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Identification of RNA Sequence Motifs Stimulating Sequence-Specific TLR8-Dependent Immune Responses¹

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The TLRs 7, 8, and 9 stimulate innate immune responses upon recognizing pathogen nucleic acids. U-rich RNA sequences were recently discovered that stimulate human TLR7/8-mediated or murine TLR7-mediated immune effects. In this study we identified single-stranded RNA sequences containing defined sequence motifs that either preferentially activate human TLR8-mediated as opposed to TLR7- or TLR7/8-mediated immune responses. The identified TLR8 RNA motifs signal via TLR8 and fail to induce IFN- α from TLR7-expressing plasmacytoid dendritic cells but induce the secretion of Th1-like and proinflammatory cytokines from TLR8-expressing immune cells such as monocytes or myeloid dendritic cells. In contrast, RNA sequences containing the TLR7/8 motif signal via TLR7 and TLR8 and stimulate cytokine secretion from both TLR7- and TLR8-positive immunocytes. The TLR8-specific RNA sequences are able to trigger cytokine responses from human and bovine but not from mouse, rat, and porcine immune cells, suggesting that these species lack the capability to respond properly to TLR8 RNA ligands. In summary, we describe two classes of single-stranded TLR7/8 and TLR8 RNA agonists with diverse target cell and species specificities and immune response profiles. *The Journal of Immunology*, 2008, 180: 3729–3738.

Toll-like receptors play an essential role in pathogen recognition and innate immune responses in mammals. In humans, 10 different TLRs were described as recognizing a variety of pathogen-associated molecular patterns from bacteria, viruses, and fungi. TLR7, 8, and 9 belong to the same subfamily of Toll-like receptors based on their genomic structure, sequence similarity, and homology (1). TLR7 and 8 are activated by small antiviral compounds such as imidazoquinolines, guanine nucleoside analogues, whole RNA from viruses, certain synthetic single-stranded oligoribonucleotides (ORNs)³ or double-stranded small interfering RNA sequences (2–8). TLR9 recognizes nonmethylated, CpG-containing DNA of bacterial or viral origin or synthetic single-stranded CpG oligodeoxynucleotides (ODNs) (9–11).

Whereas most TLRs are expressed on the cell surface, TLR7, 8, and 9 are located in intracellular endolysosomal compartments. TLR7, 8, and 9 have a unique pattern of cell type-specific expression that is thought to be responsible for different pathogen re-

sponse profiles (12). Hereby, human TLR7 and TLR8 together display a broader cell type-specific distribution than TLR9. TLR7 mRNA was mainly detected in plasmacytoid dendritic cells (pDCs) and B cells, and only low mRNA expression was observed in monocytes, macrophages, myeloid DCs (mDCs), and monocyte-derived dendritic cells (DCs). In contrast, TLR8 mRNA was found to be highly expressed in monocytes but also in mDCs, monocyte-derived DCs, and macrophages (13–18).

Specific natural viral but also eukaryotic RNA sequences have been identified that are capable of stimulating human TLR7 and TLR8 when incorporated into single-stranded synthetic ORNs. The human TLR7 and TLR8 immune response appears to be stimulated strongest with U-rich sequences (4, 19–21), although recent work has suggested that the TLR7 immune response lacks specificity for defined sequence motifs (22). TLRs are type I integral membrane glycoproteins consisting of leucine-rich repeats (LRR) in the ectodomain (ECD), and cytoplasmatic signaling domains known as Toll IL-receptor (TIR) domains. The TIR and ECD domains are joined by a single trans membrane helix, and the ECDs containing the LRRs have been inferred to be responsible for ligand recognition (23, 24).

In this study, we searched for and identified RNA sequence motifs that selectively stimulate human TLR7 and TLR8 or only TLR8. The sequence-dependent effects were analyzed on primary human immune cells and recombinant TLR-expressing cells, demonstrating that specific sequences can induce TLR7 and TLR8 effects but others stimulate only TLR8-specific signaling and, therefore, lack pDC-mediated effects like IFN- α production and pDC maturation. TLR8-dependent RNA sequences were not able to trigger TLR-mediated effects in mouse or rat immune cells, demonstrating that the murine TLR8 not only lacks the capability to respond to TLR8 small molecule ligands (25, 26) but also to TLR8 RNA ligands. However, combining poly(dT) ODN and TLR8 RNA ligands resulted in a regaining of TLR8-dependent effects, suggesting that murine TLR8 is in principle capable of signaling

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³ Abbreviations used in this paper: ORN, oligoribonucleotide; BDCA, blood DC Ag; DC, dendritic cell; DOTAP, 1,3-di-*o*-oleoyloxy-3-(trimethylammonium)propane; ECD, ectodomain; HEK, human embryonic kidney; IP-10, IFN- γ -inducible protein-10; LRR, leucine rich repeat; mDC, myeloid DC; ODN, oligodeoxynucleotide; pDC, plasmacytoid DC; poly(rI:rC), polyribonucleosine:polyribocytidylic acid; SN, supernatant.

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Table I. ORN sequences^a

ORN No.	RNA Sequence
R-1075	CCGUCUGUUGUGUGACUC
R-1263	GCCACCGAGCCGAAGGCACC
R-0001	UAUAUAUAUAUAUAUAUAUA
R-0002	UUAAUUUAUUUAUUUAUUU
R-0003	UUUAUUUAUUUAUUUAUUUA
R-0004	UUUUUUUUUUUUUUUUUUUA
R-0005	UGUGUGUGUGUGUGUGUGUG
R-0006	UUGUUGUGUGUGUGUGUGU
R-0007	UUUGUUUGUUUGUUUGUUUG
R-0008	UUUUUUUUUUUUUUUUUUUG
Control	CCGAGCCGCGCCCCC
ORN 1	
Control	CCGAGCCGAAGGCACC
ORN 2	
R-1211	dNdNdNdNdNGUUGUGdNdNdNdNdNdNdNdNdNdNd
R-1212	dNdNdNdNdNdNdNUUGUGdNdNdNdNdNdNdNdNdNdNd
R-1213	dNdNdNdNdNdNdNUUGdNdNdNdNdNdNdNdNdNdNdNdNdNdNd
R-1214	dNdNdNdNdNdNdNUUGdNdNdNdNdNdNdNdNdNdNdNdNdNdNdNd
R-1215	dNdNdNdNdNdNdNdNUUGdNdNdNdNdNdNdNdNdNdNdNdNdNdNdNd
R-1216	dNdNdNdNdNdNdNdNdNGUdNdNdNdNdNdNdNdNdNdNdNdNdNdNdNd
R-1312	GCCACCGAGCGUUGUACC
R-1321	GCCACCGAGCAUUGUGAACC
R-1322	GCCACCGAGCAUUGAGAACC
R-1323	GCCACCGAGCAUUGAGAACC
R-1324	GCCACCGAGCAAGGUGAACC

^a All ORN were phosphorothioate modified (d refers to deoxyribonucleotides). Control ORN 1 and 2 were used for motif analysis, and the underlined nucleotides were exchanged for 4-mer sequence alterations as shown in Fig. 2.

upon encountering natural RNA ligands and may have another defect.

Materials and Methods

Reagents

The TLR7 and TLR8 agonist resiquimod (R-848 or S-28463) was commercially synthesized by GLSynthesis. The CpG ODNs 2395 and 10103 were provided by Coley Pharmaceutical. ORNs were obtained from BioSpring. All oligonucleotides were controlled for identity and purity by Coley Pharmaceutical and had undetectable endotoxin levels (<0.1 endotoxin unit/ml) as measured by the *Limulus* assay (BioWhittaker). Polyriboinosinic: polyribocytidylic acid (poly(rI:rC)) was obtained from Sigma-Aldrich. Oligonucleotides were suspended in sterile endotoxin-free Tris-EDTA (ODN) (Sigma-Aldrich) or in DNase- and RNase-free water (ORN) (Invitrogen Life Technologies) and stored and handled under aseptic conditions to prevent contamination. Sequences are listed in Table I.

Reporter assay

Human embryonic kidney (HEK) cells (HEK293) containing a NF-κB-luciferase reporter construct and expressing human TLR7, TLR8, or TLR9 or without TLR expression were used as described before (25, 27). Cells were plated on 96-well plates at 1.5×10^4 /well and allowed to attach overnight. The cells were subsequently incubated for 16 h with the indicated amount of ORNs complexed to 1,2-dioleoyloxy-3-(trimethylammonium)propane (DOTAP; Roche), DOTAP alone, R-848, poly(rI:rC), or CpG ODNs and then tested for luciferase expression. Each data point was done in duplicate.

Transient transfections

HEK293T cells were plated on 96-well plates at 1.5×10^4 /well and allowed to attach overnight 1 day before transfection. Transient transfection was performed with Effectene transfection reagent (Qiagen) according to the manufacturer's protocol. To reach the appropriate level of expression of transfected genes, cells were incubated 24 h under their normal growth conditions. Stimulation of transfected cells was then performed with the indicated amounts of ORNs complexed to DOTAP (Roche), DOTAP alone, or R-848.

PBMCs

PBMC preparations from healthy male and female human donors were obtained from the Institute for Hemostaseology and Transfusion Medicine

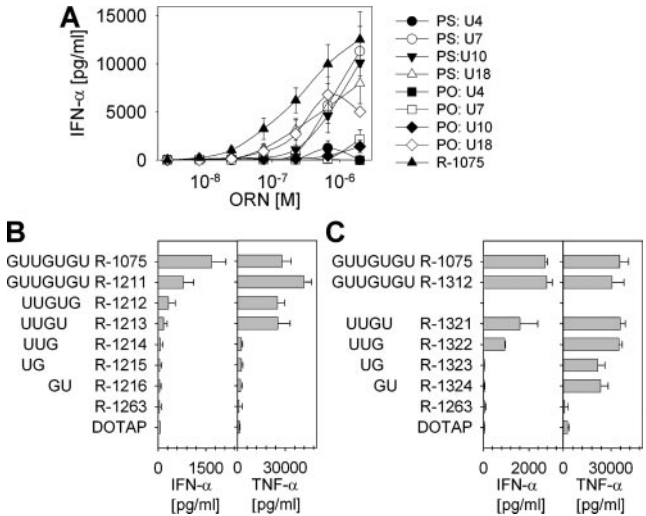


FIGURE 1. The strongest IFN-α release needs a minimum of four defined ribonucleotides. Human PBMC were stimulated with 2.0 μM ORN complexed to 25 μg/ml DOTAP or with 25 μg/ml DOTAP alone (A). After 16 h SN was harvested and IFN-α and TNF-α were measured. Sequence variants of GUUGUGU were nested within a 20-mer random nonstimulatory ODN sequence (B) or nonstimulatory ORN sequence (R-1263) (C). Sequences are listed in Table I. Data shown are mean ± SEM of three donors. One of three independent experiments is shown. PO, Phosphodiester; PS, phosphorothioate.

of the University of Düsseldorf (Düsseldorf, Germany). PBMCs were purified by centrifugation over Ficoll-Hypaque (Sigma-Aldrich). Purified PBMCs were washed twice with 1× PBS and resuspended in RPMI 1640 culture medium supplemented with 5% (v/v) heat-inactivated human AB serum (BioWhittaker) or 10% (v/v) heat inactivated FCS, 1.5 mM L-glutamine, 100U/ml penicillin, and 100 mg/ml streptomycin (all from Sigma-Aldrich).

Cytokine and chemokine production

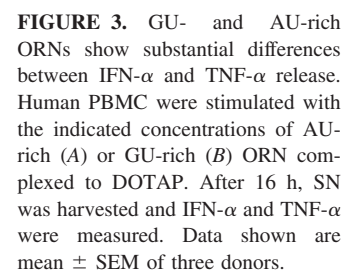
Freshly isolated PBMCs were resuspended at a concentration of 3×10^6 /ml to 5×10^6 /ml and added to 96-well round-bottom plates (200 μl/well) that had previously received nothing or R-848, ORNs complexed to DOTAP, ODNs, or DOTAP alone. Cells were cultured in a humidified incubator at 37°C for the indicated time points. Culture supernatants (SN) were collected and, if not used immediately, frozen at -20°C until required. Amounts of cytokines in the SN were assessed using commercially available ELISA kits (IL-6, IL-10, IFN-γ, or TNF-α from Hölzel Diagnostika (Dialclone) or IL-12p40 from BD Pharmingen), in-house ELISAs (IFN-γ-inducible protein-10 (IP-10) or IFN-α) developed using commercially available Abs (from BD Pharmingen or PBL Biomedical Laboratories, respectively) or Luminex technology using a Cytokine 25-plex kit (BioSource International).

Cell isolation

Human pDCs and monocytes were isolated from PBMCs by blood DC Ag (BDCA)-4 or CD14 immunomagnetic bead positive selection according to the manufacturer's recommendations (Miltenyi Biotec). Purity was confirmed by staining with mAb to CD11c, CD14, HLA-DR, CD123, BDCA-2, and BDCA-4 (all from BD Pharmingen) and was typically >90%. Human NK cells were isolated by depletion from PBMC using NK cell isolation kit II according to the manufacturer's information (Miltenyi Biotec). Purity was analyzed by staining with mAbs to CD56, CD3, CD14, and CD19 (all from BD Pharmingen). Human mDCs were isolated from PBMCs by CD1c (BDCA-1) and CD141 (BDCA-3) immunomagnetic bead positive selection according to manufacturer's protocol (Miltenyi Biotec). Cell purity was controlled by BDCA-1, BDCA-3, CD14, and CD19 staining (all from BD Pharmingen). Isolated cells were plated on 96-well plates at 5×10^5 cells/ml and stimulated for 16 h with the indicated amount of ORNs complexed to DOTAP, R-848, CpG ODN, or DOTAP alone. After 16 h SN was harvested and cytokines or chemokines were measured.

[illegible]

Blood was collected from the jugular vein by venipuncture using a 50-ml syringe containing 2 ml of 7.5% EDTA. The blood was centrifuged at $1400 \times g$ for 20 min and the white blood cell-containing buffy coat was removed and resuspended in PBS (10 mM at pH 7.4) containing 0.1% EDTA. PBMC were obtained by overlaying the buffy coat on 54% Percoll (Pharmacia Biotech) and conducting centrifugation at $2,000 \times g$ for 20 min. The PBMCs were then subjected to three washes using PBS (containing



0.1% EDTA). Stimulation of PBMCs was performed in 96-well, round-bottom plates (Nunc) using AIM-V medium supplemented with 2% FBS, 50 μ g/ml streptomycin sulfate, 10 μ g/ml gentamicin sulfate, 2 mM L-glutamine, and 50 μ M 2-ME (Sigma-Aldrich). Cells (5×10^5) were cultured with ORN in triplicate wells in a 200- μ l total volume and incubated for up to 48 h at 37°C in an atmosphere of 5% CO₂ and 95% humidity. Culture SNs were collected and assayed for cytokine production.

Multiple sequence alignment

Multiple sequence alignments were performed using ClustalW analysis (www.ebi.ac.uk/clustalw/) according to web site descriptions.

Results

Sequence-specificity of IFN- α and/or TNF- α production upon immune cell stimulation with GU- and AU-rich ORN

Previous studies suggested sequence-dependent recognition of short synthetic single-stranded U-rich RNAs by human TLR7 and TLR8 or mouse TLR7 (4, 19–21), although some reports argued against sequence-specific effects (7, 22). However, by comparing a phosphorothioate GU-rich (R-1075) sequence previously identified as a virus (HIV) genome-derived TLR7/8 ligand (4) to a homopolymeric phosphorothioate or phosphodiester poly(U) RNA sequence of the same length (18-mer), we found that the GU-rich ORN induced substantially stronger IFN- α production from human PBMCs (Fig. 1A). This was evidence in favor of a sequence-specific difference in the TLR7-dependent immune response, and we further explored the impact of sequence composition on cellular activation. To enable us to differentiate between TLR7- and/or TLR8-mediated effects, we started to study the induction of IFN- α (from TLR7-positive cells) vs TNF- α (from TLR8-positive cells) cytokine release upon the stimulation of human PBMCs with ORNs (28, 29). To observe subtle influences of sequence modifications on cytokine responses, we first determined the minimal sequence motif contained in ORN R-1075 required to stimulate immune effects (Fig. 1B). TNF- α and IFN- α production was analyzed upon stimulation with ORN R-1075, its central GU-rich sequence GUUGUGU, and different length variants of this sequence. Both the central sequence and its length variants were nested in either a random nonstimulatory DNA sequence or a nonstimulatory RNA sequence lacking U nucleotides (R-1263; see Table I). At least four nucleotides, UUGU, found in the GU-rich region (Fig. 1, B and C) were required to stimulate cytokine responses, whereas shorter RNA sequences like UUG, UG, or GU induced decreased cytokine stimulation. A combination of deoxy-ribonucleotides and ribonucleotides in the same molecule appeared to interfere with the RNA-mediated immune stimulatory effects, because nesting GU-rich RNA sequences in the random DNA sequence resulted in weaker cytokine release as compared with using the nonstimulatory RNA sequence surrounding the GU sequences (compare B and C in Fig. 1).

Our data further suggest differences in the strength of IFN- α and TNF- α induction (Fig. 1C). Whereas the 4-mer and 3-mer sequences UUGU and UUG showed only a moderate decrease of IFN- α compared with TNF- α secretion, the 2-mer sequences appeared to selectively induce TNF- α over IFN- α production. Therefore, at least one (for TNF- α production) or two U nucleotides (for TNF- α and IFN- α production) are needed to stimulate cytokine induction, and TLR7 stimulation (IFN- α production) appears more sensitive than TLR8 stimulation (TNF- α production) to subtle sequence changes.

To investigate the impact of sequence modifications on the stimulation of IFN- α and TNF- α production in more detail, 40 phosphorothioate ORNs differing in the U-containing 4-mer sequence derived from R-1075 were tested (Fig. 2 and data not shown). Surprisingly, specific sequence alterations converted an ORN from

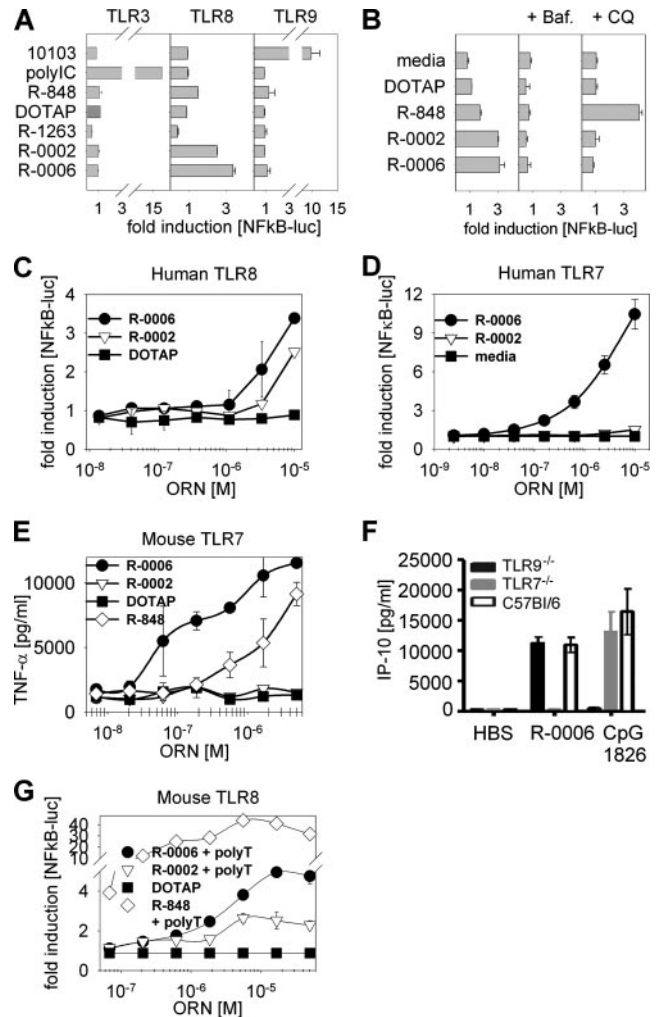


FIGURE 4. AU-rich and GU-rich ORNs stimulate human TLR7 and TLR8 signaling in a dose-dependent manner. **A**, HEK293 cells stably transfected with human TLR3, TLR8, or TLR9 containing a NF- κ B-luciferase (NF κ B-luc) reporter construct were incubated with the indicated ORN (10 μ M complexed to 50 μ g/ml DOTAP) or control stimuli (10 μ M R-848, 50 μ g/ml poly(rI:C), 3.3 μ M CpG ODN 10103, or 50 μ g/ml DOTAP) for 16 h. NF- κ B activation was measured by assaying luciferase activity. Results are given as fold induction above background (medium). **B**, Human TLR8-expressing HEK293 cells were incubated with the indicated ORN (15 μ M complexed to 75 μ g/ml DOTAP) or control stimuli (15 μ M R-848 or 75 μ g/ml DOTAP) together with 200 nM bafilomycin (Baf.) or 1 mM chloroquine (CQ) for 16 h. **C**, Human TLR8-expressing HEK293 cells were stimulated as in **A**. **D**, Human TLR7-expressing HEK293 cells containing a NF- κ B-luciferase reporter construct were stimulated as in **A**. **E**, The mouse macrophage cell line Raw264.7 was stimulated with the indicated concentrations of ORN complexed to DOTAP as above, R-848, or DOTAP alone. After 16 h, SNs were harvested and cytokine release was measured. **F**, C57BL/6 TLR7^{-/-} or TLR9^{-/-} mice ($n = 4$ /group) were injected i.v. with 100 μ g of ORN formulated with DOTAP or 100 μ g of CpG ODN 1826 and bled after 3 h. IP-10 production was measured within plasma. Values are shown as pg/ml. **G**, HEK293 cells transiently transfected with murine TLR8 were stimulated for 16 h with the indicated concentrations of ORN complexed to DOTAP (100 μ g/ml; 1/3 dilution), R-848, or DOTAP alone (100 μ g/ml) in the presence of 5 μ M poly(dT) (polyT) ODN. All data represent at least one of three or more independent experiments (for human PBMC, $n = 3$ donors).

an IFN- α and TNF- α inducer to a relatively exclusive TNF- α inducer (Fig. 2). ORNs containing A in combination with U (referred here to as AU-rich or TLR8 ORNs) such as UAAU, UAAU,

Table II. *TLR8 ORNs lack IFN- α production from PBMC and pDCs^a*

	PBMC				PDC				Monocytes				mDC			
	R-0006		R-0002		R-0006		R-0002		R-0006		R-0002		R-0006		R-0002	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
IL-1Ra	79,575	5,915	57,522	7,282	66	56	0	-1	0	0	0	0	1	1	1	1
MIP-1 β	54,803	11,617	41,704	5,410	3,621	1,892	103	86.478	5,755	1,861	4,759	1,397	968	2	606	-65
IL-6	54,510	11	51,505	1,293	350	232	12	15.565	10,646	2,485	11,871	728	1,660	211	1,149	168
TNF- α	52,524	17,313	50,402	5,202	563	87	25	25.512	19,263	1,917	18,786	12,217	1,842	328	849	205
IL-1	32,217	2,217	38,606	1,903	0	-1	0	-1	2,431	252	2,699	295	234	24	184	13
MIP-1 α	31,452	11,696	10,488	951	1,779	865	20	1.449	12,611	3,346	9,693	914	1,641	-47	1,315	-12
IL-12p40	16,792	8,648	6,060	2,605	37	18	16	0.6997	348	51	225	15	256	133	94	46
MCP-1	12,496	1,364	20,633	2,809	111	42	38	19.266	23	199	7	178	20	2	13	2
IL-8	12,075	3,363	9,785	426	33,385	11,334	7,087	3,589.1	24,194	5,920	18,269	6,952	1,740	-283	1,581	-321
IFN- α	5,065	364	10	3	1,295	300	0	-1.923	0	0	10	3	1	1	1	1
RANTES	3,773	2,345	2,030	861	0	-1	0	-1	0	0	0	0	0	-1	0	-1
IL-10	1,428	367	683	99	-2	-3	-2	-2.92	0	0	0	0	0	0	0	0
IFN- γ	1,293	786	1,460	878	-1	-1	0	-1	0	0	0	0	0	0	0	0
IP-10	1,278	136	1,066	376	1	0	0	-1.17	0	0	0	0	0	-1	0	-1
IL-2R	745	179	842	37	168	102	0	-1	0	0	0	0	28	-2	24	37
GM-CSF	675	39	688	74	0	-1	0	-1	158	9	187	4	31	3	4	3
IL-15	523	62	512	23	0	-1	0	-1	1	0	0	0	0	-5	0	-2
MIG-1	253	-23	103	1	0	-1	0	-1	0	0	0	0	0	0	0	1
IL-7	235	36	204	6	0	-1	0	-1	0	0	0	0	0	0	0	0
IL-13	93	2	89	1	0	-1	0	-1	22	2	26	5	1	2	1	3
Eotaxin	18	3	21	1	0	-1	0	-1	1	0	1	0	0	0	1	0
IL-4	13	5	12	2	1	-2	0	-2.04	0	0	0	0	0	0	0	0
IL-5	13	2	13	0	0	-1	0	-1	1	1	1	1	0	0	1	0
IL-2	5	2	5	1	0	-1	0	-1	0	2	5	1	0	2	5	1
IL-17	0	1	0	1	0	-1	0	-1	0	1	0	1	0	1	0	1

^a Human PBMCs, purified pDCs, monocytes, or mDCs were stimulated with 1.0 μ M (PBMC, pDCs, monocytes) or 2.0 μ M (mDCs) ORN complexed to DOTAP (10 μ g/ml). After 16 h SNs were harvested and cytokines were measured by Luminex technology. Data shown are mean \pm SEM of maximal activities (pg/ml) of two (PBMC and monocytes) or three (pDCs and mDCs) donors. DOTAP background values were subtracted from mean maximal activities and SEM.

AUGU, AUAU, UAUA, UAUC, AUAC, CUAC, GUAC, CUAU, or AUAU still produced high amounts of TNF- α but little to no IFN- α (Fig. 2), while ORNs containing at least two U combined with G (or C) nucleotides (referred here to as GU-rich or TLR7/8 ORNs) like UUUU, GUUC, UUGU, GUUU, UUUC, UGUU, or UCUC induced high TNF- α and IFN- α .

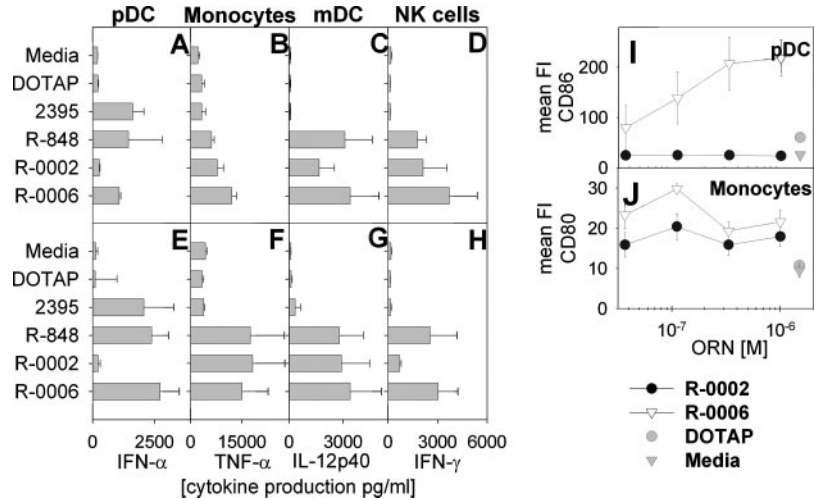
We further investigated whether the activity of ORNs containing such divergent AU or GU sequences may be altered and/or increased by nesting the identified 4-mer sequences UAUU and UUGU (strongest as 4-mers to induce TNF- α or TNF- α and IFN- α , respectively) in a longer 20-mer ORN (Table I, Fig. 3, and data not shown). All GU-rich ORNs (R-0005 to R-0008; Fig. 3B) stimulated high IFN- α and TNF- α production, although differences in the potency to induce IFN- α secretion were observed. For example, whereas ORN R-0006 appeared to induce slightly more potent IFN- α induction than the other GU-rich ORNs, ORN R-0005 lacking the UUGU motif was a less potent inducer of IFN- α secretion. In contrast, an ORN containing a homopolymeric poly(U) sequence stimulated substantially weaker IFN- α cytokine induction, and a poly(G) as well as a poly(A) or poly(C) RNA sequence did not induce an effect (data not shown). The spacing also had substantial impact on AU-rich ORNs (Fig. 3A). Whereas ORNs R-0003 and R-0004 with three or four U nucleotides bridging the A nucleotides were able to induce both TNF- α and IFN- α (resulting in principle in AUUU, UUUU, or UUUU motifs that stimulate both cytokines; Fig. 2), ORNs R-0001 and R-0002 with only one or two bridging U nucleotides stimulated TNF- α production but lacked IFN- α stimulation, indicating that the strength of the response depends on the U spacing between the motifs. Similar results as those for R-0006 and R-0002 were obtained with the same sequences in a phosphodiester backbone (data not shown).

AU-rich ORN stimulate TLR8- but not TLR7-mediated immune activation

Further characterization of AU- and GU-rich ORN-mediated effects were performed using either HEK293 cells stably transfected with human TLR3, TLR7, TLR8, or TLR9 and an NF- κ B luciferase reporter plasmid (Fig. 4, A–D). For these experiments, two representative GU- and AU-rich ORNs were used: R-0006 and R-0002 (see Fig. 3). Signaling in cells expressing TLR8 was induced by AU- and GU-rich ORNs in a sequence- and dose-dependent manner (Fig. 4, A and C), whereas only the GU-rich ORN stimulated signaling in TLR7-expressing cells (Fig. 4D). ORNs not complexed to DOTAP (data not shown) or DOTAP alone did not induce an effect. Activation of immune cells by TLR ligands such as CpG ODN or ORN has been shown to require endosomal maturation and acidification (4, 19, 30). Inhibition of TLR8-mediated signaling induced by GU- and AU-rich ORNs as well as by R-848 with bafilomycin A1 resulted in loss of NF- κ B activation (Fig. 4B). Chloroquine strongly suppressed the ORN-mediated effects but did not affect the signaling induced by R-848, at least at the concentration used (Fig. 4B). In data not shown, 10- to 100-fold higher concentrations of chloroquine were required to inhibit R-848.

Additional proof for the selectivity of the AU- vs GU-rich ORN immune response comes from experiments on murine immune cells that express functional TLR7 but lack proper TLR8-dependent activation (8, 31). To investigate whether GU- and AU-rich ORNs differ in their capacity to induce murine TLR7 stimulation, the TLR7-expressing mouse macrophage cell lines RAW264.7 and J774 were stimulated with increasing concentrations of GU- and AU-rich ORN R-0006 and R-0002 (Fig. 4E and data not shown).

FIGURE 5. Cytokine secretion from purified human immune cells stimulated by TLR7/8 and TLR8 ORNs. CD123⁺ enriched cells (A), CD14⁺ enriched cells (B), mDCs (C), NK cells (D), or human PBMC (E–H) were stimulated with the indicated ORN at 2.0 μ M complexed to DOTAP (20 μ g/ml), 2.0 μ M R-848, 0.5 μ M ODN 2395, or DOTAP alone (20 μ g/ml). After 16 h, SNs were harvested and cytokine production was measured. CD123⁺ purified pDC (I) or CD14⁺ purified monocytes (J) were incubated with the indicated amounts of ORN complexed to DOTAP or DOTAP alone. After 16 h, the cells were harvested and stained with CD123, CD11c, and HLA-DR (I) or CD14 and CD19 (J). Expression of CD86 or CD80 was acquired by flow cytometry. Data shown are mean \pm SEM of three blood donors.



Only the GU-rich ORN stimulated TNF- α production in both macrophage cell lines, whereas the AU-rich ORN completely lacked an immune stimulatory effect. TLR7 specificity of the GU-rich ORN was also demonstrated by using splenocytes from TLR7^{-/-} mice that did not respond to GU-rich ORN stimulation (Fig. 4F).

Recently, activation of murine TLR8 was demonstrated by a combination of imidazoquinoline small molecules and homopolymer poly(dT) ODN (31). A poly(dT) ODN combined with either ORN R-0002 or R-0006 on HEK-293T cells transiently transfected with murine TLR8 resulted in substantial TLR8-mediated NF- κ B signaling by both ORNs (Fig. 4G). No response was observed for ORNs and R-848 in the absence of poly(dT) ODN (data not shown). Therefore, although the AU-rich ORN did not stimulate murine TLR7, TLR8 signaling was achieved for both ORN classes when combined with a homopolymer poly(dT) ODN similar to the results observed for human TLR8.

Characterization of the TLR-mediated immune response stimulated by AU- and GU-rich ORNs

Human TLR7 and TLR9 colocalize in the same subpopulations of immune cells, B cells, and pDCs, whereas human TLR8 is strongly expressed in the myeloid compartment, e.g., monocytes, mDCs, or monocyte-derived DCs (15–17, 32, 33). Although our data strongly suggest that AU-rich ORNs do not signal through TLR7, only one major difference was observed between AU-rich and GU-

rich ORNs when evaluating cytokine production in human PBMC cultures: the TLR8 ORN R-0002 induced minimal IFN- α or IFN- α related effects compared with the TLR7/8 ORN R-0006 (Table II). These data suggest that the main effects induced upon RNA TLR7 triggering in human immune cells are IFN- α and probably IFN-dependent effects.

To further analyze motif- and cell type-specific responses stimulated by TLR7/8 and TLR8 ORNs, pDCs, monocytes, mDCs, and NK cells were purified from human PBMCs. The AU-rich ORN R-0002 completely failed to induce IFN- α secretion from human pDCs, confirming our previous observation (Fig. 5). In contrast, monocytes, mDCs, and NK cells showed somewhat comparable TNF- α , IL-12p40, and IFN- γ cytokine release for both ORNs (Fig. 5). The TLR9 ligand C-Class CpG ODN 2395 exhibited a different cytokine pattern (34). We further investigated the release of a variety of cytokines and chemokines induced from purified pDCs, monocytes, and mDCs by the TLR8 and TLR7/8 ORNs (Table II). The data confirm the above observation that no IFN- α induction was observed in pDCs with the TLR8 ORN, whereas only pDCs responded to TLR7/8 ORN stimulation by IFN- α secretion. Stimulation of most other cytokines or chemokines from pDCs, monocytes, or mDCs appeared similar for both ORN classes.

Because pDCs did not release IFN- α upon stimulation with the TLR8 ORN, we also investigated the effect of GU- and AU-rich ORNs on the up-regulation of cell surface activation markers on

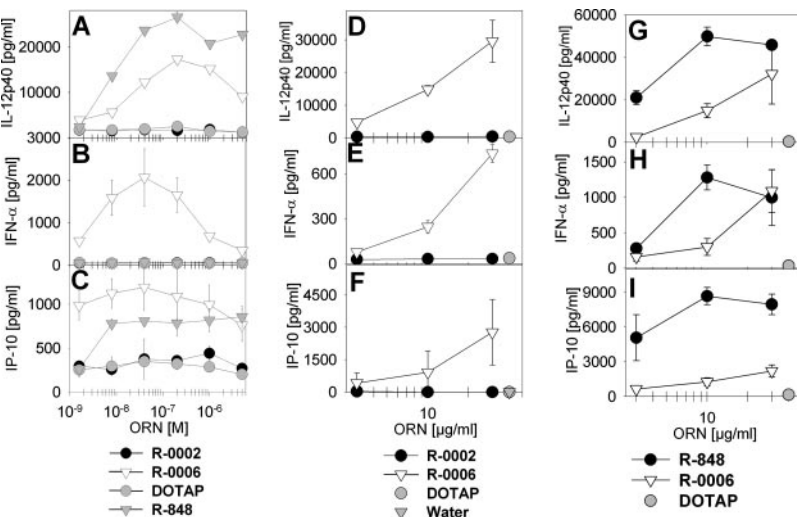


FIGURE 6. TLR8 ORNs do not stimulate mouse immune responses. A–C, CD11c⁺ cells generated from Sv129 mice were stimulated with the indicated concentrations of ORN complexed to DOTAP (50 μ g/ml), R-848, or DOTAP alone (50 μ g/ml). After 20 h, SN were harvested and cytokines were measured. Data represent one from at least three independent experiments. D–I, Sv129 mice ($n = 5$ /group) were injected i.v. with 3, 10, or 30 μ g of ORN formulated with DOTAP or R-848 and bled after 3 h. Cytokine production was measured within blood serum. Values are shown as pg/ml.

Table III. Multiple sequence alignment of TLR8 LRR3 of different species^a

Species	Amino Acid Sequence	Amino Acid Positions
Human	PNVQHQNPNPGIQSNGNLITDGAFLNLKRLRELLLEDN	95–132
Chimpanzee	PNVQHQNPNPGIQSNGNLITDGAFLNLKRLRELLLEDN	116–153
Monkey	PNVQRQNGNPGMQSNGNLITDGAFLNLKRLRELLLEDN	95–132
Dog	ANPQHLSNPNDN-KNGMNITDGAFLNLQNLNQLLEDN	95–131
Pig	AKLW--PQS----ENGMTITDGAFLNLHHLRELLLEDN	95–126
Cattle	AKSQ--SGNPAV-KKAMTITDGAFLNLKHLRELLLEDN	95–129
Rat	AKQQHPNEN----KNGMNITEGAFLSLRNLTELLLEDN	95–128
Mouse	AKQQHPNEN----KNGMNITEGALLSLRNLTVLLLEDN	98–131

^a Multiple sequence alignment was performed using the ClustalW alignment program. The table shows the amino acids of LRR3.

purified pDCs and monocytes (Fig. 5, *I* and *J*). The TLR8 ORN R-0002 did not induce CD86 expression upon culture with pDCs but up-regulated CD80 on purified monocytes. In contrast, the TLR7/8 ORN R-0006 stimulated CD86 and CD80 expression on pDCs and monocytes. Taken together, both classes of ORN ligands are capable of activating TLR8-expressing human monocytes, mDCs, and NK cells, whereas only GU-rich ORNs are capable of stimulating TLR7-expressing pDCs. In previous studies, human NK cells were demonstrated as expressing TLR8 and probably low levels of TLR7 and were the only cell type responsible for IFN- γ production upon stimulation with a TLR7/8 or TLR8 ligand, indicating direct stimulation of NK cells by ORN (21, 32, 35, 36), although one report suggested that the effects on NK cells were indirect (37).

Responses stimulated by GU- and AU-rich ORN upon stimulation of immune cells of different species in vitro or in vivo

Additional experiments aimed at evaluating the activity of the two different ORN classes on primary murine cells as well as in vivo. Results similar to the mouse macrophage cell lines were obtained in vitro; splenocytes and CD11c⁺ cells generated from Sv129 mice released a variety of cytokines and chemokines such as IL-12p40, IFN- α , IP-10, TNF- α , or IL-12p70 upon stimulation with the GU-rich ORN R-0006 or R-848, but not upon stimulation with the AU-rich ORN R-0002 (Fig. 6, *A–C*, and data not shown). In addition, in vivo studies upon i.v. administration of both ORNs complexed to DOTAP in Sv129 mice resulted in similar findings. Strong cytokine responses were detected for the TLR7/8 ligands R-848 and R-0006, but the TLR8 ligand R-0002 failed to stimulate a cytokine response (Fig. 6, *D–J*).

Protein sequence comparison of TLR8 among different vertebrates (human, monkey, chimpanzee, dog, cattle, porcine, mouse, and rat) revealed few insertions or deletions across all LRRs. However, the strongest differences are detected in LRR3 (Table III). Although the human, chimpanzee, and monkey sequences are

highly conserved, the rat, mouse, porcine, and bovine sequences exhibit deletions of four aa residues at position 106 (mouse), 103 (rat), or 102 (porcine) or an insertion of two aa at positions 105–106 (cattle). Interestingly, the porcine and bovine sequences have another deletion of two aa in the same region at position 97. Rat splenocytes are not responsive to the TLR8 ORN ligand R-0002 as observed for mouse immune cells (TNF- α , IL-6, IFN- γ , and IL-12; Fig. 7*A* and data not shown). Similar data were observed for porcine PBMC; while the TLR7/8 ORN ligand R-0006 induced high levels of IL-12, IFN- α , TNF- α , and IFN- γ , ORN R-0002 failed to stimulate cytokines (Fig. 7*B* and data not shown). In contrast, bovine PBMCs displayed responses to both ORNs (Fig. 7*C*). One recent study suggested certain chemically modified nucleic acids to be TLR7/8 or TLR8 stimulators and demonstrated that both compounds activate immune cells in nonhuman primates in a manner similar to their effects on human immune cells (38). These data suggest that the LRR3 region may play an important role in the species-specific recognition of or signaling by single-stranded RNA.

Discussion

Phylogenetic analysis indicates that TLR7, 8, and 9 belong to one subfamily of TLRs and emerged as nearest neighbors. Within this subfamily, TLR7 and TLR8 are believed to build another small functional subfamily (1, 39). Previous data demonstrated that TLR7 is activated by genomic RNA from single-stranded RNA viruses (6, 7, 40) or synthetic RNA sequences (3,4,7,19–22,41). Although some of these investigators did observe a lack of sequence specificity of TLR7-mediated responses, sequence-specific recognition of single-stranded RNA via human and murine TLR7 was suggested for sequences derived from single-stranded RNA viral genomes, U1 small nuclear RNA containing U and G nucleotides (4, 19), or certain small interfering RNA sequences (3, 41). Although all of these reports described sequence-specific effects of TLR7-dependent immune responses, no detailed analysis of the sequence selectivity of human TLR7 for single-stranded RNA was

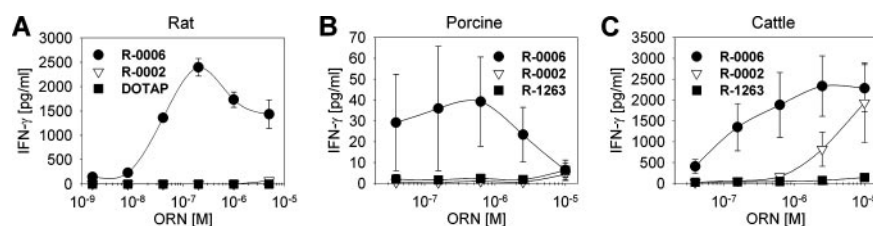


FIGURE 7. Purified rat splenocytes or porcine PBMC do not but bovine PBMC do respond to TLR8 ORNs by cytokine secretion. *A*, Splenocytes from Sprague-Dawley rats were pooled from three different rats and stimulated with the indicated ORN concentrations (complexed to 62.5 μ g/ml DOTAP; 1/5 dilution), R-848, or DOTAP alone. SNs were harvested after 20 h and cytokines were measured. *B* and *C*, Porcine (*B*) and bovine PBMC (*C*) of five individual animals were stimulated with the indicated concentrations of ORN complexed to DOTAP (20 μ g/ml, 1/3 dilution). After 48 h, SNs were harvested and IFN- γ was measured.

performed and, to our knowledge, no report investigated the potential sequence specificity of human TLR8 responses, although previously such differences were observed for small molecule TLR8 ligands (29).

Human TLR7 and TLR8 differ in their cell-type specific expression and, therefore, appear to activate divergent cell types resulting in differences in the cytokine patterns stimulated via each of these receptors. TLR7 agonists directly activate pDCs and induce IFN- α and IFN- α -regulated genes, whereas TLR8 agonists directly activate mDCs and monocytes and induce proinflammatory cytokines such as TNF- α and IL-12 (3, 4, 19, 21, 29). Target cell selectivity and diversity appear to be useful tools, in addition to recombinant cells expressing these receptors, to differentiate in vitro between potential TLR7, TLR7/8, and TLR8 RNA motifs. By comparing a variety of 4-mer RNA sequences on human primary immune cells for their potential to stimulate either IFN- α or TNF- α responses, we identified sequences stimulating both cytokines or only TNF- α , suggesting the existence of RNA motifs specific for both or the single receptors. The motif analysis defined specific GU-rich 4-mer sequences like UUGU, GUUC, GUUU, UUUC, UGUU, or UCUC activating human TLR7/8 by inducing IFN- α and proinflammatory cytokines and chemokines from cells expressing only TLR7 or both TLR7 and TLR8. In contrast, AU-rich sequences like AUGU, UAUA, AUAU, AUAC, UAAU, UUAU, CUAC, GUAC, or UAUC were found to induce the strongest TNF- α production by lacking substantial IFN- α secretion and revealed target cell and receptor selectivity by stimulating monocytes and mDCs, but not pDCs. Interestingly, our data also show that GU-rich ORNs induce substantially stronger IFN- α production from human PBMCs than a poly(U) ORN of the same length. Our motif study also suggests that human TLR7 is more sensitive to sequence modifications than human TLR8. Whereas nearly all tested RNA sequences still induced good to moderate TLR8 responses, human TLR7 appeared to prefer ORN sequences with at least two U ribonucleotides in combination with G and/or C. The selectivity for at least some similar RNA sequences may have evolutionary reasons; both TLRs lie in close vicinity to each other on the X chromosome, which suggests that they did arise from a tandem duplication of an ancestral gene. They contain two introns and three exons and more LRRs than most of the other TLRs (1, 24, 39), and the local gene order of TLR7 and TLR8 as well as the LRRs are preserved in humans and mice (42).

We investigated the sequence specificity of human TLR7 and TLR8 but did not determine in detail whether the murine TLR7 displays a comparable preference for GU-rich sequences. However, the murine TLR7 was stimulated only with a GU-rich but not an AU-rich ORN and the response required TLR7, as demonstrated by using splenocytes from TLR7-deficient mice. In contrast, AU-rich ORN in combination with a poly(dT) ODN did signal via murine TLR8. The failure of mouse TLR8 to signal upon encountering the “natural” human TLR8 ligand, single-stranded RNA, or small molecule TLR8 ligands alone (8, 31) may suggest that the activation of TLR8 requires additional factors or adaptors. In contrast to the combination of small molecule TLR8 ligands with poly(dT), AU-rich ORNs showed weaker murine TLR8-mediated NF- κ B activation. This may be explained by ORNs and ODNs being both nucleic acids and, therefore, competing for the same uptake mechanism or TLR binding site in contrast to the small molecule ligands.

TLR7, 8, and 9 are expressed in intracellular endolysosomal compartments and chloroquine as well as bafilomycin A1 suppresses TLR7/8 and TLR8 ORN signaling via TLR8. Chloroquine previously was reported to interfere with the binding between TLR9 and CpG ODN (30), and because TLR9 is highly homolo-

gous to TLR7 and TLR8 it seems likely that chloroquine may also prevent a direct binding of the ORN to these other TLRs. Our results showing that chloroquine fails to efficiently block immune stimulation by small molecule ligands such as R-848 may point either to different TLR binding sites of R-848 vs nucleic acids and chloroquine or to differences in small molecule uptake and intracellular distribution.

Rat and mouse splenocytes as well as porcine PBMCs failed to respond to AU-rich ORNs, whereas GU-rich ORNs were capable to induce immune responses in cells of these species. In contrast, bovine PBMCs responded to both GU- and AU-rich ORNs. The cellular diversity of TLR7 and 8 within human, mouse, and rat appears not to be a good explanation for differences between GU-rich and AU-rich ORN; whereas TLR7 is expressed in human pDCs and B cells and TLR8 in monocytes and mDCs, TLR7 expression can be observed in mouse myeloid and lymphoid cells (8, 43, 44). More similar to human TLR7/8 expression, rat TLR7 can be found in pDCs and both rat TLR7 and 8 are found in monocytes (45–47). Although the multiple protein sequence alignment of TLR8 among different species indicates a high similarity, differences in terms of amino acid insertions or deletions are observed, especially in the LRR3. Deletions of four aa found in the mouse, rat, and porcine LRR3 sequences compared with the human sequence may explain the differences in TLR8 RNA sequence selectivity. The observed stimulation of bovine PBMC with both ORN classes may indicate the following: 1) that bovine TLR8 responds to TLR8-specific RNA ligands; 2) that the bovine TLR8 LRR3 sequence is different than all other species; or 3) that TLR7 substitutes for the activity of TLR8. Clearly, additional experiments are needed to further investigate the stimulation or lack of activity of TLR8 between different species. Structural analysis of TLR8 will probably result in more information of a potential ligand binding site and further help to explain functional differences among vertebrates. Initial mutagenesis studies identified regions in the human TLR8 extracellular domain most probably involved in signaling (48). Specific residues in LRR8 and LRR17 appear to be absolutely required for human TLR8 activation (48), and mutation of a residue in LRR17 of human TLR9 abolished specific binding to CpG DNA (30), indicating a role for LRR17 in ligand recognition for TLR7, 8, and 9 due to the similarities between these TLRs. Unfortunately, nothing is known yet about the potential function of LRR3, but our data suggest that this region plays a role in species-specific recognition of RNA by TLR8.

It is rather difficult to speculate about a potential evolutionary reason for GU vs AU sequence selectivity of TLR7 and TLR8. Highly conserved GU-containing RNA sequences can be found in the extracistronic 3' terminal region of the genome of negative-strand viruses of the order *Mononegavirales*, including vesicular stomatitis virus, Sendai virus, and influenza virus (49). These regions are most critical for infection and the viral life cycle and contain highly conserved elements. Th1 cytokines are involved in the immune response to negative-strand viruses via TLR7 and TLR8 (5, 6, 50), and such cytokines can be found upon stimulation of human PBMCs with GU-rich ORNs derived from these sequences (21). In contrast, positive-strand alphaviruses contain repeated conserved sequence elements in the 3' nontranslated region with AU-rich 4-mer motifs like AUGU, CUGA, AUAA, and AUGC (51). Alphaviruses are described as replicating rapidly in brain and spinal cord (52) and as being involved in experimental allergic encephalomyelitis (53). Th2 cytokines are produced in the CNS early upon experimental allergic encephalomyelitis infection before T cell infiltration, suggesting that the brain may be a site of preferential production of Th2 cytokines (52, 54), although IFN- α appears to play a role in the attenuation of alphaviruses (55). Innate

immune receptors of the vertebrate immune system may have evolved to recognize specific pathogen RNA regions, resulting in differential patterns of immune responses giving rise to prevalent Th1 or Th2 responses. These responses may be at least in part driven by TLRs, leading to innate immune responses with strong (TLR7) or low (TLR8) type I IFN-dependent cytokine and chemokine production.

In summary, we have identified immune stimulatory ORN sequence motifs for human TLR7/8 and TLR8. AU-rich ORNs are TLR8 RNA ligands and mediate human TLR8 activation, whereas GU-rich ORNs are TLR7/8 RNA ligands and stimulate human TLR7 and TLR8 immune responses. However, besides the observed diverse immune effects and cellular specificities of the responses induced by the RNA TLR7/8 and TLR8 motifs, species-specific differences in the functionality of TLR8 were observed, and LRR3 in the ECD of TLR8 may play a role in this species specificity. The divergent immune modulatory responses of RNA TLR7/8 and TLR8 ligands suggests the potential of GU- and AU-rich ORNs to stimulate efficient TLR-dependent innate and adaptive immune effects that may be beneficial for different indications such as in cancer and allergic and infectious diseases.

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Disclosures

All of the authors except G.K.M. are employees of Coley Pharmaceutical Group and may have a financial interest in the study's outcome.

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