CpG Oligonucleotides with Modified Termini and Nicked Dumbbell Structure Show Enhanced Immunostimulatory Activity

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Received May 19, 2003

A series of 21 phosphodiester oligodeoxyribonucleotides containing the core sequence 5′-GACGTT-3′ or related control sequences were prepared and tested for their immunostimulatory effect on murine macrophages. The range of structural modifications tested included substituents at 3′- or 5′-termini, N3-methylation of thymidine residues, and hexaethylene glycol linkers favoring nicked or cyclic dumbbell duplexes. Lipophilic and cationic substituents at the termini failed to increase the release of TNF-α and nitric oxide, but two new types of modification were found that enhance the stimulation of RAW264.7 macrophages. One is the substitution of the 5′-terminal hydroxyl group with an amino group, and the other is the introduction of linkers favoring nicked duplexes. Even for sequences without linkers, UV-melting analysis and two-dimensional NMR showed that the core sequence 5′-GACGTT-3′ readily forms a duplex. The cyclic derivative of the most active nicked dumbbell sequence is inactive, however. Together these results suggest a recognition of both the 5′-terminus and the core of the CpG oligonucleotides by the putative receptor(s) and provide an entry into a class of modified oligonucleotides whose activity rivals that of phosphorothioates, but consists of synthetic compounds that are single stereoisomers.

Introduction

Oligodeoxyribonucleotides containing unmethylated CG dinucleotides have been found to elicit an immune response in vertebrae via stimulation of the innate immune system and B-cell activation.[1] They are commonly referred to as 'CpG oligonucleotides'. The ability of naked DNA to induce an immune response, first discovered in the context of work on tumor resistance in mice, induced by *Mycobacterium bovis* BCG,[2] has been demonstrated for DNA from a range of bacterial sources,[3] and synthetic DNA.[4] Fortunately, the immunological effects induced by oligonucleotides with unmethylated CpG motifs are also observed for human cells,[5] though recent work has shown that slightly different sequences are optimal for the stimulation of human and murine cells.[6] In both species, the recognition of microbial pathogens by the innate immune system is mediated by Toll-like receptors (TLRs). Unlike other TLR ligands, CpG DNA has to be taken up into endosomes prior to activation of receptors.[7] Toll-like receptor 9 (TLR9) was established as the key receptor for the recognition of oligonucleotides with CpG-motifs.[8] However, the details of the recognition process are not yet known.

The immune response induced by CpG oligonucleotides can help to protect hosts from pathogenic infections.[9,10] Protective and curative T_h1 responses against otherwise lethal murine Leishmaniasis have been reported.[11] The ability to bias immune responses toward a preferential T_h1 response may allow the treatment of T_h2-dominated allergic disorders in humans.[12] Further, the immunostimulatory activity of CpG-containing sequences may be used to develop oligonucleotides for the treatment of cancer[13] and asthma.[14] Perhaps most importantly, oligonucleotides with unmethylated CpG motifs may be used as adjuvants in vaccination,[15] where they provide strong costimulatory signals and favor the complete activation of T cells. Antigens used in vaccination with CpG oligonucleotides as adjuvant include, but are by far not limited to, hepatitis B surface antigen,[16,17] human immunodeficiency virus,[18] and trypanosoma.[19] The conjugation of oligonucleotides to allergens has also been reported.[20]

The extent to which structure−activity relationships have been explored for CpG oligonucleotides is limited. While sequence space has been explored extensively, leading to the identification of the 5′-PuPuCGPyPy-3′ motif, where Pu stands for purine nucleotides and Py denotes pyrimidine nucleotides, as the best studied immunostimulatory sequence in mice,[1] and the PuTCGPyPy motif for stimulation of immune cells of primates,[21] the extent to which the 'structure space' of chemical modifications has been explored is small. Much of the recent immunostimulatory work with synthetic oligonucleotides has been performed with phosphorothioate DNA, where the replacement of one nonbridging oxygen of the phosphodiester linkages with sulfur ensures greater stability toward nucleases, increased cellular uptake, and increased activity. Unfortunately, phosphorothioate oligonucleotides are complex mixtures of diastereomers and can induce a number of side effects, including splenomegaly, thrombocytopenia, complement activation, and elevation of liver enzymes.[22,23] Detailed, elegant work by Agrawal and

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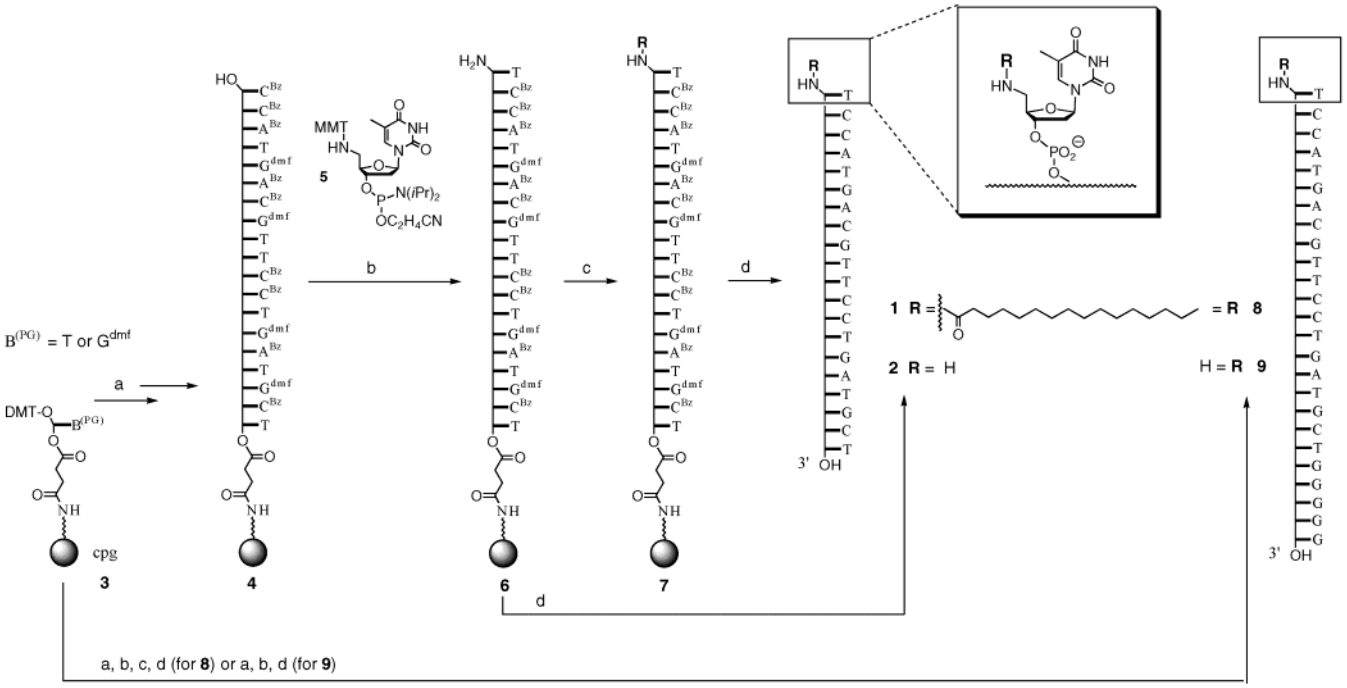
10.1021/jm030902l CCC: $25.00
Published on Web 10/14/2003

Scheme 1[a]



[a] (a) DNA synthesis via phosphoramidite protocol; (b) extension cycle with **5**; (c) $C_{15}H_{31}CO_2H$, HBTU, HOBT, DIEA; (d) NH_4OH.

co-workers has involved a range of chemical modifications, including modifications to the backbone,[24] the deoxyribose rings, the nucleobases,[25] as well as the use of abasic nucleosides,[26] and labeling[27] or linking[28] of termini. From this work it has emerged, among other things, that 5′-modifications have deleterious effects, 3′-modifications are tolerated, and that 2′-*O*-methylribonucleotides in a flanking region to the central CpG motif may increase the immunostimulatory activity of oligonucleotides.[29] Others have shown that a deazaguanosine residue in a region adjacent to the CpG motif of an inhibitory sequence does not affect activity.[30]

We have an interest in the molecular recognition of oligonucleotides, particularly recognition at the termini. We were therefore interested in studying the effect of chemical modifications at the termini on the fate and activity of sequences with the potential to elicit an immunostimulatory effect. Here we present two modifications to oligodeoxynucleotides with stereoregular phosphodiester backbones whose modification at the 5′-terminus or in the interior of the sequence enhances their immunostimulatory activity. A transition between single- and double-stranded form seems possible for the active oligonucleotides tested.

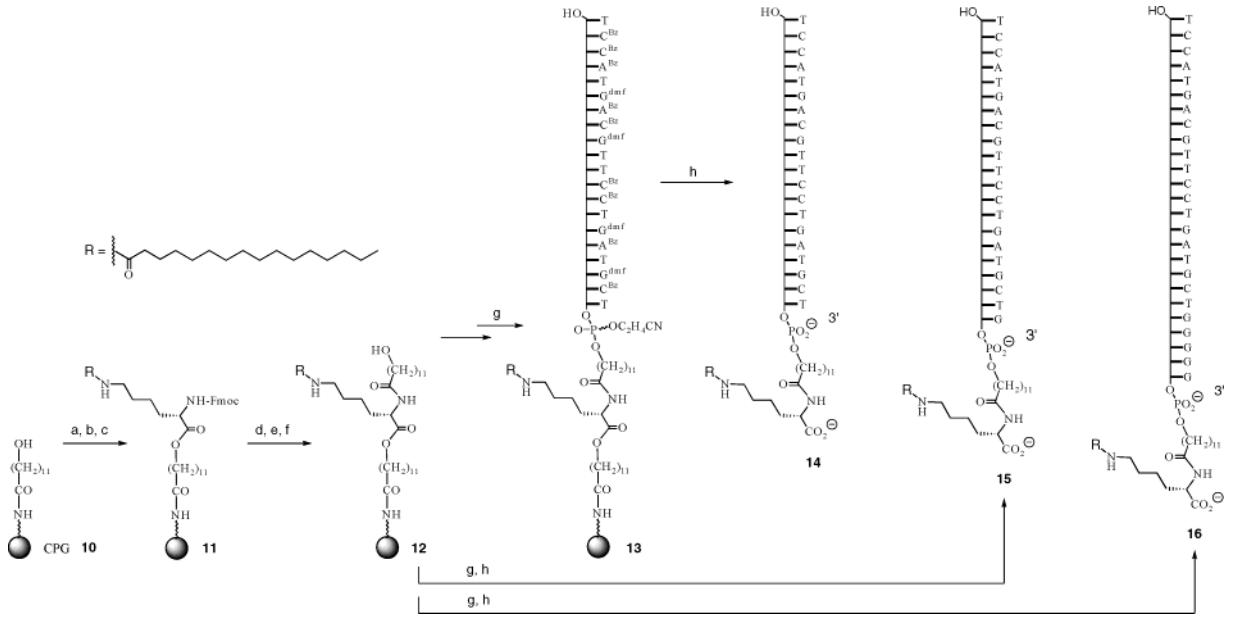
Results

The initial working model for the current work was that the core hexamer of the oligonucleotides binds to the receptor responsible for the immunostimulatory response and that the remainder of the oligonucleotide may be modified to optimize uptake and activity. The termini were considered prime sites for chemical modification since they do not block recognition of the central motif and modifying them was expected to prevent exonuclease attack. Exonucleases, i.e., not endonucleaseses, are considered the most important enzymatic activity degrading oligonucleotides in blood.[31] However, detailed in vivo work on 3′,5′-blocked oligonucleotides led to the conclusion that "blocked phosphodiester oligonucleotides are rapidly attacked by endonucleases present in mice".[32] Lipophilic moieties appended to oligonucleotides have been reported to enhance association with membranes.[33] Association with membranes may be favorable for immunostimulation by CpG oligonucleotides, since it increases the local concentration for recognition by Toll-like receptors (TLRs), which are transmembrane proteins.[34] Palmitoylation is known to favor membrane association of proteins.[35]

The first compound prepared was DNA icosamer **1** with a 5′-palmitoyl residue, linked to the nucleic acid via an amide bond (Scheme 1). This oligonucleotide contains the 5′-GACGTT-3′ motif, identified as one of the most immunostimulatory hexamers in mice, which is also statistically underrepresented in human DNA.[1,36,29] It is embedded in a sequence context optimized for immunostimulatory potency in mice.[1] In addition, derivative **2**, terminating in a free 5′-amino group, was prepared as a compound whose nonlipophilic modification was less likely to encourage association of the oligonucleotide with biological membranes. Both compounds were expected to be stable toward nuclease degradation from the 5′-terminus.

The synthesis of **1** and **2** started from commercial controlled pore glass (cpg) loaded with the 3′-terminal nucleoside residue (**3**) and involved DNA synthesis via the phosphoramidite protocol.[37,38] The oligodeoxyribonucleotides (ODNs) are presented in Scheme 1. Intermediate **4** was extended using the 3′-phosphoramidite of 5′-amino-5′-deoxythymidine (**5**)[39−41] to give **6**, followed either by deprotection (to give **2**) or the on-support acylation producing **7**, and deprotection to yield **1**. In addition, derivatives **8** and **9** were prepared using the same synthetic approach. These are identical to **1** and **2**, except for the d(G)5 tails at their 3′-termini, a

Scheme 2[a]



[a] (a) Fmoc-Lys(Boc)-OH, HBTU, HOBT, DIEA; (b) TFA; (c) palmitic acid, HBTU, HOBT, DIEA; (d) piperidine; (e) Tr-O$(CH_2)_{11}CO_2H$, HBTU, HOBT, DIEA; (f) TFA; (g) DNA synthesis cycles; (h) NH_4OH.

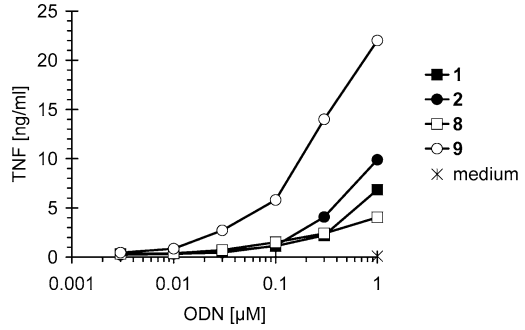


Figure 1. Dose−response curves for immunostimulation of cultured RAW264.7 macrophages with ODNs **1, 2, 8**, and **9** at the concentrations indicated. TNF-α was determined in the supernatant after 24 h, as detailed in the Experimental Section.

modification shown earlier to enhance immunostimulatory activity for all-phosphodiester oligonucleotides.[42−44] The deprotected ODNs were HPLC purified, relyophilized from water to remove excess triethylammonium acetate buffer, quantified via UV spectrophotometry, and characterized by MALDI-TOF mass spectrometry (Supporting Information). The longer and more polar sequences showed less signal intensity and increased fragmentation in MALDI-TOF mass spectra.

In an exploratory experiment, the 5′-modified oligonucleotides stimulated cytokine production in RAW264.7 macrophages. All four gave significant tumor necrosis factor alpha (TNF-α) release at submicromolar concentrations (Figure 1). Surprisingly, 5′-amino-5′-deoxy derivative **9** gave the highest activity in ELISA assays measuring TNF-α release, with half of the maximum concentration of TNF-α obtained at approximately 200 nM oligonucleotide. Palmitic acid-bearing **8** was less active by about 1 order of magnitude, and sequences **1** and **2**, lacking the d(G)5 run at the 3′-terminus, were less active still. Among the latter two compounds, the 5′-amino-5′-deoxyoligonucleotide **2** again showed a slightly elevated immunostimulatory capacity.

To reveal the extent to which 5′-amino-terminated **9** exceeded other oligonucleotides in the ability to induce release of cytokines, the study was extended to 3′-modified oligonucleotides and control sequences devoid of modifications at the termini. Antisense oligonucleotides with a lipophilic tail at the 3′-terminus have shown high activity.[45] The 3′-palmitoylated sequences were prepared as detailed in Scheme 2. Support **10**[46] was acylated using Fmoc-Lys(Boc)-OH, followed by release of the ϵ-amino group and coupling to palmitic acid. Aminoacylated support **11** thus prepared was Fmoc deprotected, coupled to ω-trityloxylauric acid,[46] and detritylated to give **12**. Standard DNA synthesis generated protected oligonucleotide **13**, which in turn gave 3′-palmitoylated **14**. Analogous syntheses gave oligonucleotides **15** and **16** with one and five additional deoxyguanosine residues at the 3′-terminus. Control compounds of unmodified DNA **17** and **18** (Figure 2) were prepared using standard phosphoramidite syntheses. The synthesis of phosphorothioate **19** involved oxidation of the phosphite intermediate with the Beaucage reagent.[47]

Dose−response curves for the stimulation of cytokine release from macrophages are shown in Figure 3. Compared to the unmodified control phosphodiesters **17** and **18**, both **2** and **9** were more active in inducing TNF-α and NO release, as measured after 24 and 48 h incubation time, respectively (Table 1 and Figure S1, Supporting Information). In terms of the concentration required for inducing half of the maximum TNF-α release, phosphorothioate **19** was active at a lower concentration. In terms of the maxima of induced effector concentration, amino-terminal compounds **2** and **9** were slightly more active than the phosphorothioate, however. In terms of both criteria, lipophilic 3′-tails (compounds **14**−**16**) failed to elicit a stronger response, both for TNF-α and nitric oxide.

Given that 5′-amino-terminal **2** and **9** showed increased activity, two derivatives of **9** were prepared that

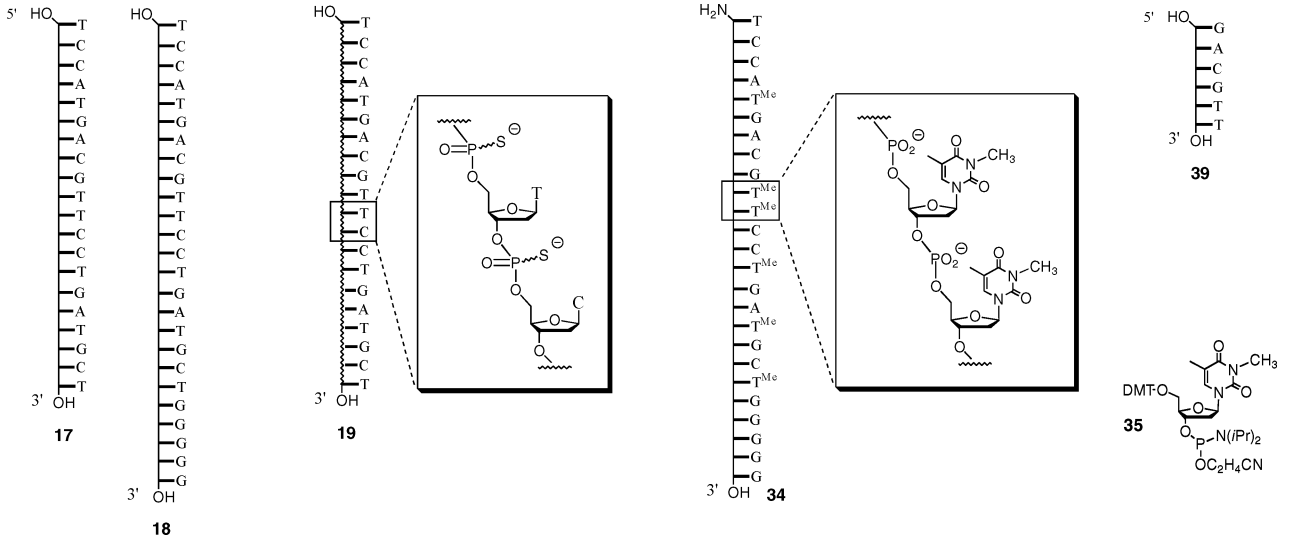


Figure 2. Schematic structural drawings of control oligonucleotides. The expansion insets highlight structural modifications introduced to **19** (phosphorothioate) and **34** (*N3*-methylated thymidine residues).

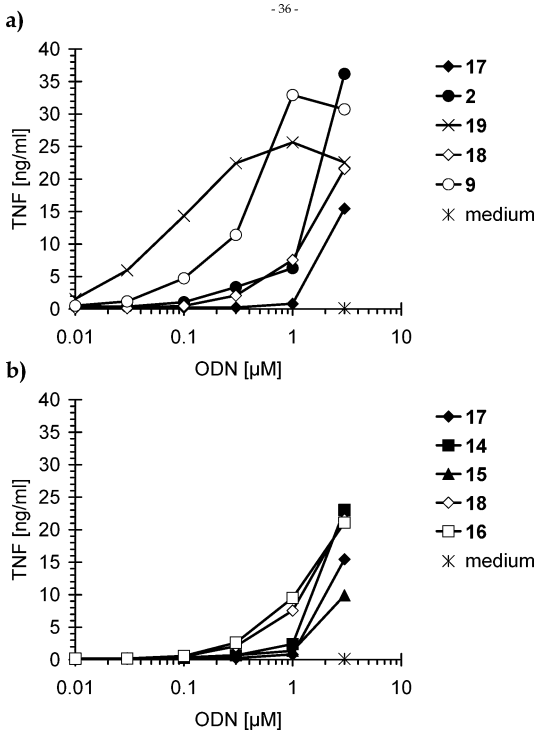


Figure 3. Dose−response curves for TNF-α release from cultured RAW264.7 macrophages after stimulation with ODNs **2**, **9**, **17**, **18**, and **19** (a), or **14**, **15**, **16**, **17**, and **18** (b).

Table 1. Release of TNF-α and NO (as measured via nitrite) from RAW264.7 Macrophages 24 h after Stimulation with the Indicated Concentration of ODNs[a]

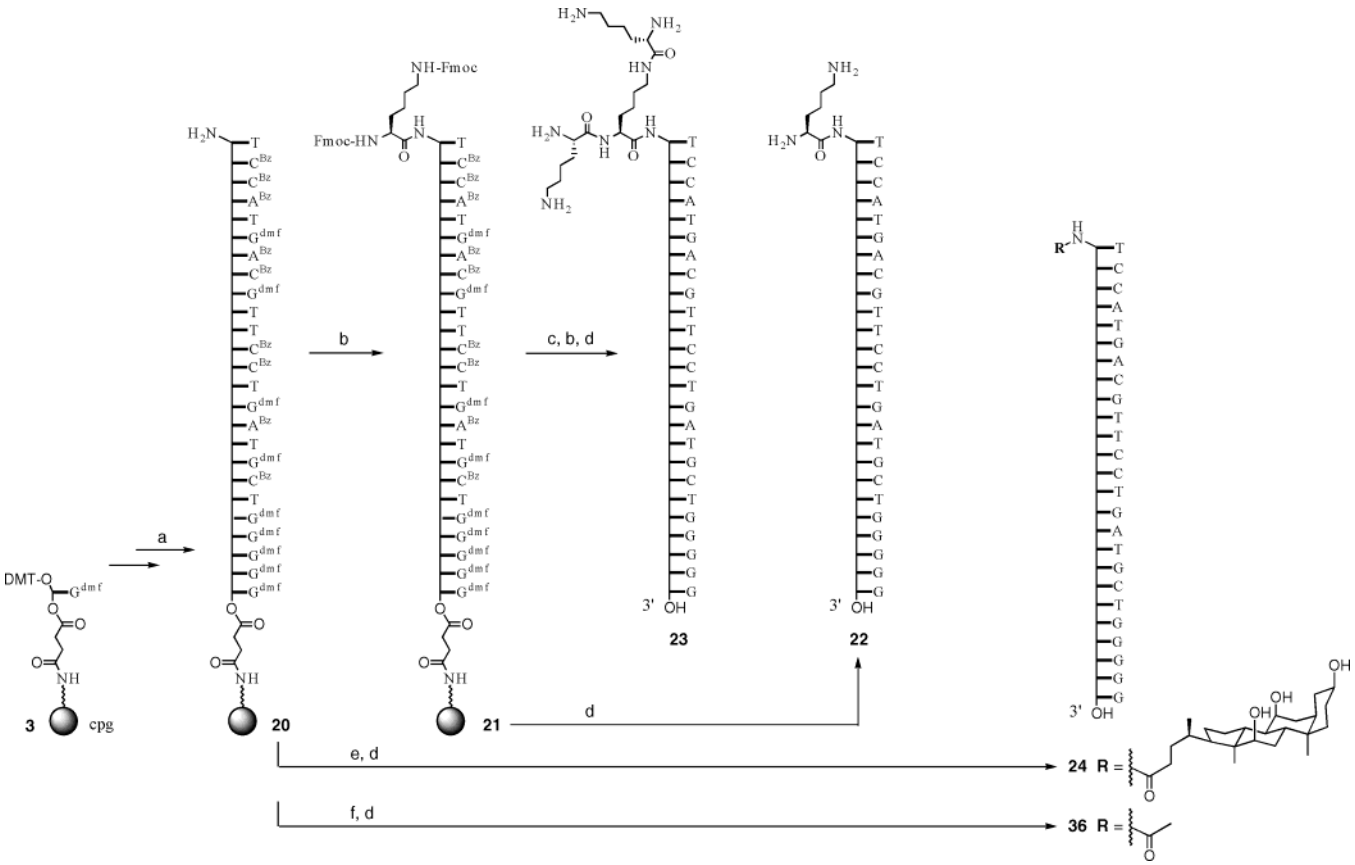
	TNF-α [% of **19**]		nitrite [% of **19**]		
ODN no.	1000 nM	100 nM	1000 nM	100nM	*n*
1	23.6 ± 9.9	4.4 ± 3.5	24.2 ± 3.9	13.1 ± 8.0	4
2	43.8 ± 15.1	5.5 ± 2.8	62.8 ± 16.4	16.4 ± 11.7	4
8	11.2 ± 3.4	5.9 ± 4.1	18.8 ± 0.9	14.4 ± 3.6	3
9	119.4 ± 7.9	48.5 ± 24.5	116.0 ± 8.1	70.6 ± 29.6	4
14	17.9 ± 9.4	1.7 ± 0.3	29.9 ± 10.7	15.4 ± 3.8	3
15	17.1 ± 16.2	3.2 ± 1.2	22.6 ± 10.2	16.0 ± 11.9	3
16	63.1 ± 20.5	11.3 ± 6.4	55.0 ± 24.7	6.2 ± 4.7	3
17	14.5 ± 6.9	1.9 ± 1.7	34.7 ± 25.3	10.3 ± 5.8	7
18	46.1 ± 10.4	21.2 ± 13.6	82.8 ± 15.7	40.7 ± 26.4	8
19	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	10
22	69.0 ± 13.2	7.1 ± 1.7	38.1 ± 24.7	15.2 ± 14.8	3
23	24.2 ± 3.4	2.6 ± 1.6	17.2 ± 14.2	4.0 ± 4.0	3
24	19.1 ± 6.5	2.8 ± 2.6	35.2 ± 31.7	7.0 ± 3.0	3
25	203.2 ± 65.8	82.1 ± 32.7	148.9 ± 19.7	79.6 ± 19.6	5
26	10.2 ± 8.3	6.4 ± 3.9	24.4 ± 6.0	17.3 ± 0.9	3
27	10.6 ± 11.3	4.6 ± 5.1	17.7 ± 10.5	9.8 ± 8.8	3
28	7.3 ± 4.4	2.9 ± 1.0	9.5 ± 5.4	12.7 ± 11.3	3
34	29.3 ± 14.1	7.5 ± 4.8	67.7 ± 5.4	14.3 ± 5.0	4
36	43.8 ± 18.5	11.6 ± 7.2	83.2 ± 6.1	29.3 ± 2.9	4
37	161.9 ± 126.0	31.6 ± 25.9	176.6 ± 106.4	27.0 ± 11.4	2
38	9.6 ± 7.7	6.3 ± 4.8	20.0 ± 7.5	14.8 ± 3.6	3

[a] The concentrations of TNF-α and nitrite detected in the supernatant were normalized to the percentage of the concentration of each effector detected after treatment with reference phosphorothioate **19** in the same assay. Data are given as mean ± one standard deviation; "*n*" indicates the number of experiments performed for a given ODN.

display additional amino groups at the 5′-terminus (Scheme 3). Starting from loaded support **3**, amine **20** was prepared via automatic DNA synthesis. Acylation with doubly Fmoc-protected lysine produced **21**. This protected 5′-lysyl-oligomer was either deprotected with aqueous ammonia to give aminoacylated **22** or Fmoc-deprotected on-support, extended by one lysyl unit at both the α- and ϵ-amino groups, and deprotected to give trilysyl-oligomer **23**. The lysyl compounds were expected to have increased immunostimulatory activity, if an unspecific interaction between cationic groups and the membranes and/or receptors was responsible for the enhancement seen for **2** over **17** and **9** over **18**. Oligolysines have also been reported to have a number of unusual effects, including enhanced uptake of proteins into cells.[48−50] Small lysine-based dendrimers displaying cationic moieties, appended to the 5′-terminus of an oligonucleotide, can also enhance the resistance of oligonucleotides to nuclease attack by favoring folding or association.[51]

In the present study, **22** and **23** proved less potent in eliciting a cytokine response from the RAW 264.7 macrophages than either **2** or **9**. At 1 μM strand concentration, both had small immunostimulatory effects, with TNF-α and NO being released at low levels (Figure S2 and Table 1). The same was true for interleukin 12 (IL12p40), another marker of proinflammatory activity,[52] whose release was also measured for these compounds (Figure S2). In these tests, trilysyl compound **23** fared more poorly than **22** in terms of activity. This suggested that an unspecific "cation effect" was not

Scheme 3[a]



[a] (a) DNA synthesis, last extension cycle with **5**; (b) Fmoc-Lys(Fmoc)-OH, HBTU, HOBT, DIEA; (c) piperidine; (d) NH_4OH; (e) cholic acid, HBTU, HOBT, DIEA; (f) Ac_2O, 2,6-lutidine, 1-methylimidazole.

causing the observed activity increase for **9**. In fact, the modification of the 5′-terminus seemed to interfere with activity, even though it is relatively far from the presumed primary recognition motif 5′-GACGTT-3′.

Parallel to the biological testing described above, experiments had been performed that evaluated the ability of oligonucleotides to form duplexes. All sequences tested contain the hexamer motif GACGTT that can form a duplex with two wobble base pairs[53] at the termini. Figure 4 shows UV-melting curves for the immunostimulatory sequences **9** and **2**, together with a control octamer duplex frequently employed in these laboratories,[54] and oligodeoxyribonucleotide $d(T)_{17}$ as single stranded control. Both **9** and **2** show the melting curve for a duplex, with the lower hyperchromicity typical of a sequence that does not engage all nucleobases in base pairing and the broader transition (lower cooperativity) of duplexes containing wobble base pairs. Therefore, we turned to testing oligonucleotides with modifications that favor duplex formation.

First, oligonucleotide **24** with a cholic acid residue at the 5′-terminus was prepared (Scheme 3). The bile acid residue is known to stabilize duplexes with target strands without disrupting the terminal T:A base pair.[55] This compound could be expected to be active in immunostimulation, if duplex formation of the 5′-terminal thymidine residue with a putative binding partner was important for biological activity. In contrast, compounds **25**−**28**, whose synthesis is shown in Scheme 4 can engage in *intra*molecular duplex formation. Their backbones contain hexaethylene glycol linkers that bridge

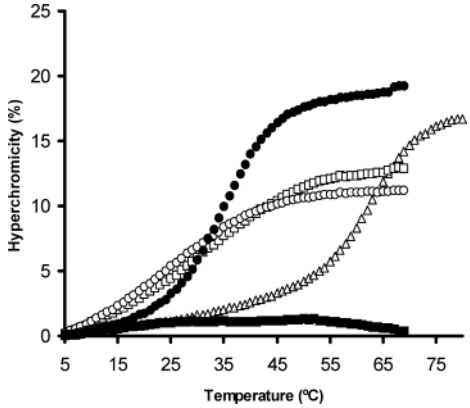
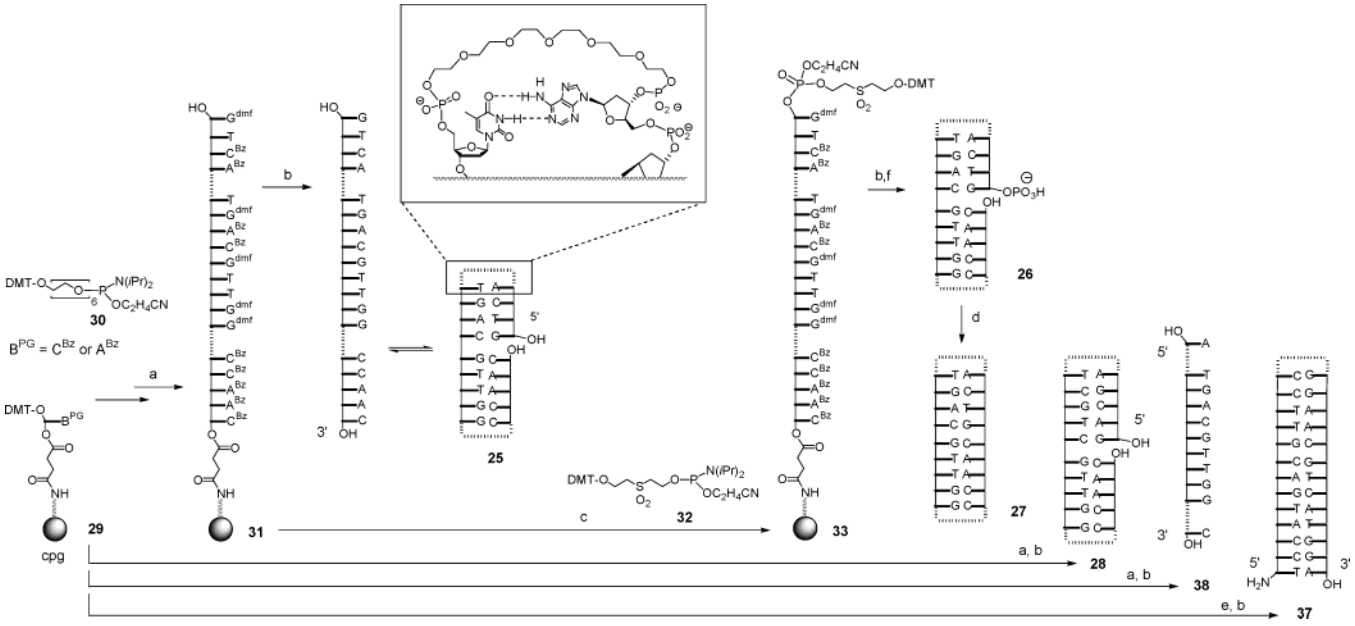


Figure 4. UV melting curves of oligonucleotide solutions. Filled circles, duplex of fully complementary duplex 5′-TGGTTGAC-3′:5′-GTCAACCA-3′ (duplex control); filled squares, $d(T)_{17}$ (single stranded control); remaining curves are immunostimulatory oligonucleotides: open squares, 25mer **9**; open circles, 20mer **2**; and open triangles, nicked cyclic duplex **25**. UV absorbance was measured at 260 nm from solution containing 150 mM NaCl, 10 mM phosphate buffer (pH 7), and the following strand concentrations: 1.5 μM each for 5′-TGGTTGAC-3′:5′-GTCAACCA-3′ and 3 μM for the remaining oligonucleotides.

the termini of intramolecular duplexes covalently. Such linkers are known to stabilize duplexes without disturbing their structure.[56] Thus, they should enable the flanking regions, which are complementary to the core, to fold back onto the core sequence, forming nicked duplexes **25**, **26**, and **28** or, after ligating the ends of the nicked duplex, a cyclic dumbbell duplex (**27**).

Scheme 4[a]



[a] (a) DNA synthesis, including extension cycles with **30**; (b) NH_4OH; (c) extension cycle with **32**; (d) EDC; (e) DNA synthesis, including extension cycles with **30** and **5**; (f) CCl_3CO_2H.

The synthesis of **25**−**28** started from commercial support **29** and proceeded via automatic DNA syntheses including phosphoramidite **30** to give compound **31**, whose deprotection gave **25** (Scheme 4). Phosphitylation of **31** with commercial **32** produced **33**, which was deprotected to give **26**. Cyclization of this 5′-phosphate to cyclic **27** was highest yielding when induced chemically with EDC. Other strategies for the synthesis of small circular oligonucleotides, namely chemical ligation of the nicked oligo with BrCN/imidazole/Ni^{2+} [57] and enzymatic ligation with T4 DNA ligase[58] were unsuccessful in our hands. The poor substrate qualities of **26** for the ligase may be due to the small size of the duplex or the poly(ethylene glycol) linkers. A procedure analogous to a literature method[59] produced **27** in 49% yield with EDC. Formation of the cyclized product was confirmed by an 18.3 °C increase in the UV melting temperature, compared to that of **25**, a shorter retention time in RP-HPLC and the MALDI-TOF mass spectrum (Figure S21, Supporting Information). Compound **28**, prepared along the same lines as **25**, was included in the study as a control compound that also forms an intramolecular nicked duplex, but lacks the core sequence 5′-GACGTT-3′. The UV melting curve of **25** (Figure 4) confirmed the duplex-stabilizing effect of the hexaethylene glycol linkages. The UV melting point at 1.4 μM strand concentration and PBS as buffer is 62 °C, i.e., well above body/assay temperature. As a result, this nicked cyclic duplex should be almost exclusively in duplex form during the assay (though some fraying of the duplex will certainly occur at the nick site). Also, as expected for an intramolecular complex, the melting point is independent of the strand concentration, whereas **9** shows the increase of the melting point with concentration expected for *inter*molecular complexes (Table S1, Supporting Information).

The testing for immunostimulatory activity of **24**−**28** gave unexpected results (Figures 5 and 6, Table 1). While **25**, with its capability of forming a nicked duplex

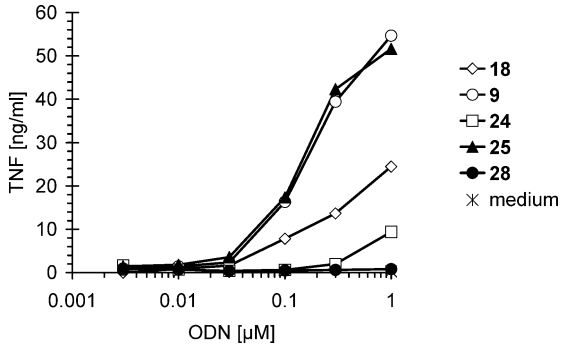


Figure 5. Dose−response curves for immunostimulation of RAW264.7 macrophages with ODNs **9**, **18**, **24**, **25**, and **28**, as measured by TNF-α release.

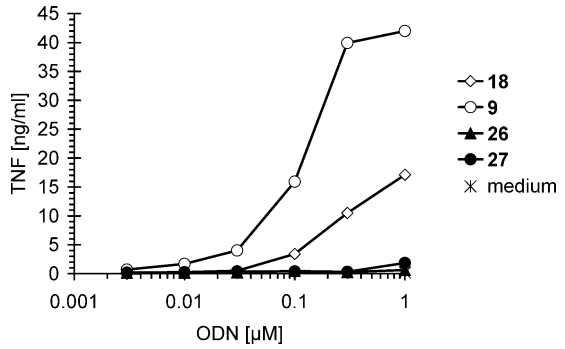


Figure 6. Release of TNF-α from murine RAW264.7 macrophages as determined 24 h after stimulation with ODNs **9**, **18**, **26**, and **27**.

was highly active, cyclic **27** was almost devoid of immunostimulatory potential, and the phosphorylated version of the nicked duplex (**26**) likewise had much reduced activity. Control sequence **28**, lacking the core sequence motif, was also inactive. This suggested that duplex formation may be advantageous during some stage of the uptake/signaling process, but that a fixed duplex structure was not advantageous, nor was a phosphorylation at the terminus tolerated. Compound

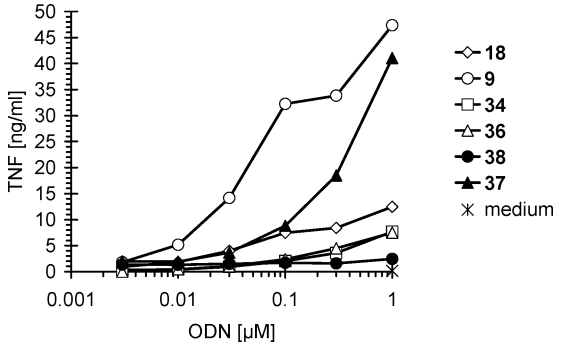


Figure 7. Dose−response curves for the release of TNF-α from RAW264.7 macrophages stimulated with ODNs **9**, **18**, **34**, **36**, **37**, and **38**.

24 with its cholic acid residue also did not improve the immunostimulatory potential of the oligonucleotide.

To better evaluate whether the critical recognition event(s) required a duplex or not (nicked duplexes, after all, are able to unfold into single strands), compound **34** (Figure 2) was prepared, which contains N3-methylated thymidine residues and is thus unable to form Watson−Crick duplexes (or bind to receptors that recognize the Watson−Crick face of this nucleobase). The synthesis of **34** involved the phosphoramidite building block of *N3*-methylthymidine (**35**),[60] prepared via methylation of thymidine with methyl mesylate in the presence of potassium carbonate, followed by DMT-protection, phosphitylation under standard conditions, and coupling to the growing chain using unmodified DNA synthesis cycles. Further, **36** (Scheme 3) was prepared as an additional control compound with a minimal modification of the 5′-terminal amino group of **9** through acetylation of the amino-terminal oligonucleotide **20** on solid support. Just like **9**, compound **36** should be resistant to nuclease attack from the 5′-terminus, but it lacks the sterically more demanding acyl side chains of other 5′-modified compounds tested. Finally, oligonucleotides **37** and **38** were synthesized on solid support (Scheme 4). The former combines the hexaethylene glycol clamp of **25** with the 5′-amino substituent in **2** and **9**, whereas the latter contains two hexaethylene glycol linkers of **25**, but lacks the full length flaps that allow for the formation of a nicked intramolecular duplex. Compound **38** is also a possible degradation product of **9** that could be generated through exonuclease attack on the unmodified termini and could therefore be expected to be active, if degradation to a non-duplex-forming strand was required for recognition by the receptor(s).

Both **34** and **36** showed much reduced activity compared to **9**, though no 'obliteration' of activity was seen (Figure 7). Compound **37** with its 5′-amino terminus was slightly less active than **9** and did not seem to benefit from a synergistic activity enhancement hoped for when introducing the duplex-favoring hexaethylene glycol cap. Still, the activity data for 26mer **37**, though currently noisy, do demonstrate that the ability to form a stable intramolecular duplex does not prevent immunostimulatory activity, as long as a free 5′-terminus is presented. This is particularly true since **37** lacks the 3′-terminal d(G)5 tail of **9** and could therefore be expected to be less active than the 25mer lacking the duplex-favoring structure.

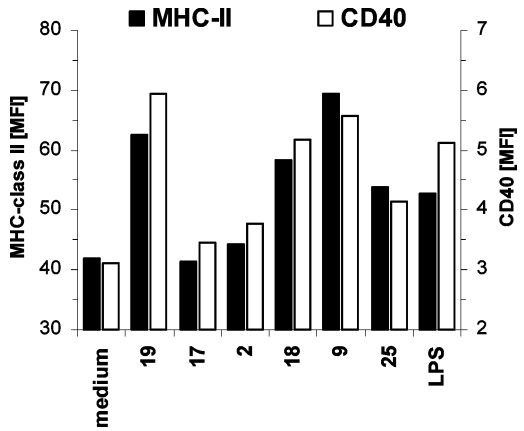


Figure 8. Results of the stimulation of bone-marrow-derived dendritic cells with either 100 nM ODN (entries for compounds **2**, **9**, **17**−**19**, and **25**), or 1 μg/mL LPS. CD11c positive cells were analyzed for MHC-class II or CD 40 expression by flow cytometry after staining with fluorophore-labeled antibodies. Mean fluorescence intensity (MFI) is shown for one representative experiment out of three experiments.

The relative ability of ODNs to induce nitric oxide generation, as measured via nitrite in the supernatant,[61] was similar to their relative ability to induce the release of TNF-α (Table 1). Figure S3 (Supporting Information) shows the result of a comparative study involving **9**, **25**, and control strands, where it can be discerned that **25** exceeds **9** in activity at all concentrations of the dose−response curve where activity was measured. This was also the case for IL-6 release from macrophages, another proinflammatory cytokine tested (Figure S4). At the same time, no release of IL-10, a non-proinflammatory cytokine, was detected when the macrophages were treated with up to 1 μM **2**, **9**, **25**, or **17**−**19** (data not shown). Exploratory experiments show that **9** inhibits the uptake of 5′-fluorescein-labeled phosphodiester oligonucleotides, suggesting that they are taken up via the same pathway (data not shown).

Besides the direct activation of murine macrophages, CpG oligonucleotides have also been reported to stimulate dendritic cells (DCs). Dendritic cell activation includes secretion of immunoregulatory cytokines as well as the up-regulation of antigen-presenting machinery, thus bridging innate with adaptive immunity. To assess the effect of those oligonucleotides found to be most immunostimulatory in macrophages on the maturation of DCs, we analyzed the expression of MHC-class II and CD40 by flow cytometry (Figure 8). As expected, lipopolysaccharide (LPS), which served as a control compound for DC maturation, as well as the standard phosphorothioate **19** induced up-regulation of MHC-class II and CD40. Of the modified oligonucleotides, both compounds with 5′-amino-5′-deoxythymidine residues (**2** and **9**) showed an increased activity when compared to their respective unmodified counterparts (**17** and **18**). Moreover, **9** appeared as effective as phosphorothioate **19** at the chosen concentration. These results foster hopes that the modified oligonucleotides also act on adaptive immune system indirectly and thus may become valuable in vaccination and the treatment of allergies, two applications previously proposed for all-phosphorothioate compounds.[12,43]

Given the unexpected activity of oligonucleotides with structures that allow for the formation of a duplex, the

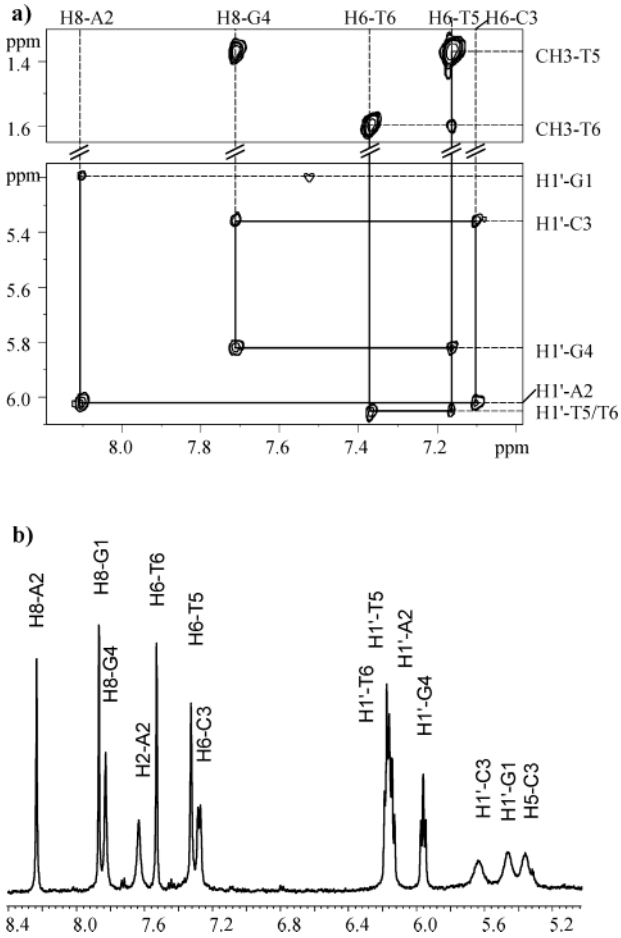


Figure 9. NMR spectra of GACGTT (**39**). (a) Expansions of a NOESY spectrum acquired at 250 ms mixing time showing cross-peaks between nucleobase and H1′ protons characteristic for duplexes. (b) Low field region of the one-dimensional ^{1}H NMR spectrum of GACGTT showing resonances of nucleobase protons and H1′-protons. Note the high field shift of H1′−G1 and the broadening of several resonances. Spectra were acquired at 283 K and 500 MHz in a solution containing 137 mM NaCl, 3 mM KCl, and 10 mM phosphate buffer at pH 7 (uncorrected for deuterium effect).

core hexamer 5′-GACGTT-3′ (**39**) was prepared separately on a 3 μmol scale, HPLC purified and subjected to NMR analysis in aqueous buffers. As expected based on the melting curve results with **9**, the NOESY spectrum of **39** shows interresidue cross-peaks indicative of duplex formation[62] (Figure 9a). Interestingly, the H1′ resonances for G1 and C3 and the H5-resonance of C3 are unusually broad (Figure 9b), suggesting that certain parts of the duplex undergo dynamic processes not observed in canonical B-form DNA duplexes (compare e.g. the ^{1}H NMR spectrum of $(TGCGCAC)_2$).[63] While G1, which is expected to form a wobble base pair with T6, could readily undergo fraying movements, the broadening of the signal for the anomeric proton of C3 is unexpected. Possibly, **39** does form a duplex, but also readily opens to expose unpaired nucleobases.

Discussion

Some structure−activity information can be gleaned from the current results. First, there is an activity enhancement achievable for phosphodiester oligonucleotides with a 5′-amino-5′-deoxy-terminus. Both **2** and **9**, though originally prepared as control compounds, are more active than their unmodified counterparts **17** and **18**. The enhancement was observed for macrophages and dendritic cells and for several indicators of immunostimulation (TNF-α, NO, IL-12p40, and IL-6). Second, introducing a structure favoring the formation of a duplex enhances activity (compare activity of **25**). All highly active compounds tested in UV-melting experiments show a clear transition, indicating their ability to form duplexes. A nick or unlinked terminus, which allows for fraying and thus the display of single-stranded DNA, is apparently critical for the activity of ODNs forming intramolecular duplexes. A static duplex, forcibly induced through cyclization (compound **27**), leads to a dramatic decrease in activity. Also, **38**, a compound expected to form from **25**, if **25** was attacked by exonucleases that degrade the vulnerable nucleotides until they encounter the oligoethylene glycol modification, was inactive. Compound **38** is unable to form an intramolecular duplex. Third, neither lipophilic palmitic acid appendages, which might have boosted activity based on an increased local concentration at membranes (compounds **1**, **8**, **14**, **15**, and **16**), nor cationic appendages (compounds **22** and **23**) enhanced the immunostimulatory activity of the ODNs. Since the appendages also introduce sterical blocks, it may be concluded that stabilization against degradation from the termini alone is not critical for activity of the oligonucleotide sequences tested.

The low immunostimulatory activity of oligonucleotides with 5′-modifications (other than the 5′-amino group) is in agreement with results of earlier work by Agrawal and colleagues.[27,64] Sterically more demanding modifications, such as the lysyl residues of **22** and **23**, seem to be particularly detrimental to activity. Smaller substituents, like the acetyl group of **36**, are more readily tolerated. This speaks in favor of a critical recognition event occurring at the 5′-terminus (vide infra). The profile of cytokines and other effectors released after immunostimulation indicate that the modified oligonucleotides still act via the same pathway as the parent compounds **17**, **18**, and **19**. As control compound **28** demonstrates, even the nicked duplex oligonucleotides are exquisitely sensitive to changes in the DNA sequence.

Next, it is interesting to consider which modifications work synergistically in enhancing activity and which do not. Thus far, we have not found a structure where intramolecular duplex formation and 5′-amino terminus combined produce a compound whose activity surpasses that of either **9** or **25**. The long, tight duplex structure of **37**, which also features a 5′-amino terminus, though active, is not more immunostimulatory than either of the modified reference compounds. Oligonucleotides with the 5′-amino modification do benefit from the known[42−44] activity enhancement for phosphodiester ODNs by the d(G)5 tail, however, so that compound **9** rivals phosphorothioate **19**, a standard both in our and other work,[65] in its activity. The UV-melting curve is largely unaffected by the $d(G)_5$ tail (compare entries for **2** and **9** in Figure 4). The deoxyguanosine appendages could, in principle, have formed G-tetrades,[66] i.e., folded DNA structures that change the shape of the oligonucleotides. The absence of an additional transition in the melting curve of **9** suggests that the activity

Table 2. UV Melting Points and Hyperchromicities for Oligonucleotide Duplexes

oligonucleotide[a]	T_m (°C)[b]	hyperchromicity (%)[b]	transition breadth (°C)[c]
9	31.4 ± 1.3	13.5 ± 1.0	23.5
2	28.2 ± 1.3	11.2 ± 1.9	17.5
17	25.8 ± 1.3	11.0 ± 1.9	20.5
18	26.8 ± 0.7	9.5 ± 1.3	18.5
19	15.6 ± 1.9	9.1 ± 0.8	9.2
25	62.8 ± 0.5	17.2 ± 1.8	15.8
27	81.1 ± 0.5	17.8 ± 1.3	13
$d(T)_{17}$	<15	----	----
TGGTTGAC GTCAACCA	36.3 ± 0.6	19.3 ± 0.7	16.5

[a] Salt concentration: 10 mM phosphate buffer, 150 mM NaCl, pH 7. [b] Average of four melting points at 3.0 μM strand concentration. [c] Half-height peak width of the first derivative of the melting curve, a measure for the cooperativity of the melting transition.

enhancement brought about by the deoxyguanosine residues relies on something other than structural changes in the DNA.

How then may these findings affect the working hypothesis for the molecular recognition of the immunostimulatory CpG oligonucleotides by the cells? One important aspect is that several lines of evidence now point toward formation of a duplex at one point of the uptake and/or recognition process. One such line of evidence is the strong activity of **25**, with its high (and concentration independent) UV melting point of 62 °C, and the discernible UV melting transitions for all active compounds studied. Another is the low activity of **34**, whose *N3*-methylation prevents duplex formation. Further, Agrawal and co-workers have shown that the core hexamer motif 5′-GACGTT-3′ is more potent than the motif 5′-AGCGTT-3′,[67] which should form a less stable duplex, since its wobble base pairs are closer to the center. Two of the four core hexamers described as the most immunostimulatory in a seminal paper on CpG oligonucleotides (GACGTC and AACGTT) are self-complementary,[1] and the remaining two (GACGTT and AACGTC) are self-complementary but for the terminal nucleotides. Also, a report on the stimulation of human peripheral blood cells by "D-type" oligonucleotides, explicitly stated that self-complementary sequences are highly active.[42] We realize that this is not true for K-type oligonucleotides or the hexanucleotides described recently,[68] and these may be interacting with other target structures. Nevertheless, there is evidence that the innate immune system of vertebrae evolved a system to detect bacterial DNA, and bacterial DNA produced via replication is double-stranded, i.e., not single stranded. In fact, some DNA sequences that activate human cells, such as GGGGGACGATCG-TCGGGGGG,[69] can form extended intermolecular duplexes, namely the decamer duplex 5′-GACGATCGTC-3′: 3′-CTGCTAGCAG-5′.

The melting of **2** and **9** is accompanied by less hyperchromicity than that of full length duplexes (Figure 4 and Table 2). This is indicative of formation of a partial duplex with single-stranded overhangs (Figure 10a). This structure can also be expected when considering the region of self-complementarity in the sequences. The hexamer duplex assumed includes two terminal G:T wobble base pair. These should have little effect on duplex stability. Melting curves for the hexamers 5′-TGCGCA-3′ and 5′-TGCGCG-3′, show virtually

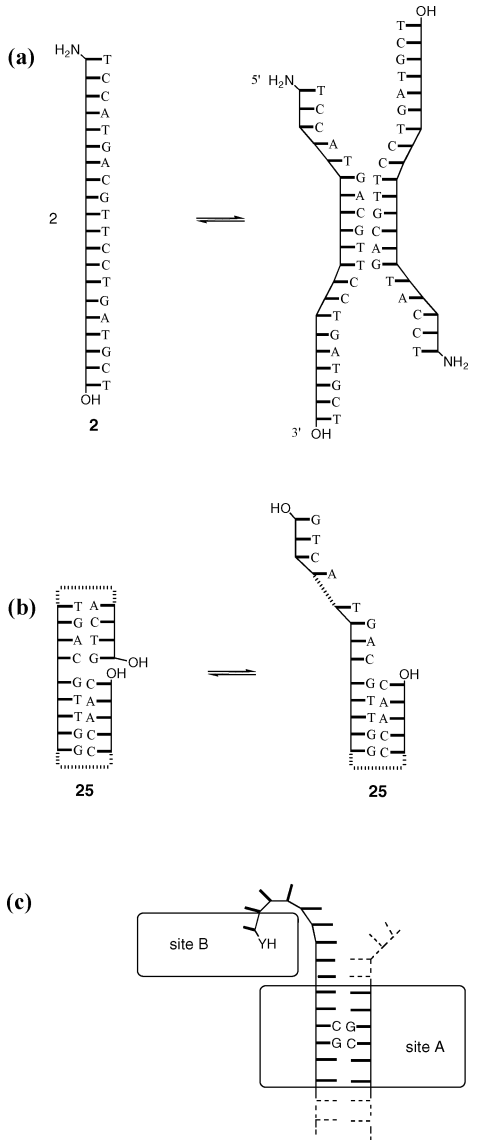


Figure 10. Cartoon of oligonucleotides **2** and **25** as they may occur in the form recognized by the putative receptor(s) (a, b), and cartoon of the presumed molecular recognition event (c).

the same melting point,[55] and wobble base pairing at the termini is well established from tRNA:mRNA duplexes leading to the degeneracy of the genetic code.[53] Since a stem-loop structure has been proposed for D-type oligonucleotides,[42] we would like to point out that the melting point elevations with increasing concentration observed for **2** and **9** (Table S1, Supporting Information) is indicative of an *inter*molecular duplex, rather than an *intra*molecular stem-loop structure, whose melting point should be concentration independent.

Direct evidence for duplex formation also comes from the NMR experiments with the core hexamer of the immunostimulatory sequences itself (Figure 9). The one-dimensional ^{1}H NMR (Figure 9b) shows broadening of some resonances, which most likely is a sign for dynamics in the duplex structure. These dynamics may make this duplex different from canonical B-form DNA and thus easier to recognize selectively. Perhaps more importantly, dynamic changes in the form of fraying lead to a conformation of **25** that displays both single- and double-stranded DNA (Figure 10b). This fraying is more likely to occur at the duplex portion involving the

5′-terminus than the one involving the 3′-terminus, since this segment contains fewer base pairs.

It is tempting to ask whether receptor(s) for uptake and/or signaling may want to recognize both a free 5′-terminus and a duplex when sensing immunostimulatory oligonucleotides, as the cartoon in Figure 10c hypothesizes (sites A and B). Recognizing two motifs may, of course, increase the selectivity of the molecular recognition and thus prevent inadvertent recognition of host DNA. The selectivity of the recognition event(s) leading to an immune response upon exposure to bacterial DNA could be difficult to guarantee, if only unmethylated CpG motifs were recognized, since these are also present (though at a lower frequency) in host DNA. Recognizing free termini simultaneously with a core motif may be beneficial, since only degradation fragments present free termini close to the core motif. They are therefore useful characteristics of the molecular pattern to be recognized, if CpG ODN-based activation involves endosomal uptake of the DNA.[7] The formation of short intermolecular duplexes, whose melting point or probability of forming is concentration dependent, is also reasonable for a signaling system: the concentration dependence of intermolecular duplex formation ensures that an 'off-signal' is generated as the endosomal degradation of the fragments continues toward strands too short for Watson−Crick duplexes. Intramolecular duplexes (compound **25**) may show higher activity than **17**, even though it lacks both an amino terminus and a $d(G)_5$-tail because it will not produce this "off" signal as readily. It may also be more than a coincidence that the most stable of all possible 16 dinucleotides in terms of duplex formation, namely the dinucleotide 5′-CG-3′ with its calculated total stacking energy of −14.59 kcal/mol,[70] is also the core sequence motif required for immunostimulatory activity.

There are other considerations that make the thought of recognition of a partial duplex interesting. Not only would the 'proofreading' that the recognition of two rather than one structural feature would provide increase fidelity, it may also have been less 'evolutionary effort' to develop a sequence-specific binding motif for a double-stranded version of the core hexamer than recognizing it in single-stranded state. A vast array of transcription factors with probe helices for the major groove of duplexes[71] can be found in eukaryotic cells.[72] Toll-like receptors are known to be able to recognize double-stranded nucleic acids, as evidenced by the recognition of double stranded RNA by TLR-3.[73] The 'code' for binding the core hexamer 5′-GACGTT-3′ could have been particularly easy to develop, since the duplex is 'labeled' with two terminal wobble base pairs.

If a free hydroxyl group at the 5′-terminus is being recognized in bacterial DNA causing immunostimulation, a hydrogen bond between the 5′-hydroxyl group as H-bond donor and an acceptor functionality in the receptor could be involved. If the receptor is, for example, a carboxylate, such as the side chain of an aspartic acid or glutamic acid residue, then the hydrogen bond from an ammonium group of a 5′-amino-5′-deoxythymidine residue would be stronger than that of the (uncharged) hydroxyl group of natural DNA, providing one possible explanation for why the 5′-amino terminal oligonucleotides show enhanced activity.

It has not escaped our attention that electron crystallography of DNA-dependent protein kinase (DNA-PK) shows a channel for the binding of double-stranded DNA plus an opening suitable for binding single-stranded DNA, and that competition experiments suggest a simultaneous binding of both species through interacting sites.[74] The catalytic subunit of DNA-PK (DNA-PKcs) has binding domains for double- and single-stranded DNA termini.[75] Duplexes with unpaired single-stranded overhangs activate DNA-PKcs most efficiently. Chu and collaborators have presented results favoring an involvement of DNA-PK in the activation of innate immunity by immunostimulatory DNA in mice.[76] Others have disputed this involvement.[65,77]

Regardless of the accuracy of the speculations on the origin of the observed effects presented in the preceding paragraphs, compounds have been found that are highly active in inducing immunostimulation. All of these compounds are stereoregular, single isomer phosphodiester that are accessible synthetically. Exploratory in vitro experiments with murine cells indicate that **9** can induce the proliferation of spleen cells, as measured by incorporation of ^{3}H thymidine, and the same compound led to the release of IL-12p40, IL-6, and TNF-α into the bloodstream of mice in exploratory in vivo experiments (data not shown). Most probably proliferation of spleen cells is due to B-cell activation, as supported by flow cytometry analysis with CD19 staining and CFSE-labeling. Together with the results presented here these findings encourage further exploration of the structure space of chemically modified oligonucleotides for immunostimulation. Some may become useful for applications, such as vaccination or the treatment of cancers and allergies.

Experimental Part

General. Anhydrous solvents were purchased over molecular sieves and were used without further modification. Reagents were the best available grade from Acros (Geel, Belgium), and Aldrich/Fluka/Sigma (Deisenhofen, Germany). The building blocks Fmoc-Lys(Fmoc)-OH, Fmoc-Lys(Boc)-OH, as well as HOBT (1-hydroxybenzotriazole) and HBTU (*O*-benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate) were from Advanced ChemTech (Louisville, KY) and were used without purification. Phosphoramidites for DNA synthesis (dA^{Bz}, dC^{Bz}, T) as well as the reagents were from Proligo (Hamburg, Germany), except for dG^{dmf} and dG^{dmf} cpg (controlled pore glass), which were from ABI (Warrington, UK). Chemical phosphorylation reagent 2-[2-(4,4′-dimethoxytrityloxy)ethylsulfonyl]ethyl-(2-cyanoethyl)-(*N*,*N*-diisopropyl)-phosphoramidite was from Glen Research (Sterling, VA). Thymidine, Beacauge reagent (3*H*-1,2-benzodithiole-3-one 1,1-dioxide) and 18-spacer phosphoramidite (18-*O*-dimethoxytritylhexaethylene glycol-1-(2-cyanoethyl)-(*N*,*N*-diisopropyl)-phosphoramidite) were from Chemgenes (Ashland, MA). Underivatized cpg (LCAA cpg, loading 77.5 μmol) was from CPG Inc. (Lincoln Park, NJ). Oligonucleotides were purified by HPLC on a 250 mm × 4.6 mm Nucleosil C4 column (Macherey-Nagel, Düren, Germany), using a gradient of CH_3CN (solvent B) in 0.1 M triethylammonium acetate, pH 7, and detection at 260 nm. Unmodified control oligonucleotides **17** and **18** and phosphorothioate **19** were purchased from MWG Biotech (Ebersberg, Germany). A sample of phosphorothioate **19** prepared and purified in-house had the same activity (within a factor of 2) in stimulating TNF-α release as the commercial sample. Lipopolysaccharide (LPS, S-form) from the wild-type strain *Salmonella enterica* sv. Minnesota, which expresses the complete core sugar and *O*-chain polysaccharide, was obtained from U. Seydel (Research Center Borstel, Germany). The LPS

preparations were lyophilized and used in the natural salt form. Yields of DNA hybrids are based on the intensity of product peak in the HPLC trace of crudes; the integration was not corrected for the absorbance caused by the solvent front. The elution program started with 0% CH_3CN for 5 min, proceeded in 40 min to the % CH_3CN given, and then proceeded to 90% CH_3CN in 5 min. Extinction coefficients of oligonucleotides were calculated through linear combination of the extinction coefficients of the nucleobases and are uncorrected for hypochromicity effects caused by duplex formation. MALDI-TOF spectra were recorded on a Bruker REFLEX IV spectrometer in negative, linear mode. The matrix mixtures for oligonucleotides were prepared from 2,4,6-trihydroxyacetophenone (THAP, 0.3 M in ethanol) and diammonium citrate (0.1 M in water) (2:1, v/v). Calculated masses are average masses; *m/z* found are those for the pseudomolecular ions ($[M - H]^-$) detected as the maximum of the unresolved isotope pattern. The accuracy of mass determination with the external calibration used is ca. 0.1%, that is, 5 Da at *m/z* 5000. DNA sequences are given 5′-to-3′ terminus; T* denotes a 5′-amino-5′-deoxythymidine residue, *T* denotes 3-methylthymidine residues.

Solid-Phase Syntheses: General Procedures. Unmodified Portion of Oligodeoxynucleotides. The following protocol was used to prepare **4** from **3** and is representative. A sample of **3** (10 mg, approximately 0.25 μmol loading according to the manufacturer) in a polypropylene reaction chamber for DNA synthesis (Prime Synthesis, Aston, PA) was extended to **4** using β-cyanoethyl phosphoramidites on a Perseptive Biosystem 8909 Expedite DNA synthesizer using the standard protocol for 1 μmol scale.

Coupling Producing Amide or Ester Linkages (General Step I). The following procedure for the coupling of palmitic acid to cpg bearing the amino-terminal DNA (precursor to compound **11**, 10 mg approximately 0.25 μmol loading) is representative. A mixture of palmitic acid (27 mg, 100 μmol), HOBT (13.5 mg, 100 μmol), and HBTU (34.1 mg, 90 μmol) was dried at 0.1 Torr, dissolved in DMF (600 μL), and treated with diisopropylethylamine (40 μL, 30.2 mg, 230 μmol), and after 10 min, the solution was injected into the column containing the cpg with the aid of two syringes. After 45 min with occasional mixing, the cpg was rinsed with DMF (2 × 2 mL) and acetonitrile (2 × 2 mL), followed by drying (0.1 Torr).

Removal of Fmoc Groups (General Step II). The following protocol for the Fmoc removal is representative. The functionalized solid support containing the Fmoc protecting group was treated with a mixture of piperidine/DMF (1:4, 1 mL) for 30 min at room temperature, followed by rinsing with acetonitrile (2 × 2 mL) and drying (0.1 Torr).

Deprotection and Release from Support (General Step III). Given below is a protocol used for the deprotection of **6** producing **2**. This protocol is representative for the final deprotection step in the preparation of all oligonucleotides. The oligonucleotide-bearing cpg (**6**, 10 mg, approximately 0.25 μmol of oligonucleotide) was transferred to a polypropylene reaction vessel and was treated with ammonium hydroxide (30% aqueous NH_3, 1 mL). The vessel was sealed, and the reaction was allowed to proceed for 16 h. Excess ammonia was then removed with a gentle air stream directed onto the surface of the solution for 30 min. The odor-free supernatant was aspired, and the support was washed with deionized water (2 × 0.5 mL). The combined aqueous solutions were filtered (4 mm syringe filter, 0.2 μm pore size, Whatman Inc, Chilton, NJ) and used directly for HPLC purification.

Acetylation (General Step IV). This protocol is for synthesis of the solid-phase-bound precursor of **36** and is representative for manual capping steps. The cpg-bound oligonucleotide (**20**, 10 mg, approximately 0.25 μmol loading) was treated with a mixture (2 mL, 1:1, v/v) of CapA solution for DNA synthesizers (acetic anhydride/2,6-lutidine/THF, 1:1:8, v/v) and CapB solution for DNA synthesizers (1-methylimidazole/THF, 84:16, v/v) for 20 min, followed by rinsing with DMF (2 × 2 mL) and acetonitrile (2 × 2 mL), and drying at 0.1 Torr.

5′-Amino-5′-deoxyoligonucleotide (6). The following protocol is for the synthesis of **6** and is representative (compounds **20** and the precursor of **37** were prepared similarly). The cpg-bound oligonucleotide (**4**, 10 mg, approximately 0.25 μmol oligonucleotide) was subjected to a DNA synthesis cycle using the phosphoramidite building block of 5′-amino-2′,5′-deoxythymidine[39−41] (**5**, 200 μL of a 0.1 M solution in CH_3CN) and reagents for a 1 μmol synthesis. The coupling time was extended to 200 s (instead of the 30 s used otherwise). The monomethoxytrityl group was removed by treating the cpg with a solution of trichloroacetic acid in dichloromethane (2 mL, 3:97, v/v) for 2 min, followed by rinsing the support with dichloromethane (2 × 2 mL) and drying (0.1 Torr). Oligonucleotide **2** was deprotected and cleaved from the solid support using General Step III.

Detritylation. Aminopropyl cpg derivatized with 12-trityloxylauric acid (precursor to compound **12**, prepared following the protocol in the preceding paragraph, 10 mg, approximately 25 μmol loading) was treated with a mixture of TFA/CH_2Cl_2 (1:1 v/v) for 2 min, followed by rinsing with acetonitrile (2 × 2 mL) and drying at 0.1 Torr.

Removal of Boc Group. The functionalized solid support containing the Boc protecting group (product of the acylation of **10** with Fmoc-Lys(Boc)-OH) was treated with a mixture of TFA/CH_2Cl_2 (1:1 v/v) for 20 min, followed by rinsing with acetonitrile (2 × 2 mL) and drying at 0.1 Torr.

Oligodeoxynucleotide with Hexa(ethylene glycol) Spacers (25). The following protocol is for the synthesis of **31** and is representative (compounds **28**, **38**, and the precursors to **26** and **37** were prepared similarly). After the synthesis of the unmodified portion, the cpg-bound oligonucleotide (40 mg, approximately 1 μmol) was coupled with 18-spacer phosphoramidite **30** with an extended coupling time of 200 s, instead of the 30 s used otherwise, followed by normal DNA synthesis to give **31**. The oligonucleotide **25** was liberated from the support using General Step III.

Phosphorothioate 19. Phosphorothioates (PS) were synthesized similar to phosphodiesters (PO) except for the oxidizing step where the Beacauge reagent[47] was used instead of standard oxidizer solution, following the recommendation of the reagent's manufacturer.

Trilysyl Oligonucleotide Hybrid (23). Fmoc-Lys(Fmoc)-OH (100 μmol, 59.1 mg) was coupled to cpg containing the amino-terminal DNA (**20**, 10 mg, approximately 0.25 μmol) using a procedure similar to General Step I. The Fmoc group was removed using General Step II. The cpg bearing the free amino groups were coupled to Fmoc-Lys(Fmoc)-OH (100 μmol, 59.1 mg) using General Step I. The coupling step was followed by deprotection (General Step III) that yielded the desired oligonucleotide **23**.

Synthesis of Oligonucleotide Containing *N3*-Methylthymidine Residues (34). The *N3*-methylthymidine phosphoramidite was synthesized under standard conditions.[78] To substitute all the thymidines of the parent sequence with *N3*-methylthymidine, this phosphoramidite was used in the thymidine channel of the synthesizer throughout the solid-phase synthesis. The cpg bearing the fully assembled strand was subjected to General Step III to yield the desired oligonucleotide.

Oligonucleotide with 3′-Modification (14). The protocol is given for the synthesis of **14** via **13** and is also representative for the preparation of **15** and **16**. The derivatized solid support was prepared using a procedure similar to one reported earlier.[46] Briefly Fmoc-Lys(Boc)-OH (100 μmol, 46.9 mg) was coupled to the lauric acid bearing support (**10**, 15 mg, approximately 0.5 μmol) using General Step I. The Boc group was removed as detailed above, and the support bearing the free ϵ-amine was coupled to palmitic acid (27 mg, 100 μmol) using General Step I. The Fmoc group was removed using General Step II and the solid support displaying the free α-amine was coupled to 12-trityloxylauric acid (45 mg, 100 μmol) using the protocol of General Step I. After the coupling, unreacted amino groups were capped using General Step IV. The solid support was detritylated, as detailed above. The solid

support thus prepared was subjected to DNA synthesis. After DNA synthesis, oligonucleotide **14** was liberated from the solid support using General Step III.

5′-Phosphorylated Oligonucleotide (26). The cpg bearing the unmodified core portion of the oligonucleotide (**31)** was extended using the phosphorylation reagent (**32**, 1 mL, 0.1 M solution in acetonitrile) on the DNA synthesizer with an extended coupling time of 200 s. The cpg bearing the extended oligonucleotide (**33**) was then subjected to General Step III to yield the title compound (**26**).

Cyclic Oligonucleotide 27. HPLC-purified phosphorylated oligonucleotide **26** (10 nmol) was taken up in MES buffer (100 μL, 50 mM, adjusted to pH 6.0 with NaOH) containing 20 mM $MgCl_2$. The reaction mixture was cooled to 0 °C and treated with 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC) (4 mg, 21 μmol). After 48 h at 4 °C, the reaction mixture was diluted to 500 μL with distilled water, filtered (0. 2 μm pore size), and purified by HPLC.

Analytical Data for Modified Oligonucleotides. 5′-T*CCATGACGTTCCTGATGCTGGGGG-3′ (**9**). Yield: 49%; HPLC: CH_3CN gradient 0% for 5 min to 20% in 45 min, t_R = 32 min, MALDI-TOF MS: calcd 7705.2, found 7704.3.

5′-T*CCATGACGTTCCTGATGCT-3′ (**2**). Yield: 47%; HPLC: CH_3CN gradient 0% for 5 min to 25% in 45 min, t_R = 32 min, MALDI-TOF MS: calcd 6059.8, found 6058.8.

5′-pal-T*CCATGACGTTCCTGATGCTGGGGG-3′ (**8**). Yield: 40%; HPLC: CH_3CN gradient 0% for 5 min to 25% in 45 min, t_R = 38min, MALDI-TOF MS: calcd 7961.1, found 7956.3.

5′-pal-T*CCATGACGTTCCTGATGCT-3′ (**1**). Yield: 15%; HPLC: CH_3CN gradient 0% for 5 min to 25% in 45 min, t_R = 36 min, MALDI-TOF MS: calcd 6315.4, found 6314.9.

5′-T*CCATGACGTTCCTGATGCTGGGGG-La-Lys(pal)-OH-3′ (**16**). Yield: 11%; HPLC: CH_3CN gradient 0% for 5 min to 25% in 45 min, t_R = 35min, MALDI-TOF MS: calcd 8347.1, found 8349.9.

5′-TCCATGACGTTCCTGATGCTG-La-Lys(pal)-OH-3′ (**15**). Yield: 4%; HPLC: CH_3CN gradient 0% for 5 min to 45% in 55 min, t_R = 47 min, MALDI-TOF MS: calcd 7028.8, found 7030.3.

5′-T*CCATGACGTTCCTGATGCT-La-Lys(pal)-OH-3′(**14**). Yield: 14%; HPLC: CH_3CN gradient 0% for 5 min to 30% in 45 min, t_R = 37 min, MALDI-TOF MS: calcd 6700.4, found 6695.8.

5′-Lys-T*CCATGACGTTCCTGATGCTGGGGG-3′ (**22**). Yield: 22%; HPLC: CH_3CN gradient 0% for 5 min to 20% in 45 min, t_R = 38min, MALDI-TOF MS: calcd 7833.1, found 7829.6.

5′-H-Lys-Lys(Lys)-T*CCATGACGTTCCTGATGCTGGGGG-3′ (**23**). Yield: 8%; HPLC: CH_3CN gradient 0% for 5 min to 20% in 45 min, t_R = 38min, MALDI-TOF MS: calcd 8085.5, found 8079.6.

5′-Chl-T*CCATGACGTTCCTGATGCTGGGGG-3′ (**24**). Yield: 14%; HPLC: CH_3CN gradient 0% for 5 min to 25% in 45 min, t_R = 36min, MALDI-TOF MS: calcd 8095.1, found 8091.7.

5′-GTCA-18spacer-TGACGTTGG-18spacer-CCAAC-3′ (**25**). Yield: 59%; HPLC: CH_3CN gradient 0% for 5 min to 25% in 45 min, t_R = 43min, MALDI-TOF MS: calcd 6188.5 found 6194.2.

5′-GACGA-18spacer-TCGTCGTTGG-18spacer-CCAAC-3′ (**28**). Yield: 28%; HPLC: CH_3CN gradient 0% for 5 min to 20% in 45 min, t_R = 34min, MALDI-TOF MS: calcd 6806.7, found 6802.1.

5′-Ac-T*CCATGACGTTCCTGATGCTGGGGG-3′ (**36**). Yield: 18%; HPLC: CH_3CN gradient 0% for 5 min to 20% in 45 min, t_R = 30min, MALDI-TOF MS: calcd 7746.1, found 7750.0.

5′-T*CCAT^{Me}GACGT^{Me}T^{Me}CCT^{Me}GAT^{Me}GCT^{Me}GGGGG-3′ (**34**). Yield: 33%; HPLC: CH_3CN gradient 0% for 5 min to 20% in 45 min, t_R = 32min, MALDI-TOF MS: calcd 7789.7, found 7792.9.

Cyclic[5′-GTCA-18spacer-TGACGTTGG-18spacer-CCAAC-3′] (**27**). Yield: 49%; HPLC: CH_3CN gradient 0% for 5 min to 20% in 45 min, t_R = 28min, MALDI-TOF MS: calcd 6253.7, found 6247.8.

5′-T*CCATGACGTTCC-18spacer-GGAACGTCATG-GA- 3′ (**37**). Yield: 24%; HPLC: CH_3CN gradient 0% for 5 min to 20% in 45 min, t_R = 36min, MALDI-TOF MS: calcd 8311.6, found 8317.0.

5′-PO_3H-GTCA-18spacer-TGACGTTGG-18spacer-CCAAC-3′ (**26**). Yield: 59%; HPLC: CH_3CN gradient 0% for 5 min to 25% in 45 min, t_R = 43min, MALDI-TOF MS: calcd 6271.7, found 6270.4.

5′-A-18spacer-TGACGTTGG-18spacer-C-3′ (**38**). Yield: 72.7%; HPLC: CH_3CN gradient 0% for 5 min to 25% in 45 min, t_R = 43min, MALDI-TOF MS: calcd 4058.9, found 4059.7.

UV Melting Experiments. UV melting experiments were performed with a Perkin-Elmer Lambda 10 spectrophotometer at 260 nm and 1 cm path length at heating or cooling rates of 1 °C/min. Solutions were 150 mM in NaCl and contained 10 mM phosphate buffer, pH 7. Prior to acquisition of the melting curves, duplexes were annealed by heating to 90 °C, followed by cooling to 5 °C at a rate of 2 °C/min. Melting temperatures were determined with the program UV Winlab 2.0 (Perkin-Elmer Inc.) and are averages of the maxima or minima of the first derivative of the 91-point smoothed curves from heating and cooling experiments. Hyperchromicities were determined by calculating the difference in adsorption between high and low-temperature baseline, dividing by the adsorption at the low-temperature baseline and expressing the result in percent of the absorption at low temperature.

Cells and Culture Conditions. RAW 264.7 cells (a kind gift from Dr. R. Schumann, Berlin, Germany) were cultured in Clicks/RPMI 1640 supplemented with 5% FCS, 50 μM β-mercaptoethanol, and antibiotics [penicillin G (100 IU/mL of medium) and streptomycin sulfate (100 IU/ml of medium)]. Bone-marrow derived dendritic cells (BMDC) from BALB/c mice were prepared as described.[79] Briefly, femurs of mice were rinsed with cell culture medium containing 10% FCS using a syringe with a 27 gauge needle. Bone marrow cells (4×10^6) were seeded into a 80 cm^2 tissue culture flask in culture medium with 40 ng/mL recombinant GM-CSF (Tebu, Frankfurt, Germany). Nonadherent cells were used at day 9 when mature DCs ($CD11c^+$, $GR\text{-}1^-$) represented >80% of the resulting cell population.

Cell Stimulation. Depending upon the experiments 1.5×10^5 cells/well (ELISA) or 0.5×10^6 cells/well (flow cytometry) were plated in 96-well or 24-well culture plates and incubated with different ODNs in the indicated concentrations. Supernatants were harvested for determination of cytokines and nitrite accumulation after 24 and 48 h. Cells were analyzed by flow cytometry after 16 h of stimulation. Each cell culture plate was stimulated with commercially available phosphorothioate **19** to obtain a reference independent of interassay variations.

Cytokine Measurement. Cytokine levels in culture supernatants were determined using commercially available ELISA kits for TNF-α, IL-6, and IL-12p40 according to the manufacturer's instructions (BD Biosciences, Heidelberg, Germany). Measurements were done in duplicate.

Determination of Nitrite Accumulation. Nitrite accumulation was measured photometrically (550 nm) by mixing equal parts of supernatant and Griess reagent (1:1 mixture of 1 g% sulfanilamide/5% H_3PO_4 and 0.1% naphthylethylenediamine dihydrochloride [NEDD]) using a standard of $NaNO_2$.

Flow Cytometry Analysis. At the end of the stimulation period cells were harvested and washed in PBS/2% FCS. Fc-receptors were blocked by incubating with anti-FcγRII/III monoclonal antibodies (clone 2.4G2, Pharmingen) and 10% normal mouse serum for 15 min on ice to avoid unspecific staining. Then cells were stained with fluorescein isothiocyanate (FITC)-conjugated anti $I\text{-}A^d/E^d$ monoclonal antibodies (clone 2G9, Pharmingen), FITC-CD40 (clone HM40-3, Pharmingen) or FITC-GR-1 (clone RB6−8C5, Pharmingen) in combination with phycoerythrin (PE)-conjugated anti-CD11c antibodies (clone HL3, Pharmingen) for 45 min on ice. After staining cells were washed and fixed in PBS/1% paraformal-

dehyde. Cells were analyzed on a Partec PAS flow cytometer (Dako, Germany). Analysis was performed by gating on FSC/SSC characteristics and CD11c positive cells. The mean fluorescence intensity (MFI) for MHC-class II and CD40 expression on the respective cells was determined.

Acknowledgment. This work was supported by the EU (grant QLK2-CT-2000-00336) and DFG (grant HE 1452/4), and the Universities of Konstanz and Karlsruhe. The authors thank Dr. Thomas Hartung for arranging a most fruitful collaboration and many helpful discussions. The authors thank Jan Rojas Stütz for help with the acquisition of MALDI-TOF mass spectra and P. Lang, A. Rapp, and J. Tuma for help with the acquisition of NMR data and their interpretation.

Supporting Information Available: Dose−response curves for selected oligonucleotides inducing NO release in macrophages, plot of the activity of **22**, **23**, **18**, and **9** in terms of TNF-α, nitrite, and IL-12p40 release, dose−response curves for the release of IL-6 for selected oligonucleotides, and MALDI-TOF mass spectra of all modified oligonucleotides. This material will be available free of charge via the Internet at http://pubs.acs.org.

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JM030902L