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How does an RNA selfie work? EV-associated RNA in innate immunity as self or danger

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ABSTRACT

Innate immunity is a first line of defence against danger. Exogenous pathogen- or microbe-associated molecular patterns (PAMPs or MAMPs) trigger innate immune responses through well-understood cellular pathways. In contrast, endogenous damage-associated molecular patterns (DAMPs) convey "danger signals" via their (mis)localization or modification. Both MAMPs and DAMPs are often communicated on or within extracellular vesicles (EVs). Despite growing evidence for the importance of EVs and their cargo in modulating innate immune responses, in some cases, it is unclear how EV-transported molecules are sensed as abnormal. In particular, EVs constitutively carry RNA, which is also abundant in the cytoplasm. How, then, would RNA convey a danger signal as a cargo of EVs? In this Perspective, we offer some thoughts on how EV-associated RNAs might raise the alarm for innate immune responses – or silence them.
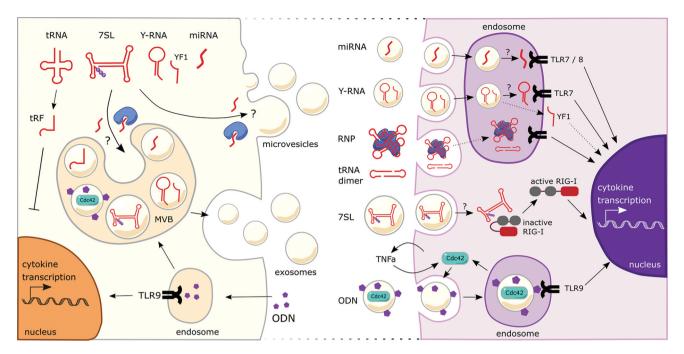
Introduction

Innate immune responses are triggered by recognition of a foreign invader or an endogenous danger [1]. In the case of the former, cellular receptors sense pathogen- or microbe-associated molecular patterns (PAMPs or MAMPs), while the latter is perceived through damage-associated molecular patterns (DAMPs). These endogenous "danger signals" are apparent because of their own (mis)localization (e.g. nuclear DNA or nuclear proteins found in the cytoplasm) or aberrant modification (like oxidized lipids). One of the most consequential sensors of these signals is the cyclic GMP-AMP synthase–stimulator of interferon genes (cGAS/STING) pathway [2]. For example, chromosomal instability in cancer cells and errors in chromosomal segregation lead to cytosolic DNA accumulation and subsequent cGAS-STING activation, promoting tumour cell invasion and metastasis [3]. Another important family of sensors is the Toll-like receptors (TLRs). In oxidative stress, oxidized phospholipids stimulate Toll-like receptor 4 (TLR4) in a manner dependent on its co-receptor, myeloid differentiation factor 2 (MD-2) [4]. This is similar to the mechanism of bacterial LPS sensing [5]. Thus, these sensors recognize "problems" with self molecules, not just foreign invaders.

Extracellular vesicles (EVs) are now known to be important carriers of both MAMPs and DAMPs. These sub-micron-sized, lipid bilayer-delimited particles are released from all investigated cell types, functioning to dispose of toxic material, provide trophic support, and shuttle molecular signals. In addition to exogenous MAMPs and the examples of endogenous PAMPs given above, EVs are also well known to carry an abundance of RNA biotypes. While microRNAs (miRNAs) [6] are by far the most studied, all other types of cellular RNAs can also be found in EVs [7]. For example, Y-RNA, 7SL and tRNA have been abundantly detected in EVs from various biological sources. In cells, 7SL forms an integral part of the signal recognition particle (SRP), which mediates the translocation of nascent proteins across the ER membrane [8], and tRNA is essential in recruiting amino acids to ribosomes during protein translation. Y-RNA subtypes (hY1, hY3, hY4 and hY5 in human; mY1 and mY3 in mouse) are components of Ro ribonucleoprotein complexes, act as scaffolds for distinct subsets of effector proteins, and may regulate RNA degradation and DNA replication [9,10]. These different RNA types may be incorporated into EVs via exosome and ectosome/

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Figure 1. Intercellular transfer of endogenous exRNAs and routes of innate immune activation. MicroRNA, Y-RNA, 7SL and tRNA have been abundantly detected in EVs from various biological sources. These RNA types may be incorporated into EVs via exosome and microvesicle biogenesis pathways, either by interactions with proteins or diffusion. Additionally, cells release exRNA that is not associated with EVs, but is found in RNPs or as stable tRNA dimers. Both miRNA and Y-RNA have been shown to stimulate endosomal TLRs, leading to cytokine production. A fragment of Y-RNA, named YF1, was shown to induce IL-10 transcription via an unresolved mechanism. RNP-associated RNA and tRNA dimers were shown to activate dendritic cells via an unknown mechanism. The 5'-triphosphate motif of 7SL was shown to activate cytosolic RNA sensor RIG-I. How these RNAs are delivered to the endosomal compartment or to the cytosol has remained unresolved. The activation of T cells was shown to be hampered by the presence of tRNA fragments, which are disposed of via EVs during T cell activation. ODNactivated macrophages transfer ODN via released EVs. Cdc42 that was concomitantly transferred stimulated the uptake of ODN-containing EVs. Possibly, TNF-α released upon sensing of ODNEV may also activate endogenous Cdc42.

microvesicle biogenesis pathways, either by interactions with proteins or diffusion, and can activate innate immune sensors such as TLRs or the cytosolic RNA sensor RIG-I. However, how do these processes occur?

How could self RNA activate the innate immune system?

In contrast with DNA, which is predominantly nuclear in healthy eukaryotic cells and thus constitutes a danger signal when found outside the cell, RNA molecules are found throughout the cell and are routinely released as extracellular RNA (exRNA) even in the healthy individual. The various exRNA carriers (EVs, lipoprotein particles, and ribonucleoprotein particles) have aroused considerable interest in recent years [11], but especially EVs because they are released without cell death and can reveal the health status of the cell of origin. Since exRNAs are released from all cells, these endogenous RNAs do not seem like good candidates as DAMPs. Yet a groundbreaking study of exRNA and innate immune signalling showed that certain host microRNAs could stimulate TLRs in the endosomal system [6]. This

interesting finding, now repeatedly confirmed for miRNAs, at least, raises some important questions. How, exactly, is endogenous exRNA recognized as a danger? What other RNAs (or other molecules) might be involved in such signalling, and how? Despite a decade of study, these pressing questions have been answered only incompletely. However, numerous new examples of seemingly "nondanger" molecules have emerged, along with ideas about how they are recognized as abnormal (summarized in Figure 1).

Does altered local abundance of RNA stimulate innate immune responses?

Perhaps the most obvious metric for interpretation of a "normal" molecule as abnormal is differential expression: the molecule of interest is perceived as being present in excess or lacking. Indeed, some RNAs appear to enhance immune responses that facilitate cancer growth, spread, and/or immune escape, possibly through altered abundance and transfer in EVs. The Y-RNA hY4, for example, was found to be enriched in plasma EVs of chronic lymphocytic leukaemia patients compared with control

individuals [12]. When transferred to monocytes, hY4 elicited responses including proinflammatory cytokine release as well as PD-L1 expression. These effects were dependent on TLR7 expression, since they were not seen for cells deficient for TLR7 or treated with signalling inhibitors (Figure 1). Additional research is needed to understand the level at which hY4 might be detected as too abundant, and how the molecule inside the EV is presented to the receptor. Furthermore, systemic inflammation can change the composition of Y-RNA subtypes in human plasma [13], with Y-RNA subtype composition differing between EVs of abundant blood cell types. Neutrophil EVs contained a distinctive Y4/Y3 ratio, which increased in plasma during inflammation and correlated with neutrophil abundance and activation. It remains to be investigated whether changes in Y-RNA subtypes in plasma trigger inflammation in EV-recipient cells.

Shielding and chemical modification of RNA: molecular context matters

The molecular context of RNA could be another means to distinguish "danger" from normal RNA. As an example, the signal recognition particle RNA RN7SL1, an endogenous RNA normally shielded by RNA-binding protein SRP9/14, was upregulated in activated stromal cells in the context of breast cancer [14]. At these abnormal levels, not all copies of RN7SL1 could be bound by the usual protein partner, and the RNA was exported in EVs as naked (or "unshielded") RNA with an exposed 5'-ppp triphosphate motif. Upon transfer to recipient breast cancer cells, this unshielded 5'-ppp on RN7SL1 activated the cytoplasmic RNA sensor RIG-I, thus promoting cancer aggression phenotypes (Figure 1). Note that a previous report found EVs to be enriched with 5'-ppp miRNAs [15]. What remains unclear is how the carrier EVs fused and delivered the unshielded cargo, as well as the level of transfer required to achieve these effects in vivo. In another example, Polymerase III-transcribed cellular RNA, in particular Y-RNA, was also shown to bind RIG-I via a triphosphate motif [16]. This was counteracted by the cellular triphosphatase Dusp11, which prevents unwarranted RNA sensing in healthy cells [16]. Whether Dusp11 is also involved in reducing RNA sensing of EV-transferred RNA cargo remains to be investigated.

To be sure, EV association may not be needed to trigger responses. In addition to being loaded into EVs [17], noncoding RNA species from MCF-7 cells, mainly dimers of tRNA-Gly-GCC halves and ribosomal RNA, are also released in non-EV fractions [18]. In this form, they can activate primary bone-marrow-derived dendritic cells and induce the release of IL-1β. It was hypothesized that these non-EV-associated RNAs may function as an immune surveillance mechanism, by which immune cells may sense damaged or dying cells (Figure 1). Furthermore, RNP-associated Y-RNA has also been shown to activate various RNA sensors [19].

Parts of the whole: differential response to RNA fragments

Differential processing of an RNA under disease conditions could also allow different modes of recognition. As an example, an EV-associated Y-RNA fragment appears to have a protective role in cardiac health [20]. EVs released from therapeutic cardiosphere-derived cells were found to contain an abundance of an hY4 5' fragment, dubbed EV-YF1. This fragment in turn increased transcription and secretion of the cardioprotective cytokine IL-10 by macrophages. EV-YF1primed macrophages prevented oxidative stress damage of cardiomyocytes in vitro, and the fragment also reduced infarct size in a rat ischemia/reperfusion model [20]. However, details of the mechanism of EV-YF1-stimulation of IL-10 remain to be elucidated. How is this fragment recognized as different from the parent molecule? Does it serve to titrate out a function of the precursor? Or does it act directly?

EV-mediated cellular depletion of specific RNA biotypes

Another way in which EVs might contribute to immune modulation is at the point of origin, by specific depletion of RNAs from the parent cell. T-cells are instrumental in adaptive immunity, and a role for tRNA fragments (tRFs) in general T-cell activation has recently been posited [21]. After a strong enrichment of tRNA was observed in T-cell EVs, chemical EV release blockage resulted in accumulation of tRFs in endosomal compartments. Furthermore, treatment of cells with antisense tRF oligos resulted in activation-prone conditions [21]. These findings suggest that tRFs tend to block – and that selective export might promote – T-cell activation, and that EVs play a role in maintaining the balance (Figure 1). Exactly how the tRFs are recognized and loaded into EVs remains uncertain, as does the trigger for enhanced EV export of tRFs during activation.

Feedback loops: modulation of EV release or uptake

Altering EV trafficking could be another route to changing the perception of carried nucleic acids. Supporting this possibility, EVs secreted from CpG

oligodeoxynucleotide (ODN)-activated macrophages transported ODN into naïve macrophages, stimulating TLR9 and enhancing the release of chemokine tumour necrosis factor–α (TNF-α) [22]. EV-associated ODN was resistant to protease, and protease and nuclease treatment of ODN-EV did not affect the release of TNF-α, suggesting that ODN may be enclosed within EVs. Furthermore, EVs from ODN-activated macrophages contained increased Cdc42 levels, which increased the uptake of EVs in recipient cells (Figure 1). Knockdown of Cdc42 in recipient cells could be restored by EV-associated Cdc42, resulting in increased EV uptake. This suggests that EV uptake in unmodified recipient cells was a result of EV-associated Cdc42, although it is possible that autocrine TNF-α signalling might also contribute. Although it is not yet clear if similar modulation might occur for RNA cargo, these results may point to an activation-induced feedback mechanism to enhance EVmediated entry of PAMPs/DAMPs into recipient cells. These findings not only shed light on the activation of innate immunity but also suggest a previously unidentified regulation strategy for these important biological pathways [22].

Release of EV RNA into endosomal and cytosolic compartments

An important unanswered question is how EV-enclosed RNA may come into contact with endosomal or cytosolic RNA sensors. EV uptake is thought to begin with interactions between specific receptors on EV and target cells followed by internalization of EVs via endocytic pathways [23]. How and to what extent internal EV cargo including RNA is delivered into the endosomal lumen or the cytoplasm has remained an unresolved and controversial issue [24]. Since most EV-associated RNA is RNase resistant [17,25], it stands to reason that it must cross the vesicular membrane in the endosome or be delivered into the cytoplasm by EV-cell fusion. It has been speculated that the acidic pH in endosomes may aid cargo delivery [26,27], much as happens for certain enveloped viruses [28]. Hypothetically, spontaneous rupture of EV membranes, EV proteins with membrane fusion capacity, and proteins that form pores in the EV membrane could additionally mediate the delivery of EV cargo. These and other mechanisms merit investigation.

Conclusion

As diligent couriers that carry a cornucopia of potential signalling molecules, the roles of EVs in innate immune regulation are just beginning to be explored.

Beyond the canonical MAMPs and PAMPs, EVs also carry RNAs that, at first glance, may seem unlikely to obtrude. Nevertheless, numerous examples of innate immune modulation by EV RNA have come to light. We have speculated here on some of the possible reasons for recognition of self RNA as a danger signal. Yet much more work is needed to establish the mechanisms and of the underlying molecular interactions. Tools and approaches might include: overexpression or depletion of ncRNAs of interest; genetic knockouts of RNA sensors or RNA-binding proteins; single-molecule visualizations of RNA localization [29]; and reporter cells to monitor EV uptake [30]. Furthermore, MISEV-compliant separation of EVs from other exRNA carriers will further our understanding of physiological functions[1,3].

Many other questions also remain. Are combinatorial signals important, such as recognition of multiple RNAs or RNA in the context of a binding protein? How is RNA (or other molecules) in the EV lumen recognized by innate immune receptors? Only when the EV disintegrates in the acidified endolysosome, exposing its contents? Or is EV-cell fusion required? Might hypothetical RNA transporters even contribute to carrying RNA across membranes, such as orthologs to the *C. elegans* RNA transporters? Finally, is the finding of EV cargo-mediated enhancement of EV trafficking limited to DNA cargo, or will parallels be found for other EV-associated molecules?

These and other questions suggest that rich learning opportunities await in the study of EVs, RNA, and innate immunity.

Disclosure statement

The authors declare no conflicts of interest.

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