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Negative Regulation of Cytoplasmic RNA-Mediated Antiviral Signaling

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Abstract

The recent, rapid progress in our understanding of cytoplasmic RNA-mediated antiviral innate immune signaling was initiated by the discovery of retinoic acid-inducible gene I (RIG-I) as a sensor of viral RNA [1]. It is now widely recognized that RIG-I and related RNA helicases, melanoma differentiated-associated gene-5 (MDA5) and laboratory of genetics and physiology-2 (LGP2), can initiate and/or regulate RNA and virus -mediated type I IFN production and antiviral responses. As with other cytokine systems, production of type I IFN is a transient process, and can be hazardous to the host if unregulated, resulting in chronic cellular toxicity or inflammatory and autoimmune diseases [2-9]. In addition, the RIG-I-like receptor (RLR) system is a fundamental target for virus-encoded immune suppression, with many indirect and direct examples of interference described. In this article, we review the current understanding of endogenous negative regulation in RLR signaling and explore direct inhibition of RLR signaling by viruses as a host immune evasion strategy.

RLR signaling and its control

RIG-I and MDA5, two so-called RIG-I-like receptor (RLR) family proteins have been identified as cytoplasmic sensors of viral RNA [1,10]. RIG-I and MDA5 belong to the DExD/H box RNA helicase family and also have two caspase activation and recruitment domains (CARD) N-terminal to the helicase region, implicated in relaying the signal downstream. Although similar, the two proteins differ in specificity of virus recognition as well as RNA binding specificity [11] as reviewed elsewhere [12]. For MDA5 neither biological substrate specificity nor exact RNA binding have been clearly specified experimentally, however MDA5 is thought to be the primary receptor for signaling initiated by cytoplasmic accumulation of the well-studied synthetic dsRNA analog, poly(I:C) [11,13]. While the helicase region represents one surface for interactions with dsRNAs, for the prototype, RIG-I, substrate recognition and binding specificity has been linked to a domain C-terminal to the helicase region. This RIG-I region has the ability to recognize 5′ tri-phosphorylated ends of double-stranded (ds) or single stranded (ss) RNA [14,15]. Structural analysis has determined that this regulatory domain contains an obligatory zinc binding module, which is conserved in both

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MDA5 and LGP2 sequence alignments [16]. A basic groove has been suggested as the 5′ triphosphate binding site and this is borne out in site-directed mutagenesis experiments [14-17].

It is commonly observed that cytokine signal transduction is transient and must be tightly regulated to prevent unwanted or inappropriate cellular responses that may lead to differentiation or apoptosis. Several mechanisms are thought to underlie the tight regulation of the RIG-I signaling response to prevent unnecessary signaling under steady state conditions. It has been demonstrated that RIG-I can autonomously control its basal activity. The 5′-triphosphate binding C-terminal region of RIG-I was initially referred to as a repressor domain (RD) [18] because its presence in RIG-I was associated with low steady-state signaling in the absence of RNA ligands. Control of basal RIG-I activity by the RD is mediated by auto-inhibition, keeping the protein in an inactive “closed conformation” [18]. RNA ligand binding is thought to induce RIG-I structural alteration to expose the CARD to downstream signaling partners [19-22]. Although structural elements of the RIG-I C-terminus are preserved in MDA5, this autoregulation function does not appear to be conserved, as MDA5 exhibits high basal signaling activity [10,18]. This dissimilarity suggests there are additional means to control MDA5 signal transduction in the absence of ligand stimulation. Currently available data suggest that while expressed modestly at steady state, MDA5 biosynthesis is highly responsive to dsRNA and interferon stimulation [18]. Accumulation of MDA5 may therefore drive signaling responses subsequent to the initial RIG-I response.

Negative regulators of RLRs

In addition to the autonomous inhibitory mechanisms mentioned above, several additional proteins have been recently described as supporting the attenuation or negative regulation of dsRNA signaling. Current experimental data suggest that there are several layers of direct and indirect feedback inhibition that individually or in combination give rise to the overall antiviral signaling profiles both generally and with tissue and cell-specific variations (Table I, Fig. 1).

LGP2

RIG-I was identified as a positive regulator of dsRNA signaling by a reporter gene based cDNA library screen. The identified reporter-activating clone encoded a truncated RIG-I encompassing only the N-terminal CARD regions, and it was found that sole expression of the CARD region of either RIG-I or MDA5 can constitutively and positively activate downstream signaling [1,23]. In contrast, a CARD-deficient C-terminal fragment, referred to as RIG-IC, Δ-CARD or C-RIG, can function as a dominant negative inhibitor. Domain structure similarity between the RIG-IC fragment and the CARD-deficient third member of the RLR family, LGP2, suggested that LGP2 may be a natural negative regulator of dsRNA signaling [18,23-25]. Indeed, expression of LGP2 negatively regulates Sendai virus and Newcastle disease virus (NDV)- mediated IFNβ gene activation, and RNAi mediated knock-down of LGP2 can enhance antiviral gene expression. Moreover, it was observed that LGP2 mRNA and protein are inducible by dsRNA or IFN treatments as well as virus infection, consistent with LGP2 functioning as a negative feedback regulator. LGP2-deficient mice were created by homologous recombination, and the absence of LGP2 resulted in a complex phenotype [26]. Experimental data support the role of LGP2 as a negative regulator for the negative strand RNA virus, vesicular stomatitis virus (VSV), as the knockout mice exhibited greater resistance to VSV infection.

Several mechanisms may account for the LGP2 inhibitory effects. As a homolog of RIG-IC, one attractive model is that LGP2 can sequester RNA ligands from recognition by RIG-I or MDA5 [23,25]. Alternatively, it was found that LGP2 can suppress IFNβ promoter activity induced by non-RNA activators, including expression of the constitutively active RIG-I N-

terminal CARD fragment or the signaling adaptor molecule IPS-1/MAVS/CARDIF/VISA [19-22] from plasmid vectors. These RNA-independent stimuli are not suppressed by the artificial dominant negative RIG-IC fragment, which implicates additional suppression activity of LGP2. Mechanistic experiments found that LGP2 co-immunoprecipitates with the adaptor protein, IPS-1/MAVS/CARDIF/VISA, and exhibits competitive binding with the downstream kinase, IKKi/IKKε for a common site in IPS-1[24]. LGP2 has also been demonstrated to associate with RIG-I to inhibit its auto-oligomerizaton via the LGP2 C-terminal region comparable to the RIG-I RD [18]. In this model, dimerization of RIG-I by 5′-triphosphorylated ssRNA, proposed to be an active form of RIG-I, [16] is replaced by a RIG-I:LGP2 hetero-oligomer.

Unexpected in view of the evidence in favor of LGP2 as a negative regulator, the LGP2-deficient mice were found to be more susceptible to infections with encephalomyocarditis virus (EMCV), indicating a role for LGP2 as a positive regulator of antiviral signaling by this picornavirus. Resistance to EMCV has been demonstrated to be controlled by MDA5 rather then RIG-I signaling, and therefore the greater susceptibility to this virus may be interpreted to indicate that LGP2 contributes to positive regulation of MDA5 mediated antiviral signaling [26]. Further mechanistic studies will be needed to clarify the functions of LGP2 in both RIG-I and MDA5 –mediated antiviral signal transduction.

A20

A20 was originally identified as a TNF-inducible gene in human umbilical vein endothelial cells [27] and was found to be induced by a wide range of stimuli including NF-κB. The anti-apoptotic protein has been known as a negative regulator of NF-κB and was recently shown to block RIG-I-mediated signaling to the IRF-3, IRF-7 and NF-κB pathways. Expression of A20 blocks the constitutively active RIG-I N-terminus from signaling IRF-3 phosphorylation, dimerization, and DNA binding [28] as well as Sendai virus, NDV and dsRNA mediated IFNβ promoter activity [29,30]. Experiments indicate that A20 intervenes downstream of the mitochondrial adaptor protein IPS-1 but upstream of the kinases (TBK1 and IKKi) that phosphorylate IRF-3. Therefore, it seems likely that A20 could also inhibit MDA5-mediated signaling, but this remains to be demonstrated formally.

A20 is a unique protein that possesses a N-terminal de-ubiquitination domain, called ovarian tumor (OTU) domain, and a C-terminal ubiquitin ligase domain. Inhibition of NF-κB signaling by A20 relies on cooperation of these two ubiquitin-related domains [31], but only the C-terminal ubiquitin ligase domain is essential for RIG-I signaling interference [28], suggesting that the A20 target protein is most likely modified by K48 ubiquitin leading to proteosomal degradation [29,30]. A20 deficient mice develop severe multi-organ inflammation [32] and A20-deficient fibroblast displayed prolonged NF-κB activity and are unable to terminate tumor necrosis factor (TNF)-induced NF-κB activation. Macrophages derived from A20 deficient mice are incapable of terminating TLR-induced NF-κB activation [32,33], indicating that A20 functions as a negative feedback regulator for several branches of the immune systems.

Pin1

It has been well established that upon virus infection, IRF-3 can be phosphorylated at two C-terminal phospho-acceptor clusters (Ser385-Ser386 and Ser396-Ser398-Ser402-Thr404-Thr405) [34-37]. These modifications are critical for IRF-3 activation [38-40]. An additional phosphorylation site, Ser339, was also found to be involved in IRF-3 regulation [41]. While the specific kinase(s) for this phosphorylation site are unknown, accumulation of phosphorylation occurs with delayed kinetics relative to activating stimuli. Due to a neighboring proline at position 340, phosphorylation of Ser 339 produces a binding site for the WW domain of the peptidyl-prolyl isomerase, Pin1. This interaction possibly subjects IRF3

to cis-trans isomerization around the Ser-Pro peptide bond. The Ser339-Pro motif is conserved evolutionarily among human, mouse, rat, and, chicken, suggesting that Pin1 activity is fundamental to IRF-3 regulation.

For other Pin 1 substrates, this type of isomerization has been shown to regulate the stability and localization of the substrate protein, with profound effects on transcriptional activation, cell cycle progression, and cell death. For example, Pin1 association with the tumor suppressor p53 generates conformational changes that enhances transactivation activity [42,43]. For IRF-3, Pin1 association was demonstrated to facilitate ubiquitin-mediated proteosomal degradation. Pin 1 deficient mouse embryonic fibroblasts (MEFs) are more hostile to replication of Newcastle disease virus (NDV) and encephalomyocarditis virus (EMCV), consistent with a negative regulation phenotype. As Pin1 inhibits at the level of IRF-3, it controls TLR3, RIG-I, and MDA5-mediated signaling by eliminating active IRF-3 pools characterized by Ser339 phosphorylation. Interestingly, Pin1 also has been reported as a positive regulator of the NF-κB component p65, which is also involved in IFNβ promoter activation [44]. Further work will be needed to determine the exact mechanisms involved in Pin1 regulation of antiviral signaling.

SIKE

Suppressor of IKKε, SIKE, has been identified as an IKKε-associated protein by yeast two-hybrid screening [45]. Further characterization of SIKE revealed that it also can associates with TBK1 and that SIKE expression can efficiently inhibit IKKε, TBK1, TRIF, TLR3 and RIG-I-mediated IFNβ and interferon stimulated response element (ISRE) promoter activation. In this study an interaction between the kinases IKKε and TBK1 with TRIF, IRF-3 and RIG-I was observed. This interaction was disrupted by expression of SIKE. This effect is specific to components of virus- and TLR-3-triggered IRF-3 activation pathway but does not disrupt components of the NF-κB pathway. Furthermore, gel filtration experiments have demonstrated that SIKE exists in a higher order complex associating with TBK1 as well as unidentified cellular protein. VSV infection or poly(I)•poly(C) treatment appear to dissociate SIKE from TBK1 as it converts these complexes to the size of monomeric and dimeric SIKE that are free from TBK1. RNAi-based knock down of SIKE without VSV infection or poly(I)•poly(C) treatment leads to increased induction of IFNβ and ISRE promoter but not NF-κB promoter activity. Accordingly, SIKE knock down decreases VSV growth while overexpression of SIKE increases VSV growth in HEK293 cells. Endogenous protein levels of SIKE protein remain unchanged during virus infection or TLR3 activation. This study suggests that SIKE functions as a suppressor of TBK1 and IKKε by sequestering TBK1 and IKKε into an inactive complex to avoid unnecessary activation of those kinases.

Atg5-Atg12 conjugate

Autophagy is a cellular process that mediates a nonspecific bulk degradation/recycling pathway that is essential for turnover of stable macromolecules, maintenance of an amino acid pool upon starvation, and the cellular response to a variety of hormonal stimuli [46]. In addition, the autophagy pathway is crucial for defense against infections with intracellular bacteria [47-49], plays a key role in recognizing signatures of viral infection through TLR7 in plasmacytoid dendritic cells, and represents a critical effecter mechanism to restrict viral replication [50,51]. However, autophagosomes have also been exploited by certain viruses as a niche for viral replication, where its membrane scaffold structure serves as a place for RNA replication [52,53]. A recent study has demonstrated that RLR signaling is negatively regulated by some autophagic proteins [54]. The small ubiquitin-like Atg12 becomes conjugated to its substrate, Atg5, to produce the Atg5-Atg12 conjugate, a key regulator of the autophagic process that associates directly with the CARD domains of RIG-I, MDA5 and IPS-1. It is postulated that Atg5-Atg12 conjugate can bridge RIG-I or MDA5 to IPS-1. The presence of the Atg5-

Atg12 conjugate in the RIG-I-IPS-1 complex might lead to an inactive status. MEFs from Atg5 deficient mice produced more IFNβ and IP10 mRNA after VSV infection or cytoplasmic delivery of poly(I)•poly(C) compared to wild type (WT) MEFs. Accordingly, absence of Atg5 leads to diminished VSV growth. In contrast, Herpes simplex virus-1 (HSV-1) –mediated induction of IFNβ and IP10 mRNA shows an opposite effect. Authors have shown that an Atg5 point mutant, which can not be conjugated to Atg12, does not inhibit IPS-1 dependent IFNα, IFNβ, and NF-κB promoter activity. In addition, Atg7 deficient MEFs, in which Atg5-Atg12 conjugation is defective, produce higher amount of type I IFNs in response to transfection of poly(I)•poly(C). Collectively, this study has indicated that Atg5-Atg12 conjugate, but not monomeric Atg5 or Atg12, acts as a suppressor of RLR signaling.

RNF125

It has recently become clear that RIG-I is regulated by ubiquitin conjugation mediated by a RING finger family protein, RNF125, an E3-ubiquitin ligase that specifies their proteosomal degradation [55]. RNF125 was identified as an UbcH8 interacting protein by yeast two-hybrid screening based on the prediction that UbcH8, which is an E2 enzyme for both ubiquitin and ISG15, might associate with the E3 ligase for RIG-I. The N-terminal region of RNF125 associates with RIG-I via the CARD and RNA helicase domain but not with the RD, and expression of RNF125 increases RIG-I ubiquitination and destabilization. Conversely, RNAi-mediated knock down of RNF125 decreases the level of ubiquitin-modified RIG-I resulting and stabilizes RIG-I. Both MDA5 and IPS-1 were also shown to be ubiquitination targets of RNF125. As a result, virus or dsRNA -mediated IFNβ production is inhibited by expression of RNF125 and enhanced by RNF125 knock-down. As RIG-I abundance decreases with the onset of the RNF125 accumulation, RNF125 also has the properties of a negative feedback regulator of RIG-I. While the exact locus for RNF125-mediated RIG-I ubiquitination is not known, its functional consequence differs from ubiquitination of RIG-I on K172 [56]. This residue of RIG-I, located in the CARD region, is a target for efficient TRIM25-induced ubiquitination that is essential for antiviral signaling. The activating ubiquitin modification is K63-linked, a type not typically associated with proteasome mediated degradation. It will be interesting to explore the potential interplay between these independent ubiquitin based regulatory mechanisms.

DUBA

An siRNA based screening for OTU deubiqitinating (DUB) family members identified DUBA, as a negatively regulator of antiviral signaling [57]. The data indicate that reduction of DUBA by siRNA knock down augments TLR3, 4, 7, RIG-I, and MDA5-mediated IFN induction. Experiments demonstrate that increased expression of DUBA had the opposite effect, indicating that DUBA acts as a negative regulator. Knockdown of DUBA did not alter NOD2, TLR2, TLR3, or TNF-dependent NF-κB reporter activity, but did increase RIG-I and MDA5-mediated responses, suggesting a more general role for IFN activation and a limited role on NF-κB activation. A biochemical approach has identified TNF receptor-associated factor 3 (TRAF3) as a DUBA interacting protein. TRAF3 is a critical adaptor molecule required for induction of type I IFNs in response to viral infection and TLR activation [58,59]. TRAF3 directly associates with IPS-1 to induce IFNs in RLR signaling by linking TBK1/IKKε kinases [60]. Expression of DUBA cleaves Lys63-linked ubiquitin chains on TRAF3 and depletion of DUBA increases ubiquitinated TRAF3. Expression of DUBA decreases interaction between TRAF3 and TBK1, suggesting that ubiquitination of TRAF3 controls the activity of the kinase-containing complex. As DUBA protein is also increased in macrophages by LPS stimulation, it may also function as part of a negative feedback circuit.

CYLD

Another OTU DUB family protein, the tumor suppressor, CYLD, has been identified as a negative regulator of RIG-I by two groups [61,62]. RNA interference targeting CYLD results in an enhancement in Sendai virus-triggered IFNβ secretion and experiments using CYLD-deficient MEFs and dendritic cells (DCs) show constitutive activation of TBK1/IKKε kinases, which is associated with hyper-induction of type I IFNs by VSV infection. The data indicate that CYLD targets RIG-I as well as TBK1 for deubiquitination that leads to inactivation of the signaling. CYLD associates with the CARD domain of RIG-I and removes K63-linked ubiquitin from the RIG-I CARDs that are conjugated by the E3 ubiquitin ligase, TRIM25. Immunoprecipitation experiments show that CYLD coprecipitates not only with RIG-I but also with TBK1 and IKKε. Interestingly, TBK1 or IKKε but not RIG-I specifically precipitates two species of CYLD in size, suggesting phosphorylation of CYLD by these kinases.

Loss of CYLD in DCs causes accumulation of ubiquitination of RIG-I indicating that RIG-I is constantly in the cycle of ubiquitination-deubiquitination with deubiquitination being a dominant event in steady state. Furthermore, the accumulation of ubiquitinated RIG-I in CYLD-deficient cells is associated with constitutive activation of TBK1/IKKε kinases. CYLD protein level is reduced in the presence of TNF and viral infection. CYLD is most likely a negative regulator that inhibits RIG-I in both, steady state and activated state, to prevent unnecessary signaling event. However, the precise mechanism that controls TRIM25-mediated ubiquitination and CYLD-mediated deubiquitination remains to be elucidated.

NLRX1

A member of nucleotide-binding domain and leucine-rich repeat containing (NLR) protein family, NLRX1 has been identified as a negative regulator of RLR signaling [63]. Combination of microscopy based and biochemical approaches has revealed that NLRX1 is a ubiquitously expressed protein that resides at the outer mitochondrial membrane where IPS-1 is located. The amino terminus of NLRX1 contains a mitochondrial-targeting sequence making it the first NLR shown to have this feature. Intriguingly, association between endogenous NLRX1 and IPS-1 is observed and the interaction is mediated by the CARD-like domain of IPS-1 and a putative nucleotide-binding domain (NBD) of NLRX1. Expression of NLRX1 results in the potent inhibition of RIG-I, MDA5, and IPS-1 -mediated IFNβ promoter activity, but does not alter TLR3-mediated signaling. Deletion of the leucine-rich repeat (LRR) abolishes the IPS-1 inhibitory activity, though it is dispensable for interaction with IPS-1. Depletion of NLRX1 by siRNA enhances IPS-1-induced IFNα, IFNβ, IL-6, RANTES, TNFα mRNA as well as virus-induced secretion of IFNβ. Mechanistic studies suggests that NLRX1 competes with RIG-I for IPS-1 interaction, implying that association between RIG-I and IPS-1 through CARD-CARD interaction is disrupted by NLRX1 in the mitochondrial compartment. Together, these results support a role of NLRX1 as a negative regulator of RLR signaling. One distinction between this negative regulator and others described here is that NLRX1 protein and mRNA levels do not change when cells are activated by expression of positive factors such as RIG-I, MDA5, IPS-1, and IRF-3/7 or even with IFNβ treatment. Therefore, NLRX1 seems to act as a inhibitor of steady state antiviral signaling rather than working as a negative feedback inhibitor.

Interestingly, NLRX1 has also been reported to be a positive regulator of the immune system that potently triggers the generation of reactive oxygen species (ROS). NLRX1 synergistically potentiates ROS production induced by TNFα, Shigella infection and double-stranded RNA treatment, resulting in amplified NF-κB-dependent and JUN amino-terminal kinases-dependent signaling [64]. Clearly this protein has several functions in immune regulation, and further research will reveal their relative contributions to overall antiviral responses.

ISG15

The ubiquitin-like IFN stimulated gene 15 (ISG15) protein has been reported to modify RIG-I [65] and can negatively regulate RIG-I signaling activity [66]. When ISG15-specific E1 (UBE1L), E2 (UbcM8), and ISG15 are expressed together in cells, robust ISG15 modification of RIG-I is observed. Expression of the ISG15 system decreases the basal level of ISRE and IFNβ promoter activity as well as NDV-mediated IFNβ promoter activity and delays cytoplasmic poly(I)•poly(C)- mediated IFNβ promoter activity. Interestingly, the basal level of RIG-I and ISG proteins but not mRNA are much higher in UBEL1-deficient murine fibroblasts than in WT cells. Accordingly, UBEL1 -/- cells produce much higher IFNβ mRNA than WT cells in response to NDV infection. As ISG15 modification system is only present in IFN stimulated cells, it is tempting to speculate that ubiquitin and ISG15 may coordinately regulate the fate of RIG-I or compete for control of RIG-I activity.

Dihydroxyacetone kinase (DAK)

Only one MDA5 specific negative regulator has been reported. The protein kinase, DAK, was found to interact with MDA5 in a yeast two hybrid screen [67]. DAK is a member of the evolutionarily conserved family of dihydroxyacetone kinases. In bacteria, DAK phosphorylates dihydroxyacetone to produce DHA phosphate, an obligatory precursor for the biogenesis of glyceryl ether phospholipids. Mammalian DAK displays dual activities as flavin adenine dinucleotide (FAD)- adenosine monophosphate (AMP) lyase and ATP-dependent Pha kinase [68,69]. However, the physiological functions of DAK in higher eukaryotes are unknown. In immunoprecipitation experiments, DAK associates with MDA5 but not RIG-I, and the CARD domain-containing fragment of MDA5 is sufficient for the association. Expression of DAK inhibits MDA5-mediated IFNβ and ISRE reporter gene activity, but not RIG-I-, IPS-1 or TLR3 activity. DAK does not inhibit MDA5-mediated NF-κB activation in reporter gene assay, suggesting that DAK selectively inhibits MDA5 mediated IRF-3 activation. The observed interaction between endogenous MDA5 and DAK is decreased when the cells are infected with Sendai virus, suggesting that the association is disrupted upon virus infection, which is similar to SIKE's dissociation from TBK1 during virus infection. Therefore DAK might keep MDA5 inactive under steady state conditions, although the detailed mechanism of DAK regulation of MDA5 remains to be elucidated.

Viral negative regulators of the RLR signaling pathway

The importance of the IFN system as a primary antiviral defense has made it a strong selective pressure for virus evolution. Though effective, the RLR signaling pathway offers many potential targets for virus evasion, interference, and antagonism, including the initiating ligand (cytoplasmic non-self RNA); the RNA sensors, RIG-I and MDA5; signaling adaptor molecules such as IPS-1 and TRAFs; kinases involved in signal propagation and transcription factor activation (e.g., TBK1, IKKs); and the transcription factors that regulate antiviral gene expression (IRF-3, IRF-7, NF-κB) [70]. The recent literature is rich with descriptions of viral interference with antiviral signaling [71-73], although for many viral proteins the exact cellular target or mode of interference awaits further description. One outstanding example, for which the mechanism of inhibition on both the TLR3 and the RLR pathways has been characterized in great detail, is the hepatitis C virus protein complex NS3/4A. The NS3/4A protease function was known to be essential for proper posttranslational cleavage of the hepatitis C virus polyprotein when it was also identified as responsible for inhibition of IRF3 phosphorylation [74]. Sequence alignments of known NS3/4A specific cleavage sites in components of TLR3 signaling revealed a potential cleavage site in TRIF/TICAM-1 (Toll-IL-1 receptor domain-containing adaptor inducing IFNβ), an adaptor molecule in the TLR3 signaling pathway. In vitro cleavage assays confirmed the predicted cleavage of TRIF into two fragments, both incapable of IFNβ induction [75]. Shortly after the identification of TRIF as an NS3/4A

substrate, NS3/4A was also shown to specifically interfere with the RLR system. This inhibition could be restored by IKKε expression, indicating a target upstream of the kinases [76]. With the identification of IPS-1 (Cardif/Visa/MAVS) [19-22] as the adaptor molecule in the RLR pathways, two groups have reported specific cleavage of IPS-1 by NS3/4A, explaining the mechanism of RLR inhibition by HCV [20,77]. The importance and effectiveness of NS3/4A mediated immune response interference was underlined by the fact that it is not unique to HCV, but homologous proteases with IPS-1 cleavage activity were found in the related Flavivirus, GB Virus B [78], as well as the Picornavirus hepatitis A Virus [79,80].

As with HCV, several viruses or their encoded proteins have been identified as inhibitors of RLR-induced signaling (Table II). However, in many cases the precise point of interference has yet to be clearly delineated. A common technique employed for determination of interference with the RLR mediated antiviral response takes advantage of the signaling activity of specific RNA ligands or ectopically expressed RIG-I N-terminal CARD region, full length MDA5, IPS-1 or downstream serine kinases as means to dissect the target for viral interference. Inhibition on the level of kinases or transcription factors will also affect TLR signaling as RLR and TLR pathways converge in later steps of their signaling cascade. Here, we focus instead on examples of viral strategies that are well described in their abilities to specifically circumvent the RLR response by RNA processing or direct interference with MDA5 or RIG-I.

RNA processing as a RIG-I evasion strategy

The specific ligand for RIG-I signaling was identified as 5′-triphosphate containing ssRNA or dsRNA [14-17]. As most viruses do not cap their genomic RNA or mRNA, 5′triphosphate ends are often detected as non-self RNA in virus infected cells. Many viral proteins have RNA binding affinity, and in some cases the same proteins act as antiviral antagonists [72]. Although it is often difficult to clearly define the specificity of multifunctional proteins, it has been suggested that RNA binding underlies the immune evasion properties of many viral IFN antagonists (Table II).

More specific than RNA sequestration from RLR detection, permanent genomic RNA processing to mask 5′end modifications was recently implicated as an IFN evasion mechanism [81,82]. Genomic RNA of representative negative-stranded RNA virus (NSV) families was tested for IFN induction. In agreement with other cases [14,15] a strong IFN response was found to be dependent on the 5′triphosphate. Interestingly, genomic RNA from members of Bunyaviridae, specifically Hantaan virus (HTNV) and Crimean-Congo hemorrhagic fever virus (CCHFV), as well as Bornaviridae, Borna disease virus (BDV), did not induce an IFN response. In all three cases, the evasion of virus-induced RIG-I signaling correlates with the presence of 5′monophosphate genomic RNA [82,83]. The exact mechanisms for removal of 5′triphosphate residues are largely unknown. Alternate mechanism have been proposed including a prime- and realign replication mechanism for HTNV [83] and programmed genome trimming for BDV [84]. In both models the 5′monophosphates suggest an endonuclease or pyrophosphatase activity.

Paramyxovirus V proteins target MDA5

To date the only viral protein known to interfere specifically with MDA5 is the paramyxovirus V protein. A wide variety of paramyxoviruses target MDA5 but not RIG-I via their V protein and can specifically block MDA5 mediated IFN induction [10,85]. MDA5 was isolated in a screen for immunoprecipitated host cell proteins that interact with the V protein of parainfluenza virus 5 (PIV5) [10]. It was demonstrated that the C-terminal domain, a highly conserved hallmark of paramyxovirus V proteins, is necessary and sufficient for the inhibition of and interaction with MDA5. The inhibition of MDA5 is a general property of V protein C-

termini and yeast-two hybrid results are suggestive of a direct protein:protein interaction. Nonetheless, the exact mechanism of interference is yet to be explained, in part due to the lack of a clear understanding of the role of MDA5 in antiviral signaling. Interestingly, despite a 30% sequence identity between MDA5 and RIG-I, no interaction was detected between RIG-I and a V protein. This strong preference for MDA5 seems in contradiction to the finding that MDA5 deficient mice exhibit normal resistance to paramyxovirus infection while RIG-I deficient mice are more susceptible [11,86]. It can be speculated that additional MDA5 functions besides IFN induction, such as the described proapoptotic activity [87], might play a role in the strong evolutionary selection for the V protein to target MDA5.

Influenza A Virus NS1 proteins target RIG-I

The nonstructural protein 1 (NS1) of influenza A is well known as an antagonist of antiviral host responses [88]. NS1 has been demonstrated to inactivate several immune effectors, including the downregulation of cellular mRNA processing [89,90], interaction and blocking of PKR [91,92] as well as RNA sequestration from 2′5′-OAS activation [93]. IFN production is highly elevated in cells infected with NS1 deficient influenza A strains compared to cells infected with wildtype virus [94], and overexpression of NS1 can block IFN production by other viruses or PRR ligands [94,95]. RIG-I and not MDA5 is responsible for influenza A detection [11,96], specifically the 5′triphosphate of influenza A genomic RNA is recognized by RIG-I [15]. Several publications demonstrated the inhibition of RIG-I mediated signaling by NS1 [15,96-98]. While initially dsRNA sequestration by NS1 was suggested as the main mechanism for prevention of PRR signaling, recently an interaction between RIG-I and NS1 was demonstrated [15,96]. Interestingly, constitutive RIG-I CARD induced signaling was also efficiently blocked by NS1 [96]. While direct interference with RIG-I may explain many actions of NS1, some findings suggest a more complicated mode of interference. For example, antiviral signaling induced by ectopic expression of IPS-1 is also inhibited by NS1 and both RIG-I and NS1 co-fractionate with IPS-1 in an insoluble cell fraction. It is unknown whether the interaction between RIG-I and NS1 is direct, but coimmunoprecipitation assays suggest that the interaction may be influenced by RNA as NS1 RNA binding mutants bound poorly to RIG-I [15]. Nonetheless NS1 mutants that are defective for RNA binding retain some ability to block IFNβ promoter transcription [99,100].

Human Metapneumovirus Glycoprotein G targets RIG-I

The newly discovered human metapneumovirus (hMPV) belongs to the family of *Paramyxoviridae* and was identified as a leading cause of respiratory tract infection among children [101]. Recombinant hMPV lacking the G gene (rhMPV-ΔG) replicated efficiently in vitro but was highly attenuated in a hamster model system [102]. The role of the gene product, glycoprotein G, a transmembrane protein, is largely unknown, however recent findings demonstrated a role in hMPV induced innate immune response. In cell culture, infection with rhMPV-ΔG lead to higher levels of type I IFNs and other cytokines compared to infection with recombinant wildtype virus [103]. Coexpression studies of RIG-I and MDA5 with the hMPV G protein revealed that the G protein can specifically block RIG-I mediated IFNβ induction. In addition, RIG-I and the G protein were shown to co-precipitate in co-expression studies as well as in hMPV infected cells [103]. As with the Influenza NS1 protein it is unknown whether the RIG-I G protein interaction is direct and what constitutes the precise mechanism of interference.

As the newly discovered cytoplasmic RLR pathways have proven their importance for innate immune responses, we expect rapid progress in the discovery of additional viral strategies to interfere with RLR signaling.

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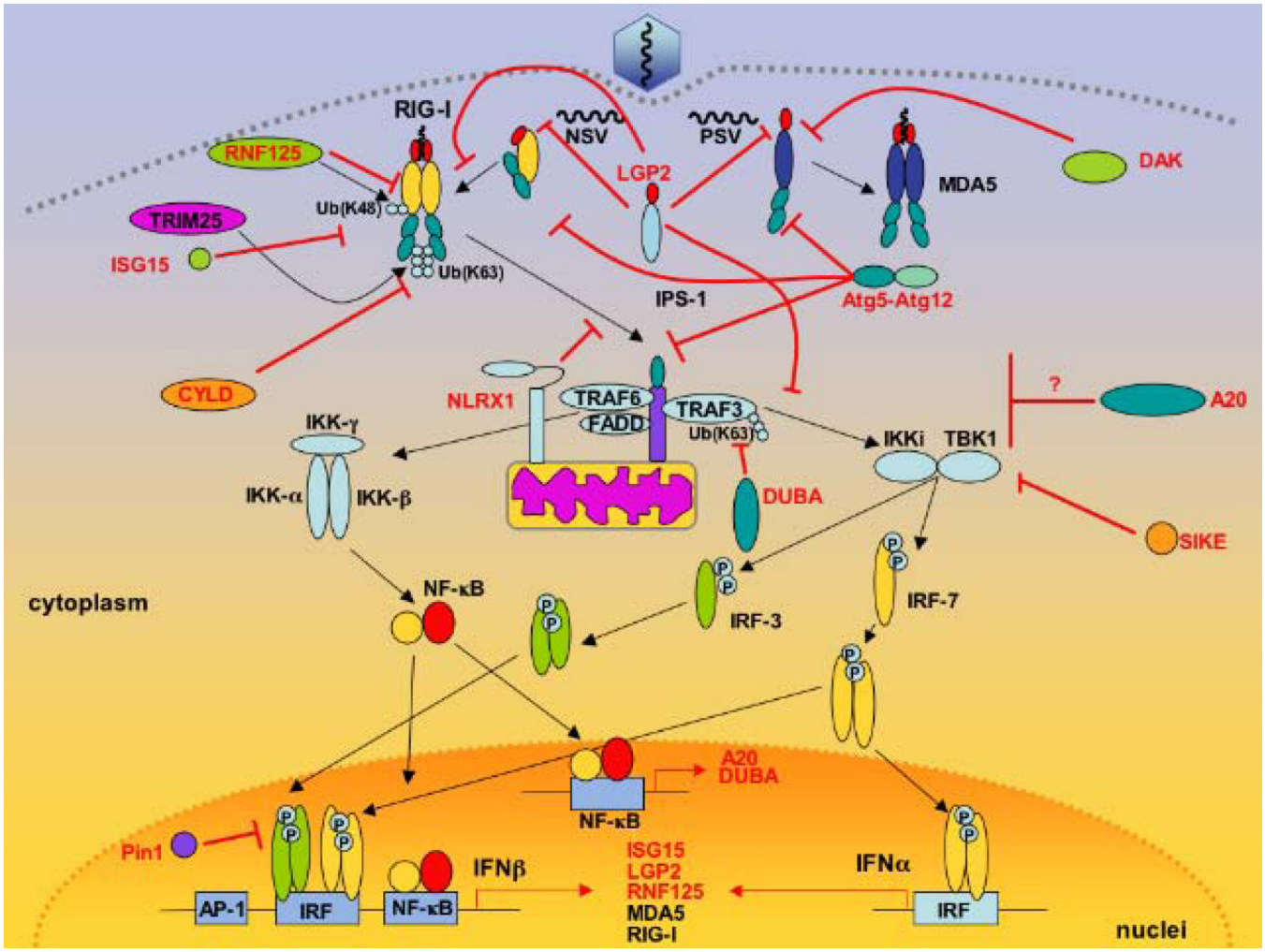

Figure 1.

RLR signaling pathway and its negative regulatory molecules. Positive signals and molecules are indicated as black arrows and black letters, respectively. Negative signals and negative regulators are indicated as red lines and red letters. For references, see text. Abbreviations; NSV: Negative stranded virus; PSV: Positive stranded virus; RIG-I: retinoic acid inducible gene-I; MDA5: melanoma differentiated-associated gene-5; LGP2: laboratory of genetics and physiology-2; RNF125: ring-finger protein 125: TRIM25: tripartite motif 25; SIKE: Suppressor of IKKε; DAK: Dihydroxyacetone kinase; FADD: Fas-Associated protein with Death Domain; DUBA: Deubiquitinating enzyme A; TRAF: tumor necrosis factor receptor-associated factor; Ub: Ubiquitin; CYLD: cylindromatosis; ATG: Autophagy-related gene; NLR: nucleotide-binding domain and leucine-rich repeat.

Table I

Cellular Inhibitors of RLR signaling

Inhibitors	Target(s)	Proposed mechanisms	Type of negative regulation	Refs.
LGP2	dsRNA, RIG-I, IPS-1 containing complex	dsRNA sequestration, Competition with IKKi for IPS-1, Inhibition of RIG-I dimerization	Negative feedback (RLRs)	[18,23-26]
A20	Unknown	deubiquitination of the target protein(s)?	Negative feedback (RLRs, TLRs etc)	[28-30]
Pin1	IRF-3	phosphorylation-dependent isomerization	Negative feedback (RLRs, TLRs)	[41]
SIKE	TBK1 and IKKe	Sequestration of kinases	Inhibition of steady state (RLRs, TLRs)	[45]
Atg5-Atg12	RIG-I, MDA5, IPS-1	Sequestration of RLRs and IPS-1	Inhibition of steady state (RLRs)	[54]
RNF125	RIG-I, MDA5, IPS-1	Proteosomal degradation of RLRs and IPS-1	Negative feedback (RLRs)	[55]
DUBA	TRAF3	deubiquitination of TRAF3	Negative feedback (RLRs, TLRs etc)	[57]
NLRX1	IPS-1	competition with RLRs for IPS-1	Inhibition of steady state (RLRs)	[63]
ISG15	RIG-I	Sequestration of RIG-I	Negative feedback (RIG-I)	[66]
DAK	MDA5	Sequestration of MDA5	Inhibition of steady state (MDA5)	[67]
CYLD	RIG-I, TBK1	deubiquitination of RIG-I	Inhibition of steady state and activated state	[61,62]

Table II

Viral Inhibitors of RLR signaling Virus	Inhibitors	Target(s)	Proposed mechanisms	Refs.
Human cytomegalovirus	TRS1	viral RNA	dsRNA sequestration, PAMP masking	[104-106]
Murine cytomegalovirus	m142/m1 43	viral RNA	dsRNA sequestration, PAMP masking	[107,108]
Vaccinia virus	E3L	viral RNA	dsRNA sequestration, PAMP masking	[109,110]
Reovirus	σ3	viral RNA	dsRNA sequestration, PAMP masking	[111,112]
Rotavirus	NSP3	viral RNA	dsRNA sequestration, PAMP masking	[113]
Rotavirus	NSP1	IRF-3	IRF-3 degradation	[114]
Herpes simplex virus	Us11	viral RNA	dsRNA sequestration, PAMP masking	[115,116]
Herpes simplex virus	ICP0	IRF-3	IRF-3 sequestration/degradation	[117-119]
Human herpesvirus 6	IE1	IRF3	Inhibition of IRF3 dimerization/IRF3 degradation	[120]
Human herpesvirus 8	vIRF	IRF3 promotor binding site	Virus encoded IRFs imitate non functional IRF	[121]
Human papillomavirus	E6	IRF-3	IRF-3 sequestration	[122]
Human papillomavirus	E7	IκB kinase complex	Inhibition of kinase complexes	[123]
Poxvirus	N1L	IκB kinase complex	Inhibition of kinase complexes	[124]
Hepatitis C virus	NS2	Unknown	Unknown	[125]
Hepatitis C virus	NS3/4A	IPS-1	IPS-1 cleavage	[20,74,76,77]
GB Virus B	NS3/4A	IPS-1	IPS-1 cleavage	[78]
Hepatitis A virus	NS3/4A	IPS-1	IPS-1 cleavage	[79,80]
West Nile	Unknown	Unknown	Unknown	[126]
Porcine reproductive and respiratory syndrome virus	Unknown	Unknown	Unknown	[127]
Mengovirus	Leader	Unknown	Inhibition of IRF-3 dimerization	[128]
Pestivirus	Npro	IRF-3	Proteasomal IRF-3 degradation	[129,130]
Hantavirus	G1	TBK1	Inhibition of TBK1	[131]
Paramyxovirus	V	MDA5	MDA5 Complex inactivation	[10,85]
Paramyxovirus	C	Unknown	Unknown	[132]
Human metapneumovirus	G	RIG-I	RIG-I complex inactivation	[102]
Human, bovine respiratory syncytial virus	NS1	Unknown	Unknown	[133,134]
Human, bovine respiratory syncytial virus	NS2	Unknown	Unknown	[133,134]
Rabiesvirus	P	TBK-1	Inhibition of TBK1	[135]
Thogotovirus	ML	IRF-3	Inhibition of IRF-3 dimerization	[136]
Borna disease virus	P	TBK-1	Inhibition of TBK1	[137]
Ebolavirus	Vp35	viral RNA, TBK1	dsRNA sequestration, PAMP masking, interference with kinase activity	[138,139]
Influenza A,B	NS1	viral RNA, RIG-I, IPS-1	dsRNA sequestration, PAMP masking, RIG-I/IPS-1 complex inactivation	[15,93-96,140]
Human immunodeficiency virus 1	TAT	viral RNA	dsRNA sequestration, PAMP masking	[141]