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, Hans-Joachim Anders


NOXXON Pharma AG, Berlin, Germany (D.E., N.S., S.Z., S.K.)
Running title: anti-Ccl2 Spiegelmers in lupus nephritis

Correspondence: Hans-Joachim Anders
Medizinische Poliklinik der LMU
Pettenkoferstr. 8a
80336 Munich
Germany
Phone: ++49-89-218075855
Fax.: ++49-89-218074860
Email: hjanders@med.uni-muenchen.de

Number of text pages: 15
Number of tables: none
Number of figures: 6
Number of references: 31
Number of words in Abstract: 248
Number of words in Introduction: 552
Number of words in Discussion: 558

Non Standard abbreviations used in the paper. CYC-Cyclophosphamide, anti-Ccl2 Sp-
anti-Ccl2 spiegelmer, Control Sp- Control spiegelmer, DPLN- Diffuse proliferative lupus
nephritis, FACS -Fluorescence-activated cell sorting.
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 MRL lpr/lpr mice with DPLN from 14 weeks of age with either vehicle, weekly 30 mg/kg CYC (full dose), monthly 30 mg/kg CYC (¼ full dose), pegylated control Spiegelmer, pegylated anti-Ccl2 Spiegelmer (3/week), pegylated anti-Ccl2 Spiegelmer plus CYC ¼ full dose and mycophenolate mofetil (MMF). At week 24, DPLN and autoimmune lung injury were virtually abolished with CYC full dose but not with CYC ¼ full dose. The CYC ¼ full dose/Spiegelmer combination was equipotent to CYC full dose on kidney and lung injury. CD3^-CD4^-CD8^- and CD3^-CD4^-CD25^+ T cells, serum IL-12p40 and TNF-α levels which were all markedly affected by CYC full dose but not by CYC ¼ full dose. No additive effects of anti-Ccl2 Spiegelmer were noted on bone marrow CFU-GM counts and 7/4 high monocyte counts, lymphoproliferation, 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 novel strategy to reduce CYC toxicity in the treatment of severe lupus.
Introduction

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 (ALMS) 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 due to the unspecific immunosuppressive effects of CYC and MMF (Appel et al., 2007). Novel drugs specifically blocking autoimmune inflammation may allow to reduce 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<sup>lpr/lpr</sup> mice with experimental SLE are protected from DPLN (Perez de Lema et al., 2005; Tesch et al., 1999). 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 therapy or transfer of transfected...
cells the truncated Ccl2 markedly reduced autoimmune tissue injury in MRL<sup>lpr/lpr</sup> mice (Hasegawa et al., 2003; Shimizu et al., 2004). However, such experimental approaches validate the Ccl2/Ccr2 axis as a target rather than representing applicable therapeutics in humans. Gene transfer causes irrepressible antagonist production and local tumors complicated the transplantation of transfected cells (Hasegawa et al., 2003; Shimizu et al., 2004).

We have recently proposed an alternative strategy to block Ccl2, i.e. with a Ccl2-binding mirror-image RNA oligonucleotide, a so-called Spiegelmer. Spiegelmers represent a new class of nuclease-resistant RNA aptamers that can bind and inhibit target molecules conceptually similar to monoclonal antibodies (Eulberg and Klussmann, 2003). The mirror-image configuration of the ribonucleotides prevents Spiegelmer digestion by nucleases (Klussmann et al., 1996). Hence, Spiegelmers are very well suited for in vitro and in vivo applications (Purschke et al., 2006; Wlotzka et al., 2002; Denekas et al., 2006; Helmling et al., 2004). We previously reported the design of anti-Ccl2 Spiegelmer (mNOX-E36), a Spiegelmer that potently neutralizes the biological functions of murine Ccl2 in vitro and in vivo (Kulkarni et al., 2007). In cell-based assays employed with THP-1 cells, Ccl2-mediated chemotaxis as well as a Ca<sup>2+</sup>-release could be inhibited, 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<sup>lpr/lpr</sup> mice. Ccl2 blockade with 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 to 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.
Methods

Preparation of anti-Ccl2 Spiegelmer mNOX-E36

The identification and molecular structure of the anti-Ccl2 Spiegelmer mNOX-E36 has been previously described in detail (Kulkarni et al., 2007). In brief, mNOX-E36 (5'-GGCGACAUAUG GUUGGG CAUG AGGCGAGGCC CUUUGAUGAA UCCGCGGCCA-3') and the control Spiegelmer revmNOX-E36 (5'-ACCGGCGCCUAAGUAGUUUC CCGGAGCGGA GUACGGGUUG GUUACAGCGG-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<sup>lpr/lpr</sup> mice were obtained from Harlan Winkelmann (Borchen, Germany) and kept under normal housing conditions with a 12 hour light and dark cycle. Water and standard chow (Ssniff, Soest, Germany) were available ad libitum. Mice were grouped in seven different groups. From an age of 14 weeks groups of 12 mice were injected for 10 weeks as follows: 5% glucose s.c. (vehicle group), 50 mg/kg (0.89 µmol/kg) revmNOX-E36 s.c. (control Spiegelmer), 50 mg/kg (0.89 µmol/kg) mNOX-E36 s.c. (anti-Ccl2 Spiegelmer), 30mg/kg/4weeks CYC i.p. (CYC ¼ full dose), 30mg/kg/week CYC i.p. (CYC full dose), 50 mg/kg mNOX-E36 plus CYC ¼ full dose (combination) and 100mg/kg/day MMF orally (Roche, Mannheim, Germany). All vehicle and Spiegelmer injections were given 3x/week. Plasma levels of mNOX-E36 were determined in samples obtained from the retro-orbital sinus 24 hours after injection from Spiegelmer-treated groups on week 1, 3, 7, and 10 of treatment. Spiegelmer plasma levels were determined as described (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 mesenteric lymphnodes to total body weight were calculated as markers of the lupus-associated lymphoproliferative syndrome. Urine albumin/creatinine ratio was determined as previously described (Pawar et al., 2006). Plasma Il-12p40 and Tnf-α levels were analysed by ELISA (Tnf-α: Biolegend Ltd, San Diego, CA, Il-12p40: OptEia BD-Pharmingen, Heidelberg, Germany). From all mice, kidneys and lungs were fixed in 10% buffered formalin, processed, and embedded in paraffin. 5 μm 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 0-4 by a blinded observer. Immunostaining was performed as previously described (Anders et al., 2002). The following primary antibodies were used: rat anti-Mac2 (macrophages, Cederlane, Ontario, Canada, 1:50), anti-mouse CD3 (1:100, clone 500A2, BD). Negative controls included incubation with a respective isotype antibody. Positive glomerular cells were counted in 15 cortical glomeruli per section. Interstitial cells were counted by high power field (hpf). Urinary albumin was assessed by ELISA (Bethyl Lab Montgomery, TX) and urinary creatinine concentrations were determined using an automatic autoanalyzer (Integra 800, Roche Diagnostics, Germany).
Evaluation of monocytopenia and bone marrow Colony Forming Units-CFU-GM counts

To evaluate therapy induced monocytopenia and myelotoxicity, we treated four groups of mice (n=5) as follows. 5% glucose s.c., 50mg/kg/3x/week anti-Ccl2 Spiegelmer s.c., CYC full dose i.p., combination of CYC ¼ full dose i.p. and 50mg/kg/3x/week anti-Ccl2 Spiegelmer s.c. for 4 weeks. After four weeks of treatment, blood was drawn from retro-orbital plexus under isoflorane anesthesia. Mice were sacrificed to get the bone marrow cells. After counting, 3*10^4 bone marrow cells derived from each group were cultured with Methocult media (M3132, Stem Cell Technology, St. Katharinen, Germany) supplemented with mGM-CSF (10ng/ml) for CFU-GM assay as per the instruction in kit insert. CFU-GM colonies were counted on day 12.

Flow cytometry

Flow cytometry was performed using a FACScalibur 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, Heidelberg, Germany) for 30min to block Fc receptors. Blood samples were incubated for 1 hour at 4°C with fluorescently labelled 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-FITC, anti-mouse CD45-PE, anti-mouse CD4-APC, anti-mouse CD8-PerCp and anti-mouse CD25-PerCp (all from BD Pharmingen). Cell density in every spleen cell single cell suspension sample was analysed by using counting beads (CALTAG, Hamburg, Germany).
**Statistical analysis**

Data were expressed as mean ± standard error of the mean (SEM). Comparison between groups were performed using univariate ANOVA and unpaired ‘t’-test. Posthoc 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<sub>lpr/lpr</sub> mice.

In order to monitor drug exposure in MRL<sub>lpr/lpr</sub> mice, mNOX-E36 plasma levels were determined in 1, 3, 6, and 10th week of treatment, i.e. the 15, 18, 21, 24th week of age. 24 hours after administration of 50 mg/kg mNOX-E36 median plasma levels were approximately 1 µM throughout the study in both of the Spiegelmer-treated groups (figure 1A). Apparently, the progressive kidney disease of MRL<sub>lpr/lpr</sub> mice did not modulate Spiegelmer pharmacokinetics and neither drug accumulation nor metabolic induction or reduction were detected. Exposure to mNOX-E36 was associated with an increase of serum Ccl2 levels in MRL<sub>lpr/lpr</sub> mice which was not observed in MRL<sub>lpr/lpr</sub> mice treated with the control Spiegelmer (figure 1B). Thus, upon subcutaneous administration to MRL<sub>lpr/lpr</sub> mice, pharmacologically relevant levels of circulating anti-Ccl2 Spiegelmer, most likely, binds and retains Ccl2 in the circulation.

Add-on therapy with anti-Ccl2 Spiegelmers improves the effects of CYC ¼ full dose on kidney disease of MRL<sub>lpr/lpr</sub> mice.

Female MRL<sub>lpr/lpr</sub> mice develop proliferative immune complex glomerulonephritis similar to DPLN in humans. We treated MRL<sub>lpr/lpr</sub> mice with CYC, MMF, Spiegelmer or vehicle from week 14 to 24 of age. This represents a therapeutic treatment protocol because at 14 weeks of age MRL<sub>lpr/lpr</sub> mice showed DPLN with an activity score index of 4.1 ± 1.1. At this age major abnormalities of the tubulointerstitial compartment were absent (not shown). After 10 weeks of treatment, vehicle- and control Spiegelmer-treated MRL<sub>lpr/lpr</sub> mice revealed DPLN associated with glomerular hypercellularity, expansion of glomerular matrix, focal tuft necrosis, and a mixed periglomerular and interstitial inflammatory cell
infiltrate (figure 2). CYC full dose and CYC ¼ full dose plus anti-Ccl2 Spiegelmer were equally potent in improving the activity and chronicity index of lupus nephritis (figures 2 and 3). Furthermore, CYC full dose-treated mice showed 100% survival and survival rates of CYC ¼ full dose plus anti-Ccl2 Spiegelmer-treated MRL lpr/lpr mice did not significantly differ from those of CYC full-dose-treated MRL lpr/lpr mice. Proteinuria as assessed by urinary albumine-creatinine ratios trended lower in these groups as compared to vehicle treatment but this did not reach statistical significance (not shown). Anti-Ccl2 Spiegelmer and CYC ¼ full dose alone as well as MMF were less potent but still significantly improved the activity and chronicity indices of lupus nephritis (figures 2 and 3). Thus, adding anti-Ccl2 Spiegelmer to a CYC ¼ full dose-based regimen is as potent as CYC full dose therapy for DPLN of MRL lpr/lpr mice.

Anti-Ccl2 Spiegelmer and CYC ¼ full dose have additive effects on the reduction of immune cell infiltrates in kidneys of MRL lpr/lpr 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 lpr/lpr mice (Tesch et al., 1999). We therefore hypothesized that the additive effects of an anti-Ccl2 Spiegelmer/ CYC ¼ full dose combination may relate to impaired macrophage and T cell recruitment in MRL lpr/lpr 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 as they were very low in all groups. CYC full dose and CYC ¼ full dose plus anti-Ccl2 Spiegelmer were equally potent in reducing the numbers of glomerular as well as interstitial Mac2-positive macrophages in kidneys of MRL lpr/lpr mice (figure 3C and 3D).
Anti-Ccl2 Spiegelmer and CYC ¼ full dose alone as well as MMF were less potent but still significantly reduced the macrophages in both compartments (figure 3C and 3D). The same was found for the numbers of interstitial CD3 positive T cells (figure 3E). Thus, the additive effect of anti-Ccl2 Spiegelmer and CYC ¼ full dose on renal pathology of MRL$^{lpr/lpr}$ mice is associated with a significant reduction of interstitial macrophages and T cells as well as of glomerular macrophages which was similar to the effect of CYC full dose.

**Anti-Ccl2 Spiegelmer and CYC ¼ full dose have additive effects on the reduction of lung injury in MRL$^{lpr/lpr}$ mice.**

Autoimmune peribronchitis is another manifestation of lupus-like systemic autoimmunity in MRL$^{lpr/lpr}$ mice. CYC full dose was more effective than CYC ¼ full dose in controlling lung injury in MRL$^{lpr/lpr}$ mice. However, CYC ¼ full dose plus anti-Ccl2 Spiegelmer were as effective as CYC full dose (figure 4). Surprisingly, MMF had no effect of lung injury in MRL$^{lpr/lpr}$ mice. Thus, the CYC ¼ full dose/Spiegelmer combination was as effective as CYC full dose on autoimmune lung injury in MRL$^{lpr/lpr}$ mice.

**Anti-Ccl2 Spiegelmer/CYC ¼ full dose combination and T cell depletion in MRL$^{lpr/lpr}$ mice.**

Systemic autoimmunity of MRL$^{lpr/lpr}$ 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 MRL$^{lpr/lpr}$ mice as compared to vehicle-treated MRL$^{lpr/lpr}$ mice (figures 5A and 5B). The effect of MMF treatment was less
evident and did only affect lymph nodes weights. Anti-Ccl2 Spiegelmer itself had no significant effect on the weight of spleens and lymph nodes in MRL<sup>lpr/lpr</sup> 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 autoimmunity and antimicrobial host defense (figures 5C and 5D). By contrast, serum IL-12p40 and Tnf-α levels were markedly decreased in MRL<sup>lpr/lpr</sup> mice treated with CYC full dose C (figures 5C and 5D). In a separate experiment groups of MRL<sup>lpr/lpr</sup> mice were treated with either vehicle, anti-Ccl2 Spiegelmer, CYC ¼ full dose plus anti-Ccl2 Spiegelmer or CYC full dose from 8 to 12 weeks of age in order to characterize immune cell subsets in spleens, bone marrows and peripheral blood by flow cytometry. CYC full dose but not CYC ¼ full dose plus anti-Ccl2 Spiegelmer significantly reduced the number of spleen cells (figure 6A). This was consistent for all spleen T cell subsets studied, i.e. CD3<sup>+</sup>, CD3<sup>+</sup>CD4<sup>+</sup>, CD3<sup>+</sup>CD8<sup>+</sup>, CD3<sup>+</sup>CD4<sup>+</sup>CD8<sup>+</sup>, and CD3<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup> cells (figure 6B). Together, anti-Ccl2 Spiegelmer and CYC ¼ full dose had no additive effects on T cell depletion as seen with CYC full dose in MRL<sup>lpr/lpr</sup> mice.

**Anti-Ccl2 Spiegelmer/CYC ¼ full dose combination and myelosuppression in MRL<sup>lpr/lpr</sup> mice.**

Full dose of CYC usually causes transient myelosuppression which renders patients susceptible to life-threatening infections. We therefore evaluated markers of myelosuppression in treated MRL<sup>lpr/lpr</sup> mice for four weeks. So as to have a single CYC injection in combination group and four weekly injections in CYC full dose group. Speigelmer and vehicle treated groups reviewed injection 3x/week. CYC full dose significantly reduced the numbers of 7/4<sup>bri</sup> monocytes in bone marrows of MRL<sup>lpr/lpr</sup> mice.
while anti-Ccl2 Spiegelmer-treated and CYC ¼ full dose plus anti-Ccl2 Spiegelmer treated MRL
lpr/lpr mice had elevated numbers of 7/4\text{bri} bone marrow monocytes (figure 6C). Anti-Ccl2 Spiegelmer- and CYC-treated MRL
lpr/lpr mice showed a trend towards lower 7/4\text{high} monocyte counts in peripheral blood but this did not reach statistical significance for any of the groups (figure 6D). CFU-GM colony assay in Methocult media supplemented with rm-GM-CSF is a test for myelosuppression, i.e. the growing capacity of hematopoetic stem cells and immune cell progenitors. CFU-GM colonies in CYC full dose -treated MRL
lpr/lpr mice were significantly reduced compared to vehicle while anti-Ccl2 Spiegelmer and CYC ¼ full dose/Spiegelmer treatment did not effect CFU-GM counts (figure 6E). Together, combination of anti-Ccl2 Spiegelmer and CYC ¼ full dose do not cause myelosuppression as seen with CYC full dose in MRL
lpr/lpr mice.
Discussion

Immunosuppressive treatment regimen for DPLN involving high dose CYC or MMF remain associated with serious and even potential life-threatening complications, i.e. infections (Appel et al., 2007; Katsifis and Tzioufas, 2004). 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; Perez de Lema et al., 2001) – is as effective as CYC full dose in suppressing DPLN and lung injury in MRL\textsuperscript{lpr/lpr} mice. Interestingly, the effect of mNOX-E36 either given alone or in combination with CYC ¼ full dose had no additive effects on T cell depletion, serum Tnf-α and Il-12p40 levels, and myelosuppression. All these parameters were severely suppressed by a CYC full dose which we show and which was previously shown by others to effectively control DPLN in experimental models of SLE including MRL\textsuperscript{lpr/lpr} mice (Smith et al., 1984; Shiraki et al., 1984; Jonsson et al., 1999; Casey, 1968; Gelfand et al., 1972). Apparently, anti-Ccl2 Spiegelmer has no anti-proliferative effect on immune cells and bone marrow-derived blood cell progenitors as the alkylating agent CYC. By contrast, the anti-Ccl2 Spiegelmer increased the numbers of bone marrow macrophages, most likely by blocking the role of Ccl2 for monocyte evasion from the bone marrow (Kulkarni et al., 2007; Serbina and Pamer, 2006). As a consequence anti-Ccl2 Spiegelmer does not affect the underlying autoimmune dysregulation of MRL\textsuperscript{lpr/lpr} mice. As we have previously shown anti-Ccl2-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 ‘autoreactive’ 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. We rather 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 renal damage, we propose that specific blockade Ccl2-dependent renal macrophage and T cell recruitment prevented glomerular and tubulointerstitial damage in MRL\textsuperscript{1pr/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{1pr/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.
Acknowledgements

The expert technical assistance of Ewa Radomska, Dan Draganovic and Jana Mandelbaum is gratefully acknowledged. We thank the NOXXON chemistry group for providing the oligonucleotides. Parts of this project were prepared as a doctoral thesis at the Faculty of Medicine, University of Munich, by O.K.
References


Footnotes

This work was supported by NOXXON PharmaAG.

D.E., N.S., S.Z., S.K. are employes of Noxxon PharmaAG. The other authors have no conflict of financial interest.
Legends for Figures

Figure 1. Plasma anti-Ccl2 Spiegelmer and Ccl2 levels in MRL<sup>lpr/lpr</sup> mice. A: Concomitant pharmacokinetic studies were performed for anti-Ccl2 Spiegelmer in female MRL<sup>lpr/lpr</sup> mice during the course of treatment from 15 to 24 weeks of age. Plasma sampling was carried out 24 hours after the first weekly injection for anti-Ccl2 Spiegelmer on week 15, 18, 21 and 24 of age. The Spiegelmer plasma levels were determined by a modification of a sandwich hybridization procedure as described in methods. Data are expressed as means of 7-12 mice in each group. B: Plasma Ccl2 levels were determined in mice of all groups at the end of the study. Data are expressed as means ± SEM of 7-12 mice in each group. *** p < 0.001 vs control.

Figure 2. Renal histopathology in MRL<sup>lpr/lpr</sup> mice. Renal sections of 24 weeks old MRL<sup>lpr/lpr</sup> mice from all groups were stained with periodic acid Schiff. Original magnification x 200. Control Sp = pegylated control Spiegelmer (revmNOX-E36), anti-Ccl2 Sp = pegylated anti-Ccl2 Spiegelmer(mNOX-E36), CYC ¼ th = monthly 30 mg/kg cyclophosphamide, CYC full = weekly 30mg/kg cyclophosphamide, MMF = 100 mg/kg mycophenolate mofetil. Crescentic or globally sclerotic glomeruli are indicated by ⭐️, tubular casts are indicated by ⚫.

Figure 3. Markers of lupus nephritis in MRL<sup>lpr/lpr</sup> mice. The activity index (A) and chronicity index (B) for DPLN were determined on PAS stained renal sections from 7-12 mice from each group as described by Austin et al., 1984. C-E: Renal sections of 24 weeks
old MRL<sup>lpr/lpr</sup> 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 (glom.) 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 ± SEM.

Control Sp = pegylated control Spiegelmer (revmNOX-E36), anti-Ccl2 Sp = pegylated anti-Ccl2 Spiegelmer (mNOX-E36), CYC ¼ th = monthly 30 mg/kg cyclophosphamide, CYC full = weekly 30mg/kg cyclophosphamide, MMF = 100 mg/kg mycophenolate mofetil. * p < 0.05, ** p < 0.01, *** p<0.001 vs vehicle, # p<0.05 vs CYC low. ## p<0.01 vs CYC low

**Figure 4. Autoimmune lung injury in MRL<sup>lpr/lpr</sup> mice.** Lung sections of 24 weeks old MRL<sup>lpr/lpr</sup> mice from all groups were stained with periodic acid Schiff. Original magnification x 100. Semiquantitative scoring of lung injury was performed as described in methods. Data represent means ± SEM. Control Sp = pegylated control Spiegelmer (revmNOX-E36), anti-Ccl2 Sp = pegylated anti-Ccl2 Spiegelmer (mNOX-E36), CYC ¼ th = monthly 30 mg/kg cyclophosphamide, CYC full = weekly 30mg/kg cyclophosphamide, MMF = 100 mg/kg mycophenolate mofetil. Peribronchial inflammation is indicated by ♬.

**Figure 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: Lymph nodes weight is expressed as mean weight per 100 mg body weight ± SEM. B: Spleen weight are expressed as mean weight per 100 mg body weight ± SEM. C and D: Serum Il-12p40 and Tnf-α levels were determined by ELISA. Data are means ± SEM from 3-12 mice in each group. Control Sp = pegylated control Spiegelmer
(revmNOX-E36), anti-Ccl2 Sp = pegylated anti-Ccl2 Spiegelmer (mNOX-E36), CYC ¼ th = monthly 30 mg/kg cyclophosphamide, CYC full = weekly 30mg/kg cyclophosphamide, MMF = 100 mg/kg mycophenolate mofetil. *p < 0.05, ** p<0.01, *** p<0.001 vs vehicle, # p<0.05 vs anti-Ccl2 Sp+ CYC (¼ th), ## p<0.01 vs anti-Ccl2 Sp+ CYC (¼ th).

Figure 6. T cell depletion and myelosuppression in MRL lpr/lpr mice. A and B: Spleens were harvested from 12 week old MRL lpr/lpr 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 ± SEM of all leukocytic cells. C-E: Bone marrow and peripheral blood samples were harvested from 12 week old MRL lpr/lpr mice (n = 5) after 4 weeks of treatment as indicated. 7/4 high positive monocytes were quantified in bone marrows (C) and peripheral blood (D) of MRL lpr/lpr mice from all groups. E: To assess drug induced myelosuppression bone marrows were cultured to quantify CFU-GM counts of hematopoietic progenitors as described in methods. Data represent mean percentages ± SEM. Anti-Ccl2 Sp = pegylated anti-Ccl2 Spiegelmer (mNOX-E36), CYC ¼ th = monthly 30 mg/kg cyclophosphamide, CYC full = weekly 30mg/kg cyclophosphamide. * p < 0.05 vs vehicle. # p < 0.05 vs. CYC ¼ dose/anti-Ccl2 Spiegelmer combination, n.s. = not significant.