

Novel immunomodulatory oligonucleotides prevent development of allergic airway inflammation and airway hyperresponsiveness in asthma

Devendra K. Agrawal^{a,*}, Jehad Edwan^a, Ekambar R. Kandimalla^b, Dong Yu^b,
Lakshmi Bhagat^b, Daqing Wang^b, Sudhir Agrawal^{b,1}

^aMedical Microbiology and Immunology and Biomedical Sciences, Center for Allergy, Asthma and Immunology,
Creighton University School of Medicine, Omaha, NE 68178, USA

^bHybridon, Inc., 345 Vassar Street, Cambridge, MA 02139, USA

Received 20 September 2003; received in revised form 20 November 2003; accepted 20 November 2003

Abstract

Oligodeoxynucleotides containing unmethylated CpG motifs (CpG oligos) have been shown to prevent development of allergic airway inflammation and airway hyperresponsiveness (AHR) in mouse models of asthma. Recently, we reported immunomodulatory oligonucleotides (IMOs) containing novel structures (immunomers) and synthetic immunostimulatory CpR (R = 2'-deoxy-7-deazguanosine) motifs show potent stimulatory activity with distinct cytokine secretion profiles. Since type 2 T cells predominate in asthma and increase in type 1 cells can prevent the differentiation of naïve T lymphocytes to a type 2 phenotype, we hypothesized that IMOs can prevent the development of allergic airway inflammation and AHR in the ovalbumin (OVA)-sensitized and challenged mouse model. We found that co-administration of novel IMOs during OVA-sensitization abrogated both early and late allergic responses (LARs). AHR to methacholine was also blocked with IMO treatment. Analysis of bronchoalveolar lavage (BAL) fluid of mice treated with IMOs demonstrated complete reduction in eosinophils, with concomitant decreases in both serum and BAL fluid IL-4, IL-5, and IL-6 levels. In addition, there was a significant reduction in serum IL-10 levels. IMOs, in general, significantly attenuated the rise in serum IgE levels. In comparison, IMOs showed a significantly more potent effect on early and late allergic response than a conventional CpG oligo in this model. These data suggest that the treatment with these novel IMOs prevents OVA-induced allergic airway inflammation and AHR in asthma in the mouse and may provide a useful agent in the treatment of human asthma.

© 2004 Elsevier B.V. All rights reserved.

Keywords: Allergic airway inflammation; Asthma; CpG oligonucleotides; IgE; Immunomodulators; Immunomodulatory oligonucleotides; Interleukin-4

1. Introduction

Many kinds of immune cells and mediators contribute to the exacerbations and progress of allergy and asthma [1]. It is therefore not surprising

* Corresponding author. Tel.: +1-402-280-2938; fax: +1-402-280-1421.

E-mail addresses: dkagr@creighton.edu (D.K. Agrawal), sagrawal@hybridon.com (S. Agrawal).

¹ Tel.: +1-617-679-5501.

that there are likewise numerous potential modalities to treat the disease. Since the dynamic of the T_H1/T_H2 phenotype as it relates to allergy and asthma has become more fully appreciated, studies have been focused to modulate this balance with the goal of suppressing type 2 T cell responses [2–4]. Largely, these efforts have focused on stimulating antigen-specific T_H1 responses, as the T-helper subsets have been shown to be polarizing and mutually antagonistic in nature [5]. In recent years, several agents such as mycobacterial antigens of *Bacillus Calmette-Guerin* (BCG) and *M. vaccae*, which can up-regulate a type 1 response [6], have shown therapeutic activity for allergen-induced asthma both in experimental animal models [7–9] and in clinical studies [10,11]. Another immunomodulator Flt3-L, which is a recently described growth factor affecting primitive hematopoietic progenitor

cells in both humans and mice [12,13], has been reported to prevent features of ovalbumin (OVA)-induced allergic airway inflammation in the mouse model [14].

More recently, attention has focused on the unmethylated CpG motifs in bacterial and synthetic DNA, thought to be at least partially responsible for the immunogenic properties of BCG and its ability to activate type 1 cytokine production [15]. The drawbacks for mycobacteria may include (but are not limited to) latent infection, loss of delayed-type hypersensitivity skin testing for mycobacterial infection. Synthetic oligodeoxynucleotides containing unmethylated CpG dinucleotides in specific sequence contexts can potentially stimulate innate immune responses and thereby acquired immunity [15–17]. Decreases in IL-4, IL-5, IgE and eosinophilia have been observed in mouse models using CpG oligos,

A Oligo sequences, structures, and modifications



B Experimental protocol

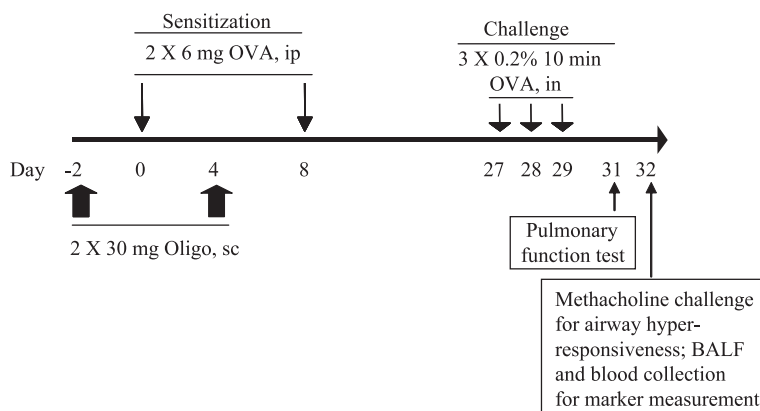


Fig. 1. (A) Sequences and structures of the immunomodulatory oligonucleotides used in the study. Sequence 1 is a conventional CpG oligo containing a mouse specific stimulatory motif (underlined). Sequences 2 and 3 have novel 3'-3'-structures linked through a glycerol linker. Additionally, sequence 3 has a synthetic CpR dinucleotide motif (R = 2'-deoxy-7-deazaguanosine). Arrows indicate 5' → 3' directionality of the CpG(R) motif. (B) Experimental protocol used in the study. BALB/c mice were sensitized to OVA by intraperitoneal injection on days 0 and 8. For immunoprevention, three groups of mice were given subcutaneous injection of oligos twice, once 2 days before and second time 4 days after the first injection of OVA. All groups of mice were challenged with OVA by inhalation on days 27, 28, and 29. Control group of mice received PBS for all injections.

either alone or conjugated to antigen [18–21]. However, CpG oligo-allergen conjugates effectively reverse established atopic eosinophilic airway disease, at least partially through redirecting a T_H2 to a T_H1 response [22].

It has been shown that bacterial and synthetic CpG DNAs activate intracellular immune signaling pathways through a pattern recognition receptor, Toll-like receptor 9 (TLR9) [23–25]. Our structure-immunostimulatory activity studies of CpG oligos identified critical structural features in the pentose sugar, phosphate backbone, nucleobases, and nucleosides required for activity [26–28]. Moreover, through the use of synthetic pyrimidine (Y) and purine (R) bases in YpG and CpR motifs, we showed requirements for functional group on cytosine and guanine in receptor recognition [29].

Importantly, our studies for the first time suggested that the receptor reads CpG oligo sequence from the 5'→3'-end and modifications that block the 5'-end abrogate immune stimulation [30,31]. In contrast, CpG oligos with two accessible 5'-ends (immunomers) show enhanced activity [32]. Recently, we showed immunomers containing synthetic CpR motif induce distinct cytokine profiles with lower IL-6 to IL-12 ratios compared with conventional CpG oligos [33]. Our preliminary in vitro studies of immunomers containing CpG and CpR motif showed potent reversal of antigen induced Th2 immune response towards a Th1 type in antigen sensitized and challenged mouse spleen cell cultures [33].

In the present study, we examined the effect of two immunomers **2** and **3** on early and late allergic response, airway hyperresponsiveness to methacholine, airway eosinophilia, serum IgE and serum and BAL fluid cytokines in a mouse model compared with a conventional CpG oligo **1**. The sequences and structures of oligonucleotides used in the study are shown in Fig. 1A. Both IMO immunomers **2** and **3** contain a novel structure. In these sequences two short nucleotide segments, containing appropriate stimulatory motifs, are attached through a glycerol linker (shown as X in the sequence) through their 3'-ends [31–33]. Immunomer **3** contains a synthetic CpR (R=2'-deoxy-7-deazaguanosine) dinucleotide [30,33] in contrast to a natural CpG dinucleotide in oligos **1** and **2** (Fig. 1A).

2. Materials and methods

2.1. Oligonucleotide synthesis and purification

CpG oligos **1–3** were synthesized on a 1 to 2 μ mol scale as described earlier [33]. Purity and molecular mass of the oligos were confirmed by CGE and MALDI-TOF mass spectrometry (Applied Biosystem's Voyager-DETM STR BiospectrometryTM Workstation), respectively. All oligos contained less than 0.075 EU/mL of endotoxin as determined by the Limulus assay (Bio-Whittaker).

2.2. Serum cytokine levels in mice

Female BALB/c mice (4–6 weeks, 19–21 g) were divided into groups of three mice. CpG DNAs were dissolved in sterile PBS and administered intraperitoneally (IP) to mice at a dose of 10 mg/kg. Blood was withdrawn from animals by retro-orbital puncture 4 h after oligo administration for cytokine assays. The IL-12 and IL-6 levels in serum samples were determined by ELISA as described previously [33].

2.3. Preparation of J774 nuclear extracts and EMSA for NF- κ B activation

J774 cells were plated at a density of 5×10^6 cells/well in six-well plates, treated for 1 h with CpG DNAs at a concentration of 10 μ g/ml and nuclear extracts were prepared as described earlier [33]. Protein concentrations were determined and the samples were either used immediately or stored frozen at -70°C . Reaction mixtures (25 μ l) containing 10 μ g of nuclear protein, 5 mM Tris (pH 7.5), 100 mM NaCl, 1 mM DTT, 1 mM EDTA, 4% glycerol (v/v), and 0.08 mg/mL salmon sperm DNA were preincubated on ice for 15 min. Then 1×10^6 cpm of 5'-³²P-labeled oligonucleotide probe (5'-AGTTGAGGGGACTTTCCC AGGC-3') was added and the binding reaction was allowed to proceed for 20 min at room temperature. DNA-protein complexes were then resolved in a 6% native polyacrylamide gel in TBE buffer (22.5 mM Tris, 22.5 mM boric acid and 0.5 mM EDTA, pH 8.3) at 140 V for 2–3 h. Gels were dried and exposed to Kodak Biomax MR film at -70°C . Films were scanned

and the images were processed using Adobe imaging software.

2.4. Sensitization and treatment

Four- to five-week-old female Balb/c mice (Harlan Laboratories, Indianapolis, IN) were housed in the animal facility of Creighton University according to the NIH guidelines and were allowed food and water ad libitum. Animals were sensitized to ovalbumin (OVA) according to the protocol previously reported [7,8] (Fig. 1B). Briefly, animals were administered with intraperitoneal (i.p.) injections of 6 µg of grade V chicken egg OVA (Sigma-Aldrich, St. Louis, MO) adsorbed to 2.25 mg Imject Alum (Pierce, Rockford, IL) and dissolved in PBS on days 0 and 8. Aerosol sensitization with 0.2% OVA for 10 min using an Ultra-Neb 90 nebulizer (DeVilbiss, Somerset, PA) were carried out on days 27, 28 and 29. Non-sensitized control mice received injection and aerosolization of PBS alone. Mice received immunomodulatory oligonucleotides (IMOs) two times by subcutaneous (s.c.) administration (30 µg/mice); first injection 2 days prior to the first sensitization with OVA and the second s.c. administration 4 days after the first OVA sensitization (Fig. 1B). Oligos were dissolved in sterile PBS.

2.5. Pulmonary functions

Pulmonary functions were measured according to the method previously reported [7–9]. Briefly, on day 31 all mice were placed in whole-body plethysmograph chambers (Buxco Electronics, Troy, NY) and baseline enhanced pause (Penh) readings taken. Penh is a dimensionless unit used to monitor airway resistance and has been positively correlated with pulmonary resistance (R_L) and change in intrapleural pressure (ΔP) as well as the response to OVA challenge. Challenge with aerosolized 0.2% OVA for 5 min was followed by recording of breathing parameters for 9 h to continuously record both the early allergic response (EAR) and late allergic response (LAR). The area-under-the-curve (AUC) was determined for each animal, using the baseline and Penh values from 0 to 15 min for the EAR and the baseline and Penh values from 1 to 5 h for the LAR

(GraphPad Prism version 3 statistical software, San Diego, CA). On day 32, 24 h following OVA challenge, mice were again placed within the chambers and challenged with increasing doses of methacholine (Sigma-Aldrich) to measure airway hyperresponsiveness (AHR). Penh values for each animal were recorded, and mean Penh values $\pm$ S.E.M. calculated.

2.6. Bronchoalveolar lavage (BAL)

BAL fluid was retrieved from each animal via cannulation of the exposed trachea and gentle flushing of the lungs with four 1 ml aliquots of PBS. Aliquots were pooled for individual animals preceding centrifugation and separation of pelleted cells and supernatant. Cytospin preparations of BAL cells were stained using Hema 3 (Biochemical Sciences, Swedesboro, NJ) differential stain and relative cell populations were determined using standard morphological criteria.

2.7. Serum immunoglobulin analysis

Blood was collected after sacrificing animals on day 32, immediately centrifuged, and serum was collected and stored at -70°C until analysis. ELISA for both total and antigen-specific IgE was conducted as previously described [7–9]. Both assays were developed with 3,3',5,5'-Tetramethyl-benzidine (TMB) substrate and read at 450 nm using a Bio-Rad microplate reader and software. Sensitivity for total IgE levels was 1 ng/ml.

2.8. Cytokine assays

Cytokines were measured in the supernatants of BAL fluid as well as in serum. Samples were analyzed by ELISA at LINCO Diagnostic Services for Cytokine measurements. Assay sensitivities for IL-4, IL-5, IL-6, IL-10, IL-12 and IFN- γ were 3, 3, 3, 3, 16, and 3 pg/ml, respectively.

2.9. Statistical analysis

Data were analyzed using GraphPad PRISM statistical analysis and graphing software. Unpaired Student's *t*-test was used to determine differences

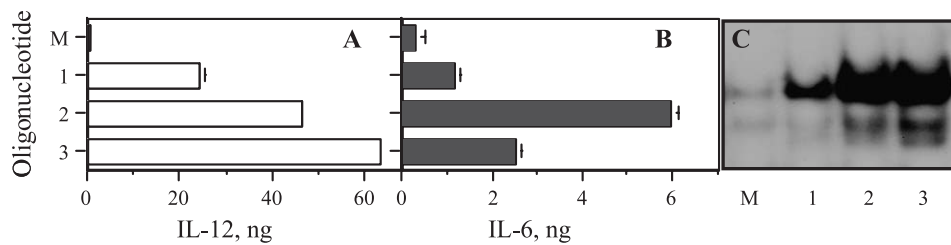


Fig. 2. In vivo serum cytokine induction by oligos 1–3 in BALB/c mice. (A) IL-12 and (B) IL-6. Data represents mean of three mice $\pm$ S.D. (C) An EMSA gel showing the bands corresponding to NF- κ B activation in J774 macrophages in response to oligo stimulation. Oligo numbers are shown at the bottom of the gel. M stands for mice or cells treated with PBS or medium alone, respectively.

between the groups and multiple group comparisons were made with ANOVA. A p value of ≤ 0.05 was considered significant.

3. Results

3.1. In vivo cytokine induction profiles of IMOs

To compare the cytokine profiles induced by IMOs in vivo, a single dose of oligos 1–2 was injected to

mice intraperitoneally at 10 mg/kg and serum IL-12 and IL-6 levels were determined after 4 h by ELISA. All three oligos induced IL-12 secretion within 4 h of oligo administration. The induction of IL-12 levels by oligos was in the order 3>2>1 (Fig. 2A). In general, a lower serum IL-6 was observed at 4 h time point with all three oligos compared with IL-12 levels (Fig. 2B). These results are consistent with our earlier in vitro studies [33], and confirm that CpR motif in immunomers induces lower levels of IL-6 compared with immunomers containing natural CpG motif.

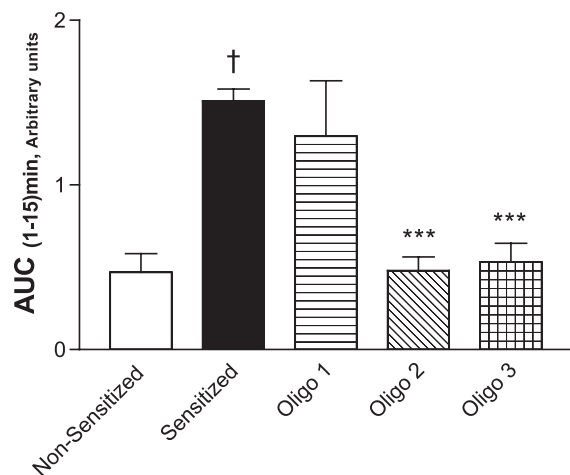


Fig. 3. Effect of IMOs on early allergic response (EAR) as measured by area under the curve (AUC). Mice were challenged with 0.2% ovalbumin on day 31 and Penh values were recorded immediately thereafter every 3 min up to first 15 min for pulmonary function. The AUC from 0 to 15 min was calculated for individual mice and expressed in arbitrary units. Data represent mean $\pm$ S.E.M. from 8 to 10 individual animals. $\dagger p < 0.001$ as compared to non-sensitized group; $***p < 0.001$ as compared to the OVA-sensitized and challenged group.

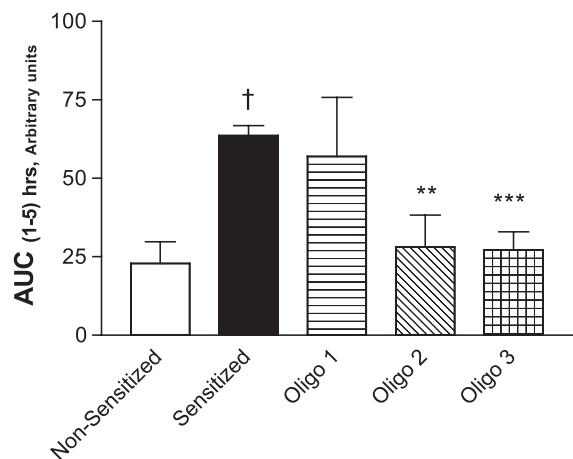


Fig. 4. Effect of IMOs on late allergic response (LAR). The pulmonary function in the same mice as in Fig. 1 was examined using barometric plethysmography from 1 to 5 h. Penh units were recorded and calculated as the AUC using the same individual baselines as used for the EAR. Data represent mean $\pm$ S.E.M. from 8 to 10 individual animals in each experimental group. $**p < 0.01$ as compared to the OVA-sensitized and challenged group; $\dagger p < 0.001$ as compared to non-sensitized group; $***p < 0.001$ as compared to the OVA-sensitized and challenged group.

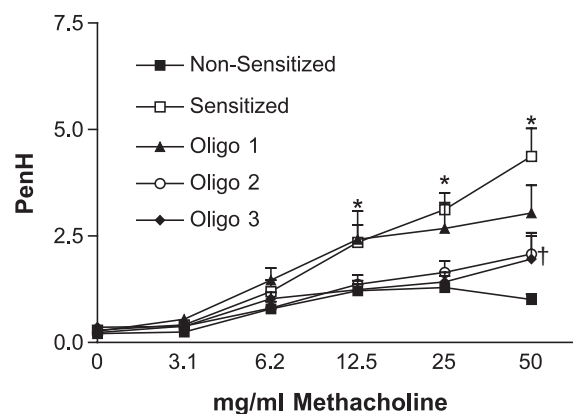
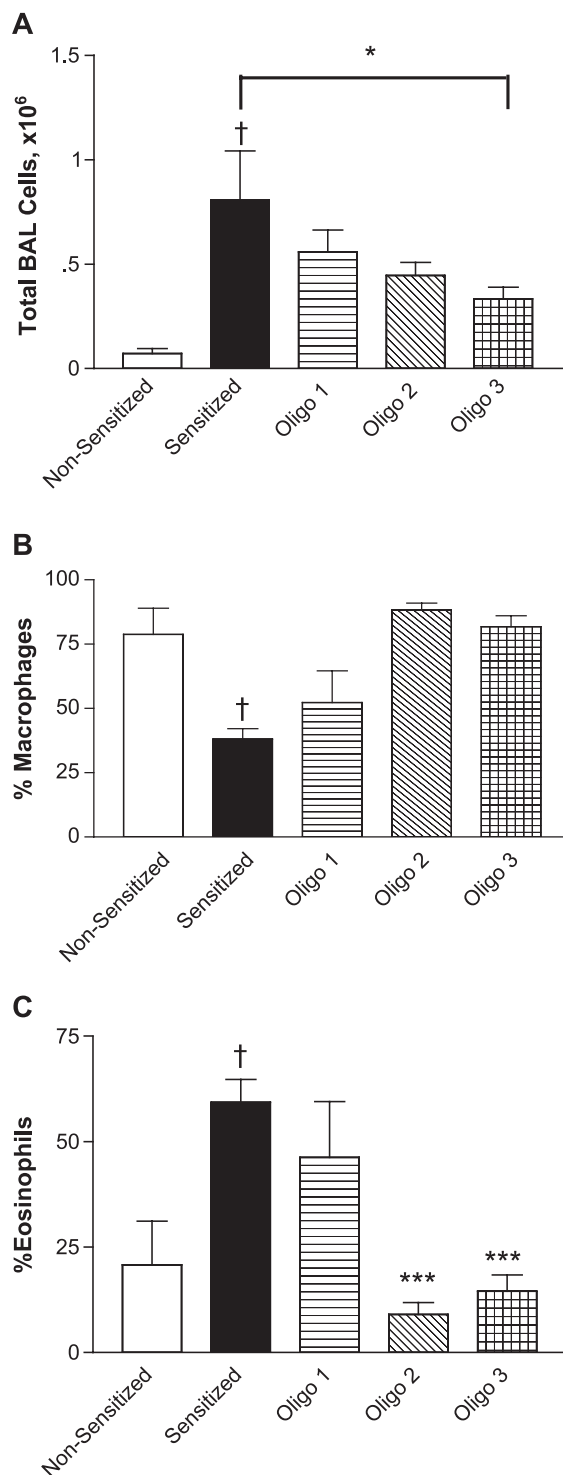


Fig. 5. Effect of IMOs on airway hyperresponsiveness (AHR) to methacholine in OVA-sensitized and challenged mice. Mice were sensitized to OVA (except the non-sensitized controls) and treated with either vehicle or IMOs s.c. Twenty-four hours following OVA challenge mice were placed into individual barometric plethysmograph chambers and exposed to increasing concentrations of aerosolized methacholine in PBS. Shown are absolute Penh means $\pm$ S.E.M. for 8–10 animals in each experimental group with comparisons to sensitized control animals. Methacholine concentration in mg/ml is shown on the abscissa and absolute Penh values are shown on the ordinate. $\dagger p < 0.001$ as compared to OVA-sensitized and challenged group; $*p < 0.05$ as compared to non-sensitized group.

3.2. Activation of NF- κ B in J774 cell cultures by IMOs

TLR9 activation by CpG DNA leads to activation of a number of intracellular signaling pathways, including the NF- κ B pathway, which plays a critical role in the up-regulation of cytokine gene expression. To further examine the differences among the three oligos, we studied the activation of the transcription factor, NF- κ B, in J774 cells following 1 h stimulation with oligos and by analysis of the nuclear extracts by EMSA. The results are presented in Fig. 2C. All three oligos showed NF- κ B activation within 1 h of stimulation.

Fig. 6. Effect of IMOs on (A) total number of cells, (B) macrophages, and (C) total eosinophilia in the BAL fluid of OVA-sensitized and challenged mice. Values represent mean $\pm$ S.E.M. from 8 to 10 individual animals in each experimental group. $\dagger p < 0.001$ as compared to non-sensitized group; $*p < 0.05$ and $***p < 0.001$ as compared to OVA-sensitized and challenged group.



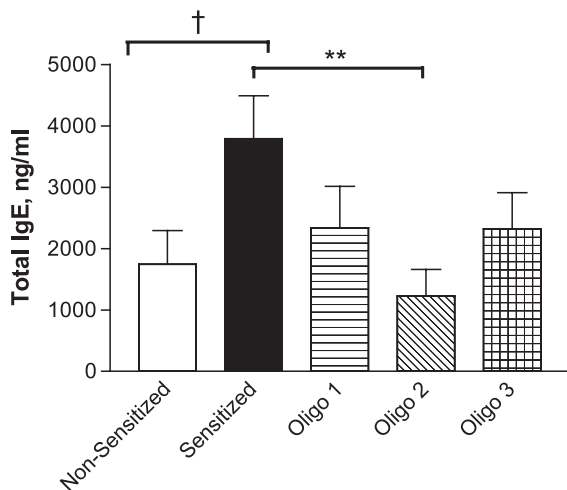


Fig. 7. Effect of IMOs on total serum IgE levels in OVA-sensitized and challenged mice. OVA/PBS group represents animals sensitized and challenged with OVA but were administered with PBS instead of oligos. PBS group is non-sensitized but challenged with ovalbumin. Mice were sensitized and treated as described in Section 2. Serum antibody production for total IgE was determined using standard ELISA. Mice were bled following methacholine challenge, 24 h following OVA challenge. Presented are means $\pm$ S.E.M. for 8–10 animals per group. † $p < 0.001$ as compared to non-sensitized group; ** $p < 0.01$ as compared to OVA-sensitized and challenged group.

However, oligos **2** and **3** showed significant activation of NF- κ B in this time period compared with oligo **1** (Fig. 2C).

3.3. IMOs suppress airway resistance during the EAR and LAR in OVA-sensitized animals

There was a significant increase in the EAR and LAR in OVA-sensitized and challenged group (Fig. 3). Conventional CpG oligo **1** had an insignificant effect on EAR as compared to the OVA-sensitized and challenged group (sensitized). However, both IMOs **2** and **3** significantly ($p < 0.001$) suppressed the early allergic response in OVA-sensitized and challenged animals (Fig. 3). Late allergic response to antigen was almost abolished by both **2** and **3** and the values in these experimental groups were not significantly different from the non-sensitized group. However, as in the case of EAR, CpG oligo **1** had no significant effect on LAR (Fig. 4).

3.4. IMOs suppress airway hyperresponsiveness to methacholine

A significant increase in airway hyperresponsiveness (AHR) to methacholine was observed in OVA-sensitized and challenged mice (Fig. 5). The AHR, as measured in absolute Penh values, was completely abolished by both **2** and **3** and the pulmonary function in animals of these experimental groups was not significantly different from the non-sensitized group at any dose (Fig. 5). CpG oligo **1** partially protected against AHR to methacholine (Fig. 5).

3.5. IMOs suppress BAL eosinophilia

While both **2** and **3** yielded apparent reductions in total BAL leukocytes, only animals treated with **3** showed significant reduction (Fig. 6A). Macrophage percentage in BALF of mice treated with **2** and **3** reached to similar levels as found in non-sensitized mice (Fig. 6B). Although we have not performed FACS analysis, these cells exhibited morphometric characteristics of macrophages. There was a significant increase in BAL eosinophilia in OVA-sensitized and challenged mice. Eosinophils were significantly reduced in animals treated with **2** and **3** (Fig. 6C).

3.6. Reduction in total serum IgE levels after treatment with IMOs

CpG oligos have been shown to reduce serum total IgE levels in treated mice. In the present study, although all three oligos had a tendency to reduce

Table 1
Effect of oligos on BALF cytokines^a in OVA-sensitized and challenged mice

Experimental group	IL-4	IL-5	IL-6
PBS	<3	4 $\pm$ 1	39 $\pm$ 9
OVA/PBS	13 $\pm$ 3	39 $\pm$ 4	83 $\pm$ 7
Oligo 1	<3*	23 $\pm$ 3**	17 $\pm$ 5*
Oligo 2	5.5 $\pm$ 1.3*	24 $\pm$ 2**	34 $\pm$ 6*
Oligo 3	<3*	14 $\pm$ 2*	27 $\pm$ 3*

^a Cytokine levels are in pg/ml and calculated as mean $\pm$ S.E.M. from five to seven animals in each experimental group.

* $p < 0.01$ as compared to the OVA-sensitized and challenged group (OVA/PBS).

** $p < 0.05$ as compared to the OVA-sensitized and challenged group (OVA/PBS).

Table 2

Effect of oligos on serum cytokines^a in OVA-sensitized and challenged mice

Experimental group	IL-4	IL-5	IL-6	IL-10
PBS	<3	10 ± 3	18 ± 5	18 ± 4
OVA/PBS	22 ± 3	33 ± 5	53 ± 9	172 ± 12
Oligo 1	<3*	6 ± 4*	27 ± 6**	32 ± 9*
Oligo 2	<3*	7 ± 2*	44 ± 4**	94 ± 12*
Oligo 3	<3*	<3*	14 ± 1*	58 ± 9*

^a Cytokine values are denoted as pg/ml and calculated as mean ± S.E.M. from five to seven animals in each experimental group.

* $p < 0.01$ as compared to the OVA-sensitized and challenged group (OVA/PBS).

** $p < 0.05$ as compared to the OVA-sensitized and challenged group (OVA/PBS).

serum total IgE levels, **2** was most effective in reducing serum total IgE levels compared with **1** and **3** (Fig. 7). Interestingly, there was no significant difference in the mean IgE levels among the three oligos.

3.7. BAL fluid and serum cytokine levels are suppressed with IMO treatment

Supernatants collected from BAL fluid were analyzed for the presence of several cytokines. Concentrations of IL-10, IL-12 and IFN- γ in BAL fluid were below the detectable level. This could be as a result of measurement of these early responsive cytokines nearly after 4 weeks of last administration of oligos to animals. Nevertheless, there was a significant increase in IL-4, IL-5, and IL-6 in the BAL fluid of OVA-sensitized and challenged mice. All three oligos significantly reduced IL-4, IL-5 and IL-6 in the BAL fluid (Table 1). In the serum of OVA-sensitized and challenged animals, there was a significant increase in IL-4, IL-5, IL-6, and IL-10 levels. Both IL-12 and IFN- γ were below the detectable levels. All three oligos significantly almost abolished the rise in serum IL-4, IL-5, and IL-6 levels (Table 2). Additionally, they also significantly reduced serum IL-10 levels.

4. Discussion

Immune modulators can shape the host response to challenge with innocuous antigens and protect against

allergic airway inflammation in asthma and possibly other allergic diseases. So far, several immunomodulators have been reported to attenuate allergic airway inflammation [5], none of them free from adverse effects. In recent years, unmethylated CpG oligos, which induce potent Th1 immune responses, have gained momentum as novel immunomodulators of allergic airway inflammation [18–22,34]. Bacterial and synthetic CpG DNAs promote B-cell proliferation, immunoglobulin production and the secretion of a number of cytokines, including IL-12, IFN- γ , IFN- α , IL-6 and TNF- α , from B-cells, monocytes/macrophages, dendritic cells and NK cells. It has been shown that a receptor, TLR9, is engaged in the recognition of CpG DNA and subsequent initiation of signaling cascade leading to the activation of the transcription factor NF- κ B. We showed that CpG oligos containing two accessible 5'-ends are more immunostimulatory than a conventional CpG oligo and the receptor recognizes a synthetic CpR motif in immunomers similar to that of the natural CpG motif [26–28,30–33].

Consistent with earlier in vitro studies [33], both oligos **2** and **3** induced higher IL-12 levels in mice. These in vivo cytokine secretion profiles are in parallel with the ability of oligos **1–3** to activate NF- κ B in J774 macrophages. Additionally, the incorporation of CpR chemical modification in oligos has been shown to induce lower levels of IL-6, but similar levels of IL-12 compared to that of an immunomer containing natural CpG motif in vitro [33]. Increased activity of immunomers also results from two accessible 5'-ends for TLR9 recognition and higher metabolic stability in vivo [33,35]. The current results further suggest that oligos containing immunomer structure are more immunostimulatory and support their comparative evaluation to prevent OVA-induced allergic asthma in mouse models.

We examined and compared the effect of novel IMOs **2** and **3**, which contain novel structure and CpG or synthetic CpR motifs, on several parameters of allergic airway inflammation. Analysis of OVA-specific airway resistance of both the EAR and LAR revealed that non-sensitized control mice had significantly lower airway resistance during either EAR or LAR ($p < 0.05$, EAR; $p < 0.001$, LAR, both compared to sensitized controls). Administration of IMOs during antigen sensitization exhibited significant protection

during both EAR and LAR. These findings were further corroborated by the effect on AHR and serum IgE analysis. Although not universally accepted [36], the LAR and AHR results have been positively linked to the presence of eosinophils in the airways [37]. Interestingly, IMOs significantly suppressed the levels of key cytokines, IL-4 and IL-5, which are involved in allergic airway inflammation, both in serum and BAL fluid. These responses were observed in samples collected 28 days after cessation of IMO treatment, supporting a stable shift in the type1/type 2 cytokine profile to a type 1 profile. IL-5 is a critical growth factor for eosinophils and also acts as a potent stimulant and chemotactic factor [38]. Indeed, we observed significantly decreased numbers of eosinophils in the BAL fluid immediately following methacholine challenge.

A direct effect of IMOs on the OVA challenge is unlikely as the last administration of IMOs was over 3 weeks prior to challenge. This suggests that the induction of a type 1 T cell bias may be more long lasting and is responsible for the decrease in asthmatic symptoms and is likely to occur via an increased type 1 response.

Treatment with CpG oligos has been shown to prevent and reverse allergic airway inflammation in experimental animals [18–22,34]. In recent studies, CpG oligos have also been shown to attenuate pathophysiological changes in the remodeled airways of chronic asthma [39,40]. These include decrease in subepithelial fibrosis and goblet cell metaplasia [39,40]. Effects of CpG oligos are dependent on specific sequences, dose, route of administration, metabolic stability, and animal species. Furthermore, immunostimulatory effects of CpG oligos between animal species and human may depend on the sequence. For example, GACGTT sequence motif elicits optimal immunostimulatory effects in mice [16], whereas GTCGTT motif produces optimal effects in isolated human cells [41].

Immunostimulatory effects of CpG oligos primarily involve the differentiation and activation of type 1 T cell response and attenuation of type 2 T cell responses. In the airways, CpG oligos directly activate dendritic cells and B cells via TLR9 to elicit their immunostimulatory effects [23–25] and the effects on NK cells could be indirect [42]. In this study, *in vivo* effect of IMOs on the release of cytokines in both

serum and BAL fluid and total serum IgE suggest their effect on antigen-presentation and activation of B cells. Indeed, recently, Liu et al. [43] reported that CpG oligos trigger anti-allergic responses by directly regulating the expression of T-bet via a TLR9-dependent signaling pathway in B cells which was synergistic with IL-12, but not dependent on IFN- γ -induced STAT1. However, effects of CpG oligos depend on structure, nucleotide sequence, and nature of the backbone. For example, CpG oligos with phosphorothioate backbone preferentially stimulate B cells to produce cytokines, secrete IgM [16,44] and also up-regulated T-bet mRNA [43]. On the other hand, CpG oligos containing G-rich and palindromic phosphodiester backbone sequences preferentially stimulate IFN- γ production from plasmacytoid dendritic cells [15,44]. Although we have not directly examined, since oligos containing CpR motifs attenuates serum IgE levels with a decrease in the concentrations of serum and BAL fluid IL-4, IL-5, and IL-6 and serum IL-10, it is very likely that CpR oligos might up-regulate transcription and translation of T-bet in the airways.

The T cell response, regulated by concurrent exposure to both OVA and IMOs, may also provide an important mediator in this apparently novel mechanism of action for the treatment of asthma. The increase in type 1 response was observed but not type 2, which may suggest several things. Okano et al. [45] observed enhanced IFN- γ and inhibited IL-5 production, without an effect on GM-CSF level, after *in vitro* stimulation of blood mononuclear cells with IL-12. Since the preponderance of dendritic cells expanded by CpG oligo treatment (the lymphoid dendritic cell population) have been shown to release IL-12 [46], this may provide an additional mechanism in our studies [33]. Secondly, Hudak et al. [47] showed that cultures of eosinophil progenitors containing only GM-CSF were still able to stimulate colony formation, approximately half as potently as the GM-CSF/IL-5 combination. These authors did find a synergistic effect of an IL-6/CpG oligo combination on proliferation in primitive bone marrow cells [47]. The levels of IL-6 have been directly linked to IgE production [48,49]. The property of IMOs to induce lower IL-6 could be an added advantage for their use in allergic-asthma [33]. As mentioned previously, naïve B cells (and later memory B cells) may have escaped CpG

oligo-induced modulation in bypassing the use of dendritic cells during the initial antigen response. These cells may have been responsible for some of the type 2-like profile observed in this study. Secondly, CpG oligo-induced dendritic cells are not a uniform group, with only a predominance of the lymphoid subset over the myeloid subset [50]. The myeloid subpopulation may further enhance allergic sensitization.

In summary, we showed that treatment with oligos containing novel structures and synthetic stimulatory motifs (CpR) in a mouse model of antigen-induced allergic airway inflammation prevents OVA-specific airway resistance of EAR, LAR, AHR to methacholine and BAL eosinophilia and decreases serum IgE and IL-4 levels. Increased effect of IMOs 2 and 3 as compared to the conventional CpG oligo 1 is likely as a result of their potent immunostimulation; distinct cytokine profile induction, and increased metabolic stability against cellular nucleases [33]. The properties of higher metabolic stability, and greater immunostimulatory activity may permit lower dosing of immunomodulators, which will be examined in an acute allergic inflammation model. Recently, we showed that immunomodulators containing natural phosphodiester backbone potentially prevent and reverse ovalbumin-induced Th2 responses in vitro and in vivo [51]. Studies examining the effects of these novel IMOs containing CpR, YpG, and YpR motifs on the reversal of acute allergic inflammation and pathophysiological changes in chronic asthma are currently underway.

References

- [1] Busse WW, Lemanski Jr RF, Asthma N. *Engl J Med* 2001; 344:350–62.
- [2] Umetsu DT, McIntire JJ, Akbari O, Macaubas C, DeKruyff RH. Asthma: an epidemic of dysregulated immunity. *Nat Immunol* 2002;3:715–20.
- [3] Janssen EM, van Oosterhout AJ, van Rensen AJ, van Eden W, Nijkamp FP, Wauben MH. Modulation of Th2 responses by peptide analogues in a murine model of allergic asthma: amelioration or deterioration of the disease process depends on the Th1 or Th2 skewing characteristics of the therapy with peptide. *J Immunol* 2000;164:580–8.
- [4] von Hertzen LC, Haahtela T. Could the risk of asthma and atopy be reduced by a vaccine that induces a strong T-helper type 1 response?. *Am J Respir Cell Mol Biol* 2000;22: 139–142.
- [5] Agrawal DK, Hopfenspirger MT, Edwan J. Immunomodulators in allergic airway inflammation in asthma. *Mod Asp Immunobiol* 2004;1:37–41.
- [6] Nahori MA, Lagranderie M, Lefort J, Thouron F, Joseph D, et al. Effects of *Mycobacterium bovis* BCG on the development of allergic inflammation and bronchial hyperresponsiveness in hyper-IgE BP2 mice vaccinated as newborns. *Vaccine* 2001; 19:1484–95.
- [7] Hopfenspirger MT, Parr SK, Hopp RJ, Townley RG, Agrawal DK. Mycobacterial antigens attenuate late allergic response, airway hyperresponsiveness, and bronchoalveolar lavage eosinophilia in a mouse model of bronchial asthma. *Int Immunopharmacol* 2001;1:1743–51.
- [8] Hopfenspirger MT, Parr SK, Townley RG, Agrawal DK. Attenuation of the late allergic response by mycobacterial antigens is independent of IgE in a mouse model of asthma. *Allergol Intern* 2002;51:21–32.
- [9] Hopfenspirger MT, Agrawal DK. Airway hyperresponsiveness, late allergic response, and eosinophilia are reversed with mycobacterial antigens in ovalbumin-prensensitized mice. *J Immunol* 2002;168:2516–22.
- [10] Shirakawa T, Enomoto T, Shimazu S, Hopkin JM. The inverse association between tuberculin responses and atopic disorder. *Science* 1997;275:77–9.
- [11] Aaby P, Shaheen SO, Heyes CB, Goudiaby A, Hall AJ, et al. Early BCG vaccination and reduction in atopy in Guinea-Bissau. *Clin Exp Allergy* 2000;30:644–50.
- [12] Lyman SD, James L, Johnson L, Brasel K, de Vries P, et al. Cloning of the human homologue of the murine flt3 ligand: a growth factor for early hematopoietic progenitor cells. *Blood* 1994;83:2795–801.
- [13] Pulendran B, Banchereau J, Burkeholder S, Kraus E, Guinet E, et al. Flt3-ligand and granulocyte colony-stimulating factor mobilize distinct human dendritic cell subsets in vivo. *J Immunol* 2000;165:566–72.
- [14] Agrawal DK, Hopfenspirger MT, Chavez J, Talmadge JE. Flt3-Ligand attenuates late phase response, airway hyperresponsiveness, and bronchoalveolar lavage eosinophilia in a mouse model of bronchial asthma. *Int Immunopharmacol* 2001;1:2081–9.
- [15] Tokunaga T, Yamamoto T, Yamamoto S. How BCG led to the discovery of immunostimulatory DNA. *Jpn J Infect Dis* 1999;52:1–11.
- [16] Krieg AM, Yi AK, Matson S, Wadsworth TJ, Bishop GA, et al. CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature* 1995;374:546–9.
- [17] Janeway Jr CA, Medzhitov R. Innate immune response. *Annu Rev Immunol* 2002;20:197–216.
- [18] Broide D, Schwartz J, Tighe H, Gifford T, Nguyen MD, et al. Immunostimulatory DNA sequences inhibit IL-5, eosinophilic inflammation, and airway hyperresponsiveness in mice. *J Immunol* 1998;161:7054–62.
- [19] Kline JN, Waldschmidt TJ, Businga TR, Lemish JE, Weinstein JV, et al. Modulation of airway inflammation by CpG oligonucleotides in a murine model of asthma. *J Immunol* 1998;160:2555–9.
- [20] Sur S, Wild JS, Choudhury BK, Sur N, Alam R, Klinman DM.

- Long term prevention of allergic lung inflammation in a mouse model of asthma by CpG oligodeoxynucleotides. *J Immunol* 1999;162:6284–93.
- [21] Broide DH, Stachnick G, Castaneda D, Nayar J, Miller M, et al. Systemic administration of immunostimulatory DNA sequences mediates reversible inhibition of Th2 responses in a mouse model of asthma. *J Clin Immunol* 2001;21:175–82.
 - [22] Santeliz JV, Van Nest V, Traquina P, Larsen E, Wills-Karp M. Amb a 1-linked CpG oligodeoxynucleotides reverse established airway hyperresponsiveness in a murine model of asthma. *J Allergy Clin Immunol* 2002;109:455–62.
 - [23] Hemmi H, Takeuchi O, Kawai T, Kaisho T, Sato S, et al. A Toll-like receptor recognizes bacterial DNA. *Nature* 2000;408:740–5.
 - [24] Bauer S, Kirschning CJ, Hacker H, Redecke V, Hausmann S, et al. Human TLR9 confers responsiveness to bacterial DNA via species-specific CpG motif recognition. *Proc Natl Acad Sci U S A* 2001;98:9237–42.
 - [25] Ashkar AA, Rosenthal KL. Toll-like receptor 9, CpG DNA and innate immunity. *Curr Mol Med* 2002;2:545–56.
 - [26] Agrawal S, Kandimalla ER. Medicinal chemistry and therapeutic potential of CpG DNA. *Trends Mol Med* 2002;8:114–21.
 - [27] Kandimalla ER, Yu D, Agrawal S. Towards optimal design of second-generation immunomodulatory oligonucleotides. *Curr Opin Mol Ther* 2002;4:122–9.
 - [28] Kandimalla ER, Zhu FG, Bhagat G, Yu D, Agrawal S. Toll-like receptor 9: modulation of recognition and cytokine induction by novel synthetic CpG DNAs. *Biochem Soc Trans* 2003;31:654–8.
 - [29] Kandimalla ER, Yu D, Zhao Q, Agrawal S. Effect of chemical modifications of cytosine and guanine in a CpG-motif of oligonucleotides: structure-immunostimulatory activity relationships. *Bioorg Med Chem* 2001;9:807–13.
 - [30] Yu D, Zhao Q, Kandimalla ER, Agrawal S. Accessible 5'-end of CpG-containing phosphorothioate oligonucleotides is essential for immunostimulatory activity. *Bioorg Med Chem Lett* 2000;10:2585–8.
 - [31] Kandimalla ER, Bhagat L, Yu D, Cong YP, Tang J, Agrawal S. Conjugation of ligands at the 5'-end of CpG DNA affects immunostimulatory activity. *Bioconjug Chem* 2002;13:966–74.
 - [32] Yu D, Kandimalla ER, Bhagat L, Tang JY, Cong YP, et al. Immunomers-novel 3'-3'-linked CpG oligodeoxyribonucleotides as potent immunomodulatory agents. *Nucleic Acids Res* 2002;30:4460–9.
 - [33] Kandimalla ER, Bhagat L, Wang D, Yu D, Zhu F.-G., et al. Divergent synthetic nucleotide motif recognition pattern: design and development of potent immunomodulatory oligodeoxyribonucleotide agents with distinct cytokine induction profiles. *Nucleic Acids Res* 2003;31:2393–400.
 - [34] Serebrisky D, Teper AA, Huang CK, Lee SY, Zhang TF, et al. CpG oligodeoxynucleotides can reverse Th2-associated allergic airway responses and alter the B7.1/B7.2 expression in a murine model of asthma. *J Immunol* 2000;165:5906–12.
 - [35] Yu D, Zhu F.-G., Bhagat L, Wang H, Kandimalla ER, et al. Potent CpG oligonucleotides containing phosphodiester linkages: in vitro and in vivo immunostimulatory properties. *Biochem Biophys Res Commun* 2002;297:83–90.
 - [36] Wilder JA, Collie DD, Wilson BS, Bice DE, Lyons CR, Lipscomb MF. Dissociation of airway hyperresponsiveness from immunoglobulin E and airway eosinophilia in a murine model of allergic asthma. *Am J Respir Cell Mol Biol* 1999;20:1326–34.
 - [37] Cieslewicz G, Tomkinson A, Adler A, Duez C, Schwarze J, et al. The late, but not early, asthmatic response is dependent on IL-5 and correlates with eosinophil infiltration. *J Clin Invest* 1999;104:301–8.
 - [38] Teran LM. Chemokines and IL-5: major players of eosinophil recruitment in asthma. *Clin Exp Allergy* 1999;29:287–90.
 - [39] Jain VV, Kitagaki K, Businga T, Hussain I, George C, et al. CpG-oligodeoxynucleotides inhibits airway remodeling in a murine model of chronic asthma. *J Allergy Clin Immunol* 2002;110:867–72.
 - [40] Ikeda RK, Nayar J, Cho JY, Miller M, Rodriguez M, et al. Resolution of airway inflammation following ovalbumin inhalation. Comparison of ISS DNA and corticosteroids. *Am J Respir Cell Mol Biol* 2003;28:655–63.
 - [41] Hartmann G, Weeratna RD, Ballas ZK, Payette P, Blackwell S, et al. Delineation of a CpG phosphorothioate oligodeoxynucleotide for activating primate immune responses in vitro and in vivo. *J Immunol* 2000;164:1617–24.
 - [42] Broide DH, Stachnick G, Castaneda D, Nayar J, Miller M, et al. Immunostimulatory DNA mediates inhibition of eosinophilic inflammation and airway hyperactivity independent of natural killer cells in vivo. *J Allergy Clin Immunol* 2001;108:759–63.
 - [43] Liu N, Ohnishi N, Ni L, Akira S, Bacon KB. CpG directly induces T-bet expression and inhibits IgG1 and IgE switching in B cells. *Nat Immunol* 2003;4:687–93.
 - [44] Verthelyi D, Ishii K, Gursel M, Takeshita F, Klinman DM. Human peripheral blood cells differentially recognize and respond to two distinct CpG motifs. *J Immunol* 2001;166:2372–7.
 - [45] Okano Y, Nakamura Y, Sano T, Azuma M, Sone S. Interleukin-12 inhibits production of interleukin-5 but not of granulocyte/macrophage colony-stimulating factor by antigen-stimulated blood mononuclear cells in allergic bronchial asthmatics. *Int Arch Allergy Immunol* 1998;115:83–90.
 - [46] Klinman DM, Yi AY, Beaucage SL, Conover J, Krieg AM. CpG motifs present in bacterial DNA rapidly induce lymphocytes to secrete interleukin-6, interleukin 12, and interferon- γ . *Proc Natl Acad Sci U S A* 1996;93:2879–83.
 - [47] Hudak S, Hunte B, Culpepper J, Menon S, Hannum C, Snipes LT. FLT3/FLK2 ligand promotes the growth of murine stem cells and the expansion of colony-forming cells and spleen colony-forming units. *Blood* 1995;85:2747–55.
 - [48] Blum AM, Metwali A, Elliott D, Li J, Sandor M, Weinstock JV. IL-6-deficient mice form granulomas in murine schistosomiasis that exhibit an altered B cell response. *Cell Immunol* 1998;188:64–72.
 - [49] Jeppson JD, Patel HR, Sakata N, Domenico J, Terada N, Gelfand EW. Requirement for dual signals by anti-CD40 and IL-4 for the induction of nuclear factor-kappa B, IL-6,

- and IgE in human B lymphocytes. *J Immunol* 1998;161: 1738–42.
- [50] Pulendran B, Smith JL, Caspary G, Brasel K, Pettit D, et al. Distinct dendritic cell subsets differentially regulate the class of immune response in vivo. *Proc Natl Acad Sci U S A* 1999;96:1036–41.
- [51] Srivastava KD, Kandimalla ER, Yu D, Agrawal S, Sampson HA, Li X. Down-regulation of Th2 cytokines in vitro by immunomodulatory oligonucleotides (IMO) containing modified CpG motifs. *J Allergy Clin Immunol* 2003;111(Part 2): S380–95 (abstract # 780).