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Novel Oligonucleotides Containing Two 3′-Ends Complementary to Target mRNA Show Optimal Gene-Silencing Activity

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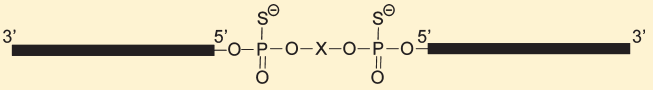
Supporting Information

ABSTRACT: Oligonucleotides are being employed for gene-silencing activity by a variety of mechanisms, including antisense, ribozyme, and siRNA. In the present studies, we designed novel oligonucleotides complementary to targeted mRNAs and studied the effect of 3′-end exposure and oligonucleotide length on gene-silencing activity. We synthesized both oligoribonucleotides (RNAs) and oligodeoxynucleotides (DNAs) with phosphorothioate backbones, consisting of two identical segments complementary to the targeted mRNA attached through their 5′-ends, thereby containing two accessible 3′-ends; these compounds are referred to as gene-silencing oligonucleotides (GSOs). RNA and/or DNA GSOs targeted to MyD88, VEGF, and TLR9 mRNAs had more potent gene-silencing activity than did antisense phosphorothioate oligonucleotides (PS-oligos) in cell-based assays and in vivo. Of the different lengths of GSOs evaluated, 19-mer long RNA and DNA GSOs had the best gene-silencing activity both in vitro and in vivo. These results suggest that GSOs are novel agents for gene silencing that can be delivered systemically with broader applicability.



INTRODUCTION

Over the last three decades, oligonucleotide-based gene-silencing agents that act through various mechanisms of action such as antisense, ribozyme, and siRNA have been studied. The use of oligodeoxynucleotides for gene silencing through an antisense mechanism of action was first described in 1978.[1] Natural phosphodiester-backbone oligonucleotides are taken up by cells efficiently and hybridize to the target mRNA effectively and selectively, but they are highly susceptible to nuclease degradation thereby limiting their gene-silencing activity.[2] To stabilize oligonucleotides against nuclease degradation, a number of backbone-modified oligonucleotides have been reported.[3−7] Oligonucleotides that were modified so that a nonbridging oxygen on phosphorus was replaced with a sulfur, referred to as phosphorothioate oligodeoxynucleotides (PS-oligos), had desirable properties such as stability in biological fluids and broad tissue disposition following systemic delivery required for antisense applications.[2,8] Studies of PS-oligos as antisense agents yielded mixed results, however, as many of the antisense sequences evaluated induced immune activation through Toll-like receptor (TLR) 9, thereby interfering with the intended mechanism of action.[9−11] To further improve the gene-silencing activity of antisense PS-oligos, we studied modifications of the 3′-ends, including a hairpin or 3′-3′-linkage.[12−14]

Antisense PS-oligoribo- (PS-RNA) and PS-2′-O-substituted-RNA have also been evaluated, but these compounds demonstrated lower activity than did antisense PS-oligos.[15,16] These RNA analogues did, however, form stable duplexes with the target mRNA and exerted minimal polyanionic-related side effects such as complement activation and prolongation of partial thromboplastin time.[17] On the basis of the results of studies of antisense PS-oligos and PS-RNA/2′-O-substituted-RNA, we created antisense compounds that have segments of PS-oligo and PS-2′-O-substituted-RNA with the desirable properties of both oligonucleotides, referred to as mixed-backbone oligonucleotides or second-generation antisense.[18,19] Second-generation antisense agents provided improved stability in biological fluids,[19,20] broad tissue disposition following systemic administration,[20] including oral delivery,[21] and reduced immune stimulatory activity.[19,20d] Currently, a number of second-generation antisense oligonucleotides targeted to various gene targets are in clinical development.[22,23]

RNA molecules with enzymatic activity, referred to as ribozymes, were first described in the early 1980s.[24] Ribozymes form base pairs with target mRNA and catalyze the hydrolysis of specific phosphodiester bonds in the target, resulting in gene silencing. A number of chemical modifications of ribozymes have been tried to improve their nuclease stability and delivery.[23] Several ribozymes have been evaluated in clinical trials without much success, mainly due to the lack of effective delivery.[25]

In the late 1990s, in a complementary approach, referred to as RNA interference (RNAi), double-stranded RNA (dsRNA) was shown to silence gene expression catalytically.[26] Later studies have shown that short dsRNAs (siRNAs) of 19−21 nucleotides long with two nucleotide overhangs at the 3′-end complementary to mRNAs efficiently silence gene expression through the RNA-induced silencing complex (RISC).[27] Further studies have shown that single-stranded RNAs complementary to mRNAs can also

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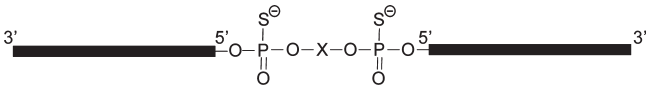

Figure 1. Structure of a gene-silencing oligonucleotide (GSO). The two DNA or RNA segments are attached at their 5′-ends via phosphorothioate linkages to a glycerol linker (X).

efficiently induce gene silencing, suggesting that both dsRNA and ssRNA act through RNAi pathway.[28] Several siRNAs targeted to various mRNAs are in clinical evaluation,[29,30] but problems with their delivery and question about the mechanism of action[31] led to discontinuation of clinical development of several other siRNA candidates.[29]

Several studies have shown that conjugation of ligands at the 3′-end of the antisense strand of siRNA leads to loss of gene-silencing activity.[32,33] Similarly, blocking the 3′-end of single-stranded RNA by conjugation of a fluorescein molecule severely limits the gene-silencing activity, suggesting that a free 3′-end is required for activity.[28a,32−34] On the basis of the understanding that the 3′-end of the oligonucleotide is required for gene-silencing activity and that the 5′-end is required for immune activation through TLR9,[32−35] we hypothesized that an oligonucleotide with two 3′-ends may provide optimal gene-silencing activity and that linking the two oligonucleotides through 5′-5′-linkage might avoid immune activation.

In the present study, we designed and synthesized novel phosphorothioate RNA and DNA complementary to targeted mRNAs with two 3′-ends, referred to as gene-silencing oligonucleotides (GSOs). GSOs contain two identical segments of RNA or DNA complementary to targeted mRNA joined via a 5′-5′-linkage thereby having two 3′-ends (Figure 1). We studied GSOs targeted to MyD88, VEGF, and TLR9 in cell-based assays and in vivo in mice.

RESULTS

Design and Synthesis of Gene-Silencing Oligonucleotides. We selected target sites on MyD88, TLR9, and VEGF mRNAs based on criteria described previously and designed complementary sequences for GSO synthesis.[11,12] Oligoribo- (RNA) and deoxynucleotide (DNA) GSOs of varying lengths (15- to 25-mer) were synthesized by linking two identical oligonucleotides complementary to target mRNA through 5′-5′-linkage, thereby generating two 3′-ends (Figure 1). Synthesis was carried out on di-DMT-glycerol-derivatized solid support using 3′-DMT-ribonucleoside-5′-phosphoramidites or 3′-DMT-deoxyribonucleoside-5′-phosphoramidites, respectively, on an automated DNA/RNA synthesizer. After completion of synthesis, crude RNA and DNA GSOs were cleaved from the solid support and deprotected using standard protocols. The crude RNA and DNA GSOs obtained were purified on anion-exchange HPLC and desalted on a C18 column. All RNA and DNA GSOs synthesized were characterized by anion-exchange HPLC and CGE for purity and by MALDI-ToF mass spectral analysis for sequence integrity (Supporting Information (SI), Tables 1, 3, 5, and 7). All GSOs contained >95% full-length compounds as determined by HPLC analysis, with the remainder comprising compounds that were one or two nucleotides shorter. The concentrations of the GSOs were determined by UV absorption measurement at 260 nm. MyD88-targeted DNA and RNA GSOs are shown in Table 1 and SI Table 2, respectively. TLR9- and VEGF-targeted GSOs are shown in SI Tables 4 and 6, respectively.

Binding Affinity of GSOs and Antisense PS-oligos to Target RNA. To study the binding affinity to target RNA, thermal melting stability of 15- to 25-mer lengths of RNA GSOs complementary to TLR9 and DNA GSOs complementary to MyD88 and their target RNA duplex were studied by UV thermal melting experiments. All GSOs showed cooperative dissociation curves as the temperature increased from 5 to 95 °C and the T_m values calculated are shown in SI Tables 1 and 3 for DNA and RNA GSOs, respectively. Thermal stability of the GSO and target RNA duplex was dependent on the length of the GSO. The T_m increased with increasing length of GSO (SI Tables 1 and 3). The T_m values of DNA GSOs and target RNA duplexes increased from 46.2 to 66.7 °C as the length of DNA GSO increased from 15- to 25-mer (SI Table 1). Similar T_m increase from 46.4 to 67.5 °C was observed for 15- to 25-mer long antisense PS-oligos and their target RNA duplexes (SI Table 1). The T_m of duplexes of 15- to 25-mer long RNA GSOs with their target RNA ranged from 65 to 75.4 °C (SI Table 3A). As expected, RNA GSOs showed higher affinity to the target RNA than DNA GSOs as reflected by higher T_m values for duplexes of RNA GSOs and their target RNA. These results suggest that 5′-5′-attachment of GSOs has minimal effect on its affinity to the target RNA and the affinity increases with increasing length.

RNA GSOs Have Length-Dependent Gene-Silencing Activity in the J774 Cell Line. We initially examined the gene-silencing activity of RNA GSOs complementary to TLR9 in the mouse macrophage J774 cell line. J774 cells were incubated in the absence or presence of different lengths (15- to 25-mer) of RNA GSOs for 48 h. Total RNA was isolated and the levels of TLR9 mRNA were determined by RT-PCR. Relative quantities of TLR9 mRNA at 25 μg/mL GSO concentration are shown in Figure 2A. A lower inhibition of mRNA expression (10%) was observed with 15-mer, and a 66% inhibition was observed with 19-mer. RNA GSOs of 21- to 25-mer length showed lower gene-silencing activity than 19-mer RNA GSO. The 19-mer RNA GSO had greater TLR9 gene-silencing activity than shorter or longer RNA GSOs, suggesting a length preference for RNA GSOs for gene-silencing activity.

In Vivo Activity of RNA GSO. Bacterial DNA-containing CpG motifs and immune modulatory oligonucleotides-containing synthetic immune stimulatory motifs are the ligands for TLR9.[36] Upon administration to mice, TLR9 agonists induce immune responses, including IL-12 induction. We evaluated the ability of a 19-mer RNA GSO to suppress TLR9-mediated immune stimulation as determined by serum IL-12 levels in C57BL/6 mice.

Mice were injected subcutaneously with 2, 5, 10, or 25 mg/kg doses of 19-mer RNA GSO in the flank, and 24 h later a TLR9 agonist[36] was administered in the opposite flank. Two hours after TLR9 agonist administration, blood was collected by retro-orbital bleeding, and serum IL-12 levels were measured by ELISA. The data are presented in Figure 2B as percent inhibition of TLR9 agonist-induced IL-12. The 19-mer RNA GSO showed a dose-dependent inhibition of TLR9-mediated activity, with a maximal inhibition of 72% at 25 mg/kg dose.

DNA GSOs for MyD88 Have Length-Dependent Gene-Silencing Activity in J774 Cells. To study if DNA GSOs containing two 3′-ends are optimal for gene silencing as is the case of RNA GSOs, we synthesized DNA GSOs of 15- to 25-mer lengths complementary to MyD88 mRNA. MyD88 is an adapter

Table 1. DNA GSO and Antisense PS-oligo Sequences of Different Lengths Complementary to Mouse and Human MyD88 mRNA[a]

	complementary sequences to mouse MyD88 mRNA (target C361)
	DNA GSOs
15-mer	3′-ACGATCTCGACGACC-X-CCAGCAGCTCTAGCA-3′
17-mer	3′-CGACGATCTCGACGACC-X-CCAGCAGCTCTAGCAGC-3′
19-mer	3′-TCCGACGATCTCGACGACC-X-CCAGCAGCTCTAGCAGCCT-3′
21-mer	3′-CGTCCGACGATCTCGACGACC-X-CCAGCAGCTCTAGCAGCCTGC-3′
23-mer	3′-GCCGTCCGACGATCTCGACGACC-X-CCAGCAGCTCTAGCAGCCTGCCG-3′
25-mer	3′-CAGCCGTCCGACGATCTCGACGACC-X-CCAGCAGCTCTAGCAGCCTGCCGAC-3′
	Antisense PS-oligos
15-mer	5′-CCAGCAGCTCTAGCA-3′
17-mer	5′-CCAGCAGCTCTAGCAGC-3′
19-mer	5′-CCAGCAGCTCTAGCAGCCT-3′
21-mer	5′-CCAGCAGCTCTAGCAGCCTGC-3′
23-mer	5′-CCAGCAGCTCTAGCAGCCTGCCG-3′
25-mer	5′-CCAGCAGCTCTAGCAGCCTGCCGAC-3′
	3′-3′-Linked PS-oligo
19-mer	5′-CCAGCAGCTCTAGCAGCCT-X-CCGACGATCTCGACGACC-5′
	3′-5′-Linked PS-oligo
19-mer	5′-CCAGCAGCTCTAGCAGCCT-X- CCAGCAGCTCTAGCAGCCT-3′
	DNA GSOs with Different Lengths of Segments
19/15-mer	3′-TCCGACGATCTCGACGACC-X-CCAGCAGCTCTAGCA-3′
19/25-mer	3′-TCCGACGATCTCGACGACC-X-CCAGCAGCTCTAGCAGCCTGCCGAC-3′
	DNA GSOs with Mismatches
19-mer-mm	3′-TCAGAAGATGTCGTCGTCC-X-CCAGCAGCTCTAGCAGCCT-3′
19-mer-MM	3′-TCAGAAGATGTCGTCGTCC-X-CCTGCTGCTGTAGAAGACT-3′
	complementary sequences to human MyD88 mRNA (target C391)
	DNA GSOs
15-mer	3′-GACCTCTGTGTTCGC-X-CGCTTGTGTCTCCAG-3′
17-mer	3′-TTGACCTCTGTGTTCGC-X-CGCTTGTGTCTCCAGTT-3′
19-mer	3′-CGTTGACCTCTGTGTTCGC-X-CGCTTGTGTCTCCAGTTGC-3′
21-mer	3′-GCCGTTGACCTCTGTGTTCGC-X-CGCTTGTGTCTCCAGTTGCCG-3′
23-mer	3′-AGGCCGTTGACCTCTGTGTTCGC-X-CGCTTGTGTCTCCAGTTGCCGGA-3′
25-mer	3′-CTAGGCCGTTGACCTCTGTGTTCGC-X-CGCTTGTGTCTCCAGTTGCCGGATC-3′
	Antisense PS-oligo
19-mer	5′-CGCTTGTGTCTCCAGTTGC-3′

[a] All sequences contain deoxyribonucleotides and were synthesized with phosphorothioate backbone. Starting nucleotide number from 3′ → 5′ on target mRNA is shown in parentheses; C stands for coding region. Underlined nucleotides represent mismatches.

molecule that is utilized by all TLRs, except TLR3, in their immune signaling pathways. Experiments were carried out in J774 cell cultures as described above using 15- to 25-mer lengths of DNA GSOs. Figure 3A shows the relative quantities of MyD88 mRNA at 3 μg/mL DNA GSOs. As observed with RNA GSOs, a 19-mer DNA GSO was consistently more potent at reducing mRNA levels (54% reduction) than were shorter (15- and 17-mers) or longer (21- to 25-mers) DNA GSOs. These results suggest that 19-mer DNA GSOs with two 3′-ends are optimal for gene silencing. Consistent with inhibition of mRNA expression, MyD88 protein expression was also inhibited by DNA GSO (Figure 3A inset). The inhibition of MyD88 protein expression was greater with 19-mer DNA GSO (50% inhibition) than 19-mer antisense PS-oligo (29% inhibition). 19-mer GSOs with five mismatch nucleotides in one branch (19-mer-mm) and five mismatches in each branch (19-mer-MM) showed only 19% and no inhibition of MyD88 protein expression, respectively (SI Figure 1), suggesting the specificity of 19-mer DNA GSO.

In addition, inhibition of gene expression mediated by the 19-mer DNA GSO was dose-dependent with an 82% inhibition at 10 μg/mL concentration (Figure 3B). These data suggest that, as observed for RNA GSOs, DNA GSOs containing two 3′-ends and carrying two 19-mer segments are capable of gene silencing more efficiently.

Length of Antisense PS-oligo Has a Limited Effect on Its Gene-Silencing Activity. We evaluated the impact of antisense PS-oligo length on gene-silencing activity. Antisense PS-oligos of various lengths (15- to 25-mers) complementary to MyD88 mRNA were synthesized and evaluated in J774 cells and in mice.

J774 cells were incubated in the absence or presence of antisense PS-oligos for 48 h, then total RNA was isolated, and the amount of MyD88 mRNA was determined by RT-PCR. Antisense PS-oligos showed lower levels of down-regulation of MyD88 mRNA (Figure 4) compared with DNA GSOs at the same dose (Figure 3B). Moreover, the length of the antisense PS-oligo had minimal influence on its gene-silencing activity. Only a moderate increase in inhibition with increasing length from 15- (25%) to 25-mer (50%) was observed (Figure 4). In addition, antisense PS-oligos injected in mice did not show length-dependent inhibition of TLR9-mediated activity (SI Figure 2).

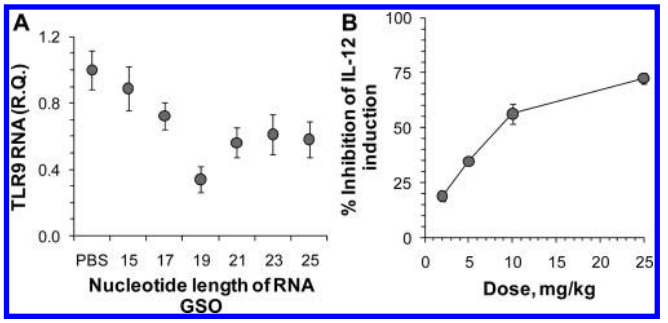

Figure 2. (A) TLR9 mRNA expression in J774 cells following treatment with different lengths of RNA GSOs targeted against mouse TLR9. J774 cells were incubated with RNA GSOs of differing lengths (25 μg/mL). Reduction in MyD88 mRNA after 48 h was measured by quantitative real-time PCR with Taqman gene expression assays. Representative results (mean ± SD) of one of three experiments are illustrated. (B) Dose-dependent inhibition of TLR9-mediated IL-12 induction by 19-mer RNA GSO in mice. Various doses of GSO in PBS were injected sc to C57BL/6 mice and 24 h later 0.25 mg/kg TLR9 agonist was injected sc in the opposite flank. Blood was collected 2 h post TLR9 agonist administration and serum IL-12 levels were determined by ELISA. Data shown are mean ± SD of three mice per group and are representative of two or three independent experiments.

DNA GSOs for MyD88 Have Length-Dependent Gene-Silencing Activity in Human PBMCs. To further confirm the results of DNA GSOs observed in J774 cells, we cultured human PBMCs with DNA GSOs complementary to human MyD88 mRNA of 15- to 25-mer long for 24 h without lipid carrier, isolated total RNA, and measured the levels of MyD88 mRNA by RT-PCR analysis. MyD88 mRNA levels decreased depending on the length of the DNA GSO, with the greatest decrease (52% inhibition) being observed with a 19-mer (Figure 5A). These results are consistent with J774 cell culture studies and confirm that the length of DNA GSO is important for optimal gene-silencing activity. In studies comparing protein inhibition in human PBMCs, a 19-mer DNA GSO showed greater inhibition of MyD88 protein (76% inhibition) than an antisense PS-oligo of the same length (44% inhibition) (Figure 5B).

We further extended our studies with DNA GSOs complementary to TLR9 mRNA and VEGF mRNA. In human PBMCs, DNA GSOs complementary to TLR9 showed length-dependent inhibition of TLR9 mRNA as well as protein expression in human PBMCs (SI Figure 3). Consistent with the data obtained with DNA GSOs complementary to MyD88, a 19-mer DNA GSO complementary to TLR9 also showed greater inhibition of TLR9 mRNA and protein expression in human PBMCs. Results similar to those obtained with MyD88 and TLR9 DNA GSOs were observed with DNA GSOs complementary to VEGF in HeLa cells (SI Figure 4A). A 19-mer DNA GSO complementary to VEGF showed dose-dependent inhibition of VEGF mRNA with a 70% inhibition at 10 μg/mL concentration (SI Figure 4B).

DNA GSOs Have Length-Dependent Activity in Vivo in Mice. To study whether the gene-silencing activity in vivo is influenced by the length of the DNA GSOs, we injected 17- to 25-mer DNA GSOs complementary to MyD88 into mice. After 24 h post-DNA GSO administration, mice were injected with a TLR9 agonist to assess the functionality of the MyD88 signaling pathway. Two hours after TLR9 agonist administration, serum IL-12 levels were measured by ELISA. The data shown in Figure 6A suggest that in vivo also 19- to 21-mer DNA GSOs are more potent in inhibiting TLR9-mediated IL-12 secretion

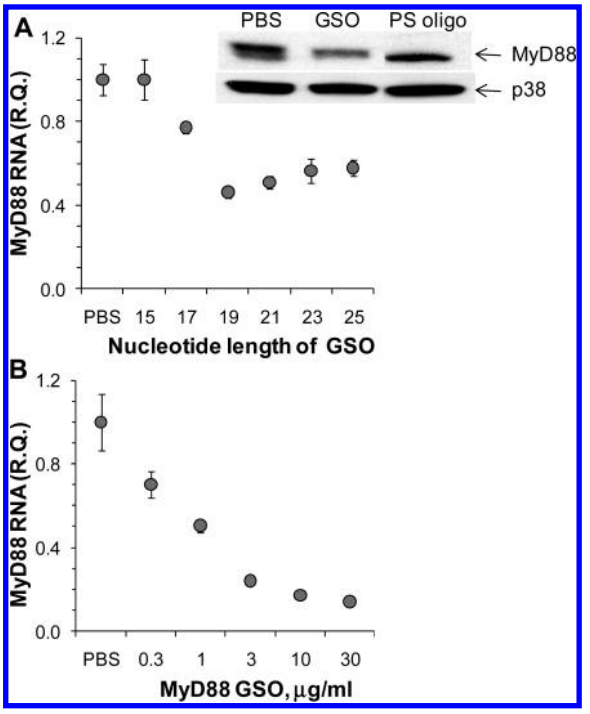

Figure 3. (A) MyD88 mRNA expression in J774 cells following treatment with various lengths of DNA GSOs targeted against mouse MyD88. J774 cells were incubated with MyD88 GSOs of different nucleotide lengths (3 μg/mL) for 48 h. Knockdown of MyD88 mRNA was measured by quantitative real-time PCR with Taqman gene expression assays as described in the Materials and Methods section. Representative results (mean ± SD) of one of three experiments are shown. (Inset) Inhibition of MyD88 protein expression in J774 cells following treatment with 3 μg/mL 19-mer DNA GSO or antisense PS-oligo. The blot for MyD88 was stripped and reprobed with a p38 antibody for equal loading. (B) Dose-dependent inhibition of mRNA expression by 19-mer DNA GSO in J774 cells. The experimental conditions were the same as described in (A).

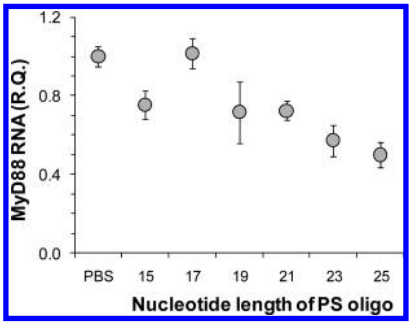

Figure 4. MyD88 mRNA expression in J774 cells following treatment with 10 μg/mL concentration of various lengths of antisense PS-oligos targeted against mouse MyD88. The experimental conditions were the same as described in Figure 2A except that the cells were incubated with 10 μg/mL of each oligonucleotide.

(67−69% inhibition) than are longer or shorter GSOs. Similar results were observed in mice with DNA GSOs complementary to TLR9, wherein the 19-mer DNA GSO showed a 70% inhibition of TLR9-mediated IL-12 induction (Figure 6B).

DNA GSOs Have Dose-Dependent Gene-Silencing Activity in Vivo in Mice. To determine whether DNA GSOs have a dose-dependent effect in vivo, we injected mice with increasing

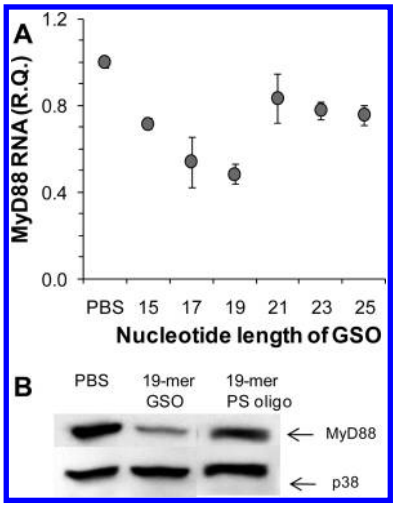

Figure 5. (A) MyD88 mRNA expression in human PBMCs following treatement with DNA GSOs targeted against human MyD88 or PBS. PBMCs were incubated with MyD88 GSOs of different lengths (10 μg/mL) for 24 h. Reduction in MyD88 mRNA was measured by quantitative real-time PCR with Taqman gene expression assays. Representative results (mean ± SD) of one of three experiments are illustrated. (B) Inhibition of MyD88 protein expression in human PBMCs following treatment with 19-mer DNA GSO, antisense PS-oligo, or PBS. PBMCs were incubated with either a 19-mer GSO or 19-mer antisense PS-oligo (3 μg/mL) targeted against MyD88 for 24 h. Whole cell lysates were processed for quantitation of MyD88 expression by Western blotting. The blot for MyD88 was stripped and reprobed with a p38 antibody for equal loading. Blot images from one experiment that are representative of three independent experiments are illustrated.

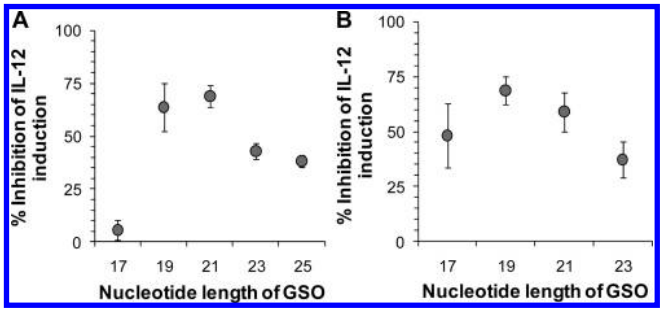

Figure 6. (A) Inhibition of TLR9-mediated IL-12 induction in mice by different lengths of DNA GSOs targeted against mouse MyD88. (B) Inhibition of TLR9-mediated IL-12 induction in mice by different lengths of DNA GSOs targeted against mouse TLR9. In both (A) and (B), GSOs were injected sc to C57BL/6 mice at a dose of 1 mg/kg (MyD88-targeted GSOs) or 2 mg/kg (TLR9-targeted GSOs) in PBS and 24 h later 0.25 mg/kg TLR9 agonist was injected sc in the opposite flank. Blood was collected 2 h post TLR9 agonist administration, and serum IL-12 levels were determined by ELISA. Data shown are mean ± SD of three mice per group and are representative of two or three independent experiments.

doses (1−5 mg/kg) of 19-mer DNA GSO complementary to MyD88 and 24 h later with a TLR9 agonist. Two hours after TLR9 agonist administration, blood was collected and analyzed for serum levels of IL-12 by ELISA. We used a 19-mer antisense PS-oligo for comparison in this study. As shown in Figure 7A, mice that received 19-mer antisense PS-oligo inhibited TLR9-mediated activity to a lesser extent (56% inhibition at 15 mg/kg) than a 19-mer DNA GSO (95% inhibition at 5 mg/kg). As observed in cell-based assays, 19-mer DNA GSO was more effective in inhibiting TLR9-mediated

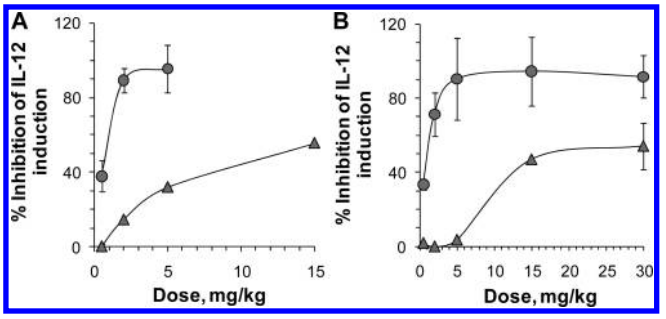

Figure 7. (A) Dose-dependent inhibition of TLR9-mediated IL-12 induction in mice by a 19-mer DNA GSO (filled circles) or antisense PS-oligo (filled triangle) targeted against mouse MyD88. (B) Dose-dependent inhibition of TLR9-mediated IL-12 induction in mice by a 19-mer DNA GSO (filled circles) or antisense PS-oligo (filled triangles) targeted against mouse TLR9. In both (A) and (B), various doses of GSO or antisense PS-oligo in PBS were injected sc to C57BL/6 mice and 24 h later 0.25 mg/kg TLR9 agonist was injected sc in the opposite flank. Blood was collected 2 h post TLR9 agonist administration, and serum IL-12 levels were determined by ELISA. Data shown are mean ± SD of three mice per group and are representative of two or three independent experiments.

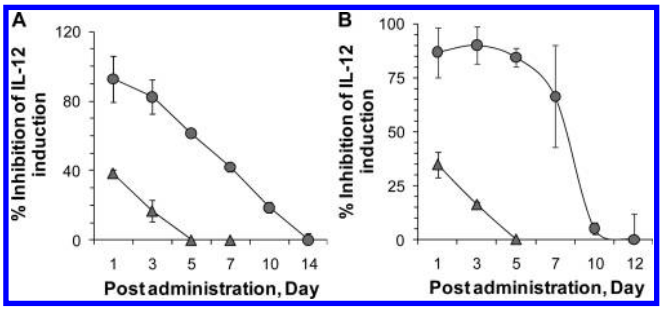

Figure 8. (A) Duration of activity of a 19-mer MyD88-targeted DNA GSO (filled circles) or antisense PS-oligo (filled triangles) on TLR9-mediated IL-12 induction in mice. (B) Duration of activity of a 19-mer TLR9-targeted DNA GSO (filled circles) or antisense PS-oligo (filled triangles) on TLR9-mediated IL-12 induction in mice. In both (A) and (B), 5 mg/kg DNA GSO or antisense PS-oligo in PBS was injected sc to C57BL/6 mice followed by sc injection of 0.25 mg/kg of TLR9 agonist in the opposite flank at the indicated time point. Blood was collected 2 h post TLR9 agonist administration, and serum IL-12 levels were determined by ELISA. Data shown are mean ± SD of three mice per group and are representative of two or three independent experiments.

IL-12 induction than antisense PS-oligo and the inhibition was dose-dependent (Figure 7A). Similar results were observed with a 19-mer TLR9-targeted DNA GSO (Figure 7B).

DNA GSOs Have a Longer Duration of Activity in Vivo in Mice. We studied the duration of activity of DNA GSO complementary to MyD88 in mice, again using a 19-mer antisense PS-oligo for comparison. The data shown in Figure 8A suggest that the DNA GSO more efficiently inhibited TLR9-mediated activity and the inhibitory effect persisted up to 10 days. In contrast, at the same dose level, the antisense PS-oligo inhibited TLR9-mediated activity to a lesser degree and the inhibition persisted for less than 5 days (Figure 8A). These results demonstrate that DNA GSO is more potent than antisense PS-oligos and can exert a prolonged inhibitory activity. Similar results were observed with a 19-mer DNA GSO complementary to TLR9 (Figure 8B).

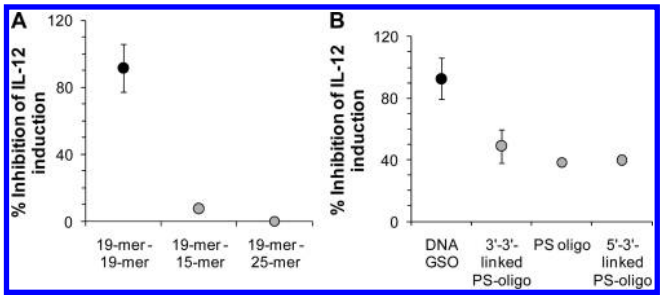

Figure 9. (A) Inhibition of TLR7-mediated IL-12 induction in mice by MyD88-targeted DNA GSOs containing 19-mer in one segment and a 19-, 15-, or 25-mer in the second segment. (B) Inhibition of TLR7-mediated IL-12 induction in mice by MyD88-targeted DNA GSO, 3′-3′-linked antisense PS-oligo, antisense PS-oligo, or 5′-3′-linked antisense PS-oligo. In both (A) and (B), 5 mg/kg of the indicated compound was injected sc to C57BL/6 mice and 24 h later 10 mg/kg RNA-based TLR7 agonist was injected sc in the opposite flank. Blood was collected 2 h post TLR7 agonist administration, and serum IL-12 levels were determined by ELISA. Data shown are mean ± SD of three mice per group and are representative of two or three independent experiments.

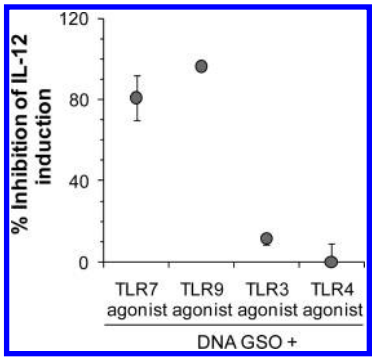

Figure 10. Specificity of MyD88-targeted GSO in mice. 19-mer GSO in PBS (2 mg/kg) was injected sc to C57BL/6 mice, and 24 h later, 10 mg/kg RNA-based TLR7 agonist, 0.25 mg/kg TLR9 agonist, 25 mg/kg TLR3 agonist, or 0.25 mg/kg TLR4 agonist was injected sc in the opposite flank. Blood was collected 2 h post TLR agonist administration, and serum IL-12 levels were determined by ELISA. Data shown are mean ± SD of three mice per group and are representative of two or three independent experiments.

DNA GSO with Two 3′-Ends and Containing 19-mer Segments Is Required for Optimal Activity. The above in vitro and in vivo studies demonstrated that a 19-mer DNA GSO is optimal for gene-silencing activity. Next, we asked whether a 19-mer is required in each segment of the DNA GSO or if a DNA GSO containing a segment of 19-mer and another segment of variable length can exert the same level of inhibition. We synthesized DNA GSOs complementary to MyD88 mRNA with a 19-mer in one segment and a 15- or 25-mer in the second segment (Table 1) and conducted a comparative study in mice. TLR7 also utilizes MyD88 for immune signaling and in this study we used an RNA-based TLR7 agonist to assess the functionality of the target protein.[37] The data shown in Figure 9A suggest that the DNA GSO containing two 19-mer segments inhibited TLR7-mediated induction of IL-12 more efficiently than did DNA GSOs containing segments of variable length. These results demonstrate that a 19-mer is optimal in both the segments of DNA GSOs.

To address whether two 3′-ends are required for GSO activity, we synthesized 3′-3′-linked and 3′-5′-linked 19-mer antisense PS-oligos with the same sequence as the 19-mer DNA GSO complementary to MyD88 (Table 1). Administration of DNA GSO, antisense PS-oligo, 3′-3′-linked antisense PS-oligo, or 3′-5′-linked antisense PS-oligo to mice, followed by administration of TLR7 agonist and analysis of serum levels of IL-12 suggest that the DNA GSO inhibited TLR7-mediated induction of IL-12 more potently than did any of the other three antisense PS-oligos (Figure 9B). These results suggest that DNA GSOs containing two 3′-ends are optimal for gene-silencing activity.

MyD88-Targeted DNA GSO Exerts Target-Specific Gene-Silencing Activity in Vivo. We evaluated the specificity of DNA GSO complementary to MyD88 against TLR3, TLR4, TLR7, and TLR9 agonist activities in mice. As expected, MyD88-targeted DNA GSO administration to mice led to inhibition of TLR7- and TLR9-, but not TLR3- or TLR4-mediated induction of IL-12 in mice (Figure 10). These results suggest that the MyD88-targeted DNA GSO specifically inhibits the expression of MyD88, thereby down-regulating the MyD88-dependent, but not other, signaling pathways.

DISCUSSION

Synthetic oligodeoxy- or oligoribo-nucleotides complementary to target mRNA can inhibit translation of specific mRNAs through various mechanisms of action, including antisense, ribozyme, and siRNA. Over the last three decades, since the introduction of the antisense approach, it has become evident that the key factors affecting gene-silencing activity are: the stability of oligonucleotides in biological fluids, systemic delivery, and the mRNA target-specific intended mechanism of action.

The design of GSOs has been prompted by the observation that the overall gene-silencing efficiency of a variety of oligonucleotide-based compounds is hampered, at least in part, by the availability of the 5′-end of the oligonucleotide to interact with TLRs such as TLR9. This interaction leads to immune activation and induction of pro-inflammatory cytokines, perturbing the physiological conditions where the oligonucleotides are expected to exert their gene-silencing effect.[35,36,38] Additionally, accessibility of the 3′-end of the antisense strand is essential for efficient gene-silencing activity of ssRNA- and dsRNA-mediated through RISC.[32−34]

To address the issues discussed above, we created GSOs comprising identical oligonucleotides complementary to the target mRNA linked through a 5′-5′-linkage, thereby blocking the accessibility of the 5′-ends for TLR interaction and also generating two 3′-ends (Figure 1). We observed no TLR9-related immune activation when an antisense PS-oligo complementary to Bcl2 mRNA containing a CpG motif, which induces immune responses,[39] was synthesized in a GSO configuration and tested in vivo in mice for IL-12 induction (data not shown).

Various lengths of RNA and DNA GSOs were synthesized using standard phosphoramidite chemistry with phosphorothioate internucleotide linkages. The phosphorothioate backbone provided stability against nuclease degradation. The GSOs synthesized were complementary to MyD88, VEGF, and TLR9. These genes have distinct biological functions and their protein products are located in different cellular compartments.

Studies of RNA GSOs of varying lengths from 15- to 25-mer complementary to TLR9 showed suppression of TLR9 mRNA in J774 cells. A 19-mer RNA GSO showed greater suppression than did other lengths of RNA GSOs (Figure 2A). Furthermore, the 19-mer RNA GSO inhibited TLR9-mediated immune responses

in a dose-dependent manner in vivo in mice following subcutaneous administration of RNA GSO (Figure 2B). The gene-silencing activity of RNA GSOs was lower than the DNA GSOs perhaps due to greater susceptibility of RNA to nuclease degradation than DNA.

DNA GSOs of varying lengths from 15- to 25-mer complementary to MyD88 suppressed MyD88 mRNA in a dose-dependent manner in J774 cells and human PBMCs (Figures 3 and 5). In both cell types, the 19-mer DNA GSO suppressed MyD88 RNA and protein to a greater extent than did other lengths of DNA GSOs. As was observed with 19-mer RNA GSO complementary to TLR9, the 19-mer DNA GSO complementary to MyD88 had more potent activity than the other lengths of DNA GSOs. Studies of various lengths of antisense PS-oligos showed no clear evidence of length dependence on gene-silencing activity (Figure 4). Similarly, in studies of DNA GSOs complementary to VEGF and TLR9, the 19-mer DNA GSO was more potent than GSOs of other lengths at suppressing the target mRNAs (SI Figures 3 and 4).

DNA GSOs complementary to MyD88 with lengths of 19- or 21-mer suppressed TLR9-mediated immune responses in mice to a greater degree than the other lengths of DNA GSOs studied (Figure 6) and the potency was greater with a longer duration of inhibition than a 19-mer antisense PS-oligo (Figure 8). The activity of 19-mer DNA GSO was compromised when the length of one of the segments of DNA GSO was either reduced to 15-mer or increased to 25-mer, suggesting that 19-mer length is optimal for gene-silencing activity of GSO (Figure 9A). In addition, DNA GSO containing mismatches in one segment showed significant loss of gene-silencing activity. A 19-mer antisense PS-oligo attached via 3′-3′-linkage or 3′-5′-linkage had lower activity than the 19-mer DNA GSO, further suggesting that the accessibility of 3′-ends is important for the activity of GSO (Figure 9B). The 19-mer DNA GSO complementary to MyD88 specifically inhibited TLR7- and TLR9-mediated immune responses (Figure 10). In contrast, it did not inhibit immune responses induced by TLR3, which does not utilize MyD88 for its signaling.

The activity of gene-silencing agents depends on a number of factors, including stability against serum nucleases, affinity to the target sequence, and efficient activation of cellular enzymatic machinery such as RNase H in the case of antisense or RISC in the case of siRNA. The current studies showed that the 19-mer RNA GSO and DNA GSO are more active than antisense PS-oligos. The greater activity of 19-mer GSOs could be due to increased binding stability against serum nucleases or increased affinity to the target mRNA. All GSOs (15- to 25-mer) contained two 3′-ends and had similar levels of stability in the presence of serum nucleases, suggesting that the greater activity of 19-mer RNA and DNA GSOs compared with other lengths of GSOs cannot be explained by stability against serum nucleases. Increased binding affinity to the target can result in increased gene-silencing activity. However, the T_m data showed that the binding affinity of both RNA GSOs and DNA GSOs to their complementary mRNA strands increased incrementally from 15-mer to 25-mer. Therefore, the 19-mer GSOs did not show an unexpected higher binding affinity to the complementary strand than the shorter or longer GSOs that would explain increased gene-silencing activity. Additionally, because both RNA and DNA GSOs have optimal activity with a length of 19-mer, the activity of the DNA GSO cannot be explained by enhanced RNase H-mediated cleavage of DNA GSO hybridized to mRNA.

The optimal 19-mer length of GSOs for higher gene-silencing activity is reminiscent of the sequence length requirements for siRNA-mediated gene-silencing. dsRNA of 19-nucleotides with two 3′-overhanging nucleotides is best suited for processing by RISC and subsequent target sequence recognition and cleavage.[27] In addition, ssRNAs complementary to target mRNAs have also been shown to mediate gene silencing through RISC.[28] It is unclear whether such a hypothesis applies to DNA GSOs as well because RISC requires oligoribonucleotides as substrates.[23−25] Nonetheless, recent studies suggest that oligodeoxynucleotides induce cleavage of target complementary mRNA in a variety of organisms through RISC complex.[40] A specific length requirement of the oligodeoxynucleotides has not been reported.

CONCLUSION

In summary, the GSOs described here are stable in biological fluids, show activity following systemic delivery without use of any lipid carrier and do not induce immune activation through TLRs. In addition, GSOs provide two accessible 3′-ends that may be conductive to efficacy. We found that of the nucleotide lengths studied, the 19-mer long RNA and DNA GSOs had the most potent gene-silencing activity in vitro and in vivo. GSOs complementary to various mRNA targets also showed similar results, suggesting broad applicability of these agents for gene silencing.

MATERIALS AND METHODS

Synthesis of Oligonucleotides. All oligonucleotides were synthesized on 5−15 μmol scale on a MerMade 6/12 DNA/RNA synthesizer using β-cyanoethylphosphoramidite chemistry. Both 3′- and 5′-phosphoramidites of dA, dG, dC, T, A, G, C, and U were obtained from ChemGenes Corporation. DiDMT-protected glyceryl linker attached to NittoPhase solid-support or controlled-pore glass solid-support was used for oligodeoxynucleotide and oligoribonucleotide synthesis, respectively. Beaucage reagent was used as an oxidant to obtain the phosphorothioate backbone modification.[41] After the synthesis, oligonucleotides were deprotected using standard protocols, purified by HPLC, and desalted on C18-column. The oligonucleotides were lyophilized and resuspended in USP-quality sterile water for irrigation (Braun) and the concentrations were determined by measuring the UV absorbance at 260 nm. All oligonucleotides synthesized were characterized by HPLC and CGE analysis for purity and MALDI-ToF mass spectrometry for molecular mass.

Thermal Melting Study. DNA GSO, RNA GSO, or antisense PS-oligo and the complementary target RNA at 1 μM concentration of each strand in 1 mL of 10 mM sodium phosphate buffer, pH 7.2, containing 150 mM NaCl were heated for 5 min at 95 °C and allowed to come to room temperature slowly. The solutions were stored at 4 °C overnight before the thermal melting temperature (T_m) was measured. The thermal melting experiments were carried out on a Perkin-Elmer Lambda 20 UV/vis spectrophotometer equipped with a Peltier temperature controller and a multicell holder. Data were collected at each degree by heating or cooling the samples at a rate of 0.5 °C/min. The data were collected and analyzed using Templab software on a personal computer attached to the instrument. Each experiment was carried out at least two times.

Cell Culture Conditions and Reagents. Polyclonal antibodies to TLR9 were obtained from Imgenex (San Diego, CA). Cell lysis buffer, MyD88 (D80F5), and p38 MAP kinase antibodies were from Cell Signaling Technology (Danvers, MA). Anti-rabbit IgG-horse radish peroxidase (HRP) conjugate was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Bio-Rad protein reagent, Ready Gels, Laemmli sample buffer, and PVDF membranes were from BioRad Laboratories

(Hercules, CA), whereas Western Lightning Plus Chemiluminescence kit was from Perkin-Elmer Life Sciences (Waltham, MA). RNeasy kits and Taqman gene expression assays and PCR reagents were purchased from Qiagen and Applied Biosystems, respectively. HyBlot CL autoradiography film was purchased from Denville Scientific (Metuchen, NJ). All other chemicals and reagents were purchased from Sigma (St. Louis, MO).

Murine macrophage-like cells, J774A.1 and HeLa cells (American Type Culture Collection, Rockville, MD) were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% heat-inactivated defined FBS (Hyclone) and antibiotics (100 IU/mL of penicillin G/streptomycin). All other culture reagents were purchased from Mediatech (Gaithersburg, MD).

Peripheral blood mononuclear cells (PBMCs) from freshly drawn healthy volunteer blood (Research Blood Components, Brighton, MA) were isolated by Ficoll density gradient centrifugation method (Ficoll Paque PLUS, GE Health Care). The culture medium used for the assay consisted of RPMI 1640 medium supplemented with 1.5 mM glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 50 μM 2-mercaptoethanol, 100 IU/mL penicillin-streptomycin mix, and 10% heat-inactivated FBS.

Monitoring Gene Expression in J774 Cells, HeLa Cells and PBMCs Treated with GSOs. J774 cells were plated overnight at a concentration of 0.7×10^6 cells/mL in 6-well culture plates. Medium was changed the next morning and the GSOs, dissolved in PBS, were added and cells were incubated for a period of 48 h.

HeLa cells were plated overnight at a concentration of 3×10^5 cells/mL in 12-well culture plates. Cells were washed and resuspended in serum free DMEM the next morning, followed by the addition of the VEGF GSO and Lipofectamine 2000 complexes (1:1 ratio). Medium was replaced with DMEM supplemented with 10% FBS after 2 h of incubation, followed by an additional 22 h of incubation.

Freshly isolated human PBMCs (10×10^6 cells/mL) in 6-well culture plates were incubated with the GSOs for 24 h. At the end of the experiment, medium was removed and the pelleted cells were washed twice with chilled PBS with protease inhibitors before being lysed and homogenized using a QIAshredder Kit.

RNA was isolated using the RNeasy Mini Kit, following the manufacturer's protocol and reverse transcribed using a High-Capacity cDNA Reverse Transcription Kit. Real Time PCR was performed on the cDNAs generated, using TaqMan Fast Universal PCR Master Mix and probes (Applied Biosystems, Carlsbad, CA) specific for mouse (Mm00440338_m1) or human (Hs00182082_m1) MyD88 or mouse (Mm00446193_m1) or human (Hs00152973_m1) TLR9, or human VEGF (Hs0000900055_ml) and the endogenous control, GAPDH (Mm99999915_gl, Hs99999905_ml) on StepOnePlus TaqMan Real-Time PCR System. Each sample was run in triplicate, and target mRNA levels in the samples were normalized using GAPDH. The expression data are shown as relative quantities of MyD88, TLR9, or VEGF in the samples treated with RNA or DNA GSOs compared with a PBS control.

Western Blotting and Quantitation of Protein Expression. For monitoring protein expression, cells were harvested at the end of the experiment, washed twice thoroughly with chilled PBS containing protease inhibitors and then suspended in cell lysis buffer. Cells were lysed on ice for 15 min, sonicated briefly, and centrifuged at 14000*g* for 20 min. The supernatants were transferred to fresh vials and stored at −70 °C until further use. Protein concentration in the samples was measured by the method of Bradford using the Bio-Rad protein assay. Samples (20−30 μg/lane) were subjected to gel electrophoresis using 10% or 4−15% gradient Tris-HCl Ready gels, and Western blotted onto PVDF membranes. After blocking in 5% nonfat milk in PBS-Tween 20 for 1 h, the membranes were incubated overnight with the appropriate primary antibody. Labeled proteins were visualized by the enhanced chemiluminescence method using HRP-coupled secondary antibodies and quantitated using a Scion Image Analysis Program (Scion Corp., Fredrick, MD). For reprobing of Western blots, the blots were washed in PBS, incubated for 30 min in the stripping buffer (Thermo Scientific). They were then washed thoroughly in PBST and probed with another primary antibody using the protocol described above.

In Vivo Activity Assessment in Mice. Female C57BL/6, 5−6 weeks old, mice were obtained from Charles River Laboratories, Wilmington, MA, and maintained in accordance with Idera Pharmaceuticals's IACUC approved animal protocols. For MyD88 and TLR9 functional inhibition studies, RNA or DNA GSOs were administered by subcutaneous (sc) injection in left flank at 0.5−30 mg/kg dosage or as indicated in the appropriate figure legend. Then a TLR9 agonist was injected in the right flank after 24 h or at indicated time point as stated in the appropriate figure legend at 0.25 mg/kg dose. The duration of inhibitory activity was studied by injection of TLR9 agonist at 1, 2, 3, 4, 5, 7, 10, 12, or 14 days post DNA GSO administration. The specificity of DNA GSOs was evaluated by injection of different TLR agonists 24 h post 5 mg/kg GSO administration, including 0.25 mg/kg TLR9 agonist,[35] 10 mg/kg TLR7 agonist (an RNA-based compound[36]), 25 mg/kg TLR3 agonist (Poly I:C, InvivoGen, San Diego, CA), or 0.25 mg/kg TLR4 agonist (LPS; lipopolysacharide, Sigma). Blood samples were collected by retro-orbital bleeding 2 h post agonist administration and IL-12 levels in the serum were determined by sandwich ELISA as described previously.[35] All reagents, including cytokine antibodies and standards, were purchased from BD Biosciences (San Diego, CA).

■ ASSOCIATED CONTENT

Supporting Information. GSO sequences and analytical characterization data. Western blot analysis of MyD88 protein expression in J774 cells; inhibition of TLR9-mediated IL-12 induction by various lengths of DNA PS-oligos targeted against mouse MyD88 in mice; TLR9 mRNA expresion in human PBMCs; VEGF mRNA expression in HeLa cells. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS USED

CGE, capillary gel electrophoresis; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; HPLC, high performance liquid chromatography; PS, phosphorothioate; PBMCs, peripheral blood mononuclear cells; TLR9, Toll-like receptor 9; MyD88, myeloid differentiation factor 88; VEGF, vascular endothelial growth factor

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