The 2′-*O*-methylation status of a single guanosine controls transfer RNA–mediated Toll-like receptor 7 activation or inhibition

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Foreign RNA serves as pathogen-associated molecular pattern (PAMP) and is a potent immune stimulator for innate immune receptors. However, the role of single bacterial RNA species in immune activation has not been characterized in detail. We analyzed the immunostimulatory potential of transfer RNA (tRNA) from different bacteria. Interestingly, bacterial tRNA induced type I interferon (IFN) and inflammatory cytokines in mouse dendritic cells (DCs) and human peripheral blood mononuclear cells (PBMCs). Cytokine production was TLR7 dependent because TLR7-deficient mouse DCs did not respond and TLR7 inhibitory oligonucleotides inhibited tRNA-mediated activation. However, not all bacterial tRNA induced IFN-α because tRNA from *Escherichia coli Nissle 1917* and *Thermus thermophilus* were non-immunostimulatory. Of note, tRNA from an *E. coli* knockout strain for tRNA (Gm18)-2′-*O*-methyltransferase (trmH) regained immunostimulatory potential. Additionally, in vitro methylation of this immunostimulatory Gm18-negative tRNA with recombinant trmH from *T. thermophilus* abolished its IFN-α inducing potential. More importantly, Gm18-modified tRNA acted as TLR7 antagonist and blocked IFN-α induction of influenza A virus–infected PBMCs.

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Abbreviations used: IAV, influenza A virus; SAM, S-Adenosyl methionine; siRNA, short interfering RNA; TLR, Toll-like receptor; tRNA, transfer RNA.

Bacterial and viral RNA are potent stimulators of the innate immune system, leading to immune cell activation and type I IFN production (Takeuchi and Akira, 2010). Generally, RNA recognition takes place in the endosome or cytoplasm and is mediated by Toll-like receptors (TLRs) and retinoic acid inducible gene I (RIG-I)–like helicases, respectively. In more detail, TLR3 recognizes double-stranded viral RNA and mRNA, whereas TLR7 and TLR8 sense viral or bacterial single-stranded RNA and short interfering RNA (siRNA; Blasius and Beutler, 2010). In contrast, cytoplasmic detection of viral and bacterial RNA is mediated by the RNA helicases RIG-I and MDA5 (melanoma differentiation–associated gene 5; Kato et al., 2006; Monroe et al., 2009).

Of note, RNA modifications in ribosomal RNA and transfer RNA (tRNA) such as 2′-*O*-methylation, base methylation (e.g., m5C, m6A, and m5U), and the occurrence of pseudouridine negatively modify the immunostimulatory potential of synthetic RNA (Karikó et al., 2005). Because these modifications are found at higher frequency in eukaryotic versus prokaryotic/viral RNA, this difference could facilitate the discrimination of foreign RNA from self-RNA. Conversely, nucleotide modifications could be exploited by pathogens for immune evasion. Accordingly, 2′-*O*-methylation of viral mRNA cap structure by virus encoded methyltransferases subvert type I IFN production (Daffis et al., 2010; Züst et al., 2011).

However, the role of RNA methylation in natural occurring RNA such as bacterial tRNA has not been analyzed. tRNAs contain ∼80

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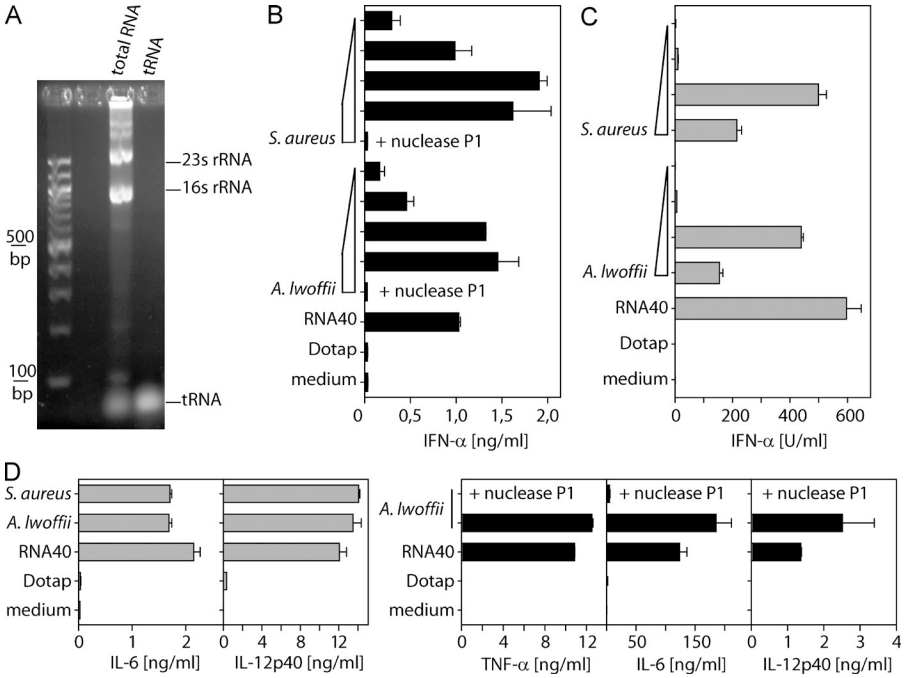

Figure 1. Bacterial tRNA induces cytokine production in human and mouse immune cells. (A) Bacterial total RNA and purified tRNA were analyzed on a 1% agarose gel and detected with ethidium bromide. One representative gel is shown ($n > 10$). (B and C) Human PBMCs (black bars; B) and WT mouse FLT3L induced DCs (gray bars; C) were stimulated with various concentrations of purified tRNA from *S. aureus* and *A. lwoffii* (10, 2, 0.4, and 0.1 µg/ml) or with 10 µg/ml tRNA digested with P1 nuclease (B). For each stimulation, tRNA was complexed to DOTAP and incubated with immune cells for 20 h with subsequent IFN-α detection by ELISA. (D) TLR2/4 double-deficient DCs (gray bars) or enriched human monocytes (black bars) were stimulated with tRNA at 2 µg/ml (±nuclease P1 treatment) and IL-6, IL12p40, and TNF production was analyzed. For B–D, one representative experiment of at least two independent experiments consisting of two PBMCs donors or two individual mice is shown ($n = 2 \pm$ SD).

nucleotides with ~10% of modified nucleotides (Björk et al., 1987) that could influence its immunostimulatory potential. In *Escherichia coli*, methylation is mediated by sequence- and position-specific methyltransferases. For example, uracil-5-methyltransferase (trmA) and guanine-7-methyltransferase (yggH/trmB) methylate specific bases such as uridine 54 (m5U54) or guanosine 46 (m7G46), respectively (Björk, 1975; De Bie et al., 2003). In contrast, the Gm18-2′-*O*-methyltransferase (spoU/trmH) methylates the 2′-*O*-position of guanosine 18 (Gm18; Persson et al., 1997). TrmH genes have been identified in *E. coli*, *Thermus thermophilus*, and *Aquifex aeolicus* (Hori et al., 2003), and a homologous gene called trm3 exists in eukaryotes. However, the function of 2′-*O*-methylation at G18 is not well understood. In general, G18 is a highly conserved residue in the D loop of tRNA and interacts with a conserved pseudouridine at position 55 (Ψ55) in the T loop to stabilize the L-shaped three dimensional structure of tRNA. Gm18 confers additional conformational rigidity on the local RNA structure. However, *E. coli* mutants lacking trmH demonstrate no obvious defects, and only in mutants lacking Gm18 and Ψ55 simultaneously is translational accuracy reduced (Urbonavicius et al., 2002).

Here, we analyzed the immunostimulatory potential of tRNA from different bacteria and identified TLR7 as specific innate immune receptor for tRNA. Interestingly, tRNA from two gram$^-$ species, *E. coli Nissle 1917* and *T. thermophilus*, was not immunostimulatory. This was the result of a specific 2′-*O*-methylation of G18 by the Gm18-2′-*O*-methyltransferase trmH. Therefore, the 2′-*O*-methylation status of G18 determines tRNA function as TLR7 activator or as antagonist. Furthermore, Gm18-methylated tRNA inhibits influenza A virus (IAV)–mediated IFN production, supporting the view that 2′-*O*-methylated tRNA has immunological function and could influence antiviral immune responses during bacterial co-infection.

RESULTS AND DISCUSSION

Bacterial tRNA induces cytokine production in human and mouse immune cells via TLR7

We isolated tRNA from total bacterial RNA of the gram$^-$ rod *Acinetobacter lwoffii* and the gram$^+$ bacterium *Staphylococcus aureus* by anionic exchange chromatography (Fig.1 A and not depicted) and investigated the immunostimulatory potential regarding IFN-α production. For stimulation experiments, tRNA was complexed to the cationic lipid DOTAP because it protects RNA from RNases and facilitates RNA uptake. Human PBMCs or WT mouse FLT3L-induced DCs responded to tRNA stimulation with type I IFN production in a concentration-dependent fashion (Fig. 1, B and C). Other cytokines, such as IL-6, IL-12p40, and TNF were also induced in TLR2/4 double-deficient DCs and enriched human monocytes (>70%; Fig. 1 D). This activation was RNA dependent because treatment with nuclease P1 abolished immune stimulation (Fig. 1, B and D). We further addressed the involvement of endosomal TLRs in the recognition of tRNA by analyzing the response of TLR-deficient DCs. Of note, the IFN induction by tRNA was TLR7 dependent because TLR7-deficient FLT3L-DCs did not respond to transfected tRNA (Fig. 2 A). In addition, the TLR7 inhibitory oligonucleotide IRS661 (Barrat et al., 2005) abrogated the tRNA-mediated IFN-α production, underscoring the involvement of TLR7 in tRNA recognition in mouse FLT3L-DCs and human PBMCs (Fig. 2 B). Single-stranded RNA and short double-stranded siRNA have been identified as ligands for TLR7 (Diebold et al., 2004; Heil et al., 2004; Hornung et al., 2005) and these structural elements are

also found in tRNA. However, the TLR7-mediated activation is somewhat unexpected because nucleoside modifications found in tRNA have been implicated in abolishing RNA immune recognition via TLR7 (Karikó et al., 2005). Our results suggest that only certain nucleoside modifications act as potent modulators of TLR7 activity.

tRNA from different bacterial species vary in their IFN-α inducing potential

To identify possible differences in immunostimulatory potential of tRNA, we extended the analysis to tRNA from additional nonpathogenic, pathogenic, and probiotic strains belonging to the group of gram$^+$ bacteria (*Lactococcus lactis* and *Bacillus subtilis*), gram$^-$ bacteria (*E. coli Nissle 1917*, *Haemophilus influenzae*, *Pseudomonas aeruginosa*, and *T. thermophilus*), or the domain archaea (*Methanothermobacter marburgensis*; Fig. 3, A and B). Interestingly, the comparison of the immunostimulatory potential of purified tRNA from nine different bacterial strains revealed that tRNA from six strains induced IFN-α in human PBMCs to similar levels as the standard TLR7 ligand RNA40. *H. influenzae*–derived tRNA showed a slightly reduced IFN-α inducing activity when compared with the other strains tested. In contrast, tRNA from *E. coli Nissle 1917* and *T. thermophilus* induced no IFN-α (Fig. 3 B).

Because methylated nucleosides within RNA are known to modulate TLR7 activation (Karikó et al., 2005; Robbins et al., 2007; Hamm et al., 2010), we hypothesized that a specific nucleoside modification in the tRNA of *E. coli* and *T. thermophilus* could account for their loss of immunostimulatory potential.

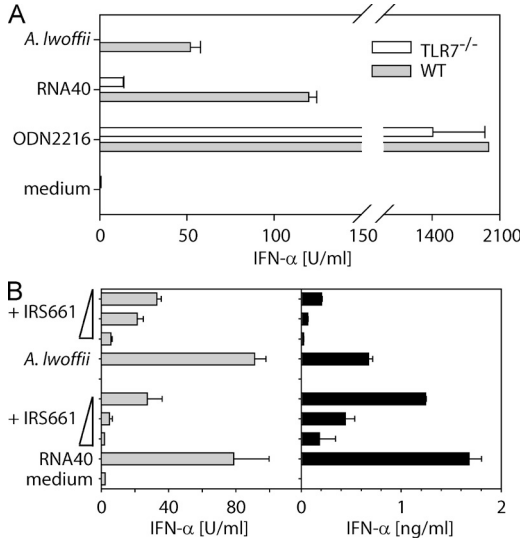

Figure 2. TLR7 mediates tRNA-induced immunostimulation. (A) WT and TLR7-deficient FLT3L-induced DCs were stimulated with 1 µg/ml RNA40 and 2 µg/ml tRNA from *A. lwoffii* complexed to DOTAP. The TLR9 ligand CpG-ODN2216 (1 µM) served as positive control. (B) Mouse FLT3L-induced DCs (gray bars) or human PBMCs (black bars) were incubated with 1 µg/ml RNA40 or 2 µg/ml tRNA from *A. lwoffii* and increasing amounts of the TLR7 inhibitory oligodeoxynucleotide IRS661 (1, 0.2, and 0.04 µM). Cells were incubated for 20 h with subsequent IFN-α detection by ELISA. For all panels, one representative experiment of at least two independent experiments is shown ($n = 2 \pm$ SD).

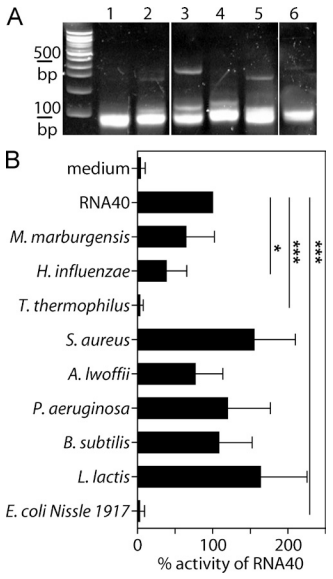

Figure 3. tRNA from different bacterial species varies in their IFN-α–inducing potential. (A) Purified tRNA from different bacterial species were visualized on a 12% polyacrylamide gel (lane 1: *B. subtilis*; lane 2: *P. aeruginosa*; lane 3: *T. thermophilus*; lane 4: *E. coli Nissle 1917*; lane 5: *L. lactis*; lane 6: *M. marburgensis*). One representative gel is shown ($n > 3$). (B) Human PBMCs were stimulated with 2 µg/ml of purified tRNA from different bacterial species as indicated and IFN-α production is depicted as percentage of activity of the TLR7 agonist RNA40. Experimental data were obtained from PBMCs preparations of 3–10 different donors per strain. Statistical analysis was performed using the paired Student's *t* test (***, $P < 0.0001$; *, $P = 0.02$).

2′-*O*-methylation at tRNA position G18 is responsible for the non-immunostimulatory character of *E. coli* tRNA via TLR7

Accordingly, we analyzed the IFN-α–inducing potential of tRNA from *E. coli* mutants devoid in tRNA-specific methyltransferases such as trmA (uracil-5-methyltransferase, m5U54), trmB (guanine-7-methyltransferase, m7G46), and trmH (Gm18-2′-*O*-methyltransferase). Interestingly, tRNA from trmH-deficient *E. coli* (*E. coli* ΔtrmH) induced IFN-α, whereas tRNA from trmA- and trmB-deficient mutants (*E. coli* ΔtrmA and *E. coli* ΔtrmB) and the parental strain did not (Fig. 4 A). Of note, the immunostimulatory potential of tRNA from *E. coli* ΔtrmH correlated with the loss of 2′-*O*-methylated guanosine (Gm) as determined by HPLC analysis of nucleosides derived from P1 nuclease-digested/phosphatase-treated tRNA (Fig. 4 B). This observation suggests a prominent role for Gm18 in the loss of immunostimulatory potential of *E. coli* tRNA. To further characterize the role of Gm18 in immune stimulation of tRNA, we generated in vitro Gm18 methylated tRNA using recombinant *T. thermophilus* trmH (Ochi et al., 2010) and tRNA purified from *E. coli* ΔtrmH. The trmH enzyme was expressed as GST fusion protein, affinity purified, and

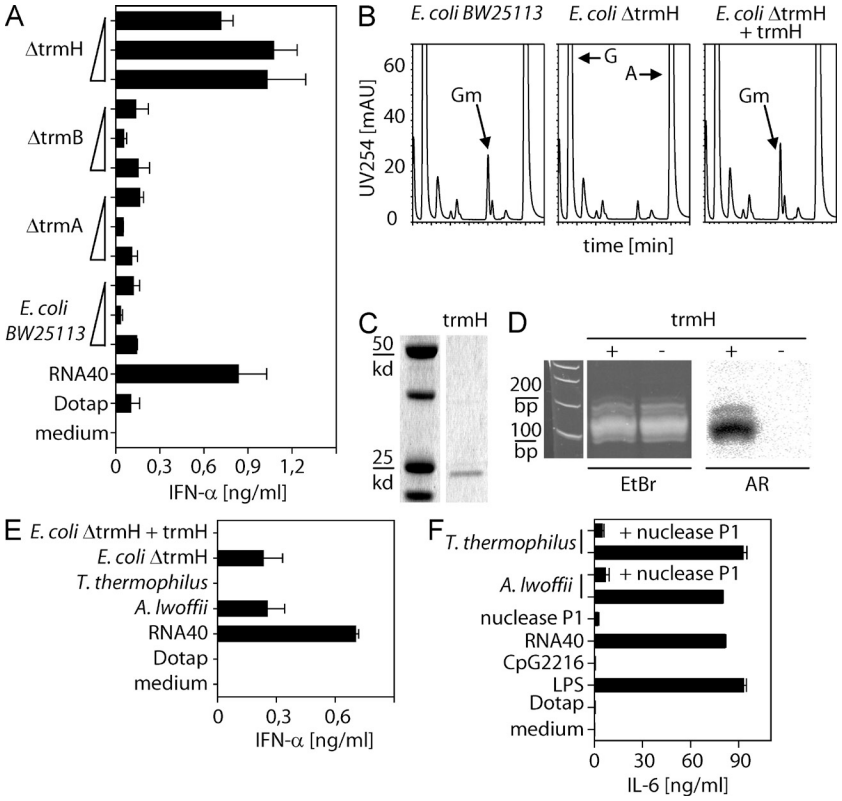

Figure 4. 2′-*O*-methylation at tRNA position G18 is responsible for non-immunostimulatory character of *E. coli Nissle 1917*. (A) Human PBMCs were stimulated with 2, 0.4 and 0.1 µg/ml of purified tRNA from *E. coli BW25113*– and *E. coli BW25113*–derived knockout mutants for trmA (uracil-5-methyltransferase, m5U54), trmB (guanine-7-methyltransferase, m7G46), and trmH (Gm18-2′-*O*-methyltransferase). PBMCs were incubated with RNA for 20 h with subsequent IFN-α detection by ELISA. Representative experimental data from one out of four PBMC preparations are shown ($n = 2 \pm$ SD). (B) HPLC analysis of P1 nuclease and phosphatase-treated tRNA from *E. coli BW25113*, *E. coli* ΔtrmH, and *E. coli* ΔtrmH methylated in vitro with *T. thermophilus* trmH. (C) SDS-PAGE of purified recombinant *T. thermophilus* trmH protein expressed in *E. coli BL21*. (D) Purified tRNA from *E. coli* ΔtrmH was incubated with ^{14}C-labeled SAM and with or without *T. thermophilus* trmH. tRNA was further visualized after PAGE by ethydium bromide (EtBr) staining and autoradiography (AR). For B–D, one representative experiment ($n \geq 2$) is shown. (E) Human PBMCs were stimulated with purified tRNA (2 µg/ml) from *A. lwoffii*, *T. thermophilus*, *E. coli* ΔtrmH, and *E. coli* ΔtrmH methylated in vitro with recombinant trmH and *T. thermophilus*. PBMCs were incubated with RNA for 20 h with subsequent IFN-α detection by ELISA. (F) Human enriched monocytes were stimulated with 100 ng/ml LPS, 1 µM CpG2216, 1 µg/ml RNA40, nuclease P1, and 2 µg/ml tRNA from *T. thermophilus* and *A. lwoffii* ± nuclease P1 digestion. After incubation for 20 h, IL-6 production was measured by ELISA. Representative experimental data from one out of four PBMCs (E) or four monocyte (F) preparations are shown ($n = 2 \pm$ SD).

released from GST by thrombin cleavage (Fig. 4 C). In vitro methylation of *E. coli* ΔtrmH tRNA with the methyl group donor S-Adenosyl methionine (SAM) and *T. thermophilus* trmH showed effective guanosine methylation as detected by HPLC analysis (Fig. 4 B). In vitro incubation of tRNA from *E. coli* ΔtrmH mutants with ^{14}C-labeled SAM and trmH further demonstrated effective tRNA methylation (Fig. 4 D) identified by PAGE and autoradiography. Most importantly, the immunostimulatory activity of *E. coli* ΔtrmH tRNA was abolished as a result of in vitro Gm18 methylation, underscoring the prominent role of Gm18 on the immunostimulatory capacity of tRNA via TLR7 (Fig. 4 E). These observations suggest an important immunomodulatory role of Gm18.

Interestingly, trmH is part of the stringent response operon triggered by nutritional deprivation and other stress conditions (Jain et al., 2006). This operon consists of five genes termed gmk, rpoZ, spot, spoU(trmH), and recG (82 min on the *E. coli* chromosome), and the first three genes are involved in formation and degradation of the alarmone guanosine penta-/tetraphosphate ((p)ppGpp; Persson et al., 1997). It is tempting to speculate that a change in growth conditions during infection and host tissue colonization may result in enhanced trmH expression and 2′-*O*-methylation of bacterial tRNAs. This may then attenuate the immune response of the host and leads to immune evasion. However, a role for Gm18 in bacterial infection as a virulence factor needs to be further examined in bacterial infection models.

TrmH genes have been identified in *E. coli*, *T. thermophilus*, and *A. aeolicus* (Persson et al., 1997; Hori et al., 2002, 2003). Database analysis using the *E. coli* trmH sequence revealed that homologous trmH genes are found in some pathogenic and non-pathogenic γ-proteobacteria and gram$^-$ thermophilic bacteria such as *Deinococcus* ssp (and *T. thermophilus*), as well as in cyanobacteria and δ-proteobacteria (e.g., *Desulfovibrio*; Fig. S1). Interestingly, the pathogenic γ-proteobacteria *H. influenzae* does not contain a gene for trmH. However, the immunostimulatory potential is reduced, suggesting that, presumably, other modifications may also influence immunostimulatory activity. Interestingly, no trmH homologous genes have been identified in gram$^+$ bacteria and the domain archaea (unpublished data), supporting our observation that isolated tRNA from those species were all inducing IFN-α. Overall, the Gm18 modification in tRNA specifically abolishes TLR7 activation and is restricted to certain gram$^-$ bacteria. To address the role of human TLR8 in the recognition of tRNA, we analyzed IL-6 production by enriched monocytes that express TLR8 but no TLR7 (Hornung et al., 2002). Of note, tRNA-driven IFN-α production by monocytes was not addressed because only TLR7 induces this cytokine and not TLR8. Interestingly, tRNA from *A. lwoffii* and *T. thermophilus* induced similar amounts of IL-6 (Fig. 4 F) and cytokine production was abolished when P1 nuclease treated tRNA was used as stimulus. In summary, these observations suggest that human TLR8 does not discriminate between the Gm18-methylated and

unmethylated form of tRNA. These results are consistent with a study that numerous 2′-*O*-methylations are necessary to efficiently block TLR8-mediated proinflammatory cytokine production (Hamm et al., 2010).

Gm18-modified tRNA acts as antagonist for TLR7-mediated IFN-α production upon tRNA and ssRNA stimulation or viral infection

We and others have shown in the past that synthetic RNA-oligonucleotides or siRNA with multiple 2′-*O*-methylations abolish immunostimulatory off-target effects via TLR7 (Judge et al., 2006; Sioud et al., 2007; Hamm et al., 2010) and act as TLR7 antagonist (Robbins et al., 2007; Hamm et al., 2010). To test if the antagonistic function of 2′-*O*-methylated RNA extended to single Gm18-modified tRNA, we exposed immune cells to a mixture of stimulatory tRNA from *A. lwoffii* and tRNA from *E. coli Nissle* or *T. thermophilus.* Interestingly, both Gm18-positive tRNAs inhibited stimulatory tRNA-driven immune activation in mouse DCs (Fig. 5 A) in a concentration-dependent manner. tRNA from *T. thermophilus* was more potent than *E. coli Nissle* tRNA, which is reflected by the frequency of Gm18-modified tRNAs in each species. Gm18-methylase from *E. coli* has a selective recognition mechanism for specific tRNAs and, therefore, only 7 out of 42 tRNAs are Gm18 modified (http://trnadb.bioinf.uni-leipzig.de/). Of note, this fraction of Gm18-positive *E. coli* tRNA is potent enough to inhibit total *E. coli* RNA-driven TLR7 stimulation (unpublished data). In contrast, *T. thermophilus* Gm18-methylase has a broad specificity and does not discriminate between tRNA species, leading to Gm18-methylation of all tRNAs (Hori et al., 2002; Ochi et al., 2010).

Furthermore, in human PBMCs *T. thermophilus* tRNA also inhibited single-stranded RNA40-driven IFN-α production. tRNA from *A. lwoffii* had no negative influence on RNA40-mediated cytokine production, underscoring the specific effect of *T. thermophilus* tRNA and ruling out an artificial inhibition by a suboptimal RNA/DOTAP ratio (Fig. 5 B). The antagonistic function of *T. thermophilus* tRNA was further tested in in vitro viral infection experiments using IAV (A/PR/8/34) and human PBMCs. Of note, in a subgroup of donors, IAV-induced IFN-α production at various multiplicities of infection (MOI) was strongly inhibited by co-incubation with tRNA (Fig. 5 C).

A well known complication of influenza infection is a super infection by bacteria (e.g., *Streptococcus pneumoniae*, *H. influenzae*, and *S. aureus*) which greatly increases morbidity and mortality. A potential mechanism for this synergism is the damage of lung epithelium during IAV infection that facilitates bacterial adhesion and invasion (Hament et al., 1999). Conversely, bacterial products such as certain proteases from *S. aureus* may boost IAV infection by cleavage activation of the viral hemagglutinin (Tashiro et al., 1987). Of note, the most common bacteria in IAV super infections are devoid of trmH. We propose that ectopic expression of trmH in *S. pneumoniae* could modulate IAV induced IFN-α production in the context of a bacterial infection.

TrmH-expressing bacteria (e.g., enteric bacteria) may also play a role in modulating the immunostimulatory activity of the gut flora, which plays a fundamental role in the development of colitis. Because probiotic bacteria such as *E. coli Nissle 1917* showed preventive effects on acute and chronic intestinal inflammation (Schultz, 2008), it is tempting to speculate that the beneficial effect of *E. coli Nissle 1917* may be a result of Gm18 tRNA-mediated inhibition of TLR7.

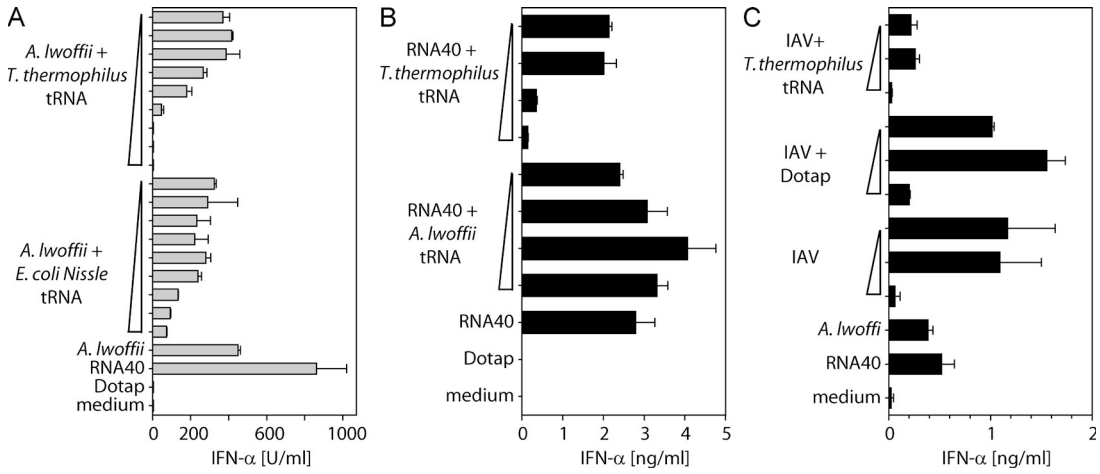

Figure 5. Gm18-modified tRNA acts as antagonist for TLR7-mediated IFN-α production upon tRNA or ssRNA stimulation and viral infection. (A) Mouse FLT3L-induced DCs were stimulated with 2 µg/ml tRNA from *A. lwoffii* and various concentrations of tRNA from *E. coli Nissle* or *T. thermophilus* (2, 1.5, 1, 0.5, 0.2, 0.1, 0.05, 0.025, and 0.0125 µg/ml). (B) Human PBMCs were stimulated with 1 µg/ml RNA40 and various concentrations of tRNA from *T. thermophilus* or *A. lwoffii* (2, 0.5, 0.125, and 0.0313 µg/ml). (C) Human PBMCs were infected with various MOI (10, 1, and 0.1) of influenza virus A/PR/8/34 (IAV) and 2 µg/ml purified tRNA from *T. thermophilus.* For each stimulation, tRNA was complexed to DOTAP and incubated for 20 h with subsequent IFN-α detection by ELISA. For A–C, representative experimental data from one out of four responsive PBMCs or two mouse cell preparations are shown ($n = 2 \pm$ SD).

Overall, we have demonstrated that the 2′-*O*-methylation status of G18 determines TLR7 activation or inhibition. Further studies will be needed to identify the role of the Gm18 modification in relation to immunity, infection or co-infection, and probiotic effects.

MATERIALS AND METHODS

Reagents. Cell culture grade phosphodiester RNA40 (5′-GCCCGUCUGUUGUGUGACUC-3′), type A CpG-ODN 2216 (5′-GsGsGGGACGATCGTCsGsGsGsGsG-3′), or inhibitory ODN IRS661 (5′-TsGsCsTsTsGsCsAsAsGsCsTsTsGsCsAsAsGsCsA-3′) were synthesized by IBA, Göttingen, or TIB MOLBIOL, respectively (s depicts a phosphorothioate-modified linkage). Mouse FLT3-ligand was obtained from an FLT3-ligand–secreting cell line (gift from H. Hochrein, Bavarian Nordic GmbH, Martinsried, Germany). DOTAP was purchased from Roche or Roth.

Bacterial strains. The following strains were cultured in medium or agar plates as indicated: *A. lwoffii F78* (provided by O. Holst and H. Heine, Leibniz-Zentrum Borstel, Borstel, Germany; 2× Lysogeny broth Lennox [LB]; Roth; 964.2×, 37°C, aerobic), *B. subtilis* (clinical isolate; Institute for Medical Microbiology and Hygiene in Marburg; Brain heart Infusion [BHI] medium; Oxoid Deutschland GmbH; CM0225, 37°C, aerobic), *E. coli Nissle 1917* (isolated from Mutaflor; Ardeypharm; LB, 37°C, aerobic), *H. influenzae b* (clinical isolate; Institute for Medical Microbiology and Hygiene in Marburg; chocolate blood; BD; 257011, 37°C, microaerophilic conditions), *L. lactis* (clinical isolate; Institute for Medical Microbiology and Hygiene in Marburg; MRS medium; Oxoid Deutschland GmbH; CM0359/0361; 37°C, aerobic), *M. marburgensis* (provided by R. Thauer and J. Moll, MPI for Terrestrial Microbiology, Marburg, Germany; mineral medium; 80% H_2/20% CO_2/0.01% H_2S, 65°C), *P. aeruginosa* (clinical isolate; Institute for Medical Microbiology and Hygiene in Marburg; MacConkey II-Agar; BD; 221172; 37°C, aerobic), *S. aureus* (clinical isolate; Institute for Medical Microbiology and Hygiene in Marburg; LB medium; 37°C, aerobic), *T. thermophilus HB27* (DSMZ; DSM7039; LB, 80°C, aerobic).

E. coli parental and methyltransferase mutant strains were obtained from the E. Coli Genetic Stock Center CGSC, Yale University, and grown at 37°C in LB medium: parental strain BW25113 (CGSC#7636; F-, Δ(araD-araB)567, ΔlacZ4787(::rrnB-3), λ-, rph-1,Δ(rhaD-rhaB)568, hsdR514), trmA (uracil-5-methyltransferase, m5U54) knockout strain JW3937-1 (CGSC#12049; F-, Δ(araD-araB)567, ΔlacZ4787(::rrnB-3), λ-, rph-1, Δ(rhaD-rhaB)568, ΔtrmA753::kan, hsdR514), trmB (guanine-7-methyltransferase, m7G46) knockout strain JW2927-1 (CGSC#10265; F-, Δ(araD-araB)567,ΔlacZ4787(::rrnB-3),λ-,ΔyggH735::kan,rph-1,Δ(rhaD-rhaB)568, hsdR514), and trmH (Gm18-2′-*O*-methyltransferase) knockout strain JW3626-1 (CGSC#11693; F-, Δ(araD-araB)567, ΔlacZ4787(::rrnB-3), λ-, rph-1,ΔtrmH755::kan, Δ(rhaD-rhaB)568, hsdR514).

RNA isolation and tRNA purification. Total bacterial RNA was isolated by the method of Chomczynski and Sacchi (2006). In brief, gram$^-$ bacteria were directly lysed in denaturing solution (solution D) containing 4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% (wt/vol) *N*-laurosylsarcosine, and 0.1 M 2-mercaptoethanol. Gram$^+$ bacteria were transformed to protoplasts by incubation with 10 mM EDTA and 1 mg/ml lysozyme for 10 min at 37°C and subsequently lysed in solution D. In general, isolated total RNA was digested with RNase-free DNase to remove traces of DNA. tRNA was isolated with Nucleobond columns DNA/RNA400 (Macherey-Nagel) according to the manufacturer's recommendations.

Mice and cells. Tlr7-deficient (Hemmi et al., 2002), TLR2/4 double-deficient (Spiller et al., 2007), and C57BL/6 WT mice (Harlan) were kept under SPF conditions in the animal facility of the University of Marburg at the Biomedizinisches Forschungszentrum. All experiments were approved by the Regierungspräsidium Giessen, Dezernat 54, Wetzlar, Germany.

FLT3-ligand–induced mixed cultures of mouse myeloid and plasmacytoid DCs were generated by seeding mouse bone marrow cells at 1.5×10^6 cells/ml and culturing with FLT3-ligand–containing supernatant for 10 d. The cells were cultivated in Opti-MEM (Invitrogen) supplemented with 1% FCS, 4 mM L-glutamine, and 10^{-5} M mercaptoethanol (PAN). Human PBMCs were isolated from buffy coats (provided by H. Hackstein and G. Bein, Institute for Clinical Immunology and Transfusion Medicine, Justus-Liebig-University Giessen, Giessen, Germany) using Ficoll density gradient centrifugation with LSM 1077 lymphocyte separation solution (PAA). Monocytes were enriched from PBMCs by elutriation to >70%.

Cell stimulation and staining. For stimulation, mouse DCs or human PBMCs were seeded at 2×10^5 cells/well. For cytokine induction, cells were incubated for 20 h with 0.5 μM CpG-ODN, 0.2 μg/ml R-848, and various tRNAs at concentration of between 0.0125 and 10 μg/ml. Complexation of tRNA with DOTAP was performed according to the manufacturer's recommendation. Shortly, RNA in 50 μl DOTAP buffer was combined with 50 μl DOTAP solution (100 μg/ml, final concentration 12.5 μg/ml) and incubated for 15 min. 100 μl of complete RPMI medium was added and the solution was mixed. 100 μl was used for stimulation of a 96-well plate that contained primary cells in 100 μl of complete medium. Mouse and human IFN-α, IL-12p40, TNF, and IL-6 were detected with reagents from Axxora, BD, and R&D Systems, respectively.

Recombinant TrmH protein and in vitro 2′-*O*-methylation. The trmH cDNA sequence was amplified with primers and cloned into pGEX-2T. Recombinant bacteria were induced with 0.1 mM IPTG for 3 h at 37°C. Cells were pelleted, lysed, and recombinant protein was purified via glutathione affinity chromatography according to the manufacturer's recommendation. In vitro methylation for HPLC analysis and cell stimulation was performed with 10 μg enzyme, 150 μg tRNA, and 1.3 mM SAM in a reaction buffer containing 50 mM Tris HCl, pH 7.5, 5 mM magnesium chloride, 6 mM β-mercaptoethanol, and 50 mM potassium chloride. Radioactive labeling of tRNA from trmH *E. coli* mutant was performed with 5 μg tRNA and 0.02 μCi ^{14}C-labeled SAM.

HPLC analysis. Aliquots of RNA equivalent to 25 μg were dissolved in 10 mM ammonium acetate, pH 5.0, containing 1 mM zinc chloride. After addition of 1.5 U nuclease P1 (from Penicillium citrinum; Sigma-Aldrich) per μg RNA samples were incubated over night at 37°C. Thereafter, samples were made alkaline by the addition of TRIS-HCl, pH 8.3 (final concentration 10 mM), and treated with shrimp alkaline phosphatase (Sigma-Aldrich) and snake venom phosphatase (Phosphodiesterase I, Type VI from *Crotalus adamanteus*; Sigma-Aldrich) at 0.01 U/μg and 0.001 U/μg RNA, respectively. After incubation at 37°C for 2 h, samples were centrifuged at 30,000 *g* for 10 min and the supernatant was harvested. HPLC analysis of digests was performed on a Dionex Ultimate 3000 System using a Supelcosil LC18 reverse phase column (250 × 4.6 mm, 5 μm) protected with a C18 guard column (4 × 2 mm; both obtained from Sigma-Aldrich). The mobile phase was: eluent A, 5 mM ammonium acetate, pH 6.0; eluent B, 40% acetonitrile. After injection of the RNA digest separation started with 100% eluent A followed by a 40-min gradient to 20% eluent B using a flow rate of 1 ml/min. Finally the column was subjected to isocratic elution with 100% eluent B for 10 min. Detection of the eluted nucleosides was continuously recorded by UV spectrometry at 254 nm.

Online supplemental material. Fig. S1 presents a phylogenetic tree of trmH genes in different bacterial species and adds valuable information regarding the limited bacterial trmH expression and Gm18 occurrence. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20111075/DC1.

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