries share several properties with brain capillaries, such as the continuity and the rarity of fenestrations (Bart et al. 2002). Therefore, the mechanical BTB consists of tight junction not only between the Sertoli cells but also between the capillary endothelial cells as seen in the blood-brain barrier. Additionally, testicular blood capillaries are specific in that they are sensitive to the endothelial damage by cadmium and gonadotropin treatments (Bergh et al. 1987). Ogawa et al. (2012) demonstrated that the testicular capillary damage by low-dosed cadmium had broken down the immune privilege in the testis.

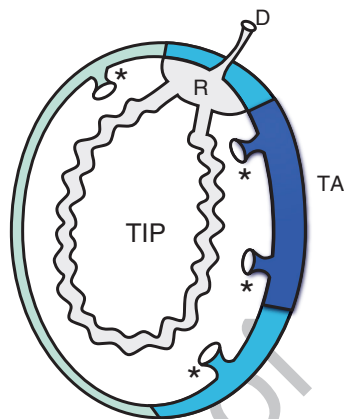
2.5.6 Lymphatic Capillary Endothelium

Testicular immune privilege had been proposed to be attributed to the absence of lymphatic drainage until evidence of the existence of afferent lymphatic vessels in the testis was obtained. Nowadays, it is known that the seminiferous tubules are

bathed in a sea of lymph. There is a possibility that the sensitization of hosts with allograft antigens is failed due to anomalous lymph drainage due to anomalous lymph drainage whereby some lymphatic trunks from the testis bypassed regional lymph nodes and opened directly into the systemic blood circulation. Classic privileged sites such as the brain and anterior chamber of the eye owe their immunosuppressive status primarily to a deficient lymphatic drainage. Indeed, experimental interruption of lymphatic drainage or production of an alymphatic skin pedicle can create an immune-privileged site for a long graft survival. In contrast, reports of immune privilege in the testis are surprising, because its interstitial tissue is well endowed with abundant lymphatic sinusoids and capillaries. Head et al. (1990) revealed a consistent and very efficient lymphatic drainage involving primarily the iliac and renal lymph nodes and to a lesser extent the external lumbar, para-aortic, and posterior gastric nodes in the rat. It was demonstrated that the ligation occlusion of the testicular lymphatics induced retardation and disturbance of spermatogenesis with decreased testosterone secretion in the rat and rabbit (Kotani et al. 1974). This suggests that the integrity of the testicular function must be dependent at least partly on a free flow of the lymph from the testis. In horseradish peroxidase-administered mice, blood-borne horseradish peroxidase was seen in the lymphatic space under the tunica albuginea with the presence of many horseradish peroxidase-endocytosing macrophages (Itoh et al. 1998a). The tunica albuginea tissue filled with many lymphatic capillaries was rapidly loaded with blood-borne horseradish peroxidase that had drained from the testicular interstitium. In the tunica albuginea tissue adjacent to the mediastinum testis, large lymphatics contained much horseradish peroxidase. In mice that had received local injection of colloidal carbon into the testes, the carbon was rapidly excluded from the testis through the lymphatics (Itoh et al. 1998b, c). However, the carbon injected into the epididymis and vas deferens flowed into the lymphatics very slowly. In other experiments, spermatozoa that traumatically leaked from epididymal ducts to the surrounding interstitium consistently accumulated, followed by formation of granulomas; however, TGC that traumatically leaked from the seminiferous tubules to the surrounding interstitium quickly disappeared with no accumulation (Itoh et al. 1999a). Intratesticular injection of isolated epididymal spermatozoa also did not induce accumulation of the injected spermatozoa. This implies that lymph flow in the testicular interstitium is much faster than in the epididymal interstitium, and the rapid kinetics of lymph flow in the testis may be also one of the causes of prevention of leukocytic infiltration leading to granulomatous formation.

In deep testicular parenchyma, the peritubular lymphatic space surrounding the seminiferous tubules is present as polygonal piles (Hamasaki and Kumabe 1994). They are joined to the adjacent piles through fenestrae to form a loose spongiform structure. In the superficial parenchyma, the peritubular lymphatic spaces communicated to the lymphatic space under the tunica albuginea on one side. The lymphatic spaces under the tunica albuginea anastomosed each other through small bypasses to form a rich network. Near the mediastinum testis, the peritubular lymphatic spaces bifurcated and narrowed on another side. The abrupt narrowing of the lymphatic spaces should restrict the flux of the lymphatic fluids (Hamasaki and Kumabe 1994).

Fig. 2.6 Localization of lymphatic endothelial cells in the testis in the mouse. The abundance of lymphatic endothelial cells is represented by the intensity of the blue color. *D* ductuli efferentes, *R* rete testis, *TA* tunica albuginea, *TIP* testicular interstitium proper between the seminiferous tubules. Asterisks indicate lymphatic capillaries just beneath the tunica albuginea



Distribution of lymphatics in the testis and the epididymis is quite regional (Hirai et al. 2010, 2012). In the epididymis, lymphatic networks are quite scarce in the initial segment and strikingly dense in the cauda compared to the caput and the corpus. In the testis, lymphatic capillaries are in and just beneath the tunica albuginea but not in the interstitium between the seminiferous tubules (Fig. 2.6). Those lymphatic capillaries just beneath the tunica albuginea run into the tunica albuginea. It was also noted that lymphatic capillaries were abundant in the thickened tunica albuginea adjacent to the epididymis, but they were scarce in the thin tunica albuginea opposite the epididymis. When normal lymphocytes were locally injected into testes, the injected lymphocytes migrated between the seminiferous tubules and then drained into the lymphatic vessels in the tunica albuginea adjacent to the rete testis (Itoh et al. 1998b, c; Hirai et al. 2012).

2.5.7 Testicular Germ Cells (TGC)

In general, antigens of TGC are immunogenic to self. Moreover, TGC secrete various inflammatory cytokines, including IL-1 and TNF-alpha (De et al. 1993; Haugen et al. 1994). However, it was shown that human spermatozoa inhibited the in vitro proliferative responses of human peripheral blood leukocytes to mitogens such as phytohemagglutinin and concanavalin A (Shearer and Hurtenbach 1982). Hurtenbach and Shearer (1982) reported that in mice given intravenous injections with 1×10^7 syngeneic TGC taken from adult mice, antigen-nonspecific suppression of cell-mediated immunity was induced. Male mice injected intravenously with syngeneic TGC exhibited reduced NK cell activity, reduced mixed lymphocyte reactivity, enhanced auto-proliferation of spleen cells, and decreased potential to generate cytotoxic T lymphocyte responses. This indicates that TGC components also exert regulatory influences on immune potential. This suppressive effect was detected as early as 4 days after injection of TGC and persisted for at least 7 weeks. The reduction of cytotoxic T cell responses and NK cell function appeared to be antigen nonspecific, but the onset of the impairment of cytotoxic T cell function was

detected earlier with hapten-modified cell autoantigens than with alloantigens. Moreover, suppressor-inducing properties of TGC from aged mice were stronger than those from young mice, showing the age-dependent potential. In addition, spermatozoa were much more effective than TGC, suggesting that more mature stages of germ cells induce significant suppression (Shearer and Hurtenbach 1982). The injection of allogeneic sperm into mice resulted in similar immunosuppression (Shearer and Hurtenbach 1982).

It was also shown that Treg for DTH to TGC were detected in the spleen cells of mice administered intravenously with 1×10^7 TGC (Sakamoto and Nomoto 1986). These Treg were sensitive to cyclophosphamide and suppress the generation of CD4⁺T cells for DTH. Therefore, TGC exhibit autoimmunogenicity when injected subcutaneously but exert immunosuppression when injected intravenously (Fig. 2.7). Also under in vitro conditions, lymphocyte proliferation was strongly reduced in the presence of TGC (Hurtenbach et al. 1980). This phenomenon may reflect mechanisms prevailing in vivo by which spermatogenesis proceeds normally in spite of the presence of the autoantigenicity of TGC and the incompleteness of BTB. In contrast, somatic cells of the testis stimulated lymphocytic proliferation under some experimental condition. Treg activated by autologous TGC in vitro were antigen nonspecific and capable of inhibiting lymphocyte proliferation against autologous and allogeneic somatic testicular cells as well as against allogeneic spleen cells. Therefore, autologous TGC are efficient inducers of tolerance by evoking Treg, whereas autologous somatic cells of the testis are immunogenic (Hurtenbach et al. 1980).

Furthermore, the seminiferous epithelium itself may participate in active immunosuppression with intra-seminiferous tubular fluid. In the fluid, proteins that inhibit complement activation exist, and they may reduce complement-mediated inflammation and complement-dependent cytolysis in the testis (Tarter and Alexander 1984). Moreover, spermatogonia produce antiviral proteins in response to IFN-alpha and IFN-gamma (Melaine et al. 2003).

Most intriguingly, Fas ligand is abundantly expressed in the meiotic and post-meiotic TGC (D'Alessio et al. 2001). Fas ligand-expressing cells may contribute to immune privilege by inducing apoptosis of Fas-bearing lymphocytes (Suda et al.

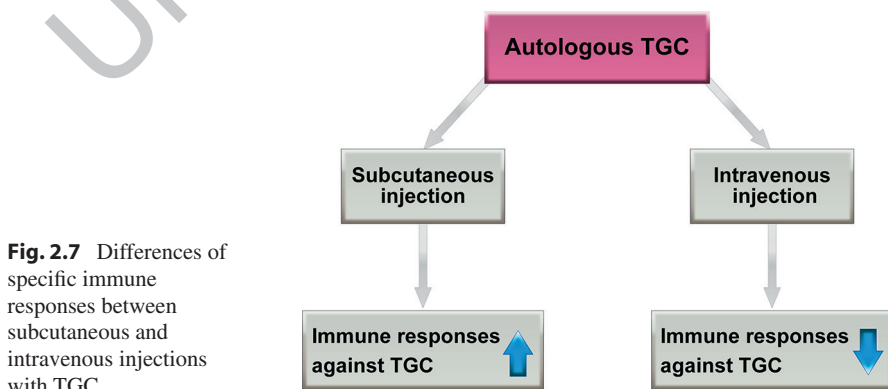


Fig. 2.7 Differences of specific immune responses between subcutaneous and intravenous injections with TGC

1993). However, it remains to be clarified whether TGC expressing Fas ligand contribute to the immune privileged status within the seminiferous tubules.

TLR2 and TLR4 on sperm recognize bacterial endotoxins and mediate apoptosis (Fujita et al. 2011). TLR3 and TLR11 in TGC also trigger innate immunity in response to ligand stimulation (Wang et al. 2012; Chen et al. 2014). Programmed death receptor-1 is a transmembrane protein, which is expressed on T cells. Programmed death ligand-1 is constitutively expressed in spermatocytes and spermatids in the seminiferous tubules and mediates T cell tolerance by activation of programmed death receptor-1 (Keir et al. 2006; Cheng et al. 2009).

2.5.8 Seminal Plasma

Seminal plasma, a mixture of aqueous extracts of prostate, seminal vesicle, Cowper's gland, vas deferens, and epididymis, exerts a potent immunosuppressive effect in vivo and in vitro (Anderson and Tarter 1982). The humoral immune responses to epididymal sperm were suppressed when sperms were incubated in the seminal plasma and then washed before immunization. Cell-mediated immunity and NK cell activity were also inhibited in the presence of the seminal plasma. Anticomplementary effects which inhibit the lytic effect of complement were also reported. Seminal plasma also suppressed mitogen-induced lymphocyte transformation. The seminal vesicle fluid and prostatic fluid can exert suppressive effects on lymphocytes independently (Saxena and Farroq 1987). Human seminal plasma at high concentrations is also lymphocytotoxic and inhibits the opsonization (Ig-mediated recognition and phagocytosis) of bacteria by phagocytic leukocytes (Alexander and Anderson (1987)). Components of human seminal plasma with well-established immunosuppressive effects include prostaglandins, polyamines, transglutaminase, proteases, opiates, transferrin, lactoferrin, macroglobulin, and microglobulin. The final product of spermatogenesis is the spermatozoon, which leaves the body of a male, travels through the reproductive tract of a female, and fuses with an oocyte. Therefore, the complex mechanisms underlying immune privilege in the testis can be regarded as a specialized microcircumstance, in which spermatozoa are adequately equipped, trained, and selected for their ability to survive as foreigners both in the males and females. Prevention of immune responses against testis-leaving spermatozoa by the seminal plasma may lead to inhibition of autoimmunity against elongated mature spermatids in the testis.

2.6 Resistance to Inflammatory Cell Responses of Various Types in the Testis

In general, the testis is resistant to induction of the interstitial inflammation. As documented earlier, sequestration of autoimmunogenic TGC by the BTB is important but not sufficient to prevent testicular autoimmunity. Lyso-glycerophosphatidylcholines in testicular interstitial fluid inhibit T cell activity, contributing to local immunosuppressive

milieu (Foulds et al. (2008)). There should be other secretory materials in testicular fluid for immunosuppression. Sertoli cells, Leydig cells, testicular macrophages, peritubular myoid cells, and capillary endothelium are testicular somatic cells that should express or release some immunosuppressive proteins. Also, some inhibitory proteins derived from TGC may be delivered to the testicular interstitium through the medium of Sertoli cells.

2.6.1 Resistance to Rejection of Allografts in the Testis

The testicular interstitium is an immunologically suppressed space in which allogenic or xenogenic tissue grafts can survive for a long time. Allografts and xenografts, including parathyroid and pancreatic islets, which would be rapidly rejected under the renal capsule, survived for prolonged periods when engrafted inside the testis (Head and Billingham 1983, 1985; Selawry et al. 1987; Bellgrau and Selawry 1990). The intratesticular grafts are resistant to the adoptive transfer of lymphocytes from rat donors primed to xenograft antigens (Bellgrau and Selawry 1990). In fact, Sertoli cells display an inherent immunosuppressive role that supports the survival of cells from other tissues, such as pancreatic islet cells, when they were co-transplanted (Selawry and Cameron 1993; Suarez-Pinzon et al. 2000). Studies on pancreatic islet cell allografts in mouse testes showed that activated T cells are destroyed, and graft antigen-specific Treg are produced when they enter the testis environment (Dai et al. 2005; Nasr et al. 2005). A blockade of androgen production in Leydig cell rapidly rejects intratesticular allografts, suggesting the role of androgens in regulating immune privilege (Head and Billingham 1985).

2.6.2 Spontaneous Occurrence of Vasculitis-Like Lesions in the Male Reproductive System

In man, both systemic and isolated vasculitis in male reproductive tissues is often asymptomatic and subclinical. Therefore, many cases have been found at autopsy. In man, vasculitis involving the male reproductive tract has been described in polyarteritis nodosa, rheumatic arteritis, Goodpasture's syndrome, Henoch-Schönlein purpura, and dermatomyositis (Silva et al. 2012, 2014). However, in laboratory mice, spontaneous testicular vasculitis was detected in only 3 out of 66 (4.5%) genetically autoimmune MRL/lpr^{+/+} mice that had systemic lupus erythematosus-like lesions, such as those in generalized lymphadenopathy, immune complex glomerulonephritis, and systemic vasculitis (Tokunaga et al. 1993). Isolated vasculitis lesions in the vas deferens and epididymis but not testis often occur in man without being a manifestation of systemic vasculitis lesions. Histological examination of normal Balb/c mice reared under specific pathogen-free conditions revealed that spontaneous vasculitis-like lesions comparable to those in man were significantly present in the epididymides and vasa deferentia of aged but not young mice (Itoh et al. 1999c). However, no significant lesions were found in the testes, ductuli efferentes, and other

organs, such as salivary glands, thyroid glands, livers, pancreases, and kidneys. These results indicate that the epididymis and vas deferens but not testis are spontaneously apt to be affected by vasculitis-like lesions with advancing age but that the lesions are not due to a systemic vasculitis disease. It is conceivable that some exogenous agents, such as bacteria and virus, may preferably reach the epididymis and vas deferens and then evoke vasculitis-like lesions in man. It has also been postulated that endogenous agents, such as autoimmunogenic germ cell antigens, may leak from the epididymal duct and vas deferens under normal conditions. This possibility supports that spontaneous exposure to the leaked autoantigens leads to local inflammation without overt manifestation of a systemic inflammation.

2.6.3 Spermatic Granulomata by Local Trauma

The chronic inflammatory lesion induced by the interaction of extravasated autoimmunogenic spermatozoa with surrounding connective tissue is known as a spermatic granulomata (a mass of leaked germ cells surrounded by many epithelioid-type macrophages) (Lyons et al. 1967). When spermatozoa are experimentally leaked from the epididymal ducts to the surrounding interstitium, they induce inflammatory cell responses followed by the formation of a spermatic granuloma. However, such lesions cannot be induced in the testis in which TGC are traumatically leaked from the seminiferous tubules (Itoh et al. 1999a). To examine the possibility that epididymal spermatozoa have inherently greater ability to form spermatic granulomata than TGC, isolated epididymal spermatozoa or TGC were locally injected into the testes and epididymides of recipient mice (Itoh et al. 1999a). The results showed that spermatic granulomata were readily formed in the epididymides after local injection of either epididymal spermatozoa or TGC. In contrast, such lesions were not formed in the testes even when epididymal spermatozoa were locally injected. This indicates that the interstitial environment in the testis exhibits resistance to the formation of spermatic granulomata. In other words, the microcircumstance of the testicular interstitium, rather than the extravasated components from the ruptured seminiferous tubules, is the main factor determining the limited formation of spermatic granulomas in the testis. Indeed, spermatic granulomata are rarely seen in the human testis but are common in the epididymis and vas deferens (Nistal et al. 1997). It is known that testicular macrophages are less activated to pathogen stimulation, and they constitutively produced anti-inflammatory cytokines (Bhushan et al. 2008, 2011; Winnall et al. 2011a, b).

2.6.4 Spermatic Granulomata by Testosterone Treatment

Testosterone, a product of Leydig cells, is an important steroid hormone for the male reproductive system and also suppresses lymphocyte proliferation. Low or moderate doses of testosterone induce hypertrophy of the epididymides, vas deferens,

prostates, seminal vesicles, and penis. However, high doses of testosterone exhibit various toxicities, such as salt and water retention leading to edema. In the reproductive system, spermatic granulomata were experimentally induced in the epididymides but not in testes of mice treated with high-dose testosterone (Itoh et al. 1999d). It is speculated that the testosterone-induced spermatic granulomata may be related to significant changes of degeneration of the epididymal rather than seminiferous epithelium, permitting spermatozoa to escape outside the epididymal ducts.

2.6.5 Reproductive Inflammation by Estrogen Treatment

Neonatal estrogen treatment in mice induced an inflammation in the ductuli efferentes, epididymis, and vas deferens, but not in the testis, provoking obstructive azoospermia in their postpubertal period (Naito et al. 2014). No morphological changes were observed until 4 weeks after the neonatal treatment. Some inflammatory cells were found in the epididymis and vas deferens after 6 weeks. Eight weeks after the treatment, inflammatory cells spread to the ductuli efferentes, and the inflammation became severe from 6 to 12 weeks after the treatment. Inflammatory cells were never seen in the testis, but cystic dilatation of the rete testis with spermatogenic disturbance was occasionally found around the mediastinum testis (Naito et al. 2014). Many inflammatory cells emigrated into the lumen of the epididymis, resulting in complete absence of spermatozoa in the vas deferens, although the spermatogenesis could be seen in the testis. Most of the inflammatory cells penetrating into the epithelial layers of epididymal ducts were neutrophils. Furthermore, many epithelioid-type macrophages and some CD4⁺, CD8⁺, or B220⁺ lymphocytes were localized in the epididymal interstitium. In the estrogen-induced inflammation, it is speculated that neonatal estrogen treatment causes an increase in expression of estrogen receptor and decrease expression of testosterone receptor in the male reproductive organs. The changes of expression of these sex hormone receptors may cause stromal-epithelial degeneration of the epididymis and vas deferens rather than the testis, resulting in inflammatory cell infiltration into the epididymis and vas deferens. It remains unknown why the major populations of infiltrating cells are neutrophils and macrophages but not lymphocytes in spite of “chronic” inflammatory responses in the estrogen-treated mice. More recently, it was found that neonatal exposure to diethylstilbestrol, an artificial estrogenic compound, also induced epididymitis in mice with 100% incidence after 5 weeks of age. Furthermore, in approximately 20% of male mice treated with diethylstilbestrol, the epididymitis was accompanied by orchitis after 12 weeks of age (Miyaso et al. 2014). The epididymal inflammation was similar to that by estrogen treatment; however, the main testicular lesion is severe degeneration of the seminiferous epithelium and interstitial edema rather than inflammatory cell responses.

2.6.6 Reproductive Inflammation by Intravenous Administration of *Bordetella pertussis* (BP)

In mice that were intravenously injected with BP, systemic inflammation involving splenomegaly was induced. It was found that the ductuli efferentes, the epididymis, the vas deferens, and the accessory glands such as the prostate, the coagulating gland, and the seminal vesicle in BP-injected mice received extravasation of leukocytes into their interstitial tissues; however, the testicular interstitium was completely free from leukocyte infiltration (Itoh et al. 1995a). This indicates that the testis is resistant to leukocyte extravasation compared with the epididymis and the prostate. The BTB composed of blood capillary endothelium may prevent the testicular interstitium from inflammatory cell responses.

Recently, a new syndrome, namely, the “autoimmune/autoinflammatory syndrome induced by adjuvants” (ASIA) has been defined (Colafrancesco et al. 2014). In this syndrome, different conditions induced by various adjuvants such as infectious fragments, hormones, aluminum, silicone, and metal are included, and the syndrome is characterized by common signs and symptoms, resulting in boosting the immune response and triggering the development of autoinflammatory phenomena (Loyo et al. 2012; Cruz-Tapias et al. 2013; Lujan et al. 2013). Reproductive inflammation induced by BP or estrogen described above may be characterized by the ASIA syndrome.

2.6.7 Spontaneous Infiltration of Eosinophils and Macrophages in Reproductive Tissues of Congenitally Lymph Node-Lacked Mice

Mice with alymphoplasia (aly/aly) mutation are characterized by a lack of lymph nodes, Peyer’s patches, and defined lymphoid follicles in the spleen (Wang et al. 2010). In male reproductive tissues, many eosinophils and macrophages spontaneously accumulate in the interstitial tissues of the epididymides and vasa deferentia, but not in the testes in this mutant mouse strain. Therefore, the testicular interstitium is resistant to infiltration of eosinophils and macrophages (Itoh et al. 1999e). In the liver and the pancreas, focal accumulation of lymphocytes and macrophages but not eosinophils was consistently found (Wang et al. 2010). It remains unclear why eosinophils specifically accumulated in the male reproductive tissues. It was noted that parenchymal and stromal injury to the epididymis and vas deferens, such as necrosis, fibrosis, granuloma, abscesses, edema, and congestion, was absent in aly/aly mice. This implies that the accumulation of eosinophils and macrophages is not an active inflammatory reaction. It might be that the systemic absence of lymph nodes may evoke abnormal lymph flow in the epididymis and vas deferens, resulting in a pool of leukocytes in their stromal tissues (Itoh et al. 1999e). In preliminary experiments involving normal mice, it was observed that locally injected colloidal carbon was rapidly excluded from the testis through the lymphatics; however, the injected carbon in the epididymis and vas

deferens flowed into the lymphatics very slowly (personal observation). This indicates that the slow kinetics of lymph flow in the epididymis and vas deferens are one of the causes of leukocytic accumulation.

2.6.8 Multiple Organ-Localized Autoimmunity

In mice that had received thymectomy on day 3 after birth, multiple autoimmune diseases involving the retina, salivary glands, thyroid, stomach, prostate, and gonads were induced (Taguchi et al. 1990). In these neonatally thymectomized mice, the incidence of autoimmune oophoritis is more than 90%; however, that of autoimmune orchitis is only 25–30% (Taguchi et al. 1980; Taguchi and Nishizuka 1981). This suggests that the testis or males are apparently resistant to autoimmune disease compared with the ovary or females. Moreover, neonatal thymectomy initially causes epididymo-vasitis during the postpubertal period before the induction of orchitis, and the incidence of orchitis remains considerably lower than that of the epididymo-vasitis, even in mature adult mice. Therefore, the testis is relatively resistant to neonatal thymectomy-induced autoimmunity among various affected organs in multiple organ-localized autoimmunities.

There are many studies on sexual dimorphism in autoimmunity (Rubtsova et al. 2015). In general, autoimmune diseases affect 5–7% of the population, and females are more susceptible to the diseases than males (Dragin et al. 2016). It is generally accepted that androgens exert suppressive effects on both humoral and cellular immunity and seem to represent natural anti-inflammatory hormones; in contrast, estrogens exert immune-enhancing activities (Cutolo et al. 2002). The autoimmune regulator (Aire) is a gene of which mutation causes multiple organ-localized autoimmune diseases (Chan and Anderson 2015). In thymi in men and mice, females expressed less expression of mRNA and protein of Aire than males after puberty. In mice, Aire expression is related to sexual hormones, as male castration decreased Aire thymic expression and estrogen receptor- α -deficient mice did not show a sex disparity for Aire expression (Dragin et al. 2016). Moreover, estrogen treatment resulted in down-regulation of Aire expression in the thymus. Therefore, estrogen induces epigenetic changes in the Aire gene in females, leading to reduced Aire expression under a threshold that increases female susceptibility to autoimmune diseases.

2.6.9 Resistance to Rejection of Transplanted Allogeneic and Xenogeneic Testis

Ectopically transplanted xenografts of testicular tissues under the kidney capsule or subderma of immunodeficient recipient animals resist rejection and survive for a long time (Bellgrau et al. 1995; Oatley et al. 2004, 2005; Rathi et al. 2005). Testicular tissue from immature donors survives better as xenograft than tissue from mature adult donors, and complete spermatogenesis can occur albeit with species-specific differences (Arreguli et al. 2008). Testis grafts derived from mice that can express

functional Fas ligand survived indefinitely when transplanted under the kidney capsule of allogeneic mice, whereas testis grafts derived from mutant *gld* mice, which express nonfunctional ligand, were surely rejected (Bellgrau et al. 1995). Fas ligand expression in the testis probably acts by inducing apoptotic cell death of Fas-expressing recipient T cells activated in response to the graft antigens. Crucially, there is little evidence for extended survival of the epididymal allograft or xenograft. Although immune privilege exists in the testis, the epididymis is much more susceptible to loss of immune tolerance (Hedger 2011b). Therefore, the ectopically transplanted epididymal graft might not be long until its rejection.

2.6.10 Successful Transplantation of Allografts and Xenografts by Co-transplantation with Sertoli Cells

Rat islet beta-cell grafts were co-transplanted with or without murine Sertoli cells in diabetic mice. Graft survival time increased when xenografts were combined with Sertoli cells (Dufour et al. 2003, 2008; Mital et al. 2010). As anti-inflammatory factor, transforming growth factor-beta-1 derived from Sertoli cells is implicated in the protection of islet beta-cell grafts after co-transplantation with Sertoli cells.

2.6.11 Successful Transplantation of Xenogeneic Spermatogenesis into the Recipient Testis

It has been demonstrated that rat spermatogenesis can occur in the seminiferous tubules of congenitally immunodeficient recipient mice after transplantation of rat spermatogonia (Clouthier et al. 1996). Experimentally immunosuppressed adult mice were also used as recipients. In other studies, the successful use of normal infant rats as recipients of hamster spermatogonia was demonstrated for xenogeneic spermatogenesis (Tanaka et al. 1997). The success of transplanting hamster spermatogonia into the infant rats may be due to that the immune function of the infants was still immature, as seen in the immunodeficient or immunosuppressed state. Later, transplantation of rat spermatogonia into immunocompetent adult mice has also succeeded (Qu et al. 2012). By this experimental system, it appeared that transplanted rat spermatogonia could undergo complete spermatogenesis in normal immune system of the recipient mice (Hirayanagi et al. 2015). This indicates that xenogeneic germ cells can be immunologically sequestered and nourished by recipient's seminiferous tubules formed by Sertoli cells, basal lamina, and peritubular myoid cells.

2.6.12 Immune Tolerance Induced by Intratesticular Antigen Priming

In a study of experimental autoimmune uveoretinitis induced by soluble retinal antigens emulsified in CFA, it was reported that an injection of the retinal antigens into the rat testes prior to immunization for induction of the autoimmune uveoretinitis

resulted in systemic tolerance and protects the animals from the disease induction (Li et al. 1997). Similar protection was also demonstrated in experimental allergic encephalomyelitis (Verajankorva et al. 2002). This phenomenon is called testicular-associated immune deviation, which is antigen specific and transferable to naïve recipients with spleen cells from the tolerized animals. This immunotolerance could be transferrable to syngeneic naïve rats by both CD4⁺ Treg and CD8⁺ Treg (Yotsukura et al. 1997). Further analyses revealed that IL-4 and IL-10 are important cytokines for the immunosuppressive effect of CD4⁺ Treg, and transforming growth factor-beta is an important immunosuppressive cytokine for CD8⁺ Treg. A striking feature of the tolerance is that orchietomy within a few hours after treatment of the testis with the retinal antigens does not fully abrogate the systemic tolerance. The tolerance induction was not affected by injuring or removing the lymphatic vessels from the testis. These results indicate that, upon the intratesticular injection with retinal antigens, a signal for retinal antigen-specific tolerance is generated quickly and migrates in blood circulation from the testis to the spleen where Treg are produced. This signal does not seem to be the retinal antigen itself because intravenous injection of the antigen is not as effective as intratesticular injection to induce the tolerance. Increased expression of transforming growth factor-beta and Fas ligand in MHC-positive interstitial cells in the testis may play an important role in the generation of the tolerance induction signal.

2.7 Five-Phased Immunoregulatory Barriers in the Testis

As described above, testicular blood capillary endothelia may function as the first barrier for inhibition of extravasation of leukocytes. Leydig cells, testicular macrophages, lymphocytes, and other leukocytes may function as the second barrier protecting the testis from inflammatory cell responses by expression and/or secretion of various anti-inflammatory materials or by cell-cell contact. Thirdly, both peritubular myoid cells and basal lamina of the seminiferous tubules may inhibit invasion of autoreactive lymphocytes by cell-cell interaction. The classic BTB formed by inter-Sertoli cell junctions functions as the fourth barrier protecting autoimmunogenic TGC from attack by autoreactive lymphocytes. Furthermore, as described above, there is a possibility that autoimmunogenic TGC themselves bathed in the seminiferous tubular fluid also exert final inhibitory effects on proliferation and activation of infiltrating lymphocytes.

2.8 Immunological Fragility of the Testicular Microcircumstance

Although multiple mechanisms and factors, including the physical structure, the local and active immunosuppressive milieu, and the systemic immune tolerance, coordinate to regulate the testicular immune-privileged state, the privileged state could be easily overcome, resulting from immunological weakness in the testis.

2.8.1 Regional Differences in the Integrity of the Immunological Shield by the BTB

The BTB at the tubuli recti and the rete testis is less complete than at the seminiferous tubules and is regarded as a susceptible site for inflammatory cell infiltration (Itoh et al. 1998a; Naito et al. 2009). The seminiferous tubules connect the tubuli recti, which opens to the rete testis (Dym and Fawcett 1970; Dym 1973, 1976; Dym and Cavicchia 1978). Three-dimensional analysis showed that 14–16 tubuli recti appeared to be connected to the rete testis in the mouse (Takahashi et al. 2007). More recently, high-performance three-dimensional reconstruction software analysis revealed the presence of 28 connection points to the rete testis in the mouse (Nakata et al. 2015). In horseradish peroxidase-administered mice, blood-borne horseradish peroxidase infiltrated into the tubuli recti and rete testis but never into the seminiferous tubules (Itoh et al. 1998a). The tubuli recti epithelial cells, which are called modified Sertoli cells, formed protruding cytoplasmic strings and actively phagocytosed degenerating spermatozoa and germ cell remnants under normal conditions (Naito et al. 2009; Tainosho et al. 2011). In general, the phagocytosis of TGC remnants within the tubuli recti is regarded as the normal mechanism for the elimination of unwanted spermatozoa (Dym and Fawcett 1970; Dym and Cavicchia 1977). However, from an immunological aspect, it should also be emphasized that the tubuli recti contains many autoantigens of TGC. Therefore, if the tubuli recti epithelial cells excrete some autoantigens to the outside of the tubuli recti latently under normal condition, the immune cells could encounter these autoantigens there (Fig. 2.5). Actually, previous studies dealt with the antigen-presenting capability of testicular macrophages and Sertoli cells in mice (Kohno et al. 1983; Housseau et al. 1997). However, it appears that class II MHC antigen-bearing testicular macrophages but not Sertoli cells at the tubuli recti could present antigens to CD4⁺ T cells. It has been demonstrated that not only the Sertoli cell junction but also the basal lamina and the peritubular myoid cells provide a selective filtration barrier. The basal lamina is developmentally formed by products of both Sertoli cells and the peritubular myoid cells. Sertoli cells of the seminiferous tubules had an almost flat and single-layered basal lamina. In contrast, the basal lamina of the modified Sertoli cells at the tubuli recti exhibited wavy and multilayered structures (Tainosho et al. 2011) (Fig. 2.3). In particular, at the middle segment of the tubuli recti, both the basal lamina and cell membrane of the modified Sertoli cells intimately interconnect with each other, similar to the sagittal suture in the human skull. This finding suggests that the modified Sertoli cells have a wide surface area at their base and therefore can effectively excrete or absorb various molecules. It may be that some TGC autoantigens are excreted into the wide gaps around the modified Sertoli cells in the normal state. Therefore, the presence of wide gaps between the modified Sertoli cells, basal lamina, and peritubular myoid cell layer at the tubuli recti might provide the microcircumstances in which lymphocytes and macrophages can easily penetrate into the tubuli recti. Furthermore, the macrophages surrounding the tubuli recti and rete testis can easily pick up the leaked autoantigens and present them to lymphocytes. At the tubuli recti, the modified Sertoli cells form a valve-like

structure, and contraction of the peritubular myoid cells prevents the reflux of spermatozoa from the rete testis into the seminiferous tubules. A few thick myoid layers were piled around the middle and terminal segments of the tubuli recti (Tainosho et al. 2011). Therefore, the wavy and multilayered structures of the basal lamina may facilitate opening or closing of the valve-like structure of the tubuli recti. This implies that a significant amount of intratubular fluid (containing TGC autoantigens) at the tubuli recti might be effectively absorbed by the modified Sertoli cells.

2.8.2 Pro-inflammatory Cytokines in the Testis Under Normal Condition

Pro-inflammatory cytokines are produced within the testis even in the absence of inflammation or immune activation events. Pro-inflammatory cytokines including IL-1 and IL-6 have direct effect on TGC differentiation and testicular steroidogenesis (Hedger and Meinhardt 2003). IL-1 is involved in the paracrine stimulation of Leydig cell steroidogenesis, and, on the contrary, IL-6 has been suggested to induce persistent testicular resistance to luteinizing hormone action and/or suppress Leydig cell steroidogenesis (Bornstein et al. 2004). IL-1 is constitutively secreted from not only testicular macrophages but also both Sertoli cells and TGC (Huleihel et al. 2001). The levels of IL-1 were increased in Sertoli cells when stimulated with lipopolysaccharide. IL-6 is expressed by TGC at different stages of differentiation, Leydig cells, and peritubular myoid cells under normal state (Potashnik et al. 2005). Both IFN-gamma and TNF-alpha are normally secreted from Sertoli cells (Terayama et al. 2011). Monocyte chemotactic protein-1, which is involved in macrophage and lymphocyte chemoattraction, was found to be expressed by peritubular myoid cells (Aubry et al. 2000). The expression was markedly stimulated by IL-1, TNF-alpha, IFN-gamma, and lipopolysaccharide. Leydig cells also expressed monocyte chemotactic protein-1 when stimulated by IL-1. Therefore, it is concluded that the pro-inflammatory cytokines in the testis could be involved in the mobilization and migration of leukocytes, leading to testicular inflammation, when the testicular immune-privileged status becomes weak under some pathological condition.

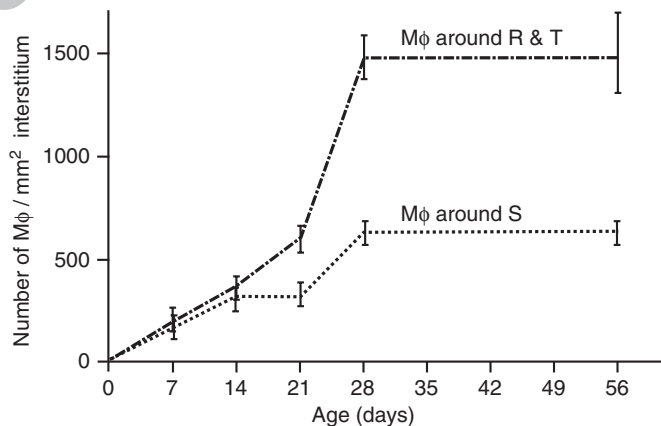
2.8.3 Specific Localization of Immune Cells in the Testis Under Normal Condition

While the testis is a remarkable immune-privileged site, it is well connected to afferent lymph nodes. Therefore, the testis has most types of immune cells, including macrophages, lymphocytes, dendritic cells, granulocytes, and mast cells. Many macrophages that are positive with F4/80, pan-macrophage antigens, preferentially accumulate around the tubuli recti under normal condition in mice (Itoh et al. 1995b, 1998a). In particular, F4/80⁺ macrophages expressing class II MHC antigens are very sparse in normal interstitium between the seminiferous tubules but form a cuff around the tubuli recti (Tung et al. 1987). To determine whether the specific accumulation of

macrophages around the tubuli recti and rete testis is a congenital or acquired phenomenon, the distribution of testicular macrophages obtained from various aged mice was investigated (Li et al. 1998; Itoh et al. 1999b). Macrophages were homogeneously distributed throughout the testicular interstitium with no specific accumulation until 2 weeks of age. However, between 3 and 4 weeks of age in the mouse, macrophages rapidly accumulated around the tubuli recti and rete testis. The density of macrophages at 4 weeks of age reached the level of the mature testes of 8-week-old mice (Fig. 2.8). Therefore, the characteristic accumulation of macrophages is an acquired phenomenon that is completed when spermatids start to differentiate in the seminiferous tubules. Therefore, the accumulation of macrophages around the tubuli recti coincides with the time of the first appearance of autoimmunogenic spermatids in the seminiferous tubules. In the tunica albuginea, some stretched- or irregular-shaped macrophages were concentrated in two regions: the inferior portion of the testis near the cauda epididymis and the upper portion adjacent to the rete testis and the tubuli recti (Fig. 2.4). These two portions are important routes for testicular lymphatic drainage from the inside to the outside of the testis. This preferential accumulation of macrophages at the tunica albuginea was also found to be an acquired phenomenon, starting and completing before puberty. It is not yet known what attracts many macrophages around the tubuli recti. It should be important to clarify whether the specific accumulation of testicular macrophages is due to the cell proliferation in situ or extravasation and arrival from the blood capillaries. In general, it is accepted that a primary function of macrophages is in the host defense system against exogenous agents, such as bacteria and viruses. Therefore, it should not be excluded that macrophages accumulate at the tubuli recti and the rete testis to protect the seminiferous epithelium from genital tract infections involving the prostates, vas deferens, and epididymis.

In contrast to the wide lumen of the rete testis, the tubuli recti lumen is occluded by tall epithelial cells called the modified Sertoli cells. They appear to form protruding cytoplasmic strings, which may serve as sensors, possibly touching the spermatozoa and monitoring their presence and antigenic expression. In addition to many macrophages in the testicular interstitium proper, a few macrophages and lymphocytes were

Fig. 2.8
Sequential changes in the density of F4/80-positive macrophages in the developing testes of the mouse. *Mφ* macrophages, *R & T* rete testis and tubuli recti, *S* seminiferous tubule



identified inside the tubuli recti but never inside the seminiferous tubules (Itoh et al. 1995b; Naito et al. 2008) (Fig. 2.4). A few cell bodies and many cell processes of the macrophages can be found in the lumen and wall of the tubuli recti in the mouse and the rabbit (Osman 1979; Itoh et al. 1995b, 1998a). The presence of phagocytosed spermatozoa within the modified Sertoli cells at the tubuli recti has been found in many species such as rat, rabbit, ram, goat, bull, and monkey (Sinowatz et al. 1979). Therefore, in the tubuli recti, a direct contact between immune cells and degraded TGC may be possible. Intratubular lymphocytes that are very close to both degraded TGC and their remnants could be occasionally found in the tubuli recti, rete testis, and epididymis, but not in the seminiferous tubules in mice and monkeys (Dym and Romrell 1975; Naito et al. 2008). Although the physiological function of these invading lymphocytes remains unknown, this microcircumstance provides a chance for evocation of autoimmune inflammation of the tubuli recti, rete testis, and epididymis under some pathological conditions. The appearance of lymphocytic infiltration around the tubuli recti was observed not only on immunological but also mechanical or chemical stimulations on the testis. In addition to the weak BTB at the tubuli recti and rete testis, the specific accumulation of many macrophages and occasional appearance of lymphocytes at the region might increase the chance of encountering testicular autoantigens.

2.8.4 Infiltration of Macrophages into the Testis in Secondary Biliary Cholestasis

Cholestasis can cause translocation of gut bacteria, endotoxemia, and systemic inflammation. The pathogenesis of hypogonadism in male patients with liver cirrhosis is complex and not well explained. In a rat biliary cholestasis model caused by common bile duct ligation, the magnitude of monocyte chemotactic protein-1 expression and CD68⁺ macrophage infiltration within the testes was progressively upregulated along with increasing duration of common bile duct ligation (Shi et al. 2015a, b). In this model, testicular apoptosis was promoted with activation of mitophagy and autophagy.

2.8.5 Mononuclear Cell Responses to Primary Testicular Cancer

From the immunological point of view, an interesting finding is that inflammatory infiltrates are common in testicular seminomas (Lehmann et al. 1986; Lehmann and Muller 1987; Bart et al. 2002). Most of these cells consist of T cells, but whether T helper cells or cytotoxic T cells predominate is unclear. Furthermore, burnout lesions of primary testicular cancers have been described. In that case, patients had metachronous metastases of a testicular cancer, but only a scar is found in the testis instead of a primary tumor. This indicates that the testis permitted infiltration of lymphocytes with the resultant damage to the seminoma. These burnout lesions

predominantly occur in disseminated seminoma and are rarely described in non-seminoma. The inflammatory T cell infiltrates can destroy the intratesticular tumor, which contradicts the opinion that cellular immune responses have less effect in the testis than in other tissues. A possible explanation could be that tumor expresses antigens that are foreign to the immune system or produce cytokines, thus inducing a cellular immune response, leading to an infiltration of lymphocytes.

Jahnukainen et al. (1995) evaluated the incidence of testicular mononuclear cell infiltrates in patients with carcinoma in situ and germ cell neoplasia. The results suggest that the incidence of mononuclear cell infiltration increases with increasing severity of testicular malignant changes. Moreover, the increased mononuclear cell infiltration is also evident in the contralateral testis where no malignant cells can be observed.

2.8.6 Frequent Involvement of Lymphocytic Leukemia in the Testis

Myelogenous, lymphocytic, and monocytic leukemia infiltration into the testis was confirmed at approximately 20% incidence, and a high-grade infiltration was most common in lymphocytic leukemia (Wakasa and Amano 1977). Blood capillaries were always increased in the area of dense infiltration of leukemic cells. Thin-walled canals located closely between the proper lamina of seminiferous tubules and the interstitial cell cluster were testicular lymphatics, which were extremely irregular in shape and occasionally did not have endothelium. Therefore, testicular lymphatics that are not a constant canal system but rather akin to narrow tissue space may allow lymphocytes to accumulate in the testis. Inhibition of testicular activity by estradiol treatment in rats that were injected intraperitoneally with rat T cell-leukemic lymphoblasts significantly decreased the proportion of the testis occupied by leukemic infiltrates (Jahnukainen et al. 1994). Daily treatment of pubertal rats with human chorionic gonadotropin did not have an effect on testicular leukemic infiltration (Jahnukainen et al. 1994). The proportion of the testis occupied by leukemic infiltrates was significantly higher in the abdominal testes of pubertal unilaterally cryptorchid rats than in the scrotal testes of leukemic control rats (Jahnukainen et al. 1994). In other experiments, it was found that mice inoculated by intramuscular route with lymphocytic leukemic cells recovered from the disseminated disease by cyclophosphamide, whereas mice inoculated by intratesticular route with the leukemic cells and then treated with cyclophosphamide died by the disseminated disease. This indicates that the testicular lymphatic sinusoidal system is equipped with a protected environment for leukemic cells against cyclophosphamide (Jackson et al. 1983).

2.8.7 Natural Autoantibodies to Testicular Antigens

It may be that some of the testicular autoantigens are physically released into the blood system and then encountered the immune system. Considering that TGC autoantigens are not immunologically sequestered but are linked to the immune

system through the tubuli recti, the presence of natural autoantibodies against TGC autoantigens is possible under normal condition. Indeed, in enzyme-linked immunosorbent assay, titers of the autoantibodies in adult normal male mice were consistently higher than those in virgin normal female mice (Itoh et al. 1989). In rats, natural anti-sperm antibodies rose between 56 and 91 days of age and were significantly higher in 91- and 128-day-old rats than at earlier intervals (Flickinger et al. 1997). The rise in anti-sperm antibodies correlated temporally with events in the postnatal development of the male reproductive system. The increase in anti-sperm antibodies is most closely following the time when spermatozoa reach the epididymis and proximal vas deferens at approximately 56 days of age. The increased serum anti-sperm antibodies only after sexual maturation also suggest that some differentiation antigens of sperm are processed and presented the immune system under normal circumstances (Flickinger et al. 1997). However, there is a possibility that the titers detected by immunosorbent assay reflect not only amounts of anti-TGC antibodies but also nonspecific binding of immunoglobulin to Fc receptors on TGC (Sethi and Brandis 1980; Kamada et al. 1991). Autoantibodies to Sertoli cells were also detectable in healthy men (Hiyoshi et al. 1991). There is another possibility that antibodies against exogenous antigens such as ubiquitous microorganism cross-react with TGC and Sertoli cell antigens.

By using SDS-polyacrylamide gel electrophoresis and immunoblotting by reacting with the sera with normal murine testicular homogenates, two immunoreactive bands corresponding to approximately 45 and 100 kDa were definitely detected in normal sera, showing the biochemical presence of natural autoantibodies against these two testicular antigens (Qu et al. 2010; Musha et al. 2013). In rats, sera from normal postpubertal animals bound several testicular proteins, including bands of >100 kDa, 82–75 kDa, 78 kDa, 68 kDa, 65 kDa, 63 kDa, 54–55 kDa, 42 kDa, 37 kDa, 35 kDa, 26 kDa, and 20–22 kDa (Flickinger et al. 1997). The majority of these autoantibodies were sperm specific. Although this phenomenon is intriguing, at present, the true reason for the presence of the natural autoantibodies remains underdetermined. There is a possibility that the spermatogenic disturbance is easily induced by these natural autoantibodies under some particular conditions where the autoantibodies can be allowed to target TGC across the seminiferous tubular wall. On the contrary, these natural autoantibodies might protect the testis from its inflammation by means of action on some regulatory immune network. In spite of habitual ideas about immanently aggressive nature of any forms of autoimmunity, autoimmune phenomena involving natural autoantibodies are permanently present in any individual and may not always reflect the potentially self-destructive activity of the immune system (Poletaev 2014).

2.9 Relation Between Acquired (Adoptive) and Innate Immune Responses in the Testis

Historically, immunology emerged as a branch of applied microbiology in which a war against aliens is one of main subjects. Probably, one of the most important evolutionally archetypical functions of the immune system is “clearance,” a prototype of

the immune system eliminating viruses, bacteria, and fungi. However, it should be noted that the clearance of degenerated and dying self-cells is more critical for maintenance of the molecular homeodynamics of the whole body. Therefore, in the testis, clearance of both endogenous cells (apoptotic TGC) and exogenous cells (various microbes) contributes to normal spermatogenesis and testosterone production.

The testis possesses properties of both remarkable immune privilege and effective local innate immunity. Teleologically, testicular immune privilege protects immunogenic TGC from attack by self-immune system, and local innate immunity is important in preventing testicular microbial infections.

It is generally assumed that TGC sequestration by the BTB is important but not sufficient to protect TGC from inflammatory attack. The testicular interstitial space outside the BTB may also provide an immunosuppressive microcircumstance. The testicular interstitium in mice is resistant to rejection of allografts and also induction of vasculitis, lymphangitis, spermatoc granuloma, and polymorphonuclear cell infiltration (Itoh et al. 2005). Lymphocyte response elicited was shown to be suppressed by proteins in fluids drained from the testicular interstitial space (Pollanen et al. 1988). Therefore, the testicular tissue outside the BTB is also protected from inflammatory cell infiltration, although many resident macrophages are normally present in the testis. Various testicular factors with the capacity to affect lymphocyte functions *in vitro* have been identified, although their physiological roles *in vivo* have not yet been fully evaluated. In local transplantation study, memory CD8⁺T cells constitute a threat to the long-term survival of transplanted organs by mediating allograft rejection despite ongoing immunosuppression. However, CD4⁺CD25⁺ Treg play a key role in the maintenance of immunologic tolerance to both self and foreign antigens by suppressing aggressive T cell responses. Indeed, islet transplantation in the testis generates much less memory CD8⁺ T cells but induces more antigen-specific CD4⁺CD25⁺ Treg than in a conventional site (Dai et al. 2005; Nasr et al. 2005). They may prevent allograft rejection and also prevent testicular autoimmunity. There is also a strong evidence for active immunoregulations by CD4⁺ Treg on autoimmune response to testicular antigens (Teuscher et al. 1990; Itoh et al. 1992).

On the other hand, innate immunity for downregulation of infectious diseases has a capability to overcome the immune privilege. The testis can be infected by various microbial pathogens derived from the circulating blood or genitourinary tract. Testicular infection is followed by initiation of effective antimicrobial innate immune responses, in which the pattern recognition receptors are involved (Zhao et al. 2014). Several subfamilies of pattern recognition receptors have been identified, and TLRs are the best characterized pattern recognition receptors in the testis (Zhao et al. 2014). Thirteen TLR members have been found in mammals. The pattern recognition receptor-initiated innate responses would be important for testicular cells to overcome immune privilege and elicit an appropriate local response against pathogen invasion. In particular, testicular innate immunity is particularly critical when systemic immunity is reduced. Pattern recognition receptors can also be activated by endogenous autoantigens released from damaged tissues and necrotic cells, termed damaged-associated molecular patterns, for triggering endogenous inflammation. TLRs initiate the innate immune response in Sertoli cells by

inducing immunoregulatory cytokines, including TNF- α , IL-1, IL-6, monocyte chemotactic protein-1, and type I IFNs (Zhao et al. 2014). Moreover, Leydig cells and TGC at different stages also express TLR. In *Chlamydia trachomatis* infection in the male genital tract such as the seminal vesicle, prostate, epididymis, and testis, chronic and mild inflammation often remains in most individuals (Mackern-Oberti et al. 2013). Recognition of chlamydial antigens is associated with TLR2 and TLR4, and *Chlamydia trachomatis* recognition by these TLR induces a local production of cytokines/chemokines, which, in turn, provoke chronic inflammation that might evolve in the onset of an autoimmune process. TAM receptors expressed on Sertoli cells are essential for phagocytic removal of apoptotic TGC, and the receptors expressed on macrophages, dendritic cells, and NK cells play critical roles in regulating innate immunity (Deng et al. 2016). It was found that TAM receptors negatively regulate TLR3 signaling in Sertoli cells. Actually, TAM^{-/-} mutant mice exhibit an excessive activation of TLR3, resulting in the upregulation of inflammatory cytokines including IL-1- β , IL-6, TNF α , and interferons α and β (Sun et al. 2010).

In summary, testicular defense mechanisms have two aspects: protection of TGC autoantigens from detrimental immune response and counteraction of invading microbial pathogens in the testis. However, the latter may sometimes involve the chronic inflammation against resident bacteria or parasites, followed by autoimmune responses against testicular antigens.

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