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Antigen-Specific Responses Accelerate Bacterial Clearance in the Bladder\textsuperscript{1}

Praveen Thumbikat,* Carl Waltenbaugh,† Anthony J. Schaeffer,* and David J. Klumpp\textsuperscript{2*†}

Urinary tract infections (UTIs) cause patient morbidity and have a substantial economic impact. Half of all women will suffer a UTI at least once, and 25% of these women will have recurrent infections. That 75% of previously infected women do not become reinfected strongly suggests a role for an adaptive immune response. The goal of this study was to characterize the adaptive immune responses to uropathogenic Escherichia coli (UPEC), the predominant uropathogen. A novel murine model of UTI reinfection was developed using the prototypic cystitis UPEC isolate NU14 harboring a plasmid encoding OVA as a unique antigenic marker. Bacterial colonization of the bladder was quantified following one or more infections with NU14-OVA. Animals developed anti-OVA serum IgG and IgM titers after the initial infection and marked up-regulation of activation markers on splenic T cells. We observed a 95% reduction in bacterial colonization upon reinfection, and splenic leukocytes showed Ag-specific proliferation in vitro. Adoptive transfer of splenic T cells or passive transfer of serum from previously infected mice protected naïve syngeneic mice from UPEC colonization. These findings support our hypothesis that adaptive immune responses to UPEC protect the bladder from reinfection and form the basis of understanding susceptibility to recurrent UTI in women.


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\textsuperscript{*}Abbreviations used in this paper: UTI, urinary tract infection; eGFP, enhanced GFP; EMB, eosin-methylene blue; L-amp, Luria broth agar with ampicillin; UPEC, uropathogenic E. coli.

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Materials and Methods

Bacterial strains

NU14 is a clinical isolate of *E. coli* originally obtained from the urine of a patient with cystitis (16). Bacteria were propagated in Luria broth at 37°C under static conditions that promote expression of type 1 pili (18). The extent of type 1 pilus expression was determined by mannose-sensitive hemagglutination (19, 20) of guinea pig erythrocytes (Cleveland Scientific).

*Generation of OVA-producing NU14*

The OVA coding sequence or TET-C (C fragment of tetanus toxoid, as a control) was expressed in NU14 (NU14-OVA and NU14-TET). This was done by transformation with the plasmids p nir15.OVA or p nir15.TET (21) by electroporation. The parental NU14 strain is ampicillin sensitive; transformants were selected by their resistance to ampicillin. Western blot analysis confirmed expression of OVA in *E. coli* after overnight culture under anaerobic conditions for induction of the nir15 promoter. Bacteria were lysed in 2× sample buffer (6% Tris-Cl (pH 6.8), 20% glycerol, 4% SDS, 2% 2-ME), and heat denatured at 100°C for 5 min. Cell lysates and purified OVA (positive control) were resolved on a 10% SDS polyacrylamide gel and transferred to nylon membranes (Pierce Biotechnology). Membranes were incubated at room temperature with 1% blocking reagent (Kirkegaard & Perry Laboratories) for 1 h and a 1/500 dilution of murine anti-OVA mAb (IgG2b; Brookwood Biomedical), for 2 h, and were detected with HRP-conjugated anti-mouse IgG (Jackson ImmunoResearch Laboratories) using the Suprasignal chemiluminescent substrate (Pierce Biotechnology).

*Flow cytometry*

Cells were washed twice in PBS/0.3% w/v BSA supplemented with 0.1% w/v sodium azide. Nonspecific binding of Abs to FcRs was blocked by preincubating cells with mAb 2.4G2 (BD Biosciences) directed against the FcRIII/II CD16/CD32 (0.5 μg/106 cells/100 μl). Cells were washed and incubated with 0.5 μg of mAb per 106 cells for 30 min at 4°C, and washed again twice. Four-color analyses were performed using a FACS Calibur (BD Biosciences). Data were analyzed using FlowJo software (Tree Star). The following reagents and mAb were obtained from BD Biosciences: PerCP-conjugated anti-CD3ε mAb 145-2C11, PE-conjugated anti-CD4 mAb GK1.5, allophyocyanin-conjugated anti-CD8α mAb 53-6.7, and FITC-conjugated anti-CD69 mAb H1.2F3.

*Cell and serum transfers*

Sera and spleen cells were harvested from NU14-OVA-infected C57BL/6 mice or instilled with saline 14 days previously. T cells were isolated using T cell enrichment columns (R&D Systems), according to the manufacturer’s instructions. Spleen cells (1×104) or enriched T cells (5×104) were resuspended in 500 μl of PBS and injected into the lateral tail veins of naive syngeneic C57BL/6 mice. Other naive C57BL/6 mice received 200 μl of undiluted preimmune or naive serum i.v. All groups were challenged tranurethrally 24 h later with NU14-OVA and sacrificed 1 day later. In one series of experiments, T cells were isolated from female enhanced GFP (eGFP) mice that had been infected with NU14-OVA and adoptively transferred into naive, syngeneic C57BL/6 mice to examine trafficking of specific T cells to the bladder.

*In vitro analyses of T cell response to OVA*

Spleen cells from naive or NU14-OVA-challenged mice were cultured in 200 μl of RPMI 1640 containing 5% FBS ≥ 20 μg of OVA or saline. At day 3, cells were centrifuged, supernatants were discarded, and plates were stored at −70°C. Briefly, the CyQUANT proliferation assay (Molecular Probes) was performed by thawing frozen cells and adding a lysis buffer containing the nucleic acid-binding CyQUANT GR dye. Proliferative response was measured as a direct increase in fluorescence measured using a GeminiXS fluorescence microplate reader (Molecular Devices) with monochromators set for 480 nm excitation and 520 nm emission maxima. Cell numbers were quantified based on a standard curve generated from known counts of spleen cells.

In vitro cytokine stimulation assays were performed on spleen cells from naive, NU14-OVA-challenged mice at day 7 postinfection or NU14-OVA-rechallenged mice 1 day after the second challenge (day 15). Briefly, spleen cells were cultured in 200 μl of DMEM/F12 supplemented with Nutridoma (a serum substitute; Roche), 1-glutamine, and gentamicin. Cells were cultured in the presence of 18 μM OVA or PBS for 1–3 days. Supernatants were collected daily and frozen at −70°C. Dilutions of the supernatant were used in a mouse Th1/Th2 ELISA as per manufacturer’s protocol (eBioscience), and results were expressed in pg/ml.

*Ig ELISA*

Total and OVA-specific Abs were detected by ELISA. Briefly, for anti-OVA ELISA, 96-well microwell plates (Corning) were coated with OVA (10 μg/ml in carbonate-bicarbonate buffer (pH 9.5)) overnight, then blocked with milk blocking buffer (Kirkegaard & Perry Laboratories). The plates were washed; serum or urine samples were incubated overnight at 4°C and washed; and bound Abs were detected using HRP-conjugated goat anti-mouse IgG (H+L) (Jackson ImmunoResearch Laboratories) or goat anti-mouse IgA and goat anti-mouse IgM (Bethyl Laboratories). The anti-OVA mAb, OVA-3 (IgG2b; Brookwood Biomedical), was used as a standard to quantitate the anti-OVA IgG in the sample, while IgM and IgA were expressed in relative absorbance units at 450 nm. Total IgG, IgM, and IgG were measured by coating plates with a capture Ab, followed by addition of serum/urine, and detected using an appropriate HRP-conjugated secondary Ab (Bethyl Laboratories). Reference mouse serum was used as a standard to quantitate total IgG. The color reaction for ELISA was developed by adding 3,3′,5′,5′-tetramethylbenzidine substrate (Pierce Biotechnology).

Immunohistochemistry and immunofluorescence

Paraffin-embedded 5-μm sections were prepared from samples fixed in 10% neutral buffered Formalin. The sections were deparaffinized using standard methods and rehydrated in graded ethanol. Nonenzymatic Ag retrieval was performed by treatment with 0.01 M sodium citrate (pH 6.0) at 92°C for 20 min, and sections were blocked with blocking solution (1% BSA, 0.1% Triton X-100 in PBS), followed by incubation with FITC- conjugated anti-CD4 (clone RM4-5; Santa Cruz Biotechnology), HRP-conjugated anti-CD8α (clone 2.43; Santa Cruz Biotechnology), or biotinylated anti-CD45RB (clone RA3-6B2; BD Biosciences) in blocking solution at room temperature in a humidified chamber. Biotinylated Ab was...
detected by incubation with streptavidin-conjugated Alexa-fluor 594 (Molecular Probes). Finally, slides were washed with PBS, mounted using diaminopropylindole mounting medium, cover slipped, and visualized using a fluorescence microscope. Serial H&E sections (Northwestern University pathology core) were also prepared and examined.

Statistical analyses
Data were analyzed using Prism software (version 4.0; GraphPad). Values are presented as mean ± SEM. The statistical significance of differences in the measured mean T cell frequencies between groups was calculated using Student’s two-tailed t test for two groups, or the one-way ANOVA, followed by Dunnett’s posttest comparison. A p value <0.05 was considered significant.

Results
NU14-OVA colonizes the bladder and undergoes normal clearance

The prototypical cystitis isolate NU14 was transformed with a plasmid encoding OVA or a control protein to generate the strains NU14-OVA and NU14-TET, respectively. The NU14-OVA strain expresses OVA under anaerobic conditions (Fig. 1A), indicating that the strain is likely to express OVA within the anaerobic environment of the bladder. The OVA-nonexpressing NU14-TET

FIGURE 2. Reduced colonization in a reinfection model of UTI. NU14-OVA was used to infect naive mice (1°), to infect mice twice at a 2-wk interval (2°), or infect mice three times at 2-wk intervals (3°). Bladders were dissected 24 h later, and homogenates were plated on EMB agar. Secondary and tertiary infected animals exhibited significantly lower bacterial numbers than primary infections. Bladders from 2° and 3° mice exhibited significantly decreased bacterial colonization relative to 1° bladders (p ≤ 0.05 and p ≤ 0.01, respectively).

FIGURE 3. Reinfection model demonstrates lymphoid infiltrate containing T and B lymphocytes. A and B, Show H&E sections of the bladders of animals 1 and 7 days after NU14-OVA infection. C and D, Show H&E sections at days 15 and 29 after reinfection once or twice, respectively, with NU14-OVA. Increasing leukocyte infiltration was evident in 3° bladders (D) that was absent in response to 1° infection (A). Within the infiltrate, 3° bladders exhibited numerous CD45+ cells (F, red) that are absent in 1° bladders (E). The infiltrate in 3° bladders also contained both CD4+ and CD8+ T cells (G and H, green, respectively). Scale bar represents 50 μm.
and NU14-OVA-infected animals.

Leukocyte aggregates were larger after a second infection (Fig. 3B). Leukocyte aggregates were not visible until 7 days postinfection (Fig. 3B).

**FIGURE 5.** UPEC infection does not result in elevation of total IgG, IgM, or IgA in serum or urine, as measured by ELISA. A, Total IgG in serum and urine remain similar between naive and NU14-OVA-infected animals. B and C, Total IgM and IgA in serum were not significantly different between naive and NU14-OVA-infected animals.

Infection with NU14-OVA evokes an Ab response

To examine a possible humoral response against UPEC, mice were infected one or more times with NU14-OVA, and serum and urine Ab responses were examined. We also characterized the specific contributions of IgM and IgG responses and examined the possible influence of UPEC infection on anti-OVA Ab titers (day 14 vs day 15). Infected mice showed no anti-OVA IgG response in serum during the first 7 days after a primary infection (data not shown), but developed significant titers by day 15 that showed no further increase at day 29 (Fig. 4A). At day 15, the urine of these animals contained 8960 ± 690.6 pg/ml anti-OVA IgG (n = 3, data not shown), but no anti-OVA IgM or IgA was detected. A rise in serum anti-OVA IgM and IgG, but not IgA, was demonstrable at both days 14 and 15, with no significant difference between the two time points (Fig. 4, B and C). Analysis of total serum and urine IgG, IgM, and IgA did not reveal significant differences between the uninfected and NU14-OVA-infected animals (Fig. 5). The ability to induce a specific IgG and IgM response suggests that UPEC, when introduced through its natural route, can evoke a host humoral immune response.

**FIGURE 4.** UPEC infection evokes a humoral immune response. OVA-specific Ab titers in serum from uninfected (naive), D14, 2° (D15), and 3° (D29), NU14-OVA-infected animals were analyzed by ELISA. A, Anti-OVA IgG titers were increased significantly in the 2° and 3° infections as compared with uninfected naive animals (p ≤ 0.05 and p ≤ 0.01, respectively). B and C; D14 and 2° (D15) serum had similar increases in anti-OVA IgG and IgM that were significantly different from naive animals (p ≤ 0.05).

**Reinfection leads to accelerated clearance of NU14-OVA from the bladder**

To assess whether an initial exposure to UPEC alters subsequent responses to challenge infection, NU14-OVA was used to infect naive mice (1°), to infect mice twice at a 2-wk interval (2°), or to infect mice three times at 2-wk intervals (3°). Bladder homogenates were prepared from all animals the day after the final infection, and bacterial CFUs were determined (Fig. 2). No further reduction in bacterial numbers was found at the 7-day time point. Subsequent time points showed bacterial numbers at or below the level of detection (data not shown). Similar bacterial counts were obtained at all time points on EMB and L-Amp agar, showing that the NU14-OVA strain retains the OVA plasmid for extended periods in vivo.

**NU14-OVA infection induces leukocyte infiltration**

Little or no leukocytic accumulation was seen in the bladder wall 24 h after primary infection with NU14-OVA (Fig. 3A), with no visible change until 7 days postinfection (Fig. 3B). Leukocyte aggregates were larger after a second infection (Fig. 3C) and appeared more pronounced after a third infection (Fig. 3D). Staining with anti-CD45R Ab revealed an accumulation of CD45+ cells in bladders of reinfeeted mice that were not observed in tissues 24 h after initial infection (Fig. 3, compare E and F). Furthermore, CD4+ and CD8+ cells were observed among the infiltrate (Fig. 3, G and H). These data show that NU14-OVA induces leukocyte infiltration and suggest that an adaptive immune response may mediate the accelerated clearance of UPEC upon reinfection.

Infection with NU14-OVA evokes an Ab response

To examine a possible humoral response against UPEC, mice were infected one or more times with NU14-OVA, and serum and urine Ab responses were examined. We also characterized the specific contributions of IgM and IgG responses and examined the possible influence of UPEC infection on anti-OVA Ab titers (day 14 vs day 15). Infected mice showed no anti-OVA IgG response in serum during the first 7 days after a primary infection (data not shown), but developed significant titers by day 15 that showed no further increase at day 29 (Fig. 4A). At day 15, the urine of these animals contained 8960 ± 690.6 pg/ml anti-OVA IgG (n = 3, data not shown), but no anti-OVA IgM or IgA was detected. A rise in serum anti-OVA IgM and IgG, but not IgA, was demonstrable at both days 14 and 15, with no significant difference between the two time points (Fig. 4, B and C). Analysis of total serum and urine IgG, IgM, and IgA did not reveal significant differences between the uninfected and NU14-OVA-infected animals (Fig. 5). The ability to induce a specific IgG and IgM response suggests that UPEC, when introduced through its natural route, can evoke a host humoral immune response.
bacterial colonization of the bladder was reduced significantly in animals that received serum, splenocytes, or T cells from previously infected mice (Fig. 7A), whereas serum or T cells from naive donors did not confer protection. We next examined whether T cells trafficked to the bladder of infected mice. C57BL/6 mice expressing an eGFP transgene were infected transurethrally with NU14-OVA and used as donors for column-enriched splenic T cells (Table I) and included both CD4+ and CD8+ T cell subsets. We examined the innate immune response to type 1 piliated E. coli infection before the age of 24 years (24). Approximately 50% of women have at least one symptomatic UTI (14), and nearly 25% of these individuals have recurrent episodes (25). In this study, we developed and characterized a novel murine model of reinfection with UPEC. Our results suggest a significant adaptive immune response to UPEC that is sufficient to protect the host against a subsequent challenge.

The innate immune response to type 1 piliated E. coli infection is initiated by bladder epithelial cell activation through LPS interaction with CD14 and TLRs (11). The release of chemokines leads to the recruitment of neutrophils that mediate bacterial clearance from the bladder. This response results in progressive resolution of bacterial colonization in different strains of mice, including the C57BL/6 strain used in this study (26). Our result in mice infected with the NU14 cystitis strain, modified to express OVA, shows that the host innate response is intact and results in spontaneous reduction in bacterial colonization over time. The use of OVA as a foreign Ag allowed for the elimination of cross-reactivity with bacterial Ags derived from other mucosal sites like the gut. Furthermore, the use of OVA for in vitro proliferation and Ab assays permitted quantitation of defined antigenic immune response as opposed to the wide array of nonspecific responses most likely against less defined bacterial Ags.

Reinfection of mice with UPEC leads to accelerated clearance of bacteria from the bladder. These findings show that initial Ag exposure results in a robust, effective bacterial clearance upon subsequent challenge. Within the constraints of our experimental system in which bladders were harvested 24 h after the last infection, multiple reinfections did not lead to sterilizing immunity of the bladder. At this juncture, we cannot rule out the possibility that longer time points beyond 24-h postinfection may result in complete clearance of UPEC. The protective response observed in this study appears to parallel the classic paradigm of adaptive immune response with an initial Ag exposure evoking an enhanced and accelerated secondary response to the same Ag.

Cellular aspects of the immune response to UTI are easily detected and are often used clinically to determine the presence of infection. Macrosopic urinalysis to detect urinary leukocyte esterase, a product of inflammatory cells and microscopic detection

Table I. CD69 induction by NU14-OVA

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<th>CD3+ Spleocytes (%)</th>
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Figure 6. In vitro stimulation of splenocytes derived from NU14-OVA-infected animals after 7 days shows release of IL-2 that is significantly different from naive animals (p ≤ 0.001) and from a second infection (D15) with NU14-OVA (p ≤ 0.01). Cytokine measurements were done using an ELISA.
of cellular response in the form of pyuria, both attest to the presence of leukocytes in UTI infection. In humans, leukocyte migration into the bladder wall has been attributed to the CXCL8 chemokine (27–31). Although much of the initial inflammatory infiltrate is comprised of neutrophils, our study demonstrates that in secondary infections there is recruitment of B and T lymphocytes to the bladder wall. Interestingly, both CD4 and CD8 T cell subsets appear to be recruited, which may have important implications with regard to the ability to clear infection. UPEC that have been demonstrated to be capable of entry and persistence in urothelial cells (12) may serve as targets for CD8 T cells through their cytotoxic activity, while extracellular UPEC may be removed by CD4-dependent mechanisms. Our study shows activation of T cells beginning as early as 5 days after bladder infection that proliferate in response to specific antigenic challenge in vitro. Furthermore, in vitro cytokine assays demonstrate an increase in IL-2 secretion from splenocytes stimulated with OVA that is consistent with the increased proliferation. However, secretion of cytokines that point to a Th1/Th2 bias was not measurable within the limits of our study, conducted 7 days after an NU14-OVA infection. We speculate that there is a lack of Th1/Th2 skewing at this early time point after NU14-OVA infection. Alternately, it is possible that activated T cells may have largely migrated to the site of infection, and as such are not present in large numbers in the spleen. The significant reduction in IL-2 secretion at day 15 following a secondary infection may further reflect this phenomenon as T cells migrate out of the spleen to the bladder to combat an active infection. Nonetheless, our results in toto suggest that UPEC infection is capable of activating a cellular response that may be required for protection.

UPEC infection elicits Ab responses in animal models and humans. Both IgG and secretory IgA have been found in urine and act as neutralizing Abs (32–34). A variety of vaccine candidates has been tested in both animal models and humans with the purpose of evoking protective humoral responses (16, 17). This study demonstrates that an initial UTI infection in mice elicits a significant systemic humoral immune response that is well developed by 14 days postinfection. Both IgG and IgM Abs specific for OVA were detectable in serum, although overall quantities of each Ig were unchanged. A second encounter with UPEC does not significantly enhance the anti-OVA humoral response, but it is possible that Ab affinity increased as often occurs upon repeated antigenic challenge. It is likely that these and other mucosal Abs play an important role in protection against UPEC infection.

Protection against UPEC infection can be adoptively transferred from immune mice to naive mice. This can be achieved by transfer of either T cells or serum, suggesting that cellular and/or humoral arms of adaptive immunity protect against UPEC reinfection. Serum used for the transfer was derived from mice 1 day after reinfection and was shown to have significant titers of OVA-specific IgG and IgM that may play a role in conferring protection. Resistance to UTI in mice has been shown previously to be enhanced by the transfer of spleen cells (T and B lymphocytes) from immunocompetent mice into T and B cell immunodeficient (SCID) mice (15). However, these investigators report that αβ T cell-deficient mice have colonization levels of UPEC equivalent to those observed in immunocompetent controls. We speculate that the differences observed in our study may be attributed to the fact that we used fully immunocompetent mice, and our reinfection model reflects the average healthy human who develops an innate and adaptive immune response to UPEC. Because the site of UPEC infection is within the bladder, it is likely that effector cells would be specifically recruited to the site of infection. The ability of adoptively transferred T cells to traffic to the bladder suggests specific recruitment that is consistent with a role for T cells in protection against UPEC infection.

The observation that transfers of immune serum and T cells thwart bacterial colonization suggests that the adaptive immune response to bladder infection is multifaceted. Immediately after infection, an innate immune response is activated and is characterized by recruitment of neutrophils and other professional phagocytes to the bladder mucosa. Urothelial cells play a critical role in this early activation mechanism through TLR4-mediated signaling and proinflammatory mediators like CXCL8 and IL-6. These immune cells mediate significant clearance of bacteria from the bladder during the first week of an infection. Concurrently, we observe the development of both cellular and humoral immune responses that peak ~2 wk after initial UPEC challenge. The cellular response appears to be driven by Ag-specific activated T cells, while the humoral immune response is characterized by Ag-specific IgG and IgM Abs in serum and urine. These separate adaptive immune responses, in combination with an innate immune response, cause significantly accelerated clearance of UPEC in subsequent infections. The similar protection against UPEC conferred by transfer of either T cells or immune serum suggests overlapping kinetics in the development of these distinct arms of immunity to UPEC. We speculate that the cellular and humoral immunity may be directed at different stages of the UPEC life cycle in UTI pathogenesis.
References


