A minimal human immunostimulatory CpG motif that potently induces IFN-γ and IFN-α production

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Recent reports have shown that immunostimulatory sequences (ISS) containing CpG motifs have minimal length requirements (≥12 bases) for the exertion of immune-enhancing function upon mammalian cells. Herein we demonstrate that short ISS (5–7 bases), which exhibit no activity on their own, induce IFN-γ and IFN-α secretion from human peripheral blood mononuclear cells when adsorbed to the surface of cationic poly(D,L-lactide-co-glycolide) microparticles (cPLGA). Utilizing this technique, we discovered a minimal ISS sequence for induction of IFN-γ and IFN-α from human cells: 5'-TCGXX-3'. These short ISS/cPLGA formulations targeted PDC in similar fashion to longer ISS ODN, the activity of which does not require (but is enhanced by) cPLGA. PDC stimulated with short ISS/cPLGA responded with enhanced uptake of ISS and elevated production of cytokines, including IFN-α. However, ISS-responsive B cells did not respond to short ISS/cPLGA, underlining the plasmacytoid dendritic cell selectivity of this formulation. These results describe a novel technique for formulating active, but very short, ISS oligodeoxynucleotide that allows for the dissection and characterization of minimal immunostimulatory CpG motifs.

Received	19/2/03
Revised	25/4/03
Accepted	20/5/03

Key words: CpG DNA / Plasmacytoid dendritic cells / Adjuvants

1 Introduction

Immunostimulatory sequence oligodeoxynucleotides (ISS ODN) are small pieces of DNA that contain an unmethylated cytosine-guanine dinucleotide within a particular sequence context (CpG motif). ISS ODN strongly stimulate the mammalian immune system by activating B cells, monocytes, and dendritic cells [1, 2], triggering the release of a variety of cytokines, and inducing potent antibody, Th1, and cytotoxic T lymphocyte (CTL) responses to antigens. The *in vivo* activity of ISS ODN as therapeutics in models of asthma and cancer and as vaccine adjuvants has been demonstrated in mice and primates [3–5]. Recently, the safety and efficacy of 1018 ISS was evaluated as a vaccine adjuvant in humans as well. The co-injection of 1018 ISS with hepatitis B surface antigen significantly enhanced the seroconversion rate and protective antibody response and was well tolerated by seronegative individuals [6].

Structure-activity studies have shown that certain oligonucleotides that have optimal immunostimulatory activity in mice can demonstrate relatively little activity in humans [7], although some sequences, such as 1018 ISS, have good activity in both species [8, 9]. The optimal consensus ISS motif for mice consists of a hexamer containing 5'-PuPuCGPyPy-3', where Pu is a purine and Py is a pyrimidine; however, the optimal ISS motif for humans has not been as clearly defined. ISS ODN that predominantly induce IFN-γ, IL-6, and B cell proliferation in human peripheral blood mononuclear cells (PBMC) generally consist of ODN with a fully modified phosphorothioate (PS) backbone and multiple CpG sequences. Various optimal human ISS motifs have been proposed, including GTCGTT [2, 10], TCGTT, and TCGTA [11]. Interestingly, ISS ODN containing these motifs described in the literature thus far generally do not induce significant levels of IFN-α from PBMC, although they perform other functions such as activation and proliferation of B cells and NK cells.

While the minimal mouse or human ISS motifs are most often described in the literature as hexameric se-

[DOI 10.1002/eji.200323948]

Abbreviations: cPLGA: Cationic poly(D,L-lactide-co-glycolide) **FAM:** Fluorescein amidite **ISS:** Immunostimulatory sequence **MFI:** Mean fluorescence intensity **ODN:** Oligodeoxynucleotide **PDC:** Plasmacytoid dendritic cell **PO:** Phosphodiester **PS:** Phosphorothioate **Pu:** Purine **Py:** Pyrimidine

0014-2980/03/0808-2114$17.50+.50/0

quences, the bases outside of the hexamer also appear to be important for activity, although their exact role is unclear [12, 13]. Most ODN with significant activity in murine systems are 15–25 nucleotides in length, while shorter sequences lack activity [14]. In human PBMC, a length of >12 nucleotides is necessary to consistently induce immune responses such as B cell proliferation and the secretion of IL-6 and IgM, whereas a minimum length of about 14–16 nucleotides is required for detectable induction of IFN-γ [11, 15]. In fact, there has been no published report of ISS activity in human cells by ODN containing fewer than 8 nucleotides, and longer ODN are usually required for robust immune stimulation (Marshall, J. D., unpublished observations). Thus, the definition of the minimal human immunostimulatory motif is made difficult when explored in the context of large oligonucleotides, which require multiple CpG motifs and flanking sequences for significant activity.

Due to the relative maturity of both antisense ODN and DNA vaccine technologies, a large number of formulation and delivery strategies for DNA have been investigated. In the course of evaluating several of these strategies for ISS ODN delivery, we found that adsorption of ISS ODN to the surface of cationic poly(D,L-lactide-co-glycolide) microspheres (cPLGA) significantly enhanced the secretion of IFN-γ and IFN-α from human PBMC compared to ISS ODN alone. Previously, polymeric microspheres were shown to be a potent delivery system for DNA vaccines [16] and at least one CpG sequence [17]. Formulation of the ODN by this technique increases their uptake and stability, while also targeting the ISS to antigen-presenting cells [18]. We have used this formulation strategy as a tool to study the immunostimulatory activity of ODN containing <8 nucleotides, eliminating the need for flanking sequences to enhance uptake and allowing for the definition of a minimal immunostimulatory sequence required for induction of IFN-γ and IFN-α from human cells.

2 Results

2.1 Enhanced response of human PBMC to ISS formulated on cPLGA

1018 ISS (22-mer) and C274 (21-mer), each containing multiple CpG motifs and a phosphorothioate backbone, induced the secretion of IFN-γ and IFN-α from human PBMC. The IFN-γ response generated by C274 was somewhat higher than that induced by 1018 ISS, but more notably, C274 induced tenfold more IFN-α than 1018 ISS [19]. Others have reported that ISS-induced IFN-γ is NK cell-derived through an as yet undefined indirect mechanism, while IFN-α is induced directly by ISS ODN from plasmacytoid dendritic cells (PDC) [2]. We observed that the induction of these cytokines was magnified by the adsorption of the ISS ODN to the surface of cPLGA microspheres (Fig. 1). The specificity of sequence recognition was not altered by the formulation, as non-CpG negative control ODN 1040 + cPLGA demonstrated no cytokine induction.

2.2 Identification of the minimal human motif for IFN-γ/IFN-α induction by short ISS

In an effort to identify the minimal sequences that still retain the ability to induce enhanced levels of IFN-γ/IFN-α from PBMC, the 21-mer ISS ODN C274 sequence was divided and synthesized as three individual heptameric sequences: TCGTCGA, ACGTTCG, and AGATGAT. Both TCGTCGA and ACGTTCG contain two CpG dinucleotides, while AGATGAT does not contain a CpG sequence and served as a negative control. None of these heptameric CpG sequences was active in the human PBMC assay when used either alone or in combination up to a concentration of 100 μg/ml (data not shown). However, TCGTCGA induced substantial amounts of IFN-γ and IFN-α from human PBMC when formulated with cPLGA (Fig. 2A, B). Surprisingly, ACGTTCG, which also contains two CpG motifs, remained inactive even when formulated with cPLGA. These observations suggested a strategy for determining the optimum size, sequence, and position of the immunostimulatory motif without the confounding effects of flanking sequences that may be needed for cellular uptake or stability, but not for TLR9-mediated activation.

Comparison of the active and inactive heptamers from C274 suggested the hypothesis that TCG is required for

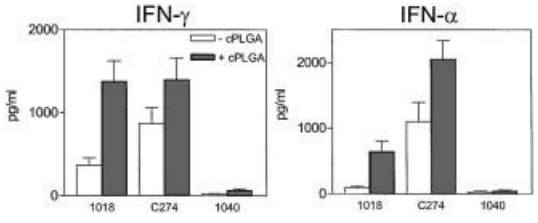

Fig. 1. cPLGA enhances the ability of ISS to stimulate IFN-γ/IFN-α production from PBMC. PBMC were stimulated for 24 h with 20 μg/ml ODN alone (white bars) or 20 μg/ml ODN complexed with 100 μg/ml cPLGA microparticles (gray bars). Data are reported as means of 27 separate donors + SEM. Statistical relevance: IFN γ: 1018 *vs*. 1040: ***; C274 *vs*. 1040: ***; 1018 *vs*. C274: *; 1018 *vs*. 1018 + cPLGA: ***; C274 *vs*. C274 + cPLGA: ns. IFN-α: 1018 *vs*. 1040: ns. C274 *vs*. 1040: **; 1018 *vs*. C274: **; 1018 *vs*. 1018 + cPLGA: **; C274 *vs*. C274 + cPLGA: *.

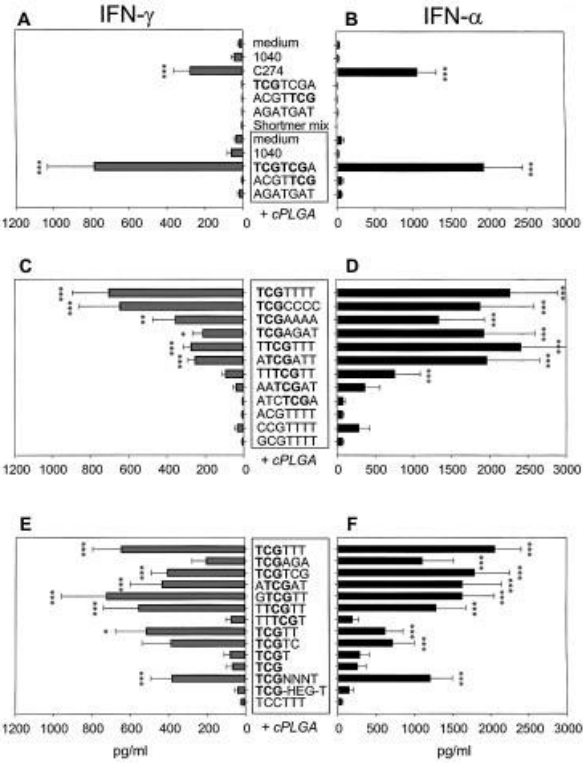

Fig. 2. Complexation with cPLGA confers potent ISS activity on short ISS ODN. PBMC were stimulated for 24 h with ISS ODN. Boxed ODN were complexed with cPLGA. Data are reported as means of 11–36 donors + SEM. Statistical relevance: each stimulatory condition compared to medium: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

human ISS activity and that the TCG motif must be located near the 5'-end (or distant to the 3'-end) of the short ISS. A series of heptamers, with a TCG motif placed in various positions throughout the sequence, was synthesized in order to study the importance of the TCG location. In addition, sequences containing ACG, CCG, and GCG were generated in order to determine the specific requirement for TCG in the immunostimulatory activity of the short ISS. As expected, none of the heptamers induced detectable IFN-γ or IFN-α in human PBMC when used as unformulated compounds (data not shown). However, for the heptamer ODN formulated with cPLGA, a TCG was absolutely necessary for immunostimulatory activity; heptamer sequences lacking a TCG (ACGTTTT, CCGTTTT, GCGTTTT) were uniformly inactive when formulated on cPLGA (Fig. 2C, D). In addition, the TCG was required to be either in the 5'-position (TCGTTTT, TCGCCCC, TCGAAAA, TCGAGAT) or the penultimate 5'-position (TTCGTTT, ATCGATT) in order to result in an optimally active ISS. Indeed, the immunostimulatory activity of the ODN was reduced as the TCG moiety was situated closer to the 3'-end (TTTCGTT, AATCGAT), until the oligonucleotides with the TCG in the penultimate 3'-position or 3'-position became completely inactive (ATCTCGA, ACGTTCG). The activity of the optimal heptameric ISS ODN adsorbed to cPLGA was comparable or superior to that observed with longer ISS ODN such as C274, which does not require cPLGA formulation for substantial IFN-γ/IFN-α activity (Fig. 2A, B).

Sequentially smaller ODN were synthesized in order to determine the minimum number of nucleotides necessary for immunostimulatory activity when adsorbed to cPLGA. A variety of hexameric and pentameric ODN were found to be active as long as they contained a TCG followed by at least two nucleotides on the 3'-end and were adsorbed to cPLGA (Fig. 2E, F). Conversely, the cPLGA-formulated trimer, 5'-TCG-3', and tetramer, 5'-TCGT-3', were inactive, further demonstrating the need for at least two nucleotides on the 3'-side of the TCG for immunostimulatory activity. Binding studies confirmed that the trimer and tetramer were adsorbed to the cPLGA at DNA loadings similar to longer ODN (data not shown); therefore, deficient binding to cPLGA was not the cause of the lack of activity of these compounds.

From the data generated above, the general human epitope was defined as 5'-TCGXX-3', where X is a nucleotide. The identity of X does not seem to be particularly important for immunostimulatory activity; rather, X appears to be necessary as a placeholder or binding site for the receptor. To further investigate this, two additional oligonucleotides were synthesized, each containing a 5'-TCG linked to a 3'-T by either three abasic (N) deoxyribose sugars (TCGNNNT) or a hexaethylene glycol (HEG) spacer (TCG-HEG-T) connected by phosphorothioate linkages. TCGNNNT/cPLGA induced IFN-γ and IFN-α from human PBMC in amounts similar to other 5'-TCG-containing heptamers formulated on cPLGA. In this case, the sugar/phosphate structure of the ODN was preserved, although there was no nucleobase present. Conversely, TCG-HEG-T/cPLGA was inactive in this assay, most likely due to the loss of the DNA-type structure in the position directly 3' to the TCG. Therefore, at least two additional residues that resemble nucleotides must be present 3' to the 5'-TCG motif for ISS activity.

2.3 Gene induction by short ISS formulated on cPLGA

To determine whether short ISS ODN/cPLGA complexes activate the immune system similarly to ISS ODN alone, the expression of an ISS-specific gene panel in human PBMC was evaluated after stimulation with C274, 1040, or a series of short ISS ODN formulated on cPLGA. The

Table 1. Short ISS/cPLGA induces similar pattern of gene expression to longer ISS[a)]

Compound	2,5-OAS	ISG-54K	IFN-γ	IFN-α	IP-10	MIG	MCP-2
Medium	1	1	1	1	1	1	1
1040	1.1 (0.3)	1.1 (0.4)	1.5 (1.0)	22.5 (37.5)	2.0 (1.1)	1.2 (1.0)	0.9 (0.3)
C274	16.1 (9.5)	27.0 (12.6)	5.2 (2.5)	632.0 (402.1)	120.9 (131.8)	13.6 (11.8)	288.1 (396.0)
TCGTCGA	1.1 (0.4)	1.2 (0.5)	1.7 (0.2)	220.0 (377.7)	1.7 (0.2)	1.5 (0.6)	1.7 (0.9)
cPLGA	0.9 (0.1)	2.0 (0.4)	3.0 (1.5)	3.0 (2.9)	1.3 (0.1)	1.2 (0.7)	1.4 (1.0)
1040/cPLGA	2.6 (1.6)	2.3 (0.7)	3.8 (2.5)	61.1 (49.1)	1.9 (1.3)	1.7 (1.4)	3.3 (2.8)
TCGTCGA/cPLGA	14.3 (2.8)	20.6 (7.9)	36.4 (50.9	1317.4 (1436.5)	49.0 (54.8)	19.3 (24.9)	126.9 (122.3)
TCGTTTT/cPLGA	20.1 (13.8)	25.2 (8.2)	66.2 (57.5)	1099.4 (722.0)	82.3 (61.5)	32.5 (26.5)	349.7 (370.9)
TCGTTT/cPLGA	21.5 (20.2)	18.6 (7.9)	32.3 (27.9)	583.3 (478.4)	63.9 (55.9)	20.0 (20.7)	242.9 (326.4)
TTCGTT/cPLGA	17.4 (16.4)	19.4 (6.7)	25.2 (23.0)	414.8 (229.0)	33.4 (28.9)	21.8 (18.7)	200.6 (223.4)
TCCTTT/cPLGA	1.7 (2.1)	2.3 (1.5)	4.1 (3.7)	1.7 (0.2)	1.7 (2.1)	1.3 (1.4)	3.9 (5.4)

[a)] PBMC from four donors were cultured for 10 h with 5 μg/ml short ISS ± PLGA and RNA was extracted and quantitated via TaqMan RT-PCR. Data are presented as the mean of fold induction over medium control (given the value of 1.0) with SEM. A second experiment using four donors gave similar results.

RNA was harvested at 10 h, converted to cDNA, and evaluated by quantitative PCR. The expression levels of each gene were normalized to ubiquitin signal and then calculated as fold-increases over medium stimulation. This ISS-specific gene panel was previously identified in our laboratory as a sensitive marker for ISS activity in human PBMC [19]. Table 1 shows that C274 and the most potent cPLGA-formulated short ODN containing the optimal ISS motif (TCGTCGA, TCGTTTT, TCGTTT, TTCGTT) induced high levels of mRNA for the cytokines IFN-γ and IFN-α, the IFN-α-inducible genes, 2,5-oligoadenylate synthetase (2,5-OAS) and interferon-stimulated gene-54K (ISG-54K), and the chemokines interferon-inducible protein-10 (IP-10), monokine induced by IFN-γ (MIG), and monocyte chemoattractant protein-2 (MCP-2). The control ODN 1040, alone or formulated on cPLGA, had little effect on gene expression. As expected, the unformulated heptamer, TCGTCGA, and the formulated short control ODN, TCCTTT, also did not significantly alter mRNA levels. These data suggest that short ISS + cPLGA transmits similar signals as standard ISS which result in comparable patterns of gene activation.

2.4 Cellular targets of short ISS formulated on cPLGA

To verify that ISS ODN and short ISS/cPLGA complexes are active on the same cell types, PDC were isolated and purified from human PBMC by immunomagnetic bead positive selection using BCDA-4. We and others have previously established that human PDC can respond to ISS stimulation with the secretion of IFN-α and TNF-α [2, 20] and that C274 is particularly potent in this respect [19]. PDC were stimulated with C274, 1040, or a series of short ISS/cPLGA complexes for 24 h, after which SN were harvested and analyzed for cytokine content. As expected, C274 induced the secretion of high levels of both IFN-α and TNF-α from PDC, while the control ODN 1040 had no effect (Fig. 3). Short ISS ODN/cPLGA complexes with the sequences TCGTCGA and TCGTTTT also directly activated the PDC to produce IFN-α and TNF-α. These same short ISS/cPLGA complexes also induced elevated IFN-α gene expression at 4 h from the PDC, independently confirming the ELISA data (data not shown).

ISS ODN are also known to directly activate a subset of B cells, causing them to proliferate and secrete IL-6. These activities were measured for purified peripheral blood B cells, which had been stimulated with a similar panel of short ISS/cPLGA complexes (Fig. 3). As expected, C274 stimulated B cells to both proliferate and release IL-6. 1040 induced minimal levels of activity in both assays, consistent with previous observations that all PS ODN can marginally activate B cells through a CpG-irrelevant pathway [21]. In contrast, the short ISS/cPLGA formulations were inert on B cells (Fig. 3), indicating that this type of complex specifically targets the PDC arm of the ISS response. Interestingly, longer ISS, such as C274, when complexed on cPLGA, are recognized by B cells, which respond with activity equivalent to that induced by the longer ISS alone (data not shown), indicating a fundamental distinction between how B cells recognize short vs. long ISS displayed on cPLGA.

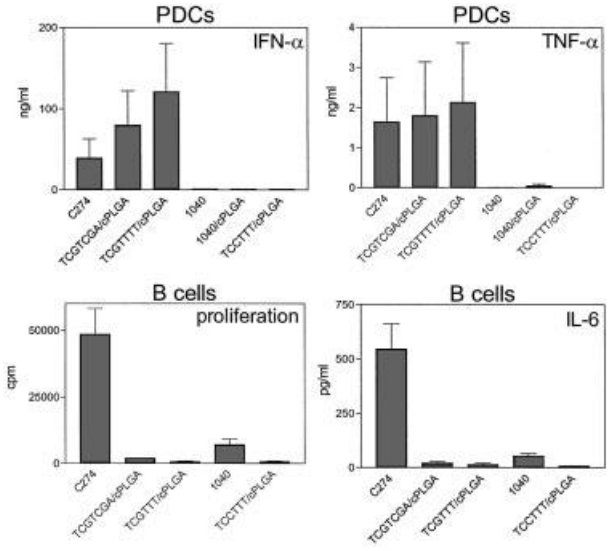

Fig. 3. Short ISS/cPLGA exerts ISS activity on PDC but is inert on B cells. MACS-purified PDC were cultured with 5 μg/ml ISS ± cPLGA for 24 h. MACS-purified B cells were cultured with 5 μg/ml ISS ± cPLGA for 72 h (proliferation) or 48 h (IL 6). Proliferation was assessed by [^{3}H]thymidine incorporation and IL-6 by ELISA. Data are reported as the means of four donors + SEM.

2.5 cPLGA enhances uptake of short ISS into PDC

Previous reports have shown that cellular uptake of ODN is sequence-independent and saturable [22]. Using ODN with a 3'-fluorescein label (FAM-ODN), we observed CpG motif-independent uptake of ODN by human monocytes, B cells, myeloid dendritic cells, and PDC (data not shown). Further studies were focused on DNA uptake by PDC within a monocyte-depleted PBMC population, because PDC are directly activated by ISS ODN while monocytes are functionally unresponsive to direct ISS stimulation yet act as a large ODN sink that can obscure ISS uptake by the much smaller PDC population. Short ODN (TCGTCGA and AGATGAT) were minimally taken up by PDC compared to longer ODN (C274 and 1040, Table 2). However, adsorption of long or short ODN on the surface of cPLGA microparticles significantly increased the number of PDC taking up FAM-ODN (% double positive for fluorescein and BCDA-4), as well as the amount of ODN taken up per cell (MFI). Short ISS formulated with cationic liposomes, another known method of increasing DNA uptake into cells [23], also yielded similar uptake and activity results in comparison to cPLGA formulations (data not shown). This suggests that greatly enhancing the uptake of short ISS into PDC through adsorption to cPLGA is responsible for the substantially increased activity of short ISS/cPLGA.

Table 2. Uptake of short ISS is enhanced by formulation with cPLGA or liposomes[a)]

ODN	% ODN-FAM positive[b)]	MFI[c)]
Medium	1.7 (1.6)	2 (<1)
C274	90.0 (5.5)	12 (2)
1040	68.0 (16.6)	8 (2)
TCGTCGA	4.8 (2.0)	3 (1)
AGATGAT	3.4 (0.9)	2 (<1)
cPLGA	1.6 (1.7)	2 (<1)
C274/cPLGA	94.3 (3.7)	72 (43)
1040/cPLGA	93.9 (4.6)	272 (137)
TCGTCGA/cPLGA	88.6 (7.8)	147 (118)
AGATGAT/cPLGA	91.5 (6.1)	325 (188)

a) Monocyte-depleted PBMC were incubated with FAM-labeled ODN alone or complexed with cPLGA for 30 min. Cells were then stained with BCDA-4-PE and analyzed via FACS for intracellular FAM-ISS content. Data are reported as the means (SD) of four donors. These data are from one of three representative experiments.

b) % of BCDA-4$^+$ PDC that were positive for ODN-FAM.

c) MFI, mean fluorescent intensity.

2.6 Short ISS ODN compete for recognition with longer ISS

If short ISS ODN bind to the same receptor that recognizes standard ISS (thought to be TLR9), then they should be able to compete with longer ISS ODN when present in excess. To examine this question, we performed ODN competition studies in which a dose titration of C274 (0.3–20 μg/ml) was conducted in the presence or absence of an excess of short ISS ODN. Since previous experiments had shown that short ISS were taken up less efficiently than the molar equivalent of longer ODN (Table 2), we used 50 μg/ml short ISS in the competition study, a concentration which we found allowed uptake into PDC comparable to tenfold lower concentrations of longer ISS (data not shown). Fig. 4 shows that an excess of either TCGTCGA or the longer ISS ODN 1018 successfully suppressed the ability of C274 to induce IFN-α from PBMC, while the short ISS ODN, ACGTTCG and AGATGAT, which lack activity even when complexed with cPLGA, did not. FACS analysis with propidium iodide indicated that excess TCGTCGA did not exhibit any toxic effects on the cells (data not shown). This suggests that TCGTCGA ODN recognize the same receptor as standard ISS but may have much

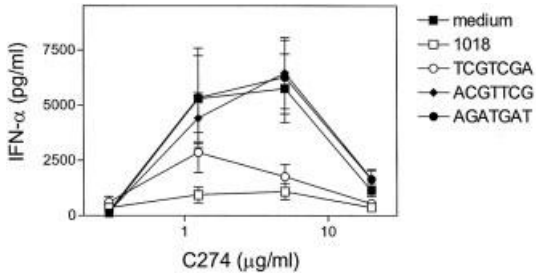

Fig. 4. Excess short ISS competes away activity of C274. PBMC were stimulated for 24 h with C274 (concentration range: 20, 5, 1.25, 0.3 μg/ml) in the absence or presence of 50 μg/ml 1018 or short ISS. Data are reported as means of ten separate donors + SEM.

lower affinity and weaker signaling power, since highly increased uptake via formulation is required for them to exert IFN-γ/α-inducing activity.

3 Discussion

Although an unmethylated CpG dinucleotide is required for biological activity, it has been shown that specific flanking bases are required for optimal activity [11, 24]. Although consensus exists for the optimal recognition motif for mouse activity (PuPuCGPyPy) [25], a comparable human motif has not yet been clearly defined. The sequence(s) recognized optimally by human TLR9 is likely similar in size to the hexameric mouse motif, but definition has been complicated by the fact that active oligonucleotides must be at least 12–15 bases long [14] and require at least two CpG motifs [10] to exhibit clear ISS activity *in vitro*. In this report, we show that complexing active ISS ODN to cPLGA microparticles significantly enhances their ability to induce IFN-γ and IFN-α production from PBMC. The specificity of ISS ODN recognition is not altered, as molecules lacking active CpG motifs remain inactive when complexed to cPLGA. The fact that ISS ODN complexed to cPLGA are not constrained by sequence length has allowed us to discover a motif of minimal length (5'-TCGXX-3') that potently stimulates for interferon production.

As has been previously reported, the optimal recognition motif for human PBMC includes a CpG dinucleotide preceded by T, rather than by two purine bases, as is the case for mouse cells [2, 10]. However, we found that the simple presence of a TCG within a short ISS sequence was not sufficient to confer activity when in cPLGA-complexed form. In fact, the position of the TCG motif was required to be at or very near the 5' end of the short ODN, allowing at least two additional bases 3' to the TCG. Sequence variation studies showed that ODN activity diminished as the TCG motif was moved farther from the 5' end and closer to the 3' end (Fig. 2). This principle is demonstrated by the inactivity of the sequence ACGTTCG, which, despite containing two CpG (one of which is a TCG), failed to meet the requirements of having the TCG near the 5' end and at least two bases on the 3' end of the TCG. Requirement for positioning of the CpG motif at the 5' end of short ISS was also observed by Tidd et al. [26], although that study examined intracytoplasmic delivery of short ISS to induce apoptosis in the MOLT-4 T cell leukemic line. We further discovered that the required 3' bases could be of any combination and could even be abasic sugar residues. This suggests that at least two nucleotide lengths of sequence nonspecific DNA are required to be present 3' of the motif, perhaps to stabilize the interaction with the TLR9 receptor. It is interesting to note that the rules we observed governing the ability of short ISS/cPLGA to stimulate optimally for IFN-α production may not fully explain the high IFN-α induction by certain longer ISS ODN, such as C274 [19]. Other sequences containing 5'-TCG, such as 2006 (TCGTCGTTTTGTCGTTTTGTCGTT), have been well documented as poor IFN-α-inducers [27]. Thus, 5'-TCG appears to be necessary but not always sufficient for high IFN-α induction by ISS of standard length.

Our data show that short heptameric ISS ODN are not taken up by PDC as efficiently as longer ODN of approximately 20 bases. However, formulating short ISS with agents that promote DNA uptake (cPLGA or liposomes) confers activity upon 5'-TCG-containing heptameric ODN. Sonehara et al. [28] described that CpG-containing palindromic hexamers were inactive on their own but were converted to active form when formulated with cationic liposomes. However, these studies were conducted with PO, not PS, ODN and ISS activity was measured as mouse splenocyte production of interferons. We have observed that short PO ISS are inactive on human cells when formulated with liposomes (data not shown), presumably due to enzymatic degradation, but are protected when complexed with cPLGA and retain ISS activity. These observations suggest a difference in nuclease production between human and mouse cells [7]. In addition, we found that both the PO and PS versions of the optimal motif (AACGTT) described by Sonehara et al. [28] as exhibiting mouse activity demonstrated no such activity on human cells, even when cPLGA- or liposome-formulated (data not shown). This suggests further mouse *vs*. human differences, *i.e.* in the specificity by which their ISS receptors recognize optimal ISS motifs.

This laboratory has found that ISS molecules such as C274 trigger a pattern of gene activation from human PBMC that includes up-regulation of message levels for

IFN-γ and IFN-α, the chemokines IP-10, MIG, and MCP-2, and the IFN-α-inducible genes 2,5-OAS and ISG-54K [19]. The finding that short ISS + cPLGA induced the same pattern of gene activation as the longer active ISS ODN demonstrates that these two types of ISS deliver comparable signals to responsive cell populations.

Although readily recognized by PDC, short ISS ODN/cPLGA complexes did not induce detectable ISS responses from B cells, despite enhancement of uptake into B cells when cPLGA-associated (data not shown). A similar disparity in the ISS response between responder cell types has been reported when comparing two major classes of ISS ODN, known variously as CpG-A and CpG-B [29]. ODN of the first type (CpG-A) induce large amounts of IFN-α from PDC and IFN-γ from NK cells but are inert on B cells [11], while ODN of the second type (CpG-B) promote B cell functions but are relatively poor for IFN-γ/IFN-α production [2]. A third and newly described class of ISS ODN, termed CpG-C and including C274, is able to induce strong ISS responses from both cell types [19]. The short ISS ODN/cPLGA complexes do not fit the chemical or sequence definitions for CpG-A ODN (PO/PS chimeric DNA backbone, polyguanosine motif), despite their similar profile of ISS activities. However, these results may indicate that multimeric presentation of ISS motifs, either by adsorption to an insoluble support for short ISS or through aggregation for CpG-A [21], preferentially amplifies IFN-α secretion from PDC over other ISS functions. These findings indicate that the motif, length, and/or presentation requirements for IFN-γ/α production may be different from the requirements for B cell activation, which in turn suggests a fundamental difference in the manner in which these two cell types recognize ISS. This may be due to differential ISS receptors between B cells and PDC, which might be represented by alternately spliced variants of TLR9, by TLR9 heterodimerized with other TLR family members, by unidentified non-TLR9 ISS receptors, or by differential intracellular compartmentalization. Although not recognized by B cells, the facts that short ISS/cPLGA complexes signal very similarly to standard ISS on PDC and that short ISS can compete away longer ISS activity suggests that short ISS interacts with the same ISS receptor as longer ODN in PDC.

In conclusion, these studies define a minimal ISS motif for the optimal induction of IFN-γ and IFN-α from human PBMC to be 5'-TCGXX-3', where X can be any nucleotide. The element of 5'-TCG has also been noted as crucial for high IFN-α induction by longer ISS as well, although other elements such as the presence of a palindrome, its length, and incorporation of other CpG motifs are also necessary [19]. We have not determined whether this is the minimal motif required for other ISS functions such as dendritic cell maturation and differentiation, and it may be that other immunomodulatory motifs also exist that target those functions. Additionally, we have described a system of formulation that can dramatically enhance the function of any ISS molecule, even those ISS ODN too short to have activity on their own. Since cPLGA microparticles are biodegradable within living systems, this formulation might serve as a potent adjuvant for ISS-based therapeutics.

4 Materials and methods

4.1 Oligodeoxynucleotides

Phosphorothioate ODN were prepared on an Expedite 8909 following manufacturers protocols, purified by RP-HPLC, and precipitated as the sodium salt. ODN sequences, here and throughout, are listed 5' to 3'. 1018: TGACTGTGAACGTTCGAGATGA; 1040: TGACTGTGAACCTTAGAGATGA; C274: TCGTCGAACGTTCGAGATGAT. 3'-Fluorescein-labeled ODN were prepared using 6-FAM-CPG (Glen Research). All ODN had <5 endotoxin units/mg of ODN as determined by Limulus amebocyte lysate assay (BioWhittaker).

4.2 Preparation of cPLGA microspheres

cPLGA (0.875 g, Resomer® RG502, Boehringer Ingelheim Chemicals) and 1,2-dioleoyl-3-trimethylammoniumpropane (0.3 g, Avanti Polar Lipids) were dissolved in methylene chloride (7.9 g) as described [16]. The cPLGA was characterized for size and surface charge: mean size (number weighted, μ) =1.4; zeta potential (mV) =32.4.

4.3 Preparation of ODN/cPLGA complexes

The ODN and cPLGA were mixed at final concentrations of 5–20 μg/ml and 100 μg/ml, respectively, for 15 min at room temperature before they were added to the culture. These concentrations were derived previously as optimal for the induction of PBMC IFN-γ.

4.4 Mononuclear cell preparation

Human PBMC were isolated as described [19] and cultured in RPMI 1640 (BioWhittaker) supplemented with 10% heat-inactivated human AB serum (Gemini) plus 50 U/ml penicillin, 50 μg/ml streptomycin, 300 μg/ml glutamine, 1 mM sodium pyruvate (BioWhittaker), and 1× nonessential amino acids (BioWhittaker). For cytokine secretion, PBMC were cultured at 0.5×10^6/well in 96-well flat-bottom plates in duplicate with ISS ODN at 20 μg/ml ± cPLGA for 24 h, determined by previous studies to be the optimal time point for ISS-induced cytokine secretion. In the competitive receptor

binding experiment, a titrated dose range of C274 (20, 5, 1.25, and 0.3 μg/ml) was premixed with 50 μg/ml of short ODN for 15 min at room temperature in media, then immediately added to culture so that the PBMC were exposed to both types of ODN simultaneously. Cell-free SN were harvested and assayed by ELISA, as described [19].

4.5 Statistical analysis

Statistical significance was calculated using an unpaired *t*-test with Welch correction, assuming parametric data with different S.D.'s, to get two-tailed *p* values (GraphPad InStat). Symbols representing significance are: ***, $p<0.001$; **, $p<0.01$; *, $p<0.05$; ns, $p>0.05$.

4.6 PDC and B cell purification and functional assays

Human PDC and B cells were isolated as described [19]. For cytokine secretion, PDC were cultured at 0.5×10^5–1×10^5/well (2×10^5–4×10^5/ml) in 96-well round-bottom plates with 5 μg/ml ISS ± cPLGA for 24 h, then SN were harvested and assayed for cytokines via ELISA. For the proliferation assay, B cells were cultured in triplicate at 1×10^5/well in 96-well round-bottom plates with 2 μg/ml ODN ± cPLGA for 72 h. At the end of the culture period, the plates were pulsed with [^{3}H]thymidine (1 μCi/well, Amersham) and incubated for an additional 8 h. Then the plates were harvested and radioactive incorporation determined using standard liquid scintillation techniques, and the data was collected in counts per minute (cpm). For IL-6 secretion, B cells were cultured at 0.5×10^6–1×10^6/well in 48-well plates with 5 μg/ml ISS ± cPLGA for 48 h, then SN were harvested and assayed for IL-6 via ELISA.

4.7 Gene expression assay and analysis

Human PBMC were cultured with ISS ± cPLGA at 2×10^6/ml for 10 h, then RNA extracted and analyzed via TaqMan RT-PCR as described [19]. Primer sequences for ubiquitin, 2,5-OAS, ISG-54 K, MIG, MCP-2, and IFN-α were synthesized by Operon and are referenced in [19]. IFN-γ and IP-10 were measured using PDAR supplied by Applied BioSystems. Threshold cycle (C_T) values for each gene were normalized to ubiquitin. The negative control for each experiment, stimulation with medium alone, was assigned a value of 1 and all data expressed as fold induction over the negative control.

4.8 DNA uptake assay

Human PBMC were depleted of monocytes by positive section with CD14-MACS beads [30]. The resultant population was cultured at 2×10^6/ml for 2 h with FAM-labeled ODN: 5 μg/ml C274 and 1040 and 1.67 μg/ml TCGTCGA and AGATGAT (to keep molar equivalence) ± cPLGA. Cells were harvested and washed three times with ice-cold 1% BSA-PBS to remove surface-associated ODN, then stained with BCDA-4-PE [30] and analyzed via FACScan. Propidium iodide staining was used to gate on living cells. Data are reported as % FAM-positive (ISS^+) cells within the BCDA-4^+ population and the MFI is also derived from that group. Liposome/ODN complexes were formulated with GenePORTER 2 Transfection Reagent (Gene Therapy Systems) according to the manufacturer's protocol.

Acknowledgements: We are grateful to F. Barrat, E. Hessel, and H. Kanzler for helpful discussion and to Charlene Lee and Sherry Kelly (of Advanced Bioscience Resources, Alameda, CA) for phlebotomy services.

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