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Immunoadjuvanticity of Novel CpG ODN (Oligodeoxynucleotide)

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Abstract

In the course of novel TLR (Toll like receptor) 9 ligand, we found novel CpG ODN (Oligodeoxynucleotide) was active in augmenting antibody in mice. However, immune mechanism of new CpG ODNs is unclear. To clarify this, we examined immunoadjuvanticity by employing *in vitro* and *in vivo* immune profiles. In brief, *in vitro* treatment of novel CpG ODN upregulated the expression of TNF-α, IL-6, and IL-12 mRNA in macrophages as well as that of IFN-γ mPNA in mouse splenocytes. In parallel, *in vivo* injection of novel CpG ODN directly activates macrophages and splenocytess, consequently upregulating MHC class II and CD86. Finally, we demonstrated anti-HBs antibody augmentation of novel CpG ODN. Collectively, this data indicates that novel CpG ODN is immunoadjuvant armed with Th1 typed immune machinery.

Keywords: CpG ODN (Oligodeoxynucleotide), Immunoadjuvanticity, TLR (Toll like receptor), Th1 (T helper 1)

TLR (Toll like receptor)9 is one of major immunosensors expressed on innate immune cells[1]. This sensor mainly might recognizes the naked DNA or splitted nuclrotide of prokaryotic or eukaryotic cells at presenting unmethylated or hypomethlated CpG nucleotide[2]. In the context, triggering TLR9 confers powerful tool in switching the progression of intractable human disease e.g., cancer, autoimmunity and infection[3,4]. Synthetic CpG ODN (oligodeoxynucleotide), TLR9 agonist is versatile immunoregulator. For instance, the immunnoadjuvant effect of CpG ODNs has been preclinically or clinically applied on tumor immunotherapy[5-7] as well as vaccination against pathogen[8] (bacteria, virus or parasite). Despite these promising benefit of CpG ODN, only a couple of CpG ODNs has been used in clinical trial[9]. This clinical restriction of CpG ODN toward human trial is due to several bottle neck; absence of convenient screening assay, difficulty to predict the structure of potent CpG ODN and difference between species[10]. To develop CpG ODN for clinical trial, we designed novel CpG ODN tailed consecutive dT in 3-end. To delineate the immune machinary of the novel CpG ODN, we examined the proliferation, the expression of cytokine mRNA and surface expression of costimulatory molecules using mouse splenocytes or peritoneal macrophages. Finally, *in vivo* model, we attempted to verify immunoadjuvant activity of novel CpG ODN through antibody titration in HBsAg-vaccinated mice.

KSK-13 Directly Induces the Proliferation of Mouse Splenocytes and Peritoneal Macrophages

The proliferation of splenocytes or peritoneal macrophages upon *in vitro* treatment of KSK-13 was tested using [^{3}H] thymidine uptake assay. In splenocytes, proliferating effect of KSK-13 was compared with other CpG ODNs. Mean splenic proliferation of KSK-13 was higher than that of KSK-12 and lower than that of KSK-2 (Figure 1).

KSK-13 Induces TNF-α, IL-6 and IL-12 mRNA Expression in Mouse Peritoneal Macrophages

To clarify that KSK13 induce the release of Th1 type immunocytokine, mouse peritoneal macrophages were stimulated with CpG ODNs including KSK -13 *in vivo* as well as *in vitro*, and cytokine mRNA profiles were examined by RT-PCR (Figure 2). To amplify the expression level of cytokine mRNA *in vivo*, PM or splenocytes from Balb/c mice intravenously injected with 100 μg CpG ODNs were shortly treated with 0.6 μg/mL same CpG ODNs *in vitro*. TNF-α mRNA expression at *in vitro* treatment of KSK-13 was enhanced when compared to that of KSK-1, non-CpG ODN and untreated control. The expression level of TNF-α mRNA was similar with

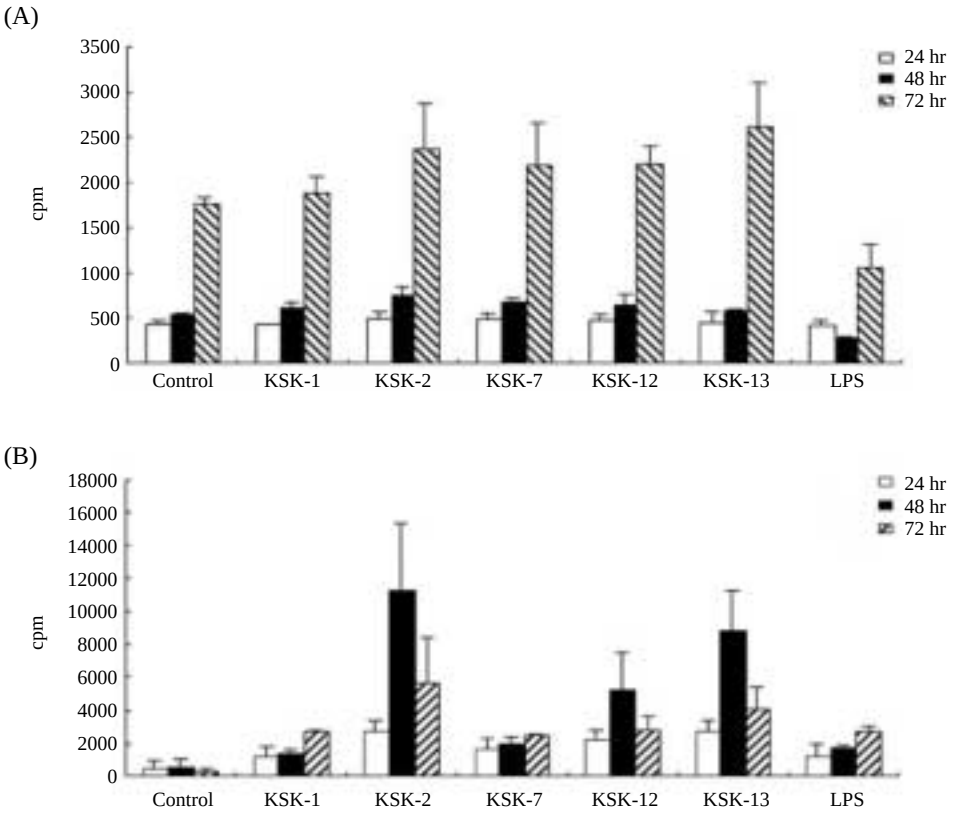

Figure 1. Comparative effect of varuous type of CpG ODNs on the proliferation of splenocytes and macrophages. 5×10^4 peritoneal macrophages or splenocytes from Balb/c were treated with or without 0.6 μg/mL of CpG ODNs for 24, 48 or 72 hours. Proliferation of the peritoneal macrophages (A) or splenocytes (B) was determined by the amount of thymidine uptake for 6 hours. All tests were duplicated. Error bars indicate standard deviation.

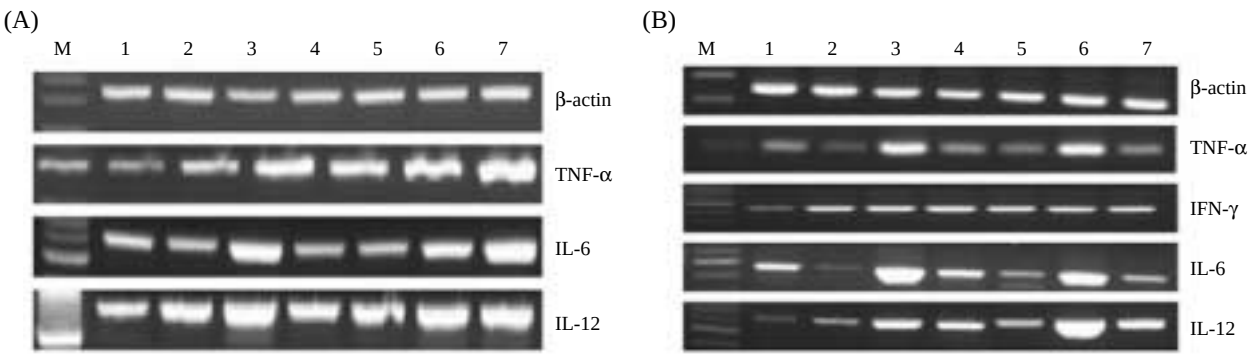

Figure 2. Expression profiles of Th1 cytokine mRNA in mouse peritoneal macrophages *in vitro* stimulated by various type of CpG ODNs. Peritoneal macrophages *in vitro* (A) or *in vivo* (B) were treated with or without 0.6 μg/mL CpG ODNs for 12 hours. Cells were harvested and total RNA was extracted by TRIzol® reagent. RT-PCR was performed with specific primers of TNF-α, IL-6 or IL-12, and electrophoresed. M: Size marker; lane 1: media only; lane2: KSK-1; lane 3: KSK-2; lane 4: KSK-7; lane 5: KSK-12; lane 6: KSK-13; lane 7: LPS. In case of figure 2A TNF-α, marker was not used.

that of KSK-2 and KSK-12. Expression of IL-6 mRNA was significantly upregulated when PM were treated with KSK-2 or KSK-13. Upregulation of IL-12 mRNA was obvious compared with untreated control but obscure compared with KSK-1. Treatment of KSK-13 prominently upregulated the expression of TNF-α and IL-6 mRNA. Interestingly, the highest expression of IL-12 mRNA is at KSK-13 treatment.

KSK-13 Increases TNF-α, IL-6 and IL-12 mRNA Expression on Mouse Splenocytes

The effect on the expression of Th1 cytokines mRNA was also examined on naïve or primed splenocytes. Treatment of KSK-13 upregulated the expre-

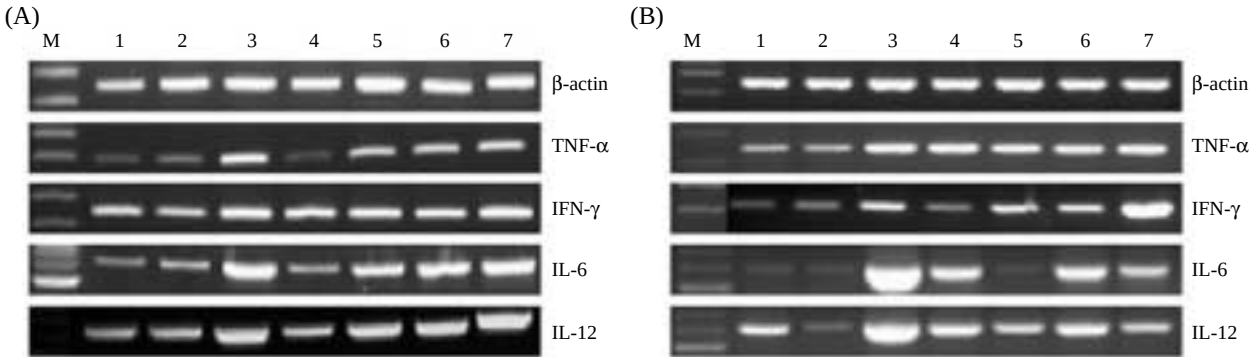

Figure 3. Expression profiles of Th1 cytokine mRNA in mouse splenocytes from mice *in vitro* (A) or *in vivo* (B) stimulated by various type of CpG ODNs. Balb/c mice were administered i.p. with 300 μL of 1% BSA-PBS with or without 100 μg of CpG ODNs. Naive or sensitized splenocytes were isolated 24 hours after injection and total RNA was extracted by TRIzol® reagent. RT-PCR was performed with specific primers of TNF-α, IL-6 or IL-12, and electrophoresed. M: Size marker; lane 1: media only; lane2: KSK-1; lane 3: KSK-2; lane 4: KSK-7; lane 5: KSK-12; lane 6: KSK-13; lane 7: LPS.

ssion of TNF-α, IL-6 and IL-12 mRNA (Figure 3A). However, the expression of IFN-γ mRNA was not significantly increased when compared with KSK-1 treatment. By contrast, *in vitro* treatment of KSK-13 upregulated the expression of IL-6 and IL-12 mRNA.

KSK-13 Induces the Expression of MHC Class II and B7.2 Molecules on Peritoneal Macrophages

The effect of KSK-13 on antigen presentation of mouse PMs was examined by expression of MHC class II and B7.2 molecules. Mice were i.p. injected with 100 μg of the indicated CpG ODNs or 25 μg of LPS. PMs were harvested 48 hrs after injection and 1×10^6 PMs were tested for expression of B7.2 and MHC class II molecules. Compared to untreated control, the expression of MHC class II molecules was shifted up when treated with KSK-13, which was medium value between the values of KSK-2 and KSK-12 (Figure 4A). KSK-7, which is KSK-12 tailed by six guanine on 3′-end, upregulated MHC class II molecules by 30.2%, which was two third lower values than KSK-12. KSK-13 also significantly upregulated the expression of B7.2 molecules by 14.2% (Figure 4B). Though KSK-7 rarely affected the expression of MHC class II molecules, co-stimulatory B7.2 molecules were significantly up regulated.

KSK-13 Induces the Expression of MHC Class II and B7.2 Molecules on Splenocytes

Treatment of KSK-13 on murine splenocytes shifted up the expression of MHC class II molecules by 22.8%, which was similar to 23.6%, at KSK-2 treatment (Figure 5A). The value was higher than that at treatment of untreated control, KSK-1 and KSK-12. The effect of KSK-13 on the expression of B7.2 molecules on splenocytes was slightly lower than that of KSK-2. We found that murine splenocytes increased the expression of B7.2 and MHC class II molecules in response to CpG ODN compared to weak response to non-CpG ODN. The upregulation rate was higher than the rate of the case of KSK-1 and KSK-12.

KSK-13 is a Potent Adjuvant of Immunization Against HBsAg in Balb/c Mice

To confirm the adjuvant effect of KSK-13, 2.5 μg of recombinant HBsAg were injected to Balb/c mice with CpG ODNs and/or alum. Alum, which is clinically widely used, was injected after emulsified with recombinant HBsAg. CpG ODNs were subcutaneoulsly injected with or without antigen. ELISA clearly verified that *in vivo* vaccination of KSK-2, KSK-12 and KSK-13 plus HBs antigen augmented the titer of anti-HBsAg Ig G upto 12,800 (Table 2). Interestingly, of groups the combined adjuvant (alum plus KSK-2; alum plus KSK-12; alum plus KSK-13) Alum plus KSK13 was optimal in synergistic four fold titre rise.

Discussion

This study indicates that novel CpG ODN is immunoadjuvant armed with Th1 typed immune machinery. This is evidenced by augmenting diverse innate or Th1 type immune response such as splenic proliferation, cytokine expression, and surface signaling or costimulatory molecule expression in fresh murine immune cells (splenocytes, peritoneal macrophages). In consistent with *in vitro* Th1 dominant activity, *in vivo* experiment using HBs antigen (Ag) vaccinated mice clearly demonstates that novel CpG ODN (KSK

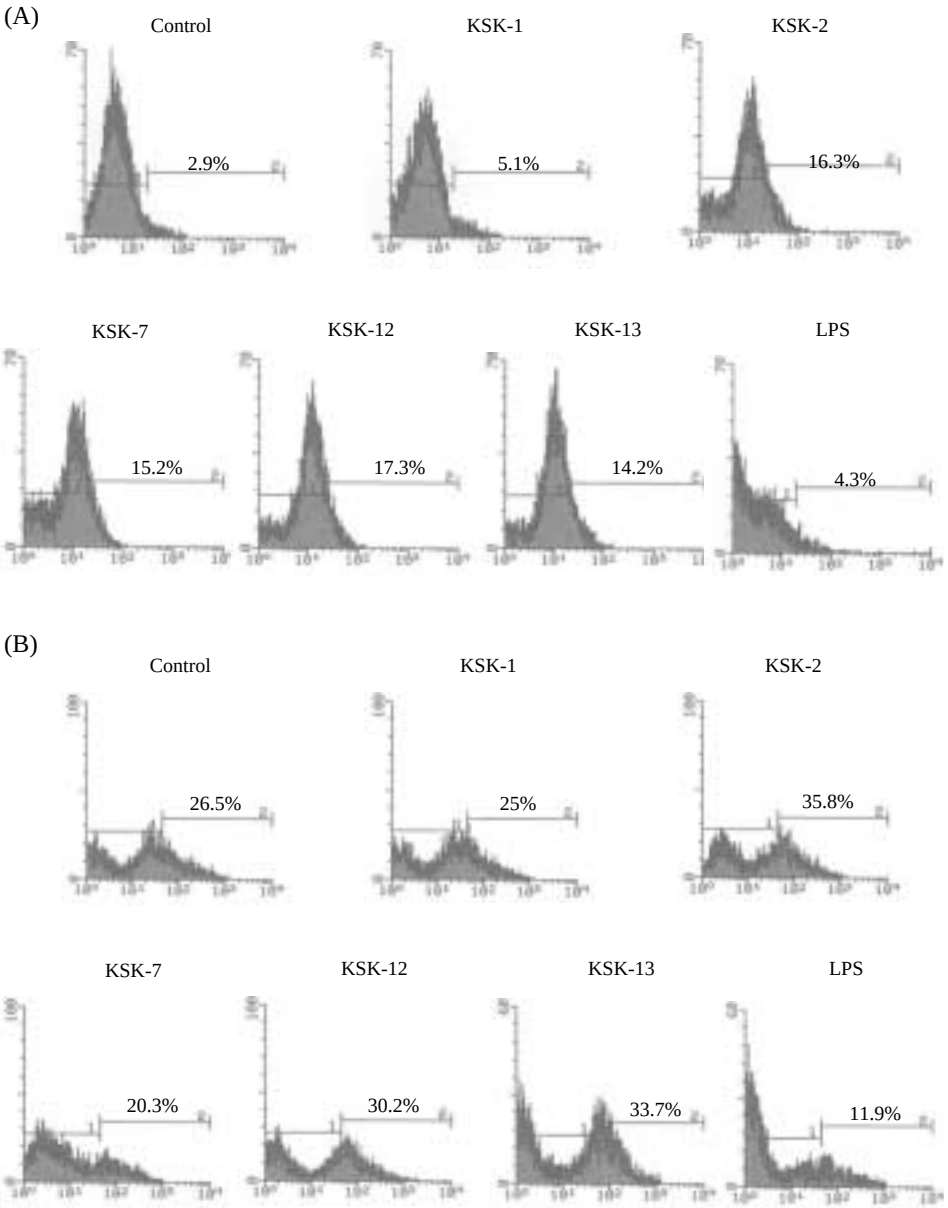

Figure 4. CpG ODNs upregulate the expression of signaling molecules in mouse peritoneal macrophages. Balb /c mice pretreated with 3% thioglycollate were given i.p. with 100 μg of CpG ODNs. Peritoneal macrophages were harvested 48 hours after injection. The cells were labeled with FITC-conjugated anti -B7.2 monoclonal antibodies (A) or MHC class II monoclonal antibodies (B).

-13) is able to potently augment anti-HBs antibody through coinjection of novel CpG ODN plus HBsAg. Surprisingly, immunoadjuvant acitivity of KSK13 is equivalent or superior to 2006 (CpG 7909), which has been on clinical trial[11]. The chemical nature of KSK13 is phosphorothioate (PS) modification of human motif[9], containing five CG dinucleotides. Such PS modification is basically required to *in vivo* immunoadjuvant effects *in vivo*[12]. Given *in vitro* experiments using peritoneal macrophages and splenocytes, major target cells of KSK-13 might be myeloid lineaged cells. However, lymphoid cells such as T cell, B cell or NK cell are not completely excluded to transduce KSK-13 signal. Emerging evidences show that TLR9 independent pathway[3] triggered by CpG ODN is possible, suggesting that TLR9 free or weakly expressing lymphoid cells would be agitated by CpG ODN assault. Despite potent activity of KSK13, the exact type of KSK13 is ambiguous. In the synthetic context of structure and activity, K or C type might be rational. In this study, we did not check cytokine quantity toward screening TLR 9 ligand. Our current data suggest that *in vitro* screening using RT-PCR coupled with *in vivo* screening would be one of rapid TLR9 ligand screening assays. Collectively, this data indicates that novel CpG ODN is immunoadjuvant

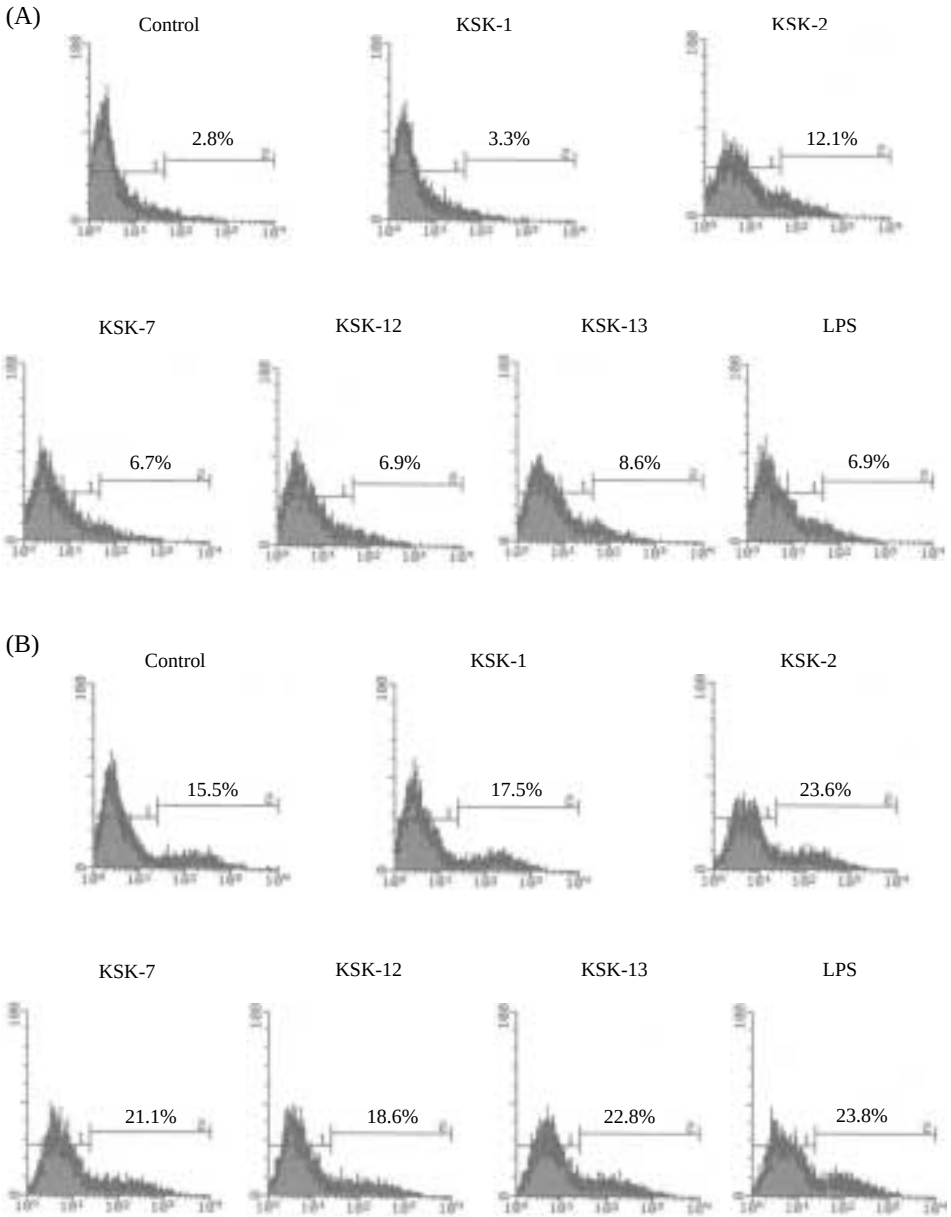

Figure 5. CpG ODNs enhance the expression of signaling molecules in mouse Splenocytes. Balb/c mice were given i.p. with 100 μg of CpG ODNs. Splenocytes were harvested 48 hours after injection. The cells were labeled with FITC-conjugated anti-B7.2 monoclonal antibodies (A) or MHC class II monoclonal antibodies (B).

armed with Th1 typed immune machinery, implying versatile application of CpG ODN in preclinical or clinical level.

Methods

Animals

Six-week-old Balb/c (H-2^d) mice were purchased from the Jackson Laboratories and housed at the Animal Care Facility of Wonju College of Medicine in accord with Federal and local regulations.

CpG ODNs

CpG ODNs were synthesized and purified by HPLC and NAP (Metabion GmbH, Germany). The sequences of CpG ODNs are described in Table 1.

Isolation of Splenocytes

Spleen from Balb/c mouse was dispersed and centrifuged at 2,000 rpm for 5 minutes. The supernatant was removed and cell pellet was resuspended in 0.84% NH_4Cl to eliminate red blood cells. Cells were washed twice with RPMI-1640 medium and resuspended in the culture medium.

Table 1. The sequence and code name of phosphorothioate CpG ODN.

ODNs	Sequence (5′ to 3′)	Code name
1982	TCCAGGACTTCTCTCAGGTT	KSK-1
1826	TCCATGACGTTCCTGACGTT	KSK-2
2006fG6run	TCGTCGTTTTCGTCGTCGTTT TGGGGGG	KSK-7
2006	TCGTCGTTTTGTCGTTTTGT CGTT	KSK-12
2006f	TCGTCG TTTTCGTCGTCGTTTT	KSK-13

Isolation of Peritoneal Macrophages (PM)

Balb/c mice were i. p. injected with 1.5 mL of 3% thioglycollate (Becton Dickinson, Cockeysville, MD). After three days, 1 mL of PBS supplemented with 2% FBS was i.p. injected and peritoneal lavage fluid was collected. After washing twice with HBSS by centrifugation at 2,000 rpm for 5 minutes, cells were resuspended in DMEM (Sigma) supplemented with 10% FBS. Cells were incubated at 37˚C for 2 hours and non-adherent cells were removed by washing with HBSS. All cells were incubated in humidified condition at 5% CO_2 at 37˚C.

Treatment of CpG ODNs

For *in vitro* experiment, 5×10^4 cells of splenocytes or peritoneal macrophages were dispensed in 6-well plate. The cells were treated with 0.6 μg/mL CpG ODNs or 0.1 μg/mL LPS for 24, 48 or 72 hrs. *In vivo* injection of CpG ODNs was performed in different methods (Dose: 10-100 μg, route: I.P, IV and S.Q) depending on the type of experiment.

Immunization of HBsAg and Sample Collection

Thirteen groups of four to six mice were prepared. For each groups, 2.5 μg of recombinant HBsAg (provided by Mogam Biotechonoly Institute) was emulsified with alum or indicated adjuvants and subcutaneously injected in lower abdomen. Twice boosting procedure was performed with same composition with a week interval. Mice were sacrificed to bleed and sera were isolated.

ELISA

Sera were collected from mice one week after last immunization. 100 μL of μg/mL HBsAg in 10 mM carbonate buffer was coated in 96-well microtiter plate at 4˚C over night. The buffer was removed and blocking buffer (PBS supplemented with 0.5% BSA and 0.1% Tween 20) was added and incubated at 37˚C for 1 hour. After washing three times with washing buffer (PBS supplemented with 0.1% Tween 20), 100 μL of serum 1 : 100-diluted with blocking buffer was added and incubated at 37˚C for 1 hour. After washing three times with washing buffer, 100 μL of HRP-conjugated anti-mouse IgG monoclonal antibody (Southern Biotechnology Associates, Inc. USA) 1 : 5,000-diluted with blocking buffer was added and incubated at 37˚C for 1 hour. After washing three times with washing buffer, 100 μL of TMB reagent was added and incubated for 30 minutes at room temperature in dark place. Absorbance was measured at 450 nm using microtiter plate reader (Elida-5, Physica Inc., New York, NY, USA). All condition of experiments was duplicated and calculated the mean for analysis.

Cell Proliferation Assay

5×10^4 splenocytes or peritoneal macrophages were cultured with or without 10 μg per mouse CpG ODN for 24, 48 or 72 hours. Cells were pulsed with 1 μCi of [^{3}H] thymidine (Perkin Elmer life science Inc, Boston, MA, USA) and then harvested 6 hours later. Amount of [^{3}H] incorporated were measured using Liquid Scintillation Analyzer (Beckman Coulter Inc, Fullerton, CA, USA). All assays were performed in triplicate.

RT-PCR

Fresh 1×10^7 cells were cultured in 6-well plate for *in vitro* stimulation of CpG ODN. Total RNA was isolated using TRIzol® reagent (Gibco BRL, Grand Island, NY, USA) as described in the manual. A total 1 μg of RNA was mixed with 2.5 μM oligo d $(T)_{16}$ (Promega, Madison, WI, USA) in 10 μL and incubated at 70˚C for 5 minutes and immediately transferred to ice-water. To the template mix, 2.5 U/μL of MMLV reverse transcriptase (Promega, Madison, WI), 1 mM of the dNTP, 1 U/μL of RNase inhibitor (Promega, Madison, WI, USA) in 10 mM Tris-HCl pH 8.3 supplimented with 50 mM $MgCl_2$ was added and reverse transcribed at 37˚C for 1 hour. The reaction was stopped by incubation at 95˚C for 10 min. For PCR, 30 μL of PCR mixture was composed in 1 μL of cDNA supplemented with 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 200 μM dNTP, 1 U Taq DNA polymerase (Takara, Tokyo, Japan) and 50 pmol of forward and reverse primers (Dae Myung, Seoul, Korea). The sequences and annealing temperatures of each primer sets were described in Table 2. After RT-PCR, PCR products were analyzed by electrophoresis in a 1.8% agarose gel contain with EtBr.

Flow Cytometry

BALB/c mice were given a single i. p. 100 μg of CpG ODN suspended in 0.01 M PBS. At 24 h after

Table 2. Sequences of PCR primers.

Mouse	Sequence (5′ to 3′)	PCR products (bp)
β-actin	TGGAATCCTGTGGCATCCATGAAAG TAAAACGCAGCTCAGTAACAGTCCG	348
TNF-α	GGCAGGTCTACTTTGGAGTCATTG CACTTCGAGGCTCCAGTCAATTCGG	307
IL-6	CTGGTGACAACCACGGCCTTCCCTA ATGCTTAGGCATAACGCACTAGGTT	600
IL-12	GAGGTGGACTGGACTCCCGA CAAGTTCTTGGGCGGGTCTG	618

Table 3. Augmentation of antibody titer through immunization with HBs antigen plus various immunologic adjuvants.

Adjuvant	Anti-HbsAgAb (IgG)
No antigen	0
No adjuvant	200
Alum	200
KSK-1	800
KSK-2	12800
KSK-7	800
KSK-12	12800
KSK-13	12800
KSK-2+alum	3200
KSK-12+alum	12800
KSK-13+alum	51200
CFA*	12800
CFA+alum	3200

a: median reciprocal titer tested by ELISA using sera post 3rd vaccination
*Complete Freund's adjuvant

injection, mice were killed and cells were isolation. In the first incubation, 15 μL of the anti-FcγRII Ab 2.4G2 (Becton Dickinson, Mountain View, CA, USA) was added to minimize nonspecific staining. Cells were stained with FITC-conjugated anti-B7.2 (Serotec, Darmstadt, Germany) or anti-MHC class II (Becton Dickinson, Mountain View, CA) for 30 min at 4˚C in PBS containing 1% BSA. Stained cells were analyzed on a FACScan (Becton Dickinson, Mountain View, CA, USA) using LYSIS II software.

Acknowledgements

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