Naturally occurring nucleoside modifications suppress the immunostimulatory activity of RNA: Implication for therapeutic RNA development

Katalin Karikó[1] & Drew Weissman[2]

Address
[1]Department of Neurosurgery
University of Pennsylvania
Rm 371 Stemmler Hall
36th & Hamilton Walk
Philadelphia
PA 19104
USA
Email: kariko@mail.med.upenn.edu

[2]Division of Infectious Diseases
University of Pennsylvania
522B Johnson Pavilion
Philadelphia
PA 19104
USA
Email: dreww@mail.med.upenn.edu

Correspondence can be addressed to either author

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DNA and RNA stimulate the mammalian innate immune system by triggering a variety of sensors, including Toll-like receptors (TLRs). TLR9 signals upon exposure to DNA, while TLR3, TLR7 and TLR8 respond to RNA. Most DNA and RNA from natural sources contain modified nucleosides. Methylation of CpG motifs in DNA blocks TLR9 signaling. The question of whether an analogous effect can be attributed to nucleoside modifications in RNA has only recently been addressed. This review characterizes a few naturally occurring nucleoside modifications of RNA and their influence on the capacity of RNA to activate immune cells and TLRs. RNAs containing modified nucleosides, and thus lacking immune-activating properties, have potential importance in clinical applications.

Keywords Autoimmune, innate immunity, interferon, nucleoside modification, RNA, TLR

Abbreviations

A adenosine, **ADAR** RNA-specific adenosine deaminase, **C** cytidine, **DC** dendritic cell, **dsRNA** double-stranded RNA, **G** guanosine, **IFN** interferon, **IL** interleukin, **MDA5** melanoma differentiation-associated gene 5, **OAS** 2'-5' oligoadenylate synthetase, **ORN** oligoribonucleotide, **PKR** RNA-dependent protein kinase, **RIG-I** retinoic acid inducible gene-I, **RNase** ribonuclease, **rRNA** ribosomal RNA, **siRNA** short interfering RNA, **SLE** systemic lupus erythematosus, **snRNA** small nuclear RNA, **ssRNA** single-stranded RNA, **TLR** Toll-like receptor, **TNF** tumor necrosis factor, **tRNA** transfer RNA, **U** uridine

Introduction

The first line of antimicrobial defense is the innate immune system, which performs its function with the help of pattern recognition receptors, namely the Toll-like receptors (TLRs). The broad classes of pathogens (eg, viruses, bacteria and fungi) express molecules that are shared across subclasses of pathogens (eg, lipopolysaccharide in Gram-negative bacteria; for a review see reference [1••]). Ten TLR family members have been identified in humans and eleven in the mouse. The ligand specificities of most TLRs have been identified, and a subset of TLRs has been shown to specialize in responding to nucleic acids. TLR9 responds to unmethylated CpG motifs that are characteristic of bacterial and certain viral DNAs [2•]. Double-stranded RNA (dsRNA), a common viral intermediate, activates TLR3 [3]. Synthetic single-stranded RNA (ssRNA) and virus-related RNA activate human TLR7 and TLR8 and murine TLR7 (Table 1) [4•,5,6••]. Based on sequence and structural similarities, TLR7, TLR8 and TLR9 form a distinct subfamily. Activation of these receptors, and also activation of TLR3 [7], depends upon endosomal acidification and leads to the production of type I interferons (IFNs) and pro-inflammatory cytokines.

While it has been known for decades that 'foreign' nucleic acids, such as bacterial DNA and viral dsRNA, are potent adjuvants of the mammalian immune system [8•], the mechanisms for nucleic acid recognition have only recently been discovered. These mechanisms can be divided into TLR-dependent and TLR-independent pathways (for a review see reference [9]). Two RNA helicases, retinoic acid inducible gene-I (RIG-I) [10••] and melanoma differentiation-associated gene 5 (MDA5) [11], are cytoplasmic sensors of RNA, while the identity of cytoplasmic DNA sensors has not yet been established (for a review see reference [12•]). dsRNA, in addition to triggering pro-inflammatory signaling cascades that lead to IFN production, also directly activates IFN-induced enzymes (Table 1), including RNA-dependent protein kinase (PKR), RNA-specific adenosine deaminase (ADAR) and 2'-5' oligoadenylate synthetase (OAS). These proteins carry out their antiviral functions in association with other enzymes, such as RNaseL, a 2'-5' oligoadenylate-dependent endonuclease, or I-RNase, which degrades inosine-containing RNA [13•].

It has been reported that hypomethylation of the cytidine in CpG motifs provides the structural basis for TLR9 activation by bacterial DNA [2•]. This finding raises the possibility that RNA-responsive TLRs, and other RNA sensors with regulatory or effector immune functions, might react differently when RNA nucleosides are modified. RNA has more than one hundred different nucleoside modifications [14•], some of which are unique to eukaryotic RNAs or prokaryotic RNAs, but most are present in both. In general, mammalian RNAs are much more abundantly modified than bacterial RNAs [15]. Thus, the extent and the quality of nucleoside modifications could alter the potency of RNA-mediated immune activation and be used as a means to differentiate between host and pathogenic RNA.

Table 1. RNA-responsive proteins of the innate immune system.

RNA sensor	Activator RNA	Function	Reference
Regulator			
RIG-I	5'-triphosphate RNA	Induction of type I IFNs	[10••,77-79]
MDA5	dsRNA		[11]
TLR3	dsRNA, *in vitro* transcript	Antiviral – viral recognition, induction of antiviral and inflammatory response	[3,39•]
TLR7	ssRNA (oligomer), polyU, *in vitro* transcript		[4•,5,6••]
TLR8	ssRNA (oligomer), *in vitro* transcript		[5,6••]
Effector			
PKR	dsRNA TNFα mRNA	Antiviral – inhibition of protein synthesis TNFα production	[80] [81]
OAS (RNaseL)	dsRNA	Antiviral – degradation of mRNA	[82]
ADAR (I-RNase)	dsRNA		[83•,84•]
Nalp3 (caspase 1)	Bacterial RNA	IL-1β production	[85]

ADAR RNA-specific adenosine deaminase, **caspase** cysteine-aspartic-acid-protease, **dsRNA** double-stranded RNA, **I-RNase** ribonuclease specific for inosine-containing RNA, **IFN** interferon, **IL** interleukin, **MDA5** melanoma differentiation-associated gene 5, **mRNA** messenger RNA, **Nalp3** leucine-rich repeat and pyrin domain-containing protein, **OAS** 2'-5' oligoadenylate synthetase, **PKR** RNA-dependent protein kinase, **RIG-I** retinoic acid-inducible gene-I, **RNaseL** latent 2'-5' oligoadenylate-dependent ribonuclease, **ssRNA** single-stranded RNA, **TLR** Toll-like receptor, **TNF** tumor necrosis factor, **U** uridine.

Nucleoside modification of RNA

Naturally, all RNA is synthesized from four basic ribonucleotides (adenosine triphosphate, cytidine triphosphate, uridine triphosphate and guanosine triphosphate), but some of the incorporated nucleosides are post-transcriptionally modified as part of the RNA maturation process. Most of the modifications involve methylation of the nucleobase or 2'-*O*-methyl formation in the ribose; however, some modifications are more complex, including addition of amino acid (eg, glycine, threonine) or sugar (eg, galactose, mannose) derivatives to the carbon or nitrogen atoms of the nucleobase. A comprehensive listing of post-transcriptionally modified nucleosides of RNA can be found in the RNA Modification Database hosted by the University of Utah [16]. Maturation-induced nucleoside modifications are considered to be irreversible and occur in conserved positions of the polynucleotide chain. In many cases, the modifying enzymes are so specialized that they perform their modification at only one or a few uniquely positioned nucleosides.

The extent and nature of modifications vary and depend on the RNA type as well as the evolutionary level of the organism from which the RNA is derived. Transfer RNAs (tRNAs) contain the most diverse set of nucleoside modifications (Table 2), and much of our knowledge of modified nucleosides comes from the study of tRNAs. The sequence motifs at which modifications are placed tend to be conserved, but most of the enzyme systems that perform the modifications remain poorly characterized. In ribosomal RNA (rRNA), which is the major constituent of total cellular RNA, fewer types of modification are found compared with tRNA, but significantly more nucleoside modifications are observed in mammalian rRNA compared with bacterial rRNA; for example, the pseudouridine (Ψ) content is approximately 10-fold greater and the 2'-*O*-methylated nucleoside content approximately 25-fold greater in human rRNA compared with bacterial rRNA. rRNA derived from mitochondria, a cellular organelle evolved from eubacteria [17], exhibits only one modification [18•].

Table 2. Distribution of different types of nucleoside modifications in RNA.

RNA		Types of modification in cells	
	% of total cellular RNA	Eukaryote	Prokaryote
tRNA	15	50	45
rRNA	80	23	18
mRNA	3 to 5	13	0
snRNA	< 2	11	–

mRNA messenger RNA, **rRNA** ribosomal RNA, **snRNA** small nuclear RNA, **tRNA** transfer RNA.

tRNA is the most heavily modified subgroup of RNA in cells. In mammalian tRNAs, up to 25% of the nucleosides can be modified, while in prokaryotic tRNAs, there are significantly fewer modifications. Mammalian messenger RNAs (mRNAs) have been demonstrated to contain 5-methylcytidine (m5C), N^6-methyladenosine (m6A), inosine and many 2'-*O*-methylated nucleosides (Nm), in addition to N^7-methylguanosine (m7G), which is part of the 5'-terminal cap structure [15,19], while bacterial mRNA contains no nucleoside modifications. Modified nucleosides (eg, m6A, m5C and Nm) have also been found in the internal regions of many viral RNAs, including influenza [20], adenovirus [21], herpes simplex virus [22], simian virus 40 [23] and respiratory syncytial virus [24]. In fact, modified nucleosides, including m6A and m5C, occur more frequently in some viral RNAs than in cellular mRNAs [21]. In experimental studies, when the sites for nucleoside modification were removed from the viral RNA, no difference in replication was observed [24], but these experiments were performed *in vitro*. If the role of the modifications were to reduce the immunogenicity of the

viral RNA, a protective effect on viral pathogenesis by the modifications would only be observed *in vivo*.

Nucleoside-modified dsRNAs as inducers of IFN: Initial observations

A series of papers published 40 years ago first demonstrated the basic observation that dsRNAs, whether they be synthetic, viral or phage isolates, induce IFN and establish an antiviral state, while ssRNAs and mammalian dsDNA do not [25-28]. Because at that time polynucleotide phosphorylase-catalyzed polymerization was the only method available to synthesize RNA, dsRNAs formed between long homopolymers were studied. Field *et al* found that polyinosinic:polycytidylic acid (poly(I:C)) was 50-fold more potent an inducer of IFN than polyadenylic:polyuridylic acid (poly(A:U)), suggesting that the composition of the nucleosides in the dsRNA play a role in activation [26]. De Clercq *et al* demonstrated that dsRNA homopolymers formed with 2'-*O*-methylated nucleotide derivatives, poly(I:Cm) and poly(A:Um), had reduced or no IFN-inducing activities (Table 3) [29,30]. Similarly, dsRNAs containing 2'-*O*-methylated nucleotide derivatives poly(I:Cm) and poly(Im:C), did not activate two IFN-induced dsRNA-dependent enzymes, OAS and PKR [31]. Methylation of the polypyrimidines at the C5-position, however, resulted in dsRNAs with opposing antiviral effects. dsRNA poly(I:m5C) was less antiviral than poly(I:C), while poly(A:m5U) possessed increased IFN-inducing and antiviral ability compared with poly(A:U) [29,32].

While nucleoside composition is an important determinant of RNA immunogenicity and certain post-synthetic nucleoside modifications are capable of suppressing various aspects of this immunogenicity, the determination of precise mechanisms suggested by these early studies has to be interpreted with caution as synthetic RNA homopolymers that form special tertiary structures (eg, triplexes, tetrads) behave differently than natural RNA sequences. In addition, because these early experiments used cultured fibroblasts and rabbits as an animal model, the target receptor(s) at which the dsRNAs acted are unknown. A more thorough understanding of the precise mechanisms by which RNA and its nucleoside modifications influence immunity has required the ability to study specific models of the immune system and the use of protocols for synthesizing synthetic RNA that more closely simulates natural RNA.

The effect of nucleoside modification on RNA-mediated immune activation

Dendritic cells (DCs) are the most powerful antigen-presenting cells of the immune system. Human DCs, when exposed to *in vitro* transcribed RNA (which contains no nucleoside modification), express multiple activation markers (major histocompatibility complex class II, CD80, CD83 and CD86) and inflammatory cytokines (eg, interleukin [IL]-12, tumor necrosis factor [TNF]α and IFNα) [33,34]. Interestingly, RNAs derived from different compartments of mammalian cells (mRNA, tRNA, nuclear, mitochondrial or total) have variable abilities to stimulate DCs. A species specificity to RNA activation is also observed when human DCs secrete high levels of IL-12 following treatment with total RNA isolated from bacterial, but not from mammalian cells [35]. Under different circumstances, however, mammalian RNA could potentiate immune responses *in vivo* when transfected with viral particles [36], or could induce IFNα when delivered to DCs [4•]. A comparative study [6••] demonstrated that minimally modified or unmodified RNA (transcribed *in vitro*, isolated from bacterial cells or isolated from mammalian mitochondria) potently induced human DCs to secrete TNFα, but total mammalian RNA and bacterial tRNA (containing more nucleoside modifications) were much less stimulatory (Figure 1). Mammalian tRNAs (the most modified naturally occurring RNAs) did not induce any detectable TNFα secretion by the DCs. The role of nucleoside modification in modulating DC responses could be discerned in a second system when results from two studies were examined in the first study, DCs were activated by RNA isolated from *Candida albicans* at the yeast but not the hyphal stage of morphological development [37]. The second study performed on the mRNA from another yeast, *Saccharomyces cerevisiae,* revealed substantial changes in the methylation level of nucleosides, especially the formation of m6A, associated with different developmental stages of this organism [38]. Thus, taken together, these studies suggest that less-modified yeast stage RNA activates DCs while more-modified hyphal RNA with identical primary sequence does not.

In vitro transcribed RNA activates human TLR3, TLR7 and TLR8 [6••,39•] and murine TLR7 [4•], while chemically synthesized oligoribonucleotides (ORNs) stimulate murine TLR7 and human TLR8 [5]. Several sequence motifs have been identified using ORNs that enhanced the potential of RNA to stimulate TLR7 or TLR8. RNAs containing GUCCUUCAA sequences and ORNs possessing stretches of oligoG-tails are stimulators of murine TLR7 and human TLR8, respectively [40•,41••]. It has also been observed that both TLR7 and TLR8 could be stimulated with GU-rich ORNs [5]. The question as to whether modification of nucleosides can impact TLR activation was addressed in experiments using both long *in vitro* transcripts and short stimulatory ORNs. Sequence composition becomes less relevant when long *in vitro* transcripts are used, because their sequences will likely contain at least some of the preferred motifs. TLR3, TLR7 and TLR8 expressed on HEK293 cells were stimulated by unmodified *in vitro* transcribed RNA, but RNAs containing some and/or any of the modifications m5C, m5U, Ψ, s2U, m5C/Ψ or m6A, did not stimulate TLR3, TLR7 and/or TLR8 (Figure 2) [6••].

The lack of TLR3 activation by m6A-containing RNA was not unexpected, as m6A destabilizes the dsRNA structure [42,43]. Approximately 80% of total cellular RNA is ribosomal (Table 2), containing a higher level of nucleoside modification in mammalian cells than in bacteria or mitochondria (3.0 versus 0.9 or 0.06%, respectively). Total RNA from mammalian cells did not stimulate any of the RNA-responsive TLRs when overexpressed on HEK293 cells; however, bacterial total RNA obtained from *Escherichia coli* activated all three human RNA responsive TLRs (Figure 3) [6••]. Mitochondrial RNA, similarly to some stimulatory ORNs [5], activated human TLR8, but not TLR7 (Figure 3), suggesting the presence of subtle differences in RNA sensitivity between these two human ssRNA-responsive TLRs when reconstituted in HEK293 cells.

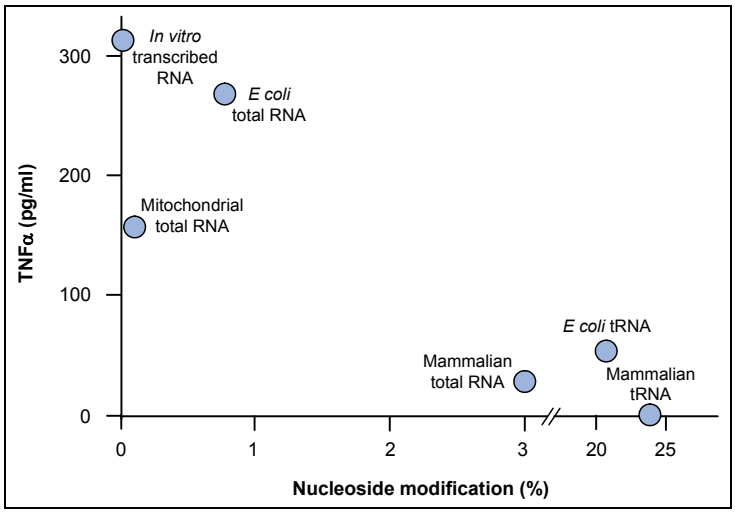
Table 3. Immune activity of RNA containing natural nucleoside modifications.

Modified nucleosides[1]	RNA[2]	Target molecule	Test system	Impact of nucleoside modification on the immune activity of RNA[3]		References
2'-*O*-methyl-nucleosides						
U or C	poly(A:U), poly(I:C)	ND	primary rabbit kidney cell	↓	anti-VSV activity	[29]
I and/or C	poly(I:C)	ND	primary human fibroblast	↓	IFN anti-VSV activity	[30]
I or C	poly(I:C)	PKR OAS	*in vitro* enzymatic assays	↓ ↓	Kinase activity 2'-5'oligoA synthesis	[31]
U and/or G C or A	dsORN	ND	human PBMC	↓ –	IFNα IFNα	[45•]
U	ssORN	ND	human PBMC	↓	IFNα, TNFα	[44•]
A	U1snRNA	TLR7	mouse DC from BM	↓	IL-6, IFNα	[86]
U	ssORN	ND	human MDDC	↓	TNFα	[6••]
N	dsORN	ND	mouse *in vivo*	↓	TNFα	[46•]
U	ssORN	RIG-I	human monocyte and PDC	↓	IFNα	[79]
m5C						
	poly(I:C)	ND	primary rabbit kidney cell	↓	Anti-VSV activity	[29]
	ssORN	ND	human PBMC	↓	IL-12	[87•]
	ssORN IVT-mRNA	ND TLR7/8 ND ND ND	human MDDC TLR-transformed HEK293 human MDDC human DC1, DC2 human DC2	↓ ↓ ↓ – ↓	TNFα IL-8 TNFα, IL-12p70 TNFα IFNα	[6••]
m6A						
	IVT-mRNA	TLR3/7/8 ND ND ND	TLR-transformed HEK293 human MDDC human DC1, DC2 human DC2	↓ ↓ – ↓	IL-8 TNFα, IL-12p70 TNFα IFNα	[6••]
s2U						
	IVT-mRNA	TLR3/7/8 ND ND ND	TLR-transformed HEK293 human MDDC human DC1, DC2 human DC2	↓ ↓ ↓ ↓	IL-8 TNFα, IL-12p70 TNFα IFNα	[6••]
	ssORN	RIG-I	human monocyte and PDC	↓	IFNα	[79]
Pseudouridine						
	ssORN IVT-mRNA	ND TLR7/8 ND ND ND	human MDDC TLR-transformed HEK293 human MDDC human DC1, DC2 human DC2	↓ ↓ ↓ ↓ ↓	TNFα IL-8 TNFα, IL-12p70 TNFα IFNα	[6••]
	ssORN	RIG-I	human monocyte and PDC	↓	IFNα	[79]
m5U						
	poly(A:U)	ND	primary rabbit kidney cell	↑	IFN	[32]
	IVT-mRNA	TLR7/8 ND ND	TLR-transformed HEK293 human DC1, DC2 human DC2	↓ ↓ ↓	IL-8 TNFα IFNα	[6••]

[1]Naturally occurring modified nucleosides, [2]RNA with phosphodiester linkage, [3]activity of modified RNA, decreased (↓), unchanged (–) or increased (↑) relative to the unmodified RNA.

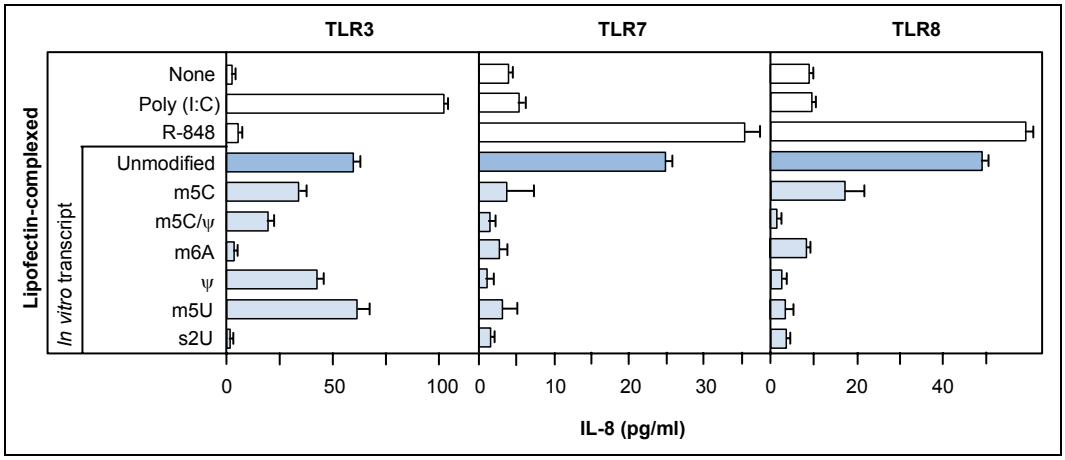
A adenosine, **BM** bone marrow, **C** cytidine, **DC** dendritic cell, **DC1** primary monocytoid dendritic cell, **DC2** primary plasmacytoid dendritic cell, **G** guanosine, **HEK293** human embryonic kidney cell, **IFN** interferon, **IL** interleukin, **IVT-mRNA** *in vitro*-transcribed messenger RNA, **m5C** 5-methylcytidine, **m5U** 5-methyluridine (ribothymidine), **m6A** N^6-methyladenosine, **MDDC** monocyte-derived dendritic cell, **N** any nucleoside, **ND** not determined, **OAS** 2'-5' oligoadenylate synthetase, **ORN** oligoribonucleotide, **PBMC** peripherial blood mononuclear cell, **PDC** plasmacytoid dendritic cell, **PKR** RNA-dependent protein kinase, **RIG-I** retinoic acid-inducible gene-I, **s2U** 2-thiouridine, **snRNA** small nuclear RNA, **TLR** Toll-like receptor, **TNF** tumor necrosis factor, **VSV** vesicular stomatitis virus, **U** uridine.

Figure 1. Production of TNFα by monocyte-derived dendritic cells incubated with lipofectin-complexed RNA transcribed *in vitro* or isolated from natural sources.



Tumor necrosis factor α (TNFα) was measured by enzyme-linked immunosorbent assay (ELISA) in the supernatant 8 h after RNA stimulation.

Figure 2. Production of IL-8 by RNA-treated HEK293 cells expressing human TLR3, TLR7 and TLR8.

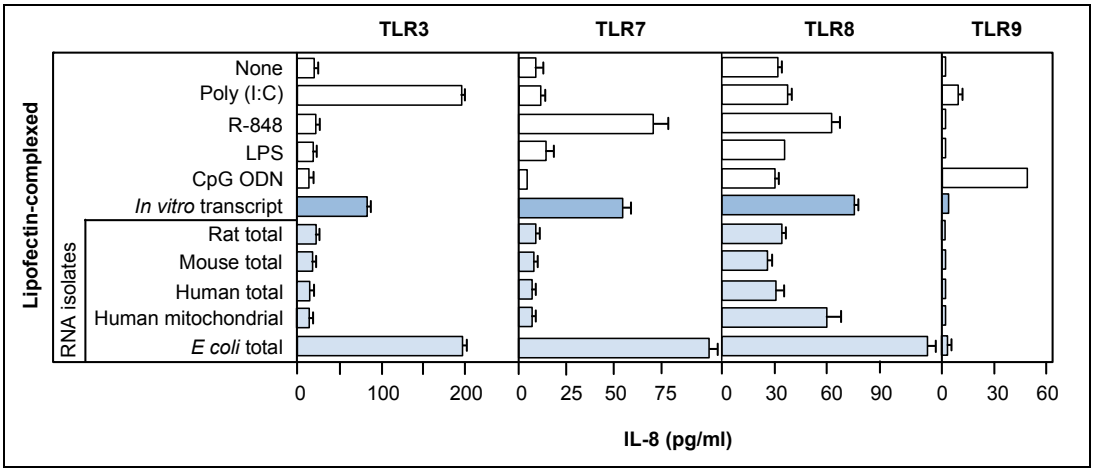


Cells were exposed to Toll-like receptor (TLR) ligands or lipofectin-complexed 1571 nucleotide-long RNA containing the indicated modified nucleosides. Interleukin (IL)-8 was measured in the supernatant 8 h after RNA stimulation.
*(Adapted with permission from Elsevier and Karikó K, Buckstein M, Ni H, Weissman D: **Suppression of RNA recognition by Toll-like receptors: The impact of nucleoside modification and the evolutionary origin of RNA.** Immunity (2005) **23**(2):165-175. © 2005 Elsevier.)*

Several studies have investigated whether 2'-*O*-methylation of nucleosides can alter the immune stimulatory effect of ss or dsORNs using human monocytes or DCs (Table 3). Modifying a single uridine in 21mer ORNs abrogates TNFα induction in DCs [6••], while modifying all of the uridines to 2'-*O*-methyluridine (Um) in 21mer ORNs abrogates TNFα induction in peripheral blood mononuclear cells (PBMCs) [44•]. Replacing all U and/or G nucleosides with their corresponding 2'-*O*-methylated derivatives diminishes the potential of dsORNs to induce TNFα and IFNα in human PBMCs, as well as in mice *in vivo* [45•,46•]. Human monocyte-derived DCs exposed to *in vitro* transcripts secrete high levels of TNFα and IL-12p70, but the introduction of m5C, m5C/Ψ, m6A, Ψ or s2U modified nucleosides ablate cytokine induction by the RNA (Figure 4) [6••]; however, these results need to be examined with the possibility that other RNA receptors, such as RIG-I and MDA5, may be involved in the recognition of RNA. For example, in primary human DCs purified directly from peripheral blood, only uridine modification appears to suppress the induction of TNFα and IFNα by RNA, while RNA containing m6A and m5C is as stimulatory as unmodified transcripts (Table 3) [6••].

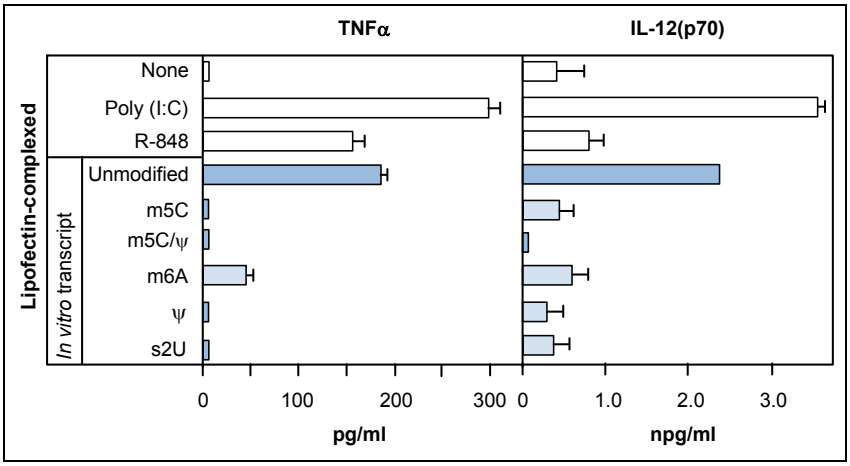
Additional data support a role for unmodified uridine in RNA immunogenicity. Koski *et al* demonstrated that poly(U) homopolymer induced IL-12 secretion in primed DCs [35]. Replacing uridines with adenosine, or modifying uridines with 2'-*O*-methylation abrogates cytokine induction [44•], while 2'-*O*-methylation of cytidines or adenosines instead of

Figure 3. Production of IL-8 by RNA-treated HEK293 cells expressing human TLR3, TLR7, TLR8 and TLR9.



Cells were exposed to Toll-like receptor (TLR) ligands or lipofectin-complexed RNA isolated from the indicated organisms or organelles. Interleukin (IL)-8 was measured in supernatants 8 h after stimulation.
(Adapted with permission from Elsevier and Karikó K, Buckstein M, Ni H, Weissman D: ***Suppression of RNA recognition by Toll-like receptors: The impact of nucleoside modification and the evolutionary origin of RNA.*** *Immunity (2005)* ***23****(2):165-175. © 2005 Elsevier.)*

Figure 4. Monocyte-derived dendritic cells treated with lipofectin-complexed 1571-nucleotide-long RNA containing the indicated modified nucleosides.



Using enzyme-linked immunosorbent assay (ELISA), tumor necrosis factor α (TNFα) and interleukin (IL)-12p70 were measured in the supernatants 8 and 16 h after RNA stimulation, respectively.
(Reproduced with permission from Elsevier and Karikó K, Buckstein M, Ni H, Weissman D: ***Suppression of RNA recognition by Toll-like receptors: The impact of nucleoside modification and the evolutionary origin of RNA.*** *Immunity (2005)* ***23****(2):165-175. © 2005 Elsevier.)*

uridines does not alter immune activation [45•]. Others have shown that even mixtures of nucleosides containing uridine were sufficient to stimulate TNFα secretion from human PBMCs [5]. Using DCs from TLR7 null mouse, TLR7 was identified as the receptor for poly(U) homopolymer [4•].

Potential role for alterations in RNA modification in autoimmune diseases

With the recent identification of human TLR7 and TLR8 as receptors for ssRNA, a series of investigations have begun to link these receptors to autoimmune disease processes [47•,48-50,51•,52••]. One could theorize that if nucleoside modification of RNA modulates signaling through these receptors, then alterations in modification could change signaling and accentuate disease processes in susceptible individuals. As the role of nucleoside modification of RNA immunity is a new field of study, no immunological disease caused by altered nucleoside modification of RNA has yet been identified. There are, however, a few diseases that are known to be associated with nucleoside-modification of RNA.

Reduced pseudouridine content in RNA, caused by mutation in two of the pseudouridine synthases, dyskerin and pseudouridine synthase 1, is associated with the human diseases dyskeratosis congenita, mitochondrial myopathy and sideroblastic anemia. These diseases are not

autoimmune, but rather the consequence of incorrect protein translation resulting from a reduced number of pseudouridines in rRNA [53•] or mitochondrial tRNA [54].

Systemic lupus erythematosus (SLE) is an autoimmune disease in which the connective tissues of the body are attacked. Drug-induced SLE occurs following administration of a class of drugs that interact with pyrimidine bases and block DNA methylation [55•,56•]. It is not known whether these drugs would also interfere with RNA methylation resulting in increased RNA immunity as a pathogenesis of the disease. This particular mechanism for the induction of lupus remains to be investigated. Interestingly, an RNA modifying enzyme, fibrillarin, which carries out the immune repressive 2'-*O*-methylation of RNA, is also an autoantigen recognized by sera from patients with autoimmune diseases (eg, SLE, systemic sclerosis, rheumatoid arthritis) [57,58,59•,60]. It is not known whether autoimmune responses against fibrillarin result from the adjuvant activity of the associated RNA or whether its enzymatic activity plays any role in the disease process.

With the identification of nucleic acid-responsive receptors, which account for four of the ten known human TLRs, studies have begun to demonstrate their involvement in the pathogenesis of a number of autoimmune diseases [61•]; for example, SLE is characterized by immune responses to multiple autoantigens including DNA and RNA and proteins associated with both nucleic acids, including Ro and La proteins that bind certain RNAs [62]. It has been demonstrated that increased TLR7 function, resulting from receptor gene duplication, accelerated the development of autoimmunity in a murine lupus model [51•,63•], while the deficiency of TLR7 in the same model decreased the severity of disease [64]. Other autoimmune diseases also have autoantigens that are found bound to RNA, including systemic sclerosis [62]. TLR3-dependent mechanisms have been implicated in autoimmune liver damage and in rheumatoid arthritis [65,66•]. As RNA activates both TLR3 and TLR7 and this activation is prevented by certain nucleoside modifications, it is possible that reduced levels of nucleoside modification in natural RNA might contribute to the development of autoimmune disease in genetically susceptible individuals.

Natural versus unnatural nucleoside analogs

Modifications of nucleosides obtained during the RNA maturation process are irreversible. Thus, when RNA eventually degrades, the modified nucleosides are discarded or catabolized [67•], but they do not enter into the nucleoside triphosphate pool and do not interfere with RNA or DNA synthesis. In contrast, unnatural nucleoside analogs including antiviral drugs (eg, fialuridine, ribavirin, AZT) and anticancer drugs (eg, 5-fluorouridine, AraC, cladribine, cytarabine, gemcitabine), enter into mammalian cells with the aid of equilibrative nucleoside transporter 1 [68,69]. Uniquely for human cells, this ubiquitously present transporter also facilitates the entry of the unnatural nucleoside analogs into the mitochondria, thus interfering with mitochondrial nucleic acid synthesis and resulting in a much more severe toxicity in humans than in rodents [70••].

The discovery that unnatural nucleoside analogs can cause lethal mitochondrial toxicity in humans that cannot be predicted by toxicity studies performed in mice or rats, is particularly important at the present time, as selected RNA molecules with diverse functions (eg, siRNAs, RNA aptamers, antisense RNA, mRNA vaccines) are now entering preclinical and clinical trials [71••,72,73•].

To enhance *in vivo* effectiveness, RNAs are often modified to contain unnatural nucleoside modifications (eg, 2'-fluoro-pyrimidines in aptamers [74] and short interfering (si)RNA [75]) that increase RNA stability; therefore, it will be highly important to test all RNA products containing unnatural nucleoside/ nucleotide modifications on human cells and to specifically measure mitochondrial toxicities in those cells. Patients undergoing treatment should also be monitored for mitochondrial depletion in the long term, as side effects of nucleoside analogs sometimes take a long time to develop [76••].

Conclusion

Since its first discovery over 40 years ago, the investigation of RNA-based immunology has been reinvigorated with the observation that three TLRs interact with RNA. The further finding that nucleoside modification alters RNA-mediated TLR signaling presents a mechanism for the long-observed differences in immunogenicity between bacterial, viral and mammalian RNAs. Further studies analyzing the effects of RNA modification on other RNA sensors and receptors are ongoing. The involvement of RNA modification in the pathogenesis of immune disorders and other diseases, and its implications in the design of therapeutics, are still being theorized, and will likely be important considerations for this field in the near future.

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•• of outstanding interest
• of special interest

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