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The testis in immune privilege

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Summary: The production, differentiation, and presence of male gametes
represent inimitable challenges to the immune system, as they are unique
to the body and appear long after the maturation of the immune system and
formation of systemic self-tolerance. Known to protect germ cells and
foreign tissue grafts from autoimmune attack, the 'immune privilege' of
the testis was originally, and somewhat simplistically, attributed to the
existence of the blood–testis barrier. Recent research has shown a pre-
viously unknown level of complexity with a multitude of factors, both
physical and immunological, necessary for the establishment and
maintenance of the immunotolerance in the testis. Besides the blood–
testis barrier and a diminished capability of the large testicular resident
macrophage population to mount an inflammatory response, it is the
constitutive expression of anti-inflammatory cytokines in the testis by
immune and particularly somatic cells, that represents an essential element
for local immunosuppression. The role of androgens in testicular immune
regulation has long been underestimated; yet, accumulating evidence now
shows that they orchestrate the inhibition of proinflammatory cytokine
expression and shift cytokine balance toward a tolerogenic environment.
Furthermore, the role of the testicular dendritic cells in suppressing
antigen-specific immunity and T-lymphocyte activation is discussed.
Finally, the active role mast cells play in the induction and amplification of
immune responses, both in infertile humans and in experimental models,
highlights the importance of preventing mast cell activation to maintain the
immune-privileged status of the testis.

Keywords: *immune privilege, testis, dendritic cells, immunosuppression, male infertility*

Introduction

At the time of puberty, after the establishment of immune
competence, male germ cells enter meiosis, beginning their
complex transition into highly specialized spermatozoa. During
the process, a myriad of surface and intracellular proteins is
expressed; yet, these new autoantigens are tolerated by the
testis. The immunogenicity of the proteins is not diminished, as
shown by their ability to induce strong autoimmune reactions
when injected elsewhere in the body (1–3); rather, it is the testis
itself that confers protection. Initial suggestions that the testis
was an immune-privileged site were substantiated experi-
mentally when histoincompatible allo- and xenografts placed
into the interstitial space of the rat testis survived and prospered
for indefinite periods of time (4–7). Similarly, ectopically
transplanted allogenic Sertoli cells not only survive but also,

when cotransplanted with allogenic pancreatic islets, resist rejection without additional systemic immunosuppression in animals (8, 9). More recently, the transplantation of spermatogonia into germ-cell-depleted testis could restore spermatogenesis, even across species borders in some instances. As transplantation of tissue fragments occurs in the interstitial space and spermatogonia are injected into the lumen of the rete testis, both compartments of the testis are permissive for allo- and xenoantigens (10, 11). There is general agreement that immune privilege is an evolutionary adaptation to protect vulnerable tissues with limited capacity for regeneration, thereby avoiding loss of function (12–14). For the testis, this protection means safeguarding reproductive capability. While protection of developing germ cells from autoimmune reactions under normal conditions is evident in all species, there are distinct interspecies differences, as shown by transplantation experiments where primate testis failed to sustain grafts of monkey thyroid or mouse testis as a recipient of human testicular xenografts (15, 16).

Notwithstanding its immune-privileged status, the testis is clearly capable of mounting normal inflammatory responses, as proven by its effective response to viral and bacterial infection. In pathological circumstances, the misbalance between the tolerogenic and the efferent limb of the testicular immune response can lead to the formation of autosperm antibodies and in rare instances, epididymoorchitis in humans. Immune infertility is now estimated to be a considerable cause of childlessness in couples seeking medical assistance (17–21). Over the years, numerous studies have highlighted the impact of the immunological response to spermatozoa in the form of antisperm antibodies (ASA) on fertility. Acting on multiple levels, ASA significantly impairs sperm's fertilizing capacity (17, 22–24), e.g. by affecting sperm motility (25, 26), the acrosome reaction (27), penetration of the cervical mucus (28), binding to the zona pellucida (29), and sperm–oocyte fusion (30). Antibodies directed to sperm antigens can be detected in seminal fluid and seminal plasma in men, as well as in cervical mucus, oviductal fluid, or follicular fluid in women. They also occur in blood serum in men and women (31), but these appear to be iso-ASA, which are not important for fertilization. Only the antibodies that are bound to the sperm are considered to be of real significance for fertility (28, 32). In 5–12% of infertile male partners, ASA is found in the seminal plasma or attached to the surface of spermatozoa (33, 34). In contrast, ASA were also detected in the semen or blood in men of proven normal fertility (35). In contrast to ASA, autoimmune responses against the developing germ cells within the human testis have not been studied very extensively. The most commonly used model for the investigation of autoimmune-based inflammatory testicular impairment is experimental autoimmune orchitis (EAO), a rodent model based on active immunization with testicular homogenate and adjuvants. EAO can be also adoptively transferred into syngeneic recipients by CD4$^+$ T cells or testis-specific T-cell lines, whereas depletion of CD4$^+$ T cells *in vivo* inhibits the disease (36). The clinical term 'orchitis' is particularly attributed to acute symptomatic disease due to local or systemic infection, whereas subacute or chronic asymptomatic inflammation of the testis including non-infectious disease is difficult to diagnose and therefore likely to be ignored (22). The classification of orchitis in humans depends on the etiology of the condition and can be caused by traumatic events as well as bacterial and viral infections, and in humans, it is usually associated with epididymitis. Orchitis may also occur in conjunction with infections of the prostate and as manifestation of sexually transmitted diseases such as gonorrhea or *Chlamydia trachomatis* (37). Urethral pathogens, i.e. *Escherichia coli*, cause bacterial epididymoorchitis (38). The most common cause of viral orchitis is mumps (39–41). On balance, these data clearly indicate that the mechanism underlying immune privilege in the testis and its disruption by pathological alterations are matters of clinical importance and hence continued scientific interest.

Structure of the testis

The testis as the male gonad has to fulfill two major functions: the generation of gametes (spermatozoa) and the production and controlled release of sex steroid hormones (primarily androgens, with testosterone being the most prominent). The testis is compartmentalized histologically and functionally, with androgen production and spermatogenesis being confined to distinct regions. Spermatogenesis takes place in the seminiferous tubules or germinal compartment and androgen is synthesized in the Leydig cells in the interstitial compartment, which is interspersed between the tubules (*Fig. 1*). In the human infant, true septa extend from the fibrous capsule (tunica albuginea), which surrounds the testis, and divide the organ into various lobules. In the adult human testis, these lobuli are present but less conspicuously, while in rodents they disappear completely.

The germinal compartment of the testis is arranged within the highly coiled seminiferous tubules, which originate and terminate at the rete testis. Each tubule is surrounded by myoid peritubular tissue that provides structural support and contains contractile elements capable of generating peristaltic waves that transport the immotile testicular spermatozoa along the tubule, through the rete testis and into the epididymis. The peritubular cells do not form a tight diffusion barrier; rather, they express a high number of cytokines, growth and differentiation factors,
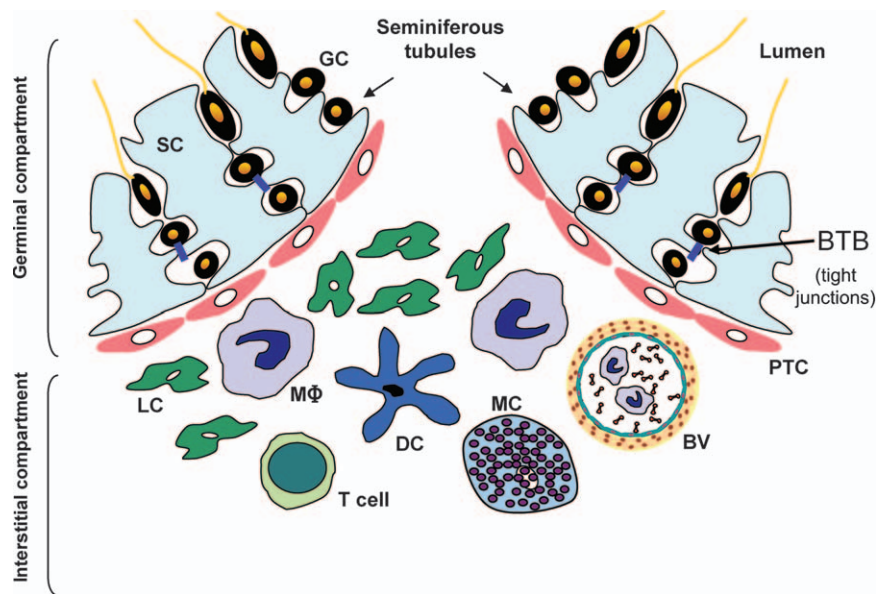
Fig. 1. Morphology of the testis. Sertoli cells (SC) traverse the whole of the tubules. Germ cells (GC) are in intimate contact with SC at all stages of their development. Together with the surrounding myoid peritubular cells (PTC), they form the germinal compartment of the testis, the seminiferous epithelium. The blood–testis barrier (BTB) is maintained by tight junctions between neighboring SC, dividing the seminiferous tubules into a basal and adluminal compartment. The interstitial space located between the tubules contains the Leydig cells (LC) and immune cells such as macrophages (MΦ), dendritic cells (DCs), mast cells (MC), and T cells as well as blood vessels (BV).

and together with the Sertoli cells secrete the components of the basal membrane, which encloses the contents of the seminiferous epithelium. The columnar Sertoli cells extend from the basal lamina toward the lumen of the tubules and constitute the main structural element of the seminiferous epithelium (Fig. 1). They are responsible for the physical support of the germ cells, in addition to providing essential nutrients and growth factors.

Mammalian spermatogenesis is a complex process of proliferation and maturation involving the initial multiplication of progenitor cells (spermatogonia) by mitosis, which is then followed by the first meiotic division. The resulting cells, now called primary spermatocytes, divide to form secondary spermatocytes, and then divide again in the second meiotic division to form the haploid round spermatids. The successful transformation of the round spermatid into the complex structure of the spermatozoon is called spermiogenesis and involves the removal of most of the spermatid cytoplasm in the form of the 'residual body', condensation of DNA in the sperm head, tail formation, and establishment of the acrosome, a cap-like, Golgi-derived sac, which covers the nucleus opposite of the tail and releases lytic enzymes during fertilization (42–44). All of the events in sperm production are highly coordinated within each region of the seminiferous epithelium and occur in a regulated cyclical manner that involves both endocrine and local (autocrine and paracrine) control mechanisms (42).

The most prominent constituents of the interstitial space of the testis are the clusters of heterogeneous, in respect to their physiological and structural features, androgen-producing Leydig cells. Possessing a microvasculature, the interstitium also contains macrophages, lymphocytes, and increasingly with age mast cells (45). Holstein and Davidoff (46) have recently described large flat fibroblastoid cells, which compartmentalize the microvessels, the Leydig cells, and part of the seminiferous tubules. These cells, justifiably named 'Co-cells' (viz. connective tissue cells/compartmentalizing cells/covering cells), appear to produce extracellular matrix components such as decorin, fibroblast surface protein, and vimentin (47), and are typically found only in the human testis. The local variability in the extracellular matrix proteins is important in cell–cell interactions within the testis [reviewed by Dym (48)], particularly as some proteins are able to bind to various classes of growth factors (49), thereby forming a reservoir that is able to modulate the bioavailability of growth factors to the respective target cells (50).

The exocrine exit of the testis is the rete testis, a complicated network of intercommunicating slit-like channels, which are lined by a flat or low columnar epithelium. The transition zone between the terminal segments of the seminiferous tubules (tubuli recti) and the rete has a special arrangement of Sertoli-like cells, often forming a valve or a plug. The blood–testis barrier (see Blood–Testis Barrier) is terminated in this area; subsequently, spermatozoa are no longer protected from autoimmune attack, a status confirmed by the observation that certain forms of autoimmune orchitis are first manifested in the rete testis (51–54).

Spermatozoa released from the seminiferous epithelium are immotile and passively transported via testicular fluid secreted by the Sertoli cells through the rete testis to the epididymis. Spermatozoa mature during transit through the epididymis, which consists of a single highly convoluted duct, and finally gain fertilizing capability. The blood–epididymis barrier is different from the blood–testis barrier and shows spatial differences and an age-dependent decrease in restricting permeability of molecules, most notably in the corpus epididymis (55). In contrast to the seminiferous epithelium, T lymphocytes and macrophages are frequently found within the epididymal epithelium and in the lumen of the epididymal duct (56–58), pointing to an immune environment that operates differently from that of the testis.

Blood–testis barrier

The existence of a blood–testis barrier was originally suspected when early studies found that certain dyes were excluded from cells inside the seminiferous epithelium (59–62). Later, ultra-structural and biochemical data confirmed and characterized this boundary to be highly specialized tight junctions (zonula occludens) between neighboring Sertoli cells capable of restrict-ing the passage of larger hydrophilic molecules, particularly proteins through the intercellular spaces. This limited access, together with the secretory activity of the Sertoli cells, ensures that the composition of the tubular or luminal fluid differs significantly from that of the interstitial fluid surrounding the seminiferous epithelium and creates a unique nurturing environ-ment for the developing meiotic and maturing postmeiotic germ cells (63–65). The blood–testis barrier comprises of various integral membrane proteins, which in turn contain a number of interesting components such as junctional adhesion molecules (JAMs), claudins 1 and 11, with claudins 3–5 and claudins 7–8 also identified in the testis (66–69), and occludin (70–72). The blood–testis barrier divides the seminiferous epithelium into two distinct compartments: the basal compartment carrying the spermatogonia, leptotene, and zygotene spermatocytes and the adluminal compartment with meiotic pachytene and secondary spermatocytes, haploid spermatids, and spermatozoa, which are all completely engulfed by cytoplasmic protrusions of the Sertoli cells. The main task of the blood–testis barrier is to protect the developing germ cells from the immune system.

Meiotic and postmeiotic germ cells, including spermatozoa (daily production: 150×10^6 spermatozoa in rat) (43, 73), express a large array of neoantigens that first appear during puberty, long after the establishment of self-tolerance. With the instigation of spermatogenesis, the blood–testis barrier is concurrently established and immediately sequesters postpubertal germ cells from the immune system. It is completely functional, and its integrity is maintained by the time the first preleptotene spermatocytes move through the junctional complex by a co-ordinated opening and closing of the barrier (74). Interestingly, as JAMs play a crucial role in leukocyte transmigration (75), it is tempting to speculate that they perform a similar role in the testis by facilitating the transfer of leptotene/zygotene early sperm-atocytes through the Sertoli cell tight junctions.

Impairment of blood–testis barrier integrity has been observed during inflammation, infection, and trauma, which ultimately results in germ cell loss (76–79). Mechanistically, elevated levels of tumor necrosis factor-α (TNF-α) and transforming growth factor-β (TGF-β), found in systemic and local testicular inflammation (23, 80–82), have been shown to perturb the assembly of the tight junctions in cultured Sertoli cells, probably by downregulating occludin expression (72, 83). Despite the junction's ability to isolate meiotic and postmeiotic germ cells from circulating antibodies and leukocytes, it is now accepted that the blood–testis barrier alone does not account for all the manifestations of the testicular immune privilege. This propo-sition supported by the findings that germ cell autoantigens are present in the basal compartment in spermatogonia and early spermatocytes, which are not protected by the blood–testis barrier (84, 85). Moreover, the blood–testis barrier is incomplete in the rete testis, a location where immense numbers of spermatozoa with newly adapted surface molecules traverse toward the epididymis, making it a particularly susceptible region for the development of autoimmune orchitis. Histopath-ological observation of mice in which EAO has been elicited by injection of a mixture of viable germ cells revealed infiltration of lymphocytes first in the tunica albuginea close to the rete testis and tubuli recti, then spreading to the interstitial space. Both locations are well outside the blood–testis barrier (52, 54). Furthermore, Head and Billingham (4) showed extended survival (i.e. no immune response/attack) of allografts that were placed under the organ capsule in the testicular interstitium, while local injury of the seminiferous epithelium caused by the routine procedure of fine-needle biopsies in humans has been found not to cause orchitis (86). Therefore, some other mechanism, besides physical separation, must exist to maintain testicular immune privilege, which requests more robust protection of the tolerogenic environment of the testis.

Endocrine regulation of testicular function and immune privilege

The endocrine regulation of the testis has recently been reviewed extensively (87, 88), as such, only a brief update

with emphasis on immune privilege is provided in this section. Testosterone, follicle-stimulating hormone (FSH), and luteinizing hormone (LH) are the most important hormones controlling testicular function. FSH and LH are both secreted by the anterior part of the pituitary and act via specific G-protein-coupled receptors. In the testis, FSH targets Sertoli cells exclusively and stimulates their proliferation during the perinatal and/or pubertal period. In the adult testis, FSH has tropic functions regulating the synthesis of a large variety of metabolites such as lactate, pyruvate, transport proteins (e.g. transferrin, androgen-binding protein), growth factors, activins, inhibins, and cytokines (22, 23, 50, 82, 89). LH, in contrast, acts on Leydig cells by binding to its cognate receptor and consequently stimulating androgen production.

Testosterone, the main androgen, is the crucial hormone for the initiation and maintenance of spermatogenesis (88, 90–93) by mediating its function via the androgen receptor expressed in Leydig cells, Sertoli cells, and peritubular cells (94–97). Interestingly, the germ cells themselves are devoid of these receptors, meaning that androgens must regulate spermatogenesis indirectly via the receptor in the testicular somatic cells, the Sertoli cells being the main target (91). Peritubular myoid cells synthesize a number of androgen-regulated factors, which can also modulate spermatogonial and Sertoli cell function (23, 98–100), and there is now accumulating evidence that peritubular-cell-secreted cytokines, such as TGF-β, macrophage chemoattractant protein 1 (MCP-1), and leukemia inhibitory factor (98, 101, 102), also directly influence leukocytes in the interstitial space. Of note, intratesticular testosterone concentration in rats is far higher than that needed for the maintenance of functional spermatogenesis. In fact, intratesticular testosterone can be reduced by 60% without any adverse effects on sperm production and would still be 10-fold greater than serum values [reviewed by Jarow and Zirkin (103), Jarow et al. (104) and Sharpe et al. (105)].

In addition to the well-known anabolic and spermatogenic effects, a role for androgens in downregulating proinflammatory cytokines has now been shown in both experimental and clinical studies. Incubation of stimulated human monocytes, macrophages, and several non-immune cell types with testosterone resulted in the suppression of adhesion molecules and cytokines, such as interleukin-1 (IL-1), IL-6, and TNF-α, and increased production of anti-inflammatory cytokines, such as IL-10 (106–110). Several reports mention improvements in human autoimmune diseases (such as lupus erythematosus and rheumatoid arthritis) following testosterone-replacement therapy (111–113) and testosterone-dependent enhanced susceptibility to infection, which in some studies could be reversed by orchiectomy (114–119). In hypogonadal men, administration of progesterone was positively associated with serum IL-6 and E-selectin concentrations, whereas the levels of the anti-inflammatory cytokine IL-10 dropped. In contrast, testosterone supplementation decreased the IL-6 levels (120). A direct connection between sex steroid levels and testicular immune privilege was shown by Head and Billingham (121), when in transplantation studies, rats pretreated with estrogen to suppress Leydig cell testosterone production promptly rejected intratesticular allografts, in direct contrast to the reaction of their untreated cohorts.

These studies indicate that high local testosterone concentrations, characteristic of the testis, seem to play an important role in the maintenance of testicular immune privilege. However, the precise manner in which testosterone mediates its anti-inflammatory functions on testicular leukocytes is as yet unknown. What can be surmised from the available data is that the conventional model of testosterone action through a nuclear (classical) receptor is extremely unlikely, as no evidence of androgen receptor expression has been found in testicular immune cells or in mature peripheral T and B lymphocytes (122). Intriguingly, testosterone has been shown to elicit a calcium influx in splenic mature T cells and macrophages, presumably via non-genomic surface receptors (123–125), although a recent report found androgen receptor expression in CD4$^+$ splenic T lymphocytes by highly sensitive reverse transcriptase–polymerase chain reaction analysis and thus did not completely rule out direct effects via nuclear androgen receptors (109). It appears likely that androgens exert their immunosuppressive function on testicular leukocytes either via non-genomic pathways or indirectly by regulating the balance of pro- and anti-inflammatory cytokine expression in the Sertoli, Leydig, and peritubular cells.

Role of leukocytes in maintenance and disturbance of testicular immune privilege

Macrophages

In the adult testes of most species, mononuclear phagocytes (monocytes and macrophages) represent a substantial cellular population of the interstitial compartment. In rats and mice, by far the two best studied species, the ratio of testicular macrophages to Leydig cells is about 1 macrophage to 4 or 5 Leydig cells (126–133). Under normal conditions, macrophages and all other leukocytes are exclusively found in the interstitial space. In humans, they are also found in the tubular wall, but never within the seminiferous epithelium (134–137). Only under pathological conditions or in the regressing testis of seasonal breeders, such as swan, can macrophages enter the

germ cell compartment where they phagocytose degenerating germ cells as the so-called 'spermatophages' (138). In the testes of men with impaired spermatogenesis of differing etiology, the number of CD68^{+} macrophages within the tubular wall has been found to be increased (134).

Testicular macrophages not only possess all the features common to macrophages at other sites but also exhibit testis-specific functions. Regarded as essential for male reproductive function, macrophages display a particularly close morphological and functional link with the predominant interstitial cell type, the Leydig cells, reflected by intimate specialized interdigitations (139). Moreover, they play an important role in Leydig cell development and the regulation of steroidogenesis in adults (130, 133, 140–146). Testicular macrophage numbers are maintained at comparatively high levels through a direct Leydig-cell-mediated mechanism rather than any influence of the seminiferous tubules, with testosterone and macrophage migration inhibitory factor (MIF) playing only a minor role, if any, in this regulation (147). In Leydig-cell-depleted testes, only 50% of the normal macrophage population persists (128). Besides their well-established interactions with Leydig cells, testicular macrophages also influence Sertoli cell function and spermatogenesis by releasing soluble mediators (148, 149). As an example, macrophage activation enhances a human chorionic gonadotropin-induced disruption of spermatogenesis in the rat (150), possibly by increasing sensitivity to IL-1 (151).

There is little doubt that macrophages play a central role in the establishment and maintenance of the immune privilege of the testis. This supposition was first substantiated by in vitro studies where testicular macrophages displayed a reduced capacity to synthesize IL-1β and TNF-α, compared with macrophages from other tissues (152–154), and exhibited immunosuppressive characteristics (153, 155). However, synthesis of granulocyte/macrophage-colony-stimulating factor and prostaglandins E2 and F2α was not inhibited in these studies (153, 154), pointing to a heterogeneous macrophage population. In a different model, macrophage IL-1 production was initiated after endotoxin challenge, but as this production was accompanied by a corresponding decrease in the expression of Sertoli-cell-derived IL-1α, bioactive IL-1 levels in total testis remained unchanged (156). Furthermore, systemic lipopolysaccharide (LPS) challenge in immature (157) and mature testes resulted in enhanced levels of the regulatory cytokine IL-6 and constitutively elevated the production of the anti-inflammatory mediators activin A and TGF-β_1 in both normal and inflamed testes (158). Interestingly, TGF-β_1 and IL-1β were mainly present in their latent, inactive form, which suggests multiple levels of regulation in a testis-specific manner (158). In spite of

the diminished capacity for expression of inflammatory cytokines such as IL-1β and TNF-α by testicular macrophages, the testis is capable of mounting an inflammatory response. There is now increasing evidence that this seemingly contradictory situation is based on the substantial contribution of the testicular somatic cells (Leydig cells, Sertoli cells, and peritubular cells) to the inflammatory response by producing various proinflammatory mediators such as MIF, inducible nitric oxide synthase (iNOS), and IL-1 isoforms (12, 133, 159–165).

In the rat testis, at least two subsets of macrophages can be discerned, one which can be differentiated by its expression of an approximately 100-kD lysosomal glycoprotein, which is recognized by the monoclonal antibody ED1 (rat homolog of human CD63), and the other more common variety, which is a surface antigen recognized by the monoclonal antibody ED2 (CD163) [reviewed by Hedger and Meinhardt (23), Hutson (130, 131), Hedger (132), and Hedger and Hales (133)]. While the ED2^{+} resident-type subset forms the majority of macrophages in rat testis, a significant proportion of ED1^{+}ED2^{−} cells is also present (about 15–20%), presumably representing circulating 'inflammatory' monocytes or recently arrived macrophages. This heterogeneity has functional implications as in the testis ED1^{+} subsets, but only few ED2^{+} resident macrophages, express MCP-1 and iNOS in untreated and LPS-challenged rats (166, 167). Recently, a possible third, as yet uncharacterized, small subset has been indicated based on its ability to synthesize IL-1β (158). Furthermore, a subpopulation of mouse testicular macrophages, isolated by density centrifugation, has been found that expresses high levels of TGF-β and exhibits a tolerogenic phenotype (155). The immunosuppressive qualities of this subpopulation were recognized by its inability to induce T-lymphocyte proliferation, a feature abrogated after adding neutralizing TGF-β antibodies, and its reduced antigen-presenting activity. Of note, in an unseparated preparation of total testicular mouse macrophages, this immunosuppressive activity prevailed (155).

These observations provide further support to the concept that at least two macrophage subsets exist in the adult testis that differ in the expression of markers and inflammatory mediators. In the rat, the ED2^{+} resident population of testicular macrophages does not participate in promoting inflammatory processes; it is believed to have an immunoregulatory role in maintaining immune privilege and tropic functions, particularly on Leydig cells. Clear evidence points out that the ED1^{+}ED2^{−} monocytes/macrophages are involved in the testicular inflammatory response, and it is the influx of ED1^{+} monocytes during acute and chronic inflammation that drastically alter the composition of the macrophage population

and shift the cytokine balance in favor of an inflammatory response with the potential to overcome the immune privilege (158, 166–168). It is noteworthy to say that the testis, at least in acute inflammatory models involving single injections of LPS, appears to possess a robust mechanism to counterbalance the influx of new ED1$^+$ monocytes/macrophages, as the increase is only temporary and resolved after 1 or 2 days (167). This mechanism is clearly not the case in chronic testicular inflammation such as autoimmune orchitis, where a strong lasting increase in macrophage numbers is characteristic (168). Obviously, other yet unknown mechanisms are in effect to override the protective mechanism, probably involving T lymphocytes and dendritic cells (DCs), with detrimental consequences for testicular function. Several important questions arise from these data. What are the local factors that recruit, then resolve ED1$^+$ monocytes to/from the testis in acute inflammation? What causes the chronicity of elevated macrophage numbers in autoimmune orchitis? Which local factors maintain the balance of ED1$^-$ED2$^+$ resident macrophages and ED1$^+$ED2$^-$ inflammatory monocytes/macrophages in the testis under normal conditions? While the mechanism that controls the resident testicular macrophage phenotype is still unknown, evidence increasingly points to the influence of the testicular somatic cells. Leydig, Sertoli, and peritubular cells are all known to produce a plethora of cytokines, and it can be speculated that these cells act in concert to first recruit and then, depending on their localization and hence the cytokine milieu, dictate macrophage type and function (23, 80, 82, 160). This hypothesis presupposes that the somatic and immune cells of the testis act together to provide an environment that protects the germ cells from autoimmune attack. In this concert, TGF-β and possibly activin A appear to play a critical role by inhibiting specific immune responses, finally minimizing the risk of autoimmune reactions to testicular self-antigens and therefore maintaining immune privilege (71, 81, 98, 155, 158). The recent study of Foulds et al. (169) shows, however, that in addition to anti-inflammatory or regulatory cytokines, other classes of molecules attribute to our understanding of the immune privilege of the testis. They isolated lysoglycerophospholines, specifically the C16 saturated and C18 unsaturated ester-linked lysophosphatidylcholines, from rat interstitial fluid and bovine follicular fluid. These compounds showed strong T-lymphocyte inhibition in vitro and can provide a rationale for the immunosuppressive activity of gonadal fluids (170–172).

Dendritic cells

DCs are a heterogeneous population that belong to the most important antigen-presenting cells (APCs) and play a major role in the initiation and orchestration of primary immune responses of both helper and cytotoxic T and B lymphocytes, the effector cells of the adaptive immune system. DCs not only activate lymphocytes but also tolerize T cells to antigens, thereby minimizing autoaggressive immune responses (173). They arise from CD34$^+$ bone marrow progenitor cells or CD14$^+$ monocytes and differentiate into at least three distinct subsets: Langerhans cells, interstitial DCs, and plasmacytoid DCs (174). DCs migrate as immature or precursor cells from the bone marrow into peripheral tissue, where upon receiving an activatory signal associated with pathogens or inflammation, they migrate to the local lymph nodes, mature, and present the antigens to T cells captured in the periphery (173). This somewhat simplistic model of the DC life cycle has been corrected in recent years to accommodate the heterogeneity of DCs found in vivo. Although it is clear that not all DC types follow this life cycle precisely, as a generalized model, it is still a valuable tool for illustrating what occurs during the initiation of immune responses (175). Immature DCs have the highest capacity to internalize antigens but low T-cell stimulatory activity, whereas mature DCs downregulate their endocytic activity and are excellent T-lymphocyte stimulators (174). Mature DCs are characterized by the upregulation of surface-T-cell costimulatory (CD40, CD80 and CD86) and major histocompatibility complex (MHC) class II molecules, the production of bioactive IL-12 and TNF-α, and changes in migratory behavior (176).

Expression of both MHC class I and II molecules occurs within the interstitial tissue of the testis, including the macrophages and Leydig cells. Our own unpublished results show that also testicular DCs express MHC II molecules. In contrast, on the developing germ cells, MHC antigens are reduced or absent. These data indicate that spermatogenic cells are able to avoid direct recognition by CD4$^+$ and CD8$^+$ T cells, which may be important for reducing the potential for antigen-specific immune responses elicited by DCs or macrophages in the seminiferous epithelium (137, 177–182). Interestingly, ejaculated human spermatozoa express MHC I and II class antigen (183–185), and the messenger RNA for human leukocyte antigen (HLA)-DRβ and HLA-DQβ have been also demonstrated in human ejaculated spermatozoa (186). The principal cells in the efferent ducts may act as APCs, since they start to express MHC II antigen after immunization with sperm antigens in rats (187) and mice (188). As the efferent duct epithelium does not express MHC II under normal conditions, this finding suggests that regulation of MHC II antigen expression in these cells may be one mechanism that protects the sperm from an autoimmune attack at this site (189).

In general, APCs are not antigen specific and present a wide range of different antigens, including the so-called danger signals. The 'danger model' proposes that stressed or damaged cells and tissues express and release heat shock proteins (Hsps) during injury caused by trauma, inflammation, pathogens, or toxins. After sensing these danger signals, DCs undergo profound morphological and physiological changes and begin to migrate from the periphery to the lymph nodes. This maturation includes a switch from antigen uptake to antigen presentation mode by signals specifically generated in the inflamed tissue (190). The activation state of the DC is crucial in determining the outcome of antigenic challenge, i.e. the development of either T-cell immunity or tolerance. Resting and activated DCs show marked differences in their expression of various costimulatory molecules. Activated DCs are potent stimulators of immune responses, an ability that is linked to their high expression of several costimulatory molecules (CD80 = B7-1, CD86 = B7-2). In contrast, resting DCs have been implicated in the generation of self-tolerance, presumably due to their reduced costimulatory capacity (191). Presentation of self-antigens by DCs is likely to play an important role in the initiation of autoimmunity and its progression toward clinically important autoimmune disease. DCs presenting autoantigens cause organ-specific, T-cell-mediated autoimmune diseases, such as type 1 diabetes, autoimmune myocarditis, multiple sclerosis, and rheumatoid arthritis (192, 193). Abnormal costimulatory phenotype and function of DCs have been shown in severe murine lupus (194). Furthermore, the functional phenotype of DCs purified from the central nervous system of mice with experimental autoimmune encephalomyelitis (EAE) is strikingly different from that of other DC populations. They are unable to prime naive T cells (195).

In spite of their potential importance in maintaining the balance of the testicular immune status between tolerant (immune privilege) and (auto-)immunogenic states, DCs in the male gonad have received little attention in the past. Cells that express DC markers or possess DC morphology have been observed in the testes of mice (129, 136), rats (121), and humans (182, 196). However, as the markers used are also found on macrophages, a reliable identification is difficult. In a recent study, the presence of DCs in normal (approximately 1×10^5 cells) and chronically inflamed testes from Wistar and Sprague–Dawley rats was determined and quantified for the first time using DC-specific markers (Ox62 and CD11c) (197). In experimentally induced EAO, DCs were found in the interstitial space of the testis and, in large numbers, in the granulomas. Although increases of between 5.5-fold (CD11c) and eightfold (Ox62) were seen compared with controls, these quantities are still significantly lower than number of macrophages found in similar circumstances (128, 147, 197).

Sainio-Pollanen et al. (198) demonstrated that the costimulatory molecules CD80 and CD86 are not expressed in the testis of normal BALB/c mice and 4-week-old non-obese diabetic (NOD) mice, a rodent model of autoimmune diabetes. As the absence of costimulatory molecules on APCs during antigen presentation has been shown to induce clonal anergy, and consequently peripheral tolerance in other systems (199), this finding would suggest that local activation of T lymphocytes is not possible in the normal testis. However, CD80 and CD86 were found in 14- to 22-week-old NOD mice (198), and our own data clearly show the detection of CD80- and CD86-positive cells in rat testis (Rival, Lustig, Meinhardt, Fijak, unpublished data), which suggests that the DC-dependent triggering of activation of naive T lymphocytes by binding to their specific antigens is at the very least possible in the rodent testis.

In light of the danger model, the recent characterization of numerous Hsps (e.g. Hsp60 and Hsp70) as testicular autoantigens could provide a mechanism for how DCs in the testis participate in the activation of autoreactive lymphocytes and in the subsequent damage of testicular tissue, thereby overcoming the immune privilege (200). Millar et al. (201) provided evidence that Hsp70, when released by necrotic cells, acts like a danger signal by enhancing the maturation of DCs, which then trigger autoimmunity (Fig. 2). Interestingly, Hsp70 did not upregulate costimulatory molecules on DCs but helped APCs become better stimulators of T cells, although the exact mechanism remains elusive. It is important to note that the release of endogenous inflammatory signals (e.g. Hsp70) requires necrotic cell death such as that resulting from infection or injury. Based on our own data and that of other autoimmune disease models, we hypothesize that immature DCs, normally involved in maintaining immune privilege, under inflammatory pathological conditions sense self-antigens like Hsp70 as danger signals and after maturation may overcome immune privilege/immune tolerance by local activation and expansion of autoreactive T cells (Fig. 2). A recent study by Serafini et al. (202) on human multiple sclerosis revealed that despite overcoming the immune privilege in the brain, most DCs maintained an immature phenotype, and the few that did acquire maturity were most likely under the influence of inflammatory cytokines and/or direct interaction with T cells. Interestingly, increased levels of TNF-α were found in orchitis testis (168), and TNF-α is one of several agents that induces the phenotypic changes characteristic of mature DCs and concurrently acts as a regulatory factor of DC migration in inflamed tissues (203).
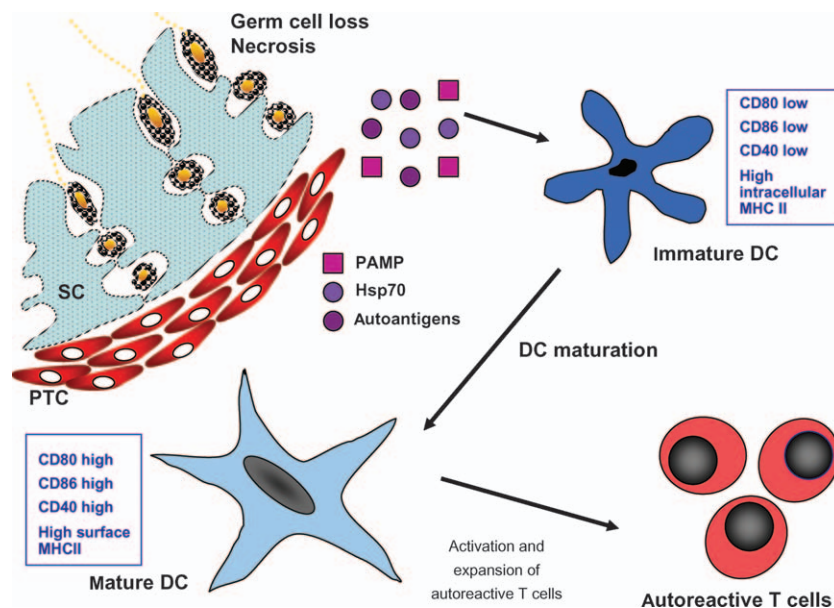
Fig. 2. Hypothetical model of the impairment of testicular immune privilege in testicular inflammation involving DCs. In chronic testicular inflammation, the number of DCs in the testis is greatly increased (197). As a consequence of a temporary or silent infection, necrosis of testicular cells can lead to the release of self-antigens [e.g. Hsp70, ODF-2, hnRNPH1 among others (200)] and/or of pathogen-associated molecular patterns (PAMPs), i.e. LPS. Following binding to Toll-like receptors, PAMPs cause the upregulation of costimulatory molecules such as CD40 on DCs. In addition, Hsp70 can act as a danger signal by enhancing maturation of DCs (201), which in turn can activate autoreactive T cells and trigger autoimmunity. ODF-2, outer dense fiber protein 2; hnRNPH1, heterogenous nuclear ribonucleoprotein H1.
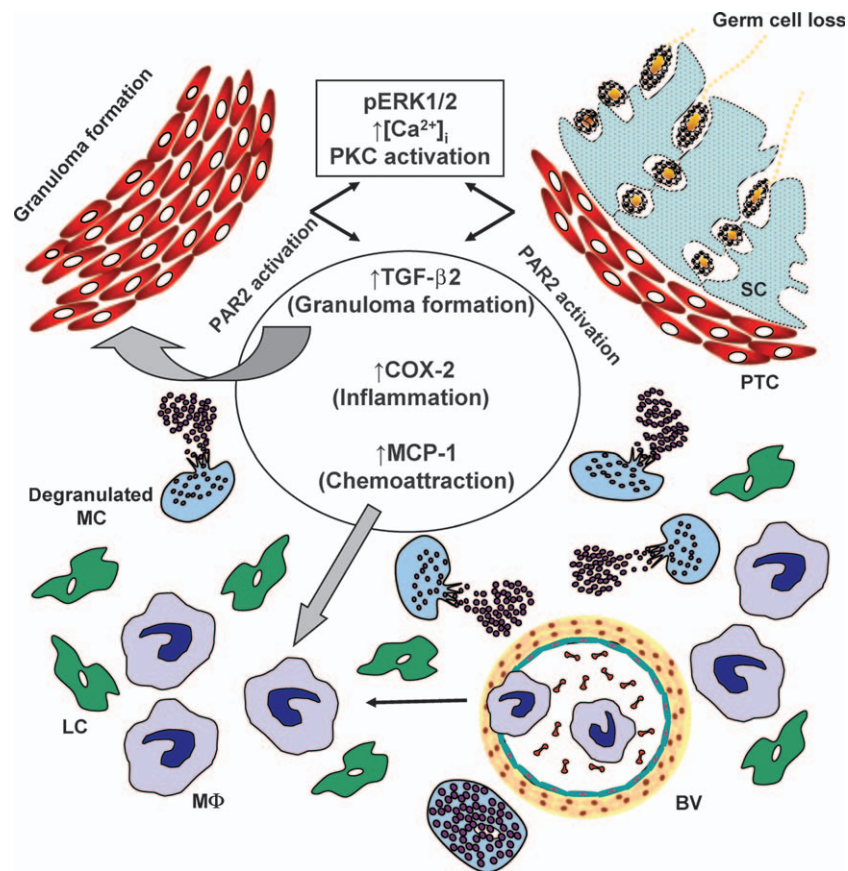
Mast cells

Mast cells are chiefly known as the primary responders in allergic and immune reactions to parasites. However, recent studies have shown the varied and complex contribution these cells make to adaptive and innate immunity, suggesting that they are also prominently involved in the regulation of immune responses, especially the development of autoimmune diseases (204, 205). The variety of potent proinflammatory mediators expressed by mast cells (e.g. histamine, IL-4, interferon-γ, TNF-α, and MIP1α) and the widespread tissue distribution of these cells make them prime candidates as modulators of autoimmune responses. Data from in vitro experiments indicate that the direct interaction with autoreactive T cells may be sufficient for mast cell activation, the induction of degranulation, and subsequent cytokine production (206). Upon activation, mast cells release numerous factors that may act as mediators and as such are capable of influencing various facets of disease induction/progression. Through the release of vasoactive amines (e.g. histamine) and cytokines (e.g. TNF-α), mast cells can profoundly affect vascular permeability, thereby opening the blood–brain barrier, which in turn provides for a greater influx of activated T cells and increased inflammatory cell traffic (207). This scenario appears to be the case in human multiple sclerosis and its rodent model (EAE), where numerous studies have found a correlation between the number and/or distribution of mast cells and the development of the disease (208–212). Increasing degranulation (211) and elevated tryptase (a mast-cell-produced proteolytic enzyme) levels in cerebrospinal fluid of multiple sclerosis patients (213) indicate the presence of activating mast cells, which are suspected in altering the blood–brain barrier and facilitating the entry of T cells into the central nervous system (207).

Mast cells are derived from CD34$^+$ hematopoietic progenitor cells and initiate their differentiation in the bone marrow under the influence of the c-kit ligand (i.e. stem cell factor) and IL-3 (214, 215). Similar to DCs and macrophages, they undergo the final stages of their differentiation and/or maturation locally, after the migration of their precursors into vascularized tissues or serosal cavities, in which they will ultimately reside (216). Mast cells are often associated with blood vessels and are found within mucosal surfaces of the gastrointestinal and respiratory tracts, in the skin, and in close proximity to peripheral nerves.

The distribution of mast cells in the testes of rats, mice, dogs, cats, bulls, boars, and deer is associated with the blood vessels in or close to the tunica albuginea (217). In contrast, human testis mast cells are localized throughout the interstitial tissue and capsule, with numbers increasing slightly during infancy, decreasing during childhood, and increasing again at puberty (218). In the mammalian testis, mast cell mediators are involved in the regulation of steroidogenesis by Leydig cells

Fig. 3. Pathomechanism of testicular inflammation involves PAR2 activation by mast cells. Testis with EAO shows loss of germ cells and massive infiltration of monocytes/macrophages in the interstitial tissue as well as granuloma formation. In models of acute inflammation activation of PAR2 in peritubular cells (PTC) by tryptase or a PAR2 agonist resulted in phosphorylation of ERK1/2, protein kinase C activation (PKC), and increased intracellular [Ca2+] concentrations. In addition, expression of MCP-1, TGF-β2, and COX-2 was elevated *in vitro* and *in vivo*. In EAO testes, mast cell numbers increase substantially, and frequent degranulation releasing mast cell tryptase in the interstitium is observed. PAR2-positive cells are PTC and monocytes/macrophages (M). Expression of MCP-1, TGF-β2, COX-2, and iNOS was found strongly elevated in EAO. Dotted lines in EAO are hypothetically derived from data obtained in the acute models. (BV = blood vessel; LC = Leydig cell; MC = mast cell; MΦ=macrophage, SC = Sertoli cell). Hypothetical model based on data of Iosub *et al.*(81). ERK 1/2, extracellular signal-regulated kinase 1 and 2.

(219). The mast cells' main secretory product, the serine protease tryptase, acts as a potent mitogen for fibroblasts (220) and enhances the synthesis of collagen (221), which in turn can result in fibrosis, sclerosis, thickening, and hyalinization of the wall of the seminiferous epithelium, all common histological features found in male infertility (222). Abnormal spermatogenesis (223, 224) and infertility have all been associated with increased numbers of testicular mast cells (225–228).

Our understanding of the role of mast cells in inflammatory disease has taken a new turn since the discovery of proteinase-activated receptor-2 (PAR2) more than a decade ago. PAR2, a member of a novel subfamily of G-protein-coupled receptors, is activated by extracellular proteolytic cleavage by serine proteases (e.g. trypsin, mast cell tryptase, and bacteria-derived enzymes), which unmask a new N-terminal sequence that in turn acts as a 'tethered' receptor-activating ligand (229).

Recently, PAR2 protein was localized in the rat testis to the peritubular cells, monocytes/macrophages and acrosome of the developing spermatids (81). In rats with EAO, PAR2 expression was found to be highly upregulated and linked to granuloma formation and tissue remodeling. Interestingly, mast cell numbers were 10-fold higher and widely distributed throughout the interstitial space, particularly around granulomas. Numerous of these mast cells were found to be degranulated, indicating they had/were releasing tryptase into the surrounding interstitial space. The increase in numbers and the redistribution of mast cells away from a location exclusively under the organ capsule are important findings, as they mean that in EAO, mast cell tryptase and its receptor PAR2 are now in close proximity, thereby facilitating an interaction between the two effectors (81). What could be the consequence in the testis? *In vivo* experimental injection of mast cell tryptase caused

elevated expression of the key inflammatory mediators MCP-1, TGF-β_2, and cyclooxygenase-2 (COX-2), largely in a PAR2-dependent mechanism (81). In vitro studies indicate that peritubular cells are responsible for the observed increase in MCP-1, TGF-β_2, and COX-2 following PAR2 challenge (81). It was hypothesized that the release of MCP-1 could at least partially account for the observed massive recruitment of inflammatory monocytes and/or macrophages to the chronically inflamed testis (Fig. 3), which have not yet adopted the testicular phenotype. Similarly, also the above mentioned testicular fibrotic disorders were related to PAR2 activation by mast cells in a COX-2-dependent mechanism (222).

We are only beginning to understand the role of mast cells in testicular immune privilege and inflammatory disease. It appears, at least in the normal rat testis, that the very restricted distribution of mast cells and their relatively low number is a mechanism meant to physically separate them from the PAR2$^+$ cells and T lymphocytes. As drugs believed to stabilize mast cells have been shown to ameliorate the severity of EAE (230–232) and as mast cell blockers have been shown to be beneficial in the treatment of idiopathic oligozoospermia and oligoasthenozoospermia (233–235), prevention of mast cell activation may be an important factor in the maintenance of an immunosuppressive phenotype not only in the testis but also in other immune-privileged sites.

Conclusions

There is now widespread agreement that the immune system, spermatogenesis and steroidogenesis, the intrinsic testicular functions, are intricately linked by a network of complex interactions. The importance of the delicate balance needed between the suppression of the immune response to protect the germ cells from autoattack on the one hand and the ability to activate an immune response to prevent damage from infection, trauma, and cancer on the other is reflected by the fact that in the human male about 12–13%, in some studies even more, of all diagnosed infertility is related to an immunological reason, while its contribution to idiopathic infertility (31% of all cases) remains unknown (18–21). The mechanisms responsible for the testes' immune privilege are still far from being understood, but it is apparent that the identified factors involved are multiple and probably redundant. Overall, long regarded as a peculiar side issue of testis function, immune privilege is now established as part of the general scheme of male gamete formation and successful reproduction. Further research in the area will not only help to improve diagnosis and treatment of immunology-based male infertility but also will open new avenues in contraceptive development and transplantation medicine.

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