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,† Svetiń Tchatalbachev,† Yongning Lu,* Suada Fröhlich,* Monika Fijak,* Vijith Vijayan,* Trinad Chakraborty,† and Andreas Meinhardt*"
mice upon treatment with both classical (LPS and IFN-γ) or alternative (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 elevated (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 polyinosinic-polycytidylic acid [poly(I:C)] to TLR3) IκBα is phosphorylated by IκK kinases, polyubiquitinated by Skp1–cullin–F-box/Kelch-like E3 ubiquitin protein ligase 2 (SCF–KlLK2), which finally destines IκBα for degradation by the 26S proteasomal complex. Degradation of IκBα exposes the nuclear localization sequence in NF-κB and leads to translocation of NF-κB to the nucleus (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 cytokines such as IL-6 and TNF-α (17).

Expression of TLR1–11 was identified in total rat testis, epididymis, 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 (249–270 g) were purchased from Harlan (Borchen, Germany) and kept under standard conditions (22 °C, 12-h light/dark cycle) according to the guidelines of the local authority (Regierungspraesidium, 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 (Danvers, MA). The Ab directed against IκBβ was purchased from Santa Cruz Biotechnology (Dallas, TX). The p38 inhibitor (SB 203580) and ERK1/2 inhibitor (U0126) were purchased from Cell Signaling Technology. Mouse monoclonal β-actin Abs (A5441), poly(IEC), 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) (Seratec, Oxford, U.K.).

**ELISA**

Shortly after isolation, TM and PM were treated with 10 μg/ml LPS and 10 μg poly(IEC) for the indicated time points in the figures. Supernatants were collected and measured by ELISA specific for TNF-α (Bioscience, 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, incubation with primary and secondary Abs, and ECL detection (GE Healthcare) 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 spectrophotometer (NanoDrop Technologies, Rockland, DE), and a quality profile with the Agilent 2100 bioanalyzer (Agilent Technologies, Waldbronn, 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 cycler (Innogen). The relative expression of target genes was normalized to that of a pool of four reference genes. PCR primer information is provided in Table I. Reactions were done in triplicates using the QuantiTect SYBR Green Kit (Qiagen). For each target and reference gene a standard dilution curve with a reference RNA sample was done and the linear equation was used to transform threshold cycle values into nanograms of total RNA. The relative fold change of target genes in the PM samples versus the TM samples was normalized by the relative expression of a pool of the following four reference genes: HMBS, PPIA, RPLP2, and SHDA (see Table I). Normalized fold change for a target gene versus every reference gene was calculated and a mean fold change of these four was the final value. In Fig. 6 mRNA expression analysis was performed as described earlier using β2-microglobulin (15).

**Immunofluorescence**

TM and PM were cultured on glass coverslips in 24-well plates (Sarstedt, Brønn, Germany) and treated with 10 μg/ml LPS or poly(IEC) 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, unspecified binding sites were blocked by incubation for 1 h in PBS containing 5% normal goat serum and 5% BSA, washed, and incubated with the primary anti-rabbit p65 Ab (1:50) at 4°C overnight. After washing, slides were incubated with the corresponding Cy3-conjugated secondary Ab (Dianova, Hamburg, Germany) for 1 h at room temperature in the dark. Nuclei were stained with TO-PRO-3 dye (Molecular Probes, Darmstadt, Germany) and treated with 10 μg/ml LPS and 85–90% as determined by immunofluorescence staining using the rat macrophage-specific Abs ED1 (CD68) and ED2 (CD163) (Seratec, Oxford, U.K.).

**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-
basic and 100 ng internal control vector (Renilla luciferase vector) using the transfection reagent GeneCellin HTC (RAW264.7) and ViaFect (vesicular macrophages) according to the manufacturer’s protocol. The plasmids were a generous gift from Dr. Stephan Immenschuh (Institute for Transfusion, Hannover Medical School, Hannover, Germany). Twenty-four hours after transfection, the cells were treated with LPS (10 μg/ml) for an additional 24 h, after which the cells were lysed and the luciferase activity was measured using the Dual-Luciferase kit (Promega) according to the manufacturer’s protocol. The firefly luciferase values were normalized with the respective Renilla luciferase values.

Flow cytometric analysis

After lysis of RBCs, freshly isolated rat TM and PM were processed for flow cytometric analysis of macrophage-specific markers by using anti-rat CD45-

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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.

IkBα 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 IkBα, the negative regulator NF-κB. Treatment with LPS and poly(I:C) induced rapid phosphorylation of IkBα 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 IkBα was observed only in PM, but not in TM, at all time points investigated. Additionally, we have examined the degradation of IkBβ after treatment with LPS in TM. The kinetics of ubiquitin-mediated degradation of IkBβ are similar to those of IkBα, albeit occurring at a slower rate. Similar to IkBα, degradation of IkBβ was not observed in LPS-treated TM (Supplemental Fig. 3A). Despite the lack of IkBα degradation, TM showed an increase in p65 phosphorylation as was evident for PM when treated with

![FIGURE 1.](http://www.jimmunol.org/) (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 increase in TM and PM. The NF-κB p65 transcriptional activity significantly increased in PM, whereas transcriptional activity of p65 did not increase in TM and PM.

**FIGURE 2.** TM and PM secrete proinflammatory cytokines upon stimulation with LPS and poly(I:C). TM and PM were treated with different doses of LPS and poly(I:C) for 24 h. Conditioned media from cells were analyzed for (A and C) TNF-α and (B and D) IL-6 levels by specific sandwich ELISA. Data are presented as the means ± SD of three to four independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

**FIGURE 3.** Downstream elements of the NF-κB signaling pathway are not activated in testicular macrophages. TM and PM (A–C) were stimulated with (A) LPS (10 μg/ml) and (B) poly(I:C) for the indicated time points. Lysed cells were subjected to Western immunoblotting analysis using Abs specific for the phospho-IκBα, IκBα, phospho-p65, p65, and β-actin (loading control). Each experiment has been performed at least three times and a representative experiment is shown. Immunoblots of phospho-IκBα, IκBα, and phospho-p65 were quantified and normalized by loading control β-actin and p65 by using Adobe Photoshop 7.0. Quantified value is indicated on the top of the respective blots. (C) TM and PM treated with LPS and poly(I:C) for 1 h were analyzed for nuclear translocation of the NF-κB p65 subunit using confocal microscopy (red, p65; blue, nuclei). p65 localized predominantly in the cytoplasm of untreated PM and translocated into the nucleus after LPS and poly(I:C) treatment. In contrast, in TM the NF-κB subunit p65 remained exclusively in the cytoplasm regardless of the experimental condition. Scale bars, 15 μm. (D) NF-κB transcriptional activity was determined in total cell lysates by using an ELISA-based NF-κB p50 and p65 transcription factor kit. Data are presented as means ± SD of three independent experiments. The unpaired Student t test was used for statistical analysis. **p < 0.01.
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.

IκBα is not ubiquitinated in TM

In the usual sequence of events on the molecular level, IκBα 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-IκBα 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-IκBα 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 IκBα and the IKK complex was examined. In PM, basal expression of IKK-α, IKK-β, IκB-ε, NEMO (IKK-γ), and RO52 was significantly higher than in TM (Fig. 4C). Interestingly, expression of SCF-βTrCP, an E3 ligase whose binding to phospho-IκBα 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 Western blot analysis using Abs specific for phospho-p38, phospho-ERK, and phospho-JNK (17, 22, 23). Phosphorylation of CREB was found in LPS- and poly(I:C)-treated TM, whereas c-Jun was phosphorylated only 15 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. MCP-1 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. 4A). 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,
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 IκBα processing. Although TLR ligands induce the phosphorylation of IκBα, the subsequent

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**FIGURE 5.** The MAPK signaling pathway is activated in stimulated TM and PM. TM and PM were stimulated with (A and C) 10 μg/ml LPS and (B and D) 10 μg/ml poly(I:C) for the indicated time points. Lysed cells were examined by Western blot analysis using Abs specific for phospho-p38, p38, phospho-ERK1/2, ERK1/2, phospho-JNK, and JNK. Each experiment has been performed at least three times with a representative experiment shown.

**FIGURE 6.** Proinflammatory mediators are produced in stimulated TM. (A) TM were stimulated with 10 μg/ml LPS for 6 h. Expression of proinflammatory cytokines and chemokines were quantified by using qRT-PCR. Results were normalized for β2-macroglobulin and expressed as means ± SD as compared with untreated control. (B) TM were stimulated with 10 μg/ml LPS and 10 μg/ml poly(I:C) for the indicated time points. Cell lysates were subjected to Western blotting using Abs specific for phospho-c-Jun, phospho-CREB, and β-actin (loading control). Each experiment has been performed at least three times with a representative experiment documented.
degradation was not observed in TM. Normally, phosphorylated IkBα is polyubiquitinated by SCF-βTrCP, which finally destines IkBα for degradation by the 26S proteosomal 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
FIGURE 8. TM produce the anti-inflammatory cytokine IL-10. (A) TM and PM were left unstimulated or stimulated with 10 mg/ml LPS for 6 h. Expression of IL-10 mRNA levels were quantified using qRT-PCR. The unpaired Student t test was employed for statistical analysis. (B) TM and PM were treated with 10 μg/ml LPS for 24 h. IL-10 protein concentrations were measured in culture supernatants by specific sandwich ELISA. Values are means ± SD of triplicates. One-way ANOVA test was employed for statistical analysis. (C) NO production in cultured supernatants was measured using Griess reagent. Values are means of two independent experiments and one-way ANOVA test was employed for statistical analysis. (D) TM and PM were cultured in 10% FCS and treated with LPS for 48 h. The cell supernatants were analyzed for the secretion of IL-12 p40 proteins by specific sandwich ELISA. The unpaired Student t test was employed for statistical analysis. (E) TM were pretreated for 30 min with SB203580 (5 μM) and CAPE (5 μM) followed by stimulation with 10 μg/ml LPS for 24 h. Cell supernatants from cells were analyzed for IL-10 secretion by specific sandwich ELISA. Data are presented as the means ± SD of three independent experiments. The one-way ANOVA test was employed for statistical analysis. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

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 suppression of the NF-κB signaling pathway at the level of IκBo ubiquitination. Moreover, TM display characteristics 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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References
Differential Activation of Pathways in Testis Macrophages


