

Delayed Chemokine Receptor 1 Blockade Prolongs Survival in Collagen 4A3-Deficient Mice with Alport Disease

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Human Alport disease is caused by a lack of the α 3-, 4-, or 5-chain of type IV collagen (COL4A). Affected humans and COL4A3-deficient mice develop glomerulosclerosis and progressive renal fibrosis in the presence of interstitial macrophages, but their contribution to disease progression is under debate. This question was addressed by treating COL4A3-deficient mice with BX471, an antagonist of chemokine receptor 1 (CCR1) that is known to block interstitial leukocyte recruitment. Treatment with BX471 from weeks 6 to 10 of life improved survival of COL4A3-deficient mice, associated with less interstitial macrophages, apoptotic tubular epithelial cells, tubular atrophy, interstitial fibrosis, and less globally sclerotic glomeruli. BX471 reduced total renal *Ccl5* mRNA expression by reducing the number of interstitial CCL5-positive cells in inflammatory cell infiltrates. Intravital microscopy of the cremaster muscle in male mice identified that BX471 or lack of CCR1 impaired leukocyte adhesion to activated vascular endothelium and transendothelial leukocyte migration, whereas leukocyte rolling and interstitial migration were not affected. Furthermore, in activated murine macrophages, BX471 completely blocked CCL3-induced CCL5 production. Thus, CCR1-mediated recruitment and local activation of macrophages contribute to disease progression in COL4A3-deficient mice. These data identify CCR1 as a potential therapeutic target for Alport disease or other progressive nephropathies associated with interstitial macrophage infiltrates.

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Current treatment strategies for progressive renal disease aim to control hemodynamic and metabolic factors of disease progression (1). However, progressive nephropathies are often characterized by mixed inflammatory cell infiltrates that contribute to disease progression by the secretion of proinflammatory and profibrotic mediators (2,3). Renal leukocyte recruitment is mediated by chemokines that are secreted by intrinsic renal cells and by infiltrating immune cells (4). Chemokines interact with their respective chemokine receptors (CCR) on intravascular leukocytes and—in concert with adhesion molecules—guide these cells through the epithelial interface into the interstitial compartment at sites of inflammation (5). Thus, specific CCR antagonists that can block leukocyte recruitment in renal injury to provide a tool to study the role of infiltrating leukocytes in kidney diseases (6), espe-

cially for those for which the contribution of leukocytic cell infiltrates for disease progression remains questionable.

Human Alport disease is caused by a genetic defect in the α 3-, 4-, or 5-chain of type IV collagen (COL4A), which affects the normal assembly of glomerular basement membranes (7). Alport disease is characterized by glomerulosclerosis and subsequent progressive tubulointerstitial injury, leading to ESRD (8). Disease progression is thought to be driven by maladaptive glomerular cell–matrix interactions and subsequent glomerulosclerotic lesions in humans and in COL4A3-deficient mice, a genetic model of human Alport disease (7,9). In human Alport disease and in COL4A3-deficient mice, disease progression is associated with considerable interstitial inflammatory monocytic cell infiltrates, but their functional role for disease progression remains unclear (8,10,11).

We studied the contribution of interstitial macrophages on disease progression of Alport disease by treating COL4A3-deficient mice with BX471, a small molecule CCR1 antagonist, that specifically blocks interstitial leukocyte recruitment in mice (12). We found that BX471 prolonged survival of COL4A3-deficient mice by reducing the amount of interstitial macrophages, renal CCL5 production, tubular atrophy, and intersti-

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tial fibrosis while preserving the peritubular microvasculature. In addition, we identified that the effects of BX471 were mediated by a specific blockade of CCR1-induced leukocyte adhesion and transmigration through activated endothelial cells *in vivo* and through blocking of CCR1-induced production of CCL5 in macrophages. Together, these data argue for a significant contribution of interstitial macrophages to the progression of renal failure in Alport disease.

Materials and Methods

Animal Studies

Mice deficient for the *Ccr1* gene were generated as described previously (13) and backcrossed into the BALB/cAnNCrI background for eight generations under specific-pathogen-free housing conditions. COL4A3-deficient mice were generated under specific-pathogen-free housing conditions as described (14). The genotype of each mouse was confirmed by PCR using genomic DNA extracted from tail tips. Both homozygous mouse strains were housed in filter top cages with a 12-h dark/light cycle and unlimited access to food and water for the duration of the study. Cages, bedding, nestlets, food, and water were sterilized by autoclaving before use. All experimental procedures had been approved by the local government authorities.

Intravital Microscopy

The surgical preparation of cremaster muscles was performed as described previously in detail (15). Throughout the procedure, the muscle was superfused with buffered Ringer's injection. Intravital microscopy was performed using an Olympus BX50 upright microscope (Olympus Microscopy, Hamburg, Germany) equipped for stroboscopic fluorescence. Epi-illumination microscopy and microscopic images as well as real-time recordings were obtained as described (15). Three hours after intrascrotal injection of CCL3 (600 ng in 0.3 ml of PBS; R&D Systems Europe Ltd, Wiesbaden, Germany), intravital microscopic analysis was performed in wild-type and CCR1-deficient mice as well as in wild-type mice that received a subcutaneous injection of the CCR1 antagonist BX471 (25 mg/kg body wt) 1 h before intrascrotal injection of CCL3. Wild-type mice that were treated with PBS were used as controls ($n = 7$ each group). Leukocyte migration parameters were determined in five postcapillary venules (inner diameter 17 to 35 μm) per animal. Rolling leukocytes were defined as those that moved slower than the associated blood flow and quantified for 30 s. Firmly adherent leukocytes were defined as leukocytes that remained stationary for at least 30 s and related to the luminal surface per 100- μm vessel length. Emigrated leukocytes were counted in regions of interest that covered a width of 75 μm on both sides of a vessel over 100- μm vessel length. For the analysis of interstitial migration of leukocytes, these regions of interest were divided into two subareas, respectively: One adjacent to the vessel (25 μm in length \times 100 μm in width) and one distant to the vessel (50 μm in width \times 100 μm in length. Blood flow velocity was measured after intra-arterial administration of fluorescence-labeled microspheres (0.96- μm FluoSpheres, Molecular Probes, Eugene, OR).

In Vitro Studies

The murine macrophage cell line J774 was maintained in RPMI 1640 (Life Technologies, Grand Island, NY) supplemented with 10% heat-inactivated FCS (PAA Laboratories, Cölbe, Germany) and 1% penicillin-streptomycin (PAA Laboratories). Cells were kept at a density of 5×10^5 cells/ml for 24 h in standard medium without supplements before being stimulated with 200 U/ml IFN- γ (Peptide, Rocky Hill,

NJ), 500 U/ml TNF- α (Immunotools, Friesoythe, Germany), and 500 ng/ml CCL3 (Cell Sciences, Canton, MA) for 24 h. In some wells, BX471 was added to a final concentration of 1 μM . Supernatants were collected and assayed for CCL5 by ELISA (DuoSet mouse; R&D Systems).

Alport Disease Model and Treatment Protocol

COL4A3-deficient mice develop glomerulosclerosis with renal fibrosis and progress to uremia-related death at approximately 10 wk of age (9,14). At the age of 6 wk, COL4A3-deficient mice were divided in two groups that received either BX471 (25 mg/kg body wt) in the vehicle 40% cyclodextrin or vehicle only by subcutaneous injections at 8-h intervals (16). This dose of BX471 does not significantly affect peripheral leukocyte counts in COL4A3-deficient mice (data not shown). Treatment was continued until death for assessment of survival or until day 63 of age (9 wk) in a subgroup of mice that were killed for histopathologic evaluation. Urine samples were obtained at weekly intervals and analyzed for protein/creatinine ratios as described (12).

Immunohistochemistry

All immunohistologic studies were performed on paraffin-embedded sections as described (12). The following rat and rabbit antibodies were used as primary antibodies: Rat anti-F4/80 (macrophages; Serotec, Oxford, UK; 1:50), anti-Ki-67 (cell proliferation; Dianova, Hamburg, Germany; 1:25), anti-ssDNA (apoptotic cells; Chemicon, Hofheim, Germany; 1:50), anti-mMECA-32 (endothelial cells; Iowa Hybridoma Bank, Iowa City, IA; 1:50), anti-mCCL5 (Peptide; 1:50), rabbit anti-laminin (gift from M. Paulsson, Cologne, Germany; 1:100), and goat anti-fibronectin (St. Cruz, Heidelberg, Germany; 1:100).

Histopathologic Evaluation

From each mouse, parts of the kidneys were fixed in 4% formalin in PBS and embedded in paraffin. Two-micrometer sections were stained with periodic acid-Schiff reagent or silver following the instructions of the supplier (Bio-Optica, Milano, Italy). Glomerular sclerotic lesions were assessed using a semiquantitative score by a blinded observer as follows: 0, no lesion; 1, <25% sclerotic; 2, 25 to 49% sclerotic; 3, 50 to 74% sclerotic; and 4, 75 to 100% sclerotic. Fifteen glomeruli were analyzed per section. The indices for interstitial volume, interstitial collagen deposition, tubular cell damage, and tubular dilation were determined as described previously (16). Interstitial laminin and fibronectin staining was graded by semiquantitative scoring of 12 different kidney sections from each animal into 0, 1+, and 2+ by a blinded observer (14). Interstitial cell counts were determined in 15 high-power fields ($\times 400$) by a blinded observer.

Real-Time Quantitative Reverse Transcription-PCR

From each animal, aliquots of the kidneys were snap-frozen in liquid nitrogen and stored at -80°C . RNA preparation and real-time reverse transcription-PCR (RT-PCR) as described (16). The following oligonucleotide primers (300 nM) and probes (100 nM) were used: Murine *Ccl5* (16); murine *Ccr1*: forward 5'-TTAGCTCCATGCCTGCCTTATA-3', reverse 5'-TCCACTGCTTCAGGCTCTTGT-3'; internal fluorescence labeled probe (FAM): 5'-ACTCACCGTACCTGTAGCCCTCATTCCC-3'; murine *Gapdh*: forward 5'-CATGGCTTCCGTGTTCTA-3', reverse 5'-ATGCCTGCTTACCACCTTCT-3'; internal fluorescence labeled probe (VIC): 5'-CCCAATGTGTCCGTCGTGGATCTGA-3'. All primers and probes were obtained from PE Biosystems (Weiterstadt, Germany).

In Vivo Assay of Renal Macrophage Recruitment

F4/80-positive macrophages were prepared by immunomagnetic selection from spleens of COL4A3-deficient mice as described previously

(12). Purity of isolated cells was verified by flow cytometry. Separated cells were labeled with PKH26 (Red Fluorescence Cell Linker Kit; Sigma-Aldrich Chemicals, Steinheim, Germany), and labeling efficacy was assessed by flow cytometry. Eight-week-old COL4A3-deficient mice received an injection of 2.0×10^5 F4/80 macrophages in 200 μ l of isotonic saline through the tail vein. Two groups of mice received injections of macrophages that had been preincubated with either 600 μ M BX471 or vehicle for 30 min. The respective mice received a single subcutaneous injection of either BX471 (25 mg/kg) or vehicle 3 h before the injection of the cells. Renal tissue was obtained after 3 h, snap-frozen, and prepared for fluorescence microscopy. The number of interstitial fluorescent cells was determined in 15 high-power fields.

Statistical Analyses

Data are presented as mean \pm SEM. Intravital microscopy data were analyzed using one-way ANOVA followed by Student-Newman-Keuls test, using SigmaStat Software (Jandel Scientific, Erkrath, Germany). Comparison of groups was performed using univariate ANOVA, and *post hoc* Bonferroni's correction was used for multiple comparisons (*in vitro* data). Paired *t* test was used for the comparison of single groups (*in vivo* data of Alport model). $P < 0.05$ was considered to indicate statistical significance. Survival curves were compared by Kaplan-Meier analysis using log-rank two-tailed testing.

Results

Delayed Onset of CCR1 Antagonism with BX471 Prolongs Survival of COL4A3-Deficient Mice

We hypothesized that CCR1 blockade can reduce interstitial leukocyte recruitment and activation during progressive renal fibrosis in COL4A3-deficient mice. Unfortunately, appropriate antibodies that allow detection of CCR1 protein by immunofluorescence or immunostaining in mice are not available at present. Thus, we first determined the expression of *Ccr1* mRNA in kidneys of 9-wk-old mice of this strain. Using real-time RT-PCR on total renal RNA samples, we found an induction of *Ccr1* mRNA normalized for *Gapdh* mRNA in kidneys of COL4A3-deficient mice compared with kidneys from wild-type control mice ($4.77\text{E-}04 \pm 4.75\text{E-}04$ in COL4A3+/+ versus $1.73\text{E-}03 \pm 7.74\text{E-}04$ in COL4A3-/-; $P = 0.015$). Next we studied the effects of BX471 on survival of wild-type and COL4A3-deficient or wild-type mice that were treated with either BX471 in vehicle or vehicle only. Vehicle-treated COL4A3-deficient mice showed a mean survival of 69 d (95% confidence interval, 64 to 74 d), whereas daily treatment with BX471 from week 6 increased mean survival to 86 d (95% confidence interval, 80 to 92 d; $P = 0.0002$; Figure 1A). Mortality of COL4A3-deficient mice was likely to be related to uremic death as within the last week of life the physical activity of COL4A3-deficient mice continuously declined until death as noted in previous studies (9,14). Wild-type control mice remained healthy until the end of the study at week 20.

CCR1 Antagonism Reduces Interstitial Macrophage Counts and Tubulointerstitial Injury in COL4A3-Deficient Mice

We hypothesized that improved survival in BX471-treated COL4A3-deficient mice was caused by prevention of renal disease progression. Thus, we performed additional studies using the same treatment protocol as before except that where

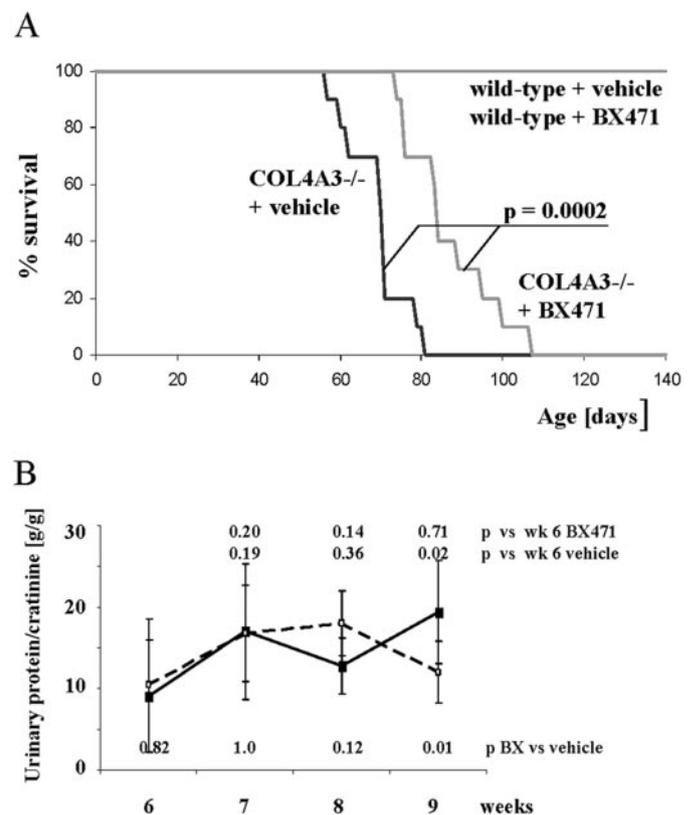


Figure 1. Survival and proteinuria of collagen 4A3 (COL4A3)-deficient mice. (A) Mice were treated with either BX471 in vehicle or vehicle alone as indicated. Survival is illustrated as Kaplan-Meier curve. (B) Urinary protein/creatinine ratios from urine samples taken at weekly intervals in vehicle-treated (black line) and BX471-treated COL4A3-deficient mice (dotted line). Values represent means \pm SEM.

COL4A3-deficient mice were killed at the age of 9 wk, we collected renal tissue.

Glomerular Injury. In vehicle-treated COL4A3-deficient mice, proteinuria increased over time until the end of the study. By contrast in BX471-treated mice, proteinuria did not increase from week 6 and showed a significant reduction of protein/creatinine ratios in urine compared with vehicle-treated mice at week 9 (Figure 1B). This was consistent with a reduced number of glomeruli with severe glomerulosclerotic lesions in BX471-injected COL4A3-deficient mice (Table 1, Figure 2). BX471 did not affect the number of Ki-67-positive proliferating glomerular cells (Table 1, Figure 2). No statistically significant differences were noted in blood urea nitrogen and serum creatinine levels in vehicle- and BX471-treated COL4A3-deficient mice at week 9.

Tubulointerstitial Injury. Vehicle-treated COL4A3-deficient mice had diffuse tubular atrophy and interstitial fibrosis as compared with age-matched wild-type mice (Figure 2, Table 1). COL4A3-deficient mice had increased numbers of Ki-67-positive proliferating and apoptotic tubular epithelial cells (Table 1, Figure 2). Interstitial damage in COL4A3-deficient mice was associated with a robust increase of interstitial F4/80-

Table 1. Serum, urinary, and histologic findings in COL4A3-deficient mice^a

	Wild-Type + Vehicle (n = 7)	COL4A3 ^{-/-} + Vehicle (n = 8)	COL4A3 ^{-/-} + BX471 (n = 10)
Renal function			
BUN (mg/dl)	23.1 ± 2.8	69.5 ± 19.8 ^b	65.9 ± 13.4 ^b
serum creatinine (mg/dl)	0.39 ± 0.05	0.52 ± 0.11 ^b	0.50 ± 0.04 ^b
Glomerulosclerosis score			
0 (no lesion)	89 ± 7	6 ± 5 ^b	20 ± 9 ^{b,c}
1 (1–24%)	11 ± 10	10 ± 4	23 ± 11 ^{b,c}
2 (25–49%)	0 ± 0	10 ± 4 ^b	12 ± 9 ^b
3 (50–74%)	0 ± 0	25 ± 9 ^b	18 ± 7 ^b
4 (75–100%)	0 ± 0	49 ± 11 ^b	27 ± 13 ^{b,c}
Cellular response (cells/glomerulus or hpf)			
glomerular Ki-67+	0.1 ± 0.1	1.0 ± 0.3 ^b	1.2 ± 0.4 ^b
interstitial F4/80+	1.9 ± 0.7	24.3 ± 2.3 ^b	17.9 ± 3.2 ^{b,c}
tubular Ki-67+	0.4 ± 0.2	1.6 ± 0.8 ^b	3.2 ± 0.7 ^{b,c}
ssDNA+	0.1 ± 0.0	1.8 ± 0.4 ^b	0.7 ± 0.4 ^{b,c}
Peritubular capillaries (capillary cross-sections/hpf)			
MECA-32 +	66.7 ± 10.3	33.8 ± 8.6 ^b	59.3 ± 9.0 ^{b,c}
Interstitial matrix deposition			
fibronectin (% hpf)	0.0 ± 0.0	1.1 ± 0.4 ^b	1.2 ± 0.4 ^b
laminin (% hpf)	0.0 ± 0.0	1.8 ± 0.3 ^b	1.3 ± 0.2 ^{b,c}

^aValues are means ± SEM. COL4A3, collagen 4A3; BUN, blood urea nitrogen; hpf, high power field.

^b*P* < 0.05 versus wild-type.

^c*P* < 0.05 BX471 versus vehicle.

positive macrophages. BX471 markedly reduced the numbers of interstitial F4/80-positive macrophages compared with vehicle-treated COL4A3-deficient mice (Figure 2, Table 1). This reduction of interstitial macrophages was associated with reduced numbers of ssDNA-positive apoptotic tubular epithelial cells. By contrast, BX471 increased the numbers of Ki-67-positive proliferating tubular epithelial cells, suggesting a role of interstitial macrophages for the balance of apoptotic cell death and tubular cell regeneration (Figure 2, Table 1). BX471 prevented the reduction in peritubular capillary cross-sections observed in untreated COL4A3-deficient mice (Table 1, Figure 2), suggesting that CCR1-dependent macrophage recruitment is involved in interstitial microvascular injury of COL4A3-deficient mice.

To confirm that BX471-induced reduction of interstitial macrophage counts is caused by blocking macrophage recruitment, we performed cell transfer studies with labeled macrophages. After injection, fluorescently labeled F4/80 macrophages localized to the interstitial compartment of 8-wk-old COL4A3-deficient mice, whereas glomeruli and perivascular fields were negative (Figure 3A). Pretreatment with BX471 significantly reduced the numbers of labeled F4/80 macrophages that infiltrated into the renal interstitium of COL4A3-deficient mice (Figure 3B).

Next we compared the degree of interstitial renal fibrosis in kidneys of mice from all groups. Vehicle-treated COL4A3-deficient mice showed an increase of the respective indices for

damaged tubular cells, tubular dilation, interstitial matrix, and interstitial volume when compared with age-matched wild-type mice (Figure 4). BX471 significantly reduced all of these markers compared with vehicle-treated COL4A3-deficient mice (Figure 4). BX471 reduced the amount of interstitial laminin deposits as compared with vehicle-treated COL4A3-deficient mice, but BX471 had no effect on interstitial fibronectin deposits (Table 1).

Renal CCL5 Expression in COL4A3-Deficient Mice

Next, we questioned whether BX471 treatment affected the production of proinflammatory mediators, *e.g.*, the CC-chemokine CCL5 in kidneys of COL4A3-deficient mice. Thus, we performed real-time RT-PCR for *Ccl5* mRNA on total renal isolates from vehicle- and BX471-treated COL4A3-deficient mice at 9 wk of age. We found that BX471 somewhat reduced renal *Ccl5* mRNA expression, although this was not statistically significant (Figure 5A). Immunostaining localized CCL5 in vehicle-treated COL4A3-deficient mice to single periglomerular and interstitial cells but not to tubular epithelial cells or glomeruli. Treatment with BX471 markedly reduced the amount of CCL5-positive cells in the renal interstitium of COL4A3-deficient mice (Figure 5B). Together, these data suggest that BX471 modulates the expression of CCL5 in kidneys of COL4A3-deficient mice, either by impairing recruitment of CCL5-producing cells to the kidney or possibly by directly inhibiting CCL5 production in resident renal macrophages.

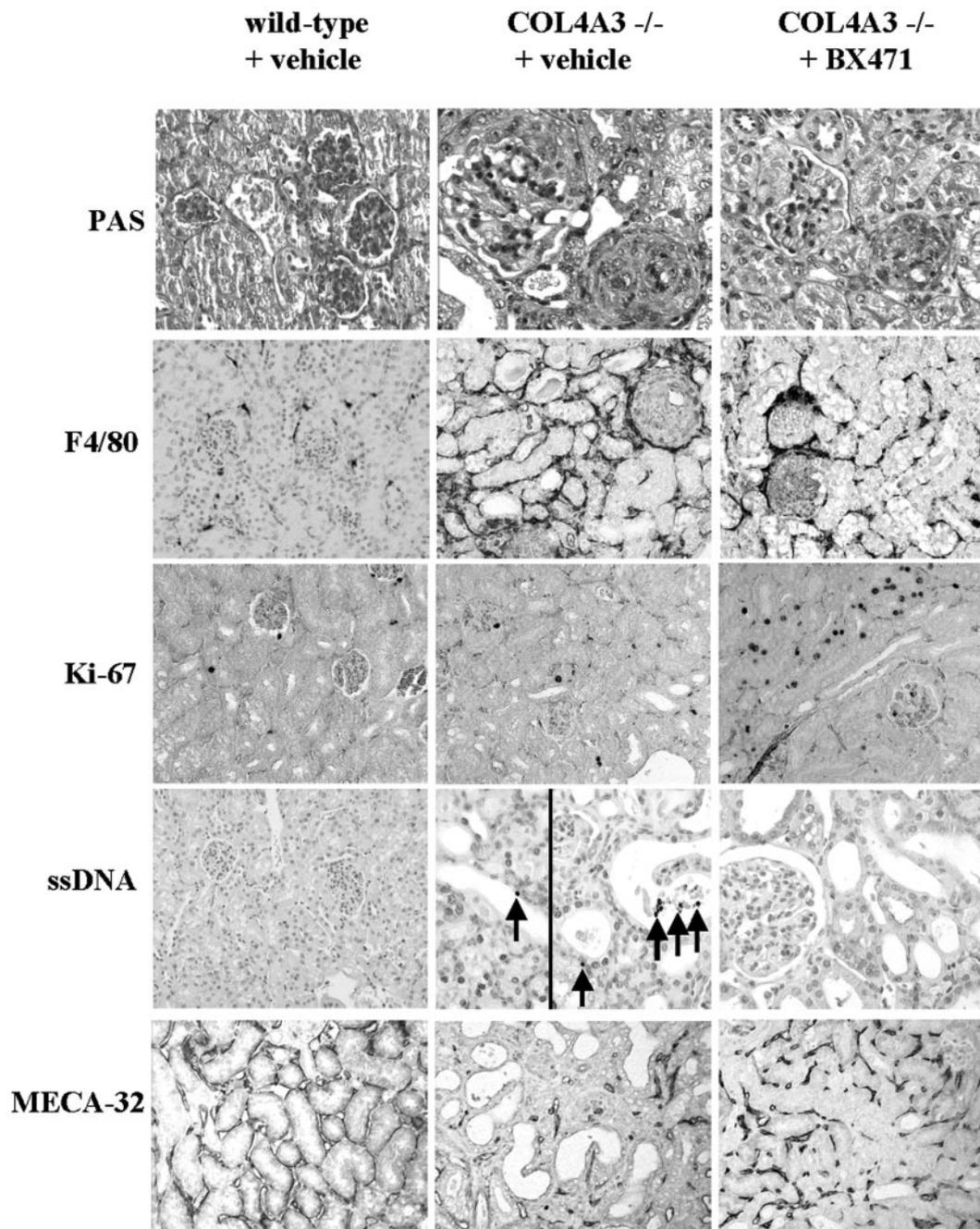


Figure 2. Renal histopathology in COL4A3-deficient mice. Renal sections of wild-type and COL4A3-deficient mice were stained with periodic acid-Schiff solution or for the indicated markers as described in the Materials and Methods section. For quantification, see Table 1. Magnification, $\times 400$.

CCL3/CCR1 Interaction Adds on Cytokine-Induced CCL5 Production by Macrophages

The role of cytokines such as TNF- α and IFN- γ for macrophage activation and CCL5 production is well established. However, it is unknown whether CCR1 ligation contributes to the activation of tissue macrophages. We evaluated CCL5 production in cultured murine J774 macrophages after incubation with various cytokine combinations for 24 h. A combination of IFN- γ and TNF- α induced marked CCL5 secretion by J774

macrophages. Adding the CCR1 ligand CCL3 to this cytokine combination increased CCL5 protein production by 50% (Figure 6). The CCR1 antagonist BX471 completely blocked the CCL3-induced increase of CCL5 secretion, indicating that CCL3 mediates its effect on CCL5 secretion through CCR1. CCL3 alone did not induce CCL5 secretion in J774 macrophages. These data suggest that CCR1 ligation by its chemokine ligand CCL3 contributes to macrophage CCL5 production, which may facilitate additional leukocyte recruitment and local inflammation *in vivo*.

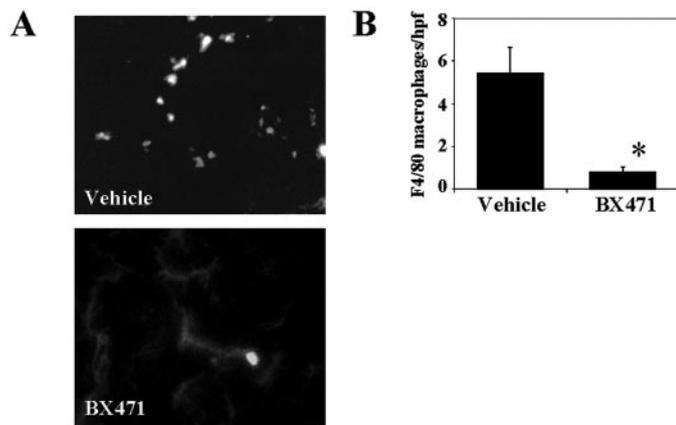


Figure 3. Renal infiltration of labeled leukocytes in kidneys of COL4A3-deficient mice. (A) COL4A3-deficient mice at 8 wk of age received an intravenous injection of PKH26-labeled F4/80 macrophages isolated from spleens of donor mice. The cells were pretreated with either vehicle or BX471 as indicated. Recipient mice received subcutaneous injections of either vehicle or BX471 before injection of the respective cells, and kidneys were obtained 3 h after injection of cells and examined by fluorescence microscopy. Fluorescence-labeled cells locate to the renal interstitium. (B) Cell counts for interstitial labeled F4/80 macrophages were determined by fluorescence microscopy from 15 high-power fields. Values are means \pm SEM. * $P < 0.001$. Magnification: $\times 400$ in A.

CCR1 Mediates Intravascular Adhesion and Transendothelial Migration of Leukocytes

To assess the role of CCR1 during the multistep recruitment process of intravascular leukocytes into inflamed tissues *in vivo*, we applied intravital microscopy of cremaster muscles in wild-type and CCR1-deficient mice, as well as in wild-type mice that were treated with the CCR1 antagonist BX471. Analysis was performed 3 h after intrascrotal administration of CCL3. This technique allowed us to visualize and quantify the four following stages of leukocyte recruitment: Rolling, adhesion, transendothelial diapedesis, and interstitial migration (Figure 7A).

Rolling Phase. In the rolling phase, transient interactions between activated endothelial cells and leukocyte surface molecules slow down circulating leukocytes. CCL3 prestimulation did not affect leukocyte rolling in wild-type mice (Figure 7B), and no statistically significant differences were detected among the four groups of mice studied.

Adherence Phase. Leukocyte arrest on activated vascular endothelial cells is mediated by chemokine-driven activation of adhesion molecules and is a prerequisite for transendothelial migration. CCL3 prestimulation significantly increased leukocyte adhesion in wild-type mice (Figure 7C). CCL3-induced increase of leukocyte adhesion was not observed in CCR1-deficient mice or in mice that were treated with the CCR1 antagonist BX471.

Transendothelial Migration Phase. After adhesion, leukocytes have to transmigrate through vascular endothelia and basement membranes to enter the interstitial compartment. CCL3 prestimulation significantly increased transendothelial

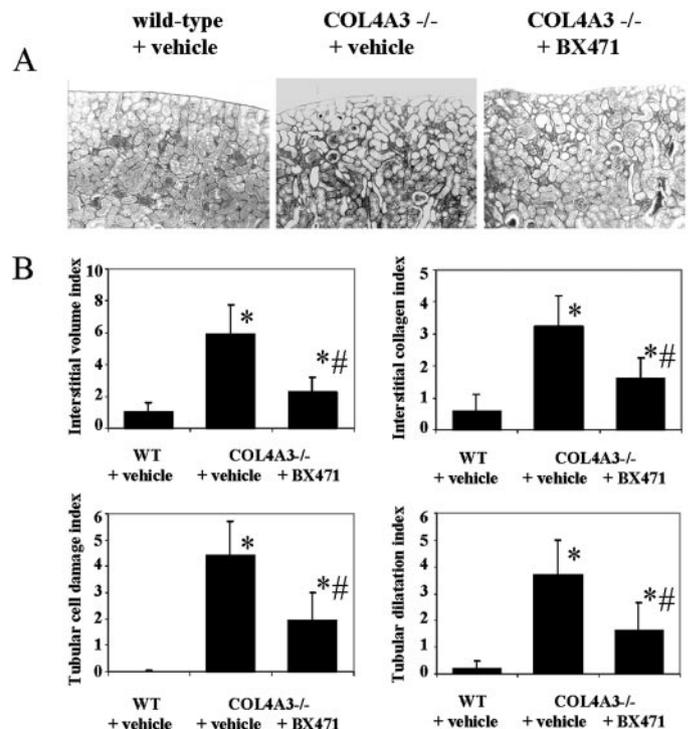


Figure 4. Renal fibrosis in COL4A3-deficient mice. (A) Renal sections of wild-type and COL4A3-deficient mice were stained with silver. Images illustrate representative sections of kidneys from the respective groups at week 9 of age. (B) Morphometric analysis of cortical renal sections was performed as described in the Materials and Methods section. Values represent means \pm SEM of the respective index in seven to 10 mice in each group. * $P < 0.001$ versus vehicle-treated COL4A3-deficient mice; # $P < 0.002$ versus vehicle-treated wild-type mice. Magnification, $\times 100$ in A.

migration of leukocytes in wild-type mice (Figure 7D). Lack of CCR1 or BX471 treatment reduced but did not completely block CCL3-induced leukocyte transmigration when compared with wild-type control mice.

Interstitial Migration Phase. In interstitial tissue compartments, leukocytes continue to migrate, but the role of CCR1 for this process is unknown. It is interesting that there was no significant difference between migration distances in cremaster muscles from either group, indicating that CCR1 does not play a role in interstitial migration of leukocytes (Figure 7E). Systemic leukocyte counts, inner diameters of the postcapillary venules studied, cell velocity, and shear rates were unaltered by CCL3 prestimulation, as these factors could compromise the comparison between CCL3-treated and control mice (data not shown).

Discussion

Because the contribution of macrophages for the progression of Alport disease is unclear, we blocked interstitial macrophage recruitment with a specific CCR1 antagonist in COL4A3-deficient mice, a mouse model for human Alport disease. CCR1 blockade was found to prolong survival in COL4A3-deficient

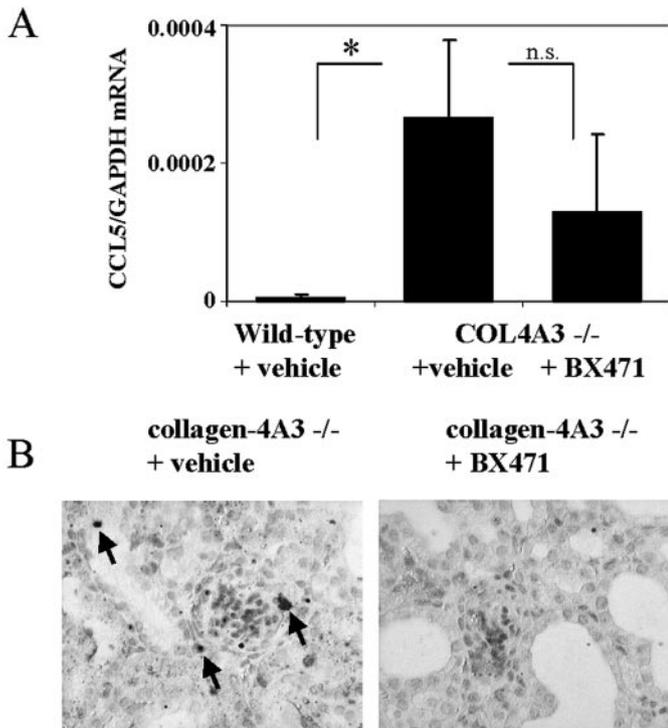


Figure 5. Renal CCL5 expression in COL4A3-deficient mice. (A) *Ccl5* mRNA expression was determined by real-time reverse transcription-PCR using total renal RNA from five to seven mice of each group. *Ccl5* mRNA levels for vehicle- and BX471-treated COL4A3-deficient mice are expressed per respective *Gapdh* mRNA expression of each kidney. **P* < 0.05. (B) Immunostaining for CCL5 was performed on paraffin-embedded renal sections as described in the Materials and Methods section. Arrows indicate CCL5-positive cells in the renal interstitium and the periglomerular area in vehicle-treated COL4A3-deficient mice. No CCL5 staining was detected in kidneys of BX471-treated COL4A3-deficient mice. Images illustrate representative sections of kidneys from the respective groups at week 9 of age. Magnification, $\times 400$ in B.

mice. Reduced interstitial macrophage infiltrates were associated with less tubular epithelial cell apoptosis, preservation of peritubular capillaries, and reduced interstitial extracellular matrix deposits. Furthermore, we show a role of CCR1 for leukocyte adhesion to vascular endothelial cells and transendothelial migration *in vivo* as well as for CCL5 production in macrophages *in vitro*. These data identify CCR1-mediated macrophage recruitment and possibly activation as a pathomechanism that contributes to the progression of renal disease in COL4A3-deficient mice.

CCR1 Antagonism Prolongs Survival of COL4A3-Deficient Mice

Interstitial macrophage infiltrates accompany progression to ESRD in COL4A3-deficient mice, but their functional significance is uncertain (10). Here we show that delayed treatment with a CCR1 antagonist reduces interstitial macrophage counts and prolongs survival of COL4A3-deficient mice. These data suggest a role for CCR1 in the progression of kidney disease in

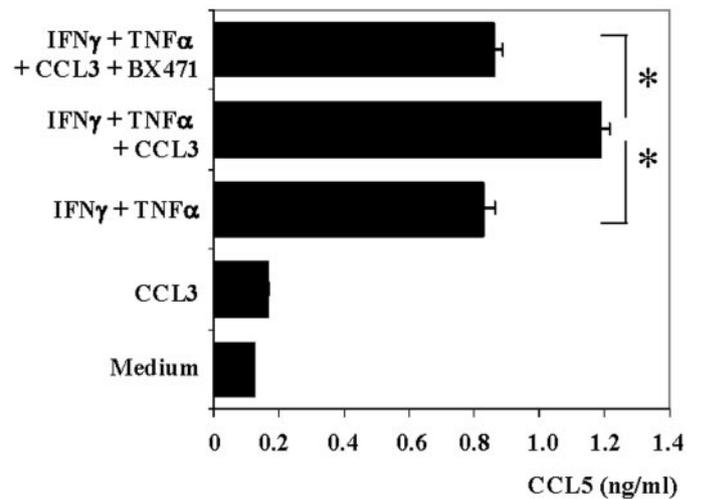


Figure 6. CCL5 production by J774 macrophages. J774 cells were stimulated with 200 ng/ml IFN- γ , 500 U/ml TNF- α , 500 ng/ml CCL3, 1 μ M BX471, or standard medium without supplements for 24 h as indicated. CCL5 protein production was determined in supernatants by ELISA. Results shown are from one of three comparable experiments, each performed in duplicate. Values are expressed as CCL5 concentrations \pm SEM. **P* < 0.05.

COL4A3-deficient mice. Macrophages are known to secrete mediators that induce apoptosis of tubular epithelial cells *in vitro* (17). In fact, a set of recent studies provided indirect evidence that interstitial macrophages may account for tubular epithelial cell apoptosis in COL4A3-deficient mice (10,11). For example, in these mice, disease progression is unrelated to TGF- β -dependent myofibroblast proliferation and interstitial matrix deposition as previously anticipated (11), but causes macrophage-induced tubular atrophy (18). Our data now provide direct evidence that lower numbers of interstitial macrophages are associated with less apoptotic tubular epithelial cells in kidneys of COL4A3-deficient mice. In addition, increased numbers of proliferating tubular cells in BX471-treated COL4A3-deficient mice suggest that lower numbers of interstitial macrophages support tubular cell regeneration in COL4A3-deficient mice. These mechanisms may also explain our finding that BX471 preserved the loss of peritubular microvasculature in COL4A3-deficient mice. Loss of peritubular capillaries is a known marker for advanced interstitial injury and thought to cause ischemia, a stimulus for fibroblast proliferation and production of extracellular matrix (19). The beneficial effects of BX471 treatment on the renal microvasculature of COL4A3-deficient mice may also contribute to our observation that BX471 reduced severe glomerulosclerotic lesions and proteinuria in COL4A3-deficient mice. All of these factors should account for prolonged survival seen with CCR1 blockade. However, serum creatinine and blood urea nitrogen levels did not differ between vehicle- and BX471-treated mice, which is consistent with the moderate effect of BX471 on survival and the high variability of these measures at 9 wk of age.

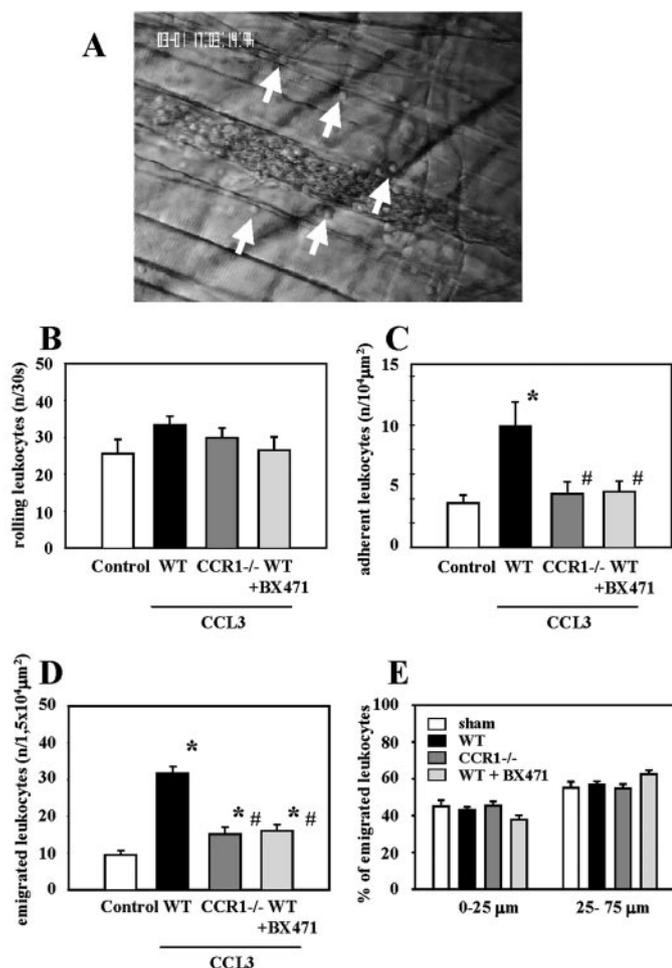


Figure 7. Intravital microscopy. (A) Microscopic image of a CCL3-stimulated cremasteric postcapillary venule that is surrounded by leukocytes emigrated into the interstitial tissue (arrows). (B through E) Quantitative analysis of the respective parameters of leukocyte-endothelial cell interactions and leukocyte emigration as determined by intravital microscopy. Values represent means \pm SEM in seven mice of each group. * $P < 0.05$ versus sham; # $P < 0.05$ versus wild-type mice. Magnification, $\times 400$ in A.

CCR1 Mediates Selected Steps during Leukocyte Recruitment and Activation

There is an ongoing debate about possible redundancy of individual chemokines for tissue inflammation (20–22). In view of our own data that suggested nonredundant functions of CCR1 for renal disease progression in COL4A3-deficient mice, we intended to identify the specific functions of CCR1 in that process. We now used the technique of intravital microscopy to study the specific roles of CCR1 during the multiple steps of leukocyte recruitment. Our finding that CCR1 is required for firm adhesion of leukocytes to activated vascular endothelium *in vivo* is consistent with previously reported data with human macrophages and T cells in an *in vitro* flow chamber model (23). CCR1 also contributes to transendothelial leukocyte migration but as lack of CCR1 or CCR1 blockade only partially impaired this process, other factors seem to be involved. *In vitro* data

from Weber *et al.* (23) argue for CCR5's being one of these factors, as BX471 in combination with a neutralizing antibody against CCR5 completely blocked transendothelial migration of human monocytes and T cells *in vitro*. Organ or compartment specificity of single chemokines or CCR is a common finding in chemokine biology. Therefore, we have to consider the possibility that our intravital microscopic data derived from the M. cremaster do not allow a conclusion about the role of CCR1 for leukocyte recruitment in mouse kidneys. We have addressed this question by injecting fluorescently labeled macrophages into COL4A3-deficient mice. BX471 blocked the recruitment of macrophages into the renal interstitium of these mice, suggesting that reduced numbers of renal macrophages observed with BX471 treatment is caused by the mechanisms identified by intravital microscopy.

CCR1 Antagonism Blocks Chemokine Expression in Macrophages

Activated resident and infiltrating macrophages are a major source of renal chemokine secretion (24), which adds on the chemokines expressed by intrinsic renal cells (reviewed in ref. 6). By this mechanism, renal macrophages support further leukocyte recruitment and renal inflammation (3). In fact, the CC-chemokine CCL5 can activate renal macrophages in such a way that kidney disease progresses even independent of the total number of renal macrophages (25). We therefore questioned whether CCR1 also contributes to the activation state of macrophages. We found that a combination of TNF- α , IFN- γ , and CCL3 increased the production of CCL5. This mimics the *in vivo* situation of an inflammatory microenvironment where CC-chemokines occur in concert with other proinflammatory cytokines. As BX471 completely blocked the CCL3-induced production of CCL5 *in vitro*, we relate this effect exclusively to CCR1 and not to other CCR that can bind CCL3. In fact, these data give rise to the hypothesis that CCR1 blockade can modulate the activation state of renal macrophages that are already present in the diseased kidney. This would occur in addition to the impaired recruitment of CCL5-producing cells, which was demonstrated by decreased numbers of CCL5-positive cells in kidneys of BX471-treated mice.

These data show that delayed onset of CCR1 blockade can modulate disease progression in COL4A3-deficient mice. This relates to the nonredundant role of CCR1 for adhesion of circulating leukocytes to activated endothelial cells. Our data also support a contribution of CCR1 to transendothelial migration and local chemokine production by macrophages. Blocking local macrophage recruitment and activation is associated with less tubular and vascular injury as well as less renal fibrosis in COL4A3-deficient mice, supporting a pathophysiologic role for interstitial macrophage infiltrates for disease progression in Alport disease.

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