Late Onset of Treatment with a Chemokine Receptor CCR1 Antagonist Prevents Progression of Lupus Nephritis in MRL-Fas(lpr) Mice

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Abstract. Slowly progressive renal injury is the major cause for ESRD. The model of progressive immune complex glomerulonephritis in autoimmune MRL[lpr/lpr] mice was used to evaluate whether chemokine receptor CCR1 blockade late in the disease course can affect progression to renal failure. Mice were treated with subcutaneous injections of either vehicle or BX471, a nonpeptide CCR1 antagonist, three times a week from week 20 to 24 of age. BX471 improved blood urea nitrogen levels (BX471, 35.1 ± 5.3; vehicle, 73.1 ± 39.6 mg/dl; $P < 0.05$) and reduced the amount of ERHR-3 macrophages, CD3 lymphocytes, Ki-67 positive proliferating cells, and ssDNA positive apoptotic cells in the interstitium but not in glomeruli. Cell transfer studies with fluorescence-labeled T cells that were pretreated with either vehicle or BX471 showed that BX471 blocks macrophage and T cell recruitment to the renal interstitium of MRL[lpr/lpr] mice. This was associated with reduced renal expression of CC chemokines CCL2, CCL3, CCL4, and CCL5 and the chemokine receptors CCR1, CCR2, and CCR5. Furthermore, BX471 reduced the extent of interstitial fibrosis as evaluated by interstitial smooth muscle actin expression and collagen I deposits, as well as mRNA expression for collagen I and TGF-β. BX471 did not affect serum DNA autoantibodies, proteinuria, or markers of glomerular injury in MRL[lpr/lpr] mice. This is the first evidence that, in advanced chronic renal injury, blockade of CCR1 can halt disease progression and improve renal function by selective inhibition of interstitial leukocyte recruitment and fibrosis.

Progressive tubulointerstitial fibrosis is the main predictor for the progression to ESRD irrespective of the trigger mechanism (1). In patients with chronic renal failure, renal histology is characterized by a mixed tubulointerstitial inflammatory cell infiltrate and increased matrix deposition leading to tubular atrophy (2). During this process, infiltrating macrophages and lymphocytes are a major source of inflammatory mediators such as cytokines, nitric oxide, and growth factors. Inhibition of leukocyte infiltration may reduce the production of such mediators and therefore may be an option to prevent or to delay ESRD.

The leukocytic cell infiltrate is triggered by locally secreted chemokines (3). *In vitro* studies suggest a role for CCR1 in

leukocyte adhesion and transendothelial migration (4), which may explain the beneficial effects of CCR1 antagonists in certain disease models, including pulmonary fibrosis (5) as well as in heart and renal transplant rejection (6,7). Using the model of unilateral ureteral obstruction in mice, we showed recently that blockade of CCR1 with the nonpeptide antagonist BX471 reduced leukocyte infiltration even when treatment was started at a time when renal fibrosis was already present (8). These data indicate that CCR1 blockade is a potential target for therapeutic intervention of progressive renal fibrosis. As chemokines are also involved in systemic immune responses (3), data from the unilateral ureteral obstruction model may not apply to renal manifestations of systemic autoimmunity, *e.g.*, lupus nephritis. In fact, lack of CCR1 has been reported to modulate the course of nephrotoxic serum nephritis in mice in association with a Th1-like immune response (9). We therefore studied the effects of therapeutic CCR1 blockade in progressive renal injury of lupus-like nephritis in MRL[lpr/lpr] mice, an autoimmune disease that leads to progressive immune complex glomerulonephritis with tubulointerstitial disease resulting in end-stage renal failure that resembles human lupus nephritis. We recently characterized the expression of chemokines and

chemokine receptors during the course of this model and found that, among other chemokine receptors, CCR1 and its chemokine ligand CCL3 are expressed in kidneys of MRL[lpr/lpr] mice (10). We hypothesized that the CCR1 antagonist BX471 might improve renal outcome during the progressive phase of disease by inhibiting renal leukocyte recruitment. This hypothesis proved to be correct for the interstitial compartment but not for the glomerular leukocyte recruitment.

Materials and Methods

Animals and Experimental Protocol

Ten-week-old female MRL[lpr/lpr] mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and housed in groups of five 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. All experimental procedures were performed according to the German animal care and ethics legislation and were approved by the local government authorities. At week 20 of age, mice were distributed into two groups ($n = 8$–10) that received subcutaneous injections three times a week until week 24 as follows: vehicle group, 50 μl of 40% cyclodextrin (#33260-7; Sigma-Aldrich, Deisenhofen, Germany) prepared as described previously (8); and BX471 group, 50 mg/kg BX471 in 50 μl of vehicle. BX471 is a nonpetide antagonists that is 10,000-fold more specific for CCR1 than for 32 other G protein–coupled receptors including the chemokine receptors CXCR3, CCR2, and CCR5 (8,10,11) (R. Horuk, personal communication). All mice were killed by cervical dislocation at the end of week 24 of age.

Evaluation of Glomerulonephritis

Blood samples were collected from each animal at the end of the study by bleeding from the retro-orbital venous plexus under general anesthesia with inhaled ether. After centrifugation, all serum samples were stored at $-80°C$ until analysis. Spot urine samples were collected from each animal at the end of the study for determination of proteinuria. The following parameters were determined using standard analytical protocols as described previously (10): Bradford assay for urine protein concentration, urease/glutamate dehydrogenase method for blood urea nitrogen (BUN) measurements (Merck Diagnostika), IgG ELISA for analysis of DNA autoantibodies using the following antibodies for detection: IgG$_1$ (Pharmingen, Hamburg, Germany; 1:100) and IgG$_{2a}$ (Dianova, Hamburg, Germany; 1:100). The left kidney from each mouse was fixed in 4% buffered formalin, processed, and embedded in paraffin. Sections for Silver and hematoxylin-eosin stains were prepared as described (8,12). The severity of the renal lesions was graded using the indices for activity and chronicity as described for human lupus nephritis (13) and previously used for this murine model (10). All morphologic evaluations were performed by a renal pathologist who was unaware of the source of the tissue.

Immunohistology

Paraffin-embedded sections were prepared as described (12). As primary antibodies, a rat anti–ERHR-3 (1:50, monocytes/macrophages; DPC Biermann, Bad Nauheim, Germany), a rat anti-CD3 (1:100, T lymphocytes, clone CD3-12; Serotec, Raleigh, NC), a mouse anti–smooth muscle actin (SMA; 1:100, myofibroblasts, clone 1A4; Dako, Carpinteria, CA), an anti–collagen I (LF-67, 1:50; provided by Dr. L.W. Fischer, National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, MD), an anti-CCL5 (1:50; Peprotech, Rocky Hill, NJ), a rabbit anti-CCL2 (1:20, rabbit antiserum, prepared as described (12)), anti–Ki-67 (1:25, cell proliferation; Dianova), anti-ssDNA (1:50, apoptotic cells; Chemicon, Hofheim, Germany) were applied. Staining for immunoglobulins was performed on acetone-fixed frozen section using anti-IgG$_1$ (rabbit, 1:50; Dianova) and anti-IgG$_{2a}$ (rabbit, 1:100; Dianova) as detection antibodies. For quantitative analysis, glomerular cells were counted in 10 cortical glomeruli per section and interstitial cells in 10 high-power fields per section selected by uniform random sampling, from each animal. For the assessment of glomerular Ig and complement deposits, 15 cortical glomeruli were analyzed from each section. Glomerular signals were scored using a semiquantitative index as follows: $0 =$ no signal, $1 =$ low signal, $2 =$ moderate signal, and $3 =$ strong signal intensity. For the quantification of interstitial collagen I immunostaining, digital pictures of 10 random high-power fields were taken using a digital camera (DC 300F; Leica Microsystems, Cambridge, UK). The area of positive staining for collagen I was measured and expressed as percentage using image analysis software (Leica Imaging Solutions, Cambridge, UK).

In Situ Hybridization

In situ hybridization for murine TGF-β1 was performed as described previously (14). The TGF-β1 probe was a gift from H.L. Moses (Department of Cell Biology, Vanderbilt University, Nashville, TN). Negative controls included hybridization performed on replicate tissue sections using the sense riboprobe.

RNA Preparation and RNase Protection Assay

Renal tissue from each mouse was snap-frozen in liquid nitrogen and stored at $-80°C$. From each animal, total renal RNA was prepared as described (12). Multiprobe template sets (mouse CC chemokines; Pharmingen, San Diego, CA) and 20 μg of total kidney RNA were used to perform RNase protection assays as described (12). Efficacy of RNase digestion was ensured by a yeast t-RNA sample in each assay. Gels were dried and exposed on phosphor screens of a Storm 840 PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Bands were quantified using the ImageQuant software (Molecular Dynamics).

Real-Time Quantitative (TaqMan) Reverse Transcription–PCR

Reverse transcription from total renal RNA was performed as described (12). Real time reverse transcription–PCR (RT-PCR) was performed on a TaqMan ABI 7700 Sequence Detection System (PE Biosystems, Weiterstadt, Germany) using a heat-activated *Taq*DNA polymerase (Amplitaq Gold; PE Biosystems) as described previously (11). Controls that were composed of ddH$_2$O were negative for target and housekeeper genes. Primers and probes were from PE Biosystems. Oligonucleotide primer (300 nM) and probes (100 nM) were used as described: murine GAPDH (8); murine collagen I (8); murine CCR1: forward 5'-TTAGCTTCCATGCCTGCCTTATA-3', reverse 5'-TCCACTGCTTCAGGCTCTTGT-3'; internal fluorescence labeled probe (FAM): 5'-ACTCACCGTACCTGTA-GCCCTCAT-TTCCC-3'; murine CCR2: forward 5'-CCTTGG-GAATGAGTAACTGTGTGA-3', reverse 5'-ACAAAGGCATAAATGACAGGATTAATG-3'; FAM: 5'-TGACAAGCACTTAGAC-CAGGCCATGCA-3'; CCR5: forward 5'-CAAGACAATCCTGATCGTGCAA-3', reverse 5'-TCCTACTC-CCAAGCTGCATAGAA-3'; FAM: 5'-TCTATACCCGATCCACAG-GAG-AACATGAAGTTT-3'; murine TGF-β1: forward 5'-CACAGT-ACAGCAAGGTCCTTGC-3', reverse 5'-AGTAGACGAT-GGGCA-GTGGCT-3'; fluorescence labeled probe (FAM): 5'-GCTTCGGCGT-

CACCGTGCT-3'; murine GAPDH: forward 5'-CATGGCCTTCCGT-GTTCCTA-3', reverse 5'-ATGCCTGCTTCACCACCTTCT-3'; internal fluorescence labeled probe (VIC): 5'-CCCAATGTGTCCGTCGT-GGATCTGA-3'.

CCR1 Expression of Leukocytes and Intrinsic Renal Cells

For assessing CCR1 mRNA expression in macrophages, T cells or intrinsic renal cells were prepared from MRL[lpr/lpr] mice as follows: Macrophages and T cells were isolated from spleens by immunomagnetic selection as described (15). Tubular segments were microdissected from RNase inhibitor–treated tissue in ice-cold PBS, as described previously for human renal biopsies (16). For isolation of primary renal fibroblasts, small pieces of renal tissue were incubated in DMEM (Invitrogen, Karlsruhe, Germany) supplemented with 10% FCS (Invitrogen), penicillin, and streptomycin for 16 d. Adherent cells were obtained by treatment with 1.5 mM EDTA (Calbiochem-Novabiochem, San Diego, CA) and were depleted of leukocytes by immunomagnetic selection using FITC anti-mCD45 (Pharmingen) and anti-FITC MicroBeads as described (Miltenyi Biotec, Bergisch Gladbach, Germany). mRNA of isolated cells was prepared by standard methods (8). Baseline CCR1 mRNA expression was determined by real-time RT-PCR as above.

In Vivo Assay of Renal T Cell Infiltration

ERHR-3–positive macrophages and CD8 T cells were prepared from spleens of MRL[lpr/lpr] mice by a previously described isolation and labeling method (15). In brief, spleen cells were isolated by immunomagnetic selection using anti-CD8 (Ly-2) and anti–ERHR-3 MicroBeads (Miltenyi Biotec). 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. Viability as assessed by trypan blue exclusion was >90%. Twenty-week-old MRL[lpr/lpr] mice received an injection of either 3.5×10^5 CD8-positive T cells or ERHR-3–positive macrophages in 200 μl of isotonic saline through tail vein. Two groups of mice received an injection of either labeled T cells or macrophages that were preincubated with 600 μM of the CCR1 antagonist BX471 for 30 min. Mice in these groups received a single subcutaneous injection of BX471 (50 mg/kg). Renal tissue was obtained after 3 h, snap-frozen, and prepared for microscopy.

Statistical Analyses

Data were expressed as mean ± SEM. Comparison of groups was performed using unpaired t test. $P < 0.05$ was considered to indicate statistical significance.

Results

Renal Disease of Autoimmune MRL[lpr/lpr] Mice at 24 Weeks of Age

Renal Function.   At 24 wk of age, MRL[lpr/lpr] mice had impaired renal function with serum BUN levels elevated to 73 mg/dl (Table 1). As a marker of glomerular damage, marked proteinuria was present (Table 1).

Glomerular Injury.   At 24 wk, kidneys of vehicle-treated MRL[lpr/lpr] mice revealed diffuse mesangioproliferative glomerulonephritis with crescents and marked proteinuria (Figure 1). Glomeruli showed few ERHR-3–positive macrophages. A mixed periglomerular inflammatory cell infiltrate that con-

sisted of ERHR-3–positive macrophages and CD3-positive lymphocytes was present around glomerular crescents (Figure 1).

Interstitial Injury.   Kidneys showed diffuse tubulointerstitial disease with tubular atrophy, inflammatory cell infiltrates, and confluent areas of interstitial fibrosis (Figure 1).

Renal Expression of CCR1 in MRL[lpr/lpr] Mice

As appropriate antibodies that allow detection of CCR1 protein by cell fluorescence or immunostaining in mice were not available, we used real-time RT-PCR to determine the expression of CCR1 mRNA in kidneys of MRL[lpr/lpr] mice. Kidneys of 24-wk-old MRL[lpr/lpr] mice showed a marked induction of CCR1 mRNA compared with MRL wild-type mice of the same age (Figure 2), a finding that is consistent with our previously reported analysis using RNase protection assays from kidneys of MRL[lpr/lpr] mice (10). For determining the source of renal CCR1 expression, tubular segments were microdissected manually from kidneys of the same MRL[lpr/lpr] mice and primary renal fibroblasts were isolated from kidneys of MRL[lpr/lpr] mice as described in Materials and Methods. Furthermore, we isolated macrophages and T cells from spleens of MRL[lpr/lpr] mice by magnetic bead isolation. Real-time RT-PCR for CCR1 was performed with RNA isolates from all types of cells prepared. Both macrophages and CD8 T cells expressed CCR1 mRNA, but CCR1 mRNA transcripts were not detected in tubular epithelial cells or renal fibroblasts (Figure 2B). These data indicate that renal CCR1 expression does not originate from renal tubular cells or interstitial fibroblasts; in contrast, CCR1 positive macrophages and T cells may contribute to renal CCR1 expression after infiltrating the kidney.

CCR1 Blockade with BX471 Does Not Affect the Humoral Immune Response in MRL[lpr/lpr] Mice

Lack of CCR1 has been reported to modulate the course of nephrotoxic serum nephritis in mice associated with a Th1-shift of the immune response (9). We therefore examined parameters of the Th1/Th2 balance of systemic autoimmunity in MRL[lpr/lpr] mice. First, we studied serum titers of DNA autoantibodies of the IgG$_1$ and IgG$_{2a}$ isotype, because an increase of IgG$_{2a}$ autoantibodies would indicate a shift toward a Th1 response (16). No difference in serum titers for DNA autoantibodies of either IgG isotypes was found between BX471- and vehicle-treated MRL[lpr/lpr] mice (Table 1). Furthermore, the amount of mesangial IgG immune complex deposits evaluated by semiquantitative scoring of renal sections showed a comparable extent of mesangial IgG$_1$ and IgG$_{2a}$ deposits in BX471- and vehicle-treated mice (Table 1). Taken together, these findings argue against a shift of the Th1/Th2 balance by CCR1 blockade with BX471.

CCR1 Blockade with BX471 Reduces Renal Damage in MRL[lpr/lpr] Mice

Renal Function.   Daily treatment with BX471 from 20 to 24 weeks of age significantly improved renal function as illustrated by a reduction of serum BUN levels compared to

Table 1. Serum, urinary, and histologic findings in MLR[lpr/lpr] mice[a]

	Vehicle ($n = 10$)	BX471 ($n = 8$)
Functional parameters		
BUN (mg/dl)	73.1 ± 39.6	35.1 ± 5.3^{b}
proteinuria (μg/mg creatinine)	2179 ± 1459	1214 ± 1047
body weight (g)	37.0 ± 2.75	36.6 ± 3.4
Histologic scores		
activity index	8.0 ± 4.6	4.0 ± 1.9
chronicity index	2.9 ± 3.6	0.1 ± 0.1^{b}
Cellular response (cells/glomerulus or hpf)		
glomerular		
EHR3+	1.2 ± 1.2	1.1 ± 0.3
CD3+	0.3 ± 0.1	0.4 ± 0.1
Ki-67+	5.8 ± 1.4	5.8 ± 1.1
interstitial		
EHR3+	15.3 ± 11.8	1.9 ± 0.4^{b}
CD3+	26.3 ± 10.8	12.9 ± 3.8^{b}
Ki-67+	7.2 ± 1.6	2.6 ± 0.9^{b}
ssDNA+	1.5 ± 0.8	0.4 ± 0.2^{b}
tubular		
Ki-67+	6.5 ± 1.0	3.3 ± 1.3^{b}
ssDNA+	1.0 ± 0.6	0.3 ± 0.3^{b}
Humoral response		
serum titers		
Anti-DNA IgG$_1$	6963 ± 4751	6162 ± 3611
Anti-DNA IgG$_{2a}$	5325 ± 2621	6349 ± 4014
IgG$_{2a}$/IgG$_1$ ratio	0.7 ± 0.6	1.0 ± 1.1
glomerular deposit score		
IgG$_1$	1.3 ± 0.4	1.2 ± 0.5
IgG$_{2a}$	0.8 ± 0.3	0.9 ± 0.4
IgG$_{2a}$/IgG$_1$ ratio	0.6 ± 0.7	0.7 ± 0.7

[a] BUN, blood urea nitrogen. Values are means $\pm$ SEM.
[b] $P < 0.05$ BX471 *versus* vehicle.

vehicle-treated MRL[lpr/lpr] mice (Table 1). In contrast, BX471-treatment did not affect proteinuria and body weight compared to vehicle-treated MRL[lpr/lpr] mice (Table 1).

Glomerular Injury. Daily treatment with BX471 did not significantly affect the extent of mesangioproliferative glomerulonephritis, the number of ERHR-3–positive glomerular macrophages, and the number of Ki-67–positive proliferating glomerular cells compared with vehicle-treated controls (Table 1). Apoptotic cells were rarely detected in glomeruli of both groups by immunostaining with an anti-ssDNA antibody (not shown). No significant difference in the activity index was found between the two groups. This index includes mostly histopathologic abnormalities of the glomerular compartment in lupus nephritis, such as proliferation of the mesangium, glomerular leukocyte infiltration, mesangial matrix, focal glomerular necrosis, and cellular crescents (13).

Interstitial Injury. In contrast to the lack of effect on glomerular inflammation and proteinuria and consistent with improved serum BUN levels, BX471 markedly reduced the extent of tubulointerstitial disease compared with vehicle-treated control mice. In BX471-treated mice, no tubular atrophy or confluent areas of interstitial fibrosis were observed. Furthermore, there was a marked reduction in periglomerular and interstitial accumulation of ERHR-3–positive macrophages and CD3 lymphocytes compared with vehicle-treated control mice (Figure 1). Immunostaining for KI-67–positive proliferating cells and ssDNA-positive apoptotic cells was performed as markers of cell turnover in the renal tubulointerstitium. BX471-treated mice showed a marked reduction of KI-67– and ssDNA-positive tubular cells as well as interstitial cells compared with vehicle-treated mice (Table 1). Immunostaining for SMA-positive myofibroblasts and interstitial collagen I deposits was performed as additional markers of interstitial fibrosis. BX471-treated mice showed a marked reduction of SMA-positive cells and collagen I deposits in the interstitium compared with those in vehicle-treated mice (Figure 3A). The latter finding was confirmed by real-time RT-PCR for renal collagen I mRNA expression, which showed a 10-fold reduction by treatment with BX471 (Figure 3B). Quantitative analysis of interstitial immunostaining for collagen I by auto-
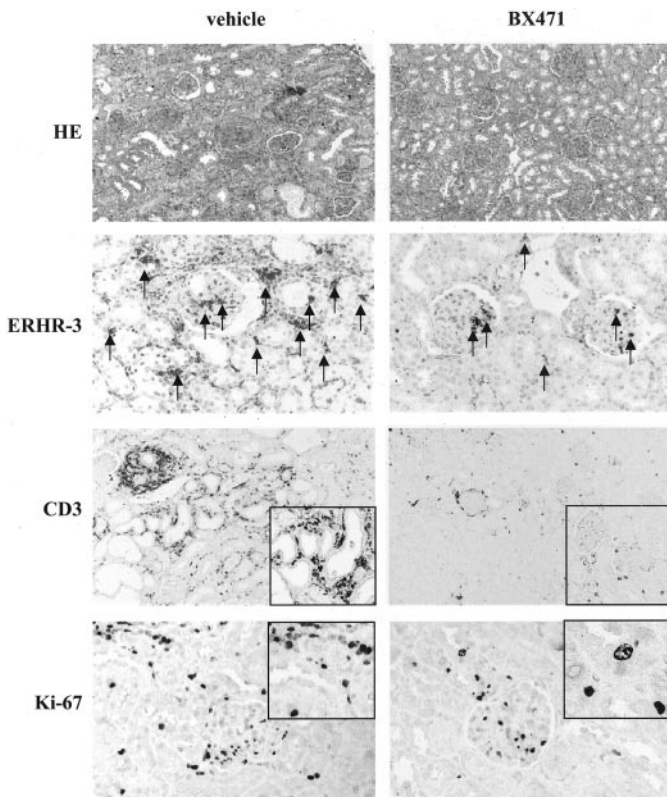
Figure 1. Renal histopathology in MRL[lpr/lpr] mice. At 24 wk of age vehicle-treated MRL[lpr/lpr] mice had proliferative glomerulonephritis (GN) and diffuse interstitial fibrosis and tubular atrophy. BX471-treated MRL[lpr/lpr] mice also had proliferative GN but no major tubulointerstitial abnormalities. HE, hematoxylin eosin staining. ERHR-3–positive macrophages and Ki-67–positive proliferating cells were present in glomeruli and the interstitium of both groups. CD3-positive cells were present only in the interstitial compartment but were not detected in glomeruli of either group. BX471 markedly reduced accumulation of ERHR-3–positive macrophages, KI-67–positive cells, and CD3-positive lymphocytes in the interstitial compartment compared with vehicle-treated mice. For quantification, see Table 1. Images illustrate representative sections of kidneys from 8 to 10 mice of respective groups at 24 wk of age. Magnifications: ×40; ×100 in inserts.

mated digital evaluation of the collagen I–positive area per high-power field demonstrated a 2.5-fold reduction in the BX471-treated group compared with vehicle-treated MRL[lpr/lpr] mice (Figure 3C). These findings are also illustrated by a significant reduction of the chronicity index between the two groups (Table 1). This index evaluates histopathologic abnormalities of the interstitial compartment in lupus nephritis, such as the extent of tubular atrophy and interstitial fibrosis (13).

CCR1 Blockade with BX471 Reduces Renal Expression of Chemokines and Chemokine Receptors in MRL[lpr/lpr] Mice

To study whether treatment with BX471 affects renal chemokine expression in MRL[lpr/lpr] mice, we performed RNase protection assays for CC chemokines from renal RNA isolates.
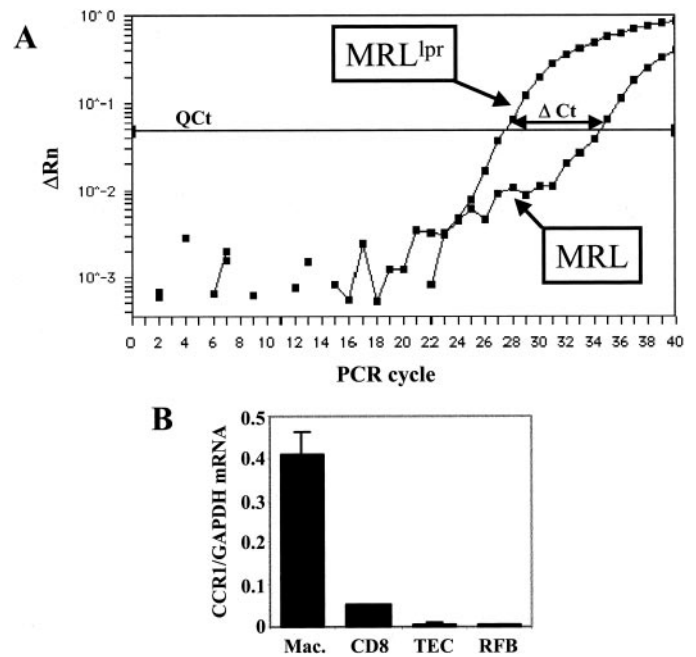

Figure 2. Renal CCR1 expression in MRL[lpr/lpr] mice. (A) Quantitative real-time reverse transcription–PCR (RT-PCR) analysis was performed on total cDNA derived from kidneys of 24-wk-old MRL[lpr/lpr] mice or MRL wild-type controls. The cDNA was amplified using primers specific for mCCR1 for 40 PCR cycles. CCR1 mRNA expression in kidneys of MRL[lpr/lpr] mice is indicated by a left shift of the amplification profile in real-time RT-PCR. The data shown are from a single mouse of each group and are representative of duplicate analysis of four mice of each group. (B) The expression of CCR1 mRNA was assessed by real-time PCR in macrophages, CD8-positive T cells, and renal cells isolated from MRL[lpr/lpr] mice as described in Materials and Methods. CCR1 mRNA levels are expressed in relation to the respective GAPDH mRNA expression. Macrophages and CD8-positive T cells expressed CCR1, in contrast to isolated renal fibroblasts and microdissected tubular segments of MRL[lpr/lpr] mice.

At 24 wk, kidneys of vehicle-treated MRL[lpr/lpr] mice contained mRNA for various CC chemokines, such as CCL5, CCL2, CCL4, and the CCR1 ligand CCL3. In contrast, kidneys of healthy MRL wild-type mice contained no detectable mRNA levels for these chemokines (Figure 4A). Treatment with BX471 reduced renal expression of CCL2, CCL3, CCL4, and CCL5 compared with vehicle-treated MRL[lpr/lpr] mice (Figure 4, A and B). To localize further the source of renal CCL2 and CCL5 expression, we performed immunostaining for these chemokines (Figure 4B). At 24 wk, CCL2 immunostaining localized to glomerular cells and interstitial cell infiltrates but not to tubular epithelial cells. Treatment with BX471 markedly reduced immunostaining for CCL2 and CCL5 in the renal interstitium of MRL[lpr/lpr] mice (Figure 4C).

To study the effect of CCR1 blockade on renal chemokine receptor expression, we performed real-time RT-PCR for the chemokine receptors CCR1, CCR2, and CCR5. Previously, we found these receptors to be progressively upregulated in kidneys of MRL[lpr/lpr] mice during progression of renal disease (10). Treatment with BX471 from 20 to 24 weeks of age
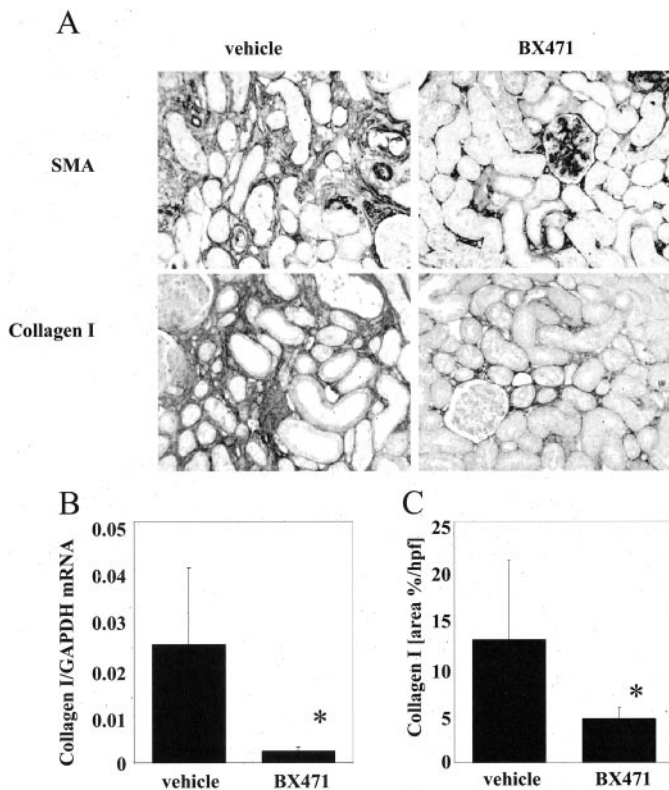
Figure 3. Renal fibrosis in MRL[lpr/lpr] mice. (A) At 24 wk of age, vehicle-treated MRL[lpr/lpr] mice had diffuse interstitial smooth muscle actin (SMA) and collagen I immunostaining in glomeruli and areas of interstitial fibrosis. In contrast, BX471-treated MRL[lpr/lpr] mice had only minimal interstitial SMA and collagen I protein expression. Images illustrate representative sections of kidneys from the respective groups at 24 wk of age. (B) The renal mRNA expression for collagen I was determined by real-time RT-PCR using total renal RNA of five to seven mice for each group. Collagen I mRNA levels for vehicle- and BX471-treated MRL[lpr/lpr] mice are expressed in relation to respective renal GAPDH mRNA; *$P < 0.02$. (C) Quantification of renal immunostaining for collagen I was performed by automated digital analysis as described in Materials and Methods. Values are expressed as collagen I–positive area per high-power field ($\times$40) in percentage and are from seven to nine mice of each group; *$P < 0.02$. Magnification, $\times$40.

reduced renal expression of the chemokine receptors CCR1, CCR2, and CCR5 compared with vehicle-treated controls (Figure 5). Taken together, late onset of treatment with BX471 reduced renal expression of chemokines and chemokine receptors in kidneys of MRL[lpr/lpr] mice.

Pretreatment of Leukocytes with the CCR1 Antagonist Blocks Their Recruitment to the Kidney in MRL[lpr/lpr] Mice

CCR1 has been shown to mediate macrophage and T cell adhesion and subsequent transendothelial migration *in vitro* under conditions of shear stress and flow (4). Thus, BX471-induced reduction of interstitial leukocyte infiltration in kidneys of MRL[lpr/lpr] mice could be related to blockade of CCR1-dependent leukocyte recruitment. We therefore studied the
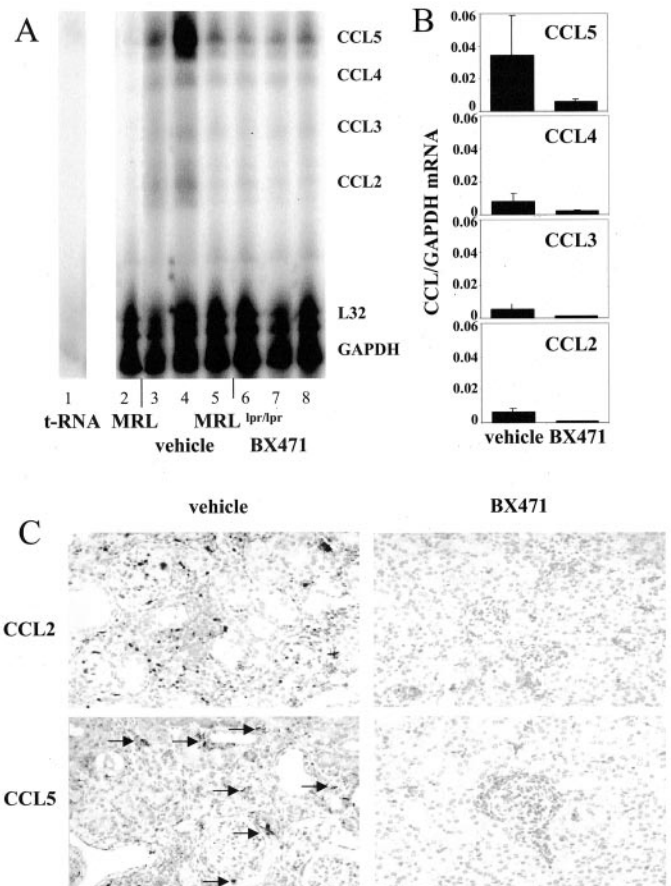

Figure 4. Renal chemokine expression in MRL[lpr/lpr] mice. (A) The renal chemokine mRNA expression was determined by RNase protection assay using total renal RNA from each group at the end of the study. The unprotected probe is shown on the left, and the protected fragments are indicated on the right. Healthy mice of the MRL strain did not reveal renal chemokine expression compared with diseased kidneys of MRL[lpr/lpr] mice. Treatment with BX471 reduced renal mRNA expression of CCL2, CCL3, CCL4, and CCL5. The illustration is representative of assays on tissue from three to four mice of each group. (B) Bands from RNase protection analysis were quantified using the ImageQuant software as described in Materials and Methods. Values are expressed as CCL per respective GAPDH mRNA expression for both groups as indicated. (C) Spatial chemokine expression in affected kidneys of MRL[lpr/lpr] mice was determined by immunostaining for CCL2 and CCL5 as described in Materials and Methods. Arrows indicate CCL5-positive cells. Images illustrate representative sections of kidneys from the respective groups at 24 wk of age. Magnification, $\times$40.

effect of BX471 on recruitment of CCR1-positive renal macrophages and T cells in MRL[lpr/lpr] mice. ERHR-3–positive macrophages and CD8-positive T cells were isolated from spleens of MRL[lpr/lpr] mice, fluorescence labeled, and incubated with BX471 or vehicle for 1 h before intravenous injection into 20-wk-old MRL[lpr/lpr] mice. Three hours later, the number of labeled cells was determined by fluorescence microscopy of frozen sections of renal tissue. All labeled macrophages and T cells that were detected in kidneys of MRL[lpr/lpr] mice localized to the interstitial area, whereas glomeruli and perivascular
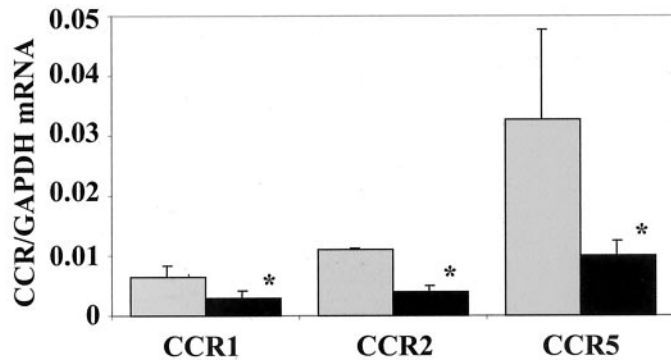
Figure 5. Renal chemokine receptor mRNA expression in kidneys of MRL$^{lpr/lpr}$ mice. (A) The chemokine mRNA expression was determined by real-time RT-PCR using total renal RNA of five to seven mice from each group. CCR mRNA levels for vehicle- (▨) and BX471-treated (■) MRL$^{lpr/lpr}$ mice are expressed in relation to respective GAPDH mRNA expression of each kidney. *$P < 0.05$.

Figure 6. Renal infiltration of labeled leukocytes in kidneys of MRL$^{lpr/lpr}$ mice. (A) MRL$^{lpr/lpr}$ mice 20 wk of age received an intravenous injection of PKH26-labeled ERHR-3 macrophages or CD8 T cells. The cells were isolated from MRL$^{lpr/lpr}$ mice, labeled, and 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. Single fluorescence-labeled cells locate to the renal interstitium. (B) Cell counts for interstitial fluorescence-labeled ERHR-3 macrophages and CD8 T cells were determined by fluorescence microscopy from 10 high-power fields and are expressed as means ± SEM. ▨, vehicle-treated cells; ■, BX471-treated cells; *$P < 0.05$. Magnification, ×400.

fields were negative for these cell types. Pretreatment with BX471 significantly reduced the amount of the injected labeled ERHR-3 macrophages and CD8 T cells that infiltrated into the interstitium of kidneys of MRL$^{lpr/lpr}$ mice (Figure 6). These data indicate that BX471 blocks CCR1-dependent macrophage and T cell recruitment into the interstitium of kidneys of MRL$^{lpr/lpr}$ mice.

Reduced Interstitial Leukocyte Infiltration in BX471-Treated MRL$^{lpr/lpr}$ Mice Is Associated with a Decrease of Renal TGF-β1 mRNA Expression

We were intrigued by the finding that the extent of renal fibrosis in BX471- and vehicle-treated MRL$^{lpr/lpr}$ mice directly paralleled the amount of interstitial leukocytes. As renal fibroblasts were negative for CCR1, the blockade of CCR1 could not directly affect fibroblast activation. In contrast, the observed reduction of renal fibrosis could be secondary to reduced secretion of profibrotic cytokines, *e.g.*, TGF-β. We therefore determined the expression of TGF-β mRNA in total renal RNA by real-time RT-PCR. Kidneys from BX471-treated mice had an 85% reduction of TGF-β mRNA expression compared with vehicle-treated MRL$^{lpr/lpr}$ mice (Figure 7A). These data show that at a late stage of nephritis in MRL$^{lpr/lpr}$ mice, BX471 reduces renal leukocyte infiltration and TGF-β mRNA expression, a cytokine that has been implicated in renal fibrosis. To determine the source of renal TGF-β, we performed *in situ* hybridization for TGF-β. In sense control kidneys of vehicle-treated MRL$^{lpr/lpr}$ mice, the *in situ* hybridization yielded only a weak diffuse deposition of silver grains (not shown). The strongest signal for TGF-β mRNA was found in areas of tubulointerstitial infiltrates (Figure 7B). The resolution of the *in situ* hybridization was not sufficient to assign the signal in the infiltrate to specific cells. In areas without prominent cell infiltration, only background signal was present, similar to incubation with sense controls. No clear tubular expression of TGF-β mRNA was apparent. These results suggest that the source of TGF-β is in the interstitial
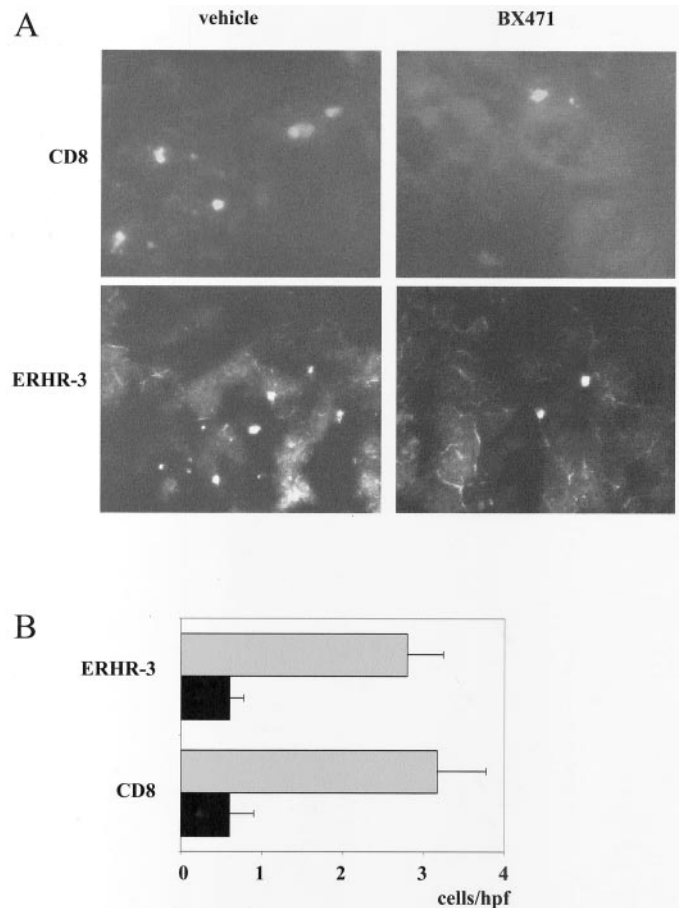
infiltrate and not the tubular cells. Furthermore, the reduction of interstitial leukocyte infiltration observed in BX471-treated MRL$^{lpr/lpr}$ mice is associated with a decrease in levels of TGF-β mRNA and protein, a cytokine that can stimulate epithelial-mesenchymal transformation, apoptosis, and collagen secretion by renal fibroblasts.

Discussion

We hypothesized that late onset of CCR1 blockade with BX471 would improve renal disease in MRL$^{lpr/lpr}$ mice by inhibiting renal leukocyte recruitment. This hypothesis proved to be correct as demonstrated by histologic evaluation of leukocyte infiltrates of the interstitial compartment and further illustrated by transfer studies with labeled macrophages and T
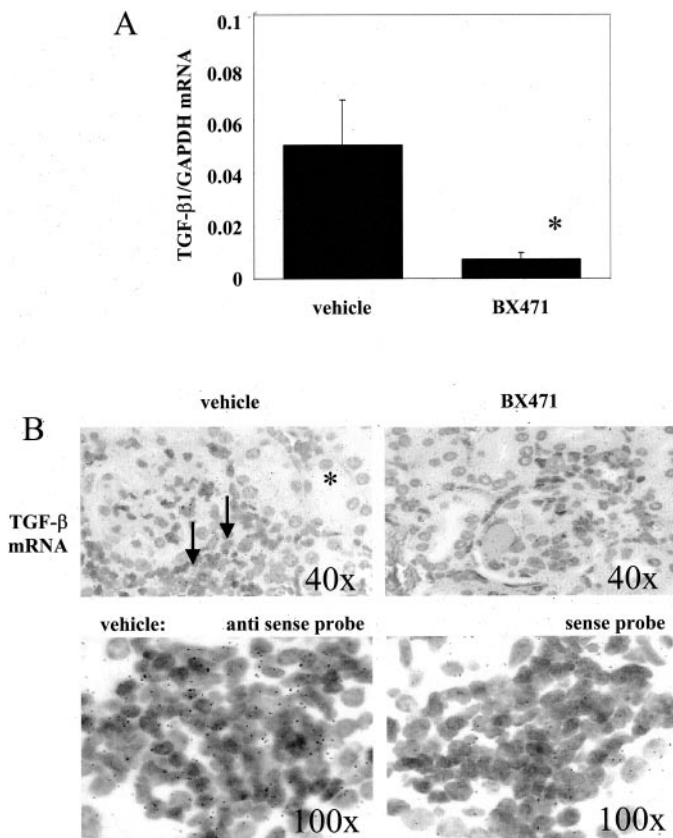
Figure 7. Renal TGF-β1 mRNA expression in MRL[lpr/lpr] mice. (A) The renal TGF-β1 mRNA expression was determined by real-time RT-PCR using total renal RNA of five to seven mice for each group of MRL[lpr/lpr] mice at the end of the study. TGF-β1 mRNA levels for vehicle- and BX471-treated MRL[lpr/lpr] mice are expressed in relation to respective GAPDH mRNA expression; *$P < 0.001$. (B) *In situ* hybridization for TGF-β mRNA in kidneys of vehicle-treated MRL[lpr/lpr] mice revealed TGF-β signals in interstitial cell infiltrates as compared with background signals in renal tubular cells or sections hybridized with sense probes, as indicated at higher magnification ($\times$100). In kidneys from BX471-treated MRL[lpr/lpr] mice, signals for TGF-β in interstitial infiltrates were hardly detectable.

cells. The BX471-induced reduction of interstitial leukocyte infiltration markedly diminished tubulointerstitial fibrosis and improved renal function despite no change in systemic autoimmunity, proteinuria, and glomerular pathology. The last may relate to the unexpected finding that glomerular leukocyte recruitment was not affected by CCR1 blockade, indicating a different role for CCR1 in the recruitment of leukocytes into the interstitial and glomerular compartments of the kidney. This is the first evidence that blockade of CCR1 can halt disease progression and improve renal function by selective inhibition of interstitial leukocyte recruitment late in the course of chronic renal failure.

BX471 Reduces Renal Damage in MRL[lpr/lpr] Mice by Blocking CCR1-Mediated Leukocyte Recruitment to the Renal Interstitium

Leukocyte migration to sites of tissue injury involves concerted interaction of adhesion molecules and chemokines and

their receptors. Our data demonstrate that CCR1 is involved in interstitial macrophage and T cell infiltration in MRL[lpr/lpr] mice. The evidence comes from the results of the histologic evaluation and from the transfer experiments using a technique of injecting labeled macrophages and T cells *in vivo*. These studies confirm that the reduction of renal leukocytes and chemokine receptor expression in BX471-treated MRL[lpr/lpr] mice relates to impaired leukocyte recruitment into the kidney. As we have recently shown that CCR1 antagonism reduces the amount of interstitial leukocytes in the mouse kidney after unilateral ureteral obstruction (8), these data indicate that interstitial leukocyte infiltration may be CCR1 dependent in other mouse models of renal disease as well. CCR1 blockade has also been shown to have beneficial effects on the outcome of other disease models and in other species. For example, BX471 improved survival and renal function after kidney transplantation in rabbits (7), delayed heart transplant rejection in rats (6), and improved functional performance of rats with experimental encephalomyelitis (18). Our data show that renal CCR1 is expressed only on the infiltrating leukocytes, and *in vitro* studies confirmed the role of CCR1 for leukocyte adhesion and transmigration through activated endothelium (4). Furthermore, these data suggest that proteinuria-induced activation of proximal tubular cells may not be sufficient to maintain progression of tubulointerstitial injury in the absence of interstitial inflammatory cell infiltrates (19). Apparently, additional signals from the infiltrating leukocytes such as proinflammatory and profibrotic cytokines are required for progression of interstitial injury. This hypothesis is supported by numerous experimental and human biopsy studies indicating roles for T cells and macrophages in local cytokine and chemokine expression and in progressive tubulointerstitial injury (20–22). Our study shows that even in chronic renal injury, preventing tubulointerstitial leukocyte infiltrate improves the disease despite unaltered glomerular damage and proteinuria. Although of interest, from this study it remains unclear whether CCR1 blockade halts or just slows disease progression in MRL[lpr/lpr] mice. However, these data indicate that CCR1-mediated leukocyte recruitment is important for interstitial inflammation in the kidney as well as in other model systems and that therapeutic blockade of CCR1 with a small molecule antagonist late in the course of immune complex glomerulonephritis can have beneficial effects on disease progression.

BX471 Reduces Renal Fibrosis in MRL[lpr/lpr] Mice

How could the reduction in mononuclear leukocyte infiltration in BX471-treated MRL[lpr/lpr] mice relate to the concomitant reduction in interstitial fibroblasts and fibrosis? The infiltrating leukocytes, via secretion of cytokines such as TGF-β, EGF, PDGF, or fibroblast growth factor, could contribute to epithelial-mesenchymal transformation, fibroblast proliferation, and collagen production (2). In fact, BX471-treated MRL[lpr/lpr] mice showed a marked reduction of renal TGF-β mRNA expression, a key cytokine for the induction of fibroblast proliferation and the development of renal fibrosis (23). To localize the site of TGF-β production, we performed *in situ* hybridization for TGF-β and localized the TGF-β production to the interstitial

cell infiltrate. Similar to the real-time RT-PCR data, *in situ* hybridization signals for TGF-β were reduced in kidneys of BX471-treated MRL$^{lpr/lpr}$ mice. The resolution of the latter method did not allow assignment of the signal to specific cells in the infiltrate, but the reduction of renal TGF-β mRNA expression with BX471 treatment corresponds to the reduction of interstitial macrophages. It therefore seems reasonable to assign the TGF-β signals to inflammatory cells, *i.e.*, the infiltrating mononuclear leukocytes, although low-level TGF-β mRNA expression may be beyond the sensitivity of the *in situ* hybridization method as the reported data about tubular TGF-β expression in mice are conflicting (24,25). TGF-β can also mediate a suppressive effect on systemic immune responses (26). In our study, reduced renal TGF-β mRNA levels were not associated with a change of systemic autoimmunity in MRL$^{lpr/lpr}$ mice. It therefore seems that the reduction in TGF-β mRNA in BX471-treated MRL$^{lpr/lpr}$ mice relates directly to the reduced renal leukocyte recruitment observed under these conditions.

BX471 Does Not Affect DNA Autoantibodies, Glomerular Macrophage Recruitment, and Proteinuria in MRL$^{lpr/lpr}$ Mice

The unchanged serum anti-DNA IgG isotype titers or glomerular IgG isotype deposits in BX471-treated mice compared with vehicle-treated controls indicate that BX471 does not induce a major shift in the Th1/Th2 balance of the systemic immune response in our lupus model. Such a shift toward the Th1 response was observed by Topham *et al.* (9) when nephrotoxic serum glomerulonephritis was induced in CCR1-deficient mice. This discrepancy may relate to different pathomechanisms of the nephrotoxic serum nephritis *versus* the MRL$^{lpr/lpr}$ lupus model. In nephrotoxic serum nephritis, a specific immune response against the planted glomerular antigen is required, whereas the MRL$^{lpr/lpr}$ lupus mouse model shows a broad polyclonal unregulated antibody production. Given this difference in pathoimmunology of the two models, the discrepancy seems less surprising, especially as in our model, the CCR1 blockade occurs after the establishment of the immune complex disease rather then during the generation of the immune response. It is interesting that we found that BX471 does not affect glomerular macrophage or T cell recruitment compared with vehicle-treated control mice, despite reduced interstitial macrophage counts with BX471. Surprising is that after injection, labeled and vehicle-treated macrophages did not localize to glomeruli, although there are clearly macrophages in glomeruli of lupus mice. Whether this observation relates to a different temporal interaction of circulating macrophages with glomerular endothelium remains unclear. Nevertheless, these data indicate that CCR1 is involved in interstitial but not in glomerular leukocyte recruitment. These data add to the growing body of evidence indicating marked differences in the mechanisms of leukocyte recruitment to the glomerular and tubulointerstitial compartment. For example, Tesch *et al.* (27) reported that CCL2/MCP-1–deficient mice were protected from tubulointerstitial but not from glomerular injury after induction of nephrotoxic serum nephritis. Furthermore, we recently showed in a model of Apoferritin-induced immune

complex glomerulonephritis in mice that Met-RANTES, a presumed CCR5 antagonist, reduced glomerular macrophage infiltration by ~50% (28). Conversely, Met-RANTES or CCR5-deficient mice showed no impairment of interstitial macrophage recruitment after unilateral ureteral ligation in mice (11). Together, these data support different roles for CCR1 and CCR5 in renal leukocyte recruitment with CCR5 involved in the glomerulus, while CCR1 is involved in the interstitial compartment. This phenomenon may relate to different adhesion molecules present on these different vascular beds, which modulate chemokine-mediated leukocyte adhesion and transmigration differentially (29,30). Further evidence for this hypothesis comes from the observation that CD3–positive lymphocytes are rarely found in the glomerulus in murine or human glomerulopathies, suggesting that glomerular endothelia may not support lymphocyte recruitment in this compartment, which again is demonstrated by the lack of recruitment of labeled CD8 T cells after intravenous injection in the present study. Together, CCR1 blockade with BX471 did not affect serum anti-DNA IgG isotype titers, glomerular immune complex deposition, glomerular macrophage recruitment, and proteinuria in MRL$^{lpr/lpr}$ mice. These data indicate that CCR1 blockade does not interfere with the humoral immune response in systemic autoimmunity of MRL$^{lpr/lpr}$ mice and that the recruitment and activation state of glomerular macrophages is not mediated by CCR1.

In summary, CCR1 mediates interstitial but not glomerular recruitment of mononuclear cells in the mouse kidney. When given late in the course of progressive renal injury in MRL$^{lpr/lpr}$ mice, BX471 improved renal function and diminished interstitial injury but did not affect glomerular damage, proteinuria, and systemic autoimmunity. These data signify the importance of interstitial injury for progressive renal dysfunction and provide the first evidence that blockade of CCR1—late in the course of chronic renal failure—can halt disease progression and improve renal function by selective inhibition of interstitial macrophage and T cell recruitment. Therefore, we propose that CCR1 blockade, currently evaluated for the treatment of multiple sclerosis in Phase II trials (31), may offer a new therapeutic strategy for lupus nephritis and perhaps for other chronic nephropathies that lead to end-stage renal failure.

Acknowledgments

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