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Suppression of immunostimulatory siRNA-driven innate immune activation by 2′-modified RNAs

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Abstract

Single-stranded (ss) and double-stranded (ds) small interfering RNAs (siRNAs) containing immunostimulatory RNA motifs can activate innate immunity through Toll-like receptor 7/8 (TLR7/8), leading to the production of proinflammatory cytokines and type I interferon. More recently, we have noted that 2′-uridine modified ss or ds siRNAs not only evade immune activation, but can suppress TLR signaling triggered by their unmodified counterparts. Here we compared the inhibitory effects of several 2′-modifications. In contrast to 2′-deoxy uridine modified ss siRNAs, 2′-*O*-methyl uridine modified ss siRNAs inhibited at nanomolar concentrations the production of TNF-α induced by a variety of immunostimulatory RNA sequences. Using oligonucleotide microarrays, we highlight the strong suppressive effect of RNA-containing 2′-*O*-methyl uridines. Indeed, nearly all of the 270 genes induced by an immunostimulatory ss siRNA were completely inhibited or downregulated by cotreatment with its 2′-*O*-methyl modified version. Also, 2′-*O*-methyl modified RNAs inhibited *E. coli* total RNA or mitochondrial RNA to induce TNF-α production in human monocytes. Collectively, these data indicate that 2′-modified RNAs, in particular those containing 2′-*O*-methyl modification, are recognized with high affinity by TLR7/8, but do not induce downstream signaling. Therefore, this new generation of TLR antagonists can be used as immunosuppressive agents to interfere with TLR signaling.

Keywords: Suppressive oligonucleotides; RNA interference; siRNA; 2′-Modified RNAs; Toll-like receptors; Innate immunity

Although the discovery of RNA interference has provided a new strategy to investigate gene function and drug target validation [1–3], several studies have shown that siRNA duplexes can exhibit off-target silencing and/or induction of the interferon pathways [4–6]. These and other emerging unwanted effects could pose a major problem in therapeutic settings. Recently, we have shown that immune activation by either single-stranded (ss) or double-stranded (ds) siRNAs is sequence-dependent and require the acidification of the endosomes [7]. Within the endosomes, siRNAs activate the immune system through TLRs, in particular TLR8 in human monocytes. In accordance with our data, two studies demonstrated sequence-dependent stimulation of the immune system by siRNAs and the TLR7 seems to be the mediator of immune stimulation in human dendritic cells [8,9].

Considering the high frequency of uridines in messenger RNAs, it is more likely that a high proportion of siRNAs will activate innate immunity. Therefore, it would be desirable to develop strategies that evade immune activation. In this respect, the incorporation of various 2′-modified nucleotides in siRNA sequences abrogated their immunostimulatory potency [10]. However, the chemical modifications that block immune activation must be chosen carefully so as not to inhibit siRNA silencing activity. Thus, finding the appropriate chemical modifications for inhibiting siRNA immune activation will be important for exploring their therapeutic applications. In a recent report, we have

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shown that replacement of only uridines with their 2′-fluoro, 2′-deoxy, or 2′-*O*-methyl modified counterparts can abrogate immune recognition of siRNAs by TLRs without blocking siRNA silencing activity [11]. Using microarray technology, we further confirmed the inhibitory effect of 2′-fluoro and 2′-*O*-methyl group on immune activation [12]. More recently, we have found that 2′-modified RNAs can bind to TLRs and block downstream signaling pathways triggered by immunostimulatory RNAs [13,14]. To address the molecular mechanisms by which 2′-modifications block immune activation, here we investigated the suppressive effects of several 2′-uridine modified siRNAs on TLR activation in human monocytes.

Materials and methods

siRNAs. The siRNAs used in this study were chemically synthesized by Ambion and Eurogentec. The sequences of the used test molecules are the following:

ss siRNA 27S, 5′-GUCCGGGCAGGUCUACUUUtt-3′;
ss siRNA 27A, 5′-AAAGUAGACCUGCCCGGACtt-3′;
ss siRNA 19S, 5′-GAGGCAAUCACCAAUAGCAtt-3′;
ss siRNA 19A, 5′-UGCUAUUGGUGAUUGCCUCtt-3′;
ss siRNA 16S, 5′-GCUGUGGCCAGCUUGUUAUtt-3′;
ss siRNA 16A, 5′-AUAACAAGCUGGCCACAGCtt-3′.

To generate ds siRNA duplexes, the sense (S) and the antisense (A) strand of each siRNA sequence were mixed at equal concentrations, heated for 1 min at 80 °C, and then annealed at 37 °C for 2 h. The annealing buffer consists of 20 mM Hepes (pH 7.4) and 150 mM NaCl.

Isolation of peripheral blood mononuclear cells. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation (Lymphoprep, Nycomed Pharm, Oslo) from buffy coats obtained from healthy adult donors. Monocytes were prepared by plastic adherence as described previously [7]. Cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum and antibiotics.

Transfection and ELISA. Freshly isolated monocytes were cultured in 96-well plate at 2×10^5/200 μl and stimulated with the test molecules for 18 h. Subsequently, culture supernatants were collected and cytokine contents were determined by ELISA. PBMCs were cultured at 3×10^6/ml in 25 cm (5 ml) and then transfected with ss siRNA-27 alone or in combination with its 2′-*O*-methyl modified version. Each experiment was performed in duplicate.

Gene profiling. Cell pellets from each experiment were collected and total RNA was prepared with Trizol. Fluorescence-labeled cDNAs were synthesized from 15 μg of the total RNA using SuperScript™ Plus Direct cDNA Labeling System (Invitrogen) according to the manufacturer's recommendations. A mixture of *Arabidopsis thaliana* spike mRNAs (SpotReport, Stratagene) was added to the samples prior to cDNA synthesis. Five micrograms of anchored oligo (dT) 20 primer was used to prime the cDNA synthesis. Labeled Alexa Fluor 555 and Alexa Fluor 647-cDNAs were purified with PCR purification kit (Quagen). The oligo microarrays used in this study were obtained from The Norwegian Microarray Consortium. The targets were 34,721 oligonucleotides (70 mer each, OPERON hum oligo v3.0) representing 34,721 genes from the Ensemble Genome Database. For details on the arrays, we refer to http://www.microarray.no/. Hybridization and data processing was performed as described previously [12]. A linear model fitting and empirical Bayes method were used for assessing differential expression. The used decide tests classify a series of related *t*-statistics as up, down or not significant, multiple testing across genes and contrasts (1) 27H; (2) 27H + 27M was used. The restriction of minimum 2-fold change and $p < 0.001$ was applied. The Venn diagrams were prepared with Limma to visualize the intersections of the significant gene sets within groups. Hierarchical clustering of genes for visualization of expression patterns was performed in MultiExperiment Viewer (MEV) http://www.tigr.org.

Results and discussion

2′-Modified RNA inhibits the activation of TLR by immunostimulatory RNAs

Although RNAi-mediated gene silencing has become a valuable strategy for functional studies and drug target discovery [2,3], we and others recently have shown that certain siRNA sequences can activate innate immunity in sequence-dependent manner through endosomal TLRs [7–9]. Such immune stimulation can be abrogated by 2′ chemical modifications. However, it remains unclear whether 2′-modified RNAs bind or not to TLRs. One way to address this question is to assess whether they compete with immunostimulatory RNAs to trigger TLR7/8 signaling. To answer this key question, we incubated for 18 h human monocytes with immunostimulatory ss siRNA27 (27H, harboring 2′-hydroxyl (OH) uridines) alone or in combination with its 2′-modified counterparts. We have tested 2′-*O*-methyl uridine (27M), 2′-fluoro uridine (27F), and 2′-deoxy uridine (27D) modifications. Subsequent to transfection, TNF-α levels in culture supernatants were determined by ELISA. In accordance with the previous data, all of the 2′-modified ss siRNA 27 did not induce TNF-α secretion, whereas the unmodified molecule (27H) did (Fig. 1A). Interestingly, co-transfection of 27H with its 27M or 27F modified versions suppressed the production of TNF-α induced by 27H. Notably, secretion of TNF-α was completely blocked by 27M, indicating a strong binding of 27M to TLR7/8 (Fig. 1A). Also, 27M abolished the immunostimulatory potency of a second ss siRNA, known as 19A (Fig. 1A). Comparable suppressive effects were obtained with 2′-*O*-methyl modified ds siRNA 27 (data not shown).

Having demonstrated that 2′-modified RNAs can compete with immunostimulatory RNAs to trigger TLR signaling, next we have evaluated whether the effects are concentration-dependent. In these experiments, monocytes were transfected with 27H alone or in combination with various concentrations of its 2′-modified versions, 27M, 27F or 27D (Fig. 1B). The 27M and 27F molecules blocked 27H-induced TNF-α production in a concentration-dependent manner. Of considerable interest, the 27M suppressed immune activation at very low concentrations when compared to 27F. Indeed, TNF-α production was reduced by half at 25 ng/ml, whereas 20 times higher concentration (500 ng/ml) was required to achieve comparable inhibition effect with 27F ($p < 0.001$). These data indicate that 27M has a high affinity to TLR7/8. In contrast, cotransfection with 27D resulted in no significant inhibition of TNF-α production. In many experiments, 27D at high concentrations slightly enhanced TLR signaling when compared to low concentrations (Fig. 1B).

To further characterize the suppressive effects of 27M, we assessed its inhibitory effect on different ss siRNA and ds siRNA immunostimulatory sequences. As seen in Fig. 1C, the 27M molecule effectively inhibited the secretion

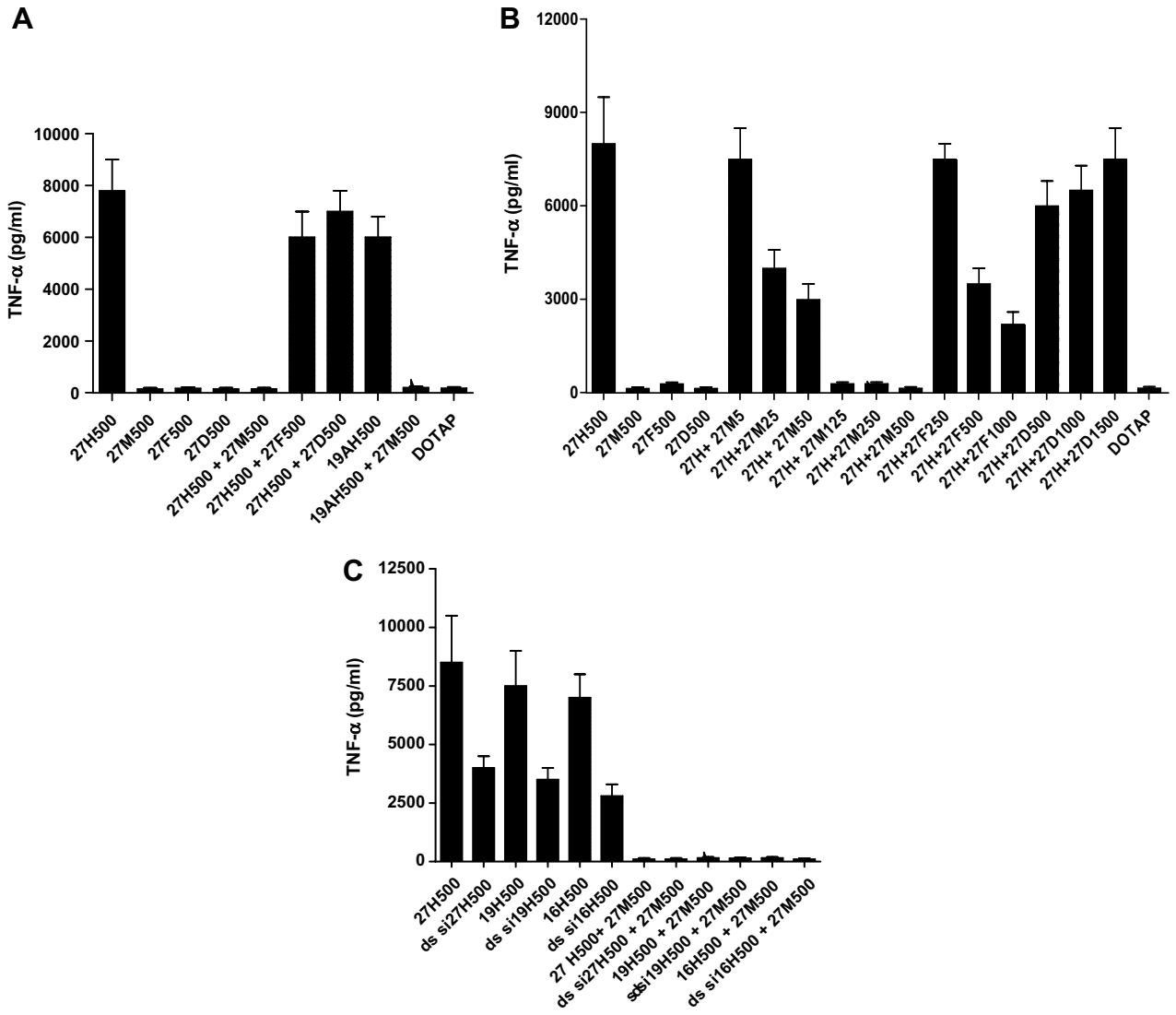

Fig. 1. Suppression of RNA-induced immune activation by 2′-modified RNAs. (A) Human monocytes were treated for 18 h with unmodified ss siRNA 27 (27H) or with its 2′-uridine modified versions, namely 2′-*O*-methyl (27M), 2′-fluoro (27F), and 2′-deoxy (27D). To test the suppressive effects of the 2′-modified versions, monocytes were cotransfected with 27H in combination with 27M, 27F, 27D, or 19AH (a second immunostimulatory ss siRNA). Subsequent to transfection, TNF-α levels in culture supernatants were measured by ELISA. All molecules were used at 500 ng/ml as indicated in the figure. (B) Concentration-dependent effects. Monocytes were transfected with 27H, 27M, 27F, or 27D at 500 ng/ml. Also, they were transfected with 27H (500 ng/ml) in combination with various concentrations (5–1500 ng/ml) of its 2′-uridine modified versions. The numbers indicate the final concentrations (ng/ml) of the tested molecules. (C) The 27M suppresses immune activation by different RNA sequences. Human monocytes were transfected for 18 h with different immunostimulatory ss siRNAs or ds siRNAs alone or in combination with 27M. Subsequently, TNF-α levels were measured by ELISA in culture supernatants. All molecules were tested at 500 ng/ml. For sequences, see Materials and methods. ds si, double-stranded siRNA.

of TNF-α elicited by a variety of immunostimulatory sequences. Similarly, 27M inhibited the activation of TLR7/8 by total RNAs obtained from *E. coli* or mitochondria (data not shown). These findings indicate that the 27M molecule has an inhibitory effect on several TLR7/8 agonists regardless of their sequences, structures, and sources.

Immunosuppressive effect of 2′-O-methyl modified ss siRNA 27 on gene expression

An "ideal" TLR7/8 inhibitor would have inhibitory effects on all genes that are under the TLR signaling. Therefore, in the next experiments we evaluated the suppressive effects of 27M on global gene expression. The transcriptome changes after transfection with 27H alone or in combination with 27M were measured relative to DOTAP-treated cells using oligo microarrays (Fig. 2A). It is intriguing that although 27H was able to alter the expression of 270 genes, its combination with 27M essentially inhibited all responses, suggesting that TLR signaling was effectively blocked. Indeed, only 9 genes were altered in both groups, while the expression of 261 genes induced by 27H alone was downregulated or completely blocked in response to co-incubation with both molecules. Notably, 10 genes exhibited altered expression specifically to 27M.

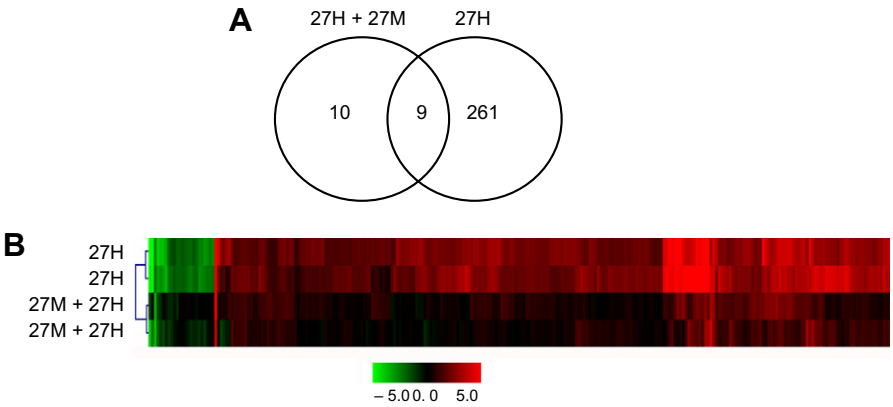

Fig. 2. Suppressive 27M inhibited 27H-mediated gene expression. The transcriptome changes after transfection with 27H alone or in combination with 27M were measured relative to DOTAP-treated cells using oligo microarrays. In these experiments, cell pellets from two independent experiments were pooled before total RNA and probe preparations. Two arrays (dye-swapped) were performed for each group. All molecules were used at 500 ng/ml. (A) A Venn diagram showing significantly ($p < 0.001$) upregulated and downregulated genes after monocyte treatment with 27H alone or 27H and 27M together. Each circle represents one treatment protocol as indicated. The intersections give a number of genes that are steadily differentially altered. (B) A cluster of 270 genes identified as differentially altered in response to 27H.

The 270 genes identified as altered in response to 27H were clustered by hierarchical clustering, sorting the genes based on similar transcriptional changes (Fig. 2B). Notably, the two treatment protocols (vertical cluster) were distinguished based on the gene expression profiles. Collectively, these data highlight the high affinity of 27M to TLR7/8 because both molecules were used at the same concentrations.

Concluding remarks

The competition data reported in the present study clearly indicate that 2′-uridine modified RNAs can bind to TLR7/8, but they do not trigger TLR signaling. Also, the data demonstrated a unexpected diversity of the possible effects of 2′-*O*-methyl, 2′-fluoro, and 2′-deoxy modified RNAs on immune suppression. The 27F molecule exhibited significantly lower suppressive effects than 27M ($p < 0.001$). Even at high concentrations, 27D did not suppress immune activation, but instead led to a slight increase in TLR signaling. In contrast to 27D and 27F molecules, 27M exhibited a strong antagonist effect on TLR7/8 signaling triggered by several immunostimulatory RNAs from different sources. The binding of 2′-*O*-methyl RNAs to TLR7/8 may inhibit either proper binding of immunostimulatory RNAs to their binding site or correct downstream signaling. The significance of our findings in physiological conditions is yet unclear. However, it is more likely that 2′-modified RNAs may block the activation of TLR7/8 by immunostimulatory RNA motifs present in endogenous RNAs synthesized in host cells.

We found earlier that 2′-modified ss and ds siRNAs are not sensed by the immune cells, including monocytes and dendritic cells [6]. Also, it has been shown that RNAs isolated from *E. coli* can activate innate immunity because prokaryotic RNAs have significantly fewer modifications than their mammalian counterparts [15]. Of interest, we found that 27 M can suppress immune activation by bacterial or mitochondrial RNAs. This observation implies that natural 2′-modifications in RNAs not only evade immune activation as suggested by Kariko and colleagues [14], but also can suppress the activation of immunity elicited by other immunostimulatory RNAs derived from microbes and/or host cells. Collectively, these findings have an important impact on the understanding of the mechanisms of self tolerance to nucleic acids and the interactions between the immune system and pathogens.

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References

[1] S.M. Elbashir, J. Harborth, W. Lendeckel, A. Yalcin, K. Weber, T. Tuschl, Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells, Nature 411 (2001) 494–498.
[2] M. Sioud, Therapeutic siRNAs, Trends Pharmacol. Sci. 25 (2004) 22–28.
[3] G.J. Hannon, J.J. Rossi, Unlocking the potential of the human genome with RNA interference, Nature 431 (2004) 371–378.
[4] D.R. Sørensen, M. Leirdal, M. Sioud, Gene silencing by systemic delivery of synthetic siRNAs in adult mice, J. Mol. Biol. 327 (2003) 761–766.
[5] A.L. Jackson, S.R. Bartz, J. Schelter, S.V. Kobayashi, J. Buchard, M. Mao, B. Li, G. Cavet, P.S. Linsley, Expression profiling reveals off-target gene regulation by RNAi, Nat. Biotechnol. 21 (2003) 635–637.
[6] M. Sioud, Innate sensing of self and non-self RNAs by Toll-like receptors, Trends Mol. Med. 12 (2006) 167–176.
[7] M. Sioud, Induction of inflammatory cytokines and interferon responses by double-stranded and single-stranded siRNAs is

sequence-dependent and requires endosomal localization, J. Mol. Biol. 348 (2005) 1079–1090.
[8] V. Hornung, M. Guenthner-Biller, C. Bourquin, A. Ablasser, M. Schlee, S. Uematsu, A. Noronha, M. Manoharan, S. Alira, A. de Fougerolles, S. Endres, G. Hartmann, Sequence-specific potent induction of IFN-alpha by short interfering RNA in plasmacytoid dendritic cells through TLR7, Nat. Med. 11 (2005) 263–270.
[9] A.D. Judge, V. Sood, J.R. Shaw, D. Fang, K. McClintock, I. MacLachlan, Sequence-dependent stimulation of the mammalian innate immune response by synthetic siRNAs, Nat. Biotechnol. 23 (2005) 457–462.
[10] D.V. Morrissey, J.A. Lockridge, L. Shaw, K. Blanchard, K. Jensen, W. Breen, et al., Potent and persistent in vivo anti-HBV activity of chemically modified siRNAs, Nat. Biotechnol. 23 (2005) 1002–1007.
[11] M. Sioud, Single-stranded small interfering RNAs are more immunostimulatory than their double-stranded counterparts: a central role for 2′-hydroxyl uridines in immune responses, Eur. J. Immunol. 36 (2006) 1222–1230.
[12] L. Cekaite, G. Furset, E. Hovig, M. Sioud, Gene expression analysis in blood cells in response to unmodified and 2′-modified siRNAs reveals TLR-dependent and independent effects, J. Mol. Biol. 365 (2007) 90–108.
[13] M. Sioud, RNA interference and innate immunity, Adv. Drug Deliv. Rev. 59 (2007) 153–163.
[14] G. Furset, Y. Fløisand, M. Sioud, Impaired expression of indoleamine 2,3-dioxygenase in monocyte-derived dendritic cells in response to TLR7/8 ligands. Immunology (2007), in press.
[15] K. Kariko, M. Buckstein, H. Ni, D. Weissman, Suppression of RNA recognition by Toll-like receptors: the impact of nucleoside modification and the evolutionary origin of RNA, Immunity 23 (2005) 165–175.