

Immunostimulatory Oligodeoxynucleotide Containing TTTCGTTT Motif from *Lactobacillus rhamnosus* GG DNA Potentially Suppresses OVA-specific IgE Production in Mice

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Abstract

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In a previous study of the immunoregulatory properties of commensal bacterial DNA, we identified the strong immunostimulatory oligodeoxynucleotide (ISS-ODN) ID35 in the genomic DNA of *Lactobacillus rhamnosus* GG (LGG). The observed effects of ID35 are because of the unique TTTCGTTT motif located at the 5' end of the ODN, which is different from the previously identified ISS motifs in humans and mice. In the present study, we used an ovalbumin (OVA)-sensitized mouse model to show that ID35 is a potent suppressor of antigen-specific immunoglobulin E (IgE) production *in vivo*. This effect was toll-like receptor 9-dependent, as GpC negID35 failed to suppress antigen-specific IgE production. ID35 activated the specific subset of CD11c⁺CD8a⁺ dendritic cells, which are associated with T-helper 1 (Th1)-type systemic responses, and effectively induced interferon-gamma (IFN- γ) production by CD4⁺ T cells in OVA-challenged mice. These immunoregulatory effects of ID35 were comparable with those induced by the murine prototype ODN 1826. Thus, ID35 is the first ISS-ODN with such a strong immunostimulatory and IgE suppressor activity to be found in immunobiotic bacterial DNA.

Introduction

Recent findings regarding the aetiology of allergic diseases have unveiled putative new strategies for allergy prevention. These strategies include using whole bacteria or bacterial substances to restore the normal T-helper (Th)1/Th2 bias, which changes during the course of the disease [1–5].

Lactobacillus rhamnosus GG (LGG) is commonly used in fermented milks and drinks, and is one of the best-studied probiotic bacteria. Studies in children have shown that LGG effectively prevents early atopic disease in patients at high risk [1]. Data obtained from these randomized trials indicate that reduced development of atopy and allergy could result from a probiotic bacteria-mediated modulation of the immune response from Th2 to Th1. Thus, these bacteria have become a putative candidate in allergy therapy. However, the active molecules associated with the immunostimulatory (ISS) and anti-allergy effects of LGG have not yet been identified.

Unmethylated CpG motifs in bacterial DNA are mitogenic for mouse immune cells [6]. In addition, CpG containing ISS oligodeoxynucleotides (ODN) are potent Th1 adjuvants, effective in both preventing and reversing Th2-biased immune deviation in allergy models [2, 3, 7, 8]. Treatment with only a single systemic dose of ISS-ODN inhibits Th2 responses in airways for 4 weeks, despite the on-going allergen challenge [4]. Recent studies on the mechanism of ISS-ODN-inhibited airway hyperresponsiveness report that ISS-ODN prevents Th2 cytokine secretion by both Th2 cells as well as immunoglobulin E (IgE)-activated cells, such as mast cells and basophils [5].

We have shown that probiotic DNA as well as probiotic DNA-derived ISS-ODN sequences can restore Th1/Th2 bias [9, 10]. Recently, we demonstrated that LGG genomic DNA is a potent inducer of murine B cell and dendritic cell (DC) immunoactivation. In the genomic DNA from LGG, we discovered ID35, which shows high activity in ISS assays in both mice and humans [11]. Interestingly, in contrast to previously reported

optimal ISS CpG motifs in humans and mice, we found that the observed effects of ID35 are because of the unique TTTCGTTT motif located at its 5' end.

In the present study, using an ovalbumin (OVA)-sensitized mouse model, we show that LGG-derived ID35 can also be beneficial in allergy prevention. The effect of ID35 on switching the Th bias to a non-allergic Th1 response and on the suppression of OVA-specific IgE production was comparable with the effect induced by murine prototype CpG 1826. We propose that bacterial DNA containing ID35 may contribute to the anti-allergic potential of LGG by modulating the immune response from Th2 to Th1.

Materials and methods

Mice. Male BALB/C mice between 6 and 8 weeks of age were purchased from Japan SLC Inc. (Hamamatsu, Shizuoka, Japan). The present study *in vivo* was conducted in accordance with the Guidelines for Animal Experimentation of Tohoku University.

ODN. The ODN were chemically synthesized by Operon (Tokyo, Japan). They were confirmed to be free of detectable endotoxin by using a *Limulus* amebocyte lysate kit (Seikagaku Co., Tokyo, Japan) according to the manufacturer's manual.

Mouse treatment. OVA-sensitized mice were used as a model. Immunization and antigen challenge of the mice were performed as follows (Fig. 1C). The mice were sensitized by i.p. injection of a saline suspension containing OVA (20 µg per mouse; Sigma Chemical Co., St Louis, MO, USA) and aluminum hydroxide (3 mg per mouse; Sigma Chemical Co.) on days 0, 7 and 14. At days 16 and 18 after immunization, mice received i.p. injections of 200 µg of CpG, GpC or phosphate-buffered saline (PBS) alone (for the control group). On day 20 (i.e. 48 h after the last injection) blood samples were collected, mice were sacrificed and the spleens were harvested for further analysis.

Luciferase assay. We constructed the murine toll-like receptor 9 (TLR9) transfectant (CHO^{mTLR9}) by using a Chinese hamster cell line (CHO K-1) [12]. The stimulation of the transfectants and data analysis were performed as previously described [13–15].

Proliferation assays. Splenocytes were prepared from the mouse spleens according to our previous method [16]. The spleen cell suspensions (2×10^5 splenocytes per well) from naïve, OVA-challenged or OVA/CpG-challenged mice were re-stimulated with OVA (100 µg/ml), incubated at 37 °C for 48 h, and radiolabelled with 9.25 kBq per well of [methyl-³H] thymidine (Amersham Biosciences, Ltd, Bucks, UK).

Flow cytometry. Splenocytes (4×10^5 cells per well) were re-stimulated with OVA (100 µg/ml) for 24 h and stained with monoclonal antibodies against CD8a,

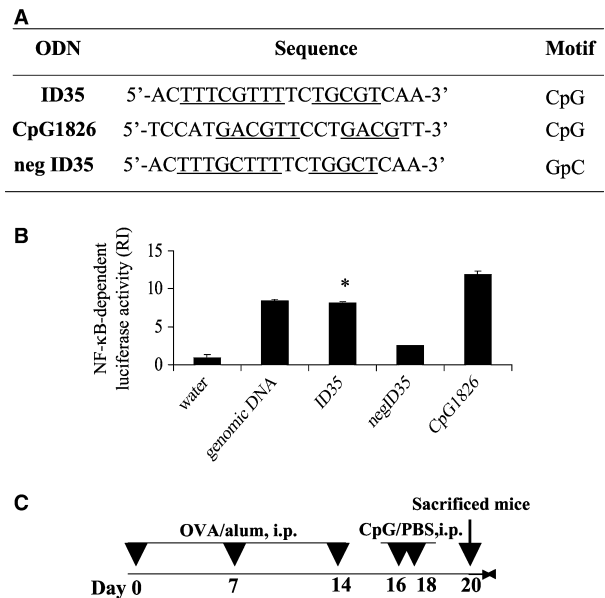


Figure 1 ID35 and genomic DNA from *Lactobacillus rhamnosus* GG (LGG) show similar stimulatory activity against a murine TLR9 transfectant. (A) Oligodeoxynucleotide (ODN) sequences. (B) CHO^{mTLR9} cells were stimulated with titrated amounts of CpG1826 to achieve relative stimulation. NF-κB-dependent luciferase activity was seen in transfected cells at 24 h after treatment with 1 µM ODN. Results are means ± SD of three or four independent experiments; **P* < 0.05 compared with identically treated cells cultured in medium only. (C) Immunization scheme. Mice were sensitized with three injections of ovalbumin (OVA)–alum suspension on days 0, 7 and 14, followed by i.p. injections of 200 µg of immunostimulatory (ISS) (ID35, 1826 or neg ID35) or phosphate-buffered saline on days 16–18.

CD11c and CD86 (PharMingen, San Diego, CA, USA). For intracellular interferon-gamma (IFN-γ) staining, the cells were stained with CD4, washed and then permeabilized with 0.5% saponin before adding PE-conjugated anti-IFN-γ monoclonal antibody (mAb; BD PharMingen) or control PE-conjugated rat IgG₁. Subsequently, the cells were analysed with a FACSCaliburTM (Becton Dickinson, Mountain View, CA, USA).

Enzyme-linked immunosorbent assay for total and antigen-specific IgE. OVA-specific IgE was evaluated as previously described [17]. Standard immune serum was obtained by s.c. immunization of mice with 20 µg of OVA adsorbed to aluminum hydroxide. Immunizations were repeated thrice at 2-week intervals. OVA-specific IgE titres were calculated by comparison with standard immune serum. Concentrations of OVA-specific IgE in the standard immune serum were arbitrarily considered as 1000 U/ml.

Real-time quantitative polymerase chain reaction (Q-PCR). RNA was prepared from splenocytes by solubilization in Trizol (Invitrogen Life Technologies, Carlsbad, CA, USA) and treatment with Turbo DNase (Ambion, Austin, TX, USA). Reverse transcription with oligo(dT) primers was performed with Superscript II (Invitrogen). Real-time Q-RT (reverse transcription)-PCR was performed on the

LightCycler-FastStart DNA Master SYBR Green I (Roche Diagnostics, Tokyo, Japan). To normalize the samples, the same amount of cDNA was used in the β -actin Q-PCR. The following primers were used: mouse β -actin, 5'-CGA TGC CCT GAG GCT CTT T-3' (sense) and 5'-TGG ATG CCA CAG GAT TCC A-3' (antisense); mouse IFN- γ , 5'-TTG GCT TTG CAG CTC TTC CT-3' (sense) and 5'-TGA CTG TGC CGT GGC AGT A-3' (antisense); mouse interleukin (IL-4), 5'-GGA GAT GGA TGT GCC AAA CG-3' (sense) and 5'-CGA GCT CAC TCT CTG TGG TGT T-3' (antisense); mouse IL-10, 5'-GAT GCC CCA GGC AGA GAA-3' (sense) and 5'-CAC CCA GGG AAT TCA AAT GC-3' (antisense).

Statistics. Statistical significance was established by using the Student's *t*-test. In the *in vivo* experiments the groups were analysed by one-way ANOVA. When the ANOVA demonstrated significant differences between data groups, it was followed by the Dunnett's test. A *P*-value <0.05 was considered significant. Statistic calculations were performed by JMP 5.1 software (SAS Cary, NC, USA).

Results

ID35 containing the TTTCGTTT motif stimulates murine TLR9 transfectant

In order to examine the affinity of ID35 to TLR9, we used the CHO K-1 cell line transfectant with murine TLR9 (CHO^{mTLR9}). ID35 at a concentration of 1 μ M signifi-

cantly increased nuclear factor kappa B (NF- κ B)-dependent luciferase activity of the stimulated transfectant. In contrast, negative control ID35 (negID35), which contained the GpC motif, was not recognized by the TLR9 transfectant, indicating that the cells specifically reacted with ID35 (Fig. 1B). The ISS capacity of ID35 to the TLR9 transfectant was comparable with that of genomic DNA from LGG. Moreover, ID35 has an ISS activity comparable with the murine prototype, CpG1826, which is known for its strong ISS activity.

ID35 activates subset of CD8a⁺ DC differentiation *in vivo*

In the present study, we examined whether or not ID35 can effectively induce specific CD11c⁺CD8a⁺ DC activation associated with Th1-type systemic responses. Splenocytes from OVA-sensitized mice treated with the ODN (Fig. 1C) were re-stimulated with OVA (100 μ g/ml), and CD86 expression on CD11c⁺CD8a⁺ DC versus CD11c⁺CD8a⁻ DC was measured. The expression of CD86 on CD11c⁺CD8a⁺ DC in the spleens of ID35-treated mice was much higher than in mice treated with negID35 or saline (Fig. 2A,B). The CD86 expression level on DC from ID35-treated mice was comparable with the level induced by CpG1826. Interestingly, CD11c⁺CD8a⁻ DC subset, was only slightly activated upon different ISS-ODN treatments as measured by CD86 expression (Fig. 2A,B). These data suggest that ID35 injection *in vivo* has the potential to activate CD11c⁺CD8a⁺ DC, which induce Th1 responses.

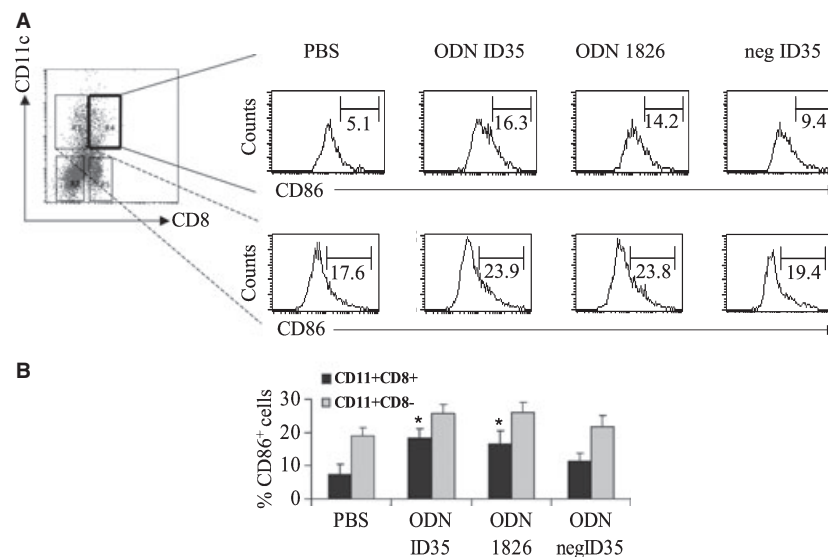


Figure 2 ID35 induces co-stimulatory molecule expression on CD8a⁺ dendritic cells (DC) from ovalbumin (OVA)-challenged mice. (A) The mice were immunized as described in Fig. 1C; spleen cell suspensions were stimulated with 100 μ g/ml of OVA for 24 h and were stained with antibodies against CD11c, CD8a and CD86. DC from OVA-challenged/ID35-treated mice expressed CD86 levels similar to OVA-challenged/1826-treated mice. Flow cytometric results are presented as histograms corresponding to cells in the CD11c⁺CD8a⁺ and CD11c⁺CD8a⁻ gates. (B) Graph depicts pooled data from two independent experiments performed as described in (A). Data are mean $\pm$ SD. **P* < 0.05, as compared with mice immunized only with OVA-challenged, phosphate-buffered saline. Three mice per group were used in these experiments.

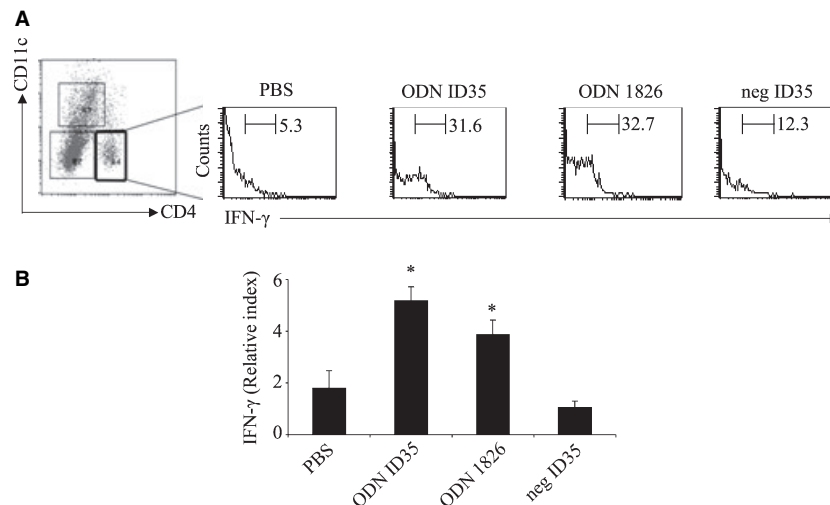


Figure 3 ID35 augments ovalbumin (OVA)-specific interferon-gamma (IFN- γ) production by CD4⁺ T cells in OVA-sensitized mice. The mice were immunized as described in Fig. 1C and splenocyte cultures were re-stimulated with OVA (100 μ g/ml) for 24 h. (A) IFN- γ production by CD4⁺ T cells was measured by intracellular IFN- γ staining. (B) By real-time polymerase chain reaction (PCR) quantification of IFN- γ expression in splenocytes derived from phosphate-buffered saline (PBS)-treated mice and CpG-immunized mice ($n = 3$ mice per group). Data are representative of two independent experiments and for real-time PCR analysis, statistical difference was calculated by ANOVA and Dunnett's test. * $P < 0.05$, for Dunnett's test with only OVA-treated, PBS-injected group of mice used as a control. Error bars $\pm$ SEM.

ID35 induces a strong Th1 response in OVA-challenged mice

To evaluate the ability of ID35 to induce Th1-biased immune responses *in vivo*, OVA-sensitized mice were injected on days 16 and 18 with 200 μ g of ID35, negID35, CpG1826 or saline. The spleens were harvested 48 h after the treatment; then, the splenocytes were re-stimulated with the antigen (OVA) and stained for CD4 and IFN- γ . CD4⁺ T cells from mice injected with ID35 produced high levels of IFN- γ (32.6% of CD4⁺ T cells) as compared with the mice treated with the negID35 (12.3% of CD4⁺ T cells) or with saline alone (5.3% of CD4⁺ T cells) (Fig. 3A). IFN- γ production by splenocytes was confirmed by transcriptional cytokine analysis (Fig. 3B). We additionally found that ID35-induced production of IFN- γ correlated with reduced IL-4 expression and enhanced IL-10 expression in the treated mice, as analysed by qPCR (Fig. 4). Thus, our results indicate that treatment of OVA-injected mice with ID35 can induce switch from allergy-associated Th2 to dominant Th1 response and this is comparable with the effect induced by CpG1826.

ID35 suppresses antigen-specific proliferation

In addition to the development of a CpG-induced Th1 response, we assumed that ODN might also suppress antigen-specific proliferation. To test this possibility, we collected spleens from OVA- or OVA/CpG-challenged mice and prepared splenocyte cultures. The splenocytes were then re-stimulated with OVA *in vitro*. Interestingly, splenocytes from the CpG-injected group proliferated

much less than splenocytes from the non-CpG-treated group (Fig. 5). As in the previous assays, the effect of ID35 was similar to that of CpG1826.

ID35 effectively suppresses IgE production

As ID35 effectively induced Th1 responses *in vivo*, we tested whether or not it can also dampen allergic inflammation by suppressing the production of OVA-specific IgE. OVA-challenged mice were treated with ID35, or saline, CpG1826, or negID35, and the serum levels of antigen-specific IgE (zFig. 6A) and total IgE (Fig. 6B) were measured. Interestingly, the ID35-treated group displayed significantly lower levels of total and antigen-specific IgE in the serum compared with the saline-treated group or negID35-treated group. Again, the effect of ID35 was comparable with that of CpG1826.

Discussion

CpG DNA has been successfully used in allergy prevention and as therapy for established disease in mice [4, 7, 8]. In the present study, we used probiotic bacteria-derived ID35 in allergy prevention studies. Our data clearly show that ID35 has sufficient potential to activate DC, elicit effective Th1 responses and suppress antigen-specific IgE production *in vivo*. This effect was CpG-dependent, as GpC ODN (negID35) was unable to elicit IFN- γ production by T cells and suppress antigen-specific IgE release in OVA-sensitized mice. Although the CpG motif is crucial for the activity, our results and results from other laboratories indicate that a small number of

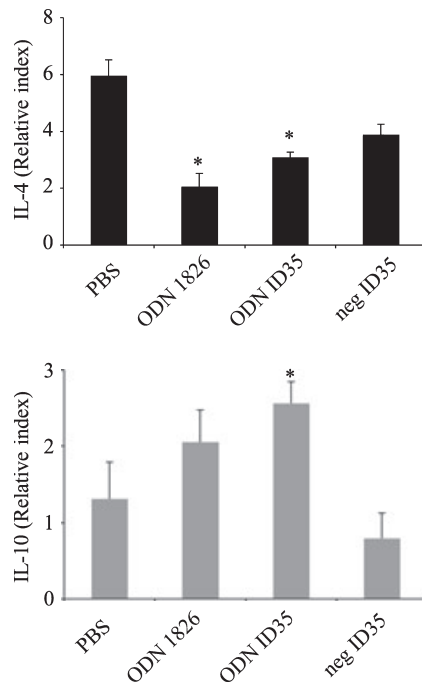


Figure 4 Decreased interleukin (IL)-4 expression and elevated IL-10 expression in ovalbumin (OVA)-sensitized mice treated with ID35. The mice were immunized as described in Fig. 1C and splenocyte cultures were re-stimulated with OVA (100 µg/ml) for 24 h. IL-4 and IL-10 gene expression in the splenocytes was analysed by real-time polymerase chain reaction (PCR). Relative index represents fold induction in cytokine gene expression in splenocytes from OVA ± immunostimulatory oligodeoxynucleotide (ISS-ODN)-treated mice over the cytokine expression in the splenocytes from naïve mice. The data are representative of two independent experiments. Statistics was determined by ANOVA and Dunnett's test. Error bars ± SEM; * $P < 0.05$, versus mice that were only OVA-challenged and phosphate-buffered saline-immunized.

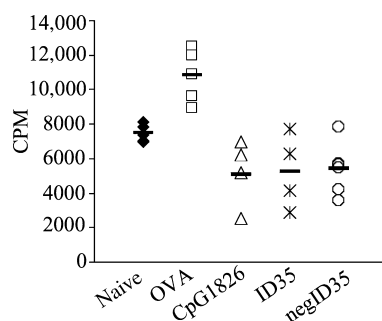


Figure 5 ID35 can effectively suppress ovalbumin (OVA)-specific proliferation. Splenocytes from phosphate-buffered saline (PBS)-treated or immunostimulatory (ISS)-treated mice were re-stimulated with OVA (100 µg/ml) and cultured in 96-well plates at 2×10^5 cells per well for 48 h. Sixteen hours before harvest, the cells were pulse-labelled with 9.25 kBq of [^{3} H]-thymidine. Cell proliferation was determined by measuring the incorporation of [^{3} H]-thymidine. Data are mean ± SE. * $P < 0.05$, as compared mice that were only OVA-challenged and PBS-immunized. Five mice per group were used in this experiment.

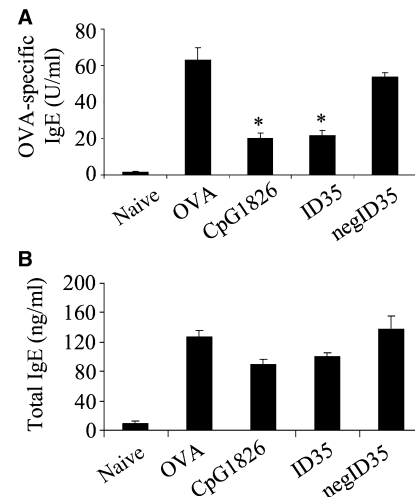


Figure 6 Decreased production of ovalbumin (OVA)-specific immunoglobulin E (IgE) and total IgE *in vivo* after ID35 treatment. The mice were immunized as described in Fig. 2C. The serum for detection of (A) total IgE and (B) OVA-specific IgE was collected on day 20. GpC ID35 (negID35) was used as a negative control. IgE levels were detected by enzyme-linked immunosorbent assay (ELISA). Each group included five mice. Statistics were determined by ANOVA and Dunnett's test. Error bars ± SEM; * $P < 0.05$, versus mice that were only OVA-challenged and phosphate-buffered saline-immunized.

the predicted ISS motifs/ODN inside the bacterial DNA elicit strong activity in the immune cells [9, 10, 18]. We have shown that the CpG-flanking sequences and the secondary structure [11, 16], together with the CpG content, are indispensable for obtaining strong ISS activity.

With respect to their ability to polarize the Th1/Th2 bias, two different subsets of DC have been described in mice. The myeloid type ($CD11c^+CD8a^-$) activates primarily Th2 T cells, whereas $CD11c^+CD8a^+$ DC activate Th1-type responses [19]. It has been recently shown *in vivo* in mice that, $CD11c^+CD8a^+$, but not $CD11c^+CD8a^-$, DC are the major cell types responsible for CpG-induced IL-12 production [20]. In the present study we showed that ID35 was able to induce activation of $CD11c^+CD8a^+$ DC (assessed by CD86 expression), but not of $CD11c^+CD8a^-$ DC. Enhanced expression of CD86 on $CD11c^+CD8a^+$ DC after ID35 treatment correlated with elevated IFN- γ production and decreased IL-4. Thus, altogether these data suggest that ID35 may specifically activate $CD8a^+$ DC subset which in turn triggers substantial Th1 response and concomitantly suppresses Th2-type cytokine production. It is likely that the enhanced IFN- γ levels after ID35 treatment would correlate with elevated IgG2a production by plasma cells which additionally might contribute to the effect exerted by CpG. However, we could not demonstrate such a correlation because of limitations in the sample volumes needed to perform these assays.

Interestingly, splenocytes from ISS-ODN/OVA-challenged mice proliferated less after *in vitro* re-stimulation

compared with splenocytes isolated from OVA-challenged mice alone, suggesting that ISS-ODN injection, together with induction of Th1 response, might trigger a mechanism which leads to suppression of antigen-specific lymphocyte clone expansion. It has been recently shown that ISS-ODN can activate plasmacytoid DC which induce differentiation of T regulatory cells (T reg) [21]. Thus, together with induction of predominant Th1 response, ID35 might activate subsets of DC able to induce T reg differentiation. In support of this notion, we found enhanced IL-10 expression in spleens of mice injected with ID35. However, additional experiments should be performed to elucidate if in our system ID35 might induce T reg.

In vivo, ID35 imparted activities similar to CpG1826, which has strong ISS activity in mice. Both ID35 and CpG1826 were equally potent in their ability to suppress allergy *in vivo*. As GACGTT has been reported as an optimal CpG motif in the mouse system, CpG1826 and CpG1668 have been accepted as a panacea for optimal ISS-ODN and widely used in mouse trials [22, 23]. Newly discovered ID35, with a core sequence of TTTCGTTT, which differs from the widely accepted optimal murine motif GACGTT, is an ODN with properties very similar to those of murine CpG1826. Although ID35 activates murine B cells and DC and strongly induces Th1-type responses, it does not include a palindrome, and only one CpG core motif is essential for its activity. TLR9 plays a critical role in the initiation of cellular activation by CpG DNA [24]. Accordingly, we showed here that ID35 exerts its ISS effect through TLR9. Despite their distinct structures, ID35 and CpG1826 suppress antigen-specific IgE production *in vivo* with equal potency. This observation, together with our previous findings [11], classify ID35 as the most potent CpG ODN to be found in probiotic DNA.

LGG has been successfully used to prevent early atopic disease in children [1]. This preventative effect has been attributed to the ability of the probiotic bacteria to modulate immune responses from Th2 to Th1. Bacterial factors, such as bacterial cell wall components and soluble factors from LGG, are involved in its immunomodulatory ability [25–27]. Our study extends these findings, as we propose that LGG DNA, consisting of ID35 and other CpG-active sequences, could be involved in the anti-allergic properties of this probiotic bacterium.

Atopic asthma and other allergic diseases are a serious health problem in industrialized societies, and no promising therapy is currently available. Pre-industrialized areas and rural populations appear relatively protected from allergic diseases. The hygiene hypothesis ascribes this protection to the effects of microbes and microbial products [28]. Building on this concept, substantial research efforts are concentrated on probiotics, which so far are the only food-grade bacteria shown to promote 'health-

stimulatory' properties [29]. Thus, LGG, which has been proven to be beneficial in the amelioration of gastrointestinal inflammation in mice and humans [29, 30], is also a good candidate for future anti-allergy therapy. Additionally, our finding of ODN with anti-allergy and strong ISS properties in the DNA from LGG indicates that probiotic DNA might be a crucial factor in the beneficial effects of LGG.

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