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Attenuation of human neutrophil migration and function by uropathogenic bacteria

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Abstract

The establishment of bacterial infections at mucosal epithelial surfaces is determined by the balance of virulence attributes of the pathogen with the activity of innate host defenses. Polymorphonuclear leukocytes (PMN) are key responders in many bacterial infections, but the mechanisms by which pathogens subvert these early responses to establish infection are largely undefined. Here, we model early interactions between human PMN and the primary cause of urinary tract infections, namely uropathogenic *Escherichia coli* (UPEC). Our objective was to define virulence phenotypes of uropathogens that permit evasion of PMN activity. We show that UPEC strains, as compared with laboratory and commensal *E. coli*, resist phagocytic killing and dampen the production of antimicrobial reactive oxygen species by PMN. Analysis of the transcriptional responses of PMN to *E. coli* strains revealed that UPEC exposure downregulates the expression of PMN genes that direct proinflammatory signaling and PMN chemotaxis, adhesion, and migration. Consistent with these data, UPEC attenuated transepithelial neutrophil recruitment in an in vitro model of acute infection and in a murine model of bacterial cystitis. We propose that these UPEC strategies are important in the establishment of epithelial infection, and that the findings are germane to bacterial infections at other epithelial surfaces.

Keywords

Escherichia coli; Neutrophil; Migration

1. Introduction

Host–pathogen interactions are characterized by the complex interplay between host defense mechanisms and attempts to evade these defenses by microorganisms. Polymorphonuclear leukocytes (PMN; neutrophils) play a critical role in the innate immune response to bacterial pathogens, engulfing and killing invading microbes via the generation of reactive oxygen species and the release of antimicrobial peptides [1]. In addition, PMN are important mediators of inflammatory signaling and cooperate with other cell types to influence the effector responses of the immune system [2]. Neutrophil migration to infected tissues is

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driven by chemoattractant signals and requires coordination of cytoskeletal rearrangements and specific interactions of surface-expressed ligands of the neutrophil with receptors on endothelial and epithelial cells [3]. While the stepwise process of neutrophil extravasation into target tissue has been well studied, the mechanisms by which bacterial pathogens modulate immune effector cell recruitment are incompletely defined.

Urinary tract infections (UTIs) are among the most common bacterial infections in humans, causing over 100 million annual infections worldwide [4]. In the United States, over 7 million urinary tract infections are diagnosed yearly, translating into health care costs exceeding \$1 billion every year [5–6]. The most frequent cause of UTIs is uropathogenic *Escherichia coli* (UPEC), which causes 85% of community-acquired UTI and 25% of cases of nosocomial UTI [5–6]. Recent work in a murine cystitis model has revealed a pathogenic cascade of events in *E. coli* UTI. Bacterial attachment to and entry into superficial facet cells of the bladder epithelium is mediated primarily by interaction of the adhesin of type 1 pili, FimH, with mannosylated uroplakins on facet cell surfaces [7–9]. UPEC rapidly multiply within superficial epithelial cells, forming intracellular biofilm-like communities [10–11], and UPEC subsequently reside in small intracellular nests that can re-emerge to cause recurrences of UTI [9,12]. Consistent with other bacterial pathogens, the inflammatory response to infection by uropathogenic *E. coli* (UPEC) is characterized by increased levels of pro-inflammatory cytokines and neutrophil influx [13]. Recent studies indicate, however, that UPEC can suppress the early secretion of inflammatory signals from uroepithelial cells in vitro [14–16], and differentiated filamentous UPEC are resistant to PMN phagocytosis in vivo [11,17]. The contribution of uroepithelial cells to PMN recruitment has been explored [18–19], yet the mechanisms by which UPEC modulate PMN recruitment and function have yet to be fully elucidated.

In this study, we examined the response of human neutrophils to uropathogenic or non-pathogenic *E. coli* in order to characterize pathogen-specific responses during Gram-negative bacterial infection. We hypothesized that UPEC downregulates neutrophil activity, a phenotype that might be important during initiation and progression of infection, or for subsequent establishment of UPEC's quiescent reservoir within the bladder; here, we chose to model the very early interactions between UPEC and PMN. Investigation of the ability of bacteria to elicit an antimicrobial response and to induce transepithelial neutrophil migration in vitro revealed active suppression of PMN responses by the pathogenic strain. A comprehensive comparative analysis of global transcription profiles from PMN exposed to bacteria was used to elucidate the underlying mechanisms of these observations. Our results indicate that uropathogenic strains elicit a less robust inflammatory response characterized by reduced expression of adhesins and molecules involved in actin polymerization. Thus, UPEC may evade the activation of the acute innate immune response in the urinary tract by suppressing neutrophil movement and antibacterial activity, providing an advantage important for establishing infection.

2. Materials and methods

2.1 Human PMN isolation

In accordance with a protocol approved by the Washington University Human Research Protection Office (HRPO), PMN were isolated from venous blood of healthy adult volunteers as described previously [20]. Scripted verbal consent for phlebotomy was obtained from study subjects, as required by the HRPO. Briefly, dextran sedimentation of erythrocytes was followed by Ficoll density-gradient centrifugation (Ficoll-Paque Plus, GE Healthcare) and hypotonic lysis of contaminating erythrocytes. PMN viability was >99% as assessed by trypan blue exclusion, and purity was >99% as determined by visualization of nuclear morphology after staining (Hema3, Fisher Scientific). Cells were resuspended in

pre-warmed RPMI 1640 medium (Gibco) buffered with 10 mM HEPES (RPMI/H; pH 7.2) at a concentration of 10^7 cells/ml and used immediately.

2.2 Bacterial strains and culture

Escherichia coli strains were cultured at 37°C in Luria-Bertani broth under static conditions for 20 h unless otherwise indicated. Strain UTI89 was isolated from a patient with cystitis [21] and CFT073 from a patient with pyelonephritis [22]; MG1655 is a well-characterized K-12 laboratory strain which is type 1 pilated [23–24]. A number of uncharacterized fecal isolates of *E. coli* from normal, healthy children (kind gift of P. Tarr; denoted FI-1 through FI-12) were also used for comparison. The FimH-deficient derivative of UTI89 was constructed as described previously [14,25].

2.3 PMN reactive oxygen species (ROS) production

The production of ROS by human PMN was measured using a kinetic assay for fluorescence of an indicator compound, 2',7'-dihydrodichlorofluorescein diacetate (DCF, Molecular Probes). Purified human PMN were incubated with 10 μ M DCF for 30 min at room temperature in PBS. The indicator-loaded PMN (10^6 cells) and *E. coli* (10^7 colony-forming units (CFU)) were combined in wells of a 96-well microtiter plate at 4°C and centrifuged for 3 min at $400 \times g$, then transferred to a microplate fluorometer (Synergy 2, BioTek). The amount of ROS generated was measured for 120 min at 37°C using excitation and emission wavelengths of 485 and 528 nm, respectively, and is reported in arbitrary fluorescent units as a percentage of the maximum fluorescence produced in response to the positive control, 5 μ M phorbol myristate acetate (PMA; Sigma). Data represent the mean and standard deviation of at least three independent assays with samples analyzed in triplicate.

2.4 Oxidative stress resistance

A disk-diffusion assay was performed to determine the sensitivity of various *E. coli* strains to the ROS, hydrogen peroxide. Cells from an overnight LB broth culture were resuspended in PBS to an OD₆₀₀ of 0.5 and a sterile cotton swab was used to spread the bacteria onto an LB agar plate. Filter paper disks (6 mm, Becton Dickinson) were added to the surfaces and 10 μ l of hydrogen peroxide (30% [vol/vol]) was spotted onto each disk. The plates were then incubated overnight at 37°C, and following growth, the diameters of inhibition zones were measured. Data represent the mean and standard error of triplicate assays. Statistically significant differences were determined by an unpaired Student's *t* test.

2.5 PMN bactericidal activity

Bacteria were grown as described above, washed once in PBS and resuspended in RPMI/H to a concentration of approximately 10^8 CFU/ml. Bacteria were combined with either PMN (10^7 cells) or an equivalent volume of sterile medium in wells of a 24-well tissue culture plate and incubated at 37°C incubator with 5% CO₂ for the length of time indicated in the text. Samples were serially diluted in sterile PBS containing a final concentration of 0.1% Triton X-100 to lyse the PMN, plated onto LB agar, and incubated at 37°C for enumeration of CFU in the original sample. An aliquot of bacteria was taken and plated as described above just prior to addition of PMN in order to determine initial input. The number of viable bacteria was calculated as a percentage of the input (which was normalized to 100%), and data represent the mean and standard deviation of at least three independent assays. Statistically significant differences were determined by an unpaired Student's *t* test.

2.6 Transcriptional profiling of host cell-bacteria interactions

To measure uropathogenic *E. coli* or eukaryotic cell gene expression during in vitro encounters, bacteria were grown as described above, washed once in phosphate buffered

saline (PBS) and resuspended in RPMI/H to a concentration of approximately 10^9 CFU/ml. The uropathogenic (UTI89) or non-pathogenic (MG1655) *E. coli* ($\sim 10^8$ CFU) were combined with either PMN (10^7 cells) or media only in wells of a 24-well tissue culture plate and centrifuged at $400 \times g$ for 5 min at 4°C . Plates were transferred to a 37°C incubator with 5% CO_2 for 60 min. The cells from each well were collected in RLT lysis buffer (Qiagen), and total RNA was isolated as described below. Transcription profiles of the PMN (with UTI89 or MG1655) relative to the media-only control were determined by DNA microarray or real-time RT-PCR as described below.

2.7 RNA isolation

For evaluation of host and bacterial transcript abundance, differential lysis was applied in order to reduce cross-contamination of eukaryotic and prokaryotic RNA samples. For each sample, cells were resuspended in lysis buffer (RLT, Qiagen) and vortexed briefly prior to centrifugation. This treatment results in near complete lysis of eukaryotic cells, but does not affect bacterial cells or transcription [26]. The supernatant containing host cell lysate was further homogenized (QIAshredder, Qiagen). Total RNA was isolated from the lysates by silica membrane binding (RNeasy, Qiagen), and contaminating chromosomal DNA was removed by DNase treatment (Turbo DNA Free, Ambion). The absence of DNA was confirmed by PCR, and the quality and quantity of RNA were determined by spectrophotometry and agarose gel electrophoresis. Criteria for inclusion in downstream applications were $\text{OD}_{260/280} \geq 2.0$ and the absence of detectable degradation. Samples intended for microarray analysis were also analyzed by a fluorescent assay involving electrophoretic separation of RNA (Agilent 2100 bioanalyzer) and rejected if the electropherogram (a diagram of fluorescence over time) showed degradation of RNA.

2.8 Global expression profiling

In collaboration with the multiplexed gene analysis core in the Laboratory for Clinical Genomics at the Washington University Siteman Cancer Center, RNA samples prepared from PMN lysates were converted to biotinylated cRNA targets and hybridized to commercially available human microarrays (U133 Plus 2.0, Affymetrix) according to standard protocols as directed by the manufacturer (Affymetrix, Foster City, CA). The expression data (.CEL file) from each GeneChip was imported into DNA-CHIP ANALYZER v1.3 (dChip) [27] for normalization, filtering, and sample comparisons. Data from these experiments were archived by the Bioinformatics Core at the Washington University Siteman Cancer Center and have been submitted to the Gene Expression Omnibus repository (accession number GSE18810). Each experiment was performed in duplicate with independent RNA samples and different PMN donors.

2.9 Real-time RT-PCR analysis

Host cell-bacteria interaction experiments and RNA preparation for real-time analysis were done with conditions identical to those used for the microarray analysis. First-strand cDNA was prepared from isolated RNA with random primers and SuperScript II reverse transcriptase (Invitrogen) according to the instructions of the manufacturer. In most cases, 50–100 ng of cDNA was used as template for real-time PCR performed with an ABI 7500 Fast thermocycler and Power SYBR Green PCR Master Mix (Applied Biosystems). Transcript abundance was normalized to the abundance of an endogenous control gene, GAPDH. Relative target expression was calculated according to the $\Delta(\Delta\text{Ct})$ method as described [28], where the fold-change in expression is equal to $2^{-\Delta(\Delta\text{Ct})}$. Data are presented as the fold-change in transcript abundance in the presence of the indicated experimental condition relative to a media-only, time-matched calibrator condition and represent the mean and standard deviation of triplicate assays performed with RNA prepared from each of at least three independent experiments. Primers were purchased from a commercial vendor

(Integrated DNA Technologies). Primer sequences are listed in Supplementary Table 1 and were designed using available genomic sequences and Primer Express software (Applied Biosystems).

2.10 Tissue culture

The bladder epithelial cell line, 5637 (derived from bladder carcinoma; ATCC HTB-9) was obtained from the American Type Culture Collection. Cells were routinely cultured in RPMI 1640 medium (Gibco) supplemented with 10% fetal bovine serum (Sigma) at 37°C in a humidified atmosphere with 5% CO₂. To prepare inverted epithelial layers, trypsinized cells were resuspended to a concentration of 3×10⁶ cells/ml, and 50 µl of the suspension was seeded on inverted Transwell inserts (0.33 cm² polycarbonate membranes with 3 µm diameter pores; catalog #3472, Corning). The cells were allowed to attach to the membranes in a humidified 5% CO₂ atmosphere at 37°C for 16 h and were then transferred to 24-well plates with 0.6 ml fresh medium in each well, and 0.1 ml medium was added to the upper reservoir. After approximately 7 days of incubation with medium refreshed every 2 days, confluent cell layers were obtained. Monolayer integrity was determined by assessing permeability to fluid as described [29].

2.11 PMN transepithelial migration assay

At the beginning of each experiment, inverted 5637 monolayers were washed three times with RPMI/H. Overnight cultures of bacteria grown in LB broth were washed once in PBS and suspended in RPMI/H at a concentration of 10⁸ CFU/ml. Transwell inserts were lifted from each well and placed in a sterile chamber with the cell layer facing up. An aliquot of the bacterial suspension (60 µl; a multiplicity of approximately 40 CFU/cell) or an equivalent volume of sterile medium was placed on top of the epithelial layers and incubated for 60 min at 37°C. The cell layers were inverted and transferred into new 24-well culture plates (Ultra Low attachment plates, Corning #3473) containing 0.6 ml of RPMI/H. An aliquot of the PMN suspension (10⁶ cells) was then added to the upper reservoir and following a 60-min incubation at 37°C, the contents of the lower reservoir were mixed by gentle pipetting and transferred to a centrifuge tube. To quantify transepithelial migration of PMN, an aliquot of this suspension was examined by microscopy and PMN enumerated using a hemacytometer. The number of PMN recruited is shown as a percentage of the input, and the data presented represent the mean and standard deviation derived from at least three independent experiments, with samples analyzed in triplicate. Any differences in recruitment elicited by the various *E. coli* strains were examined for significance using an unpaired Student's *t* test.

2.12 Myeloperoxidase activity assay

Groups of C3H/HeN mice were inoculated intraurethrally with ~2×10⁷ CFU of wild-type uropathogenic *E. coli* (UTI89, CFT073) or non-pathogenic commensal *E. coli* (MG1655, FI-10, FI-11, FI-12) in 50 µl of sterile PBS according to a previously established model of bacterial cystitis [9]. All animal procedures were approved by the animal studies committee at Washington University. A subset of animals was mock infected with sterile PBS. At one hour post infection animals were sacrificed and bladders were harvested and homogenized in 1 ml of sterile PBS. An aliquot of bladder homogenate was plated on LB agar to determine tissue bacterial burden, and 50-µl aliquots of undiluted homogenates were transferred to a 96-well plate and assayed for myeloperoxidase (MPO) activity (Cell Technology) by fluorescent detection of an MPO substrate. Samples were incubated with the reaction buffer for one hour according to the instructions of the manufacturer and relative fluorescence was measured in a microplate reader (Synergy 2; BioTek). A standard curve using purified MPO was generated for each experiment, and the amount of MPO activity in bladder samples is reported in units/ml. At least three mice were infected for each strain

tested, and samples were analyzed in triplicate. Any differences from UTI89 were examined for significance using the Mann-Whitney U test.

3. Results

3.1 UPEC are more resistant to PMN killing than non-pathogenic *E. coli*

To determine whether differences in virulence of *E. coli* were due, in part, to varied capacity of the strains to withstand innate immune effectors, we evaluated killing of uropathogenic or non-pathogenic commensal *E. coli* by human neutrophils. Strains of *E. coli* were introduced to PMN isolated from venous blood at a multiplicity of infection of 10 CFU per PMN. At various times post infection, PMN were lysed and viable bacteria were enumerated by plating and colony counting. Approximately 80% of the uropathogenic strain was recovered after 30 min of infection (Fig. 1: UTI89 and CFT073), while there was a significant reduction in the survival of non-pathogenic strains ($p < 0.05$; Fig. 1: MG1655, FI-10, FI-11, FI-12). The presence of type 1 pili did not affect the survival of UPEC during encounters with PMN, as a UTI89 FimH⁻ strain behaved similarly to the wild-type parent strain (data not shown). All strains underwent modest growth during the course of the infection, suggesting that surviving bacteria could recover and grow in the continued presence of PMN; however, the relative survival defect of non-pathogenic *E. coli* strains (MG1655, FI-10, FI-11, FI-12) persisted throughout the experiment (Fig. 1: 60 and 120 min). These observations provide strong support to the notion that evasion of PMN-mediated killing contributes to the pathogenesis of *E. coli* infections.

3.2 UPEC stimulate a reduced PMN oxidative burst and are more resistant to ROS compared with non-pathogenic *E. coli*

Given the ability of UPEC to resist PMN killing, we hypothesized that pathogenic *E. coli* strains might elicit a less robust antimicrobial response or be more resistant to ROS than commensal strains. The generation of ROS by human PMN following encounter with *E. coli* was monitored using DCF. PMN isolated from venous blood of healthy donors were loaded with this probe, which fluoresces upon exposure to intracellular ROS, and exposed to *E. coli* at a multiplicity of infection of 10 CFU per PMN. ROS production was measured in arbitrary fluorescent units at 3-min intervals over 2 h and is shown as a percentage of the maximum ROS produced by PMN exposed to PMA, a potent stimulant. PMN exposed to non-pathogenic *E. coli*, including MG1655 and several fecal commensal isolates from healthy donors, ultimately produced ~40% of the maximum ROS produced in response to the PMA control (Fig. 2A: MG1655, FI-10, FI-11, FI-12). ROS production induced by nine other uncharacterized fecal commensal isolates of *E. coli* (denoted F-1 through F-9) was found to be similar to that of the representative fecal commensal strains (data not shown). In contrast, PMN exposed to uropathogenic strains produced a significantly lower quantity of ROS, ~15% of the PMA control and equivalent to mock-infected PMN (Fig. 2A: CFT073, UTI89, PBS). As with the PMN bactericidal assay, type 1 pili were not required for this phenotype (data not shown).

Given the reduced production of ROS by UPEC exposed PMN and increased resistance of UPEC to PMN killing, it was of interest to evaluate the relative sensitivity of pathogenic and non-pathogenic *E. coli* to ROS and cytotoxicity of these strains to PMN. The non-pathogenic strains were significantly more sensitive to the ROS-generating compound, hydrogen peroxide ($p < 0.005$) than the UPEC strains as determined by a disk diffusion assay (Fig. 2B: compare UTI89 and CFT073 to MG1655, FI-10, FI-11, and FI-12). Although UPEC have been shown to induce apoptosis in human PMN at later time points [30] there was no difference in the viability of PMN exposed to any of the *E. coli* strains under the conditions used in this study (data not shown). Taken together, these data suggest that the

ability of UPEC to survive an encounter with human PMN may be a consequence of both a reduced PMN antimicrobial response and an increased resistance to antimicrobial molecules.

3.3 Gene expression in PMN exposed to non-pathogenic and uropathogenic *E. coli*

To investigate the molecular basis of enhanced UPEC survival in the presence of neutrophils and reduced stimulation of ROS production, we first used microarrays to profile global changes in human PMN gene expression following infection with UPEC or commensal *E. coli*. Multiple probe sets representing a single gene transcript were combined into one data point to reduce duplicate candidate genes. A total of 438 genes were differentially regulated in response to the non-pathogenic strain MG1655 after 60 min (Supplementary Table 2). In contrast, interaction with the UPEC strain UTI89 resulted in differential regulation of only 206 genes (Supplementary Table 3). A subset of genes from across the entire array were selected for independent confirmation of the microarray results; quantitative RT-PCR analysis gave results consistent with the microarray data, though the magnitude of differential expression varied somewhat between these two experimental methods (Supplementary Table 4). In order to characterize the uropathogen-specific PMN transcriptional program, we determined relative fold changes in PMN gene expression in response to UTI89 as compared to MG1655. This analysis revealed a significant relative decrease in expression of host genes related to immune response and cell movement when PMN were exposed to UPEC (Table 1). Specifically, UPEC infection elicited lower expression of interleukin-1, tumor necrosis factor, and an array of chemokines, as well as decreased expression of intermediates in host pro-inflammatory signaling pathways (e.g., IRAK-2, MAPK6, MAPKAPK2). Leukocyte chemoattractants, such as CCL3 (MIP-1), CXCL2 (MIP-2 α), CXCL3 (MIP-2 β) and CCL20, were also relatively downregulated by UPEC. Similarly, key host proteins related to leukocyte adhesion (e.g., ICAM-1, CD44) were expressed at lower levels following UPEC infection, as compared with MG1655 infection.

3.4 UPEC modulate expression of host genes involved in transepithelial PMN migration

The influence of UPEC on expression of molecules involved in leukocyte adhesion and chemotaxis was further elucidated using quantitative RT-PCR. We queried the relative expression, in human PMN exposed to UTI89 or MG1655, of a panel of genes whose products are known to participate in neutrophil transepithelial migration (including those found to be differentially regulated by microarray analysis). During an in vitro encounter with the commensal strain, neutrophils were found to upregulate a number of genes involved in this process (Fig. 3A: MG1655), including ICAM-1, CD43, and WASP. On the other hand, exposure to the pathogenic strain failed to induce similar expression of these genes (Fig. 3A: UTI89). The comparative transcription profile (UTI89 exposure vs. MG1655 exposure) showed a relative downregulation by UPEC of the expression of genes involved in PMN adhesion and migration (Fig. 3B). These data are consistent with a model where early recruitment of PMN to the site of UPEC infection is suppressed or delayed, providing an opportunity for the pathogen to establish a niche within host cells and thereby evade subsequent immune responses.

3.5 UPEC suppress transepithelial PMN migration

To test the hypothesis that UPEC stimulate less PMN recruitment to infected tissue, we developed a model of transepithelial neutrophil migration utilizing the Transwell system. Epithelial cells were grown to confluence on the underside of a Transwell membrane to establish an inverted monolayer. The epithelial cells were first infected with bacteria, and then human PMN were added to the upper compartment. Migration of PMN to the lower compartment was determined after 60 min and is reported as a percentage of the input. Infection with the uropathogenic strains resulted in only modest neutrophil migration,

equivalent to mock infection (Fig. 4: UTI89 and CFT073); the commensal strains elicited a more potent response, as approximately 40% of input neutrophils were detected in the lower chamber (Fig. 4: MG1655, FI-10, FI-11, FI-12). Suppression of transepithelial PMN migration did not depend on interactions involving type 1 pili, as a UTI89 FimH⁻ mutant behaved similarly to wild-type UTI89 (data not shown).

Possible mechanisms for suppression of the innate immune response, as modeled by the transepithelial neutrophil migration assay, include either failure of the uropathogenic strains to induce pro-inflammatory responses or active UPEC attenuation of pro-inflammatory signals. When bladder epithelial cells were infected with both MG1655 and UTI89, transepithelial neutrophil recruitment was minimal, suggesting UPEC suppresses host cell ability to respond to inflammatory stimuli (Fig. 4: UTI89+MG1655). Inhibition of bacterial protein synthesis with chloramphenicol prevented the suppression of PMN recruitment (Fig. 4: UTI89+Cm). Taken together, these data suggest the presence of UPEC virulence proteins that mediate suppression of transepithelial neutrophil recruitment through direct activity on PMN and/or via suppression of epithelial pro-inflammatory signaling.

3.6 UPEC suppress acute PMN infiltration in vivo

To establish an in vivo correlate to the in vitro PMN transepithelial migration assay, we adapted an established murine model of bacterial cystitis to evaluate early recruitment of host inflammatory cells to the site of infection. Myeloperoxidase is an enzyme present in the granules of PMN that oxidizes chloride ions to produce powerful bactericidal molecules such as HOCl [31]. As MPO is unique to PMN, we used the amount of enzyme activity present in bladders of infected mice or uninfected controls as a representation of the number of PMN present. Because we were interested in the acute response to the bacteria, we infected with either uropathogenic or commensal *E. coli* and harvested bladders 1 h post infection. Although there were similar numbers of bacteria present in all infected mice ($\sim 5 \times 10^3$ CFU/bladder; data not shown), the bladders infected with non-pathogenic commensal strains (Fig. 5: MG1655, FI-10, FI-11, FI-12) showed significantly more MPO activity than those infected with UPEC strains (Fig. 5: UTI89, CFT073). These data are consistent with the lower levels of MPO measured 8 hours following bladder inoculation with the UPEC strain NU14 [15]. The differences in MPO activity in these samples suggest that, consistent with the results of the in vitro transepithelial PMN migration assay, uropathogenic strains of *E. coli* elicit a less robust PMN response than commensal strains early in urinary tract infection.

4. Discussion

This study demonstrated that while *E. coli* can stimulate neutrophil defenses and pro-inflammatory pathways, pathogenic strains suppress both the migration and bactericidal activities of PMN, eliciting significantly less robust PMN responses when compared with non-pathogenic and fecal commensal strains. Our approach to further elucidating this phenotype in the context of a dynamic interaction with the innate immune system was to compare gene expression in PMN exposed to a model uropathogen, UTI89, to those generated from PMN exposed to a well-characterized non-pathogenic strain, MG1655. We found a significant increase in expression of a number of genes involved in PMN trafficking and migration when the commensal strain was used, suggesting that the pathogen modulates the intensity of the innate immune response by directly inhibiting PMN function as well as by suppressing the activation of immune and/or epithelial cells.

Previous reports demonstrate activation of epithelial cell layers and PMN in response to UPEC in a process that is dependent on IL-8 [32] and utilizes ICAM-1 and integrins [19]. Our results are consistent with these in that we did observe increases in expression of

ICAM-1 and IL-1 α in cells exposed to UPEC, though expression of these genes was significantly greater upon exposure to commensal *E. coli*. Contrary to these earlier studies, however, we did not observe a high level of neutrophil migration across UPEC-infected uroepithelial cell layers. One explanation for this difference is the model and experimental design. Earlier experiments pre-stimulated epithelial cell layers for up to 24 h prior to the addition of neutrophils in the migration assay [19]. Because we were modeling very early host-pathogen interactions, our infection was limited to one hour, and neutrophil migration after one hour in response to UPEC was in fact similar between our model and earlier studies. Further differences could be explained by the use of distinct uroepithelial cell lines. The 5637 bladder cell line was chosen for this study because it has been used extensively to model urinary tract epithelial responses to *E. coli* [7,33–35]. These cells formed confluent epithelial layers which restricted fluid movement across polycarbonate filters, and expressed a tight-junction associated protein (ZO-1) at cell-cell borders (data not shown).

A wide body of evidence supports the critical contribution of type 1 pili to UPEC pathogenesis [7,9,25]. In addition to promoting binding and invasion of bladder epithelial cells, these filaments have been shown to participate in the interaction of bacteria with other host cells, including PMN [36–38]. However, fimbrial expression was not necessary or sufficient for promoting an inflammatory response in the human urinary tract [39]. Furthermore, a number of non-pathogenic strains, including the prototype commensal strain, MG1655, bear type 1 pili, demonstrating that virulence is a complex phenotype to which many factors likely contribute. Consistent with this, type 1 pili were not required for UPEC suppression of cytokine production in bladder epithelial cells in vitro [14–15]. Under the growth conditions and at the time points we selected, an isogenic FimH⁻ derivative of our prototype UPEC strain, UTI89, did not show a significantly different phenotype from the parent strain in the assays described here. In addition, the conditions we employed did not reveal UPEC- or type 1 pili-induced PMN apoptosis, as has been reported elsewhere [30]. Though type 1 pili may ultimately be important for eliciting an inflammatory response, our data suggest a role for other bacterial factors in modulating very early interactions with the host immune system.

The bacterial factors accounting for the suppressive effects of UPEC on human neutrophils remain incompletely defined. Data presented here and elsewhere indicate that UPEC interference with eukaryotic pro-inflammatory pathways may in part relate to lipopolysaccharide structure [14,30,40]. However, alteration of neutrophil function might also arise through active secretion or presentation of a bacterial effector; exposure to chloramphenicol abrogated UPEC suppression of PMN migration, implicating a bacterial protein as the mediator of this effect. Some strains of UPEC produce cytotoxic necrotizing factor 1 (CNF1), which modulates phagocyte function in vitro through targeting Rho-family GTPases [41]. In vitro characterization suggested that CNF1 can augment the oxidative burst of human neutrophils; but these studies evaluated wild-type UPEC and isogenic *cnf1* mutants and did not include non-pathogenic comparators [42–43]. More recently, Cirl and colleagues described a new class of bacterial virulence factors, the Toll/interleukin-1 receptor-containing proteins (Tcps), which have been shown to interfere with Toll-like receptor signaling and thereby suppress innate immune responses [44]. Although Tcps have been identified in selected UPEC strains, the clinical isolate primarily used in our studies, UTI89, does not encode such proteins [45]. It is unlikely that any single virulence factor or bacterial component is responsible for the array of observations made in the present study; we are currently profiling the coordinate changes in UPEC gene expression during human PMN encounters, an effort that is likely to facilitate the identification of additional bacterial effectors involved in this dynamic interaction.

In addition to UPEC, a number of other pathogens are known to modulate neutrophil function. Some organisms, including *Helicobacter pylori* and *Salmonella enterica*, stimulate a robust oxidative burst but redirect this activity away from the phagosome where bacteria reside [46–47]. *Neisseria gonorrhoeae* interferes with activation of the NADPH oxidase complex, thereby preventing ROS generation [48–49]. While the molecular mechanisms of immunomodulation by different Gram-negative pathogens are likely to involve unique effector molecules, they may target similar signaling pathways, cytoskeletal elements, and adhesion proteins in host cells.

The early interactions between bacterial pathogens and innate effector cells such as neutrophils are likely to have a critical impact on the outcomes of host-pathogen encounters at epithelial surfaces. While it is clear that UPEC ultimately elicits inflammatory responses in the urinary tract, an initial delay or reduction in PMN arrival may allow the establishment of a protected niche, such as an intracellular bacterial community. The present work demonstrates the ability of UPEC to dampen multiple distinct antimicrobial activities of human neutrophils and elucidates the host pathways modulated by this model pathogen. Our ongoing studies will further investigate whether the capacity to downregulate PMN functions is correlated with the clinical syndromes caused by various *E. coli* strains, as well as the host and pathogen determinants that contribute to this critical molecular conversation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. UPEC are more resistant to PMN killing than non-pathogenic *E. coli*

Bacterial survival in the presence of human neutrophils was determined after incubation for the indicated time. For each time point neutrophils were lysed, and samples were plated on solid media and incubated overnight. Survival data are presented as a percentage of CFU present at time 0 and represent the mean and standard deviation of three independent experiments performed with neutrophils from different donors. An asterisk denotes a statistically significant ($p < 0.05$) reduction in survival compared to UTI89 at the same time point.

Fig. 2. UPEC elicit less PMN ROS and are more resistant to ROS than non-pathogenic *E. coli*
A. The production of reactive oxygen species (ROS) produced by human neutrophils in the presence of the indicated *E. coli* strains was measured over time. Data are presented as a percentage of the maximum amount of ROS produced in the presence of the positive control (5 μ M PMA) at 120 min and represent the mean and standard deviation from at least three independent experiments performed with neutrophils from different donors. **B.** Sensitivities of the indicated *E. coli* strains to hydrogen peroxide were determined by a disk diffusion assay. Data represent the mean and standard error of triplicate assays. An asterisk denotes a statistically significant ($p < 0.005$) increase in sensitivity compared to UTI89 and CFT073.

Fig. 3. Pathogenic *E. coli* modulate transcription in PMN adhesion and migration pathways
A. The relative transcript abundance of the indicated genes in PMN exposed to UTI89 (black bars) or MG1655 (white bars) relative to the media-only control condition as determined by RT-PCR is shown. **B.** A schematic diagram of a neutrophil-epithelial cell interaction is shown. Molecules known to be important for neutrophil migration to infected tissue are highlighted, and the gene expression changes in PMN exposed to uropathogenic *E. coli* (UTI89) relative to commensal *E. coli* (MG1655) are indicated.

Fig. 4. UPEC suppress PMN transepithelial migration in an acute inflammation model

The number of PMN recruited to the apical surface of a polarized 5637 bladder epithelial cell monolayer following infection with the indicated *E. coli* strains or an uninfected PBS control (mock) is shown. Monolayers were infected at their apical surfaces and PMN were added at the basolateral surface. The number of PMN recruited to the apical surface was enumerated at 60 min post infection and is shown as a percentage of the input PMN. An asterisk indicates that recruitment by the indicated strain was significantly different from that elicited by wild-type UTI89 ($p < 0.001$) under the same conditions. Data represent the mean and standard deviation derived from at least three independent experiments with neutrophils from different donors.

Fig. 5. UPEC suppress PMN transuroepithelial migration in vivo

The amount of myeloperoxidase (MPO) activity present in the bladder of C3H/HeN mice following intraurethral inoculation with the indicated *E. coli* strains or a PBS control (mock) is shown. Mice were infected with $\sim 2 \times 10^7$ cfu and bladders were harvested at 1 h post infection. Data points represent the mean of triplicate measurements from individual bladders, and statistically significant differences from wild-type UTI89 are indicated with an asterisk ($p < 0.01$).

TABLE 1

Genes differentially expressed in human neutrophils exposed to uropathogenic *E. coli* relative to a non-pathogenic strain^a

Gene function and symbol ^b	Entrez ID ^c	Description	Fold change ^d
Immune response, development and function			
* ABCA1	19	ATP-binding cassette, sub-family A (ABC1), member	-2.48
* B4GALT1	2683	UDP-Gal:betaGlcNAc beta 1,4-galactosyltransferase, polypeptide 1	-2.52
** CCL20	6364	chemokine (C-C motif) ligand 20	-5.08
** CCL3	6348	chemokine (C-C motif) ligand 3	-2.24
** CD44	960	CD44 molecule (Indian blood group)	-4.27
* CD48	962	CD48 molecule	-8.32
** CD58	965	CD58 molecule	-4.17
** CD83	9308	CD83 molecule	-2.02
* CDKN1B	1027	cyclin-dependent kinase inhibitor 1B (p27, Kip1)	3.22
* CFLAR	8837	CASP8 and FADD-like apoptosis regulator	-3.82
* CHRNA2	1141	cholinergic receptor, nicotinic, beta 2 (neuronal)	-2.29
* CR1	1378	complement component (3b/4b) receptor 1 (Knops blood group)	-2.34
** CREM	1390	cAMP responsive element modulator	-2.02
** CXCL2	2920	chemokine (C-X-C motif) ligand 2	-2.05
** CXCL3	2921	chemokine (C-X-C motif) ligand 3	-3.50
* DAPP1	27071	dual adaptor of phosphotyrosine and 3-phosphoinositides	2.16
* DSC2	1824	desmocollin 2	4.13
** EDN1	1906	endothelin 1	-3.30
* EP300	2033	E1A binding protein p300	-2.13
* ETS2	2114	v-ets erythroblastosis virus E26 oncogene homolog 2 (avian)	-3.04
** F3	2152	coagulation factor III (thromboplastin, tissue factor)	-10.31
* FCGR1A	2209	Fc fragment of IgG, high affinity Ia, receptor (CD64)	3.27
** FOSL1	8061	FOS-like antigen 1	-4.94
* GBP2	2634	guanylate binding protein 2, interferon-inducible	-2.15
* GGA1	26088	golgi associated, gamma adaptin ear containing, ARF binding protein 1	-2.41
* GNAI3	2773	guanine nucleotide binding protein (G protein), alpha inhibiting activity polypeptide 3	-2.38
* GNB1	2782	guanine nucleotide binding protein (G protein), beta polypeptide 1	-3.11
* GPR132	29933	G protein-coupled receptor 132	-2.70
* HEBP1	50865	heme binding protein 1	2.59
* HHX	3087	hematopoietically expressed homeobox	2.39
* HIP1	3092	huntingtin interacting protein 1	-2.43
* HMGA1	3159	high mobility group AT-hook 1	-2.08
* ICAM1	3383	intercellular adhesion molecule 1 (CD54), human rhinovirus receptor	-2.89
* ID2	3398	inhibitor of DNA binding 2, dominant negative helix-loop-helix protein	-3.04
** IL1A	3552	interleukin 1, alpha	-5.25
** IL1RN	3557	interleukin 1 receptor antagonist	-2.65

Gene function and symbol ^b	Entrez ID ^c	Description	Fold change ^d
* INDO	3620	indoleamine-pyrrole 2,3 dioxygenase	2.46
* INSIG1	3638	insulin induced gene 1	-3.18
* IRAK2	3656	interleukin-1 receptor-associated kinase 2	-3.01
** IVNS1ABP	10625	influenza virus NS1A binding protein	-2.65
** JUN	3725	jun oncogene	3.90
** KLF10	7071	Kruppel-like factor 10	-6.34
** KLF2	10365	Kruppel-like factor 2 (lung)	3.50
** LCP1	3936	lymphocyte cytosolic protein 1 (L-plastin)	-2.01
* LCP2	3937	lymphocyte cytosolic protein 2 (SH2 domain containing leukocyte protein of 76kDa)	-2.09
** LIF	3976	leukemia inhibitory factor (cholinergic differentiation factor)	-5.57
* LYPD3	27076	LY6/PLAUR domain containing 3	-2.12
** MAFG	4097	v-maf musculoaponeurotic fibrosarcoma oncogene homolog G (avian)	-3.27
* MAPK6	5597	mitogen-activated protein kinase 6	-3.08
* MAPKAPK2	9261	mitogen-activated protein kinase-activated protein kinase 2	-2.16
* MAPRE1	22919	microtubule-associated protein, RP/EB family, member 1	-2.33
** NEDD9	4739	neural precursor cell expressed, developmentally down-regulated 9	-3.43
** NFE2	4778	nuclear factor (erythroid-derived 2), 45kDa	2.93
* NFKB1	4790	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p105)	-6.15
* NFKBIB	4793	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, beta	-2.23
* NKX3-1	4824	NK3 homeobox 1	6.90
* NOD2	64127	nucleotide-binding oligomerization domain containing 2	2.44
* NP	4860	nucleoside phosphorylase	-4.06
* NR4A1	3164	nuclear receptor subfamily 4, group A, member 1	-2.79
** NR4A3	8013	nuclear receptor subfamily 4, group A, member 3	-5.37
** OLR1	4973	oxidized low density lipoprotein (lectin-like) receptor 1	-3.83
* PARVB	29780	parvin, beta	-2.53
* PHLDA1	22822	pleckstrin homology-like domain, family A, member 1	-5.07
* PHLDA2	7262	pleckstrin homology-like domain, family A, member 2	-5.87
** PIM1	5292	pim-1 oncogene	2.24
* PLAGL2	5326	pleiomorphic adenoma gene-like 2	-2.55
** PLAU	5328	plasminogen activator, urokinase	-12.07
** PLK3	1263	polo-like kinase 3 (Drosophila)	-2.24
** PTX3	5806	pentraxin-related gene, rapidly induced by IL-1 beta	-2.97
** RABGEF1	27342	RAB guanine nucleotide exchange factor (GEF) 1	-2.31
** RALGDS	5900	ral guanine nucleotide dissociation stimulator	-6.42
* SCLT1	132320	sodium channel and clathrin linker 1	-2.11
* SERPINB2	5055	serpin peptidase inhibitor, clade B (ovalbumin), member 2	-4.08
* SIRPA	140885	signal-regulatory protein alpha	-3.34
** SKAP2	8935	src kinase associated phosphoprotein 2	2.11
* SPHK1	8877	sphingosine kinase 1	-3.87
** TA-NFKBH	84807	T-cell activation NFKB-like protein	-3.83

Gene function and symbol ^b	Entrez ID ^c	Description	Fold change ^d
* TFG	10342	TRK-fused gene	-2.28
* TGIF1	7050	TGFB-induced factor homeobox 1	-3.05
** TNF	7124	tumor necrosis factor (TNF superfamily, member 2)	-5.50
* TNFAIP6	7130	tumor necrosis factor, alpha-induced protein 6	-2.45
* TNFRSF9	3604	tumor necrosis factor receptor superfamily, member 9	-2.51
* TNFSF14	8740	tumor necrosis factor (ligand) superfamily, member 14	-2.83
** VCAN	1462	versican	-4.27
* VEGFA	7422	vascular endothelial growth factor A	-5.24
* VEZF1	7716	vascular endothelial zinc finger 1	2.17
* VNN1	8876	vanin 1	2.33
* XBP1	7494	X-box binding protein 1	-4.27
Cell signalling and movement			
B4GALT5	9334	UDP-Gal:betaGlcNAc beta 1,4-galactosyltransferase, polypeptide 5	-4.51
CCRL2	9034	chemokine (C-C motif) receptor-like 2	-2.72
GSK3A	2931	glycogen synthase kinase 3 alpha	2.39
LAMB3	3914	laminin, beta 3	-3.45
NUAK2	81788	NUAK family, SNF1-like kinase, 2	2.95
TPM4	7171	tropomyosin 4	-3.06
ZEB2	9839	zinc finger E-box binding homeobox 2	-4.06

^aResults from two separate experiments were compared (fold change, 2.0 $P < 0.05$) as described in Materials and Methods.

^bGenes are classified by function (Ingenuity Pathway format). A single asterisk denotes the gene is also in the 'Cell signalling and movement' category. A double asterisk denotes the gene is also in the 'Cell signalling and movement' and the 'Cell growth and proliferation' category. Gene symbol represents the symbol of the gene represented by the probe in the array.

^cEntrez ID is the National Center for Biotechnology Information Entrez accession number of the gene represented by the probe in the array.

^dFold change is calculated by dividing the fold change for neutrophils exposed to the uropathogenic strain (UTI89) relative to neutrophils exposed to media only by the fold change for neutrophils exposed to the commensal strain (MG1655) relative to neutrophils exposed to media only.