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*J Immunol* 2012; 188:781-792; Prepublished online 19 December 2011; doi: 10.4049/jimmunol.1101231

http://www.jimmunol.org/content/188/2/781

Supplementary Material [http://www.jimmunol.org/content/suppl/2011/12/19/jimmunol.1101231.DC1](http://www.jimmunol.org/content/suppl/2011/12/19/jimmunol.1101231.DC1)

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Innate Transcriptional Networks Activated in Bladder in Response to Uropathogenic *Escherichia coli* Drive Diverse Biological Pathways and Rapid Synthesis of IL-10 for Defense against Bacterial Urinary Tract Infection

Benjamin L. Duell,*† 1 Alison J. Carey,*† 1 Chee K. Tan,* Xiangqin Cui,†‡§ Richard I. Webb,§
Makrina Totsika,* Mark A. Schembri,* Petra Derrington,‖ Helen Irving-Rodgers,#
Andrew J. Brooks,** Allan W. Cripps,* Michael Crowley,†† and Glen C. Ulett*  

Early transcriptional activation events that occur in bladder immediately following bacterial urinary tract infection (UTI) are not well defined. In this study, we describe the whole bladder transcriptome of uropathogenic *Escherichia coli* (UPEC) cystitis in mice using genome-wide expression profiling to define the transcriptome of innate immune activation stemming from UPEC colonization of the bladder. Bladder RNA from female C57BL/6 mice, analyzed using 1.0 ST-Affymetrix microarrays, revealed extensive activation of diverse sets of innate immune response genes, including those that encode multiple IL-family members, receptors, metabolic regulators, MAPK activators, and lymphocyte signaling molecules. These were among 1564 genes differentially regulated at 2 h postinfection, highlighting a rapid and broad innate immune response to bladder colonization. Integrative systems-level analyses using InnateDB (http://www.innatedb.com) bioinformatics and ingenuity pathway analysis identified multiple distinct biological pathways in the bladder transcriptome with extensive involvement of lymphocyte signaling, cell cycle alterations, cytoskeletal, and metabolic changes. A key regulator of IL activity identified in the transcriptome was IL-10, which was analyzed functionally to reveal marked exacerbation of cystitis in IL-10–deficient mice. Studies of clinical UTI revealed significantly elevated urinary IL-10 in patients with UPEC cystitis, indicating a role for IL-10 in the innate response to human UTI. The whole bladder transcriptome presented in this work provides new insight into the diversity of innate factors that determine UTI on a genome-wide scale and will be valuable for further data mining. Identification of protective roles for other elements in the transcriptome will provide critical new insight into the complex cascade of events that underpin UTI. *The Journal of Immunology*, 2012, 188: 781–792.

Urinary tract infections (UTI) are among the most common infectious diseases of humans. Up to 40% of healthy adult women experience at least one UTI episode in their lifetime (1). *Escherichia coli* is the most common cause of UTI, and the spectrum of UTI caused by uropathogenic *E. coli* (UPEC) includes asymptomatic bacteriuria, cystitis, pyelonephritis, and urosepsis. UPEC expresses multiple adhesins and virulence factors that provoke inflammation and enable bacterial colonization of the bladder as the first step in UTI pathogenesis (2–5). UPEC may also suppress innate immune responses (6–8).

The immediate steps that form the host response to UPEC in the bladder and mediate the pathogenesis of UPEC UTI in patients are not well defined. Studies have shown that UPEC triggers inflammation that consists of immune mediators, including ILs (9–12), although some of these responses, including, for example, IL-1α, occur in a pathogen-specific manner (13). Roles for TNF, NO (14), CXCR 2 (15), Tamm-Horsfall protein (16, 17), and CD44 (18) have been shown in models of human UPEC UTI, although, in most cases, the functions of these host factors at the cellular and molecular levels remain unclear. UPEC induces host cell apoptosis in cystitis patients (19–21) and macrophage inflammatory peptide 2 that is induced in response to neutrophils attracted to the bladder to counteract colonization by the bacterium (6). Specific UPEC adhesins, including type 1 and P fimbriae, trigger some of these
Processes (22, 23). Host responses that arbitrate these very early steps in the pathogenesis of UPEC UTI are largely unknown, and the overall contributions of innate immune elements beyond previously identified mechanisms of attachment are not well defined. To better understand the nature of innate immunity to UPEC in the bladder, we performed detailed genome-wide gene expression microarray profiling using the murine model of UPEC UTI to establish the global host transcriptome of mouse bladder to UPEC cystitis. The overall results of the transcriptome were interrogated using several cutting-edge bioinformatics platforms and reveal collections of innate transcriptional networks not previously recognized in bacterial UTI. Systems-level bioinformatic analyses of the transcriptional networks show that the early response to infection in the bladder is broad based and rapid and incorporates diverse biological pathways. Functional analysis of a prominent IL-1β identified within the transcriptome, IL-10, which was shared among multiple biological networks as well as the most highly activated canonical pathway, unveiled a new appreciation of function for this cytokine in the early control of acute bacterial UTI. The innate transcriptome of UPEC cystitis defined in this work will prompt renewed examination of early host determinants of acute UTI aimed at uncovering novel control and preventative strategies for bacterial UTI.

Materials and Methods

Murine model of UPEC cystitis

The murine model of UTI utilizing female C57BL/6 mice (8–10 wk; The Jackson Laboratory [Bar Harbor, ME], Animal Resources Centre [Canning Vale, WA]) based on transurethral inoculation is described elsewhere (24–27). IL-10−/− deficient B6.129P2-Ij(EII)31/j mice (002251) were purchased with appropriate control mice (000664, C57BL/6J) from The Jackson Laboratory. In addition to C57BL/6 mice, CBA mice are also used for UTI studies (28, 29), and we performed microarray assays to compare strains. Infection, mice were anesthetized by brief inhalation exposure to isoflurane, and the periurethral area was sterilized with 10% povidone-iodine, which was subsequently removed with PBS. Mice were catheterized using a sterile Teflon catheter (0.28 mm internal diameter, 0.61 mm outer diameter, and 25 mm length; Terumo, Somerset, NJ) by inserting the device directly into the bladder, and 40 μl challenge inoculum was instilled transurethrally. The catheter was removed, and mice were returned to their cages for recovery. Generally, the inoculum was retained within the bladder for <2 h, which was monitored by inclusion of 0.1% indigo in the bacterial suspension. Mice were infected with 10^7 CFU UPEC CFT073, and urine was collected at 2 h, 24 h, and 5 d postchallenge for colony counts on lysogeny broth agar (30). Bladders and kidneys within the bladder for 10 and 100 CFU cell challenge inoculum in vitro that would facilitate study of intercellular interactions (32). Human 5637 bladder urothelial cells (ATCC HTB-9), U937 monocyte-derived macrophages (ATCC CRL-1593, 21.151 C16 B cells (ATCC CRL-1649), and Jurkat T cells (ATCC TIB-152; clone E6-1) were grown in RPMI 1640 supplemented with 10% FCS and HEPES, as previously described (33–35). A total of 150,000 5637 cells, 15,000 U937 cells, and 1,500 MC116 and Jurkat cells each was seeded into wells of a 96-well cell culture plate (36). For histological analysis, 1 μg/ml mouse anti-IgG1 anti-mouse IL-10 Ab (JES5-2A5, 200 μg/ml, Santa Cruz Biotechnology, Santa Cruz, CA; confirmation assays used ab33471, Sapphire Bioscience, Waterloo, NSW, Australia). Following immersion in 1% H2O2/TBS, an anti-rat probe (Biocare Medical) was used, and sections were developed using 3,3′-diaminobenzidine (Pierce/Progen, Richlands, NSW, Australia). Sections were counterstained in Mayer’s hematoxylin, dehydrated, cleared in xylene, and mounted. Controls included Ab isotypes and staining of bladders from control (PBS-treated) mice. Serial sections were stained with H&E. For fluorescent imaging, sections were mounted in fluorescence medium (Dako). Images were acquired using an Olympus BX51 microscope, and, for whole bladder, an Olympus SXZ61 fitted with a DP71 CCD camera, and CellF acquisition software.

Histotypic human cell coculture model

We established a histotypic human cell coculture model of whole bladder in vitro that would facilitate study of intercellular interactions (32). Human 5637 bladder urothelial cells (ATCC HTB-9) (ATCC TIB-152; clone E6-1) were grown in RPMI 1640 supplemented with 10% FCS and HEPES, as previously described (33–35). A total of 150,000 5637 cells, 15,000 U937 cells, and 1,500 MC116 and Jurkat cells each was seeded into wells of a 96-well cell culture plate for infection. Equivalent cultures were seeded into wells of tissue culture-treated multwell chamber slides (Lab-Tek II 155409; Nalge Nunc International, Rochester, NY) for confocal microscopy. UPEC (multiplicity of infection 10 and 100 CFU cell^-1) was added to cocultures and incubated at 37˚C. At 2 and 5 h postinfection, supernatants were collected by centrifuging 96-well plates at 500 × g and frozen at −80˚C for subsequent IL-10 ELISA (88-7106-88; eBiosciences, San Diego, CA). For confocal microscopy, chamber slides were centrifuged (500 × g for 10 min) at 2 h, rinsed with PBS, and fixed using 4% paraformaldehyde for 45 min at room temperature (RT). Cells were stained with Alexa Fluor 594-phalloidin. Slides were viewed using an Olympus Fluoview FV1000-IX81 confocal microscope using version 1 application acquisition software.

mCherry-tagged UPEC strains

Cherry-tagged UPEC CFT073 was used to visualize the interactions between UPEC and human cells in the histotypic coculture model. The plasmid pmCherry (Clontech) was modified by introducing a tetrasaccharyl resistance cassette into the EcoRI restriction site and then used to transform CFT073 and CFT073Δm. The Δm mutation in CFT073 was constructed
using λ-Red-mediated homologous recombination, as previously described (36). The mCherry-tagged strains were then applied to the histotypic coculture model of human bladder cells, as described above, for measurement of IL-10 by ELISA. Experiments using the bacteria in multwell chamber slides were performed for confocal microscopy to study type 1 fimbriae-mediated adherence in relation to UPEC-triggered IL-10 synthesis in bladder.

Flow cytometry for identification of IL-10–secreting cells

Mixed human cell cocultures incorporating 5637 bladder urothelial cells and U937 monocytes were infected with UPEC, as above, and separated into nonadherent (monocyte-enriched) and adherent populations (urothelial enriched) after 5 h. Nonadherent cells, inclusive of several PBS rinses, were collected by centrifugation (500 × g for 5 min); adherent cells were subsequently collected by trypsinization. Cells were adjusted to 10^6 cells per sample and used in FACs analysis using CD45 as a U937 monocyte marker, or RNA isolation and subsequent qRT-PCR for human IL-10 using specific primers (Supplemental Table I). For FACs analysis, cells were resuspended in wash buffer (1% FBS in PBS), blocked (2% BSA for 1 h at RT), and centrifuged, and FcRs were blocked with 1% normal human serum in wash buffer for 1 h at RT. Cells were centrifuged and resuspended in wash buffer with mouse anti-human CD45 IgG conjugated to R-PE (NBP1-28512 [F10-89-4]; Novus Biologicals) at the concentration recommended by the manufacturer and incubated for 30 min in the dark at RT. Cells were washed twice in wash buffer, fixed in 4% paraformaldehyde overnight at 4°C, and stored at 4°C in the dark until analysis, which was performed using a LSRII Fortessa flow cytometer (BD Biosciences, North Ryde, NSW, Australia) the following day. Data were analyzed using the FACSDiva version 6 software package.

Generation of IL-10–targeting knockdown cells in U937 monocytes

We made several stable U937 cell lines containing chromosomally integrated constructs from lentiviral vectors to knock down UPEC-induced IL-10 responses and complement qRT-PCR analysis of CD45-positive cells. Lentiviruses were produced using Human Expression Arrest GIPZ lentiviral short hairpin (sh)-microRNA vectors targeting IL-10 (clone IDs V2LHS_111493, V2LHS_111458, V2LHS_226698) and a nontargeting clone (RHS4346) in conjunction with the TransLenti Viral GIPZ Packaging System (Thermo Scientific Open Biosystems), according to the manufacturer’s protocol. Normal U937 cells harvested between passages 5 and 8 were transduced in the presence of 8 g/ml puromycin, and expression of TurboGFP confirmed by fluorescence microscopy. The newly contracted U937 cell lines were designated U937-IL10-KD1, -KD3, and -KD4, and the nontargeting control line U937-IL10-NTC, and were used in culture combination, as described above.

Measurement of IL-10 in patients with UPEC UTI

Analysis of IL-10 in urine of human UTI was performed with adult patient study subjects (>18 y) encountered at the Gold Coast Hospital between August and October 2010. Patients underwent assessment for UTI because of symptoms indicating infection or as part of routine patient screening. All patients presenting with cystitis (dysuria, frequency, and/ or urgency with a concentration of a uropathogen in urine >1 × 10^5 CFU/mL) were recruited into the study. Urine samples were obtained as clean-catch voided or catheterized samples and were frozen at −80°C until assay. Control subjects were selected on the basis of not having a current UTI (i.e., no bacteriuria or symptoms indicating UTI). Urinary IL-10 was measured using ELISA. IL-10 was also measured in plasma collected from 57 patients diagnosed as having urosepsis due to UPEC, and these were compared with IL-10 levels in plasma of 60 healthy control subjects.

Statistical analysis

Most of the analysis of the microarray data was in R.2.11 using Bioconductor packages (http://www.biocoductor.org).

Data preprocessing

The raw microarray data were normalized using quantile normalization (37) and summarized using Robust Multi-array Average (38) as implemented in the affy package in Bioconductor. Differentially expressed genes were identified by analyzing the data using the MAANOVA package (39) (http://www.biocoductor.org/packages/bioc/1.6/html/maanov.html). To identify significant changes in gene expression induced by UPEC, we used a shrinkage-based t test (40) together with permutation (41) to compare the treatment group and the control group in each strain. A false discovery rate (42) of 0.05 was used to control the multiple testing issue and generate the significant gene lists. We also generated heat maps of differential genes using hierarchical-clustering algorithms implemented in the R package gplots. Gene class testing was performed using the safe package (43) in Bioconductor using default settings. Both Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway and gene ontology (GO) used as gene classes were analyzed. False discovery rate of 0.1 (44) was used as a cutoff. GoMiner and InnateDB were also used to analyze functional groups of genes activated in biological pathways, and overrepresentation analysis using Over Representation Analysis was based on fold-change and p value cutoffs of 2.0 and 0.01, respectively.

ELISA data

Unpaired Student t test was used with significance level set as a p value <0.05.

Tissue bacterial load data. Mann–Whitney U test was used to compare mean values of bacterial CFU/ml that were not normally distributed, with significance level set as a p value <0.05.

Patient data. Pearson’s χ² test was used to compare the frequency of elevated urinary IL-10 among patients and controls. Unpaired Student t test was used to compare plasma levels of IL-10 among patients and controls, with significance level set as a p value <0.05.

Ethics statement

Animals. This study was carried out in strict accordance with the national guidelines of the Australian National Health and Medical Research Council. The Institutional Animal Care and Use Committee of the University of Alabama at Birmingham and the Animal Ethics Committee of Griffith University reviewed and approved all animal experimentation protocols used in this study (permits: University of Alabama at Birmingham Animal Project 08070816 and Griffith Approval MSC14/06AE). The University of Alabama at Birmingham institution has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare (Assurance A3255-01) and is registered as a Research Facility with the United States Department of Agriculture. The animal care and use program is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (American Association for the Accreditation of Laboratory Animal Care International). Transurethral infections were performed under temporary isoflurane inhalation anesthesia.

Human subjects. This study was performed in accordance with the ethical standards of the Gold Coast Hospital, Queensland Health, Griffith University, University of Queensland, Princess Alexandra Hospital, and Helsinki Declaration. The study was approved, and the need for informed consent was waived by the institutional review boards of Queensland Health and Griffith University (MSC/18/10/HREC) and the board of the Princess Alexandra Hospital (research protocol 2008/264).

Results

Whole bladder transcriptome of UPEC cystitis

Microarray analysis of early global gene expression in bladder triggered by UPEC after transurethral challenge of C57BL/6 mice identified 1,564 genes with significantly altered expression, as follows: 914 (58.44%) were upregulated and 650 (41.56%) downregulated. These 1,564 genes represent 3.8 and 2.7% of the entire 24,000 gene set significantly upregulated and downregulated, respectively. The complete significant gene list is shown as a heat map and volcano plot in Supplemental Fig. 1 and listed according to statistical significance values in Supplemental Table II. Raw data are deposited under accession number GSE26509 in the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/). The list of genes encompassed a diverse set of regulators of metabolism, cell cycle, growth and survival, adaptive and innate immune responses, and genes central to immune regulation, such as NF-κB and ILs. During our analysis of transcriptional activation due to UPEC in the bladder, we noted 78 significant genes that encode IL family members and their receptors, including (fold change) the following: IL-1B (46.9), IL-6 (46.2), IL-1α (13.9), IL-1β (10.28), IL-10a (3.00), IL-10 (5.05), IL-18rap (3.68), and IL-23 (2.2). Other highly expressed genes were the chemokines Cxcl-10 (38.26), Cxcl-1 (22.15), Cxcl-2 (66.72), Cxcl-16 (3.19), Cxcl-3 (9.07), Cxcl-5 (3.46), and Cxcl-9 (2.67).
We next measured the expression of several IL and chemokine target genes using qRT-PCR to validate array datasets. This showed levels of gene expression of specific target genes that were highly consistent with microarray data. For example, IL-1β (195.49-fold increase), IL-1α (134.57-fold increase), IL-10 (23.67-fold increase), IL-6 (132.87-fold increase), and TNF-α (11.23-fold increase) were highly expressed according to qRT-PCR (Table I), validating array analysis. For the majority of the genes tested by qRT-PCR, including IL-1β, IL-1α, IL-10, IL-6, TNF-α, CCL2, and Cxcl-9, the responses peaked at 2 h (Table I). Various genes that were identified in microarray assays as unchanged in expression at 2 h, including Msr-1, IL-10rb, and the housekeeper genes β-actin and GAPDH, also showed no change at this time point according to qRT-PCR. Some of these, however, including Msr-1 and IL-10rb, showed significantly increased expression after 24 h, indicating a slower response to infection.

Transcriptional networks in UPEC cystitis

Gene class testing using KEGG identified 20 significant biologically relevant pathway interactions in C57BL/6 mice (Table II). These included several with significant associations with infection, including metabolism of cofactors (00670), phosphatidylinositol signaling (04070), TLR (04620), and TCR signaling (04660). Next, we analyzed expression data using GO, which identified 223 biologically relevant pathways triggered by UPEC UTI (Supplemental Table III). Among the most significant were those for MAPK activation (187), the response to bacteria-associated molecules (2237), adaptive immune responses (2250), regulation of B cell-mediated immunity (2712), chemotaxis (6935), and leukocyte adhesion (7159). A summary of gene class testing results using KEGG and GO is illustrated in Fig. 1A. GoMiner analysis also identified extensive gene activation among pathways for immune defense against bacteria, cytokine interactions, and B lymphocyte activation (Fig. 1B).

We next sought to examine the microarray dataset derived from C57BL/6 mice using an integrative approach that incorporates known biological interactions from prior studies. InnateDB bioinformatics (45) revealed a suite of 771 additional unique and significant biological pathways from REACTOME and Integrating Network Objects with Hierarchies databases (data not shown). Among these there were 68 that were overrepresented according to Over Representation Analysis. These included the TLR4 cascade (477830), biological oxidations (477446), and hematosis (477598) (Supplemental Table III). Many of these were confirmed pathways for immune defense against bacteria, cytokine interactions, and B lymphocyte activation (Fig. 1B).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Infection Period (h)</th>
<th>ΔFold UPEC</th>
<th>p Value</th>
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<td>IL-10a</td>
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<tr>
<td>GAPDH</td>
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</table>

Table II. Innate transcriptional networks triggered in the bladder by UPEC at 2 h postinfection as identified using KEGG

<table>
<thead>
<tr>
<th>KEGG No.</th>
<th>Pathway Name</th>
<th>p Value</th>
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</thead>
<tbody>
<tr>
<td>00670</td>
<td>Metabolism of cofactors and vitamins</td>
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</tr>
<tr>
<td>04070</td>
<td>Phosphatidylinositol signaling system</td>
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<td>04620</td>
<td>TLR signaling</td>
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<td>04660</td>
<td>TCR signaling</td>
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<td>04710</td>
<td>Cytokine–cytokine receptor interaction</td>
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<td>05212</td>
<td>Pancreatic cancer</td>
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<td>05217</td>
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<tr>
<td>05220</td>
<td>Chronic myeloid leukemia</td>
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</table>
using ingenuity pathway analysis (IPA), the overall results of which are shown in Supplemental Table III.

CBA mice are occasionally used as an alternate strain to C57BL/6 mice, and we analyzed gene expression in these mice to delineate conserved responses, the results of which are shown as a complete list of significant genes in Supplemental Table II. Raw data for CBA mice are deposited under accession number GSE33210 on GEO. Overall, many transcriptional network responses observed in C57BL/6 and CBA mice were shared between the mouse strains. For example, signaling pathways representing JAK–STAT (04630), T (04660) and B (04662) cell receptors, TLRs (04620), phosphatidylinositol (04070), and apoptosis (04210), identified using KEGG, were common, with highly significant p values of activation (Supplemental Table III). Collections of distinct responses were also noted, and CBA mice exhibited a larger number of activated genes. Direct comparisons illustrating the overall response of CBA mice and conserved, CBA-unique, and C57BL/6-unique gene responses are shown in Supplemental Fig. 1.

In the course of our bioinformatics analysis, we noted many IL responses that were overrepresented in activated biological pathways in response to UPEC. This included, for example, pathways for immune defense against bacteria, cytokine activity, and B cell signaling, as identified using GoMiner (Fig. 1B). Unlike IL-1β and IL-6 (46–50), many of the ILs and IL receptors in these responses (IL-2ra/b, -8ra, -10, -11, -15ra, -17c, -18rap, and -23a) have not previously been associated with UTI. Of note was IL-10, which was present in all three GoMiner pathways related to infection (Fig. 1B) and was shared in the activated T lymphocyte activation pathway, as well as eight KEGG (8107, 2794, 2791, 692, 2816, 651, 2813, 604) and eight Integrating Network Objects with Hierarchies (187, 195, 11, 51, 185, 393, 63, 121) pathways (all p = 0.005). IPA also revealed IL-10 signaling as the top hit in canonical pathway recognition and revealed the multiple genes related to IL-10 activation present within the C57BL/6 transcriptome (Supplemental Table III). As a result of these findings, we chose to study the IL-10 response further.

**Induction of bladder IL-10 in response to UPEC**

IL-10 was upregulated 5.051-fold in C57BL/6 mice at 2 h (p = 0.005), according to microarrays, and 23.67- and 2.97-fold (p = 0.001) at 2 and 24 h, respectively, according to qRT-PCR. The cognate receptor, IL-10ra, was also upregulated and was present in several biological pathways identified by InnateDB, including the cytokine–cytokine receptor pathway (Fig. 2). Both IL-10ra and IL-10rb were also significantly upregulated in CBA mice according to microarrays (Supplemental Table II). To better understand this response, we measured total IL-10 protein in bladder of C57BL/6 mice. Compared with mice that received only PBS, there was a significant increase in IL-10 protein in bladders of infected mice (492 ± 33 versus 152 ± 25 pg/ml, p < 0.0001) and a parallel increase in kidneys (Fig. 3). Urinary IL-10 was also measured, but the data were not significant (data not shown).

IHC was then performed on UPEC-colonized uroepithelium (Fig. 4A–C) and showed IL-10–secreting cells lining the luminal surface of the bladder in situ (Fig. 4D). IL-10 was not detected in sections stained with an isotype-type-matched control Ab (Fig. 4E) or in PBS-treated noninfected mice (data not shown). The presence of UPEC within these tissues expressing IL-10 was studied using mCherry-tagged CFT073. IL-10 expression in colonized uroepithelium was not consistently associated with the presence of UPEC (Fig. 4F–H). Thus, IL-10 expression forms part of the innate response to UPEC bladder colonization, but is not consistently colocalized within areas of infection.

**Early IL-10 synthesis correlates with UPEC load**

To investigate the relationship between IL-10 and bacterial burden, we analyzed the distribution of UPEC on bladder uroepithelium using mCherry-tagged UPEC. Binding of UPEC occurred in focal areas (Fig. 5A, 5B, arrows), and early IL-10 expression correlated with the highest numbers of bacteria at 2 h (Fig. 5C, 5D). Lower expression of IL-10 at 24 h (qRT-PCR data, Table I) correlated with fewer UPEC at this time point, as well as urothelial cell invasion and destruction as observed by SEM (Fig. 5E, 5F). Lower bacterial burdens were observed at 5 d (Fig. 5C). Extrusions from urothelial cells at 2 h (Fig. 5D) resembled intracellular bacterial communities (51), and elongated UPEC cells at 24 h (Fig. 5E, 5F) mirrored unique filamentous UPEC, as noted elsewhere (52). Thus, IL-10 synthesis in bladder peaks early with high UPEC loads, which are subsequently reduced, and correlate with lower IL-10 expression and urothelial cell destruction.

**Early IL-10 contributes to control of UPEC UTI**

We next analyzed whether early IL-10 in bladder has any functional role in UTI by comparing disease in immunodeficient gene-knockout mice and wild-type (WT) mice, as previously reported, for other host factors (53). Infection of IL-10−/− mice and assessment of the impact of a lack of IL-10 on disease compared with WT mice at 24 h postinfection showed that IL-10−/− mice had significantly more UPEC in the bladders, urine, and kidneys compared with WT mice (Fig. 6A–C; p = 0.013; p = 0.001; p = 0.007). Bacterial loads in IL-10−/− mice were 10-fold greater in bladder and 30-fold greater in urine and kidneys at this time point compared with WT mice. Ab depletion studies using an anti–IL-10–neutralizing Ab in WT C57BL/6 mice also demonstrated higher bacterial burdens in urine and bladders of mice following pretreatment with Ab and subsequent infection with UPEC (Fig. 6D, 6E). Mice that received anti–IL-10 Ab prior to UPEC challenge had 4.50 ± 0.20 CFU/ml in urine 24 h postchallenge compared with 3.89 ± 0.19 CFU/ml in controls, a statistically significant difference. Bladder bacterial counts in anti–IL-10–Ab-treated WT mice were also higher than controls (5.14 ± 0.26 CFU/ml versus 4.84 ± 0.24 CFU/ml), but these differences were not statistically significant based on group sizes of 12 mice. Together, these data show that early bladder IL-10 is protective in the response to UPEC and contributes to bacterial control in UTI, particularly in terms of bacteriuria.

**IL-10 is produced in a coculture model of human bladder**

We next sought to investigate whether IL-10 is produced by human cells in response to UPEC using a novel histotypic four-cell coculture model of bladder. In cocultures of urothelial cells, monocytes, T cells, and B cells (ratio 88:10:1:1), there was no detectable increase in IL-10 in response to UPEC after 2 h (data not shown). However, IL-10 was produced in UPEC-infected cultures after 5 h, with 3-fold more IL-10 secreted by infected cells compared with controls (p < 0.001; Fig. 7A). We also analyzed the effect of the number of T cells on IL-10 production because T cells often amplify IL-10 production (54). In cocultures that contained more T cells (79:10:10:1), the IL-10 response at 5 h was augmented (Fig. 7B). Confocal microscopy confirmed less binding of the mutant to bladder cells compared with the WT bacteria (Fig. 7B).
Thus, type 1 fimbriae-mediated attachment to human bladder cells is not required for UPEC-triggered IL-10 production.

**Identification of the IL-10–producing cells**

To determine the cell type that produces IL-10 in response to UPEC in the coculture model of human bladder, we used CD45 staining as a marker of monocytes and analyzed cocultures after 5 h of infection (Fig. 8A) or separated populations of nonadherent (enriched for U937 monocytes) and adherent cells (enriched for urothelial cells). Utilizing qRT-PCR, we observed a 4.1-fold increase in IL-10 mRNA in monocyte-enriched (CD45-positive) cell populations compared with controls (Fig. 8B). In contrast, IL-10 mRNA levels in urothelial-enriched (CD45-negative) cells remained unchanged after 5 h of infection (Fig. 8C).

We then measured IL-10 protein in cocultures that used various IL-10–targeting knockdown versions of U937 cells to confirm data from the qRT-PCR assays above. In infected cocultures that used any of three IL-10 knockdown U937 cells (targeting exons 1, 3, and 4), the IL-10 response was abolished compared with control cocultures that used either normal U937 cells, or a nontargeting control U937 line that was transduced with a nontargeting control vector (Fig. 9). In a fourth IL-10–targeting U937 cell line, however, the response was not changed (data not shown). Together, these data confirm that the IL-10 produced in the coculture model of human bladder in response to UPEC is derived mostly from U937 monocytes in this model.

**IL-10 is produced in human UTI**

Measurement of IL-10 in patients with UPEC cystitis by ELISA showed significantly higher urinary IL-10 levels (>10 pg/ml) among patients compared with control individuals without UTI (Fig. 10A). Low baseline levels of urinary IL-10 (<5 pg/ml) were more common among controls compared with UTI patients, but significantly more patients exhibited high levels of urinary IL-10 (>40 pg/ml) compared with controls (Fig. 10A). We also measured IL-10 in plasma of 57 patients with UPEC urosepsis and 61 healthy subjects without UTI to determine whether the urinary tract might be influenced by systemic IL-10 in urosepsis. Patients with urosepsis had significantly higher levels of IL-10 in plasma compared with controls (Fig. 10B).

**Discussion**

The very early inflammatory signaling events triggered in the bladder immediately following colonization with UPEC define the cascade of events that culminate in UTI disease or resolution of infection. Recently, innate resistance to UPEC UTI was demon-
stratified in the context of pathogen-specific signaling in murine pyelonephritis (55), and the danger-associated molecular pattern S100A8/A9 was shown to be unnecessary for effective host defense against UTI (56). The overall innate response of bladder in UPEC cystitis in the murine UTI model, however, remains largely uncharacterized. In this study, we provide a detailed genome-wide microarray analysis of whole bladder to UPEC UTI in mice using large group sizes and a nonpooled sampling approach for increased statistical power to offer a revealing new perspective on the innate response to infection. Our approach has uncovered a broad range of host factors not previously known to regulate innate defense to bacterial UTI. Several genes identified in this study are consistent with prior reports that profiled mouse bladder previously and mapped responses in foci of uroepithelium infected with intracellular UPEC pods, which displayed localized responses (50, 57). The whole tissue approach we have used gives broader definition to the total bladder transcriptome of UTI. This provides crucial new insight into the dynamic interactions of infected cells as well as adjacent bystander cells, the inflammatory infiltrate associated with UTI (46), and the local vasculature that contributes to inflammation in the bladder after bacterial colonization (20, 47, 58). Notably, many of the responses identified in the whole bladder transcriptome presented in this work are consistent with those observed in localized responses to intracellular bacterial communities in bladder urothelial cells, as previously reported (57). Of particular note were localized urothelial responses to intracellular bacterial communities such as those directed at opposing UPEC salvage of host cell iron and facilitation of glucose import and epithelial structural integrity (57). Many of these responses are consistent with the genes, such as lipocalin 2 (Lcn2) and hexokinase 2 (Hk2), identified as highly upregulated in the whole bladder transcriptome of UPEC cystitis in this study. Together, these findings emphasize the importance of iron scavenging in the lifestyle of E. coli in the bladder, as recently described (59–61), and, from the hosts’ perspective, the key role of iron sequestration, sugar metabolism, and cell integrity in the urinary tract in response to bacterial uropathogens.

The whole bladder transcriptome of UPEC cystitis reveals a complex network of interactions that encompass a broad functional variety of genes involved in metabolism, cell cycle, growth, adaptive and innate immune responses, cofactors, and signaling. Many of the identified biological pathways have not previously been associated with UTI. Leukocyte signaling triggered in the bladder appears to reflect a collection of pathways linking TLR, TCR, cytokines, JAK–STAT signaling, and BCR signaling. That...
many of these signaling pathways were activated by UPEC not only in the universal C57BL/6 mouse strain, but also the alternate CBA strain, demonstrates a high degree of conservation in many of the networks related to leukocyte activation and lymphocyte signaling during acute UTI. These observations underscore the prominence of these signaling mechanisms in the innate response to UPEC in

FIGURE 5. IL-10 expression correlates with bladder UPEC load. Synthesis of early IL-10 correlates with focal UPEC adhesion to bladder uroepithelium at 2 h, as shown in whole tissue using mCherry-tagged UPEC (A, B) and higher bacterial loads at 2 h compared with later time points (C; n = 14–18 per time point, combined from three separate experiments). Early colonization reflects UPEC bound in focal areas of adhesion around bulbous extrusions (D), which is followed by decreasing bacterial loads over time and urothelial cell destruction (E, F). Scale bars, 800 μm (A); 160 μm (B); 10 μm (F).

FIGURE 6. Early IL-10 protects against UPEC UTI. Bladders, urine, and kidneys of C57BL/6 mice and age-matched IL-10–deficient mice (B6.129P2-Il10tm1Cgn/J) challenged with UPEC were cultured after 1 d of infection (n = 12 per group, combined from two separate experiments) to compare tissue bacterial loads. Higher UPEC numbers detected in IL-10–deficient mice compared with control mice demonstrate a protective role of early bladder IL-10 expression in cystitis (A), bacteriuria (B), and pyelonephritis (C). C57BL/6 mice treated with anti–IL-10–neutralizing Ab also exhibited higher mean UPEC burdens in bladders (D) and urine (E) compared with controls, although differences in bladders were not statistically significant. Mann–Whitney U test was used for comparisons, with significance set at p < 0.05.
Data represent mean ± SD of quadruplicate samples from three separate experiments. Unpaired Student t test was used with significance at p < 0.05. Red, mCherry-tagged UPEC; green, Alexa Fluor 488 phalloidin for F-actin; blue, Hoechst 33258 for nuclei. Scale bars, 10 μm.

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FIGURE 7. UPEC-triggered IL-10 synthesis in an in vitro model of human bladder. UPEC triggers IL-10 synthesis in a histotypic coculture model of human bladder (5637 urothelial cells, monocytes, T cells, and B cells [ratio 88:10:1:1]) (A). UPEC-induced IL-10 occurs independently of type 1 fimbriae attachment because levels of IL-10 triggered by infection (B) were similar in cocultures infected with either mCherry-tagged WT UPEC (C) or CFT073fimA despite impaired binding of the mutant (D). Data represent mean ± SD of quadruplicate samples from three separate experiments. Unpaired Student t test was used with significance at p < 0.05. Red, mCherry-tagged UPEC; green, Alexa Fluor 488 phalloidin for F-actin; blue, Hoechst 33258 for nuclei. Scale bars, 10 μm.

UPEC described in this work, including MIP-2, IL-1β, IL-6 (4, 13, 46, 50), and CXCR1 (58). Interestingly, our data reveal that the induction of these mediators, which occurs amid extensive inflammatory responses, takes place with concomitant UPEC-triggered transcriptomic activation of key immunosuppressive genes. These include a suppressor of cytokine signaling socs3 and rapid induction of the gene for the master regulator of innate immunity, IL-10. We suggest that this induction of suppression-related responses may represent a proactive attempt by the host to moderate excessive inflammation, which could damage tissue locally and cause immunopathology in the bladder. Thus, the early bladder transcriptome mounted in response to UPEC appears to reflect a balance of host defense strategies designed to drive rapid and robust immune activation, but also moderate inflammatory responses by stimulating the actions of immune modulators.

A notable element within the UPEC cystitis transcriptome was broad involvement of multiple IL family members and their receptors, including IL-1, IL-2, IL-6, IL-8ra, IL-10, IL-11, IL-17cra, IL-18rap, and IL-23a. Considering the role of IL-10 as a master regulator of innate immunity, we focused on IL-10 in functional and clinical studies in patients with UPEC cystitis. IL-10 works at the crossroads of Th1-centric inflammation (54) and immunosuppression (64). Smith et al. (65) described urinary IL-10 in renal transplant patients with transplant rejection; however, the function of IL-10 in the infected human urinary tract is hitherto unknown. In our study, patients with UPEC cystitis secreted significant urinary IL-10 and produced high levels of IL-10 systemically during UPEC urosepsis. Whether the systemic IL-10 present in patients with UPEC urosepsis has coordinated effects in immune regulation, as described in other clinical conditions, is unknown (66). However, our studies in mice point to the fact that IL-10 produced in the bladder plays a functional role early in UTI disease pathogenesis. Infection of IL-10−/− mice showed an unexpected protective role for early IL-10 in UPEC UTI because these mice had more UPEC and more severe bacteriuria. Bacteriuria was also more severe in mice undergoing Ab depletion using an anti-IL-10–neutralizing Ab. These findings highlight a functional impact of early IL-10 in UPEC UTI and suggest that future investigation of novel anti-inflammatory treatments in the mouse UTI model may be worth investigating as a means of understanding the inflammation-immunosuppression nexus in acute bacterial UTI. In addition, several IL-10−/− mice also progressed to pyelonephritis, which failed to develop in control mice, suggesting additional unknown effects in the upper urinary tract.

The apparent protective role for early IL-10 in UPEC UTI in mice may reflect a moderation of pathological inflammatory responses shown to be central in many of the active biological pathways in the whole bladder transcriptome. As a comparison, for example, in protozoan and helminthic infections, IL-10 aids in disease control by reducing pathological sequelae and development of lesions in acute infections (67). It is noteworthy that IL-10 also has well-defined roles in mediating pathogenesis in other bacterial infections due to broad immunosuppressive effects; IL-10 is known to contribute to persistent infections such as tuberculosis (67, 68). These contradictory effects of IL-10 in different disease states are explained by the well-known pluripotent nature of IL-10 and the host requirement for tightly controlled inflammatory responses to defend against a diversity of microbial pathogens while concurrently minimizing host damage.

Our in vitro analysis of the human IL-10 response to UPEC using a novel coculture model of bladder incorporating urothelial cells, monocytes, and lymphocytes, which comprise a major element of the inflammatory infiltrate in the bladder response to E. coli (69), identified the source of the IL-10 as monocytes. The urothelial cell...
The coculture system used in this work has inherent limitations, such as the exclusion of polymorphonuclear leukocytes, but epithelial cell types have been cocultured with lymphocytes and monocytes previously (70, 71) and have proven useful for assessing IL-10 effects (72). The advantages of coculture models incorporating epithelial cells for the study of host–pathogen interactions are reviewed elsewhere (32). Epithelial cells in mucosal tissues are anatomically positioned in close proximity to various subepithelial cell types, including lymphocytes (73), which can contribute to cell signaling function via paracrine pathways (74). Signaling between cells may aid these responses because lymphocytes produce IL-10 in response to various bacteria such as Mycobacteria spp. (75, 76) and Helicobacter spp. (77), and intercellular interactions can be essential to drive antibacterial responses (78).

Type 1 fimbriae of UPEC induce secretion of various cytokines in the mouse UTI model (48). Surprisingly, IL-10 synthesis in human cell in vitro occurred independently of fimbriae-mediated binding in this study. This indicates that the presence of neither type 1 fimbriae nor bacterial attachment is required for IL-10 production in the mixed cell coculture model of human bladder. Other components of bacteria that may trigger IL-10 include LPS (79) and species-specific molecules such as M protein of Streptococcus pyogenes, which induces IL-10 via Tr1 cells (80). LPS triggers the production of IL-10 in noninfectious experimental models that incorporate dendritic cells and macrophage stimulation, for example (81, 82). LPS also activates key innate immune mechanisms, including TLR-4, CD14, and NF-κB (83), which were shown to be activated by UPEC within the KEGG TLR signaling pathway and the strongly induced IPA network for IL-10 signaling. In addition to UPEC-triggered synthesis of IL-10, the ligand-binding chain of the IL-10R, IL-10ra, was also triggered at the transcriptional level. Bladder cells are not known to express IL-10R, and, to our knowledge, this is the first report of IL-10ra responses in bladder tissue. Thus, this may represent an additional

![FIGURE 9.](http://www.jimmunol.org/Downloadedfrom http://www.jimmunol.org)

Stable U937 cell lines targeting IL-10 or non-targeting for knockdown in U937 monocytes confirmed that UPEC-induced IL-10 in cocultures was derived from monocytes. The IL-10 response in UPEC-infected (UPEC multiplicity of infection 10) cocultures of 5637 urothelial cells in combination with various stable U937 monocyte cell lines harboring constructs targeting exons 1, 3, and 4 of IL-10 or a nontargeting control (NTC) is shown and compared with the response of cocultures that incorporated normal U937 cells. IL-10 responses in U937 IL-10KD cocultures were not significantly different in any of three KD cell lines compared with noninfected control cultures containing normal U937 cells. An independent-samples t test was used to compare replicates (n = 12) of 5637–U937 control cocultures (containing normal U937 cells) against infected cocultures with U937 shRNA KD cell lines.

![FIGURE 10.](http://www.jimmunol.org/Downloadedfrom http://www.jimmunol.org)

IL-10 forms part of the human immune response to UPEC. In patients with UPEC UTI elevated urinary IL-10 (>10 pg/ml) was more common in individuals with UTI compared with controls (A) (n = 10/154; 6.5% versus n = 3/263; 1.1%; p = 0.006, Pearson’s χ² test). Plasma IL-10 was also elevated in patients with UPEC urosepsis (n = 57) compared with controls (n = 60) (B) (p = 0.001, unpaired Student t test, independent samples) illustrating IL-10 in the human immune response to UPEC.
mechanism of immune regulation at the bladder-bacterial interface given the key role of IL-10–dependent signaling in innate immunity (84). IL-10R is present on the surface of a variety of other human lymphoid and myeloid cells (85) and is upregulated during infection-induced inflammatory conditions such as sepsis (86). Moreover, human epithelial cells lining the colonic mucosa express IL-10 to control inflammation and maintain mucosal homeostasis (66). It is important to note, however, that our findings on IL-10R transcripts were not extended to detection of the receptor protein, which is a limitation of the current work. The role for IL-10R in addition to IL-10 in maintaining urothelial integrity is an appealing area for further investigation.

The UPEC UTI whole bladder transcriptome described in this study provides a wealth of new information for detailed study and will be an invaluable tool for further data mining and biological pathway exploration. Further molecular analysis of the role of IL-10 in the early transcriptome of UPEC UTI and investigation of the multitude of other signaling events defined to our knowledge for the first time in this study will provide opportunity for improved understanding of the innate host responses to E. coli in the context of these important human infections.

Acknowledgments

We thank Prof. John Gerrard for facilitation of clinical aspects of this study, Janice King and Yvette Hale for excellent technical assistance, and Dr. Michael Hanzal-Bayer at the Facility for Life Science Automation at In- stitute for Molecular Biosciences, University of Queensland, for performing the lentivirus transductions. We thank Dr. Joan Faoagali and other members of the microbiology laboratory at the Princess Alexandra Hospital for help with the collection of patient plasma samples. We also thank Flora Gathof for administrative assistance and Prof. David Briles for helpful discussions.

Disclosures

The authors have no financial conflicts of interest.

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