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Toll-like Receptor 7 Selective Synthetic Oligoribonucleotide Agonists: Synthesis and Structure−Activity Relationship Studies

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We previously reported a novel class of stabilized immune-modulatory RNA (SIMRA) compounds that activates TLR8 or both TLR7 and TLR8 depending on the nucleotide composition and chemical modifications incorporated. In the present study, to identify TLR7-selective agonists, we designed and synthesized novel SIMRA compounds with varying sequence compositions substituting 7-deaza-G for natural guanosine and studied immune-stimulatory activity in cell-based assays and in vivo in mice. SIMRA compounds activated NF-κB in HEK293 cells expressing TLR7 and induced cytokine production in mouse spleen cells and human PBMCs and higher levels of IFN-α in human pDCs, which correlated with TLR7 activation. Subcutaneous administration of SIMRA compounds to mice increased serum cytokine levels. TLR knockout mouse studies showed that both TLR7 and MyD88 are required for activity of SIMRA compounds. The presence of a 5′-AA/CN (A > C and N = U/C/7-deaza-G) and/or C/AUU-3′ (C > A) trinucleotide at the 5′- and 3′-ends of SIMRA compound along with a 5′-AN^{1}N^{2}UG1A-3′ (N^1 = A/C; N^2 = U/C/7-deaza-G) or UG1AZ^{1}G1Z^{2}UU (Z^1 = A < C; Z^2 = C < A) motif confers TLR7 selectivity over other sequence compositions. In conclusion, we have designed and synthesized novel SIMRA compounds that selectively act as agonists of TLR7.

Introduction

The innate immune system of mammals consists of a variety of receptors that detect invading pathogens. One family of receptors, called Toll-like receptors (TLRs[a]), recognizes pathogen-associated molecular patterns (PAMPs), such as cell wall and nucleic acid components of pathogens, and plays a key role in the innate immune system.[1−3] Of the 10 TLRs that have been identified in humans, TLR7 and TLR8 are the receptors for viral and synthetic single-stranded RNAs (ssRNAs).[4−6] Both receptors are expressed in the membranes of endosomes in different types of immune cells. In humans, TLR7 is expressed in B cells and plasmacytoid dendritic cells (pDCs) and TLR8 is expressed in myeloid DCs (mDCs) and monocytes.[7,8] Viral and synthetic RNAs rich in uridine (U) or guanidine/uridine (GU) content encapsulated in lipids activate human TLR7 and TLR8.[4,6] A recent study has identified ssRNA sequences encapsulated in lipids that selectively activate TLR8 or both TLR7 and TLR8.[9] A limitation for the preclinical and clinical application of these RNA-based agonists is that their use in vitro and in vivo requires formulation with lipids.[4,10,11]

Purine nucleoside analogues at high concentrations have been shown to act as agonists of TLR7,[12,13] and imidazoquinoline-based compounds also activate TLR7 and/or TLR8.[14,15] Imidazoquinolines have also been shown to induce immune responses via other receptors, such as the A3 adenosine receptor and the opioid growth factor receptor.[16,17] Imidazoquinoline compounds cause side effects following systemic administration, however, and are commonly used by topical application.[18−20]

In our previous studies, we showed that novel RNA structures, referred to as stabilized immune-modulatory RNA (SIMRA) compounds, act as agonists of TLR8 or TLR7/8 and induce immune responses in cell-based assays and in nonhuman primates.[21] We have observed that the nucleotide composition of SIMRA compounds and incorporation of modified nucleotides affect the selectivity toward TLR7 or TLR8. For example, incorporation of 7-deazaguanosine for guanosine or arabinocytidine for cytidine in TLR8-activating SIMRA compounds leads to activation of both TLR7 and TLR8.[21,22] Our recent studies have also shown that oligoribonucleotide-based compounds containing secondary structures act as agonists of TLR8; incorporating certain nucleotide motifs in these compounds leads to activation of both TLR7 and TLR8.[23]

RNA sequence motifs that activate TLR8 and both TLR7 and TLR8 have been identified, but the RNA sequence motifs that selectively activate TLR7 have not been characterized. In the present study, we designed and synthesized SIMRA compounds that have varying sequence compositions and contain 7-deazaguanosine in place of natural G and studied them in cell-based assays, human peripheral blood mononuclear cells (PBMCs), pDCs, and mDCs and in vivo in mice. The immune-stimulatory profiles of SIMRA compounds that selectively activate TLR7 are characterized and compared for their immunological activity with TLR8 and dual TLR7/8 agonists previously reported.

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[a] Abbreviations: CGE, capillary gel electrophoresis; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; IFN, interferon; IL, interleukin; NF-κB, nuclear factor-κB; PBMCs, peripheral blood mononuclear cells; pDCs, plasmacytoid dendritic cells; mDCs, myeloid dendritic cells; TLR, Toll-like receptor.

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Materials and Methods

Reagents. 2′-OTBDMS-3′-phosphoramidite-5′-ODMT-N-Ac-A, -N-Ac-C, -N-Ac-G, -N-^{t}Bu-7-deaza-G and -U, propanediol phosphoramidite, and controlled-pore-glass (CPG) solid support functionalized with diDMT glycerol linker were obtained from ChemGenes (Wilmington, MA). Beaucage reagent was obtained from R. I. Chemicals (Orange, CA). Other oligonucleotide synthesis reagents were obtained from Glen Research (Sterling, VA). Methylamine in water, *N*-methylpyrrolidinone, triethylamine tris(hydrogen fluoride), triethylamine, 3-hydroxypicolinic acid, and ammonium citrate dibasic were obtained from Sigma-Aldrich (St. Louis, MO). Ammonium hydroxide was obtained from J. T. Baker (Phillipsburg, NJ).

Synthesis, Purification, and Characterization of SIMRA Compounds. SIMRA compounds were synthesized on a BioAutomation (Plano, TX) Mermade 6 DNA/RNA synthesizer using phosphoramidite chemistry on CPG solid support as described previously.[21] The coupling step was carried out twice with a mixture of 0.05 M phosphoramidite solution in acetonitrile and 0.25 M ethylthiotetrazole (ETT) for 15 min. The oxidation step was carried out for 8 min with 5% Beaucage reagent in acetonitrile to obtain phosphorothioate internucleotide linkages.[24] After the synthesis, cleavage of SIMRA compounds from solid support and deprotection of amino groups were carried out using freshly prepared ammonium hydroxide/40% methylamine in water (1:1, v/v) at ambient temperature for 2 h. Solid support was removed by filtration, and the solution was evaporated to dryness in a SpeedVac. Then the 2′-protecting groups were cleaved by treating the crude oligonucleotide with 0.5 mL of freshly prepared *N*-methylpyrrolidinone/triethylamine/triethylamine tris(hydrogen fluoride) (3:1.5:2, v/v/v) reagent at 65 °C for 90 min. Deprotection reaction was quenched by adding 15 mL of 0.1 M Tris-HCl, pH 7.5 buffer. The crude oligoribonucleotide mixture was purified on an anion-exchange HPLC (buffer gradients used are given in Supporting Information). The purified SIMRA compounds were desalted, lyophilized, and characterized by HPLC, capillary gel electrophoresis (CGE), and MALDI-TOF mass spectroscopy (see Supporting Information for protocols).

Serum Stability Assay. Approximately 0.5 A_{260} units of SIMRA compounds were added to 1% human serum (Sigma-Aldrich, St. Louis, MO) in PBS and incubated at 37 °C. We removed an aliquot after 10 min, analyzed it by anion-exchange HPLC, and determined the percentage of intact full-length SIMRA compound remaining.

Human TLR7 and TLR8 Transfected HEK293XL Cell Assays. HEK293 cells stably expressing human TLR7 or TLR8 (Invivogen, San Diego, CA) were cultured and transiently transfected with the secreted form of human embryonic alkaline phosphatase (SEAP) reporter plasmid (pNifty2-Seap, Invivogen) as described previously.[19] After transfection, the medium was replaced with fresh culture medium, SIMRA compounds were added, and cultures were incubated for 18 h. Then the culture supernatants were harvested, and SEAP activity was measured by the QuantiBlue method following the manufacturer's protocol (Invivogen). The data are shown as fold increase in nuclear factor (NF)-κB activity over PBS control.

Mouse Spleen Cell Cultures. Spleen cells from C57BL/6 mice were prepared and cultured in RPMI complete medium consisting of RPMI 1640 supplemented with 10% FBS, 100 units/mL penicillin, 100 μg/mL streptomycin, and 2 mM L-glutamine (HyClone, Logan, UT). Mouse spleen cells were plated in 96-well plates at 5×10^6 cells per mL. SIMRA compounds dissolved in PBS were added at specified concentration to the cell cultures. The cells were incubated at 37 °C for 24 h, at which time the supernatants were collected for cytokine enzyme-linked immunosorbent assay (ELISA).

Human PBMC, pDC, and mDC Isolation. PBMCs from freshly drawn healthy human volunteer blood (Research Blood Components, Brighton, MA) were isolated by Ficoll density gradient centrifugation (Histopaque-1077, Sigma). pDCs were isolated from PBMCs by positive selection using the BDCA4 dendritic cell isolation kit (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions. mDCs were isolated from PBMCs by depletion of $CD19^+$ B cells followed by positive selection using the BDCA1 dendritic cell isolation kit (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions.

Human PBMC, pDC, and mDC Cultures. Human PBMCs (5×10^6 cells/mL), pDCs (1×10^6 cells/mL), and mDCs (1×10^6 cells/mL) were plated in 96-well plates. SIMRA compounds dissolved in PBS were added to the cell cultures at various concentrations. The cells were then incubated at 37 °C for 24 h. The levels of cytokines and chemokines in the culture supernatants were measured using a human multiplex kit on the Applied Cytometry Systems Luminex 100 or 200 instrument, and the data were analyzed using StarStation software, version 2.0. The required reagents were purchased from Invitrogen (Carlsbad, CA).

In Vivo Study of SIMRA Compounds in Mice. Five-to-eight-week-old female C57BL/6 mice (wild-type) (Charles River Labs, Wilmington, MA) and eight-week-old male $TLR9^{-/-}$, $TLR7^{-/-}$, and $MyD88^{-/-}$ mice with C57BL/6 background (Oriental BioService Company, Koyoto, Japan) were obtained and maintained in the animal facility of Idera Pharmaceuticals. All of the experimental procedures were performed in accordance with the approved protocols and guidelines of the Institutional Animal Care and Use Committee of Idera Pharmaceuticals. SIMRA compounds were administered subcutaneously at 10 and 25 mg/kg dose to wild-type mice ($n = 3$) and at 10 mg/kg dose to $TLR9^{-/-}$, $TLR7^{-/-}$, and $MyD88^{-/-}$ mice ($n = 3$). Two hours later, blood was collected by retro-orbital bleeding and serum cytokine levels were determined using a mouse Luminex multiplex kit or interleukin (IL)-12 ELISA kit. Luminex multiplex data were acquired on Applied Cytometry System (Dinnington, U.K.) Luminex 100/200 instrument and analyzed using StarStation software, version 2.0. The required reagents were purchased from Invitrogen (Carlsbad, CA).

Results

Design, Synthesis, and Nuclease Stability of SIMRA Compounds. SIMRA compounds **1**−**29** were designed on the basis of the results of our previous studies with two identical 11-mer oligoribonucleotide sequences attached through their 3′-ends via a glycerol linker and incorporating 7-deaza-G substitutions (Table 1).[21,22] All SIMRA compounds were synthesized using phosphoroamidite chemistry on an automated DNA/RNA synthesizer, and Becaucage reagent was used to obtain phosphorothioate internucleotide linkages. SIMRA compounds **30** and **31** reported previously by us were used as positive controls for TLR8-selective and dual TLR7/8 agonists, respectively.[21] Compound **32** served as a non-immune-stimulatory control. All SIMRA compounds were deprotected after the synthesis and purified on ion-exchange HPLC using standard protocols. Purified SIMRA compounds were desalted, lyopholized, and characterized by analytical HPLC, CGE, and MALDI-TOF mass spectroscopy (Table 1).

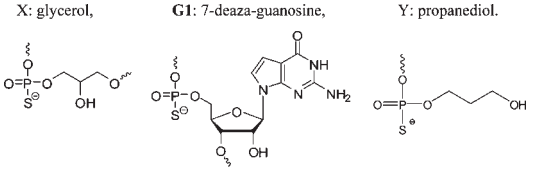
SIMRA compounds were studied for their stability against nucleases in 1% human serum in PBS. After 10 min of incubation at 37 °C, the mixture was analyzed on an anion-exchange HPLC and percentage of full-length product (FLP) remaining was determined. The stability of SIMRA compounds **1**−**29** varied depending on the sequence composition, with 63−90% FLP remaining at the end of the incubation in human serum (Table 1).

Study of SIMRA Compounds in HEK293 Cells Expressing Human TLR7. Activation of TLR7 by SIMRA compounds

Table 1. Sequences, Modifications, and Analytical Data of SIMRA Compounds Studied

SIMRA compd	sequence[a]	MALDI-ToF calcd (*m*/*z*)	MALDI-ToF found (*m*/*z*)	HPLC purity (%)	stability (% FLP remaining)[b]
1	5′-UU**G1**CU**G1**AG**1**CUU-X-UUC**G1**AG**1**UC**G1**UU-5′	7451	7451	97	83
2	5′-U**G1**AU**G1**AC**G1**AUU-X-UUA**G1**CA**G1**UA**G1**U-5′	7546	7546	97	84
3	5′-U**G1**AC**G1**AU**G1**AUU-X-UUA**G1**UA**G1**CA**G1**U-5′	7546	7543	97	86
4	5′-CUU**G1**U**G1**AG**1**CUU-X-UUC**G1**AG**1**U**G1**UUC-5′	7451	7457	98	85
5	5′-CU**G1**AG**1**AAG**1**CUU-X-UUC**G1**AA**G1**AG**1**UC-5′	7543	7542	96	72
6	5′-CU**G1**AC**G1**AG**1**CUU-X-UUC**G1**AG**1**CA**G1**UC-5′	7495	7496	95	75
7	5′-ACUU**G1**ACUU**G1**A-X-A**G1**UUCA**G1**UUCA-5′	7467	7466	97	87
8	5′-ACUU**G1**AC**G1**AUU-X-UUA**G1**CA**G1**UUCA-5′	7464	7464	96	86
9	5′-ACUU**G1**ACCUUU-X-UUUCCA**G1**UUCA-5′	7342	7342	98	72
10	5′-ACUU**G1**ACCU**G1**U-X-U**G1**UCCA**G1**UUCA-5′	7421	7419	95	63
11	5′-ACUU**G1**AA**G1**CUU-X-UUC**G1**AA**G1**UUCA-5′	7467	7465	95	79
12	5′-ACUU**G1**AACUU**G1**-X-**G1**UUCAA**G1**UUCA-5′	7467	7470	96	84
13	5′-ACU**G1**C**G1**AG**1**CUU-X-UUC**G1**AG**1**C**G1**UCA-5′	7495	7495	96	71
14	5′-ACU**G1**AG**1**AG**1**CUU-X-UUC**G1**AG**1**AG**1**UCA-5′	7543	7545	95	79
15	5′-AC**G1**U**G1**AAG**1**CUU-X-UUC**G1**AA**G1**U**G1**CA-5′	7543	7545	97	74
16	5′-AC**G1**CU**G1**AG**1**CUU-X-UUC**G1**AG**1**UC**G1**CA-5′	7495	7495	96	78
17	5′-AAUU**G1**AA**G1**CUU-X-UUC**G1**AA**G1**UUAA-5′	7511	7513	96	83
18	5′-AAUU**G1**AA**G1**CU**G1**-X-**G1**UC**G1**AA**G1**UUAA-5′	7591	7589	98	76
19	5′-AAUU**G1**AC**G1**CUU-X-UUC**G1**CA**G1**UUAA-5′	7467	7466	96	80
20	5′-AAUU**G1**AC**G1**CU**G1**-X-**G1**UC**G1**CA**G1**UUAA-5′	7543	7541	95	82
21	5′-AA**G1**U**G1**C**G1**ACUU-X-UUCA**G1**C**G1**U**G1**AA-5′	7543	7539	98	79
22	5′-AA**G1**U**G1**AC**G1**CUU-X-UUC**G1**CA**G1**U**G1**AA-5′	7543	7543	96	87
23	5′-AA**G1**U**G1**AC**G1**AUU-X-UUA**G1**CA**G1**U**G1**AA-5′	7591	7591	98	90
24	5′-AACUU**G1**AG**1**CUU-X-UUC**G1**AG**1**UUCAA-5′	7467	7467	97	82
25	5′-AACU**G1**C**G1**ACUU-X-UUCA**G1**C**G1**UCAA-5′	7465	7465	97	83
26	5′-AACU**G1**AC**G1**CUU-X-UUC**G1**CA**G1**UCAA-5′	7465	7465	96	87
27	5′-AACU**G1**AC**G1**CU**G1**-X-**G1**UC**G1**CA**G1**UCAA-5′	7541	7543	98	84
28	5′-AACU**G1**AA**G1**CUU-X-UUC**G1**AA**G1**UCAA-5′	7513	7509	98	77
29	5′-AACU**G1**AA**G1**CU**G1**-X-**G1**UC**G1**AA**G1**UCAA-5′	7589	7587	98	76
30	5′-YUGCUGCUUGUG-X-GUGUUCGUCGUY-5′	7791	7792	97	78
31	5′-U**G1**CU**G1**CUUCU**G1**-X-**G1**UCUUC**G1**UC**G1**U-5′	7399	7400	97	57
32	5′-AAAAAAAAAAA-X-AAAAAAAAAAA-5′	7690	7688	96	92

[a] All sequences have phosphorothioate backbone. [b] Stability of RNA was determined as detailed under Materials and Methods; FLP, full-length product.



1−**32** was evaluated in HEK293XL cells stably expressing human TLR7. At 50 μg/mL, SIMRA compounds **1**−**29** induced different levels of NF-κB activity (Table 2). All SIMRA compounds except **7**, **10**, and **25** induced greater than 10-fold increase in NF-κB activity in TLR7-expressing HEK293 cells. Dual TLR7/8 SIMRA compound **31** induced a 16-fold increase in NF-κB activity in TLR7-expressing HEK293 cells. TLR8-selective SIMRA compound **30** and control compound **32** did not induce NF-κB activity compared with medium in HEK293 cells expressing TLR7 (Table 2).

Specificity of SIMRA Compounds for TLR7. To study the selectivity of SIMRA compounds for TLR7, activation of TLR8 by the same compounds was also evaluated in HEK293XL cells, which stably express human TLR8. Twenty-five of the 29 SIMRA compounds induced a less than 2-fold increase in NF-κB activity in TLR8-expressing HEK293 cells (Table 2). SIMRA compounds **3**, **8**, **12**, and **14** induced increases of 2.1- to 4.9-fold in NF-κB activity in TLR8-expressing HEK293 cells. Consistent with our previous results, SIMRA compounds **30** and **31** induced 9.2- and 4.7-fold increases in NF-κB activity, respectively (Table 2). We calculated the ratios of NF-κB activity in TLR7- and TLR8-expressing HEK293 cells (Table 2). These ratios ranged from 3 to 114.5 for SIMRA compounds, compared with 3.4 and 0.1 for dual TLR7/8 and TLR8 compounds **31** and **30**, respectively, suggesting selectivity of SIMRA compounds for TLR7 in these assays. We further studied SIMRA compounds for their activity in HEK293 cells expressing mouse TLR9 or human TLR3. None of the SIMRA compounds activated either TLR3 or TLR9 (data not shown), suggesting that SIMRA compounds activate TLR7 selectively.

Requirement of 7-Deaza-G for TLR7 Activity of SIMRA Compounds. To understand the requirement of 7-deaza-G nucleoside in SIMRA compounds for TLR7 activity, we selected SIMRA compounds **8** and **11**, substituted natural Gs in place of 7-deaza-Gs (SIMRA compounds **8*** and **11***) and tested the new compounds for NF-κB activation in HEK293 cells expressing TLR7 and TLR8. As shown in Figure 1, SIMRA compounds **8** and **11**, but not **8*** and **11***, activated NF-κB in TLR7-expressing HEK293 cells and in

Table 2. Activation of HEK293XL Cells Expressing Human TLR7 or TLR8 by SIMRA Compounds[a]

SIMRA compd	fold increase in NF-κB activity ± SD		ratio of TLR7/ TLR8 activity
	TLR7	TLR8	
medium	1 ± 0.1	1 ± 0	
1	26.7 ± 0.2	0.4 ± 0	66.8
2	17.5 ± 0.2	1.6 ± 0	10.9
3	18.4 ± 0.2	4.6 ± 0.7	4.0
4	28.9 ± 0.1	1.5 ± 0.1	19.3
5	27.6 ± 0.1	0.4 ± 0.1	69.0
6	30.0 ± 0.3	0.6 ± 0.1	50.0
7	9.1 ± 0	1.4 ± 0.1	6.5
8	16.6 ± 0.1	2.1 ± 0.1	7.9
9	23.3 ± 0.4	1.8 ± 0.2	12.9
10	6.1 ± 0.1	0.7 ± 0	8.7
11	23.7 ± 0.6	2.0 ± 0.1	11.9
12	14.7 ± 0.1	4.9 ± 1.0	3.0
13	27.0 ± 0.3	1.7 ± 0	15.9
14	16.8 ± 0.3	2.4 ± 0.1	7.0
15	26.8 ± 0.8	2.0 ± 0.5	13.4
16	26.6 ± 0.9	0.9 ± 0	29.6
17	25.0 ± 0.6	1.6 ± 0.1	15.6
18	13.7 ± 0.1	0.7 ± 0	19.6
19	20.9 ± 0.1	0.3 ± 0.1	69.7
20	22.9 ± 0.4	0.2 ± 0.4	114.5
21	20.7 ± 0	0.5 ± 0.3	41.4
22	22.6 ± 0.1	0.7 ± 0.2	32.3
23	17.5 ± 0.1	0.8 ± 0	21.9
24	20.1 ± 0.2	1.0 ± 1.6	20.1
25	6.5 ± 0.1	0.3 ± 0	21.7
26	21.5 ± 0.4	0.4 ± 0.2	53.8
27	18.4 ± 0	0.4 ± 0.1	46.0
28	29.5 ± 0.3	1.0 ± 0.6	29.5
29	18.4 ± 0.3	0.6 ± 0.3	30.7
30	0.5 ± 0.4	9.2 ± 0.6	0.1
31	16.1 ± 0.3	4.7 ± 0.4	3.4
control **32**	0.3 ± 0.1	0.7 ± 0	

[a] Concentration of SIMRA compound was 50 μg/mL. Data shown are representative of three independent experiments.

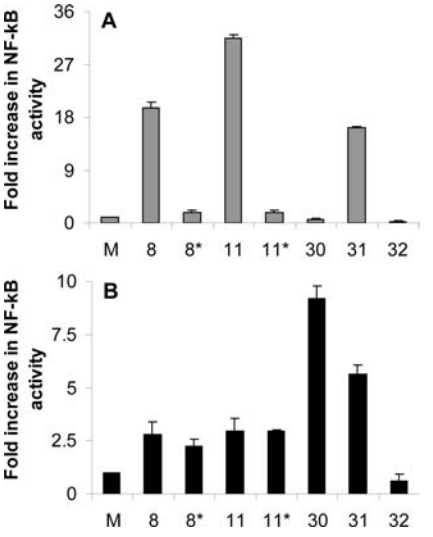


Figure 1. Human (A) TLR7 and (B) TLR8 activity of SIMRA compounds containing 7-deaza-G (SIMRA compounds **8**, **11**, and **31**) or G (SIMRA compounds **8***, **11***, and **30**) in HEK293XL cells. M indicates medium.

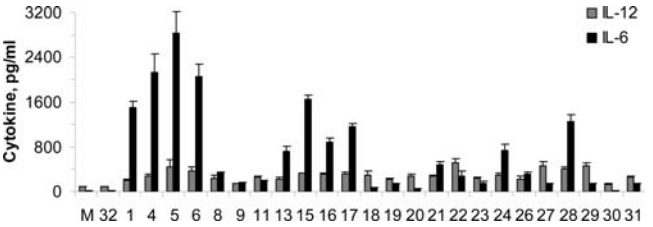


Figure 2. IL-12 and IL-6 induction in C57BL/6 mouse spleen cell cultures by SIMRA compounds at 100 μg/mL. Spleen cells were cultured in the presence or absence of SIMRA compounds for 24 h. Supernatants were collected and analyzed by ELISA. M indicates medium. Data shown are representative of three independent experiments.

TLR8-expressing HEK293 cells at similar levels. These results support our hypothesis that the presence of 7-deaza-G in these SIMRA compounds is required for TLR7 activity.

Study of SIMRA Compounds in Mouse Spleen Cell Cultures. Mice do not have a functional TLR8, and the activity observed in mouse spleen cells is the result of TLR7 activation. C57BL/6 mouse spleen cells were stimulated with 100 μg/mL SIMRA compounds for 24 h, and IL-12 and IL-6 levels were measured in the culture supernatants by ELISA. Several SIMRA compounds induced IL-12 and IL-6 (Figure 2). As expected, TLR8-selective SIMRA compound **30** induced only background levels of cytokines, similar to control SIMRA compound **32**. In general, IL-12 levels induced by SIMRA compounds correlated with their TLR7 activity in HEK293 cell assays.

Screening of SIMRA Compounds in Human PBMC Cultures for TLR7 Selective Cytokine Profiles. We selected SIMRA compounds that showed activity in TLR7-expressing HEK293 cells and induced both IL-12 and IL-6 in mouse spleen cells and tested their ability to induce cytokine secretion in human PBMCs. pDCs, which express TLR7 but not TLR8, produce interferon (IFN)-α upon activation with TLR7 agonists.[5] IL-12 and tumor necrosis factor (TNF)-α are produced by monocytes and mDCs upon stimulation with TLR8 agonists.[4] We measured the levels of these three cytokines induced by 100 μg/mL SIMRA compounds in PBMC assays to further evaluate the SIMRA compounds' selectivity for TLR7 (Table 3). All SIMRA compounds except **27**−**29** induced higher levels of IFN-α than did control compounds. SIMRA compounds **1**, **4**, **5**, **15**, and **16** induced higher levels of TNF-α and IL-12, suggesting that they may activate TLR8 to some extent in human PBMCs. Other SIMRA compounds induced no or low levels of TNF-α. As expected, dual TLR7/8 SIMRA compound **31** induced significant amounts of all three cytokines and TLR8-selective SIMRA compound **30** induced TNF-α and IL-12 but not IFN-α. Control SIMRA compound **32** induced only background levels of IFN-α, IL-12, and TNF-α in PBMCs.

Cytokine and Chemokine Induction Profiles of SIMRA Compounds in Human PBMC Cultures. SIMRA compounds **2**, **8**, **11**, **13**, **19**, **22**, **23**, and **26**, which activated TLR7 selectively in HEK293 cells, induced IL-12 and IL-6 in mouse spleen cell cultures, and induced higher levels of IFN-α and lower levels of IL-12 and TNF-α in human PBMC screening assay, were selected to further study their cytokine induction profiles in human PBMCs. Dose-dependent IFN-α, interferon-γ-induced protein (IP)-10, IL-12, TNF-α, IL-6, IL-1β, macrophage inflammatory protein (MIP)-1α, MIP-1β,

Table 3. IFN-α, IL-12, and TNF-α Induction by SIMRA Compounds in Human PBMCs[a]

	cytokine, induction ± SD (pg/mL)		
SIMRA compd	IFN-α	IL-12	TNF-α
medium	0 ± 0	5.3 ± 0	7.2 ± 0
1	145.1 ± 9.7	1366.0 ± 2.6	415.3 ± 26.8
2	182.7 ± 12.1	102.1 ± 4.8	0 ± 0
4	138.1 ± 0	1020.8 ± 6.8	403.1 ± 0
5	123.1 ± 0	1748.3 ± 0.7	818.2 ± 0
6	106.4 ± 0	518.8 ± 9.7	33.4 ± 0
8	232.9 ± 10.5	393.7 ± 18.8	114.9 ± 20.9
11	217.6 ± 11.2	442.1 ± 11.3	170 ± 4.9
13	130.6 ± 10.6	487.2 ± 5.8	43.48 ± 4.0
15	123.1 ± 0	864.2 ± 2.2	164.7 ± 3.8
16	187.9 ± 15.8	2237.4 ± 11.9	1407.0 ± 180.5
17	95.5 ± 10.7	401.4 ± 0.7	29.3 ± 0
19	192.7 ± 0	340.4 ± 9.6	8.8 ± 9.9
21	123.1 ± 0	170.6 ± 9.4	1.7 ± 0
22	204.2 ± 7.4	352.1 ± 16.1	26.2 ± 0
23	253.9 ± 19.3	400.4 ± 25.1	15.7 ± 9.7
24	106.4 ± 0	527.0 ± 19.8	74.4 ± 3.9
26	292.5 ± 27.5	249.8 ± 2.8	20.3 ± 4.2
27	0 ± 0	153.5 ± 18.7	0 ± 0
28	84.5 ± 6.4	259.6 ± 3.5	7.5 ± 0
29	28.7 ± 31.0	240.2 ± 5.1	3.5 ± 3.3
30	81.6 ± 28.1	715.3 ± 23.2	260.3 ± 79.9
31	384.9 ± 13.4	1106.7 ± 18.7	384.9 ± 0
control **32**	0 ± 0	183.2 ± 1.5	1.6 ± 0

[a] At 100 μg/mL concentration of SIMRA compounds. Data shown are representative of three or more independent experiments.

IL-10, and monocyte chemotactic protein (MCP)-1 induction by representative SIMRA compounds **13**, **19**, **22**, and **26** are shown in Figure 3. Interestingly, these compounds produced distinct cytokine induction profiles compared with TLR8 SIMRA compound **30** and TLR7/8 SIMRA compound **31**. SIMRA compounds **13**, **19**, **22**, and **26** induced higher levels of IFN-α and IP-10 and low levels of IL-12 and TNF-α. In general, SIMRA compounds **13**, **19**, **22**, and **26** induced low levels of MCP-1 at lower dose and higher levels at higher dose. The levels of representative cytokines and chemokines (IL-1β, IL-12, TNF-α, IFN-α, MIP-1α, MIP-1β, IP-10, IL-6, and MCP-1) induced in human PBMC cultures by all eight SIMRA compounds (**2**, **8**, **11**, **13**, **19**, **22**, 23, and **26**) at 50 μg/mL concentration are shown in Table 4. Control SIMRA compound **32** did not induce significant amounts of any of the cytokines and chemokines.

Cytokine and Chemokine Induction by SIMRA Compounds in Human pDC Cultures. SIMRA compounds **2**, **8**, **11**, **13**, **19**, **22**, **23**, **26**, and **30**−**32** were studied for their immune-stimulatory activity in human pDCs. The levels of IFN-α, IP-10, IL-6, and IL-12 induced by these SIMRA compounds in pDCs are shown in Figure 4. All TLR7-selective SIMRA compounds induced cytokines in pDCs; they induced particularly high levels of IFN-α. Dual TLR7/8 SIMRA compound **31** also induced higher levels of IFN-α in pDC cultures. SIMRA compound **30** induced minimal IFN-α in pDCs, consistent with the fact that it is a TLR8 agonist. Control SIMRA compound **32** did not induce any cytokine secretion in pDCs. These results suggest that SIMRA compounds stimulate pDC via TLR7 activation.

Cytokine and Chemokine Induction by SIMRA Compounds in Human mDC Cultures. SIMRA compounds **2**, **8**, **11**, **13**, **19**, **22**, **23**, **26**, and **30**−**32** were studied for their immune-stimulatory activity in human mDCs. The levels of IFN-α, IP-10, IL-12, IL-6, and TNF-α induced by those compounds in mDC are shown in Figure 5. In contrast to cytokine profiles in pDCs, the SIMRA compounds studied induced low levels of cytokines in mDCs. SIMRA compounds **30** and **31**, which activate TLR8, induced higher levels of IL-12, IL-6, and TNF-α. Control compound **32** did not induce cytokine secretion in mDCs. These results suggest that the SIMRA compounds identified here selectively activate TLR7.

Stimulation of Immune Responses by SIMRA Compounds in Vivo in Mice. SIMRA compounds **2**, **8**, **11**, **13**, **19**, **22**, **23**, **26**, and **30**−**32** were evaluated in C57BL/6 mice for their ability to induce immune responses in vivo. All SIMRA compounds studied induced higher levels of IL-12 and other cytokines 2 h after administration than did control compound (Figure 6). SIMRA compound **31**, which activates both TLR7 and TLR8, induced cytokine production in mice (Figure 6). TLR8-selective SIMRA compound **30** induced background levels of cytokines, similar to levels seen in naive mice. These results suggest that SIMRA compounds described herein induce immune responses in vivo.

Mechanism of Action of SIMRA Compounds in Vivo in Mice. To further study the specificity and the mechanism of action of SIMRA compounds, we administered a representative SIMRA compound **13** sc to wild-type (Wt), TLR9$^{-/-}$, TLR7$^{-/-}$, and MyD88$^{-/-}$ mice (n = 3) at a dose of 10 mg/kg. Blood was collected 2 h after administration, and serum IL-12 levels were determined by ELISA. The data shown in Figure 7 suggest that SIMRA compound **13** induced IL-12 secretion in wild type and TLR9$^{-/-}$ mice but not in TLR7$^{-/-}$ and MyD88$^{-/-}$ mice. These results suggest that TLR7 and MyD88 are required for SIMRA compound-induced immune responses in mice.

Discussion

Viral ssRNAs are the natural agonists of TLR7 and TLR8.[4−6] The sequence selectivity of TLR7 and TLR8 is not well understood, but ssRNA containing G/U-rich sequences encapsulated in lipids act as agonists of human TLR8.[4] Another study has shown that changing G/U to A/U in ssRNA sequences can modulate the immune-stimulatory properties of RNA for TLR7 and TLR8.[9] More importantly, to date, all these studies reported with RNA-based TLR7 and TLR8 agonists have used formulation or encapsulation with lipids for in vitro and in vivo studies in mice.

We have reported a novel class of RNA-based compounds comprising two 11-mer RNAs attached through their 3′ ends via a glycerol linker, referred to as stabilized immune-modulatory RNA (SIMRA) compounds.[21−23] Depending on the sequence composition and chemical modifications incorporated, SIMRA compounds activate immune responses through TLR8 or both TLR7 and TLR8 in cell-based assays as well as in nonhuman primates.[21] SIMRA compounds that selectively activate TLR7 have not been reported. The key advantage of SIMRA compounds is that they are stable under physiological conditions and can be administered in vivo without using lipid carriers. In the present study, we designed and synthesized 29 novel SIMRA compounds (**1**−**29**) with varying sequence compositions and containing 7-deaza-G substitutions and studied the ability of these compounds to stimulate immune responses through TLR7. RNA is rapidly degraded by endonucleases; we previously identified a few dinucleotides (UA, UC, and CA) that are more susceptible to

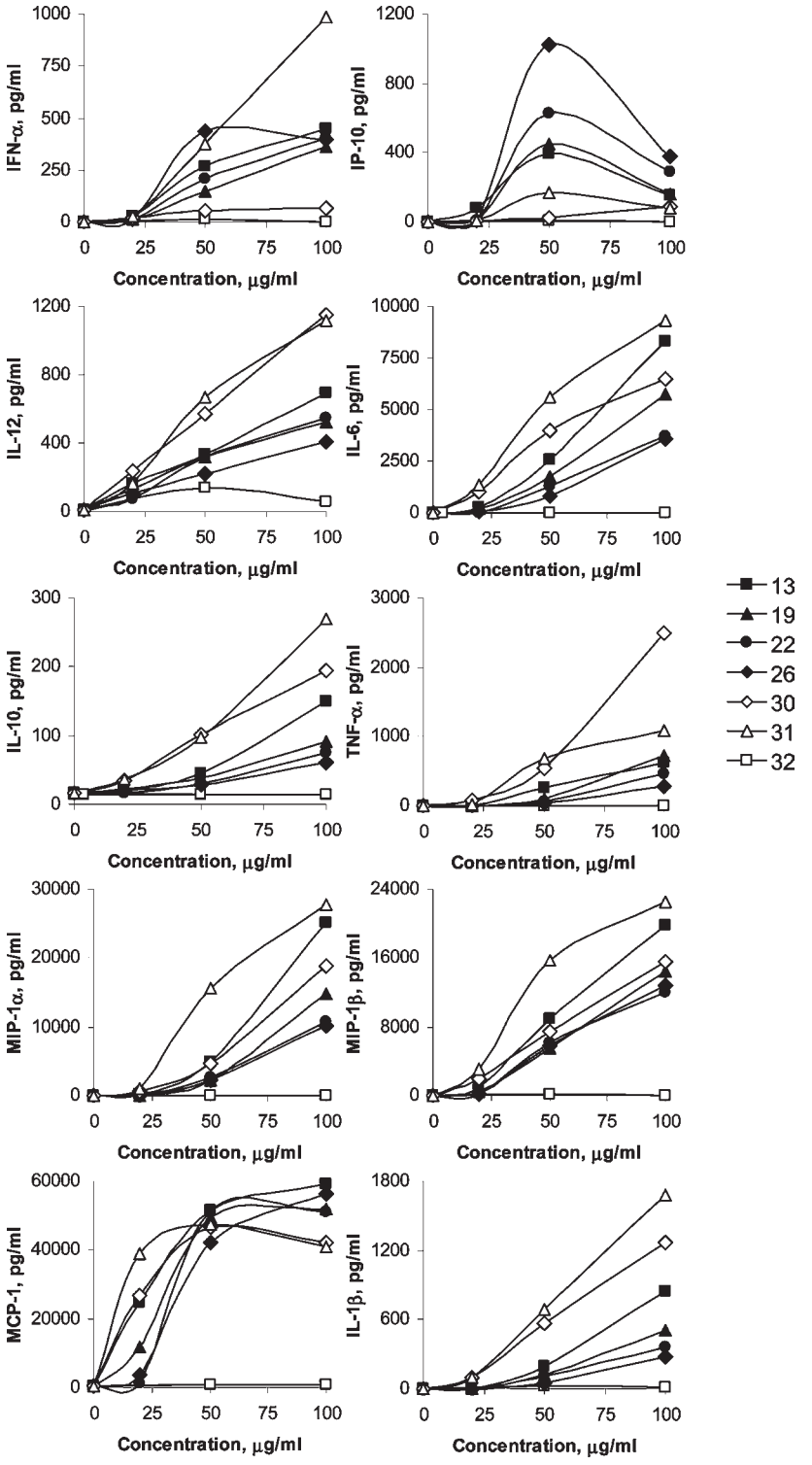


Figure 3. Dose-dependent cytokine and chemokine induction by SIMRA compounds in human PBMC cultures. Data shown are representative of four independent experiments.

degradation and avoided these dinucleotides in the design of new SIMRA compounds studied in this report.[21]

All 29 SIMRA compounds activated NF-κB in HEK293 cells expressing human TLR7. SIMRA compounds **3**, **8**, **12**, and **14**, but not others, activated NF-κB in HEK293 cells expressing TLR8. None of the SIMRA compounds activated NF-κB in TLR3- or TLR9-expressing HEK293 cells, suggesting that SIMRA compounds act via TLR7 selectively.

SIMRA compounds induced immune-stimulatory activity in mouse spleen cells, which have TLR7 but not functional TLR8. In human PBMCs, SIMRA compounds induced cytokine and chemokine production in a concentration-dependent manner. Consistent with TLR7 selectivity, SIMRA compounds induced higher levels of IFN-α and IP-10 and lower levels of IL-12, TNF-α, and IL-1β in human PBMC cultures. SIMRA compounds induced greater levels of IFN-α by human pDCs, which express TLR7. SIMRA compounds

Table 4. Cytokine/Chemokine Induction by SIMRA Compounds in Human PBMCs[a]

SIMRA compd	cytokine/chemokine induction ± SD (pg/mL)								
	IL-1β	IL-12	TNF-α	IFN-α	MIP-1α	MIP-1β	IP-10	MCP-1	IL-6
medium	0 ± 0	86 ± 0	0 ± 0	0 ± 0	23 ± 0	2 ± 0	5 ± 0	278 ± 0	0 ± 0
2	306 ± 0	434 ± 38	68 ± 3	131 ± 0	5794 ± 159	9961 ± 398	155 ± 0	55983 ± 3818	2757 ± 94
8	218 ± 19	360 ± 29	174 ± 0	106 ± 8	3617 ± 71	7330 ± 216	199 ± 19	49103 ± 1321	2379 ± 75
11	261 ± 4	354 ± 29	76 ± 4	230 ± 0	5630 ± 171	9218 ± 72	287 ± 14	52901 ± 1557	2729 ± 59
13	196 ± 12	337 ± 14	256 ± 6	266 ± 0	4889 ± 225	8985 ± 323	395 ± 21	51445 ± 5877	2577 ± 7
19	117 ± 17	320 ± 0	104 ± 5	149 ± 12	2230 ± 136	5504 ± 139	453 ± 16	49566 ± 4255	1750 ± 154
22	105 ± 0	316 ± 53	57 ± 5	209 ± 5	2544 ± 152	6231 ± 653	629 ± 2	51543 ± 4818	1303 ± 91
23	123 ± 26	174 ± 10	18 ± 1	121 ± 0	3094 ± 174	6677 ± 105	55 ± 11	46670 ± 469	1896 ± 109
26	47 ± 16	223 ± 0	39 ± 2	434 ± 3	2205 ± 128	5878 ± 345	1020 ± 87	42124 ± 3465	791 ± 2
30	568 ± 37	570 ± 33	541 ± 62	56 ± 0	4755 ± 478	5733 ± 630	24 ± 4	46717 ± 4795	3980 ± 158
31	688 ± 52	671 ± 64	688 ± 24	373 ± 26	15509 ± 1604	27555 ± 149	167 ± 18	47418 ± 46	5582 ± 120
control **32**	28 ± 12	138 ± 0	0 ± 0	12 ± 0	43 ± 13	83 ± 18	4 ± 1	724 ± 58	12 ± 0

[a] Data shown are at 50 μg/mL and representative of three or more independent experiments.

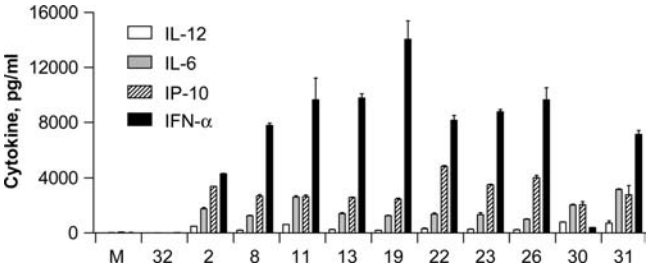


Figure 4. Cytokine and chemokine induction by SIMRA compounds at 100 μg/mL in human pDCs. M indicates medium. Data shown are representative of four independent experiments.

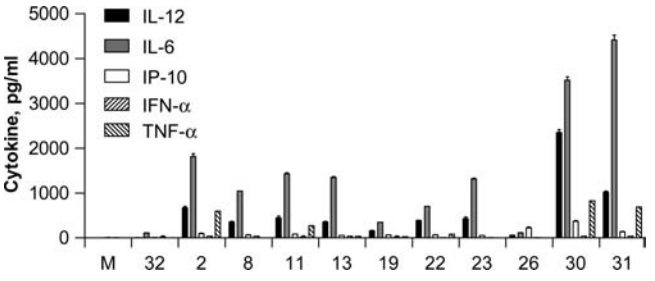


Figure 5. Cytokine and chemokine induction by SIMRA compounds at 100 μg/mL in human mDCs. M indicates medium. Data shown are representative of four independent experiments.

showed less or no immune-stimulatory activity in human mDCs, which express TLR8 but not TLR7. SIMRA compounds induced distinct immune response profiles compared with TLR8-selective and dual TLR7 and TLR8 SIMRA compounds **30** and **31**, respectively.

All SIMRA compounds tested induced potent immune responses in vivo in wild-type mice; the levels of IL-12, KC, MCP-1 as well as other cytokines and chemokines were elevated 2 h after administration. Moreover, SIMRA compound **13** induced immune-stimulatory responses in wild-type and TLR9$^{-/-}$ mice but not in TLR7$^{-/-}$ and MyD88$^{-/-}$ mice, suggesting that the SIMRA compounds studied herein induce immune responses via TLR7, which engages the adaptor molecule MyD88 for immune signaling. Mice do not have a functional TLR8. It is interesting to note that the cytokine profiles induced by SIMRA compounds were also sequence-dependent both in vitro and in vivo. This sequence-dependency should provide an opportunity to select a customized SIMRA compounds for specific applications based on cytokine profile induced.

U-Rich and U-stretch containing ssRNA encapsulated in lipids activate TLR7 preferentially.[25] However, using SIMRA compounds, we have not found any correlation between U content and TLR7 selectivity. For example, SIMRA compound **9**, which contained a U-stretch, did not show greater TLR7 activity than compounds that did not contain a U-stretch. Moreover, our results suggest that an increase in A and C content in SIMRA compounds increased TLR7 selectivity. More importantly, the presence of 7-deaza-G is required for TLR7 activation, as SIMRA compounds **8*** and **11*** without 7-deaza-G did not activate TLR7 compared with SIMRA compounds **8** and **11**. Loss or gain of TLR7 activity with subtle sequence changes suggests that maintaining appropriate nucleotide sequences with 7-deaza-G substitutions is critical for TLR7 activation. Of the 29 SIMRA compounds studied herein, SIMRA compound **9** contained two such substitutions, compounds **7**, **8**, **10**−**12**, **17**, **19**, **24**-**26**, and **28** contained four, and the remaining SIMRA compounds contained six 7-deaza-G substitutions. The presence of 7-deaza-G is required for TLR7 activation, but the number of 7-deaza-G substitutions in SIMRA compound does not influence TLR7 selectivity. We previously reported that a SIMRA compound containing all 7-deaza-G substitutions did not activate either TLR7 or TLR8.[21]

Analysis of SIMRA compound sequences was carried out to identify any common nucleotide motifs that contribute to their TLR7 selectivity. In general, SIMRA compounds that had a 5′-AA/CN (A preferred over C in second position and N = U/C/7-deaza-G) and/or C/AUU-3′ (C preferred over A in the first position) at the 5′- and 3′-ends, respectively, activated TLR7 selectively in HEK293 cells and induced higher levels of IFN-α and lower levels of IL-12 and TNF-α in human PBMC cultures, suggesting that these motifs are more favorable for TLR7 activation. Further analysis of nucleotide sequences of TLR7-selective, dual TLR7/8, and nonactive SIMRA compounds in our compound library suggested a few common sequence motifs that could lead to TLR7 selectivity of SIMRA compounds. An AN^{1}N^{2}UG1A (N^1 = A or C; N^2 = U, C, or 7-deaza-G) motif is present in SIMRA compounds **7**−**12**, **15**, **17**−**20**, **22**, **23**, **26**−**29** that selectively activated TLR7 (Table 1). A SIMRA compound containing AACUG1U did not activate TLR7 at any concentration studied, however, suggesting that a U at the sixth nucleotide position leads to loss of TLR7-mediated immune-stimulatory activity (Supporting Information Figure 1),

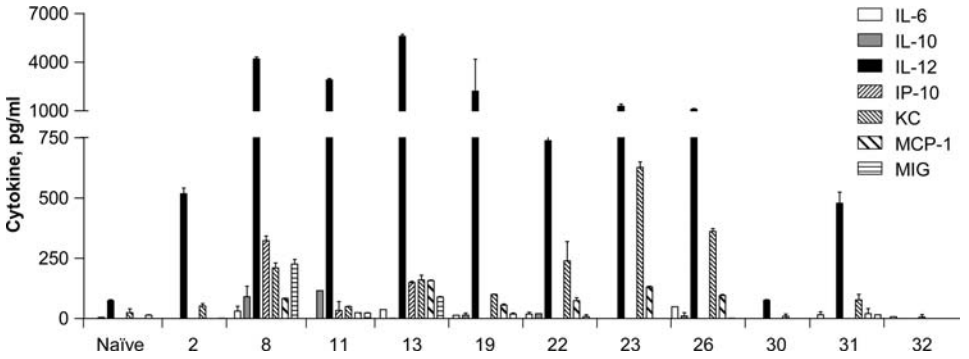


Figure 6. In vivo cytokine and chemokine induction in mice by SIMRA compounds at 25 mg/kg. Data shown are representative of two independent experiments.

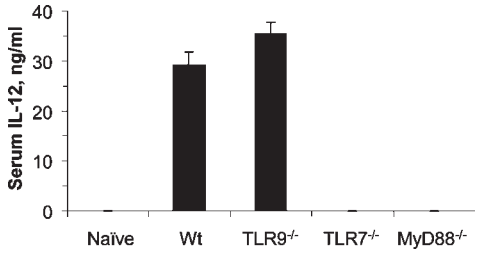


Figure 7. In vivo IL-12 induction by SIMRA compound **13** in wild-type (Wt), TLR9$^{-/-}$, TLR7$^{-/-}$, and MyD88$^{-/-}$ mice at 10 mg/kg. Data shown are representative of two independent experiments.

although C is tolerated at this position. SIMRA compounds **2**, **8**, **11**, **15**, **17**, **19**, **22**, **23**, **26**, and **28** contained a UG1AZ^{1}G1-Z^{2}UU (Z^1 = C preferred over A; Z^2 = A preferred over C) motif and substitution of the next-to-last U with a C (resulting in UG1ACG1ACU motif) led to complete loss of TLR7-mediated immune-stimulatory activity (SI Figure S1). These results suggest that the two motifs described above and containing 7-deaza-G preferentially are recognized by TLR7; even subtle changes in those preferred motifs could greatly affect TLR7 activation. SIMRA compounds **17**, **19**, **22**, **23**, **26**, and **28**, which contained both motifs, activated TLR7 to a greater degree as demonstrated by the higher in vitro and in vivo cytokine production induced by these compounds.

In the present study, we identified SIMRA compounds that selectively activate TLR7. We also observed that the immune-stimulatory profiles of SIMRA compounds can be modulated with subtle changes in nucleotide sequence composition. Two synthetic motifs were identified that selectively activate TLR7. SIMRA compounds induced Th1-type immune response profiles both in vitro and in vivo in mice. The cytokine and chemokine profiles induced by TLR7 selective SIMRA compounds were distinct from those induced by either TLR8 or dual TLR7 and TLR8 SIMRA compounds. Immune-stimulatory activity of SIMRA compounds is dependent on the presence of TLR7 and MyD88 in mice. The ability to design SIMRA compounds that selectively activate TLR7, TLR8, or both TLR7 and TLR8 and consequently induce distinct immune response profiles should provide new opportunities to develop novel immune-modulatory agents to treat infectious diseases, allergy, asthma, and cancer and as vaccine adjuvants.

Supporting Information Available: Standard purification and analysis protocols; figure showing activity of SIMRA compounds **22**, **22***, and **22****. This material is available free of charge via the Internet at http://pubs.acs.org.

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