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Agonists of Toll-like Receptor 9 Containing Synthetic Dinucleotide Motifs

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Oligodeoxynucleotides (ODNs) containing unmethylated CpG motifs activate Toll-like receptor 9 (TLR9). Our previous studies have shown that ODNs containing two 5′-ends are more immunostimulatory than those with one 5′-end. In the present study, to understand the role of functional groups in TLR9 recognition and subsequent immune response, we substituted C or G of a CpG dinucleotide with 5-OH-dC, 5-propyne-dC, furano-dT, 1-(2′-deoxy-β-D-ribofuranosyl)-2-oxo-7-deaza-8-methyl-purine, dF, 4-thio-dU, N^3-Me-dC, N^4-Et-dC, Ψ-iso-dC, and arabinoC or 7-deaza-dG, 7-deaza-8-aza-dG, 9-deaza-dG, N^1-Me-dG, N^2-Me-dG, 6-Thio-dG, dI, 8-OMe-dG, 8-O-allyl-dG, and arabinoG in ODN containing two 5′-ends. Agonists of TLR9 containing cytosine or guanine modification showed activity in HEK293 cells expressing TLR9, mouse spleen, and human cell-based assays and in vivo in mice. The results presented here provide insight into which specific chemical modifications at C or G of the CpG motif are recognized by TLR9 and the ability to modulate immune responses substituting natural C or G in immune modulatory oligonucleotides.

Introduction

Toll-like receptor 9 (TLR9) is a member of a family of 10 highly conserved innate immune receptors called Toll-like receptors present in the human.[1] TLR9 recognizes unmethylated CpG motifs, which occur at a higher frequency in the genomes of microbial pathogens, and triggers Th1-type innate immune responses.[2,3] The resulting innate immune response limits the early spread of infection and primes the development of pathogen-specific adaptive immunity. Synthetic oligodeoxynucleotides (ODN[a]) containing unmethylated CpG motifs (CpG ODN) mimic bacterial DNA and produce immune stimulatory effects through TLR9 stimulation.[3–7] The nature of the resulting immune response depends on the structure of the CpG ODN and the nucleotide sequences flanking the CpG dinucleotide. On the basis of the nucleic acid structure and the immune stimulatory profiles produced, three different classes of CpG ODN have been described in the literature: single-stranded CpG ODN, palindromic double-stranded CpG ODN, and hyperstructure-forming, poly-dG-containing CpG ODN.[5,6,8,9] The results of preclinical and clinical studies suggest that CpG ODN may be useful as vaccine adjuvants and in the treatment of asthma, allergy, infections, and cancers.[10–12]

Our studies involving the structure–activity relationships of CpG ODN have identified structural features in the pentose sugar,[13–16] phosphate backbone,[17] nucleobases,[18,19] and nucleosides[20] required for TLR9 activation. Furthermore, an accessible 5′-end is essential for TLR9 recognition and blocking the 5′-end with conjugations significantly decreases the activity.[21–23] We have shown that CpG ODNs that are attached through a 3′–3′ linkage and contain two accessible 5′-ends, referred to as immunomers, have enhanced immune stimulatory activity.[23]

TLR9 recognizes an unmethylated CpG motif in ODN. We have previously shown that certain synthetic modifications of C or G substituted within a CpG motif are also recognized by TLR9.[24–28] These substituted motifs are referred to as synthetic immune stimulatory motifs CpR and YpG. Independent studies by others have also shown that synthetic immune stimulatory motifs are recognized by TLR9.[29] To further elucidate the role of functional groups of C and G for TLR9 activation, immune stimulation, and the development of potent synthetic immunostimulatory motifs, we designed and synthesized a series of TLR9 agonists with various substitutions. These include TLR9 agonists substituted with synthetic pyrimidines, such as 5-OH-dC, 5-propyne-dC, furano-dT, 1-(2′-deoxy-β-D-ribofuranosyl)-2-oxo-7-deaza-8-methylpurine, dF, 4-thio-dU, N^3-Me-dC, N^4-Et-dC, Ψ-iso-dC, and arabinoC, in place of C in the CpG. We also used TLR9 agonists substituted with synthetic purines, such as 7-deaza-dG, 7-deaza-8-aza-dG, 9-deaza-dG, N^1-Me-dG, N^2-Me-dG, 6-Thio-dG, dI, 8-OMe-dG, 8-O-allyl-dG, and arabinoG bases, in place of G in the CpG dinucleotide. These novel agonists of TLR9 with synthetic immune stimulatory dinucleotide motifs were studied in cultures of HEK293 cells transfected with mouse TLR9 for their ability to stimulate TLR9, in mouse spleen cell cultures, in human cell-based assays, and in vivo in mice for immune stimulatory activity.

Results

Design and Synthesis of Agonists of TLR9. Our earlier studies demonstrated that CpG ODNs with two accessible 5′-ends (**1**) were more immune stimulatory than linear CpG ODNs with one accessible 5′-end (**24**).[21–23] Agonists used in the study were designed to contain two short identical oligomers linked through their 3′-ends via a glycerol linker (Table 1) and were of the same length (22 nucleotides). Agonist **1** contained a natural CpG dinucleotide (Figure 1) in a human-specific hexameric motif, GTCGTT. In **2–12**, the G in a CpG dinucleotide was replaced with one of the modified Gs shown in Figure 2A. In **13–22**, the C in a CpG dinucleotide was replaced with one of the modified Cs shown in Figure 2B. Compound **23** was a control that had the same length and structure as other agonists but without an immune stimulatory motif. All the compounds were synthesized as described in Experimental Section and characterized by MALDI-ToF mass spectral analysis for mo-

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[a] Abbreviations: IFN, interferon; IL, interleukin; IP-10, interferon-γ induced protein-10; MIG, monokine induced by γ interferon; MIP, macrophage inflammatory protein; ODN, oligodeoxynucleotide; PBMCs, peripheral blood mononuclear cells; TLR, Toll-like receptor.

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Table 1. Sequences, Chemical Modifications, and MALDI-ToF Mass Spectral Data of Agonists of TLR9

compd	sequence[a]	molecular mass[b]	
		calcd	found
1	d(5′-TCTGT**CG**TTCT-X-TCTT**GC**TGTCT-5′)	7148	7145
2	d(5′-TCTGT**CG$_1$**TTCT-X-TCTT**G$_1$C**TGTCT-5′)	7146	7141
3	d(5′-TCTGT**CG$_2$**TTCT-X-TCTT**G$_2$C**TGTCT-5′)	7148	7146
4	d(5′-TCTGT**CG$_3$**TTCT-X-TCTT**G$_3$C**TGTCT-5′)	7146	7134
5	d(5′-TCTGT**CG$_4$**TTCT-X-TCTT**G$_4$C**TGTCT-5′)	7180	7175
6	d(5′-TCTGT**CG$_5$**TTCT-X-TCTT**G$_5$C**TGTCT-5′)	7176	7172
7	d(5′-TCTGT**CG$_6$**TTCT-X-TCTT**G$_6$C**TGTCT-5′)	7176	7168
8	d(5′-TCTGT**CG$_7$**TTCT-X-TCTT**G$_7$C**TGTCT-5′)	7208	7209
9	d(5′-TCTGT**CG$_8$**TTCT-X-TCTT**G$_8$C**TGTCT-5′)	7118	7120
10	d(5′-TCTGT**CG$_9$**TTCT-X-TCTT**G$_9$**CTGTCT-5′)	7208	7209
11	d(5′-TCTGT**CG$_{10}$**TTCT-X-TCTT**G$_{10}$C**TGTCT-5′)	7260	7261
12	d(5′-TCTGT**CG$_{11}$**TTCT-X-TCTT**G$_{11}$C**TGTCT-5′)	7180	7177
13	d(5′-TCTGT**C$_1$G**TTCT-X-TCTT**GC$_1$**TGTCT-5′)	7180	7176
14	d(5′-TCTGT**C$_2$G**TTCT-X-TCTT**GC$_2$**TGTCT-5′)	7196	7221
15	d(5′-TCTGT**C$_3$G**TTCT-X-TCTT**GC$_3$**TGTCT-5′)	7226	7223
16	d(5′-TCTGT**C$_4$G**TTCT-X-TCTT**GC$_4$**TGTCT-5′)	7224	7217
17	d(5′-TCTGT**C$_5$G**TTCT-X-TCTT**GC$_5$**TGTCT-5′)	7252	7246
18	d(5′-TCTGT**C$_6$G**TTCT-X-TCTT**GC$_6$**TGTCT-5′)	7182	7183
19	d(5′-TCTGT**C$_7$G**TTCT-X-TCTT**GC$_7$**TGTCT-5′)	7175	7170
20	d(5′-TCTGT**C$_8$G**TTCT-X-TCTT**GC$_8$**TGTCT-5′)	7204	7202
21	d(5′-TCTGT**C$_9$G**TTCT-X-TCTT**GC$_9$**TGTCT-5′)	7148	7142
22	d(5′-TCTGT**C$_{10}$G**TTCT-X-TCTT**GC$_{10}$**TGTCT-5′)	7180	7176
23	d(5′-ACACACCAACT-X-TCAACCACACA-5′)	7078	7075
24	d(5′-CTATCTGTCGTTCTCTGT-3′)	5695	5687

[a] All ODN are phosphorothioates. X is glycerol linker, and **G$_1$**−**G$_{11}$** and **C$_1$**−**C$_{10}$** correspond to guanosine and cytosine modifications shown in parts A and B of Figure 2, respectively. [b] Molecular weight of the compounds was determined by MALDI-ToF mass spectrometry. "Calcd" and "found" indicate calculated and experimentally determined values.

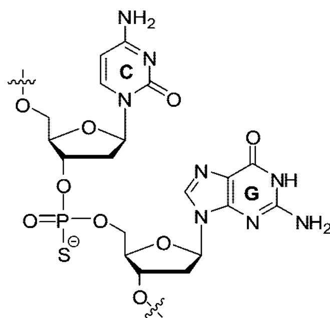

Figure 1. Chemical structure of phosphorothioate CpG dinucleotide motif showing functional groups.

lecular mass and sequence integrity (Table 1) and by HPLC, capillary, and/or slab gel electrophoresis for purity.

ODN with Two Accessible 5′-Ends Induces Greater Immune Stimulation Than with One 5′-End. The TLR9 activities of agonist **1** and linear CpG ODN **24** were compared in HEK293 cells expressing mouse TLR9. The results are presented as fold increase in NF-κB activation over PBS control. Agonist **1** produced a dose-dependent NF-κB activation greater than linear CpG ODN **24** in HEK293 cell cultures (Figure 3). Control ODN **23** had no activity, suggesting that the CpG motif is required for TLR9 activation.

The immune stimulatory activities of agonist **1** and linear CpG ODN **24** were studied in C57BL/6 mouse spleen cell cultures. Agonist **1** and linear ODN **24**, both of which contained CpG motifs, induced higher levels of IL-12 and IL-6 than did the control ODN **23** (Figure 4). However, agonist **1**, which has two accessible 5′-ends, induced higher levels of IL-12 and IL-6 than did linear ODN **24**. Both compounds induced cytokine production in a dose-dependent fashion, while control **23** failed to induce IL-12 and IL-6 at any dose tested (Figure 4). These results suggest that the presence of a CpG motif is required for immune stimulation and that ODN containing more than one accessible 5′-end induces higher levels of cytokine production in mouse spleen cell cultures. Furthermore, a comparison of the ratio of IL-12 to IL-6 induced suggests that **1** induced higher levels of IL-12 than IL-6 compared with linear ODN **24** (Figure 4C).

ODN Containing C or G Modifications in Place of CpG Motif Activates HEK293 Cells Expressing Mouse TLR9. After establishing that a CpG ODN containing two 5′-ends (**1**) was more immune stimulatory than linear ODN **24**, we examined the immune stimulatory activity of agonists **2–22**, which contained C or G modifications. These agonists of TLR9 activated NF-κB in HEK293 cells to various degrees, suggesting that the modifications were recognized by TLR9 to different extents. All of the G modifications studied stimulated TLR9 to a greater extent than did control compound **23** (Figure 5A) except **4**, which contained 9-deaza-dG in the position of G. Agonists **2**, **6**, **9**, **10**, and **12**, which contained 7-deaza-dG, N1-methyl-dG, dI, 8-O-methyl-dG, and araG, respectively, were similar in immune stimulatory activity to **1**, which has a natural dG in the CpG motif. Compounds with 7-deaza-8-aza-dG (**3**), 6-O-methyl-dG (**5**), N2-methyl-dG (**7**), and 6-thio-dG (**8**) modifications had moderate immune stimulatory activity. Agonists that contained modified C were all recognized by TLR9 (Figure 5B). Agonists **13**, **15–20**, and **22** had activity similar to **1**, and **14** and **21** showed moderate activity compared with **1**. The stimulation was dose-dependent (data not shown).

ODN Containing C or G Modifications in Place of CpG Motif Induces Cytokine Secretion in Mouse Spleen Cell Cultures. To determine the effect of C or G modifications in the CpG motif, we evaluated the agonist-induced production of cytokines in C57BL/6 mouse spleen cell cultures. As shown in parts A and B of Figure 6, agonists **1–23** that showed activity in HEK293 cell assays induced production of IL-12 and IL-6 in a dose-dependent manner. Agonists **2** and **6**, which had 7-deaza-dG and N^1-Me-dG modifications, respectively, in the place of G, and **15** and **16**, which had furano-dC and 1-(2′-deoxy-β-D-ribofuranosyl)-2-oxo-7-deaza-8-methyl-purine modifications, respectively, in the place of C induced production of

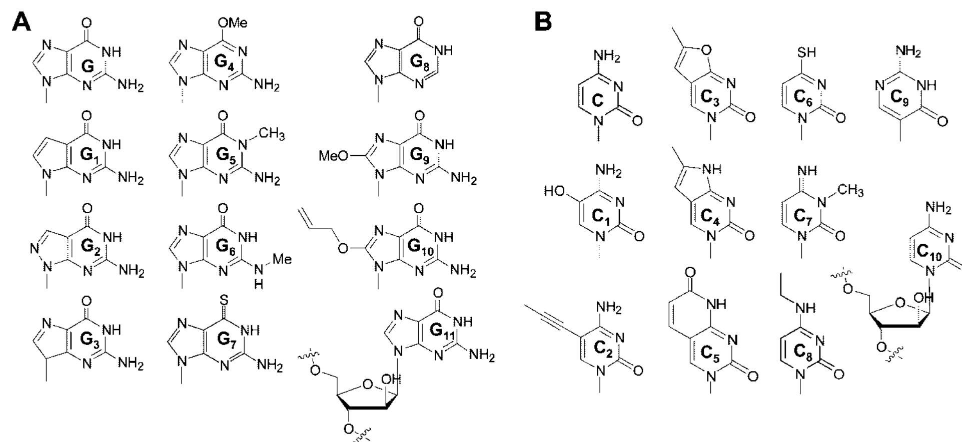

Figure 2. (A) Structures of guanine and various modifications $\mathbf{G_1}-\mathbf{G_{11}}$ used in the study. (B) Structures of cytosine and various modifications $\mathbf{C_1}-\mathbf{C_{10}}$ used in the study.

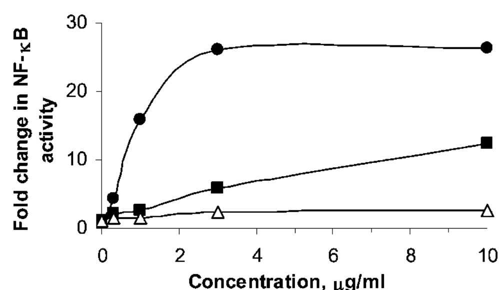

Figure 3. Activation of HEK293 cells expressing mouse TLR9 with different concentrations of agonist **1** (filled circles), linear CpG ODN **24** (filled squares), or control ODN **23** (open triangles). Data shown are representative of three independent experiments.

IL-12 at levels similar to that of **1**. On the other hand, agonists **6**, **15**, and **16** induced significantly higher levels of IL-6 than did **1**, suggesting that these modifications did not prevent binding to the receptor. Agonists **10**, **12**, **18**, **20**, and **22** induced moderate levels of IL-12 and significantly lower levels of IL-6. The rest of the agonists induced production of significantly lower levels of both IL-12 and IL-6. The plots of ratios of IL-12 to IL-6 show the differences in cytokine induction profiles in mouse spleen cell cultures by **1–22** with C or G modifications (Figure 6C).

Activation of Human PBMCs by ODN Containing C or G Modifications in the CpG Motif. We further examined the effect of C or G modifications on immune stimulation in human PBMCs as measured by induction of cytokine/chemokine production. Multiple cytokine profiles were determined by Luminex multiplex assay and IL-12, IL-6, IFN-γ, and IP-10 profiles are shown in Figures 7 and 8. All agonists induced IL-12 and IL-6 in human PBMC cell cultures because these agonists contain a human-specific stimulatory motif. All modifications studied induced greater levels of IL-12, IL-6, IFN-γ, and IP-10 than did control compound **23** (Figure 7), suggesting that these modifications were recognized by TLR9. However, agonists induced various levels of cytokines in PBMCs, suggesting that these modifications were recognized to different extents by TLR9. Among the G modifications, all agonists except **4**, **5**, and **11** induced production of IL-12, IL-6, IFN-γ, and IP-10 at levels similar to that produced by **1** (Figure 7). Agonists containing 9-deaza-dG (**4**), 6-O-methyl-dG (**5**), and 8-O-allyl-dG (**11**) modifications induced production of moderate levels of IL-12, IL-6, IFN-γ, and IP-10 compared to **1**. Among the C modifications studied, all agonists except **21** induced production of IL-12, IL-6, and IFN-γ at levels similar to that of **1** but induced lower levels of IP-10 than did **1** (Figure 8). Agonist **21**, which had a Ψ-iso-dC modification, induced moderate levels of IL-12, IL-6, IFN-γ, and IP-10 compared to **1**. Surprisingly, IP-10 was not detected in the cultures stimulated with **13**. These results together with those shown for other cytokines and chemokines (IL-1β, IL-2, IL-10, MIP-1α, MIP-1β, and MIG) for all compounds (Figure 1 of Supporting Information) suggest that different modifications induce different cytokine profiles.

Human B Cell Activation by Agonists of TLR9. ODN containing CpG motifs stimulates B cell proliferation. We examined if agonists of TLR9 containing synthetic stimulatory motifs induce B cell proliferation. Human B cells isolated from PBMCs were incubated with different concentrations of agonists, and [^{3}H]thymidine uptake was determined. The results in Figure 9A show that all compounds tested containing a modified G (**2–7**, **10–12**) induced B cell proliferation compared with control ODN **23**. Agonists **2**, **3**, **6–10** and **12** showed similar or slightly higher levels of proliferation compared with parent compound **1**. All the compounds tested containing a C modification also showed B cell proliferation similar to that of parent compound **1**. Control compound **23** without a stimulatory motif showed minimal levels of B-cell proliferation (Figure 9). Dose response data are shown in Table 1 of Supporting Information.

In Vivo Activity of TLR9 Agonists. We measured in vivo immune stimulatory profiles of the new agonists of TLR9 in mice following their subcutaneous (sc) administration to C57BL/6 mice at a dose of 1 mg/kg. The levels of serum cytokines and chemokines were determined by Luminex multiplex assay 2 h after agonist administration. The agonists that showed higher activity in mouse spleen cell culture assays induced higher levels of IL-12 in vivo (Figure 10). Control compound **23** induced no production of cytokines/chemokines, suggesting that the stimulatory motifs in agonists **1–22** were responsible for the observed immune responses in mice. The agonists of TLR9 induced production of IL-10, IP-10, KC, MCP-1, IFN-γ, IL-6, MIG, and MIP-1α to different extents in vivo (Table 2 of Supporting Information).

Discussion

ODNs containing natural CpG or synthetic dinucleotide motifs as agonists of TLR9 are being studied as potential therapeutic agents for the treatment of a wide range of diseases, including asthma, allergies, infectious diseases, and cancer.[10–12] Although a CpG motif is essential for TLR9 activation, other factors, such as an accessible 5′-end, the position of CpG

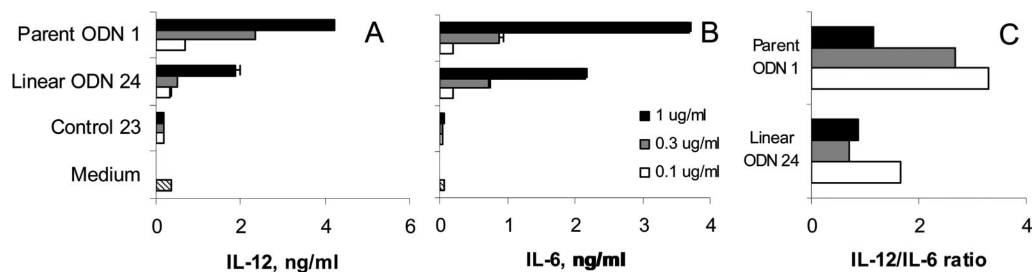

Figure 4. Induction of cytokine secretion by agonists of TLR9 in C57BL/6 mouse spleen cell cultures. C57BL/6 mouse spleen cells were cultured in the presence of agonists of TLR9 at various concentrations for 24 h, and the levels of secreted IL-12 (A) and IL-6 (B) in culture supernatants were measured by ELISA. M stands for medium-treated control, and linear ODN is linear sequence **24**. Each value is an average of three replicate wells, and the results are a representative experiment of at least two independent experiments. (C) Ratio of IL-12 to IL-6 induced by **1** and linear ODN **24**.

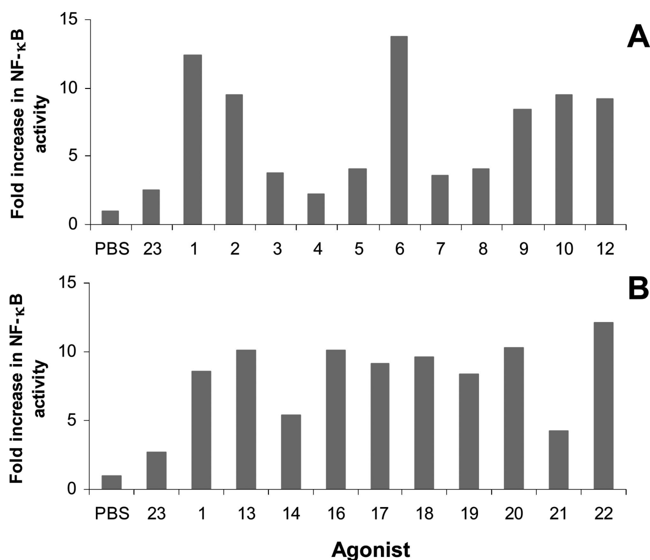

Figure 5. Activation of HEK293 cells expressing mouse TLR9 with agonists and control compounds at a concentration of 10 μg/mL and culturing for 18 h. Data shown are one representative of at least three independent experiments.

dinucleotide in the sequence, and nucleotides flanking the CpG dinucleotide, play a role in the activation of immune cells.[6–9,21–23,27] Additionally, ODN containing secondary structure-forming sequences produce higher levels of IFN-α through plasmacytoid dendritic cell activation than do those that do not contain secondary structure-forming sequences. We recently extended these findings by demonstrating that TLR9 reads CpG DNA from the 5′-end and that blocking the 5′-end through conjugations abrogates immune stimulatory activity.[21–23] Our finding that agonist **1** has greater immune stimulatory activity than does the linear CpG DNA **24** in HEK293 cells (Figure 3) and in mouse spleen cell cultures (Figure 4) supports our earlier findings that CpG DNA with more than one accessible 5′-end is more immunostimulatory than a linear CpG DNA.

Our previous studies showed that TLR9 recognizes certain synthetic purines and pyrimidines substituted in place of C or G, respectively, in a natural CpG motif and stimulates the immune system.[24–28] To further elucidate structure–immune-stimulatory activity relationships, a number of agonists (Table 1) with a range of C and G modifications (parts A and B of Figure 2) were synthesized and studied in vitro and in vivo. The results presented here provide insight into which specific chemical modifications at C or G of the CpG motif are recognized by TLR9 and the ability to modulate immune responses by substituting natural C or G with modified C or G in immune modulatory oligonucleotides.

TLR9 activation studies in HEK293 cells suggest that agonists containing 7-deaza-dG, N^1-Me-dG, dI, 8-OMe-dG, and araG modifications were well recognized. The agonists containing these modifications had activity equivalent to that of **1**, which contained a natural CpG dinucleotide, in both mouse and human cell-based assays. On the other hand, 7-deaza-8-aza-dG, 9-deaza-dG, 6-OMe-dG, N^2-Me-dG, and 6-Thio-dG modifications were recognized to a lesser extent by mouse TLR9 in HEK293 cells. Consistent with these results, compounds containing these modifications induced production of cytokines at lower levels in mouse and human cell-based assays than did parent ODN **1**.

All of the C modifications studied, except 5-propyne-dC and Ψ-iso-dC, were recognized by both mouse and human TLR9 and exhibited activity similar to that of **1**. All the modifications that were recognized by TLR9 in HEK293 cells also induced dose-dependent IL-12 and IL-6 secretion in C57BL/6 mouse spleen cell cultures except **19**, which did not induce production of IL-12 and IL-6. The modifications that were not recognized by TLR9 in HEK293 cell culture assays had no or minimal activity in mouse spleen cell cultures (Figure 6A).

The present results support our earlier observation that the nitrogen at the 7-position of G (**2**) was not required for CpG dinucleotide recognition.[24,25,27] The agonists of TLR9 containing N-glycosides were more active than the compounds containing a C-glycosidic linkage, suggesting that the structural changes caused by the C-glycosidic linkage in nucleoside or nucleotide may not be favorable for TLR9 recognition or interaction. Furthermore, incorporation of a nitrogen at the 8-position of 7-deaza-dG (8-aza-7-deaza-dG, **3**) resulted in the loss of activity. Similarly, deletion or modification of hydrogen-bond acceptor and donor functional groups at the 2- and 6-positions of G (**5**, **7**, and **9**) reduced the immune stimulatory activity in mouse spleen cell cultures. Methoxy modification of the hydrogen-bond acceptor keto group at the 6-position of G (**5**), which resulted in a lack of a hydrogen-bond donor proton at the 2-position, reduced the compound's immune stimulatory activity in human PBMC cells. On the other hand, a 6-thio modification (**8**), which retained hydrogen-bond donor functionality at the 1-position, significantly enhanced the activity. Furthermore, N1-methyl substitution (**6**), which did not change the hydrogen-bond accepting property of the keto oxygen at 6-position, did not alter the activity of the compound. These results suggest that either a hydrogen-bond acceptor group at the 6-position or a hydrogen-bond donor group at the 1-position is critical for TLR9 recognition. Methyl substitution (**7**) or deletion (**9**) of a hydrogen-bond donor functional group at the 2-position of G significantly reduced the immune stimulatory activity in mouse

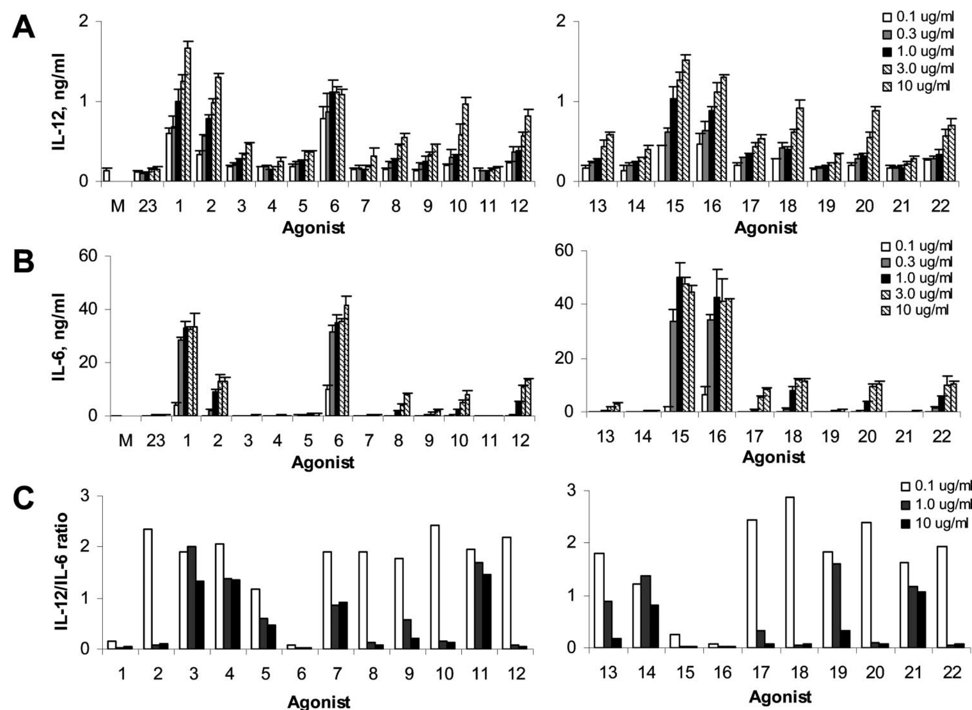

Figure 6. Dose-dependent IL-12 (A) and IL-6 (B) induction profiles of agonists **1**–**23** in C57BL/6 mouse spleen cell cultures. Spleen cells were cultured in the presence of agonists at 0.1, 0.3, 1, 3, and 10 μg/mL concentrations for 24 h as described in the Experimental Section, and the levels of secreted IL-12 and IL-6 in culture supernatants were measured by ELISA. Each value is an average of three replicate wells, and the results are of one representative experiment of at least two independent experiments. (C) Ratio of IL-12 to IL-6 induced at 0.1, 1, and 10 μg/ml concentrations of agonists **1**–**22** in C57BL/6 mouse spleen cell cultures.

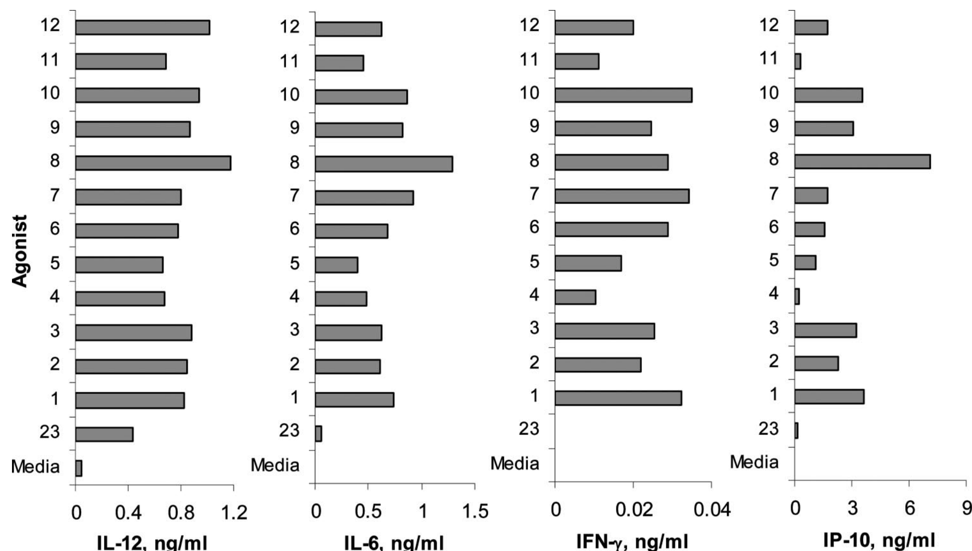

Figure 7. Cytokine induction profiles of agonists **1**–**12** and control compound **23** in human PBMC cultures. PBMCs isolated from fresh blood obtained from healthy volunteers were cultured in the presence and absence of agonists for 24 h as described in the Experimental Section. Supernatants were collected and analyzed by Luminex multiplex assay. Data shown are for one representative donor of three or more separate donors.

spleen cell cultures. However, in human PBMC cultures the same modifications showed greater activity (Figure 7), suggesting that the chemical modifications introduced further enhanced the compound's specificity for mouse or human TLR9. An O-methyl substitution at the 8-position (**10**) of G resulted in an increase in immune stimulatory activity. In contrast, an O-allyl substitution (**11**) at the same position resulted in considerable reduction in TLR9 activity, suggesting that a larger group at this position interferes with TLR9 recognition.

The presence of a methyl group at the 5-position of C in a CpG motif suppressed the immune effects of the compound.[30] Interestingly, substitution of the methyl group with a 1-propyne group at the 5-position of C (**14**) enhanced the immune stimulatory activity of the compound in human PBMC cultures. Furthermore, substitution of the propyne group with a hydrophilic hydroxyl group at the 5-position of C (**13**) further enhanced the immune stimulatory activity compared with 5-propyne-dC as well as **1**. These observations suggest that bulky hydrophilic groups are better recognized than hydrophobic groups at this position, as we have observed previously.[24] In the case of **14**, the fact that the propyne group is more hydrophobic than the methyl group, and the planar nature of

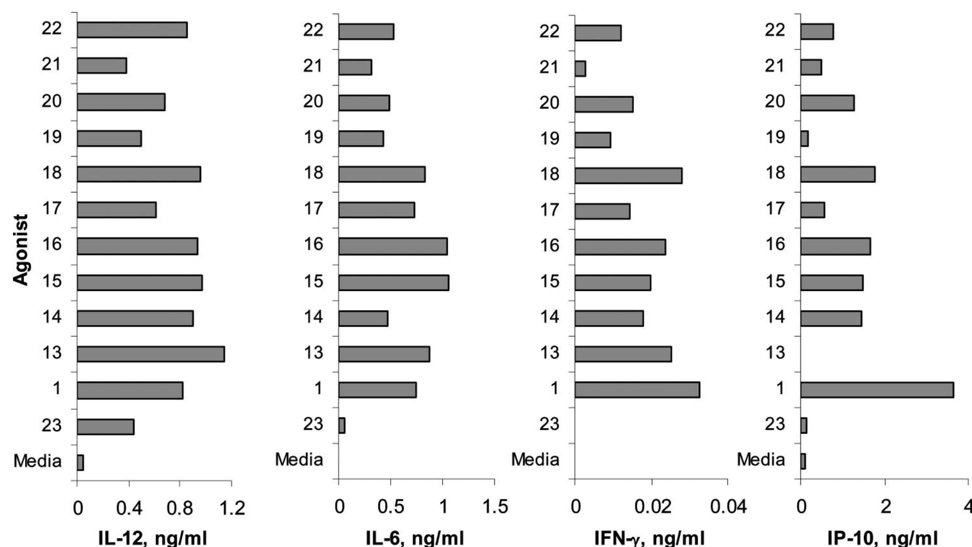

Figure 8. Cytokine induction profiles of **1** and **13–23** in human PBMC cultures. PBMCs isolated from fresh blood obtained from healthy volunteers were cultured in the presence and absence of agonists for 24 h as described in the Experimental Section. Supernatants were collected and analyzed by Luminex multiplex assay. Data shown are for one representative donor of three or more separate donors.

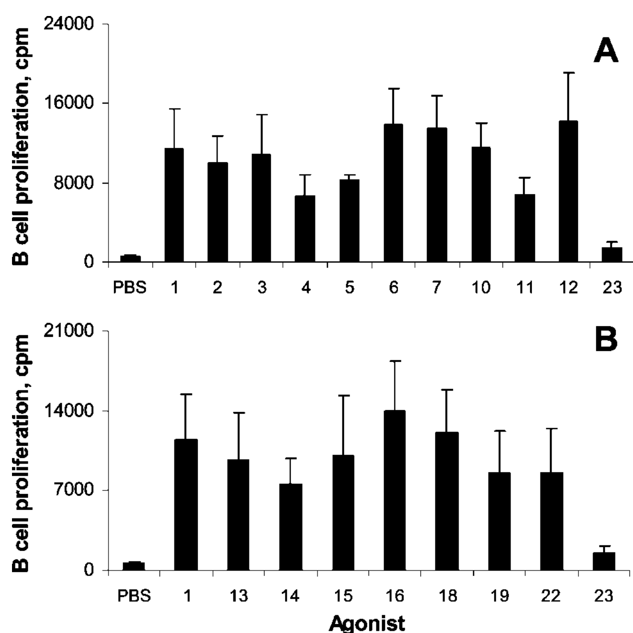

Figure 9. Human B cell proliferation induced by agonists of TLR9, **2**−**12** (A) and **13**–**22** (B), along with parent ODN **1** and control ODN **23**. Human B cells isolated from PBMC obtained from healthy human volunteers were stimulated with 3 μg/mL agonists, and [^{3}H]thymidine uptake was determined by scintillation counting. Each data point represents the average of four donors ± SD.

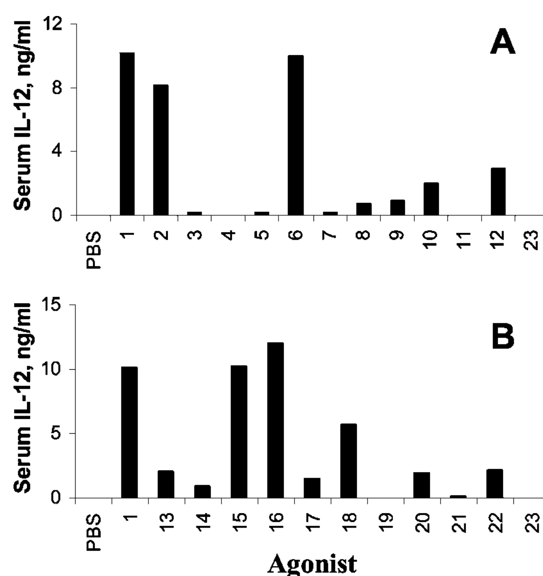

Figure 10. IL-12 secretion in C57BL/6 mice induced by agonists of TLR9, **2**–**12** (A) and **13**–**22** (B), along with parent ODN **1** and control ODN **23** following sc administration at a dose of 1 mg/kg. Blood was collected 2 h after agonist administration, and cytokine and chemokine levels in the serum were determined by Luminex multiplex assay as described in Experimental Section.

the propyne group with respect to the heterocyclic base, which allows better stacking of the bases in the ODN, may account for the better recognition and enhanced activity.

TLR9 specifically recognizes deoxyribonucleotides; substitution of C or G with ribonucleotides results in a loss of activity. The results presented here demonstrate that TLR9 recognizes arabinonucleosides in the C or G position of a CpG dinucleotide. Agonists **22** and **12**, which contain arbinoC or arabinoG in the C or G position, respectively, potently stimulated immune responses in mouse and human cell-based assays and in vivo in mice. These results confirm that TLR9 recognizes a variety of diverse nucleotide motifs.

Recent studies have shown that TLR9 exists in a dimer form and binds to single-stranded ODN. Only ODN containing the CpG motif causes the conformational changes in the receptor leading to the activation of immune signaling pathways.[31] The ability of ODN with different modifications to induce immune responses compared with that of CpG-containing ODN suggests that the new ODN binds to TLR9 and causes the conformational changes that are required to activate the immune signaling pathways. More importantly, ODN with different chemical modifications induces different cytokine and chemokine profiles than does the ODN that contains a natural CpG motif.

Taken together, the results presented here suggest that TLR9 can recognize C or G modifications, such as 5-OH-dC, furano-dT, 1-(2′-deoxy-β-D-ribofuranosyl)-2-oxo-7-deaza-8-methyl-purine, dF, 4-thio-dU, N^3-Me-dC, N^4-Et-dC, arabinoC, 7-deaza-

dG, N1-Me-dG, inosine, 8-OMe-dG, and araG. Not only does TLR9 recognize these modifications, but the activation of TLR9 by agonists containing these different modifications also results in the induction of different cytokine and chemokine profiles compared with natural CpG dinucleotides. These are the first studies in which various chemical modifications in C and G sites of a CpG motif are systematically examined in TLR9 transfected cells, mouse and human cell-based assays, and in vivo in mice to understand the ability of TLR9 to recognize these chemical modifications and induce immune responses.

Experimental Section

Synthesis and Purification of Phosphorothioate Oligdeoxynucleotides. Agonists of TLR9 comprising 2′-deoxy-7-deazaguanosine (7-deaza-dG) or 2′-deoxy-7-deaza-8-azaguanosine (7-deaza-8-aza-dG) or 2′-deoxy-9-deazaguanosine (9-deaza-dG) or 2′-deoxy-6-methoxyguanosine (6-OMe-dG) or 2′-deoxy-N^1-methylguanosine (N^1-Me-dG) or 2′-deoxy-N^2-methylguanosine (N^2-Me-dG) or 2′-deoxy-6-thioguanosine (6-Thio-dG) or 2′-deoxy-inosine (dI) or 2′-deoxy-8-methoxyguanosine (8-OMe-dG) or 2′-deoxy-8-allyloxyguanosine (8-OAllyl-dG) or arabinoguanosine (araG) modifications in the place of dG and 2′-deoxy-5-hydroxycytidine (5-OH-dC) or 2′-deoxy-5-propynecytidine (5-propyne-dC) or furano-T or 6-methyl-3-(β-D-2-deoxyribofuranosyl)pyrrolo[2,3-*d*]pyrimidin-2-one (pyrrolo-dC) or dF or 4-thio-dU or 2′-deoxy-N^3-methylcytidine (N^3-Me-dC) or 2′-deoxy-N^4-ethylcytidine (N^4-Et-dC) or pseudoiso-dC (Ψ-iso-dC) or arabinocytidine (araC) modifications in the place of dC were synthesized on a 1–2 μmol scale using β-cyanoethyl phosphoramidite chemistry on a PerSeptive Biosystem 8909 Expedite DNA synthesizer. The di-DMT-protected glyceryl linker attached to a CPG solid support was obtained from ChemGenes Corporation (Wilmington, MA). The 3′-phosphoramidites of dA, dG, dC, and T were obtained from Proligo (Denver, CO). Phosphoramidites of N-Me-dC, AraC, N-Me-dG, 7-deaza-dG, and dI were obtained from ChemGenes Corporation. Phosphoramidites of 5-OH-dC, N^4-Et-dC, and pyrrolo-dC were obtained from Glen Research (Sterling, VA). Beaucage reagent was used as an oxidant to obtain the phosphorothioate backbone modification.[32] Modified coupling protocols as recommended by the suppliers were used for modified dC and dG phosphoramidite incorporation. After the synthesis, agonists containing -dC or -dG modification were deprotected using standard protocols, purified by RP high-performance liquid chromatography (HPLC), detritylated, and dialyzed against United States Pharmacopeia-quality sterile water for irrigation (Braun). All the compounds were lyophilized and dissolved again in distilled water, and the concentrations were determined by measuring the ultraviolet (UV) absorbance at 260 nm.[33] The purity of all the compounds synthesized was determined by denaturing PAGE and RP-HPLC, and the sequence integrity was characterized by matrix-assisted laser desorption/ionization-time-of-flight (MALDI-ToF) mass spectrometry for molecular mass. The ODNs were 90–98% that of the full-length product, with some being one or two nucleotides shorter. All compounds were synthesized and purified under identical conditions to minimize endotoxin contamination.

Animals. Five-to-six-week-old C57BL/6 mice were obtained from Charles River Laboratories, Wilmington, MA, and maintained in accordance with Idera's IACUC-approved animal protocols. All the animal studies reported in the paper were carried out following Idera's IACUC guidelines and approved protocols.

Mouse Spleen Cell Cultures. Spleen cells from C57BL/6 mice were prepared and cultured in RPMI complete medium consisting of RPMI 1640 with 10% fetal calf serum (FCS), 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mM L-glutamine (HyClone, Logan, UT). Mouse spleen cells were plated in 96-well plates at 5×10^6 cells/mL. Agonists dissolved in TE buffer [10 mM Tris-HCl (pH 7.5) and 1 mM EDTA] were added to a final concentration of 0.1, 0.3, 1, 3, or 10 μg/mL to the cell cultures. The cells were then incubated at 37 °C for 24 h, and the supernatants were collected for cytokine analysis by enzyme-linked immunosorbent assays (ELISAs).

IL-12 and IL-6 levels in supernatants were measured by sandwich ELISA. The required reagents, including cytokine antibodies and standards, were purchased from BD Pharmingen (San Diego, CA). Streptavidin–peroxidase and TMB substrate were from Sigma (St. Louis, MO) and KPL (Gaithersburg, MD), respectively.

Human PBMC Isolation. Peripheral blood mononuclear cells (PBMCs) from freshly drawn healthy volunteer blood (Research Blood Components, Brighton, MA) were isolated by Ficoll density gradient centrifugation (Histopaque-1077, Sigma).

Human Cytokine ELISAs. Human PBMCs were plated in 96-well plates at a concentration of 5×10^6 cells/mL. The compounds dissolved in phosphate-buffered saline (PBS) were added to the cell cultures at a final concentration of 10 μg/mL. The cells were then incubated at 37 °C for 24 h. The levels of cytokines and chemokines in the culture supernatants were measured using a human multiplex kit on Luminex 100 (Luminex Corporation, Austin, TX), and the data were analyzed using StarStation software, version 2.0 (Applied Cytometry Systems, Sacramento, CA). The required reagents were purchased from Invitrogen (Carlsbad, CA).

Human B Cell Proliferation Assay. About 1×10^5 B cells purified from human PBMCs as described previously[27] were stimulated with different concentrations of agonists or control compound for 56 h and then pulsed with 0.75 μCi of [^{3}H]thymidine and harvested 16 h later. The incorporation of [^{3}H]thymidine was measured by scintillation counter, and the data are shown as counts per minute (cpm).

HEK293 Cell Cultures. HEK293 cells stably expressing mouse TLR9 (Invivogen, San Diego, CA) were cultured in 48-well plates in 250 μL/well DMEM supplemented with 10% heat-inactivated FBS in a 5% CO_2 incubator. At 80% confluence, cultures were transiently transfected with 400 ng/mL of the secreted form of human embryonic alkaline phosphatase (SEAP) reporter plasmid (pNifty2-Seap) (Invivogen) in the presence of 4 μL/mL of lipofectamine (Invitrogen, Carlsbad, CA) in culture medium. Plasmid DNA and lipofectamine were diluted separately in serum-free medium and incubated at room temperature for 5 min. After incubation, the diluted DNA and lipofectamine were mixed and the mixtures were incubated at room temperature for 20 min. Aliquots of 25 μL of the DNA/lipofectamine mixture containing 100 ng of plasmid DNA and 1 μL of lipofectamine were added to each well of the cell culture plate, and the cultures were continued for 4 h. After transfection, medium was replaced with fresh culture medium, agonists were added to the cultures, and the cultures were continued for 18 h. At the end of the treatment, 30 μL of culture supernatant was taken from each treatment and used for SEAP assay following the manufacturer's protocol (Invivogen). Briefly, culture supernatants were incubated with *p*-nitrophynyl phosphate substrate and the yellow color generated was measured by a plate reader at 405 nm. The data are shown as fold increase in NF-κB activity over PBS control.

Mouse Serum Cytokines. Female C57BL/6 mice ($n = 3$), 5–6 weeks old, were injected subcutaneously (sc) with agonists of TLR9 at 1 mg/kg (single dose). Serum was collected by retro-orbital bleeding 2 h after compound administration, and the levels of cytokines and chemokines in the serum were measured using a mouse multiplex kit on Luminex 100 instrument. The data were analyzed using StarStation software, version 2.0. The required reagents were purchased from Invitrogen.

Supporting Information Available: Cytokine induction profile data and human B cell proliferation data. This material is available free of charge via the Internet at http://pubs.acs.org.

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