Anti-Ccl2 Spiegelmer Permits 75% Dose Reduction of Cyclophosphamide to Control Diffuse Proliferative Lupus Nephritis and Pneumonitis in MRL-Fas(lpr) Mice

Onkar Kulkarni, Dirk Eulberg, Norma Selve, Stefan Zöllner, Ramanjaneyulu Allam, Rahul D. Pawar, Stephanie Pfeiffer, Stephan Segerer, Sven Klussmann, and Hans-Joachim Anders


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ABSTRACT

Cyclophosphamide (CYC) can control diffuse proliferative lupus nephritis (DPLN) by potent immunosuppression but remains associated with serious and life-threatening complications. Drugs that specifically target mediators of DPLN may help to reduce CYC dose and side effects. Monocyte chemoattractant protein (MCP-1)/CCL2 mediates monocyte and T cell recruitment in DPLN and Ccl2-specific L-enantiomeric RNA Spiegelmer mNOX-E36 neutralizes the biological effects of murine Ccl2 in vitro and in vivo. We injected MRLlpr/lpr mice with DPLN from 14 weeks of age with vehicle, weekly 30 mg/kg CYC (full dose), monthly 30 mg/kg CYC (one-fourth full dose), pegylated control Spiegelmer, pegylated anti-Ccl2 Spiegelmer (3/week), pegylated anti-Ccl2 Spiegelmer plus CYC one-fourth full dose and mycophenolate mofetil. At week 24, DPLN and autoimmune lung injury were virtually abolished with CYC full dose but not with CYC one-fourth full dose. The CYC one-fourth full dose/Spiegelmer combination was equipotential to CYC full dose on kidney and lung injury. CD3+ CD4+ CD8- and CD3+ CD4- CD25+ T cells and serum interleukin-12p40 and tumor necrosis factor-α levels were all markedly affected by CYC full dose but not by CYC one-fourth full dose. No additive effects of anti-Ccl2 Spiegelmer were noted on bone marrow colony-forming unit-granulocyte macrophage counts and 7/4high monocyte counts, lymphoproliferation, and spleen T cell depletion. In summary, anti-Ccl2 Spiegelmer permits 75% dose reduction of CYC for controlling DPLN and pneumonitis in MRL-Fas(lpr) mice, sparing suppressive effects of full-dose CYC on myelosuppression and T cell depletion. We propose anti-Ccl2 Spiegelmer therapy as a novel strategy to reduce CYC toxicity in the treatment of severe lupus.

Control of human diffuse proliferative lupus nephritis (DPLN) requires potent immunosuppression with either cyclophosphamide (CYC) or mycophenolate mofetil (MMF). Each of the two drugs is associated with significant morbidity and mortality. For example, in the Aspreva Lupus Management Study trial, MMF caused serious adverse effects in 27.7% and treatment-related death in 4.9% and CYC in 22.8 and 2.8% of treated patients, respectively (Appel et al., 2007). Most serious adverse effects and deaths were related to infections because of the unspecific immunosuppressive effects of CYC and MMF (Appel et al., 2007). Novel drugs specifically blocking autoimmune inflammation may allow the reduction of the toxicity of current treatment protocols either by replacing CYC and MMF or by allowing significant dose reductions when used in combination.

Cytokine antagonism is a powerful strategy to prevent tissue damage in chronic inflammation (Steinman, 2004). Beyond cytokines and interleukins, CC-chemokines represent potential targets for specific antagonism because CC-chemokines mediate immune cell activation and their recruitment to sites of inflammation (Baggiolini, 1998; Luster et al., 2005). Experimental studies revealed that Ccl2, formerly known as monocyte-chemoattractant protein-1, and its...
chemokine receptor, Ccr2, have crucial roles in autoimmune tissue injury, such as the manifestations of systemic lupus erythematosus (SLE) (Gerard and Rollins, 2001). Ccl2- or Ccr2-deficient MRL<sub>1pr/lpr</sub> mice with experimental SLE are protected from DPLN (Tesch et al., 1999; Pérez de Lema et al., 2005). Based on these studies, several groups have tried to block Ccl2 or Ccr2 with suitable antagonists in mice with experimental SLE. One example is a truncated Ccl2 that exerts inhibitory function. Expressed in situ by delayed gene experimental SLE. One example is a truncated Ccl2 that exerts inhibitory function. Expressed in situ by delayed gene

chemotaxis and a Ca<sup>2+</sup> cell-based assays employed with THP-1 cells, Ccl2-mediated release could be inhibited, with the anti-Ccl2 Spiegelmer displaying half-maximal efficacy in the low single-digit nanomolar range. In vivo studies with anti-Ccl2 Spiegelmer were carried out in female MRL<sub>1pr/lpr</sub> mice. Ccl2 blockade with the anti-Ccl2 Spiegelmer for 10 weeks starting at an age of 14 weeks significantly improved DPLN (Kulkarni et al., 2007). Although the therapeutic effect was clearly evident, it remained unclear how the efficacy of the anti-Ccl2 Spiegelmer would compare with that of CYC or MMF. Based on the specific anti-inflammatory mechanism of Ccl2 blockade, we assumed that treatment with an anti-Ccl2 Spiegelmer would not be as effective as CYC full dose for the treatment of DPLN. However, we hypothesized that therapeutic effects equivalent to CYC full dose might be achieved with a combination of less frequent CYC dosing plus anti-Ccl2 Spiegelmer, which may avoid the toxicity of CYC full dose.

**Materials and Methods**

**Preparation of Anti-Ccl2 Spiegelmer mNOX-E36.** The identification and molecular structure of the anti-Ccl2 Spiegelmer mNOX-E36 have been described previously in detail (Kulkarni et al., 2007). In brief, mNOX-E36 (5'-GGCGACAUUG GUUUGG CAUG AGGCGAGCC CUUUGAUGAA UCCGCGGCA-3') and the control Spiegelmer revmNOX-E36 (5'-ACCGGCGCCU AAGUAGUUUC GCGAGCGGA GUACGGGUUG GUUACAGGCC-3') were synthesized using standard phosphoramidite chemistry. Spiegelmers were modified with a 40-kDa polyethylene glycol moiety at the 3' terminus.

**Animals and Experimental Protocol.** Seven-week-old female MRL<sub>1pr/lpr</sub> mice were obtained from Harlan (Bicester, UK) and kept under normal housing conditions with a 12-h light/dark cycle. Water and standard chow (ssniff Spezialdiäten GmbH, Soest, Germany) were available ad libitum. Mice were grouped in seven different groups. From the age of 14 weeks, groups of 12 mice were injected for 10 weeks as follows: 5% glucose s.c. (vehicle group), 50 mg/kg s.c. (0.89 μmol/kg) revmNOX-E36 (control Spiegelmer), 50 mg/kg s.c. (0.89 μmol/kg) mNOX-E36 (anti-Ccl2 Spiegelmer), 30 mg/kg/4 weeks CYC i.p. (CYC one-fourth full dose), 30 mg/kg/week CYC i.p. (CYC full dose), 50 mg/kg mNOX-E36 plus CYC one-fourth full dose (combination), and 100 mg/kg/day MMF orally (Roche Diagnostics, Mannheim, Germany). All vehicle and Spiegelmer injections were given 3×/week. Plasma levels of mNOX-E36 were determined in samples obtained from the retro-orbital sinus 24 h after injection from Spiegelmer-treated groups on weeks 1, 3, 7, and 10 of treatment. Spiegelmer plasma levels were determined as described previously (Drolet et al., 2000). Blood samples were drawn under isoflurane anesthesia and urine was collected on week 24 from each of the survived mice for further plasma and urine analysis. Mice were sacrificed by cervical dislocation at the end of week 24 of age. All experimental procedures were performed according to the German animal care and ethics legislation and were approved by the local government authorities.

**Evaluation of Systemic Lupus.** The weight ratio of spleen and the bulk of mesenterial lymph nodes to body weight were calculated as markers of the lupus-associated lymphoproliferative syndrome. Urine albumin/creatinine ratio was determined as described previously (Pawar et al., 2006). Plasma IL-12p40 and TNF-α levels were analyzed by ELISA (TNF-α, BioLegend, San Diego, CA; IL-12p40, BD-Pharmingen, San Diego, CA). From all mice, kidneys and lungs were fixed in 10% buffered formalin, processed, and embedded in paraffin. Five-micrometer sections for periodic acid-Schiff stain were prepared following routine protocols (Anders et al., 2002). The severity of the renal lesions was graded using the indices for activity and chronicity as described for human lupus nephritis (Austin et al., 1984). The severity of the peribronchial inflammation was graded semiquantitatively from 0 to 3 by a blinded observer. Immunostaining was performed as described previously (Anders et al., 2002). The following primary antibodies were used: rat anti-Mac2 (macrophages; CEDARLANE Laboratories Ltd., Burlington, ON, Canada; 1:50) and anti-mouse CD3 (1:100, clone 500A2, BD-Pharmingen). Negative controls included incubation with a respective isotype antibody. Positive glomerular cells were counted in 15 cortical glomeruli per section. Intestinal cells were counted by a high-power field. Urinary albumin was assessed by ELISA (Bethyl Laboratories, Montgomery, TX), and urinary creatinine concentrations were determined using an automatic AutoAnalyzer (Integra 800; Roche Diagnostics).

**Evaluation of Monocytopenia and Bone Marrow Colony Forming Unit-Granulocyte Macrophage Counts.** To evaluate therapy induced monocytopenia and myelotoxicity, we treated four groups of mice (n = 5) as follows: 5% glucose s.c., 50 mg/kg/3×/week anti-Ccl2 Spiegelmer s.c., CYC full dose i.p., and combination of CYC one-fourth full dose i.p. and 50 mg/kg/3×/week anti-Ccl2 Spiegelmer s.c. for 4 weeks. After 4 weeks of treatment, blood was drawn from the retro-orbital plexus under isoflurane anesthesia. Mice were sacrificed to get the bone marrow cells. After counting, 3 × 10<sup>6</sup> bone marrow cells derived from each group were cultured with Methocult media (M3132; Stem Cell Technologies, Vancouver, BC, Canada) supplemented with murine granulocyte macrophage–colony-stimulating factor (10 ng/ml) for colony-forming unit (CFU)-granulocyte macrophage (GM) assay as per the instructions in the kit insert. CFU-GM colonies were counted on day 12.

**Flow Cytometry.** Flow cytometry was performed using a FACScan calibur machine and the previously described method for monocyte subsets analysis (Tsou et al., 2007). Blood samples and bone marrow samples were incubated with rat anti-mouse CD16/CD32 (1:500 or 1 μg/ml; BD Biosciences, San Jose, CA) for 30 min to block Fc recep-
tors. Blood samples were incubated for 1 h at 4°C with fluorescently labeled antibodies: Ly-6G-FITC (2.8 μg/ml; BD Biosciences), 7/4-PE (1:10; Serotec, Oxford, UK). Autoreactive T cells and regulatory T cells were identified, from single-cell spleenocytes suspension, by staining with anti-mouse CD3-APC, anti-mouse CD4-PE, anti-mouse CD8-PerCp, and anti-mouse CD25-PerCp (all from BD Phamingen). Cell density in every spleen cell single cell suspension sample was analyzed by using counting beads (Invitrogen, Carlsbad, CA).

Statistical Analysis. Data were expressed as mean ± S.E.M. Comparison between groups were performed using univariate ANOVA and unpaired Student’s t test. Post hoc Bonferroni’s correction was used for multiple comparisons. A value of p < 0.05 was considered to indicate statistical significance.

Results
Pharmacokinetics of Spiegelmer Plasma Levels in MRL<sup>lpr/lpr</sup> Mice. To monitor drug exposure in MRL<sup>lpr/lpr</sup> mice, mNOX-E36 plasma levels were determined in weeks 1, 3, 6, and 10 treatment, i.e., the weeks 15, 18, 21, and 24 of age. Twenty-four hours after administration of 50 mg/kg mNOX-E36, mean plasma levels were approximately 1 μM throughout the study in both of the Spiegelmer-treated groups (Fig. 1A). The progressive kidney disease of MRL<sup>lpr/lpr</sup> mice apparently did not modulate Spiegelmer pharmacokinetics, and neither drug accumulation nor metabolic induction or reduction was detected. Exposure to mNOX-E36 was associated with an increase of serum Ccl2 levels in MRL<sup>lpr/lpr</sup> mice, which was not observed in MRL<sup>lpr/lpr</sup> mice treated with the control Spiegelmer (Fig. 1B). Thus, upon s.c. administration to MRL<sup>lpr/lpr</sup> mice, pharmacologically relevant levels of circulating anti-Ccl2 Spiegelmer, most probably, bind and retain Ccl2 in the circulation.

Add-on Therapy with Anti-Ccl2 Spiegelmers Improves the Effects of CYC One-Fourth Full Dose on Kidney Disease of MRL<sup>lpr/lpr</sup> Mice. Female MRL<sup>lpr/lpr</sup> mice develop proliferative immune complex glomerulonephritis similar to DPLN in humans. We treated MRL<sup>lpr/lpr</sup> mice with CYC, MMF, Spiegelmer, or vehicle from weeks 14 to 24 of age. This represents a therapeutic treatment protocol because at 14 weeks of age, MRL<sup>lpr/lpr</sup> mice showed DPLN with an activity score index of 4.1 ± 1.1. At this age, major abnormalities of the tubulointerstitial compartment were absent (data not shown). After 10 weeks of treatment, vehicle- and control Spiegelmer-treated MRL<sup>lpr/lpr</sup> mice revealed DPLN associated with glomerular hypercellularity, expansion of glomerular matrix, focal tuft necrosis, and a mixed periglomerular and interstitial inflammatory cell infiltrate (Fig. 2). CYC full dose and CYC one-fourth full dose plus anti-Ccl2 Spiegelmer were equally potent in improving the activity and chronicity index of lupus nephritis (Figs. 2 and 3). Furthermore, CYC full dose-treated mice showed 100% survival, and survival rates of CYC one-fourth full dose plus anti-Ccl2 Spiegelmer-treated MRL<sup>lpr/lpr</sup> mice did not significantly differ from those of CYC full-dose-treated MRL<sup>lpr/lpr</sup> mice. Proteinuria as assessed by urinary albumin/creatinine ratios trended lower in these groups compared with vehicle treatment, but this did not reach statistical significance (data not shown). Anti-Ccl2 Spiegelmer and CYC one-fourth full dose alone and MMF were less potent but still significantly improved the activity and chronicity indices of lupus nephritis (Figs. 2 and 3). Thus, adding anti-Ccl2 Spiegelmer to a CYC one-fourth full dose-based regimen is as potent as CYC full-dose therapy for DPLN of MRL<sup>lpr/lpr</sup> mice.

Anti-Ccl2 Spiegelmer and CYC One-Fourth Full Dose Have Additive Effects on the Reduction of Immune Cell Infiltrates in Kidneys of MRL<sup>lpr/lpr</sup> Mice. Immune cell infiltrates contribute to renal damage in lupus nephritis (Vielhauer and Anders, 2006), and Ccl2 mediates the recruitment of T cells and macrophages to MRL<sup>lpr/lpr</sup> mice (Tesch et al., 1999). Therefore, we hypothesized that the additive effects of an anti-Ccl2 Spiegelmer/CYC one-fourth full dose combination may relate to impaired macrophage and T cell recruitment in MRL<sup>lpr/lpr</sup> mice. We performed immunostaining for Mac2-positive macrophages and CD3-positive T cells and assessed the number of glomerular and interstitial macrophages and interstitial T cells. The numbers of glomerular T cells were not analyzed because they were very low in all groups. CYC full dose and CYC one-fourth full dose plus anti-Ccl2 Spiegelmer were equally potent in reducing the numbers of glomerular and interstitial Mac2-positive macrophages and CD3-positive T cells (Fig. 3, C and D). Anti-Ccl2 Spiegelmer and CYC one-fourth full dose alone and MMF were less potent but still significantly reduced the macrophages in both compartments (Fig. 3, C and D).
same was found for the numbers of interstitial CD3 positive T cells (Fig. 3E). Thus, the additive effect of anti-Ccl2 Spiegelmer and CYC one-fourth full dose on renal pathology of MRLlpr/lpr mice is associated with a significant reduction of interstitial macrophages and T cells. The number of glomerular macrophages was evaluated as the mean number of Mac2-positive cells in 15 glomeruli (glomer.) per section (C). The numbers of interstitial macrophages (D) or T cells (E) were evaluated as mean numbers of Mac2- or CD3-positive cells in 15 high-power fields (hpf) per section. Data are expressed as means ± S.E.M. Control Sp, pegylated control Spiegelmer (revmNOX-E36); anti-Ccl2 Sp, pegylated anti-Ccl2 Spiegelmer (mNOX-E36); CYC (1/4)th, monthly 30 mg/kg cyclophosphamide; CYC full, weekly 30 mg/kg cyclophosphamide; MMF, 100 mg/kg mycophenolate mofetil. Crescentic or globally sclerotic glomeruli are indicated by a star, and tubular casts are indicated by an arrow.

Anti-Ccl2 Spiegelmer and CYC One-Fourth Full Dose Have Additive Effects on the Reduction of Lung Injury in MRLlpr/lpr Mice. Autoimmune peribronchitis is another manifestation of lupus-like systemic autoimmunity in MRLlpr/lpr mice. CYC full dose was more effective than CYC one-fourth full dose in controlling lung injury in MRLlpr/lpr mice. However, CYC one-fourth full dose plus anti-Ccl2 Spiegelmer were as effective as CYC full dose (Fig. 4). It is surprising that MMF had no effect of lung injury in MRLlpr/lpr mice. Thus, the CYC one-fourth full dose/Spiegelmer combination was as effective as CYC full dose on autoimmune lung injury in MRLlpr/lpr mice.

Anti-Ccl2 Spiegelmer/CYC One-Fourth Full Dose Combination and T Cell Depletion in MRLlpr/lpr Mice. Systemic autoimmunity of MRLlpr/lpr mice is characterized by lymphoproliferation evident from massive splenomegaly and bulks of cervical, axillary, inguinal, and mesenterial lymph nodes (Cohen and Eisenberg, 1991). CYC treatment caused a dose-dependent reduction of spleen and lymph node weights in 24-week-old MRLlpr/lpr mice compared with vehicle-treated MRLlpr/lpr mice (Fig. 5, A and B). The effect of MMF treatment was less evident and only affected lymph node weights. Anti-Ccl2 Spiegelmer itself had no significant effect on the weight of spleens and lymph nodes in MRLlpr/lpr mice. Furthermore, anti-Ccl2 Spiegelmer had no effect on the serum levels of IL-12p40 or TNF-α, two cytokines that regulate T cell and monocyte function in autoimmune and antimicrobial host defense (Fig. 5, C and D). In contrast, serum IL-12p40 and TNF-α levels were markedly decreased in MRLlpr/lpr mice treated with CYC full dose (Fig. 5, C and D). In a separate experiment, groups

![Fig. 2. Renal histopathology in MRLlpr/lpr mice. Renal sections of 24-week-old MRLlpr/lpr mice from all groups were stained with periodic acid-Schiff. Original magnification, ×200. Control Sp, pegylated control Spiegelmer (revmNOX-E36); anti-Ccl2 Sp, pegylated anti-Ccl2 Spiegelmer (mNOX-E36); CYC (1/4)th, monthly 30 mg/kg cyclophosphamide; CYC full, weekly 30 mg/kg cyclophosphamide; MMF, 100 mg/kg mycophenolate mofetil. Crescentic or globally sclerotic glomeruli are indicated by a star, and tubular casts are indicated by an arrow.](image1)

![Fig. 3. Markers of lupus nephritis in MRLlpr/lpr mice. The activity index (A) and chronicity index (B) for DPLN were determined on periodic acid-Schiff-stained renal sections from seven to 12 mice from each group as described by Austin et al. (1984). C to E, renal sections of 24-week-old MRLlpr/lpr mice were stained for Mac-2-positive macrophages and CD3-positive T cells. The number of glomerular macrophages was evaluated as the mean number of Mac2-positive cells in 15 glomeruli (glomer.) per section (C). The numbers of interstitial macrophages (D) or T cells (E) were evaluated as mean numbers of Mac2- or CD3-positive cells in 15 high-power fields (hpf) per section. Data are expressed as means ± S.E.M. Control Sp, pegylated control Spiegelmer (revmNOX-E36); anti-Ccl2 Sp, pegylated anti-Ccl2 Spiegelmer (mNOX-E36); CYC (1/4)th, monthly 30 mg/kg cyclophosphamide; CYC full, weekly 30 mg/kg cyclophosphamide; MMF, 100 mg/kg mycophenolate mofetil. *, p < 0.05; **, p < 0.01; †††, p < 0.001 versus vehicle; †, p < 0.05 versus CYC low; ††, p < 0.01 versus CYC low.](image2)
of MRL\textsuperscript{lpr/lpr} mice were treated with vehicle, anti-Ccl2 Spiegelmer, CYC one-fourth full dose plus anti-Ccl2 Spiegelmer, or CYC full dose from 8 to 12 weeks of age to characterize immune cell subsets in spleens, bone marrows, and peripheral blood by flow cytometry. CYC full dose but not CYC one-fourth full dose plus anti-Ccl2 Spiegelmer-treated MRL\textsuperscript{lpr/lpr} mice had elevated numbers of 7/4\textsuperscript{bri} bone marrow monocytes (Fig. 6C). Anti-Ccl2 Spiegelmer- and CYC-treated MRL\textsuperscript{lpr/lpr} mice showed a trend toward lower 7/4\textsuperscript{high} monocyte counts in peripheral blood, but this did not reach statistical significance for any of the groups (Fig. 6D). The CFU-GM colony assay in Methocult media supplemented with rm-GM-CSF is a test for myelosuppression, i.e., the growing capacity of hematopoietic stem cells and immune cell progenitors. CFU-GM colonies in CYC full dose-treated MRL\textsuperscript{lpr/lpr} mice were significantly reduced compared with vehicle, whereas anti-Ccl2 Spiegelmer and CYC one-fourth full dose/Spiegelmer treatment did not affect CFU-GM counts (Fig. 6E). Together, the combination of anti-Ccl2 Spiegelmer and CYC one-fourth full dose does not cause myelosuppression as seen with CYC full dose in MRL\textsuperscript{lpr/lpr} mice.

**Discussion**

The immunosuppressive treatment regimen for DPLN involving high-dose CYC or MMF remains associated with serious and even potential life-threatening complications, i.e., infections (Katsifis and Tzioufas, 2004; Appel et al., 2007). Thus, combinations of CYC with drugs that more specifically interact with autoimmune tissue injury in lupus may overcome this clinical problem.

Our data demonstrate that a combination of the anti-Ccl2 Spiegelmer mNOX-E36 and less frequent CYC full dose treatment initiated at 14 weeks of age, a time point when autoimmune tissue injury is already established (Tesch et al., 1999; Pérez de Lema et al., 2001), is as effective as CYC full dose in suppressing DPLN and lung injury in MRL\textsuperscript{lpr/lpr} mice. It is interesting that the effect of mNOX-E36, either given alone or in combination with CYC one-fourth full dose, had no additive effects on T cell depletion, serum TNF-\textalpha and IL-12p40 levels, and myelosuppression. All these parameters were severely suppressed by a CYC full dose, which we show and was shown previously by others to effectively control DPLN in experimental models of SLE including MRL\textsuperscript{lpr/lpr} mice (Casey, 1968; Gelfand et al., 1972; Shiraki et al., 1984; Smith et al., 1984; Jonsson et al., 1999). Apparently, anti-Ccl2 Spiegelmer has no antiproliferative effect on immune cells and bone marrow-derived blood cell progenitors as the alkylating agent CYC. In contrast, the anti-Ccl2 Spiegelmer increased the numbers of bone marrow macrophages, most probably by blocking the role of Ccl2 for monocyte evasion from the bone marrow (Serbina and Pamer, 2006; Kulkarni et al., 2007). As a consequence, anti-Ccl2 Spiegelmer does not affect the underlying autoimmune dysregulation of MRL\textsuperscript{lpr/lpr} mice. As we have shown previously, anti-Ccl2-
Fig. 5. Lymphoproliferation and serum cytokine levels in MRL<sup>lpr/lpr</sup> mice. A and B, spleens and the bulk of mesenteric lymph nodes were harvested from 24-week-old MRL<sup>lpr/lpr</sup> mice. A, weight of lymph nodes is expressed as mean weight per 100 mg body weight ± S.E.M. B, spleen weights are expressed as mean weight per 100 mg body weight ± S.E.M. C and D, serum IL-12p40 and TNF-α levels were determined by ELISA. Data are means ± S.E.M. from three to 12 mice in each group. Control Sp, pegylated control Spiegelmer (revmNOX-E36); anti-Ccl2 Sp, pegylated anti-Ccl2 Spiegelmer (mNOX-E36); CYC (1/4)th, monthly 30 mg/kg cyclophosphamide; CYC full, weekly 30 mg/kg cyclophosphamide; MMF, 100 mg/kg mycophenolate mofetil. *, p < 0.05; **, p < 0.01; ***, p < 0.001 versus vehicle; #, p < 0.05 versus anti-Ccl2 Sp + CYC (1/4th); ##, p < 0.05 versus anti-Ccl2 Sp + CYC (1/4th).

Fig. 6. T cell depletion and myelosuppression in MRL<sup>lpr/lpr</sup> mice. A and B, spleens were harvested from 12-week-old MRL<sup>lpr/lpr</sup> mice (n = 5) after 4 weeks of treatment as indicated. Spleen cell suspensions were quantified by flow cytometry. Total spleen cells (A) and CD3-positive T lymphocyte subsets (B) of all the groups are shown. Data represent mean percentages ± S.E.M. of all leukocytic cells. C to E, bone marrow and peripheral blood samples were harvested from 12-week-old MRL<sup>lpr/lpr</sup> mice (n = 5) after 4 weeks of treatment as indicated. C, Plasma II-12p40 (ng/ml) and D, Plasma Tnf-α (pg/ml) levels were determined by ELISA. Data are means ± S.E.M. from three to 12 mice in each group. Control Sp, pegylated control Spiegelmer (revmNOX-E36); anti-Ccl2 Sp, pegylated anti-Ccl2 Spiegelmer (mNOX-E36); CYC (1/4)th, monthly 30 mg/kg cyclophosphamide; CYC full, weekly 30 mg/kg cyclophosphamide; MMF, 100 mg/kg mycophenolate mofetil. *, p < 0.05 versus vehicle; #, p < 0.05 versus anti-Ccl2 Sp + CYC (1/4th); ##, p < 0.05 versus anti-Ccl2 Sp + CYC (1/4th).
Spiegelmer does not affect the production of DNA autoantibodies by autoreactive B cells in MRL\textsuperscript{lpr/lpr} mice (Kulkarni et al., 2007). In the present study, we show that anti-Ccl2 Spiegelmer does not modulate the CD4/CD8 double-negative “autoactive” T cell population. These data are consistent with data from other groups showing that Ccl2 blockade does not modulate the autoimmune process in itself but rather the local mechanisms of autoimmune tissue injury (Tesch et al., 1999; Hasegawa et al., 2003; Shimizu et al., 2004). This would argue against additive unspecific effects of a Spiegelmer-CYC combination on complications that relate to general immunosuppression, i.e., infections, which, however, was not obvious in our experimental setting. Rather, we observed an additive effect of the anti-Ccl2 Spiegelmer and less frequent CYC full dose on the numbers of renal macrophages and T cells, which is consistent with the role of Ccl2 for renal macrophage and T cell recruitment (Tesch et al., 1999; Hasegawa et al., 2003; Shimizu et al., 2004). Given the established role of macrophages and T cell infiltrates for established renal macrophage and T cell recruitment prevented glomerular and tubulointerstitial damage in MRL\textsuperscript{lpr/lpr} mice. However, late-onset treatment with anti-Ccl2 Spiegelmer alone was not as effective as high-dose CYC on autoimmune tissue injury in MRL\textsuperscript{lpr/lpr} mice. Higher doses of the anti-Ccl2 Spiegelmer might be more potent as the dose tested here but other than Ccl2-dependent renal macrophage and T cell recruitment contribute to autoimmune renal injury in DPLN. Hence, Spiegelmer monotherapy is unlikely to be superior to CYC full dose for the treatment of DPLN.

In conclusion, inhibition of Ccl2 in combination with CYC allows significant reduction in dosing frequency of CYC, which avoids unwarranted T cell depletion and myelosuppression despite equipotent control of autoimmune tissue damage like DPLN. This novel concept may help to reduce the serious and potentially life-threatening CYC toxicity in patients with DPLN and potentially other serious manifestations of autoimmune disease that involve Ccl2-dependent immune cell infiltrates.

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