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Role of TLR9 in Anti-Nucleosome and Anti-DNA Antibody Production in \textit{lpr} 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{lpr}/\textit{lpr}-TLR9$^{-/-}$ and TLR9$^{+/+}$ 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 TLR9$^{-/-}$ mice. In addition a higher rate of mesangial proliferation was observed in the kidney of TLR9-deficient animals. These results indicate that in C57BL/6-\textit{lpr}/\textit{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. \textit{The Journal of Immunology}, 2006, 177: 1349–1354.

\textbf{Materials and Methods}

\textbf{Mice}

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

\textbf{CpG immunization protocol}

Two-month-old B6-\textit{lpr}/\textit{lpr} and B6-\textit{lpr}/\textit{lpr}-TLR9$^{-/-}$ mice were immunized i.p. with 10 \textmu g of phosphorothioate-CpG-oligodeoxynucleotide (ODN) (5'-tcc atg acg tct ctc aag t3') endotoxin-free (InvivoGen) followed by five booster injections 2 wk apart. Two-month-old B6-\textit{lpr}/\textit{lpr} and B6-\textit{lpr}/\textit{lpr}-TLR9$^{-/-}$ were used as immunized control groups and received distilled water at each injection.

\textbf{Flow cytometry analysis and mAbs}

Splenocytes and lymph node (LN) cells from the inguinal sites were purified using Lymphocyte-M (Clinscience). 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: CD3e (CD3 e-chain), CD4 (L3T4), CD8a (Ly-2), CD19, and CD45R/B220 (RA3-6B2) and used at pretitated dilutions. After washes, cell staining was analyzed using an Epics XL (Beckman Coulter).

\textbf{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{Cribidia luciliae} (MBL), sera were diluted 1/5–1/320. After washing, bound IgG were detected using a FITC-conjugated goat anti-mouse IgG (Fc specific; Sigma-Aldrich).

\textbf{ELISAs}

Anti-dsDNA and anti-nucleosome Abs were detected by ELISAs using plasmid dsDNA-precoated plates (Varelsa; Pharmacia Diagnostics) or...
A 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 glomeruli; 1, mild with 3–10 cells per glomeruli; 2, moderate with 10–15 cells per glomeruli; and 3, severe with >15 cells per glomeruli). 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 organ 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 mice 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 exhibited 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 nuclease ELISAs. No significant difference in the frequency of anti-dsDNA auto antibodies 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 Cricetidio 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 against 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 nucleosome-binding activity was examined, none of the 3-mo-old B6-lpr/lpr-TLR9−/− mice had anti-nucleosome Abs.
of sera from B6-lpr/lpr-TLR9 animals 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 contrast to the anti-nucleosome Ab response, the 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 immunohistochemical 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−/−) 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.

FIGURE 4. Anti-dsDNA Abs in sera from B6-lpr/lpr-TLR9+/+ and B6-lpr/lpr-TLR9−/− mice. Sera were analyzed at 3 (A) and 6 mo of age (B). Titers were significantly higher in B6-lpr/lpr-TLR9−/− (white losanges) than in B6-lpr/lpr mice (black losanges) at both times of sampling. The statistical comparisons were performed using the Mann-Whitney U test. C. detection of anti-dsDNA by C. luciliae immunofluorescence. B6-lpr/lpr and B6-lpr/lpr-TLR9−/− mice sera were analyzed at 6 mo of age. Sera were considered positive for anti-dsDNA Abs when specific fluorescence was seen in the kinetoplast, a mitochondrion which exclusively contains dsDNA (original magnification, ×400). White arrows indicate the kinetoplast. Anti-dsDNA Ab titers were measured by a solid-phase ELISA using plasmid DNA as the Ag in sera from B6-lpr/lpr-TLR9+/+ and B6-lpr/lpr-TLR9−/− mice (cutoff value: OD = 0.276). D. Detection of anti-dsDNA by C. luciliae immunofluorescence at different dilutions of sera from B6-lpr/lpr and B6-lpr/lpr-TLR9−/− mice.

FIGURE 5. Immunization with CpG does not induce the production of anti-dsDNA and anti-nucleosome Abs. B6-lpr/lpr (n = 6) and B6-lpr/lpr-TLR9−/− mice (n = 6) were immunized with 10 μg of CpG followed by five booster injections. B6-lpr/lpr (n = 6) and B6-lpr/lpr-TLR9−/− mice (n = 6) were used as controls. Means of OD given by anti-nucleosome (A) and anti-dsDNA Abs (B) present in sera of B6-lpr/lpr and B6-lpr/lpr-TLR9−/− mice were measured by ELISA. For anti-dsDNA Ab detection, sera with OD >0.276 (cutoff value = mean OD of 18 controls ± 2 SD) were considered positive. For anti-nucleosome Ab detection, sera with OD >0.117 were considered positive. The arrows indicate the time of first injection and bars represent SD. WT, wild type; KO, knockout.
using the Mann-Whitney from 0 to 3 for each mouse. The statistical comparison was performed -TLR9
lpr
lpr/lpr
ment with those recently observed by Wu and Peng (23) but are
despite the persistence of anti-dsDNA. These findings are in agree-
with that the anti-nucleosome response could be strictly dependent on
TLR9, whereas the anti-DNA could not. The existence of the
TLR9-independent pathways in the anti-DNA response has been
suggested in several studies. DNA or ODN that do not contain
CpG motifs and vertebrate genomic DNA can effectively synergize
with specific Ag to costimulate murine B cells, probably via a
TLR9-independent way (30, 31). Mammalian DNA-Ig complexes,
that provide a potent activation of B cells via TLR9, can activate
dendritic cells by both TLR9-dependent and -independent path-
ways (32). Moreover, macrophage activation by endogenous
DNA, escaping to lysosomal degradation, requires TLR9-dependent
and -independent pathways (33) and is not abrogated in
MyD88-deficient mice (34). Other TLR, such as TLR3, TLR7, and
TLR8, involved in the recognition of nucleic acids of viral origin
(dsRNA and ssRNA) (35, 36) have been shown to play an impor-
tant role in the development of autoantibodies present in lupus (37,
38). In the absence of TLR9, these receptors or others that also
recognized nucleic acids, could be involved in anti-DNA Ab pro-
duction. In any case, the common feature of TLR9 deficient lupus mice expressing different genetic backgrounds is the impair-
ment of the anti-nucleosome Ab response that clearly shows the
specific requirement of TLR9 for this response.

The assessment of the renal disease in B6-lpr/lpr-TLR9−/− mice showed glomerular deposits and mesangial proliferation similar-
lly to TLR9-deficient MRL-lpr/lpr mice that also developed
substantial glomerular protein deposition (19), despite the absence
of anti-dsDNA and anti-nucleosome Abs. The authors suggest that
other specificities, anti-Sm for example, may play a pathogenic
role in their models. In our study, the persistence and even the
increased production of anti-dsDNA Abs could explain the ele-
mented mesangial proliferation in TLR9-deficient mice since it has
been clearly demonstrated that the level of anti-DNA Abs is as-
associated with the importance of nephritis (21, 22).

Our data, as do the other recent reports concerning TLR9-defi-
cient 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, protein-
uria, and lymphadenopathy than their normal counterparts. Para-
doxically, TLR9 inhibition by synthetic ODN can also have ben-
eficial 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 au-
toimmunity (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 se-
vere 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 TLR
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 cer-
tain autoantibody populations and lymphocyte proliferation. Inter-
estingly, TLR engagement may also lead to receptor inhibition (39,
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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