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Bacterial Invasion Augments Epithelial Cytokine Responses to Escherichia coli Through a Lipopolysaccharide-Dependent Mechanism

Joel D. Schilling,* Matthew A. Mulvey,* Carr D. Vincent,* Robin G. Lorenz,†† and Scott J. Hultgren2,*

One mechanism of initiating innate host defenses against uropathogenic Escherichia coli (UPEC) is the production of cytokines by bladder epithelial cells; however, the means by which these cells recognize bacterial pathogens is poorly understood. Type 1 pili, expressed by the majority of UPEC, have been shown to have a critical role in inducing the expression of IL-6 in bladder epithelial cells after exposure to E. coli. In this study, we demonstrate that type 1 pili are not sufficient to activate IL-6 production by bladder epithelial cells. Instead, it was shown that bacterial invasion mediated by type 1 pili augments bladder epithelial responses to E. coli via an LPS-dependent mechanism, leading to the production of IL-6. RNA transcripts for the LPSR Toll-like receptor 4 (TLR4) was detected in cultured bladder epithelial cells. The in vivo role of TLR4 was assessed using C3H/HeJ mice, which express a dominant negative form of TLR4. After infection with UPEC, C3H/HeJ mice have large foci of intracellular bacteria that persist within the bladder epithelium in the absence of any notable inflammatory response. These results indicate that LPS is required for bacterial invasion to enhance host responses to E. coli within the bladder. The Journal of Immunology, 2001, 166: 1148–1155.

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1 Address correspondence and reprint requests to Dr. Scott Hultgren, Department of Molecular Microbiology, Washington University School of Medicine, 660 South Euclid Avenue, St. Louis, MO 63110. E-mail address: hultgren@borcim.wustl.edu

2 Department of Molecular Microbiology, * Department of Pathology and Immunology, and † Department of Internal Medicine, Washington University School of Medicine, St. Louis, MO 63110.

3 Abbreviations used in this paper: UTI, urinary tract infection; UPEC, uropathogenic E. coli; mCD14, membrane-bound CD14; TLR, Toll-like receptor; TMP-SMZ, trimethoprim-sulfamethoxazole; H&E, hematoxylin and eosin.

Materials and Methods

Bacteria and growth conditions

AAEC185 (19), AAEC185/put2002, AAEC185/pSH2, and UTI 89 were grown static in Luria broth for 48 h at 37°C to induce the expression of type 1 pili. UTI 89 is a type 1-piliated clinical cystitis isolate kindly provided by Dr. Sol Langermann from Medimmune (M. A. Mulvey, J. D. Schilling, and S. J. Hultgren, manuscript in progress). Before all experiments, the expression of type 1 pili was confirmed by mannose-inhibitable yeast agglutination.

Cell culture

5637 epithelial cells (derived from a human bladder carcinoma, HTB-9; American Type Culture Collection, Manassas, VA) were cultured in RPMI 1640 medium (BioWhittaker, Walkersville, MD) plus 10% FBS (Sigma, St. Louis, MO) at 37°C in a water-saturated atmosphere of 95% air and 5% CO2.
IL-6 stimulation assay

5637 epithelial cells were seeded into 24-well plates at 0.3 x 10^5 to 1 x 10^5 cells per well and grown to confluency over 2 days. Forty-eight-hour bacterial cultures were pelleted by centrifugation, resuspended in PBS, and diluted to the indicated concentrations in RPMI 1640 medium plus 10% FBS plus the growth inhibiting antibiotic trimethoprim-sulfamethoxazole (TMP-SMZ) (Alpharma) at concentrations of 54 µg/ml and 270 µg/ml respectively. TMP-SMZ was used to allow the accurate determination of dose-response relationships. Bacterial suspensions were titrated at the onset of the infection to determine the number of live bacteria in every experiment. One milliliter of the bacterial suspension or 20 ng/ml of human rIL-1α (positive control for cytokine induction) (R&D Systems, Minneapolis, MN) was added per well of epithelial cells. The bacteria were spun onto the cells by low-speed centrifugation and incubated at 37°C for the indicated times. Supernatants were collected that contained cells and bacteria were removed by centrifugation, and samples were frozen at −80°C until assayed using an IL-6 ELISA (R&D Systems).

Incubation with FimCH-coated beads

FimCH-coated beads or BSA-coated beads were prepared as previously described (9). The beads were removed from storage buffer (10 ng/ml BSA without 100 µg/ml of LPS derived from L. pneumophila) and resuspended at concentrations ranging from 0.5 ng/ml to 50 ng/ml for 6 h, after which the beads were spun onto the cell cultures by low-speed centrifugation and incubated at 37°C for 6 h. The supernatants were collected and analyzed as described above.

Invasion assays and cytochalasin D treatment

5637 cells were pretreated with 0.1 µg/ml of cytochalasin D in RPMI 1640 medium plus 10% FBS for 30 min at 37°C. After the preincubation, 10 µl of 1 x 10^4 CFU/ml of bacteria in PBS was added to the cells. Bacteria were spun onto the cells and incubated at 37°C for 6 h. At the 6-h time point, supernatants were either collected for ELISA or incubated with 100 µg/ml of gentamicin to kill any extracellular bacteria. After 2 h in the presence of gentamicin, the wells were washed, lysed with 0.1% Triton X-100, and titered for viable counts of intracellular bacteria.

To determine whether intracellular E. coli could continuously stimulate epithelial IL-6 production, cells were left uninfected or infected with 20 µl of AAEC185/pSH2 (OD_{600} 0.5) for 1 h (sufficient time for significant invasion to occur (9)). Cells were then washed with PBS and incubated for 2 h with media containing 100 µg/ml of gentamicin to kill any extracellular bacteria. Subsequently, the cells were washed three times with PBS and fresh medium containing 5 µg/ml of gentamicin was added. Also, at this time, uninfected wells were left unstimulated or stimulated with 20 ng/ml of IL-1α. Supernatants were collected at 2, 6, and 24 h after the addition of the low-concentration gentamicin solution and were analyzed as described above. Intracellular titer of AAEC185/pSH2 were determined at each time point as described above.

Inhibition of LPS-mediated responses

Log increments of E. coli LPS 055:B5 (Sigma) ranging from 0.5 ng/ml to 50 µg/ml were preincubated with or without 1 µg/ml of polymyxin B sulfate (Sigma) for 15 min. 5637 cells were subsequently stimulated with the LPS preparations and the IL-6 concentration of the supernatants was determined 6 h poststimulation as described above. To analyze the inhibitory properties of detoxified LPS, 5637 cells were preincubated with or without 100 µg/ml of LPS 055:B5 for 30 min at 37°C. The cells were subsequently stimulated with LPS 055:B5 at concentrations ranging from 0.5 ng/ml to 50 µg/ml for 6 h, after which the IL-6 concentration of the supernatants was determined as described above. Bacterial suspensions were prepared as described above and left untreated, treated with polymyxin B (1 µg/ml) (17), or treated with gentamicin (100 µg/ml). Cytokine stimulation assays were performed as described above. To block epithelial recognition of LPS, 5637 cells were preincubated with 100 µg/ml of detergent LPS for 30 min at 37°C. After the pretreatment, 10 µl of 1 x 10^4 CFU/ml of E. coli bacteria in PBS were added to the cells. Bacteria were spun down onto the cells and incubated at 37°C for 6 h. The supernatants were analyzed as described above.

RT-PCR assay

THP-1 cells (TIB-202; American Type Culture Collection) were grown in RPMI 1640 medium (Life Technologies, Rockville, MD) containing 2 mM l-glutamine, 1.5g/l sodium bicarbonate, 4.5 g/l glucose, 1.0 mM sodium pyruvate, 0.05 mM 2-ME, and 10% fetal bovine serum (Sigma). Approximately 24 h before RNA isolation, 5 x 10^6 THP-1 cells were differentiated with 10 ng/ml PMA (Sigma). 5637 cells were grown to confluency as described above.

RNA was extracted from ~5 x 10^6 cells using TRIzol reagent (Life Technologies) according to manufacturer’s protocols. Subsequently, the RNA was treated with DNase I, Amplification Grade (Life Technologies) according to manufacturer’s protocols. RNA samples were stored at −80°C.

RT-PCR was performed using the Enhanced Avian RT-PCR Kit (Sigma) according to manufacturer’s protocols for the one-step method. Approximately 18 ng of RNA template was added to each reaction. Primers for TLR4 (506 bp) (20) and TLR2 (346 bp) (21) have been previously described. The products of the reverse transcriptase reaction were amplified by PCR for 35 cycles, with an annealing temperature of 52°C. Initial extension time was 45 s and every 5 cycles the extension time was increased by 30 s. PCR products were analyzed on a 1.5% agarose gel.

Mouse Infections

C3H/HeN and C3H/HeJ female mice were obtained from Harlan Sprague and Jackson Laboratory, respectively. A 48-h culture of UTL89 was pelleted and resuspended in sterile PBS to a concentration of ~2 x 10^5 CFU/ml. Mice were injected via intraperitoneal catheterization with 50 µl of the bacterial suspension (~1 x 10^6 CFU). At 10 and 48 h after infection, five mice from each group were sacrificed by cervical dislocation and the bladders were harvested. Bladders were bisected and either fixed in neutral buffered formalin for histological analysis, or homogenized in sterile 0.025% Triton X-100/PBS, and titrated for surviving bacteria. The bladder tissue was embedded in paraffin and sections were stained with hematoxylin and eosin (H&E) or with a rabbit anti-E. coli primary Ab (BioDesign, New York, NY) and goat anti-rabbit IgG-Cy3 secondary Ab (Jackson ImmunoResearch, West Grove, PA). Nuclei were counterstained with 500 ng/ml Hoechst (Sigma).

Results

Type 1 pili augment bladder epithelial IL-6 production in response to E. coli

IL-6 is a pleiotropic cytokine that has among its functions the amplification of neutrophil recruitment and the activation and differentiation of T and B cells (21–25). The rapid up-regulation of IL-6 during UTIs in humans and mice make this molecule an ideal read-out for epithelial activation in response to E. coli (26, 27). To separate the function of type 1 pili from other bacterial virulence factors, 5637 human bladder epithelial cells were infected with K12 E. coli expressing (AAEC185/pSH2) or not expressing (AAEC185) type 1 pili and the IL-6 concentration in the supernatants was determined. TMP-SMZ, a bacteriostatic antibiotic frequently used in the treatment of UTIs, was always coincubated with the bacteria to prevent growth during the assay (3). TMP-SMZ has no affect on the induction profile of IL-6 by type 1 and nonpiliated E. coli (unpublished data). Inhibition of bacterial proliferation using TMP-SMZ allowed a more detailed and accurate dissection of dose-response effects, because the number of bacteria was constant throughout the assay. As a positive control, 5637 cells were stimulated with IL-1α.

Infection of 5637 cells with AAEC185 or AAEC185/pSH2 at doses ranging from 10^3 to 10^6 CFU/ml (multiplicity of infection, 0.01–100) revealed two distinct thresholds for epithelial activation (Fig. 1A). At 1 x 10^5 CFU/ml, both AAEC185/pSH2 and AAEC185 induced 5637 IL-6 production ~5-fold over unstimulated cells (Fig. 1A). The threshold for type 1 pili-specific amplification of IL-6 expression was attainment at bacterial concentrations of ~1 x 10^5 CFU/ml. At its peak of stimulation, AAEC185/pSH2 elicited up to 4- to 7-fold more IL-6 than did AAEC185 (Fig. 1A).

Inoculation of bladder cells with concentrations of E. coli above 5 x 10^5 CFU/ml resulted in a dramatic decline of IL-6 secretion. Kinetic analysis of IL-6 production showed that from 3 to 12 h after infection, type 1-piliated E. coli induced three times more cytokine than the isogenic nonpiliated strain at an equivalent dose (~1 x 10^6 CFU/ml) (Fig. 1B). The fimH^- mutant strain
which produces nonadhesive type 1 pili, behaved identically to the parental strain AAEC185 with respect to IL-6 induction (Fig. 2).

FimH is not sufficient to induce epithelial IL-6 production

The hypothesis that FimH binding to an epithelial receptor is sufficient to induce epithelial IL-6 was investigated by using polystyrene latex beads coated with purified FimCH. Purification of FimH requires that it be coexpressed with the FimC chaperone, to prevent its misfolding and proteolytic degradation (5). In the FimCH complex, FimH has its native receptor binding structure (28). FimCH-coated beads efficiently bind to and are internalized by 5637 cells via a pathway that, like that observed with FimH-expressing bacteria, is dependent on actin polymerization, tyrosine phosphorylation, and is mannose inhibitable (9). When FimCH-coated beads were incubated with 5637 cells, no IL-6 induction was observed, suggesting that signals derived from FimH-host receptor interactions are not sufficient to activate IL-6 transcription (Fig. 2).

Cytochalasin D inhibits type 1 pilus-mediated augmentation of IL-6 production

Previous studies have demonstrated an association between bacterial invasion and epithelial cytokine production (1, 26, 29). Cytochalasin D, a drug that inhibits internalization but does not affect bacterial adherence (9), was used to prevent bacterial invasion of bladder epithelial cells. Cytochalasin D had no effect on IL-6 production when 5637 cells were incubated with IL-1α or the noninvasive, type 1 + strain AAEC185 (Fig. 3A). However, it reduced epithelial IL-6 induction by the type 1 + strain, AAEC185/pSH2, to levels similar to those seen after stimulation with the noninvasive strain (Fig. 3A). At this dose of cytochalasin D, invasion of bladder epithelial cells by AAEC185/pSH2 was reduced by 80% over the 6-hour assay (data not shown). Thus, the data show that FimH-mediated bacterial adherence is not sufficient and strongly argue that subsequent invasion is required for the augmentation of IL-6 production by epithelial cells in response to type 1-piliated E. coli.

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Induction of IL-6 by invading bacteria is transient

To determine whether cytokine stimulation occurs transiently or continuously after stimulation with type 1-piliated E. coli, AAEC185/pSH2 was allowed to invade 5637 cells for 1 h and the cells were then treated with gentamicin to kill extracellular bacteria. Subsequently, the supernatant containing dead bacteria and cellular debris was replaced with fresh medium. IL-6 production was monitored by collecting supernatants at 2, 6, and 24 h after invasion. It should be noted that IL-6 is stable for 24 h under these infection conditions (unpublished data). After an initial peak between 0 and 2 h after invasion, IL-6 production by 5637 cells plateaued between 2 and 24 h, although bacteria persisted at similar levels intracellularly (Fig. 3B, data not shown). In contrast, cells stimulated with IL-1α continued to produce IL-6 throughout this time interval (Fig. 3B). These results demonstrate that the presence of intracellular E. coli does not lead to continuous stimulation of IL-6, suggesting that epithelial cytokine induction by invasive bacteria is transient. Furthermore, the observation that IL-6 levels fail to significantly increase after 2 h postinvasion demonstrates that IL-6 is poorly induced in the absence of active bacterial internalization.

LPS is required for epithelial IL-6 production in response to type 1-piliated E. coli

The above data demonstrate that type 1 pili do not directly activate IL-6 production, but instead mediate bacterial invasion, which in turn amplifies the induction of IL-6. Therefore, we sought to identify the stimulatory bacterial molecule recognized by bladder epithelial cells. 5637 bladder epithelial cells responded to LPS in a dose-dependent manner (Fig. 4, A and C). As expected, reagents that block LPS recognition by host cells inhibited this response. Polymyxin B (Fig. 4A) and detoxified LPS (Fig. 4C) at concentrations of 1 μg/ml and 100 μg/ml, respectively, shifted the threshold of activation after stimulation with E. coli 055:B5 LPS from 5–50 ng/ml to 50–500 ng/ml. The ability of increasing concentrations of LPS to overcome the inhibition is consistent with these agents competitively inhibiting LPS-mediated responses in 5637 cells.

To investigate whether bacterial associated LPS contributes to bladder epithelial cytokine production, we used these two inhibitory reagents. Before infection, E. coli strains were preincubated with a low dose of polymyxin B (1 μg/ml). As a control for the loss of viability that can be associated with polymyxin B, bacteria

**FIGURE 4.** LPS is required for epithelial IL-6 production in response to E. coli. 5637 cells were stimulated with the indicated doses of E. coli 055:B5 LPS in the presence (■) or absence (□) of polymyxin B (A) or detoxified LPS (C). B. Cytokine production by 5637 cells was monitored after stimulation with ~10^6 CFU/ml AAEC185 or AAEC185/pSH2 in the presence of TMP-SMZ alone (□), TMP-SMZ plus gentamicin (G) (□), or polymyxin B (PM) (□). D. Similar assays were performed in the presence (□) or absence (■) of detoxified LPS. Experiments were repeated at least three times in triplicate. *p ≤ 0.05 according to a Student’s t test (determined using GraphPad Prism).
were treated with 100 μg/ml of gentamicin, a bactericidal antibiotic that has no effect on LPS. TMP-SMZ was present in all of the assays to prevent bacterial growth. Bacteria treated with gentamicin + TMP-SMZ elicited similar levels of IL-6 as bacteria incubated with TMP-SMZ alone, indicating that bacterial viability is not required for the induction of epithelial IL-6 (Fig. 4B). In contrast, treatment of both the pilated (AAEC185/pSH2) and nonpiliated (AAEC185) bacteria with polymyxin B signaling IL-6 induction by 79% and 86%, respectively, but had no effect on IL-1α induction of IL-6 (Fig. 4B and data not shown). The invasion efficiency (no. of internalized bacteria/no. of adhered bacteria) was not affected by polymyxin B (data not shown).

In addition, 5637 cells were preincubated with 100 μg/ml of detoxified *E. coli* LPS before bacterial stimulation. Detoxified LPS inhibited IL-6 stimulation by type 1-piliated and nonpiliated *E. coli* by 89% and 100%, respectively (Fig. 4D), but had no effect on IL-1α induction of IL-6 (data not shown). Bacterial adherence and invasion were unaffected by the presence of detoxified LPS (data not shown). Together these data argue strongly that LPS is the primary bacterial factor involved in both invasion-dependent and invasion-independent activation of IL-6 production.

**5637 bladder epithelial cells express TLR4, and TLR2**

TLR4 and TLR2 have been implicated in the recognition of LPS by mammalian cells; however, recent evidence suggests that TLR4 is the primary signal transducer involved in LPS signaling (14, 30, 31). RT-PCR was used to assess the expression of mRNAs for these receptors in 5637 bladder epithelial cells. Differentiated THP-1 cells (macrophage-like cells) have been shown previously to express TLR4 and TLR2 transcripts and were used as a positive control for the RT-PCR (20). TLR4 and TLR2 mRNAs were present in the differentiated THP-1 cells (Fig. 5). Similarly, 5637 bladder epithelial cells clearly expressed mRNA for TLR4 and TLR2 (Fig. 5). Thus, 5637 bladder epithelial cells express mRNAs for receptors implicated in LPS responsiveness.

**LPS-hyporesponsive mice have a severe defect in epithelial responses to type 1-piliated UPEC**

Based on our in vitro findings, LPS-hyporesponsive mice would be predicted to have defects in epithelial responses to type 1-piliated UPEC. C3H/HeJ mice have a mutation in the mammalian LPSR TLR4 and consequently fail to respond to LPS (32, 33). Others have shown that C3H/HeJ mice are unable to clear bacteria from the urinary tract and fail to generate an appropriate inflammatory response after infection with UPEC (34–38). In addition, IL-6 is not present in the urine of C3H/HeJ mice after an infection with *E. coli* (39). Thus, it would seem that TLR4-mediated host responses to bacterial LPS are critical for inducing inflammation in the urinary tract. To further substantiate our in vitro findings, we analyzed the association between internalized bacteria and the activation of the bladder epithelium in these mice.

**FIGURE 5.** 5637 bladder epithelial cells express TLR4 and TLR2 mRNAs. RT-PCR for expression of TLR4 and TLR2 mRNAs was assessed using total RNA from PMA differentiated THP-1 cells (positive control) and 5637 bladder epithelial cells. Reverse transcriptase-specific products were detected for TLR4 and TLR2 in both cell lines.

C3H/HeJ and C3H/HeN mice were infected with UTI 89, a type 1-piliated strain of UPEC, and histological analysis of the bladder epithelium using H&E and anti-*E. coli* immunofluorescence staining was performed at 10 and 48 h after infection. In this study, the recruitment of neutrophils into the epithelium was used as a measure of epithelial activation. At 10 h after infection, both mouse strains had similar numbers of bacteria in the bladder, but there were dramatic differences in the appearance of the bladder epithelium (Figs. 6 and 7, A–C, F, and G). In C3H/HeN mice, large numbers of intracellular bacteria were found within the bladder epithelium (Fig. 7, A and C). Also, at this time point, neutrophils were found to be migrating into the epithelium and specifically associating with infected cells. The influx of neutrophils seemed to correlate with the destruction of the intracellular bacterial foci (Fig. 7, A and B). Large foci of intracellular bacteria were also observed in C3H/HeJ mice, but in contrast to C3H/HeN mice, neutrophils were rarely present (Fig. 7F). By 48 h after infection, C3H/HeN mice had reduced the number of bacteria in the bladder by >90%, and no intracellular foci of bacteria in the epithelium were visible by histological analysis (Figs. 6 and 7E). Moreover, large numbers of neutrophils were present in both the epithelium and lamina propria of these mice (Fig. 7, D and E). In C3H/HeJ mice, no reduction in bacterial titers was observed at 48 h after infection (Fig. 6). Large collections of bacteria remained within the bladder epithelium (Figs. 6 and 7F) with a striking absence of neutrophils in both the epithelium and lamina propria (Fig. 7, H and I). Together these data suggest a dynamic sequence of events, whereby the bladder epithelium is activated by TLR4/bacterial interactions at sites of internalized *E. coli*.

**Discussion**

At mucosal surfaces, bacterial adherence to and/or invasion of epithelial cells are often the initial steps in the establishment of an infection. To better understand bacterial pathogenesis and innate host responses to bacteria, it will be important to characterize the bacterial and host molecules involved in the recognition of microorganisms by epithelial cells. Expression of adhesive organelles called type 1 pili by strains of *E. coli* leads to enhanced bladder epithelial cytokine production in response to infection (10) (Fig. 1). It has been suggested that direct interactions between type 1 pili and specific host receptors on bladder epithelial cells can activate host-signaling cascades leading to IL-6 transcription (11). In this
report, we tested this hypothesis by investigating the underlying mechanism of type 1 pilus-mediated augmentation of epithelial cytokine production.

Recent studies have shown that latex beads coated with the type 1 pilus adhesin, FimH, specifically bind to and are internalized by bladder epithelial cells (9). By using these adhesin-coated beads, it was determined that type 1 pilus-host receptor interactions are not sufficient to activate epithelial cytokine production independent of other bacterial factors. These results suggest that type 1 pili facilitate cytokine production through an indirect mechanism or that other components of the pilus structure contribute to cytokine induction. The finding that AAEC185/put2002 (type1 carriage of fimH) does not illicit IL-6 production above levels seen after stimulation with AAEC185 (type 1 carriage of fimH) demonstrates that other components of the type 1 pilus do not contribute to the activation of bladder epithelial cells in the absence of FimH.

Type 1 pilus-mediated invasion of bladder epithelial cells is associated with a survival advantage for UPEC in vivo (8). In this manuscript, we have shown that inhibition of bacterial invasion using cytochalasin D diminishes the boost in cytokine production observed in response to infection with type 1-piliated E. coli. Of interest, the activation of cytokine production by invasive bacteria is transient, even when the bacterial stimulus persists in the intracellular environment. These data demonstrate that type 1 pili enhance epithelial cytokine production by mediating bacterial invasion of bladder epithelial cells.

Bacterial invasion has also been implicated in the stimulation of intestinal epithelial cell cytokine production (2, 18). However, the means through which bacterial invasion leads to this phenomenon are unclear. Purified LPS is a potent inducer of cytokine production in macrophages; however, LPS is generally a poor stimulator of epithelial cytokine production. Consequently, it has been suggested that this molecule is not involved in the induction of cytokine production by these cells in response to bacterial infection (17, 18, 26). 5637 bladder epithelial cells respond to LPS in a dose dependent manner between 0.5 ng/ml and 50 ng/ml with a threshold of activation between 5 ng/ml and 50 ng/ml. However, 5637 cells required 50 ng/ml of LPS to achieve the same IL-6 response as human PBLs stimulated with 5 ng of LPS (unpublished data).

The functional role of LPS in the activation of epithelial IL-6 was further investigated using the LPS inhibitors polymyxin B and detoxified LPS. Polymyxin B is an antibiotic that binds to the lipid...
A moiety of LPS and prevents its interaction with LPS-binding protein (17, 40). Detoxified LPS is generated by mild alkaline hydrolysis of LPS removing the fatty acid side chains from lipid A, which are responsible for the toxic activity of LPS (41). Previous reports have indicated that LPS molecules with various deacylated forms of lipid A are capable of antagonizing biological responses to intact LPS by interacting with host cells (42, 43). Therefore, we reasoned that this molecule might also be able to antagonize the recognition of LPS by bladder epithelial cells. Polymyxin B and detoxified LPS, at concentrations of 1 μg/ml and 100 μg/ml, respectively, inhibited both purified LPS-mediated responses and responses to type 1-piliated and nonpiliated E. coli, demonstrating that LPS is the critical bacterial molecule recognized by 5637 bladder epithelial cells. These data indicate that bacterial invasion coupled with LPS recognition enhance epithelial responsiveness to E. coli and suggest that the role of bacterial associated LPS in epithelial cytokine production should be considered for other invasive pathogens. Interestingly, P pilus-mediated induction of cytokines from a kidney epithelial cell line has been shown to occur in an LPS-independent manner (17). P pilus do not mediate bacterial invasion of kidney or bladder epithelial cells (9). Thus, it seems that multiple mechanisms exist for the initiation of inflammation in the urinary tract.

In this report, bacterial invasion of bladder epithelial cells was demonstrated to enhance the responsiveness of bladder epithelial cells to E. coli via an LPS-dependent mechanism. It is possible that this occurs through the up-regulation of an LPSR after type 1 pilus-mediated invasion or potentially via synergy between LPS and invasion-mediated signaling cascades. However, these possibilities seem unlikely due to the observation that the coadoption of LPS and FimCH-coated beads fail to enhance the LPS responsiveness of 5637 cells (unpublished data). Furthermore, there is no delay in the augmentation of IL-6 after stimulation with type 1-piliated bacteria (Fig. 1B), suggesting that up-regulation of an LPSR is not required for this response. It is also possible that bacterial invasion leads to the clustering of an LPSR at sites of bacterial internalization or that a pool of LPSRs are located in an intracellular compartment. In support of such models, the maximal IL-6 response of 5637 cells to AAE185 (type 1) is only 25% that of the maximal cytokine response to AAE185/pSH2 (type 1), although the same amount of LPS is present in both circumstances. This observation argues that type 1 pilus-mediated invasion alters the interaction between bacterial associated LPS and the relevant LPSR or the accessibility of the LPSR. Evidence for receptor clustering has already been demonstrated for the pathogen-pattern recognition receptor TLR2, which transiently clusters around phagosomes during the internalization of yeast particles by macrophages (44).

The possibility that invasion enhances the response to an as yet unidentified bacterial molecule in an LPS-dependent manner has not been excluded. TLR4 has recently been identified as the primary mammalian LPSR (14, 32, 45–49). The observation that LPS is the stimulus for IL-6 production by 5637 bladder epithelial cells suggests that TLR4, and potentially mCD14, may be involved in the activation of epithelial cells. In contrast, C3H/HeJ mice had large numbers of neutrophils associated with foci of intracellular bacteria in the bladder epithelium. These results demonstrate that bacterial LPS and TLR4 are involved in the activation of bladder epithelial cells in response to type 1-piliated UPEC during an in vivo UTI.

Over the past decade it has become increasingly apparent that epithelial cells can participate in innate responses to pathogens (2, 18, 50). The results presented here indicate that type 1 pili augment bladder epithelial cytokine responses to E. coli by mediating bacterial invasion, not through a direct mechanism as proposed previously (11, 51). Furthermore, this study reveals that LPS is required for bacterial invasion to augment epithelial responses to E. coli, which may be a more general theme in bacterial epithelial interactions. The role of LPS in this response and the results of experiments using C3H/HeJ mice (TLR4 mutant) implicate TLRs and, in particular, TLR4 as the probable epithelial receptor involved in responses to E. coli. Consistent with this prediction, 5637 cells express TLR4 mRNA. In addition, recent studies have demonstrated that epithelial cells can express TLRs at the protein level and that mutations in the extracellular domain of TLR4 affect the LPS responsiveness of these cells (52, 53). TLRs lead to the activation of NF-κB and the subsequent production of inflammatory cytokines and chemokines, such as IL-6 and IL-8 (20, 52, 54). However, numerous other inflammatory molecules are regulated by NF-κB and future work investigating the role of TLRs in the induction of epithelial mediators will be vital to our understanding of epithelial cells as effectors of the innate immune system.

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