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Cutting Edge: $Tlr5^{-/-}$ Mice Are More Susceptible to *Escherichia coli* Urinary Tract Infection[1]

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Although TLR5 regulates the innate immune response to bacterial flagellin, it is unclear whether its function is essential during in vivo murine infections. To examine this question, we challenged $Tlr5^{-/-}$ mice transurethrally with Escherichia coli. At 2 days postinfection, wild-type mice exhibited increased inflammation of the bladder in comparison to $Tlr5^{-/-}$ mice. By day 5 postinfection, $Tlr5^{-/-}$ mice had significantly more bacteria in the bladders and kidneys in comparison to wild-type mice and showed increased inflammation in both organs. In addition, flagellin induced high levels of cytokine and chemokine expression in the bladder that was dependent on TLR5. Together, these data represent the first evidence that TLR5 regulates the innate immune response in the urinary tract and is essential for an effective murine in vivo immune response to an extracellular pathogen. The Journal of Immunology, 2007, 178: 4717–4720.

Toll-like receptors are a family of germline-encoded innate immune receptors that recognize pathogen-associated molecular patterns, such as bacterial flagellin (TLR5), LPS (TLR4), and lipopeptides (TLR1/2/6) (1). Expression of TLRs varies among cells and tissues, suggesting that individual TLRs may regulate distinct pathogen and organ-specific roles in host defense to different pathogens (1). We previously discovered that the ligand for TLR5 is bacterial flagellin, the most abundant protein in the whip-like tails of flagellated bacteria (2). We defined the TLR5 recognition site on flagellin and found that it is conserved among a wide variety of flagellated bacteria (3), although select bacterial species possess unique flagellin molecules that evade TLR5 recognition (4). TLR5 is expressed in epithelial cells of the airways, intestine, and urogenital tract, as well as on hemopoietic cells of the innate and adaptive immune system and has recently been shown to be involved in the transport of flagellated *Salmonella typhimurium* from the intestinal tract to the mesenteric lymph nodes (5).

Many important pathogenic bacteria, both Gram-positive and Gram-negative, are flagellated. Flagellated uropathogenic *E. coli* (UPEC)[4] cause 70–90% of all urinary tract infections (UTI), and their pathogenesis involves contact between bacteria and the epithelial cell surface of the urogenital tract, a site of TLR5 expression in humans (6). UPEC colonize the urethra and ascend to the bladder, where they can persist at high levels (7). In addition to cystitis in the bladder, UPEC may ascend to the kidney and cause serious complications, including pyelonephritis and bacteremia (8). *E. coli* is recognized by several TLRs, including TLRs 2, 4, 5, and 11, and likely also TLR9. Previous studies indicate that TLR4 and TLR11 regulate susceptibility to UTIs (9–11). However, it is not currently known whether TLR5 is critical for host defense to UTIs or whether there is sufficient TLR redundancy to obviate its requirement.

Materials and Methods

Mice, bacteria, and TLR agonists

$Tlr5^{-/-}$ mice (strain designation B6.129P2-$Tlr5^{tm1Aki}$) were derived and backcrossed to a C57BL/6 background for eight generations as previously described (5). Wild-type (WT) control mice were from a C57BL/6 background (The Jackson Laboratory). *E. coli* strain CFT073, from a patient with acute pyelonephritis (American Type Culture Collection), was grown in Luria-Bertani (LB) medium in static culture at 37°C for 48 h. Expression of type 1 pili was confirmed for each experiment by testing for yeast agglutination (12). Flagellin was purified from *S. typhimurium* as described in Ref. 3 and was heated to 70°C for 15 min to monomerize it. Contaminating endotoxin was removed by passage through a 100-kDa molecular mass cutoff filter (Millipore) followed by endotoxin removal on a polymixin B column (Pierce). The resulting flagellin did not show detectable endotoxin by *Limulus* assay (Cambrex). Ultrapure LPS was purchased from List Biologicals.

Real-time PCR

RNA was extracted from organs with TRIzol (Invitrogen Life Technologies), DNase treated with TURBO DNA-free (Ambion), and cDNA produced with Superscript II (Invitrogen Life Technologies). Real-time PCR was performed with TaqMan Fast (Applied Biosystems) on an Applied Biosystems Prism 7900 HT. Primer/probe sets for elongation factor 1α (EF1α) were designed with

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[4] Abbreviations used in this paper: UPEC, uropathogenic *E. coli;* UTI, urinary tract infection; EF1α, elongation factor 1α; CHO, Chinese hamster ovary; LB, Luria-Bertani; IQR, interquartile range; WT, wild type.

Primer Express 1.0 (PerkinElmer) with a 5′-FAM and 3′-TAMRA modification (Biosearch Technologies). Primer/probe sets for mouse TLR5 and cytokines/chemokines were purchased from Applied Biosystems. Threshold cycle (Ct) values were transformed by $1/2^{Ct}$, and then normalized to EF1α for each organ.

NF-κB luciferase reporter assay

Chinese hamster ovary (CHO) K1 cells stably expressing mouse TLR5 and NF-κB luciferase reporter constructs (3) were stimulated with heat-killed bacteria for 4 h and assayed for luciferase activity. Assays were done in duplicate, and the experiment was repeated three times. Percent fold induction was calculated by dividing the luciferase values for each bacterial dose by the maximal luciferase value for the bacteria in each experiment.

UTI model of infection

The Institute for Systems Biology and Osaka University Institutional Animal Care and Use Committees approved all animal protocols. Forty-eight-hour static cultures of E. coli CFT073 were resuspended in cold PBS at 1×10^9 CFU/ml. Anesthetized mice were inoculated transurethrally with 5×10^7 E. coli in 50 μl, and urethras were coated with collodion (Sigma-Aldrich) (13). Six hours postinfection, collodion was removed by blotting with acetone. At each time point, organs were homogenized in 1 ml of 0.025% Triton X-100/PBS and plated on LB-agar to enumerate CFUs.

Statistical analysis

Comparisons were made with a two-tailed Mann-Whitney U test or a Student's t test. A $p \le 0.05$ was considered to be significant. Statistics were calculated with PRISM4 (GraphPad).

Histology

Bladders and kidneys were fixed in 10% formalin-buffered saline and embedded in paraffin. Four-micrometer sections were cut, stained with H&E, and examined by a pathologist blinded to mouse genotype.

Results and Discussion

Tlr5$^{-/-}$ mice are more susceptible to E. coli *urinary tract infection*

To test our hypothesis that TLR5 is critical for host defense against E. coli UTIs, we first examined bladder and kidney tissue for TLR5 expression. We extracted RNA from tissues of C57BL/6 mice and evaluated expression levels by real-time PCR (Fig. 1A). TLR5 was expressed in both bladder and kidney, which suggested that it may regulate critical aspects of the immune response during UTI. We next examined whether *Tlr5$^{-/-}$* mice were more susceptible to urinary tract infections. When grown in static culture, uropathogenic E. coli forms type 1 pili (12) that enhance adherence to bladder epithelia and increase bladder colonization. We first determined whether growth in static culture resulted in flagellin expression. CHO cells stably expressing mouse TLR5 and a NF-κB-dependent luciferase reporter construct responded to statically grown heat-killed bacteria in a dose-dependent manner, detecting fewer than 8000 bacteria, a multiplicity of infection of ~0.1 (Fig. 1B). Control CHO cells expressing the pEF6 vector alone did not respond to bacteria (data not shown).

To test the role of TLR5 during infection in vivo, we inoculated WT and *Tlr5$^{-/-}$* mice transurethrally with 5×10^7 CFU of statically cultured E. coli. Bladder and kidneys were harvested 4 h, 24 h, 2 days, and 5 days after infection and the number of CFU in each organ was determined. At early time points, no difference was seen in bladder CFU between WT and *Tlr5$^{-/-}$* mice (Fig. 1C). In contrast, although all mice remained infected at day 5, the number of CFU per bladder was reduced in WT mice but rose dramatically in *Tlr5$^{-/-}$* mice. WT mice had a median of 475 CFU/bladder (interquartile range (IQR): 212.5–685) in comparison to *Tlr5$^{-/-}$* mice with a median of 7.7×10^5 CFU/bladder (IQR: 9.0×10^4–4.1×10^6, $p < 0.0001$ by Mann-Whitney U test) (Fig. 1C).

We next examined whether the E. coli disseminated to the kidney. Bacteria were present in the kidneys of both WT and TLR5-deficient animals, and no significant CFU differences were observed between WT and *Tlr5$^{-/-}$* mice in the kidney at early time
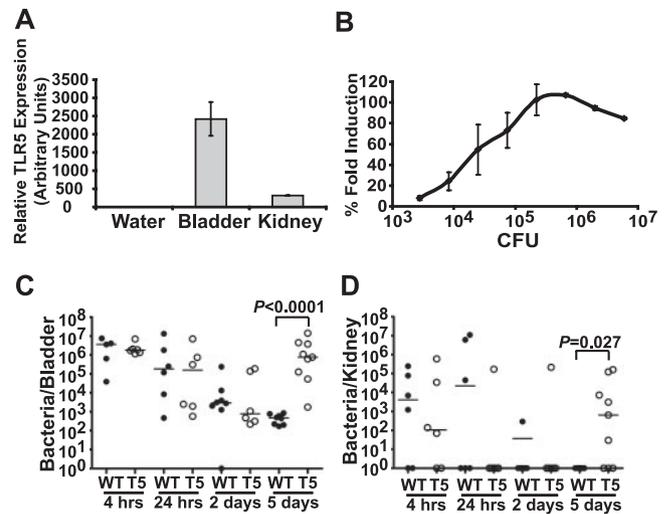
FIGURE 1. *Tlr5$^{-/-}$* mice are more susceptible to E. coli UTI. *A,* Total RNA was isolated from bladders and kidneys of two WT and two *Tlr5$^{-/-}$* mice. cDNA was prepared and real-time PCR analysis was performed. TLR5 mRNA levels are expressed as a ratio to EF1α mRNA expression. Data are representative of two experiments, each performed in triplicate. Error bars, 1 SD. *B,* NF-κB luciferase activity (percent fold induction) for CHO cells stably expressing mouse TLR5 and NF-κB luciferase reporter constructs. Cells were stimulated at a range of bacterial doses and data are from one representative experiment of three independent experiments run in duplicate. Error bars, 1 SD. *C* and *D,* E. coli bacterial counts in the bladder (*C*) and kidney (*D*) of WT and *Tlr5$^{-/-}$* (T5) mice. Mice were inoculated transurethrally with 5×10^7 E. coli CFT073 and 5 days later bladder and kidneys were removed. Organ homogenates were plated on LB agar to enumerate the CFUs per organ. Colony counts were averaged from two plates per mouse. The Mann-Whitney U test was used to determine the p values for CFU differences. Median values are depicted with a line.

points (Fig. 1D). By day 5 after infection, however, no bacteria were detected in WT kidneys, but *Tlr5$^{-/-}$* mice had a median of 6.5×10^2 CFU/kidney (IQR: 0- 6.5×10^4, $p = 0.0274$ by Mann-Whitney U test) with six of nine infected mice showing kidney counts (Fig. 1D). Together, these data suggest that *Tlr5$^{-/-}$* mice are unable to control bacterial replication and cannot clear the infection from the kidneys by day 5.

Tlr5$^{-/-}$ mice exhibit decreased inflammation at 2 days postinfection

We next examined histologic sections from WT and knockout mice by light microscopy to determine the pathologic consequences of TLR5 deficiency. Bladders and kidneys from WT and *Tlr5$^{-/-}$* mice exhibited similar levels of inflammation at 4 and 24 h after infection (data not shown). In contrast, at 2 days postinfection, *Tlr5$^{-/-}$* mice showed decreased inflammation in the bladder relative to WT mice (Fig. 2A). WT mice exhibited prominent submucosal edema and infiltration of the submucosa and epithelium by leukocytes. There was no significant inflammation in the kidneys from WT or *Tlr5$^{-/-}$* mice at the 2-day time point (data not shown).

By day 5 postinfection when TLR5-deficient mice showed increased bacterial counts, the situation was reversed, with prominent inflammation in the TLR5-deficient animals. WT mice showed minimal to no inflammation in the bladder and kidney, and bacteria were not visible in the lumen of these organs (Fig. 2, B and C). In contrast, bladders from *Tlr5$^{-/-}$* mice showed prominent submucosal edema with leukocyte infiltration into the submucosa and invasion of the epithelial layer, as well as focal microabscess formation and accumulation of leukocyte-rich exudates on the bladder surface (Fig. 2B). Bacteria were readily visible and present predominantly on the surface of the *Tlr5$^{-/-}$* urothelium without prominent evidence of enclosure within the umbrella cells of the bladder, such as the intracellular
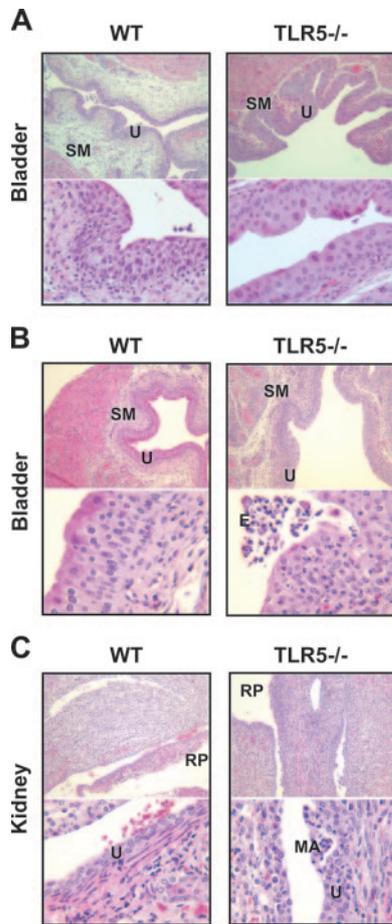
FIGURE 2. $Tlr5^{-/-}$ mice exhibit decreased inflammation at 2 days postinfection. *A–C,* H&E-stained histologic sections of mouse bladder (*A* and *B*) and renal pelvis (*C*) at 2 days (*A*) and 5 days (*B* and *C*) after infection. SM, Submucosa; U, urothelium; E, leukocyte-rich exudate; RP, renal pelvis; MA, microabscess. The panels were photographed at ×100 (*upper*) and ×400 (*lower*) magnification.

bacterial communities described by others (14). A similar but less pronounced pattern of inflammation was also present in the $Tlr5^{-/-}$ kidneys at day 5, and primarily involved the urothelium in the renal pelvis with focal extension into the interstitium of the renal medulla (Fig. 2*C* and data not shown). Together, these data indicate a turning

point at 2 days postinfection: $Tlr5^{-/-}$ mice manifest decreased inflammation that leads to overwhelming bacterial growth and more severe inflammation by day 5 after infection.

Flagellin induces early expression of proinflammatory genes in the bladder

To identify proinflammatory molecules up-regulated by TLR5 in the bladder that might lead to increased early inflammation in WT mice, we examined the in vivo response to transurethral administration of flagellin and compared this with LPS, another prominent TLR agonist present in *E. coli*. Mice were inoculated transurethrally with 30 μg of flagellin or 10 μg of ultrapure LPS in PBS, and bladders were harvested at 4 h. Real-time PCR was performed on bladder tissue for several proinflammatory cytokines and chemokines. Transurethral inoculation of flagellin up-regulated expression of KC (CXCL1), MIP2 (CXCL2), MCP-1 (CCL2), IL-6, and TNF-α mRNA, but not β-defensin 1 mRNA in WT mice (Fig. 3). As expected, $Tlr5^{-/-}$ mice did not respond to flagellin in the bladder. In contrast to flagellin, LPS delivered into the bladders of WT mice did not induce transcription of these proinflammatory genes. These results demonstrate that flagellin induces a robust TLR5-dependent innate immune response in the murine bladder that may account for its critical role in UTI pathogenesis.

Taken together, these data demonstrate that TLR5 plays a crucial role in host defense to UPEC infection by mediating flagellin-induced inflammatory responses in the bladder that limit bacterial replication in both the bladder and kidney. Two additional TLRs, TLR4 and TLR11, have also been shown to play a role in *E. coli*-induced UTI. TLR4-deficient C3H/HeJ mice exhibit a reduced inflammatory response to UPEC and exhibit significantly higher bacterial counts in the bladder and kidneys (9, 15, 16). In contrast to the inflammatory response to flagellin, we found that the bladder was relatively unresponsive to LPS. There is conflicting evidence about whether the urinary epithelium responds to LPS (12, 17–19). Our experiments comparing in vivo delivery of highly purified flagellin and LPS suggest that TLR5 and TLR4 regulate distinct bladder innate immune responses with TLR5 regulating a relatively dominant role initially. TLR11, which is a pseudogene in humans, is expressed in both kidney and bladder epithelial cells of mice (11). Infection of TLR11-deficient mice with the UPEC strain 8NU resulted in approximately equal colonization of the bladders of TLR11-deficient and WT mice, but significantly more bacteria ascended to the kidneys of TLR11-deficient mice (11). Thus, it is possible that TLR5 and TLR11 play complementary roles in the
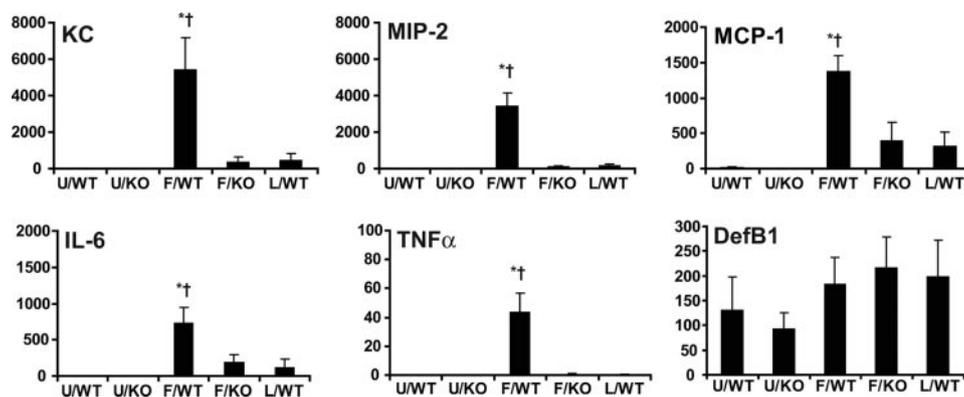
FIGURE 3. Bladder gene expression after transurethral stimulation with TLR agonists. Total RNA was isolated from bladders of unstimulated mice (U) or mice stimulated transurethrally with 30 μg of *S. typhimurium* flagellin (F) or 10 μg of LPS (L). cDNA was prepared and real-time PCR analysis was performed. Data are a combination of two experiments with $n = 2$ mice for the unstimulated WT C57BL/6 and $Tlr5^{-/-}$ (KO) mice, $n = 7$ for WT mice stimulated with flagellin, $n = 4$ for $Tlr5^{-/-}$ mice stimulated with flagellin, and $n = 7$ for WT mice stimulated with LPS. mRNA levels of cytokines and chemokines are expressed as a ratio to EF1α mRNA expression. *, $p < 0.05$ by Student's *t* test for comparison of flagellin stimulation in WT vs knockout. †, $p < 0.05$ for comparison for flagellin vs LPS stimulation in WT mice.

mouse urinary tract, with TLR5 limiting bladder replication and TLR11 primarily controlling bacterial invasion of the kidney.

Bacterial motility is important for virulence in some models of UTI, which suggests that TLR5-flagellin interactions may be important for bacterial uropathogenesis. Flagellum-negative mutants of *Proteus mirabilis* are significantly less successful at bladder colonization and do not progress to the kidneys as readily (20). Furthermore, two recent studies suggest that flagellar mutants of *E. coli* are less able to colonize the mouse urinary tract (8, 14). Flagellar-based motility may be beneficial in early colonization of the urinary tract, but may not be required for maintenance of infection (8). This is in agreement with a study that demonstrated down-regulation of flagellin genes by *E. coli* CFT073 several days after in vivo infection (21). These studies combined with our results suggest that TLR5 recognition of flagellin is an important component of the innate immune response to *E. coli* during the early stages of UTI when flagellin expression and motility contribute to colonization of the urinary tract.

In addition to TLR5 recognition of extracellular bacterial flagellin, two novel intracellular flagellin receptors have recently been described that are both members of the nucleotide-binding oligomerization domain leucine-rich repeat family (22–24). Naip5 detects flagellin from *Legionella pneumophila* that reaches the macrophage cytosol via the bacteria's type IV secretion system (24, 25). Ipaf detects cytoplasmic flagellin injected into macrophages by the type III secretion system of *S. typhimurium* (22). The roles of these additional flagellin receptors in UTI are not known, but the UPEC strain used in this study, like many other UPEC (26), does not encode a type III secretion system (27). Thus, intracellular flagellin receptors may not be able to compensate for the lack of flagellin recognition by TLR5 in this infection model.

Although TLR5 has been implicated in the innate immune response to mucosal infection (5, 28), there has been limited in vivo data to substantiate this claim. A recent study of $Tlr5^{-/-}$ mice did not find a unique role for TLR5 in defense to *Salmonella* or *Pseudomonas* (29). Our study provides the first evidence that TLR5 regulates a critical and nonredundant role in the innate immune response to a murine infection with extracellular flagellated bacteria. We have previously identified a TLR5 polymorphism present in the general population that results in a stop codon that abrogates TLR5 signaling and is associated with increased susceptibility to Legionnaire's disease (30). These murine studies support a hypothesis that individuals who possess this TLR5 variant will also be more susceptible to UTI.

Acknowledgments

Disclosures

The authors have no financial conflict of interest.

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