

Immunostimulatory oligodeoxynucleotide from *Bifidobacterium longum* suppresses Th2 immune responses in a murine model

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Summary

We have reported previously that novel immunostimulatory sequence (ISS) oligodeoxynucleotide (ODN) BL07S from a probiotic strain of *Bifidobacterium longum* inhibited immunoglobulin (Ig) E production *in vitro*. However, whether ISS-ODNs from probiotics regulate T helper type 2 (Th2)-polarized immune reactions *in vivo* remains unclear. To evaluate the inhibitory effects of ODN BL07S on type I allergic response, BALB/c mice were injected with or without ODN BL07S in the presence of ovalbumin (OVA) on days 0 and 14. Serum Ig levels (IgE, IgG1 and IgG2a) and cytokine levels (interferon (IFN)- γ , interleukin (IL)-12, IL-4, IL-5, IL-10 and IL-13) were investigated in splenocyte cultures from days 14–28. Production of OVA-specific and total IgE were significantly suppressed by administration of ODN BL07S, but not by ODN BL06S, a non-ISS-ODN. Compared to controls, ODN BL07S induced significantly lower levels of Th2 cytokines (IL-4 and IL-5) in splenocyte cultures, and significantly higher levels of serum OVA-specific IgG2a. These effects of ODN BL07S on modulation of Th2 immune response were dose-dependent. The present results demonstrate that ODN BL07S from genomic DNA of *B. longum* BB536 prevents antigen-induced Th2 immune responses *in vivo*, suggesting that ISS-ODNs from probiotics might be useful in preventing allergic disease.

Keywords: allergy, *Bifidobacterium longum* BB536, IgE, ISS-ODN, probiotics

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Introduction

Type I allergies such as allergic rhinitis and hay fever are caused by characteristic immune responses to allergens, mediated primarily by T helper type 2 (Th2) cells [1,2]. Th2 cells synthesize high levels of interleukin (IL)-4, IL-5 and IL-13, leading to the production of allergen-specific immunoglobulin (Ig) E, production of which represents a risk factor for allergic inflammatory reactions such as the release of histamine and leukotriene from mast cells [2]. Conversely, Th1 cells suppress Th2 immune responses by secreting interferon (IFN)- γ . Th1 cytokines such as IL-12 and IFN- γ induce T cell differentiation from Th0 to Th1, whereas IL-4, one of the typical Th2 cytokines, facilitates differentiation from Th0 to Th2. Estimation of IgE production and Th1/Th2 cytokine balance thus seems extremely important in the evaluation of type I allergic inflammation [3,4]. Various drugs, such as corticosteroids, epinephrine, histamine antagonists and leukotriene synthesis inhibitors, interfere with these reactions [5]. However, such drugs generally display adverse effects,

strongly suggesting the necessity for a more fundamental means of preventing allergies.

Numerous studies have reported that intake of probiotics [6] such as lactobacilli and bifidobacteria is associated with the prevention of several allergic diseases [7–12]. These reports have indicated strongly that probiotics exert the potential to modulate and regulate the immune response. We previously reported that intake of *Bifidobacterium longum* BB536-supplemented yoghurt relieved Japanese cedar pollinosis symptoms through a modulating effect on Th1/Th2 balance [13]. However, the exact components in probiotics related to this ability remain unknown. Some cell components in probiotics such as peptidoglycan, lipoteichoic acid and secreted soluble substances of organisms reportedly induce immune responses [14,15]. Immunostimulatory sequence (ISS) oligodeoxynucleotides (ODNs) [16] have already been identified from the genomic DNA of probiotics [17–20]. In general, ISS-ODNs have been found to suppress Th2 immune responses by stimulating innate immune cells via Toll-like receptor 9 (TLR9) [21] expressed on the cell

surface, consequently affecting adaptive immune systems by releasing abundant Th1 cytokines [22,23]. ISS-ODNs are thus considered therapeutically significant reagents for allergic disease. Regarding the anti-allergic actions of such ISS-ODNs, the expectations for practical use of antigen-conjugated ODNs as a vaccine against hay fever have reached the level of clinical application in humans [24]. However, the effects of ISS-ODNs derived from probiotics on allergic diseases *in vivo* have not yet been clarified.

Assays *in vitro* have shown that ISS-ODN BL07S isolated from the genomic DNA of *B. longum* BB536 decreases IgE production using splenocytes from BALB/c mice sensitized using ovalbumin (OVA) [25]. The present study therefore evaluated the effects of ODN BL07S on inhibition of IgE production and suppression of Th2 immune response *in vivo*.

Materials and methods

ODNs

ODNs used in this study comprised ODN BL07S (5'-GCGTCGGTTTCGGTGCTCAC-3') and ODN BL06S (5'-CGTTAGTGGGTTTCGCCCAT-3'), derived from the genomic sequences of *B. longum* BB536 in our previous study [25]. ODNs were synthesized chemically by Sigma-Genosys (Hokkaido, Japan), and in some cases were modified with phosphorothioate to increase resistance to nuclease degradation. ODNs were confirmed to be free of detectable endotoxin by using a Limulus amoebocyte lysate kit (Seikagaku Kogyo, Tokyo, Japan) according to the manufacturer's manual.

Mice

Specific pathogen-free 6-week-old-male BALB/c mice were purchased from Charles River Japan (Kanagawa, Japan) and housed in plastic cages at room temperature. Mice were fed a standard CRF-1 diet (Oriental Yeast Co., Tokyo, Japan) and allowed *ad libitum* access to water throughout the experimental period. All experiment protocols involving mice were performed according to the guidelines of the Prime Minister's Office in Japan (no. 6, 27 March 1980).

Experimental design

Mice were injected subcutaneously with 20 µg of OVA with (ODN group) or without (controls) ODNs dissolved in 100 µl of phosphate-buffered saline (PBS) adsorbed to an equal volume of alum solution (Pierce, IL, USA) on day 0. After 2 weeks (on day 14), second immunizations were performed under the same experimental conditions for each group. Mice in the naive group were administered alum solution only. Sera were collected for measuring levels of IgE on day 21. In the time-course study, sera were collected on days 14, 21 and 28, and spleens were harvested for culture on

these same days. Sera and spleens were collected on day 21 in the dose-response study. Endotoxin content of OVA (grade V; Sigma, MO, USA) was confirmed to be < 100 pg/mg of OVA.

Splenocyte culture

Single splenocyte suspensions were prepared in cold RPMI-1640 medium (Invitrogen, CA, USA) and pooled for each mouse at 4.0×10^6 cells/ml in complete medium [(RPMI-1640 medium containing 10% fetal calf serum (FCS; Invitrogen), 100 µg/ml penicillin and 100 U/ml streptomycin (Invitrogen)]. Cells were incubated in 24-well flat-bottomed culture plates with or without 100 µg/ml OVA at 37°C in 5% CO₂ atmosphere. Following incubation for 72 h, culture supernatants were harvested and kept at -20°C until cytokine analysis.

Induction of transforming growth factor (TGF)-β was performed as described by Nakao *et al.* [26], with some modifications. Splenocytes were suspended in AIM-V medium (Invitrogen) supplemented with 100 µg/ml penicillin and 100 U/ml streptomycin (Invitrogen) without FCS, and were cultured for 96 h with or without 100 µg/ml OVA. Culture supernatants were activated by 1 N HCl for 10 min at room temperature and neutralized to pH 7.2 with 1.2 N NaOH/0.5 M HEPES buffer (Invitrogen) before measuring TGF-β.

Flow cytometry analysis

CD4⁺CD25⁺ cells on CD3-positive splenocytes obtained from mice on day 21 were evaluated using flow cytometry analysis. Cells were stained with anti-mouse phycoerythrin (PE) CyChrome (Cy) 5-CD3, PE-CD4 and fluorescein isothiocyanate (FITC)-CD25 monoclonal antibodies (eBioscience, CA, USA). Events (≥ 100 000) were acquired on a FACSCalibur™ flow cytometer (Becton Dickinson, NJ, USA) and analysed using Cell Quest software (Becton Dickinson).

Enzyme-linked immunosorbent assay for cytokines and immunoglobulins

The following cytokines were examined in this study: IFN-γ, TGF-β, IL-4, IL-5, IL-10, IL-12 and IL-13. Levels of these cytokines in culture supernatants were quantified using enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, MN, USA) in accordance with the manufacturer's instructions. Total IgE, IgG1 and IgG2a levels were assessed using ELISA kits (Bethyl Laboratory, TX, USA) according to the manufacturer's instructions.

OVA-specific IgE, IgG1 and IgG2a were evaluated in accordance with the methods of Sato *et al.* [27]. Standard immune serum was obtained by subcutaneous immunization of mice with 20 µg of OVA adsorbed to alum, repeated

three times at 2-week intervals. OVA-specific IgE, IgG1 and IgG2a titres were calculated by comparison to standard immune serum. Concentrations of OVA-specific IgE, IgG1 and IgG2a in the standard immune serum were considered arbitrarily as 1000 U/ml.

Statistical analysis

Experimental data are expressed as mean $\pm$ standard deviation (s.d.). All statistical analysis was performed using SPSS statistical version 11.0. The significance of differences in mean values was determined using the Mann–Whitney *U*-test. Differences were considered statistically significant for values of $P < 0.05$ compared to controls.

Results

Effects of ODNs on IgE production *in vivo*

Figure 1 shows the effects of ODNs (10 μ g/mouse) on IgE production in serum of BALB/c mice sensitized with OVA. OVA-specific and total IgE levels were significantly increased in the control group compared to levels in the naive group on day 21 (total IgE: naive group, 142.4 ± 49.1 pg/ml; control group, 632.0 ± 255.6 pg/ml, $P < 0.01$; OVA-specific IgE: naive group, below limits of detection; control group, 253.7 ± 84.4 U/ml). OVA-specific and total IgE levels were significantly lower in the ODN group than in the control group on day 21. IgE levels of the ODN BL06S group used as negative controls showed almost the same levels as the control group.

Time-course response of ODN BL07S for IgE, IgG2a, IgG1 and cytokine production

The effects of ODN BL07S (10 μ g/mouse) on serum IgE levels were studied from day 14 to day 28. Production of

OVA-specific and total IgE were significantly lower in the ODN group than in the control group on both day 14 before the second immunization and on day 28 (Fig. 2a,b).

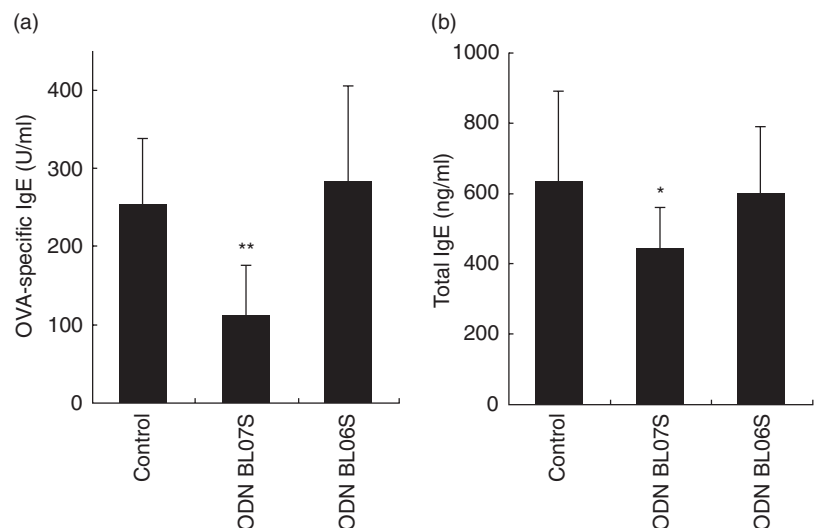
Production of serum OVA-specific IgG2a in the ODN was significantly higher on days 21 and 28 compared to controls, but total IgG2a levels were not significantly affected (Fig. 2c,d). Compared to controls, administration of ODN BL07S significantly suppressed OVA-specific and total IgG1 levels on day 21, but not on day 28 (Fig. 2e,f).

To clarify the mechanisms involved in the inhibition of IgE production by ODN BL07S, we investigated the cytokine production of splenocytes from mice restimulated with OVA. Levels of IL-12 were significantly higher in the ODN group than in controls from days 14–28 (Fig. 3a). Although a trend was seen toward increased levels of IFN- γ in the ODN group compared to controls, no significant difference was identified (Fig. 3b). Conversely, levels of IL-4 and IL-5 were significantly lower in the ODN group than in controls on days 14, 21 and 28 (Fig. 3c,d). Production of IL-13 was also significantly suppressed on days 14 and 28 (Fig. 3e). IL-12 levels in supernatants without OVA on day 21 were 76.4 ± 6.2 pg/ml, 70.2 ± 17.6 pg/ml and 80.8 ± 12.6 pg/ml in naive, control and ODN (10 μ g/mouse) groups, respectively. No significant differences were noted between groups. IL-4, IL-5, IL-13 and IFN- γ in supernatants without OVA were below the limits of detection.

Dose-response of ODN BL07S for IgE and cytokine production

To investigate the dose-response of ODN BL07S for IgE and cytokine production, ODN BL07S was administered at 0.1, 1 and 10 μ g/mouse. A dose-dependent effect was found on inhibition of serum total and OVA-specific IgE (Fig. 4). A trend toward suppression of total and OVA-specific IgE was seen at 0.1 μ g, with significant inhibition at 1 μ g. Significant effects on serum OVA-specific IgG2a, OVA-specific IgG1 and

Fig. 1. Oligodeoxynucleotide (ODN) BL07S decreased IgE production in immunized mice. Mice were immunized using ovalbumin (OVA) (20 μ g/mouse) and ODN BL07S (10 μ g/mouse) in alum at a 2-week interval. As controls, mice were administered OVA and phosphate buffered saline (PBS) in alum. Blood was collected on day 21 and IgE levels were determined using enzyme-linked immunosorbent assay. Data represent mean $\pm$ s.d. from six mice per group. (a) OVA-specific IgE; (b) total IgE. * $P < 0.05$, ** $P < 0.01$ versus controls.



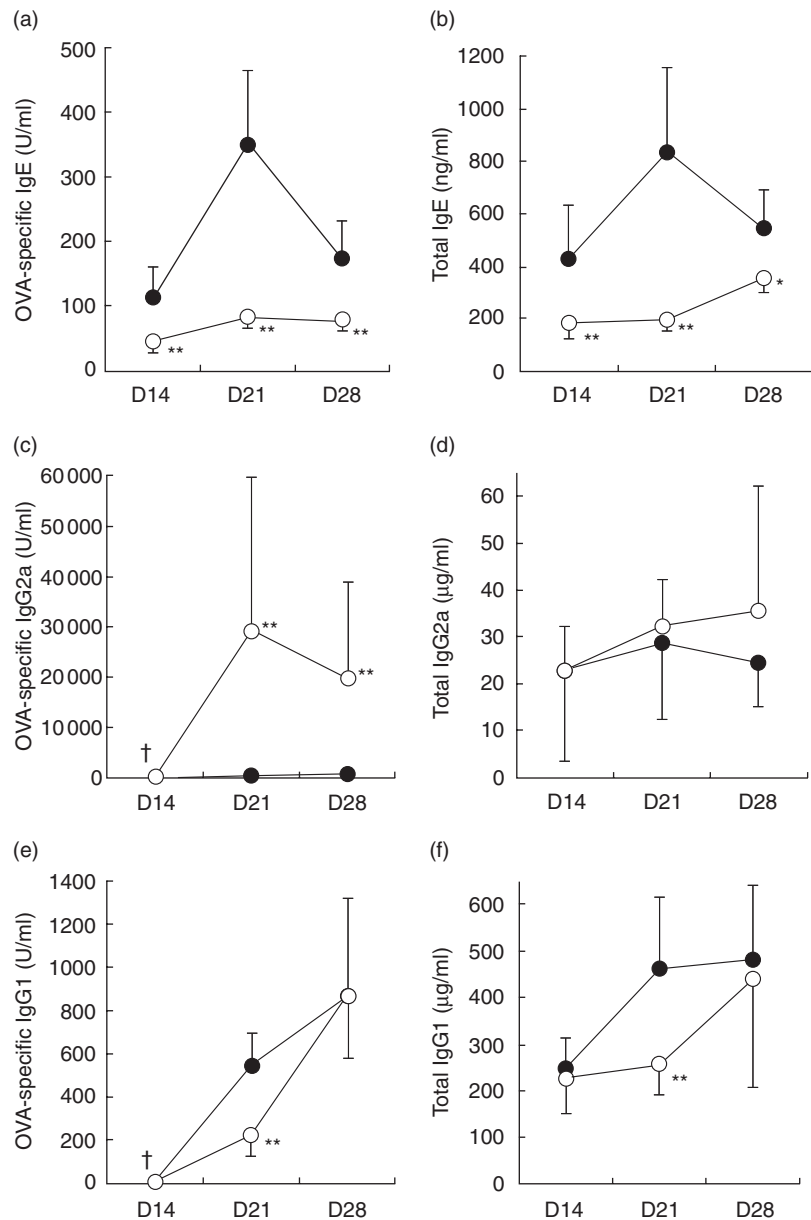


Fig. 2. Effects of oligodeoxynucleotide (ODN) BL07S on total and ovalbumin (OVA)-specific Ig levels in serum of mice sensitized with OVA. Mice were immunized as described in Fig. 1. Ig levels were determined using enzyme-linked immunosorbent assay. Data represent mean $\pm$ s. d. from eight mice per group. (●) Control group; (○) ODN BL07S group. (a) OVA-specific IgE; (b) total IgE; (c) OVA-specific IgG2a; (d) total IgG2a; (e) OVA-specific IgG1; (f) total IgG1. * $P < 0.05$, ** $P < 0.01$ versus controls. †Below the limits of detection.

total IgG1 and production of IL-12, IL-4, IL-5 and IL-13 in splenocyte cultures were found at 10 μ g, but not at lower doses (Fig. 5).

Factors related to regulatory T cells

To investigate the involvement of regulatory T cells in suppressing Th2 response, factors related to regulatory T cells were evaluated. Levels of IL-10 in cultured splenocytes were significantly lower in the ODN group (10 μ g/mouse) than in controls (Fig. 6a). Production of TGF- β was at almost the same level as in the control group (Fig. 6b). No differences in expression of CD4⁺CD25⁺ cells on CD3-positive cells (T cells) among splenocytes on day 21 were seen between naive ($1.28 \pm 0.19\%$, percentage of CD4⁺CD25⁺ T cells in

splenocytes), control ($1.34 \pm 0.23\%$) and ODN (10 μ g/mouse: $1.40 \pm 0.18\%$) groups.

Discussion

On the basis of the induction of Th1 responses, ISS-ODNs have been thought to represent effective immunomodulators of Th2-mediated diseases such as allergies. Some studies have already suggested that ISS-ODNs promoting a Th1 immune response could suppress IgE production and allergic reactions [28,29]. The present study demonstrated that ISS-ODN BL07S from genomic sequences of *B. longum* BB536 has the potential to inhibit IgE production *in vivo* in a dose-dependent manner. In addition, splenocyte cultures from mice administered ODN BL07S produce significantly

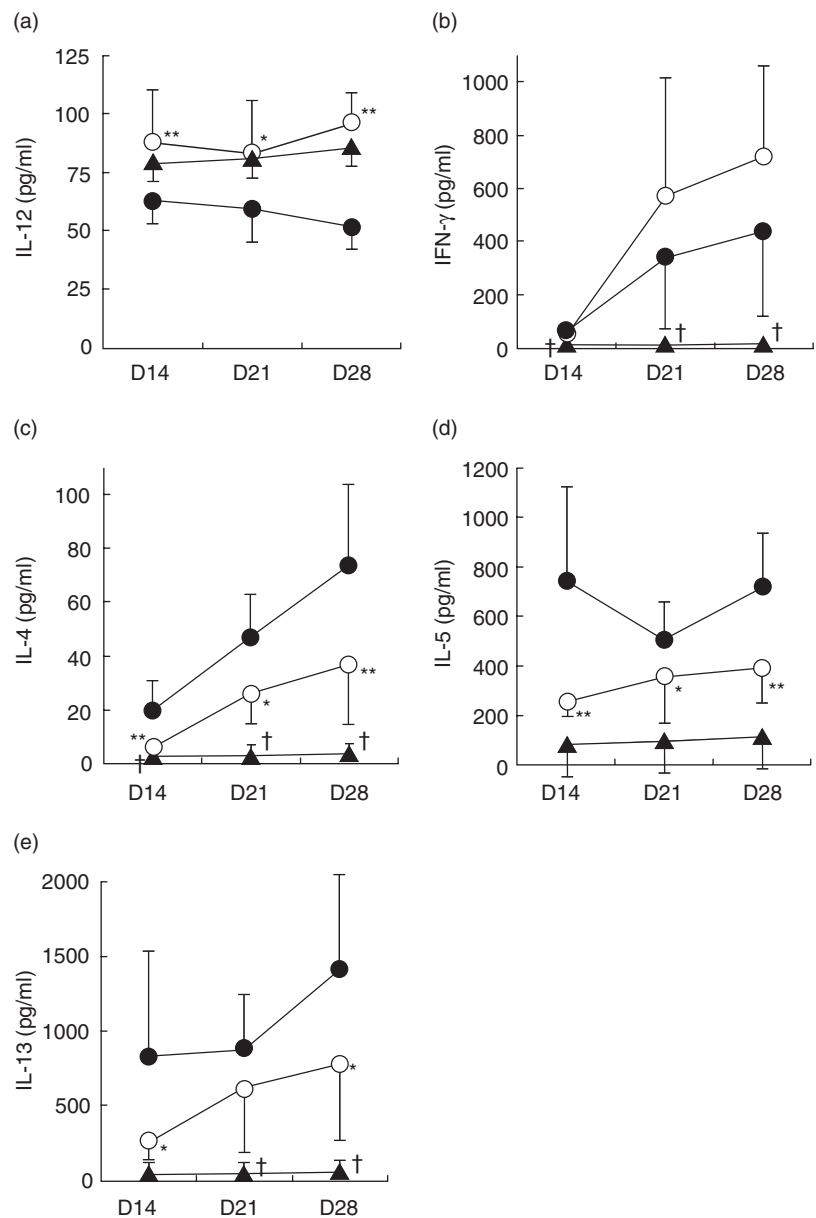


Fig. 3. Cytokine levels in supernatants of cultured splenocytes from mice sensitized with ovalbumin (OVA). Mice were immunized as described in Fig. 1. Splens were removed on days 14, 21 or 28 and single splenocytes were incubated with 100 µg/ml OVA for 72 h. Cytokine levels were determined using enzyme-linked immunosorbent assay. Data represent mean $\pm$ s. d. from eight mice per group. (●) Control group; (○) oligodeoxynucleotide (ODN) BL07S group; (▲) naive group. (a) Interleukin (IL)-12; (b) interferon (IFN)- γ ; (c) IL-4; (d) IL-5; (e) IL-13. * $P < 0.05$, ** $P < 0.01$ versus controls. †Below the limits of detection.

lower levels of Th2 cytokines compared to controls, suggesting that this ISS-ODN suppresses Th2 immune responses in an antigen-related IgE model. Our previous study [25] reported the effects of ISS-ODNs from genomic DNA of probiotics on the induction of Th1-type cytokine stimulation and inhibition of IgE production *in vitro*. These findings indicate that ODN BL07S might be beneficial in preventing IgE-related allergic diseases through suppression of the Th2 response. This is the first report to demonstrate the *in vivo* immunoregulatory abilities of ISS-ODNs from probiotics relating to the prevention of allergy.

Numerous reports have described ISS-ODNs containing mainly CpG ODNs that could reverse Th2-polarized immune responses *in vivo* [28,29]. In addition, ISS-ODNs conjugated with allergens have been expected for application as

vaccines against ragweed allergy in human trials [24]. However, these reported ODNs were designed artificially to provide strong immunostimulatory and immunomodulatory effects. CpG motifs comprising GACGTT and GTCGTT are known to represent the optimal motifs in class-A CpG ODNs for murine and human cells, respectively [30,31]. ODN BL07S represents one of the natural ODN sequences from probiotics found to be effective in regulating Th2 immune response *in vivo*. Recently, genomic sequence information for *B. longum* BB536 has been obtained (unpublished results). We identified more than 1000 sequences of mouse and human optimal motifs in the genomic sequences of *B. longum* BB536 (unpublished results). The present findings indicate that genomic sequences from probiotics might represent a great resource

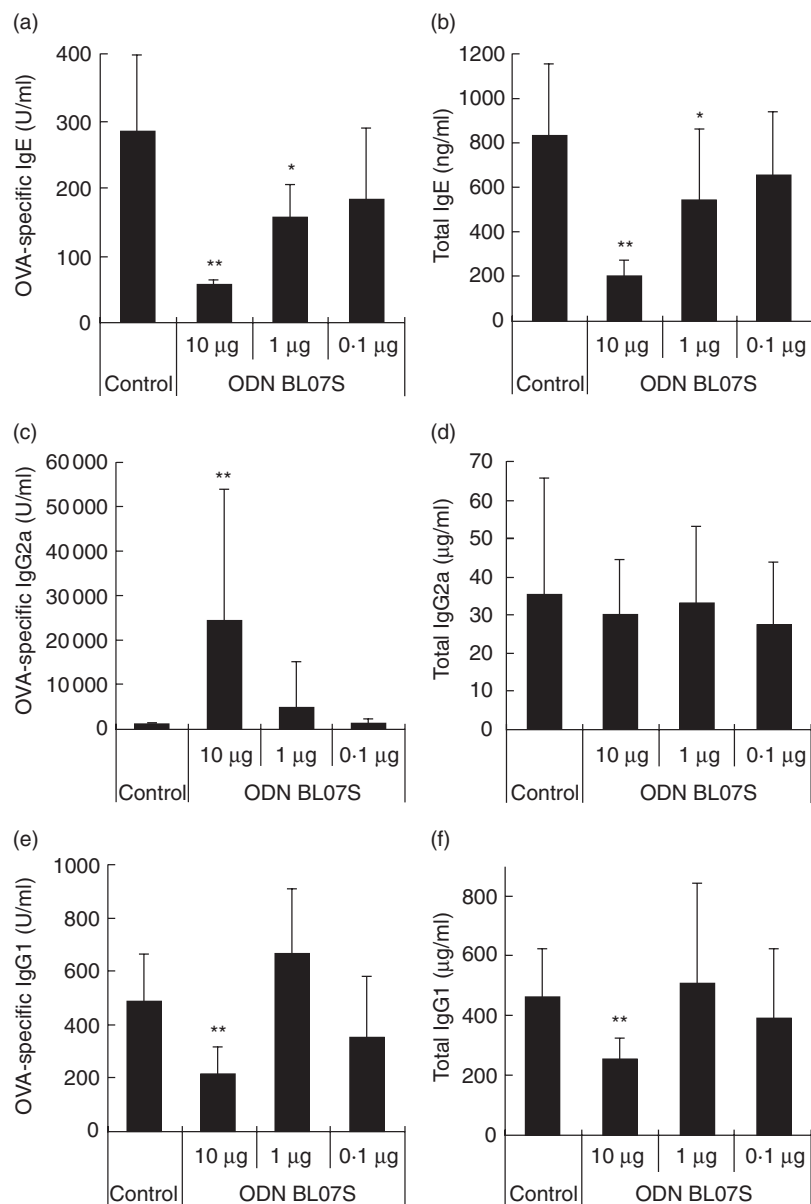


Fig. 4. Dose-response of oligodeoxynucleotide (ODN) BL07S on Ig levels in serum of mice. Mice were immunized with ovalbumin (OVA) (20 µg/mouse) and ODN BL07S (0.1 µg, 1 µg and 10 µg/mouse) in alum at a 2-week interval. Sera were collected on day 21. Ig levels in sera were determined using enzyme-linked immunosorbent assay. Results represent mean $\pm$ s.d. of serum from eight mice per group. (a) OVA-specific IgE; (b) total IgE; (c) OVA-specific IgG2a; (d) total IgG2a; (e) OVA-specific IgG1; (f) total IgG1. * P < 0.05, ** P < 0.01 versus controls.

in providing useful candidates for ISS-ODNs with anti-allergic effects from natural sequences.

Few studies have evaluated the time-course effect of ODNs in immunomodulation. In the present study, ODN BL07S was found to suppress serum total and OVA-specific IgE levels on both day 14 before second immunization and on day 28, and maximum inhibition was seen on day 21. Production of Th2 cytokines by splenocytes was inhibited from day 14. However, no differences were found in serum IgG1 and IgG2a levels or IFN- γ production by splenocytes on day 14. OVA-specific IgG2a was elevated and OVA-specific IgG1 was suppressed on day 21 by ODN BL07S, but IFN- γ production by splenocytes was not significantly altered compared to controls. These results indicate that suppression of Th2 immune response by ODN BL07S does not

require induction of IFN- γ , but induction of a Th1-skewed response may contribute to the maximum effect on day 21.

The regulatory mechanisms of Th2 response by ISS-ODNs remain unclear. In the present study, OVA-specific and total IgE production was suppressed by ODN BL07S at low doses (1 µg/mouse), but modulation of cytokine and IgG production was observed only at relatively high doses. Our *in vitro* studies indicate that ODN BL07S inhibited IgE production in splenocytes from mice sensitized with OVA, but did not need IFN- γ and IL-12 production [25]. Both *in vitro* and *in vivo* results indicate that some mechanisms, but not the Th1/Th2 balance mechanism, exist to suppress of Th2 immune response following ODN BL07S administration. Kline *et al.* [32] also reported that CpG ODNs did not require IL-12 and IFN- γ to suppress Th2 immune response

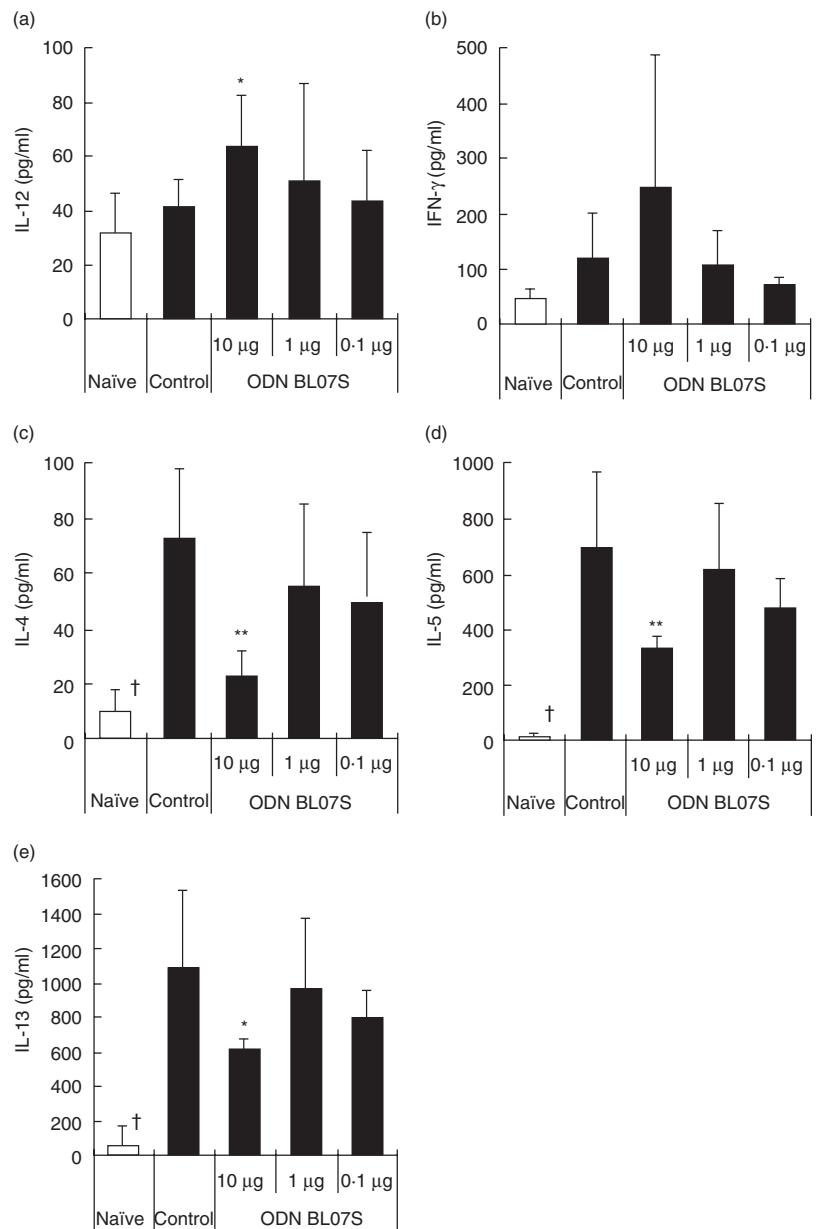
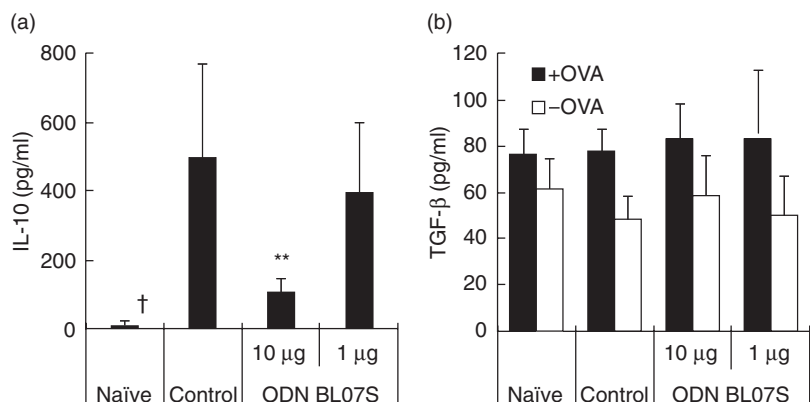


Fig. 5. Effects of oligodeoxynucleotide (ODN) BL07S dose on cytokine production of mice. Mice were immunized as described in Fig. 4. Spleens were removed on day 21 and single splenocytes were incubated with 100 μg/ml ovalbumin (OVA) for 72 h. Cytokine levels of supernatants were determined using enzyme-linked immunosorbent assay. Data represent mean $\pm$ s.d. from eight mice per group. (a) Interleukin (IL)-12; (b) interferon (IFN)- γ ; (c) IL-4; (d) IL-5; (e) IL-13. * $P < 0.05$, ** $P < 0.01$ versus controls. †Below the limits of detection.

Fig. 6. Interleukin (IL)-10 and transforming growth factor (TGF)- β levels in supernatants of cultured splenocytes. Mice were immunized with ovalbumin (OVA) (20 μg/mouse) and oligodeoxynucleotide (ODN) BL07S (1 μg or 10 μg/mouse) in alum at 2-week intervals. Spleens were removed on day 21 and single splenocytes were incubated with or without 100 μg/ml OVA. Cytokine levels of supernatants were determined using enzyme-linked immunosorbent assay. Levels of IL-10 in supernatants without OVA were below the limit of detection. Data represent mean $\pm$ s.d. of serum from six mice per group. ** $P < 0.01$ versus controls. †Below the limits of detection.



in vivo, suggesting that the Th1 response induced by CpG is not crucial in regulating Th2 responses. Recently, regulatory responses such as induction of IL-10 and TGF- β and expansion of regulatory T cells have been suggested as an alternate mechanism of suppressing Th2 responses [33]. It has been reported that human plasmacytoid dendritic cells (DCs) stimulated with CpG ODN induced CD4⁺CD25⁺ regulatory T cells [34]. The regulatory mechanism of Th2 responses by ODN BL07S was thus speculated to be associated with Th1-independent mechanisms. However, the present results indicate that ODN BL07S might not induce regulatory functions from T cells, as levels of IL-10 in splenocytes culture were lower than in controls and no changes in TGF- β level were observed. Moreover, expression of CD4⁺CD25⁺ T cells in splenocytes was almost the same between ODN and control groups. Liu *et al.* found that CpG ODN induced T-bet mRNA expression and inhibited IgE and IgG1 synthesis induced by IL-4 and anti-CD40 in purified B cells [35]. Similarly, we have reported previously that ODN BL07S inhibited IgE production in purified murine B cells [25]. However, T-bet expression is reportedly dispensable for inhibition of IgE and IgG1 production by CpG ODN [36]. The involvement of T-bet expression with IgE regulation *in vitro* remains controversial. Evaluating the CpG-mediated T cell regulatory system is extremely difficult, because TLR9 is not expressed on T cells. Further studies are required to reveal the regulatory mechanisms of Th2 response by ISS-ODNs in association with Th1-independent mechanisms, regulatory T cells or some other factors.

In conclusion, we demonstrated that an ISS-ODN from *B. longum* BB536 strain inhibits Th2 immune response *in vivo*, suggesting that ISS-ODNs derived from probiotic DNA could be novel candidates for immunopotentiators against allergic disease. Further studies on ISS-ODNs from probiotics seem likely to facilitate the development of therapeutic strategies to prevent allergic disorders.

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