Ligands to Nucleic Acid–Specific Toll-Like Receptors and the Onset of Lupus Nephritis

Rahul D. Pawar, Prashant S. Patole, Andreas Ellwart, Maciej Lech, Stephan Segerer, Detlef Schlondorff, and Hans-Joachim Anders

Medical Polyclinic, University of Munich, Munich, Germany

Lupus nephritis develops from a combination of genetic and environmental factors such as microbial infection. A role for microbial nucleic acids (e.g., via nucleic acid–specific Toll-like receptors [TLR]) was hypothesized, in this context, because microbial nucleic acids can trigger multiple aspects of autoimmunity in vitro and in vivo. Eight-week-old MRL<sup>lpr/lpr</sup> and MRL wild-type mice received an injection of piC RNA (ligand to TLR-3), imiquimod (ligand to TLR-7), or CpG-DNA (ligand to TLR-9) on alternate days for 2 wk. Only CpG-DNA triggered the onset of lupus nephritis in MRL<sup>lpr/lpr</sup> mice, as defined by diffuse proliferative glomerulonephritis associated with glomerular IgG and complement C3 deposition, proteinuria, and glomerular macrophage infiltrates. None of the compounds caused DNA autoantibody production or glomerulonephritis in MRL wild-type mice. The role of CpG-DNA to trigger lupus nephritis in MRL<sup>lpr/lpr</sup> mice was found to relate to its potent immunostimulatory effects at multiple levels: B cell IL12p40 production, B cell proliferation, double-stranded DNA autoantibody secretion, and dendritic cell IFN-α production. The induction of lupus nephritis by CpG-DNA is motif specific and could be prevented by co-injection of inhibitory DNA. In summary, among the ligands tested, CpG-DNA triggers lupus nephritis in genetically predisposed hosts. These data support the concept that systemic lupus erythematosus is triggered by pathogens that release CG-rich DNA.

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Address correspondence to: Dr. Hans-Joachim Anders, Medizinische Poliklinik, Klinikum der Universität-Innenstadt, Pettenkoferstrasse 8a, 80336 Munich, Germany. Phone: +49-89-218075846; Fax: +49-89-218075860; E-mail: hjanders@med.uni-muenchen.de

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tribute to the onset of lupus through multiple mechanisms, including the induction of lymphoproliferation, autoantibody production, cytokine, and type I IFN production as well as the control of autoreactive B and T cell subset (12,15–17). In view of these in vitro studies, we questioned whether transient exposure to dsRNA, imiquimod, and CpG-DNA would trigger lupus nephritis in vivo. In exposing young lupus-prone MRL<sup>lpr/lpr</sup> mice or nonautoimmune MRL wild-type mice to these compounds, we also addressed the role of the <i>lpr</i> mutation for TLR-mediated induction of autoimmune tissue injury in MRL mice.

Materials and Methods

Animals and Experimental Protocol

Five-week-old female MRL<sup>lpr/lpr</sup> or MRL wild-type mice were obtained from Harlan Winkelmann (Borench, Germany) and maintained throughout in filter-top cages under a 12-h light/dark cycle. Autoclaved water and standard chow (Sniff, Soest, Germany) were available ad libitum. At 8 wk of age, MRL and MRL<sup>lpr/lpr</sup> mice were distributed into groups of five mice that received intraperitoneal injections of the following compounds on alternate days: pIC RNA 50 µg (Sigma-Aldrich, Taufkirchen, Germany), 25 µg of imiquimod (SEQUOIA Research Products, Oxford, UK) in 100 µl of 0.25% sodium acetate (Merck, Darmstadt, Germany), 40 µg of CpG-DNA, 40 µg of CpC-DNA, 40 µg of G-rich DNA, or a combination of 40 µg of CpG-DNA and 40 µg of G-rich DNA (TIBMolbiol, Berlin, Germany). The sequences of the synthetic oligodeoxynucleotides (ODN) were as follows: CpG-DNA (ODN 1668) 5'-TCC ATG ACG TTC CTG ATG CT-3', CpC-DNA (ODN 1720) 5'-TCG ATG AGC TTC CTG ATG CT-3', and G-rich inhibitory DNA (ODN 2114) 5'-TCC TGG AGG GGA AGT-3'. All mice were killed by cervical dislocation at the end of week 10 of age. The experimental procedure had been approved by the local government authorities.

Evaluation of Glomerulonephritis

Blood and urine samples were collected from each animal at the end of the study period as described (18). Urine albumin concentration and serum dsDNA autoantibodies were determined by ELISA as described previously (19). Urinary creatinine concentrations were determined using an automatic autoanalyzer (Integra 800; Roche Diagnostics, Mannheim, Germany). From all mice, kidneys were fixed in 10% buffered formalin, processed, and embedded in paraffin. Two-micrometer sections for periodic acid-Schiff stains were prepared following routine protocols (data not shown). The severity of the renal lesions was graded using the indices for activity and chronicity as described for human lupus nephritis (20).

Immunostaining

Immunostaining was performed on either paraffin-embedded or frozen sections as described (18) using the following primary antibodies: Anti-mouse Mac-2 (1:50, monocytes/macrophages; BD Pharmingen, Hamburg, Germany), anti-mouse CD3 (1:100; Serotec, Oxford, UK), anti-mouse B220 (1:400, early Pro-B to mature B cells, clone RA3–6B2; BD Pharmingen), anti-mouse Ki-67 (1:100, cell proliferation; Dianova, Hamburg, Germany), anti-mouse IgG<sub>1</sub> (1:100, M32015; Caltag Laboratories, Burlingame, CA), and anti-mouse IgG<sub>2a</sub> (1:100, M32215; Caltag), anti-mouse C<sub>3</sub> (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 15 cortical glomeruli per section. Semiqualitative scoring of complement C3 deposits from 0 to 3 was performed on 15 cortical glomerular sections as described (18).

Cell Culture Conditions and Cytokine ELISA

Bone marrow–derived dendritic cells and CD19-positive B cells were isolated from MRL and MRL<sup>lpr/lpr</sup> mice, processed, and cultured as described (21). Cells were stimulated with 30 µg/ml pIC RNA, 3 µg/ml imiquimod, 1 µg/ml CpG-ODN, or medium control for 24 h, if not indicated otherwise. Cytokine levels were determined in cell supernatants using commercial ELISA kits for IL-6, IL-12p40 (OptEIA; BD Pharmingen), and IFN-α (PBL Biomedical Labs, Piscataway, NJ) following the protocols provided by the manufacturers.

B Cell Proliferation Assay

Proliferation of B cells was assessed using CellTiter 96 Proliferation Assay (Promega, Mannheim, Germany). In brief, 1 × 10<sup>5</sup> B cells were incubated in 96-well plates in 100 µl of RPMI medium that contained 10% FCS, 100 units/ml penicillin, and 100 µg/ml streptomycin (Biochrom KG, Berlin, Germany) with TLR ligands as described previously for a period of 72 h. To each well, 20 µl of CellTiter 96 Aqueous One Solution (Promega) was added and incubated at 37°C for 4 h. The optical density was measured at 492 nm using a spectrophotometric plate reader.

Statistical Analyses

Data were expressed as mean ± SEM. Comparison between TLR ligand groups was performed by one-way ANOVA using the PRISM software (GraphPad Software, San Diego, CA). Post hoc Bonferroni correction was used for multiple comparisons. The <i>t</i> test was used to compare ODN 1668 + 2114 versus ODN 1668. <i>P</i> < 0.05 indicated statistical significance.

Results

Splenomegaly and Induction of DNA Autoantibodies in Young MRL and MRL<sup>lpr/lpr</sup> Mice

At 8 wk of age, no structural abnormalities were detected in kidney and spleens of MRL wild-type mice as observed using light microscopy. Spleens of age-matched MRL<sup>lpr/lpr</sup> mice displayed lymph follicle hyperplasia with enlarged B cell zones. Renal morphology did not show any abnormalities in MRL<sup>lpr/lpr</sup> mice of this age. In MRL<sup>lpr/lpr</sup> mice, the production of autoantibodies precedes the onset of lupus nephritis (22). Given the pathogenic role of lymphoproliferation and autoantibody production for the pathogenesis of lupus nephritis, we first tested whether exposure to nucleic acid–like PAMP induces splenomegaly and the production of DNA autoantibodies in young MRL or MRL<sup>lpr/lpr</sup> mice. In both mouse strains, spleen weight increased only with exposure to CpG-DNA (Figure 1A). Serum dsDNA autoantibodies were determined by ELISA in all groups of mice at 10 wk of age. In saline-treated MRL wild-type mice, serum dsDNA antibodies were absent and none of the compounds induced significant levels of dsDNA IgG autoantibodies. By contrast, 10-wk-old saline-treated MRL<sup>lpr/lpr</sup> mice had detectable levels of total IgG and IgG<sub>1</sub> dsDNA antibodies (Figure 1B). Exposure to CpG-DNA and imiquimod increased serum levels of total IgG dsDNA antibodies. In addition, CpG-DNA increased the production of IgG<sub>2a</sub> and IgG<sub>2b</sub> dsDNA antibodies as compared with saline-treated MRL<sup>lpr/lpr</sup> mice (Figure 1B). Hence, the combination of the <i>lpr</i> mutation
and CpG-DNA induced both splenomegaly and DNA autoantibody production in MRL mice. However, the ELISA kits for detection of IgG2a and IgG2b dsDNA antibodies were observed to be more sensitive compared with the total IgG ELISA kit.

**B Cell Activation in MRL and MRL<sup>lpr/lpr</sup> Mice**

The potential of microbial nucleic acids to induce autoantibody production in MRL<sup>lpr/lpr</sup> mice may be linked to their potential to activate B cells. The proliferation of cultured B cells that were isolated from medium-treated MRL and MRL<sup>lpr/lpr</sup> mice was comparable (Figure 2A). CpG-DNA induced the proliferation of CD19<sup>+</sup> B cells that were prepared from MRL<sup>lpr/lpr</sup> but not from MRL wild-type mice. This was consistent with the marked expansion of spleen follicular B220<sup>+</sup> B cell areas in MRL<sup>lpr/lpr</sup> mice that received a injection of CpG-DNA (Figure 2B). Consistent with previously published data, the effect of imiquimod was less prominent in the absence of additional co-stimuli, and pl:C RNA did not affect the proliferation of B cells of either mouse strain. In fact, even 10-fold higher doses of imiquimod or pl:C RNA did not induce B cell proliferation in 8-wk-old MRL<sup>lpr/lpr</sup> mice (data not shown). CpG-DNA but not pl:C RNA increased the production of IL-12p40 in B cells that were prepared from both MRL and MRL<sup>lpr/lpr</sup> mice (Figure 2C). Imiquimod-induced IL-12p40 production was less potent, but in the presence of 5000 µg/ml murine IFN-α as indicated. IL-6 and IL-12p40 were measured in supernatants by ELISA after 24 h of incubation with the respective Toll-like receptor (TLR) ligands. Results shown are means ± SEM from three comparable experiments. *P < 0.05 versus medium. Magnification, ×50.

**Dendritic Cell Cytokine Release in MRL and MRL<sup>lpr/lpr</sup> Mice**

Dendritic cells coordinate adaptive immune responses during antimicrobial immunity as well as autoimmune. Therefore, we examined whether Fh3 ligand–induced, CD11c-positive, ER-HR3-negative dendritic cells that were prepared from
MRL or MRL<sup>lpr/lpr</sup> mice release proinflammatory cytokines when incubated with different dosages, pI:C RNA, imiquimod, or CpG-DNA. All three TLR agonists induced IL-12p40 and IL-6 in dendritic cells (Figure 3). Imiquimod and pI:C RNA were less potent in inducing IL-12p40 and IL-6 release in dendritic cells from MRL or MRL<sup>lpr/lpr</sup> mice, and higher dosages of imiquimod did not further increase cytokine release as a result of cytotoxicity. Imiquimod and CpG-DNA but not pI:C RNA induced IFN-α. No differences between dendritic cells from MRL and MRL<sup>lpr/lpr</sup> mice were detected except for high dosages of CpG-DNA.

**Serum IL-12p40, IL-6, and IFN-α Levels in Young MRL and MRL<sup>lpr/lpr</sup> Mice**

Circulating IL-12p40, IL-6, and IFN-α levels are markers of disease activity in lupus. Therefore, we questioned pI:C RNA-, imiquimod-, and CpG-DNA–induced changes in the respective serum levels in 10-wk-old MRL and MRL<sup>lpr/lpr</sup> mice. Serum IL-12p40, IL-6, and IFN-α levels were determined at 3, 6, 12, and 24 h after a single agonist injection in MRL and MRL<sup>lpr/lpr</sup> mice. Baseline levels of all cytokines were low in saline-treated MRL and MRL<sup>lpr/lpr</sup> mice (Figure 4). IL-12p40 was induced by imiquimod and CpG-DNA in both strains with a maximum at 6 h after injection (Figure 4). Similarly, IL-6 was induced by pI:C RNA and CpG-DNA in both strains (Figure 4). Serum IFN-α levels were low in all groups of mice and did not respond to the TLR agonists except that CpG-DNA induced the 3-h levels in MRL<sup>lpr/lpr</sup> mice (Figure 4). These data suggest that IL-12p40, IL-6, and IFN-α serum levels are induced differently upon exposure to pI:C RNA, imiquimod, or CpG-DNA.

**Lupus Nephritis in Young MRL<sup>lpr/lpr</sup> and MRL Mice**

The induction of DNA autoantibody production could be associated with respective renal immune complex deposition and subsequent lupus nephritis. Therefore, we quantified glo-
merular immune IgG1 and IgG2a deposition by immunohistochemistry. In MRL wild-type mice of all groups, glomerular IgG1 and IgG2a deposits were not detected. Saline-treated MRLlpr/lpr mice had mild glomerular IgG1 and IgG2a deposits, predominantly in a capillary staining pattern (Figure 5). In MRLlpr/lpr mice injections of pI:C RNA and imiquimod did not significantly increase the amount of glomerular IgG1 and IgG2a deposits (Figure 5). By contrast, injection of CpG-DNA was associated with a robust increase of capillary and mesangial staining of IgG1 and IgG2a (Figure 5, Table 1). Glomerular immune complexes cause glomerular damage through local complement activation. Only CpG-DNA caused marked glomerular C3 deposits in MRLlpr/lpr mice, whereas C3 staining was hardly detectable in pI:C RNA- or imiquimod-treated MRLlpr/lpr mice and absent in MRL wild-type mice (Table 1). Histologically, saline-treated MRLlpr/lpr mice revealed few hyaline deposits in the mesangium and focal mesangio proliferative lesions as compared with age-matched MRL wild-type mice (Figure 5). Diffuse global proliferative lupus nephritis was detected only in CpG-DNA–treated MRLlpr/lpr mice (Figure 5), consistent with increased numbers of Ki-67–positive proliferating glomerular cells in these mice (Table 1). Chronic lesions such as fibrous crescents, glomerulosclerosis, interstitial fibrosis, and tubular atrophy were absent in all groups of mice. These findings also were indicated by application of the respective histologic score for disease activity of lupus nephritis (Table 1). Immunostaining for CD3 lymphocytes and Mac-2 macrophages in renal sections revealed increased numbers of glomerular macrophages in CpG-DNA–injected MRLlpr/lpr mice only (Figure 5, Table 1). CD3 lymphocytes were not found in significant numbers in kidneys of MRL and MRLlpr/lpr mice. Only CpG-DNA–treated MRLlpr/lpr mice had proteinuria indicated by an elevated urinary albumin/creatinine ratio (Table 1). These data indicate that exposure to pI:C RNA, imiquimod, or CpG-DNA does not induce lupus nephritis in MRL wild-type mice. By contrast, CpG-DNA but not pI:C RNA or imiquimod can trigger lupus nephritis in MRLlpr/lpr mice, which is associated with enhanced cytokine production, B cell activation, dsDNA autoantibody production, glomerular immune complex deposition, and subsequent glomerular damage.

Inhibitory DNA Blocks the CpG-DNA–Induced Onset of Lupus Nephritis in MRLlpr/lpr Mice

The immunomodulatory effects of microbial DNA are thought to be motif specific, and inhibitory DNA sequences have been shown to suppress the immunostimulatory effects of CpG-DNA (23). We therefore treated additional groups of 8-wk-old female MRLlpr/lpr mice using the same injection protocol as before with saline, CpG-DNA (ODN 1668), GpC-DNA (ODN 1720), inhibitory DNA (ODN 2114), or ODN 1668/2114. Co-injection of ODN 2114 inhibited the CpG-DNA–induced onset of lupus nephritis in young MRLlpr/lpr mice. This was documented by a significant reduction of glomerular C3 deposits, activity index for lupus nephritis, glomerular macrophages, and albuminuria and glomerular Ki-67–positive cells (Table 2). This was consistent with a reduction of serum IL-6 and IL-12p40 levels and a trend toward reduced spleen size and IFN-α levels in ODN 1668 + 2114–treated MRLlpr/lpr mice as compared with mice that were treated with ODN 1668 (Table 2). Injections with the inhibitory ODN 2114 or the control GpC-ODN had no effect on lupus nephritis of young MRLlpr/lpr mice. These data show that the DNA-induced lupus nephritis is specific to the CpG-motif. Furthermore, inhibitory DNA elements that are delivered in synthetic ODN can prevent CpG-DNA–induced onset of lupus nephritis by suppressing CpG-DNA–induced lymphoproliferation, autoantibody production, and subsequent renal injury.

Discussion

In vitro studies suggest that microbial nucleic acids can modulate autoimmunity through multiple mechanisms, but their impact on the onset of lupus nephritis in MRLlpr/lpr mice is not known (11–13,15,17,24–26). We used MRLlpr/lpr and MRL wild-type mice to study the effects of an intermittent exposure to pI:C RNA, imiquimod, and CpG-DNA on lymphoproliferation and autoantibody production in genetically predisposed and nonpre disposed hosts. None of the compounds caused DNA autoantibody production or glomerulonephritis in MRL wild-type mice.
By contrast, CpG-DNA but not pI:C RNA or imiquimod triggered an early onset of lupus nephritis in MRL\textsuperscript{Ipr/lpr} mice.

**CpG-DNA Triggers Lupus Nephritis in Young MRL\textsuperscript{Ipr/lpr} Mice**

TLR-9 is not expressed by intrinsic renal cells (18). Hence, the potential of CpG-DNA to induce lupus nephritis in young MRL\textsuperscript{Ipr/lpr} mice is more likely to mediate its modulatory effects on immune cells at extrarenal sites (e.g., in lymphoid organs). In fact, the onset of lupus nephritis is thought to arise from glomerular immune complex deposition, complement activation, and subsequent glomerular inflammation (1). At 8 wk of age, MRL\textsuperscript{Ipr/lpr} mice are characterized by an early stage of lymphoproliferation, but serum DNA autoantibodies are still absent (22). Thus, young MRL\textsuperscript{Ipr/lpr} mice represent a model of a genetically predisposed host in which overt autoimmune tissue injury (i.e., proteinuric lupus nephritis) is absent. After exposure of MRL\textsuperscript{Ipr/lpr} mice to dsRNA, imiquimod, and CpG-DNA, prominent glomerular deposits of IgG, the complement factor C3, diffuse proliferative glomerulonephritis, and overt proteinuria were observed exclusively in CpG-DNA-treated MRL\textsuperscript{Ipr/lpr} mice. Only CpG-DNA activated immunity at multiple levels (B cell cytokine production, B cell proliferation, autoantibody production, dendritic cell cytokine production, and elevation of serum IFN-α levels). This is consistent with previous data documenting the immunostimulatory effects of CpG-DNA in other autoimmune-prone mouse strains (27,28). By contrast, dsRNA and imiquimod did not induce renal disease in MRL\textsuperscript{Ipr/lpr} mice, despite the potential of imiquimod to trigger dsDNA autoantibody production (11). dsRNA and imiquimod less potently triggered polyclonal B cell proliferation in young MRL\textsuperscript{Ipr/lpr} mice even at a 10-fold higher dosage. TLR-7 ligands were shown previously to activate more potently preactivated B cells (29) (e.g., in the presence of IFN-α [30]), an effect that we
also observed in primary B cells from MRL\textsuperscript{pr/\textit{pr}} mice. However, serum IFN-\alpha levels were undetectable or low in 8-wk-old MRL and MRL\textsuperscript{pr/\textit{pr}} mice, respectively. Polyclonal B cell proliferation is critical to trigger the onset of autoimmune tissue injury in MRL\textsuperscript{pr/\textit{pr}} mice and in human SLE (31,32). Imiquimod also failed to induce the class switch to anti-dsDNA IgG\textsubscript{2a}, which are pathogenic in lupus nephritis and specifically induced by TLR-9 signaling (33). This may explain why dsRNA and imiquimod did not induce lupus nephritis despite their stimulatory effects on serum IL-6 and IL-12p40 levels (pI:C RNA) or anti-dsDNA total IgG production (imiquimod). We recently showed that TLR-3, -7, and -9 ligation markedly aggravates the progression of advanced lupus nephritis to a comparable extent in 16-wk-old MRL\textsuperscript{pr/\textit{pr}} mice (18,19,21). This involved two additional mechanisms that apply only to MRL\textsuperscript{pr/\textit{pr}} mice in which a renal lesion is already present at the time of exposure to TLR ligands (1). Mesangial cell apoptosis that is induced by the same dosages of pI:C RNA can cause mesangiolysis in activated glomerular mesangial cells of nephritic MRL\textsuperscript{pr/\textit{pr}} mice (21,34). We did not observe mesangiolysis in young MRL\textsuperscript{pr/\textit{pr}} mice that were treated with pI:C RNA, indicating that the low basal expression of TLR-3 in mesangial cells of nonnephritic kidneys of MRL\textsuperscript{pr/\textit{pr}} mice does not support dsRNA-induced glomerular injury (34). In fact, increasing the activation state of glomerular macrophages is a potent mechanism to exacerbate glomerular injury in experimental glomerulonephritis (35–37). However, this mechanism could not operate in MRL wild-type mice or MRL\textsuperscript{pr/\textit{pr}} mice that were not treated with CpG-DNA in which glomerular macrophages were absent.

\textit{Induction of Lupus Nephritis in MRL\textsuperscript{pr/\textit{pr}} Mice by DNA is Motif Specific}

In this study, none of the ODN induced lupus nephritis in MRL\textsuperscript{pr/\textit{pr}} mice except for CpG-DNA. CpG was identified to be the critical motif in bacterial DNA that mediated its immunostimulatory effects through TLR-9 (25,38). Inhibitory DNA sequence elements counterbalance the immunostimulatory effects of CpG-DNA, but they occur at different frequencies in microbial and vertebrate DNA (39,40). Inhibitory ODN can block CpG-DNA-induced effects in vitro (12,21,23). Here we show that injections with inhibitory DNA prevent CpG-DNA-induced lupus nephritis in young MRL\textsuperscript{pr/\textit{pr}} mice. This was associated with an inhibitory DNA-related suppression of CpG-DNA-induced dsDNA autoantibody production and serum IL-6, IL-12p40, and IFN-\alpha levels. Thus, inhibitory DNA can antagonize the multiple motif-specific immunostimulatory effects of CpG-DNA in young lupus-prone MRL\textsuperscript{pr/\textit{pr}} mice.

\textit{CpG-DNA Does Not Induce Lupus Nephritis in MRL Wild-Type Mice}

MRL mice carry a number of susceptibility genes, of which some loci predispose to lymphoproliferation and DNA autoantibody production, whereas others predispose to either lupus nephritis or arthritis (41,42). Here we show that CpG-DNA-induced activation of B cells and dendritic cells does not trigger the onset of lupus nephritis in MRL mice most likely because MRL mice still are capable of controlling the number of autoreactive B cells and T cells \textit{via} Fas-induced apoptotic cell death (43). Repeated injections with CpG-DNA can cause splenomegaly and disruption of lymph follicles in nonautoimmune mice, but CpG-DNA can trigger high-affinity DNA autoantibody production in normal mice only when injected together with mammalian DNA (44,45).

\textbf{Conclusion}

Among nucleic acids and nucleic acid–like PAMP, CpG-DNA has a unique potential to trigger the onset of lupus nephritis in young autoimmune-prone MRL\textsuperscript{pr/\textit{pr}} mice. This relates to the potent immunostimulatory effects of CpG-DNA at multiple levels: B cell cytokine production, B cell proliferation, autoantibody production, dendritic cell cytokine production, and elevation of serum IFN-\alpha levels. However, CpG-DNA does not trigger the onset of lupus nephritis in the presence of Fas, despite numerous other autoimmune susceptibility genes in MRL mice. These data confirm the concept of a combination of a strong genetic predisposition in the host and external factors for the pathogenesis of lupus nephritis. In addition, these data support the concept of SLE’s being associated with chronic viral infections that continuously release CpG-DNA (e.g., Ebstein-Barr virus) rather than those that release RNA (e.g., hepatitis C virus) (5).

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