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Nucleic acid specific Toll-like receptors in lupus nephritis

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I dedicate this thesis to,
my mother Prema S. Patole and father Shivaji Vaman Patole
to whom, I owe much more than I can express here.
I would not be who and where I am today without their support,
encouragement and sacrifices.

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1. INTRODUCTION

1.1 Immune complex glomerulonephritis

The most common form of immune complex glomerulonephritis include, IgA nephropathy, post infectious glomerulonephritis and autoimmune e.g. lupus nephritis. Lupus nephritis is a form of immune-complex glomerulonephritis that remains a leading cause of morbidity and mortality in SLE. The concept that the pathogenesis of many common forms of idiopathic glomerulonephritis, such as membranous nephropathy and Immunoglobulin-A nephropathy, is related to immune complexes is widely accepted. Although anti-dsDNA was once thought to cause glomerulonephritis by forming complexes with DNA that are passively trapped in the glomeruli (1), many investigators now believe that anti-double stranded (ds)DNA antibodies are pathogenic to the kidney via direct (cross-reactivity) or indirect (via a nuclear-antigen bridge) binding to glomerular structures (2). SLE is characterized serologically by a variety of autoantibodies to deoxyribonucleic acid (DNA), ribonucleic acid (RNA), other nuclear antigens (e.g. Smith, Ro, La) and cytoplasmic antigens. The presence of anti-dsDNA antibodies has been linked most closely to pathogenicity (3), in particular the renal histological activity score (4).

1.2 Pathophysiology of lupus nephritis

Anti-double stranded (ds)DNA antibodies are thought to play a crucial role in the pathogenesis of lupus nephritis, a common form of immune complex glomerulonephritis (2, 5). Clinically, anti-dsDNA antibodies can be eluted from the kidneys of patients with active nephritis, suggesting that these antibodies might be important in the induction of

tissue damage (1, 6). In many patients with SLE, increased renal disease activity is associated with rising titers of anti-DNA antibodies, while prophylactic treatment of serologically active lupus patients with corticosteroids significantly reduces the number of subsequent disease flares (7). Accumulating data suggest that nucleosomes are the mediators of autoantibody-related glomerular immune-complex deposition, along with the major autoantigens that elicit the autoimmune response (8, 9, 10). Autoantibodies reactive to nucleosomes have been detected both in patients with lupus and in urine models, even prior to the development of anti-dsDNA and anti-histone antibodies (11). Upon renal deposition, the immune complex–mediated activation of complement through the classic pathway is traditionally believed to be a major mechanism by which tissue injury occurs (12). Further a role for self-DNA in the pathogenesis of SLE and associated lupus nephritis has been proposed. Recent studies on epigenetics, including DNA methylation and its regulatory enzymes, seem likely to contribute to elucidation of the pathogenesis of SLE and associated nephritis (13). It has been shown that methylation of CpG-motif prevents their stimulatory effect on B cells (14) and genomic DNA released by dying cells has been shown to induce the maturation of antigen-presenting cells (15). Interestingly, known inhibitors of DNA methylation can induce SLE in humans (16). Furthermore, in vertebrates inhibitory DNA sequence elements counterbalance the immunostimulatory effects of unmethylated CpG-DNA (17). Taken together these findings point towards the role of endogenous CpG DNA in the pathogenesis of SLE and associated lupus nephritis. However, the systemic pathophysiology and end organ damage during lupus nephritis that is initiated by the renal deposition of immune

complexes is driven by a complex interplay of events led by the innate and adaptive components of the immune system.

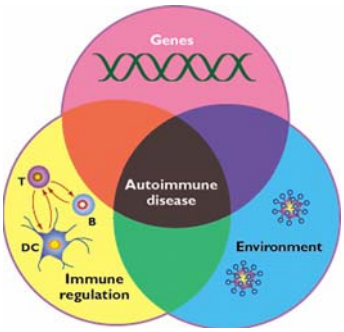
1.2.1 Overview of the immune system

The immune system is an integrated body system of organs, tissues, cells, and cell products such as antibodies that differentiates self from nonself and neutralizes potentially pathogenic organisms or substances**.** The immune system acts as a defence against foreign pathogens such as viruses, bacteria, and parasites. Immune system is often divided into the two different sections of innate and adaptive immunity (Table.1), the former encompassing unchanging mechanisms that are continuously in force to ward off noxious influences, and the latter responding to new influences by mounting an immune response.

Table 1

Property	Innate immunity	Adaptive immunity
Triggering molecules	repetitive molecular units	antigen + association structures
Recognition mechanism	direct	indirect
Onset of response	immediate (hours)	delayed (days)
Clonal expansion of responder cells	No	yes
Induces inflammation	always	often
Memory (to prevent reinfection)	No	yes

Figure. 1



The innate immune responses involve: Phagocytic cells (neutrophils, monocytes, and macrophages); cells that release inflammatory mediators (basophils, mast cells, and eosinophils); natural killer cells (NK cells); and molecules such as complement proteins, acute phase proteins, and cytokines. Examples of innate immunity are anatomical barriers, mechanical removal, chemicals, the complement pathways, phagocytosis, inflammation, fever, and the acute phase response. The adaptive immunity usually involves: Antigen-presenting cells (APCs) such as macrophages and dendritic cells (DCs); the activation and proliferation of antigen-specific B-lymphocytes; the activation and proliferation of antigen-specific T-lymphocytes; and the production of antibody molecules, cytotoxic T-lymphocytes (CTLs), activated macrophages and NK cells, and cytokines. There are two major branches of the adaptive immune responses: cell-mediated immunity and humoral immunity. Cell-mediated immunity involves the production of cytotoxic T-lymphocytes, activated macrophages, activated NK cells, and cytokines in response to an antigen and is mediated by T-lymphocytes. Humoral immunity involves the production of antibody molecules in response to an antigen and is mediated by B-lymphocytes. In this manner the innate and adaptive immune systems work in a concerted fashion to protect and organism from disease causing infectious agents. However, loss of tolerance mechanisms of the body against substances and tissues normally present in the body can lead to a condition termed as ‘autoimmune disease’. Amongst others, five clinical disorders that

are autoimmune in nature and most common in occurrence include: rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), insulin-dependent diabetes mellitus (IDDM), autoimmune thyroid disease and multiple sclerosis (MS) (18, 19, 20, 21, 22). The development of such autoimmune diseases involves mainly three factors comprising; genetic predisposition, environmental factors and immune dysregulation (Figure. 1). Among the genetic markers of predisposition to autoimmune disease are specific sets of genes for MHC molecules that both shape and regulate the specificity of the adaptive immune response. The mammalian genetic makeup determines not only how the immune system deals with antigenic challenges from the environment, but also how the immune system is regulated to remain tolerant towards self (23). During SLE loss of such self tolerance mechanisms occurs and autoreactive T cells that are necessary to activate B cells, are further stimulated to proliferate and produce autoantibodies by the elevated levels of proinflammatory cytokines (24, 25) contributing to disease in lupus nephritis. Furthermore, the autoantibody production may be enhanced further by T and B cell interaction providing anti-apoptotic signals (26, 27, 28), which may additionally contribute to autoantibody production and their renal deposition leading to tissue damage. Additionally, IL-12 levels are downregulated by IL-10, with lower levels correlating with increased disease activity and nephritis (29, 30). Further a pathogenic role for cytokines such as IL-6, IL-10 and IL-12 has been identified, in SLE and associated lupus nephritis, through a number of studies (31, 32, 33). Understanding of these and additional mechanisms involved in the pathogenesis of lupus nephritis has provided a basis for developing effective therapies.

Current therapies

Mycophenolate mofetil : (34, 35)

Rituximab (CD20 antibody): B cell depleter (36, 37)

Table 2

Biological therapies in development for SLE		
Therapy	**Mechanism of action**	**Trials**
Recombinant IL-1 receptor antagonist (Anakinra)	IL-1: potential role in development and maintenance of inflammation in SLE. IL-1RA; physiological antagonist to IL-1	Well tolerated. Principally effective (transient) for arthritic symptoms. Reduction in C3 and C4 (38, 39)
Anti-IL-10 monoclonal antibody	IL-10; pleiotropic cytokine, induces B cell differentiation	Use in NZB/WF1 mice delayed disease onset and autoantibody production (40)
		In clinical trial improved joint and cutaneous symptoms and suppressed SLE (41)
B cell tolerogens (LJP 394)	Synthetic molecule composed of multiple B cell dsDNA epitopes attached to non immunogenic carrier. Bind to anti-dsDNA receptors; modulates B cell responses, anergy and thus cessation of autoantibody production	Randomized, DBPC trial Serological improvement but minimal reduction in renal flares (42)
Anti-B lymphocyte stimulator	anti-BLys modulates B cell immune responses by (BLyS) reduction of apoptosis, interference in B cell development and differentiation	Phase I study: reduction in immunoglobulin and anti-dsDNA titres (43) Phase II trial under way
DBPC: double-blind placebo-controlled; IL-1: interleukin 1; IL-1RA: interleukin I receptor antagonist; C3: complement 3; C4: complement 4; IL-10: interleukin 10		

Adapted and modified (44).

1.2.2 Therapeutic approaches in treatment of lupus nephritis

In the past 40 years, prognosis for patients with lupus nephritis has improved, with 10-year survival now approximately 90% (45, 46). This is probably because a combination of earlier disease diagnosis and due in part to the availability of multiple serological tests for SLE, use of steroids, other immunosuppressive agents, availability of renal dialysis and transplantation. More recently, advancements in the understanding of molecular mechanisms involved in pathogenesis of SLE have translated to the development of novel therapies (Table 2), offering possible alternatives for this patient cohort (44). However, the potential for significant morbidity and mortality remains in the group of patients with partially responsive or treatment resistant disease. Thus, prompting additional research efforts for discovery of more reliable means of treating the underlying disease. One of the major concerns in this regard is the poorly known molecular mechanisms involved in the pathogenesis of SLE and associated lupus nephritis. Elucidating the path mechanisms leading to lupus and the commonly associated nephritis not only would broaden our understanding for the cause but would also enable to discover more promising preventive or curative therapeutic remedies.

1.2.3 Infections and their common association to lupus nephritis

A common association of microbial infections may be linked not only to induction but also progression of pre-existing autoimmune disease such as lupus (47). The infectious stimuli, such as viral or bacterial agents that infect individuals affected by SLE, are commonly known inducers of disease flares and may lead to organ damage and/or dysfunction as seen in immune-complex glomerulonephritis. This puts the patient at a

further risk of disease aggravation worsened due to overt immune activation during intercurrent infections. It is known that viral components can trigger disease activity in SLE or autoimmunity in general (48), but the involved mechanisms remain poorly defined. It is believed that during viral infections, pathogen recognition and subsequent induction of adaptive immune responses might interfere with the control of self-tolerance in susceptible individuals. Likewise, experimental studies with rodents suggest that injection of synthetic CpG-ODN (a bacterial DNA analogue) can exacerbate underlying autoimmune tissue injury e.g. glomerulonephritis, experimental encephalomyelitis, collagen-induced arthritis or SLE (49, 50, 51, 52). Further during infection, recognition and mounting of an appropriate immune response for combating the pathogen, is initiated by Toll-like receptors (TLRs), a family of receptors that sense conserved pathogen associated molecular patterns (PAMPs). Besides recognition of foreign antigens to initiate immune responses against the invading pathogens, TLRs are known to sense certain endogenous molecules (Table 3). By virtue of the latter property TLRs and their ability to modulate innate and adaptive immunity may be expected to affect disease activity in autoimmune SLE-associated lupus nephritis and other forms immune complex glomerulonephritis.

1.3 Toll-like receptors as immune sensors of microbial infections

Infectious microbes are recognized by our immune system so as to exert antimicrobial responses. A family of receptors that commit themselves to this cause are the TLRs (53). TLRs were originally identified in Drosophila, where they were found to play a major role in protection from fungal infections (54).

Table 3

		Potential endogenous ligands
Toll-like receptor	**Exogenous ligands***	**Ligand**
TLR1 & 2	Tri-acyl lipopeptides (bacteria)	
TLR2	Peptidoglycan (Gram-positive bacteria)	Heat shock proteins
	Lipoteichoic acid (Gram+ bacteria)	High mobility group box protein 1
	Lipoarabinomannan (mycobacteria)	
	Glycophospholipids (Trypanosomes)	
	Glycolipids (*Treponema*), Porins (*Neisseria*), Zymogen (fungi), Lipopeptides	
TLR3	Double-stranded RNA (virus), SiRNA (55)	mRNA
TLR4	Lipopolysaccharides, lipid A (Gram-negative bacteria)	Heat shock proteins
	Taxol (plant)	High mobility group box protein 1
	Protein F (respiratory syncytial virus)	Fibronectin extra domain A
	Hyphae (*Aspergillus*)	Fibrinogen
	HSP60 (*Chlamydia*)	Lung surfactant protein A
	Viral envelope proteins	Low density lipoprotein
		Heparan sulphate
		Hyaluronan fragments
TLR5	Flagellin (bacteria)	
TLR6 & 2	Di-acyl lipopeptides	
TLR7	Single-stranded RNA (viral)	
TLR8	Single-stranded RNA (viral)	
TLR9	DNA (bacteria & Herpes simplex virus), hemozoin (56)	DNA
TLR11	Uropathogenic *Escherichia coli*, profillin-like protien (57)	

Adapted and modified (53)

There are at least 11 currently identified mammalian orthologues, and these TLRs bind a remarkably diverse array of bacterial, viral, fungal and interestingly self molecular patterns (Table 3) (58). The basic structural features of the TLR family include an extracellular domain made up of multiple leucine-rich repeats and a highly conserved cytoplasmic signaling domain. The extracellular-domain forms a horseshoe-shaped solenoid with a broad hydrophobic surface that may account for the promiscuous ligand reactivities documented for TLR2 and TLR4 (58, 59). The cytoplasmic domain is shared with interleukin-1 receptor (IL-1R) family members and is commonly referred to as the Toll/IL-1R (TIR) domain. It follows that Toll and IL-1 family members use common adapter molecules, including myeloid differentiation factor 88 (MyD88), as part of their signaling cascades. Heterogeneous expression patterns of the nucleic acid specific TLRs on leukocyte subpopulations and the discovery of TLR-specific signaling pathways support specific types of immune responses for specific ligand-TLR interactions (60, 61). TLRs are differentially expressed by a range of cell types including macrophages, monocytes, dendritic cell subsets, B cells, a variety of endothelial and epithelial cell types (60). Monocytes, macrophages, and DCs routinely respond to TLR engagement by the production of proinflammatory cytokines and upregulation of costimulatory molecules (58). The B-cell response to TLR ligands is further characterized by proliferation and antibody production, and the natural IgM produced by these cells may facilitate the clearance of microbial particles as well as apoptotic or necrotic cell debris. This rapid response on the part of the innate immune system serves two important roles: first, to initially contain infectious microbes and secondly, to activate the adaptive immune response. An over-exuberant innate immune response can, however, lead to toxic shock

or other sepsis-associated complications. Among the innate immune cells, immature DCs, which are capable of capturing pathogens by phagocytosis, express several kinds of TLRs. The immature DCs mature after the recognition of microbial components via TLRs. The mature DCs in turn present pathogen-derived antigen, express co-stimulatory molecules, secrete several inflammatory cytokines including IL-12, and interact with naive T cells. The naive T cells harboring the antigen-specific T cell receptor are instructed to develop into Th1 cells, and clonally expand to exhibit effective adaptive immune responses. Likewise, IL-6 produced as a result of DC activation through TLR ligation is involved in regulatory T-cell suppression and leads to increased autoreactivity (62). These and additional findings provide an ample evidence for involvement of TLRs in linking innate immunity to adaptive immunity not only in infection associated immunity but also autoimmunity.

1.3.1 Toll-like receptors that sense nucleic acids

A diverse of bacterial, viral or fungal molecular patterns is sensed by the TLRs that signal to mount an appropriate immune response. In this context, a subfamily of TLRs namely, TLR3, TL7, TLR8, and TLR9 form an interesting group of receptors specifically recognize nucleic acid motifs.

TLR3: TLR3 is a detector of double-stranded (ds) RNA that may originate from single-stranded (ss)RNA or dsRNA viruses (63, 64). Among the monocytic immune cell subsets TLR3 is expressed on murine macrophages; whereas, in humans TLR3 is exclusively expressed on myeloid DCs (63, 65, 66). It is believed that TLR3 recognizes secondary RNA structures as synthetic RNAs, mRNA, and small inhibitory RNA (siRNA) similarly

induce the production of type I interferons and proinflammatory cytokines. Viral dsRNA induces DC maturation through TLR3 (63). In addition, TLR3 has been reported to be expressed on various cells, such as astrocytes (67), uterine epithelial cells (68), and fibroblasts (69). These cells express TLR3 constitutively at low levels and upregulate TLR3 upon exposure to dsRNA or other TLR ligands. Most cell types express TLR3 in an endosomal compartment, which supports the idea that viruses need to be processed before their RNA can be exposed to TLR3. However, fibroblasts have been reported to express TLR3 also on their outer surface membrane (69). Apparently, viral RNA can act as a natural adjuvant that promotes loss of tolerance against presented endogenous or exogenous antigens and modulates the Th1/Th2 balance of the subsequent T cell response (70).

TLR7 and TLR8: Similar to TLR3 and 9, TLR7 and 8 are expressed intracellularly in endosomes and recognize phagocytosed ligands on macrophages, DCs and B cells (71, 72). TLR7 and TLR8 both recognize viral ssRNA as well as distinct synthetic guanosine analogues (58, 73). Certain U-rich or U/G-rich oligonucleotides presented as a complex with cationic lipids (but not in free form), induce recognition and activation by means of mouse TLR7 and human TLR8 (74). Activation of TLR7 and 8 on dendritic cells lead to their maturation and production of proinflammatory cytokines so as to exert a typical antiviral response (73).

TLR9: Unmethylated cytosine-guanosine (CpG)-DNA, an important ligand for TLR9 (75) has been widely employed for several immunotherapeutic purposes (76). The CpG dinucleotide is the stimulatory motif of bacterial and viral DNA (14). CpG-DNA is a B cell mitogen and a strong activator of plasmacytoid DCs in humans (72). CpG-DNA

complexed with other proteins induces an enhanced antigen-specific humoral and cellular immune response of the Th1 type (77). TLR9 resides in the endoplasmic reticulum but redistributes to late endosomes for the interaction with ingested CpG-DNA (71). Various synthetically manufactured CpG-oligodeoxynucleotides (ODNs) represent powerful tools for research in this field. A recent study described plasmodia hemozoin as another natural ligand for TLR9, which raises doubt against the concept that TLR9 recognizes specific nucleic acid sequences (56, 78). TLR9 might recognize particle-related secondary structures rather than specific DNA sequences. This is supported by the observation that the formation of DNA nanoparticles can modulate TLR9 signalling towards production of high levels of type I interferons (79). In particular IFN-α exposure or prolonged expression of IFN-α *in vivo* induces early lethal lupus and associated nephritis in susceptible mice (80).

1.3.2 Nucleic acid-specific TLRs modulate autoimmunity

Although under normal circumstances TLRs do not recognize self-molecules, signaling through these receptors may play important role in the loss of self-tolerance and induction of autoimmunity. Several recent studies reflect a major role for nucleic acid specific TLRs, in linking innate immunity (81, 82) to adaptive immunity (83). Recently TLR 3 and 7 engagement has been shown to convert T-cell autoreactivity into overt autoimmune disease, through release of IFNα-mediated upregulation of MHC I on pancreatic tissue (84). In another study replicase-based nucleic acid vaccines that generate dsRNA, which is recognized by TLR3 have been found to break self-tolerance and convert autoreactivity into overt disease (85). Leadbetter et al. observed that, in a

mouse model of systemic autoimmune diseases, the simultaneous activation of cell-surface antigen receptors and TLR9 causes a particular subclass of self-immunoglobulin (IgG)2a, to be recognized by B cells as a pathogen and triggers T-cell-independent proliferation of B cells (77). In this study the role of mammalian DNA in activating TLR9 was confirmed using DNase treatment that rendered the immune complexes inactive. In another study, DNA with unmethylated CpG motifs efficiently stimulated anti-DNA idiotype-expressing B cells (86). Another study identified plasmacytoid DCs to be activated by immune complexes containing nucleic acid released by necrotic or late apoptotic cells and lupus IgG leading to induction of interferon (IFN)-α production (87). IFNα has been recently identified as a crucial player in induction of autoimmunity by virtue of diverse array of effects it has on innate and adaptive immunity, and the potential mechanisms involved have been discussed in a detailed review by Theofilopoulos AN et al (88) A crucial role of DCs has been identified in autoimmune disease induction where, DCs loaded with a heart-specific self peptide were shown to induce CD4+ T-cell-mediated myocarditis in nontransgenic mice. In this study, TLR3 or TLR2, 4 or 9 stimulation in concert with CD40 triggering of self peptide-loaded DCs, was shown to be required for disease induction (89). Activation of APCs by TLR9 or TLR4 engagement can break self-tolerance and trigger the development of autoimmunity even in a genetically resistant strain such as B10.S mice, transgenic for a T cell receptor specific for the encephalitogenic protein peptide, that normally are resistant to spontaneous experimental allergic encephalomyelitis (EAE) (90).

The ability of nucleic acid specific TLRs for pathogen control is widely accepted, but their role in autoimmunity is less well defined (91, 92). While, TLR9 is known to be

expressed on antigen presenting cells (APCs) and B cells, TLR3 is reported to be expressed on APCs and on various non-immune cells (67, 68, 69). Local tissue inflammatory responses through TLR activation, upon exposure to their ligands due to infection or by endogenous molecules can therefore be expected to contribute to end organ damage, such as lupus nephritis. Experimental studies with rodents suggest that exposure to synthetic CpG-ODN can exacerbate underlying autoimmune tissue injury e.g. experimental encephalomyelitis, collagen-induced arthritis or SLE (50, 51). TLR9 activation by CpG–ODN has been shown to aggravate disease activity in spontaneous immune complex glomerulonephritis of MRL*lpr/lpr* mice (49, 52). Furthermore, TLR3 activation is followed by a robust induction of IFN-responsive genes (61, 93, 94), of which IFN-α is a well-known pathogenic factor for SLE and associated nephritis (88, 95). In SLE the interaction of CpG-DNA with TLR9 is of particular interest for the reasons that: 1. CpG-DNA is a B cell mitogen that allows T cell independent B cell proliferation and autoantibody production (77). 2. Immune complexes isolated from lupus patients activate TLR9 on DCs to produce IFN-α (96). 3. CpG DNA can aggravate autoimmune tissue injury locally by activation of tissue macrophages (50). Methylation of CpG-motif prevents their stimulatory effect on B cells (14). Additionally, genomic DNA released by dying cells can induce the maturation of APCs (15) while known inhibitors of DNA methylation can induce SLE in humans (16). Furthermore, in vertebrates inhibitory DNA sequence elements counterbalance the immunostimulatory effects of unmethylated CpG-DNA (17). It is known that certain synthetic ODNs with such inhibitory motifs have shown to block CpG-DNA-induced effects (97, 98, 99). However, experimental evidence for a pathogenic role of CpG motifs in self-DNA for

lupus is lacking. These and many other studies reveal a potential role for nucleic acid specific TLRs in modulating autoimmune disease and thus play a pathogenic role. However the contribution of nucleic acid specific TLRs in leading to infectious tissue injury leading to end organ damage such as that seen with immune complex glomerulonephritis is not clear. Moreover, considering their expression on intrinsic tissue cell types apart from the immune cell repertoire my have important implications in progression rather than induction of autoimmune tissue injury.

1.4 Aim of the study

In order to test the ability nucleic acid specific TLRs, namely TLR3 and 9 to modulate autoimmunity in MRL*lpr/lpr* mice it was hypothesized that: 1. Intercurrent infection induced exposure to viral dsRNA and can aggravate immune complex glomerulonephrits through TLR3 on specific cell types and 2. Bacterial as well as endogenous CpG DNA could drive lupus and synthetic inhibitory ODN would represent an appropriate tool to block the CpGDNA/TLR9 pathway *in vivo*.

2. MATERIAL AND METHODS

2.1 Oligoribonucleotides & oligodeoxyribonucleotides (ODN)

The following synthetic nucleotides were used for in vitro or in vivo studies: Polyinosinic-cytidylic acid (pI:C RNA) and Polydeoxyinosinic-deoxycytidylic acid (pI:C DNA) (dI:dC; Sigma-Aldrich, Steinheim, Germany), a synthetically made nucleotide sequence 3′-rhodamine-labeled dsRNA from human rhinovirus strain-16.11 with sequence (5′-AUCUGGGUUGUUCCCACCCAGAUCACCUACAUGG-rhodamine -3′ and (3′-UAGACCCAACAAGGGUGGGUCUAGUGGAUGUACC- 5′) (IBA, Göttingen, Germany) on a phosphorothioate backbone. The ODNs used were: ODN 2114: 5′-TCC TGG AGG GGA AGT -3′; CpG-ODN 1668: 5′-TCC ATG ACG TTC CTG ATG CT-3′, GpC-ODN 1720, 5′-TCC ATG AGC TTC CTG ATG CT-3′ (TIB Molbiol, Berlin, Germany) and 3'-rhodamine-labeled ODN 2114 on a phosphorothioate backbone.

2.2 Animal studies

In all animal studies, the mice were housed in filter top cages under a 12-hour light and dark cycle. Autoclaved water and standard chow (Sniff, Soest, Germany) were available ad libitum. All experimental procedures were performed according to the German animal care and ethics legislation and had been approved by the local government authorities.

Effect of pI:C RNA exposure on disease activity in lupus

For studying the effect of pI:C RNA exposure on disease activity during lupus, female MRL*lpr/lpr* mice were obtained from Jackson Laboratory (Bar Harbor, ME). 16-wk-old female MRL*lpr/lpr* mice were distributed into three groups, each consisting of 12 female

mice. From weeks 16 to 18, mice of all groups received intraperitoneal injections every other day as follows: 1) 50 μg of polyinosinic-cytidylic acid (pI:C) RNA (pI:C, Sigma-Aldrich, Steinheim, Germany) in 100 μl of normal saline, 2) 50 μg of pI:C DNA (dI:dC; Sigma-Aldrich, Steinheim, Germany) in 100 μl of normal saline, and 3) 100 μl of normal saline. All mice were killed by cervical dislocation at the end of week 18 of age. For assessing renal TLR3 mRNA expression, kidneys were obtained from 5- and 20-wk-old female MRL*lpr/lpr* mice after sacrificing by cervical dislocation. The renal distribution of 3′-rhodamine-labeled dsRNA was assessed by injecting it intravenously into MRL*lpr/lpr* mice at the age of 16 wk. Renal tissue was collected 2 h later and subjected to further analysis as described below. For the identification of 3′-rhodamine-labeled viral dsRNA positive renal cells, co-staining was performed on crysections using rat anti-F4/80 (Serotec, Oxford, UK; 1:50).

Effect of ODN 2114 treatment on disease activity during lupus

To determine the effect of ODN 2114 treatment on the disease activity during lupus, ten week old female MRL*lpr/lpr* mice were obtained from Harlan Winkelmann, Borchen, Germany. The mice were divided in 2 groups (n=10) and treated with either saline or ODN 2114 on alternate days from week 11 to 24 of age. Blood and urine samples were collected from each animal under ether anaesthesia at the end of the study and urine protein/creatinine ratio and serum blood urea nitrogen concentrations were determined using standard biochemical procedures. Serum dsDNA autoantibody IgG isotype titres were determined using a modified protocol for the respective Elisa kits (Bethyl Laboratories, Inc, Texas, USA). The procedure was modified in order to coat the sample

wells in the Elisa plate with murine dsDNA, in order to detect dsDNA specific IgG isotypes, while the remaining steps were carried out using the standard protocol described below. The mice were then sacrificed by cervical dislocation and the kidney, lung, liver and lymph nodes tissue pieces were collected, and processed for RNA isolation, immunostaining or paraffin fixed for histological analysis as described below. To assess the distribution of injected ODN 2114, 100 µg 3'-rhodamine-labeled ODN 2114 were injected intravenously into MRL*lpr/lpr* mice at the age 16 weeks. Tissues were collected 2 hours later and processed for immunohistochemical staining on paraffin or cryosections as described elsewhere (Anders 2003). Co-staining was performed on cryosections using rat anti-F4/80 (Serotec, Oxford, UK; 1:50) and anti-IgG (Dianova; 1:100).

Effect of ODN 2114 treatment on CpG ODN induced toxicity

8 week old female 129Sv mice obtained from Taconic (Ry, Denmark) were used to study the effect of ODN 2114 on CpG ODN induced toxicity. In this experiment, the mice were divided into 5 different groups containing 5 animals, each of which received for 12 days, daily intraperitoneal injections of: 1) 100 µl normal saline (NS) as vehicle control, 2) CpG ODN (40µg in 100µl NS), 3) CpG ODN + ODN 2114 (40 µg in 100 µl NS), 4) ODN 2114 (40 µg in 100µl NS) and 5) GpC ODN (40 µg in 100µl NS). After completion of the treatment period the mice were sacrificed by cervical dislocation and the kidney, lung, liver and lymph nodes tissue pieces were collected, fixed with 10 % formalin and processed for periodic acid Schiff staining for the examining histomorphological changes.

In vivo B cell activation

To test the ability of pI:C RNA to induce *in vivo* B cell activation, 4-wk-old MRL*lpr/lpr* mice bred in our own animal facility, were divided in three groups of two mice each. Each mouse received 500 μg/ml pI:C RNA, CpG, or pI:C DNA intraperitoneally, and the mice were killed after 24 h and spleens were collected and processed for flow cytometry as described below. To test the ability of ODN 2114 to suppress CpG-induced in vivo B cell activation, 4-wk-old MRL*lpr/lpr* mice, were divided in 5 groups of two mice each. All mice in different groups received intraperitoneal injections of 500 μg in 100μl saline CpG ODN, DN 2114 or CpG ODN together with ODN 2114, GpC ODN or saline alone respectively, and the mice were killed after 24 h and spleens were collected. Total spleen suspension from each mouse in all experimental groups was processed for flow cytometry as described below.

Primary cell culture

For both, pI:C and ODN 2114 treatment study or spleen monocytes and bone marrow derived dendritic cells (BMDCs) female MRL*lpr/lpr* mice 8-12 week of age were used. The mice were sacrificed by cervical dislocation and spleen and bone marrow were isolated for obtaining cultures of spleen monocytes or BMDCs, respectively. The whole spleen tissue was removed and processed for splenocytes isolation and culture as described below. For obtaining the BMDCs, whole bones from the femur from the fore- and hind-limbs were separated and processed as described in detail below.

2.3 Morphological and histological analysis

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. Upon isolation the tissue pieces were placed in plastic histocassettes and dipped in formalin. The formalin solution was prepared using 500ml of 40% Formaldehyde in PBS (32.5 g Na_2HPO4 and 20 g NaH_2PO4 in 4.5 L ddH_2O water, pH 7.4). The blocks were then infiltrated and embedded with paraffin and sections were cut in ribbons and mounted on slides. 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). The sections were then treated with following primary antibodies were used: anti-TLR3 (1:50; IMG516; Imgenex, San Diego, CA), anti-TLR9 (kindly provided by Dr. Stefan Bauer, Technical University Munich, Germany, 1:50), anti–ER-HR3 (1:50; monocytes/macrophages; DPC Biermann, Bad Nauheim, Germany) (19), anti-CD3 (1:100; BD Pharmingen, Hamburg, Germany), anti–smooth muscle actin (1:100; myofibroblasts, clone 1A4; Dako, Carpinteria, CA), anti-Ki-67 (DAKO, Hamburg, Germany, 1:25), anti-collagen I (LF-67, 1:50; provided by Dr. L.W. Fischer, National Institute of Dental Research, National Institutes of Health, Bethesda, MD), anti-CCL5 (1:50; Peprotech, Rocky Hill, NJ), anti-CCL2 (1:50; Santa Cruz Biotechnology, Santa Cruz, A), and anti-IgG and anti-IgG2a (Dianova; 1:100). Negative controls included incubation with a respective isotype antibody. In the next step, a suitably labelled secondary antibody was used with respective detection system. For quantitative analysis for evaluation of glomerulonephritis glomerular cells were counted using microscope in

10 cortical glomeruli per section. The severity of the renal lesions was graded using the indices for activity and chronicity of lupus nephritis (100). Activity index included semi-quantitative score of 4 active inflammatory lesions: 1. Glomerular leukocyte infiltration, 2. Interstitial inflammation, 3. Glomerular karyorrhexis and 4. Cellular crescents; while chronicity index included semi-quantitative score of 3 inflammatory lesions: 1. Glomerular sclerosis, 2. Interstital fibrosis and 3. Tubular atrophy. Semiquantitative scoring of glomerular IgG deposits from 0 to 3 was performed on 15 cortical glomerular sections using a semiquantitative index as follows: 0 = no signal, 1 = low signal, 2 = moderate signal, and 3 = strong signal intensity. Peribronchial and pulmonary inflammation was arbitrarily graded from 0 (no inflammation) to 3 (severe inflammation).

2.4 Cell culture and stimulation experiments

Cell lines

A murine mesangial cell line was maintained under standard culture conditions (in an incubator set at 37 °C supplied with 0.5% CO_2) in Dulbecco's modified Eagle's medium (DMEM, Biochrom KG, Berlin, Germany) supplemented with 5% fetal calf serum (FCS) (Serum Supreme, BioWhittaker, Walkersville, MD, USA), penicillin100 U/ml and streptomycin 100 µg/ml as described (Complete DMEM medium). J774 mouse macrophages (American Type Culture Collection, Rockville, MD, USA) were grown in RPMI 1640 medium containing 10% heat-inactivated bovine serum, penicillin100 U/ml and streptomycin 100 µg/ml (complete RPMI medium).

Primary cultures

Spleen monocytes: Spleen monocytes were obtained from spleens of 8-12 week old female MRL*lpr/lpr* mice. Whole spleens were isolated from the mice, placed in a petri-dish containing complete RPMI medium and mashed with the help of forceps, this coarse suspension was then passed through a 30 micron steel wire mesh and collected in a sterile petri-dish. This suspension was then centrifuged at 1600 RCF for 4 min at 4 °C to obtain a pellet. The pellet thus obtained was washed with sterile PBS and the obtained pellet was resuspended in 0.83 % ammonium chloride solution so as to haemolyse the red cells. This was followed by a washing steps (2x) as mentioned above, passed through a pre-separation filter to obtain single cell suspension (Miltenyi Biotec, Germany). Finally the cells were centrifuged, supernatant was discarded and the pellet was resuspended in an arbitrary volume of complete RPMI medium and cell counts were done. A desired number of cells was plated in 6 or 12 well plates and incubated at 37 °C for 24 hrs under standard culture conditions. The culture medium was replaced with fresh complete RPMI medium so as to obtain the adherent spleen monocytes, ready to be used for stimulation experiments.

Bone marrow derived dendritic cells: Bone marrow from 8-12 week old female MRL*lpr/lpr* mice was isolated, processed and cultured using published methods (101). Bone marrow was isolated from the tibia and femurs from the fore and the hind limbs of the mice. The bones were cleaned thoroughly so as to remove any muscle tissue and the bone caps were carefully removed so as to expose the pink portion, which was cut at one of the ends of the bone. A 25-gauge needle fitted to a 1 ml syringe, filled with complete

RPMI medium, was inserted to one end of the bone so as to flush the bone marrow from the other end and collected in a sterile petri-dish. In this manner, all bones were carefully flushed to obtain bone marrow, which was then centrifuged at 1600 RCF for 4 min at 4 °C to obtain a pellet. The pellet thus obtained was washed with sterile PBS and processed for hemolysis and washes as described above. Finally the cells were centrifuged, resuspended in an arbitrary volume of complete RPMI medium with 100 ng/ml human recombinant Flt3 ligand (Immunotools, Friesoyth, Germany) and cell counts were done. To select for bone marrow derived dendritic cells (BMDCs) for stimulation experiments, bone marrow isolates were cultured for 8 days in complete RPMI with 100 ng/ml human recombinant Flt3 ligand.

B-cells: B cells were isolated from spleens of female MRL*lpr/lpr* mice using B Cell Isolation Kit (Miltenyi, Bergisch Gladbach, Germany) following the manufacturer's protocol. Total spleens were harvested as described above and magnetic labelling was performed in which the cell pellet obtained from total spleen lymphocytes were resuspended in 40µl MACS buffer (PBS pH 7.2, supplemented with 0.5 % bovine serum albumin and 2 mM EDTA) per million total cells. 10µl biotin-antibody cocktail (biotin-conjugated monoclonal antibodies against CD43 (Ly-48) (rat IgG2a), CD4 (L3T4) (rat IgG2b) and Ter-119 (rat IgG2b)) per million total cells, mixed well and incubated for 10 min at 4-8 °C. 30µl buffer and 20µl anti-biotin microbeads were added to the cell and biotin-antibody cocktail, mixed and incubated for additional 15 min at 4-8 °C. The cells were then washed by adding 10-20x labelling volume and centrifuged at 300 xg for 10 minutes and the supernatant was discarded. The cells were then resuspended in 500µl of

MACS buffer per 10 million total cells. This suspension was then applied on LS columns (Miltenyi, Bergisch Gladbach, Germany), that were pre-rinsed with 3 ml MACS buffer and the effluent was collected. The column was then washed 4 times using 3 ml MACS buffer and the remaining effluent was collected. Purity as determined by FACS analysis using CD45/B220-PE or Rat IgG2a as an isotype (BD Biosciences, Hamburg, Germany) revealed 97 % B cells after each isolation.

Stimulation experiments

For pI:C RNA treatment study; Spleen monocytes, BMDCs or mesangial cells were treated with medium control or different (indicated in figures) concentrations of pI:C RNA and pI:C DNA as a control. TLR9 ligand CpG-ODN 1668 was used as a control in selected cases. For ODN 2114 treatment study; J774 mouse macrophages were treated with with 1 μM CpG-ODN 1668, 1μg/ml LPS, with or without various concentrations of ODN 2114, while medium served as control. Similar concentrations were used in the B cell proliferation assay. In all stimulation experiments all cell types were unstarved (unless mentioned otherwise) and were incubated for a period of 24 hours under standard culture conditions and culture supernatants were collected for cytokine measurements and cells were harvested either for RNA isolation as described below or for flow-cytometric analysis.

2.5 Cytokine Elisa, nitrite and B cell proliferation assay:

Elisa: Cytokine levels in sera or cell culture supernatants were determined using commercial Elisa kits: IL-6, IL-12p70, CCL2 (all OptEiA, BD Pharmingen), IFN-α

(R&D Systems Inc, USA), and CCL5 (Duoset, R&D Systems Inc, USA) following the protocol provided by the respective manufacturers. The 96-well plate was first coated with 100µl/ well capture antibody (anti-mouse cytokine) at recommended dilution in 0.2 M Sodium phosphate buffer of specified pH and placed overnight at 4 °C. The wells were then aspirated, washed with >200 µl wash buffer (PBS pH 7 with 0.05 % Tween-20) and the plate was blocked with >200 µl/well assay diluent (PBS pH 7 with 10 % FCS) and incubated at room temperature for 1 hour. This was followed by aspiration, 2 washes as described above, and 100 µl of standard or sample was pipetted to appropriate well and the plate was incubated for 2 hours at room temperature. The plate was then aspirated, washed five times and 100 µl working detector (biotinylated anti mouse cytokine or detection antibody with avidin-horse radish peroxidase conjugate) was added to each well and incubated at room temperature for 1 hour. This was followed by an aspiration and wash step (>5 washes). The TMB substrate solution (BD Biosciences, Hamburg, Germany) was then added to each well at a volume of 100 µl and incubated for 30 minutes. The stop solution (1 M Phosphoric or 2N Sulphuric acid) was then added to each well, and absorbance was measured at prescribed wavelength (nm), using an automatic plate reader. Note: Reagents and buffers used in all experimental protocols, double distilled water (ddH_2O) was used unless stated otherwise.

Griess Assay: The Griess reagent (Promega, Mannheim, Germany) was used for the determination of nitrite in cell supernatants as a marker of NO production. The Griess Reagent System is based on the chemical reaction that uses sulfanilamide and N-1-napthylethylenediamine dihydrochloride (NED) under acidic (phosphoric acid)

conditions. A protocol provided by the manufacturer was used, in which 1ml of a 100µM nitrite solution was prepared by diluting the provided 0.1M Nitrite Standard 1:1,000 in the matrix or buffer used for the experimental samples. A 96-microtiter well plate was used for the assay and initially a series of standard dilution was prepared using 100µM as the highest concentration to 1.5µM as the lowest. The sulfanilamide and NED Solution were allowed to equilibrate to room temperature for 15-30 minutes and 50µl of each experimental sample was taken into the wells in duplicate or triplicate. Using a multichannel pipettor 50µl of sulfanilamide solution was dispensed to all experimental samples and wells containing the dilution series for the Nitrite Standard reference curve and the plate was incubate for 5–10 minutes at room temperature in dark 50µl of the NED Solution was then dispensed to all wells and the plate was incubated for 5–10 minutes at RT in dark. A purple/magenta colour began to form immediately, which was followed by measurement of absorbance within 30 minutes in a plate reader with a filter between 520–550nm. A standard was built and the nitrite concentrations in the samples was calculated from the equation obtained from the graph

B cell proliferation: Proliferation of B-cells was assessed using *CellTiter 96 Proliferation Assay* (Promega, Mannheim, Germany). The CellTiter 96 Aqueous One Solution contains a novel tetrazolium salt compound (MTS) and phenazine ethosulfate that serves as an electron-coupling reagent. The solution remains stable normally, while the MTS is bioreduced by the NADPH or NADH produced by dehydrogenase enzymes in metabolically active cells (such as proliferating cells). The isolated B-cells isolated (1 x 10^5) were incubated in 96-well plates in 100 µl complete RPMI medium and treated with

1 μM CpG-ODN 1668, 1μg/ml LPS, or various concentrations of ODN 2114 for a period of 72 hours under standard culture conditions. To each well with different treatments, 20 μl CellTiter 96 Aqueous One Solution was added and incubated at 37 °C for 4 hours and the optical density (OD) was measured at 492 nm for comparing the cell proliferation.

2.6 Flow cytometric analysis

In case of splenocytes, BMDCs and mesangial cells, surface staining was performed using PE- or FITC-labelled rat anti-CD11c, anti-MHC II, anti-CD86 or anti CD19 antibodies (BD Pharmingen, Hamburg, Germany). Anti-TLR3 antibody (1:50) was used to detect TLR3 on mesangial cells through biotinylated rabbit anti-mouse IgG antibody and streptavidin-APC (Pharmingen). A rabbit IgG (BD Pharmingen, Hamburg, Germany) was used as isotype control. For intracellular staining, mesangial cells were fixed with 1% paraformaldehyde and permeabilized with permeabilization buffer (PBS, 0,5% BSA, 0,5% saponin) at room temperature. FACS analysis was conducted using a FACScalibur machine and CellQuest software (BD biosciences, Hamburg, Germany). Primary cells were harvested using method described above, involving hemolysis, passage through pre-separation filter to obtain single cell suspension and wash steps, under cold conditions on ice. All cell types, after appropriate stimulation procedures were harvested, resuspended in 50-100 μl PBS, and incubated with the respective primary or secondary antibodies (wherever applicable) or isotype controls at prescribed dilutions for 30 minutes each, followed by a washing step, resuspended in PBS for FACS analysis. Flow cytometry of spleen cells for *in vivo* B cell proliferation and activation assay was performed as previously described. Surface staining was performed using PE-labeled rat anti-MHC II

or anti CD19 antibodies (BD Pharmingen, Hamburg, Germany). A rabbit IgG (BD Pharmingen, Hamburg, Germany) was used as an isotype control. FACS analysis was conducted using a FACSCalibur machine and CellQuest software (BD biosciences, Hamburg, Germany).

2.7 RNA isolation, cDNA synthesis and real time RT-PCR

Cultured cells

Cell lines as well as primary cell cultures in stimulation experiments were harvested for RNA isolation using RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany) according to the protocol provided. Firstly, adherent cells to be harvested from 6 well culture plates were washed with sterile PBS to remove residual medium after collection and the wash-through was aspirated. 350 µl RLT buffer containing 10µg/ml beta-mercaptoethanol was added directly over adherent cells on the plate surface and pipetted vigorously so as to lyse cells. This produced a viscous fluid, which was collected and placed in microcentrifuge tubes and frozen at –80 °C until RNA isolation. At the time of RNA isolation the samples collected were thawed, 350 µl of 70 % ethanol made in 1% Diethyl pyrocarbonate treated water (DEPC water) was added to it and mixed well. This mixture was then loaded onto RNeasy mini columns held in 2 ml collection tubes and centrifuged at 8000 xg for 30 seconds. The flow-through was discarded and the columns were loaded with 700µl of buffer RW1 and centrifuged at 8000 xg for 30 seconds. The collection tubes were discarded together with the flow-through and the columns were transferred to fresh 2 ml collection tubes and 500 µl of Buffer RPE was pipetted onto the column, was centrifuged at 8000 xg for 30 seconds and the flow-through was discarded. This step was

repeated again and the column was rendered dry by centrifugation, placed in a 150 µl fresh collection tube, 40 µl of RNase free water was pipetted directly on the silica-gel membrane and was centrifuged to collect the RNA solution. No quality check was necessary in case of the kit isolation, as empirically the RNA obtained upon kit isolation was of good quality standards.

Isolation of RNA from tissues

The RNA isolation protocol was suitably modified from Chomczynski's method (102). 3 ml of solution D containing 8µl of beta-mercaptoethanol/ml was taken in a 15 ml falcon tube, to which a small piece of tissue from which RNA had to be isolated, was placed. The tissue was homogenised using ULTRA-TURRAX T25 (IKA GmbH, Staufen, Germany) at speed level 2 and placed on ice. To this 300µl 2M sodium acetate solution was added and mixed gently, followed by addition of 3 ml Roti-Aqua-Phenol (Carl Roth GmbH, Karlsruhe, Germany) and gentle mixing. A 1.6 ml mixture of chloroform/isoamyl alcohol (49:1) was added to the contents of the falcon and vortexed for 20 seconds until a milky white suspension resulted. The falcon tube was then placed on ice for 15 min and centrifuged at 4000 xg at 4 °C. The upper phase (approximately 3 ml) was collected carefully in a fresh falcon tube, to which 3 ml isopropanol was added, incubated for 30 minutes at –20° C and centrifuged for 15 minutes at 4000 xg at 4 °C. The supernatant was then discarded carefully to avoid loss of pellet and the falcon tube was inverted on a tissue paper to drain of the remaining isopropanol and 1 ml solution. The pellet was then dissolved in 0.5 ml solution D and the solution was transferred to fresh DEPC-treated tubes and 0.8 ml Isopropanol was added to it, mixed and placed at –20 for 30 minutes.

This was followed by centrifugation for 15 minutes at 4000 xg at 4 °C; the supernatant was discarded carefully to retain the pellet. The pellet was then washed with 80 % ethanol made in DEPC water, and vortexed again for 15 minutes at 4000 xg at 4 °C. The supernatant was discarded and the tubes were inverted to drain of residual ethanol and the semi-dried pellet was dissolved in 100 µl DEPC water. A 10µl aliquot was used for the quality check and remaining RNA solution was stored at -80 °C until cDNA synthesis. The RNA was quantified and quality was determined by taking 2µl of the RNA solution diluted 50 times in DEPC water for calculating ratios 260/280 nm spectrophotometric OD measurement. The formula used was Extinction x dilution to obtain number of µg/ml of RNA per sample and a ratio value approximately close to 1.6 was considered to be of acceptable quality. Further quality check (if necessary) was performed using a denaturing RNA gel, ran at 70-100 V for 1 hour and the gel was then read on a gel documentation apparatus.

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 such as 9 µl of 5x buffer (Invitrogen, 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 ddH_2O in case of the controls. The master mix was made to a volume of 15µl and added

to 1µg/20µl 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 at placed 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 sequence-specific 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") 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. In the TaqMan® universal PCR master mix contained are 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 ddH_2O were negative for target and housekeeper genes. Oligonucleotide primer (300 nM) and probes (100 nM) (Applied Biosystems, Darmstadt, Germany) were used: murine CCL5; TLR3 accession number AF355152, forward primer 5'-CGAAAGTTGGACTTGTCATCAAATC-3', reverse primer 5'- ACTTGCCAATTG-TCTGGAAACAC-3', internal fluorescence probe 5'- CACTTAAAGAGTTCTCCC -3'. Primers and probes for murine CCL2, CCL5, and 18S rRNA were obtained as pre-developed assay reagents from PE Biosystems.

3. RESULTS

3.1 Effects of exposure to viral dsRNA on immune complex glomerulonephritis in MRL*lpr/lpr* Mice

Infection induced local tissue injury, during lupus nephritis, which is a form of immune complex glomerulonephritis, is brought about by local inflammatory cytokine and chemokine production by infiltrating immune cells as well as intrinsic renal cells. In context of viral infections, exposure of dsRNA and subsequent release of cytokines and chemokines can be expected to be mediated through TLR3, leading to local tissue injury in kidneys of MRL*lpr/lpr* mice with lupus nephritis.

3.1.1 Spleen and Kidney TLR3 Expression

Initially the expression pattern of TLR3 mRNA in kidneys and spleens of MRL*lpr/lpr* mice was determined at an early (week 5) and late (week 20) stage of autoimmune disease using real-time RT-PCR. At 5 wk of age, no structural abnormalities were detected in kidney and spleen however, TLR3 mRNA in kidneys were comparable to that in spleen, indicating that TLR3 is expressed by intrinsic renal in addition to the few resident immune cells present in kidneys of 5-wk-old MRL*lpr/lpr* mice (Figure 2). At 20 wk, spleens showed major structural abnormalities including lymphoproliferation. At this time point, kidneys of MRL*lpr/lpr* mice revealed severe glomerulonephritis and tubulointerstitial injury with interstitial and perivascular inflammatory cell infiltrates. Interestingly at 20 wk, spleen TLR3 mRNA levels were unchanged compared with wk 5. Proliferative lupus nephritis was associated with somewhat increased renal TLR3 mRNA expression, but there was no statistical difference as compared with week 5 (Figure 2).

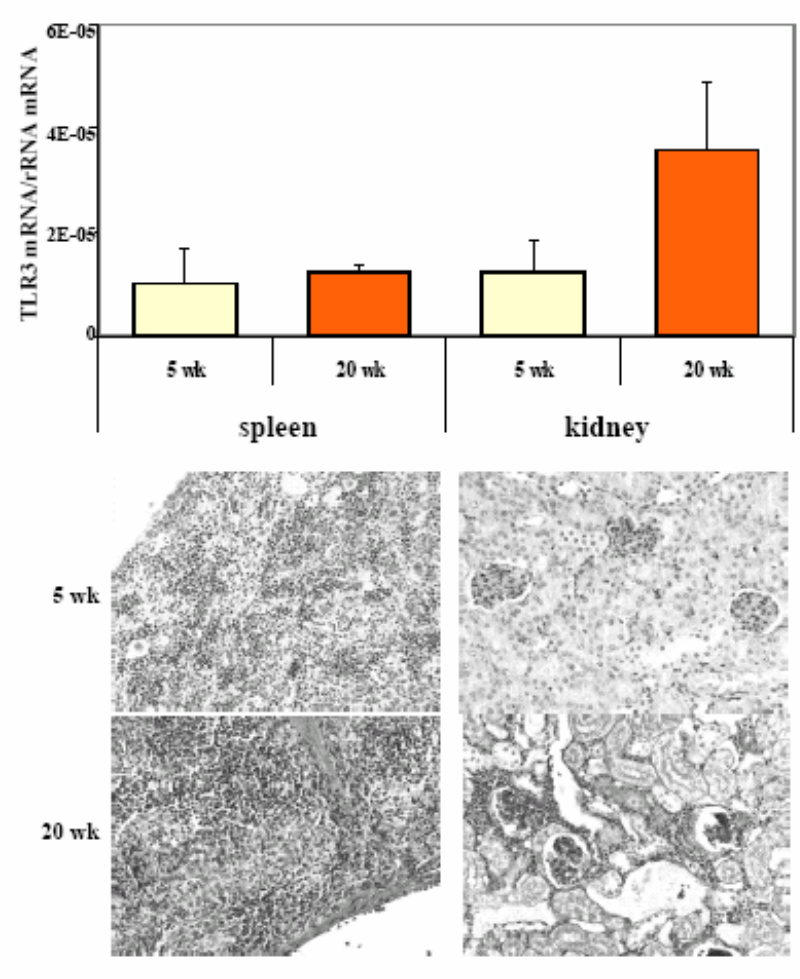


Figure 2. TLR3 mRNA expression in MRL*lpr/lpr* mice. Expression of TLR3 mRNA was assessed by real-time RT-PCR in duplicates using RNA isolated from spleens and kidneys from 7 MRL*lpr/lpr* mice each at 5 weeks and 20 weeks of age as described in methods. TLR3 mRNA expression is expressed as ratio to the respective 18S rRNA mRNA expression ± SEM (5 vs 20 weeks). At 5 weeks spleens and kidneys did not show structural abnormalities. Glomeruli (encircled) show a regular capillary network and mesangium. By contrast at 20 weeks spleens showed major structural alterations secondary to lymphoproliferative disease indicated by the malformation of spleen lymph follicles. At this time point, kidneys showed mesangioproliferative glomerulonephritis with periglomerular inflammatory cell infiltrates and tubular atrophy. (Periodic acid Schiff stain, original magnification x400).

To localize the source of renal TLR3 mRNA expression, immunostaining was performed using a polyclonal antibody specific for murine TLR3. Renal sections of 16-wk-old MRL*lpr/lpr* mice revealed positive signals in glomerular mesangial cells but not in glomerular endothelial cells or podocytes (Figure 3). Mesangial cell staining for TLR3 appeared in a speckled pattern, indicating that TLR3 is also localized in an intracellular compartment. Mononuclear inflammatory cell infiltrates were also positive for TLR3 (Figure 3).

3.1.2 Localization of labelled viral dsRNA in mice kidneys

For examining whether TLR3-positive cells take up circulating viral dsRNA *in vivo*, rhodamine-labeled viral dsRNA was injected intravenously into 16-wk-old MRL*lpr/lpr* mice. Consistent with TLR3 immunostaining in the kidney, the labelled viral dsRNA was found in speckled glomerular mesangial cell staining pattern, suggesting that injected viral dsRNA was taken up by mesangial cells into an intracellular vesicular compartment (Figure 3). Infiltrating cells showed strong granular intracellular signals for labeled viral dsRNA. Double labelling with an F4/80-specific antibody identified these cells as antigen-presenting cells of the monocyte-macrophage lineage (Figure 3). Rhodamine injected alone in MRL*lpr/lpr* mice was not found to localize in the kidney (data not shown). Analysis of spleen sections revealed viral dsRNA signals only in F4/80-positive antigen-presenting cells but not in B or T cell areas of the spleen (data not shown). Taken together, in the kidneys of MRL*lpr/lpr* mice, injected viral dsRNA co-localizes in an intracellular granular pattern with TLR3-positive cells, i.e., infiltrating mononuclear cells, but also with intrinsic renal cells predominantly in glomerular mesangial cells in an intracellular vesicular compartment.

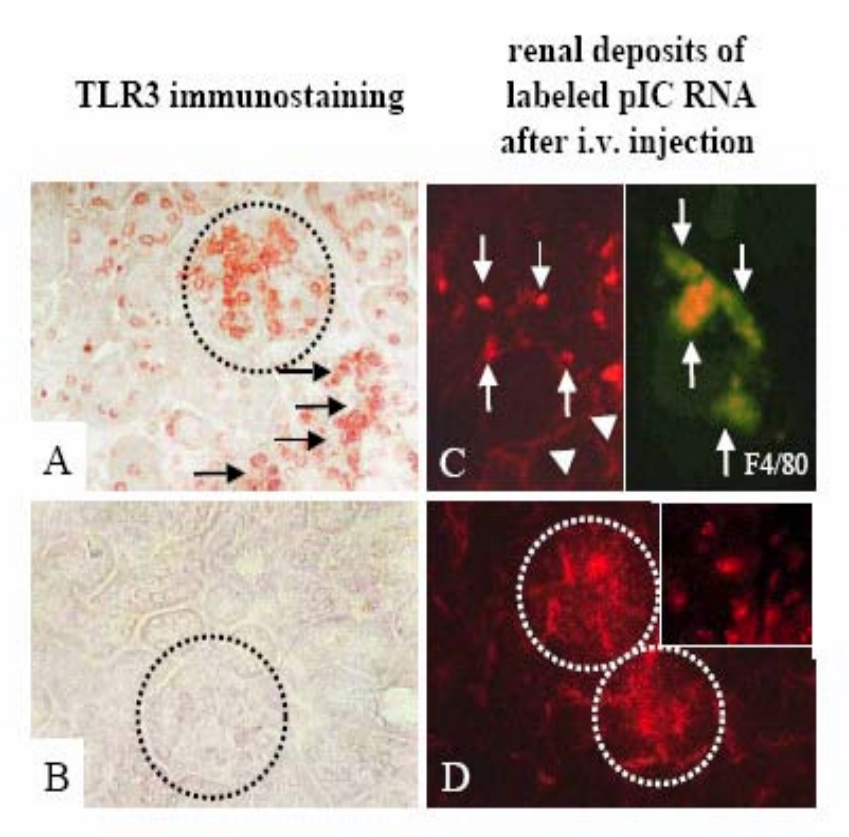


Figure 3. TLR3 in kidneys of MRL*lpr/lpr* mice. A: immunostaining for TLR3 was performed as described in the methods section. Positive staining was found in inflammatory cell infiltrates (arrows), and in glomeruli in a mesangial staining pattern (glomerulus encircled). B: negative control staining. C and D: rhodamine-labeled pI:C RNA was intravenously injected into four 16 weeks old MRL*lpr/lpr* mice and renal tissue was harvested two hours later. Fluorescence imaging of frozen sections showed uptake of labeled pI:C RNA in interstitial cells (arrows in left image of C) and in mesangial cells in glomeruli (encircled and at higher magnification in insert of D) consistent with the staining pattern for TLR3. Costaining with an FITC-labeled F4/80 antibody identified pI:C RNA–positive interstitial cells to be antigen-presenting cells of the monocytic cell lineage and illustrates the uptake of rhodamine-labeled-pI:C RNA in intracellular endosomes (arrows indicating individual endosomes in right image of C). Original magnification of all images 400x.

3.1.3 Mesangial cells express TLR3 & secrete CCL2 & IL-6

To confirm the TLR3 expression by mesangial cells, flow cytometry was performed on an established murine mesangial cell line. Under basal culture conditions, TLR3 expression was detected intracellularly after cell permeabilization, whereas only a little surface staining was detected (Figure 4A). To test the functionality of TLR3 on mesangial cells, it was examined whether the TLR3 ligand pI:C RNA can induce cytokine and chemokine secretion. Stimulation with increasing concentrations of pI:C RNA induced IL-6 and CCL2 secretion in a concentration-dependent manner (Figure 4B). In contrast, pI:C DNA or CpG DNA had no effect on IL-6 or CCL2 production. Together, these data indicate that mesangial cells express TLR3 and produce proinflammatory cytokines (e.g., IL-6) and CC-chemokines (e.g., CCL2) upon exposure to pI:C RNA in vitro.

3.1.4 Production of proinflammatory mediators in APCs

As TLR3 staining and uptake of labeled pI:C RNA in kidneys of MRL*lpr/lpr* mice also occurred in infiltrating mononuclear cells, spleen monocytes were isolated from MRL*lpr/lpr* mice and cultured. These cells were incubated with pI:C RNA, pI:C DNA, CpG-DNA, or medium for 24 h. Splenocytes showed a concentration-dependent increase in IL-12p70 and IL-6 release after exposure to pI:C RNA but not after exposure to pI:C DNA or CpG-DNA (Figure 5B). In addition, other markers of monocyte activation such as NO or the chemokine CCL5 production were determined, as molecules that can mediate tissue injury in SLE. pI:C RNA markedly induced CCL5 mRNA expression and NO production

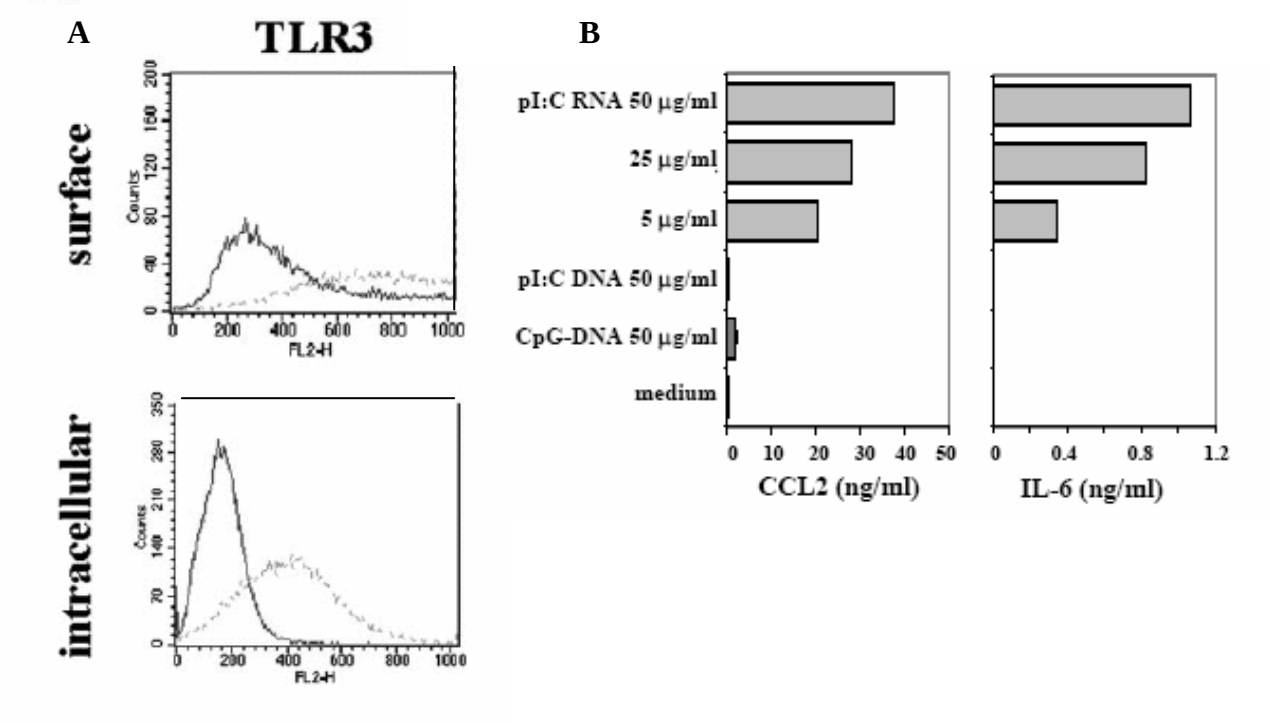


Figure 4. pI:C RNA/TLR3 interaction in cultured mesangial cells. Murine mesangial cells were cultured as described in methods. A: Flow cytometry for TLR3 before and after permeabilization for intracellular staining was performed as indicated. Expression of TLR3 (dotted line) is demonstrated by a fluorescence shift compared to the isotype control antibody (dark line). B: Cultured cells were incubated with different concentrations of either pI:C RNA, pI:C DNA or CpG-DNA or standard medium without supplements for 24 hours as indicated. IL-6 and CCL2 production was measured in supernatants by Elisa. Results shown are means ± SEM from two comparable experiments each performed in duplicate.

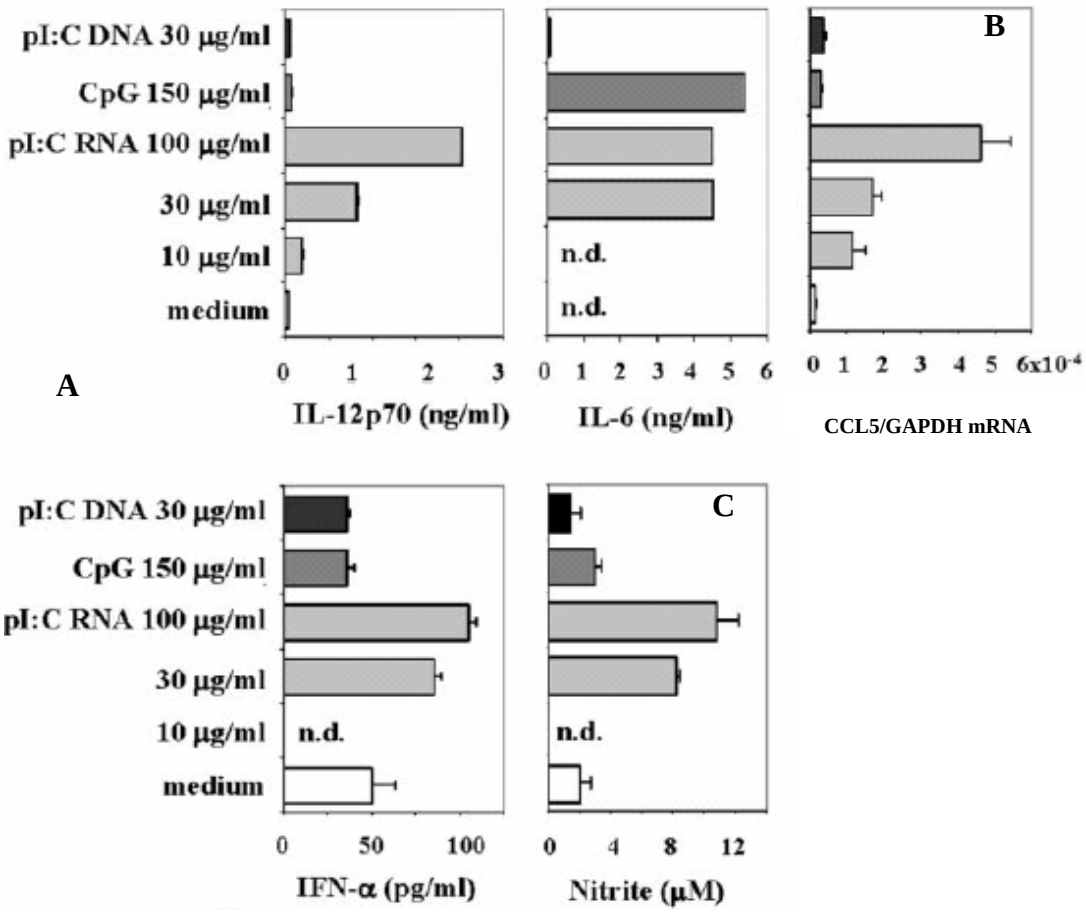


Figure 5. pI:C RNA activates spleen monocytes isolated from MRL*lpr/lpr* mice. A: Monocytes from spleens of MRL*lpr/lpr* mice and incubated with different concentrations of either pI:C RNA, pI:C DNA or CpG-DNA or standard medium for 24 hours as indicated. IL-6, IL-12p70 and IFN-α production were measured in supernatants by Elisa. Results shown are from one of three comparable experiments. n.d. = not done. B: Spleen monocytes of MRL*lpr/lpr* mice were stimulated for 12 hours as above. CCL5 mRNA expression was analysed by real-time RT-PCR as described in methods. Values are expressed as CCL5 mRNA expression in relation to respective GAPDH mRNA expression ± SEM. C: Spleen monocytes of MRL*lpr/lpr* mice were stimulated for 24 hours as above. NO production was assessed by measuring nitrite concentrations in supernatants using Griess assay as described in methods. Results (for B and C) shown are means ± SEM from three comparable experiments, each performed in duplicate.

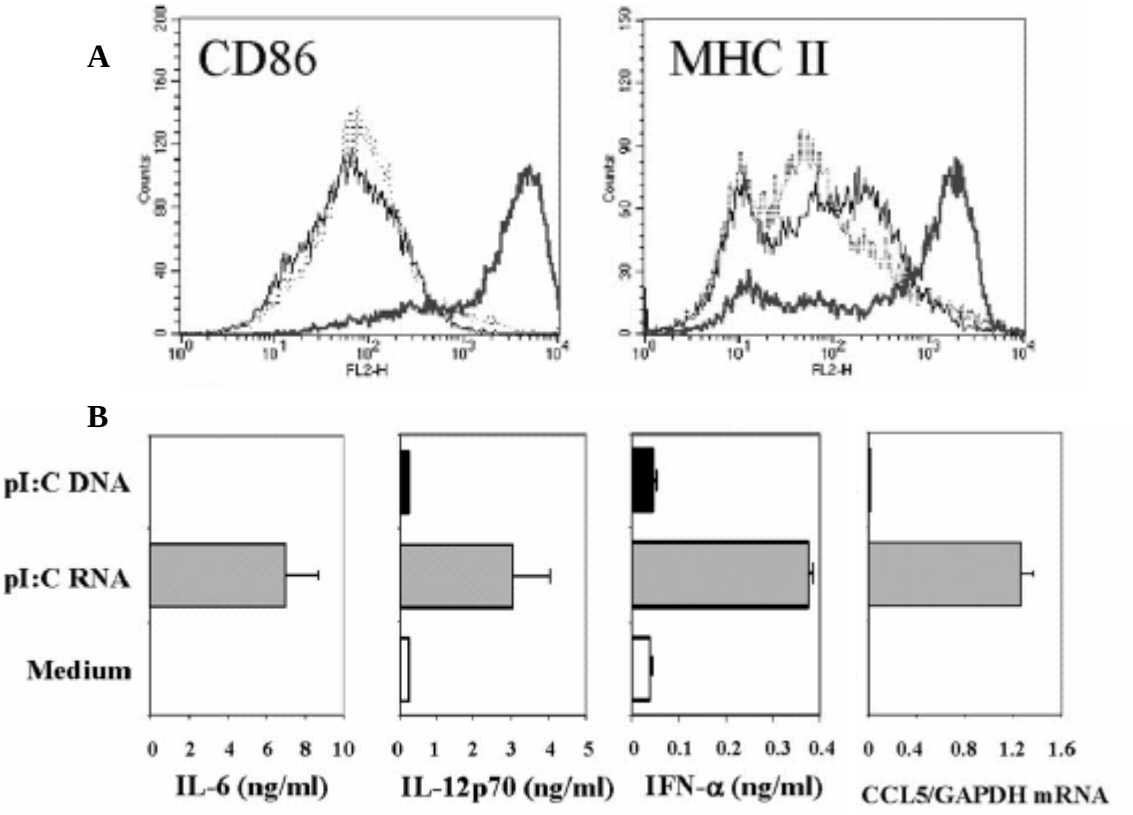


Figure 6. pI:C RNA activates DCs from MRL*lpr/lpr* mice. A and B: Flow cytometry of CD11c positive BMDCs for CD86 (A) and MHC II (B) was performed as described in methods. Cells were incubated with either pI:C RNA (bold dark line) or pI:C DNA (dotted line) before analysis. Induction of CD86 and MCH II surface expression in pI:C RNA-treated DCs is indicated by a fluorescence shift compared to the isotype control antibody (thin black line). C: Cultured cells were treated as above for 24 hours and IL-12p70, IL-6, and IFN-α production was assessed by Elisa in culture supernatants. Results shown are means ± SEM means from two comparable experiments, each performed in duplicate. D. DCs of MRL*lpr/lpr* mice were stimulated for 12 hours as above. CCL5 mRNA expression was analysed by real-time RT-PCR as described in methods. Values are expressed as CCL5 mRNA expression in relation to respective GAPDH mRNA expression ± SEM. Results shown are two comparable experiments, each performed in duplicate.

by spleen monocytes of MRL*lpr/lpr* mice compared to stimulation with pI:C DNA and CpG-DNA (Figure 5A, 5B, and 5C). Next BMDCs were prepared from MRL*lpr/lpr* mice and were incubated with pI:C RNA, pI:C DNA, or medium for 24 hours. Flow cytometric analysis for CD86 and MHC II on CD11c-positive BMDC showed a marked increase in the surface expression of both molecules with pI:C RNA, indicating BMDC maturation, which was absent, with pI:C DNA (Figure 6A). Furthermore, pI:C RNA but not pI:C DNA stimulated the secretion of IL-12p70, IL-6, and IFN-α as determined by ELISA in culture supernatants of so stimulated BMDCs (Figure 6B). These data indicate that pI:C RNA induces the production of proinflammatory mediators such as IL-12p70, IL-6, CCL5, and NO production in spleen monocytes and IL- 2p70, IL-6, and IFN-α in BMDC of MRL*lpr/lpr* mice.

3.1.5 Serum IL-6, IL-12p70 and IFN-α levels

As intermittent viral infections that can lead to immune activation and cytokine release are associated with disease flares during ongoing lupus, it was tested if viral dsRNA can induce cytokine release *in vivo*. Further having demonstrated the effect of pI:C RNA on IL-6, IL- 12p70, and IFN-α secretion in dendritic cells and macrophages isolated from MRL*lpr/lpr* mice in vitro, serum levels of these factors 6 h after intraperitoneal injection of 50 μg of pI:C RNA, 50 μg of pI:C DNA, or saline were determined in 16-wk-old MRL*lpr/lpr* mice. Injection of pI:C RNA caused an increase of serum levels of IL-12p70, IL-6, and IFN-α as compared with saline or pI:C DNA in MRL*lpr/lpr* mice (Figure 7).

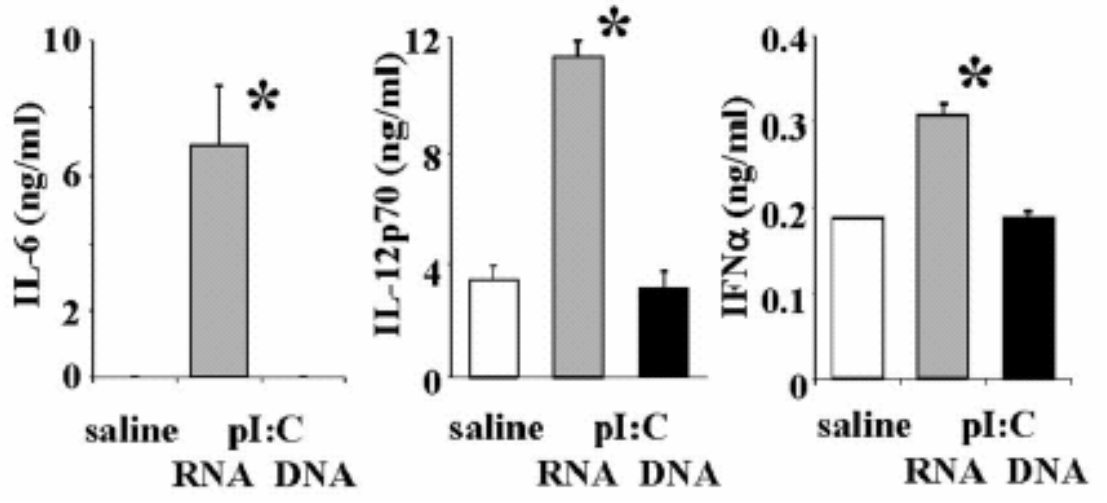


Figure 7. Serum IL-6, IL-12p70 and IFN-α levels in MRL*lpr/lpr* mice. Serum was obtained from 5-8 16 weeks old MRL*lpr/lpr* mice 6 hours after the first intraperitoneal injection of either saline, 50 mg pI:C RNA or 50 mg pI:C DNA as indicated. Serum IL-6, IL-12p70 and IFN-α levels were determined by Elisa. Data are means ± SEM. * $p<0.05$ vs saline.

3.1.6 Aggravation of renal damage and proteinuria

From the above results, one would predict that exposure to pI:C RNA would aggravate lupus nephritis in autoimmune MRL*lpr/lpr* mice. In this study lupus mice were treated with intraperitoneal injections of either 50 μg of pI:C RNA or pI:C DNA or saline on alternate days from weeks 16 to 18 of age. Saline-treated MRL*lpr/lpr* mice had diffuse proliferative glomerulonephritis with moderate mesangial hypercellularity, increase of mesangial matrix, and little periglomerular inflammatory cell infiltrates at week 18. pI:C DNA injections did not alter these histopathologic findings (Figure 8). By contrast, pI:C RNA injections induced focal segmental necrosis in glomeruli and cellular crescent formation associated with marked periglomerular inflammatory cell infiltrates (Figure 8). Aggravation of renal disease was illustrated by an increase in the activity and chronicity scores of the lupus nephritis in pI:C RNA treated MRL*lpr/lpr* mice as compared with the other groups of mice (Table 4). pI:C RNA increased the amount of glomerular ER-HR3–positive macrophages and CD3-positive lymphocytes as compared with pI:C-DNA– and saline-injected controls (Table 4). There was a trend toward increased proteinuria levels in pI:C RNA–treated mice, but this did not reach statistical significance (Table 4). In addition to the aggravation of glomerular damage, pI:C RNA injections induced tubulointerstitial damage and fibrosis. Infiltrating ERHR-3 macrophages and CD3 lymphocytes accumulated particularly in periglomerular fields and areas around glomerular crescents (Figure 8, Table 4). To assess extent of interstitial injury, immunostaining for smooth muscle actin–positive interstitial myofibroblasts and for interstitial collagen I deposits was done. Both were significantly increased in kidneys of pI:C RNA–treated mice (Figure 8, Table 4).

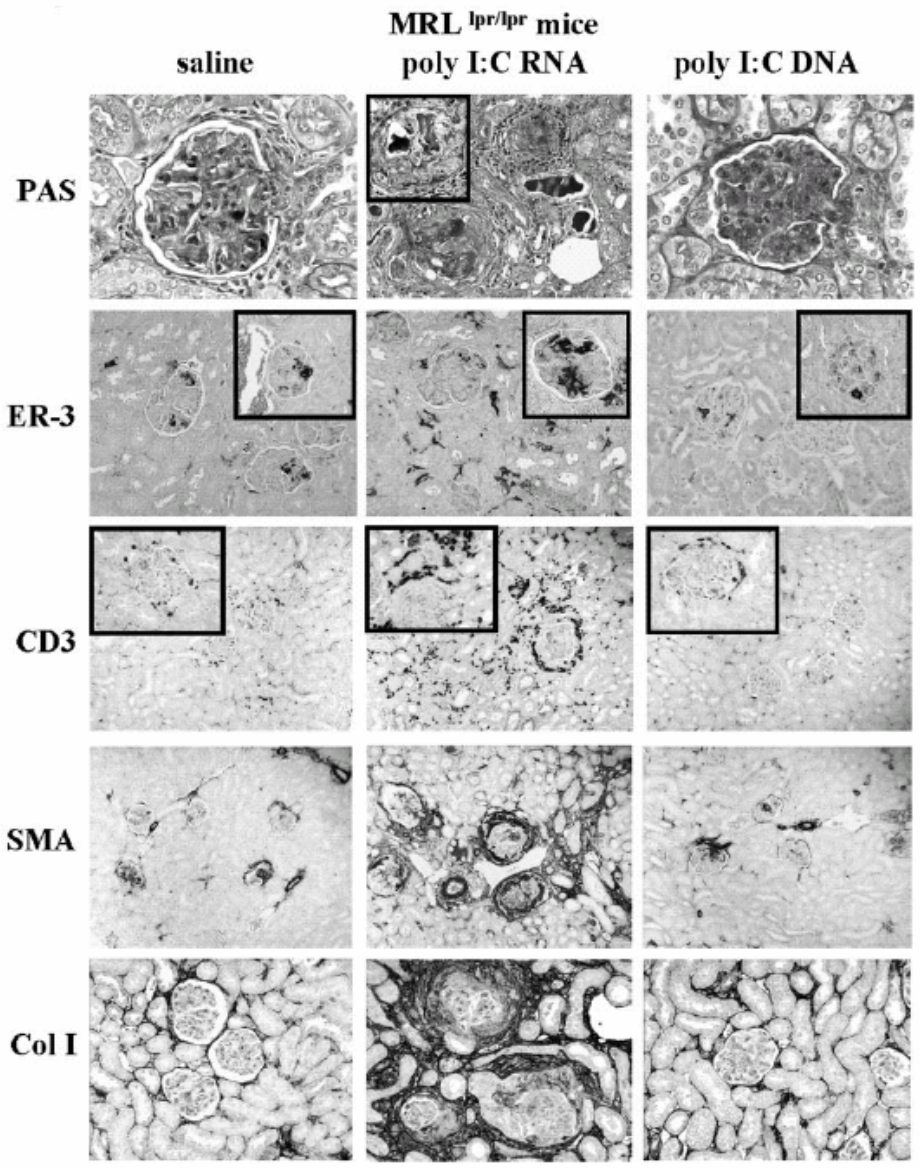


Figure 8. Renal histopathology. Renal sections of 18 weeks old MRL*lpr/lpr* mice from all groups were stained with periodic acid Schiff, and antibodies for ERHR-3 (macrophages), CD3 (lymphocytes), SMA (smooth muscle antigen for myofibroblasts), and collagen I as indicated. Insert in PAS stained sections of pI:C RNA-treated MRL*lpr/lpr* mice illustrates necrosis and aneurysm formation of glomerular capillaries not detected in other groups (Original magnification x400). Inserts in ER-HR3 and CD3-stained sections show respective glomeruli at a magnification of x530. Representative sections (n= 8-10 mice).

Taken together, exposure to pI:C RNA markedly aggravated the immune complex glomerulonephritis of MRL*lpr/lpr* mice toward a crescentic glomerulonephritis with mesangiolysis associated with marked tubulointerstitial injury.

3.1.7 Induction of renal CCL2 and CCL5 mRNA expression

On the basis of our in vitro studies with macrophages, dendritic cells, and mesangial cells, it was hypothesized that pI:C RNA would trigger local chemokine expression in nephritic kidneys of MRL*lpr/lpr* mice. In fact, kidneys of pI:C RNA– treated MRL*lpr/lpr* mice showed increased mRNA expression levels for CCL2 and CCL5 (Figure 9A). To localize renal CCL2 and CCL5 protein, immunostaining was performed (Figure 9B). At 18 wk, single spots of CCL5 and CCL2 protein were noted within the glomerular tuft and along Bowman's capsule of some glomeruli, as well as in focal interstitial areas of saline- and pI:C DNA–treated MRL*lpr/lpr* mice kidneys. By contrast, pI:C RNA–treated MRL*lpr/lpr* mice kidneys showed marked CCL2 and CCL5 staining that co-localized with interstitial leukocytic cell infiltrates and glomerular crescents (Figure 8). Thus, intermittent exposure to pI:C RNA increased local expression of CCL2 and CCL5 in areas of pronounced inflammatory cell infiltrates and tissue damage in nephritic kidneys of MRL*lpr/lpr* mice.

3.1.8 Unaffected B Cell activation and DNA autoantibody production in MRLlpr/lpr mice

Our lab previously observed that activation of TLR9 by bacterial CpG-DNA aggravated lupus nephritis in MRL*lpr/lpr* mice in association with enhanced DNA autoantibody production and glomerular immune complex deposits (49).

Table 4. Serum, urinary, and histological findings in MRL*lpr/lpr* mice

	saline	pI:C DNA	pI:C RNA
Functional parameters			
Proteinuria (μg/mg creatinine)	3118 ± 903	2646 ± 694	10405 ± 12173
Histological scores			
Activity index	8.1 ± 2.8	6.6 ± 4.2	15.6 ± 4.2 [a]
Chronicity index	1.8 ± 1.0	1.3 ± 0.3	5.4 ± 2.1[a]
Cellular response [cells/glom. or hpf]			
Glom. EHR3+ (cells/glom)	1.7 ± 0.7	1.9 ± 1.5	4.4 ± 1.6 [a]
CD3+ (cells/glom)	1.4 ± 0.4	1.5 ± 0.8	2.2 ± 0.5 [a]
Interst. EHR3+ (cells/hpf)	6.0 ± 2.9	7.2 ± 5.3	15.5 ± 4.7 [a]
CD3+ (cells/hpf)	13.7 ± 3.3	17.0 ± 6.4	35.1 ± 10.2 [a]
SMA+ (% hpf)	5.0 ± 0.8	4.2 ± 3.9	11.0 ± 1.2 [a]
Interstitial collagen (% hpf)	2.9 ± 1.5	3.1 ± 1.7	8.4 ± 4.8 [a]
Humoral response			
Serum titres			
Anti-DNA IgG	14231 ± 3519	16603 ± 3089	17718 ± 3375
Anti-DNA IgG_{2a}	10667 ± 3556	9000 ± 3500	7556 ± 3951
Glom. deposit score			
IgG	1.2 ± 0.2	1.3 ± 0.4	1.4 ± 0.2
IgG_{2a}	0.3 ± 0.2	0.4 ± 0.3	0.6 ± 0.3

Values are means ± SEM from 8-10 mice per group, [a] $p < 0.05$ pI:C RNA vs. pI:C DNA

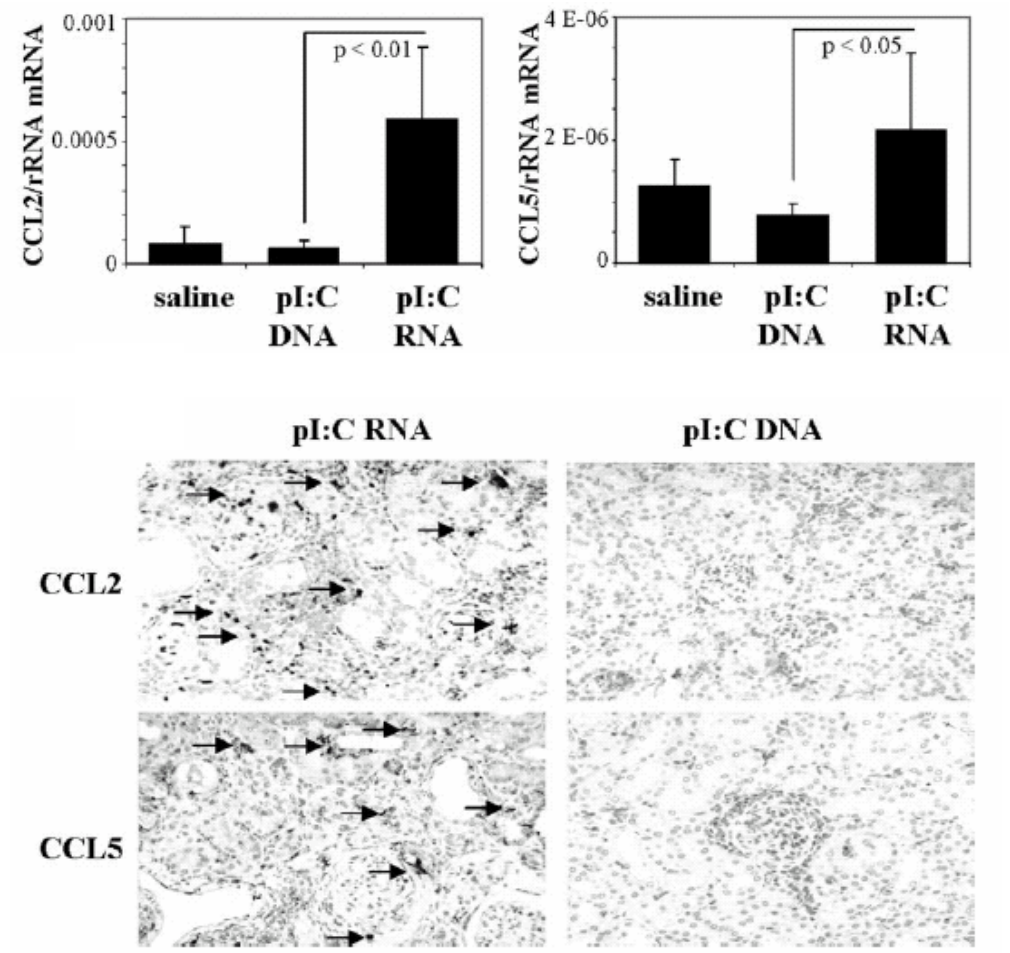


Figure 9. Renal expression of CC-chemokines. The mRNA expression of CCL2 (A) and CCL5 (B) in kidneys of 18 wk MRL*lpr/lpr* mice was determined by real-time RT-PCR using total renal RNA. mRNA levels for saline, pI:C DNA, and pI:C RNA-treated MRL*lpr/lpr* mice are expressed in relation to the respective 18S rRNA expression of each kidney. Results are means ± SEM from 3-5 mice in each group. C. Immunostaining for CCL2 and CCL5 was performed as described in methods. Injection of pI:C RNA markedly increased CCL5 expression in spatial association with increasing interstitial leukocytic cells and cellular crescents. CCL2 was stained in occasional glomerular cells and proximal tubular cells of saline- and pI:C DNA-treated MRL*lpr/lpr* mice. Injection of pI:C RNA increased interstitial CCL2 staining. Arrows indicate chemokine positive cells. Sections stained with an isotype control antibody showed no signal (not shown).

In this study, predominance of the IgG2a autoantibody and renal IgG deposits of the IgG2a isotype suggested a predominant Th1 response induced by bacterial CpG-DNA (49). Therefore, in the present study, the effects of pI:C RNA or pI:C DNA on serum anti-DNA antibody titres and glomerular IgG deposits were examined in MRL*lpr/lpr* mice. In the current study, irrespective of treatment provided all mice groups had comparable titres of total serum DNA IgG or IgG2a autoantibodies (Table 4). Consistent with these results, capillary and mesangial deposits of total IgG and of IgG2a in glomeruli were comparable in all treatment groups (Table 4). Though, human and murine B cells lack TLR3 expression, B cell activation might occur indirectly through pI:C RNA-induced activation of DCs and subsequent activation of T cells. Therefore MHC II expression on spleen B cells was determined by flow cytometry 24 h after intraperitoneal injection of 500 μg of pI:C RNA, pI:C DNA, or CpG-DNA into MRL*lpr/lpr* mice (Figure 10). In contrast to CpG-DNA, neither pI:C RNA nor pI:C DNA induced MHC II expression on CD19-positive spleen B cells, indicating that pI:C RNA does not activate B cells in MRL*lpr/lpr* mice. Together with our previous findings, these data demonstrate that bacterial CpG-DNA but not dsRNA induces B cell activation and DNA autoantibody production in autoimmune MRL*lpr/lpr* mice.

As viral infections are known to induce disease flares in individuals with autoimmune pre-disposition such as in ongoing systemic lupus, a role of viral dsRNA for

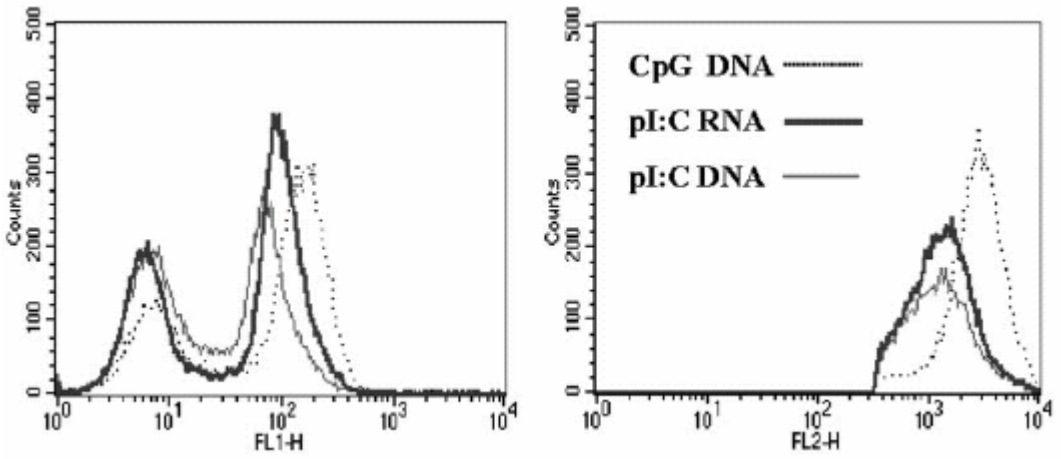


Figure 10. B cell activation in MRL*lpr/lpr* mice. 16 weeks old mice were injected with 500 ug of either pI:C RNA, CpG-DNA or GpC-DNA. After 24 hours flow cytometry for MHC II and CD19 was performed on spleen cell suspensions. Staining spleen cell suspensions for MHC II produces two peaks. Left peak represents MHC II negative T cells showing no change in number, but became a smaller proportion of the total population in CpG-ODN-injected mice as compared to pI:C RNA- and pI:C DNA-treated controls. The right peak represents MHC II positive antigen-presenting cells including B cells (Figure 9A). When gated for the CD19/MHC II double-positive cell population only the B cells of the right peak remain (Figure 9B). B cell activation is indicated by an increase in MHC II expression on CD19 positive B cells by CpG-DNA (dotted line) but not by pI:C RNA (thick line) or pI:C DNA (thin line). Images are representative for 3 mice in each group.

activating TLR3 leading to aggravation of immune complex glomerulonephrits was hypothesized. In this context viral dsRNA is known to activate DCs to secrete type I interferons and cytokines which are associated with the disease activity in SLE. The above results lead to the observations that immunostaining of nephritic kidney sections of autoimmune MRL*lpr/lpr* mice revealed TLR3 expression in infiltrating antigen-presenting cells as well as in glomerular mesangial cells. TLR3 positive cultured mesangial cells exposed to synthetic polyinosinic-cytidilic acid (pI:C) RNA *in vitro* produced CCL2 and IL-6. pI:C RNA activated macrophages and dendritic cells, both isolated from MRL*lpr/lpr* mice, to secrete multiple proinflammatory factors. *In vivo*, a single injection of pI:C RNA increased serum IL-12p70, IL-6, and IFN-α levels. A course of 50 μg pI:C RNA given every other day from week 16-18 of age aggravated lupus nephritis in pI:C-treated MRL*lpr/lpr* mice. Serum DNA autoantibody levels were unaltered upon systemic exposure to pI:C RNA in MRL*lpr/lpr* mice, as pI:C RNA, in contrast to CpG-DNA, failed to induce B cell activation. It can therefore be concluded that, viral dsRNA triggers disease activity of lupus nephritis by mechanisms different than that of bacterial DNA. In contrast to CpG-DNA/TLR9 interaction pI:C RNA/TLR3-mediated disease activity is B cell independent, but activated intrinsic renal cells e.g. glomerular mesangial cells to produce cytokines and chemokines, factors that can aggravate autoimmune tissue injury e.g. lupus nephritis.

3.2 Synthetic G-rich DNA suppresses systemic autoimmunity in MRL*lpr/lpr* mice

It is known that CpG-DNA can aggravate autoimmune tissue injury locally by activation of tissue macrophages (50). It is also known that methylation of CpG-motif prevents their stimulatory effect on B cells (14). Additionally, genomic DNA released by dying cells can induce APC maturation (15) while known inhibitors of DNA methylation can induce SLE in humans (16). Furthermore, in vertebrates inhibitory DNA sequence elements counterbalance the immunostimulatory effects of unmethylated CpG-DNA (17). Certain synthetic ODNs with such inhibitory motifs have been shown to block CpG-DNA-induced effects (97, 98, 99). Despite these facts an evidence for a pathogenic role for endogenous CpG-DNA is lacking. In order to determine the pathogenic role of endogenous CpG DNA in driving lupus, an inhibitory G-rich DNA was used to test its ability of block the CpG DNA/TLR9 pathway *in vivo* in MRL*lpr/lpr* mice.

3.2.1 ODN 2114 blocks the stimulatory activity of CpG-ODN in vitro

Initially the functional antagonism of CpG DNA induced effects, by ODN 2114, a synthetic G-rich DNA was tested *in vitro*. ODN 2114 have been reported to block CpG-ODN-induced NF-κB activation in mouse B cells (98). It was first aimed to confirm this blocking effect in murine macrophages. ODN 2114 blocked CpG-ODN-induced CCL5 (Figure 11A) and NO (Figure 11B) production in J774 monocytes in a dose-dependent manner. At equimolar concentrations of CpG-DNA and ODN 2114 the blocking effect of ODN 2114 was 100%. By contrast, ODN 2114 did not affect CCL5 or TNF-α production induced by LPS (Figures 11C and 11D). These data suggest that ODN 2114 can act as a specific antagonist for CpG-ODN-induced activation of mouse monocytes *in vitro.*

3.2.2 ODN 2114 blocks the stimulatory activity of CpG-ODN in vivo

Next the potential of ODN 2114 to block the reported CpG-ODN-induced toxicity after repeated injection was assessed in mice (103). 129Sv mice were treated with daily intraperitoneal injections of saline or either 60 μg CpG-ODN, GpC-ODN, ODN 2114, or CpG-ODN plus ODN 2114 or saline for 12 days (n=5 in each group). As previously reported CpG-ODN induced splenomegaly and lymphadenopathy as compared to saline-injected mice (103). In spleens and lymph nodes total cellularity was increased and follicles were replaced by disorganized collections of activated macrophages (Figure 12). All CpG-ODN-treated mice showed hemorrhagic ascites, as compared to saline and ODN 2114 treated controls while, ODN 2114 completely blocked CpG-ODN induced ascites. Livers of these mice revealed multifocal portal inflammatory cell infiltrates associated with large areas of necrotic and apoptotic hepatocytes (Figure 12). By contrast, all mice that received ODN 2114 injections together with CpG-ODN did not show major signs of CpG-ODN toxicity. ODN 2114 also significantly reduced CpG-ODN-induced alterations of the microarchitecture in spleens and lymph nodes (Figure 12). Mice that received injections with either ODN 2114 or GpC-ODN alone did not show any histopathological abnormalities as compared to saline injected mice (not shown), the latter excluding unspecific competition of CpG-DNA. These data imply that ODN 2114 can block CpG-ODN-induced effects *in vivo* in mice.

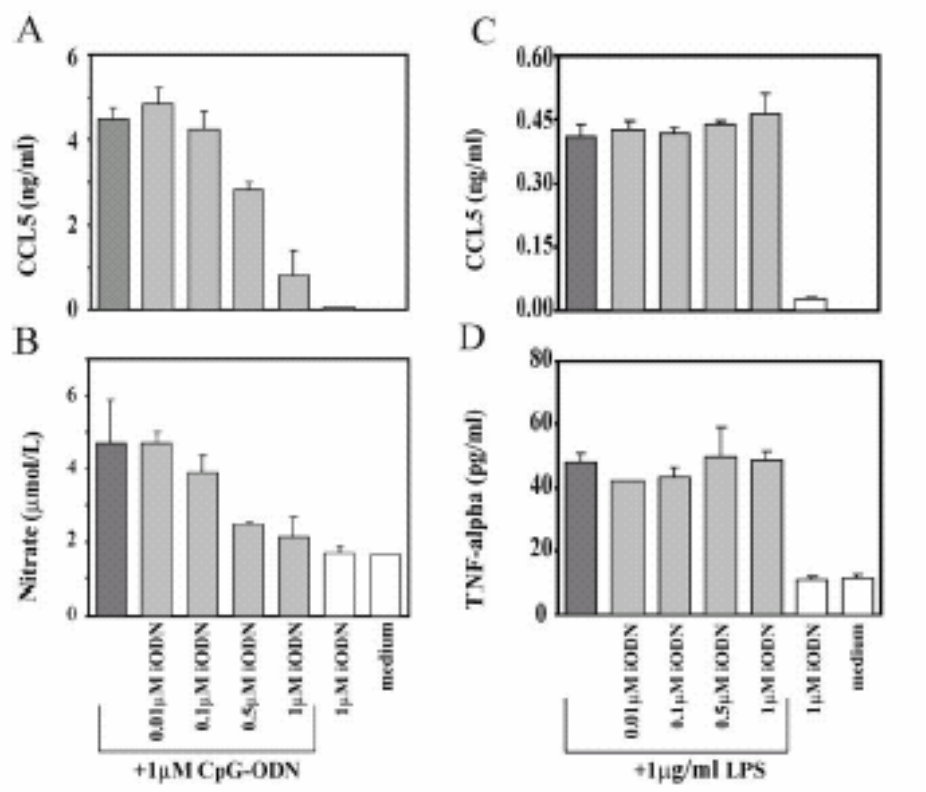


Figure 11. ODN 2114 block CpG-DNA-induced effects in vitro. Cultured J774 macrophages were incubated with either CpG-ODN or CpG-ODN with different concentrations of ODN 2114 or standard medium without supplements for 24 hours as indicated. A. CCL5 was measured in supernatants by Elisa. B. Nitrite was determined by the Griess reaction. C and D. J774 macrophages were incubated with either LPS alone or LPS with different concentrations of ODN 2114 or standard medium without supplements for 24 hours as indicated. CCL5 and TNF-α levels were measured in supernatants by Elisa. Results shown are means ± SEM from one out of two comparable experiments, each performed in duplicate.

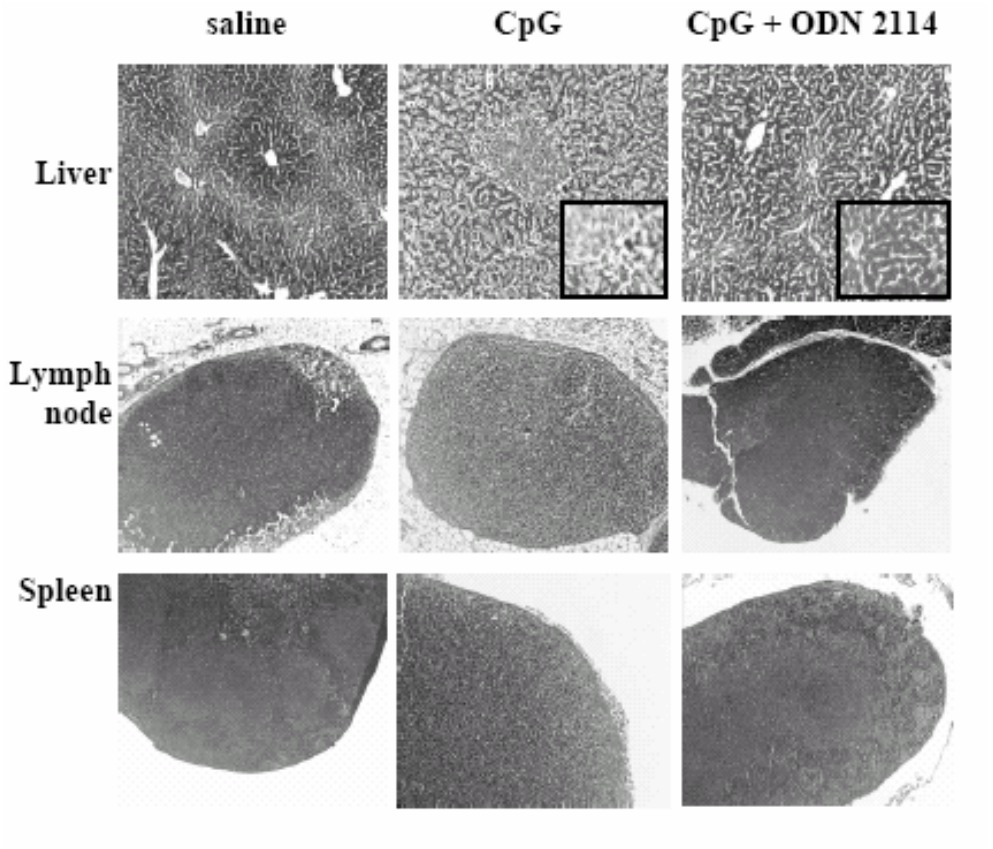


Figure 12. ODN 2114 block CpG-DNA-induced effects *in vivo*. 129Sv mice were injected intraperitoneally with saline or 60 µg either of, GpC-ODN, CpG-ODN or CpG-ODN + ODN 2114 or saline daily for 14 days (n = 5 in each group, saline not shown). Respective organs were stained with periodic acid Schiff reagent (PAS, original magnification 400x).

3.2.3 Renal distribution of labeled ODN 2114 and CpG-ODN

Both beneficial and toxic effects of CpG-ODN depend on its recognition through TLR9 (103). In mice TLR9 is known to be expressed by APCs including B cells, DCs, and monocytes (84). It has been shown that TLR9 is expressed on infiltrating monocytes but is absent on intrinsic renal cells in nephritic kidneys of autoimmune MRL*lpr/lpr* mice (49). Here it is shown that intravenous injection rhodamine-labeled CpG-DNA localizes to glomerular immune complex deposits and to CD11c positive DCs and F4/80 macrophages in nephritic kidneys of MRL*lpr/lpr* mice, consistent with the immunohistochemical staining pattern for TLR9 (49). It was hypothesized that ODN 2114 would distribute in a similar pattern in nephritic kidneys of 16 weeks old MRL*lpr/lpr* mice. In fact, rhodamine-labeled ODN 2114 was detected in a glomerular mesangial and capillary staining pattern (Figure 13A). Costaining with an antimouse-IgG antibody showed colocalization of labeled ODN 2114 with glomerular IgG deposits (Figure 13B). In some areas ODN 2114 positive granules were negative for IgG (Figure 13C). Costaining with EHRH-3 antibody identified these granules to be endosomes of glomerular macrophages but IgG-ODN2114 double positive endosomes were also observed (Figure 13D). Obviously ODN 2114 localized to intracellular endosomes similar to CpG-ODN that can interact with TLR9 after receptor redistribution from its reservoir in endoplasmic reticulum (95, 96). Thus, in autoimmune MRL*lpr/lpr* mice with lupus nephritis injected ODN 2114 distributes similar to CpG-DNA-containing immune complexes including uptake into endosomes of tissue macrophages.

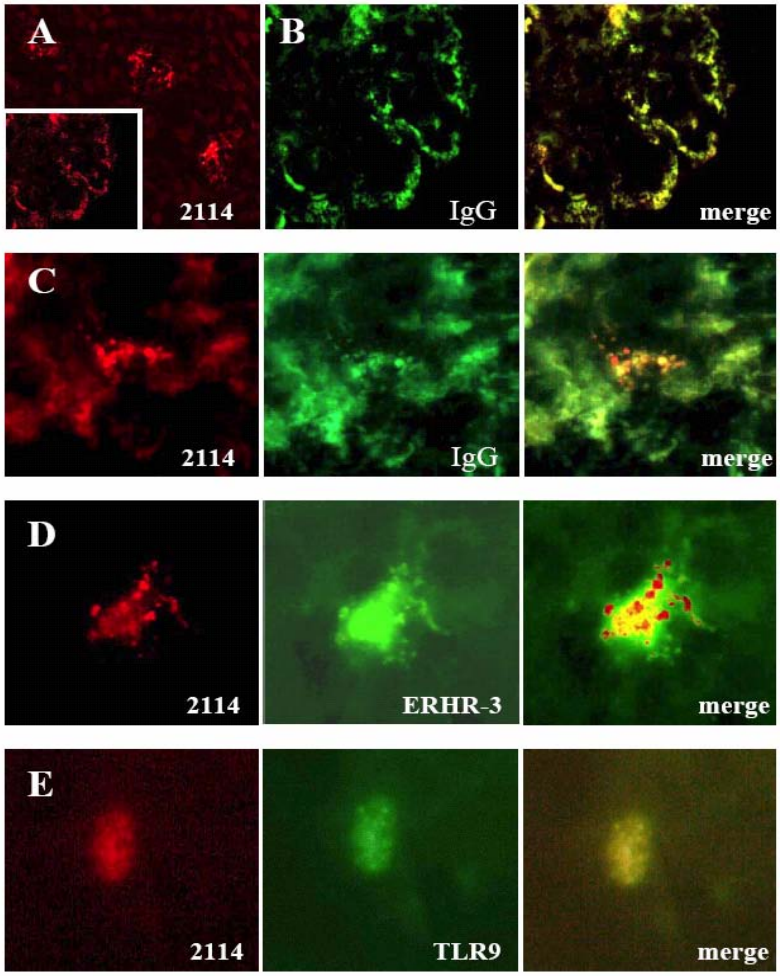


Figure 13. Localization of ODN 2114 in kidneys of MRL*lpr/lpr* mice after intravenous injection. Rhodamine-labeled ODN 2114 were I.V. injected into 16 weeks old MRL*lpr/lpr* mice and renal tissue was harvested 2hr later. A. Fluorescence imaging of frozen sections showed uptake of labeled ODN 2114 in a mesangial and capillary staining pattern (original magnification 400x). B. At higher magnification (630x) the granular deposits of ODN 2114 colocalize with IgG deposits in glomerular capillaries upon double staining. C. In some areas ODN 2114 positive but IgG negative granula are noted (original magnification 1000x). D. Costaining with an EHRH-3 antibody identified these granula to be localized within macrophages (original magnification 1000x). E. Costaining with a TLR9 antibody showed colocalization of ODN 2114 and TLR9 to an intracellular compartment (original magnification 1000x).

3.2.4 ODN 2114 reduces autoimmune tissue injury

Vertebrate DNA contains immunostimulatory and -inhibitory nucleotide sequences at a much lower ratio as bacteria (17). Thus, inhibitory sequence elements in self DNA may suppress the immunostimulatory effects of hypomethylated CpG motifs in self DNA. To test whether recognition of self-DNA via TLR9 is a pathogenic factor in SLE *in vivo*, MRL*lpr/lpr* mice were injected intraperitoneally with 40 µg/ml ODN 2114 on alternate day intervals from week 11 to 24 of age and their histopathological injury was compared with saline-treated MRL*lpr/lpr* mice at the end of the study. ODN 2114-treated MRL*lpr/lpr* mice revealed markedly reduced serum IFN-α levels as compared to saline controls (Figure 14B). This finding argues for ODN 2114-mediated suppression of autoimmunity in MRL*lpr/lpr* mice because IFN-α is known to be associated with disease activity in lupus (104). In addition, IFN-α was recently defined as a key factor in TLR-mediated autoimmunity (92). Morphometric analysis revealed less tissue injury in kidneys and lungs of ODN 2114-treated mice (Table 5). Lungs of ODN 2114-treated mice showed less peribronchiolar and perivascular inflammatory cell infiltrates compared with saline-treated controls (Figure 14C). In kidneys of MRL*lpr/lpr* mice, ODN 2114 significantly reduced numbers of interstitial ERHR-3 macrophages, CD3 lymphocytes, as well as Ki-67 positive proliferating cells in glomeruli and the tubular compartment (Table 5, Figure 14A). As a marker of glomerular injury proteinuria was also reduced in ODN 2114-treated mice, although glomerular macrophage and CD3 cell counts were similar in both groups (Table 5). Thus, injections of ODN 2114 can inhibit the immuostimulatory effect of CpG-DNA and prevent autoimmune tissue injury in MRL*lpr/lpr* mice.

Table 5. Serum, urinary, and histological findings in MRL*lpr/lpr* mice

	saline	pI:C DNA	pI:C RNA
Functional parameters			
Proteinuria (μg/mg creatinine)	3118 ± 903	2646 ± 694	10405 ± 12173
Histological scores			
Activity index	8.1 ± 2.8	6.6 ± 4.2	15.6 ± 4.2 [a]
Chronicity index	1.8 ± 1.0	1.3 ± 0.3	5.4 ± 2.1[a]
Cellular response [cells/glom. or hpf]			
Glom. EHR3+ (cells/glom)	1.7 ± 0.7	1.9 ± 1.5	4.4 ± 1.6 [a]
CD3+ (cells/glom)	1.4 ± 0.4	1.5 ± 0.8	2.2 ± 0.5 [a]
Interst. EHR3+ (cells/hpf)	6.0 ± 2.9	7.2 ± 5.3	15.5 ± 4.7 [a]
CD3+ (cells/hpf)	13.7 ± 3.3	17.0 ± 6.4	35.1 ± 10.2 [a]
SMA+ (% hpf)	5.0 ± 0.8	4.2 ± 3.9	11.0 ± 1.2 [a]
Interstitial collagen (% hpf)	2.9 ± 1.5	3.1 ± 1.7	8.4 ± 4.8 [a]
Humoral reponse			
Serum titers			
Anti-DNA IgG	14231 ± 3519	16603 ± 3089	17718 ± 3375
Anti-DNA IgG_{2a}	10667 ± 3556	9000 ± 3500	7556 ± 3951
Glom. deposit score			
IgG	1.2 ± 0.2	1.3 ± 0.4	1.4 ± 0.2
IgG_{2a}	0.3 ± 0.2	0.4 ± 0.3	0.6 ± 0.3

Values are means ± SEM from 8-10 mice per group, [a] $p < 0.05$ pI:C RNA vs. pI:C DNA

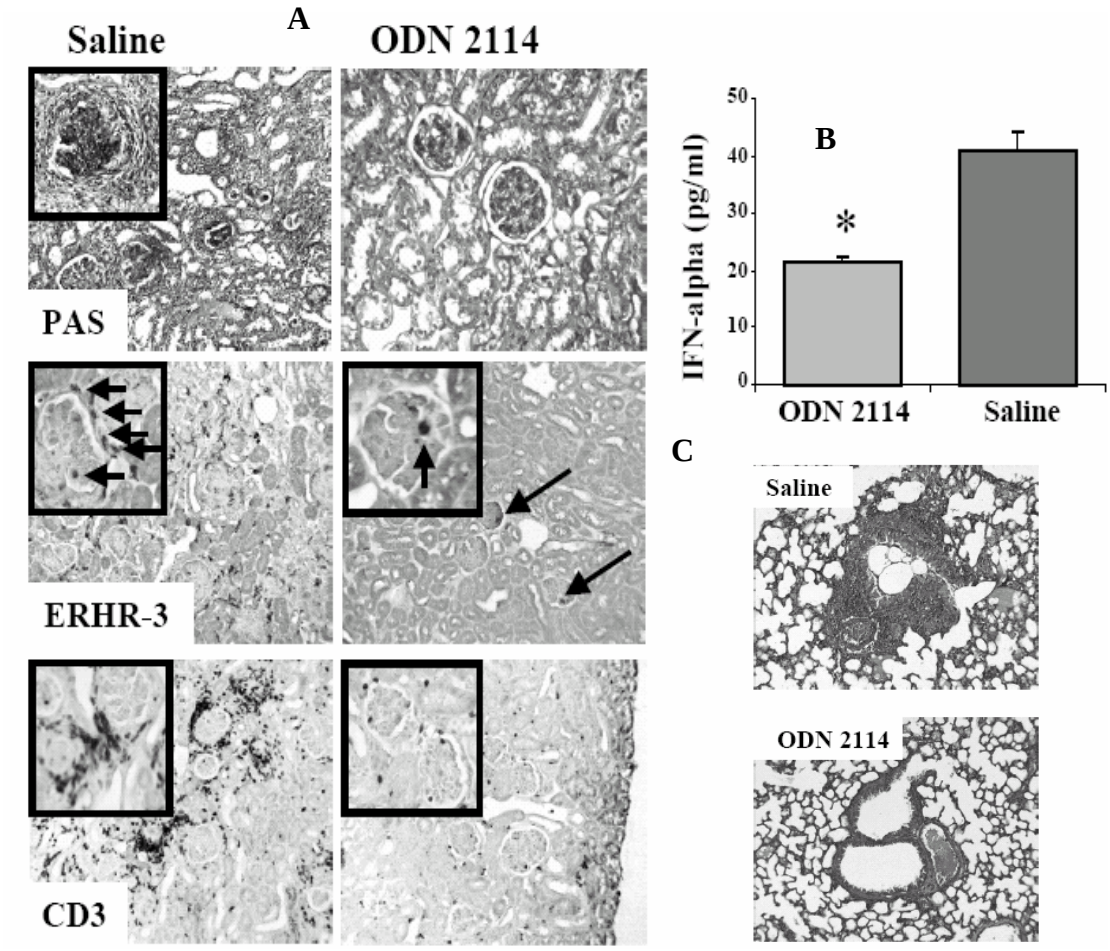


Figure 14. Autoimmune lung injury and Lupus nephritis in MRL*lpr/lpr* mice. A. Renal sections were stained with PAS, EHRH-3, and CD3 as indicated (original magnification 400x). Images are representative for 10 mice in each group. Note that ODN 2114-treated MRL*lpr/lpr* mice show less periglomerular, and interstitial inflammatory cell infiltrates as compared to saline-treated MRL*lpr/lpr* mice. B. Serum IFN-α levels in 24 weeks old female saline- or ODN 2114-treated MRL*lpr/lpr* mice were determined by Elisa (n=8 in each group), *$p<0.05$ as compared to saline. C: Lungs sections taken from 24 weeks old MRL*lpr/lpr* mice were stained with PAS. Note that ODN 2114-treated MRL*lpr/lpr* mice show less peribronchiolar and perivascular inflammatory cell infiltrates as compared to saline-injected MRL*lpr/lpr* mice (original magnification 200x).

Inhibitory ODNs have been reported to prevent organ damage induced by exogenous CpG-ODN in various disease models (98, 99). The present study reveals beneficial effects of ODN 2114 in the absence of exogenous CpG-DNA implying a pathogenic role of endogenous CpG-DNA for autoimmune tissue injury in SLE. This finding does not seem to be due to unspecific actions of ODN as in this study and others did not observe any modulatory effects of random ODN on autoimmunity in MRL*lpr/lpr* or other mouse strains (103, 49, 50).

3.2.5 Autoantibodies and renal immune complex deposits

Autoantibody production and immune complex deposition cause renal tissue injury in SLE. The effect of ODN 2114 on DNA autoantibody production and immune complex deposition was assessed in MRL*lpr/lpr* mice. At 24 weeks of age ODN 2114-treated MRL*lpr/lpr* mice revealed reduced serum levels of dsDNA-specific IgG1 and IgG2a antibodies (Table 5). This result was consistent with the observation that ODN 2114 reduced glomerular IgG1 or IgG2a deposits, a marker of renal immune complex deposition in MRL*lpr/lpr* mice (Table 5, Figure 3C). These findings are suggestive of an inhibitory effect of ODN 2114 on CpG-DNA-induced B cell activation in MRL*lpr/lpr* mice. Therefore, MHC II expression was determined on spleen B cells by flow cytometry, 24 hours after a single intraperitoneal injection of 500 µg CpG-ODN, ODN 2114 or CpG-DNA + ODN 2114 into MRL*lpr/lpr* mice. CpG-DNA-injected mice showed marked increase in MHC II expression on CD19 positive spleen B cells, as compared to mice treated with ODN 2114 alone or CpG-ODN together with ODN 2114 (Figure 3D).

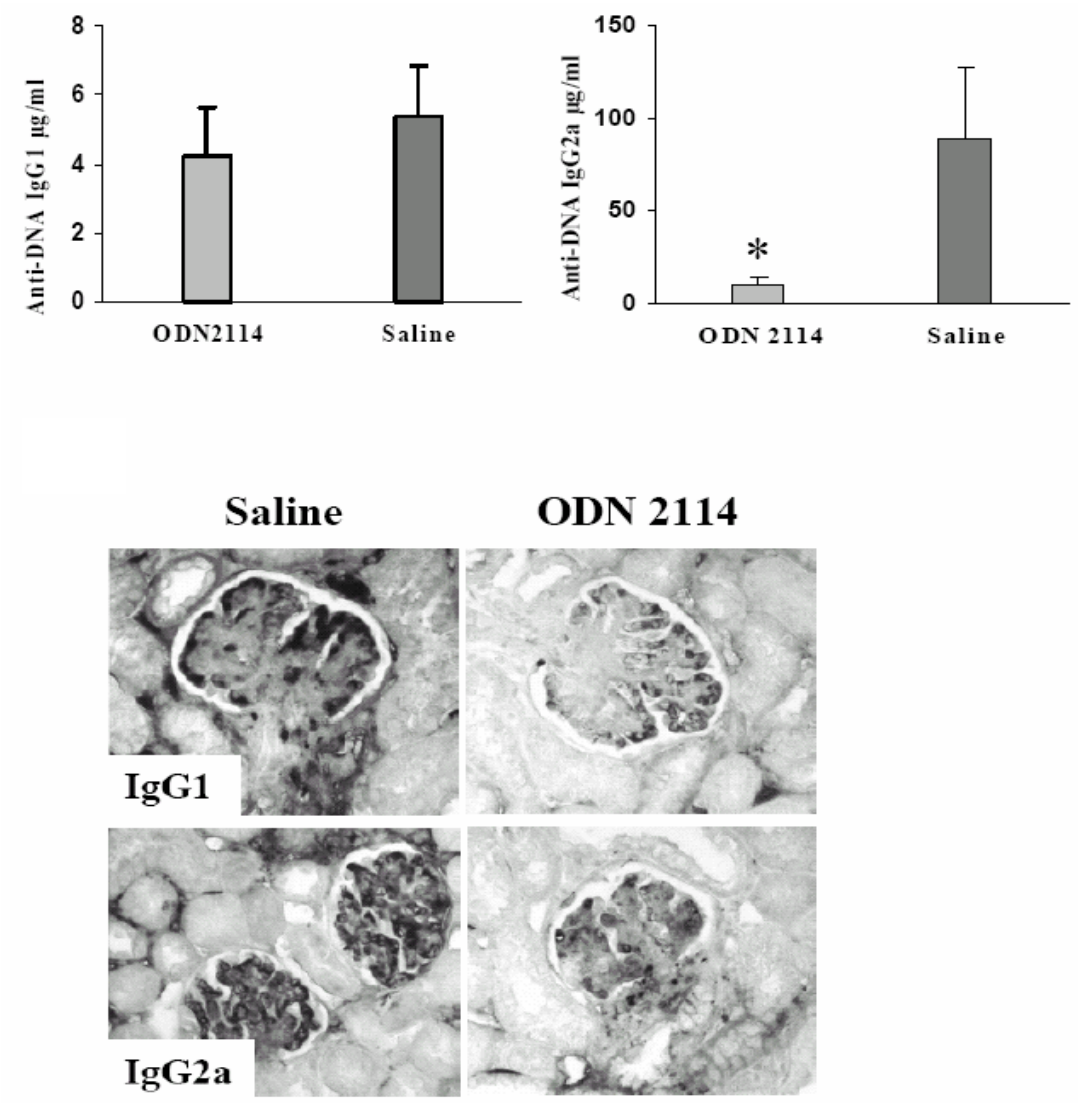


Figure 16. Serum dsDNA autoantibody levels and glomerular immune complex deposits in MRL*lpr/lpr* mice. Serum dsDNA autoantibody IgG1 (A) and IgG2a (B) levels were determined by Elisa (n=8-10). Data are means ± SEM. * $p < 0.05$ vs. saline. C. Renal sections were stained for IgG1 and IgG2a, as indicated (original magnification 400x). Note less glomerular IgG1 and IgG2a deposits in ODN 2114-treated MRL*lpr/lpr* mice. Images are representative for 10 mice in each group.

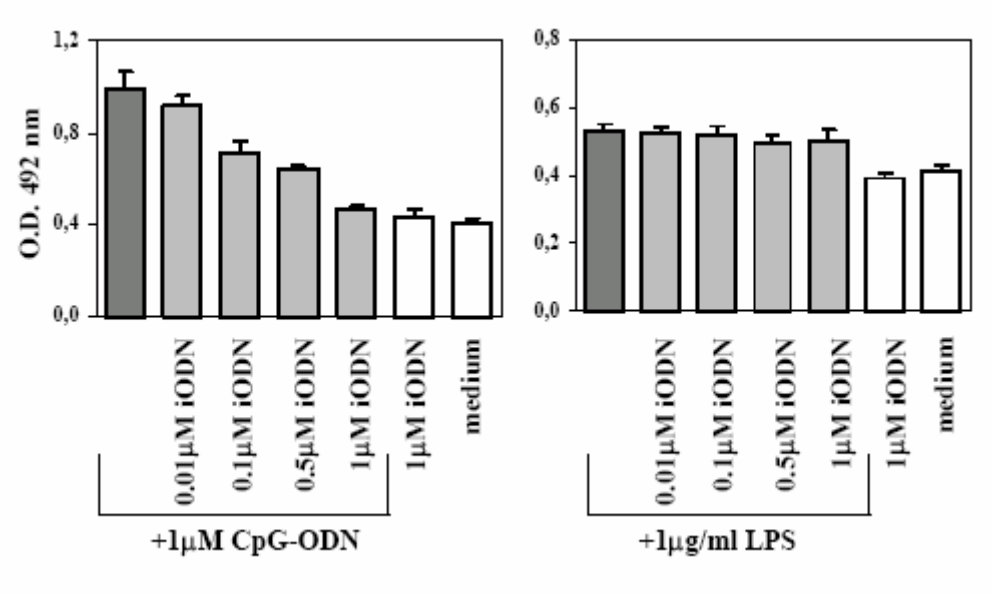


Figure 17. B-cell activation in MRL*lpr/lpr* mice. A. B-cells were isolated from MRL*lpr/lpr* mice as described in Methods. B-cells were incubated with either CpG-ODN, CpG-ODN plus different concentrations of ODN 2114 or standard medium without supplements for 72 hours as indicated. B-cell proliferation was assessed by *CellTiter 96* proliferation assay. B. B-cells were isolated as before and incubated with LPS alone or LPS with different concentrations of ODN 2114 or standard medium without supplements for 72 hours as indicated. Results are means ± SEM from one of two comparable experiments, each performed in duplicate.

These results suggest that ODN 2114 can block CpG-ODN-induced B cell activation in MRL*lpr/lpr* mice in vivo, consistent to what has been previously reported from in vitro studies with murine B cells (97). ODN 2114 reduced autoantibody production, immune complex deposition, and subsequent renal injury in MRL*lpr/lpr* mice that were not exposed to exogenous CpG-ODN. These data raise the possibility of a pathogenic role of endogenous CpG-DNA in driving autoimmunity in MRL*lpr/lpr* mice. Dying cells release DNA particles known to activate immune cells, most likely, through a TLR9-dependent mechanism (53). Methylation is another factor in masking immunostimulatory effects of vertebrate CpG-DNA (14), and DNA methyltransferase inhibitors trigger disease activity in human SLE (16). Obviously, a tight balance for controlling adjuvant activity of self DNA exists. The data from the present study supports the hypothesis that inhibitory ODN 2114 reduces autoimmunity in MRL*lpr/lpr* mice by interfering with endogenous CpG-DNA. TLR9 ligation by CpG-DNA is currently evaluated for multiple therapeutic strategies (76, 78), but may cause serious adverse effects (103, 49). Our data implicate a role for endogenous CpG-DNA in the pathogenesis in lupus and suggest that targeting TLR9 signaling, e.g. with immunosuppressive ODN, may provide a new therapeutic target for SLE.

4. DISCUSSION

4.2 Viral dsRNA aggravates immune complex glomerulonephritis in MRL*lpr/lpr* mice

Viral infections can aggravate disease activity in pre-existing SLE, but the role of viral RNA in this context is hypothetical. In this study a model of spontaneous lupus nephritis, a form of immune complex-glomerulonephritis, in MRL*lpr/lpr* mice was used to study the effects of intermittent exposure to pI:C RNA, a structural analogue to viral dsRNA. Both DNA autoantibody-dependent and independent mechanisms were examined in aggravating lupus nephritis by pI:C RNA. For the first time, this study provides evidence for expression of TLR3 on glomerular mesangial cells and for the ability of pI:C RNA to induce proinflammatory cytokine and chemokine production in these cells. The process of aggravation of autoimmune renal injury in MRL*lpr/lpr* mice that were exposed to circulating pI:C RNA is further facilitated through activation of infiltrating immune cells. Interestingly pI:C RNA–induced aggravation of lupus nephritis in MRL*lpr/lpr* mice is independent of B cell activation and DNA autoantibody production, which may relate to the cell type specific expression profile of TLR3. In MRL*lpr/lpr* mice, TLR3 expression is restricted to APCs of the monocytic cell lineage, e.g., DCs and macrophages, as well as to glomerular mesangial cells, which may play an important role in viral infection-induced local tissue injury and exacerbation of immune complex glomerulonephritis.

4.1.1 Viral dsRNA activates TLR3 on mesangial cells

Antiviral host defence requires activation of innate immunity, including the local production of type I interferons and chemokines (105, 63). The finding that injected

dsRNA localized to TLR3-positive APCs of the monocytic cell lineage and to glomerular mesangial cells in kidneys of MRL*lpr/lpr* mice suggests a role for TLR3 in local immune responses in the kidney induced by viral dsRNA. In fact, in kidneys of MRL*lpr/lpr* mice, immunostaining for TLR3 localized to glomerular mesangial cells. This is consistent with unexpected high levels of TLR3 mRNA in kidneys of these mice, which is consistent with previously published data from healthy murine and human kidneys (106, 107). The speckled staining pattern for TLR3 in mesangial cells of nephritic glomeruli of MRL*lpr/lpr* mice is also consistent with the uptake of injected and rhodamine-labeled viral dsRNA into an intracellular compartment of mesangial cells in their kidneys. Mesangial cells do not express other nucleic acid–specific TLR (TLR7, TLR8, and TLR9) (49), so the expression of a functional TLR3 in mesangial cells represents a new finding. This study with a murine mesangial cell line confirmed the intracellular localization of TLR3 analogous to its localization in APCs (108). Furthermore, some surface expression of TLR3 was detected, that has also been observed in other nonimmune cell types (e.g., human fibroblasts, intestinal epithelial cells) (69, 109). When exposed to pI:C RNA, mesangial cells expressed large amounts of IL-6 and CCL2, two proinflammatory mediators that are well known to be involved in progression of glomerulonephritis in murine disease models and in human glomerular diseases (49, 110). From these *in vitro* studies, it was expected that an increase of local expression of proinflammatory chemokines in kidneys of MRL*lpr/lpr* mice, a prediction confirmed by real-time RTPCR of total renal isolates and by immunostaining for CCL2 and CCL5 on renal sections of pI:C RNA–treated MRL*lpr/lpr* mice. pI:C RNA–induced local production of CCL2 and CCL5 is likely to be involved in the observed aggravation of

lupus nephritis in MRL*lpr/lpr* mice as both CC-chemokines contribute to the progression of lupus nephritis by mediating renal leukocyte recruitment in this disease model (111, 112) as well as in human lupus nephritis (113, 114). When leukocytes migrate to renal lesions, they by themselves become a major source of proinflammatory cytokines and chemokines (115). It was found that cells of the monocytic cell lineage located in the kidney take up circulating pI:C RNA. Studies with spleen monocytes isolated from MRL*lpr/lpr* mice support the idea that uptake of pI:C RNA by tissue macrophages contributes to the local production of proinflammatory mediators, including NO, IL-12p70, IL-6, IFN-α, and CCL5. Taken together, circulating viral dsRNA, e.g. pI:C RNA, is taken up by renal macrophages, DCs, and mesangial cells that express TLR3 in intracellular endosomes. Ligation of TLR3 activates these cell types to secrete proinflammatory mediators, including type I interferons, cytokines, and chemokines, which promote local tissue injury. This innate immune mechanism in response to viral dsRNA is detrimental in pre-existing renal inflammation such as immune complex glomerulonephritis during lupus.

4.1.2 Viral dsRNA activates APCs but elicits no B Cell response

A central role for dendritic cells (DCs) for infection-associated exacerbation of pre-existing autoimmunity is suspected for three reasons: 1) In mice and humans, DCs show constitutive expression of most TLRs in mice and humans (60, 65); 2) Ex vivo exposure of DCs to the TLR4 ligand LPS and transfer of such cells into mice that are prone to autoimmune myocarditis was sufficient to initiate overt myocarditis (89), and 3) ligation of TLR4 and TLR9 on DCs blocks the suppressor activity of regulatory T cells via

secretion of IL-6 (116). Murine macrophages and DCs express TLR3 (63), but TLR3 is restricted to DCs on human leukocytes (65). DCs constantly process self-antigens, but in the absence of co-stimulatory molecules, the presented antigen provides T cells with a signal for tolerance (117). By contrast, during viral infection, virus-associated TLR ligands stimulate DCs to upregulate co-stimulatory molecules and to secrete selected cytokines (118). Therefore, DCs are key regulators of both tolerance and antiviral immunity and therefore may be crucial for viral infection–induced exacerbation of autoimmunity, including SLE (116). In fact, among microbial products, viral dsRNA is a potent trigger for DC maturation and for the secretion of type I interferons and Th1 cytokines (93, 119, 120). pI:C RNA was found to trigger maturation and secretion of IL-12p70 and IFN-α by DCs isolated from bone marrow of MRL*lpr/lpr* mice is consistent with these studies. Although viral dsRNA–induced IFN-α production is a major mechanism of anti-viral immunity (121) elevated IFN-α levels also promote the progression of autoimmune tissue injury in SLE (122, 123). Furthermore, TLR3-induced secretion of IL-12p70 has a critical role in SLE and lupus nephritis mainly by fostering the accumulation of IFN-α producing T cells in the kidney followed by aggravation of lupus nephritis (33, 124, 125). In these studies it was observed that pI:C RNA–induced elevation of IL-12p70 serum levels was associated with acceleration of immune complex glomerulonephritis is consistent with a previous study showing aggravation of lupus nephritis by injections of recombinant IL-12 in MRL*lpr/lpr* mice (33). DC activation can induce adaptive B and T cell responses. Thus, one might assume that pI:C RNA–induced maturation of DCs would enhance humoral immunity against chromatin, an important autoantigen in SLE. It is interesting that in contrast to the TLR9 ligand CpG-DNA (52),

pI:C RNA did not affect serum levels and glomerular deposits of DNA autoantibodies. As human and murine B cells express TLR9 but not TLR3, a direct stimulatory effect of pI:C RNA was not predicted, but B cell activation could be supported by indirect mechanisms via dendritic cells and T cells. In fact, the data from this study clearly shows that in MRL*lpr/lpr* mice, systemic exposure to pI:C RNA does not provide a signal for B cell activation, consistent with the finding that serum DNA autoantibody levels and renal IgG deposits were unaffected by repetitive pI:C RNA injections. These findings are consistent with a recent observation that a series of injections with CpG-DNA but not with pI:C RNA can severely alter the morphology and functionality of mouse lymphoid organs (103). In this study it was observed that pI:C RNA induces the secretion of IL-6 in DCs and monocytes isolated from MRL*lpr/lpr* mice. IL-6 derived from antigen-presenting cells has been shown to suppress CD4+CD25+ regulatory T cells that inhibit the proliferation of autoreactive T cells (116). The role of regulatory T cells in SLE remains to be determined (126), but their number is reduced in peripheral blood of SLE patients with active disease (127, 128), indicating that disease activity of SLE may be linked to the regulatory role of this T cell population. pI:C RNA–induced IL-6 secretion therefore may modulate adaptive immunity in SLE independent of B cell responses. Overall these findings suggest that, viral dsRNA triggers disease activity of lupus nephritis by mechanisms different than that of bacterial DNA. In contrast to CpG-DNA/TLR9 interaction pI:C RNA/TLR3-mediated disease activity is B cell independent, but activated intrinsic renal cells e.g. glomerular mesangial cells to produce cytokines and chemokines, factors that can aggravate autoimmune tissue injury e.g. lupus nephritis.

4.2 Synthetic G-rich DNA suppresses systemic autoimmunity in MRL*lpr/lpr* mice

When MRL*lpr/lpr* mice are exposed to bacterial or synthetic CpG-DNA, ligation of TLR9 on immune cells leads to enhanced dsDNA autoantibody production and aggravation of lupus nephritis (52). Similarly, insufficient clearance of nuclear particles in lupus may also provide a permanent source of hypomethylated CpG motifs from self DNA (104). This mechanism may contribute to a continuous activation of B-cells and dendritic cells and perpetuate systemic lupus in humans (14, 92, 53). In the present study this hypothetical pathway by blocking potential CpG-DNA-induced immunity was tested both *in vitro* and *in vivo* with a specific antagonist, i.e. G-rich DNA. Further it was tested, if endogenous CpG-DNA were to contribute to the progression of lupus nephritis, injections of G-rich DNA should prevent disease progression in MRL*lpr/lpr* mice with experimental lupus.

4.2.1 Synthetic G-rich ODN neutralizes CpG-DNA in vitro

CpG-DNA is a strong activator of plasmacytoid DCs, macrophages, and B-cells in mice (14, 72). CpG-DNA stimulates their antigen-presentation and proinflammatory cytokine production that drive subsequent Th1 type responses (72). Lenert, et al. first used the ODN 2114 to block CpG-ODN-induced effects on murine B-cells (98). Through this study it was confirmed that this antagonistic effect is specific, as G-rich ODN 2114, totally prevented CpG-DNA-induced B-cell proliferation or CCL5 and TNF-α production by macrophages, but did not modulate LPS-related effects. This antagonism occurs proximal to NF-κB activation (98), but the specific site of interaction is yet unknown. In this study *in vivo* colocalization of injected ODN 2114 with glomerular IgG deposits was

observed which are complexed with chromatin-particles in nephritic MRL*lpr/lpr* mice. G-rich ODN represent a specific antagonist for CpG-DNA-induced B-cell and monocyte activation in mice, which is also confirmed *in vivo* by experiments in Sv129 mice. G-rich ODN 2114 blocked CpG-ODN-induced lymphoproliferation, an effect shown to be mediated through TLR9 on marginal zone B-cells and monocytes (103) Together, G-rich ODN 2114 specifically block the effects of CpG-DNA *in vitro* and *in vivo* which renders ODN 2114 a valuable tool to address the question, whether endogenous CpG-DNA can modulate systemic lupus erythematosus.

4.2.2 G-rich DNA modulates systemic autoimmunity

In MRL*lpr/lpr* mice lymphoproliferation and dsDNA autoantibody production progresses with age. G-rich ODN 2114 injections reduced spleen weight, as a marker of lymphoproliferation, and serum dsDNA autoantibody levels as compared to saline-injected MRL*lpr/lpr* mice. As the MRL*lpr/lpr* mice were not exposed to exogenous CpG-DNA the observation may be attributed to a blockade of immunostimulatory effects of endogenous CpG-DNA on B-cells. This is consistent with a study published by Leadbetter et al. showing that self chromatin-containing immune complexes stimulate B-cells isolated from MRL*lpr/lpr* mice via TLR9 (77). It is noteworthy that, suppressive ODN as well as DNase treatment of the immune complexes abrogated this effect, supporting the role for the endogenous CpG-DNA in this context (77). In this study the blocking effect of G-rich DNA on B-cell proliferation was specific for CpG-DNA, because ODN 2114 did not modulate LPS-induced B-cell proliferation.

Serum IFN-α levels depict the activation of IFN-producing plasmacytoid dendritic cells (DCs) which represents another marker for disease activity in lupus (129). Nuclear particles released from dying cells and complexed with lupus patient IgG are potent inducers of IFN-α production in plasmacytoid DCs, an effect sensitive to DNAse digestion (87). In a recent study immune complexes were isolated from sera of patients with various rheumatic diseases (98). It was found that only DNA-containing immune complexes isolated from lupus patients stimulated plasmacytoid DCs to produce cytokines and chemokines via a cooperative interaction between TLR9 and FcγRIIa (CD32). CD32 shuttles DNA-containing immune complexes into a subcellular compartment containing TLR9 (96). Only CD32 positive plasmacytoid DCs internalized DNA-immune complexes and produced large amounts of IFN-α. Results from the present study show that MRL*lpr/lpr* mice injected with G-rich DNA show lower serum IFN-α levels as compared to saline-injected MRL*lpr/lpr* mice is in favour of a blocking effect of G-rich DNA on interferon-producing plasmacytoid DCs. Thus, G-rich DNA can block B-cell proliferation, dsDNA autoantibody production, and IFN-α release in mice, which all have established etiopathogenic roles in the systemic autoimmunity of lupus erythematosus.

4.2.3 G-rich DNA prevents tissue injury in MRLlpr/lpr mice

MRL*lpr/lpr* mice injected with G-rich DNA had markedly reduced renal and pulmonary autoimmune tissue injury as compared to saline-injected mice. In part this may relate to the reduced anti dsDNA antibody production and immune complex deposition, as demonstrated for the kidney. However, injected G-rich ODN 2114 could also interact

locally with immune complexes and TLR9 positive immune cells. This issue was addressed by injecting fluorescently labeled ODN 2114 into nephritic MRL*lpr/lpr* mice. In fact, ODN 2114 localized to glomerular immune complex deposits and to intracellular compartments of infiltrating glomerular macrophages. This cellular distribution was comparable to that of TLR9 immunostaining in kidneys of MRL*lpr/lpr* mice. Thus, ODN 2114 could interfere with CpG-DNA–rich chromatin-immune complexes in the endosomes of intrarenal macrophages *in vivo*. This could reduce a proinflammatory effect of the immune complexes on macrophages and possibly DCs in kidneys of MRL*lpr/lpr* mice. Our laboratory has previously shown that exogenous bacterial or CpG-DNA markedly stimulate renal macrophages in lupus nephritis of MRL*lpr/lpr* mice and antigen-induced immune complex glomerulonephritis (49, 52). CpG-DNA stimulates macrophages to produce multiple proinflammatory mediators that contribute to the progression of renal disease. Similarly, exogenous CpG-DNA can cause macrophage-dependent arthritis or lung injury, which both can be blocked with G-rich DNA (99, 130). However, in the present study MRL*lpr/lpr* mice were not exposed to exogenous CpG-DNA, so that the beneficial effect of G-rich DNA is through blockade of endogenous CpG-DNA-mediated effects. The data from the present study supports the hypothesis that endogenous CpG-DNA–rich chromatin activates TLR9 positive immune cells – specifically B cells, macrophages, and DCs, and thus contributes to the pathogenesis of lupus. Apart from the experimental data in mice, this concept is also supported by the therapeutic properties of chloroquine, an unspecific blocker of endosomal TLR activation, in the treatment of human lupus (131). The present observation from these studies, that administration of G-rich DNA attenuates the course of the lupus-like disease

in MRL*lpr/lpr* mice also argues in favour of endogenous CpG-DNA fragments as pathophysiological contributors to disease and indicate that G-rich DNA as a potential therapeutic agent for the treatment of SLE.

5. ZUSSAMENFASSUNG

Frei oder in Immunkomplexen gebundene Nukleinsäuren können zu Immunaktivierung und damit Verschlechterung vorbestehender Autoimmunkrankheiten führen. Die Toll-like Rezeptoren TLR3 und TLR 9 erkennen virale und bakterielle Nukleinsäuren. pI:C RNA, eine synthetische Doppelstrang-RNA mit identischen immunstimulatorischen Eigenschaften wie virale dsRNA aktiviert TLR3 und führt zur m Verschlechterung einer vorbestehenden Lupusnephritis in MRL*lpr/lpr* Mäusen. Dies geschieht durch Aktivierung von TLR3 auf antigen-präsentierenden Zellen und glomerulären Mesangialzellen, was zur vermehrten lokalen Zytokin- und Chemokinproduktion führt. In gleicher Weise aggraviert CpG DNA, ein klassischer Ligand für TLR9 die Lupusnephritis dieses Mausmodells, doch die Mechanismen unterscheiden sich von den Effekten von dsRNA. Offensichtlich sind die Mechanismen Infekt-getriggerter Immunstimulation abhängig von der Zelltyp-spezifischen Expression entsprechender “pattern-recognition” Rezeptoren. dsRNA-induzierte Krankheitsaktivität ist unabhängig von einer B Zell Aktivierung und der Produktion von DNA Autoantikörpern, was hingegen einen wichtigen Mechanismus der CpG-DNA–induzierten Autoimmunität darstellt. Diese Daten fördern das Verständnis der Infekt-getriggerten Autoimmunität könnte jedoch auch eine Rolle bei anderen Immunkomplex-vermittelten Nierenerkrankungen wie z.B. der IgA Nephritis, den renalen Manifestationen der chronischen Hepatitis C Virus Infektion und der renalen Vaskulitis spielen. Darüberhinaus besteht die Frage, inwiefern endogene DNA viw TLR9 an der Pathogenese der Lupusnephritis beteiligt ist. Hier wird gezeigt, die G-reiche DNA, ein Inhibitor der CpG-DNA induzierten TLR9 Aktivierung, den Spontanverlauf der Lupusnephritis von MRL*lpr/lpr* Mäusen günstig beeinflusst. Die Blockade von TLR9

ermöglicht so ein neues Verständnis der Pathogenese der Lupusnephritis und zeigt neue Behandlungsmöglichkeiten auf.

5. SUMMARY

Nucleic acids that occur free or as immune complexes may trigger immune activation leading to aggravation of diseases with autoimmune predisposition. TLR3 and TLR 9 represent receptors that signal for viral and bacterial nucleic acids respectively. pI:C RNA, a synthetic double stranded RNA with identical properties to that viral origin activates TLR3 led to aggravation of lupus nephritis, a form of immune complex glomerulonephritis, in pre-existing lupus in MRL*lpr/lpr* mice. Exposure to pI:C RNA (a structural analogue of viral dsRNA) can aggravate lupus nephritis through TLR3 on antigen-presenting cells and glomerular mesangial cells. pI:C RNA–induced cytokine and chemokine production represents a major mechanism in this context. Likewise, CpG DNA a classical activator for TLR-9 led to disease aggravation in this mouse model albeit, through mechanisms that shared some commonality as well as differences to that observed with pI:C RNA. Apparently, pathogen associated immunomodulation relates to the cell-type-specific expression pattern of the respective pattern-recognition receptor. dsRNA-induced disease activity is independent of B cell activation and humoral antichromatin immunity in experimental SLE and therefore differs from CpG-DNA–induced autoimmunity. These findings contribute to the understanding of pathogen-associated modulation of autoimmunity but may also be involved in the pathogenesis of other types of inflammatory kidney diseases, e.g., flares of IgA nephropathy, renal manifestations of chronic hepatitis C virus infection, and renal vasculitis. Further, besides

signifying the role of foreign and self-DNA as a pathogenic factor in autoimmune disease activity in lupus, this detailed study reveals, that certain synthetic G-rich nucleic acids may potentially block nucleic acid specific TLR functions and thus prove beneficial in arresting disease activity during progressive systemic lupus. One such G-rich DNA employed in this study has proven to be beneficial and suppressed systemic lupus in MRL*lpr/lpr* mouse model. Thus, modulating the CpG-DNA - TLR9 pathway may offer new opportunities for the understanding and treatment of lupus.

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Viral Double-Stranded RNA Aggravates Lupus Nephritis through Toll-Like Receptor 3 on Glomerular Mesangial Cells and Antigen-Presenting Cells

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How viral infections trigger autoimmunity is poorly understood. A role for Toll-like receptor 3 (TLR3) was hypothesized in this context as viral double-stranded RNA (dsRNA) activates dendritic cells to secrete type I interferons and cytokines that are known to be associated with the disease activity in systemic lupus erythematosus (SLE). Immunostaining of nephritic kidney sections of autoimmune MRL$^{lpr/lpr}$ mice revealed TLR3 expression in infiltrating antigen-presenting cells as well as in glomerular mesangial cells. TLR3-positive cultured mesangial cells that were exposed to synthetic polyinosinic-cytidylic acid (pI:C) RNA *in vitro* produced CCL2 and IL-6. pI:C RNA activated macrophages and dendritic cells, both isolated from MRL$^{lpr/lpr}$ mice, to secrete multiple proinflammatory factors. *In vivo*, a single injection of pI:C RNA increased serum IL-12p70, IL-6, and IFN-α levels. A course of 50 μg of pI:C RNA given every other day from weeks 16 to 18 of age aggravated lupus nephritis in pI:C-treated MRL$^{lpr/lpr}$ mice. Serum DNA autoantibody levels were unaltered upon systemic exposure to pI:C RNA in MRL$^{lpr/lpr}$ mice, as pI:C RNA, in contrast to CpG-DNA, failed to induce B cell activation. It therefore was concluded that viral dsRNA triggers disease activity of lupus nephritis by mechanisms that are different from those of bacterial DNA. In contrast to CpG-DNA/TLR9 interaction, pI:C RNA/TLR3-mediated disease activity is B cell independent, but activated intrinsic renal cells, *e.g.*, glomerular mesangial cells, to produce cytokines and chemokines, factors that can aggravate autoimmune tissue injury, *e.g.*, lupus nephritis.

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Viral components can trigger disease activity in systemic lupus erythematosus (SLE) or autoimmunity in general (1), but the involved mechanisms remain poorly defined. It is believed that during viral infections, pathogen recognition and subsequent induction of adaptive immune responses might interfere with the control of self-tolerance in susceptible individuals. Therefore, pattern-recognition receptors that bind pathogen-associated molecular patterns may stimulate both host defense and—under certain circumstances—autoimmune disease activity.

Toll-like receptors (TLR) are a family of such pattern-recognition receptors, which can discriminate pathogens (including viruses) from self and activate suitable defense mechanisms (2,3). TLR on antigen-presenting cells also initiate and modulate adaptive immunity during infection (4). For example, most TLR ligands, including viral double-stranded RNA (dsRNA), activate dendritic cell maturation (5). This is characterized by the upregulation of MHC class II, induction of costimulatory molecules (*e.g.*, CD80, CD86), and secretion of selected cytokines, which prime subsequent T cell responses (5).

In contrast to their established role for pathogen control, the role of TLR in autoimmunity is less well defined (6,7). Systemic exposure to unmethylated CpG-DNA (ligand of TLR9) can induce experimental autoimmune encephalomyelitis (8) and aggravate the immune complex glomerulonephritis induced by apoferritin (9) and the spontaneous immune complex and lupus-like glomerulonephritis of MRL$^{lpr/lpr}$ mice (10). However, heterogeneous expression patterns of single TLR on leukocyte subpopulations and the discovery of TLR-specific signaling pathways support specific types of immune responses for specific ligand-TLR interactions (11,12). For example, TLR3, a receptor for viral dsRNA, is the only known TLR that is expressed exclusively by dendritic cells among leukocytes in humans (13). Furthermore, TLR3 is the only known TLR that depends on signaling through the adaptor molecule TRIF (Toll-IL-1 receptor domain-containing adaptor inducing IFN-β) and RNA helicase RIG-1, which is followed by a robust induction of IFN-responsive genes (12,14,15). These findings may point toward the recognition of viral dsRNA via TLR3 on dendritic cells not only as an important component of virus-induced immunity but also hypothetically as a link to viral infection–induced aggravation of preexisting autoimmunity.

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We therefore characterized the expression of TLR3 in experimental SLE and the effects of dsRNA of SLE disease activity as a model for intercurrent viral infection in SLE. We identified a novel and robust expression of TLR3 on glomerular mesangial cells *in vitro* and *in vivo*. Our data demonstrate that in SLE, upon injection, viral dsRNA is taken up by glomerular mesangial cells and infiltrating immune cells in the nephritic kidney, leading to aggravation of lupus nephritis. Thus, TLR3 on immune and nonimmune cells may contribute to viral disease–associated aggravation of autoimmune kidney diseases, *e.g.*, lupus nephritis.

Materials and Methods

Animals and Experimental Protocol

Five-week-old female $MRL^{lpr/lpr}$ mice were obtained from Jackson Laboratory (Bar Harbor, ME) and were maintained in filter-top cages under a 12-h 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. For assessing renal TLR3 mRNA expression, kidneys were obtained from 5- and 20-wk-old female $MRL^{lpr/lpr}$ mice. In addition, 16-wk-old female $MRL^{lpr/lpr}$ mice were distributed into three groups, each consisting of 12 female mice. From weeks 16 to 18, mice of all groups received intraperitoneal injections every other day as follows: (*1*) 50 μg of polyinosinic-cytidylic acid (pI:C) RNA (Sigma-Aldrich, Steinheim, Germany) in 100 μl of normal saline, (*2*) 50 μg of pI:C DNA (di:dC; Sigma) in 100 μl of normal saline, and (*3*) 100 μl of normal saline. All mice were killed by cervical dislocation at the end of week 18 of age. To assess the renal distribution of injected RNA, we used a 3′-rhodamine-labeled dsRNA from human rhinovirus strain 16.11 (5′-AUCUGGGUUGUUCCCACCCAGAUCAC-CUACAUGG-TAMRA-3′ and 3′-UAGACCCAACAAGGGUGGGUC-UAGUGGAUGUACC-5′). The viral RNA was injected intravenously into $MRL^{lpr/lpr}$ mice at the age of 16 wk. Renal tissue was collected 2 h later and subjected to further analysis as described below. For the identification of 3′-rhodamine-labeled renal cells, co-staining was performed using rat anti-F4/80 (Serotec, Oxford, UK; 1:50).

To test the ability of pI:C RNA to induce *in vivo* B cell activation, 4-wk-old $MRL^{lpr/lpr}$ mice were divided in three groups of two mice each. Each mouse received 500 μg/ml pI:C RNA, CpG, or pI:C DNA intraperitoneally, and the mice were killed after 24 h and spleens were collected. Total spleen suspension from each mouse in all experimental groups was processed for flow cytometry as described below.

Evaluation of Glomerulonephritis

Blood and urine samples were collected from each animal at the end of the study period as described (10) to determine proteinuria and creatinine using an automatic autoanalyzer (Integra 800; Roche Diagnostics, Mannheim, Germany). Serum DNA autoantibodies were determined by ELISA using the following antibodies: IgG (BD Pharmingen, Hamburg, Germany; 1:100) and IgG_{2a} (Dianova, Hamburg, Germany; 1:100). From all mice, kidneys were fixed in 10% buffered formalin, processed, and embedded in paraffin. Sections for silver and periodic acid-Schiff stains were prepared following routine protocols. The severity of the renal lesions was graded using the indices for activity and chronicity as described for human lupus nephritis (16).

Immunohistochemistry

Immunohistochemical staining was performed on paraffin-embedded sections as described (10) using the following primary antibodies: anti-TLR3 (1:50; IMG516; Imgenex, San Diego, CA), anti–ERHR-3 (1:50; monocytes/macrophages; DPC Biermann, Bad Nauheim, Germany), anti-CD3 (1:100; BD), anti–smooth muscle actin (1:100; myofibroblasts, clone 1A4; Dako, Carpinteria, CA), anti-collagen I (LF-67, 1:50; provided by Dr. L.W. Fischer, National Institute of Dental Research, National Institutes of Health, Bethesda, MD), anti-CCL5 (1:50; Peprotech, Rocky Hill, NJ), anti-CCL2 (1:50; Santa Cruz Biotechnology, Santa Cruz, CA), and anti-IgG and anti-IgG_{2a} (Dianova; 1:100). Negative controls included incubation with a respective isotype antibody. For quantitative analysis, glomerular cells were counted in 10 cortical glomeruli per section. Semiquantitative scoring of glomerular Ig deposits from 0 to 3+ was performed on 15 cortical glomerular sections as described (10).

Cell Culture Conditions

Bone marrow from $MRL^{lpr/lpr}$ mice was isolated, processed, and cultured using published methods (17). For selecting for dendritic cells, bone marrow isolates were cultured for 8 d in RPMI 1640 medium supplemented with 10% FCS and 100 units/ml penicillin, 100 μg/ml streptomycin (Biochrom KG, Berlin, Germany), and 100 ng/ml human recombinant Flt3 ligand (Immunotools, Friesoyth, Germany). Adherent spleen monocytes were isolated from spleens of 18-wk-old $MRL^{lpr/lpr}$ mice as described previously (10). Spleen monocytes were treated with medium control or 100 μg/ml pI:C RNA and pI:C DNA after 24 h. TLR9 ligand CpG-ODN no. 1668 (10) was used as a control in selected cases. After a period of 24 h, culture supernatants were collected for cytokine measurements and cells were harvested for flow cytometric analysis. Dendritic cells and spleen monocytes were stimulated as above for 24 h, and cells were harvested for RNA isolation as described previously (10). A murine mesangial cell line was maintained in DMEM (Biochrom KG) supplemented with 5% bovine serum (Serum Supreme; BioWhittaker, Walkersville, MD) and 1% penicillin-streptomycin 100 U/ml and 100 μg/ml as described (18). All cell types were incubated for 24 h without serum supplements before stimulation.

Cytokine ELISA and Griess Assay

Cytokine levels in sera or cell culture supernatants were determined using commercial ELISA kits: IL-6, IL-12p70, CCL2 (all OptEiA, BD Pharmingen), IFN-α (R&D Systems, Minneapolis, MN), and CCL5 (Duoset; R&D Systems) following the protocol of the manufacturers. The Griess reagent (Sigma) was used for the determination of nitrite in cell supernatants as a marker of nitric oxide (NO) production.

Flow Cytometry

Flow cytometry of cultured cells or splenocytes was performed as described previously (10). Surface staining was performed using PE- or FITC-labeled rat anti-CD11c, anti–MHC II, anti-CD86, or anti-CD19 antibodies (BD Biosciences). Anti-TLR3 antibody (1:50) was used to detect TLR3 on mesangial cells through biotinylated rabbit anti-mouse IgG antibody and streptavidin-APC (Pharmingen). A rabbit IgG (BD Pharmingen) was used as isotype control. For intracellular staining, cells were fixed with 1% paraformaldehyde and permeabilized with permeabilization buffer (PBS, 0.5% BSA, 0.5% saponin) at room temperature. FACS analysis was conducted using a FACScalibur machine and CellQuest software (BD).

Real-Time Quantitative (TaqMan) Reverse Transcription–PCR

Real-time reverse transcription–PCR (RT-PCR) on RNA isolated from cultured cells or renal tissue was performed as described previously (10). Controls that consisted of ddH_2O were negative for target and housekeeper genes. Oligonucleotide primer (300 nM) and probes (100 nM) were from Applied Biosystems (Darmstadt, Germany) and used as described: Murine CCL5 (9); TLR3 accession number AF355152, for-

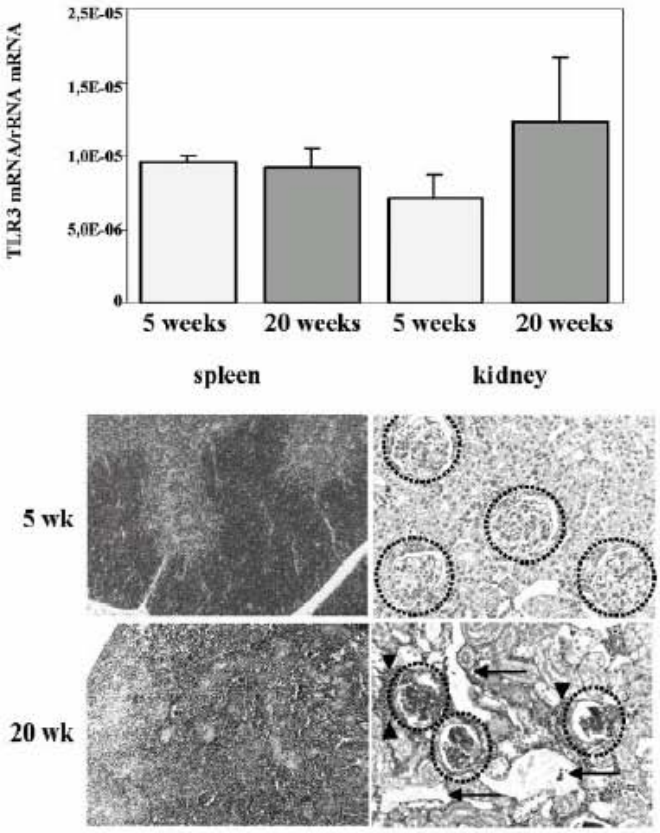

Figure 1. Toll-like receptor 3 (TLR3) mRNA expression in MRL$^{lpr/lpr}$ mice. Expression of TLR3 mRNA was assessed by real-time reverse transcription–PCR (RT-PCR) in duplicates using RNA isolated from spleens and kidneys from seven MRL$^{lpr/lpr}$ mice each at 5 and 20 wk of age as described in the Materials and Methods section. TLR3 mRNA expression is expressed as a ratio to the respective 18S rRNA mRNA expression ± SEM (5 *versus* 20 wk; $P > 0.05$). At 5 wk, spleens and kidneys did not show structural abnormalities. Glomeruli (encircled) show a regular capillary network and mesangium. By contrast, at 20 wk, spleens showed major structural alterations secondary to lymphoproliferative disease indicated by the malformation of spleen lymph follicles. At this point, kidneys showed mesangioproliferative glomerulonephritis (glomeruli encircled) with periglomerular inflammatory cell infiltrates (arrowheads) and tubular atrophy (arrows). Magnification, ×400 periodic acid-Schiff.

ward primer 5′-CGAAAGTTGGACTTGTCATCAAATC-3′, reverse primer 5′-ACTTGCCAATTGTCTGG-AAACAC-3′, internal fluorescence probe 5′-CACTTAAAGAGTTCTCCC-3′. Primers and probes for murine CCL2, CCL5, and 18S rRNA were obtained as predeveloped assay reagents from PE Biosystems (Weiterstadt, Germany).

Statistical Analyses

Data were expressed as mean ± SEM. Comparison between groups was performed using unpaired two-tailed *t* test. $P < 0.05$ was considered to indicate statistical significance.

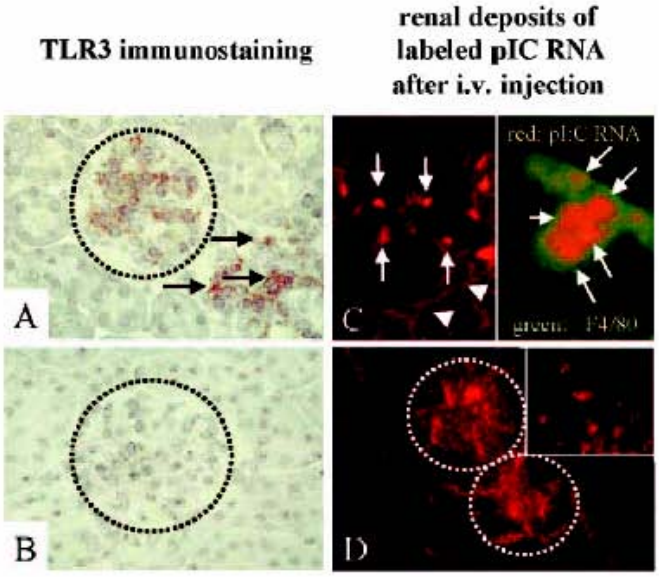

Figure 2. TLR3 in kidneys of MRL$^{lpr/lpr}$ mice. (A) Immunostaining for TLR3 was performed as described in the Materials and Methods section. Positive staining was found in inflammatory cell infiltrates (arrows) and in glomeruli in a mesangial staining pattern (glomerulus encircled). (B) Negative control staining. (C and D) Rhodamine-labeled polyinosinic-cytidylic acid (pI:C) RNA was injected intravenously into four 16-wk-old MRL$^{lpr/lpr}$ mice, and renal tissue was harvested 2 h later. Fluorescence imaging of frozen sections showed uptake of labeled pI:C RNA in interstitial cells (arrows in left image of C) and in mesangial cells in glomeruli (encircled and at higher magnification in insert of D), consistent with the staining pattern for TLR3. Co-staining with a FITC-labeled F4/80 antibody identified pI:C RNA–positive interstitial cells to be antigen-presenting cells of the monocytic cell lineage and illustrates the uptake of rhodamine-labeled pI:C RNA in intracellular endosomes (arrows indicating individual endosomes in right image of C). Magnification, ×400.

Results

Expression of TLR3 in Lupus Nephritis of MRL$^{lpr/lpr}$ Mice

We first determined the expression pattern of TLR3 in kidneys and spleens of MRL$^{lpr/lpr}$ mice and analyzed TLR3 mRNA expression levels at an early (week 5) and late (week 20) stage of autoimmune disease using real-time RT-PCR. At 5 wk of age, no structural abnormalities were detected in kidney and spleen as observed using light microscopy. At this time point, expression levels of TLR3 mRNA in kidneys were comparable to that in spleen, indicating that TLR3 is expressed by intrinsic renal in addition to the few resident immune cells present in kidneys of 5-wk-old MRL$^{lpr/lpr}$ mice (Figure 1). At 20 wk, spleens demonstrated major structural abnormalities, reflecting the typical lymphoproliferative syndrome of MRL$^{lpr/lpr}$ mice. At this time point, kidneys of MRL$^{lpr/lpr}$ mice revealed severe damage, including crescentic glomerulonephritis and tubulointerstitial injury with interstitial and perivascular inflammatory cell infiltrates. It is interesting that at 20 wk, spleen TLR3 mRNA levels were unchanged compared with week 5. Proliferative lupus

nephritis was associated with somewhat increased renal TLR3 mRNA expression, but there was no statistical difference as compared with week 5 (Figure 1). To localize the source of renal TLR3 mRNA expression, we performed immunostaining using a polyclonal antibody specific for murine TLR3. Renal sections of 16-wk-old MRL$^{lpr/lpr}$ mice revealed positive signals in glomerular mesangial cells but not in glomerular endothelial cells or podocytes (Figure 2). Mesangial cell staining for TLR3 appeared in a speckled pattern, indicating that TLR3 is also localized in an intracellular compartment. Mononuclear inflammatory cell infiltrates were also positive for TLR3 (Figure 2). The TLR3 staining pattern in nephritic MRL$^{lpr/lpr}$ mice is consistent with TLR3 expression on immunostaining in human renal biopsies (unpublished observation).

Localization of Labeled Viral dsRNA after Intravenous Injection in MRL$^{lpr/lpr}$ Mice

For examining whether circulating viral dsRNA is taken up by TLR3-positive cells *in vivo*, rhodamine-labeled viral dsRNA was injected intravenously into 16-wk-old MRL$^{lpr/lpr}$ mice. Consistent with TLR3 immunostaining in the kidney, the labeled viral dsRNA was found in speckled glomerular mesangial cell staining pattern, suggesting that injected viral dsRNA was taken up by mesangial cells into an intracellular vesicular compartment (Figure 2). Labeled single-stranded RNA did not show such a mesangial staining pattern (unpublished observation), indicating that mesangial cells take up dsRNA by a specific mechanism. Infiltrating cells showed strong granular intracellular signals for labeled viral dsRNA. Double labeling with an F4/80-specific antibody identified these cells as antigen-presenting cells of the monocyte-macrophage lineage (Figure 2). Rhodamine injected alone in MRL$^{lpr/lpr}$ mice was not found to localize in the kidney (data not shown). Analysis of spleen sections revealed viral dsRNA signals only in F4/80-positive antigen-presenting cells but not in B or T cell areas of the spleen (data not shown). Taken together, in the kidneys of MRL$^{lpr/lpr}$ mice, injected viral dsRNA co-localizes in an intracellular granular pattern with TLR3-positive cells, *i.e.*, infiltrating mononuclear cells, but also with intrinsic renal cells predominantly in glomerular mesangial cells in an intracellular vesicular compartment.

Cultured Mesangial Cells Express TLR3 and Secrete CCL2 and IL-6 upon Stimulation with pI:C RNA

To confirm the TLR3 expression by mesangial cells, we performed flow cytometry on an established murine mesangial cell line. Under basal culture conditions, TLR3 expression was detected intracellularly after cell permeabilization, whereas only a little surface staining was detected (Figure 3A). To test the functionality of TLR3 on mesangial cells, we examined whether the TLR3 ligand pI:C RNA can induce cytokine and chemokine secretion. Stimulation with increasing concentrations of pI:C RNA induced IL-6 and CCL2 secretion in a concentration-dependent manner (Figure 3B). In contrast, pI:C DNA or CpG-DNA had no effect on IL-6 or CCL2 production. Together, these data indicate that mesangial cells express TLR3 and produce

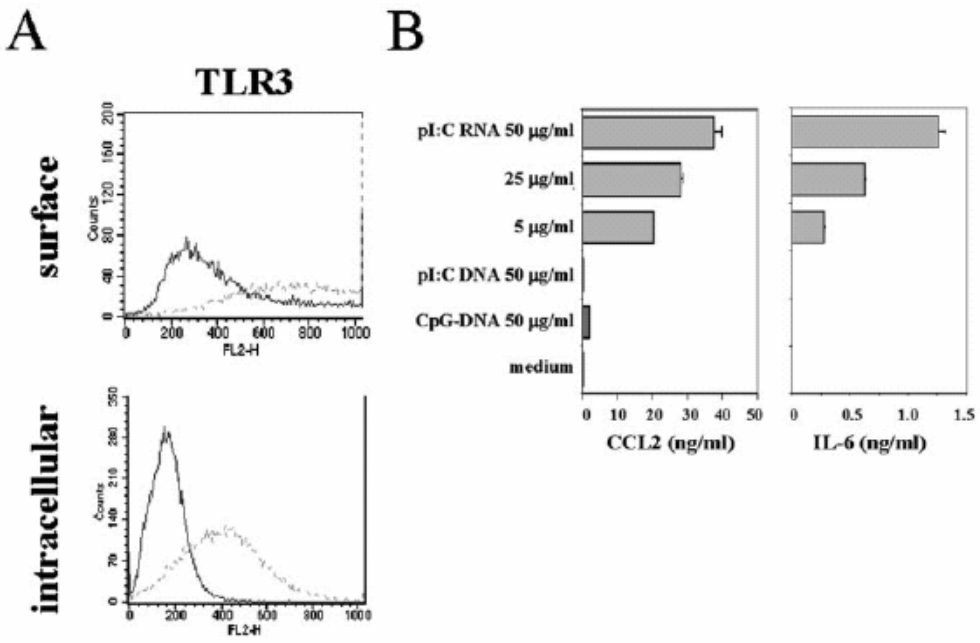

Figure 3. pI:C RNA/TLR3 interaction in cultured mesangial cells. Murine mesangial cells were cultured as described in the Materials and Methods section. (A) Flow cytometry for TLR3 before and after permeabilization for intracellular staining was performed as indicated. Expression of TLR3 (dotted line) is demonstrated by a fluorescence shift compared with the isotype control antibody (dark line). (B) Cultured cells were incubated with various concentrations of pI:C RNA, pI:C DNA, or CpG-DNA or standard medium without supplements for 24 h as indicated. IL-6 and CCL2 production was measured in supernatants by ELISA. Results shown are means ± SEM from two comparable experiments each performed in duplicate.

proinflammatory cytokines (*e.g.*, IL-6) and CC-chemokines (*e.g.*, CCL2) upon exposure to pI:C RNA *in vitro*.

pI:C RNA Induces the Production of Proinflammatory Mediators in Antigen-Presenting Cells from MRL$^{lpr/lpr}$ Mice

As TLR3 staining and uptake of labeled pI:C RNA in kidneys of MRL$^{lpr/lpr}$ mice also occurred in infiltrating mononuclear cells, we isolated spleen monocytes from MRL$^{lpr/lpr}$ mice. The cells were incubated with pI:C RNA, pI:C DNA, CpG-DNA, or medium for 24 h. Spleen monocytes showed a concentration-dependent increase in IL-12p70 and IL-6 release after exposure to pI:C RNA but not after exposure to pI:C DNA or CpG-DNA (Figure 4B). In addition, we determined other markers of monocyte activation such as the production of NO or the chemokine CCL5, two molecules that can mediate tissue injury in SLE. pI:C RNA markedly induced CCL5 mRNA expression and NO production by spleen monocytes of MRL$^{lpr/lpr}$ mice compared with stimulation with pI:C DNA and CpG-DNA (Figure 4A, 4B, and 4C).

Next we prepared bone marrow–derived dendritic cells (BMDC) of MRL$^{lpr/lpr}$ mice. BMDC were incubated with pI:C RNA, pI:C DNA, or medium for 24 h. Flow cytometric analysis for CD86 and MHC II on CD11c-positive BMDC showed a marked increase in the surface expression of both molecules with pI:C RNA, indicating BMDC maturation, which was absent with pI:C DNA (Figure 5A). Furthermore, pI:C RNA but not pI:C DNA stimulated the secretion of IL-12p70, IL-6, and IFN-α as determined by ELISA in supernatants of dendritic cells (Figure 5B). These data indicate that pI:C RNA induces the production of proinflammatory mediators such as IL-12p70, IL-6, CCL5, and NO production in spleen monocytes and IL-12p70, IL-6, and IFN-α in BMDC of MRL$^{lpr/lpr}$ mice.

pI:C RNA Injection Increases Serum IL-6, IL-12p70, and IFN-α Levels in MRL$^{lpr/lpr}$ Mice

Having demonstrated the effect of pI:C RNA on IL-6, IL-12p70, and IFN-α secretion in dendritic cells and macrophages isolated from MRL$^{lpr/lpr}$ mice *in vitro*, we studied serum levels of these factors 6 h after intraperitoneal injection of 50 μg of pI:C RNA, 50 μg of pI:C DNA, or saline into 16-wk-old MRL$^{lpr/lpr}$ mice. Injection of pI:C RNA caused an increase of serum levels of IL-12p70, IL-6, and IFN-α as compared with saline or pI:C DNA in MRL$^{lpr/lpr}$ mice (Figure 6).

pI:C RNA Aggravates Renal Damage and Proteinuria in MRL$^{lpr/lpr}$ Mice

From the above results, one would predict that exposure to pI:C RNA would aggravate tissue injury in autoimmune MRL$^{lpr/lpr}$ mice. We therefore treated groups of lupus mice with intraperitoneal injections of either 50 μg of pI:C RNA or pI:C DNA or saline on alternate days from weeks 16 to 18 of age. Saline-treated MRL$^{lpr/lpr}$ mice had diffuse proliferative glomerulonephritis with moderate mesangial hypercellularity, increase of mesangial matrix, and little periglomerular inflammatory cell infiltrates at week 18. pI:C DNA injections did not alter these histopathologic findings (Figure 7). By contrast, pI:C RNA injections induced focal segmental necrosis in glomeruli and cellular crescent formation

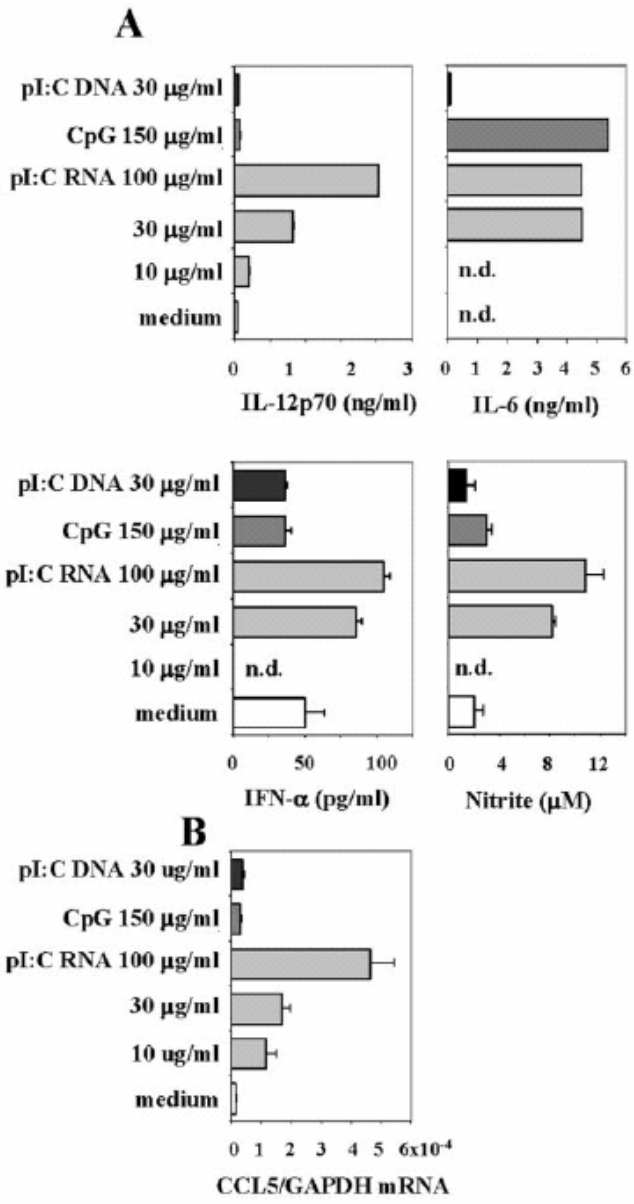

Figure 4. pI:C RNA activates spleen monocytes isolated from MRL$^{lpr/lpr}$ mice. (A) Monocytes were prepared from spleens of MRL$^{lpr/lpr}$ mice and incubated with various concentrations of pI:C RNA, pI:C DNA, or CpG-DNA or standard medium without supplements for 24 h as indicated. IL-12p70 and IFN-α production were measured in supernatants by ELISA. Results shown are from one of three comparable experiments. n.d., not done. (B) Spleen monocytes of MRL$^{lpr/lpr}$ mice were stimulated for 12 h as above. CCL5 mRNA expression was analyzed by real-time RT-PCR as described in the Materials and Methods section. Values are expressed as CCL5 mRNA expression in relation to respective glyceraldehyde-3-phosphate dehydrogenase (GAPDH mRNA) expression ± SEM. Results shown are means ± SEM from one of three comparable experiments, each performed in duplicate. (C) Spleen monocytes of MRL$^{lpr/lpr}$ mice were stimulated for 12 h as above. Nitric oxide (NO) production was assessed by measuring nitrite concentrations in supernatants after 24 h using the Griess assay as described in the Materials and Methods section. Results shown are means ± SEM from three comparable experiments, each performed in duplicate.

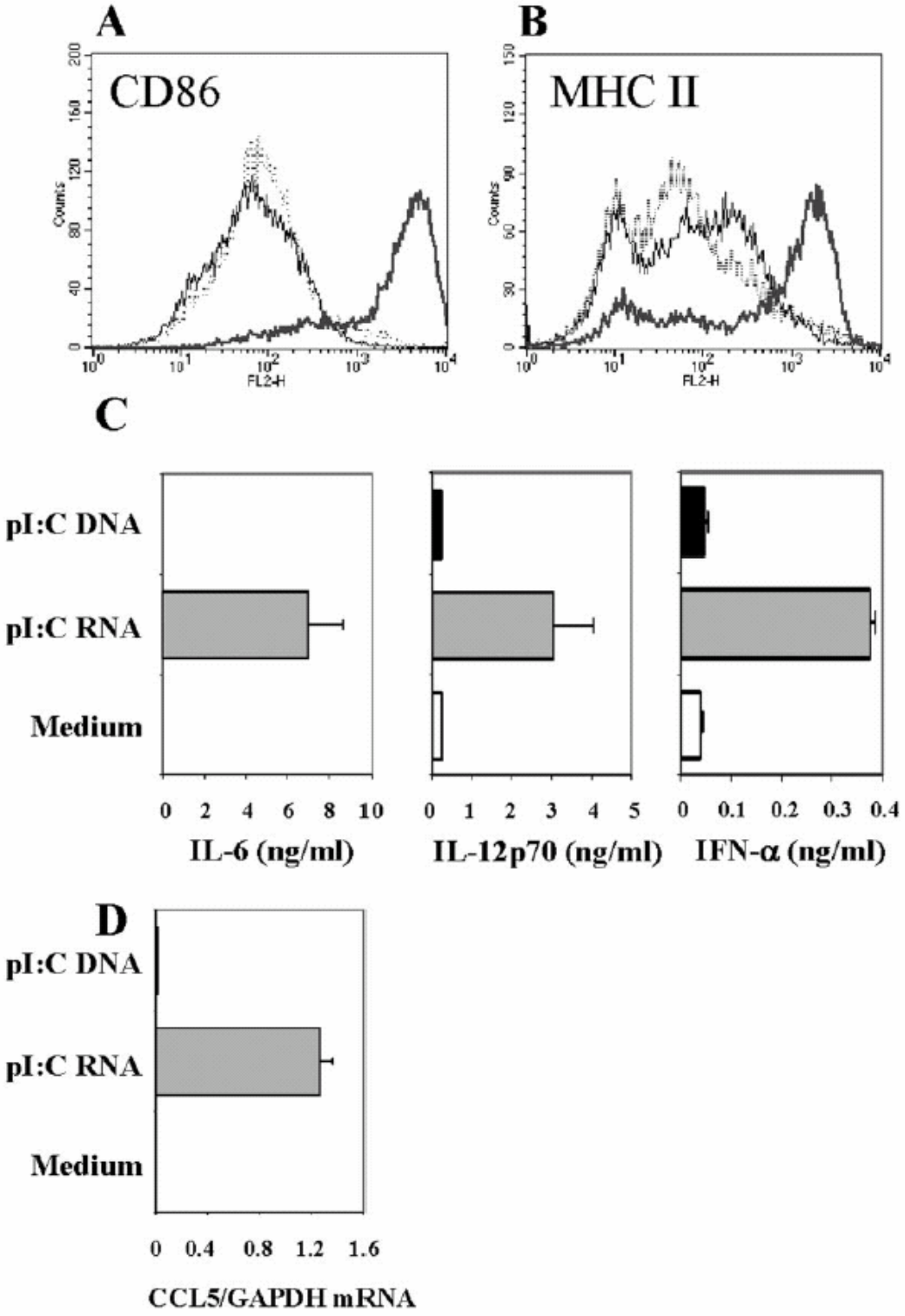

Figure 5. pI:C RNA activates dendritic cells isolated from MRL$^{lpr/lpr}$ mice. (A and B) Flow cytometry of CD11c-positive bone marrow–derived dendritic cells for CD86 (A) and MHC II (B) was performed as described in the Materials and Methods section. Cells were incubated with either pI:C RNA (bold dark line) or pI:C DNA (dotted line) before analysis. Induction of CD86 and MCH II surface expression in pI:C RNA–treated dendritic cells is indicated by a fluorescence shift compared with the isotype control antibody (thin black line). (C) Cultured cells were treated as above for 24 h, and IL-12p70, IL-6, and IFN-α production was assessed by ELISA in culture supernatants. Results shown are means ± SEM from two comparable experiments, each performed in duplicate. (D) Dendritic cells of MRL$^{lpr/lpr}$ mice were stimulated for 12 h as above. CCL5 mRNA expression was analyzed by real-time RT-PCR as described in the Materials and Methods section. Values are expressed as CCL5 mRNA expression in relation to respective GAPDH mRNA expression ± SEM. Results shown are two comparable experiments, each performed in duplicate.

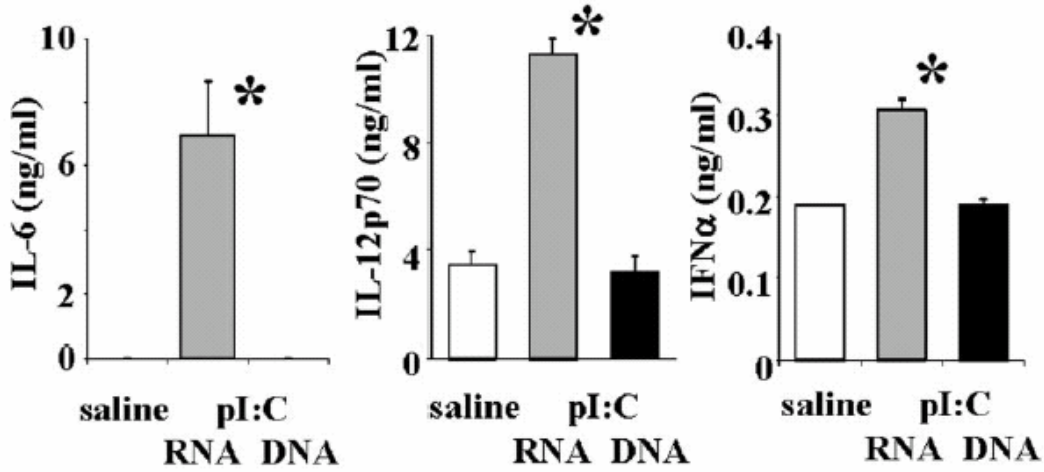

Figure 6. Serum IL-6, IL-12p70, and IFN-α levels in MRL$^{lpr/lpr}$ mice. Serum was obtained from five to eight 16-wk-old MRL$^{lpr/lpr}$ mice 6 h after the first intraperitoneal injection of saline, 50 μg of pI:C RNA, or 50 μg of pI:C DNA as indicated. Serum IL-12p70 and IFN-α levels were determined by ELISA. Data are means ± SEM. *$P < 0.05$ *versus* saline.

associated with marked periglomerular inflammatory cell infiltrates (Figure 7). Occasionally, mesangiolysis with aneurysms of corresponding glomerular capillaries became apparent, and glomeruli with segmental sclerosis were seen more often (Figure 7). Aggravation of renal disease was illustrated by an increase in the activity and chronicity scores of the lupus nephritis in pI:C RNA-treated MRL$^{lpr/lpr}$ mice as compared with the other groups of mice (Table 1). pI:C RNA increased the amount of glomerular ERHR-3–positive macrophages and CD3-positive lymphocytes as compared with pI:C-DNA– and saline-injected controls (Table 1). There was a trend toward increased proteinuria levels in pI:C RNA–treated mice, but this did not reach statistical significance (Table 1). In addition to the aggravation of glomerular damage, pI:C RNA injections induced tubulointerstitial damage and fibrosis. Infiltrating ERHR-3 macrophages and CD3 lymphocytes accumulated particularly in periglomerular fields and areas around glomerular crescents (Figure 7, Table 1). To assess the extent of interstitial injury, we performed immunostaining for smooth muscle actin–positive interstitial myofibroblasts and for interstitial collagen I deposits. Both were significantly increased in kidneys of pI:C RNA–treated mice (Figure 7, Table 1). Taken together, exposure to pI:C RNA markedly aggravated the glomerulonephritis of MRL$^{lpr/lpr}$ mice toward a crescentic glomerulonephritis with mesangiolysis associated with marked tubulointerstitial injury.

pI:C RNA Induced Renal CCL2 and CCL5 mRNA Expression in MRL$^{lpr/lpr}$ Mice

On the basis of our *in vitro* studies with macrophages, dendritic cells, and mesangial cells, we hypothesized that pI:C RNA would trigger local chemokine expression in nephritic kidneys of MRL$^{lpr/lpr}$ mice. In fact, kidneys of pI:C RNA–treated MRL$^{lpr/lpr}$ mice showed increased mRNA expression levels for CCL2 and CCL5 (Figure 8A). To localize renal CCL2 and CCL5 protein, we performed immunostaining for both chemokines (Figure 8B). At 18 wk, single spots of CCL5 and CCL2 protein were noted within the glomerular tuft and along Bowman's capsule of some glomeruli, as well as in focal interstitial areas of saline- and pI:C DNA–treated MRL$^{lpr/lpr}$ mice kidneys. By contrast, pI:C RNA–treated MRL$^{lpr/lpr}$ mice kidneys showed marked CCL2 and CCL5 staining that co-localized with interstitial leukocytic cell infiltrates and glomerular crescents (Figure 8). Thus, intermittent exposure to pI:C RNA increased local expression of CCL2 and CCL5 in areas of pronounced inflammatory cell infiltrates and tissue damage in nephritic kidneys of MRL$^{lpr/lpr}$ mice.

pI:C RNA Does Not Cause B Cell Activation and DNA Autoantibody Production in MRL$^{lpr/lpr}$ Mice

We previously observed that the activation of TLR9 by bacterial CpG-DNA aggravated lupus nephritis in MRL$^{lpr/lpr}$ mice in association with enhanced DNA autoantibody production and glomerular immune complex deposits (10). The predominance of IgG_{2a} autoantibody and renal IgG deposits of the IgG_{2a} isotype suggested a predominant Th1 response induced by bacterial CpG-DNA. We therefore investigated the effects of pI:C RNA or pI:C DNA on serum anti-DNA antibody titers and glomerular IgG deposits in MRL$^{lpr/lpr}$ mice. In the present study, all groups irrespective of treatment had comparable titers of total serum DNA IgG or IgG_{2a} autoantibodies (Table 1). Consistent with these results, capillary and mesangial deposits of total IgG and of IgG_{2a} in glomeruli were comparable in all groups (Table 1). We thought that this discrepancy of viral pI:C RNA and bacterial CpG-DNA might be related to the different expression of the respective TLR on B cells, as in patients with SLE and in MRL$^{lpr/lpr}$ mice DNA autoantibody production is linked to the activation and proliferation of B cells. Human and murine B cells lack TLR3 expression, but B cell activation might occur indirectly through pI:C RNA–induced activation of dendritic cells and T cells. We therefore determined MHC II expression on spleen B cells by flow cytometry 24 h after intraperitoneal injection of 500 μg of pI:C RNA, pI:C DNA, or CpG-DNA into MRL$^{lpr/lpr}$ mice

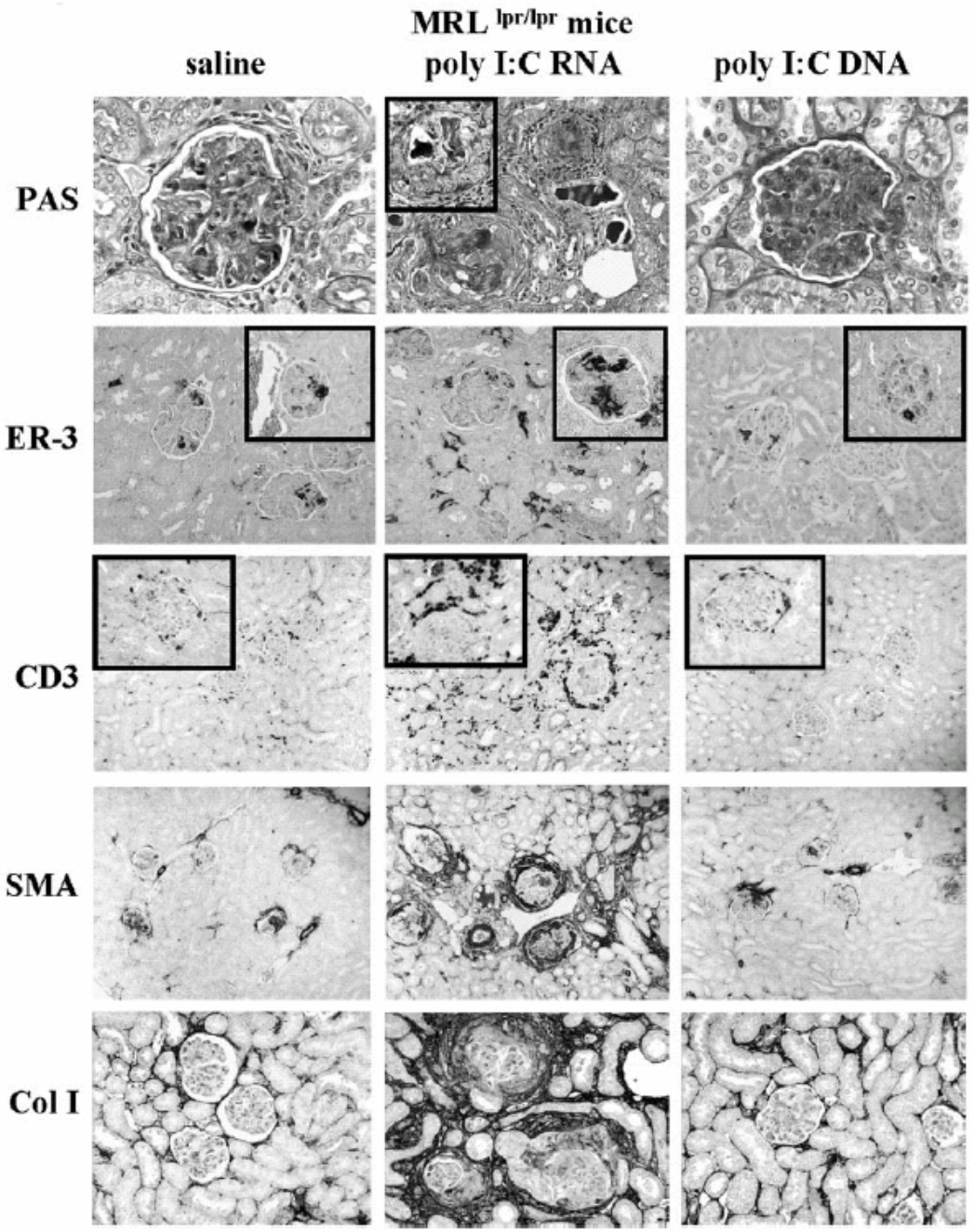

Figure 7. Renal histopathology. Renal sections of 18-wk-old MRL$^{lpr/lpr}$ mice from all groups were stained with periodic acid-Schiff (PAS) and antibodies for ERHR-3 (ER-3, macrophages), CD3 (lymphocytes), SMA (smooth muscle antigen for myofibroblasts), and collagen I as indicated. Insert in PAS-stained sections of pI:C RNA–treated MRL$^{lpr/lpr}$ mice illustrates necrosis and aneurysmal formation of glomerular capillaries that was not detected in mice of the other groups (Magnification, ×400). Inserts in ERHR-3– and CD3-stained sections show respective glomeruli at a magnification of ×530. Images are representative for eight to 10 mice in each group.

(Figure 9). In contrast to CpG-DNA, neither pI:C RNA nor pI:C DNA induced MHC II expression on CD19-positive spleen B cells, indicating that pI:C RNA does not activate B cells in MRL$^{lpr/lpr}$ mice. Together with our previous findings (10), these data demonstrate that bacterial CpG-DNA but not dsRNA induces B cell activation and DNA autoantibody production in autoimmune MRL$^{lpr/lpr}$ mice.

Discussion

Viral infections can aggravate disease activity in preexisting SLE, but the role of viral RNA in this context is hypothetical. We used the model of spontaneous immune complex-glomerulonephritis in MRL$^{lpr/lpr}$ mice to study the effects of intermittent exposure to pI:C RNA, a structural analog to viral dsRNA.

Table 1. Serum, urinary, and histologic findings in $MRL^{lpr/lpr}$ mice[a]

	Saline	pI:C DNA	pI:C RNA
Functional parameters			
proteinuria (μg/mg creatinine)	3118 ± 903	2646 ± 694	10405 ± 12173
Histologic scores			
activity index	8.1 ± 2.8	6.6 ± 4.2	15.6 ± 4.2[b]
chronicity index	1.8 ± 1.0	1.3 ± 0.3	5.4 ± 2.1[b]
Cellular response (cells/glomerulus or hpf)			
glomerular EHR3+ (cells/glomerulus)	1.7 ± 0.7	1.9 ± 1.5	4.4 ± 1.6[b]
CD3+ (cells/glomerulus)	1.4 ± 0.4	1.5 ± 0.8	2.2 ± 0.5[b]
interstitial EHR3+ (cells/hpf)	6.0 ± 2.9	7.2 ± 5.3	15.5 ± 4.7[b]
CD3+ (cells/hpf)	13.7 ± 3.3	17.0 ± 6.4	35.1 ± 10.2[b]
SMA+ (% hpf)	5.0 ± 0.8	4.2 ± 3.9	11.0 ± 1.2[b]
interstitial collagen (% hpf)	2.9 ± 1.5	3.1 ± 1.7	8.4 ± 4.8[b]
Humoral response			
serum titers			
anti-DNA IgG	14231 ± 3519	16603 ± 3089	17718 ± 3375
anti-DNA IgG_{2a}	10667 ± 3556	9000 ± 3500	7556 ± 3951
glomerular deposit score			
IgG	1.2 ± 0.2	1.3 ± 0.4	1.4 ± 0.2
IgG_{2a}	0.3 ± 0.2	0.4 ± 0.3	0.6 ± 0.3

[a]Values are means ± SEM from eight to 10 mice per group. pI:C, polyinosinic-cytidylic acid; hpf, high-power field.
[b]$P < 0.05$ pI:C RNA *versus* pI:C DNA.

We examined both DNA autoantibody-dependent and -independent mechanisms in aggravating lupus nephritis by pI:C RNA. For the first time, we provide evidence for expression of TLR3 on glomerular mesangial cells and for the ability of pI:C RNA to induce proinflammatory cytokine and chemokine production in these cells. The process of aggravation of autoimmune renal injury in $MRL^{lpr/lpr}$ mice that were exposed to circulating pI:C RNA is further facilitated through activation of infiltrating immune cells. It is interesting that the pI:C RNA–induced aggravation of lupus nephritis in $MRL^{lpr/lpr}$ mice is independent of B cell activation and DNA autoantibody production, which may relate to the specific expression profile of TLR3. In mice, TLR3 expression is restricted to antigen-presenting cells of the monocytic cell lineage, *e.g.*, dendritic cells and macrophages, as well as to glomerular mesangial cells, which may play an important role in mediating viral infection–induced exacerbation of lupus nephritis as well as other forms of glomerulonephritis.

Viral dsRNA Activates Mesangial Cells in Experimental Lupus

Antiviral host defense requires activation of innate immunity, including the local production of type I interferons and chemokines (19,20). The finding that injected dsRNA localized to TLR3-positive antigen-presenting cells of the monocytic cell lineage and to glomerular mesangial cells in kidneys of $MRL^{lpr/lpr}$ mice suggests a role for TLR3 in local immune responses in the kidney induced by viral dsRNA. In fact, in kidneys of $MRL^{lpr/lpr}$ mice, immunostaining for TLR3 localized to glomerular mesangial cells. This is consistent with unexpected high levels of TLR3 mRNA in kidneys of these mice, which is consistent with previously published data from healthy murine and human kidneys (21,22). The speckled staining pattern for TLR3 in mesangial cells of nephritic glomeruli of $MRL^{lpr/lpr}$ mice is also consistent with the uptake of injected and rhodamine-labeled viral RNA into an intracellular vesicular compartment of glomerular mesangial cells of diseased kidneys of $MRL^{lpr/lpr}$ mice. The expression of a functional TLR3 in mesangial cells represents a new finding. Mesangial cells do not express other nucleic acid–specific TLR (TLR7, TLR8, and TLR9) (9 and unpublished observation). Our studies with a murine mesangial cell line confirmed the intracellular localization of TLR3 analogous to its localization in antigen-presenting cells (23). Furthermore, we detected some surface expression of TLR3 that has also been observed in other nonimmune cell types (*e.g.*, human fibroblasts, intestinal epithelial cells) (24,25). When exposed to pI:C RNA, mesangial cells express large amounts of IL-6 and CCL2, two proinflammatory mediators that are well known to be involved in progression of glomerulonephritis in murine disease models and in human glomerular diseases (9,26). From these *in vitro* studies, we expected an increase of local expression of proinflammatory chemokines in kidneys of $MRL^{lpr/lpr}$ mice, a prediction confirmed by real-time RT-PCR of total renal isolates and by immunostaining for CCL2 and CCL5 on renal sections of pI:C RNA–treated $MRL^{lpr/lpr}$ mice. pI:C RNA–induced local production of CCL2 and CCL5 is

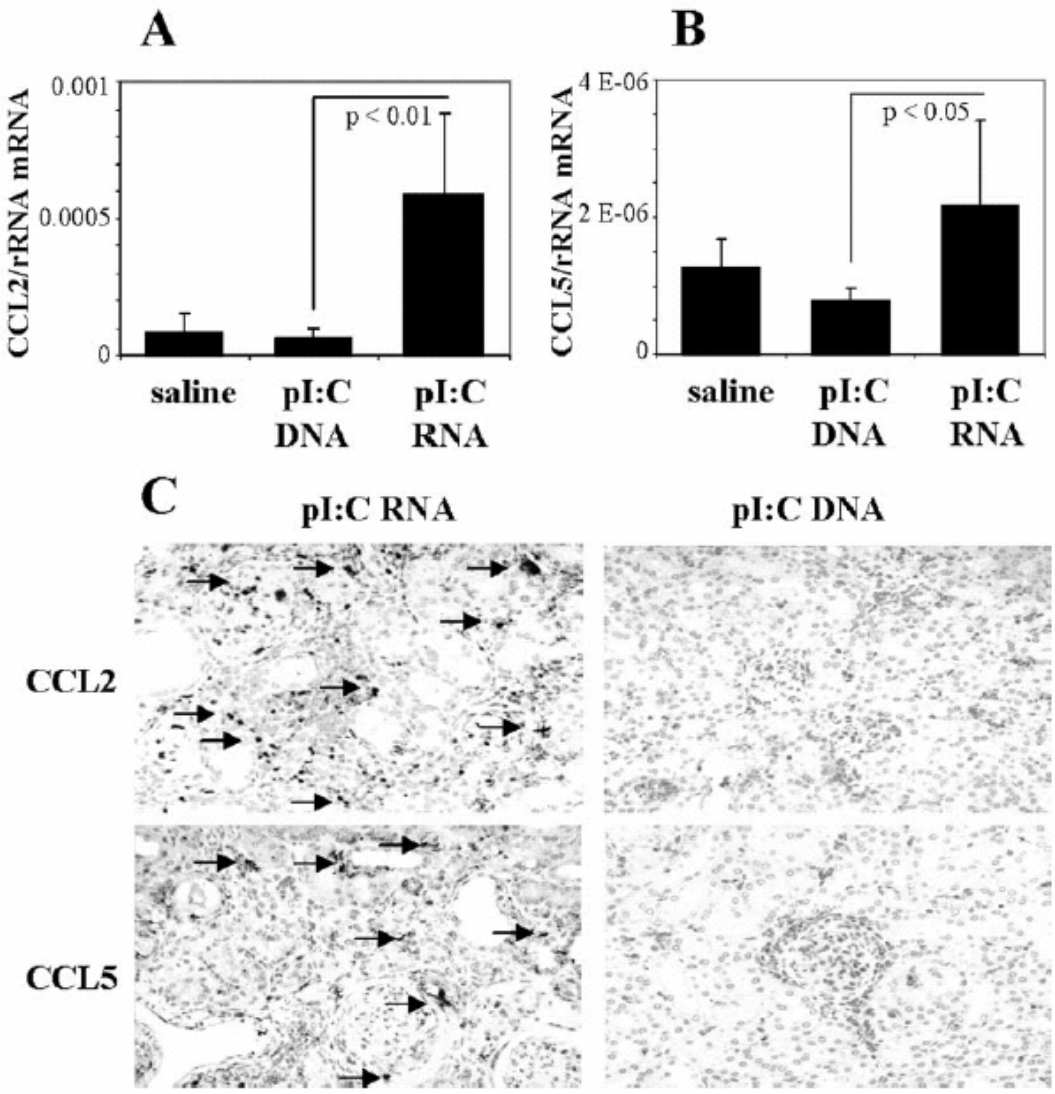

Figure 8. Renal expression of CC-chemokines. The mRNA expression of CCL2 (A) and CCL5 (B) in kidneys of 18-wk-old MRL$^{lpr/lpr}$ mice was determined by real-time RT-PCR using total renal RNA. mRNA levels for saline-, pI:C DNA–, and pI:C RNA–treated MRL$^{lpr/lpr}$ mice are expressed in relation to the respective 18S rRNA expression of each kidney. Results are means ± SEM from three to five mice in each group. (C) Immunostaining for CCL2 and CCL5 was performed as described in the Materials and Methods section. At 18 wk, CCL5 was stained in occasional cells of the glomerular tuft and along Bowman's capsule of glomeruli as well as in focal interstitial areas of saline- (not shown) or pI:C DNA–treated MRL$^{lpr/lpr}$ mice. Injection of pI:C RNA markedly increased CCL5 expression in spatial association with increasing interstitial leukocytic cells and cellular crescents. CCL2 was stained in occasional glomerular cells and proximal tubular cells of saline- and pI:C DNA–treated MRL$^{lpr/lpr}$ mice. Injection of pI:C RNA increased interstitial CCL2 staining. Arrows indicate chemokine-positive cells. Sections stained with an isotype control antibody showed no signal (not shown).

likely to be involved in the observed aggravation of lupus nephritis in MRL$^{lpr/lpr}$ mice as both CC-chemokines contribute to the progression of lupus nephritis by mediating renal leukocyte recruitment in this disease model (27,28) as well as in human lupus nephritis (29,30). When leukocytes migrate to renal lesions, they by themselves become a major source of proinflammatory cytokines and chemokines (31). In fact, we found that cells of the monocytic cell lineage that are already located in the kidney take up circulating pI:C RNA. Our studies with spleen monocytes isolated from MRL$^{lpr/lpr}$ mice support the idea that uptake of pI:C RNA by tissue macrophages contributes to the local production of proinflammatory mediators, including NO, IL-12p70, IL-6, IFN-α, and CCL5.

Taken together, circulating viral dsRNA, *e.g.*, pI:C RNA, is taken up by renal macrophages, dendritic cells, and mesangial cells that express TLR3 in intracellular endosomes. Ligation of TLR3 activates these cell types to secrete proinflammatory mediators, including type I interferons, cytokines, and chemokines, which promote local tissue injury. This innate immune mechanism in response to viral dsRNA is detrimental in preexisting renal inflammation such as lupus nephritis.

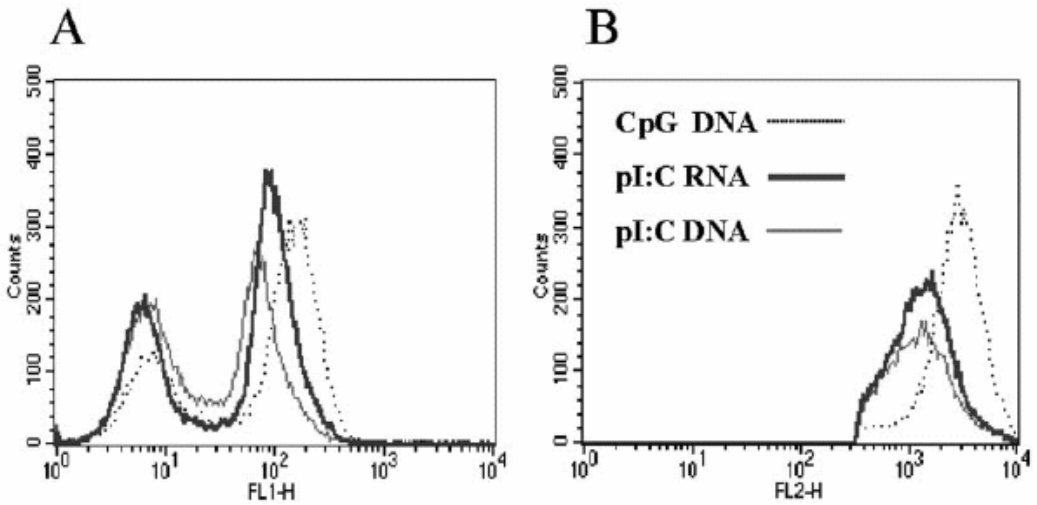

Figure 9. B cell activation in MRL$^{lpr/lpr}$ mice. Sixteen-week-old mice received an injection of 500 μg of pI:C RNA, CpG-DNA, or pI:C-DNA. After 24 h, flow cytometry for MHC II and CD19 was performed on spleen cell suspensions. Staining spleen cell suspensions for MHC II produces two peaks. Left peak represents MHC II–negative T cells showing no change in number but became a smaller proportion of the total population in CpG-ODN–injected mice as compared with pI:C RNA– and pI:C DNA–treated controls. The right peak represents MHC II–positive antigen-presenting cells including B cells (A). When gated for the CD19/MHC II double-positive cell population, only the B cells of the right peak remain (B). B cell activation is indicated by an increase in MHC II expression on CD19-positive B cells by CpG-DNA (dotted line) but not by pI:C RNA (thick line) or pI:C DNA (thin line). Images are representative for three mice in each group.

Viral dsRNA Activates Dendritic Cells but Does Not Elicit a B Cell Response in Experimental Lupus

A central role for dendritic cells for infection-associated exacerbation of autoimmunity is suspected for three reasons: (*1*) in mice and humans, dendritic cells show constitutive expression of most TLR in mice and humans (11,13); (*2*) *ex vivo* exposure of dendritic cells to the TLR4 ligand LPS and transfer of such cells into mice that are prone to autoimmune myocarditis was sufficient to initiate overt myocarditis (32), and (*3*) ligation of TLR4 and TLR9 on dendritic cells blocks the suppressor activity of regulatory T cells via the secretion of IL-6 (33). Murine macrophages and dendritic cells express TLR3 (20), but TLR3 is restricted to dendritic cells on human leukocytes (13). Dendritic cells constantly process self-antigens, but in the absence of co-stimulatory molecules, the presented antigen provides T cells with a signal for tolerance (34). By contrast, during viral infection, virus-associated TLR ligands stimulate dendritic cells to upregulate co-stimulatory molecules and to secrete selected cytokines (35). Therefore, dendritic cells are key regulators of both tolerance and anti-viral immunity and therefore may be crucial for viral infection–induced exacerbation of autoimmunity, including SLE (36). In fact, among microbial products, viral dsRNA is a potent trigger for dendritic cell maturation and for the secretion of type I interferons and Th1 cytokines (14,37,38). Our finding that pI:C RNA triggers maturation and secretion of IL-12p70 and IFN-α by dendritic cells isolated from bone marrow of MRL$^{lpr/lpr}$ mice is consistent with these studies. Although viral dsRNA–induced IFN-α production is a major mechanism of anti-viral immunity (39) elevated IFN-α levels also promote the progression of autoimmune tissue injury in SLE (40,41). Furthermore, TLR3-induced secretion of IL-12p70 has a critical role in SLE and lupus nephritis mainly by fostering the accumulation of IFN-γ–producing T cells in the kidney followed by aggravation of lupus nephritis (42–44). Our observation that pI:C RNA–induced elevation of IL-12p70 serum levels was associated with acceleration of lupus nephritis is consistent with a previous study showing aggravation of lupus nephritis by injections of recombinant IL-12 in MRL$^{lpr/lpr}$ mice (42).

Dendritic cell activation can induce adaptive B and T cell responses. Thus, one might assume that pI:C RNA–induced maturation of dendritic cells would enhance humoral immunity against chromatin, an important autoantigen in SLE. It is interesting that in contrast to our previous observation with the TLR9 ligand CpG-DNA (10), pI:C RNA did not affect serum levels and glomerular deposits of DNA autoantibodies. As human and murine B cells express TLR9 but not TLR3, a direct stimulatory effect of pI:C RNA was not predicted, but B cell activation could be supported by indirect mechanisms via dendritic cells and T cells. In fact, our data clearly show that in MRL$^{lpr/lpr}$ mice, systemic exposure to pI:C RNA does not provide a signal for B cell activation, consistent with the finding that serum DNA autoantibody levels and renal IgG deposits were unaffected by repetitive pI:C RNA injections. These findings are consistent with a recent observation that a series of injections with CpG-DNA but not with pI:C RNA can severely alter the morphology and functionality of mouse lymphoid organs (45).

We also observed that pI:C RNA induces the secretion of IL-6 in dendritic cells and monocytes isolated from MRL$^{lpr/lpr}$ mice.

IL-6 derived from antigen-presenting cells has been shown to suppress CD4+CD25+ regulatory T cells that inhibit the proliferation of autoreactive T cells (33). The role of regulatory T cells in SLE remains to be determined (46), but their number is reduced in peripheral blood of SLE patients with active disease (47,48), indicating that disease activity of SLE may be linked to the regulatory role of this T cell population. pI:C RNA–induced IL-6 secretion therefore may modulate adaptive immunity in SLE independent of B cell responses.

In summary, exposure to pI:C RNA—a structural analog of viral dsRNA—can aggravate lupus nephritis through TLR3 on antigen-presenting cells and glomerular mesangial cells. pI:C RNA–induced cytokine and chemokine production represents a major mechanism in this context. dsRNA-induced disease activity is independent of B cell activation and humoral anti-chromatin immunity in experimental SLE and therefore differs from CpG-DNA–induced autoimmunity. Apparently, pathogen-associated immunomodulation relates to the specific expression pattern of the respective pattern-recognition receptor. These findings contribute to the understanding of pathogen-associated modulation of autoimmunity but may also be involved in the pathogenesis of other types of inflammatory kidney diseases, *e.g.*, flares of IgA nephropathy, renal manifestations of chronic hepatitis C virus infection, and renal vasculitis.

Acknowledgments

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G-Rich DNA Suppresses Systemic Lupus

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Whereas the role of immune complexes in mediating renal cell and immune cell activation is well established, the contribution of sequence-specific immunomodulatory actions of the chromatin part remains unclear. Toll-like receptor-9 (TLR-9) mediates immunostimulatory effects of unmethylated microbial CpG-DNA. It was hypothesized that hypomethylated CpG-DNA in vertebrates may have similar effects and may contribute to disease progression in lupus nephritis. A synthetic G-rich DNA, known to block CpG-DNA effects, was used in this study. In macrophages, G-rich DNA suppressed CpG-DNA– but not LPS-induced production of CCL5 in a dose-dependent manner. Injections of G-rich DNA suppressed lymphoproliferation induced by CpG-DNA injections in mice. In MRL$^{lpr/lpr}$ mice with lupus nephritis, labeled G-rich DNA co-localized to glomerular immune complexes and was taken up into endosomes of TLR-9–positive infiltrating macrophages. Eleven-week-old MRL$^{lpr/lpr}$ mice that received injections of either saline or G-rich DNA for 13 wk revealed decreased lymphoproliferation and less autoimmune tissue injury in lungs and kidneys as compared with saline-treated controls. G-rich DNA reduced the levels of serum dsDNA-specific IgG2a as well as the renal immune complex deposits. This was consistent with the blocking effect of G-rich DNA on CpG-DNA–induced proliferation of B cells that were isolated from MRL$^{lpr/lpr}$ mice. As oligodeoxyribonucleotide 2114–treated MRL$^{lpr/lpr}$ mice were not exposed to exogenous CpG-DNA, these effects should relate to a blockade of CpG motifs in endogenous DNA. It is concluded that adjuvant activity of self-DNA contributes to the pathogenesis of lupus nephritis. Modulating the CpG-DNA–TLR-9 pathway may offer new opportunities for the understanding and treatment of lupus.

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The interaction of CpG-DNA and Toll-like receptor-9 (TLR-9) holds many promises for therapeutic intervention such as vaccination, anti-tumor immunity, asthma, and certain infectious diseases (1,2). However, repetitive injections of CpG-oligodeoxyribonucleotides (ODN) cause inappropriate lymphoproliferation in mice (3). Experimental studies with rodents suggest that synthetic CpG-ODN can exacerbate underlying autoimmune tissue injury, *e.g.*, glomerulonephritis, experimental encephalomyelitis, collagen-induced arthritis, or lupus nephritis (4–7). In lupus nephritis, the interaction of CpG-DNA with TLR-9 is of particular interest for the following reasons: (1) Human lupus is paradigmatic for systemic autoimmunity with polyclonal B cell proliferation; (2) CpG-DNA is a B cell mitogen that allows T cell–independent B cell proliferation and autoantibody production (8); (3) endogenous CpG-DNA may have similar effects, because immune complexes that are isolated from patients with lupus activate dendritic cells to produce IFN-α, an effect sensitive to DNAse digestion (9); and (4) CpG-DNA can aggravate autoimmune tissue injury locally by activation of tissue macrophages (4). Experimental evidence for a pathogenic role of CpG motifs in self-DNA for lupus is lacking. Methylation of CpG motif prevents their stimulatory effect on B cells (10), but CpG motifs in human DNA are methylated to only 70 to 80% (11), and genomic DNA released by dying cells can induce the maturation of antigen-presenting cells (12). It is interesting that known inhibitors of DNA methylation can induce systemic lupus erythematosus (SLE) in humans (13). Furthermore, in vertebrates, G-rich inhibitory DNA sequence elements counterbalance the immunostimulatory effects of unmethylated CpG-DNA (14). Synthetic ODN with inhibitory motifs have shown to block CpG-DNA–induced effects (15–17). Thus, we intended to test whether inhibitory ODN given in excess to mice with experimental lupus could serve as an appropriate tool to block the effects of endogenous CpG-DNA *in vivo*. We used the recently reported G-rich inhibitory ODN 2114 (16) in MRL$^{lpr/lpr}$ mice, a spontaneous model of autoimmune tissue injury with striking similarities to human lupus nephritis. We found that that injections with G-rich DNA reduced lymphoproliferation and autoimmune tissue injury in MRL$^{lpr/lpr}$ mice. This can be attributed to a specific blocking effect of G-rich DNA on CpG-DNA–induced B cell proliferation and macrophage activation. As injected G-rich DNA was found to localize to TLR-9–positive macrophages in the kidney, we conclude that injections of G-rich DNA can interfere with local as well as systemic autoimmune disease mechanisms in SLE. Thus, G-rich DNA can reduce autoimmunity in MRL$^{lpr/lpr}$ mice most likely involving TLR-9–dependent recognition of endogenous CpG-DNA.

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Materials and Methods

Phosphothioate ODN

The following ODN were used for *in vitro* or *in vivo* studies. ODN 2114 5'-TCC TGG AGG GGA AGT-3', CpG-ODN 1668 5'-TCG ATG ACG TTC CTG ATG CT-3', and GpC-ODN 1720 5'-TCG ATG AGC TTC CTG ATG CT-3' (TIB Molbiol, Berlin, Germany).

Animal Studies

Eight-week-old female 129Sv mice were obtained from Taconic (Ry, Denmark). Ten-week-old female MRL$^{lpr/lpr}$ mice were obtained from Harlan Winkelmann (Borchen, Germany) and were kept in filter-top cages under a 12-h light/dark cycle. Autoclaved water and standard chow (Sniff, Soest, Germany) were available *ad libitum*. For assessing the distribution of injected ODN 2114, 100 μg 3'-rhodamine–labeled ODN 2114 was injected intraperitoneally into 16-wk-old MRL$^{lpr/lpr}$ mice. Tissues were collected 2 h after injection and subjected to further analysis as recently described (4). Different groups of mice were treated with either saline or ODN 2114 on alternate days from weeks 11 to 24 of age. Blood and urine samples were collected from each animal at the end of the study, and urine protein/creatinine ratio, serum dsDNA autoantibody IgG isotype titers, and serum blood urea nitrogen levels were determined as described previously (4). All experimental procedures were performed according to the German animal care and ethics legislation and had been approved by the local government authorities.

Morphologic Analysis

Histologic studies were performed on paraffin-embedded sections as described (4). The severity of the renal lesions was graded using the indices for activity and chronicity of lupus nephritis (18). Peribronchial and pulmonary inflammation was graded from 0 (no inflammation) to 3 (severe inflammation). The following primary antibodies were used for immunostaining: ER-HR3 (DPC Biermann, Bad Nauheim, Germany; 1:50) (19), anti-CD3 (BD Pharmingen, Heidelberg, Germany; 1:100), anti–smooth muscle actin (Dako, Carpinteria, CA; 1:100), anti–Ki-67 (DAKO, Hamburg, Germany; 1:25), anti–TLR-9 (provided by Dr. Stefan Bauer, Technical University, Munich, Germany; 1:50 [7]). Negative controls included incubation with a respective isotype antibody. For quantitative analysis, glomerular cells were counted in 10 cortical glomeruli per section. Semiquantitative scoring of glomerular IgG deposits from 0 to 3+ was performed on 15 cortical glomerular sections as described (7).

Cell Culture Conditions

J774 mouse macrophages (American Type Culture Collection, Rockville, MD) were grown in RPMI 1640 that contained 1 mM HEPES, 10% heat-inactivated bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin (Biochrom KG, Berlin, Germany). B cells were isolated from spleens of female MRL$^{lpr/lpr}$ mice using B Cell Isolation Kit (Miltenyi, Bergisch Gladbach, Germany) following the manufacturer's protocol. Purity as determined by FACS analysis using CD45/B220-PE or rat IgG2a as an isotype (BD Biosciences, Hamburg, Germany) revealed 97% B cells after each isolation.

B Cell Proliferation Assay

Proliferation of B cells was assessed using CellTiter 96 Proliferation Assay (Promega, Mannheim, Germany). In brief, B cells (1×10^5) were incubated in 96-well plates in 100 μl RPMI medium that contained 10% FCS, 100 units/ml penicillin, and 100 μg/ml streptomycin (Biochrom KG) with 1 μM CpG-ODN 1668, 1 μg/ml LPS, or various concentrations of ODN 2114 for a period of 72 h. To each well, 20 μl of CellTiter 96 Aqueous One Solution (Promega) was added and incubated at 37°C for 4 h. The O.D. was measured at 292 nm.

Cytokine and Nitric Oxide Analysis

Cytokine levels in mice sera or cell culture supernatants were determined using commercial ELISA kits: Mouse TNF-α (Biolegend, San Diego, CA), CCL5 (R&D, Wiesbaden-Nordenstadt, Germany), and IFN-α (Perbioscience, Bonn, Germany). The Griess Reagent System (Promega) was used for the determination of nitrite in cell supernatants as a marker of nitric oxide production.

Statistical Analyses

Data were expressed as mean ± SEM. Cell culture data were analyzed using ANOVA, and *post hoc* Bonferroni correction was used for multiple comparisons. Comparison of groups of mice was performed using unpaired two-tailed *t* test. $P < 0.05$ was considered to indicate statistical significance.

Results

ODN 2114 Blocks Stimulatory Activity of CpG-ODN In Vitro

ODN 2114 have been reported to block CpG-ODN–induced NF-κB activation in mouse B cells (16). We first aimed to confirm this blocking effect in murine macrophages. ODN 2114 blocked CpG-ODN–induced CCL5 (Figure 1A) and nitric oxide (Figure 1B) production in J774 monocytes in a dose-dependent manner. At equimolar concentrations of CpG-DNA and ODN 2114, the blocking effect of ODN 2114 on nitrite production was 100%. By contrast, ODN 2114 did not affect CCL5 or TNF-α production induced by LPS (Figure 1, C and D). These data suggest that ODN 2114 can act as a specific antagonist for CpG-ODN–induced activation of mouse monocytes *in vitro*.

ODN 2114 Blocks Stimulatory Activity of CpG-ODN In Vivo

Next we assessed the potential of ODN 2114 to block the reported CpG-ODN–induced toxicity after repeated injection in mice (3). We treated 129Sv mice with daily intraperitoneal injections of saline or either 60 μg CpG-ODN, GpC-ODN, ODN 2114, or CpG-ODN plus ODN 2114 or saline for 12 d ($n = 5$ in each group). As previously reported, CpG-ODN induced splenomegaly and lymphadenopathy as compared with saline-injected mice (3). In spleens and lymph nodes, total cellularity was increased and follicles were replaced by disorganized collections of activated macrophages (Figure 2). All CpG-ODN–treated mice showed hemorrhagic ascites, as compared with saline and ODN 2114–treated controls, whereas ODN 2114 completely blocked CpG-ODN–induced ascites production. Livers of these mice revealed multifocal portal inflammatory cell infiltrates associated with large areas of necrotic and apoptotic hepatocytes (Figure 2). By contrast, all mice that received ODN 2114 injections together with CpG-ODN did not show major signs of CpG-ODN toxicity. ODN 2114 also significantly reduced CpG-ODN–induced alterations of the microarchitecture in spleens and lymph nodes (Figure 2). Mice that received injections with either ODN 2114 or GpC-ODN alone did not show any histopathologic abnormalities as compared with saline-injected mice (data not shown), the latter excluding unspe-

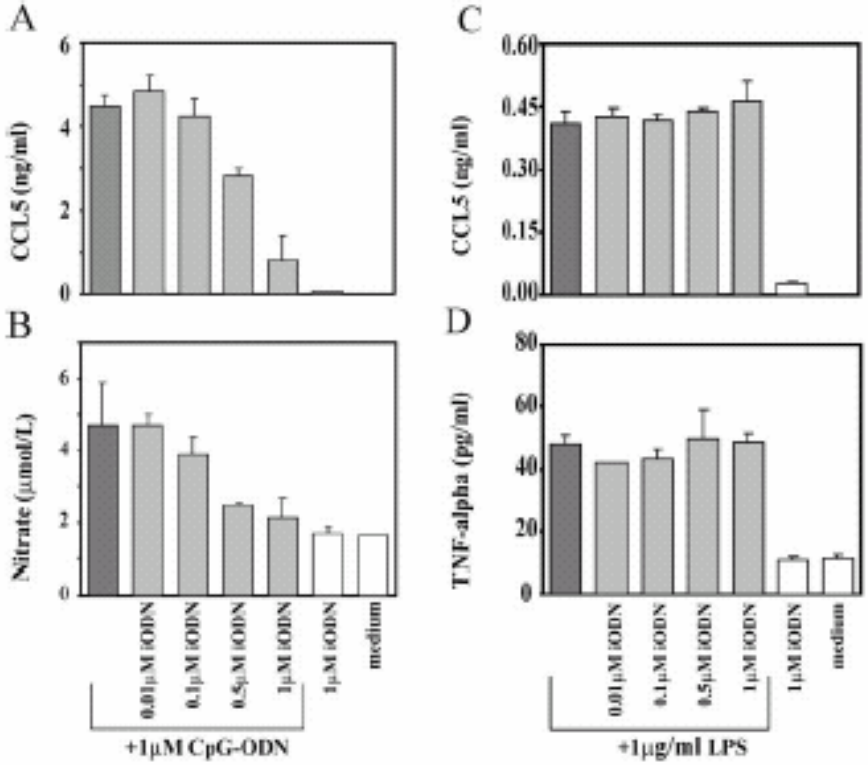

Figure 1. Oligodeoxyribonucleotides (ODN) 2114 block CpG-DNA–induced effects *in vitro*. Cultured J774 macrophages were incubated with CpG-ODN, CpG-ODN with different concentrations of ODN 2114, or standard medium without supplements for 24 h as indicated. (A) CCL5 was measured in supernatants by ELISA. (B) Nitrite was determined by the Griess reaction. (C and D) J774 macrophages were incubated with either LPS alone or LPS with different concentrations of ODN 2114 or standard medium without supplements for 24 h as indicated. CCL5 and TNF-α levels were measured in supernatants by ELISA. Results shown are means ± SEM from one of two comparable experiments, each performed in duplicate.

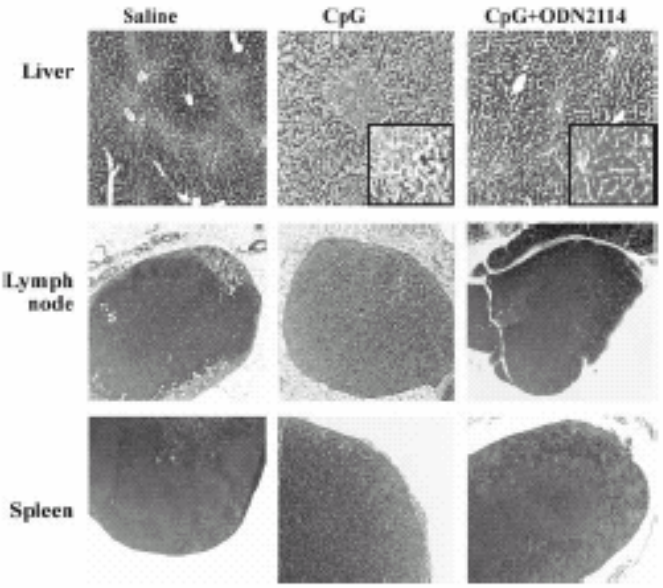

Figure 2. ODN 2114 block CpG-DNA–induced effects *in vivo*. 129Sv mice received intraperitoneal injections of saline or 60 μg of GpC-ODN, CpG-ODN, or CpG-ODN + ODN 2114 or saline daily for 14 d ($n = 5$ in each group; saline not shown). Respective organs were stained with periodic acid-Schiff (PAS). Magnification, ×400.

cific competition of CpG-DNA. These data implicate that ODN 2114 can block CpG-ODN–induced effects *in vivo* in mice.

Distribution of Labeled ODN 2114 and CpG-ODN of Autoimmune MRL$^{lpr/lpr}$ Mice

In view of our previous findings that exogenous CpG-DNA is taken up by intrarenal macrophages (7), we questioned whether injected ODN 2114 localizes likewise in nephritic kidneys of MRL$^{lpr/lpr}$ mice. Thus, we studied the distribution of rhodamine-labeled ODN 2114 in nephritic kidneys of 16-wk-old MRL$^{lpr/lpr}$ mice. After intravenous injection, ODN 2114 were detected in a glomerular mesangial and capillary staining pattern (Figure 3A). Co-staining with an anti-mouse IgG antibody showed co-localization of labeled ODN 2114 with glomerular IgG deposits (Figure 3B). In some areas, ODN 2114–positive granules were negative for IgG (Figure 3C). Co-staining with an EH-HR3 antibody identified these granules to be endosomes of glomerular macrophages but IgG–ODN 2114 double-positive endosomes were also observed (Figure 3D). Co-staining with a TLR-9 antibody confirmed co-localization of ODN 2114 with TLR-9 (Figure 3E). Nephritic MRL$^{lpr/lpr}$ mice that received an injection of rhodamine only or healthy MRL wild-type mice that received an injection of labeled ODN 2114 did not show any glomerular deposits (data not shown). These findings show that injected ODN 2114 localize to glomerular

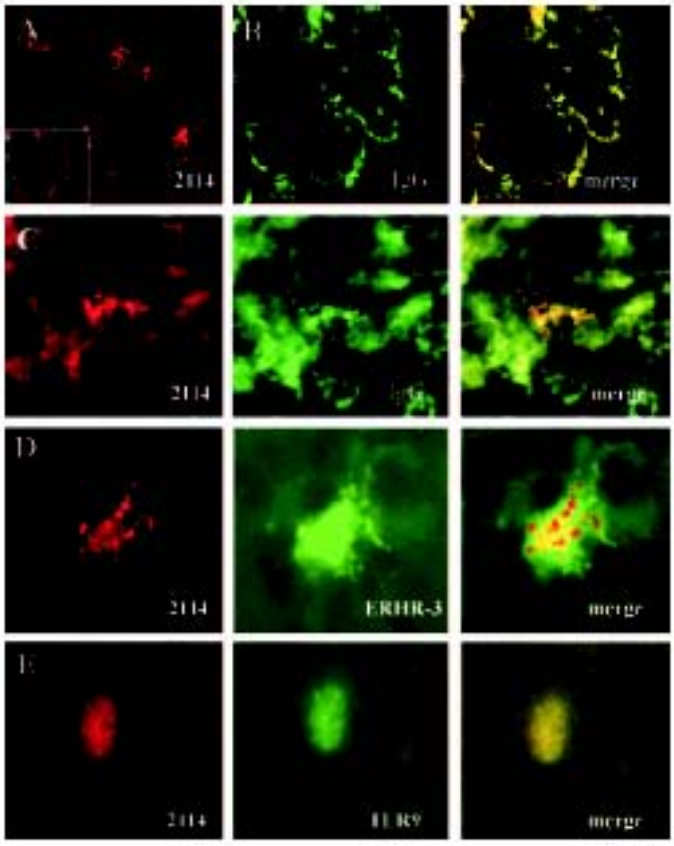

Figure 3. Localization of ODN 2114 in kidneys of MRL$^{lpr/lpr}$ mice after intravenous injection. Rhodamine-labeled ODN 2114 were injected intravenously into 16-wk-old MRL$^{lpr/lpr}$ mice, and renal tissue was harvested 2 h later. (A) Fluorescence imaging of frozen sections showed uptake of labeled ODN 2114 in glomeruli in a mesangial and capillary staining pattern. (B) At higher magnification, the granular deposits of ODN 2114 co-localize with IgG deposits in glomerular capillaries upon double staining. (C) In some areas, ODN 2114–positive but IgG-negative granula are noted. (D) Co-staining with an EHRH-3 antibody identified these granula to be localized within macrophages. (E) Co-staining with a Toll-like receptor-9 (TLR-9) antibody showed co-localization of ODN 2114 and TLR-9 to an intracellular compartment. Magnification, ×400 in A; ×630 in B; ×1000 in C through E.

mesangium and capillaries and are taken up into TLR-9–positive intracellular endosomes of glomerular macrophages.

ODN 2114 Protect MRL$^{lpr/lpr}$ Mice from Autoimmune Tissue Injury

On the basis of these data, we hypothesized that ODN 2114 might modulate macrophage function in experimental lupus. We treated MRL$^{lpr/lpr}$ mice from week 11 to week 24 of age with intraperitoneal injections of ODN 2114 on alternate-day intervals and compared markers of disease activity with saline-treated MRL$^{lpr/lpr}$ mice at the end of the study. We observed that ODN 2114 reduced spleen weight in MRL$^{lpr/lpr}$ mice as compared with saline-treated controls (Table 1). Morphometric analysis revealed less tissue injury in kidneys and lungs of ODN 2114–treated mice (Table 1). Lungs of ODN 2114–treated mice showed less peribronchiolar and perivascular inflammatory cell infiltrates compared with saline-treated controls (Figure 4). In kidneys of MRL$^{lpr/lpr}$ mice, ODN 2114 significantly reduced the number of interstitial ER-HR3 macrophages, CD3 lymphocytes, and Ki-67–positive proliferating cells in glomeruli and the tubular compartment (Table 1, Figure 5A). Proteinuria as a marker of glomerular injury was also reduced, although the altogether low glomerular ER-HR3 macrophage and CD3 T cell counts were similar in both groups (Table 1).

IFN-α is an important mediator of CpG-DNA–mediated autoimmunity (20) and a marker of disease activity in lupus erythematosus (21). Therefore, we determined serum IFN-α levels in saline and ODN 2114–treated MRL$^{lpr/lpr}$ mice. ODN 2114 treatment significantly reduced serum IFN-α levels in ODN 2114–treated MRL$^{lpr/lpr}$ mice (Figure 5B). Thus, injections of ODN 2114 that inhibit the biologic effects of CpG-DNA reduce the serum levels of IFN-α and prevent autoimmune tissue injury in MRL$^{lpr/lpr}$ mice.

ODN 2114 Block B Cell–Dependent Autoimmunity in MRL$^{lpr/lpr}$ Mice

Autoantibody production and immune complex deposition cause tissue injury in lupus. Thus, we assessed the effect of ODN 2114 on DNA autoantibody production and renal immune complex deposits in MRL$^{lpr/lpr}$ mice. At 24 wk, ODN 2114–treated MRL$^{lpr/lpr}$ mice revealed reduced serum levels of dsDNA-specific IgG_{2a} antibodies as compared with saline-treated MRL$^{lpr/lpr}$ mice (Figure 6A). This was consistent with the observation that ODN 2114 markedly reduced glomerular IgG_{2a} deposits in MRL$^{lpr/lpr}$ mice (Figure 6B, Table 1). Glomerular IgG_1 deposits were not reduced, indicating that the effect of ODN 2114 on serum IgG_1 levels relates to their specificity for dsDNA. These findings are suggestive of an inhibitory effect of ODN 2114 on CpG-DNA–induced B cell proliferation in MRL$^{lpr/lpr}$ mice. Therefore, we isolated B cells from MRL$^{lpr/lpr}$ mice and studied the effects of ODN 2114 on either CpG-DNA– or LPS-induced B cell proliferation *in vitro*. ODN 2114 blocked B cell proliferation in a dose-dependent manner when cells were exposed to CpG-DNA (Figure 7A). By contrast, ODN 2114 had no effect on LPS-induced B cell proliferation (Figure 7B). These data suggest that ODN 2114 specifically block CpG-ODN–induced B cell proliferation as well as subsequent DNA autoantibody production and renal immune complex deposits in MRL$^{lpr/lpr}$ mice.

Discussion

When MRL$^{lpr/lpr}$ mice are exposed to bacterial or synthetic CpG-DNA, ligation of TLR-9 on immune cells leads to enhanced dsDNA autoantibody production and aggravation of lupus nephritis (7). Similarly, insufficient clearance of nuclear particles in lupus may also provide a permanent source of hypomethylated CpG motifs from self DNA (22). This mechanism may contribute to a continuous activation of B cells and dendritic cells and perpetuate systemic lupus in humans (10,23,24). In this study, we addressed this hypothetical pathway by blocking potential CpG-DNA–induced immunity both *in vitro* and *in vivo* with a specific antagonist, G-rich DNA. We

Table 1. Serum, urinary, and histologic findings in kidneys of 24-wk-old MRL$^{lpr/lpr}$ mice that received injections from 11 to 24 weeks of age[a]

	Saline (n = 9)	ODN 2114 (n = 10)	p
Spleen weight	0.80 ± 0.13	0.56 ± 0.14	0.01
Lung histologic score	1.6 ± 0.5	0.9 ± 0.7	0.03
Kidney			
proteinuria (μg/mg creatinine)	16.1 ± 5.9	7.4 ± 1.9	0.05
histologic scores			
activity index	15.3 ± 3.9	6.9 ± 2.3	0.0003
chronicity index	5.1 ± 2.1	1.1 ± 1.7	0.002
cellular response (cells/glomerulus or hpf)			
glomerular			
EHR3+ (cells/glom)	0.9 ± 0.3	0.8 ± 1.3	0.84
CD3+ (cells/glom)	1.2 ± 1.0	1.7 ± 0.8	0.58
Ki-67+ (cells/glom)	5.7 ± 1.6	2.8 ± 1.2	0.01
interstitial			
EHR3+ (cells/hpf)	14.4 ± 7.4	7.0 ± 4.8	0.03
CD3+ (cells/hpf)	65.7 ± 27.2	39.1 ± 12.9	0.02
Ki-67+ (cells/hpf)	12.1 ± 5.6	7.4 ± 3.2	0.05
tubular			
Ki-67+ (cells/hpf)	4.8 ± 2.0	2.8 ± 1.1	0.04
humoral response (glomerular deposit score)			
IgG_1	1.5 ± 0.3	1.1 ± 0.2	0.047
IgG_{2a}	2.5 ± 0.3	1.7 ± 0.5	0.003

[a]Values are means ± SEM from 8 to 10 mice per group.

further argued that if endogenous CpG-DNA were to contribute to the progression of lupus nephritis, then injections of G-rich DNA should prevent disease progression in MRL$^{lpr/lpr}$ mice with experimental lupus.

Synthetic G-Rich ODN Neutralizes CpG-DNA

CpG-DNA is a strong activator of plasmacytoid dendritic cells, macrophages, and B cells in mice (10,25). CpG-DNA stimulates their antigen presentation and proinflammatory cytokine production that drive subsequent Th1-type responses (25). Lenert *et al.* (16) first used the ODN 2114 to block CpG-ODN–induced effects on murine B cells. Here we confirm that this antagonistic effect is specific, as the G-rich ODN 2114, which totally prevented CpG-DNA–induced B cell proliferation or CCL5 and TNF-α production by macrophages, did not modulate LPS-related effects. This antagonism occurs proximal to NF-κB activation (16), but the specific site of interaction is yet unknown. Competition for intracellular uptake of CpG-DNA was shown to be independent of G-rich motifs and far too weak to explain the 100% antagonism at equimolar concentrations of G-rich- and CpG-DNA (14,15). Alternatively, G-rich ODN may either compete with CpG-DNA for the CG binding site at TLR-9 (26) or modify the nanoparticle structure of nucleosomes by direct interaction with endogenous CpG-DNA (26,27). The latter would require co-localization of injected G-rich DNA chromatin particles. This may occur *in vivo* because we observed co-localization of injected ODN 2114 with glomerular IgG deposits that are complexed with chromatin-particles in nephritic MRL$^{lpr/lpr}$ mice. However, as our study was not designed to address this question, the interaction of CpG-DNA with TLR-9 on a molecular level remains to be determined. Nevertheless, G-rich ODN represents a specific antagonist for CpG-DNA–induced B cell and monocyte activation in mice. This was also confirmed *in vivo* by our experiments in Sv129 mice. G-rich ODN 2114 blocked CpG-ODN–induced lymphoproliferation, an effect shown to be mediated through TLR-9 on marginal zone B cells and monocytes (3) Together, G-rich ODN 2114 specifically block the effects of CpG-DNA *in vitro* and *in vivo*, which renders ODN 2114 a valuable tool to address the question, whether endogenous CpG-DNA can modulate lupus erythematosus.

G-Rich DNA Modulates Systemic Autoimmunity in MRL$^{lpr/lpr}$ Mice

Systemic lupus is associated with polyclonal B cell proliferation and DNA autoantibody production in humans. In MRL$^{lpr/lpr}$ mice, lymphoproliferation and dsDNA autoantibody production progress with age. Injections with G-rich ODN 2114 reduced spleen weight as a marker of lymphoproliferation as well as serum dsDNA autoantibody concentrations as compared with saline-injected MRL$^{lpr/lpr}$ mice. As the MRL$^{lpr/lpr}$ mice were not exposed to exogenous CpG-DNA, the observation may be attributed to a blockade of immunostimulatory effects of endogenous CpG-DNA on B cells. This is consistent with a study published by Leadbetter *et al.* (8) showing that self chromatin-containing immune complexes stimulate B cells iso-

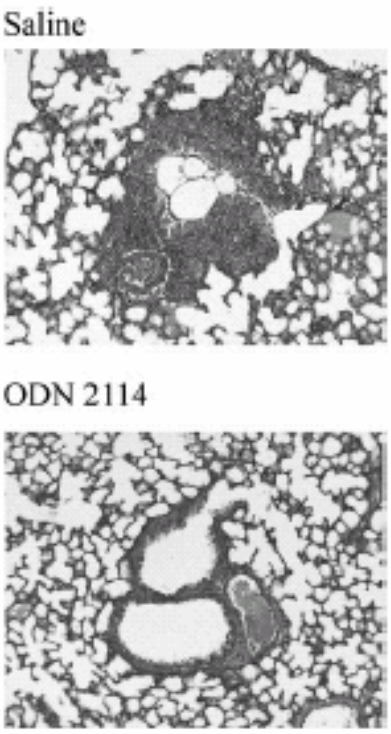

Figure 4. Autoimmune lung injury in MRL$^{lpr/lpr}$ mice. Lung sections that were taken from 24-wk-old MRL$^{lpr/lpr}$ mice were stained with PAS. Note that ODN 2114–treated MRL$^{lpr/lpr}$ mice show less peribronchiolar and perivascular inflammatory cell infiltrates as compared with saline-injected MRL$^{lpr/lpr}$ mice. Magnification, ×200.

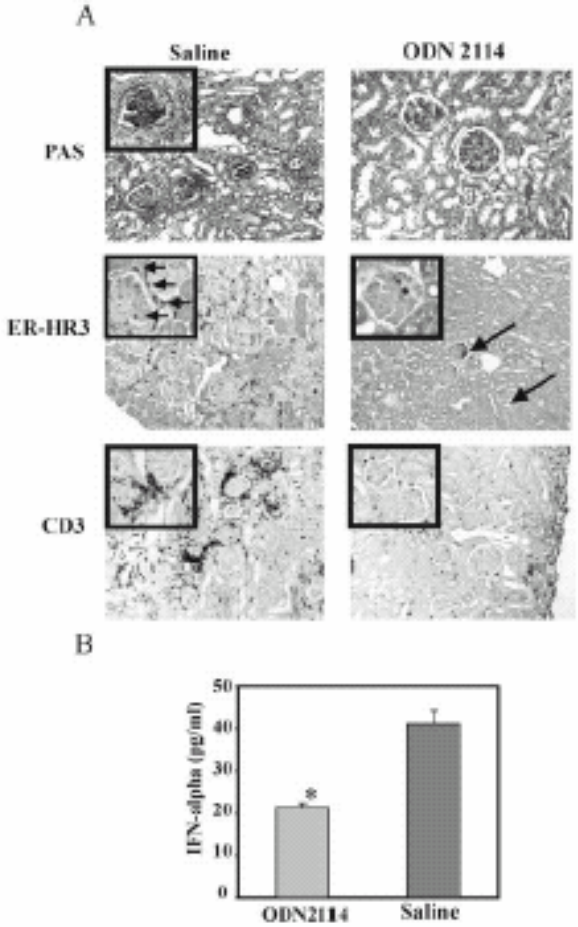

Figure 5. Lupus nephritis in MRL$^{lpr/lpr}$ mice. (A) Renal sections were stained with PAS, ERHR-3, and CD3 as indicated. Images are representative of 10 mice in each group. Note that ODN 2114–treated MRL$^{lpr/lpr}$ mice show less periglomerular and interstitial inflammatory cell infiltrates as compared with saline-treated MRL$^{lpr/lpr}$ mice. (B) Serum IFN-α levels in 24-wk-old female saline- or ODN 2114–treated MRL$^{lpr/lpr}$ mice were determined by ELISA ($n = 8$ in each group). *$P < 0.05$ as compared with saline. Magnification, ×400 in A.

lated from MRL$^{lpr/lpr}$ mice *via* TLR-9. It is noteworthy that suppressive ODN as well as DNAse treatment of the immune complexes abrogated this effect, supporting the role for the endogenous CpG-DNA in this context (8). In our study, the blocking effect of G-rich DNA on B cell proliferation was specific for CpG-DNA, because ODN 2114 did not modulate LPS-induced B cell proliferation.

Serum IFN-α levels depict the activation of IFN-producing plasmacytoid dendritic cells, which represents another marker for disease activity in lupus (28). Nuclear particles released from dying cells and complexed with lupus patient IgG are potent inducers of IFN-α production in plasmacytoid dendritic cells, an effect that is sensitive to DNAse digestion (29). In a recent study, immune complexes were isolated from sera of patients with various rheumatic diseases (16). It was found that only DNA-containing immune complexes that were isolated from lupus patients stimulated plasmacytoid dendritic cells to produce cytokines and chemokines *via* a cooperative interaction between TLR-9 and FcγRIIa (CD32). CD32 shuttles DNA-containing immune complexes into a subcellular compartment that contains TLR-9 (9). Only CD32-positive plasmacytoid dendritic cells internalized DNA-immune complexes and produced large amounts of IFN-α. Our finding that MRL$^{lpr/lpr}$ mice that received injections with G-rich DNA show lower serum IFN-α levels as compared with saline-injected MRL$^{lpr/lpr}$ mice is in favor of a blocking effect of G-rich DNA on IFN-producing plasmacytoid dendritic cells. Thus, G-rich DNA can block B cell proliferation, dsDNA autoantibody production, and IFN-α release in MRL$^{lpr/lpr}$ mice, which all have established etiopathogenic roles in the systemic autoimmunity of lupus erythematosus.

G-Rich DNA Prevents Tissue Injury in MRL$^{lpr/lpr}$ Mice

MRL$^{lpr/lpr}$ mice that received injections of G-rich DNA had markedly reduced renal and pulmonary autoimmune tissue injury as compared with saline-injected mice. In part this may relate to the reduced anti-dsDNA antibody production and immune complex deposition, as demonstrated for the kidney. However, injected G-rich ODN 2114 could also interact locally with immune complexes and TLR-9–positive immune cells. We addressed this issue by injecting fluorescently labeled ODN 2114 into nephritic MRL$^{lpr/lpr}$ mice. In fact, ODN 2114 localized to glomerular immune complex deposits and to intracellular

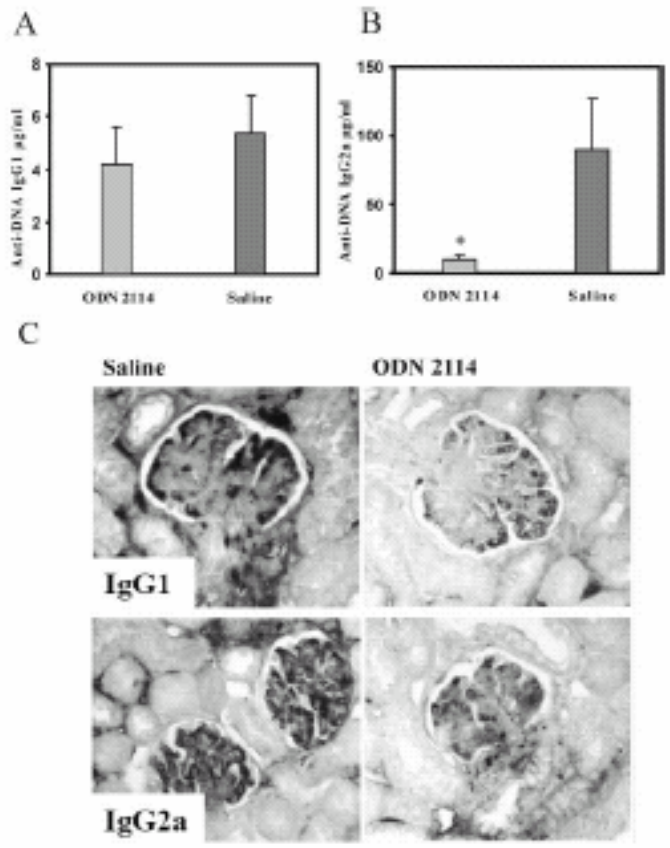

Figure 6. Serum dsDNA autoantibody levels and glomerular immune complex deposits in MRL$^{lpr/lpr}$ mice. Serum dsDNA autoantibody IgG1 (A) and IgG2a (B) levels were determined by ELISA ($n = 8$ to 10). Data are means ± SEM. *$P < 0.05$ *versus* saline. (C) Renal sections were stained for IgG1 and IgG2a, as indicated. Note less glomerular IgG1 and IgG2a deposits in ODN 2114–treated MRL$^{lpr/lpr}$ mice. Images are representative of 10 mice in each group. Magnification, ×400 in C.

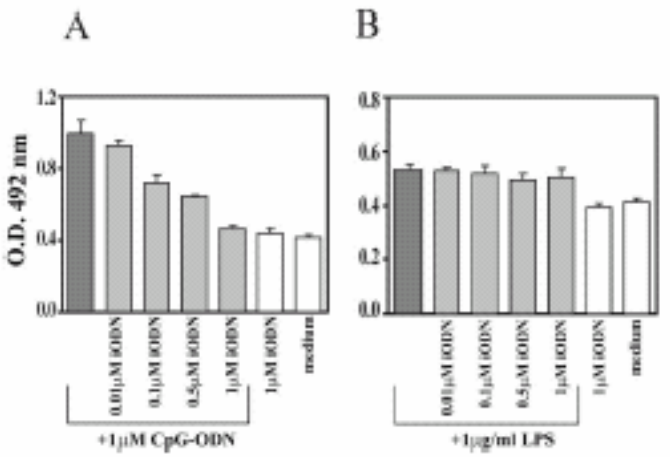

Figure 7. B cell activation in MRL$^{lpr/lpr}$ mice. (A) B cells were isolated from MRL$^{lpr/lpr}$ mice as described in Materials and Methods. B cells were incubated with CpG-ODN, CpG-ODN plus different concentrations of ODN 2114, or standard medium without supplements for 72 h as indicated. B cell proliferation was assessed by CellTiter 96 proliferation assay. (B) B cells were isolated as before and incubated with LPS alone or LPS with different concentrations of ODN 2114 or standard medium without supplements for 72 h as indicated. Results are means ± SEM from one of two comparable experiments, each performed in duplicate.

compartments of infiltrating glomerular macrophages. This cellular distribution was comparable to that of TLR-9 immunostaining in kidneys of MRL$^{lpr/lpr}$ mice. Thus, ODN 2114 could interfere with CpG-DNA–rich chromatin-immune complexes in the endosomes of intrarenal macrophages *in vivo*. This could reduce a proinflammatory effect of the immune complexes on macrophages and possibly dendritic cells in kidneys of MRL$^{lpr/lpr}$ mice. We showed previously that exogenous bacterial DNA or CpG-DNA markedly stimulate renal macrophages in lupus nephritis of MRL$^{lpr/lpr}$ mice and antigen-induced immune complex glomerulonephritis (4,7). CpG-DNA stimulates macrophages to produce multiple proinflammatory mediators that contribute to the progression of renal disease. Similarly, exogenous CpG-DNA can cause macrophage-dependent arthritis or lung injury, which both can be blocked with G-rich DNA (17,30). However, in this study, MRL$^{lpr/lpr}$ mice were not exposed to exogenous CpG-DNA, so the beneficial effect of G-rich DNA is compatible with the influence with endogenous CpG-DNA–mediated effects. Our data support the hypothesis that endogenous CpG-DNA–rich chromatin activates TLR-9–positive immune cells, specifically B cells, macrophages, and dendritic cells, and thus contribute to the pathogenesis of lupus. Apart from the experimental data in mice, this concept is also supported by the therapeutic properties of chloroquine, an unspecific blocker of endosomal TLR activation, in the treatment of human lupus (31). Our observation that administration of G-rich DNA attenuates the course of the lupus-like disease in MRL$^{lpr/lpr}$ mice also argues in favor of endogenous CpG-DNA fragments as pathophysiologic contributors to the murine disease and indicates G-rich DNA as a potential therapeutic pathway for the treatment of SLE.

Acknowledgments

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See related editorial, "More Targeted Treatments for Lupus Nephritis: Is the Future (Nearly) Here?," on pages 3146–3148.

8. CURRICULUM VITAE

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Department of Atherosclerosis, Insitut Pasteur de Lille, France

Research project: 1. Role of orphan nuclear receptor Rev-erbα in metabolic syndrome.

2. Role of RORa in metabolic syndrome and inflammation.

Principal investigator: Prof. Bart Staels

Previous professional experience:

2005 - 2006	**Post-doctoral Research Fellow (Short term)** Department of Clinical Biochemistry, Ludwig Maximilians University, Munich, Germany. Research project: Nucleic acid specific Toll-like Receptors in autoimmunity. Principal investigator: Dr. Hans-Joachim Anders
2003 - 2005	**Doctoral Research Fellow** Department of Clinical Biochemistry, Ludwig Maximilians University, Munich, Germany. Research project: Nucleic acid specific Toll-like Receptors in lupus nephritis. Principal investigator: Dr. Hans-Joachim Anders
2002 - 2003	**Sr. Research Fellow** Department: Neurodegenerative disorders, National Brain Research Center, Manesar, India. Research project: Evaluation of the Molecular Basis of Pharmacological action of traditional medicinal preparations in treatment of mental illnesses including dementia. Principal Investigator: Dr. V. Ravindranath
2001 - 2002	**Research Associate** Department: Pharmacology, Biological Research - Drug Discovery, Glenmark Research Center, Navi-Mumbai, India. Research project: Pharmacological screening of: A. Beta-3 adrenergic agonists as anti-obesity/ diabetic agents. B. Phosphodiesterase (PDE) IV-inhibitors as anti-inflammatory/ asthmatic agents.

Scholastic Achievements:

1. Post-doctoral fellowship for European Union funded project 'Diabesity' (2006-)
2. Post-doctoral fellowship for research project funded by DFG (German Research Foundation) (2005-2006)
3. Doctoral fellowship for research project funded by DFG (2003-2005)
4. Sr. Research Fellowship, Department of Biotechnology, India (2002-2003)
5. Fellowship award for M.S. course (IIT, India: GATE Pharmacy) - (1999-2000)

Membership of professional societies

1. American Society for Pharmacology and Experimental Therapeutics (pending)
2. American Physiological Society
3. North American Association for the Study of Obesity
4. Indian Academy of Neurosciences, India

Abstracts and conference proceedings: Several

List of publications:

1. **Patole PS**, Zecher D, Pawar RD, Gröne HJ, Schlöndorff D, Anders HJ. G-rich DNA suppresses systemic lupus. J Am Soc Nephrol 2005; 16: 3273-3280.
2. **Patole PS**, Schubert S, Meßmer K, Khandoga S, Khandoga A, Segerer S, Henger A, Kretzler M, Werner M, Krombach F, Schlöndorff D, Anders HJ. Toll-like Receptor-4: renal cells and bone marrow cells signal for neutrophil recruitment during pyelonephritis. Kidney Int. 2005; 68: 2582-2587.
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