Identification of a novel CpG DNA class and motif that optimally stimulate B cell and plasmacytoid dendritic cell functions

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Abstract: Recent reports have identified two major classes of CpG motif-containing oligodeoxynucleotide immunostimulatory sequences (ISS): uniformly modified phosphorothioate (PS) oligodeoxyribonucleotides (ODNs), which initiate B cell functions but poorly activate dendritic cells (DCs) to make interferon (IFN)-α, and chimeric PS/phosphodiester (PO) ODNs containing runs of six contiguous guanosines, which induce very high levels of plasmacytoid DC (PDC)-derived IFN-α but poorly stimulate B cells. We have generated the first reported ISS, C274, which exhibits very potent effects on all human immune cells known to recognize ISS. C274 is a potent inducer of IFN-γ/IFN-α from peripheral blood mononuclear cells and exhibits accelerated kinetics of activity compared with standard ISS. This ODN also effectively stimulates B cells to proliferate, secrete cytokines, and express costimulatory antigens. In addition, C274 specifically activates PDCs to undergo maturation and secrete cytokines, including very high levels of IFN-α. Sequence variation studies based on C274 were used to identify the general motif requirements for this novel and distinct class of ISS. In contrast, chimeric PO/PS CpG-containing ODNs with polyguanosine sequences exert a differential pattern of ISS activity compared with C274, perhaps in part as a result of their greatly different structural nature. This pattern is composed of high IFN-α/IFN-γ induction and low DC maturation in the absence of B cell stimulation. In conclusion, we have generated a novel class of ISS that transcends the limitations ascribed to classes described previously in that it provides excellent stimulation of B cells and simultaneously activates PDCs to differentiate and secrete large amounts of type I IFN. *J. Leukoc. Biol.* 73: 781–792; 2003.

Key Words: *human · cytokines*

INTRODUCTION

The mammalian immune system has evolved to recognize conserved, repeating microbial motifs known as pathogen-associated molecular patterns through various classes of surface-expressed and soluble pattern recognition receptors (PRRs). One family of PRRs that has recently received focused interest is the group of recognition molecules known as Toll-like receptors (TLRs), expressed primarily by antigen-presenting cells such as macrophages, dendritic cells (DCs), neutrophils, and B cells. TLRs have been demonstrated to be expressed as monomers, homodimers, or heterodimers [1]. Known ligands for TLRs include such bacterial products as Gram-positive peptidoglycans, lipopeptides, double-stranded RNA, and Gram-negative lipopolysaccharides and lipoteichoic acids, flagellin, and unmethylated CpG DNA [2–4]. Activation of TLRs by these bacterial products results in the initiation of an innate-immune response that serves as the first line of host defense. This response limits infection after microbial exposure through immediate lytic mechanisms and initiates and directs the development of adaptive immunity by controlling communication with B and T cells [5].

Much attention has focused recently on the immunobiology of immunostimulatory sequences (ISS), which largely bear CpG-containing motifs and are ligands for TLR9. Murine ISS-responsive cells include monocytes, B cells, and a subset of DCs ($CD11c^+$ $Gr\text{-}1^+$ $B220^+$) known for releasing high levels of interferon (IFN)-α when stimulated by virus [6, 7]. Experiments performed on human cells show a more limited pattern of TLR9 expression, as monocytes express very low levels, and only plasmacytoid DCs (PDCs) and B cells are substantial TLR9 expressers [8–10]. In vivo administration of ISS-based formulations to mice has been shown to result in elevated levels of total and antigen-specific immunoglobulin G (IgG)2a and decreased levels of IgG1 and IgE [11–13]; enhanced secretion of interleukin (IL)-6, IL-12, and IFN-γ by monocytes, DCs, and natural killer (NK) cells [14–16] with corresponding decreases in T helper cell type 2 immune responses [15, 17, 18]; and augmented NK cell and cytolytic T lymphocyte lytic activity [13, 14, 19]. Similarly in the human system, ISS induces IFN-γ production from NK cells [20] and IFN-α from PDCs [21], as well as stimulating B cell functions [22], NK

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lytic activity [23], DC maturation [24], monocyte activation [25], and IgE inhibition [26].

Several investigators have grouped immunostimulatory CpG motif-containing oligodeoxyribonucleotides (ODNs) into two major classes [27]. CpG-A ODNs (also known as "type D"; ref. [28]) are very potent inducers of IFN-α and IFN-γ from PDCs and NK cells, respectively [29–31]. They have chimeric DNA backbones, in that the central palindromic core, which contains at least one CpG, is phosphodiester (PO), and this region is flanked on one or both sides by phosphorothioate (PS) polyguanosine sequences. These polyguanosine sequences are required for CpG-A ODN activity, possibly as a result of their ability to form quadruplex structures, which have been reported to increase cellular uptake via receptor-mediated endocytosis [32, 33]. CpG-A ODNs demonstrate a poorer ability to induce B cell functions or to differentiate PDCs [21, 34]. Conversely, CpG-B ODNs (also known as "type K"; ref. [28]) are generally ascribed the various B cell functions (proliferation, IL-6, and IgM secretion, activation marker up-regulation) and the induction of DC maturation with little or no effect on levels of IFN-γ and IFN-α [29, 30, 35]. Studies of CpG-B versus CpG-A ODNs have thus far found them to exhibit activities that are largely mutually exclusive: B cell functions versus PDC-derived IFN-α secretion, respectively. Herein, we describe a novel ODN, C274, which allows the ISS to retain all known properties of CpG-B and CpG-A types and identify the motif requirements for a third distinct class of ISS, which we term CpG-C ODNs.

MATERIALS AND METHODS

ODN synthesis

ODNs were synthesized on an Expedite 8909 DNA synthesizer (Applied Biosystems, Foster City, CA) using phosphoramidite chemistry and the manufacturer's protocols. A 0.02 M solution of 3-amino-1,2,4-dithiazole-5-thione in 9:1 acetonitrile:pyridine was used for preparation of the PS linkages [36]. The ODNs were purified by reversed-phase high-pressure liquid chromatography (HPLC) and were isolated as the sodium salt, as described previously [37]. All ODNs had <5 endotoxin units/mg ODN, determined by *Limulus* amebocyte lysate assay (BioWhittaker, Walkersville, MD). Uppercase letters in the following sequences represent PS linkages, and lowercase letters represent PO linkages: 1018: 5′-TGACTGTGAACGTTCGAGATGA; 1040: 5′-TGACTGTGAACCTTAGAGATGA; C274: 5′-TCGTCGAACGTTCGAGATGAT; D19: 5′-GGtgcatcgatgcagGGGGG; C405: 5′-GGtgcatcgatgcagAAAGG. Additional sequences are shown (see Fig. 8).

Peripheral blood mononuclear cell (PBMC) preparation

Peripheral blood was collected from healthy volunteers by venipuncture using heparinized syringes. Buffy coats were obtained from the American Red Cross (Washington, DC). PBMCs were isolated by centrifugation through a Ficoll (Pharmacia, Uppsala, Sweden) density gradient and cultured in RPMI 1640 (BioWhitaker) supplemented with 10% heat-inactivated human AB serum (Gemini, Woodland, CA) plus 50 U/ml penicillin, 50 μg/ml streptomycin, 300 μg/ml glutamine, 1 mM sodium pyruvate (BioWhitaker), and 1× nonessential amino acids (BioWhitaker). For cytokine secretion, PBMCs were cultured at 0.5×10^6/well (2×10^6/ml) in 96-well flat-bottomed plates in duplicate with ISS ODNs at a concentration range of 0.1–20 μg/ml for 24 h, determined by previous studies to be the optimal time point for ISS-induced cytokine secretion. Cell-free supernatants (SNs) were harvested, and cytokine content was assayed by enzyme-linked immunosorbent assay (ELISA). Culture conditions (including cell concentration, ODN concentration, and period of culture) to induce optimal activity in each ISS functional assay were predetermined.

ELISA

Cell-free medium was collected from each well and assayed for cytokine content via commercial kits. IFN-γ, IL-6, and tumor necrosis factor α (TNF-α) were assayed with CytoSet antibody pairs (BioSource, Worcester, MA). Limits of maximal/minimal detection were 4000/2 pg/ml for all three assays. IFN-α was assayed with an ELISA kit (PBL Biomedical Laboratories, New Brunswick, NJ), and the limit of maximal/minimal detection was 4000/32 pg/ml. All kits and antibody pairs were used according to manufacturers' instructions. Statistical significance was calculated using one-way ANOVA, and parametric values and the Tukey multiple-comparison post-test was used to compute *P* values (GraphPad InStat, GraphPad, San Diego, CA).

B cell purification and functional assays

Human PBMCs were incubated with CD19 magnetic cell sorter (MACS) beads (Miltenyi Biotec, Auburn, CA) and were passed through a magnet, separating the $CD19^+$ B cells through positive selection [>98% $CD19^+$ as determined by fluorescein-activated cell sorter (FACS)]. For the proliferation assay, B cells were cultured at 1×10^5/well (5×10^5/ml) in 96-well round-bottomed plates. Cells were incubated in triplicate with 2 μg/ml ODN for 72 h. At the end of the culture period, the plates were pulsed with ^{3}H-thymidine (1 μCi/well; Amersham, Little Chalfont, UK) and were incubated for an additional 8 h. Then, the plates were harvested, radioactive incorporation was determined using standard liquid scintillation techniques, and the data were collected in counts per minute (cpm). For IL-6 secretion, B cells were cultured at $0.5–1 \times 10^6$/well in 48-well plates with 5 μg/ml ISS for 48 h, and then SNs were harvested and assayed for IL-6 via ELISA. For FACS analysis, B cells were cultured at $0.5–1 \times 10^6$/well in 48-well plates with 5 μg/ml ISS for 48 h, and then cells were washed and stained with anti-CD80, -CD86, -CD40, -CD54, and -human leukocyte antigen (HLA)-DP, -DQ, and -DR (PharMingen, San Diego, CA) in staining buffer [phosphate-buffered saline (PBS)+3% bovine serum albumin+0.01% NaN_3] + an equal volume of blocking buffer (10% human AB serum in PBS) for 20 min at 4°C. Cells were then washed with FACS wash buffer (isotone+0.01% NaN_3) 2× and resuspended in 100 μl propidium iodide (PI; 2 μg/ml; Sigma Chemical Co., St. Louis, MO). Samples were read on a FACScan (Becton Dickinson, Mountain View, CA), gated to exclude dead cells. Data are reported as % positive B cells or mean fluoresence intensity (MFI).

PDC purification and functional assays

PBMCs were isolated from buffy coats by Ficoll centrifugation. NK cells, T cells, and monocytic cells were depleted from PBMCs by incubating with magnetically labeled anti-CD16, anti-CD3, and anti-CD11b (Miltenyi Biotec), followed by passage over a magnetic depletion column (LD). Cells that did not bind to the column were incubated with magnetically labeled anti-BDCA-4 antibodies and then positively selected on a magnetic column (LS; >95% BDCA-2^+, CDw123^+). For cytokine secretion, PDCs were cultured at $0.5–1 \times 10^5$/well ($2–4\times10^5$/ml) in 96-well round-bottomed plates with 5 μg/ml ISS for 24 h, and then SNs were harvested and assayed for cytokines via ELISA. For FACS analysis, PDCs were cultured similarly, and then cells were washed and stained with anti-CD80 and -CD86 (PharMingen) as described above. Samples were read on a FACScan (Becton Dickinson), gated to exclude dead cells. Data are reported as % positive PDCs. The numbers of living PDCs in each culture were also enumerated via FACS analysis of PI staining.

Gene expression assay and analysis

Human PBMCs or PDCs were stimulated with ISS and cultured 4–24 h. Total RNA was extracted via the Qiagen RNeasy mini protocol (Qiagen, Valencia, CA) and was converted to cDNA using oligo-dT (Promega, Madison, WI), random hexamers (Promega), and SuperScript RT II (Invitrogen, Carlsbad, CA). cDNA was diluted 1:10, and polymerase chain reaction (PCR) was conducted using QuantiTect SYBR green PCR master mix (Qiagen) and naked primers (synthesized by Operon) or QuantiTect probe PCR master mix (Qiagen) and predeveloped Taqman assay reagents (PDAR) primers with labeled probe (Applied BioSystems). Reactions were conducted using the GeneAmp 5700 sequence detector (Perkin Elmer BioSystems, Foster City, CA). The sequences

for synthesized primers are as follows (listed 5′–3′): ubiquitin (F: CACTTGGTCCTGCGCTTGA, R: CAATTGGGAATGCAACAACTTTAT); 2,5-oligoadenylate synthetase (OAS; F: AGGGAGCATGAAAACACATTTCA, R: TTGCTGGTAGTTTATGACTAATTCCAAG); guanylate-binding protein-1 (GBP-1; F: TGGAACGTGTGAAAGCTGAGTCT, R: CATCTGCTCATTCTTTCTTTGCA); IFN-α (F: CCCAGGAGGAGTTTGGCAA, R: TGCTGGATCATCTCATGGAGG); IFN-stimulating gene (ISG)-54K (F: CTGGACTGGCAATAGCAAGCT, R: AGAGGGTCAATGGCGTTCTG); monocyte chemoattractant protein (MCP)-2 (F: CTGCTCATGGCAGCCACTTT, R: AGCAGGTGATTGGAATGGAAA); monokine induced by IFN-γ (MIG; F: CATCTTGCTGGTTCTGATTGGA, R: TGGTGCTGATGCAGGAACAG); TNF-α (F: CTTCTGCCTGCTGCACTTTG, R: CTGGGCCAGAGGGCTGAT). IFN-γ, IL-1α, IL-6, IFN-inducible protein 10 (IP-10), MCP-3, and macrophage-inflammatory protein (MIP)-3β were measured using PDARs supplied by Perkin Elmer Bio-Systems. Threshold cycle (C_T) values for each gene was normalized to ubiquitin using the Eq. $1.8^{(UBQ-GENE)}$ (100,000), where UBQ is the mean C_T of triplicate ubiquitin runs, GENE is the mean C_T of duplicate runs of the gene of interest, and 100,000 is arbitrarily chosen as a factor to bring all values above 0. The negative control for each experiment, stimulation with medium alone, is assigned a value of 1, and all data are expressed as fold-induction over the negative control.

Gel permeation chromatography (GPC)

GPC was performed on a Waters Delta 600 HPLC system using a Superdex 200 HR 10/30 column (Amersham Pharmacia). Stock solutions of the ODNs were prepared at 1.0 mg/ml in Dulbecco's PBS (DPBS; lacking calcium or magnesium) or water and were stored at 4°C for a minimum of 48 h before analysis to allow time for potential higher-order structures to form. Sample (10 μg) was injected onto the column and eluted isocratically at 0.75 ml/min using 10 mM sodium phosphate/150 mM sodium chloride/pH 7.2 buffer for 45 min. Detection was at 260 nm. A calibration curve was generated using a variety of different molecular weight single-stranded and double-stranded ODNs to approximate the molecular weight of the ODN samples and the exclusion molecular weight (ca. 200,000 Da) for the column [38].

RESULTS

C274 induces high levels of IFN-γ and IFN-α from human PBMCs and accelerates their expression compared with 1018

We have previously shown that the 22-base CpG-B ISS 1018 exhibits marked immunomodulatory activity in mice [18, 39] and have observed that it can also stimulate human PBMC secretion of IFN-γ and IFN-α in an ISS-specific manner after 24 h of culture. In contrast, other cytokines such as TNF-α and IL-6 are secreted at constitutively high levels in PBMC cultures as a result of nonspecific monocyte activation and are therefore not informative markers for ISS activity in the PBMC system (data not shown). Through sequence variation studies, we generated a novel ISS, C274, where the 5′ bases, which flank the octamer motif in 1018 (AACGTTCG), have been substituted with TCGTCG, resulting in the motif 5′-TCGTCGAACGTTCG-3′. Notably, the addition of this motif to the 1018 sequence also generated a 12-base, self-complementary region within C274 (TCGAACGTTCGA). C274 was compared with CpG-B 1018, the negative control 1040, and the CpG-A D19 for the induction of IFN-γ/IFN-α from human PBMCs derived from a large panel of healthy volunteers. As expected, 1040 induced virtually no IFN-γ or IFN-α, and 1018 induced slightly higher levels of each (**Fig. 1**). In contrast, C274 exhibited very potent activity, enhancing IFN-γ over twofold higher than 1018 and elevating IFN-α levels 12-fold higher. The CpG-A D19 also demonstrated strong enhancement of IFN-γ and IFN-α. **Figure 2** shows the results from a dose titration of ODNs from 0.1 to 20 μg/ml used to stimulate the secretion of IFN-γ and IFN-α. Although the activity of 1018 titrated away as the dose decreased, the IFN-α-inducing capability of C274 actually increased as dose decreased and became optimal at approximately 2 μg/ml (where it was 20- to 30-fold more active than 1018), after which activity decreased with dose. The bell-shaped nature of the C274-induced IFN-α response suggests negative regulatory feedback signals at high concentrations of C274. D19 did not display this negative feedback loop, which may indicate differential signaling for IFN-α secretion between C274 and D19. C274 also showed equivalent IFN-α-inducing activity to D19 when lower concentrations (~2 μg/ml) were compared. IFN-γ production decreased steadily as ISS concentration of all ODNs was lowered. Thus, the optimal concentrations of C274 required to induce IFN-γ and IFN-α appear to be distinct. As cytokine secretion by PBMCs at early time points is minimal, we also examined the induction of IFN-α and IFN-γ RNA expression by C274 (**Table 1**). It is interesting that the appearance of detectable IFN-γ and IFN-α message levels occurred much more rapidly after C274 stimulation (4 h) as compared with 1018 (10 h). Optimal expression of ISS-induced IFN-α and IFN-γ was approximately 10 h, at which time C274 induced >100-fold higher levels of IFN-α mRNA and >fourfold higher levels of IFN-γ mRNA compared with 1018. Expression of both cytokines was markedly decreased by 24 h. The optimal time point

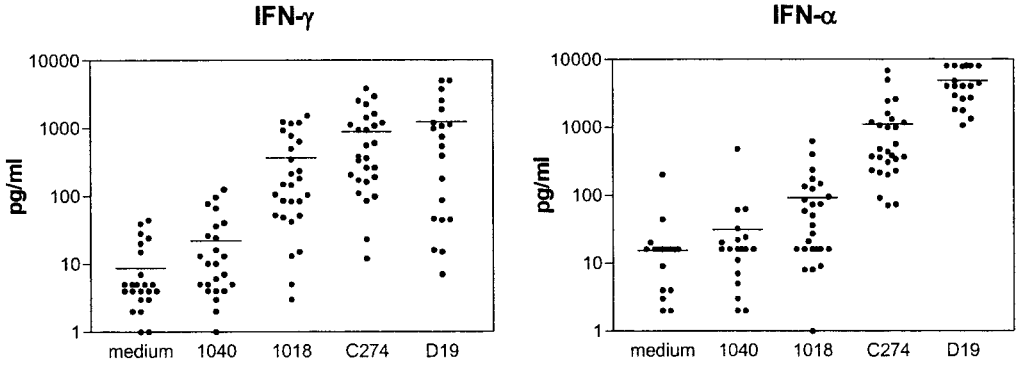

Fig. 1. C274 is a potent inducer of IFN-γ and IFN-α from PBMCs, which were isolated from 27 donors (20 for D19) and stimulated with 20 μg/ml ODN for 24 h. Cell-free SNs were assayed for IFN-γ and IFN-α content by ELISA. Data are shown as individual points for each donor, and the horizontal bars represent the mean. Statistical relevance: IFN-γ: 1040 versus 1018, $P > 0.05$; 1040 versus C274, $P < 0.01$; 1040 versus D19, $P < 0.001$; 1018 versus C274, $P > 0.05$; 1018 versus D19, $P < 0.01$; C274 versus D19, $P > 0.05$. IFN-α: 1040 versus 1018, $P > 0.05$; 1040 versus C274, $P < 0.05$; 1040 versus D19, $P < 0.001$; 1018 versus C274, $P < 0.05$; 1018 versus D19, $P < 0.001$; C274 versus D19, $P < 0.001$.

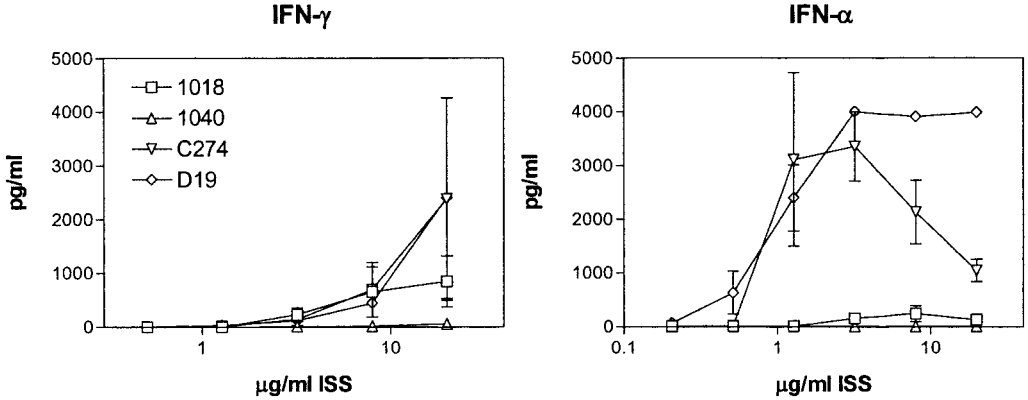

Fig. 2. Optimal concentrations of C274 to induce IFN-γ and IFN-α are different. PBMCs were isolated from four donors and stimulated with 0.2–20 μg/ml ODN for 24 h. Cell-free SNs were assayed for IFN-γ and IFN-α content by ELISA. Data are shown as means ± SEM.

and magnitude of IFN-γ/IFN-α RNA expression induced by D19 closely paralleled that of C274 (data not shown).

C274 potently enhances the expression of several chemokines and other genes

After establishing the potency of C274 in regard to IFN-γ and IFN-α induction from PBMCs, we conducted a screen for mRNA expression of numerous cytokines, chemokines, and other genes that might be indicative of the effects of C274 using the TaqMan quantitative PCR technique. We observed that C274 had no significant effect on the expressed mRNA levels of the cytokines granuloctye-colony stimulating factor (G-CSF), IL-1β, IL-6, IL-12 p40, IL-23, and TNF-α (data not shown), although IL-1α expression was decreased in the presence of C274 (**Table 2**). Furthermore, ISS stimulation had no effect on message levels of the chemokines bicinchoninic acid-1, IL-8, lymphotactin, MCP-1, macrophage-derived chemokine (MDC), MIP-1a, MIP-1b, MIP-3a, regulated on activation, normal T expressed and secreted, and thymus and activation-regulated chemokine (data not shown). However, C274 strongly up-regulated the expression of the chemokines IP-10, MCP-2, MCP-3, MIG, and MIP-3β and was considerably more potent than 1018 in this respect. As we had established C274 as a potent inducer of IFN-α expression (Table 1), we also used PCR to screen for the expression of other genes known to be enhanced by IFN-α, which in turn might be enhanced by ISS ODNs. We discovered that ISS did indeed markedly elevate message levels of the IFN-α-inducible genes 2,5-OAS, ISG-54K, and GBP-1 (Table 2). C274 also showed superiority to 1018 in the induction of these genes, which therefore indicates that 2,5-OAS, GBP-1, and ISG-54K expression can be used as qualitative indicators of ISS stimulation. The CpG-A D19 demonstrated very comparable activity with C274 in the modulation of all RNA species examined, indicating that the two ODNs may activate similar chemical messages specifically for induction of gene expression.

TABLE 1. C274 Displays Accelerated Kinetics and Higher Magnitude of IFN-α/IFN-γ mRNA Expression Than 1018[a]

		IFN-α		IFN-γ	
Stimulus	Time	Mean	SEM	Mean	SEM
Medium	2 h	1.0	0.0	1.0	0.0
1018	2 h	2.1	1.2	1.1	0.2
C274	2 h	1.2	0.2	0.8	0.2
Medium	4 h	1.0	0.0	1.0	0.0
1018	4 h	2.2	1.0	1.3	0.4
C274	4 h	55.2	21.6	8.4	4.2
Medium	10 h	1.0	0.0	1.0	0.0
1018	10 h	5.1	3.5	8.4	4.6
C274	10 h	606.5	159.7	41.1	27.8
Medium	24 h	1.0	0.0	1.0	0.0
1018	24 h	0.8	0.1	2.2	0.4
C274	24 h	0.9	0.1	4.8	1.6

[a] PBMCs from four donors were cultured for 2–24 h with 5 μg/ml ODNs, and RNA was extracted and analyzed via TaqMan reverse transcriptase (RT) PCR. Gene expression was analyzed and normalized to ubiquitin expression. Data are presented as the mean of fold-induction over medium control (given the value of 1.0) with SEM.

C274 exerts potent ISS activity on B cells

The two cell types that have been identified in human blood as expressing TLR9 and thus being directly responsive to ISS are B cells and PDCs [30]. We purified these two populations from peripheral blood to determine what activities they would exhibit in response to C274. Highly purified peripheral blood B cells were stimulated with various ISS ODNs and analyzed for effects on proliferation, cytokine secretion, gene expression, and activation status as determined by FACS analysis. **Figure 3** demonstrates that C274 has robust B cell-stimulating properties. B cell proliferation was induced by C274 at levels comparable with those of 1018. We have determined that the primary cytokine released by human B cells in response to ISS is IL-6. Also shown in Figure 3, C274 induced IL-6 at levels similar to those induced by 1018. Furthermore, we examined the RNA expression level of a wide panel of cytokines and chemokines in B cells via TaqMan PCR analysis. We found no ISS effect on the expression of G-CSF, IFN-α, IFN-γ, IL-1α, IL-1β, IL-8, IL-10, IL-12 p40, IP-10, MCP-2, MCP-3, MDC, and MIG in B cells. However, 1018 and C274 markedly enhanced RNA levels of IL-6 and TNF-α (data not shown). Finally, we measured the state of activation of the B cell population after 48 h of ISS stimulation by analyzing surface expression of CD80, CD86, CD40, CD54, and major histocompatibility complex class II. **Table 3** shows that C274 robustly enhanced the percentage of positive B cells or the MFI/B cell of all five markers in a manner comparable with 1018. In all of the various B cell-activity assays (proliferation, cytokine secretion, gene expression, activation marker expression), D19

TABLE 2. Profile of Gene Expression Modulated by C274[a]

	IL-1α		IP-10		MCP-2		MCP-3		MIG	
Stimulus	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Medium	1.0	0.0	1.0	0.0	1.0	0.0	1.0	0.0	1.0	0.0
1040	2.0	0.7	0.6	0.3	0.2	0.1	0.9	0.1	0.6	0.1
1018	1.7	0.4	2.7	0.6	28.3	21.2	3.0	1.0	3.0	0.9
C274	0.4	0.2	94.0	27.5	198.8	59.6	8.0	2.2	8.8	2.0
D19	0.2	0.1	145.4	65.1	284.8	108.7	8.5	1.4	14.5	7.0

	MIP-3β		2,5-OAS		GBP-1		ISG-54K	
Stimulus	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Medium	1.0	0.0	1.0	0.0	1.0	0.0	1.0	0.0
1040	1.2	0.3	0.7	0.2	1.0	0.1	0.7	0.1
1018	2.9	0.9	7.6	3.3	2.5	0.6	4.9	2.1
C274	6.9	1.8	16.5	2.3	5.9	0.4	27.1	2.6
D19	10.5	2.1	15.7	1.3	5.7	1.1	31.9	2.1

[a] PBMCs from four donors were cultured for 24 h with 5 μg/ml ODNs, and RNA was extracted and analyzed via TaqMan RT-PCR. Gene expression was analyzed and normalized to ubiquitin expression. Data are presented as the mean of fold-induction over medium control (given the value of 1.0) with SEM.

was completely inactive and often did not even raise activity to the minimal non-CpG-specific PS-induced activity observed with 1040 (Fig. 3 and Table 3).

C274 activates PDCs to secrete cytokines and express costimulatory molecules

We confirmed that PDCs were the only IFN-α producers in human PBMCs that respond to ISS stimulation by showing that PBMCs depleted of PDCs through blood dendritic cell antigen-4-positive selection were unable to mount an IFN-α response to any ISS, including C274 and D19 (data not shown). Accordingly, highly purified PDCs were exposed to ISS ODNs, and several different ISS activities were recorded. Measurement of secreted cytokines via ELISA (**Fig. 4**) and of cytokine RNA expression via PCR (data not shown) revealed that C274 and D19 induced extraordinarily high levels of IFN-α from PDCs, and 1018 was poor in this respect. However, 1018 was able to exert cytokine-modulating activity on PDCs, as it induced IL-6 and TNF-α to be expressed, although not to levels as high as those induced by C274 (Fig. 4). A comparison of C274 and D19 shows that C274 is superior for TNF-α and IL-6 induction from PDCs. ISS-stimulated PDCs were also analyzed via FACS for modulation of the activation markers CD80 and CD86. C274 and 1018 enhanced the expression of both markers in a comparable manner (**Fig. 5**). The non-CpG ODN, 1040, also showed some potency for activating PDCs, presumably attributable to its PS backbone. Conversely, the CpG-A ODN D19 demonstrated considerably less potency in this assay and did not even induce activation marker expression equivalent to that induced by the 1040-negative control ODN. Furthermore, we found that 1018, C274, and D19 all provided a signal that retards PDC apoptosis over a 48-h period of culture, and the non-CpG 1040 ODN failed to keep the PDC survival rate any higher than that of media alone (data not shown).

C274 and CpG-A are structurally distinct

ODNs containing at least four contiguous guanosines are known to self-assemble into quadruplex structures via guanine-guanine Hoogsteen base-pairing [32, 33], a configuration known to bind to receptors, such as the Scavenger Receptor-A, which recognizes polyanionic ligands [40]. The structures and stabilities of quadruplex ODNs have been shown to be dependent on sequence as well as on the nature and concentrations of monovalent cations such as sodium and/or potassium in the ODN diluent [41, 42]. The D19 sequence contains two distinct regions that may affect its structure: a run of six guanosines at

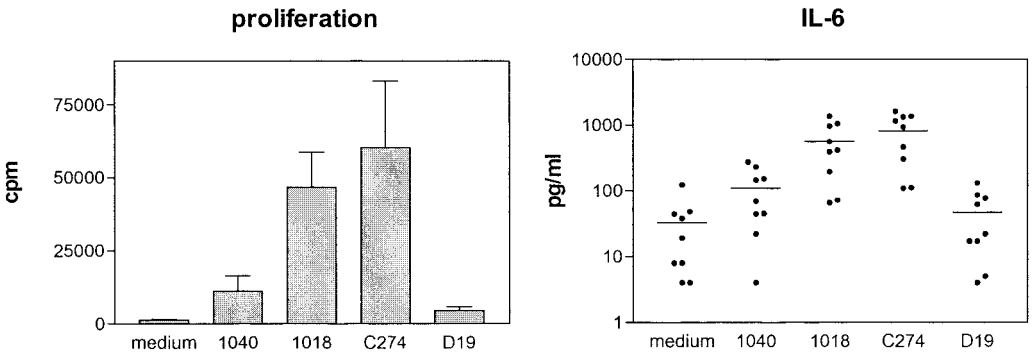

Fig. 3. C274 demonstrates robust B cell stimulatory properties. MACS-purified B cells were cultured with 5 μg/ml ODNs for 72 h (proliferation) or 48 h (IL-6). Proliferation was assessed by ^{3}H-thymidine incorporation and IL-6 by ELISA. Proliferation data are expressed as the means of four donors + SEM. IL-6 data are shown as individual points for each donor (n=7), and the horizontal bars represent the mean. Statistical relevance: IL-6: 1040 versus 1018, $P < 0.05$; 1040 versus C274, $P < 0.001$; 1040 versus D19, $P > 0.05$; 1018 versus C274, $P > 0.05$; 1018 versus D19, $P < 0.01$; C274 versus D19, $P < 0.001$.

TABLE 3. Marked B Cell Activation by C274[a]

	CD80 % Positive		CD86 % Positive		CD40 MFI		CD54 MFI		Class II MFI	
Stimulus	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Medium	4.1	2.1	22.4	7.0	36	4	340	129	39	3
1040	15.2	4.5	44.7	7.2	68	5	1238	184	87	14
1018	32.5	8.2	71.9	7.7	90	27	1607	654	108	28
C274	37.9	6.4	75.0	6.9	119	32	1782	579	144	23
D19	3.5	1.4	26.9	7.2	37	4	331	100	44	3

[a] MACS-purified B cells from four donors were cultured with ISS for 48 h and then analyzed for expression of CD80, CD86, CD40, CD54, and HLA-DR, -DP, and -DQ via FACS. Dead cells were gated out through PI staining. Data are expressed as % positive B cells for CD80 and CD86. As % positive data for CD40, CD54, and class II were very high (85–99%), the MFI data from those samples are reported.

the 3′ end, which potentially can form a quadruplex structure, and a self-complimentary 12-base central core, which may hybridize to form a double-stranded structure with Watson-Crick base-pairing. Alternatively, it has been proposed from modeling studies that D19 may exist in a stable hairpin-loop structure with the CpG exposed at the apex of the loop [43].

GPC was used to assess the secondary structure of C274 and D19. In addition, we examined C405, a sequence containing the same self-complimentary 12-base PO core as D19 but without the polyguanosine motif, which demonstrated no detectable ISS activity (data not shown), indicating the requirement of the polyguanosine motif for the activity of CpG-A. GPC separates molecules based on mass and molecular shape with large molecules eluted first and smaller molecules eluted at later retention times. The ODNs were dissolved in DPBS, which contains sodium and potassium salts at physiologically relevant concentrations, or deionized water, which significantly reduces the formation of secondary structures. The GPC profile for the PS 21-mer, C274, dissolved in DPBS or water showed a single broad peak eluting at 22.0 min (**Fig. 6, A** and **B**). This unresolved, broad peak suggests that C274 exists as a mixture of single- and double-stranded forms as a result of the 12-base palindromic PS region. The elution profiles of C405 and D19 in water showed that these ODNs also exist as mixtures of single- and double-stranded forms, eluting at 22.6 min and 21.5 min, respectively (Fig. 6, C and E). However, when C405 was dissolved in DPBS, only the double-stranded form was observed (21.4 min, Fig. 6D), and D19 in DPBS was an extremely heterogeneous mixture of secondary structures (Fig. 6F). A portion of the D19 molecules existed as very high-order aggregates, eluting as a group at the void volume of the column (11.4 min), suggesting these aggregates contain more than 30 D19 ODN strands. Other peaks observed in the GPC profile may represent quadruplex, duplex, hairpin-loop, or other structures of D19.

To better understand the role of aggregation or self-folding on the activity observed for D19, the two preparations of D19 were compared for IFN-γ/IFN-α induction from PBMCs. The water-diluted D19 exhibited significant IFN-inducing activity only at the highest concentration (20 μg/ml) and was 135-fold less active in the induction of IFN-α at 4 μg/ml and eightfold less active in the induction of IFN-γ at 20 μg/ml than D19 dissolved in DPBS buffer (**Fig. 7**). It is possible that the partial activity of D19 in water stems from some limited association that occurs within the buffered culture medium itself. Thus, the GPC analysis indicates that the most immunostimulatory preparation of D19 (in buffer) is also structurally very heterogeneous, and optimal activity is dependent on the presence of D19 aggregates. The complex aggregation state of D19 will make the characterization of D19 for preclinical and clinical development challenging. Conversely, C274 exists in a more easily characterizable, nonaggregated formation.

Identification of the motif requirements for CpG-C

The discovery that C274 had distinct biological properties from those of previously identified ISS led us to perform further sequence variation studies to define the motif requirements for this new class of ISS, which we have named CpG-C, following the current CpG-A and CpG-B nomenclature. We evaluated the role of several key features of the motif in C274 on the ODN's activity: the number and location of TCG trinucleotides at or near the 5′ end, the presence of a CpG-containing palindrome, and the number and distance between the CG dinucleotides within the palindrome. A series of uniformly modified PS ODNs were designed to test each parameter of the motif and were synthesized and assayed for their ability to induce IFN-α in human PBMC and proliferation of human B cells (**Table 4** and **Fig. 8**). For the IFN-α assay, each CpG-C ODN was titrated on human PBMCs (20, 4, and 0.8 μg/ml) to determine if the titration curve was similar to that of C274 (Fig. 2). The optimal dose for IFN-α induction by CpG-C proved to be 4 μg/ml (data not shown), and induction of IFN-α, measured as a percentage of the level induced by C274, is shown in Figure 8. The comparable activity of C583 and C582 with that of C274 demonstrated that only one TCG trinucleotide was necessary, as long as it was located at or near the 5′ end of the ODN in a sequence that also contained a CpG-containing palindrome. The necessity of the 5′-TCG was shown by the significantly lower IFN-α levels induced by C637, which lacked a 5′-TCG, compared with the almost identical sequence, C583, which contained a 5′-TCG. Additionally, a 20-base palindromic sequence with the CG dinucleotides inverted to GC, C661, was completely inactive in the IFN-α assay, showing the specificity for CpG motifs. The lack of significant IFN-α induction by 2006 showed that the presence of the 5′-TCGTCG sequence was not sufficient in the absence of a palindrome for strong IFN-α induction. In fact, we have

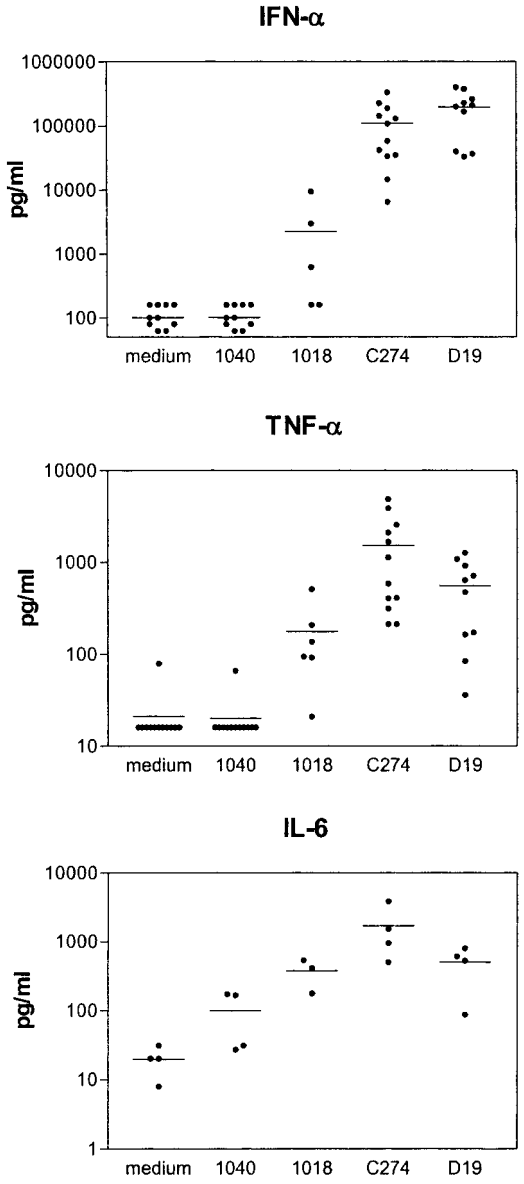

Fig. 4. C274 strongly enhances cytokine secretion, especially IFN-α, by PDCs. MACS-purified PDCs were cultured with 5 μg/ml ODNs for 24 h, and then SNs were harvested and assayed for cytokine content via ELISA. Data are shown as individual points for each donor (n=14 for medium, 1040, C274; n=8 for 1018; n=12 for D19), and horizontal bars represent the mean. Statistical relevance: IFN-α: 1040 versus 1018, $P > 0.05$; 1040 versus C274, $P < 0.01$; 1040 versus D19, $P < 0.001$; 1018 versus C274, $P < 0.05$; 1018 versus D19, $P < 0.001$; C274 versus D19, $P < 0.05$. TNF-α: 1040 versus 1018, $P > 0.05$; 1040 versus C274, $P < 0.001$; 1040 versus D19, $P > 0.05$; 1018 versus C274, $P < 0.001$; 1018 versus D19, $P > 0.05$; C274 versus D19, $P < 0.05$. IL-6: 1040 versus 1018, $P > 0.05$; 1040 versus C274, $P < 0.05$; 1040 versus D19, $P > 0.05$; 1018 versus C274, $P > 0.05$; 1018 versus D19, $P > 0.05$; C274 versus D19, $P > 0.05$.

found that although the minimum palindrome length for increased IFN-α-inducing activity is eight bases, sequences with palindromes of 12 bases or longer are optimal (data not shown). Comparison of C630, C274, C631, and C633 showed that the CG dinucleotides in the palindrome were preferably spaced 1, 2, or 3 nucleotides apart, although sequences with four nucleotide spacings retained low levels of IFN-α-inducing activity. To determine if there were sequence limitations within the palindrome, a series of C274 analogs containing all possible CpG-containing palindromic hexamers within the 12-base palindrome were prepared and tested for IFN-α-inducing ability (C593, C594, C595, C646, C640, C642, C644, and others not shown). Surprisingly, all sequences were similarly active, even those containing runs of five CG dinucleotides in a row (C642: CG spacing 0 nucleotides apart) and sequences with hexamers described previously as inactive in other sequence contexts (C644) [44]. Figure 8 additionally shows that B cell proliferation is not governed by the same stringent set of motif requirements as IFN-α induction. We found that all CpG-B and CpG-C ODNs were equivalent in the induction of B cell proliferation, except for a subgroup of CpG-C (C640, C642, C644), which contains palindromes rich with C and G. This suggests that B cells may prefer ISS motifs that are not A/T-deficient. Further investigations on the preferred motif requirements for PDCs versus B cells are underway.

DISCUSSION

Several reports have grouped CpG-containing immunostimulatory ODNs into two major categories that are distinguishable on the basis of structure and function [27]. This report is the first to describe a series of ISS ODNs, termed CpG-C, and the corresponding set of motif requirements that exerts strong, functional activities in both arms of the ISS response. Our prototypic CpG-C, C274, enhanced B cell proliferation, induced B cell activation as measured by CD80/CD86/CD40 expression, and promoted the expression of IL-6 and TNF-α. Unlike other PS ISS molecules such as CpG-B 1018, however, C274 also exhibited activity similar to the CpG-A D19 in its ability to dramatically induce the secretion of large amounts of IFN-α from PDCs, accompanied by elevated levels of IFN-γ. This demonstrates that high levels of IFN-α secretion can be achieved by non-CpG-A ODNs, an observation first made by Tokunaga and co-workers [45], who showed that PO ODNs containing certain palindromic CpG-containing hexamers could induce type I IFN from human PBMCs. However, the sequences described by Tokunaga do not induce IFN-α from human PBMCs or PDCs when prepared as PS ODNs (ref. [46] and data not shown).

It has previously been demonstrated that a variety of TCG-bearing PS ODNs, containing motifs such as AACGTTCG, GTCGTT, or even simply TCGTT, can induce B cell responses and NK lytic activity in human PBMCs [28, 47–49]. However, these studies did not show that such TCG-bearing ODNs are effective IFN-α inducers. Indeed, the CpG-B 2006, which has often been demonstrated to be a potent human ISS, has very poor ability to induce IFN-α, even from enriched PDCs, despite the presence of a 5′-TCGTCG and multiple GTCGTT motifs (refs. [8, 50]; Fig. 8). To determine whether we could enhance the activity of our prototype CpG-B, 1018, we conducted sequence variation studies that resulted in the generation of ISS C274, in which two consecutive TCG motifs have been substituted 5′ to the Dynavax octamer motif, AACGTTCG. Other ISS variants of this class were designed to identify the motif requirements for high IFN-α induction and B cell activation by fully modified PS ODNs. The key features of

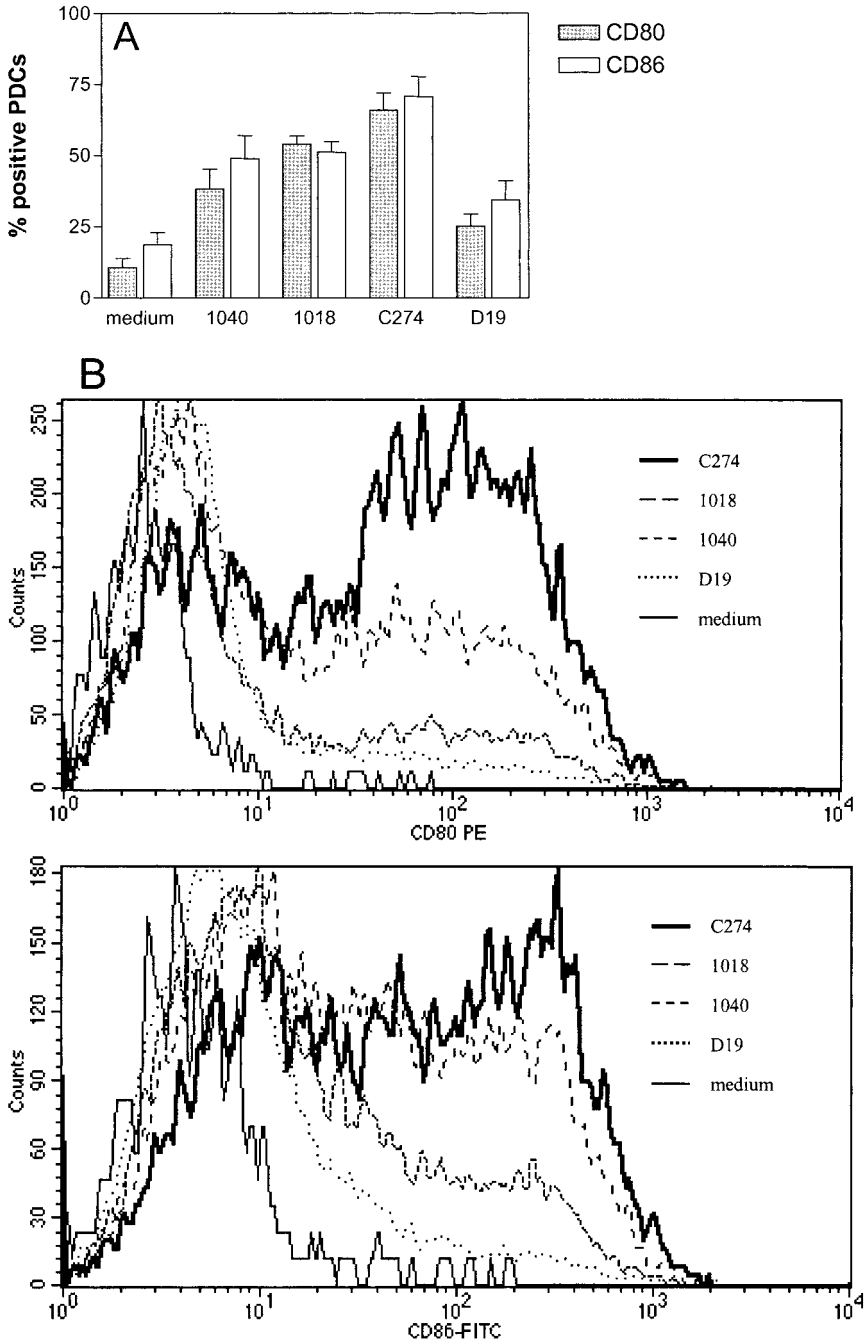

Fig. 5. C274 up-regulates CD80/CD86 expression on PDCs. (A) MACS-purified PDCs were cultured with 5 μg/ml ODNs for 24–48 h, and then cells were antibody-stained and assayed via FACS for expression of CD80/CD86 in the living cell population, gated by PI staining. Data are expressed as the means of 7–8 donors + SEM. Statistical relevance: CD80: medium versus 1040, $P < 0.01$; medium versus 1018, $P < 0.001$; medium versus C274, $P < 0.001$; medium versus D19, $P > 0.05$; 1040 versus C274, $P < 0.01$; C274 versus D19, $P < 0.001$. CD86: medium versus 1040, $P < 0.05$; medium versus 1018, $P < 0.05$; medium versus C274, $P < 0.001$; medium versus D19, $P > 0.05$; 1040 versus C274, $P > 0.05$; C274 versus D19, $P < 0.01$. (B) Histogram of one representative donor from Panel A. PE, Phycoerythrin; FITC, fluorescein isothiocyanate.

CpG-C ODNs include: one to two TCG trinucleotides at or close to the 5′ end of the ODN and a palindromic region of at least 10–12 bases, which contains at least two additional CG dinucleotides preferably spaced zero to three bases apart. It is interesting that once the requirements for a 5′-TCG and multiple CG dinucleotides optimally spaced less than four nucleotides apart within the palindrome were met, no further sequence requirements were found. Even sequences such as C642, which contain five CG dinucleotides in a row, induced high levels of IFN-α, although runs of contiguous CG dinucleotides had previously been described as CpG-neutralizing motifs [51]. Additionally, palindromic hexamers that had previously been reported as inactive, such as CCCGGG [44], induced IFN-α and B cell proliferation in the context of the longer 12-base palindrome and in conjunction with a 5′-TCG. In contrast, this motif does not appear to be necessary for optimal B cell stimulation or DC maturation, as CpG-B ODNs lacking this motif (e.g., 1018, 2006, C637) still perform those functions well.

In further examination of the capabilities of C274, we found that it did not exhibit the limitations in ISS activity, which have been demonstrated by CpG-A ODNs. Most notably, C274 is fully active on B cells, unlike D19 and other CpG-A sequences. Krug et al. [21] observed that a CpG-A ODN exhibited a partial ability to enhance CD86 expression on purified human B cells, but as no data using a non-CpG control ODN were reported, this may be a result of nonspecific stimulation from the PS portion of the CpG-A. Another group also reported marginal CpG-A induction of human B cell proliferation and IL-6 production [34]. However, these studies were performed

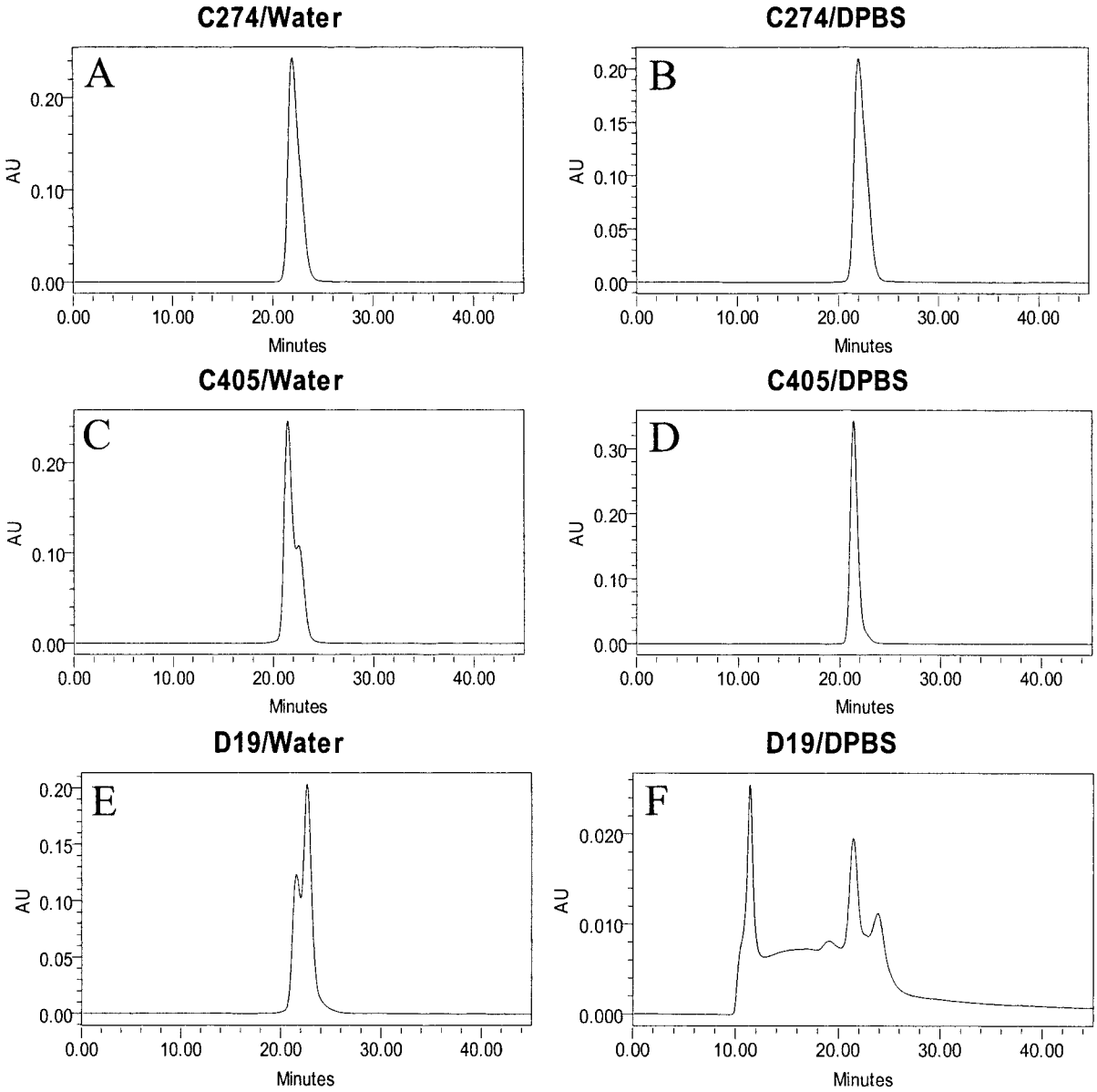

Fig. 6. GPC profiles of CpG-C versus CpG-A dissolved in water or DPBS. Solutions of the ODNs were prepared at 1.0 mg/ml in DPBS or water and were stored at 4°C for a minimum of 48 h before analysis. Sample (10 μg) was injected onto the column and eluted at 0.75 ml/min. Detection was at 260 nm. AU, Absorbance units.

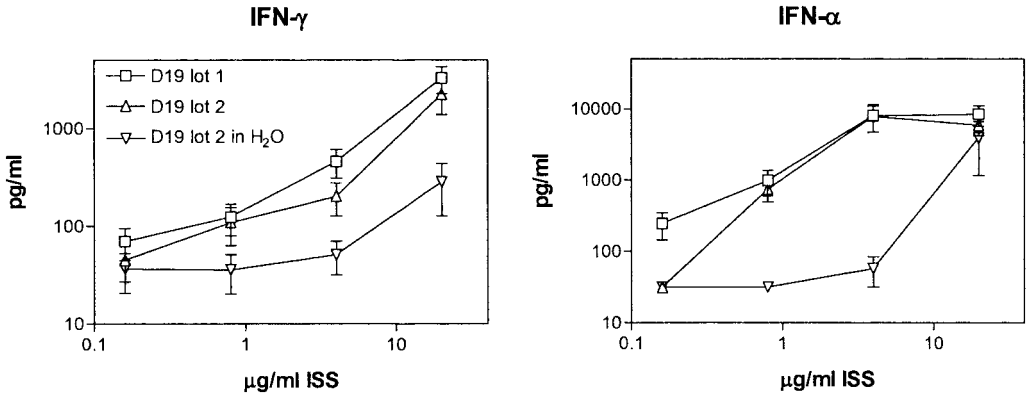

Fig. 7. The IFN-γ/α-inducing activity of D19 is largely dependent on the presence of higher order molecular weight aggregates. PBMCs were isolated from eight donors and stimulated with 0.16–20 μg/ml D19, diluted in water or buffer, for 24 h. Cell-free SNs were assayed for IFN-γ and IFN-α content by ELISA. Data are shown as means ± SEM.

TABLE 4. Sequence List of CpG-C ODNs

Code	Palindrome[a]	Sequence[b]
1018	8	TGACTGTGAACGTTCGGATGA
C274	12	TCGTCGAACGTTCGAGATGAT
C583	20	TCGAACGTTCGAACGTTCGAAT
C582	22	TTCGAACGTTCGAACGTTCGAAT
C637	22	TCAACGTTCGAACGTTCGAACGTT
C661	20	TGCTTGCAAGCTTGCAAGCA
2006	0	TCGTCGTTTTGTCGTTTTGTCGTT
C630	16	TCGACGTCGACGTCGACGTAT
C631	16	TCGTCGAAACGTTTCGACAGT
C633	16	TCGTCGAAAACGTTTTCGAGAT
C593	12	TCGTCGAGCGCTCGAGATGAT
C594	12	TCGTCGATCGATCGAGATGAT
C595	12	TCGTCGGTCGACCGAGATGAT
C646	12	TCGTCGTTCGAACGAGATGAT
C640	12	TCGTCGGGCGCCCGAGATGAT
C642	12	TCGTCGCGCGCGCGAGATGAT
C644	12	TCGTCGCCCGGGCGAGATGAT

[a] Number of nucleotides in palindrome. [b] CpG dinucleotides are bold, and palindromic sequences are underlined. Sequences are listed 5′–3′.

on PBMC populations from which B cells were later isolated and FACS analyzed, and this may have resulted in an indirect route of B cell activation rather than the direct effect of CpG-A on purified B cells investigated in our study. We additionally demonstrated that C274 apparently delivers a slightly different set of signals to the PDC than does a CpG-A ODN. For instance, although C274 and D19 elicited high IFN-α secretion from PDCs and comparably retarded the rate of PDC apoptosis, D19 induced less IL-6 and TNF-α from PDCs and substantially less up-regulation of the activation markers CD80 and CD86 on the PDC surface compared with C274. Indeed, the potency of D19 in this respect fell below even that of the non-CpG-negative control 1040, indicating that CpG-A ODNs do not activate PDCs in the same manner as CpG-B/C ODNs. This deficient ability of CpG-A to up-regulate CD80 on PDCs has also been observed by others [21]. C274 is further distinguished from D19 by the inhibition of IFN-α secretion, which is observed when the concentration of C274 rises above the optimal dose of 2–5 μg/ml (Fig. 2). D19 does not exhibit such a negative regulatory loop, as its IFN-α response curve flattens at a plateau. This disparity between the IFN-α response induced by these two ODNs coupled with their differential ability to induce DC maturation may indicate that D19 and C274 conduct qualitatively different signals to the PDC.

The differential activities of CpG-C and CpG-A may be related to structural differences between the two classes of ODNs that could lead to disparate DNA internalization pathways and/or mechanisms of action. CpG-C are single-stranded PS ODNs, which may form duplexes if they contain self-complementary regions of sufficient length but do not form high-order, secondary structures as a result of their lack of polyguanosine motifs. Conversely, CpG-A ODNs dissolved in salt-containing buffers form a heterogeneous mixture of aggregates, which range in size and include very large clusters with masses greater than 200,000 Da. This aggregate formation was dependent on several factors, including the presence of salt, the polyguanosine sequences, and the PO palindromic sequence (Fig. 6 and data not shown). Additionally, the general requirement of the polyguanosine motif for CpG-A activity [23, 33] correlates with the need for aggregation to obtain optimal IFN induction by D19 (Fig. 7). The aggregation of D19 most likely hinders degradation of the particularly susceptible PO region by nucleases.

Previous reports have shown that cellular uptake is necessary for ISS activity and that TLR9 is expressed primarily in endosomal vesicles [52]. Although it is unknown exactly how ISS ODNs enter the cell, there is general agreement that receptor-mediated endocytosis and/or pinocytosis are likely mechanisms. In contrast, large, aggregate ODNs such as CpG-A may be preferentially taken up into cells via Scavenger Receptor-A, as has been previously reported for quadruplex structures, or a similar type receptor that recognizes polyanionic ligands, thereby leading to increased uptake and/or targeting to phagocytic cells [31, 33, 53]. Therefore, it is possible that structural differences between CpG-C and CpG-A cause them to be internalized by distinct mechanisms that may lead them to disparate cellular compartments and thus similar but

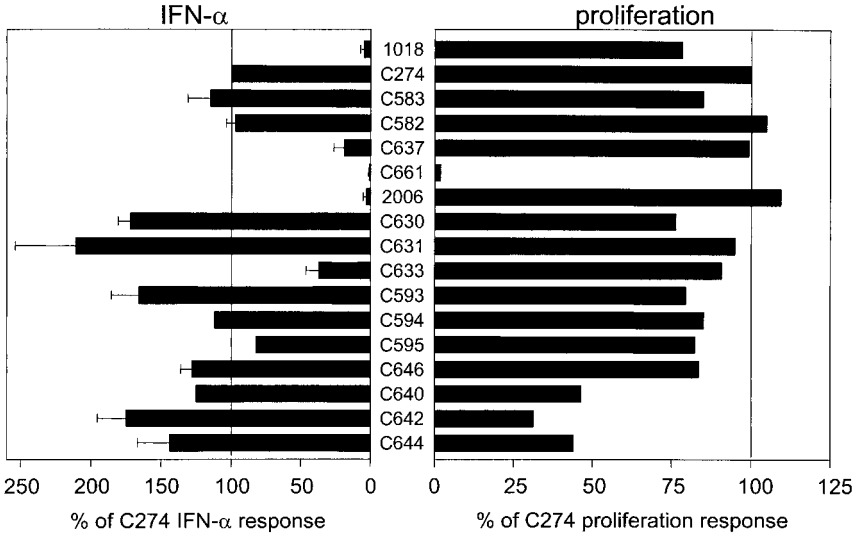

Fig. 8. CpG-C ODNs induce substantial IFN-α production and B cell proliferation. (Left) PBMCs were isolated from three to four donors and stimulated with 4 μg/ml ODN for 24 h. Cell-free SNs were assayed for IFN-α content by ELISA. Results from stimulation with C274 (7087±2529 pg/ml) are considered to be 100%, and data are reported as the percentage comparison with the C274 response. Data are means ± SD. (Right) B cells were purified from PBMCs and assayed for proliferative response to 4 days of stimulation with 5 μg/ml ODN. Results from stimulation with C274 (67,295 cpm) are considered to be 100%, and data are reported as the percentage comparison with the C274 response. Data are expressed as the means of two donors. This experiment is representative of two.

nonidentical activities. Alternatively, the differential pattern of activities of CpG-C versus CpG-A may be a result of CpG-A signaling through a heterodimeric or non-TLR9 receptor, as has been postulated in recent reports [29, 34, 50].

The presence of the polyguanosine motif in CpG-A ODNs presents formidable challenges for their clinical development [54]. The synthesis, purification, and characterization of poly-G-containing ODNs are challenging because of their tendency to aggregate. We have observed significant variations between D19 lots in regards to the proportions of the variously sized components measured by GPC, suggesting that subtle differences in the purity of the CpG-A may affect the aggregation in buffer. Additionally, it is as yet unclear which structural form(s) of D19 corresponds to its immunostimulatory activity.

In conclusion, we have identified an ISS ODN, C274, which acts similarly to a CpG-A ODN in its capacity to induce high levels of PDC-derived IFN-α but also contributes other ISS activities. Additionally, we have described the general motif requirements for this novel and distinct class of ISS CpG-C ODNs. In vivo studies have recently demonstrated marked efficacy for ISS-based vaccines as prophylactic/therapeutic tools for the prevention/treatment of a variety of disorders including asthma [39], tumors [55, 56], hepatitis B [57], herpes [58], leishmania [59], and tuberculosis [60] among others. Enhanced IFN-α production may play a key role in ISS-mediated immunomodulation. Although CpG-A ODNs can provide tremendously high levels of IFN-α, they may not activate DCs to mature and do not directly supply B cell-stimulating signals. Additionally, the activity of such ODNs is dependent on their ability to form heterogeneous populations of aggregates comprised of a variable number of CpG-A molecules, thereby making Good Manufacturing Practice-level characterization of CpG-A difficult. Conversely, C274 and the CpG-C class lack high-order, secondary structures and are therefore straightforward to manufacture and characterize. Although 1018 exhibited more limited effectiveness in vitro than C274, 1018 has been recently shown to exert immunomodulatory activity in human clinical trials of subjects with ragweed allergy or as an immunopotentiater for the hepatitis B vaccine [61, 62]. As such, C274 and other CpG-C ODNs are excellent candidates for even more potent ISS-based vaccine therapies.

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