Interactions of surface-expressed TLR-4 and endosomal TLR-9 accelerate lupus progression in anti-dsDNA antibody transgenic mice

Tai-Ping Lee[1,2], Jason C Huang[1], Chi-Jui Liu[1], Hsiu-Jung Chen[1], Yih-Horng Chen[1], Yi-Ting Tsai[1], Winnie Yang[3] and Kuang-Hui Sun[1,2,4]

[1]Department of Biotechnology and Laboratory Science in Medicine, National Yang-Ming University, Taipei 11221, Taiwan, Republic of China; [2]Department of Education and Research, Taipei City Hospital, Taipei 10629, Taiwan, Republic of China; [3]Department of Pediatric, Taipei City Hospital, Yang-Ming Branch, Taipei 11146, Taiwan, Republic of China; [4]Infection and Immunity Research Center, National Yang-Ming University, Taipei 11221, Taiwan, Republic of China

Corresponding authors: Kuang-Hui Sun and Winnie Yang. Emails: khsun@ym.edu.tw and dah06@tpech.gov.tw

Abstract

The hallmark of systemic lupus erythematosus (SLE) is the presence of high levels of anti-double-stranded DNA autoantibody (anti-dsDNA) in *sera*. In addition, pathogen infections coincide frequently with the occurrence of lupus. Our study was designed to investigate the contribution of anti-dsDNA, extracellular and intracellular Toll-like receptors (TLRs), a family of pattern-recognition receptors for sensing invading pathogens, in the pathogenesis of lupus. Although cell surface-expressed TLR4 may promote lupus progression, intracellular nucleic acid-sensing TLR9 plays either stimulatory or protective roles in different murine lupus models. To examine the role of TLR4, TLR9, and anti-dsDNA in SLE, we generated transgenic mice carrying anti-dsDNA antibody transgene and challenged the mice with TLR4- and TLR9-agonists, lipopolysaccharides (LPS), and CpG oligodeoxynucleotide (CpG ODN1826 and 2216), respectively. Splenocytes from these mice were found to secrete higher levels of interleukin-10 (IL-10) and anti-dsDNA when treated with a combination of TLR4 and TLR9 agonists (LPS + CpG). In addition, the transgenic mice were intraperitoneally administered with CpG or combined CpG and LPS to determine whether extracellular TLR4 and intracellular TLR9 activations could affect lupus progression *in vivo*. It was found that serum levels of anti-dsDNA antibodies and interferon-alpha were higher in CpG + LPS-treated transgenic mice than those in non-transgenic mice. Besides, elevated levels of proteinuria, blood urine nitrogen, and immune complex depositions in kidney were found in treated transgenic mice. Anti-dsDNA and simultaneous activation of surface-expressed TLR4 and endosomal TLR9 are crucial to promote the lupus progression.

Keywords: Systemic lupus erythematosus, anti-double-stranded DNA autoantibody, Toll-like receptors, lipopolysaccharides, CpG oligodeoxynucleotide

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Introduction

Systemic lupus erythematosus (SLE) is a complicated multifactorial autoimmune disease influenced by many genetic and environmental factors. It is known that autoantibodies are the main cause of tissue damage and can be used for diagnosis of SLE in patients.[1] Lupus nephritis is the major manifestation of SLE characterized by the presence of various circulating antinuclear antibodies (ANAs) in the lupus patients. Among the ANAs, anti-double-stranded DNA antibodies (anti-dsDNA) are the major pathogenic autoantibodies for SLE. Recently, several lines of evidence strongly suggest that autoimmunity can be triggered by environmental factors.[2] In addition, greater infection rates are found in SLE patients and higher morbidity and mortality usually come from bacterial infections.[3,4] Since simultaneous activation of extracellular and intracellular pattern-recognition receptors (PRR) is able to trigger more intense host immune responses, it is really crucial to determine whether co-engagement of extracellular and intracellular PRRs may increase disease severity in lupus.

Toll-like receptors (TLRs), a family of PRR for sensing invading pathogens, were suggested to participate in SLE pathophysiology.[5] TLRs are widely expressed in different immune cells, such as B cells, dendritic cells, macrophages, and specific types of T cells. The expression of TLR1, TLR2, TLR4, TLR5, and TLR6 mediates extracellular recognition of

microbes, while intracellular endosomes harbor the nucleic acid-sensing TLRs (i.e. TLR3, TLR7/TLR8, and TLR9).[6] TLR4 is activated by lipopolysaccharide (LPS), a cell wall component of gram-negative bacteria. A study using transgenic mice expressing surface gp96 demonstrated that TLR4 combined with commensal flora can enhance anti-dsDNA production and induce immune complex (IC)-driven glomerulonephritis.[7] Furthermore, our previous work also showed that pathogenic anti-dsDNA antibodies plus TLR4 activation were able to induce severe SLE syndromes in FVB mice.[8] However, two or more TLRs can be simultaneously activated by microbial derived TLR ligands during infections. For instance, TLR4 and TLR9 co-activation can be achieved by binding of LPS and hypomethylated CpG carrying bacteria. Therefore, we hypothesized that co-activation of extracellular and intracellular TLRs triggered by foreign pathogen binding may cooperate with each other to exacerbate lupus symptoms.

In murine lupus model, although cell surface-expressed TLR4 often promotes lupus progression, the roles of endosomal nucleic acid-sensing TLR9 are still controversial in lupus. Intracellular TLR9 binds to bacterial genome through recognition of hypomethylated CpG containing DNA sequences. Previous studies indicated TLR9 is essential for inducing antichromatin and anti-DNA autoantibody production in murine lupus.[9] Summers *et al.* found pristane-induced autoimmunity and nephropathy were dampened in TLR4 or TLR9 single knockout mice.[10] However, TLR-9-deficient animals of both the MRL/+ and the MRL/lpr backgrounds developed more severe lupus.[11] Christensen *et al*. demonstrated that increased serum levels of IgG and interferon-α (IFN-α) exacerbated disease with higher the mortality rates in $TLR9^{-/-}$ lupus prone mice.[12] Therefore, simultaneous activation of TLR4 and TLR9 in a murine model of lupus needs further exploration.

Individual conditional knockout model to study the roles of TLR4 or TLR9 was used in previous studies; however, we applied combined injection of LPS and bacterial DNA (ODN1826 and ODN2216) at the same time as a new model to investigate whether co-activation of extracellular TLR4 and endosomal TLR9 impacts on the pathogenesis of lupus. We have generated an anti-dsDNA transgenic (Tg) mouse model using a single chain variable fragment (scFv) from 9D7 hybridoma to confirm the roles of anti-dsDNA in the pathogenesis of SLE.[13–17] In this study, we injected the ligands of TLR4 and TLR9 in the 9D7 Tg mice to study the effects of combined treatment. Our data suggest that, in addition to anti-dsDNA, signaling pathways triggered by simultaneous activation of extracellular TLR4 and endosomal TLR9 can promote the progression of SLE.

Materials and methods

Mice

C57BL/6 inbred mice were purchased from the Laboratory Animal Center of National Taiwan University (Taipei, Taiwan). The autoantibody-transgenic mice were generated using our original 9D7 transgene construct by Level Biotechnology, Inc. (Taipei, Taiwan). These animals were bred and kept under specific pathogen-free environment in the Animal Center of National Yang-Ming University. All the animal experiments were performed following the regulations of the Institutional Animal Care and Use Committee of National Yang-Ming University.

Reagents

Lipopolysaccharide (LPS) from *Escherichia coli* O26:B6 strain and calf thymus dsDNA were purchased from Sigma-Aldrich, Inc. (St Louis, MO, USA). The B-type CpGODN1826 and A-type CpG-ODN2216 were obtained from InvivoGen (San Diego, CA, USA) and had the following sequence: ODN 1826, 5′-TCCATGACGTTCCTGA CGTT-3′; ODN 2216, 5′-GGGGGACGATCGTCGGGG GG-3′. The Sm/RNP (Ribonuclear protein) Complex antigen (SRC-3000) was purchased from ImmunoVision Inc. The goat anti-mouse IgG conjugated with fluorescein was purchased from Jackson ImmunoResearch (West Grove, PA, USA). The goat anti-mouse C3 was purchased from Cappel (ICN Pharmaceuticals, Aurora, OH, USA).

9D7 anti-dsDNA monoclonal antibody (9D7 mAb)

The anti-dsDNA mAb 9D7 was produced by the fusion of NS-1 myeloma cells with the spleen cells of autoimmune MRL-*lpr/lpr* mice as previously described.[13]

Transgene construction and generation of 9D7 scFv transgenic mice (9D7 Tg)

The transgene construction and generation of 9D7 Tg mice was previously described.[8] In addition, we constructed pIgH-9D7-secreted form plasmid containing FLAG tag upstream of the polyA signal.

Genotyping of 9D7 Tg mice by Southern blotting

The procedures of detection of genotyping of 9D7 Tg mice by Southern blotting were previously described.[8]

Enzyme-linked immunosorbent assay (ELISA) for anti-dsDNA and anti-Sm/RNP antibodies in sera of 9D7 Tg mice

For the anti-dsDNA ELISA, calf thymus double-stranded DNA (10 μg/mL) was diluted in 2 mM ethylenediaminetetraacetic acid (EDTA) buffer and coated onto ELISA plates overnight at room temperature. For the anti-Sm/RNP antibodies ELISA, 100 μL Sm/RNP complex Ag (10 μg/mL) diluted in 0.05 M sodium carbonate buffer was coated onto ELISA plates overnight at 4°C. The procedures of ELISA were previously described.[8]

Detection of urine proteins

The procedures of detection of urine proteins were previously described.[8]

Blood urea nitrogen (BUN) in sera

The procedures of detection of BUN were previously described.[8]

Anti-nuclear antibody (ANA)

The procedures of detection of ANA were previously described.[8]

LPS, CpG-ODN1826, and CpG-ODN2216 stimulation in vitro

Splenocytes harvested from 25-week-old mice were seeded at 1×10^6 cells/mL in 24-well tissue culture plates and cultured in 5% CO_2 at 37°C for 3 days. The cultured RPMI 1640 medium was prepared in supplement with 10% fetal calf serum (FCS), 100 U/mL penicillin, 100 μg/mL streptomycin, 1% non-essential amino acids (Gibco/Life Technologies, Foster, CA, USA), 2 mM L-glutamine (Sigma), and 50/ μM 2-mercaptoethanol, in the presence of 0.5 μM CpG1826, 2.5 μM CpG2216, 5 μg/mL LPS, or different combination of the stimulants. After incubation for 3 days, the cell supernatants were harvested and quantified for the levels of anti-dsDNA and IL-10 (R&D system, Inc.,Minneapolis, MN, USA) by ELISA.

LPS, CpG-ODN1826, and CpG-ODN2216 injection in vivo

The 29-week-old mice ($n = 4$–5) were intraperitoneally injected twice per week with phosphate buffered saline (PBS), CpG1826 (25 μg/mice), CpG2216 (25 μg/mice), or CpG and LPS (1 μg/body weight (g)) for 6 weeks. After initial injection, the blood and urine samples were collected every week. The levels of IFN-α in the *sera* were assayed by ELISA (PBL InterferonSource, Piscataway, NJ). The mice were sacrificed 6 weeks later with organ samples taken for the following studies.

Immunohistochemistry

The procedures of immunohistochemistry were previously described.[8]

Statistical analyses

The data are expressed as mean ± standard deviation (SD) and statistics was analyzed by Student's *t*-test. $P < 0.05$ was considered to be statistically significant.

Results

Generation and confirmation of 9D7 anti-dsDNA transgenic mice (9D7 Tg)

To study the roles of TLR4, TLR9, and anti-dsDNA in lupus disease progression, anti-dsDNA transgenic (Tg) mice were generated from a single-chain variable fragment (scFv) gene of 9D7 hybridoma under a mouse immunoglobulin heavy chain promoter (pIgH) in the C57BL/6 background. The coding sequences for heavy and light chain variable regions (V_H and V_L) of 9D7 mAb were described previously.[13–17] The pIgH vector was used to confine the gene expression predominantly in the B cells. To confirm our previous work,[8] the genotype and phenotype of 9D7 transgenic mice were examined by subsequent experiments. Eight 9D7-secreted founders were identified by Southern blot analysis indicating the insertion of the transgene into the genome at a

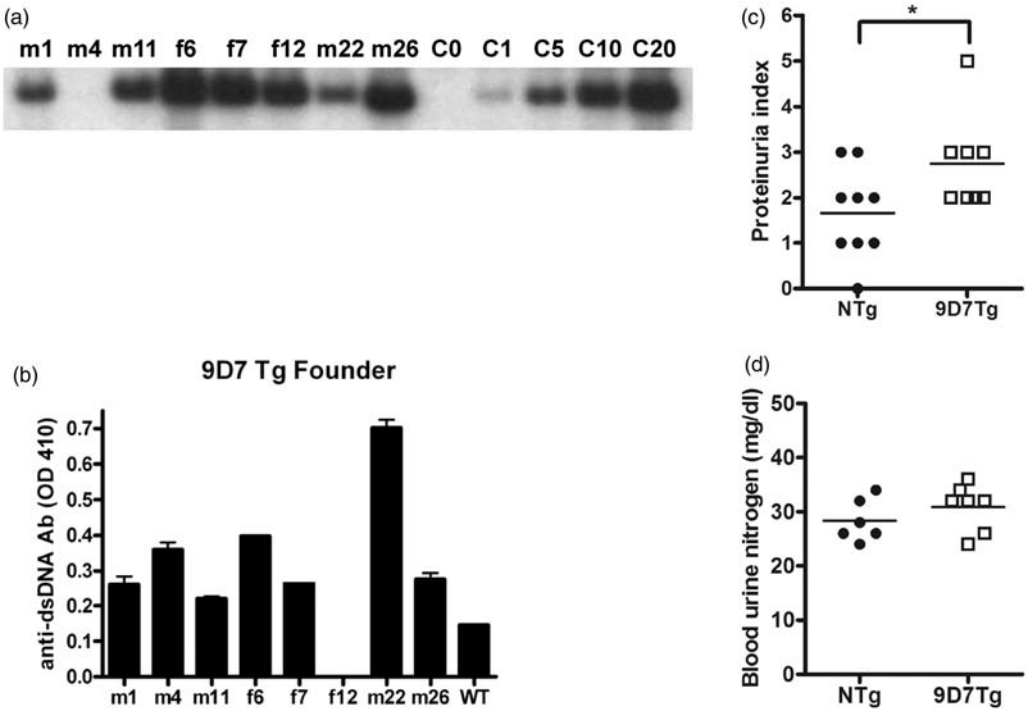

Figure 1 Generation and characterization of 9D7 scFv transgenic mice (9D7 Tg). (a) Southern blot analysis of the 9D7 scFv signals in Tg founders and the relative copy number of each transgenic line. C0–C20: standards of copy number. m: male; f: female. (b) Quantitation of anti-dsDNA antibodies in 26-week-old 9D7 scFv Tg founders and wild type (WT) mice. Urine proteins (c) and blood urea nitrogen (d) in non-Tg littermates (NTg) and 9D7 Tg mice. Mean values were indicated by a bar. *$P < 0.05$

single site (Figure 1(a)). We also found that not all of the 9D7 Tg mice produced anti-dsDNA antibodies although anti-dsDNA antibodies in the *sera* of the 9D7 Tg mice were significantly higher compared to those of the wild type mice (Figure 1(b)). The m1, f6, and m22 of 9D7-secreted Tg lines showed higher expression levels of transgenes and were used for the subsequent experiments. Next we assayed the urine protein and BUN to check renal function. The 9D7 Tg mice demonstrated slightly higher levels of proteinuria index (Figure 1(c)) and BUN (Figure 1(d)) than the non-Tg littermates. LPS injection even induced more 9D7 transgenic protein expression in the 8-week-old 9D7 mice than the non-transgenic mice (data not shown). Our data indicated that the 9D7 Tg mice are prone to develop a mild form of lupus-like syndrome.

Increased anti-dsDNA and IL-10 productions in LPS combined CpG-stimulated splenocytes of 9D7 Tg mice

To examine whether TLR4 and/or TLR9 agonists could effectively activate immune cells, splenocytes of the Tg mice were stimulated with these agonists *in vitro*. The cytokine expression levels in the culture supernatants were quantified by ELISA. We did not see any differences in the levels of IL-10 and anti-dsDNA between the NTg and 9D7 Tg mice after stimulation with LPS, CpG1826, or CpG2216 individually (Figure 2(a) and (c)). However, in the presence of LPS and CpG together, the cultured splenocytes from the 9D7 Tg mice secreted significantly more IL-10 (1645 pg/mL, Figure 2(b)) and anti-dsDNA (Figure 2(d)) than those of the NTg mice (1366 pg/mL). TLR9 was shown to have opposing inflammatory or regulatory roles in murin lupus model, but CpG induced

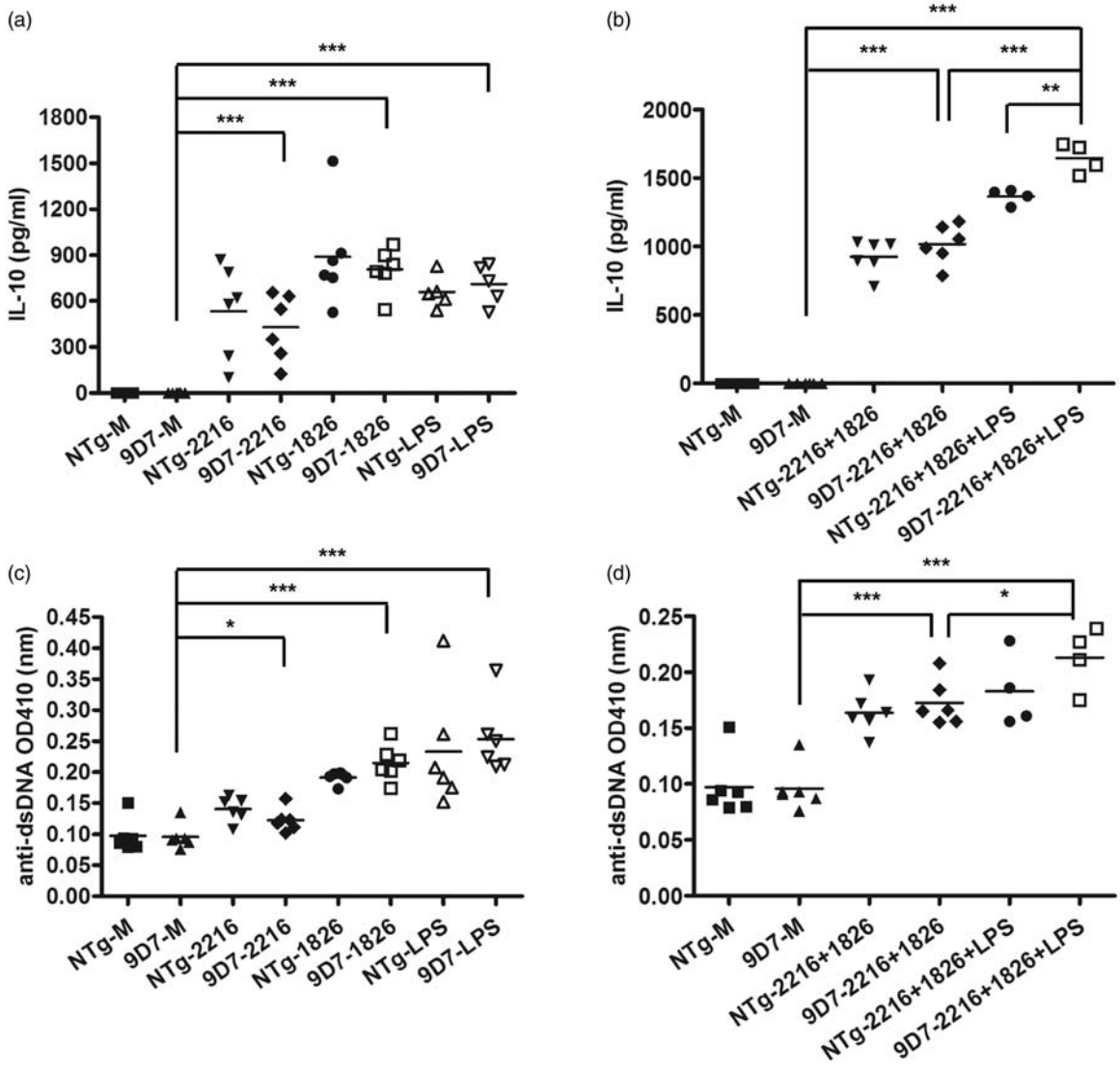

Figure 2 IL-10 and anti-dsDNA antibodies in splenocytes of 9D7 scFv Tg mice. Splenocytes of the 25-week-old NTg and 9D7 scFv Tg (9D7) mice ($n = 4$–6) were cultured with media (M), 0.5 μM CpG1826, 2.5 μM CpG2216, or 5 μg/mL LPS (a and c) or different combinations of stimulants (b and d) for 3 days. The concentrations of IL-10 (a and b) and anti-dsDNA antibodies (c and d) in the culture supernatants were determined by ELISA. Mean values were indicated by a bar. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$

comparable IL-10 secretion as LPS did in our model. Therefore, we compared the CpG alone with combined CpG and LPS for subsequent experiments to examine whether disease acceleration occurs upon simultaneous activation. These above data suggested some anergic B cells can be *in vitro* stimulated by combined treatment of extracellular and intracellular TLR4 and TLR9 agonists. Our data also imply that overproduction of IL-10 and anti-dsDNA in the 9D7 Tg mice by TLR4 and TLR9 agonists co-activation may be prone to develop systemic autoimmune disease.

Induction of lupus autoantibodies in 9D7 Tg mice after injections of LPS combined with CpG1826 and CpG2216 in vivo

To better understand whether extracellular TLR4 and intracellular TLR9 are involved in the progression of lupus-like syndrome in the 9D7 Tg mice, we injected the ligands for *in vivo* activation. Low-dose and chronic LPS injection would activate B lymphocytes to become autoantibody producing cells and have no effect on survival of C57BL/6 mice. The 29-week-old mice ($n = 4$–5) were injected with PBS, CpG1826 (25 μg/mice), and CpG2216 (25 μg/mice) twice per week or a combination of CpGs and LPS (1 μg/body weight (g)) for 6 weeks, and then the *sera* were collected for the immunopathologic index and renal function assays. When the mice were injected with LPS and CpG, the serum ANA scores (Figure 3(a) and (b)) were higher in the 9D7 Tg mice (mean score = 3) than those in the NTg mice (mean score = 2). In addition, combined injection also induced higher anti-dsDNA titers in the 9D7 Tg mice (OD = 1.76) than those in the control mice (OD = 1.36) (Figure 3(c)). Moreover, 9D7 Tg mice had elevated anti-Sm/RNP levels in *sera* when given TLR4 and TLR9 agonists (Figure 3(d)). Taken together, in agreement with *in vitro* data, signaling crosstalk by TLR4 and TLR9 agonist co-activation can enhance the secretion of lupus autoantibodies *in vivo*.

Induction of SLE syndromes in 9D7 Tg mice after injections of LPS combined CpG1826 and CpG2216 in vivo

Having observed the effects of LPS plus CpG in autoantibody induction, we further evaluated the renal function of the 9D7 Tg mice (m1, f6, and m22) by analyzing their urine protein and blood urea nitrogen levels. After receiving combined treatment with TLR4 and TLR9 agonists, the 9D7 Tg mice appeared to have considerably higher proteinuria index (Figure 4(a)) and blood urea nitrogen (BUN, 42 mg/dL) than those of the NTg (BUN, 30 mg/dL, Figure 4(b)).

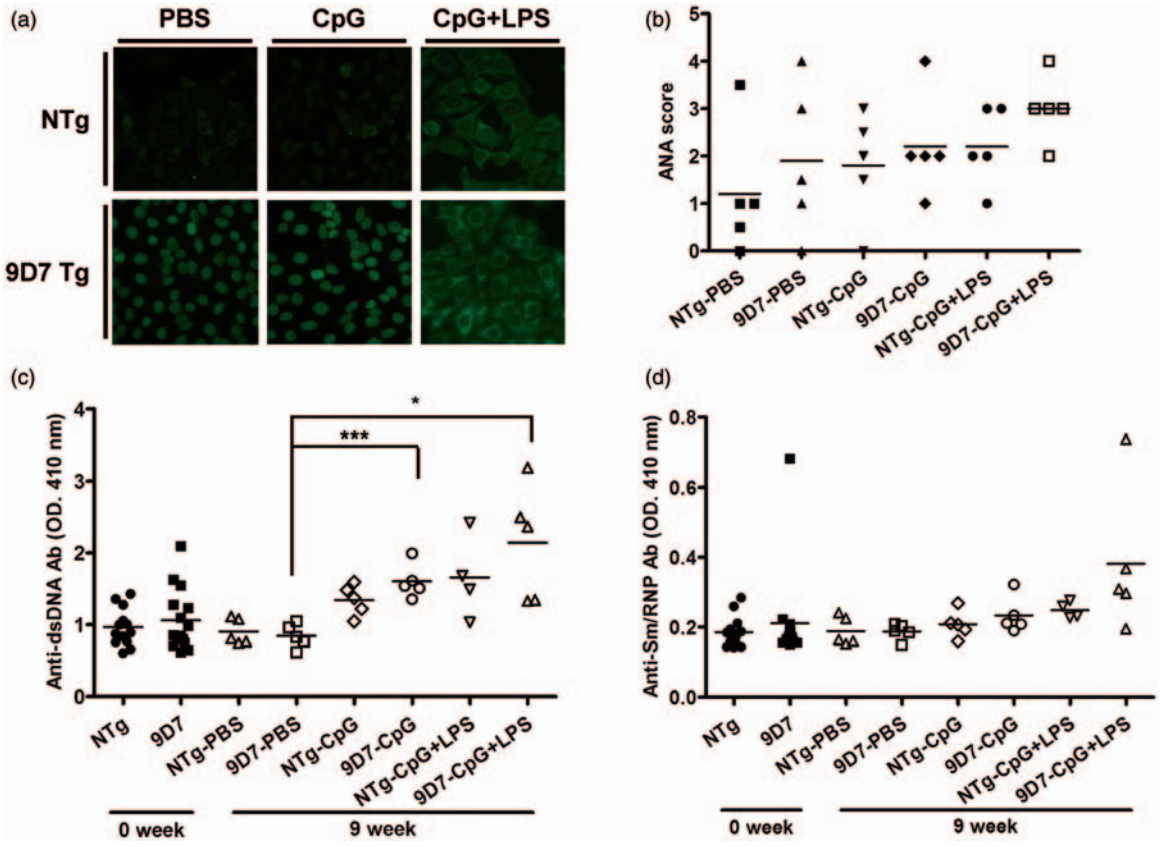

Figure 3 Higher production of antinuclear antibodies (ANA), anti-dsDNA antibodies, and anti-Sm/RNP antibodies in combined injection of 9D7 scFv Tg mice with LPS and CpG. The 29-week-old NTg and 9D7 scFv Tg (9D7) mice ($n = 4$–5) were injected with PBS twice per week, CpG1826 (25 μg/mice) and CpG2216 (25 μg/mice), or combined CpG and LPS (1 μg/body weight (g)) for 6 weeks, then the *sera* were collected upon sacrifice. (a) ANA in *sera* was detected using HEp-2 cells. (b) Signal intensity was compared using a semiquantitative score from 0 to 5+. The concentrations of anti-dsDNA (c) and anti-Sm/RNP antibodies (d) in *sera* were determined by ELISA. Mean values were indicated by a bar. $^{*}P < 0.05$; $^{***}P < 0.001$. (A color version of this figure is available in the online journal.)

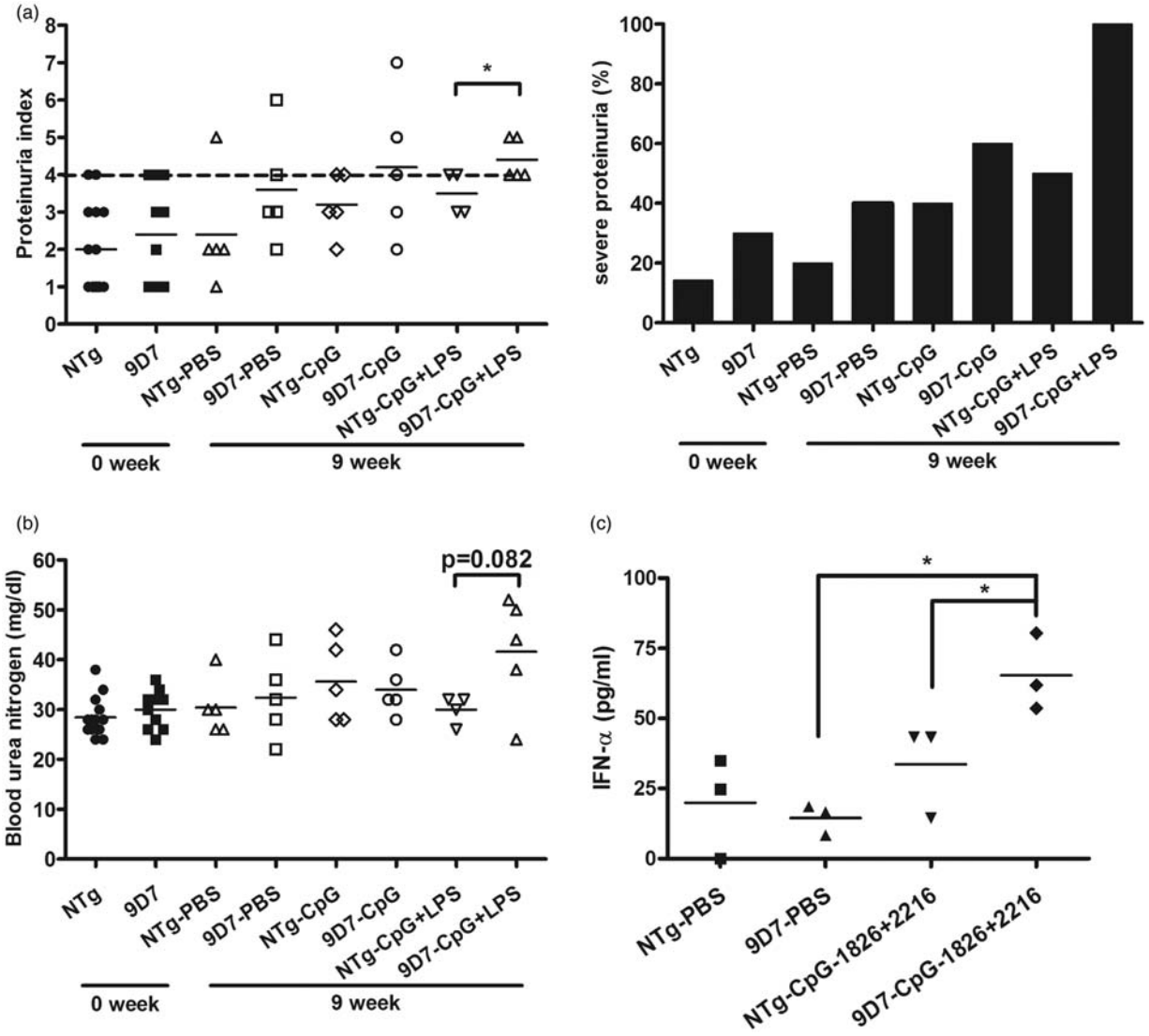

Figure 4 The clinical profiles of combined injection of 9D7 scFv Tg mice with LPS and CpG. The 29-week-old NTg and 9D7 scFv Tg (9D7) mice ($n = 4$–5) were injected with PBS twice per week, CpG1826 and CpG2216, or combined LPS and CpG for 6 weeks, then the blood and urine sample were collected. (a) Urine proteins were assayed by Medi-Test Combi 3 A. The level of proteinuria index was expressed as a scale from 0 to 7+. Mice with scores above 4+ were considered to have severe proteinuria (right panel). (b) The concentrations of blood urea nitrogen of the mice were determined using a HITACHI 7170 auto-analyzer. (c) The concentrations of IFN-α in the *sera* were determined by ELISA. Mean values were indicated by a bar. $^*P < 0.05$

All these mice (100 %) showed severe proteinuria (index above 4, over 100 mg/dL) indicating injection with combined TLR4 and TLR9 agonists accelerated the deterioration of renal function in 9D7 Tg mice (Figure 4(a), right panel). Since IFN-α is essential in the pathogenesis of SLE, we examined the IFN-α levels. The 9D7 Tg mice with TLR9 agonist injection had significantly higher IFN-α levels in *sera* (65 pg/mL) than the NTg mice (33 pg/mL, Figure 4(c)). Collectively, the anti-dsDNA transgenic mice seemed to develop more severe lupus-like syndrome after the treatments with TLR4 and TLR9 agonists.

Glomerular depositions of IgG and C3 ICs in 9D7 Tg mice after injections of LPS combined with CpG1826 and CpG2216

Lupus nephritis is the major autoimmune manifestation of SLE. Autoantibodies form circulating ICs by binding to the DNA or nucleosomes and depositions of these complexes in the tissues usually lead to inflammation. Thus, we compared the immunopathology in the 9D7 Tg and control mice. As shown in Figure 5, the results showed IgG and C3 IC depositions in the kidneys of all the 9D7 Tg mice after TLR4 and TLR9 agonist injection concordant with increased proteinuria and BUN (Figure 4(a) and (b)). The mean IgG and C3 IC deposition scores of the LPS and CpG-injected 9D7 Tg mice (mean = 2.63 and 2.49) were higher than those of the control mice with the same injection (mean = 1.5 and 1.93). Furthermore, the IC deposition scores in 9D7 Tg mice injected with CpG alone were also higher. The above data imply that combined administration with TLR4 and TLR9 agonists *in vivo* increases the autoantibody production and IC deposition in the kidney resulting to worse proteinuria index and blood urea nitrogen which accelerated the clinical SLE syndromes in the 9D7 Tg mice. The data support the idea that (Figures 3 to 5) combined

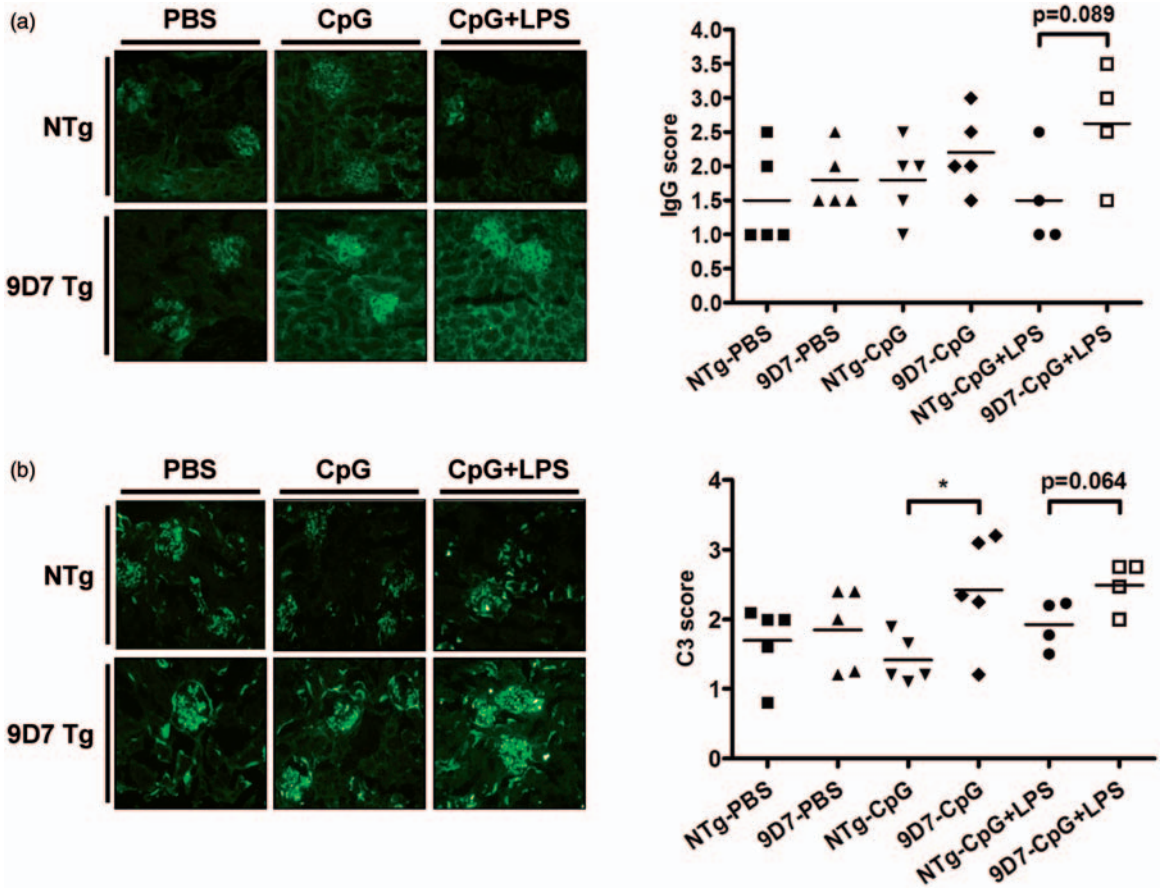

Figure 5 Glomerular immune complex (IC) depositions in 9D7 scFv Tg mice. The NTg and 9D7 scFv Tg (9D7) mice ($n = 4$–5) were injected twice per week with PBS, CpG1826 and CpG2216, or combined LPS and CpG for 6 weeks, and their kidneys were removed for staining. Kidney sections were stained with fluorescein-conjugated goat anti-mouse heavy and light chains of IgG (a) or fluorescein-conjugated goat anti-mouse C3 (b). The deposition of IgG and C3 ICs was photographed using fluorescence microscopy (magnification × 400). In the right panels of (a) and (b), the score of the sections was graded in a blinded manner by three individualities based on fluorescence intensity on a scale from 0 to 4+. Mean values were indicated by a bar. *$P < 0.05$. (A color version of this figure is available in the online journal.)

injection with TLR4 and TLR9 agonists accelerates autoantibody secretion and IC deposition in the 9D7 Tg mice which show more severe kidney disease compared with the non-transgenic littermates. These findings indicate that combined activation by extracellular TLR4 and endosomal TLR9 ligands accelerates lupus nephritis in an anti-dsDNA-transgenic 9D7 mouse model.

Discussion

Increasing body of evidence suggests the DNA antigens might not be so critical in the pathogenesis of SLE; instead, anti-DNA antibodies are more important in initiating the inflammatory processes through immunological cross-reactions with cell-surface and extracellular matrix components.[18,19] These antibodies may bind to DNA or nucleosomes to form circulating ICs and the deposition of these complexes in the tissue may elicit inflammation.[20,21] We have previously demonstrated that the 9D7 anti-dsDNA monoclonal antibody (9D7 mAb) stimulated the expression of IL-1β, IL-6, IL-8, IL-10, and TNF-α in mononuclear cells.[13] In addition, this pathogenic anti-dsDNA has been demonstrated to penetrate into living cells via electrostatic interactions and derange the cell functions *in vitro*.[14] Therefore, 9D7 mAb may have great potential in inducing pathological changes of lupus in mice. Although the 9D7 Tg mice expressed the transgene of anti-dsDNA autoantibody, only low levels of anti-dsDNA were detected in these mice (Figure 1(f)). It suggests anergic B cells may exist in 9D7 Tg mice. Additionally, accumulating evidences suggest environmental factors and pathogen infection may trigger autoimmunity.[2] This is in accordance with our data that indicated co-activation of extracellular and intracellular TLR by CpG and LPS, which mimics pathogen infection may accelerate systemic autoimmune disease in the anti-dsDNA Tg mice *in vivo*.

Previous studies have reported high levels of IL-10 can be detected in SLE patients and serum IL-10 may be a useful biomarker for SLE disease activity.[22,23] Anti-IL-10 treatment substantially delayed onset of autoimmunity in NZB/W F1 mice and SLE patients.[24,25] Previous studies demonstrated impaired production of IL-12 in SLE is negatively correlated with IL-10.[26] In our results, there were no significant differences in the expression of IL-12 under a combined injection with LPS, CpG1826, and CpG2216 between the 9D7 Tg and NTg mice (data not shown). This may be due to higher concentrations of IL-10 production (1645 pg/mL,

Figure 2(b)) when treated with both TLR4 and TLR9 agonists (LPS + CpG). On the other hand, Yin *et al.* proved MRL-*Fas*^{lpr}-IL-10$^{-/-}$ mice developed more severe lupus than their IL-10-intact littermate controls.[27] In addition, continuous overexpression of IL-10 significantly delayed antinuclear auto-antibody production and decreased clinical nephritis in a congenic model of lupus.[28] The opposing results of IL-10 may reflect it has two major effects in different periods of lupus development including a potent B-cell stimulator and anti-inflammatory effect.[29] In this study, we found that the significantly increased IL-10 may enhance activation, proliferation, and differentiation of B cells in co-activation with TLR4 and TLR9 agonists to accelerate lupus progression in the 9D7 Tg mice (Figure 2(b)).

Jacob *et al.* indicated therapeutic targeting of B-cell activating factor and/or B cells in SLE could be successful, even with high levels of IFN-α.[30] Consequently, previous works have highlighted type I IFN induction promotes systemic autoimmunity.[31] Previous studies have proved that type I interferon receptors control B-cell expression of nucleic acid-sensing Toll-like receptors, TLR9, in a murine model of lupus.[32] In addition, CpG DNA initiates a TLR9-mediated NF-κB-Rel-dependent innate pathway that cooperates with IL-10 through signal transducer and activator of transcription (STAT) proteins and IFN-responsive factors.[33] In the present study, we also found production of IL-10 in splenocytes (Figure 2(b)) and IFN-α in *sera* (Figure 4(c)) was significantly higher in CpG-treated transgenic mice than in non-transgenic mice. These results suggest IFN-α may be a potent activator of innate and adaptive immunity in SLE.

To the best of our knowledge, TLR7 and TLR9 located in endosomes are involved in the development of experimental and clinical SLE. TLR7 plays a pathogenic role in both genetic prone lupus mice[12] and pristane-treated mice,[34] but the exact role of TLR9 is even more complex.[35–38] Christensen *et al.* demonstrated, in the absence of TLR9, autoimmune disease was exacerbated, lymphocytes and plasmacytoid DCs were more activated, and serum IgG and IFN-α were increased.[12] However, activation of TLR-9 in glomerular cells by endogenous nucleic acids (nucleosomes) may amplify the inflammatory response.[37,39] In addition, blockade of TLR9 signaling in B cells impaired anti-dsDNA antibody production in mice.[40] These findings suggest that TLR9 has a dual regulatory role. In our study, activation with individual TLR4 or TLR9 agonists may accelerate systemic autoimmune disease through the overproduction of IL-10 and anti-dsDNA in the splenocytes of 9D7 Tg mice (Figure 2(a) and (c)). But, TLR9 activation alone induced lower levels of anti-Sm/RNP Abs and BUN than those of the combined TLR4 and TLR9 activation in 9D7 Tg mice (Figures 3(d) and 4(b)). De Nardo *et al.* verified complex nature of the TLR signaling crosstalk that regulates the host inflammatory reaction to bacterial infection *in vitro*.[41] In summary, we demonstrated, in anti-dsDNA transgenic mice, both TLR4 and TLR9 can cooperate with each other to induce lupus progression.

This study reveals just two risk factors, pathogenic anti-dsDNA and combined activation of extracellular and intracellular TLRs, induce SLE syndromes in normal mice. Hence, ongoing studies will determine whether block TLR4 and 9 could ameliorate lupus syndrome in anti-dsDNA lupus mice. Identification of molecular mechanisms contributing to lupus development by co-activation of extracellular TLR4 and endosomal TLR9 will open new avenues for modulating immune tolerance and suppressing disease progression.

Author contributions: TPL: conception and design, assembly of data, data analysis and interpretation, and manuscript writing; JCH: conception and design, and data analysis and interpretation; CJL: collection and/or assembly of data, and data analysis and interpretation; HJC: assembly of data, and data analysis and interpretation; YHC: collection and/or assembly of data, and data analysis and interpretation; YTT: data analysis and interpretation; WY and KHS: conception and design, assembly of data, data analysis and interpretation, manuscript writing, financial and administrative support, and final approval of manuscript.

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