Toll-like Receptors 4 and 5 Cooperatively Initiate the Innate Immune Responses to Uropathogenic Escherichia coli Infection in Mouse Epididymal Epithelial Cells¹

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ABSTRACT

Uropathogenic Escherichia coli (UPEC) may cause epididymitis and impair male fertility. The mechanisms underlying the innate immune responses to UPEC infection in the epididymis are not fully understood. This study showed that UPEC induced innate immune responses in mouse epididymal epithelial cells (EECs) through the activation of Toll-like receptor 4 (TLR4) and TLR5. Infection with UPEC significantly induced the expression of proinflammatory cytokines, including tumor necrosis factor alpha, interleukin 6, and monocyte chemoattractant protein 1, in EECs through the activation of nuclear factor kappa B. Moreover, UPEC induced the production of type 1 interferons by EECs through the activation of interferon regulatory factor 3. The UPEC-induced innate immune responses were significantly reduced in the EECs of Tlr4 or Tlr5 knockout mice. The innate immune responses were further reduced in Tlr4 and Tlr5 double-knockout EECs. Furthermore, we demonstrated that TLR4 and TLR5 cooperatively initiated the epididymal innate immune responses to UPEC infection in vivo. The results provide novel insights into the mechanisms underlying the epididymal innate immune responses to UPEC infection.

INTRODUCTION

Microbial infections of male genital tract may cause local inflammation, thus impairing male fertility [1, 2]. Uropathogenic Escherichia coli (UPEC) are the dominant bacteria that frequently lead to the pathogenesis in the male urogenital tracts [3, 4]. Previous studies on UPEC-caused pathogenesis largely focused on the urinary system, such as the bladder and kidney [5, 6]. Infection with UPEC can also cause epididymitis, which is associated with abnormal semen parameters and male infertility [7]. Several studies have examined the innate immune responses to UPEC infection in murine testicular cells [8–10]. The pathogenesis of UPEC-induced epididymitis has been recently investigated in rats [11, 12]. However, the mechanisms underlying the innate immune responses to UPEC infection in epididymal epithelial cells (EECs) have yet to be investigated.

Pattern recognition receptors (PRRs) initiate innate immune responses to microbial infections [13]. The PRRs recognize conserved pathogen-associated molecular patterns (PAMPs), thereby initiating innate immune responses and directing adaptive immunity against invading pathogens [14]. Toll-like receptors (TLRs) are the best-studied PRRs. To date, 13 TLRs have been identified in mammals. TLRs can be activated by PAMPs derived from most microbes, including bacteria, viruses, fungi, and parasites [15]. Among them, TLR4, TLR5, and TLR11 can recognize UPEC and are involved in the host defense against UPEC infection in mice [16–18]. TLRs exclusively initiate the myeloid differentiation protein 88 (MyD88)-dependent pathway, with the exception of TLR3 and TLR4 [19]. TLR3 activation exclusively initiates the Toll/interleukin 1 (IL-1) receptor domain-containing adaptor inducing interferon β (TRIF)-dependent pathway, whereas TLR4 can initiate both the MyD88- and TRIF-dependent pathways. TLR signaling pathways result in the activation of nuclear factor kappa B (NFκB) and interferon regulatory factor 3 (IRF3), which induce the expression of numerous proinflammatory cytokines, chemokines, and type 1 interferons (IFNA and IFNβ) [13]. These cytokines regulate immune responses against microbial infections and can directly restrict microbial replication.

TLRs are predominantly expressed in immune cells and initiate systemic immune responses [20]. Moreover, some TLRs are also expressed in the epithelial cells of tissues that are exposed to an environment rich in microbes, and they participate in the tissue defense against microbial pathogens. Notably, most TLRs are abundantly expressed in the male reproductive tract [21]. The functions of TLRs in mouse testicular cells have been extensively investigated [22]. Several TLRs initiate innate immune responses in Sertoli cells [23–25]. Leydig and male germ cells also express functional TLRs [10, 26, 27]. Although various TLRs are abundantly expressed in rat EECs [28], only a few studies have examined the functions of TLRs in EECs. TLR4 can be activated by lipopolysaccharides in the rat epididymis [29]. TLR2 and TLR4 initiate the innate immune responses to Staphylococcus aureus infection in EECs [30]. We recently showed that several viral sensors initiated innate antiviral responses in EECs [31]. The present study aimed to elucidate the TLR-initiated innate immune responses to UPEC infection in EECs.

MATERIALS AND METHODS

Animals

C57BL/6 mice were obtained from the Laboratory Animal Center of the Peking Union Medical College (Beijing, China). Tlr4 knockout (Tlr4−/−) mice (B6.Tlr4tm1[Karp]) and Tlr5−/− mice (B6.Tlr5tm1[Flv]) were purchased from Jackson Laboratories (Bar Harbor, ME). Tlr4 and Tlr5 double-knockout (Tlr4−/−Tlr5−/−) mice were obtained by crossing-mating Tlr4−/− and Tlr5−/− mice.
Wild-type (WT) mice were generated by backcrossing Trp55Cys/Trp55Cys mice to C57BL/6 mice. All mice were maintained in a specific pathogen-free facility with a 12L:12D cycle and were provided with food and water ad libitum. The mice were handled in compliance with the Guidelines for the Care and Use of Laboratory Animals established by the Chinese Council on Animal Care. Adult male mice at ages of 8–10 wk were used in this study.

Antibodies and Major Reagents

Rabbit anti-TLR4 (ab13556), anti-TLR5 (ab62640), anti-TLR11 (ab47079), anti-UPEC (ab68540), anti-aquaporin 9 (ab105148), and anti-cytokertatin 5 (ab53121) polyclonal antibodies, as well as rat anti-F4/80 (ab6640) monoclonal antibody, were purchased from Abcam (Cambridge, U.K.). Rabbit anti-NFKBp65 (no. 4764), anti-phospho-IRF3 (no. 4947), and anti-phospho-NFKBp65 (no. 3031) polyclonal antibodies were purchased from Cell Signaling Technology (Beverly, MA). Rabbit anti-IkBα (sc-571) and anti-IRF3 polyclonal (sc-9082) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-β-actin monoclonal antibody (A5316) was purchased from Sigma (St. Louis, MO). The horseradish-peroxidase (HRP)-conjugated and fluorescein isothiocyanate (FITC)-conjugated secondary antibodies were purchased from Zhongshan Biotechnology Co. (Beijing, China). The UPEC strain CFT073 (no. 700928) was purchased from the European Collection of Animal Cell Cultures (ECACC, Weybridge, Surrey, U.K.). The protein kinase inhibitor cocktail (Applygen Technologies Inc., Beijing, China). The primer sequences are listed in Supplemental Table S1 (Supplemental Data are available online at www.biolreprod.org).

Cell Isolation

The EECs were isolated as previously described [32]. Briefly, mice were anesthetized with CO2 and euthanized by cervical dislocation. After dissection of fat and connective tissues, the epididymides were incubated with 10 mg/ml collagenase type IV (Sigma) in F12/Dulbecco modified Eagle medium (F12/DMEM; Life Technologies Inc., Gaithersburg, MD) at room temperature for 30 min through 80-μm copper mesh. Single and aggregated EECs were collected and separated on 10% SDS-PAGE gel and subsequently electrotransferred onto PVDF membranes. Immunoblotting was performed with 1:1000 dilutions of primary antibodies at room temperature for 1 h. Bands were developed with an enhanced chemiluminescence detection kit (Zhongshan Biotechnology). β-Actin was used as the loading control.

UPEC Infection

The UPEC bacteria were cultured in Luria-Bertani media (Life Technologies) overnight at 37°C in a shaking incubator at 120 rpm. The UPECs at the exponential growing phase (OD_{600} of 0.6–1.0) were collected by centrifugation at 4000 × g for 10 min at 4°C. The pellet was washed twice with cold 1× PBS, and UPEC numbers were determined using colony-forming unit assay. The UPECs were diluted in PBS at a density of 1 × 10^8 UPECs per milliliter.

For the in vitro experiments, UPECs were inactivated by gamma irradiation based on previously described methods [36]. Primary EECs were infected with 5 × 10^7 UPECs per milliliter. For the in vivo study, 2 × 10^7 live UPECs in 10 μl of PBS were injected into the epididymis according to previously described procedures [11]. Briefly, mice were anesthetized with pentobarbital sodium (50 mg/kg), and the epididymides were surgically exposed. The epididymis was injected with UPEC or PBS via the vas deferens proximal to the cauda epididymis, using 30-gauge needles.

MTT Assay

Cell viability was assessed with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay kit (American Type Culture Collection) according to the manufacturer’s instructions. The EECs were seeded in 96-well microplates at a density of 2 × 10^5 cells per well. After 24 h, the cells were treated with UPEC and chemical inhibitors as for innate immune response induction. After the treatments, the cells were incubated with 10 μl of MTT solution for 2 h. Subsequently, 100 μl of the detergent reagent (included in the kit) was added to each well. The absorbance at 570 nm was measured with a microplate reader (BioTek, Winooski, VT). Cell viability was represented as the percentage of the absorbance values versus the controls.

Real-Time Quantitative RT-PCR

Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. RNA was treated with RNase-free DNase I (Invitrogen) to remove the genomic DNA contaminants. The absence of these DNA contaminants was confirmed by the PCR amplification of β-actin (Actb) for 35 cycles prior to reverse transcription. mRNA (1 μg) was reverse transcribed into cDNA in 20 μl reaction mixture containing 2.5 μM random hexamers, 2 μM dNTPs, 200 U of M. luteus T3 RNase H-free reverse transcriptase and in a heating block at 37°C. After reverse transcription, 2 μl of cDNA was utilized as the template for PCR amplification. Primers were designed as described in the Applied Biosystems User Bulletin No. 2 (P/N 4303859) [37]. The reaction efficiencies for all amplifications were between 95% and 100%. The primer sequences are listed in Supplemental Table S1 (Supplemental Data are available online at www.biolreprod.org).

Western Blot Analysis

The epididymides or cells were lysed with a lysis buffer containing protease and phosphatase inhibitors (Applygen Technologies Inc., Beijing, China). The protein concentrations were measured with a bicinchoninic acid protein assay kit (Pierce Biotechnology, Rockford, IL). The proteins (20 μg per well) were separated on 10% SDS-PAGE gel and subsequently electrotransferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA). The membranes were blocked with Tris-buffered saline (TBS; pH 7.4) containing 5% nonfat milk at room temperature for 1 h and were incubated with the primary antibodies overnight at 4°C. After washing twice with TBS containing 0.1% Tween-20, the membranes were incubated with the HRP-conjugated secondary antibodies at room temperature for 1 h. Bands were developed with an enhanced chemiluminescence detection kit (Zhongshan Biotechnology). β-actin was used as the loading control.

Immunohistochemistry and Immunofluorescence Staining

To prepare paraffin sections, the epididymides were fixed in Bouin solution for 24 h. The tissues were embedded in paraffin and cut into 5-μm-thick sections with a rotary microtome Reichert 820 HistoSTAT (Reichert Technologies, Depew, NY). To prepare frozen sections, the epididymides were fixed in 4% paraformaldehyde for 24 h. After cryoprotection in 30% sucrose, the tissues were cut to a thickness of 7 μm with a Leica CM1950 apparatus (Leica Biosystems, Nussloch, Germany). The sections were incubated in 1× PBS containing 3% H_2O_2 for 15 min to inhibit endogenous peroxidase activity. The slides were soaked in citrate buffer and microwave heated at 100°C for 10 min to retrieve the antigens. After blocking with 5% normal goat sera in PBS for 1 h at room temperature, the sections were incubated with the primary antibodies overnight at 4°C. The sections were washed twice with PBS and incubated with the appropriate HRP-conjugated secondary antibodies at room temperature for 30 min. The HRP activity was visualized via the diaminobenzidine method. Negative controls were incubated
with preimmune rabbit sera instead of primary antibodies. The sections were counterstained with hematoxylin and mounted with neutral balsam (Zhongshan Biotechnology).

For indirect immunofluorescence staining, EECs were cultured on Lab-Tek chamber slides (Nunc, Naperil, IL). The cells were fixed with precooled methanol at –20°C for 3 min and were permeabilized with 0.2% Triton X-100 in PBS for 15 min. After blocking with 10% normal goat sera in PBS at room temperature for 30 min, the cells were incubated with the respective primary antibodies at 37°C for 90 min. After washing thrice with PBS, the cells were incubated with appropriate FITC-conjugated secondary antibodies (Zhongshan Biotechnology) for 30 min. The slides were mounted with neutral balsam for observation under a fluorescence microscope BX-51 (Olympus, Tokyo, Japan).

Enzyme-Linked Immunosorbent Assay

The culture media were collected at 24 h after UPEC infection. The epididymis was lysed by grinding in 1× PBS, and the supernatant of the lysates was collected after centrifugation at 800 × g for 5 min. Cytokine levels were measured with ELISA kits according to the manufacturer’s instructions. Mouse tumor necrosis factor alpha (TNFA; CME0004) and interleukin 6 (IL6; CME0006) ELISA kits were purchased from Beijing 4A Biotech Company (Beijing, China). Mouse monocyte chemoattractant protein 1 (MCP1) ELISA kit (KB3817A) was purchased from Shanghai Kaibo Biochemical Reagent Company (Shanghai, China). Mouse IFN alpha Platinum ELISA kit (BMS6027) was purchased from eBioscience (San Diego, CA). The ELISA kit for IFNB (42400) was purchased from R & D Systems (Minneapolis, MN).

Statistical Analysis

All data are presented as the mean ± SEM. Statistical significance between individual comparisons was determined by Student t-test. For multiple comparisons, one-way ANOVA with Bonferroni (selected pairs) post hoc test was used. The calculations were performed with SPSS version 13.0 (SPSS Inc., Chicago, IL). A P value <0.05 was considered statistically significant.

RESULTS

Expression of TLR4 and TLR5 in EECs

Given that TLR4, TLR5, and TLR11 are involved in the host responses against UPEC infection in mice [16–18], we examined the expression of these TLRs in EECs. Primary EECs were isolated from adult C57BL/6 mice at ages of 8–10 wk, and cell purity was assessed after indirect immunofluorescence staining with specific antibodies against the marker proteins aquaporin 9 for principal cells (Fig. 1A, left), keratin 5 for basal cells (Fig. 1A, middle), and F4/80 for macrophages (Fig. 1A, right). Based on the immunostaining, we found that EECs contained >80% principal cells and approximately 12% basal cells. Only <3% macrophages were detected in the EEC preparations. It is known that mouse macrophages express most TLRs, and male germ cells express TLR11 [10]. Therefore, we determined the expression of TLR4, TLR5, and TLR11 in EECs in comparison with peritoneal macrophages and male germ cells. Real-time quantitative RT-PCR (qRT-PCR) results showed that EECs and macrophages expressed comparable mRNA levels of Tlr4 and Tlr5 (Fig. 1B, left and middle). By contrast, Tlr11 mRNA level was remarkably low in EECs compared with macrophages and male germ cells (Fig. 1B, right). Western blot analysis confirmed that TLR4 and TLR5 proteins were abundantly produced in EECs and macrophages (Fig. 1C, left and middle). TLR11 protein was not detected in EECs (Fig. 1C, right). Immunohistochemistry showed that TLR4 and TLR5 were broadly located in principal cells (arrows), basal cells (arrowheads), and some interstitial cells (asterisks) of the epididymis (Fig. 1D, left and middle). TLR11 was not evident in the epididymis (Fig. 1D, right).

UPEC-Induced Cytokine Expression in EECs

To determine the innate immune responses to UPEC infection in EECs, the expression of several inflammatory cytokines was examined. Real-time qRT-PCR results showed that the mRNA levels of the major proinflammatory cytokines, including Ifna, Il6, and Mcp1, were dramatically increased in EECs after UPEC infection in a time-dependent manner (Fig. 2A). The peak mRNA levels were detected at 4 h after UPEC infection. Uropathogenic E. coli also remarkably induced Ifna and Ifnb expression (Fig. 2B, left and middle). The plateau of Ifna and Ifnb mRNA levels appeared at 6 and 8 h after UPEC infection. By contrast, Ifng expression was not significantly affected by UPEC infection (Fig. 2B, right). A dose-dependent analysis showed that 5 × 10^7 UPECs per milliliter optimally induced the cytokine expression (Fig. 2C). The ELISA results confirmed that UPEC significantly induced the cytokine secretion by EECs at 24 h after infection (Fig. 2D).

Activation of NFKB and IRF3

Given that the expression of the inflammatory cytokines depends on the activation of NFkB and IRF3 [38], we examined NFkB and IRF3 phosphorylation in EECs. Uropathogenic E. coli evidently induced the phosphorylation of NFkB p65 (p-p65) in a time-dependent manner (Fig. 3A). The peak p-p65 level was detected at 1 h after UPEC infection. Moreover, inhibitory KBA (IKBA) was remarkably degraded in EECs at 1 h after UPEC infection. Uropathogenic E. coli also induced the phosphorylation of IRF3 (p-IRF3) in EECs (Fig. 3B). The p-p65 and p-IRF3 must be translocated into nuclei for inducing cytokine expression. Indirect immunofluorescence staining confirmed that p65 and IRF3 were both efficiently translocated into the nuclei of EECs at 1 h after UPEC infection (Fig. 3C, middle). For controls, p65 and IRF3 were exclusively located in the cytoplasm of EECs without UPEC infection (Fig. 3C, left). The dynamics of the nuclear translocation was quantitatively examined based on indirect immunofluorescence staining (Fig. 3C, right).

Involvement of NFkB and IRF3 in Innate Immune Responses

To determine the involvement of NFkB and IRF3 activation in the innate immune responses to UPEC infection, we examined the effects of the chemical inhibitors of NFkB and IRF3 activation on cytokine expression. The EECs were pretreated with the inhibitors for 2 h prior to UPEC infection. BAY11-7082 is a specific inhibitor of IKBA phosphorylation and subsequently of its degradation, which inhibited p65 phosphorylation and IKBA degradation in a dose-dependent manner (Supplemental Fig. S1). We demonstrated that 10 μM BAY11-7082 significantly inhibited p65 phosphorylation and IKBA degradation in EECs at 1 h after UPEC infection (Fig. 4A). BX795 (inhibitor of TBK1) significantly reduced UPEC-induced p-IRF3 levels (Fig. 4B). BAY11-7082 significantly inhibited the secretion of TNFA, IL6, and MCP1 by EECs at 24 h after UPEC infection (Fig. 4C). However, BAY11-7082 did not significantly affect the production of IFNA and IFNB. By contrast, BX795 significantly reduced IFNA and IFNB levels but insignificantly affected proinflammatory cytokine production. The MTT assay results showed that cell viability was not significantly reduced by UPEC and inhibitor treatments (Fig. 4D).
FIG. 1. Expression of TLR4, TLR5, and TLR11 in EECs. A) Identification of EECs. Epididymal epithelial cells were isolated from the epididymis of adult C57BL/6 mice at ages 8–10 wk. After 5 days in culture, cell purity was determined by immunofluorescence staining using specific antibodies against cell markers: aquaporin 9 for principal cells, keratin 5 for basal cells, and F4/80 for macrophages. The ratio of cell types in the primary EECs was assessed by spontaneously counting at least 300 cells.

B) TLR mRNA levels. Total RNA was extracted from EECs, peritoneal macrophages (Mφ), and male germ cells (GCs). The relative mRNA levels of Tlr4, Tlr5, and Tlr11 were determined by real-time qRT-PCR after normalization to the highest mRNA level (set as 1).

C) Protein levels of TLRs in EECs, Mφ, and GCs were determined by Western blot analysis with specific antibodies. β-Actin was used as the loading control. MW, molecular weight.

D) TLR distribution in the epididymis. Immunohistochemical staining was performed on paraffin sections with antibodies against TLR4, TLR5, and TLR11. Insets in the upper right corners of the images are negative controls, in which preimmune rabbit sera were used instead of the primary antibodies. Arrows, arrowheads, and asterisks indicate principal cells, basal cells, and interstitial cells, respectively. Images represent at least three independent experiments. Bar = 20 μm. The qRT-PCR data are the means ± SEM of three independent experiments.
FIG. 2. Uropathogenic E. coli-induced cytokine expression in EECs. Uropathogenic E. coli bacteria were inactivated using $^{60}$Co at a dose of 400 Krad. A) Expression of proinflammatory cytokines in a time-dependent manner. Epididymal epithelial cells were infected with $5 \times 10^7$ UPECs per milliliter for the specified durations. The mRNA levels of Tnfa, Il6, and Mcp1 were determined with real-time qRT-PCR. The cytokine mRNA level in EECs without UPEC infection (0 h) was set as “1,” and the relative mRNA levels (fold increase) at the indicated time points were shown. B) Uropathogenic E. coli-induced IFN expression. Epididymal epithelial cells were treated as described in A, and the relative mRNA levels of Ifna, Ifnb, and Ifng were determined. C) Uropathogenic E. coli-induced cytokine expression in a dose-dependent manner. Epididymal epithelial cells were infected with the indicated doses of UPECs, and the relative mRNA levels of cytokines were determined using real-time qRT-PCR. D) Cytokine secretion. Epididymal epithelial cells were infected with $5 \times 10^7$ UPECs per milliliter for 24 h. The cytokine levels in culture medium were measured using ELISA. Data are the mean ± SEM of three independent experiments. **P < 0.01. ND, not detectable.
Roles of TLR4 and TLR5 in EECs

We examined cytokine expression in the EECs of WT, *Tlr4*−/−, *Tlr5*−/−, and *Tlr4*−/−*Tlr5*−/− mice to determine the roles of TLR4 and TLR5 in initiating innate immune responses to UPEC infection. Real-time qRT-PCR results showed that *Tlr4* and *Tlr5* mRNAs were absent in the EECs of gene knockout mice (Fig. 5A, left and middle). Western blot analysis results confirmed the absence of TLR proteins in respective gene knockout EECs (Fig. 5A, right). TNFA, IL6, and MCP1 levels were significantly lower in the medium of *Tlr4*−/− or *Tlr5*−/− EECs than that of WT cells at 24 h after UPEC infection (Fig. 5B, left). The UPEC-induced production of TNFA, IL6, and MCP1 was further reduced in *Tlr4*−/−*Tlr5*−/− EECs. IFNA and IFNB levels were significantly decreased in *Tlr4*−/− and *Tlr4*−/−*Tlr5*−/− EECs compared with WT cells. However, WT and *Tlr5*−/− EECs produced comparable levels of IFNA and IFNB in response to UPEC infection. The knockout of *Tlr4* and *Tlr5* did not affect basal cytokine levels in the control cells without UPEC infection (Fig. 5B, right). These results indicate that TLR4 and TLR5 cooperatively mediate proinflammatory cytokine production, whereas IFNA and IFNB expression was exclusively mediated by TLR4 in EECs after UPEC infection.

UPEC-Induced Epididymal Innate Immune Responses In Vivo

To determine the epididymal innate immune responses to UPEC infection in vivo, UPECs were injected into the epididymis via the vas deferens proximal to the cauda epididymis. At 2 h after injection, the presence of UPECs in the epididymis was confirmed by Western blot analysis (Fig. 6A). Immunohistochemical staining results showed that UPECs were located in the epididymal tubules (arrows) and interstitial spaces (arrowheads) of the cauda epididymis (Fig. 6B, left). However, UPECs were only detected in the interstitial spaces of the caput epididymis (Fig. 6B, right). By contrast, UPECs were not found in the control epididymis that was injected with PBS (Fig. 6B, insets in the upper right corners). Uropathogenic *E. coli* evidently induced the phosphorylation of p65 and IRF3 in the epididymis of WT mice at 2 h after injection (Fig. 6C, left). By contrast, *Tlr4*−/− and *Tlr5*−/− mice showed remarkably low in the epididymis of *Tlr4*−/−*Tlr5*−/− mice. Immunohistochemistry results showed that p65 and IRF3 were efficiently translocated into the nuclei of principal cells (arrows) and basal cells (arrowheads) in the cauda and caput epididymis of WT mice 2 h after UPEC injection (Fig. 6D). The nuclear translocation was also observed in some interstitial cells (asterisks). By contrast, the nuclear translocation of p65 and IRF3 was not detected in the control epididymis (Fig. 6D, right).
The nuclear translocation was not evident in the epididymis of Tlr4/C0/C0/Tlr5/C0/C0 mice after UPEC infection (data not shown). Accordingly, UPEC significantly upregulated cytokine mRNA levels in the epididymis of WT mice at 6 h after injection (Fig. 6E, left). By contrast, UPEC injection faintly upregulated cytokine expression in the epididymis of Tlr4/C0/C0/Tlr5/C0/C0 mice (Fig. 6E, right). The results indicate that TLR4 and TLR5 play important roles in initiating epididymal innate immune responses to UPEC infection in vivo.

**DISCUSSION**

Uropathogenic *E. coli* bacteria are major pathogenic bacteria that infect the urogenital tract and lead to inflammation in the urinary system, including the bladder and kidney [39, 40]. Infection with UPEC may also induce epididymitis and impair male fertility [41]. Understanding the underlying mechanisms of the epididymal innate immune responses to UPEC infection can aid in establishing effective preventive and therapeutic approaches to the disease. Previous studies largely focused on the immune cell responses to UPEC infection in the epididymis [11, 12]. This study aimed to elucidate TLR-initiated innate immune responses in major tissue-specific cells, EECs. The results demonstrated that TLR4 and TLR5 cooperatively initiated the innate immune responses to UPEC infection in EECs, which suggests that EECs should be involved in the epididymal defense against UPEC infection.

Various immune cells, including macrophages, dendritic cells, and lymphocytes, reside in the epididymis under physiological conditions, and these cells are believed to constitute the front line of the epididymal defense against microbial infections [42, 43]. Expression of TLRs and their signaling molecules in the epithelial cells of the male genital tract suggests that these tissue-specific cells are equipped with innate immune machinery [21]. Epididymal epithelial cells represent the majority of the epididymal cells that make first contact with microbial pathogens from the ascending genito-urinary tract. Therefore, EECs should be involved in the epididymal innate immune responses to microbial infection. This study is the first work showing innate immune responses to UPEC infection in EECs.
should not profoundly affect the innate immune responses in primary EECs in vitro. Moreover, immunostaining results confirmed that the innate immune responses indeed occurred in the principal and basal cells after UPEC infection. Epididymal epithelial cells were infected with inactivated UPEC for in vitro experiments because live UPECs rapidly replicate in culture and are toxic to cells. The UPECs were inactivated using gamma irradiation in order to maintain major immunogenic components of bacteria [36]. By contrast, live UPECs were injected into the epididymis to mimic a natural infection for in vivo study. Infection with UPEC remarkably upregulated the expression of TNFA, IL6, and MCP1 in EECs. Although these cytokines regulate the systemic immune responses against microbial infections, they may also impair fertility. The high levels of TNFA and IL6 in semen impair sperm quality and are associated with male infertility [46]. The inflammatory cytokines are traditionally thought to be produced by immune cells. The roles of the cytokines secreted by EECs in regulating immune responses and affecting male fertility should be considered.

We demonstrated that TLR4 and TLR5 cooperatively initiated the innate immune responses to UPEC infection in EECs. Although UPECs also activate TLR11 [18], we did not detect TLR11 expression in EECs. TLR11 cannot initiate innate immune responses in human beings because functional TLR11 is absent in humans [47]. Both TLR4 and TLR5 mediate proinflammatory cytokine and chemokine induction by activating NFKB. Furthermore, TLR4, but not TLR5, also induced IFNA and IFNB expression through IRF3 activation. These results are in agreement with those showing that both TLR4 and TLR5 initiate the MyD88-dependent pathway, leading to NFKB activation, whereas TLR4, but not TLR5, also initiates the TRIF-dependent pathway that results in IRF3 activation [13]. Notably, a previous study showed that UPECs inhibited proinflammatory cytokine expression by blocking the MyD88-dependent pathway, but they induced IFNA and IFNB production via the TRIF-dependent pathway in rat testicular cells [8]. The inhibition of proinflammatory cytokine production in testicular cells favors the immunoprivileged environment in the testis. These results suggest that UPECs may trigger different signaling in the testicular and epididymal cells. The mechanisms underlying the tissue-specific innate immune responses to UPEC infection are worthy of further investigation.

We confirmed that TLR4 and TLR5 initiated the epididymal innate immune responses to UPEC infection in vivo. The UPEC-triggered epididymal innate immune responses were remarkably low in Tlr4^−/− Tlr5^−/− mice compared with WT mice. Notably, UPEC-induced cytokine expression was not completely abolished in the epididymis of Tlr4^−/− Tlr5^−/− mice, suggesting that other mechanisms may also be involved in the innate immune responses to UPEC infection. For investigating
the epididymal innate immune responses to UPEC infection in vivo, UPECs were injected into the vas deferens proximal to the cauda epididymis. Uropathogenic E. coli bacteria were detected in both the interstitial spaces and tubules of the cauda epididymis. By contrast, UPECs were only detected in the interstitial spaces of the caput epididymis. These observations...
suggest that UPECs can penetrate the epithelium of the epididymis and spread in the interstitial spaces. The absence of UPEC within the tubules of the caput epididymis remains to be explained. NFkB and IFR3 were efficiently translocated into the nuclei of EECs in both the cauda and caput epididymides of WT mice, and the cytokine expression was remarkably upregulated after UPEC injection. The cytokines produced by EECs in response to UPEC infection should be involved in the regulation of systemic immune responses because these cytokines can recruit and activate leukocytes [48]. TNFA also promotes defense production against pathogens [49]. A recent study showed that UPEC induced defense secretion in the rat epididymis, thereby limiting infection [50]. Taken together, EECs may play important roles in countering UPEC infection in the epididymis by orchestrating immune responses and directly killing invading pathogens.

In summary, the present study showed that UPEC induced innate immune responses in mouse EECs via TLR4 and TLR5 signaling. The results provide novel insights into the mechanisms underlying epididymal innate immune responses to UPEC infection. The roles of EECs in the epididymal defense against UPEC infection merit further investigation.

REFERENCES