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Role of TLR9 in Anti-Nucleosome and Anti-DNA Antibody Production in \textit{lp}r Mutation-Induced Murine Lupus$^1$

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Systemic lupus erythematosus is characterized by the production of autoantibodies directed against nuclear Ags, including nucleosome and DNA. TLR9 is thought to play a role in the production of these autoantibodies through the capacity of nuclear immunogenic particles to interact both with BCR and TLR9. To determine the role of TLR9 in SLE, C57BL/6-\textit{lp}r/lpr-\textit{TLR9}−/− and C57BL/6+−/− mice were analyzed. The abrogation of TLR9 totally impaired the production of anti-nucleosome Abs, whereas no difference was observed in the frequency of anti-dsDNA autoantibodies whose titer was strikingly higher in \textit{TLR9}−/− mice. In addition a higher rate of mesangial proliferation was observed in the kidney of \textit{TLR9}-deficient animals. These results indicate that in C57BL/6-\textit{lp}r/lpr mice, TLR9 is absolutely required for the anti-nucleosome Ab response but not for anti-dsDNA Ab production which is involved in mesangial proliferation. The Journal of Immunology, 2006, 177: 1349–1354.

**Materials and Methods**

**Mice**

C57BL/6 (B6) mice bearing homozygous Fas\textsuperscript{\textit{lp}r/\textit{lp}r} mutation (B6-\textit{lp}r/lpr) and C57BL/6-\textit{TLR9}−/− (B6-\textit{TLR9}−/−) mice were obtained from the Centre de Distribution Typage et Archivage Animal and bred in our animal facilities. C57BL/6-\textit{lp}r/lpr-\textit{TLR9}−/− (B6-\textit{lp}r/lpr-\textit{TLR9}−/−) mice were obtained from crosses between B6-\textit{lp}r/lpr and B6-\textit{TLR9}−/− mice. C57BL/6-\textit{lp}r/lpr and B6-\textit{TLR9}−/− were used as controls.

**CpG immunization protocol**

Two-month-old B6-\textit{lp}r/lpr and B6-\textit{lp}r/lpr-\textit{TLR9}−/− mice were immunized i.p. with 10 μg of phosphorothioate-CpG-oligodeoxynucleotide (ODN) (S\textsuperscript{5′}-tcc atcg tctg acgt tctg tctg-3′) endotoxin-free (InvivoGen) followed by five booster injections 2 wk apart. Two-month-old B6-\textit{lp}r/lpr and B6-\textit{lp}r/lpr-\textit{TLR9}−/− were used as unimmunized control groups and received distilled water at each injection.

**Flow cytometry analysis and mAbs**

Splenocytes and lymph node (LN) cells from the inguinal sites were purified using Lympholyte-M (Clinsciences). Cells at the interface were washed with PBS and incubated for 20 min on ice with optimal amounts of FITC- or PE-conjugated primary Abs diluted in PBS. The following Abs were obtained from BD PharMingen: CD3ε (CD3 ε-chain), CD4 (L3T4), CD8a (Ly-2), CD19, and CD45R/B220 (RA3-6B2) and used at pretitrated dilutions. After washes, cell staining was analyzed using an Epics XL (Beckman Coulter).

**Anti-nuclear Ab and anti-dsDNA immunofluorescence**

For indirect immunofluorescence assay on HEp-2 cells (BMD), sera were diluted 1/10 and incubated for 20 min. For indirect immunofluorescence assay on \textit{Crijtridia luciilae} (MBL), sera were diluted 1/5-1/320. After washing, bound IgG were detected using a FITC-conjugated goat antimouse IgG (Fc specific; Sigma-Aldrich).

**ELISAs**

Anti-dsDNA and anti-nucleosome Abs were detected by ELISAs using plasmid dsDNA precoated plates (Varelisa; Pharmacia Diagnostics) or

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3 Abbreviations used in this paper: SLE, systemic lupus erythematosus; DN, double negative; LN, lymph node; CL, cardiolipin; IIF, indirect immunofluorescence.
DNA-stripped nucleosome (Euroimmun) as the Ags on the solid phase. For anti-cardiolipin (CL), anti-ssDNA Ab and rheumatoid factor detection, plates were incubated, respectively, with 10 µg/ml bovine heart CL, 1 µg/ml calf thymus ssDNA (Sigma-Aldrich), or 10 µg/ml rabbit IgG (Jackson ImmunoResearch Laboratories). Mouse sera were diluted 1/100 and incubated for 2 h at room temperature. Bound IgG or IgM were detected either with alkaline phosphatase-conjugated goat anti-mouse IgG or anti-mouse IgM (Rockland). The cutoff values were 0.5 for anti-ssDNA, 0.276 for anti-dsDNA, 0.22 for anti-nucleosome, 0.109 for anti-CL Abs, and 0.268 for rheumatoid factor.

Proteinuria and histopathology

Urine was collected for proteinuria determination at different ages using reagent strips (Multistix; Bayer). Mice were sacrificed at 6 mo of age and renal examination was performed. Half kidneys were fixed in 4% neutral formaldehyde, sectioned, and stained with trichrome green. We analyzed the presence of interstitial lymphoid infiltration and glomerulonephritis including mesangial cell proliferation. A nephritis index was determined based on the mesangial proliferation (0, <3 cells per glomerulus; 1, mild with 3–10 cells per glomerulus; 2, moderate with 10–15 cells per glomerulus; and 3, severe with >15 cells per glomerulus). Frozen kidney sections were stained with FITC-conjugated goat anti-mouse IgG (Sigma-Aldrich). The topography and intensity of glomerular Ig deposits were assessed by two renal pathologists (P.C. and A.F.).

Statistical analysis

Weight of lymphoid organs and OD measured in mouse sera were compared using the Mann-Whitney U test. Mesangial proliferation and proteinuria observed in B6-lpr/lpr-TLR9−/− and B6-lpr/lpr-TLR9+/+ mice were compared using Fisher’s exact test. Comparison of OD measured in mouse sera at different age was performed using the Friedman test.

Results

Clinical signs of SLE in B6-lpr/lpr-TLR9−/− mice

The phenotype of B6-lpr/lpr and B6-lpr/lpr-TLR9−/− mice was analyzed from birth to 6 mo of age. As reported previously (16), the most apparent clinical sign of lpr-induced SLE was splenomegaly and generalized lymphadenopathy (Fig. 1A). In B6-lpr/lpr-TLR9−/− mice, axillary and inguinal LN weights were significantly greater (n = 12; mean = 366 ± 316 mg) than in B6-lpr/lpr (n = 11; mean = 52 ± 85.3 mg; p < 0.01, Mann-Whitney U test) (Fig. 1B). Likewise and as expected, all spleens in the lpr/lpr strain were enlarged; B6-lpr/lpr-TLR9−/− spleens were 5-fold heavier than those from B6-lpr/lpr mice (p < 0.01). However survival rates of B6-lpr/lpr-TLR9−/− (66%) vs B6-lpr/lpr mice (64%) at 24 wk of age were not different.

T and B cell phenotypes in TLR9-deficient mice

One of the major phenotype characteristic of lpr/lpr mouse is an increased number of CD4+ CD8− double-negative (DN) T cells in enlarged lymphoid organs (16, 17). Thus, we performed a phenotype analysis of cells that accumulated in LN and spleens from B6-lpr/lpr-TLR9−/− and B6-lpr/lpr mice. No difference in single-positive T cell and B cell population percentages was observed between B6-lpr/lpr-TLR9−/− and B6-lpr/lpr mice, whereas DN T cells were dramatically increased in B6-lpr/lpr-TLR9−/− mice (Fig. 2). The massive lymphadenopathy and splenomegaly observed in B6-lpr/lpr-TLR9−/− mice were accompanied by increased levels of total IgG which were significantly higher than in B6-lpr/lpr-TLR9−/− mice (mean = 30.7 ± 7 vs 5.4 ± 1.4 mg/ml; p < 0.01, Mann-Whitney U test). In contrast, total IgM were lower in B6-lpr/lpr-TLR9−/− than in B6-lpr/lpr-TLR9−/− mice (mean = 3.6 ± 1.6 mg/ml vs mean = 5.8 ± 0.7; p < 0.05).

Anti-chromatin autoantibodies in TLR9-deficient mice

Sera from B6-lpr/lpr-TLR9−/− and B6-lpr/lpr mice were collected from 1 to 6 mo of age and examined by indirect immunofluorescence (IIF) analysis on HEP-2 cells. Although 10 of 11 B6-lpr/lpr sera exhibited a homogenous staining of the nucleus, none of 14 B6-lpr/lpr-TLR9−/− sera presented this IIF pattern (Fig. 3A) since 11 of 14 bound to nucleoli (p < 0.01). The dramatic change of the nuclear staining pattern in TLR9−/− mice indicates that the abrogation of TLR9 expression either impaired the production of the autoantibody population giving the homogenous nuclear staining, demasking an antinuclear activity, or eventually changed the autoantigen binding properties of autoantibodies. Since a homogenous nuclear staining is given by autoantibodies directed against chromatin and/or its components, we performed an autoantibody analysis of wild-type and TLR9-deficient B6-lpr/lpr mouse sera by using dsDNA- and DNA-stripped nucleosome ELISAs. No significant difference in the frequency of anti-dsDNA autoantibodies was observed between B6-lpr/lpr and B6-lpr/lpr-TLR9−/− mice: they were detected in 7 of 11 B6-lpr/lpr and 13 of 16 B6-lpr/lpr-TLR9−/− mice at 3 mo of age (Fig. 3B; p = 0.160) and in 9 of 11 B6-lpr/lpr and 11 of 12 B6-lpr/lpr-TLR9-deficient mice at 6 mo of age (p = 0.466). Interestingly, the titer of IgG anti-dsDNA Abs was higher in B6-lpr/lpr-TLR9−/− (p < 0.0021; Fig. 4, A and B), whereas the titer of IgM anti-dsDNA Abs was not significantly different between these two strains of mice (p = 0.46). To confirm these results, sera were tested by IIF on Crithidia luciliae, a substrate commonly used for the detection of anti-dsDNA in SLE (18). Among 11 sera collected from 6-mo-old B6-lpr/lpr mice, 8 contained Abs staining the C. luciliae kinetoplast. Similarly, 9 of 12 six-mo-old B6-lpr/lpr-TLR9−/− mice displayed the same IIF pattern (Fig. 4C) at dilutions 1/5–1/320 (Fig. 4D). In contrast, when the nuclease-binding activity was examined, none of the 3-mo-old B6-lpr/lpr-TLR9−/− mice had anti-nucleosome Abs
(n = 16) that were detected at high levels in 10 of 11 B6-lpr/lpr mice (Fig. 3B, p < 0.0001). Although two sera from 6-mo-old TLR9-deficient mice weakly bound to nucleosome, the difference in nucleosome-binding activity between the two groups was again highly significant (p < 0.0001). Moreover, anti-histone Abs were not detected in TLR9-deficient sera (data not shown). Thus, in B6-lpr/lpr mice, TLR9 deficiency dramatically impaired anti-chromatin Ab production and dissociated the anti-dsDNA from the anti-nucleosome Ab response. These results indicate that TLR9 plays an essential role in the B cell response against nucleosome-associated proteins.

Rheumatoid factor, anti-ssDNA, and anti-CL in TLR9-deficient mice

We also looked for the presence of other autoantibody populations frequently detected in lupus mice (Fig. 3B). Rheumatoid factors were present in 93% of 3-mo-old B6-lpr/lpr-TLR9-/- and 90% of B6-lpr/lpr mice, and their titers were not different between the two groups. Anti-CL were detected in 37.5 and 18%, respectively, of B6-lpr/lpr-TLR9-/- and B6-lpr/lpr sera (p = 0.405), indicating that anti-phospholipid Ab production is not controlled by TLR9 as reported by others (19). Similarly, TLR9 deficiency did not affect the production of anti-ssDNA Abs that were present in 81 and 72.7% of sera from B6-lpr/lpr-TLR9-/- and B6-lpr/lpr, respectively. Thus, in contrast to the anti-nucleosome Ab response, the production of autoantibodies directed against IgG, CL, and ssDNA was not impaired in TLR9-deficient mice.

Activation of TLR9 with CpG in lpr/lpr mice

To determine the role of TLR9 stimulation in autoantibody production, we immunized B6-lpr/lpr and B6-lpr/lpr-TLR9-/- mice from 2 mo of age (before the appearance of IgG autoantibodies) with hypomethylated CpG-DNA, twice a month and determined the levels of anti-dsDNA and anti-nucleosome Abs. Fig. 5 shows the time course of anti-dsDNA and anti-nucleosome Ab production in B6-lpr/lpr and B6-lpr/lpr-TLR9-/- immunized mice. Anti-nucleosome Ab response did not significantly differ from that observed during the spontaneous development of the disease of TLR9 +/- mice but was blocked in TLR9-deficient mice (Fig. 5A). Thus, direct activation of TLR9 with hypomethylated CpG is not able per se to induce an anti-nucleosome Ab response in TLR9 +/- or TLR9-/- mice, as has been demonstrated in normal mice (20). We found that the absence of TLR9 did not impair the production of anti-DNA autoantibodies and immunization with CpG even induced a slight but not significant (p = 0.46) increase of anti-DNA in B6-lpr/lpr-TLR9 +/- and in B6-lpr/lpr-TLR9-/- mice (Fig. 5B). Thus, TLR9 may not be the unique receptor of CpG able to induce anti-DNA response.

Kidney disease in the absence of anti-nucleosome Abs

Proteinuria was increased in more B6-lpr/lpr TLR9-/- mice from 13 to 24 wk of age than in B6-lpr/lpr TLR9 +/- mice, but the difference was not significant (p = 0.062 at 13 wk, data not shown). Mice were killed at 6 mo of age and histological and immunochemical analyses of the kidneys were performed. In both groups, a similar interstitial lymphoid infiltration was observed. Glomerular IgG deposits that were exclusively mesangial were more intense in B6-lpr/lpr-TLR9-/- than in B6-lpr/lpr mice (Fig. 6A). A higher rate of mesangial proliferation was observed in these animals. Indeed, 11 of 12 B6-lpr/lpr-TLR9-/- mice vs 5 of 11 B6-lpr/lpr mice had mesangial cell proliferation (p = 0.024, Fisher’s exact test; Fig. 6, B and C). Our observation indicates that
glomerular IgG deposits and mesangial glomerulonephritis can occur in TLR9-deficient mice in the absence of anti-nucleosome Abs (21, 22).

Discussion

Our study shows that B6-lpr/lpr mice develop a lupus-like autoimmune disease characterized by a massive lymphoproliferation, anti-DNA autoantibodies, immune complex deposition, and glomerulonephritis and that TLR9 deficiency does not dramatically affect this phenotype. TLR9-deficient B6-lpr/lpr mice exhibited increased immune activation, as demonstrated by an augmentation of LN and splenic weights and hypergammaglobulinemia that is in agreement with previous results obtained in (TLR9−/−/H11002/H11002/H11003 MRL/Mp lpr/lpr) mice (19, 23). These observations indicate that the TLR9 signaling pathway could also exert a negative control of lymphocyte proliferation and accumulation in the context of sustained cell activation by endogenous TLR9 ligands (24, 25).

Another important observation is the change of the autoantibody specificity characterized by the loss anti-nucleosome/histone Abs in TLR9-deficient B6-lpr/lpr mice. This change in autoantibody profile is consistent with previous studies showing that TLR9 ligands can induce the production of anti-nucleosome Abs (26, 27). These findings suggest that TLR9 signaling may play a role in the regulation of autoantibody production in lupus.

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using the Mann-Whitney from 0 to 3 for each mouse. The statistical comparison was performed with those recently observed by Wu and Peng (23) but are despite the persistence of anti-dsDNA. These findings are in agreement with the importance of nephritis (21, 22).

Our data, as do the other recent reports concerning TLR9-deficient lupus mice (23), provide clear evidence for a protective role of TLR9 in the development of the lupus disease since all TLR9−/− lupus mice present more intense renal deposits, proteinuria, and lymphadenopathy than their normal counterparts. Paradoxically, TLR9 inhibition by synthetic ODN can also have beneficial effects on the disease progression in lupus nephritis (39, 40). Whereas the mechanisms responsible for this ambivalent role of TLR9 remain unclear, it should be noted that TLR9 activation could modulate the immune response from a Th1 to a Th2 pattern. Indeed, CpG-ODN have been demonstrated to induce IL-10 in lupus B cells, particularly those with the marginal zone B cell phenotype (41, 42) and then to down-regulate proinflammatory cytokines (42). IL-10-producing B cells are known to regulate autoimmunity (43) and IL-10-deficient MRL-Fas−/− mice developed severe lupus (44). Interestingly, these mice present an exacerbated disease phenotype with early appearance of skin lesions, more severe renal disease and augmented production of IgG2a anti-dsDNA autoantibodies, a phenotype resembling that observed in our TLR9-deficient lupus mice.

Taken together, our results indicate that the engagement of TLR9 expressed at the surface of B cells (or other cells of the immune system) by endogenous or exogenous ligands participate in the expression of systemic autoimmunity by selectively inducing certain autoantibody populations and lymphocyte proliferation. Interestingly, TLR engagement may also lead to receptor inhibition (39,
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40, which, along with our observations and those previously reported by others (19, 23), allows us to consider TLR as a potential target of new therapeutic agents of SLE (10).

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Disclosures

The authors have no financial conflict of interest.

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