Expression Patterns and Functions of Toll-Like Receptors in Mouse Sertoli Cells

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Toll-like receptors (TLRs) play crucial roles in mediating innate and adaptive immunity. Sertoli cells create a microenvironment that protects seminiferous tubules from autoantigens and invading pathogens. Here we examined the expression and potential function of TLR family in mouse Sertoli cells. RT-PCR, Western blotting, and flow cytometry were used to analyze gene expression. Immunofluorescence staining was used to determine activation of nuclear factor-κB. ELISA was used to detect secreted cytokines in culture medium. The phagocytosis assay was performed by Oil Red O staining for lipid droplets. We demonstrated that TLR2, TLR3, TLR4, and TLR5 are highly expressed; TLR6, TLR7, and TLR13 are expressed at relatively low level; and TLR1, TLR8, TLR9, TLR11, and TLR12 are not detected in mouse Sertoli cells. We focused our study on the roles of TLR2-TLR5 in Sertoli cells. Our data indicated that TLR2-TLR5 can be activated by their ligands in mouse Sertoli cells and subsequently increase expression of the inflammatory cytokines IL-1α, IL-6, and interferon-α, and -β. The augmented expression of the cytokines might be induced by activation of nuclear factor-κB. Notably, activation of TLR3 by its ligand, poly(I:C), specifically promoted phagocytosis of apoptotic spermatogenic cells by Sertoli cells. The TLR-induced Sertoli cell phagocytosis was found to be associated with the up-regulation of scavenger receptors. The results suggest that TLRs expressed in mouse Sertoli cells may play roles in defense against invasion of allo- and autoantigens in the seminiferous tubules. (Endocrinology 149: 4402–4412, 2008)

Toll-like receptors (TLRs) play essential roles in activating signal transduction pathways leading to the killing and clearance of pathogens. To date, 10 distinct TLRs have now been identified in humans (10) and 13 in mice (11). TLRs recognize highly conserved, pathogen-coded molecular structures termed pathogen-associated molecular patterns (12). The respective ligands of most TLRs have been revealed (13). For example, TLR2, in association with TLR1 or TLR6, recognizes different bacterial components including peptidoglycan, lipopeptide, and lipoprotein (14, 15). TLR3 recognizes double-stranded RNA that is produced by many viruses during replication and also can be activated by synthetic double-stranded RNA analog, polyinosinic-polycytidylic acid [poly (I:C)] (16). TLR4 recognizes LPS, a major component of the outer membrane of Gram-negative bacteria (17, 18). TLR5 recognizes bacterial flagellin (19). TLR7 recognizes synthetic imidazoquinoline-like molecules, guanosine analogs, single-stranded RNA, and influenza virus (20, 21). TLR8 shows the highest homology to TLR7, whereas human TLR8 mediates the recognition of imidazoquinolines and single-stranded RNA; mouse TLR8 is thought to be nonfunctional (21). TLR9 recognizes bacterial and viral CpG DNA motifs and malaria pigment hemozoin (22, 23). TLR11 responds specifically to uropathogenic bacteria (24) and profilin-like molecule from the protozoan parasite infection (25). The ligands for TLR10, TLR12, and TLR13 have not been identified yet (26, 27).

TLRs are expressed by both immune cells, such as lymphocytes, dendritic cells and macrophages, and nonimmune cell types including epithelia cells of many tissues (28). The expression and function of TLRs on epithelia cells of various tissues, such as lung, kidney, small intestine, and the reproductive tracts, have been extensively investigated (24, 29, 30). More attention has been focused on mucosal surfaces that are
in contact with an environment rich in microorganisms. In fact, the incidence of infection is low in this site despite the abundance of environmental microorganisms partial owing to TLRs-mediated immune responses. It has been demonstrated that intestinal epithelial cells express TLR1-TLR4, TLR6, and TLR9 and that gastric epithelial cells express TLR2, TLR4, and TLR5 (31–33). Human vaginal and cervical epithelial cell lines express TLR1-TLR6. As for upper reproductive tract, primary uterine epithelial cells express TLR1-TLR9 (34). Different immune molecules are produced upon stimulation of TLRs according to cell type. These previous studies indicate that the expression and function of TLRs would differ at different tissues and cell types.

Various studies have identified expression of TLRs in testis. Adult human testis expresses TLR2 and TLR4 at high levels and TLR5 and TLR6 at lower levels (35), and rat testis expresses TLR1-TLR10 (30). A recent study reported that mouse Sertoli cells express TLR2, TLR4, TLR5, and TLR6 (36), which can be activated by their agonists in Sertoli cells and may initiate testicular innate immune responses by inducing augmented secretion of the chemokine monocyte chemotactic protein-1 and increased ICAM-1 expression. This previous study focused mainly on the role of the TLR2/TLR6 complex and TLR5 and their ability to stimulate the expression of monocyte chemotactic protein-1 and ICAM-1. All these previous reports suggest that Sertoli cells may play a role in modulating locally the activity of immune competent cells. However, TLR-mediated immunological roles of Sertoli cells in testis are poorly understood. In the current study, we further investigate the expression and function of TLRs in mouse Sertoli cells. We focused on TLR2-TLR5 and their ability to activate nuclear factor-κB (NF-κB) and induce inflammatory cytokines, especially Sertoli cell phagocytotic activity mediated by TLR3. This data expand previous understanding and provide novel insight into the function of TLRs in Sertoli cells.

Materials and Methods

Animals

Mice (C57 BL/6) were maintained and breeding in the animal facility of Beijing Union Medical College (Beijing, China). All the measures taken for the mice were in accordance with approved guidelines (Guide-line for the Care and Use of Laboratory Animals) established by the Chinese Council on Animal Care.

Isolation of Sertoli cells

The procedure for isolation of Sertoli cells was based on a previous description (37) with a modification. Briefly, 3-wk-old mice were anesthetized with CO2 and then killed by cervical dislocation. The peritoneal cavity cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum on culture dishes in a humidified atmosphere containing 5% CO2 at 37 °C. After 2 h, suspending cells were removed by washing with PBS, and the macrophages attached on dishes were collected for RNA extraction and flow cytometry analysis.

RT-PCR

Total RNA was isolated using TRIzol reagent according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA). The RNA was treated with ribonuclease-free deoxyribonuclease to remove potential contamination of genomic DNA. Total RNA (0.5 μg) was reverse transcribed into cDNA in 20 μl of reverse transcriptase reaction mixture containing 2.5 μM random hexamers, 2 mM deoxynucleotide triphosphates, and 200 U Muleoney murine leukemia virus reverse transcriptase (Promega, Madison, WI). PCRs (cycles of 94 °C for 30 sec, annealing at 52–62 °C for 30 sec, and extension at 72 °C for 1 min) were performed for a number of cycles corresponding to the high end of the range in which a linear increase in products could be detected. The β-actin gene was used as the control of equal amounts of cDNA in the PCRs. The PCR products were subjected to electrophoresis in 1% agarose gels, and densitometric quantification of the bands between target genes and β-actin gene were analyzed using YLN2000 gel analysis system (Yalien, Beijing, China). The primers for PCR were shown in Table 1. Real-time RT-PCR analysis was performed with Power SYBR Green PCR master mix kit (Applied Biosystems, Foster City, CA) using an ABI PRISM 7300 real-time cycler (Applied Biosystems). The mRNA levels of target genes were normalized to β-actin. The primers used in real-time PCR are listed in Table 2.

Flow cytometry

Sertoli cells and macrophages were detached with 0.02% EDTA and washed with cold PBS plus 1% BSA. The cells were fixed and permeabilized using the Cytofix/Cytoperm kit (BD Biosciences, San Jose, CA) according to the manufacturer’s instructions. For detection of TLR3 expression, rat antitumour TLR3 polyclonal antibody and isotopic control (eBioscience, San Diego, CA) were used at 1:500 for 30 min incubation on ice. After three washes with PBS, the cells were incubated with biotin-conjugated mouse antitag IgG (0.5 μg per 106 cells in 0.2 ml PBS; eBioscience) for 15 min on ice, followed by a 15-min incubation with 1 μg/ml allophycocyanin-conjugated streptavidin (eBioscience). For detection of TLR2, TLR4, and TLR5 expression, either fluorescein isothiocyanate (FITC)-conjugated antitumour TLR2 (eBioscience), phycoerythrin-conjugated antitumour TLR4 (eBioscience) and FITC-
conjugated antimonous TLR5 (Imgenex, San Diego, CA) monoclonal antibodies, or the appropriate isotypic controls were used at 0.5 µg per 10^6 cells for 30 min incubation on ice. After washing, cells were analyzed with a BD FACScanTO flow cytometer (BD Biosciences).

ELISA

The concentrations of IL-1α, IL-6, and interferon (IFN)-α/β secreted by Sertoli cells were determined using ELISA kits (Zhongshan Biotechnology Co., Beijing, China). The assays were performed according to manufacturer’s instructions. Briefly, Sertoli cells were cultured in 24-well plates at the density of 1 × 10^6 cells/well for 24 h. The cells were treated with inhibitors of TLR2–5, 50 µg/ml TLR5 antibody (Santa Cruz Biotechnology, Santa Cruz, CA; sc-30003) for 2 h. Then the cells were stimulated with agonists of TLR2–5 for additional 24 h. The supernatants were collected for measurement of the cytokines.

Immunofluorescence staining

Sertoli cells cultured on Lab-Tek chamber slides (Nunc, Naperville, IL) were fixed with cold methanol at −20 °C for 2 min. The cells were treated with 0.3% Triton X-100 in PBS for 15 min at room temperature to increase cellular permeability. After blocking by preincubation with 10% normal goat serum in PBS at room temperature for 30 min, rabbit polyclonal anti-Actin (Santa Cruz Biotechnology) were applied at a dilution of 1:200 to increase cellular permeability. After blocking by preincubation with PBS, the cells were incubated with the FITC-conjugated goat antirabbit antibodies (Santa Cruz Biotechnology) were used at 0.5 µg per 10^6 cells/well for 30 min incubation on ice. After washing, cells were analyzed in each assay. The assay was done in triplicate. The mean ratios of lipid droplets to nuclei of Sertoli cells were analyzed by image microscope (IX-71; Olympus). Negative control cells were incubated with preimmune rabbit serum instead of primary antibodies. The assays were repeated three times, and 150 cells were counted for each assay.

Phagocytosis assay

Phagocytosis of apoptotic spermatogenic cells and residual bodies by Sertoli cells results in formation of lipid droplets in the Sertoli cells. Therefore, the lipid droplets in Sertoli cells were detected by Oil Red O (ORO) staining and used as a criterion to evaluate phagocytic ability of Sertoli cells. The procedure of phagocytosis assay was based on a previous protocol (41) with modifications. At 96 h after isolation, Sertoli cells were detached and reseeded in 24-well plates at 5 × 10^6 cells/well. Twenty-four hours later, the cells were washed three times with D-Hanks’ solution and cultured in different conditions: F12/DMEM medium containing 10% fetal calf serum, serum-free medium and serum-free medium supplemented with different TLR ligands (200 ng/ml poly(I:C), 1 µg/ml LPS, 10 ng/ml flagellin, or 10 µg/ml zymosan (InvivoGen)). Meanwhile, the apoptotic spermatogenic cells were added to the Sertoli cells at 5 × 10^6 cells/well. At 24 h after coculture, the lipid droplets in the Sertoli cells were detected by ORO staining. The area ratios of lipid droplets to nuclei of Sertoli cells were analyzed by image analyzer (Image-pro plus 6.0; Olympus) and used to assess phagocytic activity of Sertoli cells. A total of 150 Sertoli cells from three repeat wells were analyzed in each assay. The assay was done in triplicate. The mean values were presented in the results.

TABLE 1. Specific primers used for RT-PCR

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were washed two times in media without serum, and fluorescent latex beads (1 × 10^6 in 100 μl of culture medium) were added to the cultures. After 6 h coculture, Sertoli cells were washed with D-Hanks’ solution, and detached by incubating in 1 ml of D-Hanks’ containing 0.05% trypsin for 10 min at 32 C. The Sertoli cells were collected by low-speed centrifugation and washed twice with D-Hanks’ solution. The whole procedure can efficiently eliminate particles bound to the cells. Inhibition of actin involvement by 50 μg cytochalasin B (Sigma) in the phagocytic activity was used as control. The cells were counted under a fluorescent microscope (IX-71; Olympus). The ratio of the cells having internalized fluorescent beads expressed the phagocytic capacity of Sertoli cells. One hundred cells were counted in each test, and the results were presented as the mean value of three tests.

**ORO staining**

Sertoli cells cocultured with apoptotic spermatogenic cells were washed in PBS by pipetting for removing apoptotic cells and fixed with 10% formalin for 30 min. After a wash with PBS, the cells were stained with ORO (Sigma) solution (ORO-saturated solution in isopropanol-water, 3:2) for 15 min as a previous description (41). Then the cells were washed with 70% alcohol for 5 sec to remove background staining. Finally, the cells were rinsed in tap water, counterstained with Harris hematoxylin for 10 sec, and mounted in glycerol-PBS (9:1) for observation.

**Western blotting**

Total Sertoli cells lysates were prepared by lysing and scraping the cells off the culture plate with cell lysis buffer (BioDev-Technology, Beijing, China). Protein concentration was determined by using the microbicinchoninic acid method (Pierce Biotechnology, Rockford, IL). Equal amounts of proteins were subject to SDS-PAGE and subsequently electrotransferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA). After blocking with 5% nonfat dry milk in Tris-buffered saline (TBS) for 1 h, the electrotransferred membranes were incubated with first antibodies at 1:500 to 1:1000 dilutions at 4 C for overnight: goat anti-TLR2 (Imgenex); rabbit anti-TLR3 (eBioScience); rat anti-TLR4 (Santa Cruz); rabbit anti-CD36 (Santa Cruz); rabbit anti-TLR2 (Imgenex); rat anti-TLR3 (eBioScience); rabbit anti-TLR4 (eBioScience); rat anti-TLR6 (Imgenex); rat anti-TLR7 (eBioScience); polyclonal antibodies; and mouse TLR5 monoclonal antibody (Imgenex). After washing with TBS, the membrane was incubated with appropriated peroxidase-conjugated affinipured second antibodies (Zhongshan) at room temperature for 1 h. After washing with TBS, antigen-antibody complex was visualized by using an enhanced chemiluminescence detection kit (Zhongshan).

**Statistical analyses**

Data are presented as mean ± SEM for n given samples. Student’s t tests were used to determine significance between groups of cell types or treatments (e.g. treatments with TLR blockers). One-way ANOVA tests with Bonferroni corrections were used to calculate significance for multiple comparisons of different treatments (e.g. treatments with different TLR ligands). All calculations were performed with SPSS version 11.0 statistic software (SPSS Inc., Chicago, IL). *P* < 0.05 was considered significant.

**Results**

**Expression of TLRs in mouse Sertoli cells**

To examine expression pattern of TLRs in Sertoli cells, primary Sertoli cells were isolated from 3-wk-old C57 BL/6j mice, and the total RNA was extracted. By using RT-PCR, the expression of all 12 mouse TLRs at the mRNA level was examined in the primary Sertoli cells. The macrophages, which express most members of TLR family, were used as controls. As shown in Fig. 1, A and B, strong signals for TLR2, TLR4, and TLR5 were detected in Sertoli cells at a level comparable with macrophages, whereas TLR6 and TLR7 were expressed relatively weak by Sertoli cells, compared with macrophages. Notably, abundant TLR3 mRNA was detected in Sertoli cells but very weak in macrophages. The highest expressions of TLR2–5 in Sertoli cells were confirmed by real-time RT-PCR (Fig. 1C). TLR13 was also abundantly expressed in Sertoli cells, although it is lower than in macrophages. TLR8, TLR9, and TLR11 mRNA was not detected in Sertoli cells, but expressed by macrophages. TLR12 was not observed in both Sertoli cells and macrophages. Thymus cells were used as a positive control for TLR12. No homologous gene of human TLR10 has been identified in mouse (27). The results of Western blotting demonstrated the highest protein levels of TLR2–5 in Sertoli cells (Fig. 1D). The presence of TLR2–TLR5 proteins was further confirmed by flow cytometry analysis (Fig. 1E). Macrophages were used as controls for flow cytometry. Consistent with RT-PCR results, we did not detected TLR3 protein in macrophage.

**Activation of NF-κB in Sertoli cells by TLR ligands**

Although various members of the TLR family are expressed in Sertoli cells, their functions in this cell type remain to be clarified. Therefore, we examined whether TLR2–TLR5, which were expressed relatively high in Sertoli cells, can be activated by their ligands. It is known that the activation of TLR2–TLR5 results in NF-κB activation (11). NF-κB is sequestered in the cytoplasm as its inactive form and migrates into the nucleus when it is activated. Here we used immunofluorescence staining for NF-κBp65 to detect the translocation of NF-κB in Sertoli cells. To select optimal doses of agonists to active TLRs, the Sertoli cells were stimulated by different doses of zymosan, poly (I:C), LPS, and flagellin (the ligands of TLR2, TLR3, TLR4, and TLR5, respectively). Immunofluorescence staining was performed using polyclonal antibody against NF-κBp65. As shown in Fig. 2A, all four ligands-induced translocation of NF-κBp65 from cytoplasm to nuclei in a dose-dependent manner. With the selected doses of TLR ligands for highly positive effects, we performed time-dependent effects of the ligands. Compared with 0 min, when all nuclei were negative for NF-κBp65, a certain proportion of Sertoli cell nuclei were positively stained at 30 min after stimulation (Fig. 2, B and C). Poly (I:C) and LPS induced more efficiently nuclei translocation of NF-κB, compared with flagellin and zymosan, at 30 min after stimulation, with about 30 vs. 15% NF-κB-positive nuclei (*P* < 0.05). However, at 1 h after stimulation, all four ligands induced almost 100% Sertoli cell nuclei positive for NF-κBp65 (Fig. 2, B and C). It is known that NF-κB is activated through IκB degradation. To verify this process, IκB degradation in Sertoli cells was examined by immunofluorescence staining for IκBα after stimulation with TLR ligands. The results were shown in Fig. 2D. Before stimulation, Sertoli cells were positively stained for IκBα. The IκBα-positive signals were decreased at 30 min after stimulation and much weaker at 1 h. These observations indicate that TLR2–TLR5 can be activated by their ligands in Sertoli cells and thus result in NF-κB activation.
Induction of inflammatory cytokines by TLR agonists in Sertoli cells

It is known that activation of TLRs by their ligands can trigger a common signaling pathway to up-regulate inflammatory cytokines such as IL-1, IL-6, IL-12, and TNFα (26). Particularly, TLR3, TLR4, TLR7, TLR8, and TLR9 induce antiviral responses by inducing type I IFN (IFNα and IFNβ). To detect whether stimulation of TLR2, TLR3, TLR4, and TLR5 in Sertoli cells can up-regulate these inflammatory cytokines, we performed semiquantitative RT-PCR to detect mRNA of IL-1α, IL-6, IL-12, TNFα, IFNα, and IFNβ before and after treatment of Sertoli cells by TLR ligands. The results were shown in Fig. 3, A and B. At 12 h after stimulation with TLR ligands, Sertoli cells expressed about 10-fold IL-6 and 5-fold IL-1α higher than controls. The four TLR ligands induced augmentation comparable at mRNA level for IL-6 and IL-1α. However, up-regulation of IFNα and IFNβ was observed only in Sertoli cells treated by poly (I:C) and LPS. The stimulation of Sertoli cells by zymosan and flagellin did not up-regulate IFNα and IFNβ.
No IL-12 and TNFα was detected in Sertoli cells by RT-PCR before and after stimulation by TLR ligands (data not shown).

To determine whether the induction of the cytokines by TLR ligands was specifically mediated by TLRs, we used antibodies to block TLR2, TLR3, and TLR5 chemical inhibitor to block TLR4 before the presence of TLR ligands. The secretory cytokines in medium were detected using ELISA. The production of the cytokines was significantly induced by ligands of TLR2–5, and the inductions were inhibited by the blockers of the TLRs (Fig. 3C).
Increased phagocytic ability of Sertoli cells by TLR3

Sertoli cells are professional phagocytes in the seminiferous tubules to remove apoptotic germ cells and residual bodies. A previous study demonstrated that TLRs increased macrophages phagocytosis of bacteria (43). Therefore, we asked whether TLRs play a role in regulating phagocytosis by Sertoli cells. To examine this effect, Sertoli cells were cocultured with apoptotic germ cells in serum-free medium (DMEM/F12) in the presence of TLR agonists. Apoptotic spermatogenic cells were obtained through spontaneous induction during culture in vitro. To assess apoptotic rate of the cells, we stained the cells with dye mix of AO/EB. After staining, the nuclei of apoptotic cells show a yellow fluorescence, the nuclei of dead cells show an orange color, and nuclei of living cells appear a green color (Fig. 4A). Based on the procedures, we found that about 75% of spermatogenic cells are apoptotic cells; 15% of them are dead and 10% of them remain living.

Lipid droplet formation after phagocytosis was used as a criterion to evaluate phagocytic ability of Sertoli cells. At 24 h after coculture with apoptotic spermatogenic cells, the Sertoli cells were stained by ORO. Figure 4B represents images of lipid droplets in Sertoli cells cocultured with apoptotic germ cells under different stimulation. Quantitative data were shown in Fig. 4C. Poly (I:C) significantly increased lipid droplet formation by 3-fold, compared with control. In contrast, the other TLR agonists, zymosan, LPS, and flagellin, did not increase the formation of lipid droplets in Sertoli cells after coculture with apoptotic spermatogenic cells. Notably, amount of lipid droplets in the Sertoli cells stimulated by poly (I:C) in serum-free medium was comparable with that in the Sertoli cells cultured in medium containing 10% fetal calf serum (FCS). However, FCS and poly (I:C) had no additive effect on the lipid droplet formation in Sertoli cells. These observations suggest that poly (I:C) stimulates the phagocytosis of apoptotic spermatogenic cells by Sertoli cells, and factors in serum distract the effect of poly (I:C). To substantiate the specificity of poly (I:C) effect on Sertoli cell phagocytosis, the Sertoli cells were preincubated with serum-free medium containing 10 µg/ml polyclonal antibody against TLR3 (eBioscience; 24–9031) for 2 h before presence of poly (I:C) in culture. With blocking of TLR3 using the antibody, the lipid droplet formation was reduced significantly in the Sertoli cells after coculture with apoptotic spermatogenic cells (Fig. 4, B and C).

To further verify the effect of poly (I:C)/TLR3 signaling on Sertoli cell phagocytosis, a dose-dependent effect of poly (I:C) on the lipid droplet formation in Sertoli cells cocultured with apoptotic germ cells was examined. At 24 h after coculture in the presence of different concentrations of poly (I:C) (0, 2, 20, 200, 2000 ng/ml), the Sertoli cells were stained by ORO. A dramatic increased lipid droplets appeared in the Sertoli cells cultured in serum-free medium containing 200 ng/ml poly (I:C) (Fig. 4D), and no further increase in the lipid droplets was observed in the presence of more poly (I:C) (2 µg/ml). These results confirm the effect of poly (I:C) on the phagocytosis of apoptotic germ cells by Sertoli cells.

To determine whether TLR3-mediated Sertoli cell phagocytosis is specific to apoptotic germ cells or a general phenomenon, we examined the effect of poly (I:C) on Sertoli cells to ingest latex beads. As shown in Fig. 4E, there were no difference in the ingestion of latex beads between Sertoli cells cultured in serum-free medium with and without poly (I:C). The percentages of Sertoli cells ingested latex beads were 27.2% and 28.3% under two culture conditions. In contrast, treatment with cytochalasin B resulted in a marked decrease in the phagocytosis of latex beads by Sertoli cells. In macrophages, TLRs activate signal transduction pathways leading to ingest pathogens such as bacteria. To determine whether TLR3 induces the phagocytosis of bacteria by Sertoli cells, we detected uptaking of fluorescence-labeled E. coli. Neither Sertoli cells cultured with poly (I:C) nor without poly (I:C) could ingest the inactivated bacteria (data not shown). These observations suggest that TLR3 specifically promotes the phagocytosis of apoptotic spermatogenic cells by Sertoli cells but does not affect general phagocytic ability of Sertoli cells.

Increased expression of phagocytic genes in Sertoli cells stimulated by poly (I:C)

To define the molecular mechanisms underlying the TLR3-induced phagocytic activity of Sertoli cells observed in Fig. 4, we analyzed the expression of phagocytic genes in Sertoli cells stimulated by poly (I:C). Primary Sertoli cells were treated with TLR ligands for 12 h and then subjected to the extraction of total RNAs. Two scavenger receptors (CD36, SR-B1) and one receptor tyrosine kinase (Mer) are known to be involved in the engulfment of apoptotic spermatogenic cells by Sertoli cells (44–46). Therefore, the expression of these three genes was examined by semiquantitative RT-PCR in Sertoli cells treated with TLR ligands. We found that both CD36 and SR-B1 were induced by poly (I:C) at 12 h after the treatment (Fig. 5A). Compared with controls, 2.5- and 2-fold increases in the expression of CD36 and SR-B1 were detected in the Sertoli cells stimulated by poly (I:C), whereas LPS, flagellin, and zymosan did not up-regulate the expression of CD36 and SR-B1 (Fig. 5B). In contrast, Mer was expressed consistently in the Sertoli cells treated by TLR ligands. To determine whether induction of SR-B1 and CD36 transcripts correlated with increased levels of proteins, we performed Western blotting using specific antibodies against SR-B1 and CD36. We found that poly (I:C) can significantly increase expression of these proteins (Fig. 5, C and D). These data suggest that TLR3 could promote phagocytosis of apoptotic spermatogenic cells through up-regulation of SR-B1 and CD36 by Sertoli cells.

Discussion

TLRs play a crucial role in host defense against invading pathogens by mediating innate and adaptive immunity. Sertoli cells, the only somatic cell type within the seminiferous tubules, are important in the maintenance of testis as an immune privilege site in which both invading pathogen and autoantigens can be tolerated (47). The mechanisms that Sertoli cells involve in the immunity against pathogens and autoantigens have not been adequately studied. We demonstrate here that TLRs expressed in Sertoli cells may play an
FIG. 4. Effect of TLR ligands on the phagocytic activity of Sertoli cells. Sertoli cells were cultured under different conditions as described in Materials and Methods. A, The image of apoptotic spermatogenic cells after staining with AO/EB. The nuclei of apoptotic cells are yellow (open arrows), those of dead cells are orange (closed arrows), and those of living cells show green fluorescence (arrowheads). B, The images of lipid droplets in Sertoli cells after staining with ORO. C, Area ratios of the lipid droplets to nuclei of Sertoli cells were used to evaluate phagocytic ability of Sertoli cells. D, In serum-free medium, poly (I:C) stimulated phagocytic ability of Sertoli cells in a dose-dependent manner. E, Effect of poly (I:C) on the phagocytosis of latex beads (LB) by Sertoli cells. Each datum was presented as mean ± SEM of three experiments. *, P < 0.05; **, P < 0.01; #, P < 0.05. Bar, 20 μm.
CD36 and SR-B1 to subjected to Western blotting to examine CD36 and SR-B1 proteins.

Electrophoresis of the target genes to analyze the expression of CD36, SR-B1, and Mer. Electrophoresis referred to 32 cycles for CD36, 25 cycles for SR-B1, 32 cycles for Mer, and 22 cycles for β-actin were shown. The densitometric quantitation of the target genes to β-actin bands. C, Whole-cell extracts were subjected to Western blotting to examine CD36 and SR-B1 proteins after stimulation with poly (I:C). D, The densitometry of the bands of CD36 and SR-B1 to β-actin. The data are mean ± SEM of three experiments. *P < 0.05; **P < 0.01.

Phagocytosis is critical for innate immunity. A previous study demonstrated that TLR ligands promoted bacterial phagocytosis by macrophages (43). Sertoli cells are professional phagocytes in the seminiferous tubule to remove apoptotic germ cells and residual bodies (49). Therefore, it is worthwhile to determine whether the TLRs regulate phagocytic function of Sertoli cells. To address this issue, we analyzed the phagocytosis of apoptotic germ cells, latex beads, and bacteria by Sertoli cells after treatment with TLR ligands. Uptake of E. coli by Sertoli cells was not observed before and after stimulation by TLR agonists, suggesting that Sertoli cells cannot be responsible for innate immune response by directly ingesting invading pathogens. Cells undergoing apoptosis are efficiently eliminated from the organism by phagocytosis, and this phenomenon is likely to be a part of self-defense mechanisms (50). During spermatogenesis, more than 70% of spermatogenic cells are estimated to undergo apoptosis under physiological conditions (51, 52). A great deal residual bodies are formed in later stage of spermatogenesis (7). The rapid elimination of apoptotic cells and residual bodies by Sertoli cells is necessary for the normal production of sperm (53). The mechanism of this process remains to be clarified. In this study, we demonstrate that TLR3 but not other TLRs, specifically promotes phagocytosis of apoptotic spermatogenic cells by Sertoli cells. In con-
trast, activation of TLR3 does not induce Sertoli cells to ingest latex beads and bacteria. This finding provides novel insight into the mechanism underlying the phagocytosis of apoptotic spermatogenic cells by Sertoli cells.

It has been known that the phagocytosis of apoptotic germ cells by Sertoli cells is mediated by CD36 (45), SR-B1 (44) and Mer (46). In the present study, we show that poly(I:C), but not other TLR agonists, increases significantly expression of CD36 and SR-B1 in Sertoli cells. Therefore, we speculate that TLR3 promotes Sertoli cell phagocytosis of apoptotic germ cells through augmentation of CD36 and SR-B1. Induction of scavenger receptor (SR) genes including SR-A, LOX-1, and MARCO by TLRs has been reported to enhance macrophage-mediated phagocytosis of bacteria (43). Despite induction of SR genes by TLRs, each of these SR genes displays differential induction kinetics. Thus, further studies will be required to understand additional mechanisms underlying TLR-induced SR genes expression, particularly in different cell types.

Although poly(I:C) increases significantly the phagocytosis of apoptotic spermatogenic cells by Sertoli cells cultured in serum-free medium, this effect cannot be observed in Sertoli cells cultured in medium containing 10% FCS. In serum-free medium, this effect cannot be observed in these cells. Thus, further studies will be required to understand additional mechanisms underlying TLR-induced SR genes expression, particularly in different cell types.

Acknowledgments

Received December 21, 2007. Accepted May 12, 2008.

References