Inhibition of Toll-Like Receptor-7 (TLR-7) or TLR-7 plus TLR-9 Attenuates Glomerulonephritis and Lung Injury in Experimental Lupus

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Small nuclear RNA and associated lupus autoantigens activate B cells and dendritic cells via Toll-like receptor-7 (TLR-7); therefore, TLR-7 may represent a potential therapeutic target in lupus. MRL lpr mice were administered an injection of either saline or synthetic oligodeoxynucleotides with immunoregulatory sequences (IRS) that specifically block signaling via TLR-7 (IRS 661) or via TLR-7 and TLR-9 (IRS 954, which uses a active sequence from IRS 661 along with a TLR-9 inhibitory sequence) from weeks 11 to 24 of age. IRS 661 and IRS 954 both significantly reduced the weight of spleen and lymph nodes as well as serum levels of TNF as compared with saline-treated MRL lpr mice. Only IRS 661 but not IRS 954 significantly reduced serum levels of IL-12p40, anti-dsDNA IgG2a, IgG2b, and anti-Smith IgG. Both IRS localized to the kidney after intraperitoneal injection and significantly improved the activity index and chronicity index for lupus nephritis in MRL lpr mice. This was associated with significant reduction of renal glomerular and interstitial macrophage infiltrates and the number of interstitial T cells. Autoimmune lung injury was also attenuated with IRS 661 and IRS 954. These data demonstrate that TLR-7 associated with significant reduction of renal glomerular and interstitial macrophage infiltrates and the number of interstitial T cells. Autoimmune lung injury was also attenuated with IRS 661 and IRS 954. These data demonstrate that TLR-7 may represent a potential therapeutic target in systemic lupus erythematosus.

S
ystemic lupus erythematosus (SLE) is characterized by spontaneous lymphoproliferation, expansion of autoreactive B and T cells, and production of polyclonal autoantibodies against numerous nuclear antigens (1,2). Although the role of nuclear autoantigens for the pathogenesis of immune complex disease and T cell–mediated autoimmune tissue injury is generally accepted, their role as endogenous immune adjuvants remains under debate. Studies from Marshak-Rothstein’s laboratory (3,4) first suggested that chromatin or ssRNA components of SLE immunocomplexes can activate Toll-like receptor-9 (TLR-9) or TLR-7 in intracellular endosomes of B cells, respectively. Thereby, such nucleic acid–containing immune complexes were shown to activate autoreactive B cells and autoantibody production independent from T cell control. In fact, immune complexes that are prepared from sera of patients with lupus also activate TLR-9 on human dendritic cells, an effect that is sensitive to DNAse digestion (5). However, data from Medzhitov’s group (6) showed that self-DNA cannot be detected by endosomal TLR-9 unless a chimeric TLR-9 is “relocated” to the cell surface. This study rather suggested that the endosomal localization of TLR-9 represents a mechanism to prevent immune stimulation by self-DNA.

In vivo studies remain the appropriate tool to test the significance of such in vitro phenomena. For example, Tlr7 overexpression is associated with antinuclear autoantibody production and lupus-like disease in mice (7,8). Contrary, backcrossing Tlr7-deficient mice into lupus-prone MRL<sup>lpr</sup>/<sup>lpr</sup> mice is associated with less lymphoproliferation, less activation of plasmacytoid dendritic cells, and less autoimmune lung and kidney injury (9). A detailed comparison of antinuclear antibodies in Tlr7-deficient and wild-type mice revealed that TLR-7 contributes to the production of antibodies against the Smith antigen of Sm-RNP RNA (9). This is interesting because the lupus autoantigen U1snRNP RNA was identified as an endogenous ligand for TLR-7 (10,11). Furthermore, 564 Igi<sup>−/−</sup> transgenic mice produce large amounts of anti-RNA, -DNA, and -nucleosome antibodies of the IgG<sub>2a</sub> and IgG<sub>2b</sub> isotype that cause nephritis, a phenomenon that was abrogated in Tlr7-deficient mice (12).

The contribution of TLR-9 to the pathogenesis of lupus must involve different mechanisms. Several groups backcrossed Tlr9-deficient mice into lupus-prone mouse strains and found the autoimmune tissue injury to be aggravated as compared with their respective wild-type strains of mice (9,13–15). Attempts to identify the causal mechanism have not been successful, but obviously lack of TLR-9 is associated with less chromatin-specific autoantibodies and with a higher activation state of plasmacytoid dendritic cells (9). These findings challenge the
concept of a general proinflammatory role of endogenous TLR-9 ligands in the pathogenesis of lupus (16). However, targeting these TLR may still represent a valuable option for the treatment of lupus (17,18). This is indicated by the therapeutic effects of chloroquine in patients with lupus, an inhibitor of endosomal acidification and TLR activation (19). However, because TLR-7 and TLR-9 deficiency have opposing effects on experimental lupus, it becomes necessary to dissect therapeutic blockade of these TLR by specific antagonists. Such antagonists were identified by Barrat et al. (20), who characterized the inhibitory effects of synthetic oligodeoxynucleotides with immunoregulatory sequences (IRS) on TLR-7 signaling in vitro. On the basis of this evidence of a proinflammatory role of TLR-9 for lupus, we hypothesized that antagonism of TLR-7 would have beneficial effects on experimental lupus.

Materials and Methods

Phosphothioate IRS Oligodeoxynucleotides and Other TLR Ligands

The following endotoxin-free oligodeoxynucleotides (ODN; TIB Molbiol, Berlin, Germany) were used for in vitro or in vivo studies: IRS 661 5'-TGCTTGCAAGCTTGCAAGCA-3'; IRS 954 5'-TGCTCTGCCGAGGGTTGT-3'; and CpG-DNA 1668 5'-TCGATGACGTTCCTGATGCT-3'. Control ODNs were 5'-TCTTGCAGGTTAAGT-3' and 5'-TCTGGCGGAAAAGT-3'.

Studies with Spleen Monocytes

Adherent spleen monocytes were prepared from 10-wk-old female MRL+/−/− mice and kept in culture as described previously (21). Cells were stimulated with either medium or various concentrations of imiquimod (Sequoia Research Products Ltd., Oxford, UK) or CpG-DNA, Lps, and poly (I:C) (Invivogen, San Diego, CA) with or without inhibitory oligos as indicated. Supernatants were harvested after 24 h for ELISA.

Animal Studies

Ten-week-old female MRL+/−/− mice were obtained from Jackson Laboratories (Bar Harbor, MA) and were kept in filter-top cages under a 12-h light and dark cycle. Autoclaved water and standard chow (Sniff, Soest, Germany) were available ad libitum. For assessment of the distribution of injected IRS 661, 100 μg of 3’-rhodamine–labeled IRS 661 was injected intraperitoneally into 16-wk-old MRL+/−/− mice. Tissues were collected 2 h after injection and subjected to further analysis as recently described (21). Various groups of mice were treated with saline, IRS 661, or IRS 954 at a dosage of 40 μg on alternate days for weeks 11 to 24 of age. Blood samples were collected after 3 h of the last injection by retro-orbital puncture. Mice were killed by cervical dislocation under anesthesia with inhaled ether. Blood and urine samples were collected from each mouse at the end of the study period, and the urine protein/creatinine ratio was determined as described previously (9). Cytokine Levels

Serum cytokine levels and cell culture supernatants were determined using the following commercial ELISA kits for IL-6, IL-12p40 (BD OptEIA, San Diego, CA), TNF-α (Biologend, San Diego, CA), and IFN-α (Bethyl Labs, Montgomery, TX) following the protocols provided by the manufacturers.

Immunofluorescence

For qualitative analysis, glomerular cells were counted in 10 cortical glomeruli per section. Semiquantitative immunofluorescence on specific slides (BioRad Laboratories, Munich, Germany) was performed with 1:100 dilution of serum and scored for the intensity of kinetoplast DNA from 0 to 4 as described previously (9).

Anti-dsDNA. NUNC maxisorp ELISA plates were coated with polyclonal IgG (Trevena, Gaithersburg, MD) and mouse embryonic cell dsDNA. After incubation with mouse serum, dsDNA-specific IgG, IgG2a, IgG2b, and IgG3 were detected by ELISA (Bethyl Labs, Montgomery, TX).

Anti-Smith. NUNC maxisorp ELISA plates were coated with Smith (Sm) antigen (Immunovision, Springdale, AR). The Sm IgG (Y12) antibody (GeneFed, San Antonio, TX) was used for standard. A horse-radish peroxidase–conjugated goat anti-mouse IgG (Rockland, Gilbertsville, PA) was used for detection as described previously (9). The same procedure was followed for anti-Sm/RNP as for Sm-antigen except that the ELISA plates were captured with Sm-RNP complex (Immunovision) instead of Sm antigen.

Rheumatoid Factor. ELISA plates were coated with 10 μg/ml rabbit IgG (Jackson ImmunoResearch, West Grove, PA) overnight at 4°C. Serum samples were diluted 1:100; C57BL/6 10-wk mouse serum was used as negative control. Horseradish peroxidase–conjugated anti-mouse IgG was used as secondary antibody.

Flow Cytometry

Flow cytometry of splenocytes was performed as described previously (23). The following primary antibodies were used to identify T cell subsets: FITC-labeled hamster anti-mouse CD3 (clone 145–2C11; BD Biosciences, Heidelberg, Germany), allophycocyanin-labeled rat anti-mouse CD4 (clone RM4–5; BD Biosciences), peridinin chlorophyll–labeled rat anti-mouse CD8 (clone 53 to 6.7; BD Biosciences), peridinin chlorophyll–labeled rat IgG2a (clone R35–95; BD Biosciences), and allophycocyanin-labeled rat IgG2a (clone R35–95; BD Biosciences), and PE-labeled rat IgG2a (clone R35–95; BD Biosciences) were used as isotype controls, respectively.
Real-Time Quantitative (TaqMan) Reverse Transcription–PCR

Real-time reverse transcription–PCR on RNA that was isolated from cultured cells or renal tissue was performed as described previously (21). Controls that consisted of ddH₂O were negative for target and housekeeper genes. Oligonucleotide primer (300 nM) and probes (100 nM) were from PE Biosystems (Weiterstadt, Germany) and used as follows: Ccr2 forward primer 5′-CCTGGGAATGTAACGT-GTGA-3′; reverse primer 5′-ACA-AAGGCATAATGACGATTATG-3′, 6 FAM 5′-TGACAGCATTTACAGCCGCACTGCA-3′; Ccr5 forward primer 5′-CAAGCAATCTGATGCCTGCAAA-3′, reverse primer 5′-TCTCTACTCCAAGCTGTAGAAA-3′, 6 FAM 5′-CTCTATACCCGATCCAGGAACATGAAGTTT-3′. Primers for Ccl2, Ccl5, and 18s rRNA were predeveloped TaqMan assay reagent from PE Biosystems.

Statistical Analyses

Statistics were done using GraphPad Prism 4.03 version (GraphPad, San Diego, CA). Data were expressed as means ± SEM. Data were analyzed using unpaired two-tailed t test for comparison between two groups. One-way ANOVA followed by post hoc Bonferroni test was used for multiple comparisons. For nonparametric analysis of two groups, two-tailed Fisher exact test and Mann-Whitney U test were performed.

Results

IRS 661 Inhibits the Imiquimod-Induced Release of IL-6 and TNF-α and IRS 954 Inhibits the Imiquimod- and CpG-ODN–Induced Release of IL-6 and TNF-α in Spleen Monocytes from MRLlpr/lpr Mice

Oligodeoxynucleotides with IRS specifically block TLR-7– or TLR-7/TLR-9–induced cytokine production (24,25). IRS 661 dose-dependently inhibited imiquimod-induced production of IL-6 and TNF-α (Figures 1A and 2A). IRS 954 but not IRS 661 dose-dependently inhibited CpG-DNA–induced production of IL-6 and TNF-α (Figures 1B and 2B). IRS 954 but not IRS 661 showed significant dose-dependent inhibition of IL-6 and TNF-α production when the cells were incubated with imiquimod plus CpG-DNA (Figures 1C and 2C). By contrast, neither IRS affected IL-6 or TNF-α production induced by LPS or pI/C RNA (Figures 1D and 2D). The same set of experiments were repeated for the two control oligos. Neither of the control oligos showed any inhibition of the IL-6 and TNF-α release (Figures 1, E through H, and 2, E through H). These data confirm that IRS 661 and IRS 954 act as specific antagonists for either TLR-7– or TLR-7/TLR-9–induced activation of monocytes in vitro.

IRS Block Imiquimod- or CpG-ODN–Induced IL-12p40 Serum Levels in MRLlpr/lpr Mice

In view of the in vitro efficacy of IRS 661 and IRS 954 to block imiquimod- or CpG-DNA–induced cytokine production in spleen monocytes, we questioned whether these effects also apply to MRLlpr/lpr mice in vivo. Five groups of 14-wk-old MRLlpr/lpr mice (n = 5 to 8) received intraperitoneal injections with a single dose of saline, 25 μg of imiquimod, or 40 μg of CpG-DNA in the presence or absence of a single dose of IRS 661 or IRS 954 (40 μg) 30 min before the administration of the agonist. Serum IL-12p40 levels were determined after 3 h of agonist administration by ELISA. Imiquimod and CpG-DNA significantly induced the serum levels of IL-12p40 as compared with saline-injected control mice (Figure 3). IRS 661 and IRS 954 both significantly reduced the imiquimod-induced induction of IL-12p40. IRS 954 significantly reduced the CpG-DNA–induced induction of IL-12p40. Thus, 40 μg of IRS 661 and IRS 954 was observed to block TLR-7 or TLR-9 signaling in vivo.

Serum Cytokine Levels of MRLlpr/lpr Mice Administered Injections of IRS from Weeks 11 to 24

TLR-7 and TLR-9 signaling activates the expression of proinflammatory cytokines and type I interferons (26). Therefore, we examined whether injections with IRS 661 or IRS 954 from weeks 11 to 24 of age affected the serum levels of these mediators in MRLlpr/lpr mice by ELISA. In fact, both IRS 661 and IRS 954 significantly reduced the serum levels of TNF as compared with saline-injected MRLlpr/lpr mice (Table 1). IL-6 levels were also decreased with IRS 954; the trend did not reach statistical significance for IRS 661. Contrary, serum IL-12p40 levels were found to be reduced significantly only with IRS 661 injections but not with IRS 954. IFN-α levels were not affected by either IRS.

IRS 661 But Not IRS 954 Reduces Serum Autoantibody Levels in MRLlpr/lpr Mice

TLR-7 and TLR-9 were shown to have distinct effects on the production of antinuclear antibodies in MRLlpr/lpr mice (9). Therefore, we assessed whether injections with IRS 661 and IRS 954 from weeks 11 to 24 of age affected serum IgG and autoantibody levels. Serum levels of IgG1, IgG2a, IgG2b, and IgG3 were not significantly affected by either IRS. Antinuclear antibody staining on Hep2 cells revealed positive nuclear signals in each MRLlpr/lpr mouse of all groups (Figure 4A). In saline-treated MRLlpr/lpr mice, a homogeneous nuclear staining was most common. Positivity of condensed chromosomal areas of mitotic cells was indicative of autoantibodies directed against chromatin (data not shown). A speckled nuclear staining pattern was less frequent, and a cytoplasmic staining pattern was not observed. In IRS 661–treated MRLlpr/lpr mice, the homogeneous pattern was less frequent and speckled pattern was somewhat more frequent as compared with saline-treated mice. IRS 954 injections were associated with an equal distribution of homogeneous and speckled nuclear staining patterns (Figure 4A). Antinuclear antibodies specific for dsDNA were studied by the specific binding to kinetoplast DNA of Crithidia luciliae. IRS 661 but not IRS 954 significantly reduced the indirect immunofluorescence intensity of mouse serum (at a dilution of 1:100) incubated with Crithidia luciliae substrates (Figure 4B). Assessing the binding of serum to polylsine-linked dsDNA by ELISA is another method to determine the specificity of antinuclear antibodies. IRS 661 but not IRS 954 significantly reduced serum levels of dsDNA-specific IgG2a, IgG3, and anti-Sm IgG antibodies as compared with saline-treated MRLlpr/lpr mice (Table 1). Anti-Sm IgG and anti-Sm RNP IgG were reduced with IRS 661 treatment to the same extent (latter not shown).
Anti-dsDNA IgG1 and IgG3 levels as well as rheumatoid factors were not affected by either IRS. These findings are suggestive of an inhibitory effect of IRS 661 on the production of anti-dsDNA IgG2a and IgG2b antibodies, which are known to induce glomerular pathology in MRL-lpr/lpr mice by activating complement (27,28). It is interesting that glomerular deposits of IgG2a and complement factor C3c were significantly reduced in both IRS 661- and IRS 954-injected MRL-lpr/lpr mice (Figure 5, Table 2). Together these data indicate that injections with IRS 661 but not with IRS 954 reduce the production of selected autoantibodies. Glomerular complement activation was reduced with either IRS, independent of circulating IgG2a isotype levels, suggesting that IRS can modulate immune complex deposition and complement activation independent of circulating autoantibody levels in MRL-lpr/lpr mice.

Distribution of Labeled IRS 661 and IRS 954 in Kidney of Autoimmune MRL-lpr/lpr Mice
Do IRS 661 and IRS 954 mediate local effects in nephritic kidneys of MRL-lpr/lpr mice? This would require that they lo-
calize to the kidney after intraperitoneal injection, which was studied by assessment of the distribution of rhodamine-labeled IRS 661 and IRS 954 in nephritic kidneys of 16-wk-old MRLlpr/lpr mice. After injection, IRS 661 was detected in a glomerular as well as a tubular staining pattern (Figure 6A). Co-staining with an anti-mouse IgG antibody showed that labeled IRS 661 partially co-localized with glomerular IgG deposits (Figure 6A). However, tubular (Figure 6B, left) and glomerular (Figure 6B, right) IRS 661-positive granules that were negative for IgG were also observed, suggesting the uptake of IRS 661 into endosomal compartments of glomerular and tubular epithelial cells. Co-staining with a Mac-2 antibody showed that IRS 661 was also taken up by 52% of glomerular and 63% of interstitial macrophages (Figure 6C). The distribution of IRS 954 after intraperitoneal injection was identical to that of IRS 661 (data not shown). Nephritic MRLlpr/lpr mice that were administered an injection of rhodamine only did not show any renal deposits (data not shown). These findings show that injected IRS 661 and IRS 954 localize to the kidney and are taken up by glomerular cells, tubular epithelial cells, and intrarenal macrophages.

Figure 2. IRS 661 and IRS 954 specifically inhibit imiquimod- and CpG-DNA-induced TNF-α release in spleen monocytes from MRLlpr/lpr mice in vitro. Spleen monocytes from MRLlpr/lpr mice were incubated with imiquimod and IRS 661 at various concentrations as indicated or standard medium without supplements for 24 h as indicated. (A through C) Spleen monocytes were incubated with either imiquimod or CpG-DNA alone or imiquimod plus CpG-DNA. (D) Cells were stimulated with either ultrapure LPS or pIC-RNA. In A through D, TNF-α production was measured in supernatants by ELISA. Data are means ± SEM from two identical experiments. *P < 0.05 versus imiquimod or CpG stimulation, by one-way ANOVA followed by post hoc Bonferroni test.
Figure 3. IRS 661 and IRS 954 block imiquimod- and CpG-DNA-induced IL-12p40 production in vivo. Groups of 14-wk-old female MRL1pr/lpr mice (n = 5) were administered an injection of saline or a single dose of 25 μg of imiquimod (imi), 40 μg of CpG-DNA (CpG), or 40 μg of IRS as indicated. Serum IL-12p40 levels were determined after 3 h by ELISA. Data are means ± SEM. *P < 0.05 versus saline. IRS, immunoregulatory sequence.

IRS 661 and IRS 954 Reduce Lymphoproliferation and Protect MRL1pr/lpr Mice from Autoimmune Tissue Injury

On the basis of the reported phenotype of Tlr7-deficient MRL1pr/lpr mice (9) and our own data with IRS 661, a course of injections with IRS 661 from weeks 11 to 24 of age might have beneficial effects on experimental lupus of MRL1pr/lpr mice. Therefore, the trend to decreased proteinuria in IRS 661-treated mice (Figure 7), indicates that IRS-induced prevention of severe lupus nephritis was associated with decreased intrarenal inflammation. Proteinuria is a clinically important marker of lupus nephritis, but usually it shows a high interindividual variability in female MRL1pr/lpr mice. Therefore, the trend to decreased proteinuria in IRS 661- and IRS 954–treated mice did not reach statistical significance. Furthermore, we evaluated extrarenal autoimmune tissue injury in lungs of mice. Lungs of IRS-treated mice showed less peribronchiolar and

Table 1. Serum factors and lymphoproliferation in 24-wk-old MRL1pr/lpr mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Saline</th>
<th>IRS 661</th>
<th>IRS 954</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum IL-6 (pg/ml)</td>
<td>90.3 ± 31.3</td>
<td>58.1 ± 16.8</td>
<td>26.9 ± 3.1b</td>
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<tr>
<td>TNF (pg/ml)</td>
<td>23.9 ± 4.0</td>
<td>4.4 ± 2.3b</td>
<td>5.8 ± 4.4b</td>
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<tr>
<td>IL-12p40 (ng/ml)</td>
<td>5.3 ± 1.1</td>
<td>3.4 ± 0.7b</td>
<td>4.6 ± 0.7</td>
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<tr>
<td>INF-α (pg/ml)</td>
<td>35.4 ± 8.3</td>
<td>30.6 ± 7.5</td>
<td>37.5 ± 7.9</td>
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<tr>
<td>IgG1 (mg/ml)</td>
<td>38.4 ± 4.7</td>
<td>29.4 ± 4.8</td>
<td>34.4 ± 4.0</td>
</tr>
<tr>
<td>IgG2a (mg/ml)</td>
<td>12.0 ± 1.3</td>
<td>9.1 ± 1.7</td>
<td>12.9 ± 1.1</td>
</tr>
<tr>
<td>IgG2b (mg/ml)</td>
<td>9.2 ± 2.1</td>
<td>6.1 ± 1.1</td>
<td>5.8 ± 1.4</td>
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<tr>
<td>IgG3 (mg/ml)</td>
<td>3.2 ± 0.3</td>
<td>3.3 ± 0.3</td>
<td>2.9 ± 0.2</td>
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<tr>
<td>Anti-dsDNA IgG1 (μg/ml)</td>
<td>15.0 ± 6.3</td>
<td>14.7 ± 6.2</td>
<td>34.2 ± 12.7</td>
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<tr>
<td>IgG2a (μg/ml)</td>
<td>38.0 ± 7.1</td>
<td>18.9 ± 2.7b</td>
<td>56.8 ± 12.9c</td>
</tr>
<tr>
<td>IgG2b (μg/ml)</td>
<td>46.3 ± 9.5</td>
<td>18.2 ± 2.3b</td>
<td>34.8 ± 7.2c</td>
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<tr>
<td>IgG3 (μg/ml)</td>
<td>27.7 ± 3.7</td>
<td>21.8 ± 2.1</td>
<td>24.1 ± 2.2</td>
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<tr>
<td>Anti-SM IgG (μg/ml)</td>
<td>8.8 ± 3.4</td>
<td>4.4 ± 1.1b</td>
<td>10.2 ± 2.8c</td>
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<tr>
<td>Rheumatoid factor (OD [450 nm])</td>
<td>0.88 ± 0.14</td>
<td>0.86 ± 0.13</td>
<td>0.81 ± 0.13</td>
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<td>Lymph node weight (mg/g body wt)</td>
<td>16.7 ± 1.7</td>
<td>8.5 ± 1.6b</td>
<td>9.1 ± 0.6b</td>
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<td>Spleen weight (mg/g body wt)</td>
<td>8.9 ± 0.8</td>
<td>6.7 ± 0.5b</td>
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<td>CD4+ cells (% spleen)</td>
<td>18.1 ± 3.4</td>
<td>17.6 ± 4.8</td>
<td>19.4 ± 4.3</td>
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<tr>
<td>CD8+ cells (% spleen)</td>
<td>8.7 ± 1.7</td>
<td>9.7 ± 2.7</td>
<td>9.0 ± 2.0</td>
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<td>CD4–/CD8– (% spleen)</td>
<td>62.3 ± 1.4</td>
<td>51.7 ± 3.4b</td>
<td>57.5 ± 1.5b</td>
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a n = 10 per group; data are means ± SEM. IRS, immunoregulatory sequence.
bP < 0.05 IRS 661 or IRS 954 versus saline.
cP < 0.05 IRS 954 versus IRS 661 by unpaired t test.
perivascular inflammatory cell infiltrates compared with saline-treated controls (Table 2, Figure 7). Together these data show that IRS 661 and IRS 954 both reduced autoimmune tissue injury in lungs and kidneys of MRL1pr/lpr mice.

**Discussion**

Nuclear lupus autoantigens trigger autoantibody production and the release of proinflammatory mediators by activating TLR-7 and TLR-9 in B cells and dendritic cells in vitro (3-5,10,11,20,29). Blocking TLR-9 signaling with injections of specific ODN can ameliorate lupus nephritis in MRL1pr/lpr mice (21,30); therefore, we hypothesized that antagonism of TLR-7 or TLR-7 plus TLR-9 may be as effective in vivo.

Table 2. Autoimmune tissue injury in 24-wk-old MRL1pr/lpr mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Saline</th>
<th>IRS 661</th>
<th>IRS 954</th>
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</thead>
<tbody>
<tr>
<td>Kidney injury</td>
<td></td>
<td></td>
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<tr>
<td>activity index (score)</td>
<td>14.8 ± 3.2</td>
<td>5.3 ± 1.7b</td>
<td>7.0 ± 1.8b</td>
</tr>
<tr>
<td>chronicity index (score)</td>
<td>4.0 ± 1.8</td>
<td>1.0 ± 0.7b</td>
<td>1.4 ± 1.0b</td>
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<tr>
<td>glomerular C3 (score)</td>
<td>2.4 ± 0.7</td>
<td>0.7 ± 0.3b</td>
<td>0.9 ± 0.3b</td>
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<tr>
<td>IgG1 (score)</td>
<td>2.1 ± 0.2</td>
<td>2.1 ± 0.3</td>
<td>1.9 ± 0.3</td>
</tr>
<tr>
<td>IgG2a (score)</td>
<td>1.6 ± 0.6</td>
<td>0.7 ± 0.4b</td>
<td>0.7 ± 0.3b</td>
</tr>
<tr>
<td>glomerular Mac-2+ cells (cells/glomerulus)</td>
<td>6.9 ± 2.4</td>
<td>2.0 ± 1.0b</td>
<td>2.9 ± 1.5b</td>
</tr>
<tr>
<td>interstitial Mac-2+ cells (cells/hpf)</td>
<td>11.5 ± 3.2</td>
<td>3.7 ± 0.8b</td>
<td>5.2 ± 5.0b</td>
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<tr>
<td>interstitial CD3+ cells (cells/hpf)</td>
<td>23.6 ± 5.3</td>
<td>12.5 ± 2.8</td>
<td>9.2 ± 3.1b</td>
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<tr>
<td>Urinary albumin/creatinine (mg/mg)</td>
<td>4.3 ± 1.6</td>
<td>1.7 ± 0.8</td>
<td>2.6 ± 0.7</td>
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<tr>
<td>Lung injury (score)</td>
<td>2.1 ± 0.5</td>
<td>1.0 ± 0.6b</td>
<td>1.3 ± 0.6b</td>
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</tbody>
</table>

*a n = 10 per group; data are means ± SEM. hpf, high-power field.

bp < 0.05 IRS 661 or 954 versus saline.
TLR7 Blockade with IRS 661

Injections with IRS 661 were started from week 11 of age and substantially reduced autoimmune tissue lung and kidney injury at week 24 of age, which is consistent with the phenotype of Tlr7-deficient MRL^pr/lpr mice (9). These data support a role of TLR-7 for the mechanisms that foster the progression of autoimmune tissue injury in MRL^pr/lpr mice (e.g., the expansion of the CD4/CD8–double-negative autoreactive T cell population, immune complex–mediated local complement activation, and the local inflammatory response involving macrophage and T cell recruitment [1,2,31]). In fact, IRS 661 significantly reduced the number of CD4/CD8–double-negative T cells in spleen, a population that continuously expands in MRL^pr/lpr mice because of the inability to delete autoreactive and activated T cells in mice via the interaction of Fas with the Fas ligand in these mice (32). Again, our data from IRS 661–injected MRL^pr/lpr mice are consistent with the data reported from Tlr7-deficient MRL^pr/lpr mice (9).

Autoantibody production is another characteristic of MRL^pr/lpr mice and contributes to autoimmune tissue injury via immune complex formation and local complement activation. It is interesting that TLR-7 is particularly required to generate anti-Sm RNP IgG (9), and serum levels of anti-Sm RNP IgG were also reduced with injection of IRS 661. Furthermore, we found that IRS 661 reduced the serum levels of anti-dsDNA IgG_2a and IgG_2b. In fact, the glomerular deposits of IgG_2a and complement factor C3c both were reduced in IRS 661–treated mice, indicating a role for TLR-7 in the production of selected nephritogenic autoantibodies and immune complex glomerulonephritis in MRL^pr/lpr mice. By contrast, by using identical assay systems, our data and those reported by Christensen et al. (9) consistently show that blockade or lack of TLR-7 does not substantially reduce homogeneous nuclear and mitotic ANA staining of Hep2 cells. As a third mechanism, autoimmune tissue injury in MRL^pr/lpr mice is mediated by CC-chemokine–driven macrophage and T cell infiltrates. For example, in nephritidic MRL^pr/lpr mice, infiltrating macrophages and nonimmune renal cells produce CCL2 and CCL5, which triggers additional macrophage and T cell recruitment into the kidney (33). Because CC-chemokines are produced upon TLR-7 activation in macrophages, blocking intrarenal TLR-7 signaling may reduce the local production of CC-chemokines and subsequent recruitment of immune cells to the nephritic kidney. We demonstrate that when IRS 661 is injected into nephritic MRL^pr/lpr mice, it localized to tubular epithelial cells. 

Figure 6. Localization of IRS 661 and IRS 954 in kidneys of MRL^pr/lpr mice after intraperitoneal injection. Rhodamine-labeled IRS 661 and IRS 954 were intraperitoneally injected into 16-wk-old MRL^pr/lpr mice, and renal tissue was harvested 2 h later. (A) Fluorescence imaging of frozen sections showed uptake of labeled IRS 661 in glomeruli (encircled) in a mesangial and capillary staining pattern as well as in tubular epithelial cells. Co-staining with IgG showed partial co-localization of IRS 661 with glomerular IgG deposits. (B) At higher magnification, granular deposits of IRS 661 in tubular epithelial cells (left) and glomerular cells (right) can be seen. (C) Co-staining with a FITC-labeled Mac-2 antibody identified IRS 661 to co-localize with renal macrophages (arrow), whereas other renal macrophages remain IRS 661 negative. Magnification, ×400 in A and C.

Figure 7. Lupus nephritis and lung injury in MRL^pr/lpr mice. (A) Renal sections were stained with periodic acid-Schiff (PAS), Mac-2, and CD3 as indicated. Note that IRS 661– and IRS 954–treated MRL^pr/lpr mice show less periglomerular and interstitial inflammatory cell infiltrates as compared with saline-treated MRL^pr/lpr mice. (B) Lung sections taken from 24-wk-old MRL^pr/lpr mice of all groups were stained with PAS. Note that IRS 661– and IRS 954–treated MRL^pr/lpr mice show less peribronchiolar and perivascular inflammatory cell infiltrates as compared with saline-injected MRL^pr/lpr mice. Images are representative of 10 mice in each group. Magnification, ×200.
cells and intrarenal macrophages, but only the latter express TLR-7 (34). In fact, IRS 661 reduced the intrarenal production of CCL2 and CCL5 and renal macrophage and lymphocyte infiltrates in MRLlpr/lpr mice. Together, the beneficial effects of TLR-7 blockade with IRS 661 on autoimmune tissue injury of MRLlpr/lpr mice were associated with a reduction of CD4/CD8–double-negative T cells, the production of selected autoantibodies, and renal production of proinflammatory chemokines that are known to mediate immune cell recruitment into the nephritic kidney.

TLR-7/TLR-9 Blockade with IRS 954

Given the opposing effects of Tlr7 and Tlr9 deficiency in MRL1pr/lpr mice (9,14), the effects of IRS 954 were less predictable. Injections with IRS 954 from weeks 11 to 24 of age improved kidney and lung disease in MRL1pr/lpr mice, and no additive effects were observed as compared with IRS 661. The effects of IRS 954 on lupus nephritis and lung injury are comparable to what has been observed with oligonucleotide antagonists that are specific for TLR-9 only in the same lupus model (21) or in NZB/NZW mice (30). These findings support the concept that recognition of endogenous DNA and RNA molecules via TLR-7 and TLR-9 contribute to the progression of autoimmune tissue injury. This concept developed from studies that showed that lupus autoantigens in immune complexes with IgG prepared from autoimmune mice or patients with lupus can activate B cells and dendritic cells in vitro (3–5,10,11). These in vitro experiments and in vivo studies with TLR-9 antagonists are inconsistent with the aggravated phenotype of Tlr9-deficient MRL1pr/lpr mice and have caused us to question the specificity of the antagonists and the assay systems used for the analysis of serum autoantibodies (9,13–16). The data reported by Barrat et al. (20) and our own study demonstrated the specificity of IRS 954 for TLR-7 and TLR-9. Furthermore, by applying identical assay systems as in the study reported by Christensen et al. (9), we showed that IRS 954 has distinct effects on serum autoantibody levels as compared with IRS 661. For example, in contrast to IRS 661, IRS 954 did not affect serum dsDNA autoantibodies; TLR-9 antagonism provided by IRS 954 “neutralized” the suppressive effect of IRS 661 on TLR-7–mediated anti-dsDNA IgG2a, IgG2b, and anti-Sm IgG production. Furthermore, IRS 954 injections were associated with fewer mice with homogeneous nuclear staining on Hep2 cells, which is consistent with what has been observed in Tlr9-deficient MRL lpr/lpr mice (9). The finding that IRS 954 did not reduce dsDNA autoantibodies is potentially interesting because we previously found that ODN 2114, blocking TLR-9 only, reduced these antibodies in MRL1pr/lpr mice (21). Thus, the roles of TLR-7 and TLR-9 for the evolution of specific autoantibodies may be even more complex and requires a detailed analysis of immune cell subsets from TLR-7 and TLR-9 double-knockout cells, which are not yet available.

Conclusion

Our data suggest that delayed onset of oligonucleotide-based inhibition of TLR-7 reduces autoantibody production and prevents autoimmune tissue injury in experimental lupus. Combined blockade of TLR-7 and TLR-9 has no additive effects. These data support the concept that endogenous ligands of TLR-7 contribute to the pathogenesis of autoantibody production and autoimmune tissue injury in SLE and propose TLR-7 blockade as a novel therapeutic concept for lupus.

Acknowledgments

This work was supported by grants from the Deutsche Forschungsgemeinschaft (AN372/8-1, GRK 1202), the Fritz Thyssen Foundation, the EU Network of Excellence “MAIN” (FP6-502935), and the EU Integrated Project “INNOCHEM” (FP6-518167). S.S. was supported by a grant from the Else Kröner-Fresenius Stiftung and the Deutsche Forschungsgemeinschaft (SE888/4-1).

Parts of this project were prepared as a doctoral thesis at the Faculty of Medicine, University of Munich, by R.D.P.

The expert technical assistance of Dan Draganovic, Jana Mandelbaum, Ewa Radomska, and Stephanie Pfeiffer is gratefully acknowledged. We are grateful to Bruno Luckow for the generous gift of dsDNA.

Disclosures

None.

Figure 8. Renal mRNA expression of CC-chemokines and their chemokine receptors. The mRNA expression of CCL2 and CCL5 and their respective chemokine receptors CCR2 and CCR5 was determined by real-time reverse transcription–PCR from total RNA from kidneys of 24-wk-old MRL1pr/lpr mice. For analysis, RNA was pooled from 10 mice of each group. mRNA levels for saline-, IRS 661–, and IRS 954–treated MRL1pr/lpr mice are expressed in relation to the respective 18S rRNA expression of each kidney.
References


