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 T*GG AGG GG*A AGT-3′, CpG-ODN 1668 5′-TCG ATG A*CG* TTC CTG ATG CT-3′, and GpC-ODN 1720 5′-TCG ATG A*GC* 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-

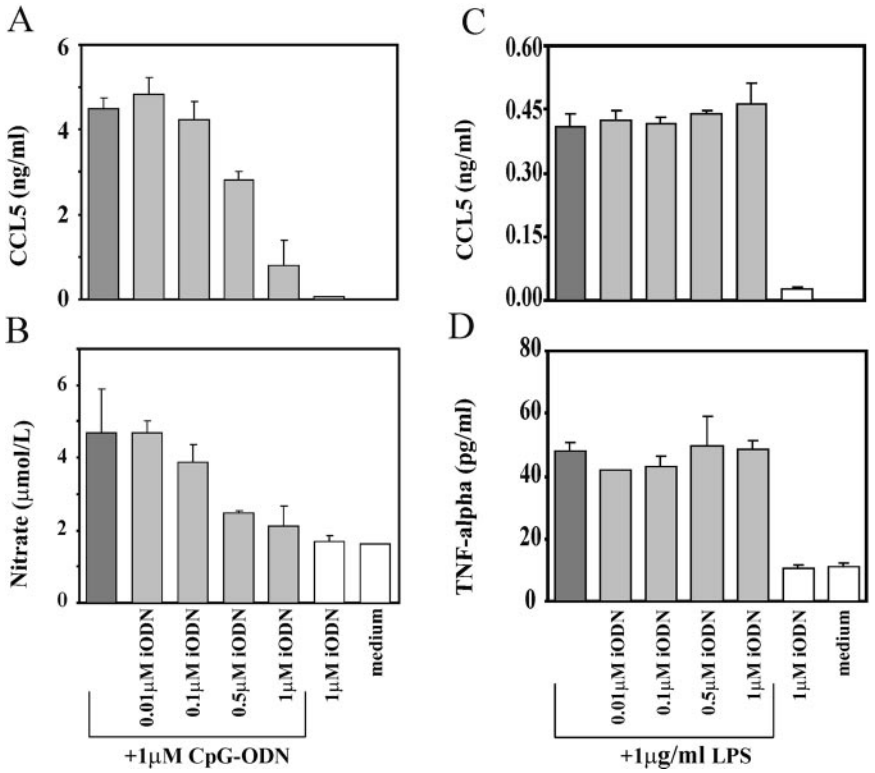

Figure 1. Oligodeoxyribonucleotides (ODN) 2114 block CpG-DNA–induced effects *in vitro*. Cultured J774 macrophages were incubated with CpG-ODN, CpG-ODN with different concentrations of ODN 2114, or standard medium without supplements for 24 h as indicated. (A) CCL5 was measured in supernatants by ELISA. (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.

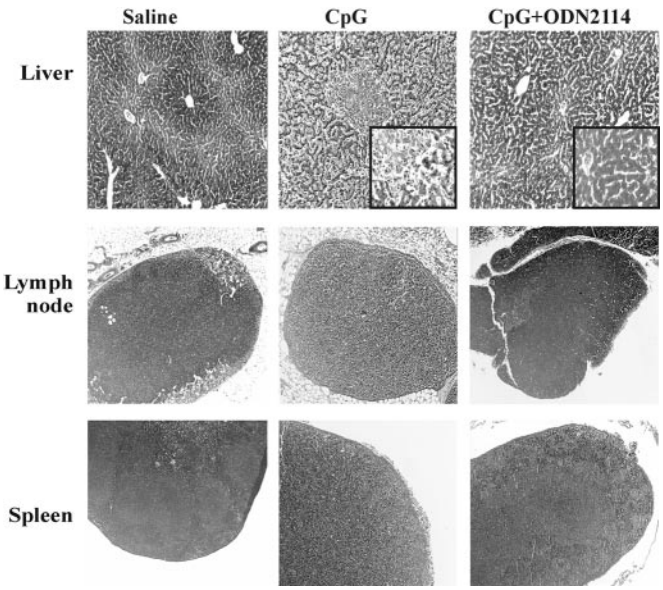

Figure 2. ODN 2114 block CpG-DNA–induced effects *in vivo*. 129Sv mice received intraperitoneal injections of saline or 60 μg of GpC-ODN, CpG-ODN, or CpG-ODN + ODN 2114 or saline daily for 14 d (n = 5 in each group; saline not shown). Respective organs were stained with periodic acid-Schiff (PAS). Magnification,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

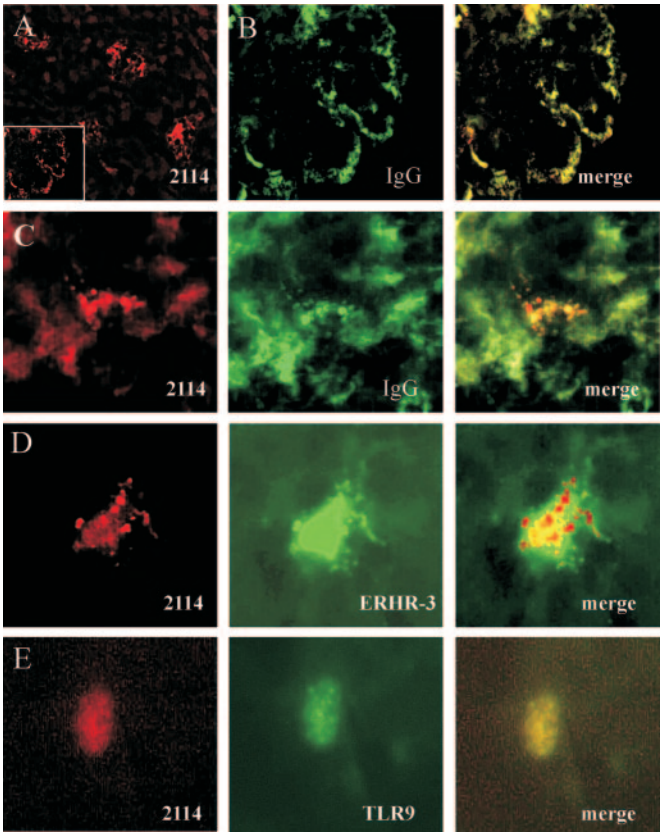

Figure 3. Localization of ODN 2114 in kidneys of MRL$^{lpr/lpr}$ mice after intravenous injection. Rhodamine-labeled ODN 2114 were injected intravenously into 16-wk-old MRL$^{lpr/lpr}$ mice, and renal tissue was harvested 2 h later. (A) Fluorescence imaging of frozen sections showed uptake of labeled 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 $MLR^{lpr/lpr}$ 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-

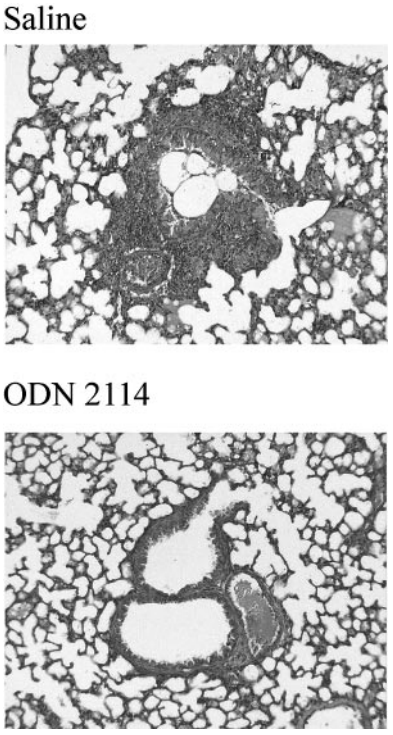

Figure 4. Autoimmune lung injury in MRL$^{lpr/lpr}$ mice. Lung sections that were taken from 24-wk-old MRL$^{lpr/lpr}$ mice were stained with PAS. Note that ODN 2114–treated MRL$^{lpr/lpr}$ mice show less peribronchiolar and perivascular inflammatory cell infiltrates as compared with saline-injected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

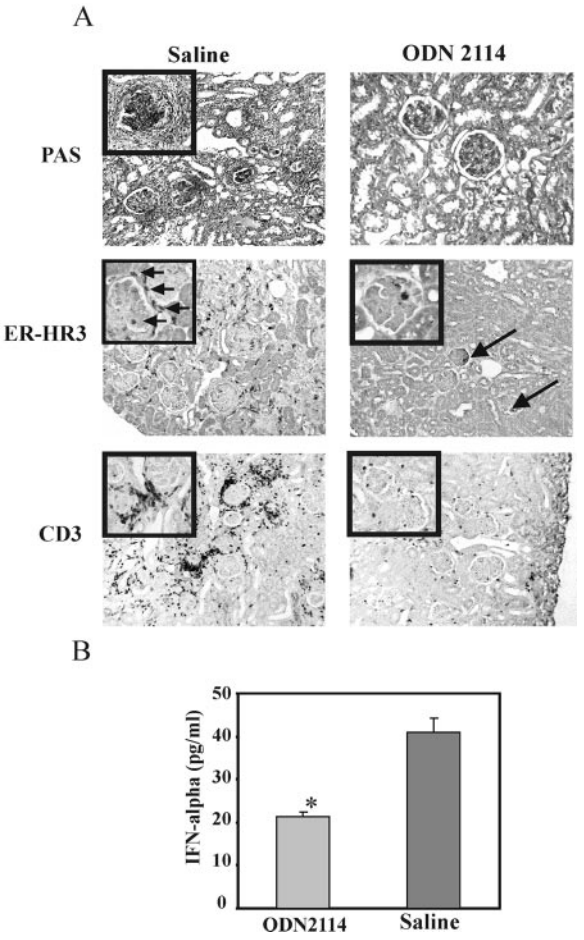

Figure 5. Lupus nephritis in MRL$^{lpr/lpr}$ mice. (A) Renal sections were stained with PAS, EHRH-3, and CD3 as indicated. Images are representative of 10 mice in each group. Note that ODN 2114–treated MRL$^{lpr/lpr}$ mice show less periglomerular and interstitial inflammatory cell infiltrates as compared 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

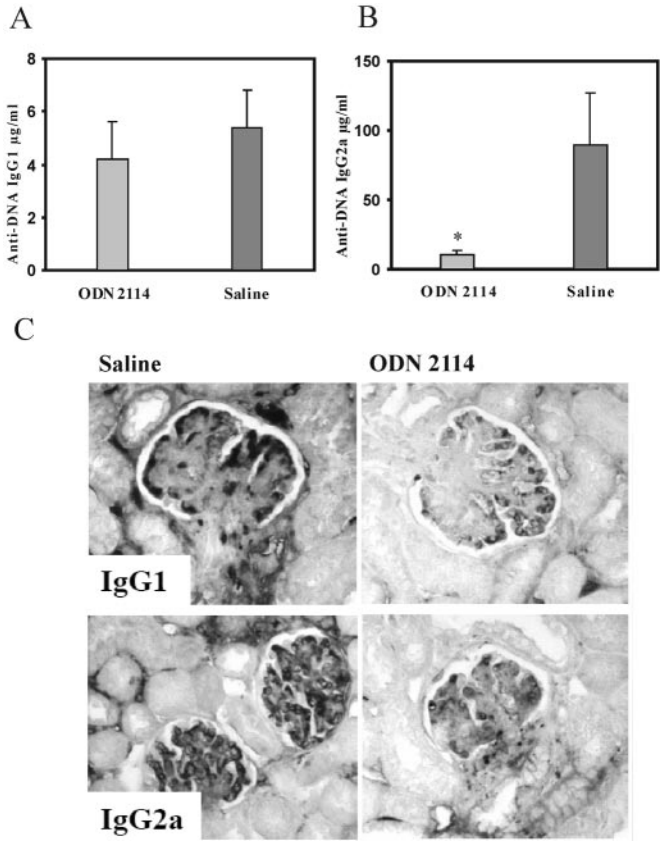

Figure 6. Serum dsDNA autoantibody levels and glomerular immune complex deposits in MRL$^{lpr/lpr}$ mice. Serum dsDNA autoantibody IgG1 (A) and IgG2a (B) levels were determined by ELISA ($n = 8$ to 10). Data are means ± SEM. *$P < 0.05$ *versus* saline. (C) Renal sections were stained for IgG1 and IgG2a, 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.

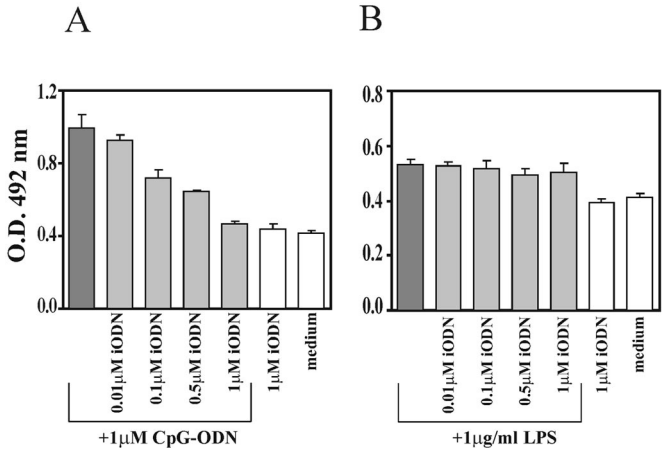

Figure 7. B cell activation in MRL$^{lpr/lpr}$ mice. (A) B cells were isolated from MRL$^{lpr/lpr}$ mice as described in Materials and Methods. B cells were incubated with CpG-ODN, CpG-ODN plus different concentrations of ODN 2114, or standard medium without supplements for 72 h as indicated. B cell 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.

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