CCR1 Inhibition Ameliorates the Progression of Lupus Nephritis in NZB/W Mice

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Systemic lupus erythematosus (SLE) is a chronic, inflammatory autoimmune disease characterized by the production of autoantibodies against nuclear Ags; immune complex deposition in multiple organs, including the kidney; complement activation; leukocyte infiltration; and tissue damage (1–3). Leukocyte infiltration features in both human and experimental lupus nephritis and is strikingly associated with a progressive loss of renal function (4, 5). This infiltration includes T cells, B cells, neutrophils, and mononuclear phagocytes, and involves the glomerular as well as the tubulointerstitial compartments of the kidney (6, 7). Infiltrating leukocytes constitute a source of inflammatory and profibrotic mediators (e.g., cytokines, chemokines, extracellular matrix proteins), which contribute to the proliferation and differentiation of resident kidney cells and to matrix deposition leading to renal injury (7–10). Thus, preventing or reducing leukocyte recruitment into inflamed kidneys should inhibit the progression of lupus nephritis.

Chemokines and their seven-transmembrane G-protein–coupled receptors are critical mediators in the inflammatory process and thus represent attractive therapeutic targets (11, 12). Many studies revealed that locally produced inflammatory chemokines (e.g., Ccl2 to Ccl5, Cxcl10 to Cxcl13) govern the complex multistep process leading to renal leukocyte infiltration and injury in different mouse models of nephropathy (13–19). In New Zealand Black/New Zealand White (NZB/W) mice, which spontaneously develop a severe autoimmune disease and more closely mimic the human SLE condition compared with other rodent models (20), we previously reported that splenic T, B, and myeloid cells from nephritic mice migrated into noninflamed syngeneic kidneys (19). This process was enhanced if the recipient kidneys were chronically inflamed, indicating that circulating leukocytes and the kidney both contribute to the inflammation in lupus nephritis through a self-maintenance mechanism. Elevated renal expression of two Ccr1 chemokine receptor ligands: Ccl3 and Ccl5, in association with mononuclear phagocytes and T cell infiltration have been reported in NZB/W mice, as well as in other models of SLE and in human lupus nephritis (16, 19, 21). Thus, Ccr1 may mediate the coordinated recruitment of inflammatory leukocytes to infiltrated B cells in the kidneys. Altogether, these findings define a pivotal role for Ccr1 in the recruitment of T and mononuclear phagocytes cells to inflamed kidneys of NZB/W mice, which in turn contribute to the progression of renal injury. The Journal of Immunology, 2014, 192: 886–896.
sites of renal injury, resulting in tubular and/or glomerular lesions and tissue fibrosis. Indeed, accumulation of CCR1-positive macrophages and lymphocytes is found mainly in interstitial lesions of renal biopsies from SLE patients (22), and Ccr1 on myeloid cells and some T cell subsets is thought to guide them to inflamed target organs such as the kidney in various rodent models of nephropathy (13, 14, 16–18, 23–28).

In contrast to the prominent role of Ccr1 in renal leukocyte infiltration in the lupus-like glomerulonephritis MRL-Fas(lpr) model (16, 17), nothing is known about the specific role of Ccr1 in the development and progression of nephritis in NZB/W mice. Our aims were to 1) compare the expression of Ccr1 and its ligands in the kidney and splenic lymphocytes of prenephritic and nephritic NZB/W mice; 2) investigate the functional relevance of Ccr1 in the renal recruitment of leukocytes in nephritic mice; and 3) assess the contribution of Ccr1 and its ligands to kidney damage using the orally available Ccr1 antagonist BL5923.

Materials and Methods

Mice and evaluation of proteinuria

NZB and NZW were purchased from Harlan Laboratories (Venray, The Netherlands). (NZB × NZW) F1 (NZB/W) mice were bred from NZB females and NZW males in our conventional animal facility. Only NZB/W female mice were used in this work. Urine samples were tested for proteinuria using Multistix 10 SG (Bayer Diagnostics, Puteaux, France) on a 0–4+ scale, corresponding to the following approximate protein concentrations: 0, negative or trace; 1+, 30 mg/dl; 2+, 100 mg/dl; 3+, 300 mg/dl; and 4+, 2000 mg/dl. Mice were considered to have severe nephritis if two consecutive urine samples scored 3+. The 25- to 30-wk-old NZB/W mice with severe proteinuria and renal inflammatory infiltrates were considered nephritic, whereas 10- to 17-wk-old mice without proteinuria and kidney inflammation were considered young and prenephritic (5, 19).

Cell labeling, syngeneic adoptive transfer and short-term treatment

Spleenic leukocytes from nephritic NZB/W donor mice were isolated using Lympholyte-M (Burlington, Ontario, Canada) density gradient centrifugation and then incubated for 30 min at 37°C with 7.2 μM CellTracker Orange 5(6)–(4-(chloromethyl) benzoyl) amino) tetramethylrhodamine (Molecular Probes, Molecular Probes, Leiden, The Netherlands). Nonadherent macrophages (CD3−CD19−, CD11b+Ly6G−), including inflammatory (SSClowLy6C+) and patrolling (SSClowLy6C−) macrophages (CD3+CD19−NK1.1−F4-80+CD11b+CD11c+), were harvested from mouse spleens as described (22, 23). Cells were labeled with 0.1 μM 2′,7′-dichlorofluorescein diacetate, processed and resuspended in PBS supplemented with 2% FCS and used for quantitative PCR, immunophenotyping, or functional assays.

Flow cytometric analyses and functional studies

Kidney and spleen leukocytes were preincubated with 5 μg/ml rat anti-mouse CD16/CD32 (Fc Blocking, e Biosciences, San Diego, CA), washed in PBS, and incubated with the following Abs: Alexa Fluor 700 (AF-700)–loaded splenic leukocytes was performed using a Transwell (Corning Costar, Tewksbury, MA) assay. Splenic leukocytes, activated macrophages (CD3−CD19−NK1.1−F4-80+CD11b+CD11c+), were harvested from mouse spleens as described (22, 23). Cells were labeled with 0.1 μM 2′,7′-dichlorofluorescein diacetate, processed and resuspended in PBS supplemented with 2% FCS and used for quantitative PCR, immunophenotyping, or functional assays.

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Quantitative RT-PCR analyses

Total cellular RNA was isolated from renal or splenic cryosections or from freshly sorted splenic myeloid, T, and B cells using the RNeasy Plus Mini Kit (Qiagen, Courtabeuf, France) according to the manufacturer’s instructions. mRNA expression was measured using poly-d(T)-15 and Moloney murine leukemia virus reverse transcriptase (Fisher Bioblock, Illkirch, France). RNA was quantified using NanoDrop technology (Thermo Scientific, Wilmington, DE). For each sample, RNA

Leukocyte preparation

To prepare cell suspensions enriched in renal leukocytes, one kidney from each mouse was digested for 1 h at 37°C in humidified air with 5% CO2, using 0.1 ml Liberase Blendzyme 3 (Roche Diagnostics, Mannheim, Germany), 0.4 mg/ml Collagenase D (Roche Diagnostics), and 400 U/ml DNase I (Sigma-Aldrich, St. Louis, MO) in DMEM supplemented with 1% BSA and 2.4 mM CaCl2. After standardized mechanical dissociation with
quality was assessed from the IS5 and 28S ribosomal RNAs, and RNA integrity was assessed using a BioAnalyzer (Agilent Technologies, Santa Clara, CA). Samples with RNA integrity numbers > 7 were processed for gene expression analyses. cDNA amplification (1 μg) was performed by quantitative real-time PCR on a LightCycler 480 instrument (Roche Diagnostics) with the LightCycler 480 SYBR Green detection kit (Roche Diagnostics) using forward (454–473) 5′-GCCATTGACCACTAGGCTT-3′ and reverse (698–705) 5′-CGGACTCATTCACTGTACC-3′ primers for β-actin (216 bp), forward (952–974) 5′-TTAGCTTCTCATGGC-3′ and reverse (1031–1051) 5′-TCCAGTTTGCAGCTCTTGTT-3′ primers for Ccr1 (57 bp), forward (120–140) 5′-GCCCTGCTGGTTCCTGGCT-3′ and reverse (357–377) 5′-GGCATTGTCGCTACAGGTGAG-3′ primers for Ccl3 (217 bp), and forward (119–138) 5′-CAGCTGCTGCCATATGCG-3′ and reverse (249–268) 5′-GGGCTGTCCAGCTAGTGAGTA-3′ primers for Ccl5 (111 bp). Reactions were performed using the following amplification scheme: 95°C for 10 min, and 40 cycles of 95°C for 20 s, 60°C for 15 s, and 72°C for 20 s. The dissociation curve method was used according to the manufacturer’s protocol (60–95°C) to verify the presence of a single specific PCR product. Relative quantification was performed with the standard curve method (33), and results were expressed as Ccr1/β-actin, Ccl3/β-actin, and Ccl5/β-actin ratios.

**Histological analyses**

Kidney sections were mounted on slides, deparaffinized, and stained with H&E, periodic acid–Schiff stain, and Masson’s trichrome. IHC was performed on paraffin sections using Abs against the pan-B and pan-T cell marker B220 (RA3-6B2; BD Biosciences), the pan–T cell marker CD3 (2356–2561; Epitomics, Burlingame, CA), the monocyte/macrophage marker F4/80 (BM40088; Interchim, San Pedro, CA), the glomerular mesangial marker CD68 (MCA9157; AbD Serotec, Oxford, U.K.), the proliferating cell marker Ki-67 (ACK02, Leica Microsystems, Buffalo Grove, IL), or the chemokine Ccl3 (AP8327b; Abgent, San Diego, CA). The sections were washed and incubated with an appropriate biotinylated secondary Ab for 1 h at room temperature, and then with streptavidin-HRP complex followed by 3,3′-diaminobenzidine or 3-amin-9-ethylcarbazole detection (LSAB kit, Dako France, Trappes, France). Sections were then counterstained with hematoxylin. Negative controls were carried out by applying the same procedure with omission of the primary Ab. Images were obtained on a Leica DMLB microscope (Leica Microsystems) equipped with standard optic objectives, at the indicated magnification, and were digitized directly with a Sony 3CCD color video camera (DVC-990P, Surrey, U.K.).

Immunohistochemical staining was interpreted and scored simultaneously by three independent investigators (F.G., M.P., and D.B.), who were blinded to the protocol. Pathological changes in the kidney sections were graded on a scale of 0 to 5, as follows: 0 = no lesions, 1 = lesions in 3%–15% of glomeruli, 2 = lesions in 15%–25%, 3 = lesions in 25%–50%, 4 = lesions in >50% of glomeruli.

**Results**

NZB/W mice with severe proteinuria had significantly higher absolute numbers of renal leukocytes than did young, prenephritic NZB/W mice (Fig. 1A). Consistent with previous reports (19, 36), these cells included CD3+ T cells, myeloid cells including F4-80+ mononuclear phagocytes, and Gr-1+ neutrophils, and, to a lesser extent, CD19+ B cells. Because the Ccl3/Ccl5–Ccr1 axis has been implicated in renal leukocyte recruitment in rodent models of nephritis (14, 18, 19, 23, 37), we used real-time PCR to quantify and compare the levels of Ccl1, Ccl3, and Ccl5 mRNAs in nephritic kidneys and nondiseased kidneys from young mice. Ccr1, Ccl3, and Ccl5 mRNA levels were significantly higher in nephritic NZB/W mouse kidneys than in those of prenephritic mice (Fig. 1B). Previous studies have shown that kidney cells do not express Ccr1 and that renal Ccr1 synthesis mainly originates from infiltrated leukocytes (17, 18). Therefore, infiltrated leukocytes alone could account for the higher levels of these mRNAs in the nephritic kidneys of NZB/W mice. Consistent with this assertion, surface Ccr1 receptor levels, that is, frequencies of Ccr1-positive cells and geometric mean fluorescence intensity values, were higher in the CD4+, CD8+, and Treg T cell subsets, proinflammatory Ly6C+ monocytes and M1 macrophages, and anti-inflammatory M2 macrophages of nephritic mice than in the leukocytes of prenephritic mice (Fig. 1C, Supplemental Fig. 1A). In contrast, patrolling Ly6C− monocytes from nephritic and prenephritic mice had a similar pattern of membrane Ccr1, whereas renal B cells exhibited very low to undetectable levels of receptors. Next, we immunostained kidney sections from nephritic and nephritic NZB/W mice to identify the source of the Ccr1 ligand Ccl3. Total Ccl3 staining was significantly greater in nephritic than in prenephritic mouse kidneys (Fig. 1D). Ccl3-positive cells were mainly infiltrated leukocytes within glomerular and tubulointerstitial compartments, although some tubular epithelial and glomerular cells stained weakly for Ccl3. Unfortunately, we were unable to use local expression of Ccr1 in the kidney resulting from the lack of a suitable Ccr1 Ab for IHC analyses. Collectively, these findings reveal that leukocyte Ccr1 and Ccl3 production is abnormally high in the kidneys of nephritic NZB/W mice.

Ccr1 is expressed by splenic T and myeloid cells from NZB/W mice

As splenomegaly is a feature of nephritic NZB/W mice and the spleen constitutes a source of effector leukocytes (19), we next assessed the levels of Ccr1, Ccl3, and Ccl5 mRNAs in spleen cytocenteses from prenephritic and nephritic NZB/W mice. Transcripts encoding all three proteins were readily detectable in the spleens of prenephritic mice (Fig. 2A). However, in accor-
dence with a previous study (15), splenic Ccr1, Ccl3, and Ccl5 mRNA levels were significantly (30–40%) higher in nephritic mice than in prenephritic mice. To identify which leukocyte subsets expressed Ccr1, T and B cells, mononuclear phagocytes, and neutrophils were sorted from the spleens of prenephritic and nephritic mice and analyzed for steady-state levels of Ccr1 mRNA. Real-time PCR analysis revealed that Ccr1 mRNA levels were highest in T and myeloid cells and that they were significantly higher in nephritic mice than in prenephritic mice (Fig. 2B). In contrast, Ccr1 transcript levels were very low in splenic B cells from both prenephritic and nephritic mice. Flow cytometric analysis of Ccr1 on the surface of these cells also corroborated the RT-PCR results. The Ccr1 geometric mean fluorescence intensity values and the fractions of Ccr1-positive cells in the splenic T cells, mononuclear phagocytes, and neutrophils of nephritic NZB/W mice were assessed by real-time PCR. Each individual sample was run in triplicate. Results are expressed as Ccr1/β-actin, Ccl3/β-actin, and Ccl5/β-actin ratios. (C) Membrane expression of Ccr1 on T cells, including CD4+, CD8+ and Tregs, B cells, Ly6C+ and Ly6C− monocytes, and M1/M2 macrophages from kidneys of prenephritic and nephritic NZB/W mice was determined by flow cytometry using a PE-conjugated goat anti-mouse Ccr1 Ab. Corresponding leukocyte subsets from the kidneys of Ccr1−/− mice were used to assess nonspecific background staining (shaded area). (D) Kidney sections from prenephritic and nephritic NZB/W mice were immunostained for Ccl3 (red, shown by white arrows) and those of prenephritic mice (Fig. 2C, Supplemental Fig. 1B). This finding was undetectable on the surface of B cells from either prenephritic or nephritic mice. Taken together, these data indicate that Ccr1 gene expression is upregulated in the splenic T and myeloid cells of nephritic mice, which led us to investigate the functional consequences of this anomaly.

Ccr1 is functional on splenic T and mononuclear phagocyte cells from NZB/W mice

We compared the in vitro Ccl3-induced migration of splenic leukocytes obtained from prenephritic and nephritic NZB/W mice, using a Transwell-based chemotaxis assay. Whereas random migration for cells from both types of mice was roughly comparable (Fig. 3A), Ccl3 (10−100 nM) stimulated T cell and mononuclear phagocyte migration across the Transwell membrane, resulting in bell-shaped dose–response patterns for cells from both types of mice. However, the migratory responses of T cells and mononuclear phagocytes from nephritic mice were greater than those of prenephritic mice at all Ccl3 concentrations tested, suggesting that the leukocytes of nephritic mice were more sensitive to Ccl3 than those of prenephritic mice. Migratory responsiveness to Ccl3 was abolished by the specific Ccr1 antagonist BL5923, indicating that this Ccl3-stimulated migration was Ccr1 dependent. These results are consistent with the higher levels of Ccr1 receptors found on the surfaces of T cells and mononuclear phagocytes from nephritic mice. In both prenephritic and nephritic NZB/W, B cells were refractory to Ccl3-promoted chemotaxis (data not shown). To exclude potential interference with Ccl3-enhanced leukocyte chemotaxis by the splenic microenvironment (e.g., production of soluble proinflammatory mediators), T cells and mononuclear phagocytes were sorted from the spleens of prenephritic and nephritic mice.
Phagocytes were sorted from the spleens of prenephritic and nephritic mice and analyzed for their ability to migrate toward a maximally effective concentration of Ccl3 (50 nM) (Fig. 3B). Ccl3-stimulated chemotaxis was enhanced in cells from nephritic mice compared with the cells of prenephritic mice, and this effect was inhibited by BL5923. Although Ccl3 also stimulated the migration of the corresponding leukocyte subsets from $Ccr1^{-/-}$ mice, this likely occurred through Ccl3 receptors other than Ccr1, such as Ccr5. Indeed, cells from $Ccr1^{-/-}$ mice remained completely insensitive to BL5923 treatment, emphasizing the specificity of this antagonist for Ccr1. Overall, these findings indicate that the increased expression of Ccr1 on NZB/W leukocytes is associated with enhanced migration to Ccl3 gradients.

Ccr1 blockade reduces the recruitment of T and macrophages to the inflamed kidney

We then investigated whether fluorescently labeled splenic leukocytes isolated from donor nephritic NZB/W mice would be recruited into the kidneys of nephritic syngeneic mice pretreated with BL5923. Short-term BL5923 treatment reduced by ~70% the numbers of donor T cells and mononuclear phagocytes, including macrophages, but not of B cells, that infiltrated the kidneys of recipient mice (Fig. 3C). These findings, combined with those displayed in Figs. 1 and 2, support the idea that abnormal renal homing of T and macrophages in nephritic mice depends on the presence of functional Ccr1 on these leukocytes.

Long-term BL5923-based treatment prolongs the lifespan of NZB/W mice

To explore whether the heightened activity of the Ccl3/Ccl5–Ccr1 axis in NZB/W mice could constitute a valid therapeutic target in lupus, we initiated long-term BL5923 treatment in NZB/W mice with already advanced nephritis. Mice with grade 3+ proteinuria were randomized to receive BL5923 or vehicle for 6 wk. In vehicle-treated mice, proteinuria continued to increase rapidly during the next 4 wk of follow-up (Fig. 4A). In contrast, proteinuria progressed more slowly to the fatal stage in BL5923-treated mice. The difference between the two groups became significant as early as 1 wk after treatment initiation and remained so until 5 wk of follow-up. In addition, BL5923 treatment markedly prolonged the lifespan of nephritic mice (Fig. 4B). Thus, taken together with its ability to inhibit renal leukocyte homing, the beneficial effects of BL5923 treatment suggest that therapeutic Ccr1 blockade slows disease progression by inhibiting the infiltration of T and myeloid cells into the inflamed kidneys of nephritic mice.
Therapeutic Ccr1 inhibition reduces renal injuries in nephritic NZB/W mice

To investigate the ability of BL5923 to hamper the progression of renal damage, we treated NZB/W mice with established severe 3+ proteinuria with either BL5923 or vehicle for 10 d and then analyzed the kidneys of the treated animals. At this endpoint, the difference in proteinuria severity between both groups was significant, but most mice from both groups were still alive (Fig. 4). Like nephritic mice (Fig. 1A), vehicle-treated mice exhibited renal accumulation of T cells, in particular the CD4+ subset, myeloid cells, and, to a lesser extent, B cells (Fig. 5, Supplemental Fig. 2A). In BL5923-treated mice, the absolute numbers of CD4+ T cells, monocytes, macrophages, and granulocytes were significantly decreased in the kidneys. Analyses of T cell and monocyte/macrophage subsets indicated that effector/memory CD4+ T cells, Ly6C+ monocytes, and both M1 and M2 macrophages were targeted by BL5923 treatment. This result was associated with lower renal levels of Ccr1, Ccl3, and Ccl5 mRNAs (Supplemental Fig. 2B) and decreased levels of serum inflammatory cytokines such as TNF-α and IL-1β (Supplemental Fig. 3). In contrast, B cells, Tregs, and Ly6C+ monocytes were not affected by Ccr1 antagonism (Fig. 5, Supplemental Fig. 2). Histological analyses confirmed these results and revealed beneficial effects of Ccr1 blockade on tubulointerstitial and glomerular injuries (Fig. 6, Table I). Indeed, kidney tissue sections from vehicle-treated mice showed marked glomerulonephritis; tubular dilation; tubular protein cast deposition; Ki-67-positive proliferation of tubular epithelial, interstitial, and glomerular cells; and infiltration of B cells, T cells, and mononuclear phagocytes in the interstitial and glomerular areas. In contrast, sections from BL5923-treated mice displayed significantly less glomerular and tubulointerstitial damage; less karyorrhexis/fibrinoid necrosis; absence of glomerular crescents, proliferating leukocytes and tubular epithelial cells; and fewer glomerular macrophages as well as interstitial T cells and mononuclear phagocytes. Note that the mean scores for the

FIGURE 3. Functional expression of Ccr1 on splenic leukocytes from nephritic NZB/W mice. (A and B) Total spleen cells (A) or sorted splenic T cells and mononuclear phagocytes (B) of NZB/W (prenephritic versus nephritic) and Ccr1−/− mice were tested for their ability to migrate in response to 10−100 nM (A) or 50 nM (B) Ccl3. Inhibition of Ccr1-mediated chemotaxis by BL5923 (10 μM) added to both chambers is shown. Transmigrated cells recovered in the lower chamber were stained with mAbs specific for CD3 and F4-80 Ags and counted by flow cytometry. Results (mean ± SD) are from three independent experiments and expressed as the percentage of input total cells that migrated to the lower chamber. (C) NZB/W mice with advanced nephritis were injected i.v. with CMTMR-labeled leukocytes isolated from spleens of donor NZB/W mice. Recipient mice were pretreated twice per day with either 60 mg/kg BL5923 or vehicle (6 h between doses) on the day before and the day of cell transfer. Kidneys were collected 18 h after injection of cells, and the infiltrated leukocytes were analyzed by flow cytometry. Results (mean ± SD) are expressed as the number of fluorescent T cells, B cells, or mononuclear phagocytes including macrophages (F4-80+CD11b+) recruited to the kidneys of recipient NZB/W mice (treated or not with 60 mg/kg BL5923; n = 11 per group) per 10⁵ transfused leukocytes and are from eight independent experiments. *p < 0.05, **p < 0.005, ***p < 0.0005 compared with leukocytes from prenephritic NZB/W or Ccr1−/− mice or with vehicle-treated NZB/W mice.

FIGURE 4. BL5923 administration delays death in nephritic NZB/W mice. Groups of 10 NZB/W mice with severe proteinuria (3+) were treated by oral gavage three times per week for 42 d with either 60 mg/kg BL5923 or vehicle. (A) Proteinuria (mean grade ± SD) was assessed once per week during the time course of the therapeutic protocol. *p < 0.05 compared with vehicle-treated NZB/W or Ccr1−/− mice. (B) Survival rate. †, End of treatment. The p value is that obtained in the log-rank test.
glomerular activity, the tubulointerstitial activity, and the chronicity of the lesions were roughly comparable between BL5923-treated and untreated mice, that is, before the onset of treatment (Table I, Fig. 6B). Vehicle- and BL5923-treated mice exhibited no substantial differences in tubular or glomerular infiltration of B cells, and BL5923 treatment did not affect systemic humoral immune responses or intraglomerular IgG deposition in nephritic NZB/W mice (Fig. 6, Supplemental Fig. 2C, 2D). Overall, these findings indicate that long-term BL5923 treatment delays tubulointerstitial and glomerular injuries in NZB/W mice, most likely by attenuating Ccr1-mediated interstitial recruitment of T and mononuclear phagocyte cells.

**Discussion**

By combining pharmacological and functional approaches, we unveiled a pathogenic role for Ccr1 in lupus nephritis in NZB/W mice. Our findings revealed elevated levels of Ccr1 receptors on peripheral mononuclear phagocytes, neutrophils, and T cells in nephritogenic NZB/W mice, which were associated with greater leukocyte chemotaxis to Ccl3. Syngeneic adoptive transfer experiments using ex vivo–labeled splenic leukocytes combined with short-term Ccr1 blockade by the orally available BL5923 antagonist identified a role for Ccr1 in the abnormal homing of macrophages and T cells into the kidney. Longer BL5923 treatment in mice with established nephritis attenuated Ccr1-driven kidney accumulation of effector/memory CD4⁺ T cells, Ly6C⁺ monocytes, and both M1 and M2 macrophages, and reduced tubulointerstitial and glomerular injuries. These processes were associated with renal disease improvement, which resulted in delayed onset of fatal proteinuria and a prolonged lifespan. To our knowledge, this is the first evidence in NZB/W mice that Ccr1 blockade can slow renal disease progression by selective attenuation of interstitial and glomerular recruitment of T and mononuclear phagocyte cells.

Our results extend previous studies indicating that functional Ccr1 receptors are present on mononuclear phagocytes, neutrophils, and T cells and that Ccr1 inactivation prevents the interstitial infiltration of Ccr1-expressing leukocytes in various nonlupus rodent models (13, 14). In this study, we unraveled that Ccr1 receptor biosynthesis is upregulated in splenic T and myeloid cells from nephritic NZB/W mice, which likely arises as a consequence of the disease process. Transcriptional expression of Ccr1 has been reported in splenic macrophages and T cells from MRL-Fas(lpr) mice (17). In line with this, our real-time PCR analyses revealed increased levels of Ccr1 mRNAs in T and myeloid cells sorted from the spleens of nephritic NZB/W mice.
FIGURE 6. Therapeutic Ccr1 inhibition reduces renal damage in nephritic NZB/W mice. Groups of eight NZB/W mice with severe proteinuria were treated as described in Fig. 5. (A) Kidney sections from vehicle- or BL5923-treated mice were stained for the indicated markers. Original magnifications ×400 (i, iii, v, vi, ix, i‘, iii‘, v‘, vii‘, and ix‘) and 31000 (ii, iv, viii, x, i‘i, iv‘, vi‘, vii‘, and x‘). Scale bars denote 50 μm and 12 μm for ×400 and ×1000 magnifications, respectively. Arrows indicate infiltrating leukocytes and proliferating cells. (B) Intermediate magnification images of representative kidney sections stained with PAS solution (i–iii). Images show inflammatory renal damage with interstitial infiltrates (arrows), glomerulonephritis, and intratubular protein cast deposition (*) in a vehicle-treated mouse (ii), and renal histology with significantly fewer glomerular and tubulointerstitial injuries in untreated or BL5923-treated mice (i and iii). Original magnification ×250. High-magnification image of representative glomeruli from a vehicle-treated mouse (v) showing glomerular crescents (*), severe endocapillary proliferation involving all the glomerular tuft (arrowhead), hyaline deposits (arrows), and karyorrhexis. Images of representative glomeruli from untreated (iv) or BL5923-treated (vi) mice, showing only mild endocapillary proliferation (arrowhead), either focal (iv) or global (vi). Original magnification ×1250. Scale bars denote 100 μm and 8 μm for ×250 and ×1250 magnifications, respectively. Results are representative of eight mice in each group.

compared with those of younger prenephritic mice. Importantly, this was corroborated at the protein level by the increased levels of Ccr1 receptors on splenic T cells, including CD4+, CD8+, and Treg subsets, proinflammatory Ly6C+ monocytes and macrophages (M1 and M2), and neutrophils from nephritic mice, and by their enhanced Ccl3-stimulated chemotaxis in vitro. Note that Ccl3 promoted typical bell-shaped dose–response chemotactic patterns of leukocytes from NZB/W mice. This finding suggests that enhanced migration of leukocytes from nephritic mice results from an increased expression of Ccr1 rather than from an impaired receptor inactivation. One can speculate that such increased expression and signaling through Ccr1 accounts for renal T and myeloid cell accumulation in nephritic NZB/W mice.

One important question that arises from these findings concerns the molecular mechanism responsible for Ccr1 upregulation in mononuclear phagocytes, neutrophils, and T cells from nephritic mice. The different cytokine and sex hormone milieu in NZB/W mice may affect the expression and activity of Ccr1. High serum concentrations of TNF-α, TGF-β, and IFN-γ have been reported in lupus nephritis, and these cytokines are known to induce Ccr1 gene expression, notably in myeloid and T cells (38, 39). Estrogens that regulate inflammation may amplify this phenomenon (40, 41). However, epigenetic (e.g., promoter hypomethylation) or posttranscriptional (e.g., microRNA) regulatory mechanisms could account for the increased steady-state levels of Ccr1 mRNAs in nephritic NZB/W mice. Further investigation is needed to determine the specific relevance of each of these factors in Ccr1 biology.

A second question that arises from our findings pertains to the cellular mechanism of Ccr1-mediated accumulation of myeloid and T cells in the nephritic kidneys of NZB/W mice. This accumulation may result from leukocyte influx, retention, and/or proliferation. Short-term treatment of nephritic NZB/W mice with BL5923 significantly reduced the number of fluorescently labeled T cells and macrophages found in the kidneys of recipient mice. These results are consistent with a Ccr1-dependent homing of leukocytes from the blood to the kidney. In line with this, in vitro and in vivo studies have reported a role for Ccr1 in T cell and monocellular phagocyte adhesion and transendothelial migration (14, 17, 18, 27, 42, 43). Thus, BL5923-induced inhibition of renal T cell and macrophage infiltration could be related to reduced Ccr1-mediated adhesion of leukocytes to activated peritubular capillaries. However, Ki-67 staining of renal tissue sections revealed fewer proliferating leukocytes in long-term BL5923-treated mice. Consistent with these results, Ninichuk and coworkers (18) showed that BL5923 treatment in vitro reduced proliferation of the murine macrophage J774 cell line in the presence of serum, whereas BL5923 treatment in vivo reduced the number of interstitial proliferating cells (which likely included some leukocytes) in nephropathy of type 2 diabetic db/db mice. This could constitute
another mechanism by which BL5923 reduces the number of tubulointerstitial and glomerular T and myeloid cells in nephritic NZB/W mice. These observations support the assertion that Ccr1 promotes leukocyte extravasation into the renal interstitial compartment and cell proliferation after kidney infiltration. In B cells, however, Ccr1 and its ligands were clearly dispensable for the homing, retention, and likely function of these cells in the nephritic kidneys of NZB/W mice. Several convergent observations support this idea. First, very low levels of Ccr1 mRNA and protein were detected in splenic and renal B cells from both prenephritic and nephritic NZB/W mice. As a consequence, these cells were insensitive to Ccl3-promoted chemotaxis. Second, short-term BL5923 treatment of nephritic NZB/W mice did not prevent the recruitment of B cells to the inflamed kidneys. Third, longer Ccr1 blockade altered neither the absolute number of B cells nor their interstitial and glomerular partitioning in the kidneys of nephritic NZB/W mice. Finally, BL5923-mediated blockade of Ccr1 did not affect systemic or renal humoral autoimmunity in nephritic NZB/W mice. These findings suggest differential chemokine receptor requirements for B cells versus T and myeloid cells in their renal homing and compartmentalization in nephritic NZB/W mice (19, 35, 44). For instance, the ligands of CXCR4 and CXCR5, which are up-regulated in the nephritic kidneys of NZB/W mice, are likely to be major contributors to this pathological process.

The third important question concerns the nature of the T or myeloid cell populations that drive kidney pathogenesis in a Ccr1-dependent manner in this model of lupus nephritis. The use of Ccr1-deficient mice or small-molecule Ccr1 antagonists efficiently reduced interstitial infiltration of T cells, mononuclear phagocytes, and/or neutrophils in different mouse models of nephropathy (13, 14, 17, 18, 23, 27, 28, 43). In particular, s.c. injections of the Ccr1 antagonist BX471 in MRL-Fas(lpr) mice led to reduced numbers of macrophages, T cells, and Ki-67 positive proliferating cells and reduced fibrosis in the interstitium. However, treatment did not modulate proteinuria or markers of glomerular injury (17). In this article, we showed that a 10-d interventional treatment using the orally available Ccr1 antagonist BL5923 resulted in decreased numbers of effector/memory CD4+ T cells, Ly6C+ monocytes, both M1 and M2 macrophages, and neutrophils in the kidneys of NZB/W mice. Concomitantly, Ccr1 blockade reduced the in situ numbers of interstitial T cells and mononuclear phagocytes, as well as glomerular macrophages, and improved tubulointerstitial, and, surprisingly, glomerular injuries. We speculate that Ccr1 inhibition reduces the renal recruitment of Ly6C+ monocytes and their differentiation toward M1-polarized macrophages, which depends on the local inflammatory environment encompassing cytokines (e.g., INF-γ, TNF-α), chemokines (e.g., Ccl3, Ccl5), and renal cell necrosis (10). Consequently, BL5923 treatment would decrease proinflammatory M1 and profibrotic M2 polarization of recruited and resident renal macrophages, which is associated with a reduction in renal cell damage, as well as a delay in the vicious circle of renal inflammation and parenchymal loss, thus resulting in prolonged survival of NZB/W mice (10, 45, 46). In contrast, Tregs and Ly6C+ monocytes were not affected by BL5923 treatment, suggesting that Ccr1 is not a dominant major receptor for their kidney infiltration and accumulation. The beneficial effect of BL5923 treatment also suggests that Tregs and Ly6C+ monocytes are not major players in kidney pathogenesis in this model of lupus nephritis. Therefore, selectively modulating the number of interstitial and glomerular mononuclear phagocytes, neutrophils, or T cells using clodronate-loaded liposomes or Ab-depleting strategies, in combination with noninvasive oral administration of BL5923, could help to determine the functional contribution of each leukocyte subpopulation to the progression of autoimmune nephropathy in NZB/W mice.

Another related finding in our work is that therapeutic Ccr1 blockade led to a reduced content of Ccr1 and its cognate ligands Ccl3 and Ccl5 in the kidneys of NZB/W mice. These findings are in line with previous studies (17, 18), which indicate that nonimmune resident kidney cells do not express Ccr1 and that Ccr1 expression originates from infiltrated T and myeloid cells. Hence, BL5923-mediated reduction of Ccr1 expression in the kidney was likely due to the attenuation of Ccr1-promoted T and myeloid cell infiltration. This process could also account for the decreased representation of Ccr1 ligands in nephritic kidneys. Indeed, we detected more Ccl3 and Ccl5 protein and mRNA in injured kidneys from nephritic mice than in control kidneys from prenephritic mice. Infiltrated leukocytes constituted the major source of renal Ccl3, whereas tubular and glomerular cells exhibited only weak Ccl3 staining. In agreement, previous studies have reported that Ccl3 and Ccl5 can be produced by infiltrating leukocytes, including mononuclear phagocytes, neutrophils, and T cells (14, 17, 47). Of interest, we observed a significant decrease in serum inflammatory cytokines TNF-α and IL-1β upon BL5923 exposure. In

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Glomerular activity index</th>
<th>Tubulointerstitial activity index</th>
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<tbody>
<tr>
<td></td>
<td>Glomerular proliferation</td>
<td>Tubular cell lesions</td>
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<tr>
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<td>2.5 (2–3)</td>
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<tr>
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<td>0.25 (0–2)</td>
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<td></td>
<td>Cellular crescents</td>
<td>Tubular distension</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0.18 (1–3)</td>
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<td></td>
<td>0.5 (0–1)</td>
<td>1.75 (1–2)</td>
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<tr>
<td></td>
<td>Hyaline deposits</td>
<td>Interstitial inflammation</td>
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<td>0</td>
<td>0.25 (0–1)</td>
</tr>
<tr>
<td></td>
<td>0.75 (0–2)</td>
<td>Tubular atrophy</td>
</tr>
<tr>
<td></td>
<td>Polymorphonuclear leukocytes</td>
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<td>Score</td>
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<td></td>
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<td></td>
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<td>3.75 (3–4)</td>
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<tr>
<td>Treatment</td>
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<td>Tubular distension</td>
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<tr>
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<td></td>
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</table>

Data are means, with ranges in parentheses. *p < 0.05, **p < 0.005, ***p < 0.0005 compared with vehicle treatment.
combination with the observation that BL5923 reduced the number of T and mononuclear phagocyte cells, which likely constitute a source of Ccr1 ligands and other inflammatory and profibrotic mediators, in damaged kidneys, these data suggest that Ccr1 contributes to leukocyte recruitment to the kidney and amplification of the inflammatory response, which involves renal cells and leukocytes themselves and participates in nephritis progression.

Finally, our findings suggest that blocking the Ccr1-mediated influx of T and myeloid cells is sufficient to improve lupus nephritis in NZB/W mice. Long-term BL5923 treatment reduced tubulointerstitial and glomerular injuries, delayed proteinuria, and prolonged the lifespan of nephritic NZB/W mice. Thus, by preventing renal infiltration of T cells, mononuclear phagocytes, and neutrophils, BL5923 might reduce detrimental inflammation in both tubulointerstitial and glomerular compartments, and result in prolonged survival. In contrast to the effects of BX471 administration in the MRL-Fas(lpr) model (17), BL5923 treatment did not improve renal fibrosis in the 10-d window of treatment in NZB/W mice. Additional work is required to decipher the functional role and renal distribution of proinflammatory (M1), as well as the balance of anti-inflammatory (M2c) and profibrotic (M2a) macrophages and their contribution to renal fibrosis (10), together with Ccr1 as a master regulator of kidney infiltration during the progression of lupus nephritis. Immunotherapies aimed at specific cell types are of interest in SLE because they could be used to improve current treatments based only on nonspecific immunosuppressive drugs. Our data provide a rationale for potentially adding BL5923 to the treatment arsenal of patients with SLE. By limiting Ccr1 activation, oral treatment with BL5923 would attenuate the aberrant interstitial and glomerular accumulation of leukocytes in the kidneys and thus reduce the severity of lupus-related nephropathy.

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Disclosures

P.L. is an employee of Novartis Pharma AG, which holds patents for BL5923. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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