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Bacterial Invasion Augments Epithelial Cytokine Responses to *Escherichia coli* Through a Lipopolysaccharide-Dependent Mechanism¹

Joel D. Schilling,* Matthew A. Mulvey,* Carr D. Vincent,* Robin G. Lorenz,^{†‡} and Scott J. Hultgren^{2*}

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.

Epithelial cells are the first lines of host defense against bacteria and other pathogens. In addition to serving as a physical barrier, epithelial cells can contribute to the initiation of innate and adaptive host defenses through the production of cytokines, chemokines, and antimicrobial peptides (1, 2). Bacterial attachment to and/or invasion of epithelial cells are often the initial steps in bacterial pathogenesis at mucosal sites. These events have also been linked to the induction of epithelial cytokine responses; however, the mechanisms by which adherence and invasion activate epithelial cells to produce inflammatory cytokines are poorly understood.

Urinary tract infections (UTIs)³ are some of the most common infectious diseases, with greater than seven million cases reported annually in the United States (3). *Escherichia coli* is the causative organism in 70–90% of UTIs (3). Nearly all uropathogenic *E. coli* (UPEC) express surface-adhesive organelles known as type 1 pili. Type 1 pili are composite structures comprised of a 7-nm thick rod joined to a thin 3-nm tip fibrillum containing the adhesin, FimH (4, 5). FimH mediates binding to mannosylated glycoproteins and has been shown to be critical in the ability of uropathogenic *E. coli* to establish bladder infections (6, 7). In addition, Fim host receptor interactions trigger the invasion of *E. coli* into bladder epithelial

cells, an event that has been proposed to facilitate bacterial persistence in the urinary tract (8, 9). However, type 1-piliated *E. coli* are also known to stimulate host defenses, such as epithelial cell apoptosis and cytokine production (8, 10). It has been proposed that type 1 pili can directly activate bladder epithelial cells leading to the production of IL-6 and IL-8 (11).

LPS is a critical factor for the induction of inflammatory cytokines in macrophages. The recognition of LPS by macrophages involves the LPS-binding protein or soluble CD14, which facilitates the transfer of LPS to membrane-bound CD14 (mCD14), a GPI-linked receptor (12, 13). The CD14-LPS complex is thought to associate with Toll-like receptor 4 (TLR4), which leads to the activation of NF- κ B and subsequently the production of proinflammatory cytokines (12, 14). In contrast, the recognition of LPS by epithelial cells is unclear. The majority of epithelial cells do not respond as well as macrophages, if at all, to soluble LPS (1, 10, 12, 15–18). Therefore, it has been assumed that LPS is not the primary molecule involved in epithelial recognition of Gram-negative pathogens. In this report, we now describe the mechanism by which type 1 pili augment the activation of bladder epithelial cell cytokine production and define the role of LPS in this response.

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.

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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.

IL-6 stimulation assay

5637 epithelial cells were seeded into 24-well plates at 0.3×10^5 to 1×10^5 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 $\mu\text{g/ml}$ and 270 $\mu\text{g/ml}$, respectively. TMP-SMZ was used to allow the accurate determination of dose-response relationships. Bacterial suspensions were titered 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-1 α (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, cell debris 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 mg/ml BSA, 0.1% sodium azide, 5% glycerol, in PBS (pH 7.4)), washed three times with PBS, and resuspended in the original volume of PBS. For experiments, 1 μl of FimCH-coated beads or BSA-coated beads was added to 5637 cells (approximately five beads/epithelial cell). The beads were spun onto the cells by low-speed centrifugation and incubated at 37°C for 6 h. The supernatants were analyzed as described above.

Invasion assays and cytochalasin D treatment

5637 cells were pretreated with 0.1 $\mu\text{g/ml}$ of cytochalasin D in RPMI 1640 medium plus 10% FBS plus TMP-SMZ for 30 min at 37°C. After the preincubation, 10 μl of 1×10^8 CFU/ml bacteria in PBS was added to the cells. Bacteria were spun onto 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 $\mu\text{g/ml}$ 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 (OD₆₀₀ 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 $\mu\text{g/ml}$ of gentamicin to kill any extracellular bacteria. Subsequently, the cells were washed five times with PBS and fresh medium containing 15 $\mu\text{g/ml}$ of gentamicin was added to the wells. Also, at this time, uninfected wells were left unstimulated or stimulated with 20 ng/ml of IL-1 α . 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 titers 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 $\mu\text{g/ml}$ were preincubated with or without 1 $\mu\text{g/ml}$ 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 $\mu\text{g/ml}$ detoxified LPS derived from LPS 055:B5 (Sigma) 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 $\mu\text{g/ml}$ 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 $\mu\text{g/ml}$) (17), or treated with gentamicin (100 $\mu\text{g/ml}$). Cytokine stimulation assays were performed as described above. To block epithelial recognition of LPS, 5637 cells were preincubated with 100 $\mu\text{g/ml}$ detoxified LPS for 30 min at 37°C. After the pretreatment, 10 μl of $1-3 \times 10^8$ CFU/ml (multiplicity of infection 1-3) bacteria in PBS were added to the cells. Bacteria were spun down onto 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). Approx-

imately 24 h before RNA isolation, 5×10^6 THP-1 cells were differentiated with 10 ng/ml PMA (Sigma). 5637 cells were grown to confluence as described above.

RNA was extracted from $\sim 5 \times 10^6$ 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) (20) 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 UTI 89 was pelleted and resuspended in sterile PBS to a concentration of $\sim 2 \times 10^9$ CFU/ml. Mice were infected via intraurethral catheterization with 50 μl of the bacterial suspension (1×10^8 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 buffer formalin for histological analysis, or homogenized in sterile 0.025% Triton X-100/PBS, and titered 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 Ig-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 effect 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-1 α .

Infection of 5637 cells with AAEC185 or AAEC185/pSH2 at doses ranging from 10^3 to 10^8 CFU/ml (multiplicity of infection, 0.01-100) revealed two distinct thresholds for epithelial activation (Fig. 1A). At 1×10^5 CFU/ml, both AAEC185/pSH2 and AAEC185 induced 5637 IL-6 production ~ 5 -fold over unstimulated cells (Fig. 1A). The threshold for type 1 pilus-specific amplification of IL-6 expression was attained at bacterial concentrations of $\sim 1 \times 10^6$ 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 5×10^7 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 ($\sim 1 \times 10^6$ CFU/ml) (Fig. 1B). The *fimH*⁻ mutant strain

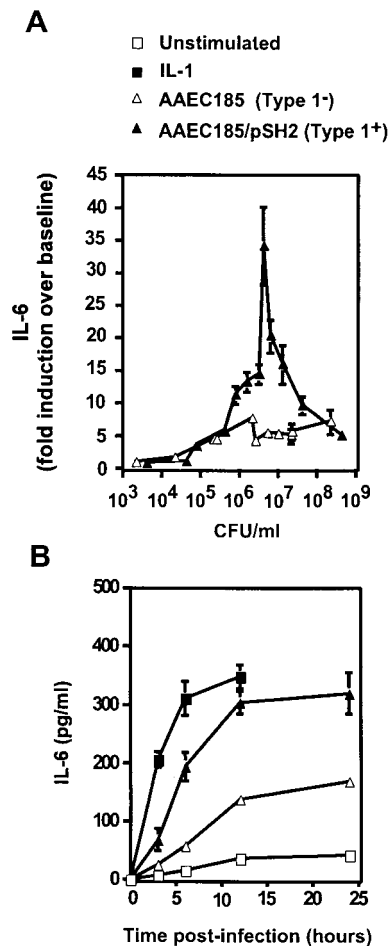


FIGURE 1. Type 1 pili augment bladder epithelial cytokine responses to *E. coli*. *A*, 5637 bladder epithelial cells were infected with the indicated concentrations of AAEC185 (type 1⁻) or AAEC185/pSH2 (type 1⁺) and IL-6 production was assessed 12 h after infection. *B*, Kinetic analysis of IL-6 production at 3, 6, 12, and 24 h postinfection. 5637 cells were stimulated with IL-1 α , AAEC185, AAEC185/pSH2, or left unstimulated. Data are presented as fold induction in all cases except kinetic plots, where the dynamics of production require the use of absolute cytokine levels. The baseline production of IL-6 from unstimulated 5637 cells ranged from 5 to 40 pg/ml during a 6-h assay; however, fold induction for the given stimulation's was consistent between experiments. Experiments were performed at least three times in triplicate.

(AAEC185/put2002), 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 re-

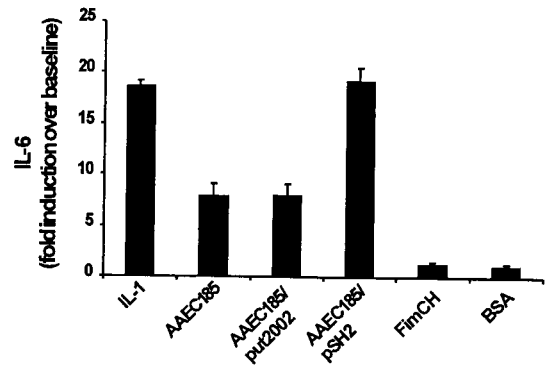


FIGURE 2. The type 1 pilus adhesin, FimH, is not sufficient to activate bladder epithelial cytokine production. 5637 cells were stimulated with IL-1 α or $\sim 10^6$ CFU/ml of AAEC185 (type 1⁻), AAEC185/put2002 (type 1⁺, *fimH*⁻), AAEC185/pSH2 (type 1⁺, *fimH*⁺), FimCH-coated beads (FimCH), or BSA-coated beads (BSA), and IL-6 concentration was determined 6 h after infection.

ceptor 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-1 α 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*.

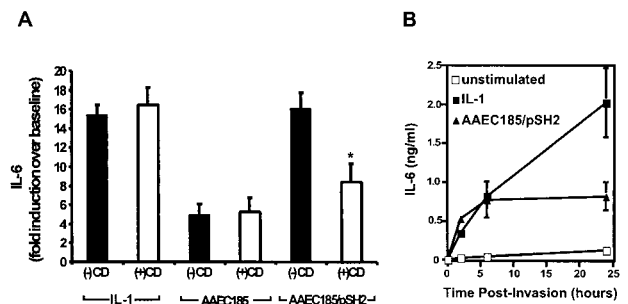


FIGURE 3. Type 1 pilus-mediated invasion of bladder epithelial cells augments IL-6 responses to type 1-piliated *E. coli*. *A*, Bladder epithelial cells were stimulated with IL-1 α , or $\sim 10^6$ CFU/ml of AAEC185 or AAEC185/pSH2 in the presence (□) or absence (■) of the invasion inhibitor cytochalasin D (CD). At 6 h after infection, IL-6 production was assessed by ELISA. *B*, IL-6 production was monitored in 5637 cells left unstimulated (□), IL-1 α stimulated (■), or stimulated with intracellular AAEC185/pSH2 (▲) at 2, 6, and 24 h postinvasion. Experiments were repeated at least three times in triplicate. *, $p \leq 0.05$ according to a Student's *t* test (determined using GraphPad Prism).

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-1 α 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 μ g/ml and 100 μ g/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 μ g/ml). As a control for the loss of viability that can be associated with polymyxin B, bacteria

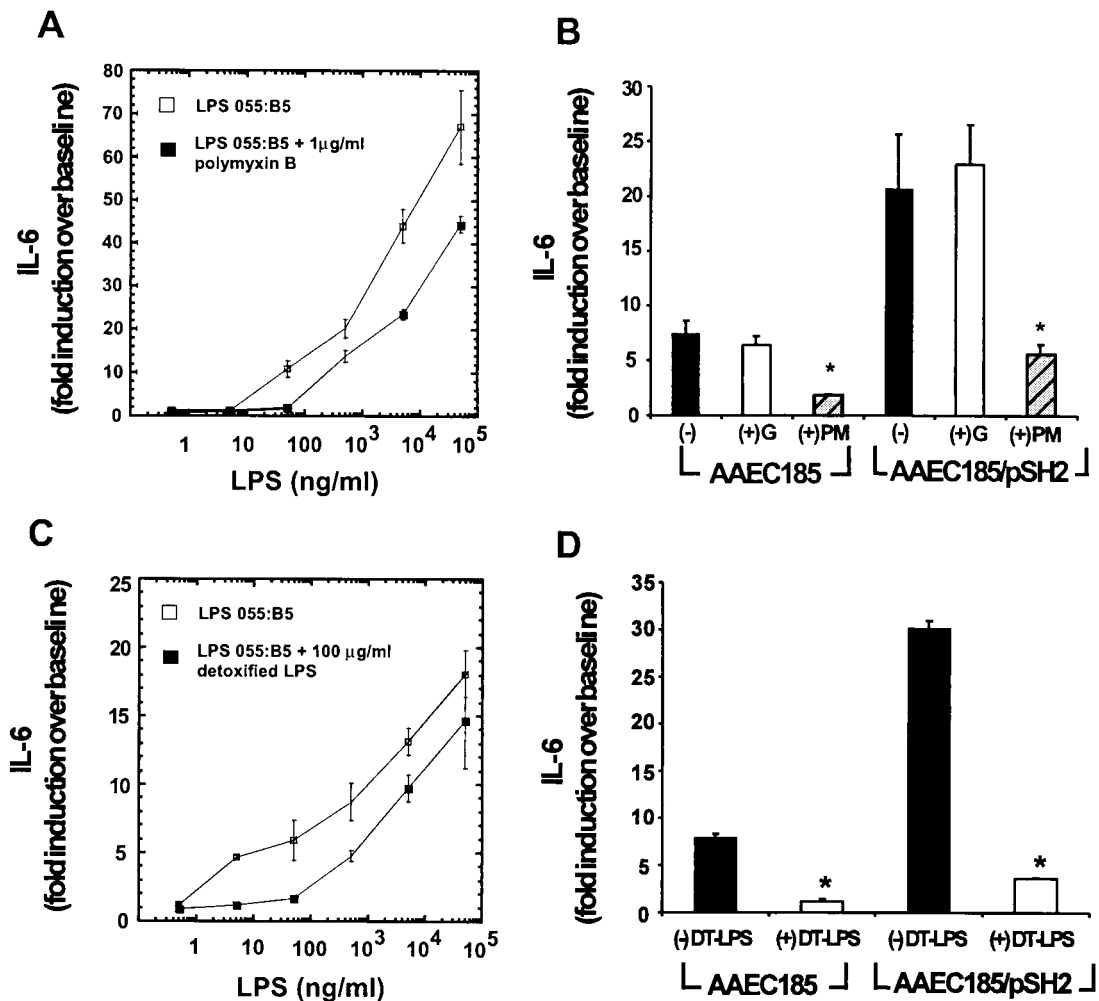


FIGURE 4. LPS is required for epithelial IL-6 production in response to *E. coli*. 5637 cells were stimulated with the indicated doses of *E. coli* 055:B5 LPS in the presence (■) or absence (□) of polymyxin B (A) or detoxified LPS (C). B, Cytokine production by 5637 cells was monitored after stimulation with $\sim 10^6$ 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. *, $p \leq 0.05$ according to a Student's *t* test (determined using GraphPad Prism).

were treated with 100 $\mu\text{g/ml}$ 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 piliated (AAEC185/pSH2) and nonpiliated (AAEC185) bacteria with polymyxin B inhibited IL-6 induction by 79% and 86%, respectively, but had no effect on IL-1 α 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 $\mu\text{g/ml}$ 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-1 α 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 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.

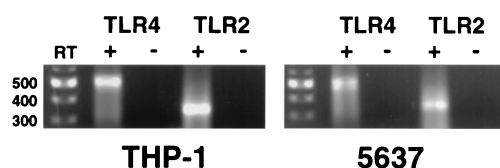


FIGURE 5. 5637 bladder epithelial cells express TLR4 and TLR2 mRNAs. RT-PCR for expression of TLR4 and TLR2 mRNAs was assessed using total RNA from PMA differentiated THP-1 cells (positive control) and 5637 bladder epithelial cells. Reverse transcriptase-specific products were detected for TLR4 and TLR2 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 7I) with a striking absence of neutrophils in both the epithelium and lamina propria (Fig. 7, H and J). 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

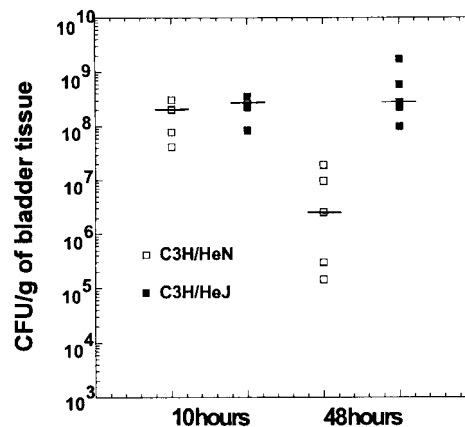
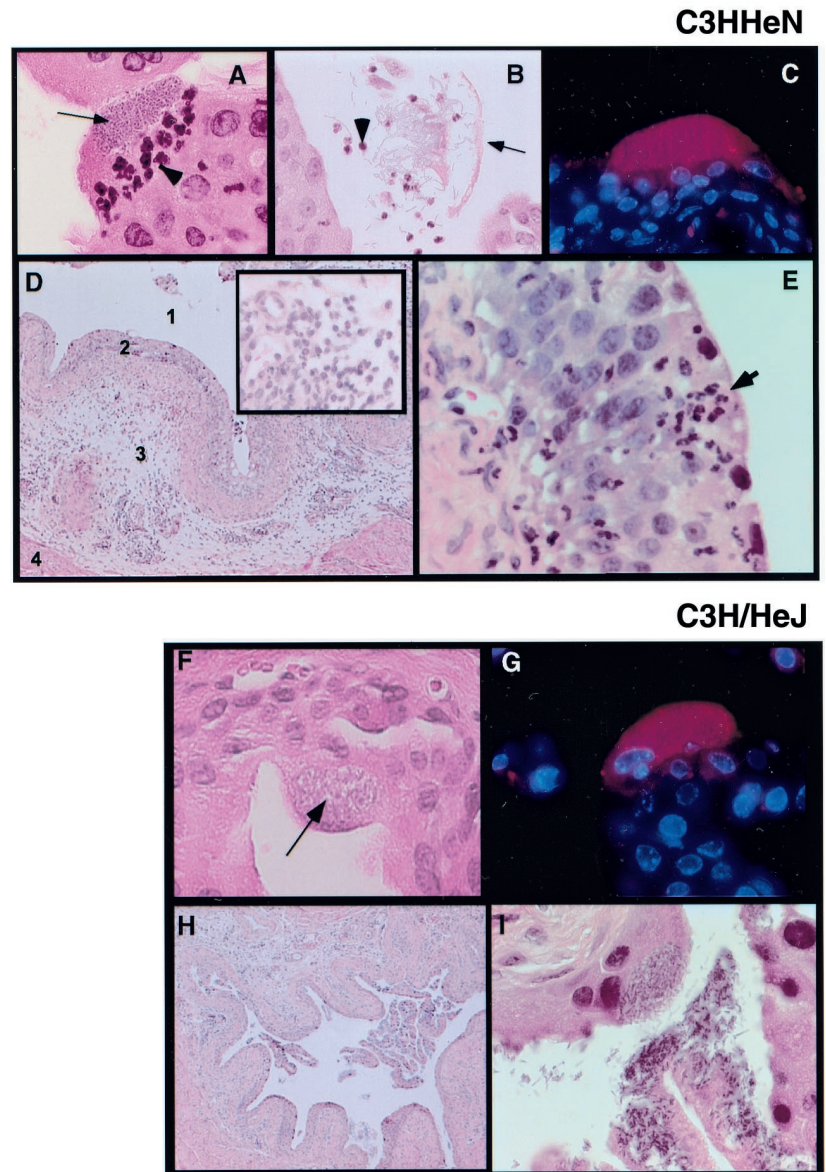


FIGURE 6. C3H/HeJ mice (TLR4 mutant) fail to clear type 1-piliated UPEC from the bladder. C3H/HeN (TLR4 normal) and C3H/HeJ (TLR4 mutant) mice were infected with 1×10^8 CFU/ml of the type 1-piliated cystitis isolate, UTI 89, and bladders were titrated at 10 and 48 h postinfection. The median titers are indicated by the black bars.

FIGURE 7. TLR4 mutant mice have distinct epithelial pathology following infection with UPEC. C3H/HeN (A–E) and C3H/HeJ (F–I) mice were infected with UTI 89, a type 1-piliated strain of UPEC, and their bladders were analyzed by H&E and fluorescence microscopy at 10 h (A–C, F, and G) and 48 h (D, E, H, and I) after infection. At 10 h after infection, C3H/HeN mice had large intraepithelial bacterial foci (arrows) (A–C) that were associated with influxing neutrophils (arrowheads) (A and B). By 48 h after infection, bacteria were no longer present in the epithelium, but neutrophils were prevalent in the lamina propria (*inset* in D) and the epithelium (arrowhead) (E). In contrast, C3H/HeJ mice had similar intraepithelial foci of bacteria at 10 h after infection (F and G), but few neutrophils (F). At 48 h after infection, the epithelium of C3H/HeJ mice was inundated with bacteria (I); however, neutrophils were not present in the lamina propria (H) or the epithelium (I). C and G were stained with a rabbit α -*E. coli* Ab and goat α -rabbit Cy3 secondary (red fluorescence). The bladder architecture is indicated in D; 1, bladder lumen; 2, bladder epithelium; 3, lamina propria; 4, smooth muscle.



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⁺, *fimH*⁻) does not illicit IL-6 production above levels seen after stimulation with AAEC185 (type 1⁻) 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 μ g/ml with a threshold of activation between 5 ng/ml and 50 ng/ml. However, 5637 cells required 50 μ g/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 $\mu\text{g/ml}$ and 100 $\mu\text{g/ml}$, 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 pili 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 coaddition of LPS and FimCH-coated beads fails 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 AAEC185 (type1⁻) is only 25% that of the maximal cytokine response to AAEC185/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 bladder epithelial cells in response to *E. coli*. Interestingly, we report here that 5637 bladder epithelial cells express mRNA for TLR4. C3H/HeJ mice express a nonfunctional form of TLR4 and consequently fail to respond to LPS, making this an ideal system to investigate the in vivo role of type 1 pilus-mediated bacterial invasion and LPS in the activation of the bladder epithelium. Previous studies have demonstrated that after infection with UPEC, C3H/HeJ mice fail to produce macrophage-inflammatory protein-2, leading to a defect in neutrophil recruitment to the urinary tract, and subsequent failure to clear bacteria (34–37). Further-

more, we have shown here that C3H/HeJ mice are unable to clear type 1-piliated UPEC from the bladder during the first 2 days after infection. These results, as with our in vitro data, state that host responses to type 1-piliated bacteria in the bladder occur through an LPS-dependent pathway. UPEC is able to invade bladder epithelial cells leading to the formation of large intracellular foci of bacteria (Fig. 7) (8) (M. A. Mulvey, J. D. Schilling, and S. J. Hultgren, manuscript in preparation). Based on the data presented here, it would be predicted that invasion of these epithelial cells in vivo would strongly activate the bladder epithelium in an LPS-dependent manner. For our analysis, neutrophil recruitment into the epithelium was used as a measure of epithelial activation. Consistent with this hypothesis were the results with the C3H/HeJ mice, which revealed a defect in the influx of neutrophils into the epithelium despite high levels of bacterial invasion of bladder epithelial cells. In contrast, C3H/HeN mice had large numbers of neutrophils associating 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- κ B 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- κ B 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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References

- Eckmann, L., M. F. Kagnoff, and J. Fierer. 1995. Intestinal epithelial cells as watchdogs for the natural immune system. *Trends Microbiol.* 3:118.
- Kagnoff, M. F., and L. Eckmann. 1997. Epithelial cells as sensors for microbial infection. *J. Clin. Invest.* 100:6.
- Hooton, T. M., and W. E. Stamm. 1997. Diagnosis and treatment of uncomplicated urinary tract infection. *Infect. Dis. Clin. North Am.* 11:551.
- Brinton, C. C., Jr. 1965. The structure, function, synthesis and genetic control of bacterial pili and a molecular model for DNA and RNA transport in Gram-negative bacteria. *Trans. NY Acad. Sci.* 27:1003.
- Jones, C. H., J. S. Pinkner, R. Roth, J. Heuser, A. V. Nicholes, S. N. Abraham, and S. J. Hultgren. 1995. FimH adhesin of type 1 pili is assembled into a fibrillar tip structure in the *Enterobacteriaceae*. *Proc. Natl. Acad. Sci. USA* 92:2081.
- Langermann, S., S. Palaszynski, M. Barnhart, G. Auguste, J. S. Pinkner, J. Burlein, P. Barren, S. Koenig, S. Leath, C. H. Jones, and S. J. Hultgren. 1997. Prevention of mucosal *Escherichia coli* infection by FimH-adhesin-based systemic vaccination. *Science* 276:607.
- Langermann, S., R. Mollby, J. E. Burlein, S. R. Palaszynski, C. G. Auguste, A. DeFusco, R. Strouse, M. A. Schenerman, S. J. Hultgren, J. S. Pinkner, et al. 2000. Vaccination with FimH adhesin protects cynomolgus monkeys from colonization and infection by uropathogenic *Escherichia coli*. *J. Infect. Dis.* 181:774.

8. Mulvey, M. A., Y. S. Lopez-Boado, C. L. Wilson, R. Roth, W. C. Parks, J. Heuser, and S. J. Hultgren. 1998. Induction and evasion of host defenses by type 1-piliated uropathogenic *Escherichia coli*. [Published erratum appears in 1991 *Science* 283:795]. *Science* 282:1494.
9. Martinez, J. J., M. A. Mulvey, J. D. Schilling, J. S. Pinkner, and S. J. Hultgren. 2000. Type 1 pilus-mediated bacterial invasion of bladder epithelial cells. *Embo. J.* 19:2803.
10. Hedges, S., M. Svensson, and C. Svanborg. 1992. Interleukin-6 response of epithelial cell lines to bacterial stimulation in vitro. *Infect. Immun.* 60:1295.
11. Svanborg, C., M. Hedlund, H. Connell, W. Agace, R. D. Duan, A. Nilsson, and B. Wullt. 1996. Bacterial adherence and mucosal cytokine responses: receptors and transmembrane signaling. *Ann. NY Acad. Sci.* 797:177.
12. Ingalls, R. R., H. Heine, E. Lien, A. Yoshimura, and D. Golenbock. 1999. Lipopolysaccharide recognition, CD14, and lipopolysaccharide receptors. *Infect. Dis. Clin. North Am.* 13:341.
13. Hailman, E., T. Vasselon, M. Kelley, L. A. Busse, M. C. Hu, H. S. Lichenstein, P. A. Detmers, and S. D. Wright. 1996. Stimulation of macrophages and neutrophils by complexes of lipopolysaccharide and soluble CD14. *J. Immunol.* 156:4384.
14. Beutler, B. 2000. Tlr4: central component of the sole mammalian LPS sensor. *Curr. Opin. Immunol.* 12:20.
15. Haziot, A., E. Ferrero, X. Y. Lin, C. L. Stewart, and S. M. Goyert. 1995. CD14-deficient mice are exquisitely insensitive to the effects of LPS. *Prog. Clin. Biol. Res.* 392:349.
16. Fearn, C., V. V. Kravchenko, R. J. Ulevitch, and D. J. Loskutoff. 1995. Murine CD14 gene expression in vivo: extramylloid synthesis and regulation by lipopolysaccharide. *J. Exp. Med.* 181:857.
17. Hedlund, M., C. Wachtler, E. Johansson, L. Hang, J. E. Somerville, R. P. Darveau, and C. Svanborg. 1999. P fimbriae-dependent, lipopolysaccharide-independent activation of epithelial cytokine responses. *Mol. Microbiol.* 33:693.
18. Svanborg, C., G. Godaly, and M. Hedlund. 1999. Cytokine responses during mucosal infections: role in disease pathogenesis and host defence. *Curr. Opin. Microbiol.* 2:99.
19. Blomfield, I. C., M. S. McClain, and B. I. Eisenstein. 1991. Type 1 fimbriae mutants of *Escherichia coli* K12: characterization of recognized afimbriae strains and construction of new fimbriae deletion mutants. *Mol. Microbiol.* 5:1439.
20. Faure, E., O. Equils, P. A. Sieling, L. Thomas, F. X. Zhang, C. J. Kirschning, N. Polentarutti, M. Muzio, and M. Arditi. 2000. Bacterial lipopolysaccharide activates NF- κ B through toll-like receptor 4 (TLR-4) in cultured human dermal endothelial cells: differential expression of TLR-4 and TLR-2 in endothelial cells. *J. Biol. Chem.* 275:11058.
21. Kopf, M., A. Ramsay, F. Brombacher, H. Baumann, G. Freer, C. Galanos, J. C. Gutierrez-Ramos, and G. Kohler. 1995. Pleiotropic defects of IL-6-deficient mice including early hematopoiesis, T and B cell function, and acute phase responses. *Ann. NY Acad. Sci.* 762:308.
22. Kopf, M., H. Baumann, G. Freer, M. Freudenberg, M. Lamers, T. Kishimoto, R. Zinkernagel, H. Bluethmann, and G. Kohler. 1994. Impaired immune and acute-phase responses in interleukin-6-deficient mice. *Nature* 368:339.
23. Romano, M., M. Sironi, C. Toniatti, N. Polentarutti, P. Fruscella, P. Ghezzi, R. Faggioni, W. Luini, V. van Hinsbergh, S. Sozzani, et al. 1997. Role of IL-6 and its soluble receptor in induction of chemokines and leukocyte recruitment. *Immunity* 6:315.
24. Xing, Z., J. Gaudie, G. Cox, H. Baumann, M. Jordana, X. F. Lei, and M. K. Achong. 1998. IL-6 is an antiinflammatory cytokine required for controlling local or systemic acute inflammatory responses. *J. Clin. Invest.* 101:311.
25. Tilg, H., C. A. Dinarello, and J. W. Mier. 1997. IL-6 and APPs: anti-inflammatory and immunosuppressive mediators. *Immunol. Today* 18:428.
26. Hedges, S. R., W. W. Agace, and C. Svanborg. 1995. Epithelial cytokine responses and mucosal cytokine networks. *Trends Microbiol.* 3:266.
27. Otto, G., J. Braconier, A. Andreasson, and C. Svanborg. 1999. Interleukin-6 and disease severity in patients with bacteremic and nonbacteremic febrile urinary tract infection. *J. Infect. Dis.* 179:172.
28. Choudhury, D., A. Thompson, V. Stojanoff, S. Langermann, J. Pinkner, S. J. Hultgren, and S. D. Knight. 1999. X-ray structure of the FimC-FimH chaperone-adhesin complex from uropathogenic *Escherichia coli*. *Science* 285:1061.
29. Wilson, M., R. Seymour, and B. Henderson. 1998. Bacterial perturbation of cytokine networks. *Infect. Immun.* 66:2401.
30. Yang, R. B., M. R. Mark, A. Gray, A. Huang, M. H. Xie, M. Zhang, A. Goddard, W. I. Wood, A. L. Gurney, and P. J. Godowski. 1998. Toll-like receptor-2 mediates lipopolysaccharide-induced cellular signalling. *Nature* 395:284.
31. Hirschfeld, M., Y. Ma, J. H. Weis, S. N. Vogel, and J. J. Weis. 2000. Cutting edge: repurification of lipopolysaccharide eliminates signaling through both human and murine toll-like receptor 2. *J. Immunol.* 165:618.
32. Poltorak, A., X. He, I. Smirnova, M. Y. Liu, C. V. Huffel, X. Du, D. Birdwell, E. Alejos, M. Silva, C. Galanos, et al. 1998. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science* 282:2085.
33. Hoshino, K., O. Takeuchi, T. Kawai, H. Sanjo, T. Ogawa, Y. Takeda, K. Takeda, and S. Akira. 1999. Cutting edge: toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. *J. Immunol.* 162:3749.
34. Hagberg, L., R. Hull, S. Hull, J. R. McGhee, S. M. Michalek, and C. Svanborg Eden. 1984. Difference in susceptibility to gram-negative urinary tract infection between C3H/HeJ and C3H/HeN mice. *Infect. Immun.* 46:839.
35. Hopkins, W. J., A. Gendron-Fitzpatrick, E. Balish, and D. T. Uehling. 1998. Time course and host responses to *Escherichia coli* urinary tract infection in genetically distinct mouse strains. *Infect. Immun.* 66:2798.
36. Haraoka, M., L. Hang, B. Frendeus, G. Godaly, M. Burdick, R. Strieter, and C. Svanborg. 1999. Neutrophil recruitment and resistance to urinary tract infection. *J. Infect. Dis.* 180:1220.
37. Shahin, R. D., I. Engberg, L. Hagberg, and C. Svanborg Eden. 1987. Neutrophil recruitment and bacterial clearance correlated with LPS responsiveness in local gram-negative infection. *J. Immunol.* 138:3475.
38. Eden, C. S., R. Shahin, and D. Briles. 1988. Host resistance to mucosal gram-negative infection: susceptibility of lipopolysaccharide nonresponder mice. *J. Immunol.* 140:3180.
39. de Man, P., C. van Kooten, L. Aarden, I. Engberg, H. Linder, and C. Svanborg Eden. 1989. Interleukin-6 induced at mucosal surfaces by gram-negative bacterial infection. *Infect. Immun.* 57:3383.
40. Scott, M. G., A. C. Vreugdenhil, W. A. Buurman, R. E. Hancock, and M. R. Gold. 2000. Cutting edge: cationic antimicrobial peptides block the binding of lipopolysaccharide (LPS) to LPS binding protein. *J. Immunol.* 164:549.
41. Ding, H. F., I. Nakoneczna, and H. S. Hsu. 1990. Protective immunity induced in mice by detoxified salmonella lipopolysaccharide. *J. Med. Microbiol.* 31:95.
42. Kitchens, R. L., R. J. Ulevitch, and R. S. Munford. 1992. Lipopolysaccharide (LPS) partial structures inhibit responses to LPS in a human macrophage cell line without inhibiting LPS uptake by a CD14-mediated pathway. *J. Exp. Med.* 176:485.
43. Kitchens, R. L., and R. S. Munford. 1995. Enzymatically deacylated lipopolysaccharide (LPS) can antagonize LPS at multiple sites in the LPS recognition pathway. *J. Biol. Chem.* 270:9904.
44. Underhill, D. M., A. Ozinsky, A. M. Hajjar, A. Stevens, C. B. Wilson, M. Bassetti, and A. Aderem. 1999. The Toll-like receptor 2 is recruited to macrophage phagosomes and discriminates between pathogens. *Nature* 401:811.
45. Medzhitov, R., P. Preston-Hurlburt, and C. A. Janeway, Jr. 1997. A human homologue of the *Drosophila* Toll protein signals activation of adaptive immunity. *Nature* 388:394.
46. Chow, J. C., D. W. Young, D. T. Golenbock, W. J. Christ, and F. Gusovsky. 1999. Toll-like receptor-4 mediates lipopolysaccharide-induced signal transduction. *J. Biol. Chem.* 274:10689.
47. Takeuchi, O., K. Hoshino, T. Kawai, H. Sanjo, H. Takada, T. Ogawa, K. Takeda, and S. Akira. 1999. Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. *Immunity* 11:443.
48. Lien, E., T. K. Means, H. Heine, A. Yoshimura, S. Kusumoto, K. Fukase, M. J. Fenton, M. Oikawa, N. Qureshi, B. Monks, et al. 2000. Toll-like receptor 4 imparts ligand-specific recognition of bacterial lipopolysaccharide. *J. Clin. Invest.* 105:497.
49. Poltorak, A., P. Ricciardi-Castagnoli, S. Citterio, and B. Beutler. 2000. Physical contact between lipopolysaccharide and Toll-like receptor 4 revealed by genetic complementation. *Proc. Natl. Acad. Sci. USA* 97:2163.
50. Diamond, G., D. Legarda, and L. K. Ryan. 2000. The innate immune response of the respiratory epithelium. *Immunol. Rev.* 173:27.
51. Hedlund, M., M. Svensson, A. Nilsson, R. D. Duan, and C. Svanborg. 1996. Role of the ceramide-signaling pathway in cytokine responses to P-fimbriated *Escherichia coli*. *J. Exp. Med.* 183:1037.
52. Cario, E., I. M. Rosenberg, S. L. Brandwein, P. L. Beck, H. C. Reinecker, and D. K. Podolsky. 2000. Lipopolysaccharide activates distinct signaling pathways in intestinal epithelial cell lines expressing Toll-like receptors. *J. Immunol.* 164:966.
53. Arbour, N. C., E. Lorenz, B. C. Schutte, J. Zabner, J. N. Kline, M. Jones, K. Frees, J. L. Watt, and D. A. Schwartz. 2000. TLR4 mutations are associated with endotoxin hyporesponsiveness in humans. *Nat. Genet.* 25:187.
54. Zhang, F. X., C. J. Kirschning, R. Mancinelli, X. P. Xu, Y. Jin, E. Faure, A. Mantovani, M. Rothe, M. Muzio, and M. Arditi. 1999. Bacterial lipopolysaccharide activates nuclear factor- κ B through interleukin-1 signaling mediators in cultured human dermal endothelial cells and mononuclear phagocytes. *J. Biol. Chem.* 274:7611.