Phosphodiester CpG-ODN ameliorates atopic dermatitis by enhancing TGF-β signaling

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Running Title: Effect of PO CpG-ODN on OVA-induced atopic dermatitis

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MATERIALS AND METHODS

Mice

Balb/c mice were purchased from Central Lab Animal Inc. (Japan). All mouse experiments were approved by the Review Board of Experimental Animals Study Committee at the Catholic University of Korea and performed according to guidelines established by the Committee.

Reagents and antibodies

The following reagents were purchased from the companies indicated in parentheses: Id2, E2A and actin, (Santa Cruz Biotechnology, Santa Cruz, CA, USA); TNF-α, IL-6, IL-4, TGF-β and IgE-Kit ELISA (BD Biosciences, San Jose, CA, USA); CD40 (BD Biosciences, San Jose, CA, USA); LPS (E. coli 0127:B8) (InvivoGen, San Diego, CA, USA); recombinant mouse IL-4 (eBioscience, San Diego, CA, USA and Creagen, Korea); TRIzol (Invitrogen, Carlsbad, CA, USA); SYBR Green I (Qiagen, Venlo, Netherlands).

CpG refers to 460 oligonucleotides (ODN) containing a phosphorothioate backbone (Genotech, Seoul, Korea). GpC refers to ODNs in which CG was substituted with GC and were used as a negative control. 1826S and 1018S CpG-ODNs were phosphorothioate forms and 46O CpG-ODN was phosphodiester form. Sequences: 1018S: 5′-TGACTGTGAAAGGTCCGAGATGA-3′, 1826S: 5′-TCCATGACGTTCCTGACGTT-3′, 46O: 5′-CTCGCACGTTGCGACTTC-3′. CpG-ODNs were synthesized and purified using HPLC by Genotech, Inc. (Daejeon, Korea).
The evaluation of hematological toxicity

ICR mice were treated with 46O CpG at concentration of 67, 200 and 600 mg/kg on every day for 4 weeks. Two week after final treatment, bloods were collected from mice and sera were isolated to measure hematological factors.

Ag-specific AD induction

Ag-specific AD in mice was induced as described previously (16) with minor modification. Briefly, mice were administered 20 μg of CpGs in 100 μl PBS intravenously (i.v). Mice were subjected to different treatment schedules. Mice received CpGs four times (i.e., on days 0, 7, 14, and 21), two times (i.e., on days 0 and 21), or once (i.e., on day 0). The animals were primed intraperitoneally (i.p.) with 10 μg chicken OVA (grade VII; Sigma-Aldrich, St. Louis, MO) precipitated with 4 mg aluminum hydroxide (alum) in a 200:l volume three times at 1-wk intervals (days 8, 15, 22). After 1 week (on day 29), mice were epicutaneously sensitized with OVA secured to the skin with a transparent dressing (Tegaderm™; 3M, St. Paul, MN, USA). One week after the first sensitization, mouse skin was re-sensitized with an OVA patch for another week. Biopsy specimens were obtained for histological examination. Splenocytes were isolated and cultured with 10 μg/ml OVA. After 48h, IL-4 in the culture media was measured by ELISA. All animal procedures were performed in accordance with the Laboratory Animals Welfare Act, the Guide for the Care and Use of Laboratory Animals, and the Guidelines and Policies for Rodent experiments provided by the institutional animal care and use committee of the School of Medicine, The Catholic University of Korea.
**B cell isolation**

B cells were purified from mouse spleen using the MACS B cell isolation kit (Miltenyi Biotec, Teterow, Germany) and the EasySep mouse B cell enrichment kit (StemCell Technologies, Vancouver B.C., Canada). Purified B cells were rested at least two hours on ice and then $2 \times 10^5$ B cells were cultured in flat-bottom 96-well plates with the indicated stimuli for 72 h. The cells were cultured in RPMI supplemented with 10% FBS, 10 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 M β-Mercaptoethanol, 100 g/ml penicillin, and 100 g/ml streptomycin. B cell proliferation was determined by incorporation of $^{3}H$-thymidine (1 Ci/well) for the last 12-16 h of culture.

**ELISA of IgE production in mouse serum**

The levels of OVA-specific mouse IgE in sera or total IgE were determined by ELISA. Briefly, the 96-well plates were coated with OVA or unconjugated anti-mouse IgE (BD Bioscience, CA) in PBS for overnight. For blocking, the wells were incubated with 5% BSA in PBS at room temperature for 1 hour. Diluted mouse sera were added into the wells and incubated at room temperature for 2 hours. For detection of OVA-specific IgE, the wells were incubated with HRP-conjugated anti-mouse IgE (BD Bioscience, CA). After washing the wells, enzymatic reaction was performed by adding a TMB substrate (Biosciences, CA) to each well. The reaction was stopped by addition of 2N H2SO4.

**ELISA and intracellular IgE staining in B cells**

B cells ($1 \times 10^5$) were stimulated for 5 days with 10 µg/ml of LPS or 5 µg/ml of α-CD40 antibody together with 10 ng/ml IL-4. The cells were treated with 1
μM CpG-ODN *in vitro*. ELISA was performed on culture supernatants to quantitate cytokine secretion. For intracellular IgE staining, B cells were stimulated for 5 days and cultured with brefeldin A for at least 6h. Intracellular IgE was assessed by flow cytometric analysis (BD Biosciences).

**RT-PCR analysis**

Total RNA was extracted from B cells using TRIzol (Invitrogen) and cDNA was synthesized from 1 μg of total RNA using reverse transcription (Qiagen). Real-time PCR analysis was performed using an Rotergene™ 6000 real time analyzer (Corbett Life Science, Australia) to measure SYBRGreen (Qiagen). Relative amounts of mRNA were normalized to GAPDH mRNA levels within each sample. The primer sequences used were: TGF-β1: F-5’-CAACAATTCCCTGGCGTTACCTTG-3’, R-5’-GAAAGCCCTGTATTCCGTCTCTTT, Smad7: F-5’: GACTCCAGGACGCTGTTGGT-3’, R-5R′CCATGGTTGCTGCATGAAC-3’ GAPDH: F-5’-TGCCCCCATGTTTGTGATTG-3’, R-5’-TGCCCCCATGTTTGTG-3’.

To analyze the GLTε mRNA level, cDNA was amplified by PCR using Taq polymerase (TAKARA, Japan). Amplified products were separated on agarose gels stained with ethidium bromide. Primer sequences and PCR conditions were as follows: GLTε: 5’-GCACAGGGGGCAGAAGAT-3A, R-ÇGTGTTGATGGAGGAGGAT-3’ (95°C for 5 min; 35 cycles of 95°C for 1 min, 60°C for 1 min, 72°C for 1 min; followed by 72°C for 10 min); GAPDH:F-5’-GCTCTTTGACGCAGGAGCG-3’, R-5’-GCTCTTTGACGCAGGAGCG-3’ (95°C for 5 min; 25 cycles of 95°C for 1 min, 60°C for 1 min, 72°C for 1 min; followed by 72°C for 10 min).
Western blotting

0.5–1 x 10^6 purified B cells were stimulated with 10 μg/ml LPS or 5 μg/ml of anti-CD40 in presence of 50 ng/ml of IL-4. 1-M CpG-ODN was used. The stimulated B cells were lysed in lysis buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1 mM Na_3VO_4, 1 mM NaF, 1 mM PMSF, 1% NP-40, and protease inhibitor cocktail). For the immunoprecipitation, 0.5% NP-40 was used. The protein concentrations of samples were determined using a BCA protein assay kit (Pierce, Rockford, IL). SDS loading buffer (50 mM Tris-Cl, pH 6.8, 5% (vol/vol) β-mercaptoethanol, 2% (wt/vol) SDS, 10% (vol/vol) Glycerol, and 0.1% (wt/vol) bromophenol blue) was added to the equal amount of cell extracts and boiled for 5–10 min. Equal amount of cell extracts were fractionated by 8–12% SDS-PAGE, transferred to polyvinylidene fluoride membrane (Pall Corporation, NY) and immunoblotted with antibodies according to the manufacturer’s recommended instructions. Primary antibodies were detected with HRP-conjugated secondary antibodies (Becton Dickinson, USA) and were visualized with chemiluminescence reagents (ECL; Pierce).

Immunoprecipitation

The stimulated B cells were lysed in lysis buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1 mM Na_3VO_4, 1 mM NaF, 1 mM PMSF, 0.5% NP-40, and protease inhibitor cocktail). A polyclonal rabbit α-ID2 antibody (C20; Santa Cruz Biotechnology) was used for immunoprecipitation from lysates of LPS/IL-4 stimulated B cells. Normal rabbit IgG (Santa Cruz Biotechnology) was used as a negative control. Lysates were loaded overnight at 4°C with 10 μg of the αID2 antibody, and then it were immunoprecipitated by incubation with protein G beads.
(Amersham Biosciences) at 4°C overnight. Western blotting was conducted with 1:1000 dilutions of αE2A and α-ID2 polyclonal antibodies (E12 and C20: Santa Cruz Biotechnology) overnight at 4°C and visualized with the ECL system.

**Statistical analysis**

Data are presented as the mean ± SD, and statistical comparisons between groups were conducted using the unpaired 2-sided Student’s t-test. All experiments were performed at least three times. *P<0.05 (*) and **P<0.01 (**) were considered statistically significant.
### Supplemental Table 1. Hematological factors

| Subject                                    | Unit       | Methods                               |
|--------------------------------------------|------------|---------------------------------------|
| RBC (Red blood cell count)                 | $10^6/\mu l$ | Flowcytometry, Isovolumetry           |
| HGB (Hemoglobin conc.)                    | g/dL       | Modified CN met- Hb method            |
| HCT (Hematocrit)                           | %          | (RBCxMCV)/10                          |
| MCV (Mean corpuscular volume)              | fl         | Histogram                             |
| MCH (Mean corpuscular hemoglobin)          | pg         | (HGB/RBC) x 10                        |
| MCHC ((Mean corpuscular Hb conc.)          | g/dL       | (HGB/(RBC x MCV)) x 1000              |
| RDW (Red cell distribution width)          | %          | Histogram                             |
| HDW (Hb conc. Distribution width)          | g/dL       | Histogram                             |
| PLT (platelet)                             | $10^3/\mu l$ | Flowcytometry                        |
| MPV (Mean platelet volume)                 | fl         | Histogram                             |
| RET (Reticulocyte)                         | %          | Flowcytometry, Isovolumetry           |
| WBC (White blood cell count)               | $10^3/\mu l$ | Flowcytometry                        |
| NEU (Neutrophil)                           | %          | Flowcytometry, Peroxidase staining    |
| LYM (Lymphocyte)                           | %          | Flowcytometry, Peroxidase staining    |
| MONO (Monocyte)                            | %          | Flowcytometry, Peroxidase staining    |
| EOS (Eosinophil)                           | %          | Flowcytometry, Peroxidase staining    |
| BASO (Basophil)                            | %          | Flowcytometry, Peroxidase staining    |
| LUC (Large unstained cells)                | %          | Noise-Lymph Histogram                 |
Supplemental Table 2. Hematological values of male mice in the main groups

| Tests | Units  | Male |  |  |  |  |
|-------|--------|------|---|---|---|---|
|       |  | G1 (0) | G2 (67) | G3 (200) | G4 (600) |  |
| RBC   | $10^6/\mu l$ | 9.00 ± 0.48 | 8.98 ± 0.41 | 8.88 ± 0.48 | 8.70 ± 0.32 |  |
| HGB   | g/dL   | 43.7 ± 1.7 | 42.9 ± 1.8 | 42.8 ± 1.7 | 42.4 ± 1.7 |  |
| HCT   | %      | 13.6 ± 0.6 | 13.5 ± 0.6 | 13.5 ± 0.7 | 13.2 ± 0.5 |  |
| MCV   | fL     | 48.6 ± 1.5 | 47.8 ± 1.6 | 48.3 ± 1.2 | 48.7 ± 1.2 |  |
| MCH   | pg     | 15.1 ± 0.5 | 15.1 ± 0.7 | 15.2 ± 0.6 | 15.2 ± 0.5 |  |
| MCHC  | g/dL   | 31.1 ± 0.8 | 31.6 ± 0.5 | 31.5 ± 1.0 | 31.3 ± 0.7 |  |
| RDW   | %      | 12.9 ± 0.4 | 13.0 ± 0.7 | 12.9 ± 0.6 | 13.2 ± 0.3 |  |
| HDW   | g/dL   | 2.15 ± 0.12 | 2.21 ± 0.18 | 2.23 ± 0.10 | 2.28 ± 0.13 |  |
| PLT   | $10^3/\mu l$ | 1400.7 ± 123.8 | 1321.9 ± 99.7 | 1330.4 ± 93.6 | 1225.3 ± 97.9** |  |
| MPV   | fL     | 5.07 ± 0.27 | 5.07 ± 0.32 | 5.11 ± 0.25 | 5.10 ± 0.26 |  |
| RET   | %      | 3.43 ± 0.30 | 3.41 ± 0.34 | 3.57 ± 0.35 | 3.55 ± 0.43 |  |
| WBC   | $10^3/\mu l$ | 1.94 ± 0.74 | 2.07 ± 0.71 | 1.94 ± 0.87 | 1.40 ± 1.01 |  |
| NEU   | %      | 1.54 ± 4.0 | 17.6 ± 4.5 | 17.3 ± 5.4 | 24.1 ± 8.4* |  |
| LYM   | %      | 80.5 ± 4.1 | 78.6 ± 4.5 | 78.4 ± 4.9 | 69.1 ± 8.1** |  |
| MONO  | %      | 1.99 ± 1.25 | 1.28 ± 0.58 | 1.13 ± 0.64 | 1.59 ± 0.92 |  |
| EOS   | %      | 1.52 ± 0.75 | 1.24 ± 0.45 | 1.86 ± 0.93 | 1.76 ± 1.01 |  |
| BASO  | %      | 0.13 ± 0.14 | 0.33 ± 0.21 | 0.24 ± 0.25 | 1.03 ± 0.75* |  |
| LUC   | %      | 0.44 ± 0.34 | 1.00 ± 0.44 | 1.09 ± 0.59 | 2.37 ± 1.00** |  |
| **N** |       | 9a) | 9a) | 9a) | 10 |  |

*/** Represents a significant difference at $p<0.05/p<0.01$ level compared with the vehicle control.

a) Specimen of one animal was not measured because of blood coagulation.
### Supplemental Table 3. Hematological values of female mice in the main groups

| Tests | Units   | Groups (mg/kg/days) | Female |
|-------|---------|---------------------|--------|
|       |         | G1 (0)              | G2 (67) | G3 (2000) | G4 (600) |
| RBC   | $10^9/\mu l$ | 9.09 ± 0.33         | 9.09 ± 0.65 | 9.72 ± 0.37 | 9.16 ± 0.38 |
| HGB   | g/dL    | 45.5 ± 1.5          | 45.2 ± 2.0 | 47.1 ± 0.9 | 45.5 ± 1.8 |
| HCT   | %       | 14.6 ± 0.6          | 14.4 ± 0.7 | 15.0 ± 0.3 | 14.4 ± 0.6 |
| MCV   | fL      | 50.1 ± 1.3          | 49.9 ± 2.0 | 48.5 ± 1.7 | 49.7 ± 1.5 |
| MCH   | pg      | 16.1 ± 0.5          | 15.9 ± 0.5 | 15.4 ± 0.5 | 15.8 ± 0.5 |
| MCHC  | g/dL    | 32.1 ± 0.7          | 31.8 ± 0.5 | 31.8 ± 0.2 | 31.8 ± 0.4 |
| RDW   | %       | 13.4 ± 0.5          | 13.3 ± 0.6 | 13.0 ± 0.2 | 13.4 ± 0.4 |
| HDW   | g/dL    | 2.20 ± 0.08         | 2.06 ± 0.10 | 2.10 ± 0.12 | 2.19 ± 0.14 |
| PLT   | $10^3/\mu l$ | 1131.8 ± 73.9       | 1199.1 ± 65.7 | 1117.1 ± 232.9 | 1143.4 ± 157.8 |
| MPV   | fL      | 5.11 ± 0.21         | 5.06 ± 0.13 | 5.16 ± 0.33 | 5.08 ± 0.34 |
| RET   | %       | 3.63 ± 0.89         | 3.54 ± 0.72 | 3.20 ± 0.45 | 3.16 ± 0.94 |
| WBC   | $10^3/\mu l$ | 3.34 ± 1.61         | 2.56 ± 1.29 | 2.60 ± 0.18 | 2.07 ± 0.93 |
| NEU   | %       | 9.5 ± 3.0           | 8.3 ± 1.5 | 1.00 ± 3.0 | 13.6 ± 4.7 |
| LYM   | %       | 86.0 ± 3.3          | 86.9 ± 2.2 | 85.5 ± 4.1 | 79.0 ± 4.9** |
| MONO  | %       | 1.33 ± 0.77         | 1.40 ± 0.52 | 1.34 ± 0.83 | 1.54 ± 1.02 |
| EOS   | %       | 2.46 ± 1.96         | 2.06 ± 0.76 | 1.63 ± 0.70 | 1.84 ± 0.78 |
| BASO  | %       | 0.12 ± 0.10         | 0.22 ± 0.13 | 0.33 ± 0.27 | 0.76 ± 0.30** |
| LUC   | %       | 30.69 ± 0.34        | 1.18 ± 0.51 | 1.30 ± 0.35 | 3.21 ± 0.72** |

| N     | 9,0)    | 10 | 9,03) | 9,04) |

*/** Represents a significant difference at p<0.05/p<0.01 level compared with the vehicle control.
a) Specimen of one animal was not measured because of blood coagulation.
Supplementary Fig. S1.