Review

Mechanisms of Non-segmented Negative Sense RNA Viral Antagonism of Host RIG-I-Like Receptors

Daisy W. Leung

Division of Infectious Diseases, *Department of Medicine, Washington University School of Medicine, St. Louis, MO 63110, USA*

Correspondence to : *dwleung@wustl.edu*
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Abstract

The pattern recognition receptors RIG-I-like receptors (RLRs) are critical molecules for cytosolic viral recognition and for subsequent activation of type I interferon production. The interferon signaling pathway plays a key role in viral detection and generating antiviral responses. Among the many pathogens, the non-segmented negative sense RNA viruses target the RLR pathway using a variety of mechanisms. Here, I review the current state of knowledge on the molecular mechanisms that allow non-segmented negative sense RNA virus recognition and antagonism of RLRs.

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Non-segmented Negative Sense RNA Viruses

Members of the *Mononegavirales* order encompass enveloped, non-segmented negative sense RNA viruses (NNSVs) that are pathogenic to humans, animals, and plants. As of 2018, the following eight viral families belong to NNSVs: *Bornaviridae*, *Mymonaviridae*, *Filoviridae*, *Nyamiviridae*, *Paramyxoviridae*, *Pneumoviridae*, *Rhabdoviridae*, and *Sunviridae* [1]. NNSVs are categorized due to their single, linear RNA genomes of reverse polarity, ranging from 8 to 19 kilobases. NNSV genomes encode for non-structural and structural proteins that are similarly organized from the 3′ end to the 5′ end. The proteins of genes common to all NNSVs include nucleoprotein (N/NP), phosphoprotein (P/X/VP35), matrix (M) protein, glycoprotein (G/GP/F/SH/HN), and the viral RNA-dependent RNA polymerase (RdRp; also known as large protein or L). Additional nonstructural proteins (NS1/NS2 in *Pneumoviridae*) and cofactors (VP30/VP24 in *Filoviridae*; M2 in *Pneumoviridae*) are also found in some NNSV genomes. Most NNSV genomes have limited coding capacity compared to other types of viruses. Perhaps as a consequence, many NNSV proteins are multifunctional and have different roles during various stages of infection, including immune inhibition and viral replication.

In addition, NNSVs share common strategies for transcription and replication in the host cell cytoplasm. The viral nucleocapsid minimally comprises the RNA genome encapsidated by N, P (or P equivalent protein), and L. It serves as the template for transcription by the viral RdRp. Viral genes are transcribed sequentially as the RdRp stops and starts at each intergenic region, which results in a decreasing gradient of mRNA transcript abundance from 3′ to 5′ end of the genome and that are capped and polyadenylated [2]. Viral mRNAs are then translated into proteins by host ribosomal machinery and, for the glycoproteins, post-translationally modified by the endoplasmic reticulum and Golgi apparatus. The viral nucleocapsid is also used by the viral RdRp to generate the positive sense antigenome prior to replication that produces more negative sense genomes [3]. These newly synthesized genomes and viral proteins are assembled together and bud into new viral particles.

Host IFN Signaling

Viral infection of host cells results in the activation of type I interferon (IFN) signaling, which triggers a program of host innate immune responses that functions as a self-defense mechanism from invading pathogens. Germline-encoded pattern recognition

receptors (PRR), including the cytoplasmic RIG-I like receptors (RLR), NOD-like receptors, and the membrane-associated Toll-like receptors, detect microbial molecules called pathogen-associated molecular patterns (PAMPs), which include viral nucleic acids [4–6]. Activation of PRRs results in conformational changes in adapter molecules, such as MAVS, TRAF, and TRIF, that then recruits downstream signaling kinases TBK1, IKKε, and NFκB (Fig. 1). These activated kinases can subsequently phosphorylate the transcription factors IRF3 and IRF7, which can homodimerize or heteriodimerize and translocate into the nucleus to induce type I IFN production, mainly IFN α/β. IFN α/β can then act in an autocrine or paracrine manner and signal through binding to the type I IFN receptor, IFNAR1/2, leading to stimulation of the JAK-STAT signaling pathway that activates the critical transcription factors STAT1 and STAT2. STAT1 and STAT2 can homodimerize or heterodimerize and accumulate in the nucleus where it induces the transcriptional activation of a large number of IFN-stimulated antiviral genes. Thus, having a robust and intact IFN response is critical for the host to combat viral infections.

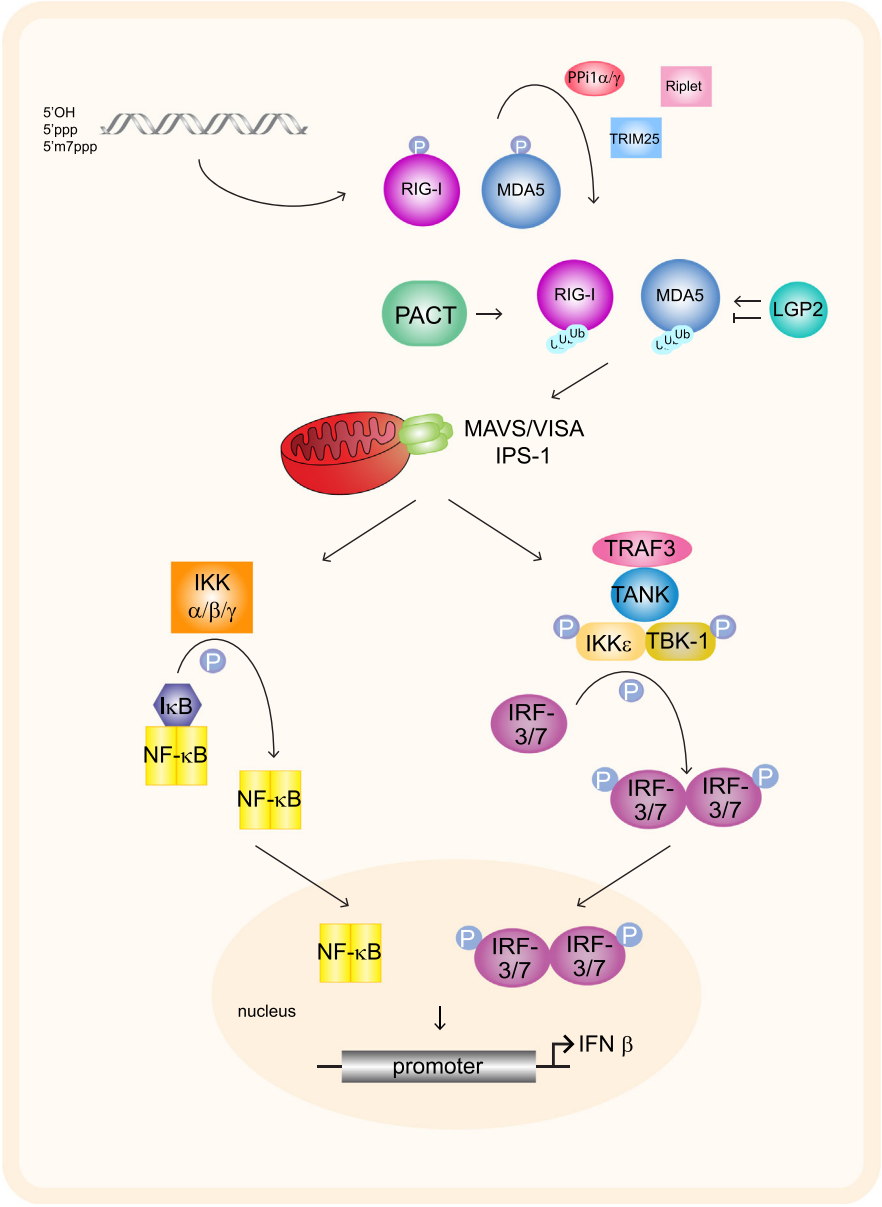

Fig. 1. Interferon-β signaling pathway regulated by RLRs. Simplified schematic illustrating the recognition of PAMPs by RIG-I and MDA5, the activation of RIG-I and MDA5, and the subsequent stimulation of downstream signaling molecules leading to interferon-β production.

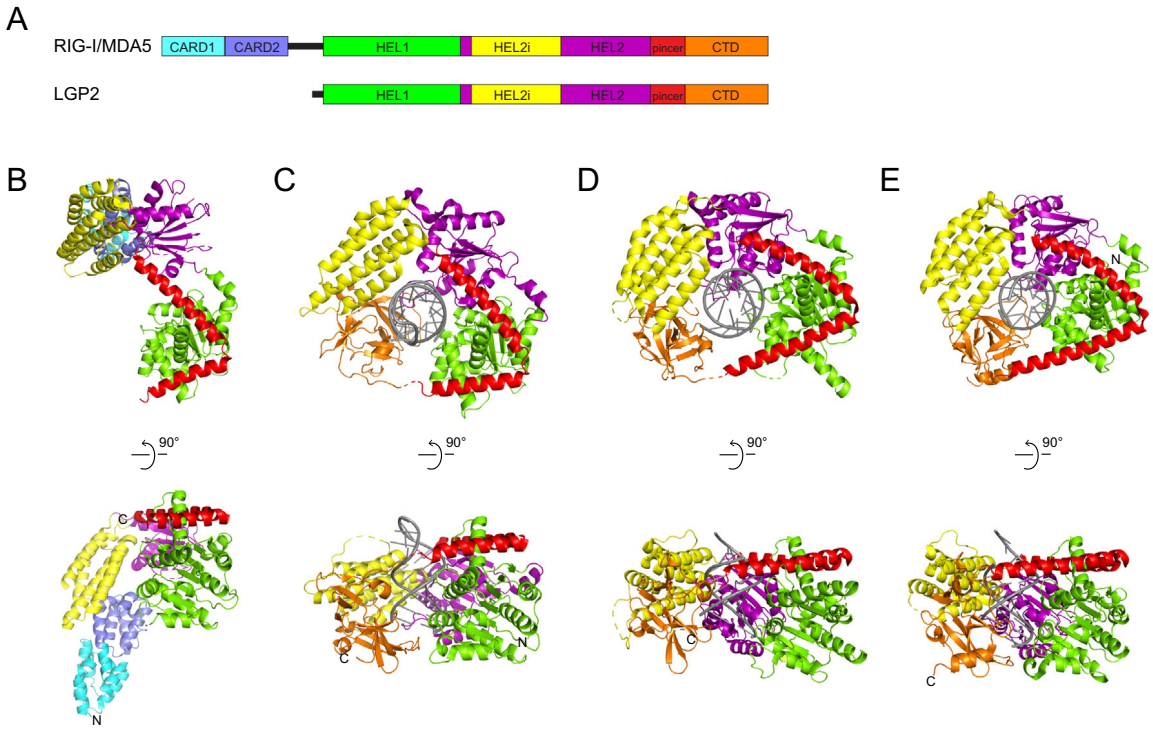

Fig. 2. PAMP binding by RLRs. (A) Domain organization of RIG-I, MDA5, and LGP2. CARD1 (cyan), CARD2 (slate), HEL1 (green), HEL2 (purple), HEL2i (yellow), pincer motif (red), CTD (orange). (B) Inactive RIG-I adopts an autoinhibited conformation where the N-terminal CARDs are sequestered from signaling. (C) RIG-I helicase bound to 24-mer blunt end hairpin RNA (gray) (PDB 5F9F) releases CARDs (not present in structure). (D) MDA5 helicase bound to 5′p 10-mer dsRNA (gray) (PDB 5JC3) releases CARDs (not present in structure). (E) LGP2 helicase, which lacks the N-terminal CARDs required for signaling, bound to 5′ppp 10-mer dsRNA (gray) (PDB 5JB2).

RLRs (RIG-I, MDA5, LGP2)

The RLRs comprise key sensors for non-self RNA detection. The RLR family includes the retinoic acid inducible gene-I (RIG-I), melanoma-associated differentiation factor-5 (MDA5), and laboratory of genetics and physiology 2 (LGP2). Each contains a super family 2 (SF2) RNA helicase domain, which binds to double-stranded RNA (dsRNA) and hydrolyzes ATP without unwinding RNA. The DExD/H box ATPase core is formed by helicase domain 1 (Hel1) and 2 (Hel2), an insertion domain (Hel2i), a regulatory pincer motif (also known as repressor domain), and the RNA binding domain at the C-terminus (CTD) (Fig. 2A). In addition, both RIG-I and MDA5 contain at the N-terminus two tandem caspase activation and recruitment domains (CARDs) that are important for interactions with other downstream CARD containing adapter proteins, notably the mitochondrial-associated antiviral signaling molecule (MAVS; also known as IPS-1/Visa/Cardif)[7–10], and with E3 ligases (such as TRIM25 and Riplet[11,12]), both of which are crucial for persistent downstream signaling. RIG-I and MDA5 function as cytosolic sensors of viral RNAs. RIG-I recognizes short 5′-pp/-ppp containing or blunt end dsRNAs and discriminates against 5′-p [13,14], whereas MDA5 recognizes long dsRNAs [15–20]. A series of structures provided insight into the regulation of RIG-I, where RIG-I signaling is modulated by autoinhibition [13,21–23]. In the autoinhibited conformation, the RIG-I N-terminal CARDs forms intramolecular interactions with the Hel2i domain, thereby preventing access to the CARDs (Fig. 2B). In the presence of dsRNA, the conformation of RIG-I changes such that the RIG-I CTD binds to ATP and endcaps dsRNA and Hel2i contacts dsRNA, presumably leading to the release of CARDs for signaling (Fig. 2C) [24–26]. The overall activated structure of the MDA5 helicase core and CTD is similar to RIG-I, forming a ring around the dsRNA linked by the pincer motif (Fig. 2D) [25,27,28]. However, the orientation of the CTD differs such that the MDA5 CTD binds to the dsRNA stem, which is compatible with MDA5 assembling on long dsRNAs and forming oligomeric filaments that are important for propagating MDA5 signaling [13,25,27–30]. LGP2 lacks the N-terminal CARDs and cannot signal downstream independently [31–37]. Instead, LGP2 appears to function as a cofactor for regulating RIG-I and MDA5 signaling [35]. LGP2 adopts mixed structural features from both RIG-I and MDA5. LGP2 binds dsRNA with high affinity. The LGP2 CTD

endcaps dsRNA like RIG-I; however, LGP2 also forms filaments that are similar to MDA5 (Fig. 2E). These features on LGP2 may provide an additional check for discrimination between self from non-self and a balanced IFN response. However, it is currently unclear if these PRRs have both overlapping and exclusive functions. While much of the work to define substrate specificity was done through biochemical studies and a limited number of cell-based studies show correlations, additional validation to define ligand specificity is required.

Evasion Mechanisms of RLR Signaling by NNSVs

Given the crucial roles RLRs have in sensing foreign RNA and to initiate innate immune responses, the activity of RLRs is tightly regulated to prevent unchecked activation that can lead to deleterious effects such as autoimmunity. Post-translation modifications, including phosphorylation, ubiquitination, and acetylation, and interactions with accessory factors provide multilayered regulation to control RLR activity. Despite this, viruses have developed diverse and often multipronged strategies to evade, subvert, or control host innate immune responses, as survival and propagation of the virus depend on its ability to replicate in the host. In many cases, viruses also target signaling downstream of the RLRs to potently inhibit IFN signaling using the same viral proteins but through distinct mechanisms. Highlighted below are some examples of how NNSVs target the critical first step in IFN signaling, the RLRs (Fig. 3).

Inhibition of PAMP Recognition

Replication intermediates or by-products of viral RNA synthesis, such as dsRNA or uncapped 5′ mRNA, are recognized by RIG-I and MDA5, which is especially risky for NNSVs as they replicate in the cytoplasm. While genome encapsidation by nucleoproteins allows for partial protection from detection by RLRs, filoviruses have developed an effective way to avoid detection, by sequestering the viral RNA. Ebola and Marburg viruses encode for a cofactor protein VP35, which is required for immune evasion and for

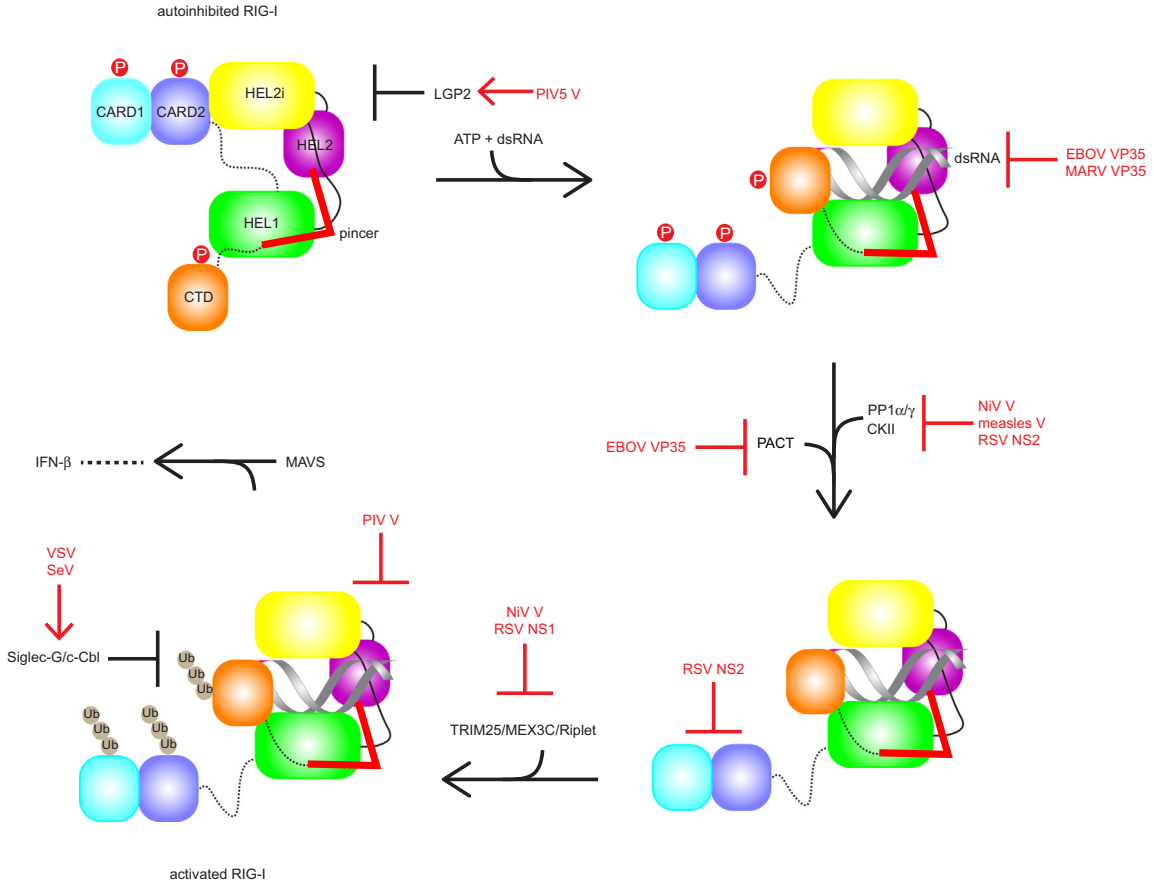

Fig. 3. Viral targeting of RIG-I activation and signaling. Simplified schematic of the activation of RIG-I and the related RLR MDA5, which results in the downstream stimulation of IFN-β production. Color scheme follows Fig. 2. Highlighted in red in this figure are points in this process that are targeted by different NNSV proteins.

viral replication. VP35 contains at the N-terminus a peptide (NPBP), which is important for binding to nucleoprotein (NP), and a coiled-coil domain that is important for mediating homo-oligomeric interactions, primarily that of a tetramer [38–42]. These are linked to the C-terminal IFN inhibitory domain (IID). The structure of Ebola VP35 IID revealed a unique mixed alpha/beta fold where the alpha helical subdomain contained residues critical for NP interactions and for viral RNA synthesis and the beta sheet subdomain contained a series of conserved basic residues that are critical for VP35 IFN inhibitory function [43–46]. The subsequent structure of Ebola VP35 IID in complex with an 8-bp dsRNA revealed that these conserved basic residues were critical for binding to the phosphodiester backbone of dsRNA (Fig. 4A). Mutational analysis of these residues, including Arg312, prevents dsRNA binding and IFN inhibition, and introduction of Lys319Ala and Arg322Ala substitutions renders a guinea pig adapted Ebola virus avirulent[47]. Furthermore, Ebola VP35 IID endcaps dsRNA, like the RLR RIG-I, facilitated through residues Phe239 and Ile340. These interactions together provide an effective mechanism for Ebola VP35 to sequester the viral genome or replication intermediates from detection by RIG-I, resulting in strong suppression of IFN production[48]. Marburg virus also encodes for the VP35 cofactor with a similar domain organization. The structure of Marburg VP35 IID is nearly identical to that of Ebola VP35 IID (backbone rmsd < 1.0 Å). However, Marburg VP35 IID preferentially binds to longer dsRNAs and binds only to the dsRNA stem without endcapping, similar to the RLR MDA5[49] (Fig. 4B). As a consequence, Marburg viral infection results in less potent inhibition of RIG-I signaling compared to Ebola viral infection [48]. Therefore, PAMP sequestration is an effective mechanism for filoviruses and the binding mode by which filoviral VP35s interact with dsRNA allows for modulation of IFN responses.

Paramyxoviruses produce an accessory V protein that is encoded in the P gene. V proteins contain an N-terminal domain that is shared with P/W/I proteins and a highly conserved C-terminal domain that is enriched in cysteine residues. Parainfluenza virus 5 (PIV5) V proteins do not directly bind to RIG-I. Instead, PIV5 V proteins promotes the formation of a complex between LGP2 and RIG-I that appears to prevent stimulation of RIG-I by viral RNAs [50,51]. The C-terminal domain of V is sufficient to bind to LGP2 and to inhibit IFN induction by RIG-I signaling. Furthermore, PIV5 V proteins were able to inhibit IFN induction by RIG-I-specific PAMPs in an LGP2-dependent manner. Similar enhancement of RIG-I inhibition by interaction with LGP2 was observed for other paramyxoviral V proteins [50].

Inhibition of RLR Dephosphorylation

Both RLRs RIG-I and MDA5 are maintained in an autoinhibited conformation that is inactive due to the constitutive phosphorylation of specific serine and threonine residues in the N-terminal CARDs (Ser8 and Thr170 on RIG-I; S88 on MDA5)[52]. Binding of viral dsRNA to the CTD relieves autoinhibition of RIG-I and MDA5 and allows for two protein phosphatases, PP1α and PP1γ, to dephosphorylate these residues and for subsequent interactions required for activation of RIG-I and MDA5.

The paramyxovirus V protein also antagonizes IFN induction through targeting of MDA5. A series of biochemical studies revealed that measles virus and Nipah virus V proteins efficiently compete with MDA5 and bind to endogenous PP1α and PP1γ [53]. Mapping of the binding interface identified a PP1-binding motif in measles V, which when mutated results in enhanced phosphorylation of MDA5 at Ser88 in primary human dendritic cells. Furthermore, the measles V protein is phosphorylated at multiple

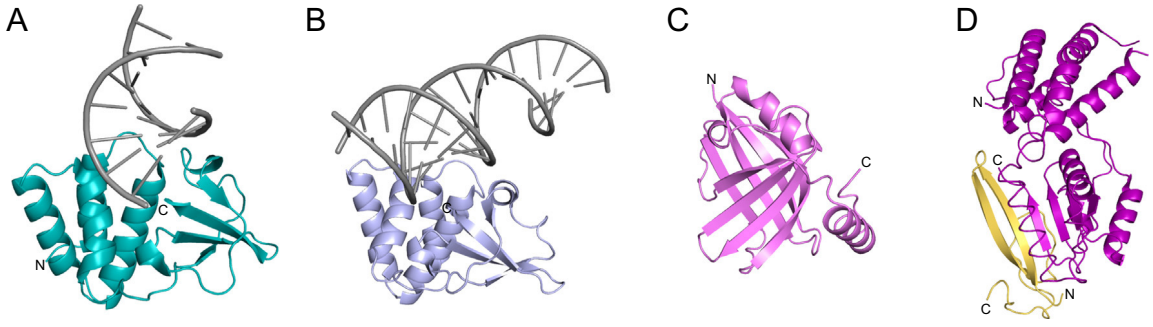

Fig. 4. NNSV targeting of RLR activity. (A) The IFN inhibitory domain of Ebola viral protein 35 (eVP35, teal) endcaps dsRNA (gray) and prevents PAMP recognition by RLRs. (B) The IFN inhibitory domain of Marburg viral protein 35 (mVP35, light blue) coats the RNA (gray) backbone to prevent PAMP recognition by RLRs. (C) Structure of respiratory syncytial virus non-structural protein 1 (NS1, pink) reveals a critical C-terminal helix that is important for interferon antagonist functions (PDB 5VJ2). (D) Unfolding of MDA5 HEL2 domain (purple) by parainfluenza virus 5 (PIV5) accessory V protein (gold, PDB 4I1S).

Ser and Thr residues that are dephosphorylated upon binding to PP1α, suggesting that measles V functions as a decoy substrate to sequester PP1 and prevent dephosphorylation of MDA5. Measles virus employs another mechanism to control PP1 activity. Measles virus binding to the cell surface receptor DC-SIGN leads to the phosphorylation of the inhibitor I-1 by Raf1 kinase that binds to the GADD34-PP1 complex to inhibit its activity [54]. However, it remains to be determined what factors dictate whether measles V targets RIG-I or MDA5 dephosphorylation.

Targeting of RLR Ubiquitination

Activation of RIG-I is also regulated by ubiquitination. Relief of autoinhibition through interactions with viral RNA releases the RIG-I CARDs for binding by TRIM25, an E3 ligase that is induced by type I IFN signaling. TRIM25 contains at its C-terminus a PRY-SPRY domain that binds to the first CARD through interactions mediated by residue Thr55. This allows for conjugation of K63-linked ubiquitin chains or binding of non-covalent K63-linked ubiquitin chains on multiple Lys residues, notably on Lys172 on the second CARD of RIG-I that is critical for RIG-I activity [11,24,55]. E3 ligases MEX3C and TRIM4 also mediates poly-ubiquitination of other Lys residues on RIG-I CARDs, including Lys48, Lys99, Lys164, and Lys169 [12,56–58]. Furthermore, the CTD of RIG-I is targeted for K63-linked ubiquitination by the Riplet E3 ligase [12,57]. Mass spectrometry analysis identified several Lys residues in the CTD (Lys849, Lys851, and Lys888) and the linker connecting the CTD to the helicase domain (Lys788) that are ubiquitinated. A more recent study suggests that Riplet is sufficient to activate RIG-I by recognizing RIG-I oligomers that are preassembled on longer dsRNAs and conjugating K83-Ub_n chains [59]. Together, ubiquitination of the CARDs and CTD is important for driving the tetramerization of RIG-I. A recent crystal structure revealed non-covalent binding of free ubiquitin at an interface that bridges two RIG-I CARDs to form the tetramer and stabilize RIG-I for binding to the downstream MAVS signaling platform to induce IFN production [24].

Both paramyxoviruses and pneumoviruses employ similar strategies to regulate ubiquitination of RLRs through interactions with TRIM25. For Nipah viruses, the C-terminal domain of the accessory V protein binds to the SPRY domain of TRIM25 in immunoprecipitation experiments and inhibits TRIM25-mediated activation of RIG-I signaling [60]. Similar binding is observed for other paramyxovirus V proteins given the high conservation of the C-terminal domain, including measles virus V and Sendai virus V proteins. In addition, Nipah virus also uses the V protein to directly bind to RIG-I and prevent TRIM25 binding [60]. Immunoprecipitation experiments demonstrated that the C-terminal domain of V binds to RIG-I CARDs, inhibiting TRIM25-mediated ubiquitination of RIG-I and preventing RIG-I interaction with MAVS. It is interesting that the same domain in the paramyxovirus V protein targets two different host molecules to inhibit virtually the same step, RIG-I ubiquitination, in the IFN signaling pathway.

Respiratory syncytial virus (RSV) encodes for small non-structural proteins, NS1 and NS2, that are unique to orthopneumoviruses and function as IFN antagonists that target different components in the IFN signaling pathway. Initial studies of NS2 suggest that NS2 binds to RIG-I through interactions with the CARDs and prevents RIG-I binding to MAVS in coimmunoprecipitation assays [61]. However, it remains unclear the exact mechanism by which NS2 binding inhibits RIG-I activation. More recently, NS1, which was revealed to be a structural paralog of RSV matrix protein (Fig. 4C), has been shown to bind to TRIM25 in a co-immunoprecipitation assay [62,63]. The TRIM25 SPRY domain is sufficient for interacting with NS1, and RSV infection results in decreased ubiquitination of RIG-I, suggesting that NS1 binds TRIM25 and inhibits RIG-I activation.

The paramyxovirus Sendai virus (SeV) and rhabdovirus vesicular stomatitis virus (VSV) utilize another mechanism to facilitate proteasomal degradation of RIG-I and MDA5. Infection with both SeV and VSV results in upregulation of Siglec-G, an immunoglobulin-like lectin [64]. Furthermore, Siglec-G-deficient mice were more resistant to VSV infection by stimulating more IFN production. Increased Siglec-G expression results in the recruitment of tyrosine phosphatase SHP2 and the E3 ubiquitin ligase c-Cbl to RIG-I. c-Cbl then mediates RIG-I degradation by facilitating K48-linked ubiquitination of RIG-I at Lys 813 in a dose-dependent manner.

Inhibition of RLR ATPase Activity

The RLRs share a common DExD/H helicase with ATPase activity. Previous studies showed that the C-terminal domain of paramyxovirus V proteins binds to MDA5 and inhibits MDA5 activation[65]. A crystal structure of parainfluenza virus 5 (PIV5) V in complex with MDA5 demonstrated that PIV V disrupts MDA5 function by inserting the β-hairpin in V CTD, unfolding the Hel2 core β sheet in MDA5, and displacing the MDA5 ATPase VI motif [66] (Fig. 4D). RIG-I lacks two residues that are present in MDA5 that are important for mediating this interaction (Glu803 and Gly805), and thus, PIV5 V does not bind to RIG-I or inhibit RIG-I activity, suggesting specific targeting of MDA5 by PIV. Interestingly, comparison of the full-length structure of PIV5 V protein bound to a different host factor (DBB1) suggests that the PIV5 V protein has high structural plasticity; it can undergo a large structural change and utilize different structural elements that are context dependent [67].

dsRNA binding domain containing proteins, such as protein kinase R activator (PACT), can enhance RIG-I activity [68,69]. PACT is a host adaptor protein containing three dsRNA binding domains and appears to promote IFN-α/β responses to viral infection and to dsRNA mediated through interactions with the CTD of RIG-I, which stimulates RIG-I ATPase activity and signaling [69]. Furthermore, there is substantial reduction of IFN-β production in PACT-depleted cells. Subsequent studies suggest that RIG-I ATPase activity helps distinguish self *versus* non-self RNAs and prevent unintentional signaling [70]. However, the mechanism by which PACT activates RIG-I remains undefined, and it remains unclear whether dsRNA binding is required to mediate the interaction between PACT and RIG-I.

The Ebola virus VP35 cofactor targets PACT-mediated activation of RIG-I signaling [71]. Binding of VP35 to PACT prevents PACT interaction with RIG-I and inhibits RIG-I ATPase activity in a dose-dependent manner. Furthermore, residues in VP35 that are important for binding to dsRNA are also important for PACT interaction as mutation of these residues (Arg312Ala, Phe239Ala, and Lys319Ala/Arg322Ala) reduces the ability of VP35 to inhibit PACT activation of RIG-I signaling.

Future Perspective

RIG-I and MDA5 are critical cellular sentinels of viral pathogens, distinguishing molecular patterns of self from non-self. They are complex multidomain molecules that are regulated at many levels, such as autoinhibition, phosphorylation, ubiquitination, acetylation [72,73], and interaction with regulatory factors, which also includes LGP2. What is becoming apparent is that for every regulatory layer that RLRs evolved to develop a high tolerance of self and to prevent inappropriate activation of RLRs, NNSVs have developed different strategies to target and antagonize RLRs, sometimes in a seemingly redundant manner. Further studies directed toward uncovering these regulatory mechanisms across the different NNSVs will be important to facilitate future therapeutic development to regulate autoimmunity or to control a subset of viral infections. In addition, these studies can uncover new ligands that can provide the basis to develop potential adjuvants that activate innate immune responses to infections.

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Abbreviations used:
NNSV, non-segmented negative sense RNA virus; IFN, interferon; PRR, pattern recognition receptor; RLR, RIG-I like receptor; PAMP, pathogen-associated molecular pattern; CARDs, caspase activation and recruitment domains; MAVS, mitochondrial-associated antiviral signaling molecule; IID, IFN inhibitory domain; RSV, respiratory syncytial virus; dsRNA, double-stranded RNA; PACT, protein kinase R activator.

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