

# Novel oligodeoxynucleotide agonists of TLR9 containing N<sup>3</sup>-Me-dC or N<sup>1</sup>-Me-dG modifications

Mallikarjuna Reddy Putta, FuGang Zhu, Yukui Li, Lakshmi Bhagat, YanPing Cong, Ekambar R. Kandimalla and Sudhir Agrawal\*

Idera Pharmaceuticals, Inc., 345 Vassar Street, Cambridge, MA 02139, USA

Received March 9, 2006; Revised May 19, 2006; Accepted May 31, 2006

## ABSTRACT

**Synthetic oligodeoxynucleotides containing unmethylated CpG motifs activate Toll-Like Receptor 9 (TLR9). Our previous studies have shown the role of hydrogen-bond donor and acceptor groups of cytosine and guanine in the CpG motif and identified synthetic immunostimulatory motifs. In the present study to elucidate the significance of N3-position of cytosine and N1-position of guanine in the CpG motif, we substituted C or G of a CpG dinucleotide with N<sup>3</sup>-Me-cytosine or N<sup>1</sup>-Me-guanine, respectively, in immunomodulatory oligodeoxynucleotides (IMOs). IMOs containing N-Me-cytosine or N-Me-guanine in C- or G-position, respectively, of the CpG dinucleotide showed activation of HEK293 cells expressing TLR9, but not TLR3, 7 or 8. IMOs containing N-Me-cytosine or N-Me-guanine modification showed activity in mouse spleen cell cultures, *in vivo* in mice, and in human cell cultures. In addition, IMOs containing N-Me-substitutions reversed antigen-induced Th2 immune responses towards a Th1-type in OVA-sensitized mouse spleen cell cultures. These studies suggest that TLR9 tolerates a methyl group at N1-position of G and a methyl group at N3-position of C may interfere with TLR9 activation to some extent. These are the first studies elucidating the role of N3-position of cytosine and N1-position of guanine in a CpG motif for TLR9 activation and immune stimulation.**

## INTRODUCTION

Toll-like receptor 9 (TLR9) is a member of the family of conserved pathogen-associated molecular pattern recognition receptors. TLR9 recognizes synthetic oligodeoxynucleotides containing unmethylated CpG motifs (CpG DNA) (1). The stimulation of TLR9 with CpG DNA leads to the activation of MyD88-dependent cellular signaling pathways, resulting in predominantly Th1-type innate and adaptive immune

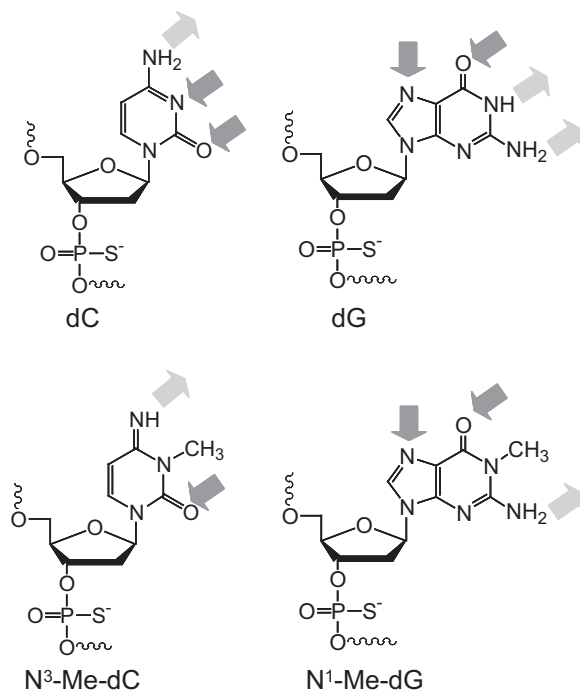
responses (2,3). The exact nature of the resulting immune response depends on the structure of the CpG DNA and on the sequences flanking the CpG dinucleotide. At least three different types of CpG DNAs [single-stranded; palindromic double-stranded; and hyperstructure-forming, poly(dG)-containing] have been described in the literature based on their structures and the immunostimulatory profiles they produce (4–7).

Our extensive investigations into the structure and immunostimulatory activity of oligodeoxynucleotides have identified critical structural features in the pentose sugar (8–11), phosphate backbone (12), nucleobases (13,14) and nucleosides (15) required for CpG DNA activity. Our studies suggest that TLR9 recognizes CpG DNA from the 5' end and that modifications that block accessibility of the receptor to the 5' end, such as linking CpG DNAs 5' end to 5' end or attaching ligands at the 5' end, significantly decrease activity (16–18). In contrast, CpG DNA that are attached through a 3'–3' linkage and contain two accessible 5' ends, referred to as immunomers, have enhanced immunostimulatory activity (16–18).

Our studies also showed that the TLR9 receptor is highly specific for deoxyribonucleotides in CpG dinucleotide and that the presence of ribonucleotides or 2'-O-alkyl ribonucleotides abrogates immune stimulation (19). We substituted synthetic pyrimidine (Y; 5-hydroxy-dC, araC, N<sup>4</sup>-methyl-dC; R', 1-(2'-deoxy-β-D-ribofuranosyl)-2-oxo-7-deaza-8-methyl-purine) and purine (R, 7-deaza-dG, araG, dI, 6-thio-dG) bases for cytosine (C) and guanine (G) in a CpG dinucleotide, in order to examine the requirement of each functional group for TLR9 activation (20–23). These studies suggest that TLR9 recognizes specific motifs, which has allowed us to develop alternate synthetic nucleotide motifs (20–23). TLR9 has a nucleotide motif recognition pattern (NMRP) and can recognize specific modified nucleotide motifs, such as YpG, CpR and R'pG (20–23). The ability of TLR9 to recognize structurally diverse nucleotide motifs to modulate the downstream cytokine and immune responses in a predictable and desirable manner is useful for the development of therapeutic agents for specific disease indications.

To further understand CpG DNA-TLR9 recognition and to continue development of potent synthetic immunostimulatory

\*To whom correspondence should be addressed. Tel: +1 617 679 5501; Fax: +1 617 679 5542; Email: sagrawal@iderapharma.com



**Figure 1.** Structures of natural 2'-deoxy-cytidine (dC) and 2'-deoxy-guanosine (dG) with hydrogen-bond acceptor (inward arrows) and donor (outward arrows) groups shown. Structures of N<sup>3</sup>-Me-dC and N<sup>1</sup>-Me-dG are shown with hydrogen-bond acceptor and donor groups for comparison. Note the loss of a hydrogen-bond acceptor functionality at the N1-position of N<sup>1</sup>-Me-dC and loss of a hydrogen-bond donor functionality at the N3-position of N<sup>3</sup>-Me-dG compared with natural dC and dG nucleotides.

motifs, we examined the effects of N<sup>3</sup>-Me-2'-deoxy-cytidine or N<sup>1</sup>-Me-2'-deoxy-guanosine (Figure 1) modifications incorporated in the C- or G-position, respectively, of immunomers, referred to as immunomodulatory oligonucleotides (IMOs).

## MATERIALS AND METHODS

### IMO synthesis and purification

IMOs with 2'-deoxy-N<sup>3</sup>-methylcytidine (N-Me-dC) or 2'-deoxy-N<sup>1</sup>-methylguanosine (N-Me-dG) modifications were synthesized on a 1 to 2  $\mu$ mol scale using  $\beta$ -cyanoethylphosphoramidite chemistry on a PerSeptive Biosystem 8909 Expedite DNA synthesizer. Di-DMT-protected glyceryl linker attached to CPG-solid-support was obtained from ChemGenes Corporation (Wilmington, MA). The 3'-phosphoramidites of dA, dG, dC and T were obtained from Prologo (Denver, CO). Phosphoramidites of N-Me-dC and N-Me-dG were obtained from ChemGenes Corporation. Beaucage reagent was used as an oxidant to obtain the phosphorothioate backbone modification (24). A modified coupling protocol recommended by the supplier was used for N-Me-dC and N-Me-dG phosphoramidite incorporation. After the synthesis, IMOs containing the N-Me-dC- or N-Me-dG modification were deprotected using standard protocols, purified by RP-high-performance liquid chromatography (HPLC), detritylated, and dialyzed against United States Pharmacopeia-quality sterile water for irrigation (Braun). The IMOs were lyophilized and dissolved again in distilled

water and the concentrations were determined by measuring the ultraviolet (UV) absorbance at 260 nm (25). The purity of all the compounds synthesized was determined by denaturing PAGE and the sequence integrity was characterized by matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) mass spectrometry for molecular mass. All IMOs were synthesized and purified under identical conditions to minimize endotoxin contamination.

### Animals

Four-to-six-week-old C57BL/6 and BALB/c mice were obtained from Taconic Farms, Germantown, NY and maintained in accordance with Idera's IACUC-approved animal protocols. All the animal studies reported in the paper were carried out following Idera's IACUC guidelines and approved protocols.

### Mouse spleen cell cultures

Spleen cells from C57BL/6 mice were prepared and cultured in RPMI complete medium consisting of RPMI 1640 with 10% fetal calf serum (FCS), 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin and 2 mM L-glutamine (HyClone, Logan, UT). Mouse spleen cells were plated in 24-well plates at  $5 \times 10^6$  cells/ml. IMOs dissolved in TE buffer [10 mM Tris-HCl (pH 7.5) and 1 mM EDTA] were added to a final concentration of 4, 14, 41, 140, 410, or 1400 nM to the cell cultures. The cells were then incubated at 37°C for 24 h and the supernatants were collected for cytokine analysis by enzyme-linked immunosorbent assays (ELISAs).

IL-12 and IL-6 levels in supernatants were measured by sandwich ELISA. The required reagents, including cytokine antibodies and standards, were purchased from BD Pharmingen (San Diego, CA). Streptavidin-peroxidase and TMB substrate were from Sigma (St. Louis, MO) and KPL (Gaithersburg, MD), respectively.

### Human PBMC isolation

Peripheral blood mononuclear cells (PBMCs) from freshly drawn healthy volunteer blood (Research Blood Components, Brighton, MA) were isolated by Ficoll density gradient centrifugation (Histopaque-1077, Sigma).

### Human cytokine ELISAs

Human PBMCs were plated in 96-well plates at a concentration of  $5 \times 10^6$  cells/ml. The IMOs dissolved in phosphate-buffered saline (PBS) were added to the cell cultures at a final concentration of 1400 nM. The cells were then incubated at 37°C for 24 h for IL-6 or 48 h for IL-10. The levels of IL-6 and IL-10 in the culture supernatants were measured by sandwich ELISA. The required reagents, including cytokine antibodies and standards, were purchased from BD Pharmingen.

### Human B-cell proliferation assay

About  $1 \times 10^5$  B-cells purified from human PBMCs as described previously (23) were stimulated with different concentrations of IMOs for 64 h, then pulsed with 0.75  $\mu$ Ci of [<sup>3</sup>H]-thymidine and harvested 8 h later. The incorporation of [<sup>3</sup>H]-thymidine was measured by scintillation counter and the data are shown as counts per minute (c.p.m.).

### HEK293 cell cultures

HEK293 cells stably expressing mouse TLR9 or human TLR3, 7 or 8 (Invivogen, San Diego, CA) were cultured in 48-well plates in 250  $\mu$ l/well DMEM supplemented with 10% heat-inactivated FBS in a 5% CO<sub>2</sub> incubator. At 80% confluence, cultures were transiently transfected with 400 ng/ml of secreted form of human embryonic alkaline phosphatase (SEAP) reporter plasmid (pNifty2-Seap) (Invivogen) in the presence of 4  $\mu$ l/ml of lipofectamine (Invitrogen, Carlsbad, CA) in culture medium. Plasmid DNA and lipofectamine were diluted separately in serum-free medium and incubated at room temperature for 5 min. After incubation, the diluted DNA and lipofectamine were mixed and the mixtures were incubated at room temperature for 20 min. Aliquots of 25  $\mu$ l of the DNA/lipofectamine mixture containing 100 ng of plasmid DNA and 1  $\mu$ l of lipofectamine were added to each well of the cell culture plate, and the cultures were continued for 4 h. After transfection, medium was replaced with fresh culture medium, IMOs were added to the cultures, and the cultures were continued for 18 h. At the end of IMO treatment, 30  $\mu$ l of culture supernatant was taken from each treatment and used for SEAP assay following manufacturer's protocol (Invivogen). Briefly, culture supernatants were incubated with p-nitrophenyl phosphate substrate and the yellow color generated was measured by a plate reader at 405 nm. The data are shown as fold increase in NF- $\kappa$ B activity over PBS control.

### Mouse serum cytokines

Female C57BL/6 mice ( $n = 3$ ) were injected subcutaneously (s.c.) with IMOs at 25 or 100  $\mu$ g dose (single-dose). Serum was collected by retro-orbital bleeding 2 and 4 h after IMO administration and IL-12 and IL-6 levels were determined by sandwich ELISA. All reagents, including cytokine antibodies and standards were purchased from BD PharMingen.

### Mouse splenomegaly assay

Female C57BL/6 mice (19–21 g) were divided into groups of three mice. IMOs were dissolved in sterile PBS and administered s.c. to mice at a dose of 5 mg/kg body weight. After 72 h, mice were euthanized and the spleens were harvested and weighed.

### OVA-sensitized mouse spleen cell culture assays

Female BALB/c mice were given intraperitoneal injections of 20  $\mu$ g of chicken ovalbumin (OVA; Sigma) in 100  $\mu$ l of

PBS mixed with 100  $\mu$ l of ImjectAlum (Pierce, Rockford, IL) on days 0 and 7, and were intranasally challenged on days 14 and 21 with 10  $\mu$ g of OVA in 40  $\mu$ l PBS. The mice were euthanized by CO<sub>2</sub> inhalation 72 h after the last challenge. Spleens were excised and single-cell suspensions were prepared as described above. IMOs were added at different concentrations to spleen cell cultures and incubated for 2 h, at which time 100  $\mu$ g/ml of OVA was added. After 72 h supernatants were collected and IL-5, IL-13, IL-12 and IFN- $\gamma$  levels were measured by ELISA as described above.

## RESULTS

### Design and synthesis of IMOs

Our previous studies showed that compounds with two 5' ends are more immunostimulatory than those with single 5' end, and IMO 1 and 6 (Table 1) have shown potent immunostimulatory activity (16–18,20–23). In the present study, we synthesized IMOs comprising CpG dinucleotides with N-Me-dC and N-Me-dG modifications (Figure 1) in place of either dC or dG, respectively, and studied the immunostimulatory activity of the resulting IMOs in various assays. IMOs 2 and 3 contained a GACGTT nucleotide motif that was recognized by mouse immune cells and IMOs 7 and 8 contained a GTCGTT nucleotide motif that was recognized by human immune cells (Table 1). Compounds 4 and 5 were control sequences in which CG was reversed to GC and N-Me-dC and N-Me-dG modifications were incorporated in the C- and G-positions, respectively. Sequence 9 was a non-CpG control compound. Incorporation of the modifications was characterized by MALDI-TOF mass analysis of the purified products, and the data are presented in Table 1.

### IMOs containing N-Me-dC or -dG in CpG motif activate TLR9

The ability of IMOs containing the N-Me-dC or -dG modification to activate TLR9 was studied in HEK293 cells stably expressing mouse TLR9. SEAP gene is used as a NF- $\kappa$ B reporter. SEAP gene is cloned downstream of a NF- $\kappa$ B-inducible composite promoter engineered with five NF- $\kappa$ B sites, resulting in a quantitative correlation (within a measurable range) of SEAP expression with NF- $\kappa$ B activation. Since SEAP is heat-stable, in SEAP reporter transfected cells, endogenous alkaline phosphatase activity is eliminated by

**Table 1.** Sequences and MALDI-TOF mass spectral analysis data of IMOs containing N<sup>3</sup>-Me-dC or N<sup>1</sup>-Me-dG modification

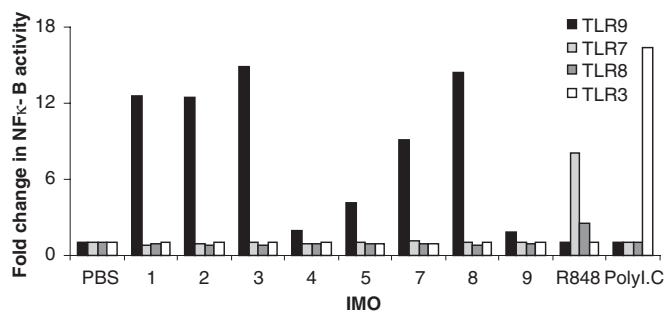
IMO no.	Sequence	Molecular weight		Motif
		Calculated	Observed	
1	d(5'-TCTGACGTCT-X-TCTTGAGTCT-5')	7164	7166	Mouse
2	d(5'-TCTGAC*GTCT-X-TCTTGC*AGTCT-5')	7194	7183	Mouse
3	d(5'-TCTGACG*TTCT-X-TCTTG*GAGTCT-5')	7194	7190	Mouse
4	d(5'-TCTGACG*TTCT-X-TCTTC*GAGTCT-5')	7194	7189	Control
5	d(5'-TCTGAG*CTTCT-X-TCTTCG*AGTCT-5')	7194	7189	Control
6	d(5'-TCTGTCTGTTCT-X-TCTTGTCT-5')	7148	7143	Human
7	d(5'-TCTGTC*GTTCT-X-TCTTGC*GTCT-5')	7175	7170	Human
8	d(5'-TCTGTGC*TTCT-X-TCTTG*GTCT-5')	7175	7171	Human
9	d(5'-ACACACCAACT-X-TCAACCACACA-5')	7078	7065	Control

C\* = N<sup>3</sup>-Me-dC; G\* = N<sup>1</sup>-Me-dG; X = Glycerol linker.

incubating samples at high temperature, allowing an accurate measurement of experimental NF- $\kappa$ B activation. The results are presented as fold increase in NF- $\kappa$ B activation over PBS control (Figure 2). IMOs 2, 3, 7, and 8, which contained N-Me-dC or -dG, activated TLR9, as shown by an increase in NF- $\kappa$ B activity. The control compounds 4 and 5, which contained N-Me-dC in the G-position or N-Me-dG in the C-position of GpC, had no or minimal activity. These results suggest that N-Me-dC is tolerated in the C-position but not the G-position and that N-Me-dG is tolerated in the G-position but not the C-position. IMOs containing natural CpG and synthetic motifs failed to activate HEK293 cells expressing TLR3, 7 or 8, suggesting that these are not the receptors for IMOs. Poly(I-C) and R848, however, activated NF- $\kappa$ B in TLR3- and TLR7- or 8-transfected HEK293 cells, respectively (Figure 2).

### IMOs containing N-Me-dC or -dG in CpG motif induce cytokine secretion in mouse spleen cell cultures

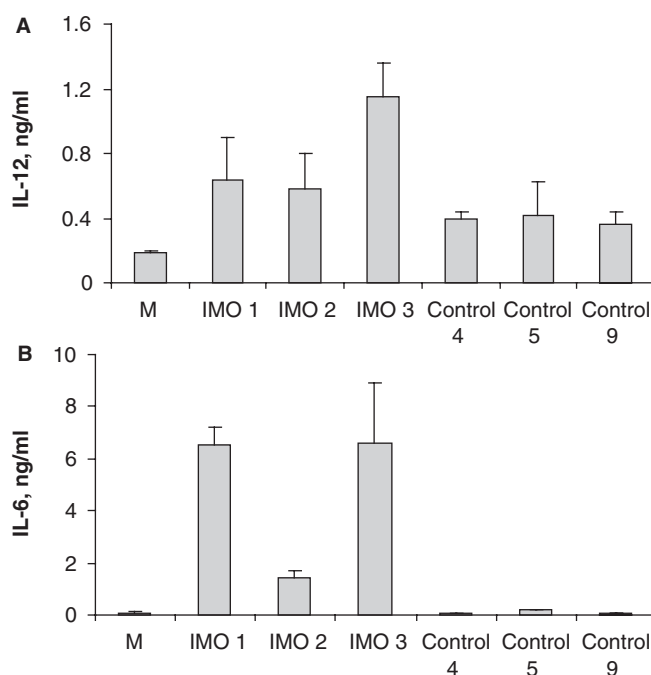
Both mouse-specific IMOs 2 and 3 induced greater production of IL-12 and IL-6 in C57BL/6 mouse spleen cell cultures than did their controls 4 and 5, respectively (Figure 3). The N-Me-dG-containing IMO 3 induced higher levels of both IL-12 and IL-6 than did the N-Me-dC-containing IMO 2. The response was dependent on the concentration of IMO (Table 2). These results suggest that the modifications are tolerated by immune cells and induce cytokine production in mouse spleen cell cultures.



**Figure 2.** Activation of HEK293 cells expressing mouse TLR9, human TLR3, TLR7 or TLR8 with IMOs and control compounds at a concentration of 1400 nM. Data shown are of one representative experiment of three independent experiments.

### IMOs containing N-Me-dC or -dG in CpG motif induce splenomegaly in mice

Oligonucleotides containing unmethylated CpG motifs induce splenomegaly in mice (19,26,27). The increase in spleen weight of mice following CpG oligo administration is a measure of immunostimulatory activity (19,26,27). Consistent with the *in vitro* data, mice injected with either IMO 2 or 3, which contained N-Me-dC and -dG, respectively, showed spleen enlargement compared with mice that received control compound 9 (Figure 4). Consistent with the *in vitro* data, mice that received IMO 3, which had the N-Me-dG modification, caused greater increases in spleen weight than did mice injected with IMO 2, which had the N-Me-dC modification.



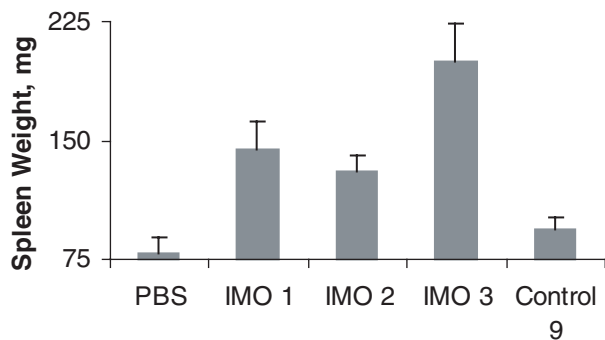
**Figure 3.** Induction of cytokine secretion by IMOs in C57BL/6 mouse spleen cell cultures. C57BL/6 mouse spleen cells were cultured in medium alone (M) or in the presence of IMOs at various concentrations for 24 h and the levels of secreted IL-12 (A) and IL-6 (B) in culture supernatants were measured by ELISA. Data shown are at 140 nM concentration of IMOs. Each value is an average of three replicate wells and the results are of one representative experiment of at least two independent experiments.

**Table 2.** Cytokine induction by IMOs in C57BL/6 mouse spleen cell cultures and *in vivo*

IMO	IL-12 (ng/ml) $\pm$ SD				IL-6 (ng/ml) $\pm$ SD				Serum IL-12 (ng/ml) $\pm$ SD <sup>a</sup>	
	14 nM	41 nM	140 nM	410 nM	14 nM	41 nM	140 nM	410 nM	1.25 mg/kg	5 mg/kg
1	0.64 $\pm$ 0.1	0.83 $\pm$ 0.05	0.63 $\pm$ 0.27	1.24 $\pm$ 0.43	0.37 $\pm$ 0.08	2.38 $\pm$ 0.47	6.51 $\pm$ 0.72	5.91 $\pm$ 1.43	NT	NT
2	0.3 $\pm$ 0.09	0.32 $\pm$ 0.14	0.58 $\pm$ 0.22	0.73 $\pm$ 0.27	0.12 $\pm$ 0.04	0.14 $\pm$ 0.04	1.4 $\pm$ 0.3	4.8 $\pm$ 0.83	27.1 $\pm$ 5.6	61.6 $\pm$ 17
3	0.4 $\pm$ 0.04	0.97 $\pm$ 0.17	1.15 $\pm$ 0.21	1.33 $\pm$ 0.28	0.82 $\pm$ 0.19	6.54 $\pm$ 1.22	6.6 $\pm$ 2.32	6.2 $\pm$ 1.03	65.0 $\pm$ 9.4	123.9 $\pm$ 22
4	0.3 $\pm$ 0.04	0.26 $\pm$ 0.04	0.39 $\pm$ 0.04	0.34 $\pm$ 0.04	0.083 $\pm$ 0.01	0.09 $\pm$ 0.01	0.08 $\pm$ 0.01	0.09 $\pm$ 0.02	1.2 $\pm$ 0.22	6 $\pm$ 0.54
5	0.25 $\pm$ 0.18	0.35 $\pm$ 0.07	0.42 $\pm$ 0.21	0.45 $\pm$ 0.03	0.08 $\pm$ 0.02	0.09 $\pm$ 0.002	0.18 $\pm$ 0.03	1.31 $\pm$ 0.25	NT	NT
9	0.24 $\pm$ 0.06	0.27 $\pm$ 0.07	0.36 $\pm$ 0.01	0.29 $\pm$ 0.06	0.07 $\pm$ 0.01	0.07 $\pm$ 0.01	0.08 $\pm$ 0.01	0.11 $\pm$ 0.02	NT	NT
Medium	0.19 $\pm$ 0.01				0.09 $\pm$ 0.03				0.48 $\pm$ 0.03	0.27 $\pm$ 0.01

<sup>a</sup>Serum IL-12 levels were measured 4 h after IMO administration as described in Materials and Methods section; NT: not tested.





**Figure 4.** Splenomegaly in C57BL/6 mice that received a 5 mg/kg dose of IMO, control compound or PBS administered s.c. Change in spleen weights were determined 72 h after IMO administration. Each bar represents the mean of three mice  $\pm$  SD.

### IMOs containing N-Me-dC or -dG in CpG motif induce cytokines *in vivo* in mice

We further examined the *in vivo* cytokine induction profiles of IMOs containing N-Me-dC or -dG modifications. IMOs 2 and 3, which contained N-Me-dC and -dG, respectively, induced dose-dependent elevation of IL-12 in mice 4 h after IMO administration (Table 2). As was seen in the splenomegaly assay, IMO 3 induced higher levels of IL-12 than did IMO 2. These results demonstrate that both the modifications (N-Me-dC and -dG) are tolerated by TLR9 but the levels of immune response are different.

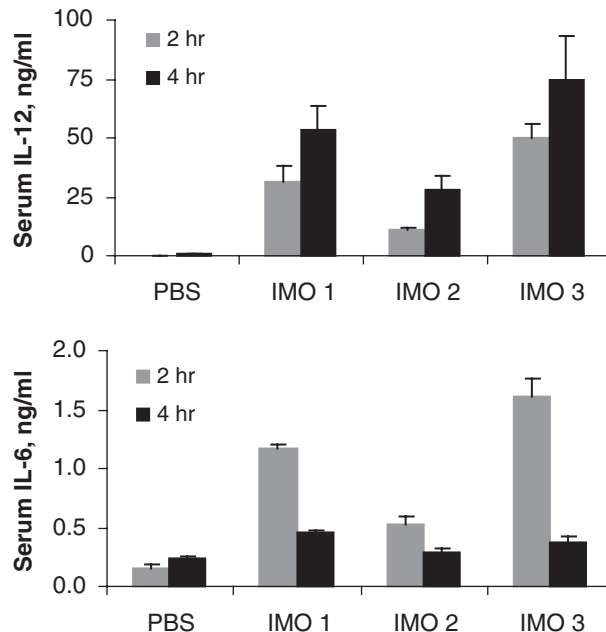
We next examined the kinetics of IL-12 and IL-6 production in mice that received s.c. injections of IMOs. The levels of IL-12 in serum were higher at 4 h than at 2 h after injection (Figure 5A). In contrast, IL-6 levels peaked at 2 h and by 4 h the levels of IL-6 returned to background levels (Figure 5B).

### IMOs containing N-Me-dC or -dG in CpG motif activate human PBMCs and B-cells

We further examined the ability of IMOs with N-Me-dC and -dG modifications to activate human PBMCs and induce cytokine production. In these assays, we used IMOs 7 and 8, which contained a human-specific motif (Table 1). Both IMOs 7 and 8 induced higher levels of IL-6 (Figure 6A) and IL-10 (Figure 6B) than did control 9, suggesting that both modifications are tolerated by human TLR9. Both IMOs 7 and 8 induced dose-dependent B-cell proliferation compared with control 9 (Figure 6C). The levels of B-cell proliferation induced by IMO 7 and 8 are comparable to natural CpG-containing IMO 6 (Figure 6C).

### IMOs containing N-Me-dC or -dG substitutions induce Th1 cytokines in OVA-sensitized and challenged mouse spleen cell cultures

In the absence of IMO treatment, OVA-sensitized and -challenged mouse spleen cells secreted high levels of Th2 cytokines (IL-5 and IL-13) and low levels of Th1 cytokines (IL-12 and IFN- $\gamma$ ), suggesting a Th2-type immune response to OVA alone (Figure 7). When spleen cells were treated with IMOs containing N-Me-dC or -dG modification, a decrease in IL-5 and IL-13 levels occurred with a concomitant increase in the levels of IL-12 and IFN- $\gamma$  (Figure 7A–D).



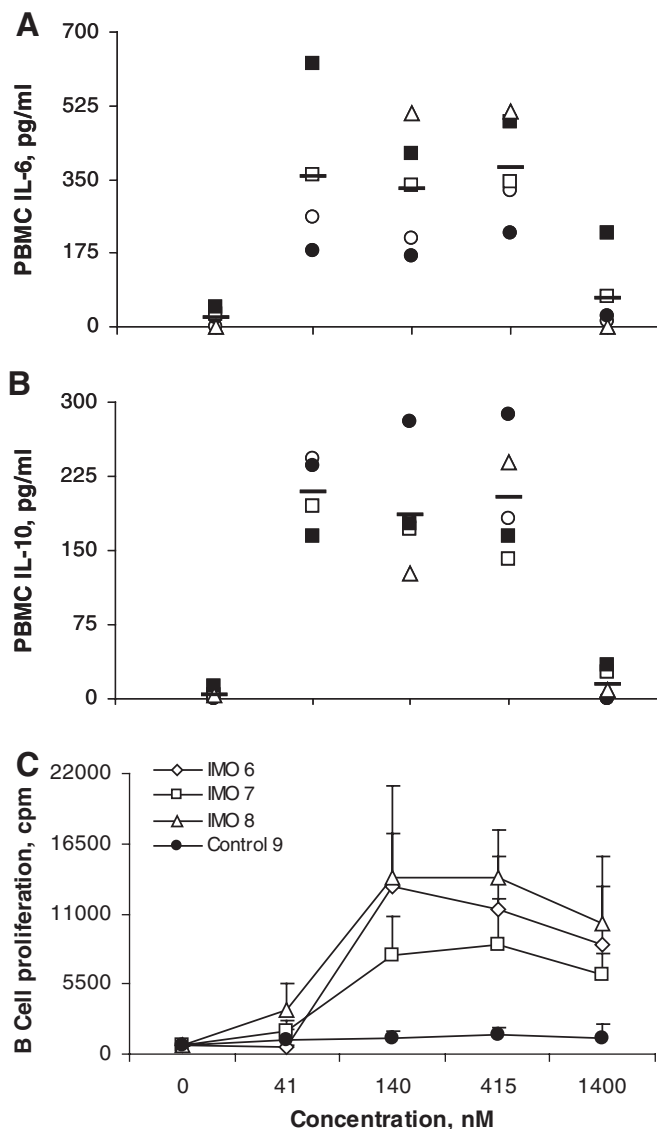
**Figure 5.** IL-12 and IL-6 secretion in C57BL/6 mice induced by IMOs following s.c. administration at a dose of 1.25 mg/kg. Blood was collected at 2 and 4 h after IMO administration and IL-12 and IL-6 levels in the serum were determined by ELISA as described in Materials and Methods. Each value is the average for three mice  $\pm$  SD.

Control 9 had no effect on OVA-induced immune responses in the spleen cell recall assay (Figure 7). These results suggest that the N-Me-dC and -dG modifications induce Th1-type cytokine profiles with reversal of the OVA-induced Th2 immune response.

## DISCUSSION

Synthetic and bacterial DNA containing unmethylated CpG motifs induce B-cell proliferation, immunoglobulin production, and the secretion of a number of cytokines, including IL-12, IFN- $\alpha/\beta/\gamma$ , IL-6 and TNF- $\alpha$ , from B-cells, monocytes/macrophages, and dendritic cells. TLR9, a receptor that belongs to a family of receptors that detect conserved pathogen-associated molecular patterns, is the receptor for CpG DNA (1). The stimulation of TLR9 with CpG DNA initiates a signaling cascade leading to the activation of the transcription factor NF- $\kappa$ B. Our recent studies showed that TLR9 recognizes not only the natural CpG motif but also a number of synthetic purines (7-deaza-dG, araG, dI, 6-thio-dG) and pyrimidines (5-hydroxy-dC, araC, N<sup>4</sup>-methyl-dC) substituted for G or C, respectively, in a CpG motif (20,21,23). Our recent studies also showed that a bicyclic synthetic nucleoside, 1-(2'-deoxy- $\beta$ -D-ribofuranosyl)-2-oxo-7-deaza-8-methyl-purine, is also tolerated by TLR9 and that this substitution for C can override TLR9 specificity for different sequences in different species (22). The results presented here elucidate the tolerance of TLR9 for a methyl substitution on N3 of C or N1 of G in a CpG motif and induction of subsequent immune stimulation.

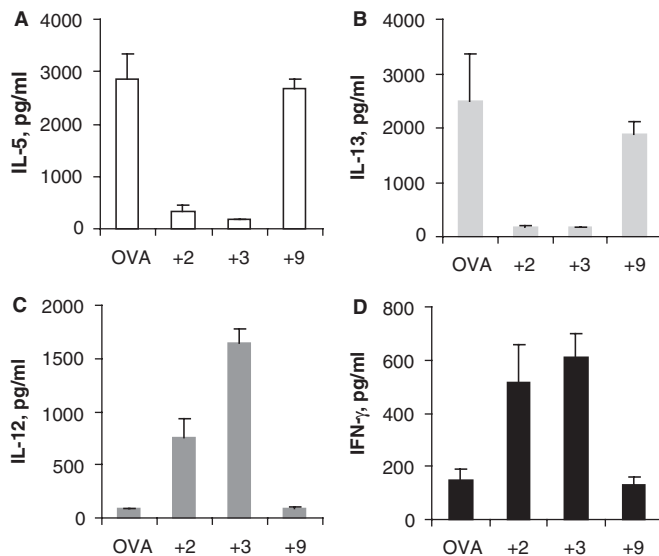
Studies in HEK293 cells stably expressing TLR9 showed that IMOs containing N-Me-dG in the G-position and N-Me-dC in the C-position of CpG, but not vice versa,



**Figure 6.** (A) IL-6 and (B) IL-10 induction profiles of IMOs in human PBMCs. PBMCs were stimulated with 1400 nM IMO for 24 h for IL-6 and 48 h for IL-10. Each data point represents data for one donor and bar represents the mean of four or five donors. (C) Human B-cell proliferation induced by IMOs. Human B-cells isolated from PBMC obtained from healthy human volunteers were stimulated with IMOs at various concentrations and  $^3\text{H}$ -thymidine uptake was determined by scintillation counting. Each data point represents the average of four donors  $\pm$  SD.

stimulated TLR9. These results indicate that IMOs containing these chemical modifications are tolerated by TLR9. TLR7 and TLR8 recognize nucleosides and related small molecules. The inability of IMOs containing N-Me-dC or -dG modifications to stimulate HEK293 cells expressing either TLR7 or TLR8 rules out the possibility that TLR7/8 is the receptor for these IMOs. Additionally, IMOs containing N-Me modifications are not recognized by TLR3, as they failed to activate HEK293 cells expressing TLR3.

In mice, macrophages, monocytes, dendritic cells and B-cells express TLR9, and synthetic DNA containing unmethylated CpG motifs activates all of these cells *in vitro* and *in vivo*. In C57BL/6 mouse spleen cell cultures,



**Figure 7.** Inhibition of (A) IL-5 and (B) IL-13 and induction of (C) IL-12 and (D) IFN- $\gamma$  production in OVA-sensitized BALB/c mouse spleen cell cultures by IMOs 2 and 3 or control 9. Spleens were obtained from BALB/c mice sensitized with OVA as described in Materials and Methods. Single spleen cells were incubated with 100  $\mu\text{g}/\text{ml}$  OVA in the absence or presence of 140 nM IMO for 72 h, and the cytokine levels in culture supernatants were measured using ELISA. The results represent the mean cytokine concentration from triplicate wells  $\pm$  SD.

IMOs containing N-Me-dC in the C-position (IMO 2) or N-Me-dG in the G-position (IMO 3) of the CpG dinucleotide induced dose-dependent IL-12 and IL-6 secretion. Control oligonucleotides with substitutions in the GpC dinucleotide had no or minimal activity. These results confirm the stimulation of mouse immune cells by the two synthetic stimulatory motifs. Moreover, a single-dose administration of IMO 2 or 3 to mice resulted in spleen enlargement, while administration of a non-CpG control compound did not, further confirming the immunostimulatory activity of the new stimulatory motifs.

IMO 3 induced higher levels of cytokines IL-12 and IL-6 *in vivo* as well as *in vitro*, compared with the natural CpG-containing IMO 1. In general, the ratio of IL-12 to IL-6 was higher for IMO 2 and 3 than for IMO 1, suggesting that there is a difference between the natural and synthetic motifs in spite of the fact that both motifs stimulate immune responses through TLR9. Similar results have been observed before with other synthetic motifs (21,23,28,29).

In humans, only B-cells and plasmacytoid dendritic cells express TLR9, and the CpG motif sequence requirement for human TLR9 is different from that for the mouse TLR9 (Table 1). We incorporated N-Me-dC and N-Me-dG modifications into the human-specific motif and studied the immunostimulatory activity of the resulting IMOs in human PBMC and B-cell cultures. Both modifications induced cytokine secretion in PBMC cultures and proliferation in B-cell cultures, indicating that the modifications were tolerated by human TLR9.

IMOs containing natural and synthetic motifs suppress allergen-induced Th2-type immune responses and tilt the immune response towards a Th1-type *in vitro* and *in vivo*

(3,29,30). In the present study, IMOs containing either the N-Me-dC or the -dG modification inhibited production of Th2 cytokines (IL-5 and IL-13) in OVA-sensitized mouse spleen cell cultures. In addition, they induced production of higher levels of Th1 cytokines (IL-12 and IFN- $\gamma$ ) than did the control compound 9.

Our previous structure-immunostimulatory activity studies revealed that the deletion or substitution of any of the functional groups on C resulted in a loss of activity (20), but an alkyl substitution on the 4-amino group did not block recognition by TLR9. A 5-methyl substitution interfered with immune stimulatory activity, but a 5-hydroxyl did not (20). Similarly, deletion or modification of hydrogen-bond acceptor and donor functional groups at the 1-, 2- and 6-positions of G reduced the immunostimulatory activity of the compound (20). However, the nitrogen at the 7-position of G was not required for recognition of CpG dinucleotides (20,21,23).

The 3-imino nitrogen of C could serve as a hydrogen-bond acceptor and the 1-imino nitrogen of G could serve as a hydrogen-bond donor (Figure 1). The substitution of a methyl group on N3 of C or N1 of G changes the hydrogen-bond acceptor and donor properties of C and G, respectively (Figure 1), and thereby the interaction of the CpG motif with TLR9. The present results indicate that the blocking of hydrogen-bond donor functionality at the N1-position of G has no bearing on the TLR9 activation by IMOs, suggesting that N1 is not involved in hydrogen bonding with the receptor. In fact, the data suggest that the bulkier hydrophobic group at the N1-position of G might facilitate stronger binding to the receptor, resulting in more potent immunostimulatory activity than that of the natural CpG-containing IMO.

A methyl substitution on N3 of C resulted in the loss of (i) a hydrogen-bond acceptor functionality at the N3-position and (ii) a hydrogen on the 4-amino group (Figure 1). These structural changes influenced the immunostimulatory activity of IMOs containing the N-Me-dC modification compared with IMOs containing N-Me-dG, suggesting that TLR9 tolerated N-Me-dC modification to a lesser extent than N-Me-dG.

We have shown here that the N1 of G in a CpG motif does not interfere with the recognition of TLR9 and, in fact, a methyl substitution on N1 of G increases the immunostimulatory activity of the compound and results in induction of a distinct cytokine profile. The N3-hydrogen of C could be involved in hydrogen bonding with the TLR9 receptor and a methyl substitution at that site decreases, but does not block the stimulatory activity. We have shown that IMOs with synthetic motifs have potent immunostimulatory activity in mouse spleen cell cultures, human cell-based assays and cultures, and *in vivo* in mice. Additionally, these synthetic motifs can shift the Th2-type immune response induced by allergen towards a Th1-type response *in vitro*. These are the first studies elucidating the role of N3-position of cytosine and N1-position of guanine for TLR9 activation.

## ACKNOWLEDGEMENTS

Funding to pay the Open Access publication charges for this article was provided by Idera Pharmaceuticals.

*Conflict of interest statement.* None declared.

## REFERENCES

- Hemmi,H., Takeuchi,O., Kawai,T., Kaisho,T., Sato,S., Sanjo,H., Matsumoto,M., Hoshino,K., Wagner,H., Takeda,K. *et al.* (2000) A Toll-like receptor recognizes bacterial DNA. *Nature*, **408**, 740–745.
- Messina,J.P., Gilkeson,G.S. and Pisetsky,D.S. (1991) Stimulation of *in vitro* murine lymphocyte proliferation by bacterial DNA. *J. Immunol.*, **147**, 1759–1764.
- Klinman,D.M. (2004) Immunotherapeutic uses of CpG oligodeoxynucleotides. *Nature Rev. Immunol.*, **4**, 249–258.
- Krieg,A.M., Yi,A.K., Matson,S., Waldschmidt,T.J., Bishop,G.A., Teasdale,R., Koretzky,G.A. and Klinman,D.M. (1995) CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature*, **374**, 546–549.
- Sato,Y., Roman,M., Tighe,H., Lee,D., Corr,M., Nguyen,M.D., Silverman,G.J., Lotz,M., Carson,D.A. and Raz,E. (1996) Immunostimulatory DNA sequences necessary for effective intradermal gene immunization. *Science*, **273**, 352–354.
- Verthelyi,D., Ishii,K.J., Gursel,M., Takeshita,F. and Klinman,D.M. (2001) Human peripheral blood cells differentially recognize and respond to two distinct CPG motifs. *J. Immunol.*, **166**, 2372–2377.
- Marshall,J.D., Fearon,K., Abbate,C., Subramanian,S., Yee,P., Gregorio,J., Coffman,R.L. and vanNest,G. (2003) Identification of a novel CpG DNA class and motif that optimally stimulate B cell and plasmacytoid dendritic cell functions. *J. Leukocyte Biol.*, **73**, 781–792.
- Zhao,Q., Yu,D. and Agrawal,S. (1999) Site of chemical modifications in CpG containing phosphorothioate oligodeoxynucleotide modulates its immunostimulatory activity. *Bioorg. Med. Chem. Lett.*, **9**, 3453–3458.
- Zhao,Q., Yu,D. and Agrawal,S. (2000) Immunostimulatory activity of CpG containing phosphorothioate oligodeoxynucleotide is modulated by modification of a single deoxynucleoside. *Bioorg. Med. Chem. Lett.*, **10**, 1051–1054.
- Yu,D., Kandimalla,E.R., Zhao,Q., Cong,Y. and Agrawal,S. (2002) Immunostimulatory properties of phosphorothioate CpG DNA containing both 3'-5'- and 2'-5'-internucleotide linkages. *Nucleic Acids Res.*, **30**, 1613–1619.
- Agrawal,S. and Kandimalla,E.R. (2001) Antisense and/or immunostimulatory oligonucleotide therapeutics. *Curr. Cancer Drug Targets*, **1**, 197–209.
- Yu,D., Kandimalla,E.R., Zhao,Q., Cong,Y. and Agrawal,S. (2001) Immunostimulatory activity of CpG oligonucleotides containing non-ionic methylphosphonate linkages. *Bioorg. Med. Chem.*, **9**, 2803–2808.
- Yu,D., Kandimalla,E.R., Zhao,Q., Cong,Y. and Agrawal,S. (2001) Modulation of immunostimulatory activity of CpG oligonucleotides by site-specific deletion of nucleobases. *Bioorg. Med. Chem. Lett.*, **11**, 2263–2267.
- Yu,D., Kandimalla,E.R., Zhao,Q., Bhagat,L., Cong,Y. and Agrawal,S. (2003) Requirement of nucleotide bases proximal to a CpG-motif for immunostimulatory activity of synthetic oligonucleotides. *Bioorg. Med. Chem.*, **11**, 459–464.
- Yu,D., Kandimalla,E.R., Cong,Y., Tang,J., Tang,J.Y., Zhao,Q. and Agrawal,S. (2002) Design, synthesis, and immunostimulatory properties of CpG DNAs containing alkyl-linker substitutions: role of nucleosides in the flanking sequences. *J. Med. Chem.*, **45**, 4540–4548.
- Yu,D., Zhao,Q., Kandimalla,E.R. and Agrawal,S. (2000) Accessible 5' end of CpG-containing phosphorothioate oligodeoxynucleotides is essential for immunostimulatory activity. *Bioorg. Med. Chem. Lett.*, **10**, 2585–2588.
- Kandimalla,E.R., Bhagat,L., Yu,D., Cong,Y., Tang,J. and Agrawal,S. (2002) Conjugation of ligands at the 5' end of CpG DNA affects immunostimulatory activity. *Bioconjug. Chem.*, **13**, 966–974.
- Yu,D., Kandimalla,E.R., Bhagat,L., Tang,J.Y., Cong,Y., Tang,J. and Agrawal,S. (2002) 'Immunomers'—novel 3'-3'-linked CpG oligodeoxyribonucleotides as potent immunomodulatory agents. *Nucleic Acids Res.*, **30**, 4460–4469.
- Zhao,Q., Tamsamani,J., Iadarola,P.L., Jiang,Z. and Agrawal,S. (1996) Effect of different chemically modified oligodeoxynucleotides on immune stimulation. *Biochem. Pharmacol.*, **51**, 173–182.
- Kandimalla,E.R., Yu,D., Zhao,Q. and Agrawal,S. (2001) Effect of chemical modifications of cytosine and guanine in a CpG-motif of oligonucleotides: structure-immunostimulatory activity relationships. *Bioorg. Med. Chem.*, **9**, 807–813.
- Kandimalla,E.R., Bhagat,L., Wang,D., Yu,D., Tang,J., Wang,H., Huang,P., Zhang,R. and Agrawal,S. (2003) Divergent synthetic

- nucleotide motif recognition pattern: design and development of potent immunomodulatory oligodeoxyribonucleotide agents with distinct cytokine induction profiles. *Nucleic Acids Res.*, **31**, 2393–2400.
22. Kandimalla, E.R., Bhagat, L., Zhu, F.G., Yu, D., Cong, Y.P., Wang, D., Tang, J.X., Tang, J.Y., Knetter, C.F., Lien, E. *et al.* (2003) A dinucleotide motif in oligonucleotides shows potent immunomodulatory activity and overrides species-specific recognition observed with CpG motif. *Proc. Natl Acad. Sci. USA*, **100**, 14303–14308.
  23. Kandimalla, E.R., Bhagat, L., Li, Y., Yu, D., Wang, D., Cong, Y.P., Song, S.S., Tang, J.X., Sullivan, T. and Agrawal, S. (2005) Immunomodulatory oligonucleotides containing a cytosine-phosphate-2'-deoxy-7-deazaguanosine motif as potent Toll-like receptor 9 agonists. *Proc. Natl Acad. Sci. USA*, **102**, 6925–6930.
  24. Iyer, R.P., Egan, W., Regan, J.B. and Beaucage, S.L. (1990) 3H-1,2-Benzodithiole-3-one 1,1-dioxide as an improved sulfurizing reagent in the solid-phase synthesis of oligodeoxyribonucleoside phosphorothioates. *J. Am. Chem. Soc.*, **112**, 1253–1254.
  25. Puglisi, J.D. and Tinoco, I., Jr (1989) Absorbance melting curves of RNA. *Meth. Enzymol.*, **180**, 304–325.
  26. Branda, R.F., Moore, A.L., Mathews, L., McCormack, J.J. and Zon, G. (1993) Immune stimulation by an antisense oligomer complementary to the *rev* gene of HIV-1. *Biochem. Pharmacol.*, **45**, 2037–2043.
  27. Zhao, Q., Tamsamani, J., Zhou, R.Z. and Agrawal, S. (1997) Pattern and kinetics of cytokine production following administration of phosphorothioate oligonucleotides in mice. *Antisense Nucleic Acid Drug Dev.*, **7**, 495–502.
  28. Agrawal, D., Edwan, J., Kandimalla, E.R., Yu, D. and Agrawal, S. (2004) Novel immunomodulatory oligonucleotides (IMOs) prevent development of allergic airway inflammation and airway hyperresponsiveness in Asthma. *Int. Immunopharmacol.*, **4**, 127–138.
  29. Wang, D., Li, Y., Yu, D., Song, S.S., Kandimalla, E.R. and Agrawal, S. (2004) Immunopharmacological and antitumor effects of second-generation immunomodulatory oligonucleotides containing synthetic CpR motif. *Int. J. Oncol.*, **24**, 901–908.
  30. Zhu, F.G., Kandimalla, E.R., Yu, D., Tang, J.X. and Agrawal, S. (2004) Modulation of ovalbumin-induced Th2 responses by second-generation immunomodulatory oligonucleotides in mice. *Int. Immunopharmacol.*, **4**, 851–862.