Differential Activation of Inflammatory Pathways in Testicular Macrophages Provides a Rationale for Their Subdued Inflammatory Capacity

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Differential Activation of Inflammatory Pathways in Testicular Macrophages Provides a Rationale for Their Subdued Inflammatory Capacity

Sudhanshu Bhushan,* Svetlin Tchatalbachev,† Yongning Lu,* Suada Fröhlich,* Monika Fijak,* Vijith Vijayan,* Trinad Chakraborty,† and Andreas Meinhardt*

Spermatogenic cells express cell-specific molecules with the potential to be seen as “foreign” by the immune system. Owing to the time difference between their appearance in puberty and the editing of the lymphocyte repertoire around birth, local adaptations of the immune system coined immune privilege are required to confer protection from autoattack. Testicular macrophages (TM) play an important role in maintaining testicular immune privilege and display reduced proinflammatory capacity compared with other macrophages. However, the molecular mechanism underlying this macrophage phenotype remained elusive. We demonstrate that TM have a lower constitutive expression of TLR pathway–specific genes compared with peritoneal macrophages. Moreover, in TM stimulated with LPS, the NF-kB signaling pathway is blocked due to lack of IκBα ubiquitination and, hence, degradation. Instead, challenge of TM with LPS or polynosinic-polycytidylic acid induces MAPK, AP-1, and CREB signaling pathways, which leads to production of proinflammatory cytokines such as TNF-α, although at much lower levels than in peritoneal macrophages. Pretreatment of TM with inhibitors for MAPKs p38 and ERK1/2 suppresses activation of AP-1 and CREB signaling pathways and attenuates LPS-induced TNF-α secretion. High levels of IL-10 production and activation of STAT3 by LPS stimulation in TM indicate a regulatory macrophage phenotype. Our results suggest that TM maintain testicular immune privilege by inhibiting NF-kB signaling through impairment of IκBα ubiquitination and a general reduction of TLR cascade gene expression. However, TM do maintain some capacity for innate immune responses through AP-1 and CREB signaling pathways. The Journal of Immunology, 2015, 194: 000–000.

Immune privilege is defined as a special status of tissues in the body that tolerate allo- and autoantigens. Beside the testis, immune-privileged sites include the anterior chamber of the eye, the brain, and temporarily the placenta/uterus during pregnancy as well as tumor-draining lymph nodes (1, 2). The rat testis was identified as an immune-privileged organ when allografts transplanted into the male gonad survived prolonged periods of time without evidence of rejection (3). Testicular immune privilege seems logical, as the first appearance of a large number of neoantigens in developing germ cells occurs only after establishment of self-tolerance and thus requires specific adaptations of the immune system to avoid rejection and ultimately infertility by chronic orchitis. Tolerance is conferred by the testis itself, as autoimmunity is rapidly elicited when testicular autoantigens are injected under the skin (4). Data accumulated during the last decade now imply that local active immunosuppression rather than simple sequestration of autoantigens by the blood/testis barrier is central in the establishment and maintenance of testicular immune privilege. Mechanisms implicated in the control of immune privilege include high levels of intratesticular androgens, and particularly immunosuppressive properties of local cells such as Sertoli cells in the seminiferous epithelium as well as regulatory T cells (Tregs) and macrophages in the interstitial space (1, 5, 6). One way how Sertoli cells induce testicular immune tolerance is by generation of Tregs (Foxp3⁺ Tregs) in a mechanism that triggers Notch/Jagged signaling pathways in these cells (7, 8).

Testicular macrophages (TM) constitute the principal population of immune cells in the testis of most species (1, 9). TM are morphologically and functionally closely linked to the androgen-producing Leydig cells in the testicular interstitial space (10). There is strong evidence that TM play a vital role in testis development and adult function, as the absence of macrophages from the testis leads to disordered testicular development. Most notably, op/op mice deficient in the macrophage growth factor M-CSF have poorly developed testes, with very few TM, aberrant steroidogenesis, and deficient sperm production, whereas the development of Leydig cells and androgen production in testes depleted of macrophages by various other means is similarly inhibited (11).

TM, similar to other macrophages, show normal phagocytic and bactericidal function. However, by contributing to immune privilege they display a clearly diminished innate immune response following challenge with LPS compared with macrophages in other tissues (for review, see Refs. 12, 13). Recently, Winnall et al. (14) reported that the immune response of TM is skewed toward an alternatively activated macrophage type similar to the M2 type in

*Department of Anatomy and Cell Biology, Justus Liebig University Giessen, 35392 Giessen, Germany; †Department of Medical Microbiology, Justus Liebig University Giessen, 35392 Giessen, Germany

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Address correspondence and reprint requests to Prof. Andreas Meinhardt, Department of Anatomy and Cell Biology, Justus Liebig University Giessen, Alaweg 123, 35392 Giessen, Germany. E-mail address: Andreas.Meinhardt@anatomic.med.uni-giessen.de

The online version of this article contains supplemental material.

Abbreviations used in this article: CAPE, caffeic acid phenethyl ester; CSN, COP9 signalosome; PM, peritoneal macrophage; poly(IC), polynosinic-polycytidylic acid; qRT-PCR, quantitative RT-PCR; SCF–β-TrCP, Skp1–cullin–F-box/β-transducin repeat–containing protein; TM, testicular macrophage; TRAM, Toll/IL-1R domain–containing adapter-inducing IFN-β–related adapter molecule; Treg, regulatory T cell; TRIF, Toll/IL-1R domain–containing adapter-inducing IFN-β.
mice upon treatment with both classical (LPS and IFN-γ) or alter-
native (IL-4) activation ligands, although still a reduced capacity for proinflammatory gene expression was observed. In support of these observations, incubation with uropathogenic *Escherichia coli* instead of LPS increased mRNA expression of the proinflammatory cytokines IL-6 and TNF-α; however, protein levels were not ele-
vated (15). In the same study, activation of the NF-κB signaling pathway is prevented by maintaining stable levels of the NF-κB inhibitor IκBα (15).

Generally, recognition of microbes by macrophages depends on binding of conserved molecular patterns such as LPS to members of the TLR family. All TLRs activate a common signaling pathway that culminates in the activation of NF-κB and AP-1. In resting cells, NF-κB p50/p65 heterodimers are retained in the cytoplasm by the inhibitory molecule IκBα. Upon binding to the ligand (e.g., LPS to TLR4 or polynsinosic-polycytidylid acid [poly(I:C)] to TLR3) IκBα is phosphorylated by IKK kinases, polyubiquitinated by Skp1–cullin–F-box/b, and bound to the cytosol by the inhibitory molecule IκBα. Upon binding to the ligand (e.g., LPS to TLR4 or polynsinosic-polycytidylid acid [poly(I:C)] to TLR3) IκBα is phosphorylated by IKK kinases, polyubiquitinated by Skp1–cullin–F-box/b, and bound to the cytosol by the inhibitory molecule IκBα. Dephosphorylation of IκBα and release from the cytosol by the proteasome (16). Alternatively, activation of the AP-1 signaling pathways is mainly mediated by MAPKs such as p38, ERK, and JNK. Activation of both NF-κB and AP-1 signaling pathways leads to increased expression of proinflammatory cyto-
kines such as IL-6 and TNF-α (17).

Expression of TLR1–11 was identified in total rat testis, epi-
didymis, and vas deferens (18), whereas TLR1–9 were found in human testis samples (19). Besides the occurrence of all TLRs, isolated rat TM also synthesize CD14, MD2, and the adaptor protein MyD88, all of which are required for the initiation of TLR4-mediated signaling (20). Although TM are equipped with the necessary machinery to sense pathogens and mount proinflammatory immune responses, there is little understanding at what molecular level the immune response of TM is discerned from that of other macrophages and how this mechanistically contributes to immune privilege by reduced capacity for proinflammatory gene expression.

**Materials and Methods**

**Animals**

Adult male Wistar rats (240–270 g) were purchased from Harlan (Borchen, Germany) and kept under standard conditions (22°C, 12-h light/dark cycle) with pelleted food and water ad libitum. The experiments were performed according to the guidelines of the local authority (Regierungspräsidium, Giessen, Germany) and conform to the Code of Practice for the Care and Use of Animals for Experimental Purposes (permission GI 20/23 no. A 31/2012).

**Abs and chemicals**

Abs directed against p38 (no. 9212), phospho-p38 (no. 9211), ERK1/2 (no. 9102), phospho-ERK1/2 (no. 9106), JNK1/2 (no. 9252), phospho-JNK1/2 (no. 9251), IκBα (no. 4814), phospho-IκBα (no. 9246), p65 (no. 3034), phospho-p65 (no. 3036), phospho-CREB (no. 9198), phospho-STAT3 (no. 9145), and phospho-c-Jun (no. 3270) were purchased all from Cell Signaling Technology. Mouse monoclonal β-actin Abs (A5441), polyclonal (LC), NF-κB inhibitor, caffeic acid phenethyl ester (CAPE), MG132, and LPS (from *E. coli* 0127:B8) were purchased from Sigma-Aldrich (Steinheim, Germany).

**Cell isolation**

TM were isolated as described previously (20). Briefly, two adult rat testes were decapsulated and the seminiferous tubules gently separated. Tubular fragments were allowed to sediment for 5 min, and cells in the supernatant were pelleted, resuspended, and then seeded into culture plates. After 30 min at 32°C, TM adhered to the culture dish and contaminating cells were removed by extensive washing. Peritoneal macrophages (PM) were isolated by peritoneal lavage as previously described (20). Briefly, PM were seeded into culture plates at 37°C for 30 min and washed vigorously to remove the contaminating cells. Purity of TM and PM was ~85–90% as determined by immunofluorescence staining using the rat macrophage-
specific Abs ED1 (CD68) and ED2 (CD163) (Serotec, Oxford, U.K.).

**ELISA**

Shortly after isolation, TM and PM were treated with 10 μg/ml LPS and 10 μg poly(I:C) for the indicated time periods in the figures. Supernatants were collected and measured by ELISA specific for TNF-α (Biosecience, San Diego, CA), IL-10 (BD Biosciences, San Jose, CA), and IL-6 (Duoset; R&D Systems, Wiesbaden, Germany) following the manufacturers’ instructions.

**Western blot**

After treatment, TM and PM were washed with ice-cold PBS and lysed with SDS-PAGE sample buffer. Lysed cells were gently sonicated on ice (10 s, one pulse; Bandelin Sonopuls, Berlin, Germany) and heated at 90°C for 10 min. Equal amounts of protein were separated on 10% SDS-PAGE and transferred to a nitrocellulose membrane (Hybond ECL, 0.2 μm; GE Healthcare, Buckinghamshire, U.K.). Blocking of unspecified binding, in-
cubation with primary and secondary Abs, and ECL detection (GE Health-
care) occurred as previously described (20). Membranes were stripped and reprobed with an anti-actin Ab to assess equal loading.

**RNA isolation**

Total RNA was isolated using the RNeasy mini kit and the RNase-free DNase I set (Qiagen, Hilden, Germany) following the manufacturer’s protocol. The RNA was recovered in RNase-free water, heat denatured for 10 min at 65°C, quantified with the NanoDrop ND-1000 UV-Vis spec-
trophotometer (NanoDrop Technologies, Rockland, DE), and a quality profile with the Agilent 2100 bioanalyzer (Agilent Technologies, Wald-
bron, Germany) was collected.

**Real time RT-PCR**

First-strand cDNA was synthesized with 400 ng purified RNA using SuperScript II (Invitrogen) and a mixture of T21 and random nonamer primers (Metabion, Martinsried, Germany) following the instructions for the reverse transcription reaction recommended for the QuantiTect SYBR Green kit (Qiagen). Real-time quantitative PCR was performed with a QuantiTect SYBR Green Kit (Qiagen) on an ABI Prism 7700 real-time cyt

**Immunofluorescence**

TM and PM were cultured on glass coverslips in 24-well plates (Sarstedt, Nümbrecht, Germany) and treated with 10 μg/ml LPS or poly(I:C) for 1 h. After treatment cells were washed three times with PBS and fixed with 4% paraformaldehyde in PBS for 30 min at room temperature. Cells were permeabilized with 0.2% Triton X-100 for 10 min. Subsequently, unspecific

**Luciferase reporter assay**

RAW264.7 macrophages and primary rat testicular macrophages were seeded on 12-well plates. After overnight culture, the cells were transfected with 1 μg NF-κB luciferase reporter plasmid or the empty vector pGL3.
After lysis of RBCs, freshly isolated rat TM and PM were processed for flow cytometric analysis. The flow cytometric analysis of macrophage-specific markers was performed by using anti-rat CD45-PE/Cy7 (BioLegend, no. 202213), anti-rat CD163–Alexa Fluor 647 (AbD Serotec, no. MCA342A647), and anti-rat CD68-allophycocyanin-Vio770 (Miltenyi Biotec, Bergisch Gladbach, Germany, no. 130-103-366). Background staining was evaluated using appropriate isotype controls, that is, PE/Cy7 mouse IgG1 (BioLegend, no. 400125), REA control-allophycocyanin-Vio770 (Miltenyi Biotec, no. 130-104-634), and Alexa Fluor 647 mouse IgG1 (BioLegend, no. 406617). All incubation steps were conducted at 4°C for 30 min. Briefly, 1 × 10^6 cells were incubated in HBSS with anti-CD16/32 (Fc block; BD Biosciences), after which CD45 and CD163 Abs were added. Cells were washed with HBSS buffer. For intracellular staining of CD68, cells were fixed and permeabilized using an intracellular fixation and permeabilization buffer set (eBioscience). Data were collected for 20,000 events using a MACSQuant analyzer 10 flow cytometer (Miltenyi Biotec) and analyzed with FlowJo software version X (Tree Star, Ashland, OR).

### Table 1. Information of primer sequence, annealing temperature, and amplicon size

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HMBS, hydroxymethylbilane synthase; iNOS, inducible NO synthase; IP-10, IFN-γ-inducible protein 10; IRAK, IL-1R-associated kinase; IRF3, IFN regulatory factor 3; β2M, β2-microglobulin; PPIA, peptidylprolyl isomerase A; RPLP2, ribosomal protein, large P2; SDHA, succinate dehydrogenase α subunit; SOCS, suppressor of cytokine signaling.
Measurement of NF-κB (p50 and p65) transcription activity

The transcription activity of NF-κB subunits p50 and p65 was measured with a transcription factor kit for NF-κB p50 and p65 (Thermo Scientific, Waltham, MA) following the manufacturer’s instructions. Briefly, TM and PM were treated with LPS (10 μg/ml) for 1 h and total proteins were isolated by RIPA extraction buffer. Binding of NF-κB subunits to their respective consensus sequences was measured by using luminometry.

NO measurement

TM and PM were treated with LPS (10 μg/ml) for 24 h. Subsequently, cell culture supernatants were collected and NO was quantified using a Griess reagent system (Promega) according to the manufacturer’s instructions.

Results

TM express low levels of mRNAs for TLR and NF-κB signaling pathway genes

In rats, TM are characterized by the expression of surface markers CD68 (ED1) and CD163 (ED2). Analysis of macrophage-specific markers on freshly isolated TM and PM revealed substantial differences in the protein levels of CD68 and CD163 between different cell subtypes. PM showed significantly higher levels of CD68 (ED1). In contrast, TM displayed significantly higher expression of CD163 (ED2) as compared with PM (Supplemental Fig. 1).

To determine whether the subdued inflammatory response observed in rat TM (21) is due to an aberrant expression of TLR signaling pathway genes, the mRNA expression of representative genes (Table I) was compared in TM and PM by quantitative RT-PCR (qRT-PCR). In contrast to PM, TM displayed profoundly lower basal expression of TLR pathway–specific genes such as CD14, LBP, MD2, MyD88, Toll/IL-1R domain–containing adaptor-inducing IFN-β (TRIF), TRAM (TRIF-related adaptor molecule), MAL, TNFR-associated factor 6, TAK1, RIP1, and NIK (Fig. 1, Table I). Expression of SARM (negative regulator of TLR3 signaling) and RP105 (negative regulator of MD2/TLR4 signaling) were significantly higher in TM (Fig. 1B, Table I). Additionally, we have checked the expression of TLR3 and TLR4 protein by Western blot analysis. Levels of TLR3 protein are comparable in both cell types, whereas TLR4 protein was abundantly expressed in PM and only faintly detectable in TM (Supplemental Fig. 2). The basal expression of TLR4-triggered proinflammatory cytokines genes TNF-α and IL-6 were significantly lower in TM than PM (Fig. 1D, Table I). Increasing concentrations of LPS and poly(I:C) induced the secretion of TNF-α and IL-6 protein in a dose-dependent manner in both TM and PM in vitro (Fig. 2). However, levels of secreted TNF-α and IL-6 were much lower in TM.

IκBα degradation is blocked in LPS- and poly(I:C)-treated TM

Activation of the classical NF-κB signaling pathway requires the phosphorylation and subsequent degradation of IκBα, the negative regulator NF-κB. Treatment with LPS and poly(I:C) induced rapid phosphorylation of IκBα in a similar pattern in both TM and PM with the phosphorylation being detected as early as 15 min and sustained up to 120 min (Fig. 3A, 3B). However, degradation of IκBα was observed only in PM, but not in TM, at all time points investigated. Additionally, we have examined the degradation of IκBβ after treatment with LPS in TM. The kinetics of ubiquitin-mediated degradation of IκBβ are similar to those of IκBα, albeit occurring at a slower rate. Similar to IκBα, degradation of IκBβ was not observed in LPS-treated TM (Supplemental Fig. 3A). Despite the lack of IκBα degradation, TM showed an increase in p65 phosphorylation as was evident for PM when treated with

FIGURE 1. (A–C) Gene expression profiles for TLR signaling pathway genes in TM and PM. (D) Basal mRNA expression of TLR signaling pathways genes and proinflammatory cytokines IL-1α, IL-1β, IL-6, and TNF-α were quantified in TM and PM by using qRT-PCR. The expression of each target gene is normalized by four reference genes in TM and PM samples. For each gene the mean of expression level of PM is divided by the respective mean of TM samples and results were presented as the PM/TM ratio and expressed as mean ± SD (n = 3). The unpaired Student t test was employed for statistical analysis. *p < 0.05, **p < 0.01, ***p < 0.001.
LPS or poly(I:C) (Fig. 3A, 3B). However, immunofluorescence revealed that translocation of p65 was observed only in PM, but not in TM (Fig. 3C). Next, we investigated the transcriptional activity of NF-κB subunits following treatment with LPS in TM and PM. The NF-κB p65 transcriptional activity significantly increased in PM, whereas transcriptional activity of p65 did not
change in TM (Fig. 3D). Inactivation of NF-κB signaling pathways in TM was further confirmed by NF-κB luciferase reporter assay. In agreement with our above results, no significant changes in luciferase activity of p65 transcription factor were evident (Supplemental Fig. 3B). Interestingly, transcriptional activity of the p50 subunit was not observed in both TM and PM. In summary, these results indicate that TM are unable to activate the NF-κB signaling pathway.

IkBα is not ubiquitinated in TM

In the usual sequence of events on the molecular level, IkBα requires phosphorylation as a prerequisite for ubiquitination and final degradation by the 26S proteasome. Whereas LPS- and poly(I:C)-challenged PM showed strong polyubiquitination of phospho-IkBα in the presence of the proteasome inhibitor MG132, the same effect was barely detected in TM (Fig. 4A, 4B).

To determine whether the failed polyubiquitination of phospho-IkBα in TM was due to aberrant expression of ubiquitinating and deubiquitinating enzymes, the gene expression of various members of the ubiquitination/deubiquitination complex involved in the activation and repression of IkBα and the IKK complex was examined. In PM, basal expression of IKK-α, IKK-β, IκB-ε, NEMO (IKK-γ), and ROS2 was significantly higher than in TM (Fig. 4C). Interestingly, expression of SCF-βTrCP, an E3 ligase whose binding to phospho-IkBα is leading to recognition by the 26S proteasome and degradation, was 25-fold higher in TM than PM (Fig. 4C).

LPS and poly(I:C) activate MAPKs in TM

To investigate whether poly(I:C) and LPS activate TLR3 and TLR4 downstream signaling pathways in TM and PM, we examined the activation of the three MAPKs (p38, ERK1/2, and JNK) by detecting their dually phosphorylated (Tyr/Thr) forms by Western blot analysis by using phospho-specific Abs (Fig. 5). LPS strongly stimulated a rapid but persistent activation of both p38 and ERK1/2 MAPKs in both TM and PM. In contrast to PM, poly(I:C) activates both p38 and ERK1/2 in TM, although the level of activation was much weaker than for LPS (Fig. 5A, 5B). In poly(I:C)-treated TM, activation of p38 was observed at 30 min, whereas increased phosphorylation of ERK1/2 was seen at 60 min of treatment. The levels of phosphorylated JNK compared with total JNK remained largely the same in both LPS- and poly(I:C)-treated TM (Fig. 5). However, in PM, activation of JNK was observed after 15 min of treatment.

LPS and poly(I:C) activate the AP-1 and CREB signaling pathways in TM

Despite the lack of activation of the NF-κB signaling pathway, TM were able to respond with the production of a number of cytokines and chemokines following challenge with LPS or poly(I:C) (Fig. 6A, Table I). Gene expression of IL-1α, IL-1β, IL-6, TNF-α, COX-2, and inducible NO synthase was strongest in LPS-treated TM, whereas IFN-γ–inducible protein 10 mRNA levels were substantially elevated in poly(I:C)-treated TM. MCP1 mRNA expression was similar in both LPS- and poly(I:C)-treated cells (Fig. 6A). Pending on upstream activation of the MAPK p38, regulation of proinflammatory cytokine production can also be mediated by AP-1 and CREB transcription factors besides NF-κB (17, 22, 23). Phosphorylation of CREB was found in LPS- and poly(I:C)-stimulated TM, whereas c-Jun was phosphorylated only in LPS-treated cells (Fig. 6B).

LPS and poly(I:C) secrete proinflammatory cytokines through the AP-1 and CREB signaling pathways in TM

In support of the above-mentioned data, the p38 inhibitor SB203580 dose-dependently diminished activation of c-Jun and CREB (Fig. 7A) and suppressed TNF-α secretion in LPS-stimulated TM (Fig. 7B). In contrast, CAPE, a specific inhibitor of NF-κB, did not cause inhibition of TNF-α secretion. Notably, treatment of PM with CAPE significantly suppressed TNF-α release (Fig. 7B). Similar results were observed for the ERK1/2 inhibitor U0126 (data not shown). These findings indicate that in TM, MAPKs play a key role in inducing the secretion of proinflammatory cytokines through the AP-1 and CREB signaling pathways.

TM show characteristics of regulatory macrophages

Contrary to PM, treatment of TM with LPS resulted in a significant upregulation of the mRNA and protein secretion of the anti-inflammatory/regulatory cytokine IL-10 (Fig. 8A, 8B). Moreover, LPS-stimulated production of NO as a marker of classically activated M1 macrophages was substantially higher in PM than in TM (Fig. 8C). Similarly, secretion of the proinflammatory cytokine IL-12, a marker for M1 macrophages, was significantly increased in PM (Fig. 8D). To further examine an alternative phenotype of TM, we performed immunoblot analysis with a phospho-specific STAT3 Ab on LPS-treated TM and PM. In contrast to PM, treatment with LPS causes phosphorylation of STAT3 (Supplemental Fig. 4). In Fig. 7B, we have observed that secretion of TNF-α is mediated through MAPK signaling pathways. To determine the role of these signaling pathways in secretion of IL-10, TM were pre-treated with the p38 inhibitor SB203580 followed by LPS challenge. Inhibition of p38 MAPK significantly abrogates the secretion of IL-10. Likewise, IL-10 secretion also was attenuated by the EKR inhibitor. In contrast, CAPE, a NF-κB–specific
inhibitor, did not cause any significant changes in the secretion of IL-10 (Fig. 8E). Taken together, these results suggest that under inflammatory conditions, TM display the phenotype of a regulatory macrophage, a characteristic that helps protecting the developing germ cells from cytotoxic proinflammatory mediators.

Discussion
TM contribute to the immune privilege of the testis by diminished secretion of proinflammatory cytokines (1, 9). However, the molecular mechanisms involved in this phenomenon are largely unknown. Their understanding could contribute to better knowledge of organ-specific immune suppression in the testis that when disturbed leads to inflammation/infection-based male infertility, which paradoxically represents the second most prevalent etiology in the male (24, 25). It is therefore of importance to unravel the mechanisms by which TM display a diminished inflammatory response to protect the developing germ cells from cytotoxic effects of excessive proinflammatory cytokine production, but still maintain an adequate responsiveness to infection and inflammation.

In this study, we provide two lines of evidence for the subdued inflammatory response of TM at the molecular level. First, TM show a significantly reduced expression of CD14, MD2, and of adaptor molecules of TLR signaling pathways such as MyD88, MAL, TRAM, and TRIF, which could explain decreased expression of proinflammatory cytokines by inefficient signal transduction. In agreement, TM expressed increased levels of mRNA for SARM and RP105, negative regulators for TLR3 and TLR4 signaling pathways, respectively (26, 27). Similar mechanisms have been reported in intestinal macrophages, where reduced expression of TLR signaling molecules and proinflammatory cytokine genes contribute to intestinal homeostasis needed to tolerate the commensal microbes of the gut (28).

Second, a striking contrast of TM in comparison with PM is their inability to activate the NF-κB signaling pathway upon treatment with the classical TLR ligands LPS and poly(I:C). In this study, we have shown that inhibition of NF-κB signaling principally takes place at the level of IkBa processing. Although TLR ligands induce the phosphorylation of IkBa, the subsequent
FIGURE 7. LPS-induced activation of AP-1 and CREB is p38-dependent. (A) TM were pretreated for 30 min with the selective p38 inhibitor SB203580 with the indicated doses and then stimulated with 10 μg/ml LPS for 30 min. Cell lysates were subjected to Western blot analysis using Abs specific for phospho-p38, p38, phospho-c-JUN, phospho-CREB, and β-actin (loading control). Each experiment has been performed at least three times and a representative experiment is shown. (B) TM and PM were pretreated for 30 min with CAPE and SB203580 with the indicated dose and then stimulated with 10 μg/ml LPS for 24 h. Conditioned media from cells were analyzed for TNF-α protein levels by specific sandwich ELISA. Data are presented as the means ± SD of two to three independent experiments. The one-way ANOVA test was employed for statistical analysis. *p < 0.05, ***p < 0.001, ****p < 0.0001.

degradation was not observed in TM. Normally, phosphorylated IkBα is polyubiquitinated by SCF-βTrCP, which finally destines IkBα for degradation by the 26S proteasomal complex (16). Our data suggest that intrinsic mediators in TM may interfere at the level of ubiquitination of phosphorylated IkBα. Because degradation of IkBα is prevented at the level of ubiquitination, we hypothesize that the COP9 signalosome (CSN) (29) may play a role in IkBα ubiquitination in TM. The association between CSN and IkBα suggests that the turnover of IkBα protein is dependent on CSN, leading to increased amounts of stable cytoplasmic IkBα and thereby inhibiting NF-κB activation (30). Thus far, transfection experiments in delineating the role of the COP9 signalosome in stabilizing IkBα have been hindered in TM by their limited numbers, the short period of survival in culture, and the multimeric protein composition of the CSN, but the data in this study encourage challenging experiments along this line.

LPS triggers the TLR signaling pathway through MyD88 adaptor molecules ultimately resulting in the activation of the NF-κB and MAPK (p38 and ERK) pathways (31, 32). Activated MAPKs phosphorylate the transcription factors AP-1 (c-Jun) and CREB, which subsequently leads to the secretion of proinflammatory cytokines such as IL-6, TNF-α, as well as the expression of the anti-inflammatory cytokine IL-10 (33, 34). In this study, we have clearly demonstrated that LPS induces the activation of MAPKs (p38 and ERK1/2) and subsequent activation of AP-1 and CREB observed by its phosphorylation. We hypothesize that in the absence of NF-κB signaling the residual cytokine expression is regulated by AP-1 and CREB signaling pathways. In line with this hypothesis, the treatment of TM with the MAPK inhibitors SB203580 and U0126 blocks AP-1 and CREB phosphorylation and more importantly the LPS-induced secretion of TNF-α. MAPK-mediated activation of AP-1 and CREB signaling pathways is also required for the production of IL-10 (Fig. 8D). Hence, AP-1 and CREB signaling pathways could play an important role in limiting excessive inflammation. In addition to MAPK pathways, the expression of IL-10 is regulated by the NF-κB signaling pathway. Upon stimulation with inflammatory stimuli, NF-κB p50 subunit homodimerizes and then translocates to the nucleus and exclusively binds to the IL-10 promoter. Moreover, IL-1R–associated kinase 1 binding protein 1 promotes translocation of p50/p50 homodimers over to p50/p65 heterodimers and induces the production of IL-10 in macrophages (35, 36). In this study, we have not observed the activation of the NF-κB signaling pathway, and hence production of IL-10 is mediated mainly through the AP-1 and CREB signaling pathways.

Macrophages are polarized to M1, M2, or regulatory macrophages upon stimulation with respective stimuli. Macrophages develop toward M1 macrophages upon stimulation with IFN-γ and microbial stimuli such as LPS (37). Conversely, Th2 cytokines IL-4 and IL-13 promote polarization toward M2 macrophages, particularly during allergy and parasitic infections (38). However, in the recent past a third macrophage subclass called regulatory macrophages has been described with similar properties as those of M2 macrophages. Macrophages are polarized to regulatory macrophages by stimulation with TLR ligands in the presence of the immune complex and PGs and are characterized by secretion of immunosuppressive cytokines such as IL-10 and the low-secretion proinflammatory cytokine IL-12 (39). Rat TM produce high basal levels of PGE2, and treatment with LPS significantly increases the concentration of PGE2 (40). In this study, we have shown that upon treatment with LPS, TM exhibit a regulatory macrophage phenotype by
producing a large amount of IL-10, low secretion of IL-12, and failure to induce NO release. The main physiological function of regulatory macrophages is to dampen proinflammatory responses by producing large quantities of IL-10, thus maintaining tissue homeostasis. IL-10 exerts anti-inflammatory activity by activation of STAT3 signaling pathway and through heme oxygenase-1 (41). In the present study, we have shown that LPS induced the activation of STAT3 signaling pathways in TM and hence provide evidence that the activation of IL-10/STAT3 signaling pathways could play a role in inhibiting the secretion of proinflammatory cytokines, at least partially. The role of IL-10 in maintaining homeostasis of tissues is demonstrated by the development of spontaneous colitis in IL-10-deficient mice (34). Additionally, mutations in IL-10 and IL-10 receptors result in severe infantile inflammatory bowel disease (34). Of note, intestinal macrophages are potent producers of IL-10, which in turn increases the Treg population by maintaining stable Foxp3 expression (33). It is tempting to speculate that IL-10 secreted by TM in vivo helps to maintain the testicular population of Tregs, which are known to be relevant for the establishment of testicular immune privilege (42). In the future, it will be worthwhile to study the role of TM in the induction of Tregs and elucidate the molecular mechanism of immune tolerance in testis to protect the developing germ cells from profound inflammatory response.

In conclusion, the results obtained from this study suggest that TM maintain immune privilege of testis by profound downregulation of inflammatory genes and as a unique mechanism by suppressing the NF-κB signaling pathway at the level of IκBα ubiquitination. Moreover, TM display characteristic of regulatory macrophages, a subset known to control and dampen inflammatory immune response. Although TM are unable to induce NF-κB signaling, they maintain some capacity to respond to inflammatory stimuli by secreting proinflammatory cytokines through the AP-1 and CREB signaling pathways. These properties of TM provide them with the ability to adequately respond to microbial challenge while protecting the sensitive germ cells from the negative consequences of a high-magnitude immune response.

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Disclosures
The authors have no financial conflicts of interest.

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