A modified dinucleotide motif specifies tRNA recognition by TLR7

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ABSTRACT

RNA can function as a pathogen-associated molecular pattern (PAMP) whose recognition by the innate immune system alerts the body to an impending microbial infection. The recognition of tRNA as either self or nonself RNA by TLR7 depends on its modification patterns. In particular, it is known that the presence of a ribose methylated guanosine at position 18, which is overrepresented in self-RNA, antagonizes an immune response. Here, we report that recognition extends to the next downstream nucleotide and the effectively recognized molecular detail is actually a methylated dinucleotide. The most efficient nucleobases combination of this motif includes two purines, while pyrimidines diminish the effect of ribose methylation. The constraints of this motif stay intact when transposed to other parts of the tRNA. The results argue against a fixed orientation of the tRNA during interaction with TLR7 and, rather, suggest a processive type of inspection.

Keywords: RNA modification; TLR7; immunostimulation; innate immunity; ribose methylation

The innate immune system guards against microbial infections by a variety of alerting pathways that rely on recognition of pathogen-associated molecular pattern (PAMP) by pattern recognition receptors (PRR). Depending on its structure and content of nucleoside modifications, DNA and RNA may serve as PAMPs that can be recognized by a large variety of PRRs (Barbalat et al. 2011; Desmet and Ishii 2012). Among those, cytosolic receptors include RIG-I-like receptors (Mafee and Valvassori 1981; Yoneyama et al. 2004), further members of the DExD/H-box helicase superfamily (Zhang et al. 2011), inflammasome activators AIM2 (Burckstummer et al. 2009; Fernandes-Alnemri et al. 2009; Hornung et al. 2009) and NLRP3 (Kanneganti et al. 2006), cyclic GMP-AMP synthase (cGAS) (Ablasser et al. 2013; Sun et al. 2013), and STING (Ishikawa et al. 2009). Furthermore, endosomal sensing of phagocytosed microbes and thereof derived nucleic acids can be mediated by Toll-like receptors (TLRs), including TLR3 (Alexopoulou et al. 2001), TLR7 (Diebold et al. 2004; Heil et al. 2004), TLR8, TLR9 (Hemmi et al. 2000), and TLR13 (Dalpke and Helm 2012; Hidmark et al. 2012; Oldenburg et al. 2012). The principles of differential recognition of self and nonself RNA are a point of outstanding interest in the field. While the number of receptors by itself documents the importance of this issue, details of recognition on the molecular level remain elusive. Since RNA of self and nonself origin is composed of the same four basic ribonucleotide building blocks, selective recognition and discrimination may rely on particular features of sequence (Heil et al. 2004; Vollmer et al. 2005; Diebold et al. 2006; Forsbach et al. 2008), secondary/tertiary structure (Lan et al. 2009), and processing states of the 5′ and 3′ extremities, including, e.g., 5′-triphosphates (Hornung et al. 2006). Whereas it is now accepted that TLR7 recognition by RNA requires certain sequence orders (Hornung et al. 2005), an exact stimulatory motif is still ill-defined. Finally, a more recently discovered discriminatory property is post-transcriptional modification of nucleic acids. Several examples pertaining to the latter have demonstrated both the capability for effective inspection and intricate interaction by the innate immune system, as well as the importance of a deeper understanding of its molecular details (Krieg et al. 1995; Kariko et al. 2005; Robbins et al. 2007; Dalpke and Helm 2012).

Recently, several groups, including us, reported on the recognition of tRNA by the endosomal TLR7 (Gehrig et al. 2012; Jockel et al. 2012). In these studies, naturally occurring nucleoside modifications within tRNA have clearly been identified.
as structural anti-determinants for innate immune recognition. Immune activation via TLR7 can be triggered by an unmodified tRNA, as well as by many bacterial tRNAs that contain few modifications in comparison to the eukaryotic tRNA counterparts. The relative abundance of modifications in eukaryotic tRNA appears to be a key feature (Madore et al. 1999; Machnicka et al. 2013), but the picture is rather incomplete, since many eukaryotic modifications also occur in bacteria. A case in point is the Gm18 modification, which was shown to act as an antagonist in TLR7-mediated signaling, even when present in bacterial tRNA. Gm denotes a ribose methylation on the 2′-hydroxyl function of a guanosine residue in the structural core of the typical L-shaped tRNA. Its primary function is likely a structural stabilization of this region (Motorin and Helm 2010; Ochi et al. 2010; Ishida et al. 2011), whereas a function in immune recognition is assumed to have evolved as a secondary feature. Whereas this modification is found in many archaea, bacteria, and eukaryotes, it occurs only in a subset of five tRNA species from *Escherichia coli* (Jühling et al. 2009).

Upon substitution of Gm and its downstream neighbor nucleoside, we observed highly interesting effects, which persist even when the modification is transplanted into parts of *E. coli* tRNA^{Tyr} where it does not naturally occur. We constructed a number of modivariants (Madore et al. 1999) by splint ligation of synthetic oligoribonucleotides and tested the corresponding TLR7-mediated response. Therefore, peripheral blood mononuclear cells (PBMCs) from at least five human donors were stimulated with DOTAP encapsulated tRNA preparations. An ELISA assay (Gehrig et al. 2012) was used measuring interferon α (INF-α) by plasmacytoid dendritic cells (pDC) to determine TLR7 activity.

Mutations at position 18, surprisingly, revealed that the nature of the nucleobases is less important (Fig. 1A); all variations of nucleobases in combination with a methylated ribose and a downstream purine lead to efficient and highly significant silencing.

Cytidine 18 is an exception to some degree in that it does lead to silencing but less efficiently and with strong variation among the five donors. We previously reported similar but less pronounced effects of ribose-methylated cytidine in synthetic siRNA, where permethylated ribose modification of adenosine and uridine, but not of cytidine, decreased immunostimulation (Eberle et al. 2008). We conclude that the ribose methylation itself is recognized along with structural features other than the nucleobase #18. This unexpected finding prompted us to investigate permutations of the nucleoside at position 19, i.e., directly downstream from the methylated ribose. These experiments revealed that ribose methylation at position 18 by itself is not sufficient but highly efficient in combination with a downstream purine (Fig. 1B). This combination corresponds to the native occurrences of Gm18 in tRNA (Jühling et al. 2009), such as Gm18-G19. The presence of pyrimidines at the +1 position is clearly detrimental to the immunosilencing effect. The effect is milder for uridine (GmU19) but quantitative for cytidine (GmC19).

Together, these results imply a functional [DmR] motif (D = all but C; R = purine) featuring a methylated ribose in a dinucleotide unit with a 3′ purine. To verify this hypothesis, we moved the ribose methylation downstream by 1 nt unit within the native tRNA sequence. The resulting construct is identical in sequence and molecular weight to the immunosilent parent Gm18 tRNA and differs only in the position of the ribose methylation by ~5 Å. The direct comparison in Figure 1C shows that the silencing by ribose methylation is negatively affected in this construct, again with noticeable donor variation. Strikingly, as also shown in Figure 1C, it can be functionally recovered by reconstituting the intact methylated dinucleotide signal [DmR] via insertion of a guanosine 3′ to the ribose methylation at position 20.

The above results correspond to a transposition of the [DmR] motif by 1 nt toward the 3′, and the fact that it remains functionally intact upon transposition triggers the question if it can be moved to a completely different structural context within the same tRNA scaffold. Hence, the [DmR] motif was transplanted into two other loop regions of the tRNA: position 34, where it naturally occurs in eukaryotic tRNAs such as tRNA^{Phe} (Jühling et al. 2009), and position 57, where it has not been detected so far. Figure 1, D and E shows that the results of permutation studies of methylated nucleosides at these positions strongly resemble those conducted at position 18, with only minor variations.

The common denominators of permutation studies at all three locations, i.e., the D-loop, the anticodon loop, and the T-loop, are as follows: (1) Purine bases on 3′ side of the methylated ribose produce maximum silencing efficiency; (2) pyrimidines are generally detrimental to silencing; (3) with cytidine more so than uridine; and (4) donor variation is in evidence, which is especially noticeable for dinucleotides containing pyrimidines.

The observed ranking of stimulation efficiency stays intact at different concentrations, as illustrated in Figure 2, where a titration with increasing amounts of the four principle modivariants Gm18, Am18, Um18, and Cm18 is normalized to the effect of the unmodified in vitro transcript. Furthermore, it was verified that the nonstimulatory effect is also an inhibitory effect, by cotransfection of a selection of modivariants with stimulating in vitro transcript (Fig. 3). Note the correlation of immunosilent modivariants (Fig. 1) with immunosuppressing modivariants in Figure 3. Indeed, the relative suppression of IFN-α secretion by modivariants adhering to the above mentioned common denominators is faithfully reproduced in the assays shown in Figure 3, which now represent actual immunosuppression experiments. Rather than just being immunosilent, i.e., not recognized by TLR7, the modivariants antagonize the immunooactivating properties of the unmodified in vitro transcript.

We conclude that we have identified a dinucleotide motif which includes a post-transcriptional methylation as the...

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decisive structural element for an inhibition of TLR7-mediated immune activation. This bears some resemblance to the reported effects of the presence of 5-methylcytidine in CpG motifs on recognition of DNA by TLR9, which is also an endosomal nucleic acid-dependent PRR (Hemmi et al. 2000). While the extent of further similarities is unclear, the structure of tRNA used in our case helps to further our understanding of the actual recognition event somewhat. When comparing the X-ray structure (Liu et al. 2008) of a 40-mer double-stranded RNA complexed to TLR3, a tRNA molecule...
spans a distance long enough to sustain a receptor dimerization as observed with TLR3. However, structures of TLR8 alone and in complex with a small molecule effector strongly show that recognition, dimerization, and downstream signaling can, in principle, be affected by molecules that do not span the entire width of the receptor (Tanji et al. 2013). In keeping with this, TLR7 signaling is actively antagonized by the presence of our [DmR] motif at several positions within the tRNA structure, implying several different functional orientations of the tRNA during TRL7 recognition. This, in turn, means that any such interaction cannot be of completely static nature. In this respect, our data point to a processive type of RNA inspection, where TLR7 scans different regions of the tRNA molecule until stuck at the dinucleotide motif.

MATERIALS AND METHODS

Synthesis of modivariants

Splinted ligation was performed by annealing three synthetic fragments of RNA (IBA Göttingen; 5′ fragment: GGUGGGGUUCCC GAGCCGCAAAAG; middle fragment: GGAGCAUGUUCGAU CUCUCUCACACACACCA; 3′ fragment: ACUUCGAAGGUUCGAAU CUCUUCCCAACC). Corresponding in sequence to unmodified E. coli [tRNA] or its modivariants/mutants onto a reverse complementary oligodeoxynucleotide (IBA Göttingen; TGGTGGGGGGGAAGGATTCGAACCTTCGAAGTCTGTGACGGCAGATTT ACAGTCTGCTCCCTTTGGCCGCTCGGGAACCCCACC). Appropriate fragments (4 nmol) were 5′-phosphorylated by incubating in KL buffer (50 mM Tris-HCl [pH 7.4], 10 mM MgCl2) supplemented with 5 mM ATP, 5 mM DTT, and 0.75 units/µL T4 polynucleotide kinase (PNK, Fermentas) in a final volume of 150 µL in the thermomixer at 37°C for 1 h. To the phosphorylation reaction mixture, an equimolar amount of the 5′ fragment and the DNA splint were added, as well as KL buffer, ATP (5 mM), and DTT (5 mM), leading to a final volume of 500 µL and an 8 µM concentration of each fragment. The RNA fragments were hybridized to the DNA splint by heating to 75°C in the thermomixer for 4 min and letting the reaction mixture cool down to room temperature for 15 min.

Then, T4 DNA ligase (1.5 units/µL; Fermentas) and T4 RNA ligase 2 (22 ng/µL) were added, and the ligation was performed in the thermomixer at 16°C overnight. Template DNA was removed by addition of 1.5 units/µL DNase I (Fermentas), followed by 1 h of incubation at 37°C. The tRNAs were purified from ligation mixtures by denaturing PAGE, excised and eluted from the gel, and precipitated with ethanol. Concentrations were calculated from absorption at 254 nm, as determined on a Nanodrop ND-1000 spectrometer after resuspension in water.

Stimulation of peripheral blood mononuclear cells with tRNAs

Human PBMCs were isolated from heparinized blood of healthy donors upon informed consent by standard Ficoll-Hypaque density-gradient centrifugation (Ficoll 1.078 g/mL) (Eberle et al. 2008). PBMCs were resuspended in complete medium prepared of RPMI 1640 supplemented with heat-inactivated (1 h, 56°C) 10% FCS. Five hundred nanograms of tRNA sample was diluted in a volume of 50 µL. The RNA was encapsulated with 2 µL of 1 mg/mL DOTAP (N-[1-(2, 3-dioleoyloxy)propyl]-N, N, N-trimethylammonium methysulfate) (Roth) by mixing with serum-free medium and incubation for 10 min. Cells were plated on top of the transfection mixture at 4 × 10^5 cells/well in a 96-well flat-bottom plate. PBMCs were incubated in a humidified 5% CO2 atmosphere at 37°C for 16 h. As an internal positive control, PBMCs were stimulated with the TLR9-specific stimulus CpG2216 (1 µM) and the TLR7/8-agonist R848 (1 µg/mL) (InvivoGen). Cell-free supernatant was analyzed for secretion of IFN-α using a sandwich ELISA (Bender MedSystems).

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REFERENCES

Ablasser A, Goldeck M, Cavar T, Deimling T, Witte G, Rohl I, Hopfner KP, Ludwig J, Hornung V. 2013. cGAS produces a 2′-5′-linked cyclic dinucleotide second messenger that activates STING. Nature 498: 380–384.

Alexopoulou L, Holt AC, Medzhitov R, Flavell RA. 2001. Recognition of double-stranded RNA and activation of NF-κB by Toll-like receptor 3. Nature 413: 732–738.

Bakalar B, Ewald SE, Mouchess ML, Barton GM. 2011. Nucleic acid recognition by the innate immune system. Annu Rev Immunol 29: 185–214.

Burkstummer T, Baumann C, Bluml S, Dixit E, Durnerburg G, Jahn H, Planovsky M, Bilban M, Colinge J, Bennett KI, et al. 2009. An orthologous proteomic-genomic screen identifies AIM2 as a cytoplasmic DNA sensor for the inflammasome. Nat Immunol 10: 266–272.

Cantara WA, Crain PF, Rozenski J, McCloskey JA, Harris KA, Zhang X, Helm M. 2012. RNA mediated Toll-like receptor stimulation activates caspase-1 through cryopyrin/Nalp3. Nature 460: 233–236.

Diebold SS, Kaitho T, Hemmi H, Akira S, Reis e Sousa C. 2006. A receptor for bacterial RNA. Nature 438: 266–270.

Diebold SS, Massacrier C, Akira S, Conzelmann KK, Schlee M, et al. 2006. Synthetic oligoribonucleotides containing secondary structures act as agonists of Toll-like receptors 7 and 8. Biochim Biophys Acta 1762: 443–448.

Eberle F, Giessler K, Deck C, Heeg K, Peter M, Richert C, Dalpke AH. 2010. tRNA stabilization by modified nucleotides. Biochemistry 49: 4934–4944.

Heil F, Hemmi H, Hochrein H, Aumannberger F, Kirschning C, Akira S, Lipford G, Wagner H, Bauer S. 2004. Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. Science 303: 1526–1529.

Hemmi H, Takeuchi O, Kawai T, Sato S, Sanjo H, Matsumoto M, Hoshino K, Wagner H, Takeda K, et al. 2000. A Toll-like receptor recognizes bacterial RNA. Nature 408: 740–745.

Ishikawa H, Ma Z, Barber GN. 2009. STING regulates intracellular DNA-mediated, type 1 interferon-dependent innate immunity. Nature 461: 788–792.

Ishida K, Kunibayashi T, Tomikawa C, Ochi A, Kanai T, Hirata A, Iwashita C, Horii H. 2011. Pseudouridine at position 55 in tRNA controls the contents of other modified nucleotides for low-temperature adaptation in the extreme-thermophilic eubacterium Thermus thermophilus. Nucleic Acids Res 39: 2304–2318.

Jockel S, Nees G, Sommer R, Zhao Y, Cherkasov D, Hori H, Ehm G, Schnare M, Nain M, Kaufmann A, et al. 2012. The 2′-O-methylation status of a single guanosine controls transfer RNA-mediated Toll-like receptor 7 activation or inhibition. J Exp Med 209: 235–241.

Jühling F, Morl M, Hartmann RK, Sprinzl M, Stadler PF, Pütz J. 2009. RNAdb 2009: Compilation of tRNA sequences and tRNA genes. Nucleic Acids Res 37(Database issue): D159–D162.

Kanegangi TD, Ozoren N, Body-Malapel M, Amer A, Park JH, Franchi L, Whitfield J, Barchet W, Colonna M, Vandenabeele P, et al. 2006. Bacterial RNA and small antiviral compounds activate caspase-1 through cryopyrin/Naip3. Nature 440: 233–236.

Kariko K, Buckstein M, Ni H, Weissman D. 2005. Suppression of RNA recognition by Toll-like receptors: the impact of nucleoside modification and the evolutionary origin of RNA. Immunology 23: 165–175.

Krieg AM, Yi AK, Matson S, Waldschmidt TJ, Bishop GA, Teasdale R, Koretzky GA, Klinman DM. 1995. CpG motifs in bacterial DNA trigger direct B-cell activation. Nature 374: 546–549.

Lan T, Putta MR, Wang D, Mai D, Yu V, Dandimalla ER, Agrawal S. 2009. Synthetic oligoribonucleotides-containing secondary structures act as agonists of Toll-like receptors 7 and 8. Biophy J 96: 2140–2147.

Liu L, Botos I, Wang Y, Leonard JN, Shioach J, Segal DM, Davies DR. 2008. Structural basis of toll-like receptor 3 signaling with double-stranded RNA. Science 320: 379–381.

Machnicka MA, Milanowska K, Osman Ogлу O, Purta E, Kurkowski M, Olchowik A, Januszewski W, Kalinowski S, Dunin-Horkawicz S, Rother KM, et al. 2013. MODOMICS: A database of RNA modification pathways—2013 update. Nucleic Acids Res 41(Database issue): D262–D267.

Madore E, Florentz C, Giegé R, Sekine S, Yokoyama S, Lapointe J. 1999. Effect of modified nucleotides on Escherichia coli RNA polymerase structure and on its aminoacylation by glutamyl-tRNA synthetase. Predominant and distinct roles of the mmn5 and s2 modifications of U34. Eur J Biochem 266: 1128–1135.

Maeve MF, Valvassori GE. 1981. Radiology of the craniofacial anomalies. Laryngol Clin North Am 14: 939–988.

Morton Y, Helm M. 2010. tRNA stabilization by modified nucleotides. Biochemistry 49: 4934–4944.

Ochi A, Makabe K, Kuwajima K, Hori H. 2010. Flexible recognition of the tRNA G18 methylation target site by TrmH methyltransferase through first binding and induced fit processes. J Biol Chem 285: 9018–9029.

Oldenburg M, Kruger A, Fersl R, Kaufmann A, Nees G, Sigmund A, Bathe K, Lauterbach H, Suter M, Dreher S, et al. 2012. TLR13 recognizes bacterial 235-RNA devoid of erythromycin resistance-forming modification. Science 337: 1111–1115.

Robbins M, Judge A, Liang L, McClintock K, Yaworski E, MacLachlan M. 2007. 2′-O-methyl-modified RNAs act as TLR7 antagonists. Mol Ther 15: 1663–1669.

Sun L, Wu J, Du F, Chen X, Chen ZJ. 2013. Cyclic GMP-AMP synthase is a cytosolic DNA sensor that activates the type I interferon pathway. Science 339: 786–791.

Tanji H, Ohuta U, Shibata T, Miyake K, Shimizu T. 2013. Structural reorganization of the Toll-like receptor 8 dimer induced by agonistic ligands. Science 339: 1426–1429.

Vollmer J, Thuk S, Schmitz C, Hann M, Jurk M, Forbach A, Akira S, Kelly KM, Reeves WH, Bauer S, et al. 2005. Innate stimulation mediated by autoantigen binding sites within small nuclear RNAs involves Toll-like receptors 7 and 8. J Exp Med 202: 1573–1585.

Yoneyama M, Ikuczki M, Nakanawa T, Shinobu N, Imazumi T, Miyagishi M, Taira K, Akira S, Fujita T. 2004. The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. Nat Immunol 5: 730–737.

Zhang Z, Kim T, Bao M, Facchinetti V, Jung SY, Ghaffari AA, Qin J, Cheng G, Liu YJ. 2011. DDX1, DDX21, and DDX36 helicases form a complex with the adaptor molecule TRIF to sense dsRNA in dendritic cells. Immunity 34: 866–878.