# Chemokine Receptor Ccr2 Deficiency Reduces Renal Disease and Prolongs Survival in MRL/lpr Lupus-Prone Mice

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MRL/MpJ-$Fas^{lpr}$/J (MRL/lpr) mice represent a well-established mouse model of human systemic lupus erythematosus. MRL/lpr mice homozygous for the spontaneous lymphoproliferation mutation (lpr) are characterized by systemic autoimmunity, massive lymphadenopathy associated with proliferation of aberrant T cells, splenomegaly, hypergammaglobulinemia, arthritis, and fatal immune complex–mediated glomerulonephritis. It was reported previously that steady-state mRNA levels for the chemokine (C-C motif) receptor 2 (Ccr2) continuously increase in kidneys of MRL/lpr mice. For examining the role of Ccr2 for development and progression of immune complex–mediated glomerulonephritis, Ccr2-deficient mice were generated and backcrossed onto the MRL/lpr genetic background. Ccr2-deficient MRL/lpr mice developed less lymphadenopathy, had less proteinuria, had reduced lesion scores, and had less infiltration by T cells and macrophages in the glomerular and tubulointerstitial compartment. Ccr2-deficient MRL/lpr mice survived significantly longer than MRL/lpr wild-type mice despite similar levels of circulating immunoglobulins and comparable immune complex depositions in the glomeruli of both groups. Anti-dsDNA antibody levels, however, were reduced in the absence of Ccr2. The frequency of $CD8^+$ T cells in peripheral blood was significantly lower in Ccr2-deficient MRL/lpr mice. Thus Ccr2 deficiency influenced not only monocyte/ macrophage and T cell infiltration in the kidney but also the systemic T cell response in MRL/lpr mice. These data suggest an important role for Ccr2 both in the general development of autoimmunity and in the renal involvement of the lupus-like disease. These results identify Ccr2 as an additional possible target for the treatment of lupus nephritis.

Systemic lupus erythematosus is an autoimmune disease with immune complex–mediated glomerulonephritis as a major manifestation and determinant of the disease outcome (1). The MRL/lpr mouse carries a mutation in the apoptosis-related Fas gene, resulting in autoreactive lymphocyte proliferation, and is considered as a mouse model that closely mimics the human disease with lymphadenopathy; splenomegaly; hypergammaglobulinemia with anti-dsDNA antibodies that lead to tissue deposition; and injury in various organs, including lung and kidney (2,3). These pathophysiologic processes are mediated by immune complex deposition, complement activation, and infiltration of inflammatory leukocytes (4,5). The renal leukocyte infiltrates consist mostly of macrophages and T lymphocytes. These cells are major contributors to the development of progressive renal insufficiency, ultimately resulting in the death of the MRL/lpr mice.

Numerous studies in both human and experimental murine lupus have provided evidence that chemokines play a considerable role during the development and progression of the renal disease. We and others have proposed that deposition of immune complexes and complement activation leads to local generation of chemokines in the kidney followed by the influx of macrophages and T cells (6). Initially, this may be restricted to the glomerulus with subsequent expansion to the tubulointerstitial compartment and development of progressive renal insufficiency (7). This hypothesis is supported by studies with MRL/lpr mice carrying a targeted deletion of the Ccl2 (murine MCP-1) gene (5) or treated with antagonists for Ccl2 (8,9). In both cases, an attenuation of the nephritis has been observed. Furthermore, Ccl2-deficient MRL/ lpr mice showed a marked reduction of inflammatory lesions, including in the kidney, leading to prolonged survival (5). The chemokine receptor Ccr2, which represents the only known receptor for Ccl2, is considered to play a role not only in the local

infiltration of macrophages and T cells but also in the development and bias of the overall immune response (10). Surprisingly, Ccr2 and Ccl2 have opposite effects on Th1 *versus* Th2 polarization. Ccr2-deficient mice show decreased IFN-γ secretion, *i.e.*, a Th1 defect (11), whereas Ccl2-deficient mice show predominantly a defect of Th2 cytokine secretion (12). Possibly because of these differences, conflicting results have been obtained with Ccl2- and Ccr2-deficient mice in several disease models (13). These may relate in part to the fact that in addition to Ccl2, other Ccr2 ligands exist (Ccl7/MCP-3, Ccl8/MCP-2, and Ccl12/MCP-5) (13) and that other, yet unidentified, receptor(s) for Ccl2 may exist (14).

Although Ccr2-deficient mice have been studied in many disease models (15), data about the role of Ccr2 in lupus nephritis are still missing. This report describes the generation and analysis of Ccr2-deficient MRL/lpr mice with a main focus on the development and progression of lupus nephritis.

## Materials and Methods
### Animals
MRL/MpJ-*Fas^lpr*/J (briefly MRL/lpr) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and subsequently maintained as a breeding colony in our animal facility. All mice were housed under specific pathogen free conditions. All animal experiments were performed in compliance with current guidelines and approved by local authorities.

### Generation of Ccr2-Deficient MRL/lpr Mice
A targeting vector (Figure 1A) was constructed and electroporated into E14–1 embryonic stem (ES) cells. A total of 192 ES cell clones that were resistant to G418 and Ganciclovir were screened by Southern blot analysis (Figure 1B). Homologous recombination within the 3′-homology arm was confirmed by long-range PCR (Expand Long Template PCR System; Roche Applied Science (Penzburg, Germany), forward primer 5′-CAGAAGAACGGCATCAAGGTGAAC-3′, reverse primer 5′-TGTGGAACAAACTTTATGCTGGGG-3′; Figure 1C). Two positive ES cell clones were microinjected into C57BL/6 blastocysts. Resulting chimeras were mated with C57BL/6 wild-type (wt) mice for germline transmission. A more detailed description is available upon request. The resulting heterozygous Ccr2-deficient mice were backcrossed for seven generations onto the MRL/lpr background and then intercrossed to obtain $Ccr2^{-/-}$ MRL/lpr mice that were used for all experiments. For survival analysis, four additional Ccr2-deficient mice that were backcrossed for 10 generations were included. All experiments were performed with virgin female mice homozygous for the lpr mutation.

### Genotype Analysis
All mice were genotyped by PCR using genomic DNA that was isolated from tail snips and the following primers: Ccr2 forward 5′-AAAGGAAATGGAAGACAATAATATG-3′; Ccr2 reverse 5′-AAAG-GCAAACTGTCACTTACTTTAC-3′; EGFP reverse 5′-GTCAGGGTG-GTCACGAGG-3′; Fas forward 5′-AAAGGTTACAAAAGGTCACCC-3′; Fas reverse 5′-GGTGCAGCCAGAAGCTAG-3′; lpr reverse 5′-GTTCCTCTTAGCATCTCTCTGC-3′.

### Collection of Blood, Urine, and Tissue Samples and Determinations of Blood Urea Nitrogen, Creatinine, and Urinary Albumin
Blood and spot urine samples were obtained and determinations were performed as described previously (6,16). After a final bleeding step, anesthetized mice were killed by cervical dislocation. One kidney was snap-frozen in liquid nitrogen and used exclusively for isolation of total RNA. The other kidney was used for histologic and immunohistologic analyses.

### Histopathology
A renal pathologist performed in a blinded manner the histopathologic analysis as described previously (17). Glomerular lesions were graded semiquantitatively from 0 to 3+ for hypercellularity, mesangial matrix expansion, and necrosis (0, absence; 1, mild; 2, moderate; 3, severe), as well as for the percentage of sclerotic glomeruli and presence of crescents (0, <10%; 1, 10 to 25%; 2, 25 to 40%; 3, >40%). Tubulointerstitial lesions were also graded from 0 to 3+ for peritubular and pericapillary mononuclear cell infiltrate, tubular damage, interstitial fibrosis, and vasculitis. Finally, perivascular lymphoproliferative mononuclear cell infiltrate was graded from 0 (absence) to 3+ (maximal intensity). Global glomerular and tubulointerstitial lesion scores were calculated for each mouse from the sum of all parameters.

### Immunofluorescence and Immunohistochemistry
Direct immunofluorescence was performed on 5-μm ether/ethanol-fixed cryostat sections using FITC-conjugated rabbit anti-mouse IgG and goat anti-mouse complement C3 antibodies (16). CD3-, ER-HR3–, Mac-2–, and Ki-67–positive cells were detected using 4-μm-thick paraffin sections from renal tissue (17,18). IgG of the corresponding isotype served as negative control. For quantification, positively stained cells were counted either within 20 glomeruli or within 10 high-power fields (×630) of tubulointerstitial tissue and expressed as cells per glomerular cross-section or high-power field.

### RNase Protection Assay
Cytokine, chemokine, and chemokine receptor mRNA expression was analyzed in kidney RNA from wt and $Ccr2^{-/-}$ MRL/lpr mice using RPA template sets mCK-3, mCK-5b, and mCR-5 (BD Biosciences, Heidelberg, Germany) (19). Integrity of RNA samples was confirmed by agarose gel electrophoresis. For chemokine receptors, 30 μg/lane total kidney RNA was used; for cytokines and chemokines, 10 μg/lane was used. Bands were quantified by phosphorimaging (Storm 840 PhosphorImager; Molecular Dynamics, Sunnyvale, CA) using ImageQuant software.

### Real-Time Reverse Transcriptase–PCR
Spleen IFN-γ mRNA levels were quantified by real-time RT-PCR as described (20) using a predesigned probe and primer set from Applied Biosystems (Darmstadt, Germany). The normalized expression values were multiplied by a factor of $10^5$.

### Analysis of Peripheral Blood by Flow Cytometry and ELISA
Blood was collected from the retro-orbital plexus of anesthetized mice and peripheral blood leukocytes were stained after erythrocyte lysis with antibodies against CD3 (clone 17A2), CD4 (clone RM4–5), CD8β (clone H35–17.2), CD19 (clone 1D3), CD49b (clone DX5), B220 (clone RA3–6B2), and Gr-1 (clone RB6–8C5; all PharMingen, Heidelberg, Germany). Samples were acquired (25,000 leukocytes) using a high-throughput sampler on a FACS Calibur (BD Biosciences, San Jose, CA) and analyzed using FlowJo software (Tree Star, Ashland, OR).

Plasma levels of Ig isotypes were determined by standard sandwich ELISA using goat anti-mouse Ig antibodies and alkaline phosphatase (AP) conjugates (SouthernBiotech, Birmingham, AL). Antibodies against double-stranded DNA were detected by indirect ELISA on plates coated with calf thymus DNA (5 μg/ml; Sigma-Aldrich, Taufkirchen, Germany) and AP-conjugated goat anti-mouse polyvalent immunoglobulins antibody
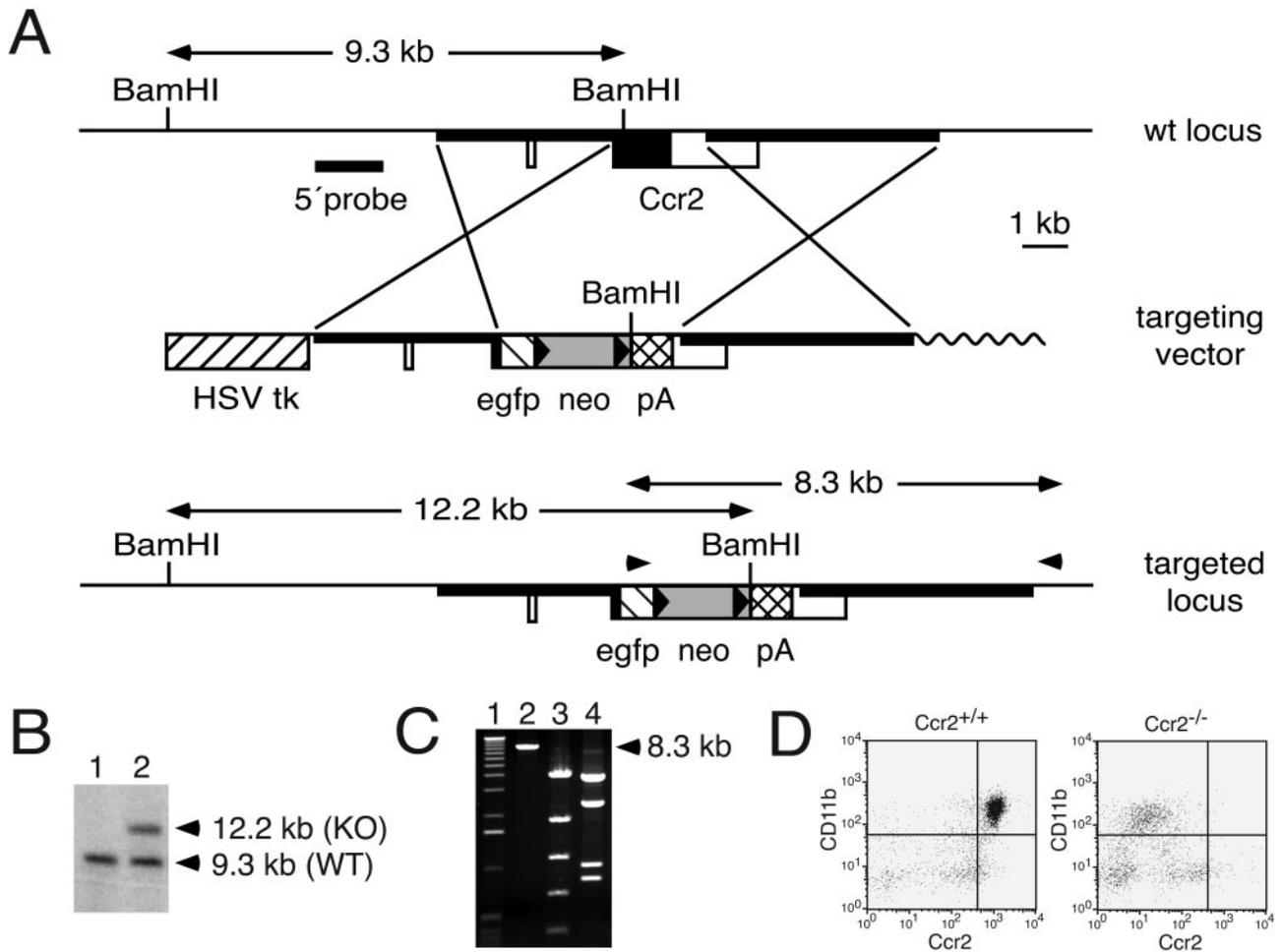
*Figure 1.* Generation and identification of Ccr2-deficient mice. (A) Schematic representation of the targeting strategy. The murine Ccr2 wild-type (wt) locus, the targeting vector and the targeted Ccr2 locus are shown together with selected restriction sites relevant for Southern blot analysis. □, 5′ and 3′ untranslated regions; ■, coding region of the Ccr2 gene. The homology arms are depicted as thick lines, the floxed neomycin resistance gene (neo) for positive selection as a gray box with black triangles at both ends, the Herpes simplex virus thymidine kinase gene (HSV tk) for negative selection as a hatched box, the enhanced green fluorescence reporter gene (egfp) also as a hatched box, and the SV40 polyadenylation signal (pA) as a cross-hatched box. The wavy line represents prokaryotic sequences in the targeting vector. The sizes of relevant restriction fragments and PCR fragments before and after homologous recombination are depicted. Arrowheads indicate the position of primers used for long-range PCR analysis. (B) Southern blot analysis of wt embryonic stem cells (lane 1) and a successfully targeted ES cell clone (lane 2) using the 5′ external probe depicted in A. (C) Long-range PCR to prove homologous recombination within the 3′ arm of homology. An 8.3-kb PCR fragment indicates homologous recombination. Lane 1, 1-kb ladder; lane 2, PCR product; lane 3, PCR product digested with *Hind*III; lane 4, PCR product digested with *Bam*HI. (D) Analysis of peripheral blood leukocytes (PBL) from wt and Ccr2-deficient mice by flow cytometry. PBL were stained with antibodies directed against CD11b and Ccr2 and gated for monocytes.

(Sigma-Aldrich). Serum from MRL/lpr mice was used in 1:1250 to 1:10,000 dilution as positive control in autoantibody assays.

MC-21 antibody was used to stain Ccr2 on peripheral blood leukocytes (21). Detection was performed with PE-conjugated polyclonal goat anti-rat IgG (P64; Biomeda, Foster City, CA). For simultaneous CD11b staining, a FITC-conjugated mAb (clone M1/70, BD Biosciences) was used.

## Statistical Analyses

Data are shown as means ± SD and were analyzed either by two-tailed unpaired $t$ test (parametric data), Mann-Whitney $U$ test (nonparametric data), or log-rank test (survival). $P < 0.05$ was considered significant.

## Results

### Generation of Ccr2-Deficient MRL/lpr Mice

Ccr2-deficient mice were generated using the targeting strategy outlined in Figure 1. They were viable, fertile, and indistinguishable from their wt littermates. Absence of Ccr2 protein on the cell surface of blood monocytes was demonstrated by flow cytometry (Figure 1D). Ccr2-deficient mice were backcrossed for seven generations onto the inbred strain MRL/lpr and then intercrossed to obtain Ccr2$^{-/-}$ MRL/lpr mice. Because the contribution of the MRL/lpr genetic background in these mice exceeds 99%, wt MRL/lpr mice were used as controls.

## Body Weight, Splenomegaly, and Lymphadenopathy

The body, spleen, and right axillary lymph node weights of $Ccr2^{+/+}$ and $Ccr2^{-/-}$ MRL/lpr mice were determined at the age of 14 and 20 wk (Figure 2). There was no difference in body weight between wt and $Ccr2^{-/-}$ MRL/lpr mice. In contrast, the spleen weights were significantly reduced in $Ccr2^{-/-}$ MRL/lpr mice at 14 wk but not at 20 wk. Lymph node weights were significantly reduced in $Ccr2^{-/-}$ MRL/lpr mice at 14 and 20 wk.

## Time Course of Blood Urea Nitrogen and Albuminuria

No statistically significant differences in blood urea nitrogen (BUN) were observed between wt and Ccr2-deficient MRL/lpr mice at 8, 14, and 20 wk of age (Figure 3). Urinary albumin excretion increased between weeks 8 and 20 in MRL/lpr wt mice with a marked interanimal variability and was significantly reduced in Ccr2-deficient MRL/lpr mice at 8 and 14 wk (Figure 3).

## Histopathologic and Immunohistochemical Findings

At the age of 14 wk, $Ccr2^{+/+}$ MRL/lpr mice showed enlarged hypercellular glomeruli, an increase in mesangial matrix, and a mild peritubular mononuclear cell infiltrate (Figure 4A). In addition, prominent perivascular lymphoid cell accumulations were present at this time point, which may be related to the lymphoproliferative process of these mice (16,17). At the age of 20 wk, $Ccr2^{+/+}$ MRL/lpr mice showed locally and focally variable signs of intraglomerular necrosis, sclerosis, and crescent formation and peritubular mononuclear cell infiltration with increased overall tubular damage and focal tubular atrophy and intratubular protein cast formation (Figure 4A). In addition, mild signs of tubulointerstitial fibrosis could be observed. No vasculitis was detected. These lesions were significantly reduced in $Ccr2^{-/-}$ MRL/lpr mice (Figure 4).

## Immune Complex Deposition

IgG and C3 depositions were analyzed by immunofluorescence within the glomeruli of 14- and 20-wk-old $Ccr2^{+/+}$ and



Figure 3. Effect of Ccr2 deficiency on blood urea nitrogen (BUN) and proteinuria. BUN levels were measured in serum samples from wt and Ccr2-deficient MRL/lpr mice (week 8 wt $n = 8$, KO $n = 6$; week 14 wt $n = 14$, KO $n = 19$; week 20 wt $n = 13$, KO $n = 21$). Albuminuria was determined by ELISA in urine samples from wt and Ccr2-deficient MRL/lpr mice (week 8 wt $n = 20$, KO $n = 8$, $P = 0.018$; week 14 wt $n = 15$, KO $n = 23$, $P = 0.0014$; week 20 wt $n = 14$, KO $n = 19$).
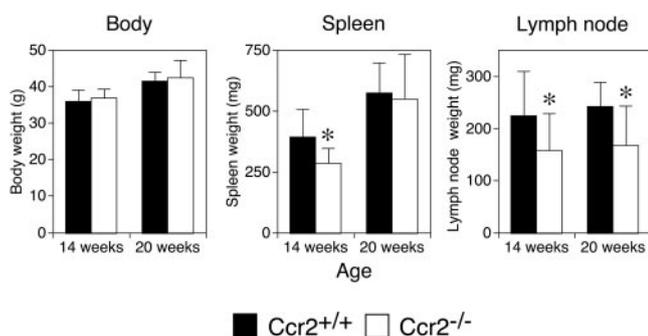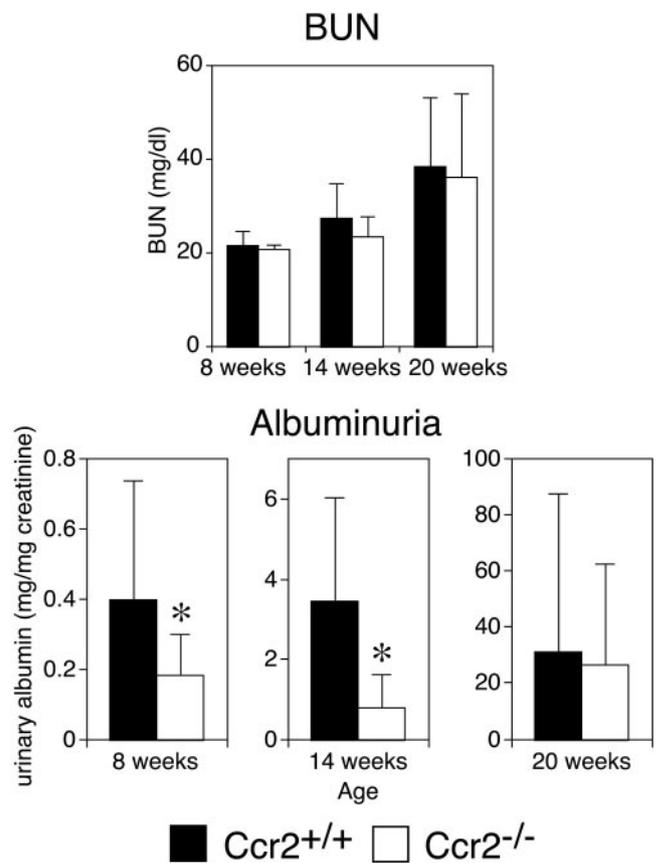


Figure 2. Effect of Ccr2 deficiency on body weight, splenomegaly, and lymphadenopathy. The body weight, the spleen weight, and the weight of the right axillary lymph node were determined in 14- and 20-wk-old wt and Ccr2-deficient MRL/lpr mice (body: week 14 wt $n = 20$, knockout [KO] $n = 15$; week 20 wt $n = 12$, KO $n = 17$; spleen: week 14 wt $n = 13$, KO $n = 15$, $P = 0.006$; week 20 wt $n = 12$, KO $n = 10$; lymph node: week 14 wt $n = 17$, KO $n = 15$, $P = 0.031$; week 20 wt $n = 12$, KO $n = 10$, $P = 0.016$).

$Ccr2^{-/-}$ MRL/lpr mice. No differences in immune complex and complement C3 depositions were observed between wt and Ccr2-deficient MRL/lpr mice (Figure 5A).

## Kidney T Cell and Macrophage Infiltration

Infiltration of the kidney by T lymphocytes and macrophages was analyzed by immunohistochemical staining for CD3 and ER-HR3 (Figure 5B). At 14 and 20 wk of age, low numbers of T cells and macrophages were found in the glomerular compartment, which were significantly reduced in Ccr2-deficient MRL/lpr mice at 20 wk of age (Figure 5C). The number of glomerular macrophages was also determined by Mac-2 staining, which detects a broader range of macrophage subpopulations than ER-HR3 staining (22). As expected, the number of Mac-2$^+$ cells per glomerulus was an order of magnitude higher for both groups and reduced in Ccr2-deficient mice (week 20 $13.4 \pm 4.7$ versus $9.5 \pm 5.0$; wt $n = 9$, KO $n = 15$; $P = 0.034$ one-tailed unpaired $t$ test, $P = 0.068$ two-tailed test). In the tubulointerstitial compartment, both CD3$^+$ T cells and ER-HR3$^+$ macro-
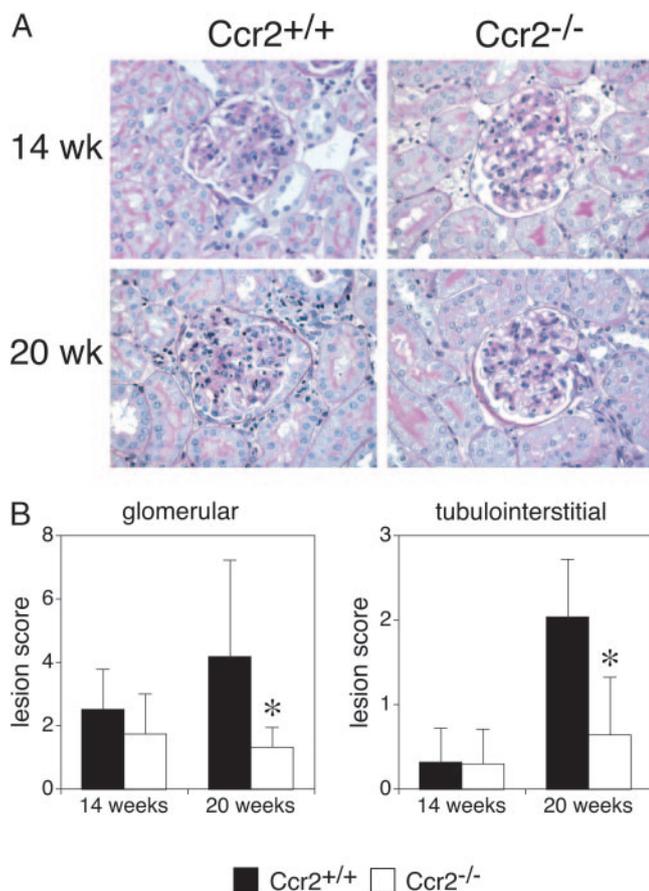
*Figure 4.* Effect of Ccr2 deficiency on glomerular and tubulointerstitial damage. (A) Representative periodic acid-Schiff–stained kidney sections from 14- or 20-wk-old wt and Ccr2-deficient MRL/lpr mice. (B) Glomerular and tubulointerstitial lesion scores for 14- or 20-wk-old wt and Ccr2-deficient MRL/lpr mice (14 wk wt $n$ = 15, KO $n$ = 10; 20 wk wt $n$ = 25, KO $n$ = 14, glomerular scores $P$ = 0.001, tubulointerstitial scores $P$ = 0.006). Magnification, ×630 in A.

phages were significantly reduced in Ccr2-deficient MRL/lpr mice at both time points (Figure 5, B and C).

### Intraglomerular Cell Proliferation

Intraglomerular cell proliferation was analyzed by immunohistochemical staining for Ki-67. A prominent signal was observed mainly in glomeruli of 14-wk-old $Ccr2^{+/+}$ MRL/lpr mice, consistent with a more acute/proliferative phase of glomerulonephritis (Figure 5B). At 20 wk, fewer Ki-67–positive cells were detected within the glomeruli, and proliferative lesions were replaced by necrosis and sclerosis. Ccr2-deficient MRL/lpr mice had fewer Ki-67–positive cells per glomerulus, at both 14 and 20 wk of age as compared with age-matched $Ccr2^{+/+}$ MRL/lpr mice (Figure 5C).

### Expression of Proinflammatory Cytokines, Chemokines, and Chemokine Receptors

Multiprobe RPA was used to analyze mRNA expression of selected cytokines, chemokines, and chemokine receptors in kidneys of 14- and 20-wk-old $Ccr2^{+/+}$ and $Ccr2^{-/-}$ MRL/lpr
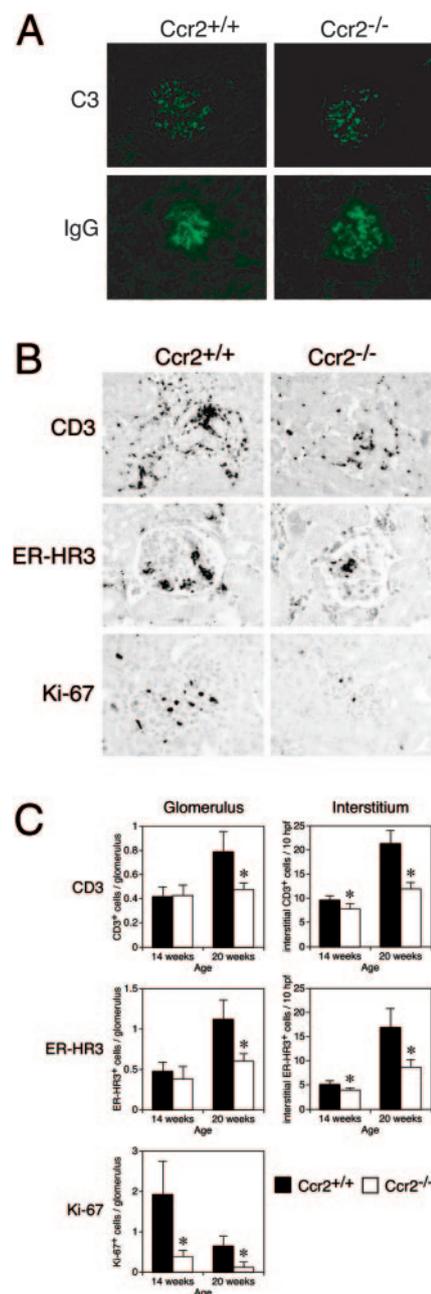


*Figure 5.* Effect of Ccr2 deficiency on immune complex deposition, kidney infiltration, and cellular proliferation. (A) Analysis of C3 and IgG immune complex deposition in glomeruli from 14-wk-old wt and Ccr2-deficient MRL/lpr mice by immunofluorescence. (B) Immunohistochemical analysis of T cell and macrophage infiltration and cellular proliferation in kidneys from 14- and 20-wk-old wt and Ccr2-deficient MRL/lpr mice. The Ki-67 pictures are from 14-wk-old animals; the other ones are from 20-wk-old animals. For illustrative purposes, pictures were chosen showing glomeruli with a higher number of infiltrated macrophages than the mean values obtained from all analyzed glomeruli (see C). (C) Quantitative analysis of infiltration and cellular proliferation in kidneys from wt and Ccr2-deficient MRL/lpr mice at 14 and 20 wk of age (wt $n$ = 4 to 6, KO $n$ = 4 to 6; glomerulus week 20: CD3 $P$ = 0.016, ER-HR3 $P$ = 0.0095; tubulointerstitium week 20: CD3 $P$ = 0.0008, ER-HR3 $P$ = 0.0033; Ki-67 week 14 $P$ = 0.019, week 20 $P$ = 0.020). Magnification, ×400 in A.
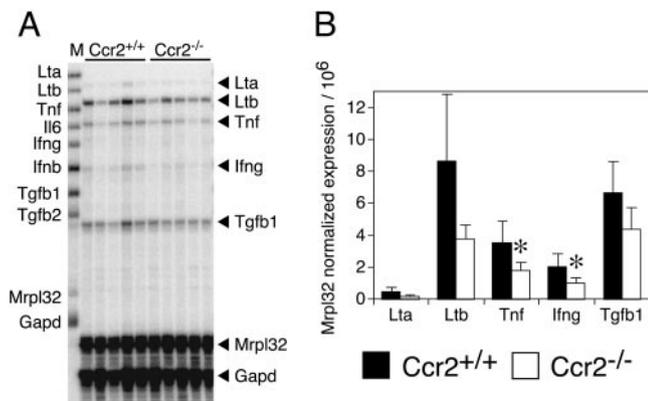
*Figure 6.* Effect of Ccr2 deficiency on renal cytokine expression. Expression of cytokines was analyzed by multiprobe RPA using total kidney RNA from 20-wk-old wt and Ccr2-deficient MRL/lpr mice (wt $n = 5$, KO $n = 5$). (A) PhosphorImager scan. (B) Quantitative evaluation (TNF-$\alpha$ $P = 0.038$, IFN-$\gamma$ $P = 0.039$).

mice. The results of 20-wk-old mice are shown in Figures 6, 7, and 8. Although the expression of all detected genes was somewhat lower in Ccr2$^{-/-}$ mice, a statistically significant reduction was observed only for the cytokines TNF-$\alpha$ and IFN-$\gamma$ (Figure 6); the chemokine Xcl1 (Figure 7); and the chemokine receptors Ccr1, Ccr3, and Ccr5 (Figure 8). To examine whether the IFN-$\gamma$ response was generally lower in Ccr2$^{-/-}$ MRL/lpr mice, we used real-time RT-PCR to quantify IFN-$\gamma$ mRNA levels in spleens from 20-wk-old Ccr2$^{+/+}$ and Ccr2$^{-/-}$ MRL/lpr mice. No differences were observed between both groups (wt 16.2 $\pm$ 7.8, $n = 6$; KO 15.0 $\pm$ 11.6, $n = 7$; $P = 0.833$).

*Circulating Immunoglobulins*

Circulating Ig isotype levels and anti-dsDNA antibodies were determined in 14- and 20-wk-old wt and Ccr2-deficient MRL/lpr mice (Figure 9). We noticed a three- to four-fold decrease in IgG2a between weeks 14 and 20 in all mice. With the exception of anti-dsDNA antibodies (Figure 9B), no signif-
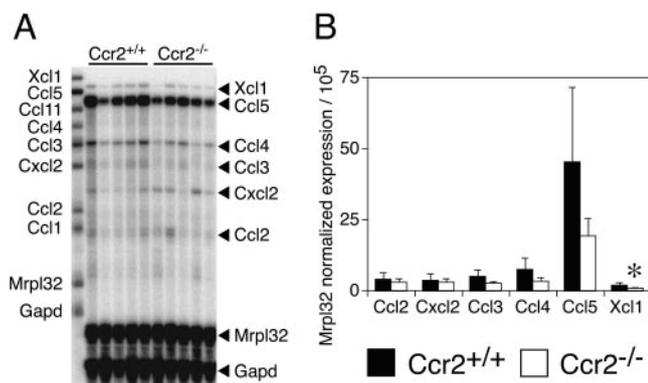
icant differences between wt and Ccr2-deficient MRL/lpr mice were observed (Figure 9A).

*Immunoscreen of Peripheral Blood*

Flow cytometry was used to measure the frequency of selected leukocyte subpopulations in peripheral blood from 14- and 20-wk-old Ccr2$^{+/+}$ and Ccr2$^{-/-}$ MRL/lpr mice. Lymphadenopathy in MRL/lpr mice is characterized by a marked accumulation of CD4$^-$ CD8$^-$ B220$^+$ T cells (double-negative T cells), which can comprise >90% in older animals. As expected, an increase of B220$^+$ cells was observed in parallel to an increase of CD3$^+$ T cells between weeks 14 and 20 (Figure 10). Whereas no differences between wt and Ccr2$^{-/-}$ MRL/lpr mice were observed with respect to the percentage of overall CD3$^+$ T cells, B220$^+$ cells, CD19$^+$ B cells, Gr-1$^+$ cells, or DX5$^+$ NK cells at both time points, the frequency of CD4$^+$ T cells was significantly lower in Ccr2-deficient MRL/lpr mice at 14 wk ($P = 0.007$). Furthermore, the percentage of CD8$^+$ T cells was markedly reduced at both 14 ($P = 0.00008$) and 20 wk ($P = 0.00012$; Figure 10).

*Survival Analysis*

Ccr2$^{+/+}$ MRL/lpr mice showed a median (50%) survival time of 25.7 $\pm$ 2 wk (95% confidence interval 21.8 to 29.6), which was increased to 38.7 $\pm$ 10.6 wk (95% confidence interval 18.0 to 59.4) in Ccr2$^{-/-}$ MRL/lpr mice. The statistical analysis of the survival data revealed a significant prolongation of survival in Ccr2-deficient MRL/lpr mice (Figure 11).

## Discussion

The various roles of Ccr2 and its ligand Ccl2 have been examined in numerous experimental studies. Discrepant results have been obtained concerning the general immune response in Ccl2$^{-/-}$ (12) *versus* Ccr2$^{-/-}$ mice (23). With regard to infiltration of T cells and monocytes/macrophages at sites of
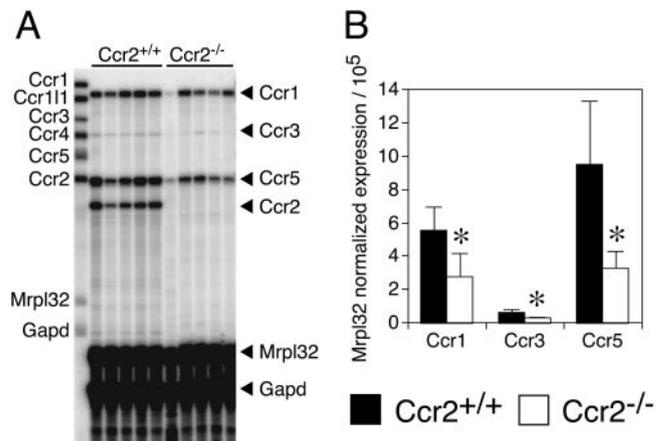


*Figure 8.* Effect of Ccr2 deficiency on renal chemokine receptor expression. Expression of chemokine receptors was analyzed by multiprobe RPA using total kidney RNA from 20-wk-old wt and Ccr2-deficient MRL/lpr mice (wt $n = 5$, KO $n = 5$). (A) PhosphorImager scan. (B) Quantitative evaluation (Ccr1 $P = 0.012$, Ccr3 $P = 0.006$, Ccr5 $P = 0.020$).



*Figure 7.* Effect of Ccr2 deficiency on renal chemokine expression. Expression of chemokines was analyzed by multiprobe RPA using total kidney RNA from 20-wk-old wt and Ccr2-deficient MRL/lpr mice (wt $n = 5$, KO $n = 5$). (A) PhosphorImager scan. (B) Quantitative evaluation (Xcl1 $P = 0.038$).
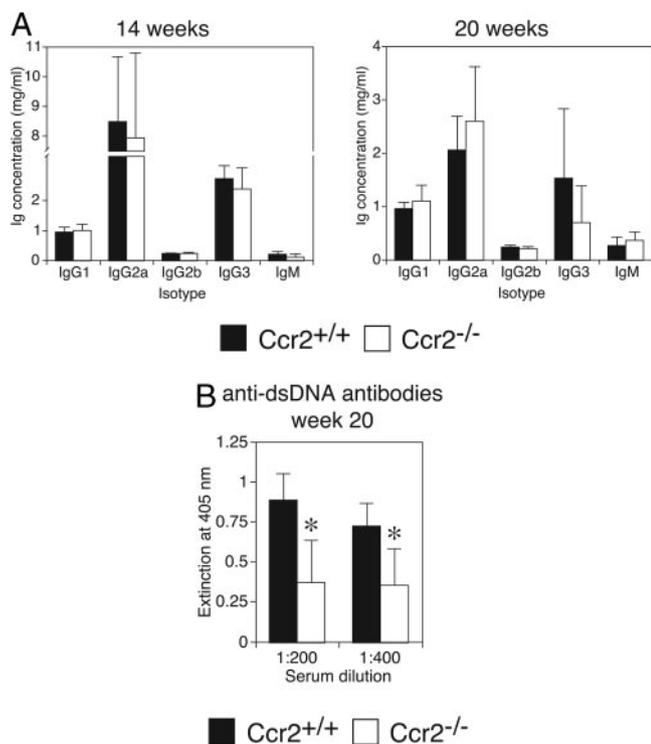
*Figure 9.* Effect of Ccr2 deficiency on circulating Ig isotype levels. (A) Serum from wt (week 14 $n = 7$, week 20 $n = 6$) and Ccr2-deficient MRL/lpr mice (week 14 $n = 8$, week 20 $n = 7$) was analyzed by ELISA for Ig isotype levels. (B) Serum from 20-wk-old wt ($n = 6$) and Ccr2-deficient MRL/lpr mice ($n = 7$) was analyzed by ELISA for anti-dsDNA antibodies (1:200 $P = 0.002$, 1:400 $P = 0.008$).



*Figure 10.* Effect of Ccr2 deficiency on leukocyte subsets in peripheral blood. Peripheral blood from wt (week 14 $n = 7$, week 20 $n = 6$) and Ccr2-deficient MRL/lpr mice (week 14 $n = 8$, week 20 $n = 7$) was analyzed by flow cytometry using antibodies directed against CD3, CD4, CD8, and B220.

immune injury, a more uniform picture is emerging. Our results obtained with the model of systemic lupus in Ccr2-deficient MRL/lpr mice support a role for this receptor in the development of the systemic autoimmune parameters, as well as in the local leukocyte infiltrates, especially in the kidney, the major target organ in lupus. Consistent with a role of Ccr2 in the general immune response, Ccr2$^{-/-}$ MRL/lpr mice developed less lymphadenopathy and had a lower percentage of CD4$^+$ and CD8$^+$ peripheral T lymphocytes (Figure 10).

In keeping with an important role for Ccr2 in the infiltration of T cells and macrophages in the kidney, Ccr2-deficient MRL/lpr mice showed reduced glomerular and tubulointerstitial CD3$^+$ T cell and ER-HR3$^+$ and Mac2$^+$ macrophage infiltrates and less glomerular cell proliferation, with a corresponding reduction in proteinuria and renal disease. This occurred despite unchanged hyperimmunoglobulinemia and comparable glomerular immune complex and complement deposits. There was, however, a reduction in plasma anti-dsDNA antibody titers at 20 wk of age. Finally, these changes were associated with a prolongation of overall survival of Ccr2$^{-/-}$ MRL/lpr mice as compared with Ccr2$^{+/+}$ MRL/lpr controls. These effects cannot be attributed to differences in genetic background between the Ccr2$^{+/+}$ and Ccr2$^{-/-}$ MRL/lpr mice, as the knockout strain had been backcrossed for seven generations and therefore represents an incipient congenic strain. Our re-
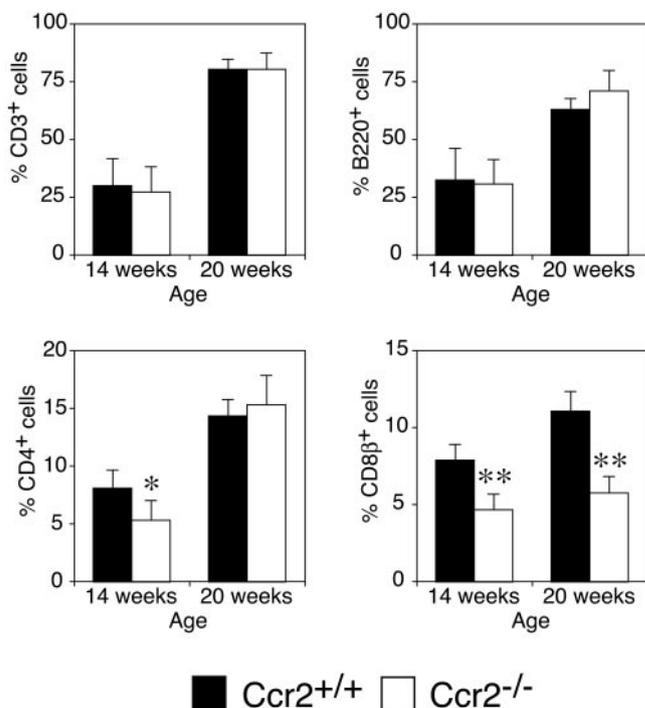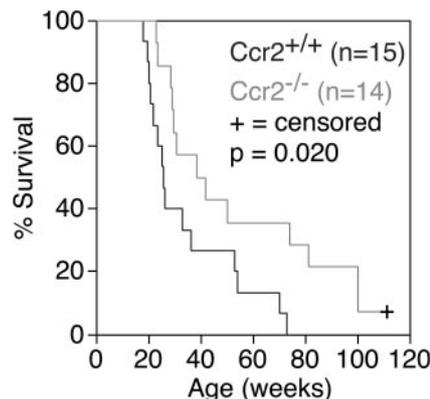


*Figure 11.* Effect of Ccr2 deficiency on survival. Kaplan-Meier plot showing the survival curves for wt and Ccr2-deficient MRL/lpr mice. One Ccr2-deficient mouse is still alive (August 12, 2005) and 111 wk old.

sults support roles for Ccr2 in the development of the autoimmune disease, especially the generation of activated T cells and anti-dsDNA antibody production, and in the development of renal injury, thereby influencing disease progression and survival of MRL/lpr mice.

The phenotypes of Ccr2-deficient mice in diverse disease models have been summarized recently (15). Depending on the model used, the disease parameters and the outcome in Ccr2$^{-/-}$ mice were worse (24), unchanged (25), or improved (26). With some

exceptions, autoimmune (27–29) and inflammatory disease models (30) were generally improved in $Ccr2^{-/-}$ mice, whereas most infectious models had a more severe outcome (31,32). Overall, these diverse studies support a role for Ccr2 in leukocyte recruitment to the site of immune injury.

A potential shift of the Th1–Th2 response in $Ccr2^{-/-}$ mice has been discussed, but, depending on the model used, conflicting results have been obtained (11,32–35). On the basis of the lack of difference in the Ig isotypes in $Ccr2^{-/-}$ MRL/lpr mice compared with those in the $Ccr2^{+/+}$ MRL/lpr mice, our data seem not to support a major shift in Th1–Th2 balance in this model. Furthermore, IFN-$\gamma$ mRNA levels were unchanged in spleens from wt and Ccr2-deficient mice at 20 wk. We did observe, however, decreased IFN-$\gamma$ mRNA levels in kidneys from Ccr2-deficient mice, which would indicate a defect in Th1 response, as previously reported for $Ccr2^{-/-}$ mice (11). The reduced IFN-$\gamma$ mRNA levels in the kidneys of $Ccr2^{-/-}$ MRL/lpr mice that were observed by us are consistent with the results of Tesch *et al.* (5) in Ccl2-deficient MRL/lpr mice and of Hasegawa *et al.* (8) obtained with a Ccl2 antagonist in MRL/lpr mice. IFN-$\gamma$ plays a major role in the development of the renal disease in MRL/lpr mice, as demonstrated by marked improvement of disease parameters in IFN-$\gamma$–deficient MRL/lpr mice (36,37). Infiltrating T cells and macrophages can be the source of the IFN-$\gamma$ (38,39). Because there was also less inflammatory infiltrate in the kidneys, we cannot differentiate between attributing the lower renal IFN-$\gamma$ to fewer IFN-$\gamma$–producing cells in the kidney and/or less IFN-$\gamma$ produced per infiltrating macrophage. The latter possibility is less likely, however, because Peters *et al.* (40) could show that the reduced IFN-$\gamma$ levels that were observed in Ccr2-deficient mice were caused by an *in vivo* trafficking defect of IFN-$\gamma$–producing cells and not by diminished production. Furthermore, we observed comparable IFN-$\gamma$ mRNA levels in spleens from both groups. Similar considerations apply to the lower mRNA levels for TNF. Irrespective of the exact mechanism, the lower IFN-$\gamma$ levels in the kidney could contribute to the relative protection of the kidney to the development of immune nephritis.

The reduced lymphadenopathy noted in the Ccr2-deficient MRL/lpr mice is comparable to data obtained in Ccl2-deficient MRL/lpr mice (5). The reason for the attenuation—but not normalization—of the size of the lymphatic organs in both the $Ccr2^{-/-}$ and $Ccl2^{-/-}$ MRL/lpr mice remains speculative but possibly could relate to Ccl2- and Ccr2-mediated cell migration and sequestration in lymph nodes and spleen (41) or Ccl2-dependent apoptosis of bone marrow precursor cells as reported by Reid *et al.* (42) in $Ccr2^{-/-}$ mice.

The reduction in size of the lymphatic organs in the $Ccr2^{-/-}$ MRL/lpr mice was associated with a reduction of circulating $CD8^+$ T cells in the peripheral blood, consistent with the reported role of Ccr2 in T cell migration and the enhanced expression of Ccr2 on activated $CD8^+$ T cells (43). The lower number of $CD8^+$ T cells that we observed in the blood of $Ccr2^{-/-}$ MRL/lpr mice is consistent with the recent observation of fewer CD62low CD44high $CD8^+$ T cells in the spleen of $Ccr2^{-/-}$ mice that received islet transplants (44). The reduction in $CD8^+$ T cells observed by us in the blood of Ccr2-deficient

MRL/lpr mice and by Abdi *et al.* (44) in the spleens of mice that received transplants suggests a regulatory role of Ccr2 in the activation of $CD8^+$ T cells during the immune-mediated disease. A previous report from our group has shown that in peripheral blood of BALB/c mice with apoferritin-induced glomerulonephritis, only 4% of the $CD4^+$ and 3% of the $CD8^+$ T cells expressed Ccr2, but 63 and 30% of these cell types that were isolated from inflamed kidneys were positive for Ccr2 (21). In the context of the MRL/lpr mouse, our finding of reduced peripheral $CD8^+$ T cells in $Ccr2^{-/-}$ MRL/lpr mice potentially could point toward a contributory role of T cells in the development of nephritis (45,46).

Despite the reduction in lymphadenopathy in either the $Ccr2^{-/-}$ (these data) or the $Ccl2^{-/-}$ MRL/lpr mice (5), the polyclonal hyperimmunoglobulinemia persisted in both models. Thus, the autoreactive B cell expansion of the MRL/lpr mouse seems to be independent of Ccr2 or its ligand. Consistent with the unchanged circulating antibody levels, the glomerular deposition of Ig and complement were unchanged by Ccr2 deficiency in the MRL/lpr mice. Together, these results add to the growing number of studies in MRL/lpr mice showing that local immune complex deposition and complement activation alone seem to be insufficient to induce a full-blown glomerular inflammatory response. This has been observed with elimination of the receptor for the Ig FcR (47); for the cytokines IFN-$\gamma$ (37), IL-12 (48), and CSF-1 (49); and for the chemokine Ccl2 (5,8) or the respective receptors, *e.g.*, Ifngr1 (50) or Ccr2 (this report).

The mitigation of the immune-mediated renal disease may be a reason for the improved survival. As previously reported by others and us, glomerular chemokine production follows immune complex deposition and precedes the leukocyte infiltration, which in turn precedes the development of proteinuria (5,6). At 20 wk of age, $Ccr2^{-/-}$ MRL/lpr mice showed reduced numbers of infiltrating $ER$-$HR3^+$ macrophages as well as $CD3^+$ T cells in the glomerular and tubulointerstitial compartments. As expected, this was associated with significantly lower histopathologic scores. Similar results have been reported by Hasegawa *et al.* (8) using a Ccl2 antagonist and by Tesch *et al.* (5) using $Ccl2^{-/-}$ MRL/lpr mice, with the exception that in the latter study, no reduction in glomerular T cell infiltrate was observed. The difference in glomerular T cell infiltrate between the $Ccr2^{-/-}$ and the $Ccl2^{-/-}$ mice could be related to the reduced peripheral $CD8^+$ T cells noted in the $Ccr2^{-/-}$ mice and to the high percentage of $Ccr2^+$ T cells infiltrating kidneys with immune complex disease as previously reported by us (21). Furthermore, the diminished T cell infiltration may not necessarily reflect a direct role for Ccr2 in T cell migration but could also be a consequence of the reduced macrophage infiltration leading to less secondary T cell recruitment (46). The reduced Ccr1, Ccr3, and Ccr5 mRNA levels in kidneys of $Ccr2^{-/-}$ MRL/lpr mice may simply reflect the decrease in number of infiltrating macrophages and T cells that express—in addition to Ccr2—Ccr1, Ccr3, or Ccr5 (21). Taken together, the ligand-receptor pair Ccl2 and Ccr2 seems to play a significant role in glomerular and in tubulointerstitial macrophage and T

cell infiltration in MRL/lpr lupus nephritis and contribute to its progression toward terminal renal insufficiency.

Surprisingly, the reduced renal pathology of the $Ccr2^{-/-}$ MRL/lpr mice at 20 wk of age was not consistently associated with a reduction in BUN levels or proteinuria. In contrast, the mild proteinuria at 14 wk of age was significantly reduced in Ccr2-deficient mice, but this did not persist as proteinuria progressed. The reasons for this discrepancy between the improved histopathology and the unchanged proteinuria remain unclear.

In conclusion, our results in Ccr2-deficient MRL/lpr mice suggest a role for this receptor in the development of lupus-like disease manifestations consisting of lymphoproliferation, activation of T cells, generation of anti-dsDNA antibodies, renal disease with chemokine and cytokine production, and mononuclear cell infiltration, resulting in proteinuria and progressive renal insufficiency. The reduction in the development of the lupus-like disease in the $Ccr2^{-/-}$ MRL/lpr mice leads to prolongation of life, pointing toward a role for Ccr2 in the development and progression in immune complex–mediated renal disease.

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