CXCR3 Mediates Renal Th1 and Th17 Immune Response in Murine Lupus Nephritis


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Infiltration of T cells into the kidney is a typical feature of human and experimental lupus nephritis that contributes to renal tissue injury. The chemokine receptor CXCR3 is highly expressed on Th1 cells and is supposed to be crucial for their trafficking into inflamed tissues. In this study, we explored the functional role of CXCR3 using the MRL/MpJ-Fas<sup>−/−</sup> (MRL/lpr) mouse model of systemic lupus erythematosus that closely resembles the human disease. CXCR3<sup>−/−</sup> mice were generated and backcrossed into the MRL/lpr background. Analysis of 20-wk-old CXCR3<sup>−/−</sup> MRL/lpr mice showed amelioration of nephritis with reduced glomerular tissue damage and decreased albuminuria and T cell recruitment. Most importantly, not only the numbers of renal IFN-γ-producing Th1 cells, but also of IL-17-producing Th17 cells were significantly reduced. Unlike in inflamed kidneys, there was no reduction in the numbers of IFN-γ- or IL-17-producing T cells in spleens, lymph nodes, or the small intestine of MRL/lpr CXCR3<sup>−/−</sup> mice. This observation suggests impaired trafficking of effector T cells to injured target organs, rather than the inability of CXCR3<sup>−/−</sup> mice to mount efficient Th1 and Th17 immune responses. These findings show a crucial role for CXCR3 in the development of experimental lupus nephritis by directing pathogenic effector T cells into the kidney. For the first time, we demonstrate a beneficial effect of CXCR3 deficiency through attenuation of both the Th1 and the newly defined Th17 immune response. Our data therefore identify the chemokine receptor CXCR3 as a promising therapeutic target in lupus nephritis. The Journal of Immunology, 2009, 183: 4693–4704.

Systemic lupus erythematosus (SLE) is a chronic remitting and relapsing multisystem autoimmune disease that can affect the skin, joints, kidneys, lungs, nervous system, serous membranes, and/or other organs of the body. Renal involvement (lupus nephritis) is common in SLE and of central importance to the long-term prognosis. Typical morphological findings in human and experimental lupus nephritis are glomerular Ig deposits and the recruitment of mononuclear inflammatory cells. Infiltrating T cells, in particular, are thought to contribute to renal tissue damage in glomerular inflammation and, thus, to progressive loss of renal function. To date, diffuse and proliferative forms of glomerulonephritis, such as those found in SLE, have been believed to be predominantly Th1 cell mediated. However, the Th1 paradigm has recently been challenged by the identification of a new population of Th cells producing IL-17, which have been termed Th17 cells. Increasing evidence suggests that this newly identified T cell population is also crucial for the development and perpetuation of autoimmune disease. Two recent studies, including one by our own group, could show a role for Th17 cells in mouse models of glomerulonephritis.

Before T cells can exert their detrimental effects on renal function in lupus nephritis, they have to reach the site of injury. The molecule families of chemokines and chemokine receptors are key regulators of directional leukocyte trafficking under homeostatic and inflammatory conditions. By detecting chemokine concentration gradients via corresponding chemokine receptors, inflammatory cells are guided toward the focus of inflammation, where they exert their effector functions. Data from previous in vitro studies, experimental models, and studies in humans have highlighted the role of chemokines and their receptors in renal inflammation, including lupus nephritis. However, the functional importance of chemokine receptors in the recruitment of different T cell subsets into the kidney and the resulting clinical impact are still largely unknown. Chemokine receptors are expressed by effector T cells in a lineage-specific manner. Th1 cells express CXCR3, CCR5, and CXCR6, whereas Th2 cells express CCR3, CCR4, and CCR8.

Our group previously generated CXCR3-deficient mice and demonstrated that CXCR3<sup>−/−</sup> animals were partly protected from immunologically mediated injury by a reduction in renal T cell trafficking in a model of acute glomerulonephritis. This model of glomerulonephritis is an acutely induced “one-hit”
model that involves an inflammatory response with a certain time course. In contrast, many human autoimmune diseases such as lupus nephritis develop over a long time and are caused by multiple and chronic inflammatory stimuli.

The function of CXCR3 in lupus nephritis has not yet been determined. This study was therefore performed to define the role of CXCR3 in MRL/MpJ-Fas<sup>lpr</sup> (MRL/lpr) mice, a well-established mouse model of human lupus nephritis. CXCR3<sup>−/−</sup> mice were backcrossed into the MRL/lpr background to address two major issues, as follows: 1) What is the potential impact of CXCR3 deficiency on the clinical course of MRL/lpr nephritis? 2) What is the effect on renal Th1 and Th17 immune responses?

Materials and Methods

**Animals**

MRL/MpJ-Fas<sup>lpr</sup> (MRL/lpr) and MRL/MpJ (MRL) mice were purchased in 2003 from The Jackson Laboratory and subsequently maintained as breeding colonies in our animal facility. CXCR3<sup>−/−</sup> C57BL/6 mice were generated as published by us (22). The CXCR3 gene is located on the X chromosome. Male mice hemizygous for the CXCR3 mutation are CXCR3 deficient, and are therefore referred to as CXCR3<sup>−/−</sup>. All mice were housed under specific pathogen-free conditions. CXCR3<sup>−/−</sup> mice were backcrossed for at least eight generations into the MRL/lpr background to obtain CXCR3<sup>−/−</sup> MRL/lpr animals. To minimize genetic background differences between the animals, the CXCR3 wild-type MRL/lpr mice used in this study were derived from the same litter as the CXCR3<sup>−/−</sup> MRL/lpr animals. MRL mice were used as healthy controls. Only male animals were used for the experiments. Animal experiments were performed according to national and institutional animal care and ethical guidelines, and had been approved by local committees.

**Patients and tissue samples**

Renal biopsy samples were obtained from the archive of the Department of Pathology of the University Hospital of Hamburg-Eppendorf. Only patients in whom lupus nephritis of World Health Organization types III and IV had been diagnosed in our center were included in the study. Specimens of the control group derived from patients without autoimmune kidney diseases. The study was conducted with approval from the local ethics committee.

**Genotyping**

All mice were genotyped by PCR using genomic DNA that was prepared from tail biopsies. The following primers were used: CXC-EX2-F<sub>1</sub>, 5'-TTG CTT CTG AGT TGG GCT-3'; CXC-UTR-R<sub>1</sub>, 5'-TGG GGC TGA GCT GCT AGG AT-3'; Neo<sup>5</sup>-R<sub>1</sub>, 5'-CTA AAG GCC ATG CTC CAG ACT GCC-3' (for genotyping of the CXCR3 locus); Fas forward, 5'-AAAGGTACAAAAAGGTACCC-3'; Fas reverse, 5'-GGTGCGAC CCAAGCACTAG-3' and lpr reverse, 5'-GTTCCTCTTAGCATCTCTC TGC-3' (for genotyping of the fas mutation).

Additionally, a genome-wide screening with 201 autosomal single nucleotide polymorphism (SNP) markers, including the H-2 loci, as well as 16 SNP markers on the X chromosome, was conducted by the JAX Genome Scanning Service at The Jackson Laboratory.

**Functional studies**

For urine collection, mice were housed in metabolic cages for 6 h one day before sacrifice. Urinary albuminuria was determined by standard ELISA analysis (mice-albumin kit; Bethyl Laboratories). At the time of sacrifice, blood was drawn for blood urea nitrogen (BUN) measurement.

**Real-time RT-PCR analysis**

Total renal RNA from the cortex of frozen murine kidneys was prepared, as described previously (23). RNA from archived formalin-fixed and paraffin-embedded biopsies were extracted from two 10-μm-thick sections of each patient. The High Pure FFPE RNA kit (Roche) was used as published (23). Biopsy specimens from the same patients were used for immunohistochemistry. Real-time PCR was performed for 40 cycles (initial denaturation, 95°C, 10 min; denaturation, 15 s, 95°C; primer annealing and elongation, 1 min, 60°C) with 1.5 μl of cDNA samples in the presence of 2.5 μl of specific murine and human primers (primer sequences are available upon request) and 12.5 μl of 2× Platinum SYBR Green qPCR Supermix (Invitrogen) using an ABI Prism Sequence Detection System 7000 (Applied Biosystems). To account for small RNA and cDNA variability, an 18S rRNA PCR was run in parallel. All samples were run in duplicate and normalized to 18S rRNA, as described recently (24).

**In situ hybridization**

In situ hybridization procedures were performed, as described previously (24). In brief, probes for monokine induced by IFN-γ (Mig/ CXCL9, IFN-γ-inducible protein 10 (IP10)/CXCL10, IFN-γ-inducible T cell α chemotactant (IFAT/CXCL11), and CXCR3 used for in situ hybridization were prepared by in vitro transcription of subcloned cDNA with incorporation of 35S-labeled UTP. The 526-bp Mig/CXCL9 probe corresponds to nt 18–344 of sequence M34815, the 298-bp IP10/ CXCL10 probe corresponds to nt 264–554 of sequence NM_021274, the 235-bp IFAT/CXCL11 probe corresponds to nt 56–291 of sequence NM_019494, and the 286-bp CXCR3 probe corresponds to nt 1139–1425 of sequence NM_009910 (GenBank accession numbers http://www.ncbi.nlm.nih.gov/GenBank). 

**Morphological examinations**

Light microscopy was performed by routine procedures, as described previously (23). The percentage of glomeruli with crescents and capillary necrosis was assessed by a nephropathologist in at least 70 glomeruli per mouse in a blinded fashion using periodic acid-Schiff (PAS)-stained paraffin sections. 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, as previously described (22).

**Immunohistochemistry**

Paraffin-embedded sections (2 μm) were stained with the following Abs directed against the T cell marker CD3 (A0452; DakoCytomation): the monocyte-specific marker F4/80 (BM8; BMA); a marker for glomerular monocytes MAC-2 (M338; Cedarlane Laboratories); C3 (Cappel Laboratories; Organon Teknika); and mouse IgG1, IgG2a, IgG2b, or IgG3 (all Jackson ImmunoResearch Laboratories). Tissue sections were deparaffinized in the Vextastain ABC-AP kit (Vector Laboratories). Immunohistochemistry was performed using the Vectastain ABC-AP kit (Elite Kit, Vectastain PK-6102; Vector Laboratories), Tissue sections were developed with diaminobenzidine (DAB) for 5 min and visualized with 0.05% hydrogen peroxide and 0.01% nickel ammonium sulfate in 0.01 M Tris-HCl pH 7.6. Tissue sections were counterstained with 0.5% methyl green. Control sections were incubated with non-immune serum.

**Electron microscopy**

Light microscopy was performed by routine procedures, as described previously (23). The percentage of glomeruli with crescents and capillary necrosis was assessed by a nephropathologist in at least 70 glomeruli per mouse in a blinded fashion using periodic acid-Schiff (PAS)-stained paraffin sections. 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, as previously described (22).

**Isolation of leukocytes from renal, lymphatic, and intestinal tissues**

Previously described methods were used for renal cell isolation from murine kidneys (26). 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. Cell suspensions were centrifuged at 4000 rpm for 5 min. Single-cell suspensions were separated using Percoll density gradient (70 and 40%) centrifugation (27). The leukocyte-enriched cell suspension was aspirated from the Percoll interface. For single-cell suspension of spleens and lymph nodes, a leukocyte-lysis buffer (all Jackson ImmunoResearch Laboratories) was used for red blood cell lysis. Single-cell suspensions contained 95% viable cells with 98% purity (all Jackson ImmunoResearch Laboratories).
for at least eight generations, which resulted in male MRL/lpr CXCR3−/− and MRL/lpr CXCR3 wild-type littermates. B, PCR screening for the CXCR3 mutation resulted in a band at 628 bp in wild-type animals, at 421 bp in CXCR3−/− animals, and in both bands in heterozygous mice. Screening for the fas mutation resulted in a band at 179 bp in MRL control animals and at 217 bp in MRL/lpr mice. C, FACS analysis showed absence of CXCR3 on CD3+ splenocytes from MRL/lpr CXCR3−/− mice.

nodes, organs were minced and sequentially passed through 70- and 40-μm nylon meshes. After lysis of erythrocytes with ammonium chloride, cells were washed in RPMI 1640 medium for 0.5 min. Supernatants were filtered through a 70-RPMI 1640 medium, and then washed twice by shaking in complete RPMI. Small intestines were stirred at 37°C for 20 min in complete RPMI 1640 medium for 0.5 min. Supernatants were filtered through a 70-μm nylon sieve, and lymphocytes were enriched using Percoll density gradient centrifugation. After intraepithelial lymphocyte isolation, the cell viability was assessed by trypan blue staining.

Intraepithelial lymphocytes and lamina propria lymphocytes were isolated, as previously described (28). Briefly, after the excision of Peyer’s plaques, small intestines of the individual mice were cut open and washed several times with HBSS (without Ca2+ and Mg2+) and re-suspended in RPMI 1640 with 10% FCS. After 30 min of incubation, brefeldin A (10 ng/ml; Sigma-Aldrich) and ionomycin (1 μg/ml) were added. After several washing steps and staining of cell surface markers, cells were incubated with Cytofix/Cytoperm (BD Biosciences) at 4°C for 20 min to permeabilize cell membranes. Then, intracellular IL-17 and IFN-γ were stained using rat anti-mouse IL-17 Ab (PE; TC11-18H10; BD Pharmingen) and an anti-IFN-γ Ab (FTC; XMG1.2; BD Pharmingen). Experiments were performed with a BD Biosciences FACScalibur or FACScanto System using the CellQuest Professional or Diva software.

Assessment of the humoral immune response

Serum levels of IgG isotopes were determined by sandwich ELISA using goat anti-mouse IgG Ab as capture Ab and HRP-conjugated Abs for detection of IgG1, IgG2a, IgG2b, and IgG3 isotopes (Dianova). Serial dilutions of serum samples are indicated in Fig. 5B. Abs against dsDNA were detected by indirect ELISA at the indicated dilutions on plates coated with calf thymus DNA (5 μg/ml; Sigma-Aldrich) and alkaline phosphatase-conjugated goat anti-mouse polyvalent Ig Ab (Sigma-Aldrich). Serum from MRL/lpr mice was used in 1/100–1/12,500 dilution as positive control in autoantibody assays.

Statistical analysis

Results are expressed as individual data points. The horizontal line represents the mean. Differences between individual experimental groups were compared by Kruskal-Wallis test with post hoc analysis by Mann-Whitney U test. Those experiments that did not yield enough independent data for statistical analysis due to the experimental setup were repeated at least three times.

Results

Generation and characterization of MRL/lpr CXCR3−/− mice

The targeting strategy outlined in Fig. 1A was used to generate CXCR3−/− mice with a complete deletion of exon 2 of the CXCR3 gene (22). Using PCR analysis, all animals were genotyped for the fas mutation and CXCR3 status, as described in Materials and Methods (Fig. 1B). To confirm the absence of the CXCR3 protein, FACS analyses were performed, which showed CXCR3 expression on ~25% of CD3+ T cells from spleens of MRL/lpr mice, whereas CD3+ T cells from MRL/lpr CXCR3−/− mice were negative (Fig. 1C).

To assess the H-2 class I and II haplotypes, which differ between MRL and C57BL/6 mice, FACS analyses were performed. These showed that leukocytes from peripheral blood of MRL/lpr wild-type and MRL/lpr CXCR3−/− mice solemnly express the MRL
characteristic β allele, whereas no expression of the C57BL/6 characteristic β allele could be detected (supplemental Fig. 1). Furthermore, SNP analysis of the complete autosome showed >99% MRL/lpr background in CXCR3 intact as well as CXCR3 knockout MRL/lpr littersmates with no residual C57BL/6 or 129sv/PAS genomic DNA background. Screening of the X chromosome showed complete MRL/lpr background with the exception of the regions surrounding the CXCR3 gene. Here MRL/lpr CXCR3 knockout mice still have fragments of the C57BL/6 background (supplemental Table I). MRL/lpr CXCR3<sup>−/−</sup> mice were viable, fertile, and showed no developmental defects in comparison with MRL/lpr mice (data not shown).

**Chemokine and chemokine receptor expression in MRL/lpr nephritis**

Real-time quantitative PCR was performed to compare intrarenal expression of CXCR3 and its ligands Mig/CXCL9, IP10/CXCL10, and ITAC/CXCL11 between MRL/lpr mice and nonnephritic MRL controls at five different time points (Fig. 2A). mRNA expression of the receptor and all three ligands was already increased in MRL/lpr mice at 8 wk of age and continued to increase until the last time point analyzed, i.e., at 25 wk of age.

Analysis of chemokine mRNA from kidneys of 20-wk-old nephritic mice showed a lower, but statistically insignificant expression level of all three CXCR3 ligands in knockout animals as compared with wild-type mice. Analysis of monocyte-attracting chemokines showed a significantly less pronounced increase in MCP1/CCL2 expression in CXCR3<sup>−/−</sup> animals, whereas expression of RANTES/CCL5 was similar in both types of mice (Fig. 2B).

To localize intrarenal chemokine and chemokine receptor expression, we performed in situ hybridization. Strong Mig/CXCL9, IP10/CXCL10, and ITAC/CXCL11 expression was found in interstitial inflammatory infiltrates, inside glomeruli, and in the periglomerular area. No difference was seen in the expression pattern between MRL/lpr and MRL/lpr CXCR3<sup>−/−</sup> mice. CXCR3 mRNA was expressed in an overlapping pattern with the three chemokine ligands in MRL/lpr mice, whereas CXCR3<sup>−/−</sup> mice showed no staining (Fig. 2C).

**Renal T cell and monocyte recruitment**

To investigate renal T cell infiltration, immunohistochemical staining for the pan T cell marker CD3 was performed. For analysis of compartment-specific monocyte infiltration, two different immunohistochemical staining methods (MAC-2 and F4/80) were used, because MAC-2 is suitable for monocyte detection in glomeruli, but not in the tubulointerstitium. In contrast, F4/80 is more sensitive for the detection of interstitial monocytes, but less specific for monocytes in the glomeruli. Representative staining patterns are shown in Fig. 3A.

Quantification of CD3<sup>+</sup> cells revealed a significant increase in glomerular T cells in MRL/lpr wild-type mice when compared with MRL controls of the same age. MRL/lpr CXCR3<sup>−/−</sup> mice, however, showed significantly fewer glomerular T cell infiltrates when compared with MRL/lpr wild-type mice. Unlike in MRL controls, tubulointerstitial T cell infiltration was also highly increased in MRL/lpr wild-type mice. As in the glomeruli, interstitial T cell infiltration was significantly reduced in MRL/lpr CXCR3<sup>−/−</sup> mice when compared with MRL/lpr wild-type mice (Fig. 3B).
In comparison with control mice, both MRL/lpr wild-type and MRL/lpr CXCR3<sup>−/−</sup> mice showed a significant increase in tubulointerstitial and glomerular monocyte recruitment. Although there was a decrease in infiltrating glomerular monocytes in MRL/lpr CXCR3<sup>−/−</sup> mice when compared with MRL/lpr wild-type mice, the reduction was not statistically significant. In contrast, tubulointerstitial monocyte infiltration in MRL/lpr CXCR3<sup>−/−</sup> mice was significantly reduced as compared with MRL/lpr wild-type mice (Fig. 3C).

**Assessment of renal histological injury**

PAS staining of renal tissue at the time of sacrifice showed severe glomerular damage with crescent formation and capillary necrosis in MRL/lpr wild-type mice, as has previously been described for this model. Glomerular lesions in MRL/lpr CXCR3<sup>−/−</sup> mice, however, were significantly reduced (Fig. 4A). Electron microscopic examination confirmed the light microscopic findings. In MRL control mice, there was only mild segmental expansion of the mesangium, with few mesangial deposits. Glomerular basement membranes and podocytes appeared normal. MRL/lpr wild-type mice showed severe mesangial expansion with increased matrix deposition, increased cell numbers, activated mesangial cells, and electron-dense deposits. Capillary lumina frequently contained leukocytes. The glomerular basement membranes showed irregularities that included newly formed matrix and frequent subepithelial and few subendothelial deposits. Podocytes displayed extensive effacement of foot processes. MRL/lpr CXCR3<sup>−/−</sup> mice, in comparison, showed only mild expansion of the mesangium, but a similar amount of mesangial electron-dense deposits. Peripheral glomerular basement membranes and podocyte foot processes were mostly well preserved. There were no subepithelial or subendothelial deposits (Fig. 4B).

To quantify renal tissue damage, kidney sections from 20-wk-old MRL/lpr CXCR3<sup>−/−</sup>, MRL/lpr wild-type, and healthy MRL control mice were evaluated for the presence of glomerular crescents and capillary necrosis. The frequency of crescents was significantly decreased in MRL/lpr CXCR3<sup>−/−</sup> mice compared with MRL/lpr wild-type animals. Furthermore, glomeruli of MRL/lpr CXCR3<sup>−/−</sup> mice showed a significantly lower frequency of capillary necrosis. MRL control mice did not show any crescents or capillary necrosis (Fig. 4C).

**Assessment of functional renal impairment**

Albuminuria normalized to urinary creatinine was assessed at 10, 15, and 20 wk of age. MRL/lpr wild-type mice showed a markedly increased albumin to creatinine ratio at 15 and 20 wk of age compared with MRL control mice. However, the albumin to creatinine ratio was dramatically reduced in MRL/lpr CXCR3<sup>−/−</sup> mice when compared with MRL/lpr wild-type mice at these time points (Fig. 4D). Furthermore, BUN levels were assessed at the time of sacrifice (20 wk of age). BUN levels of MRL/lpr wild-type mice were significantly higher than those of nonnephritic MRL controls. BUN levels in MRL/lpr CXCR3<sup>−/−</sup> mice tended to be lower than in MRL/lpr wild-type mice, but differences were not statistically significant (Fig. 4E).

**Renal Th1 and Th17 immune responses**

To assess the role of Th1 and Th17 immune responses in MRL/lpr nephritis and the effects of CXCR3 deficiency, we performed intracellular staining for the Th1 prototype cytokine IFN-γ and for IL-17, the characteristic Th17 cytokine. Analyses showed a slightly reduced percentage of IFN-γ-producing CD3<sup>+</sup> and CD4<sup>+</sup> T cells in kidneys of MRL/lpr CXCR3<sup>−/−</sup> mice as compared with their MRL/lpr wild-type counterparts. It is of note, however, that in comparison with MRL/lpr wild-type mice, the total number of infiltrating T cells was significantly reduced, which confirms our data from the previous immunohistochemical studies. Furthermore, a large number of IL-17<sup>+</sup> intrarenal T cells was seen in MRL/lpr wild-type mice, whereas these IL-17-producing T cells were almost completely absent in MRL/lpr CXCR3<sup>−/−</sup> mice (Fig. 5A). No significant numbers of IFN-γ- or IL-17-expressing T cells
were found in MRL control animals (data not shown). Interestingly, no double-positive IL-17- and IFN-γ-producing renal T cells were detectable, underscoring the dichotomy of these T cell subtypes (data not shown). To test whether the reduction in IFN-γ- and IL-17-producing T cells was due to impaired trafficking of these cells, six-color FACS analyses (CXCR3, CD3, CD4, CD8, IFN-γ, and IL-17) of infiltrating renal cells and splenocytes were performed. Results showed that CXCR3 was predominantly expressed in both organs on CD4+ Th cells, whereas most CD8+ and double-negative (DN) T cells did not express this receptor (Fig. 6A).

Comparative results were seen with regard to IFN-γ-positive cells. More than half of renal IFN-γ-expressing CD4+ T cells carried the CXCR3, whereas most IFN-γ-positive CD8+ and DN T cells did not. CXCR3 was also found to be present on renal IL-17-expressing T cells. Approximately one-third of CD4+ and CD8+ T cells and one-half of DN IL-17-expressing T cells were CXCR3 positive (Fig. 6B). Numbers of CXCR3-positive IFN-γ-producing T cells in the spleen were smaller than in the kidney, but analysis of subtypes showed the same pattern (Fig. 6C). As expected, no significant IL-17 production was observed in splenocytes (data not shown).

Characterization of renal and systemic CXCR3-positive cells

To further validate whether the general reduction of T cells, as well as the Th1 and Th17 subsets, observed in CXCR3 knockout mice was due to impaired trafficking of these cells, six-color FACS analyses (CXCR3, CD3, CD4, CD8, IFN-γ, and IL-17) of infiltrating renal cells and splenocytes were performed. Results showed that CXCR3 was predominantly expressed in both organs on CD4+ Th cells, whereas most CD8+ and double-negative (DN) T cells did not express this receptor (Fig. 6A).

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Renal and systemic immune responses

To investigate whether CXCR3 deficiency induces alterations in the humoral immune response, we performed immunohistochemical staining for mouse IgG and complement C3 in kidneys of...
20-wk-old MRL control, MRL/lpr wild-type, and MRL/lpr CXCR3−/− mice. As shown in Fig. 5A, no difference was found in the degree of glomerular IgG deposition between MRL/lpr wild-type and MRL/lpr CXCR3−/− mice. Further analyses of IgG subclasses did not show any difference in Ab deposition of IgG1, IgG2a, IgG2b, or IgG3 either (supplemental Fig. 2). C3 deposition,
however, was slightly reduced in MRL/lpr CXCR3−/− mice when compared with their wild-type counterparts. MRL control mice did not show relevant IgG or C3 deposition (Fig. 7A).

To assess the systemic humoral immune response, we analyzed the isotype pattern of IgG Abs in the serum of nephritic mice using ELISA. Quantification of IgG isotypes revealed no bias for either
Th1 (IgG2a, IgG3)- or Th2 (IgG1)-type Ab production in MRL/lpr or CXCR3−/− mice compared with MRL/lpr wild-type mice. Abs of the IgG2b isotype were also present in comparable amounts in MRL/lpr CXCR3−/− mice and MRL/lpr wild-type mice (Fig. 7B). Abs against dsDNA typically present in SLE were significantly up-regulated in MRL/lpr wild-type animals compared with MRL control mice. However, the titers in MRL/lpr wild-type animals were comparable to those of MRL/lpr CXCR3−/− mice (Fig. 7C).

Because MRL/lpr mice spontaneously develop a lymphoproliferative disease with marked enlargement of secondary lymphoid organs, we measured the spleen weight. In comparison with MRL control mice, both MRL/lpr wild-type and MRL/lpr CXCR3−/− mice showed a similar enlargement of the spleen (Fig. 7D). To examine whether CXCR3 deficiency results in a general defect during ontogenesis of the immune system, we analyzed the architecture of secondary and primary lymphatic organs at the time of sacrifice. As can be seen in Fig. 7E, the spleen and lymph node architecture was unaltered in CXCR3−/− MRL/lpr mice, and showed clear segregation of CD3+ T cell, CD19+ B cell, and CD11b+ monocyte/macrophage regions. The vast majority of cells in the thymus of both wild-type and CXCR3−/− mice were CD3+ T cells (thymocytes), with a smaller population of CD11b+ monocytes/macrophages.

Detection of CXCR3 and IP-10/CXCL10 in human lupus nephritis

To investigate whether the chemokine receptor CXCR3 might also be involved in renal T cell trafficking in human lupus nephritis, we performed serial and double staining for CD4 and CXCR3 in renal biopsies from patients with World Health Organization type IV lupus nephritis. Representative stainings are shown in Fig. 8, A and B, demonstrating that a large portion of infiltrating CD4+ T cells

**FIGURE 7.** Renal and systemic immune responses. A, Immunohistochemical staining for mouse IgG showed identical deposition in both MRL/lpr wild-type and CXCR3−/− animals, whereas C3 deposition was slightly reduced. MRL control kidneys did not contain relevant amounts of IgG or C3 (original magnification: ×400). B, Quantification of IgG subclasses did not reveal any differences between MRL/lpr wild-type and CXCR3−/− animals. C, Analysis of dsDNA Abs showed significant up-regulation in nephritic animals as compared with MRL controls (n = 6). The levels in MRL/lpr wild type (n = 12) were comparable to those detected in CXCR3−/− (n = 11) animals. D, Comparison of spleen weight demonstrated a similar degree of lymphoproliferation in both MRL/lpr wild-type (n = 11) and CXCR3−/− mice (n = 8). Spleen weights of MRL control animals (n = 3) were significantly lower than in the two other groups. E, Triple immunostaining of lymphatic organs for the T cell marker CD3 (blue), the B cell marker CD19 (green), and the monocyte marker CD11b (red) showed no difference between MRL/lpr wild-type and CXCR3−/− animals. The microarchitecture with separate T cell, B cell, and monocyte zones was completely preserved (original magnification: 100-fold). Symbols represent individual data points, and vertical lines represent means.
expresses the CXCR3 on their surface. No significant CXCR3 positivity was seen on resident kidney cells.

To analyze intrarenal expression of CXCR3 and its ligand IP10/CXCL10, RNA was isolated from renal biopsy specimens obtained from nine lupus patients with World Health Organization type IV and three patients with World Health Organization type III proliferative lupus nephritis. Renal biopsies from five patients without renal autoimmune disease served as controls. mRNA analysis revealed a significantly higher IP10/CXCL10 mRNA expression in the group of lupus patients compared with the healthy control group (Fig. 8C).

**Discussion**

In a wide range of human inflammatory diseases such as glomerulonephritis (31, 32), renal transplant rejection (25, 33), rheumatoid arthritis (34), and multiple sclerosis (35), the chemokine receptor CXCR3 has been localized to infiltrating effector T cells. The three CXCR3 ligands, Mig/CXCL9, IP-10/CXCL10, and ITAC/CXCL11, have been shown to be expressed in an overlapping pattern with CXCR3-bearing Th cells in these diseases, arguing for a decisive role in the recruitment of CXCR3+ cells (25, 31, 34, 35). Data from experimental models further support the functional importance of the CXCR3 receptor in T cell trafficking. Involvement of CXCR3 was reported in murine models of heart allograft rejection (36), autoimmune type 1 diabetes mellitus (37), and acute renal ischemic injury (38, 39). CXCR3+/− mice were largely protected from organ damage in these immunologically mediated diseases.

Beneficial effects have also been shown in two models of acute glomerulonephritis. Using a rat model of renal endothelial microvascular injury (24), our group was able to demonstrate that Ab neutralization of the CXCR3 ligand IP10/CXCL10 had beneficial effects. Moreover, we recently showed amelioration of renal disease in CXCR3−/− mice with nephrotoxic serum nephritis (NTN) (22). These observations have meanwhile been confirmed by a study conducted by another group that shows improved renal outcome in mice deficient in the CXCR3 or its ligand Mig/CXCL9 in NTN. Surprisingly, however, IP10/CXCL10 deficiency did not alter renal pathology (40). There is still a lack of evidence to support a protective role of interference with the CXCR3 receptor-ligand axis in chronic renal autoimmune disease, such as the lupus nephritis of MRL/lpr mice. As in NTN, IP10/CXCL10 deficiency did not lead to improvement of the clinical course of glomerulonephritis in this murine model of human lupus nephritis (40). The influence of CXCR3 deficiency has not been studied to date.

The beneficial effects of CXCR3 deficiency in glomerulonephritis have generally been attributed to impaired trafficking of Th1-polarized T cells to the kidney. However, no definite evidence has yet been found that the number of intrarenal T cells producing the Th1 prototype cytokine IFN-γ is reduced in CXCR3−/− mice. Furthermore, CXCR3 has recently been found to be also expressed on the Th17 cell population (19, 20). There are only sparse data on the role of the Th17 response in glomerulonephritis and no data at all on the effect of CXCR3 deficiency on Th17 cell trafficking. In this study, we show for the first time that CXCR3 deficiency improves renal outcome in the MRL/lpr model of lupus nephritis, not only by reducing the number of renal Th1 cells, but also by almost completely abolishing Th17 cell infiltration.
In agreement with published studies (40, 41), renal mRNA expression of all three CXCR3 ligands, Mig/CXCL9, IP-10/ CXCL10, and ITAC/CXCL11, as well as CXCR3 mRNA, increased with progression of nephritis in MRL/lpr animals from 10 wk of age. At the time of sacrifice (20 wk of age), intrarenal chemokine levels of all CXCR3 ligands were slightly reduced in MRL/lpr CXCR3−/− mice. By contrast, MCP-1/CCL2 expression was significantly less enhanced in MRL/lpr CXCR3−/− mice. As shown by in situ hybridization, all three CXCR3 ligands were abundantly expressed inside inflamed glomeruli and in the periglomerular area. No significant difference was found in the distribution pattern between wild-type and knockout mice.

Despite similar intrarenal expression of the CXCR3 ligands, the total numbers of infiltrating T cells were significantly reduced inside the glomeruli and in the tubulointerstitium of CXCR3−/− mice. The number of tubulointerstitial monocytes/macrophages was also lower in CXCR3−/− mice, which may be secondary to substantially reduced renal expression of the monocyte-attracting chemokine MCP-1/CCL2. Because the MCP-1/CCL2 production by resident renal cells is stimulated by IL-17 (11), reduced expression might be a result of impaired renal Th17 cell infiltration. We cannot exclude, however, that CXCR3 expression on activated monocytes also contributes to the trafficking of this pathogenic cell population into the nephritic kidney, as was recently reported (40).

In accordance with reduced T cell and monocyte infiltration, renal tissue damage was substantially less pronounced in CXCR3−/− mice. Glomerular crescent formation and capillary necrosis were significantly reduced in CXCR3−/− mice as compared with their wild-type counterparts. Analysis by electron microscopy confirmed these data. Amelioration of the renal morphological damage led to direct improvement of renal function. In CXCR3−/− mice, albuminuria was substantially decreased and the BUN level tended to be lower.

To test the hypothesis that CXCR3 deficiency interferes with trafficking of Th1 and maybe also Th17 cells into the kidney, we performed FACS analyses of isolated renal leukocytes. CXCR3 expression on renal infiltrating cells was found predominantly on the CD4-positive Th cell subset. Approximately 60% of IFN-γ-producing Th1 cells were positive for CXCR3. Interestingly, we also found that approximately one-third of infiltrating IL-17-producing Th17 cells expressed high levels of CXCR3 on their surface. In agreement with these observations and our immunohistochemical studies, significantly reduced total numbers of intrarenal T cells, IFN-γ-secreting Th1, and IL-17-producing Th17 cells were found. However, the relative contribution of IFN-γ-secreting renal T cells to the total number of T cells was only slightly reduced. This might be due to alternative mechanisms of Th1 cell migration into the inflamed kidney, possibly via the chemokine receptors CCR5 and CXCR6, which are also known to be expressed on Th1-polarized cells (42). Alternatively, CXCR3 is likely to be involved in the trafficking of non-IFN-γ-producing T cells because we could show that a significant number of IFN-γ-negative cells also express CXCR3. In this case, one could hypothesize that similarly impaired migration of these cells restores the balance between infiltrating IFN-γ-positive and IFN-γ-negative cells. Th17 cells are one of the candidate cell populations in this process. Intrarenal T cells with high expression of the Th17-defining cytokine IL-17 were clearly present in nephritic wild-type mice. Strikingly, this population of highly pathogenic T effector cells was completely absent in CXCR3−/− animals. Because only about one-third of Th17 cells express CXCR3, other yet to be defined mechanisms probably contribute to this observation. It is likely that reduced recruitment of CXCR3-positive cells leads to less tissue damage and inflammation, which in turn reduces influx of further immune cells, including Th17 cells, independent of their CXCR3 expression.

To investigate whether a reduction in intrarenal IFN-γ- and IL-17-producing T cells might be due to an impaired cellular immune response in CXCR3−/− mice, we analyzed spleens and renal lymph nodes. In CXCR3−/− MRL/lpr mice, the total number and percentage of IFN-γ-producing T cells in these tissues were even slightly increased, showing their general ability to mount a physiological Th1 response. In accordance with published data (30), no relevant IL-17 production was found in either lymph nodes or spleens. We therefore analyzed two intestinal lymphocyte populations in which Th17 cells are constitutively present (30). FACS analyses showed a higher number and frequency of IL-17-producing T cells in lamina propia as well as in intraepithelial lymphocytes in CXCR3−/− mice, whereas the Th17 population in MRL/lpr wild-type animals was identical with that found in healthy control mice. These data demonstrate that a reduction in IL-17-producing T cells inside inflamed kidneys is not due to a general defect in the differentiation of Th17 cells, but rather argues for impaired trafficking of these cells in CXCR3−/− mice.

The fact that systemic Th1 and Th17 immune responses are intact in CXCR3−/− animals highlights the potential use of CXCR3 blockade as therapeutic strategy in inflammatory kidney diseases. In contrast, neutralization of the shared p40 subunit of IL-12 and IL-23, the only other molecule known to simultaneously interfere with pathologic Th1 and Th17 responses, results in general immune defects, and is therefore likely to cause significant unwanted side effects.

Because CXCR3 deficiency might have effects on the humoral immune response (40), we evaluated glomerular and systemic IgG levels as well as glomerular complement deposition. By immunohistochemistry, no relevant difference was seen between MRL/lpr CXCR3−/− and wild-type mice with respect to glomerular binding of IgG and its subtypes. Deposition of complement C3 appeared slightly reduced in CXCR3−/− animals. Although the quantity of immune complexes was similar between the groups, electron microscopy revealed a differential pattern of deposition. The majority of Ig deposition in both strains of mice located to the mesangial area. However, subepithelial and subendothelial deposits, which are characteristic of more aggressive forms of lupus nephritis, were regularly present in the MRL/lpr wild-type mice and greatly reduced in CXCR3 knockout animals.

Analysis of systemic dsDNA Abs and IgG1, IgG2a, IgG2b, and IgG3 showed similar levels in both groups of animals. Amelioration of renal disease in CXCR3−/− lpr mice therefore seems to be more a result of an impaired cellular, rather than an impaired humoral immune response.

To assess possible general defects in the ontogenesis of the immune system in CXCR3−/− mice, we examined primary and secondary lymphatic organs. Spleens of both CXCR3 wild-type and CXCR3−/− mice showed the same degree of lymphoproliferation as assessed by measurement of the organ weights. Even more important, the tissue microarchitecture of spleen, lymph nodes, and thymus was unaltered in MRL/lpr CXCR3−/− mice as compared with their wild-type counterparts, arguing against general immune defects in CXCR3−/− animals.

To verify the genetic background of our experimental mice, screening of the complete genome was conducted. Importantly, analyses confirmed that the complete autosome, including all known lupus susceptibility loci as well as the H-2 region, was of the MRL/lpr genetic background. The X chromosome was also shown to be of MRL/lpr background, except fragments surrounding the CXCR3 locus, which remained of the C57BL/6 background. It is highly unlikely, however, that this small amount of residual non-MRL/lpr background results in specific impairment of cellular trafficking, whereas the renal humoral and the systemic humoral and cellular immune response is unchanged.
To investigate whether CXCR3 and its ligands are also involved in human lupus, we analyzed renal biopsies from patients with active lupus nephritis. In this study, we could demonstrate high intrarenal mRNA expression of CXCR3 and its ligand IP10/CXCL10 as well as CXCR3-bearing Th cells infiltrating the inflamed kidneys. These findings are in line with recently published data (43) and suggest that CXCR3-mediated renal T cell trafficking is not only important in murine, but also in human lupus nephritis.

Taken together, our data show for the first time that not only Th1, but also Th17 effector T cells mediate glomerulonephritis in MRL/lpr mice. Deficiency of the chemokine receptor CXCR3 leads to significant morphological and functional improvement of nephritics kidneys. These protective effects are mediated by interference with trafficking of both Th1 and Th17 cells into the inflamed kidneys. Moreover, we provide further evidence that CXCR3-bearing T cells are not only of importance in experimential, but also in human lupus nephritis. Therefore, CXCR3 blockade is a promising new strategy, especially in the light of the recently developed small molecular inhibitors.

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Disclosures
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