Toll-Like Receptor-7 Modulates Immune Complex Glomerulonephritis

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Viral infections may trigger immune complex glomerulonephritis *via* Toll-like receptors (TLR), as certain TLR trigger immunity upon recognition of viral nucleic acids. On the basis of previous findings regarding viral double-stranded RNA and TLR3 in experimental lupus erythematosus, a similar role for TLR7 that recognizes viral single-stranded RNA was hypothesized. Immunostaining of kidney sections of nephritic MRL$^{lpr/lpr}$ mice revealed TLR7 expression in infiltrating ER-HR3–positive macrophages and few CD11c-positive dendritic cells but not in glomerular mesangial cells as observed for TLR3. This finding was consistent with the distribution pattern of intravenously injected single-stranded RNA in nephritic MRL$^{lpr/lpr}$ mice. TLR7 ligation activated monocytes and dendritic cells, both isolated from MRL$^{lpr/lpr}$ mice, to secrete IFN-α, IL-12p70, IL-6, and CCL2. *In vivo*, a single injection of the TLR7 ligand imiquimod increased serum levels of IL-12p70, IFN-α, and IL-6. A course of 25 μg of imiquimod given every other day from week 16 to 18 of age aggravated lupus nephritis in MRL$^{lpr/lpr}$ mice. This was associated with increased glomerular immune complex deposits as well as interstitial expression of CCL2 in imiquimod-treated MRL$^{lpr/lpr}$ mice. Different types of viral nucleic acids seem to modulate systemic autoimmunity through specific interactions with their respective TLR. Different TLR expression profiles on immune cell subsets and nonimmune parenchymal cell types determine the molecular mechanisms involved in viral infection–associated exacerbation of lupus nephritis and possibly other types of immune complex glomerulonephritis.

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Viral infection can trigger disease activity in immune complex glomerulonephritis, *e.g.*, lupus nephritis, but the molecular mechanisms remain poorly understood. It is believed that viral compounds that induce immune responses also interfere with the control of autoimmunity in susceptible individuals. Thus, pattern-recognition receptors that recognize such viral compounds may stimulate both antiviral immunity and, under certain circumstances, autoimmune disease activity.

Toll-like receptors (TLR) are a family of such pattern-recognition receptors, and a subgroup of TLR activates antiviral immunity upon recognition of viral compounds (1,2). For example, various viral nucleic acids ligate a group of TLR on dendritic cells and induce dendritic cell maturation characterized by the upregulation of MHC class II, induction of costimulatory molecules, and secretion of type I interferons (3,4). In contrast to their established role for pathogen control, the role of TLR in autoimmunity or lupus nephritis is less well defined (5,6). Systemic exposure to unmethylated CpG-DNA (ligand of TLR9), can aggravate the immune complex glomerulonephritis induced by apoferritin (7), as well as the lupus-like immune complex glomerulonephritis of MRL$^{lpr/lpr}$ mice (8). In both disease models, aggravation was associated with enhanced autoantibody production and intrarenal monocyte activation, consistent with the expression of TLR9 on B cells and monocytic antigen-presenting cells. 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 (9). For example, viral double-stranded RNA (dsRNA) activates TLR3, which is expressed by glomerular mesangial cells and monocytic antigen-presenting cells but not by B cells (10,11). As a consequence, exposure to viral dsRNA aggravates lupus nephritis in MRL$^{lpr/lpr}$ mice by inducing mesangiolysis as well as enhanced local inflammatory tissue damage, but circulating DNA autoantibodies remain unaffected (11). As another viral nucleic acid, single-stranded RNA (ssRNA) or synthetic guanine nucleoside analogs are recognized by TLR7 and in humans possibly by TLR8 (12–14). Whereas TLR7 signals for antiviral immunity upon infection with ssRNA viruses, its role in autoimmunity remains unknown (15). On the basis of the known expression profiles of TLR3, TLR7, TLR8, and TLR9 on immune cell subsets, TLR7 follows the distribution of TLR9 rather than TLR3 (16). Furthermore, whereas TLR7, TLR8, and TLR9 signal through the adaptor molecule myeloid differentiation factor 88, TLR3 is the only known TLR that depends on Toll-IL-1 receptor domain-containing adaptor inducing IFN-β and RNA helicase retinoic acid–inducible gene 1 (RIG-1) (17,18). Despite this different

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signaling pathways, ligation of all of these viral nucleic acid–specific TLR induces a robust induction of IFN-α (3,19,20). IFN-α is a critical mediator of both antiviral immunity and autoimmune tissue injury (21). The recognition of viral ssRNA *via* TLR7 may represent an important mechanism of virus-induced autoimmunity, *e.g.*, in lupus. Thus, we characterized the expression of TLR7 in experimental lupus and studied the effects of TLR7 ligation on lupus disease activity as a model for intercurrent viral infection in systemic lupus erythematosus (SLE).

Materials and Methods

Animals and Experimental Protocol

Ten-week-old female $MRL^{lpr/lpr}$ mice were obtained from Harlan Winkelmann (Borchen, Germany) and kept 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 had been approved by the local government authorities. For assessing renal TLR mRNA expression, kidneys were obtained from 5- and 20-wk-old female $MRL^{lpr/lpr}$ mice. In addition, 16-wk-old female $MRL^{lpr/lpr}$ mice were distributed into three groups that received intraperitoneal injections every other day as follows: (*1*) 25 μg of imiquimod, a compound of the imidazoquinadine family and TLR7 agonist (Sequoia Research Products Ltd, Oxford, UK) (13) in 100 μl of 10% DMSO (Sigma-Aldrich, Steinheim, Germany); (*2*) 100 μl of 10% DMSO; or (*3*) 100 μl of normal saline. All mice were killed by cervical dislocation at the end of week 18 of age. For assessing the renal distribution of ssRNA, 3′-rhodamine–labeled ssRNA40, known to ligate mTLR7 (12), was injected intravenously into $MRL^{lpr/lpr}$ mice at the age of 16 wk. Renal tissue was collected 2 h later and subjected to further analysis as described below.

Evaluation of Glomerulonephritis

Blood and urine samples were collected from each animal at the end of the study period as described (8) to determine proteinuria and creatinine using an automatic autoanalyzer (Integra 800; Roche Diagnostics, Mannheim, Germany). Serum DNA autoantibodies were determined by ELISA using the anti-mouse IgG_1 and IgG_{2a} antibodies (Bethyl Laboratories Inc., Montgomery, TX; 1:100). From all mice, kidneys were fixed in 10% buffered formalin, processed, and embedded in paraffin. Five-micrometer sections for silver and periodic acid-Schiff stains were prepared following routine protocols. The severity of the renal lesions was graded using the indices for activity and chronicity as described for human lupus nephritis (22).

Immunostaining

Immunostaining was performed on either paraffin-embedded or frozen sections as described (8) using the following primary antibodies: Anti-mouse TLR7 (1:50, IMG581; Imgenex, San Diego, CA), anti-mouse ER-HR3 (1:50, monocytes/macrophages; DPC Biermann, Bad Nauheim, Germany), anti-mouse CD11c (1:50, clone HL3, BD Pharmingen, Heidelberg, Germany), anti-mouse CD3 (1:100, clone 500A2; BD Pharmingen), anti-mouse smooth muscle actin (1:100, myofibroblasts, clone 1A4; Dako, Carpinteria, CA), anti-mouse CCL5 (1:50, clone VL1; Peprotech, Rocky Hill, NJ), anti-mouse CCL2/MCP-1 (1:50, polyclonal; Santa Cruz Biotechnology, Santa Cruz, CA), anti-mouse IgG_1 (1:100, clone H143.225.8; Dianova, Hamburg, Germany), anti-mouse IgG_{2a} (1:100, clone R19–15; Dianova), and anti-mouse C3c (1:200, GAM/C3c/FITC; Nordic Immunological Laboratories, Tilburg, Netherlands). 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 and C3c deposits from 0 to 3 plus was performed on 15 cortical glomerular sections as described (8).

Cell Culture Conditions and Cytokine ELISA

Bone marrow–derived dendritic cells and plastic-adherent spleen monocytes were isolated from $MRL^{lpr/lpr}$ mice, processed, and cultured as described (23,24). Spleen monocytes were treated with medium control or imiquimod 3 μg/ml, RNA40 along with DOTAP (Roche, Mannheim, Germany) 30 μg/ml, and pI:C RNA 30 μg/ml after 24 h of incubation. TLR9 ligand CpG-ODN no. 1668 at a concentration of 1 μg/ml was used as a control in selected cases. After a period of 24 h, culture supernatants were collected for cytokine measurements and cells were prepared for flow cytometric analysis. Dendritic cells and spleen monocytes were stimulated as above for 24 h, and cells were harvested for RNA isolation as described previously (23). 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). A murine mesangial cell line was maintained in DMEM (Biochrom KG, Berlin, Germany) supplemented with 2.5% FCS and 1% penicillin-streptomycin 100 U/ml and 100 μg/ml, respectively (25). Cells were incubated for 24 h without serum supplements before stimulation. Cytokine levels were determined using commercial ELISA kits following the protocol provided by the manufacturers: IL-6, IL-12p70, CCL2 (all OptEiA, BD Pharmingen), and IFN-α (PBL Biomedical Labs, Piscataway, NJ).

Flow Cytometry

Flow cytometry of cultured cells or splenocytes was performed as described previously (11). The following primary antibodies were used to detect TLR on mesangial cells and macrophages: Anti-mouse TLR3 (1:50, IMG516; Imgenex, San Diego, CA), TLR7 (1:50), and anti-mouse TLR9 (5G5, provided by Dr. Stefan Bauer, Technical University, Munich, Germany). A biotinylated rabbit anti-mouse IgG antibody and streptavidin-APC (BD Pharmingen) were used for detection, and a rabbit IgG (BD Pharmingen) was used as isotype control.

Real-Time Quantitative (TaqMan) Reverse Transcription–PCR

Real-time reverse transcription–PCR (RT-PCR) on RNA that was isolated from renal tissue was performed as described previously (11). Controls that consisted of ddH_2O were negative for target and housekeeper genes. Oligonucleotide primer (300 nM) and probes (100 nM) were from Applied Biosystems (Darmstadt, Germany) and used as described: TLR3, no. AF355152, forward 5′-CGAAAGTTGGACTTGTCATCAAATC-3′, reverse 5′-ACTTGCCAATTGTCTGGAAACAC-3′, 6 FAM 5′-CACTTAAAGAGTTCTCCC-3′; TLR7, no. AY035889, forward 5′-TGCCACC-TAATTTACTAGAGCTCTATCTTTAT-3′, reverse 5′-TAGGTCAAGAACTTGCAACTCATTG-3′, 6 FAM 5′-CCAAGAAAATGATTTTAATAAC-3′; TLR9, no. NM 031178, forward 5′-CAATCTGACCTCCCTTCGAGTACTT-3′, reverse 5′-GCCACATTCTATACAGGGATTGG-3′, 6 FAM 5′-ATTGCCGTCGCTGCGACCATG-3′. Primers and probes for murine 18S rRNA were obtained as predeveloped assay reagents from Applied Biosystems.

Statistical Analyses

Data were expressed as mean ± SEM. Comparison between groups was performed using univariate ANOVA. *Post hoc* Bonferroni correction was used for multiple comparisons. $P < 0.05$ was considered to indicate statistical significance.

Results

Expression of TLR7 in Lupus Nephritis of MRL$^{lpr/lpr}$ Mice

We first determined the expression pattern of TLR7 in kidneys and spleens of MRL$^{lpr/lpr}$ mice and compared TLR3, TLR7, and TLR9 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 (data not shown). At this time point, expression levels of TLR7 and TLR9 mRNA were low as compared with that in spleen. By contrast, kidney TLR3 mRNA levels were comparable to that in spleen of 5-wk-old MRL$^{lpr/lpr}$ mice, consistent with the known TLR3 mRNA expression by mesangial cells (Figure 1). At 20 wk, proliferative lupus nephritis was associated with increased renal TLR7 and TLR9 mRNA expression as compared with week 5 (Figure 1). To localize the source of renal TLR7 mRNA expression, we used a polyclonal antibody specific for murine TLR7 and performed double staining for either ER-HR3 or CD11c in renal sections of 16-wk-old MRL$^{lpr/lpr}$ mice. Approximately 30% of interstitial ER-HR3 macrophages and CD11c dendritic cells (ratio 90%:10%, respectively) stained positive for TLR7 (Figure 2A). Staining for TLR7 appeared in a speckled pattern, indicating that TLR7 is localized in an intracellular compartment. Intrinsic renal cells were negative for TLR7, and the macrophages that were found to be present in the glomerular compartment were rarely positive for TLR7. Together these data suggest that in nephritic kidneys of MRL$^{lpr/lpr}$ mice, TLR7 is expressed mainly by infiltrating interstitial macrophages but not by intrinsic renal cells.

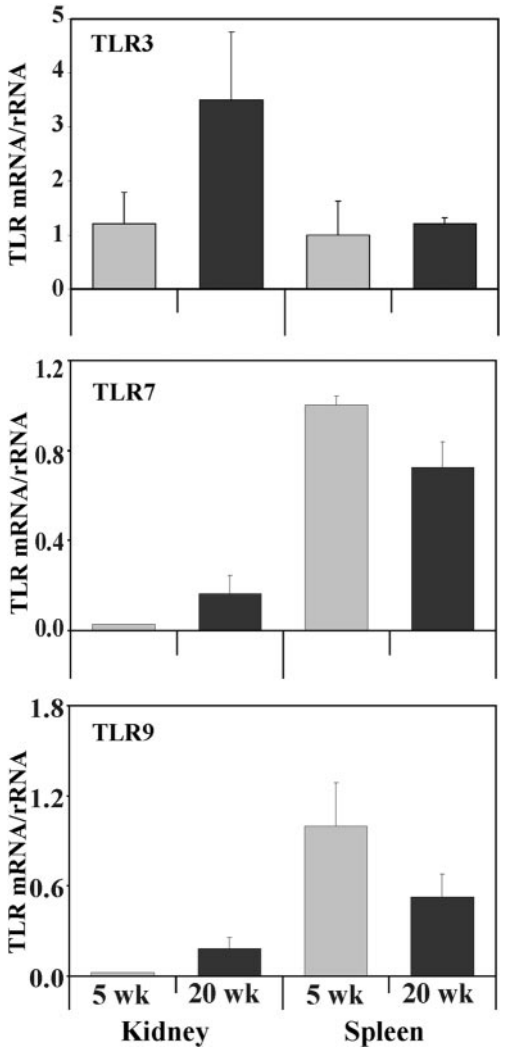

Figure 1. Toll-like receptor 7 (TLR7) expression in MRL$^{lpr/lpr}$ mice. Expression of TLR mRNA was assessed by real-time reverse transcription–PCR (RT-PCR) in duplicate using RNA isolated from spleens and kidneys from seven MRL$^{lpr/lpr}$ mice each at 5 and 20 wk of age as described in Materials and Methods. TLR mRNA expression is expressed as a ratio to the respective 18S rRNA mRNA expression ± SEM.

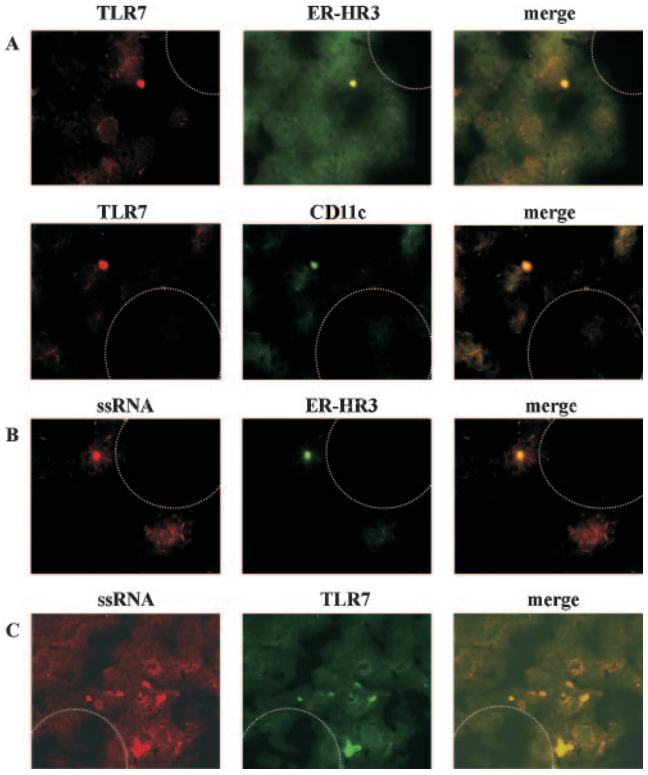

Figure 2. TLR7 immunostaining and uptake of labeled single-stranded RNA (ssRNA) in kidneys of MRL$^{lpr/lpr}$ mice. (A) An mTLR7-specific antibody was used on renal sections of 18-wk-old nephritic MRL$^{lpr/lpr}$ mice. A PE-labeled secondary antibody was used for detection. Positive signals co-localized with ER-HR3–positive macrophages or CD11c-positive dendritic cells, both detected by a FITC-labeled secondary antibody. (B) Rhodamine-labeled ssRNA40 was injected intravenously into 18-wk-old MRL$^{lpr/lpr}$ mice, and renal tissue was harvested 2 h later. Fluorescence imaging of frozen sections showed uptake of ssRNA40 (red) into ER-HR3–positive macrophages (green). (C) Co-staining of rhodamine-labeled cells (red) for TLR7 (green) demonstrates uptake of RNA40 into TLR7-positive cells. Magnification, ×530.

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

To examine whether circulating ssRNA localizes to nephritic kidneys of MRL$^{lpr/lpr}$ mice, we injected rhodamine-labeled ssRNA intravenously into 20-wk-old MRL$^{lpr/lpr}$ mice. Consistent with TLR7 immunostaining in the kidney, the labeled ssRNA was found in infiltrating cells in a granular intracellular staining pattern (Figure 2B). Double labeling with an ER-HR3-specific antibody identified these cells as renal macrophages (Figure 2B). Rhodamine that was injected into MRL$^{lpr/lpr}$ mice did not localize in the kidney (data not shown). Double labeling for TLR7 confirmed that injected ssRNA was taken up into TLR7-positive cells (Figure 2C). Taken together, in kidneys of MRL$^{lpr/lpr}$ mice, injected ssRNA co-localizes in an intracellular granular pattern with TLR7-positive cells, *i.e.*, infiltrating mononuclear cells, but not with intrinsic renal cells.

Cultured Mesangial Cells and Macrophages Respond to Nucleic Acids According to Their Respective TLR Expression Profile

To confirm the respective TLR7 expression, we used established murine cell lines for macrophages and mesangial cells. Under basal culture conditions, mesangial cells expressed TLR3 mRNA, whereas mRNA for TLR7 and TLR9 were not detected (Figure 3A). By contrast, J774 macrophages expressed all three receptors (Figure 3A). The subcellular localization of TLR3, TLR7, and TLR9 was assessed by flow cytometry. In mesangial cells, TLR3 and in macrophages TLR3, TLR7, and TLR9 were expressed intracellularly, whereas surface expression was absent (Figure 3B). Next, we questioned whether mesangial cells and macrophages respond to microbial nucleic acids that correspond to their specific TLR expression profile. We stimulated both cell types with synthetic mimics of microbial nucleic acids: pI.C RNA (TLR3), imiquimod and RNA40 (TLR7), and CpG-ODN (TLR9). Consistent with their respective TLR expression profile, mesangial cells produced the CC-chemokine CCL2 only after exposure to mimics of viral dsRNA, whereas J774 macrophages responded to all TLR agonists tested (Figure 3C). Together, these data indicate that monocytes but not mesangial cells express TLR7 in an intracellular compartment and produce CCL2 upon exposure to ssRNA or imiquimod *in vitro*.

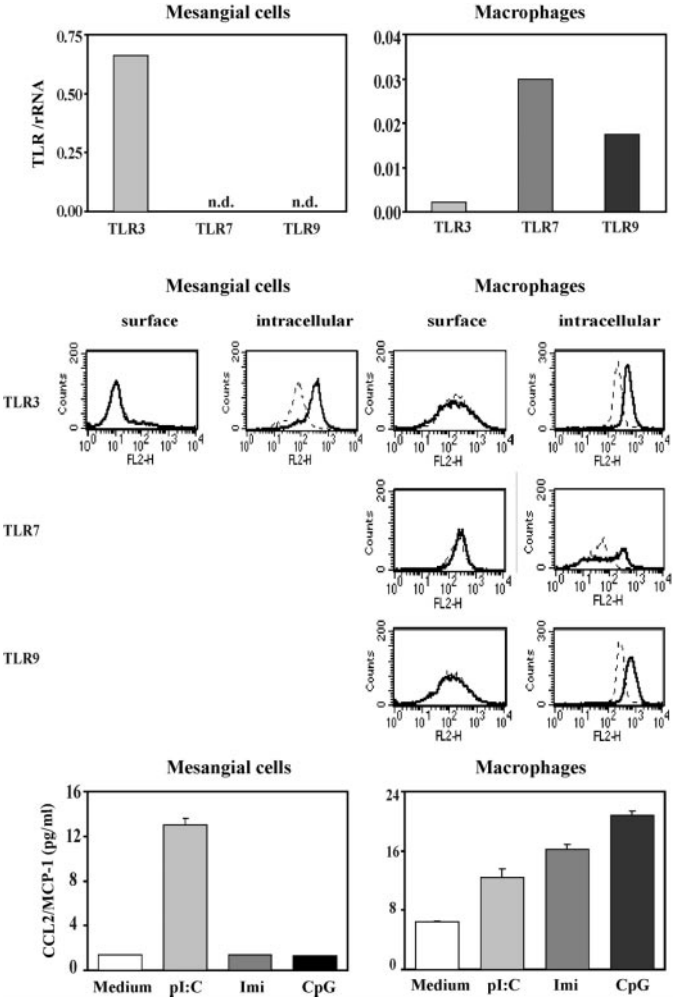

Figure 3. Nucleic acids activate cultured mesangial cells and J774 macrophages consistent with their respective TLR expression profile. Murine mesangial cells and J774 macrophages were cultured as described in Materials and Methods. (A) Expression of TLR mRNA was assessed by real-time RT-PCR in duplicate using RNA that was isolated from cultured cell lines. TLR mRNA expression is expressed as a ratio to the respective 18S rRNA mRNA expression ± SEM (*$P < 0.05$). (B) Flow cytometry for TLR before and after permeabilization for intracellular staining was performed as indicated. Expression of TLR (solid line) is demonstrated by a fluorescence shift compared with the isotype control antibody (dotted line). (C) Cultured cells were incubated with pI:C RNA, imiquimod (IMI), RNA40, CpG-DNA, or standard medium without supplements for 24 h as indicated. CCL2 production was measured in supernatants by ELISA. Results shown are representative of two comparable experiments each performed in duplicate.

TLR7 Agonists Induce Production of Proinflammatory Mediators in Monocytes and Dendritic Cells Isolated from MRL$^{lpr/lpr}$ Mice

Because TLR7 seems to be expressed by ER-HR3–positive macrophages and CD11c-positive dendritic cells in nephritic kidneys of MRL$^{lpr/lpr}$ mice, we intended to characterize the factors produced by these cells in response to agonists of TLR7 in comparison with other nucleic acid–like TLR agonists. Spleen monocytes and bone marrow dendritic cells were isolated from MRL$^{lpr/lpr}$ mice as described in Materials and Methods. The ER-HR3–positive group comprised approximately 90% of the spleen monocytes, which were found to be negative for CD11c. Likewise, approximately 85% of the bone marrow–derived dendritic cells were found to be positive for CD11c, which were found to be negative for ER-HR3 as determined by flow cytometry (data not shown). Both cell types were incubated with pI:C RNA, RNA40, imiquimod, and CpG-DNA. In spleen macrophages both RNA40 and imiquimod induced production of IL-6, IL-12p70, IFN-α, and CCL2, respectively (Figure 4). In bone marrow–derived dendritic cells, the observed responses were similar except for increased IL-12p70 and IFN-α production upon exposure to pI:C RNA (Figure 4) as compared

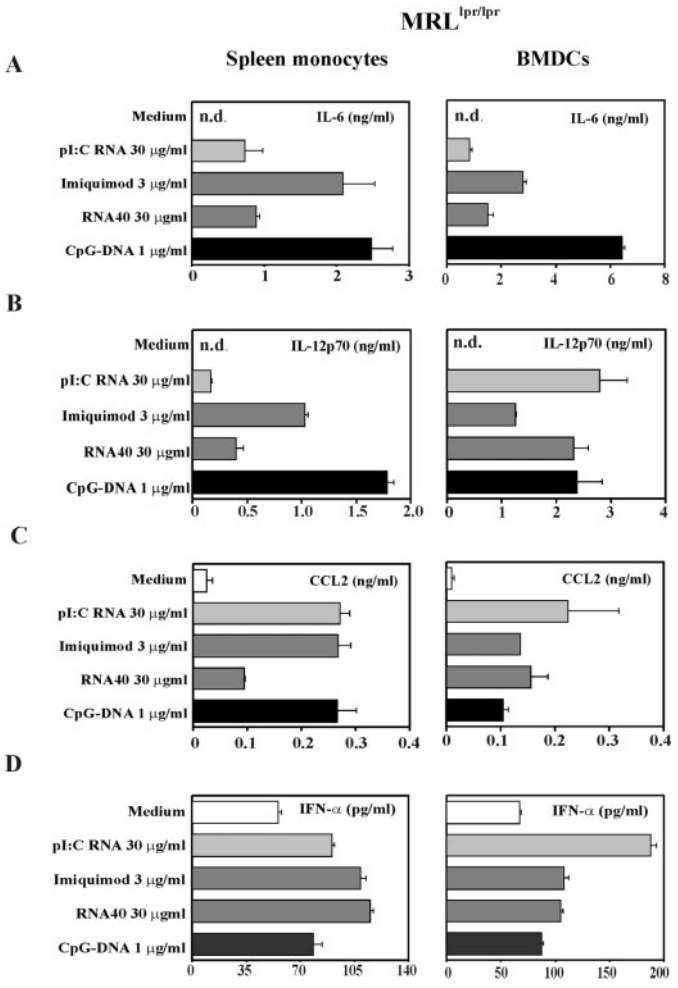

Figure 4. TLR7 agonists activate ER-HR+ monocytes and CD11c+ dendritic cells that were isolated from MRL$^{lpr/lpr}$ mice. ER-HR3+/CD11c− monocytes and ER-HR3−/CD11c+ dendritic cells (DC) were isolated from spleens and bone marrow (BM) of MRL$^{lpr/lpr}$ mice and incubated with pI:C RNA, RNA40, imiquimod, CpG-DNA, or standard medium for 24 h as indicated. IL-6 (A), IL-12p70 (B), CCL2/MCP-1 (C), and IFN-α (D) were measured in supernatants by ELISA. Results shown are from one of three comparable experiments. For each experiment, cells were pooled from three mice. Values represent means ± SEM; n.d., nondetectable.

with stimulation of spleen monocytes. These data suggest that TLR7 agonists induce the production of IL-12p70, IL-6, CCL2, and IFN-α in both macrophages and dendritic cells isolated from MRL$^{lpr/lpr}$ mice.

Imiquimod Increases Serum IFN-α, IL-6, and IL-12p70 Levels in MRL$^{lpr/lpr}$ Mice

Circulating IFN-α, IL-6, and IL-12p70 levels are markers of disease activity in lupus. Thus, having demonstrated the effect of TLR7 ligation on IFN-α, IL-6, and IL-12p70 secretion in antigen-presenting cell subsets that were isolated from MRL$^{lpr/lpr}$ mice *in vitro*, we next studied serum levels of these factors 6 h after intraperitoneal injection of 25 μg of imiquimod, vehicle, or saline into 16-wk-old MRL$^{lpr/lpr}$ mice. Injection of imiquimod significantly increased serum levels of IL-12p70, IL-6, and IFN-α in MRL$^{lpr/lpr}$ mice as compared with vehicle-injected controls (Figure 5).

Imiquimod Aggravates Autoimmune Tissue Injury in MRL$^{lpr/lpr}$ Mice

From the above results, one would predict that ligation of TLR7 would be associated with more severe autoimmune tissue injury in MRL$^{lpr/lpr}$ mice. We therefore treated groups of lupus mice with intraperitoneal injections of 25 μg of imiquimod, vehicle, or saline on alternate days from weeks 16 to 18 of age. Saline-treated MRL$^{lpr/lpr}$ mice had diffuse proliferative glomerulonephritis with moderate mesangial hypercellularity, increase of mesangial matrix, and few periglomerular inflammatory cell infiltrates at week 18 (Figure 6A). Vehicle injections did not alter these histopathologic findings. By contrast, imiquimod injections increased mesangial matrix deposits with focal segmental sclerosis in glomeruli and cellular crescent formation associated with marked periglomerular inflammatory cell infiltrates (Figure 6A). Mesangiolysis was not observed. Aggravation of renal disease was illustrated by an increase in proteinuria and the activity and chronicity scores of the lupus nephritis in imiquimod-treated MRL$^{lpr/lpr}$ mice as compared with the other groups of mice (Table 1). There was a trend toward increased glomerular macrophages and CD3 cells in imiquimod-treated MRL$^{lpr/lpr}$ mice, but this did not reach statistical significance (Table 1). In addition to the aggravation of glomerular damage, imiquimod injections induced tubulo-

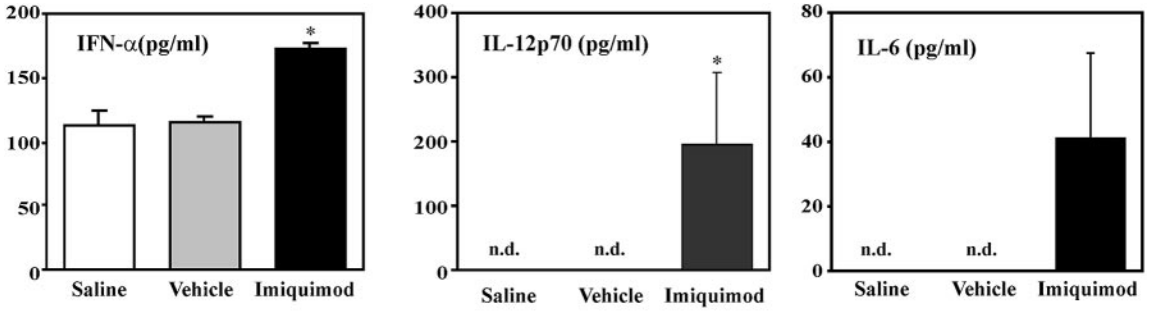

Figure 5. Serum IFN-α, IL-12p70, and IL-6 levels in MRL$^{lpr/lpr}$ mice. Serum was obtained from 16-wk-old MRL$^{lpr/lpr}$ mice 6 h after the first intraperitoneal injection of saline, vehicle, or 25 μg of imiquimod as indicated ($n = 5$ to 10). Serum levels were determined by ELISA. Data are means ± SEM. *$P < 0.05$ *versus* saline.

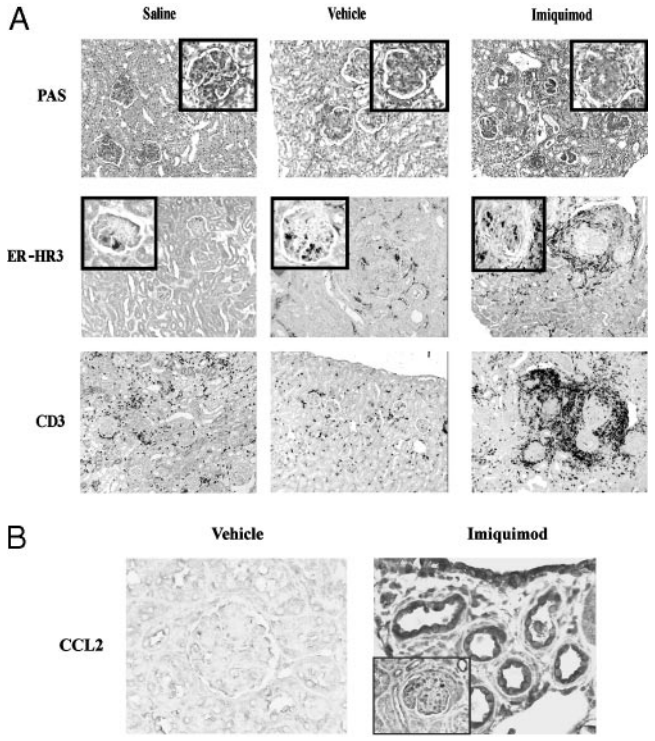

Figure 6. Renal histopathology. (A) Renal sections of 18-wk-old $MRL^{lpr/lpr}$ mice from all groups were stained with periodic acid-Schiff (PAS) and antibodies for ER-HR3 (macrophages), CD3 (lymphocytes), and smooth muscle antigen for myofibroblasts (SMA) as indicated. Insert in PAS-stained sections of imiquimod-treated $MRL^{lpr/lpr}$ mice illustrates glomerular tuft necrosis and crescent formation not detected in mice of the other groups. Inserts in ER-HR3– and CD3-stained sections show respective glomeruli. Images are representative of eight to 10 mice in each group. (B) Renal sections of 18-wk-old vehicle- or imiquimod-treated $MRL^{lpr/lpr}$ mice were stained for CCL2/MCP-1. Arrows indicate CCL2-positive glomerular and interstitial cells. Note CCL2 positivity also in tubular epithelial cells in imiquimod-treated mice. Images are representative of five mice in each group. Magnification, ×400 in A; ×530 in A inserts; ×400 in B.

interstitial damage. Infiltrating ER-HR3 macrophages and CD3 lymphocytes accumulated particularly in periglomerular fields and areas around glomerular crescents (Figure 6A, Table 1). On the basis of our *in vitro* studies with macrophages and dendritic cells, we hypothesized that TLR7 ligation would trigger local chemokine expression in nephritic kidneys of $MRL^{lpr/lpr}$ mice. Thus, we performed immunostaining for CCL2. At 18 wk, single spots of CCL2 protein were noted within the glomerular tuft and along Bowman's capsule of some glomeruli, as well as in focal interstitial areas in kidneys of vehicle-treated $MRL^{lpr/lpr}$ mice (Figure 6B). By contrast, imiquimod-treated $MRL^{lpr/lpr}$ mice showed marked CCL2 staining that co-localized with tubular epithelial cells, interstitial leukocytic cell infiltrates, and glomerular crescents. Taken together, imiquimod aggravated autoimmune tissue injury in $MRL^{lpr/lpr}$ mice associated with increased local expression of CCL2 in areas of inflammatory cell infiltrates and tissue damage in nephritic kidneys of $MRL^{lpr/lpr}$ mice.

Imiquimod Increases Renal Immune Complex Deposition in $MRL^{lpr/lpr}$ Mice

We previously observed that the activation of TLR9 as well as TLR3 aggravated lupus nephritis in $MRL^{lpr/lpr}$ mice. Unlike TLR3, TLR9 activation induced a marked increase of DNA autoantibody production and glomerular immune complex deposits (8,11). Thus, we investigated the effects of TLR7 ligation on serum dsDNA autoantibody levels and glomerular IgG deposits in $MRL^{lpr/lpr}$ mice. Imiquimod somewhat increased serum total IgG as well as IgG_1- and IgG_{2a}-dsDNA autoantibodies as compared with vehicle-treated $MRL^{lpr/lpr}$ mice, although this was not statistically significant (Figure 7A). However, imiquimod-treated $MRL^{lpr/lpr}$ mice showed increased glomerular capillary and mesangial deposits of total IgG, IgG_1, and IgG_{2a} (Figure 7B, Table 1). This was associated with increased glomerular capillary and mesangial deposits of complement factor C3c in imiquimod-treated $MRL^{lpr/lpr}$ mice (complement deposit score 2.4 ± 0.3 [imiquimod] *versus* 1.3 ± 0.1 [vehicle]; $P < 0.01$; Figure 7C). Together, imiquimod-induced aggravation of lupus nephritis in $MRL^{lpr/lpr}$ mice is associated with increased glomerular immune complex deposition.

Discussion

Viral infections can aggravate disease activity in preexisting SLE, but the role of viral ssRNA in this context is hypothetical. We used the model of spontaneous lupus-like immune complex glomerulonephritis in $MRL^{lpr/lpr}$ mice to study the effects of intermittent exposure to imiquimod, a synthetic TLR7 ligand with immunostimulatory effects comparable to viral ssRNA (12–14). We provide evidence for an intracellular expression of TLR7 on renal macrophages and dendritic cells in nephritic lesions of $MRL^{lpr/lpr}$ mice, which corresponds to the distribution of nucleic acid–specific TLR, *i.e.*, TLR3 and TLR9 in these cell types (8,11). Spleen monocytes and bone marrow–derived dendritic cells produce proinflammatory cytokines, chemokines, and type I interferons upon ligation of TLR7. Obviously, the immunostimulatory effects of ssRNA relates to the cell type–specific expression pattern of TLR7, which clearly differs from the viral dsRNA-specific TLR3. These data may provide a new understanding of viral infection-induced exacerbation of lupus nephritis as well as other types of glomerulonephritis.

TLR7 Is Expressed by Renal Macrophages in Experimental Lupus

Antiviral host defense requires activation of innate immunity, including the local production of type I interferons and chemokines (26). The finding that injected ssRNA localized to TLR7-positive macrophages in kidneys of $MRL^{lpr/lpr}$ mice suggests a role for TLR7 in activating renal macrophages in the kidney. For example, our studies with spleen monocytes that were isolated from $MRL^{lpr/lpr}$ mice argue in favor of TLR7 activation and its effect on tissue macrophages contributing to the local production of proinflammatory mediators, including IL-12, IL-6, CCL2, IFN-α, which are known to contribute to the

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

	Saline	Vehicle	Imiquimod
Functional parameters			
proteinuria (mg/mg creatinine)	5.4 ± 2.5	3.3 ± 0.9	17.8 ± 6.2[b]
Histologic scores			
activity index	6.8 ± 2.6	8.8 ± 4.0	14.6 ± 5.0[b]
chronicity index	1.3 ± 0.5	1.4 ± 1.7	4.3 ± 2.2[b]
Cellular response (cells/glomerulus or hpf)			
glomerular ER-HR3+ (cells/glomerulus)	2.0 ± 0.6	2.1 ± 0.9	2.6 ± 0.9
CD3+ (cells/glomerulus)	1.2 ± 0.5	1.3 ± 0.6	1.7 ± 0.5
interstitial ER-HR3+ (cells/hpf)	6.2 ± 2.9	8.3 ± 4.5	19.4 ± 7.4[b]
CD3+ (cells/hpf)	13.7 ± 3.3	18.3 ± 4.3	41.0 ± 21.5[b]
SMA+ (% hpf)	5.0 ± 0.6	4.8 ± 0.9	8.5 ± 1.0[b]
Glomerular IgG deposit score			
IgG	1.2 ± 0.2	1.4 ± 0.2	2.0 ± 0.4[b]
IgG_1	0.9 ± 0.2	0.9 ± 0.4	1.8 ± 0.5[b]
IgG_{2a}	1.2 ± 0.3	1.4 ± 0.3	2.1 ± 0.5[b]

[a]Values are means ± SEM. Hpf, high-power field; SMA, smooth muscle antigen.
[b]$P < 0.05$ imiquimod *versus* vehicle.

progression of lupus nephritis (27–31). As TLR7-positive macrophages were mainly detected in the renal interstitium, the aggravation of glomerular injury in imiquimod-treated $MRL^{lpr/lpr}$ mice should be secondary to increased glomerular immune complex deposition. Furthermore, a low number of CD11c-positive dendritic cells were found to express TLR7 in the kidney. A recent study has demonstrated the antigen-presenting phenotype of this cell population in glomerulonephritis (32). In fact, TLR7 ligation is a potent trigger for dendritic cell maturation (14). The functional role of intrarenal CD11c-positive dendritic cells for the progression of nephritis in $MRL^{lpr/lpr}$ mice remains unclear. However, our *in vitro* data with CD11c-positive bone marrow–derived dendritic cells suggest that this cell type contributes to local production of proinflammatory mediators, (IL-6, IL-12, CCL2, and IFN-α). For example, IL-6 that is derived either from renal macrophages or CD11c-positive dendritic cells may suppress regulatory T cells that control autoreactive T cells (33). In fact, in our study, exposure to imiquimod increased serum levels of the aforementioned mediators and aggravated autoimmune tissue injury in kidneys and lungs of the $MRL^{lpr/lpr}$ mice. This was associated with enhanced renal production of the chemokine CCL2 and increased interstitial macrophage and T cell infiltrates. On the basis of similar effects of TLR7, TLR3, and TLR9 ligands on cytokine and chemokine production by monocytes and dendritic cells, these data add to the concept of how nucleic acid–specific TLR on antigen-presenting cells can modulate autoimmunity, *e.g.*, lupus nephritis (5,34).

TLR7 Is not Expressed by Intrinsic Renal Cells

Immunostaining for TLR7 revealed that TLR7 is not expressed by intrinsic renal cells. In addition, labeled ssRNA did not localize to intrinsic renal cells after injection. These observations were consistent with our findings in cultured mesangial cells, which did not express TLR7 mRNA and did not respond to imiquimod. So far, TLR7 has not been reported to be expressed in any nonimmune cell type (35). Thus, the expression of TLR7 seems to be restricted to antigen-presenting cells in mice and humans (16). These data indicate that imiquimod-induced aggravation of nephritis in $MRL^{lpr/lpr}$ mice is unrelated to direct activation of intrinsic renal cells.

Effect of TLR7 Ligation on Humoral Immunity in Experimental Lupus

Lupus is characterized by polyclonal autoantibody production and B cell proliferation, the latter expressing TLR7 at intermediate levels (16). TLR7 ligation with repeated injections of imiquimod in $MRL^{lpr/lpr}$ mice was associated with a trend toward increased serum dsDNA autoantibody levels, a significant increase in glomerular immune complex deposits, and complement activation. The last may explain why imiquimod aggravated glomerular injury in $MRL^{lpr/lpr}$ mice despite having no effect on glomerular macrophage counts. The finding that imiquimod had only a moderate effect on autoantibody production is supported by a recent study that showed that B cells do not respond to TLR7 ligands unless their B cell sensitivity is enhanced by dendritic cell–derived IFN-α (36). Obviously, TLR7 ligands alone cannot induce B cell activation because IFN-producing dendritic cells control TLR7 sensitivity of B cells. We assume that imiquimod had a moderate effect on B cell–dependent production of dsDNA autoantibodies in $MRL^{lpr/lpr}$ mice because B cells localize in close proximity to IFN-producing dendritic cells in lymphoid organs and imiquimod injection increased serum IFN-α levels in $MRL^{lpr/lpr}$ mice. In fact, imiquimod and ssRNA were shown to induce IFN-α production in bone marrow–derived dendritic cells that were isolated from $MRL^{lpr/lpr}$ mice. Unlike CpG-TLR9–mediated activation of B cells, TLR7 ligation requires additional co-fac-

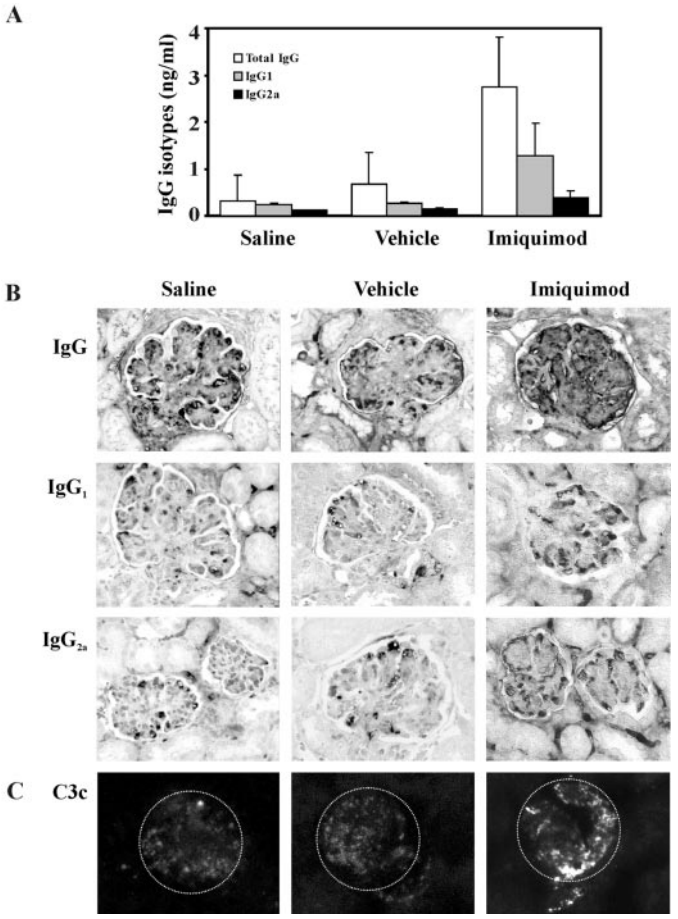

Figure 7. DNA autoantibodies and immune complex deposits in $MRL^{lpr/lpr}$ mice. (A) Serum double-stranded DNA autoantibody levels were determined by ELISA ($n = 5$ to 10). Data are means ± SEM. $P > 0.05$ *versus* vehicle for IgG and both IgG isotypes. (B and C) Immunostaining for total IgG, IgG_1, IgG_{2a}, and complement factor C3c were performed on renal sections of $MRL^{lpr/lpr}$ mice as described in Materials and Methods. Magnification, ×400.

tors, *e.g.*, IFN-α, to mount B cell activation and subsequent DNA autoantibody production. Thus, TLR7 ligation has a moderate effect on autoantibody production in $MRL^{lpr/lpr}$ mice.

On the basis of this and previous studies, three different mechanisms by which nucleic acid–specific TLR can contribute to the exacerbation of immune complex glomerulonephritis seem to exist: Viral dsRNA activates production of proinflammatory mediators through TLR3 expressed by glomerular mesangial cells as well as macrophages and dendritic cells (11). Viral dsRNA does not activate autoantibody production in $MRL^{lpr/lpr}$ mice, because B cells lack TLR3 expression. TLR7 and TLR9 both are expressed on renal macrophages, dendritic cells, and B cells (8,37). However, here we show that TLR7 can activate B cells only in the presence of additional co-factors, which result in an intermediate increase of autoantibody production and glomerular immune complex deposition. In addition, all three nucleic acid–specific TLR induce dendritic cell maturation toward an antigen-presenting phenotype as well as production of proinflammatory cytokines, chemokines, and type I interferons by renal macrophages. In summary, various types of nucleic acids induce specific patterns of immune responses that relate to the cell type–specific expression and function of the respective TLR. These findings contribute to the understanding of the broad clinical spectrum and the lack of universal serum markers of infection-associated disease activity of lupus nephritis and possibly other types of immune complex glomerulonephritis.

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