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Morphological plasticity promotes resistance to phagocyte killing of uropathogenic *Escherichia coli*

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Abstract

Uropathogenic *Escherichia coli* proceed through a complex intracellular developmental pathway that includes multiple morphological changes. During intracellular growth within Toll-like receptor 4-activated superficial bladder epithelial cells, a subpopulation of uropathogenic *E. coli* initiates Sula-mediated filamentation. In this study, we directly investigated the role of bacterial morphology in the survival of uropathogenic *E. coli* from killing by phagocytes. We initially determined that both polymorphonuclear neutrophils and macrophages are recruited to murine bladder epithelium at times coincident with extracellular bacillary and filamentous uropathogenic *E. coli*. We further determined that bacillary uropathogenic *E. coli* were preferentially destroyed when mixed uropathogenic *E. coli* populations were challenged with cultured murine macrophages in vitro. Consistent with studies using elliptical-shaped polymers, the initial point of contact between the phagocyte and filamentous uropathogenic *E. coli* influenced the efficacy of internalization. These findings demonstrate that filamentous morphology provides a selective advantage for uropathogenic *E. coli* evasion of killing by phagocytes and defines a mechanism for the essential role for Sula during bacterial cystitis. Thus, morphological plasticity can be viewed as a distinct class of mechanism used by bacterial pathogens to subvert host immunity.

Keywords

URINARY TRACT INFECTIONS; UROPATHOGENIC ESCHERICHIA COLI; PHAGOCYTOSIS; INNATE IMMUNITY

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1. Introduction

Pathogens use multiple mechanisms to actively modulate host innate immune effectors to prevent recruitment, activation and/or internalization by professional phagocytes [reviewed in [1]]. *Flectobacillus*, *Comamonas acidovorans*, and *Betaproteobacteria* use morphological plasticity to mediate evasion of predation in marine environments [reviewed in [2]]. Although there are a large number of potential bacterial morphologies in nature, each bacterial species is limited to a small subset of morphological forms, suggesting that bacterial morphology plays a role in the adaptation to each environmental niche [3] including within host tissues.

The uropathogenic *Escherichia coli* (UPEC) lifestyle involves successive morphological and physiological changes during a complex intracellular developmental cycle that is critical for the establishment of lower urinary tract infections (UTI) [4-8]. The majority of UPEC within an intracellular bacterial community exhibit morphological plasticity with a transition from a bacillary to a coccoid morphology. A subpopulation of UPEC exhibits further morphological plasticity through inhibition of cell division, resulting in a filamentous shape [6,9]. An acute UTI proceeds through multiple rounds of intracellular growth and development, which culminates in the establishment of a latent quiescent reservoir within the bladder epithelium [9,10]. The intracellular developmental model observed in mice was subsequently validated in human cystitis: evidence for the UPEC developmental cycle was observed in the urine of women seeking treatment for cystitis caused by UPEC, *Klebsiella pneumoniae*, or *Enterobacter aerogenes* [11]. Moreover, filamentous forms of UPEC, *K. pneumoniae*, *E. aerogenes*, and *Proteus mirabilis* were observed in human urine more than twice as frequently as other stages of the developmental cycle [11], indicating that morphological plasticity is commonly manifested during human urinary tract infections.

The observation that multiple uropathogens exhibit distinct morphological stages during cystitis makes this an ideal model system to investigate the role of bacterial morphology during infection. Moreover, there is both genetic and microscopic evidence to indicate that morphological plasticity plays an important role in UPEC during infection. The cell division inhibitor of the SOS DNA damage repair system, Sula, is essential for UPEC persistence as well as filamentation during murine cystitis in immunocompetent hosts [6,9], particularly during the transitory extracellular stage of the lifecycle. Plasticity of UPEC morphology (from bacillary to filamentous) as well as subsequent restoration of cell division (from filamentous to bacillary daughter cells) is essential for ensuing rounds of intracellular bacterial community formation as well as for establishment of quiescent intracellular reservoirs [9]. Time-lapse fluorescence video microscopy studies of live, infected murine bladder explants suggested that infiltrating phagocytes readily internalized bacillary forms of UPEC, while filamentous morphotypes appeared to be resistant to phagocytosis [6]. Filamentation was not observed in murine strains defective in Toll-like receptor 4 (TLR4) signal transduction, indicating that filamentation occurs in response to activated host innate immune effectors. Moreover, Sula is dispensable in the absence of TLR4 signal transduction [9], which indicates that the requirement for Sula is closely aligned with survival of innate immune responses. However, the molecular mechanisms responsible for this requirement for Sula in the face of the innate immune response are poorly understood.

In this study, we directly examined the role of bacterial morphology on the ability of phagocytes to internalize and kill UPEC in vitro. Flow cytometric methods were adapted for the identification and quantitative comparison of morphologically distinct UPEC populations. We observed that primary murine macrophages and neutrophils as well as cultured murine macrophages preferentially consumed bacillary UPEC, resulting in enrichment of the filamentous morphotype when both morphotypes were present. These

findings demonstrate that UPEC filamentation is a strategy to subvert phagocytosis during infection.

2. Materials and methods

2.1. Bacterial strains and cultivation

UTI89, a prototypic strain of UPEC, was isolated from a patient with cystitis [12]. Green fluorescent protein (GFP) expression was from pCOMGFP [13]. The BL21 (λ DE3)*lon::Tn10* mutation (Promega, Madison, WI) was introduced into UTI89/pCOMGFP by P1 transduction [14], with selection on 25 μ g/ml tetracycline and 125 μ g/ml ampicillin (both from Fisher Bioreagents, Fair Lawn, NJ). The UTI89 Δ *sulA::kan* strain was previously described [9].

2.2. Cell culture

RAW 264.7 cells (American Type Culture Collection [ATCC] designation TIB-71, Manassas, VA), a murine macrophage cell line, were cultured in Dulbecco's modified Eagle's medium (DMEM; Cellgro, Manassas, VA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Invitrogen, Carlsbad, CA) at 37°C with 5% atmospheric CO₂. T24 cells (ATCC designation HTB-4, Manassas, VA), derived from a human transitional cell carcinoma, were maintained in Roswell Park Memorial Institute 1640 medium (Hyclone, Logan, UT) containing 10% heat-inactivated FBS at 37°C with 5% atmospheric CO₂.

2.3. Experimental UTI and flow cytometry of immune cell infiltrate

Seven- to nine-week-old female C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) were transurethrally inoculated with 10⁷ colony-forming units (CFU) of UPEC as previously described [6,15]. At indicated time points, bladders were aseptically harvested, bisected, and digested in buffer containing 1.19 Wunsch units/ml of collagenase (Sigma-Aldrich, St. Louis, MO), 66.7 mM NaCl (Fisher Bioreagents, Fair Lawn, NJ), 6.71 mM KCl (Fisher Bioreagents, Fair Lawn, NJ), 101 mM (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (Sigma-Aldrich, Saint Louis, MO), and 4.78 mM CaCl₂ (Fisher Bioreagents) at a pH 7.6 for 30 minutes at 37 °C. Bladder cell suspensions were washed with FACS buffer [2% Donor Calf Serum (Mediatech, Inc., Manassas, VA) in Dulbecco's phosphate buffered saline without calcium and magnesium (DBPS; Fisher Bioreagents, Fair Lawn, NJ)] and treated with anti-murine CD16/32 (eBiosciences, San Diego, CA) to minimize non-specific antibody staining. Specific markers were identified by incubation with antibodies against anti-Gr-1-fluorescein isothiocyanate (RB6-8C5), anti-CD11b-phycoerythrin (M1/70), anti-CD11c-allophycocyanin (N418), anti-F4-80-biotin and streptavidin-allophycocyanin (BM8) (all from eBioscience, San Diego, CA). Debris was removed by passage through a 70- μ m nylon strainer (Becton Dickinson, Franklin Lakes, NJ), and staining with propidium iodide (Calbiochem, Gibbstown, NJ) was used to exclude dead cells. Data were collected with a FACSCalibur cytometer (Becton Dickinson, San Jose, CA) and analyzed with Flowjo software (Tree Star, Ashland, OR). All animal experiments were performed using accredited conditions for animal welfare approved by the Institutional Animal Care and Use Committee (Welfare Assurance Number A3544-01) at The Research Institute at Nationwide Children's Hospital, AR06-00119.

2.4. Thioglycollate-induced peritonitis

Isolation of primary murine macrophages and neutrophils from the peritoneal cavity was performed essentially as described [16]. Briefly, 1 ml of 3% thioglycollate broth (Fluka Analytical) was injected i.p. into seven- to nine-week-old female C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME). 5-6 h after thioglycollate injection, mice were anesthetized

with 3% isoflurane solution (Baxter Healthcare Corporation, Deerfield, IL) and were sacrificed by cervical dislocation. Peritoneal exudate cells (PECs) (primarily macrophages and neutrophils) (S. Fig. 1) were collected by i.p. injection of 10 ml of ice-cold DPBS without magnesium and calcium (Mediatech, Inc., Manassas, VA), centrifuged at $400 \times g$ for 10 min at 4°C , re-suspended in 1 ml of ACK lysis buffer (Invitrogen, Carlsbad, CA) to remove red blood cell contamination, washed once with DMEM containing 10% heat-inactivated FBS (DMEM-S) and viable cell counts were determined by trypan blue staining. PECs were re-suspended in ice-cold DMEM-S and kept on ice until use in the UPEC survival assay (see section 2.9.1).

2.5. Induction of UPEC filamentation

2.5.1. Mitomycin C induction—UPEC cultures were grown statically overnight at 37°C at 5% atmospheric CO_2 in DMEM-S. Overnight cultures were diluted 1:500 into fresh DMEM-S and grown for 2-3 h. Alternatively, early stationary phase cultures of UPEC were grown in DMEM-S as described above and were diluted 25-fold in DMEM-S containing a final concentration of 24% glycerol and frozen at -80°C . Frozen aliquots were thawed in 37°C water bath, centrifuged at $16,100 \times g$ for 5 min, and were diluted 1:250 in pre-warmed DMEM-S and grown statically for 1 h at 37°C with 5% CO_2 . Aliquots of approximately 10^5 CFU of early log-phase UPEC were added to DMEM-S media in 12 well plates (Corning, Lowell, MA) containing either 400 ng/ml mitomycin C (MMC) (Fisher Bioreagents, Fair Lawn, NJ). Growth was continued for an additional 3 h at 37°C at 5% CO_2 to allow for sufficient time to generate filamentous UPEC greater than $10 \mu\text{m}$ in length.

2.5.2 Innate immune antimicrobial induction—UPEC were grown as described above (2.5.1). During the early logarithmic growth phase of UPEC, monolayers of cultured RAW 264.7 mouse macrophages were stimulated with 10 ng/ml LPS *E. coli* O111:B4 (Sigma-Aldrich, Saint Louis, MO). Approximately 10^5 UPEC were added to 1×10^6 LPS-activated RAW 264.7 cells (innate immune antimicrobial induction). Co-cultures were then incubated for an additional 3 h at 37°C at 5% CO_2 to allow for sufficient time to generate filamentous UPEC greater than $10 \mu\text{m}$ in length. In order to prevent intracellular killing and contamination of cellular debris during co-culture with RAW 264.7 cells, UPEC cultures were grown in the upper chamber of a Transwell insert containing a $3\text{-}\mu\text{m}$ pore size (Becton Dickinson Labware, San Jose, CA).

2.6. Flow cytometric determination of bacterial morphology

In order to determine bacterial morphology by flow cytometry, UPEC were cultured in the presence of MMC or murine macrophages [(described above (2.5.1, 2.5.2)]. Bacillary and filamentous bacterial populations were distinguished on the basis of forward scatter area (FSC-A), side scatter area (SSC-A), and GFP fluorescence area (GFP-A) parameters. Bacterial cell lengths were determined from fluorescence micrographs using ImageJ software (National Institutes of Health, Bethesda, MD; <http://rsbweb.nih.gov/ij>). The strategy used to identify each of the morphological populations is outlined in Supplemental Figs. 2 and 3.

2.7. Filter centrifugation

UTI89 $\Delta lon::Tn10$ /pCOMGFP were grown overnight statically in DMEM-S as described above (2.5.1). Bacteria were subcultured 1:1000 in 30 ml of fresh DMEM-S and grown statically at 37°C with 5% CO_2 for 1.5 h. Cultures of bacteria were divided into two equal portions; one portion was treated with 400 ng/ml MMC for 4 h, and the other remained untreated. Cultures were then harvested, fixed in phosphate buffered saline (PBS; Fisher Bioreagents, Fair Lawn, NJ) with 3% paraformaldehyde (Electron Microscopy Sciences,

Hatfield, PA) for 15 minutes at room temperature. Populations enriched for filamentous or bacillary UPEC were separated by centrifugation through a 5.0- μ m PVDF Ultrafree-CL Centrifugal filter device (Millipore, Billerica, MA) at $863 \times g$ for 5 min. The filamentous population was further enriched by the addition of 1 ml of PBS to the filter cup for additional rounds of centrifugation. This enrichment process was repeated three times to ensure that rods and filaments were adequately separated. Samples of each of the filtrates and filtrands were stored and analyzed by both microscopy and flow cytometry.

2.8. Epithelial surface-based UPEC survival of phagocytes

Cultures containing confluent monolayers of human bladder carcinoma cells, T24, (ATCC designation HTB-4) (1×10^5) were seeded in triplicate with approximately 10^6 UPEC (a mixture of both bacillary and filamentous morphotypes). Approximately 10^5 non-stimulated RAW 264.7 cells were added to each well at a multiplicity of infection of 1:10. Binding of bacteria and phagocytes was facilitated by centrifugation at $754 \times g$ for 5 min at room temperature to promote phagocyte-bacterial interaction upon the surface of the T24 cells. Finally, the cells were returned to the incubator at 37°C at 5% CO_2 for 1 h for the completion of phagocytosis. Eukaryotic cells were disrupted by lysis buffer [0.8% sarkosyl, 1% glucose, and 8 mg/ml 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (Fisher Bioreagents, Fair Lawn, NJ)], washed once in FACS buffer, transferred to 12×75 mm polystyrene round bottom tubes (Becton Dickinson, Franklin Lakes, NJ), and analyzed on an LSR II cytometer (Becton Dickinson, Franklin Lakes, NJ). Samples were excited with an argon 488 nm laser; fluorescence was detected with the 505 long pass and 530/30 bandpass filters. For accurate enumeration of bacteria, fluorescence signals were amplified in the logarithmic mode. Debris from cell lysis was removed from analysis using the FSC parameters to set the electronic threshold. Data were analyzed using Flowjo software (Tree Star, Ashland, OR).

2.9. UPEC survival of phagocyte killing

2.9.1. In solution

Human primary neutrophils: Isolation of neutrophils from peripheral blood from healthy adults, induction with mitomycin C, co-incubation of bacteria with isolated neutrophils, and microscopy were performed as previously described [9]. This study was conducted according to the principles expressed in the Declaration of Helsinki and with approval from the Institutional Review Board at The Research Institute at Nationwide Children's Hospital, # IRB06-00603, approved 11/7/2007. Samples were placed on glass slides and imaged with an Axiovert 200M inverted epifluorescence microscope equipped with an AxioCam MRM CCD camera (Carl Zeiss Inc., Thornwood, NY).

Mouse primary neutrophils and macrophages: Approximately 5×10^5 primary exudate cells (PECs) were added to 5-ml polystyrene round bottom tubes (Becton Dickinson, Franklin Lakes, NJ) containing MMC-induced UPEC at multiplicities of infection from 1:0.1 to 1:10. Binding of bacteria and phagocytes was facilitated by placing the tubes in a 37°C water bath at 180 RPM for 40 min. Eukaryotic cells were disrupted by lysis buffer [0.1% Triton X-100 PBS (Fisher Bioreagents, Fair Lawn, NJ) and analyzed on an LSR II cytometer (Becton Dickinson, Franklin Lakes, NJ) as described above (2.8).

2.9.2. Upon Epithelial Surface—T24 cells were seeded onto 18-mm cover glasses (Fisher Bioreagents, Fair Lawn, NJ) inside a 12-well plate and were allowed to reach confluency. The phagocytosis assay was performed as described above (2.8). T24 cells were incubated with $1 \mu\text{g/ml}$ of wheat germ agglutinin-Alexa Fluor 594 in PBS (Invitrogen, Carlsbad, CA) for 10 min to permit visualization of epithelial cell surfaces. Wells were

stained with 0.5 µg/ml Hoechst 34580 (Invitrogen, Carlsbad, CA) for 10 min to visualize cellular DNA. The cover glasses were removed from the wells, mounted on microscope slides, and imaged with an Axiovert 200M inverted epifluorescence microscope equipped with an AxioCam MRM CCD camera (Carl Zeiss Inc., Thornwood, NY).

3. Results

3.1. Robust influx of neutrophils and macrophages are coincident with UPEC egress to the bladder surface

Recognition of bacteria by the superficial bladder epithelial cells induces the production of nitric oxide and other reactive species [17] to directly combat bacteria as well as the secretion of pro-inflammatory cytokines [18,19] to assist in the recruitment of neutrophils, monocytes, macrophages, and dendritic cells to the bladder [19-23] for continued control of bacteria. Microscopic examination of live explanted bladders demonstrated that filamentous UPEC appear to be resistant to internalization by infiltrating phagocytes [6], but the types and numbers of phagocytes (e.g., neutrophils, macrophages) present during bacterial egress were not determined. We initially sought to identify the phagocytic cells that would participate in the clearance of UPEC following egress when UPEC are extracellular on the luminal surface of the bladder.

To quantitatively characterize the influx of phagocytes in response to UPEC, we used flow cytometry to determine the number and type of immune cells present in single-cell suspensions of the bladder (Fig. 1A). As expected, neutrophils (Gr-1^{hi} CD11b⁺) were absent in the bladders of naïve mice (Fig. 1A). In contrast, robust recruitment of neutrophils was evident in the bladder 16 h post infection ($p < 0.0001$) (Fig. 1B). As observed with other nonlymphoid organs such as the liver and kidneys [24], resident macrophages (CD11b⁺ F4-80^{med}) were observed in the naïve bladder (Fig. 1B). After introduction of UTI, a robust influx of macrophages was also observed at 16 h post infection ($p < 0.003$; Fig. 1B). We conclude that both neutrophils and macrophages are recruited during bacterial cystitis and could potentially be responsible for clearance of extracellular UPEC.

3.2. Filamentous and bacillary UPEC are distinguished by flow cytometry

Previous studies proposed that filamentous UPEC provides a survival advantage from phagocytosis based upon qualitative examination through video microscopy of live explanted bladders. To quantitatively assess the effect of bacterial morphologies on phagocytosis in vitro, we developed a flow cytometric technique that could distinguish and enumerate bacillary and filamentous morphotypes independent of microscopic or culture techniques. Flow cytometry has been successfully utilized to characterize heterogeneous populations of microorganisms [25,26], determine total bacterial protein content [27], monitor responses of bacteria to antibiotics [28,29], and discriminate filamentous *E. coli* exposed to ciprofloxacin [30].

Our requirement for a culture-independent methodology was twofold. First, a bacterial filament, regardless of length, is a single colony-forming unit; so cultures would not provide information regarding the biomass of each individual filament, which could be important in protection from phagocytosis. Second, we inactivated the Lon protease to prevent recovery from filamentation [9] during the assay essentially rendering the filamentous population non-culturable. To verify that flow cytometry was sufficiently sensitive to distinguish bacillary and filamentous UPEC morphotypes, we treated cultures of UTI89Δlon/pCOMGFP with mitomycin C (a DNA-damaging agent known to induce the SOS response) to produce Sula-mediated filamentous morphotypes [9]. As observed in vivo where all morphological changes arise within the same epithelial cell, both bacillary and filamentous

morphologies were present in the same culture tube (Fig. 2D, treated). Moreover, UPEC is exposed to lethal levels of DNA damage during intracellular residence within the superficial bladder epithelial cells [31], indicating that the SOS response is necessarily induced as part of the intracellular lifestyle during cystitis. Therefore, this culture system provides the unique opportunity to assess the role of morphology using bacteria from the same genetic stock under growth conditions that reflect conditions observed in vivo in the absence of physiological changes invoked by mixing morphotypes produced under distinct growth conditions (SOS-induced filaments vs. naïve bacillary UPEC). Forward- and side-scatter comparisons of UPEC grown in the presence of mitomycin C provided a method to identify populations of bacteria differing in length by as little as two-fold (Fig. 2).

To validate the appropriate assignment of the two populations, we purified bacillary and filamentous UPEC by filter centrifugation prior to flow cytometric analysis. Bacillary UPEC (<3 µm in length) readily passed through a 5-µm pore filter and were enriched from 75.5% of total events prior to filter centrifugation (Fig. 3A and 3B, region I) to 96.7% of total events post filter centrifugation (Fig. 3D, E). In contrast, filamentous UPEC were retained by the filter and were enriched from 22.1% (Fig. 3B, region II) to 74.2% of total population after filter centrifugation (Fig. 3G, H). Therefore, we can readily distinguish and enumerate these different UPEC morphologies by flow cytometry.

Furthermore, we utilized GFP fluorescence intensity as an indicator for viability as well as an additional correlate of cell length. The presence of GFP fluorescence indicates bacterial viability, as ethanol-killed UPEC exhibit similar forward and side scatter properties to untreated bacteria but exhibit no GFP fluorescence (data not shown) [32]. We predicted that filamentous UPEC would exhibit more intense GFP fluorescence per flow cytometric event due to the proportional increase in overall bacterial volume. We found that filamentous populations exhibited more robust fluorescence intensity for GFP (mean fluorescence intensity [MFI] 11,421) compared to bacillary populations (GFP MFI 1,397) (Fig. 3C). These data demonstrate that flow cytometry is sufficient for quantitative identification and enumeration of UPEC bacillary and filamentous morphologies as well as viability based upon light scattering and fluorescence properties. The strategies used for distinction of each morphotype by flow cytometry are delineated further in Supplemental Figs. 2 and 3.

3.3. Murine phagocytes preferentially kill bacillary UPEC

Previous studies indicated that extracellular, filamentous UPEC become the predominant bacterial morphology on the bladder surface of C3H/HeN mice following egress from the infected superficial epithelial cell [6]. We theorized that enrichment for filamentous UPEC results from a selection process whereby filamentation is induced by exposure to host effectors, then bacillary UPEC are preferentially consumed by host phagocytes [6]. To test this hypothesis, we developed an in vitro survival assay recapitulating the enrichment of filamentous bacteria on an epithelial surface by the addition of phagocytes as observed previously (Supplemental Mov. 1) [6]. In contrast to a glass or plastic surface, the presence of epithelial cells provides a host-relevant substratum for bacterial attachment that also promotes phagocyte motility [33]. Since both neutrophils and macrophages are present during UPEC egress (Fig. 1), we used cultured RAW 264.7 murine macrophages as model phagocytes for our in vitro assay [34]. To quantitatively ascertain whether bacterial morphology affects resistance to phagocytic killing, cultures of UT189 *lon*/pCOMGFP were treated with mitomycin C, resulting in a mixed population of morphotypes (5-22% of the filamentous form) (Fig. 4A). We then determined the ratio of bacillary and filamentous morphotypes before addition to epithelial cells (input) and after phagocytosis (output). Importantly, co-incubation of UPEC with either epithelial cells or RAW 264.7 cells alone or in combination did not affect UPEC morphology during the time frame of the phagocytosis assay (data not shown). Following application of RAW 264.7 cells and mitomycin C-

induced UPEC populations to bladder epithelial cells, the filamentous population increased to 70-85% of all viable bacteria in the assay ($p < 0.0001$ vs input; Fig. 4C). Bacillary and filamentous populations used in these experiments were produced from the same strain exposed to the same growth conditions in a single culture tube; therefore, we can exclude the possibility that differences in growth conditions led to the observed preferential killing of bacillary UPEC.

To exclude any potential influences uniquely attributable to a single cell line (RAW 264.7), phagocyte (macrophages) or epithelial surface (T24 cells), we modified the assay by utilizing primary peritoneal macrophages and neutrophils and performing the phagocytosis assay in the absence of epithelial cells (Fig. 4D-F). Indeed, mixtures of peritoneal macrophages and neutrophils also exhibited preferential killing of bacillary UPEC independently of an epithelial cell surface. These results clearly demonstrate that filamentous morphology confers a survival advantage for UPEC against internalization and destruction by both murine macrophages and neutrophils.

Finally, we evaluated preferential killing under conditions where UPEC filamentation was induced utilizing host phagocytes that are known to encounter UPEC in vivo. Previous studies have shown that interferon- λ primed RAW 264.7 cells can induce intracellular *Salmonella enterica* serovar Typhimurium to filament in a MEK kinase- and NADPH oxidase-dependent manner [35]. Additional antimicrobial effector molecules possessed by macrophages that are known to elicit filamentation include inducible nitric-oxide synthase [36] and cathelicidin-related antimicrobial peptide [37]. To determine if stimulated RAW 264.7 cells are sufficient to induce UPEC filamentation extracellularly, we co-cultured LPS-stimulated RAW 264.7 cells with UPEC and monitored filamentation induction by flow cytometry. We found that LPS-activated RAW 264.7 cells were able to induce UPEC filamentation (Fig. 4G), albeit at a lower percentage compared to MMC (Fig. 4A, D). More importantly, when these mixtures of bacillary and filamentous morphologies were subjected to the epithelial-based phagocytosis assay with naïve RAW 264.7 cells as phagocytes, the bacillary morphotype was again preferentially killed (Fig. 4G-I).

3.4. Visualization of epithelial phagocytosis assay

To confirm our previous finding that filamentous morphology contributes to resistance to phagocytosis and killing, we utilized time-lapse fluorescence microscopy to observe interactions of UPEC morphotypes with phagocytes upon a bladder epithelial surface. UTI89 $\Delta lon/pCOMGFP$ was treated with mitomycin C and subjected to the epithelial-based in vitro phagocytosis assay. When a mixture of bacillary and filamentous UPEC was incubated with cultured murine macrophages (Fig. 5A), filamentous UPEC were enriched on the epithelial surface after 20 min (Fig. 5B) whereas bacillary UPEC were consumed by macrophages (Fig. 5C). These observations further demonstrate that filamentous morphology provides UPEC with a survival advantage over the bacillary morphotype and are consistent with the results of our flow cytometric-based phagocytosis assay.

3.5. Mechanism for subversion of internalization

Previous studies have demonstrated that the target geometry of the initial interaction of target particle and phagocyte directly influence efficacy of internalization [38]. Due to the similarity in dimensions of the target polystyrene particles used in previous reports [38] and our filamentous UPEC, we hypothesized that target geometry would participate in the resistance of filamentous UPEC to internalization and killing. In order to test this hypothesis, we employed time-lapse phase microscopy to monitor the interactions of individual filamentous UPEC and primary murine and human phagocytes in solution [9]. In classifying over 150 initial interactions between filamentous UPEC and mouse neutrophils,

we found that ~75% of these were non-polar (Fig. 5D-F). A similar predilection was observed in interactions of UPEC filaments with human neutrophils (Fig. 5G-I). Additionally, we determined by time-lapse phase video microscopy that the site of initial interaction of human neutrophils with filaments (polar or non-polar) dictated the efficiency of the subsequent phagocytic event (Fig. 5 and Supplemental Mov. 2). When the initial encounter between human neutrophils and filamentous UPEC occurred at the filament pole, the phagocyte was largely successful in the internalization and killing of the pathogen (Fig. 5D-F, arrows). In contrast, neutrophils that initially engaged the longitudinal portion of the filamentous morphotype (Fig. 5D-F, arrowheads) were comparatively unproductive in internalization and killing. Thus, the overall shape of the bacteria and the initial point of engagement with the neutrophil dictated the outcome of the host-pathogen interaction, as described for polystyrene particles [38].

4. Discussion

The contribution of morphological plasticity to virulence is not as widely investigated in bacteria as it is in fungal pathogens [39,40]. Here, we demonstrate that filamentous morphology provides a survival advantage when bacillary and filamentous UPEC encounter phagocytes relevant to epithelial infection. Importantly, these results provide evidence that the bias of phagocytes to consume one morphotype over another dramatically alters the relative abundance of UPEC morphotypes present in a given population.

Morphological plasticity has been described for other bacterial pathogens, but its contribution to the virulence of these organisms is unclear. However, filamentous forms of *Haemophilus influenzae* [41], *Legionella pneumophila* [42], *Mycobacterium tuberculosis* [43], *Salmonella typhimurium* [44] and *Yersinia pestis* [45] have been observed in animal and/or in vitro models of disease, though specific biological roles of the filamentous forms have yet to be determined. In addition, a recent study reported that both filamentous and multiple cell chain morphotypes of *Listeria monocytogenes* were significantly less susceptible to phagocytic uptake and survival in human neutrophils compared to a coccobacillary morphotype [46], but the contribution of these morphotypes to the pathogenic lifestyle of *Listeria* is not described. Mutants of *Candida albicans* that fail to form hyphae display attenuated virulence, and agents that block hyphal formation are currently in development for treatment of candidal infection [47,48]. Morphologic plasticity has also been studied in predator-prey interactions occurring in freshwater and marine environments, in which protist predation disproportionally reduces bacterial species of the edible size range, leaving behind inedible forms [49]. In aquatic environments, filamentation is included as a strategy for subversion of predation along with other phenotypic traits including high-speed motility, toxin production, and cell miniaturization [49]. However, the mechanisms by which the marine organisms survive predation are not fully described.

We provide multiple lines of evidence that morphological plasticity can be considered as another mechanism for subversion of host innate immunity by UPEC during cystitis [6,9]. Filamentous forms have been demonstrated in both urine samples from patients seeking treatment for UTIs [11] and are prevalent in the murine model for human cystitis [4,6,8]. Moreover, there is now genetic [9], in situ [6], and quantitative in vitro evidence (presented here) that filamentation is a direct response to innate immune effectors and a means for subversion of internalization and killing by phagocytes. The observation that the initial interaction of the phagocyte with the filamentous morphotype dictates efficacy of internalization is consistent with previous findings that internalization of polystyrene particles is strongly dependent upon the shape of the target and the initial site of phagocyte attachment [38]. The resistance of filamentous forms of UPEC to phagocytosis, described

here, defines at least one mechanism that underlies the requirement for the Sula cell division inhibitor during UPEC pathogenesis [9].

Further studies are required to specify the host signals that elicit filamentation in UPEC and to better characterize the physiological changes that accompany UPEC filamentation within the host. As is the case for candidiasis, novel agents that target either initiation of, or recovery from, filamentation by UPEC might provide a therapeutic adjunct in acute and recurrent urinary tract infections.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Kinetics of neutrophil and macrophage influx to the bladder during UTI

Female C57BL/6J mice were transurethally inoculated with UTI89; bladders were harvested at time points indicated and processed for the magnitude of neutrophils and macrophage/monocyte populations. Contour plot analysis of representative bladder cell suspensions (naïve and 16-h infection) depicting the presence of neutrophil and monocytes/macrophages populations (A). Magnitude of neutrophils (■) and macrophage/monocytes (●) present at each time point during experimental cystitis are expressed as a percentage of total viable leukocytes (B). Each symbol represents a single animal, and horizontal lines represent the mean of all animals. An unpaired t-test was used to determine statistical significance (* $p < 0.003$, ** $p < 0.0001$).

Figure 2. Filamentous and bacillary UPEC are distinguished by flow cytometry

Fluorescence microscopic images of untreated (A) and MMC-treated (D) cultures of UTI89 $\Delta lon::Tn10/pCOMGFP$ correlate with contour plots from flow cytometric analysis of untreated (B) and MMC treated (E) cultures. The strategy for flow cytometric designation of each morphotype is described in Supplemental Fig. 1. The lengths of 100 individual bacteria were measured from microscopic images of untreated (C) or MMC-treated bacteria (F) using ImageJ.

Figure 3. Filtration-based enrichment of UPEC morphotypes

Mitomycin C-treated UTI89 Δlon /pCOMGFP was observed by differential interference contrast microscopy (A) and physiological properties by flow cytometry (B, C). The overall size and granularity of the population was determined by comparison of side scatter (SSC) and forward scatter (FSC) properties (B). The fluorescence intensity differs between the populations of bacillary (blue) and filamentous (red) morphotypes (C). Enrichment of the two morphotypes was observed by differential interference contrast microscopy (D, G) and flow cytometry (E, F, H, I) following centrifugation through a 5- μ m pore filter.

Figure 4. Mouse phagocytes preferentially kill bacillary UPEC

UTI89 Δlon /pCOMGFP treated with mitomycin C (MMC)) (A-C, D-F) or co-cultured with LPS-activated murine RAW 264.7 macrophages (G-I) were subjected to an in vitro epithelial-cell based phagocytosis assay utilizing RAW 264.7 cells as phagocytes (A-C, G-H) or to in vitro phagocytosis assay utilizing peritoneal exudate cells (PECs) as phagocytes (D-F) and examined by flow cytometry. Contour plots from representative assays before phagocytosis (input) (A, D, G) and after phagocytosis (output) (B, E, H) demonstrate the distribution of bacterial morphologies by FSC and SSC parameters. Percentages of bacilli (red) and filaments (gray) before (input) and after phagocytosis (output) of UPEC cultures treated with MMC (C, F) or co-cultured with LPS-activated RAW 264.7 macrophages (I) from triplicate wells from four independent experiments are presented as the mean $\pm$ S.E. A two-tailed student's *t* test was used to determine statistical significance (***p* < 0.003, ****p* < 0.0001).

Figure 5. Effect of target geometry on phagocytosis efficiency

Cultured murine macrophages (blue) were incubated with mitomycin C-induced UTI89 Δ lon/pCOMGFP (green) upon the surface of bladder epithelial cells (red stained with WGA Alexa 594) and monitored by fluorescence microscopy before (A) and after phagocytosis (B, C). Mitomycin C-induced UTI89/pCOMGFP were incubated with primary murine neutrophils and visualized by fluorescence microscopy (D-F). Mitomycin C-induced UTI89/pCOMGFP were incubated with primary human neutrophils and visualized by light microscopy (G-I). Phagocyte-bacteria interactions that initially occurred upon pole of the filament are indicated with arrows and non-polar interactions with arrowheads (D-I). Scale bars = 10 μ m.