RESEARCH ARTICLE

Subinhibitory Antibiotic Therapy Alters Recurrent Urinary Tract Infection Pathogenesis through Modulation of Bacterial Virulence and Host Immunity

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ABSTRACT **The capacity of subinhibitory levels of antibiotics to modulate bacterial virulence *in vitro* has recently been brought to light, raising concerns over the appropriateness of low-dose therapies, including antibiotic prophylaxis for recurrent urinary tract infection management. However, the mechanisms involved and their relevance in influencing pathogenesis have not been investigated. We characterized the ability of antibiotics to modulate virulence in the uropathogens *Staphylococcus saprophyticus* and *Escherichia coli*. Several antibiotics were able to induce the expression of adhesins critical to urothelial colonization, resulting in increased biofilm formation, colonization of murine bladders and kidneys, and promotion of intracellular niche formation. Mice receiving subinhibitory ciprofloxacin treatment were also more susceptible to severe infections and frequent recurrences. A ciprofloxacin prophylaxis model revealed this strategy to be ineffective in reducing recurrences and worsened infection by creating larger intracellular reservoirs at higher frequencies. Our study indicates that certain agents used for antibiotic prophylaxis have the potential to complicate infections.**

IMPORTANCE **Antibiotics are the mainstay treatment for bacterial infections; however, evidence is emerging that argues these agents may have off-target effects if sublethal concentrations are present. Most studies have focused on changes occurring *in vitro*, leaving questions regarding the clinical relevance *in vivo*. We utilized a murine urinary tract infection model to explore the potential impact of low-dose antibiotics on pathogenesis. Using this model, we showed that subinhibitory antibiotics prime uropathogens for adherence and invasion of murine urothelial tissues. These changes in initial colonization promoted the establishment of chronic infection. Furthermore, treatment of chronically infected mice with subtherapeutic ciprofloxacin served to exacerbate infection. A part of these changes was thought to be due to suppression of mucosal immunity, as demonstrated through reductions in cytokine secretion and migration of leukocytes into the urinary tract. This work identifies novel risk factors associated with antibiotic therapy when dosing strategies fall below subtherapeutic levels.**

Received 2 March 2015 **Accepted** 6 March 2015 **Published** 31 March 2015

Citation Goneau LW, Hannan TJ, MacPhee RA, Schwartz DJ, Macklaim JM, Gloor GB, Razvi H, Reid G, Hultgren SJ, Burton JP. 2015. Subinhibitory antibiotic therapy alters recurrent urinary tract infection pathogenesis through modulation of bacterial virulence and host immunity. mBio 6(2):e00356-15. doi:10.1128/mBio.00356-15.

Editor Julian E. Davies, University of British Columbia

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The use of antibiotics to treat and prevent bacterial infections has made an unprecedented impact on improving human health. However, recent studies demonstrating the capacity of antibiotics to modify bacterial phenotypes at levels below the MIC are challenging the paradigm that their effects are benign and have raised the possibility that they may actually enhance bacterial virulence. Transcriptional analyses suggest that subinhibitory levels of antibiotics induce the differential expression of as many as 5 to 10% of the genome, modulated through the activities of both general stress responses and specific signaling systems (1–5), including the enhancement of toxin and biofilm production by potential human pathogens (3, 6–14). In addition, antibiotics, including macrolides and fluoroquinolones, are now recognized for their capacity to suppress proinflammatory host responses (15, 16). Thus, we hypothesize that modulation of gene expression in both the host and the pathogen during prophylactic treatment with a low dose of an antibiotic could alter the course of bacterial pathogenesis. Clinical studies have suggested that subinhibitory antibiotic therapies and dosing strategies are risk factors for severe complication of infection, risk of breakthrough infection, and future recurrence episodes (17–21). Although the etiology of therapy failure in many of these cases remains unknown, we propose that

inappropriate antibiotic treatment may be a contributing factor. The objective of our study was to explore this phenomenon in the context of a subinhibitory dosing regimen, namely, antibiotic prophylactic management for recurrent urinary tract infection (rUTI).

UTIs are very common, accounting for 10.5 million outpatient and emergency room visits during 2007 in the United States alone (22). They are a significant cause of morbidity in women throughout their life span and in infant boys and older men, with serious sequelae, including treatment failure, frequent recurrences, pyelonephritis with sepsis, and renal damage in young children (23, 24); however, upwards of 44% of women will experience a recurrence, with ~10% of these caused by a relapse of the original index episode strain (25). Such recurrent or persistent infections are generally managed by using long-term, low-dose prophylactic regimens. In general, UTIs can be readily treated with short-course antibiotic therapy (~3 to 7 days). However, following therapy, recurrent or persistent infections may develop that are often managed using long-term, low-dose prophylactic regimens. While prophylaxis is usually effective in reducing symptoms during its application (26), it does not alter the long-term risk of recurrence, as infection rates return to pretreatment levels following therapy (27) and pathogens become more resistant to future treatment (28). Thus, it is critical to understand the effectiveness of antimicrobial prophylaxis in managing recurrent infections and whether the benefits of such treatments outweigh the risks of promoting resistance and potential increased virulence.

Uropathogenic *Escherichia coli* (UPEC) and *Staphylococcus saprophyticus* collectively cause ~95% of all uncomplicated UTIs (23, 29) and are often associated with recurrent and chronic disease necessitating long-term prophylaxis management. Type 1 fimbriae and uro-adherence factors (Uafs) are critical in establishing UPEC and *S. saprophyticus* UTIs, respectively (30, 31). In particular, type 1 fimbriae are important in promoting the invasion of superficial umbrella cells on the luminal surface of the bladder epithelium and formation of intracellular bacterial communities, whereby UPEC rapidly replicates within the cytoplasm during acute infection to establish a foothold in the bladder (32–37). While the fate of intracellular *S. saprophyticus* is unknown, intracellular UPEC proliferation results in the formation of large, multicellular aggregates, dubbed intracellular bacterial communities (IBCs) (32). Within this niche, uropathogens are able to subvert host immune cells and rapidly build up in numbers, thus facilitating the progression toward chronic infection in mice. In addition, UPEC has been shown to be able to survive antibiotic therapy by forming dormant quiescent intracellular reservoirs (QIRs) that can serve as seeds for recurrent infection (38–41).

We hypothesized that subinhibitory antibiotic-dependent virulence modulation and immune suppression *in vitro* would translate to actual changes in disease outcome *in vivo*. Thus, we investigated various classes of subinhibitory antibiotics for their capacity to influence bacterial virulence traits in two uropathogenic clinical isolates, *S. saprophyticus* 15305 and *E. coli* UTI89. We also attempted to characterize the signaling networks involved with these responses in UPEC by using expression analyses. Changes in virulence and pathogenesis were assessed *in vivo* by inoculating ciprofloxacin-primed mice (who had received 1/4 the minimal inhibitory dose of ciprofloxacin) with *S. saprophyticus* or UPEC and determining acute and chronic infection characteristics, including bacterial persistence within the urinary tract and establishment of intracellular bacterial reservoirs. The effect of inadequate (with 1/50 the empirical therapeutic dose) ciprofloxacin dosing in chronically infected or resolved mice was also investigated, in addition to characterizing immune responses in this host. Lastly, the efficacy of prophylaxis was assessed for its ability to prevent persistent UTIs and clear UPEC from host urine and tissues in addition to the intracellular niche.

RESULTS

Subinhibitory antibiotics induce adhesin expression and biofilm formation in uropathogens. Challenge of both *S. saprophyticus* and UPEC with subinhibitory ciprofloxacin, ampicillin, or gentamicin resulted in denser biofilm production (Fig. 1A). In addition, enhanced planktonic aggregation was observed with both organisms (see Fig. S1 in the supplemental material). For UPEC, motility was suppressed (Fig. 1B), as determined in a soft agar motility assay, and type 1 fimbriation was increased, as determined via a guinea pig erythrocyte hemagglutination assay (Fig. 1C). Similarly, subinhibitory antibiotics increased sheep erythrocyte hemagglutination with *S. saprophyticus*, suggesting increased exposure or the presence of adhesin uro-adherence factor A (UafA) (Fig. 1D). Since capsular polysaccharide can interfere with UafA-ligand interactions, hemagglutination was also evaluated in an acapsular strain to discern whether our observations were due to increased UafA expression or decreased capsular polysaccharide abundance. We found that subinhibitory antibiotics also increased hemagglutination in the acapsular strain, indicative of increased UafA surface exposure (Fig. 1D).

Antibiotic-induced adhesin expression is SOS dependent in UPEC. RNA sequencing analysis confirmed significant induction of both a *fimH*-like adhesin, *fmlD* (4.84-fold), and the suspected uropathogenic adhesin/invasin *hek* (2.47-fold) (Table 1). Interestingly, changes in type 1 fimbrial transcripts were not observed via RNA sequencing, nor were any changes observed in the proportion of the population that had the invertible *fim* promoter in the ON orientation (data not shown). As the SOS-responsive elements *lexA* and *recA* were also highly induced with treatment (4.08- and 3.37-fold, respectively), we assessed their contributions to adhesin expression and biofilm formation by using SOS-deficient *E. coli* UTI89 strains. These strains demonstrated a modest suppression in biofilm formation (Fig. 2A) and type 1 fimbria-dependent hemagglutination (Fig. 2B) and did not respond to subinhibitory ciprofloxacin or gentamicin treatment (Fig. 2C and D). Expression analysis using quantitative PCR (qPCR) confirmed *fmlD* and *hek* induction with ciprofloxacin challenge in wild-type UPEC; however, transcription was not altered in the *recA*-deficient strain (Fig. 2E). Ampicillin increased biofilm mass in all strains tested, but only at concentrations near the MIC (Fig. 2C and D).

Subinhibitory antibiotics prime uropathogens for urothelial colonization. Ciprofloxacin priming prior to infection resulted in higher overall bladder and kidney titers for both *S. saprophyticus* 15305 and *E. coli* UTI89 at 24 h postinfection (p.i.) (Fig. 3A). Almost all murine kidneys inoculated with primed uropathogens lacked signs of bacterial clearance. Ciprofloxacin priming also resulted in increased risk of severe UTI, manifesting as persistent bacteriuria, high bacterial bladder titer, and chronic inflammation, an outcome referred to as chronic cystitis, at 14 days p.i. (Fig. 3B). Chronic cystitis represents unchecked luminal bacterial replication and is defined histologically by urothelial hyperplasia

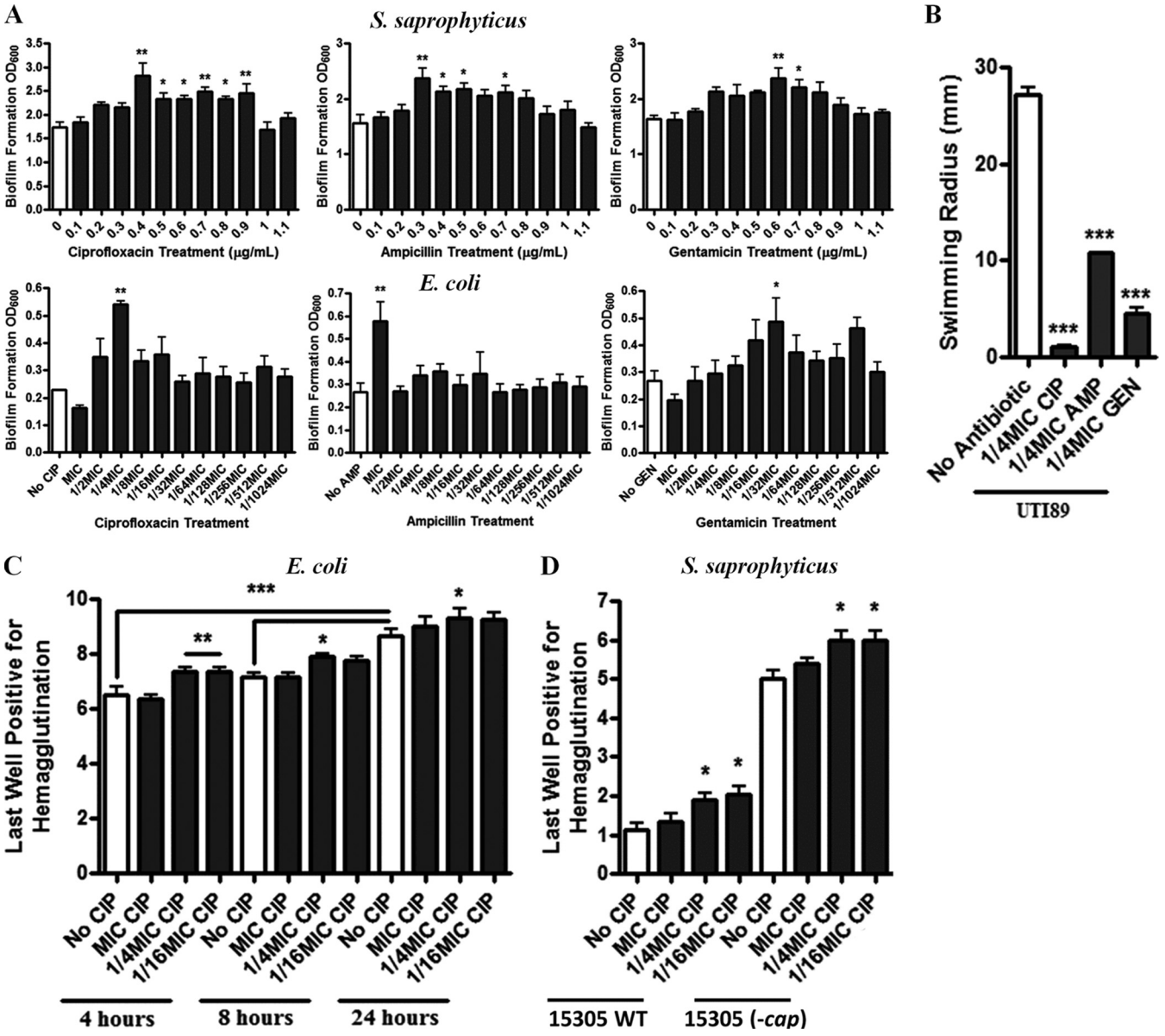

FIG 1 Subinhibitory antibiotics prime uropathogens for colonization. (A) *S. saprophyticus* 15305 and *E. coli* UTI89 biofilm formation in the presence of various antibiotic concentrations at 24 h. (B) Swimming motility of *E. coli* UTI89 in the presence of subinhibitory antibiotic concentrations. (C) Type 1 fimbria-dependent hemagglutination of *E. coli* UTI89 exposed to various subinhibitory concentrations of ciprofloxacin over time. (D) UafA-dependent hemagglutination of *S. saprophyticus* 15305 and the acapsular C1 strain following exposure to various concentrations of ciprofloxacin. Means from at least three independent experiments are shown. Significance was determined using a one-way ANOVA and Dunnett's or Bonferroni's multiple comparison test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

and submucosal lymphoid aggregates, a histological pattern similar to that seen in humans experiencing chronic UTI (33, 42, 43). The bladder colonization threshold for assessing the risk of chronic cystitis is the presence of *E. coli* UTI89 loads of $>10^4$ CFU/ml at 14 days p.i. or later (such data do not exist for *S. saprophyticus*) (44). Considering this threshold, priming resulted in 100% of mice presenting with a high risk of chronic *E. coli* UTI89 cystitis. Although there are no established infection thresh-

TABLE 1 Subinhibitory ciprofloxacin induces urothelium adhesin gene expression[a]

Gene	Fold change ($\log_2$)	Median effect size	Functional annotation
fmlD	2.275	1.891	Fimbrial, FimH-like protein, mannose binding
Hek	1.304	3.475	Hek adhesin/virulence factor
lexA	2.03	5.635	Repressor of LexA
recA	1.751	3.75	DNA repair, SOS induction

[a] Significant changes in differentially expressed transcripts of interest in *E. coli* UTI89 after 4-h exposure to 1/4 MIC ciprofloxacin.

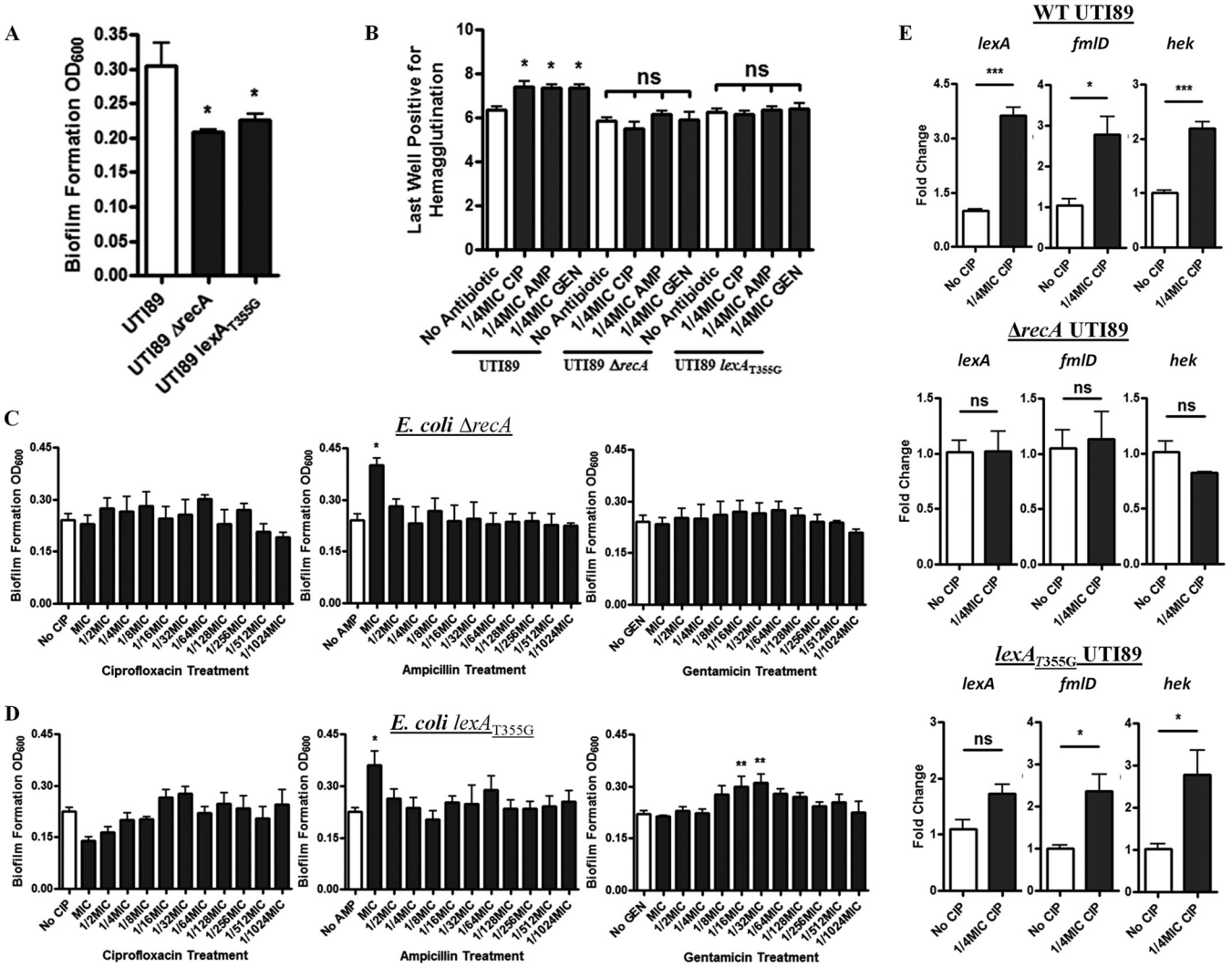

FIG 2 SOS activation is essential for antibiotic-primed adhesion. (A) Biofilm formation of *E. coli* UTI89 and SOS-deficient strains after 24 h. (B) Type 1 fimbria-dependent hemagglutination of wild-type and SOS-deficient strains of *E. coli* UTI89. (C and D) Biofilm formation of *E. coli* SOS-deficient strains Δ*recA* (C) and $lexA_{T355G}$ (D) after 24 h in the presence of various antibiotics at subinhibitory concentrations. (E) Quantitative real-time PCR analysis of wild-type and SOS-deficient strains of *E. coli* UTI89; the reported data are the fold changes in expression of *lexA*, *hek*, and *fmlD* after 4 h of exposure to 1/4 MIC ciprofloxacin. Means from at least three independent experiments are shown. Significance was determined using a one-way ANOVA and Dunnett's multiple comparison test. ns, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

olds for *S. saprophyticus* 15305, it is noteworthy that 100% of mice inoculated with untreated pathogen completely resolved their infection, while 50% maintained low, but measurable, bladder titers with ciprofloxacin priming. Among primed *S. saprophyticus* 15305-treated mice, 100% maintained at least some level of infectious kidney titer, while in control groups only 60% exhibited the same result.

Antibiotic-driven colonization promotes tissue invasion and IBC formation. Changes in IBC formation during the early stages of infection were assessed for *E. coli* UTI89 by staining for *lacZ* expression using 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). This technique indicated significantly more IBCs in ciprofloxacin-primed groups, indicative of more numerous urothelial invasion events (Fig. 4A). IBC morphology was further explored using a green fluorescent protein (GFP)-expressing *E. coli* UTI89 strain and confocal microscopy. Appearance of untreated *E. coli* UTI89 was unremarkable, as IBCs demonstrated characteristic tight, globular clustering (Fig. 4B). However, examination of ciprofloxacin-primed *E. coli* UTI89 revealed larger, diffuse IBCs with atypical morphology (Fig. 4C). Early filamentation was also noted in several of the IBCs belonging to the ciprofloxacin-treated *E. coli* UTI89 group (Fig. 4D). Volumetric analysis of the IBCs confirmed that ciprofloxacin priming significantly increased overall size 2.0-fold (Fig. 4E). The ability for ciprofloxacin to induce filamentation was investigated further, as this process might contribute to observed early IBC evacuation, which has been shown to lead to spreading of the infection to neighboring cells (45). Cultures of *E. coli* UTI89 were treated with subinhibitory ciprofloxacin and imaged using transmission electron microscopy (TEM). Control *E. coli* UTI89 cells were unremarkable, appearing to be ~1 to 2 μm in length (see Fig. S2A in the supplemental material). Most cells in the ciprofloxacin treatment

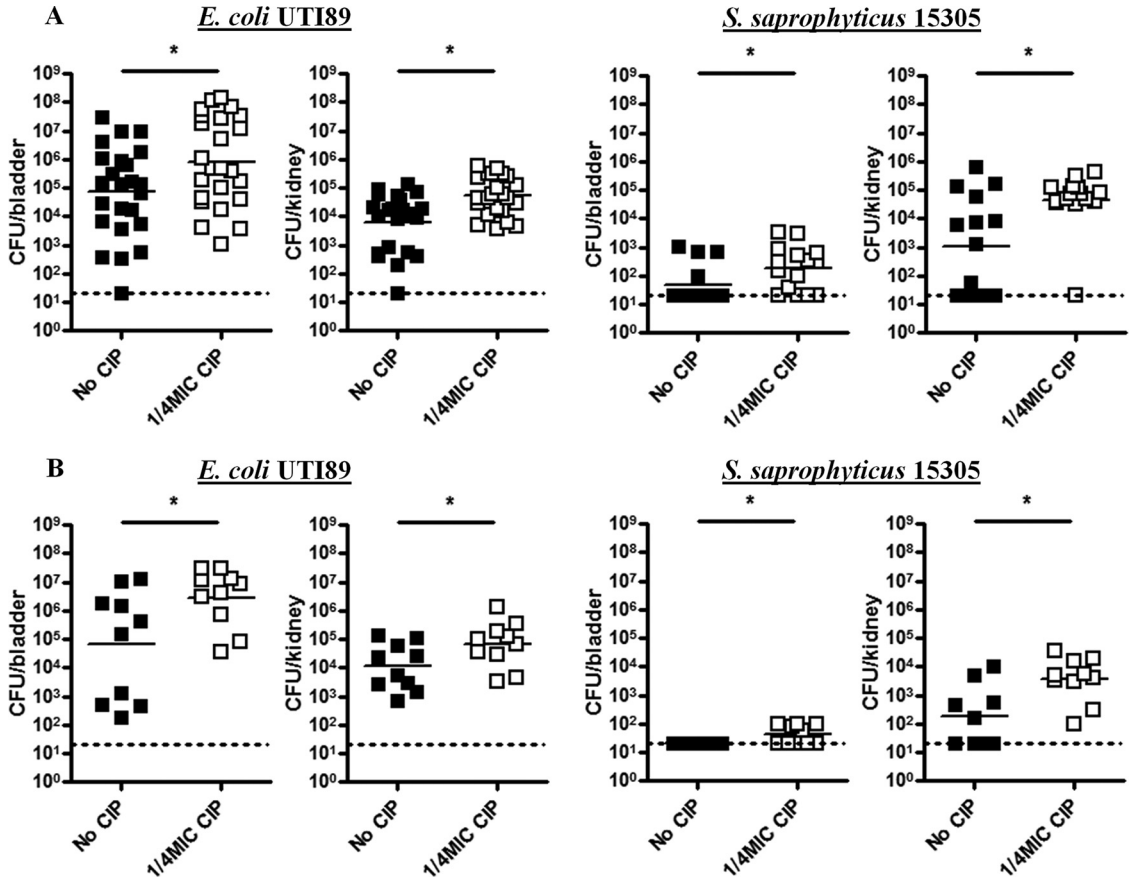

FIG 3 Antibiotic priming improves uropathogen colonization. *S. saprophyticus* 15305 and *E. coli* UTI89 titers in C3H/HeN mouse bladders and kidneys at 24 h (A) or 14 days (B) postinfection. The dotted line indicates the limit of detection. Means from two independent experiments are shown. Significance was determined using the Mann-Whitney test (Gaussian approximation). *, $P < 0.05$.

group appeared similar in nature; however, ~10% of the overall population presented with the observed filamenting phenotype, sometimes increasing to >10 μm in length (see Fig. S2B).

Subtherapeutic ciprofloxacin dosing induces recurrence and severe infection in mice. The effects of oral subinhibitory ciprofloxacin therapy were considered in two groups of mice that had been previously inoculated with *E. coli* UTI89 and had either (i) naturally resolved their infection (described as a $<10^4$ CFU/ml bacterial urine load >30 days p.i.) or (ii) maintained chronic urine titers (described as $>10^4$ CFU/ml for >30 days p.i.). Prior to the initiation of oral antibiotic therapy, the urine titers for all mice were measured over 3 days in the absence of antibiotics to determine the baseline bacterial burden. Supplementation of ciprofloxacin into drinking water at 1/50 the empirical therapeutic concentration caused 80% of previously resolved mice to develop significant infection ($>10^4$ CFU/ml) after 3 to 6 days (Fig. 5A). Although 1/25 the empirical therapeutic dose had no effect, 1/10 the empirical therapeutic dose resulted in retraction of bacterial urine loads to the limit of detection, suggesting that the observed antibiotic-dependent increases were not due to the appearance of resistant mutants (data not shown). In chronically infected mice, supplementation with 1/50 the empirical therapeutic dose for 3 days significantly increased mean UPEC urine titers by 26-fold (Fig. 5B). Again, treatment with 1/10 the therapeutic dose was sufficient to decrease bacteriuria to the limit of detection (data not shown).

Ciprofloxacin modulates human and murine mucosal immune responses. Luminex profiling demonstrated induction of the cytokines interleukin-6 (IL-6) and IL-8 in T24 bladder cells infected with *S. saprophyticus* 15305, but this induction was suppressed to below baseline levels with the application of subinhibitory ciprofloxacin (Fig. 6A). IL-8 secretion in human 5637 bladder cells treated with *S. saprophyticus* 15305 or lipopolysaccharide (LPS) was also reduced by ciprofloxacin, slightly by gentamicin, but not affected by ampicillin, as determined in an enzyme-linked immunosorbent assay (ELISA) (Fig. 6B). Thus, we hypothesized that these observed immunomodulatory effects suppress the secretion of proinflammatory mediators and decrease immune cell infiltrate *in vivo*. Urine sediment polymorpohonuclear leukocytes (PMNs) taken 3.5 h after UPEC inoculation was reduced in mice receiving subtherapeutic ciprofloxacin (Fig. 6C). Ciprofloxacin-dependent modulation of cytokine secretion was assessed using a Luminex bead-based multiplex assay of extracted bladder tissue homogenates. A total of seven urinary-relevant cytokines were assessed and included IL-1β, IL-6, keratinocyte chemoattractant (KC, also called IL-8), granulocyte colony-stimulating factor (G-CSF), IL-17, IL-10, and tumor necrosis factor alpha (TNF-α) (46). All produced reliable profiles with the exception of TNF-α (data not shown). Changes in IL-17 secretion were unremarkable in either the control or ciprofloxacin treatment groups (Fig. 6D). Similarly, KC was not significantly impacted by ciprofloxacin, although there was a trend of reduced secretion when antibiotic was

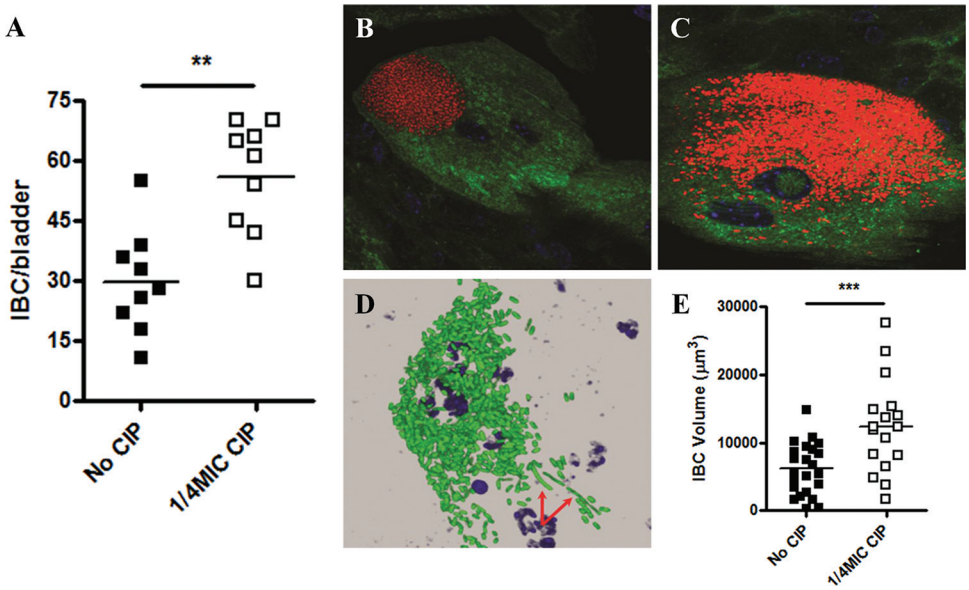

FIG 4 Antibiotic priming increases the invasive capacity of UPEC. (A) *E. coli* UTI89 IBC quantity at 6 h p.i. of C3H/HeN bladders. (B and C) Representative confocal images of control (B) and antibiotic-pretreated (C) *E. coli* UTI89 IBCs in C3H/HeN mouse bladders 6 h p.i. (red, GFP/UTI89; blue, Syto9/nuclei; green, WGA/cell). (D) Early pathogen evacuation from an IBC via filamentation (arrows) in a ciprofloxacin-pretreated sample. (E) Volumetric analysis of confocal images is depicted for both control and antibiotic-pretreated IBCs. Means from at least two independent experiments are shown. Significance was determined using a one-way ANOVA, Dunnett's multiple comparison test, or the Mann-Whitney test. *, $P < 0.05$; **, $P < 0.01$.

present (Fig. 6D). The proinflammatory mediators IL-1β and IL-6 were suppressed, while secretion of the anti-inflammatory cytokine IL-10 was increased (Fig. 6D). In contrast, the proinflammatory cytokine G-CSF was significantly increased in the presence of ciprofloxacin and *E. coli* UTI89 infection (Fig. 6D).

Prophylactic intervention is ineffective in curtailing recurrence and increases intracellular UPEC burden. The possibility that low-dose prophylaxis promotes virulence and enhances pathogenesis was investigated in the C57BL/6J model of rUTI, where frequent recurrent infections originate from QIRs (33, 39, 47). Following inoculation with nonprimed UPEC, changes in bacterial urine load were assessed at least every 2 days to track the course of infection using a novel prophylaxis therapy model. Establishment of infection after 24 h and response to trimethoprim-sulfamethoxazole ("co-trimoxazole") intervention were observed (see Fig. S3 in the supplemental material). The following 7-day antibiotic-free period revealed dynamic changes in *E. coli* UTI89 urine titers, with frequent recurrences observed. Initiation of prophylactic therapy did not appear to improve the frequency of infection resolution over the 7-day treatment period, as 2/20 mice in this group presented with clinically significant bacteriuria while 3/20 in the untreated group did so at the study's end (Fig. 7A). Both bladder and kidneys demonstrated greater bacterial loads in the prophylaxis group, although this difference was not significant (Fig. 7B). However, comparison of bladder titers revealed prophylaxis significantly increased the presence of low-level bladder CFU

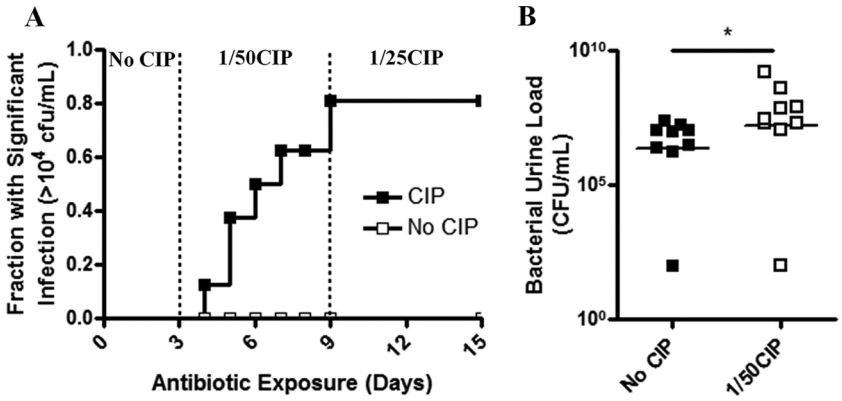

FIG 5 Subtherapeutic ciprofloxacin increases bacterial burden and recurrence frequency. (A) The fraction of resolved C3H/HeN mice presenting with clinically significant ($>10^4$ CFU/ml) bacterial urine titers following subtherapeutic ciprofloxacin dosing over time ($n = 10$ mice). The antibiotic dosing period is indicated on the upper *x* axis as fractions of the therapeutic dose. (B) Urine titers of chronically infected mice receiving either no antibiotic or subtherapeutic ciprofloxacin for 3 days. Means from at least two independent experiments are shown. Significance was determined using a log rank test (panel A; $P = 0.0131$) or Mann-Whitney test (panel B; $P = 0.0142$).

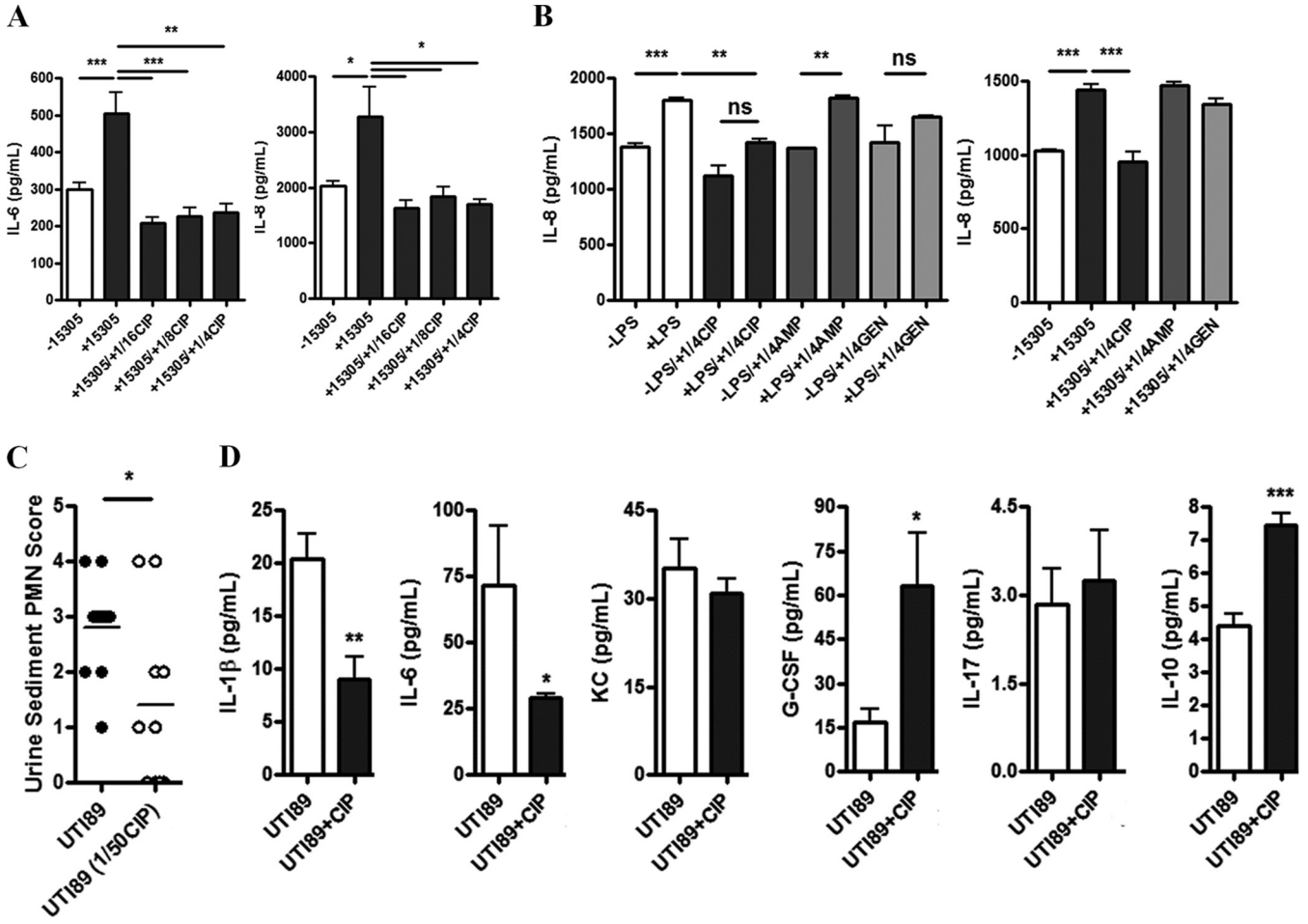

FIG 6 Ciprofloxacin suppresses urinary cytokine secretion and PMN infiltration. (A) IL-6 and IL-8 cytokine expression profiles for T24 bladder cells infected with *S. saprophyticus* 15305 exposed to various subinhibitory levels of ciprofloxacin. (B) Release of IL-8 from 5637 bladder cells following treatment with either LPS or *S. saprophyticus* 15305 in the presence of 1/4 MIC of either ciprofloxacin, ampicillin, or gentamicin, as indicated. (C) PMN counts collected from murine urine sediments 3.5 h after *E. coli* UTI89 inoculation. Mice were treated with or without 1/50 the subtherapeutic ciprofloxacin concentration in their drinking water. (D) Murine bladder cytokine secretion 3.5 h p.i. with *E. coli* UTI89 in the presence or absence of 1/50 the subtherapeutic ciprofloxacin concentration in drinking water. Means from at least two independent experiments are shown. Significance was determined using a one-way ANOVA and Bonferroni's multiple comparison test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

as determined via *ex vivo* gentamicin protection; this was likely indicative of more numerous QIRs (Fig. 7C).

DISCUSSION

Subinhibitory antibiotics promote SOS-dependent virulence factor expression. We have demonstrated that subinhibitory antibiotics enhance adherence and biofilm production in uropathogens. However, unlike previous studies, which focused on single pathogen-antibiotic combinations, we found that several agents targeting distinct cellular processes were capable of similar effects in unrelated pathogens and that these changes translated to enhanced virulence *in vivo*. Thus, in addition to increasing the risk of resistant infection, subinhibitory antibiotic therapy may also pose an additional risk of promoting the expression of virulence factors. We further characterized the nature of adherence and determined that adhesins critical to uropathogenesis were induced in both *S. saprophyticus* 15305 and *E. coli* UTI89 by subinhibitory concentrations of antibiotics. RNA sequencing of subinhibitory ciprofloxacin-treated UPEC revealed that *fmlD* and *hek* induction coincided with SOS activation. Further phenotypic and qPCR investigations of strains deficient in SOS revealed the importance of this stress response system to adhesin gene expression and enhanced adherence, as deficient strains no longer responded to treatment. This finding provides a general mechanism by which diverse classes of antibiotics might enhance virulence via a system essentially ubiquitous across bacterial species. Investigations further characterizing the poorly established role of the SOS in virulence regulation are warranted (48).

Subinhibitory ciprofloxacin priming enhances pathogenesis in a murine model of UTI. Previous results have demonstrated that antibiotics are capable of priming organisms for downstream colonization, primarily through the upregulation of adhesins (49–51). In mice, ciprofloxacin treatment prior to infection shifted the equilibrium toward infection persistence in bladders and kidneys. This effect was most dramatic in the kidneys, where both uropathogens showed no signs of clearance. The results imply that infection severity could be worsened by the application of ciprofloxacin prophylactically, a practice that is widely conducted (52–

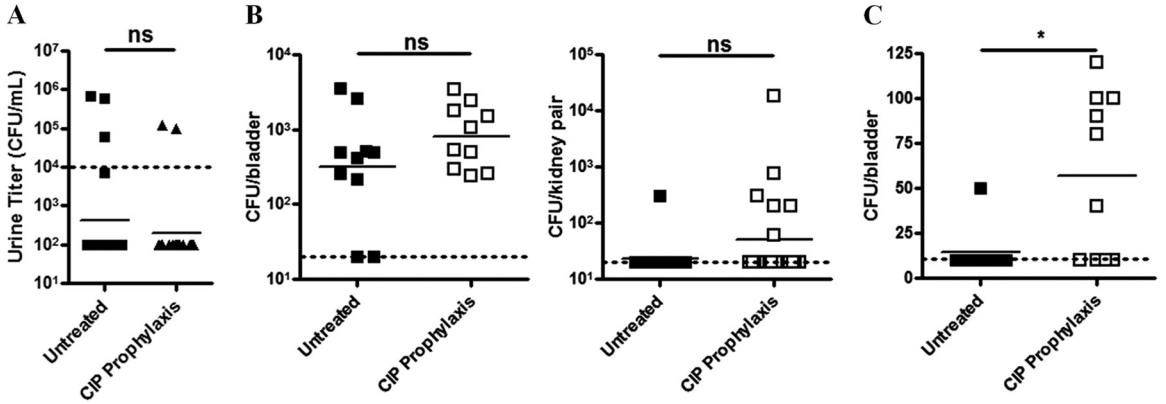

FIG 7 Low-dose ciprofloxacin therapy increases intracellular UPEC reservoirs. (A and B) *E. coli* UTI89 titers in C57BL/6 mouse urine (A) or bladders and kidneys (B) following a 1-week prophylactic treatment regimen with either ciprofloxacin or vehicle. (C) Gentamicin protection assays indicated the presence of intracellular *E. coli* reservoirs. The dotted line indicates the limit of detection. Means from at least two independent experiments are shown. Significance was determined using an unpaired, two-tailed *t* test. ns, not significant; *, $P = 0.0148$.

54). *S. saprophyticus* 15305 poorly colonized the bladders of mice but showed a similar infective capacity as *E. coli* UTI89 in the kidneys. The absence of UafA ligand in the mouse bladder likely accounts for this (55–57). C3H/HeN mice inoculated with 10^7 CFU of *E. coli* UTI89 and then presenting with $>10^4$ CFU/ml at 14 days p.i. are known to be at very high risk of chronic UTI (44). The increased titers observed following ciprofloxacin priming suggested that these organisms were better equipped to colonize the host than were the untreated counterparts. The host-pathogen interactions during the first 24 h p.i. are thought to play a significant role in infection establishment or resolution, suggesting that increased titers at this time have some predictive value in determining the severity of UTI (33). The data obtained at 14 days p.i. with *E. coli* UTI89 validated this finding, as ciprofloxacin priming prior to inoculation was sufficient to increase the frequency of chronic cystitis to 100%, compared to 60% in untreated groups. While no such data exist that describe the infection thresholds of *S. saprophyticus* 15305, it is noteworthy that 100% of mice resolved infection when inoculated with untreated *S. saprophyticus* 15305, while 50% of ciprofloxacin-primed inoculated mice maintained low-grade bladder colonization. Although lifelong infection carriage needs to be evaluated in this model, the data are highly suggestive that organism virulence is enhanced by the presence of subinhibitory antibiotic levels, such that there is a direct impact on persistence within host tissues.

The capacity for ciprofloxacin to promote chronic UPEC infections is likely dependent on its ability to induce invasion of urothelial cells. Type 1 fimbriae are known to be critical in internalization and IBC formation (58–60); their expression in response to ciprofloxacin and subsequent chronic infection development is likely paramount. Although changes in type 1 fimbria transcript abundance was not observed, posttranscriptional modification or capsule downregulation and enhanced FimH exposure might contribute to the observed changes in antibiotic-induced hemagglutination (61, 62). Increased numbers of IBCs are associated with an increased risk of chronic cystitis (36). The enhanced IBC-forming capacity observed in ciprofloxacin-primed *E. coli* UTI89 indicated that establishment of an intracellular niche is an important step in developing chronic infection in the C3H/HeN model of chronic cystitis. Ciprofloxacin also altered the morphology of IBCs, resulting in a dispersed phenotype compared to that of untreated controls. The characteristics of these IBCs, such as increased numbers of bacterial filaments, are reminiscent of those observed during later stages of infection (63), suggesting that ciprofloxacin priming alters IBC development, resulting in an earlier dispersal and fluxing of the bacteria from the IBC biomass and their spread to neighboring cells. Ciprofloxacin-triggered filamentation could thus hasten the spread to distal tissues before exfoliation can occur, promoting the establishment of quiescent intracellular reservoirs in the bladder that can serve as seeds for recurrent UTI (39). Thus, ciprofloxacin-induced changes in *E. coli* UTI89 might contribute to increased urothelial adherence, immune evasion, and invasion potential (45, 64). These processes might further help uropathogens subvert aspects of early host immunity by rapidly gaining access to the intracellular environment, and at a higher frequency. Combined, alterations in these pathogenic mechanisms are likely responsible for driving the dynamics of the host-pathogen equilibrium in favor of bacterial persistence.

Subtherapeutic ciprofloxacin augments infection severity and recurrence risk in chronically infected and resolved mice. Two experimental groups were evaluated for the influence of subtherapeutic ciprofloxacin treatment on (i) the worsening of prognosis in chronically infected mice and (ii) the predisposition of previously inoculated but resolved mice for clinically significant recurrences. In both cases, subtherapeutic ciprofloxacin was associated with increased *E. coli* UTI89 urine titers. The effects of subtherapeutic ciprofloxacin dosing on modulating cytokine expression could, in part, account for increased pathogen urine load in chronically infected mice (discussed below). However, the ability of ciprofloxacin to trigger clinically significant recurrences in resolved mice was unexpected and might occur through reemergence of *E. coli* from QIRs. As fluoroquinolones inhibit phosphodiesterase activity in mammalian cells (65, 66), a resulting accumulation of cyclic AMP has been proposed to trigger exocytosis of intracellular UPEC (67). Although chronically infected mice were housed in separate cages from resolved mice, cross-infection between mice experiencing recurrences within the same cage cannot be ruled out. Interestingly, there were mice that never experienced recurrences with ciprofloxacin therapy, indicating

urinary clearance and providing an argument against cross-infection. These findings are the first to directly associate subtherapeutic antibiotic therapy with increased infection risk *in vivo*. If translatable to the clinic, noncompliance with antibiotic prescriptions, or failure to follow strict intraoperative redosing guidelines, could contribute to infection complications (19, 20).

Ciprofloxacin modulates aspects of host immunity. Ciprofloxacin was found to have an immunomodulatory effect in urothelial tissues, which might account for the changes in bacterial urine titers observed in mice during subtherapeutic treatment. The effect was noted in both human bladder epithelial cell lines in addition to murine bladder tissues extracted following infection. Low doses of ciprofloxacin were sufficient to depress the release of IL-6 and IL-8 in T24 and 5637 bladder cells, suggesting that residual levels left over following therapy might predispose to infection later on. IL-6 and IL-8 are both important proinflammatory mediators for host cell immunity during UTIs, and suppression of either cytokine has important implications on neutrophil chemotaxis to sites of infection (68–70). This was confirmed in mice, where there were significantly depressed PMN infiltrates in urine sediments when animals were treated with subtherapeutic ciprofloxacin. In addition to IL-6 and IL-8 suppression *in vivo*, the proinflammatory mediator IL-1β, produced by activated macrophages, is an important early response element to UPEC infection, and this cytokine was suppressed with ciprofloxacin treatment. Alternatively, the anti-inflammatory cytokine IL-10 was upregulated with infection and ciprofloxacin presence. IL-10 is important in urothelial protection during UTI as it limits macrophage activation (71–73). Interestingly, ciprofloxacin dramatically increased the expression of G-CSF. This cytokine increases neutrophil migration from the bone marrow, and UPEC is known to trigger its upregulation during UTIs (46). G-CSF presence could be effective in increasing levels of circulating neutrophils; however, the local suppression of chemotactic cytokines in the bladder would diminish the effect at sites of infection. As serum was not collected from these mice, ciprofloxacin augmentation of systemic responses was not assessed. G-CSF also has immunomodulatory effects on macrophages and attenuates IL-1β production, leading to less efficient bacterial clearance, which further corroborates our findings (66, 74, 75). The capacity for ciprofloxacin to induce one cytokine and suppress another while having no effect on others might depend on the augmentation of immune populations and their activity within the bladder during infection. Further characterization of cytological profiles of immune population changes in response to ciprofloxacin would corroborate this hypothesis.

Ciprofloxacin prophylaxis is not associated with improved outcome and increases the intracellular bladder reservoir in mice. We were able to demonstrate therapeutically relevant effects of ciprofloxacin treatment on UPEC pathogenesis using a murine model of prophylaxis. The results indicated that the effect of prophylaxis on bacterial urine loads was negligible and not effective in preventing recurrence risk. Further assessment of sacrificed mice for augmentation in bladder and kidney *E. coli* UTI89 loads revealed a trend toward higher titers when ciprofloxacin was provided. The most important finding from these studies was in revealing the propensity for prophylaxis to enhance UPEC tissue invasion in bladders. These results mirror clinical observations, in that while prophylaxis might assist in decreasing UTI symptoms, they inexorably do not alter the long-term risk of recurrence and may in fact encourage future episodes by promoting the persistence or accumulation of intracellular reservoirs (28, 52, 76). The presence of intracellular UPEC has been associated with recurrence risk in both humans (77) and mice (36) in the past. Further studies are warranted to determine the longevity of these antibiotic-induced bacterial reservoirs and whether they result in future recurrence episodes.

Clearly, there is a need to reevaluate the effectiveness of prophylactic strategies in patients with highly recurrent UTIs, especially when treatment length is short or when individuals are noncompliant. The findings presented herein likely extend beyond recurrent UTI management, raising important questions regarding the appropriateness of even well-defined antibiotic therapeutic approaches in other disease milieux. For example, empirical therapy for emergent bacteremia may compromise some patients when pathogens are resistant and thus exposed to only subinhibitory antibiotic levels. The issues to overcome may include ensuring appropriate Gram-positive and -negative coverage, but also only using agents which are known to not induce the release of potentially deadly toxins. This study highlights the need to validate and optimize current regimens and ensure their use is based upon empirical evidence of efficacy rather than anecdotal observation, theory, or instinct on what is thought to work.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Organisms were grown in lysogeny broth (LB) supplemented with antibiotics when appropriate. MIC determinations were performed via the broth microdilution method as per CLSI protocols. Enumeration of bacterial CFU was performed using LB agar (Bacto agar; BD) plates prepared as per the manufacturer's instructions and supplemented with antibiotics when required. Cultures were grown statically unless stated otherwise. If shaking was implemented, it was done so at 200 rpm. The UPEC strain used in this study was a kanamycin-resistant derivative of the human cystitis isolate UTI89: att_{HK022}::*COM-GFP* (*E. coli* UTI89) (78). *S. saprophyticus* 15305 was purchased from the American Type Culture Collection (Manassas, VA). SOS-deficient strains *E. coli* PAS0209 (Δ*recA*) and PAS0211 ($lexA_{T355G}$; also referred to as $lexA_{G85D}$ or the Ind$^-$ mutant) derived from the UTI89 background were provided by Sheryl Justice (The Research Institute at Nationwide Children's Hospital, Columbus, OH). The acapsular *S. saprophyticus* C1 strain derived from the 15305 background was provided by Toshiko Ohta (Graduate School of Comprehensive Human Science, University of Tsukuba, Tsukuba, Japan).

Biofilm formation assays. Overnight (24-h) cultures of *S. saprophyticus* 15305 or *E. coli* strains UTI89, Δ*recA*, and $lexA_{T355G}$ grown in LB were subcultured 1,000-fold in fresh medium. The wells of a 96-well plate were prepared by loading various subinhibitory concentrations of either ciprofloxacin, ampicillin, gentamicin, or vehicle in 100 μl of LB. Organisms were added at 10 μl per well, and plates were sealed and then incubated for either 24 or 48 h at 37°C. If incubation periods continued for longer than 24 h, wells were washed and replaced with fresh LB containing the appropriate concentration of antibiotic. Following these incubation periods, wells were washed, stained with crystal violet, and decolorized with 70% ethanol. Biofilm abundance was determined by measuring the optical density at 600 nm (OD_{600}).

***In vitro* human cell line assays.** Please refer to the further description of our methods provided in the supplemental material for details used regarding our *in vitro* human cell line assays..

Hemagglutination titers. Alsevers guinea pig red blood cells (RBCs; Colorado Serum Company, Denver, CO), for *E. coli* hemagglutination, or sheep RBCs (Fisher Scientific), for *S. saprophyticus* hemagglutination, were prepared as previously described (79, 80). *E. coli* UTI89, Δ*recA*, or $lexA_{T355G}$ and *S. saprophyticus* 15305 or C1 were subcultured 1,000-fold

from overnight (24-h) cultures into fresh LB medium containing 1/4 MIC of relevant antibiotics or vehicle for 4 h. Bacteria were then pelleted at 6,500 rpm and resuspended in phosphate-buffered saline (PBS) to an OD_{600} of 1.0. One milliliter of the resulting suspension was transferred to a 1.5-ml microcentrifuge tube and centrifuged at 6,500 rpm for 2 min using a Sorvall Legend Microbiology 21 centrifuge (Thermo Scientific) to pellet. Supernatants were then aspirated, and pellets were resuspended in 100 μl of PBS. V-bottom, 96-well plates were prepared by transferring 25 μl of PBS into each well. Additionally, 4% (wt/vol) α-methyl-D-+-mannopyranoside (Sigma) was prepared in a second plate in a similar manner and served as a measure of mannose-resistant hemagglutination. In duplicate, 25 μl of each suspension was transferred to the first column of the plate and serially diluted 2-fold into each subsequent well to create a dilution gradient of organisms. Twenty-five microliters of the prepared blood suspension was added per well and mixed by gently tapping the plate. Plates were then sealed, covered, and placed at 4°C for 2 to 3 h prior to analysis. Titers were read by determining the last well to display RBC agglutination.

Soft agar motility assay. Soft agar plates (0.3% agar) were prepared by loading with either 1/4 MIC levels of antibiotic or vehicle as previously described (81). *E. coli* UTI89, Δ*recA*, or $lexA_{T355G}$ was subcultured 1,000-fold in fresh LB without antibiotic, incubated at 30°C with shaking (200 rpm) for 4 h, and adjusted to an OD_{600} of 1.0 before being spot plated onto the surface of antibiotic-loaded or unloaded soft agar plates. Organisms were also pretreated with 1/4 MIC levels of relevant antibiotics for 4 h prior to plating on unloaded soft agar plates. The radius of the resulting swimming zone (in millimeters) was measured 24 h postincubation.

Mouse infection protocols. Murine infections models (82) routinely made use of 6- to 8-week-old female C3H/HeN or C57BL/6 mice obtained from Harlan (Harlan Sprague Dawley Inc., Indianapolis, IN) or Jackson (Jackson Laboratory, Bar Harbor, ME), respectively. All animal studies were conducted in accordance with the Guide for the Care and Use of Laboratory Animals under Animal Welfare Assurance A3381-01 at the Washington University School of Medicine, St. Louis, MO. The Washington University School of Medicine Animal Study Committee approved animal protocol 20120216 (expiration, 01/11/2016).

E. coli UTI89 and *S. saprophyticus* 15305 used for infections were inoculated into 10 ml LB directly from 80°C freezer stocks, grown statically overnight at 37°C, subcultured (100-fold) in fresh LB medium with or without 1/4 MIC ciprofloxacin, and grown for another 4 h at 37°C (83). These cultures were then centrifuged for 10 min at 3,000 × *g*, resuspended in 10 ml of PBS, and diluted to an OD_{600} of 0.35 (~2.0×10^8 CFU/ml). Fifty microliters of this suspension (~1×10^7 to 2×10^7 CFU) was used to inoculate the bladders of mice via transurethral catheterization as previously described (82). Urine bacterial load (titer) was determined via serial dilution in PBS and spot plating (10 μl per drop, 50 μl total per dilution, 1 plate per sample) during the course of infection where indicated. At the indicated times, mice were sacrificed, and bladders and kidneys were aseptically removed and processed for microscopy or mechanically homogenized for CFU titration.

The effect of subtherapeutic ciprofloxacin dosing on the murine response to UTI was investigated in previously infected mice. The animals were initially infected with a dose (~10^8 CFU) of *E. coli* UTI89 and were left for at least 30 days to either spontaneously resolve infection or develop persistent high-titer bacteriuria (>10^4 CFU/ml for ≥28 days p.i.), which is an accurate indicator of the presence of chronic cystitis (44). The urine titers of mice were additionally monitored for 3 days prior to initiation of subtherapeutic ciprofloxacin supplementation. The empirical therapeutic ciprofloxacin dose in mice was estimated based on human data (40 μg/ml ciprofloxacin administered daily). Ciprofloxacin was supplemented into the drinking water for *ad libitum* consumption, and water intake was monitored and did not differ between antibiotic-supplemented and control groups, indicating ciprofloxacin was well tolerated and did not alter palatability (data not shown). Dosing ranges of <1/25 the empirical therapeutic dose were found to not significantly decrease bacterial urine titers over a 3-day period (data not shown). Following optimization of dosing parameters, such that UPEC was not negatively influenced by the level of ciprofloxacin present, 1/50 the empirical ciprofloxacin therapeutic dose was supplemented into the drinking water and replaced each day for 3 to 6 days. The urine titer of each mouse was monitored daily for changes as described elsewhere. At either 3 or 6 days, the dose was further increased to 1/25 the empirical therapeutic dose for another 3- to 6-day period, with urine titers again determined daily. Lastly, the dose was increased further to 1/10 the empirical therapeutic dose for 3 days to clear infection and ensure that spontaneous development of ciprofloxacin-resistant mutants did not occur.

Evaluation of intracellular bacterial populations within the bladders. *E. coli* UTI89 IBC enumeration, visualization, and volumetrics were performed using *lacZ* staining and confocal microscopy as previously described (36, 84). For IBC enumeration, animals were sacrificed 6 h p.i., and bladders were excised, bisected longitudinally, and placed onto a silicone bladder pinning pad. They were fixed with 3% paraformaldehyde (Sigma), washed thrice with 2 mM $MgCl_2$ (Sigma), 0.01% sodium deoxycholate (Sigma), and 0.02% Nonidet P-40 (Roche, Mississauga, ON, Canada) in PBS. Staining was performed using 25 mg/ml X-Gal (Sigma) and a solution containing 1 mM potassium ferrocyanide and 1 mM potassium ferricyanide (Sigma). After an incubation period of 16 h at 30°C, bladders were visualized under an Olympus SZX12 dissecting microscope (Olympus, America, Center Valley, PA).

For IBC visualization, infections and bladder fixation were conducted as described elsewhere, with the exception that *E. coli* UTI89 carrying the GFP-expressing plasmid pANT4 was utilized. Fixed bladders were washed and counterstained with nuclear ToPro3 (Molecular Probes) and recombinant wheat germ agglutinin (r-WGA) to outline superficial umbrella cells (1:700 dilution for each). Bladders were imaged using a Zeiss LSM 510 Meta laser scanning inverted confocal microscope (Thornwood, NY). IBCs were rendered in three-dimensions (3D) via reconstructive *z*-stacking of images, and volumes were determined by using Volocity 4 image analysis software (PerkinElmer, Waltham, MA).

Murine and human tissue cytokine profiling and urine sediment analysis. Mice were supplemented with 1/50 the empirical ciprofloxacin therapeutic dose or vehicle for 3 days, then inoculated with 10^7 nontreated *E. coli* UTI89 and sacrificed at 3.5 h p.i. Bacterial urine load and immune cell infiltrate were determined at the time of sacrifice, and bladders and kidneys were excised and snap-frozen in liquid nitrogen for future cytokine profiling. Murine urine immune cell sediment analysis was carried out using the CytoPro 7620 cytocentrifuge (Wescor, Logan, UT) as per the manufacturer's instructions. Stained urine sediments were examined by light microscopy on an Olympus BX51 light microscope, and the average number of PMN per 400× magnification field (high-powered field) was calculated by counting at least five fields and using a semiquantitative scoring system as previously described (44).

The cytokine expression profiles for murine bladders were determined using an xMAP fluorescent bead-based technology (Luminex Corporation, Austin, TX). Cytokines were liberated from tissues by using homogenization in extraction buffer containing 20 mM Tris-HCl (pH 7.5; Sigma), 150 mM NaCl (Sigma), 1-mM phenylmethylsulfonyl fluoride (PMSF; Sigma), 0.05% Tween 20 (Sigma), and a protease inhibitor cocktail (100-fold dilution; Roche). Conversely, supernatants from previous *in vitro* human T24 tissue cell line experiments were also extracted and analyzed. Total protein levels were assessed using a bicinchoninic acid (BCA) kit (Thermo Scientific) as per the manufacturer's instructions and diluted when required. Levels of cytokines IL-1β, IL-6, KC, G-CSF, IL-17, IL-10, and TNF-α were measured using multiplexed immunoassay kits according to the manufacturer's instructions (Bio-Rad Laboratories, Inc., Hercules, CA). Cytokine levels (in picograms per milliliter) were automatically calculated from standard curves using Bio-Plex Manager software (v. 4.1.1; Bio-Rad).

ELISAs were used to determine IL-8 expression levels in human 5637 bladder cells. Infections were carried out as previously described, with the

exceptions that LPS (Sigma) was added at 5 μg/ml in place of *S. saprophyticus* 15305 and ampicillin and gentamicin were also tested at 1/4 MIC levels in addition to ciprofloxacin. Four hours post-5637 cell infection, supernatants were extracted and cytokines were quantified using the human CXCL8/IL-8 Quantikine ELISA kit (R&D Systems, Minneapolis, MN) as per the manufacturer's instructions.

Model of ciprofloxacin prophylaxis. C57BL/6 mice were inoculated as previously described with ~10^7 CFU of nonprimed *E. coli* UTI89. Infections were allowed to develop for 24 h prior to initiation of a 3-day regimen of normal co-trimoxazole therapy (270 μg/ml replaced daily; Qualitest Pharmaceuticals, Huntsville, AL) (40). Following therapy cessation, mice were provided a 7-day rest period prior to initiation of ciprofloxacin prophylaxis. They then received either a typical prophylactic dose of ciprofloxacin (1/4 the empirical therapeutic dose; 100 μl of a 1-μg/ml solution) or vehicle (distilled H_2O) daily via oral gavage for 7 days. Following prophylactic therapy, animals were sacrificed, and kidneys and bladders were excised for determinations of bacterial titers. In some cases, *ex vivo* gentamicin protection assays were conducted on bladders for enumeration of the intracellular population. Urine was taken at least every 2 days throughout the course of the experiment to allow tracking of infection.

Gene expression analyses. For details on the methods used for the gene expression analyses, please refer to the supplemental material.

Statistical analyses. Statistical analyses were conducted using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA). Significance was determined using one-way analysis of variance (ANOVA) and Dunnett's multiple comparison test or Bonferroni's multiple comparison test, a Mann-Whitney test with Gaussian approximation, a log-rank test, or a two-tailed or unpaired *t* test. Each test used for the various experiments is described in detail in the corresponding figure legend. Statistical analyses for the differential abundance of mapped mRNA reads from RNA sequencing were conducted by the ALDEx2 R package version 2.0.6 (85, 86). A $\log_2$ median effect size (calculated by using ALDEx, as the mean ratio of the difference between groups versus the maximum difference within groups) of at least 1.5 (representing at least a 2.25-fold-greater difference between groups versus within groups) was required for genes to be considered differentially expressed. In addition, a >1% overlap in the distributions between the two conditions was permitted for inclusion (85, 87). This approach ensures that the within-group variance is consistently and substantially smaller than the between-group difference when small sample sizes are used.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00356-15/-/DCSupplemental.

Figure S1, TIF file, 8.1 MB.
Figure S2, TIF file, 9.1 MB.
Figure S3, TIF file, 28.2 MB.
Text S1, DOCX file, 0.02 MB.

ACKNOWLEDGMENTS

We thank Sheryl Justice for providing the SOS-deficient strains, Shannon Seney for conducting Luminex cytokine quantitation assays, and David Carter for preparing the libraries for RNA sequencing.

Lee W. Goneau was supported by a Frederick Banting and Charles Best Canadian Institutes of Health Research Doctoral Research Award.

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