Uropathogenic *Escherichia coli* Block MyD88-Dependent and Activate MyD88-Independent Signaling Pathways in Rat Testicular Cells

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Uropathogenic Escherichia coli Block MyD88-Dependent and Activate MyD88-Independent Signaling Pathways in Rat Testicular Cells

Sudhanshu Bhushan,* Svetlin Tchatalbachev,† Jörg Klug,* Monika Fijak,* Charles Pineau,‡ Trinad Chakraborty,† and Andreas Meinhardt2*†

Uropathogenic Escherichia coli (UPEC) is the most common etiological cause of urogenital tract infections and represents a considerable cause of immunological male infertility. We examined TLR 1–11 expression profiles in testicular cells and the functional response to infection with UPEC. All testicular cell types expressed mRNAs for at least two TLRs and, in particular, synthesis of TLR4 was induced in testicular macrophages (TM), Sertoli cells (SC), peritubular cells (PTC), and peritoneal macrophages (PM) after UPEC exposure. Even though MyD88-dependent pathways were activated as exemplified by phosphorylation of mitogen-activated protein kinases in TM, SC, PTC, and PM and by the degradation of IκBα and the nuclear translocation of NF-κB in PTC and PM, treatment with UPEC did not result in secretion of the proinflammatory cytokines IL-1α, IL-6, and TNF-α in any of the investigated cells. Moreover, stimulated production of these cytokines by nonpathogenic commensal E. coli or LPS in PM was completely abolished after coincubation with UPEC. Instead, in SC, PTC, TM, and PM, UPEC exposure resulted in activation of MyD88-independent signaling as documented by nuclear transfer of IFN-related factor-3 and elevated expression of type I IFNs α and β, IFN-γ-inducible protein 10, MCP-1, and RANTES. We conclude that in this in vitro model UPEC can actively suppress MyD88-dependent signaling at different levels to prevent proinflammatory cytokine secretion by testicular cells. Thus, testicular innate immune defense is shifted to an antiviral-like MyD88-independent response. The Journal of Immunology, 2008, 180: 5537−5547.

The mammalian testis is compartmentalized, with androgens synthesized in the interstitial compartment by Leydig cells while the production of spermatozoa is localized in the seminiferous epithelium, a cord-like structure surrounded by myoid peritubular cells capable of expressing a high number of cytokines and growth and differentiation factors. Within the seminiferous epithelium the columnar Sertoli cells support the development of germ cells from spermatogonia via spermatocytes and spermatids to spermatozoa (1). Anatomically, the seminiferous epithelium is a continuation of the urethra. The excurrent ducts provide a gateway for ascending microbial infections that can manifest themselves as urethritis, prostatitis, epididymitis, or more often as combined epididymo-orchitis. It is therefore not surprising that in 13−15% of all cases of male infertility, infection and inflammation of the genital tract are causes or cofactors (2–4). The testis is an immune-privileged organ protecting the auto-antigens of the meiotic and haploid germ cells, which first appear after the establishment of self-tolerance at the time of puberty (5). Immune privilege is attributed to tissues such as the eye, brain, testis, and fetal-maternal interface, where even minor infections can threaten organ integrity and function (5). This under-representation of innate immune function, however, can enable microorganisms to infect and colonize the testis, causing subsequent impairment of spermatogenesis. On the cellular level, testicular macrophages in the interstitial space, Sertoli cells, and to a lesser extent peritubular cells have been implicated in both the maintenance of immune privilege and a primary role in local defense responses (5–7).

Bacteria eliciting epididymo-orchitis are either sexually transmitted or originate from common urinary tract infections (8–11). Uropathogenic Escherichia coli (UPEC) are among the most frequently isolated microbial agents in epididymo-orchitis and in men (12–14). Albeit models of experimental bacterial epididymo-orchitis in rats are documented (15, 16), until now only very few investigations have focused on the effects of E. coli on testicular cells. As a consequence, neither the mechanisms of protection against E. coli infection of the testis nor the way the bacteria eventually evade the testicular immune response directed against them is understood.

The innate immune defense against pathogens hinges on recognition of pathogen-associated molecular patterns by members of...
The TLR family, which recently have been found also in the testis (17). TLR4 recognizes LPS, a component of the cell wall of Gram-negative bacteria such as E. coli. Binding of LPS to TLR4 leads to the activation of cellular signals resulting in a proinflammatory response characterized by the expression of TLR-response genes such as IL-1, IL-6, and TNF-α. Common TLR signaling is mediated through an adaptor protein, MyD88, which recruits other intermediate molecules that in turn activate mitogen-activated protein (MAP) kinases such as p38, JNK, and ERK1/2. Furthermore, IκBα, the cytoplasmic anchor of the transcription factor NF-κB, is rapidly degraded in response to proinflammatory stimuli. This degradation liberates NF-κB for translocation into the nucleus, where it directs transcription of the TLR response gene products.

Table I. Information on sequences of forward (FP) and reverse primers (RP) used in RT-PCR, annealing temperatures, gene accession numbers and amplicon sizes

<table>
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<tr>
<th>Gene</th>
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<th>Annealing Temperature</th>
<th>GenBank Accession No.</th>
<th>Amplicon Size (bp)</th>
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<td>101</td>
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</tbody>
</table>

*Primers for RANTES, IFNα, and IFNβ were purchased from Qiagen. Details of these primers are found at www.qiagen.com/GeneGlobe.
defensins (18–20). In addition to the common MyD88-dependent pathway, TLR3 and TLR4 can use an alternative MyD88-independent signaling pathway where nuclear translocation of the transcription factor IFN-α and β IFN-γ regulated factor 3 (IRF-3) activates the expression of genes typical for antiviral responses such as IFN-α and β IFN-γ induced protein 10 (IP-10), MCP-1, and RANTES (21, 22).

The objective of this study was to determine how two types of bacteria (uropathogenic E. coli and nonpathogenic commensal E. coli) interact with testicular cells that are known to play an important role in pathogen recognition and defense signaling (5, 23).

**Materials and Methods**

**Animals**

Male Wistar rats were purchased from Charles River Laboratories. Sertoli cells and peritubular cells (PTC) were isolated from 19- to 21-day-old rats while peritoneal macrophages and testicular macrophages were isolated from 249- to 270-g adult male rats. Animals were housed for 1 day after delivery at a 12-h light:dark cycle and access to water and food pellets ad libitum. Animals were sacrificed by isoflurane inhalation. Experimental procedures were approved by the local authority (Regierungspraesidium Giessen, Giessen, Germany) and confirm to the Code of Practice for the Care and Use of Animals for Experimental Purposes.

**Isolation of cells**

Testicular PTC and Sertoli cells (SC) were isolated from 40 testes essentially as described previously (24) except that digestion with trypsin (Roche Diagnostics) was stopped already after 15–20 min. The PTC fraction was resuspended in RPMI 1640 medium supplemented with 10% FCS (PAA Laboratories), and cells were divided into five 75-cm² culture flasks and cultured for 3 days at 37°C in 5% CO₂. PTC were briefly trypsinized (0.05% trypsin and 0.02% EDTA) and split 1:2. This procedure was repeated twice to obtain a pure population of PTC. Subsequently, 5 × 10⁶ cells/well were plated onto 6-well plates. SC were resuspended in serum free RPMI 1640 medium, and 2 × 10⁶ viable cells/well were plated onto 6-wells plates and incubated at 32°C in 5% CO₂. Purity of PTC and SC cell preparations was estimated >95% by immunofluorescence using Abs directed against smooth muscle actin (for PTC from DakoCytomation) and vimentin (for SC from Sigma-Aldrich).

Leydig cells, spermatogonia, pachytene spermatocytes, round spermatids, and residual bodies were prepared from rat testes exactly as described previously (25). For isolation of testicular macrophages (TM) two testes were decapsulated into 10 ml of ice-cold endotoxin-free DMEM:F12 medium (PAA Laboratories). The seminiferous tubules were gently separated using straight Semken forceps following the method described by Hayes et al. (26). The volume was adjusted to 50 ml and the tubule fragments were allowed to settle for 5 min before the supernatant was recovered and centrifuged at 300 × g for 10 min at 4°C. The interstitial cell pellet was resuspended in 5 ml DMEM:F12 and the concentration was adjusted to 5 × 10⁶ cells/ml. Cells (2 × 10⁶ cells/well) were plated in 6-well plates and incubated at 32°C for 30 min. Contaminating cells were removed by extensive washing, taking advantage of the rapid adherence of macrophages. Purity of TM was determined to be >90% by immunofluorescence using Abs directed against the monocyte/macrophage markers ED-1 and ED-2 (Serotec). Germ cells, mainly spermatooza, constituted the contaminants. Peritoneal exudate cells were harvested from Wistar rats by lavage with 50 ml of DMEM:F12 medium (PAA Laboratories). Retrieved cells were sedimented at 300 × g for 10 min at room temperature. The cell pellet was resuspended in 10 ml DMEM:F12 medium, and 2 × 10⁶ cells/well were seeded onto 6-well plates and incubated at 37°C for 30 min to allow peritoneal macrophages (PM) to adhere. Nonadherent cells were removed by washing thoroughly three times with DMEM:F12. Like TM, the purity of PM was >90% as determined by combined ED-1 and ED-2 immunofluorescence.
UPEC and ERK1/2.

Potential changes in the total amount of kinases following treatment and equal loading of samples were assessed by detecting total levels of p38, JNK, and PTK. Cell lysates were separated by electrophoresis on 10% SDS-polyacrylamide gels and blot membranes were treated with Abs specific for phosphorylated p38, p38, phosphorylated JNK (P-JNK), and phosphorylated extracellular regulated kinases 1/2 (P-ERK1/2), respectively, as indicated on the left. Potential changes in the total amount of kinases following treatment and equal loading of samples were assessed by detecting total levels of p38, JNK, and ERK1/2. β-Actin levels served as loading control. NPEC has no effect on MAP kinase phosphorylation (data not shown).

**Bacterial strains**

Uropathogenic *E. coli* strain CFT073 (American Type Culture Collection no. 700928; GenBank accession nos. AE014075 and NC_004431) characterized by Welch et al. (27) was obtained from U. Dobrindt, University of Würzburg, Würzburg, Germany. Commensal nonpathogenic *E. coli* (NPEC) strain 470, a human colon isolate (microbial collection of Institute of Medical Microbiology, University of Giessen, Giessen, Germany) was used as a control. Both strains were propagated overnight in Luria-Bertani medium. For infection experiments, overnight cultures of both *E. coli* strains were diluted 1/50 in fresh Luria-Bertani medium and grown to early exponential phase (OD600 = 0.5–1.0) at 37°C in a shaking incubator. The concentration of viable bacteria was calculated using standard growth curves for both strains. Bacteria (2 × 10^7/) were centrifuged at 4,500 x g for 8 min at room temperature. The pellet was washed twice at room temperature with PBS to remove bacterial growth medium and resuspended in 10 ml of DMEM or RPMI 1640 medium. One hundred microliters of this suspension (2 × 10^7 CFU/) was used to infect cells in each well of a 6-well cell culture plate (multiplicity of infection (MOI) = 20; the MOI is defined as the number of infectious agents (bacteria) per one target cell).

The ability of UPEC to elicit orchitis in vivo was demonstrated by injecting 50 μl of a bacterial suspension (1 × 10^9 CFU/ml) in the vas deferens close to the epididymis as described previously (28). Seven days after injection, orchitis was evident by smaller testes, reduced testis weight, reddening, edema formation, and by histological analysis. Testicular interstitial fluid was collected 7 days after UPEC infection by cutting a small incision in the testicular capsule opposite of the rete testis following an established protocol (29). By using a surgical microscope, great care was taken to ensure that seminiferous tubules remained intact. Testicular homogenates and interstitial fluid were plated on agar plates without antibiotics. PBS-injected rats served as control. After 16 h numerous colonies were visible on agar plates from UPEC-infected rats and bacteria were identified as *E. coli* using standard color test. This provides strong evidence that in ascending canalicular infections not only Sertoli cells but also PTC and TM become exposed to UPEC.

**Western blot analyses**

Abs directed against phosphorylated p38, p38, phosphorylated ERK1/2, ERK1/2, phosphorylated JNK1/2, and JNK1/2 were purchased from Cell Signaling Technology. TLR4 and IκBoκs Abs were obtained from Santa Cruz Biotechnology, and the mouse monoclonal β-actin Ab was purchased from Sigma-Aldrich.

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**FIGURE 2.** Detection of TLR4 protein after infection of TM, PM, SC, and PTC with UPEC and commensal NPEC. After infection for the indicated periods of time, cell lysates were separated by electrophoresis on 10% SDS-polyacrylamide gels and immunoblots were decorated with a TLR4 Ab. Equal loading of protein was assessed by β-actin detection.

**FIGURE 3.** Activation of MAP kinases following UPEC infection. Isolated TM, PM, SC, and PTC were infected with UPEC for the indicated periods of time. Cell lysates were separated by electrophoresis on 10% SDS-polyacrylamide gels and blot membranes were treated with Abs specific for phosphorylated p38 (P-p38), phosphorylated JNK (P-JNK), and phosphorylated extracellular regulated kinases 1/2 (P-ERK1/2), respectively, as indicated on the left. Potential changes in the total amount of kinases following treatment and equal loading of samples were assessed by detecting total levels of p38, JNK, and ERK1/2. β-Actin levels served as loading control. NPEC has no effect on MAP kinase phosphorylation (data not shown).
Cells were lysed in lysis buffer (10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100 (v/v), 0.25% Igepal CA-630 (v/v), 1 mM sodium orthovanadate, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, and protease inhibitor mixture (Sigma-Aldrich)) by incubation on ice for 30 min. Lysates were cleared by centrifugation (16,000 g for 15 min at 4°C), and the protein concentration in the recovered supernatant was determined by Bradford protein assay (Bio-Rad). Twenty micrograms of protein from each sample were fractionated by electrophoresis on 10% SDS-polyacrylamide gel and transferred to nitrocellulose membrane (Hybond-ECL (0.2 μm); GE Healthcare). Membranes were blocked with 5% nonfat dry milk for 1 h in TBS (20 mM Tris-HCl (pH 7.6) and 150 mM NaCl) containing 0.1% Tween 20 (v/v) (TBS/Tween) before overnight incubation with anti-\( \text{IκB}\alpha \) Ab. Detection of \( \beta\)-actin served as loading control. For detection of bound primary Abs, membranes were treated with a peroxidase-conjugated secondary Ab at room temperature for 1 h. After three washes with TBS/Tween, bands were visualized using ECL (GE Healthcare).

**Immunofluorescence**

PM, TM, SC, and PTC were grown on glass coverslips in 12-well cell culture plates and infected with UPEC and NPEC (MOI = 20). After 3 h of incubation of PM, TM, and SC and 6 h of incubation of PTC, cells were fixed with 4% paraformaldehyde for 30 min and subsequently permeabilized with 0.2% Triton X-100 in PBS for 15 min. Unspecific protein interactions were blocked by incubation for 1 h in PBS containing 5% normal goat serum and 5% BSA. Rabbit polyclonal anti-human IRF-3 Ab (Santa Cruz Biotechnology) or mouse monoclonal anti-human \( p65 \) Ab diluted in TBS with 5% milk (w/v) and incubated on ice for 1 h. Membranes were washed three times with TBS and subsequently incubated with appropriate secondary antibody conjugated with a fluorophore (Cy3, Cy5, Cy2) for 1 h. Nuclei were counterstained with Cy5-conjugated TO-PRO-3. For detection of bound primary Abs, membranes were treated with a peroxidase-conjugated secondary Ab at room temperature for 1 h. After three washes with TBS/Tween, bands were visualized using ECL (GE Healthcare).
I/100 in PBS containing 3% BSA and 0.05% Tween 20 was added overnight at 4°C. Cy3-labeled secondary Ab (Chemicon) was incubated for 1 h. Cy5-labeled To-PRO-3 (Molecular Probes) was used for nuclear counterstaining. Slides were visualized with a TCS SP2 confocal laser-scanning microscope (Leica Microsystems).

Cytokine ELISA

PM, TM, SC, and PTC were stimulated with 10 μg/ml LPS (from E. coli 0127:B8; Sigma-Aldrich) or UPEC, and cell culture supernatants were collected at the indicated time points (see Fig. 5). Secreted IL-1α, IL-6, and TNF-α were measured in triplicate from three individual experiments using sandwich ELISA (BioSource International).

Results

Differential mRNA expression pattern of TLR1–11, nucleotide-binding oligomerization domain (NOD), and MyD88 in rat testicular cells

Basal mRNA expression of the pattern recognition receptors of the TLRs 1–11, the TLR adaptor protein MyD88, and the nucleotide-binding oligomerization domains NOD1 and 2 were determined in various types of isolated testicular cells and PM (Fig. 1 and Table II). Most testicular somatic and germ cell types expressed at least two TLRs, while TM and DC synthesize the mRNA for all tested TLRs as well as NOD1, NOD2, and MyD88 (not DC) constitutively (Fig. 1). PM were found to also express most tested genes; however, very weak expression of TLR9 –11 and NOD2 was noted (Fig. 1 and Table II). TLR4, which recognizes LPS, is weakly expressed in SC and more strongly in spermatocytes. TLR 7 and 8, recognizing single-stranded RNA, and TLR9, binding unmethylated CpG-DNA, were detected in TM and DC but not in any other testicular cell type. Of note, TLR3, which recognizes viral double-stranded RNA, is highly expressed in all cell types except spermatids and LC. Expression of the TLR adaptor molecule MyD88 as well as the mRNA of the cytoplasmic surveillance proteins NOD1 and NOD2 was

FIGURE 5. UPEC suppress LPS- and NPEC-induced cytokine responses. A and C. Isolated cells were infected with UPEC (MOI = 20) for the indicated periods of time. IL-6 and TNF-α concentrations in culture supernatants were measured by sandwich ELISA. UPEC infection caused no secretion of IL-6 and TNF-α from cultured PM, TM, SC, and PTC. C. Control. B and D. PM were left untreated (C, control), incubated with 10 μg/ml LPS or, alternatively, with LPS plus UPEC (MOI = 20) for 3 h. IL-6 and TNF-α concentrations in culture supernatants collected after 3 h were measured by sandwich ELISA. IL-1α concentrations showed the same trend regarding IL-6 and TNF-α levels in all cell types (data not shown). TM, PTC, and SC were also treated with 10 μg/ml LPS but failed to induce IL-1α, IL-6, and TNF-α secretion (data not shown). Values are means ± SD of triplicates.

FIGURE 6. Translocation of IRF-3 to the nucleus was observed in all cell types (SC, PTC, TM, and PM) after UPEC infection. After NPEC infection, IRF-3 was predominantly stained in the cytoplasm of all testicular cells (TM, SC, and PTC) and PM. C, Control.
highest in TM. Overall, expression of pattern recognition receptors in testicular cells was most prominent in SC, PTC, and TM. Based on these findings, the ability of UPEC to reach all three cell types in an in vivo infection model (data not shown) and the roles of SC and PTC as well as TM in testicular immune regulation prompted us to select these cells for the subsequent in vitro investigations.

**UPEC induces TLR 4 expression in testicular cells**

SC, PTC, TM, and PM were infected with UPEC and commensal NPEC for up to 8 h. Using immunoblotting, TLR4 protein could not be detected in the lysates of noninfected cells of all tested cell types (Fig. 2). UPEC induced de novo synthesis of TLR4 protein after 2 h of exposure in SC, TM, and PM, whereas PTC showed a delayed accumulation after 6 h with a further increase after 8 h. TLR4 levels were sustained for one more hour in SC, TM, and PM (Fig. 2). Interestingly, NPEC did not cause any induction of TLR4 protein within 3 h of treatment in SC, TM, and PM, although LPS is produced by both *E. coli* strains (Fig. 2). Of note, SC, TM, and PM showed massive cell death when incubation time exceeded 3 h, excluding later time points from assessment. Cell death of PTC

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**FIGURE 7.** Analysis of relative mRNA expression of MCP-1, IP-10, RANTES, IFN-α, and IFN-β in PM, TM, SC, and PTC using quantitative real-time RT-PCR. Results were normalized using 18S rRNA as endogenous control and are shown as fold changes relative to uninfected controls. Cells were incubated with UPEC for the indicated periods of time. Values are means ± SD of triplicates. ND, Nondetectable; C, control.
was delayed as most PTC detached only after 8 h. In contrast, following NPEC incubation all four cell types showed no visible sign of cell death by 3 h (SC, PM, and TM) or 8 h (PTC) (data not shown).

**Differential activation of MAP kinase pathways following incubation with UPEC**

SC displayed the most prominent and sustained reaction with the phosphorylation of all three MAP kinases starting 15 min (phosphorylated p38) or 60 min (JNK and ERK1/2) after UPEC exposure. Levels remained elevated up to 180 min (Fig. 3). TM showed activation of p38 after 15 min that peaked after 30 min. Transient phosphorylation of ERK1/2 occurred in TM after 30 min, whereas JNK was unaffected. A rapid response was visible in PM, with sustained phosphorylation of p38 and ERK1/2 noticeable after 5 min. In clear contrast to TM, neither phosphorylated nor unphosphorylated JNK and ERK1/2 were detectable 60 min after exposure in PM.

Table III. Comprehensive summary of results of in vitro studies after UPEC infection

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<th>Cell Type</th>
<th>PM</th>
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<td>TLR 1–11</td>
<td>TLR 1–5; 10–11</td>
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<td>–</td>
<td>–</td>
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<td>UPEC induced cytokine production (IL-1α, IL-6, TNF-α)</td>
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<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td>LPS-induced cytokine production</td>
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</table>
IL-6, and TNF-α/H9251 whereas IFN-α, IP10, and RANTES were also elevated after UPEC treatment, MCP-1, and RANTES (Fig. 7). In PTC, mRNA levels of MCP-1, and RANTES (Fig. 7). SC mRNA expression of MCP-1 and IP-10 was elevated and in addition of UPEC did not induce IL-1 and TNF-α/H9251 up to 3 h), and PTC (infected with UPEC for up to 8 h) were measured by specific ELISA (Fig. 5, A and C). Surprisingly, the addition of UPEC did not induce IL-1α (data not shown), IL-6, and TNF-α (Fig. 5) production within the maximal incubation period (Fig. 5, A and C). Incubation of PM with NPEC or LPS caused a significant increase of IL-1α (data not shown), IL-6, and TNF-α (Fig. 5, B and D) concentrations in culture supernatants. Remarkably, the production of these cytokines could be completely suppressed by coinoculation with UPEC (Fig. 5, B and D). In contrast to PM, the TM, PTC, and SC showed a lower response of IL-1α, IL-6, and TNF-α to stimulation with LPS or NPEC (data not shown). However, TM and SC are not completely unresponsive. SC mRNA expression of MCP-1 and IP-10 was elevated and in TM a marked increase of cyclooxygenase-2, TGF-β, and MCP-1 transcription was observed following LPS challenge. This indicates a selective rather than general unresponsiveness of TM to inflammatory challenge (data not shown).

UPEC induce IRF-3 nuclear translocation and MyD88-independent transcription of type I IFNs and chemokines in TM, PM, SC, and PTC

Nuclear translocation of IRF-3 is a prerequisite for the activation of a TLR4-MyD88-independent signaling pathway. Using confocal microscopy, all cells examined (TM, PM, SC, and PTC) revealed IRF-3 translocation from the cytoplasm to the nucleus subsequent to incubation with UPEC, whereas IRF-3 remained predominantly cytoplasmic in all four cell types when infected with NPEC or untreated (Fig. 6).

SC reacted most comprehensively to UPEC challenge with strongly increased expression of mRNA for IFN-α and -β, IP-10, MCP-1, and RANTES (Fig. 7). In PTC, mRNA levels of MCP-1, IP10, and RANTES were also elevated after UPEC treatment, whereas IFN-α or -β mRNA was not detectable by RT-PCR. In TM, mRNA expression of RANTES was unaffected by UPEC while IP-10 and IFN-α and -β expression increased after 1 h, but at later time points mRNA expression decreased. MCP-1 expression in TM also was inhibited with a 5-fold negative change at 2 h postincubation. Similarly, mRNA expression of type I IFN (α and β) and IFN-inducible genes IP-10 and RANTES increased after 1 h of incubation with UPEC in PM but diminished later (Fig. 7).

IRF-3 activation together with the subsequent elevated transcription of type I IFN (α and β) and IFN-inducible genes MCP-1, IP-10, and RANTES in almost all cells indicates that UPEC activates MyD88 independent signaling pathway in infected testicular cells (Table III).

Discussion

It is well established that bacterial and viral infections of the male reproductive tract contribute significantly to impaired fertility. The direct pathologic effects resulting from the bacterial invasion are often aggravated by the inflammatory process. It is therefore important to understand the mechanisms by which immunocompetent cells in the testis recognize infectious agents and how bacterial pathogens try to diminish an inflammatory response by interfering with testicular defense pathways.

TLRs are the front-line sensor molecules in the initiation of innate immunity against invading pathogens (9, 19). In view of the paradoxical immune status of the testis with its susceptibility to infection and local inflammation (5, 6, 30), it is surprising that few studies have examined the role of TLRs in this organ. Only recently was expression of TLR 1–9 shown in human and rat testis, epididymis, and vas deferens (17, 31). Indication of a central role of SC in the recognition of microorganisms came from Riccioli et al. (7), who reported expression of TLR2–6 mRNA in mouse SC and subsequent activation of the NF-κB pathway following stimulation with TLR2/TLR5 agonists (7). However, to date a comprehensive investigation of the cellular profile of TLR expression in the testis has been lacking. In this study we show that rat TM and DC express all known TLRs while other testicular somatic cells and germ cells were usually positive for varying sets of one to six TLRs. Notably, TLR3 transcription was prominent in all testicular cells, except LC and spermatids, where only weak expression was evident. This indicates a strong capacity for the recognition of double-stranded viral RNA in the testis. Palladino et al. (17) demonstrated the presence of TLR 1–10 in total testis, which we did not see for TLR4 and 7–9. However, isolated TM and testicular DC expressed TLR4 and 7–9, indicating different sensitivities in the assays. Therefore, the TLR expression profiles reported here are mostly in agreement with previous TLR studies investigating the total testis of rat and other species (17, 31, 32).

Based on the relevance of UPEC in pathogen-associated-fertility disturbances, our study focused on the in vitro response of testicular cells to infection with this strain (11, 33, 34). SC, PTC, and TM, all centrally implicated in the testicular defense system, were found to express TLR4 (5, 6). TLR4 is a well-studied sensor of LPS, a major component of the Gram-negative bacteria cell wall. It is therefore surprising that incubation with the pathogen or commercial E. coli did not result in the release of the proinflammatory cytokines IL-1α, IL-6, and TNF-α despite the partial activation of the MyD88-dependent pathway, which is known to lead to their synthesis (Fig. 5 and Table III). The lack of an inflammatory response in testicular cells as shown in our study could not be attributed to a lack of a particular recognition receptor, because basal levels of TLR4 mRNA were detected in all cell types and increased amounts of the receptor protein were observed in all four cell types upon treatment with UPEC (Fig. 2). Also TLR4 mRNA was coexpressed by either TLR2 or TLR5 or both of them in all cell lines. Thus, recognition of additional bacterial products like lipoproteins (TLR2) and flagellin (TLR5) seems very likely (Fig. 1 and Table II). Our data suggest that the lack of proinflammatory cytokine production is likely due to active suppression of the MyD88-dependent NF-κB pathway by UPEC at various levels of the signaling cascade, depending on the cell type infected. All four cell types (SC, PTC, TM, and PM) respond to incubation with
UPEC, but not to incubation with NPEC, with elevated TLR4 protein levels. In PTc and PM, downstream signaling after UPEC exposure is initiated as the MAP kinases p38 and JNK are activated (Table III). These two kinases normally trigger transcription of proinflammatory genes, including IL-1, IL-6 and TNF-α, by activation of the nuclear transcription factors AP1 and NFAT. Stimulation of the MyD88 pathway in PTc and PM culminates in degradation of IkBα with subsequent transfer of the p65 unit of NF-κB to the nucleus. Despite activation of both MAP kinases and NF-κB, the transcription of proinflammatory genes is blocked. In SC and TM the MAP kinases are activated, but not the NF-κB pathway, thus supporting the observation that the lack of proinflammatory cytokine secretion is not related to the lack of pathogen recognition but more likely to virulence factors that block downstream transmission of proinflammatory signals. It seems that in TM and SC, NF-κB activation is already blocked before or at the point of activation of IkBα kinases. In PTc and PM, however, the MyD88/NF-κB-dependent cytokine production must be abrogated at the start of gene transcription or at the posttranscriptional level (Table III).

Further support for an UPEC-induced, cytokine-suppressing mechanism in the testis is derived indirectly from our observation that coinoculation of PM with UPEC fully inhibited LPS- or NPEC-induced production of IL-1α, IL-6, and TNF-α. Similarly, IL-6 synthesis was found abrogated by UPEC strain UTI89 in bladder epithelial cells (35) with inhibition of the classical NF-κB pathway, albeit without evidence showing at which level this may occur (36). From comparative genomic analysis, virulence factors of UPEC are known to be encoded on pathogenicity islands. Several of the identified genes were found to be involved in the pathogen’s fitness and adaptability (37). It can be speculated that a variety of these virulence-associated genes are candidates for blocking MyD88-dependent signaling as detected in our study. Differently from PM, all testicular cells were unresponsive to both LPS and NPEC challenge in terms of IL-1, IL-6, and TNF-α secretion; however, we could detect up-regulation of MCP-1, TGF-β, and cyclooxygenase-2 at least in TM (data not shown). In contrast, Okuma at al (38), reported an LPS-dependent increase of TGF-β and cyclooxygenase-2 in human keratinocytes (H929). Although this discrepancy can be attributed to differences in the experimental approaches, namely a much higher concentration of LPS (125 μg/ml vs 10 μg/ml in our study) was applied for longer periods of time (48 vs 3 h) in addition to the use of adult SC compared with immature SC in our study. We note, however, that in our experiments even prolonged incubation up to 24 h did not induce p65 translocation to the nucleus (S. Bhushan, unpublished observations). Recent analysis of TLR4 signaling demonstrate that LPS can induce a similar response as seen by stimulating TLR3, which senses double-stranded viral RNA, by producing type I IFN such as IFN-α, IFN-β, and IFN-response genes like MCP-1, IFN-γ-inducible protein 10 (IP-10), and RANTES (39). This so-called MyD88-independent pathway involves another adapter molecule, TOLL/IL-1R domain-containing adapter inducing IFN-β (TRIF), which activates the TRIF-related adapter molecule (TRAM), causing phosphorylation of IRF-3 in infected cells. After transfer to the nucleus, phosphorylated IRF-3 induces an antiviral and apoptotic response in various cells that is characterized by the production of endogenous type I IFNs and chemokines (40–45). This shows that upon initial triggering by a pathogen, antiviral and antibacterial pathways can merge by using common adapter molecules. In fact, infection of rat testicular somatic cells with Sendai virus, closely related to the mumps virus causing orchitis in humans, resulted in elevated levels of MCP-1, IP-10, and IFN-α mRNA (46, 47), a response very similar to that after UPEC challenge in this study. In the process of investigating a potential activation of the TLR4-TRIF/IRF-3 pathway, we found MyD88-independent nuclear translocation of IRF-3 in all testicular cell types after treatment with UPEC, but not with NPEC. Furthermore, up-regulation of IP-10, RANTES, and MCP-1 in PTc as well as that of IFN-α or -β and IP-10 in TM and SC clearly indicated that UPEC infection fully activated the TRAM-TRIF-IRF-3 alternative pathway, resulting in transcription of these genes. This up-regulated synthesis of chemokines may provide an explanation for the observed influx of leukocytes in bacterial orchitis (48).

In summary, UPEC-dependent inhibition of the NF-κB pathway suppresses production of proinflammatory cytokines in the testis. However, subsequent inflammation is regulated by effective activation of the MyD88-independent pathway (Table III). In light of the immune-privileged status of the testis, this is the first analysis providing insight that cytokine secretion is abrogated at different stages in the signaling pathways in different cellular populations of the testis.

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Disclosures
We have no financial conflict of interest.

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