Natural history of renal scarring in susceptible mIL-8Rh−/− mice

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Background. Urinary tract infections (UTIs) cause end-stage renal disease (ESRD) but the molecular mechanisms have remained unclear. Recently, the interleukin (IL)-8 receptor was shown to control disease susceptibility in mice and low IL-8 receptor expression was observed in pyelonephritis-prone patients.

Methods. Intravesical *Escherichia coli* infection was established in mIL-8Rh−/− or Balb/c control mice. Survival, bacterial persistence, and histology were used as measurements of disease severity.

Results. Within 2 days, 19/30 mIL-8Rh−/− mice developed lethal infection with bacteremia. Surviving mice remained infected and developed progressive renal damage with pathologic neutrophil accumulation and abscess formation first under the pelvic epithelium and then throughout the tissue. Recruited immune effector cells were unable to remove the dying neutrophils and frustrated macrophages formed foam cell aggregates. As a result, there was successive destruction of the mucosal barrier, medulla and cortex and necrosis of the renal papilla. The mIL-8Rh+/+ mice all survived and infection was cleared within a few days without symptoms or tissue pathology.

Conclusion. mIL-8Rh−/− mice develop acute bacteremic pyelonephritis and renal scarring due to a dysfunctional neutrophil response. The tissue damage resembles human disease, and these mice offer a model system to study the molecular mechanisms of renal scarring.

Urinary tract infections (UTIs) remain a major health problem despite the success of modern antibiotics. Acute pyelonephritis is accompanied by bacteremia in about 30% of adults and gram-negative sepsis causes significant mortality in all age groups [1, 2]. In addition, progressive kidney infection is an important cause of tissue damage and renal failure [3]. The severity of acute disease reflects the virulence of the infecting *Escherichia coli* (*E. coli*) strain but there is no virulence factor associated with progressive disease and renal scarring. Rather to the contrary, many isolates from patients with renal scarring seem to lack the pyelonephritis-associated virulence traits, suggesting that host susceptibility factors are at least as important for disease progression as the properties of the infecting strain [4–6].

There have been many attempts to identify such host defense defects but until recently, no molecular explanation of disease susceptibility had been offered. Mechanical dysfunctions like vesicoureteric reflux are known to increase the damaging effect of acute pyelonephritis [7, 8], but reflux does not explain qualitative differences in tissue responsiveness and inflammation. The variation in mucosal receptors for bacterial fimbriae influences the selection of the infecting *E. coli* strain, but differences in receptor expression do not explain the tendency to develop renal scarring [5, 9, 10]. Human leukocyte antigen (HLA) typing has been attempted, but without success [11]. Recently, however, the expression of interleukin (IL)-8 chemokine receptors was shown to control bacterial clearance from infected kidneys in the murine UTI model. Furthermore, we found reduced expression of the human IL-8 receptor in a group of children prone to acute pyelonephritis [12].

The antimicrobial defense of the urinary tract relies on innate immunity [13]. There is no preexisting mucosal immune response in the uninfected urinary tract and yet, bacteriuria is cleared within hours or days. After intravesical infection, bacteria adhere to the renal pelvic epithelium and trigger a chemokine response, which recruits neutrophils from the circulation to the mucosal lining and the neutrophils then cross the epithelial barrier into the lumen [14, 15]. This exit from the tissues is crucial for bacterial clearance and tissue integrity, and is supported by chemokines like CXCL8 (IL-8) and their receptors.

In mIL-8Rh−/− mice, the neutrophils are trapped under the mucosal lining, and eventually a massive neutrophil infiltrate builds up throughout the tissues [16, 17]. The tendency to develop renal scarring may possibly be attributed to the dysfunctional neutrophil response in the mIL-8Rh−/− mice, but the cellular infiltrate and tissue response has not been examined.

Few if any of the existing animal models mimic the development of renal scarring in humans. In the absence of host susceptibility genes, resistant animals have been used and surgical procedures have been required to establish reflux or tissue injury prior to infection [18, 19]. The mIL-8Rh is the first example of a host susceptibility gene and the mutant mice offer a unique model of human disease without mechanical or pharmacologic manipulations. This study examined the natural disease progression from mucosal infection of the urinary tract to renal scarring in mIL-8Rh−/− mice.

METHODS

Bacteria

E. coli 1177, serotype O:1K:1H:7, was isolated from a child with acute pyelonephritis [20]. The strain is virulent in the UTI mouse model and evokes a strong inflammatory response [21]. It expresses P and type 1 fimbriae, but is hemolysin-negative. *E. coli* 1177 was maintained in deep agar and passaged on Tryptic soya agar (TSA) plates. For experimental infection, *E. coli* 1177 was cultured on TSA plate, harvested by centrifugation, and resuspended to a concentration of 10^9 cfu/mL. The bacterial concentration was confirmed by viable counts.

Mice

The mice were bred in the animal facilities at the Department of Microbiology, Immunology and Glycobiology, University of Lund. Breeding pairs of Balb/c-*CmKar2^{tm1Mwm}* (mIL-8Rh−/−) were purchased from Jackson Laboratories (Bar Harbor, ME, USA). Congenic Balb/c mice were used as controls. mIL-8Rh−/− mice fail to express the murine IL-8 receptor homologue [22] due to the insertion of a neomycin gene.

Genotyping by polymerase chain reaction (PCR)

The wild-type or mutant mIL-8Rh genotype was confirmed by PCR on DNA extracted from tail clippings of individual mice as described earlier (Fig. 1A) [12].

Experimental UTI

Following anesthesia, 8- to 12-week-old female mice were infected by intravesical inoculation with *E. coli* 1177 (10^9 cfu/mL, 100 μL) through a soft polyethylene catheter (outer diameter 0.61 mm) (Clay Adams, Parsippaniy, NY, USA) as earlier described [23]. The catheter was with-

drawn, and the mice were allowed food and water ad libitum. Animals were sacrificed at the designated time intervals after infection, or when they developed symptoms of severe disease.

Bacterial numbers were determined by viable counts on kidney homogenates. One kidney was placed in a sterile plastic bag containing 5 mL of phosphate-buffered saline (PBS), and homogenized in a Stomacher 80 homogenizer (Seward Medical, UAC House, London, UK). Homogenates were diluted in sterile PBS, 0.1 mL of each dilution was plated on TSA, and the number of colonies was scored after overnight culture at 37°C. The contralateral kidney was placed in 4% paraformaldehyde (PAF) and stored for histologic analysis.

Blood was obtained by cardiac puncture and injected into blood culture flasks containing liquid medium. In parallel, a drop of blood was cultured directly on McConkey agar and blood agar plates. Bacterial numbers were semiquantitatively determined after overnight culture at 37°C.

The study was approved by the Animal Experiment Ethics Committee at Lund District Court, Sweden.

Histologic evaluation of acute and chronic infection

The fixed tissue samples were dehydrated by over night incubation in alcohol, followed by xylen, and placed in Histowax (Histolab Products, Västra Frölunda, Sweden) according to standard methods. The samples were embedded in paraffin, and 4 to 5 μm sections were cut and placed on glass slides. Sections were deparaffinized and stained with hematoxylin-eosin. Masson trichrome staining was used for detection of fibrosis.

The density of neutrophils, lymphocytes, and foam cells was semiquantified as 0 (no cells), + (1 to 20 cells), ++ (many) or +++ (abundant). The fibrosis was semiquantified from 0 (no fibrosis) to +++ (abundant fibrosis). Three individuals investigated samples independently.

RESULTS

mIL-8Rh−/− mice develop lethal infections with bacteremia

UTI was established in mIL-8Rh−/− mice by intravesical injection of a human pyelonephritis *E. coli* isolate (Fig. 1B). Within 2 days, 19/30 mice died, ten on day one and nine on day two. Another two mice died on day 28. Balb/c mice, in contrast, did not develop symptomatic or lethal infections during the experimental period. The results show that the mIL-8Rh mutation impairs host resistance to kidney infection and that systemic, life-threatening disease develops as a consequence. The frequency of *E. coli* bacteremia was estimated at 40% in a separate group of mIL-8Rh−/− mice. The results suggest that the mIL-8Rh is essential to limit the progression from local to
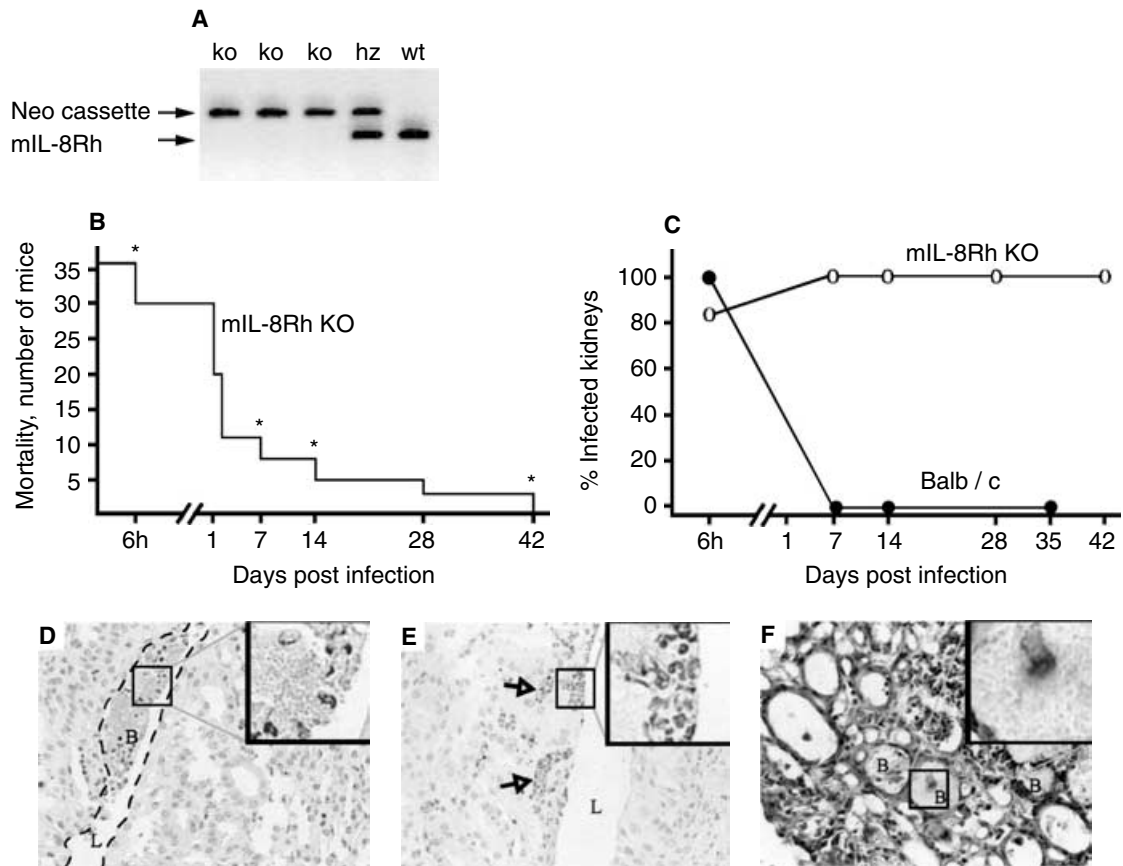
Fig. 1. Susceptibility to infection in mice. (*A*) IL-8Rh genotype as determined by polymerase chain reaction (PCR) using primers to neomycin gene (−/−) and to *mIL-8Rh* (wt). Hz is heterozygous. (*B*) Mortality after intravesical infection of mIL-8Rh mice. Asterisks denote times of sacrifice. (*C*) Clearance of kidney infection in Balb/c and mIL-8Rh−/− mice. (*D*) Bacteria in the pelvic lumen 7 days postinfection. (*E*) Bacteria in mucosal abscesses 7 days postinfection. (*F*) Bacteria in the lumen of collecting ducts 14 days post infection. L is lumen and B is bacterial mass. Hematoxylin-eosin staining (original magnification ×200).

systemic infection, and to resist the systemic phase of gram-negative infection.

mIL-8Rh−/− mice show impaired clearance of the infection from the kidneys

The kinetics of infection was compared between the mIL-8Rh−/− and Balb/c mice (Fig. 1C). The Balb/c mice were resistant to infection (Fig. 1C) and the kidneys obtained on days 7, 14, and 35 after infection were sterile (<50 cfu/tissue). The −/− mice, in contrast, remained infected until day 42, with bacterial numbers varying from 2×10^3 to 1×10^9 cfu/kidney. Bacteria were detected in tissue sections from the mIL-8Rh−/− mice (Fig. 1D to F). After 7 days, they were localized to the lumen of the renal pelvis (Fig. 1D) and together with neutrophils in the microabscesses in the tip of the papilla (Fig. 1E). After 14 days, the bacteria were found in the tubuli of the renal papilla (Fig. 1F). After 28 days, the renal papilla contained abscesses with bacterial aggregates.
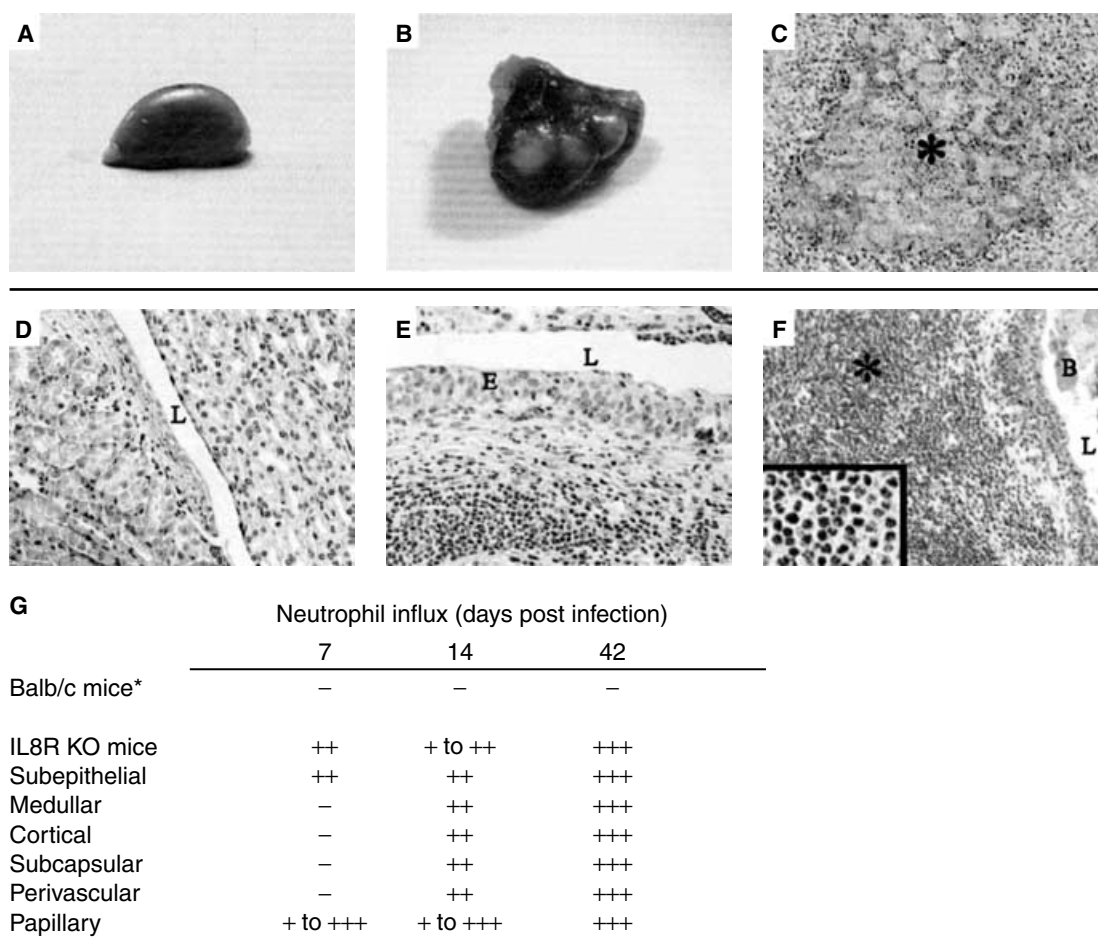
Macroscopic signs of pyelonephritis

The kidneys were inspected for signs of acute pyelonephritis-like hyperemia, edema, and subcapsular abscesses or for signs of renal scarring-like irregularities in overall contour, reflecting retractions due to fibrosis. Macroscopic changes were only seen in the −/− mice. After 7 days, some of the kidneys were enlarged, red and swollen. Two weeks postinfection, macroscopic, subcapsular abscesses were visible on the surface of two kidneys. After 6 weeks, some kidneys were irregular and subcapsular abscesses were visible (Fig. 2B). Occasionally, the entire kidney was fibrotic and destroyed (Fig. 2C). The Balb/c mice did not develop any macroscopic signs of acute pyelonephritis or renal deformity (Fig. 2A), confirming the importance of the mIL-8Rh mutation as a cause of disease.

Neutrophils accumulate in renal tissue

The neutrophil cell infiltrate was characterized by histology (Fig. 2D to G). After 7 days, massive numbers of neutrophils had accumulated in under the renal pelvis epithelium (Fig. 2E) and some neutrophils formed microabscesses within the epithelium. By day 14 postinfection, the subepithelial neutrophil accumulation had continued but, in addition, neutrophils were seen around

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	Neutrophil influx (days post infection)		
	7	14	42
Balb/c mice*	–	–	–
IL8R KO mice	++	+ to ++	+++
Subepithelial	++	++	+++
Medullar	–	++	+++
Cortical	–	++	+++
Subcapsular	–	++	+++
Perivascular	–	++	+++
Papillary	+ to +++	+ to +++	+++

* These mice showed a transient neutrophil response during the first days after infection, but by day 7, infection had cleared and the neutrophil response had vanished.

Fig. 2. Abscess formation and neutrophil accumulation. (*A*) Normal kidney morphology. (*B*) mIL-8Rh−/− Kidney morphology 42 days postinfection. (*C*) Abscesses (∗) in the renal medulla 14 days postinfection. (*D*) Kidney parenchyma of an uninfected mIL-8Rh−/− mouse. (*E*) Subepithelial neutrophil accumulation 7 days postinfection. (*F*) Neutrophil accumulation after 28 days. Asterisk marks the site of the close up. (*G*) Kinetics of neutrophil accumulation in the kidney of mice. L is lumen, B is bacterial mass, E is epithelium. Hematoxylin-eosin staining [original magnification ×200 (C to E) and ×100 (F).

blood vessels and deeper in the kidney tissue. After 42 days, neutrophils were abundant throughout the kidneys. In the Balb/c mice, there was no evidence of neutrophil accumulation on day 7 or at later time points. The results confirmed the difference in neutrophil trafficking between the mIL-8Rh−/− and the Balb/c mice. The massive accumulation of neutrophils in renal tissues of the mIL-8Rh−/− mice was a probable cause of the tissue destruction.

Lymphocytic infiltrates in the kidneys of mIL-8Rh−/− mice

Lymphocytes and plasma cells were recruited into the kidneys of the mIL-8Rh−/− mice (Fig. 3A to D) but not in the Balb/c controls. The kinetics are summarized in Fig. 3C. After 7 days, the occasional lymphocyte was

observed in perivascular areas and after 14 days, there were numerous lymphocytes in this area. By day 42, lymphocytes had infiltrated the entire kidney (Fig. 3B). The infiltrate included plasma cells, which formed Russell bodies (Fig. 3D), representing the end stage of these cells. Some foam cells could be detected after 28 days, and after 42 days foam cells were visible in every sample (Fig. 3E). Thus the immune effector cells were unable to rescue the tissues.

Infection destroys renal tissue architecture

The overall effects of infection on the tissue structure were evaluated at low magnification (Fig. 4). The early changes included microabscesses (Fig. 1E) and thickening of the epithelium after 7 and 14 days, respectively (Fig. 4G and H). Larger abscesses were found after 14
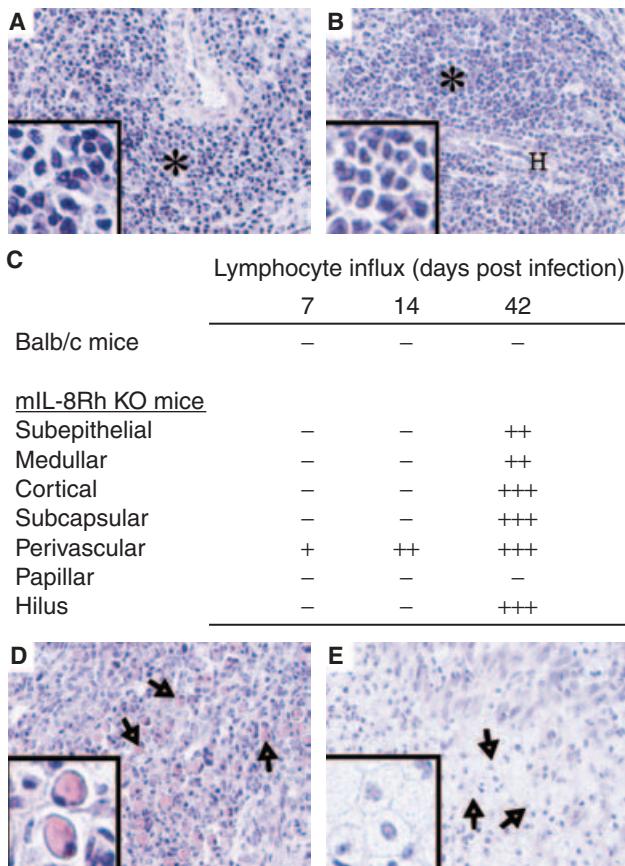
Fig. 3. Lymphocyte infiltration in mIL-8Rh−/−mice (*A*) Asterisk marks lymphocyte infiltrate 28 days postinfection. (*B*) Lymphocyte infiltration (∗) 42 days postinfection. (*C*) Kinetics of lymphocyte infiltration. (*D*) Russel bodies (arrows). (*E*) Foam cells (i.e., xanthoma cells, arrows). Close up magnifications are from the same picture. H is high endothelial like vessel. Hematoxylin-eosin staining (original magnifications ×200).

C	Lymphocyte influx (days post infection)		
	7	14	42
Balb/c mice	−	−	−
mIL-8Rh KO mice			
Subepithelial	−	−	++
Medullar	−	−	++
Cortical	−	−	+++
Subcapsular	−	−	+++
Perivascular	+	++	+++
Papillar	−	−	−
Hilus	−	−	+++

Fig. 5. Epithelial proliferation and tissue fibrosis. Proliferating epithelium (E) in the renal pelvis (*A*) and ureter (*B*). (*C*) Small fibrotic areas (blue, ∗) in uninfected kidneys (*D*) Limited fibrosis 7 days postinfection (∗). (*E*) Thickened and fibrotic smooth muscle cell layer after 28 days (∗). (*F*) Massive subepithelial fibrosis after 42 days (∗). L is lumen, E is epithelium. Hematoxylin-eosin staining (A and B), Masson trichrome staining (C to F) (original magnification ×100).

and 28 days (Figs. 2C and 4I), and by 28 days, the renal papilla was necrotic and the medullar region was filled with inflammatory cells with almost no normal structure left (Fig. 4D and I). After 42 days, the entire kidney tissue was destroyed (Fig. 4E and J). The pelvic and urethral epithelium became thickened and convoluted, like a glandular structure and there was evidence of cellular proliferation (Fig. 5A and B). The epithelium was one of the rare remaining landmarks in the tissue (Fig. 4J). As the tubular epithelial cells died, the pelvic epithelium was shed together with neutrophils, forming debris in the lumen. Thus, infection caused progressive renal tissue destruction in mIL-8Rh−/− mice, involving the renal papilla, cortex, and medulla.

Destroyed tissue is replaced by fibrotic tissue

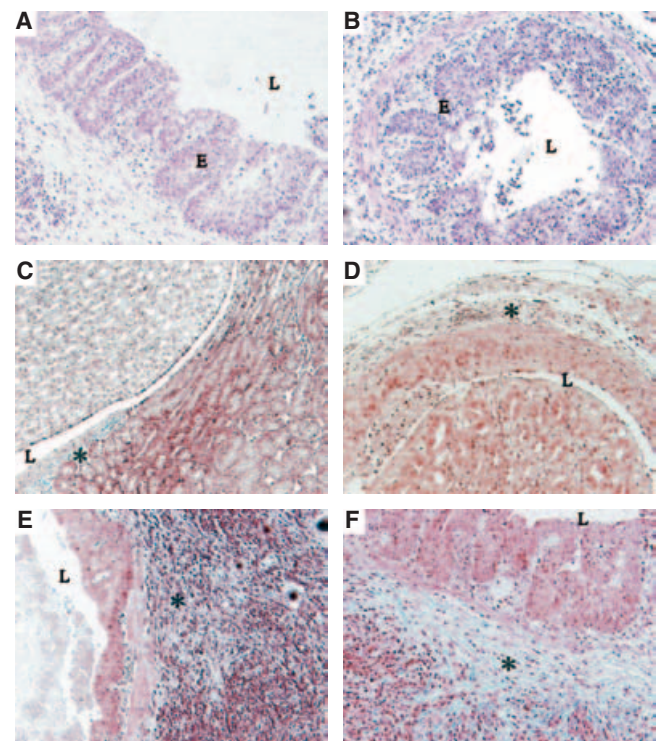Fibrotic tissue was identified with Masson trichrome staining. In the Balb/c control mice, a low degree of nat-ural fibrosis was seen around the ureter and in the pelvic area. There was no increase in fibrosis after infection of the control mice. At day 28, small areas of diffuse fibroses were seen under the transitional epithelium of the pelvis (Fig. 5E). Furthermore, the perivascular space, the subcortical areas, and the ureter were surrounded by fibrotic tissue. After 42 days, significant fibrotic areas were found in the subepithelial space (Fig. 5F). Thus, a significant part of the renal tissue was replaced by fibrotic scar tissue.

DISCUSSION

Gram-negative septicemia and renal scarring are the most serious consequences of acute pyelonephritis. The molecular basis of host susceptibility has remained unclear until recently when the mIL-8Rh−/− mouse was shown to develop acute pyelonephritis and renal scarring [12]. The present study was undertaken to establish the natural disease history in mice, including acute morbidity and chronic tissue damage. The mice were shown to develop acute, lethal disease, and more than 50% succumbed to systemic infection within 2 days. The lethal infections were accompanied by bacteremia,
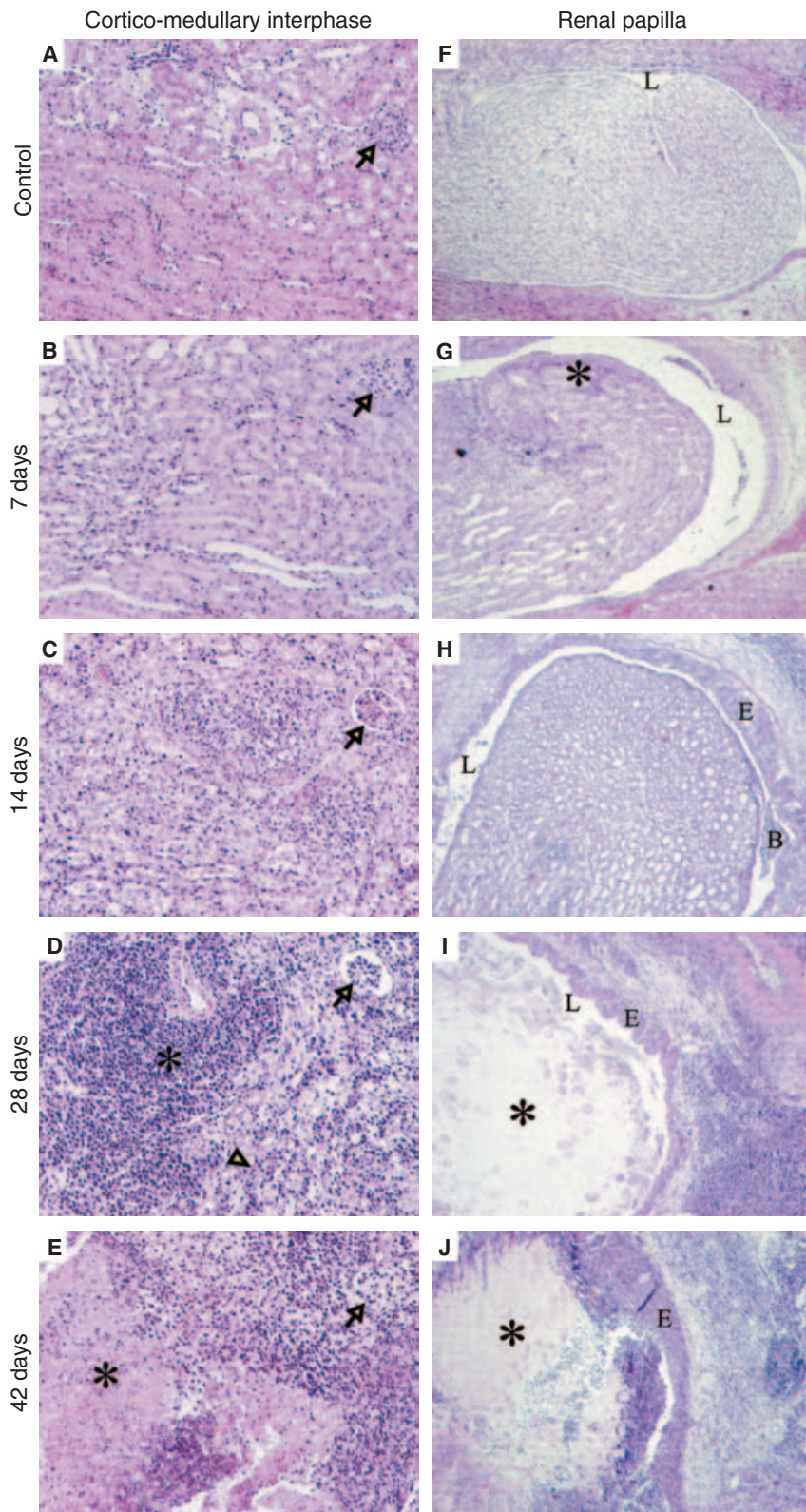
Cortico-medullary interphase

Renal papilla

Fig. 4. Development of tissue damage and papillary necrosis. Representative pictures of the border between medulla and cortex (*A* to *E*) and the renal papilla (*F* to *J*). Arrows indicate glomeruli in each section. (A and F) are uninfected control kidneys (B) 7 days postinfection the structure is normal. (C) Fourteen days postinfection there neutrophil accumulation and some abscesses (compare Fig. 2C). (D) Twenty-eight days postinfection the inflammatory cell infiltrate (∗) is abundant and the tubular structure is almost completely destroyed. Also some Russel bodies can be seen (arrowhead) and the glomerulus is shrunken (arrow). (E) Forty-two days postinfection the tissue is destroyed and replaced by abscesses and inflammatory cells. (F) Noninfected papilla of a mIL-8Rh−/− mouse kidney. (G) Neutrophil infiltration (∗) 7 days postinfection. (H) Thickening of the pelvic epithelium and bacterial mass in the lumen 14 days postinfection. (I) The papilla replaced with necrotic abscess (∗) 28 days postinfection. (J) The structure of the entire kidney is destroyed and the papilla is necrotic (∗). L is lumen, E is epithelium, B is bacterial mass. Hematoxylin-eosin staining [original magnification ×100 (A to E) and ×40 (F to J)].

suggesting that the mice developed urosepsis. Surviving animals developed chronic infection and progressively destructive inflammation of the kidneys. A pathologic accumulation of neutrophils in renal tissue was the major cause of the tissue damage and recruited lymphocytes and macrophages were unable to cope with the massive neutrophil infiltrate. The mIL-8Rh−/− mice offer the first animal model of renal scarring in a susceptible host.

The mIL-8Rh model demonstrates that impaired host susceptibility is sufficient to develop renal scarring. The renal scarring process has previously been studied in a number of animal models but most species are resistant to UTI and it has been necessary to use surgical manipulations or other invasive procedures that lower the susceptibility of the urinary tract. For example, vesicoureteric reflux or obstructions have been created by surgical means [18, 19]. Other approaches include direct injection of bacteria into renal tissue, thus combining infection with trauma [24]. In the primate model, obstruction was used to create a more chronic infection, and the use of anti-inflammatory agents and oxygen radical scavengers was shown to improve the fate of renal tissues [25]. Anatomic malformations are not a prerequisite for renal scarring and thus neither of these models resembles the human situation. Renal scarring developed in the mIL-8Rh−/− mouse following mucosal, noninvasive infection and without obstruction or prior tissue damage. Reflux may play a facilitating role to establish kidney infection as mice are a naturally refluxing species, but mice do not have severe obstructive forms of reflux [26]. The importance of the host defense defect is also illustrated by comparing the Balb/c and the mIL-8Rh−/− mice, which have the same degree of reflux but a completely different disease history.

Surviving mIL-8Rh−/− mice remained persistently infected and bacteria were visible in tissue sections from different parts of the kidneys. During the first week, the mass of bacteria was in the lumen of the renal pelvis and in microabscesses along the mucosa. Subsequently, bacteria were seen in the renal papilla, and especially the collecting ducts. This is surprising, as the environment of the papilla should be hostile due to the high osmolarity of the urine, but uropathogens have been shown to express osmoprotective proteins [27]. This localization confirms that reflux is not essential, as bacteria that enter the renal tissue with the help of reflux are expected to break the mucosal fold at the far end of the papilla and cause polar disease [28]. The infection the renal papilla provides a direct mechanism of injury to the machinery that is responsible for the concentration of urine and may explain how damage to the renal concentrating capacity can occur during acute pyelonephritis [29].

The pathologic neutrophil accumulation was the key feature of the scarring process. In resistant mice, the neutrophils migrated to the pelvic epithelium and they crossed into the lumen. In the mIL-8Rh−/− mice, the exit of neutrophils was prevented, however. Initially, the mice did not have pyuria, but later the infection damages the mucosal barrier and both bacteria and neutrophil aggregates are found in the lumen. It is perplexing that the massive neutrophil infiltrate does not remove the bacteria, but neutrophils of the mIL-8Rh−/− mice probably have an activation deficiency. The initial failure to clear the infection may depend on neutrophils not reaching bacteria adjacent to the mucosa, but subsequently, the phagocytosis defect may explain bacterial persistence [30].

Aging neutrophils are meant to undergo apoptosis, and recruited macrophages should scavenge the apoptotic cells. In the present study, macrophages were seen surrounding the neutrophil aggregates, but mostly around the microabscesses, apparently unable to deal with the massive number of dying or dead cells in the interior of the abscess. The neutrophils outnumbered the macrophages and efficient elimination by the macrophages would therefore be difficult based purely on stoichiometry. The macrophages developed into foam cells, which represent the frustrated end stage of cells that have saturated their phagocytic capacity. The lymphocytes appeared much later than the neutrophils and were outnumbered. There was no evidence that the recruitment of macrophages, lymphocytes, or plasma cells succeeded to scavenge the dying cells, or to prevent tissue damage. The inefficiency of specific immunity is consistent with earlier data from immunodeficient mice but the specificity of the immune response was not investigated here [31, 32]. We propose that the dying neutrophils and the frustrated tissue response causes the progressive tissue pathology.

The mIL-8Rh−/− mice provide a relevant model of human disease. Epidemiologic studies have shown that renal scarring develops in a subgroup of the children who suffer from acute pyelonephritis but there has been no explanation of their susceptibility and no diagnostic or prognostic tools have been available. The mIL-8Rh deficiency has a human counterpart as pyelonephritis-prone children have reduced CXCR1 expression and new *CXCR1* single nucleotide polymorphisms in were detected [12] [Lundstedt et al (manuscript in preparation)]. In addition, the renal pathology in the mIL-8Rh−/− mice shows many features that resemble renal scarring in humans. We conclude that deficient IL-8R expression is a risk factor for the development of acute pyelonephritis and renal scarring and that this host factor should be further explored in the risk assessment of children with UTI.

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