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Toll-like receptor-4: Renal cells and bone marrow cells signal for neutrophil recruitment during pyelonephritis

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Background. The molecular mechanisms of pathogen recognition that initiate infective pyelonephritis are poorly understood. Toll-like receptor-4 (TLR4) mutant mice infected with uropathogenic *Escherichia coli* lack renal CXCL2 mRNA expression, subsequent neutrophil recruitment, and renal abscess formation.

Methods. We used a bone marrow transplant approach in order to investigate the contribution of TLR4 in intrinsic renal cells or bone-marrow–derived immune cells to neutrophil recruitment during infective pyelonephritis.

Results. Both chimera either expressing mutant *tlr4* in intrinsic renal cells and wild-type *tlr4* in bone marrow–derived cells or vice versa showed an impaired response to uropathogenic *E. coli* infection in terms of leukocyturia and renal abscess formation when compared to *tlr4* wild-type mice with congenic bone marrow transplants.

Conclusion. These data suggest that TLR4 is required on both intrinsic renal cells (e.g., tubular epithelial cells) and bone marrow–derived immune cells for the control of ascending uropathogenic *E. coli* infection by initiating chemokine-driven renal neutrophil recruitment.

Toll-like receptors (TLRs) are germ line–encoded pathogen-pattern recognition receptors that recognize a variety of microbial products, including lipopolysaccharide (LPS), lipoprotein, peptidoglycan, and bacterial DNA [1, 2]. The virulence of pathogens is often determined by their interaction with epithelial surfaces, being considered as a barrier to the entry of pathogens [3, 4]. TLR4 and TLR11 are expressed by kidney tubular epithelial cells in mice [5, 6], but only TLR4 is expressed in tubular epithelial cells of the human kidney [6, 7]. The LPS hyporesponsive C3H/HeJ and C57BL/10ScCr mice lack functional TLR4 expression which is a critical component of the LPS receptor complex [8, 9]. LPS is present in the wall of uropathogenic *Escherichia coli*. Uropathogenic *E. coli* are responsible for the vast majority of urinary tract infections, including pyelonephritis. The vigorous inflammatory response in the kidney is thought to be a result of a direct interaction between uropathogenic *E. coli* and epithelia of the urinary tract [10]. Thus, we hypothesized that the expression of TLR4 on renal cells may contribute to the initiation of antibacterial immunity during renal infection with uropathogenic *E. coli* in vivo.

Key words: pyelonephritis, Toll-like receptor-4, UPEC, neutrophils, bone marrow transplant.

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METHODS

Cell culture experiments

Primary cells were isolated from C3H/HeN and C3H/HeJ mice and grown as previously described [5]. Cells were incubated for 24 hours with medium control, 1 μg/mL ultrapure LPS (Invitrogen, San Diego, CA, USA) or heat-incativated uropathogenic *E. coli* CFT073.

RNA isolation and real-time reverse transcription-polymerase chain reaction (RT-PCR)

RNA isolation and RT was performed as described [11]. The following primers (300 nmol/L) and probes (100 nmol/L) (PE Biosystems, Weiterstadt, Germany) were used: TLR4 forward primer 5′-TTCAGAACTT CAGTGGCTGGATT-3′, reverse primer 5′-CCATGCC TTGTCTTCAATTGTTT-3′; 6 FAM 5′-ATCCAGGTG TGAAATT-3′-TAMRA; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward primer 5′-CATGG CCTTCCGTGTTCCTA-3′, reverse primer 5′-ATGCC TGCTTCACCACCTTCT-3′; 6 FAM 5′-CCCAATGTG TCCGTCGTG-GATCTGA-3′-TAMRA; CXCL2 predeveloped TaqMan® primer assay (PE Biosystems)

(Mm00436450_m1); RT PCR for TLR11 5′-TTGATG TATTCGTGTCCCACTGC-3′ and 5′-CCACTCTTTCT CTCCTCTTCCTCG-3′.

Generation of chimeric mice and induction of bacterial pyelonephritis

Female C3H/HeN wild-type mice and *tlr4* dominant-negative C3H/HeJ mice were obtained from Charles River (Sulzfeld, Germany). Six-week-old mice were lethally irradiated with 9.4 Gy of total body irradiation (GC40-107) (MSD Nordion, Ottawa, Canada), reconstituted with bone marrow (3.0×10^6 cells) either from a congenic or an allogenic donor and maintained for 8 weeks to allow for complete engraftment of the donor bone marrow. Ablation of recipient hematopoetic cells and full reconstitution of donor marrow (>95%) was confirmed by flow cytometry for H2-k in peripheral blood from five sentinel mice (H-2b) as described [12]. Pyelonephritis was induced by intravesical inoculation of 4×10^8 colony-forming units uropathogenic *E. coli* CFT073 in 20 μL normal saline. Liquid collodion (MMD0014) (Mavidon, At Erica, The Netherlands) was placed on the urethra for 6 hours for urethral obstruction and mice were sacrificed 36 hours after infection.

Urinary leukocyte and renal bacteria counts

Leukocyte counts were assessed from 5 μL fresh uncentrifuged urine in 10 high power fields. Kidney homogenates were diluted in phosphate-buffered saline (PBS) and plated on Luria Bertani (LB) agar to quantitate colony-forming units.

Histologic evaluation

Kidneys were fixed in 10% buffered formalin and embedded in paraffin as described [11]. The following primary antibodies were used: rat anti-ER-HR-3 (monocytes/macrophages) (DPC Biermann, Bad Nauheim, Germany) (1:50), rat anti-CD3 (Serotec, Düsseldorf, Germany) (1:50), rat anti-GR-1 (BD Pharmingen, Hamburg, Germany) (1:100). To count interstitial cells 10 high power fields (400×) were analyzed by a blinded observer.

Intravital microscopy

Intravital microscopy after surgical preparation of cremaster muscles was performed as previously described in detail [13]. At $3\frac{1}{2}$ hours after intrascrotal injection of either LPS (0.05 μg/kg) or CXCL2 (0.01 μg/kg), intravital microscopic analysis was performed in *tlr4* wild-type and *tlr4* mutant mice ($N = 7$ each). Definitions for rolling, adherent, transmigrating, and interstitial migrating leukocytes were as previously described [13].

Statistical analysis

Data were expressed as mean ± SEM. Comparison of groups was performed using analysis of variance (ANOVA) and post hoc Bonferroni's correction was used for multiple comparisons. Unpaired Student *t* test was used for the comparison of single groups (intravital microscopy and RT-PCR data). Data were normalized by identifying outlyers using box-and-whisker's plot analysis where appropriate. A value of $P < 0.05$ was considered to indicate statistical significance.

RESULTS AND DISCUSSION

Mouse tubular epithelial cells produce CXCL2 upon exposure to LPS or heat-inactivated uropathogenic *E. coli*

We hypothesized that the expression of TLR4 on intrinsic renal cells may contribute to antibacterial immunity in infective pyelonephritis. Thus, we first isolated tubular epithelial cells from kidneys of wild-type mice and *tlr4* mutants and studied CXCL2 mRNA expression 24 hours after incubation with medium, ultrapure 1 μg/mL LPS, or heat-inactivated uropathogenic *E. coli*. Tubular epithelial cells did not express CXCL2 mRNA under basal culture conditions (Fig. 1A). LPS and uropathogenic *E. coli* both induced CXCL2 mRNA levels in tubular epithelial cells macrophages isolated from wild-type mice. By contrast, cells isolated from *tlr4* mutant mice did not respond to ultrapure LPS, but showed some induction of CXCL2 mRNA when exposed to heat-inactivated uropathogenic *E. coli* (Fig. 1A). CXCL2 is the murine homologue of a group of human CXC-chemokines, including GRO-α, -β, -χ, and interleukin (IL)-8, that mediate neutrophil recruitment and antibacterial immunity during urinary tract infection in mice and humans [14–16]. These data indicate that among the multiple ligands of uropathogenic *E. coli* LPS is the dominant trigger for CXCL2 mRNA expression via TLR4 in tubular epithelial cells and macrophages.

TLR4 is required for LPS-induced leukocyte recruitment in vivo

We used intravital microscopy to study the functional role of TLR4 and CXCL2 for the recruitment of intravascular leukocytes in vivo. *Tlr4* mutant mice showed impaired leukocyte adhesion and transendothelial migration after intrascrotal injection of nonultrapure LPS as compared to wild-type control mice (Fig. 1B). By contrast, leukocyte adhesion and transendothelial migration were comparable in both mice strains after intrascrotal injection of CXCL2 (Fig. 1B). These data show that TLR4 is required for LPS-induced leukocyte recruitment in vivo, although in nonultrapure LPS preparations signaling through other TLRs may occur. These data are consistent with the concept that LPS recognition by TLR4 is a proximal event and that subsequent CXCL2-mediated leukocyte recruitment is independent of TLR4 in vivo [14, 17].