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*J Immunol* 2006; 176:3080-3086; doi: 10.4049/jimmunol.176.5.3080

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Antigen-Specific Responses Accelerate Bacterial Clearance in the Bladder

Praveen Thumbikat,* Carl Waltenbaugh,† Anthony J. Schaeffer,* and David J. Klumpp2*†

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 naive 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. The Journal of Immunology, 2006, 176: 3080–3086.

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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Received for publication May 13, 2005. Accepted for publication December 28, 2005.

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This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases Training Grant in Urology T32DK002716 (to P.T.).

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.
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 pnr15.OVA or pnr15.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 nir 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 Supersignal chemiluminescent substrate (Pierce Biotechnology).

**Mice**

Female C57BL/6 mice were obtained as specific pathogen free from commercial suppliers (Harlan Sprague-Dawley; The Jackson Laboratory) and housed in barrier conditions. GFP-transgenic C57BL/6-Tg(CAG-EGFP)C14-Y01-FM131Ob mice (gift of M. Okabe, Osaka University, Osaka, Japan) express GFP under the control of the actin promoter and have been described previously (22). After a 2-wk acclimatization period, 6- to 10-wk-old mice were anesthetized with isoflurane and inoculated transurethrally with 10^8 CFU of _Salmonella_ in saline under conditions that minimize reflux to the kidneys (23). During the process of developing and characterizing the model, we have confirmed, under these inoculation conditions, the lack of kidney infection by plating kidney homogenates onto nutrient agar. The results show that infection is localized to the urinary bladder and does not ascend to the kidneys. To ensure that the inoculum was retained within the bladder, mice were deprived of water for 30 min before anesthesia, the pelvic region was massaged before instillation to displace any urine previously collected in the bladder, and mice were maintained under anesthesia for 30 min following instillation to minimize voiding activity. At intervals after initial challenge, mice were rechallenged and euthanized the following day. At the time of sacrifice, blood was collected by cardiac puncture for serum separation. Urine was recovered by catheterization at the time of sacrifice and stored at −70°C for subsequent determination of anti-OVA Abs. Splenocytes and lymph node were harvested and processed for isolation of lymphocytes. Bladders were harvested for determination of colonization. Bladder mucosa was preserved in sterile saline, serial dilutions were plated onto eosin-methylene blue (EMB) agar, and discrete colonies within the range of 20–200 were counted for calculation of CFU/bladder. EMB agar is selective for Gram-negative enteric rods, but Luria broth agar with ampicillin (L-amp) plates are selective for pnr15.OVA and were used routinely in duplicate plating to confirm that the plasmid was retained in each experiment. All in vivo experiments were conducted following the guidelines of the Northwestern University Institutional Animal Care and Use Committee.

Isolation of spleen and lymph node cells

Spleens and inguinal, renal, and lumbar lymph nodes were removed aseptically from euthanized mice and passed through a 70-μm nylon mesh to obtain single cell suspensions. Erythrocytes were lysed using a mouse erythrocyte lysing kit (R&D Systems). Isolated lymphocytes were resuspended in appropriate buffer for subsequent processing.

Flow cytometry

Cells were washed twice in PBS/0.3% w/v BSA supplemented with 0.1% w/v sodium azide. Nonpecific binding of Abs to FcRs was blocked by preincubating cells with mAb 2.4G2 (BD Biosciences) directed against the FcεRII/II CD16/CD32 (0.5 μg/10^6 cells/100 μl). Cells were washed and incubated with 0.5 μg of mAb per 10^6 cells for 30 min at 4°C, and washed again twice. Four-color analyses were performed using a FACSCalibur (BD Biosciences). Data were analyzed using FlowJo software (Tree Star). The following reagents and mAb were obtained from BD Biosciences: PerCP-conjugated anti-CD3e mAb 145-2C11, PE-conjugated anti-CD4 mAb GK1.5, aliphophycocyanin-conjugated anti-CD8a 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 × 10^6) or enriched T cells (5 × 10^6) 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 transurethrally 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 monochromaters 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), l-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 IgGs. The color reaction for ELISA was developed by adding 3,3’,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), anti-CD8a conjugated anti-CD8 (clone 2.43; Santa Cruz Biotechnology), or biotinylated anti-CD45R Ab (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 diamino propylidene 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 1. NU14-OVA is cleared by an innate response. A, Immunoblotting indicates OVA expression in NU14. NU14-OVA expresses the foreign Ag that is not expressed in the control strain NU14-TET. Purified OVA was run as a control. B, Bladders were harvested at various times postinfection, and tissue homogenates were plated onto selective medium. NU14-OVA exhibits gradual reduction in bladder colonization levels over 7 days postinfection. Horizontal bars indicate the mean, with each experiment being repeated at least three times with similar results.

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.
NU14-OVA infection activates T cells

We next sought to determine whether UPEC infection elicited specific T cell responses. Female C57BL/6 mice received transurethral infection with NU14-OVA or PBS, and 5 days later their splenic lymphocytes were analyzed by flow cytometry. Primary
bladder infection greatly increased CD69 expression by CD3+ spleen (T) cells (Table I) and included both CD4+ and CD8+ T cell subsets. We examined the in vitro lymphocyte proliferative response to OVA following infection of mice with NU14-OVA or NU14-TET (Table II). Splenocytes from NU14-OVA-infected animals showed significant proliferation in response to OVA stimulation that was not observed in spleens of mock-infected controls or mice infected with NU14-TET. The use of OVA as the stimulating foreign Ag allowed for the determination of specific responses not likely to have been compromised by pre-existing immune responses to antigenic epitopes shared by normal enteric and uropathogenic strains of E. coli. These data suggest that UPEC infection leads to the generation of Ag-specific T cell responses. We also examined the cytokine response of in vitro OVA-stimulated spleen cells derived from naïve and NU14-OVA-infected animals. Our results show enhanced secretion of IL-2 by spleen cells derived from NU14-OVA-infected animals 7 days after infection (Fig. 6). This significant rise in IL-2 secretion in these animals is consistent with the increased proliferation of splenocytes from NU14-OVA-infected animals (Table II). IL-4, IL-10, and IFN-γ production was not detected, suggesting a lack of Th1/Th2 skewing at this early time point or an exodus of skewed T cells from the spleen to the site of infection. Our data suggest that UPEC infection leads to cellular activation consistent with the development of an adaptive immune response.

Transfer of protection with T cells or serum
To examine the protective role of humoral and cell-mediated responses, naïve mice were inoculated transurethrally with NU14-OVA, 24 h after receiving transfer of serum, splenocytes, or column-enriched splenic T cells from preimmune syngeneic hosts. 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 naïve 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. Bladder sections from naïve recipient mice can be seen with GFP+ T cells (Fig. 7B) within the bladder 24 h after NU14-OVA infection. In contrast, adoptive transfer of T cells from uninfected female eGFP mice did not result in significant numbers of eGFP T cells in the bladders of donor mice (data not shown). Taken together, these data suggest that both humoral and cell-mediated mechanisms confer enhanced host resistance to bladder infection.

Discussion
One in three women will be afflicted with at least one urinary tract 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 that may represent the response of 75% of patients who successfully resist future infection 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. Macroscopic urinalysis to detect urinary leukocyte esterase, a product of inflammatory cells and microscopic detection

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**Table I.** CD69 induction by NU14-OVA

<table>
<thead>
<tr>
<th>Group</th>
<th>Mock (n = 3)</th>
<th>NU14-OVA (n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3+CD69+</td>
<td>1.6 ± 0.2</td>
<td>63.5 ± 4.8</td>
</tr>
<tr>
<td>CD4+CD69+</td>
<td>0.7 ± 0.1</td>
<td>50.4 ± 3.9</td>
</tr>
<tr>
<td>CD8+CD69+</td>
<td>0.5 ± 0.2</td>
<td>13.2 ± 0.9</td>
</tr>
</tbody>
</table>

**Table II.** OVA induces proliferation of NU14-OVA leukocytes

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Saline (×10⁶)</th>
<th>OVA (×10⁶)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mock</td>
<td>3</td>
<td>2.2 ± 0.1</td>
<td>1.9 ± 0.1</td>
<td>0.0422</td>
</tr>
<tr>
<td>NU14-OVA</td>
<td>3</td>
<td>2.0 ± 0.1</td>
<td>2.8 ± 0.2</td>
<td>0.0153</td>
</tr>
<tr>
<td>NU14-TET</td>
<td>3</td>
<td>3.0 ± 0.4</td>
<td>2.5 ± 0.4</td>
<td>0.1164</td>
</tr>
</tbody>
</table>

**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 CXCL-8 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


