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Lack of TCRαβ$^+$ CD8$^+$ and TCRγδ$^+$ Lymphocytes Ameliorates LPS Induced Orchitis in Mice – Preliminary Histological Observations*

Leopold ŚLIWA, Barbara MACURA, Monika MAJEWSKA-SZCZEPANIK, and Marian SZCZEPANIK

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The inflammation of the reproductive system can affect reproduction causing partial or complete infertility. It is well known that lipopolysaccharide (LPS) triggers an inflammatory response in the whole organism, including immunologically privileged organs, e.g. the testicles. Adult male TCRα-/-, TCRδ-/-, CD1d-/- and β$_2$m-/- on B10.PL (H-2^u) and B10.PL control mice were intraperitonealy (i.p.) injected with lipopolysaccharide (LPS). The animals were killed 24h and 10 days post LPS treatment and their gonads were prepared for microscopic examination. Histological changes in the testes after LPS injection were found only in control B10PL and CD1d-/- mice. The experiments revealed disturbances in Leydig's glands structure, blood vessel dilatation in the interstitial tissue as well as degeneration of seminal tubule epithelium, disruption of spermatogenesis and subsequent decrease of sperm cell number in the tubule lumen. These changes were noticed mainly 10 days after LPS treatment. Lack of either TCRαβ+ CD8+ or TCRγδ+ lymphocytes diminishes the response of testicular macrophages to LPS whereas the absence of CD1d-dependent NKT cells does not affect macrophage reactivity.

Key words: Lipopolysaccharide (LPS), knock-out mice, testis, inflammation, infertility.

Leopold ŚLIWA, Barbara MACURA, Monika MAJEWSKA-SZCZEPANIK, Marian SZCZEPANIK, Chair of Medical Biology, Faculty of Health Sciences, Jagiellonian University, Medical College, Kopernika 7, 31-034 Kraków, Poland.
E-mail: leosliwa@cm-uj.krakow.pl

A basic cause of decreased fertility or infertility in male animals is functional disorder of the testicles. This may result from current or prior infection and inflammatory response in the reproductive glands (ADAMOPOULOS *et al*. 1978; BUCH & HAVLOVEC 1991).

In the experimental model an inflammatory reaction can be induced *in vivo* by administration of lipopolysaccharide (LPS) isolated from gram negative bacteria. LPS causes various pathophysiological reactions including fever, leukopenia, tachycardia, tachypnoe, hypotension, disseminated intravascular coagulation and multiorgan failure including the reproductive glands (HEINE *et al*. 2001). It was found that the administration of LPS to males may obstruct testicular steroidogenesis and also affect the course of spermatogenesis (WALLGREN *et al*. 1993). However, the direct effects of LPS and the observed inflammatory reaction on the course of spermatogenesis in the spermatic ducts of a testicle are not fully understood. It was only found that administration of LPS causes degenerative changes in the cytoarchitecture of seminiferous epithelium in rats (O'BRYAN *et al*. 2000) and BALB/c mice (ŚLIWA *et al*. 2009). Similar changes were observed in seminal tubules and Leydig's cells. These *in vivo* studies were fully confirmed by *in vitro* experiments showing that testicular macrophages exposed to LPS stimulate Leydig's cells to inhibit testosterone production and finally suppress spermatogenesis (BRYNIARSKI *et al*. 2004).

At present it is not known how these changes proceed in mice which selectively lack different T cell populations. Previously, it was found that macrophages isolated from TCRα-/- and β$_2$m-/- mice on B10.PL (H-2^u) background produce elevated levels of reactive oxygen intermediates (ROi'S) and nitric oxygen (NO) and express higher production of pro-inflammatory cytokines

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such as TNF-α (tumor necrosis factor - α), IL-6 (interleukin-6), and IL-12 (interleukin-12) (BRYNIARSKI *et al*. 2005a). Additionally, it was shown that macrophages isolated from TCRα-/-, TCRδ-/- and $β_2$m-/- mice possess an increased ability to induce a cell mediated immune response. TCRαβ+ CD8+ and TCRγδ+ lymphocytes inhibit the capability of peritoneal macrophages to induce contact hypersensitivity (MAJEWSKA *et al*. 2009). These data may suggest that macrophages are under negative regulation of different T cell populations.

Thus, the aim of our current work was to determine the role of different T cell populations in negative regulation of inflammatory response in the male gonad caused by LPS activated testicular macrophages.

Material and Methods

Mice

Six to eight week old TCRα-/-(mice lacking TCRαβ lymphocytes), TCRδ-/- (mice lacking TCRγδ lymphocytes), CD1d-/- (mice lacking CD1d restricted NKT lymphocytes), and $β_2$m-/- (mice lacking CD8 lymphocytes) on B10.PL (H-2^u) and B10.PL control mice were used in experiments. Animals originated from the breeding unit of the Department of Medical Biology, Jagiellonian University, College of Medicine. All mice were kept under pathogen-free conditions using filter-topped microisolator cages and sterile equipment and fed autoclaved food and water.

Experiments were carried out according to guidelines of the Animal Use and Care Committee of Jagiellonian University (dated 16.03.2004).

Treatment with lipopolysaccharide and histological evaluation

Control B10.PL mice and the following knock out mice: TCRα-/-, TCRδ-/-, CD1-/-, and $β_2$m-/- were intraperitonealy (i.p.) injected with one dose of 100 μg of LPS from *E. coli* 026; B6 (Sigma Chemical Co., St Louis MI) in 1 ml of sterile PBS. All experimental groups contained 10 male mice. The animals were killed by cervical dislocation 24h or 10 days post LPS treatment and their gonads were removed and fixed with Bouin's fluid for 24h. Serial 7 μm paraffin-embedded sections were routinely stained with hematoxyline and eosin. The effect of LPS treatment was determined under a light microscope in 20 cross section of testis per animal from experimental and control groups. The histological evaluation focused on qualitative, descriptive and subjective observations, not on quantitative measurements.

Results

Histological structures of B10PL and Balb/c testes including cytoarchitecture of genital epithelium do not differ much. The only difference seems to be reduced epithelial thickness caused by lower cell numbers during consecutive stages of spermatogenesis (mainly spermatocytes I) and therefore a lower number of spermatozoa in the seminiferous tubule lumens. There is also a decreased number of Leydig cells and macrophages in the interstitial tissue of these animals compared with the BALB/c strain (Fig. 1a).

Genital epithelium in all tested knock out mice is thin, with a small number of spermatocytes I, however all spermatogonial stages can be distinguished. Relatively small numbers of spermatozoa are localized in the lumens of seminiferous tubules in the testes. Interstitial tissue tightly fills spaces between tubules, however, there is a significantly lower number of macrophages in comparison with wild type mice. Fig. 1d shows a testis section of CD1d-/- mouse, but similar changes were observed in all tested knock- out mice.

LPS administration caused inflammation in testes of B10.PL (control group) and CD1d-/- (knock-out group) mice. We detected changes in genital epithelium organization, spermatozoa production in seminiferous tubules and also changes in interstitial tissue structure accompanied by macrophage infiltration and enlarged volume of blood vessels, even with local lesions.

In testis of B10.PL mice (control group) the inflammatory reaction with histological changes was first observed 24h after *i.p.* injection of LPS. Twenty four h after LPS injection blood vessels between the seminiferous tubules and within interstitial glands were widened. The lumen of blood vessels was enlarged particularly in the parts of the organ covered by the testicle. Leydig's glands were enlarged, mainly because of an increased number of macrophages within the interstitial tissue. The structure of the walls of seminal ducts and cytoarchitecture of seminiferous epithelium did not reveal any irregularities (Fig. 1b).

On the 10th day of the inflammatory reaction, degenerative changes were profound. In the testicle a significant number of widened blood vessels can be found. Exudates occurring in their vicinity frequently disrupt the cohesion of the basic membrane of seminiferous tubules. There are numerous macrophages within Leydig's glands. The germinal epithelium in tubules reveals signs of degeneration. The germinal epithelium contains mostly spermatogonia and primary spermatocytes. There are few spermatydes and spermatozoa. The epithelium is frequently exfoliated towards the lumen of

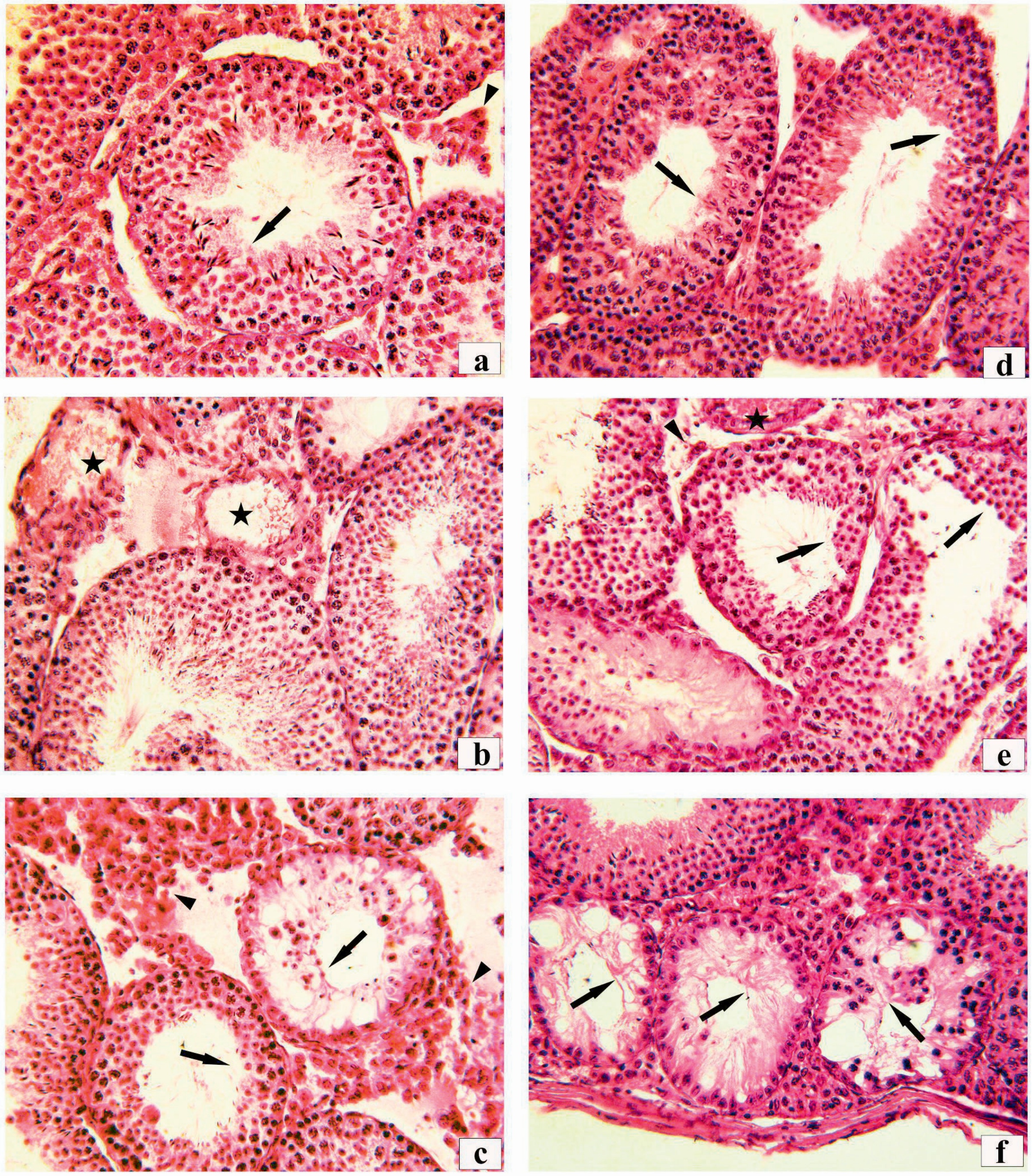

Fig. 1. Section through the seminiferous tubules of male mice from the B10 PL (a-c) and CD1d-/- groups (d-f); (a), (d) control group without LPS injection; (b), (e) 24 hours after LPS injection, and (c), (f) 10 days post LPS injection. Magnification about 450 ×. Arrows indicate normal cytoarchitecture of seminiferous epithelium in control animals and its degenerative changes during inflammation (a, c-f). Widened blood vessels during inflammation (b, e) are marked with an asterisk. Arrowheads shows Leydig's glands in control (a) and enlarged Leydigs glands with numerous macrophages in animals 10 days post LPS administration (c).

the ducts and is much thinner than in the control group. Fragmentation of Sertoli's cells occurs, which, in the form of round fragments of cytoplasm, separate towards the lumen of seminiferous tubules. In some of the tubules there are only spermatogonia located on the basic membrane; in this case, the interior of a duct is filled with a fibrous substance which does not contain cells (Fig. 1c).

In testes of knock- out mice degenerative changes characteristic of inflammatory response in testes induced by LPS administration were found only in CD1d-/- mice. Initial degeneration of genital epithelium was observed in seminiferous tubules of treated males 24 h post LPS injection. The epithelium became thin and partly peeled. It was difficult to distinguish consecutive stages of spermatogenesis in such changed epithelium. In the tubule lumens, only a few normal spermatozoa were detected. In the interstitial tissue of Leydig's glands, numerous macrophages were present as well as apoptotic cells. Other characteristic symptoms of inflammation were intensified including damage of blood vessels (Fig. 1e).

The degenerative symptoms on day 10 were more severe when compared to those found 24 h after LPS injection. The advanced destruction of genital epithelium cytoarchitecture in seminiferous tubules with almost complete lack of spermatozoa in lumens were the main symptoms of the acute inflammatory reaction. In the interstitial tissue the inflammatory state was manifested also by the presence of intracellular transudates and numerous lesions of testicular blood vessels (Fig. 1f).

On the other hand there was no detectable inflammation in testis of male TCRα-/-, TCRδ-/- and β_2m-/- mice 24 h and 10 days after LPS administration. The structure of testes in TCRα-/-, TCRδ-/- and β_2m-/- did not show any differences compared to non-treated mice.

Discussion

Many host cells, including epithelial cells, macrophages, neutrophiles, natural killer cells, dendritic cells and macrophages belong to the first line of defense against infection by sensing conserved microbial structures called pathogen associated molecular patterns (PAMP) through Toll-like receptors (TLRs) (MEDVEDEV *et al*. 2006). Recognition of microbial structures by TLRs triggers intracellular signaling pathways, leading to the production of pro- and anti-inflammatory cytokines and expression of co-stimulatory molecules by antigen presenting cells (SZCZEPANIK 2007). Lipopolysaccharide (LPS), the most studied PAMP, initiates its biological function via TLR4. LPS elicits an immune reaction which is responsible for many of the harmful effects seen in septic shock patients which results in multiorgan failure including the central nervous system (MÜLLER--LOENNIES *et al*. 2007; SURA *et al*. 2006).

Our previous work showed that administration of LPS causes degenerative changes in cytoarchitecture of seminiferous epithelium and alterations in interstitial tissue structure accompanied by macrophage infiltration in BALB/c mice (ŚLIWA *et al*. 2009). Additionally, experiments employing knock out mice lacking different T cell populations and research using in vitro depleted T cells (MAJEWSKA *et al*. 2009; ODYNIEC *et al*. 2004) suggest that macrophages are under regulation of different T cell populations.

Thus we determined the role of different T cell populations in the regulation of the inflammatory response in the male gonad caused by LPS activated testicular macrophages.

Our data show that B10.PL mice, similarly to other wild type mice, develop an inflammatory response in the testis after LPS treatment. However, inflammation was not observed in TCRα-/-, TCRδ-/- and β_2m-/- mice that lack TCRαβ, TCRγδ and CD8 T cells respectively. As shown previously macrophages play an important role in orchitis induced by LPS (BRYNIARSKI *et al*. 2005b). Lack of macrophage activation by LPS and subsequent orchitis in T cell deficient mice might suggest that Tαβ+ CD8+ and TCRγδ+ lymphocytes play an important role in LPS induced macrophage activation.

On the other hand CD1d-/- mice treated with LPS develop orchitis accompanied by macrophage activation. This data might suggest that CD1d dependent NKT lymphocytes are not involved in macrophage activation after LPS treatment even though NKT cells express TLR4 receptor (ASKENASE *et al*. 2005).

In summary, both TCRαβ+ CD8+ and TCRγδ+ lymphocytes regulate the biological activity of testicular macrophages in response to LPS whereas CD1d dependent NKT cells do not seem to be involved in this phenomenon. Further experiments are required to better understand the interplay between different T cell populations and testicular macrophages.

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