

Treatment with phosphodiester CpG-ODN ameliorates atopic dermatitis by enhancing TGF- β signaling

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Synthetic oligodeoxynucleotides (ODNs) containing unmethylated CpG phosphorothioate (PS CpG-ODN) are known to decrease IgE synthesis in Th2 allergy responses. Nonetheless, the therapeutic role of PS CpG-ODN is limited due to cytotoxicity. Therefore, we developed a phosphodiester (PO) form of CpG-ODN (46O) with reduced toxicity but effective against allergies. In this study, we first compared the toxicity of 46O with CpG-ODNs containing a PS backbone (1826S). We also investigated the therapeutic efficacy and mechanism of 46O injected intravenously in a mouse model of ovalbumin (OVA)-induced atopic dermatitis (AD). To elucidate the mechanism of 46O underlying the inhibition of IgE production, IgE- and TGF- β -associated molecules were evaluated in CD40/IL-4- or LPS/IL-4-stimulated B cells. Our data showed that the treatment with 46O was associated with a lower hematological toxicity compared with 1826S. In addition, injection with 46O reduced erythema, epidermal thickness, and suppressed IgE and IL-4 synthesis in mice with OVA-induced AD. Additionally, 46O induced TGF- β production in LPS/IL-4-stimulated B cells via inhibition of Smad7, which suppressed IgE synthesis via interaction between Id2 and E2A. These findings suggest that enhanced TGF- β signaling is an effective treatment for IgE-mediated allergic conditions, and 46O may be safe and effective for treating allergic diseases such as AD and asthma. [BMB Reports 2021; 54(2): 142-147]

INTRODUCTION

Allergic diseases, such as asthma and atopic dermatitis (AD), are closely associated with increased serum immunoglobulin E (IgE) levels (1). IgE binding to the high-affinity receptor (Fc ϵ RI) in mast cells and basophils induces allergic response. Inhibition of IgE synthesis is an important factor in treating allergic diseases (2, 3).

Transforming growth factor- β (TGF- β) is a cytokine that acts via the Smad2 and Smad3 pathway to induce cell survival, proliferation, and migration during multiple biological processes, including immune responses, fibrosis, wound healing, and carcinogenesis (4). In an AD model of Smad3-deficient mice, allergen-induced skin inflammation and IgE production were increased (5). Moreover, TGF- β is induced by NF- κ B activation via TLR9 signaling in prostate cancer cells (6).

CpG-oligodeoxynucleotides (CpG-ODNs) are toll-like receptor 9 (TLR9) agonists, which enhance the anti-cancer efficacy of monoclonal antibodies or cancer vaccines when used as immune adjuvants in various animal models of cancer (7-9). Most TLR9-expressing immune cells, mainly B cells and dendritic cells absorb CpG-ODNs (10). Studies reported that CpG-ODNs prevent T helper 2 (Th2) allergic responses such as antigen-induced asthmatic responses, allergic rhinitis, eczema, and AD (11). CpG-ODNs inhibit the synthesis of interleukin (IL)-4, IL-13, and IL-5 by Th2 cells and trigger Th1 response via induction of interferon (IFN)- α and - γ , as well as IL-12. In addition, CpG-ODNs inhibit IgE production in B cells (12). However, the mode of action remains unclear.

Synthetic CpG-ODNs mimic the immunostimulatory activity of bacterial DNA, and contain a partial or complete phosphorothioate (PS) backbone, which protects ODN from degradation by nucleases in the body such as DNase, instead of the archetypal phosphodiester (PO) backbone (8). However, CpG-ODNs with PS backbones induce splenomegaly via B cell proliferation (7), but also trigger the release of TNF- α , IL-6, and IL-1 β by modulating NF- κ B activation via TLR9 signaling (13). Therefore, patient treatment with PS CpG-ODNs is very limited. However, CpG-ODNs containing a PO backbone (PO

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CpG-ODNs) are more easily degraded by DNase than PS CpG-ODNs. Thus, it is necessary to develop PO CpG-ODNs with an anti-allergic effect and low toxicity.

We sought to identify natural CpG sequences with a PO backbone, and 46O was identified in a previous study (14, 15). In this study, we show that this compound is less toxic and therefore useful for treating allergic diseases. More interestingly, we demonstrated that the anti-allergic effect of 46O may result from enhanced TGF- β signaling via suppression of Smad7, which down-regulated IgE production via induction of Id2/E2A complexes in LPS/IL-4-stimulated B cells. These results imply that induction of TGF- β is a useful strategy for treating allergic diseases.

RESULTS

Low toxicity of 46O compared to 1826S *in vivo*

Previous studies reported that CpG-ODNs with a PS backbone, such as 1826S, exhibit immunotoxicity and hepatotoxicity, leading to conditions such as splenomegaly, liver necrosis, and hemorrhagic ascites (16). A low-toxic CpG-ODN with a phosphodiester backbone was synthesized and designated as 46O (14). To determine the toxicity, 46O was subcutaneously injected into the mice at concentrations of 67, 200 and 600 mg/kg every day for 4 weeks and hematological toxicity was observed at 2 weeks after final treatment with 46O (Supplementary Table 1). The results showed that 46O had no hematological toxicity (Supplementary Table 2 and 3). In addition, 46O and 1826S were injected four times into the peritoneal cavity of mice (Fig. 1A). The 1826S CpG-ODN (1826S), which contains a PS backbone, was used as a positive control *in vivo*. The sizes of the spleen and lymph node increased with 1826S treatment. In contrast to 1826S, 46O-treated mice showed reduce spleen and lymph node masses (Fig. 1B). Moreover, the expression of TNF- α was highly induced in the serum of 1826S-treated mice compared with 46O-treated mice (Fig. 1C).

B cell proliferation by CpG-ODNs is a key mediator of splenomegaly *in vivo*, and therefore we investigated B cell proliferation via thymidine incorporation *in vitro*. B cells were isolated from spleen and incubated for 24 h after treatment with 46O or 1826S. As shown in Fig. 1D, 1826S treatment increased B cell proliferation, while 46O treatment did not. We next measured CpG-ODN-induced IL-6 synthesis in B cells by ELISA. As shown in Fig. 1E, treatment with 46O failed to induce IL-6 production, unlike 1826S. Thus, these results indicate that 46O is less immunotoxic than 1826S.

Prevention of antigen (Ag)-induced AD by 46O treatment

Studies have reported that CpG-ODNs are effective in treating Ag-induced AD (17). However, the preventive role of CpG-ODNs in Ag-induced AD remains unknown. Therefore, we investigated CpG-mediated AD prevention. We modified the protocol of the Ag-induced AD mouse model established by Kootiratrakam et al. (17). Before sensitization, 46O and 1826S

were administered once, twice, or four times intravenously (Fig. 2A). Balb/c mice were intraperitoneal injected for three times of OVA in alum and along with an occlusive patch of OVA as shown in Fig. 2A. Biopsy specimens were obtained from patch-applied skin one day after completion of epicutaneous sensitization for two weeks. The patch-applied skin was photographed and the injection of 46O and 1826s reduced the skin inflammation phenotype (Fig. 2B). Topical application of OVA in OVA-primed mice indeed induced epidermal hyperplasia and spongiosis with a dense dermal infiltration compared with control mice as analyzed by histological examination of the biopsy samples. Interestingly, treatment with 46O and 1826S reduced OVA-induced epidermal hyperplasia and infiltration of effector cells. This effect was more pronounced when the mice were treated with CpG-ODNs four times compared to

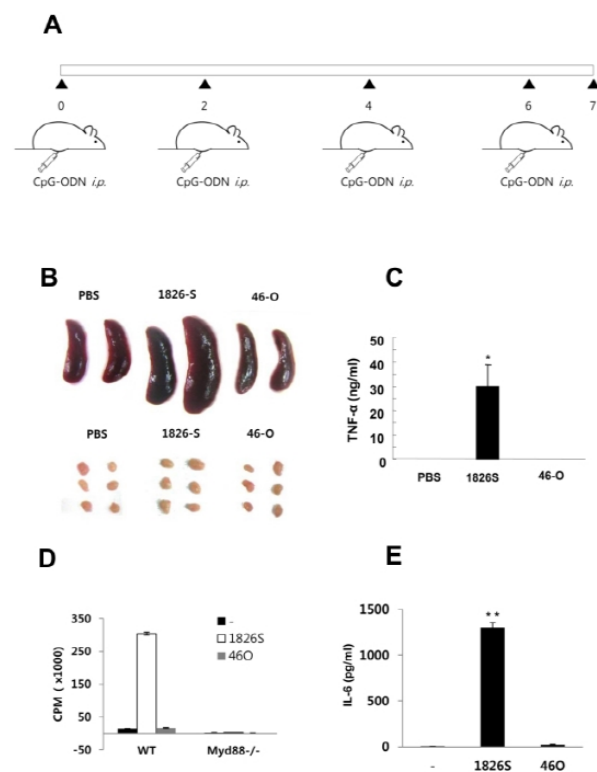


Fig. 1. Toxicity of 46O CpG-ODNs. (A) Experimental protocol for the analysis of toxicity induced by CpG-ODNs. (B) The spleen and lymph nodes of mice treated with 46O or 1826S CpG-ODN. (C) TNF- α expression in the serum of 46O or 1826S CpG-ODN-treated mice was determined by ELISA. (D) [^{3} H]-thymidine incorporation assays of splenic B cells derived from 46O or 1826S CpG-ODN-treated mice for 24 h. (E) IL-6 production in splenic B cells of 46O or 1826S CpG-ODN-treated mice was measured in the culture supernatant by ELISA; * $P < 0.05$ and ** $P < 0.01$, versus PBS-treated mice.

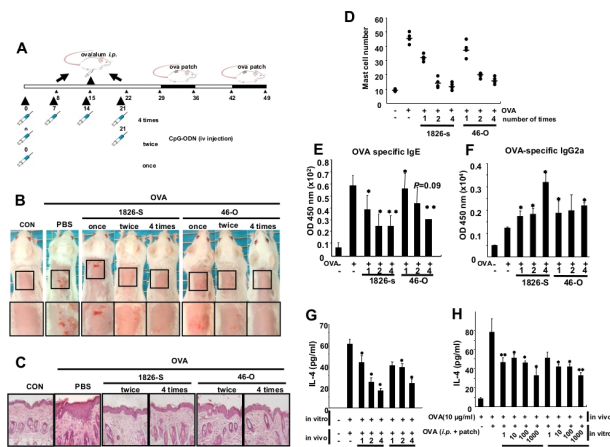


Fig. 2. Treatment with 46O CpG-ODN prevents Ag-induced AD and IgE secretion. (A) Experimental protocol for *in vivo* studies to test the efficacy of CpG-ODNs in an Ag-induced AD mouse model. (B) Phenotypical presentation of OVA patch sites. (C) Hematoxylin and eosin staining of the skin (200× magnification). (D) Mast cell infiltration was analyzed by toluidine blue staining and counted per 400× field by two independent researchers. Expression of OVA-specific (E) IgE and (F) IgG2a secretions in the serum of experimental mice was analyzed by ELISA. (G) Splenocytes were isolated from experimental mice and cultured with 10 µg/ml OVA protein for 48 h. IL-4 production was analyzed by ELISA. (H) *In vitro* analysis of IL-4 synthesis in OVA-activated splenocytes following 46O treatment. Splenocytes were purified from PBS-treated mice and cultured in the presence or absence of 1826S and 46O. After 48 h, IL-4 was measured in the cell-free culture medium by ELISA; *P < 0.05 and **P < 0.01, versus no treatment with CpG-ODN. Data are expressed as mean ± standard deviation.

twice (Fig. 2C). Besides IgE and IL-4 production, mast cells play an important role in allergic diseases (2). The mast cells in the skin were quantified by toluidine blue staining. The number of mast cells per 400× microscopic field in the infiltrated dermal area was five-fold higher in OVA-induced AD skin, and this induction was decreased by both CpG-ODNs in a dose-dependent manner (Fig. 2D).

IgE inhibition by 46O treatment in antigen-induced AD

IgE is an important factor in AD pathogenesis (18). Therefore, we investigated whether pre-treatment with 46O prevented IgE production. We collected sera at the time of skin biopsy and then measured the total and OVA-specific IgE production by ELISA. Similar to the histological results, pre-treatment with 46O or 1826S significantly decreased OVA-specific IgE in a CpG-ODN dose-dependent manner (Fig. 2E). However, these compounds increased OVA-specific IgG2a (Fig. 2F).

Since IL-4 plays an important role in Th2-mediated diseases such as AD, we analyzed OVA-specific IL-4 production *in vitro*. We isolated splenocytes from the biopsy specimens and then cultured them with 10 µg/ml OVA protein. After 48 h,

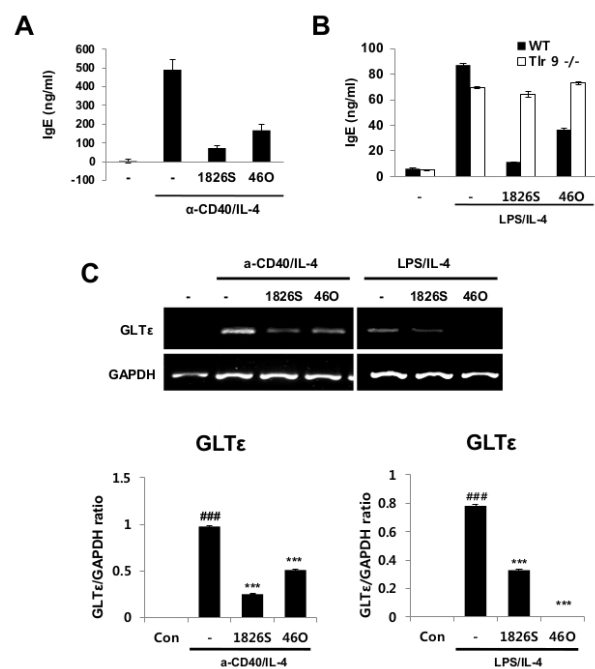


Fig. 3. Inhibitory effect of 46O on IgE production. (A) B cells isolated from the spleen of Balb/c mice were cultured for 5 days with CPG-ODNs, in the presence of LPS/IL-4 or anti-CD40/IL-4. The level of IgE was determined by ELISA. (B) Inhibition of IgE synthesis by 46O via TLR9. B cells were purified from spleens of Balb/c or TLR9 knockout mice and then cultured with 1826S or 46O in the presence of LPS/IL-4 or anti-CD40/IL-4 for 5 days. IgE levels were assessed by ELISA. (C) Level of Cε GLT in B cells. Splenic B cells were activated by LPS/IL-4 or anti-CD40/IL-4 and then cultured with CpG-ODNs, including 1826S or 46O, for 3 days. The level of Cε GLT mRNA was quantified by RT-PCR; *P < 0.05 and **P < 0.01, versus positive control. Data are representative of three independent experiments.

IL-4 was measured in the culture medium. The level of IL-4 secretion decreased in splenocytes derived from CpG-ODN-treated mice compared with PBS-treated control mice (Fig. 2G) in a dose-dependent manner. Additionally, splenocytes isolated from PBS-treated mice were cultured with CpG-ODNs *in vitro*. As shown in Fig. 2H, 46O treatment inhibited IL-4 secretion in isolated splenocytes from PBS-treated mice *in vitro*. These results showed that 46O is not only a potential therapeutic agent but also prevents Ag-induced AD.

Inhibition of IgE production in B cells by 46O

IgE synthesis in B cells requires two signals (19). The first signal is provided by cytokine IL-4 and the second signal is provided by ligation of CD40 on B cells. Lipopolysaccharide (LPS) can mimic CD40 ligation *in vitro*. To evaluate the inhibitory effect of 46O on IgE synthesis, B cells were cultured for 5 days with LPS or CD40 ligand (CD154) in the presence of IL-4, and the IgE production in the culture media was

measured. As shown in Fig. 3A, IgE synthesis was decreased significantly by both 1826S and 46O treatment in CD40/IL-4- or LPS/IL-4-treated B cells. However, IgE synthesis was not decreased by CpG-ODNs in TLR9^{-/-} B cells (Fig. 3B). Additionally, we also determined the transcription level of GLTε, which is responsible for IgE class switching. Our data demonstrate that the level of GLTε mRNA was reduced by 46O and 1826S in CD40/IL-4- or LPS/IL-4-treated B cells (Fig. 3C).

Inhibition of IgE production via 46O-induced Id2/E2A complex formation in LPS/IL-4-activated B cells

To investigate the inhibitory mechanism of 46O in IgE secretion, we investigated whether this compound regulated TGF-β signaling because TGF-β suppressed IgE levels in AD-like skin lesions and serum in the NC/Nga mouse model (20). TGF-β expression was measured in culture media using ELISA. Fig. 4A and B showed that treatment with 46O and 1826S induced TGF-β secretion from CD40/IL-4- and LPS/IL-4-treated B cells. The level of TGF-β mRNA was also increased by treatment with both CpG-ODNs (Fig. 4C). Next, we analyzed the Smad7 mRNA level via quantitative real-time PCR because Smad7 is a known suppressor of TGF-β signaling (21). B cells were treated with LPS or CD40 ligand (CD154) in the presence of IL-4 for 3 days and RNA was extracted from B cells. As shown in Fig. 4D, the expression of Smad7 mRNA was decreased by both 46O and 1826S in LPS/IL-4- and CD40/IL-4-stimulated B cells. Since both CpG-ODNs slightly suppressed Smad7 expression in CD40/IL-4-activated B cells, we investigated the role of CpG-ODN and TGF-β signaling in IgE production by LPS/IL-4-activated B cells. LPS/IL-4-activated B cells were incubated with or without LY364947, a TGF-β inhibitor. Treatment with LY364947 abrogated the 46O-induced IgE suppression in B cells (Fig. 4E). However, LY364947 could not reverse the 1826S-induced IgE suppression, suggesting that 46O, but not 1826S inhibits IgE synthesis via TGF-β signaling in LPS/IL-4-activated-B cells.

Finally, we performed immunoprecipitation with an anti-Id2 antibody using lysates of 46O-treated B cells to elucidate the mechanism by which 46O inhibits IgE production via TGF-β up-regulation. E2A binds to the E-Box in the ε germline promoter, which promotes class switch recombination (CSR) to IgE. Id2 acts as a negative regulator of basic helix-loop-helix (bHLH) transcription factors, such as E2A, by antagonizing E2A activity (22). To establish Id2 and E2A complex formation, B cells were cultured with or without 46O in the presence of LPS/IL-4, and the cell lysates were harvested. As shown in Fig. 4F, 46O treatment enhanced Id2/E2A complex formation, as well as Id2 expression in LPS/IL-4-activated B cells. Therefore, our data demonstrate that 46O inhibited IgE synthesis by increasing Id2 expression and binding to E2A.

DISCUSSION

Synthetic PS CpG-ODNs contain a partial or complete PS

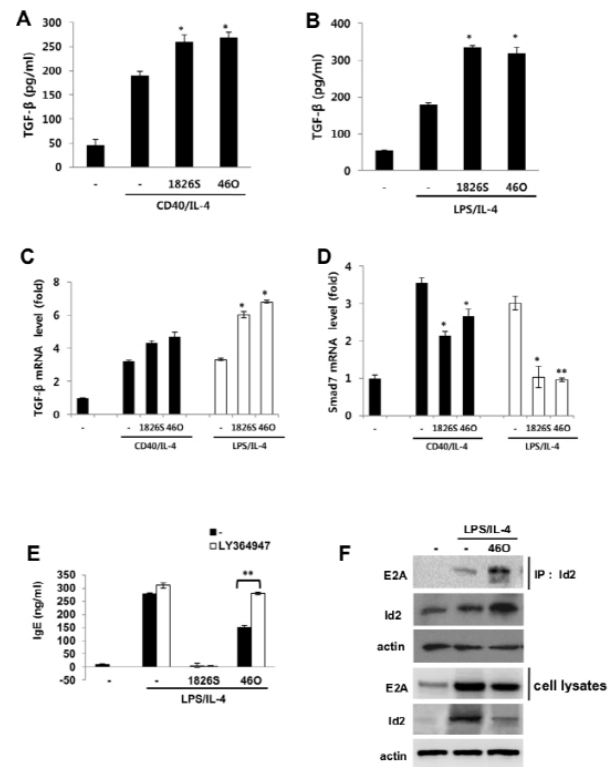


Fig. 4. Inhibition of IgE by 46O mediated via TGF-β induction in LPS/IL-4-activated B cells. TGF-β induction by CPG-ODNs, including 1826S and 46O, in B cells treated with (A) anti-CD40/IL-4 and (B) LPS/IL-4. B cells were cultured for 4 days. TGF-β expression in the culture supernatant was measured by ELISA. (C) Induction of TGF-β mRNA level in B cells. (D) Inhibition of Smad7 mRNA level by CpG-ODNs, including 1826S and 46O. B cells were cultured in the presence of anti-CD40/IL-4 or LPS/IL-4 for 3 days. Levels of TGF-β and Smad7 mRNA were analyzed by quantitative real-time PCR. (E) LY364947 reverses 46O-induced IgE suppression in LPS/IL-4-stimulated B cells. IgE in B cells was measured in cultured media for 5 days using ELISA. (F) Immunoprecipitation (IP) of Id2/E2A complexes. B cells were cultured with or without 46O in the presence of LPS/IL-4 for 5 days. B cell lysates were immune precipitated with anti-Id2 antibody or the respective control. *P < 0.05 and **P < 0.01, versus positive control. Data are representative of three independent experiments.

backbone instead of the archetypal PO backbone to protect the ODN from degradation by DNases in the body (8). CpG-ODNs are strong Th1 response inducers while inhibiting Th2 responses, and therefore CpG-ODNs are considered as adjuvant treatment for Th2-mediated allergy (7). Despite significant advances in treating Th2 allergic disease using these compounds as therapeutic adjuvants, the clinical use of PS CpG-ODNs is restricted by side effects such as megalosplenia, pro-inflammatory cytokine release, and lymphoid follicle destruction (23). Therefore, the development of less toxic CpG-

ODNs is necessary. In the present study, we showed that 46O, which carries a PO backbone, did not induce megalosplenia and secreted lower levels of pro-inflammatory cytokines than 1826S, a PS CpG-ODN.

CpG-ODNs inhibit IgE synthesis in B cells, a process that requires two signals (19). The first signal is provided by IL-4, which activates transcription at a specific Ig locus. The second signal is provided by the ligation of CD40 on B cells, which in turn activates DNA switch recombination. This signal can be also provided by LPS instead of CD40 *in vitro*. Liu *et al.* showed that PS CpG-ODN directly up-regulates T-bet expression in CD40/IL-4-stimulated B cells and T cells, resulting in IgE suppression (24). However, it remains to be elucidated how PO CpG-ODN inhibits IgE production in LPS/IL-4-stimulated B cells.

In this study, we revealed a novel mechanism involving PO CpG-ODN-induced IgE inhibition via TGF- β signaling in LPS/IL-4-stimulated B cells. Oral administration of high doses of TGF- β inhibits serum IgE in an OVA-induced allergy model (25). Another study demonstrated that CpG-ODN promotes IL-8 and TGF- β 1 via NF- κ B activation in prostate cancer cells (26). TGF- β signaling inhibits IgE synthesis and AD (20). Consequently, TGF- β signaling was investigated as a possible target of 46O in IgE inhibition. Indeed, 46O-induced TGF- β signaling inhibited IgE synthesis in B cells activated by stimulation with LPS/IL-4 or CD40/IL-4. To elucidate the mechanism of TGF- β induction by 46O, we investigated Smad7 expression, which plays an essential role in the negative feedback regulation of TGF- β signaling by inhibiting TGF- β receptors (21). Here, we showed that Smad7 mRNA was suppressed dramatically by 46O treatment in LPS/IL-4-stimulated B cells. LY364947, a TGF- β inhibitor, rescued 46O-induced IgE suppression; however, this response was not mediated by 1826S in LPS/IL-4-treated B cells. These results suggest that the inhibitory mechanism of 46O differs from that of 1826S and only 46O inhibits IgE synthesis via TGF- β signaling in LPS/IL-4-activated B cells. Thus, 1826S and 46O may have different modes of action and the inhibitory mechanism of 1826S may be mediated via modulation of other signals rather than TGF- β . However, a comprehensive study is required to delineate the inhibitory mechanism of 1826S on IgE production. Finally, we investigated the regulation of Id2/E2A complex formation in 46O-treated B cells to further elucidate the mechanism by which 46O inhibits IgE via TGF- β up-regulation. E2A binds to the E-Box within the ϵ germline promoter, which promotes IgE class switch recombination (CSR) (27) and is negatively regulated by Id2 via Id2-E2A interaction. Further, E2A binds strongly to E-boxes within the ϵ germline promoter in Id2-deficient mice (28). Interestingly, TGF- β can induce Id2 (29). Therefore, we investigated whether 46O induced Id2/E2A interaction via TGF- β induction. As expected, 46O inhibited IgE CSR by increasing Id2 via enhanced TGF- β signaling in LPS/IL-4-stimulated B cells.

We previously reported the therapeutic effects of topical

application of 46O in allergic Th2 responses using a mouse model of AD (30). In this study, we demonstrated for the first time the detailed mechanism of the preventive effects of 46O against allergic diseases. Several studies have reported that OVA stimulation, together with aluminum, induces release of OVA-specific IgE, which represents a switch to induction of allergic Th2 responses (31). In the present study, 46O was administered once, twice, or four times intravenously before sensitization. Treatment with 46O inhibited IL-4 secretion in IgE-secreting plasma cells isolated from PBS-treated mice *in vitro* as well as OVA-specific synthesis of IgE and IL-4 in sera. Moreover, the toxicity of 46O, as measured by the occurrence of splenomegaly and TNF- α production, was significantly lower compared to that of 1826S. These results indicate that 46O pre-treatment can prevent the Th2 response in allergic diseases without cytotoxicity.

Taken together, we demonstrated that 46O treatment prevents allergic responses by up-regulating TGF- β via suppression of Smad7, which inhibits IgE synthesis by inducing Id2/E2A complex formation in LPS/IL-4-stimulated B cells. Therefore, our data suggest that 46O may be a safe and effective therapeutic candidate for treatment of allergic diseases such as AD and asthma.

MATERIALS AND METHODS

Detailed information is provided in the Supplementary Information.

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CONFLICTS OF INTEREST

The authors have no conflicting interests.

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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 460 CpG-ODN was phosphodiester form. Sequences: 1018S: 5'-TGACTGTGAACGTTTCGAGATGA-3', 1826S: 5'-TCCATGACGTTCTGACGTT-3', 460: 5'-CTCGCACGTTGCCGACTTC-3'. CpG-ODNs were synthesized and purified using HPLC by Genotech, Inc. (Daejeon, Korea).

The evaluation of hematological toxicity

ICR mice were treated with 460 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 μ g of CpGs in 100 μ l 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 μ g chicken OVA (grade VII; Sigma-Aldrich, St. Louis, MO) precipitated with 4 mg aluminum hydroxide (alum) in a 200:1 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, mice 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 μ g/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 2×10^5 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 ^3H -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 H_2SO_4 .

ELISA and intracellular IgE staining in B cells

B cells (1×10^5) were stimulated for 5 days with 10 $\mu\text{g}/\text{ml}$ of LPS or 5 $\mu\text{g}/\text{ml}$ of α -CD40 antibody together with 10 ng/ml IL-4. The cells were treated with 1

μ M 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 μ g of total RNA using by 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- β 1: F-5'-CAACAATTCCTGGCGTTACCTTGG-3', R-5'-GAAAGCCCTGTATTCCGTCTCCTT, Smad7: F-5': GACTCCAGGACGCTGTTGGT-3', R-5R' CCATGGTTGCTGCATGAACT-3' GAPDH: F-5'-TGCCCCCATGTTTGTGATG-3', R-5'-TGCCCCCATGTTTGTGATG-3'.

To analyze the GLT ϵ 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: GLT ϵ : 5'-GCACAGGGGGCAGAAGAT-3A, R-CGTTGAATGATGGAGGAT-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'-CATCTTCCAGGAGCCAGACC-3T, R-5-RTCCACCACCCTGTTGCTGTA-3C ' (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 × 10⁶ 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 Na₃VO₄, 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 Na₃VO₄, 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 α E2A 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 $\pm$ 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)	$10^6/\mu\text{l}$	Flowcytometry, Isovolumetry
HGB (Hemoglobin conc.)	g/dL	Modified CN met- Hb method
HCT (Hamatocrit)	%	(RBCxMCV)/10
MCV (Mean corpurscular volume)	fL	Histogram
MCH (Mean corpurscular hemoglobin)	pg	(HGB/RBC) x 10
MCHC ((Mean corpurscular 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)	$10^3/\mu\text{l}$	Flowcytometry
MPV (Mean platelet volume)	fL	Histogram
RET (Reticulocyte)	%	Flowcytometry, Isovolumetry
WBC (White blood cell count)	$10^3/\mu\text{l}$	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

Hematological Values					
Male					
Tests	Units	Groups (mg/kg/days)			
		G1 (0)	G2 (67)	G3 (200)	G4 (600)
RBC	$10^6/\mu\text{l}$	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	$10^3/\mu\text{l}$	1400.7 ± 123.8	1321.9 ± 99.7	1330.4 ± 93.6	$1225.3 \pm 97.9^{**}$
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	$10^3/\mu\text{l}$	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	$24.1 \pm 8.4^*$
LYM	%	80.5 ± 4.1	78.6 ± 4.5	78.4 ± 4.9	$69.1 \pm 8.1^{**}$
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	$1.03 \pm 0.75^*$
LUC	%	0.44 ± 0.34	1.00 ± 0.44	1.09 ± 0.59	$2.37 \pm 1.00^{**}$
N		9 ^{a)}	9 ^{a)}	9 ^{a)}	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

Hematological Values					
Female					
Tests	Units	Groups (mg/kg/days)			
		G1 (0)	G2 (67)	G3 (2000)	G4 (600)
RBC	10 ⁶ /μl	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 /μl	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 /μl	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 ^{a)}	10	9 ^{a,)}	9 ^{a,)}

*/** 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.

