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Distinct and Orchestrated Functions of RNA Sensors in Innate Immunity

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Summary

Faithful maintenance of immune homeostasis relies on the capacity of the cellular immune surveillance machinery to recognize 'nonself', such as the presence of pathogenic RNA. Several families of pattern-recognition receptors exist that detect immunostimulatory RNA and then induce cytokine-mediated antiviral and proinflammatory responses. Here we review the distinct features of *bona fide* RNA sensors – Toll-like receptors and RIG-I-like receptors in particular – with a focus on their functional specificity imposed by cell type-dependent expression, subcellular localization, and ligand preference. Furthermore, we highlight recent advances on the roles of NOD-like receptors and DEAD-box or DEAH-box RNA helicases in an orchestrated RNA-sensing network and also discuss the relevance of RNA sensor polymorphisms in human disease.

eTOC Blurb:

Liu and Gack summarize recent advances on the physiological role of classical and emerging RNA sensors and co-sensors in innate immunity, their 'cross-talk' to form an orchestrated RNA-sensing network, and the relevance of polymorphisms in RNA sensors for human disease.

eTOC Blurb:

Recent advances identify several RNA sensors and co-sensors of innate immunity which are critical for the detection of viral pathogens. Gack and colleague review the individual roles of these sensors, their 'cross-talk' to form an orchestrated RNA-sensing network, and the relevance of polymorphisms in RNA sensors for human disease.

Introduction

Loss of homeostatic control of the immune response is a key determinant of human disease pathogenesis. Exogenous insults such as microbial infection or danger signals from within the cell (*e.g.* during cell injury) are rapidly recognized by the innate immune system, which elicits host mechanisms to resolve the threats. Failures in tightly regulating these defense

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DECLARATION OF INTERESTS

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mechanisms can lead to detrimental consequences such as chronic infection or autoimmunity. Two major innate immune defense programs are the type I interferon (IFN) and IL-1-mediated proinflammatory responses. While type I IFNs signal through the IFNα/β (IFNAR) receptor to upregulate IFN-stimulated gene (ISG) products that have antiviral or immunomodulatory activities, IL-1β of the IL-1 cytokine family engages the cognate IL-1 receptor to induce inflammation during microbial infections. Accumulating evidence shows extensive 'cross-talk' between type I IFN and IL-1 responses in microbial infections and autoinflammatory disorders, wherein either cooperative or negatively-regulatory circuits have been reported (Mayer-Barber et al., 2014; Mayer-Barber and Yan, 2017).

The production of type I IFNs and IL-1β relies on the activation of a set of sensors of the innate immune system, collectively known as pattern-recognition receptors (PRRs). These PRRs survey different cellular compartments and have evolutionarily gained the capability to distinguish foreign or abnormal molecules from host-cell components. Viral RNA species represent a pivotal class of pathogen-associated molecular patterns that activate these sensors. Depending on their cellular localization, which is determined by the route of viral entry and site of virus replication, viral RNA-ligands are recognized by one or more specific PRRs which subsequently mount coordinated host responses. Recent advances have expanded the repertoire of immunostimulatory RNA to certain RNA species of host origin, in particular different types of cellular noncoding RNAs. These host RNAs become immunostimulatory when chronically-upregulated, mislocalized, and/or misprocessed during viral infection or in disease settings such as autoimmunity. Accordingly, the recognition of cellular immunostimulatory RNAs triggers innate immune responses to combat viral infection, or it can cause chronic inflammation. The vast majority of immunostimulatory RNAs – both pathogen-and host-derived RNAs – reside in the endosome and cytoplasm, where also most RNA sensors are localized. Intriguingly, recent studies show that PRR-mediated RNA sensing can also occur in the nucleus and mitochondrion (Cao et al., 2019a; Liu et al., 2018; Wang et al., 2019; Zhang et al., 2020), highlighting an orchestrated multi-compartmental RNA-sensing paradigm.

Here, we summarize our current knowledge on classical and emerging RNA sensors and co-sensors of the innate immune system, with an emphasis on their functional distinction imposed by cell type-dependent expression, subcellular localization, and ligand specificity. We also highlight their physiological roles in type I IFN and IL-1β responses as well as their interdependent regulation. Finally, we discuss the relevance of polymorphisms in genes encoding RNA sensors for human disease conditions.

Toll-like Receptors

Toll-like receptors (TLRs) represent well-characterized PRRs in mammals whose identification preceded the other PRR families. They are named after the *Drosophila* Toll proteins which are involved in *Drosophila* embryo development and antimicrobial peptide expression (O'Neill et al., 2013). To date, the mammalian TLR family contains ten (TLR1–10) and twelve (TLR1–9, TLR11–13) members in human and mouse, respectively, among which TLR3, TLR7, TLR8, and TLR13 sense RNA. TLRs primarily localize to the cell surface and/or endosomes, and share a common structural architecture consisting of a

leucine-rich repeats (LRR)-containing domain at the amino (N)-terminus, a transmembrane domain, and a carboxyl (C)-terminal cytoplasmic Toll/IL-1 receptor (TIR) domain (Jin and Lee, 2008). Upon ligand binding to the LRR-containing ectodomain, TLRs predominantly form homodimers that are characterized by an “m”-shaped architecture. Unlike that of most TLRs, the apo form LRR domain of TLR8 has been crystallized as a pre-existing inactive homodimer, but not a monomer (Choe et al., 2005; Tanji et al., 2013; Zhang et al., 2016). In the dimer, the cytoplasmic TIR domains of TLRs are orientated in such a way that allows for the recruitment of downstream adaptor proteins for signaling transduction (Figure 1). The myeloid differentiation primary-response protein 88 (MyD88) is the key adaptor of all TLRs except for TLR3. It contains a TIR domain which mediates homotypic interactions with the TIR domain of TLRs. The recruitment of MyD88 triggers the assembly of the ‘Myddosome’, which is mediated by homotypic interactions between the death domains (DD) of MyD88 and also the DD–DD interactions between MyD88 and IL-1R-associated kinases (IRAK) such as IRAK1, IRAK2 and IRAK4 (Gay et al., 2011). This higher-order complex serves as a signaling platform for the recruitment of TNF receptor-associated factor 6 (TRAF6), which then activates TGFβ-activated kinase 1 (TAK1). TAK1 leads to the activation of mitogen-activated protein kinases (MAPKs), nuclear factor (NF)-κB, and IFN-regulatory factor 5 (IRF5) (Bergstrom et al., 2015; Kawai and Akira, 2010; Takaoka et al., 2005). TLR3 signals exclusively through the TIR-domain-containing adapter-inducing interferon-β (TRIF) (Hoebe et al., 2003; Oshiumi et al., 2003; Yamamoto et al., 2003). TRIF interacts with TRAF3 to trigger the TANK-binding kinase 1 (TBK1)–IκB kinase ε (IKKε) axis for IRF3 activation, or associates with TRAF6 and receptor-interacting protein kinase 1 (RIPK1) to activate NF-κB and MAPKs via TAK1 (Kawai and Akira, 2010). In plasmacytoid dendritic cells (pDCs), TLR7 activates IRF7 exclusively through a MyD88–IRAK1/4–TRAF6 axis (Honda et al., 2004; Kawai et al., 2004; Uematsu et al., 2005). The activation of NF-κB, activator protein 1 (AP1), and IRF3 or IRF7 ultimately leads to the expression of type I and III IFNs as well as pro-inflammatory cytokines (Figure 1).

The RNA-sensing TLRs are known to recognize their ligands with defined specificity, which has also been supported by structural studies (Table 1). TLR3 contains two RNA-binding sites that recognize double-stranded (ds)RNA in a sequence-independent manner (Alexopoulou et al., 2001). The minimal dsRNA length required for TLR3 activation *in vitro* has been shown to be 39–48 bp, which conforms to the length of dsRNA co-crystallized with the TLR3 ectodomains (Leonard et al., 2008; Liu et al., 2008). In cells engineered to express intracellular (but not cell surface-bound) TLR3, a minimal length of 90 bp is required for activation (Leonard et al., 2008). TLR3 has also been shown to be activated by small-interfering RNAs (siRNA) that are ~20 bp in length (Kariko et al., 2004a; Kleinman et al., 2008), though the underlying mechanism of siRNA recognition by TLR3 remains unknown. TLR3 plays an important role in the innate immune response to viral pathogens such as West Nile virus (WNV), influenza A virus (IAV), and poliovirus (Abe et al., 2012; Daffis et al., 2008; Le Goffic et al., 2006; Wang et al., 2004); however, the physiological TLR3 ligands in these contexts remain uncharacterized, except for poliovirus-derived RNA harboring stem-loop core structures (Tatematsu et al., 2013). In myeloid DCs, TLR3 also responds to phagocytosed RNA of viral (and likely also host) origin (Kariko et al., 2004b; Tatematsu et al., 2013), and phagocytosed viral RNA enhances cross-priming of cytotoxic T

cells (Schulz et al., 2005). TLR7 and TLR8 are highly homologous RNA sensors with an RNA ligand preference for single-stranded (ss)RNA (Diebold et al., 2004; Heil et al., 2004; Lund et al., 2004). In humans, TLR7 is primarily expressed in pDCs and B cells, while TLR8 is expressed in myeloid DCs, monocytes, and monocyte-derived DCs (Gorden et al., 2005). Both TLRs contain two RNA ligand-binding sites within their LRR domain, designated site 1 and site 2, both of which are indispensable for TLR7 and TLR8 activation. The site 1 is a highly conserved binding site for nucleosides, with TLR7 and TLR8 having a preference for guanosine (G) and uridine (U), respectively (Tanji et al., 2015; Zhang et al., 2016; Zhang et al., 2018b). The oligonucleotide-binding activity is conferred by site 2. While TLR8 binds the UG dinucleotide, TLR7 requires at least a 3-mer motif containing a U in the second position. Further analysis reveals that the site 2 ssRNA sequence preference is UU(U/C) > UU(G/A) > XUX (where X stands for non-U) (Tanji et al., 2015; Zhang et al., 2016; Zhang et al., 2018b). However, it remains unclear how exactly physiological ssRNAs activate TLR7 and TLR8 by coordinating both binding sites. To date, TLR7 has been shown to mediate type I IFN responses to several RNA viruses, such as IAV, vesicular stomatitis virus (VSV), and human immunodeficiency virus-1 (HIV-1) (Diebold et al., 2004; Heil et al., 2004; Lee et al., 2007; Lund et al., 2004). The detailed ligand characteristics of a few microbial and cellular TLR7 agonists have been identified, which include HIV-derived GU-rich ssRNA, microRNA let-7, and multiple siRNA sequences (Heil et al., 2004; Hornung et al., 2005; Lehmann et al., 2012). The orphan receptor TLR13 is a mouse-specific endosomal TLR that is predominantly expressed in DCs and macrophages. It recognizes bacterially and virally derived RNAs, leading to proinflammatory and type I IFN responses through NF-κB and IRF7, respectively (Hidmark et al., 2012; Shi et al., 2011). Furthermore, the bacterial 23S ribosomal RNA (rRNA) has been identified to be a physiological ligand of TLR13. This RNA encodes a highly conserved 13-nt sequence within the ribozyme catalytic domain that mediates TLR13 activation *in vitro* and *in vivo* (Li and Chen, 2012; Oldenburg et al., 2012). Structural analysis of the TLR13 ectodomain in complex with the 23S rRNA-derived 13-nt ssRNA reveals that a unique stem-loop structure is crucial for TLR13 binding in a sequence- and conformation-specific manner. A similar RNA motif activating TLR13 is found in the VSV genome (Shi et al., 2011; Song et al., 2015) (Table 1).

The RNA-sensing TLRs are subjected to regulation by multiple mechanisms. Among those, regulation of TLR subcellular compartmentalization represents a key regulatory mechanism (Miyake et al., 2017; Pelka et al., 2016), and mislocalization of TLRs has been shown to lead to autoimmunity (Fukui et al., 2011; Kono et al., 2009). TLR trafficking from the endoplasmic reticulum (ER) to endolysosomes is primarily mediated by the multipass-transmembrane protein Unc-93 homolog B1 (UNC93B1), which directly interacts with TLRs (Kim et al., 2008; Lee et al., 2013; Tabeta et al., 2006). UNC93B1 can also direct TLR3 and TLR7 to the cell surface (Kanno et al., 2015; Pohar et al., 2013). Moreover, UNC93B1 has been shown to stabilize TLR3 and TLR7 prior to their endosomal trafficking in mouse DCs and macrophages (Pelka et al., 2018), and to limit TLR7 activation by recruiting syntenin-1 which facilitates TLR7 sorting into exosomes (Majer et al., 2019). Another major regulatory mechanism for TLR7 and TLR8 is proteolytic cleavage. Proteolytic processing by compartment-specific proteases, such as the furin-like propeptide convertases, ultimately ensures proper maturation of TLR7 and TLR8 in endosomes (Hipp et

al., 2013; Ishii et al., 2014). The protease cleavage sites locate to the site 2 (within a loop structure called ‘Z-loop’) that is involved in ssRNA binding (Zhang et al., 2016). A failure in Z-loop cleavage inhibits TLR8 dimerization and renders it inactive (Hipp et al., 2013; Tanji et al., 2016; Tanji et al., 2013). Phosphorylation has also been shown to regulate the activation of RNA-sensing TLRs. Activation of TLR3 and TLR8 has been shown to require phosphorylation of several tyrosine residues located in their TIR domains, although the kinases responsible for phosphorylation remain controversial (Leifer and Medvedev, 2016). Moreover, TLR3 has been shown to undergo N-linked glycosylation, and chemical inhibition of glycosylation or mutational perturbation of the predicted glycosylation sites impairs TLR3-mediated NF-κB activation (Sun et al., 2006).

RIG-I-like Receptors

The family of retinoic-acid inducible gene-I (RIG-I)-like receptors (RLR) encompasses three members: RIG-I, melanoma differentiation-associated protein 5 (MDA5), and laboratory of genetics and physiology 2 (LGP2) (Andrejeva et al., 2004; Cui et al., 2001; Yoneyama et al., 2004). They belong to the SF2 helicase superfamily whose members mostly act as NTP-dependent RNA helicases. RLRs predominately localize to the cytoplasm, with a small portion of RIG-I also localized to the nucleus (Liu et al., 2018). RIG-I and MDA5 are the two signaling-competent RLR members with a common domain architecture that consists of a central helicase flanked by an N-terminal tandem caspase activation and recruitment domain (CARDs) and a C-terminal domain (CTD). LGP2 contains the helicase domain and CTD but lacks the N-terminal CARDs (Saito et al., 2007), and thus mainly acts as a regulator of RIG-I and MDA5 due to a lack of signaling competency (Bruns and Horvath, 2015). In the absence of RNA ligands, RIG-I resides in an auto-inhibitory conformation in which the CARDs are sequestered through an interaction of CARD2 with the Hel-2i subdomain of the helicase. In contrast, MDA5 is believed to adopt an open, quite flexible conformation in the absence of stimulatory RNA. RNA binding to the CTD and helicase of RIG-I and MDA5 liberates the CARDs for downstream protein association (Kolakofsky et al., 2012) (Figure 1). The exposed CARDs interact with several regulatory enzymes *(e.g.* ubiquitin E3 ligases and phosphatases), which posttranslationally modify the CARDs, making them fully signaling-active. RLRs in their signaling-primed forms then interact with members of the 14–3-3 protein family, specifically 14–3-3ε and 14–3-3η which, respectively, mediate the translocation of RIG-I and MDA5, to their common adaptor protein MAVS (mitochondrial antiviral signaling protein). MAVS predominately localizes to the outer mitochondrial membrane and has also been found at mitochondria-associated membranes of the endoplasmic reticulum (MAMs) and peroxisomes (Chow et al., 2018a; Horner et al., 2011; Tan et al., 2018). MAVS contains a C-terminal transmembrane domain and an N-terminal CARD domain involved in the homotypic interaction with the CARDs of RIG-I and MDA5. Activated MAVS recruits TRAF2, TRAF5, TRAF6, and TRADD which – via the TRAF3–TANK–TBK1 axis – ultimately leads to IRF3 or IRF7 activation and the induction of type I and III IFNs. Additionally, MAVS signaling induces NF-κB activation for pro-inflammatory cytokine expression through the FADD–RIP1–IKK axis (Liu et al., 2013; Michallet et al., 2008) (Figure 1). RLR signaling also ‘cross-talks’ with other sensing pathways such as TLRs, NLRs, and the cGAS-STING pathway (Barber, 2015; Zevini et al.,

2017). Conceptually, PRR crosstalk can occur at the transcriptional level whereby activation of an immediate sensor, via autocrine or paracrine IFNAR signaling, transcriptionally upregulates other PRRs, which usually are themselves ISGs. Furthermore, different sensors can collaborate at the level of downstream signaling molecules. For example, TLR7 and RLRs have been shown to pair respectively with IRF5 and IRF3 to induce partitioned gene expression and immune cytokine production in pDCs, leading to polarized immune response outcomes (Chow et al., 2018b). Moreover, RLRs have recently been shown to also suppress viral replication in a signaling-independent manner (Sato et al., 2015; Weber et al., 2015).

Àctivation of RIG-I and MDA5 requires multiple steps which include, besides RNA binding, the multimerization and posttranslational modifications (PTMs) of the sensors. Both receptors form ATP-mediated filaments on dsRNA, however with distinct mechanisms [(described in detail elsewhere) (Reikine et al., 2014)]. Upon filament formation, the 'head-to-tail' stacking of monomeric RIG-I or MDA5 brings the nearby CARDs into proximity to oligomerize into a 'lock-washer'-like helical assembly. This helical oligomer then induces the formation of prion-like MAVS filaments (Hou et al., 2011; Peisley et al., 2014; Wu et al., 2014). Multiple PTMs that regulate either RNA binding or CARD signaling by RLRs have been identified (Chiang and Gack, 2017). Acetylation of RIG-I at K909, which is located within its CTD, inhibits RNA binding and RNA-dependent RIG-I oligomerization. As such, deacetylation of the RIG-I CTD by histone deacetylase 6 (HDAC6) is necessary for RIG-I activation (Choi et al., 2016; Liu et al., 2016). Serine or threonine phosphorylation of the CTD and CARDs prevents aberrant activation of RIG-I and MDA5 in uninfected cells (Gack et al., 2010; Maharaj et al., 2012; Sun et al., 2011; Takashima et al., 2015; Wies et al., 2013). Dephosphorylation by cellular phosphatases PP1α or PP1γ upon RNA stimulation is required for RIG-I and MDA5 activation (Wies et al., 2013). In the case of RIG-I, dephosphorylation of the CARDs by PP1 induces K63-linked polyubiquitination, which is required for RIG-I activation by promoting its oligomerization (Gack et al., 2007; Jiang et al., 2012). RIG-I undergoes covalent K63-linked ubiquitination not only in the CARDs but also CTD (Gack et al., 2007; Oshiumi et al., 2009; Oshiumi et al., 2013). In addition, noncovalently-bound K63-polyubiquitin at the CARDs has been shown to activate RIG-I *in vitro* (Zeng et al., 2010); however, the relevance of noncovalent K63-polyubiquitin in RIG-I activation in infected cells is less well established. Over the past years, several E3 ubiquitin ligases have been proposed to be involved in RIG-I K63-ubiquitination; however, only tripartite motif-containing protein 25 (TRIM25) and ring finger protein 135 (RNF135, also known as Riplet) have been demonstrated to directly catalyze RIG-I K63-ubiquitination and to mediate antiviral innate immune responses (Gack et al., 2007; Oshiumi et al., 2009; Rehwinkel and Gack, 2020). For TRIM25 in particular, multiple cellular interacting proteins (*e.g.* NLRP12, caspase 12, and NDR2) and host noncoding RNAs have been shown to modulate the TRIM25-mediated RIG-I CARD ubiquitination (Chen et al., 2019; Lin et al., 2019; Liu et al., 2019; Sanchez et al., 2018; Wang et al., 2010). TRIM25 has also been shown to be a major target of viral evasion strategies (Chan and Gack, 2016). In the context of full-length RIG-I, the current model is that K63-linked ubiquitination of the RIG-I CTD at K788 by Riplet relieves the auto-repression of RIG-I, which serves as a prerequisite for subsequent K63-linked ubiquitination at K172 within the CARD by TRIM25 (Gack et al., 2007; Oshiumi et al., 2013). Of note, a recent study claims that Riplet is the only required

E3 ligase for the K63-linked ubiquitination and activation of RIG-I (Cadena et al., 2019). However, the data from this study contradict an abundance of work by several independent groups which demonstrates that TRIM25 directly ubiquitinates RIG-I (both in cells and *in vitro*) and is necessary for efficient RIG-I signaling in response to a variety of viruses [(reviewed in detail elsewhere) (Rehwinkel and Gack, 2020)]. The requirement of TRIM25 and/or Riplet in RIG-I signaling may be cell-type-or virus-specific, or temporally-distinct. Of note, Riplet has also been recently shown to regulate type I IFN responses independently of RIG-I by acting at the step of IRF3 activation (Vazquez et al., 2019). Whether K63-linked ubiquitination is necessary for MDA5 activation is less well understood. Unanchored K63-polyubiquitin chains have been reported to promote MDA5 CARD oligomerization (Jiang et al., 2012), presumably through a mechanism similar to that for RIG-I. TRIM65 has been identified as an E3 ubiquitin ligase that catalyzes covalent K63-linked ubiquitination of the MDA5 helicase (at K743), thereby triggering MDA5 activation during EMCV infection (Lang et al., 2017). Recently, ZCCHC3 has been identified as a coreceptor for both RIG-I and MDA5, which facilitates dsRNA binding and TRIM25-mediated K63-linked ubiquitination of both RLRs (Lian et al., 2018). The activities of many signaling proteins downstream of RLRs are also regulated by PTMs such as phosphorylation and different types of polyubiquitination, and E3 ligases of the TRIM protein family have emerged as key regulators of RLR signal transduction (van Gent et al., 2018). Alternative splicing represents another mechanism of RLR regulation. A splice variant of RIG-I which carries a short deletion within the first CARD has been shown to lose TRIM25 binding and K63-linked ubiquitination, thereby acting as a dominant-negative inhibitor of RIG-I signaling (Gack et al., 2008). Similarly, an N-terminally truncated form of MAVS produced from leaky ribosomal scanning dampens RLR signaling (Brubaker et al., 2014).

LGP2 is a major regulator of RLR signaling where it can act both positively and negatively. Several mechanisms for inhibition of RIG-I by LGP2 have been proposed, including competitive RNA binding (Murali et al., 2008; Rothenfusser et al., 2005; Yoneyama et al., 2005), competition with IKKε for MAVS interaction (Komuro and Horvath, 2006), blockage of RIG-I multimerization (Saito et al., 2007), and inhibition of the TRIM25-mediated K63-linked ubiquitination of RIG-I (Quicke et al., 2019). Studies in *Lgp2*$^{-/-}$ mice reveal a role of LGP2 in sensitizing MDA5-dependent antiviral responses to encephalomyocarditis virus (EMCV) infection (Satoh et al., 2010; Venkataraman et al., 2007), which requires the ATPase activity of LGP2 (Bruns et al., 2013; Satoh et al., 2010). The intimate cooperation of LGP2 with MDA5 during EMCV infection has further been substantiated by the fact that LGP2 associates with MDA5-stimulatory RNA (Deddouche et al., 2014). LGP2 facilitates the interaction of MDA5 with dsRNA as well as the formation of shorter MDA5 filaments (which also contain LGP2), which have been shown to be more immunostimulatory than long MDA5-only filaments (Bruns et al., 2014). Structurally, LGP2 preferentially interacts with dsRNA in an ‘end-capping’ mode. This is reminiscent of RIG-I binding but with relaxed ligand specificity. LGP2 can also form filaments on dsRNA in a less cooperative way than does MDA5. At a limiting concentration, LGP2 potentiates MDA5 signaling in response to poly(I:C), which is likely due to promoting MDA5 nucleation and oligomerization on dsRNA (Bruns et al., 2014; Uchikawa et al., 2016).

RLR ligands and their features

RIG-I and MDA5 play critical roles in sensing RNA derived from RNA viruses, leading to the induction of type I and III IFN responses. Accumulating evidence demonstrates that RIG-I and MDA5 can act distinctly or cooperatively to detect certain viruses. Negative-strand RNA viruses of the *Paramyxoviridae*, *Orthomyxoviridae*, *Rhabdoviridae*, *Bunyaviridae*, and *Filoviridae* families are mainly sensed by RIG-I, whereas positive-strand RNA viruses of the *Picornaviridae* family are sensed by MDA5 (Kato et al., 2008; Kato et al., 2006; Loo et al., 2008). The sensing of *Reoviridae* and *Flaviviridae* family members is mediated by both RIG-I and MDA5, highlighting the collaboration of the two receptors in antiviral immunity (Errett et al., 2013; Loo et al., 2008). Over the past decade, the RNA-ligand specificity of RIG-I has been extensively studied. Two canonical RNA characteristics are generally required for optimal RIG-I activation (Table 1). First, the RIG-I ligand requires a relatively short (~10 to 19 bp in length) dsRNA region that is usually blunt-ended (Schlee and Hartmann, 2016). RIG-I ligands also tolerate wobble base pairs, mismatches, and bulge elements as those contained in the panhandle structures of the RNA genomes of negative-strand RNA viruses (Liu et al., 2015; Schlee et al., 2009). While RNA recognition by RIG-I is generally believed to be sequence-independent, certain motifs in RNA ligands have also been proposed to confer, or facilitate, RIG-I binding. These include the poly(U/UC) tract in the 3’ UTR of the hepatitis C virus (HCV) genomic RNA (Saito et al., 2008), the GA-rich motifs embedded in the multi-branch loop structures of the cellular long noncoding RNA lnc-Lsm3b (Jiang et al., 2018), as well as AU-rich motifs found in the *L* gene mRNA of measles virus (Runge et al., 2014), Sendai virus (SeV) defective-interfering genome (Xu et al., 2015) and Kaposi’s sarcoma-associated herpesvirus (KSHV)-derived RNA transcripts (Zhang et al., 2018a). Second, typical RIG-I agonists harbor a 5’ di-or tri-phosphate group (Goubau et al., 2014; Hornung et al., 2006; Pichlmair et al., 2006; Schmidt et al., 2009), which is commonly present in the (sub)genomes and replication intermediates of negative-strand RNA viruses but is absent in most cellular RNAs. RIG-I ligands that lack a 5’ di-/triphosphate moiety have also been reported, such as RNase L-cleaved cellular RNA bearing 5’-OH and 3’-monophosphates (Malathi et al., 2007), RNase III-digested poly(I:C) (Kato et al., 2008), cellular RNA cleaved by inositol-requiring enzyme 1 (IRE-1) (Eckard et al., 2014), and exogenous circular RNA (Chen et al., 2017). The structural basis for RIG-I activation by these ‘non-canonical’ ligands warrants further investigation.

Accumulating evidence highlights the importance of RIG-I in sensing DNA viruses, such as herpes simplex virus type 1 (HSV-1), Epstein–Barr virus (EBV), KSHV, and adenovirus. RNAs detected by RIG-I during DNA virus infection can be from either viral or host origin and, further, are often RNA polymerase (Pol) III transcripts, which have been shown to activate RIG-I (Ablasser et al., 2009; Chiu et al., 2009). The 5S ribosomal RNA pseudogene transcript 141 (RNA5SP141) has been shown to activate RIG-I during HSV-1, EBV and also IAV infection. Viral infection induces the nucleus-to-cytoplasm relocalization of RNA5SP141, and viral host shutoff mechanisms downregulate specific proteins that normally associate with RNA5SP141. Consequently, RNA5SP141 accumulates in the cytoplasm of infected cells and becomes liberated for RIG-I binding (Chiang et al., 2018). During KSHV infection, viral downregulation of the cellular triphosphatase DUSP11 (Dual

specificity phosphatase 11) leads to the accumulation of 5'-triphosphorylated vault RNAs, which then activate RIG-I (Zhao et al., 2018). Furthermore, Pol III-driven small RNA transcripts derived from EBV (EBER RNAs) and adenovirus (VA RNAs) induce type I IFN responses in a RIG-I-dependent manner (Ablasser et al., 2009; Minamitani et al., 2011) (Table 1).

The characteristics of MDA5 ligands are much less well-understood than those of RIG-I agonists. MDA5 is preferentially activated by long irregular dsRNA which presumably exists in a complex higher-order configuration (Kato et al., 2008; Pichlmair et al., 2009). Partial digestion of synthetic poly(I:C) by RNase III shows that dsRNA longer than 300 bp activates MDA5 (Kato et al., 2008). The replication of DNA viruses, positive-strand RNA viruses, and dsRNA viruses is generally believed to produce long dsRNAs (Weber et al., 2006), which are potential sources of MDA5 ligands. Furthermore, parainfluenza virus 5 (PIV5) and measles virus (which harbor negative-strand RNA genomes) also elicit MDA5-mediated type I IFN responses, which has been shown to be mediated by an association of MDA5 with the mRNA of the viral *L* gene (Luthra et al., 2011; Runge et al., 2014).

Recent studies indicate a role for MDA5 in sensing endogenous dsRNA. dsRNAs of ~300 bp in length formed through hybridization of inverted repeat transposable *Arthrobacter luteus* (*Alu*) elements have been identified as MDA5 ligands in Aicardi-Goutières syndrome (AGS)-associated type I IFN induction (Ahmad et al., 2018). Furthermore, mitochondrial dsRNA has been shown to activate MDA5 in cells deficient of mitochondrial RNA helicase SUV3 and polynucleotide phosphorylase (PNPase) (Dhir et al., 2018) (Table 1).

NLRs and Their Roles in Viral RNA Sensing

The nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) are a group of intracellular proteins that mount immune responses to a variety of microorganisms and exogenous or cell-derived danger signals (Wen et al., 2013). They are characterized by the presence of a NOD domain which mediates dNTPase activity and NLR protein oligomerization. In addition, NLRs contain an N-terminal effector domain and a C-terminal LRR domain (Kanneganti et al., 2007). While the array of LRR motifs is responsible for ligand binding, the effector domain mediates binding to distinct downstream signaling proteins (Ting et al., 2008). Similar to RLRs, NLRs are in a monomeric state prior to activation in which an intramolecular interaction between the LRR and NOD domains prevents signaling. Ligand recognition stimulates conformational rearrangements that induce NLR oligomerization and the exposure of effector domains for protein recruitment (Lechtenberg et al., 2014). Multiple members of the 22 known human NLRs serve as scaffold proteins for the assembly of inflammasomes, which are large multiprotein complexes that catalyze caspase-1-mediated processing of pro-IL-1β and pro-IL-18 into their mature forms for secretion.

Other PRRs are known to 'cross-talk' with NLRs. For example, TLR signaling induces NF-κB-mediated transcriptional upregulation of NLRP3 and pro-IL-1β to prime inflammasome activation (Swanson et al., 2019). Recent studies show that some NLRs, in complex with specific DEAH-box RNA helicases, can sense viral dsRNA and then trigger type I IFN-

and/or inflammasome-dependent antiviral responses. Furthermore, several NLR members function as regulators of other RNA-sensing pathways, in particular the RLR-MAVS pathway (Figure 2).

NOD2 (also known as NLRC2) has been shown to recognize viral genomic ssRNA during RSV infection and to mediate IRF3-dependent antiviral responses via MAVS in both hematopoietic and non-hematopoietic cells. *Nod2*$^{-/-}$ mice were impaired in type I IFN responses to RSV infection and exhibited more severe pathology (Sabbah et al., 2009). NLRP6, which is highly expressed in intestinal epithelial cells and exerts anti-bacterial defenses, also plays a central role in type I and III IFN-mediated antiviral immunity in the intestine (Wang et al., 2015). *Nlrp6*$^{-/-}$ mice that were orally infected with EMCV or murine norovirus 1 had higher viral titers and increased mortality compared to control mice. Mass spectrometry analysis of affinity-purified NLRP6 identifies the RNA helicase DHX15 as an interaction partner. The NLRP6-DHX15 complex interacts with long viral dsRNA in EMCV-infected cells and then is recruited to MAVS to transcriptionally induce type I and III IFNs as well as ISGs, which ultimately leads to virus restriction (Wang et al., 2015). Another intestinally-expressed NLR member, NLRP9b, is critical for the restriction of rotavirus infection *in vivo* (Zhu et al., 2017). NLRP9b together with the RNA helicase DHX9 senses short dsRNA and then triggers ASC-and caspase 1-mediated IL-18 production. In addition, the NLRP9b-DHX9-ASC axis induces pyroptosis through gasdermin D activation (Zhu et al., 2017) (Figure 2).

Several NLR proteins are key regulators of RLR signaling. NLRP12 functions as a critical checkpoint in RIG-I-mediated signaling by modulating nondegradative and degradative polyubiquitination events of the sensor (Chen et al., 2019). NLRP12 interacts with the E3 ligase TRIM25, and this interaction prevents K63-linked ubiquitination of the RIG-I CARDs and thereby binding to the adaptor MAVS. On the other hand, NLRP12 promotes RNF125-mediated K48-linked ubiquitination of RIG-I and thereby its degradation by the proteasome machinery. Both actions of NLRP12 attenuate RIG-I-mediated antiviral and proinflammatory cytokine responses. Myeloid-cell-specific *Nlrp12*$^{-/-}$ mice exhibited more robust immune signaling to RNA virus infection, in particular VSV, or upon stimulation with 5′ppp-dsRNA (Chen et al., 2019). Another NLR family member, NLRX1, regulates RLR signaling by acting more downstream in the pathway, at the step of MAVS activation (Allen et al., 2011; Moore et al., 2008). NLRX1 was found to prevent the association of MAVS and RLRs, thereby impeding type I IFN induction. During HCV infection, NLRX1 was also shown to recruit hnRNPE2 to induce MAVS protein degradation (Qin et al., 2017). NLRX1 together with TUFM (mitochondrial Tu translation elongation factor) and the classical autophagy proteins, ATG5, ATG12 and ATG16L1, increases the cellular autophagic response, which in turn further limits RLR signaling (Lei et al., 2012). NLRX1 was shown to have itself RNA-binding capacity *in vitro* (Hong et al., 2012); however, the impact of RNA binding on NLRX1's role in the RLR pathway remains largely elusive. Another interconnection of the RLR and NLR pathways has been shown to be mediated by the NLRP3 inflammasome, which is important for *in vivo* host defenses against many pathogens including certain viral infections (*e.g.* IAV) (Swanson et al., 2019). MAVS was shown to function as a second critical adaptor of the NLRP3 inflammasome (besides the well-known

adaptor ASC) by positioning NLRP3 at mitochondria, which ultimately promotes IL-1β production (Subramanian et al., 2013) (Figure 2).

At least two NLR proteins have been shown to regulate RNA-sensing pathways by targeting critical kinases downstream of innate immune sensors. NLRP14, which is specifically expressed in germ cells, interacts with TBK1, promoting its degradation (Abe et al., 2017). This action limits RNA sensing and DNA sensing by the RIG-I-MAVS and cGAS-STING axis, respectively, which, if aberrantly activated in germ cells, would have deleterious consequences for fertilization. NLRC5, which is the largest NLR protein, inhibits both NF-κB activation and RLR-mediated type I IFN induction (Cui et al., 2010). NLRC5 interacts with IKKα and IKKβ, inhibiting their kinase activity and ultimately suppressing NF-κB-dependent cellular responses. In addition, NLRC5 has been shown to bind to the sensors RIG-I and MDA5, blocking IRF3-dependent cytokine expression (Cui et al., 2010). Somewhat conflicting results have been reported for cytokine responses in *Nlrc5*$^{-/-}$ mice (Kumar et al., 2011; Tong et al., 2012), suggesting a pathogen-specific or temporal role for NLRC5 in type I IFN-driven innate immune responses (Figure 2).

RNA helicases, hnRNPs, and ZBP1: Emerging RNA Sensors and Co-Sensors

RNA helicases are critical orchestrators of RNA metabolism and also play emerging roles in innate immune sensing. The SF1 helicase ZNFX1 has been shown to localize to mitochondria where it binds viral RNA and mediates MAVS-dependent, but RLR-independent, type I IFN responses (Wang et al., 2019). Apart from RLRs, other SF2 superfamily members such as DEAD-box or DEAH-box helicase proteins, have been shown to sense RNA (Figure 3). Characteristic features of these RNA helicases are an Asp-Glu-Ala-Asp (DEAD) or Asp-Glu-Ala-His (DEAH) motif within their helicase domains, which also contain highly conserved motifs that confer NTP hydrolysis and RNA unwinding capabilities. Two principal ways of how DEAD-box or DEAH-box helicases participate in RNA sensing have been proposed: They either act as direct RNA sensors that function independently of other PRRs, or they serve as co-sensors of RLRs or NLRs potentiating their activation.

The DEAD-box protein DDX1 has been identified as a poly(I:C)-binding protein in mouse myeloid DCs. It constitutively complexes with DHX36 through DDX21 to induce type I IFN and proinflammatory responses in a TRIF-dependent but TLR3-independent manner (Zhang et al., 2011a). Whereas the ablation of RLR expression leads to a major loss of type I IFN production in response to poly(I:C), silencing each component of the DDX1–DDX21–DHX36– TRIF complex further reduces type I IFN responses, suggesting an RLR-independent sensing pathway. DHX9 has been found to associate with poly(I:C) *in vitro* and to mediate cytokine responses to poly(I:C) or RNA virus infection in myeloid DCs in a RIG-I-independent but MAVS-dependent manner (Zhang et al., 2011b). DHX9 also interacts with specific NLR members to aid in viral RNA sensing and inflammasome activation (as described above) (Zhu et al., 2017). DHX33 has been shown to sense RNA and then trigger NLRP3 inflammasome activation and MAVS-dependent type I IFN induction in human

macrophages and mouse myeloid DCs, respectively (Liu et al., 2014; Mitoma et al., 2013). DHX33 preferentially binds dsRNA, such as poly(I:C) or reovirus genomic RNA (Mitoma et al., 2013). DHX33 also associates with RNase L-cleaved RNAs bearing 2',3'-cyclic phosphoryl termini in human macrophages, which triggers NLRP3-dependent IL-1β production during VSV and IAV infections (Chakrabarti et al., 2015). As with DHX9, DHX33-mediated type I IFN induction in myeloid DCs has been shown to require MAVS, but not RLRs.

Several helicase proteins have been identified to function as RLR co-sensors. DDX3 associates with MAVS and RLRs to strengthen type I IFN responses (Oshiumi et al., 2010), while DDX60 interacts only with RLRs, but not with MAVS, to facilitate the induction of type I IFNs and ISGs (Miyashita et al., 2011; Oshiumi et al., 2015). Both DDX3 and DDX60 bind to synthetic RNA *in vitro*; however, whether their RNA-binding ability is required for their potentiating effects on RLR signaling remains unclear. A recent study proposes that DDX3 can act as a direct RNA sensor that functions independently of RLRs. In human monocyte-derived DCs and macrophages, DDX3 associates with abortive HIV-1 RNA and endogenous cellular transcripts lacking 3' poly(A) tails, and then triggers type I IFN responses in a MAVS-and TRAF3-dependent manner (Gringhuis et al., 2017). In response to poly(I:C), DHX15 interacts with MAVS, thereby activating IRF3, MAPKs and NF-κB (Lu et al., 2014; Mosallanejad et al., 2014). DHX15 has been shown to sense specifically RNA virus infection (*i.e.* SeV and reovirus), but does not detect poly(dG:dC) or DNA virus infection (*i.e.* HSV-1) (Lu et al., 2014). DHX15 directly binds poly(I:C) in an ATPase-independent manner, and the RNA-binding affinity of DHX15 is enhanced by DHX9 interaction (Lu et al., 2014). DHX15 also cooperates with specific NLR proteins to facilitate type I and III IFN responses during EMCV infection (Wang et al., 2015). In the context of RLR signaling, DHX15 has been shown to interact with the RIG-I CARDs, leading to RIG-I activation (Pattabhi et al., 2019). DHX29 is reported to bind poly(I:C) *in vitro* and to interact with RIG-I and MAVS. Individual silencing of DHX29 and RIG-I leads to a comparable reduction in type I IFN gene expression, suggesting a shared signaling axis (Sugimoto et al., 2014). A recent study proposes that DHX29 functions as a co-sensor for MDA5, but not for RIG-I. Upon interaction, DHX29 promotes MDA5 activation by enhancing its ability to bind RNA and to form filaments (Zhu et al., 2018).

Heterogeneous nuclear ribonucleoproteins (hnRNPs) are nuclear or nucleocytoplasmic RNA-binding proteins that have recently been implicated in RNA sensing (Figure 3). Traditionally, hnRNPs are known to regulate RNA metabolism at either the transcriptional or translational level (Geuens et al., 2016). At least three hnRNP members have been identified to participate in the negative regulation of RLR signaling. Upon VSV infection, hnRNPE2 (also known as PCBP2) translocates from the nucleus to the cytoplasm, where it interacts with MAVS at the mitochondrial outer membrane to mediate MAVS degradation by the E3 ubiquitin ligase AIP4 (You et al., 2009). Such virus-induced nucleus-to-cytoplasm redistribution has also been observed for hnRNPM which, when present in the cytoplasm, interacts with RLRs to impair their RNA-binding activities (Cao et al., 2019b). Silencing of hnRNPC, a cancer biomarker, leads to the accumulation of endogenous dsRNA enriched in *Alu* elements. These RNAs induce RIG-I-mediated type I IFN responses, which, in turn, inhibit cancer cell proliferation and tumorigenesis (Wu et al., 2018). A recent study proposes

hnRNPU (also known as SAFA) as a nuclear RNA sensor (Cao et al., 2019a). HnRNPU associates with HSV-1-derived dsRNA in the nucleus and mediates type I IFN induction. HnRNPU also interacts with SMARCA5 and TOP1, two key components of the SWI/SNF nucleosome remodeling complex, to activate enhancers and superenhancers that drive the transcription of antiviral and proinflammatory genes. hnRNPU deficiency reduces the residual type I IFN response in cells lacking MAVS, TBK1 or IRF3, indicating a nuclear signaling axis directly acting on type I IFN transcription.

ZBP1 (also known as DAI) contains two N-terminal Z-DNA-binding domains (Zα1 and Zα2) and was originally identified as a DNA sensor that mediates type I IFN responses to synthetic DNA or HSV-1 infection (Takaoka et al., 2007). More recently, ZBP1 has been shown to function as an RNA sensor activating cell death pathways in response to viral infection. During IAV infection in mice, ZBP1 associates with IAV (sub)genomic RNA through the Zα2 domain, and engages RIPK3 to recruit either the RIPK1–FADD–caspase-8 complex for the activation of apoptosis, or MLKL for necroptosis induction (Kuriakose et al., 2016; Nogusa et al., 2016; Thapa et al., 2016). Structural analyses support that the ZBP1 Zα2 domain can accommodate either dsDNA or dsRNA in a ‘Z’ conformation (Thapa et al., 2016), and IAV infection has recently been shown to generate Z-RNA (Zhang et al., 2020). Of note, Z-RNA ‘signature’ is also known to be present within the transcripts of endogenous retroelements (*e.g. Alu*), which engage the Zα domain of ADAR1-p150 (the IFN-inducible isoform of ADAR1) for RNA editing (Herbert, 2019). ZBP1-associated IAV RNAs show similarity to those associated with RIG-I during IAV infection (Baum et al., 2010; Thapa et al., 2016), suggesting a set of RNA-PAMPs that is collaboratively sensed by the two sensors. These two sensors likely act in a sequential mode since ZBP1-induced cell death has been shown to require RIG-I-mediated type I IFN induction (Kuriakose et al., 2016). Further supporting a role for ZBP1 in RNA recognition, ZBP1 has been found to associate with nascently synthesized viral (and likely also host) RNA, but not DNA, during murine cytomegalovirus (MCMV)-induced necroptosis (Maelfait et al., 2017; Sridharan et al., 2017). While ZBP1 localizes primarily to the cytoplasm of uninfected cells, MCMV and IAV infections induce nuclear accumulation of ZBP1 (Maelfait et al., 2017; Sridharan et al., 2017; Zhang et al., 2020). ZBP1 also directly binds IAV-derived Z-RNA in the nucleus, within which it initiates necroptosis (Zhang et al., 2020).

Whereas these recent advances on the involvement of RNA helicases, hnRNPs, and ZBP1 in RNA sensing are exciting, additional studies are needed to confirm the physiological contribution of these proteins to innate immunity, and whether they primarily act by directly detecting RNA or by regulating RNA-dependent signaling through indirect mechanisms.

RNA Sensor Polymorphisms in Associated Autoinflammation and Infection

It has long been proposed that mutations that lead to aberrant nucleic sensing or constitutive activation of innate immune receptors may cause autoimmune or autoinflammatory diseases in humans. Indeed, common to several types of autoimmune diseases is an inborn excessive production of IFN-α/β, thus prompting the term ‘type I interferonopathies’. On the other hand, mutations that ablate the proper functioning of innate immune sensors are expected to decrease the host organism’s defense against pathogens. Recent research demonstrates that

specific mutations in members of the RLR and TLR protein families are associated with several inherited autoimmune disorders, or an increased susceptibility to infections.

Gain-of-function mutations in the RLR members *IFIH1* (encoding MDA5) and *DDX58* (encoding RIG-I) have been shown to be causative of several rare, inherited autoimmune diseases: classical and atypical Singleton-Merten syndrome (SMS) as well as specific types of Aicardi-Goutières-syndrome (AGS) and Systemic Lupus Erythematosus (SLE). Common to these diseases are constantly elevated type I IFN amounts; yet, each disease is caused by a distinct RLR polymorphism and hence a specific underlying mechanism of RLR activation.

AGS is a multi-symptom autoimmune disease that most consistently affects the brain and the skin. AGS is caused by mutations – autosomal recessive or autosomal dominant ones – in one of several genes that are involved in RNA-or DNA-metabolizing processes: *TREX1, ADAR1, SAMHD1* and *RNASEH2 (subunit A, B or C).* Additionally, multiple AGS-associated missense mutations in *IFIH1* have been identified (p.Arg337Gly, p.Arg779Cys, p.Gly495Arg, p.Asp393Val, p.Arg720Gln, p.Arg779His, p.Ala452Thr, p.Leu372Phe), all of which are monoallelic and located within close proximity to the RNA-binding or ATP-binding sites in the MDA5 helicase domain (Oda et al., 2014; Rice et al., 2014). *In vitro*, these MDA5 mutations lead to heightened type I IFN induction as compared to wildtype MDA5, and several mechanisms (*e.g.* more effective RNA-binding ability, constitutive MDA5 activation without the need of RNA) have been proposed (Oda et al., 2014; Rice et al., 2014). Recent reports indicate a mechanistic interplay between MDA5 and ADAR1 in AGS. Whereas loss-of-function of *ADAR* (lack of A to I conversion) triggers aberrant activation of MDA5 by recognition of unmodified endogenous RNAs, such as *Alu* elements (Ahmad et al., 2018; Liddicoat et al., 2015; Pestal et al., 2015), MDA5 variants found in AGS individuals show reduced susceptibility to inhibition by RNA duplex structural irregularities as compared to wildtype MDA5; this ultimately leads to the formation of signaling-competent filaments by mutant MDA5 on *Alu*-dsRNAs (Ahmad et al., 2018).

Genetic mutations in several different genes that encode critical components of the type I IFN pathway have been associated with SLE, a chronic autoinflammatory disease with a multitude of symptoms including arthritis, skin rashes, nephritis, and neuroinflammation. At least two polymorphisms were found in the *IFIH1* gene, p.Ala946Thr and p.Arg779His, the latter found by whole-exome sequencing in a patient with severe early-onset SLE (Cen et al., 2013; Gono et al., 2010; Molineros et al., 2013; Van Eyck et al., 2015). Both polymorphisms are proposed to lead to ‘hyper-active’ states of MDA5, which is consistent with the fact that SLE patients exhibit elevated amounts of type I IFNs.

Moreover, a missense mutations (p.Arg822Gln) in the gene encoding MDA5 has been found to be causative of SMS (Rutsch et al., 2015), which is an extremely rare, autosomal-dominant inherited disease that is characterized by dental and skeletal abnormalities and cardiac calcifications along with an enhanced type I IFN signature gene pattern. The Arg822Gln mutation is located in the HEL2 domain of MDA5 and is predicted to trigger conformational alterations that may increase the formation and/or stability of MDA5 filaments on dsRNA, resulting in constitutive activation of the sensor. Apart from this ‘classical’ type of SMS, a more recently discovered form of SMS called ‘atypical’ SMS has

been found to be caused by monoallelic mutations in the *DDX58* gene that encodes RIG-I (p.Cys268Phe and p.Glu373Ala), which are located in the ATPase motifs of the sensor (Jang et al., 2015). Functional characterization shows that these RIG-I mutations have deficient ATPase activity and increased binding to self RNA, which ultimately leads to aberrant innate immune signaling and transcriptional induction of IFN-β (Lassig et al., 2015).

Single nucleotide polymorphisms (SNPs) in the TLRs 3, 7 and 8 have been linked to an increased susceptibility to certain viral or bacterial infections. A rare variant of TLR3 (p.Pro554Ser), which is defective in activating IRF3 and NF-κB, has been identified in two patients that exhibited encephalitis caused by HSV-1 infection (Zhang et al., 2007). Two other TLR3 SNPs (p.Asn248Ile and p.Leu412Phe), with the latter being widely prevalent, locate in the LRR domain of TLR3 and were found to dampen TLR3 signaling activity *in vitro* by reducing its cell surface localization (Ranjith-Kumar et al., 2007). The p.Leu412Phe SNP was further proposed to increase cell survival responses in siRNA-based treatment of angiogenic disorders (Kleinman et al., 2008). Several SNPs in TLR7 – both coding changing and synonymous ones – were shown to affect chronic HCV infection or disease outcome (*i.e.*, fibrosis, inflammation) (Askar et al., 2010; Schott et al., 2007). For example, p.Gln11Leu in TLR7 decreased IFN-λ expression in chronic HCV-infected individuals but had no direct effect on viral loads (Askar et al., 2010). HIV-infected individuals carrying the same TLR7 variant (p.Gln11Leu) showed an accelerated progression of HIV-induced disease and higher viral loads (Oh et al., 2009). Of note, the TLR7 Gln11Leu polymorphism has also been linked to a predisposition to SLE especially in males from East Asian origin (Shen et al., 2010). Furthermore, at least two variants of TLR8 have been linked to higher susceptibility to pulmonary tuberculosis and Crimean-Congo hemorrhagic fever (Davila et al., 2008; Engin et al., 2010).

Concluding Remarks

Molecular insight into the activation and regulation of RNA sensors in innate immunity is important for understanding their implications in microbial infection and sterile inflammation. With the continuous effort to identify additional RNA sensors, what remains less well understood is the physiological relevance and contribution of emerging sensors to antimicrobial host defenses and/or pathology in relation to the known *bona fide* sensors. For example, the physiologic role of DEAD-box or DEAH-box RNA helicases, whose contribution to the type I IFN response relies strictly on MAVS but appears to be independent of RLRs, is not very well understood. It is conceivable that the relative contribution of DEAD-box or DEAH-box RNA helicases to antiviral type I IFN induction is dictated by a distinct temporal expression pattern. Furthermore, for many RNA sensors, especially the recently identified ones, physiological ligands and their detailed characteristics remain to be determined.

Since many of the emerging RNA sensors also have important roles in cellular RNA metabolism or exert direct antiviral functions, it will be important to determine the contribution of their direct RNA-sensing capacity to antiviral immunity. Furthermore, the molecular details of how different RNA sensors ‘cross-talk’ have just begun to be elucidated. This information will, more broadly, enhance our understanding of how RNA sensing, RNA

metabolism, and the direct activities of these proteins on viral nucleic acid cooperate in ‘nucleic acid immunity’.

Viral strategies to evade TLR and RLR signaling have been extensively investigated, which include *(1)* sequestration, masking, or modifying viral RNA, *(2)* antagonism of RNA sensors through direct binding or manipulation of their regulatory PTMs, and *(3)* inhibition of downstream signaling molecules through a variety of mechanisms (Chan and Gack, 2016). As such, exploration of how viruses evade detection by DEAD-box or DEAH-box RNA helicases and other emerging RNA sensors will be an important avenue for future research. Moreover, specific agonists of RLRs and TLRs showed promise in the treatment of cancers or infectious diseases (Barrat et al., 2016; Rehwinkel and Gack, 2020). Therefore, investigating the molecular features of RNA ligands may aid in developing therapeutic strategies to combat microbial infection, cancer, and autoinflammatory diseases.

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Highlights:

- RLRs and several TLRs sense microbial or cellular RNA in several cell compartments.
- NLRs can directly sense RNA or they regulate other RNA-sensing pathways.
- DEAD/DEAH-box helicases, hnRNPs and ZBP1 are emerging RNA sensors or co-sensors.
- RNA sensor polymorphisms are implicated in autoinflammation and infection.

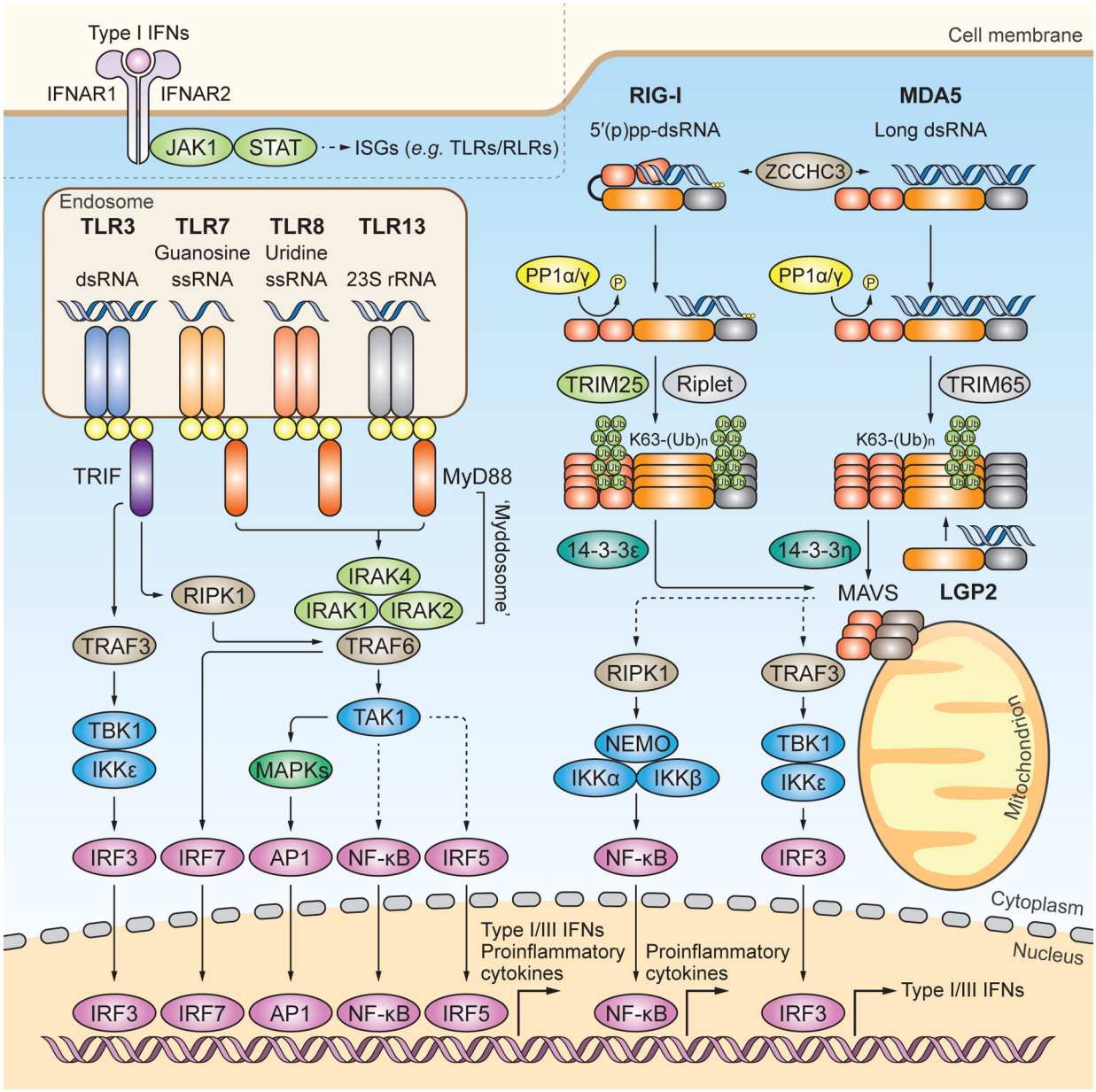

Figure 1. TLR and RLR signaling pathways

RNA-sensing TLRs predominately localize to the endosome and include TLR3, TLR7, TLR8 and TLR13. TLR3 recognizes dsRNA. TLR7 and TLR8 contain one binding site with specificity for guanosine and uridine, respectively, and the other binding site for ssRNA. TLR13 recognizes bacterial 23S rRNA. All RNA-sensing TLRs form homodimers upon activation. TLR7, TLR8 and TLR13 engage MyD88 as an adaptor protein to form the 'Myddosome', which is a multi-protein complex of MyD88, IRAK1, IRAK2 and IRAK4. The 'Myddosome' recruits TRAF6 and TAK1 to activate MAPKs, NF-κB, and IRF5. TLR3 signals through the adaptor protein TRIF to activate IRF3 via the TRAF3-TBK1–IKKε axis, or to activate MAPKs and NF-κB via the TRAF6-RIPK1-TAK1 axis. In plasmacytoid DCs, TLR7 activates IRF7 through TRAF6. It is noted that, for simplicity, this cell-type specific

pathway is not illustrated separately. Nuclear translocation of activated transcription factors ultimately drives the expression of proinflammatory cytokines and type I and III IFNs. The RLR family members RIG-I and MDA5 localize to the cytoplasm and preferentially recognize 5’ di-or tri-phosphorylated short dsRNA and long dsRNA, respectively. ZCCHC3 facilitates RNA binding of RIG-I and MDA5. Structurally, RIG-I is held in an auto-repressed, closed conformation, while MDA5 likely adopts an open and flexible conformation. Cognate RNA-ligand binding induces conformational changes in RIG-I and MDA5 that expose their N-terminal CARDs, which are then subjected to posttranslational modifications (PTMs). PP1α or PP1γ-mediated dephosphorylation and TRIM25-mediated K63-linked ubiquitination at the CARDs induce further activation of RIG-I, which subsequently engages the chaperon protein 14–3-3ε for cytosol-to-mitochondrial translocation. Riplet modifies the C-terminal domain (CTD) of RIG-I with K63-linked-polyubiquitin chains. TRIM65 mediates K63-linked ubiquitination of MDA5 in its helicase domain, which induces MDA5 activation. The cytosol-to-mitochondria translocation of MDA5 is facilitated by 14–3-3η. Both RIG-I and MDA5 interact with and activate MAVS on mitochondria (and also other organelles), which subsequently activates IRF3 via TBK1 or IKKε and NF-κB via the IκB kinase (IKK) complex. LGP2 has been shown to facilitate MDA5 activation, whereas it inhibits antiviral signaling by RIG-I. Type I IFNs induced by TLR or RLR signaling bind to the type I IFN receptor (consisting of IFNAR1 and IFNAR2) and activate the Janus kinase (JAK)–signal transducer of activators of transcription (STAT) pathway to transcriptionally upregulate many ISGs including TLRs and RLRs themselves.

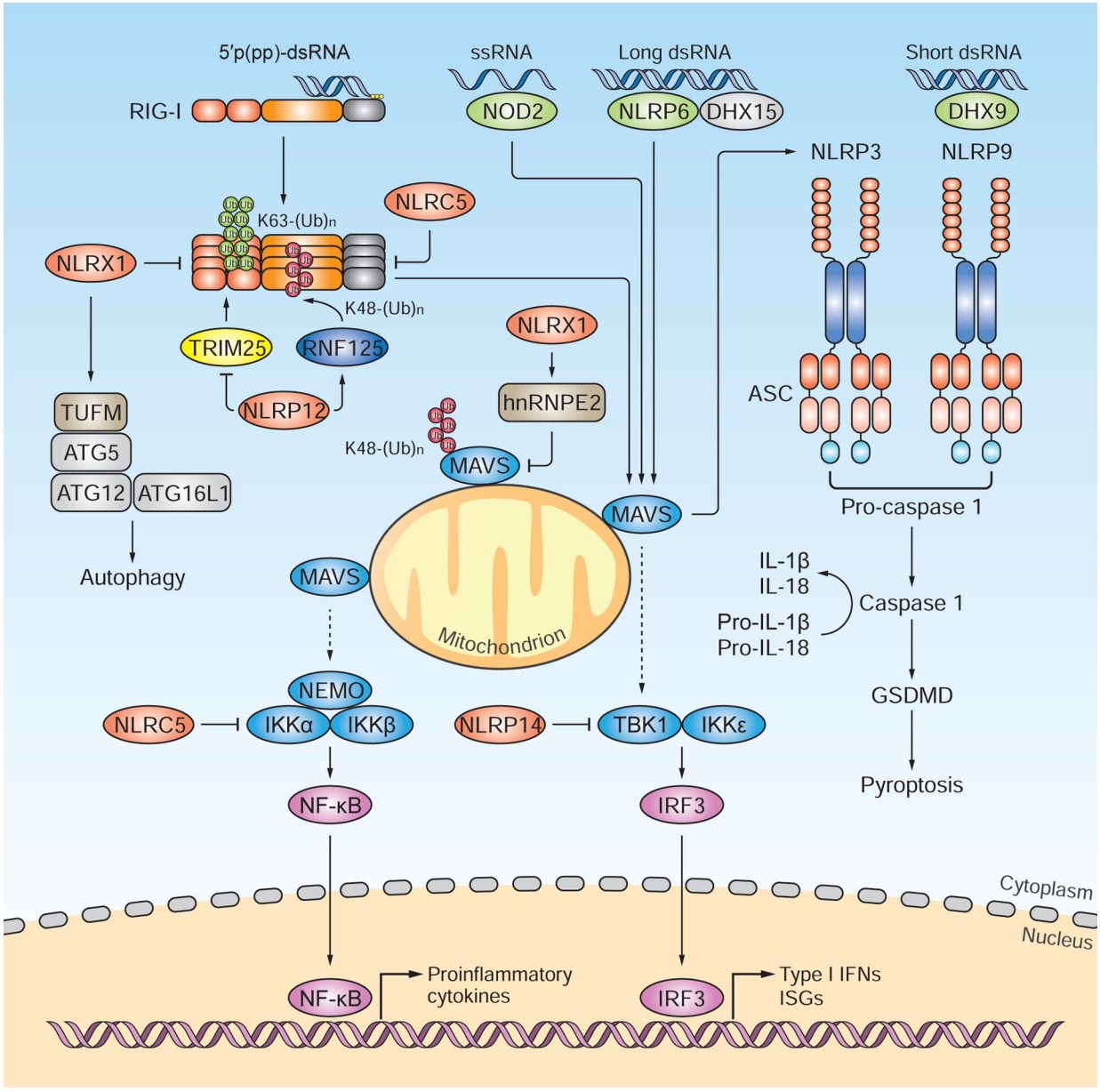

Figure 2. RNA sensing and regulatory roles of NLRs

NLRs have been shown to sense viral RNA and trigger type I IFN-and/or inflammasome-dependent host responses, or they regulate other innate sensing pathways such as the RLR pathway. Of note, NLR proteins are expressed and/or function in a highly cell-type-specific or tissue-specific manner, which is not illustrated for simplicity. NOD2 recognizes the ssRNA genome of RSV and mounts MAVS-dependent antiviral responses in hematopoietic and non-hematopoietic cells. NLRP6, in complex with the DEAH-box RNA helicase DHX15, binds viral dsRNA from EMCV and induces MAVS-dependent type I IFN responses in intestinal epithelial cells. Another intestinally-expressed NLR member, NLRP9b, when complexed with DHX9, senses short dsRNA from rotavirus and triggers ASC-and caspase 1-mediated IL-18 production. Additionally, the NLRP9b-DHX9 complex activates caspase-1-dependent pyroptosis through gasdermin D (GSDMD). NLRP12

attenuates RIG-I signaling by counteracting the TRIM25-mediated K63-linked ubiquitination of RIG-I. Moreover, NLRP12 promotes RNF125-mediated K48-linked ubiquitination of RIG-I, which leads to its proteasomal degradation. NLRX1 prevents the RLR-MAVS interaction and also recruits hnRNPE2 to induce MAVS degradation. Furthermore, NLRX1 interacts with TUFM, which promotes autophagy via ATG5, ATG12 and ATG16L1, thereby dampening RLR signaling. NLRP3 interacts with MAVS, and the mitochondrial positioning of NLRP3 promotes inflammasome activation and IL-1β production. NLRC5 and NLRP14 negatively regulate RLR signaling by targeting IKK-mediated and TBK1-mediated innate immune responses, respectively. NLRC5 also binds directly to RLRs and thereby blocks IRF3-dependent cytokine induction. NLRs that promote cytokine responses are shown in green; those that dampen cytokine induction are indicated in red.

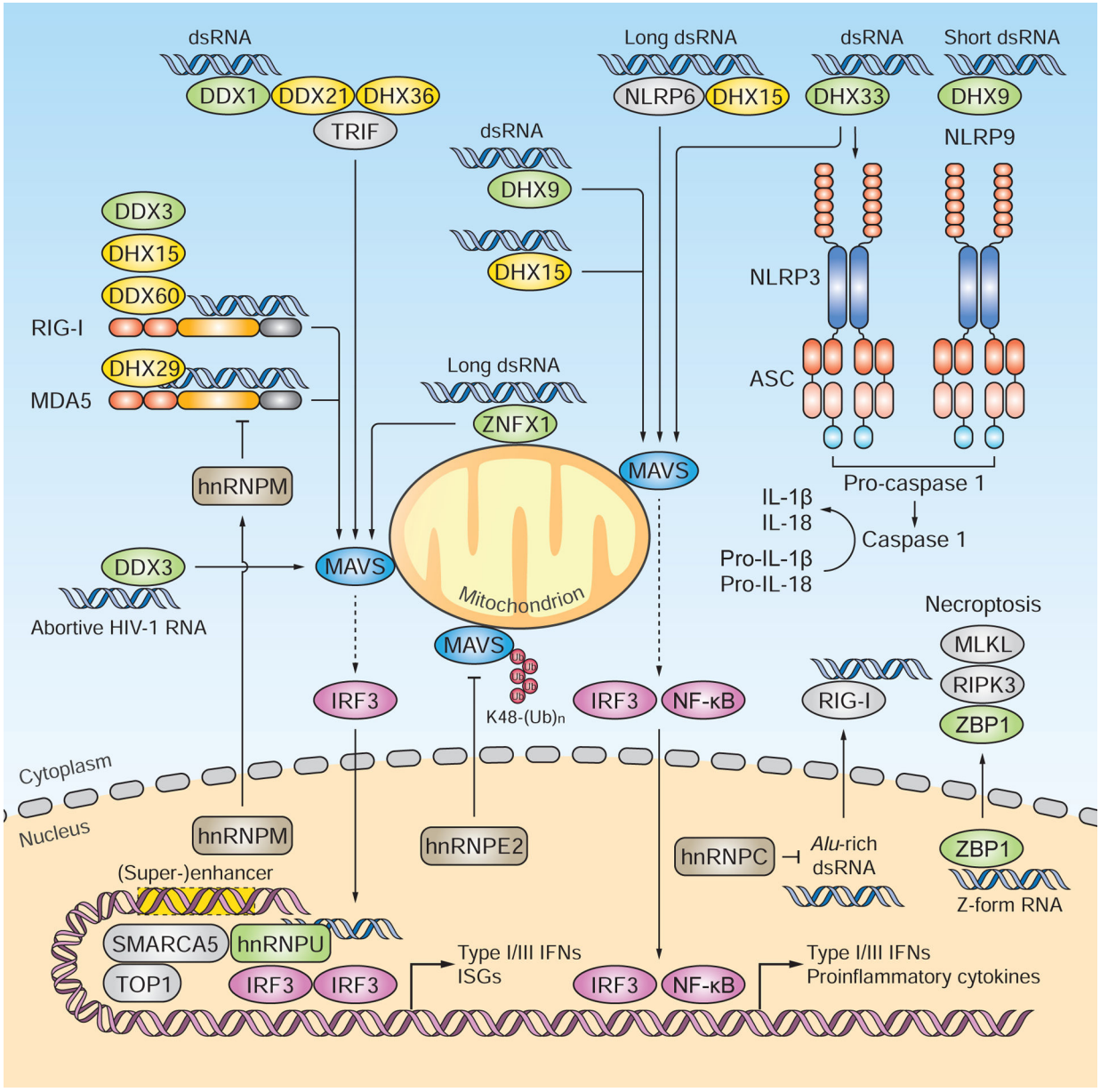

Figure 3. RNA helicases, hnRNPs and ZBP1 as emerging RNA sensors and co-sensors

The DEAD-box or DEAH-box family proteins DDX1, DHX9 and DHX33 have been shown to sense RNA. DDX1, in complex with DDX21 and DHX36, recognizes poly(I:C) and induces type I IFN and proinflammatory responses via TRIF and MAVS. DHX9 associates with poly(I:C) and mediates MAVS-dependent cytokine responses in myeloid DCs. DHX9 also facilitates NLRP9b binding to short dsRNA [*e.g.* low-molecular-weight poly(I:C)] and thereby activates inflammasomes. DHX33 senses dsRNA [*e.g.* poly(I:C), reovirus genomic RNA, and RNase L cleavage products] and triggers NLRP3-mediated inflammasome activation and MAVS-dependent type I IFN induction. Multiple DEAD-box or DEAH-box helicases serve as RLR co-sensors. Most of these helicases (*e.g.* DDX3, DDX60, DHX15 and DHX29) interact with RLRs and potentiate their activation. DDX3 was found to also directly bind abortive HIV-1 RNA and to induce MAVS-dependent type I IFN responses.

The hnRNP family members hnRNPE2, hnRNPM and hnRNPC negatively regulate RLR signaling. Upon virus-induced nucleus-to-cytoplasm translocation, HnRNPE2 interacts with MAVS and mediates its degradation. HnRNPM undergoes nucleus-to-cytoplasm redistribution and impairs the RNA-binding activity of RLRs. HnRNPC protects cells from accumulating RIG-I-stimulatory endogenous *Alu*-rich dsRNA. HnRNPU directly associates with HSV-1-derived dsRNA in the nucleus and, together with SMARCA5 and TOP1, activates the enhancers and super-enhancers that drive the transcription of antiviral and proinflammatory genes. ZBP1 senses both viral and host ‘Z-form’ dsRNA and then induces necroptosis through RIPK3 and MLKL. Green signifies proteins that are reported to function as direct RNA sensors; yellow indicates proteins that serve as RLR or NLR co-sensors.

TABLE 1.

TLR and RLR ligands

Sensors	Ligand characteristics	Synthetic RNA	Microbial RNA	Host RNA	Key references
TLR3	dsRNA	• dsRNA ≥ 39–48 bpds • dsRNA ≥ 90 bp (intracellular TLR3 only) • siRNA	Viral and bacterial RNA *(e.g.* long stem-loop containing poliovirus- derived RNA)	Phagocytosed host RNA (context-dependent)	(Kariko et al., 2004a; Kariko et al., 2004b; Kleinman et al., 2008; Leonard et al., 2008; Liu et al., 2008; Tatematsu et al., 2013)
TLR7	Site 1: guanosine Site 2: ssRNA	• UU(U/C) > UU(G/A) > XUX (X = non-U) • siRNA	Viral and bacterial RNA (e.g. HIV GU-rich RNA)	Let-7 miRNA	(Heil et al., 2004; Hornung et al., 2005; Lehmann et al., 2012; Tanji et al., 2015; Zhang et al., 2016; Zhang et al., 2018b)
TLR8	Site 1: uridine Site 2: ssRNA	UG dinucleotide	Viral and bacterial RNA	N/A	
TLR13	Stem-loop ssRNA	≥ 13 nt	• Bacterial 23S rRNA • VSV ssRNA genome	N/A	(Li and Chen, 2012; Oldenburg et al., 2012; Song et al., 2015)
RIG-I	5’-di- or triphosphate and blunt-ended dsRNA	Short dsRNA of ≥ 10–19 bp	• Sub(genomic) RNA of negative-strand RNA viruses *(e.g.* IAV panhandle structure) • Reovirus and yeast L-A totivirus genomic RNA	N/A	(Baum et al., 2010; Goubau et al., 2014; Hornung et al., 2006; Liu et al., 2015; Pichlmair et al., 2006; Schlee et al., 2009; Schmidt et al., 2009)
	‘Non-canonical’	• RNase Ill-digested poly(I:C) of ≤ 300 bp • Pol III transcripts of poly(dA-dT)	• HCV 3’ UTR • Measles virus *L* gene mRNA; KSHV RNA transcripts • EBV-encoded small RNAs (EBERs); adenovirus-associated RNA (VA-RNAs)	• 5S ribosomal RNA pseudogene 141 • Vault RNA • lnc-Lsm3b • RNase L cleavage products; IRE-1 cleavage products • Circular RNA	(Ablasser et al., 2009; Chen et al., 2017; Chiang et al., 2018; Chiu et al., 2009; Eckard et al., 2014; Jiang et al., 2018; Kato et al., 2008; Malathi et al., 2007; Minamitani et al., 2011; Runge et al., 2014; Saito et al., 2008; Zhang et al., 2018a; Zhao et al., 2018)
MDA5	Long irregular dsRNA	Poly(I:C) of > 300 bp	• Parainfluenza virus 5 and measles virus *L* gene mRNA	• Inverted repeat transposable *Alu* elements (~300 bp)	(Ahmad et al., 2018; Deddouche et al., 2014; Dhir et al., 2018; Kato et al., 2008; Luthra et al., 2011; Pichlmair et al., 2009; Runge et al., 2014)

Sensors	Ligand characteristics	Synthetic RNA	Microbial RNA	Host RNA	Key references
			• EMCV *L* antisense RNA	• Mitochondrial dsRNA	
LGP2	dsRNA; no end specificity	dsRNA that is blunt-ended or has overhangs with or without 5’-triphosphate moiety	EMCV *L* antisense RNA	N/A	(Deddouche et al., 2014; Uchikawa et al., 2016)

Abbreviations: HIV, human immunodeficiency virus; VSV, vesicular stomatitis virus; IAV, influenza A virus; HCV, hepatitis C virus; KSHV, Kaposi’s sarcoma-associated herpesvirus; EBV, Epstein-Barr virus; IRE-1, inositol-requiring enzyme 1; EMCV, encephalomyocarditis virus; *Alu*, *Arthrobacter luteus*; N/A, not available.