CARDIOVASCULAR, PULMONARY, AND RENAL PATHOLOGY

Lack of P2X₇ Receptors Protects against Renal Fibrosis after Pyelonephritis with α -Hemolysin—Producing *Escherichia coli*

Jacob R. Therkildsen,* Mette G. Christensen,* Stine J. Tingskov,[†] Julia Wehmöller,* Rikke Nørregaard,[†] and Helle A. Praetorius*

From the Departments of Biomedicine* and Clinical Medicine,[†] Aarhus University, Aarhus, Denmark

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Address correspondence to
Helle A. Praetorius, M.D.,
Ph.D., Department of Biomedicine,
Aarhus University, Ole
Worms Alle 4, Build 1160,
8000 Aarhus C, Denmark. E-
mail: hp@biomed.au.dk.

Severe urinary tract infections are commonly caused by sub-strains of *Escherichia coli* secreting the pore-forming virulence factor α -hemolysin (HlyA). Repeated or severe cases of pyelonephritis can cause renal scarring that subsequently can lead to progressive failure. We have previously demonstrated that HlyA releases cellular ATP directly through its membrane pore and that acute HlyA-induced cell damage is completely prevented by blocking ATP signaling. Local ATP signaling and P2X₇ receptor activation play a key role in the development of tissue fibrosis. This study investigated the effect of P2X₇ receptors on infection-induced renal scarring in a murine model of pyelonephritis. Pyelonephritis was induced by injecting 100 million HlyA-producing, uropathogenic *E. coli* into the urinary bladder of BALB/cJ mice. A similar degree of pyelonephritis and mortality was confirmed at day 5 after infection in P2X₇^{+/+} and P2X₇^{-/-} mice. Fibrosis was first observed 2 weeks after infection, and the data clearly demonstrated that P2X₇^{-/-} mice and mice exposed to the P2X₇ antagonist, brilliant blue G, show markedly less renal fibrosis 14 days after infection compared with controls ($P < 0.001$). Immunohistochemistry revealed comparable early neutrophil infiltration in the renal cortex from P2X₇^{+/+} and P2X₇^{-/-} mice. Interestingly, lack of P2X₇ receptors resulted in diminished macrophage infiltration and reduced neutrophil clearance in the cortex of P2X₇^{-/-} mice. Hence, this study suggests the P2X₇ receptor to be an appealing antifibrotic target after renal infections. (*Am J Pathol* 2019, 189: 1201–1211; <https://doi.org/10.1016/j.ajpath.2019.02.013>)

The Gram-negative *Escherichia coli* is usually a commensal component of the normal intestinal flora. However, *E. coli* is also responsible for a large number of extraintestinal infections¹ and is particularly prone to induce urinary tract infections (UTIs)² that may ascend to the kidney and cause pyelonephritis.³ The *E. coli* subtypes that cause severe UTI vary serotypically from the *E. coli* found in the normal intestinal flora^{4–6} and regularly produce a wide fan of virulence factors.^{4–8} Of the various virulence factors associated with UTI, α -hemolysin^{9,10} (HlyA) is the most abundant and found in approximately 50% of clinical isolates of *E. coli*.¹ Patients experiencing recurring pyelonephritis as a result of malformation in the urinary system are prone to develop renal fibrosis, which may result in renal failure.¹¹ Unfortunately, sufficient antibiotic treatment of UTIs after the onset of symptoms (within 24 hours) does not prevent

this renal scarring.¹² Therefore, it is paramount to investigate the therapeutic potential for signal transduction pathways that interfere with infection-induced renal fibrosis.

It is interesting that the virulence factor HlyA is intimately associated with ATP release and purinergic signaling. HlyA is known to cause lysis, primarily of erythrocytes, by inserting as a pore into the plasma membrane. ATP has been demonstrated to be released early after membrane insertion of HlyA,¹³ long before any cell lysis can be detected. Moreover, HlyA-induced lysis is amplified substantially by this ATP release and subsequent activation of the ligand-gated, nonselective cation channels P2X₁ and P2X₇.^{14,15} Inhibition of these channels

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prevents HlyA-induced cell damage in erythrocytes, and, interestingly, new data show that there is a clear association between high expression of P2X₁ receptors on erythrocytes and the decrease in hematocrit and hemoglobin observed in patients with acute sepsis.¹⁶

ATP is a well-documented pathogen- and damage-associated molecular pattern molecule with a primarily proinflammatory profile and, thus, is active in the early inflammation, which is a prerequisite for tissue fibrosis. ATP acts as a find me signal for neutrophil and macrophage recruitment to the wound/infection site¹⁷ and directly stimulates IL-13 and transforming growth factor- β release from lymphocytes and macrophages, which further activates local fibroblasts.^{17,18} The activated fibroblasts subsequently form the collagen necessary to support wound healing, continuously assisted by M2 macrophages and IL-13/IL-4 release.^{17,19} Moreover, IL-1 β plays an important profibrotic role during inflammation.²⁰ Many pathogen- and damage-associated molecular patterns stimulate macrophages to synthesize pro-IL-1 β ,²¹ which is cleaved and activated by caspase 1,²² mediated via the Nod-like receptor protein 3 inflammasome.²³ IL-1 β has a variety of effects, including contribution to epithelial-to-mesenchymal transition.^{24,25} The P2X₇ receptor has been established to play a crucial role in activation of the inflammasome and, thus, IL-1 β activation and secretion²⁶ and has been strongly implicated in tissue fibrosis.^{27–33}

We hypothesized that P2X₇ receptor activation may be a potential target for reducing renal fibrosis in response to severe urinary tract infections. Confirmed pyelonephritis with uropathogenic *E. coli* for at least 5 days resulted in moderate renal fibrosis in a murine model. Mice that lacked the P2X₇ receptor or were continuously treated with a P2X₇ receptor antagonist showed a markedly lower degree of fibrosis. This effect is likely to result from reduced macrophage infiltration in the P2X₇-deficient kidney and, thus, secondary fibrosis.

Materials and Methods

Animals

P2X₇^{−/−} mice back bred over 15 generations into a BALB/cJ background were kept and bred at the Institute of Biomedicine, Aarhus University (Aarhus, Denmark). The P2X₇^{−/−} mice were matched with either P2X₇^{+/+} littermates from heterozygous breeding or age-matched BALB/cJ mice from Janvier Labs (Saint-Berthevin, France). The P2X₇^{−/−} mice were originally developed by GlaxoSmithKline (London, UK). Animal experiments with the P2X₇ antagonist, brilliant blue G (BBG), were performed on BALB/cJ mice from Janvier Labs. All animal experiments were conducted in line with the Federation of European Laboratory Animal Science Associations guidelines and the Danish national legislations. Permission to conduct these experiments was obtained from the Danish Animal Experiments Inspectorate (Dyreforsøgstilsynet, Glostrup, Denmark; 2014-15-0201-00316).

Murine Pyelonephritis Model

Female P2X₇^{+/+} and P2X₇^{−/−} mice or female BALB/cJ mice were injected with 50 mg/kg per day of saline (for up to 14 days). The mice were anesthetized by s.c. injection of ketamine (0.1 mg/g body weight) and xylazine (0.01 mg/g body weight). BBG was chosen as an antagonist because of its blue color, which allows us to detect the plasma value of the compound, as previously described.³⁴ The abdomen was shaved and the mice were placed on their backs on a heating plate (38°C), resulting in a rectal temperature of approximately 36.5°C. After disinfection with iodine, a small sagittal incision was placed in the skin above the urinary bladder. The urinary bladder was carefully located, punctured, and emptied of its content (urine was kept for culture). Bacteria (10⁸) diluted in sterile 0.9% NaCl (20 μ L) were injected into the urinary bladder. Both the peritoneum and skin were sutured with Ethilon polyamide 6.0 (Abena, Aabenraa, Denmark), and the mice were administered buprenorphine hydrochloride [Temgesic (Indivior, Berkshire, UK); 0.1 μ g/g body weight] subcutaneously. By this procedure, the mice developed fulminant pyelonephritis after 24 hours, as determined in a separate concentration-response experiment (24 hours) and culture of homogenized kidneys. Pyelonephritis was confirmed on day 5. The infection was quantified by scoring blood plates streaked with equal samples of homogenized renal tissue, as follows: 0, no bacterial growth; 1, <5 *E. coli* colonies per agar plate; and 2, $\geq$ 5 *E. coli* colonies per agar plate or a noncountable number of colonies covering >10% of the plate. The infection status for experiments lasting for 14 days was assessed by spot urine sample at day 5 after surgery.

Under anesthesia, the abdomen was opened and the left kidney was ligated and removed for mincing and culture. After this procedure, a blood sample was collected with a heparinized syringe from the inferior vena cava, immediately centrifuged at 2938 $\times$ g for 10 minutes, and divided into plasma and erythrocyte fractions. Plasma (50 μ L) was stored at −80°C for cytokine measurements. Finally, the animals were perfusion fixed with 4% paraformaldehyde through the left ventricle of the heart at 0.2 bar.

Bacteria

The hemolytic *E. coli* strain ARD6 (O6:K13:H1), obtained from Statens Serum Institute (Copenhagen, Denmark), was grown on agar plates containing lysogeny broth and kept for up to 1 month at 4°C. A fresh preparation of *E. coli* was cultured overnight from one colony in 4 mL lysogeny broth medium (37°C, 250 rpm) for every experiment. The overnight culture was centrifuged and resuspended in sterile saline twice. The preparation contained approximately 10% dead cells, assessed by a cell viability kit (BD Biosciences, Kongens Lyngby, Denmark) and flow cytometry (Accuri C6; BD Biosciences).

Cytokine Measurements

Cytokine levels (IL-1 β , IL-6, keratinocyte chemoattractant, and tumor necrosis factor- α) in urine and plasma samples were measured (CBA Flex Set; BD Biosciences) by flow cytometry (Accuri C6), following the manufacturer's manual (BD Cytometric Bead Array, Mouse/Rat Soluble Protein Master Buffer Kit; BD Biosciences).

Measurement of Tissue Fibrosis

Fibrosis was quantified by identifying the number of fibrotic areas in picrosirius red (Sigma-Aldrich, Copenhagen, Denmark) stained kidney tissue (divided by area) by light microscopy (blinded counting, J.R.T.). To ensure that the fibrotic areas, in fact, were collagen and not artifacts, the identified areas were checked using plane-polarizing filters.

Semiquantitative Immunoblotting

Renal cortex was isolated and homogenized (240 seconds, 50 Hz in TissueLyser LT; Qiagen, Hilden, Germany) in a dissecting buffer (0.3 mol/L sucrose, 25 mmol/L imidazole, and 1 mmol/L EDTA, pH 7.2) containing the following: phosphatase inhibitor cocktails 2 and 3 (Sigma-Aldrich, St. Louis, MO) and complete miniprotease inhibitor cocktail tablets (serine, cysteine, and metalloprotease inhibitor; Roche, Basel Switzerland). The homogenized tissue was centrifuged at $4500 \times g$ at 4°C for 10 minutes. The supernatant was added Laemmli buffer containing 2% SDS, and the protein content was measured by a Pierce BCA protein assay kit (Roche, Basel, Switzerland). The protein samples were size separated on a 12% Criterion TGX Precast Gel (Bio-Rad Laboratories, Copenhagen, Denmark) and electroblotted onto a nitrocellulose membrane (Hybond ECL; GE Healthcare, Hatfield, UK). The blots were blocked with 5% nonfat dry milk in phosphate-buffered saline (PBS) with Tween 20 (80 mmol/L Na₂HPO₄, 20 mmol/L NaH₂PO₄, 100 mmol/L NaCl, and 10% Tween 20, pH 7.4). After washing with PBS with Tween 20, the blots were incubated overnight at 4°C with primary antibodies against fibronectin (Ab2413; Abcam, Cambridge, UK), kidney injury molecule-1 (AF1817; R&D Systems, Minneapolis, MN) or α -smooth muscle actin (M0851; Dako A/S, Glostrup, Denmark). The antigen-antibody complex was visualized with horseradish peroxidase-conjugated secondary antibodies for 1 hour at room temperature (P447, P448, and P449; diluted 1:4000; Dako A/S) using an enhanced chemiluminescence system (Amersham ECL Plus, Little Chalfort, UK). All Western blot analyses were normalized to total protein, as measured using Stain-Free technology (Bio-Rad Laboratories).³⁵

Histology, Immunohistochemistry, and Microscopy

The organs were dehydrated after 1 hour in 4% paraformaldehyde by replacing the liquid every 2 hours with 70%

ethanol solution, followed by 96% ethanol and 99% ethanol and finally by xylene. Then, the organs were embedded in paraffin, cut into sections (2 or 5 μ m thick) with a Leica 2165 rotating microtome (Leica, Wetzlar, Germany), and fixed on objective glass (Thermo Fisher Scientific, Waltham, MA). For quantification of fibrosis, sections (5 μ m thick) were dewaxed in xylene and rehydrated again in graded ethanol, stained, and inspected by light microscopy ($\times 10$ magnification; Leica DMRE; Leica) equipped with a Leica DM300 digital camera.

For immunohistochemistry, tissue sections (2 μ m thick) were dewaxed in xylene, rehydrated again in graded ethanol, and stained, followed by heat-induced epitope retrieval. The sections were boiled in a TEG buffer for 10 minutes in a microwave, followed by cooling on ice. Reactive aldehyde residues, produced during the antigen retrieval, were then blocked by incubation in PBS with addition of 50 mmol/L NH₄Cl for 30 minutes. Unspecific antibody binding was blocked by incubation in PBS with 1% bovine serum albumin (BSA), 0.2% gelatin, and 0.05% saporin (10 minutes). The sections were labeled with either the primary antibody, NIMP-R14 (ab2557; diluted 1:500; Abcam), or anti-CD68 (ab125212; diluted 1:900; Abcam), diluted in PBS with 0.1% BSA and 0.3% Triton X-100 for 1 hour at 21°C, followed by 4°C overnight. The tissue was washed with PBS with 1% BSA, 0.2% gelatin, and 0.05% saporin (10 minutes). For immunofluorescence, Alexa 488-conjugated, goat anti-rat IgG (Invitrogen, Carlsbad, CA) was used as secondary antibody, diluted 1:1000 in PBS with 0.1% BSA and 0.3% Triton X-100 at room temperature. Coverslips were mounted with a hydrophilic mounting medium containing antifading reagent (Glycergel; Dako A/S). The sections were imaged on a Nikon TE-2000 microscope (Nikon, Tokyo, Japan) equipped with monochromator and super-video graphics array charge-coupled device (Visitech International, Sunderland, UK) with a plan fluo20/numerical aperture 0.45 objective. For peroxidation staining on hematoxylin and eosin, sections were incubated for 1 hour with horseradish peroxidase-conjugated secondary antibody in PBS with 0.1% BSA and 0.3% Triton X-100, washed, and visualized with diaminobenzidine in 35% H₂O₂ for 10 minutes. The sections were counterstained with Mayer's hematoxylin and rinsed in running tap water before mounting with coverslips using Eukitt (CellPath, Newtown, UK). The sections were inspected by light microscopy (Leica DMRE), equipped with a Leica DM300 digital camera.

Statistical Analysis

Data are presented as means $\pm$ SEM. Data were tested for normal distribution by Kolmogorov-Smirnov test. When normally distributed, the data were tested by two-way analysis of variance with multiple comparison design or *t*-test, in the case in which only two groups were compared. In the cases in which the results were not normally distributed, the data were tested by Kruskal-Wallis test, followed by Dunn's post hoc test. Kaplan-Meier plots were used to assess

Figure 1 Murine pyelonephritis model. **A:** The figure shows bacterial growth on blood agar plates of homogenized kidney from P2X₇^{-/-} and P2X₇^{+/+} mice exposed to α -hemolysin-producing *Escherichia coli*. **B:** The quantification of the bacterial growth from kidney homogenates. The ordinate shows the number of blood plates in percentage. The bars illustrate the counts for three groups: plates with 0 colonies, plates with 1 to 5 colonies, and plates with >5 colonies for both P2X₇^{+/+} and P2X₇^{-/-} mice.

mortality rates, and the data in Figure 1B are tested by a χ^2 test. $P < 0.05$ was considered statistically significant. All statistical analyses were performed using GraphPad Prism version 6 (GraphPad Software, San Diego, CA).

Results

Renal Infection and Cytokine Levels 5 Days after Infection

The murine pyelonephritis model was established to provide fulminant infection of the renal parenchyma after 24 hours

of infection. Mice with spot urine positive for *E. coli* after 24 hours of inoculation with HlyA-producing *E. coli* in the urinary bladder were followed up for either 5 or 14 days. Pyelonephritis on day 5 after infection was confirmed as growth of HlyA-producing *E. coli* after seeding homogenized kidney on a blood plate. The degree of pyelonephritis was similar in P2X₇^{+/+} and P2X₇^{-/-} mice (Figure 1). Figure 1A shows representative blood plates of homogenized renal tissue. The degree of infection was similar in P2X₇^{+/+} ($n = 17$) and P2X₇^{-/-} ($n = 14$) mice ($P = 0.7575$) (Figure 1B). Mice contaminated with bacteria other than HlyA-producing *E. coli* were excluded from the study (four mice), as were mice that had cleared their UTI during the first 5 days of observation (10 P2X₇^{+/+} and 17 P2X₇^{-/-} mice). Figure 2 illustrates the survival of the included experimental groups, for a total of 131 mice. Less than 20% of the mice died during the 5 days of observation, and the few occurrences happened early and were likely associated with the surgical procedure. The mortality was similar in P2X₇^{+/+} and P2X₇^{-/-} mice ($P = 0.1627$).

The P2X₇ receptor is strongly associated with activation and secretion of IL-1 β ,²⁶ and one would expect P2X₇ receptor-deficient mice to exhibit lower levels of proinflammatory cytokines. This is certainly not the case in a murine model of urosepsis. On the contrary, the P2X₇^{-/-} mice show a massive cytokine storm and, indeed, exceedingly high IL-1 β compared with control mice.³⁴ This was likely the result of activation of the caspase 8 pathway during sepsis, induced by HlyA-producing *E. coli*.³⁴ Figure 3 shows the levels of proinflammatory cytokines in plasma and urine, collected 5 days after infection. In P2X₇^{+/+} mice, pyelonephritis caused a marked increase in IL-6 in both urine and plasma (Figure 3), whereas IL-1 β only was statistically significant in urine (Figure 3). Surprisingly, the surgery in itself was enough to trigger a substantial increase in both IL-1 β and IL-6 in the P2X₇^{-/-} mice. The surgery causes this difference

Figure 2 Survival: murine pyelonephritis model. The figure shows the Kaplan-Meier plot of the survival data collected from P2X₇^{-/-} and P2X₇^{+/+} mice exposed to sterile saline or α -hemolysin-producing *Escherichia coli*. No statistically significant difference is observed in survival in the four groups. $n = 22$ wild type-control (WT-CTRL); $n = 50$ WT-*E. coli*; $n = 20$ knockout (KO)-CTRL; $n = 39$ KO-*E. coli*.

Figure 3 Cytokine levels in plasma and urine 5 days after infection. Plasma levels of tumor necrosis factor (TNF)- α (A), IL-1 β (B), IL-6 (C), and keratinocyte chemoattractant (KC; D). Urine levels of TNF- α (E), IL-1 β (F), IL-6 (G), and KC (H). Data are expressed as means $\pm$ SEM (A–H). $n = 10$ wild type–control (WT-CTRL) (A–H); $n = 14$ WT–*Escherichia coli* (A–H); $n = 9$ knockout (KO)–CTRL (A–H); $n = 10$ KO–*E. coli* (A–H); $n = 6$ BGG–*E. coli* (A–H). * $P < 0.05$. BGG, brilliant blue G.

because P2X₇^{-/-} mice that have not been exposed to surgery have normal circulating proinflammatory cytokines.³⁴ However, the level of proinflammatory cytokines in the P2X₇^{+/+} and P2X₇^{-/-} mice exposed to HlyA-producing *E. coli* was not statistically different. This means that any difference there may be between the P2X₇^{+/+} and P2X₇^{-/-} mice was not explained by a defective IL-1 β release pathway in the P2X₇-deficient mice.

Pyelonephritis-Induced Renal Fibrosis

The degree of renal fibrosis was evaluated by picrosirius red staining of perfusion-fixed kidneys on days 5 and 14 after infection. None of the kidneys showed any defined fibrotic areas after 5 days of infection (Supplemental Figure S1). The kidneys from both P2X₇^{+/+} and P2X₇^{-/-} mice, infected with HlyA-producing *E. coli*, stained more pronounced with picrosirius red than when injected with saline only. More important, these apparent differences in staining intensity could not be quantified with regular light microscopy. However, an increased number of nucleated cells were observed around the collecting duct in the renal medulla of the mice with pyelonephritis, consistent with infiltration by immune cells. This was observed to a similar extent in wild-type and P2X₇^{-/-} mice.

Development of fibrosis was observed 14 days after the initial infection (Figure 4). Mice exposed to pyelonephritis with HlyA-producing *E. coli* exhibited areas of intensive red staining, with well-defined collagen fibers in the cortex (Figure 4), whereas the medulla did not show any sign of

fibrosis (Figure 4). To determine whether the degree of fibrosis varied between the groups, the sections were blinded (J.R.T.) and four sections from each kidney were analyzed from seven different animals for each group (Figure 5). The quantification clearly revealed that wild-type mice subjected to HlyA-producing *E. coli* had a markedly higher number of fibrotic regions in the renal cortex (1.153 ± 0.076 areas/mm²) compared with vehicle-treated mice (0.071 ± 0.031 areas/mm²; $P < 0.0001$). Strikingly, the renal infection produced much less fibrosis in P2X₇^{-/-} mice after pyelonephritis with HlyA-producing *E. coli* (0.296 ± 0.036 areas/mm²; $P < 0.0001$). Despite the marked reduction of fibrotic areas in the P2X₇^{-/-} mice subjected to *E. coli*, the degree of fibrosis was still slightly higher than in wild-type mice exposed to saline only ($P < 0.05$). Similarly, daily injections with BGG reduced the development of fibrosis in mice exposed to HlyA-producing *E. coli* (0.381 ± 0.0353 areas/mm²; $P < 0.001$), although the degree of fibrosis was higher than in wild-type mice treated with BGG alone (0.095 ± 0.044 areas/mm²; $P < 0.01$). Thus, the data show that the lack of P2X₇ receptors or blocking activation of the receptors markedly reduced fibrotic development in response to renal infection with uropathogenic *E. coli*.

Neutrophil Granulocyte Migration in Response to Pyelonephritis

Neutrophil granulocytes are essential for the acute inflammatory response to infection. Therefore, it was tested whether

Figure 4 Sporadic cortical fibrosis in response to a single infection with α -hemolysin (HlyA)–producing *Escherichia coli* in mice. **A–D**: Picrosirius red staining of renal cortex from P2X₇^{+/+} mice exposed to sterile saline (**A**), P2X₇^{+/+} mice exposed to HlyA-producing *E. coli* (**B**), P2X₇^{−/−} mice exposed to HlyA-producing *E. coli* (**C**), and P2X₇^{+/+} mice exposed to HlyA-producing *E. coli* and the P2X₇-antagonist brilliant blue G (BBG) (**D**). **E–H**: Picrosirius red staining of renal medulla from P2X₇^{+/+} mice exposed to sterile saline (**E**), P2X₇^{+/+} mice exposed to HlyA-producing *E. coli* (**F**), P2X₇^{−/−} mice exposed to HlyA-producing *E. coli* (**G**), and P2X₇^{+/+} mice exposed to HlyA-producing *E. coli* and the P2X₇-antagonist BBG (**H**). Scale bars = 200 μ m (**A–H**). Original magnification, $\times 2.4$ (**B**, right panel).

there was any discrepancy in the neutrophil infiltration between P2X₇^{+/+} and P2X₇^{−/−} mice. **Figure 6**, A and C, shows infiltration of neutrophil granulocytes in the cortex of wild-type mice injected with HlyA-producing *E. coli* (2.96 ± 0.13 cells/mm²) compared with wild-type controls (2.06 ± 0.26 cells/mm²) 5 days after surgery ($P < 0.05$). A similar pattern was observed in P2X₇^{−/−} mice, in which HlyA-producing *E. coli* resulted in 2.52 ± 0.11 neutrophils/mm², which was statistically significantly higher than in P2X₇^{−/−} mice injected with saline (1.69 ± 0.15 cells/mm²; $P < 0.05$). Interestingly, there was no observed difference ($P > 0.05$) between wild-type mice injected with saline (2.61 ± 0.22 cells/mm²) and those injected with HlyA-producing *E. coli* (2.73 ± 0.24 cells/mm²) after 14 days of infection (**Figure 6**, B and D). However, P2X₇^{−/−} mice exposed to HlyA-producing *E. coli* still showed an elevated number of infiltrating neutrophils 14 days after infection (3.96 ± 0.20 cells/mm²; $P < 0.05$) compared with P2X₇^{+/+} mice exposed to HlyA-producing *E. coli*. This is consistent with a slower clearance of neutrophils from the cortex in the P2X₇^{−/−} mice in connection with pyelonephritis. A similar pattern of reduced clearance of neutrophils was observed in BBG-treated mice subjected to either saline (2.17 ± 0.19 cells/mm²) or HlyA-producing *E. coli* (4.20 ± 0.18 cells/mm²; $P < 0.01$). Notably, there was not an observed increase in neutrophil infiltration in the medulla as a result of the induced infection.

Macrophage Infiltration in Response to Pyelonephritis

Normally, neutrophils are cleared by macrophages at later stages of inflammation. Therefore, macrophage infiltration was tested in the kidney in response to pyelonephritis (**Figure 7**). **Figure 7** shows a substantial increase in infiltrating

macrophages in the renal cortex of P2X₇^{+/+} mice exposed to HlyA-producing *E. coli* for 5 days (0.94 ± 0.26 cells/mm²) compared with controls (0.23 ± 0.10 cells/mm²; $P < 0.05$). No infiltration of macrophages was observed in the renal medulla in connection with the infection. Exposing P2X₇^{−/−} mice to HlyA-producing *E. coli* for 5 days did not increase the macrophage infiltration in the cortex (0.27 ± 0.09 cells/mm²) or the medulla compared with P2X₇^{−/−} mice injected with saline (0.23 ± 0.07 cells/mm²). The macrophage infiltration was even more pronounced 14 days after infection (**Figure 7**), when wild-type mice exposed to HlyA-producing *E. coli* had 2.829 ± 0.8074 macrophages/mm², which was statistically significantly higher than mice exposed to saline (0.29 ± 0.06 cells/mm²; $P < 0.001$). Interestingly, the P2X₇^{−/−} group injected with *E. coli* (0.40 ± 0.13 cells/mm²) did not show any statistically significant increase in the number of macrophages compared with the wild-type controls (0.29 ± 0.06 cells/mm²), consistent with a lag of macrophage migration to the cortex. Similarly, mice treated with BBG showed the same degree of macrophage infiltration regardless of whether they were injected with *E. coli* (0.44 ± 0.09 cells/mm²) or not (0.19 ± 0.08 cells/mm²). Taken together, these data demonstrate a striking reduction of macrophage infiltration in response to infection if the P2X₇ receptor is lacking or blocked.

Discussion

Urinary tract infections are exceedingly common and affect up to one in three women younger than 24 years.³⁶ Uncomplicated urinary tract infections rarely give any secondary problems. If, however, urinary tract infections are repeated or severe, there is a long-term increased risk of developing renal fibrosis and, potentially, reduced renal function, particularly in children.^{37,38} Consistent with this,

Figure 5 Quantification of cortical fibrosis in response to a single infection with α -hemolysin (HlyA)-producing *Escherichia coli* in mice. The figure shows the quantification of fibrotic areas by picrosirius red staining of renal cortex from P2X₇^{+/+} and P2X₇^{-/-} mice exposed to sterile saline or HlyA-producing *E. coli* as well as P2X₇^{+/+} mice treated with the P2X₇ receptor inhibitor brilliant blue G (BBG) exposed to sterile saline or HlyA-producing *E. coli*. The ordinate shows the number of fibrotic areas. $n = 10$ wild type—control (WT-CTRL); $n = 14$ WT-*E. coli*; $n = 9$ knockout (KO)—CTRL; $n = 10$ KO-*E. coli*; $n = 6$ BBG-*E. coli*. ** $P < 0.01$, **** $P < 0.0001$.

the murine pyelonephritis model, in which HlyA-producing *E. coli* are inoculated in the urinary bladder of mice, does not cause massive, global renal fibrosis. In this model, the mice developed pyelonephritis rapidly, 24 hours after the inoculation, which lasted at least up to day 5. This condition was generally well tolerated by the mice, which spontaneously cleared their urinary tract infection within 14 days of observation. During this period, mice exposed to HlyA-producing *E. coli* selectively developed sporadic cortical fibrosis with local assembly of collagen fibers arranged in parallel, primarily in association with proximal tubule segments. This was not seen in the vehicle control mice. Although the picrosirius red staining was consistently more marked in mice exposed to bacteria at day 5 after inoculation, no structural changes or fibrotic areas were observed at that point. The fibrosis likely developed later, between day 5 and day 14 after inoculation.

Strikingly, P2X₇^{-/-} mice exposed to HlyA-producing *E. coli* showed little signs of cortical fibrosis at day 14 compared with wild-type mice. Notably, the P2X₇^{+/+} and P2X₇^{-/-} mice showed the same degree of pyelonephritis, estimated from the number of colony-forming units on blood plates after seeding homogenized kidneys from either of the genotypes. The finding that lack of functional P2X₇ receptors resulted in less fibrosis in response to the same degree of infection was confirmed using the P2X₇ receptor antagonist, BBG, which also markedly reduced the number of fibrotic regions in the cortex. These data were substantiated by immunoblot analysis of the matrix protein fibronectin, polymerization of which is implicated as essential in fibrosis. A marked up-regulation of fibronectin was observed in the renal cortex in response to pyelonephritis in wild-type mice ($P < 0.05$) (Supplemental Figure S2B).

Apparently, the baseline fibronectin levels are higher in the P2X₇^{-/-} mice compared with the P2X₇^{+/+} mice ($P < 0.001$), but the fibronectin level decreased drastically in P2X₇^{-/-} mice on infection ($P < 0.01$). This may easily reflect the reduced fibrosis observed in these mice, particularly considering that inhibition of fibronectin has recently been demonstrated to reduce fibrosis and improve cardiac output in a model of cardiac fibrosis.³⁹ A statistically significant increase was not observed in cortical expression of kidney injury molecule-1 in the mouse kidney in response to infection, consistent with pyelonephritis with HlyA-producing bacteria not causing massive renal damage. As a curiosity, it can be mentioned that the kidney injury molecule-1 expression is higher in the P2X₇^{-/-} mice ($P < 0.0001$) and that this is reduced under infection ($P < 0.0001$) (Supplemental Figure S2C). Similarly, no support was found for increased transdifferentiation of fibroblasts into myofibroblasts in the cortex using the protein levels of α -smooth muscle actin as an indicator (Supplemental Figure S2A), which may reflect the transient nature of transdifferentiation. The finding that lack of P2X₇ receptors resulted in less tissue fibrosis is in alignment with previous studies that have pointed out a marked role of the P2X₇ receptor in tissue fibrosis,^{29,31,33} even in the kidney.⁴⁰ However, it is exceedingly interesting that lack of P2X₇ receptor function reduced infection-induced fibrosis because it occurs episodically and, thus, only requires an antifibrotic treatment of a short interval during infection.

If P2X₇ receptor antagonists are to be used as antifibrotic treatment, it is paramount that they do not interfere with the outcome or potentiate the infection. Thus, P2X₇^{+/+} and P2X₇^{-/-} mice showed the same degree of pyelonephritis on inoculation with a fixed number of bacteria in the urinary bladder and low mortality in both groups. This means that there is no immediate potentiation of UTI in the absence of functional P2X₇ receptors. This is important because a previous study showed that P2X₇^{-/-} mice are exceedingly more sensitive to urosepsis with HlyA-producing *E. coli*.³⁴ It must be stressed that this results from an intrinsic immune deficiency in the P2X₇^{-/-} mouse that is not found in wild-type mice treated with P2X₇ receptor antagonists.³⁴ Moreover, the similar degree of pyelonephritis in the P2X₇^{+/+} and P2X₇^{-/-} mice allows us to conclude that the apparent difference in fibrosis lies in the host response to the infection.

The level of proinflammatory cytokines can be considered as a readout for the host response to a given infection. At day 5 after the infection, both plasma and urine levels of IL-1 β and IL-6 were elevated in P2X₇^{+/+} mice exposed to HlyA-producing *E. coli* compared with vehicle controls, whereas tumor necrosis factor- α and keratinocyte chemoattractant (the mouse ligand for the IL-8 receptor) are unchanged. Notably, the plasma levels of tumor necrosis factor- α , IL-1 β , IL-6, and keratinocyte chemoattractant were similar in P2X₇^{+/+} and P2X₇^{-/-} mice on day 5 after infection, suggesting that the difference in fibrosis cannot be explained by an impeded acute inflammation associated with cytokine release, at least not as measured in plasma and urine. It was surprising that the P2X₇^{-/-} mice that were only subjected to

Figure 6 Infection-induced cortical infiltration of neutrophil granulocytes. The figure shows representative images of anti-NIMP-R14 stained neutrophil granulocytes in renal cortex stained 5 days after infection from P2X₇^{+/+} exposed to vehicle, P2X₇^{+/+} exposed to α -hemolysin (HlyA)-producing *Escherichia coli*, and P2X₇^{-/-} exposed to HlyA-producing *E. coli* (A); the same groups are shown 14 days after infection (B). The blinded quantification of the data (J.R.T.) is shown for 5 days (C) and 14 days (D) after the infection in P2X₇^{+/+} and P2X₇^{-/-} mice exposed to sterile saline or HlyA-producing *E. coli*. $n = 6$ in all groups (A–D). * $P < 0.05$, ** $P < 0.01$. Scale bars = 50 μ m (A and B). BBG, brilliant blue G.

the surgical procedure and injection of control solution showed elevated urine values of IL-1 β , IL-6, and keratinocyte chemoattractant. The P2X₇^{-/-} mouse is remarkably immunologically responsive and shows massively elevated

levels of proinflammatory cytokines and activation of caspase 8 in response to *E. coli* infection compared with P2X₇^{+/+} mice.³⁴ The data presented in the current work show that the surgery in itself is sufficient to elevate the

Figure 7 Infection-induced cortical infiltration of macrophages. The figure shows representative images of anti-CD68-stained renal cortex stained from P2X₇^{+/+} exposed to vehicle (A), P2X₇^{+/+} exposed to α -hemolysin (HlyA)-producing *Escherichia coli* (B), and P2X₇^{-/-} exposed to HlyA-producing *E. coli* (C). The blinded quantitation (J.R.T.) of anti-CD68-positive cells is shown at 5 days (D) and 14 days (E) after the infection in P2X₇^{+/+} and P2X₇^{-/-} mice exposed to sterile saline or HlyA-producing *E. coli*. The arrows indicate stained macrophages. $n = 6$ in all groups (A–E). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. Scale bars = 50 μ m (A–C). BBG, brilliant blue G.

levels of proinflammatory cytokines in the urine of P2X₇^{-/-} mice. The P2X₇^{-/-} mouse used in this study was originally generated by GSK (Brentford, UK) on a C57/bl6 background and is not a full P2X₇ receptor knockout mouse. The mouse still retains the splice variation *p2x7k*, which is mainly found in the central nervous system, the lung, and the spleen.⁴¹ It should be emphasized that none of the available P2X₇ receptor-deficient mice are complete knockouts, but that the GSK P2X₇^{-/-} mice on a BALB/c background nevertheless have been demonstrated to have a marked phenotype (eg, in bone homeostasis).⁴² There is not any survey over the general expression of the GSK P2X₇^{-/-} mice on a BALB/cJ background; however, it was previously tested that the intracellular Ca²⁺ response to high concentrations of ATP is markedly reduced in peritoneal macrophages isolated from P2X₇^{-/-} mice on a BALB/cJ background compared with their P2X₇^{+/+} controls.⁴³ Thus, at least the macrophages lack functional P2X₇ receptors, which is in line with the lack of expression shown in osteoclasts from GSK P2X₇^{-/-} on a BALB/cJ background.⁴⁴ It cannot be excluded that a functional residual P2X₇ receptor function in these mice may explain elevated levels of proinflammatory cytokines in the P2X₇^{-/-} mice. Collectively, however, these data allow us to conclude that the reduced fibrosis observed in P2X₇^{-/-} mice does not result from a reduced release of proinflammatory cytokines.

Another possible explanation for the P2X₇-dependent discrepancy in renal fibrosis could be a difference in recruitment of immune cells in response to pyelonephritis. Neutrophil granulocytes are recruited early into the tissue after infection. The P2X₇ receptor has not, to our knowledge, been directly implicated in migration of neutrophils. However, one study suggests that P2X₇ receptor activation on resident macrophages could cause cellular release of macrophage inflammatory protein-2, which works as a chemoattractant for neutrophils.⁴⁵ Neutrophils were recruited to the kidney during pyelonephritis, although the response was similar in P2X₇^{+/+} and P2X₇^{-/-} mice at day 5 after infection. Interestingly, the neutrophils tended to linger in the cortex of mice with pyelonephritis if they lacked P2X₇ or were exposed to BBG. Recent data suggest that a reduced number of neutrophils are associated with increased fibrosis⁴⁶ and, thus, the prolonged increased number of neutrophils in P2X₇^{-/-} or BBG-treated mice may be implicated in the reduced fibrosis in these mice. This could, in principle, result from the neutrophil-mediated release of metalloprotease 8 to balance the lay down of collagen types 1 and 3 during the fibrotic process.

The pattern of neutrophil expression was mirrored by the tissue macrophages. Pyelonephritis with uropathogenic *E. coli* clearly increased the number of macrophages in the cortex. Lack of P2X₇ receptors or treatment with BBG completely prevented this accumulation. Macrophage accumulation in the renal medulla was slightly prolonged compared with neutrophils, which fits with a differentiation of macrophages from circulating monocytes.⁴⁷ These data clearly demonstrate that

infection-induced renal fibrosis can be damped by preventing the massive up-regulation of tissue macrophages in the inflamed tissue in a P2X₇ receptor-dependent manner. Normally, macrophages are essential for removing senescent or damaged neutrophils⁴⁸ from the tissue, preventing the release of potentially tissue-damaging proteases. Thus, one could speculate that the reduced numbers of macrophages prevent excessive renal fibrosis by tipping the balance between macrophage-induced stimulation of matrix generation and neutrophil-associated matrix degradation.

Clearly, these results show that lack of P2X₇ receptor function reduces the immediate fibrotic processes in the kidney that occur after infection, without having any major effect on the clearance of the bacterial infection. These data propose that P2X₇ receptor inhibition may potentially be used alongside antibiotics in severe urinary tract infections in the prevention of later progressive renal failure. However, it will be pressing to perform a proper interventional study of P2X₇ receptor antagonist(s) in the prevention of renal fibrosis after fulminant pyelonephritis has developed.

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Supplemental Data

Supplemental material for this article can be found at <http://doi.org/10.1016/j.ajpath.2019.02.013>.

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