

Identification of modifications in microbial, native tRNA that suppress immunostimulatory activity

Stefanie Gehrig,¹ Mariel-Esther Eberle,² Flavia Botschen,¹ Katharina Rimbach,² Florian Eberle,² Tatjana Eigenbrod,² Steffen Kaiser,³ Walter M. Holmes,^{4,5,6} Volker A. Erdmann,⁷ Mathias Sprinzl,⁸ Guillaume Bec,⁹ Gérard Keith,⁹ Alexander H. Dalpke,² and Mark Helm^{1,3}

¹Department of Chemistry, Institute of Pharmacy and Molecular Biotechnology; and ²Department of Infectious Diseases–Medical Microbiology and Hygiene; Heidelberg University, 69117 Heidelberg, Germany

³Institute of Pharmacy and Biochemistry, Johannes Gutenberg University Mainz, 55128 Mainz, Germany

⁴Department of Microbiology and Immunology, ⁵Department of Biochemistry and Molecular Biology, and ⁶Institute for Structural Biology and Drug Discovery, Virginia Commonwealth University, Richmond, VA 23284

⁷Institute for Chemistry and Biochemistry, Free University Berlin, 14195 Berlin, Germany

⁸Institute of Biochemistry, University of Bayreuth, 95440 Bayreuth, Germany

⁹Architecture et Réactivité de l'ARN, Institut de Biologie Moléculaire et Cellulaire du Centre National de la Recherche Scientifique, Université de Strasbourg, 67084 Strasbourg, France

Naturally occurring nucleotide modifications within RNA have been proposed to be structural determinants for innate immune recognition. We tested this hypothesis in the context of native nonself-RNAs. Isolated, fully modified native bacterial transfer RNAs (tRNAs) induced significant secretion of IFN- α from human peripheral blood mononuclear cells in a manner dependent on TLR7 and plasmacytoid dendritic cells. As a notable exception, tRNA^{Tyr} from *Escherichia coli* was not immunostimulatory, as were all tested eukaryotic tRNAs. However, the unmodified, 5'-unphosphorylated in vitro transcript of tRNA^{Tyr} induced IFN- α , thus revealing posttranscriptional modifications as a factor suppressing immunostimulation. Using a molecular surgery approach based on catalytic DNA, a panel of tRNA^{Tyr} variants featuring differential modification patterns was examined. Out of seven modifications present in this tRNA, 2'-O-methylated G_m18 was identified as necessary and sufficient to suppress immunostimulation. Transplantation of this modification into the scaffold of yeast tRNA^{Phe} also resulted in blocked immunostimulation. Moreover, an RNA preparation of an *E. coli trmH* mutant that lacks G_m18 2'-O-methyltransferase activity was significantly more stimulatory than the wild-type sample. The experiments identify the single methyl group on the 2'-oxygen of G_m18 as a natural modification in native tRNA that, beyond its primary structural role, has acquired a secondary function as an antagonist of TLR7.

CORRESPONDENCE

Mark Helm:
mhelm@uni-mainz.de

Abbreviations used: hh, hammerhead; IVT, in vitro transcript; MTase, methyltransferase; pDC, plasmacytoid DC; siRNA, small interfering RNA; TLR, Toll-like receptor; tRNA, transfer RNA.

Microbial nucleic acid recognition has been identified as a common theme in innate immunity involving a variety of receptors (Toll-like receptors [TLRs] 3, 7, 8, and 9, RIG-I, Mda5, Nalp3, AIM2, and IFI16; Kumar et al., 2011). Generally, three principles have been elucidated that allow for differentiation of foreign and self-nucleic acids: spatial restrictions, sequence composition, and nucleotide modifications.

RNA is abundantly modified with >100 different known chemical modifications that are

introduced posttranscriptionally (Czerwonec et al., 2009; Grosjean, 2009; Cantara et al., 2011). Because the extent of modifications depends on the RNA species as well as its evolutionary origin, and because eukaryotic RNA in general is more abundantly modified, nucleotide modifications might conceivably allow discrimination of self and nonself (Koski et al., 2004;

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A.H. Dalpke and M. Helm contributed equally to this paper.

Karikó et al., 2005). Indeed, random incorporation of naturally occurring m^5C , m^6A , m^5U , s^2U , or pseudouridine into in vitro transcripts (IVTs) of messenger RNAs abrogated stimulation of TLRs (Koski et al., 2004). Similarly, multiple studies showed that incorporation of 2'-O-Me nucleotides into small interfering RNA (siRNA) suppressed unwanted immunostimulation (Robbins et al., 2007; Sioud et al., 2007; Eberle et al., 2008). As a point in case, 2'-O-Me is a regularly occurring modification in natural RNA that occurs in higher frequencies in eukaryotic RNA than in bacterial or mitochondrial RNA (Karikó and Weissman, 2007; Jühling et al., 2009).

However, the aforementioned concepts have not yet been tested with natural, full-length RNA species of defined sequence. Because access to pure native RNA species is limited, all previous studies, when specifically analyzing single modifications, made use either of short synthetic oligoribonucleotides or of IVTs. Moreover, investigations are biased toward self-RNA, and most interpretations have not been cross-validated with nonself-RNA. A point in case is again given by 2'-O-Me: although a suppressive effect on secretion of IFN- α from PBMCs is documented in RNAs of artificial or eukaryotic sequence, effects in foreign RNAs are not understood. This is particularly relevant because 2'-O-Me occurs in bacteria as well (Persson et al., 1997; Hori et al., 2002; Czerwonec et al., 2009; Cantara et al., 2011). Conceivably, the ensemble of other bacterial

modifications as well as positioning and structural context of such modifications is probably of additional importance.

Therefore, we investigated native bacterial RNA species to analyze nucleotide modifications in their natural context. We used purified transfer RNA (tRNA) species of bacterial origin, and the resulting TLR7-mediated stimulation, as measured by ELISA-based detection of IFN- α secretion from PBMCs, was compared with purified eukaryotic tRNA species. We report the development of a method to produce tRNA hybrids that allows dissecting single modification and analyzing their immunostimulatory potential.

Using such a molecular surgical approach, we unequivocally identify 2'-O-Me G18 (G_{m18}) as a highly efficient suppressive modification in bacterial native tRNA^{Tyr}. This single methyl group is necessary and sufficient for immunosuppression in a classical tRNA macromolecule, as could be shown by transplantation experiments into otherwise highly stimulatory IVTs. Analysis of eukaryotic tRNAs reveals that G_{m18} is not the singular discriminator of self- versus nonself-tRNA. However, increased stimulation by total tRNA preparations from *Escherichia coli* mutants lacking the G_{m18} methyltransferase (MTase) *tmmH* suggests a potential role of this enzyme as a virulence factor.

RESULTS AND DISCUSSION

As opposed to previous approaches identifying nucleotide

modifications with effects on immunostimulation in self-RNA, we set out to identify such moieties in nonself-RNA. Although previous studies have relied on ill-defined preparations of total RNA,

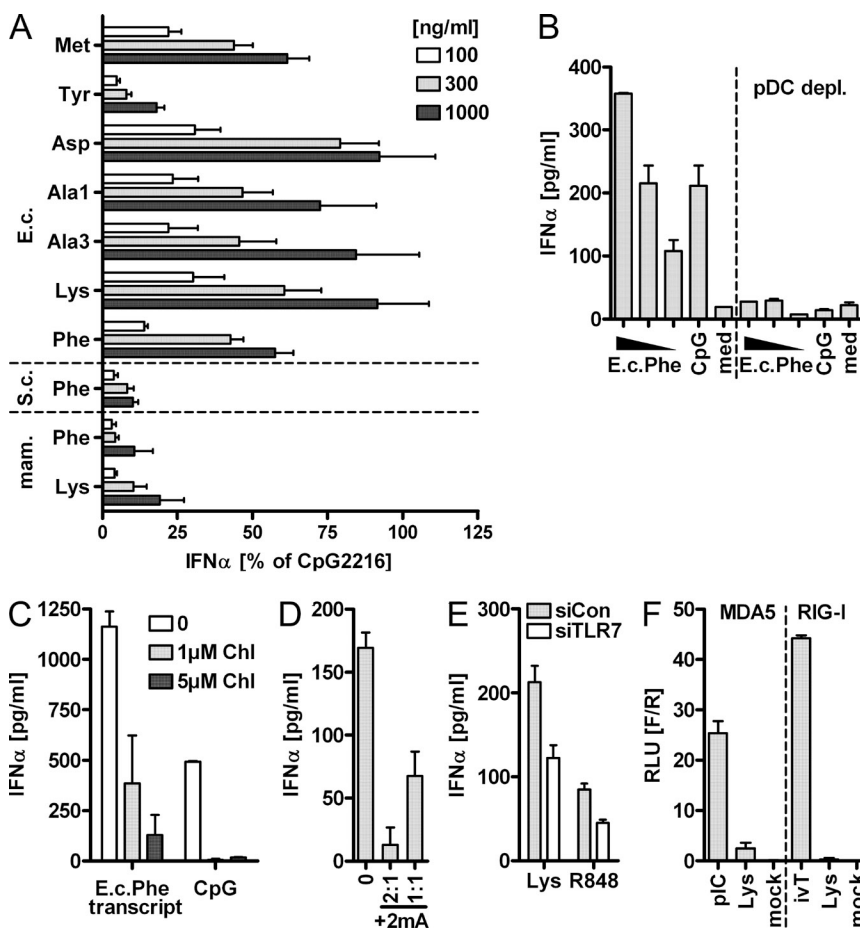


Figure 1. Native *E. coli* tRNA^{Tyr} lacks immunostimulation in PBMCs. (A) PBMCs were stimulated with *E. coli* (E.c.) tRNAs, tRNA^{Phe} from eukaryotic *S. cerevisiae* (S.c.), mammalian (mam.) tRNA^{Phe}, or mammalian tRNA^{Lys}, and analyzed for secretion of IFN- α . Data are normalized to 1 μ M CpG2216 ($n = 3-10$; mean + SEM). (B) PBMCs or PBMCs depleted of pDCs were stimulated with *E. coli* tRNA^{Phe} and analyzed as in A (mean of triplicate values + SD). (C) PBMCs were preincubated with 1 or 5 μ M chloroquine (Chl) and stimulated with 1 μ g/ml IVT of *E. coli* tRNA^{Phe}. (D) PBMCs were stimulated with 300 ng/ml *E. coli* tRNA^{Lys} in the absence or presence of a 1:1 or 1:2 ratio of the TLR7-blocking RNA oligonucleotide 2mA. (E) PBMCs were treated with siRNA against TLR7 and subsequently stimulated with 1 μ g/ml *E. coli* tRNA^{Lys} or 1 μ g/ml R848 and tested for IFN- α secretion (one of two experiments is shown; mean + SD). (F) Huh7.5 cells harboring an IRF3 reporter gene were transfected with either RIG-I or MDA5. Cells were stimulated with 1 μ g/ml *E. coli* tRNA^{Lys}, 0.2 μ g/ml of 400-bp ds IVT RNA or poly (dI:dC) was used as control, and reporter gene activity was measured ($n = 2$; mean + SD). RLU, raw light units.

we based our investigations on the fact that native bacterial tRNAs contain a large number of modifications at precise and well-known positions.

Native *E. coli* tRNA^{Tyr} lacks immunostimulatory activity in PBMCs

We first tested a panel of native modified bacterial tRNAs. Importantly, tRNA accounts for around 10% of the bacterial RNA population and thus is the second most abundant RNA species. Considering that the majority of the ribosomal RNA population is complexed in ribosomal particles and thus likely to be shielded from TLR recognition, tRNA is probably a relevant RNA species for immune recognition. PBMCs were tested for secretion of IFN- α upon stimulation with RNA. To account for donor variance, experimental results were normalized to stimulation with the TLR9 ligand CpG2216. Various tRNA species from *E. coli* in general showed strong immunostimulation (Fig. 1 A). In contrast, tRNA^{Phe} from *Saccharomyces cerevisiae*, a heavily modified eukaryotic tRNA, induced only minor amounts of IFN- α when compared side by side with *E. coli* tRNA^{Phe}. Similarly, mammalian tRNA^{Phe} and tRNA^{Lys} were devoid of immunostimulatory activity. Among the strongly stimulatory tRNA species from *E. coli*, i.e., Met, Ala1, Ala3, Phe, Lys, and Asp (sequences in Jühling et al. [2009]), only moderate differences could be evidenced. In surprising contrast to these bacterial tRNAs, native *E. coli* tRNA^{Tyr} was not stimulatory.

We verified that the observed IFN- α response was caused by stimulation of TLR7 in plasmacytoid DCs (pDCs). Thus, depletion of pDCs from PBMCs abolished tRNA-mediated IFN- α secretion (Fig. 1 B). The observed tRNA activity was abolished either by blocking endosomal maturation with chloroquine or by inhibition of TLR7 with an antagonistic RNA oligonucleotide (2mA; Fig. 1, C and D). Furthermore, partial knockdown of TLR7 in PBMCs by siRNA clearly decreased tRNA-mediated induction of IFN- α in proportion to RNA interference efficiency by a known TLR7 ligand (Fig. 1 E). Contributions by other RNA-sensing entities were largely excluded. No activation of RIG-I but minimal stimulation of MDA5 could be observed in Huh7.5 cells expressing the respective receptors (Fig. 1 F), thus ruling out a major contribution of intracellular MDA5 recognition. Moreover, siRNA knockdown of IPS1, the downstream adaptor of MDA5 and RIG-I, did not reduce tRNA stimulation (not depicted).

Lack of activity of *E. coli* tRNA^{Tyr} is caused by naturally occurring nucleotide modifications

We compared the lack of immunostimulatory activity of *E. coli* tRNA^{Tyr} with the immune response to an unmodified version, obtained by in vitro transcription. Of special importance, IVTs were generated from a precursor with a self-excising hammerhead (hh) leader sequence, resulting in a 5'-OH end, effectively excluding the presence of 5'-triphosphates, whose known stimulatory properties might activate RIG-I (Hornung et al., 2006). Processed tRNA

IVTs were detected and purified by PAGE. The IVTs of tRNA^{Tyr} and tRNA^{Met} were directly compared with the native tRNA preparations (Fig. 2 A). Of note, the IVT of tRNA^{Tyr} strongly induced IFN- α , thus arguing for suppressive activity within the native tRNA. Testing yet further IVTs of various tRNAs (*E. coli* Phe, *E. coli* Ala1, and *S. cerevisiae* Phe) showed that in general all IVTs are stimulatory, and among the tested bacterial tRNAs, only tRNA^{Tyr} showed a significant difference between native and IVT tRNA (Fig. 2 B). Comparison of native and IVT *E. coli* tRNA^{Tyr} clearly indicates that the silencing effect must be caused by one or more of the seven posttranscriptional nucleotide modifications present in the native tRNA, namely s⁴U8, G_m18, Q34, ms²i⁶A37, Ψ 39, T54, and Ψ 55 (Fig. 3 A).

Molecular surgery identifies 2'-O-Me at G18 to suppress IFN- α induction by *E. coli* tRNA^{Tyr}

To deconvolute the contribution of single modifications or groups thereof, we synthesized modivariants, i.e., differentially modified tRNA^{Tyr} species, in a two-step procedure of molecular surgery as depicted in Fig. 3. By judicious choice of the targeting sequences (Hengesbach et al., 2008), the RNA cleavage activity of an 8-17 DNAzyme was directed between nucleotides 24 and 25 to generate defined tRNA

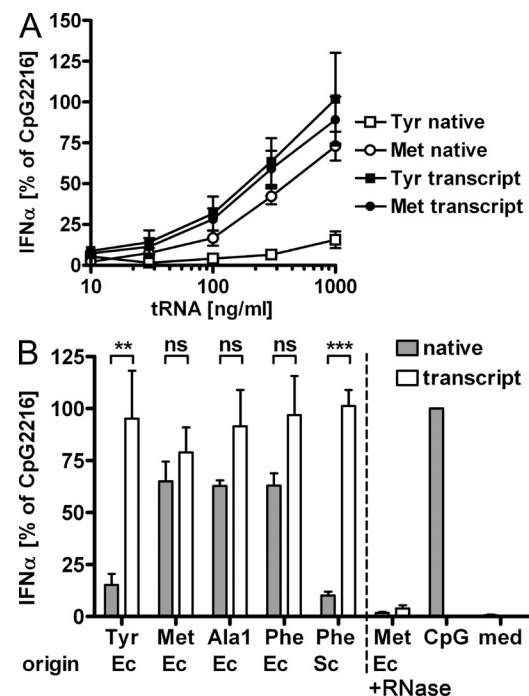


Figure 2. IVT tRNAs are stimulatory. (A) TLR7 response of native *E. coli* tRNAs (open symbols) and IVTs (closed symbols) of tRNA^{Tyr} (squares) and tRNA^{Met} (circles) normalized to CpG2216 ($n = 4-6$; mean + SEM). (B) Comparison at 1 μ g/ml of native tRNAs (closed bars) and the corresponding IVTs (open bars) of *E. coli* (Ec) tRNAs for Tyr, Met, Ala1, and Phe and *S. cerevisiae* (Sc) tRNA^{Phe} ($n = 4-7$; mean + SEM; IVT tRNA^{Phe} Sc, $n = 2$). The control consist of an RNase A digest of native tRNA^{Met} and its IVT. med, medium control. **, $P < 0.01$; ***, $P < 0.001$.

fragments carrying modifications from native tRNA^{Tyr} or their unmodified counterparts from the corresponding IVT (Fig. 3, B and C). Repeated thermocycling was used to maximize cleavage, and the resulting fragments were isolated from reactions on a preparative scale (i.e., 800 pmol). After phosphorylation on their 5' end and removal of cyclic 3'-phosphates by treatment with T4-PNK, reassembly of permuted fragments was achieved by hybridization onto a full-length cDNA. This resulted in the formation of nicked DNA–RNA hybrids, which are ligation-competent complexes for T4-DNA ligase (Fig. 3, D and E; Kurschat et al., 2005). After ligation, the DNA splint was removed by enzymatic digestion, and the full-length modivariant tRNAs mv#1 and mv#2 were purified by PAGE in final quantities of ~2 µg.

Analysis of these modivariants in the IFN- α secretion assay revealed that the modifications suppressing immunostimulation are situated in the 5' half of the tRNA (Fig. 4 A): mv#1, which contains the five modifications Q34, ms²ⁱA37, Ψ 39, T54, and Ψ 55 of the 3' fragment, was nearly as strongly stimulatory as the IVT, whereas mv#2, containing s⁴U8 and G_m18, was nearly as silent as the native tRNA^{Tyr}. Next,

modivariants G_m18 and s⁴U8 were generated from synthetic fragments including only G_m18 or s⁴U8 in the 5' end of the tRNA. We observed that s⁴U8 did not show any effect (Fig. 4 A), G_m18 alone was sufficient to completely ablate any immunostimulatory properties of the 85-nt full-length bacterial tRNA^{Tyr}, thus emerging as the singular effective modification in this tRNA.

Different modification events lead to immunosilencing of eukaryotic tRNA

Because G_m is common in eukaryotic tRNAs as well, the question arises of whether this modification represents a fundamental principle in the recognition of tRNA by TLR7. Native tRNA^{Phe} from yeast, which showed no immunostimulation (Fig. 1), lacks G_m18 (Jühling et al., 2009) but bears other modifications that obviously act in a suppressive manner. Using a similar molecular surgery approach as for tRNA^{Tyr}, we set out to identify these. However, tRNA^{Phe} is more heavily modified than tRNA^{Tyr}, and the accompanying increase in thermostability protected native tRNA from DNase cleavage, whereas the IVT was an efficient substrate

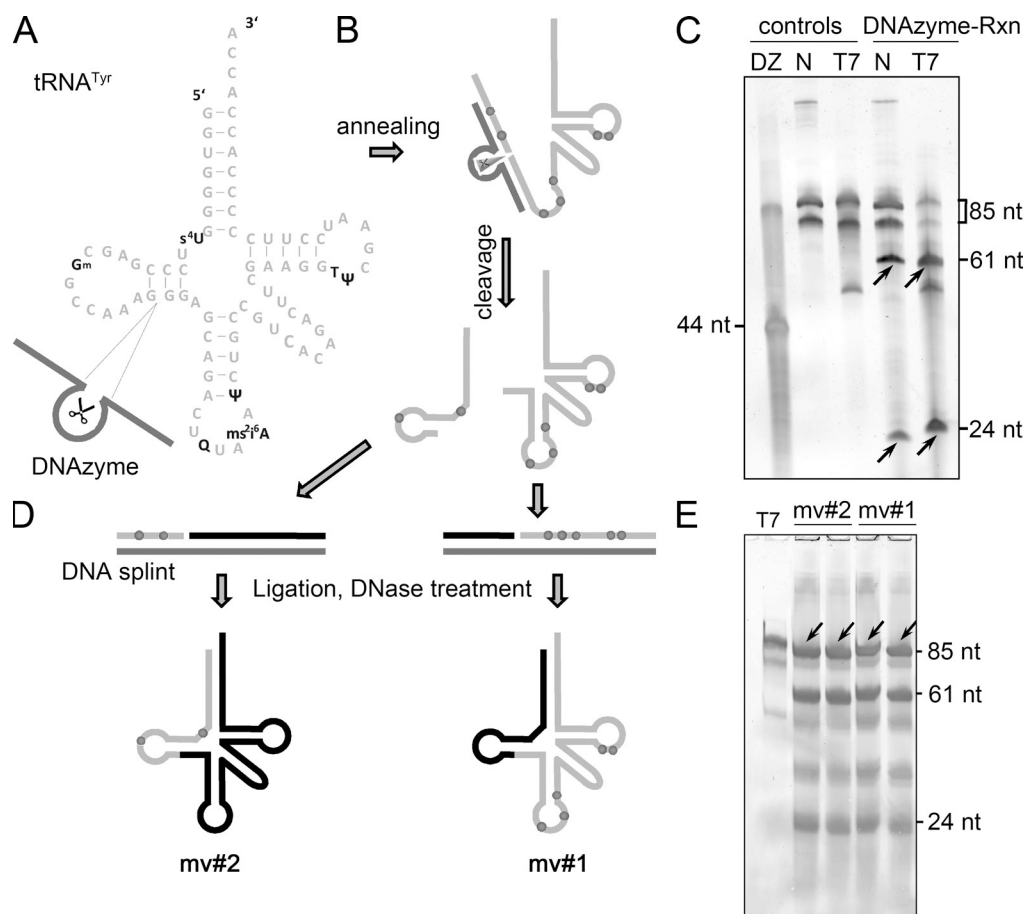


Figure 3. Molecular surgery approach based on catalytic DNA. (A and B) A DNAzyme was engineered to cut between residues 24 and 25 of tRNA^{Tyr} (85 nt), generating fragments of 61 nt and 24 nt. (C) Fragments of cleaved native tRNA^{Tyr} and its IVT were purified by PAGE (indicated by arrows). (D) Isolated unmodified (black lines) and modified fragments (gray lines with dots indicating modifications) were annealed on splint cDNA to yield modivariants mv#1 and mv#2 after ligation and digestion of cDNA. (E) PAGE purification of modivariants (indicated by arrows).

(not depicted). We therefore resorted to fragments resulting from chemical cleavage of the tRNA by hydrochloric acid, which causes a depurination of the wybutosine base 37, followed by acid cleavage (Sprinzl et al., 1976). From the resulting fragments, several modivariants were synthesized in small amounts and tested, which pointed to the anticodon as possible

location of silencing modifications with a potential similar to G_m18 (Fig. S1). According to these observations and the results from tRNA^{Tyr}, we constructed modivariants carrying single 2'-O-Me modifications found in native tRNA^{Phe} (G_m34 and C_m32 ; Fig. 4 B). C_m32 was without effect, but G_m34 efficiently suppressed TLR7 recognition of tRNA^{Phe}. Because certain modivariants carrying modifications in the 3' part of the tRNA were not synthetically accessible, the possibility that further modifications influence TLR7 recognition could not be ruled out. Indeed, fully modified tRNA^{Phe} was yet less stimulatory than its G_m34 modivariant (Fig. 4). Of note, mammalian tRNA^{Lys}₃ does not contain any G_m at all and must thus rely on yet a different modification.

G_m contains a methylation of the ribose 2'-OH, which has been described as a factor lowering TLR-mediated immunostimulation that results in IFN secretion (Karikó et al., 2005; Karikó and Weissman, 2007; Robbins et al., 2007; Sioud et al., 2007; Eberle et al., 2008). However, the previously discovered effect of U_m nucleotides (Robbins et al., 2007) was much less pronounced; indeed, to silence a 21mer (single-stranded) siRNA, all uridines in this RNA were 2'-O-Me. Tluk et al. (2009) have then described a silencing effect of 2'-O-Me of all four major nucleotides and at various positions within short self-RNA or related sequences. The sequences of the latter were derived by insertion of an immunostimulatory 5'-UUGU-3' tetranucleotide sequence. Although such modifications did indeed silence the TLR7-mediated IFN- α response, their provenance was not native, i.e., neither was the position of the natural modification maintained, nor was the investigation extended to a full-length natural RNA. Previously published results included a density of at least one 2'-O-Me residue per ~15–20 nt (Karikó et al., 2005). In contrast, we have here identified a silencing modification that acts on a significantly larger, full-length RNA that is of physiological significance and unrelated to self-RNA.

Molecular transplantation experiments demonstrate that bacterial and eukaryotic tRNA scaffolds are interchangeable

The IVT of tRNA^{Phe} from *S. cerevisiae* was as strongly stimulatory as all other tRNA IVTs (Fig. 1), suggesting that there are no cryptic details inherent in eukaryotic tRNA sequences that might govern TLR7 recognition on a fundamental level. To clearly demonstrate that bacterial and eukaryotic tRNA scaffolds are interchangeable, transplantation experiments were performed in which both identified effective G_m modifications (G_m18 from prokaryotic and G_m34 from eukaryotic tRNA) were assayed in a nonnative tRNA context. Transplantation by molecular surgery of G_m18 into the scaffold of the tRNA^{Phe} IVT efficiently silenced the strong response provoked by the IVT (Phe G_m18 in Fig. 4 B). This shows that the TLR7 response to eukaryotic self-tRNA may be silenced by G_m18 also in a tRNA context in which it does not naturally occur. Similarly, in the analogous transplantation of the eukaryotic modification into a bacterial tRNA context, the G_m34 modification also silenced the tRNA^{Tyr} response, albeit with somewhat reduced efficiency (Tyr G_m34 in Fig. 4 C).

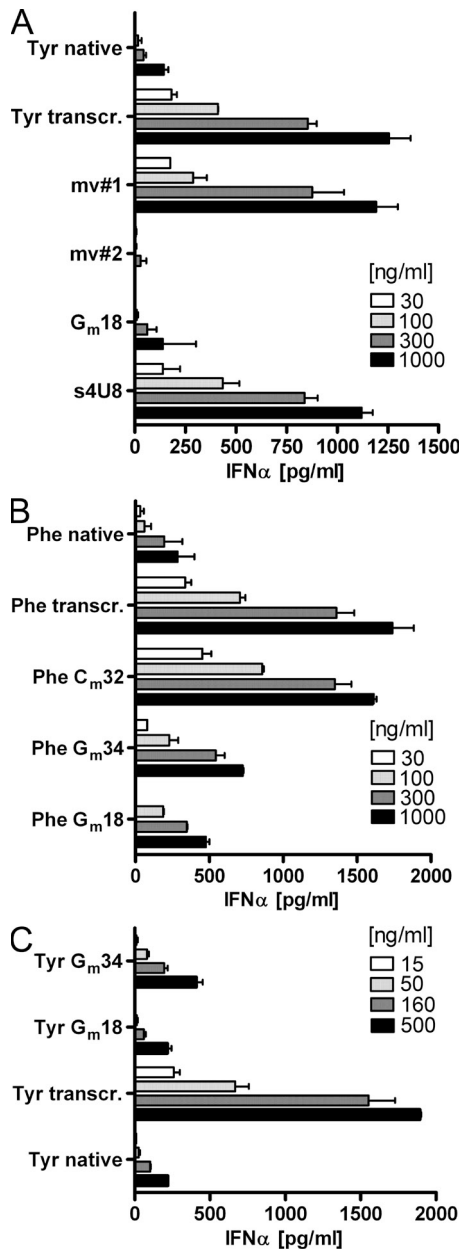


Figure 4. Unique effect of G_m18 in suppression of immunostimulation. (A) PBMC stimulation with native *E. coli* tRNA^{Tyr}, its IVT, and modivariants mv#1 and mv#2, as well as modivariants G_m18 and s⁴U8 ($n = 4$; mean + SD). (B) Stimulation of PBMCs with native *S. cerevisiae* tRNA^{Phe}, its IVT, and variants bearing G_m18 or single, naturally occurring 2'-O-Me residues at different positions (C_m32 and G_m34 ; $n = 2$; mean + SD of triplicates). (C) Stimulation of PBMCs with variants of *E. coli* tRNA^{Tyr} with positionally altered G_m ($n = 3$; mean + SD).

G_m18 reduces immunostimulatory activity of total bacterial tRNA preparations

In *E. coli*, the gene product of *trmH* has been identified to act as 2'-O-MTase, which is crucial for tRNA G_m18 modification (Persson et al., 1997). *trmH* deficiency results in loss of G_m18. As G_m18 occurs in 13 out of 45 tRNAs in *E. coli*, loss of G_m18 might increase the immunostimulatory properties of whole tRNA preparations. Surprisingly, total tRNA from WT *E. coli* was only weakly stimulatory, although the majority of tRNAs did not possess the G_m18 modification. We speculated that G_m18 might not only lack immunostimulation but even suppress otherwise activating tRNA. This notion is strongly supported by the finding that total tRNA from a *trmH* mutant ($\Delta trmH::kan$; Persson et al., 1997) elicited a significantly stronger IFN- α response (Fig. 5 A). This result implies an antagonistic property of G_m18-containing tRNAs, which appear to silence the TLR7-mediated response to the remainder of the total tRNA population. We therefore decided to evaluate the antagonistic properties of G_m18-containing tRNA^{Tyr} in a titration experiment with stimulatory, G18-unmethylated tRNA. Increasing amounts of *E. coli* tRNA^{Tyr} were added to stimulatory *E. coli* tRNA^{Phe} (Fig. 5 B). Indeed, *E. coli* tRNA^{Tyr} decreased IFN- α secretion induced by tRNA^{Phe} in an inhibitory manner.

At this point, we can state that self-tRNA is guarded against TLR7 activation (which was the most important receptor triggered by tRNA in our study setting) not by a singular principle, but by a variety of at least three different posttranscriptional modifications, of which we have characterized G_m18 and G_m34 in detail, and another one is contained in mammalian tRNA^{Lys}₃. Systematic investigation of all of the >40 tRNA species in humans must be postponed to future research. We have already shown (Fig. 4) that C_m32

does not affect recognition of the tRNA^{Phe} scaffold by TLR7, indicating that not all 2'-O-methylations are effective in this matter. Naturally occurring 2'-O-Me residues in tRNAs include positions 4, 44, or 54, which are thus prime candidates for further investigations (Jühling et al., 2009; Cantara et al., 2011).

G_m18 in bacterial tRNA is likely to have evolved from selection pressure unrelated to the mammalian innate immune system. Indeed, the G_m18 modification is found in bacterial, archaeal, and eukaryotic tRNAs alike, where it is part of a class of modifications in the structural core of the tRNA deemed to be responsible for structural and metabolic stabilization (Motorin and Helm, 2010). Lack of a growth phenotype, i.e., normal growth in modification-deficient mutants, as observed for *trmH* mutants in *E. coli* (Persson et al., 1997), is a common feature of such modifications, which are thought to contribute to a network of cooperative structural effects. Although a structural effect still awaits biophysical confirmation (Kumagai et al., 1980; Björk, 1995; Motorin and Helm, 2010), a widespread occurrence supports the notion that an effect of G_m18 on TLR7 recognition is a secondary function acquired late in evolution.

Most interestingly, a potential physiological importance of G_m18 for the immunological recognition of bacterial RNA could be demonstrated by using an *E. coli* mutant that lacked the 2'-O-MTase *trmH*. Whole tRNA preparations from mutant bacteria showed increased immunostimulation as compared with WT bacteria. This result verifies that G_m18 in a physiological setting is important to shape bacterial immunogenicity for innate immune cells. Our results show that among natural tRNA modifications, G_m18 plays an outstanding role in modulating the tRNA's immunostimulatory properties. The fact that *trmH* mutants are more immunostimulatory although only a minority of tRNAs (Persson et al., 1997) bear a G_m18 modification (Fig. 5 A) and decreased immunostimulation of tRNA upon addition of G_m18-modified species (Fig. 5 B) indicates a dominant inhibitory effect, which de facto categorizes tRNA^{Tyr} as a TLR7 antagonist.

Assuming a secondary role of bacterial G_m18 in avoiding a mammalian immune response, a correlation between the G_m content in bacterial tRNA from various species and the adaptation of the respective prokaryote to mammals might reveal evolutionary immunological pressure that selected for this modification. Certainly, differences in G_m18 frequencies between different bacteria can be observed, e.g., *Haemophilus influenzae* is lacking *trmH* (Fleischmann et al., 1995), but extensive systematic analysis of G_m content in bacterial tRNA is needed to address this point. It remains unknown whether bacteria can regulate their G_m18 modification pattern to cope with immune recognition by a mammalian host. Interestingly, many of the stimulatory *E. coli* tRNAs present in total tRNA, including, e.g., tRNA^{Phe} and tRNA^{Lys}, possess the relevant target residue G18 but are no substrates for TrmH and remain unmethylated. This is not untypical for tRNA MTases, which normally require a limited set of structural and sequence elements for substrate recognition (Motorin and Helm, 2011). Although the precise substrate requirements for TrmH are yet

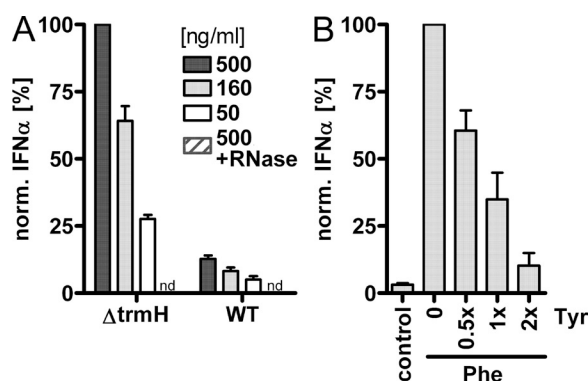


Figure 5. Increased immunostimulation by tRNA lacking G_m18.

(A) PBMCs were stimulated with whole tRNA preparations from either WT *E. coli* or a deletion mutant of the G_m18 MTase *trmH* ($\Delta trmH$). RNA preparations (500 ng/ml) were also digested with RNase A. To account for donor variance, the IFN- α response at 500 ng/ml RNA from $\Delta trmH$ was used for normalization. nd, not detectable ($n = 4-6$; mean $\pm$ SEM; RNase A digests, $n = 2$). (B) PBMCs were stimulated with a fixed concentration (830 ng/ml) of tRNA^{Phe}, and tRNA^{Tyr} was added in a ratio as indicated ($n = 3$ donors with each donor stimulated by tRNA^{Phe} alone set to 100% to account for donor variance; mean $\pm$ SEM).

unknown, they are obviously present in tRNA^{Tyr} but not in tRNA^{Phe} and tRNA^{Lys}. In turn, this points to the substrate specificity of TrmH as a potentially important contribution to the overall immunostimulation of total tRNA from *E. coli* in particular and from any given potential pathogen in general. Future research must elucidate whether broader substrate specificity and a correspondingly higher fraction of G_m18-containing tRNAs leads to a further decrease in TLR7 stimulation and, possibly, increased virulence of the respective bacterium. Also, future tests must include less canonical tRNA structures such as initiator tRNA, human mitochondrial tRNAs, or mutated tRNAs with deliberate mutations of tertiary structure to evaluate whether our findings apply to substrates without the classical tRNA structure context.

MATERIALS AND METHODS

tRNA preparation

Native tRNA^{Tyr}, tRNA^{Phe}, tRNA^{Ala}, tRNA^{Ala}₂, tRNA^{Met}, tRNA^{Asp}, and tRNA^{Lys}₁ from *E. coli* had been purified as described previously (Holmes et al., 1975) and were repurified by PAGE after long-term storage. *S. cerevisiae* tRNA^{Phe} and certain batches of tRNA^{Tyr}₁ were obtained from Sigma-Aldrich. Mammalian tRNA^{Phe} and tRNA^{Lys}₃ were isolated as described previously (Keith and Dirheimer, 1978; Bénas et al., 2000). Fragments of yeast tRNA^{Phe} originated from chemical cleavage via depurination of the wybutosine nucleotide at position 37, followed by strand scission under acidic conditions. These fragments had been isolated earlier (Sprinzl et al., 1976).

Total tRNA lacking G_m18 was prepared from strain CF4409, an *E. coli* K-12 derivative in which *spoU/tmH* 2'-O-MTase (Persson et al., 1997) had been deleted ($\Delta tmH::kan$). The parental strain CF897 was used as a control (Xiao et al., 1991). All strains were provided by M. Cashel (National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD). Bacteria were grown at 37°C for 6 h in Luria-Bertani medium supplemented with 50 µg/ml kanamycin. Bacteria were pelleted, frozen at -80°C overnight, and then lysed in 40 mg/ml lysozyme for 20 min. Total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. RNA was separated by denaturing PAGE (10% acrylamide and 50% urea). The bands containing the tRNA were cut and shaken in 0.3 M sodium acetate overnight. tRNA was precipitated overnight and resuspended in RNase-free water. The concentration was controlled via UV absorbance.

tRNA synthesis by transcription

Unmodified full-length tRNAs were obtained by in vitro transcription with T7 RNA polymerase as described previously (Fechter et al., 1998) using a PCR-amplified template coding for the T7 promoter, an hh ribozyme, and the tRNA sequence. Synthetic oligonucleotides (IBA) used for transcription of *E. coli* tRNAs were as follows: forward primer (for all tRNAs), 5'-CGC-GCGAAGCTTAATACGACTCACTATA-3'; template Tyr, 5'-TGG-TGGTGGGGGAAGGATTTCGAACCTTCGAAGTCTGTGACGGCA-GATTACAGTCTGCTCCCTTTGGCCGCTCGGGAACCCACC-GACGGTACCGGGTACCCTTCGTCCTCACGGACTCATCAGGG-TGGGGTTTCTCCCTATAGTGAGTCGTATT-3'; reverse primer Tyr, 5'-TGGTGGTGGGGG-3'; template Ala1, 5'-TGGTGGAGCTATGCGGGATCGAACCGCAGACCTCCTGCGTGCAAAGCAGGCGCTCTCCCAGCTGAGCTATAGCCCCGACGGTACCGGGTACCGTTTCGTCCTCACGGACTCATCAGGGGGCTATATCTCCCTATAGTGAGTCGTATT-3'; reverse primer Ala1, 5'-TGGTGGAGCTATGCG-3'; template Phe, 5'-TGGTGGCCGGACTCGGAATCGAACCAAGGACACGGGG-ATTTTCAATCCCCTGCTCTACCGACTGAGCTATCCGGGCGAC-GGTACCGGGTACCGTTTCGTCCTCACGGACTCATCAGGCCCGG-ATATCTCCCTATAGTGAGTCGTATT-3'; reverse primer Phe, 5'-TGG-TGCCCGGAC-3'; template Met, 5'-TGGTGGCTACGACGGGATT-

CGAACCTGTGACCCCATCATTATGAGTGATGTGCTCTAACCA-ACTGAGCTACGTAGCCGACGGTACCGGGTACCGTTTCGTCCT-CACGGACTCATCAGGGCTACGTATCTCCCTATAGTGAGTCGT-ATT-3'; and reverse primer Met, 5'-TGGTGGCTACGAC-3'.

Upon transcription, tRNA precursor IVTs are generated, which self-process into full-length tRNAs by autocatalytic cleavage of the hh leader sequence creating a hydroxyl group at the 5' end of the tRNA. RNAs were purified by denaturing PAGE, band excision, and elution of the processed tRNA. *S. cerevisiae* tRNA^{Phe} was transcribed from a linearized plasmid containing the tRNA^{Phe} gene (p67YF0; Sampson and Uhlenbeck, 1988), and 5 µg tRNA was 5'-dephosphorylated with 0.6 U/µl shrimp alkaline phosphatase in shrimp alkaline phosphatase buffer (10 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, and 0.1 mg/ml BSA) in a final volume of 50 µl for 1 h at 37°C and purified by phenol-ether extraction.

Synthesis of modivariants

DNAzyme catalyzed fragmentation of *E. coli* tRNA^{Tyr}. DNAzyme cycling reactions of 250-µl final volume contained 150 mM KCl, 10 mM MgCl₂, 50 mM Tris-HCl, pH 7.4, 20 µg of either native or in vitro IVT *E. coli* tRNA^{Tyr}, and a 2× molar excess of DNAzyme (5'-AGTCTGCTCTGT-CAGCGACACGAATTTGGCCGCTCGGGAACCC-3'). The mixture was subjected to 20 repetitions of temperature cycling: 30 s at 85°C, a temperature decrease by 0.5°C/s to 37°C, and 3 min at 37°C. DNAzyme was digested by directly adding DNase I (4–6 U/µg DNA substrate; Fermentas) to the reaction mixture and incubating for 1 h at 37°C.

Phosphorylation and dephosphorylation. For 5'-phosphorylation and 3'-dephosphorylation, 200 pmol of each fragment was incubated in Buffer A (50 mM Tris-HCl, pH 7.6, 10 mM MgCl₂, 5 mM DTT, 0.1 mM spermidine, and 0.1 mM EDTA) supplemented with 2 mM ATP and 0.8 U/µl T4-PNK (Fermentas) in a final volume of 17–24 µl for 1 h at 37°C. The reaction mixture was directly used for subsequent steps.

Splinted ligation. Ligation of prephosphorylated fragments was performed in a final volume of 50 µl, at final concentrations of 50 mM Tris-HCl, pH 7.4, 10 mM MgCl₂, 4 µM of each oligonucleotide, and 0.9 equivalents of DNA splint (5'-TGGTGGTGGGGGAAGGATTTCGAACCTTCGAAGTCTGTGACGGCAGATTTACAGTTTACAGTCTGCTCCCTTTGGCCGCTCGGGAACCCACC-3'). After heating for 4 min to 75°C and slow subsequent cooling to room temperature for 10 min, T4-DNA ligase (Fermentas) was added to 4 U/µl, and the mixture was incubated overnight at 16°C. The DNA splint was digested, and the ligation product was PAGE purified as described in tRNA synthesis by transcription. For the construction of modivariants Tyr G_m18, G_m34, and s⁴U8, synthetic oligonucleotides corresponding to nucleotides 1–24, 25–53, and 54–86 of the natural tRNA sequence and carrying appropriate 2'-O-Me or s⁴U modifications were used.

Synthesis of *S. cerevisiae* tRNA^{Phe} modivariants. Complementing fragments to the chemically derived native ones were generated via in vitro transcription from T7 hh templates as described in tRNA synthesis by transcription using the same forward primer, template 1, 5'-TGGTGGCA-ATTCTGTGGATCGAACACAGGACCTCCAGATCGACGGTACCGG-GTACCGTTTCGTCCTCACGGACTCATCAGGATCTGGACTCTCC-CTATAGTGAGTCGTATT-3'; reverse primer, 5'-TGGTGGCAATTCT-GTGGATCGAAC-3'; template 2, 5'-CTTCAGTCTGGCGCTCTCC-CACTGAGCTAAATCCGCGACGGTACCGGGTACCGTTTCGTCCTCACGGACTCATCAGGCGGATTATCTCCCTATAGTGAGTCGT-ATT-3'; and reverse primer, 5'-CTTCAGTCTGGCGCTCT-3'. Modivariants Phe G_m18, C_m32, G_m34, and C_mG_m were constructed of synthetic oligonucleotides corresponding to nucleotides 1–36 of the natural tRNA sequence, and carrying appropriate 2'-O-Me modification, nucleotides 37–52 and 53–76. Native fragments were 3'-dephosphorylated and 5'-phosphorylated as described in Phosphorylation and dephosphorylation. Ligation was performed as described in Splinted ligation, but at 37°C and using the DNA splint 5'-TGGTG-CGAATTCTGTGGATCGAACACAGGACCTCCAGATCTTCAGTCT-GGCGCTCTCCCACTGAGCTAAATCCGCATTTTGGGTACC-3'.

Stimulation of PBMCs with tRNAs

Human PBMCs were isolated from heparinized blood of healthy donors by standard Ficoll-Hypaque density-gradient centrifugation (Bicoll 1.078 g/ml; Eberle et al., 2008). PBMCs were filtered through a 100- μ m cell strainer and resuspended in complete medium prepared of RPMI 1640 supplemented with heat-inactivated (1 h, 56°C) 2% autologous serum. Cells were plated at 4×10^5 cells/well in a 96-well flat-bottom plate. 1 μ g tRNA sample was diluted in a volume of 5 μ l. The RNA was encapsulated with 2.5 μ l of 1 mg/ml DOTAP (N-[1-(2, 3-dioleoyloxy)propyl]-N, N, N-trimethylammonium methylsulfate) by mixing with serum-free medium and incubation for 10 min. PBMCs were stimulated in a humidified 5% CO₂ atmosphere at 37°C for 16 h. As internal positive control, PBMCs were stimulated with 1 μ M of the TLR9-specific stimulus CpG2216. In inhibition experiments, two different tRNAs were mixed as indicated and added together to the cells. Cell-free supernatant was analyzed for secretion of IFN- α using a sandwich ELISA (Bender MedSystems).

Where indicated, cells were pretreated with 1 or 5 μ M chloroquine (Sigma-Aldrich) for 30 min or with RNA oligonucleotide 2mA (5'-GMAM-ACUUCMAGGGUCMAGCUUGCCG-3') to block endosomal TLRs or TLR7, respectively (Eberle et al., 2009). PBMCs were also tested when depleted of BDCA-4-positive pDCs through magnetic cell separation (MACS; Miltenyi Biotec) according to the manufacturer's protocol. Induction of TNF by LPS stimulation was not affected through pDC depletion or any of the inhibitors. Analysis of stimulation of RIG-I or MDA5 was essentially performed as described previously (Eberle et al., 2009) by overexpressing these receptors in otherwise inactive Huh7.5 cells harboring an IRF3 reporter.

Knockdown of TLR7 in PBMCs to verify engagement of TLR7 by tRNAs was performed as follows: in brief, 2.5 pmol siRNA was incubated with Lipofectamine RNAiMax in OptiMem in 96-well plates. PBMCs were plated after 20 min of incubation (10^5 cells/well) and placed at 37°C, 5% CO₂. After 24 h, cells were stimulated with the respective tRNA or control.

Statistical analysis

Data were analyzed by the Prism 5 program (GraphPad Software). Significant differences were assessed by the Student's *t* test. In all figures, the *p*-value is indicated by *, *P* < 0.05; **, *P* < 0.01; and ***, *P* < 0.001.

Online supplemental material

Fig. S1 shows the preparation of modivariants mv#3 and mv#4 of *S. cerevisiae* tRNA^{Phe}, similar to the scheme in Fig. 3 except that acid hydrolysis was used instead of a DNAzyme to site-specifically cleave native tRNA, and also shows stimulation data of these modivariants mv#3 and mv#4, as well as of one more construct tRNA^{Phe}G_mC_m, which point to the identification of 2'-O-Me modifications in the anticodon loop of tRNA^{Phe} as being important for silencing of the IFN- α response to this tRNA. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20111044/DC1>.

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