CXCL9, but not CXCL10, Promotes CXCR3-Dependent Immune-Mediated Kidney Disease

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ABSTRACT

Chemokines are instrumental in macrophage- and T cell–dependent diseases. The chemokine CCL2 promotes kidney disease in two models of immune-mediated nephritis (MRL-Faslpr mice and the nephrotoxic serum nephritis model), but evidence suggests that multiple chemokines are involved. For identification of additional therapeutic targets for immune-mediated nephritis, chemokine ligands and receptors in CCL2−/− and wild-type (WT) MRL-Faslpr kidneys were profiled. The focus was on intrarenal chemokine ligand/receptor pairs that were highly upregulated downstream of CCL2; the chemokine CXCL10 and its cognate receptor, CXCR3, stood out as potential therapeutic targets. However, renal disease was not suppressed in CXCL10−/− MRL-Faslpr mice, and CXCL10−/− C57BL/6 mice were not protected from nephrotoxic serum nephritis compared with WT mice. Because CXCR3 engages with the ligand CXCL9, CXCR3+/−, CXCL9−/−, and CXCL10−/− B6 mice were compared with WT mice with nephrotoxic serum nephritis. Kidney disease, measured by loss of renal function and histopathology, was suppressed in both CXCR3−/− and CXCL9−/− mice but not in CXCL10−/− mice. With nephrotoxic serum nephritis, CXCR3−/− and CXCL9−/− mice had fewer intrarenal activated T cells and activated macrophages. Both IgG glomerular deposits and antigen-specific IgG in serum were reduced in these mice, suggesting that although CXCR3 and CXCL9 initiate nephritis through cell-mediated events, renal inflammation may be sustained by their regulation of IgG. It is concluded that specific blockade of CXCL9 or CXCR3 may be a potential therapeutic target for human immune-mediated kidney diseases.


Chemokines are instrumental in the recruitment, migration, and effector functions of immune cells during inflammation.1–4 Chemokine ligands engaging with their cognate receptors promote the influx of leukocytes into the kidney, a hallmark of nephritis.5,6 Although multiple chemokines share the same receptor, they are not necessarily redundant.7 Because chemokine ligand/receptors are induced during inflammation, these molecules are appealing therapeutic targets for nephritis.

Macrophages (Mø) and T cells in the kidney mediate inflammation.8 MRL-Faslpr mice and nephrotoxic serum nephritis (NSN) are two distinct T cell– and Mø-dependent mouse models of immune-mediated nephritis.9 We previously determined that CCL2 (monocyte chemoattractant protein-1) is
pivotal in promoting renal disease in these models. Using CCL2−/− MRL-Faspr mice, we established that tubular/interstitial and glomerular disease is suppressed.10 By comparison, tubular/interstitial but not glomerular disease is suppressed in CCL2−/− mice during NSN.10,11 In each model, Mø and T cells are no longer recruited to sites in the interstitium adjacent to tubular epithelial cells (TEC), the major source of CCL2 but rich in CCL5 (RANTES), a chemokine capable of inciting local renal inflammation in MRL-Faspr mice.12 This suggests that multiple chemokines dictate the tempo and locale of kidney disease.

To identify the chemokines along with CCL2 that are instrumental in T cell– and Mø-mediated nephritis, we extensively profiled kidneys of CCL2−/− and wild-type (WT) MRL-Faspr mice during the development of lupus nephritis. We identified a highly expressed chemokine ligand/receptor pair instrumental in attracting T cells during inflammation, CXCL10 (IP-10)/CXCR3.13–16 We report that CXCR3 is expressed on intrarenal activated Mo in addition to T cells during experimental immune-mediated kidney disease. Furthermore, the expression of CXCR3 on T cells and Mø serves as a mediating their recruitment into the kidneys expressing CXCL9 during NSN. Finally, we determined that CXCR3 and one, CXCL9, but not another, CXCL10, of its ligands promote T cell– and Mø-dependent nephritis. Thus, CXCL9 and CXCR3 are potential therapeutic targets for immune-mediated kidney illnesses.

RESULTS

Multiple Chemokine Ligand/Receptors Are Upregulated in MRL-Faspr Nephritic Kidneys

To identify potential therapeutic chemokine ligand/receptor targets in MRL-Faspr mice, we compared intrarenal chemokine ligand/receptor transcripts in mice before (2 mo of age) and after (5 mo of age) onset of nephritis. The majority of intrarenal chemokine ligands (18 of 23) and chemokine receptors (10 of 16) we evaluated increased with advancing nephritis (Supplemental Figure 1). We focused on the groups with the highest increase in intrarenal transcript expression. Within this group of chemokine ligands, we detected an increase in CXCL9 (nine-fold), CXCL9 (45-fold), CXCL11 (10-fold), CXCL13 (133-fold), CCL5 (16-fold), CCL20 (seven-fold), and CX3CL1 (two-fold), and within the group of chemokine receptors, we detected an increase in CXCR3 (nine-fold), CXCR4 (six-fold), CXCR5 (42-fold), CCR2 (10-fold), and CX3CR1 (six-fold; Supplemental Figure 1). Of note, the chemokine ligand/receptor transcript levels in the MRL-Faspr and B6 kidneys at 2 mo of age were similar.

Identifying Chemokine Ligand/Receptors Other than CCL2 that Are Expressed in Nephritic MRL-Faspr Kidneys

CCL2 promotes lupus nephritis in MRL-Faspr mice.10 To identify chemokines other than CCL2 that are central to MRL-Faspr nephritis, we compared chemokine ligand/receptor transcripts in CCL2−/− and WT MRL-Faspr kidneys. Most (>80%) chemokine ligand/receptor transcripts that were upregulated in WT MRL-Faspr nephritic kidneys were suppressed in CCL2−/− MRL-Faspr kidneys (Supplemental Figure 2). One possible interpretation is that within the hierarchical pattern of chemokine ligand/receptor expression regulating immune responses, CCL2 is proximal in the chemokine cascade leading to nephritis in MRL-Faspr mice; therefore, our goal was to focus on the chemokine ligand/receptors that maybe expressed “downstream” of CCL2 in MRL-Faspr kidneys during nephritis.

Mø and TEC Are Sources of Intrarenal CXCL10 Expression during Lupus Nephritis in MRL-Faspr Mice

CXCL10 engaging with its receptor CXCR3 is a potent T cell chemoattractant.15 Because CXCL10 is among the most highly upregulated chemokines and may be downstream of CCL2, we explored the role of CXCL10 in MRL-Faspr mice. Alveolar Mø express CXCL10,17 and TEC are a rich source of multiple chemokines in MRL-Faspr mice; therefore, Mø and TEC were prime candidates in our attempt to identify sources of CXCL10. We detected CXCL10 in TEC (Figure 1A), leukocytes (Figure 1A, inset), and endothelial cells (data not shown) in MRL-Faspr mice with nephritis. CXCL10−/− MRL-Faspr kidneys served as negative controls. The frequency of CXCL10+ Mø increased (two-fold) in MRL-Faspr kidneys from 3 to 6 mo of age with advancing nephritis as determined by FACS analysis (Figure 1A). By comparison, the frequency of CXCL10+ T cells (CD4+, CD8+) in MRL-Faspr kidneys, before and during nephritis, was minimal (approximately 2%; data not shown). Similarly, the expression of CXCL10 in primary TEC derived from MRL-Faspr mice increased (more than nine-fold) as determined by real-time PCR (Figure 1A). Of note, CXCL10+ Mø and TEC in MRL-Faspr kidneys were similar to B6 kidneys (3 mo of age). Thus, Mø and TEC are intrarenal sources of CXCL10 during nephritis in MRL-Faspr mice.

Frequency of CXCR3+ T Cells and Mø Is Increased in MRL-Faspr Nephritic Kidneys

Because there is an increase in the frequency of intrarenal CXCL10+ Mø and TEC, we probed for the expression of CXCR3. The frequency of CD4+, CD8+, and B220+ (unique double-negative) T cells and Mø (CD68+) expressing CXCR3 in the kidneys of MRL-Faspr mice increased with nephritis (6 mo of age) as compared with non-nephritic MRL-Faspr and B6 mice (2 mo of age; Figure 1B). The increased frequency of CXCR3+ T cells and Mø was not limited to the kidney; these leukocytes increased in their spleens (p < 0.05; data not shown). Taken together, an increase in intrarenal and extrarenal CXCR3+ T cell subsets and Mø is associated with a rise in intrarenal CXCL10 in nephritic MRL-Faspr mice.
Nephritis, Systemic Disease, and Survival Are not Altered in CXCL10+/− MRL-Faslpr Mice

To determine whether CXCL10 is central to lupus nephritis and the systemic illness, we compared CXCL10+/− and WT MRL-Faslpr strains (6 mo of age). The severity of glomerular, interstitial, and perivascular pathology was similar in CXCL10+/− and WT MRL-Faslpr mice (Figure 1C). Similarly, we did not detect a difference in the number (CD68+, CD4+, CD8+, B220+ cells) and activation stages (CD69+; IFN-γ) of infiltrating leukocytes and renal function (Figure 1C). Furthermore, we did not detect a difference in survival in CXCL10+/− MRL-Faslpr mice (data not shown). Thus, despite enhanced intrarenal expression of CXCL10 and CXCR3 during nephritis, CXCL10 is not central to disease in MRL-Faslpr mice.

CXCR3 and Its Ligands, CXCL9, CXCL10, and CXCL11, Are Upregulated during NSN

Our results suggest that CXCL10 is not central to lupus nephritis; however, it is possible that CXCR3 engaging with other ligands (CXCL9, CXCL11) promotes T cell– and Mø-mediated renal diseases. To explore this possibility, we selected a rapidly induced T cell– and Mø-dependent nephritis, NSN. Using this model, we evaluated the expression of CXCR3 and its ligands. Intrarenal CXCR3, CXCL10, CXCL9, and CXCL11 transcripts increased during NSN (Figure 2A). Thus, as in the MRL-Faslpr nephritic kidneys, intrarenal CXCR3 and its ligands are upregulated during NSN.

To identify the leukocyte populations expressing CXCR3 and its ligands, we probed CD68+ and T cells (CD4+, CD8+) for CXCR3 expression. Of note, a similar percentage of intra-
renal CD68+ and CD11b+ cells expressed CXCR3; therefore, we refer to these CD68+ cells as Mø (data not shown). The frequency of intrarenal CD4+, CD8+ T cells, and Mø expressing CXCR3 increased during NSN compared with untreated mice (Figure 2B). We verified CXCR3 antibody (Ab) specificity using CXCR3+/− mice with NSN. Although it is widely appreciated that CXCR3 is expressed on activated T cells, reports of CXCR3+/− Mø are limited.15,18 To evaluate further CXCR3+/− Mø, we determined that there is an increased frequency of CXCR3+/− bone marrow macrophages (BMMø), (F4/80+/−; Figure 2C) and CD11b+ (data not shown) after stimulation with LPS (Figure 2C) and IFN-γ (data not shown) in WT mice. The specificity of CXCR3 Ab was verified by using CXCR3+/− BMMø and an isotype control. Because activated Mø induce apoptosis of renal parenchymal cells,11 we evaluated the frequency of activated Mø during NSN. Intrarenal CXCR3+/− activated Mø increased during NSN as compared with untreated mice (Figure 2D). Thus, intrarenal CXCR3+/− activated Mø and T cells are upregulated during NSN.

We next determined the intrarenal locale of the ligands (CXCL9, CXCL10) binding to CXCR3 during NSN. Because there is a functional loss of CXCL11 protein as a result of a missense mutation at 36 bp (G/A) and a single-nucleotide deletion mutation at 39 bp (C/−) in the gene for CXCL11 in B6 mice (Genbank sequence accession no. NT_109320; A.D.L. et al., unpublished observations), we evaluated CXCL9 and CXCL10. CXCL9 is expressed on the majority of TEC and absent on CXCL9+/− kidneys during NSN and in untreated mice (Figure 2E). In addition, a small population of leukocytes express CXCL9 (Figure 2E). These intrarenal CXCL9+ leukocytes that increased during NSN are CD4+ and CD68+ cells (Figure 2E). Similarly, CXCL10 is expressed primarily on TEC and to a lesser extent on leukocytes and is absent in CXCL10+/− control kidneys during NSN and in untreated mice (Figure 2E). The increase in intrarenal CXCL10+ leukocytes during NSN is in CD4+, CD8+, and CD68+ cells (Figure 2E). Although the frequency of these intrarenal CXCL10+ leukocytes is

Figure 2. T cells and activated Mø increase during NSN, whereas TEC are a primary source of CXCL9 and CXCL10 during NSN (day 14). (A) Expression of CXCR3 and its ligands CXCL10, CXCL9, and CXCL11 during NSN were evaluated by real-time PCR. (B) The frequency of intrarenal T cells (CD4+ and CD8+) and Mø expressing CXCR3. Note representative FACS plots of CXCR3 expression on T cells and Mø in WT and control CXCR3+/− mice during NSN. (C) The frequency of cultured BMMø expressing CXCR3 rises in response to stimulation with LPS. To verify staining specificity, we analyzed CXCR3−/− BMMø stimulated with LPS and B6 BMMø stimulated with LPS and stained with an isotype control. These data are representative of three samples and two additional experiments that used isotype controls alone. (D) Intrarenal activated Mø increased during NSN. We determined the total number of intrarenal activated (CD86+) CXCR3+ Mø by multiplying the frequency by the total number of Mø. (E) CXCL9 and CXCL10 are mainly expressed by TEC during NSN (n = 4/group). Intrarenal CXCL9+ CD4+ and CD68+ cells and CXCL10+ CD4+, CD8+, and CD68+ cells increase during NSN. We analyzed 0.5 to 1 × 10⁶ cells (B, D, and E) and 2.5 × 10⁵ cells (C) by FACS analysis. Data are means ± SEM.
somewhat higher than in CXCL9−/− cells, it was substantially (>2×) lower than in CXCR3+ cells (Figure 2, B and E). Thus, intrarenal CXCR3 is primarily expressed on activated T cells and Mø, whereas the ligands CXCL9 and CXCL10 are largely generated by TEC during NSN. Of note, CXCL9 and CXCL10 expression and CXCR3+ leukocytes are rarely detected in glomeruli

Figure 3. CXCR3−/− and CXCL9−/− but not CXCL10−/− mice are protected from loss of renal function and renal pathology during NSN (day 14). (A) The rise in proteinuria, blood urea nitrogen (BUN; day 14), and serum creatinine (day 14) was suppressed in CXCR3−/− and CXCL9−/− but not CXCL10−/− mice as compared with WT mice during NSN. Data are means ± SEM; n = 4 to 9/group. (B) Tubular and glomerular pathology is reduced in CXCR3−/− and CXCL9−/− mice as compared with WT mice. In contrast, tubular and glomerular pathology is similar in CXCL10−/− mice and WT mice during NSN. Representative photomicrographs are shown. Data are means ± SEM; n = 7 to 9/group. Magnification, ×40.
during NSN (data not shown). This pattern of enhanced CXCR3 and its cognate ligand expression during NSN is consistent with our findings in MRL-Faslpr kidneys.

**CXCR3 and CXCL9 but not CXCL10 Promote NSN**

To determine whether CXCR3 regulates intrarenal T cell– and Mø-mediated renal disease via ligands other than CXCL10, we compared CXCR3−/−, CXCL9−/−, and CXCL10−/− mice with WT mice during NSN. Because CXCR3−/−, CXCL9−/−, and CXCL10−/− mouse strains do not express a functional CXCL11, for simplicity, we refer to each strain as the single unique “knockout.” CXCR3−/− mice were protected from the loss of renal function (Figure 3A). By comparison, renal function in CXCL10−/− mice was similar to WT mice during NSN (Figure 3A). Consistent

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**Figure 4.** Intrarenal activated T cells and activated Mø are suppressed in CXCR3−/− and CXCL9−/− mice as compared with WT mice during NSN (day 14). (A) The number of CD4+ and CD8+ T cells and Mø detected by immunostaining is diminished within glomeruli (intra-, periglomerular) and in the interstitium in CXCR3−/− and CXCL9−/− mice as compared with WT mice during NSN. (B) We detected a suppression of activated (CD69+) CD4+ and CD8+ T cells and activated (CD86+) Mø in CXCR3−/− (top) and CXCL9−/− (bottom) mice as compared with WT mice during NSN. We analyzed 0.5 to 1.0 × 10⁶ cells by FACS (n = 6 to 9/group). (C) We evaluated kidneys from CXCR3−/− and WT mice for the presence of activated Mø (CD68+, TNF-α+) during NSN using immunofluorescence. Representative photomicrographs illustrate CD68+ cells expressing TNF-α+ (circled) in the CXCR3−/− compared with WT kidneys. Data are means ± SEM; n = 4/group.
with preserving renal function, renal pathology in CXCR3−/− but not in CXCL10−/− mice was reduced (Figure 3B). Tubular and glomerular pathology were blunted in CXCR3−/− but not in CXCL10−/− mice. Thus, CXCR3 but not CXCL10 promotes NSN, suggesting that another CXCR3 ligand is instrumental in immune-mediated nephritis.

To determine whether CXCL9, another ligand for CXCR3, promotes immune-mediated nephritis, we compared CXCL9−/− and WT mice during NSN. Renal function (proteinuria, blood urea nitrogen, and serum creatinine) was improved in CXCL9−/− mice (Figure 3A). Similarly, renal pathology (glomerular and tubular) was suppressed in CXCL9−/− mice (Figure 3B, Supplemental Figure 3). Notably, renal function (Figure 3A) and pathology (data not shown) in CXCR3−/− and CXCL9−/− mice did not return to baseline (untreated mice). This suggests that CXCR3 and one, CXCL9,
but not another, CXCL10, of its ligands promote immune-mediated nephritis.

**Intrarenal Activated T Cells and Mø Are Decreased in CXCR3<sup>−/−</sup> and CXCL9<sup>−/−</sup> Mice during NSN**

Because CXCR3 is expressed on intrarenal T cells (CD4<sup>+</sup>, CD8<sup>+</sup>) and Mø during NSN, we hypothesized these intrarenal leukocytes decrease in CXCR3<sup>−/−</sup> as compared with WT mice. Fewer CD4<sup>+</sup>, CD8<sup>+</sup>, and Mø were detected in the glomeruli (intra-, periglomerular) and interstitium in CXCR3<sup>−/−</sup> mice (Figure 4A). A similar decrease in CD4<sup>+</sup> T cells and Mø occurred in the CXCL9<sup>−/−</sup> kidneys, whereas we did not detect a reduction in these leukocytic populations in CXCL10<sup>−/−</sup> mice (data not shown).

CXCR3 is expressed on activated T cells<sup>19</sup> and Mø; therefore, we compared these intrarenal leukocytes in CXCR3<sup>−/−</sup> mice with WT mice during NSN. We detected a decreased frequency of activated (CD69<sup>+</sup>) CD4<sup>+</sup> and CD8<sup>+</sup> T cells and activated (CD86<sup>+</sup>) Mø in CXCR3<sup>−/−</sup> mice during NSN (Figure 4B). In support of this decrease in activated Mø, the frequency of TNF-α<sup>+</sup> Mø was diminished (Figure 4C, circled, immunofluorescence). Similarly, CXCL9<sup>−/−</sup> mice had a reduced frequency of activated T cells and Mø (Figure 4B). Thus, CXCR3 engaging with CXCL9 fosters an intrarenal accumulation of activated CD4<sup>+</sup>, CD8<sup>+</sup> T cells and Mø during NSN.

**Serum Antigen-Specific IgG Isotypes and Glomerular IgG Deposits Are Suppressed in CXCR3<sup>−/−</sup> and CXCL9<sup>−/−</sup> Mice during NSN**

Elevated Ig and Ig class switching are hallmarks of autoimmune disease<sup>20,21</sup>; therefore, we determined whether the attenuated severity of kidney disease in CXCR3<sup>−/−</sup> and CXCL9<sup>−/−</sup> mice was related to a reduction in circulating levels of antigen-specific IgG isotypes during NSN. For this purpose, we measured the anti-sheep and –rabbit IgG isotypes in the sera of CXCR3<sup>−/−</sup> and CXCL9<sup>−/−</sup> mice, respectively, in comparison with WT mice during NSN. We detected a suppression of IgG and IgG1 and not IgG2b at multiple titers (2.0 to 8.0 × 10<sup>5</sup>) in CXCR3<sup>−/−</sup> and CXCL9<sup>−/−</sup> mice (Figure 5A). Because serum isotypes were attenuated in CXCR3<sup>−/−</sup> and CXCL9<sup>−/−</sup> mice, we hypothesized that the reduced renal pathology resulted from decreased deposition of IgG within glomeruli of CXCR3<sup>−/−</sup> and CXCL9<sup>−/−</sup> mice. We detected a reduction in IgG deposits at multiple dilutions (3.0 to 7.5 × 10<sup>5</sup>) in CXCR3<sup>−/−</sup> and CXCL9<sup>−/−</sup> glomeruli (Figure 5B).

Contrastingly, circulating anti-sheep IgG isotypes and glomerular IgG deposits in CXCL10<sup>−/−</sup> did not differ from WT mice. Taken together, this suggests CXCR3 engaging with CXCL9 may promote renal disease by increasing systemic IgG and IgG deposits within glomeruli.

**DISCUSSION**

Chemokines are tempting therapeutic targets for a wide range of diseases, because they are specifically upregulated during inflammation. Mø and T cells are instrumental in mediating renal inflammation; thus, chemokines that regulate Mø and T cells within the kidney are potential therapeutic targets for nephritis. Therefore, we extensively profiled MRL-<i>Fas<sup>pr</sup></i> kidneys before and during Mø- and T cell–dependent lupus nephritis. Although previous reports claimed that a few chemokine ligands (four of nine) and chemokine receptors (three of six) are upregulated in MRL-<i>Fas<sup>pr</sup></i> kidneys, we now report that the majority of chemokine ligands (18 of 23) and chemokine receptors (10 of 16) that we profiled were upregulated in MRL-<i>Fas<sup>pr</sup></i> mice with lupus nephritis.<sup>6,22</sup> This difference in our findings may be related to the larger panel of chemokine ligand/receptors in our experiments and the detection system (ribonuclease protection assay<sup>23</sup> versus real-time PCR, our study). We previously established that CCL2 is required to promote Mø- and T cell–dependent lupus nephritis in MRL-<i>Fas<sup>pr</sup></i> mice.<sup>10</sup> We now report that the rise in the panel of chemokine ligand/receptors that increase in MRL-<i>Fas<sup>pr</sup></i> mice is suppressed in CCL2<sup>−/−</sup> MRL-<i>Fas<sup>pr</sup></i> mice. This may suggest that CCL2 is a “proximal master switch” in the chemokine cascade; however, increased expression of a specific chemokine during disease does not necessarily indicate that it promotes inflammation; it may not have an impact or even thwart inflammation. Therefore, we investigated the impact of CXCR3 and its ligands, CXCL9 and CXCL10, during Mø and T cell immune–mediated nephritis (NSN). Of note, we could not analyze the impact of CXCL11 during NSN because the protein for this remaining CXCR3 ligand is not expressed in B6 mice. We now report that CXCR3 and CXCL9 but not CXCL10 are central to Mø- and T cell–dependent kidney disease.

The impact of CXCL10 in autoimmune and kidney disease is controversial. Whereas Ab blockade of CXCL10 in experimental insulin-dependent diabetes<sup>23</sup> and adjuvant arthritis<sup>24</sup> abrogates disease, deleting CXCL10 does not diminish experimental autoimmune encephalomyelitis, although it lowers disease threshold.<sup>25</sup> Thus, the consequences of CXCL10 engagement with CXCR3 in autoimmune diseases are complex and yet not fully understood. Furthermore, the impact of CXCL10 on glomerular and tubulointerstitial renal disease is unclear. In induced forms of renal injury, puromycin aminonucleoside– and anti-nephrin Ab–induced nephropathy,<sup>26</sup> Ab blockade of CXCL10 exacerbated proteinuria and podocyte injury. Conversely, Ab blockade of CXCL10 in a model of renal endothelial injury reduced interstitial T cell infiltration and improved renal function; however, pathology was not evaluated.<sup>27</sup> We now report that despite the abundant protein expression of intrarenal CXCL10 on TEC and leukocyte infiltrates, CXCL10 is not instrumental in two distinct models of immune-mediated renal disease, MRL-<i>Fas<sup>pr</sup></i> mice and NSN. This indicates that CXCL10 alone is not central to immune-mediated kidney disease.

Because it is possible that other ligands alone or together with CXCL10 promote immune-mediated kidney disease, we compared CXCR3<sup>−/−</sup> and CXCL9<sup>−/−</sup> mice during NSN with WT mice. CXCR3 and CXCL9 but not CXCL10 are central to immune-mediated kidney disease. This is the first report indi-
cating that CXCL9−/− mice are protected from nephritis. Our findings are in agreement with recent studies reporting that CXCR3−/− mice develop less severe nephritis than WT mice during NSN.28 In fact, CXCR3 has been shown to incite non-immune-induced renal inflammation because CXCR3 orchestrates the recruitment of T cells instrumental in mediating renal ischemic injury.16 Thus, CXCR3 may be a potential therapeutic target for a broad array of kidney diseases. Our findings are intriguing: although CXCL9 and CXCL10 protein expression is similar in locus and intensity during NSN, the functional impact of these two CXCR3 ligands differ. This differential impact on nephritis may be related to distinct bioavailability of CXCL9 and CXCL10. The preferential contribution of one CXCR3 ligand to disease pathology has been seen in other inflammatory models of disease, such as CXCL10 in mouse hepatitis virus and Dengue virus encephalitis.29,30 and CXCL9 in herpes simplex virus-1 ocular infection and cytomegalovirus infection.31,32 Regardless of the exact mechanisms, our findings indicate that selective blockade of CXCL9 and CXCR3 is a potential therapeutic strategy to combat immune-mediated renal diseases.

Although it is well appreciated that activated T cells express CXCR3, we now report that activated and resting Mø express CXCR3 during kidney disease. The frequency of intrarenal CD68+ cells along with CD4+ and CD8+ T cells expressing CXCR3 increased during NSN. This rise in CXCR3+ Mø in the kidney is consistent with studies reporting that a low percentage of CXCR3+ monocytes in the normal circulation increases in patients with rheumatoid arthritis.33 Furthermore, we determined that whereas some primary cultured BMMø express CXCR3, the frequency increases after IFN-γ stimulation and is even more robust after LPS stimulation. This suggests that whereas low numbers of resting Mø express CXCR3, the increase in CXCR3+ Mø is a reflection of a rise in activated Mø during renal inflammation. Consistent with this concept, the total number of intrarenal activated CXCR3+ Mø increases during NSN as compared with untreated mice. Furthermore, CXCR3 is responsible for recruiting these activated Mø, because there is a decrease in intrarenal activated Mø, along with activated CD4+ and CD8+ T cells, in CXCR3−/− during NSN. This suggests that activated Mø bearing CXCR3 are central to inducing renal injury, because activated Mø release mediators that initiate TEC apoptosis.11 Thus, we provide the first evidence that CXCR3 is instrumental in recruiting activated Mø, as well as T cells, into the kidney, that are responsible for inciting renal injury.

Humoral and cell-driven immune mechanisms mediate kidney disease.20 We investigated whether the suppression in renal disease in the CXCR3−/− and CXCL9−/− mice during NSN was related to a decrease in pathogenic Ab within the kidney. We detected a reduction in total serum antigen-specific IgG and IgG1 Ab in CXCR3−/− and CXCL9−/− mice during NSN. Our finding is reminiscent of reduced Ab against a bacterial pathogen (Francisella tularensis) in CXCL9−/− mice; however, our data are not in agreement with the recent study of CXCR3−/− mice, indicating that there is no difference in total mouse anti-sheep IgG or anti-sheep IgG1 during NSN (day 8).28 This difference may be related to the assay methods, the day of analysis, or other variables related to inducing NSN. Nevertheless, our data support the concept that CXCR3 and CXCL9 enhance Ab production during immune responses. The regulation of serum IgG and glomerular deposits by CXCR3 and CXCL9 remains unclear. We speculate that distinct patterns of CXCR3+ CD4+ T cells and CXCL9 expression in peripheral centers during inflammation and/or the expression of CXCR3 on a subset of memory B cells and plasma cells may regulate serum IgG and, in turn, glomerular deposits.35,36 Notably, the decline in antigen-specific IgG in CXCR3−/− and CXCL9−/− serum correlates with diminished IgG deposits within glomeruli; therefore, suppressed renal pathology and improved renal function may, in part, result from a reduced intrarenal deposition of pathogenic Ab in CXCR3−/− and CXCL9−/− mice during NSN. In addition, we did not detect CXCL9 and CXCL10 expression and CXCR3+ leukocytes in glomeruli of WT mice during NSN. Thus, the reduction in proteinuria in CXCR3−/− and CXCL9−/− mice during the later stages of NSN may be related to the suppression of humoral immune responses. This is consistent with findings indicating that although the generation of autologous murine IgG against the nephrotoxic Ab is not essential for disease initiation, it may contribute to sustaining renal dysfunction.37 This may indicate that although CXCR3 and CXCL9 promote the initiation of NSN via cell-mediated events, their regulation of the production and deposition of pathogenic Ab may be responsible for sustaining immune-mediated renal disease.

In conclusion, CXCR3 and one, CXCL9, but not another, CXCL10, of its ligands mediate activated T cells and Mø recruitment into the kidney and enhance Ab deposition in glomeruli that may, in turn, promote immune-mediated kidney disease. This suggests that blocking CXCR3 and CXCL9 is a potential therapeutic target for human immune-mediated kidney diseases.

CONCISE METHODS

Mice

We purchased MRL/MpJ-Faslpr/Faslpr (MRL-Faslpr) and C57BL/6 (B6) from the Jackson Laboratory (Bar Harbor, ME). Mice were housed and bred in our pathogen-free animal facility. CXCL9 null (−/−), CXCL10−/−, CXCR3−/− (B6 background), and CCL2−/− (MRL-Faslpr background) mice were generated as described previously.5,10,34,38 We generated CXCL10−/− MRL-Faslpr mice using a backcross-intercross scheme. MRL-Faslpr mice were mated with CXCL10−/− (B6 background) mice to yield heterozygous F1 offspring. We intercrossed F1 mice and screened the progeny for disease. Our finding is that there is no difference in total mouse anti-sheep IgG or anti-sheep IgG1 during NSN (day 8).28 This difference may be related to the assay methods, the day of analysis, or other variables related to inducing NSN. Nevertheless, our data support the concept that CXCR3 and CXCL9 enhance Ab production during immune responses. The regulation of serum IgG and glomerular deposits by CXCR3 and CXCL9 remains unclear. We speculate that distinct patterns of CXCR3+ CD4+ T cells and CXCL9 expression in peripheral centers during inflammation and/or the expression of CXCR3 on a subset of memory B cells and plasma cells may regulate serum IgG and, in turn, glomerular deposits.35,36 Notably, the decline in antigen-specific IgG in CXCR3−/− and CXCL9−/− serum correlates with diminished IgG deposits within glomeruli; therefore, suppressed renal pathology and improved renal function may, in part, result from a reduced intrarenal deposition of pathogenic Ab in CXCR3−/− and CXCL9−/− mice during NSN. In addition, we did not detect CXCL9 and CXCL10 expression and CXCR3+ leukocytes in glomeruli of WT mice during NSN. Thus, the reduction in proteinuria in CXCR3−/− and CXCL9−/− mice during the later stages of NSN may be related to the suppression of humoral immune responses. This is consistent with findings indicating that although the generation of autologous murine IgG against the nephrotoxic Ab is not essential for disease initiation, it may contribute to sustaining renal dysfunction.37 This may indicate that although CXCR3 and CXCL9 promote the initiation of NSN via cell-mediated events, their regulation of the production and deposition of pathogenic Ab may be responsible for sustaining immune-mediated renal disease.

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Mice

We purchased MRL/MpJ-Faslpr/Faslpr (MRL-Faslpr) and C57BL/6 (B6) from the Jackson Laboratory (Bar Harbor, ME). Mice were housed and bred in our pathogen-free animal facility. CXCL9 null (−/−), CXCL10−/−, CXCR3−/− (B6 background), and CCL2−/− (MRL-Faslpr background) mice were generated as described previously.5,10,34,38 We generated CXCL10−/− MRL-Faslpr mice using a backcross-intercross scheme. MRL-Faslpr mice were mated with CXCL10−/− (B6 background) mice to yield heterozygous F1 offspring. We intercrossed F1 mice and screened the progeny for disrupted and intact CXCL10 and the Faslpr mutation in tail genomic DNA. The DNA was assessed by PCR using oligonucleotide primers that recognized the normal CXCL10 gene: Sense (5′-TCC CTC CCG TAA CCA CAC AGT AAA T-3′) and antisense (5′-GCG GAT AGA TTT CAC TTT GG-3′) and antisense (5′-TTT CAC TTT GG-
3'). Gel analysis of the PCR products identified the CXCL10 and Neo gene fragments at 342 and 640 bp, respectively. The Fas<sup>br</sup> mutation was identified as previously reported. After five generations of backcross matings (N5), we analyzed and compared CXCL10<sup>−/−</sup> MRL-Fas<sup>br</sup> mice with age- and gender-matched WT MRL-Fas<sup>br</sup> littermates. The number of mice analyzed is specified in each figure. We compared these strains at the N5 generation, because we previously established that there are sufficient MRL-Fas<sup>br</sup> background genes to result in consistent phenotypic changes characteristic of MRL-Fas<sup>br</sup> mice. Use of mice in our study was reviewed and approved by the Standing Committee on Animals in the Harvard Medical School in adherence to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

**Inducing NSN**

We prepared nephrotoxic serum by immunizing sheep with a particulate fraction of mouse glomerular basement membrane from B6 mice kidneys, as described previously. To induce NSN, we primed CXCL10<sup>−/−</sup>, CXCR3<sup>−/−</sup>, and WT male mice (6 wk of age) by injecting 0.5 mg of sheep IgG in Freund’s complete adjuvant (Sigma Chemical Co., St. Louis, MO) subcutaneously in each flank. We challenged these mice 7 d later with an intravenous injection of nephrotoxic serum (15 μl/g body wt). We used nephrotoxic serum from rabbits to immunize CXCL9<sup>−/−</sup> and WT mice as described previously. Mice were killed 14 d after challenge and analyzed. The number of mice in each group is specified within each figure.

**Interrenal Chemokine and Chemokine Receptor Transcripts**

We analyzed the expression of chemokines (CXCL10, CXCL9, CXCL11, CXCL12, CCL13, CCL5, CCL28, CCL22, CCL24, CCL11, CCL17, CX3CL1, CCL25, CCL2, CCL8, CCL7, CCL3, CXL2, CCL28, CCL4, CCL28β, CCL27, CCL21, CXCL16, and CXCL1) and chemokine receptors (CXCR2, CXCR3, CXCR4, CXCR5, CXCR6, CCR1, CCR2, CCR3, CCR4, CCR5, CCR6, CCR7, CCR8, CCR9, CCR10, and CXCR1) in MRL-Fas<sup>br</sup> mice by using real-time, two-step, quantitative PCR, as described previously. Primers were designed as previously reported. To detect CXCL10 expression during NSN, we used primers designed by Applied Biosystems ( Foster City, CA). To detect CXCL9, CXCL11, and CXCR3 expression during NSN, we used the following primers: glyceraldehyde-3-phosphate dehydrogenase sense 5'-CAT GGC CTC CAA GGA GTA AG-3' and antisense 5'-CTT AGG CCC CTC CGT TTA TT-3'; CXCL9 sense 5'-TCC TTT TGG GCA TCA TCT TC-3' and antisense 5'-TTC CCC CTC TTT TGC TTT TT-3'; CXCL11 sense 5'-AGT AAC GGC TTC GAC AAA GT-3' and antisense 5'-GCA GTC CTG TCC AAG ACA GCA GA-3'; and CXCR3 sense 5'-TGA GAC AAC TGA GGC CTC CTA-3' and antisense 5'-TCT TGC TCC CCA GAT GAT G-3' (Invitrogen, Carlsbad CA). We evaluated expression as previously reported.

**Isolation of TEC and BMMø**

We isolated and cultured TEC from B6 and MRL-Fas<sup>br</sup> mice as previously reported, and we isolated and cultured BMMø from B6 mice as described previously.

**Flow Cytometry**

We prepared and stained single-cell suspensions from kidneys, spleens, or primary cultured TEC/BMMø as described previously. We collected 0.5 to 1.0 × 10<sup>6</sup> total kidney or spleen cells and 0.5 to 1.0 × 10<sup>6</sup> of cultured cells using a FACS Calibur (Becton Dickinson, San Jose, CA) and analyzed data using Flowjo software (Tree Star, Palo Alto, CA).

**Antibodies**

We used the following Ab from eBioscience (San Diego, CA) for FACS analysis: FITC-conjugated anti-CXCL4 (L3T4), anti-CXCL8 (53-6.7), anti-CXCL27, anti-CXCL21, anti-CXCL16, and anti-CXCL1 Ab (R&D Systems, Minneapolis, MN), and rabbit anti-mouse CXCL10 Ab (PeproTech, Rocky Hill, NJ). The following isotype-specific Ab were used for controls: Rat-IgG2a (BR2a), rat-IgG2b (KLH/G2b-1-2), and rabbit-IgG (eBioscience). As secondary PE- or allophycocyanin-conjugated Ab, we used goat anti-rabbit (Jackson ImmunoResearch Laboratories; West Grove, PA) and biotin-conjugated rabbit anti-goat Ab (Vector Laboratories, Burlingame, CA). To detect biotin-conjugated secondary Ab, we used streptavidin PE or allophycocyanin (Jackson ImmunoResearch Laboratories). We identified renal proximal tubules by binding of fluorescein-conjugated lotus lectin (Vector Laboratories).

**Histopathology**

We fixed kidneys in 10% formalin and prepared and stained paraffin sections with the periodic acid Schiff reagent. Slides were coded before grading the renal pathology.

**MRL-Fas<sup>br</sup> Mice.** We evaluated renal (glomerular, tubular, interstitial, and perivascular) pathology on a scale of 0 (normal) to 3 (severe) as described previously.

**NSN.** We evaluated CXCL9<sup>−/−</sup>, CXCL10<sup>−/−</sup>, CXCR3<sup>−/−</sup>, and B6 kidneys for glomerular and tubular damage as described previously. We stained cryostat-cut kidney sections for the presence of Mø with anti-CXCL6 Ab (FA-11; Serotec) and for T cells with anti-CXCL4 Ab (RM4-5), anti-CXCL8 Ab (53-6.7), and anti-CXCL27, anti-CXCL21, and anti-CXCL16 Ab (R&D Systems, Minneapolis, MN). We used the following Ab from eBioscience (San Diego, CA) for FACS analysis: FITC-conjugated anti-CXCL4 (L3T4), anti-CXCL8 (53-6.7), anti-CXCL27, anti-CXCL21, and anti-CXCL16 Ab (R&D Systems, Minneapolis, MN), and rabbit anti-mouse CXCL10 Ab (PeproTech, Rocky Hill, NJ). The following isotype-specific Ab were used for controls: Rat-IgG2a (BR2a), rat-IgG2b (KLH/G2b-1-2), and rabbit-IgG (eBioscience). As secondary PE- or allophycocyanin-conjugated Ab, we used goat anti-rabbit (Jackson ImmunoResearch Laboratories; West Grove, PA) and biotin-conjugated rabbit anti-goat Ab (Vector Laboratories, Burlingame, CA). To detect biotin-conjugated secondary Ab, we used streptavidin PE or allophycocyanin (Jackson ImmunoResearch Laboratories). We identified renal proximal tubules by binding of fluorescein-conjugated lotus lectin (Vector Laboratories).

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ing isotype-specific Ab were used for controls: Rat-IgG2a (R35–95), rat IgG2b (R35–38), rabbit IgG (eBioscience), and goat IgG (Southern Biotechnology, Birmingham, AL). The secondary Ab for immunostaining was biotin-conjugated rabbit anti-rat IgG (Vector Laboratories). The immunostaining was analyzed by counting for the presence of CD68⁺, CD4⁺, CD8⁺, and B220⁺ cells within and surrounding glomeruli and in the interstitium in 10 randomly selected high-power fields. Of note, we have determined that the B220⁺ cells in the kidney of MRL-Fas⁺/⁻ mice are the unique double-negative (CD4⁻CD8⁻ double-negative) T cells and are not B cells.52

To determine the number of activated Mø in the kidney during NSN, we fixed frozen kidney sections in paraformaldehyde, stained them with rat anti-mouse TNF-α-PE (MP6-XT22; eBioscience) and anti-CD68-FITC (FA-11; Serotec), and analyzed these sections using a colorimetric analysis kit (Infinity; Thermo Electron, Melbourne, Australia) and serum creatinine using the creatinine reagent kit and a colorimetric analysis kit (Infinity; Thermo Electron, Melbourne, Australia) and serum creatinine using the creatinine reagent kit and a colorimetric analysis kit (Infinity; Thermo Electron, Melbourne, Australia).40

The data are means ± SEM and were analyzed by GraphPad Prism 4.0 (GraphPad, San Diego, CA). We used the nonparametric Mann-Whitney U test to determine differences among groups. Survival curves were compared and analyzed using the log-rank test.

Renal Function
We assessed urine proteins semiquantitatively by dipstick analysis as described previously.43 We measured blood urea nitrogen levels using a colorimetric analysis kit (Infinity; Thermo Electron, Melbourne, Australia) and serum creatinine using the creatinine reagent kit and a colorimetric analysis kit (Infinity; Thermo Electron, Melbourne, Australia).40

Survival
We compared survival of the CXCL10⁻/⁻ MRL-Fas⁺/⁻ and WT MRL-Fas⁺/⁻ mice using similar numbers of males and females from birth to 10 mo of age.

Serum Anti-Sheep and Anti-Rabbit IgG Isotypes
We measured the levels of mouse anti-sheep IgG Ab (Sigma Chemical Co.) and anti-rabbit IgG (Jackson ImmunoResearch Laboratories) by ELISA using sera collected at day 14 during NSN as described previously.53 We detected bound mouse anti-sheep/anti-rabbit IgG, IgG1, and IgG2b using peroxidase-conjugated rabbit anti-mouse IgG Ab (1:5000; Southern Biotechnology).

IgG Deposits within the Kidney
We evaluated IgG deposits within renal glomeruli as described previously.54 We evaluated stained sections by scoring 20 glomeruli as either positive or negative and graded the amount (severity) of deposits in 20 positive glomeruli per specimen on a scale of 0 to 3 using multiple dilutions (1:3000, 1:4500, 1:6000, and 1:7500) of the detection Ab (fluorescein-conjugated goat anti-mouse IgG; MP Biomedicals, Aurora, OH).40

Statistical Analyses
The data are means ± SEM and were analyzed by GraphPad Prism 4.0 (GraphPad, San Diego, CA). We used the nonparametric Mann-Whitney U test to determine differences among groups. Survival curves were compared and analyzed using the log-rank test.

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DISCLOSURES
None.

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