

Modulation of ovalbumin-induced Th2 responses by second-generation immunomodulatory oligonucleotides in mice

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Abstract

Oligodeoxynucleotides containing unmethylated CpG dinucleotides (CpG DNAs) prevent development of T-helper type 2 (Th2) immune responses and reverse established allergic responses in mouse models. We recently reported that second-generation immunomodulatory oligonucleotides (IMOs) containing novel structures (immunomers) and a synthetic immunostimulatory CpR ($R = 2'$ -deoxy-7-deazguanosine) motif induce the production of distinct cytokine secretion profiles *in vitro* and *in vivo*. In the present study, we evaluated IMOs containing CpG and CpR motifs to modulate allergen-induced Th2 immune responses in prevention and treatment models. Mice sensitized and challenged with ovalbumin (OVA) were treated with a CpG DNA or an IMO by administration either at the time of OVA sensitization (co-administration; prevention) or after establishment of an allergic response (treatment). Spleens, blood, and lungs were collected and analyzed for immune responses. Spleen-cell cultures harvested from OVA-sensitized mice showed a significant decrease in Th2 cytokine levels with a concomitant increase in Th1 cytokine levels only when CpG DNA or IMOs were co-administered with OVA. The co-administration of CpG DNA or IMOs during OVA sensitization significantly reduced serum OVA-specific and total IgE levels in mice. The mice who received CpG DNA or IMOs co-administered with OVA showed a small reduction in serum OVA-specific and total IgG1 levels and a significant increase in serum OVA-specific and total IgG2a levels. Similar results were found in mice with established allergic responses who received IMO treatment. IMO treatment also resulted in strong inhibition of inflammatory cell infiltration and goblet cell hyperplasia in the lungs compared with untreated mice lungs. These data demonstrate that IMOs prevent antigen-induced Th2 immune responses when co-administered to mice during OVA sensitization and that IMOs reverse established allergic responses induced by OVA.

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1. Introduction

An allergic asthma response is characterized by activation of T-helper type 2 (Th2) lymphocytes. The responses induced by Th2 lymphocytes play a major role in the pathogenesis and propagation of allergic inflammation in asthma [1]. The Th2 cytokine IL-5 increases the generation and survival of eosinophils,

Abbreviations: ELISA, enzyme-linked immunosorbent assay; HE, hematoxylin and eosin; IFN, interferon; Ig, immunoglobulin; IL, interleukin; IMO, immunomodulatory oligonucleotide; i.n., intranasal; OVA, ovalbumin; PAS, periodic acid Schiff; s.c., subcutaneous; Th, T-helper; TLR, Toll-like receptor.

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leading to increased airway eosinophilia [2]. Other Th2 cytokines (IL-4, IL-9, and IL-13) also play critical roles in allergic inflammation by inducing production of allergen-specific IgE, mast-cell proliferation, endothelial-cell adhesion-molecule expression, and airway hyper-responsiveness [3]. Corticosteroids are currently the only widely used treatment for allergic asthma. Steroid treatment is effective only in minimizing the manifestations of inflammation, however, and does not cure the disease. Continuous therapy is required to prevent the progression of allergic asthma [4].

Decreased exposure to bacteria may be partially responsible for the increased incidence of, severity of, and mortality due to allergic diseases such as asthma, atopic dermatitis, and rhinitis in the developed countries [5]. This hypothesis is supported by evidence that bacterial infections or products can inhibit the development of allergic disorders in experimental animal models and clinical studies [6–9]. Bacterial DNA or synthetic oligodeoxynucleotides containing unmethylated CpG dinucleotides in certain sequence contexts (CpG DNA) potentially stimulate innate immune responses and thereby acquired immunity [10–12]. The immune response to CpG DNA includes activation of innate immune cells, proliferation of B cells, induction of Th1 cytokine secretion, and production of immunoglobulins (Ig) [13–16]. The activation of immune cells by CpG DNA occurs via Toll-like receptor 9 (TLR9), a molecular pattern recognition receptor [17]. CpG DNAs induce strong Th1-dominant immune responses characterized by secretion of IL-12 and IFN- γ . CpG DNAs alone or as allergen conjugates decrease production of IL-4, IL-5, and IgE and reduce eosinophilia in mouse models of allergic asthma [18–22]. CpG DNAs also effectively reverse established atopic eosinophilic airway disease by converting a Th2 response to a Th1 response [23–29].

While the presence of the CpG dinucleotide is critical for the immunostimulatory activity of the oligodeoxynucleotide, a number of other characteristics of CpG DNAs, such as the flanking sequences, accessibility to the 5' -end, and the presence of secondary structures, also play significant roles in determining the immunostimulatory activity [30,31]. We recently showed that two CpG DNAs linked through their 3' -ends (3'–3'), referred to as immunomers,

were more potent immunostimulators than the conventional CpG DNAs [32–37]. In contrast, CpG DNAs linked through their 5' -ends had no activity, suggesting that an accessible 5' -end was required for activity [32–34].

Our previous studies also showed that CpG DNAs containing a synthetic CpR (R = 2' -deoxy-7-deazaguanosine) motif activated immune cells [38]. Immunomers containing CpR motifs (second-generation immunomodulatory oligonucleotides; IMOs) induced higher IL-12 and lower IL-6 secretion in mouse spleen cell cultures than did those with CpG motifs [35]. Immunomers containing CpR dinucleotides inhibited IL-5 production and induced IFN- γ production in antigen-specific recall response in vitro [35]. In a previous study, we showed that administration of immunomers at the time of ovalbumin (OVA) sensitization potentially prevented OVA-induced allergic airway inflammation and airway hyper-responsiveness to methacholine in mice [39]; the effects of immunomers on OVA-induced Th2 cytokine and antibody responses were not studied. In the current study, we investigated whether these novel immunomers containing mouse-specific CpG and CpR motifs can prevent development of or reverse established Th2-dominant immune responses and whether these compounds modify antigen-specific antibody responses in mice sensitized with chicken egg OVA.

2. Materials and methods

2.1. Animals

Female BALB/c mice, 5–6 weeks old, were obtained from Taconic Farms (Germantown, NY) and maintained on an OVA-free diet. Groups of five mice were used in the immunization study, which was approved by the Hybridon Institutional Animal Care and Use Committee.

2.2. Oligodeoxynucleotide synthesis and purification

Conventional CpG DNA and IMOs (Fig. 1A) were synthesized, purified, and analyzed as previously described [35]. The purity of full-length CpG DNA and IMOs ranged from 93% to 98% with the rest being shorter by one or two nucleotides ($n - 1$

A. CpG DNA and IMO sequences and modifications

Number	Sequence ^a	Modification	Motif
1	5'-CTATCTGACGTTCTCTCTGT-3'	Conventional	CpG
2	5'-TCTGACGTTCT-X-TCTTGCAGTCT-5'	Immunomer	CpG
3	5'-TCTGACRITCT-X-TCTTRCAGTCT-5'	Immunomer	CpR

^a: X is glycerol linker and R is 2'-deoxy-7-deazaguanosine. All are oligodeoxynucleotides with phosphorothioate backbone modification

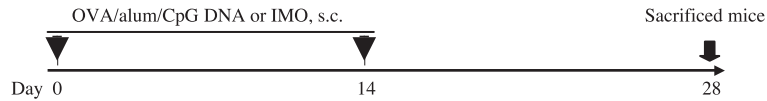
B. Prevention protocol**C. Therapeutic protocol**

Fig. 1. (A) Sequences of conventional CpG DNA and IMOs. (B) Prevention protocol. (C) Therapeutic protocol.

and $n-2$) as determined by capillary gel electrophoresis and/or denaturing polyacryl amide gel electrophoresis. All CpG DNAs contained less than 0.2 EU/ml of endotoxin as determined by the Limulus assay (Bio-Whittaker).

2.3. Prevention protocol

Each mouse was sensitized by subcutaneous (s.c.) injection of 20 μ g chicken ovalbumin (OVA; grade V, Sigma, St. Louis, MO) in 100 μ l PBS mixed with an equal volume of alum solution (Imject-Alum, Pierce, Rockford, IL) on days 0 and 14 (Fig. 1B). The naïve group of mice received alum injection only. CpG DNA or IMO (10 μ g) dissolved in the OVA/alum mixture was administered to mice on days 0 and 14. Fourteen days after the last immunization, mice under anesthesia with isoflurane (Abbott Laboratories, North Chicago, IL) were bled by retro-orbital puncture and then sacrificed to remove the spleens.

2.4. Therapeutic protocol

Each mouse was sensitized by intraperitoneal (i.p.) injection of 20 μ g OVA/alum solution as described

above on days 0 and 14 and challenged intranasally (i.n.) with 10 μ g OVA in 40 μ l PBS on days 28, 29, and 30 (Fig. 1C). IMO 3 (60 μ g) dissolved in 200 μ l PBS was injected s.c. to mice on days 33, 37, 40 and 43. Blood samples from mice under anesthesia were collected 2 h after the first injection of IMO on day 33 by retro-orbital puncture and serum was harvested for cytokine assays. Each mouse was challenged i.n. with 10 μ g OVA in 40 μ l PBS on day 44. Mice were bled and the lungs and spleens were removed 24 h after the last OVA challenge.

2.5. Spleen-cell culture

Single spleen-cell suspensions were prepared in cold RPMI 1640 medium (Sigma) and pooled for each experimental group at 5×10^6 cells/ml in RPMI 1640 medium containing 10% FCS (HyClone, Logan, UT) and 100 μ g/ml penicillin and 100 U/ml streptomycin (HyClone). Spleen cells (0.2 ml/well) were incubated in 96-well flat-bottom culture plates (Costar, Cambridge, MA) in the presence of 100 μ g/ml OVA at 37 °C in a 5% CO₂ atmosphere. Following a 72-h incubation, culture supernatants were harvested for cytokine assays.

2.6. Cytokine measurements

Levels of IL-5, IL-10, IL-12, and IFN- γ were determined by enzyme-linked immunosorbent assay (ELISA) with mouse antibodies from BD Biosciences (San Diego, CA) as previously described [35]. IL-4 and IL-13 levels were assessed with mouse DuoSet ELISA kits (R&D System, Minneapolis, MN) following the manufacturer's instructions.

2.7. Immunoglobulin measurements

Levels of OVA-specific and total IgE, IgG1, and IgG2a were evaluated in mouse serum by ELISA. For OVA-specific Ig detection, 96-well ELISA plates (Immulon 2; Dynatech, Chantilly, VA) were coated with 10 $\mu\text{g/ml}$ OVA in PBS, pH 9.6. For total Ig detection, the plates were coated with 1 $\mu\text{g/ml}$ anti-mouse IgE (clone R35-72) and 0.2 $\mu\text{g/ml}$ anti-mouse IgG1 (clone R35-72) or 1 $\mu\text{g/ml}$ anti-mouse IgG2a (clone R11-89). After incubation at 4 °C overnight, the plates were blocked with 1% BSA/PBS, pH 7.4, at room temperature for 1 h. Serial dilutions of serum (1:10 for IgE, 1:100 for IgG2a, and 1:1000 for IgG1) was added to the plates and incubated at room temperature for 2 h. The plates were washed and 100 $\mu\text{l/well}$ of biotinylated anti-mouse IgE (clone R35-116) at 0.25 $\mu\text{g/ml}$, IgG1 (clone A85-1) at 0.1 $\mu\text{g/ml}$, or IgG2a (clone R19-15) at 0.25 $\mu\text{g/ml}$ was added to plates and incubated at room temperature for 2 h. All antibodies were obtained from BD Biosciences. Plates were washed and 100 $\mu\text{l/well}$ of 0.25 $\mu\text{g/ml}$ streptavidin–peroxidase (Sigma) was added for 1-h incubation at room temperature. Assays were developed in 100 $\mu\text{l/well}$ of TMB Substrate Solution (KPL, Gaithersburg, MD) followed by 100 $\mu\text{l/well}$ of Stop Solution (KPL). A_{450} was measured using a microplate reader (Bio-Tek Instruments, Winooski, VT), and the data were analyzed with KC Junior software (High Point, NC).

2.8. Lung histology

The lungs removed on day 45 were fixed in neutral formalin and sent to Mass Histology Service (Warwick, RI) for processing and hematoxylin and eosin (HE) and periodic acid Schiff (PAS) staining. The lung tissue sections were observed under a light microscope and photographed with a digital camera.

2.9. Statistical analysis

Statistical analysis was performed using analysis of variance (ANOVA). OVA-immunized and CpG DNA- or IMO-treated groups were compared using Student's *t*-test. Results were expressed as the mean $\pm$ S.E.M. All comparisons were two tailed and the statistical significances were shown as * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.005$.

3. Results

3.1. Study in prevention model

Several groups have shown that CpG DNA can prevent allergic inflammation in animal models. In the current study, we examined the ability of a conventional CpG DNA (1; Fig. 1A) and two novel IMOs (2 and 3) to prevent OVA-induced allergic inflammation in mice. IMOs 2 and 3 contained a novel structure in which two short CpG DNA sequences were attached through their 3' -ends, referred to as immunomers (Fig. 1A). In addition to novel structure, IMO 3 contained a synthetic immunostimulatory motif CpR (R = 2' -deoxy-7-deazaguanosine), while CpG DNA 1 and IMO 2 contained the natural CpG motifs. All three test compounds contained mouse-specific motif shown underlined in each sequence (Fig. 1A). The prevention protocol used is shown in Fig. 1B.

3.2. Effect of conventional CpG DNA and IMOs on the inhibition of Th2 cytokines, IL-4, IL-5, and IL-13 in antigen-specific recall immune response

To determine the ability of CpG DNAs to alter the Th2-dominant immune response in mice injected with OVA/alum, we injected the conventional CpG DNA 1 or IMO 2 or 3 along with OVA/alum on days 0 and 14. The spleen cells were isolated from mice on day 28 and incubated with OVA for 72 h (antigen recall). Spleen cells from mice injected with OVA/alum only produced higher levels of Th2-associated cytokines, such as IL-4 (~ 2-fold), IL-5 (130-fold), and IL-13 (28-fold), than did naïve mice (Fig. 2A–C). Spleen cells from mice that received CpG DNA or IMOs and OVA secreted significantly less of these cytokines, particularly IL-5 and IL-13; IL-5 was decreased by 20–97% and IL-13

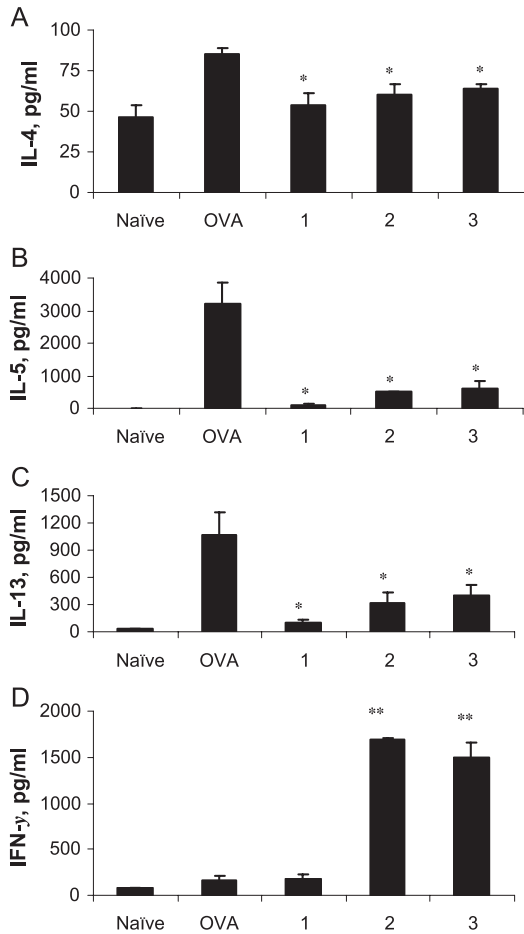


Fig. 2. Inhibition of IL-4, IL-5, and IL-13 and induction of IFN- γ production in mice immunized with OVA plus CpG DNA or IMOs. The spleens were removed on day 28 and single spleen cells were incubated with 100 μ g/ml OVA for 72 h, and the cytokine levels in culture supernatants were measured using ELISA. The results represent the mean cytokine concentration from five mice per group ($\pm$ S.E.M.) * and ** represent $p < 0.05$ and 0.01 , respectively, compared with OVA-sensitized and -challenged group.

was decreased by 60–95%. These results suggest that CpG DNA and IMOs have an inhibitory effect on Th2-associated cytokine secretion.

3.3. Effect of CpG DNA and IMOS on IFN- γ production in antigen-specific recall immune response

In the same antigen-recall experiments, significantly higher levels of the Th1 cytokine IFN- γ were produced by spleen cells from mice injected with

OVA/alum and IMO 2 or 3 than by cells from naïve or OVA/alum-injected mice (Fig. 2D). Spleen cells from mice injected with OVA/alum and conventional CpG DNA 1 produced levels of IFN- γ comparable to those produced by naïve spleen cells. These results demonstrate that treatment of OVA/alum-injected mice with IMOs can change the immune response from Th2-dominant to predominantly Th1 type, as reflected by the cytokines produced by spleen cells.

3.4. The effect of CpG DNA and IMOs on OVA-specific and total IgE production

To assess the effects of CpG DNA and IMOs on IgE production, we examined serum levels of OVA-specific and total IgE 14 days after the last OVA/alum injection. Sensitization with OVA/alum resulted in high levels of OVA-specific IgE production in mice (Fig. 3A); levels of OVA-specific IgE were significantly lower (comparable to those found in naïve mice) in mice that received CpG DNA or IMOs along with OVA/alum. Total serum IgE levels were also low in mice injected with OVA/alum and CpG DNAs (Table 1).

3.5. Effect of CpG DNA and IMOs on OVA-specific and total IgG1 and IgG2a production

To assess the effects of CpG DNA on IgG1 and IgG2a production, we examined serum levels of OVA-specific and total IgG1 and IgG2a. Animals that received injections of OVA/alum produced high levels of OVA-specific IgG1 and insignificant levels of OVA-specific IgG2a (Fig. 3B and C). Injection of CpG DNA or IMOs to mice either had no effect or reduced the levels of OVA-specific IgG1 compared with levels found in OVA/alum-injected mice (Fig. 3B). OVA-specific IgG2a antibody levels were significantly higher in the serum of mice who received CpG DNA or IMOs than in those who received only OVA/alum (Fig. 3C). These IgG1 and IgG2a levels translated into higher ratios of OVA-specific IgG2a/IgG1 in mice injected with OVA/alum plus CpG DNA, especially with IMOs 2 and 3, than in mice who received only OVA/alum (Table 1).

Similar results were found for serum total Ig production (Table 1). Serum from mice injected with OVA/alum and CpG DNA or IMOs had lower levels

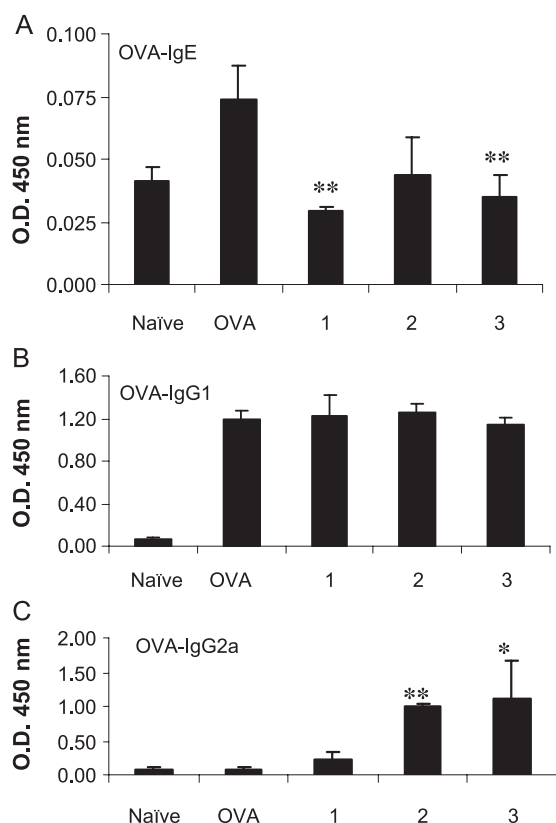


Fig. 3. Effect of CpG ODN or IMO coadministration on OVA-specific IgE (A), IgG1 (B), and IgG2a (C) production in the serum of OVA-immunized mice. Mice were immunized s.c. with OVA in alum with or without CpG DNA or IMO on days 0 and 14, and bled on day 28. Serially diluted serum was assessed for OVA-specific antibody levels using ELISA. The results represent the mean of serum from five mice per group ($\pm$ S.E.M.), where * and ** represent $p < 0.05$ and $p < 0.01$ respectively, compared with OVA-sensitized group.

of total IgG1 and higher levels of total IgG2a than did mice that received OVA/alum only. The ratios of total IgG2a/IgG1 were found particularly high in mice who received OVA/alum and IMO 2 or 3 (Table 1).

3.6. Study in therapeutic model

Both IMOs 2 and 3 potently prevented allergic airway hyper-responsiveness at a level comparable to that achieved with conventional CpG DNA 1. We therefore tested the therapeutic potential of IMO 3, which contained a novel structure as well as a

synthetic CpR stimulatory motif, in a mouse model of asthma. Mice were sensitized and challenged with OVA and treated with IMO 3 72 h after the OVA challenge on days 33, 37, 40, and 43 (Fig. 1C).

3.7. Effect of IMO 3 treatment on OVA-induced allergic lung inflammation

To determine the effects of IMO 3 treatment on the local immune response, the histology of the lungs of naïve and OVA/alum-sensitized and -challenged mice with or without IMO 3 treatment was examined 48 h after the last injection of IMO 3. OVA-sensitized and -challenged mice that were not treated with IMO 3 showed severe inflammatory cell infiltration and goblet cell hyperplasia compared with naïve mice (Fig. 4A and B). On the contrary, IMO 3-treated mice had less inflammatory cell infiltration and less airway hyperplasia than did untreated mice (Fig. 4C). These results demonstrate the ability of IMO 3 to reverse OVA-induced lung inflammation in mice sensitized and challenged with OVA.

3.8. Effect of IMO 3 treatment on Th2 and Th1 cytokines in antigen-specific recall immune response

To determine the ability of IMO 3 to reverse Th2-dominant immune responses in mice sensitized and challenged with OVA, we isolated spleen cells

Table 1
Serum total immunoglobulins and ratios of IgG2a and IgG1 in prevention study

Group	IgE (ng/ml)	IgG1 (ng/ml)	IgG2a (ng/ml)	IgG2a/ IgG1	OVA-IgG2a/ IgG1
Naïve	7 $\pm$ 2.8	479 $\pm$ 183	306 $\pm$ 30	0.64	1.2
OVA	30.3 $\pm$ 4.9	2191 $\pm$ 448	418 $\pm$ 167	0.19	0.08
OVA + 1	4.7 $\pm$ 2.3***	1344 $\pm$ 514	416 $\pm$ 77	0.31	0.18
OVA + 2	5.7 $\pm$ 1.7***	734 $\pm$ 316**	1367 $\pm$ 356*	1.86	0.79
OVA + 3	4.7 $\pm$ 2.9***	1168 $\pm$ 266*	991 $\pm$ 740	0.85	0.97

Mouse serum levels of total IgE, IgG1, and IgG2a were measured and expressed as mean $\pm$ S.E.M.

* $p < 0.5$ compared with the group administered with OVA only.

** $p < 0.01$ compared with the group administered with OVA only.

*** $p < 0.005$ compared with the group administered with OVA only.

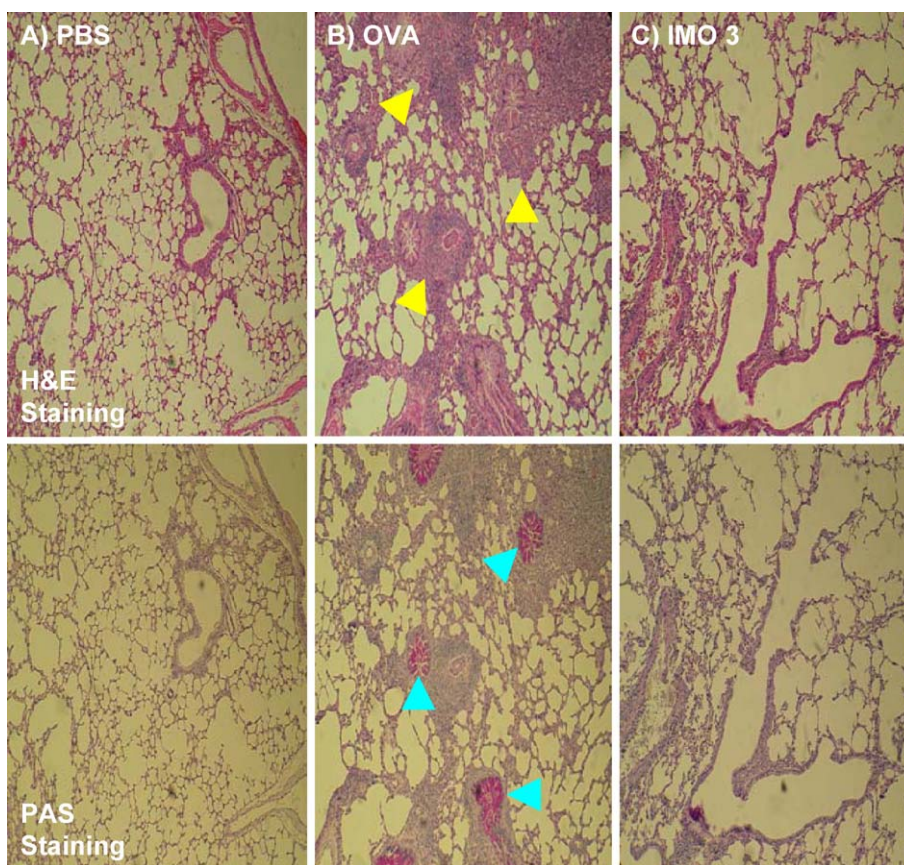


Fig. 4. Effect of treatment with IMO 3 on lung inflammation in mice sensitized and challenged with OVA. The photomicrograph (magnification $\times 100$) displays lung histology with hematoxylin and eosin (HE) (top panels) and periodic acid Schiff (PAS) (bottom panels) stains. Severe perivascular and peribronchial infiltration of inflammatory cells (HE) and intensive mucous secretion (PAS) were indicated with arrows in OVA-treated lung tissue (B). Both inflammation and mucus secretion were reduced in the IMO 3-treated group (C) compared with the OVA-treated group.

from mice on day 45 and incubated them with OVA for 72 h. After re-stimulation of spleen cells with OVA, we observed marked differences in the production of Th2 cytokines (IL-4, IL-5, and IL-13) among the treatment groups, as was the case in the prevention model (Table 2). Spleen cells from mice injected with OVA only produced high levels of Th2-associated cytokines (Table 2). Mice treated with IMO 3 produced significantly less of the OVA-induced Th2 cytokines (Table 2). In the antigen-recall experiments, only low levels of IL-12 and IFN- γ were induced in cultures of spleen cells from naïve and OVA/alum-sensitized and -challenged mice (Table 2). Spleen cells from mice treated with IMO 3 following the OVA challenge produced

significantly higher levels of IL-12 and IFN- γ (Table 2).

3.9. Effect of IMO 3 treatment on serum IL-12 and IFN- γ levels in mice sensitized and challenged with OVA

To assess the immediate effects of IMO 3 treatment, serum IL-12 and IFN- γ levels were measured in blood withdrawn 2 h after the first injection of IMO 3 on day 33. OVA-sensitized and -challenged mice who received IMO 3 produced significantly higher levels of the Th1 cytokines IL-12 and IFN- γ (Fig. 5) than did naïve or OVA-sensitized and -challenged mice (Fig. 5). These results suggest that IMO 3 inhibited OVA-

Table 2

Cytokines levels in spleen cell cultures and serum total immunoglobulin levels in treatment study

Cytokine/total Ig	PBS	OVA	IMO 3
<i>In spleen cell cultures (pg/ml)</i>			
IL-4	51 ± 33	241 ± 23	73 ± 17***
IL-5	37 ± 3	3148 ± 231	869 ± 63***
IL-13	70 ± 8	12031 ± 3118	590 ± 161***
IL-10	283 ± 26	1288 ± 186	253 ± 84*
IL-12	103 ± 57	33 ± 35	178 ± 68*
IFN-γ	332 ± 43	293 ± 35	593 ± 35*
<i>Serum total immunoglobulins (ng/ml)</i>			
IgE	0.054 ± 0.01	8.87 ± 1.7	3.6 ± 2.2*
IgG1	0.4 ± 0.1	147 ± 39	44 ± 24*
IgG2a	127 ± 14	162 ± 6	686 ± 10*

Spleens and blood were collected on day 45, cytokines were measured in spleen cell cultures and the levels of total Ig subtypes in serum were determined as described in the text and expressed as mean ± S.E.M.

* $p < 0.5$ compared with the group sensitized and challenged with OVA but not treated with IMO 3.

*** $p < 0.005$ compared with the group sensitized and challenged with OVA but not treated with IMO 3.

induced Th2 cytokine production and shifted the cytokine profile toward a Th1 type. Only low levels of Th2 cytokines (IL-4, IL-5, and IL-13) were found in the serum of mice treated with IMO 3 compared with untreated mice (data not shown).

3.10. Effect of treatment with IMO 3 on OVA-specific and total serum IgE production in OVA-sensitized and -challenged mice

To assess the effects of IMO 3 on IgE production, we examined serum levels of OVA-specific and total IgE. Mice that were sensitized and challenged with OVA but not treated with IMO 3 had high levels of OVA-specific IgE in serum (Fig. 6A), while mice treated with IMO 3 had significantly lower levels of OVA-specific IgE. Total serum IgE levels were also low in mice that received IMO 3 treatment (Table 2).

3.11. Effect of IMO 3 treatment on OVA-specific and total IgG1 and IgG2a production in OVA/alum-sensitized and -challenged mice

To assess the effect of IMO 3 treatment on IgG1 and IgG2a production, we examined serum levels of

OVA-specific and total IgG1 and IgG2a. Without IMO 3 treatment, mice sensitized and challenged with OVA had high serum levels of OVA-specific IgG1 and insignificant levels of OVA-specific IgG2a (Fig. 6B and C). OVA-sensitized and -challenged mice treated with IMO 3 had slightly lower levels of OVA-specific IgG1 than mice who did not receive IMO 3 (Fig. 6B). We observed an increase in OVA-specific IgG2a antibody levels in serum of the mice treated with IMO 3 (Fig. 6C). Similar results were found for serum total Ig production (Table 2): mice sensitized and challenged with OVA and treated with IMO 3 had low serum levels of total IgG1 and higher levels of total IgG2a than did mice that were not treated with IMO 3.

4. Discussion

OVA with alum is commonly used to establish a Th2-dominant immune response in various mouse and rat models. The Th2 immune response includes increased IL-4, IL-5, and IL-13 production, elevated serum levels of total and antigen-specific IgE, IgG1, and lower levels of IgG2a. Conventional CpG DNAs, such as CpG DNA 1 used in the present study, prevent

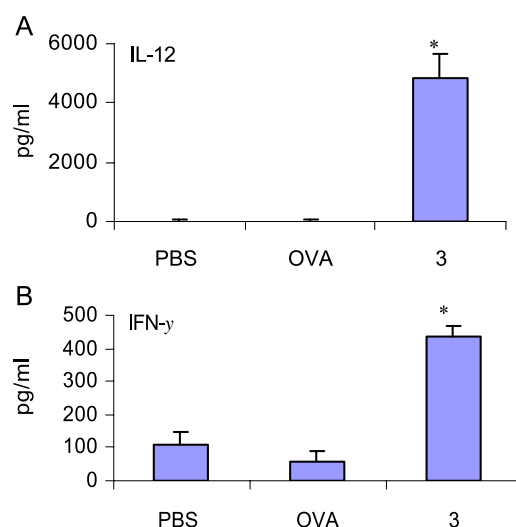


Fig. 5. Effect of IMO 3 treatment on serum IL-12 (A) and IFN-γ (B) levels in mice sensitized and challenged with OVA. * indicates $p < 0.05$ compared with OVA-sensitized and -challenged group.

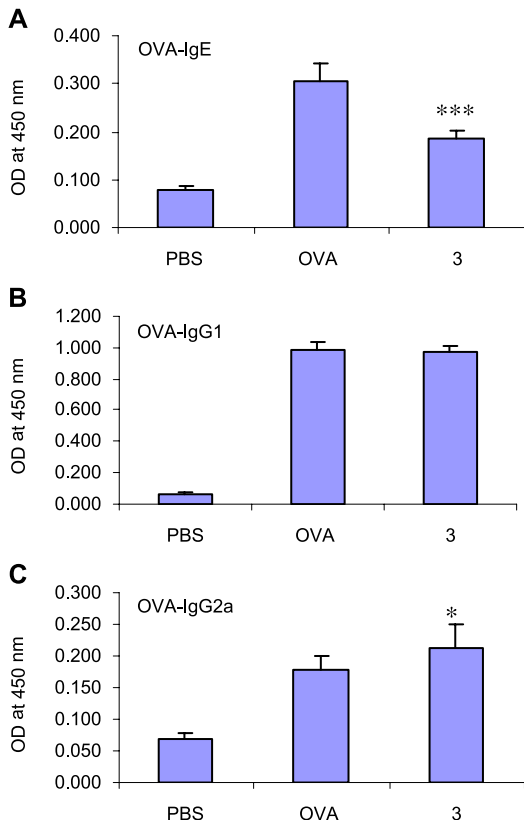


Fig. 6. Effect of IMO 3 treatment on serum OVA-specific IgE (A), IgG1 (B), and IgG2a (C) production in mice sensitized and challenged with OVA. Results represent the mean of serum from five mice per group ($\pm$ S.E.M.). * and *** indicate $p < 0.05$ and $p < 0.005$, respectively, compared with IMO 3 untreated group.

and reverse established Th2-dominant immune responses in mice [20,24,35,39–41]. Consistent with earlier reports, the co-administration of conventional CpG DNA 1 or IMOs 2 or 3 with OVA/alum to mice reduced IL-4, IL-5, and IL-13 production and induced IFN- γ production in spleen-cell cultures subjected to antigen re-stimulation. Furthermore, CpG DNA and IMOs inhibited antigen-specific and total IgE and enhanced IgG2a production in these mice.

In previous studies, injection of OVA/alum and CpG DNA induced a lymphocyte antigen-recall response in mice characterized by low levels of Th2-associated cytokines, IgE and IgG1, and high levels of Th1-associated cytokines and IgG2a [42,43]. Co-administration of CpG DNA with other kinds of antigens, such as *S. masoni* egg and hen egg lyso-

zyme, also resulted in reversal of the Th2 response to a Th1-dominant response in in vitro and in vivo studies [44,45]. In the present study, conventional CpG DNA 1 and both IMOs 2 and 3 effectively prevented development of a Th2 immune response and allowed a strong Th1 response.

While Th2 cytokines trigger an Ig isotype switch towards production of IgE and IgG1, the Th1 cytokine IFN- γ induces production of IgG2a by B-lymphocytes [46]. Mice injected with OVA/alum and IMOs produced lower levels of IL-4, IL-5, and IL-13 and higher levels of IFN- γ , accompanied by lower IgE and IgG1 and higher IgG2a levels, than did mice injected with OVA/alum alone. This result suggests the existence of a close link between Th1 cytokine induction and immunoglobulin isotype switch in mice that received antigen and CpG DNAs [42–45].

Serum antigen-specific and total IgE levels were significantly lower in mice receiving OVA/alum and CpG DNA than in mice receiving OVA/alum alone. In contrast, OVA-specific IgG1 levels were insignificantly changed and total IgG1 levels were only slightly decreased compared with mice injected with OVA/alum alone. The different response may result from different mechanisms involved in the control of IgE and IgG1 class switch, though both isotypes are influenced by IL-4 and IL-13. For example, IL-6 promotes B lymphocytes to synthesize IgG1 in the presence of IL-4 [47].

Our previous in vitro and in vivo studies described subtle differences in the immune responses induced by conventional CpG DNA 1 and IMOs 2 and 3, which contain CpG or synthetic CpR motifs [35,39]. In the present study, conventional CpG DNA and the two IMOs potentially prevented OVA-induced Th2 responses with the induction of Th1-type immune responses in mice, but subtle differences existed in the responses elicited with the two types of compounds. All three CpG DNAs studied reduced OVA-induced IL-4, IL-5, and IL-13 levels to a similar extent compared with those achieved in mice immunized with OVA/alum alone. IMOs 2 and 3 induced higher IFN- γ levels than found in mice that received OVA/alum alone or OVA/alum and CpG DNA 1. As well as inducing higher levels of IFN- γ , IMOs 2 and 3 also induced significantly higher serum levels of OVA-specific and total IgG2a than did conventional CpG DNA 1. These

results are consistent with the ability of IMOs 2 and 3 to abrogate both early and late allergic responses and to block airway hyper-responsiveness to methacholine in OVA-sensitized mice [39].

Consistent with the effects observed in the prevention model, in established allergic model IMO 3 suppressed production of Th2-type cytokines IL-4, IL-5, and IL-13 and induced production of Th1 cytokines IL-12 and IFN- γ in spleen cell cultures re-stimulated with OVA, suggesting that IMO 3 can reverse established allergen-induced immune responses. Serum OVA-specific and total IgE and IgG1 levels were lower and IgG2a levels were higher in OVA-sensitized and -challenged mice treated with IMO 3 than in those sensitized and challenged with OVA but not treated with IMO 3. Lung tissue inflammation was noticeably less in IMO 3-treated mice than in untreated mice, suggesting that IMO 3 treatment influenced not only the systemic immune response but also the local immune response. The immediate systemic immune response was Th1-type, with serum IL-12 and IFN- γ levels increasing within 2 h of IMO 3 injection. These studies in mouse models of allergic asthma support the idea that IMOs containing CpR stimulatory motifs potentially prevent and reverse allergen-induced immune responses as do conventional CpG DNAs [23,24].

In summary, we have shown that IMOs can prevent and reverse antigen-specific Th2-type immune responses in vivo. These results further demonstrate that a synthetic CpR motif is recognized by immune cells and, like the CpG motif, can shift an ongoing allergen-specific Th2 response towards a Th1 response. However, subtle differences are noted between the two motifs, suggesting that this may allow further optimization of IMOs with substitution of appropriate chemical moieties [30,31]. IMOs 2 and 3, which contained two 5' -ends, were more immunostimulatory than was conventional CpG DNA 1, which had single 5' -end, supporting the concept that TLR9 reads the CpG DNA sequence from the 5' -end [32–34]. The 3' –3' linkage present in IMOs also makes these compounds more stable against degradation by cellular nucleases than CpG DNA; increased stability may allow less frequent dosing for compounds used therapeutically. Thus, IMOs have several characteristics, including a potent ability to induce a Th1-type immune response and greater in vivo stability, which make them attractive

candidates to be used as antigen-specific immunotherapy for Th2-dominant inflammatory diseases, such as allergic asthma.

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