Association of O-antigen serotype with the magnitude of initial systemic cytokine responses and persistence in the urinary tract

Running title: LPS Serotype, cytokine modulation and persistence

Dennis J. Horvath Jr., Ashay S. Patel, Ahmad Mohamed, Douglas W. Storm, Chandra Singh, Birong Li, Jingwen Zhang, Stephen A. Koff, Venkata R. Jayanthi, Kevin M. Mason, Sheryl S. Justice

1The Research Institute at Nationwide Children’s Hospital, Columbus, Ohio
2Nationwide Children’s Hospital, Columbus, Ohio
3The Ohio State University School of Medicine, Columbus, Ohio

#Correspondence: Sheryl S. Justice, Center for Microbial Pathogenesis, The Research Institute at Nationwide Children’s Hospital, 700 Children’s Drive, Columbus OH, 43205

Sheryl.justice@nationwidechildrens.org

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Abstract

Urinary tract infection (UTI) is one of the most common ailments requiring both short term and prophylactic antibiotic therapies. Progression of infection from the bladder to the kidney is associated with more severe clinical symptoms (e.g. fever, vomiting) as well as dangerous disease sequelae (e.g. renal scarring, sepsis). Host-pathogen interactions that promote bacterial ascent to the kidney are not completely understood. Prior studies indicate that the magnitude of proinflammatory cytokine elicitation \textit{in vitro} by clinical isolates of uropathogenic \textit{E. coli} (UPEC) inversely correlates with the severity of clinical disease. Therefore, we hypothesize that the magnitude of initial proinflammatory responses during infection defines the course and severity of disease. Clinical UPEC isolates obtained from patients with a non-febrile UTI elicited high systemic proinflammatory responses early during experimental UTI in the murine model and were attenuated in bladder and kidney persistence. Conversely, UPEC isolates obtained from patients with febrile-UTI elicited low systemic proinflammatory responses early during experimental UTI and exhibited prolonged persistence in the bladder and kidney. Soluble factors in the supernatant from saturated cultures as well as the lipopolysaccharide (LPS) serotype correlated with the magnitude of proinflammatory responses \textit{in vitro}. Our data suggest that the structure of the O-antigen sugar moiety of the LPS may determine the strength of cytokine induction by epithelial cells. Moreover, the course and severity of disease appear to be the consequence of the magnitude of initial cytokines produced by the bladder epithelium during infection.
Importance

The specific host-pathogen interactions that determine the extent and course of disease are not completely understood. Our studies demonstrate that modest changes in the magnitude of cytokines observed using in vitro models of infection translate into significant ramifications for bacterial persistence and disease severity. While many studies have demonstrated that modifications of the LPS lipid A moiety modulate the extent of TLR4 activation, our studies implicate the O-antigen sugar moiety as another potential rheostat for the modulation of proinflammatory cytokine production.
Introduction

The plasticity of the *Escherichia coli* genome has led to the evolution of strains that can serve as keystone commensal organisms in the microbiota as well as the development of multiple virulent pathotypes that cause both intestinal and extraintestinal infections. Comparison of the genetic content of over 180 strains has revealed that the core *E. coli* genome is composed of about 1,700 genes with an additional pan genome of over 16,000 genes (1). It has become increasingly apparent that the plasticity of the *E. coli* genome allows acquisition of virulence traits from diverse genetic origins and, as such, the potential to attain the same phenotypic trait using different mechanisms (2). Given this vast variation in genetic material available, it has been difficult to assign specific subsets of genes to an individual pathotype, particularly given that many features are shared (1). Within the urinary tract, *E. coli* can manifest as: transient contamination by non-pathogenic commensals, asymptomatic carriage, symptomatic infection of the bladder (cystitis), severe infection of the kidney (pyelonephritis) that can result in urosepsis and death. While there has been significant interest in identification of genes that are critical for colonization and infection in the urinary tract (3-9), genes that exclusively differentiate strains as commensal, asymptomatic, cystitis or pyelonephritis are yet to be identified (2, 10-12). For example, flagella appear to be dispensable for cystitis but are important for pyelonephritis (13-15). However, the presence of flagellar genes alone is not discriminatory, as motility is a core *E. coli* function. Our understanding of the molecular events that regulate the progression and severity of disease is limited by the lack of discriminatory bacterial markers associated with each clinical manifestation.
Human and mouse studies have demonstrated that the magnitude of host immune responses have a dramatic consequence on the outcome of disease (16-21). For example, human polymorphisms in Toll-like receptor 4 (TLR4) increase susceptibility to asymptomatic carriage (16), while polymorphisms in CXCR1 and IRF3 increase susceptibility to acute pyelonephritis (17, 18). In addition, cyclooxygenase-2 is critical for establishment of chronic and recurrent UTI (21). Although there is clear evidence that host immune responses influence disease progression, human genetics is likely not the only factor that directs the disease course given the statistics that a woman has a 50% chance of having a UTI in her lifetime. A role for bacterial traits in this process is supported by observations that absence of cytotoxic necrotizing factor, α-hemolysin or the outer membrane chaperone SurA changes the magnitude of immune responses induced by isogenic wild type strains (22-25). Current evidence supports a model whereby the magnitude of host immune responses, mediated either by host polymorphisms and/or bacterial traits influences the disease manifestation observed clinically.

Interleukin-6 (IL-6) is present in human urine during acute UTI (26, 27). Prior studies indicate that independent UPEC isolates elicit varying degrees of IL-6 produced by cultured human bladder epithelial cells in vitro (25, 28, 29). Moreover, we observe an inverse association between the amount of IL-6 induction in vitro and clinical severity of UTI (28), suggesting that this phenotype may discriminate between isolates that cause cystitis and pyelonephritis. We extended these observations and, for the first time, demonstrated a correlation between the magnitude of IL-6 elicited from cultured human bladder epithelial cells in vitro with the magnitude of systemic IL-6 in during
experimental UTI in the murine model. We further determined that the initial extent of systemic proinflammatory responses was associated with enhanced persistence of UPEC in the kidney during experimental UTI. In addition, the extent of IL-6 elicitation was associated with the LPS O-antigen serotype of the strain. Taken together, our experimental evidence provides insight into the molecular mechanisms that demarcate an infection that will be cleared in the bladder from an infection that will progress to a more severe and persistent disease in the kidney.

Materials and Methods

Collection of *E. coli* strains isolated from non-febrile and febrile UTIs

UPEC isolates were obtained from the urine of patients presenting to the urology service at Nationwide Children's Hospital's with UTIs. The ChildLab clinical microbiology laboratory at Nationwide Children's Hospital identified the bacterial species and determined a bacterial burden of at least $10^5$ colony forming unit/milliliter (cfu/ml) of urine for each isolate (28, 30). Patient gender, age and urinary tract diagnosis/etiology are indicated in Table 1. Patients were placed into one of four diagnostic and etiologic categories: 1) Neurogenic Bladder Group (NGB); 2) Vesicoureteral Reflux Group (VUR); 3) Bladder and Bowel Dysfunction (BBD) and 4) No Underlying UTI Etiology Group as previously described (28). Isolates were categorized as causing febrile UTI (clinical pyelonephritis) when patients presented with flank pain, leukocytosis, body temperature $>38.5^\circ\text{C}$, and nausea and/or vomiting (28). This study was performed with the approval of the Institutional Review Board for human studies (OHRP Assurance No. FWA00002860) at the Research Institute at Nationwide Children's Hospital (IRB12-
UTI89 is a prototypic non-febrile UPEC isolate obtained from a woman with cystitis (31).

**Murine model of human UTI**

Based upon the magnitude of cytokine elicitation induced by these strains *in vitro* as part of previous studies (28) and empirical determination that these isolates exhibit similar infection kinetics *in vivo* as part of this study, PEDUTI177 and PEDUTI914 *E. coli* isolates were selected as representatives of the non-febrile (NF-UTI) isolates. PEDUTI175 and PEDUTI939 *E. coli* isolates were selected as representatives of the febrile (F-UTI) isolates. In addition, the isolates represent each of the four diagnostic and etiologic categories (Table 1). UTI89 and these four pediatric isolates were statically grown in LB broth (Fisher Scientific, Pittsburgh, PA) to saturation at 37 °C. The presence of type 1 pili was confirmed by mannose-sensitive agglutination using *Saccharomyces cerevisiae* (32). Seven- to nine-week-old female C3H/Hen mice (Harlan Laboratories, Indianapolis, IN) were anesthetized with 3% isoflurane and inoculated transurethrally with 50 µl containing 1 x 10⁷ viable bacteria as described (33). At the indicated time points post inoculation, the mice were humanely sacrificed for aseptic retrieval of bladder and kidney pairs for tissue homogenization and bacterial enumeration. Serum was collected at the time of tissue harvest and the magnitude of proinflammatory cytokines was determined using a Mouse Inflammation Cytokines Bead Array (BD Biosciences, San Jose, CA)(34). The serum cytokines were determined using two dilutions of the serum. The cytokines were evaluated on three independent occasions. All animal experiments were performed using accredited conditions for animal welfare approved by the Institutional Animal Care and Use Committee (Welfare...
Microscopic evaluation of intracellular communities

Infected murine bladders were harvested at 6 hours post inoculation, bisected, splayed and fixed in 4% paraformaldehyde (EM Sciences, Hatfield, PA) in phosphate buffered saline (PBS; Sigma, St. Louis, MO) as described (33, 35, 36). For visualization of pediatric *E. coli* isolates, bladders were treated with 0.1% triton X-100 (Fisher Scientific, Pittsburg, PA) in PBS to permeabilize the epithelial plasma membranes. Pediatric UPEC strains were visualized with the addition of rabbit anti-*E. coli* polyclonal antisera (US Biological, Salem, MA) diluted 1:200 in PBS/0.1% Triton X-100 at 23°C for 1 hour. Bladders were washed with PBS for 3 times before the addition of Goat anti-Rabbit secondary IgG conjugated to Alexa-594 (Life Technologies, Carlsbad, CA) diluted 1:200 in PBS/0.1% Triton X-100 at 23°C for 1 hour. Residual antibodies were removed by washing with PBS for 3 times. Bladders infected with UTI89/pANT4 (37) required no specific staining for visualization of bacteria as this strain constitutively produces the green fluorescent protein. Bacterial and host DNA was visualized by the addition of Hoechst 34580 (Invitrogen, Carlsbad, CA) for 10 minutes. Bladders were mounted with ProLong Gold antifade reagent (Invitrogen). Images were acquired using an Axiovert 200 M inverted epifluorescence microscope equipped with a motorized stage, an Axiocam MRM CCD camera and the Apotome component to improve fluorescence resolution (Carl Zeiss, INC, Thornwood, NY). The intensity of the fluorescent images was uniformly adjusted to all pixels within the image using the levels function in Adobe Photoshop (Adobe Systems Incorporated; San Jose, CA).
Preparation of conditioned medium from UPEC isolates

All UPEC isolates (20 NF-UTI, 22 F-UTI, UTI89) were grown to saturation in RPMI 1640 (HyClone Laboratories, Logan, Utah) supplemented with 10% heat-inactivated fetal bovine serum (Sigma, St. Louis, MO) at 37 °C overnight without shaking. Bacteria were removed from the saturated cultures by centrifugation and the supernatants were further clarified by passage through a 0.22 µm filter (EMD Millipore, Billerica, MA) to generate "conditioned medium".

To determine the contribution of shed LPS to the magnitude of cytokine elicitation, the conditioned medium obtained from UTI89 and the UPEC isolates was depleted of LPS by two sequential passages over a Detoxi-gel Endotoxin Column (Thermo Scientific, Rockford, IL) according to manufacturer’s recommendation. The absence of viable bacteria in the clarified conditioned medium was verified by plating on LB agar (Fisher Scientific).

The contribution of outer membrane vesicles to the magnitude of cytokine elicitation was determined by further clarification of the conditioned medium to remove the outer membrane vesicles by ultracentrifugation at 38,000 x g for 1 hour as previously described (38).

Elicitation of interleukin 6 (IL-6) from cultured human bladder epithelial cells in vitro

Use of conditioned medium to elicit cytokine production: T24 bladder epithelial cells (derived from human bladder carcinoma; ATCC HTB-4; Manassas, VA) were grown in 24 well plates containing RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum at 37 °C in humidified atmosphere with 5% carbon
dioxide. Confluent epithelial cell monolayers were washed with warm media and then overlaid with 500 µl of clarified conditioned medium and returned to the incubator for 2 hours. Culture supernatants were removed, clarified at 20,000 x g for 5 minutes and frozen at -80 °C.

**Use of live bacteria to elicit cytokine production:** T24 human bladder epithelial cell monolayers were grown in 24 well plates as described above. After washing with warm media, cells were infected with viable UPEC, grown in RPMI as described above, at a multiplicity of infection 10 bacteria per epithelial cell and returned to the incubator for 2 hours. Culture supernatants were removed, clarified at 20,000 x g for 5 minutes and frozen at -80 °C.

The magnitude of IL-6 accumulation within the epithelial cell culture supernatant was determined by enzyme-linked immunoabsorbant assay (eBioscience, San Diego, CA) as previously described (25, 28). The triplicate values were averaged and plotted as individual points for each isolate tested. Due to daily variation in results, representative studies are depicted. Where indicated, purified *E. coli* LPS 0111:B4 (Sigma-Aldrich, Saint Louis, MO) was added to a final concentration of 1.25 µg/ml.

**LPS serotype of strains was determined by PCR**

Identification of the O-antigen LPS serotypes was determined by PCR (Table 1) using previously defined primers (39, 40) with genomic DNA purified from each isolate (Qiagen, Carlsbad, CA).

**Statistical analysis**
The significance of the results was determined using a two-tailed Mann-Whitney U-test, chi-square, or one-way ANOVA as indicated (GraphPad Software, La Jolla, California).

Results

Magnitude of serum cytokines is associated with persistence during experimental UTI

Genetic differences between independent UPEC isolates as well as the multiplicity of infection can influence the kinetics of infection (25, 29, 41-43). Therefore, we sought to identify independent UPEC isolates from our library that exhibit similar infection kinetics during the first few hours of infection. These isolates would provide a means to determine the concordance of the cytokine profiles observed in vitro with those produced in response to experimental UTI. To this end, the kinetics of the initial stages of infection was first evaluated in the well-established mouse model of human UTI (33) using four pediatric UPEC isolates. Two representatives for each of the non-febrile and febrile isolates were chosen to increase the reliability of the results. There was no significant difference in the bacterial burden of either the urinary bladder or the kidney pairs 6 hours after introduction of any of the four representative UPEC isolates into the bladder (6 hours; Figure 1A, B). Furthermore, the development of intracellular bacterial communities within superficial bladder epithelial cells were similar amongst these four representative UPEC isolates and as compared with the prototypical cystitis isolate, UTI89 (Figure 1C, D, E) (37, 44, 45). Therefore, as evidenced by the similarity in intracellular community development and bacterial burden, these isolates are
appropriate for comparison of the immunomodulation capacity of each strain on initial proinflammatory responses during infection *in vivo*.

The magnitude of systemic proinflammatory responses induced by each of the independent UPEC isolates during experimental UTI was evaluated. Consistent with the *in vitro* observations (28), mice infected with the two representative non-febrile UPEC isolates (high elicitation of IL-6 *in vitro*) displayed a statistically significant increase in the magnitude of systemic IL-6 as compared with mice infected with the two representative febrile UPEC isolates (low elicitation of IL-6 *in vitro*) (Figure 2). Although not as pronounced as in the case of IL-6, a statistically significant increase in IL-2, IL-4, IL-10, IL-17A and tumor necrosis factor (TNF) was also observed in the serum of mice infected with the two representative non-febrile UPEC isolates as compared with the two representative febrile UPEC isolates (Figure 2). Therefore, the immunomodulation of cytokine production observed *in vitro* is recapitulated during infection in the host.

To evaluate the consequences of modulation of proinflammatory responses on the manifestation of disease, the persistence of each of the four representative UPEC isolates was followed throughout the course of an acute infection. The bacterial burden of bladders infected with either of the representative febrile UPEC isolates was significantly higher than that of bladders infected with either of the non-febrile isolates at 16 hours post inoculation (Figure 3A). This time point coincides with the maximal influx of PMNs and macrophages into the bladder (46). By 24 hours, bacteria were not recovered from either the bladder or the kidneys of mice infected with the two representative non-febrile isolates (Figure 3B, C; solid line). The time to clearance of UPEC from both the bladders and the kidneys infected with either of the representative
febrile isolates (Figure 3B, C; dashed line) significantly increased as compared with those tissues infected with either of the representative non-febrile isolates. Therefore, the magnitude of systemic cytokines inversely correlated with persistence in the urinary tract. 

Magnitude of IL-6 elicitation by conditioned medium is associated with febrile and non-febrile UTI

The concordance of the in vitro and in vivo cytokine profiles provided credence for the use of the in vitro system as an appropriate high throughput first approach for the identification of bacterial factors that modulate the proinflammatory responses. The immunosuppression of cytokine responses by UPEC was abolished when LPS O-antigen synthesis was disrupted (25, 43). In addition, the binding of LPS by the TLR-4 receptor is the predominant signal for proinflammatory responses to bacteria in the urinary tract (42, 47-49). LPS and other bacterial metabolites accumulate in the culture medium during exponential growth. The clarified culture supernatant is termed “conditioned medium” and has been used previously with mutants defective in assembly of LPS O-antigen (25, 43). Conditioned medium was obtained from each of the 42 isolates within our library to stimulate cultured human bladder epithelial cells in vitro. The inverse correlation of the magnitude of IL-6 pro-inflammatory responses and clinical disease severity observed with the complete panel of viable bacteria (28) was recapitulated with the conditioned media (Figure 4) (p>0.0001). Moreover, the magnitude of IL-6 elicitation from all non-febrile isolates was indistinguishable from the conditioned medium of the prototypical and well characterized isolate obtained from a woman with non-febrile cystitis (UTI89, gray circle)(Figure 4). Therefore, this evidence

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suggests that one or more constituents of the culture medium contribute to the immunomodulation associated with UPEC isolates.

**Contribution of LPS to cytokine elicitation in conditioned medium**

To begin to define the bacterial factors that contribute to modulation of epithelial proinflammatory responses, we used two approaches to deplete constituents from the conditioned medium. Elicitation of human bladder epithelial cells with conditioned medium from UTI89 was abolished to baseline following a two stage depletion of LPS (p=0.008) (Figure 4A) with the use of Detoxi-gel Endotoxin resin. Additional passages over the Detoxi-gel Endotoxin Column did not have any additional effect on the magnitude of cytokine elicitation (data not shown). Reconstitution of the depleted conditioned medium with commercially available LPS resulted in similar levels of IL-6 elicitation as compared to the commercial LPS alone (Figure 5A), suggesting that the depletion of the LPS abolishes immune stimulation. Bacterial LPS is shed in the form of micelles and outer membrane vesicles. Under these experimental conditions, outer membrane vesicles (and any associated proteins) could have been depleted due to the content of LPS. The magnitude of cytokine elicitation was significantly reduced when outer membrane vesicles were removed by centrifugation (p=0.03)(Figure 5B). Extraction of residual LPS following removal outer membrane vesicles resulted in IL-6 production that was indistinguishable from uninfected cells (Figure 5B). Centrifugation of the conditioned medium after LPS depletion did not significantly affect the extent of IL-6 elicitation (Figure 5B), suggesting that outer membrane vesicles were removed during depletion of the LPS.
Since the depletion of LPS by column chromatography was sufficient to remove the elicitation factors from the conditioned medium of UTI89, we used this methodology to evaluate the conditioned medium of all the UPEC isolates. As observed with UTI89, the conditioned medium from the clinical isolates failed to elicit IL-6 production following depletion with the Detoxi-gel resin (four representative febrile and four non-febrile isolates depicted in Figure 6), suggesting that the molecules that modulate the immune response in the conditioned medium from the clinical isolates are associated with LPS and/or outer membrane vesicles.

**LPS serotype and disease severity**

The common bacterial factor depleted from the conditioned medium using both approaches is LPS. Therefore, a potential correlation between the LPS O-antigen serotype and clinical disease severity was evaluated. The LPS serotype was successfully determined for 37 of the 42 UPEC isolates (Table 1). We observed a bias for the presence of certain serotypes with disease presentation (Figure 7A). Serotypes O2, O16 and O25b were primarily obtained from children with febrile UTI. Conversely, serotypes O21, O25a and O75 were primarily obtained from children with non-febrile infection. Our results are consistent with a prior study of 343 UPEC strains that demonstrated an enrichment for serotypes O2 and O16 with pyelonephritis and a lack of association for disease severity with O18 (50).

**LPS serotype and magnitude of cytokine elicitation**

To further investigate the association of LPS serotype with the magnitude of cytokine elicitation, IL-6 was quantified from cultured human bladder epithelial cells viable bacteria. Within our collection, certain serotypes were excluded from further
evaluation due to statistical limitations (O1, O4, O6, O7, O8) (Figure 7A). Therefore this analysis included 33 of the isolates from the library. With the exception of serotypes O18 and O25b, the magnitude of cytokines elicited for each independent clinical isolate was very similar within the same serotype (Figure 7B). The association of the degree of the cytokine elicitation with the LPS serotype of the strain was statistically significant (p=0.0001)(Figure 7B). Taken together, serotypes O21, O25a and O75 elicit high IL-6 secretion and represent a low risk for progression to febrile infection. In contrast, serotypes O2, O16 and O25b elicit low IL-6 secretion and represent a high risk for progression to a febrile infection. This association was further explored through the evaluation of combinatorial exposure of human bladder epithelial cells to outer membrane vesicles displaying similar protein profiles but exhibiting different LPS serotypes (Figure S1). Consistent with observations with whole cells (Figure 7B), we observed differences in the magnitude of IL-6 elicitation when bladder epithelial cells were exposed to outer membrane vesicles from two representative non-febrile isolates (PEDUTI177, PEDUTI914) and two representative febrile isolates (PEDUTI175, PEDUTI 939) (Figure S2). When the vesicles were mixed, the IL-6 elicitation was representative of one of the two isolates, suggesting that the cells respond to the vesicles produced by only one of the LPS serotypes tested. Therefore, the LPS serotype appears to correlate with the magnitude of IL-6 responses, bacterial persistence, as well as the severity of clinical disease.

Discussion

The specific host-bacterial interactions that transform the disease course leading to enhanced bacterial persistence and disease severity are not completely understood.
In our study, we use the same genetic host background (e.g. T24 bladder cells, C3H/HeN mice) to focus only on bacterial attributes that modulate the immune response to elucidate the molecular events that determine disease outcomes. During experimental UTI, we demonstrated that mice infected with UPEC isolates obtained from patients with febrile UTI displayed decreased systemic immune responses and increased bacterial persistence in the urinary tract as compared with mice infected with UPEC isolates obtained from patients with non-febrile UTI. This observation suggests that the rapid clearance of bacteria during experimental UTI is likely a consequence of the high cytokine elicitation that has been observed *in vitro* (25, 29, 43, 51). Prior studies indicate that bacterial persistence is promoted by two sequential instillations of the prototypical cystitis strain, UTI89, into the bladder 1-6 hours apart (52). However, increasing the time between the two instillations (>24 hours) nullifies the benefit of superinfection. In light of our data, we suggest that immunosuppression of epithelial responses from the first inoculum could protect the second inoculum from early innate immune responses. We further propose that the enhanced survival of the febrile isolates correlates with a reduction in the recruitment of immune cells combined with a decrease in epithelial production of extracellular and intracellular antibacterial agents (3, 32, 53). Thus, suppression of the TLR4 cascade provides multiple mechanisms to ensure a more hospitable environment for the expansion of UPEC strains in the urinary tract. Our data not only support the use of *in vitro* systems to investigate epithelial proinflammatory responses to UPEC (25, 28, 29, 42, 43), but also demonstrate that modulation of the initial inflammatory responses, mediated at the urothelium, has a
direct impact on disease progression. As such, these studies provide mechanistic insight into the molecular events that contribute to disease severity.

Bacterial LPS is a very diverse class of molecules. LPS is readily remodeled through modifications of the lipid A moiety (54), the portion that directly interacts with the TLR4 receptor (55). The ability of lipid A modifications to alter the magnitude of inflammatory responses has been exploited to identify optimal vaccine adjuvants (54, 56). Moreover, lipid A mimetics are being investigated as potential therapeutics to enhance or antagonize TLR4 signaling (57-59). Although each strain appears to only produce one O-antigen, as a species, *E. coli* can assemble ~158 different sugar moieties onto the lipid A/core complex. Although modifications to the O-antigen within a particular strain participate in pathogenesis (54), a specific role for the structure of the sugar moiety in pathogenesis is unclear. Given the diversity in LPS O-antigen, the use of isogenic strains is not easily amenable to the direct evaluation of the variations in O-antigen sugar structure during disease. Therefore, our use of a panel of *E. coli* isolates provided a first line of experimental evidence that the LPS O-antigen moiety is associated with the magnitude of cytokine production. Although our *in vitro* study focused only on the induction of IL-6, our *in vivo* evidence suggests that multiple cytokines and chemokines may be modulated by UPEC during UTI. Future studies will include evaluation of an increased repertoire of LPS serotypes to further elucidate the molecular interactions that determine the magnitude of TLR4 responses.

Outer membrane vesicles are produced by a wide variety of bacteria and contribute to pathogenesis and immune modulation (38, 60-62). We observed that outer membrane vesicles retained the ability to modulate the production of IL-6 from cultured
bladder epithelial cells. In addition to LPS, proteins and nucleic acids are constituents of
outer membrane vesicles. Proteins that are known to regulate proinflammatory
responses (e.g. hemolysin, SurA substrates) (23-25) are packaged into UPEC outer
membrane vesicles (63). Therefore, further biochemical characterization of outer
membrane vesicles could identify additional bacterial traits that contribute to the
diversity of cytokine responses observed for some LPS serotypes (i.e. O18, 25b).

In summary, we provide experimental evidence that bacterial persistence
correlates with the extent of immunosuppression during the initial stages of infection.
Moreover, the LPS serotype, by virtue of the association with magnitude of cytokine elicitation, is associated with the severity of disease.
References.


Figure 1. Colonization and microscopic examination of UPEC clinical isolates associated with febrile and non-febrile UTI. Female mice (n= 4 per cohort) were inoculated with $10^7$ colony forming units (CFU) of either a UPEC non-febrile isolate (NF-UTI; PEDS177 gray square; PEDS914 black square) or febrile isolate (F-UTI; PEDS175 gray circle; PEDS939 black circle). The serotype of each strain is indicated in the legend. Organs indicated were harvested at 6 hours post infection for homogenization and enumeration of bacteria as total CFU per organ. Statistical significance was determined using two-tailed Mann-Whitney U test. Additional bladders were bisected, splayed, fixed and stained for microscopic examination of intracellular bacterial communities (green) within the epithelial cells (blue nuclei). Representative intracellular communities of UTI89, PEDUTI177 and PEDUTI939 are presented (C-E). Scale bar = 10 µm.

Figure 2. Non-febrile UTI isolates elicit higher magnitude of systemic cytokines. The magnitude of serum cytokines (pg/ml) 6 hours after intraurethral introduction of UPEC clinical isolates associated with febrile and non-febrile UTI. The serum obtained from each individual mouse was measured independently on two separate occasions. The averages were plotted. Statistical significance was determined using two-tailed Mann-Whitney U test.

Figure 3. Persistence of UPEC clinical isolates associated with febrile and non-febrile UTI. Female mice (n=8 per cohort) were inoculated with $10^7$ CFU of either a UPEC non-febrile isolate (NF-UTI; PEDS177 gray square; PEDS918 black square) or febrile isolate (F-UTI; PEDS175 gray circle; PEDS939 black circle). The serotype of each
strain is indicated in the legend. Organs indicated were harvested at 16 hours post infection for homogenization and enumeration of bacteria. Statistical significance was determined using two-tailed Mann-Whitney U test. Bacterial persistence in the bladders (B) and the kidneys (C) of mice infected with non-febrile (solid line; n=9 mice each for PEDS177 and PEDS914 per time point) and febrile (dotted line; n=8 mice each for PEDS175 and PEDS175 per time point) UPEC isolates is presented as a percentage for 48 hours after inoculation. Statistical significance was determined by Chi square analysis. 

Figure 4. Magnitude of IL-6 production elicited by conditioned medium is associated with disease presentation. Immortalized human bladder carcinoma cells were stimulated with conditioned medium produced by 35 independent isolates. The IL-6 accumulated in the culture supernatant was quantified by ELISA after 2 hours incubation. Each data point is the average value for the triplicate quantification of each independent isolate. UPEC strains were categorized as non-febrile (NF-UTI; squares) or febrile (F-UTI; triangles) according to the clinical symptoms at the time of isolation from the urine. 95% CI and median values are represented for each group. Statistical significance was determined using a two-tailed Mann Whitney U test.

Figure 5. Depletion of LPS and vesicles from conditioned medium abolishes cytokine elicitation by UTI89. (A) IL-6 elicitation of human bladder epithelial cells was quantified following exposure to conditioned medium (CM), conditioned medium following LPS depletion (dCM), LPS depleted conditioned medium with reconstitution of 1.25 µg/ml commercially available LPS (dCM+LPS), and commercially available LPS alone (LPS
(B) IL-6 elicitation of UTI89 conditioned medium after removal (-) of vesicles and/or LPS. All samples were normalized using the magnitude detected from parallel non-treated cells. Statistical significance was determined using a two-tailed Mann Whitney U test. (**, p<0.008; *, p=0.03)

Figure 6. Depletion of LPS abolishes cytokine elicitation of UPEC isolates. Four representative isolates of the non-febrile and febrile isolates evaluated for elicitation of IL-6 in the presence (black bars; +) and absence (gray bars; -) of LPS in the conditioned media are depicted. The serotype and strain name are indicated on the X-axis. Statistical significance was determined using a two-tailed Mann Whitney U test. (*, p<0.05)

Figure 7. Association of LPS serotype with clinical disease severity and cytokine responses. (A) Distribution of LPS serotypes from patients that present with non-febrile (dark gray) or febrile (light gray) UTI. The LPS serotype could not be determined (ND) by this method in 5 of the isolates (11%). (B) IL-6 elicitation was determined following infection of cultured bladder epithelial cells with intact viable *E. coli*. Each data point represents the average of three independent replicates. Statistical significance was determined using a one-way ANOVA (p=0.0001).
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