Review Article

Extracellular tRNA-derived RNAs as emerging activators of endosomal Toll-like receptors: a narrative review

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Background and Objective: The innate immune system deploys various pattern-recognition receptors (PRRs), including Toll-like receptors (TLRs), to detect the invasion of pathogens and initiate protective responses. TLR7 and TLR8 are located within the endosome of immune cells and are activated by single-stranded RNAs (ssRNAs). In addition to foreign ssRNAs from bacteria and viruses, endogenous self-ssRNAs, such as microRNAs (miRNAs), have been shown to activate TLR7 and TLR8, but such endogenous ssRNA ligands have not yet been fully elucidated. This scientific knowledge gap is partly derived from the technical limitations of standard RNA-seq, particularly its inability to capture non-miRNA-short non-coding RNAs (sncRNAs) lacking 5'-phosphate and 3'-hydroxyl ends. However, recent advances in our understanding of "previously-hidden" sncRNAs, captured by RNA-seq using T4 polynucleotide kinase-treated RNA samples, have widened the pool of candidate ssRNA molecules that could act as endogenous ligands of ssRNA-sensing immune receptors. This has indeed been exemplified in the recent finding that, during immune response, transfer RNA (tRNA)-derived sncRNAs in macrophages are packaged into extracellular vesicles (EVs), and those tRNA-derived extracellular (ex-) sncRNAs can function as endogenous ligands of TLR7 when delivered to the endosome of recipient cells. In this review, we highlight advances in our understanding of the functional roles of tRNA-derived ex-sncRNAs as emerging activators of endosomal TLRs.

Methods: We searched PubMed for articles relevant to our topic and published prior to September 2022.

Key Content and Findings: tRNA-derived ex-sncRNAs could act as endogenous modulators of the immune system by activating endosomal TLRs in diverse pathological conditions, ranging from bacterial infection to cancers and neurological disorders.

Conclusions: tRNA-derived ex-sncRNAs may constitute a significant class of bioactive circulating RNAs, and they are promising candidates as biomarkers in various diseases. Further research is required to realize their full implication in human health and disease.

Keywords: tRNA-derived RNA; transfer RNA half (tRNA half); extracellular RNA; endosomal TLR; Toll-like receptor 7 (TLR7)

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Introduction

One of the first lines of defense against invading pathogens begins when the pattern-recognition receptors (PRRs) of the innate immune system detect pathogen-associated molecular patterns (PAMPs), leading to initiation of protective responses (1,2). Toll-like receptors (TLRs) are the most extensively studied PRRs, and are expressed in both innate immune cells (e.g., macrophages and dendritic cells) and non-immune cells (e.g., epithelial cells and fibroblast cells) (2-4). Among the 10 TLRs characterized in humans,

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Table 1 Search information

Items	Specification
Date of search	August 31, 2022
Databases and other sources searched	PubMed
Search terms used	tRNA, tRNA half, tRF, tsRNA, exRNA, circulating RNA, immune response, TLR, TLR7, TLR8, neurodegeneration, cancer
Timeframe	1986–2022
Inclusion and exclusion criteria	Research and review manuscripts written in English were included, and articles written in a language other than English were excluded
Selection process	Both authors performed the search for suitable articles

TLR1, TLR2, TLR4, TLR5, TLR6, and TLR10 localize to the cell surface (surface TLRs), while TLR3, TLR7, TLR8, and TLR9 localize to endosomes and other intracellular compartments (endosomal TLRs). Each TLR specifically recognizes a different PAMP, ranging from polysaccharides and lipopeptides to nucleic acids (2-4). Endosomal TLRs are nucleic acid-sensing TLRs, and among those, TLR7 and TLR8 recognize single-stranded RNAs (ssRNAs) as their ligands (2-5). ssRNA recognition by these TLRs results in the recruitment of the adaptor protein MyD88, leading to NF-κB-mediated transcription and downstream induction of interferon and cytokine production (2-4). While "foreign" ssRNAs from bacteria or viruses have been extensively studied as the ligands recognized by TLR7 and TLR8 (5-10), "endogenous" ssRNA ligands from host cells have not been fully characterized yet, and indeed the idea of endogenous ligands of PRRs remains controversial, as it complicates the traditional view of the role of PRRs in distinguishing self from non-self (11).

Once endogenous ssRNA molecules are packaged into extracellular vesicles (EVs), they can be delivered into the endosomes of recipient cells, where they can be sensed by TLR7 or TLR8. This has been observed with certain extracellular (ex-) microRNAs (miRNAs), which, upon delivery to recipient cell endosomes, become ligands for TLR7 and TLR8 and activate the downstream pathway (12,13). This miRNA-mediated TLR7/TLR8 activation is relevant not only to immune response (14,15) but has also been demonstrated to play roles in neurodegeneration (12), tumor growth and metastasis (16,17), autoimmunity (18), and pathobiology of various other diseases (19). Because miRNAs are the best-studied short non-coding RNAs (sncRNAs), it is natural that they have dominated current research on both ex-sncRNAs and endogenous ssRNA ligands of TLRs. However, recent advances in our understanding of "previously-hidden" sncRNAs have widened the pool of candidate ssRNA molecules that could act as endogenous ligands of ssRNA-sensing immune receptors, exemplified in the recent finding that transfer RNA (tRNA)-derived sncRNAs can function as endogenous ligands of TLR7 (20). We present this article in accordance with the Narrative Review reporting checklist (available at https://exrna.amegroups.com/article/view/10.21037/exrna-22-22/rc).

Methods

To identify articles suitable for this review, we conducted a literature search via PubMed using search terms that included "tRNA", "tRNA half", "tRF", "tsRNA", "exRNA", "circulating RNA", "immune response", "TLR", "TLR7", "TLR8", "neurodegeneration", and "cancer". Articles written in English and published prior to August 31, 2022 were considered. These details are compiled in *Table 1*.

Most ex-sncRNAs are uncaptured by standard RNA-seq

Cellular sncRNA molecules generally possess either a hydroxyl group (OH), a monophosphate (P), or a 2',3'-cyclic phosphate (cP) at their termini (*Figure 1A*), and the terminal states of each sncRNA are determined by the catalytic machinery underlying the RNA cleavage that produces them (21,24). Although next-generation sequencing of RNA molecules (RNA-seq) has become a common tool to characterize RNA expression profiles, most sncRNA sequencing studies to date have relied on a standard small RNA-seq method in which 5'- and 3'-adaptors (AD) can be ligated only to 5'-P and 3'-OH ends of RNAs, respectively.

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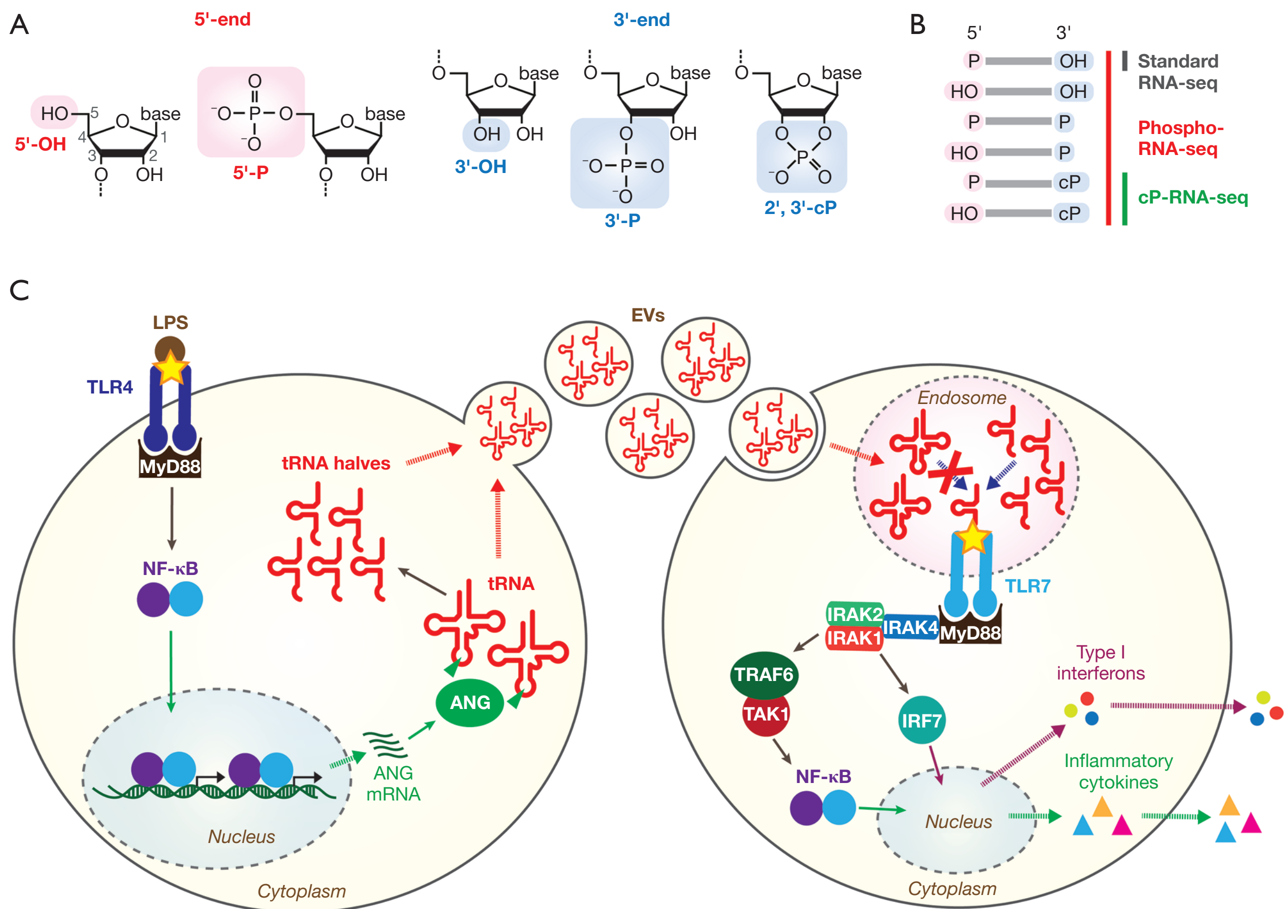

Figure 1 Infection-induced ex-5'-tRNA halves activate TLR7. (A) Chemical structures of general sncRNA termini [modified from our previous review (21)]; (B) targeted sncRNAs in standard RNA-seq, Phospho-RNA-seq, and cP-RNA-seq; (C) surface TLR stimulation culminates in activation of NF-κB, leading to upregulation of ANG, which cleaves the anticodon-loops of tRNAs. The resultant 5'-tRNA halves are secreted into EVs, and then the ex-5'-tRNA halves are delivered into endosomes in recipient cells and activate TLR7. Following TLR7 activation, signal transduction proceeds through MyD88, leading to activation of a protein complex that includes members of the IRAK family and the TRAF family, along with IKKα (22). Activation of IRAKs, particularly IRAK1 and IRAK2, and of TRAF6 leads to activation of IRF7 and its subsequent translocation into the nucleus, where it promotes transcription of IFN (23). Formation of a protein complex including TRAF6 and the TAK1 leads to downstream activation of NF-κB via degradation of its inhibitor. Liberated NF-κB can then migrate to the nucleus, where it induces expression of inflammatory cytokines and promotes cellular immune response. TLR, Toll-like receptor; IRAK, IL-1R associated kinase; TRAF, tumor necrosis factor receptor-associated factor; IKKα, IκB kinase α; IRF7, interferon regulatory factor 7; IFN, type I interferon; TAK1, transforming growth factor-β-activated kinase 1.

While this standard method is suitable for efficient amplification and sequencing of 5'-P/3'-OH-containing sncRNAs, such as miRNAs, it cannot capture RNAs with other terminal structures (i.e., 5'-OH, 3'-P, or cP) (*Figure 1B*). Due to this limitation, non-miRNA-sncRNAs lacking the 5'-P/3'-OH ends have been significantly underrepresented in many of the current sncRNA analyses, and thus comprise a largely unexplored component in the transcriptome.

This notion is especially important when it comes to the sequencing analyses of ex-sncRNAs. Human plasma samples contain many ex-sncRNAs which lack 5'-P or 3'-OH and are therefore uncaptured by standard RNA-seq (25). To sequence ex-sncRNAs with any given set of terminal phosphate states, Giraldez *et al.* developed Phospho-RNA-seq (25) (*Figure 1B*), which begins with the treatment of

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RNA samples with T4 polynucleotide kinase (T4 PNK). Because T4 PNK possesses both 5'-phosphorylation (convert 5'-OH to 5'-P in the presence of ATP) and 3'-dephosphorylation (convert 3'-P/cP to 3'-OH) activities, the T4 PNK treatment leaves all RNA species with the 5'-P/3'-OH-ends, thus rendering them available for 5'- and 3'-AD ligation in subsequent small RNA-seq procedure. Phospho-RNA-seq has been successfully used to profile the expression of ex-sncRNAs in human plasma samples and their tissue specific signatures (25). Other human plasma sequencing studies also showed that the addition of T4 PNK treatment to the sequencing procedure significantly altered the proportion of the reads of the sncRNAs derived from tRNAs, messenger RNAs (mRNAs), ribosomal RNAs (rRNAs), and other non-coding RNAs (26,27), corroborating that standard RNA-seq without T4 PNK treatment cannot fully capture these ex-sncRNAs. Our study on EVs secreted from human monocyte-derived macrophages (HMDMs) further confirmed the necessity of T4 PNK treatment in ex-sncRNA sequencing (20). T4 PNK treatment was required to amplify the majority of the cDNAs derived from the EV ex-sncRNAs. Treatment with a mutant T4 PNK, which lacks 3'-dephosphorylation activity, resulted in dramatic reduction of cDNA yield, suggesting that most ex-sncRNAs in HMDM EVs are 3'-P- or cP-containing RNAs, and miRNAs and other 3'-OH containing RNAs comprise only a small part of those ex-sncRNA species packaged into the EVs (20). Based on these findings, recent ex-sncRNA sequencing studies have been utilizing T4 PNK treatment to make terminal formation of RNA molecules consistently 5'-P and 3'-OH, allowing for the capture of the full range of ex-sncRNAs.

tRNA-derived sncRNAs as a component of the expanding sncRNA world

It is well established that tRNAs, canonically understood as adaptor molecules of translational machinery, are also a source of abundant functional sncRNAs (28-31). The tRNA-derived sncRNAs are generated from mature tRNAs or their precursor transcripts, not as random degradation products but as specific functional molecules, and can be classified into two groups: tRNA halves and tRNA-derived fragments (tRFs) (28-31). The 30–35-nt tRNA halves are generally much more abundant than tRFs and are produced by endonucleolytic cleavage of the tRNA anticodon-loop. In mammalian cells, angiogenin (ANG), a member of the RNase A superfamily, is one of the major enzymes responsible for the anticodon cleavage (32,33). Because ANG-mediated cleavage leaves a cP and 5'-P in 5'- and 3'-cleavage products (34), respectively, 5'-tRNA halves contain a 5'-P (from mature tRNAs) and a cP, while 3'-tRNA halves possess a 5'-OH and an amino acid (AA) at the 3'-end (from mature tRNAs), which has been experimentally validated (35). These terminal formations make tRNA half molecules the aforementioned "hidden class" of sncRNAs that are uncaptured by standard RNA-seq. Due to the presence of a cP in 5'-tRNA halves, a specific sequencing method for cP-containing RNAs, termed cP-RNA-seq (*Figure 1B*), has been utilized for the expressional characterization of 5'-tRNA halves (20,35-39). ANG-mediated tRNA cleavage can be induced by various biological factors/phenomena such as stress stimuli (32,33,38) and sex hormone signaling pathways (35). Although ANG is bound by its inhibitor ribonuclease/angiogenin inhibitor 1 (RNH1) in the cytoplasm, under stress conditions, degradation of RNH1 and/or translocation of ANG to stress granules, where it dissociates from RNH1, enable ANG-mediated tRNA cleavage (40-42). tRNA halves function in stress granule formation (43), regulation of translation (32,44), and promotion of cell proliferation (35); they are also associated with various disorders such as neurodegeneration (45,46). tRNA halves can further serve as direct precursors for shorter sncRNAs such as Piwi-interacting RNAs (piRNAs) (36,39).

tRFs are generally shorter than tRNA halves and can mainly be subclassified into 5'-tRFs, 3'-tRFs, and internal (i)-tRFs (28-31). While 5'- and 3'-tRFs are derived from 5'- and 3'-parts of mature tRNAs, respectively, i-tRFs are derived wholly from internal parts of mature tRNAs. Although Dicer and ANG are known to be involved in the biogenesis of some tRFs (47,48), detailed regulatory mechanisms and other biogenesis factors for tRFs remain elusive, leaving terminal phosphate states of tRFs undefined. This further emphasizes the importance of inclusive sequencing methods such as Phospho-RNA-seq for the comprehensive profiling of tRNA-derived sncRNAs. While many tRFs have been shown to function as miRNAs or piRNAs by binding to AGO or PIWI proteins (29,36,39), tRFs further have various functions, such as regulating mRNA stability or translation, preventing cell apoptosis, and promoting viral infection, and their dysregulation is involved in various diseases (28,30,31,49).

tRNA-derived sncRNAs as abundant ex-RNAs

tRNA-derived sncRNAs are increasingly recognized as an abundant class of ex-RNAs, released from cells under

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diverse conditions and packaged into EVs (50-52) or bound to RNA-binding proteins (RNPs) (53) or lipoprotein particles (LPPs) (54). Functional roles of some of these tRNA-derived ex-sncRNAs have also been identified. For example, ex-5'-tRNA halves and ex-5'-tRFs contained in the vesicles deriving from the epididymis, termed epididymosomes, are transferred to maturing sperm (55) and regulate gene expressions in embryonic stem cells and embryos (55-57). tRNA derived ex-sncRNAs further appear to have potential as biomarkers in various disease contexts (58). For example, tRNA-derived ex-sncRNAs are differentially accumulated in breast cancer (59), prostate cancer (60), lung cancer (61), and chronic kidney disease (62), compared to healthy individuals. For further discussion of tRNA-derived ex-sncRNAs, we recommend a review from Tosar and Cayota (63). Experimental results on tRNA-derived ex-sncRNAs have continuously accumulated; more recent studies have provided further evidence that tRNA-derived ex-sncRNAs could be useful prognostic and diagnostic biomarkers in breast cancer (64-66), and have also shown their differential accumulation in metastatic hypopharyngeal cancer (67), systemic lupus erythematosus (68), and ischemic kidney injury (69).

Infection-induced ex-tRNA halves activate TLR7

We recently identified a novel role of ex-tRNA halves as activators of endosomal TLR (20). We demonstrated that infection of HMDMs with *Mycobacterium bovis* BCG and surface TLR activation by treatment with lipopolysaccharide (LPS) or peptidoglycan (PGN) upregulate the NF-κB-mediated transcription of *ANG*, leading to accumulation of tRNA half molecules in HMDMs and their secreted EVs. In sequencing data of T4 PNK-treated RNAs from the EVs, 5'-tRNA halves comprised over 96% of tRNA-derived reads [in EV #1 library (20)], while 3'-tRNA halves (1.3%), 5'-tRFs (0.87%), 3'-tRFs (0.63%), and i-tRFs (0.99%) were minor species. The mechanisms underlying the specific and selective packaging of tRNA-derived sncRNAs into EVs are unknown, but specific RNA binding proteins, such as YBX1 that binds to 5'-tRNA halves (44) and functions in RNA sorting into EVs (70), could be involved in the mechanisms. Most ex-5'-tRNA halves were derived from a focused subset of cytoplasmic tRNAs, including tRNA$^{ValCAC/AAC}$, tRNA^{GlyGCC}, tRNA^{HisGUG}, and tRNA^{GluCUC}, which in aggregate were the sources of approximately 90% of the identified ex-5'-tRNA halves. One of the most remarkable characteristics of ex-tRNA halves is their abundance. The ex-5'-tRNA^{HisGUG} half was >210 times more abundant than miR-150, one of the most abundant miRNAs in HMDM-derived EVs (20). While it has been demonstrated that ex-miRNAs can act as ligands for endosomal TLRs, the much greater abundance of ex-tRNA halves suggests that they might constitute a more significant, biologically relevant class of endogenous ligands of these immune receptors. Indeed, the EV-contained ex-tRNA halves were experimentally confirmed to be delivered into endosomes in recipient HMDMs where the 5'-tRNA^{HisGUG} half strongly activates TLR7 (20). The activity of the 5'-tRNA^{HisGUG} half is as high as that of HIV-derived ssRNA40, a widely used positive control ssRNA known as a strong activator of endosomal TLRs (5), suggesting that 5'-tRNA^{HisGUG} half could have the capacity to produce an immune response.

Expressional induction and secretion of tRNA halves are not limited to cell culture settings but have been further observed in actual pathological situations. By developing a sensitive multiplex tRNA half quantification method, we revealed an approximately 1,000-fold enrichment of plasma ex-5'-tRNA halves in patients infected with *Mycobacterium tuberculosis* (20,71). A dramatic increase in the levels of serum ex-5'-tRNA halves has also been observed in LPS-injected mice and monkeys and in patients experiencing active, but not quiescent, hepatitis B virus infection (72). Because upregulation of 5'-tRNA half expression has been reported upon infection with respiratory syncytial virus (73,74), *Rickettsia* (75), and hepatitis B and C viruses (76), it seems possible that induction of 5'-tRNA halves and their secretion as ex-5'-tRNA halves could be a universal phenomenon among infectious diseases. These studies suggest a novel role of ex-5'-tRNA half molecules as “immune activators,” but further studies are required to fully unveil the immune response pathways mediated by tRNA-derived ex-sncRNAs.

Future perspectives

The study of endogenous ex-sncRNA ligands of immune receptors is still at an initial stage and remains an area of some controversy. Likewise, we are just beginning to appreciate the previously hidden classes of sncRNAs not detected by standard RNA-seq methods. We expect that the 5'-tRNA^{HisGUG} half is just the first example of an immune receptor-simulating tRNA-derived ex-sncRNA, and other tRNA halves and tRFs could be found to be similarly immunostimulatory through the TLR7 or TLR8 axis. The 5'-tRNA^{HisGUG} half strongly activates TLR7, but not

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TLR8 (20), probably because of differences in ligand specificity for TLR7 and TLR8 (6,77,78). Future exploration of immune stimulatory tRNA-derived sncRNAs should include review of studies looking into the characteristics of ssRNA ligands of TLR7 and TLR8.

Beyond tRNA-derived sncRNAs, our first genome-wide identification of cP-containing sncRNAs revealed abundant expression of rRNA- and mRNA-derived sncRNAs in various tissues (37), and, indeed, not only mature rRNAs (79) but also rRNA- and mRNA-derived sncRNAs have been shown to be abundantly secreted as ex-RNAs (26,80,81). These other unexplored classes of ex-sncRNAs should further be investigated as potential activators of TLR7 and TLR8.

Endosomal TLR3 also recognizes RNA molecules, but it binds to double-stranded RNAs (dsRNAs) (82). Although ssRNAs containing partial stem structures can also be TLR3 ligands, at least 40–50-base-pairs (bp) length of dsRNA molecule is required to ensure stable complex formation with TLR3 (83,84), and more than 90-bp length of dsRNA is required to activate TLR3 signal transduction in dendritic cell maturation (85). Therefore, ex-sncRNAs shorter than mature tRNAs are unlikely to participate in the TLR3 signaling. However, exRNAs more broadly should be considered as potentially major endogenous ligands of TLR3, as exemplified in a study showing that dsRNAs released from necrotic polymorphonuclear neutrophils induce inflammatory response in macrophages (86).

Although mature tRNAs have been reported to be incorporated into EVs (70,87), unlike the 5'-tRNA^{HisGUG} half, the full-length tRNA^{HisGUG} was incapable of stimulating endosomal TLR (20), possibly due to its rigid secondary and tertiary structures. Therefore, shortening mature tRNA^{HisGUG} into less-rigid 5'-half molecules by anticodon-cleavage is crucial to produce immunostimulatory sncRNAs. ANG is highly enriched in the EVs derived from aggressive brain tumor cells (52), while another RNase A superfamily member, RNase 1, has recently been shown to cleave non-vesicular tRNAs (88). Further research on immune responsive ribonucleases which cleave tRNAs, rRNAs, mRNAs, or other substrate RNAs to produce immune responsive ex-sncRNAs is necessary to fully understand the regulation and functional consequences of immune response mediated by these ex-sncRNAs.

TLR7 and TLR8 are involved in various biological processes and diseases (89). The symptom severity of COVID-19 is associated with TLR7 mutations (90-92). The genetic polymorphisms in TLR7 and TLR8 genes, which reduce the TLR activity, increase susceptibility to Mtb infection (93), and upregulation and stimulation of TLR7 in macrophages suppress Mtb growth (94,95). TLR7 and TLR8 have been implicated in the progression of Parkinson's disease (96) and in Alzheimer's disease (12,97,98), possibly playing a role in promoting neuroinflammation and autoimmunity. A recent study also linked a specific gain-of-function mutation in TLR7 with severe systemic lupus erythematosus (99). Furthermore, missense mutations in TLR7 have been reported in the tumors of a subset of esophageal adenocarcinoma patients (100), and cellular immune response through activation of TLR7 and TLR8 can be directed against tumors (101). Despite these known linkages between TLR7/TLR8 and pathobiology of diseases, the endogenous ligands of these ssRNA-sensing TLRs remain poorly understood. The growing evidence of TLR7 and TLR8 involvement in the pathogenesis of non-infectious diseases highlights the need for a better understanding of the endogenous ligands of these receptors. Further research into tRNA-derived ex-sncRNAs, aided by upgraded sequencing methods, is necessary to identify these ligands and to gain a fuller understanding of the interplay between ex-RNAs, TLRs, and the immune system in the body's response to diverse pathologies.

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Footnote

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Reporting Checklist: The authors have completed the Narrative Review reporting checklist. Available at https://exrna.amegroups.com/article/view/10.21037/exrna-22-22/rc

Conflicts of Interest: Both authors have completed the

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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