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

Prashant S. Patole,* Hermann-Josef Gröne,† Stephan Segerer,* Raluca Ciubar,* Emilia Belemezova,* Anna Henger,* Matthias Kretzler,* Detlef Schlöndorff,* and Hans-Joachim Anders*

*Nephrological Center, Medical Policlinic, Ludwig-Maximilians-University, Munich, and †Division of Molecular and Cellular Pathology, German Cancer Research Center, Heidelberg, Germany

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 MRL1pr/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 MRL1pr/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 MRL1pr/lpr mice. Serum DNA autoantibody levels were unaltered upon systemic exposure to pI:C RNA in MRL1pr/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.


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 MRL1pr/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-I, 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.
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<sup>lpr/lpr</sup> 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<sup>lpr/lpr</sup> mice. In addition, 16-wk-old female MRL<sup>lpr/lpr</sup> 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 (pC) RNA (Sigma-Aldrich, Steinheim, Germany) in 100 μl of normal saline, (2) 50 μg of pC 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′-AUCUGUGUGUUGUCCUCACGAGAUCAUGAUGUAAAAG-3′) and 3′-UAGACCCAACAAGGGUGGGUC-UAGUGGAGUACCA-5′). The viral RNA was injected intravenously into MRL<sup>lpr/lpr</sup> 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 pC RNA to induce cell activation, 4-wk-old MRL<sup>lpr/lpr</sup> mice were divided in three groups of two mice each. Each mouse received 500 μg/ml pC RNA, CpG, or pC 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 analyzer (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<sub>2a</sub> (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

**Immunohistochemistry**

Immunohistochemical staining was performed on paraffin-embedded sections as described (10) using the following primary antibodies: anti-TLR3 (1:50; IMG516; Imegenx, San Diego, CA), anti–ERHR-3 (1:50; 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<sub>2a</sub> (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<sup>lpr/lpr</sup> 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<sup>lpr/lpr</sup> mice as described previously (10). Spleen monocytes were treated with medium control or 100 μg/ml pC RNA and pC 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 described 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 Pharningen), 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 (TagMan) 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<sub>2</sub>O 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-
ward primer 5'-CGAAAGTGGACTTGTCATCAAATC-3', reverse primer 5'-ACTTGCCAATTGTCTGG-AAACAC-3', internal fluorescence probe 5'-CACTTAAAGACCTCCC-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.

Results

Expression of TLR3 in Lupus Nephritis of MRL<sup>1pr/1pr</sup> Mice

We first determined the expression pattern of TLR3 in kidneys and spleens of MRL<sup>1pr/1pr</sup> 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<sup>1pr/1pr</sup> mice (Figure 1). 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 mesangioliproliferative glomerulonephritis (glomeruli encircled) with periglomerular inflammatory cell infiltrates (arrowheads) and tubular atrophy (arrows). Magnification, ×400 periodic acid-Schiff.

Figure 1. Toll-like receptor 3 (TLR3) mRNA expression in MRL<sup>1pr/1pr</sup> 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<sup>1pr/1pr</sup> 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 mesangioliproliferative glomerulonephritis (glomeruli encircled) with periglomerular inflammatory cell infiltrates (arrowheads) and tubular atrophy (arrows). Magnification, ×400 periodic acid-Schiff.

Figure 2. TLR3 in kidneys of MRL<sup>1pr/1pr</sup> 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<sup>1pr/1pr</sup> 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.

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.
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<sup>lpr/lpr</sup> 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<sup>lpr/lpr</sup> mice is consistent with TLR3 expression on immunostaining in human renal biopsies (unpublished observation).

Localization of Labeled Viral dsRNA after Intravenous Injection in MRL<sup>lpr/lpr</sup> 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<sup>lpr/lpr</sup> 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<sup>lpr/lpr</sup> 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<sup>lpr/lpr</sup> 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\textsuperscript{lpr/lpr} Mice

As TLR3 staining and uptake of labeled pI:C RNA in kidneys of MRL\textsuperscript{lpr/lpr} mice also occurred in infiltrating mononuclear cells, we isolated spleen monocytes from MRL\textsuperscript{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\textsuperscript{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\textsuperscript{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-\alpha 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-\alpha in BMDC of MRL\textsuperscript{lpr/lpr} mice.

pI:C RNA Injection Increases Serum IL-6, IL-12p70, and IFN-\alpha Levels in MRL\textsuperscript{lpr/lpr} Mice

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

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

From the above results, one would predict that exposure to pI:C RNA would aggravate tissue injury in autoimmune MRL\textsuperscript{lpr/lpr} mice. We therefore treated groups of lupus mice with intraperitoneal injections of either 50 \( \mu \)g of pI:C RNA or pI:C DNA or saline on alternate days from weeks 16 to 18 of age. Saline-treated MRL\textsuperscript{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\textsuperscript{lpr/lpr} mice. (A) Monocytes were prepared from spleens of MRL\textsuperscript{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-\alpha 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\textsuperscript{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 relative to 

\[ \frac{\text{GAPDH mRNA}}{\text{CCL5 mRNA}} \]

SEM. Results shown are means \( \pm \) SEM from one of three comparable experiments, each performed in duplicate. (C) Spleen monocytes of MRL\textsuperscript{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 \( \pm \) SEM from three comparable experiments, each performed in duplicate.
Figure 5. pI:C RNA activates dendritic cells isolated from MRL<sup>lpr/lpr</sup> 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<sup>lpr/lpr</sup> 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.
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 MRLlpr/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 MRLlpr/lpr mice toward a crescentic glomerulonephritis with mesangiolysis associated with marked tubulointerstitial injury.

pI:C RNA Induced Renal CCL2 and CCL5 mRNA Expression in MRLlpr/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 MRLlpr/lpr mice. In fact, kidneys of pI:C RNA–treated MRLlpr/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 MRLlpr/lpr mice kidneys. By contrast, pI:C RNA–treated MRLlpr/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 MRLlpr/lpr mice.

pI:C RNA Does Not Cause B Cell Activation and DNA Autoantibody Production in MRLlpr/lpr Mice

We previously observed that the activation of TLR9 by bacterial CpG-DNA aggravated lupus nephritis in MRLlpr/lpr mice in association with enhanced DNA autoantibody production and glomerular immune complex deposits (10). The predominance of IgG2a autoantibody and renal IgG deposits of the IgG2a 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 MRLlpr/lpr mice. In the present study, all groups irrespective of treatment had comparable titers of total serum DNA IgG or IgG2a autoantibodies (Table 1). Consistent with these results, capillary and mesangial deposits of total IgG and of IgG2a 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 MRLlpr/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 MRLlpr/lpr mice.
Figure 7. Renal histopathology. Renal sections of 18-wk-old MRL<sup>lpr/lpr</sup> 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<sup>lpr/lpr</sup> 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<sup>lpr/lpr</sup> 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<sup>lpr/lpr</sup> 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<sup>lpr/lpr</sup> mice to study the effects of intermittent exposure to pI:C RNA, a structural analog to viral dsRNA.
responses in the kidney induced by viral dsRNA. In fact, in induced aggravation of lupus nephritis in MRLlpr/lpr mice is infiltrating immune cells. It is interesting that the pI:C RNA– circulating pI:C RNA is further facilitated through activation of ing cells of the monocytic cell lineage, TLR3. In mice, TLR3 expression is restricted to antigen-present-duction, which may relate to the specific expression profile of independent mechanisms in aggravating lupus nephritis by pI:C excretion exacerbation of lupus nephritis as well as other forms of may play an important role in mediating viral infection–in- 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 gramulonephritis.

Viral dsRNA Activates Mesangial Cells in Experimental Lupus

Antiviral host defense requires activation of innate immu-nity, 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<sup>lpr/lpr</sup> mice suggests a role for TLR3 in local immune responses in the kidney induced by viral dsRNA. In fact, in kidneys of MRL<sup>lpr/lpr</sup> 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<sup>lpr/lpr</sup> mice is also consistent with previously published data from known to be involved in progression of glomerulonephritis 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 specific TLR (TLR7, TLR8, and TLR9) (9 and unpublished finding. Mesangial cells do not express other nucleic acid–dependent receptors expressing high levels of TLR3 mRNA in kidneys of these mice, 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.

**Table 1. Serum, urinary, and histologic findings in MRL<sup>lpr/lpr</sup> mice**

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

<sup>a</sup>Values are means ± SEM from eight to 10 mice per group. pI:C, polyinosinic-cytidylic acid; hpf, high-power field.

<sup>b</sup>p < 0.05 pI:C RNA versus pI:C DNA.

We examined both DNA autoantibody-dependent and -inde-pendent 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 pro-duction in these cells. The process of aggravation of autoim-mune renal injury in MRL<sup>lpr/lpr</sup> 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<sup>lpr/lpr</sup> 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.
likely to be involved in the observed aggravation of lupus nephritis in MRL<sup>lpr/lpr</sup> 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<sup>lpr/lpr</sup> 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.
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 (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*pr/pr* 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*pr/pr* 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*pr/pr* 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. plC RNA–induced IL-6 secretion therefore may modulate adaptive immunity in SLE independent of B cell responses.

In summary, exposure to plC RNA—a structural analog of viral dsRNA—can aggravate lupus nephritis through TLR3 on antigen-presenting cells and glomerular mesangial cells. plC 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

The work was supported by a grant from the Deutsche Forschungsgemeinschaft (AN372/4-1) and the Fritz Thyssen Foundation to H.J.A. S.S. is supported by a grant from the EU gemeinschaft (AN372/4-1) and the Fritz Thyssen Foundation to H.J.A. S.S., H.J.A., and D.S. were supported by a grant from the EU Gemeinschaft (AN372/4-1) and the Fritz Thyssen Foundation to H.J.A. S.S., H.J.A., and D.S. were supported by a grant from the EU Gemeinschaft (AN372/4-1) and the Fritz Thyssen Foundation to H.J.A. S.S., H.J.A., and D.S. were supported by a grant from the EU Gemeinschaft (AN372/4-1) and the Fritz Thyssen Foundation to H.J.A. S.S., H.J.A., and D.S. were supported by a grant from the EU Gemeinschaft (AN372/4-1) and the Fritz Thyssen Foundation to H.J.A. S.S., H.J.A., and D.S. were supported by a grant from the EU Gemeinschaft (AN372/4-1) and the Fritz Thyssen Foundation to H.J.A. S.S., H.J.A., and D.S. were supported by a grant from the EU Gemeinschaft (AN372/4-1) and the Fritz Thyssen Foundation to H.J.A.

References


