Spiegelmer Inhibition of CCL2/MCP-1 Ameliorates Lupus Nephritis in MRL-(Fas)lpr Mice

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ABSTRACT
The monocyte chemoattractant protein CCL2 is crucial for monocyte and T cell recruitment from the vascular to the extravascular compartment at sites of inflammation. CCL2 is expressed in human lupus nephritis and was shown to mediate experimental lupus; therefore, CCL2 antagonists may be beneficial for therapy. This study describes the L-enantiomeric RNA oligonucleotide mNOX-E36, a so-called Spiegelmer that binds murine CCL2 with high affinity and neutralizes its action in vitro and in vivo. The mirror image configuration of the Spiegelmer confers nuclease resistance and thus excellent biostability. mNOX-E36 does not induce type I IFN via Toll-like receptor-7 or cytosolic RNA receptors, as recently shown for certain synthetic D-RNA. Autoimmune-prone MRL<sup>pr/pr</sup> mice that were treated with a polyethylene glycol form of mNOX-E36 from weeks 14 to 24 of age showed prolonged survival associated with a robust improvement of lupus nephritis, peribronchial inflammation, and lupus-like inflammatory skin lesions. Thus, mNOX-E36–based inhibition of CCL2 represents a novel strategy for the treatment of autoimmune tissue injury, such as lupus nephritis.


Blocking proinflammatory mediators has become a successful approach for the treatment of chronic inflammation. In addition to TNF and IL, CC-chemokines are important candidates for specific antagonism because CC-chemokines mediate leukocyte recruitment from the intravascular space to sites of inflammation. There is strong evidence that CCL2, formerly known as monocyte chemoattractant protein-1, and its respective chemokine receptor CCR2 have a crucial role in autoimmune tissue injury such as the clinical manifestations of systemic lupus erythematosus. For example, MRL<sup>pr/pr</sup> mice deficient either for the Ccl2 or the Ccr2 gene are protected from lupus-like disease manifestations. Hence, the CCL2/CCR2 axis may represent a promising therapeutic target (e.g., for lupus nephritis). In fact, delayed gene therapy or transfer of transfected cells, both resulting in in situ production of an NH<sub>2</sub>-truncated CCL2, markedly reduced autoimmune tissue injury in MRL<sup>pr/pr</sup> mice. However, such experimental approaches cannot be used in humans because of irrepressible antagonist production and tumor formation.

The biologic functions of proteins can be blocked by aptamers (three-dimensional nucleic acid structures that can be designed to bind to target molecules conceptually similar to antibodies). However, the therapeutic use of oligonucleotide-based antagonists is limited by their susceptibility to nuclease digestion resulting in low biostability. Spiegelmers represent a new class of nuclease-resistant RNA oligonucleotides. Spiegelmers are L-enantiomeric aptamers in which the mirror-image configuration of the Spiegelmer confers nuclease resistance and thus excellent biostability.
Configuration of the ribonucleotides prevents nuclease degradation. Hence, Spiegelmers show excellent biostability without any further chemical modifications, which renders them very well suited for in vitro and in vivo applications. We therefore hypothesized that Spiegelmer-based blockade of CCL2 would be suitable for the treatment of lupus nephritis and other disease manifestations of systemic lupus erythematosus. Here we report the identification of the Spiegelmer mNOX-E36 that specifically inhibits murine CCL2 (mCCL2) in vitro in the absence of type I IFN induction in dendritic cells, recently described for natural and synthetic RNA. We further show that late onset (i.e., nonprophylactic therapy) with CCL2 Spiegelmer effectively improves lupus nephritis, autoimmune peribronchitis, and lupus-like skin disease in MRL1pr/lpr mice, independent of any previous problem associated with therapeutic CCL2/CCR2 blockade such as tumor formation or irrepressible antagonist production in situ.

RESULTS

Identification and Characterization of mNOX-E36
High-affinity aptamers to D-mCCL2 were identified after 11 rounds of in vitro selection. Alignment of the aptamers revealed one family of relatives that differed by point mutations. As determined by comparative binding analysis, the most prevalent sequence was also the best binding molecule. Sequential deletions from the 5' and 3' ends, respectively, enabled the truncation to the final candidate mNOX-E36. A secondary structure prediction using the software mfold proposes three helical regions, as well as three bulges and a large central loop spanning positions 18 to 27 (Figure 1A). The dissociation constant of D-mNOX-E36 and biotinylated D-mCCL2 (1 to 76) was determined in a pull-down assay at 37°C and calculated to 157 ± 48 pM (Figure 1B). Migration of mCCL2-stimulated THP-1 cells was functionally prevented by mNOX-E36 with an IC50 of approximately 3 nM (Figure 1C). Whereas the polyethylene glycol (PEG)ylated molecule mNOX-E36–3PEG had very similar inhibitory efficiency, the control Spiegelmer was not inhibitory up to a concentration of 1 μM (data not shown).

Pharmacokinetics of Spiegelmer Plasma Levels in MRL1pr/lpr Mice
For monitoring drug exposure in MRL1pr/lpr mice, Spiegelmer plasma levels were determined at weekly intervals. The median plasma levels of mNOX-E36 and mNOX-E36–3PEG were approximately 300 nM and 1 μM throughout the study, respectively (Figure 3). Thus, PEGylation increased the plasma levels of mNOX-E36 and the progressive kidney disease of MRL1pr/lpr mice did not modulate the pharmacokinetics of both Spiegelmers. In addition, neither drug accumulation nor metabolic induction or reduction was obvious.

Anti-CCL2 Spiegelmer Blocks the Emigration of Monocytes from the Bone Marrow
Monocyte emigration from bone marrow during bacterial infection was shown to involve chemokine receptor CCR2, but the role of CCR2 in the context of autoimmunity remains hypothetical. To verify the effect of CCL2 blockade on monocyte migration with Spiegelmer treatment, we performed a single-dose study of the anti-CCL2 Spiegelmer (50 mg/kg), control Spiegelmer (Co, 50 mg/kg), and 5% glucose (control). Anti-CCL2 Spiegelmer significantly reduced the percentage of the circulating CCR2-positive cells 6 h after injection, compared with the vehicle-treated group.
By contrast, the control Spiegelmer had no effect (Figure 4A).

Furthermore, we examined the CCR2-positive monocyte population in peripheral blood and bone marrow in 24-wk-old MRL\(^{pr/pr}\) mice that had been treated with anti-CCL2 Spiegelmer or saline from weeks 14 to 24 of age. Anti-CCL2 Spiegelmer increased CCR2-positive cells from 13 to 26% in the bone marrow and reduced this population from 26 to 11% in the peripheral blood, respectively (Figure 4B). These data support a novel role for CCL2 for the evasion of CCR2-positive cells from the bone marrow during autoimmune disease of MRL\(^{pr/pr}\) mice.

**Anti-CCL2 Spiegelmer Improves Survival and Kidney Disease of MRL\(^{pr/pr}\) Mice**

Female MRL\(^{pr/pr}\) mice develop and subsequently die from proliferative immune complex glomerulonephritis with striking similarities to diffuse proliferative lupus nephritis in humans. We treated MRL\(^{pr/pr}\) mice with anti-CCL2 Spiegelmers, nonfunctional Co-Spiegelmers (Co-/Co-PEG), or vehicle from weeks 14 to 24 of age, thus using a therapeutic dosage regimen. In fact, at 14 wk of age, MRL\(^{pr/pr}\) mice revealed proliferative glomerulonephritis with an activity index of 4.1 \(\pm\) 1.1 and a chronicity index of 0.1 \(\pm\) 0.2 (data not shown). At this age, major abnormalities of the tubulointerstitial compartment could not be detected. After 10 wk of treatment, vehicle-, Co-, or Co-PEG–treated MRL\(^{pr/pr}\) mice showed diffuse proliferative glomerulonephritis characterized by glomerular macrophage infiltration and a mixed periglomerular and interstitial inflammatory cell infiltrate consisting of glomerular and interstitial Mac2-positive macrophages and interstitial CD3-positive lymphocytes (Figure 5A, Table 1).

Anti-CCL2 Spiegelmer improved the activity and chronicity index of lupus nephritis as well as the aforementioned markers of renal inflammation (Table 1). Non-PEGylated anti-CCL2 Spiegelmer was less effective on the chronicity index and interstitial macrophage and T cell counts (Table 1). Advanced chronic kidney disease was further illustrated by tubular atrophy and confluent areas of interstitial fibrosis in vehicle- and Co-Spiegelmer-treated mice (Figure 5A). We used morphometry to quantify these changes and found that PEGylated and non-PEGylated anti-CCL2 Spiegelmers reduced interstitial volume, tubular cell damage, and tubular dilation, all being markers of the severity and prognosis of chronic kidney disease.
These histomorphologic markers of lupus nephritis were associated with significant mortality and marked albuminuria at 24 wk of age (Figure 6A, Table 1). In fact, PEGylated but not non-PEGylated anti-CCL2 Spiegelmers improved the parameter 50% mortality. Thus, anti-CCL2 Spiegelmers treatment reduced the number of renal macrophage and T cell infiltrates, improved the symptoms of lupus nephritis, and increased (renal) survival of MRL<sup>lpr/lpr</sup> mice. To study whether treatment with PEGylated or non-PEGylated anti-CCL2 Spiegelmer affects intrarenal inflammation in MRL<sup>lpr/lpr</sup> mice, we performed real-time reverse transcriptase–PCR for the proinflammatory chemokines CCL2 and CCL5 which were previously shown to be progressively upregulated in kidneys of MRL<sup>lpr/lpr</sup> mice during progression of renal disease.<sup>22</sup> Treatment with both anti-CCL2 Spiegelmers reduced renal expression of CCL2 and CCL5 mRNA compared with vehicle-treated controls (Figure 6B).

### Anti-CCL2 Spiegelmers Reduce Extrarenal Autoimmune Tissue Injury in MRL<sup>lpr/lpr</sup> Mice

Skin and lungs are also commonly affected from autoimmune tissue injury in MRL<sup>lpr/lpr</sup> mice. In vehicle-treated mice, autoimmune lung disease was characterized by moderate peribronchiolar and perivascular inflammatory cell infiltrates, and skin lesions were observed in 60% of mice (Figure 7, Table 1). Compared with vehicle- and Co-Spiegelmer-treated MRL<sup>lpr/lpr</sup> mice, administrations of both anti-CCL2 Spiegelmers reduced peribronchial inflammation and skin disease (Figure 7, Table 1). Hence, the effects of CCL2-specific Spiegelmers are not limited to lupus nephritis but extend to other manifestations of autoimmune tissue injury in MRL<sup>lpr/lpr</sup> mice.

### Anti-CCL2 Spiegelmers and the Lymphoproliferative Syndrome and dsDNA Autoantibodies in MRL<sup>lpr/lpr</sup> Mice

Female MRL<sup>lpr/lpr</sup> mice are known to develop a lymphoproliferative syndrome characterized by massive splenomegaly and bulks of cervical, axillary, inguinal, and mesenterial lymph nodes. Both anti-CCL2 Spiegelmers had no effect on the weight of spleens and lymph nodes in MRL<sup>lpr/lpr</sup> mice (Table 2). Autoimmunity in MRL<sup>lpr/lpr</sup> mice is characterized by the production of autoantibodies against multiple nuclear antigens including dsDNA. In 24-wk-old MRL<sup>lpr/lpr</sup> mice, serum dsDNA IgG, IgG<sub>1</sub>, IgG<sub>2a</sub>, and IgG<sub>2b</sub> autoantibodies were present at high levels. Both anti-CCL2 Spiegelmers both had no effect on either of these DNA autoantibodies (Table 2). Thus, anti-CCL2 Spiegelmers do not affect lymphoproliferation and anti-dsDNA IgG production in MRL<sup>lpr/lpr</sup> mice.

**DISCUSSION**

Aptamers can neutralize biologic functions of target molecules conceptually similar to antibodies. Unlike aptamers,
Table 1. Renal function and histologic findings in MRL<sup>lpr/lpr</sup> mice<sup>a</sup>

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Vehicle</th>
<th>Control Spiegelmer</th>
<th>Anti–CCL2 Spiegelmer</th>
<th>PEGylated Control Spiegelmer</th>
<th>PEGylated Anti–CCL2 Spiegelmer</th>
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<tr>
<td>Renal disease</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>U alb/creat (µg/mg)</td>
<td>15.6 ± 10.1</td>
<td>4.3 ± 1.4</td>
<td>6.8 ± 1.8</td>
<td>3.8 ± 0.5</td>
<td>2.4 ± 0.7</td>
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<td>IgG&lt;sub&gt;1&lt;/sub&gt; (glomerular score)</td>
<td>1.5 ± 0.4</td>
<td>1.6 ± 0.2</td>
<td>1.0 ± 0.5</td>
<td>1.7 ± 0.2</td>
<td>1.7 ± 0.5</td>
</tr>
<tr>
<td>IgG&lt;sub&gt;2a&lt;/sub&gt; (glomerular score)</td>
<td>0.9 ± 0.3</td>
<td>1.0 ± 0.3</td>
<td>1.0 ± 0.3</td>
<td>1.1 ± 0.3</td>
<td>1.0 ± 0.3</td>
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<tr>
<td>activity index (score)</td>
<td>17.4 ± 4.9</td>
<td>17.8 ± 4.2</td>
<td>10.3 ± 5.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>17.4 ± 2.7</td>
<td>9.4 ± 4.2&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>chronicity index (score)</td>
<td>6.0 ± 2.0</td>
<td>7.2 ± 2.6</td>
<td>2.6 ± 2.5</td>
<td>5.4 ± 1.0</td>
<td>1.6 ± 1.8&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>Mac&lt;sup&gt;2+&lt;/sup&gt; (cells/glomerulus)</td>
<td>13.4 ± 2.0</td>
<td>12.6 ± 0.9</td>
<td>8.5 ± 2.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>13.6 ± 2.3</td>
<td>8.2 ± 3.5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Mac&lt;sup&gt;2+&lt;/sup&gt; (cells/hpf)</td>
<td>20.3 ± 8.1</td>
<td>20.6 ± 6.7</td>
<td>10.8 ± 5.1</td>
<td>19.3 ± 3.7</td>
<td>7.7 ± 4.0&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>CD3&lt;sup&gt;&lt;/sup&gt; (cells/hpf)</td>
<td>44.6 ± 14.7</td>
<td>39.4 ± 7.5</td>
<td>23.8 ± 10.2</td>
<td>36.2 ± 3.1</td>
<td>19.0 ± 8.0&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Lung injury (score)</td>
<td>1.6 ± 0.8</td>
<td>1.6 ± 0.4</td>
<td>0.6 ± 0.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.4 ± 0.4</td>
<td>0.4 ± 0.5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Skin lesions (% of mice)</td>
<td>60</td>
<td>60</td>
<td>28</td>
<td>80</td>
<td>28</td>
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<sup>a</sup>Data are means ± SEM. Hpf, high-powered field; PEG, polyethylene glycol; U alb/creat, urinary albumin/creatinine ratio.

<sup>b</sup>P < 0.05 anti–CCL2-Spiegelmer versus respective control Spiegelmer.

Figure 6. Survival and renal chemokine expression of MRL<sup>lpr/lpr</sup> mice with experimental lupus. (A) Survival of mice of the various treatment groups was calculated by Kaplan-Meier analysis. (B) Renal mRNA expression for the CC-chemokines CCL2 and CCL5 was determined by real-time reverse transcriptase–PCR using total renal RNA pooled from five mice of each group. RNA levels for each group of mice are expressed per respective 18s rRNA expression. Co, control Spiegelmer.

Spiegelmer-based oligonucleotides are nuclease resistant and thus biostable without further modifications,<sup>11</sup> which makes them very well suited for in vivo applications. We generated the Spiegelmer mNOX-E36, which binds with high affinity to murine CCL2 and blocks its function in vitro at low nanomolar concentrations. The PEGylated form of mNOX-E36 displayed higher plasma levels compared with the non-PEGylated mNOX-E36, but progressive renal failure in MRL<sup>lpr/lpr</sup> mice did not affect their plasma levels.

Synthetic oligonucleotides may induce IFN-α in dendritic cells<sup>17,18</sup> through innate pattern recognition receptors in cytosolic or endosomal compartments such as Toll-like receptor-7, retinoic acid-inducible protein-1, or melanoma differentiation-associated gene-5.<sup>16–19,23</sup> IFN-α is an important mediator of autoimmunity.<sup>24,25</sup> The induction of IFN-α would hamper the use of mNOX-36 in lupus nephritis. However, Spiegelmers did not induce IFN-α production in dendritic cells, because not recognized by RNA-specific pattern recognition receptors.

Evidence for a pathogenic role of the CCL2/CCR2 axis in lupus-associated tissue injury already exists. CCL2- and CCR2-deficient MRL<sup>lpr/lpr</sup> mice are protected from kidney, lung, and skin pathology.<sup>5,6</sup> Thus, CCL2 and CCR2 both are crucial for the recruitment of cytokine-producing leukocytes to peripheral tissues. Our data demonstrate that even non-prophylactic late-onset treatment with anti-CCR2 Spiegelmer at 14 wk of age—a point at which autoimmune tissue injury is already established<sup>5,6</sup>—is effective to the same extent as CCL2 or CCR2 deficiency, although such knockout animals lack CCL2/CCR2 from birth.

The consistency of the data from CCL/CCR2 deficiency and therapeutic CCL2 blockade may relate to the predominant role of the CCL2/CCR2 axis during the late leukocyte recruitment–dependent autoimmune tissue injury rather than the early development of autoimmunity in MRL<sup>lpr/lpr</sup> mice. In fact, both CCL2-deficient and anti-CCR2 Spiegelmer–treated MRL<sup>lpr/lpr</sup> mice did not affect the lymphoproliferative syndrome, DNA autoantibodies, and renal immune complex deposition, all being early disease markers.<sup>6</sup> Our findings are consistent with other studies that initiated blockade of the CCL2/CCR2 axis after onset of autoimmunity. Hasegawa et al.<sup>7</sup> injected 5 × 10<sup>6</sup> cells that were...
transfected with an NH₂-truncated CCL2 analog subcutaneously into 12-wk-old female MRLlpr/lpr mice which was associated with an improved pathology at 20 wk. However, the cell transfer approach caused local tumors of considerable size. Similar results were obtained by Shimizu et al. who injected 7ND, a NH₂-terminal deletion mutant of the Ccl2 gene, into skeletal muscles of 16-wk-old female MRLlpr/lpr mice.

Ccl2 blockade is thought to target CCL2-dependent leukocyte adhesion to activated endothelia and transendothelial migration of leukocytes at sites of inflammation. A recent study found that CCR2 is also required for the evasion of monocytes from the bone marrow into the intravascular compartment during bacterial infection. Our finding that CCL2 blockade has a similar effect on monocyte homing to the bone marrow during systemic autoimmunity extends this finding two-fold: First, monocyte homing to the bone marrow involves CCL2, possibly acting through CCR2, and second, this mechanism seems to apply to systemic inflammation caused by infection and autoimmunity.

The Spiegelmer-based inhibition of CCL2/monocyte-chemoattractant protein-1 offers a new and promising way to treat lupus nephritis. The compound mNOX-E36 not only shows efficacy in MRLlpr/lpr mice but also demonstrates its suitability in terms of pharmacokinetic profile as well as its absence of immunostimulatory adverse effects.

**CONCISE METHODS**

Peptides and Nucleic Acids

Recombinant mCCL2 was obtained from R&D Systems (Wiesbaden, Germany). C-terminally biotinylated murine all-D-CCL2 (1 to 76; QP-DAVNAPLT CCYSFTSKMI PMSRLESYKR ITSSRCPKEA VVFVT- KLKRE VCADPKKEWV QTYIKNLDRN QMRSEP) was custom synthesized by Bachem (Bubendorf, Switzerland). Oligonucleotides were synthesized at NOXXON by using standard phosphoramidite chemistry. L-Phosphoramidites were from ChemGenes Corp. (Wilmington, MA). The DNA library containing 40 internal random positions had the sequence 5′/H11032-GAATTCTAAT ACGACTCACT ATAGGAAGAG ATGGCGAC-N40-GCCATTCGAA CCGTG-3′/H11032 and was amplified with the forward primer 5′/H11032-GAATTCTAAT ACGACTCACT ATAGGAAGAG ATGGCGAC-3′/H11032 and the reverse primer 5′/H11032-CACGGTTCGAATGGC-3′/H11032.

For in vivo application, the mCCL2-binding Spiegelmer mNOX-E36 (5′-GGCGACAUUG GUUGGGCAUG AGGCGAGGCC CUUUGAUGAAUCCGCGGCCA-3′/H11032) and the nonfunctional control Spiegelmer PoC (5′-UAAGGAAACU CGGUCUGAUG CGGUAGCGCU GUGCA-GAGCU-3′/H11032) were used nonmodified or modified with 40-kD PEG at the 3′ and 5′ terminus, respectively. The PoC Spiegelmer is representative for most Spiegelmers identified at NOXXON to date, and its size completely matches the human-specific anti-CCL2 Spiegelmer NOX-E36, which is intended for clinical development. In vivo doses of anti-CCL2 Spiegelmer in mg/kg apply to the combined total weight of the oligonucleotide part plus the coupled PEG moiety.

**Spiegelmer In Vitro Selection and Truncation**

With the use of a library of approximately 6 × 10¹⁴ RNA molecules, Spiegelmers to murine d-CCL2 were identified after 11 rounds of in vitro selection. The best binding RNA sequence was truncated to give the final candidate mNOX-E36 5′-GGGCGACAUUGGUUGGCGCACUGAGCGAGGCCCUUUGUAUGAAUCCCGGCCA-3′.
Determination of Binding Affinities and Inhibition of mCCL2-Induced Chemotaxis In Vitro
The affinity of Spiegelmers to murine δ-CCL2 was measured in a pull-down assay format at 37°C using a constant amount of radioactively labeled Spiegelmer and a dilution series of biotinylated δ-CCL2. The percentage of binding was plotted against the concentration of biotinylated murine δ-CCL2, and dissociation constants were calculated assuming a 1:1 stoichiometry (GRAFIT; Erithacus Software, Surrey, UK).

The inhibitory activity of mNOX-E36 on CCR2 activation in cultured cells was determined by measurement of mCCL2-stimulated chemotaxis using THP-1 human acute monocytic leukemia cells (DSMZ, Braunschweig, Germany) that express the CCL2 receptor CCR2. The migration of THP-1 cells was stimulated with 0.5 nM mCCL2 (R&D Systems) in the presence of increasing mNOX-E36 concentrations. Overnight-grown THP-1 cells (3 x 10^5) were trans-ferred to each insert of a transwell plate that contained 600 μl of stimulation medium. A total of 100 μl of δ-CCL2 (0.5 μM) was added to a dilution series of biotinylated Spiegelmer and a dilution series of biotinylated δ-CCL2. The percentage of binding was plotted against the concentration of biotinylated δ-CCL2, and dissociation constants were calculated assuming a 1:1 stoichiometry (GRAFIT; Erithacus Software, Surrey, UK).

Immunostimulatory Effects of Spiegelmers In Vitro
pDC were generated by incubation with Flt-3 ligand from bone marrow–derived dendritic cells of MRL1pr/pr mice as described. pDC were incubated with 10 μg of various types of synthetic RNA in 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP; Roche, Mannheim, Germany; i.e., δ- and l-enantio-meric phosphorothioates RNA40, RNA41, and RNA42, respecti-vely27), as well as various concentrations of the Spiegelmers in DOTAP for 24 h. Supernatants were analyzed for IFN-α by ELISA (PBL Biomedical Labs, Piscataway, NJ).

Animals and Experimental Protocol
Seven-week-old female MRL1pr/pr mice were obtained from Harlan Winkelmann (Borchen, Germany) and kept under normal housing conditions in a 12-h light and dark cycle. Water and standard chow (Ssniff, Soest, Germany) were available ad libitum. At an age of 14 wk, 10 mice were killed for determination of the extent of renal injury at the time of starting the intervention. Additional groups of 12 mice each received subcutaneous injections of Spiegelmers of 4 ml/kg in 5% glucose as vehicle three times per week: mNOX-E36, 25 mg/kg; mNOX-E36–3’PEG, 50 mg/kg; non-functional control Spiegelmer PoC, 25 mg/kg; PoC-PEG, 50 mg/kg; or vehicle. The plasma levels of mNOX-E36 and mNOX-E36–3’PEG were determined weekly 3 or 24 h after injection as described previously. All experiments were approved by the local government authorities.

Evaluation of Systemic Lupus
Skin lesions were recorded by a semiquantitative score. Urine albumin/creatinine ratios and serum dsDNA autoantibody IgG isotype titers were determined as described previously. Paraffin sections (5 μm) for silver and periodic acid-Schiff stains were prepared following routine protocols. The severity of the renal lesions was graded using the indices for activity and chronicity as described for human lupus nephritis, and morphometry of renal interstitial injury was conducted as described previously. The severity of the peribronchial inflammation was graded semiquan-titatively from 0 to 4. Immunostaining was performed as described previously. The following primary antibodies were used: Rat anti-Mac2 (macrophages; Cederlane; Hornby, ON, Canada; 1:50), anti-mouse IgG1 (1:100, M32215; Caltag). Glomerular Ig deposits were counted in 15 cortical glomeruli per section. Glomerular IgG deposition was scored from 0 to 3 on 15 glomerular sections.

RNA Preparation and Real-Time Quantitative (TaqMan) Reverse Transcriptase–PCR
Renal tissue from each mouse was snap-frozen in liquid nitrogen and stored at −80°C. From each mouse, total renal RNA preparation and reverse transcription were performed as described previously. Primers and probes were from PE Biosystems (Weiterstadt, Germany). Oligonucleotide primer (300 nM) and probes (100 nM) were used as listed in Table 3.

Flow Cytometry
Flow cytometry was performed using a FACSCalibur machine and the previously characterized MC21 anti-mCCR2 antibody. A biotinyl-
ated anti-rat IgG antibody (BD Biosciences) was used for detection. A rat IgG2b (BD Biosciences) was used as isotype control.

Statistical Analyses

Data were expressed as means ± SEM. Comparison between groups were performed using univariate ANOVA (post hoc Bonferroni correction was used for multiple comparisons) and t test. P < 0.05 indicated statistical significance.

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DISCLOSURES

W.P., D.E., N.S., K.B., and S.K. are employees of NOXXON Pharma AG. H.J.A. received consultancy fees from NOXXON Pharma AG.

REFERENCES


Table 3. Primers and probes used for real-time RT-PCR

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<th>Gene Target</th>
<th>Sequence</th>
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<tr>
<td>Ccl2</td>
<td>Predeveloped TaqMan assay reagent from PE Biosystems</td>
</tr>
<tr>
<td>Ccl5</td>
<td>Predeveloped TaqMan assay reagent from PE Biosystems</td>
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<td>Ccr1</td>
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