Role of therapeutic blockade of CCL2 in a mouse model of SLE and lupus nephritis

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DECLARATION

I here by declare that the present work embodied in this thesis was carried out by me under the supervision of OA PD Dr. Hans Joachim Anders, Internist-Nephrologe-Rheumatologie, Medizinische Poliklinik-Innenstadt Klinikum der Universität München. This work has not been submitted in part or full to any other university or institute for any degree or diploma.

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Date: Onkar P. Kulkarni
Place: München
Dedicated to

‘My loving parents’

Without whom i would have not reached so far

” बाबा हा प्रबंध तुमच्याशी “
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1. Introduction
Systemic lupus erythematosus (SLE) is characterised by the production of antibodies to components of the cell nucleus in association with a diverse array of clinical manifestations. The basic pathological features of SLE are that of inflammation and blood vessel abnormalities, which include band or occlusive vasculopathy, vasculitis, and immune complex deposition. The best characterised organ pathology is in the kidney. By light and immunofluorescence microscopy, renal biopsies in patients with SLE display mesangial cell proliferation, inflammation, basement membrane abnormalities, and immune complex deposition, with immunoglobulins and complement components (Tumlin JA. 2008). On electron microscopy, these deposits can be visualised in the mesangium and the subendothelial or subepithelial surface of the basement membrane. Current therapeutic regimen for lupus nephritis mainly comprised of medications which target abnormalities of immune regulation e.g. immunosuppressant B and T cell targeting drugs. Corticosteroids, antimalarial drugs and other therapies are being practiced along with immunosuppressants (Houssiau FA, Ginzler EM. 2008).

Even though we are yet to narrow down the exact aetiology of lupus pathogenesis, immunologists have enough idea about how the end stage organ damage happens? Researchers have been able to identify some of the inflammatory mediators which play important role in chronic tissue inflammation in lupus nephritis. With this information, we assume that reducing inflammatory mediators derived tissue destruction along with low exposure to immunosuppressants; can be an excellent approach to lupus nephritis treatment. In this study we evaluated the effectiveness of blocking CC-chemokine ligand 2 (CCL2) in a murine model of lupus nephritis using a novel tool to neutralize CCL2 in vivo. Before going in to details of the study, we will try to summarise known details of Lupus nephritis, about its histology, pathogenesis, role of different immune cell types (T cells, B cells), cytokines, chemokines and brief information about lupus therapy in this introductory segment.

1.1 Lupus nephritis
Lupus nephritis is one form of immune complex glomerulonephritis. The major immunological features of lupus are, loss of self tolerance to autoantigens, the presence of autoreactive B and T cells, with polyclonal activation of B-cells, the consequent production of autoantibodies by plasma cells, and the release of cytokines.
Anti-double stranded DNA antibodies (anti-dsDNA) are probably the most pathogenic type of antibody (Ab) produced. Other antibodies which bind to nucleosomes, laminin and collagen type IV also contribute to nephritis. The formation of immune complexes, the activation of the complement pathway and the defective clearance of immune complexes are also likely to play an important part in disease pathogenicity.

1.1.1 Histology
The International Society of Nephrology (ISN) and the World Health Organization (WHO) systems classify the various forms of lupus nephritis according to light, immunofluorescent and electron microscopic changes.

As shown in Table 1, the WHO system of nomenclature identifies six different classes of lupus nephritis, with classes III and IV being the “proliferative” forms of the disease. By definition, patients with WHO class III have less than 50% of the volume of an individual glomerulus or less than 50% of the total number of Glomeruli with endocapillary proliferation. While many patients with severe class III may exhibit focal necrosis (karyorrhexis) or extracapillary proliferation (crescents), these findings are not required for staging a particular biopsy as class III or class IV. The overall prevalence of class III is 25% to 30%. In general, class III is associated with higher titers of anti-DNA antibodies, low complement levels, and active extra-renal manifestations of SLE. WHO class IV (diffuse proliferative) lupus nephritis shares many similarities with class III but generally demonstrates more extensive and aggressive histopathology.

At the histology level, class IV is defined by the presence of endocapillary proliferation in greater than 50% of glomeruli. While not required for the diagnosis, patients with class IV lupus nephritis often demonstrate extensive crescents and karyorrhexis. At the clinical level, patients with class IV lupus nephritis frequently demonstrate severe extra-renal manifestations, including lupus cerebritis and lupus pneumonitis.
### Table 1: World Health Organization (WHO) nomenclature for classifying the various forms of lupus nephritis.

<table>
<thead>
<tr>
<th>WHO Class</th>
<th>Class I</th>
<th>Class II</th>
<th>Class III</th>
<th>Class IV</th>
<th>Class V</th>
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<tr>
<td><strong>Name</strong></td>
<td>Normal</td>
<td>Mesangial</td>
<td>Focal</td>
<td>Diffuse</td>
<td>Membranous</td>
<td>Sclerosing</td>
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<tr>
<td>Light microscopy</td>
<td>Normal</td>
<td>Mesangial proliferation</td>
<td>&lt;50% Glomeruli endocapillary proliferation</td>
<td>&gt;50% Glomeruli endocapillary proliferation</td>
<td>Thickenened capillary loops</td>
<td>Interstitial Fibrosis</td>
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<td>Immuno-fluorescent microscopy</td>
<td>Normal</td>
<td>IgG mesangial staining</td>
<td>Karyorrhexis Crescents</td>
<td>Karyorrhexis Crescents</td>
<td>Absent proliferation/ crescents</td>
<td>IgG mesangial Subepithelial</td>
</tr>
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<td>Electron microscopy</td>
<td>Immune complex deposits</td>
<td>Mesangial dense deposits</td>
<td>Mesangial subendothelial deposits</td>
<td>Mesangial subendothelial Subepithelial</td>
<td>Mesangial Subepithelial</td>
<td>Variable</td>
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</tbody>
</table>

Adopted from Tumlin JA 2008

Membranous lupus nephritis (WHO class V) is present in between 10% to 20% of renal biopsies and is characterized by thickened capillary loops and mesangial expansion, but without significant crescent formation or endocapillary proliferation. However, the histopathology of class V is more diffuse than other forms of lupus nephritis and can be subdivided into three other forms. Patients with class Vb exhibit membranous features in conjunction with mesangial proliferation, while class Vc and Vd demonstrate focal or diffuse endocapillary proliferation.

### 1.1.2 Pathogenesis of lupus nephritis

Search of the precise immunopathogenesis of lupus nephritis has remained a challenge to many research groups all around the globe. This has led us to lots of conceptual theories which are published in recent times. Progression to lupus nephritis in SLE is thought to be dependent on the loss of self-tolerance and the formation of autoantibodies that deposit in the kidney to induce nephritis. Mechanistic studies in human and several murine models of SLE have shown that a variety of predisposing factors in the host must be present for these events to result in renal pathology.
1.1.2.1 Predisposing factors associated with lupus nephritis

Genetical factors
Genetic susceptibility to lupus is inherited as a complex trait and studies have suggested that several genes could be important. Genes that contribute to the pathogenesis of systemic lupus are classified as follows: 1) Genes that cause break in tolerance for the self-antigens. 2) Genes that lead to immune dysregulation (loss of control of regulatory lymphocytes over the autoreactive lymphocytes). It is also suggested that multiple mutations, inherited or somatic, may be needed before a self-reactive clone B and T lymphocytes bypasses sequential tolerance check points resulting in emergence of autoimmune disease (Goodnow, 2007).
Hormonal factors
SLE is a disease affecting women of childbearing age and there have been many anecdotal reports of exogenous oestrogens exacerbating lupus or increasing the risk of developing this disorder. Oral contraceptive use in the Nurses Health Study (Sanchez-Guerrero J.1997) was associated with a slightly increased risk of disease with a relative risk for users versus never users of 1.9. Sex hormones are shown to affect T cells and B cells. Oestrogen has multiple effects of immune system. Oestrogen upregulates Bcl2, thus blocking tolerance induction of naive B cells (Bynoe MS. et al.2000). Either an increase in oestrogen or prolactin can break tolerance of high-affinity DNA-reactive B cells. Oestrogen, in this model, promotes the survival and activation of the T-independent marginal zone B-cell subset (Grimaldi CM. 2006a). Thus, oestrogen may facilitate the maturation of pathogenic naive autoreactive B cells, whereas hampering a potentially protective autoreactive B-cell repertoire (Grimaldi CM. et al.2006b). In SLE T cells oestrogen increases expression of CD40L (Rider V. et al.2000). Oestrogen can activate dendritic cells (Hughes GC., Clark EA.2007). Prolactin has some effects that mirror oestrogen in the immune system and other effects that oppose it. For example, an increase in oestrogen or prolactin can break tolerance of high-affinity DNA-reactive B cells (Grimaldi CM.2006a). In murine SLE models, hyperprolactinemic mice have elevated albuminuria, regardless of oestrogen levels (Elbourne KB. et al.1998).

Environmental factors
Ultraviolet (UV), UV-A and UV-B, light has been classified as the classic environmental precipitant of SLE. Drugs like minocycline, and anti-TNF biologics, can lead to drug-induced lupus erythematosus (DILE). Sulfonamide antibiotics can induce idiopathic SLE. Subacute cutaneous lupus erythematosus (SCLE) is associated with thiazides, calcium channel blockers and angiotensin-converting enzyme inhibitors. Epstein-Barr viral infection has been strongly associated with SLE, in both children and adults in multicase SLE families. Amongst the other factors which are also associated with SLE are toxic exposures to silica and mercury (Mok et al.2003).

We just had a brief overview of various predisposing factors of lupus nephritis, but what triggers the immune dysregulation? We know that SLE T cells and B cells lose the tolerance for self antigen, but what is the source of these self antigens? To understand this theory we will review the phenomena of apoptosis in the next section.
1.1.2.2 Apoptosis

Cell death is the most likely phenomenon to supply autoantigens. There are two main forms of cell death, namely apoptosis and necrosis. Whether cells die through apoptosis or necrosis is determined by the initial stimulus and the microenvironment. Apoptosis is an active, programmed and regulated cellular process which appears under both physiological and pathological conditions in all tissues. In states of high-rate tissue turnover like embryogenesis and development, apoptosis plays a critical role in the maintenance of a balance between old and new cells. Morphological and biochemical changes of dying cells are extremely important for their clearance from tissues by the scavenger system. If these cells are not cleared on time, they lose their membrane integrity and become secondarily necrotic; thereby releasing high amounts of modified nuclear and cytoplasmic material. Apoptosis is a tightly regulated process of programmed cell death that regulates the late phase of immune responses. Disordered regulation of both apoptosis and the clearance of apoptotic products have been implicated in the pathogenesis of SLE and lupus nephritis (Kamradt et al. 2001).

Under normal immune circumstances, activity against self antigens is prevented by several mechanisms, including the Apo-1/Fas pathway of apoptosis, which was shown to be involved in the process of immune tolerance by deletion of unwanted autoreactive T cells and B cells (Nagata S., Golstein P. 1995). Defective Fas function therefore has the potential to lead to accumulation of lymphocytes, particularly autoreactive lymphocytes. This is the basic mechanism by which MRL1pr/1pr mice develop autoimmune syndrome.

Cell death by necrosis, on the other hand, occurs when external factors strike cells. A violent interruption of their vital functions and finally a disruption of the plasma membrane are the consequences. This phenomenon is often triggered by an infectious agent, heat, ischaemia, low ATP levels or a mechanical injury (Rahman A., Isenberg DA. 2008). No matter if cells die through apoptosis or necrosis, they must quickly be eliminated from tissues in order to prevent further damage. Early apoptotic cells are cleared by phagocytosis without eliciting either inflammation or immune response. Necrotic cells induce inflammation and favour the initiation of immune responses (Green DR. et al. 2009). There is growing evidence for a clearance deficiency of early apoptotic cells in mouse models of SLE (Taylor PR. et al. 2000, Licht R. et al. 2004) and in humans (Herrmann et al. 1998, Baumann et al. 2002). In later stage of apoptosis, apoptotic cells are called as secondary necrotic cells. Secondary necrotic
cells can release DNA-containing nucleosomes together with dangerous inflammatory signals towards immune system cells (Rovere P. et al. 2000). It has been shown that the high mobility group B1 (HMGB1) protein, which is attached to the chromatin of apoptotic cells, remains immobilized even under conditions of secondary necrosis, while in the case of primary necrotic cells it is released and acts as an inflammatory cytokine (Voll RE. et al. 2008). In clinical observations human SLE subjects had increased levels of circulating apoptotic mononuclear cells and dermal keratinocytes. The SLE patients had greater levels of circulating products of apoptosis such as nucleosomes than the controls. Autoantibodies associated with SLE reacted against the granzyme-cleaved nuclear products presented in the surface membrane blebs of apoptotic cells. While increased rates of apoptosis and/or reduced clearance of neoantigens created by apoptosis might lead to increased autoantibody production, one mechanism by which direct renal damage might occur in lupus nephritis is by increased rates of apoptosis among resident cells. In a murine model of lupus nephritis, caspase inhibitor therapy reduced glomerular injury (Seery JP. et al. 2001). These combined observations suggest that disordered regulation of apoptosis might contribute to the lupus nephritis phenotype at different stages in the progression of the disease.

So we know that apoptotic bodies are one of the major sources of auto antigens. If we look at the physiology of cell, every cell undergoes apoptosis at one point of time in its life cycle. But at the same time in a healthy individual clearing mechanisms are appropriately placed to clear the debris. In addition to that healthy immune system is tolerant to self. But in SLE the immune system is deregulated and increased generation of autoreactive B and T cells takes place along with the reduction of regulatory T cells. The regulatory T cells lose its control on the expansion of T cells. Circulating autoantigens or immune-complexes which are non antigenic in healthy conditions, are recognised as danger signals which further lead to excessive activation of immune system in SLE. (Goodnow.2005, 2007; Marshak-Rothstein.2006, Mok et al.2003). In the next parts of the introduction we will try to have an overview of the T cells and B cells characteristics in lupus nephritis.

1.1.2.3 Role of T cells

Among the cells that participate in the initiation, progression and perpetration of the disease, T lymphocytes play a key role in all stages, also because the production of
pathogenic autoantibodies in SLE is a T-cell-dependent process (Datta SK, et al. 1987). As major contributors to the disease, T cells in SLE display multiple abnormalities that reflect and partly explain some aspects of the complex disease process.

T cells are functionally and phenotypically heterogeneous, and it has therefore been useful to classify and divide these cells into subgroups. One major distinction is based on the surface expression of CD8 on the T cells that have cytotoxic functions and CD4 on the T cells that provide help to other cells (although each subset may also display the opposite function, under specific circumstances). Another important distinction for T cells is between naive (or virgin) T cells and memory T cells, in reference to the experience of the cell with the antigen after its migration from thymus. T cells can also be divided into regulatory T cells and effector T cells. Regulatory T cells are typically hyporesponsive to stimulation with antigen and suppress the activation or effector activities of other immune cells. Differently from regulatory T cells, effector T cells proliferate in response to antigen stimulation, secrete cytokines and help the function of cytotoxic T cells or B cells for the production of antibodies. Effector T cells are divided into T helper (Th)1, Th2 and Th17 cells, depending on the major cytokines that they produce. Th1 cells mainly make IL-2 and IFN-γ (which favour the elimination of pathogens), Th2 cells produce IL-4 and IL-13 (which associate with allergy and parasitic infection) and Th17 cells make the proinflammatory cytokine IL-17.

The CD8+ T cells in SLE are impaired, and activated CD4+ helper T cells produce elevated amounts of cytokines and help B cells to secrete autoantibodies that form immune complexes which can bind to and/or remain trapped in tissue, with subsequent inflammation and organ damage. Most of the disturbed T cell homeostasis in SLE seems to depend on aberrant mechanisms of peripheral control, and it is generally thought that central tolerance (that during thymic development allows the deletion of autoreactive T cells before they migrate to the periphery) may not be affected or can only partly influence T-cell autoimmunity in SLE (Wither J., Vukusic B.1998, Fatenejad et al. 1998). On the contrary, several mechanisms of peripheral immune tolerance are abnormal in SLE T cells, including: a) resistance to the induction of anergy (Xu L. et al.2004), b) reduced apoptosis and impaired clonal deletion of autoreactive T cells (Budagyan et al. 1998), c) increased spontaneous signalling and decreased threshold for the activation of T cells, and d) reduced number and/or
function of regulatory T cells (in addition to indirect factors such as an abnormal cytokine production that contribute to immune deviation).

SLE T cells display spontaneously increased activation associated with a reduced threshold of activation to self antigens, yet they are hypo-responsive to further antigenic stimulation (Murashima et al. 1990, Dawisha et al. 1994), furthermore they are resistant to apoptosis (Budagyan et al. 1998), show increased survival after activation, and have many altered intracellular signalling pathways. At the molecular level, T cell receptor (TCR) stimulation in lupus T cells associates with an increased signalling protein phosphorylation and a sustained increase in free intracellular Ca$^{++}$ (Vassilopoulos et al. 1995). Lupus T-cell deficiencies in signaling pathways include a decreased expression of the CD3 $\zeta$ chain, a decreased expression of protein kinase (PK)C, a reduced expression of the p65-RelA subunit of the transcription factor NF-κB, a decreased activity of PKA, a decreased phosphatase activity of CD45 (Takeuchi et al. 1997), a reduced levels of the intracellular signalling protein Lck in lipid rafts (Jury et al. 2003) and a defective phosphorylation of Cbl-b, a factor that negatively regulates transmembrane signaling (Yi et al. 2000). Other abnormalities include an increased binding of the transcriptional inhibitor cyclic AMP response element modifier (pCREM) to the IL-2 promoter (Tenbrock et al. 2003). IL-2 abnormalities have a central relevance in the activity and function of T cells in SLE. IL-2 is a pivotal regulator of T-cell responses, and it is reduced in lupus mice and in some patients with SLE. Additional molecular mechanisms that could contribute to impaired T-cell functions in SLE include histone acetylation and methylation, as shown by the finding that treatment with histone deacetylase inhibitors can suppress murine lupus (Mishra et al. 2003).

### 1.1.2.4 Role of B cells

The role of the B cell in the pathogenesis of immune mediated glomerulonephritis (GN) has traditionally been viewed as limited to that of antibody producer. However, it is increasingly appreciated that B cells contribute to the pathogenesis of GNs in many other ways. They can function as potent antigen-presenting cells (APCs), and in this role, their ability to clonally expand makes them highly efficient activators of antigen-specific T cells. More recent evidence suggests that B cells also play a role in the production of lymphangiogenic factors; thus, the B cell may orchestrate the local expansion of lymphatics required to support a florid immune response. Furthermore, B
cells may regulate T cells and dendritic cells (DCs) through the production of cytokines or regulatory antibodies.

Evidence for an antibody-independent role for B cells is suggested by animal models, that is, an MRL<sup>lpr/lpr</sup> lupus prone mouse with nonsecretory plasma cells developed evidence of autoimmune disease (Chan OT. et al. 1999), yet B-cell-deficient MRL<sup>lpr/lpr</sup> mice did not (Shlomchik et al. 1994). B cells producing autoantibodies in SLE have undergone extensive clonal expansion, suggesting that the antibodies are produced in response to chronic stimulation of B cells by antigen and costimulatory autoreactive CD4<sup>+</sup> T cells—therefore suggesting an important role for the autoreactive T cell in addition to B lymphocytes. Another B-cell-related functions likely to be important in the pathogenesis of SLE is cytokine release, particularly proinflammatory IL-10, tumor necrosis factor (TNF)-α, and IL-6, all of which are produced in high levels in SLE, and BlyS/BAFF (Blymphocyte stimulator/B-cell activating factor; a TNF-family cytokine that promotes B-cell maturation and survival and plasma cell differentiation) (Martin F., Chan AC. 2006). The role of the B lymphocyte as an APC is also likely to be essential in the development of autoimmunity. In experimental models of autoimmune arthritis, the APC function of B cells was essential for the development of disease, while the antibody-secreting function was not (O’Neill SK. et al. 2005, Chan OT. et al. 1999). Ultimately, activated B cells can aggregate into ectopic lymph node-like structures containing plasmablasts, memory B cells, and plasma cells are observed in sites with chronic inflammation.

**1.1.2.5 Anti-DNA antibodies**

Antibodies to DNA were first described in 1957 (Holborow EJ. et al 1957). They constitute a subgroup of antinuclear antibodies that bind ssDNA, dsDNA, or both. They might be IgM antibodies or any of the subclasses of IgG antibodies. Anti-dsDNA antibodies are thought to play a crucial role in the pathogenesis of lupus nephritis (Hahn BH. 1998). In many patients with SLE, increased renal disease activity is associated with rising titres of anti-DNA antibodies. Antibodies to ssDNA and dsDNA are part of the normal repertoire of natural autoantibodies; most of these are low-affinity IgM antibodies that react weakly with several self-antigens. However, these natural antibodies can undergo an isotype switch (from IgM to IgG) that increases their potential to be pathogenic. In addition, somatic mutations in the encoding
immunoglobulin genes might result in the production of high-affinity IgG antibodies to DNA.

It is indisputable from histopathological analyses that immune aggregates are present at sites of injury in glomeruli. Whether these are derived from circulating immune complexes or from an \textit{in situ} combination of antigen and antibody were once debatable. Although anti-dsDNA was once thought to cause glomerulonephritis by forming complexes with DNA that are passively trapped in the glomeruli, many investigators now believe that anti dsDNA antibodies are pathogenic to the kidney via direct (cross-reactivity) or indirect (via a nuclear antigen bridge) binding to glomerular structures. A series of studies by Chan \textit{et al} (1995, 1997, and 2002) have shed light on the potential pathogenicity of anti-DNA binding to glomerular cells in lupus nephritis. Subsets of anti-DNA antibodies from patients with SLE were shown to bind to human mesangial cells and endothelial cells, and the cellular binding of these autoantibodies correlated with disease activity (Chan \textit{et al} 1995, 1997, and 2002). In addition to cell binding, there is convincing evidence to show that anti-dsDNA penetrate into living cells. Administration of certain anti-dsDNA antibodies to nonautoimmune mice in vivo leads to cell penetration and intranuclear Ig deposits in the kidney and other organs (Madaio \textit{et al} 1998). Apart from glomerular cells, renal tubular cells, hepatocytes, neuronal cells, fibroblasts and mononuclear cells are all susceptible to penetration by anti-dsDNA antibodies. One intracellular effect of anti-DNA antibodies is to enhance cell growth and proliferation, or conversely induce apoptosis. Madaio \textit{et al} (1996) reported that nuclear localizing anti-DNA antibodies bind to DNAse I in living cells and inhibit the activity of this enzyme, making the cells more resistant to apoptosis. This observation might explain the finding of glomerular hypercellularity in mice injected with penetrating antibodies. In contrast, the cytopathic effects of anti-dsDNA antibodies to induce cell death have been demonstrated by others. (Hsieh \textit{et al} 2001). Up-regulation of apoptosis by anti-DNA antibodies would be consistent with the observation that anti-DNA antibodies can enhance cleavage of DNA (Kubota \textit{et al} 1996). Apart from their effect on cell viability; anti-dsDNA antibodies might influence pro-inflammatory pathways upon their binding to cell membrane antigens and/or entry into the cell cytosol.
1.1.2.6 Nucleosomes

Accumulating evidence suggests that autoantibody interactions occur with nucleosomes (complexes of DNA and histone-containing pairs of histone peptides around which dsDNA are wrapped twice. Antibodies reactive to nucleosomes have been detected both in SLE patients and in murine lupus models, even prior to the development of anti-dsDNA and antihistone antibodies (Monestier M.1997). These antibodies are immunoglobulin G in isotype, and bind to glomerular basement membrane via nucleosomes (Tax et al.1995). Generation of nucleosomes in vivo requires apoptosis (Casciola-Rosen L., Rosen A.1997). These nucleosomes and intracellular debris appear as blebs on apoptotic cell surfaces, and might incite T cell-driven stimulation of B cells. The injection of syngeneic apoptotic cells into normal mice has been shown to generate antinuclear antibodies and immune deposition in kidneys (Mevorach et al.1998). Data generated by transmission electron microscopy, immune electron microscopy (IEM), and co-localization IEM analyses using experimental antibodies specific for dsDNA, histones, transcription factors, or laminin identified exposed glomerular basement membrane-associated nucleosomes as target structures for nephritogenic autoantibodies in vivo (Kalaaji M. et al.2006a, 2006b, 2007). Furthermore, terminal deoxynucleotidyl-transferase (TdT)-mediated dUTP nick end-labeling (TUNEL) assay and activated caspase 3 staining demonstrated that murine lupus nephritis was linked to the accumulation of apoptotic cells in glomeruli and chromatin in glomerular capillary membranes and in the mesangial matrix (Kalaaji M. et al.2006b, 2007). These data suggest that apoptotic nucleosomes can be released, bound to glomerular membranes, but not cleared. In this situation, they may be targeted by pathogenic anti-nucleosome antibodies. It would be reasonable to assume that nucleosomes are one of the major autoantigens in lupus nephritis and might well be the primary inciting stimulus for the ‘antigen-driven’ IgG immune response.
Figure 2: A, a typical transmission electron microscopy observation in lupus nephritis is glomerular basement membrane (GBM)-associated EDSs. B, bound antibodies are stained with gold particles. C, the EDSs are shown as dark unique structures. In D, it is shown how nucleosomes bind glomerular capillary membranes or the mesangial matrix (GBM) where they are observed as EDSs (Mortensen et al 2008).

1.1.2.7 Complement
An intimate but paradoxical relationship exists between the complement system and SLE. Kidney is an important source of complement synthesis. Activation of complements by immune complexes through classical pathway is supposed to be the main mechanism of tissue injury. Wang et al. demonstrated decreased proteinuria and renal disease in NZB/NZW F1 mice, a mouse model of SLE, after treatment with a blocking anti-C5 antibody (Wang Y. et al. 1996). The involvement of the complement system in SLE occurs in three steps, known as the ‘waste disposal hypothesis’. The first step is the failure to clear autoantigens i.e. defective waste disposal (Walport MJ 2001). This is the stage at which complement deficiency might have a pathogenic role. The second step is the uptake of autoantigen by immature dendritic cells in the presence of inflammatory cytokines, which causes these cells to mature into antigen-
presenting cells, allowing the presentation of autoantigens to T cells. The third step is the pathogenesis of lupus nephritis provision of help by T cells to autoreactive B cells, which have taken up autoantigen by means of their immunoglobulin receptors. Such B cells mature into plasma cells that secrete autoantibodies. It is likely that in the majority of patients, SLE develops only in the presence of abnormalities in more than one of these steps. At the same time hereditary deficiencies in the complement components of the classical pathway increase the risk of lupus and lupus like disease. Deficiencies in C1, C2, C4 and CR1 predispose an individual to the development of SLE. C1q-deficient mice developed renal injury with vascular thrombosis, proteinuria and renal failure (Robson et al. 2001). Animal studies have produced conflicting data as to whether C3 confers protective or harmful effects (Einav S. et al. 2002, Sekine H. et al. 2001). At present, it is difficult to reconcile the potentially positive and negative impacts of complement in SLE. It is difficult to ascribe the role of complement in lupus as a bystander effect, especially when complement has several potent effector mechanisms to induce inflammation and tissue damage.

1.1.2.8 Role of cytokines
The role of TNF-α in lupus is controversial. This cytokine may be protective in patients with lupus, since giving TNF-α to lupus-prone NZB/W F1 mice delayed the development of lupus (Jacob CO., McDevitt HO. 1988). The protective effect is specific to that mouse strain, and the mechanism is unknown. In some patients with rheumatoid arthritis who were treated with anti–TNF-α antibodies, anti–dsDNA antibodies developed (Charles PJ. et al. 2000), and lupus erythematosus developed in a few of these patients (Mohan AK. et al. 2002). Serum levels of IL-10 are consistently high in patients with lupus, and they correlate with the activity of the disease (Houssiau FA. et al. 1995). IL-10 has a number of biologic effects, including stimulation of polyclonal populations of B lymphocytes. Blocking this cytokine could reduce the production of pathogenic autoantibodies.
Serum levels of interferon-α are also elevated in patients with active lupus (Rönnblom L., Alm GV. 2003), and microarray studies showed that 13 genes regulated by interferon were up-regulated in peripheral-blood mononuclear cells from patients with lupus, as compared with similar cells from healthy controls (Baechler EC. et al. 2003). In studies of lupus-prone NZB/W F1 mice, nephritis developed 15 to 20 weeks earlier in mice continuously exposed to interferon-α from a young age than in control mice not subject to this exposure (Mathian A. et al. 2005). Anti-interferon drugs may be the next anticytokine agents to be developed as treatments for patients with lupus.

The B-lymphocyte stimulator (BLyS) is a member of the TNF-ligand superfamily. It promotes the proliferation and survival of B lymphocytes. Circulating levels of BLyS are elevated in several other conditions, including rheumatoid arthritis and Sjögren’s syndrome, as well as in lupus. The overexpression of BLyS has been detected in both humans with lupus and lupus-prone mice (Cancro MP. et al. 2009).

IL-6 is yet another important cytokine which, among many other effects, induces the expression of acute phase proteins and also leads to increased antibody secretion by B-lineage cells (Naka T. et al. 2002).

TGFβ could exert a bidirectional effect similar to the effects of the classical Th2 cytokines, with less inflammation on the one hand, but more fibrosis on the other. TGFβ may also play a role for regulatory T cell action. Increased glomerular TGFβ
was found present and locally produced in samples of adults as well as children with lupus nephritis (Iwano M. et al. 1994, Yamamoto T. et al. 1996). In MRL<sup>pr/pr</sup> mice, where TGFβ is likewise over expressed, additional TGFβ was beneficial with regard to autoantibody formation, kidney disease and survival (Raz E. et al. 1995), but its role in fibrosis has been suggested to be critical.

1.1.2.9 Other inflammatory mediators of cell migration in chronic inflammation

So far we have seen how the immunological reaction is build up in lupus nephritis. Apoptotic bodies are a source of self antigen, which in turn leads to release of pathogenic antibodies into the circulation. Pathogenic antibodies then form immune complexes and along with nucleosomes get deposited in various organs. This is where the inflammatory mediators contribute to the pathology of lupus. Initiation of inflammatory reaction is characterised by expression of cytokines, chemokines, and hyper-infiltration of immune cells. In chronic inflammatory conditions like lupus nephritis leukocytes leave the intravascular compartment and exit into the interstitium. A series of distinct molecular interactions between the monocyte and the lining vascular endothelium are essential in this process. Certain molecules presented by vascular endothelial cells on their luminal surface represent the ‘signs’ that indicate the monocyte to slow down. Transient interactions of E- and L-selectins of the endothelium and the monocyte surface reduce the monocyte flow velocity and appears like cells slowly rolling on a sticky surface upon flow chamber or in vivo microscopy. These transient interactions are required to eventually enable the binding of chemokines that are presented on the luminal surface of endothelial cells with their respective chemokine receptors on the monocyte. This process is necessary to activate members of the β<sub>2</sub>-integrin family such as leukocyte function-associated antigen-1 (LFA-1) (αMβ<sub>2</sub>, CD11a/CD18) and Mac-1 (αMβ<sub>2</sub>, CD11b/CD18) on the macrophage surface. Upon activation these can interact with endothelial counter-receptors such as ICAM-1 and surface-associated fibrinogen which enables full adhesion appearing as monocyte arrest on microscopy. The subsequent monocyte transmigration into the interstitium involves additional molecules that either facilitate transcellular or paracellular migration, the latter requiring the opening and closing of endothelial cell-endothelial cell interactions including tight junctions.
### Table 2: Mediators of cell migration as novel anti inflammatory targets.

<table>
<thead>
<tr>
<th>Target</th>
<th>Drug</th>
<th>Company (Status of the compound)</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Selectins</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>antagonist</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>antagonist</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P- and E-selectin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mAb against P-selectin</td>
<td></td>
<td>Design Labs (Phase II)</td>
<td>Mocco J (2002)</td>
</tr>
<tr>
<td></td>
<td>CY1747 (PB 1.3)</td>
<td>Epimmune (Preclinical 2001)</td>
<td>Lefer DJ (2000)</td>
</tr>
<tr>
<td>mAb against L-selectin</td>
<td>DREG200</td>
<td>Boehringer (Preclinical 2001)</td>
<td>Lefer DJ (2000)</td>
</tr>
<tr>
<td><strong>β₂ integrins (CD18)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>the β₂ integrins</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Humanized mAb against</td>
<td>Erlizumab</td>
<td>Genentech Roche Phase II (halted 2001)</td>
<td>Baran KW (2001)</td>
</tr>
<tr>
<td>β₂ integrins</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Murine mAb against the</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α₁ integrin (CD11a)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Humanized mAb against</td>
<td>Efazulimab</td>
<td>Genentech Xoma Awaiting FDA and EMEA (2003)</td>
<td></td>
</tr>
<tr>
<td>α₁ integrin</td>
<td></td>
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<tr>
<td>integrin</td>
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<td>integrin</td>
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<tr>
<td>integrin</td>
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<tr>
<td><strong>α₄ integrins (CD49d)</strong></td>
<td>Natalizumab</td>
<td>Elan, Biogen, Approved</td>
<td>Miller DH (2003)</td>
</tr>
<tr>
<td>mAb against α₄ integrin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Small peptide antagonist</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>of α₄β₁ (CD29/CD49d)</td>
<td>MLUPUS NEPHRITIS02</td>
<td>Genentech Millenium Phase II 2003</td>
<td>van Assche G (2002)</td>
</tr>
<tr>
<td>and α₂β₂ integrins</td>
<td>(LDP02)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α₄β₂ integrin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antagonist of α₄β₁</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>integrin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antisense nucleotide to</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ICAM-1</td>
<td>A205804</td>
<td>Abbott Icos Preclinical 2002</td>
<td>Zhu D (2001)</td>
</tr>
<tr>
<td>Inhibitors of ICAM-1 and</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E-selectin expression</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Others</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mAb PECAM-1</td>
<td>mAb PECAM-1</td>
<td>preclinical</td>
<td>Vaporiyan, A (1993)</td>
</tr>
<tr>
<td>mAb JAM-A</td>
<td>mAb BV-11</td>
<td>preclinical</td>
<td>Khandoga, A (2005)</td>
</tr>
<tr>
<td>soluble JAM-C (sJAM-C)</td>
<td>soluble JAM-C</td>
<td>preclinical</td>
<td>Scheiermann (2009)</td>
</tr>
<tr>
<td>anti-CD99 monoclonal</td>
<td>mAb CD99</td>
<td>preclinical</td>
<td>Dufour EM (2008)</td>
</tr>
<tr>
<td>antibody</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
1.1.2.10 Role of chemokines

All types of renal cells can express chemokines upon stimulation. The regulatory role of chemokines for leukocyte recruitment during inflammatory tissue injury has gained most interest over the last decade. In the kidney, all types of renal cells have been shown to produce inflammatory chemokines upon various kinds of injury, including CCL2, CCL3, CCL4, CCL5 or CXCL10. Local production of these chemokines initiates recruitment of macrophages, natural killer cells and T-cell subsets, which leads to subsequent glomerulonephritis in the glomerular compartment and to interstitial nephritis in the tubulointerstitium. Secreted chemokines bind to endothelial surfaces or interstitial matrix components and mediate leukocyte migration through their corresponding CCR on the surface of the leukocyte. The specificity of recruiting a particular leukocyte subset is provided by specific surface expression patterns of CCR. For example, neutrophils but not macrophages express CXCR1, which facilitates recruitment upon recognition of the neutrophil attractant chemokine CXCL8. Glomerular and interstitial leukocyte recruitment is regulated by different chemokines. CCL5 is critical for glomerular macrophage recruitment, as CCL5 blockade reduces glomerular macrophage counts during immune complex glomerulonephritis (Anders HJ. et al. 2003), but CCL5 blockade or lack of the CCL5 receptor CCR5 has no effect on interstitial macrophage and T-cell accumulation after unilateral ureteral obstruction (Eis V. et al. 2004).

In contrast, CCR1 is critical for interstitial macrophage and T-cell recruitment during progressive lupus nephritis, but CCR1 blockade does not affect the number of glomerular macrophages in murine model of lupus nephritis (Anders HJ. et al. 2004).

In this study Anders et al. (2004) could show prevention of disease progression with late onset of CCR1 antagonist treatment which explains a role of CCR1 other than mediating leukocyte recruitment. Another target for therapeutic intervention in kidney disease is CCL2. Deletion of the CCL2 gene dramatically reduced tubulointerstitial injury in mice with nephrotoxic serum nephritis or lupus nephritis of MRL1pr/lpr mice (Tesch GH. et al. 1999a,1999b).
Figure 4: Hypothetical model of the role of chemokines and chemokine receptors during progressive renal diseases. (A) Normal renal tissue. (B) Initiation phase. (C) Amplification phase. (D) Progression phase. (Segerer et al. 2000)

Gene transfer of a truncated human CCL2 protein demonstrated beneficial effects in renal fibrosis after unilateral ureteral obstruction in mice (Wada T. et al. 2004), protein-overload disease in rats (Shimizu H. et al. 2003), ischaemic acute renal failure in mice (Furuichi K. et al. 2003) and in MRL<sup>1nr/1pr</sup> mice with lupus nephritis (Hasegawa H. et al. 2003). Epidemiological studies in renal transplant recipients with mutations in chemokine or CCR genes support the relevance of such rodent data in human kidney disease (Fischereder M. et al. 2001, Kruger B. et al. 2002). These data suggest that interstitial fibrosis, the common final pathway of most chronic nephropathies, may be susceptible for therapeutic blockade of selected chemokines or chemokine receptors

CCL2 in nephritis

There is an increasing body of evidence that the CC chemokine monocyte chemoattractant protein-1 (CCL2) plays a major role in the pathogenesis of progression of renal failure. This is based on observations both in various animal models of renal damage and in different types of human renal disease. Locally produced CCL2 seems to be particularly involved in the initiation and progression of
tubulointerstitial damage. The latter has been documented in experiments using transgenic mice with nephrotic serum-induced nephritis: compared with wild-type mice, CCL2-deficient mice exhibit less tubulointerstitial lesions, but they exhibit no differences in glomerular lesions (Tesch GH. et al.1999a). There is, however, evidence that CCL2 also plays a role in the progression of glomerular lesions, since glomerular expression of CCL2 correlates with the degree of renal damage in inflammatory (Panzer U. et al. 2001) and non-inflammatory (Taal MW. et al. 2000) models of glomerular injury. Furthermore, in humans with crescentic glomerulonephritis, CCL2 is not only expressed in tubular epithelial cells and leukocytes infiltrating the tubulointerstitium, but also in crescents and parietal epithelium (Segerer S. et al.2000). In experimental crescentic glomerulonephritis, administration of antibodies to CCL2 decreases the extent of proteinuria, reduces glomerulosclerosis and improves renal dysfunction. CCL2, a potent chemoattractant for monocytes and for T cells, is secreted from many kinds of renal cells, including endothelial, mesangial, tubular epithelial and interstitial cells, and macrophages, in response to stimulation with proinflammatory cytokines and immune complexes. A significant number of T cells and macrophages infiltrate the kidneys of patients with lupus nephritis. Overexpression of CCL2 in renal tissue parallels mononuclear cell accumulation (Tesch GH. et al.1999b, Perez de Lema G. et al. 2001). In biopsy samples from patients with lupus nephritis, the presence of CCL2 has been observed in endothelial cells, macrophages, mesangial cells, and tubular epithelial cells (Rovin BH. et al.1994). In addition, it has been reported that elevated urinary CCL2 excretion reflects lupus nephritis disease activity (Noris M et al. 1995). These findings suggest that overexpression of CCL2 plays an important role in the pathogenesis of lupus nephritis in humans and in animal models. In lupus nephritis, production of CCL2 in the many types of renal cells described above is triggered by the deposition of ICs and complement activation, and subsequently leads to mononuclear cell infiltration. Moreover, after CCL2 production by local cells, the infiltrating macrophages become a source of CCL2, resulting in an amplification loop.

1.1.3 Biology behind therapy for lupus nephritis
Since lupus nephritis is a chronic disease with no known cure, its treatment is restricted to dealing with symptoms, that is, management of inflammatory reactions by suppression of the activity of the patient’s immune system. This is done mainly with steroids alone or in combination with other drugs. Mild or remittent disease may
sometimes be left untreated, but patients should avoid sun exposure. If required NSAIDs or anti-malarial may be used.

**Figure 5**: Current therapy and novel therapeutic approaches for lupus.

Anti-malarial and immunosuppressants are used preventively to reduce incidence of flares, the process of the disease and to lower the need of steroid use. In more severe cases, corticosteroids and immunosuppressant, drugs that modulate immune system, are used to control the disease and prevent flares. Since immunosuppressant drugs can have serious side effects, they are mainly used only for patients with severe systemic disease or organ failure (e.g. nephritis). Cyclophosphamide (CYC), MMF, azathioprine are the first line immunosuppressant drugs for treatment of lupus nephritis. Beside steroids hydroxychloroquine or azathioprine is sometimes used to
control lupus during pregnancy since these drugs appear to have fewer risks to the foetus than other drugs.

Lupus patients are more susceptible to infections, since both lupus and its treatments, in particular corticosteroids and immunosuppressive drugs, affect the immune system. Infections can induce lupus flare, which further increases the risk of infection. Finally lupus appears to increase the risk of cancer, especially non-Hodgkin’s lymphoma, which affects the lymphoid organs. Immunosuppressive drugs used to treat lupus can also enhance the risk of cancer. Thus the main goal of research is to find drugs to treat lupus more specifically, without systemically suppressing the immune system.

Treatments, that are more specific in modifying particular subsets of immune cells (e.g. T/B cells), or the activity of cytokine they secret have been gaining attention. Biological agents targeting B cells are under development including a fully humanised anti-CD20 antibody (Rituximab), an anti-CD22 antibody (Epratuzumab) and a number of molecules blocking BlyS have been developed. The human anti-BlyS antibody (Belimumab) is currently in a phase III study. BlyS (also called BAFF) is a soluble mediator that plays a role in the regulation of B-cell homeostasis and differentiation. The three known receptors for BlyS are differentially expressed at various stages of B-cell and plasma cell development suggesting that the consequences of BlyS blockade may differ from B-cell depletion by anti-CD20 treatment (Treml et al. 2006).

A variety of co-stimulatory ligand/receptor pairs have been described that modulate the interaction of dendritic cells and T cells, or T cells and B cells, respectively. The first family of these to be discovered was the B7 family. CTLA-4-Ig is a drug that blocks the interaction of CD80 and CD86 on antigen-presenting cells with CD28 on T cells and has negative regulatory effects. CTLA-4-Ig has been approved for the treatment of RA after recent successful RCTs (Todd et al. 2007). It has also been demonstrated to block nephritis in a mouse model of SLE (Cunnane et al. 2004) and human studies are ongoing. There were 3 trials with anti-CD40L mAb IDEC-131, enrolling in global 110 lupus patients, although not all had with nephritis (Huang et al. 2002, Davis Jr et al. 2001) and one with BG9588 which included 28 patients with active proliferative lupus nephritis (Boumpas et al. 2003). In summary the results failed to demonstrate significant drug benefit compared to placebo in terms of time to renal flare, SLE disease activity index (SLEDAI) and serologic markers (complement and anti-DNA Ab) (Kalunian et al. 2002). The study with BG9588 was prematurely
terminated due to the increased occurrence of thromboembolic events despite concurrent prophylactic anticoagulation (Boumpas et al. 2003, Kawai et al. 2000).

A very different approach is to try modulating the autoimmune process in SLE in an antigen-specific way. Abetimus is a molecule composed of a series of linked oligonucleotides. The concept behind Abetimus is that the drug will block the binding of anti dsDNA antibodies to their autoimmune targets and/or will tolerise B cell with antigen-specificity for DNA (Alarcon-Segovia et al. 2003). Results in clinical trials have been very modest so far. Edratide represents a similar approach. Edratide is a peptide derived from the antigen-binding region of a human monoclonal anti-dsDNA antibody (Sharabi et al. 2007). It has been proposed that this molecule can modulate the function of DNA-reactive B cells through idiotype–anti-idiotype interactions.

Even though T cells and B cells targets seem to promising, clinical trail results for these therapies do not look encouraging (see table). This leaves scope for the development of new strategies to encounter lupus nephritis.

**Table 3: SLE trail data for new biological therapies**

<table>
<thead>
<tr>
<th>Target</th>
<th>Treatment</th>
<th>In mouse model</th>
<th>In humans</th>
</tr>
</thead>
<tbody>
<tr>
<td>T cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD40 and CD40-L</td>
<td>BJ9588</td>
<td>Effective</td>
<td>Trial stopped for side effects (Boumpas DT. et al. 2003)</td>
</tr>
<tr>
<td>ICOS and ICOS-L</td>
<td>Anti-CD154</td>
<td>Effective</td>
<td>End points not met (Kalunian KC. et al. 2002)</td>
</tr>
<tr>
<td>B cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD22</td>
<td>Epratuzumab</td>
<td></td>
<td>Effective (Dorner T. et al. 2006)</td>
</tr>
<tr>
<td>CD20</td>
<td>Rituximab</td>
<td></td>
<td>End points not met (Explorer Trail)</td>
</tr>
<tr>
<td>Anti metabolite</td>
<td>MMF</td>
<td></td>
<td>End points not met (ALMS study)</td>
</tr>
<tr>
<td>Multivalent DNA</td>
<td>Abetimus</td>
<td></td>
<td>End points not met (Cardiel MH. et al. 2008)</td>
</tr>
<tr>
<td>Anti-BAFF</td>
<td>Belimumab</td>
<td></td>
<td>End points not met (Wallace DJ. et al. 2009)</td>
</tr>
</tbody>
</table>

**Targeting CCL2 in lupus**

As we have reviewed in early part of this introduction CCL2 presents an exciting target to attenuate lupus nephritis particularly in controlling the inflammatory cascade of the lupus pathogenesis. To block CCL2 and its receptor (CCR2) signalling pathway Shimizu et al. (2004) used the strategy of anti-CCL2 gene therapy involving the
transfection of a mutant human CCL2 gene into skeletal muscle. This mutant human CCL2, designated 7ND, lacks NH$_2$-terminal amino acids 2–8 and has been shown to work as a dominant-negative inhibitor of human CCL2. Histological findings of kidneys in treated mice with 7ND showed that protection against renal injury resulted from reduced infiltration of leucocytes. 7ND gene therapy was shown to prolong the life span of MRL$^{lpr/lpr}$ mice (Shimizu S et al. 2004). Another group (Hagesawa et al. 2003) injected CCL2 antagonist- or TARC antagonist-transfected MRL/N-1 cells subcutaneously into MRL$^{lpr/lpr}$ mice ages 7 weeks (before the onset of lupus nephritis) and 12 weeks (at the early stage of the disease). After 8 weeks, mice bearing the CCL2 antagonist showed markedly diminished infiltration of macrophages and T cells, glomerular hypercellularity, glomerulosclerosis, crescent formation, and vasculitis compared with control mice. Authors concluded that the effect was due to decreased production of interferon-$\gamma$ and IL-2 in the kidney. However, such experimental approaches cannot be used in humans because of irrepresible antagonist production and tumor formation (Shimizu S et al. 2004, Hagesawa et al. 2003).

Following these impressive findings we thought of targeting CCL2 blockade with a clinically applicable approach. Spiegelmer technology based aptamers rose to the occasion as these are safe, biostable and specifically binding to the target.

1.2. Spiegelmers: Next generation aptamers

To achieve CCL2 antagonism we used a RNA-aptamers (Spiegelmer), a patented technology of NOXXON Pharma (Berlin). RNA-aptamer binds to the active site of target chemokine and makes them biologically non-functional.

An aptamer is a nucleic acid structure that can bind to a target molecule conceptually similar to an antibody that recognizes an antigen. Aptamers have binding characteristics similar to peptides or antibodies, with affinities in the low nanomolar to the picomolar range. However, there are several drawbacks to aptamers as useful therapeutic products. As relatively small molecules, aptamers demonstrate circulating half-lives in vivo in the order of minutes. This situation can be addressed by attaching large inert molecules to aptamers (e.g. polyethylene glycol) to reduce their elimination via the kidney and increase their presence in the circulation. Still, aptamers, as natural nucleic acid polymers, are prone to rapid degradation by nucleases that are present in all tissues in the body.
Spiegelmers are biostable aptamers, that have all of the diverse characteristics of aptamers and possess a structure that prevents enzymatic degradation. While conventional aptamers are created from the natural D-nucleotides, which are recognized by the nucleic acid degrading enzymes, Spiegelmers are synthesized from mirror image L-oligonucleotide and hence they can not be degraded by any nucleases since there are no such enzymes in the body capable of interacting with these unnatural molecules. Spiegelmer technology is based on the simple concept that if an aptamer binds its natural target, the mirror image of the aptamer will identically bind the mirror image of the natural target (Figure 6). The process of aptamer selection is carried out against the mirror image target protein; an aptamer against this unnatural mirror image is obtained. More important, this Spiegelmer is now resistant to nuclease degradation. Spiegelmers should not be confused with antisense RNAs in that they do not directly interfere with transcription or translation of their target molecules. They are designed to bind specifically to extracellular proteins, either a receptor or its ligand, similar to the behavior of a monoclonal antibody, aptamer or peptide. Spiegelmers appear to be non-immunogenic, even under the most inductive conditions for antibody formation in rabbits. These molecules are termed “Spiegelmer” from the German word “Spiegel” meaning “mirror”. Spiegelmers possess the high affinity binding characteristics of the best aptamers and antibodies in the low nanomolar and picomolar range, while defying enzymatic degradation that severely limits the utility of aptamers (Klussmann S. et al. 1996). Data indicate that Spiegelmers are stable in human plasma for over 60 hours at 37 °C (Figure 7.) while non-modified RNA aptamers are degraded in seconds under the same conditions (left panel, Figure 7). Results in animals indicate that a similar stability can be expected in vivo as well.
Figure 6: Representation of Spiegelmer generation. L-enantiomer of the target is processed to get the selective binding D-aptamer. Highly selective D-aptamer for mirror image (L-target) is then amplified and then mirrored to get L-aptamer which has the selective binding property for natural D-target. (Taken from www.noxxon.net)

Figure 7: Representation of Spiegelmer stability. Left panel with D-enantiomeric RNA which degrades in seconds when incubated in human plasma at 37°C., while L-enantiomeric RNA is stable even at 60 hrs of incubation in human plasma (right panel)
Summary of Spiegelmer Properties

• high binding specificity to their target
• comparable binding affinity as antibodies (low nanomolar-picomolar)
• stable in human plasma and after injection into animals
• non-immunogenic
• low toxicity (target-based)
• synthesized using standard chemistry (scalable)
• no biological contaminants
• easy to formulate (polar) with excellent solubility
1.3 MRL\textsuperscript{lpr/lpr} mice: mouse model of lupus nephritis

MRL\textsuperscript{lpr/lpr} mice lack functional expression of the apoptosis-inducing receptor Fas, thereby accelerating the manifestation of the autoimmune disease, which also develops in wild-type MRL\textsuperscript{lpr/lpr} mice, albeit, with a slower kinetic. MRL\textsuperscript{lpr/lpr} mice show systemic autoimmunity, massive lymphadenopathy associated with proliferation of aberrant CD3\textsuperscript{+}B220\textsuperscript{-}CD4\textsuperscript{-}CD8\textsuperscript{-} T cells and immune complex-derived glomerulonephritis. Starting at approximately 3 months of age, levels of circulating immune complexes, such as those from spontaneously generated anti-dsDNA antibodies, rise dramatically in MRL\textsuperscript{lpr/lpr} but not in wild-type MRL controls. Lymph node weight of MRL\textsuperscript{lpr/lpr} increases approximately 75-fold over controls and renal pathology occurs extensively in MRL\textsuperscript{lpr/lpr} at approximately 4 months of age. In MRL/lpr mice, a proliferative glomerulonephritis is seen, with mononuclear cell infiltration, endothelial and mesangial cell proliferation, and crescent formation (Cohen PL., Eisenberg 1991). Finally, female MRL\textsuperscript{lpr/lpr} mice die at an average of 17 weeks of age and males at 22 weeks.

\textbf{Figure 8:} MRL\textsuperscript{lpr/lpr} mice life span and progression of autoimmune phenotype.
2. Research hypothesis/objectives

1. The role of CCL2/CCR2 axis in chronic inflammatory conditions is well known. But so far there is no therapy in the clinics which attenuate the inflammation mediated by CCL2, even though lots of efforts are being made. Gene therapy against CCL2 can suppress nephritis in murine model of lupus nephritis (Hagesawa et al. 2004). But these kinds of approaches are not clinically applicable, as these represent irreversible antagonism and have been reported for side effects. Considering these facts in mind our first objective was to evaluate the efficacy of an anti-CCL2 Spiegelmer (mNOX-E36).

2. Second objective was to compare the efficacy of Anti-CCL2 Spiegelmer (mNOX-E36) with that of cyclophosphamide and MMF.

3. Immunosuppressants like CYC and MMF effectively control rapidly progressing lupus. But with high exposure of immunotherapies there is always a possibility of adverse effects. In clinical practice lots of patients experienced infections, gonadal toxicities and other side effects which are even fatal in some cases. That is why lots of efforts are being made to discover novel ways to combat lupus nephritis. T cell and B cells based therapies did not show any promising results. Considering all these findings we thought of combining anti-CCL2 Spiegelmer with low dose of CYC and then comparing it high dose of CYC for its efficacy as well as safety.
3. Materials and Methods

3.1 Materials

Equipments

Balances:
Analytic Balance, BP 110 S  Sartorius, Göttingen, Germany
Mettler PJ 3000  Mettler-Toledo, Greifensee, Switzerland

Cell Incubators:
Type B5060 EC-CO₂  Heraeus Sepatech, München, Germany

Centrifuges:
Heraeus, Minifuge T  VWR International, Darmstadt, Germany
Heraeus, Biofuge primo  Kendro Laboratory Products GmbH, Hanau, Germany
Heraeus, Sepatech Biofuge A  Heraeus Sepatech, München, Germany

ELISA-Reader
Tecan, GENios Plus  Tecan, Crailsheim, Germany

Fluorescence Microscopes
Leica DC 300F  Leica Microsystems, Cambridge, UK
Olympus BX50  Olympus Microscopy, Hamburg, Germany

Spectrophotometer
Beckman DU® 530  Beckman Coulter, Fullerton, CA, USA

TaqMan Sequence Detection System
ABI prism™ 7700 sequence detector  PE Biosystems, Weiterstadt, Germany

Other Equipments
Cryostat RM2155  Leica Microsystems, Bensheim, Germany
Cryostat CM 3000  Leica Microsystems, Bensheim, Germany
Homogenizer ULTRA-TURRAX
T25  IKA GmbH, Staufen, Germany
Microtome HM 340E  Microm, Heidelberg, Germany
pH meter WTW
Thermomixer 5436
Vortex Genie 2™
Water bath HI 1210

WTW GmbH, Weilheim, Germany
Eppendorf, Hamburg, Germany
Bender&Hobein AG, Zurich, Switzerland
Leica Microsystems, Bensheim, Germany

Chemicals and materials
Chemicals for the molecular biology techniques
RNeasy Mini Kit
RT-PCR primers
Qiagen GmbH, Hilden, Germany
PE Biosystems, Weiterstadt, Germany

Cell culture
DMEM-medium
RPMI-1640 medium
FSC
Dulbecco’s PBS (1×)
Trypsine/EDTA (1×)
Penicillin/Streptomycin (100×)
Biochrom KG, Berlin, Germany
GIBCO/Invitrogen, Paisley, Scotland, UK
Biochrom KG, Berlin, Germany
PAA Laboratories GmbH,
Cölbe, Germany
PAA Laboratories GmbH,
Cölbe, Germany
PAA Laboratories GmbH,
Cölbe, Germany

Antibodies
rat anti-Mac2
anti-CD3
anti-CD4
anti-CD8
anti-CD25
anti-CD45
anti-7/4
anti-Ly6G
Cederlane, Ontario, Canada
BD Pharmingen, Hamburg, Germany
BD Pharmingen, Hamburg, Germany
BD Pharmingen, Hamburg, Germany
BD Pharmingen, Hamburg, Germany
BD Pharmingen, Hamburg, Germany
Abd-Serotec,
BD Pharmingen, Hamburg, Germany
### Miscellaneous

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<td>Plastic histosettes</td>
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<td>SuperFrost® Plus</td>
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### Chemicals

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<td>HCl (5N)</td>
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<td>Merkaptoethanol</td>
<td>Roth, Karlsruhe, Germany</td>
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<td>SSC (Saline-sodium citrate Puffer)</td>
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<td>Tissue Freezing Medium</td>
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<td>Oxygenated water</td>
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<tr>
<td>Xylol</td>
<td>Merck, Darmstadt, Germany</td>
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3.2. Methods

3.2.1 Methods Part I

3.2.1.1 Grouping and treatment
Seven week old female MRL$^{lpr/lpr}$ mice were obtained from Harlan Winkelmann (Borchen, Germany) and kept in filter top cages under a 12 hour light and dark cycle. Water and standard chow (Sniff, Soest, Germany) were available ad libitum. All experimental procedures were approved by the local government authorities. At the age of 14 week, female MRL$^{lpr/lpr}$ mice were distributed into seven groups that received subcutaneous injections every alternate day as follows:

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Dosage level $^a$(mg/kg)</th>
<th>Dose volume (ml/kg)</th>
<th>Treatment Schedule</th>
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</thead>
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<tr>
<td>1</td>
<td>Vehicle (5% Glucose)</td>
<td>0</td>
<td>4</td>
<td>3 times/week</td>
<td>12</td>
</tr>
<tr>
<td>2</td>
<td>POC</td>
<td>25</td>
<td>4</td>
<td>3 times/week</td>
<td>12</td>
</tr>
<tr>
<td>3</td>
<td>POC-PEG</td>
<td>50</td>
<td>4</td>
<td>3 times/week</td>
<td>12</td>
</tr>
<tr>
<td>4</td>
<td>mNOX-E36</td>
<td>25</td>
<td>4</td>
<td>3 times/week</td>
<td>12</td>
</tr>
<tr>
<td>5</td>
<td>mNOX-E36-PEG</td>
<td>50</td>
<td>4</td>
<td>3 times/week</td>
<td>12</td>
</tr>
<tr>
<td>6</td>
<td>mNOX-E36 (PKa)</td>
<td>25</td>
<td>4</td>
<td>3 times/week</td>
<td>12</td>
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<tr>
<td>7</td>
<td>mNOX-E36-PEG (PKa)</td>
<td>50</td>
<td>4</td>
<td>3 times/week</td>
<td>12</td>
</tr>
</tbody>
</table>

Pka = Satellite animals for pharmacokinetics; $^a$ = in vivo doses of mNOX-E36 and POC-PEG in mg/kg apply to the combined total weight of the oligonucleotide part plus the coupled PEG moiety. Thus, mNOX-E36 has to be multiplied with the factor 0.29.

All Spiegelmer samples were provided by Noxxon Pharma AG, in ready to use formulation.
3.2.1.2 Route and rationale of test material administration
The chosen route of administration was subcutaneous, as the animals tolerate a large number of subcutaneous administrations better than numerous intraperitoneal injections, handling is easier and because by this route unPEGylated Spiegelmer is present longer in the circulation.

3.2.1.3 Test substance and formulation

<table>
<thead>
<tr>
<th>Spiegelmer</th>
<th>Sequence</th>
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<tr>
<td>mNOX-E36</td>
<td>5’-GGCGACAUUG GUUGGGCAUG</td>
</tr>
<tr>
<td></td>
<td>AGGCGAGGCC CUUUGAUGAA</td>
</tr>
<tr>
<td></td>
<td>UCCGCCGCCA-3’</td>
</tr>
<tr>
<td>mNOX-E36-PEG</td>
<td>40 kD PEG</td>
</tr>
<tr>
<td></td>
<td>5’-GGCGACAUUG GUUGGGCAUG</td>
</tr>
<tr>
<td></td>
<td>AGGCGAGGCC CUUUGAUGAA</td>
</tr>
<tr>
<td></td>
<td>UCCGCCGCCA-3’</td>
</tr>
<tr>
<td>Control Spiegelmer</td>
<td>Sequence</td>
</tr>
<tr>
<td>POC</td>
<td>5’-UAAGGAAACU CGGUCUGAUG</td>
</tr>
<tr>
<td></td>
<td>CGGUAGCGCU GUGCAGAGCU-3’</td>
</tr>
<tr>
<td>POC-PEG</td>
<td>40 kD PEG</td>
</tr>
<tr>
<td></td>
<td>5’-UAAGGAAACU CGGUCUGAUG</td>
</tr>
<tr>
<td></td>
<td>CGGUAGCGCU GUGCAGAGCU-3’</td>
</tr>
</tbody>
</table>

For in vivo application, the mNOX-E36 and the non-functional control Spiegelmer POC were used non-modified or modified with 40-kD PEG at the 3’ and 5’ terminus, respectively. The test substance (Spiegelmer solution) was dissolved in isotonic 5 % glucose solution.

3.2.1.4 Mortality, clinical signs, skin score and body weight
Mortality was recorded every week throughout the study. Animals were observed daily for clinical signs. In addition, MRL<sup>lpr/lpr</sup> mice of all groups were checked daily for cutaneous lupus manifestations, which typically occur in the facial or neck area. The severity was graded from 0 – 0.5 (no lesion), 1 (mild lesion), 2 (moderate) to 3 (severe). After 10 weeks of treatment, satellite animals for pharmacokinetics were kept alive in order to monitor survival rate. Body weight was recorded once a week during weeks 14 to 24 of age (10 weeks of treatment).
3.2.1.5 Pharmacokinetic analysis
In week 1, 5 and 10 of treatment, blood samples for pharmacokinetic analysis were collected in satellite animals at the following times:
- mNOX-E36 (25 mg/kg) at 0.083, 0.33, 0.66, 1, 3, 9, 12 and 24 hours after injection;
- mNOX-E36-P (50 mg/kg) at 3, 6, 9, 12, 24, 30, 36 and 48 hours after injection.
To correlate the pharmacokinetic alteration with the progression of the disease, additional samples were taken 3 hours post administration for unPEGylated mNOX-E36 (25 mg/kg) and 24 hours post administration for mNOX-E36 (50 mg/kg), after the first dose of the week. All samples were stored at –20 °C until analysed by method described below.

For immobilization of the Spiegelmer an L-RNA capture probe (CP) which is complementary (base-pairing) to one end of the analyte Spiegelmer is covalently coupled to a 96-well plate. After hybridization (base-pairing) of the analyte Spiegelmer to this capture probe, a second (biotinylated) L-RNA probe ("detect probe", DP) is hybridized (base-pairing) to the second end of the analyte. After unbound complexes have been removed, the complex of Spiegelmer and detect probe is detected by a streptavidine/alkaline phosphatase conjugate converting a chemiluminescence substrate.

3.2.1.6 Glomerular filtration rate
Preparation of 5% FITC-inulin solution
5% FITC-inulin was dissolved in two ml of 0.9% NaCl -- facilitated by heating the solution in boiling water.

Intravenous injection and blood collection
Mice were anesthetized using Isoflurane, which for approximately 20 seconds. 5% FITC-inulin (3.74μl /g body weight) was injected retroorbitally under anesthesia within 10 seconds. Under general anaesthesia, blood was drawn from the retro orbital plexus at 5, 10, 15, 20, 35, 60 and 90 minutes post administration.

Determination of fluorescence of the sampled plasma
Since pH significantly affects FITC fluorescence value, each plasma sample was buffered to pH 7.4, by mixing 10 μl of plasma with 40 μl of 500 mM HEPES (pH 7.4). The titrated samples were then loaded onto a 96-well plate, 50μl sample/well. Fluorescence was determined with 485 nm excitation, and read at 538 nm emission.
Calculation of GFR

A two-compartment clearance model may be employed for the calculation of GFR. In the two-compartment model used, depicted in Figure 9, the initial, rapid decay phase represents redistribution of the tracer from the intravascular compartment to the extracellular fluid. Systemic elimination also occurs, but the distribution process is relatively dominant during this initial phase. During the later, slower decay in concentration of the tracer systemic clearance of the tracer from the plasma predominates. At any given time ($t_X$), the plasma concentration of the tracer ($Y$) equals to $Ae^{-\alpha t_X} + Be^{-\beta t_X} + \text{Plateau}$.

![Figure 9: Representation two phase regression curve](image)

The parameters of above equation could be calculated using a non-linear regression curve-fitting program (GraphPad Prism, GraphPad Software, Inc., San Diego, CA.). GFR was calculated using the equation:

$$GFR = \frac{I}{(A/\alpha + B/\beta)},$$

Where $I$ is the amount of FITC-inulin delivered by the bolus injection; $A$ (Span1) and $B$ (Span2) are the $y$-intercept values of the two decay rates, and $\alpha$ and $\beta$ are the decay constants for the distribution and elimination phases, respectively.

3.2.1.7 Plasma, urine and tissue collection

After 10 weeks of treatment (on the day of sacrifice), blood samples were collected under ether anaesthesia 2 to 3 hours after the last administration. Blood was drawn by retroorbital bleeding using heparinised capillaries. Plasma was separated by centrifugation at 10,000 rpm for 5 minutes.

Urine samples were collected when the animals were 16, 21 and 24 weeks of age.
At study termination (at the end of the 10-week treatment period), organ weights of spleen and lymph nodes were determined. Spleen, lymph nodes, kidney, lung, liver, and salivary gland tissue samples were collected from each mouse for histology, RNA analysis.

3.2.1.8 Histology of kidney and lungs

Morphological and histological processing

From all mice, the tissues were isolated from kidneys or spleens and placed in plastic histocassettes and immediately fixed in 10% buffered formalin (formaldehyde in PBS) for overnight and next day processed, with automatic tissue-processor (Thermo-Shandon), which processes tissues in following manner- 70% ethanol (for 5 hours), 96% ethanol (2 hrs), 100% ethanol 3.5 hrs, xylene (2.5 hrs), paraffin (4 hrs). Then the histocassettes were taken out and the paraffin blocks were prepared with hot liquid paraffin using a machine (Microm- EC 350). The prepared blocks were allowed to cool down and later separated from the steel holders. These blocks were later used for making fine sections for different stainings (PAS or immunohisto). The fine sections (5μm) were prepared using the microtome (Microm- HM340E) and mounted gently on a glass frosted slide. Such slides were later allowed to dry for 2 hours in an oven at 50°C temperature. Deparaffinisation followed by dehydration was carried out by incubating the sections in xylene, 100% absolute ethanol, 95%, 80% and 50% ethanol followed by rinsing with PBS (2 changes, 3 minutes each). 5 μm sections for silver and periodic acid-Schiff stains were prepared following routine protocols for histopathology. From each mouse tissue collected as mentioned above, 2 μM thick paraffin-embedded sections were cut and processed for immunohistochemical staining performed on paraffin-embedded sections.

Analysis of glomerulonephritis

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). Activity index was considered as the sum of individual scores of the following items considered to represent measures of adaptive lupus nephritis: glomerular proliferation, leucocyte exudation, karyorrhexis/fibrinoid necrosis (X2), cellular crescents (X2), hyaline deposits, and interstitial inflammation. The maximum score was 24 points for activity index. Chronicity index was considered to be the sum of individual scores of the following items considered to represent measures of chronic irreversible lupus
nephritis: glomerular sclerosis, fibrous crescents, tubular atrophy and interstitial fibrosis. The maximum score was 12 points for the chronicity index.

**Immunostaining**

For immunostaining sections of formalin-fixed and paraffin-embedded tissues were dewaxed and rehydrated. Endogenous peroxidase was blocked by 3 % hydrogen peroxide and antigen retrieval was performed in Antigen Retrieval Solution (Vector, Burlingame, CA USA) in an autoclave oven. Biotin was blocked using the Avidin / Biotin blocking kit (Vector, Burlingame, CA USA). Slides were incubated with the primary antibodies for one hour, followed by biotinylated secondary antibodies (anti-rat IgG, Vector, Burlingame, CA USA), and the ABC reagent (Vector, Burlingame, CA USA). Slides were washed in phosphate buffered saline between the incubation steps. 3′3′Diaminobenzidine (DAB, Sigma, Taufkirchen, Germany) with metal enhancement was used as detection system, resulting in a black coloured product. Methyl green was used as counterstain, slides were dehydrated and mounted in Histomount (Zymed Laboratories, San Francisco, CA USA). The following primary antibodies were used: rat anti-MAC-2 (macrophages, Cederlane, Ontario, Canada, 1:50), anti-mouse CD3 (1:100, clone 500A2, BD), anti-mouse IgG1 (1:100, M32015, Caltag Laboratories, Burlingame, CA USA), anti-mouse IgG2a (1:100, M32215, Caltag Laboratories, Burlingame, CA USA). Negative controls included incubation with a respective isotype antibody. For quantitative analysis glomerular cells were counted in 15 cortical glomeruli per section. Glomerular Ig deposits were scored from 0 – 3 on 15 cortical glomerular sections. The indices for interstitial volume, interstitial collagen deposition, tubular cell damage, and tubular dilatation were determined by superposing a grid containing 100 (10 x 10) sampling points on photographs of 10 nonoverlapping cortical fields of silver-stained tissue (x 20) of each kidney.

**3.2.1.9 Biochemical analysis of plasma and urine samples**

Urine albumin (Bethyl Laboratories Inc, Montgomery, TX USA) and creatinine were determined using diagnostic kits. Plasma samples were analysed for cytokines (BD Pharmingen kits for IL12p40, IL6 and CCL2) and immunoglobulin (Bethyl Laboratories Inc, Montgomery, USA) using ELISA kits. Plasma creatinine, albumin, urea nitrogen and cholesterol were analysed by SynLab GmbH & Co. KG, Augsburg, Germany.
Analysis of plasma autoantibodies

Serum dsDNA autoantibodies were determined by using commercial ELISA kits (Bethyl Labs, Montgomery, TX, USA) using the following antibodies: anti-mouse IgG1 and IgG2a following the manufacturer’s protocol with some modifications as follows:

1) Sample wells in NUNC maxisorp 96 well flat bottom ELISA plate were coated with 50 μg/ml (dilute in PBS) of the aqueous solution of 100 μl of Poly-L-Lysine (Trevigen, Gaithersburg, MD, USA) for 1 hour at room temperature.
2) Washed once with washing buffer, tap dried on blotting paper and coated the sample wells with 1 μg/ml of dsDNA, which was diluted in SSC buffer (1X), pH 7.
3) For standard wells, capture antibody in coating buffer was added as described in the protocol.
4) The plate was incubated overnight at 4°C.
5) Next day the plate was washed with the wash buffer 3 times and then blocked with blocking solution and the protocol was followed as provided by the bethyl labs from this blocking step onwards.
6) Standard dilutions and samples were added in respective wells in required dilution and incubated at room temperature for 2 hours.
7) Plate was washed 3 times and then secondary antibody at given dilution was added as per the protocol and incubated for 1 hour at room temperature.
8) Plate was again washed as before and TMB substrate (1:1 mixture of substrate A and B) was added in each well and incubated in dark for 5-20 minutes.
9) The reaction was stopped with 1 M H2SO4 and absorbance was measured at 450 nm.

Protocol for ELISAs (in brief)

1) The NUNC ELISA plate wells were captured overnight at 4°C with the capture antibody in coating buffer.
2) Next day the plates were washed 3 times with the washing buffer as given in protocol for 3 times and blocked with the blocking solution or assay diluent for 1 hour or as specified.
3) Again the washings were repeated 3 times followed by addition of standards; samples and sample diluent (blank) into the wells of tap dried plate and incubated at RT for 2 hours
4) This was followed by washings for 5 times or as specified
5) Then HRP/AP conjugated secondary antibody diluted in assay diluent was added. Incubate the plate as specified.
6) The wells were washed again for 5-7 times or as specified and incubated with the 100 μl of substrate A and B (1:1 mixture) for 25-30 min in dark to develop colour. The reaction was stopped by addition of 100 μl 1 M H₂SO₄.
7) The reading of the absorbance was taken at 450 nm and the reference wavelength was 620 nm using a spectrophotometer (TECAN-Genios Plus).

3.2.1.10 RNA isolation, cDNA synthesis and real time RT-PCR
Renal tissue from each mouse was snap frozen in liquid nitrogen and stored at -80 °C. Real time RT-PCR was performed in pooled samples (5 animals from each group) on a TaqMan ABI 7700 Sequence Detection System (PE Biosystems, Weiterstadt, Germany) using a heat activated TaqDNA polymerase (Amplitaq Gold, PE Biosystems). Controls comprising ddH₂O were negative for target and housekeeper genes. Primers and probes were from PE Biosystems, Weiterstadt, Germany. Oligonucleotide primer (300 nM) and probes (100 nM) were used (primers and probes used are mentioned below). From each animal total renal RNA preparation and reverse transcription were performed as described.

Isolation of RNA from tissues
When animals were sacrificed on termination of the study, small parts of tissue from each mouse were preserved in RNA-later and stored at -20 °C until processed for RNA isolation. RNA isolation was carried out using RNA isolation kit from Qiagen (Germany). In short, tissues (30 mg) preserved in RNA-later were homogenized using blade homogenizer for 30 seconds at 14500 rpm in lysis buffer (600 µl) containing β-mercaptoethanol (10 µl/ml). The homogenate was centrifuged at 15000 rpm for 3 min. and 350 µl of supernatant was transferred to fresh DEPC-treated tube to this equal amount (350 µl) of 70 % ethanol was added and whole mixture was loaded on RNA column and processed for RNA isolation as per the manufacturer’s instruction. Isolated RNA was stored at -80 °C until further used.

RNA quantification and purity check
For quantification isolated RNA samples were diluted in DEPC water (2 μl of RNA + 98 μl of DEPC water, 50 times dilution) and absorbance was measured at two wavelengths as 260 nm and 280 nm.
Amount of RNA (μg/μl) = O.D. at 260 nm * 40 * 50 (dilution factor)/ 1000

The ratio of optical densities at 260 nm and 280 nm is an indicator for RNA purity (indicative of protein contamination in the RNA samples). Only samples with a ratio of 1.8 or more were considered to be of acceptable quality.

RNA integrity check
Further quality check (if necessary) was performed using a denaturing RNA gel. In short 2 % Agarose gel with Ethidium-bromide was casted, RNA samples were mixed with RNA loading buffer (4:1 ratio) (Sigma) and were loaded on the gel. Electrophoresis was carried out at constant volt (70-100 V) using MOBS running buffer for 1 hour and the gel was read on a gel documentation apparatus under UV lamp. RNA samples showing a single bright band were considered to be of good quality. Loss of RNA integrity could be detected as smear formation in the agarose gel (Figure 10).

![Figure 10: Representative agarose gel for RNA integrity check.](image)

cDNA synthesis and real-time RT-PCR
The RNA samples isolated according to the procedure detailed above were diluted in DEPC water to a concentration of 1μg/20μl. A master mix was prepared with reagents including 9 μl of 5x buffer (Invitogen, Karlsruhe, Germany), 1μl of 25mM dNTP mixture (Amersham Pharmacia Biotech, Freiburg, Germany), 2 μl of 0.1 M DTT (Invitrogen, Karlsruhe, Germany), 1 μl of 40U/μl RNasin (Promega, Mannheim, Germany), 0.5μl of 15μg/ml linear acrylamide (Ambion Ltd, Cambridgeshire, UK), 0.5μl of Hexanucleotide (Roche, Mannheim, Germany), 1μl of Superscript (Invitrogen, Karlsruhe, Germany) or ddH2O in case of the controls. The master mix was made to a volume of 15μl and added to 2μg/30μl RNA samples were taken in separate DEPC
treated microcentrifuge tubes, which were mixed and placed at 42 °C on a thermal shaker incubator for 1 hour. After 1 hour the cDNA samples were collected and stored at -20 °C until use for real-time RT-PCR analysis.

The cDNA samples prepared as described above were diluted 1:10 a dilution for the real-time RT-PCR. The real-time RT-PCR was performed on a TaqMan® ABI Prism 7000 or 7700 (Applied Biosystems, Darmstadt, Germany). The quantitative PCR for mRNA is based on the employment of sequencespecific primers and likewise sequence-specific probes. The latter is tagged at both ends with a fluorescent molecule. The quencher absorbs TAMRA (at the 3'-End) the fluorescence of the other reporter tagged material such as FAM or VIC at the 5'-End. The TaqMan® universal PCR master mix (Applied Biosystems, Darmstadt, Germany) contained Taq polymerase possessing a 5'→3' polymerase activity and a 5'→3' exonuclease activity. During the elongation phase of the PCR, specifically bound probe was hydrolyzed by the exonuclease and the 5'-tag was set free. With every newly synthesized DNA strand fluorescent tag material was set free and the resulting fluorescence was measured at 488nm. The resulting fluorescence signal is directly proportional to the quantity of DNA synthesized. The CT value (Cycle Threshold value) was computed for each sample. This is the cycle number, with which the reporter fluorescence signal breaks through a user-defined threshold. The TaqMan® universal PCR master mix containing, the forward primers and Reverse primers (final concentration of 300 nM) and the probe (final concentration of 100 NM) was placed on ice. TaqMan® universal PCR master mix contained PCR buffers, dNTPs and the AmpliTaqGold® previously mentioned (Taq polymerase without 3'→5' exonuclease activity). 18μl of the mastermix was pipetted into each well of a 96-well plate and 2μl of template (DNA dilution) was added to each of these wells. The plate was sealed and centrifuged at 280 xg and analyzed using TaqMan® ABI PRISM 7000 or 7700. For the TaqMan® RT-PCR the following temperature settings were used: The first incubation was carried out for 2 minutes at 50°C followed by 95°C for 10 minutes so as to activate the polymerase. Templates were amplified during 40 cycles each comprising 15 seconds incubation at 95°C followed by 1 minute incubation at 60°C. The RT-PCR for the housekeepers (18S rRNA or GAPDH) was carried out under similar conditions. The CT values were recorded using the ABI PRISM Sequence Detection software (version 1.0) and the results were evaluated in relation the respective housekeepers. In all cases controls consisting of ddH2O were negative for target and housekeeper genes. Oligonucleotide
primer (300 nM) and probes (100 nM) were from Applied Biosystems (Darmstadt, Germany): Primers for CCL2, Ccl5, and 18s rRNA was predeveloped TaqMan assay reagent from PE Biosystems.

**Primers and probes used for real-time RT-PCR**

<table>
<thead>
<tr>
<th>Target</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<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>
</tr>
<tr>
<td>CCR1</td>
<td>Forward primer: 5'-TTAGCTTCCATGCTTGCTTATA-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse primer: 5'-TCCACTGCTTCAGGCTTTGT-3'</td>
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<tr>
<td></td>
<td>6 FAM: 5'-ACTCACCCTACCTGTAGCCCTACTTCCC-3'</td>
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<td>CCR2</td>
<td>Forward primer: 5'-CCTTGGGAATGAGTAACTGTGTGA-3'</td>
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<td>Reverse primer: 5'-ACAAAGGCCATAAT-GACAGGATAT-3'</td>
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<td></td>
<td>6 FAM: 5'-TGACAAGCACCATTAGACCAGGCATGCA-3'</td>
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<td>CCR5</td>
<td>Forward primer: 5'-CAAGACAATCTCTGATGCTGCAA-3'</td>
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<td>Reverse primer: 5'-TCTACTCCAAGCTGCATAGAA-3'</td>
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<td>6FAM: 5’TCTATACCCGATCCACACGG-AGAAATGAGTTT-3’</td>
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<td>TGF-β1</td>
<td>Forward primer: 5'-CACAGTACAGCAAGGTTCCTGC -3'</td>
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<td>collagen I-α1</td>
<td>Forward primer: 5'-TGCTTTCTGCCCAGGAAG -3'</td>
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<td></td>
<td>Reverse primer: 5'-GGATGCCCATCTGCTCA - 3’</td>
</tr>
<tr>
<td></td>
<td>6 FAM: 5’ - CCAGGCTCTCCGGTGTCACATCT -3’</td>
</tr>
<tr>
<td>18s rRNA</td>
<td>predeveloped TaqMan assay reagent from PE Biosystems</td>
</tr>
</tbody>
</table>

**3.2.1.11 FACS analysis**

Total blood and bone marrow samples were obtained from mice of all groups at the end of the study for FACS analysis.

**Bone marrow cells isolation**

Tibia and femur bones from the fore and hind limbs of MRL<sup>lpr/lpr</sup> mice were isolated after sacrificing 3 mice from each group, using all sterilized materials and sterile
conditions for the isolation in an aseptic hood with HEPA filters. Bones were freed from the adhering tissues and both the ends of each bone were punctured with the 24 gauge needle and then the RPMI media was flushed (using sterile surgical syringe with needle) through the bones to isolate bone marrow in a sterile petri plate containing the 2-3 ml of ice-cold media (RPMI+ 10 %FCS + 1 % PS) on ice. Then it was homogenously resuspended using 1 ml pipette. This cell suspension was taken in a 15 ml Falcon tube using 1 ml pipette and centrifuged at 1600 rpm at 4°C for 6 min. and washed with 1x PBS (cell culture grade) for 2 times. Then it was passed through the 40 μm sterile filters to obtain single cell suspension and centrifuged as before. The supernatant was decanted and the pellet was resuspended in 4 ml of sterile 0.3M NH4Cl and kept at room temperature for 5 min. Then it was centrifuged as above and supernatant was decanted and pellet was resuspended in ice cold (cell culture use) PBS and washed 2 times with PBS as before. The pellet was resuspended in RPMI media (1-2 ml).

Whole blood collection
Blood sample was drawn after 2-3 hrs of last injection of Spiegelmer and vehicle in respective groups in an ependrof tube containing EDTA.

Staining bone marrow and blood samples for FACS
Bone marrow the cells were resuspended to approximately 1-5x10^6 cells/ml in ice cold PBS, 10%FCS, 1% sodium azide. Use ice cold reagents/solutions and at 4°C as low temperature and presence of sodium azide prevented the modulation and internalization of surface antigens which could produce a loss of fluorescence intensity. 100μl of whole blood sample was used for staining.

1) 100 μl of cell suspension /100μl of whole blood was added to each tube.
2) 0.1-10 μg/ml of the primary antibody (MC21, Mack et.al 2001) was added. Dilutions for primary antibody were made in 3% BSA/PBS.
3) Incubated for at least 30 min at 4°C in the dark.
4) Samples were then washed 3-times by centrifugation at 400 g for 5 min and resuspend them in ice cold PBS.
4) Biotinylated anti-rat IgG antibody (BD Biosciences GmbH, Heidelberg, Germany) diluted in 3% BSA/PBS at the optimal dilution was added in the samples (secondary antibody) and then resuspended the cells in this solution.
5) Incubated for at least 40 minutes at 4°C in the dark.
6) The cells were then washed 3-times by centrifugation at 400 g for 5 min and resuspend them in ice cold PBS, 3% BSA, 1% sodium azide.

7) Then the cells were treated with optimum amount of streptavidin-PE.

8) Store the samples for 40 min at 4°C in the dark.

9) The cells were then washed 3-times by centrifugation at 400 g for 5 min and resuspend them in ice cold PBS, 3% BSA, 1% sodium azide.

10) Bone marrow cells were ready for the analysis. Blood samples were then lysed with BD lysis buffer (BD Biosciences). Followed by two washings before analysis.

**FACS acquisition**

Stained samples were gated for monocyte region according FSC and SSC plot. Gated cells were then plotted against FL2 (for PE fluorescence).

### 3.2.1.12 Immunostimulatory effect of Spiegelmer (*in vitro*)

**Isolation and preparation of murine bone marrow derived dendritic cells (BMDCs)**

Tibia and femur bones from the fore and hind limbs of MRL<sup>lw/lp</sup> mice were isolated after sacrificing 4-5 mice, using all sterilized materials and sterile conditions for the isolation in an aseptic hood with HEPA filters. Bones were freed from the adhering tissues and both the ends of each bone were punctured with the 24 gauge needle and then the RPMI media was flushed (using sterile surgical syringe with needle) through the bones to isolate bone marrow in a sterile Petri plate containing the 2-3 ml of ice-cold media (RPMI+ 10 %FCS + 1 % PS) on ice. Then it was homogenously resuspended using 1 ml pipette. This cell suspension was taken in a 15 ml Falcon tube using 1 ml pipette and centrifuged at 1600 rpm at 4°C for 6 min. and washed with 1x PBS (cell culture grade) for 2 times. Then it was passed through the 40 μm sterile filters to obtain single cell suspension and centrifuged as before. The supernatant was decanted and the pellet was resuspended in 4 ml of sterile 0.3M NH₄Cl and kept at room temperature for 5 min. Then it was centrifuged as above and supernatant was decanted and pellet was resuspended in ice cold (cell culture use) PBS and washed 2 times with PBS as before. The pellet was resuspended in RPMI media (2-4 ml) and the no. of cells were counted using Neubar’s chamber under the microscope after suitable dilution. Adequate no. of cells (5 million) in 12 well plates (NUNC cell culture plates), each well containing 1.5 ml of complete RPMI media containing 100 ng/ml recombinant murine Flt3 ligand (Immunotools, Friesoyth, Germany). The plates were
incubated at 37°C for 8 days to obtain a lineage of mixture of plasmacytoid dendritic cells and conventional dendritic cells (pDC and cDC). After 4 days of incubation 1 ml of fresh media (with 100 ng/ml Flt3 ligand) was added in each well. After 8 days of the incubation, 2 ml of fresh media was replaced for the old media by careful removal only from the top of each well.

**Stimulation of BMDCs**

Then the cells were stimulated with ligands or media as followed. 10 μg D- and L-enantiomeric phosphorothioates RNA40 (TLR7 agonistic sequence), RNA41 and RNA42 (control sequences), and various concentrations of mNOX-E36 (12.5, 25 and 50 μg/mL) and POC-PEG control (25 and 50 mg/mL) in 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP; Roche, Mannheim, Germany). The plates were again incubated for 24 hrs at 37°C. After incubation the supernatants were carefully isolated in microcentrifuge tubes. The supernatants were separated from the residual amount of cells by centrifuging it at 6000 rpm for 10 min. And immediately stored them at –20°C. These supernatants were analyzed for the murine IFN-α by (ELISA (PBL, Biomedical Labs, and Piscataway, NJ USA)

3.2.1.13 **In-situ hybridization (distribution of mNOX-E36)**

The paraffin-embedded tissue sections were deparaffinised in glass coplin jars at room temperature (RT) in used xylene, fresh xylene, used 100% ethanol, fresh 100% ethanol, used 95%, 70%, 50% ethanol and phosphate buffered saline (PBS), respectively (1× 5 min each). Sections were fixed again 20 min in 4% (w/v) paraformaldehyde in PBS at RT and flushed in PBS. The tissue then was partially digested for 30 min with 20 μg/ml proteinase K in TE buffer at RT and flushed with 2 mg/ml glycine in PBS at RT and PBS, respectively. Pre-hybridisation was carried out in a humidified container for 2 h at 40°C in pre-hybridisation buffer. Slides were washed once in pre-warmed 2× SSC for 5 min. The hybridisation with biotinylated L-RNA probes was performed in a humidified container overnight with 0.2 μM L-RNA probe in pre-hybridisation buffer at 40°C.

The next day slides were washed three times for 15 min (1) with pre-warmed 2× SSC, (2) with 2× SSC at RT, and (3) with TBS. Hybridised probes were detected with streptavidin alkaline phosphatase 1:1000 in TBS for 2 h at RT. Slides were washed again twice for 10 min (1) with TBS and (2) with 10 mM Tris/HCl, pH 9.8; 1 mM MgCl₂. 5-Bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT)
alkaline phosphatase substrate solution (Sigma B1911) was then added and slides were incubated for 30-120 min at RT. The reaction was stopped with deionized H₂O and tissue sections were counterstained with Nuclear Fast Red (Sigma N3020) for 10 min at RT. The tissue sections were then dehydrated and embedded in Canada balm or Entellan (Merck) for long-term storage.

3.2.1.14 Statistical analysis
Statistics were done using GraphPad Prism (4.03 version). Data were expressed as mean ± SEM. Data were analysed using unpaired two-tailed t-test for comparison between two groups. One-way ANOVA followed by post-hoc Bonferroni’s test was used for multiple comparisons. For nonparametric analysis of two groups Two-tailed Mann Whitney U test were performed.
3.2.2 Methods Part II

3.2.2.1 Grouping and treatment
Seven week old female MRL\textsuperscript{lp/lpr} mice were obtained from Harlan Winkelmann (Borchen, Germany) and kept in filter top cages under a 12 hour light and dark cycle. Water and standard chow (Sniff, Soest, Germany) were available \textit{ad libitum}. All experimental procedures were approved by the local government authorities. At the age of 14 week, female MRL\textsuperscript{lp/lpr} mice were distributed into seven groups that received injections as follows:

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Dosage level\textsuperscript{a} (mg/kg)</th>
<th>Dose volume (ml/kg)</th>
<th>Treatment Schedule</th>
<th>no</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Vehicle (5% Glucose)</td>
<td>0</td>
<td>4</td>
<td>3times/week</td>
<td>16</td>
</tr>
<tr>
<td>2</td>
<td>mNOX-E36-PEG</td>
<td>50</td>
<td>4</td>
<td>3times/week</td>
<td>12</td>
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<tr>
<td>3</td>
<td>revmNOX-E36-PEG</td>
<td>50</td>
<td>4</td>
<td>3times/week</td>
<td>16</td>
</tr>
<tr>
<td>4</td>
<td>CYC ¼ th full dose</td>
<td>30</td>
<td>4</td>
<td>once/ 4 weeks</td>
<td>16</td>
</tr>
<tr>
<td>5</td>
<td>CYC full dose</td>
<td>30</td>
<td>4</td>
<td>once weekly</td>
<td>12</td>
</tr>
<tr>
<td>6</td>
<td>mNOX-E36-PEG</td>
<td>50</td>
<td>4</td>
<td>3times/week</td>
<td>12</td>
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<tr>
<td></td>
<td>plus CYC ¼ th full dose</td>
<td>30</td>
<td>4</td>
<td>once/4 weeks</td>
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<tr>
<td>7</td>
<td>MMF</td>
<td>100</td>
<td>4</td>
<td>once daily</td>
<td>12</td>
</tr>
</tbody>
</table>

\textsuperscript{a} = \textit{in vivo} doses of mNOX-E36 and revmNOX-E36-PEG in mg/kg apply to the combined total weight of the oligonucleotide part plus the coupled PEG moiety. Thus, mNOX-E36 has to be multiplied with the factor 0.29.

All Spiegelmer samples were provided by Noxxon Pharma AG, in ready to use formulation. Cyclophosphamide (CYC) was procured from sigma Aldrich. Mycophenolate mofetil (MMF) was purchased from Roche (Manheim, Germany)

3.2.2.2 Route and rationale of test material administration
The chosen route of administration was subcutaneous, as the animals tolerate a large number of subcutaneous administrations better than numerous intraperitoneal injections, handling is easier and because by this route unPEGylated Spiegelmer is present longer in the circulation. So mNOX-E36 and controls were administered
subcutaneously (4 mL/kg) three times a week (Monday, Wednesday and Friday), CYC intraperitoneally once per month or once weekly and MMF orally at daily intervals.

3.2.2.3 Test substance and formulation

<table>
<thead>
<tr>
<th>Spiegelmer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>mNOX-E36-PEG</td>
<td>40 kD PEG</td>
</tr>
<tr>
<td></td>
<td>5’-GGCGACAUUG GUUGGGCAUG AGGCGAGGCC CUUUGAUGAA UCCGCGGCCA-3’</td>
</tr>
<tr>
<td>Control Spiegelmer</td>
<td></td>
</tr>
<tr>
<td>revmNOX-E36-PEG</td>
<td>40 kD PEG</td>
</tr>
<tr>
<td></td>
<td>5’-ACCGCGCUCUAAGUAGUUUC CCGGAGCGAGUACGGGUUG GUUACAGCGG-3’</td>
</tr>
</tbody>
</table>

For *in vivo* application, the mNOX-E36 and the non-functional control Spiegelmer were used as modified with 40-kD PEG at the 5’ terminus. The test substance (Spiegelmer solution) was dissolved in isotonic 5% glucose solution.

3.2.2.4 Pharmacokinetic analysis

In weeks 1, 3, 7 and 10 of treatment, blood samples for pharmacokinetic analysis were collected 24 hours after injection from mNOX-E36 treated groups and determined as previously described.

3.2.2.5 Plasma, urine and tissue collection

After 10 weeks of treatment (on the day of sacrifice), blood samples were collected under ether anaesthesia 2 to 3 hours after the last administration. Blood was drawn by retroorbital bleeding using heparinised capillaries. Plasma was separated by centrifugation at 10,000 rpm for 5 minutes. Urine samples were collected when the animals were 24 weeks of age.

At study termination (at the end of the 10-week treatment period), organ weights of spleen and lymph nodes were determined. Spleen, lymph nodes, kidney, lung, liver, and salivary gland tissue samples were collected from each mouse for histology, RNA analysis and Cryo analysis.
3.2.2.6 Biochemical analysis of plasma and urine samples

Urine albumin (Bethyl Laboratories Inc, Montgomery, TX USA) and creatinine were determined using diagnostic kits. Plasma samples were analysed for cytokines (BD Pharmingen kits for IL12p40, IL6 and CCL2), TNF-α (BioLegend Ltd, San Diego, CA, USA) and immunoglobulin (Bethyl Laboratories Inc, Montgomery, USA) using ELISA kits.

3.2.2.7 FACS analysis

Spleen cells were obtained from mice of all groups at the end of the study for FACS analysis.

Isolation and preparation of murine spleen cells

Spleen was isolated from the MRL<sup>lw/lw</sup> mice after sacrificing it, using all sterilized materials and sterile conditions for the isolation in an aseptic hood with HEPA filters. Spleen was collected in a sterile petri plate containing 2-3 ml of ice-cold media (RPMI+ 10 %FCS + 1 % PS) on ice. Then it was mashed using forceps in the petri plate itself and broken into fine pieces of tissues. Then the cell suspension was passed through 70 μm plastic filter (BD biosciences) using the 1 ml pipette and collected in another sterile plate. This single cell suspension was later collected in a 15 ml Falcon tube with pipette and centrifuged at 1600 rpm at 4°C for 6 min. The supernatant was decanted and the pellet was resuspended in 4 ml of sterile 0.3M NH4Cl and kept at room temperature 5 min. Then it was centrifuged at 1600 rpm at 4°C for 6 min. Supernatant was decanted and pellet was resuspended in ice cold (cell culture use) PBS and centrifuged at 1600 rpm at 4°C for 6 min.

Staining of spleen cells for FACS

Spleen cells were resuspended to approximately 1-5x10<sup>6</sup> cells/ml in ice cold PBS, 10%FCS, 1% sodium azide. Use ice cold reagents/solutions and at 4°C as low temperature and presence of sodium azide prevented the modulation and internalization of surface antigens which could produce a loss of fluorescence intensity.

1) 100 μl of cell suspension was added to each tube.
2) 0.1-10 μg/ml of the flurochrome labelled antibody was added. Dilutions for primary antibody were made in 3% BSA/PBS. following antibodies were used ; 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, Heidelberg, Germany)
3) Incubated for at least 45 min at 4°C in the dark.
4) Samples were then washed 3-times by centrifugation at 400 g for 5 min and resuspend them in ice cold PBS.
5) Store the samples for 40 min at 4°C in the dark.

**FACS acquisition**

Stained samples were gated for lymphocytes region according FSC and SSC plot. Gated cells were then plotted against FITC (FL1) to identify CD3 positive cells population. These cells then gated and plotted in another dot plot (FL3-CD8-PerCp vs FL4-CD4.APC) to identify CD3+CD4-CD8- (autoreactive T cells). In other staining gated CD3 positive cells were plotted (FL3-CD25-PerCp vs FL4-CD4.APC) to identify CD3+CD4+CD25+ (regulatory T cells). Cells number / suspension was determined by using Caltag counting beads as per the manufacture’s method.

**3.2.2.8 Statistical analysis**

Statistics were done using GraphPad Prism (4.03 version). Data were expressed as mean ± SEM. Data were analysed using unpaired two-tailed t-test for comparison between two groups. One-way ANOVA followed by post-hoc Bonferroni’s test was used for multiple comparisons. For nonparametric analysis of two groups Two-tailed Mann Whitney U test were performed.
3.2.3 Methods Part III

3.2.3.1 Grouping and treatment

Seven week old female MRL<sup>lpr/lpr</sup> mice were obtained from Harlan Winkelmann (Borchen, Germany) and kept in filter top cages under a 12 hour light and dark cycle. Water and standard chow (Sniff, Soest, Germany) were available <i>ad libitum</i>. All experimental procedures were approved by the local government authorities. At the age of 8 week, female MRL<sup>lpr/lpr</sup> mice were distributed into four groups that received injections as follows:

**Table: 6 Treatment protocol III**

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Dosage level a (mg/kg)</th>
<th>Dose volume (ml/kg)</th>
<th>Treatment Schedule</th>
<th>no</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Vehicle (5% Glucose)</td>
<td>0</td>
<td>4</td>
<td>3 times/week</td>
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</tr>
<tr>
<td>2</td>
<td>mNOX-E36-PEG</td>
<td>50</td>
<td>4</td>
<td>3 times/week</td>
<td>5</td>
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<tr>
<td>3</td>
<td>CYC full dose</td>
<td>30</td>
<td>4</td>
<td>once/week</td>
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<tr>
<td>4</td>
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<td></td>
<td>plus CYC low</td>
<td>30</td>
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<td>once/4 weeks</td>
<td></td>
</tr>
</tbody>
</table>

a = <i>in vivo</i> doses of mNOX-E36 in mg/kg apply to the combined total weight of the oligonucleotide part plus the coupled PEG moiety. Thus, mNOX-E36 has to be multiplied with the factor 0.29.

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The chosen route of administration was subcutaneous, as the animals tolerate a large number of subcutaneous administrations better than numerous intraperitoneal injections, handling is easier and because by this route unPEGylated Spiegelmer is present longer in the circulation. So mNOX-E36 and controls were administered subcutaneously (4 mL/kg) three times a week (Monday, Wednesday and Friday), CYC intraperitoneally once per month or once weekly.
3.2.3.3 Test substance and formulation

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<tr>
<td></td>
<td>AGGCAGGCCC CUUGAUGAA</td>
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<tr>
<td></td>
<td>UCCGGGGCCA-3’</td>
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</tbody>
</table>

For in vivo application, the mNOX-E36 was used as modified with 40-kD PEG at the 5´ terminus. The test substance (Spiegelmer solution) was dissolved in isotonic 5% glucose solution.

3.2.3.4 Blood samples for FACS

To evaluate the effect of treatments of blood cell count, blood samples were drawn every week.

3.2.3.5 FACS analysis

Bone Marrow cells, Spleen cells and whole blood samples were obtained from mice of all groups at the end of the study for FACS analysis.

Isolation and preparation of bone marrow cells and murine spleen cells:

Bone marrow cells were isolated as described in methods part-I. Spleen cells were isolated as described in methods part-II

Staining of procedure for FACS

Blood samples and Bone marrow samples were stained for monocytes and neutrophills by using anti mouse 7/4 –PE and anti mouse Ly6G-FITC antibodies.

Spleen cells were resuspended to approximately 1-5x10^6 cells/ml in ice cold PBS, 10% FCS, 1% sodium azide. Use ice cold reagents/solutions and at 4°C as low temperature and presence of sodium azide prevented the modulation and internalization of surface antigens which could produce a loss of fluorescence intensity.

1) 100 μl of cell suspension was added to each tube.
2) 0.1-10 μg/ml of the flurochrome labelled antibody was added. Dilutions for primary antibody were made in 3% BSA/PBS. following antibodies were used ; 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, Heidelberg, Germany)
3) Incubated for at least 45 min at 4°C in the dark.
4) Samples were then washed 3-times by centrifugation at 400 g for 5 min and resuspend them in ice cold PBS.
5) Store the samples for 40 min at 4°C in the dark.

**FACS acquisition**

For bone marrow and Blood samples granulocyte and monocytes region according to FSC and SSC plot was gated. Gated cells then plotted (FL2-7/4-PE vs FL1-Ly6G-FITC). 7/4 brd cells were monocytes and 7/4+Ly6G+ cells were evaluated as neutrophils. 7/4 med cells were mixed leukocytes (Tsou et al.2007).

Stained spleen cell samples were gated for lymphocytes region according FSC and SSC plot. Gated cells were then plotted against FITC (FL1) to identify CD3 positive cells population. These cells then gated and plotted in another dot plot (FL3-CD8-PerCp vs FL4-CD4.APC) to identify CD3+CD4-CD8- (autoreactive T cells). In other staining gated CD3 positive cells were plotted (FL3-CD25-PerCp vs FL4-CD4.APC) to identify CD3+CD4+CD25+ (regulatory T cells)

Cells number / suspension were determined by using Caltag counting beads as per the manufacture’s method.

**3.2.3.6 Colony forming units (CFU-GM)**

To determine the ability of bone marrow cells to form granulocyte colony in culture, isolated bone marrow cells were cultured in presence of conditioned media for granulocyte growth (methocult media) supplemented with mGM-CSF (10ng/ml) for 12 days. After ten days, granulocyte colonies were counted using microscopic analysis.

**3.2.3.7 Statistical analysis**

Statistics were done using GraphPad Prism (4.03 version). Data were expressed as mean ± SEM. Data were analysed using unpaired two-tailed t-test for comparison between two groups. One-way ANOVA followed by post-hoc Bonferroni’s test was used for multiple comparisons. For nonparametric analysis of two groups Two-tailed Mann Whitney U test were performed.
4. Results

4.1. Results part- I

Lupus nephritis is a chronic renal disease. Loss of self tolerance and impaired clearance of autoantigens leads to deposition of immune complexes in renal tissue, which initiates the inflammatory cascade. Lupus nephritis is characterised by the induced expression of cytokines and chemokines in renal tissue. So far the therapy for lupus nephritis is targeted towards modulating immune reaction, but we hypothesised that controlling inflammatory cascade in combination with immunomodulators will help to reduce the side effects of therapy without compromising its effect. In this study first we looked at the efficacy of blocking CCL2 mediated tissue injury in mouse model of lupus nephritis (MRL^{lpr/lpr}). We looked at anti-CCL2 Spiegelmer for its pharmacokinetics, and pharmacodynamic effects. Results of the first part are summarised below.

4.1.1 Pharmacokinetics of the anti-CCL2 Spiegelmer in MRL^{lpr/lpr} mice

4.1.1.1 Bioavailability

In intervention studies for chronic renal disease determination of plasma levels of injected substance is essential as kidney being one of the important excretory organs. In order to monitor drug exposure in MRL^{lpr/lpr} mice, Spiegelmer plasma levels were determined at weekly intervals. The median plasma levels of mNOX-E36 and mNOX-E36-3’PEG were approx. 300 nM and 1 µM throughout the study, respectively (Figure 11). Thus, PEGylation increased the plasma levels of mNOX-E36 and the progressive kidney disease of MRL^{lpr/lpr} mice did not modulate the pharmacokinetics of both Spiegelmers. In addition, neither drug accumulation nor metabolic induction or reduction was obvious.
Figure 11: Pharmacokinetics of anti-mCCL2 Spiegelmers in chronic kidney disease. Concomitant pharmacokinetic studies were performed for mNOX-E36 and mNOX-E36-PEG in female MRL

plasma level [nM]

Figure 4.1.1.2 Distribution

Anti-CCL2 Spiegelmer showed an excellent bioavailability profile. Bio-distribution is one of the key factors which can predict untoward effects or non specific activity of injected drug. To determine distribution of mNOX-E36, tissues were fixed in formalin and paraffin-embedded. From these fixed tissues 5 µm sections were prepared and mounted on silanized glass slides. These sections were then processed to detect Spiegelmer by in situ hybridisation. Spiegelmer was found to be distributed mainly in tubulointerstitial area of renal tissue (Figure 12). CCL2 has been shown to be expressed in this region. Selective binding of the Spiegelmer with its target was seen even systemically when we looked at the plasma levels of CCL2 (data shown in later parts of the results). This explains the selective binding of mNOX-E36 to murine CCL2 and its distribution to high CCL2 expressed area of tissue.
4.1.2 Survival rate

End stage kidney failure is one of the major causes of death in SLE patients. In MRL<sup>lpr/lpr</sup> mice the average median survival time of female mice is around 22 weeks. CCL2 antagonist (unPEGylated mNOX-E36 and mNOX-E36) treatment led to prolonged median survival time, compared to respective controls. The median survival time for mice treated with the vehicle control and POC controls was around 21 – 23 weeks, and for mice treated with unPEGylated mNOX-E36 (25 mg/kg) it was 26 weeks (Figure 13). The PEG form of the drug (50 mg/kg mNOX-E36) was found to be even more effective in prolonging survival, with median survival of more than 30 weeks (Figure 13). In summary, mNOX-E36 improved the 50 % mortality at the 5 % level of statistical significance compared to the vehicle-control MRL<sup>lpr/lpr</sup> mice.
Figure 13: Survival of mice of the various treatment groups was calculated by Kaplan-Meier analysis.

4.1.3 Renal parameters

4.1.3.1 Albumin / creatinine ratio

Albumin / creatinine ratio is an important clinical parameter for renal disease. In lupus nephritis it increases with the progression of kidney inflammation. Treatment with a CCL2 antagonist was expected to inhibit progression of renal disease in MRL\(^{lpr/lpr}\) mice, which can be indicated by the reduction of albumin / creatinine ratio, when compared to untreated or POC-treated groups. In week 16 of age, the CCL2 antagonist unPEGylated mNOX-E36 at a dose of 25 mg/kg, administered three times per week, delayed the onset of renal inflammation, indicated by a statistically significant (p < 0.05, Figure 14) reduction in proteinuria compared to vehicle control. In week 24 of age, there was no significant reduction in the albumin / creatinine ratio, but a definite trend indicating protection in all groups treated with mNOX-E36 or unPEGylated mNOX-E36 could be seen when compared to the vehicle control (Figure 14). Some of the diseased mice from control group died before the final estimation of proteinuria might explain, why the protective effect could not reach the significance at week 24.
Figure 14: Urine albumin (mg/dl) to creatinine (mg/dl) ratio was measured on week 16, 21 and 24 of age. Data represented in Mean ± SEM.

4.1.3.2 Glomerular filtration rate

Progressing inflammation of the kidneys results in a loss of filtering ability. In MRL<sup>lpr/lpr</sup> mice, the GFR decreases with age. In this study, the GFR was determined by the ability of mice to excrete FITC-labelled inulin. Mice treated three times per week with mNOX-E36 or unPEGylated mNOX-E36 showed a higher GFR compared to the vehicle control or POC-treated group (not statistically significant).

Figure 15: Glomerular filtration rate (GFR) was evaluated by determining plasma clearance of Inulin-FITC. Plasma concentration of Inulin was measured 5, 10, 15, 20, 35, 60 and 90 min after retro-orbital injection. GFR was then calculated using Two phase decay kinetics (Graph Pad prism 4.0)
4.1.3.3 Renal histology

Female MRL<sup>lpr/lpr</sup> mice develop and subsequentially die from diffuse proliferative immune complex glomerulonephritis, with striking similarities to diffuse proliferative lupus nephritis in humans. MRL<sup>lpr/lpr</sup> mice showed diffuse proliferative glomerulonephritis characterised by glomerular macrophage infiltration and a mixed periglomerular and interstitial inflammatory cell infiltrate consisting of glomerular and interstitial MAC-2 positive macrophages and interstitial CD3-positive lymphocytes.

**Activity and chronicity index**

Even at 14 wk of age, MRL<sup>lpr/lpr</sup> mice revealed proliferative glomerulonephritis with an activity index of 4.1 ± 1.1 and a chronicity index of 0.1 ± 0.2. At all dose groups, treatment with mNOX-E36 and unPEGylated mNOX-E36 improved the activity and chronicity index (Figure 16) of lupus nephritis, markers of renal inflammation. The improvement of activity and chronicity index was statistically significant at the 5 % level (p = < 0.05, vs. respective POC, Mann Whitney test) after treatment with 50 mg/kg of mNOX-E36 or 25 mg/kg of unPEGylated mNOX-E36. Administration of 25 mg/kg of unPEGylated mNOXE36 was less effective on the chronicity index than treatment with 50 mg/kg of mNOX-E36 (Figure 16).
Figure 16: Renal histopathology in MRL<sup>lpr/lpr</sup> mice. Renal sections of 24-wk-old MRL<sup>lpr/lpr</sup> mice were stained with periodic acid-schiff. Disease activity and chronicity was evaluated by scoring method established by Austin et al. 1984 Images are representative for seven to 12 mice in each group. § p < 0.05 versus respective control Spiegelmer. Magnifications: x100

**Cellular infiltration**

**CD3 positive cells (interstitial)**

Immune cell infiltrates contribute to renal damage in lupus nephritis and CCL2 mediates the recruitment of T cells and macrophages to MRL<sup>lpr/lpr</sup> mice (Tesch et al., 1999). Cellular infiltration is characteristic for glomerulonephritis in MRL<sup>lpr/lpr</sup> mice. Treatment with 50 mg/kg of mNOX-E36 or 25 mg/kg of unPEGylated mNOX-E36 three times per week reduced CD3 positive cell infiltration significantly (p < 0.01 or < 0.05, Figure 17) when compared to vehicle control, respectively.
**Macrophages (interstitial and glomerular)**

Macrophages are important inflammatory cells, responsible for tissue destruction. Lupus nephritis in MRL^{lpr/lpr} mice leads to significant renal macrophage infiltration. Treatment with mNOX-E36 and unPEGylated mNOX-E36 at all dose groups reduced the macrophage infiltration (MAC-2 positive cells) in interstitial as well as glomerular compartment. This effect was statistically significant for 50 mg/kg of mNOX-E36 (p < 0.05, interstitial and glomerular, Figure 18) and for 25 mg/kg of unPEGylated mNOX-E36 (p < 0.05, glomerular, Figure 18) when compared to vehicle control.

![Renal histopathology in MRL^{lpr/lpr} mice](image)  
*Figure 17: Renal histopathology in MRL^{lpr/lpr} mice. Renal sections of 24-wk-old MRL^{lpr/lpr} mice were stained with antibodies for CD3 T cells (interstitial). Images are representative for seven to 12 mice in each group. * P< 0.05, ** P<0.01 versus respective control Spiegelmer. Magnifications: x100*
Figure 18: Renal histopathology in MRL<sup>lpr/lpr</sup> mice. Renal sections of 24-wk-old MRL<sup>lpr/lpr</sup> mice were stained with antibodies for Mac-2 cells (interstitial and glomerular). Images are representative for seven to 12 mice in each group. * P< 0.05 versus respective control Spiegelmer Magnifications: x400

**Morphometry**

Tubular atrophy and confluent areas of interstitial fibrosis are associated with advanced chronic kidney disease. 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. Compared to vehicle control, the treatment with 25 mg/kg of unPEGylated mNOX-E36 or 50 mg/kg of mNOX-E36 three times per week reduced interstitial fibrosis (p < 0.01), tubular cell damage (p < 0.01) and tubular dilation (p < 0.01, Figure 19)
**IgG deposits**

CCL2 plays an important role in chronic inflammation as in lupus nephritis. But it has no effect on autoimmune parameters which supports our aim to develop a small molecule antagonist which attenuates the inflammation without affatcing the immunity. Treatment with mNOX-E36 or unPEGylated mNOX-E36 reduced the cellular infiltration and severity of glomerular alterations at the light microscopic level but there was no change in extent of IgG depositions in glomeruli of all dose groups (data shown in Figure 20).
Figure 20: Renal histopathology in MRL\textsuperscript{1pr/1pr} mice. Renal sections of 24-wk-old MRL\textsuperscript{1pr/1pr} mice were stained for IgG. Images are representative for seven to 12 mice in each group.

4.1.4 Extra-renal autoimmune tissue injury in MRL\textsuperscript{1pr/1pr} mice

4.1.4.1 Skin lesion
Cutaneous lupus manifestations, which typically occur in the facial or neck area, were less common in mNOX-E36 (50 mg/kg) or unPEGylated mNOX-E36-treated (25 mg/kg) mice being statistically significant at the 1 % level (p < 0.01, vs. POC-PEG, see Figure 21). The percentage of animals with skin lesions was determined to demonstrate variability within the group. In control groups, more than 60 % (80 % in 50 mg/kg POC-PEG) of the animals showed skin lesions in Week 24. The severity was graded from 0 (no lesion), 1 (mild lesion), 2 (moderate) to 3 (severe). The percentage of animals with skin lesions was determined to see the variability within the group.
4.1.4.2 Lung histology

After sacrifice, lung tissue of all mice was prepared and scored semi-quantitatively. Treatment with unPEGylated mNOX-E36 and mNOX-E36 at all dose groups reduced peribronchiolar inflammation with mean severity grades from 0.42 to 1.2 (controls, severity grades from 1.4 to 1.6) in MRL\textsuperscript{Ipr/Ipr} mice. After the administration of 50 mg/kg of mNOX-E36 this effect was statistically significant at the 5 % level when compared to the vehicle control (Figure 22).
Figure 22: Extrarenal autoimmune tissue injury in MRL<sup>lpr/lpr</sup> mice. At 24 wk of age, lung tissue was prepared from MRL<sup>lpr/lpr</sup> mice of all groups and scored semiquantitatively as described in the concise methods section. Treatment with anti-CCL2 Spiegelmers reduced peribronchiolar inflammation in MRL<sup>lpr/lpr</sup> mice. Images are representative of seven to 11 mice in each group. Magnification: x100. * p<0.05 vs control group.

4.1.4.3 Splenomegaly and lymphadenopathy
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. Splenomegaly and lymphadenopathy are two of the typical morphological clinical signs for lupus in MRL<sup>lpr/lpr</sup> mice. Treatment with unPEGylated mNOX-E36 and mNOX-E36 did not show any effect on spleen (Figure 23) or lymph node weights (Figure 23). Thus, treatment with Spiegelmers did not affect lymphoproliferation in MRL<sup>lpr/lpr</sup> mice.
Figure 23: At week 24 of age MRL<sup>lpr/lpr</sup> mice show massive splenomegaly and lymphadenopathy. Data represents organ weight/100 gm body weight of spleen and mesenteric lymphnodes of the groups under observation.

4.1.5 Systemic parameters

4.1.5.1 Plasma IgGs
Autoimmunity in MRL<sup>lpr/lpr</sup> mice is characterised by the production of autoantibodies against multiple nuclear antigens including dsDNA. In 24-week old MRL<sup>lpr/lpr</sup>, plasma dsDNA IgG, IgG1, IgG2a, and IgG2b autoantibodies were present at high levels. unPEGylated mNOX-E36 and mNOX-E36 both had no effect on either of these DNA autoantibodies. Thus, treatment with mNOX-E36 did not affect anti-dsDNA IgG production in MRL<sup>lpr/lpr</sup> mice.

Table 7: Plasma IgG subtypes.

<table>
<thead>
<tr>
<th></th>
<th>Vehicle</th>
<th>mNOX-PoC</th>
<th>mNOX-E36</th>
<th>mNOX-PoC-P</th>
<th>mNOX-E36-P</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG&lt;sub&gt;1&lt;/sub&gt;</td>
<td>11.7 ± 3.6</td>
<td>7.5 ± 2.8</td>
<td>11.7 ± 3.4</td>
<td>8.2 ± 1.2</td>
<td>6.6 ± 1.2</td>
</tr>
<tr>
<td>IgG&lt;sub&gt;2a&lt;/sub&gt;</td>
<td>2.0 ± 0.3</td>
<td>1.9 ± 0.3</td>
<td>2.1 ± 0.1</td>
<td>2.0 ± 0.2</td>
<td>1.9 ± 0.2</td>
</tr>
<tr>
<td>IgG&lt;sub&gt;2b&lt;/sub&gt;</td>
<td>17.6 ± 5.6</td>
<td>18.9 ± 5.2</td>
<td>22.6 ± 3.3</td>
<td>28.3 ± 2.5</td>
<td>20.6 ± 3.8</td>
</tr>
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</table>

Data presented as Mean ± SEM
4.1.5.2 Plasma CCL2

UnPEGylated mNOX-E36 and mNOX-E36, but none of the control Spiegelmers, were found to increase the plasma concentrations of CCL2, indicating that CCL2-specific Spiegelmers retain soluble CCL2 in the intravascular compartment. Plasma CCL2 was measured in samples collected 3 hours post administration on the day of sacrifice. These data showed that pegylation increases the plasma levels of unPEGylated mNOX-E36, which most effectively retains soluble CCL2 in the intravascular compartment (Figure 24). It also explains the selectivity of the treatment for murine CCL2.

![Figure 24](image)

Figure 24: Plasma CCL2 levels in MRL<sup>1pr/1pr</sup> mice. Plasma CCL2 levels were determined in mice of all groups at the end of the study. Data are expressed as means ± S.E.M.

4.1.6 Emigration of monocytes from the bone marrow

Monocyte emigration from bone marrow during bacterial infection was shown to involve chemokine receptor CCR2 (Serbina et al.2006), 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 (50 mg/kg), and 5% glucose (control).
Figure 25: Flow cytometry for CCR2 in peripheral blood and bone marrow of MRL^{lpr/lpr} mice. (A) To study the effects of anti-CCL2 Spiegelmer on leukocyte evasion from the bone marrow, we performed flow cytometry for CCR2 on bone marrow and peripheral blood in 24-wk-old vehicle- or anti–CCL2 Spiegelmer–treated MRL^{lpr/lpr} mice. Data are mean percentage of CCR2^{+} cells ± SEM in either bone marrow or peripheral blood in five mice of each group. (B) Effect of single dose of anti-CCL2 Spiegelmer (50 mg/kg), Spiegelmer (Co, 50 mg/kg), and 5% glucose on circulating CCR2^{+} cells. Data are mean percentage of CCR2^{+} F4/80^{+} cells ± SEM in blood 6 h after injection.

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 25B). Furthermore, we examined the CCR2-positive monocyte population in peripheral blood and bone marrow in 24-wk-old MRL^{lpr/lpr} 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 25A). These data support a novel role for CCL2 for the evasion of CCR2-positive cells from the bone marrow during autoimmune disease of MRL^{lpr/lpr} mice.
4.1.7 RT-PCR analysis

In order to study whether treatment with unPEGylated mNOX-E36 and mNOX-E36 affects intrarenal inflammation in MRL \(^\text{lpr/lpr}\) mice, real-time RT-PCR was performed in pooled samples for the proinflammatory chemokines CCL2 and CCL5, which are progressively up regulated in kidneys of MRL \(^\text{lpr/lpr}\) mice during progression of renal disease. Treatment with Spiegelmers, unPEGylated mNOX-E36 and mNOX-E36, from Week 14 to 24 of age reduced renal expression of CCL2 and CCL5 mRNA compared to vehicle-treated controls. mRNA levels for CCL2 (Figure 26) and CCL5 (Figure 26) were expressed per respective 18s rRNA expression.

![Graph of CCL2 mRNA/18s rRNA](image)

**Figure 26:** Renal chemokine expression of MRL \(^\text{lpr/lpr}\) mice with experimental lupus: 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.

4.1.8 Body weight development

After subcutaneous treatment with mNOX-E36 or unPEGylated mNOX-E36, MRL \(^\text{lpr/lpr}\) mice gained body weight at all dose groups. In week 24, the mean body weight gains of MRL \(^\text{lpr/lpr}\) mice treated with 16.66 or 50 mg/kg mNOX-E36 and with 8.33, 25 mg/kg or 25 mg/kg (once weekly) unPEGylated mNOX-E36 were +5 % or −3 % and ±0 %, −1 % or +6 % higher or lower when compared to the vehicle control.
4.1.9 Immunostimulatory effect of Spiegelmer (in vitro)

The therapeutic use of Spiegelmers would be hampered by induction of type 1 interferon via innate RNA recognition receptors. To exclude this mechanism, plasmacytoid dendritic cells (pDC) were prepared from bone marrow of MRL\textsuperscript{lpr/lpr} mice and incubated with the anti-CCL2 Spiegelmer and various synthetic RNA. D-enantiomeric RNA40 but not L-enantiomeric RNA40 induced IFN-\(\alpha\) production in pDC (Figure 27). Furthermore, IFN-\(\alpha\) release was absent upon exposure to control D- and L-RNA42 as well as high concentrations of the anti-CCL2 Spiegelmer. Hence, Spiegelmers do not induce IFN-\(\alpha\) production in pDC. In summary, D-RNA-40-P (P-thioate Backbone) (TLR7 agonist) has shown to induce IFN-\(\alpha\) (immunostimulation), whereas L-RNA-40-P did not show any induction. mNOX-E36 also did not show significant induction of IFN (Figure 27). Hence, this provides a compound as L-enantiomeric RNA aptamers, without immunostimulatory effect.

![Figure 27: Spiegelmers do not induce IFN-\(\alpha\) release in plasmacytoid dendritic cells. Plasmacytoid dendritic cells were generated from bone marrow dendritic cells of MRL\textsuperscript{lpr/lpr} mice by incubation with Flt-3 ligand and incubated with 10 \(\mu\)g each of d- and l-enantiomeric phosphorothioates RNA40 (TLR7 agonistic sequence), RNA42 (control sequence) in 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), and various concentrations of mNOX-E36. IFN-\(\alpha\) production was measured by ELISA. Data are means ± SEM of two independent experiments](image-url)
4.2 Results Part- II

In the first part of this study we evaluated the efficacy and pharmokinetics of anti-CCL2 Spiegelmer in MRL\textsuperscript{lpr/lpr} mice. Treatment with mNOX-E36 improved the renal phenotype in MRL\textsuperscript{lpr/lpr} mice and had no effect on parameters which defines autoimmunity. It was clear that this L-enantiomeric aptamer specifically attenuates CCL2-derived inflammation. In the second part of our study we compared the efficacy of the anti-CCL2 Spiegelmer with that of known immunomodulators like CYC and MMF which are already in clinical use as a therapy for LN. Looking at the pathogenesis of lupus nephritis, we assumed that the efficacy will not be comparable, but we assumed if we combine the anti-CCL2 Spiegelmer with a low dose of cyclophosphamide, we will be able to reduce the side effects of high exposure of CYC. But is the combination therapy as effective as high dose CYC? Results of the second part of the study will answer this question. Results are summarised below.

4.2.1 Pharmacokinetics of Spiegelmer in MRL\textsuperscript{lpr/lpr} mice

In order to monitor drug exposure in MRL\textsuperscript{lpr/lpr} mice, mNOX-E36 plasma levels were determined in the 1\textsuperscript{st}, 3\textsuperscript{rd}, 6\textsuperscript{th}, and 10\textsuperscript{th} week of treatment, i.e. the 15\textsuperscript{th}, 18\textsuperscript{th}, 21\textsuperscript{st}, and 24\textsuperscript{th} 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 28). Apparently, the progressive kidney disease of MRL\textsuperscript{lpr/lpr} mice did not modulate Spiegelmer pharmokinetics and neither drug accumulation nor metabolic induction or reduction was detected.
4.2.2 Renal parameters

4.2.2.1 Renal Histology

Activity and chronicity index

At 14 weeks of age MRL\textsuperscript{lp/lp} mice showed diffuse proliferative lupus nephritis 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\textsuperscript{lp/lp} mice revealed diffuse proliferative lupus nephritis associated with glomerular hypercellularity, expansion of glomerular matrix, focal tuft necrosis, and a mixed periglomerular and interstitial inflammatory cell infiltrate (Figure 30). Full dose CYC and the combination of mNOX-E36 and \(\frac{1}{4}\) full dose CYC were equally potent in improving the activity (Figure 29) and chronicity index (Figure 29) of lupus nephritis, markers of renal inflammation. This effect was statistically significant at the 0.1 % level (\(p < 0.001\) vs. vehicle control). Furthermore, combination of mNOX-E36 with \(\frac{1}{4}\) full dose CYC was significantly (\(p < 0.05\)) more effective when compared with \(\frac{1}{4}\) full dose CYC treatment alone. \(\frac{1}{4}\) full dose CYC alone, mNOX-E36 alone as well as MMF were less potent but still significantly improved the activity (\(p < 0.05\) vs. vehicle control).
control), but only ¼ full dose CYC alone and mNOX-E36 alone improved the chronicity indices (p < 0.001 or p < 0.05 vs. vehicle control) of lupus nephritis (Figure 29). Thus, adding mNOX-E36 to a low dose (30 mg/kg/4 weeks) CYC-based regimen is as potent as full dose CYC therapy for diffuse proliferative lupus nephritis in MRL<sup>lpr/lpr</sup> mice.

**Figure 29:** Markers of lupus nephritis in MRL<sup>lpr/lpr</sup> 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) Control Sp, pegylated control Spiegelmer (rev-E36-P); anti-CCL2 Sp, pegylated anti-CCL2 Spiegelmer (-E36-P); CYC low, monthly 30 mg/kg cyclophosphamide; CYC high, 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
Figure 30: Renal histopathology in MRL<sup>lpr/lpr</sup> mice. Renal sections of 24-week-old MRL<sup>lpr/lpr</sup> mice from all groups were stained with periodic acid Schiff. Original magnification, x200. Control Sp, pegylated control Spiegelmer (rev-E36-P); anti-CCL2 Sp, pegylated anti-CCL2 Spiegelmer (E36-P); CYC low, monthly 30 mg/kg cyclophosphamide; CYC high, 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.
Cellular infiltration

Cellular infiltration is characteristic for glomerulonephritis in MRL\textsuperscript{lpr/lpr} mice. Additive effects of an anti-CCL2 Spiegelmer and ¼ full dose CYC combination may relate to impaired macrophage and T cell recruitment in MRL\textsuperscript{lpr/lpr} mice. Immunostaining was performed for Mac2-positive macrophages and CD3-positive T cells and the number of glomerular and interstitial macrophages as well as interstitial T cells was assessed. The numbers of glomerular T cells were not analysed as they were very low in all groups.

CD3 positive cells (interstitial)

All treatments reduced CD3 positive cell infiltration significantly (p < 0.001 vs. vehicle control, Figure 31), while treatment with full dose CYC or the combination of mNOX-E36 and ¼ full dose CYC seemed to be equally potent in reducing the numbers of interstitial CD3 positive cell in kidneys of MRL\textsuperscript{lpr/lpr} mice than treatment with ¼ full dose CYC alone and mNOX-E36 as well as MMF.

![Figure 31](image-url)

**Figure 31:** Renal sections of 24-week-old MRL\textsuperscript{lpr/lpr} mice were stained for CD3-positive T cells. Numbers of interstitial T cells were evaluated as mean numbers of CD3-positive cells in 15 high-power fields (hpf) per section. Data are expressed as means ± S.E.M. Control Sp, pegylated control Spiegelmer (rev-E36-P); anti-CCL2 Sp, pegylated anti-CCL2 Spiegelmer (E36-P); CYC low, monthly 30 mg/kg cyclophosphamide; CYC high, weekly 30 mg/kg cyclophosphamide; MMF, 100 mg/kg mycophenolate mofetil. *, p < 0.05; **, p < 0.01; ***, p < 0.001 versus vehicle; ##, p < 0.01 versus CYC low.
Macrophages (glomerular and interstitial)

Full dose CYC and the combination of mNOX-E36 and ¼ full dose CYC were equally potent (p < 0.001 vs. vehicle control) in reducing the numbers of glomerular Mac-2 positive macrophages in kidneys of MRL\textsuperscript{lpr/lpr} mice (Figure 32). Additionally, combination of mNOX-E36 with ¼ full dose CYC was significantly more effective in reducing the numbers of glomerular Mac2-positive macrophages than treatment with ¼ full dose CYC alone (p < 0.01). ¼ full dose CYC alone and mNOX-E36 alone as well as MMF were less potent but still significantly (p < 0.01 and p < 0.001 vs. vehicle control, respectively) reduced the macrophages in glomeruli and the interstitium (Figure 32). The number of interstitial Mac2-positive macrophages in kidneys of MRL\textsuperscript{lpr/lpr} mice were significantly reduced by full dose CYC and to a lower extend equally reduced by ¼ full dose CYC and combination therapy with mNOX-E36 (p < 0.001 vs. vehicle control, Figure 32). Treatment with mNOX-E36 alone and MMF were less potent but still significantly at the 5% level (p < 0.05 vs. vehicle control). Thus, the additive effect of mNOX-E36 and ¼ full dose CYC on renal pathology of MRL\textsuperscript{lpr/lpr} mice was associated with a significant reduction of interstitial and glomerular macrophages which was similar to the effect of full dose CYC.

![Graph](image)

**Figure 32**: Renal sections of 24-week-old MRL\textsuperscript{lpr/lpr} mice were stained for Mac-2-positive macrophages. The number of glomerular macrophages was evaluated as the mean number of Mac2-positive cells in 15 glomeruli (glom.) per section. The numbers of interstitial macrophages (D) were evaluated as mean numbers of Mac2-positive cells in 15 high-power fields (hpf) per section. Data are expressed as means ± S.E.M. Control Sp, pegylated control Spiegelmer (rev-E36-P); anti-CCL2 Sp, pegylated anti-CCL2 Spiegelmer (E36-P); CYC low, monthly 30 mg/kg cyclophosphamide; CYC high, weekly 30 mg/kg cyclophosphamide; MMF, 100 mg/kg mycophenolate mofetil. *, p < 0.05; **, p < 0.01; ***, p < 0.001 versus vehicle; ++, p < 0.01 versus CYC low.
4.2.3 Extra-renal autoimmune tissue injury in MRL$^{lpr/lpr}$ mice

4.2.3.1 Lung histology

Autoimmune peribronchitis is another manifestation of lupus-like systemic autoimmunity in MRL$^{lpr/lpr}$ mice. After sacrifice, lung tissue of all mice was prepared and scored semiquantitatively. Full dose CYC (30 mg/kg/week) was significantly (p < 0.001) more effective than ¼ full dose CYC (30 mg/kg/4 weeks) and mNOX-E36 in reducing peribronchiolar inflammation in MRL$^{lpr/lpr}$ mice. However, ¼ full dose (30 mg/kg/4 weeks) CYC plus anti-CCL2 Spiegelmer were as effective as full dose of CYC and significantly (p< 0.05) more effective than treatment with mNOX-36 alone (Figure 33 and Figure 34). Daily oral treatment with MMF had no effect on lung injury in MRL$^{lpr/lpr}$ mice. Thus, the ¼ full dose (monthly) CYC/Spiegelmer combination was as effective as full dose (weekly) administrations of 30 mg/kg of CYC on autoimmune lung injury in MRL$^{lpr/lpr}$ mice.

![Figure 33: Semiquantitative scoring of lung injury was performed as described Materials and Methods. Data represent means ± S.E.M. Control Sp, pegylated control Spiegelmer (rev-E36-P); anti-CCL2 Sp, pegylated anti-CCL2 Spiegelmer (E36-P); CYC low, monthly 30 mg/kg cyclophosphamide; CYC high, weekly 30 mg/kg cyclophosphamide; MMF, 100 mg/kg mycophenolate mofetil. * p<0.05,** p<0.01,*** p<0.001 vs vehicle control.](image-url)
Figure 34: Autoimmune lung injury in MRL<sup>lpr/lpr</sup> mice. Lung sections of 24-week-old MRL<sup>lpr/lpr</sup> mice from all groups were stained with periodic acid Schiff. Original magnification, x100. Control Sp, pegylated control Spiegelmer (rev-E36-P); anti-CCL2 Sp, pegylated anti-CCL2 Spiegelmer (E36-P); CYC low, monthly 30 mg/kg cyclophosphamide; CYC high, weekly 30 mg/kg cyclophosphamide; MMF, 100 mg/kg mycophenolate mofetil.
4.2.3.2 Splenomegaly and lymphadenopathy

Splenomegaly and lymphadenopathy are two of the typical morphological clinical signs for lupus in MRL<sup>lpr/lpr</sup> mice. CYC treatment caused a dose-dependent reduction of spleen (p < 0.01, full dose CYC) and lymph node (p < 0.001, full and ¼ full dose CYC) weights in 24-week old MRL<sup>lpr/lpr</sup> mice as compared to vehicle-treated MRL<sup>lpr/lpr</sup> mice (Figure 35). The effect of MMF treatment was less evident and did only reduce significantly (p < 0.001) weight of lymph nodes. Treatment with mNOX-E36 did not show a significant effect on spleen or lymph node weights (Figure 35) and no additional effect of mNOX-E36 could be noted when given in combination with ¼ full dose of CYC. Thus, mNOX-E36 did not affect lymphoproliferation in MRL<sup>lpr/lpr</sup> mice.

![Figure 35: Lymphoproliferation in MRL<sup>lpr/lpr</sup> mice. Spleens and the bulk of mesenteric lymph nodes were harvested from 24-week-old MRL<sup>lpr/lpr</sup> mice. Weight of lymph nodes is expressed as mean weight per 100 mg body weight ± S.E.M. Spleen weights are expressed as mean weight per 100 mg body weight ± S.E.M. Control Sp, pegylated control Spiegelmer (rev-E36-P); anti-CCL2 Sp, pegylated anti-CCL2 Spiegelmer (E36-P); CYC low, monthly 30 mg/kg cyclophosphamide; CYC high, 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 vehicle (t Test).](image-url)
4.2.4 Plasma cytokines

4.2.4.1 Plasma IL-12p40 and TNF-α

Amongst others factors, T cell and monocyte function in autoimmunity and antimicrobial host defense is regulated by the two cytokines IL-12 and TNF-α. Treatment with mNOX-E36 had no effect on the plasma levels of IL-12p40 or TNF-α. By contrast, plasma IL-12p40 and TNF-α levels were markedly (p < 0.001, IL-12p40 or p < 0.01, TNF-α vs. vehicle control) decreased in MRL<sup>lpr/lpr</sup> mice treated with full dose CYC (Figure 36). ¼ full dose CYC alone and in combination of mNOX-E36 as well as MMF alone were less potent but still decreased plasma IL-12p40 (p < 0.05 vs. vehicle control). Thus, the anti CCL2 Spiegelmer did not affect plasma cytokine levels of IL-12p40 or TNF-α in MRL<sup>lpr/lpr</sup> mice.

![Figure 36](image)

Figure 36: Plasma cytokine levels in MRL<sup>lpr/lpr</sup> mice. Plasma 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 (rev-E36-P); anti-CCL2 Sp, pegylated anti-CCL2 Spiegelmer (E36-P); CYC low, monthly 30 mg/kg cyclophosphamide; CYC high, weekly 30 mg/kg cyclophosphamide; MMF, 100 mg/kg mycophenolate mofetil. *, p < 0.05; **, p < 0.01; ***, p < 0.001 versus vehicle
4.2.4.2 Plasma CCL2

mNOX-E36, but not the control Spiegelmer revmNOX-E36, was found to increase statistically significant (p < 0.001 vs. vehicle control) the plasma concentrations of CCL2 (Figure 37), indicating that CCL2-specific Spiegelmers retain soluble CCL2 in the intravascular compartment. Plasma CCL2 was measured in samples collected 2 to 3 hours post administration on the day of sacrifice. These data showed that pharmacologically relevant levels of circulating mNOX-E36, most likely, binds and retains CCL2 in the intravascular compartment.

![Graph showing Plasma CCL2 levels in MRL^lpr/lpr mice.](image)

**Figure 37:** Plasma CCL2 levels in MRL^lpr/lpr mice. Plasma CCL2 levels were determined in mice of all groups at the end of the study. Data are expressed as means ± S.E.M. Control Sp, pegylated control Spiegelmer (rev-E36-P); anti-CCL2 Sp, pegylated anti-CCL2 Spiegelmer (E36-P); CYC low, monthly 30 mg/kg cyclophosphamide; CYC high, weekly 30 mg/kg cyclophosphamide; MMF, 100 mg/kg mycophenolate mofetil. *** p< 0.001 vs control.

4.2.5 Body weight development

After subcutaneous treatment with mNOX-E36 or revmNOX-E36, MRL^lpr/lpr mice gained body weight at all dose groups as expected; animals in the groups treated with immunosuppressants full dose CYC and MMF lost weight. Mice on ¼ full dose CYC and combination therapy showed only minimal or no weight loss at all. In week 24, the mean body weight gains of MRL^lpr/lpr mice treated with 50 mg/kg mNOX-E36 were (+3.84 g) similar to mean body weight gain in the vehicle group (+5.2 g). In contrast, animals in the full dose CYC and MMF showed strong loss of mean body weight (-6.38 g and -6.52 g, respectively).
4.3 Results Part III

We have been able to show that mNOX-E36 treatment improves the renal phenotype in MRL\textsuperscript{lpr/lpr} mice. Combining anti CCL2 therapy with low dose CYC shows comparable efficacy with that of high dose CYC. But what is the effect of combination therapy on overall immunity? is it equally immunosuppressive? or it spares the side effects of high dose CYC without hampering the efficacy. To evaluate this, in a separate experiment four satellite groups of MRL\textsuperscript{lpr/lpr} mice were treated for four weeks with vehicle, Spiegelmer, Spiegelmer in combination with ¼ full dose CYC, full dose CYC alone from 8 to 12 weeks of age in order to characterise immune cell subsets in spleen, bone marrow and peripheral blood by flow cytometry (FACS).

4.3.1 Splenomegaly and lymphadenopathy

![Graph showing spleen weights and cells per spleen](image)

**Figure 38**: Spleen weights are expressed as mean weight per 100 mg body weight ± S.E.M. Spleen cells are expressed as million/spleen. anti-CCL2 Sp, pegylated anti-CCL2 Spiegelmer (E36-P-50); CYC(30), weekly 30 mg/kg cyclophosphamide; Combi; mNOX-E36 plus CYC (low) *, p < 0.05 versus vehicle;

Full-dose CYC but not ¼ full dose CYC + mNOX-E36 significantly (p < 0.05 vs. vehicle control) reduced the total number of spleen cells and spleen weight. (Figure 38). This was consistent for all spleen T cell subsets studied, i.e. CD3+, CD3+CD4+ CD3+CD8+, CD3+CD4-CD8-, and CD3+CD4+CD25+ cells (Figure 39). Thus, mNOX-E36 + ¼ full dose CYC had no additive effects on T cell depletion as seen with full dose CYC treatment in MRL\textsuperscript{lpr/lpr} mice.
Figure 39: T cell depletion in MRL<sup>lpr/lpr</sup> mice. 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. CD3-positive T lymphocyte subsets of all the groups are shown. Data represent mean percentages ± S.E.M. of all leukocytic cells. anti-CCL2 Sp, pegylated anti-CCL2 Spiegelmer (E36-P-50); CYC(30), weekly 30 mg/kg cyclophosphamide; Combi; mNOX-E36 plus CYC (low) *, p < 0.05 **, p < 0.01 versus vehicle;

Full dose of CYC usually causes transient myelosuppression which renders patients susceptible to life-threatening infections. Full dose CYC significantly (p < 0.05 vs. vehicle control and ¼ full dose CYC + mNOX-E36 combination) reduced the numbers of 7/4 (bri) monocytes in bone marrows of MRL<sup>lpr/lpr</sup> mice while mNOX-E36-treated and combined ¼ full dose CYC + mNOX-E36-treated MRL<sup>lpr/lpr</sup> mice had significantly (p < 0.05 vs. vehicle control) elevated numbers of 7/4 (bri) bone marrow monocytes (Figure 40). In peripheral blood, test item- and CYC-treated MRL<sup>lpr/lpr</sup> mice showed a trend towards lower 7/4 (bri) monocyte counts but this did not reach statistical significance for any of the groups (Figure 40).
4.3.2 Effect on blood and bone marrow

Figure 40: Myelosuppression in MRL<sup>lpr/lpr</sup> mice. Bone marrow and peripheral blood (every week) samples were harvested from 12-week-old MRL<sup>lpr/lpr</sup> mice (n = 5) after 4 weeks of treatment as indicated. 7/4<sup>high</sup>-Positive monocytes were quantified in bone marrows (B) and peripheral blood (A) of MRL<sup>lpr/lpr</sup> mice from all groups. Data represent mean percentages ± S.E.M. of all leukocytic cells. anti-CCL2 Sp, pegylated anti-CCL2 Spiegelmer (E36-P-50); CYC(30), weekly 30 mg/kg cyclophosphamide; Combi; mNOX-E36 plus CYC (low). *, p < 0.05; **, p < 0.01 versus vehicle; *** p < 0.001 vs combination.
4.3.3 CFU-GM colony assay

**Figure 41**: Number of CFU-GM colonies / 3*10^4 BM cells. Data represented as Mean ± SEM. anti-CCL2 Sp, pegylated anti-CCL2 Spiegelmer (E36-P-50); CYC (30), weekly 30 mg/kg cyclophosphamide; Combi; mNOX-E36 plus CYC (low). ** p< 0.01 vs control.

CFU-GM colony assay in Methocult media supplemented with m-GM-CSF is a test for myelosuppression, i.e. the growing capacity of haematopoietic stem cells and immune cell progenitors. CFU-GM colonies in full dose CYC-treated mice were significantly reduced on day 12 of incubation (p < 0.01 vs. vehicle control and p < 0.05 vs. ¼ full dose CYC + mNOX-E36), while mNOX-E36 and ¼ full dose CYC + mNOX-E36 treatment did not affect CFU-GM counts (Figure 41). Thus, mNOX-E36 + ¼ full dose CYC treatment did not cause myelosuppression as seen with full dose CYC in MRL^lpr/lpr mice.
5. Discussion

**CCL2 and Lupus nephritis**

Apart from activation of auto-reactive T cells, B cells and production of auto-antibodies, end stage tissue damage is an important aspect of lupus pathogenesis. Tissue inflammation in chronic disease is characterised by hyper infiltration of immune cells (T cells, macrophages). Thus targeting molecules (CCL2, CCL5, ICAM, integrins) which participate in cell migration would be effective in preventing end stage tissue damage. 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 (Tesch et al. 1999, Perez de Lema et al. 2005) Thus, CCL2 and CCR2 both are crucial for the recruitment of cytokine-producing leukocytes to peripheral tissues. 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 (Serbina NV, Pamer EG. 2006).

Hasegawa et al (2003) injected 5 x 10<sup>6</sup> cells that were transfected with an NH<sub>2</sub>-truncated CCL2 analogue subcutaneously into 12-wk-old female MRL<sup>lpr/lpr</sup> mice which was associated with an improved pathology at 20 wk. Similar results were obtained by Shimizu et al. (2004) who injected 7ND, a NH<sub>2</sub>-terminal deletion mutant of the CCL2 gene, into skeletal muscles of 16-wk-old female MRL<sup>lpr/lpr</sup> mice. In both of the studies CCL2 blockade improves the renal pathology without affecting the systemic autoimmunity parameters, as none of the above studies showed reduction in autoantibodies. This makes CCL2 blockade as attractive approach to attenuate chronic inflammation without interfering with immunity. However, the cell transfer approach caused local tumours of considerable size (Hasegawa et al. 2003). The caveat for the use of this gene therapy–like approach in humans is that CCL2 antagonism cannot be abandoned once initiated.

**Spiegelmers: Clinically applicable aptamers**

Cell transfer approach (induction of local tumours) and gene therapy (irreversible antagonism) are not clinically applicable in humans. Aptamers can neutralize biologic functions of target molecules conceptually similar to antibodies. But aptamers are not biostable and can induce immune reaction though recognition by TLRs. Development of safer biological aptamers is motivating many groups working all over the globe.
Spiegelmer (L-enantiomeric aptamer) is one of such invention. Unlike aptamers, Spiegelmer-based oligonucleotides are nuclease resistant and thus biostable without further modifications (Klussmann et al. 1996), which makes them very well suited for in vivo applications. We evaluated anti-CCL2 Spiegelmer (mNOX-E36), which binds with high affinity to murine CCL2 and blocks its function in vitro at low nanomolar concentrations. Spiegelmers, being L-enantiomeric, are not recognised by nucleases. Because of these structural benefits they remain in systemic circulation for long time and have better pharmacokinetics than conventional aptamers. We analysed systemic Spiegelmer levels after subcutaneous injection in MRL^{lpr/lpr} mice. Blood samples were taken after 3hrs and 24hrs for mNOX-E36 and mNOX-E36-Peg respectively. The PEGylated form of mNOX-E36 displayed higher plasma levels compared with the non-PEGylated mNOX-E36. Levels determined for both form of drugs were sufficient for the in vivo application. Progressive renal failure in MRL^{lpr/lpr} mice did not affect their plasma levels.

We analysed CCL2 levels at termination of the studies at 24 weeks age of MRL^{lpr/lpr} mice. Groups treated with anti-CCL2 Spiegelmers (not control Spiegelmers) showed higher levels of CCL2. This defines the specificity of Spiegelmer to neutralise murine CCL2. Spiegelmers upon interacting with systemic CCL2 neutralises it and keeps it in circulation in biologically inactive form. This biological inactivation was seen with decreased percentage of CCR2 positive monocytes in peripheral blood. Contrary to peripheral blood, percentage of CCR2 positive blood cells in bone marrow was found to be elevated in MRL^{lpr/lpr} mice treated with anti-CCL2 Spiegelmer. We looked at the in vivo-distribution of mNOX-E36 in MRL^{lpr/lpr} after subcutaneous injection. mNOX-E36 was seen in tubulointerstitial compartment of renal tissue. CCL2 is known to be expressed in high levels in this compartment of renal tissue. All these observations show that mNOX-E36 specifically binds to CCL2 and neutralises it in vivo.

Synthetic oligonucleotides may induce IFN-α in dendritic cells (Heil et al. 2004, Hornung et al. 2005) 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 (Heil et al. 2004, Hornung et al. 2005) IFN-α is an important mediator of autoimmunity (Banchereau J, Pascaul V. 2006) 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 the L-
enantiomeric configuration of Spiegelmers is probably not recognized by RNA-specific pattern recognition receptors.
All these properties make anti-CCL2 Spiegelmer (mNOX-E36), a clinically applicable aptamer.

**Anti-CCL2 Spiegelmer (mNOX-E36) in lupus nephritis**
Our data demonstrate that even non prophylactic late-onset treatment with anti-CCL2 Spiegelmer at 14 wk of age, a point at which autoimmune tissue injury is already established (Tesch et al.1999, Perez de Lema et al.2005), 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 CCL2/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$^{lpr/lpr}$ mice. In fact, both CCL2-deficient and anti-CCL2 Spiegelmer–treated MRL$^{lpr/lpr}$ mice did not affect the lymphoproliferative syndrome, DNA autoantibodies, and renal immune complex deposition, all being early disease markers (Tesch et al.1999). Treatment with anti-CCL2 Spiegelmer reduced inflammatory cell infiltration in renal tissue, improved renal phenotype, and improved survival in MRL$^{lpr/lpr}$ mice. Our findings are consistent with other studies that initiated blockade of the CCL2/CCR2 axis after onset of autoimmunity.

CCL2 blockade is thought to target CCL2-dependent leukocyte adhesion to activated endothelia and transendothelial migration of leukocytes at sites of inflammation (Luster et al.2005). Our finding that CCL2 blockade has a similar effect on monocyte homing to the bone marrow during systemic autoimmunity extends this findings 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.

**Current lupus therapy regimen and novel approaches**
The aim of the treatment in lupus nephritis is to suppress the inflammation in renal tissue and to preserve the structure and function of the kidney, avoiding the progression to renal failure. This objective must be achieved without developing significant adverse events. Currently, the therapy for severe lupus nephritis is based on the use of high-dose corticosteroids and immunosuppressive drugs. Several
randomized controlled studies demonstrated that, for patients with severe lupus nephritis, a regimen including CYC is more effective than treatment with only glucocorticoids (Steinberg AD et al.1991, Austin HA et al. 1986). Noteworthy, these randomized trials of the United States National Institutes of Health (NIH) failed to demonstrate significant differences among the different regimens containing immunosuppressive drugs (daily oral CYC, monthly intravenous (IV) CYC, daily oral azathioprine (AZA), combined low-dose oral CYC plus AZA). Nevertheless, monthly IV CYC became the gold standard of treatment for severe lupus nephritis since then. A recent study (by Houssiau et al.2002) has showed that, as far as induction of remission and prevention of end-stage renal failure is concerned, a 12-week course of IV CYC given at a fixed dose of 500 mg fortnightly and followed by AZA for 2 years is as effective as an abbreviated NIH protocol consisting on six monthly IV CYC pulses of 0.5 g/m² (increasing the dose according to the leukocyte count) followed by two quarterly pulses, after a median follow-up of 72 months. Unfortunately, the use of IV CYC is not exempt from pitfalls. Despite the treatment with CYC, a significant proportion, up to 50%, of patients with lupus nephritis (Korbet SM et al.2000, Ginzler EM et al.2005) do not achieve a complete remission. In the studies of the United States NIH, a longer duration of the therapy with CYC reduced the cumulative probability of relapse, when comparing 24 versus 6 months of treatment (Boumpas DT et al.1992), but longer therapy with CYC was associated with increased gonadal toxicity. In addition, CYC, even with intravenous administration, has a significant toxicity in terms of hemorrhagic cystitis, gonadal failure and infections.

For the above mentioned reasons, the need for less toxic and more effective alternatives to CYC in the treatment of lupus nephritis is clear. Because costimulation sustains the abnormal activity of T cells and helps the production of autoantibodies, it represents an obvious target in SLE. Thus, several approaches aim at decreasing the deleterious effects of T cells by blocking costimulation in SLE. CD28/CTLA-4 (Abatacept) and CD80/86, CD40 and CD40 ligand (L)(BJ9588), ICOS and ICOS-L (anti ICOS antibody) have been targeted to normalise activity of T cells.

Several different B cell targeting strategies have been identified and are currently under investigation. These include targets directly associated with the B cell, such as B cell surface molecules (CD20, rituxumab) (CD22, Epratuzumab) and more indirect targets, such as the inhibition of costimulatory ligands present on the T cell.
**Figure 42:** Mechanisms of current immunotherapies. BCDT: B-cell depletion therapy; Ag: antigen; Ab: antibody. Influence of immunotherapeutic drugs indicated by red arrows. Anti-BLyS acts on BLyS, a B-cell survival factor. Costimulatory molecules CD40 and B7 are present on both APC and B-cells. For ease of use, the thicker arrows are only indicated for anti-CD40, CTLA4-Ig on APC.

All these approaches are meant to target reduced autoantibody production. None of the novel approaches have shown any promising clinical data so far.

Complexities of the disease prevent us from relying solely on anti-inflammatory treatment approach for lupus nephritis. We hypothesised that blocking inflammatory mediators along with low exposure of immunosuppressant like cyclophosphamide, can improve the renal pathology as effectively as high dose of immunosuppressant. CCL2 plays an important role in inflammatory phase of lupus. Anti-CCL2 Spiegelmer showed good therapeutic benefit in murine model of LN. So we combined anti-CCL2 Spiegelmer (mNOX-E36) with low dose of cyclophosphamide. This way we thought of finding a novel way to reduce immunosuppressant exposure and it also signifies the probable role of CCL2 blockade in LN treatment.
Combining CCL2 blockade with low dose cyclophosphamide

Immunosuppressive treatment regimen for diffuse proliferative lupus nephritis involving high dose CYC or MMF remain associated with serious complications in lupus nephritis patients, i.e. infections (Katsifis et al. 2004, Appel et al. 2007). Thus, combinations of CYC with drugs that more specifically interact with autoimmune tissue injury in lupus may overcome this problem. 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 diffuse proliferative lupus nephritis. 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.

It was demonstrated that a combination of mNOX-E36 and monthly (¼ full dose) CYC treatment initiated at 14 weeks of age and continued for 10 weeks was as effective as weekly (full dose) CYC treatment in suppressing diffuse proliferative lupus nephritis and lung injury in MRL<sup>lpr/lpr</sup> mice. The effect of mNOX-E36 either given alone or in combination with a ¼ full dose CYC had no additive effects on T cell depletion, serum TNF-α and IL-12p40 levels, and myelosuppression. All these parameters were significantly suppressed by full dose CYC therapy. Anti-CCL2 Spiegelmer had 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. As a consequence, mNOX-E36 had no effect on the underlying autoimmune dysregulation in MRL<sup>lpr/lpr</sup> mice. Furthermore, mNOX-E36 did not modulate the CD4/CD8 double negative ‘autoreactive’ T cell population, indicating that mNOX-E36 did not modulate the autoimmune process in itself but rather the local mechanisms of autoimmune tissue injury. Additionally, an additive effect of combined mNOX-E36 / ¼ full dose CYC therapies on the numbers of renal macrophages and T cells was observed which is consistent with the role of CCL2 in renal macrophage and T cell recruitment. However, late onset treatment with mNOX-E36 alone was not as effective as high dose CYC therapy on autoimmune tissue injury in MRL<sup>lpr/lpr</sup> mice. As expected, the non-functional control Spiegelmer was not
effective in this lupus nephritis model. The animals tolerated the treatment well and there was no influence on body weight development due to Spiegelmer treatment. We could see significant renal protection with anti-CCL2 Spiegelmer treatment in MRL\textsuperscript{1pr/lpr} mice. But translation of murine efficacy in human clinical efficacy has always been a tough task for researchers. Even lupus nephritis phenotype in MRL\textsuperscript{1pr/lpr} mice differs with that of human disease. So our data could be encouraging for us but may/may not differ in terms of efficacy in humans. Anti-CCL2 Spiegelmer (NOX-E36) is already in clinical trials for diabetic nephropathy and lupus nephritis as indications. Recently phase-I data for pharmacokinetic and pharmacodynamic evaluation has been released by NOXXON AG on their website. NOX-E36 in human showed good Pka behaviour and could be able to reduce CCR2 positive monocytes in a dose dependent manner as pharmacodynamic parameter. It is just a data from healthy individuals from phase-I study, data from patients study (phase-II) is still pending.
6. Summary and conclusion

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 in murine models of lupus nephritis; therefore, CCL2 antagonists may be beneficial for therapy. That is why our first aim for this study was to find out the efficacy of novel tool i.e. anti-CCL2 Spiegelmer in MRL\textsuperscript{lpr/lpr} mice. This study showed the L-enantiomeric RNA oligonucleotide mNOX-E36, an anti-CCL2 Spiegelmer that binds murine CCL2 with high affinity and neutralizes its action in vitro and in vivo. CCL2 antagonism in MRL\textsuperscript{lpr/lpr} mice was shown to be effective earlier by some groups using different approaches to block CCL2 e.g. injecting transfected cells with mutant CCL2 (Hagesawa \textit{et al}.2003), gene therapy (Shimizu \textit{et al}.2004), and by using CCL2 knockout mice in MRL\textsuperscript{lpr/lpr} background (Tesch \textit{et al}.1999). Our experimental approach is more clinically suitable as 1) the mirror image configuration of the Spiegelmer confers nuclease resistance and, thus, excellent biostability and 2) 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.

Distribution of anti-CCL2 Spiegelmer was confined to the tissue regions where CCL2 is expressed in MRL\textsuperscript{lpr/lpr} mice. \textit{In situ} hybridisation located Spiegelmers in the tubulointerstitial compartment of the kidney where CCL2 is known to be expressed at high levels in these mice. Plasma levels of CCL2 where found to be high in the circulation as Spiegelmer tend to bind CCL2 to neutralize it biologically and keep it in the circulation. This showed the specificity of anti-CCL2 Spiegelmer for murine CCL2 \textit{in vivo}.

Autoimmune-prone MRL\textsuperscript{lpr/lpr} 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. Improvement of lupus nephritis was seen with reduced activity index and chronicity index compared to control mice. As anti-CCL2 Spiegelmer acts by inhibiting the influx of inflammatory cells to injured tissues, we found a profound reduction in macrophages and T cell infiltration in renal tissue. But at the same time Spiegelmer treatment had no effect of autoimmunity parameters in
these mice. Thus, mNOX-E36-based inhibition of CCL2 represents a novel strategy for the treatment of lupus nephritis.

Cyclophosphamide (CYC) can control diffuse proliferative lupus nephritis by potent immunosuppression but remains associated with serious and life-threatening complications. Drugs that specifically target mediators of diffuse proliferative lupus nephritis may help to reduce CYC dose and side effects. In the second part of the study we wanted, 1) To compare the efficacy of anti-CCL2 Spiegelmer with that of existing therapies for lupus nephritis, 2) To evaluate the efficacy of combining anti-CCL2 Spiegelmer and low dose of CYC with that of high dose of CYC, 3) To compare the safety profile of this combination with high dose CYC.

To achieve that we injected MRL lpr/lpr mice with diffuse proliferative lupus nephritis 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, diffuse proliferative lupus nephritis 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 equipotent to CYC full dose on kidney and lung injury. Serum IL12p40 and TNF-α levels were all markedly affected by CYC full dose but not by CYC one-fourth full dose.

In other experimental design with four groups, we injected MRL lpr/lpr mice with vehicle, anti-CCL2 Spiegelmer, anti-CCL2 Spiegelmer plus CYC (once/4 weeks) and CYC (once/week). We did the treatment for one month before terminating the experiment. CYC full dose was associated with reduction in spleen weights, CD3+CD4-CD8- T cells and CD3+CD4+CD25+ T cells. No additive effects of anti-CCL2 Spiegelmer were noted on bone marrow colony-forming unit-granulocyte macrophage counts and 7/4(high) monocyte counts, lymphoproliferation, and spleen T cell depletion.

In summary, anti-CCL2 Spiegelmer permits 75% dose reduction of CYC for controlling diffuse proliferative lupus nephritis and pneumonitis in MRL lpr/lpr mice, sparing suppressive effects of full-dose CYC on myelosuppression and T cell depletion.
Conclusion

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 MRL<sup>lpr/lpr</sup> mice but also demonstrates its suitability in terms of pharmacokinetic profile as well as its absence of immunostimulatory adverse effects. Furthermore, combination of anti-CCL2 Spiegelmer and CYC therapy permits significant dose reduction of CYC and could be proposed as novel strategy to reduce CYC toxicity in the treatment of severe lupus.
7. Zusammenfassung


Die Verteilung des anti-CCL2-Spiegelmers war auf die CCL2-exprimierten Gewebe in MRL\(^{lpr/lpr}\)-Mäusen begrenzt. Durch \textit{in situ}-Hybridisierung konnten die Spiegelmere im tubulointerstitiellen Kompartement der Niere lokализiert werden, wo CCL2 bekanntermaßen vermehrt exprimiert wird. Im Plasma konnten ebenfalls hohe CCL2-Spiegel nachgewiesen werden, da Spiegelmere dazu tendieren CCL2 zu neutralisieren und weiter zirkulieren. Dies zeigte die Spezifität des anti-Spiegelmers für murines CCL2 \textit{in vivo}.

MRL\(^{lpr/lpr}\)-Mäuse, ein murines SLE Modell, wurden mit einer pegylierten Form des mNOX-E36 im Alter von 14 bis 24 Wochen behandelt. Diese Mäuse zeigten ein verlängertes Überleben, welches mit einer deutlichen Verbesserung der

Cyclophosphamid (CYC) kann die diffuse proliferative Lupusnephritis durch potente Immunsuppression kontrollieren. Diese Therapie ist aber mit schwerwiegenden und lebensbedrohlichen Komplikationen verbunden. Medikamente, die spezifisch Mediatoren der diffusen proliferativen Lupusnephritis erreichen, könnten helfen, die CYC-Dosis und damit Nebenwirkungsraten zu reduzieren. Im zweiten Teil dieser Studie wollten wir 1) die Wirksamkeit des anti-CCL2-Spiegelmers mit bereits existierenden Therapien der Lupusnephritis vergleichen, und 2) die Wirksamkeit des anti-CCL2-Spiegelmers in Kombination mit niedrig-dosiertem CYC evaluieren und 3) die Nebenwirkungsraten dieser Kombinationsstudie mit der der hoch-dosierten CYC-Behandlung vergleichen.

Dazu injizierten wir MRL
\textsuperscript{Lpr/Lpr}-Mäusen mit nachgewiesener diffuser proliferativer Lupusnephritis im Alter von 14 Wochen jeweils den Trägerstoff, 30 mg/kg CYC wöchentlich (hoch-dosiert), 30 mg/kg CYC monatlich (ein Viertel der hoch-dosierten CYC-Gabe), pegyliertes Kontroll-Spiegelmer, pegyliertes Anti-CCL2-Spiegelmer (3mal/Woche) sowie pegyliertes Anti-CCL2-Spiegelmer in Kombination mit CYC (ein Viertel der hoch-dosierten CYC-Gabe) und Mycophenolat Mofetil. Im Alter von 24 Wochen war die proliferative Lupusnephritis und die autoimmune Lungenbeteiligung durch die Behandlung mit der hoch-dosierten CYC-Gabe komplett aufgehoben, was jedoch nicht mit der Behandlung mit niedrig-dosierten CYC (ein Viertel der hoch-dosierten CYC-Gabe) erreicht werden konnte. Die Behandlung der Kombination aus niedrig-dosiertem CYC (ein Viertel der hoch-dosierten CYC-Gabe) und Spiegelmer erzielte vergleichbare Ergebnisse bezüglich der Nieren- und Lungenbeteiligung wie die die Behandlung mit hoch-dosiertem CYC. IL12p40- und TNF-α-Werte im Serum
waren in der hoch-dosierten CYC-Gruppe deutlich beeinflusst, jedoch nicht in der Gruppe mit der niedrig-dosierten CYC-Gabe.


Zusammenfassend ermöglicht die anti-CCL2-Spiegelmer-Therapie eine 75%ige Dosis-Reduzierung von CYC, um die diffuse proliferative Lupusnephritis und Pneumonitis in MRL<sup>lpr/lpr</sup>-Mäusen zu behandeln, während die suppressiven Eigenschaften der hoch-dosierten CYC-Gabe mit Knochenmarksuppression und T-Zell-Depletion ausgespart werden.

**Fazit**

Die Spiegelmer-basierte Hemmung von CCL2 offenbart eine neue und vielversprechende Therapieoption für die Behandlung der Lupusnephritis. Das Präparat mNOX-E36 zeigt nicht nur Wirksamkeit im MRL<sup>lpr/lpr</sup>-Mausmodell, sondern demonstriert auch seine Eignung im Hinblick auf das pharmakokinetische Profil und das Fehlen immunstimulatorischer Nebenwirkungen. Darüber hinaus ermöglicht die Kombinationstherapie mit anti-CCL2-Spiegelmer und CYC eine signifikante Dosisreduktion von CYC und könnte somit als neue Therapiestrategie zur Toxizitäts-Reduktion von CYC bei der Behandlung des progressiv verlaufenden Lupus vorgeschlagen werden.
8. References


9. Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>BM</td>
<td>basement membrane</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>BUN</td>
<td>blood urea nitrogen</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
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<tr>
<td>CCL2</td>
<td>chemokine C-C motif ligand 2</td>
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<tr>
<td>CCL5</td>
<td>chemokine C-C motif ligand 5</td>
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<tr>
<td>CCR1</td>
<td>chemokine C-C motif receptor 1</td>
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<tr>
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<tr>
<td>CCR3</td>
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<td>CXCR7</td>
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</tr>
<tr>
<td>CX3CR1</td>
<td>chemokine C-X3-C motif receptor 1</td>
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<tr>
<td>CKD</td>
<td>chronic kidney disease</td>
</tr>
<tr>
<td>CT</td>
<td>cycle threshold</td>
</tr>
<tr>
<td>ddH$_2$O</td>
<td>double distilled water</td>
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<tr>
<td>DEPC</td>
<td>diethyl pyrocarbonate</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>et al.</td>
<td>et alii = and others</td>
</tr>
<tr>
<td>e.g.</td>
<td>exempli gratia = for instance</td>
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<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylendiamintetra acetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-Linked Immunosorbent Assay</td>
</tr>
<tr>
<td>ESRD</td>
<td>end-stage renal disease</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescence activated cell sorting</td>
</tr>
<tr>
<td>FCS</td>
<td>fetal calf serum</td>
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FITC  fluorescein isothiocyanate
GBM  glomerular basement membrane
GFR  glomerular filtration rate
HE  Hematoxylin-Eosin
hpf  high-power-field
ICAM-1  intercellular adhesion molecule-1
i.e.  id est = in other words
IL  interleukin
IFN-γ  interferon-γ
kDa  kilo Dalton
MCP  monocyte chemoattractant protein
MCP-1  monocyte chemoattractant protein-1
MCP-2  monocyte chemoattractant protein-2
min  minute/minutes
ml/min  milliliter/minute
M/M  monocytes / macrophages
mNOX-E36  anti-CCL2 Spiegelmer
mRNA  messenger ribonucleic acid
n.d.  nondetectable
NF-κB  nuclear factor-κB
O.D.  optical density
PAS  Periodic acid Schiff
PBS  phosphate buffered saline
PCR  polymerase chain reaction
pDCs  plasma dendritic cells
POC  scrambled sequence of RNA
(Control Spiegelmer for mNOX-E36)
RANTES  regulated on activation normal T cell expressed and secreted
RNA  ribonucleic acid
Rnase  ribonuclease
RPM  revolutions per minute
RPMI Medium  cell culture medium
rRNA  ribosomal ribonucleic acid
RT  room temperature
<table>
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<th>Abbreviation</th>
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<tr>
<td>RT-PCR</td>
<td>real-time reverse transcription-polymerase chain reaction</td>
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<tr>
<td>s</td>
<td>second</td>
</tr>
<tr>
<td>TGF-β</td>
<td>transforming growth factor-β</td>
</tr>
<tr>
<td>Th1</td>
<td>T helper cell type 1</td>
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<td>TNF-α</td>
<td>tumor necrosis factor-α</td>
</tr>
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<td>units</td>
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<tr>
<td>VCAM-1</td>
<td>vascular cell adhesion molecule-1</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
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<td>vs</td>
<td>versus</td>
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<tr>
<td>v/v</td>
<td>volume/volume</td>
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Appendix

1. FACS buffer:
   Sterile DPBS  500 ml
   Na Azide  500 mg (0.1 %)
   BSA  1 g (0.2 %)

2. Paris Buffer:
   20 mM Tris-HCL, 125 mM NaCl, 10 mM KCl, 10 mM Sodium acetate,
   5 mM Glucose.
   For 1000 ml:
   Tris-HCL (MW 121.14)  2.4228 g
   NaCl (MW 58.44)  7.31 g
   KCl (MW 74.56)  0.74556 g
   Sod. Acetate (MW 82.03)  0.8203 g
   D-Glucose (MW 180.16)  0.9 g

3. 10X HBSS (Hank’s Balanced Saline Solution) with Ca, Mg:
   For 1000 ml
   KCl  4 g
   KH₂PO₄  0.6 g
   NaCl  80 g
   Na₂HPO₄.2H₂O  0.621 g
   NaHCO₃  3.5 g
   CaCl₂  1.4 g  (or CaCl₂.2H₂O  1.854 g)
   MgCl₂.6H₂O  1 g
   MgSO₄.7H₂O  1 g
   D-Glucose  10 g
   Dissolve in 900 ml of distilled water and adjust to pH 7.4 with 1N HCl
   or 1N NaOH. Make up the volume with distilled water to 1000 ml.

4. 10X HBSS (Hank’s Balanced Saline Solution) without Ca, Mg:
   For 1000 ml
   KCl  4 g
   KH₂PO₄  0.6 g
5. DNAse stock solution (1 mg/ml):

DNAse (type III) 15000 U/6 mg (Sigma D5025)

To prepared 1 mg/ml solution: Add 6 ml of 50 % (w/v) Glycerol in 20 mM Tris-HCl (pH 7.5), 1 mM MgCl₂.

Can be kept at – 20 ⁰C for several weeks.

Caution: Solution is stable only for 1 week at 4 ⁰C.

6. 50 % Glycerol in 20 mM Tris-HCl (pH 7.5), 1 mM MgCl₂:

a. 0.48 g of Tris-HCl in 100 ml of distilled water, adjust pH to 7.4 (= 40 mM)

b. 50 ml of Glycerol 100 % + 50 ml of 40 mM Tris-HCl (20 mM)

c. Add 100 ul of 1M MgCl₂ solution.

7. Collagenase / DNAse solution:

1 mg/ml Collagenase, 0.1 mg/ml DNAse in 1X HBSS (with Ca, Mg)

For 10 ml:

Collagenase (type I) (Sigma C0130) 10 mg

1 mg/ml DNAse stock solution 1 ml

HBSS (with Ca, Mg) 9 ml

To be preheated in 37 ⁰C water bath before use.

Caution: Prepare freshly every time (Stable only for few days)

8. Collagenase solution:

1 mg/ml Collagenase in 1X HBSS (with Ca, Mg)

For 10 ml:

Collagenase (type I) 10 mg

HBSS (with Ca, Mg) 10 ml

To be preheated in 37 ⁰C water bath before use.

Caution: Prepare freshly every time (Stable only for few days)
9. EDTA 2 mM:
   EDTA 7.44 mg in 10 ml HBSS (without Ca, Mg)
   To be preheated in 37°C water bath before use.

9. MACS Buffer
   PBS pH 7.2
   0.5% bovine serum albumin
   2 mM EDTA

10. Citrate buffer 10X
    110 mM Sodiumcitrate
    in ddH₂O
    with 2N NaOH to pH 6

11. PBS
    2.74 M NaCl
    54 mM KCl
    30 mM KH₂PO₄
    130 mM Na₂HPO₄
    in ddH₂O
    Adjust pH to 7.5 with HCl
Curriculum vitae

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Research publications


Previous education

2. **(1998-2002) Bachelor** of Pharmaceutical sciences was completed at College of Pharmacy Solapur, Shivaji University, India.

**Additional Research Experience in R & D**


**Awards and Scholarships**

1. First prize at ‘Fellows night’ nephrology forum, Munich-2008.
2. Awarded with ‘Teaching assistance ship for LMU research students’ in year 2009.

**Abstracts and conference proceedings**

1. Paul-Martini-Workshop 2006, Toll-like receptor-based drug development, Universität-Bonn, Bonn, attended on September 15-16 2006,

**References**

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**Declaration** -The above stated particulars are true to the best of my knowledge.

(Mr. Onkar P. Kulkarni)