3730 J. Med. Chem. 2010, 53, 3730–3738
DOI: 10.1021/jm100177p

Journal of Medicinal Chemistry Article

Impact of Nature and Length of Linker Incorporated in Agonists on Toll-Like Receptor 9-Mediated Immune Responses

Mallikarjuna Reddy Putta, Dong Yu, Lakshmi Bhagat, Daqing Wang, Fu-Gang Zhu, and Ekambar R. Kandimalla*

Idera Pharmaceuticals, Inc., 167 Sidney Street, Cambridge, Massachusetts 02139

Received February 10, 2010

Oligodeoxynucleotides containing unmethylated CpG motifs act as ligands of Toll-like receptor 9 (TLR9). We previously reported a novel class of TLR9 agonists, referred to as immune-modulatory oligonucleotides (IMOs), in which two 11-mers of the same sequence are attached via their 3′-ends through a 1,2,3-propanetriol linker and contain a synthetic immune-stimulatory motif, Cp7-deaza-dG. In the present study, we have examined the impact of length, nature, and stereochemistry of the linker incorporated in agonists for TLR9 activation. The new linkers studied include (*S*)-(−)-1,2,4-butanetriol, 1,3,5-pentanetriol, *cis*,*cis*-1,3,5-cyclohexanetriol, *cis*,*trans*-1,3,5-cyclohexanetriol, 1,3,5-tris(2-hydroxyethyl)isocyanurate, tetraethyleneglycol, and hexaethyleneglycol in place of 1,2,3-propanetriol linker. Agonists with various linkers are studied for TLR9-mediated immune responses in HEK293 cells, human cell-based assays, and in vivo in mice. Results of these studies suggest that C3−C5 linkers, 1,2,3-propanetriol, (*S*)-(−)-1,2,4-butanetriol, or 1,3,5-pentanetriol, are optimal for stimulation of TLR9-mediated immune responses. Rigid C3 linkers with different stereochemistry have little effect on immune stimulation, while linkers longer than C5 reduced TLR9-mediated immune stimulation.

Introduction

Bacterial and synthetic DNA containing unmethylated CpG[a] motifs are ligands for Toll-like receptor (TLR) 9, a receptor that belongs to a family of innate immune receptors called TLRs.[1] TLR9 is expressed intracellularly in the endosomal compartments of human B cells and plasmacytoid dendritic cells (pDCs).[2] TLR9 activation by its ligands results in predominantly Th1-type immune responses.[3,4] Modulation of TLR9-mediated immune responses may prove useful in treating various diseases, including cancers, allergies, asthma, and infectious diseases and as an adjuvant with vaccines.

Using data from extensive structure−activity relationship studies of agonists of TLR9, we have delineated structural features in the pentose sugar, phosphate backbone, and nucleobases of DNA and identified a number of synthetic immune-stimulatory motifs that induce TLR9-mediated immune responses.[5,6] The sequences flanking the immune-stimulatory motif[7] and the presence of secondary structures also play a role in TLR9-mediated immune stimulation.[8] Our studies have shown that an accessible 5′-end is required for the activity of TLR9 agonists and blocking the 5′-end accessibility of ligands results in the loss of immune-stimulatory activity.[9−11] Furthermore, the presence of more than one accessible 5′-end enhances the immune-stimulatory activity of TLR9 agonists.[10] By combining novel structures and synthetic immune-stimulatory motifs, we have created a portfolio of agonists of TLR9 containing two 5′-ends, referred to as immune-modulatory oligonucleotides (IMOs).[5,6,11−13] Currently, IMOs are being evaluated in clinical studies for the treatment of cancers, infectious diseases, asthma, and allergies.[5]

A C3 linker, such as 1,2,3-propanetriol (commonly referred to as glycerol throughout the paper), which approximates the natural internucleotide distance, is commonly used in TLR9 agonits.[6,11−13] We do not yet understand the effect of the linker's length, nature, and spatial orientation in the agonist on TLR9-mediated immune responses. In the present study, we have incorporated novel acyclic and cyclic linkers, (*S*)-(−)-1,2,4-butanetriol, 1,3,5-pentanetriol, *cis*,*cis*-1,3,5-cyclohexanetriol, *cis*,*trans*-1,3,5-cyclohexanetriol, 1,3,5-tris(2-hydroxyethyl)-isocyanurate, tetraethyleneglycol, and hexaethyleneglycol (Figure 1) into an agonist (Table 1). These novel TLR9 agonists were studied in cultures of TLR9-transfected HEK-293 cells, human cell-based assays, and in vivo in mice to demonstrate the impact of the linkers on TLR9-mediated immune-stimulatory activity.

Results and Discussion

Design of TLR9 Agonists. TLR9 agonists containing two 11-mer identical sequences were attached via their 3′-ends through a non-nucleosidic linker such as 1,2,3-propanetriol (glycerol; L_1).[6,11−13] To evaluate the effect of length, nature, and spatial orientation of the linker on TLR9-mediated immune-stimulatory activity, we designed and synthesized

*To whom correspondence should be addressed. Phone: 617-679-5536. Fax: 617-679-5532. E-mail: ekandimalla@iderapharma.com.

[a]Abbreviations: CGE, capillary gel electrophoresis; CpG, deoxycytidine-phosphate-deoxyguanosine; DCM, dichloromethane; DIC, *N*,*N*′-diisopropylcarbodiimide; DMAP, 4-(dimethylamino)pyridine; DMTCl, 4,4′-dimethoxytrityl chloride; ELISA, enzyme-linked immunosorbent assay; EtOAc, ethyl acetate; FBS, fetal bovine serum; IFN, interferon; IMO, immune-modulatory oligonucleotide; LCAA-CPG, long chain alkyl amine controlled pore glass; NF-κB, nuclear factor-κB; PBMCs, peripheral blood mononuclear cells; TBAF, tetrabutylammonium fluoride; SEAP, secreted alkaline phosphatase; TBDMS, *t*-butyldimethylsilane; TEA, triethylamine; THF, tetrahydrofuran; TLR, Toll-like receptor.

 Published on Web 04/02/2010

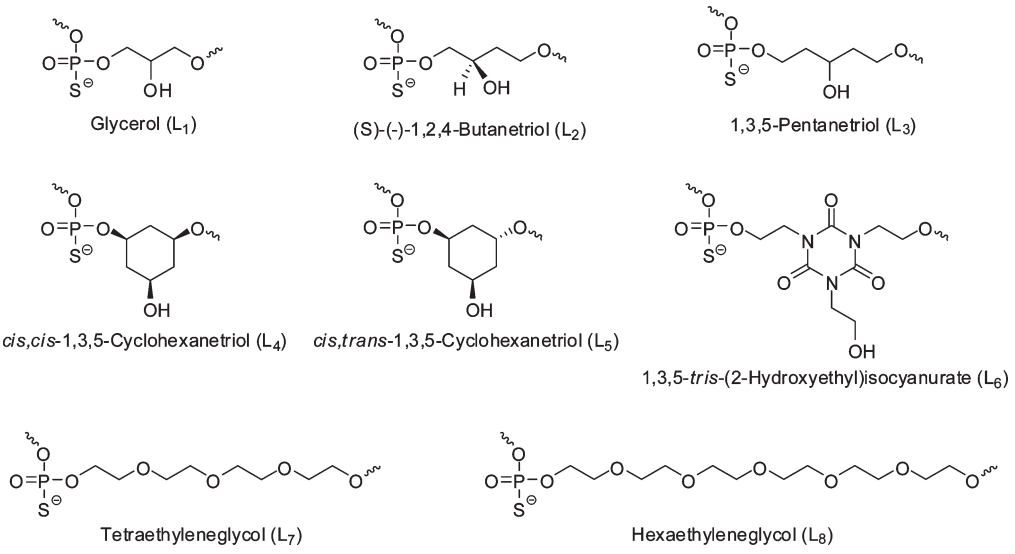


Figure 1. Structures of linkers used in the study.

Table 1. Sequences, MALD-TOF Mass Spectral, and HPLC Purity Data of TLR9 Agonists Containing Different Linkers

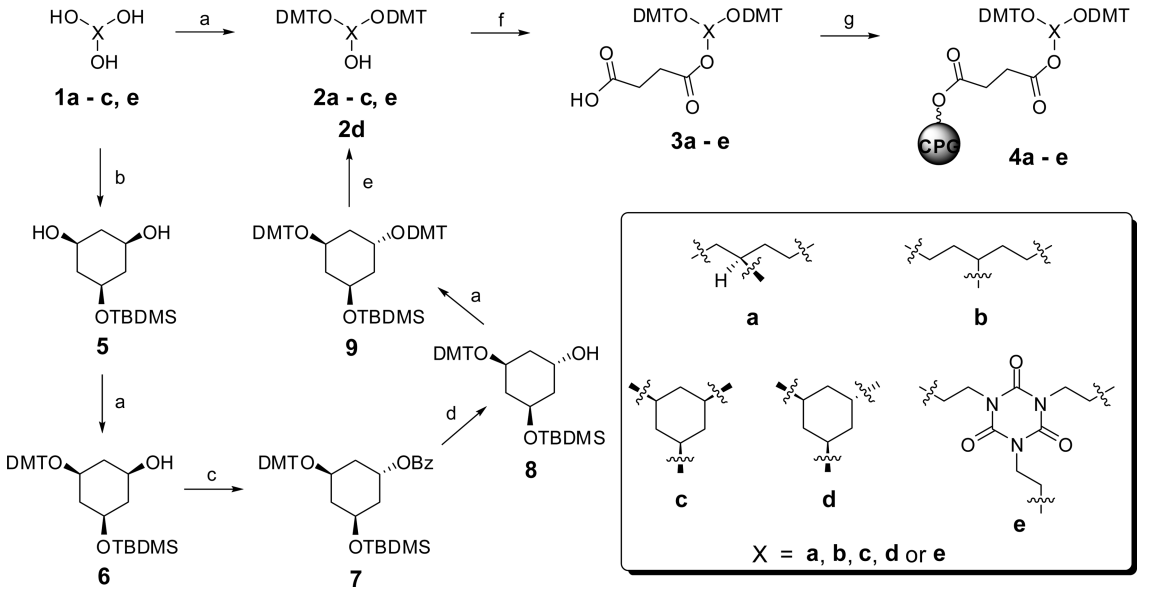
agonist	sequence[a]	mol wt[b] calcd	mol wt[b] obsd	purity (% FLP)[c] (IE-HPLC)
11	5′-TCTGTC**R**TTCT-**L**$_1$-TCTT**R**CTGTCT-5′	7146	7141	96
12	5′-TCTGTC**R**TTCT-**L**$_2$-TCTT**R**CTGTCT-5′	7160	7167	98
13	5′-TCTGTC**R**TTCT-**L**$_3$-TCTT**R**CTGTCT-5′	7174	7178	98
14	5′-TCTGTC**R**TTCT-**L**$_4$-TCTT**R**CTGTCT-5′	7186	7187	96
15	5′-TCTGTC**R**TTCT-**L**$_5$-TCTT**R**CTGTCT-5′	7186	7187	98
16	5′-TCTGTC**R**TTCT-**L**$_6$-TCTT**R**CTGTCT-5′	7315	7315	98
17	5′-TCTGTC**R**TTCT-**L**$_7$-TCTT**R**CTGTCT-5′	7248	7258	95
18	5′-TCTGTC**R**TTCT-**L**$_8$-TCTT**R**CTGTCT-5′	7336	7341	95
Control **10**	5′-ACACACCAACT-**L**$_1$-TCAACCACACA-5′	7078	7065	96

[a] **R** = 7-deaza-dG; $\mathbf{L}_1$ = 1,2,3-propanetriol (glycerol); $\mathbf{L}_2$ = (*S*)-(−)-1,2,4-butanetriol; $\mathbf{L}_3$ = 1,3,5-pentanetriol; $\mathbf{L}_4$ = *cis*,*cis*-1,3,5-cyclohexanetriol; $\mathbf{L}_5$ = *cis*,*trans*-1,3,5-cyclohexanetriol; $\mathbf{L}_6$ = 1,3,5-tris(2-hydroxyethyl)isocyanurate; $\mathbf{L}_7$ = tetraethyleneglycol; $\mathbf{L}_8$ = hexaethyleneglycol. [b] Molecular weight of compounds as calculated (calcd) and determined (obsd) by MALDI-TOF mass spectral analysis. [c] FLP = full length product; IE-HPLC = anion-exchange HPLC; similar purities were also observed by capillary gel electrophoresis (CGE).

novel acyclic and cyclic linkers. As shown in Figure 1, flexible, rigid, and hydrophilic linkers (*S*)-(−)-1,2,4-butanetriol (L_2), 1,3,5-pentanetriol (L_3), *cis*,*cis*-1,3,5-cyclohexanetriol (L_4), *cis*,*trans*-1,3,5-cyclohexanetriol (L_5), 1,3,5-tris-(2-hydroxyethyl)isocyanurate (L_6), tetraethyleneglycol (L_7), and hexaethyleneglycol (L_8) were investigated in the present study. The C3 linker (L_1) approximates the internucleotide distance found in the deoxyribose/ribose unit of a nucleotide. The acyclic C4 (L_2) and C5 (L_3) linkers are one and two carbon atoms longer than the C3 linker, respectively. The cyclic linkers L_4 and L_5 have spacing similar to that of the C3 linker (L_1) but provide more rigid structures with different orientations of the two attaching groups. Longer linkers with a more rigid central core (L_6) and hydrophilic nature (L_7 and L_8) were also incorporated into agonists to evaluate the distance required between two 5′-ends for optimal stimulation of TLR9-mediated immune responses.

Synthesis of Linkers and Solid Support Derivatization. Solid-support functionalized linkers were required in order to incorporate the new linkers into agonists. The long chain alkyl amine-controlled pore glass (LCAA-CPG) derivatized linkers were synthesized starting from corresponding triols **1a**−**c**,**e** as shown in Scheme 1. (*S*)-(−)-1,2,4-Butanetriol (**1a**), *cis*,*cis*-1,3,5-cyclohexanetriol (**1c**), and 1,3,5-tris(2-hydroxyethyl)isocyanurate (**1e**) were obtained from a commercial source, and 1,3,5-pentanetriol (**1b**) was prepared by $LiAlH_4$ reduction of diethyl 3-hydroxy glutarate.[14] Selective protection of two hydroxyls of triol **1a**−**c**,**e** with 4,4′-dimethoxytrityl chloride (DMTCl) in the presence of 4-(dimethylamino)pyridine (DMAP) afforded bis-DMT alcohols **2a**−**c**,**e** in good yields. Bis-DMT *cis*,*trans*-1,3,5-cyclohexanetriol (**2d**) was prepared from *cis*,*cis*-1,3,5-cyclohexanetriol (**1c**) as shown in Scheme 1. To make *trans*-configuration of the two strands of the agonist, it was critical to achieve *trans*-configuration specifically at the DMT-protected hydroxyls of cyclohexanetriol. The configuration at one of the hydroxyls of **1c** was inverted by the Mitsunobu reaction, by orthogonally protecting the remaining hydroxyls, to achieve the *cis*,*trans* configuration (Scheme 1). The *cis*,*cis*-triol **1c** was selectively converted into mono *t*-butyldimethylsilane (TBDMS)-ether **5** in high yield (∼94%) in the presence of a mixture of NEt_3 and NaH as the bases, which tremendously reduces the formation of bis and tris-TBDMS ethers.[15,16] The second hydroxyl of **5** was protected with DMTCl, to produce **6**, and the configuration at the third hydroxyl was inverted by the Mitsunobu reaction using benzoic acid to obtain **7** with the desired *cis*,*trans*-configuration. Debenzoylation of **7** with K_2CO_3 in methanol produced **8**, which was subsequently reacted with DMTCl in the presence of DMAP to produce fully protected *cis*,*trans*-triol **9**. TBDMS

Scheme 1[a]



[a] Reagents and conditions: (a) DMTCl, DMAP, pyridine, 0 °C−rt; (b) TBDMSCl, Et_3N, NaH, THF, rt; (c) PPh_3, benzoic acid, DEAD; (d) K_2CO_3, MeOH, rt; (e) TBAF, THF; (f) succinic anhydride, DMAP, pyridine; (g) LCAA-CPG, DMAP, DIC, pyridine, acetonitrile.

was removed using tetrabutylammonium fluoride (TBAF) in tetrahydrofuran (THF) to obtain bis-DMT protected *cis*,*trans*-1,3,5-cyclohexanetriol **2d**. To facilitate the loading of the linker on to the solid support, the third hydroxyl of each linker was converted into its succinate **3a**−**e** in high yields. The succinates **3a**−**e** were then attached to an amino-functionalized solid support in the presence of *N*,*N*′-diisopropylcarbodiimide (DIC)/DMAP in pyridine/acetonitrile mixture, producing linker-derivatized solid supports **4a**−**e**. We found a 50 μmol/g loading to be ideal for enhanced nucleotide couplings and improved final product yields. Hydrophilic tetraethyleneglycol and hexaethyleneglycol linkers were procured from a commercial source as phosphoramidites.

Synthesis of TLR9 Agonists. All TLR9 agonists (**10**−**18**) used in this study were oligodeoxynucleotides with phosphorothioate internucleotide linkages. To evaluate the effect of the linker on TLR9-mediated immune-stimulatory activity, glycerol linker agonist **11** was substituted with L_2−L_8 (Figure 1 and Table 1). Agonists **12**−**16** with L_2−L_6 linkers were synthesized on an automated DNA synthesizer[10−12] using solid supports derivatized with bis-DMT linkers **4a**−**e**. Although the agonists **17** and **18** with L_7 and L_8 linkers contained identical sequences, they were synthesized in a linear synthetic strategy[11] utilizing commercially available linker phosphoramidites. The sequence preceding the linker was synthesized using a 5′-dT solid-support and 5′-phosphoramidites; regular 3′-phosphoramidites were used after the linker coupling to obtain the agonist with two 5′-ends. Compound **10** was used as a negative control that had the same length and structure as other agonists but lacked an immune-stimulatory dinucleotide motif. All compounds were synthesized as described in Experimental Section, characterized by MALDI/TOF mass spectral analysis for their sequence integrity and by capillary gel electrophoresis and IE-HPLC for purity (Table 1).

Activation of TLR9 in HEK293 Cells by Agonists Containing Different Linkers. Agonists containing a CpR dinucleotide activate the immune system through TLR9.[6] Studies

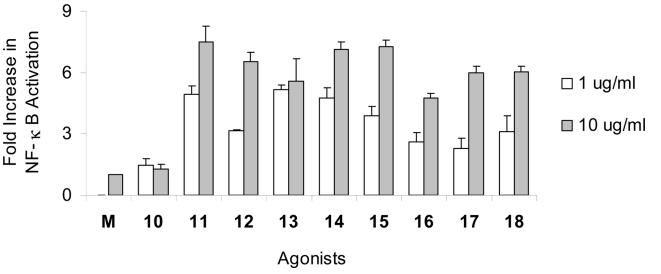


Figure 2. Activation of HEK293 cells expressing mouse TLR9 with agonists **11**−**18** and control compound **10** at 1 and 10 μg/mL. Data shown are of one representative experiment of three independent experiments.

were performed in HEK293 cells stably expressing mouse TLR9 to evaluate the impact of linkers on TLR9 agonist activity. As shown in Figure 2, agonists with different linkers induced a dose-dependent activation of NF-κB greater than that of control compound **10**. Although there was no significant difference in NF-κB activation at a 10 μg/mL concentration of agonists, the difference was apparent at 1 μg/mL (Figure 2). Agonists **11**−**15** with C3−C5 linkers produced similar levels of NF-κB activation at 1 μg/mL. The ability of agonists **16**−**18** to activate NF-κB with the increased length of the linker was decreased compared with **11** (Figure 2). As shown in Figure 2, cyclic C3 linkers (agonists **14** and **15**) produced similar levels of NF-κB activation at 10 μg/mL, but the *cis*,*cis*-linker (agonist **14**) produced slightly higher activation than did the *cis*,*trans*-linker (agonist **15**) at 1 μg/mL, suggesting that the nature of the linker plays a role in TLR9-mediated immune responses. The variation in NF-κB activation by agonists with different length linkers suggests that the linker's length or the distance between the two 5′-ends plays a role in TLR9-mediated immune stimulation.

Induction of Cytokine Secretion in Mouse Spleen Cell Cultures by TLR9 Agonists Containing Different Linkers. To further understand the impact of linkers on TLR9-mediated immune-stimulatory activity, we evaluated the

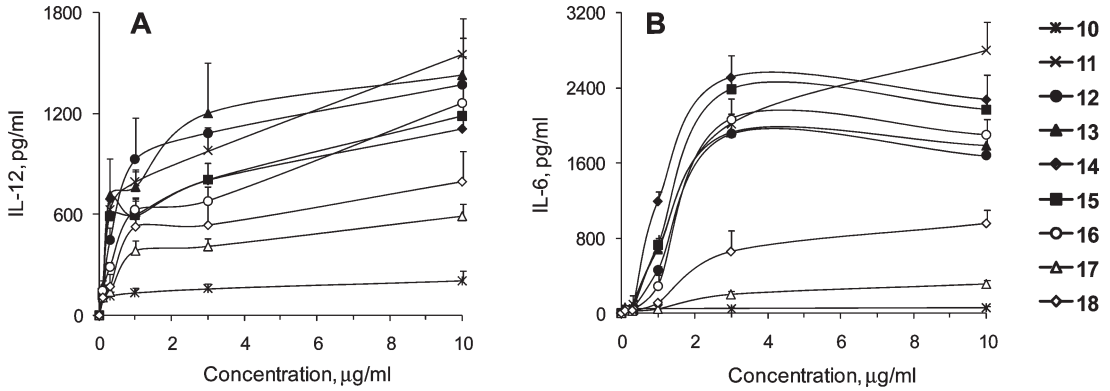


Figure 3. Dose-dependent (A) IL-12 and (B) IL-6 induction by agonists **11**−**18** and control compound **10** in C57BL/6 mouse spleen cell cultures. Spleen cells were cultured in the presence of agonists at 0.1, 0.3, 1, 3, and 10 μg/mL concentrations for 24 h as described in the Experimental Section, 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.

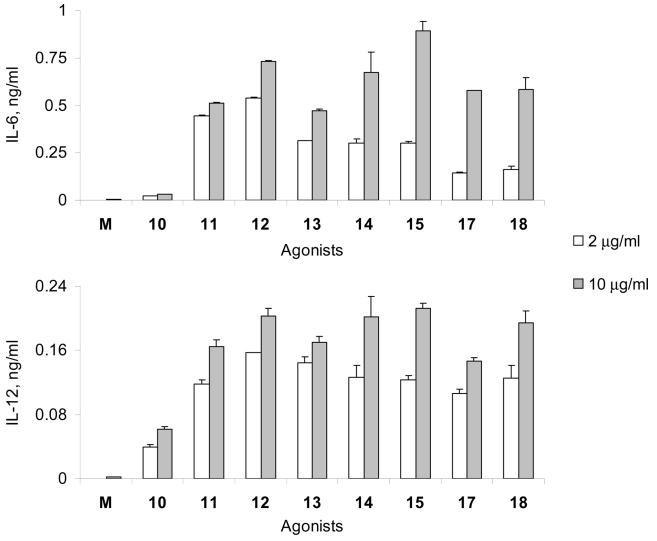


Figure 4. Cytokine induction profiles of agonists **11**−**15**, **17**, **18**, and control compound **10** containing different linkers in human PBMC cultures. PBMCs were stimulated with 2 and 10 μg/mL agonists for 24 h, and the supernatants were collected and cytokine levels were measured by Luminex multiplex assay. The data shown are for one donor and are representative of three independent experiments.

agonist activity in C57BL/6 mouse spleen cell cultures. In general, all agonists with different linkers induced dose-dependent IL-12 and IL-6 production (Figure 3). Similar to the results observed in HEK293 cell assays, the linkers affected cytokine induction by agonists in mouse spleen cell cultures. Incorporation of longer linkers, such as tetra or hexaethyleneglycol (**17** and **18**), significantly reduced IL-6 and IL-12 production compared with agonists containing other linkers (**11**−**16**). Agonists **14** and **15**, containing *cis*,*cis*- and *cis*,*trans*-linkers, respectively, induced both IL-12 and IL-6 to a similar extent. However, these agonists induced slightly lower levels of IL-12 and slightly higher levels of IL-6 compared with agonists containing linear C3−C5 linkers (agonists **11**−**13**). These results suggest that the linker plays a role in TLR9-mediated cytokine induction and the ability of the agonist to induce cytokines can be modulated by incorporating linkers with appropriate length and nature.

Activation of Human PBMCs by TLR9 Agonists Containing Different Linkers. We measured agonist-induced cytokine and chemokine production in human PBMCs to further elucidate the impact of linkers on the ability of agonists to induce TLR9-mediated immune stimulation. Multiple cytokine and chemokine profiles were analyzed using the Luminex multiplex assay. All agonists, containing different linkers, induced production of a number of cytokines/chemokines at higher levels than did control compound **10**, suggesting that these linkers are tolerated by human TLR9. However, variation in cytokine/chemokine induction profiles (Figure 4 and Supporting Information) with different linkers suggests that the linker plays a role in TLR9-mediated immune stimulation. At 2 μg/mL, agonists containing both cyclic and acyclic C3−C5 linkers (agonists **11**−**15**) induced similar levels of IL-6, but agonists **17** and **18** containing tetra or hexaethyleneglycol linkers induced significantly lower IL-6 levels. Conversely, agonists containing other linkers induced comparable levels of IL-12 at 2 μg/mL. Agonist **16** induced low levels of IL-12 and IL-6 (data not shown). These results provide evidence that the length of linkers plays a role in TLR9-mediated cytokine/chemokine induction and that this induction by agonists can be modulated by incorporating the appropriate linker.

Human B Cell Activation by TLR9 Agonists Containing Different Linkers. We examined human B cell proliferation induced by TLR9 agonists containing different linkers to evaluate the effect of the linker on TLR9-mediated B cell activation. All agonists induced dose-dependent B-cell proliferation compared with control compound **10** (Figure 5). Similar to previous observations, agonists **17** and **18** containing tetra and hexaethyleneglycol linkers, respectively, induced slightly lower levels of B cell proliferation than did agonists containing other linkers (Figure 5). The B cell proliferative ability of agonist **16** was similar to that of **17** and **18** (data not shown). Agonists containing either acyclic or cyclic C3−C5 linkers induced almost identical levels of B cell proliferation. These results suggest that TLR9 recognizes synthetic agonists containing new linkers to different extents and that the different agonists induce distinct B cell responses; specifically, C5 and *cis*,*trans*- linkers are more favorable for TLR9-mediated B cell proliferation.

Impact of Linkers on In Vivo Activity of TLR9 Agonists. Agonists with CpR dinucleotides induce production of serum cytokines in vivo. Therefore, immune-stimulatory profiles of TLR9 agonists containing new linkers were studied in C57BL/6 mice at a dose of 1 mg/kg. Agonists were administered subcutaneously (sc), and serum IL-12 levels were measured

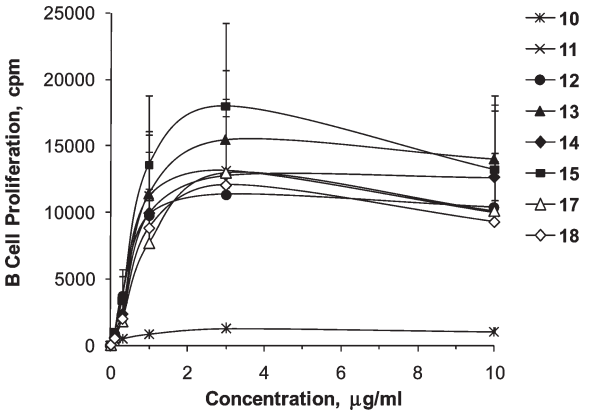


Figure 5. B-cell proliferation induced by agonists **11**−**15**, **17**, **18**, and control compound **10** containing different linkers. Human B-cells isolated from PBMCs obtained from healthy human volunteers were stimulated with agonists at various concentrations and [^{3}H]-thymidine uptake was determined by scintillation counting and the data are shown as counts per minute (cpm). The data shown are mean ± SD of four donors.

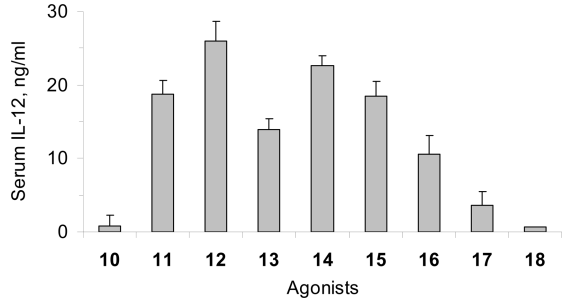


Figure 6. IL-12 secretion in C57BL/6 mice induced by agonists **11**−**18** and control compound **10** following sc administration at a dose of 1 mg/kg. Blood was collected 2 h after agonist administration and IL-12 in the serum was determined by ELISA as described in Experimental Section. Each value is an average of three mice ± SD.

by ELISA in blood samples collected 2 h after administration of the agonist. As shown in Figure 6, all agonists studied, except **18**, induced higher levels of IL-12 than did the control compound **10**. IL-12 levels induced by these agonists varied widely, suggesting that linkers play a role in TLR9-mediated immune responses. The agonists that exhibited higher activity in mouse spleen cell culture assays also induced higher levels of IL-12 in vivo (Figure 6). Agonist **13** containing a C5 linker induced slightly lower levels of IL-12 than did agonist **11** containing a C3 linker. Similarly, agonist **14** containing a *cis*,*cis*-linker induced slightly higher levels of IL-12 than did agonist **15** containing a *cis*,*trans*-linker (Figure 6). Agonist containing cyanurate linker (agonist **16**) induced lower levels of IL-12 than C5- linker containing agonist **12**. Consistent with in vitro studies, incorporation of tetra (agonist **17**) or hexaethyleneglycol (agonist **18**) linkers greatly reduced the in vivo activity of the agonist, suggesting that linkers longer than five carbon atoms are not optimal for TLR9-mediated immune responses. These in vivo results demonstrate that the length and nature of the linker play a role in TLR9 recognition and subsequent immune stimulation.

The results presented here suggest that dCp7-deaza-dG dinucleotide motif is recognized by TLR9 leading to potent TLR9-mediated immune responses. Our previous studies showed that an accessible 5′-end of oligonucleotide is required for TLR9-mediated immune responses.[9,10,12,13] Moreover, TLR9 agonists consisting of two 5′-ends bind to TLR9 strongly[17] and induce rapid and greater TLR9-mediated immune responses compared with agonists containing single 5′-end.[9,10,12,13] Recent studies have shown that TLR9 exists in dimer form and the binding of agonists to the receptor brings conformational changes in the endodomain of the receptor leading to the recruitment of adoptor molecules and the subsequent activation of immune signaling pathways.[18] Non-CpG oligonucleotides also bind to TLR9 but are not capable of bringing conformational changes in the endodomain, failing to activate immune signaling pathways.[18] The results of the present studies suggest that TLR9 agonists containing two 5′-ends effectively bind to the two monomeric units of TLR9 and stabilize dimerization of the receptor leading to potent induction of TLR9-mediated immune responses. This notion is further supported by the observation that various levels of immune responses are induced by agonists containing different lengths and natures of linkers.

Conclusions

We have synthesized solid-support functionalized novel non-nucleoside linkers and successfully incorporated the new linkers into TLR9 agonists. The new compounds have been systematically studied both in vitro and in vivo to understand the impact of length and nature of the linker present in agonists on TLR9-mediated immune stimulation of agonists. Compounds with C3−C5 linkers and rigid linkers with different stereochemistry have minimal impact on TLR9 agonist-induced immune stimulation. Incorporation of longer and more flexible linkers, such as cyanurate, tetra, and hexaethyleneglycol linkers, significantly reduced the immune-stimulatory activity of agonists. These results demonstrate that the length and nature of the linker in TLR9 agonists play a role in the recognition of TLR9 and subsequent TLR9-mediated signaling, resulting in varying immune response profiles. The ability to modulate TLR9-mediated immune responses in a desired fashion using different linkers allows researchers to design novel TLR9 agonists for treating specific disease indications as required.

Experimental Section

Chemistry. General Methods. Unless otherwise noted, all commercially available starting materials, reagents, and solvents of anhydrous quality were obtained from Sigma-Aldrich (St. Louis, MO) and used without further purification. Thin layer chromatography (TLC) was performed on silica gel 60 F_{254} coated on aluminum sheets and visualized by UV light or by a 5% phosphomolybdic acid (PMA) solution. Flash column chromatography was performed on silica gel 60 (mesh size 0.040−0.063 mm and 230−400 mesh ASTM) which was obtained from EMD Chemicals (Gibbstown, NJ), and the required solvents were purchased from J. T. Baker (Phillipsburg, NJ). NMR spectra were performed on Varian 400 MHz Unity Inova instrument. Chemical shifts (δ) are in ppm relative to TMS and the coupling constants (J) are in Hz. HRMS (ES+/TOF) was performed on the Micromass LCT-TOF mass spectrometer. Long chain alkyl amine controlled pore glass (LCAA-CPG; 120−200 mesh, 500 Å, 90−120 μmol/g NH_2 groups) was obtained from Millipore (Lincoln Park, NJ).

General Procedure: Synthesis of *Bis*-DMT Protected Linkers 2a−c,e. Triol **1a**−**c** and **e** (20 mmol) and DMAP (250 mg, 2 mmol) were dissolved in dry pyridine (100 mL), cooled to 0 °C, and maintained under argon atmosphere. DMTCl (14.91 g,

44 mmol) in pyridine (150 mL) was added dropwise to the above stirring solution. After the addition, the reaction mixture was allowed to reach room temperature (~4 h) and continued stirring for overnight. TLC [hexanes/ethyl acetate (EtOAc) 3:1 containing 0.5% triethylamine(TEA)] indicated completion of the reaction. Pyridine was evaporated to dryness, residue was dissolved in EtOAc (500 mL) and washed successively with saturated NH_4Cl solution (500 mL) and brine (500 mL) solution. EtOAc layer was dried over anhydrous $MgSO_4$ and rotoevaporated to dryness. The residue was purified on silica gel flash column chromatography using hexane/EtOAc (3:1) containing 0.5% TEA to give bis-DMT alcohol **2a**−**c**,**e** as a white foam.

Compound **2a** (13.08 g, 92%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 1.65−1.89 (m, 2H, −CH(OH)CH_2CH$_2$−), 2.75 (bs, 1H,OH), 3.05−3.20 (m, 1H, −CH(OH)CH$_2$CH_2−), 3.24−3.42 (m, 1H, −CH(OH)CH$_2$CH_2−), 3.47−3.71 (m, 1H, −CH(OH)CH$_2$-CH$_2$−), 3.82 (d, 12H, −OCH_3), 3.87−4.17 (m, 2H, −CH_2CH-(OH)CH$_2$CH$_2$−), 6.80−6.87 (m, 8H, Ar−H), 7.16−7.45 (m, 18H, Ar−H). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 33.30, 35.00, 55.59, 61.37, 62.03, 67.00, 67.72, 70.12, 71.76, 72.12, 81.76, 86.29, 86.66, 127.00, 128.10, 128.18, 129.46, 130.00, 130.29, 131.35, 136.44, 138.08, 145.00, 145.29, 145.34, 147.66, 158.71, 158.86, 158.96, 159.32. HRMS (ESI/TOF+) calcd for $C_{46}H_{46}O_7$ (M + Na)$^+$ 733.3141, found 733.3133.

Compound **2b** (10.44 g, 72%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 1.65−1.81 (m, 4H, −CH_2CHCH_2−), 3.16−3.31 (m, 4H, DMTO-CH_2−), 3.78 (s, 12H, −OCH_3), 3.95−4.01 (m, 1H, −CH−), 6.81 (d, J = 8.8, 8H, Ar-H), 7.17−7.42 (m, 18H, Ar-H). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 37.22, 55.38, 61.81, 69.58, 86.54, 113.27, 126.88, 128.01, 128.26, 130.15, 136.44, 145.16, 158.55.

Compound **2c**[19] (8.54 g, 58%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 1.04−1.13 (m, 3H, 2,4 and 6-CH_2−), 1.24−1.28 (m, 3H, 2,4 and 6-CH_2−), 1.66 (d, 1H, 1-OH), 2.84−2.93 (m, 1H, −CH-OH), 3.10−3.18 (m, 2H, 3 and 5-CH−), 3.78 (d, 12H, −OCH_3), 6.78 (d, J = 8.8, 8H, Ar-H), 7.16−7.42 (m, 18H, Ar-H). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 41.42, 42.67, 55.37, 66.16, 67.91, 86.28, 113.12, 126.83, 127.81, 128.53, 130.41, 137.39, 146.29, 158.53. HRMS (ESI/TOF+) calcd for $C_{48}H_{48}O_7$ (M + Na)$^+$ 759.3298, found 759.3335.

Compound **2e**[20] (9.0 g, 52%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 2.73 (bs, 1H, −OH), 3.36−3.44 (m, 2H, −NCH_2CH$_2$OH), 3.76−3.89 (m, 4H, −NCH_2CH$_2$ODMT), 4.07 (m, 2H, −NCH$_2$CH_2OH), 4.13 (m, 4H, −NCH$_2$CH_2ODMT), 3.80 (s, 12H, −OCH_3), 6.74−6.86 (m, 8H, Ar-H), 7.15−7.39 (m, 18H, Ar-H).

***trans*-1,3-Bis-(4,4-dimethoxytrityloxy)-*cis*-5-hydroxy-cyclohexane (2d).** To a solution of **9** (4.25 g, 5 mmol) in THF (20 mL) at 0 °C was added TBAF (1 M in THF, 20 mL, 20 mmol) over 5 min, and stirring was continued at room temperature for 6 h. The reaction was diluted with dichloromethane (DCM; 100 mL) and quenched with saturated NH_4Cl (100 mL) solution. The organic layer was separated, and the aqueous layer was extracted with DCM (2 × 100 mL). The combined organic phase was dried over anhydrous $MgSO_4$ and concentrated. Flash column chromatography of the residue on silica gel using 5:1 hexanes/EtOAc mixture containing 0.5% TEA gave pure **2d** (3.34 g, 91%) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.90−1.04 (m, 3H, 3HCH_{ax}) 1.26 (m, 1H, HCH_{eq}), 1.35−1.45 (m, 3H, 2HCH_{eq} and HC-OH), 2.14 (m, 1H, HCOH), 3.73 (d, J=4, 12H, 4× −OCH_3), 4.03 (m, 1H, HCODMT), 4.12 (m, 1H, HCODMT), 6.80 (m, 8H, ArH), 7.14−7.30 (m, 14H, ArH), 7.36 (dd, 4H, J = 7 and 1.2, ArH). ^{13}C NMR (100 MHz, $CDCl_3$): δ 40.37, 40.45, 55.37, 59.54, 67.19, 67.34, 69.87, 113.16, 113.25, 113.38, 126.85, 126.93, 127.82, 127.94, 128.06, 128.48, 128.61, 129.34, 130.54, 136.93, 137.07, 137.39, 137.48, 146.06, 146.41, 158.62, and 158.69. HRMS (ESI/TOF+) calcd for $C_{48}H_{48}O_7$ (M+Na)$^+$ 759.3298, found 759.3280.

General Procedure: Synthesis of Bis-DMT Protected Linker Succinates 3a−e. Succinic anhydride (0.75 g, 7.5 mmol) was added in portions to a stirring solution of bis-DMT alcohol **2** (5 mmol) and DMAP (0.61 g, 5 mmol) in dry pyridine (50 mL) at room temperature. The reaction mixture was stirred for overnight and rotoevaporated to dryness. The residue was dissolved in DCM (250 mL) and successively washed with ice cold 10% citric acid solution (2 × 250 mL) and water (250 mL). The DCM layer was dried over anhydrous $MgSO_4$, concentrated to 50 mL volume using rotoevaporator, and purified on silica gel flash column chromatography using 0 → 2% methanol in DCM containing 0.5% TEA. Pure product was white foam obtained as triethylammonium salt of succinate.

Compound **3a** (3.28 g, 81%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 1.32 (t, 9H, J = 7.6,−N(CH$_2$CH_3)$_3$), 1.92 (m, 2H, −CHCH_2CH$_2$−), 2.94−3.22 (m, 10H, −CH_2CHCH_2CH$_2$− and −N(CH_2CH$_3$)$_3$), 3.75 (d, 12H, −OCH_3), 5.21−5.31 (m, 1H, −CH$_2$CHCH$_2$CH$_2$−), 6.76 (m, 8H, Ar-H), 7.16−7.40 (m, 18H, Ar-H). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 8.89, 30.71, 31.82, 45.50, 55.49, 59.71, 64.81, 71.20, 86.19, 113.36, 126.91, 128.00, 128.06, 128.45, 130.34, 136.36, 136.68, 145.32, 145.41, 158.60, 158.65, 172.86, 177.03. HRMS (ESI/TOF+) calcd for $C_{50}H_{50}O_{10}$ (M+Na)$^+$ 833.3302, found 833.3312.

Compound **3b** (3.42 g, 83%). ^{1}H NMR (CDCl3, 400 MHz): δ 1.20 (t, J = 7.6, 9H, −N(CH$_2$CH_3)$_3$), 1.80−1.85 (m, 4H, −CH_2CHCH_2−), 2.40 (s, 4H, −COCH_2CH_2CO−), 2.90 (q, 6H, −N(CH_2CH$_3$)$_3$), 3.03−3.11 (m, 4H, 1 and 5-CH_2−), 3.78 (s, 12H, −OCH_3), 5.20−5.26 (m, 1H, −CH−), 6.81 (d, J = 8.8, 8H, Ar-H), 7.17−7.42 (m, 18H, Ar-H). ^{13}C NMR (CDCl3, 100 MHz): δ 9.09, 30.94, 31.77, 34.45, 45.08, 52.92, 55.32, 59.73, 69.73, 86.00, 113.12, 126.71, 127.85, 128.32, 130.11, 136.54, 145.22, 158.40, 173.07, and 177.71. HRMS (ESI/TOF+) calcd for $C_{51}H_{52}O_{10}$ (M + Na)$^+$ 847.3458, found 847.3480.

Compound **3c**[19] (3.26 g, 78%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 1.17 (t, J = 7.6, 9H, −N(CH$_2$CH_3)$_3$), 1.17−1.25 (m, 2H, 4-CH_2−), 1.36−1.51 (m, 4H, 2 and 6-CH_2−), 2.37−2.47 (m, 4H, −COCH_2CH_2CO−), 2.86 (q, 6H, −N(CH_2CH$_3$)$_3$), 2.99−3.10 (m, 2H, 3 and 5-CH−), 3.78 (d, 12H, −OCH_3), 3.98−4.08 (m, 1H, 1-CH−), 6.76 (dd, J = 8.8, 8H, Ar-H), 7.15−7.37 (m, 18H, Ar-H). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 9.38, 31.16, 31.96, 39.02, 41.76, 45.18, 55.37, 67.68, 86.24, 113.12, 126.79, 127.77, 128.42, 130.33, 130.38, 137.15, 137.26, 146.28, 158.54, 172.58, and 178.16. HRMS (ESI/TOF+) calcd for $C_{52}H_{52}O_{10}$ (M + Na)$^+$ 859.3458, found 859.3434.

Compound **3d** (3.31 g, 79%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 0.72−0.99 (m, 3H, 3HCH_{ax}), 1.20t, J = 7.2, 9H, −N(CH$_2$-CH_3)$_3$), 1.24 (m, 1H, HCH_{eq}), 1.47 (m, 1H, HCH_{eq}), 1.76 (m, 1H, HCH_{eq}), 2.40−2.49 (m, 4H, −COCH_2CH_2CO−), 2.95 (q, 6H, −N(CH_2CH$_3$)$_3$), 3.70 (d, J=8.2, 6H, 2× −OCH_3), 3.76 (s, 6H, 2× −OCH_3), 3.85 (m, 1H, HCODMT), 4.08 (m, 1H, HCOCO−), 4.79 (m, 1H, HCODMT), 6.73 (m, J = 8.3, 4H, Ar-H), 6.79 (dd, J=8.8 and 3.3, 4H, Ar-H), 7.14−7.27 (m, 12H, Ar-H), 7.35 (dd, 4H, J=8.8 and 3.8, Ar-H), 7.46 (d, 2H, J=7.2, Ar-H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 8.9, 30.14, 45.15, 55.44, 68.05, 69.12, 86.44, 86.66, 113.24, 126.73, 126.85, 127.86, 128.37, 128.61, 130.48, 130.64, 137.00, 137.39, 137.48, 146.15, 146.55, and 158.61. HRMS (ESI/TOF+) calcd for $C_{52}H_{52}O_{10}$ (M + Na)$^+$ 859.3458, found 859.3456.

Compound **3e** (4.44 g, 92%). ^{1}H NMR (CDCl3, 400 MHz): δ 1.41 (t, J = 7.6, 9H, −N(CH$_2$CH_3)$_3$), 2.47−2.68 (m, 4H, −COCH_2CH_2CO−), 3.10 (q, 6H, −N(CH_2CH$_3$)$_3$), 3.75−3.89 (m, 4H, −NCH_2CH$_2$ODMT), 3.80 (s, 12H, −OCH_3), 4.10−4.35 (m, 6H, NCH$_2$CH_2ODMT and NCH_2CH$_2$O−), 4.40−4.51 (m, 2H, NCH$_2$CH_2O−), 6.83 (d, (m, 8H, Ar-H), 7.14−7.31 (m, 18H, Ar-H).

General Procedure: Preparation of Bis-DMT Linker Loaded Controlled-Pore Glass Solid Supports 4a−e. A solution of succinate **3** (1 mmol), DMAP (0.4 g, 3.3 mmol) and DIC (5 mL) in 1:6 mixture of pyridine/acetonitrile (105 mL) was added to controlled-pore glass solid support (25 g), and the slurry was shaken for 24 h at room temperature. Solution was filtered off, and the solid support was washed with acetonitrile

containing 5% pyridine (2 × 100 mL) followed by acetonitrile (3 × 250 mL). Cap A (acetic anhydride in pyridine/THF, 89 mL) and Cap B (1-methylimidazole in THF, 100 mL) solutions were added to solid support and shaken for 4 h. Solutions filtered off and capping reaction was repeated one more time. Finally, solid support was washed thoroughly with acetonitrile containing 5% pyridine, followed by acetonitrile and dried under high vacuum for overnight to get dry solid supports **4a**−**d**. Loading was determined by treating a known amount of linker derivatized solid support with 3% trichloroacetic acid in DCM and assayed DMT content by measuring absorbance at 498 nm.

(1*S*,3*R*,5*S*)-1-Hydroxy-3-(4,4-dimethoxytrityloxy)-5-(*tert*-butyldimethylsilanyloxy)-cyclohexane (6). DMTCl (9.83 g, 29 mmol) in dry pyridine (100 mL) was added dropwise to a stirring solution of *cis*-diol **5**[15,16] (6.66 g, 27 mmol) and DMAP (3.54 g, 29 mmol) in dry pyridine (100 mL) at 0 °C. After the addition, reaction mixture allowed to slowly reach room temperature (∼4 h), and stirring was continued overnight. TLC (hexanes/EtOAc 2:1 mixture containing 0.5% TEA) indicated the completion of the reaction and pyridine was rotoevaporated to dryness. The residue was dissolved in DCM (500 mL) and washed successively with saturated aqueous NH_4Cl solution (500 mL), brine (500 mL), and water (500 mL). The organic layer was dried over anhydrous $MgSO_4$ and rotoevaporated to dryness. The residue was purified on silica gel flash column chromatography using hexanes/EtOAc 3:1 mixture containing 0.5% TEA. Pure compound **6** was obtained as a pale-yellow solid (6.37 g, 43%). ^{1}H NMR (400 MHz, $CDCl_3$): δ −0.90 (d, J = 8.8, 6H, Me_2Si), 0.80 (s, 9H, TBDMS *tert*-butyl), 1.15−1.36 (m, 5H, 3HCH_{ax}, HCH_{eq}, HCO*H*), 1.83 (m, 1H, HCH_{eq}), 2.01 (m, 1H, HCH_{eq}), 3.18−3.26 (m, 1H, *H*CODMT), 3.29−3.45 (m, 2H, *H*C-OTBDMS and *H*C-OH), 3.79 (s, 6H, 2× −OCH_3), 6.81 (dd, 4H, J = 8.8 and 2.0, Ar-*H*), 7.14−7.32 (m, Ar-*H*), 7.39 (dd, 4H, J = 8.8 and 4.4, Ar-*H*), 7.50 (d, 2H, J = 7.2, Ar-*H*). ^{13}C NMR (100 MHz, $CDCl_3$): δ −4.68, −4.54, 18.28, 25.99, 43.03, 43.07, 44.84, 55.37, 66.34, 66.64, 67.88, 86.44, 113.22, 126.93, 127.91, 128.53, 129.41, 130.46, 130.54, 137.30, 137.49, 146.30, and 158.68. HRMS (ESI/TOF+) calcd for $C_{33}H_{44}O_5Si$ (M + Na)$^+$ 571.2856, found 571.2849.

(1*R*,3*R*,5*R*)-1-Benzoyloxy-3-(4,4-dimethoxytrityloxy)-5-(*tert*-butyldimethylsilyloxy)-cyclohexane (7). To a solution of compound **6** (4.4 g, 8 mmol) and triphenylphosphine (2.62 g, 10 mmol) in anhydrous THF (75 mL) was add benzoic acid (1.22 g, 10 mmol) in one portion and maintained under nitrogen atmosphere. The solution was cooled to −10 °C in an ice−salt mixture with stirring, and diisopropyl azodicarboxylate (2.42 g, 12 mmol) in anhydrous THF (25 mL) was added dropwise over a period of 30 min while maintaining the temperature well below 0 °C. The reaction mixture was stirred until it reaches gradually to room temperature in about 5 h. Then solvent was rotoevaporated under vacuum and the residue was purified on a silica gel flash column chromatography using 9:1 hexanes/EtOAc containing 0.5% TEA to afford pure product **7** (4.91 g, 94%) as white solid. ^{1}H NMR ($CDCl_3$): δ −0.03 (d, 6H, J = 1.2, Me_2Si), 0.83 (s, 9H, TBDMS *tert*-butyl), 1.34−1.52 (m, 4H, 3HCH_{ax} and 1HCH_{eq}), 1.94−2.05 (m, 2H, 2HCH_{eq}), 3.72 (d, 6H, J = 6.6, 2× −OCH_3), 3.67−3.82 (m, 2H, *H*C-ODMT and *H*C-OTBDMS), 5.24 (t, 1H, *H*C-OBz), 6.73 (t, 4H, J = 8.8 Ar-*H*), 7.14−7.23 (m, Ar-*H*), 7.36 (dd, 4H, J = 8.8 and 6.0, Ar-*H*), 7.45 (m, 4H, Ar-*H*), 7.57 (m, 1H, Ar-H), 7.80 (d, 2H, J = 8.2, Ar-*H*). ^{13}C NMR (100 MHz, $CDCl_3$): δ −4.56, −4.42, 18.35, 26.04, 37.32, 39.30, 44.11, 55.33, 66.46, 67.62, 70.69, 86.54, 113.17, 126.87, 127.84, 128.48, 128.52, 129.57, 130.40, 130.47, 130.80, 133.00, 137.31, 146.31 158.62 and 165.39. HRMS (ESI/TOF+) calcd for $C_{40}H_{48}O_6Si$ (M + Na)$^+$ 675.3118, found 675.3099.

(1*R*,3*R*,5*S*)-1-Hydroxy-3-(4,4-dimethoxytrityloxy)-5-(*tert*-butyldimethylsilyloxy)-cyclohexane (8). To a solution of compound **7** (4.7 g, 7.2 mmol) in anhydrous methanol (75 mL) was added anhydrous potassium carbonate (1.1 g, 8 mmol), and the reaction mixture was stirred at room temperature for 24 h. Methanol was rotoevaporated, and the residue was dried under vacuum. The residue was dissolved in DCM (250 mL), and insoluble material was filtered off. The DCM solution was washed with brine (250 mL) and dried over anhydrous $MgSO_4$. Solvent was rotoevaporated to dryness, and the resulting residue was purified on silica gel flash column chromatography using 9:1 hexanes/EtOAc containing 0.5% TEA to afford pure product **8** (3.79 g, 96%) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ −0.05 (d, 6H, J = 2.8, Me_2Si), 0.83 (s, 9H, TBDMS *tert*-butyl), 1.24−1.35 (m, 3H, 3HCH_{ax}), 1.66 (m, 1H, HCH_{eq}), 1.83 (m, 1H, HCH_{eq}), 3.67−3.75 (m, 1H, *H*C-OTBDMS), 3.78 (s, 6H, 2× −OCH_3), 3.81−3.89 (m, 1H, *H*C-ODMT), 4.04 (bs, 1H, *H*C-OH), 6.81 (d, 4H, J = 8.8 Ar-*H*), 7.16−7.28 (m, 3H, Ar-*H*), 7.40 (dd, 4H, J = 8.8 and 2.2, Ar-*H*), 7.51 (m, 2H, Ar-*H*). ^{13}C NMR (100 MHz, $CDCl_3$): δ −4.61, −4.45, 0.21, 11.73, 18.36, 26.08, 40.36, 41.64, 44.29, 46.42, 55.39, 66.00, 67.19, 67.26, 86.40, 113.14, 126.89, 127.85, 128.63, 129.34, 130.59, 137.58, 137.61, 146.40, and 158.66. HRMS (ESI/TOF+) calcd for $C_{33}H_{44}O_5Si$ (M + Na)$^+$ 571.2856, found 571.2851.

***trans*-1,3-bis-(4,4-Dimethoxytrityloxy)-*cis*-5-(*tert*-butyldimethylsilyloxy)-cyclohexane (9).** DMTCl (3.42 g, 10.1 mmol) in dry pyridine (50 mL) was added dropwise to a stirring solution of **8** (3.7 g, 6.74 mmol) and DMAP (1.23 g, 10.1 mmol) in dry pyridine (50 mL) at room temperature. After stirring for 24 h at room temperature, pyridine was rotoevaporated to dryness. The residue was dissolved in DCM (500 mL) and washed successively with NH_4Cl (500 mL), brine (500 mL), and water (500 mL). The organic layer was dried over anhydrous $MgSO_4$, rotoevaporated, and the residue was purified by silica gel flash column chromatography using 0→10% gradient of EtOAc in hexanes containing 0.5% TEA to get pure compound **9** (4.7 g, 82%) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ −0.18 (d, J = 11.6, 6H, Me_2Si), 0.73 (s, 9H, TBDMS *tert*-butyl), 1.12−1.28 (m, 3H, 3HCH_{ax}) 1.71 (m, 1H, HCH_{eq}), 1.83−2.01 (m, 1H, HCH_{eq}), 3.56−3.65 (m, 1H, *H*CODMT), 3.86 (m, 1H, *H*C-OTBDMS), 4.04−4.14 (m, 1H, *H*CODMT), 3.71 (d, J = 7.2, 6H, 2× −OCH_3), 3.78 (d, J = 2, 6H, 2× −OCH_3), 6.74 (d, 4H, J = 7.7, Ar-*H*), 6.81 (dd, 4H, J = 9.2 and 1.8, Ar-*H*), 7.12−7.30 (m, 12H, Ar-*H*), 7.43 (dd, 4H, J = 6.5 and 2.2, Ar-*H*), 7.53 (d, 2H, J = 7.6, Ar-*H*). ^{13}C NMR (100 MHz, $CDCl_3$): δ −4.42, 0.22, 14.42, 18.23, 26.08, 39.96, 40.18, 44.24, 55.29, 55.37, 66.61, 68.08, 68.78, 86.30, 86.40, 113.10, 113.13, 113.38, 126.74, 126.79, 127.79, 128.50, 128.70, 129.34, 130.41, 130.75, 137.20, 137.33, 137.62, 137.72, 146.21, 146.74, and 158.55. HRMS (ESI/TOF+) calcd for $C_{54}H_{62}O_7Si$ (M + Na)$^+$ 873.4163, found 873.4138.

Synthesis of TLR9 Agonists 10−18. All agonists were synthesized on a 2−10 μmol scale, as DMT-off, using β-cyanoethyl-phosphoramidite chemistry[9,10,21,22] on a MerMade 6 synthesizer (Bioautomation, Inc., Plano, TX). Unmodified 3′-phosphoramidites were obtained from Glen Research (Sterling, VA). Glycerol loaded solid support, 7-deaza-dG, ethylene glycol linker phosphoramidites, and all 5′-phosphoramidites were obtained from ChemGenes Corporation (Wilmington, MA). Beaucage reagent was used as an oxidizing agent to obtain the phosphorothioate backbone modification.[23] A modified coupling protocol recommended by the supplier was used for 7-deaza-dG and 5′-phosphoramidites incorporation. After the synthesis, oligodeoxynucleotides were cleaved from the solid support and deprotected using standard protocols. Crude oligodeoxynucleotides were purified on preparative anion-exchange HPLC (Waters Delta 600 system) equipped with Source 15Q (GE Healthcare Biosciences-AB, Uppsala, Sweden) column (250 mm × 20 cm). The mobile phases used were (A) 25 mM Tris-HCl, pH 7.5 buffer containing 20% ACN and (B) 25 mM Tris-HCl, pH 7.5 buffer containing 20% ACN and 2 M NaCl. After purification, oligodeoxynucleotides were desalted on C18 reversed phase-HPLC column and dialyzed against United States Pharmacopeia-quality sterile water for irrigation (B. Braun Medical, Inc., USA). All oligodeoxynucleotides synthesized were lyophilized, reconstituted in distilled water, and the concentrations were determined by measuring UV

absorbance at 260 nm. The purity of TLR9 agonists synthesized was found to be ≥95% and the rest being shorter by one or two nucleotides ($n-1$ and $n-2$) as determined by analytical anion-exchange HPLC (DNA Pack100 column), capillary gel electrophoresis (P/ACE MDQ system, Beckman Coulter, Inc., Brea, CA) and denaturing PAGE. The sequence integrity was characterized by MALDI-TOF mass spectrometry (Micro MX, Waters Co., USA). All of the compounds were tested for endotoxin by *Limulus* assay (Bio-Whittaker), and the endotoxin levels were found to be < 0.5 EU/mL.

HEK293 Cell Cultures. HEK293 cells stably expressing mouse TLR9 (Invivogen, San Diego, CA) were cultured in 96-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 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 6 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 agonist treatment, 20 μL of culture supernatant was taken from each well and used for SEAP assay following manufacturer's protocol (Invitrogen). Briefly, culture supernatants were incubated with QuantiBlue substrate, and the blue color generated was measured by a plate reader at 620−645 nm. The data are shown as fold increase in NF-κB activity over PBS control.

Human PBMC Isolation and Cytokine Multiplex Assay. Peripheral blood mononuclear cells (PBMCs) were isolated from freshly drawn healthy volunteer blood (Research Blood Components, Brighton, MA) by Ficoll density gradient centrifugation (Ficoll Paque PLUS, GE Health Care). PBMCs were plated in 96-well plates at a concentration of 5×10^6 cells/mL. The agonists dissolved in phosphate-buffered saline (PBS) were added to the cell cultures at a final concentration of 2 or 10 μg/mL. The cells were then incubated at 37 °C for 24 h. The levels of cytokines/chemokines in the culture supernatants were measured by Luminex multiplex assay using human 25-plex kits from Invitrogen.

Human B Cell Proliferation Assay. About 1×10^5 B-cells purified from human PBMCs as described previously[12] were stimulated with different concentrations of agonists for 56 h, 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 (Microbeta Trilus, Perkin-Elmer, USA). The data are shown as counts per minute (cpm).

In Vivo Studies in Mice. Four- to six-week-old C57BL/6 mice were obtained from Charles River Laboratories (Wilmington, MA). All the animal studies reported in the paper were carried out in accordance with Idera's IACUC-approved animal protocols and guidelines. Female C57BL/6 mice ($n = 3$) were injected sc with agonists at 1 mg/kg dose (single-dose). Blood was collected by retro-orbital bleeding 2 h after agonist administration and serum IL-12 levels were determined by sandwich ELISA.

Acknowledgment. We thank Dr. Sudhir Agrawal for support, encouragement, and suggestions.

Supporting Information Available: Table showing cytokine and chemokine induction in human PBMCs by agonists of TLR9 containing different lengths of linkers. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Hemmi, H.; Takeuchi, O.; Kawai, T.; Kaisho, T.; Sato, S.; Sanjo, H.; Matsumoto, M.; Hoshino, K.; Wagner, H.; Takeda, K.; Akira, S. A Toll-like receptor recognizes bacterial DNA. *Nature* **2000**, *408*, 740–745.

(2) Latz, E.; Schoenemeyer, A.; Visintin, A.; Fitzgerald, K. A.; Monks, B. G.; Knetter, C. F.; Lien, E.; Nilsen, N. J.; Espevik, T.; Golenbock, D. T. TLR9 signals after translocating from the ER to CpG DNA in the lysosome. *Nature Immunol.* **2004**, *5*, 190–198.

(3) Roman, M.; Martin-Orozco, E.; Goodman, J. S.; Nguyen, M. D.; Sato, Y.; Ronaghy, A.; Kornbluth, R. S.; Richman, D. D.; Carson, D. A.; Raz, E.; Immunostimulatory, D. N. A. sequences function as T helper-1-promoting adjuvants. *Nat. Med.* **1997**, *3*, 849–854.

(4) Klinman, D. M. Immunotherapeutic uses of CpG oligodeoxynucleotides. *Nat. Rev. Immunol.* **2004**, *4*, 249–258.

(5) Agrawal, S.; Kandimalla, E. R. Synthetic agonists of Toll-like receptors 7, 8, and 9. *Biochem. Soc. Trans.* **2007**, *35*, 1461–1467.

(6) Agrawal, S.; Kandimalla, E. R. Modulation of Toll-like receptor 9 responses through synthetic immunostimulatory motifs of DNA. *Ann. N.Y. Acad. Sci.* **2003**, *1002*, 30–42.

(7) (a) Yu, D.; Kandimalla, E. R.; Cong, Y.; Tang, J.; Tang, J. Y.; Agrawal, S. Design, synthesis, and immunostimulatory properties of CpG DNAs containing alkyl-linker substitutions: Role of nucleosides in the flanking sequences. *J. Med. Chem.* **2002**, *45*, 4540–4548. (b) Yu, D.; Kandimalla, E. R.; Zhao, Q.; Bhagat, L.; Cong, Y.; Agrawal, S. Requirement of nucleotide bases proximal to a CpG-motif for immunostimulatory activity of synthetic oligonucleotides. *Bioorg. Med. Chem.* **2003**, *11*, 459–464. (c) Yu, D.; Kandimalla, E. R.; Zhao, Q.; Cong, Y.; Agrawal, S. Immunostimulatory properties of phosphorothioate CpG DNA containing both 3′-5′- and 2′-5′-internucleotide linkages. *Nucleic Acids Res.* **2002**, *30*, 1613–1619. (d) Yu, D.; Kandimalla, E. R.; Zhao, Q.; Cong, Y.; Agrawal, S. Immunostimulatory activity of CpG oligonucleotides containing non-ionic methylphosphonate linkages. *Bioorg. Med. Chem.* **2001**, *9*, 2803–2808. (e) Yu, D.; Kandimalla, E. R.; Zhao, Q.; Cong, Y.; Agrawal, S. Modulation of immunostimulatory activity of CpG oligonucleotides by site-specific deletion of nucleobases. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 2263–2267.

(8) (a) Kandimalla, E. R.; Bhagat, L.; Cong, Y. P.; Pandey, R. K.; Yu, D.; Zhao, Q.; Agrawal, S. Secondary structures in CpG oligonucleotides affect immunostimulatory activity. *Biochem. Biophys. Res. Commun.* **2003**, *306*, 948–953. (b) Cong, Y. P.; Song, S. S.; Bhagat, L.; Pandey, R. K.; Yu, D.; Kandimalla, E. R.; Agrawal, S. Self-stabilized CpG DNAs optimally activate human B cells and plasmacytoid dendritic cells. *Biochem. Biophys. Res. Commun.* **2003**, *310*, 1133–1139. (c) Yu, D.; Putta, M. R.; Bhagat, L.; Dai, M.; Wang, D.; Trombino, A. F.; Sullivan, T.; Kandimalla, E. R.; Agrawal, S. Impact of secondary structure of Toll-like receptor 9 agonists on interferon-α induction. *Antimicrob. Agents Chemother.* **2008**, *52*, 4320–4325.

(9) (a) Yu, D.; Zhao, Q.; Kandimalla, E. R.; Agrawal, S. Accessible 5′-end of CpG-containing phosphorothioate oligodeoxynucleotides is essential for immunostimulatory activity. *Bioorg. Med. Chem. Lett.* **2000**, *10*, 2585–2588. (b) Kandimalla, E. R.; Bhagat, L.; Yu, D.; Cong, Y.; Tang, J.; Agrawal, S. Conjugation of ligands at the 5′-end of CpG DNA affects immunostimulatory activity. *Bioconjugate Chem.* **2002**, *13*, 966–974. (c) Putta, M. R.; Zhu, F. G.; Wang, D.; Bhagat, L.; Dai, M.; Kandimalla, E. R.; Agrawal, S. Peptide conjugation at the 5′-end of oligodeoxynucleotides abrogates Toll-like receptor 9-mediated immune stimulatory activity. *Bioconjugate Chem.* **2010**, *21*, 39–45.

(10) Yu, D.; Kandimalla, E. R.; Bhagat, L.; Tang, J. Y.; Cong, Y.; Tang, J.; Agrawal, S. "Immunomers"-novel 3′-3′-linked CpG oligodeoxyribonucleotides as potent immunomodulatory agents. *Nucleic Acids Res.* **2002**, *30*, 4460–4469.

(11) (a) Kandimalla, E. R.; Yu, D.; Zhao, Q.; Agrawal, S. Effect of chemical modifications of cytosine and guanine in a CpG-motif of oligoribonucleotides: Structure−immunostimulatory activity relationships. *Bioorg. Med. Chem.* **2001**, *9*, 807–813. (b) Yu, D.; Putta, M. R.; Bhagat, L.; Li, Y.; Zhu, F.; Wang, D.; Tang, J. X.; Kandimalla, E. R.; Agrawal, S. Agonists of Toll-like receptor 9 containing synthetic dinucleotide motifs. *J. Med. Chem.* **2007**, *50*, 6411–6418. (c) Kandimalla, E. R.; Bhagat, L.; Wang, D.; Yu, D.; Zhu, F. G.; Tang, J.; wang, H; Huang, P.; Zhang, R.; Agrawal, S. Divergent synthetic nucleotide motif recognition pattern: design and development of potent immunomodulatory oligodeoxynucleotide agents with distinct cytokine induction profiles. *Nucleic Acids Res.* **2003**, *31*, 2393–2400. (d) Putta, M. R.; Zhu, F. G.; Li, Y.; Bhagat, L.; Cong, Y.; Kandimalla, E. R.; Agrawal, S. Novel oligodeoxynucleotide agonists of TLR9 containing

N^3-Me-dC or N^1-Me-dG modifications. *Nucleic Acids Res.* **2006**, *34*, 3231–3238.
(12) Kandimalla, E. R.; Bhagat, L.; Li, Y.; Yu, D.; Wang, D.; Cong, Y. P.; Song, S. S.; Tang, J. X.; Sullivan, T.; Agrawal, S. Immunomodulatory oligonucleotides containing a cytosine-phosphate-2′-deoxy-7-deazaguanosine motif as potent toll-like receptor 9 agonists. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 6925–6930.
(13) Kandimalla, E. R.; Bhagat, L.; Zhu, F. G.; Yu, D.; Cong, Y. P.; Wang, D.; Tang, J. X.; Tang, J. Y.; Knetter, C. F.; Lien, E.; Agrawal, S. A dinucleotide motif in oligonucleotides shows potent immunomodulatory activity and overrides species-specific recognition observed with CpG motif. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 14303–14308.
(14) Hollenstein, M.; Leumann, C. J. Synthesis and incorporation into PNA of fluorinated olefinic PNA (F-OPA) monomers. *Org. Lett.* **2003**, *5*, 1987–1990.
(15) Fielden, J.; Sprott, J.; Cronin, L. Design and sterospecific synthesis of modular ligands based upon *cis*-1,3-*trans*-5-substituted cyclohexanes. *New J. Chem.* **2005**, *29*, 1152–1158.
(16) Hilpert, H.; Wirz, B. Novel versatile approach to an enantiopure 19-nor, des-C, D vitamin D3 derivative. *Tetrahedron* **2001**, *57*, 681–694.
(17) Knetter, C. F.; Kandimalla, E. R.; Yu, D.; Agrawal, S.; Espevik, T.; Lien, E. 5′-Accessibility of CpG DNA is necessary for strong interaction with TLR9 and activation of receptor signaling. Presented at 38th Annual Meeting of the Society for Leukocyte Biology, Oxford, England, September 21−24, 2005.
(18) Latz, E.; Verma, A.; Visintin, A.; Gong, M.; Sirois, C. M.; Klein, D. C.; Monks, B. G.; McKnight, C. J.; Lamphier, M. S.; Duprex, W. P.; Espevik, T.; Golenbock, D. T. Ligand-induced conformational changes allosterically activate Toll-like receptor 9. *Nat Immunol.* **2007**, *8*, 772–779.
(19) De Napoli, L.; Di Fabio, G.; Messere, A.; Montesarchio, D.; Musumeci, D.; Piccialli, G. Synthesis and characterization of new 3′-3′ linked oligodeoxyribonucleotides for alternate strand triple helix formation. *Tetrahedron* **1999**, *55*, 9899–9914.
(20) Iyer, R. S.; Su, S. H.; Inamdar, A.; Kalra, K. L. Multifunctional linking reagents for synthesis of branched oligomers. U.S. Patent 5,916,750, **1999**.
(21) Kandimalla, E. R.; Agrawal, S.; Venkataraman, G.; Sasisekharan, V. Single strand targeted triplex formation: parallel-stranded DNA hairpin duplexes for targeting pyrimidine strands. *J. Am. Chem. Soc.* **1995**, *117*, 6416–6417.
(22) Chaix, C.; Iyer, R. P.; Agrawal, S. 3′-3′-linked oligonucleotides: Synthesis and stability studies. *Bioorg. Med. Chem. Lett.* **1996**, *6*, 827–832.
(23) Iyer, R. P.; Egan, W.; Regan, J. B.; Beaucage, S. L. 3*H*-1,2-Benzodithiole-3-one 1,1-dioxide as an improved sulfurizing reagent in the solid-phase synthesis of oligodeoxyribonucleoside phosphorothioates. *J. Am. Chem. Soc.* **1990**, *112*, 1253–1254.