

Suppression of RNA Recognition by Toll-like Receptors: The Impact of Nucleoside Modification and the Evolutionary Origin of RNA

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Summary

DNA and RNA stimulate the mammalian innate immune system through activation of Toll-like receptors (TLRs). DNA containing methylated CpG motifs, however, is not stimulatory. Selected nucleosides in naturally occurring RNA are also methylated or otherwise modified, but the immunomodulatory effects of these alterations remain untested. We show that RNA signals through human TLR3, TLR7, and TLR8, but incorporation of modified nucleosides m5C, m6A, m5U, s2U, or pseudouridine ablates activity. Dendritic cells (DCs) exposed to such modified RNA express significantly less cytokines and activation markers than those treated with unmodified RNA. DCs and TLR-expressing cells are potently activated by bacterial and mitochondrial RNA, but not by mammalian total RNA, which is abundant in modified nucleosides. We conclude that nucleoside modifications suppress the potential of RNA to activate DCs. The innate immune system may therefore detect RNA lacking nucleoside modification as a means of selectively responding to bacteria or necrotic tissue.

Introduction

The innate immune system is the first line of defense against invading pathogens (Medzhitov, 2001). This system utilizes TLRs to recognize conserved pathogen-associated molecular patterns and orchestrate the initiation of immune responses. TLRs are germ line-encoded signaling receptors with extracellular leucine-rich repeats and intracellular signaling domains. In humans, ten distinct TLR family members have been identified, and corresponding microbial ligands for most have been identified. Several TLRs recognize and respond to nucleic acids. DNA containing unmethylated CpG motifs, characteristic of bacterial and viral DNA, activate TLR9 (Hemmi et al., 2000). Double-stranded (ds)RNA, a frequent viral constituent, has been shown to activate TLR3 (Alexopoulou et al., 2001; Wang et al., 2004), single-stranded (ss)RNA activates mouse TLR7 (Diebold et al., 2004), and RNA oligonucleotides with phosphorothioate internucleotide linkages are ligands of human TLR8 (Heil et al., 2004). Based on structural and sequence similarities, TLR7, TLR8, and TLR9 form a subfamily. Activation of these receptors depends upon endosomal acidification and leads to interferon production. Human TLR7 and TLR8 are stimulated by the syn-

thetic antiviral compound R-848 (Jurk et al., 2002), but a natural ligand has not been identified.

It has been known for decades that selected DNA and RNA molecules have the unique property to activate the immune system. It was discovered only recently that secretion of interferon in response to DNA is mediated by unmethylated CpG motifs acting upon TLR9 present on immune cells (Hemmi et al., 2000). For years, bacterial and mammalian DNA were portrayed as having the same chemical structure, which hampered the understanding of why only bacterial, but not mammalian, DNA is immunogenic. Recently, however, the sequence and structural microheterogeneity of DNA has come to be appreciated. For example, methylated cytidine in CpG motifs of DNA has proven to be the structural basis of recognition for the innate immune system. In light of this finding and given that multiple TLRs respond to RNA, a question emerges as to whether the immunogenicity of RNA is under the control of similar types of modification. This possibility is not unreasonable given that RNA undergoes nearly one hundred different nucleoside modifications (Rozenki et al., 1999). Importantly, the extent and quality of RNA modifications depend on the RNA subtype and correlate directly with the evolutionary level of the organism from which the RNA is isolated. Ribosomal RNA, the major constituent (~80%) of cellular RNA, contains significantly more nucleoside modifications when obtained from mammalian cells versus bacteria. Human rRNA, for example, has ten times more pseudouridine (Ψ) and 25 times more 2'-O-methylated nucleosides than bacterial rRNA, whereas rRNA from mitochondria, an organelle that is a remnant of eubacteria (Margulis and Chapman, 1998), has very few modifications (Bachelier and Cavaille, 1998). Transfer RNA is the most heavily modified subgroup of RNA. In mammalian tRNAs, up to 25% of the nucleosides are modified, whereas there are significantly less modifications in prokaryotic tRNAs. Bacterial mRNA contains no nucleoside modifications, whereas mammalian mRNAs have modified nucleosides such as 5-methylcytidine (m5C), N6-methyladenosine (m6A), inosine and many 2'-O-methylated nucleosides in addition to N7-methylguanosine (m7G), which is part of the 5'-terminal cap (Bokar and Rottman, 1998). The presence of modified nucleosides was also demonstrated in the internal regions of many viral RNAs including influenza, adeno, and herpes simplex; surprisingly, modified nucleosides were more frequent in viral than in cellular mRNAs (Bokar and Rottman, 1998). A substantial number of nucleoside modifications are uniquely present in either bacterial or mammalian RNA, thus providing an additional molecular feature for immune cells to discriminate between microbial and host RNA. Considering that cells usually contain five to ten times more RNA than DNA, presence of such distinctive characteristics on RNA could make them a rich molecular source for sampling by the immune system, a notion becoming evident by the identification of multiple TLRs signaling in response to RNA. The role

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of nucleoside modifications on the immunostimulatory potential of RNA, however, is not known.

In recent years, we have investigated the immunomodulatory effect of RNA on human DCs. These studies demonstrated that in vitro-transcribed RNA activates/matures DCs (Weissman et al., 2000) partially by a mechanism in which double-stranded regions of the RNA signal through TLR3 (Kariko et al., 2004). Recently, it was noted that in vitro transcripts, or total RNA derived from bacteria, but not from eukaryotic cells, can prime DCs for high-level IL-12 secretion (Koski et al., 2004). The molecular basis for the discrimination between these various RNAs is not fully understood. In this report, we sought to determine whether naturally occurring nucleoside modifications modulate the immunostimulatory potential of RNA and the role TLRs might play in this process.

Results

Naturally Occurring RNAs Are Not Equally Potent Activators of DCs

We recently demonstrated that RNA transcribed in vitro or released from necrotic mammalian cells activates human DCs (Kariko et al., 2004). In an independent study, we also determined that although in vitro-transcribed RNAs are effective, eukaryotic mRNA and tRNA did not stimulate cultured human DCs (Koski et al., 2004). This finding prompted us to investigate the immunostimulatory potential of different cellular RNA subtypes. The objective was to identify the likely RNA components from necrotic cells that activated DCs in the original experimental setting. We first isolated RNA from different subcellular compartments (cytoplasm, nucleus, and mitochondria). These RNA isolates as well as total RNA, tRNA, and polyA-tail-selected mRNA, all from mammalian sources, were complexed to lipofectin and added to monocyte-derived DCs (MDDCs) generated with GM-CSF and IL-4. We determined that mammalian total, nuclear, and cytoplasmic RNA and mRNA all induced TNF- α secretion, although at very low levels relative to RNA synthesized in vitro by T7 RNA polymerase (RNAP) (Figure 1). Interestingly, mammalian tRNA did not induce any detectable level of TNF- α , whereas mitochondrial (mt)RNA was the most potent RNA type to stimulate MDDCs. Considering that mtRNA shares more characteristics with bacterial RNA than with other mammalian RNA types, it was not surprising to find that bacterial total RNA was also a very potent activator of MDDCs (Figure 1). Bacterial tRNA, which is modified but to a lesser extent than mammalian tRNA, induced a low level of TNF- α , whereas tRNAs from other sources (yeast, wheat germ, and bovine) were nonstimulatory (Figure 1 and data not shown). Similar results were observed when RNAs from other mammalian sources were tested as well as when the RNA repertoires were analyzed by using GM-CSF + IFN- α -generated MDDCs (data not shown). When RNA samples were digested with Benzonase, capable of cleaving both ssRNA and dsRNA, RNA signaling was abolished in MDDCs, verifying that RNA is the active component that triggers TNF- α secretion (Figure 1). These findings demonstrate that from the view of immunostimulation not all natu-

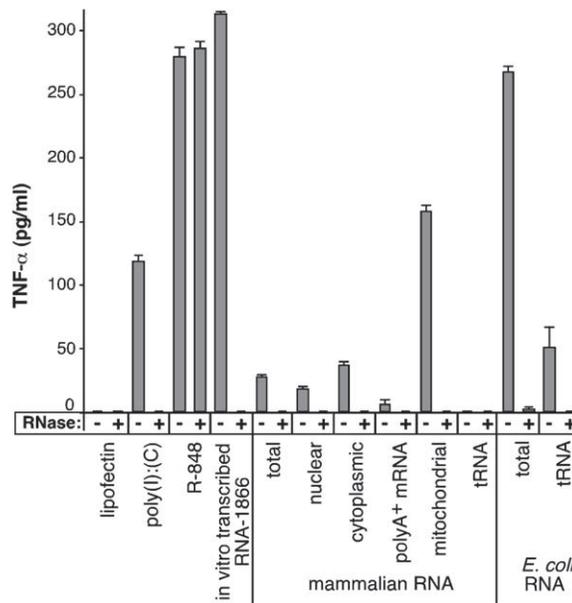


Figure 1. Production of TNF- α by MDDCs Transfected with Natural RNA

Human MDDCs were incubated with lipofectin alone, or complexed with R-848 (1 μ g/ml), or RNA (5 μ g/ml) from 293 cells (total, nuclear, and cytoplasmic RNAs), mouse heart (polyA⁺ mRNA), human platelet mitochondrial RNA, bovine tRNA, bacterial tRNA, and total RNA (*E. coli*) with or without RNase digestion. After 8 hr, TNF- α was measured in the supernatants by ELISA. Mean values \pm SEM are shown. The results are representative of three independent experiments.

rally occurring RNAs are equal. The activation potentials of RNAs seem to have an inverse correlation with the extent of their nucleoside modification, because bacterial RNA and mtRNA that contain only a few modifications are the most potent activators of DCs, whereas extremely modified tRNAs have little to no activity.

In Vitro-Transcribed RNA Stimulates Human TLR3, TLR7, and TLR8, but Most of the Nucleoside-Modified RNAs Are Not Stimulatory

Naturally, all RNA is synthesized from four basic ribonucleotides, ATP, CTP, UTP and GTP, but some of the incorporated nucleosides are modified posttranscriptionally in almost all types of RNA. The extent and nature of modifications vary and depend on the RNA type as well as the evolutionary level of the organism from where the RNA is derived. Because findings with natural RNA suggested that nucleoside modifications might influence the ability of RNA to activate DCs, we set out to further investigate this possibility. First, to obtain RNA with selected modifications, we performed in vitro transcription reactions in which one or two of the four nucleotide triphosphates (NTPs) were substituted with a corresponding nucleoside-modified NTP. Several sets of RNA with different primary sequences ranging in length between 0.7 and 1.9 kb and containing either none, one, or two types of modified nucleosides were

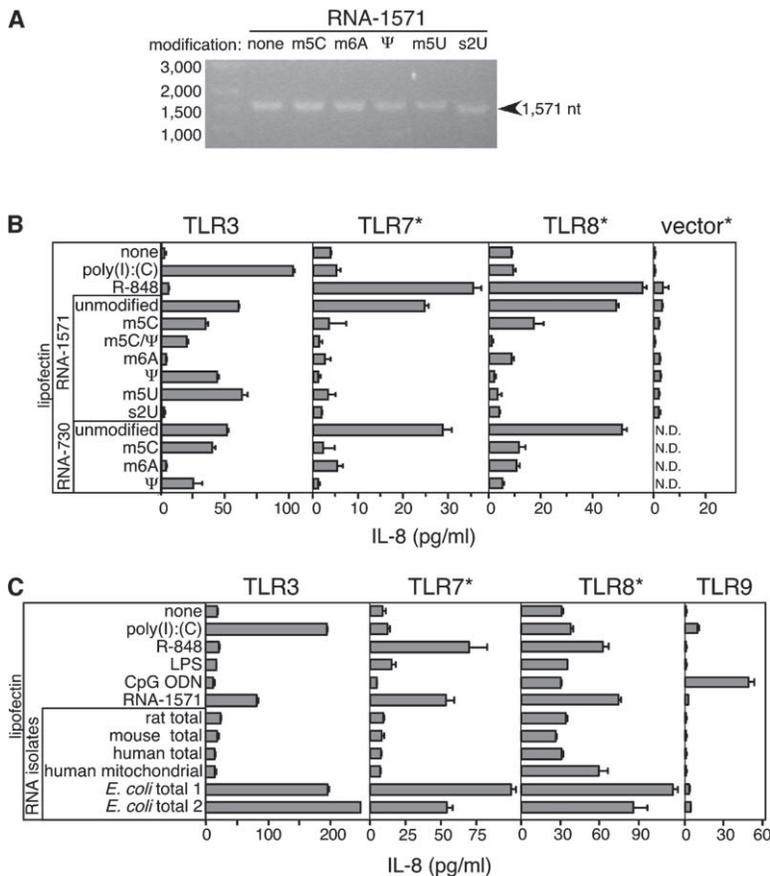


Figure 2. TLR-Dependent Activation by RNA
(A) Aliquots (1 μ g) of in vitro-transcribed RNA-1571 without (none) or with m5C, m6A, Ψ , m5U, or s2U nucleoside modifications were analyzed on denaturing agarose gel followed by ethidium bromide-staining and UV illumination.

(B) 293 cells stably expressing human TLR3, TLR7, TLR8, and control vectors were treated with lipofectin alone or complexed with R-848 (1 μ g/ml) or the indicated RNA (5 μ g/ml). Modified nucleosides present in RNA-730 and RNA-1571 are noted. 293-ELAM-luc cells were used as control cells, but other controls gave similar results.

(C) CpG ODN-2006 (5 μ g/ml), LPS (1.0 μ g/ml), and RNA isolates were obtained from rat liver, mouse cell line (TUBO) and human spleen (total), or from two different *E. coli* sources. 293-hTLR9 cells served as control. After 8 hr, IL-8 was measured in the supernatants by ELISA.

Mean values \pm SEM are shown. Cell lines transformed to express hTLR3-targeted siRNA are indicated with an asterisk. The results are representative of four independent experiments. N.D., not determined.

transcribed. Modified RNAs analyzed by denaturing gel electrophoresis were indistinguishable from their non-modified counterparts in such that all were intact and migrated as expected based on their sizes (Figure 2A).

We and others recently demonstrated that in vitro-transcribed RNA activates human TLR3 (Kariko et al., 2004) and murine TLR7 (Diebold et al., 2004), whereas chemically synthesized oligoribonucleotides (ORNs) stimulate murine TLR7 and human TLR8 (Heil et al., 2004). Therefore, to determine whether modification of nucleosides influences the RNA-mediated activation of TLRs, we utilized human 293 cell lines stably transformed to express human TLR3, TLR7, or TLR8 and monitored TLR activation through IL-8 release. First, TLR3-transformed cells were treated with lipofectin-complexed RNA (RNA-1571, RNA-730, or RNA-1866) and, as expected based on previous studies (Kariko et al., 2004), high levels of IL-8 secretion were measured. RNA containing m6A or s2U modifications did not induce detectable levels of IL-8 (Figure 2B and data not shown). The presence of other nucleoside modifications such as m5C, m5U, Ψ , or m5C/ Ψ in the RNA had a less remarkable suppression or no effect at all on the potential of RNA to activate TLR3 (Figure 2B).

We previously noted that the parental 293 cells express a low level of endogenous TLR3 (Kariko et al., 2004). Therefore, the unwanted expression of endogenous TLR3 was eliminated by stably transfecting the 293-hTLR8 cell line with a plasmid expressing TLR3-

specific short hairpin (sh)RNA. This newly generated cell line, which did not respond to poly(I):(C), was used for further study (Figure 2B). When the 293-hTLR8 cells expressing TLR3-targeted shRNA were transfected with in vitro-transcribed RNAs, they secreted large amounts of IL-8; however, transfecting RNA containing any of the nucleoside modifications did not stimulate these cells (Figure 2B). In some experiments, we observed that m6A modification in the RNA permitted a limited amount of IL-8 release. Control cells (293, 293-pUNO null, 293-TLR3-sh, and 293-hTLR9) did not respond to RNA transfection. (Figures 2B and 2C and data not shown). To rule out that clonal artifacts were responsible for RNA-induced stimulation, at least three separate clones for each TLR-expressing cell line were analyzed and gave similar results.

In a previous study, human TLR8, but not human TLR7, was shown to signal in response to guanosine- and uridine-rich ssRNA oligomers with phosphorothioate internucleotide linkages (Heil et al., 2004). Given that TLR7 and TLR8 share R-848 as a ligand, we sought to determine whether long RNA with natural phosphodiester internucleotide linkages was also a shared ligand for these human TLRs. All of the in vitro-transcribed RNAs induced IL-8 at levels comparable to R-848 when transfected into 293-hTLR7 cells expressing TLR3-targeted shRNA. However, transfection of RNA containing modified nucleosides resulted in no induction of IL-8 (Figure 2B). Experiments performed on

cell lines expressing hTLR7 from different constructs gave similar results. Overall, these experiments demonstrate that RNA activates human TLR3, TLR7, and TLR8 and that nucleoside modifications limit the capacity of RNA to stimulate these TLRs. Specifically, m6A and s2U modifications suppress the ability of RNA to stimulate TLR3, whereas m6A, m5C, m5U, s2U, and Ψ modifications block stimulation of TLR7 and TLR8.

In the next set of experiments, RNAs isolated from natural sources were tested. First, RNA from different mammalian species were transfected into 293 cells stably expressing human TLR3, TLR7, or TLR8 (TLR7 and TLR8 cell lines also expressed TLR3-targeted shRNA). None of these RNAs induced substantial IL-8 secretion. However, bacterial total RNA, obtained from two different *E. coli* sources, induced robust IL-8 secretion (Figure 2C). These results and additional experimental evidence, first that bacterial RNA transfected to 293-hTLR9 did not induce IL-8 secretion (Figure 2C) and second that LPS and unmethylated DNA (CpG ODN), the potential contaminants in bacterial RNA isolates, did not activate the tested TLRs (Figure 2C), together indicate that bacterial RNA is an activator of TLR3, TLR7, and TLR8. Mitochondrial RNA isolated from human platelets also stimulated human TLR8, but not TLR3 or TLR7 (Figure 2C). Collectively, these data directly demonstrate that RNA that is scarce in modified nucleosides, such as those isolated from bacteria or mitochondria, stimulate selected human TLRs, whereas total mammalian RNA abundant in nucleoside modifications are non- or minimally stimulatory.

Modified Nucleosides Reduce the Capacity of RNA to Induce Cytokine Secretion and Activation Marker Expression by DCs

RNAs containing modified or unmodified nucleosides were tested on DCs. A representative data set obtained with MDDCs and IFN- α -generated MDDCs (Figures 3A and 3B) demonstrates that nucleoside modifications diminish the ability of RNA to induce TNF- α and IL-12 secretion. Results were similar (data not shown) when other sets of RNA with the same base modifications but different primary sequences and lengths were tested or when the RNAs were further modified by adding 5' cap structure and/or 3' end polyA-tail or by removing the 5' triphosphate moiety, which was previously reported to promote interferon production (Kim et al., 2004). RNAs of different length and sequence induced varying amounts of TNF- α from DCs, typically less than a 2-fold difference (Figure 3C). However, we detected more variability when MDDCs from different donors were used. In most of the experiments, MDDCs responded to RNA treatment as presented in Figure 3A, but ~25% of the time, the presence of m6A reduced the RNA-mediated MDDC activation more potently than m5C or Ψ did. Under those circumstances, the relative sensitivity of MDDCs to poly(I):(C) and R-848 treatments also differed. (Figure S1 available in the Supplemental Data with this article online). This variability was not observed for primary DCs described below. By using Northern analysis we also confirmed that cellular uptake and stability of the transfected RNAs were not influenced by the nucleoside modifications (data not shown).

To determine whether primary blood DCs responded to RNA in a manner similar to cytokine-generated DCs, we purified primary monocytoïd (DC1, BDCA-1⁺) and plasmacytoïd (DC2, BDCA-4⁺) DCs from peripheral blood. Both cell types produced TNF- α when exposed to R-848, but only DC1 responded to poly(I):(C), though at a very low level, demonstrating absence of TLR3 activity in DC2. Transfection of in vitro transcripts induced TNF- α secretion in both DC1 and DC2 (Figure 3D). Data with modified RNA revealed that only transcripts in which uridine was replaced with m5U, Ψ , or s2U were not stimulatory, whereas RNAs containing m5C and m6A were almost as potent inducers of cytokines as the corresponding unmodified RNAs. This was unexpected because DC2s do not express TLR3 or TLR8 and as such should resemble the response observed in 293-hTLR7 cells. To determine whether m5C and m6A exert a dominant stimulatory effect, transcripts with m6A/ Ψ double modification were tested and found to be nonstimulatory, whereas the mixture of RNA with single type of modification (m6A + Ψ) was a potent cytokine inducer. This suggested that primary DCs likely have an additional RNA signaling entity that recognizes m5C- and m6A-modified RNA and whose signaling is inhibited by modification of U residues.

FACS analysis of MDDCs treated with RNA-1571 and its modified versions revealed that modified nucleosides such as m5C, m6A, Ψ , s2U, and m6A/ Ψ decrease the ability of RNAs to induce cell surface expression of CD80, CD83, CD86, and MHC class II (Figure 4). Collectively, these results demonstrate that the capacity of RNA to induce DCs to mature and secrete cytokines depends on the subtype of DC as well as on the characteristics of nucleoside modification present in the RNA with the general tendency of modifications blocking stimulation.

Suppression of RNA-Mediated Immune Stimulation Is Proportional to the Number of Modified Nucleosides Present in RNA

To ideally define the importance of nucleoside modifications that are components of natural RNA would require the construction of RNAs in vitro that accurately model the extent and diversity of nucleoside modifications of native RNAs and the ability to selectively remove modifications present in natural RNA isolates, both of which are beyond current technology. Most of the nucleoside-modified RNA utilized in the present study contained one type of modification amassing ~25% of the total nucleotides in the RNA. Because the ratio of any one particular modified nucleoside, though variable, is much lower than 25% in native RNAs, we asked what is the minimal frequency of any one particular modified nucleoside that is sufficient to limit the immunostimulatory potential of RNA. To answer this question, two approaches were used to generate RNA with limited numbers of modified nucleosides. First, we transcribed RNA in vitro in the presence of decreasing amounts of m6A, Ψ , or m5C and increasing amounts of the corresponding unmodified NTPs. We expected the incorporation of modified nucleoside phosphates into RNA to be proportional to the ratio contained in the transcription reaction, because prior RNA yields ob-

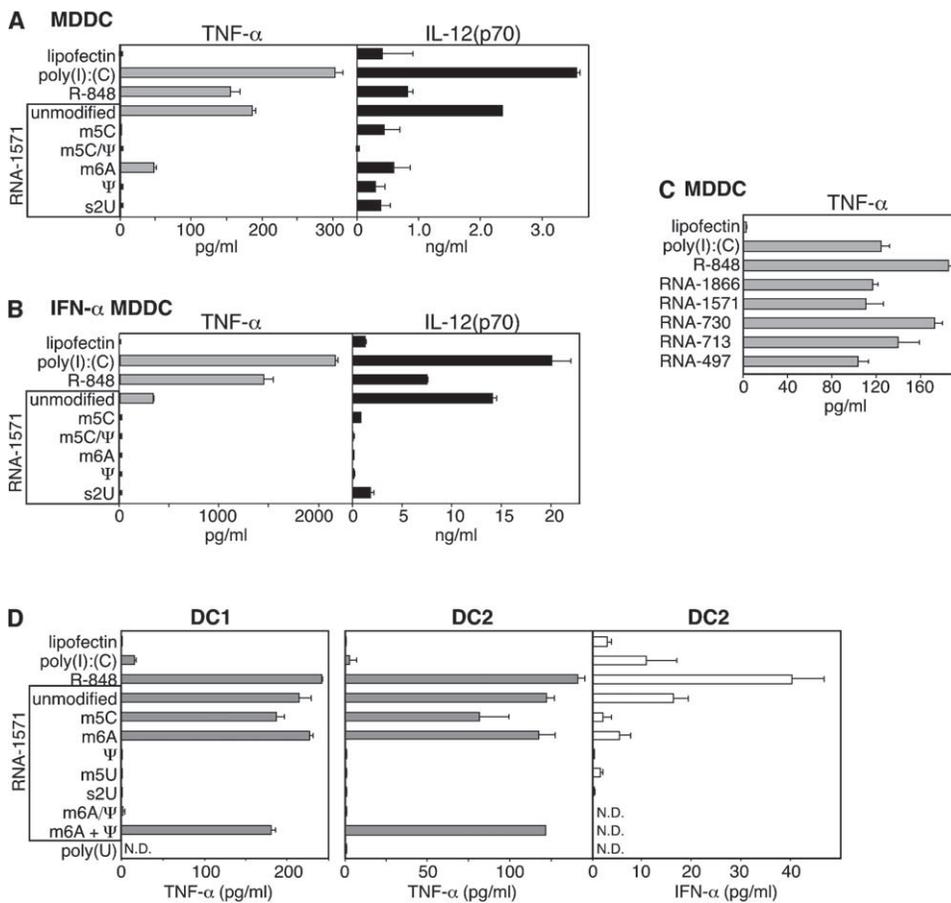


Figure 3. Cytokine Production by RNA-Transfected DCs

MDDC (A and C), IFN-α MDDCs (B), and primary DC1 and DC2 (D) were treated for 8–16 hr with lipofectin alone or complexed with R-848 (1 μg/ml) or the indicated RNA (5 μg/ml). Modified nucleosides present in RNA-1571 are noted. TNF-α, IL-12(p70), and IFN-α were measured in the supernatant by ELISA. Mean values ± SEM are shown. The results are representative of ten (A and C), four (B), and six (D) independent experiments. N.D., not determined.

tained with T7 RNAP suggested the enzyme utilizes NTPs of m6A, Ψ, or m5C almost as efficiently as the basic NTPs. HPLC analysis confirmed this notion, showing for example, that after digestion of RNA transcribed in the presence of UTP:ΨTP in a 50:50 ratio, nearly equal amounts of incorporated UMP and ΨMP were released (Figure 5A). When RNA-1571 with increasing amounts of modified nucleoside content were transfected into MDDCs, we detected that the presence of an increasing amount of modified nucleosides proportionally inhibited the capacity of RNA to induce TNF-α (Figure 5B). The presence of 0.2%–0.4% m6A, Ψ, or m5C in the RNA, which corresponds to approximately three to six modified nucleosides per one molecule of the 1571 nt-long RNA, was sufficient to cause detectable inhibition of cytokine secretion (Figure 5B). When RNAs with modified nucleoside levels of 1.7%–3.2%, which correspond to 14–29 modifications per molecule, were tested, the RNA could maintain only half of its capacity to induce expression of TNF-α. When similar transfection experiments were performed on TLR-expressing 293 cells, usually a higher percent

(~2.5%) of modified nucleoside content was required to inhibit RNA-mediated signaling events (data not shown).

In the second approach, we utilized chemically synthesized 21-mer ORNs with phosphodiester internucleotide linkages and 5' monophosphate and identical primary sequences but with modified nucleosides such as m5C, Ψ, or 2'-O-methyl-U (Um) in a single position (Figure 6A). Results obtained after transfection of MDDCs with the synthetic ORNs demonstrated that short unmodified ORNs were capable of inducing significant TNF-α secretion, but the presence of a single nucleoside modification was sufficient to abolish this effect (Figure 6B). Repeating the experiments on TLR-transformed 293 cells expressing TLR3-targeted siRNA, we found that control ORN induced 293-hTLR8 cells to secrete IL-8, whereas those containing modified nucleosides did not. When testing ORNs on hTLR3- or hTLR7-expressing cell lines, however, we saw no IL-8 secretion under any conditions (data not shown). Finally, by using Northern assay, we tested the 21-mer chemically synthesized ORNs along with 31-mer in vitro transcripts for their ability to induce TNF-α mRNA in MDDCs. ORN5

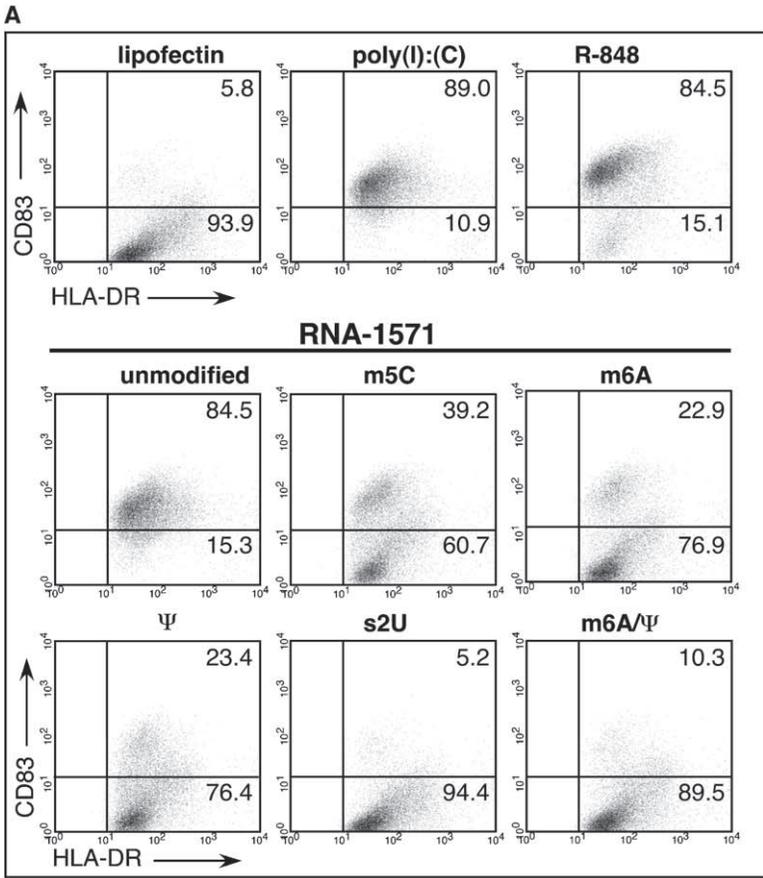


Figure 4. Activation of DCs by RNA
MDDCs were treated for 20 hr with lipofectin alone or complexed with R-848 (1 μg/ml) or the indicated RNA (5 μg/ml). Modified nucleosides present in RNA-1571 are indicated. (A) CD83 and HLA-DR staining is shown. (B) TNF-α was measured in the supernatants by ELISA (the asterisk represents cells that were cultured in 30-fold larger than usual volume of medium for flow cytometry). Mean fluorescence of CD80 and CD86 was determined by flow cytometry. Data are representative of four independent experiments.

B

	TNF-α* pg/ml	CD80 mean fluorescence	CD86 mean fluorescence
lipofectin	0	7.6	55.3
poly(I):(C)	45.6	59.4	257.4
R848	48.3	55.2	235.4
RNA-1866			
unmodified	26.7	52.7	246.4
m5C	0	16.4	108.6
m6A	0	12.4	78.4
Ψ	0	12.0	87.5
s2U	0	8.0	62.7
m6A/Ψ	0	8.6	68.4

and ORN6 (31-mers) caused robust induction, whereas the 21-mer ORN1 control induced less TNF-α mRNA, although still well detectable, particularly in cells exposed to the protein synthesis inhibitor cycloheximide, which is also known to block degradation of selected mRNAs. More importantly, ORNs containing a single modified nucleoside induced less TNF-α mRNA, and consistently, ORN2-Um, the 2'-O-methylated ORN, was the least stimulatory (Figure 6C). Taken together, these results demonstrate that RNA-mediated immune stimulation is suppressed proportionally by the number of modified nucleosides present in RNA. Modification, when present in a single or very few positions, depending on the length of RNA, was sufficient to inhibit the

stimulatory effect of RNA on MDDC and 293 cells expressing individual TLRs.

Discussion

We demonstrate here that a variety of natural RNAs had different capacities to activate immune cells. The most potent RNAs were those that had the least number of modified nucleosides; therefore, we hypothesize that nucleoside modification suppresses the immune-stimulatory effect of RNA. In a quest to prove this, several novel lines of evidence were discovered about RNA-mediated immune activation. Initially, we established that RNA is a ligand for human TLR7. Next, by using

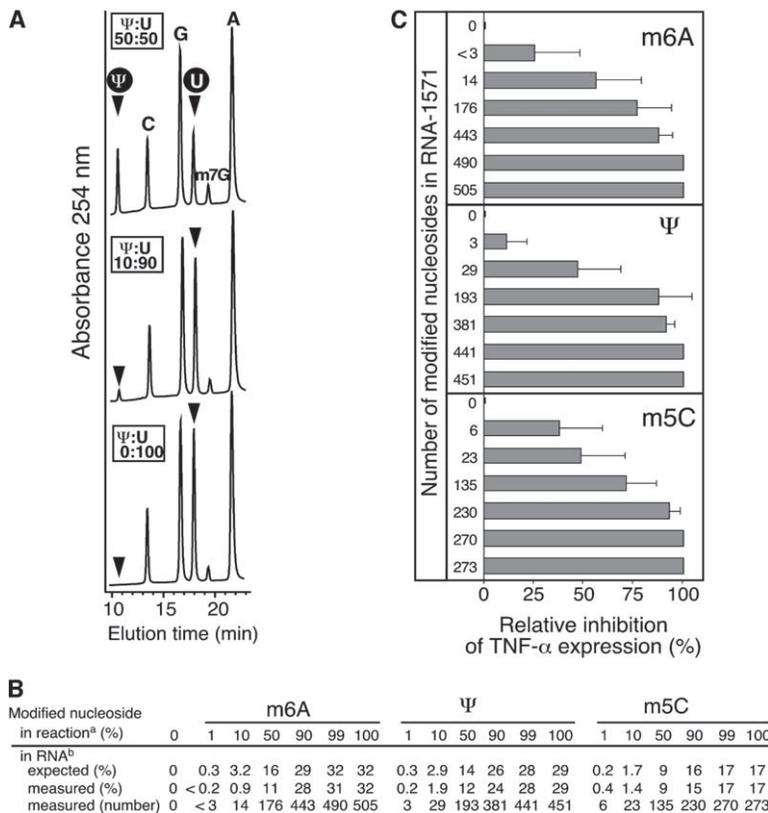


Figure 5. Analyzing RNA Containing Different Amounts of Modified Nucleosides

Capped RNA-1571 containing m6A, Psi, or m5C was transcribed under conditions in which the relative ratio of m6ATP, PsiTP, or m5CTP to the corresponding unmodified NTP was 0%, 1%, 10%, 50%, 90%, 99%, and 100%.

(A) All transcripts were digested to monophosphates and analyzed by reversed-phase HPLC to determine the relative amount of modified nucleoside incorporation. For simplicity, only symbols for the nucleosides are shown. Representative absorbance profiles obtained by RNA transcribed in the presence of pseudouridine- and uridine-triphosphates (Psi:U) at the indicated ratios are shown. Elution times are noted for 3'-monophosphates of pseudouridine (Psi), cytosine (C), guanosine (G), uridine (U), 7-methylguanosine (m7G), and adenoside (A). (B) Modified nucleoside content of RNA-1571. The expected percentage of m6A, Psi, or m5C in RNA-1571 was calculated based on the relative amount of modified NTP in the transcription reaction and the nucleoside composition of RNA-1571 (expected percentage). The values for measured modified nucleoside content (in percentage) were determined based on relative values obtained after quantitation of the HPLC chromatograms. Based on these measured values and on the nucleoside content of RNA-1571 (A: 505, U: 451, C: 273, and G: 342), the number of m6A, Psi, or m5C per molecule of RNA-1571 was calculated. "a" represents values

(%) for m6ATP, PsiTP, and m5CTP relative to ATP UTP and CTP, respectively. "b" represents values for m6A, Psi, and m5C monophosphates relative to all NMPs.

(C) MDDCs were transfected with lipofectin-complexed capped RNA-1571 (5 μg/ml) containing the indicated amount of m6A, Psi, or m5C. After 8 hr, TNF-α was measured in the supernatants by ELISA. Data are expressed as relative inhibition of TNF-α. Mean values ± SEM obtained in three independent experiments are shown. The number of m6A, Psi, or m5C per molecule of RNA-1571 was calculated as indicated in (B).

RNA bearing modified nucleosides such as m5C, m5U, s2U, m6A, Psi, or 2'-O-methyl-U, all constituents of natural RNA, we showed that modifying U, A, and C nucleosides, in general, suppresses the capacity of RNA to activate cytokine-generated DCs, as measured by secretion of TNF-α and IL-12 and by expression of CD80, CD83, CD86, and HLA-DR. Interestingly, only uridine modifications, such as m5U, s2U, or Psi, but not m5C or m6A, could abolish the capacity of RNA to activate primary, blood-derived DCs. Distinct TLRs responded differently to RNA containing different modified nucleosides. RNA with m6A and s2U modifications did not activate TLR3, and those with m5C, m5U, s2U, m6A, or Psi did not activate TLR7 or TLR8, whereas unmodified RNA could activate all these human TLRs. Finally, we show that RNA-mediated immune stimulation is suppressed proportionally with the number of modified nucleosides present in RNA and that even a few modifications are sufficient to exert a suppressive effect.

Nucleoside modification is the foundation of the most ancient "immune" mechanism. Bacteria methylate selected nucleosides in their own genome, which enables them to distinguish and destroy an invader's unmodified DNA with restriction enzymes. During evolution, the discrimination between host and pathogen based

on characteristics of DNA methylation remains an important component of the immune system. In mammalian DNA, cytosines in CpG motifs are mostly methylated, but the lack of such modification in the genomes of microbial pathogens is recognized by TLR9, which then mediates the induction of the mammalian innate immune response (Hemmi et al., 2000).

Despite the fact that the immune stimulatory activity of RNA was discovered decades before such was identified for DNA and that RNA contains numerous modified nucleosides (Rozenski et al., 1999), the effect of nucleoside modifications on RNA immunity has not been explored. From the standpoint of immune activation, RNA and DNA share many characteristics. We have shown that RNA, similarly to DNA, is more immunogenic when derived from bacteria than from mammalian cells (Figures 1 and 2C) (Koski et al., 2004). Similar to mammalian DNA, mammalian RNA also exerts a limited but detectable level of immune activation (Figure 1). Others have reported that mammalian RNA induces IFN-α when delivered to immune cells (Diebold et al., 2004). To explain why mammalian RNAs are immunogenic, it was reasoned that in those experiments transfected RNA entered the endosomal compartments of immune cells, therefore the immune system might

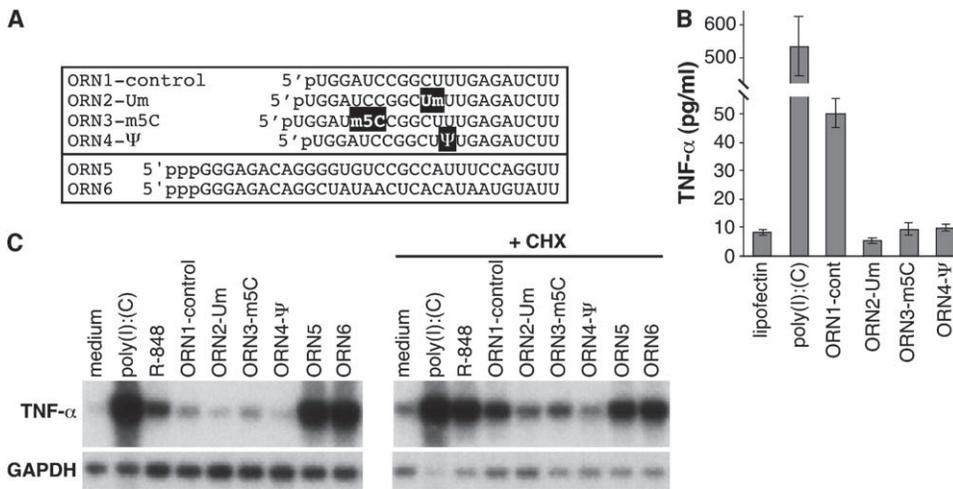


Figure 6. TNF-α Expression by RNA-Transfected DCs

(A) Sequences of oligoribonucleotides (ORNs) synthesized chemically (ORN1-4) or transcribed in vitro (ORN5-6) are shown. Positions of modified nucleosides Um (2'-O-methyluridine), m5C, and Ψ are highlighted. Human MDDCs were transfected with lipofectin alone (medium), R-848 (1 μg/ml), or with the indicated RNA (5 μg/ml) complexed with lipofectin. Where noted, cells were treated with 2.5 μg/ml cycloheximide (CHX). After 8 hr incubation, TNF-α was measured in the supernatant by ELISA (B). Mean values ± SEM are shown. The results are representative of three independent experiments. RNA isolated from the cells were analyzed by Northern blot (C).

discriminate between self and nonself RNA based on cellular location rather than some unique pathogen-associated molecular pattern (Croizat and Beutler, 2004). It has been shown, however, that the human innate immune system can also discriminate between molecular features of eukaryotic and bacterial mRNA and recognize mRNA devoid of polyA-tail as stimulatory (Koski et al., 2004). In this report, we observed potent immune stimulation with bacterial, but not with mammalian, total RNA and concluded that this was due to the difference in their modified nucleoside content. This is supported by the observation that the major mass of total RNA is rRNA, and modified nucleosides are abundant in mammalian, but not in bacterial, rRNA: 3% versus 0.8% (Bachelierie and Cavaille, 1998). We also observed that suppression of RNA-mediated immune stimulation correlated with the level of this difference in modified nucleoside content (Figure 5C). The present study now identifies nucleoside modification as a novel feature of RNA recognized by the innate immune system, specifically by TLR3, TLR7, and TLR8 (Figure 2). We observed earlier that RNA from necrotic cells activated DCs, whereas RNA from apoptotic cells did not (Kariko et al., 2004). Based on the results presented in this report, we now propose that mammalian RNA, especially the least-modified mtRNA, likely contributed to the observed effect. The immune potential of mammalian RNA might also explain why degradation of RNA during apoptosis is so critical. Fragmentation of genomic DNA is a well-established process and used consistently as a technique to define apoptosis itself. Although less described, a well-orchestrated degradation of cellular RNA also occurs in apoptotic, but not necrotic, cells. Interestingly, the most immunogenic mtRNA degrades at a very early stage of apoptosis (Crawford et al., 1997), hours before the breakdown of cytoplasmic RNA, DNA laddering (Houge et al., 1995),

or morphological changes associated with apoptosis could be detected.

Both RNA and DNA are central immunogenic determinants in the autoimmune disease of systemic lupus erythematosus (SLE), which is characterized by production of autoantibodies directed against DNA, RNA, and proteins associated with nucleic acids (Ronnlblom et al., 2003). In the development of SLE, studies now have established the involvement of TLR9 activation by mammalian DNA that bears hypomethylated CpG motifs (Boule et al., 2004). Another prominent target molecule in SLE is U1 small nuclear RNA, which has been recently shown to activate TLR3 (Hoffman et al., 2004), suggesting the potential involvement of an RNA-sensitive TLR in the disease process.

Nucleosides in native RNA become modified post-transcriptionally as part of their maturation process. Almost one hundred different types of modified nucleosides have been identified in RNA, but the physiological significance of these alterations is not well understood. Most of the modifications occur nonrandomly at positions conserved across diverse species, implying that they are important. Surprisingly, however, even the extensively modified tRNA could function without any modification (Sampson and Uhlenbeck, 1988), thus leaving the role of nucleoside modification very puzzling.

Pseudouridine is the most abundant modified nucleoside in RNA. It is generated by isomerization of uridines. We have demonstrated here that pseudouridine along with the other uridine modifications m5U and s2U uniquely suppress the capacity of RNA to activate primary DCs (Figure 3D). This finding implies that unmodified uridine probably contributes to the immune stimulatory action of RNA. Indeed, several points of evidence support this suggestion. In an earlier study, poly(U) was identified as the only homopolymer capable of inducing IL-12 in primed DCs (Koski et al., 2004). By using DCs

from TLR7 null mice, Diebold et al. identified TLR7 as the responding receptor for poly(U) treatment (Diebold et al., 2004). Others have shown that even nucleoside mixtures with uridine are sufficient to stimulate PBMCs to secrete TNF- α (Heil et al., 2004). Of interest, we did not find that poly(U) or any other RNA homopolymer activated primary DCs or human TLR3, TLR7, or TLR8 when transformed 293 cells expressing these receptors were used for testing. Presence of Ψ in RNA promotes base stacking, thereby stabilizing RNA duplex regions (Charette and Gray, 2000), which might explain why Ψ -modified RNA could potentially activate TLR3 (Figure 2B).

N6-methyladenosine (m6A) is the only base-modified nucleoside that is present in all RNA types, including rRNA, tRNA, and snRNA, as well as in mRNAs of cellular and viral origins. The methylation in m6A interferes with Watson-Crick base pairing, thus, its presence destabilizes RNA duplexes (Kierzek and Kierzek, 2003). This characteristic of m6A might explain why RNA containing m6A did not stimulate TLR3 (Figure 2B). m6A is present in mRNA of mammalian cells and RNA of viruses that replicate in the nucleus such as influenza, adenovirus, HSV, SV40, and RSV (Bokar and Rottman, 1998). In general, m6A modifications were found internally, mostly in coding sequences, and viral mRNA usually contained significantly more m6A than cellular mRNA (Bokar and Rottman, 1998). Interestingly, Rous sarcoma virus replicated similarly with and without m6A when tested in cell culture (Kane and Beemon, 1987), therefore no function could be assigned to m6A in this viral mRNA. It is tempting to speculate that the presence of m6A in viral RNA might serve the virus by allowing it to avoid immune activation. This suggestion is strengthened by considering that the frequency of m6A modifications found in viral mRNAs, up to eight per a 1.8 kb-long segment of influenza RNA (Narayan et al., 1987), is sufficient to suppress the capacity of RNA to activate DCs (Figure 5). Because those early studies with viruses were performed in cell culture and not in animals, the immune suppressive effect of m6A might have been missed.

RNA containing either m6A or m5C stimulated primary DC1 and DC2 as potently as the corresponding nonmodified RNA (Figure 3D). This was an unexpected finding, because DC2 express only TLR7 (Ito et al., 2002; Matsumoto et al., 2003) and thus resemble 293-hTLR7 cells that did not respond to any of the modified RNA (Figure 2B). Because all tested RNA were delivered by transfection to the cells, where they could interact with many different RNA binding proteins, it is possible an RNA receptor is uniquely present in primary DCs, but not in 293 cells or MDDCs. Such an RNA sensor could likely recognize U-rich RNA patterns even in the presence of the m6A and m5C nucleoside residues, but not when the U residues are masked by modifications. Support for this hypothesis was provided by the observations that RNA containing both m6A and Ψ modifications on the same strand did not activate DC1 or DC2, whereas mixtures of RNA containing either m6A or Ψ modification on separate strands potently activated these cells (Figure 3D). In this regard, there are already examples for single- and double-stranded RNA-responsive cytoplasmic receptors such as FADD,

RIG-1, and PKR that function in the innate immune system independently from TLRs (Sen and Sarkar, 2005).

By using TLR7-expressing cell lines, we demonstrate (Figure 2) that in vitro-transcribed RNA and bacterial RNA, but not dsRNA, are ligands for human TLR7. This finding is in discordance with results obtained by Heil et al. (2004), who showed that human TLR7 was nonresponsive to RNA oligomers with phosphorothioate linkage. Differences in the stimulating RNA, such as long RNA versus short ORNs with phosphorothioate linkage, likely account for the conflicting result. We observed that all in vitro-transcribed RNAs, regardless of their primary sequence, as well as bacterial RNA activated TLR7 expressing 293 cells, demonstrating that natural RNA is a ligand for this receptor.

In summary, we demonstrate that selected natural RNA isolated from mammalian and bacterial cells and RNA transcribed in vitro or synthesized chemically activate human DCs and stably transformed 293 cells expressing human TLR3, TLR7, or TLR8. Such activation was reduced or completely eliminated with RNA containing naturally occurring modified nucleosides, such as m5C, m6A, m5U, pseudouridine, or 2'-O-methyl-U. Insights gained from this study could advance our understanding of autoimmune diseases where nucleic acids play a prominent role in the pathogenesis, determine a role for nucleoside modifications in viral RNA, and give future directions into the design of therapeutic RNAs.

Experimental Procedures

Plasmids and Reagents

Plasmids pTEVluc (D. Gallie, UC Riverside), pT7T3D-MART-1 (ATCC, Manassas, VA), pUNO-hTLR3 (InvivoGen, San Diego, CA), and pSVren (Kariko et al., 2004) were obtained. Human TLR3-specific siRNA, pTLR3-sh was constructed by inserting synthetic ODN-encoding shRNA with 20 nt-long homology to human TLR3 (nt 703–722, accession: NM_003265) into plasmid pSilencer 4.1-CMV-neo (Ambion, Austin, TX). pCMV-hTLR3 was obtained by first cloning TLR3-specific PCR product (nt 80–2887; accession NM_003265) into pCRII-TOPO (Invitrogen, Carlsbad, CA), then released with NheI-HindIII cutting and subcloning to the corresponding sites of pcDNA3.1 (Invitrogen). Cells were treated with the following reagents: LPS (*E. coli* 055:B5) (Sigma Chemical Co, St. Louis, MO), CpG ODN-2006, and R-848 (InvivoGen).

Cells and Cell Culture

Human embryonic kidney 293 cells (ATCC) were propagated in DMEM supplemented with glutamine (Invitrogen) and 10% FCS (Hyclone, Ogden, UT) (complete medium). 293-hTLR3 and 293 pUNO null cell lines were generated by transforming 293 cells with pUNO-hTLR3 and pUNO null. Cell lines 293-hTLR7, 293-hTLR8, and 293-hTLR9 (InvivoGen) were grown in complete medium supplemented with blasticidin (10 μ g/ml) (InvivoGen). Cell lines 293-ELAM-luc and TLR7-293 (M. Lamphier, Eisai Research Institute, Andover, MA) and TLR3-293 cells were cultured as described (Kariko et al., 2004). Cell lines 293, 293-hTLR7, and 293-hTLR8 were stably transfected with pTLR3-sh and selected with G-418 (400 μ g/ml) (Invitrogen). Neo-resistant colonies were screened, and only those that did not express TLR3, determined as lack of IL-8 secretion in response to poly(I):(C), were used. Cell lines were used as soon as possible, because shRNA-mediated suppression of TLR3 became leaky over time. Leukopheresis samples were obtained from HIV-uninfected volunteers through an IRB-approved protocol. DCs were produced as described previously and treated with GM-CSF (50 ng/ml) + IL-4 (100 ng/ml) (Weissman et al., 2000) or IFN- α (1000 U/ml) (Santini et al., 2000) (R&D Systems, Minneapolis, MN) in AIM

V medium (Invitrogen). Primary myeloid and plasmacytoid DCs (DC1 and DC2) were obtained from peripheral blood by using BDCA-1 and BDCA-4 cell isolation kits (Miltenyi Biotec Auburn, CA), respectively.

RNA

In Vitro-Transcribed RNA

By using *in vitro* transcription assays (MessageMachine and Mega-Script kits; Ambion), the following long RNAs were generated by T7 RNAP as described (Kariko et al., 1998) (note: the names of templates are in parentheses), the number in the name of the RNA specifies the length: RNA-1866 (NdeI-linearized pTEVluc) encodes firefly luciferase and a 50 nt-long polyA-tail, RNA-1571 (SspI-linearized pSVren) encodes *Renilla* luciferase, RNA-730 (HindIII-linearized pT7T3D-MART-1) encodes the human melanoma antigen MART-1, RNA-713 (EcoRI-linearized pT7T3D-MART-1) corresponds to antisense sequence of MART-1, and RNA-497 (BglII-linearized pCMV-hTLR3) encodes a partial 5' fragment of hTLR3. To obtain RNA bearing nucleoside modification, the transcription reaction was assembled with the replacement of one (or two) of the basic NTPs with the corresponding triphosphate-derivative(s) of the modified nucleotide 5-methylcytidine, 5-methyluridine, 2-thiouridine, N6-methyladenosine, or pseudouridine (TriLink, San Diego, CA). In each transcription reaction, all four nucleotides or their derivatives were present in equimolar (7.5 mM) concentration. In selected experiments, 6 mM m7GpppG cap analog (New England BioLabs, Beverly, MA) was also included to obtain capped RNA. To obtain RNA containing increasing amounts of m6A, Ψ, or m5C, the transcription reaction was performed in a reaction mix in which the ratio of one particular modified NTP relative to the corresponding unmodified NTP was 0%, 1%, 10%, 50%, 90%, 99%, and 100%. By using DNA oligodeoxynucleotide templates and T7 RNAP (Silencer siRNA construction kit, Ambion), ORN5 and ORN6 were generated.

Natural and Synthetic RNA

Mitochondria were isolated from outdated platelets (obtained from the University of Pennsylvania Blood Bank under an IRB approved protocol) by using a fractionation lyses procedure as described by the manufacturer (Mitochondria Isolation Kit; Pierce, Rockford, IL). RNA was isolated from the purified mitochondria, cytoplasmic and nuclear fractions of 293 cells, unfractionated 293 cells, rat liver, mouse cell line TUBO, and DH5 α strain of *E. coli* by Master Blaster (Bio-Rad, Hercules, CA). Bovine tRNA, wheat tRNA, yeast tRNA, *E. coli* tRNA, poly(A)⁺ mRNA from mouse heart, and poly(I):(C) were purchased from Sigma; total RNA from human spleen and *E. coli* RNA were purchased from Ambion. Oligoribonucleotide 5'-monophosphates were synthesized chemically (Dharmacon, Lafayette, CO).

Aliquots of RNA samples were incubated in the presence of Benzonase nuclease (1 U per 5 μ l of RNA at 1 μ g/ μ l for 1 hr) (Novagen, Madison, WI). Aliquots of RNA-730 were digested with alkaline phosphatase (New England Biolab). Generally, RNA samples were analyzed by denaturing agarose or polyacrylamide gel electrophoresis for quality assurance. Assays for LPS in RNA preparations using the Limulus Amebocyte Lysate gel clot assay were negative with a sensitivity of 3 pg/ml (University of Pennsylvania, Core Facility).

HPLC Analysis

Nucleoside monophosphates were separated and visualized via HPLC (Greenwood and Gentry, 2002). Briefly, first to release free nucleoside 3'-monophosphates, 5 μ g aliquots of RNA were digested with 0.1 U RNase T2 (Invitrogen) in 10 μ l of 50 mM NaOAc and 2 mM EDTA buffer (pH 4.5) overnight, then the samples were injected into an Agilent 1100 HPLC by using a Waters Symmetry C18 column (Waters, Milford, MA). Buffer A consisted of 30 mM KH₂PO₄ and 10 mM tetraethylammonium phosphate (PicA reagent, Waters), pH 6.0. Buffer B was acetonitrile. At a flow rate of 1 mL/min, a gradient from 100% buffer A to 30% buffer B was run over 60 min. Nucleotides were detected by using a photodiode array at 254 nm. Identities were verified by retention times and spectra. Relative percentage of modified versus the corresponding unmodified nucleosides in RNA was determined for each transcript.

Treatment of Cells

Parental 293, 293-hTLR7, and 293-hTLR8 cells, all expressing TLR3-specific siRNA, and 293-hTLR9, TLR3-293 were seeded into

96-well plates (5×10^4 cells/well) and cultured without antibiotics. On the subsequent day, the cells were exposed to R-848 or RNA with prior complexing to lipofectin (Invitrogen) as previously described (Kariko et al., 1998). The RNA was removed after 1 hr, and the cells were further incubated in complete medium for 7 hr. Supernatants were collected for IL-8 measurement.

DCs in 96-well plates ($\sim 1.1 \times 10^5$ cells/well) were treated with R-848, lipofectin alone, or complexed with RNA for 1 hr when the medium was replaced by fresh medium. Cells and medium were harvested at the end of an 8–20 hr incubation; cells were harvested for either RNA isolation or flow cytometry, whereas the collected culture medium was subjected to cytokine ELISA. The levels of IL-12 (p70) (BD Biosciences Pharmingen, San Diego, CA), IFN- α , TNF- α , and IL-8 (Biosource International, Camarillo, CA) were measured in supernatants by ELISA. Cultures were performed in triplicate to quadruplicate and measured in duplicate.

Analysis of DC Activation

DCs treated as described above were analyzed by flow cytometry after 20 hr. DCs were stained with CD83-phycoerythrin mAb (Research Diagnostics Inc, Flanders, NJ), HLA-DR-Cy5PE, and CD80 or CD86-fluorescein isothiocyanate mAb and analyzed on a FACScalibur flow cytometer by using CellQuest software (BD Biosciences).

Northern Blot Analysis

RNA was isolated from MDDCs after an 8 hr incubation following treatment as described above. Where noted, cells were treated with 2.5 μ g/ml cycloheximide (Sigma) 30 min prior to the stimulation and throughout the entire length of incubation. RNA samples were processed and analyzed on Northern blots as described (Kariko et al., 2004) by using human TNF- α and GAPDH probes derived from plasmids (pE4 and pHcGAP, respectively) obtained from ATCC.

Supplemental Data

Supplemental Data include one figure and are available with this article online at <http://www.immunity.com/cgi/content/full/23/2/165/DC1/>.

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