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Extracellular miRNAs as activators of innate immune receptors

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ABSTRACT

Since the discovery of the existence of microRNAs (miRNAs) in body fluids, the fascinating hypothesis that extracellular miRNAs may play a role in cell-to-cell signalling started to make its own way. In this review, we summarize the current knowledge that supports the role of miRNAs in the regulation of the immune response by an unconventional mechanism based on the activation of intracellular innate immune sensors of nucleic acids, namely the Toll-like receptors (TLRs). Such a mechanism of action has been now described to amplify and influence the pathogenesis of several inflammation-dependent pathological conditions, including cancer growth and metastasis, neurodegeneration, autoimmunity and cardiovascular diseases. The available data suggest that we have only begun to touch upon a complex system that is likely to involve many receptors and molecules. These findings may help to understand the pathogenesis of immune-mediated diseases and provide the basis for the identification of new potential therapeutic targets.

1. Introduction

The presence of cell-free miRNAs was first described ten years ago in plasma and readily demonstrated in virtually all biological fluids [1–3]. Extracellular miRNAs were found to be remarkably stable in harsh conditions, including high RNAse concentrations and extreme pH variations, mostly because of their association with proteins, such as those of the AGO family that protect them from degradation [4,5], leading to long term storage. Most of the extracellular miRNAs exist as “naked” ribonuclein particles, while a minor fraction (1–5%) associate with extracellular vesicles (EVs) [5,6]. The origin, composition and biological significance of the extracellular miRNA pool remain a matter of debate. Extracellular miRNAs are released upon cell damage or death and may as such represent cell remnants devoid of any particular function. However, compelling evidence has emerged that cells can operate specific sorting of secreted miRNAs, suggesting that these molecules may possess specific biological functions. A current view of the complex and still controversial biology of extracellular miRNAs, including their relationship with EVs, has been extensively reviewed elsewhere [7]. Here, we will concentrate on the role of extracellular miRNAs as soluble messengers, focussing on the unconventional hypothesis that they might be involved in the activation of inflammatory and immune responses via the triggering of innate pattern recognition receptors (PRRs).

2. The cell-to-cell communication theory

The discovery of miRNAs in body fluids, together with the evidence that miRNAs co-purified with EVs, raised the fascinating hypothesis that extracellular miRNAs could mediate paracrine, or even endocrine, cell-to-cell signalling. Indeed, EV-associated miRNAs were shown to penetrate into target cells to trigger effects consistent with the function of intracellular miRNAs, such as post-transcriptional regulation of the expression of specific mRNAs [8,9]. Compelling evidence in support of such a mechanism of action is provided by the constantly growing number of reports demonstrating repression of target mRNAs in cells treated with EV fractions enriched in specific miRNAs. This function covers an impressive range of donor/target cell types and is involved in several pathological processes, from cancer spreading and chemosensitivity, to atherosclerosis and cardiovascular diseases, to neurodegeneration [10–12], but also encompasses physiological phenomena, such as the induction and regulation of immune responses [13–15]. The regulation of the transcriptome of recipient cells by extracellular miRNAs in these different settings has been recently and exhaustively reviewed elsewhere [16–20] and will not be further analysed here due to space constraints.

However, a conclusive proof of the physiological relevance of distant cell regulation by extracellular miRNAs is still lacking and some arguments supporting the existence of this phenomenon *in vivo* have been questioned [21]. First, the concentration of extracellular miRNAs in plasma is very low, in the order of hundreds of femtomoles per litre,

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List of abbreviations

AGO	Argonaute
cDCs	conventional dendritic cells
DAMPs	damage-associated molecular patterns
dsRNA	double strand RNA
EVs	extracellular vesicles
GVHD	graft versus host disease
HDL	high-density lipoprotein
IFNs	interferons
IRF	interferon-regulatory factor
MDA5	melanoma differentiation-associated protein 5
mRNA	messenger RNA
miRNA	microRNA
NAs	Nucleic acids
NF-kB	nuclear factor kB
PAMPs	pathogen-associated-molecular patterns
pDCs	plasmacytoid dendritic cells
PRRs	pattern recognition receptors
RIG-I	retinoic acid-inducible gene I
RLRs	retinoic acid-inducible gene I-like receptors
SLE	systemic lupus erithematosus
SR-BI	scavenger receptor class B type 1
ssRNA	single strand RNA
TERF1	telomeric repeat binding factor 1
TLRs	Toll-like receptors

among which the amount of any single miRNA would represent only a tiny fraction, well below the concentration of any known intercellular messenger [22]. In addition, since the majority of extracellular miRNAs are not encapsulated into EVs [5,6], they could hardly penetrate the cell membrane, at least according to the current paradigms. Other studies found that the ratio of exosome/miRNA molecules is very high in many body fluids, with only one copy of a given miRNA every 100 exosomes [23]. Altogether, these results suggest that EVs may not carry a biologically relevant number of miRNAs to exert cell-to-cell regulation. This may be true especially at the post-transcriptional level, where target mRNA suppression requires a threshold miRNA concentration of several hundreds copies per every recipient cell [24]. This calculation, however, needs to be carefully considered based on the still limited information available concerning the mechanisms of secretion, sorting, packaging, target cell recognition and molecular activity of extracellular miRNAs *in vivo*. For instance, it was suggested that the calculated concentration of total extracellular miRNAs in body fluids and on a "per exosome basis" should be "adjusted" taking into account that only selected miRNAs or so far unidentified subclasses of EVs could be involved in intercellular regulation [25,26]. In addition, the effective "final" miRNA concentration required to inhibit target mRNAs is currently difficult to predict and might be much lower than hypothesized, since it depends not only on the absolute number of miRNAs, but also on other factors such as the strength and the number of miRNA binding sites, the abundance of target mRNAs and the fact that each mRNA can be regulated by many different miRNAs [24,27,28].

Indeed, it can be envisioned that cell-to-cell regulation by extracellular miRNAs *in vivo* might happen under defined circumstances or conditions [29]. For example, cell-to-cell miRNA transfer could have a significant role in paracrine intercellular regulation, allowing to locally overcome the threshold concentration of target-specific miRNAs. Also, endocrine regulation of distant cells may rely on a so far overlooked regulatory role for free-miRNAs, that are much more abundant as compared to EV-associated miRNAs, or on the existence of mechanisms of EV "selection" by acceptor cells, which would allow to select miRNA-bearing EVs among the many "empty" EVs. Finally, mechanisms different from mRNA targeting and able of a certain degree of amplification may start to emerge. One such mechanism will be addressed in the following paragraphs.

3. Innate immune RNA sensors

Nucleic acids (NAs) of microbial origin represent a relevant class of pathogen-associated-molecular patterns (PAMPs), i.e. invariant

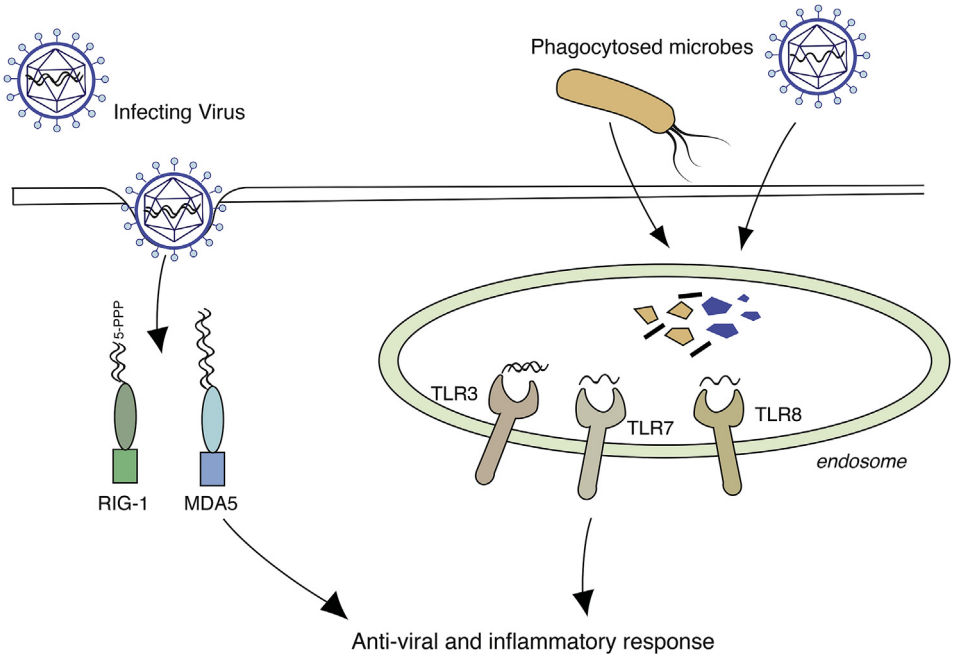

Fig. 1. Innate immune RNA sensors.
Different RNA sensors expressed by immune cells can recognize bacterial and viral RNAs. The cytosolic RIG-I and MDA5 bind dsRNA from infecting virus; while the endosomal receptors TLR3, TLR7 and TLR8 recognize fragments of both ssRNA and dsRNA from phagocytosed microbes.

microbial structures that activate the inflammatory and immune response against pathogens by triggering a heterogeneous group of germline-encoded PRRs [30]. Two main families of RNA-sensing PRRs have been identified: the retinoic acid-inducible gene I-like receptors (RLRs) and the Toll-like receptors (TLRs) (Fig. 1). RLRs are intracellular proteins that bind and signal the presence of cytosolic double strand RNA (dsRNA), such as transcription products from RNA viruses. They are ubiquitously expressed at low levels and are rapidly induced upon viral infection or interferon stimulation [31]. RLRs comprise two main receptors: RIG-I, that preferentially binds short dsRNA harbouring 5′-triphosphate ends, and MDA5, which displays preference for long dsRNA [32,33]. TLRs are a family of type I transmembrane proteins comprising members capable of sensing a wide variety of PAMPs [34]. At contrast with RLRs, RNA-recognizing TLRs are located within the

A

Table summarizing research on extracellular miRNAs as TLR ligands

Context	miRNA	Source	Receptor	Recipient cell type	Readout	Biological meaning	Year of publication	Reference
Cancer	miR21, miR29a	Lung cancer cells	Murine TLR7	Macrophages	Pro-inflammatory cytokine production	Prometastatic inflammatory response	2012	PMID: 22753494
	miR21, miR29, miR147	Synthetic (DOTAP)	Human TLR8	Peripherapheral blood mononuclear cells	Pro-inflammatory cytokine production	?		
	miR21	Neuroblastoma	Human TLR8	Monocytes	Upregulation of miR155	Chemoresistence	2015	PMID: 25972604
Neurological diseases	let-7b	Dying neurons	Murine TLR7	Neurons, Microglia	Neurodegeneration, pro-inflammatory cytokine production	Neurodegeneration	2012	PMID: 22610069
	miR21	Brains from monkeys with SIV (Macrophages? Microglia?)	Murine TLR7	Neurons	Neurotoxicity	SIV-induced neurological disease?	2015	PMID: 26154133
Nociception	let-7b	Activated nociceptor neurons, Intraplantar injection	Murine TLR7 (coupled to TRPA1)	Nociceptor neurons	Induction of rapid inward currents and action potentials	Pain	2014	PMID: 24698267
Autoimmunity	let-7b	Synovial fluid	Murine TLR7	Macrophages	M1 polarization	Arthritic joint inflammation	2016	PMID: 26662519
	miR21, miR574, let-7b	Synthetic (DOTAP), Plasma of SLE patients	Human TLR7	pDCs	Type-I IFN and pro-inflammatory cytokine production	Role in the onset of interferonopaties?	2018	PMID: 29769437
GVHD	miR29a	Serum of GVHD patients	Murine TLR7, Human TLR8	Dendritic cells	Pro-inflammatory cytokine production	Induction of GVHD	2017	PMID: 28159900
Ischemia	miR34a, miR122, miR133a, miR142, miR146a and miR208	Synthetic (Lipofectamine)	Murine TLR7	Macrophages, Cardiomiocytes	Pro-inflammatory cytokine production, Leukocite migration	Inflammation, tissue damage?	2017	PMID: 28768728

SIV: simian immunodeficiency virus; GVHD: graft versus host disease; pDCs: plasmacytoid dendritic cells; IFN: Interferon

B

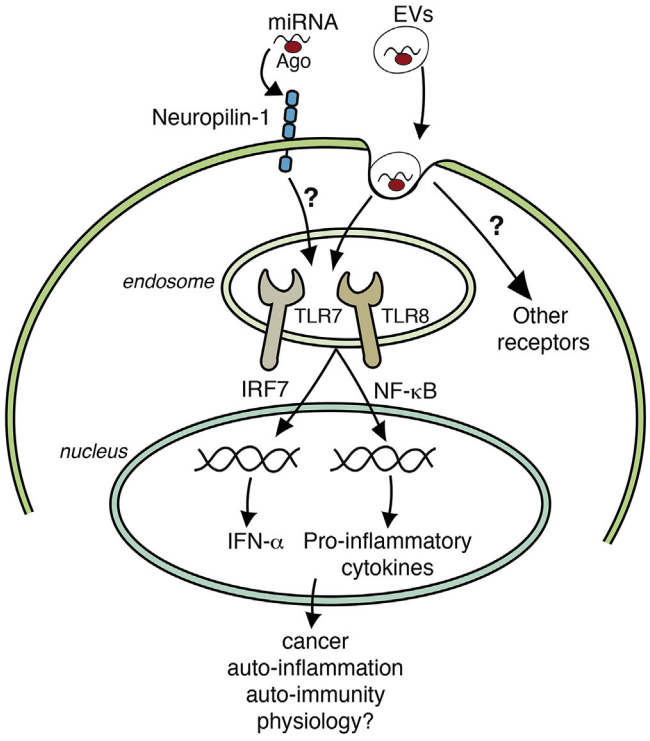

Fig. 2. Extracellular miRNAs activate TLR7 and TLR8.
A) Table summarizing research characterizing cell activation via TLR triggering by extracellular miRNAs. B) Envisioned mechanism of TLR triggering by extracellular miRNAs in immune cells. EV-associated extracellular miRNAs enter the cells by endocytosis and clathrin-dependent mechanisms, while free miRNA may use neuropilin-1, that recognizes AGO2, and other so far unidentified receptors. Once in the cell, miRNAs may reach endosomal compartments and bind TLR7 or TLR8, leading to NF-κB and IRF7 activation with the consequent production of pro-inflammatory cytokines and IFN-α.

endolysosomal membrane and recognize NAs released upon microbial digestion [35]. They comprise TLR3, detecting dsRNA, and TLR7 and TLR8, two highly homologous proteins that signal in response to viral ssRNA. Very recently, structural data have clarified that TLR7 and TLR8 are activated by degradation products of ssRNAs rather than by intact molecules as was originally assumed (reviewed in Ref. [36]). These two receptors are often mutually expressed in immune cells, which may explain their conservation in evolution, despite their functional and structural similarity. TLR7 is typically expressed by plasmacytoid dendritic cells (pDCs) and B cells, whereas TLR8 is expressed in monocyte/macrophages and conventional dendritic cells (cDCs) [37–39]. Upon ligand recognition, both RLRs and TLRs trigger intracellular signalling cascades leading to the activation of IRF3 and IRF7 transcription factors and the master antiviral response mediated by type I interferons (IFNs) as well as the activation of the pro-inflammatory transcriptional factor NF-κB (reviewed in Refs. [40,41]). In addition to RLRs and TLRs, a number of other intracellular RNA-helicases (distantly related to RLRs) and RNA-binding proteins were shown to associate with RNA molecules with some specificity and suggested to work as RNA sensors of viral infection, but their biology and ligand specificities remain largely to be elucidated [42–45].

Altogether, these RNA-sensors allow the recognition of a large variety of microbial RNAs that characterizes different stages of interaction with host cells [41]. For example, the endolysosomal localization of TLRs allows a prompt activation of antiviral immunity before and independently of cell infection, which gives the body a crucial advantage for viral elimination. However, PRRs can be triggered also by mammalian endogenous molecules arising under certain conditions that, by analogy to PAMPs, have been termed DAMPs (damage-associated molecular patterns). Thus, it is of fundamental importance to maintain a fine balance between a sensitive and robust immune response against microbial NAs and tolerance toward self-NAs. Indeed, dysregulated interferon production and untimely inflammation pave the way to the development of chronic inflammatory and autoimmune disorders [46–48]. Several “safety measures” have evolved to prevent unwanted PRR activation. According to a recent classification, these mechanisms operate with four main modalities: i) “ligand pattern”, i.e. molecular differences between PAMPs and DAMPs; ii) “PRR subcellular sequestration”, i.e. physical segregation discriminating PRRs and DAMPs; iii) “ligand quantity in specific niches”, meaning that physiological amounts of DAMPs are tolerated, but exceeding quantities may end up in PRR activation; and iv) “PRR sensitivity threshold”, meaning that changes in the expression or regulation of PRRs modulate the sensitivity to DAMPs [49]. The “ligand pattern” mechanism applies to sensors of dsRNA, a molecular species that does not exist in steady state eukaryotic cells and is produced only during viral infection. The remaining NA-sensing PRRs display limited or no selectivity for non-self NAs. For this reason, all the other mechanisms must be at play to keep RNA sensors “safe”. Again, the sequestration of TLRs to endolysosomes is a key strategy to prevent activation by self-RNA and the generation of autoinflammation [50]. Indeed, in homeostatic conditions, free self-NAs are readily degraded and cannot reach the endosomal compartments. However, TLRs are engaged by self-NAs if they are protected from degradation, such as upon association with specific antibodies or poly-cathionic antimicrobial peptides [51,52]. Also, the accumulation of endogenous NAs, either depending on aberrant supply (e.g. as a result of cell damage or death [47,48]) or on defect in the degradation machinery (e.g. as in DNAse I deficiencies [53,54]) may result in TLR7 and TLR8 triggering. Thus, if under homeostatic conditions RNA-sensors are efficiently prevented to recognize self-RNAs by different mechanisms acting in concert, this equilibrium can be readily subverted even by subtle changes in any of these safety mechanisms.

Extracellular miRNAs represent ideal candidate ligands of ssRNA-sensors in virtue of the fact that are released by virtually all cells in the body, are resistant to degradation and may be encapsulated in EVs, facilitating cell entry. In addition, extracellular miRNA are deregulated in a variety of pathologies ranging from cancer, to autoimmune and cardiovascular diseases [55–58].

4. EXTRACELLULAR miRNAs AS TLR LIGANDS

An unconventional mechanism of cell-to-cell regulation mediated by extracellular miRNAs is represented by their interaction with innate immune receptors (Fig. 2), as initially proposed by two distinct research groups. In 2012, Fabbri et al. [59] using a murine experimental model, identified two miRNAs (miR21 and miR29a) secreted by tumor cells capable of inducing the production of pro-inflammatory and pro-metastatic cytokines by binding to murine TLR7 and human TLR8. Almost simultaneously, Lehmann et al. [60] found that let-7b, a miRNA that is found upregulated in the cerebrospinal fluid of patients with Alzheimer's disease, activated murine microglia and bone-marrow derived macrophages in a TLR7-dependent way. Intrathecal injection of let-7b in wild-type mice, but not in TLR7 knockout mice, induced neurodegeneration. These studies demonstrated for the first time that miRNAs could play a regulatory role in a pathological context by acting as paracrine agonists of TLRs.

Additional work in cancer showed that the interaction between miR21 and TLR8 represents the core of a complex cross-talk mechanism between neuroblastoma and innate immune cells, leading to increased resistance to chemotherapy [61]. Indeed, cancer cells can activate monocytes via the release of exosomal miR21, which triggers TLR8 and NF-κB. In turn, activated monocytes release exosomal miR155, which once entered into neuroblastoma cells can increase resistance to chemotherapy both *in vitro* and in *in vivo*, possibly by targeting the mRNA of TERF1, a component of the shelterin complex and inhibitor of telomerase. This work unveiled that the positive correlation between two oncogenic miRNAs (miR21 and miR155) is mediated by the presence of TLR8-positive cells in the tumor microenvironment.

Let-7b was also found to be markedly upregulated in synovial fluid of patients with rheumatoid arthritis and responsible for inducing macrophage polarization to the proinflammatory M1 phenotype acting via murine TLR7 ligation [62]. In addition, joint swelling and M1 macrophages were found to be absent in TLR7-deficient mice tested in a model of inflammatory arthritis.

The central nervous system, and neurons in particular, appear to be relevant targets of extracellular miRNAs. Indeed, in addition to neurodegeneration and neurotoxicity [60,63], TLR7 triggering by extracellular let-7b was also described to induce rapid inward currents and action potentials in nociceptor neurons, thus resulting in pain when administered to mice by intraplantar injection [64].

Further studies suggested also a detrimental role of extracellular miRNAs in graft versus host disease (GVHD). A functional significance for increased levels of miR29a in the serum of GVHD patients was identified by showing the ability of this miRNA to activate dendritic cells via murine TLR7 and human TLR8. In addition, the treatment with an anti-miR29a in a mouse model of acute GVHD ameliorated the clinical features of the disease [65].

Extracellular miRNAs were also implicated in the induction of undesired immune responses in cardiovascular diseases. Several miRNAs were upregulated in the serum of a murine model of myocardial ischemia. Among these, miR34a, miR122, miR133a, miR142, miR146a and miR208 were found to activate murine macrophages, cytokine production and leucocyte migration through TLR7 signalling [66].

We have recently extended these observations to the human setting in spite of the fact that miRNAs were initially believed unable to target human TLR7 [59]. Exosomes purified from systemic lupus erythematosus (SLE) plasma promoted activation, maturation and survival of human pDCs [67], the main source of type I IFNs [39]. These effects were recapitulated when pDCs were stimulated with miRNAs purified from SLE patient exosomes or with the corresponding synthetic miRNAs. By the use of reporter cells transected with defined TLRs it was possible to demonstrate that pDCs activation by miRNAs is dependent

on TLR7 and TLR8 activation. Consistent with previous work, pDC activation did not occur indiscriminately but displayed a certain degree of sequence specificity. Indeed, it was previously reported that short interfering RNAs containing sequences known as “immunostimulatory motif” or “interferon induction motif” (such as the UGUGU sequence) [68] were able to induce broad activation of innate immune responses, including the production of type I IFNs and cytokines. In agreement with this observation, among the miRNAs found to be upregulated in SLE patients, those containing such motif (e.g. miR21, miR574, let-7b) were able to activate pDCs *in vitro*, while control miRNA devoid of this motif (e.g. miR16, miR210, miR31) failed to do so. Other studies identified GU-rich elements or an elevated number of U ribonucleotides as important patterns for the activation of innate responses by miRNAs [59,60]. Although, a precise TLR-activating RNA sequence was never defined [69–71]. A possible explanation for this resides in recent structural studies demonstrating that both TLR7 and TLR8 associate with degradation products of RNA instead of binding to full length ssRNA molecules with TLR7 working as a dual sensor for guanosine and uridine-containing ssRNAs, while TLR8 as a uridine sensor (reviewed in Ref. [36]). Interestingly, it was recently found that guanosine and uridine represent the most abundant ribonucleotides in human miRNAs, often present as GU dinucleotide, and that at least 50 miRNAs bearing a UGUGU motif could be detected [72]. Thus, it is conceivable to assume that, *in vivo*, many miRNAs possess the structural features required to bind and trigger TLRs. Such redundancy might overcome the theoretical limitation of miRNAs to induce cell-to-cell regulation based on their low concentration in extracellular fluids. This possibility is also supported by experiments showing activation of pDCs by mixture of miRNAs used at suboptimal concentrations [67]. At present, however, it remains difficult to predict which miRNAs, or combination of miRNAs, will result in TLR activation *in vivo*.

Our results demonstrate that miRNAs protected by a liposomal vehicle effectively can reach the endosome in pDCs [67]. However, the mechanism underlying endosomal versus cytoplasmic delivery of EV-associated miRNAs still need to be addressed. We speculate that in pDCs, and more generally in innate immune cells, EVs could also be endocytosed, bringing their content in contact with endosomal TLRs. In support of this view, nanoparticles were shown to be selectively phagocytosed by pDCs [73]. In our hands, miRNAs administered *in vitro* to pDCs in the absence of liposomal vehicles do not induce TLR activation [67], in line with the prevailing view that cell-to-cell communication is mediated by EV-associated miRNAs. By contrast, in another study, inflammatory neuronal degeneration was obtained by injecting free miRNAs [60]. Interestingly, it was demonstrated that SR-BI, the high-density lipoprotein (HDL) receptor, plays a role in the transport and uptake of certain HDL-bound miRNAs that retained mRNA targeting capabilities in recipient cells [74]. Although this mechanism may have a limited physiological impact because of the relatively small fraction of HDL-bound miRNAs [21], this work indicated that cellular miRNA-receptors may exist and play a role. Recently, it was shown that extracellular miRNAs may enter the cells via the binding of AGO proteins to neuropilin-1 while retaining the capability to induce biological effects such as proliferation and migration of cancer cells and tube formation by human endothelial cells [75]. Since neuropilin-1 is abundantly expressed by pDCs [76], our system will allow to investigate if neuropilin-1 uptake might have a role in endosomal TLR triggering by free extracellular miRNAs (Fig. 2B).

5. Concluding remarks

The ability of miRNAs to play a role in cell-to-cell-regulation is still ill-defined. In addition to their possible action in the regulation of specific target mRNAs, miRNAs also appear to act as paracrine activators of TLRs. However, several issues are still open.

For instance, one main question is about the mechanisms that regulate miRNA delivery to exosomes versus cytoplasm. Another open question is if miRNAs can act as broad activators of innate immune receptors. Based on current knowledge, TLR7 and TLR8 are at the moment the most obvious candidates, but other PRRs still need to be investigated in this context. It is conceivable that additional miRNA receptors may exists within the group of RNA-sensors whose ligand specificity still remains undefined or in the large family of orphan receptors [41].

At a different level, a missing piece in the puzzle is the understanding of the biological significance of miRNA-mediated PRR triggering. Is this a pathway that characterizes only pathological conditions or also a way to regulate physiological intercellular communication? To elucidate this issue we will need to better understand how PRRs are protected from miRNA recognition in steady state conditions and what can convert the extracellular pool of miRNAs in strong PRR agonists and mediators of disease amplification. Additional work is needed to provide the answer to these challenging questions.

Conflict of interest statement

Authors declare that no conflict of interest exists.

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