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CCR5 Deficiency Aggravates Crescentic Glomerulonephritis in Mice


The chemokine receptor CCR5 is predominantly expressed on monocytes and Th1-polarized T cells, and plays an important role in T cell and monocyte recruitment in inflammatory diseases. To investigate the functional role of CCR5 in renal inflammation, we induced a T cell-dependent model of glomerulonephritis (nephrotoxic serum nephritis) in CCR5−/− mice. Induction of nephritis in wild-type mice resulted in up-regulation of renal mRNA expression of the three CCR5 chemokine ligands, CCL5 (15-fold), CCL3 (4.9-fold), and CCL4 (3.4-fold), in the autologous phase of the disease at day 10. The up-regulated chemokine expression was paralleled by infiltration of monocytes and T cells, followed by renal tissue injury, albuminuria, and loss of renal function.

Nephritic CCR5−/− mice showed a 3- to 4-fold increased renal expression of CCL5 (61.6-fold vs controls) and CCL3 (14.1-fold vs controls), but not of CCL4, in comparison with nephritic wild-type mice, which was accompanied by augmented renal T cell and monocyte recruitment and increased lethality due to uremia. Furthermore, CCR5−/− mice showed an increased renal Th1 response, whereas their systemic humoral and cellular immune responses were unaltered. Because the CCR5 ligands CCL5 and CCL3 also act via CCR1, we investigated the effects of the pharmacological CCR1 antagonist BX471. CCR1 blockade in CCR5−/− mice significantly reduced renal chemokine expression, T cell infiltration, and glomerular crescent formation, indicating that increased renal leukocyte recruitment and consecutive tissue damage in nephritic CCR5−/− mice depended on functional CCR1.

In conclusion, this study shows that CCR5 deficiency aggravates glomerulonephritis via enhanced CCL3/CCL5-CCR1-driven renal T cell recruitment. The Journal of Immunology, 2008, 181: 6546–6556.

The infiltration of leukocytes into the kidney is a hallmark of human and experimental crescentic glomerulonephritis. In particular, monocytes and effector T cells of the Th1 type are thought to play a central role in immune-mediated tissue damage, which ultimately leads to progressive loss of kidney function (1).

The molecular family of chemokines and their receptors are among the main regulators of directional leukocyte trafficking under homeostatic and inflammatory conditions. According to the position of two cysteine residues near the N terminus, chemokines are classified into four subfamilies named C, CC, CXC, and CX3C chemokines (2). By detecting chemokine concentration gradients via corresponding chemokine receptors, inflammatory cells are guided toward the focus of inflammation, where, in the case of autoimmune disease, they exert their fatal effector functions (3).

The specific composition of the inflammatory infiltrate is achieved by differential expression patterns of chemokine receptors on leukocyte subsets (4, 5).

The chemokine receptor CCR5 is predominantly expressed on Th1-polarized T cells, monocytes, and NK cells (6, 7). Recruitment of CCR5-positive T cells and monocytes is regarded as a typical feature in a variety of human autoimmune diseases, including rheumatoid arthritis, multiple sclerosis, and glomerulonephritis (5, 8–10). In human crescentic anti-neutrophil cytoplasmatic Ab-associated glomerulonephritis, anti-glomerular basement membrane glomerulonephritis, and lupus nephritis, up-regulated renal expression of the CCR5 ligands CCL3, CCL4, and CCL5 has been demonstrated (11), and renal CCR5-positive infiltrates are a common feature of lupus nephritis and IgA nephropathy (12). Furthermore, application of the modified CCL5 analogues Met-RANTES and aminooxypentane-RANTES with mainly antagonistic effects on CCR5, CCR1, and CCR3 led to a reduction in leukocyte infiltration and improvement of the disease outcome in various experimental models of glomerulonephritis (13, 14). In contrast, aggravation of glomerular damage was recently reported in a model of immune complex-mediated glomerulonephritis in Met-RANTES- and aminooxypentane-RANTES-treated animals (15). No experimental data are yet available on selective CCR5 blockade in experimental models of glomerulonephritis. Likewise, it remains unclear how CCR5 signaling contributes to cell-mediated renal tissue damage. The expression of CCR5 on monocytes and effector Th1 cells is presumed to play a central role in the recruitment of these cells into inflamed renal tissue, and thus in cell-mediated kidney injury. Therefore, blockade of CCR5 might represent a new therapeutic approach for the treatment of renal autoimmune disease. This is of
particular interest because a pharmacologic antagonist for human CCR5 has recently become available and has been tested in the clinical setting of HIV therapy (16).

Nephrotoxic serum nephritis (NTN)3 in mice is a well-characterized experimental model of human crescentic glomerulonephritis (17). Induction of NTN by injection of a polyclonal nephrotoxic sheep Ab initially results in glomerular complement activation and renal infiltration of neutrophils (18). This heterologous phase (<day 4) is followed by an autologous phase of adaptive immune response directed against the planted glomerular sheep Abs, leading to Th1 cell- and monocyte-mediated kidney injury (19–21).

The aim of the present study was to determine the contribution of CCR5 signaling to the formation of inflammatory infiltrates during the course of crescentic glomerulonephritis. We hypothesized that selective blockade of CCR5 might be sufficient to reduce renal T cell and monocyte recruitment. To address this question, we induced NTN in wild-type (WT) and CCR5−/− mice.

Materials and Methods

Animals

CCR5−/− mice (C57BL/6 background; 129P2-Ccr5tm1Kuz/J) were purchased from The Jackson Laboratory, and CCR5+/−/− genotype was confirmed by PCR analysis in each animal. Knockout mice underwent embryo transfer to meet the general standards of our institution. Age-matched C57BL/6 WT controls (10–12 wk old) also derived from the same breed in our animal facility. All animals were raised in specific pathogen-free conditions. Animal experiments were performed according to national and institutional animal care and ethical guidelines, and were approved by local committees.

Animal experiments

NTN was induced in 10- to 12-wk-old male C57BL/6 CCR5−/− and C57BL/6 WT mice by i.p. injection of 2.5 mg of nephrotoxic sheep serum per gram of mouse body weight, as described (22). Controls were injected i.p. with an equal amount of nonspecific sheep IgG. Altogether, nephritis was induced in 33 WT and 17 CCR5−/− animals. In experiments with CCR1 blockade, mice received s.c. injections of the CCR1-specific antagonist BX471 from day 3–10 after induction of NTN. Mice were dosed with 50 mg/kg BX471 in 50–60 μl vehicle at 8-h intervals. Control mice received an equal amount of the vehicle 2-hydroxypropyl-β-cyclodextrin (cyclodextrin; Sigma-Aldrich) (23). Solution of BX471 in 40% cyclodextrin was prepared, as previously described (24).

Functional studies

For urine sample collection, mice were housed in metabolic cages for 6 h. Albuminuria was determined by standard ELISA analysis (mouse-albumin kit; Bethyl Laboratories). Blood samples for blood urea nitrogen (BUN) measurement and assessment of systemic Ab response were obtained at the time of sacrifice. Urinary creatinine and BUN were measured by standard laboratory methods.

Real-time RT-PCR analysis

Total RNA of renal cortex was prepared according to standard laboratory methods. Real-time PCR was performed for 40 cycles (initial denaturation: 95°C, 10 min; denaturation: 95°C, 15 s; primer annealing and elongation: 60°C, 1 min) with 1.5 μl of cDNA samples in the presence of 2.5 μl (0.9 μM) of specific primers (primer sequences are available upon request) and 12.5 μl of 2× Platinum SYBR Green qPCR Supermix (Invitrogen) in an AbiPrism Sequence Detection System 7000 (Applied Biosystems). All samples were run in duplicate and normalized to 18S rRNA to account for small RNA and cDNA variability (25).

In situ hybridization

In situ hybridization procedures were performed, as described previously (26). In brief, CCL5 and CCR5 cRNA probes were prepared by in vitro transcription of subcloned cDNA. The CCL5 probe corresponds to nt 736–975 of cDNA sequence NM_009917. Antisense hybridization probe and sense control were labeled with 35SUTP (20 μCi/ml; Amersham), and free nucleotides were separated with a Sephadex G-50 column (Quickspin columns; Roche). In situ hybridization was performed on 12-μm cryosections of renal tissue using 5 ng of the 35S-labeled antisense and sense RNA probes, respectively. After exposing sections overnight to Kodak Biomax MR x-ray films, they were treated with Kodak NTB-3 nuclear track emulsion and exposed for 3 wk, followed by development in Kodak D19 and fixation with Kodak Unifix. Finally, sections were stained with Mayer’s Hemalum.

Morphological examinations

Light microscopy and immunohistochemistry were performed by routine procedures. Crescent formation and glomerular sclerosis (deposition of Periodic acid Schiff (PAS)-positive material) were assessed in 50 glomeruli per mouse in a blinded fashion in PAS-stained paraffin sections. As a measure for tubulointerstitial injury, interstitial area was estimated by point counting three independent areas of renal cortex per mouse in low magnification fields (×200), as previously described (26). Paraffin-embedded sections (2 μm) were either stained with an Ab directed against the pan-T cell marker CD3 (A0452; DakoCytomation), the monococyte-specific markers F4/80 (BM8; BMA Biomedicals) and MAC-2 (M5/38; Cedarlane Laboratories), C3 (Cappel Laboratories; Organon Teknika), or sheep IgG and mouse IgG (both Jackson ImmunoResearch Laboratories). Tissue sections were developed with the Vectastain ABC-AP kit (Vector Laboratories), MAC-2- and CD3-positive cells in 50 glomerular cross-sections (gcs) and F4/80- and CD3-positive cells in 30 tubulointerstitial high power fields (hpf) per kidney were counted by light microscopy in a blinded fashion. For electron microscopy, kidney tissue was fixed in 4% paraformaldehyde in 0.1 M cacodylate buffer, embedded in araldite, cut with an Ultratome E ultramicrotome (Reichert Jung), and stained with uranyl acetate and lead citrate. Specimens were examined with a Zeiss EM 109 electron microscope.

Ag-specific humoral immune response

Mouse anti-sheep IgG Ab titers were measured by ELISA using sera collected 10 days after induction of the nephritis, as described recently (27). In brief, ELISA microtiter plates were coated with 100 μl of 100 μg/ml sheep IgG (Sigma-Aldrich) in carbonate-bicarbonate buffer overnight at 4°C. After blocking with 1% BSA in TBS (Sigma-Aldrich), the plates were incubated with serial dilutions of mouse serum (1/100 to 1/12,500) for 1 h at room temperature. Bound mouse IgG was detected using peroxidase-conjugated goat anti-mouse IgG (Biozol) at 1/1,000, tetramethylbenzidine peroxidase substrate, and absorbance readings (at 450 nm) on a spectrophotometer. Lack of cross-reactivity of the secondary Ab with sheep IgG was demonstrated by omitting the primary Ab. IgG isotypes (IgG1, IgG2a, and IgG2b) were measured using the ELISA technique already described. The bound mouse IgG isotypes were detected using peroxidase-conjugated rabbit anti-mouse IgG1, IgG2a, and IgG2b Abs (Zymed Laboratories-In vitrogen) at a dilution of 1/1,000.

Renal leukocyte isolation

Previously described methods for renal cell isolation from murine kidneys were used (22). In brief, kidneys were finely minced and digested for 45 min at 37°C with 0.4 mg/ml collagenase D (Roche) and 0.01 mg/ml DNase I in DMEM (Roche) supplemented with 10% heat-inactivated FCS (In Vitrogen). Cell suspensions were sequentially filtered through 70- and 40-μm nylon meshes and washed with HBSS without Ca2+ and Mg2+. Renal cell suspensions were either stained with an Ab directed against the pan-T cell marker CD3 (A0452; DakoCytomation), the monococyte-specific markers F4/80 and MAC-2 (BMA Biomedicals), or Mac-2 (M5/38; Cedarlane Laboratories), C3 (Cappel Laboratories; Organon Teknika), or sheep IgG and mouse IgG (both Jackson ImmunoResearch Laboratories). Tissue sections were developed with the Vectastain ABC-AP kit (Vector Laboratories), MAC-2- and CD3-positive cells in 50 glomerular cross-sections (gcs) and F4/80- and CD3-positive cells in 30 tubulointerstitial high power fields (hpf) per kidney were counted by light microscopy in a blinded fashion. For electron microscopy, kidney tissue was fixed in 4% paraformaldehyde in 0.1 M cacodylate buffer, embedded in araldite, cut with an Ultratome E ultramicrotome (Reichert Jung), and stained with uranyl acetate and lead citrate. Specimens were examined with a Zeiss EM 109 electron microscope.

Flow cytometry

For T cell differentiation, isolated cells were stained for 25 min at 4°C with fluorochrome-labeled Abs specific for CD3 (allophycocyanin; 17A2; R&D Systems) and CD4 (PE; GK1.5; Miltenyi Biotec). Before Ab incubation, unspecific staining was blocked with normal mouse serum (Sigma–Aldrich).

3 Abbreviations used in this paper: NTN, nephrotoxic serum nephritis; BUN, blood urea nitrogen; gc, glomerular cross-section; hpf, high power field; PAS, Periodic acid Schiff; WT, wild type.
Staining of intracellular IFN-γ was performed, as recently described by Korn et al. (29). In brief, splenocytes or isolated renal leukocytes were activated by incubation at 37°C, 5% CO2 for 5 h with PMA (50 ng/ml; Sigma-Aldrich) and ionomycin (1 μg/ml; Calbiochem-Merck) in RPMI 1640 with 10% FCS. After 30 min of incubation, brefeldin A (10 μg/ml; Sigma-Aldrich) was added. After several washing steps and staining of cell surface markers, cells were incubated for 20 min at 4°C in Cytofix/Cytoperm (BD Biosciences) to permeabilize cell membranes. Then, intracellular IFN-γ was stained using a rat anti-mouse IFN-γ Ab (FITC; XMG1.2; BD Biosciences) (28). Experiments were performed on a BD Biosciences FACSCalibur system using the CellQuest Professional software.

Statistical analysis

Results are expressed as mean ± SD. Differences between individual experimental groups were compared by Kruskal-Wallis test with post hoc analysis by Mann-Whitney U test. For survival analysis, Kaplan-Meyer plot with log-rank test was used. Experiments that did not yield enough independent data for statistical analysis due to the experimental setup were repeated at least three times.

Results

Renal phenotype of CCR5−/− mice

To examine whether CCR5 deficiency leads to structural or functional renal defects, we analyzed 40-wk-old C57BL/6 CCR5−/− and C57BL/6 WT mice with respect to histological and functional alterations (Fig. 1). Gross morphology in PAS-stained kidney sections (Fig. 1A) and fine structure of the glomerular filtration barrier (Fig. 1B) were well preserved in CCR5−/− mice. Furthermore, functional analysis revealed no differences in BUN and urinary albumin excretion between WT and CCR5−/− mice under homeostatic conditions (Fig. 1C). Induction of experimental glomerulonephritis in WT and CCR5−/− mice

NTN in C57BL/6 WT and C57BL/6 CCR5−/− mice was induced by i.p. injection of nephrotoxic sheep serum. Specific glomerular binding and linear deposition patterns of the polyclonal nephrotoxic Ab were demonstrated by immunohistochemical staining of sheep IgG on renal tissue 48 h after serum injection (Fig. 2). Intensity and distribution of sheep IgG staining did not differ between WT and CCR5−/− mice. A critical step in the induction of the heterologous phase of NTN is the binding and activation of complement (18). To evaluate whether this initial inflammatory insult is altered in CCR5−/− mice, we performed immunohistochemistry for complement factor C3 in the heterologous phase of NTN at 48 h (Fig. 2). Nephritic kidneys of both WT and CCR5−/− mice showed intensive glomerular and tubulointerstitial binding of C3, demonstrating unchanged and effective induction of NTN in CCR5−/− mice.

Expression of chemokines and chemokine receptors during nephrotoxic nephritis

To gain further insight into the role of CCR5 and its ligands CCL3, CCL4, and CCL5 in the initiation and maintenance of
cell-mediated tissue damage, we performed real-time RT-PCR analysis on renal cortex of healthy and nephritic WT and CCR5−/− mice (Fig. 3).

Basal renal chemokine expression levels were low and did not differ significantly between healthy WT and CCR5−/− mice. Likewise, no early up-regulation of CCR5 ligands could be detected in the heterologous phase of nephritis at 48 h (data not shown).

However, mRNA expression of all CCR5 ligands showed significant up-regulation in the autologous phase at day 10 when compared with nonnephritic controls (nephritic WT and CCR5−/− mice, p < 0.01; Fig. 3A). Interestingly, at this time point, renal CCL3 (WT, 4.9-fold; CCR5−/−, 14.1-fold; p < 0.01) and CCL5 expression (WT, 15-fold; CCR5−/−, 61.6-fold; p < 0.01) was significantly higher in nephritic CCR5−/− mice when compared with nephritic WT mice. CCL4 expression was not differentially regulated in nephritic WT or CCR5−/− mice (WT, 3.4-fold; CCR5−/−, 5.5-fold; Fig. 3A).

To address the question as to whether increased induction of chemokines in nephritic CCR5−/− mice is specific to CCR5 ligands, we analyzed mRNA levels of the CCR5-independent chemokine CCL2. The monocyte-attracting chemokine CCL2 was already slightly up-regulated in the heterologous phase at 48 h (WT, 5.1-fold; CCR5−/−, 3.9-fold; p < 0.01 vs nonnephritic controls) and showed strong induction in the autologous phase at day 10. However, in contrast to CCL3 and CCL5, no differential expression of CCL2 could be demonstrated between nephritic WT and CCR5−/− mice (WT, 54.2-fold; CCR5−/−, 59.7-fold; Fig. 3A).

Induction of nephritis also resulted in significant up-regulation of CCR5 mRNA during the autologous phase in WT mice compared with nonnephritic controls (6.7-fold; p < 0.01 vs nonnephritic controls; Fig. 3A). No CCR5 mRNA was detectable in CCR5−/− mice.

Analysis of spleen chemokine expression revealed that increased production of CCL3 and CCL5 in nephritic CCR5−/− mice was specific to the kidney. Total spleen mRNA levels of CCL2, CCL3, CCL4, and CCL5 were not differentially regulated between nephritic WT and CCR5−/− mice. A comparison showed that they were similar to mRNA levels of nonnephritic controls immunized with nonspecific sheep IgG (data not shown).

Localization of CCR5 and its ligand CCL5 in nephrotoxic nephritis
To identify the renal compartments expressing the most abundantly induced CCR5 ligand CCL5 and the CCR5 receptor, we performed in situ hybridization experiments (Fig. 3B). mRNA expression of CCL5 in both nephritic WT and CCR5−/− mice...
at day 10 was predominantly detected in periglomerular and tubulointerstitial infiltrates. Sporadically, strong expression was also detectable in epithelial cells of markedly dilated tubules, whereas intraglomerular CCL5 signals were apparently restricted to cellular crescents and single infiltrating cells in the glomerular tuft. Arterioles and peritubular capillaries were largely negative for CCL5 mRNA expression. No differential CCL5 expression pattern was found in nephritic WT and CCR5<sup>−/−</sup> mice. Similar to CCL5, CCR5 mRNA was mainly expressed in infiltrating cells in periglomerular and tubulointerstitial compartments and in glomerular crescents. No CCR5 mRNA expression could be found in resident renal cells. CCL5 and CCR5 mRNAs were only detected in low levels in nonnephritic control mice. Nephritic CCR5<sup>−/−</sup> mice showed no specific expression of CCR5 mRNA above background level.

**Increased renal recruitment of monocytes and T cells in CCR5<sup>−/−</sup> mice**

To investigate the effects of CCR5 deficiency on T cell and monocyte recruitment, kidney sections were stained immunohistochemically for tubulointerstitial and glomerular T cells (CD3) and macrophages (F4/80 and MAC-2, respectively) (Fig. 4A).

No relevant tubulointerstitial infiltration of monocytes and only a slight increase in T cell numbers were found at 48 h, which is in line with the lack of monocyte- and T cell-attracting chemokine expression during the heterologous phase of nephritis (data not shown). Up-regulated chemokine expression in the autologous phase at day 10, mainly in the tubulointerstitium and in the periglomerular region (as shown by in situ hybridization), was paralleled by infiltration of T cells (nonnephritic controls, 2.8 ± 1.6/hpf; nephritic WT, 12.8 ± 10.8/hpf) and macrophages (nonnephritic controls, 1.4 ± 1.1/hpf; nephritic WT, 10.8 ± 6.8/hpf; Fig. 4B) mainly into these intrarenal compartments.

Quantification of CD3- and F4/80-positive tubulointerstitial cells in nephritic CCR5<sup>−/−</sup> mice at day 10 revealed a significant increase in renal infiltration of T cells (WT, 12.8 ± 10.8/hpf; CCR5<sup>−/−</sup>, 26.4 ± 7.2/hpf; p < 0.05) and macrophages (WT, 10.8 ± 6.8/hpf; CCR5<sup>−/−</sup>, 41.4 ± 28.6/hpf; p < 0.01) compared with nephritic WT mice (Fig. 4B). Glomerular infiltration of T cells (nonnephritic controls, 0.16 ± 0.23/gcs; nephritic WT, 0.34 ± 0.4/gcs; nephritic CCR5<sup>−/−</sup>, 0.77 ± 0.63/gcs) and of MAC-2-positive (nonnephritic controls, 0.62 ± 0.2/gcs; nephritic WT, 1.79 ± 0.84/gcs; nephritic CCR5<sup>−/−</sup>, 2.24 ± 0.41/gcs) monocytes in diseased animals was less pronounced and did not differ between nephritic CCR5<sup>−/−</sup> and nephritic WT mice (Fig. 4B).

**Aggravated renal tissue damage in CCR5<sup>−/−</sup> mice**

Examination of PAS-stained kidney sections of nephritic mice in the autologous phase at day 10 showed severe focal glomerular and tubular alterations with complete destruction of regular tissue structures (Fig. 5A). Glomerular changes included hypercellularity and formation of cellular crescents, capillary aneurysms, and intraglomerular deposition of PAS-positive material. In addition to massive leukocyte infiltrates, the tubulointerstitial compartment showed tubular dilatation, necrosis and atrophy, and protein casts and tubular protein reuptake due to proteinuria. Glomerular and tubulointerstitial damage was particularly pronounced in CCR5<sup>−/−</sup> mice. To quantify renal tissue damage, PAS-stained kidney sections were evaluated for the presence of crescents,glomerular sclerosis (deposition of PAS-positive material), and tubulointerstitial injury (Fig. 5B), as described previously (22, 26). The frequency of glomerular crescents at day 10 was significantly increased in CCR5<sup>−/−</sup> mice compared with WT controls (nonnephritic controls, 0.5 ± 1.3%; WT, 13.6 ± 8.9%; CCR5<sup>−/−</sup>, 28.9 ± 7.8%; p < 0.01). Nephritic kidneys showed a high percentage of glomerulosclerosis (nephritic controls, 0%; nephritic WT,
27.3 ± 16.6%; p < 0.01; Fig. 5B) and considerable tubulointerstitial injury, as indicated by a significant increase in interstitial area (nonnephritic controls, 7.7 ± 2.3%; nephritic WT, 16.6 ± 5.9%; p < 0.01). Interstitial injury appeared pronounced in nephritic CCR5−/− mice (CCR5−/−, 21.1 ± 8.1%), but was not statistically significant, whereas glomerulosclerosis was similar in both groups (CCR5−/−, 31.3 ± 9.9%).

**CCR5 deficiency deteriorates renal function in nephrotoxic nephritis**

After 10 days, mice were sacrificed for assessment of renal function (Fig. 5C). BUN of nephritic mice were significantly elevated at day 10 compared with nonnephritic controls (p < 0.01). In the heterologous phase at 48 h, there was no difference in impairment of renal function between WT and CCR5−/− mice (data not shown). During the T cell-mediated autologous phase at day 10, however, we found a significant increase in BUN in nephritic CCR5−/− mice (50 ± 8 mg/dl) compared with nephritic WT mice (42 ± 10 mg/dl; p < 0.05; Fig. 5C).

As an indicator of renal tissue damage, urinary albumin excretion was monitored (Fig. 5C). WT and CCR5−/− mice showed a markedly increased albumin to creatinine ratio at day 10 after induction of nephritis (nonnephritic controls, 0.04 ± 0.03; WT, 47.9 ± 36; CCR5−/−, 28.4 ± 16.5; p < 0.01 vs nonnephritic controls). There was no statistically significant difference in albumin excretion between nephritic CCR5−/− and nephritic WT mice.

**Increased lethality of nephrotoxic nephritis in CCR5−/− mice**

Induction of NTN is usually well tolerated in WT mice. They show only minor behavioral changes, and only a few animals succumb to the disease. In the setting of CCR5 deficiency, however, nephritic mice showed more severe clinical impairment. In addition, the lethality rate was significantly increased from day 4 after serum injection (Fig. 5D). Induction of NTN in CCR5−/− mice was lethal in 30% compared with 3% in WT mice within the first 10 days (p < 0.01; log-rank test).

Two CCR5−/− mice suffering from terminal disease were sacrificed at days 6 and 7, respectively. Their BUN levels were 381 and 219 mg/dl, respectively, clearly demonstrating that loss of renal function was the cause of increased lethality in CCR5-deficient mice. Nephritic CCR5−/− mice sacrificed at day 10 therefore represented a select population of mice that survived the early

**FIGURE 5.** Renal tissue damage and impairment of renal function. A, Representative photographs of PAS-stained kidney sections of WT and CCR5−/− mice at day 10 of NTN. Nephritic mice show glomerular crescents (arrows) and glomerular sclerosis (arrowheads) (magnification: upper panel, ×200; lower panel, ×400). B, Quantification of glomerular (left) and tubulointerstitial (right) tissue damage in nephritic WT mice (n = 11), nephritic CCR5−/− mice (n = 6) at day 10 of NTN, and nonnephritic WT controls (n = 13). C, Left, BUN levels of nephritic WT mice (n = 16), nephritic CCR5−/− mice (n = 7) at day 10 of NTN, and nonnephritic WT controls (n = 12). Right, Albuminuria of nephritic WT mice (n = 9), nephritic CCR5−/− mice (n = 7) at day 10 of NTN, and nonnephritic WT controls (n = 13) expressed as urinary albumin to creatinine ratio. Symbols represent individual data points, and the horizontal lines indicate mean values (*, p < 0.05). D, Kaplan-Meyer survival analysis of WT (n = 33) and CCR5−/− mice (n = 17) with NTN (**, p < 0.01).
autologous phase of the disease. Compared with nephritic WT mice, the differences observed in renal function are thus probably even more pronounced than documented.

Unaltered humoral and cellular systemic immune responses in CCR5−/− mice

To address the question as to whether CCR5 deficiency induces alterations in Ab production directed against the nephritogenic Ag, we performed immunohistochemistry for mouse IgG on kidney sections 10 days after induction of nephritis (Fig. 6A). The amount of deposition of glomerular mouse IgG and the distribution patterns were similar in WT and CCR5−/− kidneys.

For a more precise description of Ab-specific humoral immune responses, we analyzed by ELISA the isotype pattern of Ab response directed against sheep IgG in the serum of nephritic mice (Fig. 6B). There was no significant difference in sheep IgG-specific Ab titers of total mouse IgG at day 10 of NTN. Furthermore, the analysis of IgG isotypes revealed no bias for either Th1-type (IgG2a) or Th2-type (IgG1 and IgG2b) Ab production in nephritic CCR5−/− compared with WT mice.

To analyze the systemic cellular Th1 response, we isolated spleen cells from CCR5−/− and WT mice at day 10 of NTN (Fig. 6C). Spleen cells were restimulated by incubation with PMA and ionomycin. IFN-γ production by CD4-positive T cells was assessed by intracellular cytokine staining and subsequent FACS analysis. The percentages of IFN-γ-positive T cells in spleens of nephritic CCR5−/− mice were not different from those of nephritic WT mice. Consistent with this finding, real-time RT-PCR analysis of spleen mRNA from nephritic CCR5−/− and WT mice revealed no difference in the expression of the Th1 cytokines IFN-γ and TNF-α (data not shown).

Increased renal Th1 response in CCR5−/− mice

Because the local Th1 response is assumed to play a central role in immune-mediated tissue damage during NTN, we quantified renal infiltration of IFN-γ-producing CD4-positive T cells. Renal leukocytes were isolated from CCR5−/− and WT kidneys at day 10 of NTN (Fig. 7A). Both immunohistochemical quantification and FACS analysis showed an approximate 5-fold increase in T cell recruitment into nephritic WT kidneys compared with nonnephritic controls. The numbers of infiltrating CD4-positive T cells in nephritic CCR5−/− kidneys were even higher. Most importantly, however, infiltration of IFN-γ-producing CD4-positive T cells, as determined by intracellular cytokine staining after restimulation with PMA/ionomycin, was increased by ~40% in nephritic CCR5−/− compared with nephritic WT kidneys. Furthermore, renal mRNA expression of the Th1 cytokine TNF-α was increased in nephritic CCR5−/− mice compared with WT controls (WT, 6.2-fold; CCR5−/−, 19.2-fold; p < 0.05; Fig. 7B). The expression levels of IFN-γ tended to be higher in CCR5−/− kidneys (WT, 2.6-fold; CCR5−/−, 14.1-fold), but failed to reach statistical significance because of considerable variability among the animals. Induction of the anti-inflammatory cytokine IL-10 was not regulated differently in nephritic CCR5−/− or WT mice (WT, 30.8-fold; CCR5−/−, 56-fold). Renal mRNA expression of the transcription factor FoxP3, which is a marker for regulatory T cells, was also similar in nephritic CCR5−/− and WT mice. Levels of mRNA for the Th2 cytokine IL-4 were below detection limits in nephritic and control animals (data not shown).

CCR1 blockade improves the outcome in nephritic CCR5−/− mice

Because CCL3 and CCL5 may attract leukocytes by interaction with their alternate receptor CCR1, increased renal CCL3 and CCL5 levels in nephritic CCR5−/− mice might lead to increased T cell and monocyte recruitment via a CCR1-dependent mechanism. To test this hypothesis, we treated nephritic WT and CCR5−/− mice with the CCR1-specific antagonist BX471 during the autologous phase of NTN (days 3–10). The elevated
renal production of CCL3 and CCL5 in nephritic CCR5−/− mice was abolished by CCR1 blockade (Fig. 8A). At day 10 of NTN, renal mRNA expression of CCL5 in BX471-treated CCR5−/− mice was significantly reduced compared with vehicle-treated CCR5−/− mice (nephritic CCR5−/− + Vh, 19-fold; nephritic CCR5−/− + BX471, 7.9-fold; p < 0.01). Expression of CCL3 was also reduced, though not reaching a statistically significant level (nephritic CCR5−/− + Vh, 37.8-fold; nephritic CCR5−/− + BX471, 10.3-fold). Importantly, expression levels of CCL3 and CCL5 in BX471-treated CCR5−/− mice were not different from that seen in vehicle- or BX471-treated nephritic WT mice. Compared with previous experiments, up-regulation of CCL5 mRNA expression in nephritic WT mice was slightly reduced. This difference was caused by a ∼2-fold increase in basal CCL5 levels of nonnephritic WT mice treated with the vehicle 2-hydroxypropyl-β-cyclodextrin (cyclodextrin), which serve as a reference value for calculation of the relative increase. Cyclodextrin is widely used to improve water solubility of lipophilic drugs for pharmacological application in animals and humans. As it has been described (23) that cyclodextrin treatment can lead to minor histopathological changes in the kidneys, which are reversible and do not impair renal function, this slight up-regulation of CCL5 expression in controls might be a side effect of the vehicle treatment.

Double blockade of CCR5 and CCR1 by application of BX471 to nephritic CCR5−/− mice significantly reduced renal T cell recruitment in comparison with nephritic vehicle-treated CCR5−/− and WT mice (CD3-positive T cells: nonnephritic controls, 4.9 ± 3.2/hpf; nephritic WT + Vh, 23.6 ± 5.8/hpf; nephritic CCR5−/− + Vh, 51.3 ± 5.1/hpf; nephritic CCR5−/− + BX471, 11.3 ± 4.7/hpf; p < 0.01; Fig. 8B). Furthermore, excessive renal monocyte infiltration in nephritic CCR5−/− mice was inhibited by CCR1 blockade (F4/80-positive monocytes: nonnephritic controls, 1.2 ± 0.4/hpf; nephritic WT + Vh, 20.2 ± 12.2/hpf; nephritic CCR5−/− + Vh, 48.9 ± 3.4/hpf; nephritic CCR5−/− + BX471, 23.0 ± 9.4/hpf; p < 0.01; Fig. 8B). Most importantly, double blockade of the two chemokine receptors effectively protected mice from immune-mediated kidney injury, as demonstrated by a reduced number of crescentic glomeruli in CCR5−/− mice receiving BX471 injections compared with nephritic vehicle-treated CCR5−/− and WT mice (nonnephritic controls, 0.9 ± 1.6; nephritic WT + Vh, 20.0 ± 3.9/hpf; nephritic CCR5−/− + Vh, 44.4 ± 8.1/hpf; nephritic CCR5−/− + BX471, 13.3 ± 6.2/hpf; p < 0.05; Fig. 8C).

In line with the previous experiments, the lethality rate was increased in nephritic vehicle-treated CCR5−/− mice (25%), whereas no mouse deceased in the group of BX471-treated CCR5−/− animals. This positive selection of surviving vehicle-treated CCR5−/− mice showed an insignificant tendency to increased BUN levels (nonnephritic controls, 40.9 ± 6.3 mg/dl; nephritic WT + Vh, 60.4 ± 18.4 mg/dl; nephritic CCR5−/− + Vh, 67.6 ± 1.3 mg/dl; p = 0.073, NS). BUN levels in nephritic BX471-treated CCR5−/−, however, were similar to nephritic vehicle-treated WT mice (nephritic CCR5−/− + BX471, 57.1 ± 15.3 mg/dl; Fig. 8D). The albumin to creatinine ratio was also significantly decreased in nephritic BX471-treated CCR5−/− animals compared with nephritic vehicle-treated WT mice (Fig. 8D).

In nephritic WT mice, CCR1 blockade significantly reduced T cell infiltration (WT + BX471, 14.3 ± 7.3/hpf; p < 0.05 vs nephritic WT + Vh), but it did not prevent monocyte infiltration and failed to improve markers of renal tissue damage and parameters of renal function (Fig. 8, B–D).
Discussion

Studies of the past few years have highlighted the role of chemokines and their receptors as main regulators of leukocyte recruitment in renal inflammation (30, 31). The chemokine receptor CCR5 is expressed on monocytes and activated Th1 cells, and might therefore play an important role in directional trafficking of these leukocyte subsets (4–7).

Because of the lack of data on the functional role of CCR5 in renal autoimmune inflammation, we induced a T cell-mediated model of crescentic glomerulonephritis in CCR5-deficient and WT mice. The main finding of our study is that CCR5−/− mice developed an exacerbated form of crescentic glomerulonephritis associated with significantly increased lethality due to uremia (30 vs 3% in WT mice). The high susceptibility of CCR5−/− mice to severe kidney injury was caused by excessive renal up-regulation of the CCR5 ligands CCL3 and CCL5, resulting in enhanced CCR1-driven monocyte and T cell infiltration with aggravated tissue damage and deterioration of renal function.

The excessive up-regulation of CCR5 ligands in CCR5−/− mice along with augmented cellular infiltration and a worse disease outcome has been described in models of cutaneous Arthus reaction (32) and T cell-mediated liver injury (33). In Con A-induced hepatitis, application of the mainly antagonistic CCL5 analog Met-RANTES to CCR5−/− mice reduced the recruitment of CCR1-positive NK cells to the liver (34). This observation suggests facilitated CCR1-dependent hepatic leukocyte infiltration, which is driven by increased intrahepatic levels of shared CCR5/CCR1 ligands in the setting of CCR5 deficiency.

To test this hypothesis, we blocked CCR1 signaling in nephritic mice by application of the CCR1-specific small molecule antagonist BX471 (35). BX471 has been used for treatment in a variety of disease models, including murine lupus nephritis (36) and unilateral ureter obstruction, a model of renal fibrosis (24, 37). In our experiments, double blockade of CCR5 and CCR1 almost abolished renal T cell recruitment and consistently protected from autoimmune tissue damage. Furthermore, the CCR1 antagonist protected CCR5−/− mice against deterioration of renal function and lethality. This indicates a predominant role for these two chemokine receptors in renal Th1 cell trafficking.

Monocyte infiltration was normalized to levels of nephritic WT mice by treatment of CCR5−/− mice with the CCR1 blocker. However, double receptor blockade did not lead to further inhibition of monocyte infiltration below the level of nephritic WT animals, suggesting that other chemokine receptors (e.g., CCR2) compensate for the lack of functional CCR5 and CCR1 in this setting.

Beneficial effects of selective CCR1 blockade by BX471 treatment or ccr1 gene deletion with reduction in tubulointerstitial leukocyte infiltration and organ damage have been shown in murine lupus nephritis (36) and renal fibrosis (24, 37). Although BX471 treatment significantly reduced renal T cell infiltration in our experiments, it did not protect nephritic WT mice against kidney injury. The unfavorable results obtained in CCR1−/− mice with NTN (38) support the theory that single blockade of CCR1, like single blockade of CCR5, is not a successful strategy in crescentic glomerulonephritis.

The mechanisms underlying excessive up-regulation of CCR5 ligands in nephritic CCR5−/− kidneys remain unclear. Interestingly, however, CCR1 blockade led to normalization of CCL3 and CCL5 levels. We showed by in situ hybridization that inflammatory infiltrates are the main source of CCL5 in NTN. Increased renal CCL5 mRNA expression could therefore be secondary to increased cellular infiltration and decreased to WT levels by reducing the inflammatory infiltrate, as was the case in our CCR1 blockade experiments. However, one could also speculate that CCR5 signaling in chemokine-producing cells is needed to regulate the expression of its own ligands in terms of a negative feedback loop. It has been demonstrated that stimulated T cells and NK cells of CCR5−/− mice show increased IFN-γ production (34, 39), but no dysregulation of CCR5 ligand production has been reported to date (39).

Another reason for a worse outcome in nephritic CCR5−/− mice might be an altered systemic or local immune response. Previous experiments have shown that CCR5−/− mice produce equal amounts of total IgG in response to challenge with a T cell-dependent Ag (39, 40). Zhou et al. (39) reported increased IgG isotype 1 production in CCR5−/− mice, but these results have not been confirmed by others (40). In our experiments, total IgG and IgG isotypes directed against sheep IgG, the nephritogenic Ag,
were consistently similar in CCR5−/− and WT mice. Furthermore, number and frequency of IFN-γ-producing Th1-polarized cells in the spleen were constant in CCR5−/− mice, arguing against defective T cell priming, which has been demonstrated for allogeneic CCR5−/− T cells in a model of heart transplantation (40). Despite an unchanged systemic immune response, we found an increased local Th1 response in CCR5−/− mice, which was reflected by an elevated number and a higher frequency of renal IFN-γ-producing CD4+ T cells and by increased renal production of the Th1 prototype cytokine TNF-α. This is consistent with the finding of an augmented cutaneous delayed-type hypersensitivity reaction in CCR5−/− mice (39). The mechanisms underlying these observations are still unclear and require further study.

The role of a human mutation leading to the loss of functional CCR5 due to a 32-bp deletion in the encoding gene (CCR5Δ32) in glomerulonephritis is under debate. Our results of improved renal survival of heterozygous CCR5Δ32 carriers in IgA nephropathy (41) could not be reproduced by others (42). Large differences in study populations, endpoints, and time periods studied could account for these different results. It is noteworthy, however, that because of the low frequency of homozygous CCR5Δ32 allele carriers in the Caucasian population (~1%), both studies included only low numbers of homozygous patients, and therefore did not allow statistical analysis of this completely CCR5-deficient group. In the control group of patients with intact CCR5, 27% developed end-stage renal disease. In the group of heterozygous CCR5Δ32 patients who have reduced CCR5 expression on leukocytes, only 19% developed end-stage renal disease during followup (41, 42). In striking contrast, however, four of the seven (57%) homozygous and completely CCR5-deficient patients developed end-stage renal disease (41, 42), indicating that CCR5 deficiency does not protect against initiation and progression of IgA nephropathy, but might even exacerbate this form of glomerulonephritis. Whether CCR5 deficiency in humans is generally disadvantageous in renal autoimmune disease, as shown in this study for mice, cannot be answered on the basis of these data. This issue, however, is especially important because a CCR5-specific antagonist has recently become available for HIV therapy (16) and could also represent a new therapeutic option in fatal autoimmune disease. Our present study emphasizes the potential danger that lies in a blockade of a single chemokine receptor. In view of counterregulatory mechanisms and a high degree of redundancy in the chemokine system, we suggest that a therapeutic strategy for human autoimmune disease should include blockade of a group of chemokine receptors involved in the recruitment of pathogenic leukocyte subsets.

Disclosures

The authors have no financial conflict of interest.

References


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