Activation of toll-like receptors by non-coding RNAs and their fragments (Review)

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Abstract. Non-coding RNAs are a diverse group of RNAs that are generally not translated into proteins, and control gene expression and other cellular processes through a myriad of mechanisms. Non-coding RNAs are fragmented to regulate cellular processes such as ribosomal RNA maturation, reverse transcription and degradation of nascent transcripts, among other functions. Non-coding RNAs and their fragments interact with Toll-like receptors (TLRs) to induce the activation of the innate and adaptative immune system, which are important for counteracting viral and bacterial infections as well as for triggering ischemia-related cytotoxic effects in the brain. As the study of these interactions progresses, novel functions are being elucidated, such as their participation in viral reactivation in the brain. Due to their importance as pattern recognition receptor families, TLRs may be potential therapeutic targets for the treatment of autoimmune diseases, viral diseases and cancer. TLR activators are currently used for the treatment of different types of cancer and several other biomolecules are still under investigation to progress towards clinical use. The ncRNAs and their fragments also function as ligands for TLRs, but further study of non-coding RNAs and their action on TLRs will allow the elucidation of new TLR agonists and antagonists to establish successful immunotherapies. The aim of the present review is to show the existing evidence on TLR activation by ncRNAs and their fragments, with special emphasis on the diseases in which they are involved and on the potential of the study of these interactions for the identification of therapeutic targets and development of therapies.

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1. Introduction

The majority of the human genome consists of regions that do not code for proteins and within these regions are the non-coding RNAs (ncRNAs), which are broadly classified as small ncRNAs (sncRNAs) and long ncRNAs (lncRNAs). Since their discovery, several studies have reported roles of ncRNAs as master regulators of gene expression by acting through innumerable mechanisms (1-4). Previous studies have shown that microRNAs (miRNAs; miRs), RNYs-a class of ncRNAs that are components of the Ro60 ribonucleoprotein particle-lncRNAs and fragments derived from certain ncRNAs physically interact with Toll-like receptors (TLRs), which results in oncogenesis (5), the induction and suppression of inflammatory processes in a neurodegenerative context (6), the induction of nephritis and lung disease (7), the elimination of bacterial infections (8) and the reactivation of the human immunodeficiency virus (HIV) (9). In addition to this, Rodríguez-Corona *et al* (10) predicted the physical interaction of RNYs and their fragments with TLRs and other cellular receptors, which indicate a range of signaling pathways and cellular processes that could be regulated by ncRNAs.

TLRs recognize unique microbial patterns known as pathogen-associated molecular patterns (PAMPs) (11-15) and damage-associated molecular patterns (DAMPs) that lead to the activation of the innate immune system. PAMPs have been associated with molecules derived from pathogenic bacteria and viruses (proteins and lipopeptides, LPS, RNA species, DNA and flagellin) that activate pattern recognition receptor (PRR) families; however, PRRs also respond to molecules from commensal organisms (16). Meanwhile, DAMPs are molecules associated with tissue damage (heat shock proteins, transcription factors, native RNA and DNA, tissue matrix components, such as hyaluronan and fibronectin and humoral proteins such as fibrinogen), which activate PRRs, particularly TLRs (17). To date, 10 TLRs have been identified in humans

(TLR1-10) (18) and 12 in mice (TLR1-9, TLR11-13) (19), which are expressed on the cell surface or in intracellular vesicles. Due to the importance of TLR activation in the induction of the innate and adaptive immune response, several studies have focused on the development of activators and inhibitors of these receptors for the treatment of various diseases (20-27). Currently, biomolecules are used for the treatment of bladder cancer (28,29) and several other biomolecules are still under study to establish their use in clinical practice (20-22). Previous studies reviewed information on the activation of TLRs by miRNAs (30) and by sncRNAs, proposing the sequential activation hypothesis in autoimmune diseases (22,31). The purpose of the present review was to describe the protective or harmful effects that activation of TLRs by ncRNAs has, including the activation of these receptors by lncRNAs and by fragments derived from ncRNAs. The present review described the involvement of TLR-ncRNAs/fragments interactions in other diseases such as cancer and viral infections, as well as in neurodegenerative and neurocognitive diseases, and discussed advances in the generation of TLR agonists and/or antagonists or ncRNA mimics or inhibitors for the treatment of these diseases. Advances in the study of ncRNAs/fragments-TLRs interaction could lead to the development of clinically useful immunotherapies in the future.

2. TLRs activation by ncRNAs

Currently, there is relatively little information about the physical interaction of ncRNAs with cellular receptors. Well-studied interactions include those between miRNAs and TLRs 7/8 and ribosomal RNAs (rRNAs) and RNYs with TLRs 13/2 and 3/7/8, respectively (5,7,32-37) and recent evidence indicates TLRs interaction with lncRNAs (38,39). In addition, Yang *et al* (40) demonstrated the miRNA-1 interaction with the pore-facing G-loop of Kir2.1 via the core sequence AAGAAC, which does not include the seed region of this miRNA. To the best of our knowledge, there is no evidence for the interaction of other ncRNA with TLRs or other cellular receptors. However, based on the existing evidence, it is possible that other ncRNAs can interact with a variety of cellular receptors (10).

miRNAs. miRNAs are sncRNAs with length of ~22 nucleotides (nt) in mature form (41-43). A study by Hansen *et al* (44) demonstrated the presence of miRNAs, with a size of 80-100 nt, known as Agotrons, which are bound to and stabilized by Argonaute (Ago) proteins in the cytoplasm. Although Agotrons also repress gene expression by interacting with the 3'UTR region of their target mRNA, their main function could be related to giving specificity to free Ago proteins by certain RNA species.

miRNAs are produced by diverse biogenic pathways, which seems to indicate that cells 'secure' the control of gene expression by miRNAs using diverse molecular elements for their synthesis and processing (45-47). The best characterized function of miRNAs is their binding to the 3'UTR region of their target mRNAs via the seed region (48); however, there are organisms that do not express Ago proteins (49), suggesting different modes of action for these RNAs. Indeed, miRNAs interact with specific promoters (50,51), regulate gene expression post-transcriptionally at the nuclear level (52), and act as ligands for Kir2.1 (40) and for TLRs 7 and 8.

miRNA-mediated activation of TLRs in cancer. TLRs recognize both PAMPs and DAMPs, where DAMPs are secreted by cells in response to tissue damage or cell death and their excessive release is associated with autoimmune diseases and cancer (48,49). In relation to this, an increase in the production of DAMPs (also called 'alarmins') has been observed in different types of cancer as (e.g., pancreatic cancer, breast cancer, lung cancer, among others) a response to cell death and chronic inflammation (53,54).

The involvement of TLRs in cancer is a complex issue as it involves several factors such as: i) Expression of specific TLRs (55,56); ii) expression of TLRs in specific cell strains (57); iii) mutagenesis (58); and iv) TLR adaptor proteins (59), among others. In spite of the aforementioned issues, certain ligands of TLRs 2,4,7 have been used for the treatment of various types of cancer (28,29,60) and several ligands have antitumor effects (61-63). The efficiency of TLR ligands as immunotherapeutic agents lies predominately in the initiation of T-cell immunity: Antigen uptake, processing and presentation, dendritic cell maturation and T-cell activation (64).

Activation of TLRs by miRNAs and their relation to cancer was first demonstrated by Fabbri *et al* (5), who demonstrated that activation of TLRs 7/8 by miRs-21 and -29a induced a prometastatic inflammatory response and promoted tumor growth and metastasis in a NF-κB-dependent manner. Similar to the observation of Heil *et al* (65), Fabbri *et al* (5) identified that GU-rich motifs were required for the activation of TLRs by miRNAs. Notably, survival of TLR7$^{-/-}$ mice was markedly longer compared with that of wild-type mice injected with Lewis lung carcinoma cells. In addition, depletion of tumor exosomes markedly attenuated the induction of tumor necrosis factor-α (TNF-α), interleukin (IL)-6 and early activation antigen CD69. This demonstrated the impact of activation of TLRs 7/8 by specific miRNAs and suggested the use of miRNA inhibitors to attenuate the effects in lung cancer; however, further studies are needed to translate these experimental results into clinical use for humans.

Studies subsequent to that of Fabbri *et al* (5) reported a relationship between TLRs-miRNAs, but superficially (32-34). For instance, He *et al* (32) demonstrated that TLR7 activation, in a c-Jun N-terminal kinase-dependent manner, by miR-21 induced myoblasts apoptosis in cancer cachexia. More recently, a feedback loop was demonstrated between prostaglandin 2 (PGE2) and the activation of TLRs 7/8 by circulating miR-574-5p (33). These authors suggested that decreased intracellular expression levels of both miR-574-5p and PGE2 were associated with the activation-dependent antitumor effects of TLRs 7 and 8; however, this needs to be validated experimentally. Finally, miR-16-5p was overexpressed in gastric cancer and its function as a ligand of TLRs was predicted using bioinformatics, but its biological impact is unknown. In addition, it was also postulated that regulatory networks, lncRNAs-miRNAs-mRNAs, were modified in gastric cancer (34).

Currently, understanding of TLR activation by miRNAs and their relationship with cancer is limited. Some differentially expressed miRNAs that function as ligands of TLRs have been identified in cancer (5,32-34), which could function as circulating biomarkers for the detection in various types of

cancer. The activation of TLRs by various ligands is known to have both pro- (66-68) and anti-tumor effects (69-71) and this depends on several factors, which should be further studied in depth in each type of cancer. The identification and establishment of all the molecular events underlying the activation of TLRs by miRNAs will likely result in the establishment of activators and/or inhibitors useful in clinical practice.

miRNAs and TLRs in myocardial ischemia. Free RNA concentration increases shortly after a transient myocardial ischemia event and the presence of RNases attenuates necrotic cell-induced cytokine production in cardiomyocytes and immune cells and reduces myocardial infarction after transient ischemia (72,73). Feng *et al* (12) reported that certain circulating miRNAs facilitated the production of specific cytokines, an effect that was markedly attenuated by: i) Mutating the uridines of miRs-133a, -146a and -208a by adenosines; ii) treatment with RNases; iii) eliminating the expression of TLR7 and that of the signal transduction adapter innate immune system (myeloid differentiation primary response 8, MyD88); or iv) using TLR7 antagonists. Similarly, the exposure of murine cardiomyocytes to extracellular vesicles (EVs) containing miR-146a-5p induced the production of macrophage inflammatory protein 2 (MIP-2), IL-6 and TNF-α in a TLR7-dependent manner. These effects were observed *in vivo*, and cytokine production and activation of the innate immune response, which was attenuated in mice lacking TLR7 (74).

Although there are still numerous studies that should be carried out, it is reasonable to consider that the increased expression of circulating RNA, particularly miRNAs, could be useful for the diagnosis of a myocardial ischemia event. It would be potentially beneficial to investigate whether the expression of other ncRNAs is also increased during these ischemic events and whether they function as TLR ligands. The search for and establishment of inhibitors of these miRNAs or TLR7 could be clinically useful for the treatment of this disease.

Involvement of miRNAs and TLRs in sepsis. Sepsis is a serious clinical condition that leads to organ dysfunction in the body caused by an uncontrolled host response to infection characterized by systemic inflammation, which is partially mediated by TLRs and certain miRNAs (75,76). Xu *et al* (75) demonstrated that specific circulating miRNAs that are secreted into EVs have a proinflammatory effect and stimulated the production of IL-6, TNF-α, interleukin-β and MIP-2. These effects were attenuated by miR-34a, miR-122 and miR-146a inhibitors, as well as in cells that did not express TLR7 or MyD88. Notably, injection of EVs from septic mice into wild-type mice promoted peritoneal neutrophil migration and this decreased in mice that did not express MyD88. Similarly, intrathecal administration of exogenous miR-146a triggered pulmonary inflammation, activated endothelium and increased endothelial permeability in a TLR7-dependent manner, indicating the involvement of miR146a-TLR7 in sepsis-associated acute respiratory distress syndrome (76).

Based on the aforementioned studies, several therapeutic strategies could be developed for the treatment of this clinical condition, such as the establishment of miRNA inhibitors, TLR7 antagonists or MyD88 inhibitors. In particular, some of these therapeutic strategies may be effective for the treatment of sepsis-associated acute respiratory distress syndrome.

miRNAs and TLRS in neonatal morbidity. Several lines of evidence indicate that certain miRNAs (miR-21a, miR-29a, miR-146a-3p and Let-7b) function as endogenous danger signals by activating TLRs 7/8, which are viral single strand RNA (ssRNA) sensors. Exposure to bacterial lipopolysaccharides (LPS) from human fetal membrane (FM) explants and wild-type mouse FM increased the expression of miR-146a-3p (77). In women with preterm birth and chorioamnionitis, increased expression of this miRNA was also observed and TLR8 activation by miR-146a-3p induced the IL-8 and IL-1β production in an LPS-dependent manner (77).

Involvement of miRNAs/TLRs 7/8 in coronavirus disease 2019 (COVID-19). COVID-19 is an infectious disease caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), producing symptoms such as fever, cough, dyspnea, myalgia and fatigue, among others (78). In severe cases, it is characterized by pneumonia, acute respiratory distress syndrome, sepsis and circulatory shock (79,80). In order to improve the understanding of the etiology of the inflammatory processes triggered by SARS-COV-2, Wallach *et al* (81) investigated whether RNA fragments of SARS-COV-2 could activate TLRs. In fact, several SARS-COV-2 RNA fragments, the majority of which were 22 nt in length, activated TLRs 7/8 and induced the release of cytokines from macrophages and microglia to induce the human immune response against the virus. Regarding this, Liao *et al* (82) observed that the SARS-CoV-2 spike protein activated platelets and induced the expression and secretion of miR-21 and let-7b in EVs to activate TLRs 7 and 8. The activation of these receptors induced p47phox phosphorylation and the NADPH oxidase activation in neutrophils, resulting in reactive oxygen species (ROS) production and the formation of hyperactive neutrophil extracellular traps, which was related to disease severity. Therefore, increased circulating expression of miRs-21 and -let-7b could be used as predisposing factors and the TLR7/8-miRNA axis as a therapeutic target for severe COVID-19.

TLR activation by miRNAs in the brain. Several studies indicate the aberrant release of miRNAs in neurodegenerative diseases, such as Alzheimer's (83-85) and Parkinson's disease (86-88); however, the physiological significance of this is currently unknown. To investigate the possible relationship of miRNAs and the establishment and/or progression of these diseases, Wallach *et al* (6) demonstrated that miRNAs secreted by cortical neurons induced the secretion of cytokines and chemokines by microglia and macrophages, which had a neurotoxic effect mediated by TLRs 7 and 8 (Fig. 1A and B). miRs 100-5p and 298-5p were internalized within microglia and activated endosomal TLR8 in a miRNA sequence-dependent manner, but not on its secondary structure (Fig. 1C). In a similar manner, these miRNAs also activated neuronal TLR7 and decreased cell viability in a dose-dependent manner (Fig. 1D). The presence of these miRNAs in the cerebrospinal fluid induced neurodegeneration and accumulation of microglia in the mouse cerebral cortex through TLR7 activation (Fig. 1E) (89). Similarly, in a murine model of sepsis, it was observed that the activation of TLR7/MyD88 by specific miRNAs activated the inflammatory processes in the brain and induced neuronal apoptosis (90).

In addition, TLR7 and miR-let7b co-localize in hippocampal CA1 neurons, suggesting the TLR7 activation by this

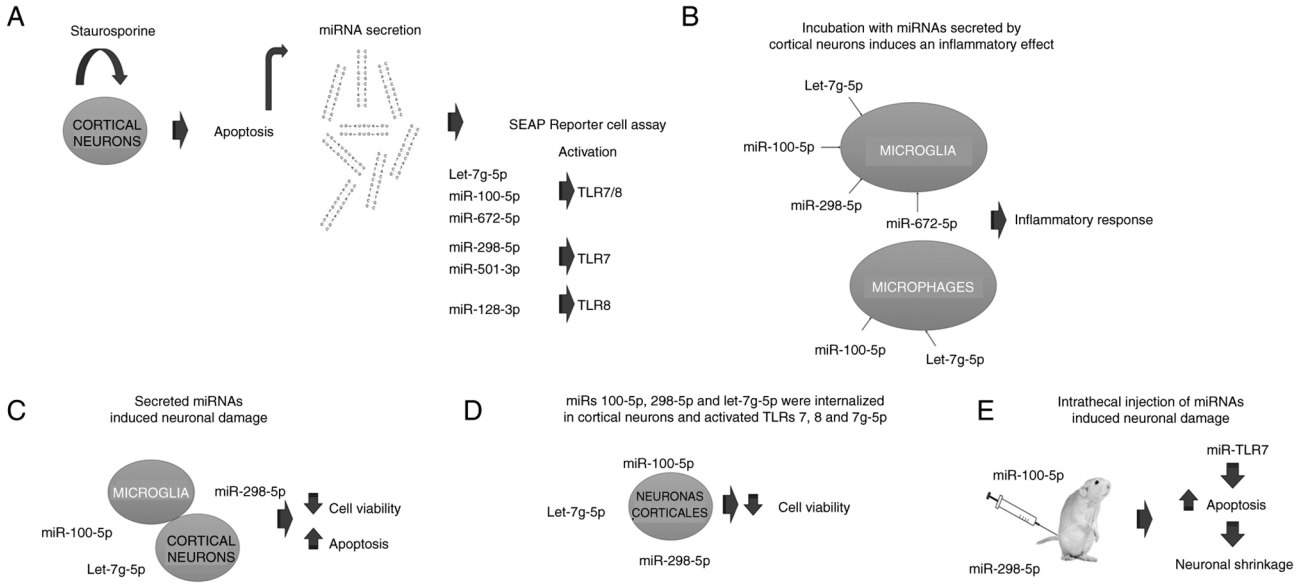

Figure 1. miRNAs secreted by cortical neurons activate TLRs 7 and 8 and induced neuronal damage (6,89). (A) Apoptotic cortical neurons induce the secretion of specific miRNAs, which activate particular TLRs *in vitro*. (B) Incubation of microglia and macrophage cell cultures with miRNAs secreted by cortical neurons have a neurotoxic effect. (C) The miRs-100-5p, -298-5p and let-7g-5p are secreted by cortical neurons and internalized by microglia. These miRNAs activate endosomal TLR8, induce apoptosis and decrease cell viability. (D) The miRNAs secreted by cortical neurons act on neuronal TLRs 7 and 8 to decrease neuronal viability by increasing apoptosis. This effect was markedly greater in the presence of microglia. (E) *In vivo,* intrathecally injected miRNAs increase apoptosis of cortical neurons and consequently cause a decrease in neuronal number. TLR, toll-like receptor; miRNA, microRNA; SEAP, secreted embryonic alkaline phosphatase.

miRNA. Markedly reduced levels of both TLR7 and miR-let7b increased hippocampal-dependent memory and attenuated inflammatory cytokine production (91). These results demonstrated for the first time that specific miRNAs secretion, in a particular physiological context, can act on specific TLRs in the brain. It has been postulated that these miRNAs could be important in the establishment and/or development of neurodegenerative diseases, as well as in neurocognitive disorders; however, further studies are needed to ascertain this. An improved understanding of the mechanisms underlying the activation of TLRs by miRNAs could help to establish treatments to ameliorate neurodegenerative diseases or neurocognitive disorders

Sequence characteristics of miRNAs in TLR activation. Early work that demonstrated the ssRNA detection by TLRs was carried out by Heil *et al* (65). The authors reported that HIV-derived ssRNA, rich in guanosine (G) and uridine (U), induced the secretion of interferon-α and proinflammatory and regulatory cytokines in response to murine TLR7 activation and human TLR8. Similarly, miRs-21 (GUUG) and -29a (GGUU) have a GU motif in the nucleotide region 18-21, where base number 20 (U) was very important for the activation of human TLR8 (1). Meanwhile, Wu *et al* (92) identified the 'UGUUAU' motif and certain uridines and cytosines of miR-20a-5p as determinants for TLR7 activation and cytokine secretion. The shortest sequence to induce TLR7 activation was 10 nt in length (ssRNA), including the 'UGUUAU' motif. The activation of TLR7 promoted the secretion of multiple proinflammatory molecules, which was dependent on PI3K, MAPK and NF-κB1. The administration of miR-20a-5p in wild-type mice increased leukocyte migration and this was attenuated in both TLR7 knockout mice and in mice administered with miR-20a-5p but with the 'UGUUAU' motif mutated.

TLR activation by miRNAs and other types of ncRNAs is a focus of ongoing research and existing evidence indicates that the recognition of miRNAs by TLRs is primarily dictated by GU-rich motifs, where the sequence and position of nucleotides within the mature miRNA sequence is a determinant for TLR activation (5).

TLR8 is activated by the binding of ssRNA degradation products at two different sites (93). At the first binding site, uridine mononucleoside (Kd, 55 μM) binds to TLR8 by stacking interactions between TLR8 and the aromatic moiety of ligands and hydrogen bonds (94). The second site of TLR8 is recognized by the UG dinucleotide, which increases the affinity of uridine for TLR8 by 50-fold. TLR8 and TLR7 receptors have functional and sequence similarities, so some structural features of TLR8 can be applied to those of TLR7. Regarding this, TLR7 has an activation mode similar to that of TLR8, in which guanosine and its derivatives, instead of uridine, bind to TLR7 and this binding is also modulated by oligonucleotide binding at a second site (95,96).

Similar to TLRs 7/8, TLR13 is activated by ssRNA 13, derived from the 23S ribosomal subunit, by binding at a site formed by the interaction of the N- and C-terminal regions of this receptor (97). The binding of ssRNA13 to TLR13 depends on the formation of a stem-loop structure (G2057 and C2064) and the establishment of hydrogen bonds between specific nucleotides (A2058, A2060 and G2061) and the TLR13 leucine rich regions LRR2-LRR25. Notably, the interaction of ssRNA13 with TLR13 was pH-dependent, with a preference for acid pH.

miRNAs retain the viral consensus motifs for TLR interaction and activation, but this does not rule out other sequences and mechanisms involved in TLR activation by ssRNA. Ligand binding to a first site of TLRs 7 and 8 increases the affinity of

another ligand at a second site, which may occur by allosteric events. More evidence is needed to know whether activation of TLRs by miRNAs also occurs in this way.

Interactions of RNYs with TLRs. RNYs are highly conserved sncRNAs in vertebrates (98) and have also been detected in prokaryotes (99) and viruses (100). In humans, four RNYs have been identified: RNY1, RNY3, RNY4 and RNY5, and ~1,000 pseudogenes (101). The physiological importance of YRNAs was partially identified by studying their interaction with proteins and by bioinformatics approaches (6). Functions regulated by RNYs include control of the immune system (102,103), DNA replication (99), development (98), cell proliferation (104), enhanced translation efficiency and virus assembly and retrotransposition control (105).

Circulating ribonucleoprotein complexes associated with RNYs predominantly induce immune activation and several of these effects depend on signaling pathways mediated by TLRs. A previous study reported that each of the TLRs do not respond in the same way to the RNY activation and have diverse physiological effects (Table I) (7). For instance, TLR7 activation by RNY1 induced nephritis, but that of TLR3 by RNY3 did not. It has also been shown that the activation of a particular TLR by a specific RNY can induce inflammation in a given tissue and repress it in another. Regarding this, TLR3 or TLR7 activation by U1 RNA induced lung disease, but that of TLR7 by RNY1 did not have the same effect (7). In addition, it has been shown that RNYs that are not bound to Ro60 or La proteins have a greater effect on TLR activation, because these proteins recognize the hairpin structure and the 5'-phosphate group of RNYs, which are important sites for their interaction with TLRs (35).

Based on the aforementioned studies, the TLR-RNY interactions seem to depend on the type of TLR and RNY involved as well as on the cellular tissue. In certain cases, this activation was related to inflammatory processes, but the opposite effect was observed in others. It is necessary to identify which other molecular components and signaling pathways are determining these biological differences.

In pediatric patients with astrocytoma, Rodríguez-Corona *et al* (10) demonstrated differential circulating expression of RNYs (Fig. 2A-C) and the interaction of RNYs with cellular receptors was predicted. Bioinformatics analysis [HIPPIE database (cbdm-01.zdv.uni-mainz.de/~mschaefer/hippie/), by using the following commands: NETWORK QUERY; direct interaction; GO (biological process); show KEGG effect; and PANTHER database (pantherdb.org), by using the following commands: ID List; *Homo sapiens*; Functional classification viewed in gene list; Pathway)] indicated the RNY interaction with receptors other than TLRs to regulate a myriad of cellular pathways, such as apoptosis, angiogenesis, cholecystokinin receptor and p53 signaling pathways, as well as Parkinson's disease (Fig. 3). Identifying the target organs of RNYs and understanding the biological processes that are being modified by acting on TLRs or on other types of receptors may be important to understand.

rRNA interactions with TLRs. rRNA is the most abundant RNA species in organisms and, similar to miRNAs and RNYs, rRNAs also interact with TLRs. TLR13 is known to recognize a specific sequence of the bacterial ribosomal 23S subunit, which induces the production of infection-fighting cytokines (8,36,97,106); however, the 23S ribosomal subunit derived from erythromycin-resistant *Staphylococcus aureus* (*S. aureus*) strains or methylated oligoribonucleotides, which mimics MLS antibiotic resistance, did not stimulate TRL13 (36). This observation demonstrates a mechanism of antibiotic resistance and parallels host cell 'detection' systems, whereby exposure of bacteria to antibiotics establishes epigenetic marks that render them less or totally inert to antibiotics and also evade detection by TLR13. Similarly, fish TLR13a and b receptors were activated by bacterial 23S rRNA and induced the immune response by stimulating specific effector proteins (Table II) (106).

Li and Chen (36) demonstrated that the Sa19 sequence (ACGGAAAGACCCC) of the 23S subunit is a ligand of murine TLR13, which acts mainly against infection by Gram-positive bacteria. The Sa19 sequence and mitochondrial 16S rRNA (mt-rRNA) stimulate peripheral blood mononuclear cells in a MyD88-dependent manner. Sa19 and *S. aureus* and *Escherichia coli* (*E. coli*) derived sequences and mitochondrial (mt)-RNA also activate human monocytoid THP1 cells via TLR8. In addition to the 23S and 16S subunits, Krüger *et al* (37) reported that the 5S subunit from *S. aureus* and *E. coli*, as well as mt-rRNA and P-/DAMPs stimulators, activate human TLR8. In addition to the UGG motif, the UAA and UGA motifs are required for TLR8 activation.

In addition to the 23S and 16S ribosomal subunits, the 18S ribosomal subunit is involved in TLR activation. The interaction of the 18S rRNA subunit with Pam2 (Pam2 CSK4), ligand of TLRs 2 and 6, increased the affinity of Pam2 for TLR2 to act synergistically. TLR2 activation stimulated the transcriptional expression of inflammatory genes and TNF-α release from macrophages, which was NF-κB-dependent (107). Usually, the acute cellular responses to numerous signals are additive and, in certain cases, the response to simultaneous stimuli is notably greater than the sum of the responses to each stimulus separately; this is known as synergism. This has biological significance since the cell will only respond when two or more signals are present simultaneously. Therefore, it will be necessary to identify whether all TLRs act synergistically, and which ligand combinations and which mechanisms govern the regulation of specific cellular processes.

Although TLRs are a primary detection system that fight diverse infections, there is also evidence of their participation in the beneficial action exerted by lactic acid bacteria in organisms (e.g. anti-inflammatory effects and activation of the intestinal immune system) (108-110). In mice, these bacteria have immunomodulatory effects that are mediated, at least in part, by IL-12 and TLR13. Since humans do not express this receptor, it could be considered that the effects of this type of bacteria are mediated by another TLR. Nishibayashi *et al* (111) demonstrated that the 23S and 16S rRNA subunits of *Enterococcus faecalis* Ec-12 strain activated TLR8 and induced IL-12 production.

Organisms have 'engineered' molecular elements that allow them to fight infectious agents. The bacterial rRNA binding site to the TLR is also involved in antibiotic resistance, which ensures evasion of these two defense systems (36). Conversely,

Table I. Activation of specific TLRs by ncRNAs and their fragments.

A, ncRNAs: miRNAs

First author/s, year	ncRNA type	Activated TLR	Experimental model	Biological function	(Refs.)
Fabbri *et al*, 2012	miR-21 and miR-29a	7,8	Cancer	Induction of prometastatic inflammatory response: Promotion of tumor growth and metastasis.	(5)
Feng *et al*, 2017	miR-133a, miR-146a and miR-208a	7	Myocardial ischemia	Production of the cytokines MIP-2, TNFα and IL-6. Migration of peritoneal neutrophils and monocytes.	(12)
Borchert *et al*, 2007	miR-146a-5p	7	Myocardial ischemia	Production of MIP-2, IL-6 and TNFα and activation of the innate immune response.	(46)
Chung *et al*, 2011	miR-34a, miR-122 and miR-146a	7	Sepsis	Proinflammatory and production of MIP-2, IL-6, IL-β and TNFα: Peritoneal neutrophil migration.	(47)
Wallach *et al*, 2020	miR-340-3p and miR-132-5p	7,8	Neuronal injury	Release of cytokines and chemokines from microglia: Neurodegenerative effect.	(6)
Mardente *et al*, 2012	miR-100-5p and miR-298-5p	7,8	Neurodegenerative disease model	Secretion of cytokines and chemokines from microglia and macrophages: Increased phagocytosis. Activation of autonomic apoptosis of cortical neurons. miRNAs in cerebrospinal fluid: Neurodegeneration and accumulation of microglia in mouse cerebral cortex.	(54)
Hao *et al*, 2018	miR-20a-5p and miR-148b-3p	7	Central nervous system pathology	Secretion of proinflammatory molecules dependent on PI3K, MAPKs and NF-κβ. Migration of leukocytes.	(56)

B, ncRNAs: rRNAs

Oldenburg *et al*, 2012; Huang *et al*, 2023; Chen *et al*; 2014; Huang *et al*, 2022	23S rRNA	13	Bacterial infections, action of lactic acid bacteria	Activation by bacterial infections, regulates the beneficial actions of lactic acid bacteria.	(8,34, 72,76)
Shimada *et al*, 2020	23S (Sa19) and 16S mt-rRNA	8	Bacterial infections	Activation of PBMCs in a MyD88-dependent manner.	(74)
Chen *et al*, 2014	18S rRNA	2	Inflammation	Activation of differentiated human monocytoid THP-1 cells.	(72)

Table I. Continued.

First author/s, year	ncRNA type	Activated TLR	Experimental model	Biological function	(Refs.)
B, ncRNAs: rRNAs					
Alvarez-Carbonell *et al*, 2017	Bacterial rRNA	3	HIV-associated neurocognitive disorders	Synergic activation of TLR2 stimulated transcriptional expression of inflammatory genes and TNF-α release from macrophages.	(9)
C, ncRNAs: RNYs					
Greidinger *et al*, 2007	RNY3 RNY1 RNY4 RNY5	3,7,8 7, 8 7 7	Autoimmunity	RNY3 was a strong activator strong activator of TLRs 3,7, and 8. TLR3 activation by RNY3 did not induce nephritis. RNY4 was a strong activator of TLR7. TLR7 activation by RNY5 induced nephritis.	(7)
Rodriguez-Corona *et al*, 2023	RNYs 1,3-5	5,7,8,10	Pediatric astrocytoma	The interaction of RNYs 1,3-5 with TLRs 5,7,8,10 was predicted. Alzheimer disease-amyloid secretase, B cell activation, ubiquitin proteasome, angiogenesis, apoptosis, endothelin and dopamine receptor mediated-signaling pathways.	(10)
D, ncRNA fragments: tRNAs					
Deng *et al*, 2024	5'-tRNAHisGUG	7	Bacterial infections	TLR7 activation	(91)
Wu *et al*, 2021	5'-tRNAVal CAC/AAC	7	Bacterial infections	Elimination of *Mycobacterium tuberculosis* in a GUUU sequence-dependent manner.	(92)
E, ncRNA fragments: s-RNYs					
Onafuwa-Nuga *et al*, 2005	RNY1-5p, RNY3-5p and RNY4-5p	7	Atherosclerosis	Induction of apoptosis and NF-κB-mediated inflammation	(100)
Rodriguez-Corona *et al*, 2023	s-RNYs loop domains	3,7,10	Pediatric astrocytoma	Bioinformatic prediction.	(10)

TLR, Toll-like receptor; MyD88, myeloid differentiation primary response 88; PMBCs, peripheral blood mononuclear cell; ncRNA, non-coding RNA; HIV, human immunodeficiency virus; miR, microRNA; RNY, a class of non-coding RNAs that are components of the Ro60 ribonucleoprotein particle; s-RNYs, fragments derived from RNYs; tRNA, transfer RNA; rRNA, ribosomal RNA.

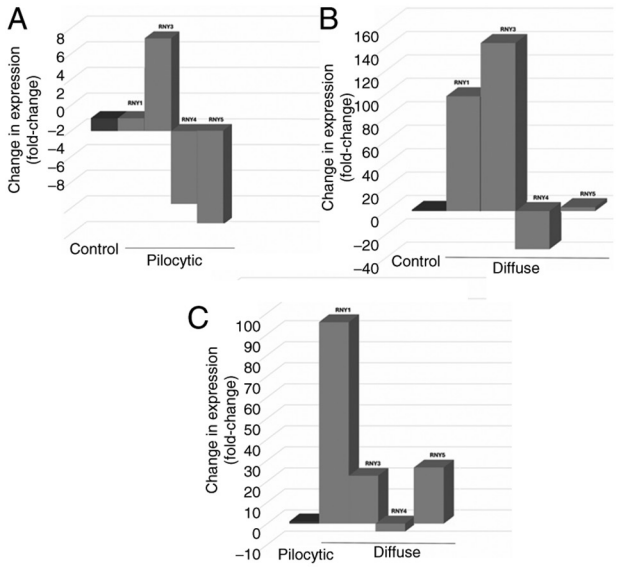

Figure 2. Circulating RNY expression in pilocytic and diffuse astrocytoma. (A) Pilocytic vs. control samples. (B) Diffuse vs. control samples. (C) Pilocytic vs. diffuse samples. RNYs are a class of non-coding RNAs that are components of the Ro60 ribonucleoprotein particle. Data obtained from Rodríguez-Corona *et al* (10).

the infected cell detects the infectious agent by means of TLRs and, additionally, it secretes mtRNA to ensure the innate immune system response to the infection (37). In short, each organism fights for its survival by generating innumerable 'attack' or 'defense' mechanisms to ensure its survival.

i) rRNAs and TLRs in the brain. Alvarez-Carbonell *et al* (9) demonstrated the reactivation of HIV in microglia cultures, an effect that was mediated by TLR3 activation by poly (I:C) and bacterial rRNA. The activation of other TLRs revealed a minor effect on the reactivation of this virus, and the activation of TLRs 2/1, 4-6 induced the NF-κB-mediated pathway, but the activation of TLR3 induced IRF3. Diverse effects were observed in human monocytes and rat microglia. These results demonstrate that the activation of TLRs may have functions other than the immune system control. As aforementioned, both, the biological effect and the potency of this effect, depends on the type of TLR activated and by the ligand and the coactivators that may or may not be present in a given tissue.

lncRNAs control TLR activity. lncRNAs are RNAs with a size of 200-1,000 kb, which exert a variety of cellular functions by diverse mechanisms (112,113). It is established that the activation of TLRs 7 and 9 is associated with immune dysfunction and severe disease states, and that blockade of these receptors improved the health status of mice susceptible to develop lupus (7-9,20). Similarly, Yang *et al* (38) demonstrated that lnc-Atg1611 binds to TLR7 and activates the MyD88-mediated signaling pathway in immune cells and deletion of this lncRNA attenuated TLR7-related autoimmune phenotypes in murine models. The binding of lnc-Atg1611 to TLR7 is complex, as on one side it binds to TLR7 and on the other to MyD88, thus functioning as a 'scaffold', which induces structural changes in the TLR7 stem-loop and induces signal transduction (113). The authors suggested that self-RNAs, particularly lnc-Atg1611, may be potential therapeutic targets to control the development of autoimmune disorders. In this regard, Mussari *et al* (20) identified an antagonist (7f) that has a high affinity for TLR7 and, to a lesser extent, for TLR9, which blocks cytokine production mediated by TLRs 7 and 9 and markedly decreased proteinuria, antibody production and IL-10 secretion.

i) lncRNAs and TLRs in cancer. Anaplastic thyroid carcinoma (ATC) is a rare and aggressive type of thyroid cancer that spreads rapidly to other parts of the body. In thyroid cancer, the lncRNA C5AR1 shows increased expression and correlates positively with short patient survival (39). Molecularly, C5AR1 activates TLRs 1 and 2 receptors and MyD88, which results in tumor growth and lung metastasis of ATC cells in a murine model and inhibition of the expression of this lncRNA, considerably decreased the oncogenic effects regulated by its interaction with TLRs (39). Regarding this, miR-355-5p negatively regulated C5AR1 expression, which could be used as a therapeutic tool to downregulate the levels of this lncRNA and attenuate the oncogenic effects produced by C5AR1-TLRs 1/2-MyD88 signaling.

These results demonstrate that several ncRNAs can function as ligands for TLRs and exert oncogenic effects. The identification of all ncRNAs and molecular elements regulating the TLR-mediated signaling at a specific point in time will allow for the understanding of the interaction mechanisms underlying TLR activation and/or inhibition to develop immunotherapies. lncRNAs have been identified as positive or negative regulators of TLRs, affecting immune response and drug sensitivity; however, further studies are needed to understand the mechanisms by which these ncRNAs control TLR pathways.

3. Interaction of ncRNA-derived fragments with TLRs

Since 1971, the production of fragments (~50 nt) originating from the 16S ribosomal subunit has been observed (114). Similarly, Borek *et al* (115) reported a higher rate of turnover and excretion of transfer RNA (tRNA) 'degradation products' in patients with cancer relative to individuals without cancer. Subsequent studies continued to report the presence of these 'degradation products' (116-120). It was not until 2008 that Thompson *et al* (121) reported that these RNA fragments have specific biological functions in addition to controlling the levels of full-length sequence transfer RNA (tRNAs). To date, it is acknowledged that all known ncRNAs are fragmented and that this appears to occur by specific biogenic pathways; however, the molecular elements involved in these machineries are not yet known.

Fragments derived from tRNAs. Several types of fragments generated from tRNAs have been identified and they are classified into two categories: tRNA halves (tiRNAs; 31-40 nt) and tRNA-derived fragments (14-30 nt) based on the nuclease cleavage sites in mature or premature tRNAs (116,117).

Several physiological functions have now been described for transfer RNA-derived small RNAs (tsRNAs) (122,123), including TLR7 activation. Pawar *et al* (118) demonstrated that mycobacterium infection activates TLRs expressed on the surface of human monocyte-derived macrophages, which promoted the production and secretion of 5'-tRNA

Table II. Differential activation of intracellular pathways mediated by TLR13a and b.

Co-expression in 293T cells	Signaling pathway activated
TLR13a/MyD88	Increased NF-κB-mediated pathway activation. Low effect on AP1 pathway activation. Significant activation of the IFN-β-mediated pathway
TLR13b	Activation of the AP1-mediated pathway.

TLR, toll-like receptor; MyD88, myeloid differentiation primary response 88; AP1, activator protein 1.

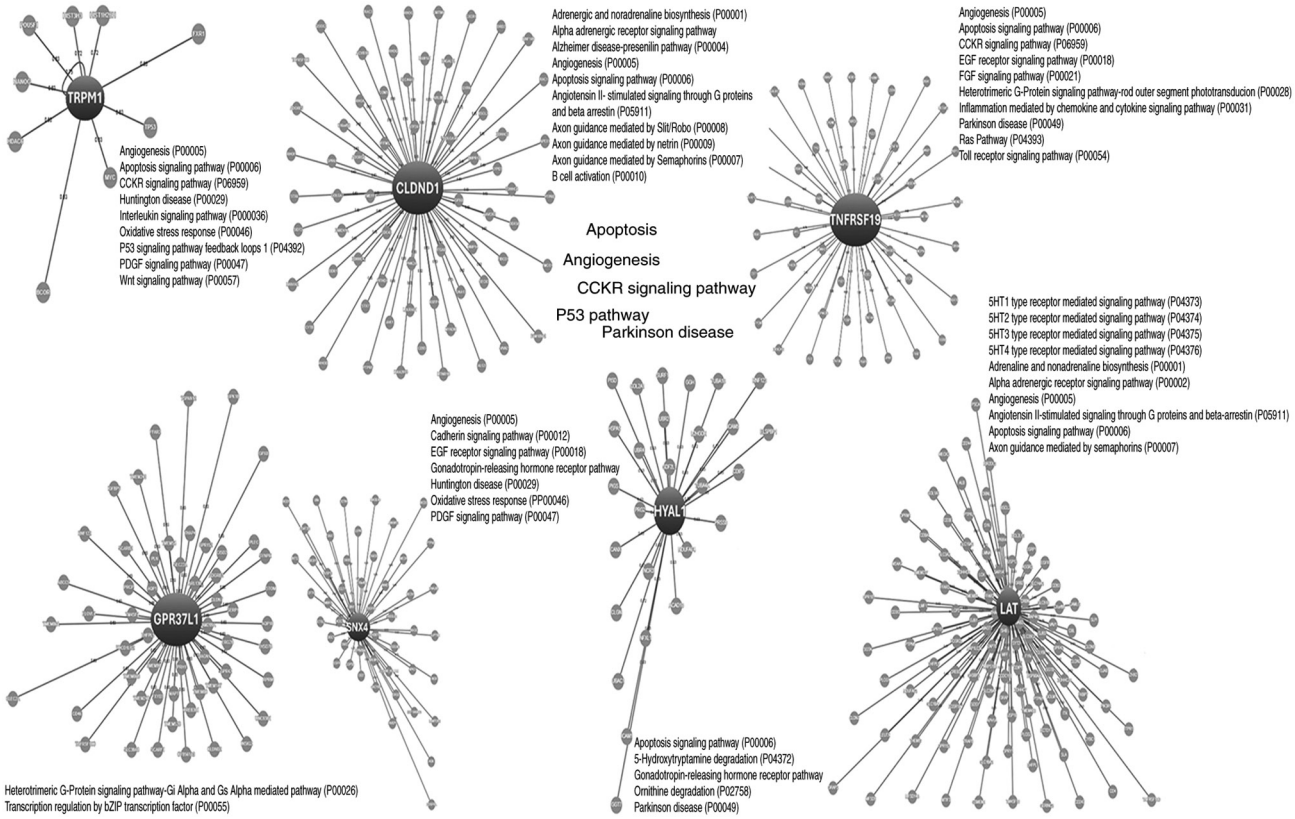

Figure 3. Predicted functions for circulating RNYs in pediatric patients with astrocytoma. Differential expression of the four human RNYs was detected in exosomes of pediatric patients with astrocytoma. Bioinformatic analyses indicate that RNYs potentially interact with various protein receptors. The establishment of protein-protein interaction networks for these receptors [HIPPIE database (cbdm-01.zdv.uni-mainz.de/~mschaefer/hippie)] and subsequent analysis with the PANTHER database (pantherdb.org) showed the potential involvement of RNYs-receptors in the control of apoptosis, angiogenesis, cholecystokinin receptor and p53 signaling pathways, and in Parkinson's disease. RNYs are a class of non-coding RNAs that are components of the Ro60 ribonucleoprotein particle. Data obtained from Rodríguez-Corona *et al* (10). The data were analyzed with the Hippie and Panther databases. Interactome images were obtained from the Hippie database.

halves in EVs. Specifically, the 5'-tRNA^{HisGUG} half was recognized and endocytosed by recipient cells to subsequently activate endosomal TLR7. Later, the authors demonstrated TLR7 activation by the 5'-tRNA$^{ValCAC/AAC}$ where its GUUU terminal sequence was determinant for the activation of this receptor and for the elimination of bacterial infection. The mutation of this tRNA-half showed that the GUUU sequence was determinant for TLR7 activation. Notably, this motif is present in other RNAs that also stimulate this receptor (124).

To date, tsRNAs are the best characterized fragments and are known to be involved in rRNA maturation, reverse transcription, adaptation of parasites to their environment and as ligands for TLRs. The tsRNA-TLR interaction is key for eliminating *Mycobacterium tuberculosis* infection and certainly for other types of bacteria and viruses, so understanding the mechanisms underlying the tsRNA-TLR regulatory axis may be important for generating drugs to mitigate these types of infections, which is particularly important due to the growing resistance to antibiotics (125,126).

Fragments derived from RNYs and lncRNAs. RNY fragments (s-RNYs) are generated from stem terminal regions during apoptosis (119) from miRNAs or Piwi-interacting RNAs (127) or by RNase L activity or by activation of the innate immune system mediated by Poly: I:C (120). Specific

biogenic pathways have been proposed for the generation of s-RNYs, which retain specific sequences from their parental RNYs to regulate particular cellular processes (Table I). Currently, limited information exists regarding TLR activation by s-RNYs. To the best of our knowledge, there is only one study which revealed that the release of s-RNYs (sRNYs1-5p, 3-5p and 4-5p)/Ro60 by macrophages resulted in the activation of TLR7 and in the induction of caspase-3-dependent apoptosis and NF-κB-mediated inflammation. The full form of RNYs/Ro60 did not have the same effect, indicating specific functions in this cellular context for the s-RNYs (128). Related to this, Rodriguez-Corona *et al* (10) predicted the RNYs and their fragments interactions with TLRs and with other receptors (Table I).

RNYs and their fragments have a central role in immune system responses and at least part of these responses depend on RNYs/s-RNYs-TLRs interactions. Studies indicate that the action of s-RNYs is not necessarily the same as that of parental RNYs (10,128); therefore, it is necessary to identify the molecular traits that are determining these differences.

In relation to lncRNAs, Giraldez *et al* (129) demonstrated the presence of fragments of mRNAs and lncRNAs in blood plasma that are missed by standard sequencing techniques. The detection of these fragments in plasma has potential for use as biomarkers and their functions need to be gradually elucidated.

4. Conclusion

The discovery of ncRNAs, considerably changed the understanding and approach to the study of life sciences. Initial studies demonstrated the interfering function that miRNAs (22,23) and small-interfering RNAs (siRNAs) (97) have; subsequent studies have shown the diversity of functions and mechanisms of action by which ncRNAs may act.

Up to now, several studies have demonstrated that TLR activity is mediated by ncRNAs and their fragments (5,7,8,32,36,38); however, several questions arise regarding the molecular mechanisms underlying this activation and the physiological effect this may have. The majority of data indicate that activation of TLRs by ncRNAs or their fragments primarily induces the innate immune system, protecting organisms from microbial and viral infections (11-15); however, this activation is also associated with oncogenic (5,39), neurodegenerative effects (6,90) and viral reactivation (9). Although in the majority of cases the end result is the activation of the immune system, each ncRNA and its fragments activate specific intracellular signaling pathways, resulting in the control of specific biological effects (113,118,128). Uncovering the molecular mechanisms involved in these differences will allow for improved understanding of the ncRNAs/fragment-TLR relationship and the biological functions that are mediated by these interactions. Regarding this, Wallach *et al* (6) reported that secondary structure formation did not determine the binding of miRNAs to TLRs; however, miRs-21, -93 and 296 can adopt hairpin and/or homoduplex structures, depending on the miRNA concentration and ionic conditions, which determined their specificity for target mRNAs (130,131). Similarly, there is evidence indicating that secondary structures of mature siRNAs influenced the efficiency of siRNA-mRNA interaction, where unstructured siRNAs exert a greater silencing effect than those with secondary structures. Belter *et al* (131) observed that miRNA hairpins resemble those of the anti-Tn-C aptamer, indicating that miRNAs can directly regulate their targets in an RISC-independent manner, making them highly specific regulators, more so than previously considered.

TLRs regulate both the innate and adaptive immune system and are mainly associated with autoimmune disorders, chronic inflammation, chronic viral infections and cancer, making these receptors a target for the development of immunotherapeutic strategies. Currently, there are several TLR ligands used as vaccine adjuvants to increase the efficacy of vaccines, and TLR agonists and antagonists have been identified for the treatment of chronic viral infections such as hepatitis B virus (HBV), HIV (23,132), autoimmune diseases (20,21,133) or for the treatment of cancer (25-27). For instance, TLR7 agonists have been tested in clinical trials for HBV infection and a TLR9 ligand as an adjuvant in a vaccine against HBV (132). The activation of specific TLRs inhibited HIV replication and reduced viral spread; however, TLR activation also led to the reactivation of latent HIV in cell cultures. This may be important for the development of anti-HIV therapies, since reactivation of latently infected reservoir cells could facilitate the recognition by cytotoxic immune cells, which has already been observed in clinical studies. Therefore, immunotherapy against chronic HBV or HIV infection is promising and antiviral drugs have been approved for the clinical treatment of HBV and HIV (134).

In previous years, several groups have focused on designing oligonucleotides that antagonize TLRs 7-9 and have been proposed as potential treatments for autoimmune and inflammatory diseases (20-22). Of these antagonists, IMO-8400 reduced moderate-to-severe plaque psoriasis in patients participating in a phase IIa clinical trial (21). Other research groups have focused on developing small molecules that function as antagonists of TLRs 7-9. For example, the 7f antagonist of TLRs 7 and 9 has a strong potency and high selectivity for these receptors, emphasizing the use of small molecules for the treatment of autoimmune diseases (20). Meanwhile, TAC5, an antagonist of endosomal TLRs, reduced inflammation and prevented the progression of psoriasis and systemic lupus erythematosus in mice, indicating its therapeutic potential for their treatment (22). Despite this, there is a need to evaluate the sensitivity and specificity as well as the side effects that the application of these molecules may have. For example, a study conducted by Christensen *et al* (133) reported that TLR9 knockout mice developed more aggressive disease pathology; however, dual TLR 7 and 9 knockout demonstrated protection.

For cancer treatment, several TLR3 agonists were observed to induce cancer regression by restoring immunogenicity to chemotherapy (25,26). Similarly, intratumoral injection of the TLR7 and 8 receptor agonist MEDI9197 suppressed tumor growth and increased survival in murine models by regulating the immune response and by increasing the expression of associated genes in innate and adaptive immunity (27,135,136). Notably, the antitumor effects of MEDI9197 were most potent when this drug was applied in combination with a programmed death ligand 1 inhibitor (135). Although no tumor response was observed, the application of MEDI9197 or in combination with durvalumab and/or palliative radiation induced local and systemic immune activation in patients with solid tumors (136).

In addition, sugar-conjugated TLR7 ligands increased autoimmunostimulatory activity, demonstrating potential for application as adjuvants in vaccines and cancer therapies (137).

The activation of TLRs to induce innate and adaptive immunity and to treat various diseases has great clinical potential, as there is considerable experimental evidence indicating their application for the treatment of viral and immune diseases and different types of cancer. Nevertheless, the translation of experimental observations into effective clinical application is a major challenge and further work is still needed to increase the therapeutic effect and increase the precision of treatments.

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Authors' contributions

RREG and MAVF contributed to the study conception and design and conducted the literature review and wrote the manuscript. All authors read and approved the final version of the manuscript. Data authentication is not applicable.

Ethics approval and consent to participate

Not applicable.

Patient content for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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