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Uropathogenic *Escherichia coli* Superinfection Enhances the Severity of Mouse Bladder Infection

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Abstract

Urinary tract infections (UTIs) afflict over 9 million women in America every year, often necessitating long-term prophylactic antibiotics. One risk factor for UTI is frequent sexual intercourse, which dramatically increases the risk of UTI. The mechanism behind this increased risk is unknown; however, bacteriuria increases immediately after sexual intercourse episodes, suggesting that physical manipulation introduces periurethral flora into the urinary tract. In this paper, we investigated whether superinfection (repeat introduction of bacteria) resulted in increased risk of severe UTI, manifesting as persistent bacteriuria, high titer bladder bacterial burdens and chronic inflammation, an outcome referred to as chronic cystitis. Chronic cystitis represents unchecked luminal bacterial replication and is defined histologically by urothelial hyperplasia and submucosal lymphoid aggregates, a histological pattern similar to that seen in humans suffering chronic UTI. C57BL/6J mice are resistant to chronic cystitis after a single infection; however, they developed persistent bacteriuria and chronic cystitis when superinfected 24 hours apart. Elevated levels of interleukin-6 (IL-6), keratinocyte cytokine (KC/CXCL1), and granulocyte colony-stimulating factor (G-CSF) in the serum of C57BL/6J mice prior to the second infection predicted the development of chronic cystitis. These same cytokines have been found to precede chronic cystitis in singly infected C3H/HeN mice. Furthermore, inoculating C3H/HeN mice twice within a six-hour period doubled the proportion of mice that developed chronic cystitis. Intracellular bacterial replication, regulated hemolysin (HlyA) expression, and caspase 1/11 activation were essential for this increase. Microarrays conducted at four weeks post inoculation in both mouse strains revealed upregulation of IL-1 and antimicrobial peptides during chronic cystitis. These data suggest a mechanism by which caspase-1/11 activation and IL-1 secretion could predispose certain women to recurrent UTI after frequent intercourse, a predisposition predictable by several serum biomarkers in two murine models.

Introduction

Nearly nine million people present each year to primary care physicians with a urinary tract infection (UTI), costing nearly $2 billion yearly [1,2]. Women suffer the majority of these infections, with the lifetime risk approaching 50% [3]. Furthermore, 25–40% of these women will suffer recurrent UTI (rUTI), with 1.5 million women referred to urology clinics and often requiring prophylactic antibiotics to prevent recurrence [4–6]. Uropathogenic *E. coli* (UPEC) are responsible for >80% of community-acquired UTI and 50% of nosocomial UTI [7,8]. In the absence of antibiotic therapy, up to 60% of women experience symptoms and/or bacteriuria lasting months after initial infection [9–12], implying that cystitis is not always self-limiting. Furthermore, if the infection persists without adequate treatment, the organisms have the capacity to ascend the ureters, causing pyelonephritis and sepsis [13]. Antibiotic resistant organisms further complicate infection and threaten to increase the likelihood of chronic UTI, pyelonephritis and potentially bacteremia [14,15]. UTIs are increasingly being treated with fluoroquinolones, which in turn has led to a rise in resistance and the spread of multi-drug resistant microorganisms globally, which is a looming worldwide crisis [16,17]. It is therefore imperative to understand the molecular mechanisms that underlie this problematic disease in order to develop novel therapies.

Sexual intercourse is one of the most significant risk factors predisposing otherwise healthy women to UTI. Early studies demonstrated that sexual intercourse led to a 10-fold increase in bacteria/ml of urine and a subsequently increased predisposition to developing a UTI within 24 hours thereafter [5,18–21]. More recent studies have shown that the frequency with which a woman has sexual intercourse dramatically impacts the likelihood of developing both acute and rUTI [4,22,23]. Schols et. al found a direct association between the number of episodes of sexual intercourse in a given month and the risk of developing rUTI. However the significance of the timing between these episodes of sexual intercourse is unknown. Are evenly spaced episodes associated with an equal risk or, instead, does an episode prime...
the bladder for rUTI if another insult follows within a sensitive period? To address this question, we developed a model of sequential infection in mice to explore the hypothesis that a sensitive period exists after an initial bacterial insult to the bladder in which the likelihood of developing severe, chronic infection is dramatically increased.

Murine models of UTI have been used to decipher complexities of this disease in naive individuals. UPEC are capable of colonizing multiple body habitats and niches, including both intracellular and extracellular locations within the bladder, as well as in the gastrointestinal (GI) tract and the kidneys. Selective pressure and bacterial population bottlenecks during colonization impact the ultimate fate of disease [24–27]. Adhesive pili assembled by the chaperone/usher pathway (CUP), such as type 1 pili, contain adhesins at their tips that function in adherence and invasion of host tissues and in biofilm formation on medical devices. Upon introduction of UPEC into the bladder, bacteria bind to either mannosylated uroplakin plaques or β1-23 integrin receptors on the epithelial surface of the bladder via the type 1 pilus FimH adhesin [28–30]. Upon internalization, UPEC can be exocytosed as part of a TLR4 dependent innate defense process [31]. In addition to expulsion of individual bacteria, the host can exfoliate superficial facet cells to shed attached and invaded bacteria into the urine for clearance [29]. A small fraction of invaded bacteria escape into the host cell cytoplasm, where they are able to subvert expulsion and innate defenses by replicating into biofilm-like intracellular bacterial communities (IBCs) [24,32]. UPEC eventually flux out of these communities with a substantial proportion existing as neutrophil resistant filaments [33,34]. Importantly, evidence of IBCs and bacterial filaments have been observed in women suffering acute UTI, one to two days post self-reported sexual intercourse, but not in healthy controls or infections caused by Gram-positive organisms, which do not form IBCs [21]. IBCs have also been observed in urine from children with an acute UTI [35]. Additionally, IBC formation and the innate immune response of cytokine secretion and exfoliation have been observed in all tested mouse strains, but the long-term outcome of infection differs [36–38].

There are two main, mutually exclusive, outcomes to acute infection in C3H/HeN mice: either chronic bacterial cystitis (chronic cystitis), which is characterized by persistent high tier bacteriuria (>10^8 CFU/ml) and high titer bacterial bladder burdens (>10^4 CFU) two or more weeks after inoculation, accompanied by chronic inflammation [37,39], or resolution of bacteriuria [37]. Mice that resolve infection may harbor small populations of dormant UPEC called Quiescent Intracellular Reservoirs (QIRs) [40]. Other mouse strains exhibit varied proportions of these two outcomes. C57BL/6j mice resolve bacteriuria within days and thus are resistant to chronic cystitis, but are susceptible to QIR formation [40,41]. In contrast, other TLR4-responsive C3H background sub-strains and closely related CBA/J and DBA/2j mice experience persistent high-tier bacteriuria and bladder colonization by UPEC in the presence of chronic inflammation lasting at least four weeks post-infection (wpi). During chronic cystitis, persistent lymphoid aggregates and urothelial hyperplasia with lack of superficial facet cell terminal differentiation accompany luminal bacterial replication [37]. These same histological findings of submucosal lymphoid aggregates and urothelial hyperplasia have been observed in humans suffering persistent bacteriuria and chronic cystitis [42]. Since murine chronic cystitis predisposes to recurrent chronic UTI after antibiotic-mediated bacterial clearance, this is also a relevant model to interrogate the mechanism of recurrent cystitis [37]. In mouse models of UTI, mice initially experience urinary frequency and dysuria as determined by reaction to noxious stimuli and nerve responses during acute infection [43,44]; however, during chronic cystitis bacterial replication may exist in an asymptomatic carrier state as studies have not been conducted to determine whether dysuria persists. Interestingly, higher serum levels of interleukins (IL) 5 and 6, keratinocyte cytokine (KC/CXCL1), and granulocyte colony-stimulating factor (G-CSF) in C3H/HeN mice at 24 hours post infection (hpi) predicted the development of persistent bacteriuria and chronic cystitis thereafter, suggestive of a host-pathogen checkpoint during acute infection that predicts long term outcome [26,37]. In women with an acute UTI, increased amounts of serum CXCL1, M-CSF, and IL-8 correlated with subsequent rUTI, suggesting a similar checkpoint [45].

In this manuscript, we developed a superinfection model to mimic the clinical scenario of frequent sexual intercourse whereby sequential inocula are introduced within a brief period of time. C57BL/6j mice are resistant to chronic cystitis when singly infected; however, 30% of C57BL/6j mice developed chronic cystitis when superinfected 24 hours after the initial infection. Serum elevations of IL-6, KC, and G-CSF prior to superinfection predicted the development of persistent bacteriuria in C57BL/6j mice similar to singly infected C3H/HeN mice. Superinfecting C3H/HeN mice 1–6 hours after the initial inoculation increased the proportion of mice experiencing chronic cystitis. In order for this elevation to occur, we found that the initial UPEC inoculum (the “priming” inoculation) must be alive, invasive, capable of intracellular replication, and able to regulate hemolysin expression. Inhibition of the caspase 1/11 inflammasome prior to priming reduced bacterial CFU at four wpi relative to DMSO-treated mice. Microarray analysis of mouse bladders four wpi revealed that both C57BL/6j and C3H/HeN mice secreted antimicrobial peptides and IL-1 during chronic infection. In contrast to C3H/HeN mice, immunoglobulin expression was upregulated in C57BL/6j mice experiencing chronic cystitis. This immunoglobulin expression was absent in C57BL/6j mice that resolved infection and in C3H/HeN mice. Our data suggest mechanisms whereby certain women may be susceptible to rUTI after frequent sexual intercourse dependent on intracellular bacterial replication and the host immune response.

Author Summary

Urinary tract infections (UTIs) affect millions of women each year resulting in substantial morbidity and lost wages. Approximately 15 million women are referred to urology clinics suffering from chronic recurrent UTI on a yearly basis necessitating the use of prophylactic antibiotics. Frequent and recent sexual intercourse correlates with the development of UTI, a phenomenon referred to clinically as “honeymoon cystitis.” Here, using superinfection mouse models, we identified bacterial and host factors that influence the likelihood of developing chronic UTI. We discovered that superinfection leads to a higher rate of chronic UTI, which depended on bacterial replication within bladder cells combined with an immune response including inflammasome activation and cytokine release. These data suggest that bacterial inoculation into an acutely inflamed urinary tract is more likely to lead to severe UTI than bacterial presence in the absence of inflammation. Modification of these risk factors could lead to new therapeutics that prevent the development of recurrent UTI.

Superinfection Enhances Severe Bladder Infection
Results

Time-sensitive enhancement of infection

Studies suggest that a host-pathogen checkpoint within the first 24 hpi determines UTI outcome in C3H/HeN mice [26,37]. In addition, the chronic inflammation observed in mice experiencing chronic cystitis was found to predispose to rUTI after re-infection [37]. Thus, we hypothesized that superinfecting mice during this period of acute inflammation would increase the proportion of mice experiencing chronic cystitis. We transurethrally infected 7–8 week old female C3H/HeN mice with $10^7$ CFU UTI89 or PBS as the priming inoculation and superinfected them 1–2, 6, or 24 hours thereafter. Enumeration of bacterial CFU at one wpi as an initial screen revealed a dramatic increase in the proportion of mice experiencing chronic cystitis in mice superinfected 1–6 hours after priming compared to singly infected or PBS treated mice (Fig. 1A). We used a cutoff of $10^6$ CFU to demarcate mice experiencing high-titer bacterial infection at one week. Importantly, we did not observe a significant increase in CFU when a single inoculum was doubled ($2 \times 10^7$ CFU). Superinfection at 24 hpi had no effect on bacterial titers at one week, suggesting that the factors predisposing to increased susceptibility to chronic cystitis upon superinfection wane over time [26]. However, inoculation with PBS followed by UTI89 24 hpi did lead to high titers in 60% of mice. While this result is perplexing, it possibly reflects that sacrifice six days post infection was not sufficient to delineate the typical bimodal distribution of outcomes [37]. The process of catheterization also induces inflammation, which may not have resolved by 6 dpi [46]. We conducted all subsequent C3H/HeN superinfections one hour after priming.

Since early severe inflammatory responses predispose to chronic cystitis [37], we hypothesized that the initial inoculum primed the bladder by initiating an innate immune response to intracellular bacteria that predisposed to a higher proportion of mice experiencing chronic cystitis upon superinfection. We utilized a panel of UTI89 mutants in fimH, ompA, and kps that have been shown to differ in their ability to: i) invade and form IBCs and ii) persist during chronic cystitis in co-infection experiments [47,48]. Mature IBCs caused by WT bacteria are clonally derived from a single invasive event [24]. The mannose-binding pocket of FimH

Fig. 1. Superinfections of C3H/HeN mice. A) Mice were infected with $10^7$ CFU UTI89, $2 \times 10^7$ CFU UTI89, or PBS and re-infected with UTI89 at the indicated time points. One week total bladder titers are shown. Percentage of mice likely to develop chronic cystitis is displayed at the top of the column based on a CFU cutoff of $10^6$. Asterisks indicate $p<0.05$ from the PBS control and singly infected mice. B–D) Mice were infected with the indicated strain or PBS and re-infected one hour later. Urine titers were determined over time and four-week bladder titers are displayed. The fraction of mice with chronic cystitis is displayed at the top of each column. Red data points indicate resolved infection. Horizontal bars indicate median values. The dashed line at 20 CFU represents the LOD, and the dashed line at $10^5$ (A) or $10^7$ CFU (B–D) represents the chronic cystitis cutoff for urine and bladder titers. Panel A reflects 2–4 experiments with 5–9 mice per group. Panel B is 2–7 experiments with 4–5 mice per group. Panel C–D are 3 experiments with 4–5 mice per group. Statistical comparisons were determined using Fisher’s exact test based on the fraction of mice experiencing chronic cystitis.

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is invariant among sequenced UPEC [47], and the binding pocket mutant, FimH::Q133K, is defective in mannose-binding and can neither invade the bladder epithelium nor form IBCs. FimH undergoes compact and elongated conformational changes wherein the receptor binding domain bends approximately 37° with respect to the pilin domain. The mannose-binding pocket is deformed in the compact conformation whereas the elongated conformation is mannose binding proficient [49,50]. Several residues outside the mannose-binding pocket (positions 27, 62, 66 and 163) are under positive selection in clinical UPEC isolates compared to fecal strains [47] and have been shown to function in modulating the conformational changes between the elongated and compact states [48]. FimH::A27V/V163A predominantly adopts a high-mannose-binding, elongated conformation. Its expression results in: i) a 10-fold reduction in intracellular CFU one hpi and ii) a defect in the ability to form IBCs at six hpi. FimH::A62S shifts the equilibrium towards the compact conformation. Expression of this allele results in: i) a 10-fold reduction in intracellular CFU one hpi and ii) a 10-fold reduction in IBC formation compared to WT UTI89 [47,48]. UTI89ΔompA forms half the number of IBCs as UTI89 [51], and UTI89Δkps is defective in IBC formation. UTI89Δkps can replicate intracellularly and the IBC defect can be rescued by coinoculation with WT UTI89, which results in mixed strain, non-clonal, IBCs [52].

We primed mice with these strains and superinfected one hpi with WT UTI89 and assessed bacteruria at days 1, 7, 14, and 21 and enumerated bladder titers at 28 dpi. Mice were designated as having chronic cystitis if they had urine bacterial titers greater than 10⁴ CFU/ml at each time point and bladder titers greater than 10⁴ CFU at sacrifice [37]. We found that the FimH::A27V/V163A allele was incapable of priming the bladder for the development of chronic cystitis (p<0.05 relative to WT superinfection). In contrast, FimH::A62S did not significantly differ from PBS or WT superinfection; therefore, it may be capable of priming, though to a lesser degree. UTI89ΔompA and UTI89Δkps were both able to prime the bladder for enhanced chronic cystitis relative to PBS when superinfected one hpi with WT UTI89 (p<0.05 and p<0.01 respectively; Fig. 1C). We also primed with heat-killed UTI89 and found that live, but not heat killed, UTI89 were capable of priming the bladder indicating that bacterial products such as LPS were insufficient (Fig. 1B). These data indicate that live and invasive UTI89 capable of at least some degree of intracellular replication are required for the priming to enhance the incidence of chronic cystitis upon superinfection of UTI89. Taken together these data suggest that priming begins during invasion and early IBC formation.

UPEC hemolysin and caspase 1/11 activation are essential

One of the most potent host defenses to eliminate adherent and invaded UPEC is superficial facet cell exfoliation [29]. The process of exfoliation is activated in part by the bacterial expression of hemolysin (HlyA) [53] (Nagamatsu et al. in review). UTI89ΔpxR overexpresses HlyA, leading to exfoliation and attenuation in our murine model of cystitis (Nagamatsu et al. in review). The UTI89ΔpxRΔhlyA double mutant was not attenuated, suggesting that the in vitro defect was due to increased hemolysin expression (Nagamatsu et al. in review). The ability of UPEC to rapidly build up in numbers in the form of IBCs and then disperse to neighboring cells may be part of a mechanism to subvert an exfoliation response. Thus, fine-tuning the expression of HlyA during acute bladder infection may serve to maximize UPEC persistence and give UPEC a fitness edge against the host innate inflammatory response. Interestingly, in C3H/HeN mice, UTI89 ΔhlyA is not attenuated throughout infection and causes chronic cystitis comparable to UTI89; however, other reports suggest deletion of HlyA in UPEC CFT073 decreases virulence [54]. We investigated the role of hemolysin in priming the bladder for chronic cystitis upon superinfection by utilizing UTI89ΔhlyA or UTI89ΔpxRΔhlyA as the initial inoculation followed by WT UTI89 one hpi. Both of these strains were statistically significantly different when compared to WT UTI89 as the priming inoculum. Therefore, we conclude that neither was capable of priming the bladder for enhanced chronic cystitis (Fig. 1D). Thus, too high or low expression of hemolysin abolished the ability of UTI89 to prime for enhanced chronic cystitis implying that an optimal level of hemolysin expression is critical for priming the bladder for enhanced chronic cystitis.

HlyA-mediated exfoliation is in part due to its ability to trigger degradation of paxillin, a scaffold protein that modulates the dynamics of cytoskeletal rearrangements [55]. HlyA can also trigger cell death in human bladder epithelial cells and release of IL-1β via caspase-4 (the murine ortholog is caspase-11) activation and caspase-1-dependent IL-1β secretion via activation of the NLRP3 inflammasome pathway, which orchestrates additional cell death (Nagamatsu et al. in review). We hypothesized that inflammasome and caspase 1/11 activation were essential for superinfection. Thus, mice were treated intravesically with a dose of caspase 1/11 inhibitor or DMSO one hour prior to priming and a second dose with the priming inoculum to test this hypothesis (Fig. 2A). Providing two doses of the inhibitor was previously shown to be effective in dampening in vivo inflammatory responses. In vitro, the inhibitor dramatically reduced downstream elements of inflammasome activation, IL-1β and IL-1β secretion, when bladder cells were infected with UTI89 (Nagamatsu et al. in review). Caspase 1/11 inhibition significantly reduced median bladder titers at four weeks after superinfection relative to the DMSO control group (Fig. 2B). We also saw a trend of caspase 1/11 inhibition in reducing the proportion of WT superinfected mice experiencing chronic cystitis to single infection levels (Fig. 2B). DMSO also reduced the proportion of mice experiencing persistent bacteriuria and chronic cystitis, but to a lesser degree than caspase 1/11 inhibition (Fig. 2B vs. Fig. 1B-D), suggesting an anti-inflammatory role of DMSO alone. Intriguingly, DMSO was recently found to inhibit the NLRP3 inflammasome [56]. Taken together, these data implicate hemolysin and the NLRP3 inflammasome in the priming response to enhanced chronic cystitis.

We further investigated whether chemical exfoliation could enhance the proportion of mice experiencing chronic cystitis prior to a single infection. We utilized the cationic protein, proteamine sulfate, which has previously been used to exfoliate the superficial facet cell layer of the urothelium [40,57]. A 10 mg/mL dose delivered intravesically in 50 μL PBS was shown to exfoliate 65% of the facet cell layer 12 hours after treatment while an additional booster dose of 50 mg/mL led to 95% exfoliation [40]. We utilized these concentrations to initiate, but likely not complete, the process of exfoliation one hour prior to infection with UTI89. We did not observe a significant increase in the proportion of mice experiencing chronic cystitis over PBS pretreatment (Fig. 2C). Thus, these data suggest that at least partial IBC formation in conjunction with caspase 1/11 activation primes the bladder for enhanced chronic cystitis, but chemical initiation of exfoliation is not sufficient. Taken together, these data suggest that exfoliation per se might not play a significant role in impacting the likelihood of enhanced chronic cystitis but instead may reflect a downstream marker of the priming event.
Superinfection leads to chronic cystitis in a resistant mouse strain

C57BL/6J mice typically rapidly resolve bacteriuria and are resistant to chronic cystitis upon single inoculation with UPEC [37,38]. Five to ten percent of the time after inoculation with UTI89, C57BL/6J mice experience persistent bacteriuria, but this is generally due to kidney infection without concomitant high titer bladder infection [37,41]. This degree of kidney infection is not infectious dose dependent and therefore likely due to ureteric reflux of the bacteria during experimental inoculation [37]. We investigated whether superinfecting C57BL/6J mice during acute infection would stimulate an immune response leading to chronic cystitis. We inoculated bladders with PBS or 10⁷ CFU of UTI89 followed by superinfection with UTI89 1, 6, 24, 48 hours or one week after initial infection and collected urine at days 1, 7, 14, and 21 dpi followed by enumeration of bladder and kidney titers at 28 dpi (Fig. 3). A 24 hpi superinfection resulted in 35% of mice sustaining persistent bacteriuria with bladder titers >10⁴ CFU at four weeks compared to 0% in the singly infected group (Fig. 3A). Kidney titers were also increased in the mice with persistent bacteriuria, but we did not observe a significant increase in the proportion of mice with kidney infection greater than 10⁴ CFU (Fig. 3B). These data suggest that at 24 hours after infection the bladders of C57BL/6J mice were primed to develop chronic cystitis upon superinfection. We investigated whether an ascending kidney infection plays a role in predisposing these mice to chronic cystitis by inoculating PBS into the bladder, either 24 hours before or after infection with UTI89, to stimulate a bladder and ureter stretch response or potentially increase reflux of bacteria into the kidneys, respectively. We determined the percentage of mice with persistent bacteriuria and those with bladder and kidney titers greater than 10⁴ CFU at sacrifice (Table 1). We found in all conditions that persistent bacteriuria was a 100% predictor of kidney titers >10⁴ CFU at four wpi. Persistent bacteriuria also predicted bladder titers greater than 10⁴ CFU at four wpi in C57BL/6J mice superinfected 24 hpi with UTI89. For the group

Fig. 2. Role of caspase 1/11 and exfoliation in C3H/HeN superinfections. A) Inoculation protocol shown for caspase inhibition studies of panel B. B) Four-week total bladder titer based on inhibitor or vehicle. C) Mice were inoculated with PBS, UTI89 or the indicated dose of protamine sulfate in 50 µL PBS and inoculated one hour later with UTI89. Urine was collected weekly and overall bladder titers are shown at four weeks. Panel B represents 5 experiments with n = 5–10 mice per group. Panel C represents 2 experiments with n = 5–10 mice per group. B–C) Observations in red indicate resolved infection. Percent of mice with persistent bacteriuria and chronic cystitis is shown at the top of each column. For Panel B and C, a Fisher’s Exact Test was used to determine significance between proportions of mice experiencing chronic cystitis. Mann-Whitney U Test was used to compare median CFU values in Panel B.

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of mice inoculated with PBS before the initial UTI89 infection, persistent bacteriuria did not correlate with high bladder titers suggesting these bacteria were only replicating in the kidneys. Serially infecting with two inocula of UTI89 trended towards increased persistent bacteriuria and chronic cystitis compared to the group inoculated with UTI89 followed by PBS at 24 hpi (P = 0.066; Table 1 and Fig. 4A). Kidney titers of UTI89 superinfected mice were significantly higher than when PBS was used to prime or superinfect perhaps suggesting that repeat infection may also increase susceptibility to pyelonephritis (Fig. 4B). Thus, a 24 hpi superinfection of WT UTI89 led to increased rates of persistent bacteriuria and chronic cystitis; however, bladder/ureter stretch or kidney ascension at 24 hpi may contribute to this increase.

C3H/HeN mice that progress to chronic cystitis upon single inoculation can be predicted by elevated serum levels of IL-5, IL-6, KC, and G-CSF at 24 hpi [37]. We hypothesized that similar elevations would predict sensitization to chronic cystitis in C57BL6/J if they were subsequently superinfected. Thus, we determined levels of 23 serum cytokines from C57BL/6/J mice 24 hrs after initial inoculation with PBS or UTI89 prior to superinfection. We then superinfected a subset of the mice initially infected with UTI89 (superinfection in Fig. 5) leaving the other mice untouched (UTI89 group). All mice were evaluated with urine titers over 28 d and sacrificed to enumerate bladder titers. We stratified the superinfected mice based on outcome four weeks later as determined by persistent bacteriuria and chronic cystitis. We found elevations of serum KC (Fig. 5A), IL-6 (Fig. 5B), and G-CSF (Fig. 5C) in mice that progressed to chronic cystitis relative to those that resolved infection or were mock-infected with PBS. Therefore, higher levels of these cytokines correlate with chronic cystitis that develops later if mice are superinfected. At the time we obtained serum, the single infection and superinfection groups were identical, and no statistical differences existed among them. These data demonstrate that a subset of C57BL/6J mice respond to an initial infection in a way that results in higher specific serum cytokine levels and primes them to develop chronic cystitis if an additional insult is delivered 24 hpi.

Response to infection differs between C3H/HeN and C57BL/6J

During chronic cystitis of singly-infected C3H/HeN mice, the bladder epithelium is hyperplastic and normal terminal differentiation of the superficial facet cell layer, including the expression of surface uroplakins, does not occur [37]. In this environment, the bacteria are able to persist extracellularly by an unknown

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Fig. 3. C57BL/6J mice are susceptible to chronic cystitis when superinfected 24 hpi. A–B) Mice were transurethrally infected with PBS or UTI89 and re-infected at the indicated timepoints with UTI89. Urine was tracked weekly and four-week total bladder (A) and kidney pair (B) titer is displayed. N = 2–4 experiments with 4–5 mice per group. Statistical differences determined by Fisher’s Exact test.

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### Table 1. Characteristics of C57BL/6J superinfections.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Incidence % (n)</th>
<th>Persistent Bacteriuria</th>
<th>Resolved Bacteriuria</th>
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<tr>
<td>UTI89</td>
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<td>PBS</td>
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**Notes:**
- All mice infected 24 hours after initial infection.
- Defined as >10^4 CFU/mL bacteria in clean catch urine throughout four-week infection.
- Median values listed.
- Photographs were used for imaging purposes.
- CUP pili may play a role.

**Discussion**

We have developed models of bacterial superinfection of the urinary tract, which may provide insight into the connection...
between recent and frequent sexual intercourse and the susceptibility to the development of chronic UTI [5,22]. Our results demonstrate that superinfection resulted in increased susceptibility to chronic cystitis in both susceptible and resistant mouse genetic backgrounds, but the time window for priming differed between strains. We have previously shown that chronic cystitis predisposes to severe rUTI upon a subsequent infection weeks to months after clearance of the first infection with antibiotics [37]. Clinically, millions of women take post-coital and prophylactic antibiotics so as not to develop rUTI [64]. Therefore, if clinically applicable, our results detailed here may partially explain why frequent sexual intercourse is such a strong risk factor for UTI. The necessity of prophylactic antibiotics could be obviated if the risk factors and bacterial traits identified here can be altered in the clinical population of women suffering chronic rUTIs.

Frequent sexual intercourse is among the most important risk factors for rUTI in young women [22]. Peri-urethral carriage of the causal strain and sexual intercourse immediately precede the development of a rUTI [5]. Sexual intercourse likely introduces mixed populations of bacteria into the urinary tract, with E. coli being the most common [18]. In this environment, UPEC invade bladder tissue and replicate, forming IBCs and bacterial filaments, which have been observed in human urine in 40% of patients suffering acute UTI, 24–48 hours after reported sexual intercourse [21]. These data may provide mechanistic insight as to the frequent clinical observation that recent and frequent sexual intercourse over a brief period of time leads to increased rates of rUTI [23]. Furthermore, elevated levels of serum CSF1, CXCL-1, and CXCL-8 in women with acute UTI were associated with a higher rate of rUTI [45]. Using C3H/HeN and C57BL/6J mice,

Fig. 4. UTI89 Superinfection of C57BL/6J mice increases bladder and kidney infection. A–B) mice were infected with PBS or UTI89 Kanr and re-infected 24 hrs later with PBS or UTI89 Spect. Urine was tracked over four weeks and total bladder (A) and kidney pair titer (B) is displayed. Number above columns indicates number of mice with persistent bacteriuria with bladder (A) or kidney (B) titer>10^4 CFU. Data represents 3–8 experiments with n = 4–29 mice per group. Panels also include data reproduced from Fig. 3. Statistical differences determined by Fisher’s Exact test. doi:10.1371/journal.ppat.1004599.g004

Fig. 5. Serum cytokine signature of C57BL/6J mice with persistent bacteriuria. Serum was obtained 24 hrs after initial inoculation prior to superinfection. Levels of 23 cytokines were determined and cytokines showing significant differences between resolved and chronic superinfected mice are shown. Levels of KC (A), IL-6 (B), and G-CSF (C) shown in pg/mL. Data represent 4–6 experiments with n = 4–29 mice per group. Statistical differences determined by One-Way ANOVA overall and Mann-Whitney U test for pairwise comparisons. doi:10.1371/journal.ppat.1004599.g005
we have shown that superinfection during the period of acute infection dramatically increases the proportion of mice that experience chronic cystitis with inoculations of $10^7$ UPEC (Fig. 1A and 3A). The bacterial characteristics responsible for frequent recurrences are beginning to be assessed [65]. Hemolysin is expressed by 50% of UPEC isolates, but is more likely to be associated with symptomatic UTI [66]. It is possible that hemolysin-mediated exfoliation and caspase 1/11 activation leads to UTI-associated symptoms. In our studies, we found that an increase in priming for chronic cystitis correlated with the bacterial ability to invade and replicate within the bladder tissue (Fig. 1B–C), and through hemolysin to activate caspase 1/11 leading to IL-1 secretion and bacterial replication (Fig. 1D and 2B). Activation of caspase 1/11 has been shown to contribute to epithelial cell death in vitro and exfoliation in vivo in C3H/HeN mice, suggesting that caspase-mediated exfoliation may expose the underlying epithelium upon which UPEC replicates during chronic cystitis (Nagamatsu et al. in review). Inhibition of caspase 1/11 protected superinfected mice from chronic cystitis (Fig. 2), suggesting a role for cytokines downstream of caspase activation including IL-1α and IL-1β, identified in our microarray of four-week bladders (Fig. 6; S1 Table). A microarray analysis revealed that in C3H/HeN and C57BL/6j mice, 11/20 of the most upregulated genes during chronic cystitis were the same. Differences between the responses to infection in these mouse strains may result from the dramatic increase in kidney infection or QIR presence in C57BL/6j relative to C3H/HeN mice [37,40]. Further, this data supports the hypothesis that a muted inflammatory response to UPEC infection is more likely to lead to resolution [26]. Also, our studies suggest that serum biomarkers such as IL-6, KC, and G-CSF may predict a predisposition to rUTI (Fig. 5) [37]. Recently, it was demonstrated that cytokines involved in immune cell chemotaxis and maturation (the human homolog of KC included) during acute UTI enhanced the likelihood to develop chronic cystitis, treatments with soluble compounds such as mannosides that block the ability of UPEC to invade the tissue or compounds that might alter FimH conformation hold promise as effective means to prevent or treat rUTI [67–70]. These analyses may allow us to identify high-risk patients for more aggressive therapy and/or anti-virulence compounds to limit this troubling disease.

**Materials and Methods**

**Bacterial strains**

All WT bacterial strains utilized were derivatives of UTI89, including tagged, isogenic UTI89 isolates, kanamycin resistant UTI89 attHK022::COM-GFP, kanamycin resistant UTI89 with re-integrated UTI89 FimH, spectinomycin resistant UTI89 att::PSSH10-1, and chloramphenicol resistant UTI89 [24,47,71]. FimH mutant strains, ΔompA, ΔhlyA, ΔΔhlyR were all previously published [47,31,32][Nagamatsu et al. in review].

**Mouse infections**

Bacteria for infection were prepared as previously described [72]. Six to seven week old female C3H/HeN (Harlan) or C57BL/
6J (Jackson) were transurethrally infected with a 50 µL suspension containing $5 \times 10^5 - 2 \times 10^7$ CFU of UTI89 or relevant mutant in PBS under 3% isofluorane. Protramine Sulfate (Sigma) was dissolved in PBS and caspase 1/11 inhibitor Ac-YVAD-CMK (BACHEM) was dissolved in DMSO and transurethrally inoculated into the bladder. At indicated timepoints after infection, mice were anesthetized and infected again. Venous blood was obtained at 24 hpi, just prior to re-infection, by submandibular puncture and centrifuged at max speed at 4°C in Microtainer serum separation tubes (BD) and stored at −20°C until use. Cytokine expression was measured using the Bio-Plex multiplex cytokine Group 1 bead kit array (Bio-Rad), which measures 23 cytokines. Expression was measured using the Bio-Plex multiplex cytokine separation tubes (BD) and stored at −20°C until use. Cytokine data, values out of the range of the instrument were not included for analysis. Fisher’s exact test was utilized to determine differences between groups for rates of chronic cystitis. One-way ANOVA was utilized to determine whether any cytokine differences were apparent and pairwise assessment of median values was determined by Mann-Whitney test. Unless otherwise indicated, p<0.05 was considered significant. Analyses were performed in Graphpad Prism 5.0.

Scanning electron microscopy
Mice were infected as described above. Bladders were aseptically harvested, bisected, and splayed. Bladders were fixed in 2.0% glutaraldehyde in 0.1M sodium phosphate buffer overnight. Bladders were then washed three times with 0.1M sodium phosphate buffer and de-ionized water before being fixed in 1.0% osmium tetroxide. Bladders were washed and then critical point drying was performed with absolute ethanol and liquid carbon dioxide. Sputter coating was performed with gold-palladium using a Tousimis Samsputter-2a. Images were obtained on a Hitachi S-2600H operated at 20 kV accelerating voltage.

Statistical analysis
Datapoints below the limit of detection (LOD) were set to the LOD for graphical representation and statistical analysis. For cytokine data, values out of the range of the instrument were not included for analysis. Fisher’s exact test was utilized to determine differences between groups for rates of chronic cystitis. One-way ANOVA was utilized to determine whether any cytokine differences were apparent and pairwise assessment of median values was determined by Mann-Whitney test. Unless otherwise indicated, p<0.05 was considered significant. Analyses were performed in Graphpad Prism 5.0.

Supporting Information
S1 Fig. Bacteria replicate on the bladder surface during chronic cystitis. Bladders of C3H/HeN and C57BL6 mice were splayed four wpi and fixed in glutaraldehyde. A–B Chronic C3H/HeN bladders. C–D Chronic C57BL/6J bladders. E Mock infected C57BL/6J bladder shown for comparison. (TIFF)

S1 Table Genes with highest fold change of chronic cystitis versus resolved. (PDF)

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Author Contributions
Conceived and designed the experiments: DJS TJH SJH. Performed the experiments: DJS MSC TJH. Analyzed the data: DJS MSC TJH SJH. Contributed reagents/materials/analysis tools: MSC TJH. Wrote the paper: DJS SJH. Edited the manuscript: MSC TJH.

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