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CpG penta- and hexadeoxyribonucleotides as potent immunomodulatory agents

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Abstract

We demonstrate a new design for immunomodulatory CpG DNA containing two sequences each with as few as five or six-nucleotides joined together via 3′–3′ linkers. These do not require the –PuPu(Py)CGPyPy– hexameric motif generally found essential for CpG DNA immune stimulation. These novel, short-immunomers show potent immunostimulatory activity manifested by IL-12 and IL-6 secretion in murine spleen cell and PBMC cultures and splenomegaly in vivo. Short-immunomers show strong activation of NF-κB and stress-activated signaling pathways and induce cytokines in J774 cell cultures. The same sequences also induce cytokines in healthy human PBMC cultures whereas conventional CpG DNA requires different optimal sequences for murine and human immune cells. Additionally, short-immunomers inhibit IL-5 secretion and induce IFN-γ secretion in conalbumin-sensitized mouse spleen cell cultures, suggesting reversal of established Th2 responses to Th1 type responses. Short-immunomer also inhibits growth of MCF-7 human tumor xenograft in nude mice. This is the first report of activity with such short DNA sequences and also of sequences lacking hexameric motifs proposed in earlier studies.

Keywords: Anti-cancer; Th1 immune response; IFN-γ,IL-12; IL-6; Immunomodulatory; 3′–3′-linked; Oligonucleotides; Phosphorothioate

The presence of CpG dinucleotides in certain sequence contexts in bacterial and synthetic oligodeoxyribonucleotides (CpG DNAs) are known to activate vertebrate innate immune and B cells [1–4]. The activation of immune cells by CpG DNA induces secretion of a number of cytokines, including IFN-γ, IL-12, TNF-α, and IL-6, and stimulates expression of costimulatory surface molecules [5–8]. The immunomodulatory use of CpG DNAs is being explored broadly in anti-tumor, anti-infectious, anti-asthmatic, and anti-inflammatory applications and for adjuvant activity in immunotherapy [9–11].

The presence of a CpG dinucleotide and the sequences flanking the dinucleotide play a critical role in determining the immunostimulatory activity of DNA [3,12–14]. CpG dinucleotides in palindromic or non-palindromic hexameric sequences ($P_1P_2CGP_3P_4$) are required for immune stimulation. Further, PuPuCGPyPy and PuTCGPyPy motifs optimally activate murine and human immune systems, respectively [3,15]. A pathogen associated molecular pattern recognition receptor, TLR9, specifically recognizes CpG dinucleotides in DNA and activates intracellular immune signaling pathways [16].

Our structure-activity studies suggest that the receptor has high specificity for deoxyribonucleotides in the CpG dinucleotide as substitution by ribonucleotides or 2′-*O*-alkyl ribonucleotides eliminates immune responses [17]. We also identified a number of critical structural and functional group requirements within the pentose sugar [17–21], phosphate backbone [17,22], nucleobases [23,24], and nucleosides [25] of CpG DNA for immunostimulatory activity. Moreover, we investigated the

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role of functional groups on cytosine and guanine in receptor recognition through synthetic pyrimidine (Y) and purine (R) bases in YpG and CpR motifs to replace CpG [26].

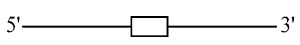
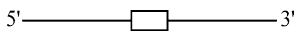
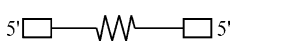
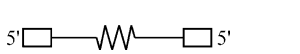
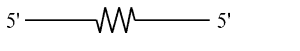
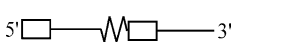
Recently, we showed that two CpG DNAs linked through their 5′-ends (5′–5′) have less immunostimulatory activity than a 5′ → 3′ CpG DNA containing the same number of CpG dinucleotides [27–29]. In contrast, an increased immunostimulatory activity is observed when they are linked through their 3′-ends (3′–3′) [27–30]. Based on these observations, we postulated that the CpG receptor(s) may read the sequence beginning at an accessible 5′-end [27–30].

Our recent studies to understand the structural requirement of flanking nucleosides P_1–P_4 in the motif $P_1P_2CGP_3P_4$ revealed that a nucleobase is absolutely required for C, G, P_3, and P_4 [24]. Surprisingly, an abasic nucleoside is permitted at either P_1 or P_2 depending on the neighboring base [24]. In the present study, we deleted the sequence up to P_1 on the 5′-side and sought the shortest oligomers that retained the activity of the parent CpG DNAs. Two identical strands of five or six nucleotides were joined through their 3′-ends using appropriate linkers. The resulting short-immunomers activated immune cells to produce cytokines in cell culture assays. They also reversed Th2 immune responses to Th1 type in allergen-sensitized mouse spleen cell cultures and showed anti-tumor activity in nude mice bearing MCF-7 human breast cancer xenograft. Moreover, unlike CpG DNA where different sequences are optimal for the receptors of human and mouse immune cells, short-immunomers, surprisingly, require no such sequence adjustment. Most importantly, it is not necessary to have a $P_1P_2CGP_3P_4$ hexameric motif for immune stimulation, especially in immunomers.

Materials and methods

Synthesis and purification. CpG DNAs and immunomers were synthesized on a 1–2 μmol scale using β-cyanoethylphosphoramidites on a PerSeptive Biosystem's 8990 Expedite DNA synthesizer as described earlier [29]. The phosphoramidites of dA, dG, dC, and T were obtained from PE Biosystems. C3-linker phosphoramidite was obtained from Glen Research Corporation. Immunomers were synthesized on solid supports carrying DiDMT protected glyceryl linker obtained from ChemGenes using parallel synthesis strategy as described earlier [29]. Beaucage reagent was used as an oxidant to obtain the phosphorothioate backbone modification [31]. Immunomers were deprotected using standard protocols, purified by HPLC, and dialyzed against USP quality sterile water for irrigation (Braun). The immunomers were lyophilized and dissolved again in distilled water and the concentrations were determined from UV absorbance at 260 nm. All immunomers were characterized by CGE and MALDI-TOF mass spectrometry (Applied Biosystem's Voyager-DE STR Biospectrometry Workstation) for purity and molecular mass, respectively (Table 1). The purity of full-length immunomers ranged from 89% to 95% with

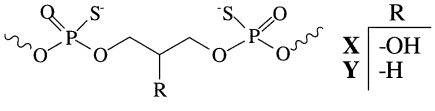
Table 1
Sequences and chemical modifications of immunomers

No.	Sequence[a]	Structure[b]	Length[c]	Molecular weight	
				Found	Calculated
1	5′-CTATCTGA$\overrightarrow{CGT}$TCTCTGT-3′		18-mer	5705	5704
2	5′-CTATCTG$\overrightarrow{TCG}$TTCTCTGT-3′		18-mer	5694	5695
3	5′-$\overrightarrow{TCG}$TTG-**Y-X-Y**-G$\overleftarrow{TTG}$CT-5′		6-mer	4308	4313
4	5′-$\overrightarrow{TCG}$TT-**Y-X-Y**-$\overleftarrow{TTG}$CT-5′		5-mer	3620	3623
5	5′-CTGTTG-**Y-X-Y**-GTTGTC-5′		6-mer	4310	4313
6	5′-$\overrightarrow{TCG}$TTGT-**Y**-$\overrightarrow{TCG}$TTGT-3′		15-mer	ND	4630

[a] Arrows indicate 5′ → 3′ directionality of CpG dinucleotide; chemical structures of **X** and **Y** linkers are shown below.

[b] Line drawings of immunomer stuructures, box represents the position of CpG dinucleotide in the sequence, plain and spiked lines represent nucleotide sequence and linker position, respectively.

[c] Length of each segment in immunomers excluding linkers.



the rest being shorter by one or two nucleotides (n-1 and n-2) as determined by CGE and/or denaturing PAGE. All CpG DNAs contained less than 0.075 EU/mL of endotoxin as determined by the Limulus assay (Bio-Whittaker).

Cell culture conditions and reagents. Spleen cells from 4 to 8-week-old BALB/c, C57BL/6, or C3H/HeJ mice were cultured in RPMI complete medium as described earlier [17,32]. Murine J774 macrophages (American Type Culture Collection, Rockville, MD) were cultured in Dulbecco's modified Eagles medium supplemented with 10% (v/v) FCS and antibiotics (100 IU/mL penicillin G/streptomycin). All other culture reagents were purchased from Mediatech (Gaithersburg, MD).

PBMC isolation from fresh murine and human blood. Peripheral blood mononuclear cells (PBMCs) from freshly drawn C3H/HeJ mouse or healthy human volunteer blood were isolated by Ficoll–Paque density gradient centrifugation method (Histopaque-1077, Sigma) as described earlier [24].

Establishment of Th2 immune response in mice. Four to six-week-old AKR/J male mice were obtained from Jackson Labs (Bar Harbor, Maine). The mice were given intraperitoneal injections containing 200 μg conalbumin (Sigma) with ImjectAlum adjuvant (Pierce) in 100 μL PBS on days 0 and 7, and intranasally challenged on days 14 and 21. The mice were sacrificed 72 h after the last challenge by CO_2 inhalation. Spleens were excised and single cell suspensions were prepared as described above. Spleen cells were treated with immunomers at different concentrations for 2 h followed by treatment with 50 μg/mL conalbumin. Supernatants were harvested after 72 h and IL-5 and IFN-γ levels were measured by ELISA as described below.

Cytokine ELISAs. Mouse spleen or J774 cells were plated in 24-well dishes using 5×10^6 or 1×10^6 cells/mL, respectively. The CpG DNA dissolved in TE buffer (10 mM Tris–HCl, pH 7.5, 1 mM EDTA) was added to a final concentration of 0.03, 0.1, 0.3, 1.0, 3.0, or 10.0 μg/mL to the cell cultures. The cells were then incubated at 37 °C for 24 h and the supernatants were collected for ELISA assays. The experiments were performed two or three times for each CpG DNA in triplicate for each concentration. The secretion of IL-12 and IL-6 was measured by sandwich ELISA as described previously [30]. The required reagents, including cytokine antibodies and standards, were purchased from PharMingen.

Mouse splenomegaly assay. 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 subcutaneously (SC) to mice at a dose of 5 mg/kg. The mice were sacrificed and the spleens were harvested and weighed as described previously [17,32].

Preparation of J774 cell nuclear extracts and EMSA. NF-κB activation in J774 cells treated with CpG DNAs was carried out and analyzed by EMSA as described previously [30].

Preparation of J774 cell lysates and Western blotting. To detect the phosphorylated and total p38 MAP kinase by Western blotting, J774 cells were grown in serum-reduced medium (0.5% FCS) for 24 h and then stimulated with immunomers for 30 min. After stimulation, cells were washed with cold PBS and lysed in 2% SDS sample buffer as per the protocol provided by Cell Signaling Technology. Crude lysates were resolved on 10% SDS polyacrylamide ReadyGels (Bio-Rad) and blotted onto nitrocellulose membranes. Membranes were probed with a phospho-p38 MAP kinase (Thr 180/Tyr 182) antibody and visualized using Enhanced Chemiluminescence Kit (PE Life Sciences). The blots were then stripped and reprobed with an antibody to p38 MAPK that detects total levels of endogenous p38 MAPK protein. All antibodies were purchased from Cell Signaling Technology.

In vivo nude mice model and treatment plan. The animal use and care protocols were approved by the Institutional Committee on Animal Use and Care of the University of Alabama at Birmingham. Female athymic nude mice (nu/nu, 4–6 weeks old) were obtained from Frederick Cancer Research and Development Center (Frederick, MD, USA) and inoculated with MCF-7 cells as described previously [30]. The animals bearing MCF-7 xenograft (50–100 mg) were randomly divided into various treatment groups and treated by subcutaneous injection with short-immunomer **3** at a dose of 0.5 mg/kg or saline (control) on days 1, 3, and 5 every week. The mice were monitored by general clinical observation as well as by body weight and tumor growth. Tumor growth was recorded with the use of calipers, by measuring the long and short diameters of the tumor. Tumor mass (in grams) was calculated using the formula $1/2a \times b^2$, where "a" and "b" are the long and short diameters (in centimeters), respectively.

Results

Design and synthesis of short-immunomers

Two 18-mer CpG DNA sequences **1** and **2**, containing a mouse specific 'GACGTT,' or human specific 'GTCGTT' hexameric sequence motif, respectively (Table 1) are used as positive controls in the study. These sequences have been shown to induce immunostimulatory activity in earlier studies [23–30]. The immunomers used in the present study contain two identical segments that are tethered through their 3′-ends via a non-nucleosidic glyceryl-linker (see Table 1). Earlier we showed that immunomers containing 11-mer segments exhibit greater activity than their parent CpG DNAs [29]. Our results also showed that immunomers containing shorter segments had activity if suitable linkers are placed appropriately between the segments [29]. In the present study, we used a glyceryl linker in combination with two flanking C3-linkers (Table 1). Immunomers **3** and **4** contained six- (TCGTTG) or five-nucleotide (TCGTT) segments, respectively, which only partially meet the PuPu(Py)CGPyPy motif rule. Since they contained two identical sequence segments, we synthesized them in parallel on an automated DNA synthesizer using solid support with diDMT-glyceryl linker as reported earlier [29]. Parallel synthesis of immunomers **3** and **4** involved seven and six coupling cycles, respectively, while oligos **1** and **2** required 17-coupling cycles. An immunomer (**5**) with the same length as that of immunomer **3** but without a CpG dinucleotide was used as a control. Additionally, oligo **6** containing two copies of 5′-TCGTTGT-3′ linked through a C3-linker (15-mer) was synthesized, in 15 coupling cycles, as a control (Table 1).

Activity of short-immunomers in murine spleen cell and PBMC cultures

All immunomers showed a concentration-dependent induction of two typical cytokines, IL-12 and IL-6, in BALB/c, C57BL/6, and C3H/HeJ mouse spleen cell cultures. As expected, CpG DNA **1**, containing a mouse specific sequence motif, generally showed greater activity than CpG DNA **2** with a human specific sequence motif (Table 2). At lower concentrations, immunomers **3** and **4** generally induced levels of cytokines lying between

Table 2
In vitro cytokine secretion in murine spleen cell, and PBMC cultures and in vivo spleen enlargement

No.	Spleen weight (mg)[b]	Spleen cell cultures[c]						PBMC cultures[d]	
		BALB/c		C57BL/6		C3H/HeJ		C3H/HeJ	
		IL-12 (pg/mL)	IL-6 (pg/mL)	IL-12 (pg/mL)	IL-6 (pg/mL)	IL-12 (pg/mL)	IL-6 (pg/mL)	IL-12 (pg/mL)	IL-6 (pg/mL)
1	153 ± 10.0	1775 ± 139	9774 ± 798	889 ± 71	7257 ± 132	931 ± 148	5465 ± 140	6541 ± 254	1602 ± 175
2	138 ± 10.3	612 ± 6	3211 ± 287	266 ± 12	3605 ± 404	157 ± 55	2099 ± 234	3838 ± 92	482 ± 93
3	146 ± 9.7	1532 ± 196	6086 ± 268	831 ± 114	11218 ± 631	650 ± 89	3503 ± 53	2327 ± 373	1095 ± 106
4	125 ± 9.8	1711 ± 126	6606 ± 322	884 ± 45	8410 ± 309	783 ± 42	3456 ± 307	4400 ± 703	536 ± 26
5	NT	53 ± 4	ND	77 ± 6	281 ± 57	24 ± 5	ND	ND	ND
M/V[a]	75 ± 2.4	47 ± 1	7 ± 1	71 ± 12	108 ± 38	24 ± 6	10 ± 3	25 ± 8	5 ± 3

NT and ND stand for not tested and not detected, respectively.
[a] Medium or vehicle (PBS) control.
[b] Average spleen weight of three mice per group obtained after 48 h of subcutaneous administration of single dose of 5 mg/kg immunomer.
[c] At a concentration of 3.0 μg/mL immunomer.
[d] At a concentration of 10.0 μg/mL immunomer.

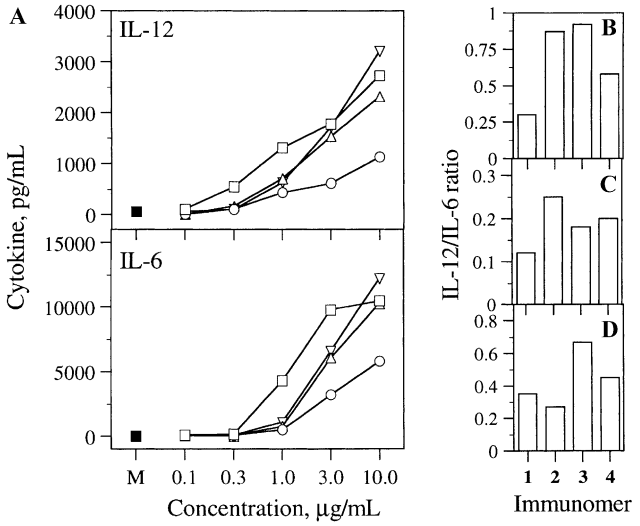


Fig. 1. (A) Concentration-dependent cytokine induction in BALB/c mouse spleen cell cultures. Squares, **1**; circles, **2**; triangles, **3**; and reversed-triangles, **4** in both IL-12 and IL-6 panels. The ratio of IL-12 to IL-6 induced in (B) BALB/c, (C) C57BL/6, and (D) C3H/HeJ mice spleen cell cultures at 3 μg/mL concentration of immunomers, **1**–**4**.

those found with **1** and **2** in BALB/c mouse cells (Fig. 1). However, at higher concentrations the activities of **3** and **4** matched or exceeded that of **1** even though the short immunomers do not contain the mouse specific 'GACG TT' motif.

Levels of IL-12 and IL-6 induced by **1**–**5** at concentrations of 3.0 μg/mL in the three strains of mouse spleen cell cultures are shown in Table 2. Levels vary with mouse strain and depend on sequence (**1** vs **2**) and the structure of the CpG DNA. Control immunomer **5** produced cytokine levels similar to background showing the need for the CpG dinucleotide. In general, a higher IL-12 to IL-6 ratio was found for short-immunomers compared with CpG DNAs **1** and **2** (Fig. 1B). The results obtained in LPS non-responsive strain, C3H/HeJ mouse spleen cell cultures suggest that immunomer activities are not due to LPS contamination acting on the TLR4 receptor.

Previously, we showed that the presence of multiple CpG dinucleotides in oligonucleotides does not increase activity significantly over that due to a single copy. Here, control oligo **6** contains two copies of a CpG containing heptamer joined $5' \rightarrow 3'$ through a C3-linker. Oligo **6** induced 501 ± 21 pg/mL IL-12 and 514 ± 164 pg/mL IL-6 at a concentration of 3.0 μg/mL in C57BL/6 mouse spleen cell culture assays compared with 2833 ± 341 and 2870 ± 760 pg/mL IL-12, and 24276 ± 4740 and 14256 ± 3304 pg/mL of IL-6 by **1** and **3**, respectively, at the same concentration. These results confirm that the activity seen with **3** and **4** is not as a result of the presence of two copies of CpG dinucleotide, but because of their optimal structure for receptor recognition.

In BALB/c mouse bone marrow derived dendritic cell and macrophage cell cultures, short-immunomer **3** induced similar levels of IL-12, IFN-γ, but significantly less IL-6, TNF-α, and nitric oxide (NO) compared with CpG DNA **1** (data not shown).

Spleen cells consist of different subsets of cell population than peripheral blood mononuclear cells (PBMCs). To examine, if this difference in cell population could result in different activities, we isolated PBMCs from C3H/HeJ mouse peripheral blood and tested for the ability of short-immunomers to induce IL-12 and IL-6 secretion. The results obtained at 10 μg/mL concentration of immunomers are presented in Table 2. Immunomers **3** and **4** showed a similar trend of cytokine induction as in spleen cells.

In vivo activity of short-immunomers as determined by increase in spleen weight or splenomegaly

To further test the immunostimulatory activity, a single dose of 5 mg/kg immunomers **1–4** was injected subcutaneously to BALB/c mice and the spleen weights of mice (3 per group) were measured after 48 h [17,32,33]. Average weights of 153, 138, 146, and 125 mg (all ± 10 mg) were found for **1–4**, respectively, while PBS-treated controls gave 75 ± 6 mg. Treatment with **6** containing two copies of TCGTTGT gave 123 ± 7 mg after 72 h recapitulating the lower activity of this compound in cell culture. These results further confirm the immunostimulatory activities found in vitro.

Influence of the nucleotide preceding CG dinucleotide on immunostimulatory activity

Recently, we showed that the activity of the $P_1P_2CGP_3P_4$ motif containing an abasic deoxynucleoside substitution at P_1 is influenced by the nature of the nucleoside (A, C, G, or T) present at P_2 [24]. Since we completely deleted P_1 in short-immunomers, we evaluated the effect of different nucleosides at P_2. Analogs of **3** were synthesized having A, G, or C, instead of T at each 5′-end. In general, those with A, G, or C showed lower or no induction of cytokines compared to **3** (IL-12 secretion in BALB/c spleen and J774 cell cultures is shown in Fig. 2).

Short-immunomers activate NF-κB and p38 MAP kinase pathways in J774 cell cultures

CpG DNA activates the NF-κB and p38 MAP kinase signaling pathways, which play a critical role in stimulating cytokine gene expression [34,35]. To see if short-immunomers work by the same mechanism, we studied activation of NF-κB in J774 cell nuclear extracts (Fig. 3A). CpG DNA **1**, which has mouse specific sequence, activated NF-κB as expected (lane 4) [25], as did LPS in lane 5 (Fig. 3A). In contrast, CpG DNA **2**, containing human specific sequence, failed to induce NF-κB (lane 2), suggesting specificity of the receptor for CpG DNA sequence. Immunomers **3** and **4**, which have two 5′-accessible ends and five-nucleotide homology to the human specific motif, GTCGTT, activated NF-κB (lanes 6 and 7) to the same extent as **1**, suggesting that short immunomers are recognized as mouse specific sequences.

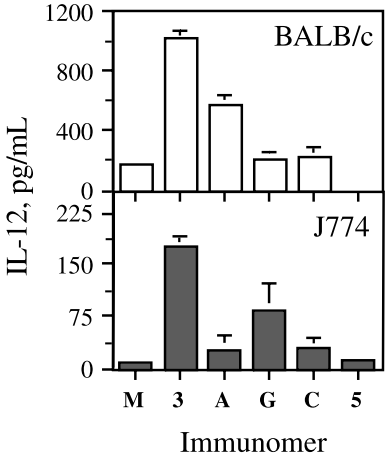


Fig. 2. Effect of the nucleotide preceding CpG dinucleotide in short immunomers as determined by the induction of IL-12 secretion in BALB/c mouse spleen and J774 macrophage cell cultures at a concentration of 10.0 μg/mL immunomers.

Additionally, to examine the activation of stress kinase pathways by short-immunomers, we examined p38 MAP kinase activity in J774 macrophages after

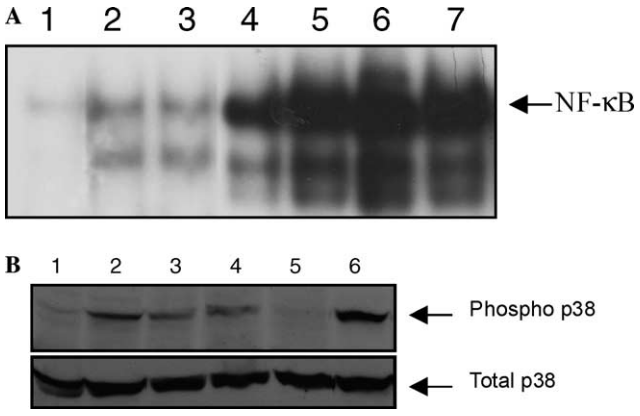


Fig. 3. (A) A gel showing activation of NF-κB in J774 macrophages after stimulation for 1 h with 10.0 μg/mL immunomers. Lane 1, media treated control; lane 2, human specific CpG DNA **2**; lane 3, control non-CpG DNA **5**; lane 4, mouse specific CpG DNA **1**; lane 5, LPS at 0.1 μg/mL; lane 6, immunomer **3**; and lane 7, immunomer **4**. (B) p38 phosphorylation in J774 macrophages following activation with immunomers for 30 min at 10.0 μg/mL concentration. Lane 1, media treated control; lane 2, mouse specific CpG DNA **1**; lane 3, immunomer **3**; lane 4, immunomer **4**; lane 5, human specific CpG DNA **2**; and lane 6, LPS at 0.1 μg/mL. Total p38 content is shown in lower panel.

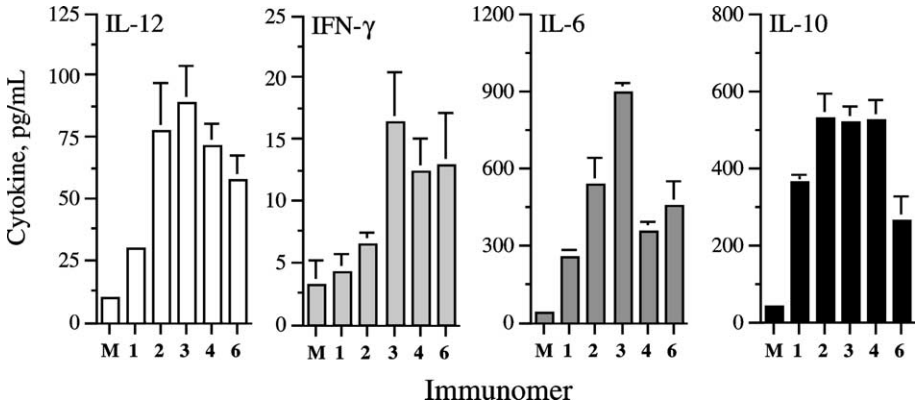


Fig. 4. Induction of IL12, IFN-γ, IL-6, and IL-10 secretion in human peripheral blood mononuclear cell (hPBMC) cultures at 1.0 μg/mL concentration of short-immunomers after 72 h treatment. See Materials and methods for experimental details.

treatment with immunomers (Fig. 3B). Both **3** and **4** activated stress-activated pathways as shown by the presence of phosphorylation product in J774 cell lysates within 30 min of treatment (lanes 3 and 4) as did **1** (lane 2) and LPS (lane 6). In contrast, **2** containing human specific CpG motif failed to activate the stress-activated pathway. Consistent with the activation of NF-κB, **1**, **3**, and **4**, but not **2** and **5**, induced IL-12 and IL-6 in J774 cell cultures (data not shown).

Immune-response in human PBMCs

Short-immunomers were further tested for their ability to stimulate human PBMCs to secrete cytokines IL-12, IL-6, IL-10, and IFN-γ. Representative data obtained in a single healthy donor PBMC cultures at 1 μg/mL concentration are shown in Fig. 4. As expected, mouse specific CpG DNA **1** induced lower cytokine production than did human specific **2**. Short-immunomers **3** and **4** gave similar or higher levels of cytokine induction than did CpG DNA **2**. CpG DNA **6** showed lower cytokine induction than did **2**, **3**, and **4** despite containing as many CpG dinucleotides as **3** and **4**.

Effect of short-immunomer on IL-5 and IFN-γ secretion in allergen-sensitized spleen cell cultures

To assess the effects of treatment of short-immunomers on Th2 cytokines associated with allergic airway responses, we measured IL-5 and IFN-γ secreted in spleen cell cultures obtained from AKR/J mouse challenged with conalbumin [36,37]. In the absence of immunomer treatment, conalbumin-sensitized spleen cells secreted markedly higher levels of IL-5 and low IFN-γ, suggesting predominantly a Th2 type response (Fig. 5A). When the allergen-primed spleen cells were treated with immunomers, a concentration-dependent decrease

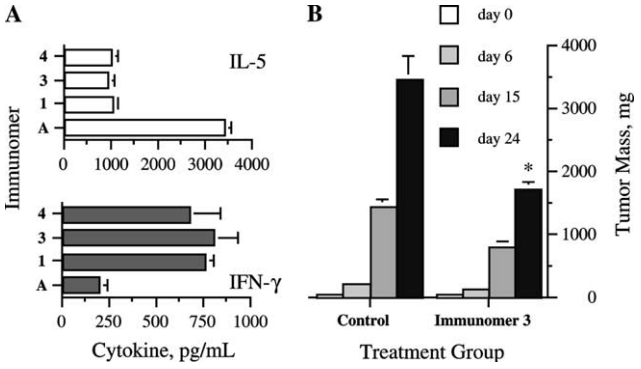


Fig. 5. (A) Induction of IL-5 (top) and IFN-γ (bottom) secretion in conalbumin-sensitized AKR/J mice spleen cell cultures at 10.0 μg/mL concentration of immunomers. **A** stands for allergen (conalbumin)-sensitized but not treated with immunomers. (B) Anti-tumor activity of immunomer **3** in nude mice bearing MCF-7 human breast cancer xenograft. Control represents the group of mice treated with saline. Each group had six mice. Immunomer was given subcutaneously on days 1, 3, and 5 every week. * Represents statistically significant value ($p < 0.01$).

in IL-5 and increase in IFN-γ secretion were observed. Fig. 5A shows IL-5 (top plot) and IFN-γ (bottom plot) secretion levels at 10 μg/mL concentration of immunomers. These data suggest that short-immunomers not only induce Th1 type cytokine secretion but potently reverse Th2 responses in vitro.

Anti-tumor activity of short-immunomer in nude mice bearing human breast cancer MCF-7 xenograft

PS-CpG DNAs and PO-immunomers have been shown to induce potent anti-tumor activity in vivo [30,38,39]. To examine if the potential in vitro activity of short-immunomers can be translated into in vivo anti-tumor activity, we administered short-immunomer **3** subcutaneously at a dose of 0.5 mg/kg three times a week to nude mice bearing MCF-7 breast cancer xenografts that express wild-type p53. At the relatively low dose,

immunomer **3** gave 51% inhibition of MCF-7 tumor growth on day 24 compared with the saline control ($p < 0.01$) (Fig. 5B). These preliminary anti-tumor studies further suggest that short-immunomers exhibit potent anti-tumor activity in vivo as a result of potent immune stimulation.

Discussion

Potential applications exist for various CpG DNA molecules to treat a broad spectrum of diseases including cancer [30,38,39], allergy, asthma [36,37], and infections [40,41]. Additionally, as a result of their ability to induce strong Th1 type immune responses, CpG DNAs have been shown useful as potent adjuvants [42,43]. Recently, we proposed a novel design of CpG DNAs called immunomers in which two CpG DNA molecules are attached through their 3′-ends, based on our earlier observations that an accessible 5′-end of CpG DNA is required for immune stimulation [27–30]. These studies allowed us to postulate that the receptor reads the CpG DNA sequence from the 5′-end to the 3′-end and any sequence or structural changes placed towards the 5′-end have a greater effect on immune stimulation than those placed in the 3′-flanking sequence [27–30]. In fact, the immunomer design allowed us to distinguish immunostimulatory effects of phosphorothioate and phosphodiester CpG DNAs [30]. In the present study, for the first time, we showed short-immunomers, containing pentamers and hexamers with only a partial sequence match to the [PuPu(Py)CGPyPy] motif that is necessary for activating immune cells [3,15], strongly activate immune responses and induce cytokine secretion in mouse and human cell cultures.

TLR9, a member of the Toll-Like Receptor family, has been shown to be important for CpG DNA recognition and subsequent activation of signaling pathways [16]. Following activation of TLR9, a number of cellular events are initiated including recruitment of MyD88, IRAK, and TRAF6, and activation of IκB kinase, MAP kinase, the stress kinases N-terminal c-Jun kinases JNK-1 and -2, and p38, leading to the activation of multiple transcription factors ATF-2, AP-1, and NF-κB [44]. The transcription factors activated ultimately stimulate the synthesis of a number of regulatory cytokines and expression of costimulatory molecules. The results obtained in J774 cell cultures with short-immunomers clearly show that NF-κB and p38 MAP kinase pathways are activated as is the case with mouse specific CpG DNA. However, human specific CpG DNA failed to show activity in J774 cell cultures as expected because the murine receptor does not recognize this sequence. These studies suggest that a CpG dinucleotide appropriately presented to the receptor as in the case of short-immunomers can potentially activate immune cells without requiring a PuPuCGPyPy hexameric core sequence.

Our previous results showed that CpG DNAs lacking an accessible 5′-end fail to activate NF-κB and induce cytokine secretion, suggesting that the receptor is unable to recognize the molecule lacking an accessible 5′-end, even though it has two appropriate CpG dinucleotides [27–29]. The immunomers having two free 5′-ends might be recognized by the receptor at both 5′-ends leading to the enhanced activity [28–30]. The present results with short-immunomers further validated this hypothesis. CpG DNA **6**, with two CpG dinucleotides, is no more immunostimulatory than those with one [29]. Possibly binding of the first CpG by a receptor may preclude binding of the second by a second receptor, because the molecule has only a single accessible 5′-end.

Short-immunomers **3**, **4**, and **6** have a five-nucleotide sequence, TCGTT, that matches the last five nucleotides of the human specific hexameric sequence motif GTCGTT or last four nucleotides of the mouse specific hexameric sequence motif GACGTT. In spite of this difference in sequence, **3** and **4**, unlike **1** and **2**, showed potent activity in both murine and human cultures where they appear to be recognized to similar extents. **6** is linked $5' \rightarrow 3'$ and showed lower activity in murine cell cultures and similar activity in human cell cultures as that of human specific oligo **2**, suggesting this oligo is recognized differently by human and murine immune cells. Therefore, the new short immunomer design developed here may give rise to CpG DNAs that are recognized by a variety of vertebrate species without sequence optimization. However, further studies currently underway are required in different vertebrate species to validate this concept.

Oligonucleotides are primarily degraded by 3′-exonucleases [45]. Short-immunomers lacking a free 3′-end are more resistant to these enzymes in vivo [30]. Oligonucleotides can be degraded from 5′-ends at a significantly slower rate. The slow removal of one (T) or two (T and C) nucleotides from the 5′-end of short-immunomer results in inactive metabolites that no longer elicit immunostimulatory activity. Our studies with 5′-truncated immunomers suggest that at least one nucleotide (preferably T) is required on the 5′-side adjacent to CpG dinucleotide (unpublished data) and we showed that a CpG dinucleotide is required for immunostimulatory activity. This is of particular importance where a brief immune activation is desired rather than a prolonged effect.

In conclusion, we proposed a novel design of CpG DNAs in which pentamers or hexamers are joined by 3′–3′-linkers. These do not necessarily contain the hexameric core sequences generally necessary for immunostimulatory activity. As a result of the unique structure and appropriate placement of CpG dinucleotides, they showed potent immunostimulatory activity in

both murine and human cell culture systems, suggesting the possibility of overriding species-dependent receptor specificity for CpG DNA sequences. In spite of their unique structure, short length, and lack of the usual hexameric CpG motif, these immunomers acted on the same pathways as other CpG DNAs and perhaps bacterial CpG DNA, although subtle differences are noted in cytokine induction profiles. Short-immunomers may reverse antigen induced Th2 immune responses to Th1 type immune responses through inhibition of IL-5 secretion and induction of IFN-γ secretion. Treatment with short-immunomers inhibited growth and establishment of breast tumors in nude mice. Further, short-immunomers might provide sustained therapeutic and safety profiles because of their unique structure, length, and location of immunoactive CpG dinucleotide in the sequence. As CpG DNA-based immunomodulatory agents are being vigorously exploited for various therapeutic indications including infectious diseases affecting millions of people worldwide, short-immunomers may prove to be an economically feasible design because of their short synthetic cycles, in addition to their potent biological and safety profiles.

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