Renal scar formation and kidney function following antibiotic-treated murine pyelonephritis

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Key words: pyelonephritis, renal scarring, fibrosis, urinary tract infection, chronic kidney disease, hydronephrosis

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SUMMARY STATEMENT

A new model of antibiotic-treated severe pyelonephritis offers a novel platform to study the molecular pathogenesis of pyelonephritis, response to antibiotic therapy, and sequelae including fibrosis and renal scarring.

LIST OF ABBREVIATIONS

BUN:  blood urea nitrogen
CKD:  chronic kidney disease
CRO:  ceftriaxone
UTI:  urinary tract infection
UPEC:  uropathogenic Escherichia coli
ABSTRACT

We present a new preclinical model to study treatment, resolution, and sequelae of severe ascending pyelonephritis. Urinary tract infection (UTI), primarily caused by uropathogenic *Escherichia coli* (UPEC), is a common disease in children. Severe pyelonephritis is the primary cause of acquired renal scarring in childhood, which may eventually lead to hypertension and chronic kidney disease in a small but important fraction of patients. Preclinical modeling of UTI utilizes almost exclusively females, which (in most mouse strains) exhibit inherent resistance to severe ascending kidney infection; consequently, no existing preclinical model has assessed the consequences of recovery from pyelonephritis following antibiotic treatment. We recently published a novel mini-surgical bladder inoculation technique, with which male C3H/HeN mice develop robust ascending pyelonephritis, highly prevalent renal abscesses, and evidence of fibrosis. Here, we devised and optimized an antibiotic treatment strategy within this male model to more closely reflect the clinical course of pyelonephritis. A 5-day ceftriaxone regimen initiated at the onset of abscess development achieved resolution of bladder and kidney infection. A minority of treated mice displayed persistent histologic abscess at the end of treatment, despite microbiologic cure of pyelonephritis; a matching fraction of mice 1 month later exhibited renal scars featuring fibrosis and ongoing inflammatory infiltrates. Successful antibiotic treatment preserved renal function in almost all infected mice, as assessed by biochemical markers 1 and 5 months post treatment; hydronephrosis was observed as a late effect of treated pyelonephritis. An occasional mouse developed chronic kidney disease, generally reflecting the incidence of this late sequela in humans. In total, this model offers a platform to study the molecular pathogenesis of pyelonephritis, response to antibiotic therapy, and emergence of sequelae including fibrosis and renal scarring. Future studies in this system may inform adjunctive therapies that may reduce the long-term complications of this very common bacterial infection.
INTRODUCTION

Urinary tract infection is a common affliction across the human lifespan, regularly affecting infants and young children in the first years of life (Foxman, 2003; Foxman and Brown, 2003; Foxman, 2010; Arshad and Seed, 2015). Ascension of uropathogens to the kidneys can lead to pyelonephritis, which even with successful antibiotic treatment may carry long-term repercussions for the patient, including the development of renal scarring, hypertension, and eventual progression to end-stage renal disease (Jacobson et al., 1989; Martinell et al., 1996; Wennerstrom et al., 2000; Levey and Coresh, 2012). Ascending bacterial infection of the renal parenchyma in humans elicits severe tubulointerstitial inflammation (Goluszko et al., 1997; Svensson et al., 2005; Mak and Kuo, 2006; Svensson et al., 2011; Olson et al., 2016; Li et al., 2017). This innate inflammatory response, perhaps as much as bacterial processes per se, may largely underlie renal damage resulting from UTI, and is correlated with loss of functional renal tissue (scarring) and the development of fibrosis (Miller and Phillips, 1981; Bille and Glauser, 1982; Anders and Schaefer, 2014; Suarez-Alvarez et al., 2016). However, the mechanisms of how and if pyelonephritic scars contribute to chronic kidney disease (CKD) are unknown (Salo et al., 2011; Toffolo et al., 2012; Nevéus, 2013). Further, it is unclear if the location, severity, or timing of renal fibrosis influences progression to CKD.

An understanding of the link between infection-related fibrosis and subsequent development of CKD has been hindered largely by the lack of robust murine models of ascending severe upper-tract UTI in immunocompetent hosts (Hopkins et al., 1998; Svensson et al., 2005; Hannan et al., 2010; Svensson et al., 2011). While cystitis can be induced by transurethral catheterization in females of many mouse strains, most are resistant to severe pyelonephritis with abscess formation after bladder inoculation (Hopkins et al., 1998; Hannan et al., 2010; Tittel et al., 2011; Hains et al., 2014; Schwartz et al., 2015; Olson et al., 2016). Several previous studies have utilized direct injection of uropathogens into the kidneys (Miller and Phillips, 1981; Santos et al., 1994; Mussalli et al., 1999), but these models bypass ascension of the ureter and clearly do not recapitulate natural arrival of UPEC in the collecting system. Other reports have induced female murine pyelonephritis with serial, high-CFU transurethral inoculations, but did not note gross abscess or severe nephropathy (Tittel et al., 2011; Bowen et al., 2013; Hains et al., 2014; Schwartz et al., 2015). Substantial recent work has been performed in C3H/HeN mice, which are recognized to feature vesicoureteral reflux (Hopkins et al., 1998; Bowen et al., 2013), reflecting a primary risk factor for upper-tract UTI in
children (Feld and Mattoo, 2010; Hoberman et al., 2014). In the C3H/HeN mouse strain, a minority of females (historically used almost exclusively in preclinical UTI work, as the bladders of male mice cannot reliably be accessed by catheter) develop pyelonephritis – without abscess formation – following bladder inoculation with UPEC, while most females resolve infection (Hannan et al., 2010). We previously found using a novel mini-surgical bladder inoculation technique that male C3H/HeN mice, unlike females, develop nearly 100% penetrant severe pyelonephritis and renal abscesses following ascending infection, and fail to spontaneously resolve UTI (Olson et al., 2016). Furthermore, infected males exhibit fibrosis and progressive renal disease during later stages of infection (Olson et al., 2016).

In addition, prior studies have examined the generation of inflammation and fibrosis only during ongoing, active infection (Svensson et al., 2005; Svensson et al., 2011; Bahat et al., 2014; Olson et al., 2016; Li et al., 2017), while human patients with pyelonephritis typically would receive antibiotic treatment, such as a cephalosporin or fluoroquinolone, upon recognition of symptoms and appropriate laboratory testing (Warren et al., 1999; Gupta et al., 2011). Thus, we here extended our new preclinical model of UTI in C3H/HeN males to test the efficacy of antibiotic treatment in severe pyelonephritis and in early or established renal abscesses, and to examine long-term sequelae of infection following antimicrobial treatment.

**RESULTS**

**Ceftriaxone (CRO) achieves microbiologic cure of pyelonephritis in C3H males.** There exist no optimal preclinical models of antibiotic-treated severe pyelonephritis and the immediate or long-term detrimental sequelae of disease. Therefore, we employed mini-surgical inoculation of the bladders of male C3H/HeN mice to model the resolution and sequelae of severe pyelonephritis. By 14 days post infection (dpi) with uropathogenic *Escherichia coli* (UPEC) strain UTI89, over 90% of surgically infected C3H/HeN males develop grossly evident, bilateral renal abscess (Olson et al., 2016). In our efforts to model antibiotic treatment, we first attempted multiple CRO dosing schemes starting 14 dpi in male C3H/HeN mice; these strategies failed to effectively treat the advanced abscesses established in kidneys by that time point (Supplementary Figure S1). Further, we felt it likely that patients would more commonly present earlier in the course of pyelonephritis. In C3H/HeN males, abscesses rapidly become evident between 5 and 6 dpi and are fully formed in nearly 100% of males by 7 dpi (Olson et al., 2016) (and Olson, unpublished data). Therefore, we
next elected to initiate CRO or placebo (PBS) administration (given by subcutaneous injection every 12 hours [q12 h] for 5 d) beginning 5 dpi, harvesting organs upon euthanasia 24 h after the final CRO dose (i.e., 11 dpi; Figure 1A). Bladder inoculation with UPEC in male C3H/HeN mice resulted in robust bladder (Figure 1B) and kidney (Figure 1C) infection in both start-of-treatment controls (5 dpi) and mock-treated animals. Ceftriaxone treatment significantly reduced bladder (Figure 1B; \( P<0.0001 \)) and kidney (Figure 1C; \( P<0.0001 \)) bacterial burdens compared to mock-treated animals. Ceftriaxone-treated mice continued to harbor \( 10^2 \)-\( 10^4 \) colony-forming units (CFU) of UPEC in their bladders (Figure 1B), despite resolving bacteriuria (Figure 1D); this finding is consistent with prior reports of UPEC reservoirs persisting within bladder tissue following antibiotic treatment (Mysorekar and Hultgren, 2006; Hannan et al., 2010; Blango et al., 2014; Olson et al., 2016). The majority of CRO-treated mice completely resolved kidney infection (Figure 1C). Trials of other treatment regimens beginning 5 dpi, including increased CRO duration, dose, or frequency, did not further affect organ bacterial burdens (Supplemental Figure S2) compared to the 5-d, q12 h regimen.

**CRO treatment beginning 5 dpi sterilizes existing abscesses and halts further abscess formation.** Among mice sacrificed at the start of treatment (5 dpi), all of which had high kidney bacterial burdens (Figure 1B), a minority (4 of 15, 27%) demonstrated grossly and microscopically evident abscess formation (Figure 2A), matching our previous report at the same time point (Olson et al., 2016). By 11 dpi (24 h post treatment completion), all (10 of 10) UPEC-infected, mock-treated males displayed gross renal abscess formation (Figure 2B). Thus, abscess development was progressive during this 6-day interval in the absence of antibiotic treatment. In contrast, the abscess frequency observed 11 dpi in CRO-treated mice (4 of 14, 29%; \( P=0.0006 \) vs mock-treated; Figure 2C) was equivalent to 5-dpi start-of-treatment controls, illustrating that timely antibiotic therapy interrupted abscess progression. As noted above, these CRO-treated mice with evident abscess exhibited low kidney bacterial burdens (at or near the lower limit of detection; Figure 1A) and did not have ongoing bacteriuria (Figure 1D). These data suggest that CRO treatment beginning 5 dpi arrested renal abscess development and neutralized the burgeoning UPEC population within the renal parenchyma. As expected, control mice (mock-infected with PBS and treated with CRO) displayed healthy kidney architecture 24 h post treatment (Figure 2D).
Convalescent outcomes in treated pyelonephritis. While the majority of CRO-treated mice demonstrated microbiologic cure of pyelonephritis 1 d post treatment, a few maintained very low residual UPEC burdens (Figure 1B and C). It was unclear if such UPEC remaining in the bladder or kidney post CRO treatment would reemerge to cause recrudescence infection. To specify outcomes in treated pyelonephritis, we treated UPEC-infected mice with CRO or PBS for 5 days beginning 5 dpi and quantified organ bacterial burdens 4 weeks post-treatment. Mock-treated mice all exhibited high bladder bacterial burdens (typical of chronic cystitis) and kidney burdens at this later time point (Figure 3A), consistent with the near-complete prevalence of these severe UTI outcomes in C3H/HeN males that we reported previously (Olson et al., 2016). All CRO-treated mice resolved renal and bladder infection (Figure 3A; \( P=0.0007 \) and \( P=0.0003 \), respectively, vs mock-treated controls). No CRO-treated mice displayed urine UPEC titers \( >10^4 \) CFU mL\(^{-1}\) at biweekly samplings (Supplemental Figure S3), but a minority maintained low-level colonization of the bladder (Figure 3A), again consistent with quiescent reservoir formation as previously reported (Mysorekar and Hultgren, 2006; Hannan et al., 2010; Blango et al., 2014; Olson et al., 2016). Remarkably, gross renal scars were found in several CRO-treated mice at necropsy 4 weeks post treatment (Figure 3B, arrowheads). Affected kidneys demonstrated broad-based, U-shaped cortical scarring with retraction of the renal parenchyma, matching the pathological descriptions of human pyelonephritic scars (Smith, 1962; Paueksakon and Fogo, 2014). The fraction of mice displaying grossly visible renal scars 4 weeks following CRO treatment (28%) was equivalent to the fraction of mice demonstrating abscess at either start of treatment (5 dpi) or 1 day post treatment (11 dpi) (Figure 3C). Collectively, these data indicate that the tissue destruction associated with microscopic abscess formation is spatially and pathologically associated with the subsequent development of renal scars.

Renal scars, despite resolution of infection, harbor progressive inflammation. Histopathological analysis of UPEC-infected, CRO-treated kidney sections by Gomori trichrome staining revealed extensive cortical scars 4 weeks post treatment, with collagen deposition in some scars extending from the renal capsule to the medulla (Figure 4A). The strictures we observed on gross inspection of recovered kidneys (Figure 3B) were also evident microscopically, and the renal capsule was substantially thickened overlying the scar (Figure 4B). Fibrosis in these scars followed patterns similar to those observed at earlier stages of abscess development 5 and 11 dpi in infected, CRO-treated mice (see Figure 2). No scars were observed in mock-infected, CRO-treated animals. More striking was the...
presence of a cellular infiltrate within the scar (Figure 4C, D), despite all tested animals resolving renal infection (Figure 3A) and exhibiting sterile urine cultures. Collections of inflammatory cells (primarily lymphocytes) and fibroblasts were embedded within the area of fibrosis (Figure 4D). These data indicate that the development and maturation of renal scars is an active process that continues following successful microbiologic cure of infection with antibiotics.

**Successful CRO treatment restores renal function.** Human patients that develop acute pyelonephritis typically manifest baseline renal function following resolution of infection; renal scars and other adverse sequelae of resolved infection are identified in a minority of patients, and some are not evident clinically until later in life (Jacobson et al., 1989; Martinell et al., 1996; Wennerstrom et al., 2000; Shaikh et al., 2010; Levey and Coresh, 2012). In the UPEC-infected, CRO-treated mice that resolved infection and lacked evidence of renal scarring 4 weeks post treatment, histopathological analysis of Gomori trichrome-stained kidney sections showed no increase in interstitial fibrosis compared to mock-infected controls, and normal overall renal architecture similar to mock-infected animals (Figure 5A, B). However, we frequently observed areas of glomerular sclerosis in these UTI89-infected, CRO-treated, scar-free animals (Figure 5B). We also performed serial measurements of blood urea nitrogen (BUN) as a biochemical marker of renal function in UPEC-infected mice. These data demonstrate that BUN remained stable in mice receiving CRO treatment, while ongoing infection (i.e., mock treatment) was associated with an increase in BUN (Figure 5C).

**Long-term outcomes reflect those observed in human patients.** Children who develop renal scars following UTI may be followed into adulthood, when signs of CKD may manifest in a minority of such patients (Jacobson et al., 1989; Martinell et al., 1996; Wennerstrom et al., 2000; Levey and Coresh, 2012). Therefore, we surgically inoculated male C3H/HeN mice with either PBS (mock) or UTI89, treated with CRO for 5 days beginning 5 dpi, and then observed these mice until 30 weeks of age (i.e., 5 months post treatment). Successful antibiotic treatment preserved normal renal function in the majority of UPEC-infected mice at this longer interval; mean serum creatinine (Figure 6A), BUN (Figure 6B), and urine protein (Figure 6C) were not significantly higher than in mock-infected mice. However, in one UPEC-infected mouse (Figure 6) that developed particularly notable bilateral scars, these three biochemical markers were markedly higher, indicating the development of CKD.
Surprisingly, we found grossly visible bilateral hydronephrosis in 80% of the UPEC-infected, CRO-treated mice allowed to age to 30 weeks. These animals had bilaterally dilated ureters (Figure 7A), and expansion of the renal pelvis was visible on bisection of the kidneys. Histopathology confirmed these findings, with infected animals displaying enlarged, dilated renal pelvis and calyces, flattening of the pelvic epithelia, atrophy and thinning of the renal cortex, and expanded ureters (Figure 7B). These abnormal features were uniformly absent in mock-infected, CRO-treated animals at 30 wpi (Figure 7C). There was no evidence of hydronephrosis at 11 or 38 dpi in any mock- or UPEC-infected, mock- or CRO-treated mice (Figures 2, 3, 4). Fibrotic scars in the parenchyma near the renal pelvis, with persistent inflammatory infiltrates, were evident microscopically at 30 weeks in UPEC-infected, CRO-treated mice (Figure 7D, as seen at 38 dpi), but not in mock-infected controls. Histologic examination of the bladders of UPEC-infected, CRO-treated mice at this long time point revealed epithelial changes reflecting bladder remodeling and chronic inflammatory infiltrates, but no evidence of obstructive lesions at the ureterovesical junction (Figures 7E, F).

DISCUSSION

Here, we developed a new model to enable preclinical studies of the resolution and sequelae of antibiotic-treated upper-tract UTI. To do so, we leveraged the mini-surgical inoculation technique that allows infection of C3H/HeN males, which develop nearly 100% penetrant severe pyelonephritis and renal abscess following bladder inoculation with UPEC. While antibiotic treatment alone was not successful when initiated at later time points (presumably because of very advanced infection, and consistent with clinical experience in patients with established abscesses), CRO treatment initiated 5 dpi achieved resolution of infection, aborting the abscess development that begins at that time. An appropriate minority of these infected, successfully treated animals developed renal scars by 1 month post treatment; these scars comprised fibrosis and ongoing inflammatory cellular infiltrates. At longer follow-up, an even smaller proportion demonstrated biochemical evidence of CKD.

Roughly one third of infected male C3H/HeN mice exhibited abscess 5 dpi (at the start of treatment) or demonstrated sterile abscess post treatment, and a similar fraction of mice demonstrated renal scarring 1 month after successful antibiotic treatment. Current limitations
of live-animal imaging preclude a definitive link between the anatomic locations of initial abscess and ultimate renal scar. However, we can reasonably posit that abscess development, with associated renal parenchymal necrosis and replacement with inflammatory infiltrates, gives way to scar formation in this spatially identical region of a given kidney. Post-antibiotic, sterile abscesses featured inflammation and tissue destruction similar to descriptions of active abscess 5 dpi (Figure 2), and inflammation persisted in renal scars at later time points (Figure 5), consistent with reports from human pathology (Bernstein and Arant, 1992). This temporal and spatial association argues that the immune responses to UPEC introduction and the inflammatory processes surrounding micro- and macroabscess formation lay the mechanistic foundation for scar development. This hypothesis is also supported by recent findings in the C3H/HeOuJ mouse strain (Li et al., 2017). It follows that if these mechanisms can be understood at a molecular level, future targeted therapeutic modalities may attenuate or alter the nature of renal inflammation, and/or impact the inflammatory modulators released from pyelonephritic scars (Haraoka et al., 1994; Pohl et al., 1999; Nevéus, 2013; Bahat et al., 2014), ultimately reducing risk for CKD.

Our preclinical model of renal scarring following successful antibiotic treatment of ascending upper-tract UTI fills a substantial gap in the field and reproduces the outcomes observed in patients, particularly children, with pyelonephritis. Only a small percentage of humans presenting with upper-tract UTI develop renal scars following resolution of acute infection, and it is unclear why some individuals develop these scars while others do not (Hewitson, 2009; Shaikh et al., 2010; Strohmeier et al., 2014). Estimates of the risk of renal scarring after pyelonephritis in children vary, but range between 8% and 40%, with a meta-analysis concluding that ~15% of such children have demonstrable evidence of scarring at follow-up (Jakobsson et al., 1994; Shaikh et al., 2010). The model described in the present work approximates this proportion, with 27-29% of mice developing renal scars. Clinical studies have shown that early and aggressive antibiotic treatment minimizes the risk of renal scar formation (Miller and Phillips, 1981; Winter et al., 1983; Shaikh et al., 2016); our studies reinforce this point, suggesting that minor delays in the start of antimicrobial treatment could substantially influence whether permanent renal damage occurs or if pyelonephritis instead resolves without complication.

This work was limited to the study of an adult male murine model, employed to overcome the shortcomings of previous female models of pyelonephritis. The sex ratio in UTI among
infants favors females, but approximates 2:1 over the first two years of life. A number of studies indicate that male cases outnumber female neonatal UTI within the first 6 months after birth (Winberg et al., 1974; Ginsburg and McCracken, 1982; Wettergren et al., 1985; Kanellopoulos et al., 2006; Wong et al., 2010; Ismaili et al., 2011; Park et al., 2011; Bonadio and Maida, 2014). Further, several reports suggest that male sex may be a prognostic factor for the development of renal scarring in infants that develop febrile UTI (Marra et al., 2004; Soylu et al., 2008; Mattoo et al., 2015). This matches a growing body of evidence that male sex may be an indicator for worse morbidity, mortality, or sequelae from pyelonephritis (Nicolle et al., 1996; Efstathiou et al., 2003; Foxman et al., 2003; Ki et al., 2004; Olson et al., 2016). Additionally, the clinical data supporting an age-related influence on risk for scarring after childhood pyelonephritis are variable, but on balance may indicate a slight predisposition for developing renal scars in older children presenting with pyelonephritis (Benador et al., 1997; Ataei et al., 2005; Shaikh et al., 2014). Future work with our ceftriaxone treatment model in female models of ascending UTI and in male mice of varying age could further define the influences of sex and age, respectively, on post-pyelonephritic fibrosis and sequelae of infection.

Our experiments also reveal translationally appropriate rates of long-term sequelae, both scarring and CKD. To clearly delineate the association and mechanistic pathways leading to CKD specifically in this model, future studies will require substantially larger sample sizes. Alternatively, the incidence of the CKD outcome might be augmented by infecting with a greater inoculum or by initiating treatment somewhat later in infection (e.g., 7 dpi), when a larger proportion of C3H/HeN males will have established abscesses (Olson et al., 2016). Most non-infectious murine models of CKD require some combination of unilateral or subtotal nephrectomy, ureteral obstruction, and injury or insult to the remaining kidney (Davies et al., 2003; Davies et al., 2005; Manson et al., 2011; Agapova et al., 2016); one could imagine attempting to increase the incidence of post-pyelonephritic CKD by introducing a similar unilateral or partial nephrectomy procedure prior to UPEC infection and antibiotic treatment. Measurement of blood pressure will help to correlate renal scars in mice with hypertension, which is more common as a sequela of human pyelonephritis than CKD. Continued work along these lines will provide further evidence to define the relationship between pyelonephritic scar formation and risk for CKD.
While it is accepted that UTI risk is enhanced in individuals with hydronephrosis associated with vesicoureteral reflux (VUR) or urodynamic obstruction (Feld and Mattoo, 2010; Hoberman et al., 2014), there is, to our knowledge, no conclusive paradigm in which pyelonephritis and/or severe cystitis causes or reveals the presence of hydronephrosis. Several early studies did speculate that infection might cause an increase in VUR, although the evidence for cystitis either promoting VUR or having no effect on VUR is relatively weak (Hanley, 1962; Howerton and Lich, 1963; Tanagho et al., 1965; Gross and Lebowitz, 1981; Garin et al., 1998). The hydronephrosis we observed in long-term follow-up of infected and successfully treated C3H/HeN males – mice known to have preexisting VUR – suggests that incident UTI may worsen such reflux, either temporarily or in a more protracted way. Future studies that optimize models of severe pyelonephritis in non-refluxing backgrounds such as C57BL/6 (Tittel et al., 2011; Hains et al., 2014) may ascertain the contribution of VUR to the phenotypes we observed in C3H/HeN males. Other potential causes of bilateral hydronephrosis in these recovered males, such as bilateral ureteral or ureterovesical obstruction, were not observed but are not completely excluded by the present data. Papillary blunting and hydronephrosis were not seen in UPEC-infected, mock-treated animals, or at earlier time points immediately following antibiotic treatment, suggesting that these findings may arise in association with long-term remodeling and fibrosis responses in the bladder, ureter, and/or kidney. In any case, fibrosis is evident much earlier in the course of recovery than is hydronephrosis; it is therefore unlikely that hydronephrotic injury is the initiator of fibrosis in this model, though it may contribute to progression of fibrosis. Other recent studies have begun to illuminate not only macroscopic, but microscopic and molecular imprints left by severe UTI on the urinary tract and its cellular constituents (O'Brien et al., 2015; Schwartz et al., 2015; O'Brien et al., 2016). These studies may support the concept of a vicious cycle in patients with urodynamic abnormalities, who are already predisposed to UTI but whose urodynamics may also regress with repeated UTI.

In summary, we report a novel system for modeling the complications arising from severe UTI. Susceptible hosts develop renal abscess upon ascending UPEC infection and, despite successful antibiotic therapy, a translationally relevant proportion of mice ultimately develop renal scars after treatment. This model promises to address the relationships between the development of pyelonephritis, timing and effectiveness of antibiotic therapy, and sequelae including renal scarring and chronic kidney disease, as well as to illuminate the soluble and
cellular inflammatory components responsible for ongoing renal damage following microbiologic resolution.

**MATERIALS AND METHODS**

**Bacteria.** Uropathogenic *Escherichia coli* strain UTI89 was isolated from a patient with cystitis (Chen et al., 2009). For surgical infections, bacteria were grown statically in Luria-Bertani (LB) broth for 16 h at 37°C. The cultures were centrifuged for 10 min at 7,500 × g at 4°C before resuspension in sterile phosphate-buffered saline (PBS) to a final density of $4 \times 10^8$ CFU ml$^{-1}$.

**Introduction of murine UTI.** All animal procedures received prior review and approval from the Institutional Animal Care and Use Committee at Washington University. A widely used female murine model of cystitis with transurethral inoculation via catheter has been described in methodologic detail (Mulvey et al., 1998; Hung et al., 2009; Hannan and Hunstad, 2016), but this approach is technically precluded in male animals. A recently developed surgical approach (Olson et al., 2016) was used to initiate infection in male mice. Eight-week-old male C3H/HeN mice (Envigo, Indianapolis, IN) were maintained under inhalation anesthesia with 3% isoflurane via vaporizer and nose cone. Briefly, anesthetized mice were positioned supine, shaved, and the ventral abdomen was sterilized with 2% chlorhexidine solution. A vertical, midline incision (3 mm in length) was made directly overlying the bladder, first through the abdominal skin and then through the peritoneum. The bladder was exposed, aseptically emptied, and punctured with a 30-gauge, 0.5-inch needle adapted to a 1-mL tuberculin syringe containing the bacterial inoculum. Fifty microliters containing $1-2 \times 10^7$ CFU was introduced to the bladder lumen over 10 s, the bladder was allowed to expand for a further 10 s, and the needle was then withdrawn. The peritoneum and the skin were closed with simple, interrupted sutures, and the animal was awakened in fresh air.

**Ceftriaxone treatment.** We adapted CRO treatment regimens reported previously in female murine models to clear UTI and to provide circulating drug levels similar to those seen in patients treated with CRO (Lepeule et al., 2012; Tratselas et al., 2014). At 120 h after surgical infection, male C3H/HeN mice received 125 mg kg$^{-1}$ CRO dissolved in sterile water by subcutaneous injection; mock-treated animals received an equivalent volume of PBS.
Mice received similar subcutaneous injections of CRO or PBS every 12 h for 5 days (i.e., 10 doses in total). Bladders and kidneys were aseptically harvested 24 h after the last treatment to allow for clearance of residual CRO from the tissues (see Figure 1A).

**Determination of urine and tissue bacterial loads.** Where indicated, we noninvasively obtained post-infection, clean-catch urine samples using gentle suprapubic pressure for serial dilution and plating to enumerate CFU mL\(^{-1}\) urine. At the indicated time points, mice were euthanized via CO\(_2\) asphyxiation, and bladders and kidney pairs were aseptically removed and homogenized in 1 ml or 0.8 ml sterile PBS, respectively. We plated serial dilutions of tissue homogenates on LB agar to enumerate bacterial loads. Where indicated, infection in C3H/HeN mice was classified as “chronic” if all urine and endpoint bladder titers contained \(>10^4\) CFU ml\(^{-1}\), while “resolved” mice demonstrated endpoint bladder burdens and at least one urine time point \(<10^4\) CFU ml\(^{-1}\) (Hannan et al., 2010).

**Blood and urine chemistries.** Serum or urine was analyzed on the day of blood draw for blood urea nitrogen (BUN), serum creatinine, or urine protein by standard autoanalyzer laboratory methods performed by the Department of Comparative Medicine veterinary facility at Washington University.

**Tissue histopathology.** Infected bladders and kidneys were bisected and fixed in 10% neutral buffered formalin for 24 h. Fixed tissues were embedded in paraffin, sectioned, and stained with hematoxylin and eosin or Gomori trichrome stain before light microscopy. Whole-kidney images (Figure 7B, C) were acquired with the AxioScan Z1 Automated Slide Scanning System and digitally tiled by the acquisition software.

**Statistical analysis.** Statistics and graphing were performed using Prism 7 (GraphPad Software, La Jolla, CA). Organ bacterial loads and other numerical data were compared by the nonparametric Mann-Whitney U test. 2×2 comparisons were analyzed using the Fisher exact test. \(P\) values <0.05 were considered significant.
ACKNOWLEDGMENTS
None.

COMPETING INTERESTS
DAH serves on the Board of Directors of BioVersys AG, Basel, Switzerland. All other authors have no competing interests to declare.

AUTHOR CONTRIBUTIONS
PDO, KAH, and DAH designed experiments. PDO, LKM, AL, KLB, KMT, and ALD performed experiments. PDO, KAH, and DAH interpreted data. PDO and DAH wrote the manuscript, with input and review from all other authors.

FUNDING
This work was supported by National Institutes of Health grants P50-DK064540 to DAH, T32-AI007172 to PDO, and F30-DK104446 to PDO. LKM is supported by the Olin Fellowship for Women and a National Science Foundation Graduate Research Fellowship (DGE-1143954).
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Figure 1. Ceftriaxone (CRO) treatment eliminates renal bacterial burden in C3H/HeN mice with pyelonephritis. (A) Male C3H/HeN mice were surgically infected with UTI89 or PBS and then treated with CRO or PBS for 5 days starting 5 dpi. Bladders (B) or kidneys (C) were aseptically homogenized and plated to enumerate CFU 24 h after the last CRO injection. Organ titers in 5-dpi start-of-treatment controls (triangles) were equivalent to
mock-treated mice 11 dpi (filled circles), while CRO treatment (open circles) significantly reduced bladder bacterial loads and sterilized the infected kidneys. Mock-infected mice (diamonds), as expected, bore no bacteria in bladders or kidneys. Data shown reflect the aggregate of three independent experiments (total n = 7-15 per condition, *** \( P<0.001 \) by Mann-Whitney U test). Dashed lines in all panels represent the limit of detection; bars indicate the geometric mean. (D) In male C3H/HeN mice surgically infected with UTI89, then either mock-treated (with PBS) or treated with CRO (grey circles) starting 5 dpi, urine bacterial titers on the indicated days are shown. Solid lines connect corresponding urine titers from each individual mouse.
Figure 2. Ceftriaxone (CRO) treatment reduces prevalence of renal abscess. (A) A minority (27%) of infected C3H/HeN males sacrificed before start of treatment already exhibited abscess formation at this time point (representative image among n = 15 mice). (B) 100% of UTI89-infected C3H/HeN males receiving mock treatment (PBS) displayed abscess 1 d post treatment (representative image among n = 10 mice). (C) The prevalence of abscess in UTI89-infected, CRO-treated mice (29%) matched that prior to treatment, indicating that CRO treatment prevented further abscess formation but did not reverse tissue damage already present in abscessed kidneys, even though microbiologic cure was achieved (representative image among n = 14 mice). (D) Mock-infected, CRO-treated control mice exhibited normal
kidney architecture 1 day after treatment conclusion (representative image among n = 7 mice). Gomori trichrome staining; scale bar, 200 µm.
Figure 3. Renal scars develop following durably successful ceftriaxone (CRO) treatment. Male C3H/HeN were surgically infected with PBS or UTI89 and then treated with PBS or CRO. (A) Bladders and kidneys were aseptically harvested, homogenized, and CFU enumerated at 38 dpi (28 days post-treatment). Ceftriaxone treatment significantly reduced bacterial burden and resulted in sterile kidney titers 38 dpi (aggregate of two experiments, total n = 5-8 per condition; ** P<0.01, *** P<0.001 by Mann-Whitney U test).
(B) Following autopsy, gross renal scars were observed in 27% of UPEC-infected, CRO-treated mice 38 dpi. A representative image of a scarred left kidney is shown. (C) The percentage of animals exhibiting abscess 5 dpi (pre-CRO treatment; n = 15) or 11 dpi (24 h following CRO treatment; n = 14) is matched by the percentage exhibiting renal scars 4 weeks post CRO treatment (n = 11) (p = NS by Fisher exact test for each pairwise comparison).
Figure 4. Post-pyelonephritic scars demonstrate ongoing active inflammation. Gomori trichrome staining of UPEC-infected mice (n = 11) with grossly evident scars but negative organ bacterial titers 28 days after completion of CRO treatment demonstrated collagen deposition as well as replacement and retraction of cortical tissue (A, B; scale bar, 200 µm). This was accompanied by dramatic capsular thickening over the scar and a cellular infiltrate (C, inset from B; scale bar, 50 µm) that morphologically consisted primarily of lymphocytes (D; scale bar, 20 µm).
Figure 5. CRO treatment preserves normal short-term renal function. (A) Gomori trichrome staining of renal cortex illustrates healthy kidney histology, 28 d post treatment, in mock-infected mice treated with CRO (representative image from n = 10 mice). (B) UPEC-infected mice that resolved pyelonephritis via CRO treatment and lacked gross scars demonstrated minor glomerular sclerosis, but displayed otherwise normal kidney architecture (representative image from n = 14 mice). Scale bar for A and B, 50 µm. (C) Blood urea nitrogen (BUN) rose over a 30-day interval in UPEC-infected mice (n = 4-5 per condition) that did not receive antibiotics (*P = 0.029 by Mann-Whitney U test, 5 d vs 30 d), but remained at baseline in mice that were treated with CRO (P = NS by Mann-Whitney U test, 5 d vs 30 d).
Figure 6. Successful CRO treatment preserves long-term renal function in most UPEC-infected mice. Mock-infected mice (PBS; filled circles) and UPEC-infected mice (inverted triangles) were treated with CRO for 5 d beginning 5 dpi and aged to 30 weeks (2-3 experiments with total n = 10-15 per condition). Serum was analyzed for creatinine (SCr; A) and blood urea nitrogen (BUN; B), and urine protein was measured (UPro; C). Most UPEC-infected mice demonstrated normal serum creatinine, BUN, and urine protein 30 wpi (p = NS by Mann-Whitney U test for each analyte), though one UPEC-infected mouse that displayed notable bilateral scars also had marked elevation in these biochemical markers.
Male C3H/HeN were treated with CRO for 5 d beginning 5 dpi and aged to 30 weeks as in Figure 6. The majority of UPEC-infected, CRO-treated males exhibited grossly visible hydronephrosis with dilated ureters (A, white arrowheads). Histopathology of Gomori trichrome-stained sections confirmed dilated collecting structures and proximal ureter in UPEC-infected, CRO-treated mice (B; scale bar, 500 µm), while mock-infected, CRO-treated mice displayed normal kidney architecture without discernible hydronephrosis (C; scale bar, 500 µm). As was also noted 4 weeks post treatment, UPEC-infected, CRO-treated animals 30 wpi displayed fibrotic scars with and continued active inflammation, despite resolving infection (D; scale bar, 50 µm). Complete sectioning of the bladders from these animals (E and F; scale bars, 200 µm) did not reveal evidence of obstruction at the ureterovesical junction; E shows ureter (ur) as it approaches the wall of the bladder (bl), while F is a serial section showing patency of the ureterovesical junction (magnified in inset). Images representative of 2 experiments, total n = 10 mice per condition.
Supplementary Figure S1. Antibiotic treatment begun 2 weeks post infection (wpi) fails to sterilize the urinary tract. Male C3H/HeN mice were infected with UTI89 as described in Materials and Methods. Beginning 2 wpi, mice received ceftriaxone 125 mg/kg SQ every 12 h for 5 d (A) or an equivalent volume of PBS (B). Shown for each condition are urine bacterial loads (colony-forming units [CFU]/mL) prior to the start of treatment (2 wpi) and at the conclusion of treatment, and the bacterial loads (CFU/organ) in bladders and kidneys upon sacrifice 20 dpi. A solid line connects the data points for each individual mouse.
**Supplementary Figure S2.** Ceftriaxone dosing every 6 h yields microbiologic cure similar to the twice-daily regimen. C3H/HeN male mice were infected with UTI89 as described in Materials and Methods, and ceftriaxone 70 mg/kg SQ every 6 h was commenced 5 dpi. Shown are urine titers (colony-forming units [CFU]/mL) pre-treatment (5 dpi) and at 6 and 7 dpi, as well as bladder and kidney bacterial loads (CFU/organ) at sacrifice 11 dpi. A solid line connects the data points for each individual mouse.
Supplementary Figure S3. Ceftriaxone treatment rapidly sterilizes urine cultures. Shown are bacterial loads (colony-forming units [CFU]/mL) in urine cultures at the indicated time points in UPEC-infected C3H male mice receiving a 5-day course of ceftriaxone (CRO; red) or control (PBS; black) beginning 5 dpi. A solid line connects the data points for each individual mouse.