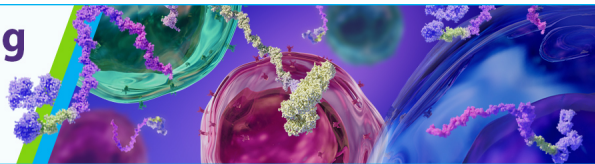


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Human Peripheral Blood Cells Differentially Recognize and Respond to Two Distinct CpG Motifs^{1,2}

Daniela Verthelyi, Ken J. Ishii, Mayda Gursel, Fumihiko Takeshita, and Dennis M. Klinman³

Oligodeoxynucleotides (ODN) that contain unmethylated CpG dinucleotides trigger a strong innate immune response in vertebrates. CpG ODN show promise as vaccine adjuvants, anti-allergens, and immunoprotective agents in animal models. Their transition to clinical use requires the identification of motifs that are optimally stimulatory in humans. Analysis of hundreds of novel ODN resulted in the identification and characterization of two structurally distinct "clusters" of immunostimulatory CpG ODN. One cluster ("D") preferentially stimulates IFN- γ production by NK cells, whereas the other ("K") stimulates cell proliferation and the production of IL-6 and IgM by monocytes and B cells. The distinct immunostimulatory properties of K and D ODN can improve the design of CpG-based products to achieve specific therapeutic goals. *The Journal of Immunology*, 2001, 166: 2372–2377.

The mammalian immune system responds to bacterial infection by mounting a rapid inflammatory response that limits the early spread of the microorganism while facilitating the emergence of pathogen-specific immunity (1). Although immune recognition of proteins and lipids derived from bacteria is well documented (1), the immune system's ability to recognize and respond to "CpG motifs" present in bacterial DNA was discovered only recently (2–5). In mice, bacterial DNA and synthetic oligodeoxynucleotides (ODN)⁴ expressing CpG motifs stimulate the production of polyreactive Ig, cytokines, and chemokines (3, 4, 6). Multiple cell types are activated, including lymphocytes, NK cells, and professional APCs (4, 7–11). Animal studies indicate that CpG ODN may be useful as vaccine adjuvants, anti-allergens, and immunoprotective agents (12–14). In mice, optimally stimulatory ODN contain an unmethylated CpG dinucleotide flanked by two 5' purines (Pu) and two 3' pyrimidines (Py) (2, 4). However, immune recognition of CpG motifs varies between species, which is consistent with evolutionary divergence in CpG recognition (15). Indeed, human PBMC respond poorly to ODN that are optimally active in mice (16).

To identify motifs that activate human cells, several hundred novel ODN were synthesized and studied. The results demonstrate that human PBMC recognize and respond to two structurally distinct clusters of CpG motifs. One cluster preferentially induced cell proliferation, IgM production by B cells, and IL-6 secretion by monocytes/dendritic cells, whereas the other stimulated IFN- γ release by NK cells. By selectively employing these two types of ODN, the immune system can be manipulated to support specific therapeutic ends.

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² The assertions herein are private ones of the authors and are not to be construed as official or as reflecting the views of the Food and Drug Administration.

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⁴ Abbreviations used in this paper: ODN, oligodeoxynucleotides; Pu, purine; Py, pyrimidine; ELISPOT, enzyme-linked immunospot.

Materials and Methods

Cells

Normal PBMC were obtained from the National Institutes of Health Department of Transfusion Medicine (Bethesda, MD). The human myeloma cell line RPMI 8226 (CCL-155; American Type Culture Collection, Manassas, VA) and the NK-92 human NK cell line (a kind gift of Dr. J. Ortaldo, National Cancer Institute, Frederick, MD) were grown in RPMI 1640 supplemented with 10% FCS, penicillin/streptomycin, L-glutamine, HEPES, sodium pyruvate, and 2-ME in a 5% CO₂ in-air incubator. Medium for NK-92 cells was supplemented with IL-2 (200 IU/ml; R&D Systems, Minneapolis, MN) and IL-15 (15 ng/ml; Endogen, Boston, MA).

Oligodeoxynucleotides

ODN were synthesized at the Center for Biologics Evaluation and Research core facility. All had <0.1 endotoxin U/ml endotoxin at ODN concentrations of 1 mg/ml.

Antibodies

Abs against human IFN- γ (Endogen), IL-6 (R&D Systems), and IgM (Serotec, Oxford, U.K.) were used for ELISA and enzyme-linked immunospot (ELISPOT) assays. FITC- and/or CyChrome-labeled Abs against human CD3, CD4, CD14, CD11c, CD16, CD56, CD83, HLA-DR, IL-6, and IFN- γ were obtained from BD Pharmingen (San Diego, CA) or BD Biosciences (San Jose, CA) and used as recommended by the manufacturer. Neutralizing Abs to IL-12 were obtained from R&D Systems, and Abs to IL-18 were kindly provided by Dr. Howard Young (National Cancer Institute).

Mononuclear cell preparation

Mononuclear cells were separated by density gradient centrifugation over Ficoll-Hypaque as described (17). Cells were washed three times and cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FCS for 72 h at 5×10^5 cells/well in the presence of 1–3 μ M ODN.

ELISA and ELISPOT assays

Ninety-six-well microtiter plates (Millipore, Bedford, MA) were coated with anti-cytokine Ab or anti-IgM and blocked with PBS-5% BSA (17, 18). Cytokines and Ig in culture supernatants or secreted by individual cells were detected colorimetrically using biotin-labeled Abs followed by phosphatase-conjugated avidin and then phosphatase-specific colorimetric substrate. Standard curves were generated to quantitate ELISA results using known amounts of recombinant cytokine or purified IgM. The detection limit of the assays was: 6 pg/ml for IFN- γ , 20 pg/ml for IL-6, and 10 ng/ml for IgM. Stimulation index was calculated by the formula: (value for stimulated cells – background)/(value for unstimulated cells – background). In cases where cytokine/Ig production was below assay sensitivity, the lower limit of detection was used to calculate the stimulation indices. All assays were performed in triplicate.

Proliferation assays

A total of 10^5 PBMC/well were incubated with $3 \mu\text{M}$ of ODN for 68 h, pulsed with $1 \mu\text{Ci}$ of [^3H]thymidine, and then harvested 4 h later. The proliferation index represents the fold difference between stimulated and unstimulated cells. All assays were performed in triplicate.

Intracellular cytokine staining and flow cytometry

PBMC were cultured for 8 h (K type) or 24 h (D type) with $3 \mu\text{M}$ of various ODN. Brefeldin A ($20 \mu\text{g/ml}$) was added to the cultures after 2 or 12 h, respectively. Cells were harvested with warm PBS-0.02% EDTA and washed. PBMC (1×10^6 /sample) were fixed and permeabilized using the Fix & Perm cell permeabilization kit (Caltag, Burlingame, CA) as recommended by the manufacturer. Cells were then stained with PE-conjugated anti-IL-6 or anti-IFN- γ plus specified FITC- or CyChrome-conjugated Abs against cell surface markers for 30 min in darkness. After labeling, the cells were washed twice, and 40,000 events per sample were analyzed by FAC-Scan flow cytometry (BD Biosciences). CellQuest software (BD Biosciences) was used for data analysis.

Statistical analysis

Statistically significant differences were determined using a two-tail nonparametric Mann-Whitney U test and nonparametric ANOVA.

Results

Response of human PBMC to CpG ODN

Novel ODN were studied for their ability to stimulate human PBMC to proliferate and/or secrete Ig or cytokines. As seen in Fig. 1, two structurally distinct ODN classes were identified that stimulated PBMC from >95% of the donors. Those of the K type stimulated significantly greater cell proliferation ($p < 0.0001$) and induced higher levels of IL-6 (240 vs 85 pg/ml ; $p < 0.01$) and IgM (695 vs 20 ng/ml ; $p < 0.0001$) than D ODN. In contrast, D ODN were stronger inducers of IFN- γ (70 vs 13 pg/ml ; $p < 0.05$).

Type D ODN

Modifications were introduced in various regions of D ODN to identify the critical sequences and structures that account for the

ability of these ODN to induce IFN- γ . To standardize results from the large number of subjects and experiments included in the analysis, the magnitude of each response is presented as fold increase over cells from the same subject incubated in medium alone. The general magnitude of these responses was comparable to that shown in Fig. 1. D-type ODNs contain an unmethylated CpG dinucleotide (Fig. 2). Inversion, replacement, or methylation of the CpG reduces or abrogates reactivity (Fig. 2A, *line 1* vs *lines 2–6*, and *line 7* vs *line 8*; $p < 0.0001$). D ODN are stimulatory only if the CpG dinucleotide and its immediate flanking regions are composed of phosphodiester (shown in gray) rather than phosphorothioate nucleotides (Fig. 2B, *line 1* vs *line 2*; $p < 0.001$). Because phosphorothioate-modified nucleotides confer resistance to exonuclease digestion, they are incorporated at the ends of the ODN to improve activity (Fig. 2B, *lines 1* and *5* vs *lines 3* and *4*; $p = 0.07$). Unless otherwise stated, all D ODN studied are phosphorothioate/phosphodiester chimeras.

The level of immune stimulation induced by D ODN is influenced by the bases flanking the CpG dinucleotide. Self-complementary hexamers consisting of a PuPyC $\underline{\text{G}}$ PuPy are the most active, as exemplified by ATCGAT and ACCGGT (Fig. 2C, *lines 1* and *2*). Substituting a Pu for a Py, or vice versa, significantly reduces or eliminates ODN activity (circled nucleotides in Fig. 2C, *lines 1* and *2* vs *lines 6–13*; $p < 0.0001$). By comparison, hexamers that maintained the PuPyC $\underline{\text{G}}$ PuPy sequence but are nonself-complementary induce lower levels of IFN- γ production (Fig. 2C, *lines 1* and *2* vs *lines 3–5*; $p < 0.001$).

Sequential deletion experiments show that the minimum length of an active D ODN is ~ 18 bp (Fig. 2D; $p < 0.01$). This finding suggests that sequences outside the central hexamer might influence D ODN activity. Indeed, stimulation is maximal when the three bases on each side of the CpG-containing hexamer form a self-complementary sequence (Fig. 2E, *lines 1* and *2* vs *lines 3* and *4*; $p < 0.0001$). Computer modeling of D ODN suggests that these

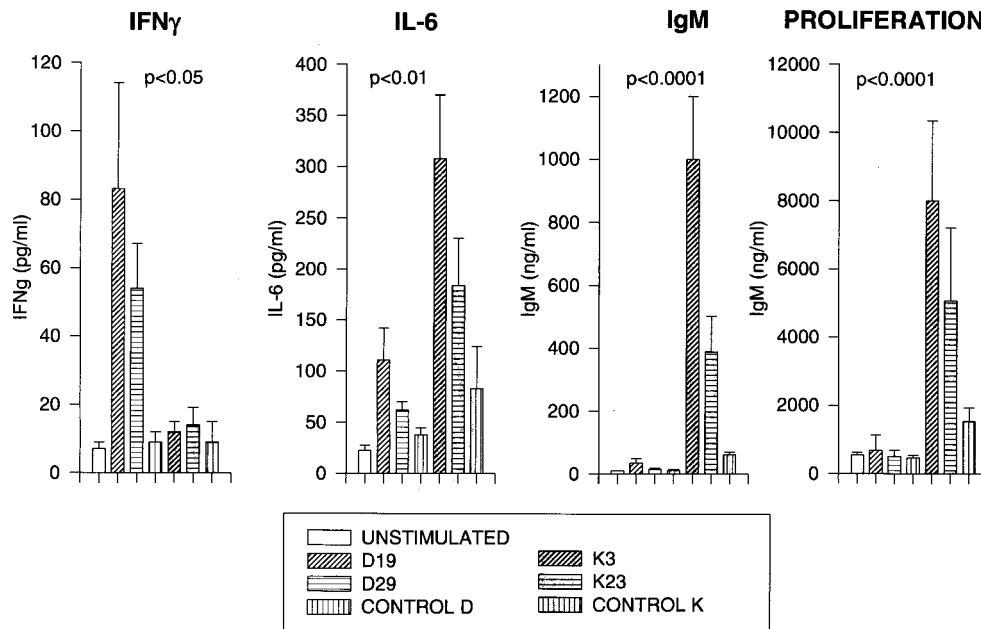
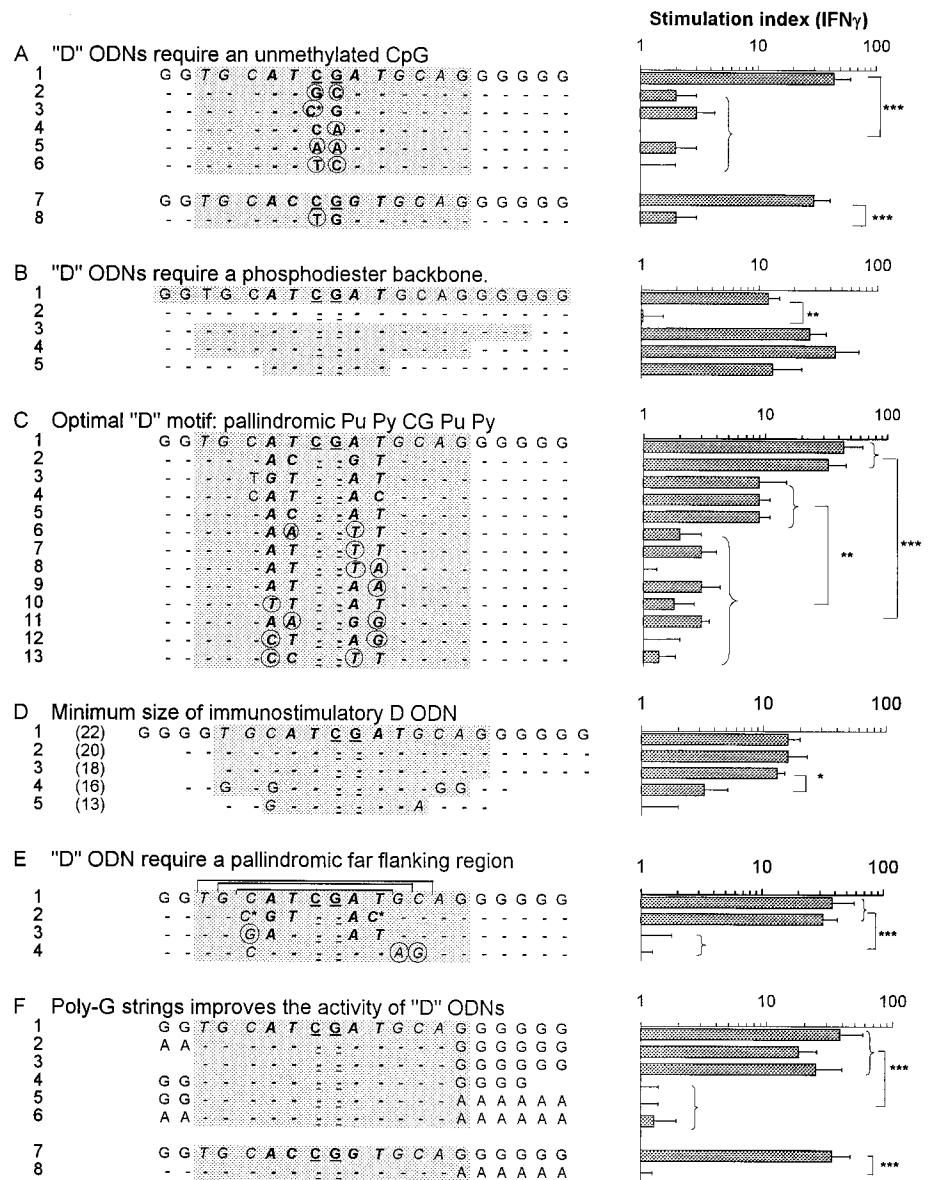


FIGURE 1. Response of PBMC to K and D ODN. PBMC from 35 donors were stimulated for 72 h with K or D ODN ($3 \mu\text{M}$). D19 (GGTGCATC-GATGCAGGGGG) and D29 (GGTGCACCGGTGCAGGGGG) exemplify the response of PBMC to ODN that selectively induce IFN- γ , whereas K3 (ATCGACTCTCGAGCGTTCTC) and K23 (TCGAGCGTTCT) exemplify ODN that induce IgM and IL-6 secretion and cell proliferation but little IFN- γ . Control ODN have the CG dimer reversed; therefore, control D (GGTGCACGCGTGCAGGGGG) and control K (TGCAGGCTTCTC). Bases in bold-face type are phosphodiester, and those in normal type are phosphorothioate. Cytokine and Ig concentrations in supernatants were determined by ELISA, and cell proliferation was assessed by [^3H]thymidine uptake. All assays were done in triplicate. Statistical significance was determined by the nonparametric Mann-Whitney U test.

FIGURE 2. Rules governing D ODN induced immune activation. The ODN shown here are representative of 120 ODN used to characterize the structural requirements of D ODN. CGs are underlined; immunostimulatory motifs are in bold; extended motifs are in italics; methylated bases have an asterisk; dots indicate identity; and shaded backgrounds identify phosphodiester-linked bases. Important base changes in the sequence are circled. Data is expressed as stimulation indices, representing the fold increase in cytokine secretion relative to unstimulated cells from the same donor. Bars represent the mean and SE of 20 different experiments. The ODN shown do not induce significant levels of IgM or proliferation. Statistical significance was determined by the non-parametric Mann-Whitney *U* or non-parametric ANOVA: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.



self-complementary base sequences help form a stem-loop structure with the CpG dinucleotide at the apex at 37°C (not shown). The ends of the ODN also contribute to its activity, with the inclusion of more than four Gs at the 3' end significantly improving function (Fig. 2F, lines 1–3 vs lines 4 and 5; $p < 0.001$). Thus, changes in any of the three areas (the central hexamer, the region flanking the hexamer, or the poly G tail) influence ODN activity.

Type K ODN

K ODN trigger cell proliferation and the secretion of IgM and IL-6, but little IFN- γ (Fig. 1). These ODN have a phosphorothioate backbone and at least one unmethylated CpG dinucleotide (Table IA). As with D ODN, eliminating the CpG dinucleotide significantly reduces immune activation (Table IA, line 3 vs line 4; $p < 0.02$). Incorporating multiple CpGs in a single ODN increases immune stimulation (Table IA, lines 1–3). To determine the minimum length of a stimulatory K ODN, nucleotides were sequentially deleted from each end. ODN at least 12 bases long consistently induce strong immune cell activation, whereas shorter ODN are relatively less active (Table IB, lines 1–5 vs lines 6–10).

CpG motifs at the 5' end were the most stimulatory (Table IC, line 2 vs line 3 and line 4 vs line 5), although at least one base upstream of the CpG was required (Table IC, line 1 vs line 6). Indeed, a thymidine in the immediate 5' position (Table ID, lines 1 and 2 vs lines 3–5 and line 6 vs line 7) and a 3' TpT or a TpA (Table IE, lines 1 and 2 vs lines 3 and 4 and lines 5 and 6 vs line 7) yielded the most active K ODN. Modifications >2 bp from the CpG dinucleotide had relatively less effect on ODN activity (data not shown).

Cellular targets of K and D ODN

The phenotype of the cells stimulated to produce cytokine was determined by combined cell surface and intracytoplasmic staining. As seen in Table IIA, D ODN selectively stimulated CD3⁻CD16⁺CD56⁺CD14⁻ cells to produce IFN- γ , consistent with the direct activation of NK cells. The effect appears to be direct because D ODN do not induce a significant increase in IL-12 secretion (data not shown). Moreover, studies using neutralizing anti-IL-12, which reduce the production of IFN- γ by PBMCs stimulated with PHA (44% $p < 0.05$) or with bacillus Calmette-Guérin

Table I. Rules governing K ODN-induced immune activation^a

Assay Subject No.		Stimulation Index					
		IL-6		Proliferation		IgM	
		1	2	1	2	1	2
A. Multiple CpGs induce more stimulation							
1	ATCGACTCTCGAGCGTTCTC	50	57	13	30	138	71
2	TCGAGCGTTCTC	35	40	15	37	19	79
3	TCGAGGCTTCTC	28	12		25	8	22
4	TGCAGGCTTCTC	1	0	5	7	5	4
B. Minimum size of stimulatory K ODN							
1	TCGACTCTCGAGCGTTCTC (20)	20	23	37	72	>100	100
2	ACTCTCGAGCGTTCTC (16)	20	18	30	46	>100	100
3	TCTCGAGCGTTCTC (14)	16	40	28	41	>100	100
4	TCGAGCGTTCTC (12)	23	15	13	25	>100	80
5	CTCGAGCGTTCT (12)	21	14	16	21	95	92
6	TCGAGCGTTCT (11)	10	4	15	35	45	78
7	TCGAGCGTTC (10)	6	5	17	40	35	82
8	TCGAGCGTT (9)	1	7	5	7	32	25
9	TCGAGCGT (8)	1	5	1	13	25	12
10	TCGAGCG (7)	1	1	1	5	9	4
C. CpG motifs located at the 5' end of the ODN are most stimulatory							
1	TCGAGCGTTCTC	12	40	52	59	80	>100
2	TCGAGGCTTCTC	6	12	51	61	>100	>100
3	TGCTTCGAGCTC	4	3	12	16	20	60
4	GCGAGGCTTCTC	5	12	18	14	>100	>100
5	TGCAGCGAGCTC	5	2	4	4	1	16
6	CGAGCGTTCTC	<1	<1	1	1	<1	2
D. Optimization of the 5' CpG flanking region							
1	TCGATGCTTCTC	5	12	60	67	100	9
2	TCGAGGCTTCTC	6	12	51	61	160	5
3	ACGAGGCTTCTC	3	3	18	23	110	11
4	GCGAGGCTTCTC	3	1	18	14	72	6
5	CCGAGGCTTCTC	4	1	15	25	25	7
6	TGCTTCGAGCTC	3	1	12	16	60	40
7	TGCAGCGAGCTC	2	1	4	4	16	8
E. Optimization of the 3' CpG flanking region							
1	TCGTTTGTCTC	8	8	28	31	>100	>100
2	TCGTATGTACTC	8	8	26	32	2	33
3	TCGGATGAGCTC	6	8	9	20	28	41
4	TCGAATGCTCTC	3	5	14	22	6	14
5	TTGTTTCGTTCTC	3	4	15	14	19	26
6	TTGTTTCGTACTC	2	4	14	13	15	72
7	TTGTTTCGAGCTC	2	4	6	4	6	16
8	TTGTTTCGAACTC	2	2	11	5	1	1

^a Over 200 novel ODN were synthesized, and their ability to activate PBMC from multiple donors was examined. PBMC were stimulated for 72 h in the presence of ODN (1 μ M added at time 0). Cytokine and Ab secretion in the supernatants were assessed by ELISA, and proliferation was determined by [³H] uptake. Examples of general findings are presented in this table. Results are expressed as fold increase over unstimulated cells.

(77%; $p < 0.05$), did not decrease the IFN- γ production induced by CpG-ODN (data not shown).

By comparison, K ODN stimulated CD14⁺, CD11c⁺, and CD83⁺ cells to produce IL-6, indicating that they were of monocyte/dendritic cell lineage (Table IIB). K ODN also stimulated a fraction of CD19⁺ B cells to release IL-6.

To confirm these findings, human NK, T, and B cell lines were tested for their responsiveness to K and D ODN. The NK-92 cell line responded exclusively to D ODN by secreting IFN- γ , whereas the human RPMI 8226 B cell line was stimulated by K ODN to release IL-6 (Fig. 3). Non-CpG ODN did not stimulate either cell line.

Discussion

This report is the first to demonstrate that two structurally distinct types of CpG ODN stimulate different cellular elements of the human immune system to mount divergent responses. K-type ODN induce monocytes/dendritic cells to produce the proinflammatory cytokine IL-6 and B cells to proliferate and secrete IgM, whereas D-type ODN support NK cell production of IFN- γ .

Interest in the immunostimulatory properties of CpG ODN has grown rapidly since their ability to stimulate murine cells to secrete Ig and cytokine was first reported (2–5). Research in mice established that CpG ODN can function as vaccine adjuvants, as anti-allergens, and as immunoprotective agents (12–14). Several clinical trials are under way to examine their safety and efficacy for these applications (19).

Although earlier works examined the response of human cells to CpG ODN (7, 16, 20–23), the current report is the first to establish that different elements of the human immune system respond to two structurally distinct CpG motifs and to establish the “rules” governing recognition of these motifs. Previous studies generally examined the response of a small number of donors to a few DNA sequences that frequently contained multiple CpG motifs (7, 16, 20–22, 24). This limited their ability to identify the structural basis underlying PBMC stimulation (20, 22). The use of costimulatory factors (such as IL-2) to augment ODN-mediated responses, or cationic lipids to bypass normal cellular uptake, also obscured the unique effects of ODN (20, 25). However, recent work by Hartmann et al. (16, 24) identified multiple TCG repeats as contributing to the stimulation of B and NK cells.

Table II. Phenotype of PBMC stimulated by CpG ODN to secrete cytokines^a

Cell Surface Marker	% Positive Cells
A. Phenotype of PBMC activated by D ODN to produce IFN- γ	
CD16	91
CD56	99
CD3	5
CD14	<1
B. Phenotype of PBMC activated by K ODN to produce IL-6	
CD83	93
CD14	70
CD11c	69
CD19	13
CD16	<1

^a Freshly isolated PBMC were stimulated with K (8 h) or D (24 h) ODN. Cells were fixed, permeabilized, and stained with PE-anti-IL-6 or anti-IFN- γ . Results are representative of 4–10 experiments.

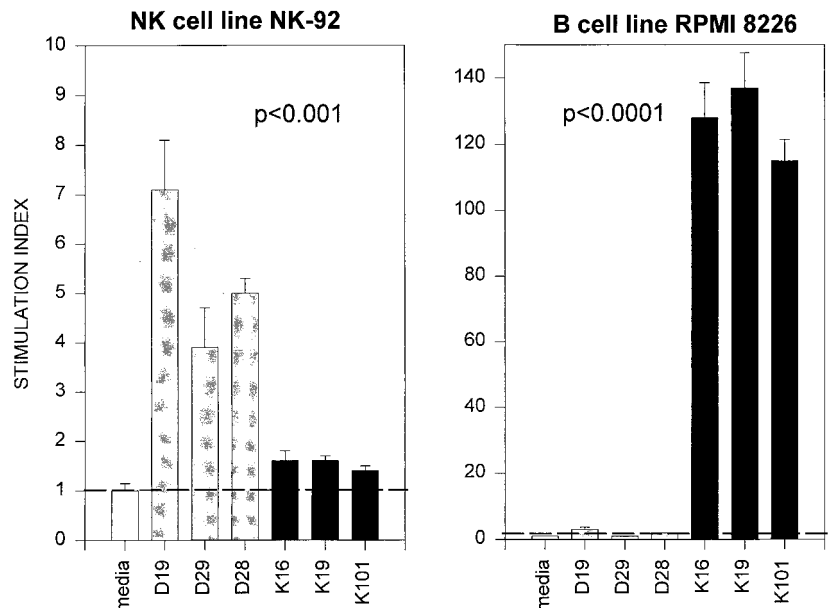
Our work significantly extends those findings by establishing that human PBMC respond to two structurally distinct types of ODN and by describing the key structural elements of each. These results were derived from the study of a large number of normal donors and were confirmed by analysis of monoclonal cell lines. Unmethylated CpG dinucleotides were critical components of both types of ODN because methylation, inversion, or substitution of this dinucleotide reduced or eliminated activity. The use of degradation-resistant phosphorothioate nucleotides improved ODN function, although D motifs were most effective when the CpG and flanking regions were composed of phosphodiester nucleotides. This confirms the findings of Ballas et al. (7) that pure phosphorothioate ODN stimulate human NK cells poorly. Consistent with previous reports, pure phosphorothioate ODN lacking CpG motifs were modestly stimulatory on B cells from some subjects (26).

The CpG motifs at the heart of K and D ODN differ. The optimal K motif contains a thymidine immediately 5' and a TpT or TpA 3'. These findings corroborate and extend the observation by Hartmann et al. (24) and Yamamoto et al. (27) that ODN encoding a 5' TCGTCGTT octamer stimulate the expression of CD86 on B cells. By comparison, optimally active D ODN contain Pu-Py dimers on each side of the CpG. These structural differences may underlie the functional differences between D vs K ODN.

D ODN also differed from K ODN by being longer and requiring that the CpG-flanking regions be self-complementary. Two-dimensional computer modeling suggests that the self-complementary sequence facilitates the formation of a hairpin loop that exposes the CpG at the apex. It is likely that this stem-loop structure contributes to the recognition of D ODN because IFN- γ production declines when the length or binding strength of the palindrome is reduced (Fig. 2E, and data not shown). The increased stimulation of NK cells by ODN-containing palindromes is reminiscent of early findings by Yamamoto et al. (27) involving stimulatory structures in bacterial DNA. The inclusion of poly Gs at the 3' end of D ODN may also confer a structural benefit or may simply improve the efficiency of cellular uptake (28).

ELISA analysis shows that significant amounts of cytokine are secreted into the culture supernatant of ODN-activated cells. K and D ODN activate distinct cell types. Flow cytometric analysis showed that K ODN activate monocytes and B cells to secrete IL-6, whereas D ODN stimulate NK cells to secrete IFN- γ . Studies currently under way suggest that the differential stimulation is not due to differential uptake of the ODN, because monocytes and NK cells take up both types of ODN (M. Gursel, K. Ishii, D. Verthelyi, and D. Klinman, manuscript in preparation). Furthermore, it appears that the ODN activate their target cells directly because 1) CpG ODN can stimulate cloned cell lines to secrete cytokines; 2) cytokine mRNA appears within minutes of ODN stimulation (4); and 3) ELISPOT studies show that the CpG ODN induced rapid increases (2- to 5-fold) in IL-6- and IFN- γ -secreting PBMC (5 and 18 h after stimulation, respectively (data not shown)). Moreover, flow cytometric analysis of cells stimulated to secrete IFN- γ by CpG ODN were CD3⁻ even after 72 h of stimulation, indicating that the increased IFN- γ in supernatants is not the result of a secondary activation of T cells. Although it is possible that the induction of IFN- γ secretion by NK cells is mediated by increased IL-12 or IL-18, our studies using neutralizing anti-IL-12 or anti-IL-18 confirmed the observations reported by Iho et al. (25) that induction of IFN- γ by NK cells is not mediated by these cytokines. It should be noted that culture conditions can influence the relative magnitude of the cytokine response induced by K or D. Thus, care must be taken to eliminate lots of FCS that contain factors that synergize with ODN activity.

FIGURE 3. Cell type-dependent response to CpG ODN. Fold increase in IFN- γ production by NK-92 cells and IL-6-secreting cell number by RPMI 8226 cells in response to ODN (3 μ M for NK cells and 1 μ M for B cells). The number of cells secreting IL-6 was determined by ELISPOT, and the secretion of IFN- γ was determined at 72 h in culture supernatants by ELISA. Sequences: D19, D29 (see Fig. 1), D28 (GGTGGTCGATGCAGGGGG), K16 (TCGACTCTCGAGCGTTCTC), K19 (ACTCTCGAGCGTTCTC), K101 (CTCGAGCGTTCT). Statistical significance determined by nonparametric Mann-Whitney *U* and nonparametric ANOVA tests.



Although the immunostimulatory properties of CpG DNA were only recently described, clinical trials exploring their utility as vaccine adjuvants and anti-allergens have been initiated (19). The distinct immunostimulatory properties of K and D CpG ODN should allow for the design of DNA-based products that support specific therapeutic goals. For example, agents designed to treat allergy or control infection by Th1-sensitive pathogens might benefit from the inclusion of D ODN, whereas vaccines dependent on strong humoral immune responses might benefit from the use of K ODN as adjuvants.

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