

# Extracellular RNA Sensing by Pattern Recognition Receptors

Megumi Tatematsu<sup>a,b</sup> Kenji Funami<sup>a</sup> Tsukasa Seya<sup>a</sup> Misako Matsumoto<sup>a</sup>

<sup>a</sup>Department of Vaccine Immunology, Hokkaido University Graduate School of Medicine, Sapporo, Japan;

<sup>b</sup>Dr. von Hauner Children's Hospital, Department of Pediatrics, University Hospital, LMU Munich, Munich, Germany

## Keywords

Endosome · Extracellular RNA · Innate immune signaling · RNA uptake · Toll-like receptor

## Abstract

RNA works as a genome and messenger in RNA viruses, and it sends messages in most of the creatures of the Earth, including viruses, bacteria, fungi, plants, and animals. The human innate immune system has evolved to detect single- and double-stranded RNA molecules from microbes by pattern recognition receptors and induce defense reactions against infections such as the production of type I interferons and inflammatory cytokines. To avoid cytokine toxicity causing chronic inflammation or autoimmunity by sensing self-RNA, the activation of RNA sensors is strictly regulated. All of the Toll-like receptors that recognize RNA are localized to endosomes/lysosomes, which require internalization of RNA for sensing through an endocytic pathway. RIG-I-like receptors sense RNA in cytosol. These receptors are expressed in a cell type-specific fashion, enabling sensing of RNA for a wide range of microbial invasions. At the same time, both endosomal and cytoplasmic receptors have strategies to respond only to RNA of pathogenic microorganisms or dying cells. RNA are potential vaccine adjuvants for immune enhancement against cancer and provide a benefit for vaccina-

tions. Understanding the detailed molecular mechanisms of the RNA-sensing system will help us to broaden the clinical utility of RNA adjuvants for patients with incurable diseases.

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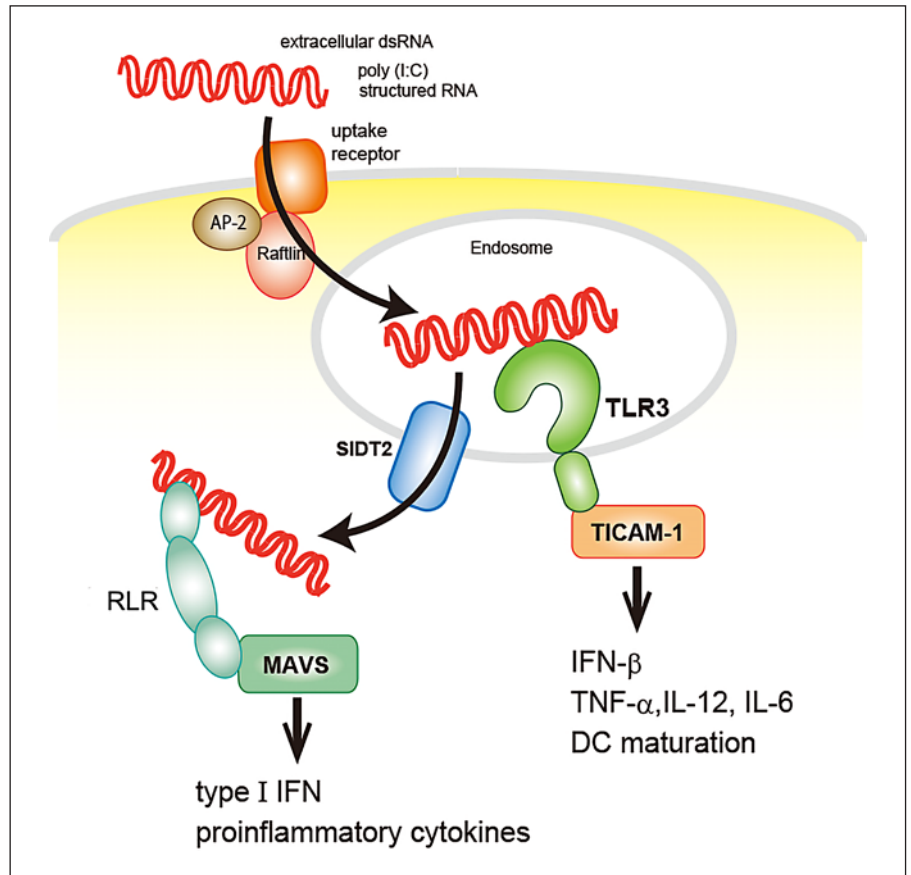
## Introduction

Three different types of pattern recognition receptor (PRR) families, i.e., Toll-like receptors (TLR), RIG-I-like receptors (RLR), and nucleotide-binding and oligomerization-like receptors, play key roles in host defense against microbial infection and tissue injury by recognizing the molecular patterns of microorganisms or damaged and dead cells. Nucleic acids are well-studied ligands for these PRR.

Both single-stranded (ss) and double-stranded (ds) RNA can be recognized by PRR in mammalian cells as pathogen-associated molecular patterns or damage-associated molecular patterns. PRR activation leads to the induction of innate immune responses, including the production of type I interferons and inflammatory cytokines. Because excessive production of cytokines can cause

Dr. Megumi Tatematsu  
Dr. von Hauner Children's Hospital, KUBUS Research Center, LMU Munich  
Lindwurmstrasse 2a  
DE-80337 Munich (Germany)  
E-Mail Megumi.Tatematsu@med.uni-muenchen.de

Prof. Misako Matsumoto  
Department of Vaccine Immunology  
Hokkaido University Graduate School of Medicine  
Kita 15, Nishi 7, Kita-ku, Sapporo 060-8638 (Japan)  
E-Mail matumoto@pop.med.hokudai.ac.jp



**Fig. 1.** Internalization of extracellular dsRNA. dsRNA in the extracellular milieu bind to uptake receptors on the cell surface and are endocytosed, mediated by Raftlin, in a clathrin-dependent manner. dsRNA activate TLR3 in endosomes and then SIDT2 urge them to exit from the endosome to the cytosol. In the cytosol, RLR recognize dsRNA.

harmful chronic inflammation or auto immune responses, hosts have developed strategies to avoid sensing of self-RNA by RNA sensors.

TLR are type I transmembrane proteins. As all nucleic acid-sensing (NAS) TLR are localized to endosomal compartments, extracellular nucleic acids have to be endocytosed to gain access to NAS TLR. Host cells have an apparatus to selectively take up extracellular RNA into endosomes/lysosomes. In contrast, RLR are cytosolic sensors; RIG-I recognizes 5'-di-/triphosphate blunt-ended dsRNA and 5'-triphosphate ssRNA, which are unique patterns to pathogens, and MDA5 recognizes long dsRNA. Non-self-RNA can be segregated from self-RNA by its structures.

Uptake of extracellular RNA is the first step in recognition by endosomal TLR. To date, some cell type-specific uptake mechanisms have been reported. However, although extracellular RNA activate RLR-mediated signaling, the mechanisms via which RNA reach cytosolic sensors is unclear. One component that may be involved is SIDT2, which was recently reported to play a role in traf-

ficking of dsRNA from the endosome to the cytosol after TLR3 activation [1] (Fig. 1). In this review, we summarize how RNA trigger innate immunity through RNA sensors and how subsequent immune responses are regulated.

### RNA Species Activate Innate Immunity

RNA-sensing receptors detect various RNA structures of microorganisms and self-RNA (Table 1). During viral infection, positive-stranded RNA viruses and dsDNA viruses produce dsRNA. These RNA byproducts, as well as genomic RNA, accumulate in the cytoplasm of infected cells and are released into the extracellular space when infected cells are dying.

TLR3 senses dsRNA and structured RNA, which contains a partial stem in secondary structures of ssRNA, originating from viruses or from stressed or necrotic cells [2–5]. The synthetic dsRNA analog poly(I:C), which activates both TLR3 and MDA5, is widely used as an experimental TLR3 ligand.

**Table 1.** RNA-sensing pattern recognition receptors

RNA sensor	Expression	Localization (signal initiation)	Ligands
TLR3	cDC, macrophages, neural cells, fibroblasts, epithelial cells	endosome	dsRNA (>40 bp), poly(I:C), structured RNA
TLR7	pDC, B cells	endosome	ssRNA, R848, CL097
TLR8	monocytes, macrophages, mDC	endosome	ssRNA, R848, CL095, CL097
RIG-I	ubiquitous	cytoplasm	short dsRNA, 5' di-/triphosphate RNA, RNase L cleavage product, circular RNA
MDA5	ubiquitous	cytoplasm	long dsRNA (>1 kb)

Endocytosed and phagocytosed ssRNA are sensed by TLR7 and TLR8. These 2 receptors have a high homology and are both activated by GU-rich ssRNA and the synthetic chemical compounds imidazoquinolines [6, 7]. It has been reported that bacterial RNA stimulates TLR7/8, whereas fungal RNA stimulates TLR7 [8–12]. Viruses invade mammalian cells using endosomal or phagosomal pathways and release their genomic RNA into endosomal compartments, triggering TLR7/8 responses.

dsRNA are sensed by RLR when they are present in the cytoplasm. Especially RIG-I recognizes RNA with 5'-triphosphate or diphosphate ends. Because RNA polymerase III produces transcription products with 5' triphosphate ends from dsDNA, RIG-I activation by dsDNA in the cytoplasm is thought to occur via an RNA polymerase III-dependent mechanism [13, 14]. However, this mechanism of viral nucleic acid sensing has yet to be confirmed. Furthermore, RNase L cleavage product from self-RNA and circular viral RNA have been reported to activate RIG-I, although it is unclear which motifs in these RNA species are recognized [15–18].

RIG-I and MDA5 are activated by dsRNA with different lengths; RIG-I detects short dsRNA (minimum 18–19 bp), whereas MDA5 detects long dsRNA (>1,000 bp) [19]. Similarly, in the laboratory, low- and high-molecular-weight poly(I:C) can be used for different purposes. Mammalian cells also produce short dsRNA of self-origin as miRNA duplexes. Although miRNA can bind to RIG-I, miRNA Dicer processing creates a 2-nucleotide overhang at the 3' ends of miRNA duplexes [20] that prevents RIG-I from activating the signaling cascade [21]. Thus, the structure of RNA species allows RLR to discriminate between self- and non-self-RNA.

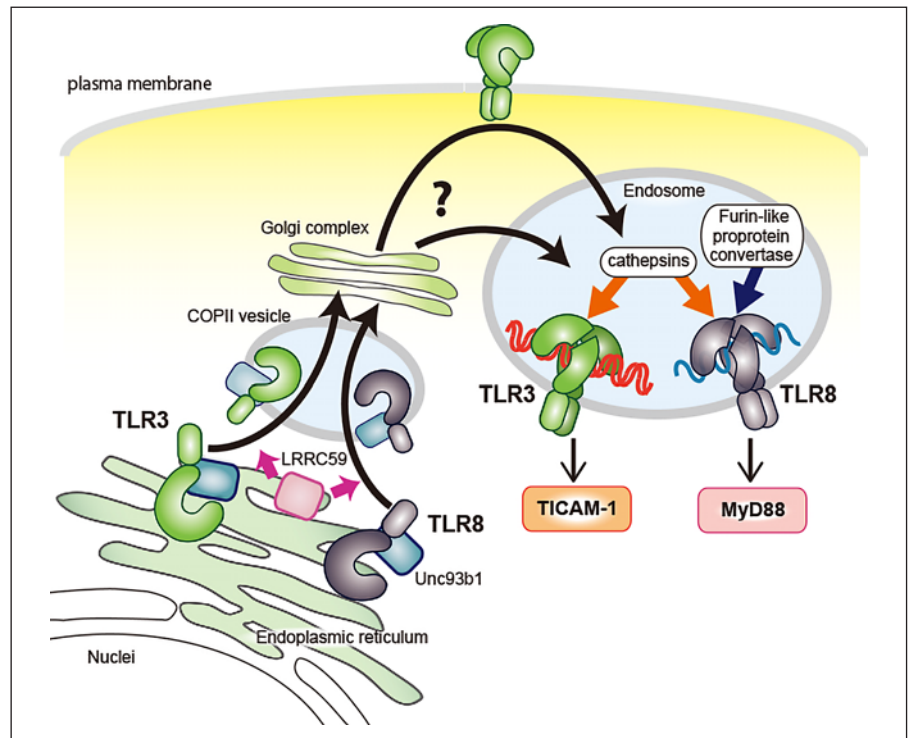
### RNA Sensing in the Endosomal Compartment

TLR3, TLR7, and TLR8 can detect extracellular dsRNA or ssRNA in the endosome or lysosome and initiate immune responses. Therefore, endosomal localization must be strictly regulated to avoid autoimmune reactions caused by self-RNA sensing.

TLR are secreted from the endoplasmic reticulum (ER) and transported to the Golgi apparatus by COPII-coated vesicles in a similar manner to other transmembrane proteins. UNC93B1 is known to play a key role in the ER-to-Golgi trafficking of NAS TLR [22, 23]. UNC93B1 binds to the transmembrane domains of TLR in the ER and aids in their translocation from the ER to the Golgi apparatus. Additionally, LRRC59, a type II transmembrane ER protein, binds to UNC93B1 and promotes the exit of TLR3 and TLR8 from the ER [24] (Fig. 2).

Different regions of TLR3, TLR7, and TLR8 are responsible for their intracellular localization. The linker region between the transmembrane and Toll-interleukin-1 receptor (TIR) domains of TLR3, the transmembrane domain of TLR7, and both the transmembrane and TIR domains of TLR8 are required for endosomal targeting [25–27]. Regarding post-Golgi trafficking, TLR7 is sorted from the Golgi apparatus directly to the endosome through the noncanonical endosomal sorting complexes required for transport pathway [28, 29]. However, the mechanisms of post-Golgi trafficking of TLR3 and TLR8 are not known. Considering that TLR3 is expressed on the cell surface of human fibroblasts and some epithelial cells, trafficking via the plasma membrane is conceivable, at least in cells with surface TLR3 [25, 30]. The detailed mechanism via which NAS TLR are targeted to the endosome/lysosome remains to be determined.

**Fig. 2.** Trafficking and maturation in the endosome of TLR3 and TLR8. UNC93B1 interacts with NAS TLR and regulates the COP II-mediated exit from the ER to the Golgi apparatus. Also, LRRC59 promotes the ER-to-Golgi delivery of TLR3 and TLR8. NAS TLR are translocated to the endosomal/lysosomal compartments and cleaved by proteases maturing to recognize ligands effectively.



NAS TLR are matured through proteolytic processing to bind ligands. This also contributes to the prevention of autoimmunity by limiting the activation site to the endolysosomal compartment. In mouse macrophages and dendritic cells (DC), TLR7 is processed by asparagine endopeptidase and pH-dependent cathepsins in a stepwise manner [31–33], whereas furin-like proprotein convertase cleaves human TLR7 and TLR8 [34, 35]. Murine TLR7 is thought to be digested at the asparagine endopeptidase-sensitive asparagine residue located between leucine-rich repeat (LRR) 14 and LRR15. However, this putative asparagine cleavage site is located in the C-terminal half of TLR8, suggesting that asparagine endopeptidase is not involved in TLR8 proteolysis. After processing, the N- and C-terminal halves of TLR7 and TLR8 re-associate, and both fragments contribute to ligand recognition. Although TLR3 is cleaved by cathepsins B and H at LRR12 [36], and the N- and C-terminal halves of TLR3 are associated [37], it is unclear whether this is necessary for ligand recognition since intact TLR3 binds dsRNA [38].

Acidic pH is important for the function of TLR3 [39]. TLR3 binds dsRNA more effectively in low-pH environments than at pH 7.0 [40]. The ligand-binding sites of TLR3 are located in the N- and C-terminals of the ectodo-

main. Upon the binding of dsRNA to both binding sites, TLR3 forms a dimer or oligomer that initiates signal transduction. For TLR3 dimerization, 40–50 bp of dsRNA are required [41]. However, short dsRNA between 21 and 30 bp can also trigger the formation of less stable TLR3 dimers [42]. The flexible recognition of RNA structures by TLR3 might allow a variety of RNA to be TLR3 ligands.

TLR3 and TLR7 are also expressed on the plasma membrane in some cell types including immune cells. Cell surface TLR3 are detected in mouse CD8-positive DC and marginal zone B cells as well as human fibroblasts, and TLR3-mediated signaling can be blocked by anti-TLR3 antibodies [30, 43], suggesting that cell surface TLR3 may have a role in the TLR3 response. However, TLR3 binds to dsRNA efficiently in acidic pH and delivers signals from endosomes, indicating that ligand recognition occurs mainly in endosomes. Also, cell surface expression of mouse TLR7 has been shown in splenic DCs and bone-marrow derived macrophages. Anti-TLR7 antibody inhibits TLR7 responses in vitro and in vivo [44]. TLR3 and mouse TLR7 need to be processed in the endosome, so trafficking between endosomes and the plasma membrane should regulate the cell surface expression of mature TLR3 and mouse TLR7. Cell type-specific surface



expression of these TLR regardless of endosomal expression levels indicates the existence of unknown mechanisms of trafficking to control expression sites.

On the other hand, human TLR7 can mature before reaching the endosome since it is proteolytically processed by furin-like proprotein convertase which does not require an acidic pH for its activity. In THP-1 cells, the lack of biological activity of TLR7 forcedly expressed on the cell surface has been demonstrated using chimeric protein of the TLR7 ectodomain and the TLR4 transmembrane and cytosolic domains as well as full-length TLR7 fused to a fragment of Ist2, a yeast protein with a plasma membrane-targeting motif. Both of these proteins containing a TLR7 ectodomain failed to respond to R837, a chemical ligand of TLR7, although they were properly processed to N- and C-terminal fragments [45]. Thus, the actual function of NAS TLR on the cell surface remains to be elucidated.

### Internalization of Extracellular RNA into the Endosome and Cytosol

Extracellular dsRNA are internalized by cells via clathrin-dependent endocytosis [46]. Raftlin, a lipid raft protein that is known to positively regulate B-cell receptor signaling, mediates the endocytosis of poly(I:C) bound to a clathrin heavy chain [47]. However, raftlin is not involved in all endocytic events and the molecular mechanism of raftlin-mediated nucleic acid internalization is not yet known. It is thought that a specific receptor for dsRNA uptake is expressed on the cell surface and that the relationship between this receptor and raftlin plays a key role in this interaction.

To date, several plasma membrane proteins have been shown to participate in dsRNA uptake in different species and cell types. Although the class A scavenger receptor has been reported to play a role in poly(I:C) internalization [48], it is not involved in TLR3- or RLR-mediated innate immune signaling [49]. CD11b has also been reported to be involved in poly(I:C) uptake by mouse macrophages but it contributed only partially to this function [50]. It seems that multiple mechanisms participate in RNA internalization.

The receptor for advanced glycation end-products (RAGE) has been shown to be a cell surface uptake receptor for CpG oligodeoxynucleotides [51] and was recently shown to function as an RNA uptake receptor as well [52]. In HEK293 cells, RAGE is expressed on the cell surface and enhances the signaling of TLR3, TLR7, TLR8, and

TLR13 upon stimulation with their respective ligands, which include dsRNA or ssRNA. Additionally, CD14 directly binds to and mediates internalization of dsRNA in bone marrow-derived macrophages [53]. However, the dsRNA uptake receptor in human myeloid DC, which do not express CD14, is yet to be determined. Since dsRNA must be internalized to reach endosomes, binding to these uptake receptors is the first step for RNA to access RNA sensors and trigger immune responses.

The discovery of a novel machinery that mediates endocytosis of dsRNA and its transport from endosomes to the cytosol is important for our understanding of the host reaction to extracellular RNA that have been internalized into cells. SIDT2, a mammalian ortholog of the *Caenorhabditis elegans* dsRNA transporter SID-1, localizes to endolysosomes and functions as a transporter of dsRNA, promoting escape from intracellular compartments [1]. Sixty minutes after its addition, poly(I:C) accumulates in the endosomal compartments of *Sidt2* knockout bone marrow-derived DC, whereas poly(I:C) is diffuse in wild-type (WT) cells. Notably, there was no difference in the uptake of dsRNA between knockout and WT cells. Moreover, SIDT2 positively regulates MDA-5-mediated defense against encephalomyocarditis virus infection. Additionally, SIDT2 has been reported to be involved in uptake of ssRNA in a human cell line [54]. Further investigations are required to uncover the cell type specificity and selectivity of RNA structures in SIDT2-mediated RNA transport (Fig. 1).

### RNA-Sensing Signaling Pathways

TLR3 induces TICAM-1 (TRIF)-dependent pathways, resulting in the production of type I interferon and pro-inflammatory cytokines via interferon regulatory factor (IRF) 3 and NF- $\kappa$ B activation [55]. TICAM-1 interacts with tumor necrosis factor receptor-associated factors (TRAF) and receptor-interacting protein (RIP) 1 [56]. The recruitment of TRAF3 leads to the activation of TANK-binding kinase (TBK) 1, followed by IRF3 phosphorylation and activation of NF- $\kappa$ B by TRAF6 and RIP1. Type I interferon induction by TLR3 is mediated by IRF3. This pathway also causes apoptosis via caspase 8. Additionally, the activation of DC and the induction of foreign antigen cross-presentation with dsRNA as an adjuvant are important components of the TLR3-TICAM-1 pathway in antitumor immunity [57]. The TLR3-TICAM-1-IRF3-IFN- $\beta$  axis is indispensable for poly(I:C)-induced cross-presentation in DC. The TLR3-specific ligand AR-

NAX induces IL-12 and IFN- $\beta$  production by professional antigen-presenting DC and cross-primes antigen-specific CD8<sup>+</sup> T cells, leading to antitumor immunity without inflammatory cytokine production [58].

All TLR, with the exception of TLR3, trigger MyD88-dependent signal transduction. IL-1 receptor-associated kinases, TRAF6, and IRF7 are recruited to Myd88, resulting in NF- $\kappa$ B activation by TRAF6 and type I interferon induction by IRF7.

RLR are ubiquitously expressed in various types of cells and induce robust proinflammatory cytokines and type I interferons. RIG-I and MDA5 bind RNA ligands via their helicase domains, whereas the C-terminal and caspase activation and recruitment (CARD) domains bind to MAVS, an adaptor molecule localized to the mitochondrial-associated membrane, upon ligand recognition. Upon recognition of RNA by RIG-I, the C-terminal domain of RIG-I plays a crucial role in sensing 5' di- and triphosphates of dsRNA, forming a pocket for binding of the ligand ends [59, 60]. Although MDA5 does bind short dsRNA, the binding of long dsRNA enables MDA5 to form a helical filament and initiate signal transduction via MAVS [61, 62]. Oligomerized MAVS forms a signaling complex with TRAF3, I $\kappa$ B kinase, and TBK1, leading to the activation of NF- $\kappa$ B and IRF3/7. RLR activation is controlled by several regulatory mechanisms [63–65] and aberrant activation of the MDA5-MAVS pathway can trigger autoimmune disorders [66].

### Antiviral Immunity Mediated by RNA Sensors

TLR3 is expressed in immune and nonimmune cells, including conventional DC, macrophages, fibroblasts, and epithelial cells. Based on experiments with *Tlr3* knockout mice, TLR3 appears to be involved in immunity against mouse cytomegalovirus [67], encephalomyocarditis virus [68], coxsackievirus [69], and poliovirus [70]. In the case of West Nile virus infection, TLR3 may both benefit and inhibit the virus [71, 72]. Additionally, *Tlr3* knockout mice infected with influenza virus A reportedly had higher survival rates than WT mice [73]. It is thought that WT mice produce lethal levels of inflammatory mediators upon IVA infection. Since IVA is a negative-stranded RNA virus, it barely generates stable dsRNA during replication [74], indicating that structured RNA, but not dsRNA, activates TLR3.

In human clinical cases, TLR3-mediated signaling has been shown to be critical for protection against herpes simplex encephalitis in children. Gene mutations in *Tlr3*,

*Unc93b1*, *Ticam-1*, *Traf3*, and *Irf3* have been reported in patients with herpes simplex encephalitis [75–80]. Similarly, TLR3-TICAM-1 pathway-dependent interferon production has been shown to be crucial for human host defense mechanisms against herpes simplex virus 1 infection.

TLR7 is predominantly expressed by plasmacytoid DC, whereas TLR8 is expressed by monocytes, macrophages, and myeloid DC. TLR7 produces type I interferons in plasmacytoid DC infected with ssRNA viruses such as human immunodeficiency virus (HIV)-1, Sendai virus, and flaviviruses [81–84]. TLR8 is also activated in HIV-1 infection [82]. Unlike TLR7, TLR8 activation mainly results in the production of inflammatory cytokines due to the types of cells in which it is expressed. A recent study demonstrated that recognition of live bacteria by TLR8 induced human monocytes to produce large amounts of IL-12, which drives a follicular helper T-cell response in naive human CD4<sup>+</sup> T cells [85].

It is known that RIG-I and MDA5 play different roles during viral infection due to their different ligand specificity. Genomic dsRNA from dsRNA viruses and replication byproducts of positive-stranded ssRNA viruses activate both RIG-I and MDA5. Nonsegmented negative-stranded ssRNA viruses produce copy-back defective interfering particles that can be sensed by RIG-I [86]. Additionally, the genomes of segmented negative-stranded ssRNA viruses provide panhandle structures that can be recognized by RIG-I but not by MDA5 [87].

RIG-I is activated by a variety of viruses, including vesicular stomatitis virus, Newcastle disease virus, IVA, IVB, hepatitis C virus, and the Ebola virus [88–94]. In contrast, MDA5 responds to infection with encephalomyocarditis virus, Theiler's virus, and Mengo virus [95, 96]. The Sendai virus, the West Nile virus and the dengue virus induce responses by both RIG-I and MDA5 [97–99]. RLR have been thought to play a role mainly in infected cells because they detect RNA in the cytosol. However, SIDT2-mediated RNA translocation from endosomes to the cytosol may explain how RLR in non-infected bystander cells induce immune responses. Likewise, exosomes may help RNA translocation in some situations including cancer and infections [100, 101].

### Concluding Remarks

RNA sensors play different roles in diverse infections and tissue injuries. PRR activated by RNA are expressed on different cell types and they are localized to different

areas of cells. This diversity allows the host to tailor its response to diverse infections with microbes that employ different infection routes and strategies to evade host defenses. Multiple levels of regulation of RNA-sensing systems maintains appropriate immune responses to ssRNA and dsRNA from pathogenic microbes and damaged cells and avoids excessive inflammatory responses. Indeed, inappropriate TLR activation is related to the pathogenesis and/or progression of autoimmune diseases such as systemic lupus erythematosus and rheumatoid arthritis.

In clinical applications, RNA species are considered to be potential adjuvants for cancer immunotherapies and vaccinations. Therefore, it is important to understand the common and unique mechanisms that underlie the activation and regulation of RNA sensors. For example, SIDT2 may be a novel target for selective activation of TLR by RNA trapping in endosomes. New insights into the molecular mechanisms of RNA sensing will uncover

novel strategies for the optimization of therapeutic RNA species, lead to clinical benefits, and further our understanding of innate immunity in mammals.

## Disclosure Statement

The authors declare no conflict of interests.

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