2′-O-methylation within prokaryotic and eukaryotic tRNA inhibits innate immune activation by endosomal Toll-like receptors but does not affect recognition of whole organisms

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ABSTRACT

Bacterial RNA has emerged as an important activator of innate immune responses by stimulating Toll-like receptors TLR7 and TLR8 in humans. Guanosine 2′-O-methylation at position 18 (Gm18) in bacterial tRNA was shown to antagonize tRNA-induced TLR7/8 activation, suggesting a potential role of Gm18 as an immune escape mechanism. This modification also occurs in eukaryotic tRNA, yet a physiological immune function remained to be tested. We therefore set out to investigate the immune modulatory role of Gm18 in both prokaryotic and eukaryotic microorganisms, Escherichia coli and Saccharomyces cerevisiae, and in human cells. Using RiboMethSeq analysis we show that mutation of trmH in E. coli, trm3 in S. cerevisiae, and CRISPR/Cas9-induced knockout of TARBP1 in H. sapiens results in loss of Gm18 within tRNA. Lack of Gm18 across the kingdoms resulted in increased immunostimulation of peripheral blood mononuclear cells when activated by tRNA preparations. In E. coli, lack of 2′-O-methyltransferase trmH also enhanced immune stimulatory properties by whole cellular RNA. In contrast, lack of Gm18 in yeasts and human cells did not affect immunostimulation by whole RNA preparations. When using live E. coli bacteria, lack of trmH did not affect overall immune stimulation although we detected a defined TLR8/RNA-dependent gene expression signature upon E. coli infection. Together, these results demonstrate that Gm18 is a global immune inhibitory tRNA modification across the kingdoms and contributes to tRNA recognition by innate immune cells, but as an individual modification has insufficient potency to modulate recognition of the investigated microorganisms.

Keywords: immune stimulation; bacterial RNA; TLR; RNA modification; 2′-O-methylation

INTRODUCTION

To ensure a rapid immune response against a broad range of invading pathogens, cells of the host’s innate immune system are equipped with a limited set of pattern recognition receptors (PRRs) that sense highly conserved microbial structures, so-called pathogen-associated molecular patterns (PAMPs). Bacterial RNA has been identified as a potent activator of innate immune responses and was shown to be sensed by Toll-like receptors (TLRs) localized in the endolysosome (Koski et al. 2004; Eberle et al. 2009; Eigenbrod et al. 2015). Within human peripheral blood

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mononuclear cells (PBMCs), monocytes recognize microbial RNA in a TLR8 dependent manner, triggering the release of proinflammatory mediators including TNF, IL-12p40, and IFN-β. In contrast, plasmacytoid dendritic cells (DCs) sense bacterial RNA via endosomal TLR7, culminating in the production of IFN-α (Diebold et al. 2004; Heil et al. 2004; Ablasser et al. 2009; Bergstrøm et al. 2015; Saitoh et al. 2017).

As both, bacterial and host RNA, are composed of the same building blocks, a reliable discrimination of self and non-self by the innate immune system is critical to ensure induction of an adequate antimicrobial defense in case of pathogen encounter while at the same time avoiding aberrant immune stimulation by host (“self“) RNA. Indeed, several auto-immune disorders including systemic lupus erythematosus have been associated with abnormal recognition of self nucleic acids (Barrat et al. 2005; Kono et al. 2009). Apart from compartmentalization—i.e., localization of nucleic acid sensing TLRs in the endolysosome to which self-RNA has limited access—and differences in the RNA sequence composition, another determinant for self/non-self discrimination has been attributed to RNA modification profiles. As such, both the extent and type of posttranscriptional RNA modifications differ between self and microbial RNA. In this regard, various studies have highlighted the importance of ribose 2′-O-methylation as a potent modification impeding immune stimulation by endosomal TLRs (Karikö et al. 2005; Robbins et al. 2007; Sioud et al. 2007; Gehrig et al. 2012; Jöckel et al. 2012). 2′-O-methylation not only impairs TLR7 and TLR8 activation but also acts as a TLR7/8 antagonist, thus inhibiting immune cell stimulation by otherwise immunogenic RNA species. Subsequent studies identified a DmDN (D = all but C; N = all) and a DmRC (D = all but C; R = G, A) motif as most efficient in antagonizing TLR7 and TLR8, respectively (Schmitt et al. 2017). Of note, 2′-O-methylation of cytosine consistently failed to impede TLR7/8 activation in previous studies (Robbins et al. 2007; Eberle et al. 2008; Kaiser et al. 2014).

Although the highest abundance of 2′-O-methylation has been described in eukaryotic tRNA, this modification is also found in certain bacterial tRNAs (Boccaletto et al. 2018). Specifically, tRNA 2′-O-methylation of guanosine at position 18 (Gm18), which is present in five tRNA species in Escherichia coli (E. coli), has been demonstrated to be necessary and sufficient to suppress immune stimulation via TLR7 by whole E. coli tRNA preparations. Total tRNA preparations derived from an E. coli mutant lacking Gm18 2′-O-methyltransferase activity (trmH knockout) elicited a stronger TLR7 response than the respective wild-type samples (Gehrig et al. 2012; Jöckel et al. 2012). These observations raised the hypothesis that Gm18, apart from its structural function, may also serve as an immune escape mechanism for E. coli or other microorganisms carrying this specific modification. Yet, this assumption is, thus far, exclusively based on experiments involving the transfection of purified tRNA preparations into immune cells. How E. coli tRNA 2′-O-methylation indeed shapes bacterial immunogenicity under physiological conditions, i.e., during infection with the whole, living microorganism, remains elusive. Moreover, Gm18 is present in the tRNA of lower and higher eukaryotes, including Saccharomyces cerevisiae (S. cerevisiae) as well as human cells. Its physiological significance for immune recognition via endosomal TLRs across the different kingdoms has not been studied so far. Given that eukaryotic RNA generally shows a much higher degree of posttranscriptional RNA modifications than prokaryotic RNA (Motorin and Helm 2011; Motorin 2015) Gm18 might play a more redundant role in eukaryotes as compared to bacteria but experimental evidence is lacking.

The aim of the present study was therefore to determine the role of Gm18 within tRNA in pro- and eukaryotic organisms, specifically E. coli, S. cerevisiae, and human cells, for modulating innate immune recognition. It should be tested whether Gm18 exerts an immune modulatory function across kingdoms and how this modification affects immune stimulation in general. We show that Gm18 deficiency augmented PBMC activation by purified total tRNA preparations from all investigated organisms. We also show for the first time an impact of Gm18 on the immune stimulatory properties of human tRNA. However, an impact of Gm18 in tRNA on immune activation by whole cellular RNA was only visible for E. coli but not for eukaryotes, presumably due to dominant differences in 2′-O-methylation in ribosomal RNA. In neither microbe did a knockout of the Gm18 methyltransferase impact the overall immune reaction, although we identified an RNA-TLR8 regulated gene signature in response to E. coli in general. Thus, Gm18 is a global immune modulatory tRNA modification but it does not seem to play a role as an immune escape mechanism during infection with the investigated microorganisms. The results suggest the existence of a threshold for Gm content in an invading pathogen’s cellular RNA that governs TLR7/8 mediated immune responses.

RESULTS

Yeast tRNA fractions show inverse correlation of Gm content with type I interferon secretion by human peripheral blood mononuclear cells

First we intended to test whether 2′-O-methylation might affect the immune stimulatory potency of S. cerevisiae tRNA as had been described before for E. coli. As a starting point we investigated fractions of tRNA isoacceptors that had been obtained in counter current distribution experiments (Heitzler et al. 1992), a technique which separates tRNA according to their relative lipophilicity. Fractions were analyzed for their relative Gm, Am, Um, and Cm
content by LC-MS/MS and compared to their IFN-α–inducing properties upon stimulation of human PBMCs. Due to highly cell-type specific receptor and cytokine expression profiles, IFN-α secretion within PBMCs is commonly used as a read-out for TLR7-dependent plasmacytoid DC activation. Gm-rich tRNAs were predominantly eluted in fractions 1–6 and 13–15, while relative Am levels showed a peak in fractions 13–15 (but were absent in the first ten fractions). Um was found prominently in fractions 3–6 and Cm in fractions 1–8 and 18–21 (Fig. 1A). Introduction of Gm, Am, and Um into synthetic RNA previously had been described to affect immune recognition whereas Cm consistently failed to impede TLR7/8 activation (Robbins et al. 2007; Eberle et al. 2008; Schmitt et al. 2017). When the individual tRNA fractions were tested for induction of type I IFN from PBMCs, they varied strongly in their IFN-α–inducing capacity (Fig. 1B), suggesting a negative correlation between 2′-O-methylation and IFN induction also in eukaryotic RNA. In a first approximation a correlation matrix showed a significant negative correlation between Gm content and IFN-α secretion (Fig. 1C), whereas Am, Cm, and Um content showed no relation. A closer examination confirmed a general negative impact of Gm on IFN secretion (Fig. 1D). We do not exclude that other modifications might contribute to immune modulation, e.g., Am and Um may affect the lowered IFN-α levels observed in fractions 13–15 and fractions 3–6, respectively.

To confirm these starting observations of a negative regulatory function of Gm in S. cerevisiae we used a targeted, independent experimental approach: An isogenic mutant strain deficient in trm3, the trmH homolog in yeast, was analyzed. Total tRNA and whole cellular RNA preparations were purified from WT and mutant S. cerevisiae. We used unbiased RiboMethSeq analysis (Galvanin et al. 2019) to study the impact of loss of trm3 on the yeast tRNA modification pattern: Thereby we confirm the presence of Gm18 in eight tRNA isoacceptors corresponding to five tRNA species in WT samples (Boccaletto et al. 2018). trm3 deficient cells showed a lack of Gm18 in total tRNA extracts (Fig. 2A). We also used a complementary LC-MS/MS approach that demonstrated a residual Gm content in these preparations; this can be explained by the presence of Gm at position 34 in tRNA\textsuperscript{Thr} (Fig. 2B; Boccaletto et al. 2018). In line with the results obtained with tRNA fractions, specific lack of Gm18 in trm3 mutant S. cerevisiae significantly enhanced TLR7 stimulatory properties of total tRNA preparations, although IFN-α secretion was generally lower as compared to stimulation with bacterial RNA, the latter serving as positive control (Fig. 2C). Thus, a physiological role of Gm18 modified tRNA in eukaryotes could be demonstrated. However, whole cellular RNA from both WT and Δtrm3 S. cerevisiae failed to induce any TLR7 activation (Fig. 2C). We also tested ΔtrmH E. coli lacking the corresponding Gm tRNA methyltransferase. Gm18 deficiency was also confirmed by RiboMethSeq and LC-MS/MS analysis. For ΔtrmH E. coli, not only enhanced IFN-α release by total tRNA, as published previously (Gehrig et al. 2012; Jöckel et al. 2012), but also by whole bacterial RNA preparations (Fig. 2D–F) was observed. Thus, in both microorganisms the presence of a few Gm18 modified tRNA isoacceptors was sufficient to manipulate recognition of whole tRNA preparations confirming a dominant-inhibitory effect of this modification. Yet, whole

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**FIGURE 1.** IFN-α production by PBMCs upon stimulation with fractionated tRNAs from S. cerevisiae inversely correlates with the Gm content. (A) Relative levels of 2′-O-methylated nucleotides quantified by LC-MS/MS in S. cerevisiae fractions that were generated as described in the Materials and Methods section. Values were normalized to highest relative amount of each modified nucleotide. (B) Human PBMCs (400,000/well in a total volume of 200 µL) were stimulated overnight in duplicate wells with fractionated and DOTAP-encapsulated tRNAs (c = 1 µg/mL). IFN-α release was measured in cell-free supernatants by ELISA. Bars represent pooled data from three individual donors +SEM. (C) A correlation matrix of IFN-α secretion and relative amount of Gm, Cm, Am, Um was calculated for Pearson’s r including 95% confidence interval. (***) P < 0.001 (D) Individual correlations of IFN-α and Xm, normalized to the highest respective value are shown. For Gm, goodness of fit was calculated using an appropriated non-linear dose response model (GraphPad Prism).
WT strains and 2 MS analysis of isolated and S. cerevisiae RNA (2019) Vol. 25, No. 7

PRRs, PBMCs were infected with a variety of PAMPs activating potentially a large set of immune stimulation by purified RNA species but also by in-cell-free RNA preparations showed differing influence by loss of Gm18 in tRNA.

Gm18 deficiency does not alter cytokine production in PBMCs upon infection with living E. coli and S. cerevisiae

In order to investigate whether Gm18 not only affects immune stimulation by purified RNA species but also by infection with the whole organism which is composed of a variety of PAMPs potentially activating a large set of PRRs, PBMCs were infected with E. coli and S. cerevisiae WT and Gm18 deficient mutant strains at various multiplicities of infection (MOIs) (Fig. 3A–D). Although total cellular RNA preparations isolated from E. coli ΔtrmH-induced cytokine release was quantified in cell-free supernatants by ELISA. Each data point represents the mean value of one donor stimulated in duplicate wells. Error bars indicate mean ± SD of three to five individual donors.

(*) P < 0.05; (**) P < 0.001.

Gene expression profiling reveals TLR8-dependent pathways in E. coli sensing

Next, we aimed to more broadly explore a potential impact of RNA sensing in general and of Gm18 in particular on E. coli mediated immune activation, as ΔtrmH E. coli gave differing results for whole RNA versus whole bacterial cell stimulation (Figs. 2, 3). To this end, microarray-based gene expression profiling was performed in trans-differentiated BLaER1 cells that recognize bacterial and archaeal RNA in a TLR8-dependent manner (Fig. 3E,F). Thus, the data do not provide evidence for a role of Gm18 as an immune escape mechanism under physiological conditions of infection within the context of the here analyzed living whole microorganisms.

RNA preparations showed differing influence by loss of Gm18 in tRNA.

Visualizations of the raw data including IL-6, TNF and IFN-β (Supplemental Table 1A,B).
Role of Gm18 in innate immunity

were significantly different between WT and Δtmh E. coli (Fig. 4C), indicating that Gm18 does not influence overall immune stimulation by E. coli. Yet, it is well known that E. coli possesses a very bioactive LPS triggering TLR4 and indeed the gene signature induced by E. coli showed a large overlap with LPS, which was included as positive control (Fig. 4D).

We next asked the question to what extent an RNA-TLR8 axis contributed to E. coli–induced cell activation in general. To this end, a gene expression analysis was performed in WT and TLR8−/− BlaER1 cells upon infection with WT E. coli. Interestingly, 50 genes were significantly differentially regulated (40 down and 10 up) in TLR8−/− as compared to WT cells, including IFN-β, IL-12p35 and three members of the small proline-rich protein family (SPRRs) whose function in innate immunity is as yet insufficiently defined (Fig. 4E; Supplemental Table 1C). These results thus clearly point toward an RNA-TLR8 dependent gene signature in E. coli sensing even in the presence of a strong LPS response. Microarray data were further validated by qPCR, confirming a strong reduction of IFN-β and partial impairment of IL-12p35 and IL-12p40 expression in TLR8−/− cells upon E. coli exposure, while induction of TNF and IL-6 were not altered (Fig. 4F and data not shown). Together, these data demonstrate that despite the existence of TLR8-dependent pathways in E. coli recognition, Gm18 does not affect innate immune activation by this pathogen and thus does not serve as an immune escape mechanism under the here tested physiological conditions.

Gm18 deficiency in human cells enhances immune cell activation by total tRNA

In a next step, we further wanted to analyze the influence of Gm18 on immune stimulation in the human system (higher eukaryote). Therefore, TARBP1, the proposed human homolog of tmH, was targeted by CRISPR/Cas9 in HEK293 cells, a cell line that is easy to transfect and to genetically manipulate. Successful knockout of TARBP1 was confirmed by sequencing, demonstrating biallelic frameshift–inducing insertions resulting in altered amino acid sequences (Fig. 5A), as well as on the protein level (Fig. 5B). Lack of Gm18 in tRNA derived from TARBP1-deficient cells was then analyzed by RibomethSeq analysis (Fig. 5C). Indeed we can show that TARBP1 in human cells has the proposed 2′-O-methyltransferase activity. However, surprisingly, Gm18 was detected in only three isoacceptors corresponding to two tRNA species (Ser, Gln) in HEK WT cells but not in further tRNAs. Yet, despite the relatively low abundance of Gm18 in human tRNA, preparations derived from TARBP1−/− cells still caused a slight but statistically significant increase in immune stimulation by total tRNA preparations (Fig. 5D). In contrast, whole cellular RNA was in general nonstimulatory and no differences were observed between RNA from WT and TARBP1−/− cells. This pattern was similar to the results obtained with S. cerevisiae (Fig. 2C) but different to E. coli (Fig. 2F). We speculated that within whole cellular RNA the high content of ribosomal RNA might explain the differences. Indeed, when testing rRNA preparations we observed a clear discrimination of prokaryotic rRNA, which was stimulatory, from eukaryotic rRNA, that was inactive (Fig. 5E), indicating a dominant contribution of immune silencing modifications present in rRNA.

DISCUSSION

RNA modifications have been recognized as critical modulators of RNA-mediated innate immune responses and a number of studies have highlighted the TLR7/8 antagonistic properties of ribose 2′-O-methylation in this context (Robbins et al. 2007; Hamm et al. 2010; Jung et al. 2015; Schmitt et al. 2017). Yet, it was unknown whether Gm in its natural context plays a comparable immune-modifying role across different kingdoms. We now show that deletion of Gm18 by knockout of the modifying enzymes tmH,
trm3, and TARBP1 in prokaryotes, lower and higher eukaryotes results in an increased immune stimulation by tRNA. Using the unbiased RiboMethSeq approach to study frequencies of Gm, this immune modulatory effect could be observed although only a few isoacceptors had a Gm modification, thus demonstrating a potent antagonistic activity of naturally occurring Gm18.

Apart from its role in microorganisms, we also investigated the significance of Gm18 in the human system. Surprisingly, RiboMethSeq analysis identified Gm18 in only three isoacceptors corresponding to two tRNA species (Ser and Gln). This finding was rather unexpected as human tRNA is generally believed to show the highest degree of posttranslational RNA modifications. Yet, despite its low abundance, Gm18 deficiency still enhanced cytokine release by total tRNA, pointing toward a potential role of this modification for immune modulation.

Mitochondrial tRNA, which accounts for ∼13 ± 5% of total tRNA in our study, may at least partially contribute to the overall immune stimulation observed by tRNA preparations (Karikö et al. 2005). Despite an effect of TARBP1 that could only be observed for tRNA but not whole RNA, links to a physiological contribution of Gm18 to self/foreign discrimination have been published: Rare single-nucleotide variants were found in TARBP1 in exome sequencing studies associated with autoimmune psoriasis (Tang et al. 2014) and TARBP1 is differentially regulated between healthy patients and those with autoimmune SLE or ANCA vasculitis (Lyons et al. 2010). Moreover, HIV has been recently shown to use TARBP1 to recruit 2′-O-methyltransferase activity to its RNA to hide the RNA from immune recognition (Ringeard et al. 2019).

Human and S. cerevisiae rRNA and total cellular RNA preparations lacked immune stimulatory properties entirely, suggesting the presence of relevant immune silencing modifications within rRNA. Of note, rRNA from S. cerevisiae and especially from Homo sapiens has been reported to be heavily 2′-O-methylated on both guanosine and adenosine residues by the 2′-O-methyltransferases NOP1 in yeast and its homolog fibrillarin in humans (Jansen et al. 1991; Tollervey et al. 1991; Marchand et al. 2016; Erales et al. 2017). Studying fibrillarin and rRNA 2′-O-methylation would be interesting but is challenging as NOP1 was described to be crucial for cell viability in yeast (Tollervey et al. 1991). Interestingly, antifibrillarin antibodies are detected in the serum of patients suffering from

FIGURE 4. E. coli induces an RNA/TLR8-dependent gene signature which is not affected by Gm18. (A) BLaER1 cells were stimulated overnight with LPS, TLR8-specific ligand TL8-506 (TL8), and bacterial RNA from S. aureus. Levels of IL-6 and TNF were measured in cell-free supernatants by ELISA. (B–D) BLaER1 cell were stimulated with whole, live E. coli WT, E. coli ΔtrmH (MOI 10), or LPS for 5 h, and gene expression profiling was performed using an Illumina HT12 microarray. (B) Heatmap depicts 2304 significantly differentially regulated genes (defined as log2 fold-change >1 or <−1 and P < 0.05; mean of three independent experiments) for each condition as compared to the nontreated control. For each condition, data of three independent biological replicates were pooled. (C) Scatter plot displaying log2 intensity values of genes regulated by E. coli WT versus E. coli ΔtrmH relative to the unstimulated control. (D) Venn diagram of E. coli WT and LPS regulated genes. (E) Volcano plot of 482 regulated genes of BLaER1 WT compared to BLaER1 TLR8−/− upon stimulation with E. coli WT. Significantly differentially regulated genes (defined as log2 fold-change >1 or <−1 and P < 0.05) are highlighted in green and red. All microarray data were analyzed using Chipster software. (F) WT and TLR8−/− BLaER1 cells were stimulated for 5 h with TLR8-specific ligand TL8-506, LPS, E. coli WT, and E. coli ΔtrmH (MOI 10), and relative cytokine expression was determined by qPCR. Values represent mean data (+SEM) of three independent experiments. (***)) P < 0.001.
autoimmune diseases (Van Eenennaam et al. 2002; Barrat et al. 2005; Hagberg and Rönnblom 2015).

The fact that 2′-O-methylation is also naturally occurring in a subset of tRNA isoacceptors at position 18 (Gm18) in E. coli gave rise to the hypothesis that this modification, beyond its structural role, may have acquired a secondary function as an immune escape mechanism in organisms bearing this modification (David 2012; Gehrig et al. 2012; Jöckel et al. 2012). Yet, this idea was based on the transfected of purified tRNAs into PBMCs, a situation not adequately reflecting the physiological conditions of infection with the live microorganism. In the present study, we demonstrate that Gm18 deficiency in E. coli indeed enhances immune stimulation by isolated total tRNA and even whole cellular RNA preparations, yet does not interfere with innate immune sensing of live E. coli by both PBMCs and BLaER1 cells. Similar results were obtained for S. cerevisiae. These results therefore argue against a role of Gm18 as a relevant immune escape mechanism under physiological conditions at least in the microorganisms tested. As tRNA constitutes only 10%–20% of total E. coli RNA with only five tRNA species being Gm18 methylated, it is conceivable that the ratio of Gm18 methylated to unmethylated RNA might not be sufficient to suppress immune activation during infection when rRNA is also present. Due to the dominant action of LPS when studying E. coli (Fig. 4D), a role of Gm18 might be more prominent in other bacteria. Another influencing factor might be accessibility of different RNA species.

The results are compatible with a model of a certain threshold for Gm content in an organism’s cellular RNA to modulate TLR7/8 mediated immune responses: In S. cerevisiae and human cells, the overall Gm content is high, especially due to its abundance in rRNA, and exceeds a certain threshold, thus resulting in lack of immune recognition. Deletion of Gm only in tRNA does not increase sensing of the whole organism, as the overall Gm content is still sufficiently high (tRNA). In contrast in E. coli, Gm levels are generally low due to the lack of RNA 2′-O-methylation, resulting in immune stimulation by all RNA species. However, when only tRNA is analyzed, a situation of Gm enrichment is achieved which now reveals the antagonistic function of this modification.

E. coli is known to be recognized in a predominantly TLR4-dependent manner (Takeuchi et al. 1999; Roger et al. 2009), in line with a large overlap of the E. coli- and LPS-induced gene expression profiles observed in our study. Yet, microarray-based analysis of WT and TLR8−/− BLaER1 cells clearly identified an additional TLR8- and thus RNA-dependent gene signature triggered by E. coli infection, including regulation of classical proinflammatory mediators like IFN-β and IL-12 as well as proteins with less well-defined functions in innate immunity (e.g., small proline-rich proteins, SPRRs). Interestingly, an RNA-dependent induction of specific cytokines by E. coli was also described by the groups of L. Sander and J. Blander who raised the concept of bacterial RNA as
so-called “vita-PAMP”: Due to the rapid degradation of RNA after bacterial cell death, RNA was proposed to signify microbial viability and thus a persistent infectious threat to the host, culminating in more robust innate and adaptive immune responses (Sander et al. 2011; Barbet et al. 2018; Ugolini et al. 2018). Indeed, the here observed genes that are strongly dependent on TLR8 (e.g., IL-12 and IFN-β) have also be described by the Blander and Sander groups. While initial investigations emphasized the relevance of E. coli RNA for the induction of IFN-β and activation of the NLRP3 inflammasome with subsequent production of bioactive IL-1β (Sander et al. 2011), recent studies highlighted the contribution of microbial viability sensing via TLR8 for T follicular cell differentiation and antibody responses in a manner dependent on IL-12 and on an IFN-β/IL-1β axis (Barbet et al. 2018; Ugolini et al. 2018). It might therefore be interesting to analyze T helper cell differentiation instead of global gene expression of monocytes by stimulation with trmH mutants as those might affect only a small, yet important part of the gene signature.

In summary, the findings of this study demonstrate that Gm18 is an immune inhibitory RNA modification across organisms of different kingdoms, yet it does not serve as an immune evasion mechanism in the studied organisms. The results also identify an impact of Gm18 on immune stimulatory properties within human tRNA; in conjunction with published data on differential TARBP1 regulation and SNPs in autoimmune diseases a potential contribution in tolerance to self-RNA can be envisioned.

MATERIALS AND METHODS

Reagents

RPMI 1640 containing stable glutamine was purchased from Anprotec; FCS was obtained from Gibco; Ficoll (1.078 g/mL), DPBS w/o Ca2+ and Mg2+ from Pan Biotech; TRIzol from Anprotec; FCS was obtained from Gibco; Ficoll (1.078 g/mL), DPBS w/o Ca2+ and Mg2+ from Pan Biotech; TRIzol from Thermo Fisher Scientific; R848 and TL8-506 from InvivoGen; DOTAP (N-[1-(2, 3-dioleoyloxy)propyl]-N, N, N-trimethylammonium methylsulfate) from Roth; peptone (Sigma-Aldrich), yeast extract (AppliChem) Nanosept MF, bio-inert membrane, purple, 0.45 µm were from Pall Laboratory; and ammonium acetate was from J.B. Baker.

Preparation of RNA samples from E. coli, S. cerevisiae, and HEK cells

WT (parental strain: BW25113) and trmH deficient (JW3626-1) Escherichia coli (provided from E. coli Genetic Stock Center CGSC, Yale University) were grown in Luria-Bertani (LB) medium (Merck) and harvested within the mid-log phase growth, pelleted, and the cell wall was digested by lysozyme (c = 40 mg/mL, 20 min at 37°C). WT (parental strain: Y00000) and trm3 deficient (Y03809) S. cerevisiae (purchased from Euroscarf, Johann Wolfgang Goethe-University, Frankfurt, Germany) were grown in YPD-Medium (20 g peptone; 10 g yeast extract; ad 975 mL dH2O; pH 6.5), harvested in the mid-log phase growth, pelleted and the cell wall was digested by Lyticase (100 U, 20 min at 37°C). HEK cells were harvested using Trypsin-solution and pelleted at 1300 rpm for 15 min. Total RNA of all species was isolated using TRIzol reagent according to the manufacturer’s protocol. The obtained RNA was further separated by agarose gel electrophoresis, and RNA bands corresponding to tRNA and/or rRNA were excised. Gel slices were transferred to a 2 mL reaction tube and mashed, and 500 µL ammonium acetate 0.5 M was added. Samples were stored for 1 h at −80°C and shaken overnight at room temperature at 500 rpm to extract RNA. The next day, samples were transferred to a Nanosept filter and flow through was mixed with 1250 µL ethanol to precipitate RNA. RNA preparations were stored for 1–2 h at −80°C and centrifuged for 60 min at 10,000 g, 4°C. Pellets were washed in 70% ethanol and RNA was dissolved in nuclease-free water. Purity of the RNA preparations was validated by determining the 260/230 nm and 260/280 nm extinction ratio by NanoDrop (Thermo Scientific). Only RNA preparations with ratios >1.8 were used for stimulation experiments.

Fractionation of S. cerevisiae tRNA

The fractions of tRNA yeast used in experiments shown in Figure 1 were generated several decades ago using fractions counter current distribution, an old separation technique that separated tRNA according to their relative lipophilicity. Despite long-term storage, analysis of the fractions by high-resolution capillary electrophoresis (QIAxcel, Qiagen) did not indicate significant degradation. According to a mapping of the tRNA distribution (Heitzler et al. 1992), the relevant fractions 1–6 contain Gm-containing tRNAs accepting the amino acids Phe (Gm34), Ser, Tyr, Trp, and Leu (all Gm18) and fractions 13–15 contain His (Gm18, Am4) and Val (no Gm). Fractions 4–5 contained Ser (Um44) (Boccaletto et al. 2018). All analyses on the 2’-O-methylation status of the preexisting tRNA fractions using LC-MS and RiboMethSeq analysis, as described below, were performed during the course of the present study.

LC-MS measurements of Gm, Am, Um, and Cm levels

tRNA samples were digested into nucleotides using 0.6 U nuclease P1 from P. citrinum (Sigma-Aldrich), 0.2 U snake venom phosphodiesterase from C. adamanteus (Worthington), 2 U FastAP (Thermo Scientific), 10 sU benzonase (Sigma-Aldrich), 200 ng Pentostatin (Sigma-Aldrich), and 500 ng Tetrahydrodouridine (Merck-Millipore) in 25 mM ammonium acetate (pH 7.5; Sigma-Aldrich) overnight at 37°C. The nucleosides were then spiked with internal standard (13C stable isotope-labeled nucleosides from S. cerevisiae, SIL-IS) and subjected to analysis. Technical triplicates with 100 ng digested RNA and 50 ng internal standard were analyzed via LC-MS (Agilent 1260 series and Agilent 6460 Triple Quadrupole mass spectrometer equipped with an electrospray ion source [ESI]). The solvents consisted of 5 mM ammonium acetate buffer (pH 5.3; solvent A) and LC-MS grade acetonitrile (solvent B; Honeywell). The elution started with 100% solvent A with a flow rate of 0.35 mL/min, followed by a linear gradient to 8% solvent B at 10 min and 40% solvent B after
20 min. Initial conditions were regenerated with 100% solvent A for 10 min. The column used was a Synergy Fusion (4 μm particle size, 80 Å pore size, 250 × 2.0 mm; Phenomenex). The UV signal at 254 nm was recorded via a diode array detector (DAD) to monitor the main nucleosides. ESI parameters were as follows: gas temperature 350°C, gas flow 8 L/min, nebulizer pressure 50 psi, sheath gas temperature 350°C, sheath gas flow 12 L/min, capillary voltage 3000 V. The MS was operated in the positive ion mode using Agilent MassHunter software in the dynamic MRM (multiple reaction monitoring) mode. For quantification, a combination of external and internal calibration was applied as described previously (Kellner et al. 2014).

**RiboMethSeq analysis**

Total tRNA RiboMethSeq analysis was performed essentially as described previously (Marchand et al. 2017). Briefly, about 100 ng of total tRNA either from *E. coli*, *S. cerevisiae*, or human HEK cells were randomly fragmented in bicarbonate buffer for different times (6–10 min for *E. coli*, 8–12 min for *S. cerevisiae*, and 14 min for human HEK, at 95°C). The reaction was stopped by ethanol precipitation as previously described in Marchand et al. (2016). RNA fragments were then subjected to end-repair and converted to a library using NEBNext Small RNA Library kit (ref#E7330S, New England Biolabs) following the manufacturer’s instructions. Library sequencing was performed using Illumina HiSeq 1000 sequencer in a single read mode for 50 nt. Analysis of human tRNA 2′-O-methylation was performed by trimming of raw sequencing reads using Trimmomatic v0.32, followed by end-to-end alignment by Bowtie 2.0 to nonredundant collection of human intronless tRNA genes. Reference sequence was created by collapsing of identical tRNA sequences and deduction of the consensus sequences from closely related species. The resulting reference allowed unambiguous alignment of >80% of tRNA reads. Further data analysis was done by previously described bioinformatic pipeline (Galvanin et al. 2019). Briefly, RiboMethSeq scores were calculated from the cleavage profiles derived from accumulated 5′- and 3′-end counts. Candidate 2′-O-methylated sites in tRNA species were identified by values of Score A >0.5 and Score Mean >0.90, followed by visual inspection of the coverage profiles to exclude irregularly cleaved regions. MethScore (Score C2) was used to quantify the 2′-O-methylation level. Differential heatmaps for figures were constructed by normalizing of MethScore levels for every given position to the average value observed in the series of samples. Positive (shown in red) values indicate higher methylation compared to the average (undermethylation), while negative (shown in green) positions have MethScore below the average (undermethylation).

**Generation of TARBP-1 deficient HEK cells using CRISPR–Cas9**

TARBP1-deficient HEK cells were created via a S. pyogenes derived CRISPR/Cas9 system using a NGG PAM sequence. gRNAs were designed by using a Blue Heron webpage (www .blueheronbio.com) with a GC content between 60% and 80% and blasted to control for off target effects. gRNAs were custom synthesized by Eurofins Genomics and cloned into a GFP-encoding vector backbone using the restriction enzyme BbsI (Chen et al. 2013). Plasmids were kindly provided by Professor Dr. Dirk Grimm, BioQuant, University of Heidelberg, Heidelberg, Germany. The TARBP1′−/′ clone used in the current study was generated using the following gRNA: fw: 5′-CACCCGGCGCGCAAGGGCGGC-3′, rev: 5′-AACCGTGGCGCGCGGTTCCTGGCGCGGTC-3′. Nucleotides highlighted in gray indicate the overhang required for cloning into the BbsI digested vector. Resulting plasmids were transfected into HEK293 cells using Lipofectamine 2000, and GFP-positive cells were sorted into 96-well plates 24 h posttransfection. After 1–2 wk, single clones were analyzed for successful targeting of TARBP1 by both Sanger sequencing (primer sequences for PCR and sequencing: fw 5′-ACCTCTGGCCTTA CGTCTAAATC-3′, rev 5′-ACGGCTGCTAGCATACTTAC-3′) and western blot analysis (anti-TARBP1 antibody [EPR8727] Abcam). Sequences were analyzed using CRISP-ID software (Dehairs et al. 2016).

**Isolation and stimulation of human peripheral blood mononuclear cells (PBMCs)**

Human PBMCs were isolated from heparinized blood of healthy volunteers upon informed consent and approval by the local ethical committee (admission number: S-716/2017) by standard Ficoll-Hypaque density gradient centrifugation (Ficoll 1.078 g/mL). PBMCs were resuspended in RPMI 1640 supplemented with 2% heat-inactivated human serum. For transfection experiments, all RNAs were encapsulated with DOTAP at a ratio of 3 μL DOTAP per 1 μg of RNA in serum-free medium according to the manufacturer’s protocol. Unless otherwise indicated, cells were stimulated with RNA preparations at a final concentration of 500 ng/mL, and transfection was performed at a density of 2 × 10⁶ PBMCs per well in a 96-well flat bottom plate at a final volume of 100 μL. Cells were incubated overnight in a humidified 5% CO₂ atmosphere at 37°C. For cytokine measurement, levels of TNF, IL-6 (BD), and IFN-α (eBioscience) were determined in cell-free supernatants by ELISA.

**Trans-differentiation and stimulation of BLaER1 cells**

WT and TLR8−/− B-cell like BLaER1 cells, the latter generated as previously described (Vierbuchen et al. 2017), were kindly provided by Holger Heine, Research Center Borstel, Germany. BLaER1 cells were trans-differentiated into monocyte/macrophage-like cells by seeding 1 million cells/well into a six-well in 3 mL/well RPMI 1640 medium supplemented with 10% FCS, 1% Pen/Strep, 10 ng/mL IL-3, 10 ng/mL M-CSF, and 150 nM β-Estradiol. Plates were incubated at 37°C and 5% CO₂. At days 2 and 5 of trans-differentiation, cells were collected by pipetting up and down, centrifuged at 1300 rpm for 10 min, and fresh medium with the aforementioned supplements was added. After 1 wk, BLaER1 cells were sorted using the AutoMACS magnetic cell sorter system and the human CD19 MicroBeads kit according to manufacturer’s instructions (Miltenyi Biotech) to deplete residual CD19+ BLaER1 cells. CD19 negative, trans-differentiated monocyte/macrophage like BLaER1 cells were resuspended in RPMI 1640 supplemented with 10% FCS. For experiments involving measurement of IL-6 and TNF in cell-free supernatants by ELISA (BD), 1 × 10⁵ cells were transferred into a 96-well flat bottom plate and stimulated overnight in duplicate wells with LPS (c = 100 ng/mL), TL8-506
were rested overnight at 37°C, 5% CO2 prior to stimulation to avoid preactivation of BLaER1 cells due to MACS sorting, cells were seeded in a 48-well plate in a final volume of 250 µL. To minimize bacterial contamination, penicillin/streptomycin was added to kill extracellular bacteria. (MOI 10) for 5 h in duplicate wells. At 1.5 h postinfection, penicillin-streptomycin was added to kill extracellular bacteria.

Microarray analysis and quantitative real-time PCR

For microarray analysis and quantitative real-time PCR, total RNA was extracted 5 h poststimulation or infection using the RNeasy Mini Kit including an on-column DNase treatment according to the manufacturer’s instructions (Qiagen). For microarray-based gene expression profiling, biotin-labeled cRNA of three independent experiments was hybridized to a whole-genome HumanHT-12 array from Illumina according to the manufacturer’s instructions, and Microarray scanning was carried out using an iScan array scanner. As a test for significance, a Bayes test was used on the bead expression values of the groups of interest. The average expression value was calculated as mean of the measured expressions of beads together with the standard deviation of the beads. Genes with a P-value < 0.05 were selected and log2 fold expression changes of differentially regulated genes were calculated. All analyses were performed using Chipster Software. Microarray data have been uploaded to GEO (accession number GSE125797). For quantitative real-time PCR, total RNA was reverse transcribed using 10 µL isolated RNA in a total reaction volume of 20 µL with a High Capacity cDNA RT Kit (Applied Biosystems) according to the manufacturer’s instructions. For quantitative real-time PCR analysis, SYBRGreen PCR Master Mix Fast (Applied Biosystems) was used. noRT and nontemplate controls as well as melt curve analysis served to specify control. Relative expression of target gene mRNA (for Primers see Table 1) was calculated by \( \Delta \Delta C_t \) compared to the indicated housekeeping gene.

Statistical analysis

Statistical significance between groups was determined by the two-tailed Student’s t-test or two-way ANOVA with Bonferroni posttest. For experiments comparing immune stimulation by RNA species derived from WT cells as compared to the respective Gm18 deficient mutant, significance was calculated using the linear mixed-effects model with Tukey’s rank test to account for donor variation. Differences were considered significant for (*) \( P < 0.05 \), (**) \( P < 0.01 \), (***) \( P < 0.001 \). Analyses were performed using Graph Pad Prism 7 (Graph Pad Software) or using R version 3.3.0 with the package nlme.

Supplemental material is available for this article.

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TABLE 1. List of primer sequences used for quantitative real-time PCR

| Target      | Forward (5′–3′) | Reverse (5′–3′) |
|-------------|----------------|----------------|
| β-actin     | AGAGCTACGGATGCAGCGAC | AGCAGTGTATTGGGATCAG |
| GAPDH       | ACGGATTGGTGATCGTGGGC | TGTGACGCTGTTGGGATCAG |
| IFN-β       | ATGACCAAAAGTGTCCTCC | GGAATCCAAAGCAAGTGTAGGCTC |
| IL-12p35    | CTCCTG3GACCACCTCAGTTCG | GGTGAAAGCGATGGGAAACTT |
| IL-12p40    | CGGGAGCTGCTACACTCTC | CCTGATCCCTCAGGGCAGAC |
| TNF         | GCCGAGCGAGCGATCATCTTC | TGAGGTACAGCGCCTCTGATGG |
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