



CpG ODN mediated prevention from ovalbumin-induced anaphylaxis in mouse through B cell pathway

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Abstract

Allergic inflammation is induced by type 2 T helper cell (Th2) and Th2 cytokines such as interleukin (IL)-4, IL-5 and IL-13. These cytokines induce the production of allergen-specific immunoglobulin (Ig)E by B cells, and the ensuing degranulation of mast cells via IgE cross-linking leads to most clinical manifestations of allergic diseases. We examined the ability of immunomodulatory unmethylated CpG oligodeoxynucleotides (ODN), which are potent inducers of Th1 cytokines, to prevent allergic symptoms in mice immunized and sensitized with allergen. Coadministration of CpG ODN with ovalbumin (OVA) before OVA sensitization substantially prevented mice from allergic anaphylaxis representing enhanced circulating concentrations of OVA-specific IgE and histamine, and decreased body temperature. Although CpG ODN provokes an abundance of Th1-skewing cytokines, including IL-12, interferon (IFN)- α and IFN- γ , administration of CpG ODN in IFN- γ deficient mice inhibited IgE production and prevented from OVA-induced anaphylaxis, indicating a dispensable role of IFN- γ in mediating these protective effects. *In vitro* analysis revealed that CpG ODN inhibited class switching from IgM to IgE and IgG1 in response to CD40 and IL-4 in B cells, and this effect did not correlate with up-regulation of IFN- α production. These results imply a B cell-intrinsic, T cell-independent mechanism by which CpG ODN directly acts on B cells and inhibits IgE and IgG1 production leading to cause prevention from allergic symptoms.

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1. Introduction

Although the prevalence of allergic diseases has steadily increased over the last several decades, thus far no effective prevention measure exists. So it is very important to develop novel therapeutic approaches and effective prevention measures.

One potential explanation for the prevalence of allergic diseases is that with a declining incidence of many infectious diseases due to vaccination, the use of antibiotics, and other public health practices, a major source of type 1 T helper cell (Th1) immune provocation has been lost, with a consequent increase in the Th2 bias of immune responses toward environmental allergens [1]. This theory has been referred to as the "hygiene hypothesis". While still unproved, the "hygiene hypothesis" offers an attractive explanation for current trends in the prevalence of allergic diseases and offers a provocative rationale for consideration of DNA-based therapeutics for treatment of allergic diseases. For more than a century it has been known that bacterial extracts have immunostimulatory effects that can be used to fight disease. Important work over the past 10 years by a number of investigators have proved that many of these effects are mediated by unmethylated CpG dinucleotides in the DNA of bacteria [2]. Since the immunological activities of immunostimulatory DNA-based therapeutics are dependent on their content of specific immunostimulatory DNA sequences (CpG motifs) which are abundant in many microbial genomes but are rare in mammalian DNA [2,3], immunostimulatory DNA-based therapeutics are likely to elicit immunity, in part, by mimicry of natural infectious exposures. If the sterilization of the environment we live in proves to be a contributing factor in the rising prevalence of allergic diseases, as the hygiene hypothesis proposes, then immunostimulatory DNA-based therapeutics represent novel and rational approaches for their treatment.

Cellular recognition of CpG-containing DNA predominantly requires the Toll-like receptor 9 (TLR9) [2], which stimulates a myeloid differentiation marker 88 (MyD88)-dependent signaling pathway [2].

It has been accepted that allergic diseases are mediated by the over-production of Th2 type cytokines (particularly, interleukin (IL)-4 and IL-5) [4,5]. Cytokines that support Th1 cell responses (such as interferon (IFN)- γ and IL-12) dampen the diseases process [6,7]. Therefore, one aim of treating allergic diseases with immunomodulators would be to try to change the Th-cell balance in allergic individuals: that is, from the dominant Th2 type to a Th1 type response. CpG oligodeoxynucleotides (ODN) (synthetic ODN that contain non-methylated CpG motifs) have this effect, eliciting the production of IFN- γ and IL-12 while reducing the production of IL-4 [8,9]. It has been reported that administration of CpG ODN prevents the symptoms of asthma and allergic conjunctivitis [9–13]. However, it is still largely unknown about the mechanism which underlies CpG ODN mediated protective effects on allergic diseases. So far it is widely believed that the application of CpG ODN induces the production of IFN- γ and IL-12, thereby, leading to the development of antigen-specific Th1 cells and interfering with the development of allergen-specific Th2 cells. The generation of allergen-specific Th1 cells is also believed to be an important prerequisite of CpG ODN mediated inhibition of allergic responses. But, as T cells do not express Toll-like receptor 9 (TLR9), it is unclear how CpG ODN achieves its regulatory effects on T helper cells.

The present study was conducted to investigate the mechanism of CpG ODN mediated protective effect on allergic diseases by using a mouse anaphylaxis model. On the contrary to the widely believed notion, our results show that

CpG ODN mediated prevention from ovalbumin (OVA)-induced anaphylaxis through the inhibition of immunoglobulin (Ig)E and IgG1 production of B cells, rather than convert Th2 immune response to Th1 response.

2. Materials and methods

2.1. Mice

Wild type C57BL/6 mice were purchased from Japan SLC Inc. (Hama-matsu, Japan). C57BL/6 background IFN- γ deficient (IFN- $\gamma^{-/-}$) mice [14] and myeloid differentiation factor (MyD) 88 deficient (MyD88 $^{-/-}$) mice [15] were kindly provided by Dr. Y. Iwakura (Institute of Medical Science, University of Tokyo, Tokyo, Japan) and Dr. S. Akira (Institute of Microbial Diseases, Osaka University, Osaka, Japan) respectively. All mice were kept under specific pathogen-free conditions throughout, and all experiments were approved by and performed in compliance with the guidelines of the Institute of Medical Science, University of Tokyo, and only female mice (8–13 weeks) were used.

2.2. Materials

OVA (grade V) and alum were purchased from Sigma (St. Louis, MO), *Bordetella pertussis* was obtained from Nakalai Tesque (Kyoto, Japan). Endotoxin-free (<1 ng/mg) phosphorothioate CpG ODN (CpG-B-ODN) and non-CpG ODN (ODN which contain GpC motif) were purchased from Biognostik (Gottingen, Germany). CpG-A-ODN was obtained from Hokkaido System Science Co., LTD (Sapporo, Japan). The CpG-B-ODN (1826) used in these studies has the sequence 5'-T**C**C*A*T*G*A*C*G*T*T*C**C**T*G*A*C*G*T**T**3'. The non-CpG ODN (1745) has the sequence 5'-T**C**C*A*A*T*G*A*G*C*T*T*C**C**T*G*A*G*T**C**T*3'. The CpG-A-ODN (D19) has the sequence 5'-G*G*TGCATC-GATGCAG*G*G*G*G*G-3' (*=phosphorothioated). Except indicate specifically, CpG ODN used in these series of experiments is referred to CpG-B-ODN (1826).

2.3. Induction of anaphylaxis

Mice were sensitized i.p. with 300 μ g of OVA plus 2×10^9 *B. pertussis* and 1.0 mg of alum and were challenged i.v. with 100 μ g of OVA 14 days later. After the challenge injections were given, the mice were observed for 2 h and signs of anaphylaxis shock were recorded. Rectal temperature was measured with a Digital Thermometer (Shibaura Electronics CO.LTD., Osaka, Japan) just before challenge and every 10 min for 2 h after the OVA challenge. At the same time, mouse clinical symptom was assessed and quantified on a 5-point scale: 0, no signs of shock; 1, mild shock including itching, ruffling of fur, dyspnea, and decrease in spontaneous activity; 2, moderate shock including prostration, sluggish gait, and slightly activity after prodding; 3, severe shock including complete paresis, no activity following prodding with or without convulsion; 4, death within 30 min.

2.4. Measurement of serum OVA-specific IgE and IgG2a

Serum OVA-specific IgE and IgG2a were measured by enzyme-linked immunosorbent assay (ELISA). Plates were coated for 6 h at 37 °C with 100 μ l 0.1 M NaHCO₃ containing 100 μ g OVA per milliliter. The plates were blocked for 2 h at 37 °C with 200 μ l 3% bovine serum albumin in phosphate buffered saline. Plates were washed, and 100 μ l of diluted serum were applied and kept overnight at 4 °C. The amount of bound antibody (Ab) was analyzed using biotinylated anti-mouse IgE, biotinylated anti-mouse IgG2a and avidin-horseradish peroxidase conjugate (BD Biosciences PharMingen, San Diego, CA). Plates were read in a microplate autoreader (Wako Pure Chemical Industries, Ltd, Osaka, Japan) at 450 nm. Serum titers for all Abs

were expressed as relative ELISA units (U), referring to a laboratory standard sera pool.

2.5. Measurement of plasma histamine

Two minutes after the OVA challenge, mice were bled, and plasma was collected into EDTA containing tubes on ice for histamine analysis using a commercial ELISA kit according to the manufacturer's recommendations (Immunotech, Westbrook, ME). Mice used to analyze histamine release were not used in the assessment of clinical signs.

2.6. Prophylactic treatment protocol

Wild type C57BL/6 mice were untreated or received subcutaneous injections with 20 µg of OVA alone, or 20 µg OVA+CpG ODN (1826) (100 µg), or 20 µg OVA+non-CpG ODN (1745) (100 µg), or CpG ODN (1826) (100 µg) alone in 200 µl of normal saline solution in the base of the tail on 2 occasions 7 days apart before anaphylaxis induction. Seven days after the last prophylactic treatment, anaphylaxis was induced by sensitization and challenged as described above.

C57BL/6 background MyD88^{-/-} and IFN-γ^{-/-} mice were untreated or treated with 20 µg OVA+CpG ODN (1826) (100 µg), following the protocol used in wild type mice.

2.7. Measurement of splenocyte cell culture cytokines

Sensitized with or without prophylactic pretreatment mice spleens were harvested, teased to prepare single cell suspensions, and resuspended in RPMI-1640 medium (Gibco BRL, Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Sigma-Aldrich, Co., St. Louis, MO), 2 mM glutamine, 50 µM of 2-mercaptoethanol, and 50 IU/ml penicillin and 50 µg/ml streptomycin (complete media). Splenocytes were incubated at 5×10⁵ cells per well in 96-well plates in a final volume of 200 µl of complete media with 100 µg/ml OVA with or without CpG ODN (100 µg/ml) or non-CpG ODN (100 µg/ml) at 37 °C in a 5% CO₂ atmosphere. Culture supernatants were harvested at 72 h and analyzed by using ELISA. Samples were tested for the presence of IFN-γ and IL-4. All monoclonal antibodies (mAb) specific for mouse IFN-γ and IL-4 used for capture and detection were purchased from BD Biosciences Pharmingen. ELISA was performed following the instruction of BD Biosciences Pharmingen.

In some experiments, naive mice spleen cells were stimulated with CpG-B-ODN (1826) (100 µg/ml) or CpG-A-ODN (D19) (100 µg/ml). After 48 h, culture supernatants were collected and measured for the presence of IL-12p40 and IFN-α. All mAbs specific for mouse IL-12p40 used for capture and detection were purchased from BD Biosciences Pharmingen. IFN-α was measured by using a commercial available mouse IFN-α ELISA kit (PBL Biomedical Laboratories, Piscataway, NJ).

2.8. Culture of purified B cells for isotype switching study

Spleen cells were prepared from 7- to 10-week old mice, and B cells were purified by the magnetic depletion of cells of other types with CD43-specific microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) as described [16]. Purified B cells contained >97% B220⁺CD19⁺ double positive cells as assessed by fluorescence analysis using a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA).

Purified B cells (1×10⁵ cells per 200 µl per well) were cultured in a 96-well plate in RPMI 1640 medium supplemented with 8% heat-inactivated FCS, penicillin (50 U/ml), and streptomycin (50 µg/ml). Purified B cells were stimulated with monoclonal anti-mouse CD40 (2 µg/ml) (R&D Systems, Minneapolis, MN) and recombinant murine IL-4 (25 ng/ml) (PeproTech EC, London, UK) with or without CpG ODN

(CpG-B-ODN) (1826) (50 µg/ml). In some experiments, CpG-A-ODN (D19) (50 µg/ml) was used. Cells were collected at day 4 of the culture to analyze surface Igs expression of B cells by using a FACSCalibur flow cytometer. Supernatants were harvest at day 8 of the culture to measure Ig production from B cells by using ELISA according to procedures previously described [17]. Living cell number was counted at the time of culture set up and at day 4 of the culture by trypan blue dye. The viability of cultured B cells was also confirmed by propidium iodide (PI) staining and fluorocytometric analysis.

2.9. Fluorocytometric analysis

The expression levels of IgE, IgG1, IgG2a and IgM on the surface of stimulated B cells were determined by FACS staining using the following mAbs: fluorescein isothiocyanate (FITC)-anti mouse IgE, FITC-anti mouse IgG1, FITC-anti mouse IgG2a and FITC-anti mouse IgM. All of these antibodies were purchased from BD Biosciences Pharmingen. Fluorescence intensity was measured by using a FACSCalibur flow cytometer.

2.10. Statistical analysis

Differences between groups were evaluated by student's *t* test. Statistical analysis was performed on the Excel program for Macintosh.

3. Results

3.1. CpG ODN mediated prevention from OVA-induced anaphylaxis

The efficacy of CpG ODN+allergen (OVA) prophylactic treatment in protecting against the development of anaphylaxis was evaluated.

Because allergic reactions are characterized by an increase in allergen-specific serum IgE levels, the levels of serum OVA-specific IgE after OVA sensitization in CpG ODN+OVA treated group and control groups were examined. As shown in Fig. 1a, the anti-OVA IgE levels of mice treated with CpG ODN+OVA were significantly lower than those of mice that remained untreated, as well as mice treated with OVA, or non-CpG ODN+OVA. Pretreatment of mice with CpG ODN alone did not have a significant effect on serum anti-OVA IgE levels.

Mice receiving OVA sensitization without prophylactic treatment or pretreated with OVA alone, or non-CpG ODN+OVA showed physical signs of anaphylaxis after the OVA challenge. The severity of anaphylaxis was scored as 3–4. In contrast, considerably less severity (clinical score 1–2) was seen after challenge in mice pretreated with CpG ODN+OVA. The pretreatment with CpG ODN alone did not have a significant effect on clinical scores (Fig. 1b).

Because decreased core body temperature is inversely correlated with the severity of systemic anaphylaxis in mice [18], this parameter was also used to objectively measure the consequence of anaphylaxis. Mean rectal temperatures in the CpG ODN+OVA treated mice were significantly higher than those in the untreated group. The pretreatment with CpG ODN alone did not have a significant effect on ameliorating body temperature. Body temperature of mice pretreated with OVA and non-CpG ODN+OVA was not obviously different from that of mice in the untreated group (Fig. 1c).

Mast cell degranulation and histamine release are major mechanisms underlying anaphylactic reactions. So we measured

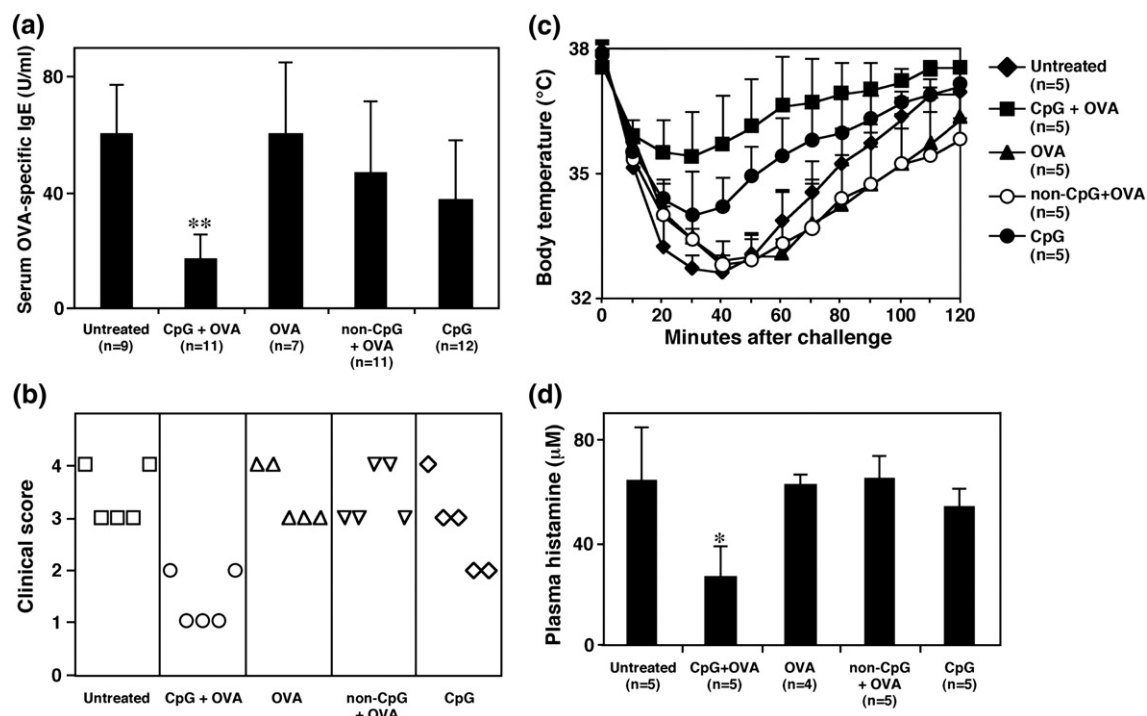


Figure 1 CpG ODN + OVA treatment obviously ameliorated anaphylaxis. (a) CpG ODN + OVA treatment inhibited serum OVA-specific IgE. Two days before challenge, serum was obtained for antibody determination by ELISA. The value represents the mean and SD in each group. ** $P < 0.01$ vs. all other groups by Student's t Test. (b) Protection against OVA-induced anaphylactic reactions by CpG ODN + OVA pretreatment. Anaphylactic symptoms were observed for 2 h following the challenge. (c) CpG ODN + OVA treatment ameliorated anaphylaxis shock. Means and SD of rectal temperatures are shown for 2 h following the challenge. (d) CpG ODN + OVA treatment reduced plasma histamine levels. Two min after intravenous OVA challenge, plasma was collected for histamine determination. Results represent mean values and SD in each group. * $P < 0.05$ vs. all other groups by Student's t Test. The number of mice used under each condition is indicated as ($n=x$). This is a representative of three experiments with similar results.

plasma histamine levels in sensitized and challenged mice 2 min after intravenous OVA challenge. Plasma histamine levels were markedly elevated in mice receiving sensitization and challenge without prophylactic treatment (untreated group). Plasma histamine levels in the CpG ODN + OVA treated group were significantly lower than those in the untreated group. The pretreat-

ment of CpG ODN alone did not have a significant effect on decreasing of plasma histamine levels. The pretreatment of OVA or non-CpG ODN + OVA did not reduce histamine levels (Fig. 1d).

To evaluate the importance of CpG ODN mediated signals in preventive effect of CpG ODN + OVA pretreatment on anaphylaxis, we further analyzed using $MyD88^{-/-}$ mice. As shown in

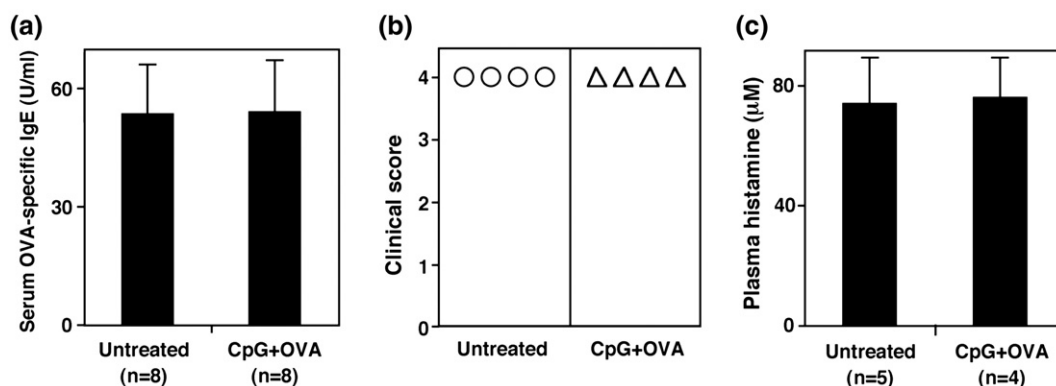


Figure 2 CpG ODN + OVA treatment did not ameliorate anaphylaxis in $MyD88^{-/-}$ mice. $MyD88^{-/-}$ mice were untreated or pretreated, sensitized, and challenged the same as wild type mice which was described in the Materials and methods section. (a) CpG ODN + OVA treatment did not inhibit serum OVA-specific IgE in $MyD88^{-/-}$ mice. The value represents the mean and SD in each group. (b) CpG ODN + OVA treatment did not decrease clinical symptoms in $MyD88^{-/-}$ mice. (c) CpG ODN + OVA treatment did not reduce plasma histamine levels in $MyD88^{-/-}$ mice. Results represent mean values and SD in each group. This is a representative of two experiments with similar results.

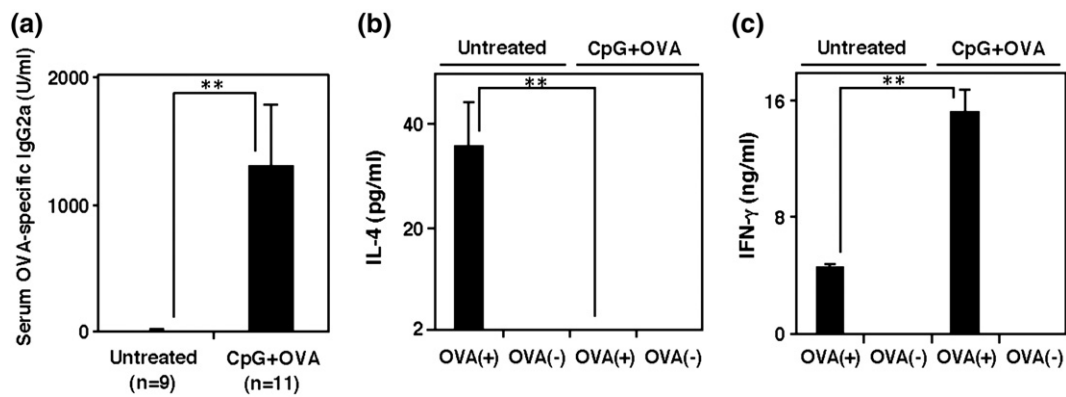


Figure 3 CpG ODN+OVA induced OVA-specific Th1 immune responses. (a) CpG ODN+OVA treatment significantly enhanced serum OVA-specific IgG2a. Mice were untreated or pretreated, sensitized, and challenged as described in the Materials and methods section. Two days before challenge, serum was obtained for antibody determination. The number of mice used under each condition is indicated as (n=x). The value represents the mean and SD in each group. ** $P < 0.01$ by Student's t Test. (b, c) CpG ODN+OVA treatment significantly inhibited IL-4 and greatly enhanced IFN- γ production *in vitro*. Mice were untreated or pretreated, sensitized, and challenged as described in the Materials and methods section. Seven days after sensitization, splenocytes were harvested and cultured with or without OVA. Supernatants were harvested at 72 h for cytokine determination. Results represent mean values and SD in each group. The minimum detectable dose of mouse IL-4 is 2 pg/ml according to the manufacturer's description. ** $P < 0.01$ by Student's t Test. One representative experiment out of three is shown.

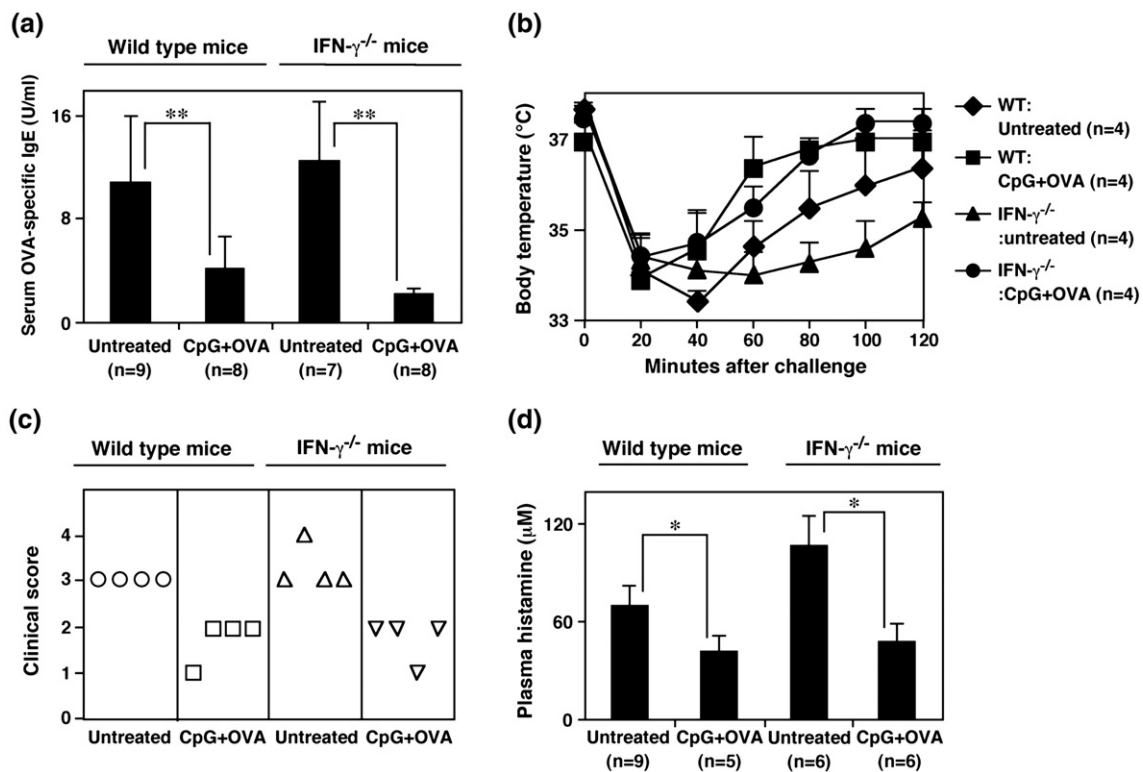


Figure 4 CpG ODN+OVA treatment ameliorated anaphylaxis in IFN- $\gamma^{-/-}$ mice as it did in wild type mice. Wild type and IFN- $\gamma^{-/-}$ mice were untreated or pretreated, sensitized, and challenged as described in the Materials and methods section. (a) CpG ODN+OVA treatment inhibited serum OVA-specific IgE both in wild type and IFN- $\gamma^{-/-}$ mice. The value represents the mean and SD in each group. ** $P < 0.01$ by Student's t Test. (b) CpG ODN+OVA treatment ameliorated anaphylaxis shock both in wild type and IFN- $\gamma^{-/-}$ mice. Means and SD of rectal temperatures are shown for 2 h following the challenge. (c) CpG ODN+OVA treatment decreased anaphylaxis symptoms both in wild type and IFN- $\gamma^{-/-}$ mice. (d) CpG ODN+OVA treatment inhibited plasma histamine levels both in wild type and IFN- $\gamma^{-/-}$ mice. The number of mice used under each condition is indicated as (n=x). Results represent mean values and SD in each group. * $P < 0.05$ by Student's t Test. This is a representative of three experiments with similar results.

Fig. 2, pretreatment of MyD88^{-/-} mice with CpG ODN+OVA before OVA sensitization neither inhibited serum OVA-specific IgE levels (Fig. 2a), nor decreased clinical score (Fig. 2b), nor reduced plasma histamine levels (Fig. 2c). In summary, CpG ODN mediated signaling through MyD88 is essential for preventive effect of CpG ODN+OVA pretreatment on anaphylaxis.

3.2. Investigation of mechanism of CpG ODN mediated prevention from OVA-induced anaphylaxis

3.2.1. CpG ODN induced Th1 immune responses

Because anaphylaxis is associated with Th2 biased immunity, we examined whether CpG ODN+OVA pretreatment prevented the development of Th2 biased immunity after OVA sensitization regarding OVA-specific IgE antibody production and splenic cytokine profiles. Serum OVA-specific IgE levels in the group of mice pretreated with CpG ODN+OVA were significantly lower than those in the untreated group of mice (Fig. 1a). Mice pretreated with CpG ODN+OVA produced markedly higher levels of OVA-specific IgG2a than the untreated control group of mice (Fig. 3a). Compared to spleen cells from mice received only OVA sensitization without prophylactic pretreatment, spleen cells from mice pretreated with CpG ODN+OVA followed by OVA sensitization produced lower IL-4 and higher IFN- γ upon OVA stimulation *in vitro* (Fig. 3b and c).

3.2.2. CpG ODN mediated prevention from OVA-induced anaphylaxis in IFN- γ ^{-/-} mice

To examine whether the protection conferred by CpG ODN+OVA pretreatment on anaphylaxis is dependent on Th1 cytokines, effects of IFN- γ on the CpG ODN+OVA treatment was examined by using IFN- γ ^{-/-} mice. As shown in Fig. 4, pretreatment of IFN- γ ^{-/-} mice with CpG ODN+OVA before OVA sensitization significantly inhibited OVA-specific IgE production (Fig. 4a), and

decreased anaphylaxis clinical symptoms at similar extent to what were observed in wild type mice (Fig. 4b and c). Furthermore, CpG ODN+OVA pretreatment also reduced plasma histamine levels in IFN- γ ^{-/-} mice as it did in wild type mice (Fig. 4d). These results indicate that effects of CpG ODN+OVA pretreatment on prevention from OVA-induced anaphylaxis appear to be IFN- γ ^{-/-} independent.

3.2.3. CpG ODN and Ig class switching of B cells

As *in vivo*, pretreatment with CpG ODN+OVA significantly inhibited serum OVA-specific IgE in OVA-sensitized mice, we examined whether CpG ODN could influence isotype switching of B cells to IgE. Compared to B cell culture stimulated with IL-4 and anti-CD40 mAb *in vitro*, addition of CpG ODN to the culture stimulated with IL-4 and anti-CD40 mAb potentially inhibited the IgE and IgG1 production (Fig. 5a and b). Moreover, the addition of CpG ODN to the B cell culture stimulated with IL-4 and anti-CD40 mAb also markedly inhibited surface expression of IgE and IgG1 on B cells, while enhanced surface expression of IgM on B cells (Fig. 6). The B cells expressing surface IgG2a were undetectable in both groups of culture (Fig. 6). These results suggest that CpG ODN suppresses IgE and IgG1 production of B cells by inhibiting isotype switching from IgM to IgE and IgG1. Because addition of CpG ODN did not decrease the viability of cultured B cells (Fig. 5c), these data show that CpG ODN effectively inhibits IL-4/CD40-induced, Th2-related IgE and IgG1 production through a direct effect on B cells.

3.3. Comparison of the efficacy of CpG-A-ODN and CpG-B-ODN in their preventive effect on anaphylaxis

The basic CpG ODN motif consists of unmethylated CpG dinucleotides within particular flanking bases, however, modifications of the backbone and/or additional flanking sequences result in

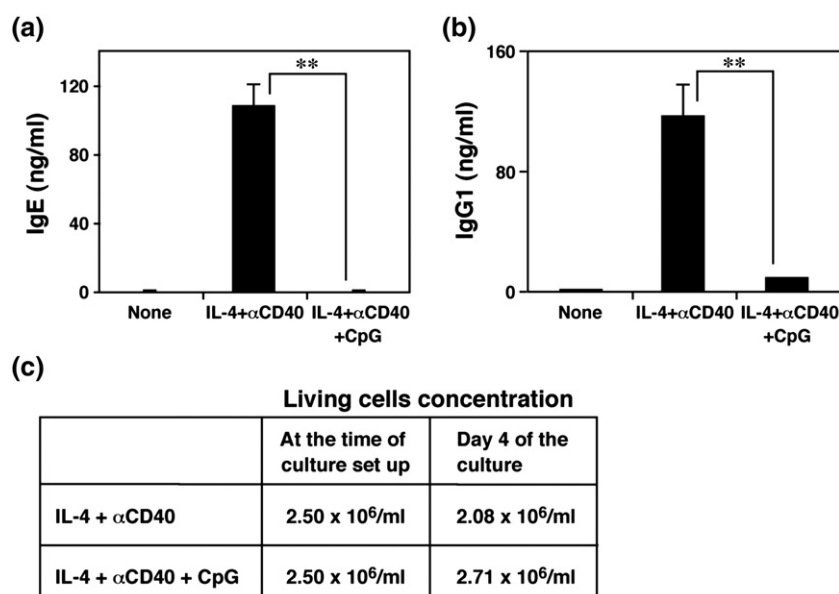


Figure 5 CpG ODN directly inhibited IgE and IgG1 production of B cells. (a, b) Purified mouse splenic B cells were stimulated with monoclonal anti-mouse CD40 and recombinant murine IL-4 with or without CpG ODN. Secretion of IgE and IgG1 induced by IL-4 and anti-CD40 was significantly inhibited by CpG ODN treatment. The value represents the mean and SD in each group. ** $P < 0.01$ by Student's *t* Test. (c) Addition of CpG ODN did not reduce the viability of cultured B cells. Living cells number was counted at the time of culture set up and day 4 of the culture by using trypan blue dye. The viability of cultured B cells was also confirmed by PI staining and fluorocytometric analysis.

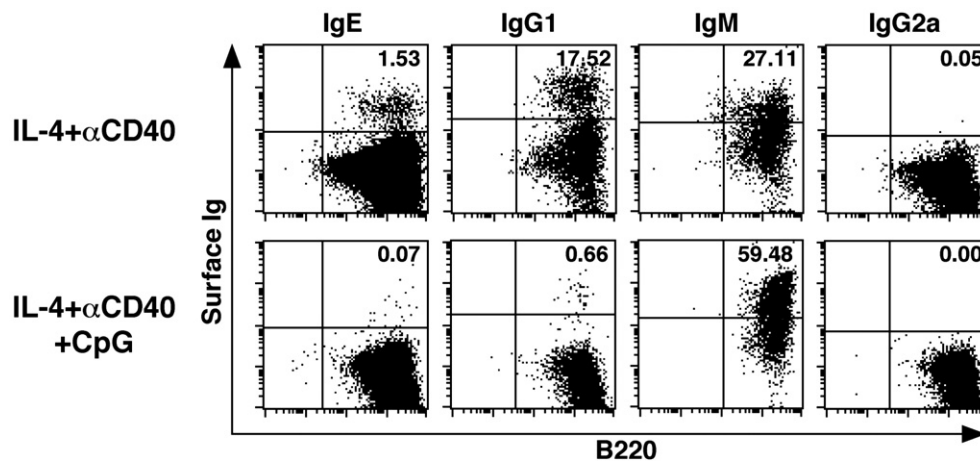


Figure 6 CpG ODN obviously inhibited surface IgE and IgG1 expression of B cells, while enhanced surface IgM expression of B cells. Mouse splenic B cells were purified and stimulated as described in Fig. 5 and in the Materials and methods section. At day 4 of the culture, B cells surface expression of immunoglobulins was examined by FACS.

classes of CpG ODN [19,20]. In order to select the most effective and ideal class of CpG ODN for CpG ODN-based immunotherapy, the efficacy of CpG-A-ODN and CpG-B-ODN in their preventive effect on anaphylaxis was compared. As shown in Fig. 7, both

CpG-A-ODN+OVA and CpG-B-ODN+OVA pretreatment significantly inhibited serum OVA-specific IgE (Fig. 7a), ameliorated anaphylaxis shock (Fig. 7b), decreased anaphylaxis clinical symptom (Fig. 7c), and significantly reduced plasma histamine

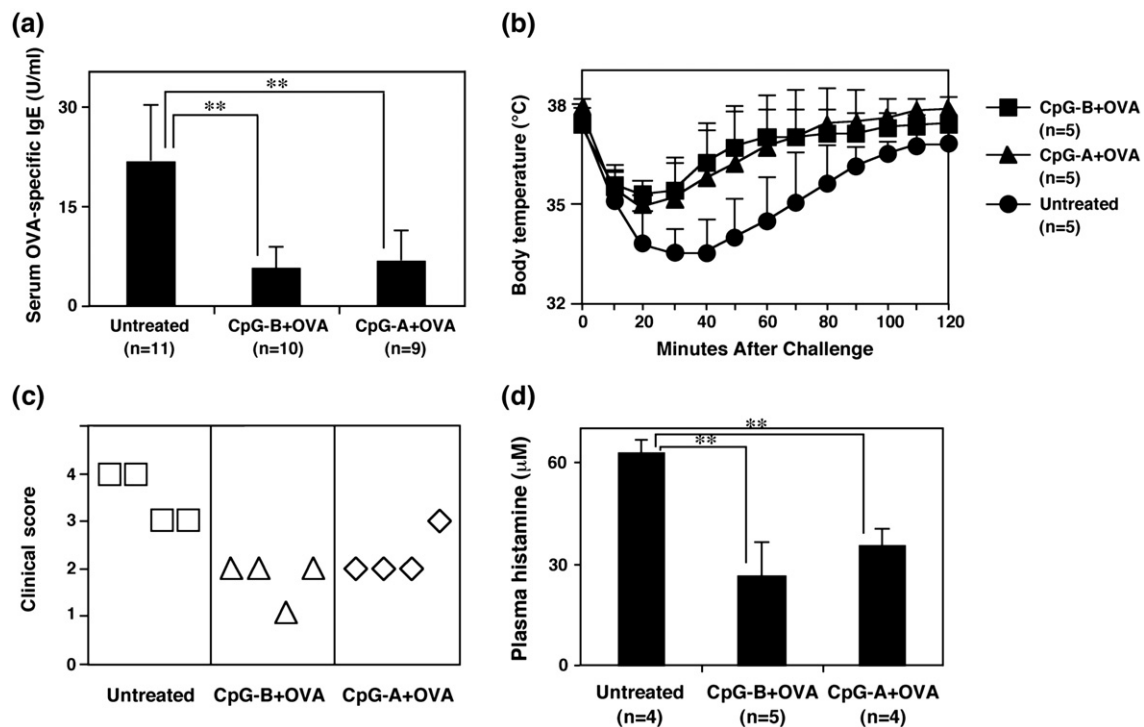


Figure 7 Both CpG-B-ODN+OVA and CpG-A-ODN+OVA treatment ameliorated anaphylaxis. Mice were untreated or pretreated, sensitized, and challenged as described in the Materials and methods section. (a) Both CpG-B-ODN+OVA and CpG-A-ODN+OVA treatment significantly reduced serum OVA-specific IgE. Two days before challenge, serum was collected for antibody determination by ELISA. The value represents the mean and SD in each group. $**P < 0.01$ by Student's *t* Test. (b) Both CpG-B-ODN+OVA and CpG-A-ODN+OVA treatment ameliorated anaphylaxis shock. Means and SD of rectal temperatures are shown for 2 h following the challenge. (c) Both CpG-B-ODN+OVA and CpG-A-ODN+OVA treatment inhibited anaphylaxis symptoms. Anaphylaxis symptoms were observed for 2 h following the challenge. (d) Both CpG-B-ODN+OVA and CpG-A-ODN+OVA treatment reduced plasma histamine levels. Two min after intravenous challenge, plasma was collected for histamine determination. Results represent mean values and SD in each group. $**P < 0.01$ by Student's *t* Test. The number of mice used under each condition is indicated as ($n=x$). One representative experiment out of three is shown.

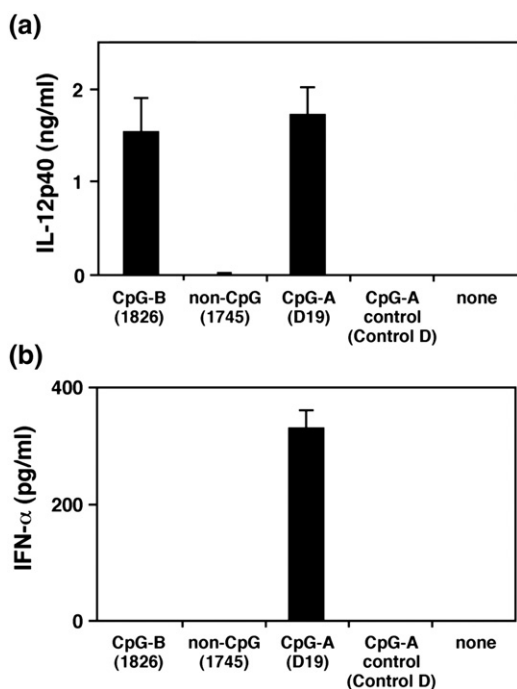


Figure 8 Comparison of CpG-A-ODN and CpG-B-ODN in cytokine production. Naive mice spleen cells were stimulated with CpG-A-ODN or CpG-B-ODN. Supernatants were harvested at 48 h for cytokine determination. The value represents the mean and SD in each group. (a) CpG-A-ODN and CpG-B-ODN induced a similar amount of IL-12p40 production. (b) IFN- α was only detected in CpG-A-ODN stimulated culture supernatant. Results represent mean values and SD in each group. One representative experiment out of three is shown.

levels (Fig. 7d). There was no significant difference of the efficacy between CpG-A-ODN and CpG-B-ODN.

In order to further examine the mechanism of the protective effects offered by CpG-B-ODN and CpG-A-ODN, profiles of cytokine production by splenic cells *in vitro* were compared. Both CpG-A-ODN and CpG-B-ODN induced a similar amount of IL-12p40. The striking difference between two groups was IFN- α production. CpG-A-ODN stimulation induced an obvious IFN- α production, while CpG-B-ODN did not induce IFN- α production (Fig. 8).

Next, effects of CpG-A-ODN and CpG-B-ODN on Ig production *in vitro* by splenic B cells were compared. Compared to the purified B cells stimulated with IL-4 and anti-CD40 mAb, addition of CpG-A-ODN or CpG-B-ODN greatly inhibited IgE and IgG1 production (Fig. 9a and b). Neither CpG-A-ODN nor CpG-B-ODN decreased the viability of cultured B cells (Fig. 9c). Furthermore, compared to stimulation with IL-4 and anti-CD40 mAb, addition of CpG-A-ODN or CpG-B-ODN efficiently inhibited surface expression of IgE and IgG1 on B cells, and enhanced surface expression of IgM (Fig. 10). These results suggest that both CpG-A-ODN and CpG-B-ODN suppress IgE and IgG1 production of B cells by inhibiting isotype switching from IgM to IgE and IgG1.

4. Discussion

The present series of experiments demonstrate that pretreatment with CpG ODN and allergen (OVA) markedly reduced subsequent OVA-induced anaphylaxis.

Our results also showed that pretreatment with CpG ODN alone did not have a significant effect on preventing OVA-induced anaphylaxis. It has been reported that although CpG ODNs stimulate essentially all murine B cells, they preferentially stimulate activated and/or memory B cells, as

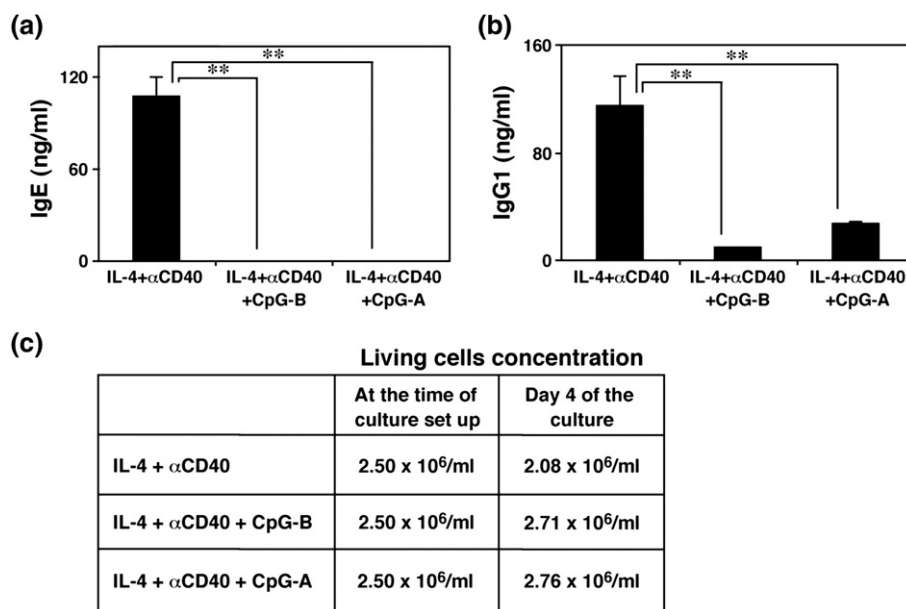


Figure 9 Comparison the effect of CpG-A-ODN and CpG-B-ODN on B cells. Purified mouse splenic B cells were stimulated as described in the Materials and methods section. (a, b) Both CpG-A-ODN and CpG-B-ODN significantly inhibited IgE and IgG1 production of B cells induced by IL-4 and anti-CD40. The value represents the mean and SD in each group. $**P < 0.01$ by Student's *t* Test. (c) Neither CpG-A-ODN nor CpG-B-ODN reduced the viability of cultured B cells. Living cells number was counted at the time of culture set up and at day 4 of the culture by trypan blue dye. The viability of cultured B cells was also confirmed by PI staining and fluorocytometric analysis.

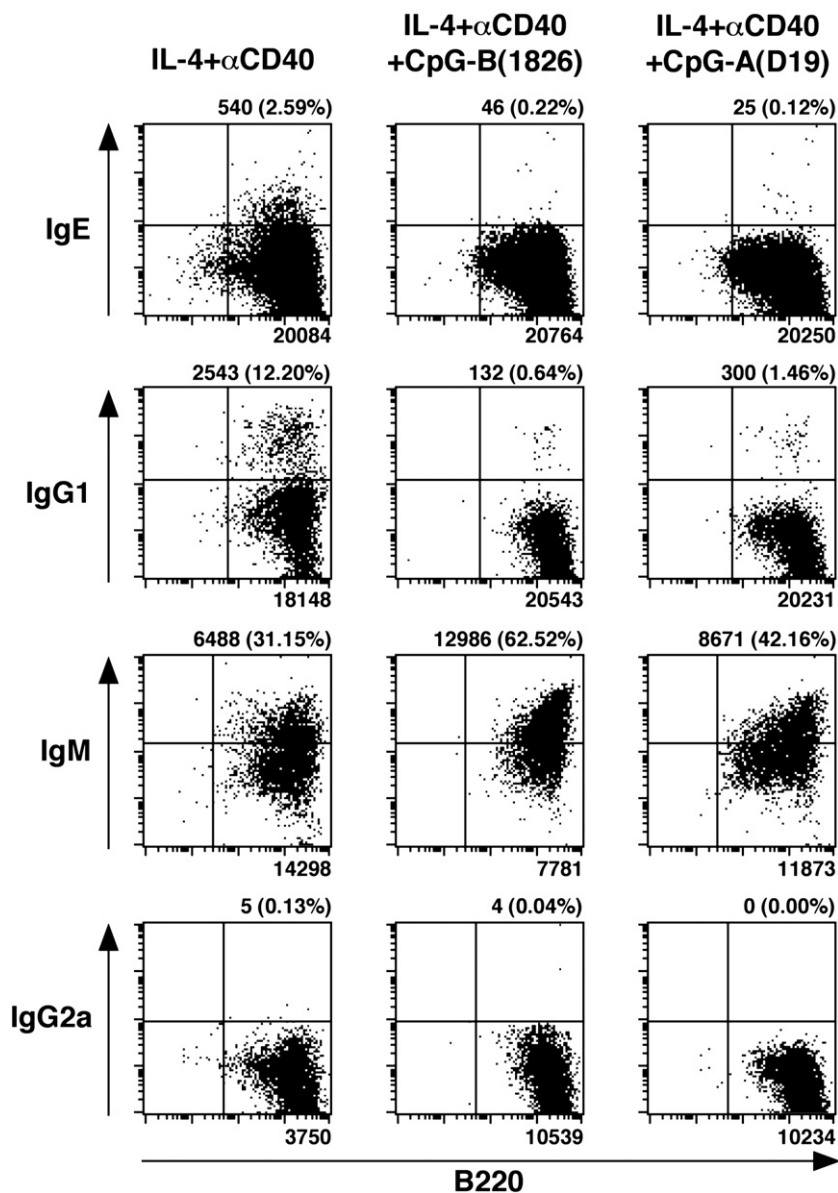


Figure 10 Both CpG-B-ODN and CpG-A-ODN inhibited B cells surface expression of IgE and IgG1, while enhanced B cells surface expression of IgM. Mouse splenic B cells were purified and stimulated as described in the Materials and methods section. At day 4 of the culture, B cells surface expression of Igs was examined by FACS. The numbers written on the upper and lower side of each FACS figure indicate the actual cell numbers of upper right (UR) quadrant and lower right (LR) quadrant respectively. The numbers in the parenthesis indicate the percentage of upper right quadrant cells in total cells. Data represent four mice in each group. One representative experiment out of three is shown.

opposed to naive cells, reflecting the preferential expression of TLR9 on activated cells [21]. According to this view, in our specific protocol, as mice were pretreated twice with CpG ODN+OVA, CpG ODN may preferentially stimulate OVA-specific B cells. Combining our results of B cell culture, at this time point, CpG ODN may inhibit OVA-specific B cell differentiation into IgE-producing B cells. When mice pretreated with CpG ODN+OVA are sensitized with OVA, OVA-specific B cells will undergo secondary response, leading to rapid proliferation and differentiation and production of specific antibody the same as produced in the primary response. While in the case of CpG ODN treated mice, OVA-specific B cells could not be preferentially stimulated before

sensitization. Therefore, the inhibition of isotype switch from IgM to IgE in OVA-specific B cells may not be induced as efficiently as in CpG ODN+OVA treated group.

Since only coadministration of CpG ODN and OVA was effective, we next sought to evaluate the importance of CpG ODN transduced signals in CpG ODN+OVA mediated preventive effect on anaphylaxis. Previous report showed that CpG induced IgG2a class switching through TLR9 in B cells [22]. Moreover it is well known that the interaction of CpG ODN and TLR9 stimulates MyD88 dependent signaling pathway [2]. Therefore, we used MyD88^{-/-} mice to evaluate the importance of CpG ODN transduced signals. The results from MyD88^{-/-} mice showed that abolition of CpG ODN transduced

signals completely disrupted CpG ODN+OVA mediated preventive effect. This suggests that CpG ODN transduced signals through MyD88 play an indispensable role in the protective effect of CpG ODN+OVA pretreatment. Pretreatment with OVA alone did not show any protection of mice against the development of anaphylaxis.

As to mouse anaphylaxis model establishment, we follow the protocol of active systemic anaphylaxis induction which has been proved to be an IgE-dependent anaphylactic reaction [23]. In order to collect more parameters such as body temperature, clinical score to evaluate anaphylaxis more objectively, we decreased allergen dose at sensitization from 500 μ g to 300 μ g. It has been reported that down-regulation of allergen-specific IgE response protects from anaphylaxis [18].

What we are interested in is the mechanism by which CpG ODN could mediate protective effect on allergic diseases. Currently it is widely believed that the application of CpG ODN induces the production of IL-12, IFN- γ and type I IFN, thereby, leading to the development of antigen-specific Th1 cells and interfering with the development of allergen-specific Th2 cells. The generation of allergen-specific Th1 cells is also believed to be an important prerequisite of CpG ODN mediated inhibition of allergic responses. We think it is necessary to clarify whether CpG ODN-induced Th1 responses are causal in inhibiting allergic disease.

In the present prophylactic treatment model, CpG ODN+OVA treatment induced a shift in the isotype of antibody following sensitization. The decreased OVA-specific IgE and enhanced OVA-specific IgG2a *in vivo*, and inhibited antigen-specific IL-4 and elevated antigen-specific IFN- γ production demonstrate that CpG ODN+OVA pretreatment indeed induced Th1 immune responses. Intriguingly, our results show that CpG ODN+OVA pretreatment is also effective in prevention of anaphylaxis in IFN- $\gamma^{-/-}$ mice, indicating that the CpG ODN-induced IFN- γ is not involved in its preventive effect. These results further suggest that the Th1 response induced by CpG ODN may not be mediating the beneficial effect of CpG ODN, although the participation of Th1 cytokine, such as IL-12, cannot be denied in the prevention effect of anaphylaxis offered by CpG ODN. In this regard, there are recent studies which demonstrate that adoptively transferred allergen-specific Th1 cells do not inhibit Th2-mediated airway hyperreactivity in mouse models of asthma [24,25]. It is also reported that *in vitro*, CpG-C stimulated plasmacytoid dendritic cells could induce plasma cell differentiation in purified B cells in the absence of T-cell help [26]. Thus, while it is clear that CpG ODN induces Th1 responses, and at the same time inhibits Th2 responses and allergic diseases, our results suggest that these events are just associated and not causal. On the other hand, the present studies also provide evidence that CpG ODN effectively inhibits IgE and IgG1 production induced by IL-4 and CD40 via a direct action on B cells. The latter mechanism is more likely responsible for ameliorating IgE-mediated allergic responses.

The present results also show that both CpG-A-ODN+OVA and CpG-B-ODN+OVA pretreatment ameliorated anaphylaxis shock *in vivo*. And *in vitro* experiments, both CpG-A-ODN and CpG-B-ODN suppress IgE and IgG1 production of B cells. As different sequence CpG ODNs should be regarded as different reagents, the present studies may not represent all of CpG-A-ODN and CpG-B-ODN. Nevertheless, our results show

that the specific CpG ODNs: D19 and 1826, which belong to CpG-A-ODN and CpG-B-ODN, respectively, exhibit similar protective effect on anaphylaxis *in vivo* and probably through a similar pathway as suggested by *in vitro* experiments.

A striking characteristic of CpG-A-ODN is the induction of IFN- α production. And it is known that type I IFN stimulates the development of Th1 cells. IFN- α can increase production of IFN- γ by natural killer cells [27] and induce Th1 clonal expansion [28]. In addition, IFN- α prevents IL-4-mediated suppression of Th1 cells [29]. IFN- β stimulates Th1 clonal proliferation [30] and induces IFN- γ and IL-12 from peripheral lymphocytes [31]. In the present studies, CpG-A-ODN (D19) indeed induced IFN- α production, however, the protective effect on anaphylaxis was not elevated in CpG-A-ODN group. These results suggest that IFN- α does not play an important role in CpG ODN mediated protective effect. Moreover, therapy with CpG motifs in a murine model of allergic airway inflammation in IFN- $\beta^{-/-}$ mice shows that IFN- β is not required for CpG ODN mediated suppression of IgE and airway eosinophilia [32]. These results may further support the conclusion of the present studies that Th1 immune response induced by CpG ODN does not play a critical role in CpG ODN mediated protective effect on allergic responses. On the contrary, CpG ODN directly suppressed IgE and IgG1 production of B cells by inhibiting isotype switching from IgM to IgE and IgG1 may underlie its beneficial effect on allergic diseases.

So far the studies of the mechanism of the beneficial effect of CpG ODNs on allergic diseases such as asthma [8,9,33,34] and allergic conjunctivitis [12] have mainly focused on their ability to induce Th1 responses. However, our results of IFN- $\gamma^{-/-}$ mice suggest that CpG ODN induced IFN- γ -producing Th1 cells are not involved in its protective effect. As far as we know, this is the first paper which evaluates the importance of IFN- γ and Th1 response in CpG ODN+allergen mediated protection in an anaphylaxis model. Furthermore, although it has been reported that *in vitro* CpG ODN represses ϵ germline transcripts while inducing γ 2a germline transcripts [35], we provide the information about the actual antibody production *in vivo*. We think *in vivo* information is indispensable, because in an *in vivo* setting, it is likely that additional T cell-dependent signals and/or cytokines further regulate the production of class-switched antibody. Moreover, we compared the therapeutic effect of CpG-A-ODN and CpG-B-ODN on allergic disease. Our *in vivo* studies presented here add a new dimension to what was previously known about the mechanisms regulating anaphylaxis. We think our present work about therapeutic effect of different CpG ODNs may provide some useful information for future immunotherapeutic strategy design.

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