Sequences derived from self-RNA containing certain natural modifications act as suppressors of RNA-mediated inflammatory immune responses

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Abstract

The ability of the host to distinguish between self and foreign nucleic acids is one of the critical factors contributing to the recognition of pathogens by Toll-like receptors (TLRs). Under certain circumstances, eukaryotic self-RNA may reach TLR-containing compartments allowing for self-recognition. Specific modifications were previously demonstrated to suppress immune activation when placed at several positions in an immune stimulatory RNA or silencing RNA (siRNA). However, we show that even a simple natural modification such as a single 2′″-O-methylation at different nucleotide positions throughout a sequence derived from a self-RNA strongly interferes with TLR-mediated effects. Such a single modification can even have an inhibitory effect in vitro and in vivo when placed in a different than the immune stimulatory RNA strand acting as suppressive RNA. Several safeguard mechanisms appear to have evolved to avoid cellular TLR-mediated activation by self-RNAs that may under other circumstances result in inflammatory or autoimmune responses. This knowledge can be used to include as few as a single 2′″-O-methyl modification at a specific position in a siRNA sense or anti-sense strand to avoid TLR immune effects.

Introduction

The recognition of molecules expressed by specific pathogens is mediated by several families of pattern recognition receptors (PRRs). Toll-like receptors (TLRs) are one of the best characterized class of PRRs and belong to the IL-1-receptor (IL-1R) superfamily that recognizes pathogen-associated molecular patterns (1). Ligation of TLRs triggers innate immune responses to bacterial, viral or fungal threats. Natural pathogen-derived ligands have been identified for most of the 11 reported mammalian TLRs. Pathogen nucleic acids such as single-stranded, non-methylated, CpG-containing DNA of bacterial or viral origin have been found to activate TLR9 (2). Other TLRs sensing nucleic acids include TLR7 and TLR8 recognizing single-stranded viral RNA (3, 4), whereas double-stranded viral RNA was demonstrated to target TLR3 (5). In addition to the TLRs, cytoplasmic receptors such as retinoic acid-inducible gene-I (RIG-I) or the cellular RNA helicase melanoma differentiation-associated gene-5 (mda-5) participate in the recognition of pathogen double-stranded RNA (6–8). All these receptors have a cell type- and compartmental-specific distributions (9–11). Human TLR7 and TLR9 are expressed in the same sub-populations of immune cells, B cells and plasmacytoid dendritic cells (pDCs), whereas strongest expression of human TLR8 is found within monocytes. The cell type-specific expression usually results in specific cytokine profiles upon TLR7 (strong IFN-α production from pDC) or TLR8 [strong tumor necrosis factor (TNF)-α production from monocytes] activation. The combined responses of endolysosomal (TLR3, 7, 8 and 9) and cytoplasmic (RIG-1, mda-5) PRRs result in an efficient cell type and pathogen molecule-dependent defense mechanism of the host.

Eukaryotic RNAs are heavily modified, and ~100 different post-transcriptionally modified nucleotides have been reported (12, 13). These encompass complex nucleotide
Inhibition of RNA-dependent TLR stimulation

Several nucleotide modifications found in natural RNAs appear to interfere with their reported TLR-dependent immune modulatory effects (15). Long natural-modified RNAs are significantly less stimulatory than their unmodified counterparts. In addition, double-stranded silencing RNAs (siRNAs) that trigger immune responses via signaling through TLR7 and TLR8 lose their activity if they contain several 2'-modifications (18-20). Specific modifications can also affect TLR3 signaling responding to viral double-stranded RNA molecules (21). However, nucleotide modifications are usually found in clusters, leaving a significant number of RNA nucleotides in natural RNAs such as rRNAs unmodified (12). If specific modifications would as predicted help eukaryotic cells to differentiate between self and foreign RNA (15, 22), how may this be achieved for the non-modified self-RNA regions?

In this study, the impact of natural occurring modifications in eukaryotic U1 snRNA on its TLR-dependent immune effects was investigated. By introducing a single 2'-O-methyl modification in a given sequence contained in the U1 snRNA, we surprisingly found that immune modulation is decreased independent of the position of the modified nucleotide. Using a simplified oligoribonucleotide (ORN) sequence with a defined stimulatory GU-rich region, we further demonstrate that the 2'-O-methyl modification imposes its negative effect also when placed upstream or downstream of the immune modulatory region itself. Moreover, a simple 2'-O-methyl modification can transform a stimulatory into a suppressive RNA sequence, suggesting that modified RNA can act itself as an inhibitor of TLR7 and TLR8 immune responses. In summary, our data extend the understanding of how a eukaryotic organism discriminates between self and pathogen RNA to avoid inflammatory responses induced by endogenous self-RNA.

Methods

Reagents

ORN were obtained from BioSpring (Frankfurt, Germany) and were controlled for identity and purity by Coley Pharmaceutical GmbH and had undetectable endotoxin levels (<0.1 EU ml⁻¹) measured by the Limulus assay (BioWhitaker, Verviers, Belgium). ORN were suspended in sterile endotoxin-free DNase- and RNase-free water (Life Technologies, Eggenstein, Germany) and were stored and handled under aseptic conditions to prevent contamination. Sequences are listed in Table 1. Chloroquine was obtained from Sigma (Deisenhofen, Germany) and 1,3-dioleoylxylo-3-(trimethylammonium) propane (DOTAP) from Roche (Mannheim, Germany).

PBMC isolation and purification of cell subsets

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

Table 1. ORN sequences used for the study

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<thead>
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<th>ORN</th>
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<tr>
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<tr>
<td>AU R-1301</td>
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<td>Influenza 2′OMe A R-2540</td>
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<td>Influenza 2′OMe U R-2541</td>
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</tr>
<tr>
<td>R-0006</td>
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</table>

ORN in upper case are completely phosphorothiate (PS), ORN in lower case have phosphodiester (PO) linkages. Underlined nucleotides are 2′-O-methylated.
Inhibition of RNA-dependent TLR stimulation

(1) PBMCs were purified by centrifugation over Ficoll-Hypaque (Sigma). 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, 100 U ml−1 penicillin and 100 mg ml−1 streptomycin (all from Sigma).

Measurement of cytokine and chemokine production

Freshly isolated PBMCs were re-suspended at a concentration of 3 × 10^6 ml−1 to 5 × 10^6 ml−1 and added to 96-well round-bottomed plates (200 µl per well), which had previously received nothing or ORN complexed to DOTAP or DOTAP alone. Cells were cultured in a humidified incubator at 37°C for the indicated time points. Culture supernatants (SNs) were collected and, if not used immediately, were frozen at −20°C until required. Amounts of cytokines in the SNs were assessed using commercially available ELISA kits (IFN-γ or TNF-α, Diaclone; or IL-12p40, BD Pharmingen), an in-house ELISA (IFN-α) developed using commercially available antibodies (from BD Pharmingen or PBL, New Brunswick, NJ, USA, respectively), or the Luminex technology using a cytokine 25-Plex (BioSource, Camarillo, CA, USA).

Reporter assays

Human embryonic kidney cells (HEK293) containing a NFκB luciferase reporter construct and expressing human TLR7 or TLR8 or without TLR expression were used as described before (23). Cells were plated on 96-well plates at 1.5 × 10^4 per well and allowed to attach overnight. Cells were subsequently incubated for 16 h with the indicated amount of ORN complexed to DOTAP (Roche) and then tested for luciferase expression. Each data point was done in duplicate.

In vitro murine assays

Naive sv129 mouse splenocytes were used for all in vitro assays. Animals were anesthetized with isoflurane and euthanized by cervical dislocation. Spleens were removed under aseptic conditions and placed in PBS + 0.2% BSA (Sigma, St Louis, MO, USA). Spleens were then homogenized and splenocytes were re-suspended in RPMI 1640 (Life Technologies) medium supplemented with 2% normal mouse serum (Cedarlane Laboratories, Ontario, Canada), 2 mM L-glutamine, penicillin-streptomycin solution (final concentration of 1000 U ml−1 and 1 mg ml−1, respectively) and 5 × 10^−5 M β-mercaptoethanol (all from Sigma). Splenocytes pooled from the spleens of five mice were plated in 96-well round-bottomed plates (5 × 10^5 cells per well). Each splenocyte sample was plated in quadruplicate and the cells were incubated in a humidified 5% CO₂ incubator at 37°C for 20 h. SNs were harvested and a commercially available assay kit for IL-6 or IL-12p40 (mouse OptEIA kit; PharMingen, Mississauga, Ontario, Canada) was used according to the manufacturer’s instructions to assay cytokine levels.

In vivo murine assays

Female Balb/c or sv129 mice (6–8 weeks of age) were used for the in vivo experiments; sv129 mice were purchased from Charles River Canada (Quebec, Canada), and mice were housed in micro isolators at the animal care facility of Pfizer Vaccine Ottawa (Kanata, Canada). All studies were conducted under approval of the institutional animal care committees and in accordance with the guidelines set forth by the Canadian Council on Animal Care. For post-injection experiments, phosphorothioate-modified suppressive S-Class ODN 2088 [5'-TCCTGGCGGGAAGT-3' (24)] was administered intra-peritoneally to Balb/c mice (n = 5 per group). After 1 h, animals were injected subcutaneously with ORN HIV formulated with DOTAP. Plasma was collected at 3 h post subcutaneous injections for ELISA assays. Suppressive ORN R-1862 or control ORN R-1507 were formulated with DOTAP and, for post-injection experiments, were administered intravenously to sv129 mice (n = 5 per group). After 1 h, animals were injected intravenously with ORN HIV formulated with DOTAP. Plasma was collected at 3 h post intravenous injections with ORN HIV for ELISA assays. For co-injection experiments, suppressive ORN R-1862 or control ORN R-1507 were formulated with DOTAP in the presence of ORN HIV. ORN were administered intravenously to sv129 mice (n = 5 per group) and plasma was collected at 3 h post intravenous injections for ELISA assays.

Results

Single 2′-methylcylation in self-RNA suppresses TLR-dependent effects

Self-RNAs such as U1 snRNA in immune complexes or upon enforced uptake activate human immune cells via TLR7 and TLR8 (25, 26). The cytokine response by the U1 snRNA is essentially dependent on the RNA itself as incubation with RNAse decreases IFN-α induction to the background levels (25). Three out of only five modified nucleotides in the U1 snRNA are 2′-O-methylated, two at the very 5′ end and one in stem loop II (Table 2) (27). To investigate a potential impact of these 2′ modifications on immune stimulation, we used short ORN with sequences of different lengths derived from the 5′ end or stem loop II of the U1 snRNA covering modified U1 snRNA regions and that were previously examined for their immune stimulatory effects (25). These sequences were either unmodified or modified at the ‘natural’ modified sites (see Tables 1 and 2). Compared with the unmodified ORN, all 2′-O-methylated ORN stimulated significantly lower IFN-α levels, and there was no considerable difference between ORN R-1301 (the 5′ U1 snRNA

Table 2. U1 snRNA sequence and positions of nucleotide modifications

| 5′-m7GpppAUAUAACUCCUCUCGAUCUGCaGCGGGGAGAUAAACAUAGUCGAAAGGUGGUUUUCCCGGGGGAGGCUUAUCCAUUGCAGCAUCUGCGAAGUGCGACUUGGUGCAGGACUCUGGUCCGCGCUUCCCUUG3′ |

Underlined are sequences used for oligonucleotide synthesis. Highlighted are U1 snRNA modifications: A™; U™; 2′OMe modification; Ψ: Pseudouridine.
sequence with both A and U 2'-O-methylated) and ORN R-1334 (2'-O-methylated at the U) or R-1335 (2'-O-methylated at the A) each with only one modification (Fig. 1A). In contrast, the IL-12 and TNF-α responses showed more pronounced differences between the modified and unmodified ORN (Fig. 1B and C). These data cannot be explained by an impact of the 2'-O-methyl modification on the potential formation of higher ordered RNA structures known to be stabilized by 2'-O-methyl modifications, since none of the ORN formed secondary structures (data not shown).

From these data, although demonstrating that a single 2'-O-methyl modification is sufficient to reduce the cytokine induction, it was unclear if a modification at any position in the ORN would have a suppressive effect. Therefore, additional ORNs with 2'-O-methylation at various other positions of the 5' U1 snRNA sequence were tested for their IFN-α inducing activity (Fig. 1D and Table 1). Although most modifications again interfered with cytokine induction, an ORN with a 3' U modification (R-1636) showed only a minimal reduction of IFN-α stimulation. The 5’ snRNA ORN R-1300 contains Us at various positions throughout its sequence. The U and G content of an ORN determines its immune stimulatory activity (4, 25, 28), but from these data it appears as if either 2'-O-methylation affects immune stimulation in a position-dependent way or not all Us are equally important for mediating a cytokine response.

A single 2'-O-methyl modification inside or outside of an immune modulatory GU-rich RNA sequence suppresses RNA-mediated immune effects

To better understand a potential position dependency of the 2' modification on immune activation, we used a non-stimulatory control ORN sequence (R-1507) and introduced a GU-rich 4mer sequence 5’-UUGU-3’ (in R-1505) that is sufficient to stimulate cytokine production from human PBMC (Table 1 and Fig. 2) (29). Whereas ORN R-1507 was inactive in all assays, ORN R-1505 showed immune stimulatory effects. Single 2'-O-methyl modifications at each position of the GU-rich 4mer sequence in the parent ORN R-1505 resulted in substantial loss of IFN-α stimulation (Fig. 2A), highlighting the importance of all positions of this 4mer sequence for IFN-α induction. In contrast, the effect on TNF-α production was less pronounced, but a clear difference in the importance of the nucleotide positions was observed (Fig. 2B). Whereas ORN with a 2' modification at the first U and fourth G (positions 1 and 3 of the 4mer) of the parent ORN R-1505 still stimulated substantial although somewhat reduced TNF-α secretion, ORN modified at the second and third U (positions 2 and 4 of the 4mer) induced significantly lower cytokine levels. Similar results for IFN-α and TNF-α were obtained with PO ORN (Fig. 2C and D), with the PO form of R-1505 stimulating only somewhat higher cytokine levels compared with the PS form. These data suggest that
at least with these defined RNA sequences, putative TLR8-mediated immune effects (e.g. TNF-α production) may be more forgiving to RNA modifications than TLR7-mediated effects (e.g. IFN-α production).

In addition to the modifications in the 4mer GU sequence, we tested ORN with single 2'-modifications outside of this sequence (Fig. 3A and B). Surprisingly, all but the ORN with C modifications induced background IFN-α stimulation. ORN with a 2'-O-methyl-C (R-1628 and R-1632) at different positions had similar not statistically significant IFN-α activity as the parent ORN. The same result was in principal observed for TNF-α induction (Fig. 3B), although the G modification still mediated substantial TNF-α secretion, whereas the two ORN with an A modification adjacent to the GU-rich 4mer sequence had strongly decreased effects.

To further investigate the obvious lack of a negative effect of 2'-O-methyl-C modifications on immune stimulation, we generated additional C-modified ORN derived from R-1505 (Table 1 and data not shown). As above, most C-modified ORN had activities not significantly different to the parent ORN, whereas modifications at any other position completely (IFN-α) or strongly (TNF-α) inhibited cytokine secretion (Fig. 3C and D).

Removal of the 2'-OH at the As adjacent to the 4mer GU-rich sequence by introducing deoxynucleotides in contrast to the introduction of 2'-O-methyl residues did not alter the immune modulatory effects, indicating that the 2' residue itself is involved in the suppression (data not shown).

2' methylation suppresses RNA-dependent immune responses even when not in the same immune stimulatory RNA sequence

Our data demonstrate that 2’-O-methylation interferes with RNA-mediated immune stimulation when placed in an immune stimulatory ORN. The possibility exists that 2’-modified RNA suppresses RNA immune activation even when co-incubated with a stimulatory unmodified ORN. Therefore, human PBMCs were stimulated with ORN R-1300 derived from the U1 snRNA and the same sequence with a 2'-O-methyl modification at the 5’ U (R-1334) was added. The modified ORN indeed suppressed IFN-α secretion stimulated by ORN R-1300 in a dose-dependent manner (Fig. 4A and B, and data not shown) and the suppression was similar as observed with chloroquine. Similar results were obtained with the same modified sequence with a PO backbone, although the PS ORN was significantly more efficient most probably due to its enhanced nuclease stability (Fig. 4B).

We further wanted to explore if a 2’-O-methylated ORN is also capable to suppress immune stimulatory effects induced by ORN derived from virus RNA. Indeed, when using a stimulatory PO or PS sequence derived from the 5’ untranslated region of the HIV genome (ORN HIV, R-1075) [Table 1 and (4)], the addition of the 2’-O-methylated U1 snRNA ORN (modified at the 5’ U, R-1334, compare Fig. 4A and B) completely suppressed IFN-α secretion (Fig. 4C). Moreover, the same phosphorothioate HIV sequence...
containing 2'-O-methylated bases lost its immune stimulatory effects (Table 1 and Fig. 4D). The same result was observed for two other ORN derived from the 3' untranslated regions of the Sendai and influenza virus [Table 1 and (30)] with a single modification at U, A or G (Fig. 4E). Although pathogen RNA is hypomethylated, we wanted to investigate if similar to the U1 snRNA 2'-O-methylation of the HIV sequence would result in a suppressive ORN. Therefore, the HIV ORN was cultured with increasing concentrations of the same sequence with 2'-O-methylated bases (Table 1, R-1238). As observed for the methylated U1 snRNA sequence, the 2'-O-methylated HIV ORN did decrease the IFN-α production induced by the HIV ORN to the background levels (Fig. 4F).

Characterization of the 2'-O-methyl-mediated suppression

We generated an ORN with the sequence of the non-stimulatory ORN R-1507 (see Fig. 2), but with a single 2'-O-methyl modification at a central A (R-1862, see Table 1). The non-modified control ORN R-1507 did have a suppressive effect, although only at the highest concentrations tested (Fig. 5A). In contrast, a snRNA-derived modified ORN (R-1334) was a significantly stronger inhibitor and the same sequence as ORN R-1507 but with the 2'-O-methyl modification (R-1862) was strongest to significantly suppress IFN-α induction even at low concentrations (Fig. 5A). The difference became even more apparent in the suppression of TNF-α secretion where the unmodified ORN was non-inhibitory at the concentrations tested, and the 2' modified ORN did suppress TNF-α secretion, although at higher concentrations compared with the IFN-α inhibition (Fig. 5B).

ORN R-1862 contains a single 2'-O-methyl modification at an A, so that we also wanted to explore if 2' modifications at other positions are inhibitory. Indeed, ORN with single 2'-O-methyl modifications at a G (R-1991) or even 2' methylated in the 4mer GU-rich sequence (R-1993) suppressed IFN-α induction by the HIV-derived ORN (Fig. 5C). A modified ORN with a 2'-O-methyl-C (Fig. 5C, R-1992) appeared not as potent as the other ORN, although a significant inhibition of the IFN-α response could not be observed. Similar results were observed for other cytokines such as IL-12p40 and IFN-γ (data not shown). In principal, the lower inhibitory activity of C-modified ORN may have similarities to the finding of a lack of a negative effect of 2' C methylation in an immune stimulatory ORN (Fig. 3).

To further characterize the suppressive effects mediated by 2'-O-methyl-modified ORN, we wanted to investigate the time dependency of the addition of the suppressive ORN (R-1862) to confer the inhibitory effect (Fig. 5D). Similar to the formerly described suppressive ODN (S-Class ODN) (31) delayed addition of suppressive ORN R-1862 to the stimulatory HIV ORN still inhibited IFN-α induction. There was no substantial difference between the addition of inhibitory ORN R-1862 up to 3 h before or after the stimulatory HIV-derived ORN.

We performed cross-titration experiments to further investigate the suppression of IFN-α secretion by the unmodified
Fig. 4. ORN with 2'-O-methyl modifications suppress cytokine induction stimulated by unmodified ORN. (A) Human PBMCs were incubated for 24 h with 1.0 μM of the stimulatory snRNA 5’ ORN (R-1300) complexed to DOTAP (DO) or in the presence of the indicated concentrations of chloroquine (CQ) or the same sequence with a 2'-O-methyl modification or DO alone and IFN-α measured. (B) Human PBMCs were cultured as in (A) with 1.0 μM of the snRNA ORN, and the indicated concentrations of the 2'-O-methyl modified PS snRNA ORN or the same sequence with a PO backbone were added. (C) The stimulatory ORN HIV, derived from the 5’ untranslated region of HIV, was used with a PS (ORN HIV, R-1075) or PO (ORN HIV PO) backbone at 1.0 μM complexed to DOTAP to stimulate human PBMC. The indicated concentrations of the 2'-O-methyl-modified snRNA sequence (snRNA 5’ 2’OMe 5’ U) was added and after 24 h IFN-α was measured. (D) Human PBMCs were cultured for 24 h with the indicated concentrations of the PS HIV ORN or the same sequence containing 2'-O-methyl modifications complexed to DOTAP and IFN-α measured. (E) Human PBMCs were cultured as in (D) with increasing concentrations of Sendai-derived or influenza-derived ORN and the same sequences each with a single 2'-O-methyl modification were added. (F) ORN HIV at 1.0 μM was used to stimulate human PBMC as in (A) and the indicated concentrations of the same sequence with 2'-O-methyl modifications were added. IFN-α was measured after 24 h. Mean ± SEM of one representative out of at least one to two independent experiments (n = 3 donors). Significance of differences at 2 μM (A–C) to the addition of R-1334 were determined for data points as indicated by Student’s t-test (*P < 0.01, **P < 0.001).
Fig. 5. Strong suppressive activity of ORN depends on the presence of 2'-O-methyl modification. (A and B) Human PBMCs were incubated for 24 h with 0.25 μM HIV ORN complexed to DOTAP (DO) or in the presence of the indicated concentrations of the unmodified non-stimulatory ORN R-1507, the same sequence with a single 2'-O-methyl modification (R-1862), or the snRNA-derived 2' modified ORN (snRNA 5' 2'OMe 5' U), or DOTAP alone and cytokines measured. (C) Human PBMCs were cultured as in (A and B) with 0.25 μM HIV ORN, and the indicated concentrations of R-1507-derived ORN with single 2'-O-methyl modifications at different positions or a R-1505-derived ORN with all nucleotides in the GU-rich 4mer motif 2'-O-methylated. (D) Human PBMCs were cultured as in (C) with or without (medium, Med) 0.25 μM HIV ORN, and the 2'-O-methylated ORN R-1862 complexed to DOTAP was added at different time points before or after or at the same time as the stimulatory ORN.
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In these experiments, we used the GU-rich immune stimulatory TLR7, 8 agonist ORN R-0006 (29) and measured IFN-α production from PBMC upon addition of increasing concentrations of R-1507 and R-1862 (Fig. 5E). As observed for the U1 snRNA and HIV ORN, the suppression of IFN-α secretion was strongest for the 2′-O-methylated compared with the unmodified ORN and was concentration dependent, although the unmodified ORN also resulted in some degree of IFN-α inhibition. However, in contrast to the results obtained in the cytokine secretion experiments, there was no statistically significant difference between the modified and unmodified ORN in suppressing NFκB signaling in TLR7 and TLR8 recombinant cells (Fig. 5F and G).

To further investigate the suppressive effect of the 2′-O-methylated ORN R-1862 versus the unmodified control ORN R-1507, we investigated their effect on a panel of different cytokines and chemokines (Fig. 6). The unmodified non-stimulatory ORN at the concentration tested did partially suppress the response induced by ORN HIV, although the 2′-O-methylated ORN was partially significantly stronger and inhibited cytokine secretion to ∼100%. The differences observed between the strength of suppression by unmodified ORN R-1507 versus R-1862 are also reflected in Fig. 5(B) where ORN R-1507 only partially affected TNF-α secretion, but had a stronger effect on IFN-α secretion, probably indicating a higher sensitivity of TLR7 to 2′ modification as suggested above.

2′-O-methyl inhibition is also observed on murine immune cells

The above data demonstrate that 2′-O-methyl modifications negatively affect responses induced by immune stimulatory ORN in vitro on human immune cells. To further evaluate the immune suppressive effect, the mouse macrophage cell line RAW264.7 or mouse spleenocytes were used (Fig. 7 for spleenocytes and data not shown). In contrast to human immune cells, murine cells appear to express only one receptor (TLR7) that is stimulated by ligands identified to induce human TLR7 responses (29, 32). It is possible that murine TLR8 needs additional co-factors or signal transduction molecules allowing for TLR8-dependent signaling stimulated by RNA (29). Similar to the data on human PBMC, the 2′-O-methylated ORN R-1862 was significantly more potent in inhibiting cytokine secretion stimulated by the HIV-derived ORN (Fig. 7A and B). In contrast, the non-modified ORN R-1507 suppressed only at substantially higher concentrations.

We also wanted to investigate the suppressive effect of 2′-O-methyl-modified ORN on in vivo activation of the TLR7 pathway. As a first step, we used the well-defined inhibitory S-Class ODN 2088 (24) to significantly suppress in vivo cytokine secretion induced by the HIV ORN (Fig. 7C). Animals were pre-treated intra-peritoneally with ODN 2088 and 1 h after were injected subcutaneously with the HIV ORN complexed to DOTAP. The IP-10 plasma levels found 3 h after injection of the HIV ORN were decreased to the background levels when the S-Class ODN was pre-injected. However, the same protocol used for the 2′-O-methyl-modified ORN did not result in an inhibitory effect (data not shown). Therefore, we choose to inject the stimulatory and inhibitory ORN via the same intravenous route of injection (Fig. 7D–F). We tested two different protocols, either as above injected ORN R-1862 or R-1507 complexed to DOTAP for 1 h before the HIV ORN (HIV ORN post injected) or complexed the HIV ORN and ORN R-1862 or R-1507 to DOTAP and injected the two ORN together (HIV ORN co-injected). When the HIV ORN was administered after the modified or unmodified ORN, no significant effect on IP-10, IL-6 or IL-12 induction could be observed (Fig. 7D–F). However, co-injection of the 2′-O-methyl-modified ORN with the stimulatory HIV ORN resulted in partially significant and complete inhibition of the immune modulatory effects otherwise induced by the HIV ORN. Similar to the human PBMC data, the non-modified ORN also resulted in partial inhibition, although not to the background levels.

Discussion

The immune system appears to be enabled to discriminate between self and invading DNAs because they differ markedly in their CpG dinucleotide content and methylation. Bacterial and many viral DNAs compared with vertebrate DNAs not only contain a higher frequency of CpG dinucleotides that are recognized by TLR9 but also contain unmodified CpG dinucleotides, and methylation at the C5 position in the CpG dinucleotide greatly decreases immune stimulatory effects (33, 34). In contrast, enforced uptake and distribution of self-DNA to TLR9-expressing endosomal compartments lead to TLR9-dependent immune activation, demonstrating that the segregation of self-DNA and TLR9 plays a critical role (35, 36).

TLR7 and TLR8 respond to pathogen RNA and are expressed in endosomal compartments making it difficult for self-RNAs to reach these receptors (37–39). In contrast, self-RNAs coated with cationic liposomes or in immune complexes are taken up in TLR7, 8-containing compartments resulting in immune activation (25, 40, 41). The host must have evolved an additional mechanism to be enabled to discriminate between self and foreign RNAs reaching endosomal compartments. Indeed, certain naturally occurring modifications in RNAs can limit their detection by TLRs (15). In contrast to other eukaryotic RNAs, U1 snRNA contains only few modifications concentrated at the 5′ end that

(E) Cross-titration studies were performed using the GU-rich ORN R-0006 at the concentrations indicated, and increasing concentrations (0.005, 0.05, 0.5 and 5.0 μM) of the non-stimulatory unmodified ORN R-1507 or the same sequence with a 2′-O-methyl modification (R-1862) were added. PBMCs were cultured for 24 h and IFN-α measured. Mean ± SEM of at least one experiment (n = 3 donors). (F and G) HEK293 cells stably transfected with human TLR7 or TLR8 were incubated with the indicated concentrations of ORN R-0006 complexed to DOTAP for 16 h in the presence of increasing concentrations of unmodified ORN R-1507 or 2′-O-methyl modified ORN R-1862. NFκB activation was measured by assaying luciferase activity. Results are given as fold induction above background (medium). Significance of differences at 0.4 and 2 μM (A and G) to the addition of R-1334 (A) or R-1862 (C) were determined for data points as indicated by Student’s t-test (*P < 0.01, **P < 0.001). No statistical significant difference was observed for (F and G) between the addition of R-1862 and R-1507.
appear to have a role in pre-mRNA splicing (42). Comparison of the immune stimulatory effects of isolated 2'-O-methyl-modified snRNA sequences and the unmodified same sequences demonstrate that simple modifications such as 2'-O-methyl modification, and cytokines measured by Luminex. Given is the mean percentage ± SD of remaining cytokine-inducing activity of ORN R-1075 after co-culture. Significance of differences were determined by Student's t-test (*P < 0.01, **P < 0.001).
RNAs are degraded in apoptotic cells and, for example, U1 snRNA is cleaved near the 5' end resulting in two truncated apoptotic RNA molecules (42). In our experiments, the modified 5' end of the U1 snRNA not only lacked immune modulatory effects but also suppressed the stimulation induced by another RNA sequence. This observation could be even extended to other RNA sequences with only one 2'-O-methyl modification. The inhibition appeared to be stronger for IFN-α and related responses than for other cytokines similar to the effect imposed by a 2'-O-modification in the same RNA sequence. After 1 h, animals were injected intravenously with ORN HIV (100 μg) complexed to DOTAP (200 μg) (post-injection). In addition, mice (n = 5) were injected intravenously with 100 μg of ORN HIV complexed together with 100 μg ORN R-1507 or R-1862 to DOTAP (400 μg) (co-injection). Plasma was collected at 3 h post intravenous injections for cytokine assays by ELISA. Significance of differences between R-1862 and R-1507 in (C) ODN 2088 were determined by Student's t-test (*P < 0.01, **P < 0.001).

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In summary, the detection of pathogen RNAs mainly depends on endosomal and cytoplasmatic pathogen receptors. Although it is not yet completely clear how cytoplasmatic receptors such as RIG-I distinguish between self and viral RNAs, RNA modifications may also have an impact on RIG-I activation (7). In contrast, for endoplasmatic receptors such as TLR7, 8 and 9, several mechanisms to avoid self-stimulation appear to have evolved. On the one hand, the intracellular localization of TLRs usually ensures the...
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recognition of viral but not self-RNAs. However, under certain circumstances such as during defective apoptotic clearance or in immune complexes (45), self-RNAs can reach these compartments in principle allowing for self-recognition. In these cases, another safeguard mechanism appears to involve the inhibition of TLR7 and TLR8 responses by RNA modifications found more frequently in self than pathogen RNAs. Although RNA modifications in eukaryotic RNAs are clustered in conserved regions, modified RNA sequences appear to impose a suppressive effect even when placed on another RNA strand, helping eukaryotic cells to tolerate their own nucleic acids.

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Abbreviations

DOTAP 1,3-dioleoylxylo-o-3-(trimethylammonium)propane
mda-5 melanoma differentiation-associated gene-5
ODN Oligodeoxyribonucleotide
ORN oligoribonucleotide
PDC plasmacytoid dendritic cell
PO phosphodiester
PRR pattern recognition receptor
RIG-I retinoic acid-inducible gene-I
siRNA silencing RNA
SN supernatant
SnRNA small nuclear RNA
TLR Toll-like receptor
TNF tumor necrosis factor

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