Immunity
Minireview

Regulation of Mitochondrial Antiviral Signaling Pathways

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Mitochondrial antiviral immunity involves the detection of viral RNA by intracellular pattern-recognition receptors (PRRs) belonging to the RIG-I-like helicase family. The convergence of these and other signaling molecules to the outer mitochondrial membrane results in the rapid induction of antiviral cytokines including type-1 interferon. Here, we discuss recent studies describing new molecules implicated in the regulation of this antiviral response.

It was the late Charles Janeway who first hypothesized that there exists a family of pattern-recognition receptors (PRRs) that are expressed by cells of the innate immune system and that are capable of recognizing specific pathogen-associated molecular patterns (PAMPs) and initiating antimicrobial immune responses (Janeway, 1989). Since this time, the identification of the human toll-like receptor (TLR) and RIG-like helicase (RLH) families have made it clear the extraordinary insightfulness and accuracy of Janeway's predictions. The first family of molecules identified which fit Janeway's PRR model was the TLRs. This family of transmembrane proteins, expressed mostly at the cell surface and endosomes, survey the extracellular environment for PAMPs derived from a wide range of microbes including protozoa, bacteria, fungus, and virus. Upon PAMP recognition, TLRs transduce this extracellular danger signal into an appropriate intracellular response through direct interactions of the TLR toll-interleukin 1 receptor (TIR) domain with a cytoplasmic TIR-containing adaptor molecule, such as MyD88, TRIF (also known as TICAM-1), TRAM, or TIRAP. The ensuing downstream signaling cascade results in the activation of an array of transcriptional responses to induce a plethora of immune and inflammatory cytokines.

In 2005, a new pathway and TLR-independent response to pathogen was uncovered with the discovery of RIG-like helicase (RLH) proteins. This family of strictly intracellular PRRs is composed of three molecules: retinoic acid inducible gene-I (RIG-I), melanoma differentiation-associated gene-5 (MDA-5), and laboratory of genetics and physiology 2 (LGP2). RIG-I and MDA-5 are prototypical PRR molecules. The ligands for RIG-I and MDA-5 are viral "nonself" nucleic acids, and like TLR signaling, a classical ligand-receptor-adaptor model of PRR signaling has been proposed for RLH-mediated signaling, culminating in the induction of antiviral type-1 interferon cytokines, such as IFN-β and IFN-α. RLH-mediated activation of type-1 interferon and other proinflammatory cytokines results in autocrine and paracrine stimulation of cellular pathways leading to the rapid transcription of antiviral genes whose actions include the inhibition of viral infection and replication. Because viruses have evolved mechanisms designed to hijack host machinery for transcription of their own genes, it is not surprising that as a countermeasure the host genome has evolved to include cytoplasmic antiviral PRRs, such as RLHs, which are capable of responding to a viral infection by triggering host antiviral responses. A complex network of signaling molecules involved in regulating the RLH pathway is now known (Takeuchi and Akira, 2008), and the discovery of the mitochondria as a membrane platform for RLH-mediated signaling highlights the novelty and excitement of this new field.

Viral Recognition by RIG-like Helicases

A few TLRs, such as TLR3, TLR7-TLR8, and TLR9, are capable of detecting viral PAMPs. These receptors respond to viral nucleic acid either at the cell surface or within endosomal compartments. In addition, it is thought that TLRs are responsible for most of the type-1 interferon production from immune cells such as dendritic and natural killer cells. In contrast, RLH proteins recognize viral nucleic acid strictly from within the cytosol and unlike TLRs are expressed in both immune and nonimmune cells. Therefore, although at first glance the existence of these two classes of antiviral PRRs appears redundant, the expression of each in disparate cell types and compartments would suggest quite unique roles in the initiation, maintenance, and fine-tuning of host antiviral responses. In fact, TLR3-deficient mice do not have impaired overall innate and adaptive immune responses to different infectious viral models including lymphocytic choriomeningitis virus (LCMV), vesicular stomatitis virus (VSV), murine cytomegalovirus (MCMV), and reovirus (Edelmann et al., 2004). In contrast, mice lacking RLH-mediated signaling exhibit severely impaired antiviral responses to several viruses (Sun et al., 2006). Thus, the existence of RLH molecules in nonimmune cells would suggest that unlike TLRs, this antiviral defense is available within those cells most probably confronted with an initial viral infection, such as those lining the respiratory and reproductive tracts.

Mitochondrial Antiviral Signaling

Four independent groups simultaneously discovered a previously uncharacterized CARD-containing adaptor protein that was essential for RLH-mediated antiviral signaling. This signaling adaptor was named the mitochondrial antiviral signaling adaptor (MAVS), also known as IPS-1, VISA, and Cardif (Kawai et al., 2005; Meylan et al., 2005; Seth et al., 2005; Xu et al., 2005).

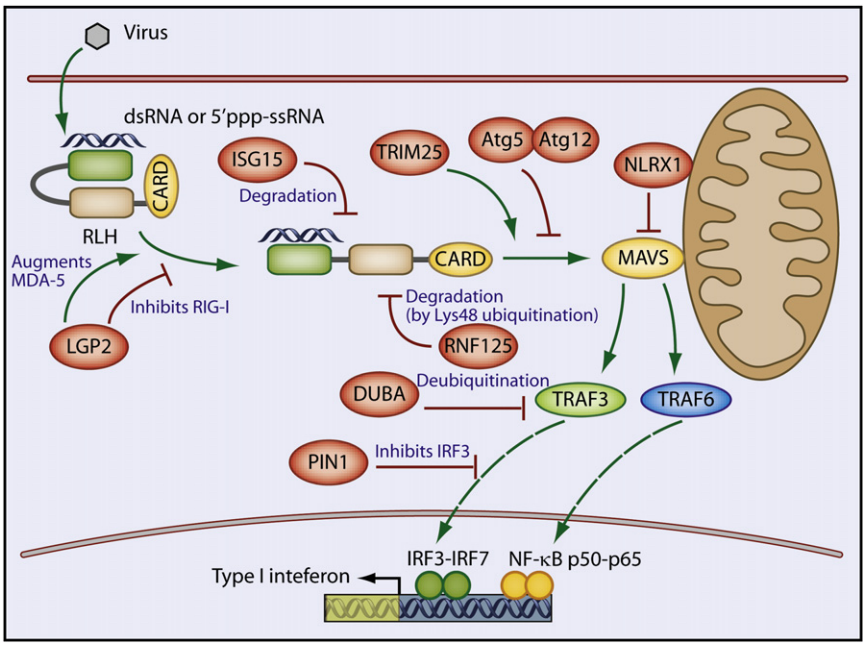

Figure 1. Regulators of RLH Signaling
Intracellular signaling responses to viral infection begins with the recognition of the viral intermediates, double-stranded RNA (dsRNA), or 5′ triphosphate single-stranded RNA (5′ppp-ssRNA) by the RIG-like family of helicases (RLH). Upon ligand binding, RIG-I undergoes a conformation change allowing for the interaction of the RIG-I CARD domain with the CARD-containing mitochondrial antiviral signaling adaptor (MAVS), also known as IPS-1, VISA, and Cardif. MAVS signals through TRAF3 or TRAF6 to activate kinases leading to the nuclear translocation of IRF3-IRF7 and NF-κB, respectively, and resulting in the transcription of type-1 interferons. Several regulatory molecules (shown in red) regulate this response and the inhibitory (brown lines) and stimulatory (green arrows) effects of these molecules on RLH signaling are illustrated. LGP2 inhibits RIG-I and augments MDA-5-mediated responses. Ligand-activated RIG-I is regulated through proteosomal degradation after ISGylation (ISG15) or lysine 48 ubiquitination (RNF125). Conversely, lysine 63 ubiquitination (TRIM25) of RIG-I augments RIG-I-MAVS interactions and enhances downstream signaling, whereas RIG-I-MAVS interactions are inhibited by an autophagy regulator (Atg5-Atg12). The NLR protein NLRX1 functions as an inhibitor of MAVS signaling from within the mitochondria, and the removal of ubiquitin moieties from TRAF3 by the deubiquitinase DUBA prevents TRAF3 associations with downstream kinases, thereby inhibiting RLH signaling. At the most distal level, phoshorylated IRF3 is targeted for proteosomal degradation by the peptidyl-prolyl isomerase (PIN1).

Overexpression of MAVS results in the potent induction of type-1 interferons including IFN-β and IFN-α. Furthermore, mice lacking MAVS exhibit severely reduced type-1 interferon production in response to several RIG-I and MDA-5 mediated viral infections (Sun et al., 2006). Therefore, MAVS functions as the essential signaling adaptor required for RLH signaling and as expected contains an N-terminal CARD domain required for signaling. The remainder of the protein contains a proline-rich region (PR) and a C-terminal transmembrane region (TM). Upon viral challenge, the MAVS CARD associates with the CARDs of RIG-I or MDA-5 (Seth et al., 2005). Similar to the TIR-TIR homotypic interaction in TLR signaling, this CARD-CARD interaction is an essential component in RLH-mediated signaling. However unlike TLR signaling, this receptor-adaptor interaction does not occur at the plasma membrane; rather, Seth et al. (2005) discovered that MAVS localizes to the mitochondrial outer membrane via its C-terminal transmembrane domain. In fact, mutation of the TM domain or the targeting of MAVS to other cellular membranes such as the endoplasmic reticulum completely abolished RLH signaling (Seth et al., 2005). This was the first report linking the mitochondria to type-1 interferon responses after viral recognition and identified a new intracellular surface for the assembly of cytoplasmic PRR-signaling complexes.

RLH-mediated signaling downstream of MAVS requires the tumor necrosis factor (TNF)-receptor-associated factor (TRAF) family members. The MAVS proline-rich domain contains a TRAF3-binding site required for association with TRAF3 and essential for MAVS-mediated activation of type-1 interferon, but not NF-κB (Takeuchi and Akira, 2008). Conversely, mutation of a MAVS TRAF6-binding site showed marked reduction in MAVS-mediated NF-κB activity, suggesting that TRAF6 mediates MAVS activation of NF-κB (Xu et al., 2005). MAVS signaling downstream of TRAF6 is thought to involve the activation of the canonical IκB kinase (IKK) complex consisting of IKKγ-(NEMO):IKKα:IKKβ, resulting in the phosphorylation of the inhibitor of NF-κB (IκBα) and its subsequent release for translocation into the nucleus. In contrast, MAVS signaling downstream of TRAF3 involves the formation and activation of a different signaling complex consisting of MAVS, the TRAF-family-member-associated NF-κB activator (TANK), and a noncanonical class of IKKs including (TANK)-binding kinase 1 (TBK1) and inducible IκB kinase (IKK-í or IKK-ε). The activation of TBK1 and IKK-í results in the phosphorylation and subsequent dimerization and nuclear translocation of the transcription factors IRF3 and IRF7. Therefore, intrinsic to the function of MAVS is the bifurcation of RLH-mediated signals into NF-κB and IRF pathways. Because it is known that both NF-κB and IRF3 and IRF7 are required for the assembly of an enhancesome complex required for the induction of IFN-β and IFN-α promoters, the essential role of MAVS in the activation of both of these transcription factors further emphasizes the importance of this mitochondrial adaptor protein in type-1 interferon signaling.

Regulators of RLH Signaling

Several new molecules have emerged which function as regulators of RLH signaling. Thus far, many of these molecules function as negative regulators, presumably in order to maintain a tight control over virus-initiated IFN production (Figure 1). In fact, RIG-I-mediated production of IFN can, in turn, increase the transcription of RIG-I itself, thus setting into motion an IFN amplification loop, which if left unchecked, could become deleterious to the host.

One regulator of this IFN positive-feedback loop can be found within the RLH family. Unlike RIG-I and MDA-5, LGP2 is devoid of the N-terminal CARDs required for activating MAVS-dependent signaling events. However, similar to RIG-I, LGP2 is inducible by IFN-β, virus, or dsRNA and can bind viral RNA through a RIG-I-like C-terminal domain (Yoneyama et al., 2008). Another intriguing property of LGP2 is its ability to associate with MAVS despite the loss of CARDs. Thus, LGP2 has been shown to compete with MAVS for binding of the downstream signaling kinase IKK-í (Komuro and Horvath, 2006). Moreover, LGP2 contains a RIG-I-like repression domain (RD) capable of inhibiting RIG-I multimerization and signaling. Consistent with a negative regulatory role on RIG-I signaling, LGP2-deficient mice are resistant to vesicular stomatitis virus infection. However, MDA-5-mediated responses are dampened in LGP2-deficient mice because these mice are defective in IFN-β production after infection with encephalomyocarditis virus. Therefore, LGP2 appears to play different roles in the regulation of RLH-mediated responses through inhibition of RIG-I and augmentation of MDA-5 signaling (Venkataraman et al., 2007).

The IFN-inducible ubiquitin ligase RNF125 was found to conjugate lysine 48-linked polyubiquitin chains to RIG-I or MDA-5 and cause the proteosomal degradation of either protein (Arimoto et al., 2007). Another recent report describes the control of RIG-I antiviral signaling by an ubiqutination-like process called ISGylation. This involves the targeted degradation of RIG-I after IFN-induced conjugation of the ubiquitin-like protein ISG15. It has been proposed that this mechanism represents a negative regulatory loop that fine-tunes RIG-I-mediated antiviral responses (Kim et al., 2008). Ubiquitin-mediated proteosomal degradation as a means of IFN-negative regulation has been also identified at the level of the transcription factor IRF3. Upon transfection with dsRNA, the peptidyl-prolyl isomerase (Pin1) interacts with phosphorylated IRF3, resulting in the proteasomal degradation of IRF3 (Saitoh et al., 2006).

Despite the above examples, not all posttranslational modifications within the RLH pathway are inhibitory. For example, a member of the tripartite motif (TRIM) protein family, TRIM25, functions as an E3 ubiquitin ligase of RIG-I. The SPRY domain of TRIM25 delivers a lysine 63-linked ubiquitin moiety to the N-terminal CARDs of RIG-I, which strengthens interactions with MAVS and enhances downstream signaling to IFN-β. In congruence with these findings, TRIM25-deficient mice are severely impaired in RIG-I-dependent antiviral responses (Gack et al., 2007). Furthermore, it has been shown that the removal of ubiquitin moieties from specific targets can have a modulatory effect on RLH signaling. The deubiquitinizing enzyme A (DUBA) cleaves Lys-63-linked polyubiquitin chains from TRAF3 resulting in the dissociation of TRAF3 from TBK1, and such a dissociation effectively squelches RLH-mediated type-1 interferon production. The removal of endogenous DUBA by siRNA produced an elevated IFN-β and IFN-α response to poly:IC or Sendai virus, whereas NF-κB activation was predominantly unaffected (Kayagaki et al., 2007). Consistent with these findings, a close family member of DUBA, A20, and known inhibitor of NF-κB signaling, also inhibits RLH signaling. A20 is thought to inhibit upstream of the TRAF3-TBK1-IKK-ε signaling complex because only constitutively active RIG-I signaling was abolished by A20 overexpression (Lin et al., 2006). Further research on the kinetics of these posttranslational modifications is needed to shed light on the role of these regulatory mechanisms during the course of a viral infection.

One report has implicated the Atg5-Atg12 conjugate, an essential component of the autophagy pathways, in the regulation of MAVS. In the absence of viral infection, the Atg5-Atg12 conjugate interacts directly with the MAVS CARD and weakly associated with RIG-I and MDA-5 CARDs. Thus, it is thought that Atg5-Atg12 intercalates between the CARDs of RLHs and MAVS, thereby interfering with RLH-MAVS interactions and inhibiting downstream signal transmission resulting in the reduction of type-1 interferon production. In fact, Atg5-deficient mice exhibit a hyperproduction of dsRNA-mediated type-1 interferon (Jounai et al., 2007). Because the Atg5-Atg12-MAVS interaction occurred in the absence of stimulus, it has been proposed that this regulatory mechanism functions to maintain cellular homeostasis under resting conditions (Jounai et al., 2007).

Another molecule with an inhibitory function on MAVS signaling is found within the NLR (nucleotide-binding domain and leucine-rich-repeat-containing) protein family (Ting et al., 2008). NLRX1 was found to interact with MAVS and block RIG-I-MAVS interactions and signaling after Sendai virus infection. Furthermore, siRNA-mediated abrogation of NLRX1 expression allowed for a more robust type-1 interferon and inflammatory cytokine expression that occurred after viral infection and that was accompanied by an increase in viral clearance from these cells. However, unlike the Atg conjugate, NLRX1 protein localizes to the mitochondria (Moore et al., 2008; Tattoli et al., 2008). Thus, this is the first report describing regulation of RLH signaling from within the mitochondria and links the rapidly emerging NLR family with RLH-mediated signaling. Reactive oxygen species (ROS) production is increased with NLRX1 overexpression (Tattoli et al., 2008). However, the physiological relevance of this finding in the context of endogenous NLRX1 and antiviral immunity is unclear. It is quite possible that NLRX1 functions both as a constitutive inhibitor on RLH-mediated responses and positive regulator of ROS. In vivo studies utilizing NLRX1-deficient mice should shed more light on the importance of NLRX1 to regulation of these responses.

It has been speculated that the NLR family function as cytoplasmic PRRs. However, data on NLRs' ability to directly bind PAMPs are lacking. In fact, NLRX1 appears to function as an indirect regulator of PRRs through its constitutive interactions with MAVS. This method of indirect sensing of host cell signaling is more consistent with the "guard hypothesis" as described for the NLR-like plant NBS-LRR (nucleotide-binding site and leucine-rich repeat) disease resistance (R proteins) (Van der Biezen and Jones, 1998). This guard model predicts that plant R proteins detect and respond to pathogen infections indirectly through surveillance of the specific host effector protein targeted by the pathogen. For example, rather than direct binding of the *Pseudomonas syringae* effector protein AvrRpt2, the plant NBS-LRR protein RPS2 functions to "guard" the receptor RIN4, which has been shown to physically associate with AvrRpt2 (DeYoung and Innes, 2006). We propose that NLRX1 functions to "guard" signaling via MAVS and thus indirectly regulates the function of the PRR, in this case, RLH (Figure 2). This is the most direct evidence that some NLRs might function as modulators of pathogen responses (MOPRs), rather than as classical PRRs. Curiously, this is reminiscent of the NLR protein CIITA

	Arabidopsis thaliana	Mammalia	
Ligand	*Pseudomonas syringae* AvrRpt2	Viral dsRNA 5′ppp-ssRNA polyI:C	MHC class II promoters
Receptor complex	RIN4	RIG-I-MAVS MDA5-MAVS	RFX CREB NF-Y
Guard protein	RPS2	NLRX1	CIITA

Figure 2. NLRX1 Is a Guard Protein on RLH Signaling Responses
The guard hypothesis predicts that the plant NBS-LRR disease resistance proteins prevent unwarranted intracellular signaling through indirect regulation of PAMP receptors. Similar to the plant guard hypothesis, NLRX1 appears to function as a "guard" on RLH-MAVS signaling through binding of the essential adaptor, MAVS. Therefore, NLRX1 is an example of NLRs functioning as modulators of pathogen responses (MOPRs) rather than classical PRRs. Interestingly, another well-known NLR protein, the class II transactivator (CIITA), exerts its regulatory function on MHC class II promoters indirectly through interactions with cofactors known to bind DNA.

(class II transactivator), which is a transcription coactivator for class II MHC genes but does not directly bind DNA; rather, CIITA modifies transcription indirectly through proteins that directly contact DNA (Figure 2). It remains to be determined whether other NLRs are consistent with this guard hypothesis or play a role in regulating RLH-mediated IFN responses.

Viral Evasion of RLH Signaling

Coevolution of host and pathogen applies selective pressures within each organism, resulting in the emergence of subversive physiological mechanisms. In the context of the host, RLH-mediated activation of antiviral immunity is designed to provide a defense against viral infection. Conversely, virus would need to acquire mechanisms to evade or suppress the production of antiviral IFNs by this pathway to establish a productive infection. Indeed, many viral genomes are known to encode for proteins that are designed to target host innate immune responses.

One such viral countermeasure is found within the paramyxoviridae family that includes simian virus 5, human parainfluenza virus 2, mumps virus, Sendai virus, and Hendra virus. Each of these RNA viruses encode for a V protein that binds to MDA-5 and inhibits dsRNA or MDA-5 induction of IFN-β promoter activity (Takeuchi and Akira, 2008). However, another report showed that RIG-I, and not MDA-5, was essential for the production of IFNs in response to paramyxovirus; therefore, these viruses may also function to inhibit RIG-I signaling through an unknown mechanism (Takeuchi and Akira, 2008). Another RNA virus, hepatitis A virus (HAV), potently inhibits RLH-mediated IFN production. This virus targets the RIG-I pathway downstream of RIG-I and upstream of the TBK1-IKK-ε complex by cleaving MAVS utilizing a virally encoded protease, 3ABC (Yang et al., 2007). Consistent with HAV, a nonstructural protein produced by hepatitis C virus (HCV), NS3/4A, colocalizes with MAVS at the mitochondria and specifically targets MAVS as a means to inhibit IFN production. NS3/4A contains serine-protease activity that proteolytically cleaves MAVS at the cysteine-508 resulting in the loss of MAVS mitochondrial localization (Takeuchi and Akira, 2008). Because MAVS localization to the mitochondria is essential for RLH-mediated signaling, this is an effective strategy employed by HCV to counteract host immunity and may explain the persistent and chronic nature of HCV infection in humans. Interestingly, NS3/4A also cleaves TRIF, the essential adaptor of TLR3-signaling, thereby extending the IFN inhibitory properties of HCV to TLR-mediated responses. Influenza A virus suppresses IFN production through the actions of a virally encoded nonstructural protein 1 (NS1). This protein was found to inhibit IFN-β production by blocking RIG-I-mediated recognition of triphosphate RNA (Takeuchi and Akira, 2008). In addition, NS1 was found to complex with RIG-I and associate with MAVS at the mitochondria, thereby disrupting downstream activation of IRF-3 (Mibayashi et al., 2007).

Concluding Remarks and Future Perspectives

We have summarized the current knowledge of mitochondrial antiviral signaling and highlighted several molecules encoded by the host and virus which function to regulate this pathway. However, all of the aforementioned reports have focused on MAVS-mediated IFN responses to RNA virus. It is known that many pathogenic viruses including small pox, herpes viruses, and Epstein barr virus utilize dsDNA for replication. One recent report suggests that the sensing of dsRNA and dsDNA involve a common pathway including RIG-I and MAVS (Cheng et al., 2007). In addition, at least one cytosolic sensor for DNA was predicted as the DNA-dependent activator of IFN regulatory factors (DAI) (Takaoka et al., 2007). However recent in vivo studies have questioned DAI as essential for viral DNA signaling (Ishii et al., 2008). Therefore, the search continues for a definitive cytoplasmic viral DNA receptor, although it is likely that many of the signaling molecules and regulatory mechanisms underlying RLH-mediated responses will overlap with viral DNA pathways. Moreover, future studies on viral DNA signaling are likely to unearth yet-undiscovered molecules and regulations specific for this response. Therefore, as with TLR research, our knowledge of host intracellular pathways involving cytoplasmic receptors and possibly the mitochondria will probably expand in complexity over time. However, with each new molecule and regulatory mechanism identified comes the exciting possibility of future therapies designed to tip the balance of host-pathogen interactions in favor of host protection.

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