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Immunotoxicological Effects of Mouse CpG Oligodeoxynucleotides in Lupus-prone NZB/NZW F1 Mice

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Abstract

Despite wide therapeutic use of CpG ODN against infection, allergy and cancer, the safety and toxicity of CpG ODNs were poorly delineated. Thus, we investigated whether optimal dosing of CpG ODN would affect immunotoxicological parameters in NZB/NZW F1 mice. Comparisons were made among control, non-CpG ODN and mouse CpG ODN (10 μg)-treated groups for 4 weeks. To gauge the immunotoxicity of CpG ODNs, we measured nonspecific parameters, degree of lupus nephritis, proteinuria, or autoantibody, and cytokine expression in mRNA level of lymphocytes. We found that there were no significant differences among groups in nonspecific immunotoxicological profiles and in evaluation profiles of glomerulonephritis. However, titer of anti-dsDNA and anti-cardiolipin antibodies in mouse CpG ODN group rose three or eight-fold higher than in control group. Collectively, CpG ODN might be clinically less immunotoxic in terms of clinical profiles in lupus-prone NZB/NZW F1mice, in spite of high autoantibody titer in CpG ODN treated groups.

Keywords: CpG ODN, Lupus, Immunotoxicity, NZB/NZW F1 mice

CpG oligodeoxynucleotides (CpG ODNs), mimicking bacterial DNA fragments are directly sensed by Toll-like receptor 9 (TLR9), coordinate innate and adaptive immune response and release Th1-type or proinflammatory cytokines such as interferons, and chemokines[1-6,7]. In that context, CpG ODN as vaccine adjuvant or immunotherapeutic widely confers protection or treatment against infection, allergy and cancer in preclinical and clinical fields[1,8,9].

Despite wide therapeutic usage of CpG ODN, the safety and toxicity of CpG ODNs were veiled. While CpG ODNs comprises nucleotides, these chemical structures might provoke autoimmunity or hypersensitivity via Th1 polarized immunity[1,8,10,11]. Accordingly, since unmethylated CpG ODN could function as vaccine adjuvant, CpG ODN theoretically might induce polyclonal B cell activation, autoantibody (Ab) against self DNAs or protein, thereby forming immune complexes. These immune complexes might be deposited in vital organs such as kidney, consequently leading to damage or malfunction.

However, these potential immunotoxicological profiles and mechanisms of CpG ODNs remain unsettled. To clarify this, we investigated whether optimal dosing of CpG ODN treatment would affect immunotoxicological parameters reflecting autoimmunity status in NZB/NZW F1 mice, spontaneous lupus model. Here autoimmune parameters encompass 1) nonspecific immunotoxicological profiles: body weight, organ weight, and routine hematology and/or serology, 2) specific autoimmune organ profile: lupus nephritis and proteinuria level, 3) autoantibody marker: titer of auto-dsDNA Ab and auto-cardiolipin Ab in lupus-prone mice, and 4) cytokine expression in mRNA level with lymphocyte activation status in spleen.

Body Weight and Spleen Organ Weight Ratio

In lupus mice treated with non-CpG ODN, mouse CpG ODN, or saline only for control group, body weight (Figure 1A) and spleen/BW ratio (Figure 1B) were not different among three groups. After CpG ODN injection for 4 weeks, the size of spleen was barely enlarged.

Hematological Parameters

To investigate hematological or serological impact of CpG ODN, we measured leukocytes, erythrocytes and platelet counts, and simultaneously BUN, Cr, BUN /Cr ratio, SGOT, and SGPT for kidney and liver function. In mouse CpG ODN 10 μg group, even though WBC counts and differentials were slightly decreased, there were no statistically significant (Table 1). Erythrocytes (Table 2) and platelet count (Table 3) of mouse CpG ODN10 μg group were in normal range. In renal function test, BUN and BUN/Cr ratio were within normal range, but Cr of mice in all three groups were below 0.3 mg/dL, indicating that renal function was not aggravated (Table 4). For liver function test, we selected serum glutamic oxaloacetic transaminase (SGOT) and serum glutamic pyruvic transaminase (SGPT). All NZB/NZW F1 mice had a high SGOT value in base line (Table 5).

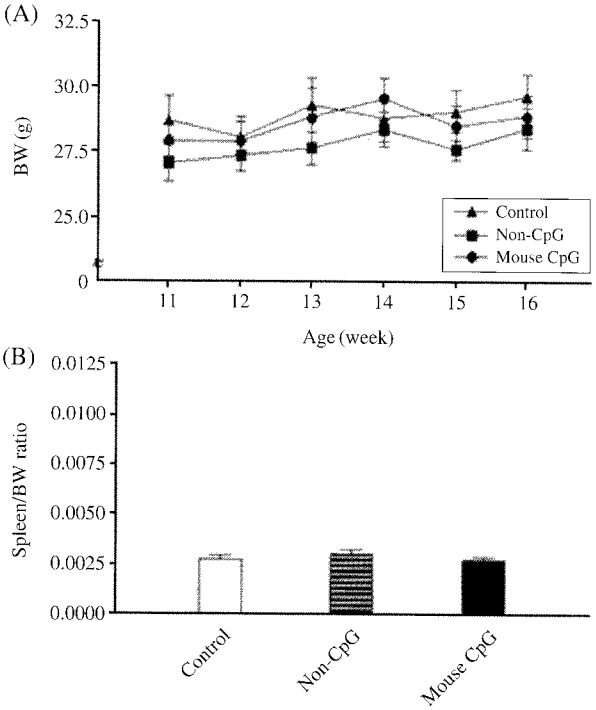

Figure 1. Body weight (A) and spleen/BW ratio (B) in CpG ODN treated-lupus prone NZB/NZWF1 mice. After sacrifice, each mouse was measured spleen organ weight. Data are shown as mean±SD and each group was an individual measurement (n=18).

Proteinuria

To determine the renotoxic effect in NZB/NZW F1 following CpG ODN injection, we measured weekly spot urine to test protein level. Ten mg/mL concentration of albumin solution was used to draw a standard curve in ELISA (Figure 2). Proteinuria is a sensitive marker to identify glomerulonephritis, and if protein level in urine rises more than 3.33 mg/mL, it refers to serious renal damage. All three groups revealed low protein level below 0.75 mg/mL.

Renal Histopathology

H&E and six different immunofluorescence (IF) stainings: IgG, IgM, IgA, C1q, C3, and fibrosis in mesangium showed that both control and mouse CpG ODN groups (Figure 3) revealed normal glomeruli

Table 1. Leukocyte counts in NZB/NZW F1 female mice treated CpG ODNs (n=18).

	Normal range	Control (n=6)	Non-CpG (n=6)	Mouse CpG (n=6)	X^2	*P*
		MEAN (SD)				
WBC (K/μL)	1.8-10.7	5.84 (0.741)	6.66 (2.011)	4.93 (0.889)	5.485	0.064
NE (K/μL)	0.1-2.4	1.38 (0.322)	1.59 (0.560)	1.00 (0.191)	5.626	0.060
LY (K/μL)	0.9-9.3	3.94 (0.425)	4.63 (1.411)	3.57 (0.750)	4.082	0.130
MO (K/μL)	0.0-0.4	0.32 (0.197)	0.31 (0.147)	0.22 (0.054)	1.139	0.566
EO (K/μL)	0.0-0.2	0.15 (0.119)	0.10 (0.034)	0.10 (0.052)	0.154	0.926
BA (K/μL)	0.0-0.2	0.05 (0.033)	0.03 (0.018)	0.04 (0.026)	0.908	0.635

Table 2. Erythrocyte counts in NZB/NZW F1 female mice treated CpG ODNs (n=18).

	Normal range	Control (n=6)	Non-CpG (n=6)	Mouse CpG (n=6)	X^2	*P*
		MEAN (SD)				
RBC (M/μL)	6.36-9.42	8.96 (1.464)	9.25 (0.590)	10.55 (2.3530)	1.509	0.470
Hb (g/dL)	11.0-15.1	12.12 (1.839)	12.28 (1.042)	11.95 (1.1000)	0.390	0.823
HCT (%)	35.1-45.4	48.22 (7.787)	50.47 (2.590)	56.13 (11.513)	1.847	0.397
MCV (fL)	45.4-60.3	53.82 (1.393)	54.62 (1.162)	53.38 (1.6190)	2.380	0.304
MCH (pg)	14.1-19.3	13.78 (3.099)	13.33 (1.603)	11.58 (1.4320)	5.522	0.630
MCHC (g/dL)	30.2-34.2	25.53 (5.161)	24.38 (2.420)	21.68 (2.6780)	3.528	0.171
RDW (%)	12.4-27.0	18.07 (0.753)	17.90 (0.632)	19.25 (1.3920)	4.882	0.087

Table 3. Platelet counts in NZB/NZW F1 female mice treated CpG ODNs (n=18).

	Normal range	Control (n=6)	Non-CpG (n=6)	Mouse CpG (n=6)	X^2	*P*
		MEAN (SD)				
PLT (K/μL)	592-2972	650.83 (166.150)	560.67 (236.873)	657.33 (189.908)	0.641	0.726
MPV (fL)	5.0-20.0	4.97 (0.28000)	5.10 (0.42400)	5.75 (2.08500)	0.063	0.969

Table 4. Serum renal function tests in NZB/NZW F1 female mice treated CpG ODNs (n=18).

	Normal range	Control (n=6)	Non-CpG (n=6)	Mouse CpG (n=6)	X^2	*P*
		MEAN (SD)				
BUN (mg/dL)	9.2-29.2	18.68 (3.1910)	20.26 (6.0370)	18.67 (4.3480)	0.456	0.796
Cr (mg/dL)	0.4-1.4	0.30 (0.0000)	0.30 (0.0000)	0.30 (0.0000)	0.000	1.000
BUN/Cr ratio	6.57-73	62.28 (10.636)	68.72 (20.125)	62.22 (14.492)	0.456	0.796

Table 5. Serum liver function tests in NZB/NZW F1 female mice treated CpG ODNs (n=18).

	Normal range	Control (n=6)	Non-CpG (n=6)	Mouse CpG (n=6)	X^2	*P*
		MEAN (SD)				
SGOT (mg/dL)	17-44	75.00 (13.624)	74.67 (18.662)	78.00 (23.108)	0.026	0.987
SGPT (mg/dL)	17-78	19.67 (2.3380)	19.00 (2.4490)	21.33 (1.9660)	3.769	0.152

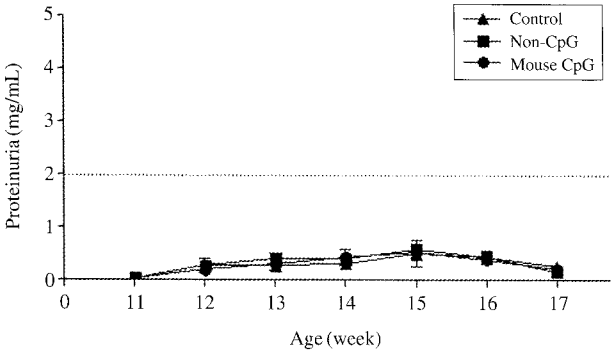

Figure 2. Proteinuria test in CpG ODN treated-lupus prone NZB/NZWF1 mice. At each time point, spot urine collected from each mouse was tested for the presence of protein in urine by Bradford assay. Data are shown as mean±SD and each group is an individual measurement (n=18).

Class I type, according to WHO classification lupus nephritis.

Antibody Test: Anti-dsDNA and Anti-cardiolipin Abs

We traced the presence of anti-dsDNA and anti-cardiolipin Abs for autoimmunity. Figure 5A showed the time course of anti-dsDNA Abs production in NZB/NZW F1 mice received with mouse CpG ODN 10 μg for 4 weeks. Anti-dsDNA Ab level was augmented in mouse CpG ODN group only. In mouse CpG ODN group, anti-dsDNA Ab was detected in sera from the final sacrifice, and rose three-fold higher than control group (Figure 4B). In parallel, anti-cardiolipin titers was measured in final blood sera taken after 5 weeks of each CpG ODN injection. Anti-cardiolipin Ab titer in mouse CpG ODN group rose eight-fold high compared to control group (Figure 4C).

IgG1 and IgG2a Abs

To verify switching of IgG1 Ab subclass such as IgG1 and IgG2a, we assessed pre- and post-CpG ODN treatment sera of 5 week-aged NZB/NZW F1 mice by using ELISA. In mouse CpG ODN group, pre-post difference of IgG1 was not marked when compared to other group (Figure 5A), whereas pre-post difference of IgG2a titer appeared the biggest among all group (Figure 5B). This switching IgG2a might be owing to CpG ODN-triggered Th1 immune response.

Immunocytes Distribution of Spleen

To determine the subpopulation of splenic immunocytes such as B/T lymphocytes, NK cell, monocytes, DC, flow cytometric analyses were used. Figure 6A and 6B revealed that the proportion of B cells in mouse CpG ODN-treated group was lower than that in control group. By contrast, the proportion of B cells in mouse CpG ODN-treated group was higher than that in control group. As for the proportion of NK cells, monocytes, or granulocytes marker, CD11c+, there

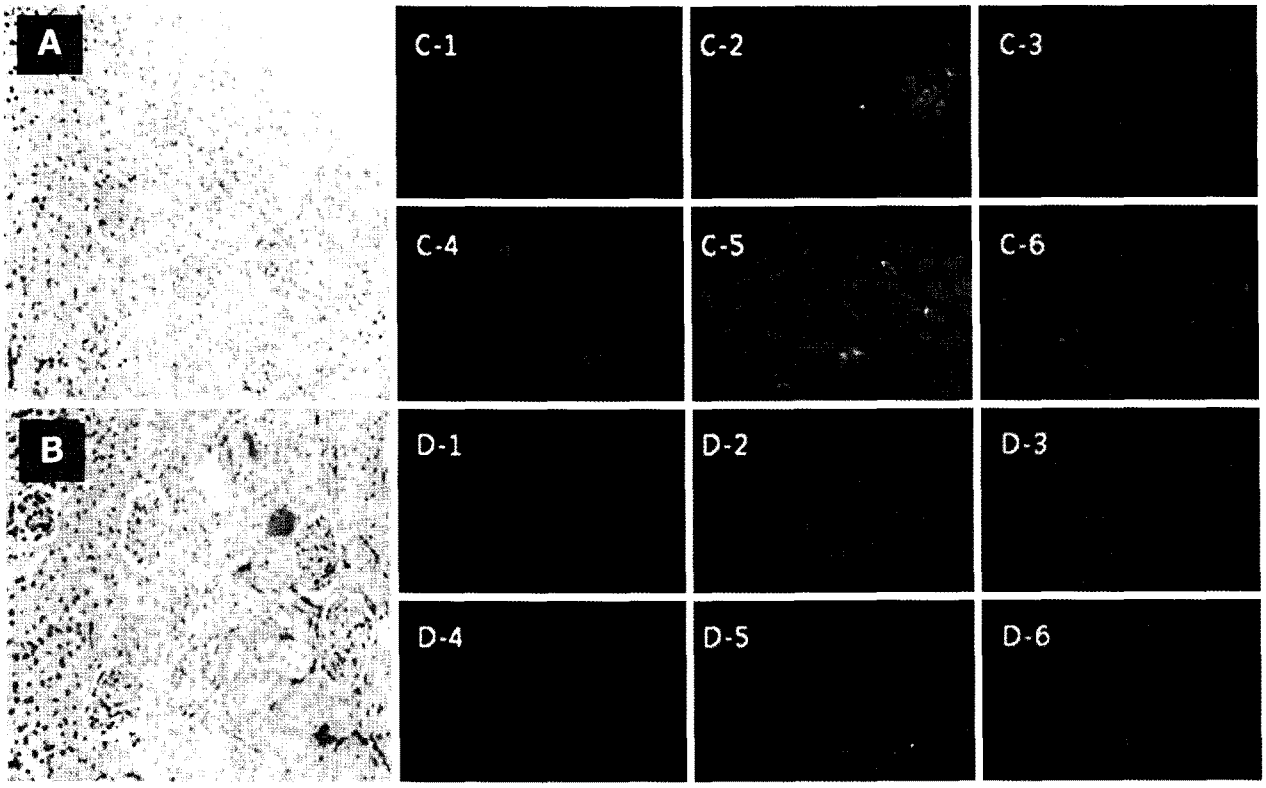

Figure 3. Renal histopathology of control and mouse CpG ODN groups. Renal sections of 16-week-old NZB/NZWF1 mouse were stained. Cortical sections from representative mice of control and mouse CpG ODN 10ug group were stained with H&E (A, B) and IF: IgG (C-1, D-1), IgM (C-2, D-2), IgA (C-3, D-3), C1q (C-4, D-4), C3 (C-5, D-5), and fibrosis (C-6, D-6). Both control and Mouse CpG ODN treated mice revealed normal glomeruli without immune deposit IgG, IgM, IgA, C1q, C3 and fibrosis in mesangium. Original magnification ×400.

were little differences among all test groups (Figure 6B). Meanwhile, the proportion of I-A/I-E (MHC class II marker)+cells in mouse or non-CpG ODN group was lower than that in control group (Figure 6C). The proportion of costimulator B7-1 (makers for antigen presenting cell, dendritic cell, activated B cell and macrophage)+cells was lower in mouse CpG ODN group than that in control group (Figure 6D). Taken together, CpG ODN treatment renders B cell population in spleen of lupus prone mice to decline, albeit T cell population to expand. These results were contradictory to the known effect (B cell proliferation and recruitment) of mouse CpG ODN.

Cytokine Expression in mRNA Level from Splenocytes

Previous study showed that as the course of SLE disease progressed, the mRNA expression of IL-10, IFN-γ, TNF-α, IL-6 or IL-1 rose in splenocytes or serum albeit decline of IL-12 expression[12,13]. Our RT-PCR showed that in IFN-γ and IL-10 cytokine expression in mouse CpG ODN group were slightly lower than control mice group, whereas TNF-α cytokine expression was slightly higher (data not shown).

Discussion

While CpG ODN as promising immunotherapeutic has been widely used or adopted in preclinical or clinical settings[1,8,9], still the safety and toxicity of CpG ODN have been poorly understood. Moreover, there are scanty documented about the immunotoxicities of CpG ODN. Of potential immunotoxicities, basic immunotoxicological profiles as well as autoimmunity has been suggested as a primary priority owing to rarity of the evidenced document. In this point, potential immunohazard induced by CpG ODN should be resolved toward wider or safer usage of CpG ODN in biomedicine. Considering these, we designed *in vitro* and *in vivo* experiment using autoimmune lupus prone mice in optimal dosing (CpG ODN 10 μg) for 4 weeks. In terms of therapeutic dose, the basis of optimal dosing and their schedule lies on our previous and cumu-

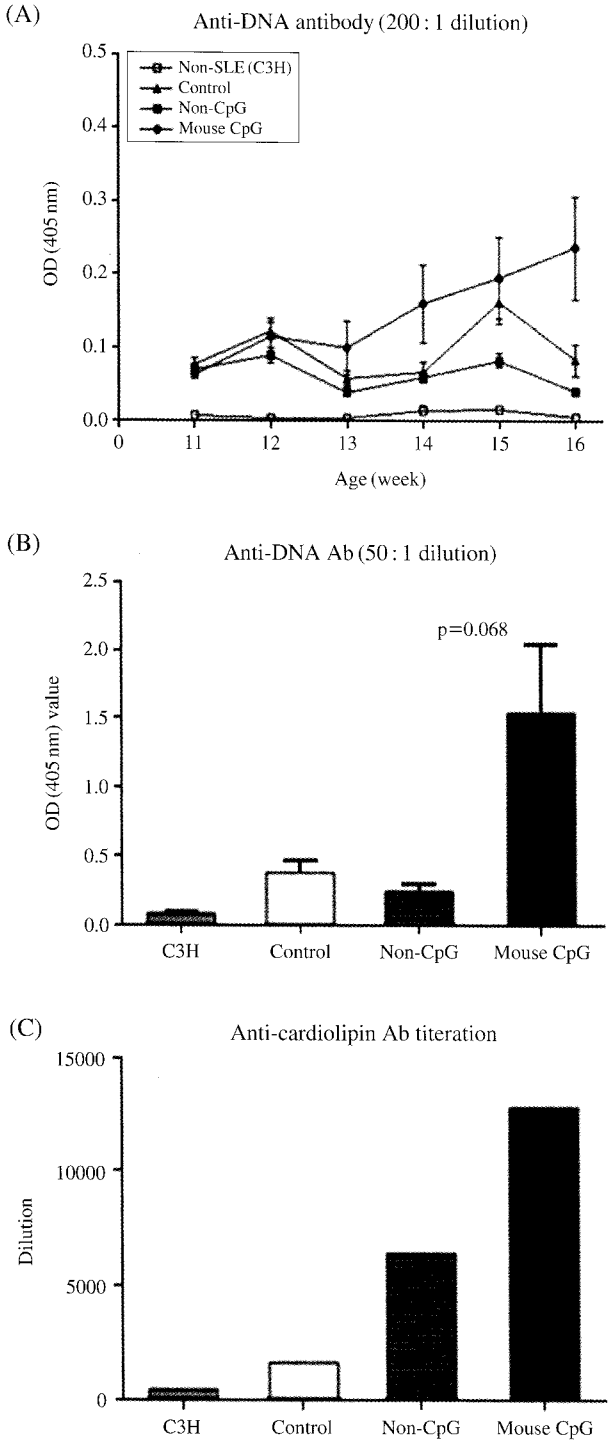

Figure 4. Anti-dsDNA (A, B) and anti-cardiolipin titers (C) auto-Abs production in CpG ODN treated lupus prone NZB/NZWF1 mice. We used autoantibody-free C3H mice (negative control) for same periods. At each time point, sera collected from the blood of each mouse were tested for the presence of IgG Ab agaisnt dsDNA by ELISA (A). Anti-cardiolipin titers was measured in final blood sera taken after 5 weeks of each CpGODN (C). Data are shown as mean ± SD and each group is an individual measurement (n=21).

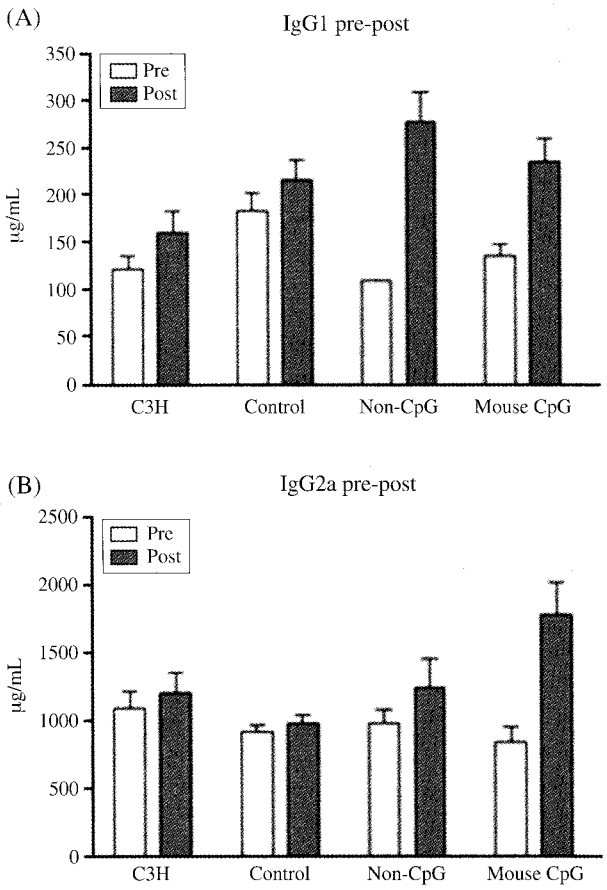

Figure 5. IgG1 (A) and IgG2a (B) production in CpG ODN treated-lupus prone NZB/NZWF1 mice. We used auto-Ab free C3H mice as negative control for same periods. Before treated and after 4 weeks later sera were measured for the presence of IgG1 and IgG2a by ELISA. Data are shown as mean ± SD and each group is an individual measurement (n=21).

lative data as well as mimicking or translating the schedule of current clinical CpG ODN-based immunotherapy[14-17].

This study indicates that optimal dosing of CpG ODN might be less immunotoxic in lupus-prone NZB/NZW F1 female mice. There were no significant differences among control, non-CpG ODN and mouse CpG ODN groups: in nonspecific immunotoxicological profiles (body weight, organ/BW ratio, and WBC, RBC, SGOT, and SGPT) and in evaluation profiles of glomerulonephritis (BUN, Cr, BUN/Cr ratio, renal histopathology and proteinuria). These phenomena are partly consistent with some reports that CpG ODN revealed non-toxicity in *in vivo* experiments[18-20]. However, titer of anti-dsDNA and anti-cardiolipin Abs in mouse CpG ODN group rose three or eight-fold higher than in control group. This auto-Abs induction might

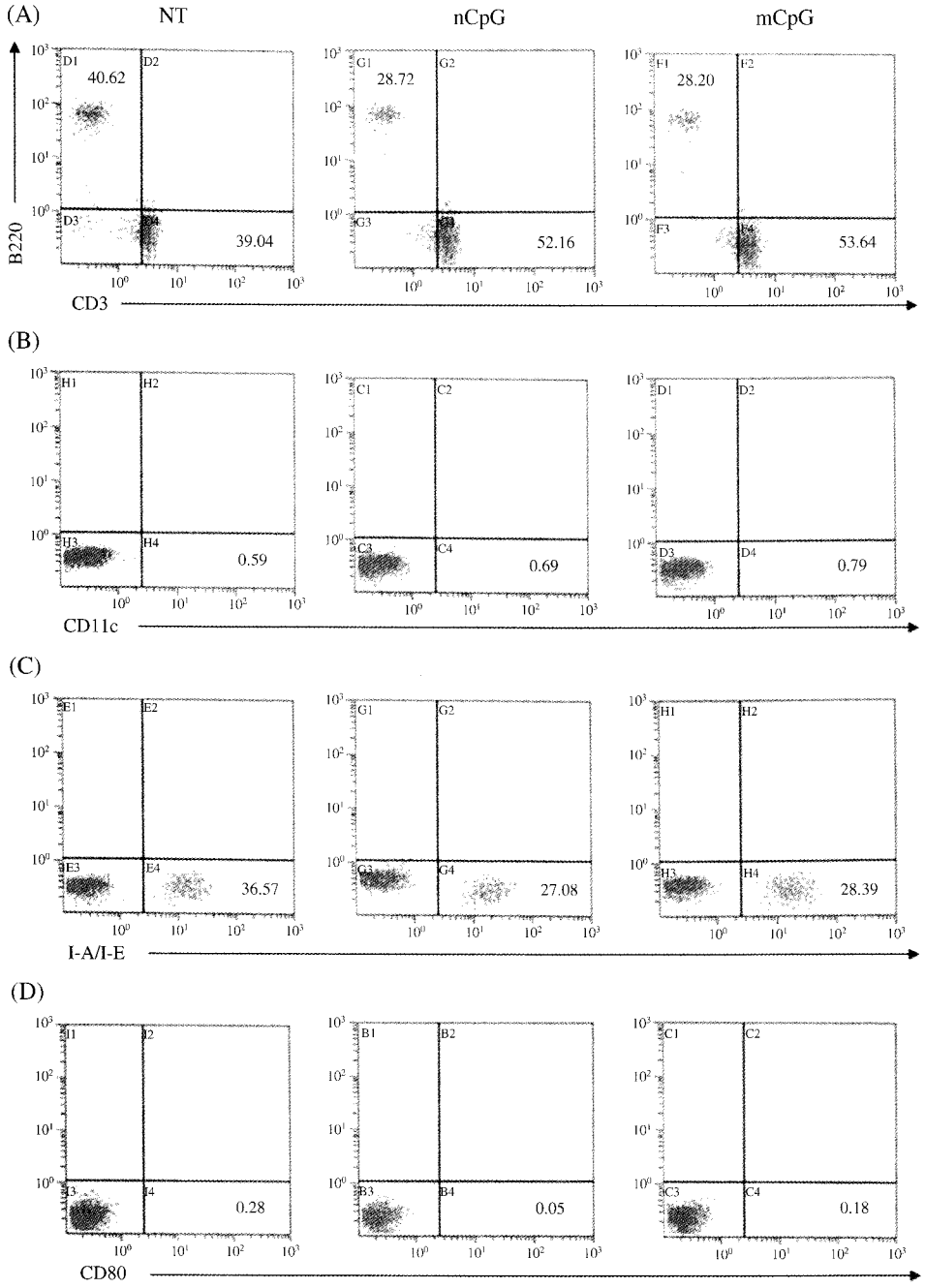

Figure 6. Flow cytometric analyses for B and T cell (A), NK cell (B), MHC class II (C), and B7-1 molecule (D) on splenocytes in CpG ODN treated-SLE prone NZB/-NZWF1 mice. After sacrificed each mouse, we freshly isolated splenocytes and stained with PE-anti-CD45R/B220 and FITC-anti-CD3, FITC-anti-CD11c, FITC-anti-I-A/I-E, or FITC-anti-CD80. Each data was shown as representative sample of individual measurements in each group.

be due to potent adjuvanticity of test CpG ODN, thus validating our optimal dosed model.

Next, CpG ODN might induce B cell depletion or functional modulation of B and T lymphocytes in our lupus mice model. In SLE disease, the B cells were key player in developing autoimmunity and disease progress because large numbers of B cells spontaneously produce pathogenic and high-affinity auto-Ab[21]. Several lines of evidences showed that in autoimmune patients and NZB or NZB/NZW F1 mice, increased numbers of B-1 cells are found in patients with autoimmune disease[22-26]. In our experiment, we speculate that reduced B cells might be due to apoptotic activity via TLR9 ligation. Additionally as we consider emerging data, it might be possible that CpG ODN could induce apoptosis of transformed or dysfunctional B cell *in vitro* as well as *in vivo*. If B cell is depleted, we consider that progress of SLE disease can be attenuated by CpG ODN.

Collectively, these results indicate that CpG ODN

might be clinically less immunotoxic autoimmune indexes on lupus-prone NZB/NZW F1 female mice in spite of high titer of autoantibodies in CpG ODN treated groups. Further experiments are warranted to identify autoimmunological profiles such as sublymphocytes and autoantibodies according to escalated dosing of CpG ODN for long period.

Materials & Methods

Animal Treatment

Twelve weeks of aged NZB/NZW F1 female mice purchased from JungAng Experimental Laboratories (Seoul, Korea) were divided into 3 treatment groups of six animals each. Control group received the Vehicle saline alone, Non-CpG ODN group received 1982 CpG ODN and mouse CpG ODN group received 1826 CpG ODN intraperitoneally administered 10 μg at 12, 13, 14, and 16 weeks of age. The animals were acclimated in a controlled-environment animal room (temperature, 21±3°C; humidity 30-70%; photoperiod, 12-h light/dark cycle) and were fed on commercial pellets and tap water.

Drug

The CpG ODNs were completely phosphorothioate-modified. These were provided by MWG-Biotech AG (Ebersberg, Germany). The sequences were 1982 CpG ODN (Non-CpG motif): 5′ TCCAGGACTTCTCTCAGGTT 3′, 1826 CpG ODN (mouse motif): 5′ TCCATGACGTTCCTGACGTT 3′. All CpG ODNs contained <0.1 EU/mL of endotoxin as determined by the Limulus assay (Bio-Whittaker, USA).

Hematology and Serum Chemistry

Blood samples were taken from the orbital of mice into Mricrotainer Brand tubes (BD, USA) for whole blood and empty tube for serum examinations, The blood counts were measured by HEMAVET 950FS (Drew scientific Inc., USA). Sera were separated and measured by DRI-CHEM 3500i (FujiFilm, Japan).

Auto-dsDNA and Cardiolipin Abs Levels in Serum

The brief measurement procedures are as follows: For anti-dsDNA Ab or For anti-cardiolin (CL), ELISA 96well microtiter plates were coated overnight at 4°C with calf thymus dsDNA (Sigma, USA) or Bovine Heart cardiolipin sodium salt (Sigma aldrich, USA) antigen at a concentration of 10 μg/mL; 200 μL blocking buffer (5% bovine serum albumin-and 0.05% Tween 20-containing phosphate buffered saline) was placed in each well, and incubated for 1 h at 37°C; collected serum diluted 3,000 or 10,000 times with diluents (0.5% bovine serum albumin-and 0.05% Tween 20-containing phosphate buffered saline) for IgG was added at 100 μL/well, and incubated for 2 h at 37°C; alkaline phosphate-conjugated goat anti-mouse IgG (Southern Biotech, USA) diluted 3,000 and 10,000 times, respectively, with the diluents was added at 100 μL/well, and incubated for 2 h at 37°C; p-nitropheyl-phosphate liquid substrate solution (Sigma, USA) was added at 100 μL/well, and incubated for 15 min at room temperature. The optical density of the test solution was read at 405 nm, and the absorbance value was used.

IgG1 and IgG2a Levels in Serum

The amounts of IgG1 and IgG2a level were measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits (Koma biotech, Korea). Color reaction developed was measured as OD unit at 450 nm on Emax, ELISA reader (Molecular Devices, USA).

Lymphocyte Subsets in the Spleen

Splenocytes from each group NZB/NZW F1 mice were obtained aseptically and purified using 0.7 μm mesh (BD Falcon, USA). A direct immunofluorescence technique was used to measure FITC- or PE-conjugated CD45R/B220+ cells, CD3+ cells (BD Pharmigen, USA), or CD11c+ cells, CD80+ cells, and I-A/I-E+ cells (eBioscience, USA) in spleen of NZB/NZW F1 mice. Data on 50,000 events were acquired and processed using a Cytomics FC 500 (Beckman Coulter, Germany).

Proteinuria

We collected weekly fresh spot urine samples from all NZB/NZW F1 mice of each treated group as protocols. To quantify protein concentration in fresh urine we used Bradford assay in every week. Color reaction developed was measured as OD unit at 595 nm on Emax, ELISA reader (Molecular Devices, USA).

Semiquantitative RT-PCR

After collecting splenocytes from all of NZB/NZW F1 mice, we isolated mRNA using TRIzol reagent (Invitrogen Life Technologies Inc., USA) and obtained cDNA as described in the manual. For PCR, 25 μL of PCR mixture was composed in 2 μL of cDNA supplemented with 10 mM Tris-Hcl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 5 μM dNTP, 1U Taq DNA polymerase (Takara, Japan) and 20 pmol of forward and reverse primers (COSMO, Korea). The sequences and annealing temperatures of each primer sets were described as follows: β-actin 348 bp (sense: TGG AAT CCT GTG

GCA TCC ATG AAA C, antisense: TAA AAC GCA GCT CAG TAA CAG TCC G), IL-10 251 bp (sense: AGA AAT CAA GGA GCA TTT GA, antisense: CTG CAG GTG TTT TAG CTT TT), IFN-γ 281 bp (sense: TTG GAT ATC TGG AGG AAC TG, antisense: CCT CAA ACT TGG CAA TAC TC), TNF-α 307 bp (sense: GAG TGA CAA GCC TGT AGC CCA TGT TGT AGC A, antisense: GCA ATG ATC CCA AAG TAG ACC TGC CCA GAC). After RT-PCR, PCR products were analyzed by electrophoresis in a 1.2% agarose gel contain with ethidium bromide. Amplified DNAs were quantified using the FlourS-MultiImager system (Biorad, Germany).

Histopathology

Renal tissue was fixed in 10% neutral buffered formaldehyde in saline, and 3 μm, paraffin-embeded sections were stained with hematoxylin and eosin (H&E) and period acid-Schiff stain (PAS). In brief, after preparing 2-3 μm thick frozen sections for detection of IgG, IgM, IgA, C1q, C3, and Fibrinogen deposits (n= 4) in glomeruli, each section was incubated with FITC-labelled rabbit anti-human/mouse IgG, IgM, IgA, C1q, C3, and Fibrinogen Abs (Dako, USA). The severity of the renal lesion was graded by WHO classification of lupus nephritis by physician of pathology department.

Statistically Analysis

All of the data were expressed as Mean±SD and analyzed using PRIZM4 software (GraphPad Software, SanDiego, USA). We used the Kruskal-Wallis for differences over three groups, Mann-Whitney U test for inter-group differences, and Spearman correlation to identify correspondence of lupus disease in all mice parameters.

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References

1. Krieg, A. M. CpG motifs in bacterial DNA and their immune effects. *Annu Rev Immunol* **20**:709-760 (2002).
2. Takeda, K. & Akira, S. Toll-like receptors in innate immunity. *Int Immunol* **17**:1-14 (2005).
3. Iwasaki, A. & Medzhitov, R. Toll-like receptor control of the adaptive immune responses. *Nat Immunol* **5**:987-995 (2004).
4. Akira, S., Takeda, K. & Kaisho, T. Toll-like receptors: critical proteins linking innate and acquired immunity. *Nat Immunol* **2**:675-680 (2001).
5. Chu, R. S., Targoni, O. S., Krieg, A. M., Lehmann, P. V. & Harding, C. V. CpG oligodeoxynucleotides act as adjuvants that switch on T helper 1 (Th1) immunity. *J Exp Med* **186**:1623-1631 (1997).
6. van Duin, D., Medzhitov, R. & Shaw, A. C. Triggering TLR signaling in vaccination. *Trends Immunol* **27**:49-55 (2006).
7. Klinman, D. M., Yi, A. K., Beaucage, S. L., Conover, J. & Krieg, A. M. CpG motifs present in bacteria DNA rapidly induce lymphocytes to secrete interleukin 6, interleukin 12, and interferon gamma. *Proc Natl Acad Sci USA* **93**: 2879-2883 (1996).
8. Klinman, D. M. Immunotherapeutic uses of CpG oligodeoxynucleotides. *Nat Rev Immunol* **4**:249-258 (2004).
9. Bhattacharjee, R. N. & Akira, S. Modifying toll-like receptor 9 signaling for therapeutic use. *Mini Rev Med Chem* **6**:287-291 (2006).
10. Anders, H. J. *et al.* Activation of toll-like receptor-9 induces progression of renal disease in MRL-Fas(lpr) mice. *FASEB J* **18**:534-536 (2004).
11. Anders, H. J. A Toll for lupus. *Lupus* **14**:417-422 (2005).
12. Anders, H. J. *et al.* Bacterial CpG-DNA aggravates immune complex glomerulonephritis: role of TLR9-mediated expression of chemokines and chemokine receptors. *J Am Soc Nephrol* **14**:317-326 (2003).
13. Aringer, M. & Smolen, J. S. Cytokine expression in lupus kidneys. *Lupus* **14**:13-18 (2005).
14. Cho, H. C. *et al.* Cancer immunotherapeutic effects of novel CpG ODN in murine tumor model. *Int Immunopharmacol* **8**:1401-1407 (2008).
15. Link, B. K. *et al.* Oligodeoxynucleotide CpG 7909 delivered as intravenous infusion demonstrates immunologic modulation in patients with previously treated non-Hodgkin lymphoma. *J Immunother* **29**:558-568 (2006).
16. Friedberg, J. W. *et al.* Combination immunotherapy with a CpG oligonucleotide (1018 ISS) and rituximab in patients with non-Hodgkin lymphoma: increased interferon-alpha/beta-inducible gene expression, without significant toxicity. *Blood* **105**:489-495 (2005).
17. Cooper, C. L. *et al.* CPG 7909, an immunostimulatory TLR9 agonist oligodeoxynucleotide, as adjuvant to Engerix-B HBV vaccine in healthy adults: a double-blind phase I/II study. *J Clin Immunol* **24**:693-701 (2004).
18. Gilkeson, G. S. *et al.* Modulation of renal disease in autoimmune NZB/NZW mice by immunization with bacterial DNA. *J Exp Med* **183**:1389-1397 (1996).
19. Gilkeson, G. S. *et al.* Effects of bacterial DNA on cytokine production by (NZB/NZW)F1 mice. *J Immunol* **161**:3890-3895 (1998).
20. Hofmann, M. A. *et al.* Phase 1 evaluation of intralesionally injected TLR9-agonist PF-3512676 in patients with basal cell carcinoma or metastatic melanoma. *J Immunother* **31**:520-527 (2008).
21. Lipsky, P. E. Systemic lupus erythematosus: an autoimmune disease of B cell hyperactivity. *Nat Immunol* **2**:764-766 (2001).
22. Hayakawa, K., Hardy, R. R., Parks, D. R. & Herzenberg, L. A. The "Ly-1 B" cell subpopulation in normal immunodefective, and autoimmune mice. *J Exp Med* **157**:202-218 (1983).
23. Wofsy, D. & Chiang, N. Y. Proliferation of Ly-1 B cells in autoimmune NZB and (NZB × NZW)F1 mice. *Eur J*

Immunol **17**:809-814 (1987).
24. Sidman, C. L., Shultz, L. D., Hardy, R. R., Hayakawa, K. & Herzenberg, L. A. Production of immunoglobulin isotypes by Ly-1+ B cells in viable motheaten and normal mice. *Science* **232**:1423-1425 (1986).
25. Burastero, S. E., Casali, P., Wilder, R. L. & Notkins, A. L. Monoreactive high affinity and polyreactive low affinity rheumatoid factors are produced by CD5+ B cells from patients with rheumatoid arthritis. *J Exp Med* **168**:1979-1992 (1988).
26. Viau, M. & Zouali, M. B-lymphocytes, innate immunity, and autoimmunity. *Clin Immunol* **114**:17-26 (2005).