Chemokine Receptor CCR1 But Not CCR5 Mediates Leukocyte Recruitment and Subsequent Renal Fibrosis after Unilateral Ureteral Obstruction

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Abstract. As chemokine receptor CCR1 and CCR5 expression on circulating leukocytes is thought to contribute to leukocyte recruitment during renal fibrosis, the authors examined the effects of unilateral ureteral obstruction (UUO) in mice deficient for CCR1 or CCR5. Analysis of UUO kidneys from CCR1-deficient mice revealed a reduction of interstitial macrophages and lymphocytes (35% and 55%, respectively) compared with wild-type controls. CCR1-deficient mice had reduced CCR5 mRNA levels in UUO kidneys, which correlated with a reduction of CCR5+ T cell infiltrate as determined by flow cytometry. Interstitial fibroblasts, renal TGF-β1 mRNA expression, interstitial volume, and collagen I deposits were all significantly reduced in CCR1-deficient mice. In contrast, renal leukocytes and fibrosis were unaffected in CCR5-deficient mice with UUO. However, if treated with the CCR1 antagonist BX471, CCR5-deficient mice showed a similar reduction of renal leukocytes and fibrosis as CCR1-deficient mice. To determine the underlying mechanism labeled macrophages and T cells isolated from either wild-type, CCR1-deficient, or CCR5-deficient mice were injected into wild-type mice with UUO. Three hours later, renal cell recruitment was reduced for CCR1-deficient cells or cells pretreated with BX471 compared with CCR5-deficient or wild-type cells. Thus, CCR1 but not CCR5 is required for leukocyte recruitment and fibrosis after UUO in mice. Therefore, CCR1 is a promising target for therapeutic intervention in leukocyte-mediated fibrotic tissue injury, *e.g.* progressive renal fibrosis.

Chronic inflammation and tissue fibrosis are common causes of progressive organ dysfunction. In the kidney, the extent of leukocyte infiltration and tubulointerstitial fibrosis are strong prognostic factors for the degree of renal insufficiency and the progression to end-stage renal disease (1). Interstitial fibrosis is characterized by the accumulation of interstitial T cells, macrophages, and fibroblasts that contribute to extracellular matrix production and tubular atrophy (2). Strategies that specifically block the infiltration of leukocytes and thereby reduce interstitial inflammation may provide a potential option to reduce progressive renal fibrosis and to prevent end-stage renal disease (3).

Infiltration of circulating leukocytes is triggered by locally secreted chemokines (4), and chemokine-mediated leukocyte infiltration is thought to contribute to the initiation and progression of renal disease (5). Studies on progressive renal fibrosis using the model of unilateral ureteral ligation (UUO) in the mouse have demonstrated that increasing amounts of the chemokine receptors CCR1 (ligands CCL3 and CCL4) and CCR5 (ligands CCL5 and CCL4) were expressed on infiltrating macrophages and T cells in parallel to the development of renal fibrosis (6). CCR1 and CCR5 are thought to mediate the migration of T cells and macrophages into inflamed tissues (4,7). Evidence for the role of CCR1 in renal disease comes from a study that showed that the CCR1 antagonist BX471 was similar to cyclosporin in its ability to prevent renal allograft rejection and to improve survival in rabbits (8). Furthermore, we have recently shown that BX471 reduced leukocyte accumulation and renal fibrosis after UUO in mice (9). However,

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when CCR1-deficient mice were injected with nephrotoxic serum they showed enhanced glomerular T cell infiltration, crescent formation, and proteinuria in association with an enhanced Th1-response, indicating CCR1 is also involved in the regulation of systemic immune responses (10). Conflicting results have also been found with Met-RANTES or AOP-RANTES, two antagonists that can block CCR5. AOP-RANTES reduced macrophage infiltration in Thy1.1 antibody-induced glomerulonephritis in rats (11), and Met-RANTES improved rat renal allograft rejection (12). However, both antagonists aggravated immune complex glomerulonephritis in mice, which was associated with antagonist-induced modulation of macrophage function toward a proinflammatory phenotype (13).

Although the multiple roles of CCR1 and CCR5 activation on leukocytes may show different outcomes in systemic immune responses, their specific roles for localized leukocyte recruitment *in vivo* at sites of restricted inflammation or tissue injury remain do be determined. *In vitro* studies have suggested different roles of CCR1 and CCR5 for leukocyte recruitment. CCR1 but not CCR5 was found to be required for the initial adhesion of human monocytes and T cells to activated endothelium, whereas CCR5 seemed to be involved in the subsequent transendothelial cell migration (14).

We therefore hypothesized that both CCR1 and CCR5 might be involved in macrophage and T cell infiltration and the development of renal fibrosis after UUO in mice. Using a multipronged approach involving combinations of CCR1-deficient and CCR5-deficient mice, transfer of leukocytes from these animals into wild-type mice with UUO, and a specific CCR1 antagonist, we could clearly demonstrate that CCR1 but not CCR5 is required for leukocyte recruitment in this model. We conclude that CCR1 but not CCR5 may be a valuable target for therapeutic intervention for chronic nephropathies accompanied by leukocyte-mediated progressive interstitial fibrosis.

Materials and Methods

Animal Studies

CCR1-deficient mice were generated as described (15). CCR1-deficient mice were backcrossed into the C57BL/6 background for eight generations under specific pathogen-free housing conditions. Details about the generation of CCR5-deficient mice will be described elsewhere (Luckow *et al.*, manuscript in preparation). In brief, almost the entire coding region of the murine CCR5 gene was deleted by homologous recombination in E14–1 ES cells using standard gene-targeting methods. ES cells with a targeted deletion of the CCR5 gene were identified by Southern blot analyses and subsequently used to generate chimeric mice by morula aggregation. After germ line transmission, the resulting heterozygous CCR5 knockout mice were backcrossed for five generations into the inbred strain C57BL/6 (Charles River, Germany) and then intercrossed to obtain homozygous CCR5 knockout mice. Deletion of CCR1 or CCR5 was confirmed by repeated genotyping in all individual mice by PCR analyses using genomic DNA prepared from tail snips as described (15). After UUO was performed, all mice were housed in groups of 7 to 9 mice in filter top cages with a 12-h dark/light cycle and unlimited access to food and water. Cages, bedding, nestlets, food, and water were sterilized by autoclaving before use. For all experiments, 8- to 12-wk-old animals were used. One group of CCR5-deficient mice were treated with the CCR1 antagonist BX471 (50 mg/kg body wt, thrice daily, subcutaneously) from day 0 to day 10 as described (9). Sex-matched and age-matched controls were obtained from Charles River, Sulzfeld, Germany. Tissue was obtained at day 10. Contralateral kidneys served as intraindividual control. All experimental procedures were performed according to the German animal care and ethics legislation and had been approved by the local government authorities.

Morphologic Evaluation

Tissue sections from the obstructed and the contralateral kidneys of each mouse were fixed in 4% formalin in PBS and embedded in paraffin. Two-micrometer sections were stained with periodic acid-Schiff reagent and silver following the instructions of the supplier (Bio-Optica, Milano, Italy). To count interstitial cells, 15 high power fields (hpf, ×400) were analyzed by a blinded observer. Positive cells were counted per hpf omitting positive cells in glomerular fields. Quantitation of the interstitial volume (I_{Vol}), interstitial collagen deposition (I_{col}), and tubular dilatation (I_{Tdil}) was performed as described previously (9).

Immunohistochemistry

All immunohistologic studies were performed on paraffin-embedded sections as described (6). The following rat and rabbit antibodies were used as primary antibodies: rat anti-F4/80 (1:50; Serotec, Oxford, UK), rat anti-CD3 (1:50; Serotec), rabbit anti-human TGF-β crossreacting with mouse TGF-β (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-FSP1 (1:500; kindly provided by Dr. E. G. Neilson, Vanderbilt University, Nashville, TE).

In Situ Hybridization

In situ hybridization for murine TGF-β1 was performed as described previously (16). The TGF-β1 probe was a gift from H.L. Moses (Department of Cell Biology, Vanderbilt University, Nashville, TN) as described. Negative controls included hybridization performed on replicate tissue sections using the sense riboprobe.

Isolation of Renal Cells for FACS Analysis

A preparation of isolated renal cells including infiltrating leukocytes was obtained as described previously (6) from obstructed and contralateral kidneys. For flow cytometry, the resulting cell suspensions were incubated with 5 μg/ml monoclonal antibodies against murine CCR5 or the isotype control rat IgG_{2b} (Pharmingen, Hamburg, Germany), as described (17). To identify CD8, T cells samples were incubated with a Cy-chrome-labeled anti-mCD8 antibody (Pharmingen).

In Vivo Assay of Renal Leukocyte Infiltration

F4/80 macrophages and CD8 T cells were prepared from spleens of CCR1 −/−, CCR5 −/−, and wild-type mice by a previously described isolation and labeling method (18). In brief, spleen T cells and F4/80 macrophages were isolated by immunomagnetic selection using the following antibodies: CD8a (Ly-2) MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany), FITC rat anti-mouse F4/80 (Serotec, Düsseldorf, Germany), and Anti-FITC MicroBeads (Miltenyi). 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 to be >97%. Viability assessed by trypan

blue exclusion was >90%. Wild-type mice underwent surgery for UUO and were injected with either 7.5×10^5 F4/80 positive cells or CD8 positive T cells in 200 μl of isotonic saline into a tail vein 10 d after surgery. One group of mice was injected with CCR5-deficient cells that were preincubated with 600 μM CCR1 antagonist BX471 for 30 min (9). Mice of this group received a single subcutaneous injection of BX471 (50 mg/kg) at the time of injection. Renal tissue was obtained after 3 h, snap frozen, and prepared for microscopy. Interstitial cells from UUO and contralateral kidneys were analyzed as above.

Isolation of Cells for Real-Time RT-PCR

To assess CCR1 and CCR5 mRNA expression in renal fibroblasts and tubular epithelial cells, renal tissue samples were obtained 10 d after UUO. Tubular segments were microdissected from RNase inhibitor treated tissue in ice-cold PBS, as described previously for human renal biopsies (19). For isolation of primary renal fibroblasts, small pieces of renal tissue were incubated in DMEM (Invitrogen Corporation, Karlsruhe, Germany) supplemented with 10% FCS (Invitrogen), penicillin, and streptomycin for 21 d. Adherent cells were lifted with 1.5 mM EDTA (Calbiochem-Novabiochem, San Diego, CA) and were depleted for leukocytes by immunomagnetic selection using FITC anti-mCD45 (Pharmingen) and anti-FITC MicroBeads as described (Miltenyi). F4/80-positive macrophages and CD8-positive T cells were obtained from wild-type mice as described above. mRNA of isolated cells was prepared by standard methods as described (9)

Real-Time Quantitative RT-PCR

Pieces of kidney from each animal were snap frozen in liquid nitrogen and stored at −80°C. RNA preparation and real-time RT-PCR on a TaqMan ABI 7700 Sequence Detection System (PE Biosystems, Weiterstadt, Germany) were performed as described (9). Controls consisting of ddH_2O were negative for target genes and the housekeeper gene GAPDH. The following oligonucleotide primers (300 nM) and probes (100 nM) were used. Murine CCR1: forward 5'-TTAGCTTCCATGCCTGCCTTATA-3', reverse 5'-TCCACTGCTTCAGGCTCTTGT-3', internal fluorescence labeled probe (FAM) 5'-ACTCACCGTACCTGTAGCCCTCATTTCCC-3', the probe is located in the deleted region of the CCR1 gene in CCR1-deficient mice; murine CCR5: forward 5'-CAAGACAATCCTGATCGTGCAA-3', reverse 5'-TCCTACTCCCAAGC-TGCATAGAA-3'; FAM 5'-TCTATACCCGATCCACAGGAG-AACATGAAGTTT-3', the probe is located in the deleted region of the CCR5 gene in CCR5-deficient mice, CCR5 specificity of primers and probe were tested on CCR plasmids; murine TGF-β1: forward 5'-CACAGTACAGCAAGGTCCTTGC-3', reverse 5'-AGTAGACGATGGGCAGTGGCT-3', FAM 5'-GCTTCGGCGTCACCGTGCT-3'; murine GAPDH: forward 5'-CATGGCCTTCCGTGTTCCTA-3', reverse 5'-ATGCCTGCTTCACCACCTTCT-3', internal fluorescence labeled probe (VIC) 5'-CCCAATGTGTCCGTCGTGGATCTGA-3'. All primers and probes were obtained from PE Biosystems. For the expression, CCR levels in isolated cells values are expressed as ratio of respective CCR mRNA to GAPDH mRNA expression. Expression levels of CCR in the UUO model were analyzed accordingly, showing the aforementioned ratio in each animal separately for UUO and contralateral kidneys (CLK). For comparison of mRNA expression, levels between different groups the ratio of UUO to CLK is shown.

RNase Protection Assay

Total spleen RNA was isolated from three C57BL/6 wild-type mice, five CCR1−/− mice, and four CCR5−/− mice. Multiprobe RNase protection assay was performed with the RiboQuant multiprobe template set for murine CC chemokine receptors (mCR-5) obtained from Pharmingen using 10 μg of total spleen RNA per lane as described (6). Unfortunately, the CCR1-specific RPA probe from the multiprobe template set is located outside of the deleted region of the CCR1 gene in CCR1-deficient mice and therefore not suited to differentiate between wild-type and knockout transcripts.

Statistical Analyses

Data are presented as mean ± SD. Comparison of groups was performed using univariant ANOVA and post-hoc Bonferroni correction was used for multiple comparisons. Paired *t* test was used for the comparison of single groups (FACS data). A value of $P < 0.05$ was considered to indicate statistical significance.

Results

Chemokine Receptor Expression in Renal Cells

As we intended to study the role of CCR1 and CCR5 in the fibrotic kidney, we first determined the expression of CCR1 and CCR5 by real-time RT-PCR using total renal RNA. There was a marked increase of mRNA of both chemokine receptors in obstructed compared with unobstructed kidneys (Figure 1A). To characterize whether intrinsic renal cells contribute to renal CCR1 and CCR5 expression, we performed real-time RT-PCR for both receptors on renal fibroblasts and tubular epithelial cells. Neither cell type expressed mRNA for CCR1 or CCR5 (Figure 1B). CCR1 and CCR5 were examined on naïve macrophages and T cells, as these receptors may contribute to recruitment of these cells to the kidney after UUO. Both F4/80 macrophages and T cells expressed CCR1 and CCR5 mRNA. In macrophages, the expression of CCR1 mRNA was much higher than that of CCR5 mRNA. In contrast T cells expressed CCR5 mRNA to a greater extent than CCR1 mRNA (Figure 1B). To further characterize the expression of CCR5 protein on infiltrating T cells, FACS analysis on renal cell isolates was performed. The ratio of CD4/CD8-positive among the CD3-positive lymphocytes infiltrating the kidney was 1:1; 93 ± 3% of CD8 T cells infiltrating the obstructed kidney were positive for CCR5. Flow cytometry of F4/80 macrophages for CCR5 was not feasible due to unspecific binding of isotype antibodies. Deficiency of CCR1 was not compensated by higher expression levels for CCR2 and CCR5 and *vice versa* as analyzed by RNase protection assays with spleen cell mRNA from all mouse strains (Figure 1C), confirming data reported by others (10). These data indicate that both CCR are expressed by infiltrating leukocytes but not by intrinsic interstitial cells such as tubular epithelial cells or fibroblasts in the UUO kidneys. Furthermore, lack of either CCR1 or CCR5 is not followed by upregulation of the other CCR on immune cells and *vice versa*.

CCR1 But Not CCR5 Is Required for Leukocyte Infiltration after UUO

At day 10 after UUO, a prominent cell infiltrate of CD3-positive lymphocytes and F4/80-positive macrophages was localized to the peritubular interstitium in wild-type mice by immunostaining (Figure 2A). CCR1-deficient but not CCR5-

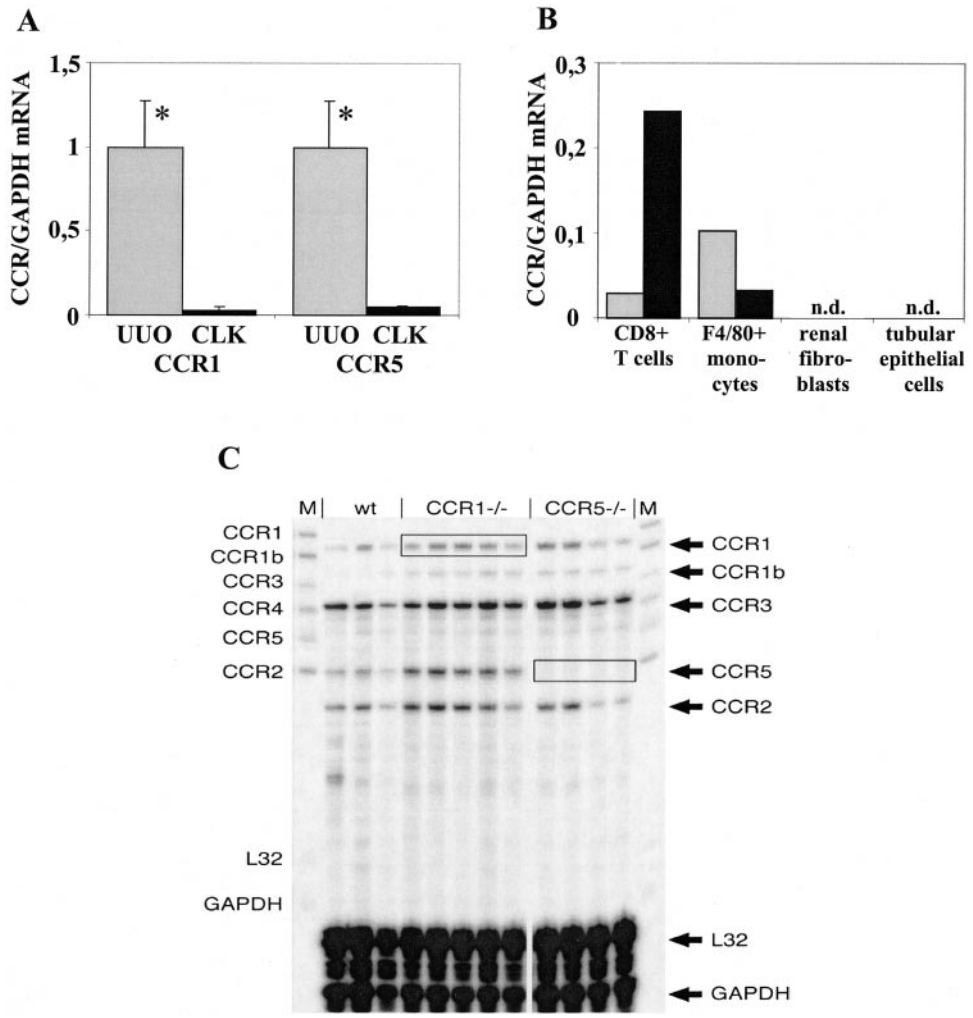

Figure 1. Chemokine receptor mRNA expression. (A) Renal expression of CCR1 and CCR5 mRNA was determined by real-time RT-PCR from total renal isolates 10 d after unilateral ureteral obstruction (UUO). CCR mRNA expression of obstructed kidneys (UUO) and contralateral unobstructed kidneys (CLK) is expressed as ratio to the respective GAPDH mRNA expression ($n = 5$). CCR mRNA expression in UUO kidneys is set as 1. * $P < 0.05$. (B) The expression of CCR1 (gray) and CCR5 (black) mRNA in different cell types was determined in duplicate by real-time RT-PCR and expression levels are expressed as ratio to the respective GAPDH mRNA expression. (C) Expression of CC chemokine receptor mRNA in splenocytes determined by RNase protection assay. The two lanes designated M show the undigested templates, the arrows indicate the positions of the RNase digested templates. The lower box shows the absence of the corresponding message in CCR5 −/− mice. A CCR1 signal was obtained from the CCR1 −/− mice (upper box) because the probe used for CCR1 RPA was located outside of the region that has been deleted in the CCR1 −/− mice; therefore, a positive signal was obtained. It is obvious from the gel, that deletion of either CCR1 or CCR5 has no major effect on the expression of the other closely related chemokine receptors. Similar results have been obtained for the ligands of CCR5 using RPA. WT, wild-type; CCR −/−, mice negative for respective chemokine receptor expression; n.d., not detected.

deficient mice showed a reduction of interstitial CD3-positive lymphocytes and of interstitial F4/80-positive macrophages in obstructed kidneys 10 d after UUO compared with UUO kidneys of wild-type control mice (Figure 2B). However, if treated with BX471, CCR5-deficient mice revealed a reduction of interstitial CD3-positive lymphocytes and of interstitial F4/80-positive macrophages in obstructed kidneys similar to what was observed in CCR1-deficient mice (Figure 2B).

FACS analysis of isolated renal cells revealed that this decrease was accompanied by a significant reduction of CCR5/CD8-positive T cells in UUO kidneys of CCR1-deficient mice compared with wild-type controls (93 ± 3% *versus* 50 ± 17%; $P = 0.036$; Figure 3A). When CCR5-positive CD4 cells were evaluated in one experiment, they were also reduced by 33%. Due to the lack of appropriate antibodies against mCCR1 that work in FACS or immunostaining, we were unable to demonstrate the amount of CCR1-positive cells in UUO kidneys. We therefore used real-time RT-PCR to determine the amount of CCR1 and CCR5 mRNA expression in UUO kidneys of all groups. Corresponding to reduced renal leukocyte counts, CCR1-deficient mice revealed a significant reduction of renal CCR5 mRNA expression (Figure 3B). In line with their geno-

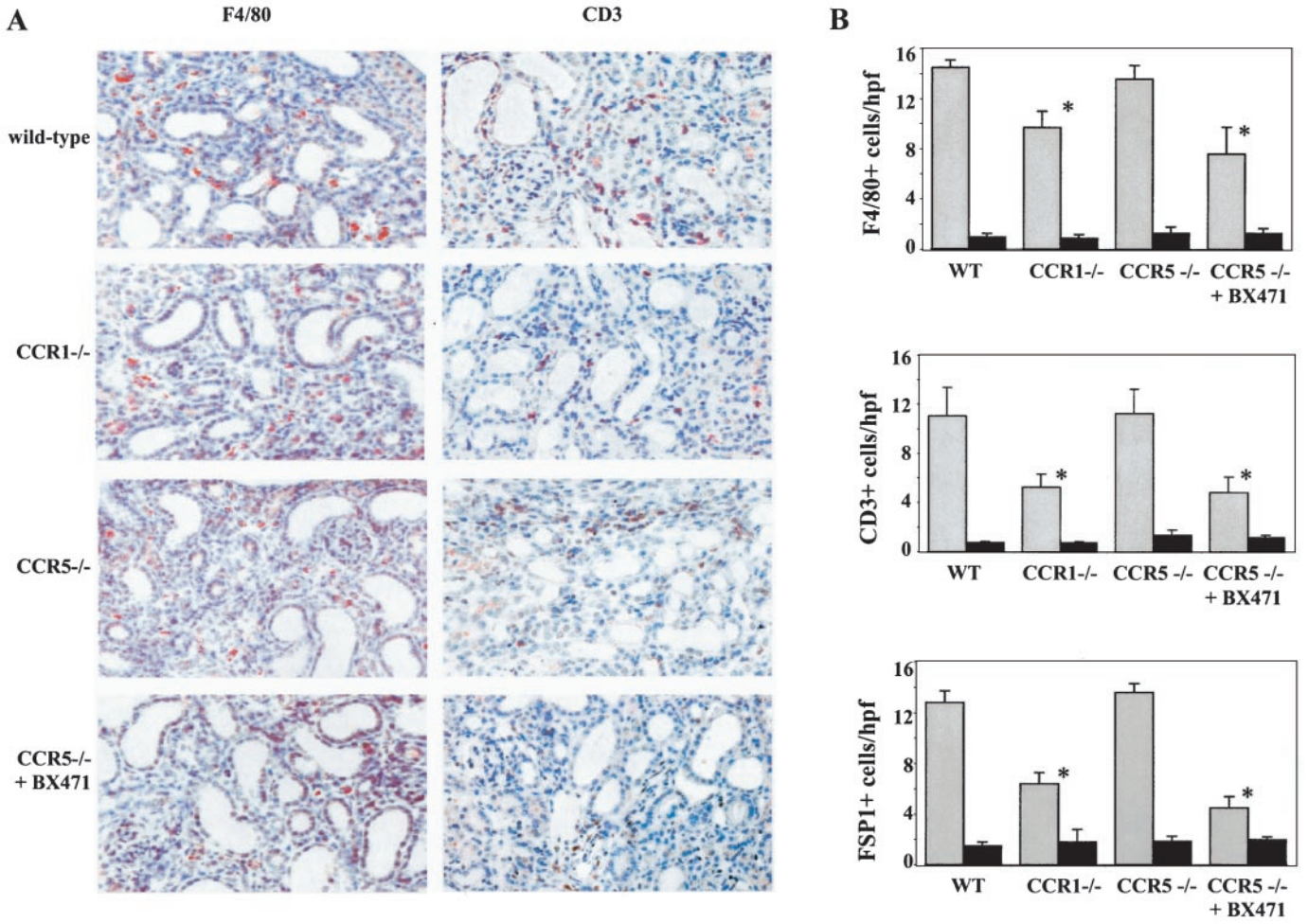

Figure 2. Accumulation of leukocytes after UUO. (A) Immunohistochemistry. Cortical renal sections were stained for F4/80-positive macrophages (left) and CD3-positive lymphocytes (right). Images illustrate representative sections of UUO kidneys from the respective groups 10 d after UUO (original magnification, ×400). (B) Quantitative analysis of F4/80-positive macrophages, CD3-positive T cells, and FSP1-positive fibroblasts 10 d after UUO in obstructed (gray bars) and contralateral unobstructed kidneys (black bars). Values are means ± SD of cell counts per 10 high power fields (hpf) per kidney from 5 to 7 mice in each group. WT, wild type; CCR −/−, mice negative for respective chemokine receptor expression. BX471 was administered in CCR5-dificient mice as described in methods. * $P < 0.05$.

type, CCR5 mRNA expression was not detectable in CCR5-deficient mice. However, in analyzing renal CCR1 mRNA expression, we found that UUO kidney CCR1 mRNA levels of CCR5-deficient mice were comparable to those of wild-type control mice. In contrast, when treated with the CCR1 antagonist BX471, CCR1 mRNA expression levels of UUO kidneys from CCR5-deficient mice were markedly reduced compared with those of wild-type mice (Figure 3B). In line with their genotype CCR1 mRNA expression was not detectable in UUO kidneys of CCR1-deficient mice.

CCR1 But Not CCR5 Mediates Renal Infiltration of Macrophages and T Cells In Vivo

On the basis of previous *in vitro* studies, we investigated the role of CCR1 and CCR5 for renal T cell and macrophage recruitment *in vivo* and used the approach of injecting purified and fluorescence-labeled leukocytes from the various knockout strains intravenously into wild-type mice at day 10 after UUO. The tissue was analyzed 3 h after injection. CD8-positive T cells or F4/80-positive macrophages purified from wild-type mice both accumulated in the interstitium of UUO kidneys (Figure 4A). When the same cells were isolated from CCR1-deficient mice, there was a 66% reduction of interstitial F4/80 positive macrophages and a 35% reduction of interstitial CD8 T cells in the UUO kidney after injection compared with wild-type controls (Figure 4B). In contrast, macrophages and T cells isolated from CCR5-deficient mice accumulated to the same extent in UUO kidneys as cells isolated from wild-type animals. When cells were isolated from CCR5-deficient mice and both the cells and the recipient mice were pretreated with BX471 before injection of the labeled cells, there was a 35% reduction of labeled interstitial F4/80-positive macrophages and a reduction of 35% of interstitial CD8-positive T cells in UUO kidneys compared with injection of untreated CCR5-negative cells or cells from wild-type controls (Figure 4B). No cells were detected in unobstructed contralateral kidneys from

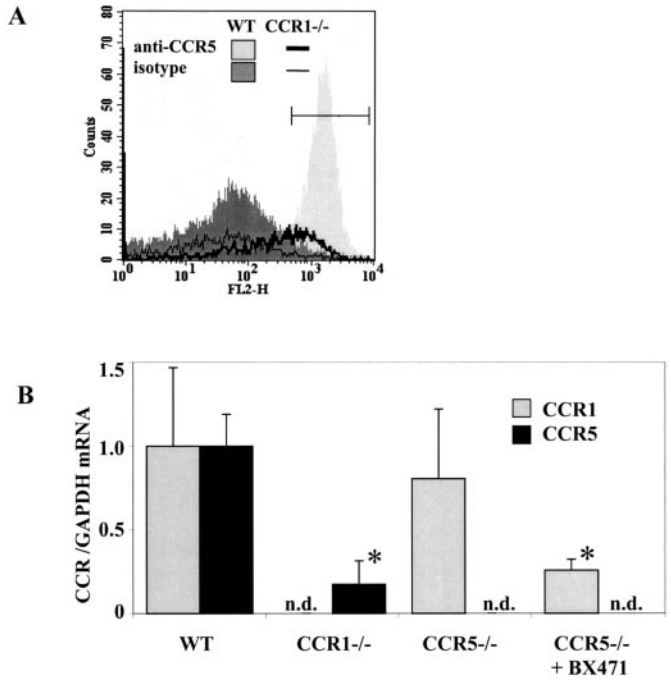

Figure 3. Renal chemokine receptor expression after UUO. (A) Flow cytometry analysis of CCR5 expression on CD8-positive T cells isolated from UUO kidneys of CCR1-deficient and wild-type mice 10 d after UUO. Staining with the CCR5 antibody is illustrated as ░ (CCR1+/+) or thick black line (CCR1−/−) and IgG2b isotype control as ▓ (CCR1+/+) and thin black line (CCR1−/−), respectively. Data shown are from obstructed kidneys of one animal of each group and are representative of four independent samples. (B) Real-time RT-PCR was performed using total renal RNA of UUO kidneys of 4 to 5 mice 10 d after UUO. Levels of mRNA expression for CCR1 (gray) and CCR5 (black) in UUO kidneys are expressed in relation to renal GAPDH mRNA expression and to the respective ratio of the unobstructed contralateral kidney. The expression of control mice is set as 1. Primers for CCR1 were located in the deleted region of the CCR1 gene. Compared with kidneys of wild-type (WT) mice, CCR1-deficient mice revealed a significant reduction of CCR5 mRNA expression ($P = 0.03$). As to be expected, CCR1 mRNA expression was not detectable in CCR1-deficient mice. Renal CCR1 mRNA expression in CCR5-deficient mice was not reduced compared with wild-type controls. CCR5-deficient mice treated with BX471 revealed a significant reduction of CCR1 mRNA expression (* $P < 0.04$). BX471 was administered in CCR5-deficient mice as described in Materials and Methods. As to be expected, CCR5 mRNA was not detectable in CCR5-deficient mice. WT, wild-type; CCR −/−, mice negative for respective chemokine receptor expression; n.d., not detected; * $P < 0.05$.

all groups, indicating that the injected cells only localize to the UUO kidneys (not shown). These data indicate that, in the mouse, CCR1 but not CCR5 is involved in the infiltration of circulating macrophages and T cells into the kidney after UUO.

Lack of CCR1 But Not of CCR5 Is Associated with a Reduction of Interstitial Fibrosis after UUO

As a cellular marker of fibrosis, the amount of FSP1-positive fibroblasts was assessed by immunohistochemistry. Fibroblasts accumulated in the peritubular interstitium in areas of marked tubular dilatation in the obstructed kidney 10 d after UUO compared with contralateral unobstructed kidneys (Figure 5). In CCR1-deficient but not in CCR5-deficient mice, the amount of interstitial FSP1-positive fibroblasts in UUO kidneys was significantly reduced compared with wild-type controls. In contrast, when treated with BX471, CCR5-deficient mice revealed a reduction of interstitial FSP1-positive fibroblasts in UUO kidneys by 65%, similar to that of CCR1-deficient mice (Figure 2B).

Morphometric analysis of silver-stained renal sections revealed a marked increase of tubular dilation, interstitial volume, and collagen deposition in obstructed kidneys compared with the respective unobstructed contralateral kidneys (Figure 5A). CCR1-deficient but not CCR5-deficient mice revealed a reduction of interstitial volume and collagen deposition, respectively (Figure 5B). In contrast, when treated with BX471, interstitial volume and collagen were also reduced in UUO kidneys of CCR5-deficient mice similar to that of CCR1-deficient mice (Figure 5B). The extent of tubular dilation in UUO kidneys was comparable in all groups as expected after persistent UUO (Figure 5B). No changes in interstitial cell counts or morphometric parameters were observed in contralateral kidneys.

Reduced Interstitial Leukocyte Infiltration in CCR1-Deficient Mice Is Associated with a Decrease of Renal TGF-β1 mRNA Expression

We were intrigued by the finding that the extent of renal fibrosis after UUO in CCR1-deficient and wild-type mice directly paralleled the amount of interstitial leukocytes. As renal fibroblasts were negative for CCR1, the lack or blockade of CCR1 is unlikely to directly affect fibroblast activation. In contrast, the observed reduction of renal fibrosis could be secondary to reduced secretion of profibrotic cytokines. We therefore determined the expression of TGF-β1 mRNA in total renal RNA by real-time RT-PCR. UUO kidneys from mice that lack CCR1 had a marked reduction of TGF-β1 mRNA expression compared with wild-type mice (Figure 6A). In contrast, lack of CCR5 did not affect renal TGF-β1 mRNA expression. However, if treated with BX471, CCR5-deficient mice revealed a significant reduction of renal TGF-β1 mRNA expression compared with untreated CCR5-deficient mice (Figure 6A). To determine the source of renal TGF-β1, we performed *in situ* hybridization and immunostaining for TGF-β. In unobstructed contralateral kidneys of wild-type mice, the *in situ* hybridization yielded only a weak diffuse deposition of silver grains not different from the sense controls (Figure 6B). The strongest signal for TGF-β mRNA was found in areas of tubulointerstitial infiltrates (Figure 6B). The resolution of the *in situ* hybridization did not allow assignment of the signal in the infiltrate to specific cells. In areas without prominent cell infiltration, only the background signal was present, similar to unobstructed control kidneys. No clear tubular expression of TGF-β mRNA was apparent. Immunostaining for TGF-β protein was also restricted to the interstitial cell infiltrates of UUO kidneys from wild-type mice (Figure 6B). In unobstructed

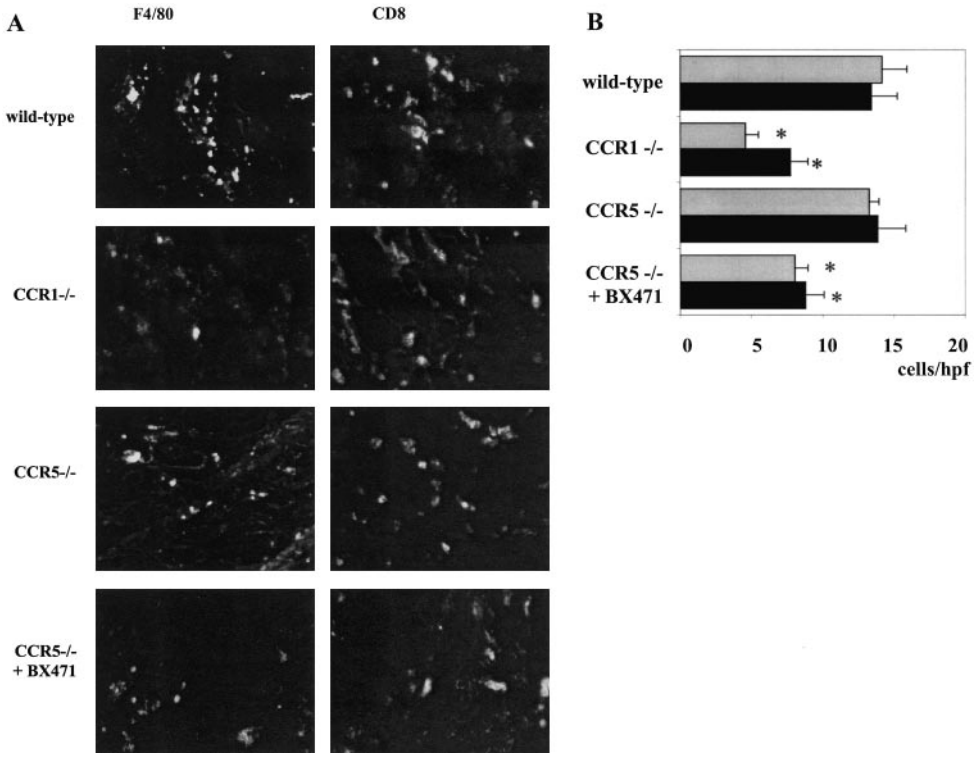

Figure 4. Renal infiltration of leukocyte subsets. (A) Wild-type mice were injected intravenously 10 d after UUO with PKH26-labeled F4/80$^+$ macrophages (left lane) or CD8$^+$ T cells (right lane) that were isolated from wild-type mice, CCR1-deficient mice, CCR5-deficient mice, and CCR5-deficient mice pretreated with BX471. Kidneys were obtained 3 h after injection and underwent fluorescence microscopy (original magnification, ×400). (B) Cell counts for labeled F4/80$^+$ macrophages (gray bars) or labeled CD8$^+$ T cells (black bars) were determined by fluorescence microscopy from 10 hpf and are expressed as means ± SD. WT, wild type; CCR −/−, mice negative for respective chemokine receptor expression. BX471 was administered in CCR5-deficient mice as described in Materials and Methods. * $P < 0.05$.

contralateral kidneys, only a weak background immunostaining for TGF-β was detected. UUO kidneys of CCR1-deficient mice had fewer interstitial TGF-β positive cells as detected by immunostaining, which correlated with less tubulointerstitial deposition of silver grains by *in situ* hybridization for TGF-β mRNA. (Figure 6B) and with the lower degree of cell infiltrate in these mice (Figure 2). In contrast, lack of CCR5 did not affect tubulointerstitial expression of TGF-β mRNA and protein (Figure 6B).

These data indicate that the source of TGF-β is in the interstitial infiltrate and not the tubular cells. Furthermore, the reduction of interstitial leukocyte infiltration observed in CCR1-deficient mice is associated with a decrease of TGF-β1 mRNA and protein, a cytokine that can stimulate epithelial-mesenchymal transformation, apoptosis, and collagen secretion by renal fibroblasts.

Discussion

We used the UUO model to study the role of CCR1 and CCR5 for leukocyte infiltration and renal fibrosis. Using combinations of genetically generated CCR1-deficient and CCR5-deficient mice, their respective leukocytes for transfer, and the CCR1 antagonist BX471, we could unequivocally demonstrate that lack or blockade of CCR1 effectively reduced the infiltration of macrophages and T cells into the UUO kidney and subsequent renal fibrosis, whereas lack of CCR5 had no effect.

CCR1 Is Required for Leukocyte Infiltration after UUO

Here we show that CCR1 is involved in renal macrophage and T cell recruitment after UUO in mice. Other studies using CCR1-deficient mice have documented its role for neutrophil migration in the defense of certain infectious organisms such as *Toxoplasma gondii*, *Paramyxovirus*, and *Aspergillus fumigatus* (15,20,21). In the context of progressive fibrotic disease states, a recent study reported the effects of a neutralizing antibody against murine CCR1 in the bleomycin-induced pulmonary fibrosis model (22). Antibodies against CCR1 but not against CCR2 reduced pulmonary mononuclear cell infiltration 10 d after induction of disease. We have recently shown in the UUO model in mice that the small molecule CCR1 antagonist BX471 reduces the amount of interstitial leukocytes (9). Previous *in vitro* studies demonstrated that CCR1 mediates leukocyte adhesion to activated endothelium of human T cells and monocytes under conditions of shear stress and flow (8,17). In addition, our studies with labeled leukocyte subsets *in vivo* clearly confirmed a role of CCR1 for macrophage and T cell recruitment into the UUO kidney in mice. As the UUO model does not involve a systemic immune response, our data are not

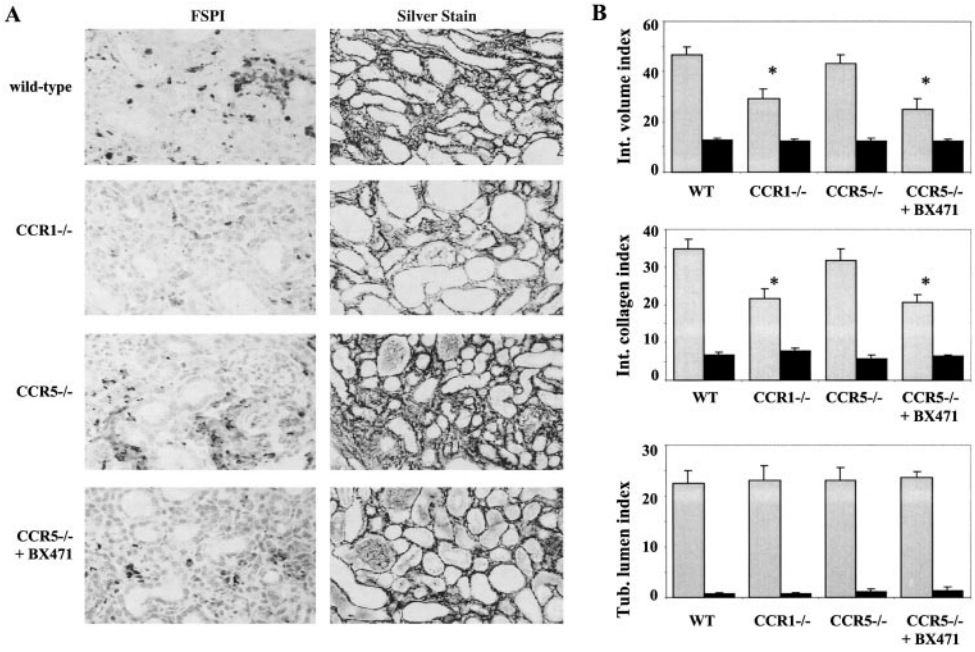

Figure 5. Morphometric analysis of tubulointerstitial injury and renal fibrosis after UUO. (A) Cortical renal sections were either stained with an FSP1-specific antibody (left) or underwent silver staining (right) as described in the Materials and Methods section. Images illustrate representative sections of UUO kidneys 10 d after UUO in mice of the respective group as indicated (original magnification, ×400). (B) The indices for interstitial volume, interstitial collagen deposition, and tubular dilatation were determined in obstructed kidneys (gray bars) and unobstructed contralateral kidneys (black bars) 10 d after UUO by quantitative morphometry as decribed in the methods section. Values represent means ± SD from 10 hight power fields of UUO (gray bars) and unobstructed contralateral kidneys (black bars). Five to seven mice were evaluated in each group. WT, wild-type; CCR −/−, mice negative for respective chemokine receptor expression. BX471 was administered in CCR5-deficient mice as described in Materials and Methods. * $P < 0.05$.

in conflict with a report that lack of CCR1 enhances Th1 responses and glomerular injury during nephrotoxic serum nephritis (10). The data on nephrotoxic nephritis would indicate that CCR1 is also involved in systemic immune responses. The fact that genetically generated CCR1 deficiency and intermittent CCR1 blockade with a specific antagonist had the same effect on renal inflammatory infiltrates and fibrosis after UUO provides powerful evidence for the role of CCR1 for macrophage and T cell recruitment after UUO and the concomitant fibrosis. Our results are consistent with the beneficial effects of CCR1 blockade on the functional outcome in renal and heart transplantation, pulmonary fibrosis, or experimental encephalomyelitis and argue for an important role of CCR1-mediated leukocyte recruitment in these models (8,23,24).

Lack of CCR5 Expression Does Not Affect Leukocyte Infiltration and Renal Fibrosis after UUO

CCR1 and CCR5 are coexpressed to different extents on macrophages and T cells. Although T cells expressed only low levels of CCR1 mRNA, the absence of CCR1 or its blockade resulted in significant reduction of renal CCR5-positive T cells in the UUO kidneys, arguing for a role of CCR1 in the recruitment of CCR5-positive cells. In contrast, despite expression of high CCR5 mRNA levels on T cells in wild-type mice, the lack of CCR5 itself did not influence renal leukocyte recruitment after UUO. Interestingly, CCR5 is involved in recruitment of leukocyte subsets in other disease models. For example, lack of CCR5 has been shown to attenuate disease activity in a model of *Aspergillus fumigatus conidia*–induced asthma, which was associated with a decrease of T cells but not of macrophages in bronchial lavage fluids (25). In a model of dextran sodium sulfate–mediated colitis, improvement of colitis in CCR5-deficient mice was also associated with impaired intestinal lymphocyte recruitment compared with wild-type controls, whereas macrophage recruitment did not depend on CCR5 expression (26). Furthermore, lack of CCR5 improved outcome after cardiac transplantation in fully MHC-mismatched mice, which was associated with reduced host T cell and macrophage recruitment to the rejected allograft (27). Interestingly, in a model of pulmonary *Cryptococcus neoformans*-infection, 12-wk survival was markedly reduced in CCR5-deficient mice, although no defects in lung leukocyte recruitment were observed (28). However, in this model, CCR5-deficient mice showed impaired leukocyte recruitment into the brain, leading to a reduction in cerebral elimination of cryptococcal polysaccharide (28). It was concluded that CCR5-mediated leukocyte trafficking is organ-specific during host defense against *C. neoformans*. In contrast, macrophage recruitment did not depend on CCR5 in experimental encephalomyelitis (29) and a model of peripheral nerve injury induced by sciatic nerve axotomy (30). The lack of an effect of CCR5 elimination on inflammatory infiltration after UUO cannot be

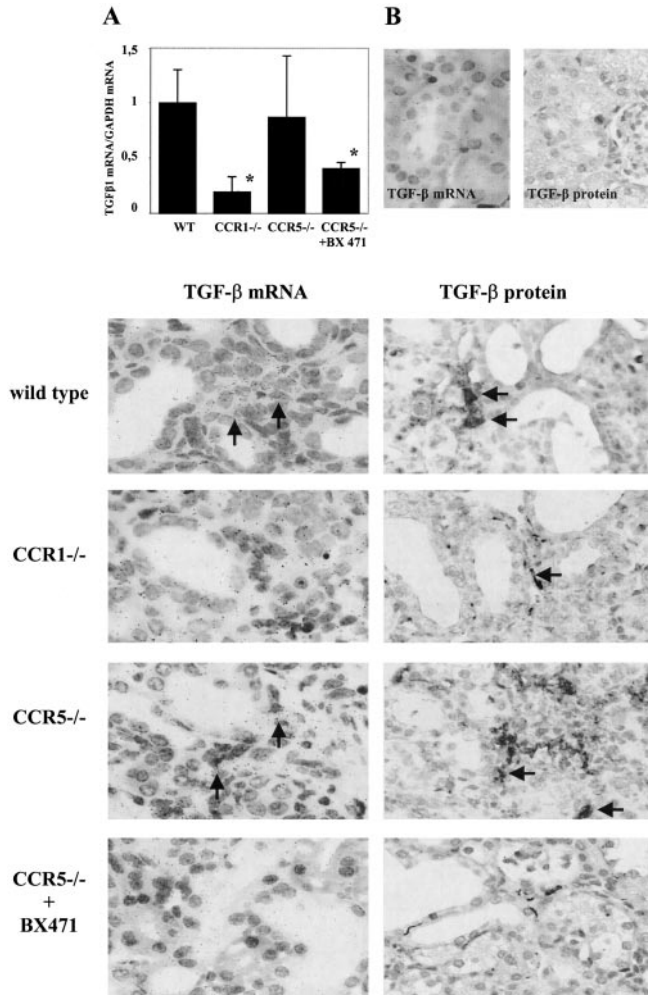

Figure 6. Renal TGF-β expression. (A) Total renal mRNA from UUO kidneys 10 d after UUO was analyzed for TGF-β1 mRNA expression by real-time RT-PCR. Levels of mRNA expression for TGF-β1 is expressed in relation to renal GAPDH mRNA expression as indicated under Materials and Methods. TGF-β1 mRNA expression of wild-type mice was set as 1 and values represent means $\pm$ SD from 5 mice per group. WT, wild-type; CCR $-/-$, mice negative for respective chemokine receptor expression. BX471 was administered in CCR5-dificient mice as described in Materials and Methods. * $P < 0.05$. (B) *In situ* hybridization for TGF-β mRNA (left column) and immunostaining for TGF-β protein (right column) in wild-type mice with UUO revealed marked TGF-β signals in interstitial cell infiltrates but only background signals in renal tubular cells of obstructed kidneys. In obstructed kidneys of CCR1-deficient mice, a reduction of TGF-β signals in interstitial infiltrates was noted. In contrast, obstructed kidneys of CCR5-deficient mice revealed similar TGF-β–positive interstitial cell infiltrates as wild-type controls. In contrast CCR1 blockade by BX471 in CCR5-deficient mice resulted in reduced inflammatory infiltrates and TGF-β signals. Immunostaining for TGF-β protein and with *in situ* hybridization using sense primers for TGF-β mRNA were negative in unobstructed contralateral kidneys (see panel upper right).

explained by lack of the chemokine ligand CCL5 in UUO kidneys. The chemokine ligand CCL5 for CCR5 is markedly expressed in UUO kidneys (6). Thus the interaction of CCL5-CCR5 may have functions other than cell recruitment in UUO. In contrast, our data clearly demonstrate the nonredundant role of CCR1 (ligands CCL3 and CCL4) for renal leukocyte recruitment after UUO in mice. The finding that CCR1 is important for renal leukocyte recruitment *in vivo* is consistent with *in vitro* data showing that CCR1 but not CCR5 is essential for the initial leukocyte attachment to endothelium (14). Initial attachment mediated by CCR1 is therefore critical for renal leukocyte recruitment. We therefore conclude that the role of CCR5 for leukocyte recruitment may vary in different disease models, which may relate differential chemokine expression patterns or to additional functions of CCR5 in these models.

Lack of CCR1 on Leukocytes Reduces Renal Fibrosis after UUO

How could the reduction in mononuclear leukocyte infiltration in CCR1-deficient mice or with CCR1 blockade relate to the concomitant reduction in interstitial fibroblasts and fibrosis? The infiltrating leukocytes could via secretion of, *e.g.* cytokines such as TGF-β, contribute to epithelial-mesenchymal transformation, fibroblast proliferation, and collagen production (2). In fact, CCR1-deficient mice showed a marked reduction of renal TGF-β1 mRNA expression, a key cytokine for the induction of fibroblast proliferation and the development of renal fibrosis (31). To localize the site of TGF-β production, we therefore performed both *in situ* hybridization and immunostaining for TGF-β. Both methods localized the TGF-β production to the interstitial cell infiltrate. Compared with real-time RT-PCR data, both *in situ* hybridization and immunostaining signals for TGF-β were reduced in UUO kidneys of CCR1-deficient mice and in mice treated with the CCR1 antagonist. The resolution of both methods did not allow assignment of the signal to specific cells in the infiltrate. Clearly, however, the TGF-β is not coming from the tubular epithelial cells or from the interstitium in areas without inflammatory cell infiltrate. It therefore appears reasonable to assign the TGF-β signals to inflammatory cells, *i.e.*, the infiltrating mononuclear leukocytes. This interpretation is also consistent with another report that localized TGF-β to infiltrating leukocytes in the same model using the same TGF-β antibody (32). It appears that the reduction in TGF-β mRNA and immunostaining in the UUO kidney from CCR1-deficient mice or with CCR1 receptor blockade relates directly to the reduced number of infiltrating cells observed under these conditions and is less likely a result of TGF-β production per infiltrating cell. In fact, *in vitro* studies with peritoneal macrophages from mice of all three strains expressed comparable amounts of TGF-β mRNA after stimulation with LPS (unpublished data).

In summary, CCR1 appears to play an important role in mediating the infiltration of mononuclear cells after UUO. Lack of CCR1 reduced the infiltration of macrophages and lymphocytes in the obstructed kidney and the resultant interstitial fibrosis. In contrast, lack of CCR5 had no effect on leukocyte infiltration in the UUO model. Previous studies have demonstrated a similar effect with a CCR1 antagonist; we therefore propose that CCR1 blockade may offer a new therapeutic strategy to reduce renal leukocyte infiltration and re-

sulting fibrosis in chronic nephropathies leading to end-stage renal disease or even leukocyte-mediated progressive tissue fibrosis in general.

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