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Sertoli Cells Initiate Testicular Innate Immune Responses through TLR Activation[1]

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TLRs play a crucial role in early host defense against invading pathogens. In the seminiferous epithelium, Sertoli cells are the somatic nurse cells that mechanically segregate germ cell autoantigens by means of the blood-tubular barrier and create a microenvironment that protects germ cells from both interstitial and ascending invading pathogens. The objective of this study was to examine TLR expression and their functional responses to specific agonists in mouse Sertoli cells. We measured the expression of TLR2, TLR4, TLR5, and TLR6 mRNAs and confirmed by FACS analysis the presence of proteins TLR2 and TLR5 on which we focused our study. Stimulation of Sertoli cells with macrophage-activating lipopeptide-2, agonist of TLR2/TLR6, and with flagellin, agonist of TLR5, induces augmented secretion of the chemokine MCP-1. To assess the functional significance of MCP-1 production following TLR stimulation, conditioned medium from either macrophage-activating lipopeptide-2 or flagellin-treated Sertoli cells was tested for in vitro chemotaxis assay, and a significant increase of macrophage migration was observed in comparison with unstimulated conditioned medium. Moreover, we studied the role of NF-κB and of MAPKs in regulating TLR-mediated MCP-1 secretion by using inhibitors specific for each transduction pathway and we demonstrated a pivotal role of the IκB/NF-κB and JNK systems. In addition, TLR2/TLR6 and TLR5 stimulation induces increased ICAM-1 expression in Sertoli cells. Collectively, this study demonstrates the novel ability of Sertoli cells to potentially respond to a wide variety of bacteria through TLR stimulation. *The Journal of Immunology,* **2006, 177: 7122–7130.**

The mammalian testis is composed of two main compartments: the interstitium, which contains the Leydig cells, responsible for testosterone production, macrophages, fibroblasts, blood and lymphatic vessels; and the seminiferous tubules, which are bordered by the peritubular smooth muscle cells (PSMC)[4] and composed of the various generations of differentiating germ cells that are associated to the somatic Sertoli cells (1). Obviously, the protection of germ cells from infections in the seminiferous tubules is of primary importance, because bacterial infections can seriously damage reproductive functions (2). A source of testicular infection is represented by some bacterial species, which are able to induce sexually transmitted diseases: *Neisseria gonorrheae*, *Candida albicans*, and *Ureaplasma urealyticum* (3). Several microbial pathogens are able to invade and colonize reproductive tract and semen. However, the testicular defense to infections, particularly to bacteria, is poorly defined at the molecular level. Innate immunity should play a crucial role against bacterial invasion of the urethra, epididymis, and testis to protect and preserve spermatozoa. Anatomically, the epididymis is the continuation of the urethra, exposing the testis to a permanent risk of microbial ascending infections.

Two barriers may be fundamental for the protection of both spermatogenesis and androgen production. The first line of testicular defense against pathogens from the bloodstream is represented by the testicular macrophages and Leydig cells (4, 5). In fact, the latter cell type is responsible for recruitment and control of the testicular macrophage population producing different proinflammatory mediators (6). The second line of defense involves the PSMC, lining the seminiferous tubules and Sertoli cells, all of which have been shown to express high levels of IFN-$\alpha\beta$ in response to viral infection (7). Moreover, Sertoli and PMSC constitutively express the antiviral protein PKR, and this expression is stimulated by IFN-$\alpha\beta$ and IFN-γ (8).

β-defensins are small cationic peptides possessing broad antimicrobial activities. The existence of multiple β-defensin isoforms in the male reproductive tract of humans, rats, mice and dogs has long been demonstrated (3, 9–12). The canine β-defensin, expressed by Sertoli cells, could be the primary endogenous antibiotic line of defense against bacterial invasion in testis and other epithelial surfaces (i.e., lung and small intestine) (3).

Other important mediators of microbial recognition are TLRs. TLRs recognize conserved pathogen-associated molecular patterns and structural subunits, including microbial cell wall (e.g., peptidoglycan), cell membrane (e.g., LPS), and virulence proteins (e.g., flagellin) (13), that are absent in the host. Recognition of pathogen-associated molecular patterns by TLRs ensures that pathogens with these conserved structures rarely escape surveillance by the innate immune system (14). Following activation, TLRs trigger inflammatory responses and dendritic cell maturation, which result in the eradication of invading pathogens. Maturation of dendritic cells is essential for the induction of pathogen-specific adaptive immune responses, thus indicating that TLRs link innate and adaptive

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[4] Abbreviations used in this paper: PSMC, peritubular smooth muscle cell; TIR, TLR/IL-1; SCCM, Sertoli cell conditioned medium; β-gal, β-galactosidase; CTR, untreated; MALP-2, macrophage-activating lipopeptide-2.

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immunity (15). TLRs are expressed on effector cells, including T and B lymphocytes, dendritic cells, macrophages, and epithelial cells. To date, 10 members of TLRs have been identified in human and 13 in mice (16). Genetic studies have revealed the specific ligands of each TLR. TLR2, in concert with TLR1 or TLR6, recognizes various bacterial components (peptidoglycan, lipopeptide, lipoprotein of Gram-positive bacteria and mycoplasma lipopeptide) (17, 18). In particular, TLR2/1 and TLR2/6 discriminate respectively triacyl lipopeptide and diacyl lipopeptide (19). TLR3 recognizes dsRNA that is produced by many viruses during replication. TLR4, in association with a coreceptor CD14 and the extracellular molecule MD-2, recognizes LPS, the major component of the outer membrane of Gram-negative bacteria (20, 21). TLR5 recognizes a highly conserved structure specific to bacterial flagellin (22). TLRs, after binding with their specific ligands, trigger a common signal transduction pathway involving cytoplasmic TLR/IL-1 (TIR) domains required for starting intracellular signaling (23). TLRs interact through their TIR domains with cytosolic adaptors also containing TIR domains such as MyD88, TIR domain-containing adaptor protein (TIRAP/Mal), TIR domain-containing adaptor inducing IFN-β (Trif), and Trif-related adaptor molecule (TRAM) (16). Interaction between TLRs and TIR domains leads, through a cascade of events, to NF-κB and MAPK activation, ultimately resulting in the induction of inflammatory cytokines.

Several papers have investigated TLR expression on epithelial cells, in particular on mucosal surfaces that are in contact with an environment rich in microorganisms. In fact, the incidence of infection is low despite the abundance of environmental microorganisms, probably because mucosal host defense mechanisms create a hostile environment for potential pathogens. One of the best studied sites in this regard is the gastrointestinal mucosa. It has been demonstrated that intestinal epithelial cells express TLR1–TLR4, TLR6, and TLR9, and that gastric epithelial cells express TLR2, TLR4, and TLR5 (24–27). Within the human female reproductive tract, vaginal and cervical epithelial cell lines express TLR1–TLR6, but lack TLR4, while primary endocervical cells express TLR1, TLR2, TLR3, and TLR6 (28). Moreover, as for upper reproductive tract, the expression of TLR1–TLR9 was demonstrated in primary uterine epithelial cell line (29). Interestingly, different immunologically relevant molecules are produced upon stimulation of TLRs according to the cell type. Altogether, these data can lead to the hypothesis that nonsterile sites (i.e., mouth, colon, or vagina) would require a response system different from that of sterile sites (bladder, kidney, or fallopian tubes, testis). It is conceivable that at nonsterile sites epithelial cells display a less efficient reactivity than at sterile sites where the appearance of even a low load of microorganisms would be deleterious, and therefore should be rapidly detected and eliminated. The pattern of expression of TLRs would then differ at sterile vs nonsterile sites (30).

In testis, Sertoli cells play a key role in the physiology of spermatogenesis because they supply a metabolic aid to developing germ cells and play a crucial role in the regulation of testicular immune privilege as responsible for the integrity of the blood-testis barrier, that physically segregates the adluminal compartment containing immunogenic germ cells (31, 32). Further on, they respond to stimulation with TNF-α activating MAPKs and NF-κB, resulting in the induction of the adhesion molecules ICAM-1 and VCAM-1, of the apoptotic molecule Fas and increasing the secretion of IL-6 (33–36). Hence, Sertoli cells possess the machinery necessary to start an inflammatory response to microorganisms. We have previously demonstrated (37) that Sertoli cells respond not only to inflammatory cytokines, but also to LPS by up-regulating the adhesion molecule ICAM-1 and the secretion of IL-6. In view of the light shed by the recent identification of TLRs, and considering the protective function of Sertoli cells toward germ cells, we sought to investigate whether Sertoli cells express TLRs and which responses are induced by their activation, in terms of secretion of cytokines, chemokines and/or immunological surface adhesion molecules. Our study shows the novel ability of testicular somatic cells to affect inflammation and to prevent infection.

Materials and Methods

Materials

DNase, collagenase, and recombinant murine TNF-α were purchased from Roche Biochemicals; trypsin was from Difco; U0126, SB203580, and lactacystin were from Calbiochem, SP600125 was from Tocris Bioscience. Flagellin, macrophage-activating lipopeptide (MALP), and LPS were from Apotech and InvivoGen. Cell viability after exposure to all the inhibitors tested was checked by the trypan blue dye exclusion test and was found to be not affected even at the highest concentrations used.

Animals

The animals used were 15-day-old CD1 mice (Charles River Laboratories). Animals were kept in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and were killed by CO_2 asphyxia.

Seminiferous tubule isolation and Sertoli cell cultures

Seminiferous tubules from prepuberal and adult mice were prepared, after removal of tonaca albuginea, by a digestion with HBSS supplemented with 0.1% collagenase to remove interstitial tissue. To avoid interstitial contaminations, the tubules were isolated under Wild M3C microscope (Leica) before RNA extraction.

Sertoli cells were prepared from CD1 mice according to Refs. 38 and 39. Purity of Sertoli cell cultures was ~96%, and the cultures were routinely checked for possible contamination by macrophages and PSMC (40).

At the fourth day of culture, Sertoli cell monolayers were treated for different times with various TLR agonists, with or without pretreatment with the various drugs to be tested.

RT-PCR and real-time PCR analyses

Total RNA was extracted using TRIzol reagent (Invitrogen Life Technologies). Samples were processed following the manufacturer's protocol.

First-strand complementary DNA was made in 20 μl of reverse transcriptase buffer (Invitrogen Life Technologies) using 3 μg of total RNA in the presence of SuperscriptII (50 U), random primers (250 ng), and $MgCl_2$ (25 mM) (Invitrogen Life Technologies). The reaction quality was checked by PCR with specific primers for β-actin mRNA amplification. Two microliters of cDNA from the reverse transcription reaction were used as a template for the PCR. Each 50-μl PCR mixture also contained 25 pmol specific primers, nucleosides 5′-triphosphate (0.2 mM) each, $MgCl_2$ (1.5 mM), 10× PCR buffer (5 μl), and 2.5 U of TaqDNA polymerase (Invitrogen Life Technologies). PCR products were amplified using the primers as reported previously (41). Conditions for amplification were 94°C for 45 s, 58°C for 45 s, and 72°C for 40 s for 35 cycles in the T1 Thermocycler (Whatman Biometra).

For real-time PCR analysis, 1 μg of total cDNA was used for each real-time reaction; analysis were performed in triplicate for each sample. cDNA was mixed with 0.5 μM of both forward and reverse primers and 10 μl of 2× Master mix (DyNAmo HS SYBR Green qPCR kit; Finnzymes) to a final reaction volume of 20 μl. Reactions were performed on Opticon2 DNA Engine (MJ Research) with a hot start step of 95°C for 15 min followed by 40 amplification cycles (95°C, 10 s; 58°C, 20 s; and 72°C, 30 s), by a melt step from 65 to 95°C and by a final 72°C extension for 10 min. Amplification efficiency for each primers pair was determined by amplification of a linear standard curve (from 0.16 to 100 ng) of total cDNA assessed by UV spectrophotometry. The standard curves showed good linearity and amplification efficiency (98–99%) for each primers pair of both experimental (TLRs) genes and reference (β-actin) gene. As negative controls, each sample was previously run with β-actin primers without reverse transcription to detect genomic DNA contamination; moreover, blank controls were assayed in each reaction and for each primer pair to detect DNA contamination of the reagents. Experimental samples were compared with a gene specific standard curve to determine the amount of specific cDNA present in the standard reaction. Standards were prepared

from highly purified PCR products amplified from positive controls; concentration and purity of the standards was assessed by A_{260}/A_{280} spectrophotometric measurement. The standard curve ranged linearly from 5 attograms (5×10^{-18}) to 5 picograms (5×10^{-12}). Total cDNA levels were standardized by normalizing to the β-actin control and presented as the total levels of cDNA detected (femtograms of specific cDNA/μg total retrotranscribed mRNA). Additionally, data are presented as $n°$ of fold increase (ratio of the experimental gene value/actin gene value) vs the control sample.

Western immunoblotting

Total Sertoli cells lysates were prepared by lysing and scraping the cells off the culture plate with cell lysis buffer (New England Biolabs) containing 1 μg/ml leupeptin and 1 mM PMSF (Sigma-Aldrich).

Protein concentration was determined by using the micro bicinchonic acid method (Pierce). Equal amounts of proteins (15 μg) were subject to SDS-PAGE and then transferred onto nitrocellulose. The filters were saturated with 5% nonfat dry milk in Tris-buffered saline. Rabbit polyclonal Abs against IκB-α and IκB-β were from Santa Cruz Biotechnology. Phosphospecific anti-p38, phosphospecific anti-ERK, and phosphospecific IκBα were purchased from New England Biolabs. Phosphospecific anti-p65 and phosphospecific anti-JNK were from BioSource International; Ab against α-tubulin was from Sigma-Aldrich. The secondary Abs were HRP conjugates (Zymed Bio-Rad). After incubating with the first and secondary Abs, the membranes were washed three times for 15 min with TBS containing 0.1% Tween 20. Ab detection was performed by using the chemiluminescence system (ECL Advance Western blotting detection kit; Amersham Biosciences).

Flow cytometry

Control and treated Sertoli cells were detached with 0.02% EDTA and washed with cold PBS plus 1% BSA. For detection of ICAM-1 expression on Sertoli cell surface, we used the hamster IgG anti-ICAM-1 mAb (BD Biosciences). Specific mAb or the appropriate isotypic control mAb were used at 0.5 μg/10^6 cells for 30 min on ice. For detection of TLR2 and TLR5, cells were fixed and permeabilized using the Cytofix/Cytoperm Kit (BD Biosciences) according to the manufacturer's instructions.

Either mouse anti-TLR2 (eBioscience) and anti-TLR5 (Imgenex) mAbs or the appropriate isotypic control mAb were used at 0.5 μg/10^6 cells for 30 min on ice. Cells were gated using forward vs side scatter to exclude dead cells and debris. After washing, cells were analyzed with a Coulter Epics XL flow cytometer (Beckman Coulter). Fluorescence of 10^4 cells per sample was acquired in logarithmic mode for visual inspection of the distributions and in linear mode for quantifying the expression of the relevant molecules by calculating the mean fluorescence intensity.

Luciferase assay

NF-κB luciferase reporter vector (pNF-κB-Luc) was obtained from Stratagene. Sertoli cells were cotransfected by means of LipofectAMINE plus reagent (Invitrogen Life Technologies) with pNF-κB-Luc and a β-galactosidase (β-gal) vector to normalize for transfection efficiency. Transfection was stopped after 5 h. After 18 h, cells were rinsed with fresh medium before stimulation with 100 ng/ml flagellin and 10 ng/ml MALP-2 for 6 h. Cells were lysed 48 h posttransfection using reporter lysis buffer (Promega). Luciferase activity was assayed with a Berthold luminometer using a luciferase assay kit (Promega) according to the manufacturer's instructions. Transfection efficiency was evaluated by 5-bromo-4-chloro-3-indolyl β-D-galactoside staining for each experiment and was always >30%. Data were normalized to protein concentration. As a positive control, a p65 vector was cotransfected with pNF-κB-Luc.

Cytokine secretion

Sertoli cell conditioned medium (SCCM) from untreated cells (CTR) and from cells treated with Flagellin or MALP-2 for 24 h were assayed using CBA mouse inflammation kit and MCP-1 Flex Set (BD Biosciences) according to the manufacturer's instructions. Beads were analyzed with a Coulter Epics XL flow cytometer (Beckman Coulter).

Chemotaxis

A murine macrophage cell line Raw 264.7 (a gift from Dr. S. Barca, Italian National Institute of Health, Istituto Superiore di Sanità, Rome, Italy), was used in in vitro transmigration experiments. Briefly, cells were assayed for their ability to migrate through a polyvinyl pyrrolidone filter with a pore diameter of 8 μM (Whatman) using Boyden Chambers (Neutroprobe). A total of 200,000 cells per well was added to the upper compartment of the chamber, and SCCM was placed in the lower part. The assembled chambers were incubated in a humidified incubator at 37°C with 5% CO_2 for 16 h for the analysis of Raw migration. Nonmigrated cells were removed from the filter, and the filter was then fixed with 90% ethanol and stained with Coomassie blue. The membranes were mounted on microscope slides, and five random microscopic fields from the bottom surface of each filter were recorded with AxioCam Zeiss. The cells on recorded fields were analyzed and counted using MetaMorph software (Molecular Devices).

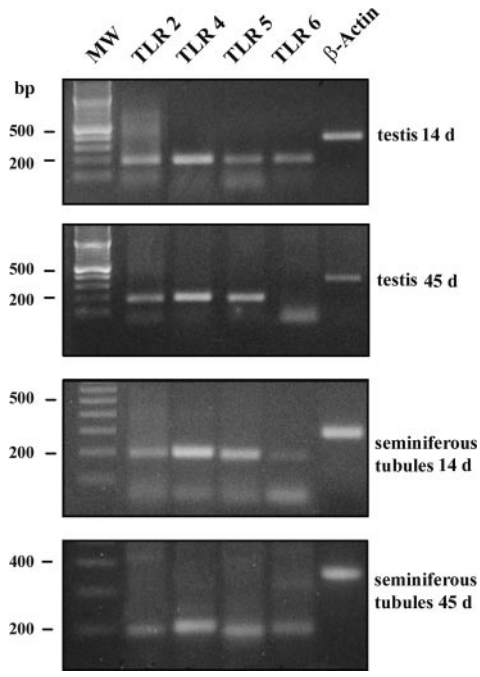

FIGURE 1. TLRs mRNA expression in murine seminiferous tubules and testis. Real-time PCR for TLR2 (196 bp), TLR4 (200 bp), TLR5 (192 bp), and TLR6 (189 bp) on whole testis and on seminiferous tubules from prepuberal (14 days old) and adult mice (45 days old). Electrophoresis referred to 35 PCR cycles for TLRs and 22 PCR cycles for β-actin are shown.

Statistical analysis

The data are presented as the mean ± SEM. Statistical evaluation was performed according to ANOVA or two-tailed paired t test. The p values were calculated using ANOVA with Bonferroni's posttest for analysis of real-time PCR data. Values of $p < 0.05$ were considered statistically significant.

Results

TLR mRNA expression in mouse testes and in seminiferous tubules

The testicular expression of TLRs, recognized by a broad variety of bacteria, was first analyzed at the mRNA level on extracts from whole testes obtained from prepuberal (14 days of age) and adult (45 days of age) mice. By using RT-PCR, we observed that TLR2, TLR4, TLR5, and TLR 6 are present in prepuberal testis; similar expression was revealed in adult testes with the exception of TLR6, which is not expressed (Fig. 1).

Testicular interstitium is composed mainly by Leydig cells, fibroblasts, and macrophages, whereas seminiferous tubules contain myoid cells, Sertoli cells, and differentiating germ cells. To investigate which cell types express TLRs, seminiferous tubules free of interstitial contaminants were obtained.

Fig. 1 shows that seminiferous tubules express TLR2, TLR4, TLR5, and TLR6 mRNA. The pattern of expression of various TLRs was similar in seminiferous tubules of prepuberal and adult mice. The presence of TLR6 signal in adult tubules, but not in

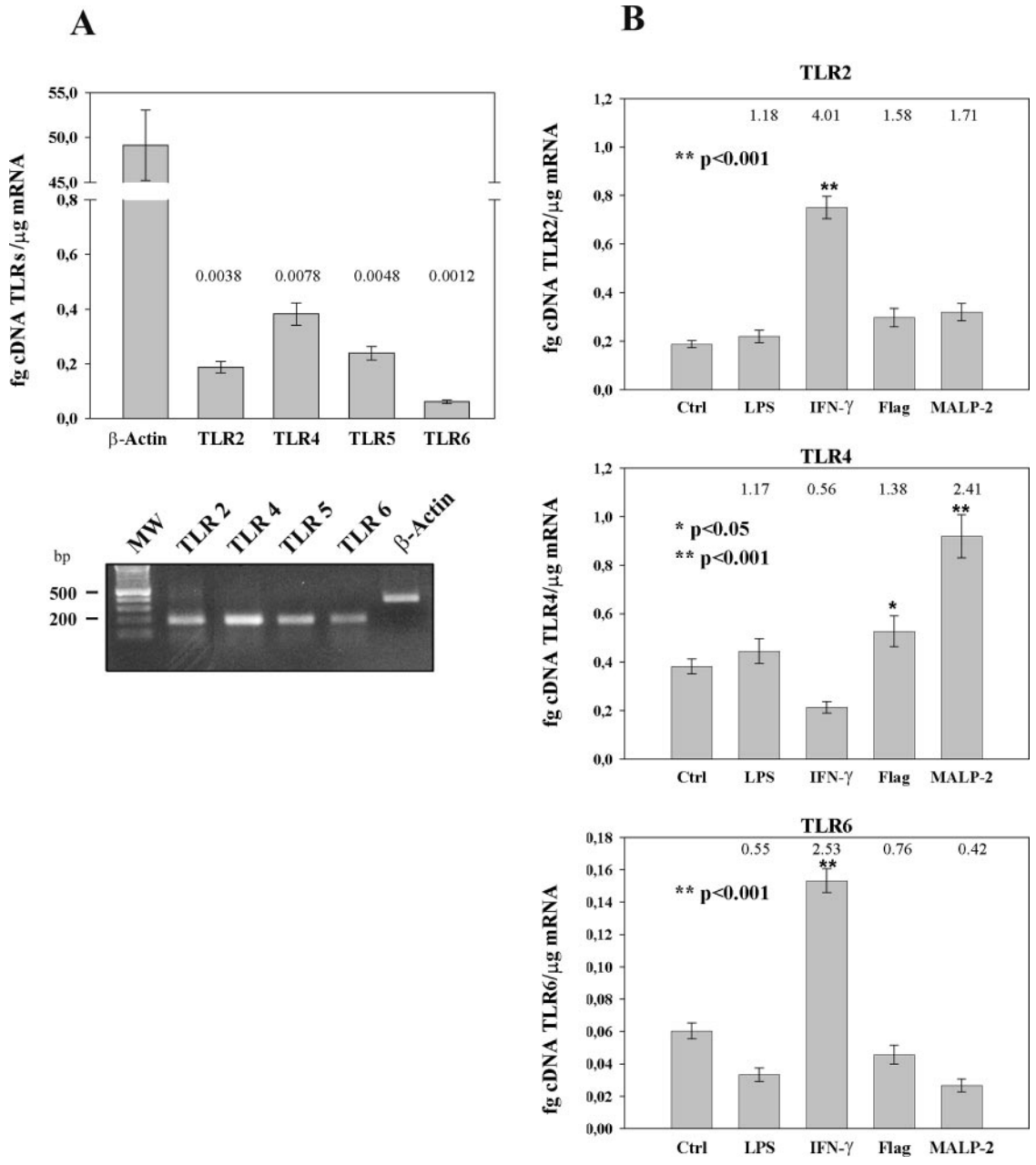

FIGURE 2. Expression of TLR mRNAs in Sertoli cells in basal condition and following IFN-γ stimulation and TLR ligation. *A*, TLR expression in Sertoli cell cultures by real-time PCR. Basal expression (control medium), of indicated TLR was measured by real-time PCR in femtograms (fg), and quantities were normalized vs housekeeping gene (β-actin). *Lower panel*, Electrophoresis referred to 35 PCR cycles for TLRs and 22 PCR cycles for β-actin are shown. *B*, Sertoli cell cultures were stimulated for 24 h with IFN-γ or TLR agonists, as indicated. Then, the cells were lysed, RNA was isolated and quantitative real-time PCR was conducted using specific primers. Asterisks, Significant increases in expression compared with the unstimulated cells. The numbers above the bar graphs represent the fold increase/decrease in TLRs expression compared with the unstimulated Sertoli cells. These data are representative of at least three separate experiments.

adult testis, suggests that TLR6, although at minimal level, is expressed exclusively in the tubules and not in the interstitium. Moreover, the expression of tubular TLR6 both in immature and in mature animals, differing in germ cell composition suggests the possible involvement of tubular somatic cells.

Expression and regulation of TLRs in mouse Sertoli cells

Since the Sertoli cell has been described as "the sentinel" cell capable both of silencing and inducing testicular immune response, we studied the expression pattern of TLRs in purified mouse Sertoli cells, to determine the potential role of these barrier cells in mediating innate immune response. Quantitative real-time PCR and semiquantitative RT-PCR (Fig. 2*A*) show that TLR2, TLR4, TLR5, and TLR6 are expressed. TLR expression is known to be regulated by the proinflammatory cytokine IFN-γ and by TLR agonists themselves (41). We then analyzed TLR mRNA levels following stimulation for 24 h with IFN-γ and with the agonists of TLRs: MALP-2 (TLR2/6), LPS (TLR4), and flagellin (TLR5). RNA was extracted and analyzed by quantitative real-time PCR. Stimulation levels were determined by comparison with expression in unstimulated Sertoli cells. Results shown in Fig. 2*B* demonstrate that IFN-γ up-regulates TLR2 and TLR6, and that MALP-2 and flagellin increase TLR4, whereas none of the treatments affect TLR5 expression (data not shown).

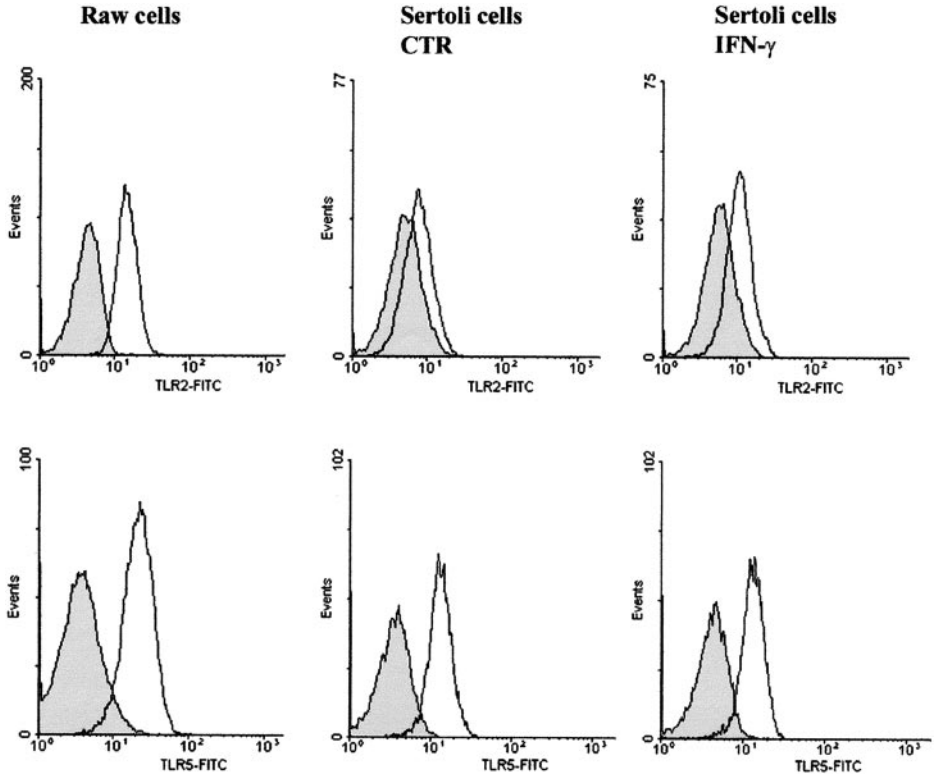

FIGURE 3. FACS analysis of TLR2 and TLR5 expression on mouse Sertoli cells. Flow cytometric analysis was performed on Sertoli cells, untreated (CTR) or stimulated with 500 U/ml IFN-γ for 24 h, then were fixed and permeabilized as described in *Materials and Methods*. The gray area represents the isotypic control Ab fluorescence. Raw 246.7 cells were used as positive control. The diagrams are representative of at least three independent experiments.

Then, we investigated in mouse Sertoli cells the presence, at the protein level, of TLR2, which is reported to be associated with TLR6 (42), and of TLR5. Flow cytometric analysis, performed with polyclonal-specific Abs, shows (Fig. 3) that Sertoli cells express both TLR2 and TLR5, with levels of TLR5 comparable to that of the positive control RAW cell line. Moreover, IFN-γ significantly increases TLR2 protein expression, whereas it fails to affect TLR5 cell surface expression (Fig. 3), according to the data obtained by real-time PCR. TLR6 was not detected (data not shown), as expected, given the very low expression in real-time PCR.

Flagellin and MALP-2 activate MAPKs and NF-κB in Sertoli cells

Currently, the signaling pathways activated by different TLRs appear to use identical components (43, 44). To study whether specific TLR2/6 and TLR5 agonists trigger signal transduction pathways in Sertoli cells, we stimulated cell cultures for different times with MALP-2, that activates TLR2 in association with TLR6 (42), and with flagellin, TLR5 agonist. Whole lysates were subjected to Western blot analysis to study MAPK activation and IκB degradation. Fig. 4 shows that 100 ng/ml flagellin and 10 ng/ml MALP-2 activate MAPKs and induce the degradation of both IκBα and IκBβ. Kinetics of activation of each MAPK is similar: JNK, p38, and ERK phosphorylation peaks at 30′ and decreases after 1 h stimulation. IκBα degradation starts at 15′ and is completed within 30 min, whereas IκBβ degradation has different kinetic with the two agonists. In flagellin-treated Sertoli cells, as expected, IκBβ is degraded slightly later, starting at 30′, and remains degraded still after 1 h, whereas IκBα is resynthesized after 1 h. It is, in fact, well known that NF-κB activation can up-regulate IκBα gene expression, inducing its rapid resynthesis, whereas the IκBβ gene is not regulated by NF-κB (45). On the contrary, in MALP-2-treated Sertoli cells, IκBβ degradation is earlier than IκBα degradation.

Moreover, both MALP-2 and flagellin induce also p65 phosphorylation, which represents an NF-κB activation mechanism independent from IκBα degradation (Ref. 46 and Fig. 4).

To verify NF-κB activation induced by MALP-2 and flagellin, Sertoli cells were transfected with a pNF-κB-Luc and then stimulated for 6 h with either MALP-2 or flagellin. Cell lysates

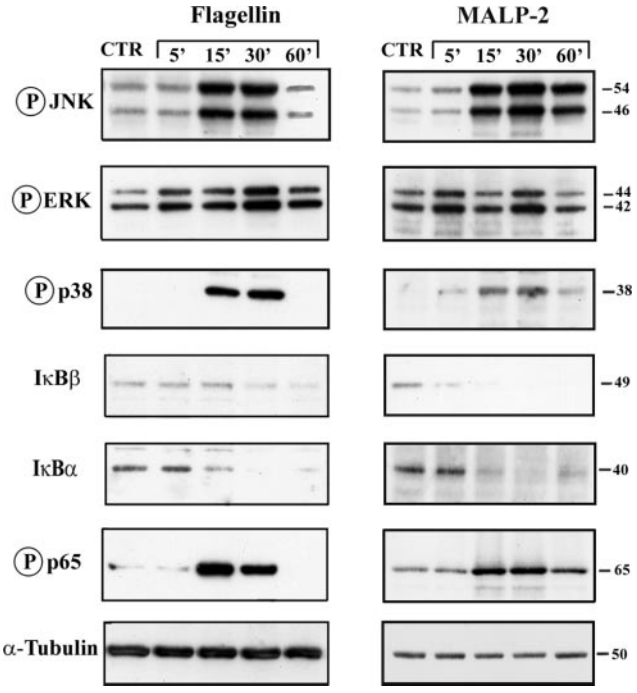

FIGURE 4. Flagellin and MALP-2 activate MAPKs and induce IκBα/IκBβ degradation. Sertoli cells were stimulated with 100 ng/ml flagellin (*A*) and 10 ng/ml MALP-2 (*B*) for different times. Whole-cell lysates (15 μg) were subjected to Western blot analysis using polyclonal Abs against the phosphorylated forms of JNK, ERK, p38, p65, and against IκBα and IκBβ. The same filter was reincubated with an anti-α-tubulin Ab as control of equal amount of protein loaded. This Western blot is representative of three independent experiments.

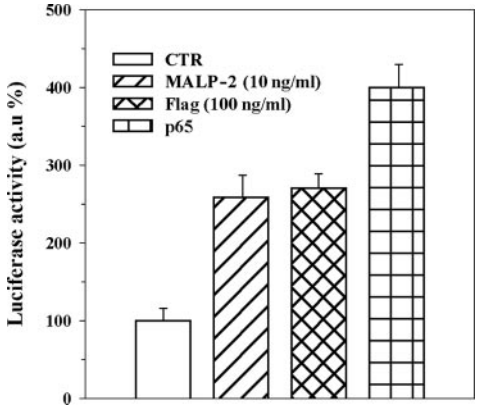

FIGURE 5. Flagellin and MALP-2 activate NF-κB. Sertoli cells were cotransfected with pNF-κB-Luc and β-gal. Cells were subsequently treated 24 h with 100 ng/ml flagellin or 10 ng/ml MALP-2. p65, Sertoli cells cotransfected with pNF-κB-Luc, β-gal, and a p65 vector as a positive control. Transfection efficiency was evaluated by 5-bromo-4-chloro-3-indolyl β-D-galactoside staining, and data were normalized to protein concentration. Each point represents the mean of triplicate samples of three experiments and is expressed as average ± SEM. Asterisk, Statistically significant difference according to ANOVA.

were assayed for luciferasic activity. Both MALP-2 and flagellin induce a significant NF-κB activation, as shown by the increase of luciferasic activity (Fig. 5).

Flagellin and MALP-2 induce ICAM-1 expression in Sertoli cells

After detecting the activation of signal transduction mediated by flagellin and MALP-2 treatments, we investigated whether these agonists were biologically functional. In particular, we analyzed their ability to induce up-regulation of cytokines, chemokines, and adhesion molecules involved in leukocyte transmigration through endothelium for the defense of seminiferous tubules from infections.

To study a possible effect on ICAM-1 expression, Sertoli cells were stimulated with MALP-2 or flagellin for 24 h, and surface expression of ICAM-1 was analyzed by flow cytometric analysis. Fig. 6 shows that both agonists up-regulate ICAM-1.

Up-regulation of MCP-1 secretion following TLR2/6 and TLR5 stimulation depends on JNK and NF-κB

We next evaluated the effect of MALP-2 and flagellin on the secretion of cytokines and chemokines by Sertoli cells. Cell cultures were left untreated or stimulated with either MALP-2 or flagellin for 24 h, and conditioned medium was collected and assayed for secreted MCP-1, IL12p70, IL-6, TNF-α, IFN-γ, and IL-10, using the CBA mouse inflammation kit by flow cytometry (see *Materials and Methods*).

MALP-2 and flagellin were ineffective in the induction of IL12p70, IL-6, TNF-α, IFN-γ, and IL-10 (data not shown), whereas both induced a strong induction of MCP-1 (Fig. 7).

After demonstrating that MAPKs and NF-κB are activated, we sought to investigate which transduction pathway might be involved in MCP-1 induction. To this aim, Sertoli cells were pretreated with specific MAPKs and NF-κB inhibitors for different times, according to the inhibitor used, and then stimulated with MALP-2 or flagellin. After 24 h, SCCM was assayed for MCP-1 levels. Results shown in Fig. 7, demonstrate that pretreatment with SB203580, inhibitor of p38 pathway, and with U0126, ERK inhibitor, induced a significant decrease in MCP-1 induction by flagellin, whereas they were almost ineffective in inhibiting

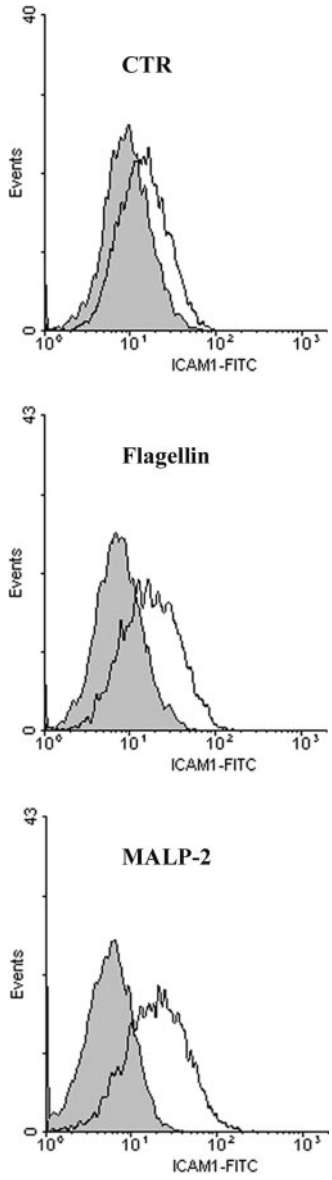

FIGURE 6. Flagellin and MALP-2 induce ICAM-1 expression. Flow cytometric analysis of ICAM-1 expression was performed on Sertoli cells, untreated (CTR) or stimulated with 100 ng/ml flagellin or 10 ng/ml MALP-2 for 24 h. Cells were labeled with FITC-conjugated hamster IgG anti-ICAM-1 mAb. The diagrams are representative of at least three independent experiments.

MCP-1 induction by MALP-2, indicating that p38 and ERKs are dispensable for MCP-1 induction by MALP-2 and are partially involved in flagellin-mediated MCP-1 induction (Fig. 7). To study the role of JNK and NF-κB, Sertoli cells were preincubated with the JNK inhibitor SP600125 and with MG-132, a proteasome inhibitor that inhibits IκBα degradation and subsequently NF-κB activation. Data presented in Fig. 7 show that SP600125 and MG-132 totally inhibit MCP-1 induction by both flagellin and MALP-2. Thus, we can conclude that MCP-1 induction by both agonists is regulated by JNK and NF-κB-mediated intracellular signals.

SCCM from MALP-2- and flagellin-treated Sertoli cells induces migration of mouse monocyte-macrophage RAW cell line

We performed experiments to assess the ability of MCP-1 secreted by Sertoli cells upon MALP-2 and flagellin stimulation to induce

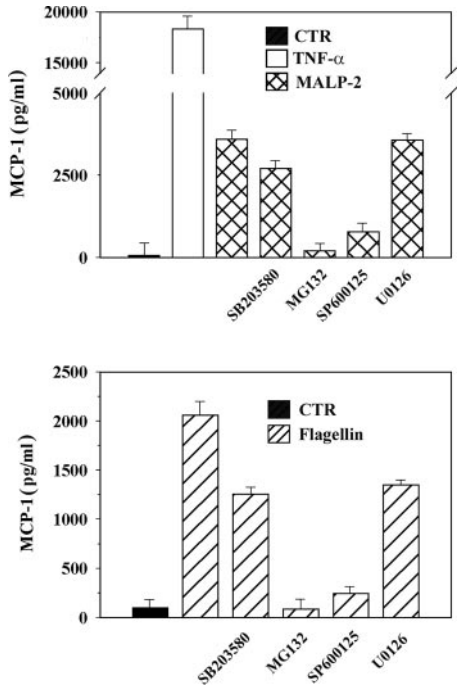

FIGURE 7. Effect of flagellin and MALP-2 on MCP-1 secretion and analysis of the transduction pathways involved. MCP-1 was assayed in SCCM collected after 24 h of flagellin (100 ng/ml) or MALP-2 (10 ng/ml) stimulation in absence or in presence of MAPK or NF-κB inhibitors. CTR is conditioned medium from untreated cells, and conditioned medium from cells stimulated with TNF-α (20 ng/ml) was used as positive control.

chemotactic migration of RAW cells across an 8-μm pore filter. SCCM previously assayed for MCP-1 levels (data not shown) was placed below the filter upon which Raw cells were plated. After 16 h, the cells adhering to the lower surface of the filter were counted in five random fields (see *Materials and Methods*). As shown in Fig. 8, SCCM from Sertoli cells treated with MALP-2 (containing the highest levels of MCP-1, see Fig. 7) induces maximal transmigration of RAW cells, compared with SCCM, without any pretreatment used as the control. Flagellin induces a lower, yet significant, effect on RAW cell transmigration. Enhanced secretion of MCP-1 by Sertoli cells, subsequent to TLR activation, is therefore paralleled by augmented migration of macrophages.

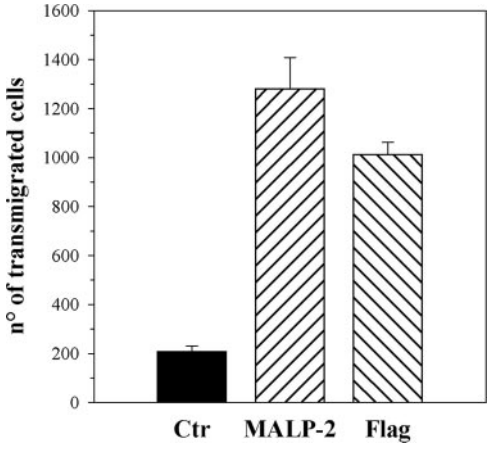

FIGURE 8. MCP-1 secreted by Sertoli cells induces RAW macrophage cell line chemotaxis. SCCMs from untreated cells or from cells stimulated with MALP-2 or flagellin for 24 h were collected. Raw cells were exposed to such SCCMs for 16 h. Chemotactic activity was assayed using Transwell cell culture chambers as described in *Materials and Methods*. Values are data from a representative experiment, which was replicated three times. Each assay was performed in triplicate, and the number of migrating cells in five random fields was counted for each membrane. Results are shown as number of migrated cells found on the lower surface of the filter. Each value represents the mean $\pm$ SEM; $n = 3$.

Discussion

The testis is considered to be an immunologically privileged site where both allo- and autoantigens can be tolerated. An unbalance of this condition can determine an autoimmune reaction, leading to a permanent disruption of spermatogenesis (47). In contrast, infections of the testis are usually overcome, indicating that an active and efficient immune response against pathogens can be locally generated. TLRs, recently discovered regulators of both the innate response to pathogens and the specific immune reaction, have so far not been adequately studied in the testis. In particular, no information is available about TLR expression in the seminiferous tubules.

We have analyzed the TLR expression pattern in the mouse prepuberal and adult testis by using semiquantitative RT-PCR and found that mRNA expression levels of each TLR are similar to those already reported in adult human testis (48), with main expression of TLR2 and TLR4 and lower levels of TLR5 and TLR6. However, our data are in contrast with a previous paper that reported the absence of TLR4 and TLR5 in the adult mouse testis by RT-PCR (49); this discrepancy can be explained considering that we performed PCR with a higher number of cycles (35 vs 30). Moreover, we used a different strain of mice, CD1 nonsyngenic mice, and it has been demonstrated that in nonsyngenic strains of mice, the expression of TLR mRNA molecules in various organs, such as ovary, is higher than in the syngenic strains (49).

In our experiments, we analyzed TLR expression in seminiferous tubules selectively isolated from the interstitial compartment which contains macrophages and blood vessels. We detected the presence of TLR2, TLR4, TLR5, and TLR6, which, among the TLRs, are those that mediate the innate immune response against all species of bacteria: Gram-negative (TLR4), Gram-positive (TLR2/TLR6) and flagellates (TLR5). The pattern of TLR expression in seminiferous tubules was found to be age independent, strongly suggesting that the cells equipped with these receptors are not germ cells, a population that differs with age in quantity and composition.

Sertoli cells, the only somatic cell type of the seminiferous epithelium, have been implicated in the maintenance of immune tolerance (31, 32). We demonstrate here that murine Sertoli cells express a panel of TLRs, such as TLR2, TLR4, TLR5, and TLR6, possibly involved in the bactericidal defense mechanisms of the testis. Treatments with IFN-γ or TLR agonists induce a significant increase of TLR2, TLR4, and TLR6 mRNA, analogous to data reported in mouse microglia (41). Moreover, the surface protein expression of TLR2 and TLR5 was detected in basal conditions and TLR2 protein was up-regulated by IFN-γ.

Following stimulation with proinflammatory cytokines Sertoli cells are able to express ICAM-1 and to bind lymphocytes as well as to secrete IL-6 (37), suggesting their role also in promoting inflammation in the testis. These cells might therefore play a master role, not only in the endocrine regulation of spermatogenesis, but also in modulating locally the activity of immune competent cells.

Our data show that flagellin and MALP-2, respectively agonists of TLR5 and TLR2/6, induce a strong up-regulation of Sertoli cell membrane ICAM-1, an adhesion molecule for lymphocyte ligation. ICAM-1 is typically expressed by endothelial cells, but is

occasionally inducible by inflammatory stimuli in a set of nonvascular cells (50). This molecule is considered responsible for adhesion to endothelial cells and migration of leukocytes from the bloodstream to inflamed tissues (51). These data suggest that Sertoli cells are able to respond not only to cytokines, but also directly to bacterial motifs by expressing a molecule that is strongly involved in lymphocyte recruitment.

Among the cytokines/chemokines tested, only MCP-1 secretion in conditioned medium was stimulated by both flagellin and MALP-2 treatment. MCP-1 is a potent chemokine synthesized by several cell types, e.g., inflammatory cells, such as monocytes, and various nonleukocytic cells, including endothelial cells and vascular smooth muscle cells (52). MCP-1 stimulation of monocytes results in their chemotaxis (53).

It has been demonstrated that MCP-1 is induced in rat Sertoli cells by inflammatory cytokines, whereas germ cells do not express it at all (54). Some authors demonstrated that, in the testicular fluid of rats with orchitis, there was a significant increase in MCP-1, compared with control group (55). In fact, testicular inflammation in vivo causes an influx of new monocytes and macrophages in the testis and chemokines are involved in leukocyte trafficking (6). In this paper, we demonstrate that flagellin and MALP-2 activate macrophage migration through membrane in Boyden chambers analogous to monocyte transmigration through endothelium, which primes host innate immune response. Moreover, higher MCP-1 levels detected in SCCM following treatment with MALP-2 correlate with a major number of cells transmigrated. Our results are in agreement with the data described in a previous paper showing an increase in the number of testicular macrophages stimulated by MCP-1 in an LPS inflammatory model (56).

To sum up, Sertoli cells respond to two TLR agonists with a strong up-regulation of chemokine MCP-1 secretion (innate response) and of ICAM-1 expression (adaptive response). These findings suggest a potential role of Sertoli cell in innate and adaptive immune response mediated by TLR specific activation.

The testis is to be considered a sterile site; hence, stimulation of TLRs is expected to induce a strong inflammatory response because even low loads of pathogens might be dangerous for the homeostasis of the organ. In contrast, a strong inflammatory response can also lead to tissue damage, which, in this case, would result in loss of spermatogenesis. TLR signals that initiate innate immune responses to pathogens must be tightly regulated, particularly in this organ, to prevent excessive inflammatory damage. It is then important to know the transduction pathways involved in the regulation of inflammatory responses. It is well known that both TLR2/6- and TLR5-activated pathways induce MAPK and NF-κB activation (16). Our data show that in Sertoli cells flagellin and MALP-2 activate JNK, ERK, and p38. Moreover, NF-κB is activated both by IκBα and IκBβ degradation. IκBα is involved in the early phase of NF-κB activation, whereas IκBβ is responsible for NF-κB delayed activation following flagellin stimulation. These data are in agreement with our previous data showing a similar pattern of NF-κB activation by TNF-α in Sertoli cells (36). We further demonstrate that flagellin and MALP-2 induce NF-κB activation also by inducing p65 subunit phosphorylation, thus suggesting the hypothesis that the inflammatory response activated by TLR agonists strongly involves NF-κB.

To gain insight into the transduction pathways involved in MCP-1 induction, we pretreated Sertoli cells with specific MAPK and NF-κB inhibitors before stimulation with MALP-2 or flagellin. Our data show that the up-regulation of MCP-1 induced by MALP-2 and flagellin is NF-κB and JNK pathway dependent, as the pretreatment with inhibitors of NF-κB and JNK completely abrogates the secretion of MCP-1. Nevertheless, in cells pretreated with SB203580 and U0126, respectively p38 and ERK inhibitors, before flagellin stimulation, we detected a significant decrease of MCP-1 ($\sim$50%). We can hypothesize that either p38 and ERK are directly involved in MCP-1 induction or indirectly through NF-κB activation. In fact, as demonstrated in recent papers, NF-κB activation can be partially dependent on p38 and/or ERK (57, 58).

The testis is an organ that is seldom reached by infective agents (via both systemic and ascendant routes); virtually all cases of spontaneous autoimmune orchitis are associated with an infectious disease. The host's immune response appears to play a key role in tissue destruction due to infections. Therefore, pathogens induce orchitis by disrupting the mechanisms of immune privilege in the testis (59). Sertoli cells are involved in the negative, as well as the positive, regulation of immune responses. In both phenomena, accessory cells and lymphocytes interact by means of two basic mechanisms: paracrine messages through cytokines and specific surface interactions (60). TLRs expression on Sertoli cells might represent an important mechanism for driving specific inflammatory responses to bacterial infections. Thus, the activation of TLRs might induce the switch from a tolerogenic phenotype toward immune cells to a bactericidal and inflammatory one.

Testicular inflammation is associated locally with disruption of spermatogenesis. After an infection, a dysregulated inflammatory response can lead to disruption of epithelial barriers, which, together with other mechanisms, contributes to the development of sepsis. The inflammatory molecules produced by Sertoli cells after TLRs activation, such as ICAM-1and MCP-1, might be involved in the pathogenesis of a septic stage, likely damaging the integrity of the blood-tubular barrier. Since it has been demonstrated that MyD88 is involved in the generation of systemic inflammatory response in a murine model (61), it might be interesting to study in vivo the role of different TLRs in the development of local and systemic long-term inflammation.

In conclusion, on the basis of our data, we feel that further detailed studies focused on immune function of the Sertoli cell will provide new insight to understand how Sertoli cell is poised to respond to infections activating different inflammatory pathways.

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Disclosures

The authors have no financial conflict of interest.

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