Interplay between vesicoureteric reflux and kidney infection in the development of reflux nephropathy in mice

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SUMMARY

Vesicoureteric reflux (VUR) is a common congenital defect of the urinary tract that is usually discovered after a child develops a urinary tract infection. It is associated with reflux nephropathy, a renal lesion characterized by the presence of chronic tubulointersitial inflammation and fibrosis. Most patients are diagnosed with reflux nephropathy after one or more febrile urinary tract infections, suggesting a potential role for infection in its development. We have recently shown that the C3H mouse has a 100% incidence of VUR. Here, we evaluate the roles of VUR and uropathogenic *Escherichia coli* infection in the development of reflux nephropathy in the C3H mouse. We find that VUR in combination with sustained kidney infection is crucial to the development of reflux nephropathy, whereas sterile reflux alone fails to induce reflux nephropathy. A single bout of kidney infection without reflux fails to induce reflux nephropathy. The host immune response to infection was examined in two refluxing C3H substrains, HeN and HeJ. HeJ mice, which have a defect in innate immunity and bacterial clearance, demonstrate more significant renal inflammation and reflux nephropathy compared with HeN mice. These studies demonstrate the crucial synergy between VUR, sustained kidney infection and the host immune response in the development of reflux nephropathy in a mouse model of VUR.

INTRODUCTION

Primary vesicoureteric reflux (VUR) is a congenital defect of the ureterovesical junction (UVJ), affecting up to 1% of children (Burger and Smith, 1971; Chapman et al., 1985; Sargent, 2000). The UVJ consists of the intravesical ureter, which is the portion of the ureter that inserts through the bladder wall, and the trigone, or bladder musculature. During voiding, the trigone compresses the intravesical ureter against the bladder wall, occluding the UVJ and preventing the retrograde flow of urine. If the length of the intravesical ureter is too short or there is a defect in the trigone musculature, the UVJ will not be occluded and VUR will occur. VUR is a risk factor for urinary tract infections (UTIs) and reflux nephropathy (RN), a specific renal lesion characterized by chronic tubulointerstitial inflammation, fibrosis and destruction of nephrons. RN is a common cause of end-stage renal failure in children (Ardissino et al., 2003; Craig et al., 2000; Canadian Institute for Health Information, 2011) and in adults (US Renal Data System, 2011; Canadian Institute for Health Information, 2011). Although most cases of VUR improve either spontaneously or from surgical correction of the UVJ defect, many patients are left with RN, a

condition that is poorly understood. Furthermore, despite intervention strategies, there has been no improvement in the incidence of end-stage renal failure attributed to RN (Craig et al., 2000). Therefore, there is a fundamental need to re-explore the biology of VUR and RN to understand which patients are at risk for RN and to target therapeutic interventions (Jacobson et al., 1992; Wallace et al., 1978; Jacobson et al., 1989; Goonasekera et al., 1996).

VUR promotes the ascent of bacteria from the bladder into the renal pelvis and predisposes affected individuals to kidney infections (Hodson and Edwards, 1960; Risdon, 1987), which are defined histologically as 'pyelo-nephritis'. 'Pyelitis' refers to the initial inflammation that is observed secondary to neutrophil influx into the submucosa, the collecting system and the renal pelvis, and 'nephritis' refers to the more extensive renal inflammation that encompasses other tubular segments and the interstitial compartment (Johnson et al., 1992). Reactive oxygen metabolites released by neutrophils in response to bacterial infection can, in excess, be toxic and induce renal scarring or fibrosis in animal models (Roberts et al., 1982). A large proportion of individuals with VUR will develop 'acquired RN', which is characterized by chronic tubulointerstitial inflammation, fibrosis and the destruction of nephrons after one or more kidney infections (Swerkersson et al., 2007; Lenaghan et al., 1976; Bailey, 1973), and some will even progress to end-stage renal failure (Craig et al., 2000) [North American Pediatric Renal Trials and Collaborative Studies (NAPRTCS) 2011 Annual Dialysis Report (https://web.emmes.com/study/ped/annlrept/annualrept2011.pdf)]. Other patients with VUR and kidney infections will never develop RN (Pennesi et al., 2008). Patients have also been diagnosed with RN and no history of UTIs (Nguyen et al., 2000). The mechanism for how RN arises in these individuals is unclear, but it has been suggested that some of these patients might have 'congenital RN', in which a developmental defect leads to abnormal formation of the UVJ and

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TRANSLATIONAL IMPACT

Clinical issue

Vesicoureteric reflux (VUR) is a congenital disease that affects approximately 1% of children. It is associated with reflux nephropathy, a renal lesion that is characterized by inflammation and fibrotic scarring of the kidneys and that can lead to end-stage renal disease and the need for renal transplant. VUR is also a risk factor for urinary tract infections. Therefore, affected individuals are often given prophylactic antibiotics to prevent the occurrence of cystitis and/or pyelonephritis. Importantly, however, the relationship between VUR and infection in the development of reflux nephropathy is not clear. Although the results of some studies in a minipig surgical model of VUR suggest that sterile reflux alone is enough to induce renal nephropathy, the results of other studies implicate infection in the development of this lesion.

Results

In this study, the authors investigate the role of infection in the progression from VUR to reflux nephropathy in two C3H mouse substrains (C3H/HeN and C3H/HeJ) that spontaneously develop VUR. Reflux alone fails to induce reflux nephropathy in these mice, but the authors show that inoculation with uropathogenic *Escherichia coli* leads to sustained kidney infection and the subsequent development of reflux nephropathy. Thus, urinary tract infection, specifically kidney infection, is necessary for the development of reflux nephropathy in C3H/HeN mice. Notably, transient kidney infection is not sufficient to induce reflux nephropathy in B6 mice, which do not develop VUR. Finally, the authors show that sustained kidney infection leads to more marked renal inflammation and reflux nephropathy in C3H/HeJ mice, which have a defect in innate immunity and bacterial clearance, than in C3H/HeN mice.

Implications and future directions

These findings clearly demonstrate the role of infection in the progression from VUR to reflux nephropathy in the C3H mouse model. The knowledge that kidney infection is necessary for the development of reflux nephropathy in VUR-susceptible animals should impact treatment strategies for VUR patients. Although prophylactic antibiotic therapy is widely administered to pediatric VUR patients to prevent the occurrence of urinary tract infections, it has been unclear whether this strategy reduces the sequelae of VUR. These findings suggest that the use of more aggressive therapies to prevent urinary tract infections and to target inflammation once infection has occurred might help to reduce the incidence of these sequelae, including end-stage renal disease.

Fig. 1. HeN mice are VUR-susceptible at birth and at 8 weeks of age.
Methylene blue dye is visible in the ureters of post-natal day 1 (A) and 8-week-old (B) HeN mice but not B6 mice (A,B). Dye was instilled into bladders and ureter ascent was noted. Dashed lines outline ureter and bladder structures. (C) Incidence of VUR in HeN and B6 newborn and 8-week-old female mice. Newborn HeN mice (*n*=29) exhibited 93% incidence of VUR, whereas B6 mice (*n*=30) had no incidence of VUR. 8-week-old female HeN mice (*n*=13) had 100% incidence of bilateral VUR, whereas only one B6 mouse exhibited unilateral VUR (*n*=13). (D) H&E staining of kidneys from juvenile C3H/HeN mice reveals normal pathology and no inflammation. Scale bar: 200 μm.

the kidney such that there is histological evidence of focal or diffuse renal hypoplasia. Another mechanism that has been proposed is that sterile reflux by itself leads to elevated intrarenal pressure and acquired RN. Interestingly, the majority of patients that develop acquired RN are females, whereas individuals with congenital RN are predominantly male (reviewed by Mattoo, 2011).

To explore the dual roles of urinary tract infection and VUR in the development of acquired RN, we have studied a mouse model of VUR, the C3H mouse that we recently reported (Murawski et al., 2010). We report that VUR promotes and sustains kidney infection following bladder inoculation with uropathogenic *Escherichia coli* (UPEC) and this leads to RN in the C3H mouse model. Reflux of sterile urine in the absence of infection does not lead to RN. This is the first study to examine the factors that lead to RN in a mouse model with spontaneous VUR.

RESULTS

C3H/HeN mice are susceptible to VUR but do not spontaneously develop RN

Previously, we determined that the HeJ mouse is a model of VUR that recapitulates what is observed in most affected children: the
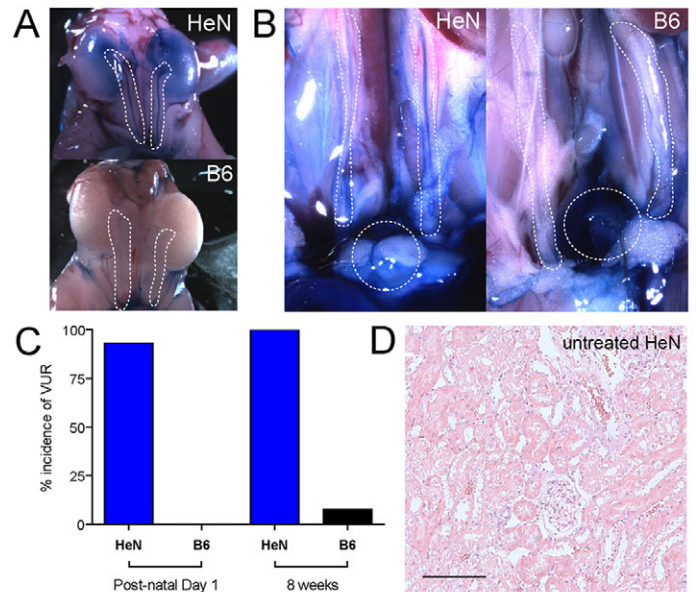
mice exhibit a delay in urinary tract formation that leads to a short intravesical ureter and they have normally formed kidneys (Murawski et al., 2010). To confirm the presence of VUR in a related C3H substrain, the HeN mouse, which, unlike the HeJ mouse, has an intact Toll-like receptor 4 (*TLR4*) gene and normal innate immunity, we instilled methylene blue dye in pup bladders and evaluated the retrograde flow of dye from the bladder towards the kidneys (Fig. 1A). Almost all of the HeN pups demonstrated VUR (93%, 27/29), whereas none of the B6 pups demonstrated VUR (*n*=30) (Fig. 1C). We also tested juvenile (8-week old) female HeN and B6 mice and found that all HeN mice exhibited VUR (*n*=13), whereas only one B6 mouse (*n*=13) demonstrated unilateral reflux at an elevated hydrostatic pressure, suggesting that even older B6 mice continue to be resistant to VUR (Fig. 1B,C). These data demonstrate that the HeN mouse is VUR-susceptible in both the newborn and juvenile stages of life.

VUR is associated with RN, a renal lesion characterized by tubulointerstitial inflammation and fibrosis (Yoshioka et al., 1990; Yoshioka et al., 1987). Previous studies in the pig model have suggested that the reflux of sterile urine is sufficient to induce RN (Paltiel et al., 2000; Ransley et al., 1984). We evaluated kidneys from juvenile HeN and B6 mice for signs of RN and found no abnormalities in kidney pathology for HeN mice (Fig. 1D) or in non-refluxing B6 controls (supplementary material Fig. S1). This suggests that RN does not develop from the reflux of sterile urine.
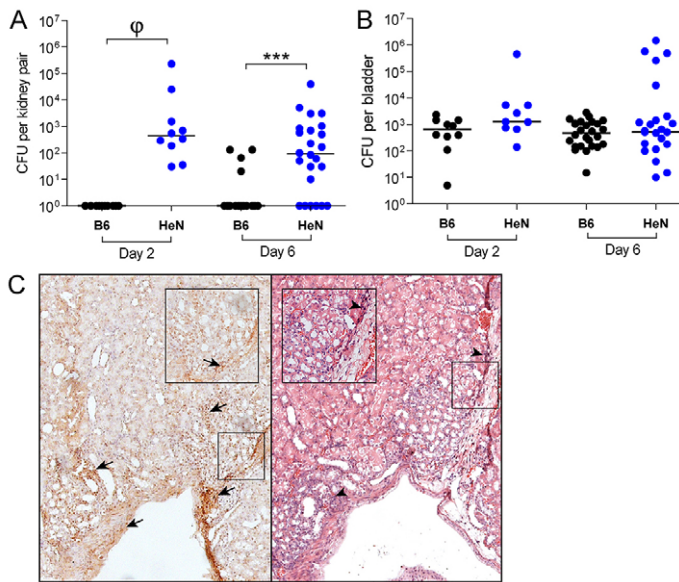
Fig. 2. HeN mice develop kidney infection after bladder inoculation.
Following induced bladder infection with UPEC strain CI5 via catheter, mice were sacrificed at 2 days or 6 days post-infection, and CFU counts were determined for combined kidney pairs (A) and bladders (B). (A) HeN mice (*n*=10) exhibit kidney infection at 2 days post-infection, whereas B6 mice do not (*n*=10). HeN mice (*n*=24) have a significantly higher kidney bacterial burden than B6 mice (*n*=26) at 6 days post-infection. (B) HeN and B6 bladders do not carry significantly different bacterial burdens at 2 days or 6 days post-infection. (A,B) Median bars indicated. All statistics calculated with Mann-Whitney *U*-test: Φ, significance cannot be calculated; ****P<0.0001.
(C) Immunostaining against the FimH molecule of UPEC strain CI5 was performed on HeN kidneys following bladder infection. Arrows indicate the presence of bacteria at 6 days post-infection in HeN kidneys on the left, whereas the adjacent section on the right is stained with H&E and reveals neutrophils in regions where UPEC is present. Arrows indicate areas of immunostaining for UPEC CI5; arrowheads indicate neutrophil influx. Boxed areas are shown at higher magnification in the insets.

C3H/HeN mice develop kidney infections following bladder inoculation

We determined whether the reflux of infected urine from the bladder leads to kidney infection and RN. To induce bladder-confined infection, we instilled 10^8 colony-forming units (CFU) of UPEC in a small volume into the bladders of juvenile HeN and B6 mice (supplementary material Fig. S2). At 2 days post-infection, HeN kidneys were heavily colonized (median: 4.4×10^2 CFU/kidney pair, *n*=10), whereas no bacteria were detectable in B6 kidneys (Fig. 2A). Kidney infection persisted at 6 days in HeN mice (median: 92.0 CFU/kidney pair, *n*=24) (Fig. 2A). Four B6 mice (*n*=26, median: 0.0 CFU/kidney pair) exhibited small numbers of renal bacteria at 6 days, likely due to isolated and transient ascending infection. As previously observed (Mulvey et al., 2001; Mysorekar and Hultgren, 2006), both B6 and HeN bladders exhibited sustained infection at 2 and 6 days post-infection (Fig. 2B). To confirm UPEC infection in HeN kidneys, we performed immunohistochemistry on kidney sections using an antibody raised against the FimH antigen of UPEC. We detected regions with large expression of the UPEC antigen, particularly in the tubulointerstitial compartment in HeN kidneys (Fig. 2C). Antigen was not detected in B6 kidneys (data not shown). The sections also revealed neutrophil influx consistent with an intense inflammatory reaction. These studies suggest that VUR promotes prolonged kidney infection post bladder-inoculation.

C3H/HeN mice with kidney infections have histological signs of renal inflammation and RN

To determine whether HeN mice with kidney infections develop renal inflammation and fibrosis, the hallmark signs of RN, we examined sections of kidneys at 6 days post-challenge that were stained with hematoxylin and eosin (H&E) and with Masson's trichrome (MT) (Fig. 3A). Infected HeN kidneys showed significant inflammation with a loss of delineation between the cortex and the medulla and extensive neutrophil infiltration extending from the renal pelvis to the tubulointerstitial compartment (Fig. 3A). These histological findings were not observed in saline-treated HeN or UPEC-inoculated B6 mice (Fig. 3A), or in saline-treated B6 mice (supplementary material Fig. S3). MT staining also revealed the presence of large collagen deposits in infected HeN kidneys. We quantified the degree of inflammation and fibrosis using an adapted scoring system (supplementary material Table S1) (Hopkins et al., 1998; Kaneto et al., 1994). Infected HeN kidneys had significantly higher inflammation (median: 1.00) and fibrosis (median: 1.00) scores than saline-treated HeN (inflammation: median 0.00; fibrosis: median 0.00) and UPEC-inoculated B6 (inflammation: median 0.00; fibrosis: median 0.00) mice (Fig. 3B,C). In summary, HeN mice develop significant RN following kidney infection.

VUR sustains kidney infection

To investigate whether VUR sustains kidney infection, we developed a protocol to induce kidney infections in VUR-resistant B6 mice as well as VUR-susceptible HeN mice. We artificially and temporarily induced reflux during bacterial challenge by instilling bacteria into the bladder with a larger volume of inoculum, forcing bacteria to enter the ureters and kidneys (supplementary material Fig. S2). Kidney and bladder bacterial burdens were compared in HeN and B6 mice at 6 hours, 2 days and 6 days post-challenge. At 6 hours, UPEC was present in similar numbers in both B6 and HeN kidneys (B6: median 1.7×10^3 CFU/kidney pair, *n*=6 versus HeN: median 3.5×10^3 CFU/kidney pair, *n*=7, *P*=0.3964), indicating that comparable levels of kidney infection were achieved with this protocol (Fig. 4A). However, by 2 days, B6 mice had cleared their kidney infections, whereas HeN mice did not (B6: median 1.0 CFU/kidney pair, *n*=10 versus HeN: median 4.3×10^2 CFU/kidney pair, *n*=8, *P*<0.0001) (Fig. 4A). Significant kidney infection was sustained in HeN mice even at 6 days, whereas B6 kidneys remained mostly clear of infection (HeN: median 2.4×10^3 CFU/kidney pair, *n*=9 versus B6: median 1.0 CFU/kidney pair, *n*=5, *P*=0.0289) (Fig. 4A). Additionally, we observed that bladder bacterial numbers were equivalent at 6 hours post-challenge (B6: median 1.8×10^5 CFU/bladder, *n*=6; HeN: median 1.5×10^4 CFU/bladder, *n*=5; *P*=0.1255) and this continued at 6 days post-infection (B6: 4.0×10^2 CFU/bladder, *n*=5; HeN: 2.0×10^4 *n*=9; *P*=0.153), although at 2 days post-infection the HeN bladder bacterial load was significantly higher than that of B6 mice (*P*=0.0025) (Fig. 4B); this could be due to an overall diminished
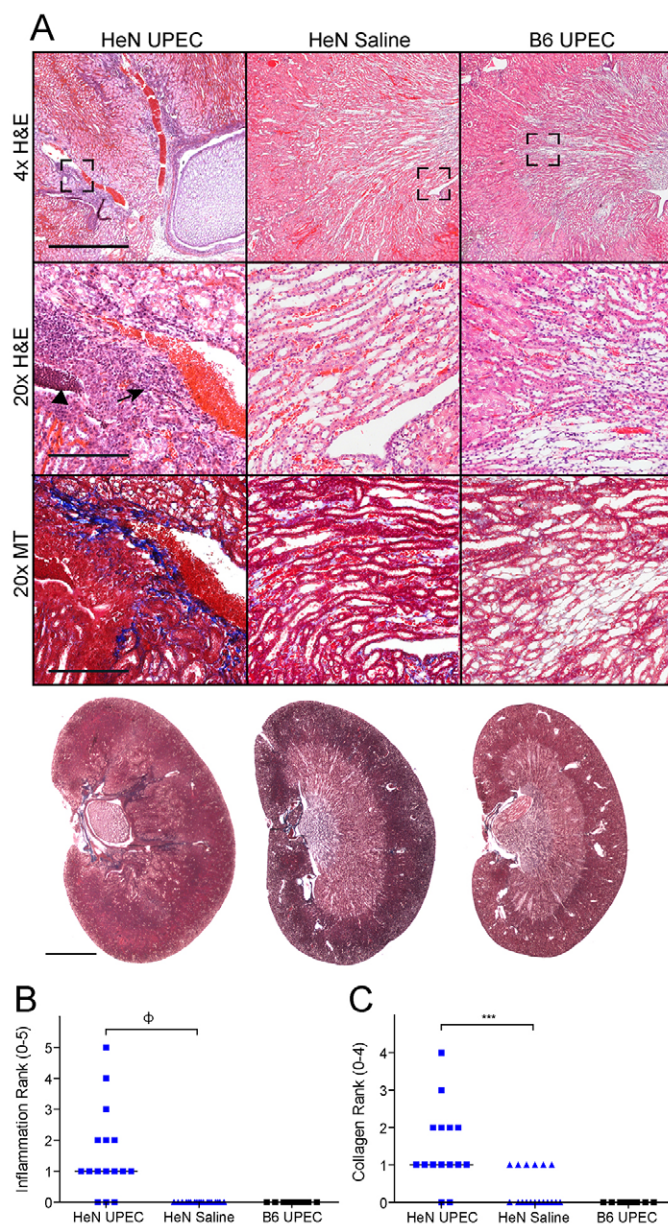
Fig. 3. HeN mice develop RN after bladder inoculation. (A) Infected HeN kidneys exhibit RN, whereas saline-treated HeN kidneys and bladder-infected B6 kidneys do not. Paraffin-embedded kidneys at 6 days post-infection were stained with H&E (first and second rows) or MT for collagen deposition (blue) (third row and lowest row showing whole kidney images). Large blue collagen deposits and a loss of kidney architecture are evident at 20× magnification (third row, left panel) and in whole kidney images in infected HeN kidneys but not saline HeN kidneys (third row, middle panel) and bladder-infected B6 kidneys (third row, right panel). Inflammation and neutrophil influx are evident in infected HeN kidneys but not saline HeN or bladder-infected B6 kidneys (first and second rows). Images are representative of all kidneys examined (HeN UPEC: n=16; HeN saline: n=18; B6 UPEC: n=10). Boxed areas in the first row are shown at higher magnification in the second row. Arrow indicates neutrophil influx in the interstitium; arrowhead indicates casts of neutrophils in renal tubules. (B,C) Infected HeN kidneys have significantly more inflammation (B) and collagen deposition (C) based on rank scoring than HeN saline-treated or B6 infected kidneys (see supplementary material Table S1 for scoring scales) (Student's t-test: Φ, significance cannot be calculated; ***P=0.0009). Scale bars: 4×, 1 mm; 20×, 0.2 mm; whole kidney, 2 mm.
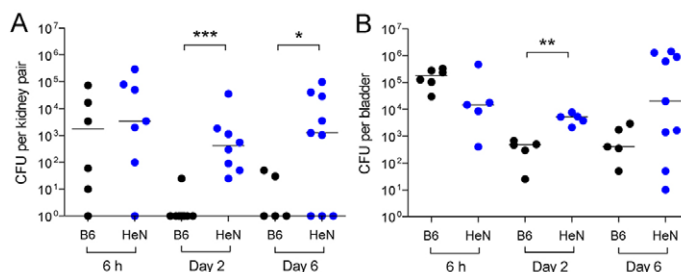

Fig. 4. HeN but not B6 mice exhibit sustained renal infection following induced kidney inoculation. HeN and B6 mice were infected via catheter instillation in a manner to directly induce kidney infection and sacrificed at 6 hours, 2 days or 6 days post-infection to quantify CFU counts in kidneys and bladders. (A) Both HeN (n=7) and B6 (n=6) kidneys are infected at 6 hours post-infection with no significant difference in kidney burden. At 2 days post-infection, B6 mice (n=10) have cleared kidney infections, and HeN mice (n=8) have significantly higher bacterial burdens, which persist at 6 days post-infection (B6: n=5, HeN: n=9). (B) CFU counts in infected HeN and B6 bladders at 6 hours and 6 days post-infection are not significantly different; however, HeN bladders have significantly higher bacterial burdens at 2 days post-infection. Median bars indicated. All statistics calculated with Mann-Whitney U-test: *P=0.0289, **P=0.0025, ***P<0.0001.

number of bacteria in the urinary tract due to kidney clearance by 2 days. Overall, these observations suggest that VUR promotes and sustains kidney infection through the reflux of bacteria from the bladder to the kidneys.

To determine whether sustained kidney infection is required for the development of RN or whether a single acute episode is sufficient to induce this pathology, we compared kidney histology in B6 and HeN mice at 6 days (Fig. 5A,B) and 14 days (supplementary material Fig. S4) post-challenge via direct kidney inoculation. At 6 days, HeN kidneys exhibited significantly more inflammation (HeN: median 3.00, n=8 versus B6: median 1.00, n=8, P=0.0323) and collagen deposition (HeN: median 2.00, n=8 versus B6: median 1.00, n=8, P=0.0177), and this trend remained at 14 days post-infection for inflammation (HeN: median 1.50, n=8 versus B6: median 0.0, n=8, P=0.0308) and collagen deposition (HeN: median 2.00, n=8 versus B6: median 1.00, n=8, P=0.0029) (Fig. 5C,D). Saline-treated kidneys did not exhibit signs of inflammation or collagen deposition (data not shown). Cumulatively, these data suggest that VUR sustains kidney infection, and this leads to the pathological changes of RN. B6 mice probably exhibit less severe signs of inflammation or fibrosis following induced kidney infection because they quickly clear bacteria and do not experience re-infection of the kidneys in the absence of VUR.

Inflammation and fibrosis following bacterial clearance

There is little information about the natural history of inflammation and kidney fibrosis in RN once the infection is cleared. We observed that most HeN mice eventually clear the kidney infection by 14 days post-challenge (data not shown). The elimination of bacteria from the kidneys as part of a pathogen-specific immune response in the host allows us to examine the degree of kidney inflammation and fibrosis following clearance of infection. Because the presence of bacteria is crucial for RN, we examined another refluxing C3H mouse substrain, HeJ, for comparison. The HeJ mouse possesses a point
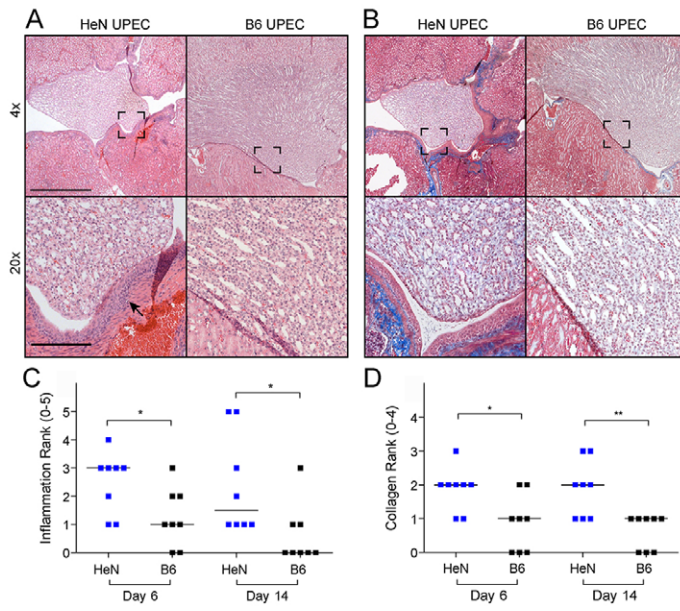
Fig. 5. Sustained kidney infection is required for the development of RN. HeN and B6 mice were infected via catheter instillation to induce kidney infection and sacrificed at 6 days (A-D) or 14 days (C,D) post-infection. (A) H&E staining and (B) MT staining reveal the presence of inflammation and collagen deposition in infected HeN kidneys but not infected B6 kidneys at 6 days post-infection. Neutrophil influx is evident in HeN kidneys (A, lower left panel, arrow), as well as collagen deposition (B, lower left panel). Boxed areas in the first row are shown at higher magnification in the second row. Scale bars: 4×, 1 mm; 20×, 200 μm. (C) When quantified, HeN kidneys exhibit significantly more inflammation at both 6 days and 14 days post-infection than do B6 mice (HeN: n=8, B6: n=8, unpaired t-test: 6 days *P=0.0323, 14 days *P=0.0308). (D) Similarly, HeN kidneys also have significantly more collagen present at 6 days and 14 days post-infection compared with B6 mice (HeN: n=8, B6: n=8, Student's t-test: *P=0.0177, **P=0.0029).
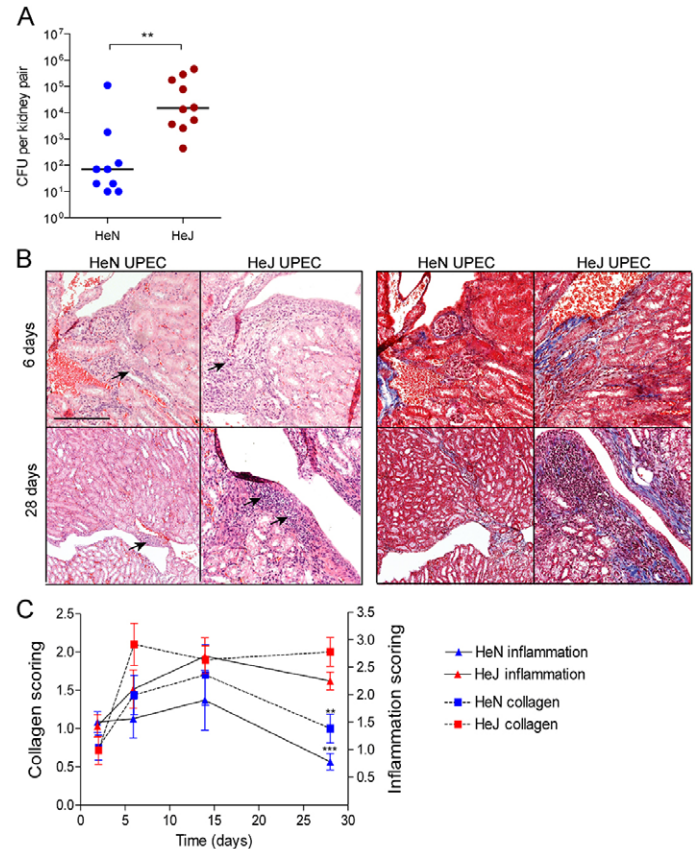

Fig. 6. Sustained renal infection perpetuates RN. HeN and HeJ mice were infected with UPEC via catheter to induce bladder infection, and mice were sacrificed at 6 days post-infection to determine kidney and bladder CFU counts. (A) HeJ mice harbor significantly higher kidney bacterial burdens than HeN mice (median bar indicated; HeN: n=9, HeJ: n=10, Mann-Whitney U-test: **P=0.0013), but bladder burden is equivalent for both HeJ and HeN mice (data not shown). (B) H&E (left block) and MT (right block) stainings reveal the presence of inflammation and collagen deposition in both HeN and HeJ kidneys at 6 days post-infection (top row) but, at 28 days, there is more inflammation and fibrosis in HeJ compared with HeN kidneys (bottom row). Arrows indicate areas of neutrophil influx. Scale bar: 0.4 mm. (C) Quantification reveals a significant difference in scoring for inflammation (***P<0.0001) and collagen (**P=0.0022) between HeN and HeJ mice at 28 days post-infection (HeN: n=14; HeJ: n=10).

mutation in the *TLR4* gene that results in a defective neutrophil response (Haraoka et al., 1999) and limited clearance of bacteria compared with HeN mice (Shahin et al., 1987; Haraoka et al., 1999; Svanborg Edén et al., 1984). Importantly for our studies, HeN and HeJ mice reflux to the same degree (Murawski et al., 2010). Consistent with the literature, HeJ mice carried a significantly higher renal bacterial burden than HeN mice (HeJ: $1.5\times10^4\pm6.2\times10^4$ CFU/kidney pair, n=10 versus HeN: $7.0\times10^2\pm2.5\times10^2$ CFU/kidney pair, n=9, P=0.0013) at 6 days post-challenge (Fig. 6A). The kidney infection persisted in HeJ mice at 14 days, whereas, in HeN mice, it was cleared (data not shown). We then examined RN progression post bladder-challenge. At 2 days, both inflammation and collagen deposition levels were significantly elevated compared with uninfected controls in both HeN and HeJ mice (data not shown); uninfected HeN and HeJ mice did not exhibit signs of RN (supplementary material Fig. S1), nor did saline-treated HeJ mice (supplementary material Fig. S3). By 6 days, inflammation and collagen deposition were comparable in both substrains (Fig. 6C). By 28 days, inflammation and collagen deposition in HeN mice were significantly lower than in HeJ mice (P<0.0001). This suggests that the severity of RN depends on the duration of bacterial infection and the ability of the host immune response to clear infection.

DISCUSSION

This is the first report to describe the crucial interplay between VUR and sustained kidney infection in the development of RN in a naturally occurring animal model of VUR: the inbred C3H mouse (Fig. 7). We demonstrate that VUR alone is not sufficient for the development of RN. An additional insult is required: sustained bacterial infection of the kidneys. We show that, unlike non-refluxing B6 mice, HeN mice develop sustained kidney infections following infection of the bladder, owing to reflux. Finally, we report that, when infecting bacteria are cleared from the kidneys, inflammation and collagen deposition levels are reduced compared with levels in kidneys with ongoing infection, clearly demonstrating the role of the host immune response in modulating the severity of RN. Together, these factors contribute to the pathogenesis of RN.
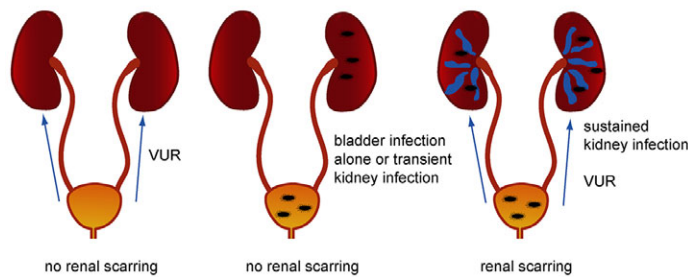
Fig. 7. Model for the synergistic roles of VUR and sustained kidney infection in the development of RN. RN does not develop in the presence of VUR alone (left image) or transient kidney infection or bladder infection alone (middle image). RN requires the presence of both VUR and sustained kidney infection to induce renal scar formation (right image).

The ability to investigate VUR, infection and RN pathogenesis in a naturally occurring mouse model of reflux is noteworthy, because most of the previous studies have reported on a minipig model involving surgical resection of the UVJ to induce VUR (Coulthard et al., 2002; Paltiel et al., 2000; Pohl et al., 1999; Ransley et al., 1984; Ransley et al., 1987; Risdon et al., 1994). In this model, the role of infection in RN has been difficult to ascertain. Some of the pig studies have reported that sterile VUR alone is sufficient to induce RN (Paltiel et al., 2000; Ransley et al., 1984; Ransley et al., 1987), whereas other studies have reported that infection with UPEC is required to induce RN (Coulthard et al., 2002; Pohl et al., 1999). There is a lack of appropriate controls in these studies, making the results difficult to interpret. Given the lack of consistency with results from surgical VUR models, we found it crucial to explore renal scarring in a model with naturally occurring VUR. The C3H mouse recapitulates the most common pediatric clinical presentation: specifically, VUR in the presence of normally formed kidneys.

Our observations in this naturally occurring mouse model of reflux suggest that intrarenal pressure associated with sterile VUR is not sufficient to trigger RN. Our studies also suggest that a single bout of kidney infection in the absence of VUR is not sufficient to cause significant levels of renal inflammation and fibrosis, because B6 mice subjected to a single kidney infection failed to exhibit these signs (Fig. 5). Instead, sustained UPEC infection is required for the development of RN (Figs 3, 5). In an immunocompetent host, the kidneys respond to infection by mounting an immediate innate immune response that includes neutrophil influx, which rapidly clears infecting bacteria (Shahin et al., 1987). In our study, kidney infections were largely cleared from B6 mice by 2 days post-challenge, which is presumably not sufficient time for the recruited inflammatory cells to elicit a cytotoxic reaction of the magnitude required to initiate fibrosis. Kidneys from B6 mice after a single infection showed little to no signs of inflammation or fibrosis at 6 days after challenge, in contrast to kidneys from HeN mice, which showed sustained infection and signs of RN (Fig. 5). Thus, RN is evident only in the presence of sustained kidney infection.

In our studies, we subjected refluxing C3H mice to one bladder infection with UPEC strain CI5 (which is a non-hemolytic strain). Our histopathology findings demonstrate interstitial inflammatory infiltration and tubulointerstitial fibrosis, which are well-described

and essential features seen in all cases of RN (Morita et al., 1990; Weiss and Parker, 1939; Laberke, 1987; Risdon et al., 1993). In more severe cases of RN, secondary glomerular pathology is also seen with glomerular sclerosis and periglomerular fibrosis noted (Morita et al., 1990; Torres et al., 1980). Once the disease is more severe and encompasses the glomeruli, one would expect that affected individuals would begin to show proteinuria and a decrease in the glomerular filtration rate as measured by serum creatinine. The pathology described in the literature is generally skewed towards the most severe cases; for example, the Risdon study (Risdon et al., 1993) describes the histology from patients with RN who underwent nephrectomy. As has been previously outlined (Farris and Colvin, 2012), there are many methods to assess renal fibrosis, and although none are considered the gold standard, MT is one suggested stain. The findings we present in the mouse are consistent with human RN, which consists of a spectrum of pathological findings from mild to severe. We would anticipate that, if the mice were subjected to multiple bladder infections and to more virulent bacterial strains, even more severe pathological findings would be detected.

The intrinsic susceptibility of C3H mice to reflux could explain why these strains are widely known to exhibit 'more severe' or 'chronic' UTIs compared with other mouse strains following experimental infection (Hopkins et al., 1998; Hannan et al., 2010). Various studies to elucidate the underlying basis have been undertaken with varying conclusions, but a quantitative trait loci (QTL) mapping study in C3H/HeJ mice did reveal loci associated with increased susceptibility to bladder and kidney infections on chromosomes 4 and 6, respectively (Hopkins et al., 2009). We previously identified a significant QTL for VUR susceptibility on chromosome 12 (Murawski et al., 2010). These studies demonstrate that C3H mice can be used to model genetic predispositions to both UTI and VUR, perhaps reproducing what is seen in some affected children.

Our work demonstrates that the severity of RN correlates with the presence of sustained infection, such that when kidney infection is cleared, there is less inflammation and fibrosis compared to what is seen in the presence of sustained infection. Both our work and previous studies have demonstrated that HeJ mice maintain a higher renal bacterial burden than HeN mice (Shahin et al., 1987). Unlike HeN mice, HeJ mice do not clear kidney infections by 14 days (data not shown) or even by 4 weeks (Hannan et al., 2010). The fact that HeN mice exhibited significantly less RN than HeJ mice at 28 days post-challenge suggests that the severity of RN correlates with bacterial clearance. These findings correlate with a previous study in which mIL-8Rh knockout (KO) mice sustained higher renal bacterial burdens than did wild-type mice and eventually developed fibrosis and renal scarring (Hang et al., 2000). Thus, both infection and the host immune response are likely to be crucial factors that dictate the severity of RN. The C3H/HeN model of VUR could therefore be used to evaluate targeted therapies to prevent the development and progression of RN.

MATERIALS AND METHODS
Mouse strains and VUR assay in newborn and juvenile mice
The incidence of VUR was examined in newborn and juvenile (8 weeks) mice from the following inbred strains: C3H/HeN (HeN) (Charles River, Wilmington, MA), C3H/HeJ (HeJ) and C57BL/6J

(B6) (Jackson Laboratories, Bar Harbor, ME), as previously described (Murawski et al., 2010). Briefly, mice were euthanized and dissected via midline incision to expose the urinary tract. Methylene blue dye was injected into the bladder using a pressure gradient, and VUR was defined by the presence of dye moving retrogradely from the bladder into the ureters and renal pelvis. All mouse work was performed in accordance with guidelines established by the Canadian Council on Animal Care (CCAC) and the Office of Laboratory Animal Welfare in the US Department of Health and Human Services.

Bacterial culture and mouse infections

Uropathogenic *E. coli* (UPEC) strain CI5, a clinical pyelonephritis isolate, was utilized for all murine infections (Song et al., 2007a; Song et al., 2009; Song et al., 2007b). Overnight cultures were inoculated from frozen stock in Luria broth [Becton Dickinson and Company (BD), Franklin Lakes, NJ] and grown at 37°C. Optical density (OD) was determined, and cultures were washed and diluted in PBS. Juvenile (8-week old) female C57BL/6J, C3H/HeN or C3H/HeJ mice were anesthetized and catheterized with polyethylene tubing (inner diameter: 0.28 mm) (BD), and UPEC was instilled from a 1 ml tuberculin syringe with a 30G1/2 needle. The use of 8-week-old mice was necessitated by technical limitations for the catheterization procedure, but, as demonstrated in Fig. 1, juvenile HeN mice are fully VUR-susceptible. Additionally, owing to technical considerations, female mice were utilized because of ease of catheterization. To induce bladder-only infection, 10^8 bacteria in 30 μl were slowly instilled over 20 seconds into the bladder, whereas 10^8 bacteria in 50 μl were quickly instilled over 4 seconds to induce kidney infection (Chan et al., 2013) (protocol schematic shown in supplementary material Fig. S2). Mice were euthanized by CO_2 asphyxiation, and bladders and kidneys were isolated, homogenized and plated on MacConkey agar to quantify CFUs. Statistics were calculated with the Mann-Whitney *U*-test.

Kidney pathology

Kidneys were dissected, fixed in 4% paraformaldehyde and paraffin-embedded. Serial sections of each kidney (3 and 7 μm) were stained with either H&E or MT to visualize adjacent kidney sections. A subset of slides from the most proximal to midline areas of the kidney were examined for inflammation and fibrosis, and were scored using two adapted scales (supplementary material Table S1) (Kaneto et al., 1994; Hopkins et al., 1998). All specimens were examined without knowledge of infective protocol. Statistics were calculated with Student's *t*-test.

Immunohistochemistry and immunofluorescent detection of UPEC

Immunohistochemical staining was performed to detect the presence of UPEC antigen in the kidneys. Kidney sections of 3 μm were blocked with 1% BSA and incubated overnight with a primary antibody specific to the FimH adhesin protein of UPEC CI5 (Bishop et al., 2007). Following incubation, slides were treated with 0.6% hydrogen peroxide for 15 minutes to block endogenous peroxidase activity, and a biotinylated secondary antibody (Vector, Burlington, Ontario, CA) was applied. Slides were treated with DAB (Vector) and counterstained with Mayer's hematoxylin (Sigma-Aldrich, St Louis, MO).

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