REVIEW ARTICLE

Host–pathogen checkpoints and population bottlenecks in persistent and intracellular uropathogenic *Escherichia coli* bladder infection

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Received 4 November 2011; revised 10 February 2012; accepted 5 March 2012. Final version published online April 2012.

DOI: 10.1111/j.1574-6976.2012.00339.x

Editor: Christoph Dehio

Keywords
cystitis; urinary tract infection; UPEC; type 1 pili; intracellular bacterial communities; antibiotic resistance.

Abstract

Bladder infections affect millions of people yearly, and recurrent symptomatic infections (cystitis) are very common. The rapid increase in infections caused by multidrug-resistant uropathogens threatens to make recurrent cystitis an increasingly troubling public health concern. Uropathogenic *Escherichia coli* (UPEC) cause the vast majority of bladder infections. Upon entry into the lower urinary tract, UPEC face obstacles to colonization that constitute population bottlenecks, reducing diversity, and selecting for fit clones. A critical mucosal barrier to bladder infection is the epithelium (urothelium). UPEC bypass this barrier when they invade urothelial cells and form intracellular bacterial communities (IBCs), a process which requires type 1 pili. IBCs are transient in nature, occurring primarily during acute infection. Chronic bladder infection is common and can be either latent, in the form of the quiescent intracellular reservoir (QIR), or active, in the form of asymptomatic bacteriuria (ASB/ABU) or chronic cystitis. In mice, the fate of bladder infection, QIR, ASB, or chronic cystitis, is determined within the first 24 h of infection and constitutes a putative host–pathogen mucosal checkpoint that contributes to susceptibility to recurrent cystitis. Knowledge of these checkpoints and bottlenecks is critical for our understanding of bladder infection and efforts to devise novel therapeutic strategies.

The problem: recurrent cystitis is common and antibiotic resistance is rising among uropathogens

Overview

Urinary tract infections (UTI) are among the most common bacterial infectious diseases afflicting humans (Hooton & Stamm, 1997; Foxman, 2003), resulting in close to $2.5 billion in healthcare costs in the year 2000 in the United States alone (Griebling, 2007). Women are at greatest risk: nearly half will have a UTI in their lifetime and annually over 15 million women suffer from UTI in the United States with each episode causing serious deterioration in the quality of life (Foxman, 1990, 2002). Recurrent cystitis is a major concern as 20–30% of women with an acute infection will have a recurrence within 3–4 months (Foxman, 2002). The elderly and prepubertal children are also particularly susceptible to recurrent and chronic cystitis (Foxman, 2002; Conway *et al.*, 2007), and renal scarring is a potentially serious complication in infants (Huland & Busch, 1984). Risk factors for uncomplicated cystitis in premenopausal adult women include frequent sexual activity, exposure to

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spermicides, a history of childhood UTI, being a nonsecretor of ABH blood-group antigens, and a maternal history of UTI (Sheinfeld *et al.*, 1989; Scholes *et al.*, 2000). This last risk factor indicates that host genetic factors and perhaps other vertically transmissible traits such as microbiota may play a significant role in susceptibility to UTI. In postmenopausal women, chronically recurrent cystitis can be particularly troubling, requiring long-term prophylactic antibiotic use and is associated with a history of premenopausal UTI and bladder voiding abnormalities (Raz *et al.*, 2000). Additional subpopulations at risk for what is termed complicated UTI include patients with spinal cord injury, patients undergoing urethral catheterization, diabetics, and individuals with underlying urologic abnormalities (Sedor & Mulholland, 1999; Foxman, 2002; Mittal & Wing, 2005). Bladder infections are also common in other mammalian species, including domesticated animals such as dogs, cattle, horses, and swine (Dowling, 1996). Interestingly, host genetic factors have been implicated in the relative susceptibility of dogs to UTI, as some breeds are particularly susceptible to recurrent cystitis (Norris *et al.*, 2000; Ling *et al.*, 2001).

Clinical manifestations of UTI

Clinically, UTI are defined by the presence of bacteria in the urine, known as bacteriuria. The source of the bacteria cannot be directly determined by noninvasive means, so clinicians rely on patient symptoms for diagnosis. Symptoms of lower UTI, that is, affecting the urethra and urinary bladder, include frequent urination, burning sensation and pain during urination (dysuria), suprapubic pain and/or lower abdominal discomfort, and cloudy and/or bloody urine that is often foul smelling. Upper UTI, that is, affecting the ureters and kidney, are usually diagnosed by the presence of bacteriuria and pyuria that is accompanied by flank pain and fever. These symptoms are, in part, a result of the mucosal inflammatory response to bacterial colonization of the bladder (cystitis) and/or the kidney (pyelonephritis). Uncomplicated cystitis accounts for most symptomatic UTI (Hooton & Stamm, 1997), and this review will focus entirely on bladder infections. Pelvic region pain responses leading to symptomatology in cystitis are complex, and a recent study of referred pain responses in uropathogenic *Escherichia coli* (UPEC)-infected mice suggests that lipopolysaccharide (LPS) plays an important role in inducing or suppressing pain responses to infection (Rudick *et al.*, 2010). UTI, however, are not always symptomatic. Patients with asymptomatic bacteriuria (ASB, also abbreviated as ABU) have high levels of bacteriuria without the classical hallmark symptoms of UTI, that is, dysuria and foul-smelling urine with variable suprapubic pain. ASB commonly occurs in the elderly (Nicolle *et al.*, 2005), in pregnant women (Celen *et al.*, 2011), in young girls (Kunin & Paquin, 1967; Lindberg *et al.*, 1975), and in patients with diabetes (Renko *et al.*, 2011). While there is no benefit to treatment of ASB in the elderly (Raz, 2003), in younger women ASB is a strong predictor of future symptomatic UTI (Hooton *et al.*, 2000), and pregnant women with ASB are aggressively treated with antibiotics because of the unique risks that UTI presents for both the mother and child (Alarcon *et al.*, 2004; Goldenberg *et al.*, 2005).

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UPEC are by far the most common cause of UTI, and these Gram-negative bacteria, whose primary niche is the large bowel of vertebrate animals, are responsible for approximately 80% of community-acquired infections and 25% of nosocomial infections (Ronald, 2002). *Staphylococcus saprophyticus* is recovered from 10% to 15% of community-acquired infections, followed in prevalence by *Klebsiella*, *Enterobacter*, *Proteus*, and *Enterococcus* species. While antibiotics have historically been very successful in resolving bladder infections, the increasing prevalence of antibiotic resistance among these uropathogenic strains is a major concern (Gupta *et al.*, 2001, 2005). In particular, increasing resistance to first-line empiric therapies such as trimethoprim-sulfamethoxazole has resulted in more frequent use of fluoroquinolones as the first-line therapy for cystitis, which in turn has led to increasing resistance against this class of antibiotics (Schaeffer, 2002; Hooton *et al.*, 2004). Furthermore, fluoroquinolones have significant toxicity, being associated with tendonitis and even spontaneous tendon rupture, particularly in the elderly and in those with impaired renal function (Huston, 1994). The emergence of UTI caused by multidrug-resistant (MDR) strains (Karlowsky *et al.*, 2006; Aypak *et al.*, 2009; Kallen *et al.*, 2010) threatens to make chronic and recurrent UTI an even more common problem. Nosocomial infections caused by bacteria with very high rates of antibiotic resistance, such as *Pseudomonas aeruginosa* and *Enterococcus* species, have become much more prevalent and can be life-threatening (Merle *et al.*, 2002). Prior to the widespread use of antibiotic therapy to cure cystitis, reports of chronic symptomatic UTI abounded (Nickel, 2005). Placebo-controlled studies have demonstrated that about half of women remain bacteriuric for weeks after an acute episode of cystitis if not treated with antibiotics, despite overall improvement of symptoms (Mabeck, 1972; Ferry *et al.*, 2004). However, even if antibiotic therapy is successful in the short-term, many individuals suffer from persistent problems with chronic, recurrent cystitis, necessitating the use of long-term prophylactic antibiotics (Raz *et al.*, 2000;

Foxman, 2002; Conway *et al.*, 2007). Murine studies have demonstrated and human studies suggest that differences in the nature of the early innate host responses to UTI between individuals are significant determinants of disease outcome and susceptibility to recurrence (Frendeus *et al.*, 2000; Ragnarsdottir *et al.*, 2007; Fischer *et al.*, 2010; Hannan *et al.*, 2010). Thus, understanding the mechanisms contributing to acute cystitis and progression to chronic and/or recurrent cystitis, using human genomic studies and robust animal models of disease, is of critical importance to develop new therapeutic and prophylactic strategies (Schaeffer *et al.*, 2010; Dielubanza & Schaeffer, 2011). In this review, we will highlight recent studies that have shed light on the remarkable complexity of lower UTI pathogenesis primarily by the model uropathogen UPEC, paying particular attention to the roles of intracellular bacteria and persistent infections, and propose how this understanding could potentially change the way we treat and prevent bladder infections.

UPEC: a model organism for studying host–pathogen interactions in bladder infection

Host–pathogen interactions and population bottlenecks

The mammalian urinary tract has developed formidable innate defenses to infection, which have been reviewed in detail elsewhere (Schilling *et al.*, 2001a; Song & Abraham, 2008; Ragnarsdottir *et al.*, 2011). To successfully colonize the urinary tract, pathogens such as UPEC must be capable of surmounting or evading these mucosal barriers to infection or they will not be able to persist. These host–pathogen interactions at each stage of infection constitute potential transmission bottlenecks that reduce bacterial diversity, allowing only a fraction of clones to persist (Fig. 1). While pathogenesis typically selects for clones that are fit to transit these bottlenecks, the latter are typically stochastic to some degree and therefore can have unpredictable impacts upon virulence of evolving pathogens (Bergstrom *et al.*, 1999). It is critical to understand these population bottlenecks to devise new and effective strategies to treat and prevent infectious disease.

Mucosal barriers of the bladder: normal urination

In the urinary bladder, evolution has provided mammals with a means for storing urine so that micturition may occur intermittently. The cost of this adaptation is that urine stasis is present in the bladder for long periods of time between voiding, making it a weak link in the anatomical barriers to ascending infection, as increased susceptibility to recurrent cystitis is seen in patients with urine voiding defects of the bladder (Raz *et al.*, 2000; Foxman, 2002). However, prior to colonizing the bladder, uropathogens such as UPEC must ascend the urethra against the flow of urine and anatomical or functional urologic defects that compromise normal urination, such as urinary incontinence, predispose healthy postmenopausal women to recurrent cystitis (Raz *et al.*, 2000). In healthy premenopausal women, sexual activity, which can result in retrograde forces up the urethra, and use of spermicides, which can alter the vaginal and periurethral flora, are the most important risk factors for recurrent cystitis (Scholes *et al.*, 2000). As few models of urethral ascension exist and this stage of pathogenesis is poorly understood, in this review, we will focus primarily on the role of bladder colonization in cystitis.

Mucosal barriers of the bladder: the urothelium

Despite the long periods of urine stasis in the bladder, adherence to the mucosa is still required for microorganisms to avoid being eliminated during intermittent, but high shear force micturition (Thomas *et al.*, 2002). The mucosal epithelium of the lower urinary tract, known as the urothelium, is a critical barrier to UTI. It extends from the proximal urethra to the renal pelvis and consists of a unique pseudostratified epithelial layer comprising a layer of basal and transitional cells covered by a layer of superficial umbrella (or facet) cells, which are large, flat, terminally differentiated epithelial cells (Wu *et al.*, 2009). The luminal surface of the urothelium is lined by the asymmetric unit membrane, consisting of a quasi-crystalline array of uroplakin integral membrane proteins within the outer leaflet of the plasma membrane of the superficial umbrella cells. These uroplakin plaques are composed of four uroplakin proteins, UPIa, UPIb, UPII, and UPIIIa, three of which are highly glycosylated (Wu *et al.*, 1994). They form a permeability barrier preventing the resorption of urine solutes across the urothelium and limiting the availability of receptors for bacterial adherence factors (Sun *et al.*, 1996; Hu *et al.*, 2002). Furthermore, the urothelium is coated by an extracellular proteoglycan mucin layer that also reduces permeability and opposes intimate bacterial colonization, in part due to the high negative charge of the sulfated and carboxylated glycosaminoglycans (Parsons *et al.*, 1990; Hurst & Zebrowski, 1994; Nickel & Cornish, 1994). However, UPEC and other uropathogens that express mannose-binding type 1 pili are able to subvert these physical barriers to adherence by binding to the high mannose

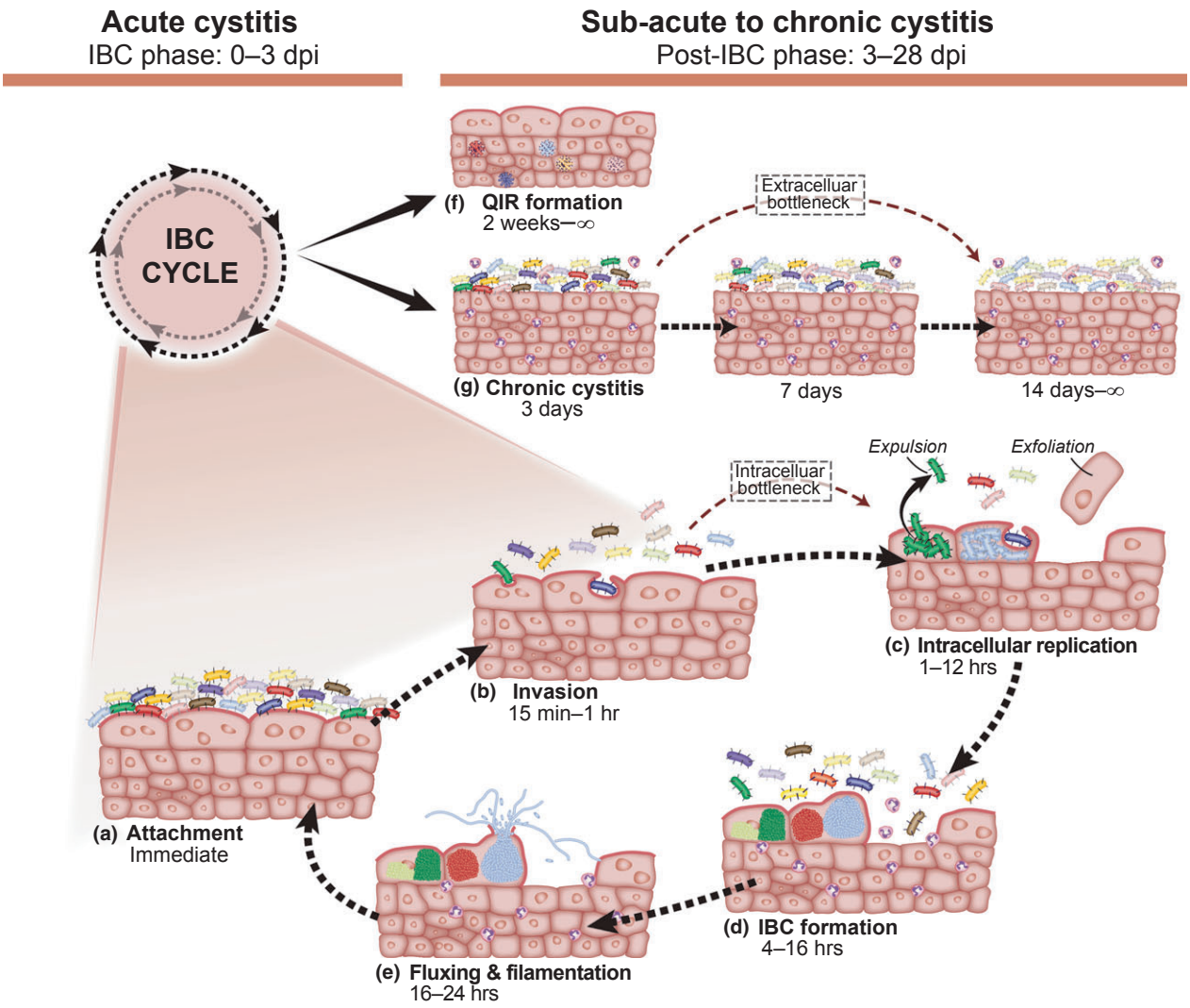

Fig. 1. Hypothetical model for UPEC population bottlenecks in acute and chronic cystitis. This model depicts a hypothetical situation where two virulent and clonal UPEC populations, the Solids and the Pastels, are introduced into the urinary bladder. Individual bacteria within each clonal population are identical, but are represented by different Solid and Pastel tones to demonstrate the population bottleneck encountered during acute cystitis. Successful passage through this acute bottleneck is linked to clonal expansion via the IBC cycle (a–e) during acute cystitis (Schwartz *et al.*, 2011). If these clones are equally fit to proceed through the IBC cycle, the loss of 'diversity' is purely stochastic, affecting both the Solids and Pastels equally. We postulate that the dominant role of type 1 pili in acute cystitis in naïve mice, including a critical role in IBC formation, explains why few UPEC genes have been demonstrated to contribute to acute virulence using isogenic knockout strains. Indeed, most virulence genes identified so far in animal models of cystitis directly impact upon type 1 pili production or the ability of the strain to process through the IBC cycle. Likewise, QIR formation (f) during acute infection results in stochastic loss of diversity and in the latent QIRs that persist in the urothelium after acute infection resolves, the Solids and Pastels persist equally well (Mysorekar & Hultgren, 2006; Hannan *et al.*, 2010; Schwartz *et al.*, 2011). However, in chronic cystitis (g), the urothelium is hyperplastic and cannot support IBC formation (Hannan *et al.*, 2010). Therefore, UPEC transitions to an extracellular niche, and either remains adherent to the urothelium or clustered with host cells in the bladder lumen. Therefore, loss of diversity is more gradual and will only occur if one clonal population has enhanced fitness for chronic persistence in the bladder. In this example, the Pastels are more fit than the Solids in competing for limited resources and eventually take over the bladder.

glycoprotein UPIa, in mice and humans, via the type 1 pilus cognate adhesin protein FimH (Zhou *et al.*, 2001; Xie *et al.*, 2006). Although soluble factors normally found in the urine, such as Tamm-Horsfall protein, protect the bladder in part by competing for type 1 pili binding (Bates *et al.*, 2004), if UPEC are allowed to adhere to the urothelium, this initiates the acute pathogenic cycle of bladder infection.

Type 1 pili mediate UPEC adherence to the urothelium

Experimental studies

UPEC are a genetically diverse pathotype of *E. coli* that have evolved by pathoadaptive mutation and horizontal gene transfer to effectively colonize the mammalian urinary tract. UPEC elaborate a number of bacterial factors that contribute to their ability to colonize the urinary tract. Foremost among these are adhesive fibers known as pili (fimbriae), such as type 1 pili, which are formed on the bacterial outer membrane by an assembly process called the chaperone/usher pathway (CUP; Sauer *et al.*, 1999; Barnhart *et al.*, 2000; Remaut *et al.*, 2008; Phan *et al.*, 2011). These pili contain adhesins at their tips that are thought to play an important role in host–pathogen interactions. Each sequenced UPEC strain has been found to encode a multitude of CUP operons (Welch *et al.*, 2002; Chen *et al.*, 2006; Brzuszkiewicz *et al.*, 2006). Some CUP adhesins are known to recognize specific receptors with stereochemical specificity. For example, FimH, the type 1 pilus tip adhesin, has been shown to bind mannosylated UPIa (Zhou *et al.*, 2001), as well as N-linked oligosaccharides on β1 and α3 integrins (Eto *et al.*, 2007), and the pattern recognition receptor Toll-like receptor 4 (TLR4) (Mossman *et al.*, 2008), all of which are expressed on bladder epithelial cells. Type 1 pili have been shown by several groups to be critical for infection in a murine model of cystitis (Hagberg *et al.*, 1983; Abraham *et al.*, 1985b; Hultgren *et al.*, 1985; Alkan *et al.*, 1986; Connell *et al.*, 1996; Bahrani-Mougeot *et al.*, 2002; Wright *et al.*, 2007). The expression of type 1 pili is phase variable, regulated by an invertible element that overlaps with the promoter (Abraham *et al.*, 1985a). During UPEC infection, the type 1 pilus-associated tip adhesin, FimH, mediates adherence to and invasion of superficial umbrella cells of the urothelium (Hultgren *et al.*, 1985; Mulvey *et al.*, 1998). Studies by Sokurenko and colleagues suggest that type 1 binding to mannose is facilitated by conditions of high shear force because of an allosteric catch-bond mechanism (Thomas *et al.*, 2002; Le Trong *et al.*, 2010). Vaccines directed against type 1 pili and the tip adhesin, FimH, have been shown to be protective in both murine and primate models of acute cystitis, whether delivered mucosally or systemically (Langermann *et al.*, 1997, 2000; Poggio *et al.*, 2006). Most importantly, type 1 pili have been shown to be required for binding of UPEC to human urothelial tissue culture cells, and $FimH^+$, but not $FimH^-$, bacteria have been demonstrated to adhere to human bladder tissue *in situ* (Langermann *et al.*, 1997; Martinez *et al.*, 2000; Hung *et al.*, 2002). The nonadherent ASB strain 83972 has been used to therapeutically treat patients with chronically recurrent cystitis (Wullt *et al.*, 2001; Bergsten *et al.*, 2005, 2007) and provided the opportunity to test the role of UPEC adhesins in the human urinary tract (Bergsten *et al.*, 2007). This interesting study concluded that the expression of P, but not type 1, pili was important for colonization of the human urinary tract, largely based upon the differences in acute inflammation induced after inoculation of ASB 83972 carrying plasmids encoding either of these pili (Bergsten *et al.*, 2007). However, as these patients had a history of complicated UTI with significant bladder dysfunction and long-standing chronic bladder inflammation, the applicability of these findings to uncomplicated cystitis is uncertain. Thus, in summary, while the experimental evidence overwhelmingly supports a critical role for type 1 pili in bladder cell adherence by UPEC, this role remains to be clearly demonstrated in human patients with cystitis.

Human clinical studies

While the role of type 1 pili has been clearly demonstrated in diverse experimental models of acute UPEC UTI using both mouse and human tissue, evidence from clinical studies is mostly circumstantial. Several studies of UTI in women have investigated type 1 pilus expression by UPEC in an immunostaining analysis of infected urine sediments. They found that the frequency of type 1 pilus positive specimens from patients with acute UTI ranged from 40% to 76% (Pere *et al.*, 1987; Kisielius *et al.*, 1989; Lichodziejewska *et al.*, 1989), comparable to what was found in acutely infected mice (Hultgren *et al.*, 1985). Possible reasons for the relative lack of type 1 pilus expression in urine include that these bacteria represent the *fim* phase OFF nonadherent fraction or that they originate from the upper urinary tract. Indeed, type 1 piliated bacteria identified in urine collected from either mice or women with acute UTI were mainly associated with urothelial cells, and nonpiliated bacteria were mostly planktonic (Hultgren *et al.*, 1985; Kisielius *et al.*, 1989). While these early studies suggested that the expression of type 1 pili during *in vivo* infection is phase variable and tightly regulated, they do not preclude a critical role for type 1 pili in UTI pathogenesis. Although type 1 pilus genes are found in most *E. coli*, including nonpathogenic strains, they are not uniformly expressed. For example, members of the O157:H7 enterohemorrhagic *E. coli* pathotype do not express type 1 pili because of mutation of the *fim* switch regulatory region (Roe *et al.*, 2001; Shaikh *et al.*, 2007). Furthermore, there is strong evidence that the tip adhesin FimH has undergone pathoadaptive mutation in UPEC clinical isolates (Sokurenko *et al.*, 1994, 1995, 1998; Schembri *et al.*, 2000; Weissman *et al.*, 2006),

with several amino acid residues found to be under positive selection (Chen *et al.*, 2009). Mutation of these residues resulted in reduced virulence in a murine model of cystitis, strongly suggesting that FimH plays an important role *in vivo* during human UTI. In humans, the severity of UTI was increased and the immunological response was greater in children with infections caused by type 1 piliated UPEC strains (Connell *et al.*, 1996). Thus, the clinical data appear to support a role for type 1 pili in cystitis in at least a significant subset of patients. Thus, a recent paper from the Mobley laboratory concluded that 'molecular Koch's postulates of microbial pathogenesis have been satisfied for the type 1 fimbria of UPEC' (Snyder *et al.*, 2006).

Is there an alternate bladder adhesin?

Investigations to unearth a novel bladder adhesin that is required for cystitis have not borne fruit. A signature-tagged mutagenesis study was unable to find an adhesin other than type 1 pili that was required for *in vivo* infection in mice when type 1 pili were expressed (Bahrani-Mougeot *et al.*, 2002). *Escherichia coli* UTI are common in many mammalian species that receive routine veterinary care, including dogs, cats, cattle, and pigs. *Escherichia coli* isolates from dogs with UTI are indistinguishable from human UPEC isolates (Johnson *et al.*, 2001). In fact, UPEC strains have been found to transmit between people and their pets within a household (Johnson *et al.*, 2008), supporting the contention that UPEC are not host-adapted, but can easily pass between biological niches. Mice are also naturally susceptible to UTI caused by *E. coli*, as we have found that laboratory mice sometimes have pre-existing cystitis (T. J. Hannan and S. J. Hultgren, unpublished data), and experimental UPEC infection in mice closely mimics human disease (Rosen *et al.*, 2007; Hung *et al.*, 2009). Furthermore, the genomic data available now for several UPEC strains strongly indicate that UPEC are a genetically diverse pathotype, without a common virulence plasmid or pathogenicity-associated island that is required for infection (Brzuszkiewicz *et al.*, 2006; Lloyd *et al.*, 2009b). A leading research group in the field recently concluded that 'the varied virulence profile of *E. coli* strains causing acute cystitis suggests that diverse bacterial strains, expressing type 1 fimbriae trigger a convergent host response, involving pathways that give rise to the characteristic symptoms of acute cystitis' (Norinder *et al.*, 2011). Therefore, the evidence strongly supports a requirement of these adhesive fibers in initializing uncomplicated cystitis in patients with normally differentiated bladder urothelium, regardless of the piliation state of the bacteria in other urogenital niches or during later stages of infection, thus making type 1 pili an attractive target for anti-infective compounds or vaccines.

UPEC invade bladder urothelial cells

Uroplakin-dependent invasion pathways

UPEC invasion of superficial umbrella cells of the urothelium is a critical event in acute lower urinary tract disease. Early electron microscopy studies in rodent models of UPEC UTI observed what appeared to be intracellular bacteria within the superficial umbrella cells of the bladder urothelium during acute infection (Fukushi *et al.*, 1979; McTaggart *et al.*, 1990). *Ex vivo* treatment of acutely infected bladders with gentamycin was unable to sterilize the tissue, further suggesting that UPEC could reside intracellularly (Mulvey *et al.*, 1998). Subsequent *in vitro* studies have significantly expanded our understanding of UPEC invasion of urothelial cells. Type 1 pili-mediated binding to urothelial cells initiates signal transduction cascades that result in activation of Rho-GTPases and internalization by a zippering mechanism that involves actin rearrangement (Martinez *et al.*, 2000; Martinez & Hultgren, 2002). FimH is sufficient for invasion, as FimH-coated beads can also be internalized by urothelial cells (Martinez *et al.*, 2000). The receptor for type 1 pili appears to depend upon the differentiation state of the urothelial cells. Mature superficial umbrella cells express complexes of uroplakins on their luminal surface, and FimH has been shown to bind to mannosylated UPIa (Zhou *et al.*, 2001). *In vivo*, UPIa localizes to lipid rafts and disruption of lipid rafts abrogates UPEC invasion (Duncan *et al.*, 2004). Klumpp and colleagues utilized an immortalized normal human urothelial cell line with cell surface uroplakin expression to investigate the role of uroplakin binding on bacterial invasion. They found that UPIIIa, the only one of the four major uroplakins with a potential cytoplasmic signaling domain, undergoes phosphorylation subsequent to FimH binding to the uroplakin receptor complex via UPIa, resulting in an increase in intracellular calcium and enhanced invasion (Thumbikat *et al.*, 2009; Wang *et al.*, 2009).

Uroplakin-independent invasion pathways

In contrast, in urothelial cancer cell lines such as 5637 cells, uroplakins are not typically expressed on the cell surface. In these poorly differentiated urothelial cell lines, FimH binds to mannosylated $\alpha3\beta1$ integrins (Eto *et al.*, 2007). Increases in intracellular calcium inhibits UPEC invasion of 5637 cells (Eto *et al.*, 2008). The specific mechanism of invasion into immature urothelial cells has been reported to involve components of clathrin-coated

pits such as clathrin and the cargo adaptor protein AP-2 (Eto *et al.*, 2008). Caveolae and lipid rafts have also been reported to be necessary for invasion (Duncan *et al.*, 2004). Involvement of these two, traditionally independent, endocytic mechanisms suggests that multiple invasion pathways may occur in UPEC-infected 5637 cells. However, there is emerging evidence that clathrin localization in lipid rafts regulates some internalization pathways (Stoddart *et al.*, 2002), and disruption of lipid rafts abrogates UPEC invasion (Duncan *et al.*, 2004). Actin rearrangement during zippering of endocytic bacteria into 5637 cells involves focal adhesion kinase, phosphotidylinositol-3-kinase, and Rac1 and Cdc42 members of the Rho family of GTPases (Martinez *et al.*, 2000; Martinez & Hultgren, 2002; Song *et al.*, 2007a), as well as the action of microtubules (Dhakal & Mulvey, 2009).

Urothelial cells can expel invasive UPEC

After internalization, UPEC can reside within Rab27b/CD63/Caveolin-1 positive fusiform vesicles that resemble secretory lysosomes and are also involved in regulating the surface area of the apical plasma membrane, only to be expelled by a mechanism that requires TLR4, cyclic AMP, Rab27b, and caveolin-1 (Mulvey *et al.*, 2001; Bishop *et al.*, 2007; Song *et al.*, 2007a; Song *et al.*, 2009). Treatment of mice 2 h after UPEC infection with the drug forskolin, which increases cytosolic cyclic AMP, is accompanied by a reduction in the intracellular bacterial burden in the bladder (Bishop *et al.*, 2007). UPEC expulsion is thought to involve a TLR4-mediated mechanism and TLR4 signaling incompetent C3H/HeJ mice have higher intracellular bacterial burdens than C3H/HeN mice (Song *et al.*, 2007a). Taken together, these findings suggest that this exocytic mechanism occurs in mature superficial umbrella cells *in vivo* and may be an important early innate defense against invasive infection of the bladder.

Intracellular bacterial communities and acute UTI pathogenesis

UPEC escape the endocytic vesicle

Several research groups have now found that during experimental infection of the murine bladder, UPEC may elude expulsion from superficial umbrella cells and escape into the cytoplasm, where they can replicate rapidly while aggregating into intracellular bacterial communities (IBCs; Anderson *et al.*, 2003; Nicholson *et al.*, 2009; Blango & Mulvey, 2010; Li *et al.*, 2010; Wieser *et al.*, 2011). The mechanism of escape into the cytoplasm is unclear. Hindering the investigation of this critical step in UPEC pathogenesis is the fact that IBC formation does not typically occur in undifferentiated urothelial cells. In these cells, bacterial replication occurs in a limited fashion within a membrane bound vesicle, in some cases forming bacterial inclusions in structures that resemble multivesicular bodies (Mulvey *et al.*, 2001). However, treatment of undifferentiated urothelial cells with either membrane or actin destabilizing agents allows either bacterial escape from or alteration of the phagocytic vesicle such that robust bacterial proliferation with properties of IBCs may ensue (Eto *et al.*, 2006; Berry *et al.*, 2009). This suggests that the actin network, which has been shown to be much denser in undifferentiated urothelial cells compared with superficial umbrella cells, helps to restrict bacterial escape from the vesicle and/or proliferation. Furthermore, the difference in FimH receptors in undifferentiated ($\alpha3\beta1$ integrins) and differentiated (UPIa) urothelial cells may result in different UPEC invasion pathways and phagocytic trafficking. The classical UPEC virulence factor and pore-forming toxin, α-hemolysin, which is highly expressed in IBCs (Reigstad *et al.*, 2007), does not seem to play a role in vesicular escape *in vivo*, as a mutant UPEC strain lacking the α-hemolysin gene forms IBCs as well as the wild-type strain (Hannan *et al.*, 2008).

UPEC replicate within the urothelial cytoplasm

Upon escape from the vesicle and access to the urothelial cell cytoplasm, UPEC are able to replicate quickly with a doubling time of 30–35 min (Fig. 1a–e; Justice *et al.*, 2004). As bacterial replication within IBCs occurs in an intracellular niche that is protected from many of the innate immune defenses against luminal bladder colonization, such as phagocytosis by neutrophils (Justice *et al.*, 2004), approximately half of bladder bacteria appear to be intracellular at 12 h postinfection (hpi; Mulvey *et al.*, 1998). In this niche, they are also protected from antibiotics, particularly the first-line drug trimethoprim-sulfamethoxazole, which has increased efficacy against UTI because it concentrates in the urine but is relatively cell impermeant (Blango & Mulvey, 2010; Cusumano *et al.*, 2011). A recent study demonstrated that 16 antibiotics capable of killing the virulent cystitis isolate UTI89, *in vitro*, many of which could also eliminate intracellular UTI89 within bladder epithelial tissue culture cells, are ineffective in eliminating UTI89 from bladder tissue during *in vivo* infection (Blango & Mulvey, 2010). Thus, harboring antibiotic-tolerant bacteria within IBCs or a persistent intracellular niche (Eto *et al.*, 2006; Mysorekar & Hultgren, 2006; Blango & Mulvey, 2010) may provide a nidus for surviving pathogens to cause a relapse (treatment failure) or recurrent cystitis, respectively, once antibiotics are removed.

UPEC aggregation into IBCs requires type 1 pili expression and resembles biofilm formation

At 6 hpi, the number of IBCs detected in the murine bladder has been reported to range from 3 to 700 (median: ~40) after infection of 7–10-week-old C3H/HeN mice with 10^7 colony-forming units (CFU) of the UPEC strain UTI89 (Justice *et al.*, 2006a; Wright *et al.*, 2007; Rosen *et al.*, 2008b; Chen *et al.*, 2009; Cusumano *et al.*, 2010; Schwartz *et al.*, 2011). UPEC aggregation into IBCs requires continued type 1 pili expression after invasion (Wright *et al.*, 2007). Amino acid residues in FimH that are under positive selection in UPEC isolates from human patients have been found to function in IBC formation, independent of mannose-binding (Chen *et al.*, 2009). Directed mutations in two of these residues (A27V/V163A), which are not located near the mannose-binding pocket, in a virulent UPEC strain disrupted *in vivo* urothelial invasion and IBC formation, but did not affect *in vitro* binding to mannose or urothelial cells. Subsequent maturation of the IBC is accompanied by production of structural components otherwise associated with UPEC biofilm such as antigen 43 and a polysaccharide-rich matrix (Anderson *et al.*, 2003). In addition to antigen 43, the secreted amyloid fiber curli and several other UPEC autotransporter proteins including UpaC, UpaG, and UpaH have been implicated in biofilm growth; however, their role in IBC formation remains to be elucidated (Ulett *et al.*, 2007; Valle *et al.*, 2008; Cegelski *et al.*, 2009; Allsopp *et al.*, 2010, 2012). IBC maturation involves a partially defined differentiation program. Within 12–16 h, rapidly replicating bacteria first take on a coccoid morphology and then as the IBC matures, the bacteria become more rod shaped again and begin to flux away from the IBC and emerge from the dying urothelial cells, often in filamentous form, into the lumen to colonize and invade neighboring cells (Justice *et al.*, 2004). Deletion of the cell division inhibitor gene, *sulA*, abolishes the ability of UTI89 to filament, a property that has been associated with resistance to neutrophil attack. The *sulA* mutant is capable of forming early IBCs in the first 6–8 h, but subsequent rounds of IBC formation no longer occur. As a consequence, bladder colonization is reduced at 24 h postinfection, suggesting that UPEC filamentation is necessary for virulence after the first round of IBC formation in the immunocompetent host (Justice *et al.*, 2006b). Thus, the IBC pathway resembles biofilm formation in that both aggregation and dispersal of UPEC are critical for bacterial persistence.

IBCs are clonal

Microscopy studies using a co-inoculum of green fluorescent protein expressing (GFP^+) UPEC and nonexpressing (GFP^-) UPEC have demonstrated that IBCs are clonal, originating from a single invasive bacterium (Schwartz *et al.*, 2011). IBCs were either entirely GFP^+ or GFP^-, and when two IBCs occurred in the same superficial umbrella cell, they did not mix together but were generally distinct. An exception to this was observed when polysaccharide capsule-deficient mutants of the K1 UPEC strain UTI89 were co-inoculated with wild-type UTI89. In this case, K1 capsule-deficient UTI89 mutants were markedly deficient in their ability to aggregate and form IBCs, but could become incorporated into wild-type IBCs when both a mutant and a wild-type bacterium had invaded the same superficial umbrella cell (Anderson *et al.*, 2010). This suggests that K1 capsule contributes to UPEC aggregation within the superficial umbrella cell cytoplasm, likely by providing the polysaccharide-rich matrix of the intracellular biofilm, as the defect can be complemented *in trans*. Although the capsule-deficient mutants were able to replicate within the cytoplasm of the superficial umbrella cells, the lack of a biofilm-like organization appeared to leave the bacteria much more susceptible to neutrophil infiltration and phagocytosis. As UPEC strains vary in the composition of their capsule, it is unclear whether disruption of capsule formation in other UPEC K serotypes also impact upon IBC formation.

Chaperone/usher pathway and outer membrane structures in acute pathogenesis

Like other chaperone-usher pili, type 1 pili, which are necessary for urothelial adherence, invasion, and IBC formation, are anchored at the outer bacterial membrane (Waksman & Hultgren, 2009). Therefore, secretion of pilus subunits into the periplasm, proper folding of these subunits within the periplasm, and translocation of the subunits across the outer membrane usher, which catalyzes their insertion into the growing pilus, are all processes required for proper pilus assembly and UTI pathogenesis. SurA is a bacterial periplasmic peptidyl-prolyl isomerase that facilitates insertion of porins such as OmpA into the outer membrane (Lazar & Kolter, 1996). Studies have shown that SurA is required for normal insertion of the FimD outer membrane usher (Watts & Hunstad, 2008). Thus, strains lacking the *surA* gene are impaired in urothelial adherence, invasion, and IBC formation (Justice *et al.*, 2006a). OmpA is necessary for IBC maturation independent of type 1 pili production, suggesting that it plays a role in intracellular aggregation or survival (Nicholson *et al.*, 2009). OmpT confers resistance to antimicrobial peptides in human urine (Hui *et al.*, 2010). Other periplasmic protein folding catalysts such as the disulfide bond (Dsb) enzymes are also required for

full virulence of UPEC and other Gram-negative pathogens (Heras *et al.*, 2009). DsbA is a periplasmic oxidoreductase involved in the correct folding of important virulence factors such as P fimbriae and flagella (Dailey & Berg, 1993; Jacob-Dubuisson *et al.*, 1994). Deletion of the *dsbA* gene in UPEC results in attenuation in an acute mouse bladder infection model (Totsika *et al.*, 2009). Thus, strategies aimed at inhibiting the function of periplasmic proteins necessary for proper folding and targeting of outer membrane proteins may represent novel approaches to treat UPEC infections.

Central metabolic pathways

Recently, studies have revealed that proper regulation of central metabolism pathways, such as the tricarboxylic acid cycle, are essential for acute UPEC virulence and IBC formation in the urinary tract, but not for planktonic growth in urine (Alteri *et al.*, 2009a; Kostakioti *et al.*, 2009; Hadjifrangiskou *et al.*, 2011). The QseBC two-component system is found in many Gram-negative pathogens and appears to play a critical role in regulating virulence factor expression (Sperandio *et al.*, 2002; Clarke *et al.*, 2006; Bearson & Bearson, 2008; Rasko *et al.*, 2008; Bearson *et al.*, 2010; Khajanchi *et al.*, 2011; Wang *et al.*, 2011). Deletion of the sensor kinase QseC in UPEC and other pathogens interferes with dephosphorylation of the cognate QseB response regulator unleashing an uncontrolled positive feedback loop of excess QseB expression that causes pleiotropic effects in the bacterial cell, including reduced virulence factor expression, such as type 1 pili, and reduced virulence and IBC formation *in vivo* (Kostakioti *et al.*, 2009; Hadjifrangiskou *et al.*, 2011). Gene expression, metabolomic, and directed mutation analysis surprisingly found that the altered virulence factor regulation in a *qseC* deletion mutant was because of overactivation of QseB leading to pleiotropic defects in central metabolism (Hadjifrangiskou *et al.*, 2011). In particular, two different mutants unable to complete the TCA cycle phenocopied the *qseC* deletion mutant strain. Another study found that peptide transport, the TCA cycle, and gluconeogenesis are each essential for *in vivo* virulence of UPEC in the bladder, but not for *in vitro* growth in urine (Alteri *et al.*, 2009a). Glycolysis was not required for *in vivo* virulence, suggesting that glucose is not a major carbon source for UPEC *in vivo*. While glucose is typically absent in the urine and thought to be readily available in the host cell cytoplasm, it does not seem to be available to the rapidly replicating UPEC within the IBC, as the latter readily stain with X-gal at 6 hpi indicating β-galactosidase expression (Justice *et al.*, 2006a), which only occurs in the presence of lactose when glucose levels are low (Jacob & Monod, 1961; Wilson *et al.*, 2007). Moreover, expression analysis of host cell mRNA obtained from urothelial cells by laser capture microdissection found that genes involved in glucose import, such as hexokinase (*Hk2*) and glucose transporter 1 (*Glut1*), were upregulated in urothelial cells that were in close proximity to an IBC (Reigstad *et al.*, 2007). Taken together, these studies support the hypothesis that rapid bacterial replication in the IBC quickly expends available urothelial glucose such that other carbon sources, such as lactose and amino acids, are preferentially utilized by UPEC. It is unclear how lactose is available to the bacteria, as it is not typically present in the eukaryotic cell cytoplasm. These findings suggest that the *in vivo* defects of the QseC deletion mutant are not solely because of reduced virulence factor expression, for example, type 1 piliation, but also indicate a requirement for these central metabolic pathways, *per se*, in IBC formation.

Iron acquisition

Iron acquisition is another critical requirement for bacterial virulence as bacteria need iron to grow and the host has many mechanisms for sequestering iron during infection (Williams & Carbonetti, 1986; Valdebenito *et al.*, 2005; Skaar, 2010). A number of iron acquisition-associated genes common to all *E. coli* are under positive selection in UPEC strains isolated from the urinary tract (Chen *et al.*, 2006). UPEC typically have multiple, seemingly redundant iron acquisition systems, and they have been shown to be highly upregulated in the IBC, when compared with UPEC grown either under aerobic or under anaerobic conditions *in vitro* or during cecal colonization of gnotobiotic mice (Reigstad *et al.*, 2007). Urothelial cells in close proximity to the IBC upregulate genes for the transferrin receptor and for lipocalin 2, both of which are involved in preventing bacterial acquisition of iron (Reigstad *et al.*, 2007). However, UPEC can scavenge iron from heme and a deletion mutant lacking the heme transporter ChuA, which is highly expressed in the IBC, but not elsewhere, forms significantly smaller IBCs *in vivo* (Reigstad *et al.*, 2007). Genes involved with the synthesis of all three siderophores produced by the UPEC strain UTI89 (enterobactin, salmochelin, and yersiniabactin) are also highly expressed in the IBC (Reigstad *et al.*, 2007). Metabolomic studies have revealed that UPEC clinical isolates preferentially synthesize the siderophores yersiniabactin and salmochelin, but not enterobactin or aerobactin, when grown under iron-limiting conditions compared with fecal *E. coli* strains isolated from the same patient (Henderson *et al.*, 2009). Both salmochelin and enterobactin production have been associated with resistance to the

antibacterial effects of lipocalin 2 (Raffatellu *et al.*, 2009; Bachman *et al.*, 2011). However, in the pyelonephritis isolate CFT073, which does not synthesize yersiniabactin, aerobactin appears to play an important role in bladder fitness, suggesting that these two siderophores may have overlapping functions (Garcia *et al.*, 2011). UPEC strains that cause ASB also produce multiple siderophores, with the best-characterized ASB strain 83972 known to produce enterobactin, salmochelin, aerobactin, and yersiniabactin (Watts *et al.*, 2012). Thus, bacterial iron-acquisition by multiple redundant systems has been selected for in UTI.

IBC formation proceeds through several rounds during acute infection

Bacterial cycling through IBCs does not typically proceed indefinitely (Fig. 1). Although several cycles of IBC formation can occur, in mice that spontaneously resolve infection each successive round is associated with slower bacterial replication and smaller IBCs, eventually resulting in small collections of intracellular bacteria (Justice *et al.*, 2004). This deceleration of the IBC cycle coincides with the exfoliation response of the superficial umbrella cells to infection. As mature umbrella cells are lost and immature underlying cells differentiate to take their place, these maturing cells, which are smaller, cannot support large IBCs (Justice *et al.*, 2004). Smaller IBCs have also been observed in immature umbrella cells when mice are infected several hours after chemical exfoliation of the bladder (Mysorekar & Hultgren, 2006). This restriction in IBC formation in immature umbrella cells may contribute toward the resolution of infection.

IBCs are commonly produced by numerous *Enterobacteriaceae* and are found in human UTI

The IBC pathway has been observed to occur in all mouse strains tested and in experimental infections in C3H/HeN mice with 15 of 18 human UPEC isolates from a clinical study of UTI, including some without common putative UPEC virulence factors such as hemolysin (Garofalo *et al.*, 2007). The remaining three strains unable to form IBCs were also found to be unable to invade the mouse urothelium. The IBC pathway is not confined to infection of mice with UPEC. We have found that other important Gram-negative uropathogens that express type 1 pili, such as *Klebsiella pneumonia, Enterobacter* spp., and *Citrobacter freundii*, also utilize the IBC pathway in a FimH-dependent manner (D. A. Rosen and S. J. Hultgren, unpublished data; Rosen *et al.*, 2008b, c). Importantly, translational studies in women with acute episodes of recurrent cystitis with UPEC found evidence of IBCs in 18% of urine sediments (Fig. 2), but never in urines from healthy controls or when the causative agent of the UTI was a Gram-positive pathogen (Rosen *et al.*, 2007). This level of detection of exfoliated IBCs in the urine sediment is similar to the sensitivity of this method in detecting IBC formation in mice during early acute infection (T. J. Hannan and S. J. Hultgren, unpublished data). Together, these findings suggest that the IBC pathway is a universal mechanism for establishment of UTI in mammalian bladders by Gram-negative uropathogens that express type 1 pili and have the capability to invade the urothelium.

IBC formation constitutes a stringent population bottleneck during acute cystitis

The predominance of the IBC pathway in UPEC pathogenesis during early acute cystitis (Mulvey *et al.*, 1998) and the finding that each IBC arises from a single bacterial clone during experimental cystitis (Schwartz *et al.*, 2011) together raised the hypothesis that the ability to replicate within superficial umbrella cells and form IBCs constituted a stringent population bottleneck, restricting the diversity of persisting bacteria. To test this, Seed and colleagues generated a set of 40 isogenic mutants of the cystitis strain UTI89, each of which contain a unique genetic 'tag' that is engineered into the lambda bacteriophage attachment site and can be detected by multiplex PCR (Schwartz *et al.*, 2011). By co-inoculating all 40 tagged strains simultaneously, the relative diversity of the bacterial population during experimental infection was assessed in different host niches over time. They found that bacterial diversity in the urinary bladder as early as 6 hpi roughly correlated with the number of IBCs formed, suggesting a founder effect where those clones that form IBCs become the dominant clones within the overall bacterial population later in infection. In agreement with this, by 24 hpi bacterial diversity was significantly reduced in both the bladder and kidneys and the largest percentage of tags found in the urine, bladder lumen, and kidneys were also found in the gentamycin-protected (intracellular) bladder niche at both 24 hpi and 7 dpi, but not at 6 hpi, suggesting that the dominant clones during acute and subacute UTI originated from first-generation IBCs. As these tagged clones were isogenic and equally fit, this population bottleneck is at least partially stochastic (Fig. 1) and likely represents a weak link in UPEC pathogenesis that can be targeted by novel anti-infective drugs that prevent IBC formation.

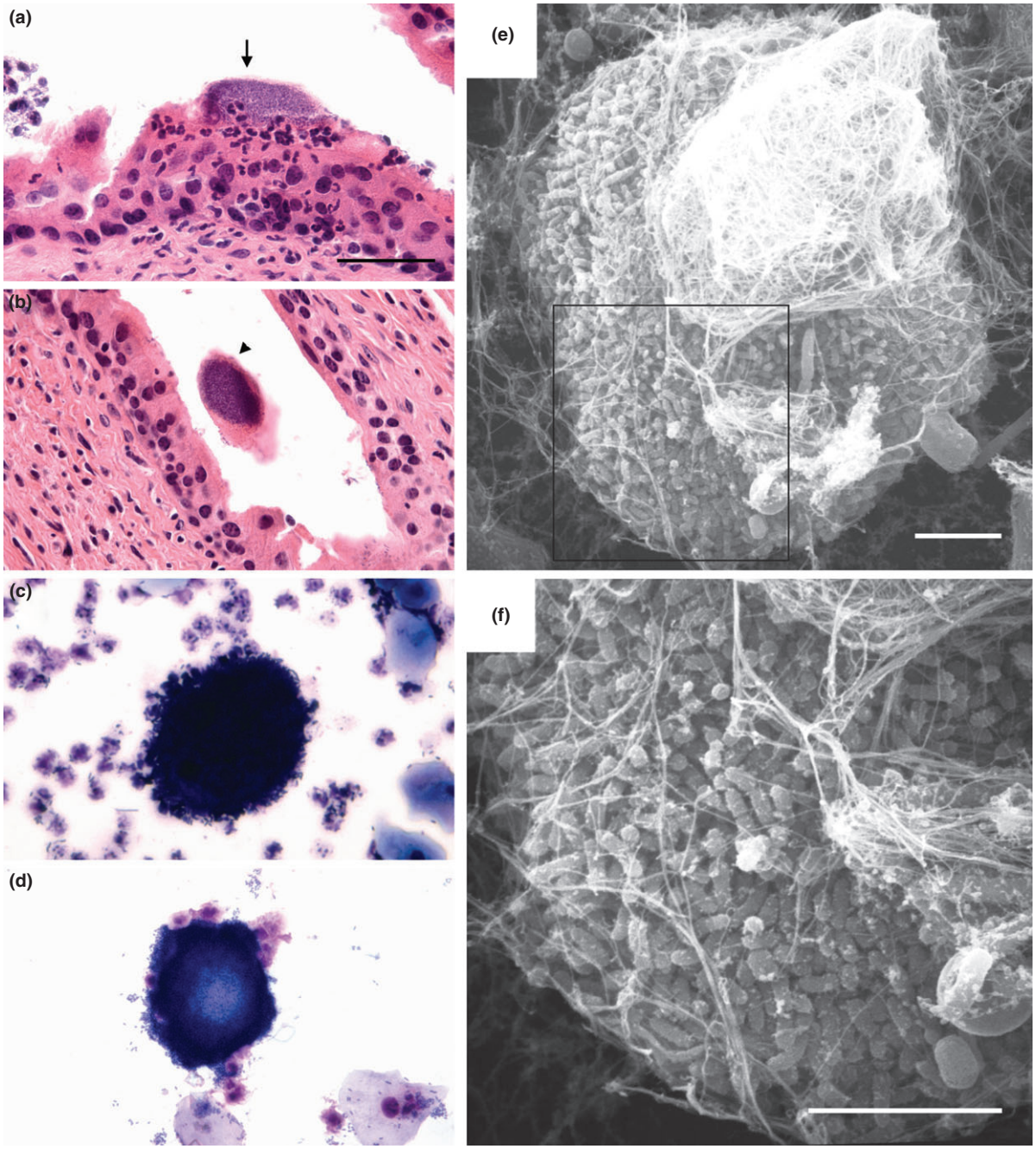

Fig. 2. IBC-like biofilms were found in the urines of women suffering from an episode of acute uncomplicated cystitis with UPEC (Rosen *et al.*, 2007). (a–d) An *E. coli* strain was isolated from a human patient with recurrent cystitis. Urine cytology from this patient was positive for the presence of IBC-like structures and bacterial filaments, which are a hallmark of bacterial emergence from the IBC. (a, b) this strain was inoculated into the bladders of C3H/HeN mice where it progressed through the IBC pathogenic cycle. Several IBCs were observed by Hematoxylin & Eosin staining in the mouse bladder at 30 hpi (a, arrow). IBCs could also be seen exfoliated into the bladder lumen (b, arrowhead). (c, d) urine collected from mice at this time point was positive for IBCs (c). These IBCs were similar in morphology and size to those formed by the same *E. coli* isolate in the original human urine specimen (d). (e, f) scanning Electron Microscopy analysis of cystitis urines deemed positive for IBCs and filaments captured large bacterial biofilm-like collections (e, inset shown in f) composed of bacteria with a smaller, more coccoid morphology than typical *E. coli*. Scale bars: 50 μm (a–d) and 5 μm (e, f). Figure modified from Rosen *et al.*, 2007.

Diabetes and UTI

The acute population bottleneck likely exists because UPEC colonization is counteracted by robust innate mucosal immune responses to infection. For example, people with diabetes are at particular risk for ASB and symptomatic upper UTI (Patterson & Andriole, 1997). While the reasons for this association are unclear, the propensity toward ASB suggests that the bladder environment is altered in these individuals. In experimental UTI studies in mice with streptozocin-induced diabetes, UPEC ID_{50} values are over 200-fold lower than in nondiabetic mice, and these diabetic mice are exquisitely sensitive to persistent high titer bladder and kidney infections at inocula well below those needed to consistently establish infection and form IBCs in nondiabetic mice (Rosen *et al.*, 2008a). Thus, the diabetic state in mice appears to alter the host population bottleneck during acute infection, potentially lessening the requirement for IBC formation and favoring an ASB-like colonization of the urinary bladder. Importantly, these differences are much less dramatic in C3H/HeJ mice, which lack TLR4 signaling and have muted inflammatory responses, suggesting that defects in the innate inflammatory response contribute to the increased susceptibility of streptozocin-treated mice to UTI.

Mucosal immune responses to lower urinary tract infection

Bladder innate immune defense

Innate immune signaling has long been known to play a significant role in host defense against Gram-negative pathogens and has been reviewed extensively elsewhere (Ragnarsdottir *et al.*, 2011). For example, inducible factors such as complement (Springall *et al.*, 2001) and the antimicrobial peptides such as cathelicidins (Chromek *et al.*, 2006) are secreted by the host into the urine and can have antimicrobial effects. Foremost among the innate responses to UPEC is the signaling of the LPS-sensing pattern recognition receptor, TLR4. The requirement for TLR4 signaling in controlling bladder infection is complex (Hagberg *et al.*, 1984; Hopkins *et al.*, 1996, 1998; Hannan *et al.*, 2010). Mice deficient in TLR4 signaling are generally considered susceptible to an ASB-like persistent bladder colonization that is not directly harmful to the host (Svanborg *et al.*, 2006). However, these mice also uniformly develop severe ascending infection to the kidneys and are very susceptible to urosepsis and death (Hagberg *et al.*, 1984; Haraoka *et al.*, 1999; Fischer *et al.*, 2006, 2010; Hannan *et al.*, 2010). Svanborg and colleagues have demonstrated that children with ASB, which does not typically result in severe ascending infection, are more likely to have certain *Tlr4* promoter polymorphisms that are associated with reduced TLR4 expression while maintaining normal TLR4 signaling (Ragnarsdottir *et al.*, 2007, 2010).

Bladder TLR4 signaling in bladder host defense

Studies utilizing murine bone marrow chimeras found that the TLR4-dependent innate immune responses have both stromal (radioresistant) and hematopoietic (radiosensitive) components (Schilling *et al.*, 2003), suggesting that urothelial cells and/or other radioresistant cells play an important role in initiating inflammation. Although urothelial cells express TLR4, the adaptor protein CD14 is also required for mounting responses to LPS and is not thought to be expressed in normal, uninfected and untransformed urothelial cells (Samuelsson *et al.*, 2004; Smith *et al.*, 2011). However, both FimH and the ceramide linkage of the P pilus globoside receptors have been shown to induce TLR4 signaling, independent of LPS (Fischer *et al.*, 2006). Nevertheless, the picture that is emerging is that the normal, uninflamed urinary bladder is a fairly tolerant (privileged) mucosal site, relying on physical defenses to clear transient colonization by less virulent bacteria while maintaining the integrity of the mucosal barrier.

UPEC invasion of urothelial cells enhances the innate immune response

Some UPEC strains are more invasive and gain entry into host niches that are not privileged: the urothelial endosome and cytoplasm. UPEC invasion of a bladder cancer cell line greatly enhances urothelial interleukin 6 (IL-6) production in an LPS-dependent manner (Schilling *et al.*, 2001b). CD14 gene expression is robustly induced at 2 hpi in the mouse bladder (Duell *et al.*, 2012), and it may be that invasion of normal urothelial cells induces CD14 expression, thereby potentiating the TLR4 responses to infection. TLR4 can signal through two different downstream pathways: via MyD88 or TRIF. The former is generally considered to predominate in TLR4 signaling initiated at the cell surface, whereas the latter is typically associated with intracellular endosomal TLR4 signaling (Casanova *et al.*, 2011). However, a recent study using nonpiliated *E. coli* expressing either type 1 or P (pyelonephritis-associated) pili with a plasmid system found that type 1 piliated bacteria favored MyD88-dependent signaling, whereas P piliated bacteria also induced TRIF-dependent signaling (Fischer *et al.*, 2006). MyD88-dependent TLR4 signaling in response to UPEC infection of bladder epithelial cancer cell lines that express CD14 primarily results in activation and nuclear localization of the transcription factor NF-κB and proinflammatory cytokine production (Song & Abraham, 2008).

TLR5-mediated signaling is also MyD88-dependent and contributes to the acute inflammatory response to UPEC infection (Andersen-Nissen *et al.*, 2007). As a result, C57BL/6 mice lacking TLR5 are more susceptible to cystitis and pyelonephritis. The TLR5 ligand, flagellin, is expressed by most UPEC and is associated with motility, which contributes to ascension of the urinary tract (Lane *et al.*, 2005; Wright *et al.*, 2005). Recently, one group reported that normal human ureter cells grown in tissue culture respond to flagellin, but not LPS, suggesting that TLR5 may play a critical role in early urothelial inflammatory responses to UPEC infection (Smith *et al.*, 2011).

Early effector responses in the urinary tract

An additional MyD88- and TRIF-independent TLR4 response pathway involving the second messengers Ca^{2+} and cAMP has been discovered in urothelial cells (Song *et al.*, 2007b). This pathway leads to a more immediate IL-6 response from the urothelium and also mediates expulsion of endosomal UPEC (Song *et al.*, 2007a). The urothelium not only produces IL-6, but also IL-8 in response to UPEC infection (de Man *et al.*, 1989; Hedges *et al.*, 1991; Ko *et al.*, 1993; Godaly *et al.*, 2000). IL-6 is a cytokine that initiates broad proinflammatory effects within the urinary tract, and IL-8 is a chemotactic cytokine, which attracts granulocytes to the urothelium. Granulocytes play a critical role in controlling UPEC infection as depletion of neutrophils in TLR4-competent C3H/HeN mice results in very severe UTI that is worse than that seen in TLR4-incompetent C3H/HeJ mice, where neutrophil responses are present but muted (Haraoka *et al.*, 1999). Mast cells also play a critical role in early effector responses, producing large amounts of histamine, eicosanoids, and TNFα within the first hour of infection with $FimH^+$ UPEC (Malaviya *et al.*, 1999; Malaviya & Abraham, 2000; Malaviya *et al.*, 2004). Importantly, mast-cell-deficient mice have muted granulocyte recruitment and are compromised in their ability to clear acute infection (Malaviya *et al.*, 2004). Dendritic cells and macrophages are also present in the uninfected mouse bladder and increase in number and become activated consequent to UTI (Hirose *et al.*, 1992; Schilling *et al.*, 2003). While these mononuclear cells are thought to play an important role in host defense against UTI, experimental studies have failed to demonstrate a specific role for these cells during infection (Engel *et al.*, 2006). TRIF-dependent TLR4 signaling, which activates the IRF3/IRF7 complex and induces type 1 interferon production, has recently been demonstrated in response to UPEC. FimH directly induces type 1 interferon production in the vaginal mucosa (Ashkar *et al.*, 2008), and others have found that TLR4-dependent type 1 interferon production plays a role in controlling experimental pyelonephritis by UPEC (Fischer *et al.*, 2010). Currently, it is unclear how these early innate responses interact to affect bladder mucosal immunity.

UPEC actively dampens the innate immune response

While the urothelial inflammatory response is augmented by *E. coli* invasion of urothelial cells (Schilling *et al.*, 2001b), UPEC have adapted to an intracellular pathway by actively blocking TLR4 signaling, NF-κB activity, and proinflammatory cytokine production in urothelial cells compared with K-12 strains (Hunstad *et al.*, 2005; Billips *et al.*, 2007, 2008; Lloyd *et al.*, 2009a; Yadav *et al.*, 2010). This NF-κB blockade also results in exfoliation of the superficial umbrella cells of the urothelium by a mechanism that resembles apoptosis, thereby removing infected urothelial cells (Mulvey *et al.*, 1998, 2000). Exfoliation is dependent upon type 1 pili production and blockade with a pan-caspase inhibitor increased the bacterial burden during acute infection (Mulvey *et al.*, 1998). However, exfoliation comes with a significant cost, unveiling deeper layers of the urothelium and whether this works to the advantage of the host or the bacteria is probably a function of timing and mouse strain. Host cell exfoliation prior to IBC maturation, as commonly happens in C57BL/6J mice, removes infected cells before the bacteria have emerged, but loss of urothelial integrity after or coincident with UPEC emergence from IBCs could facilitate bacterial colonization of deeper layers of the urothelium.

Adaptive immune cell responses

While the critical roles of innate immune cells in controlling UPEC UTI is well established, the role of adaptive immune cells and the mechanisms by which UPEC are eliminated by the host if the innate responses fail to control acute infection are less well understood. The fact that recurrent cystitis is such a troublesome problem suggests that at least some fraction of the susceptible human population does not develop long-lasting protective adaptive immunity consequent to natural UPEC infection of the urinary bladder (Kantele *et al.*, 1994). The reasons for this are unknown. Yet, 75% of women with a prior cystitis episode do not have a recurrence within 6 months of the initial UPEC UTI (Foxman *et al.*, 2000). Furthermore, several studies suggest that adaptive responses may play a role in limiting acute infection upon re-exposure to UPEC, at least in some genetic backgrounds (Hopkins *et al.*, 1993; Langermann *et al.*, 1997; Thumbikat *et al.*, 2006). The T helper ($CD4^+$) cell adaptive responses to UPEC infection are unknown, although $CD8^+$ T cells are reportedly recruited to UPEC-infected bladders 24 hpi (Sivick &

Mobley, 2010). In BALB/c mice, $CD4^+$ and $CD8^+$ T cells and IgA+ B cells only infiltrate the bladder upon infection and may play an important role in directing the immune response and providing humoral and cellular immunity (Hirose *et al.*, 1992). These host responses typically polarize toward either a type 1 (T_H1), a type 2 (T_H2), or an IL-17-mediated (T_H17) T helper lymphocyte response that are classically thought to target intracellular pathogens, eukaryotic parasites, and extracellular bacterial infections, respectively. A number of other T helper cell and innate lymphoid cell populations have been described in mucosal tissue, with pro- and anti-inflammatory properties (Cella *et al.*, 2009; Monticelli *et al.*, 2011). In a C57BL/6J model of UTI, both γ-interferon signaling and γδ-T cells, but not αβ-T cells, were shown to contribute to clearance of UTI, suggesting that a helper T-cell-independent type 1 host response to UPEC infection mediates clearance (Hopkins *et al.*, 1993; Jones-Carson *et al.*, 1999). γδ-T cells have been found to produce IL-17 during acute infection, contributing to bacterial clearance in C57BL/6J mice (Ingersoll *et al.*, 2008; Sivick *et al.*, 2010). The presence of γδ-T cells has also been reported in the uninfected bladder of BALB/c mice, and they accumulate in both the bladder and kidney in response to experimental UTI (Matsukawa *et al.*, 1994). Further work is needed to understand the role of lymphoid cells in innate and adaptive immunity of the urinary bladder.

UPEC infection and urothelial renewal

UPEC binding and invasion also elicit rapid and robust innate responses by the host urothelium, such that UPEC infection may have significant implications for normal epithelial renewal and the course of UTI (Mysorekar *et al.*, 2002). Gene expression analysis of C57BL/6J mouse bladders early in UPEC infection identified a key regulator of urothelial proliferation and differentiation, bone morphogenetic protein 4 (Bmp4), which is a member of the TGFβ superfamily of secreted signaling molecules (Mysorekar *et al.*, 2002). Bmp4 is antiproliferative and promotes differentiation of epithelial cells. In the unperturbed bladder, it is made constitutively by stromal cells in the lamina propria, and normal urothelial turnover is slow, taking many months (Mysorekar *et al.*, 2009). However, in response to UPEC infection, Bmp4 expression is sharply downregulated, resulting in activation of the urothelial stem cell niche, which rapidly replenishes urothelial cells lost to exfoliation. Activation of the stem cell niche and eventual restoration of the umbrella cell layer after UPEC infection was disrupted upon ablation of the Bmp4 receptor and did not occur after chemical exfoliation with protamine sulfate. Urothelial stem cells also initiate feedback signals that affect their proliferation as they secrete Sonic hedgehog (Shh) in response to UPEC infection, which in turn activates the urothelial stem cell niche by inducing stromal cells to secrete Wnt signals (Shin *et al.*, 2011). This suggests that innate immune signaling in response to UPEC infection of the bladder modulates Bmp4, Shh, and Wnt signaling and activates the urothelial stem cell niche.

Models of UPEC persistence in the urinary bladder

Latent persistence

UPEC invasion of urothelial cells is critical not only for the establishment of acute infection through IBC formation, but also for chronic persistence within an intracellular reservoir (Mulvey *et al.*, 1998, 2001; Schilling *et al.*, 2002; Fig. 1f). Upon resolution of active infection and elimination of bacteriuria in C57BL/6J mice, UPEC remain within the murine urothelium inside LAMP1-positive vesicles (Eto *et al.*, 2006; Mysorekar & Hultgren, 2006). These rosettes of typically 4–10 nonreplicating bacteria can remain viable for months in the murine host without eliciting a measurable inflammatory response. Therefore, these rosettes have been termed the quiescent intracellular reservoir (QIR). Upon epithelial turnover, these bacteria are capable of emerging to seed a new acute infection (Mysorekar & Hultgren, 2006) and may represent a nidus for recurrence months after the acute infection. While a low titer infection suggestive of a bacterial reservoir is found in many mouse strains, BALB/c mice have persistent titers that can be tenfold higher than those seen in C57BL/6J mice, while maintaining sterile urines (Hannan *et al.*, 2010). We have found that BALB/c mice have increased number of QIRs, as well as small collections of what appear to be extracellular bacteria within the urothelium surrounded by granulocytic inflammation (T. J. Hannan and S. J. Hultgren, unpublished data). It is possible that these apparent microabscesses result from bacterial emergence from QIRs in deeper layers of the urothelium, raising the hypothesis that bacterial emergence from intracellular reservoirs may participate not only in chronic, recurrent UTI (rUTI) but also in the etiology of other chronic bladder diseases, such as overactive bladder (OAB) and interstitial cystitis (IC).

Intramacrophage survival

Recently, it was demonstrated that some UPEC strains are able to survive within primary mouse bone marrow-derived macrophages (BMM) up to 24 h postinfection (Bokil *et al.*, 2011). In this study, UPEC strain UTI89 was localized to a $Lamp1^+$ vesicular compartment within BMMs. Intracellular survival was also demonstrated in

human monocyte-derived macrophages, suggesting that UPEC may subvert macrophage antimicrobial pathways. Interestingly, the Crohn's disease-associated adherent and invasive *E. coli* (AIEC) strain LF82, which is closely related to UPEC and can invade intestinal epithelial cells by a type 1 pili-dependent mechanism, can also survive within the macrophage phagolysosome (Bringer *et al.*, 2006). It is possible that intramacrophage survival could contribute to the pathology and/or chronicity of UPEC UTI, and perhaps also dissemination from the bladder to distal sites.

Chronic cystitis

Mouse models of chronic UTI have been described (Hopkins *et al.*, 1996, 1998), but until recently these were poorly understood. A comparative approach, screening several mouse strains for chronic bladder infection (chronic cystitis), found that all TLR4-responsive C3H background strains screened as well as the closely related strains, CBA/J and DBA/2J, were susceptible to chronic cystitis in an infectious dose-dependent manner with the human cystitis isolate UTI89 (Hannan *et al.*, 2010). Chronic cystitis was defined by the presence of high titer ($> 10^4$ CFU mL^{-1}) persistent bacteriuria and high titer ($> 10^4$ CFU per organ) bladder colonization at 4 wpi. Chronic cystitis was typically accompanied by urothelial hyperplasia with loss of terminal urothelial differentiation as indicated by a lack of uroplakin surface expression, and chronic inflammation, including the presence of lymphoid aggregates and isolated lymphoid follicles. These lesions closely resemble follicular cystitis in human patients with persistent bacteriuria or rUTI (Hansson *et al.*, 1990; Schlager *et al.*, 2011). Biomarkers of chronic cystitis in mice were identified at 24 hpi, including elevated serum IL-5, serum and urine IL-6, G-CSF (Csf2) and KC (CXCL1), weight loss, and severe pyuria. The presence of these biomarkers correlated with the development of severe bladder inflammation with urothelial necrosis at 24 hpi, suggesting that acute immunopathology predisposed to chronic bacterial infection. This appeared to be the case, as C3H/HeN mice immunosuppressed with high-dose dexamethasone therapy were dramatically protected against chronic cystitis, despite having similar levels of bacterial infection at 24 hpi. Immunodeficient C3H strains lacking lymphocytes (severe combined immunodeficient, or SCID, mice) or TLR4 signaling (C3H/HeJ) also had reduced incidences of chronic cystitis.

Recurrent cystitis

The consequences of chronic cystitis can be serious even after clearance of infection with antibiotic therapy (Hannan *et al.*, 2010). We found that the development of chronic cystitis lasting 14 or 28 days, but not 1 day (grouping mice by serum KC as a biomarker of chronic cystitis) prior to antibiotic therapy to clear the infection dramatically sensitized these individuals to recurrent chronic cystitis with severe acute symptomatology upon challenge with a differently marked UPEC strain. Those mice that had spontaneously resolved infection were protected from challenge. Thus, these studies have identified an early host–pathogen checkpoint that not only determines the outcome of acute infection, but also dictates susceptibility to recurrent cystitis (Fig. 3). The mechanism of this acute checkpoint and whether similar biomarkers can be identified in women with recurrent cystitis is unclear. However, this is the first model of recurrent cystitis that mimics two important aspects of the clinical problem: the lack of protective immunity and the presence of exacerbated symptoms. Recently, it was found that a TLR4 polymorphism, which is associated with decreased TLR4 signaling and cytokine production *in vitro,* significantly decreased the risk of recurrent cystitis in premenopausal women (Hawn *et al.*, 2009). Thus, understanding the mechanisms of chronic and recurrent cystitis in C3H mice may elucidate important bladder mucosal immune responses to bacterial infection and aid in the development of vaccines targeting UPEC.

IBC formation occurs primarily during acute cystitis

In TLR4-responsive mice with chronic cystitis, neither IBCs nor QIRs are observed at 4 wpi. The reasons for the cessation of IBC formation during subacute and chronic infection are unknown, but may be due to the lack of terminally differentiated superficial umbrella cells as a result of the urothelial hyperplasia that accompanies chronic cystitis. A number of findings suggest that this may be the case. First, as we have discussed, undifferentiated urothelial cells do not appear to support IBC formation *in vitro* because of actin-gating of the phagocytic vesicle and perhaps also because of different invasion pathways. Second, complete chemical exfoliation of the umbrella cells by infusion of a high dose of protamine sulfate into the urinary bladder prior to infection abrogates IBC formation (Mysorekar & Hultgren, 2006). However, low-dose protamine sulfate treatment, which only partially exfoliates the umbrella cells, results in increased numbers of IBCs within the remaining umbrella cells, likely indicating the antiadherent properties of the proteoglycan layer, which is also removed by this treatment (Mysorekar & Hultgren, 2006). Finally, TLR4 signaling-defective C3H/HeJ mice can develop a form of chronic bladder infection that is accompanied by biofilm-like colonization of the urothelium and continued IBC formation as late as 4 weeks postinfection (Hannan *et al.*, 2010). As these

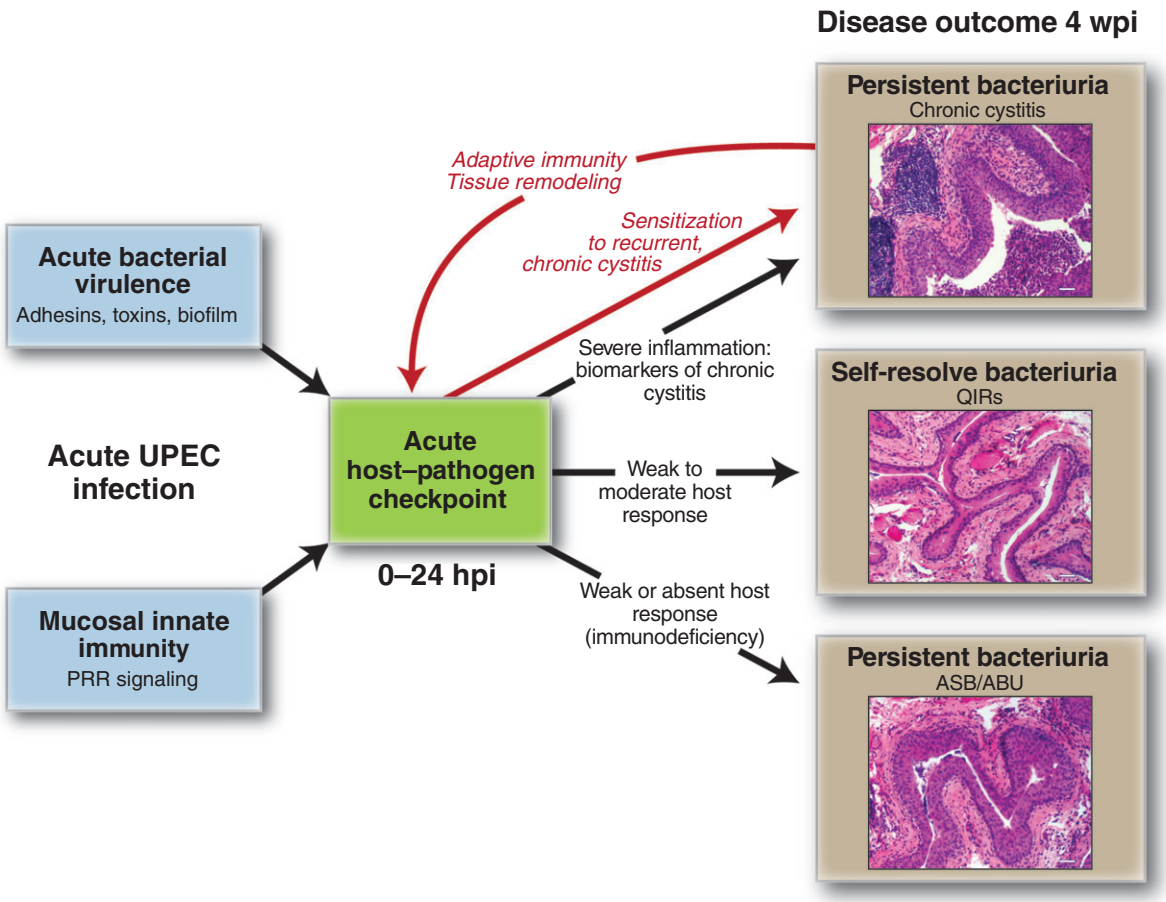

Fig. 3. Model of host–pathogen checkpoints in chronic and recurrent cystitis. We hypothesize that an acute host–pathogen mucosal checkpoint exists early in UPEC infection of the bladder that determines the outcome of infection. 'Inputs' into this checkpoint (blue boxes) include bacterial virulence and host innate immune signaling, such as pattern recognition receptor (PRR) signaling (e.g. TLR4 signaling). The bladder mucosa integrates these signals (green box), which include the intensity and invasiveness of UPEC infection and the character of PRR signaling, and responds. These responses or 'outputs' in naïve mice (green arrows) include the biomarkers of chronic infection, the severity of bladder inflammation, and the character and extent of urothelial cell death and exfoliation. These outputs correlate with disease outcome (brown boxes). Severe or weak mucosal responses can each lead to persistent bladder infection with (chronic cystitis in TLR4-responsive C3H mice) or without ('asymptomatic' bladder infection in C3H/HeJ mice) inflammation, respectively. In turn, the adaptive mucosal response that accompanies the development of chronic cystitis in C3H mice increases the sensitivity of the acute host–pathogen checkpoint, even after clearance of the infection with antibiotics, predisposing to severe recurrent and chronic cystitis after challenge infection (red arrows). QIRs; ASB/ABU.

mice have muted bladder inflammatory responses, the urothelium is only mildly hyperplastic, and it is in the few remaining terminally differentiated, strongly uroplakin-positive superficial umbrella cells that IBCs are found. As C3H/HeJ mice have been proposed to be a model for ASB in humans, this raises the interesting question of whether this condition is caused in part by low-level persistent cycling of bacteria through IBCs.

During chronic cystitis, UPEC selection is driven by competition for limited resources

Although IBCs or QIRs are not observed in TLR4 competent mice with chronic cystitis, the bacteria are not exclusively extracellular as *ex vivo* assays indicate that approximately 10–20% of bladder bacteria are in a gentamycin-protected niche, likely intracellular within urothelial cells, macrophages, and/or neutrophils (Hannan *et al.*, 2010; Schwartz *et al.*, 2011). Therefore, the selection pressure within the host during chronic cystitis is very different from that seen during acute infection, where bacteria cycle through IBCs. As researchers have struggled to identify functions for many classical UPEC virulence factors, we hypothesize that perhaps many of these genes are conserved because they confer a fitness advantage during polymicrobial inoculations into the bladder and chronic persistence while vying for limited resources in a restricted host niche (Fig. 1g). To test this, we have

performed 2–4 week *in vivo* competitive infection experiments where urines are collected longitudinally throughout the course of infection and plated with antibiotic selection to identify the kinetics of competitive advantage. As a proof of principle, we tested the fitness of a UTI89 mutant in which a large UPEC genomic island, PAI II_{UTI89}, that encodes several adhesins (P and F17-like pili, Hek) and toxins (CNF1, α-hemolysin, CdiA) was deleted while leaving the flanking *LeuX* tRNA intact (ΔPAI II_{UTI89}). In a previous study, this mutant was able to invade the urothelium and form IBCs similar to wild type (Hannan *et al.*, 2008) and it is capable of causing chronic cystitis in single infection studies (T. J. Hannan and S. J. Hultgren, unpublished data). Consistent with our hypothesis, we found that the ΔPAI II_{UTI89} deletion strain dropped out completely by day 14 postinfection when coinoculated with wt UTI89, suggesting that this island confers a fitness advantage for long-term persistence (T. J. Hannan and S. J. Hultgren, unpublished data). These results suggest that the chronic cystitis model has particular relevance for understanding bacterial virulence and immune evasion strategies in the context of long-term persistence in the face of an active inflammatory response. Furthermore, they provide evidence for the presence of a more gradual population bottleneck during chronic cystitis, which limits bacterial diversity by selecting those clones most fit to compete for limited resources in a hostile host environment.

Asymptomatic bacteriuria

ASB appears to be multifactorial, as both host and pathogen factors appear to play an important role in its development (Mabbett *et al.*, 2009; Ragnarsdottir *et al.*, 2011). On the one hand, bacterial studies suggest that ASB isolates are often functionally different from symptomatic UTI isolates (Klemm *et al.*, 2006, 2007). Specifically, they lack the ability to express UPEC virulence factors such as pili. Indeed, many ASB isolates fit this model, where the adhesin and other putative virulence genes present in these strains have been found to have undergone decay (Zdziarski *et al.*, 2008). Without adhesins or flagella, they are unable to invade the urothelium or ascend the ureters, events that lead to enhanced inflammation (Schilling *et al.*, 2001a, b; Lane *et al.*, 2007). Recent data suggest that ASB isolates are able to persist in the bladder in the absence of canonical adherence mechanisms because of their enhanced ability to grow in urine and form biofilms (Roos *et al.*, 2006; Ferrieres *et al.*, 2007). The ability to acquire iron via the production of siderophores also contributes to growth in urine and bladder colonization by ASB isolates (Roos *et al.*, 2006; Watts *et al.*, 2012). On the other hand, clinical studies discovered that ASB in children is associated with decreased expression of TLR4 on neutrophils and thus hyporesponsiveness of these and possibly other cells to bacterial LPS (Hagberg *et al.*, 1984; Ragnarsdottir *et al.*, 2007). Concordant with these studies, it was recently demonstrated in mice that bladder instillation of LPS into the urinary bladder is sufficient to either increase or decrease, depending on the source of the LPS, pelvic area pain sensitivity in a TLR4 dependent manner (Rudick *et al.*, 2010). LPS from a virulent cystitis strain, NU14, increased sensitivity, whereas LPS from the ASB strain, 83972, which lacks type 1 and P pili, decreased pain sensitivity. In mouse models of UPEC infection, TLR4 nonresponsive C3H/HeJ mice are exquisitely sensitive to pyelonephritis, but relatively resistant to cystitis (Hagberg *et al.*, 1984; Hannan *et al.*, 2010). Although chronic bladder infection can occur in C3H/HeJ mice, it only occurs in a fraction of mice with chronic pyelonephritis and is accompanied by very mild bladder inflammation (Hopkins *et al.*, 1996, 1998; Hannan *et al.*, 2010). Surprisingly, at lower infectious doses, these immunosuppressed mice were significantly more efficient at clearing bladder infection than the closely related C3H/HeOuJ strain, which exhibits a robust inflammatory response to UPEC (Hannan *et al.*, 2010). These findings provide further evidence for a necessary role for severe acute inflammation in the development of chronic cystitis.

Some urinary tract diseases traditionally considered 'noninfectious' may be associated with persistent bacteria

Overactive bladder

'Noninfectious' diseases of the bladder are also common. These include bladder cancer (Brandau & Bohle, 2001), OAB disorder (Milsom *et al.*, 2001), and the debilitating bladder pain condition known as IC/painful bladder syndrome, which is estimated to possibly affect as many as one in every five women and one in 20 men during their lifetimes (Parsons *et al.*, 2007). Traditionally, the underlying pathophysiology of OAB, a condition that affects 17% of people over 40 (Milsom *et al.*, 2001), is thought to be increased contractility of the detrusor muscle, although the etiology is uncertain. However, recent advances have led to an awareness of the role of the urothelium, which releases ATP and other neuromodulators, in the pathogenesis of the OAB syndrome (Birder *et al.*, 2010). The main therapy for OAB syndrome is anticholinergic medications, which reduce detrusor contractility and ameliorate the urgency/urge incontinence. Unfortunately, long-term studies show that only 17% of patients achieve

'cure' (Morris *et al.*, 2008) from these medications; symptoms tend to wax and wane over the years in the remainder. A history of childhood bedwetting or a family history of OAB symptoms are poor prognostic factors, leading to the suspicion that a subset of these patients have a genetic component to their condition (Morris *et al.*, 2004). Patients not responding to therapy are termed 'refractory' (Nitti *et al.*, 2010).

Overactive bladder and low-count bacteriuria with UPEC

Classic teaching has been that UTI should be excluded to make a diagnosis of OAB. In patients with symptomatic cystitis, the symptoms of dysuria and bacteriuria are often accompanied by increased frequency of micturition, urgency, nocturia, and episodic urge incontinence during an acute UTI. On the other hand, patients presenting with the main complaints of frequency/urgency/nocturia/urge incontinence without dysuria and bacteriuria ($>10^5$ CFU mL^{-1}) are considered to have the OAB syndrome (Walsh & Moore, 2011). However, recent evidence has demonstrated pyuria (Horsley *et al.*, 2011) and low-count ($\geq 10^3$ CFU mL^{-1}) bacteriuria on catheter specimens in patients diagnosed with OAB (Walsh *et al.*, 2011a; Walsh & Moore, 2011). Furthermore, recent evidence indicates that a portion of women with refractory OAB experience recurrent episodes of bacterial cystitis that manifest as acute exacerbations of the frequency/urgency/nocturia/urge incontinence symptoms, without the classical dysuria or foul-smelling urine usually associated with bacteriuria. Previously, successful anticholinergic medicines often fail in efficacy during an episode of bacterial cystitis. Preliminary studies have suggested that combining the anticholinergic agents with antibiotics could lead to improved outcomes (Gill *et al.*, 2011). Bladder biopsies in such patients reveal histological evidence of 'chronic cystitis' (Lunawat *et al.*, 2009). A study of midstream urine specimens from 50 refractory OAB women and 50 controls obtained positive urine cultures ($\geq 10^3$ CFU mL^{-1}) from 39% of those with urge incontinence compared with 6% of controls ($P < 0.0001$), with the majority of these UTI caused by *E. coli*, and associated pyuria (Walsh *et al.*, 2011b). A later study of catheterized urine specimens from patients with newly diagnosed OAB revealed bacteriuria in 13%, the majority culturing *E. coli* with pyuria, vs. 6% of controls ($P = 0.07$; Walsh *et al.*, 2011a). Thus, it appears that in this subset of refractory OAB, up to one-third of patients may exhibit bacterial cystitis, albeit without expression of the dysuria or foul-smelling urine symptoms that have been considered the classic hallmarks of UTI.

Interstitial cystitis/painful bladder syndrome

A further clinical problem known as 'Painful Bladder Syndrome' includes patients that also present with the symptoms of frequency, urgency, nocturia and more importantly suprapubic pain, which is often relieved by micturition. Urge incontinence rarely occurs, because patients void so frequently, driven by pain. This syndrome encompasses a wide clinical spectrum of severity; on the severe end of the spectrum patients are found to have typical 'Hunner's ulcers' at cystoscopy with petechial hemorrhages and raised mast cell counts upon biopsy – these patients are considered to have 'IC'. On the lesser end of the spectrum, patients have a small bladder capacity but rarely demonstrate the above cystoscopic features; such patients are defined by the International Continence Society as having 'Bladder Oversensitivity'. While patients with classic IC must by definition have sterile urine, those with bladder oversensitivity are found to have bacterial cystitis on catheter specimens in up to 32% of cases (Walsh *et al.*, 2011b). Thus, evidence is now emerging that classically 'noninfectious' lower urinary tract conditions may exhibit bacterial cystitis in a substantial proportion of cases.

The emergence of antibiotic-resistant strains

Overview

The emergence of MDR Gram-negative bacterial pathogens represents a major threat to human health. Not only is the increase in resistance of Gram-negative bacteria occurring at an unprecedented rate, but there are also few new antibiotics active against Gram-negative bacteria in the drug development pipeline (Livermore, 2009). The increased resistance of Gram-negative bacteria is primarily because of antibiotic resistance genes carried on plasmids that can spread efficiently within bacterial populations. Bacterial strains and plasmids can also be transported rapidly across the globe as a result of increased human travel and migration. To a great extent, this dissemination remains undetected, with resistant strains carried in the normal human flora and only becoming evident when they are the source of endogenous infections such as UTI.

The spread of antibiotic and MDR UPEC strains worldwide

MDR UPEC are extremely common, with many strains now recognized as belonging to specific clones (Stamm, 2001). For example, the trimethoprim-sulfamethoxazole

(TMP-SMZ) resistant *E. coli* 'clonal group A' was identified a decade ago and shown to be widespread across the United States (Manges *et al.*, 2001). Physician concerns about resistance to TMP-SMZ resulted in more frequent use of fluoroquinolones and nitrofurantoin as empirical treatment for cystitis (Hooton, 2003), which in turn led to a consistent stepwise increase in resistance rates to ciprofloxacin among *E. coli* isolates causing UTI (Karlowsky *et al.*, 2006). Over the last 5 years, *E. coli* clone O25:H4-ST131 (*E. coli* ST131) has emerged globally as an important MDR extraintestinal pathogen. *Escherichia coli* ST131 is a major cause of urinary tract and bloodstream infections within the community as well as in hospitals and long-term care facilities in Europe, Asia, Africa, North America, and Australia (Cagnacci *et al.*, 2008; Nicolas-Chanoine *et al.*, 2008; Johnson *et al.*, 2009b; Pitout *et al.*, 2009; Platell *et al.*, 2010; Sidjabat *et al.*, 2010). *Escherichia coli* ST131 are also major contributors to 'the CTX-M pandemic'; a recent worldwide increase in *E. coli* uropathogens that produce CTX-M type ('active on CefoTaXime, first isolated in Munich') extended spectrum β-lactamases (ESBLs; Canton & Coque, 2006). *Escherichia coli* ST131 are commonly identified among *E. coli* producing CTX-M-15; currently the most widespread CTX-M ESBL enzyme worldwide (Coque *et al.*, 2008; Nicolas-Chanoine *et al.*, 2008). *Escherichia coli* ST131 strains are commonly resistant to multiple classes of antibiotics, including oxyimino-cephalosporins (i.e. cefotaxime and ceftazidime), monobactams, and fluoroquinolones (i.e. ciprofloxacin; Simner *et al.*, 2011; Johnson *et al.*, 2009a, b, 2010a). Thus, infections caused by this clone are generally associated with limited treatment options.

UPEC ST131 strains are a significant cause of UTI and are virulent in a murine model of UTI

The virulence capacity of *E. coli* strains within the ST131 clonal group remains to be properly examined. Two clinical studies have reported transmission of *E. coli* ST131 strains causing pyelonephritis and septic shock between family members (Ender *et al.*, 2009; Johnson *et al.*, 2010c). Infection with *E. coli* ST131 has also been associated with poor prognosis in renal transplant patients (Johnson *et al.*, 2010b) and pyomyositis in patients with hematologic malignancies (Vigil *et al.*, 2010). Additionally, several studies have reported the carriage of *E. coli* ST131 by companion animals (Platell *et al.*, 2010; Johnson *et al.*, 2009a; Ewers *et al.*, 2010). In a single study that examined *E. coli* ST131 virulence in a mouse sepsis infection model, four (out of four) strains caused rapid death (Nicolas-Chanoine *et al.*, 2008). The same study also demonstrated strong biofilm formation by two (out of two) strains using a microfermentor assay. Most *E. coli* ST131 strains belong to the B2 phylogenetic group, with only a few virulence genes uniformly present in all strains – that is, the *fimH* adhesin of type 1 fimbriae, the secreted autotransporter toxin (*sat*), the aerobactin receptor (*iutA*), the uropathogenic-specific protein (*usp*), and the pathogenicity island marker (*malX*; Nicolas-Chanoine *et al.*, 2008; Johnson *et al.*, 2009a, b, 2010a, b; Coelho *et al.*, 2011). Recently, the genome sequence of the *E. coli* ST131 strain EC958 was reported (Totsika *et al.*, 2011). EC958 is a member of the pulse field gel electrophoresis (PFGE) defined UK epidemic strain A, which represents one of the major pathogenic lineages (PFGE strains A–E) causing UTI across the UK (Lau *et al.*, 2008). EC958 is resistant to eight antibiotic classes, including oxyimino-cephalosporins, fluoroquinolones, and sulfonamides and contains many virulence genes commonly associated with UPEC. Interestingly, EC958 contained a transposon insertion in the *fimB* gene, which encodes the recombinase responsible for the 'on' switching of the *fim* promoter and thus expression of type 1 fimbriae. This insertion was also identified in the majority of other ST131 strains examined from the United Kingdom and Australia, indicating that the mutation is common among *E. coli* ST131 strains (Totsika *et al.*, 2011). EC958 was still able to mediate phase switching of the *fim* promoter, suggesting that the regulation of type 1 fimbriae gene expression is altered in this strain as well as in the majority of other *E. coli* ST131 strains. The induction of type 1 fimbriae expression by EC958 resulted in enhanced adherence, invasion and intracellular survival to/within T24 bladder epithelial cells, and also led to increased colonization of the mouse bladder during acute infection. We are currently investigating the molecular mechanisms that coordinate the expression of type 1 fimbriae in EC958 and the contribution of intracellular survival to infections caused by this globally disseminated MDR *E. coli* clone.

The rise of carbapenemase-expressing UPEC strains

In 2008, a new type of carbapenemase referred to as New Delhi metallo-β-lactamase-1 (NDM-1) was identified in a strain of *K. pneumoniae* that caused a UTI in a 59-year-old Swedish man of Indian origin (Yong *et al.*, 2009). Since then, NDM-1 has been identified in a range of *Enterobacteriaceae* (and other bacteria) isolated from patients in multiple countries including India, Pakistan, Bangladesh, the United Kingdom, the United States, the Netherlands, Australia, Kenya, and Canada (Hsueh, 2010). The gene encoding the NDM-1 (bla_{NDM-1}) is located on plasmids of varying sizes that carry additional antibiotic resistance determinants and can transfer to other bacteria, directly conferring multi- or even extreme-

drug-resistant phenotypes (Kumarasamy *et al.*, 2010; Walsh, 2010; Nordmann *et al.*, 2011). Recently, there have been several reports demonstrating the acquisition of the bla_{NDM-1} gene by *E. coli* ST131 strains (Poirel *et al.*, 2010; Peirano *et al.*, 2011). Carbapenem antibiotics are considered as a 'last-line' of therapy against MDR Gram-negative pathogens, and thus the presence of the bla_{NDM-1} gene in an *E. coli* clone that has already demonstrated its capacity to rapidly disseminate across the globe highlights the need for the development of alternative anti-infective strategies.

Novel UPEC vaccines and antibacterial/antivirulence therapies

FimH: a promising target for therapies to prevent and treat UPEC cystitis

The alarming rise in MDR strains highlights the urgent need to design novel, clinically applicable therapeutics and vaccines to improve the lives of women suffering from recurrent and chronic cystitis. As discussed previously, during acute infection, UPEC face an immediate population bottleneck caused by extensive host extracellular clearance mechanisms that is counteracted by bacterial adherence to and invasion of the urothelium and IBC formation within urothelial cells in order for the bacteria to survive and become founders of an acute infection and/or to successfully persist (Schwartz *et al.*, 2011). Furthermore, the ability to form many IBCs correlates with the triggering of an innate host–pathogen checkpoint that predisposes to chronic and severe recurrent cystitis. Thus, the ability to transition from planktonic growth in the urine to intimate colonization and intracellular replication constitutes a population bottleneck and thus an opportunity for therapeutic and prophylactic intervention (Mecsas, 2002). It has been shown that FimH is critical to the ability of virulent strains of UPEC to survive this bottleneck (Wright *et al.*, 2007). Furthermore, IBCs are present in the urine of women with UTI, but only by uropathogens that express FimH (Rosen *et al.*, 2007), and FimH is under positive selection in clinical isolates of UPEC consistent with its critical role in human UTI (Ronald *et al.*, 2008; Chen *et al.*, 2009). For these reasons, vaccines and therapeutic agents have been developed that target FimH.

Overview of UTI vaccines

Numerous UTI vaccines have been proposed over the past 20 plus years. These include systemic and mucosal vaccines, using heat-killed UPEC or recombinant proteins, including fimbrial adhesins and outer membrane proteins (Uehling *et al.*, 1994; Langermann *et al.*, 1997, 2000; Schmidhammer *et al.*, 2002; Poggio *et al.*, 2006; Durant *et al.*, 2007; Hopkins *et al.*, 2007; Alteri *et al.*, 2009b; Wieser *et al.*, 2010; Wieser *et al.*, 2012). Despite these efforts, so far only two vaccines targeting a broad array of UPEC strains, one using a mixture of 10 heat-killed UPEC strains and the other recombinant FimH, have been demonstrated to significantly protect naïve animals against cystitis, which accounts for approximately 90% of UTI, after UPEC challenge (Uehling *et al.*, 1994; Langermann *et al.*, 1997, 2000; Hopkins *et al.*, 2007). While the heat-killed vaccine showed promise in reducing the frequency of rUTI in sexually active women aged 20–50 years in Phase II clinical trials when applied vaginally (Hopkins *et al.*, 2007) to the best of our knowledge it has not been licensed. Recently, Mobley and colleagues proposed a strategy to use iron acquisition receptors found in the outer membrane of UPEC as the antigens for a mucosal vaccine (Alteri *et al.*, 2009b). One such antigen, the aerobactin receptor IutA, induced protection against bladder infection, but the *iutA* gene is only found in some UPEC clonal groups. Thus, a vaccine including multiple iron acquisition receptors may impart broader protection.

Immunoactive therapy

Although not a vaccine in the traditional sense, Uro-Vaxom, which is approved for use in a small number of countries, has been shown in numerous placebo-controlled double-blind studies to be effective in preventing rUTI in human patients with a history of chronic rUTI, similar to prophylactic antibiotic therapy (Tammen, 1990; Bauer *et al.*, 2002; Naber *et al.*, 2009). Uro-Vaxom is composed of bacterial extracts from 18 *E. coli* strains and is taken orally once daily for 3 months and then needs to be 'boosted' by additional regimens every 6–12 month thereafter (Cruz). It is considered to work as an immunoactive agent. A recent study demonstrated that oral treatment of mice with Uro-Vaxom for 10 days resulted in increased IL-6 and gamma interferon in the urinary bladder, but upon LPS stimulation the bladders displayed markedly reduced inflammation (Lee *et al.*, 2006). As the timing of this study suggests that this anti-inflammatory effect is not mediated by the adaptive immune system, it raises the interesting hypothesis that Uro-Vaxom is inducing LPS tolerance as a mechanism for protecting patients against rUTI (Fig. 3; Beeson, 1946; Hawn *et al.*, 2009).

Vaccines targeting FimH

Two groups have published studies investigating the efficacy of vaccines targeting FimH. The recombinant FimCH vaccine is comprised of full-length FimH stably

bound to its periplasmic chaperone FimC. Studies in naïve mice and primates after systemic vaccination with FimCH showed dramatic protection against experimentally induced cystitis (Langermann *et al.*, 1997, 2000). A similar vaccine using a truncated form of recombinant FimH resulted in similar protection whether administered systemically or intranasally, despite the fact that the mucosal route induced higher vaginal wash FimH-specific IgA levels (Poggio *et al.*, 2006). The lack of correlation between degree of protection and urogenital IgA levels suggest that either IgA is not important for protection or that systemic vaccination induces IgA levels that are sufficient for protection. Recently, structural data and *in vitro* mannose-binding studies have suggested that certain monoclonal FimH antibodies, and to a lesser extent polyclonal sera, lock FimH in a 'high-affinity' binding conformation (Le Trong *et al.*, 2010; Tchesnokova *et al.*, 2011). The authors suggest that this makes FimH a poor vaccine candidate, because antibody binding could theoretically increase the adherence of the bacteria to the urothelium. However, *in vivo* studies in both mice and primates conducted by two separate groups clearly demonstrate that FimH vaccination confers protection against UPEC cystitis. One possible explanation for this discrepancy is that antibody coating of FimH *in vivo* interferes with UPEC type 1 pilus adherence to UPIa, as the pilus tip appears to be 'buried' in the central cavity of the hexagonal uroplakin complex (Mulvey *et al.*, 1998). Alternatively, opsonization may interfere with UPEC invasion of urothelial cells and/or IBC formation. Furthermore, independent of these effects on urothelial interactions, opsonization of bacteria activates bactericidal complement pathways and promotes phagocytosis by innate immune cells. An interesting question yet to be addressed is whether vaccination of C3H/HeN mice sensitized to chronic and recurrent cystitis are similarly protected, as potentially these mice more closely resemble the patient population that would be targeted to prevent recurrent cystitis.

The development of biarylmannose-derivative FimH antagonists

The mannose-binding pocket of FimH is comprised of amino acid residues that are invariant in all strains of UPEC (Hung *et al.*, 2002). Mutations in these residues disrupt mannose binding and attenuate virulence (Sokurenko *et al.*, 1998; Hung *et al.*, 2002; Chen *et al.*, 2009). The X-ray crystal structures of FimH bound to α-D-mannose and mannose derivatives called mannosides (Hung *et al.*, 2002; Bouckaert *et al.*, 2005; Wellens *et al.*, 2008; Han *et al.*, 2010) were used to rationally design biarylmannose-derivative FimH binding inhibitors with excellent cellular potency and low molecular weight (Han *et al.*, 2010). Surprisingly, although heptyl mannose bound within a so-called 'tyrosine gate' outside of the mannose-binding pocket the biarylmannose derivatives docked to one side of this gate, making π-π stacking interactions with one of the tyrosines. Using a reiterative process of structure-based design, combinatorial chemistry, and *in vitro* cell-based screening of hundreds of compounds, we have developed highly potent mannosides (Han *et al.*, 2010). By structure and ligand-based lead optimization, pharmacokinetic properties including oral bioavailability were improved and after evaluation of efficacy in treating established experimental UTI, we further developed lead candidate mannoside compounds for use in experimental and preclinical translational studies (Cusumano *et al.*, 2011). These advances have led to the first demonstration of the capacity of our mannoside derivatives to treat an established UTI when delivered orally (Fig. 4; Cusumano *et al.*, 2011). Mice with established chronic cystitis of 2 weeks duration were treated with a single dose of mannoside by oral gavage and bladder titers were reduced approximately 100- (50 mg kg^{-1} dose) and 1000- (100 mg kg^{-1}) fold within 6 h of treatment, whereas treatment with trimethoprim-sulfamethoxazole (TMP-SMZ) in the drinking water only reduced titers 50-fold during this time. This demonstrates that mannosides are not only fast acting, but they also have efficacy against an established chronic infection.

Independently, Ernst and colleagues have developed similar biarylmannose-derivative FimH antagonists (Klein *et al.*, 2010; Schwardt *et al.*, 2011). These compounds have nanomolar affinity and the biaryl moieties of these compounds dock outside of the tyrosine gate similar to those compounds described earlier. Treatment of C3H/HeN mice with their lead compound prior to UPEC infection dramatically protected them from acute cystitis, as bladder titers at 3 hpi were 10 000-fold lower with treatment (Klein *et al.*, 2010). They then endeavored to develop mannosyl-triazoles to enhance the flexibility of the compounds to enable binding within the tyrosine gate in hopes of attaining a higher binding affinity. While these mannosyl-triazole compounds did dock within the tyrosine gate and mostly had higher affinities for FimH, the cellular potency was decreased. Thus, further optimization of the mannoside compounds from both groups is necessary, not only to improve potency, but also to improve pharmacokinetics before moving into human preclinical trials.

Mannosides prevent acute cystitis caused by divergent and antibiotic-resistant strains

In addition to treating an established UTI, we have also shown that our lead mannoside compound has a potent efficacy in preventing acute UTI caused by divergent

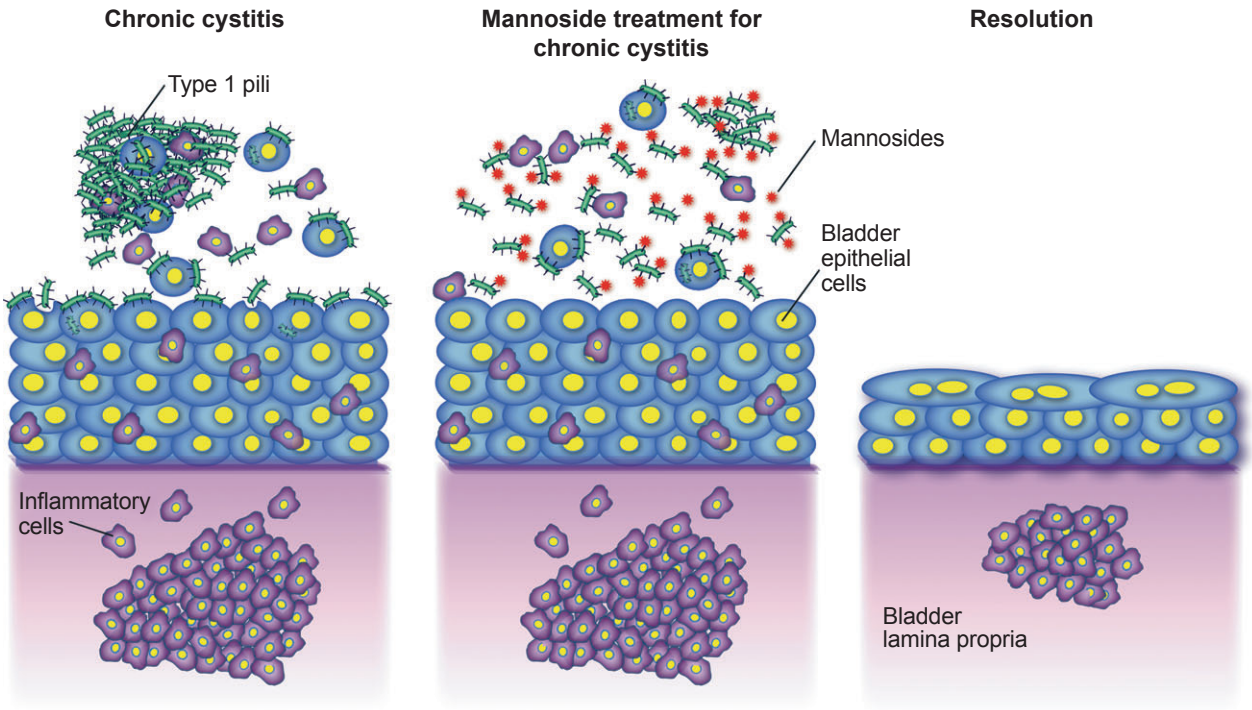

Fig. 4. Mannosides successfully treat existing chronic infections.

strains, including the TMP-SMZ resistant strain PBC-1 (Cusumano *et al.*, 2011). This likely is due to the fact that all FimH variants have an invariant binding pocket to which mannosides bind in a conformation that represents neither the canonical low- or high-affinity conformation identified by Sokurenko and colleagues (Han *et al.*, 2010; Le Trong *et al.*, 2010) In this study, bladder titers in C3H/HeN mice were reduced approximately 100-fold at 6 hpi and the intracellular niche was eliminated, as UPEC were unable to invade the urothelium and form IBCs. The lead mannoside compound also acted synergistically with TMP-SMZ, which concentrates in the urine. This synergy appears to be a result of preventing UPEC invasion of urothelial cells and compartmentalization of UPEC to the bladder lumen, thus exposing bacteria to TMP-SMZ concentrations well above the minimal inhibitory concentration (MIC) of even a clinically resistant strain, resulting in bacterial cell death (TMP reached 9 mg mL^{-1} in the urine, well above the 256 μg mL^{-1} MIC of the resistant strain, PBC-1). It is likely that during current standard treatment, TMP-SMZ reaches tissue concentrations above the MIC needed for killing of sensitive strains but fails to reach tissue levels needed for killing PBC-1. Thus, in the absence of mannoside, residence of PBC-1 in an intracellular niche, such as in IBCs during acute infection, likely protects it from antibiotic killing. Currently, treatment of UTI typically requires a 3–10 day course of antibiotics or, in the case of chronic, recurrent cystitis, daily prophylaxis. Thus, our mannoside compounds have the potential to shorten this course and/or increase the efficacy of TMP-SMZ resulting in fewer treatment failures, if clinically translatable. Translated to clinical practice, mannosides could be a cost-effective treatment that lowers the clinical antibiotic resistance rate, which is currently as high as 30% in some studies (van der Starre *et al.*, 2010) and may also reduce the use of fluoroquinolones, thus decreasing resistance. In addition, the unique mechanism of mannoside action, that is, inhibiting the function of the extracellular FimH pilus tip adhesin, negates the need for the compound to cross the bacterial outer membrane for efficacy, which thus would circumvent the development of resistance because of porin mutations or efflux.

Conclusions

UTI and associated bladder diseases are a major problem, particularly when the consequences are chronic and debilitating in nature. Previously thought to be an exclusively extracellular disease, bladder infections caused by UPEC are now recognized to be complex pathogenic events with distinct acute and chronic phases of infection, each of which have intracellular and extracellular niche components. Yet in spite of this knowledge, molecular details

regarding UPEC persistence and the complex range of syndromes associated with UTI are sorely lacking. Increasing antibiotic and multidrug-resistance is compounding the clinical problem, making it even more imperative that we gain a complete understanding of UPEC pathogenesis including molecular details of the host–pathogen factors that influence disease outcomes and sequelae. In tackling this problem, we propose to consider UPEC pathogenesis as a series of host–pathogen checkpoints and population bottlenecks. We hypothesize that an acute host–pathogen checkpoint exists early in acute infection that determines disease outcome. In this model, an initial severe and chronic infection with a highly virulent UPEC strain has the potential to sensitize individuals to recurrent infection, such that much less virulent UPEC strains are now capable of causing symptomatic rUTI. Such a model could explain, in part, the large degree of heterogeneity among UPEC isolates. Understanding the molecular mechanisms of this putative checkpoint will be critical for developing new therapeutic strategies to prevent rUTI. Likewise, we hypothesize that UPEC population bottlenecks not only offer an opportunity for therapeutic intervention, such as with the mannoside compounds, but also help us to understand bacterial survival strategies and host defense mechanisms that are in play during acute infection and chronic persistence.

Acknowledgements

We thank Chia Hung for contributing to Fig. 4, and Jim Janetka, Drew Schwartz, and Sarah Rowland for critical reading of this manuscript and helpful discussion. This work was supported by the National Institutes of Health and Office of Research on Women's Health Specialized Center of Research (DK64540, DK51406, AI48689, AI29549, AI49950 and AI95542 to S.J.H., and a Mentored Clinical Scientist Research Career Development Award K08 AI083746 to T.J.H.); the Australian National Health and Medical Research Council (grants 631654, APP1012076, APP1005315, APP1033799 to M.A.S.); an Australian Research Council Future Fellowship award to M.A.S. (FT100100662), and a University of Queensland Early Career Researcher Grant to M.T.

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