Ascension and Adhesion of Uropathogenic Escherichia coli during Pyelonephritis

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Ascension and Adhesion of Uropathogenic *Escherichia coli* during Pyelonephritis

by

Lisa Kristine McLellan

A dissertation presented to
The Graduate School
of Washington University in
partial fulfillment of the
requirements for the degree
degree of Doctor of Philosophy

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*It is good to have an end to journey toward, but it is the journey that matters in the end.*

- *Ursula K. Le Guin.*

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Lisa K. McLellan

*Washington University in St. Louis*

*January 2021*
Dedicated to my parents -

Words cannot express my gratitude and my love.
Abstract of the Dissertation

Ascension and Adhesion of Uropathogenic *Escherichia coli* during Pyelonephritis

By

Lisa Kristine McLellan

Doctor of Philosophy in Biological and Biomedical Sciences

Molecular Microbiology and Microbial Pathogenesis

Washington University in St. Louis, 2021

Professor David A. Hunstad, Chair

The ability of uropathogenic *Escherichia coli* (UPEC) to successfully establish an infection within the urinary tract relies on the pathogen to be able to navigate the complex interactions between pathogen and host. During a urinary tract infection (UTI), UPEC must ascend, adhere, and form bacterial communities in various infection niches, from the bladder (cystitis) to the kidneys (pyelonephritis). Each of these steps is critical for bacterial survival, and host and pathogen factors required at each stage can vary depending on host sex and anatomic niche. Preclinical modeling of pyelonephritis and sex effects on UTI has been limited due to the propensity for kidney infections in standard female murine catheterization models to resolve spontaneously, and technical barriers to infection of male mice *via* catheter. As a result, host and bacterial factors involved in pyelonephritis and male UTI remain incompletely defined.

Here, we employed newly developed *in vivo* and *in vitro* models to begin addressing this gap in our understanding of UTI pathogenesis. In these models, we found that male and
androgenized murine hosts with vesicoureteral reflux develop severe pyelonephritis, leading to the formation of penetrant renal abscesses. We also demonstrated the presence and localization of kidney bacterial communities (KBCs) that nucleate renal abscess formation, and identified KBC formation as a key pathogenic stage in pyelonephritis.

We sought to define UPEC community behavior during abscess formation, as ascension into the kidney has been thought to impose a severe population bottleneck. Using a set of “barcoded” isogenic UPEC isolates and confocal microscopy, we defined the spatial and temporal dynamics of UPEC populations during experimental ascending pyelonephritis in mice. Further, we found that KBCs were clonal, and thus ascension into the nephron is a relatively uncommon event accomplished by single bacteria within a nephron unit.

Once UPEC arrives within a nephron, it must adhere in order to withstand the shear force of urine flow and to enable replication and ultimately KBC formation. Type 1 pili and the tip adhesin FimH have long been recognized to be critical for UPEC adhesion to bladder epithelium. However, we demonstrated an essential role for type 1 pili in colonizing the kidney during pyelonephritis and showed that mannosides (novel small-molecule inhibitors of FimH) can limit the severity of experimental pyelonephritis. Further, by performing a lentiviral CRISPR screen in cultured renal collecting duct epithelial cells, we identified desmoglein-2, a mannosylated, surface-expressed tight junctional protein, as the first known kidney receptor for FimH. Using cellular assays and biochemical approaches, we confirmed that desmoglein-2 binds FimH and that this interaction is critical for UPEC binding to collecting duct cells.

Effective and personalized future treatment of UTIs will rely on a deep mechanistic understanding of host-pathogen interactions mediating each step of the virulence cascade, in all urinary tract niches and in both host sexes. The present work suggests that androgen modulation
may represent a new therapeutic or preventive strategy for UTI in selected males and in women with hyperandrogenic conditions, such as polycystic ovary syndrome. Further, identification of the FimH-Dsg2 interaction in the kidney indicates that mannosides and vaccines that target FimH binding can be further developed as non-antibiotic approaches to pyelonephritis.
Chapter 1. Introduction to the Dissertation


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Abstract

The clinical syndromes comprising urinary tract infection (UTI) continue to exert significant impact on millions of patients worldwide, most of whom are otherwise healthy women. Antibiotic therapy for acute cystitis does not prevent recurrences, which plague up to one fourth of women after an initial UTI. Rising antimicrobial resistance among uropathogenic bacteria further complicates therapeutic decisions, necessitating new approaches based on fundamental biological investigation. Here, we highlight contemporary advances in the field of UTI pathogenesis and how these might inform both our clinical perspective and future scientific priorities.
A Pervasive and Persistent Problem

Urinary tract infections (UTIs) are among the most common bacterial infections, affecting 150 million people worldwide each year (1-3). Although both men and women may become infected, UTIs are traditionally thought of as a disease of women, among whom 50% will be affected across their lifespan (2). Approximately 25% of women presenting with a first episode of bacterial cystitis go on to suffer recurrent UTI within 6 months, some having 6 or more infections in the year following the initial episode (2, 4). Current therapeutics are suboptimal, as the prevalence of multidrug-resistant uropathogens is increasing and antibiotic treatment for acute infection does not preclude recurrences (2, 5, 6). These recalcitrant infections can become a significant health problem and diminish quality of life for affected men and women.

Bacterial infections of the urinary tract (UT) present clinically with a variety of signs and symptoms and may be caused by an array of organisms (see Figure 1). Here, we focus primarily on uropathogenic Escherichia coli (UPEC) as the etiologic agent of UTI, as UPEC is responsible for >80% of all community-acquired infections (2). Other etiologies include Staphylococcus, Klebsiella, Enterobacter, Proteus, and Enterococcus; these organisms become particularly relevant during catheter-associated and healthcare-associated infections (7, 8). The pathogenic cascade of UPEC cystitis has been extensively studied in recent years, largely in cell-culture and mouse models, as mice recapitulate many facets of the bladder epithelial environment (reviewed in (9)). Through these studies, unprecedented light has been shed on the molecular and cellular basis of infection. Further, recent years have seen the advent of several new mouse models, enabling the study of complicated UTIs (pyelonephritis, renal abscess, catheter-associated UTI) and recurrent cystitis. In addition, recent data suggest that the normal, healthy bladder is not
always sterile, and a picture of the urinary microbiome is emerging. Such advances promise to further illuminate molecular mechanisms of virulence in UPEC (reviewed in (10)) and other uropathogens, as well as the intricacies of the host immune response. With these approaches, we are poised to address heretofore unanswered questions with clinical relevance to UTI treatment and prevention.

**Molecular Pathogenesis of UTI**

Infection of the urinary tract begins when UPEC, likely introduced after colonization of the periurethral area by gastrointestinal tract flora (11-13), accesses and ascends the urethra by incompletely defined mechanisms. Upon reaching the urinary bladder, UPEC bind to superficial epithelial (facet) cells in a type 1 pili-dependent manner (14) (see **UPEC Virulence Factors: Adhesins**). A subset of adherent bacteria are then internalized into facet cells (15, 16), a dynamic process that likely relies on the normal cycling of apical membrane segments in these cells (17). Countering this key pathogenic activity, bladder epithelial cells undertake active expulsion of internalized UPEC. Recent data show that UPEC are capable of neutralizing the lysosome, and that this neutralization is sensed by a lysosomal membrane protein called mucolipin TRP channel 3 (TRPML3), activating pathways that direct exocytosis of UPEC-containing lysosomes (18). Through a distinct mechanism, activation of Toll-like receptor 4 (TLR4) by internalized UPEC leads to specific ubiquitination of TRAF3, enabling its interaction with a guanine-nucleotide exchange factor that directs assembly of the exocyst complex, thereby also accomplishing expulsion of intracellular bacteria (19).

Using strategies that are poorly understood, UPEC can gain access to the bladder epithelial cell cytoplasm, thereafter developing clonal, biofilm-like masses termed intracellular
bacterial communities (IBCs) (15, 20). As part of the host response, most superficial epithelial (facet) cells are exfoliated (21), liberating IBCs into the urine and ridding the body of thousands of bacteria. Shed IBC-bearing cells are observed in the urine of infected women and children, supporting their clinical relevance (22, 23). After 16-24 h in murine UTI models, a subset of UPEC in remaining IBCs adopt a neutrophil-resistant, filamentous morphology and escape the IBCs, subsequently re-invading naïve bladder epithelial cells (24). Some of these bacteria will go on to infect immature bladder epithelium which is exposed after exfoliation, later forming quiescent intracellular reservoirs, which avoid immune clearance and resist systemic antibiotic treatment (25-27). These persistent UPEC may re-emerge, in response to currently undefined signals, to cause the recurrent cystitis that is so common clinically.

A significant gap in our understanding is the mechanism by which UPEC escape the initial vacuole (after internalization) to reach the cytoplasm, where the IBC is formed. Unlike other Gram-negative pathogens that escape an endosome, UPEC do not encode a type III secretion system to deliver virulence factors (28). Further, the bottleneck imposed by IBC formation in the bladder precludes classical in vivo mutant screens, and no in vitro model for IBC formation has been wholly accepted by the field (14, 15). As a result, surrogate methods have been used to illuminate requirements for IBC formation. For example, since IBCs exhibit many characteristics of biofilms, one group performed a transposon screen for genes necessary for in vitro biofilm formation, using polyvinyl chloride as substrate as well as sampling the pellicle of standing broth cultures. This screen yielded genes with functions in attachment, motility, LPS synthesis and modification, metabolism, as well as bacterial cell maintenance (29). In other studies, murine UTI models have shown that single-gene mutants of UPEC exhibit defects in specific steps of the IBC pathogenic cascade, as in the case of OmpA, a major outer
membrane porin. OmpA deletion does not inhibit UPEC binding to superficial epithelial cells or internalization; however, once within the cytoplasm of these cells, UPEC ΔompA cannot complete the intracellular pathway and, as assessed by dwindling organ bacterial loads and confocal microscopy, fails to progress past very early stages of IBC formation in mice (30). Similarly, UPEC harboring a deletion of the small non-coding RNA Hfq cannot replicate within cultured human bladder epithelial cells, despite exhibiting normal levels of binding and invasion (31). Defining the roles of relevant host factors (exemplified by the exocytosis studies mentioned above) will also help to elucidate the mechanism by which UPEC accesses the critical cytoplasmic niche. Answering these questions will require collaborative and broad-based efforts involving cell biology, bacterial physiology, biochemistry, and optimized in vitro or ex vivo models.

Following escape into the cytoplasm, the bacteria find themselves occupying an environment very different from the nutrient-poor bladder lumen. Transcriptomic analyses of UPEC in different models (such as during murine UTI or bacterial growth in urine) have suggested that various metabolic pathways are essential for pathogenesis; these include sialic acid transport/metabolism, gluconeogenesis, the tricarboxylic acid (TCA) cycle, iron uptake, ethanolamine and phosphate metabolism, as well as amino acid metabolism (32-35) (reviewed in (36)). Although this work has provided broad insight into the metabolic activities required to cause UTI, we are on the verge of being able to specifically interrogate UPEC populations in defined niches and times during infection. UPEC survival and growth at distinct spatiotemporal points during infection could rely on very different metabolic sources. Intracellular survival presumably requires a unique set of metabolic capabilities, but the precise needs are incompletely defined. Metabolism of a chromogenic substrate during cystitis provides
circumstantial evidence that UPEC can utilize β-galactosidase, perhaps reflecting a glucose-limited milieu during this intracellular step (37). Transcriptional profiling from whole mouse bladders 6 h post infection with UPEC strain UTI89 was posited to reflect mostly bacteria that are internalized and actively forming IBCs (38). This analysis found that 2.3% of the UPEC genome was differentially regulated within the bladder at this time point (6 h), when compared to the statically grown UPEC broth culture that was used as inoculum. Genes associated with alternative carbon source utilization pathways, such as \( \text{lacZ} \) and \( \text{srlA} \) for galactose and sorbitol utilization, respectively, were upregulated; deletion of \( \text{lacZ} \) was subsequently found to impair virulence (38). Genes associated with iron acquisition were also highly expressed, including siderophores (secreted bacterial proteins that chelate extracellular iron and return it to the bacterial cell). In contrast, tryptophan and cysteine synthetic genes were downregulated, reflecting an abundance of these amino acids within the IBC niche (38). A more specific understanding of bacterial metabolism within pathogenic niches could reveal points of potential intervention, to halt infection and/or eliminate reservoirs that seed recurrent UTIs. Of note, the central metabolic pathways in \( E. coli \) do not necessarily represent all uropathogenic species; other pathogens with distinct metabolism may respond to different nutritional cues during infection (32).

Comparatively less is known about the molecular pathogenesis of infection in the kidney. In traditional animal models (female mice of most strains), severe kidney infection (including renal abscess formation) is quite uncommon, hampering the study of this entity. Attenuation in mouse models of kidney infection has been observed with UPEC mutants lacking specific virulence factors, such as type 1 pili, P pili, flagella, α-hemolysin, and cytotoxic necrotizing factor 1 (CNF1) (3, 10). Further, host genetics appear to play a role in susceptibility to acute
pyelonephritis. For example, data suggest that innate immune defenses play a critical role in control of urinary pathogens (39). Increased risk of acute pyelonephritis and renal scarring have been linked to polymorphisms reducing expression of IRF3 (encoding a pro-inflammatory transcription factor) or CXCR1 (encoding IL-8 receptor) in certain UTI-prone patient populations (39-41). Compared to the understanding of bacterial cystitis, the understanding of pyelonephritis remains limited and, consequently, a fertile area of study.

UPEC Virulence Factors: Adhesins

UPEC contains a mosaic of virulence factors that work in concordance with host background in order to establish infection. An extensive review of UPEC virulence factors has been done elsewhere (3). Here, however, we discuss chaperone-usher pathway (CUP) pili, major adhesive structures used by UPEC to establish tropisms within various environmental and host niche. CUP pili are part of a broadly conserved molecular paradigm for Gram-negative bacterial secretion of heteropolymeric surface structures. Expression of pili on the surface of bacteria involves delivery of structural subunits by the periplasmic chaperone to the outer membrane usher for assembly through the outer membrane. On the bacterial surface, the pilus structure includes the tip adhesin, adapters, and repeating subunits of the helical pilus rod. Each subunit has its immunoglobulin-like fold completed by a strand provided by the next subunit, reflecting a process called donor-strand complementation (DSC) (42). The energetic favorability provided by this final structure drives assembly on the periplasmic side of the usher, as the periplasm is devoid of ATP (43). The adhesin subunit is located at the binding tip of the pilus and is comprised of a pilin domain and a lectin domain. The lectin domain binds sugars with stereochemical specificity, providing the tropism that characterizes each CUP pilus (reviewed in
(44-46)). A given UPEC strain can encode up to 16 CUP operons; for many of these, a specific function has yet to be elucidated. Specifically, the genome of UPEC strain UTI89 contains approximately 10 CUP pili operons (8-9 complete operons and 2 incomplete operons; Figure 2) (47, 48). Here, we discuss two of the best studied and most relevant CUP pili to UTI pathogenesis: type 1 pili and P pili.

**Type 1 pili**

Type 1 pili (encoded by the *fim* operon) have long been considered the major urovirulence factor during cystitis. One of the first pathogenic steps of cystitis is binding of the UPEC type 1 pilus adhesin (FimH) to α-D-mannosylated uroplakins on the apical (luminal) surfaces of superficial epithelial (facet) cells of the bladder (49-51). This binding event allows to bacteria to avoid elimination during urine voiding and activates a signaling cascade that leads to UPEC internalization into the facet cells, where IBCs are subsequently formed in the facet cell cytoplasm. Within the IBCs, type 1 pili then participate in interbacterial interactions, forming biofilm-like communities (20, 21, 52, 53).

Recent work has identified structural variants that help to illuminate the function of type 1 pili as a molecular tether in the bladder. The tip adhesin FimH can transition between tensed and relaxed conformational states (with low and high binding affinity to mannose, respectively) to modulate UPEC binding. A balance between these states allows optimal epithelial binding by UPEC within the bladder environment (52, 54). The variant adhesin FimHA27V/V163A displays higher *in vitro* affinity for mannose than wild-type FimH, but UPEC expressing this variant are attenuated during murine cystitis. This could reflect that higher-affinity binding to mannose causes less frequent interactions with the bladder epithelium (catch-bond mechanism) or that the
variant more effectively binds decoy receptors such as Tamm-Horsfall glycoprotein (48, 54, 55). Regardless of mechanism, the attenuation of this variant in the bladder demonstrates the functional importance of these FimH conformational states.

The importance of type 1 pili during bladder infection is highlighted through both mouse models and human studies. Lack of the pilus and adhesin causes near-complete abrogation of acute cystitis in mice (16, 20, 21, 53). In humans, UPEC attached to shed urothelial cells express type 1 pili, while expression is relatively lacking in bacteria recovered unbound in urine (56-59). Given the importance of the FimH-mannose interaction, novel therapeutics are being developed to target bacterial attachment (see Next-generation Therapeutics).

**P pili**

P pili have been considered the major adhesin during pyelonephritis. P pili (encoded by the *pap* operon) bind α-D-galactopyranosyl-(1,4)-β-D-galactopyranoside and appear to contribute to pyelonephritis in some species (60, 61). P pili are enriched in the genomes of UPEC isolates from children with acute pyelonephritis (62, 63). Allelic variation in the P pilus adhesin PapG correlates with differing affinity for various glycolipid receptors that are differentially expressed in the kidneys of humans and model animals, including mice (44, 64). Specifically, the mouse does not significantly express the human PapGII receptor, the globoside Gb04 (64). As noted above, female mice of most strains are generally resistant to severe kidney infection, or it is self-limited (see Emerging, Clinically Relevant Models for UTI); therefore, understanding the role of various adhesins in pyelonephritis remains a fertile area for study.
Immune Control and Pathogen Evasion

After ascending the urethra, bacterial pathogens are challenged by innate defenses within the bladder. Arrival in the bladder triggers a TLR4-dependent, lipopolysaccharide (LPS)-stimulated inflammatory response from bladder epithelial cells and resident leukocytes, culminating in activation of the NF-κB pathway, which promotes expression of inflammatory cytokines and neutrophil chemoattractants (65). This inflammatory milieu engenders massive neutrophil influx into the bladder tissue and lumen, reflecting a diagnostic hallmark of UTI. The importance of this neutrophil influx in controlling UPEC infection has been well established (e.g., (66-69)). Production of polysaccharide capsule by UPEC, particularly of the K2 or K1 serotype, may provide some protection against complement-mediated killing (70). Further, many other soluble factors (e.g., antimicrobial peptides, complement, lipocalin-2, lysozyme, lactoferrin) are also released by host cells into the urinary space, potentially creating a less hospitable environment for arriving bacteria (71, 72). Antimicrobial peptides likely protecting the urinary tract include defensins, the human cathelicidin LL-37, and ribonuclease 7 (73-76). These molecules may exert direct antimicrobial activity, augment innate cellular recruitment, or function to alter the environmental niche to make it less favorable for uropathogens (e.g., by sequestering siderophores and the critical nutrient iron from the bacteria) (77). One such host factor, lipocalin-2 (neutrophil gelatinase-associated lipocalin (NGAL)), is secreted into the urine during UTI and acts as a predictive biomarker for UTI in febrile infants, young children, and women with recurrent UTI, as well as limiting bacterial clearance in mouse models (77-79). Host transcriptional regulators such as hypoxia-inducible factor 1α are also expressed in response to bacteria, potentially boosting innate defense components such as nitric oxide, cathelicidin, and β-defensin 2 (77, 80). The humoral pattern recognition molecule pentraxin 3 (PTX3) has been
shown to help control UTI by serving as an opsonin and promoting bacterial uptake by neutrophils; UTI-prone children and adult cystitis patients who had suffered recurrent UTI as children exhibited polymorphisms in *PTX3* (81), suggesting that the cellular and soluble components of innate immunity can influence disease outcomes. As uropathogens ascend into the kidneys, the bacteria encounter additional innate defenses. In addition to *IRF3* and *CXCRI* (see Molecular Pathogenesis of UTI), the roles of the cytokines IL-6 and IL-8 during ascending pyelonephritis has been a major focus of study (39). IL-6 stimulates antimicrobial peptide expression and monocyte proliferation (82-84). In children with pyelonephritis, high IL-6 serum and urine concentrations correlated with the presence of renal scarring (85, 86). As discussed above, IL-8 expression facilitates neutrophil transmigration to infected areas and polymorphisms reducing expression of its receptors (CXCR1 and CXCR2) are linked to a risk of acute pyelonephritis (41, 87). Thus, an effective, controlled innate response is critical for effective clearance of uropathogens (39, 88).

Formation of IBCs is a key means by which bacteria subvert neutrophil activity, as arriving neutrophils accurately locate IBC-bearing facet cells but cannot access the bacteria within (65, 89). UPEC can subvert and delay the innate immune response, and thus the arrival of neutrophils, in multiple ways (reviewed in (65)). For example, liberation of the effector YbcL by UPEC leads to a measurable dampening of neutrophil infiltration into the bladder (90-92). Further, UPEC induces host expression of genes such as *IDO*, which, via generation of kynurenine metabolites, can attenuate neutrophil migration across infected bladder epithelia, both in a Transwell system and in mice (93, 94). Some UPEC strains, such as CFT073, can also disrupt host signaling by producing TIR domain-containing proteins such as TcpC; this virulence factor interacts with host adaptor MyD88 to disrupt TLR4 signaling, also reducing urinary IL-1β.
in mice and inhibiting the NLRP3 inflammasome in macrophages (95, 96). While robust innate defenses are able to repel most bacterial challenges, this inflammatory response may represent a double-edged sword. In murine cystitis, excessive inflammation and resulting bladder tissue damage predisposes the host to worse infection outcomes, including chronic cystitis (88, 97, 98).

As mucosal barriers such as the bladder epithelium are repeatedly assaulted with bacteria, they are generally tolerant to transient microbial presence, and innate defenses are key to prevent infection. However, clinical syndromes such as recurrent UTI raise questions about the importance of adaptive immunity in bladder protection. Pro-inflammatory cytokines that also elicit adaptive immune effects, such as IL-17, are prominently secreted during the acute phase of murine experimental UTI (99, 100). CD8+ T cells are recruited to the bladder as early as 24 h post infection, but the precise roles of these and other adaptive immune cell populations are unknown (101). Regarding humoral immunity, the prevalence of recurrent UTI in the female population suggests that a lasting protective immune response is not established following cystitis, at least in this subpopulation of women (102). Upper-tract UTI (pyelonephritis) may elicit a more robust serological response, although it is not clear if antibodies generated against UPEC antigens here would subsequently reach the bladder to provide protection against future cystitis. In total, the mechanisms by which adaptive immunity might help to control UPEC infection are substantially understudied in comparison with the innate immune system. Understanding the basis of functional adaptive immunity against UTI could have major implications for recurrent UTIs and vaccine development, as further discussed below.
The Present and Future of UTI Diagnostics

For decades, the diagnosis of UTI has relied on culturing urine samples and looking under the microscope for white blood cells. Providers also utilize point-of-care dipstick tests to assay the presence of leukocyte esterase, nitrites, and other compounds. Even in combination with careful symptom history and risk factor ascertainment, these tests offer only 50-85% sensitivity and 80-90% specificity (103). Further, community diagnosis of UTI is typically made on clean-catch urine samples, raising the possibility of contamination and rendering some positive cultures difficult to interpret (including “false-positives”). In the age of “omics,” widespread mass spectroscopy, point-of-care molecular detection, bacterial genomic sequencing, and other tools, the time is right to move toward better UTI diagnostics. These might rely on a combination of host immune and metabolic markers, as well as on the detection of uropathogens and their components (DNA, proteins, etc.). For example, if sample preparation challenges could be circumvented, direct mass spectrometry on infected urine might be useful, detecting bacteria promptly in urine without the need to wait for growth on solid media (104). Alternatively, rapid molecular identification of *E. coli* at the substrain level, as well as prediction of antibiotic resistances, might enable more efficient selection of antibiotics for treatment (105, 106). Ultimately, improved and accurate diagnostics for UTI should translate into more effective care for patients, less frustration and speculation on the part of providers, and an overall reduction in antibiotic use.

Next-generation Therapeutics

Put simply, UTI therapies are in need of innovation. For decades, finite courses of antibiotics have been prescribed for women with UTIs, often in the absence of culture data; such
empiric treatment is effective at resolving acute symptoms, but clearly fails to eliminate recurrence risk (2). In addition, the rise of multidrug-resistant uropathogens (e.g., (107)) mandates definitive therapeutic selection based on actual patient culture and susceptibility results and/or local and institutional antibiograms. As the rate of resistance development (especially among Gram-negative uropathogens) has overtaken the pace of new antibiotic development, fundamentally new approaches are needed (108). Further, prophylactic antibiotics are incompletely effective in preventing infection (109), and in one mouse study, subtherapeutic levels of ciprofloxacin were shown to augment murine UTI (110). To move forward in the therapeutic realm, we must extend our molecular understanding of both the pathogen and the host. Contemporary development of novel UTI therapeutics has focused on interfering with pathogen binding to bladder epithelium or other key pathogen processes; the development of vaccines based on bacterial components; and the modulation of host responses, specifically promoting exfoliation to eradicate chronically resident bacteria from the bladder.

An emerging example in which basic biology of the host-pathogen interaction has informed therapeutics development is that of mannosides and pilicides, compound families which target in distinct ways the crucial step of bacterial adherence to host cells. Pilicides interfere with the chaperone-usher pathway, preventing the assembly of adhesive pili on the bacterial surface and thereby abolishing epithelial binding (111, 112). In contrast, mannosides serve as competitive inhibitors, occupying the binding pocket of the type 1 pilus adhesin FimH with affinities that are orders of magnitude higher than those of the mannosylated uroplakins decorating the bladder epithelial surface (113). The oral bioavailability and efficacy of mannosides in preventing UTI in mice portend substantial potential utility in the clinic (114, 115). Beyond uncomplicated cystitis, mannosides have also shown efficacy in mouse models for
prevention of catheter-associated UTI (as reflected by diminished bladder and catheter colonization) (116). Mannosides are being rationally optimized to exhibit more drug-like pharmacokinetic properties, such as improved metabolic stability and bioavailability (113, 117). Agents such as these, so-called “anti-virulence” compounds that block specific molecular steps in pathogenesis, apply much less selective pressure on pathogenic bacteria, thereby reducing the rate of resistance development (118). Further, due to their known mechanism of action, such agents can be used as tools to further probe the biology of host-pathogen interactions (119). Recent structural “snapshots” of bacterial pilus assembly via the chaperone-usher pathway (see Figure 3) may illuminate additional routes to inhibition (45, 120-123), with potentially much broader impact as this bacterial secretion pathway also underlies virulence factor production by diverse bacterial pathogens (e.g., *Yersinia pestis*). Direct application to the bladder luminal surface of nanoparticles, perhaps coated with the FimH adhesin (124), has also been explored in mice as a means to accomplish targeted delivery of novel therapeutics to the host (125).

Successful vaccination against UPEC and other uropathogens could have monumental impact on the lives of those at risk for complicated UTIs or who suffer from recurring episodes. Multiple groups have worked to identify specific UPEC factors for potential use as vaccine antigens. Candidate antigens include the FimH adhesin, siderophores such as yersiniabactin (126), and other immunodominant proteins identified in mouse models (127, 128) (reviewed in (3, 44)). Two important considerations may hinder the effectiveness of vaccine candidates against UTI. First, as strains of *E. coli* (expressing type 1 pili, iron acquisition systems, and other factors) are present in the normal gut microbiota, vaccination could potentially alter the populations of proteobacteria in the gut. Second, as noted above, it is not clear how much antibody (IgG) in the healthy urinary tract should reach the bladder lumen. Therefore, elicitation
of serum antibodies against UPEC antigens may be more effective in preventing pyelonephritis, as antibodies may be more readily delivered to the kidney. Further studies into the correlates of adaptive immunity in both the upper and lower urinary tract are needed to advance these efforts.

Another strategy for the management of acute or recurrent UTI may be to modulate or enhance host responses to UTI. As noted earlier, an exuberant inflammatory response predisposes the host to chronic cystitis (88, 98). In fact, in a mouse UTI model, inhibiting this response using an oral anti-inflammatory COX-2 inhibitor yielded better outcomes without actually targeting the bacteria (and thereby applying no selective pressure). These findings corroborated small clinical trials in women, in which symptomatic improvement at 4 and 7 days with ibuprofen alone was equivalent to oral antibiotics (129-131). Further, as the bladder exfoliation accompanying acute UPEC cystitis is not complete, bacteria within quiescent reservoirs may re-emerge to seed recurrent infection. Advanced, more efficacious exfoliants are being designed to unearth these quiescent reservoirs (132, 133). Once these bacteria are forced to emerge, they may be more susceptible to the actions of standard antibiotics. Therefore, combined exfoliant-antimicrobial strategies might rid the host of the UPEC reservoirs that underlie some recurrent UTIs (133).

Finally, in considering updated UTI therapeutics, one must consider an impending paradigm shift regarding the “normal” state of the bladder – which has long been assumed to be sterile (134). Enhanced culture techniques, as well as metagenomics on catheter-collected samples, have detected urinary bacteria in the healthy and asymptomatic state in women (135). Interactions between these apparent commensals and soluble mediators such as antimicrobial peptides may alter susceptibility to UTI (136). Specific microbiome structures may also be related to conditions traditionally thought to be non-infectious, such as stress or urgency.
incontinence (137, 138) and interstitial cystitis/chronic bladder pain. As the urinary microbiome becomes more extensively defined, we will have to account for it in considering the pathogenesis of UTI, as well as in choosing therapies for symptomatic patients.

**Emerging, Clinically Relevant Models for UTI**

Although many uncomplicated UTIs can resolve spontaneously or with antibiotic treatment, more complicated forms of UTI have not, until recently, been reflected in animal models. The majority of preclinical work in the last two decades on cystitis and pyelonephritis has relied on transurethral inoculation of UPEC into the bladder in female mice (139, 140). Emerging mouse models may enable additional clinically relevant questions to be addressed.

**Catheter-associated UTI (CAUTI)**

Prolonged urinary catheter usage is a risk factor for UTI, due largely to the ability of bacteria to establish a biofilm on the catheter that resists clearance by host defenses and antibiotics. CAUTIs represent the most common nosocomial infections and are associated with increased hospital length of stay, morbidity, and mortality (141, 142). As UPEC are less prominent in the epidemiology of CAUTI, other organisms such as *Enterococcus faecalis* have emerged as model organisms for study (7). Insertion of a urinary catheter elicits an inflammatory environment in the bladder, which is manifested histologically as exfoliation, edema of the lamina propria and submucosa, urothelial thinning, and mucosal lesions (7). This damaged mucosa and the catheter itself offer surfaces for bacterial adhesion (143). Recent data indicate that enterococcal adherence to urinary catheter material is mediated by fibrinogen, a host protein that is released into the bladder lumen and deposited on the catheter following insertion. *E.*
faecalis then bind fibrinogen via the pilus tip adhesin EbpA, subsequently forming a biofilm on the catheter (144, 145). These pathogenic events can be modeled in C57BL/6 mice in which a short length of silicone catheter material is transurethrally deposited in the bladder, followed by introduction of *E. faecalis* (143, 146). A structural understanding of bacterial adhesin association with catheter material and proteinaceous deposits may enable the design of new strategies to counteract catheter-associated UTI.

**Recurrent UTI**

Approximately 20-30% of women with acute cystitis go on to develop recurrent UTI (rUTI), and those who do suffer on average 2-3 additional UTIs in the year following an initial episode (2). The subsequent UTI might arise from reinoculation of the urethra with flora from the gastrointestinal tract, or from re-emergence of a bladder epithelial reservoir. In a genomic study, isolates from four patients with rUTI were analyzed by whole-genome sequencing (11). In two patients, the same UPEC clone dominated both gut and urinary tract habitats at the initial and subsequent infection; in the other two, a new clone had established dominance in both habitats at the time of recurrent UTI. Further, isolates causing subsequent UTI in these patients, when introduced into mice and compared with their initial infecting strain, exhibited increased fitness in both the gut and the urinary tract, demonstrating that fitness in these two important niches is not mutually exclusive (11). More recent models of gut colonization by UPEC may illuminate the movement of uropathogens from one body site to another and suggest strategies to eliminate UPEC from the gut as a route to preventing recurrent UTI (147).

In a newly developed mouse model of rUTI, the C3H mouse strain – known to feature vesicoureteral reflux, a major risk factor for upper-tract UTI in humans (148) – can be sensitized
to later infection. Following an initial infection (experimentally resolved by treatment with antibiotics) and upon subsequent re-challenge with a later infection, these “sensitized” mice were more likely than naïve mice to suffer persistent bacteriuria and chronic cystitis (88). A leading hypothesis for recurrent UTIs is that an exuberant inflammatory response to initial infection causes bladder remodeling that predisposes the host to recurrent infection or more inflammatory outcomes (4, 88, 98, 142). This model may enable a mechanistic understanding of apparent predisposition to recurrent infection, in turn informing therapies that could interfere with or dampen this process.

**Male and Complicated UTI**

The higher prevalence of UTI in females is chiefly attributed to anatomic factors in women, such as shorter urethral length, shorter distance from the anus to urethral meatus, and permissiveness of the vaginal and perineal environments to microbial colonization (13, 142). However, males at both ends of the age spectrum (mainly infants <1 year of age and elderly men with prostatic hypertrophy) exhibit a higher incidence of UTI, and other conditions in males (diabetes, spinal cord injury, catheter use) also promote UTI (149). Among individuals with upper-tract UTI (pyelonephritis), males exhibit greater morbidity and mortality than females (150), suggesting that non-anatomical differences may be at work in these more severe infections. Further, there is a higher UTI incidence in women with polycystic ovary syndrome (a common hyperandrogenic state), and this risk normalizes upon antiandrogen treatment (151-153), implicating a role for androgens in UTI risk in females as well.

Until recently, essentially all cystitis and pyelonephritis studies have been performed in female mice, as the male mouse bladder has not been reliably accessible by catheter. Of note,
instillation of uropathogens into the urethra of male mice elicits prostatic infection (154-156). In a recently developed model of UTI, a small abdominal incision is made and bacteria are inoculated via needle into the bladders of male and female mice, permitting direct sex comparisons (157). This inoculation method recapitulates the IBC cascade of acute cystitis established in studies with catheter-infected females. Interestingly, once anatomic barriers are bypassed in this way, male mice experience more severe infection than females, mirroring epidemiologic data observed clinically in men; indeed, male C3H mice uniformly develop severe pyelonephritis and renal abscesses that are seen much less frequently in female mice (Figure 4) (157). Further, this susceptibility to pyelonephritis was potentiated in androgenized, catheter-infected female mice (158). Of note, within the abscesses of male C3H/HeN mice, UPEC formed dense, biofilm like bacterial communities termed kidney bacterial communities (KBCs) (158). These KBCs are thought to contribute to the pathogenesis of upper-tract UTI (see Chapter 2). Nonsurgical inoculation techniques for male mice have also been advanced recently (156, 159). These new models open doors to study sex differences in UTI pathogenesis and host response, as well as sequelae of severe pyelonephritis and abscess formation; these latter phenotypes are relevant to febrile UTI in children, following which renal scarring is a common complication.

**Concluding Remarks**

Urinary tract infections continue to be among the most common bacterial infections in humans, eliciting millions of antibiotic prescriptions annually. Available therapies have not evolved significantly in recent years, do not prevent recurrences, and are challenged by rising antibiotic resistance. Creative approaches to treatment, including the development of anti-virulence therapeutics, should be prioritized. In addition, the field lacks a thorough
understanding of protective host immunity related to UTI, if such is generated after natural infection (especially pyelonephritis) or can be elicited via vaccination. Given the broad range of organisms that can cause UTI and the unavoidable nature of some risk factors (e.g., urinary catheters), even highly effective novel interventions will not completely mitigate the impact of these infections on human health. However, the common pathogenic themes in Gram-negative community-onset UTI make this subset of infections a particularly important epidemiologic target.

**Conflicts of Interest**

D.A.H. serves on the Board of Directors of BioVersys AG, Basel, Switzerland.

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Figure 1. Clinical features and virulence mechanisms in cystitis and pyelonephritis. UTIs can present clinically in a variety of ways, most often reflecting cystitis (infection of the bladder) or pyelonephritis (infection of the kidney). Uropathogenic *Escherichia coli* (UPEC) are the most common cause of UTI (especially among community-onset infections), among other pathogens. Selected virulence factors associated with the pathogenesis of UPEC cystitis or pyelonephritis are shown and include adhesins, siderophores, toxins, siderophores, capsule, and other systems (see text for details). UT: urinary tract.
Figure 2. Various CUP pili present in UTI89, UPEC cystitis isolate. Current known CUP pili encoded within the genome of UTI89 and their associated function. Percentages represent percent of *E. coli* genomes containing the designated operons (47). Figure kindly provided by the laboratory of S.J. Hultgren.
Figure 3. Ribbon representation of the chaperone-adhesin-usher complex for assembly of type 1 pili from *Escherichia coli*. The periplasmic chaperone FimC (green) delivers structural subunits to the outer membrane usher (FimD, red) for assembly. Subunits shown represent the pilus tip structure and include the adhesin FimH (purple) and adapters FimG (yellow, within the barrel of FimD) and FimF (gray). Each subunit has its immunoglobulin-like fold completed by a strand provided by the next subunit, in a process called donor-strand complementation (DSC). The energetic favorability provided by this final structure drives assembly on the periplasmic side of the usher, as the periplasm is devoid of ATP. Protein Database PDB# 4J3O; adapted from (123).
Figure 4. Tubular inflammation, dropout, and edema during severe *Escherichia coli* pyelonephritis in C3H/HeN male mice. Gomori trichrome stain (photo from P. Olson; *Trends Mol Med* cover image) (160).
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Chapter 2. Androgen Exposure Potentiates Formation of Intratubular Communities and Renal Abscesses by *Escherichia coli*


*PDO and LKM contributed equally to this work*

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Abstract

Females across the lifespan and certain male populations are susceptible to urinary tract infections (UTI). The influence of sex on UTI is incompletely understood, in part because preclinical modeling has been performed almost exclusively in female mice. Here, we employed established and new mouse models of UTI with uropathogenic *Escherichia coli* (UPEC) to investigate androgen influence on UTI pathogenesis. Susceptibility to UPEC UTI in both male and female hosts was potentiated with 5α-dihydrotestosterone, while males with androgen receptor (AR) deficiency and androgenized females treated with the AR antagonist enzalutamide were protected from severe pyelonephritis. In androgenized females and in males, UPEC formed dense intratubular, biofilm-like communities, some of which were sheltered from infiltrating leukocytes by the tubular epithelium and by peritubular fibrosis. Abscesses were nucleated by small intratubular collections of UPEC first visualized 5 days post infection and briskly expanding over the subsequent 24 h. Male mice deficient in Toll-like receptor 4, which fail to contain UPEC within abscesses, were susceptible to lethal dissemination. We conclude that AR activation imparts susceptibility to severe upper-tract UTI in both female and male murine hosts. Visualization of intratubular UPEC communities illuminates early renal abscess pathogenesis and the role of abscess formation in preventing dissemination of infection. In addition, our data suggest that androgen modulation may represent a novel therapeutic route to combat recalcitrant or recurrent UTI in a range of patient populations.
Introduction

Bacterial infection of the urinary tract represents one of the most common human infectious diseases and imposes a large economic burden (1, 2). Ascension of uropathogens to the kidneys, resulting in pyelonephritis or urosepsis, is associated with mortality and threatens lifelong morbidity, including renal scarring and attendant risks of hypertension and chronic kidney disease, despite appropriate initial antibiotic treatment (3, 4). These contemporary challenges to the human host manifest in an era in which the primary causative agent of UTI, uropathogenic *Escherichia coli* (UPEC), displays unprecedented global prevalence and breadth of antimicrobial resistance (5).

Preclinical modeling of severe pyelonephritis has been constrained by a lack of susceptible female murine models and technical inability to access the male mouse bladder via catheter (6, 7). To begin to decipher sex differences in UTI, we recently developed a minimally invasive surgical technique enabling direct bladder inoculation and comparison of UTI pathogenesis and outcomes in both male and female hosts. After equal intravesical inoculation with UPEC, male mice of multiple genetic backgrounds, compared with females, developed more severe renal infection – including extremely high kidney UPEC burdens and 90% prevalent renal abscess in male C3H/HeN mice (7). The C3H background is known to exhibit vesicoureteral reflux (VUR) (8) – a translationally relevant feature, as VUR is a major risk factor for upper-tract UTI, particularly in affected children. Micro- and macroscopic abscess formation is a pathological feature of pyelonephritis in humans (9), and the severity of inflammation correlates with subsequent renal scarring in several models of renal injury (10, 11).

We previously reported that castration of male C3H/HeN mice prior to induction of UTI mitigated the development of chronic cystitis, severe pyelonephritis, and renal abscess, while
ovariectomy had no effect on these outcomes in females. Exogenous testosterone replacement in castrated male mice restored severe UTI phenotypes (7). It remained unknown if androgen-driven UTI susceptibility extends to females, in whom UTI is much more prevalent overall (1, 12, 13). Here, we used specific androgen receptor (AR) agonists and antagonists as well as genetic models to conclusively demonstrate the involvement of AR activation in susceptibility to UTI in both female and male hosts. The androgenized phenotypes enabled us to detail the formation of intratubular UPEC communities – dense, biofilm-like bacterial masses filling multiple tubular segments at the center of developing abscesses. These UPEC communities were sheltered from intense neutrophilic infiltrates by intact tubular epithelia, which exhibited peritubular fibrosis. Mice lacking functional TLR4 (the innate immune sensor of bacterial lipopolysaccharide) failed to contain UPEC within abscesses and were prone to mortality from urosepsis. These experiments reveal the importance of androgen exposure in UTI susceptibility across both host sexes, and enable the future dissection of niches and mechanisms exploited by UPEC for growth and persistence in the kidney during pyelonephritis and renal abscess formation.

**Materials and Methods**

**Bacteria**

Uropathogenic *E. coli* strain UTI89, a type 1-piliated clinical cystitis isolate (14), was grown in static Luria-Bertani (LB) broth for 16-18 h at 37°C. Cultures were centrifuged for 10 min at 7,500 × g at 4 °C before resuspension in sterile phosphate-buffered saline (PBS) to a final density of ~4 × 10⁸ CFU/mL.
Surgical and catheterization murine models of UTI

All animal protocols received prior approval from the Washington University Institutional Animal Care and Use Committee. Experiments were conducted in C3H/HeN (Envigo, Indianapolis, IN), C3H/HeJ, C57BL/6 A^w^J/A^w^J (or its isogenic Tfm mutant), or C57BL/6 (all from Jackson Laboratories, Bar Harbor, ME) strains. Mini-surgical bladder inoculations were carried out as described previously (7). Mice aged 8-9 wk were anesthetized with inhaled 3% isoflurane, and the abdomen was shaved and sterilized with 2% chlorhexidine solution. A 3-mm longitudinal, midline incision was made over the bladder through the skin and peritoneum. The bladder was aseptically emptied before injection of 50 µL containing 1-2 × 10^7 CFU into the bladder lumen over 10 s. The bladder was allowed to expand for an additional 10 s before the needle was removed, and the peritoneum and skin were closed with sutures. Certain female-only experiments, as indicated, were replicated with bladder inoculation by catheter (15-17).

Castration and androgen treatment

For surgical castration, male mice aged 4 wk received preoperative subcutaneous buprenorphine SR (0.05 mg/kg) prior to inhaled 3% isoflurane anesthesia. The scrotum was depilated with Nair (Church & Dwight Co., Ewing, NJ) and the operative field was disinfected with 2% chlorhexidine solution. A 1-cm ventral, midline incision was made in the scrotum, the tunica was pierced, and the testis and vas deferens were mobilized. The spermatic cord on each side was clamped and ligated with 4-0 Vicryl above the epididymis, and the testis and vas deferens were removed. The skin incision was closed with Vetbond (3M Animal Care Products, St. Paul, MN). Four days following castration or sham operation, 60-day extended-release pellets
containing 25 mg testosterone, 25 mg DHT, or placebo (Innovative Research of America, Sarasota, FL) were implanted in sterile fashion at the posterior neck, using a small incision and blunt dissection to create a subcutaneous pocket. UTI was induced 4 weeks later (i.e., at 8-9 wk of age).

Antiandrogen treatment

Female C57BL/6 mice, 5 weeks of age, received subcutaneous DHT or placebo pellets as described above. Beginning 3 d later, mice received enzalutamide 50 mg/kg daily for 6 d per wk by oral gavage, or vehicle (1% carboxymethylcellulose, 0.1% Tween-80, 5% DMSO). Ten days later, UTI was induced via catheter inoculation, as described above. Treatment continued until sacrifice 2 wpi.

Serum analyte measurements

Blood was collected by submandibular puncture, retroorbital aspiration, or cardiac puncture (if collection was coincident with euthanasia) into Microtainer serum separation tubes (Becton Dickinson, Franklin Lakes, NJ). Samples were allowed to clot for 90 min at room temperature (RT) before centrifugation at 10,000 × g. Serum testosterone was measured by enzyme immunoassay at the Ligand Assay and Analysis Core, University of Virginia Center for Research in Reproduction (Charlottesville, VA). Blood urea nitrogen (BUN) was measured by the veterinary laboratory at Washington University School of Medicine.
**Determination of urine and tissue bacterial loads**

Where indicated, we obtained post-infection, clean-catch urine samples using gentle suprapubic pressure to enumerate CFU/mL urine. At the indicated time points, mice were euthanized via CO₂ asphyxiation, and bladders and kidney pairs were aseptically removed and homogenized in 1 ml or 0.800 ml sterile PBS, respectively. To ascertain prostate bacterial loads, the male urogenital system was removed *en bloc* and the prostate was micro-dissected under a dissecting microscope as previously described (18). Serial dilutions of tissue homogenates or urine were plated on LB agar to enumerate bacterial loads. Where indicated, cystitis in C3H/HeN mice was classified as “chronic” if all urine and endpoint bladder titers contained >10⁴ CFU/mL.

**Tissue histopathology, immunofluorescence, and immunohistochemistry**

Infected bladders and kidneys were bisected and fixed in 10% neutral buffered formalin for 24 h. Fixed tissues were embedded in paraffin, sectioned, and stained with H&E or Gomori trichrome. Alternatively, kidneys were frozen, sectioned with a cryostat, and similarly stained. For immunofluorescence, unstained slides were deparaffinized in xylenes and rehydrated in isopropanol, washed in water, and boiled in 10 mM sodium citrate.Slides were blocked in 1% BSA, 0.3% Triton-X 100 in PBS for 30 min at RT, and incubated with rabbit anti-*E. coli* antibody (E3500-06C, US Biological, Salem, MA) for 1 h at RT or overnight at 4 °C. After washing in PBS, sections were stained with AlexaFluor 488-conjugated goat anti-rabbit IgG (Life Technologies, Grand Island, NY) and SYTO 61 red fluorescent nucleic acid stain (Molecular Probes, Eugene, OR). Images were acquired on an Olympus FV1200 confocal microscope. For immunohistochemistry, unstained slides were deparaffinized in xylenes, rehydrated in a series of ethanol washes, boiled in 10mM sodium citrate, and blocked in 1%
BSA, 0.3% Triton-X 100 in PBS for 1 h at RT. Slides were incubated with rat anti-Ly6G antibody (BE0075-1, BioXCell, West Lebanon, NH) for 2 h at RT, then stained with biotinylated goat anti-rat IgG (BA-9400, Vector Laboratories, Burlingame, CA) for 1 h at RT; or incubated with rabbit anti-AR antibody (ab74272, Abcam, Cambridge, MA) for 2 h at RT, followed by biotinylated goat anti-rabbit IgG (BA-1000, Vector Laboratories) for 30 min at RT. For each target, slides were then treated for 30 min with Vectastain Elite ABC reagent and developed using DAB substrate kit (Vector Laboratories). Slides were then counterstained with hematoxylin, rehydrated, mounted, and visualized.

Statistics
Organ bacterial loads and numerical data were compared by the nonparametric Mann-Whitney U test. Survival analysis was performed with the Mantel-Cox log-rank test. Fisher exact test was used for 2×2 comparisons. P values <0.05 were considered significant.

Results
Androgen exposure aggravates UTI severity in female C3H/HeN mice. To test whether androgens could amplify UTI severity and enhance abscess development in female mice, as seen previously in males (7), we implanted slow-release subcutaneous testosterone or placebo pellets in female C3H/HeN mice 4 wk prior to mini-surgical bladder inoculation with UPEC. Testosterone pellet implantation significantly increased serum testosterone compared with placebo-treated females (Figure 1A), mirroring serum testosterone levels seen in males of similar age (19, 20). Multiple prior studies show that female C3H/HeN mice develop a bimodal distribution of bladder UPEC titers at 2-4 weeks post infection (wpi), whereby a minority (20-
40% of animals display chronic, active infection with ongoing inflammation and persistently high bladder bacterial loads while the majority of these females resolve infection (6, 7, 21). Consistent with these reports, a minority (36%) of placebo-treated females here exhibited chronic cystitis 2 wpi (i.e., bladder bacterial loads > 10^4 CFU; Figure 1B, circles). In contrast, 64% of androgenized females exhibited chronic cystitis 2 wpi (Figure 1B, triangles). When CFU burdens were compared numerically, testosterone-treated females exhibited significantly higher bladder (P=0.0102) and kidney (P=0.002) bacterial loads 2 wpi compared to placebo-treated controls (Figure 1B). More strikingly, androgen exposure led to development of grossly visible renal abscess in 9 of 14 females (64%), compared to 0 of 14 placebo controls (P=0.0006). These data illustrate that elevated circulating testosterone predisposes to severe UTI independent of biological sex. Of note, while intravesical inoculation with UPEC does induce acute and chronic prostatitis in C3H/HeN males (Figure 2), our data in androgenized females exclude the prostate as a driver of androgen-induced severe UTI.

Androgen receptor activation drives UTI severity. We next aimed to identify the hormonal pathway(s) by which testosterone acts to induce severe UTI. Testosterone supplementation could perturb levels of other hormones in the hypothalamic-pituitary-gonadal (HPG) axis via its negative feedback on gonadotropin releasing hormone (GnRH), luteinizing hormone (LH), and follicle-stimulating hormone (FSH). In this scenario, increased GnRH, LH, and/or FSH could be protective in castrated males and non-androgenized females, while decreased secretion of these hormones in androgenized hosts could promote UTI susceptibility. Refuting this possibility, we previously found that while orchiectomy dramatically attenuated UTI, ovariectomy in females (which would activate the HPG axis similarly to castration in males(22)) had no influence on
UTI outcome (7). This finding, in conjunction with our ability to facilitate severe UTI in females via androgen treatment (Figure 1B), eliminates alteration in GnRH or gonadotropin levels as a mechanism for increased UTI severity in androgenized hosts.

We next focused on the two canonical pathways of testosterone activity and relevant receptors. First, testosterone can undergo peripheral conversion by aromatase into estradiol, which could then activate the estrogen receptor. We therefore tested whether an aromatase-resistant and (compared with testosterone) more potent and specific AR agonist, 5α-dihydrotestosterone (DHT), would enhance UTI severity. We implanted slow-release DHT or placebo pellets 4 d after castration or sham operation in C3H/HeN males, then surgically infected mice 4 wk later. Consistent with our previous results, castration attenuated bladder ($P=0.041$) and kidney ($P=0.0063$) bacterial burdens 2 wpi in placebo-treated males (Figure 3A). Treatment with exogenous DHT reversed the effects of castration (Figure 3A), recapitulating the complementation seen with testosterone (7). Organ bacterial burdens in castrated, DHT-complemented males were similar to those in sham-operated, placebo-treated males, with most animals in both groups developing bilateral renal abscess. Likewise, infection of DHT-treated female C3H/HeN mice led to significantly higher bladder bacterial burdens ($P<0.0001$) and frequency of chronic cystitis (87% vs 27%; $P=0.0025$) 2 wpi, compared with placebo-treated females (Figure 3B). DHT treatment also induced gross abscess formation in 73% of these females (vs 0% with placebo, $P<0.0001$) and dramatically increased kidney titers (Figure 3B, $P<0.0001$). Thus, peripheral aromatization of testosterone does not mediate susceptibility to severe UTI. Of additional note, in female-only experiments we replicated these results with UPEC inoculations via the traditional catheter route, demonstrating that the observed severe UTI
phenotypes and their androgen dependence are not attributable to the mini-surgical infection methodology.

Independent of aromatization, androgens classically engage the AR, though alternative signaling pathways independent of AR have recently emerged (23, 24). We confirmed AR expression in both tubular epithelium and infiltrating leukocyte populations 2 wpi in male C3H kidneys (Figure 4). To demonstrate AR involvement in our observed phenotypes, we first adopted a genetic approach using C57BL/6 A<sup>tm</sup> testicular feminization (Tfm) mice, which encode a frameshift mutation disrupting the steroid-binding domain of the AR, rendering these animals insensitive to classical AR signaling (25, 26). Wild-type (WT) males exhibited kidney titers significantly higher than WT females (Figure 5A; \(P=0.016\)), as we previously observed (7). Importantly, the majority of Tfm males resolved renal infection (Figure 5A), with kidney bacterial burdens significantly lower than WT males (\(P=0.0043\)) but indistinguishable from WT females (\(P=0.50\)). The Tfm mutation did not have a significant effect on bladder bacterial loads 24 hpi (Figure 6).

We also demonstrated direct AR involvement via pharmacological inhibition with enzalutamide (27), a second-generation antiandrogen with higher potency and improved specificity over earlier agents (28, 29). We implanted C57BL/6 females with DHT or placebo pellets 2 wk prior to UTI, and 3 d later initiated enzalutamide or vehicle treatment. Enzalutamide administration conferred strong protection against severe UTI outcomes, with significantly reduced bacterial loads in the kidneys and bladder (\(P<0.0001\) vs vehicle; Figure 5B, C). In total, these data specify that AR activation induces susceptibility to severe UTI in both host sexes and across multiple genetic backgrounds.
Severe upper-tract UTI in the androgenized host manifests as abscess nucleated by intratubular UPEC communities. As modeling of UTI has until now been limited to female mice, and females of most strains are resistant to severe pyelonephritis and renal abscess formation (6, 7, 30, 31), the field lacks a detailed understanding of the pathogenesis of ascending renal abscesses. However, the high incidence of abscess formation in androgenized C3H/HeN hosts (7) (Figure 1B, 3B) now allows interrogation of the anatomic and temporal details of abscess development following intravesical UPEC inoculation. Histological examination of kidneys 3 days post infection (dpi) from mice exhibiting urine and bladder bacterial titers >10⁴ CFU (predictive of pyelonephritis (7)) demonstrated only scant neutrophilic inflammation near the renal pelvis, with no visible abscess (Figure 7A) and no apparent tissue alteration in the renal cortex or medulla (Figure 7B). Visualized bacteria were limited to the renal pelvis and calyces (Figure 4C). By 5 dpi, a minority of infected mice (4 of 15, 27%; Figure 7A) exhibited gross abscess at necropsy. Sectioning of entire kidneys revealed infrequent small, intraluminal collections of bacillary UPEC distributed from the renal medulla (Figure 7D) to the cortex (Figure 7E, 8). The tubules housing these UPEC communities reflected cellular injury and were surrounded by small numbers of infiltrating neutrophils, without intraluminal inflammation (Figure 7E, F). By 6 dpi, 90% of infected males exhibited renal abscess at necropsy (Figure 7A, G), equivalent to the frequency observed 2 wpi (7). Thus, ascending renal abscesses in this model develop precipitously between 5 and 6 dpi, signifying rapid UPEC replication and neutrophil recruitment in this interval.

Kidney sections from C3H/HeN males 2 wpi revealed UPEC populations in the centers of dense neutrophilic lesions appearing predominantly in the cortex (Figure 9A, 9B, 10). At this advanced stage, visible UPEC were located within the intratubular space, adopting
coccobacillary morphology in tightly packed communities (Figure 9B, C), in contrast to their more loosely associated appearance 5 dpi (Figure 7F). Recruited neutrophils had largely destroyed or replaced much of the surrounding tubular architecture (Figure 9A-D) and also formed casts within surrounding tubules (Figure 9E), as seen in human pyelonephritis and as reported in other mouse models (8, 32, 33). We often observed multiple, discrete intratubular bacterial collections in close proximity within the same neutrophilic lesion (Figure 9D), and at low power, parenchymal involvement distinctly followed a wedge-shaped pattern while remote areas of kidney were spared (Figure 9F). In conjunction with findings at earlier time points (Figure 7), and consonant with recently published data in C3H/HeOuJ mice (32), these results indicate that ascending UPEC colonize an individual papillary collecting duct pyramid and multiple interconnected nephrons via robust intraluminal replication to nucleate abscess formation within a given segment of the kidney. Consistent with our prior data (34), serum BUN was not significantly elevated in these C3H males 2 wpi (Figure 11). Additionally, abscesses rarely develop in non-androgenized female C3H/HeN mice (6, 7, 35), but we did identify an abscess with similar bacterial intratubular community morphology in one such mouse (out of >30 examined 2 wpi; Figure 12).

Abscess formation prevents lethal dissemination but shelters UPEC intratubular communities. In the abscessed region of kidney 2 wpi, renal parenchyma was largely replaced by intense neutrophilic infiltrate (Figure 9). Light and fluorescence microscopy captured breaching of the UPEC-harboring tubules by neutrophils (Figure 13A, B), and trichrome staining of male C3H/HeN kidneys 2 wpi indicated that the infected tubules were fibrotic (Figure 13C, D). These findings suggest that for at least some duration, the tubular structure and
subsequent UPEC-provoked peritubular fibrosis shield the intratubular UPEC community from phagocytic attack.

It was previously reported that mice with functional deficiency of Toll-like receptor 4 (TLR4), which exhibit markedly delayed and diminished innate responses to UTI (36, 37), do not develop renal abscesses even following direct renal injection of UPEC (6, 38, 39). We therefore tested how TLR4 deficiency would impact sex differences in ascending renal infection by surgically infecting male and female C3H/HeJ mice, isogenic with C3H/HeN except for a TLR4 mutation that renders HeJ mice insensitive to lipopolysaccharide (40). Most infected C3H/HeJ females survived through the period of observation, while many infected C3H/HeJ males succumbed to infection between 3 and 7 dpi (P=0.0274; Figure 14A). Interestingly, the timing of demise in these C3H/HeJ males coincided with the window of abscess development we observed in C3H/HeN males (Figure 7). Abscess was not observed in C3H/HeJ males at necropsy despite the presence of intratubular UPEC communities (Figure 14B). In addition, at 3 dpi (preceding any deaths), infected male C3H/HeJ mice displayed significantly higher renal (P=0.001) and bladder (P=0.001) bacterial burdens compared to female C3H/HeJ mice (Figure 14C).

These outcomes in C3H/HeJ males are in sharp contrast to infected C3H/HeN males, which uniformly survive despite persistence (for at least 20 wpi) of high renal bacterial burdens and abscesses (34). To further assess the cause of this sex difference in mortality, we infected male or female C3H/HeN mice and cultured spleen, liver and blood 3 dpi (preceding any deaths). Dissemination of infection was significantly more prevalent in males, as reflected by higher bacterial loads in spleen (P<0.001), liver (P<0.001), and blood (P<0.05) (Figure 14D). Persistence of observed sex differences in UTI outcomes in the absence of functional TLR4
suggests that these differences are not attributable to androgen effects on TLR4 signaling, expression, or activation. Collectively, these data support a model in which abscess formation with peritubular fibrosis enables the immunocompetent host to contain renal infection and prevent lethal systemic disease; however, this host strategy also permits UPEC to maintain a sheltered intratubular niche for replication and survival following ascension into the kidney.

**Discussion**

In this study, we show that AR activation in either host sex underlies increased susceptibility to pyelonephritis and renal abscess formation, a paradigm with significant translational implications. We further demonstrate that UPEC establishes intraluminal communities within kidney tubules, arising rapidly in the androgenized host within a narrow temporal window to nucleate nascent renal abscesses. A subset of these *kidney bacterial communities* (KBCs) were protected from phagocytosis, as the fibrotic tubular epithelium encapsulating these communities appeared to hinder phagocyte entry.

Abscesses were focal or multifocal, occurring within regions of pyelonephritis that followed the pattern of a collecting duct unit (32) and located adjacent to other, uninvolved segments of renal parenchyma. This observation suggests that among UPEC that reach the renal pelvis, only a fraction are ultimately successful in accessing and persisting within selected nephrons. Beyond the process of uropathogen ascension through the urethra or from the bladder to the kidney, it is equally important to illuminate this “third ascension” of uropathogens from the renal pelvis and calyces to more proximal segments of the nephron. Although some studies have implicated immunologic factors influencing ascension and persistence in the collecting duct
(41-43), the virulence mechanisms and host factors which enable tubular ascension and intraluminal replication are incompletely defined.

Even in a single micrograph of a developing abscess, one can see areas of complete destruction of cortical and tubular architecture, infected tubules that have been invaded partially or completely by neutrophils (consistent with neutrophil casts seen in other recent studies (8, 32)), and intratubular KBCs that have not yet been successfully breached by phagocytes. These persisting KBCs are apparently shielded for a more prolonged time by tubular epithelium and peritubular fibrosis. These images thus offer snapshots of various stages of the infiltration and phagocytic activity of host immune cells en route to a fully formed abscess. In other disease models, including ischemic kidney injury, transmigration of neutrophils across the tubule epithelium appears to be unimpaired (44-46). Fruitful future investigations in the present model will illuminate pathogen strategies or host factors (e.g., local ischemia (47, 48), anatomic features such as the basement membrane, or specific cellular programs that promote post-infection fibrosis) that impede neutrophil migration into the abscess community. Importantly, while male C3H/HeN mice are unable to resolve renal infection, their survival indicates that abscess formation successfully restricts the dissemination of infection. In contrast, congenic males with impaired innate responses fail to form abscesses surrounding bacterial communities and ultimately succumb to disseminated infection. We therefore propose a model whereby the pathology of abscess formation is a double-edged sword: bacteria are contained via inflammation and fibrosis, preventing overwhelming systemic disease, but some tubules represent a privileged site for bacterial replication where, at least for a time, phagocytes within the abscess lesion are unable to readily access the expanding UPEC community.
These KBCs display notable parallels to earlier descriptions of intracellular bacterial communities within bladder epithelium (49-52), though the microenvironment is presumably quite different in these two niches. The thematic similarity of UPEC community morphology in bladder and kidney hints that UPEC may use conserved programming to proliferate and to ultimately subvert the innate cellular response through biofilm formation within these niches (53). Beyond the recognized advantage of biofilms in limiting antibiotic diffusion, the location of KBCs also likely impairs antibiotic action, perhaps by occluding local transit of antibiotics in the urinary space, inducing restriction of blood flow to UPEC-infected areas (47, 48), or limiting diffusion from the blood space through injured and fibrotic tubular epithelia. These features correlate with the clinical need for prolonged antibiotic therapy in severe pyelonephritis and renal abscess.

AR activation clearly induced susceptibility to severe pyelonephritis and abscess development in both host sexes; genetic or pharmacologic inhibition of AR signaling attenuated severe UTI. Further investigations will specify the AR-regulated gene networks that mediate UTI susceptibility in androgenized hosts, in renal epithelium and/or local or circulating immune cells. Androgen signaling has been implicated in immune cell function in other systems. For example, monocyte migration and production of TNFα were amplified by androgen exposure in a mouse model of wound healing (54); overall, mouse models and in vitro studies of neutrophils and macrophages have not yet painted a complete and unified picture regarding androgen influence (55-58). Further, the persistence of a sex difference in C3H/HeJ mice, which lack functional TLR4 (the primary instigator of inflammatory responses in the kidney), suggests that the effect of androgens on UTI pathogenesis does not reside entirely in the hematopoietic compartment.
We previously saw no appreciable phenotype following estrogen depletion (7), and here have ruled out a contribution from peripheral aromatization. This is in contrast to a current paradigm, supported by some human and mouse studies, in which estrogen levels are believed to influence UTI pathogenesis (59-63). Instead, we argue that even modest elevations in circulating testosterone (which often parallel increases in estradiol (64-66)) in female and susceptible male patients may have a greater effect on frequency or severity of UTI. Certainly, the human population with the highest circulating testosterone, adolescent males (67), exhibit the lowest rates of UTI (12), but the urogenital anatomy (compared with that of women) comprises the key defense against UTI in otherwise healthy men (7). In fact, when these anatomic barriers are compromised or bypassed, epidemiologic data reflect increased morbidity and mortality in men who do develop complicated UTI, as compared with women (68-71). In a related vein, male infants (e.g., those under 6 months of age) presenting with UTI outnumber their female counterparts, with male UTI rates falling steadily from the neonatal period to late infancy (72-80). This epidemiologic phenomenon closely parallels the postnatal surge in testosterone in male infants that reaches pubertal levels shortly after birth, then steadily wanes to a prepubertal baseline by 6-9 months of age (81-84). Thus, while UTI risk in male infants is commonly ascribed to indistinct “urodynamic immaturity,” we speculate that testosterone activity may actually underlie this risk, especially in the two thirds of such infants who (after first febrile UTI) lack demonstrable VUR or obstruction (85).

The present finding that androgen-mediated UTI receptivity affects females (as well as males) extends the translational implications of our work. In murine models of polycystic ovarian syndrome (PCOS; a common hyperandrogenic state in young women), mice overexpressing LH (and consequently testosterone) develop spontaneous pyelonephritis and
associated renal damage (86-89). Most clinical studies of PCOS have not specifically ascertained UTI incidence in these women; however, where data are available, women with PCOS exhibited increased UTI rates compared to healthy females (90-92), and these rates fell in affected women following antiandrogen therapy (90). An untold number of women without overt hyperandrogenism might be at higher UTI risk because of circulating testosterone levels near the upper limits of the “normal” range, a potential causative relationship that mandates further study. We present proof of concept that the course of severe UTI can be mitigated by AR antagonism, suggesting that antiandrogen therapy may represent an avenue for adjunctive therapy in treatment-refractory cases of complicated UTI.

Conflicts of Interest

DAH serves on the Board of Directors of BioVersys AG, Basel, Switzerland. All other authors have no potential conflicts of interest to disclose.

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Figures

Figure 1. Androgen exposure aggravates UTI severity in females. Female C3H/HeN mice were subcutaneously implanted with placebo (filled circles) or long-release testosterone pellets (filled triangles) 4 wk prior to induction of UTI. Testosterone was measured via enzyme immunoassay at time of infection; each sample was measured in duplicate and recorded as the mean. As anticipated, testosterone pellets increased the serum testosterone to levels physiologically relevant to normal males (A, ***P<0.0001 vs placebo). Organ harvest 2 wpi (B) revealed higher bacterial loads in C3H/HeN females receiving testosterone, in both the bladders (*P=0.0102) and kidneys (**P=0.002). Dotted line indicates limit of detection.
Figure 2. Male C3H/HeN mice develop continued prostrate infection after surgical bladder inoculation. Mini-surgical inoculation of the bladder with UPEC strain UTI89 in C3H/HeN males results in durable prostate infection. Shown are bacterial loads in homogenates of whole prostate harvested at the indicated time points (aggregate of 3 experiments with total n = 14-15 per condition). Bars indicate geometric mean, and dotted line indicates limit of detection.
Figure 3. Testosterone influence on UTI severity is not mediated via aromatization.

Bladder and kidney bacterial burdens were measured 2 wpi (A) in sham-operated C3H/HeN males treated with placebo pellets (open circles), castrated males treated with placebo pellets (open triangles), and castrated males receiving pellets of 5α-dihydrotestosterone (DHT), a potent androgen not susceptible to conversion to estrogen via the action of aromatase (inverted triangles). As seen previously, castration was protective against severe UTI (*P<0.041 in bladder, **P<0.0063 in kidney vs sham-operated). Treatment of castrated males with exogenous DHT reversed this effect (**P<0.0001 vs castrated males receiving placebo); DHT-complemented males exhibited organ bacterial burdens equivalent to sham-operated, placebo-treated males (A) and similarly developed gross renal abscess. (B) In an analogous way, treatment of C3H/HeN females with DHT prior to initiation of UTI was associated with significantly higher bladder and kidney bacterial burdens 2 wpi (**P<0.0001 vs placebo). Dotted lines indicate limit of detection.
Figure 4. The androgen receptor is expressed on epithelial and hematopoietic cells in infected male C3H/HeN kidneys. Immunohistochemistry demonstrates androgen receptor (AR) expression by both epithelial and hematopoietic cells in the infected kidney. AR staining of (A) an uninflamed area and (B) a heavily inflamed area of UPEC-infected kidney in a C3H/HeN male 2 wpi shows expression in most epithelial cells, particularly in the nuclei, and in most of the infiltrating leukocytes. Panels (C) and (D) represent control images of similar areas of kidney, with hematoxylin counterstain but with no primary antibody. Scale bar in lower right, 50 µm, applies to all images.
Figure 5. Androgen receptor activation enhances severity of UTI. (A) Renal bacterial loads 2 wpi in genotypes comprising males (open circles) or females (filled circles). Included strains are WT males (AR+/Y), androgen receptor-deficient (Tfm) males (AR^{Tfm}/Y), and WT females (AR^{+/+}). The renal bacterial loads of functionally AR-deficient (TFM) males were significantly lower than in WT males (**P<0.01) and were equivalent to those in WT females. In fact, the kidneys of most AR-deficient males were sterile 2 wpi (below the limit of detection, indicated by the dotted line). (B, C) Female C57BL/6 mice were implanted 2 wk prior to infection with a long-release subcutaneous pellet of DHT or placebo; 3 d later (11 d prior to infection), oral gavage with enzalutamide (Enz) or vehicle (Veh) was initiated and continued until sacrifice 2 wpi. As observed in C3H mice (Figures 1 and 2), DHT treatment of C57BL/6 females conferred susceptibility to severe UTI, as evidenced by significantly higher bladder (B) and kidney (C).
bacterial loads 2 wpi compared with non-androgenized females (**$P<0.01$). In addition, treatment of DHT-treated mice with Enz sharply reduced bacterial loads in both bladder and kidneys compared with those receiving vehicle (***$P<0.001$).
Figure 6. In C57BL/6 mice, bladder titers are not significantly impacted by the Tfm mutation. Bladder bacterial loads in C57BL/6 mice with the indicated genotypes. In contrast to the kidney, the Tfm mutation had a less evident effect on bladder titers at this early time point.
Figure 7. Timeline of renal abscess formation in C3H/HeN mice. (A) Proportion of male C3H/HeN mice exhibiting gross abscess formation at necropsy at the indicated intervals (n = 8-15 per time point). At 3 dpi, histology demonstrates normal renal architecture (B) and bacteria within the renal pelvis (C). At 5 dpi, immunofluorescence and H&E staining identify small collections of bacillary UPEC in the urinary space from the medulla (D; anti-*E. coli* green) to the cortex (E, F; anti-*E. coli* green in E inset). By 6 dpi, histologic evidence of abscess is fully established, with necrotic lesions, loss of tubular architecture and replacement by neutrophilic infiltrate (G, arrowheads). Scale bars: B, 100 µm; C, 50 µm; D, 25 µm; E, 100 µm; F, 20 µm; G, 200 µm.
Figure 8. Bacillary UPEC colonize tubules and form developing colonies 5 dpi. At 5 dpi in infected C3H/HeN males, immunofluorescence microscopy demonstrates bacillary UPEC (green) colonizing renal tubules. Scale bar, 20 μm.
Figure 9. UPEC establish biofilm-like communities in a protected intraluminal niche.

Neutrophilic abscesses were seen 2 wpi predominantly in the cortex (A), centered on a nidus of infected tubules (examples at A arrowheads, and B). Infected tubules at this time point were filled with coccobacillary UPEC, as seen by immunofluorescence with anti-\textit{E. coli} antibodies (green in C; host nuclei are stained red). In many infected kidneys, multiple infected tubules were identified within a single large abscess lesion (D). The lumina of some tubules were occupied by neutrophil casts (E). Overall, the pattern of inflammation followed that of a wedge of nephrons associated with an infected papillary collecting duct (duct of Bellini), generally outlined by the gray dashed lines in (F). Scale bars: A, 200 µm; B, 50 µm; C, 25 µm; D, 50 µm;
Figure 10. The infiltrate within renal abscesses is composed primarily of neutrophils. The cellular infiltrate surrounding kidney bacterial communities within renal abscesses observed 2 wpi in UPEC-infected C3H/HeN males is comprised predominantly of neutrophils (left panel), as revealed by immunohistochemistry with anti-Ly6G antibody. An uninvolved area of the same kidney is shown for comparison (right panel). Scale bars, 100 µm.
Figure 11. Serum BUN measurements show no difference between infected and mock-infected mice. Serum BUN measurements pre-inoculation (Pre), 7 dpi, and 14 dpi in C3H/HeN males infected via mini-surgical bladder inoculation with UPEC strain UTI89 or mock infected with PBS (Mock). No significant differences were observed. All males shown here had high-titer pyelonephritis with abscess at 14 dpi (data not shown). In prior published work (34), infected C3H/HeN males exhibited elevated BUN not until 30 dpi.
Figure 12. Abscess within female C3H/HeN mice. Abscess identified 2 wpi in a single non-androgenized female C3H/HeN mouse, demonstrating an H&E appearance analogous to abscesses seen in a majority of male C3H/HeN mice. Features include intratubular UPEC communities separated by tubular epithelia from intense neutrophilic infiltrate, which has replaced much of the nearby cortical architecture. Scale bar, 100 µm.
Figure 13. Tubular epithelium and peritubular fibrosis restrict infection but protect UPEC from infiltrating phagocytes. The process by which tubular architecture is destroyed within the abscess by 2 wpi in C3H/HeN males was captured in images showing infiltrating neutrophils breaching the epithelium of tubules harboring UPEC communities (A, H&E; B, anti-*E. coli* in green, nuclear stain in red). Gomori trichrome staining 2 wpi revealed that infected tubules were fibrotic (C), compared with kidneys from mock-infected C3H/HeN males at the same time point (D). Scale bars, 50 μm.
Figure 14. UPEC-infected TLR4-deficient males fail to form renal abscesses and are vulnerable to lethal dissemination. (A) Neutrophil recruitment and abscess formation were necessary for containment of infection; when the TLR4-deficient C3H/HeJ strain was used, male mice were much more likely to succumb to infection ($n=10$ per group, $*P=0.0274$), an outcome not seen in immunocompetent C3H/HeN mice. Surviving C3H/HeJ males failed to form abscesses surrounding UPEC-infected tubules (B; scale bar, 20 µm). Male C3H/HeJ mice developed significantly higher bladder and kidney titers than females 3 dpi (C, $***P=0.001$). In addition, male C3H/HeJ mice were significantly more susceptible than females to dissemination, the likely cause of death, as evidenced by bacterial loads 3 dpi in spleen, liver and blood (D, $***P<0.001$, $*P<0.05$). Dotted lines indicate limit of detection.
References


Chapter 3. Population Dynamics During Formation of Kidney Bacterial Communities by Uropathogenic Escherichia coli


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Abstract

Uropathogenic Escherichia coli (UPEC), the primary etiologic agent of urinary tract infections (UTIs), encounters a restrictive population bottleneck within the female mammalian bladder. Its genetic diversity is restricted during establishment of cystitis because successful UPEC must invade superficial bladder epithelial cells prior to forming clonal intracellular bacterial communities (IBCs). Here, we aimed to understand UPEC population dynamics during ascending pyelonephritis, namely formation of kidney bacterial communities (KBCs) in the renal tubular lumen and nucleation of renal abscesses. We inoculated the bladders of both male and female C3H/HeN mice, a background which features vesicoureteral reflux; we have previously shown that in this model, males develop severe, high-titer pyelonephritis and renal abscesses much more
frequently than females. Mice were infected with 40 isogenic, PCR-tagged (“barcoded”) UPEC strains, and tags remaining in bladder and kidneys were ascertained at intervals following infection. In contrast to females, males maintained a majority of strains within both the bladder and kidneys throughout the course of infection, indicating only a modest host-imposed bottleneck on overall population diversity during successful renal infection. Moreover, the diverse population in the infected male kidneys obscured any restrictive bottleneck in the male bladder. Finally, using RNA-in situ hybridization following mixed infections with isogenic UPEC bearing distinct markers, we found that despite their extracellular location (in the urinary space), KBCs are clonal in origin. This finding indicates that even with bulk reflux of infected bladder urine into the renal pelvis, successful ascension of UPEC to establish the tubular niche is an uncommon event.

Introduction

Within the unique environments of the mammalian host, expansion of a few bacteria into a large multicellular community is a complex process in which the bacteria must adapt to local conditions and survive mechanical, immunologic, and other stresses. These host forces may impose narrow bottlenecks in which the founding population undergoes a dramatic reduction in genetic diversity as it circumvents some facet of the host environment. Conversely, in the absence of such a bottleneck, the infecting population retains more genetic diversity, potentially allowing for more biological and phenotypic diversity (e.g., virulence and community behaviors) during infection.

Uropathogenic Escherichia coli (UPEC), the primary etiologic agent of urinary tract infections (UTIs), encounters such a restrictive population bottleneck as it establishes clonal, biofilm-like intracellular bacterial communities (IBC) within the superficial epithelial cells (facet
cells) of the bladder (1-6). During this pathogenic process, UPEC first bind these facet cells, and a subset of bacteria are then internalized. Within an infected facet cell, a single founder bacterium must avoid expulsion by the cell (7-9) and subsequently replicate in the cytoplasm, ultimately giving rise to a clonal IBC (1-3, 10). Indeed, a UPEC inoculum of $10^7$ colony-forming units (CFU) during experimental cystitis in C3H/HeN and C57BL/6 female mice results in the formation of on average $\sim 40-100$ IBCs (1, 10-14). Bacteria within the IBC are protected from host defenses and antibiotic treatment, while those remaining in the bladder lumen are susceptible to neutrophil attack and to elimination via micturition (15-18). Bacteria within IBCs later re-emerge to infect naïve cells, initiating subsequent rounds of IBC formation (15, 19, 20). Thus, the infecting population becomes dominated by founder clones that successfully completed the IBC cycle (1, 10, 19).

Bacteria in the bladder lumen may also ascend the ureters to establish infection in the kidneys (pyelonephritis). Colonization of the kidney represents another event in which a population bottleneck might occur. While cystitis is clinically quite common (annual incidence of community-acquired cystitis is 3-13% in females and 0.5-3% in males), only $\sim 1\%$ of cystitis cases progress to pyelonephritis, which carries risks for hospitalization, sepsis, and renal abscess formation (21). In fact, 10-30% of pyelonephritis cases result in hospital admission, conferring $2-4$ billion in medical costs in the US annually (21). A common risk factor (primarily in childhood) for developing pyelonephritis is vesicoureteral reflux (VUR), identified in 30-45% of young children who present with febrile UTI (22-25). Pyelonephritis in children increases risk for renal scarring and associated lifelong morbidities such as hypertension and end-stage renal disease (24, 26, 27), making it important to understand the pathophysiology and dynamics of upper-tract UTI.
The mouse is a highly suitable model for the study of UTI; however, preclinical modeling of UTIs has been performed almost exclusively in female mice, which in most backgrounds resolve upper-tract UTI without antibiotic treatment (28, 29). As a result, our molecular understanding of UPEC pathogenesis arises mostly from studies of the female bladder, while mechanisms of UPEC colonization of the kidneys remain a fertile area of study. More recently, we and others have developed models by which male mice can also undergo bladder inoculation (12, 30). Over a time course after equivalent bladder inoculation, males harbor significantly higher bladder and kidney bacterial loads than do females. Indeed, male C3H/HeN mice (a background with documented VUR (31-34)) uniformly develop severe pyelonephritis and >90% exhibit renal abscesses; these outcomes are observed in >70% of androgenized females (12, 35, 36) but only rarely in naïve females. As renal abscesses are being nucleated, tubules are occupied by biofilm-like collections of UPEC, which we termed kidney bacterial communities (KBCs) (35, 37). These intratubular (luminal) UPEC colonies are first visible microscopically 5 days post infection (dpi); thereafter, UPEC multiply rapidly and attract a robust neutrophil response, yielding fully formed KBCs within early abscesses by 7 dpi (35).

Here, we employed this murine model and a set of “barcoded” UPEC isolates to interrogate the population dynamics of renal tubular colonization by UPEC. We found that in contrast to the female bladder, which imposes a marked bottleneck related to the requirement for intracellular invasion by UPEC, UTI in male mice features much less stringent restriction of genetic diversity among the infecting population, as measured in both the kidneys and bladder. Despite this overall diversity, we found that individual KBCs each were clonal, as revealed by in situ hybridization after mixed infection with distinctly tagged UPEC strains. These data indicate that while ascension
to the kidney does not constrain the bacterial population as a whole, pyelonephritis is initiated by individual bacteria that successfully colonize a group of contiguous nephron segments.

**Materials and Methods**

**Bacterial strains and growth**

UPEC strain UTI89 was isolated from a patient with cystitis (10). UTI89 HK::Kan\(^R\), UTI89 HK:Chl\(^R\), as well as a set of 40 isogenic, “barcoded” strains of UTI89 with PCR-detectable tags, were previously published (1). This strain set was generated using the λ Red recombinase system (38). Individual strains were grown statically at 37 °C for 18 h in Luria-Bertani (LB) broth, then combined in equal mixture based on optical density (OD\(_{600}\)). Bacteria were pelleted at 7,500 × \(g\) at 4 °C for 10 min, then resuspended to an OD\(_{600}\) of 1.0 (~4 × 10\(^8\) CFU/mL) in sterile phosphate-buffered saline (PBS) for inoculation into mice. Type 1 pili expression was confirmed in all inocula by agglutination of guinea pig erythrocytes (Colorado Serum Company).

**Mouse infections**

All animal protocols received prior approval from the Institutional Animal Care and Use Committee at Washington University. Male C3H/HeN mice (Envigo) aged 8-9 weeks were infected as previously described (12). Briefly, mice were anesthetized with inhaled 3% isoflurane, and the lower abdomen was shaved and sterilized with 2% chlorhexidine solution. A 3-mm midline abdominal incision was made through the skin and peritoneum, exposing the bladder. The bladder was aseptically emptied before 50 µl of inoculum (1-2 × 10\(^7\) CFU in PBS) was injected into the bladder lumen via 30-gauge needle over 10 s. The bladder was allowed to
expand for an additional 10 s before the needle was removed. The peritoneum and skin incisions were closed with simple, interrupted sutures. At the time of surgery, mice were given sustained-release buprenorphine (1 mg/kg SQ) for analgesia. At the indicated time points, mice were euthanized by CO₂ asphyxiation. Bladders and kidney pairs were steriley removed and homogenized in 1 mL or 0.8 mL PBS, respectively. Homogenates were serially diluted and plated on LB agar for CFU enumeration or fixed for histology.

Detection of “barcoded” UTI89 strains

Methods of detecting the “barcoded” UTI89 strains have been described previously (1). Briefly, 100 µL of each organ homogenate was spread onto LB agar and incubated overnight at 37 °C. To collect genomic DNA, 2 mL of sterile water was added to bacterial lawns and scraped using bent, sterile glass pipettes. Genomic DNA was extracted via the Wizard genomic DNA purification kit; concentration was assayed by NanoDrop, and samples were diluted to 100 ng/µL.

Multiplex PCR was performed using 50 ng of genomic DNA, 1× Taq buffer (Invitrogen), 2.5 mM MgCl₂, 0.2 mM dNTP, 100 pmol each of primers BP-8C (cgtgcgcagctctctattttcct) and BP-8K (gcttcaaaagctctgaagttcctatac), 2.5 U Taq DNA polymerase (Invitrogen), and a set of 3 BP-xxF primers at final concentrations of ~66.6 pmol/primer. BP-xxF primer sets are shown in Table 1.

PCR reactions were cycled as follows: (1) initial denaturation of 94 °C for 3 min, (2) 10 cycles of 94 °C for 30 s, 62 °C for 30 s with a 1 °C decrease per cycle, and 72 °C for 30 s, (3) 30 cycles of 94 °C for 15 s, 55 °C for 15 s, and 72 °C for 30 s, and (4) a final 7-min extension at 72 °C. The reaction products were run on 2.5% Tris-borate-EDTA (TBE) agarose gels. The
presence or absence of a band representing each individual strain in the multiplex PCR was
determined by eye, by a scientist blinded to sample identity. Thirty-seven of the 40 tags were
routinely detected in the inoculum. Three tags were statistically underrepresented in PCR of the
inoculum pool and therefore were excluded from all analyses. Ambiguous bands were
adjudicated by analysis in an independent multiplex PCR replicate.

**Histology and RNA in situ hybridization**

Infected bladders and kidneys were bisected and fixed in 10% neutral buffered formalin
for 24 h. Fixed tissues were embedded in paraffin, sectioned, and processed for RNA in situ
hybridization (RNA ISH) using Advanced Cell Diagnostics protocols and reagents. Slides were
probed with RNAscope probes B-KanR (Catalog # 812371) and B-\textit{E.coli}-CAT-C2 (Catalog #
879281-C2). Detection was then performed using the RNAscope Multiplex Fluorescent Reagent
Kit v2 (ACD document # 323100-USM). Images were acquired on an Olympus FV1200
confocal microscope or with a Zeiss Axio Scan.Z1 digital slide scanner (for digitally tiled
images).

**Statistical analysis**

Statistical analysis was performed using Prism (GraphPad Software Inc., La Jolla, CA).
Differences were analyzed with the unpaired, two-tailed, nonparametric Mann-Whitney U test. P
values <0.05 were deemed significant. Quantitative Venn diagrams were created using the Eulerr
package in R Studio, with circle dimensions adjusted slightly to enable visual display of all
niches.
Results

Temporal course of male C3H/HeN kidney bacterial loads. First, we temporally detailed the development of severe pyelonephritis in male C3H/HeN mice, infecting them with UPEC strain UTI89 and measuring organ bacterial loads at different time points post infection. Bladder bacterial loads were high immediately following inoculation (5 min post infection [mpi]; geometric mean $7.5 \times 10^6$ CFU/bladder) and remained consistent throughout 14 days post infection (dpi; Figure 1A). In contrast, initial kidney titers were substantially lower than bladder titers, with a geometric mean of $3.9 \times 10^4$ CFU/kidney pair 5 mpi (Figure 1B). Kidney titers significantly increased 3 dpi and then decreased modestly (but significantly) to $\sim 10^6$ CFU/kidney pair 7 and 14 dpi (Figure 1B). On the basis of this pattern, we reasoned that if a bottleneck were present during kidney infection in this model, it was occurring between 3 and 7 dpi (in contrast to the female mouse bladder, in which a marked bottleneck is observed 24 hpi (1)).

The outcomes of experimental pyelonephritis in this model are substantially more severe in males than in females (12, 35). Here we observed that bacterial titers reaching the kidney 5 mpi were equivalent between male and female C3H/HeN mice (Figure 2). Therefore, initial ascension to the kidney (e.g., a sex difference in VUR) does not explain the sex differences we have observed in pyelonephritis severity.

Establishment of ascending pyelonephritis in male C3H/HeN mice does not impose a narrow bottleneck at the population level. In mice and in humans with properly functioning vesicoureteral junctions, retrograde flow of urine to the kidneys is precluded, thereby likely restricting the diversity of bacteria that can reach the kidney. In the absence of this anatomic protection (i.e., in C3H/HeN mice and humans with VUR (24, 39)), further bottlenecks might be
imposed during colonization of the collecting system and nephron. To investigate this, we infected male C3H/HeN mice with a set of 40 isogenic, PCR-barcoded isolates of UTI89 (1). Overall organ bacterial loads with this UTI89-derived strain set (Figure 3) matched our earlier data with the root strain (Figure 1). Using multiplex PCR, we assayed the proportion of isolates remaining at each time point, separately interrogating three niches (bladder, left kidney, and right kidney) to determine correlations among the tags detected in these locations. As expected, at 5 mpi, all tags were detected in the bladder (Figure 4A), and most were detected in the kidneys (Figure 4B). Next we looked 1 dpi, when the bladder IBC cascade is occurring and in female mice a significant bladder bottleneck can be discerned (1, 2, 12, 15). In male C3H/HeN mice, a majority of isolates (bladder, 94%; kidneys, 97% and 94%) were detected 1 dpi (Figure 4). This sharply contrasted with published data in C3H/HeN females, in which 25% of tags remained in the bladder and 60% in the kidneys 1 dpi (1). At 7 dpi in male C3H mice, we observed a slight narrowing of diversity, with 70%, 74%, and 72% of tags remaining in the bladder and the left and right kidneys, respectively; these proportions were unchanged 14 dpi (Figure 4). This course aligns temporally with the modest reduction in total kidney bacterial load observed between 3 and 7 dpi (Figure 1). Of note, no significant differences were seen between left and right kidneys at any time point (Figure 4).

To confirm that the difference between our male data and published female data was not attributable to the mini-surgical inoculation method, we also infected a small cohort of female C3H/HeN mice using the same technique. Total organ bacterial loads in females were equivalent to those in males 5 mpi, but organ titers fell in females 7 and 14 dpi (Figure 5), consistent with prior data (12, 28). As was true in males (Figure 4), at 5 mpi in females 100% of tags were detected in the bladder (Figure 6), and most tags were detected in the left and right kidneys (Figure 6B,C).
At 7 dpi in females, a sharp bottleneck was observed in the bladder (48% of tags) and kidneys (23% and 20%, respectively); by 14 dpi this bottleneck was even more pronounced (bladder, 36% of tags; kidneys, 11% and 12%; Figure 6). These data are consonant with those previously reported in females infected via catheter (1).

**Shared distribution of bacterial clones among urinary tract niches is persistent in males.** We next sought to understand whether the same isolates were found within all three niches, or if each niche was occupied by a unique set of UPEC clones. Using the multiplex PCR data from male C3H/HeN mice (Figure 4), we coded each strain and tracked them within each of the studied niches. The proportion of tags common to all three niches was 71%, 88%, 64%, and 58% of isolates at 5 mpi and 1, 7, and 14 dpi, respectively (Figure 7). The initial rise in shared proportion, followed by a slight narrowing, reflects a mild diversity bottleneck predicted by the overall bacterial loads in the male kidney (Figure 1) and recapitulates trends in the total number of tags across these time points (Figure 4).

Meanwhile, a majority of isolates (78%) in female C3H/HeN mice occupied all three niches 5 mpi (Figure 8A), again indicating that initial ascension to the kidney does not impose a sex-discrepant population bottleneck (see also Figure 2). However, by 7 and 14 dpi in females, the proportion of isolates common to all three niches had fallen sharply (Figure 8B,C), in agreement with earlier work in females (1).

**Kidney bacterial communities (KBCs) arise from clonal expansion of founder bacteria within individual tubules.** Formation of IBCs in females imposes a bottleneck on bladder population diversity because only a small minority of bacteria successfully complete the IBC
cascade; each IBC in this model is formed from a single founder bacterium that has invaded a facet cell (1). While morphologic similarities exist between bladder IBCs and renal tubular KBCs, clonality in the IBC is a result of prerequisite intracellular invasion (1, 2, 15, 40). This is in contrast to KBCs which form in the extracellular space (tubular lumen) (35). Therefore, we hypothesized that KBCs would be the product of multiple bacterial clones reaching the tubular lumen. To interrogate the clonal composition of KBCs, we infected C3H/HeN males with a 1:1 mixture of UTI89 HK::KanR and UTI89 HK::ChlR (Figure 9). We determined the composition of the KBCs 2 wpi by RNA in situ hybridization (RNA ISH), detecting expression of KanR or ChlR. Of note, this method was chosen after standard immunofluorescence microscopy was judged to be inadequately specific for these markers and challenged by autofluorescence in the kidney. Surprisingly, visualized KBCs were always composed of either UTI89 HK::KanR or UTI89 HK::ChlR, but never both (of 87 KBCs visualized across 9 mice infected in four independent experiments), all were monochromatic; Figure 9). Within an individual kidney section, groups of KBCs formed from a single bacterial strain were located in spatially distinct areas of abscess (Figure 9). This finding indicates that at least in this preclinical model of ascending pyelonephritis, the KBC arises from growth and expansion of a founder bacterium that has ascended the nephron to establish a given intratubular focus of infection.

**Discussion**

We conclude that the more robust kidney infection observed in males reflects a greater number of successful founder bacteria (i.e., tagged strains) establishing renal colonization in the male host, despite initial inoculation that is equivalent between sexes. The kidney thus represents a niche, distinct from the bladder tissue, where successful clones can establish themselves and
replicate. These kidney-occupying UPEC clones, then, continuously descend from the kidney and contribute to ongoing pathogenesis in the bladder (and, if VUR is present, can likely exchange clones with the contralateral kidney). The harboring of a more genetically diverse infection in the kidneys in males – “upstream” of the bladder – thereby overcomes the population bottleneck that would otherwise be evident during initial colonization of the bladder. Meanwhile, the modest narrowing of genetic diversity in the male kidney between 3 and 7 dpi likely reflects a transition from bulk retrograde flow (into the renal pelvis with VUR) to molecular interactions that drive colonization of the nephron (35), potentially coupled with the arrival of phagocytes. In other words, arrival in the kidney in the setting of VUR does not impose a strict bottleneck on the bacterial population, while ascension into the nephron is a comparatively uncommon event accomplished by individual bacteria that express the requisite traits (e.g., for motility or attachment). This model predicts that KBCs are clonal in nature – a conclusion supported by our RNA ISH data. Of note, while IBCs in the bladder are clonal due to the process of UPEC internalization into facet cells, we have not observed an intracellular stage associated with KBC formation in our model (1, 35).

This work furthers our understanding of UPEC population dynamics during UTIs, demonstrating that the susceptible kidney offers a distinct niche for success of bacterial clones and can overcome the bottleneck in genetic diversity that is imposed during cystitis. Future studies should aim to identify additional molecular determinants of host-pathogen interaction that permit establishment and persistence of upper-tract UTI. Specifically, the lack of sharp genetic restriction in the kidney may enable unbiased approaches (e.g., with transposon libraries) to discover such kidney-specific UPEC virulence factors.
Conflicts of Interest

D.A.H. serves on the Board of Directors for BioVersys AG, Basel, Switzerland. All other authors have no conflicts to declare.

Acknowledgements

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Figures

Figure 1. Male C3H/HeN mice develop high-titer cystitis and pyelonephritis. Male C3H/HeN mice were infected with UTI89, and organs were harvested at the indicated intervals. (A) Bladder bacterial loads remained constant across the time points studied. (B) Kidney bacterial loads were statistically highest 3 dpi. Horizontal bars indicate geometric mean, and dotted line indicates limit of detection. *p<0.05, ***p<0.001.
Figure 2. Vesicoureteral reflux (VUR) is equivalent among male and female mice.

C3H/HeN mice were infected with UTI89 by direct surgical inoculation of the bladder. There was no difference noted in bladder titers (A) or kidney titers (B) between males and females. Horizontal bars indicate geometric mean, and dotted line indicates limit of detection. Male data shown here are the same as those 5 mpi in Figure 1.
Figure 3. Male C3H/HeN bacterial loads with UTI89-derived set of “barcoded” isolates.

Male C3H mice were infected with 40 PCR-tagged (“barcoded”) isogenic UPEC isolates. Bladder (A) and kidney (B) titers were enumerated at the indicated time points. Horizontal bars indicate geometric mean, and dotted line indicates limit of detection. *p<0.05, **p<0.01, ***p<0.001.
Figure 4. Male mice maintain a wide diversity of isolates in the bladder and kidneys. We infected C3H/HeN male mice with 40 PCR-tagged (“barcoded”) isogenic UPEC isolates. Bacterial genomic DNA was extracted from bacterial outgrowths of organ homogenates. Tags remaining in the bladder (A) and kidneys (B) were detected by multiplex PCR. *p<0.05, **p<0.01.
Figure 5. Male and female C3H/HeN bacterial loads with UTI89-derived set of “barcoded” isolates. Male (black circles) and female (blue triangles) C3H mice were infected with 40 PCR-tagged (“barcoded”) isogenic UPEC isolates. Bladder (A), left kidney (B), and right kidney (C) titers were enumerated at the indicated time points. Horizontal bars indicate geometric mean, and dotted line indicates limit of detection. Male data displayed here are the same as in Figure 3, and male statistical comparisons were omitted here for clarity. *p<0.05, **p<0.01, ***p<0.001.
Figure 6. In contrast to males, female C3H mice display a marked population bottleneck in the bladder and kidneys. We infected C3H/HeN male and female mice with 40 PCR-tagged ("barcoded") isogenic UPEC isolates. Bacterial genomic DNA was extracted from bacterial outgrowths of organ homogenates. Tags remaining in the bladder (A), left kidney (B), and right kidney (C) of males (black circles) and females (blue triangles) were detected by multiplex PCR. Male data displayed here are the same as in Figure 4; statistical comparisons among male groups are omitted here for clarity. *p<0.05, ***p<0.001.
Figure 7. Niche distribution of UPEC in infected male C3H/HeN mice. Using the multiplex PCR data gathered from bladders and kidneys, we quantified the sharing of tags across niches at 5 mpi (A; n=4), 1 dpi (B; n=5), 7 dpi (C; n=9), and 14 dpi (D; n=10). Venn diagrams represent the average proportion of tags detected in the indicated niches. Niche colors: yellow, bladder; hot pink, left kidney; blue, right kidney. Overlap colors: orange, bladder and left kidney; green, bladder and right kidney; purple, left and right kidneys; rose, all three niches. Labels indicate measured proportions in each niche, while colored regions approximate these proportions (smallest regions enlarged for visual clarity).
Figure 8. Niche distribution of UPEC in infected female C3H/HeN mice. Using the multiplex PCR data gathered from bladders and kidneys, we quantified the sharing of tags across niches at 5 mpi (A; n=4), 7 dpi (B; n=5), and 14 dpi (C; n=4). Venn diagrams represent the average proportion of tags detected in the various niches. Niche colors: yellow, bladder; hot pink, left kidney; blue, right kidney. Overlap colors: orange, bladder and left kidney; green, bladder and right kidney; purple, left and right kidneys; rose, all three niches. Labels indicate measured proportions in each niche, while colored regions approximate these proportions (smallest regions enlarged for visual clarity).
**Figure 9. KBCs arise from clonal expansion of individual bacteria.** (A) Experimental scheme for inoculation of C3H/HeN male mice with UTI89 HK::Kan$^R$, UTI89 HK::Chl$^R$, or an equal mixture of both, followed by KBC examination 2 wpi via RNA ISH and confocal microscopy. As controls, mice infected only with UTI89 HK::Kan$^R$ displayed staining only with the Kan$^R$ probe (B, green), and mice infected only with Chl$^R$ displayed staining only with the Chl$^R$ probe (C, red), although both probes were applied to slides. (D-E) After mixed infection, KBCs displayed staining for either Kan$^R$ or Chl$^R$; no mixed KBCs were seen. Representative images selected from 87 analyzed KBCs across 4 independent experiments; scale bars, 10 µm. (F) Digitally tiled scans of entire slides after mixed infection revealed Kan$^R$ and Chl$^R$ KBCs within spatially separate areas of abscess in the same kidney; representative image shown. Colored arrowheads indicate respectively stained KBCs; scale bar, 200 µm.
## Tables

**Table 1.** BP-xxF primer sets and sequences for detection of “barcoded” UTI89 strains

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Chapter 4.  A Host Receptor Enables Type 1 Pilus-Mediated Pathogenesis of \textit{Escherichia coli} Pyelonephritis


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Abstract

Type 1 pili have long been considered the major virulence factor enabling colonization of the urinary bladder by uropathogenic *Escherichia coli* (UPEC). The molecular pathogenesis of pyelonephritis is less well characterized, due to previous limitations in preclinical modeling of kidney infection. Here, we demonstrate in a recently developed mouse model that beyond bladder infection, type 1 pili also are critical for establishment of ascending pyelonephritis. Bacterial mutants lacking the type 1 pilus adhesin (FimH) were unable to establish kidney infection in male C3H/HeN mice. We developed an *in vitro* model of FimH-dependent UPEC binding to renal collecting duct cells, and performed a CRISPR screen in these cells identifying desmoglein-2 as a primary renal epithelial receptor for FimH. The mannosylated extracellular domain of human DSG2 bound directly to the lectin domain of FimH *in vitro*, and introduction of a mutation in the FimH mannose-binding pocket abolished binding to DSG2. In infected C3H/HeN mice, type 1-piliated UPEC and Dsg2 were co-localized within collecting ducts, and administration of mannoside FIM1033, a potent small-molecule inhibitor of FimH, significantly attenuated bacterial loads in pyelonephritis. Our results broaden the biological importance of FimH, specify the first renal FimH receptor, and indicate that FimH-targeted therapeutics will also have application in pyelonephritis.

Introduction

Bacterial adhesion to urinary tract epithelium is a critical step in establishing urinary tract infection (UTI). Strains of uropathogenic *Escherichia coli* (UPEC), the main causative agents of UTIs, carry in their genomes a variable repertoire of adhesive pili assembled via the canonical chaperone-usher pathway (CUP) (1, 2). Given their importance in host-pathogen interactions,
UPEC adhesins including CUP pili have received considerable attention as mediators of pathogenesis and potential therapeutic targets (2-5). Within the mammalian bladder, UPEC employ type 1 pili tipped with the FimH adhesin, which binds α-D-mannose with stereochemical specificity. FimH-mediated UPEC binding to mannosylated uroplakins that coat the luminal surfaces of superficial uroepithelial (facet) cells is a key event in the pathogenesis of cystitis (6-11). UPEC can subsequently be internalized into facet cells, where they replicate and establish intracellular bacterial communities (IBCs) (12-16). Bacteria within IBCs are protected from phagocytosis and other host defenses, and this community behavior within the bladder is a critical component of UPEC cystitis (17, 18).

Less is known about the host-pathogen interactions enabling establishment of upper-tract UTIs, including pyelonephritis and renal abscess. Another CUP pilus, the P pilus, is tipped with the PapG adhesin, which has been implicated in human pyelonephritis (2, 19-21). However, its species-specific glycolipid receptor is absent in many animal models, including mice (22). Preclinical modeling of UTIs has been performed predominantly in female mice, and in a majority of murine backgrounds, upper-tract UTI resolves spontaneously and without significant sequelae (23, 24). Historically, female mice have been utilized as an experimental UTI model, which is typically initiated by catheter-directed inoculation of the mouse bladder, and reliable catheter access to the male mouse bladder is technically challenging (25-28). We previously developed an inoculation technique for initiating UTI in both male and female mice; when normal anatomic protections in males were thus bypassed, males evidenced more severe UTI, reflected throughout the course of infection by higher bladder and kidney bacterial loads, leukocytic infiltration, and inflammation (29). Of note, these findings aligned with human epidemiologic data showing higher morbidity from complicated UTI in men, and higher UTI
incidence in women with polycystic ovary syndrome (a common hyperandrogenic state) (30-36). In C3H/HeN mice (a background inherently featuring vesicoureteral reflux, a primary risk factor for upper-tract UTI in humans), males and androgen-exposed females developed severe pyelonephritis and renal abscesses (29, 37). Renal infection was nucleated by collections of UPEC occupying collecting ducts and more proximal segments of the nephron, which we termed kidney bacterial communities (KBCs) (37).

The consistent development of severe pyelonephritis in this model enables detailed investigation of bacterial and host factors involved in the establishment of ascending upper-tract UTI. We identified a previously unappreciated role in the kidney for the mannose-binding adhesin FimH, which tips type 1 pili and is known to represent a major urovirulence factor within the bladder. High-affinity mannosides, which neutralize FimH function and are in development for the treatment of cystitis (4, 5, 38, 39), here significantly lowered bacterial burdens in the kidneys of mice with established pyelonephritis. To specify host factors required for upper-tract UTI, we performed a CRISPR/Cas9 screen in immortalized murine renal epithelial cells and identified desmoglein-2 (Dsg2) as a candidate receptor for FimH. The lectin domain of FimH (but not FimH with a point mutation that abolishes mannose binding) directly bound to the extracellular domain of human desmoglein-2 (DSG2). Finally, we observed co-localization of UPEC with Dsg2 in the collecting ducts of mice with ascending pyelonephritis. Our studies demonstrate that disruption of FimH binding to renal tubular epithelium represents a potential therapeutic intervention during pyelonephritis.
Materials and Methods

Bacterial strains and growth

For mouse infections and for binding studies with IMCD-3 cells, bacteria were grown statically at 37 °C for 18 h in Luria-Bertani (LB) broth. For the CRISPR screen, type 1 pili were induced by static growth at 37 °C for 18 h, then 1:100 subculture and static growth for an additional 18 h. Bacterial strains were all previously published, with mutations generated using the λ Red Recombinase system (40) (see Table 1). Bacteria were pelleted at 7,500 × g at 4 °C for 10 min, then resuspended to an OD₆₀₀ of 1.0 (~4 × 10⁸ colony-forming units [CFU]/mL) in sterile phosphate-buffered saline (PBS) for mouse infections, or in DMEM F-12 (Invitrogen 1132-0033) supplemented with 10% fetal bovine serum (FBS; VWR 97068-085) for tissue culture experiments.

Mouse infections

All animal protocols complied with relevant ethical regulations and received prior approval from the Institutional Animal Care and Use Committee at Washington University (approval number 20180159). Male C3H/HeN mice (Envigo) aged 8-9 weeks were infected as previously described (29). Briefly, mice were anesthetized with inhaled 3% isoflurane, and the lower abdomen was shaved and sterilized with 2% chlorhexidine solution. A 3-mm midline abdominal incision was made through the skin and peritoneum, exposing the bladder. The bladder was aseptically emptied before 50 µl of inoculum (1-2 × 10⁷ CFU in PBS) was injected into the bladder lumen via 30-gauge needle over 10 s. The bladder was allowed to expand for an additional 10 s before the needle was removed. The peritoneum and skin incisions were closed with simple, interrupted sutures. At the time of surgery, mice were given sustained-release
buprenorphine (1 mg/kg SQ) for analgesia. At the indicated time points, mice were euthanized by CO₂ asphyxiation. Bladders and kidney pairs were steriley removed and homogenized in 1 mL or 0.8 mL PBS, respectively. Homogenates were serially diluted and plated on LB agar for CFU enumeration.

**Immunofluorescence microscopy**

Infected bladders and kidneys were bisected and fixed in 10% neutral buffered formalin for 24 h. Fixed tissues were embedded in paraffin, sectioned, and stained. Unstained slides were deparaffinized in xylenes, rehydrated in isopropanol, boiled in 10 mM sodium citrate, and blocked in 1% bovine serum albumin (BSA), 0.3% Triton-X 100 in PBS for 1-2 h at room temperature (RT). Tissue culture cells were fixed in methanol at -20 °C for 10 min and then blocked in 3% BSA for 1 h. Slides were incubated with primary antibodies for 4 h or overnight at RT and then (after washing in PBS) with secondary antibodies for 1 h at RT. Slides were then mounted with ProLong Gold antifade reagent (Invitrogen) and images acquired on an Olympus FV1200 confocal microscope. Primary antibodies utilized were: rabbit anti-type 1 pili (1:500 dilution), rabbit anti-*E. coli* (1:1000 dilution, E3500-06C; US Biological), goat anti-*E. coli* (1:100-1:500 dilution, B65109G; Meridian Life Science), mouse anti-AQP2 conjugated with AlexaFluor 488 or 647 (1:50-1:200 dilution, sc-515770; Santa Cruz Biotechnology), mouse anti-desmoglein 2 (1:100 dilution, 6D8, MCA2272T, BioRad), rabbit anti-desmoglein 2 (1:100, A303-758A, Bethyl Labs). Secondary antibodies utilized were AlexaFluor 488-conjugated goat anti-rabbit IgG (1:500 dilution, A11008; Life Technologies), AlexaFluor 647-conjugated chicken anti-rabbit IgG (1:200, A-21443; ThermoFisher), AlexaFluor 594-conjugated donkey anti-goat IgG (1:200, ab150132; abcam), Cy5-conjugated donkey anti-mouse IgG (1:250, 715-175-151;
JacksonImmuno), AlexaFluor 488-conjugated goat anti-rabbit IgG (1:1000, 111-095-144; Jackson Immuno). Nuclear staining was with DAPI (1:10,000) or SYTO 61 (1:1000, Molecular Probes).

**Tissue culture**

Murine intramedullary collecting duct cells (IMCD-3; ATCC CRL-2123) were cultured in DMEM F-12 supplemented with 10% FBS. Medium included 0.1 mg/mL penicillin-streptomycin (Gibco 15140-122) until the day before an experiment, when medium was changed to the above without antibiotics. Cells were maintained at 37 °C in a humidified atmosphere with 5% CO₂.

**In vitro binding assays**

The day before infection, IMCD-3 cells were seeded in 24-well plates. Medium was removed and cells were washed with PBS supplemented with Mg²⁺ and Ca²⁺ (PBS-MgCa; Sigma-Aldrich D8662). After inoculation with UPEC at a multiplicity of infection (MOI) of 20, plates were centrifuged at 400 × g for 3 min, then returned to the incubator. To enumerate bound CFU, after 45 min, wells were washed 5 times with PBS-MgCa, then lysed with 0.1% Triton X-100 (Sigma T9284). To enumerate internalized CFU, after 60 min, cells were washed with PBS-MgCa, treated with medium containing 50 μg/mL gentamicin (Thermo Fisher 15750060), and incubated at 37 °C for 90 min, then lysed with 0.1% Triton X-100. Lysates were serially diluted and plated to LB agar. Binding efficiencies were calculated in comparison to input wells, in which cells were inoculated with UPEC, incubated for 45 min, then lysed by addition of Triton X-100, and the well contents plated to LB agar for enumeration of input CFU.
Flow cytometry

The day before infection, IMCD-3 cells were seeded in 6-well plates. Cells were infected with UPEC (at MOI 20-150) for 45 min, then washed as described above; cells were liberated with 0.05% trypsin-0.02% EDTA (Gibco), pelleted, and fixed in 4% paraformaldehyde (Electron Microscopy Sciences) for 30-60 min at RT. Cells were then blocked in 3% BSA for 1 h at RT. Cells were stained with primary rabbit anti-\textit{E. coli} antibody (E3500-06C; US Biological) or isotype control (Rabbit serum; Sigma-Aldrich R9133) and secondary AlexaFluor 488-conjugated donkey anti-rabbit IgG (Invitrogen A21206) for 1 h each. Samples were stored in 3% BSA until analyzed on a Becton Dickinson (BD) LSR II Fortessa flow cytometer. Gating strategy is shown in Figure 1.

Generation of virus and viral constructs

Viruses were generated by transfecting 293T cells with lentiviral cDNA (VSV-G, pMD2.G, Addgene #12259; psPAX2, Addgene #12260; and construct of interest) using Opti-MEM (Thermo Fisher 31985062) and TransIT LT1 transfection reagent (Mirus MIR2300). Virus was harvested 48 h post transfection.

Library preparation and CRISPR screen

IMCD-3 cells were transduced with lentivirus containing Cas9 (pXPR_101, Addgene #52962) by spinoculation (1 mL of Cas9 lentivirus onto $3 \times 10^6$ cells), then selected with 5 µg/mL blasticidin (ThermoFisher A1113903). Cas9 activity was assayed by transducing cells with lentivirus containing pXPR_011 (Addgene #59702), which expresses eGFP and a sgRNA targeting eGFP, and selecting with 5 µg/mL puromycin (ThermoFisher A1113803). Loss of GFP
expression in cells expressing the eGFP guide was assessed by flow cytometry as described above. After Cas9 activity was confirmed, a Brie mouse sgRNA library (Brie pXPR_003, Batch 3, Lot #m-AA89-20171107; generated by the Broad Institute) was transduced at MOI 0.5 into IMCD-3 Cas9 cells by spinoculation and selected with 5 µg/mL puromycin for 10 d until screening.

For screening, 5 × 10^6 IMCD-3 cells were seeded into eighty 15-cm dishes the day before infection. Cells were infected with UTI89 at MOI 150 and incubated at 37 °C for 45-75 min, then fixed and stained for flow cytometry as described above. Samples were placed at 4 °C overnight on a tube roller; the following day, the 5% least FITC-positive cells were collected on a Sony iCyt Synergy BSC sorter. Tubes containing these low-FITC sorted cells were centrifuged, resuspended in 250 µl PBS, and stored at -20 °C. A total of 6 × 10^8 mock-treated cells were harvested, separated into aliquots of 8 × 10^7 cells, resuspended in 2 mL PBS, and stored at -20 °C until genomic DNA preparation. DNA was extracted using QIAamp DNA Blood Maxi kit (Qiagen 51192) for mock samples and QIAamp DNA FFPE Tissue kit (Qiagen 56404) for experimental samples.

**Sequencing and bioinformatics**

Illumina sequencing was performed as described previously (41). Briefly, genomic DNA was aliquoted into multiple wells of a 96-well plate (up to 10 µg of DNA in 50 µL total volume). Samples were sequenced on an Illumina HiSeq 2000. Barcodes in the P7 primer were deconvoluted, and the sgRNA sequence was mapped to a reference file of sgRNAs in the Brie library. To normalize for different numbers of reads per condition, read counts per sgRNA were normalized to 10^7 total reads per sample; this normalized value was then log2 transformed. We
used the hypergeometric distribution method to rank sgRNAs and calculate gene p-values using the probability mass function of a hypergeometric distribution (https://portals.broadinstitute.org/gpp/public/analysis-tools/crispr-gene-scoring-help). We considered candidate genes those having an average log fold change $>0.5$ and a false discovery rate $>2.5$ (41, 42). For input of the hypergeometric distribution ranking, we subtracted the log$_2$ normalized read values of the uninfected unsorted IMCD-3 Brie library from the log$_2$ normalized read values of the 5% lowest UPEC-bound sorted cells. We used R Studio to visualize the results of the hypergeometric distribution analysis.

**Generation of Dsg2 knock-down clones (C4 and F11)**

Dsg2 knock-down clones C4 and F11 were among hundreds generated by the Genome Engineering and iPSC Center at Washington University School of Medicine. IMCD-3 cells were nucleofected with Cas9 and a Dsg2-specific sgRNA (5’ GGAACTACGCATCAAAGTTCTGG 3’). Single-cell clones were isolated by FACS and expanded in 96-well plates. Cells were harvested, and genomic DNA was amplified and subjected to targeted deep sequencing of a ~400-bp amplicon flanking the gRNA target. Clones were screened for frameshifts by sequencing the target region with Illumina MiSeq at ~1500× coverage. We obtained no clones that completely lacked Dsg2 expression. Frameshift mutations identified in knock-down clones C4 and F11 are detailed in Table 2.
**Quantitative PCR**

RNA was isolated from tissue culture cells using the Qiagen RNeasy kit. qPCR was performed using the Applied Biosystems TaqMan RNA-to-Ct 1-Step Kit (ThermoFisher 4392938) and the probes listed in Table 3 (Integrated DNA Technologies).

**Immunoblot**

Samples were separated by SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore) using overnight wet transfer (48mM Tris pH 9.2, 38mM glycine, 20% methanol, 0.05% SDS). After blocking for 6-8 h with 5% milk in PBS-T, blots were probed with rabbit anti-desmoglein-2 (1:250 dilution, A303-758A Bethyl Labs) and mouse anti-β-actin (1:100,000 dilution, 3700; Cell Signaling) in PBS-T with 5% milk overnight. Blots were then secondarily probed with HRP-conjugated sheep anti-mouse IgG (1:1000 dilution, NA931V; GE) and HRP-conjugated donkey anti-rabbit IgG (1:1000 dilution, NA934V; GE) for 1 h. Gels were developed using Clarity and Clarity Max Western ECL substrates (1705060 and 1705062; BioRad). Western blot images were analyzed with ImageJ.

**Protein production and biolayer interferometry (BLI)**

Methods for purification of full-length FimH and the lectin domain (FimHLD; amino acids 1-160) have been described previously (10). Briefly, untagged FimHLD was purified from *E. coli* periplasmic extracts using ion-exchange and size-exclusion chromatography, dialyzed into sterile PBS, and stored at 4 °C until use.

Expression constructs for 6×His-tagged human DSG2 EC1-5 (amino acids A1-A553) or DSG2 EC1-3 (amino acids A1-N332) (kind gifts of O. Harrison and L. Shapiro (43)) were
transfected (1 µg/mL of culture) into Expi293F cells (ThermoFisher) using Opti-MEM. Six days post transfection, the supernatant containing the 6×His-tagged DSG2 ectodomains was collected, dialyzed into 20mM Tris, 150mM NaCl pH 8 overnight at 4 °C, batch purified by nickel-nitrilotriacetic acid (Ni-NTA) affinity chromatography, and then further purified by HiLoad 16/600 Superdex 200 size-exclusion chromatography (GE Healthcare). Protein was concentrated and was exchanged into a final buffer of sterile PBS, then stored at 4 °C until use in binding experiments. DSG2 EC1-5 was also expressed in Expi293F GnTI- cells (which generate proteins lacking complex N-linked glycans but decorated only with high mannose) and purified as described above.

All BLI experiments were performed in 10 mM HEPES (pH 7.4), 150 mM NaCl, 1 mM CaCl2, 3 mM EDTA, and 0.005% P20 surfactant with 1% BSA at 25 °C using an Octet-Red96 device (Pall ForteBio). DSG2 variants were biotinylated using EZ-Link NHS-PEG4-Biotin (Thermo Fisher) per manufacturer’s instructions. Biotinylated DSG2 was loaded onto streptavidin biosensors (ForteBio), then incubated with different concentrations of FimHLD or FimHQ133K (10 µM to 156 nM) for 15 min followed by a 20 min dissociation. Real-time data were analyzed using BIAevaluation 3.1 (GE Healthcare), and kinetic curves were fitted using a global 1:1 binding algorithm with drifting baseline.

**Mannoside treatment**

Male C3H/HeN mice were infected as described above. Beginning 5 dpi, mice were given mannoside FIM1033 (gift of Fimbrion Therapeutics), 8 mg/kg in sterile PBS with 4% DMSO, by intraperitoneal injection every 8 h for 24 or 48 h. Mock-treated mice were injected with 4% DMSO in sterile PBS. Mice were sacrificed 6 h post last treatment dose.
Statistical analysis

Statistical analysis was performed using Prism 8 (GraphPad Software). Differences were analyzed with the unpaired, two-tailed, nonparametric Mann-Whitney U test. P values <0.05 were deemed significant.

Results

Type 1 pili are required for both bladder and kidney infection in male C3H/HeN mice.

Type 1 pili have long been implicated in pathogenesis of UPEC cystitis, but only recently have new experimental models enabled interrogation of their role in pyelonephritis. We infected male C3H/HeN mice with either wild-type UTI89 (a prototypic UPEC strain), or UTI89ΔfimH, an isogenic mutant lacking the type 1 pilus adhesin (16, 44). Two weeks post infection (wpi), UTI89ΔfimH was sharply attenuated compared to wild-type in bladder and kidney bacterial loads (p<0.0001; Figure 2A) and in incidence of visible renal abscess (1/16 vs 13/13; p<0.0001). We confirmed that UTI89ΔfimH reached the kidney normally following bladder inoculation, as kidney bacterial loads 24 hours post infection (hpi) were equivalent to wild-type (Figure 3). An analogous defect was confirmed using the urosepsis UPEC isolate CFT073, whose isogenic CFT073ΔfimH mutant failed to colonize the kidney 2 wpi (Figure 4). Using immunofluorescence microscopy, we demonstrated type 1 pili expression by UPEC in KBCs located within abscesses 2 wpi (Figure 2B). As UTI89 carries ten full or partial CUP operons (45), we similarly tested deletion mutants in each of the annotated CUP pili. No other CUP pilus mutants exhibited a defect in bladder or kidney infection in male C3H mice 2 wpi, after inoculation either alone or in competition with wild-type UTI89 (Figure 5 and data not shown).

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The pilin and lectin domains of FimH together exist in an equilibrium between tense and relaxed states with differential mannose binding affinity. Conversion between these states is governed in part by a catch-bond mechanism (9, 46-48). In the relaxed state, the FimH lectin domain samples conformational ensembles, allowing it to act as a molecular tether, with the mannose binding pocket in a conformation able to bind mannose tightly; in the tense state, the mannose pocket is open and thus binds mannose only weakly (8). In the bladder, the ability of FimH to transition between these states allows optimal epithelial binding by UPEC (8, 49, 50). We infected male C3H/HeN mice with wild-type UTI89 or with UTI89ΔfimH complemented with FimHA27V/V163A, a variant that predominantly adopts the relaxed conformation (high mannose-binding state). Paradoxically, UPEC expressing this variant are attenuated during cystitis in female mice (8, 49, 50). Here, we found similarly that UTI89 FimHA27V/V163A also failed to establish pyelonephritis, with significantly lower bacterial loads in kidneys (p=0.001) as well as bladder (p<0.0001) compared to wild-type UTI89 (Figure 6A). Further, the type 1 pilus rod, formed from FimA subunits, normally can unwind from a tightly coiled helix to a more linearized rod; this action is hypothesized to help UPEC withstand the shear force of urine flow (51-53). A variant in the type 1 pilus helical rod, FimAA22R, requires less force to adopt the unwound form, but UPEC expressing this variant are attenuated in the female mouse bladder (54). We found that UTI89 expressing FimAA22R was also attenuated in the kidneys (p=0.0038) and bladders (p<0.0001) of male C3H/HeN mice 2 wpi (Figure 6B). Thus, the conformational dynamics of type 1 pili, critical in establishing bladder infection, are similarly important in ascending pyelonephritis. Collectively, our data demonstrate that beyond their well-described role in establishing bladder infection, UPEC type 1 pili are also important in initiating ascending pyelonephritis and renal abscess.
Murine collecting duct cells display type 1 pili-dependent UPEC binding. The collecting duct is the first nephron segment encountered by ascending UPEC. Consistent with prior findings (55, 56), ascending UPEC were located within the collecting ducts in C3H/HeN mice 5 dpi (Figure 7A). Therefore, we chose mouse intramedullary collecting duct (IMCD-3) cells to establish an in vitro model of type 1 pili binding to kidney epithelium. In standard bacterial binding assays, UTI89 ΔfimH was significantly attenuated compared to wild-type UTI89 (Figure 7B), recapitulating what we observed in in vivo infection. Following IMCD-3 cell infection and anti-E. coli antibody staining, we confirmed by flow cytometry the in vitro binding defect of ΔfimH (Figure 7C,D). Furthermore, UPEC binding to collecting duct cells was significantly inhibited by methyl α-D-mannopyranoside and to an even greater extent by mannoside FIM1033 (Figure 8). Thus, the in vivo requirement for type 1 pilus function is reflected in in vitro binding of UPEC to IMCD-3 cells.

A CRISPR screen identifies candidate type 1 pilus receptor desmoglein-2. Although type 1 pili are well known as critical virulence factors in the bladder (6-11), they have not been implicated in ascending pyelonephritis, and a cognate receptor has not been identified. To screen for host genes participating in type 1 pili-dependent binding of UPEC to collecting duct epithelium, we performed a genome-wide CRISPR-Cas9 screen using the Brie sgRNA library in IMCD-3 cells (Figure 9A), with 4× nominal coverage of each gene within the mouse genome (41). The pooled library of edited cells were inoculated with UPEC, fixed and stained, and subsequently flow-sorted for the population of cells with the lowest fluorescence (Figure 9A). Genomic DNA was extracted from sorted cells, subjected to Illumina sequencing, and analyzed using the probability mass function of a hypergeometric distribution to identify candidate genes
statistically associated with bacterial binding (Table 4) (41, 42). Among these candidates (Figure 9B), we focused on genes encoding proteins that would be localized to the cell surface and could be available to interact with bacterial pili (57, 58). The screen revealed deficient UPEC binding to cells edited with guides targeting desmoglein-2 (Dsg2; Figure 9B), which encodes a mannosylated cell junctional protein displayed on kidney tubular epithelia throughout the nephron (43, 57, 59). By fluorescence microscopy of IMCD-3 cells inoculated with UPEC, we observed co-localization of UPEC with Dsg2 (Figure 9C).

**FimH binds DSG2 in vitro.** Desmoglein-2 is a member of the cadherin superfamily and in humans and mice comprises five extracellular cadherin (EC) domains, a single transmembrane segment, and an intracellular domain (Figure 10A). To determine whether desmoglein-2 binds directly to FimH, we generated two forms of recombinant 6×His-tagged human DSG2 (EC1-5, amino acids A1-A553) (43) in wild-type Expi293F cells, as well as from Expi293F GnTI- cells (which lack complex N-glycans and instead exhibit uniform GlcNAc2Man5 glycosylation). We also purified the lectin domain of FimH (FimHLD, amino acids 1-160) from *E. coli* (Figure 11). By biolayer interferometry (BLI), FimHLD bound to DSG2 EC1-5 from wild-type cells with an apparent binding affinity of 1.6 µM (Figure 10B and Figure 12). In comparison, DSG2 EC1-5 purified from GnTI- cells exhibited higher binding to FimHLD (K_D,apparent = 484 nM, p=0.008; Figure 10B), consistent with preferential binding of FimHLD to highly mannosylated structures. In contrast, the FimH^{Q133K} mutant, which lacks mannose binding activity (10), did not bind to either variant of DSG2 EC1-5 (p = 0.036; Figure 10B). Finally, FimHLD bound only minimally to DSG2 EC1-3 (amino acids A1-N332; p = 0.015 vs EC1-5; Figure 10B). Taken together, these
data show that FimH binds the extracellular portion of DSG2 in a mannose-dependent manner, and that DSG2 domains EC4 and/or EC5 mediate FimH binding.

To validate Dsg2 as a UPEC receptor, we edited IMCD-3 cells with CRISPR-Cas9 to achieve specific deletion of Dsg2. Though hundreds of edited clones were screened, no clones entirely lacking Dsg2 expression were obtained, suggesting that complete Dsg2 deletion may be physiologically detrimental. Of note, germline Dsg2 deletion in mice is lethal (60). We did identify two gene-edited clones (namely C4 and F11) that exhibited sharply reduced Dsg2 transcript expression (Figure 13A); by quantitative immunoblot of cell lysates, these knockdown clones expressed significantly less Dsg2 protein than wild-type IMCD-3 cells (Figure 13B; median C4/WT ratio 4.3%, p=0.0022; median F11/WT ratio 4.7%, p=0.0087). By flow cytometry, median binding of C4 and F11 cells by UPEC was decreased by 33% and 46% compared with WT IMCD-3 cells (p=0.0235 and 0.0032, respectively; Figure 13C,D).

**Targeting the Dsg2-FimH interaction during in vivo infection.** In the kidneys of UPEC-infected C3H/HeN mice, we identified UPEC within Dsg2+, aquaporin-2+ collecting ducts 5 dpi (Figure 14A). Across multiple sections from several infected mice, of 239 intratubular UPEC colonies visualized 5 dpi, 228 (95%) resided in Dsg2+ tubules.

Finally, we hypothesized that if the Dsg2-FimH interaction was important for pyelonephritis, mannosides could be employed therapeutically in our mouse model. Male C3H/HeN mice were inoculated with UTI89, and early KBCs were allowed to form over 5 days (37, 61); mice were then treated with mannoside FIM1033 (formerly termed 29R(5)) (Figure 14B). Compared with mock-treated mice, bladder and kidney bacterial loads were significantly reduced by mannoside treatment for 24 h (kidney p=0.0434, bladder p=0.0255; Figure 14C) and
even more so with treatment for 48 h (kidney \( p=0.0042 \), bladder \( p=0.0003 \); **Figure 14D**). These *in vivo* data support a model in which desmoglein-2 on collecting duct epithelium serves as a receptor for UPEC FimH during pyelonephritis *in vivo*.

**Discussion**

In this study, we reveal the importance of type 1 pili in host-pathogen interaction during ascending pyelonephritis and identify desmoglein-2 as the first candidate receptor for FimH on renal tubular epithelium. Our findings in male C3H mice confirm that type 1 pili are essential for cystitis (as in female mice (6-11)), but more importantly provide both *in vitro* and *in vivo* evidence of the function of FimH in bacterial infection of the kidney. Furthermore, our ability to mitigate kidney infection with mannosides suggests that type 1 pilus-directed therapeutics currently under development for recurrent cystitis (2, 4, 5, 38, 39) may also be useful in pyelonephritis.

Compared to bacterial cystitis, significantly less is known about the pathogenesis of UPEC pyelonephritis, primarily due to the fact that female mice of most backgrounds resolve kidney infection spontaneously (23, 24). Work in humans has identified genetic factors that confer susceptibility to pyelonephritis and renal scarring, including polymorphisms reducing *IRF3* or *CXCR1* (encoding IL-8 receptor) expression, in certain UTI-prone kindreds (62-65). Previously, P pili have been considered the major adhesin in kidney infection (2, 19, 21). This work has been complicated by allelic variation in the P pilus adhesin PapG correlated with differing affinity for various glycolipid receptors that are differentially expressed in the kidneys of humans and model animals, including mice (2, 66). While P pili are enriched in the genomes of pyelonephritis-associated UPEC isolates for children with acute pyelonephritis, a significant
proportion of UPEC isolates from women with acute and recurrent UTI lack P pili, suggesting a role for other adhesin(s) during ascending infection (21, 67-69).

Recent works have employed new mouse models (including C3H/HeOuJ mice, male C3H/HeN mice, and androgenized female mice) which now enable detailed study of the pathogenesis of kidney infection and abscess formation (29, 37, 55). In our model of ascending UTI, type 1 pili were shown to be essential for maintenance of renal infection, through both genetic mutation of the pilus and pharmacological inhibition with mannosesides. Other work has hinted at a role for type 1 pili and mannose targets in kidney infection (70-73). For instance, signaling through C5a receptor 1 (C5aR1), which regulates inflammatory cell recruitment in UPEC infection, may also enhance presentation of mannosylated glycoproteins by primary renal tubular epithelial cells (71, 72). Meanwhile, experiments in which tubular infection was initiated via microinjection of UPEC directly into rat nephrons posited a role for type 1 pili in interbacterial interactions and biofilm formation (70). While our work indicates that FimH mediates renal epithelial binding (in agreement with prior staining of kidney sections using purified adhesins (21, 74)), the present work does not exclude a role for type 1 pili in interbacterial interactions and/or biofilm formation within the kidney. Inhibition of any of these interactions by administration of mannosesides might exert therapeutic benefit in pyelonephritis.

Using a CRISPR-Cas9 screen, we identified desmoglein-2 as a receptor for UPEC during ascending infection. DSG2 is a member of the cadherin family of Ca^{2+}-binding proteins, involved in intercellular junctions via the desmosome (75, 76). Its identified roles in mammalian disease states are limited; of note, cardiomyocyte-specific conditional knockout of Dsg2 in mice phenocopies human arrhythmogenic cardiomyopathy, which is correlated with loss-of-function DSG2 mutations (77, 78). Interestingly, other members of the desmoglein family also act as
receptors for unrelated microbes (79). Dsg2 has been specifically implicated as a receptor for adenovirus serotypes 3, 7, 11, and 14 (80, 81); of note, serotype 11 is most commonly associated with hemorrhagic cystitis in renal and other transplant recipients (82). Along the length of the nephron, DSG2 expression is highest within the collecting duct and decreases as one ascends to more proximal segments of the tubule (59). As a family, desmogleins (and other cadherins) exhibit a unique form of mannosylation in which α-D-mannose (the binding target of type 1 pili) is present as a novel O-linked glycosylation modification (43). Prior studies have demonstrated FimH binding to N-linked glycans containing a terminal mannose (7, 10, 83, 84). While N-linked glycans are present on each EC domain of DSG2 (43), our data suggest that FimH interaction with native DSG2 is mediated through EC4 and/or EC5 (Figure 10). Of particular interest is the possibility that the cadherin-specific O-linked α-D-mannose on EC4 may represent the preferred binding target. While FimH canonically binds mannose, existing studies have not aimed to interrogate whether the spatial arrangement of nearby amino acid residues may also influence binding affinity. Future work, including determination of the structural basis of DSG2-FimH binding, will further address this question.

One limitation of this study is that we were unable to obtain a complete Dsg2 knockout cell line, indicating that complete absence of Dsg2 may confer a significant defect in in vitro growth of IMCD-3 cells. It is becoming better appreciated that CRISPR-generated knockout cell clones display phenotypic plasticity, and residual low-level protein expression often persists (85). Therefore, it is difficult to precisely quantify what proportion of UPEC binding is to Dsg2 as opposed to other mannosylated cell-surface receptors. It is possible that Dsg2 knock-down leads to compensatory production of other proteins; that Dsg2 knock-down reveals the participation of more minor binding partners for type 1 pili; or that the amount of residual Dsg2
in the knock-down clones provides sufficient receptors for UPEC to bind a modest proportion of cells. The present data do not distinguish among these possibilities.

Our results indicate that Dsg2 may not be the sole receptor for UPEC within the kidney, but rather the most important receptor among others on renal tubular epithelium. The apical surfaces of bladder epithelial facet cells are coated with a comparatively restricted set of proteins, largely four uroplakins (86). Among these, uroplakin Ia bearing N-linked oligomannose is thought to be the primary receptor important for UPEC binding of intact bladder epithelium (6-8, 10, 11, 87). In contrast, the renal tubular epithelium bears a wider variety of surface proteins, a number of which might be mannosylated. Of note, CRISPR knock-down of the glycosyltransferase Dad1 on IMCD-3 cells also conferred a UPEC binding defect (Figure 9B), suggesting that this enzyme may play a role in mannosylation of Dsg2 and/or other potential receptors for UPEC type 1 pili. Additionally, multiple innate immune genes were statistically enriched in the CRISPR screen (Figure 9B), hinting that innate response pathways may alter receptor expression and thereby influence UPEC binding; this too represents an avenue for future study.

The present work highlights the importance of type 1 pili and the mannosylated epithelial receptor desmoglein-2 in UPEC colonization of renal tubules. As the type 1 pilus adhesin FimH is already targetable by small-molecule inhibitors and vaccines in patients with cystitis (3, 68, 88), these therapeutics may prove useful in prevention or treatment of upper-tract UTI as well. Finally, desmoglein-2 is expressed widely across many epithelia, suggesting that it may serve as a FimH receptor in other UTI-relevant niches, perhaps as a secondary receptor in the urinary bladder or in the gut.
Conflicts of Interest

D.A.H. serves on the Board of Directors for BioVersys AG, Basel, Switzerland. J.W.J. and S.J.H. are inventors on US patent US8937167 B2, which covers the use of mannose-based FimH ligand antagonists for the treatment of disease. J.W.J. and S.J.H. have ownership interest in Fimbrion Therapeutics. All other authors have no competing interests to declare.

Acknowledgements

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Figure 1. Gating strategy for flow cytometry experiments. Samples were gated on single cells. Representative samples of IMCD-3 cells treated with media alone (red) and WT UTI89 (blue) shown.
Figure 2. Type 1 pili are required for bladder and kidney infection. (A) Male C3H/HeN mice were infected with UTI89 (closed circles) or UTI89ΔfimH (open diamonds), and organs were harvested 2 wpi. Bacterial loads after WT infection were significantly higher than with ΔfimH in both the bladder and kidneys (****p<0.0001). Horizontal bars indicate geometric mean, and dotted line indicates limit of detection. n=13-16 mice per group over 3 independent experiments. (B) Immunofluorescence microscopy of C3H/HeN male kidney 2 wpi with UTI89, showing expression of type 1 pili within kidney bacterial communities (magnified in inset; green, anti-type 1 pili; red, SYTO 61 nuclear stain). Scale bar, 20 μm.
Figure 3. UTI89ΔfimH are present within the bladders and kidneys of male C3H/HeN mice 24 hpi. Male C3H/HeN mice were infected with UTI89 (closed circles) or UTI89ΔfimH (open diamonds), and organs were harvested 24 hpi. No significant difference in bacterial loads between WT and ΔfimH were observed, indicating that ΔfimH reaches the kidney normally after inoculation of the bladder. Horizontal bars indicate geometric mean, and dotted line indicates limit of detection. n = 9 mice per condition over 2 independent experiments.
Figure 4. Urosepsis isolate (CFT073) lacking FimH also displays fitness defect 2wpi. Male C3H/HeN mice were infected with CFT073 (closed circles) or CFT073ΔfimH (open diamonds), and organs were harvested (A) 24 hpi or (B) 2 wpi. No significant differences in bacterial loads were observed 24 hpi; ΔfimH was attenuated significantly 2 wpi in both the bladder (*p = 0.0482) and kidneys (****p<0.0001). Horizontal bars indicate geometric mean, and dotted line indicates limit of detection. (A) n=10 mice per condition over 2 independent experiments; (B) n=13-14 mice per condition over 3 independent experiments.
Figure 5. Knockouts of the other CUP pili in UTI89 do not cause a colonization defect.

Male C3H/HeN mice were infected with UTI89 (closed circles) or isogenic, individual UTI89 CUP pili mutants (open diamonds), and organs were harvested 2 wpi. None of these CUP pili mutants displayed defects in bladder or kidney colonization. Horizontal bars indicate geometric mean, and dotted line indicates limit of detection. n=6-18 mice per experimental condition over 6 independent experiments.
Figure 6. Functional variants of type 1 pili are ineffective in establishing kidney infection.

Male C3H/HeN mice were infected with UTI89 (closed circles) compared to type 1 pili variants. Bladders and kidneys harvested 2 wpi yielded higher bacterial loads of wild-type (WT) UTI89 compared to (A) UTI89 FimHA27V/V163A (open diamonds) (bladder ****p<0.0001, kidney ***p=0.0001) or (B) UTI89 FimAA22R (open diamonds) (bladder ****p<0.0001, kidney ***p=0.0038). Horizontal bars indicate geometric mean, and dotted line indicates limit of detection. (A) n=13-19 mice per condition over 3 independent experiments; (B) n=10-15 mice per condition over 2 independent experiments.
Figure 7. Murine collecting duct cells display type 1 pili-dependent UPEC binding. (A) Early intratubular colonies of UPEC (green) were localized 5 dpi in male C3H/HeN mice collecting duct tubules (AQP2+, red; DAPI, blue; scale bar, 10 µm). (B) To intramedullary collecting duct (IMCD-3) cells, WT UTI89 (filled circles) displayed significantly higher levels of bacterial binding than ΔfimH (open diamonds; ****p<0.0001) (MOI 20). Horizontal bars indicate geometric mean. n= 12 samples per group over 4 independent experiments. (C, D) Binding of IMCD-3 cells by UTI89 or ΔfimH was quantified by flow cytometry after gating on single cells. (C) FITC signal (anti-*E. coli*) was evident in IMCD-3 cells infected with wild-type UTI89 (navy blue) compared with medium alone (green); infection with ΔfimH yielded no signal (orange). (D) Cells infected with UTI89 displayed significantly higher median fluorescence intensity (MFI) than those treated with medium alone or ΔfimH (****p<0.0001). n=8-15 samples per group over 6 independent experiments.
Figure 8. Methyl α-D-mannopyranoside and mannoside FIM1033 inhibit UPEC binding to collecting duct epithelial cells. (A) FITC signal (anti-*E. coli*) on IMCD-3 cells infected with wild-type UTI89 (navy blue) compared to infection with UTI89 ΔfimH (orange) or with wild-type UTI89 in media containing 2% methyl α-D-mannopyranoside (teal). (B) Binding of IMCD-3 cells by UTI89 (closed circles) or ΔfimH (open diamonds) after addition of methyl α-D-mannopyranoside was quantified by flow cytometry after gating on single cells. Significance is shown in comparison to WT UTI89 without methyl α-D-mannopyranoside (**p<0.01, ***p<0.001). n=6-9 samples per condition over 3 independent experiments. (C) FITC signal (anti-*E. coli*) on IMCD-3 cells infected with wild-type UTI89 (navy blue) compared to infection with UTI89 ΔfimH (orange) or with wild-type UTI89 in media containing FIM1033 (0.1µM, light pink; 1µM, dark pink; 10µM, maroon). (D) Binding of IMCD-3 cells by WT UTI89 (closed
circles) with or without addition of FIM1033, or by Δ*fimH* (open diamonds), was quantified by flow cytometry after gating on single cells. Horizontal bars indicate geometric mean, and significance is shown in comparison to WT UTI89 without FIM1033 (***(p<0.0001). n=9 samples per condition over 3 independent experiments.
Figure 9. A lentiviral CRISPR screen identifies candidate type 1 pilus receptor desmoglein-2. (A) Schematic of screen design. To screen for host genes responsible for type 1 pilus-dependent binding to this cell line, we transduced the Brie library of mouse guide RNAs into IMCD-3 cells bearing Cas9, providing 4× nominal coverage of each gene within the mouse genome. Cells were then bound (MOI 150) by UTI89 and sorted by fluorescent labeling, isolating cells unbound by bacteria. Genomic DNA was extracted and sequenced, identifying candidate genes that may be required for UPEC binding to IMCD-3 cells. (B) Volcano plot results from sorted and sequenced cells. Colored dots represent genes having an average log₂ fold change >0.5 and a –log₁₀(p-value) >2.5. (C) After in vitro binding, desmoglein-2 (red) on IMCD-3 cells co-localized with UPEC (green); scale bar, 5 µm. Lower panel demonstrates co-localization in orthogonal view of z-stack projection.
Figure 10. The FimH lectin domain directly binds DSG2 EC1-5 in a mannose-specific manner. (A) Schematic of human DSG2, with five extracellular cadherin domains (EC1-5; blue) followed by a single transmembrane (TM) segment and the cytoplasmic (desmoglein) portion of the protein (gray). O-linked mannoses are represented by green dots, N-linked glycosylation sites with terminal mannose shown by yellow dots. (B) Normalized BLI signal (mean ± SD) of DSG2 ectodomains (immobilized at 1 µg/mL) binding to FimHLD or FimHQ133K (10 µM). Relative BLI signals were normalized to amount of captured WT DSG2 EC1-5. DSG2 EC1-5 and DSG2 EC1-3 were purified from wild-type Expi293F cells, and DSG2 EC1-5 was also purified from Expi293F GnTI- cells (which lack complex N-glycans and instead exhibit uniform GlcNAc2Man5 glycosylation). *p=0.036 (EC1-5 WT vs Q133K), **p=0.008 (EC1-5 WT vs Q133K).
WT vs GnTI-), *p=0.015 (EC1-5 vs EC1-3). n= 3-5 independent experiments per group.

**Figure 11. Purification of 6×His-tagged DSG2 EC1-5 from Expi293F cells.** (A) Coomassie blue-stained gel of FimHLD and FimH_{Q133K}. (B) Coomassie blue-stained gel after metal-affinity purification of DSG2 EC1-5 and EC1-3 from cell supernatant. DSG2 EC1-5 was expressed in both WT Expi293F cells as well as in Expi293F GnTI- cells (lacking complex glycans). In right panel, due to its immunoglobulin folds, addition of the reducing agent DTT alters the apparent molecular weight (MW) of DSG2 EC1-5, from ~70 kDa to ~75 kDa.
Figure 12. Biolayer interferometry tracings for binding of FimH^{LD} to immobilized DSG2 EC1-5. Biolayer interferometry (BLI) tracings for binding of FimH^{LD} to immobilized DSG2 EC1-5 expressed from WT Expi293F cells (left panel) or Expi293F GnTI- cells (right panel). A 1:1 binding model (red lines) was used to fit experimental curves (black lines). Representative curves shown from 5 independent BLI runs per condition; affinity values represent the mean ± SD of two independent experiments.
Figure 13. Dsg2 knock-down cells exhibit a UPEC binding defect. (A) qPCR of desmoglein-2 normalized to GAPDH in IMCD-3 Dsg2 knock-down clones C4 and F11 (C4/WT *p= 0.0458, F11/WT *p= 0.0449). n=3 independent samples per group, error bars represent standard error of the mean. (B) Quantitative immunoblot for Dsg2 in clones C4 and F11, normalized to β-actin (median C4/WT ratio 4.3%, * p=0.0022; median F11/WT ratio 4.7%, **p=0.0087). Inset shows representative blot. n=6 independent samples per group. (C, D) Binding of UTI89 to IMCD-3 cells or to clones C4 and F11 (MOI 150) was quantified by flow cytometry after gating on single cells. (C) Histogram of FITC shift (anti-E. coli) in infected IMCD-3 cells. Medium alone (green) or infection with ΔfimH reflects no shift (orange); clones C4 and F11 (light and dark pink respectively) exhibited significantly decreased UPEC binding compared to WT IMCD-3 cells (navy blue). (D) Wild-type IMCD-3 cells infected with UTI89 displayed significantly higher median fluorescence intensity (MFI) than clones C4 and F11 (C4/WT *p= 0.0235; F11/WT **p=0.0032). n=15 samples per group over 5 independent experiments.
**Figure 14. Mannoside treatment attenuates bladder and kidney infection.** (A) Schematic of mannoside treatment experiments. Male C3H/HeN mice were infected with UTI89; after 5 d, mice were administered mannoside 29R (8 mg/kg/dose IP) or vehicle (mock treatment) every 8 h for either 24 or 48 h. (B) Intratubular colonies of UPEC (teal) were observed 5 dpi in male C3H/HeN mice within desmoglein-2 positive (Dsg2+, red) collecting ducts (aquaporin-2 [AQP2]+, green). DAPI, blue; scale bar, 10 µm. Mannoside treatment for 24 h (C) significantly reduced bacterial loads in the bladder (*p= 0.0255) and kidneys (*p=0.0434) (n=8-10 mice per group over 2 independent experiments), while an even more significant effect was seen with 48 h treatment (D; bladder ***p=0.0003, kidneys **p=0.0042; n=8 mice per group over 2 independent experiments).
### Tables

**Table 2.** Strains used in this study.

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<tr>
<th>Strain</th>
<th>Description</th>
<th>Reference</th>
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<td>UTI89</td>
<td>Prototypic UPEC isolate used for mouse infections and <em>in vitro</em> binding</td>
<td>(16)</td>
</tr>
<tr>
<td>UTI89 ∆fimH</td>
<td>Type 1 pilus adhesin mutant in UTI89</td>
<td>(16)</td>
</tr>
<tr>
<td>CFT073</td>
<td>Pyelonephritis/urosepsis isolate</td>
<td>(89)</td>
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<tr>
<td>CFT073 ∆fimH</td>
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<td>(89)</td>
</tr>
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<td>UTI89 FimH&lt;sub&gt;WT&lt;/sub&gt;</td>
<td>Kan-resistant comparator for FimH&lt;sub&gt;A27V/V163A&lt;/sub&gt;</td>
<td>(8)</td>
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<td>UTI89 FimH&lt;sub&gt;A27V/V163A&lt;/sub&gt;</td>
<td>Functional variant of the FimH adhesin</td>
<td>(8)</td>
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<td>UTI89 FimA&lt;sub&gt;WT&lt;/sub&gt;</td>
<td>Spc-resistant comparator for FimA&lt;sub&gt;A22R&lt;/sub&gt;</td>
<td>(54)</td>
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<td>(90)</td>
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<td>UTI89ΔpapC</td>
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<td>(83, 90)</td>
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<td>UTI89ΔyagW</td>
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Table 3. Frameshift mutations within Dsg2 in IMCD-3 clones C4 and F11 (two allelic variants present in each clone are shown).

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### Table 4. Probes and primers for qPCR.

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<th>Probe and Primers (P1/P2)</th>
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<td></td>
<td>P2: 5'-CATCTCTTGTCCACCGT-3'</td>
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<td>NM_007393</td>
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<td>P2: 5'-GTGGAGTCACTGGAACATGTAG-3'</td>
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Table 5. CRISPR screen statistics for candidate genes those having an average log fold change >0.5 and a false discovery rate >2.5. Full data set of CRISPR screen analyzed ranks and statistics is available upon request.

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<th>Gene Symbol</th>
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References


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Chapter 5. Concluding Remarks and Future Directions

Introduction

Despite the importance of urinary tract infections (UTIs) in human health globally, we still lack a full understanding of the host and pathogenic factors involved in pyelonephritis. Previous technical barriers within the UTI field meant that preclinical studies historically have focused largely on the female murine bladder (1-4), which has indeed been fruitful. However, leveraging the development of new in vivo and in vitro models, the current body of work delineates the dynamics of kidney colonization by uropathogenic Escherichia coli (UPEC; the chief etiological agent of UTIs). We identified new host-pathogen interactions suitable for targeting by novel, non-antibiotic therapeutics, including androgen receptor antagonists as well as inhibitors targeting the interaction of the type 1 pilus adhesin FimH with host receptors. Collectively, this body of work provides fundamental understanding of key host-pathogen molecular interactions during pyelonephritis that will spawn and shape future biological and biomedical studies and lead to translatable interventions.

Androgens play a key role in the development of severe pyelonephritis.

We sought a mechanistic understanding of the role of androgens in the development and severity of pyelonephritis. While UTIs are typically considered a disease of women, UTIs in males are neither non-existent nor benign. Thus, to delineate the role of sex and sex hormones in the pathogenesis of UTI, we employed new mouse models of UTI in which males develop severe pyelonephritis in comparison to females. We described that exposure of female mice to
androgens enabled development of severe pyelonephritis, mimicking increased UTI risk in women with polycystic ovary syndrome (PCOS), a common hyperandrogenic state (5-7). Treatment of androgenized female mice with FDA-approved androgen receptor antagonists reversed this phenotype. We also demonstrated the presence of biofilm-like bacterial communities, termed kidney bacterial communities (KBCs), as a key pathogenic stage in pyelonephritis. This work implies that androgen modulation may represent a new therapeutic or preventive strategy for male UTI, in women with recurrent UTI (rUTI) and PCOS (8), and possibly in rUTI patients without clinical PCOS but with testosterone levels above the mean. Going forward, the cellular and molecular mechanisms by which androgen exposure modulates UTI must be identified, and other host factors that promote severe UTI outcomes should be investigated.

**Identify additional host factors involved in UPEC-induced pyelonephritis**

As we have determined that androgens in both male and female mice impart susceptibility to pyelonephritis (8, 9), we now desire to understand what androgen-responsive elements are activated, especially at the host-pathogen interface, to promote severe pyelonephritis? Specifically, it is critical to understand what cell types are involved. For example, it is possible that androgens act specifically on the non-hematopoietic compartment within the bladder and/or kidneys, upregulating receptors or otherwise making the androgenized epithelium more conducive to bacterial attachment or replication. Another possibility is that androgens shape the immune response to UPEC ascension in a way that hampers bacterial clearance and enables severe pyelonephritis. The androgen receptor (AR) is expressed widely in many cell types in male and female mammals, including leukocytes and most if not all cell types
in the kidney (10, 11). Of note, we have also demonstrated high expression of the AR within neutrophil-dominated renal abscesses (8). Thus, it is most likely that fundamental sex differences impact the host-pathogen interaction and cellular responses in pyelonephritis, thereby influencing pathogenicity, resolution, and subsequent renal scarring.

As immune cells have been shown in other experimental systems to act differently in androgenized versus estrogenized hosts, a critical next step is understanding the influences of sex on susceptibility to pyelonephritis. As demonstrated in preclinical models with female C3H mice, an overzealous immune response early in infection leads to chronic cystitis and worsens infection outcome (12, 13). During human infection, bacterial infection of the renal parenchyma elicits marked tubulointerstitial inflammation (14-18). This inflammatory response is similarly reflected in our models, with established abscesses composed primarily of neutrophils destroying the surrounding renal parenchyma (8, 9, 19). Work in our lab and others has begun to delineate an altered immune response to UPEC in the presence of androgens (20-23). Specifically, our lab has demonstrated that while androgenized mice with UTI exhibit high levels of myeloid cell recruitment, UPEC are unable to be cleared from androgenized kidney. Androgen exposure increased the expression of activin A (a driver of renal scarring) in myeloid cells, helping to perpetuate scar formation; in part, this was attributable to prolonged polarization of macrophages toward a pro-fibrotic M2a phenotype (20). This work begins to link androgen influences on the innate immune response with increased susceptibility to severe pyelonephritis (8, 19, 20). Continued mechanistic studies will provide further understanding of hormonal effects during upper-tract UTI and illuminate ways to mitigate sequelae of severe pyelonephritis.

Further, androgens also influence the expression of other, non-immune genes that could act in the development of upper-tract UTI. For example, expression of desmoglein-2, identified
in this work to be a receptor for UPEC FimH within the kidney (Chapter 4), is predicted to be influenced by various hormones and receptors (24, 25); however, the influence of various sex hormones on Dsg2 expression differs across model systems and target organs (24-27).

Additionally, we are beginning to understand that other androgen-responsive non-hematopoietic genes influence host outcomes. Indeed, the process of fibrosis, which is initiated during abscess formation, is responsive to the influence of androgens. The presence of testosterone was found to increase local production of TGFβ1 before the initiation of experimental UTI. This testosterone-induced surge of TGFβ1 increased the presence of mesenchymal stem cells and activated myofibroblasts, priming the kidney for fibrosis and collagen deposition (21). In other non-infectious renal injury models, including unilateral ureteral obstruction (UUO), androgens enhance the severity of injury and scarring (28, 29). Together, these data paint a complex picture of androgens acting on cells of both hematopoietic and non-hematopoietic origin, interacting to create the inflammatory environment and tissue damage we observe in UPEC-infected males and androgenized females.

We are now poised to elucidate these pathways further. Recently emerged technologies now permit detailed interrogation of sex-specific host responses in the kidney to ascending infection with UPEC. Specifically, future collaborative studies led by the Hunstad lab will employ single-nucleus RNA sequencing, using techniques developed in the Humphreys lab (28-30), on kidneys from UPEC-infected and uninfected male, female, and androgenized female C3H mice. These studies will help to define how both chromosomal and hormonal sex influence the transcriptional responses of epithelial cells and of resident and recruited leukocyte subsets during ascending UPEC infection of the kidney. The results will provide a much more detailed
picture of the cellular and molecular pathways that comprise the host response to UPEC infection of the mammalian kidney, in both host sexes.

**Ascending UTI imposes a modest bottleneck on genetic diversity of infecting UPEC.**

During infection of the female mouse urinary tract, UPEC undergo a restrictive population bottleneck – first through the development of clonal intracellular bacterial communities (IBCs) and second through an ascension bottleneck in the kidney (resulting in female mice resolving kidney infection) (31). However, in male mice that develop severe pyelonephritis, we found that ascending pyelonephritis imposes only modest narrowing of genetic diversity among infecting UPEC. Using a set of “barcoded” isogenic UPEC isolates (31) and confocal microscopy, we defined the spatial and temporal dynamics of UPEC populations during experimental ascending pyelonephritis in mice. These analyses demonstrated relative preservation of infecting clones from 1-7 days post arrival in the kidney. Further, we found that kidney bacterial communities (KBCs) were clonal, despite being located in the urinary (extracellular) space and thereby lacking the invasion requirement that defines clonal IBCs in the bladder. Thus, at least in the present model, each KBC arises from growth and expansion of a single bacterial clone that ascended the nephron to establish a given intratubular focus of infection. These data further indicate that while bulk ascension into the kidney does not impose an acute bottleneck upon the bacterial population, individual nephron ascension is a comparatively uncommon event accomplished by single bacteria within a nephron unit.
Interrogate pathogenic factors using signature-tagged mutagenesis

In female mice, large-scale signature-tagged mutagenesis screens to identify novel UPEC virulence factors have been precluded by the marked bottleneck imposed during the bladder IBC cascade and by spontaneous resolution of kidney infection in females of most mouse backgrounds (31). However, as our male kidney model does not impose such a bottleneck (Chapter 3), we can take multiple complementary approaches in order to delineate bacterial factors mediating kidney infection – namely high-throughput transposon-based screening techniques such as insertion-site sequencing (INSeq), as well as hybrid capture-enhanced bacterial RNA sequencing (RNAseq). These studies, funded by a recently awarded R01, are commencing in collaboration with the Earl group at the Broad Institute. Additionally, we developed an in vitro model of pyelonephritis using inner medullary collecting duct (IMCD-3) cells that accurately recapitulated the requirement for type 1 pili in renal tubular adherence (Chapter 4). Therefore, both INSeq and RNAseq could also be employed within this in vitro system to specify other UPEC factors that might be required for infection in vivo. Our ability to use contemporary RNA and DNA sequencing methods in both these models will enable the identification of novel, host-sex-specific UPEC virulence factors important in colonization, persistence, and disease causation in the kidney.

Of particular interest, the UPEC adhesin profile and toxin profile during pyelonephritis remain to be further delineated. Unlike during female cystitis, in which type 1 pili are required immediately upon arrival in the bladder (32-35), our male model showed that type 1 pili were more critical at later time points (Chapter 4). This suggests a role for other bacterial products in bulk persistence within the renal pelvis, potentially including adherence in this initial kidney niche. However, our initial screen of chaperone-usher pathway pili (CUP pili) did not implicate
an alternative CUP pilus in UPEC pyelonephritis (recognizing that the P pilus receptor is
minimally if at all expressed in the mouse kidney (36-38)). Additionally, among several
canonical UPEC toxins we previously examined (α-hemolysin, cytotoxic necrotizing factor 1,
OmpT), none were required for infection in this model (P. Olson, unpublished data; (39, 40)).
Therefore, an unbiased screen to illuminate additional virulence factors during pyelonephritis
will be essential to further understanding host-UPEC interactions in the kidney.

**UPEC adhesion to renal tubular epithelium is mediated by UPEC FimH
binding to host desmoglein-2 (Dsg2).**

Type 1 pili of UPEC have long recognized to be critical for bacterial adhesion to the
bladder (32, 34, 35, 41, 42). Using the male mouse model of UTI, we demonstrated an essential
role for type 1 pili in colonizing the kidney during pyelonephritis. We showed that mannosides
(novel small-molecule inhibitors of type 1 pili) can limit the severity of experimental
pyelonephritis. Further, by performing a lentiviral CRISPR screen in cultured renal epithelial
cells, we were able to identify host factors implicated in UPEC binding in the kidney. This
screen identified a strong candidate type 1 pilus receptor on renal epithelium, namely
desmoglein-2, a mannosylated, tight junctional protein (43, 44). Using both cellular assays and
biochemical approaches, we confirmed that desmoglein-2 binds type 1 pili and that this
interaction is critical for UPEC binding. Because we have now identified this key molecular
interaction, mannosides and other therapeutics that target type 1 pilus binding can be further
developed as non-antibiotic treatments for pyelonephritis. Future work will further interrogate
the structure and *in vivo* interactions of desmoglein-2 in the kidney, as described below.
Interrogate the structural and *in vivo* interactions of type 1 pili and desmoglein-2

We described the ability of the type 1 pilus adhesin, FimH, to bind purified human desmoglein-2 (DSG2) ectodomains (EC1-5). This interaction was mannose specific, as FimH<sub>Q133K</sub>, a non-mannose-binding mutant, was unable to bind DSG2. Further, the interaction was eliminated when the extracellular domain was truncated (DSG2<sub>ΔEC4-5</sub>), suggesting that domains EC4 and/or EC5 mediate FimH binding. EC4 displays a unique, cadherin-specific form of mannosylation in which α-D-mannose (the canonical binding target of type 1 pili) is present as a novel O-linked glycosylation modification (43). As prior studies all have demonstrated FimH binding to N-linked glycans containing terminal mannose, it is intriguing to speculate that the O-linked mannose on EC4 may represent the preferred binding target (41, 42, 45, 46).

To interrogate the specific binding site of FimH, and specifically test the hypothesis that FimH interacts with O-linked mannose present on DSG2 EC4, multiple approaches are proposed. First, we can narrow to specific domains or perhaps peptide sequences that are responsible for UPEC binding by expressing DSG2 truncates or individual cadherin domains. Second, as FimH binding is mannose specific, point mutations targeting glycosylated residues within DSG2 EC4-5 can eliminate specific glycans. This could help to specify the relative contributions of various mannose sites to FimH binding. As it is possible that binding of FimH can occur at multiple sites along DSG2, employing an array of these complementary methods (expressing specific domains, point mutations, blocking antibodies, treatment with specific glycosidases, etc.) will be maximally informative in determining the binding affinity and domain specificity of the DSG2-FimH interaction. Of note, in the same lentiviral screen, we also sorted for cells that displayed higher binding of UPEC. This high binding population displayed many glycosyltransferases.
(Figure 1), and thus could be further used to interrogate the effect of various sugars on UPEC binding.

Additionally, future studies will interrogate in depth the structural basis of this interaction. Unlike at the bladder epithelial surface, where only a limited set of proteins (uroplakins) line the luminal surface and are thus available for binding, the renal tubular epithelium presumably expresses a wider variety of potentially mannosylated surface proteins. Our understanding of how regional amino acid and protein context impact FimH binding to mannose is currently minimal. What features would make one mannosylated protein residue a suitable (or high-affinity) receptor for FimH while others may bind poorly or not at all? It is unclear whether any mannosylated peptide – as long as it is sterically available to FimH – is capable of binding UPEC. While current dogma holds that FimH binds mannose specifically, no work has been done to understand if the protein scaffold plays any role in binding affinity or specificity. Therefore, an X-ray or cryoEM structure of the FimH-DSG2 complex may in fact expand our understanding of the range of biological interactions mediated by FimH.

**Determine the role of UPEC with Dsg2 with alternate in vivo niches**

While we know that UPEC co-localize with Dsg2 in vivo during kidney infection, we hope to study Dsg2 in conditional knockout mice to directly understand the how the expression of Dsg2 in different niches correlates to UPEC binding. We are currently generating mice with specific deletion of Dsg2 from renal tubular epithelia, which will allow us to further interrogate this mannosylated host protein as a type 1 pilus receptor in the kidney. As germline Dsg2 knockout is embryonic lethal in mice (47), we are currently generating relevant conditional tools in the C3H background (C3H Dsg2<sup>fl/fl</sup>) to demonstrate the contribution of Dsg2 to the in vivo
pathogenesis of pyelonephritis. Crossing of Dsg2 floxed mice with various cell type-specific Cre mice (also being made in the C3H background) will enable us to interrogate where and when the FimH-Dsg2 interaction is most impactful. Specifically, we are creating C3H Ksp-Cre mice for both conditional and inducible knockout; here, the Cre recombinase is driven by the kidney-specific cadherin (aka Cdh16) promoter, expressed in renal tubular epithelia (48, 49).

Desmoglein-2 is expressed widely across mammalian epithelia (43, 44), thus raising the possibility that Dsg2 acts as a receptor for UPEC FimH in other niches relevant to UTI. Specifically, the exfoliated bladder epithelium is a potential site for UPEC-Dsg2 interactions. While uroplakin 1a is the canonical FimH receptor in the naïve bladder (41), the host response to initial bladder infection causes the uroplakin-coated superficial facet cells to be exfoliated (32, 50-52). Underlying epithelial cells may express uroplakins at lower levels, but additional receptors likely also enable UPEC adherence to these newly exposed cells. In fact, Dsg2 is highly expressed on the bladder epithelial layers beneath the facet cells (Figure 2) (44). Further, UPEC have anecdotally been observed by scanning electron microscopy to line up along facet cell junctions (S.J. Hultgren, personal communication), where Dsg2 might be most highly expressed and available for interaction. These observations raise the distinct possibility that Dsg2 plays a role in UPEC adherence within the bladder – either in the exfoliated bladder or as a secondary receptor during initial bladder colonization.

**Investigate the role of the innate immune response in UPEC kidney infection.**

It is clear that the innate immune response plays a critical role in pyelonephritis (53, 54). Renal abscesses are packed with infiltrating neutrophils, and C3H/HeJ mice (which lack a functional TLR4 response) subjected to experimental pyelonephritis succumb to urosepsis (8).
Further, in the CRISPR/Cas9 lentiviral screen for UPEC binding, there was an unpredicted but noticeable innate immune signature, including the genes *Xbp1, Il6,* and *Ifnb,* among others. The screen was conducted after 45 minutes of binding, selecting for cells that specifically displayed less binding of bacteria. Thus, future studies will interrogate how facets of the epithelial innate immune response to UPEC challenge may actually promote binding of the pathogen.

One hypothesis is that epithelial cells, sensing the arriving pathogen, activate programs that function to reinforce tissue barriers, including intercellular junctions. In such a process, junctional proteins such as desmoglein-2, if expressed more highly, represent a means for increased bacterial binding to occur. Interestingly, 2 weeks post infection, we observed high expression of Dsg2 on many cell types within kidney abscesses (Figure 3). It is unclear whether activated neutrophils actively express Dsg2 or whether the destruction of renal parenchyma has exposed Dsg2 from other cellular sources (55). Immune cells do not canonically express Dsg2, and neutrophils show only low-level expression under normal conditions (44, 56). However, Dsg2 expression is predicted to be under the influence of immune signaling pathways (24, 25), raising the possibility that immune cells within the abscess do promote Dsg2 expression. An alternative hypothesis is that the major injury and cell death in renal parenchymal or epithelial cells exposes their Dsg2. Of note, in the intestinal tract, Dsg2 is cleaved intracellularly in response to injury or inflammation and subsequently released from cell surfaces (55). If this process were also occurring in renal abscesses, it might have the effect of reducing receptor availability on the epithelial surface, or to liberate a decoy molecule for FimH binding, as with Tamm-Horsfall protein in the urinary space (46, 57, 58). A third possibility is that immune activation results in the expression of alternative FimH receptors that were not specifically
detected in the screen. These all represent experimentally testable and nonexclusive hypotheses to be addressed in future work.

**Concluding remarks**

The present body of work defined essential host factors (androgens and desmoglein-2) and pathogen factors (type 1 pili and FimH) that are required for UPEC establishment of severe pyelonephritis. By employing new mouse models of UTI, we determined how androgenization of female mice leads to worsened kidney infection and demonstrated the presence of kidney bacterial communities as a key pathogenic stage in upper-tract UTI. We established that on a population level, high bacterial titers maintained overall genetic diversity, overshadowing the strict bottleneck that occurs in the bladder. However, at an individual nephron level, KBCs were formed by the clonal expansion of bacteria within a nephron unit. We then illuminated the dynamics and CUP pili adhesive profile of UPEC during renal abscess formation, revealing an essential role for type 1 pili in UPEC colonization of the renal epithelium during pyelonephritis. By generating a CRISPR-Cas9 lentivirus library in cultured renal epithelial cells, we screened for host factors implicated in UPEC binding in the kidney. This screen identified a receptor on renal epithelium for type 1 pili, desmoglein-2, and demonstrated that mannosides (novel small-molecule inhibitors of type 1 pili) may be useful in treating pyelonephritis. In total, this work provides novel insights into the pathogenesis of pyelonephritis that could lead to more comprehensive, non-antibiotic therapeutic treatments. While the work advances our fundamental understanding of pyelonephritis, it also opens multiple new avenues of study in the UTI field to further illuminate UPEC pathogenesis and inform treatment of upper-tract UTI.
Figures

Figure 1. A lentiviral CRISPR screen identifies multiple glycosyltransferase enzymes involved in increasing UPEC binding. To screen for host genes responsible for type 1 pili-dependent binding to this cell line, we transduced the Brie library of mouse sgRNAs into IMCD-3 cells bearing Cas9, providing $4^\times$ nominal coverage of each gene within the mouse genome. Cells were then bound (MOI 150) by UTI89 and sorted by fluorescent labeling, isolating cells highly bound by bacteria. Genomic DNA was extracted and sequenced, identifying candidate genes that may be required for UPEC binding to IMCD-3 cells. Volcano plot results from sorted and sequenced cells. Colored dots represent genes having an average log$_2$ fold change $>0.5$ and a $-\log_{10}(p\text{-value}) >2.5$. 
Figure 2. Desmoglein-2 expression on bladder epithelia. Immunohistochemistry for DSG2 expression in normal human bladder, showing strong expression in uroepithelium. Inset (lower right) shows comparatively less expression in facet cell layer. From proteinatlas.org (CAB025122) (44).
**Figure 3. Desmoglein 2 expression within renal abscess.** An image from the kidney of a C3H/HeN male mouse 2 wpi shows UPEC (green) within a KBC being infiltrated by immune cells (blue DAPI-stained nuclei). The immune cell infiltrate comprising the abscess can be seen highly expressing Dsg2 (red), suggesting a role of the innate immune system in local $dsg2$ expression. Scale bar, 20 µm.
References


