

Strong Cytosine-Guanosine-Independent Immunostimulation in Humans and Other Primates by Synthetic Oligodeoxynucleotides with PyNTTTTGT Motifs

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Synthetic oligodeoxynucleotides (ODNs) containing cytosine-guanosine (CpG) motifs stimulate B and plasmacytoid dendritic cells of the vertebrate immune system. We found that in primates strong stimulation of these cells could also be achieved using certain non-CpG ODNs. The immunostimulatory motif in this case is a sequence with the general formula PyNTTTTGT in which Py is C or T, and N is A, T, C, or G. Assays performed on purified cells indicated that the immunostimulatory activity is direct. The use of a nuclease-resistant phosphorothioate backbone is not a necessary condition, since phosphodiester PyNTTTTGT ODNs are active. It was also demonstrated that ODN 2006, a widely used immunostimulant of human B cells, possess two kinds of immunostimulatory motifs: one of them mainly composed of two successive TCG trinucleotides located at the 5' end and another one (duplicated) of the PyNTTTTGT kind here described. Even though PyNTTTTGT ODNs are mainly active on primate cells, some of them, bearing the CATTGT motif, have a small effect on cells from other mammals. This suggests that the immunostimulatory mechanism activated by these ODNs was present before, but optimized during, evolution of primates. Significant differences in the frequency of PyNTTTTGT sequences between bacterial and human DNA were not found. Thus, the possibility that PyNTTTTGT ODNs represent a class of pathogen-associated molecular pattern is unlikely. They could, more reasonably, be included within the category of danger signals of cell injury. *The Journal of Immunology*, 2003, 171: 3697–3704.

Synthetic oligodeoxynucleotides (ODNs)² containing unmethylated cytosine-guanosine dinucleotides (CpG-ODNs), within a given context, stimulate cells of the vertebrate immune system (1). In vitro, CpG-ODNs can directly activate B cells and plasmacytoid dendritic cells (2–6). Moreover, they can activate, albeit indirectly, monocytes, macrophages, NK, and memory T cells (7–10). In vivo, CpG-ODNs are potent adjuvants, promoting cellular and humoral immune responses specific for a variety of Ags (11–15). CpG-ODNs have also been proved to be effective in animal models of cancer and allergy (16–19). Thus, CpG-ODNs can be visualized as valuable new immunological drugs with a broad spectrum of possible uses. Several clinical trials using CpG ODNs are currently in progress (1).

Regarding structural features necessary for immune activation, it can be said that optimal immune activation requires, in addition to unmethylated CpG dinucleotides, flanking dinucleotides of different composition according to the vertebrate animal species. For example, the optimal CpG motif for mice has been reported to be GACGTT (2), and that for humans and other primates is GTCGTT (20). CpG-ODNs with phosphorothioate bonds including this last motif have been reported to be among the most active in sheep, goats, horses, pigs, dogs, cats, chickens, and cotton rats (21). This

suggested a high degree of evolutionary conservation in recognition mechanisms for immunostimulatory ODNs.

Differences between content and context of CpGs in microbial and vertebrate DNAs suggested that immune recognition of CpG motifs triggers protective pathways analogous to those activated by pathogen-associated molecular patterns that are characteristic of the innate immune system (1). However, the panorama may be more complicated, since we have now found that direct stimulation of B cell and plasmacytoid dendritic cells could be reached by a very efficient CpG-independent pathway that is fully operative mainly in animals of the order *Primate*.

Materials and Methods

Oligodeoxynucleotides

Desalted phosphodiester or phosphorothioate ODNs were purchased from Operon Technologies (Alameda, CA), Annovis (Aston, PA), or Oligos ETC (Bethel, ME). ODNs were suspended in depyrogenated water, assayed for LPS contamination using the *Limulus* test, and kept at -20°C until used. Purity was assessed by HPLC and PAGE assays. ODN preparations were used if purity was $>97\%$ and LPS levels were undetectable.

Antibodies

Abs for flow cytometry or ELISA were purchased from Serotec (Raleigh, NC).

PBMC

Blood was obtained by venipuncture from healthy donors or monkeys at the animal facility of the Centro Medico de Investigaciones Clínicas (Buenos Aires, Argentina) using heparin as anticoagulant. PBMC were isolated by Ficoll-Hypaque (Sigma-Aldrich, St. Louis, MO) density gradient centrifugation. Briefly, blood samples diluted 1/2 in RPMI 1640 medium (PAA Laboratories, Linz, Austria) supplemented with 2.0 mM L-glutamine, 50.0 $\mu\text{g}/\text{ml}$ gentamicin, and 20 mM HEPES were centrifuged at $1000 \times g$ for 40 min at 20°C . PBMC were isolated, washed, and suspended in medium supplemented with 10% FCS.

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² Abbreviations used in this paper: ODN, oligodeoxynucleotide; CpG, cytosine-guanosine/dinucleotide.

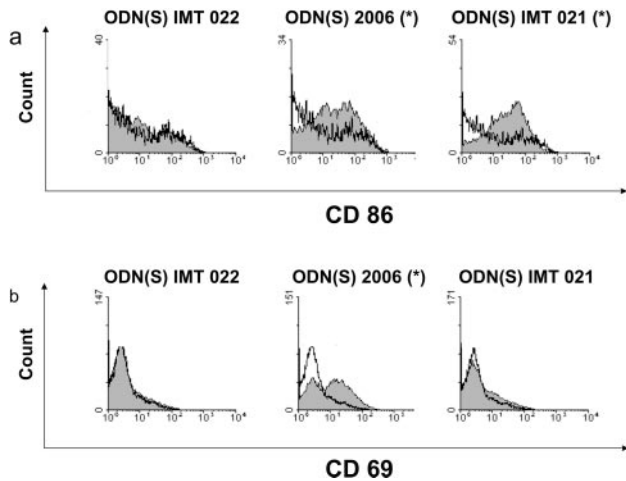


FIGURE 1. Induction of CD86 on CD19 cells and CD69 in CD56 cells by phosphorothioate CpG and non-CpG ODNs. Human PBMC were cultured for 48 h with the indicated ODN and then stained with fluorescent anti-CD19/anti-CD86 (a) or cultured for 24 h with the indicated ODN and then stained with fluorescent anti-CD56/anti-CD69 (b). Flow cytometric results are presented as histograms corresponding to cells in the CD19- or CD56-positive gate. □, Cells cultured in the absence of ODN; ■, cells cultured in the presence of ODN. ODN (S), phosphorothioate ODN. Each experiment was repeated four times using PBMCs from different donors. Results were similar in all cases to those presented here. Statistical significance was evaluated by Student's *t* test. *, Statistically significant differences ($p < 0.05$) compared with cells cultured in the absence of ODN.

Purification of cells

B lymphocytes and plasmacytoid dendritic cells were purified from human PBMC using MACS (Miltenyi Biotec, Bergish Gladbach, Germany). Primary B cells were prepared by an indirect system using Abs against CD2, anti-IgE, CD4, CD11b, CD16, and CD36 to deplete T cells, NK cells, monocytes, granulocytes, platelets, and erythroid precursor cells from PBMCs. Plasmacytoid dendritic cells were positively isolated using an anti-blood dendritic cell Ag-4 Ab. Cell purity was >96% according to flow cytometric assays.

Cell proliferation assays

PBMC were cultured in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated FCS, 2.0 mM L-glutamine, and 50.0 μ g/ml gentamicin. Cells (1×10^5 cells/well) were incubated in 96-well microtiter plates (NUNC, Copenhagen, Denmark) at 37°C in a 5% CO₂ humidified atmosphere for 72 h. PBMC were stimulated with ODNs at 0.375 μ g/ml unless otherwise stated. Eighteen hours before cell harvest, 1 μ Ci of [³H]thymidine (Amersham Pharmacia Biotech, Piscataway, NJ; sp. act., 25 Ci/mmol) was added to each well. Cells were harvested onto glass-fiber filters, and ³H incorporation was measured by scintillation counting. The SDs of quadruplicate wells were <10%.

Table I. Induction of PBMC proliferation and IL-6 and IgM secretion by non-CpG variants of ODN 2006^a

ODN (S)	Sequence	Proliferation Index			IL-6 Secretion (pg/ml)			IgM Secretion (ng/ml)		
		Avg.	<i>n</i>	SD	Avg.	<i>n</i>	SD	Avg.	<i>n</i>	SD
2006	TCGTCGTTTTGTCGTTTTGTCGTT	13.56	4	2.15	222.5	4	83.4	776	4	214
IMT501	T <u>AGT</u> AGTTTTGTA <u>GT</u> TTTTGTA <u>GT</u>	5.04	4	2.23	60.8	4	54.0	567	4	199
IMT502	TGGT <u>GG</u> TTTTGTCGTTTTGTCGTT	6.86	4	2.84	76.6	4	91.4	555	4	216
IMT503	T <u>TGT</u> TGTTTTGTTGTTTTGTTGTT	8.47	4	2.09	150.2	4	71.4	640	4	120
IMT504	TC <u>ATC</u> ATTTGTCATTTTTGTCATT	13.98	4	1.88	298.6	4	49.2	952	4	222
IMT505	TCC <u>TC</u> TTTTGTCCTTTTTGTCCTT	13.08	4	2.53	226.1	4	17.6	799	4	242
IMT506	TC <u>T</u> TCTTTTGTCCTTTTTGTCCTT	12.62	4	2.09	242.7	4	61.3	857	4	131
None		1.00	4		<30.0	4		315	4	138

^a The averages (Avg.) shown are the mean of the results of *n* PBMCs from different donors. (S), phosphorothioate. Changes with respect to ODN 2006 are underlined. The ODN concentration was 0.375 μ g/ml in proliferation assays, 1.5 μ g/ml in IgM assays, and 6 μ g/ml in IL-6 assays.

IL-6 assay

PBMC (3×10^5 /well) or purified B cells (2×10^5) were cultured as described above with ODNs for 24 h. Phosphorothioate ODNs were added (6 μ g/ml) at time zero of incubation. Phosphodiester ODNs were added as follows: 30 μ g/ml at time zero, 30 μ g/ml after 4 h, and 30 μ g/ml after 16 h of incubation. After incubation, supernatants were collected, and IL-6 levels were measured by ELISA. Briefly, 96-well microtiter plates (NUNC) were coated with anti-IL-6 Abs and blocked with RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated FCS. IL-6 was detected colorimetrically using biotin-labeled Abs, followed by peroxidase-conjugated streptavidin and then peroxidase-specific colorimetric substrate. Standard curves were generated using known amounts of recombinant IL-6. The detection limit of these assays was 30 pg/ml. All assays were performed in duplicate.

IgM assay

PBMC (3×10^5 /well) or purified B cells (10^5 /well) were cultured as described above with ODNs (1.5 μ g/ml) for 72 h. After this, supernatants were collected, and IgM was assayed by ELISA. Briefly, 96-well microtiter plates (NUNC) were coated with anti-IgM Abs and blocked with RPMI 1640 medium. IgM was detected colorimetrically using peroxidase-labeled Abs, followed by peroxidase-specific colorimetric substrate. Standard curves were generated using known amounts of purified IgM. The detection limit of these assays was 50 ng/ml. All assays were performed in duplicate.

Flow cytometry

Staining of surface Ags was performed as previously described (22). Anti-CD19 (clone LT19), CD86 (clone BU63), CD4 (clone S 3.5), CD40 (clone LOB 7/6), MHC class I (clone W6/32), and MHC class II (clone WR 18) Abs (Serotec, Raleigh, NC). Flow cytometric data for 20,000 cells/sample were acquired on a FACScan (BD Biosciences, San Jose, CA). Data were analyzed using the computer program Win MDI, 2.8, Interface Flow Cytometry Application (Joseph Trotter, copyright 1993–1998).

Statistical analysis

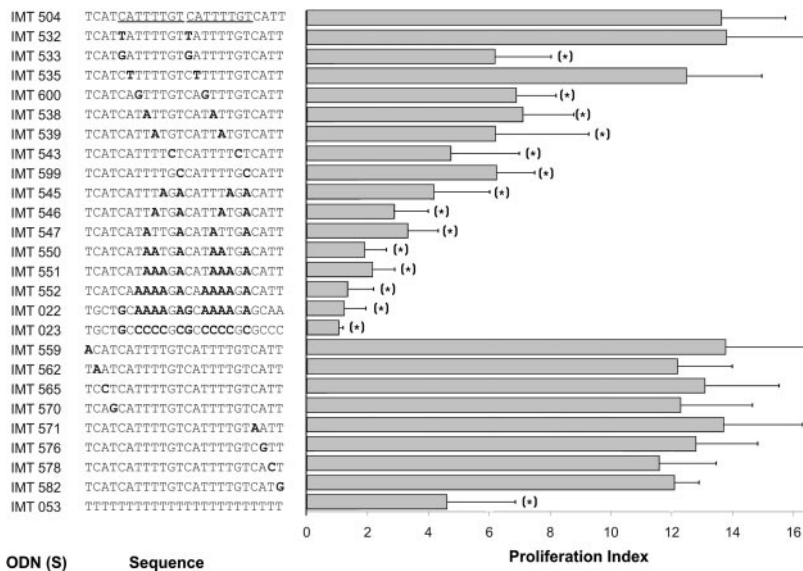
Statistical significance of differences was evaluated by Student's *t* test. Differences were considered significant for $p < 0.05$.

Results

Identification of non-CpG ODNs with immunostimulatory activity

During the course of a study aimed to investigate the mechanisms of direct human B cell activation by CpG ODNs, we surprisingly found that inversion of the CpGs groups present in ODN 2006 described by Hartmann and Krieg as strongly active on human cells (6) did not significantly suppress the immunostimulatory activity on PBMC as measured by immunoproliferation and IL-6 and IgM secretion (not shown). Furthermore, ODN IMT021 containing the inverted CpG groups was able to increase the expression of the costimulatory molecule CD86 on CD19-positive cells (B cells) as efficiently as ODN 2006 (Fig. 1a). However, ODN

FIGURE 2. Influence of nucleotide changes in the activity of the immunomodulatory PyNTTTTGT motif. Nucleotide changes (in bold) were introduced in the phosphorothioate ODN IMT504 that have two PyNTTTTGT motifs (underlined). Each experiment was repeated four times using PBMCs from different donors. Results represent the means and SDs of these experiments. Statistical significance was evaluated by Student's *t* test. *, Statistically significant differences ($p < 0.05$) compared with cells cultured in the presence of ODN IMT504.



IMT021 was poorly effective compared with ODN 2006 to stimulate the expression of CD69 on CD 56-positive cells (NK cells; Fig. 1*b*). This strongly suggested that in human cells, a CpG-independent B cell activation pathway different from the one that leads to NK cell activation might exist. Therefore, in ODN 2006 two independent motifs may coexist, one acting on the pathway for B cell activation and the other acting on the pathway for NK cell activation.

To investigate the composition of the B cell activation motif we first systematically altered the CpGs present in ODN 2006 (Table I). The Gs of the CpGs within ODN 2006 are not necessary for full stimulation of cell proliferation and IL-6 and IgM secretion. However, modification of the Cs of the CpGs is detrimental even though immunostimulatory activity remains significant. These results clearly indicate that stimulation of PBMC cell proliferation and IL-6 and IgM secretion by ODN 2006 is not necessarily associated with the integrity of the CpG groups.

The analysis of hundreds of ODNs allowed us to define a core sequence responsible for optimal non-CpG immunostimulatory activity on human B cells. This motif is PyNTTTTGT, wherein Py is C or T, and N is any deoxynucleotide. Fig. 2 shows the influence of some changes in nucleotides within the two motifs (CATT TGT) present in the phosphorothioate ODN IMT504. As shown, simultaneous changes in only one of the critical nucleotides of the two motifs results in a 40–60% loss in the proliferation activity, while two changes result in a 60–90% loss. On the other hand, changes outside the motifs do not produce significant changes. Similar results were obtained measuring IL-6 secretion and IgM production (not shown). A 24-nt poly T (ODN IMT053) also had a considerable activity (~30% of the most active ODNs). This is consistent with the results described by Vollmer et al. (23). It is worth noting that a poly T chain possesses a motif that differs in only one base from the optimal immunostimulatory PyNTTTTGT motif here reported. Therefore, a phosphorothioate poly T chain could be considered as a phosphorothioate ODN bearing a defective PyNTTTTGT motif.

PyNTTTTGT ODNs stimulate expression of costimulatory molecules on CD19+ (B) cells

As previously shown in Fig. 1*a*, the non-CpG ODN IMT021 is able to stimulate the expression of CD86 on CD19+ human PBMC. To extend this observation to other important cell surface

markers, human PBMC were incubated with ODN IMT 504 (motif: CATTTTGT), ODN 2006 as a positive control, and ODN IMT022 as a negative control (Fig. 3). As shown, ODN IMT504 is as active as ODN 2006 for stimulation of the expression of CD40, MHC I, and MHC II on CD19+ (B) cells.

The PyNTTTTGT ODN IMT504 is quantitatively as active as the CpG ODN2006 in proliferation, IL-6 secretion, and CD40 expression assays on human PBMC

The above-presented experiments demonstrated that PyNTTTTGT ODNs have a number of immunostimulatory activities that at least qualitatively are very similar to those displayed by the human prototype CpG ODN2006. Thus, to investigate whether PyNTTTTGT

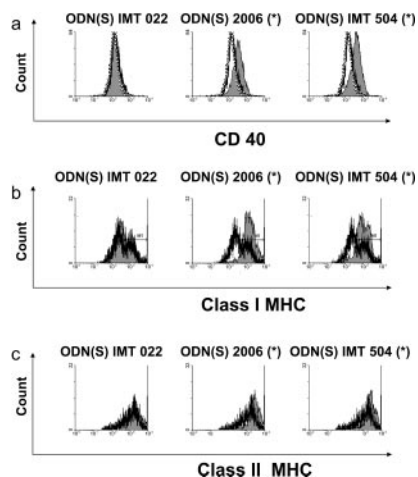


FIGURE 3. Induction of CD40, MHC I, and MHC II on CD19 cells by phosphorothioate PyNTTTTGT ODNs. Human PBMC were cultured for 24 h with the indicated PyNTTTTGT ODNs or controls (ODN 2006 and ODN IMT022) and then stained with fluorescent anti-CD19/anti-CD40 (*a*), anti-CD19/anti-MHC I (*b*), or anti-MHC II (*c*). Flow cytometric results are presented as histograms corresponding to cells in the CD19-positive gate. □, Cells cultured in the absence of ODN; ■, cells cultured in the presence of ODN. ODN (S), phosphorothioate. Each experiment was repeated four times using PBMCs from different donors. Results were similar in all cases to those presented here. Statistical significance was evaluated by Student's *t* test. *, Statistically significant differences ($p < 0.05$) compared with cells cultured in the absence of ODN.

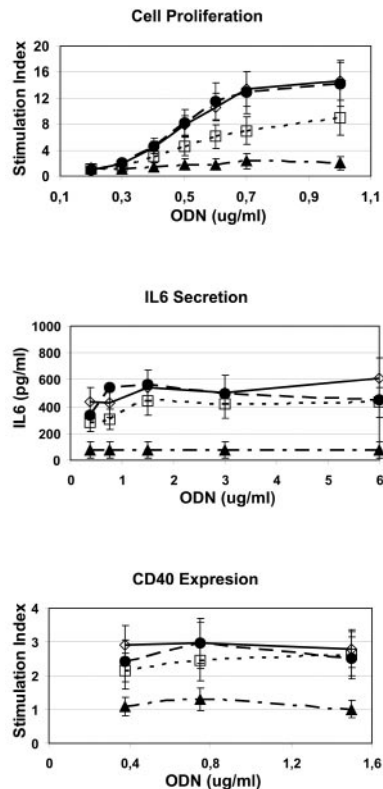


FIGURE 4. Comparison of PyNTTTTGT and CpG ODNs based on dose-response curves. Human PBMC were incubated with increasing concentrations of phosphorothioate ODNs IMT504 (\diamond), IMT021 (\square), IMT022 (\blacktriangle), and 2006 (\bullet) at the indicated concentrations. Each experiment was repeated four times using PBMCs from different donors. Curve points represent the means and SDs of these experiments.

ODNs are also quantitatively similar to human CpG ODNs we performed dose-response experiments. Fig. 4 shows the results obtained using proliferation, IL-6 secretion, and CD40 expression assays. As shown, the curves corresponding to PyNTTTTGT ODN IMT 504 are very similar to those corresponding to CpG ODN2006 in all three assays. In contrast, ODN IMT021, which has a PyNTTTTGT motif defective in one nucleotide, has less activity at the lower concentrations assayed. On the other hand, the negative control ODN IMT022 remained as such under all assayed conditions.

B cell stimulation by PyNTTTTGT ODNs is direct

To investigate whether the immunostimulatory activity of CNTTTTGT ODNs on B cells is direct, human B cells were purified. Table II and Fig. 5 show that the immunostimulatory activ-

ity on these purified cells is comparable to that observed using human PBMC. These results indicate that immunostimulation by PyNTTTTGT ODNs on human B cells is direct.

B cell stimulation by phosphodiester PyNTTTTGT ODNs

The use of a phosphorothioate backbone in immunostimulatory PyNTTTTGT ODNs is convenient because of its relative nuclease resistance. However, Table III and Fig. 6 show that the use of this backbone is not an absolute requirement, as phosphodiester PyNTTTTGT ODNs are also active. Despite this, a higher concentration should be used to obtain the same effect induced by the corresponding phosphorothioate ODNs. This has also been observed for CpG ODNs (20). On the other hand, the activities of the phosphodiester polyT ODN IMT053 and the phosphodiester ODN IMT021 are very poor. This suggests that in a phosphodiester backbone, a canonical PyNTTTTGT motif is strongly required. In contrast, in a phosphorothioate backbone, specificity seems to be relaxed.

Stimulation of plasmacytoid dendritic cells by PyNTTTTGT ODNs

Fig. 7 shows stimulation of purified plasmacytoid dendritic cells by PyNTTTTGT ODNs. As shown, ODNs (S) IMT021 and IMT504 are able to stimulate the expression of CD86, CD40, and MHC class I surface molecules on plasmacytoid dendritic cells as well as ODN 2006. None of these phosphorothioate ODNs is able to stimulate IFN- α production. However, both ODN IMT504 and ODN 2006 with a phosphodiester backbone are able to stimulate the production of this cytokine (not shown).

ODN 2006 has two independent immunostimulatory motifs

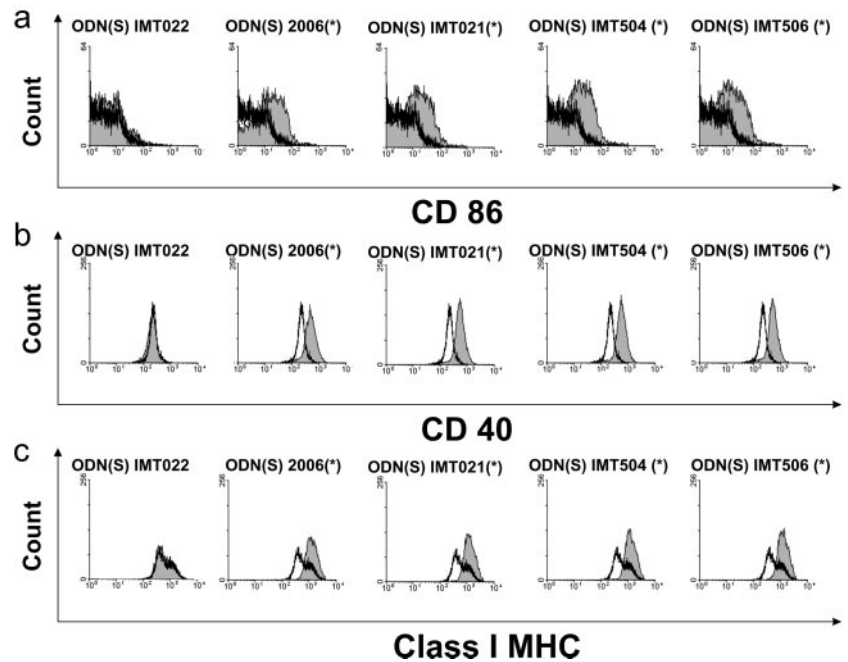
Phosphorothioate PyNTTTTGT ODNs are unable both to stimulate the expression of CD69 on CD56-positive cells (NK cells) in cytometric assays and to stimulate lytic activity in vitro assays for cytotoxicity (not shown). In contrast, ODN 2006 is positive in both kinds of assays. On the other hand, replacement of the Ts corresponding to the two PyNTTTTGT motifs present in ODN 2006 by As (ODN IMT518) does not result in the loss of B cell activation or NK cell stimulation activity (Table IV). In contrast, a similar change in ODN IMT504 results in loss of its immunostimulatory activity on B cells (see ODN IMT552 in Fig. 2). Furthermore, replacement in ODN IMT518 of the Ts present in the TCG trinucleotides by As (ODN IMT519) results in the loss of all immunostimulatory activity. In this case B cell stimulation can be restored by restitution of four of the Ts involved in the PyNTTTTGT motifs (ODN IMT517). However, this change does not result in restoration of NK stimulatory activity. On the other hand, the results obtained by replacement of the four CpG dinucleotides (one at the time) present in ODN 2006 (ODNs IMT584, IMT585, IMT586, and IMT587) by GpCs indicate that the two main TCGs

Table II. Induction of proliferation and IL-6 and IgM secretion on purified B cells by phosphorothioate PyNTTTTGT ODNs^a

ODN (S)	Sequence	Proliferation Index			IL6 Secretion (pg/ml)			IgM Secretion (ng/ml)		
		Avg.	n	SD	Avg.	n	SD	Avg.	n	SD
IMT022	TGCTGCAAAAGAGCAAAAGAGCAA	1.54	5	0.62	1019	5	665	451	5	173
IMT021	TGCTGCTTTTGTGCTTTTGTGCTT	22.16	5	5.67	9588	5	729	856	5	203
IMT504	TCATCATTTGTGTCATTTTGTGTCATT	46.45	5	7.48	11002	5	884	1216	5	238
IMT506	TCTTCTTTTGTCTTTTGTCTTTT	29.63	5	6.92	10266	5	888	1291	5	327
2006	TCGTCGTTTGTGTCGTTTGTGTCGTT	50.22	5	7.03	8300	5	907	1255	5	317
None		1.00	5		972	5	544	448	5	196

^a The averages (Avg.) shown are the mean of the results of N PBMCs from different donors. (S), phosphorothioate. The ODN concentration was 0.375 µg/ml in proliferation assays, 1.5 µg/ml in IgM assays, and 6 µg/ml in IL-6 assays.

FIGURE 5. Induction of CD86, CD40, and MHC I on purified B cells by phosphorothioate PyNTTTTGT ODNs. Human purified B cells were cultured for 24 h with the indicated phosphorothioate PyNTTTTGT ODNs or controls (ODN 2006 and ODN IMT022) and then stained with fluorescent anti-CD19/anti-CD86 (a), anti-CD19/anti-CD40 (b), or anti-CD19/anti-MHC I (c). Flow cytometric results are presented as histograms corresponding to cells in the CD19-positive gate. □, Cells cultured in the absence of ODN; ■, cells cultured in the presence of ODN. ODN (S), phosphorothioate ODN. Each experiment was repeated four times using PBMCs from different donors. Results were similar in all cases to those presented here. Statistical significance was evaluated by Student's *t* test. *, Statistically significant differences (*p* < 0.05) compared with cells cultured in the absence of ODN.



involved in NK stimulation are those located at the 5' end of the ODN.

These results confirm that two independent immunostimulatory motifs coexist in ODN 2006: one (duplicated) of the PyNTTTTGT type here described, with a capacity for direct human B cell activation, and another that involves mainly the two successive TCG trinucleotides located at the 5' end, with a capacity for both B and NK cell stimulation. Probably this last motif is the one responsible for the highly conserved (within mammals) immunostimulatory activity reported for ODN 2006 (21) (see below).

Species specificity of the PyNTTTTGT immunostimulatory motif

Table V shows the immunostimulatory activity of some PyNTTTTGT ODNs on PBMC from different mammal species. As shown, these ODNs were active in animals within the order *Primate*, while ODN 2006 was active in all of them as previously reported (21). However, we observed a small, but reproducible, immunostimulatory activity (represented by ± in Table V) on PBMC of non-primate mammals using ODN IMT504, which possesses two

CATTTTGT motifs. Furthermore, spleen cells of mice are not stimulated by PyNTTTTGT ODNs (not shown).

PyNTTTTGT ODNs in non-human primates

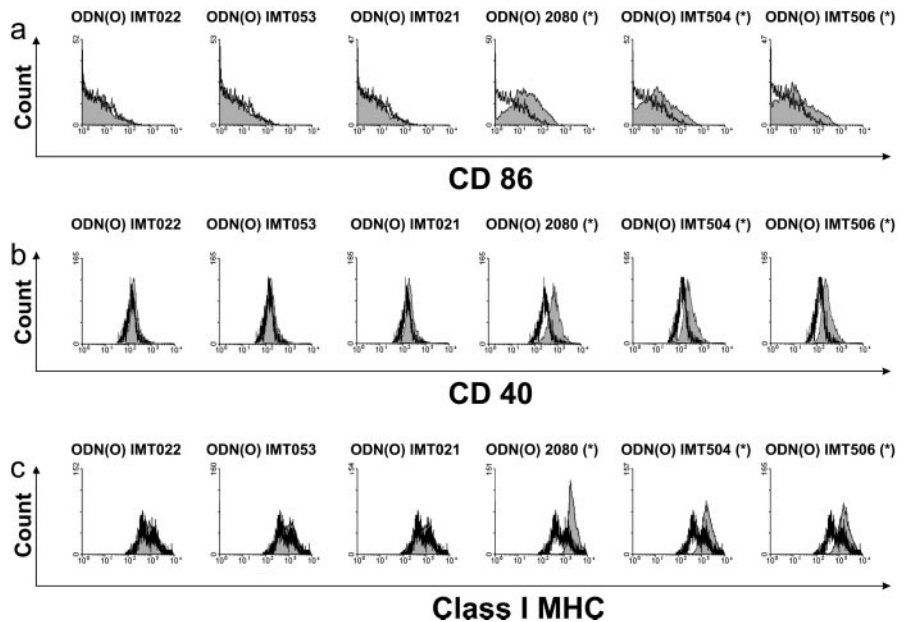
Assays performed on PBMC of two species of monkeys, *Macacca fascicularis* and *Cebus apella*, demonstrated a much more marked influence of the second position of the immunostimulatory motif than that observed using human PBMC (Fig. 8). The most active non-CpG ODN on *M. fascicularis* cells (ODN IMT504) has an A in this position, while ODN IMT 506, which has a T in the same position, shows low activity. Also, ODN IMT504 is clearly more active than ODN IMT506 on *C. apella* cells. In both cases the activity of ODN IMT021 is poor. According to these results, the most effective non-CpG immunostimulatory motif acting on B cells within the order *Primate* seems to be PyPuTTTGT, wherein Py is C or T, and Pu is A or G. However, no other immunological marker, besides immunoproliferation, was measured in monkeys, and conclusions are limited by this fact.

Table III. Induction of IL-6 secretion by phosphorothioate or phosphodiester PyNTTTTGT ODNs^a

ODN (O)	Sequence	IL-6 Secretion (pg/ml)		
		Avg.	n	SD
IMT 022 (S)	TGCTGCAAAAAGAGCAAAAAGAGCAA	38	4	17
IMT 022 (O)	TGCTGCAAAAAGAGCAAAAAGAGCAA	27	4	12
IMT 053 (S)	TTTTTTTTTTTTTTTTTTTTTTTTTTT	123	4	21
IMT 053 (O)	TTTTTTTTTTTTTTTTTTTTTTTTTTT	24	4	24
IMT 021 (S)	TGCTGCTTTTGTGCTTTTGTGCTT	182	4	51
IMT 021 (O)	TGCTGCTTTTGTGCTTTTGTGCTT	35	4	12
IMT 504 (S)	TCATCATTTTGTCAATTTGTCAAT	285	4	39
IMT 504 (O)	TCATCATTTTGTCAATTTGTCAAT	210	4	43
IMT 506 (S)	TCTTCTTTTGTCTTTTGTCTTT	228	4	70
IMT 506 (O)	TCTTCTTTTGTCTTTTGTCTTT	250	4	32
2006 (S)	TCGTCGTTTTGTCGTTTTGTCGTT	216	4	84
2006 (O)	TCGTCGTTTTGTCGTTTTGTCGTT	228	4	40
None		28	4	14

^a The averages (Avg.) shown are the mean of *n* PBMCs from different donors. (S), phosphorothioate; (O), phosphodiester. Phosphorothioate ODNs were added (6 μg/ml) at time zero of incubation. Phosphodiester ODNs were added as follows: 30 μg/ml at time zero, 30 μg/ml after 4 h, and 30 μg/ml after 16 h of incubation.

FIGURE 6. Induction of CD86, CD40, and MHC I in CD19 cells by phosphodiester PyNTTTTGT ODNs. Human PBMC were cultured for 48 h with the indicated phosphodiester PyNTTTTGT ODNs or controls (ODN 2080 (TCGTCTGTTCCCCCCCCCCCC) and ODN IMT022) and then stained with fluorescent anti-CD19/anti-CD86 (*a*), anti-CD19/anti-CD40 (*b*), or anti-CD19/anti-MHC I (*c*). Flow cytometric results are presented as histograms corresponding to cells in the CD19-positive gate. □, Cells cultured in the absence of ODN; ■, cells cultured in the presence of ODN. ODN (O), phosphodiester ODN. Each experiment was repeated four times using PBMCs from different donors. Results were similar in all cases to those presented here. Statistical significance was evaluated by Student's *t* test. *, Significant differences ($p < 0.05$) compared with cells cultured in the absence of ODN.



Discussion

It has been known for some time that the innate immune system responds to single-stranded ODNs containing specific sequences (24). The structural requirements for immunostimulation were defined as a central unmethylated CpG dinucleotide flanked by less critical base sequences (2). We now report the discovery of a potent immunostimulatory human non-CpG motif and its action on B lymphocytes and plasmacytoid dendritic cells. The general composition of the novel non-CpG motif for humans is PyNTTTTGT, wherein Py is C or T, and N is A, T, C, or G. ODNs containing this motif strongly stimulated human B cells to proliferate, secrete IL-6 and IgM, and express increased levels of the cell surface immunologically relevant molecules CD86, CD40, MHC I, and MHC II.

The activity of the most widely used PyNTTTTGT ODN during this study, i.e., IMT504, was very similar to that corresponding to the prototype human CpG ODN2006 in proliferation, IL-6 secretion, and CD40 expression assays performed at different concentrations on PBMC. This is important because these assays measure the activation of different pools of cells. For example, the B cell population that proliferates in human PBMC incubated with ODN 2006 corresponds to memory B cells (25). In contrast, in the same conditions up-regulation of costimulatory molecules seems to occur in most, if not all, CD19-positive cells (our own results). Thus, ODN504 and ODN2006 activate more than one pool of B cells in a similar (qualitative and quantitative) fashion. Assays performed on purified B lymphocytes cells indicated that the immunostimulatory activity of

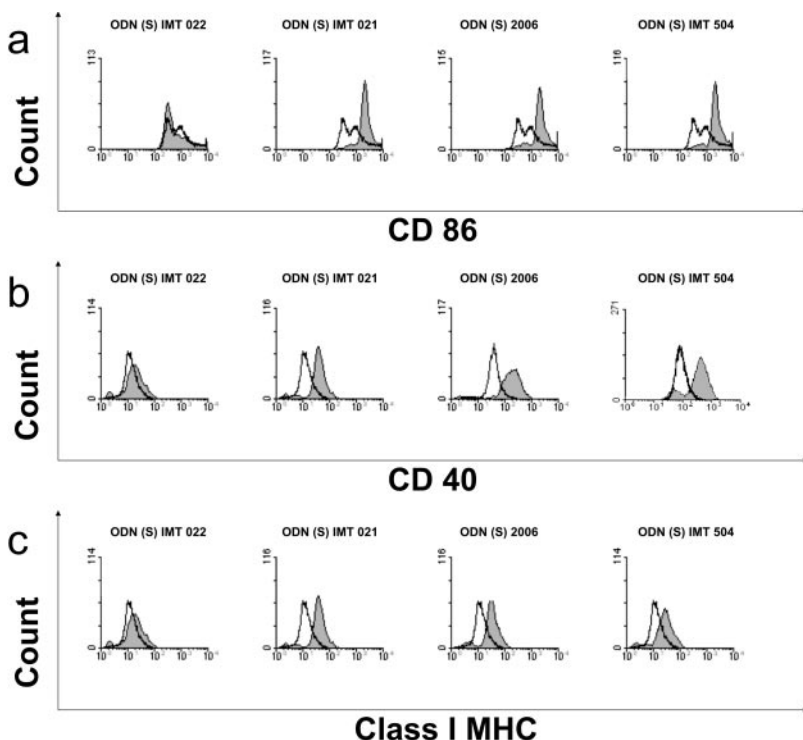


FIGURE 7. Induction of CD86, CD40, and MHC I on plasmacytoid dendritic cells by phosphorothioate PyNTTTTGT ODNs. Plasmacytoid dendritic cells were cultured for 24 h with the indicated phosphorothioate PyNTTTTGT ODNs or controls (ODN 2006 and ODN IMT022) and then stained with fluorescent anti-CD4, CD11c/anti-CD86 (*a*); anti-CD4, CD11c/anti-CD40 (*b*); or anti-CD4, CD11c/anti-MHC I (*c*). Flow cytometric results are presented as histograms corresponding to cells in the CD4-positive, CD11c-negative gate. □, Cells cultured in the absence of ODN; ■, cells cultured in the presence of ODN. ODN (S), phosphorothioate ODN. Each experiment was repeated four times using PBMCs from different donors. Results were similar in all cases to those presented here. Statistical significance was evaluated by Student's *t* test. *, Statistically significant differences ($p < 0.05$) compared with cells cultured in the absence of ODN.

Table IV. *B cell and NK stimulation by ODNs derived from the ODN(S) 2006^a*

ODN (S)	Sequence	B Cell Stimulation (proliferation index)	NK Cell Stimulation (CD69 on CD56 cells)
2006	TCGTCGTTTTGTCGTTTTGTCGTT	+	+
IMT 517	<u>ACG</u> ACGTTTTG <u>ACG</u> TTTTG <u>ACG</u> TT	+	-
IMT 518	TCGTCG <u>AAAA</u> GTCG <u>AAAA</u> AGTCGAA	+	+
IMT 519	<u>ACG</u> ACG <u>AAAA</u> GACG <u>AAAA</u> AGCGAA	-	-
IMT 587	TCGTCGTTTTGTCGTTTTGTCGTT	+	+
IMT 586	TCGTCGTTTTGTCGTTTTGTCGTT	+	±
IMT 585	TCGTCGTTTTGTCGTTTTGTCGTT	+	-
IMT 584	<u>TGCT</u> CGTTTTGTCGTTTTGTCGTT	+	-

^a Experiments were performed in four independent PBMCs. Each assay was performed in quadruplicate. Changes with respect to ODN 2006 are underlined. A proliferation index of 4 or more was considered positive for B cell stimulation. The proliferation index of negative ODNs was <2. A mean fluorescence intensity of 60% or more with respect to ODN 2006 was considered positive for NK cell stimulation. The percent mean fluorescence intensity for ODN IMT586 was ~40% with respect to ODN 2006 and was considered as ±. The mean fluorescence intensity for negative ODNs was <10% with respect to ODN 2006. Differences between positive and negative ODNs were statistically significant ($p < 0.01$) by Student's *t* test.

the PyNTTTTGT ODNs on these cells is direct. Also, PyNTTTTGT ODNs stimulated purified plasmacytoid dendritic cells, as demonstrated by expression assays of the CD86, CD40, and MHC I surface markers.

During this study, the fact that the widely used ODN 2006, a very effective immunostimulatory ODN in humans and other animals (20–21), possesses two independent immunostimulatory motifs was unmasked. One of these coincident with the general non-CpG motif PyNTTTTGT here described, and the other one is strongly dependent on two successive TCG trinucleotides present at the 5' end of the ODN. Both motifs have immunostimulatory activity on human B cells, but NK cell activation was solely dependent on the integrity of the two successive TCG trinucleotides present at the 5' end of the ODN 2006.

Some of these observations are coincident with those previously reported by Liang et al. (26). For example, these authors pointed out the importance of TCG trinucleotides to reach maximal activity of phosphorothioate ODNs on human B cells. Also, while we were writing this paper, a report by Vollmer et al. (23) was published describing CpG-independent immunostimulation by phosphorothioate poly T ODNs. However, the non-CpG ODNs described by these authors have a much lower activity than those reported here, and an efficient non-CpG motif was not defined. It is worth noting that a poly T chain possesses the sequence TTTTTTTT, which can be considered a motif with one mismatch with respect to the general formula PyNTTTTGT. On the other hand and in contrast to the absolute requirement for a phosphorothioate backbone of the non-CpG ODNs described by Vollmer et al. (23), phosphodiester PyNTTTTGT ODNs are active.

ODN 2006 has been found to be very active in several species of mammals (21). However, ODNs containing exclusively the

PyNTTTTGT immunostimulatory motif described here are mainly active within the order *Primate*. Therefore, the universal mammalian immunostimulatory activity associated to ODN 2006 seems to be related to the two successive TCG trinucleotides present at the 5' end of the ODN. This last motif is at least in part coincident with the one present in K-type CpG ODNs, also named B-type CpG ODNs (1, 27, 28), that strongly stimulate human B cells interacting with the Toll-like receptor 9 (29, 30). Differences in the spectrum of immunostimulatory activities and species specificity of PyNTTTTGT ODNs and K (or B) CpG ODNs suggest that they may act through different receptors. The presence of a small, but reproducible, immunostimulatory activity of at least one ODN bearing two CATTTTGT motifs in non-primate mammals indicates that the immunostimulatory mechanisms activated by PyTTTTGT ODNs were present before, but optimized during, the evolution of primates.

The results here presented gave rise to the following question. Is the PyNTTTTGT motif here described a pathogen-associated molecular pattern, as has been postulated for CpG motifs? In regard to this, it can be said that our efforts to find differences between microbial and human DNA related to this particular sequence were unsuccessful. Therefore, we hypothesized that the PyNTTTTGT motif may be a danger signal released by necrotic cells, as seems to be the case for other cellular components such as heat shock proteins (31).

Further research is needed to clarify the biological role of single-stranded immunostimulatory DNA. In the meantime, identification of new motifs acting on the human immune system through different cells and pathways strongly fuels the field of possible clinical applications of immunostimulatory DNA.

Table V. *Species specificity of PyNTTTTGT ODNs^a*

ODN (S)	Sequence	Species			
		Human	Monkey ^b	Swine	Sheep
2006	TCGTCGTTTTGTCGTTTTGTCGTT	+	+	+	+
IMT 504	TCATCATTGTCATTTGTCATT	+	+	±	±
IMT 505	TCCTCCTTTGTCCTTTGTCCT	+	±	-	-
IMT 506	TCTTCTTTTGTCTTTTGTCTTT	+	±	-	-

^a Experiments were performed in four PBMCs from different donors. Each assay was performed in quadruplicate. A proliferation index of 4 or more was considered positive. A proliferation index between 2 and 3 was considered ±, and an index below 2 was negative. Differences between positive and negative ODNs were statistically significant ($p < 0.01$) by Student's *t* test. Differences between ± and negative ODNs were not statistically significant.

^b *C. apella* and *M. fascicularis*.

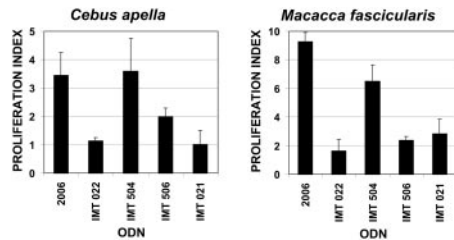


FIGURE 8. Stimulation of PBMC proliferation by PyNTTTTGT ODNs in non-human primates. *C. apella* or *M. fascicularis* PBMC were cultured for 72 h with phosphorothioate PyNTTTTGT ODNs or controls (ODN 2006 and ODN IMT022; 6 μ g/ml). Eighteen hours before cell harvest, [3 H]thymidine was added. The cultures were harvested onto glass-fiber filters, and 3 H incorporation was measured by scintillation counting. Data represent the mean and SD of four PBMCs from four different animals. Assays were performed in quadruplicate.

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