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Caspase-8 and FLIP regulate RIG-I/MDA5-induced innate immune host responses to picornaviruses

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Abstract

Picornaviruses are small, nonenveloped, positive-stranded RNA viruses, which cause a wide range of animal and human diseases, based on their distinct tissue and cell type tropisms. Myocarditis, poliomyelitis, hepatitis and the common cold are the most significant human illnesses caused by picornaviruses. The host response to picornaviruses is complex, and the damage to tissues occurs not only from direct viral replication within infected cells. Picornaviruses exhibit an exceptional ability to evade the early innate immune response, resulting in chronic infection and autoimmunity. This review discusses the detailed aspects of the early innate host response to picornaviruses infection mediated by RIG-I-like helicases, their adaptor, mitochondrial antiviral signaling protein, innate immune-induced apoptosis, and the role of caspase-8 and its regulatory paralog, FLIP, in these processes.

Keywords

apoptosis; caspase-8; FLIP; innate immunity; MAVS; MDA5; mitochondria; picornavirus; RIG-I

The *Picornaviridae* family

The *Picornaviridae* family is divided into 12 genera. The most important human pathogens within the picornavirus family are poliovirus, coxsackieviruses and human rhinoviruses, which all belong to the *Enterovirus* genus. The hepatitis A virus (HAV) belongs to the *Hepatovirus* genus, and the most important animal pathogenic picornavirus, foot-and-mouth disease virus, is the prototypic member of the genus *Aphthovirus*. Poliovirus is the best known of the picornaviruses, and survived as an endemic pathogen in the human population for thousands of years; until the late 19th century it spread epidemically by the oral–fecal route throughout the world [1]. Poliovirus replicates in the intestine, and infection leads to poliomyelitis; an inflammation and neuronal necrosis of motor neurons, which project their axons outside the CNS through the anterior horn of the spinal cord and control muscle function. In the final stage of infection, poliovirus may result in permanent paralysis [2]. The

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coxsackieviruses are subdivided into two groups, A and B, which differ in their tissue tropism and pathogenesis in humans. The group B coxsackieviruses (CVB) include six serotypes (B1–B6) that are associated with myocarditis, meningitis, encephalitis, pancreatitis and Type 1 diabetes [3], with the CVB3 strain most commonly associated with cardiovirulence [4–7]. Similar to other enteroviruses, CVB are resistant to low pH and pass unaffected through the gastric acidity of the stomach and replicate in mucosal cells of the small intestine prior to their dissemination to produce systemic infection [8]. Furthermore, the rhinoviruses, with an estimated billion cases of the common cold in the USA each year, are the most infectious viral agents in humans [9]. They are also one of the most prevalent causes of asthma exacerbations, as they can spread from the ciliated epithelial cells of the upper respiratory tract into the lower airways [10].

The sphere-shaped, nonenveloped virions of picornaviruses are approximately 28 nm in diameter and contain an RNA genome of 7000–9000 nucleotides. As an example CVBs possess a positive-stranded RNA genome of approximately 7.4 kb, of which 10% comprises a 5′ untranslated region (UTR) containing 741 nucleotides of a highly structured RNA sequence covalently linked to a virally encoded protein VPg (virus protein genome-linked). This is followed by a single long reading frame [11], which is responsible for both viral protein translation and viral genome replication [12–14]. The nucleotide sequence of 5′ UTRs from different CVB serotypes differ significantly; however, the overall higher-order RNA structures are sufficiently similar; replacement of one 5′ UTR with another permits replication and function in cell culture [12,15]. Picornaviruses start the infectious cycle with cognate receptor recognition, followed by cell entry, during which the virus undergoes uncoating and release of its genome. Picornaviruses utilize a number of diverse surface molecules as receptors, including immunoglobulin-like molecules and adhesion proteins. The receptor of human poliovirus, an immunoglobulin-like protein known as CD155, and the poliovirus receptor, which plays a consistent key role in expediting the enteric phase of poliovirus infection, has been shown to be expressed in the human gut on enterocytes and in Peyer’s patches [16]. The major human receptor for rhinoviruses is the ICAM-1 (CD54), which is expressed on respiratory epithelial cells [17]. CVB3 binds to two cellular receptors, the glycoproteins CAR and DAF, following which CVB3 undergoes an altered particle formation that lacks viral RNA [18,19], an intermediate step in the entry and uncoating process. The particles are capable of binding to liposomes and forming membrane channels [18]. The pore structure is thought to facilitate externalization of the RNA from the capsid and entry into the cytoplasm. All six CVB serotypes bind CAR [20,21] and the extracellular domain of the molecule is adequate to promote virus entry [21]. The DAF receptor is more widely distributed in tissues and cells than CAR and it protects cells against complement-mediated lysis [22]. Unlike CAR, DAF is present in nonadherent cells such as leukocytes, and may be responsible for the infection and replication of CVB3 in B cells, dendritic cells and activated T cells [23,24].

The expression of the viral genome by each member of the *Picornaviridae* family transpires cap independently, through the 5′-UTR internal ribosome entry site (IRES), which directs translation of a single open reading frame that encodes a polyprotein approximately 250 kDa in size [25]. The global organization of viral proteins in the polyprotein is highly conserved (VP4-VP2-VP3-VP1-2A-2B-2C-3A-3B-3C-3D). Upon cleavage by viral proteases, the polyprotein yields 11 mature proteins through various independently functioning intermediates [1]. For example, in CVB3 the single open reading frame is divided into three regions within a polyprotein of 2185 amino acids encoding for 11 structural and nonstructural proteins. The 3′ end contains another nontranslated region (NTR) region of 100 nucleotides in length. Replication of the genome is facilitated by the viral RNA-dependent RNA polymerase 3D, which generates a negative sense RNA that acts as a template for synthesis of viral genomic RNA. The viral genome is polyadenylated at the 3′

end and acts as mRNA for synthesis of the monocistronic polyprotein. This is processed by two viral proteases 2A and 3C, into four structural proteins (VP1–4), which form the capsid and seven nonstructural proteins, which are involved in viral replication and protein processing. Replication occurs in the cytoplasm in association with intracellular membranes, and the release of progeny virions for poliovirus, coxsackievirus and rhinoviruses occurs via cell lysis. However, the mechanism of hepatitis A virion release is not yet known [26].

The innate immune responses to picornaviruses

The innate immune response provides rapid protection during the early stages of viral infection, before there is complete activation of the adaptive immune response. This is achieved through recognition of pathogen-associated molecular patterns, which are conserved structural motifs specific for microbial pathogens and are generally not expressed by host cells [27,28]. The pathogen-associated molecular patterns are recognized by nonpolymorphic pattern recognition receptors (PRR) of the innate immune system that rapidly signal for the initiation of the antiviral response. The RIG-I-like receptors (RLR) and the Toll-like receptors (TLR) are the two principal groups of PRRs in picornaviruses recognition [29]. The innate signaling via TLR was recently reviewed by Kembell *et al.*, and therefore is not discussed herein [30]. The major cytoplasmic recognition receptors of viral RNA are RIG-I and MDA5 [31], both of which are cytosolic, but were also shown to associate with stress granules [32,33]. These viral RNA receptors activate IKK ϵ (inhibitor of NF- κ B kinase ϵ) and TBK1 [33,34] via the adaptor protein MAVS, also known as IPS-1, CARDIF or VISA [35–37]. The MAVS complex formation leads to the phosphorylation of IRF3, resulting in IRF3 dimerization and translocation into the nucleus [38], where it induces type I IFN transcription (Figure 1). Secreted type I IFN binds to the type I IFN receptor of neighboring cells and induces expression of a variety of IFN-stimulated genes, thereby amplifying its own signal transduction. The regulation of RLR signaling involves crosstalk with the NOD-like receptor pathway and caspase-8 [39].

Although type I IFN can be induced in nearly all cell types [40], the immune response will fluctuate according to the cell type infected. Interestingly, the viral interference with innate response was also shown to be dependent on the different cell types infected [41]. For example, poliovirus replication, which is in some measure resistant to type I IFN, occurs only in the CNS of receptor-transgenic mice; however, in mice that lack the receptor for type I IFN, replication will also occur in the pancreas, liver and spleen [42]. Since poliovirus resistance was shown to correlate with the amount of virus infecting each cell [43], this represents a possibility that there are cell-specific antiviral consequences of IFN, which by an unknown mechanism, cannot be inhibited by poliovirus and/or other picornaviruses. Further differences in the RLR innate immune cell response to picornaviruses infection are contingent on the specific RIG-I-like helicases being expressed in particular cells. Although RIG-I and MDA5 were shown to be expressed in most of the cells [44], the latest findings indicate that there is considerable alteration in RIG-I and MDA5 expression between diverse subsets of dendritic cells [45]. How the lack of RIG-I-like helicases in CD8 α^+ dendritic cells [45] would affect picornaviruses needs to be elucidated.

Of particular interest is how the RLR pathway and apoptosis are controlled by the apoptotic protein caspase-8 and its regulatory protein FLIP during picornavirus infection. Caspase-8 and -10 in humans, are cysteine proteases that initiate apoptotic cell death in response to cell-surface death receptor activation such as Fas (CD95) [46]. However, increasing data now implicate caspase-8 and -10 in nonapoptotic functions [47,48]. Today, caspase-8 has been implicated in the regulation of most PRR signaling pathways. Among the nonapoptotic functions of caspase-8 are the regulation of innate and adaptive immunity signaling pathways and the inhibition of an inflammatory form of cell death termed necroptosis [48].

FLIP was originally discovered as a protein in certain viruses (v-FLIP), and the mammalian cellular homolog (c-FLIP) was identified shortly after [49,50]. Multiple c-FLIP splice variants have been described, with the two main forms being c-FLIP short form (c-FLIP_S, 26 kDa) and long form (c-FLIP_L, ~55 kDa). Both splice variants have death effector domains by which they bind Fas-associated death domain (FADD) at the Fas-induced death-inducing signaling complex and inhibit caspase-8 activation. Interestingly, reduction in c-FLIP expression has been demonstrated to effectively reduce the ventricular myocardial function of patients with end-stage heart failure, as well as in rodents after myocardial infarction or picornavirus infection [51,52].

Picornaviruses have evolved exquisite mechanisms to target RLR pathways to promote enhanced replication and spread within the host. Understanding the pivotal role of RLRs in immune regulation and signaling crosstalk in antiviral immunity can provide new insights into therapeutic strategies for the control of virus infection. The resulting immune response must be transient and tightly regulated to prevent uncontrolled responses that may have deleterious effects on the host, such as promoting allergy, cell death or autoinflammation [53].

Interaction of picornaviruses with MDA5

The current view of cytoplasmic RNA-mediated innate immune signaling involves the differential activation of the cytoplasmic RNA helicases RIG-I, MDA5 and LGP2, which form the RLR family. RIG-I and MDA5 consist of a conserved ATP helicase core, which is connected at the N-terminus to two caspase activation and recruitment domains (CARD), and at the C-terminus to a Zn²⁺-containing regulatory domain, which also contains a viral RNA binding site [54]. Upon binding and activation by viral uncapped dsRNA or triphosphorylated RNA, RIG-I and MDA5 are recruited through the CARD domains to the MAVS located on the outer membrane of the mitochondria (Figure 1) [35–37,55].

While a wide variety of RNA viruses, including rhabdoviruses, paramyxoviruses, influenza and flaviviruses are recognized by RIG-I [32,56–58], picornaviruses are believed to be recognized predominantly by MDA5 [31,59–61]. The explicit ligand for MDA5 is poorly defined, and is thought to be long dsRNA (>1–2 kb in length) [31,62]. The specific sequence/secondary structure of picornaviral RNA for MDA5 recognition is not known. Recently, it was reported that 2'-O-methylation of mRNA protects viral RNA from recognition by MDA5 and thus prevents MDA5-dependent production of type I IFN in virus-infected cells [63,64]. Furthermore, it has been shown that the distinction between 2'-O-methylated mRNA and nonmethylated mRNA is not only responsible for the MDA5-dependent induction of type I IFN, but also responsible for IFN-induced proteins with tetra-ricopeptide repeats (IFIT), which are IFN-stimulated genes implicated in the regulation of protein translation [63]. Members of the picornavirus family do not encode the methyltransferases necessary for this chemical modification, but have evolved alternative 5' ends of their RNA. As mentioned earlier, picornaviruses covalently attach VPg proteins to the 5' terminus of their genomic RNA [65]. Although the replication of encephalomyocarditis virus was shown not to be restricted by IFIT proteins [63], infection with this virus (along with other picornaviruses) is sensed through the MDA5 pathway [59]. This suggests that VPg at the 5' end of picornavirus RNA does not prevent MDA5 mediated recognition. It was proposed by Thiel's group that encephalomyocarditis virus evades host restriction via IFIT proteins through the use of IRES at the 5' end of the viral RNA [64]; however, the mechanism of this interference is not known.

The mechanism of cap-independent translation has been studied extensively, above all in poliovirus, which now, therefore, serves as a standard for the entire family of enteroviruses

[66,67]. The recognition of the specific features of the 5' NTR by cellular translational factors, ribosomes, and other cellular proteins leads to assembly of an initiation complex directly downstream of the start codon [68]. The key for efficient viral replication and virulence is the structural integrity of the 5' NTR. Mutations in the 5' NTR of CVB3 and other picornaviruses were shown to significantly decrease the viral reproduction [12], vary cell tropism [69] and diminish virulence [70]. For example, modifications in domain II of the 5' NTR of CVB3 led to the identification of a cardiovirulence determinant that reduces replication efficiency in cardiomyocytes [71]. It would be interesting to determine whether 5' terminal deletions, especially those that occur naturally in the human heart after CVB2 infection [72], manifest different affinity for MDA5 in comparison to wild type viral RNA. Furthermore, the impact on MDA5 recognition of the long homopolymeric poly(C) tracts within the 5' UTR from cardioviruses, such as the Mengo and encephalomyocarditis viruses, warrants elucidation. It was shown that deletion or shortening of these tracts negatively influences virulence, but does not impact the engagement in translation [73]. Whether the short-tract strains are less effective at inducing an important protective element within the host's defense system, or whether they are defective in some critical step within a specific tissue, remains to be determined. Numerous differences in the sequence of domain II of viral RNA between noncardiovirulent strains and cardiovirulent strains suggests that the pathogenicity of the coxsackieviruses depends upon the structure of this domain [11]. Whether or not the RNA structure in the 5' NTR holds key elements to control innate immune responses mitigated via RIG-I/MDA5 interactions is an important question to be investigated.

Experiments performed in mice deficient in the gene encoding MDA5 were shown to be more vulnerable to infection with encephalomyocarditis virus and to secrete less type I IFN [31,59]. The role of MDA5 in innate response to CVB3 infection remains controversial. The two groups that described the role of the MDA5 in CVB3 infection came to opposite conclusions about the increase of type I IFN secretion and the level of CVB3 replication, indicating that although it is clear that MDA5 plays an important role in regulating CVB3 replication, the outcome is transient and might not be reconciled by type I IFN [60,61].

The fact that MDA5 is cleaved during poliovirus infection supports the role of this protein during picornavirus infection [74]. Cleavage of MDA5 was shown to not be dependent on the virus, but rather to be induced by cellular proteases, as it was shown to be blocked by general caspase and proteasome inhibitors, but not by point mutations in 2Apro and 3Cpro [74]. Since MDA5 was shown to be cleaved during the induction of apoptosis [75], it was proposed that poliovirus-induced apoptosis is responsible for the cleavage of MDA5 [74]. Cleavage of MDA5 during poliovirus infection could be a mechanism to evade the innate immune response, by attenuating the production of IFN or increasing virus replication; however, the data published show neither an increase in IFN nor suppression of viral replication in cells that were exposed to general protease inhibitors [74], indicating that picornavirus-induced cleavage of MDA5 has yet to be identified.

Early studies on MDA5 have shown that overexpression of the murine MDA5 revealed its ability to increase the rate of cell apoptosis through a caspase-8 cleavage site between the CARD and helicase domains. Upon induction of apoptosis by FasL, cleavage of the full-length cytoplasmic MDA5 allows the helicase domain to translocate into the nucleus and accelerate DNA fragmentation [75]. Although the exact mode of action of cleaved MDA5 is unknown, it was speculated that the helicase domain of MDA5, once freed from the CARD domains, acts on chromatin architecture, allowing easier access of DNase CAD to DNA and thereby accelerating the degradation of the genomic DNA [75]. Therefore, it seems plausible to suggest that the MDA5 cleavage and consequent induction of apoptosis during picornavirus infection could be a protective mechanism, limiting viral replication and spread

of the virus. The lack of involvement of the type I IFN system in the induction of apoptosis by MDA5 during poliovirus infection is surprising, especially given that the transcription factor IRF3, which has a major role in the type I IFN pathway, was reported to be involved in apoptosis induction by encephalomyocarditis virus [76].

The role of FLIP in regulating the RLR pathway is particularly interesting, because MDA5 was shown to recognize long dsRNA that is also made by DNA viruses [77], some of which have usurped the short form of c-FLIP, which is responsible for blocking caspase-8 activity [78]. It would be interesting to find out when and where the caspase-8-dependent cleavage of MDA5 occurs and if this cleavage is dependent on the interaction of MDA5 with MAVS. Our preliminary data show that transgenic mice expressing the short form of FLIP not only suppress the cleavage of MDA5 and apoptosis during CVB3 infection, but also reduce type I IFN production and enhance viral replication [Buskiewicz IA. Cellular FLIP-short increases myocarditis susceptibility to coxsackievirus B3 (2012), Submitted]. Previous studies in c-FLIP_L transgenic mice had revealed the opposite phenotype, so clearly c-FLIP_S and c-FLIP_L behave very differently during CVB3 infection. This may explain why certain DNA viruses consistently express only the short form of c-FLIP.

Interaction of picornaviruses with RIG-I

Mice deficient in the gene encoding RIG-I were shown to be resistant to infection with encephalomyocarditis virus and showed no alteration in IFN production [31]. This coincides with the fact that RIG-I was shown to be stimulated by RNA containing 5'-triphosphate, and picornaviruses carry a modified protein VPg that acts as the primer for RNA replication and also allows the virus to evade detection by RIG-I. Evolution studies indicate that both RIG-I and MDA5 helicases evolved independently from each other, and the exceptional domain arrangement in both proteins is not a consequence of gene duplication, but is rather due to the distinct sensitivity of RIG-I and MDA5 to viral infection [79]. In contrast to MDA5, RIG-I orthologs are not found in fish, suggesting that MDA5 evolved before RIG-I [79].

Strikingly, RIG-I undergoes proteolytic cleavage by the viral proteinase 3Cpro in cells infected with poliovirus, rhinoviruses, echovirus and encephalomyocarditis virus, indicating that RIG-I could play an important role in picornavirus infection. Furthermore, recent studies revealed that some viruses, such as West Nile virus and dengue virus, as well as vaccine strains of measles virus, are redundantly sensed by both RIG-I and MDA5 [80–82], indicating that picornaviruses could be also recognized by both helicases. Our preliminary studies in search of a coxsackievirus RNA motif interacting with RIG-I and MDA5 indicate that RIG-I is able to recognize coxsackievirus RNA, and although the recognition is fast as analyzed by stopped-flow kinetic analysis, the RIG-I protein stays in the complex with coxsackievirus RNA for a much shorter time than with 5'-ppp-RNA [Buskiewicz IA, Unpublished Data]. The fact that RIG-I could contribute to picornavirus sensing indicates that picornaviruses encode RNA sections positioned throughout the genome and antigenome that are efficient at stimulating RIG-I. It is known that besides the 5'-triphosphate RNA, the RIG-I helicase recognizes short, approximately 1 kb sections of dsRNA [32,33]. Poly(U) and respective poly(A) of greater than 50 nucleotides in length was shown to be a major determinant that confers RIG-I binding and signaling for the genome of HCV [83]. Analogous sequences also appear in the genomes of picornaviruses, and dsRNA intermediates of replication of poly(A)–poly(U), which could serve as substrates for RIG-I, were shown in poliovirus-infected cells [84,85]. Furthermore, although picornavirus genomes are obscured by VPg, it is possible that RIG-I is sensing picornaviral RNA when VPg is not attached. It was recently shown that once the picornavirus enters the cell, VPg is removed by an 'unlinkase' protein before translation starts, leaving the 5' end as monophosphate [86–88]. The unlinked, single-stranded picornaviral RNA with an

unobstructed 5' end may then be directly recognized by RIG-I. However, since it was shown that RIG-I can recognize dsRNA with one strand monophosphorylated [89], it is more likely that the real substrate for RIG-I is the 'unlinked' picornavirus RNA bound to a negative-sense strand. Owing to the fact that the levels of negative-sense RNA are very low in the cell, it is also possible that RIG-I is sensing a more abundant RNA structure, such as the IRES site. These highly structured RNA domains contain many double-stranded regions that may be ligands for RIG-I. It would be interesting to know when and how picornaviral RNA interacts with RIG-I, and if such an interaction colocalizes to replication complexes of picornaviruses. It might be possible that RIG-I recognizes picornaviruses at early stages while they are still associated with actin [90]. Deletion analysis has shown that the association of RIG-I with the cytoskeleton was dependent on CARD domains. The actin cytoskeleton is commonly modulated by invading viruses to facilitate cellular uptake and/or trafficking. Because this modulation often occurs very early in the virus life cycle, the role of RIG-I in picornavirus recognition could be transient. The main issue is whether this transient interaction brings RIG-I to interact with MAVS. It seems plausible that RIG-I may be as important to picornavirus infection as MDA5.

Role of MAVS in innate-induced responses to picornaviruses

Recognition of picornavirus RNA during infection by the RIG-I-like helicases involves an interaction with the mitochondrial antiviral signaling protein MAVS, which eventually leads to the initiation of antiviral and inflammatory responses mediated by type I IFN and NF- κ B pathways [35–37,55]. MAVS colocalizes with the outer membrane of mitochondria [35], peroxisomes [91], autophagosomes [92,93] and the endoplasmic reticulum (ER) [94], where the sequestration of a multiprotein complex facilitates the management of downstream signaling (Figure 1). MAVS is a 540-amino acid protein, which contains three discrete functional domains: a N-terminal CARD domain; a central proline-rich domain and a C-terminal transmembrane domain. The CARD domain of MAVS encloses polar surfaces at opposite ends, which are postulated to facilitate homotypic CARD–CARD interactions between MAVS and RIG-I helicases [95]. Although there are many other CARD domain-containing proteins, no interaction of MAVS CARD domains with proteins other than RIG-I helicases were reported to date [95]. The proline-rich domain contains multiple well-conserved motifs for TRAF family proteins [96], which were shown to positively and negatively regulate IFN antiviral responses [97]. The C-terminal transmembrane domain of MAVS is anchored to the mitochondrial outer membrane and has significant structural resemblance to other membrane proteins located at the mitochondria, such as Bcl-2 or TOM20 [35]. The deletion of the C-terminal domain depletes MAVS from mitochondria into the cytosol, which in turn ablates MAVS signaling to NF- κ B and IFN- β [35,55]. The importance of the mitochondrial localization of MAVS is further emphasized by the fact that HCV employs the viral protease NS3/4A to cleave MAVS off the mitochondrial membrane, thereby suppressing type I IFN [36,98]. Similar to the CARD domains of MAVS, the transmembrane domains were also shown to homodimerize [99,100], suggesting that downstream signaling is conditional on MAVS self-association. It was recently reported that MAVS forms functional prion-like aggregates after viral infection, and that these aggregates are required for the activation of the transcription factor IRF3 and subsequent production of type I IFN [101]. Intriguingly, both the transmembrane domains and the CARD domains of MAVS were shown to be indispensable for the formation of protease-resistant prion-like fibrils, which convert MAVS into functional aggregates [101]. MAVS was also shown to interact with mitofusin proteins [102–104] involved in the regulation of mitochondrial dynamics, which not only were shown to control mitochondrial web arrangement and metabolism in the cell, but also to regulate RLR signaling. MAVS interaction with mitofusin protein 1, for example, was shown to be essential for the recruitment of RIG-I and MAVS-enriched mitochondria to the environs of viral replication [104].

MAVS has also been shown to contribute to the coordination of metabolism [105] and apoptotic functions during viral infection [106,107]. During viral infection there is an increased need for energy, therefore, the fact that MAVS is localized to the mitochondria would suggest that this molecule, together with other regulatory molecules, could influence the metabolic output of the cell during viral infection, and, therefore, enhance the innate immune response. On the other hand, innate response-induced apoptosis could play a protective role for the host cell during viral infection by preventing the virus from completing its replication and producing infectious progeny viruses [108]. It is known that type I IFN induction stimulates apoptotic responses, since more than 15 IFN-stimulated genes that are induced during viral infection have proapoptotic functions [109]. However, the molecular mediators that activate host cell apoptosis during viral infection are not as well known. At the time of the initial discovery of MAVS, the authors speculated that MAVS might have a role in apoptosis induction [35]. The elegant study by Lei *et al.* showed that during viral infection MAVS is responsible for apoptosis induction, and that this function is independent of its function in initiating type I IFN production [110]. MAVS-induced cell death requires mitochondrial localization, is caspase dependent, and is inhibited by HCV NS3/4A protein [110]. These observations were confirmed with experiments showing that overexpression of MAVS induces apoptosis by activation of caspase-3, -8 and -9 [111,112]; however, the exact mechanism of how MAVS activates these caspases is not known.

Picornaviruses require intact cells for viral progeny release [113]. Therefore, they possess effective mechanisms to regulate host cell death in order to avoid premature termination of their own replication by subverting the apoptotic machinery of their hosts with the aim of increasing viral production and dissemination [114]. Picornaviruses have developed highly specialized strategies to target the MAVS adaptor molecule as a powerful means to eliminate not only the antiviral signaling but also apoptosis. The polioviral 2Apro and 3Cpro were shown to promote degradation of MAVS in HeLa cells [115] and mediate poliovirus induced apoptosis [116,117]. Furthermore, the viroporin 2B of poliovirus, which is coupled with the viral replication complex and virus-engendered membranes was shown to localize not only with ER and Golgi compartments [118,119], but also to interact with mitochondria and induce its perinuclear reorganization, which further leads to caspase-dependent cell death [120]. The exceptional ability and speed with which poliovirus induces apoptosis and suppresses MAVS is paralleled by its exceptionally quick replication and complete virion formation [121,122]. Similarly to poliovirus, infection of HeLa cells with the enteroviruses echovirus 7 and enterovirus 71 was shown to suppress the expression of MAVS [90]. HAV on another hand disrupts RLR host signaling by affecting the MAVS protein through a mechanism that parallels that of the serine NS3/4A protease of HCV, but differs in its use of a stable, catalytically active 3ABC polyprotein processing intermediate [123]. MAVS cleavage by HAV with its own 3Cpro cysteine protease occurs after the 3ABC precursor is stably targeted to the mitochondria and interestingly the cleavage of MAVS requires both the protease activity of 3Cpro and a transmembrane domain in 3A. Lack of the 3A domain that directs 3ABC to mitochondria diminishes the ability of mature 3Cpro to cleave MAVS [123]. 2A as well as 3C mediate virus-induced apoptosis, which interestingly does not suppress viral replication and rather promotes viral progeny release [124]. The 2B protein of HAV was also shown to interfere with MAVS and RLR signaling by affecting the downstream kinases IKK ϵ and TBK1, although the mechanism of this interference is not known [125]. It is assumed that the effects of 2B on MAVS and the kinases indirectly result from interactions of 2B with cellular membrane structures, which is an interesting hypothesis since MAVS is known to interact with membranes including those of mitochondria. Similar to HAV, human rhinovirus 1a 2Apro and 3Cpro proteases lead to the degradation of MAVS in cells [115]. Interestingly, caspases support the 2A 3C-mediated inhibition of MAVS signaling by further cleavage of MAVS [115]. Moreover, rhinovirus's

protein 2B was shown to suppress secretion of cytokines, including IFN, by localizing to the Golgi membranes, resulting in inhibition of the secretory pathway [126]. Similar to HAV and rhinovirus, CVB3 3Cpro cleaves MAVS to escape host immunity, but it does not need to be localized to the mitochondrial membrane via a transmembrane domain within the 3A viral protein [90]. Rather, CVB3 3A is known to localize to the ER membrane, where it disrupts ER–Golgi vesicular trafficking [127,128]. 3Cpro was previously shown to cleave glutamine–glycine (Q–G) bonds and exhibit proteolytic activity for glutamine–alanine (Q–A) bonds in both the viral polyprotein and cellular targets [129]. During CVB3 infection, 3Cpro cleaves MAVS at residue Q148 within the proline-rich region, which mediates its interaction with several downstream signaling molecules to induce type I IFN secretion [37,55,96]. Interestingly, cleavage of MAVS by 3Cpro, which has nothing to do with the anchoring of the protein to the mitochondrial membrane, nonetheless causes delocalization of MAVS to the cytoplasm [90]. The exact mechanism of the cleaved MAVS release from mitochondria is not known. However, it is possible that 3Cpro action is orchestrated with the function of CVB3's B2 protein. The B2 protein of CVB3 was shown to manipulate intracellular Ca^{2+} homeostasis and influence mitochondrial potential, which could lead to the release of MAVS from mitochondria [130,131]. It is intriguing that CVB3 3Cpro cleaves MAVS in the proline-rich region, as this part of the protein was initially shown to be dispensable for type I IFN signaling, and is instead responsible for sequestering a protein complex that regulates the RIG-I pathway and NF- κ B, as was observed for Toll/IL-1 receptor domain-containing adaptor inducing IFN- β [90]. The proline-rich region sequesters proteins such as RIP1 and caspase-8, responsible for necroptosis, apoptosis and innate response regulation. Interestingly, our ultra-high resolution, spatial analysis of caspase-8 following CVB3 infection shows that active caspase-8 colocalizes with mitochondria, and that the colocalization is MAVS dependent. The biochemical analysis of caspase-8 interaction with MAVS at the mitochondria during CVB3 infection indicates that caspase-8 activity with MAVS is in fact of a nonapoptotic nature (Figure 2). Unquestionably, it will be interesting to find out if proteins, that modulate the RIP1 caspase-8 function interfere with picornavirus evasion strategies at MAVS.

Picornaviruses, especially enteroviruses, are known to induce autoimmune responses. Altered expression or viral ablation of MAVS could potentially not only inhibit antiviral cells signaling, but also correspondingly stimulate cell into autoimmune disease. It is interesting that a recently identified uncommon allelic variant of the *MAVS* gene not only affects the RLR signaling upon viral infection in humans, but also plays role in a development of systemic lupus erythematosus [132]. Screening of nonsynonymous single nucleotide polymorphisms in the *MAVS* gene led to identification of one critical loss-of-function variant in the *MAVS* C79F allele, which was related to low type I IFN secretion and absence of anti-RNA-binding protein autoantibodies [132]. The identified C79F single nucleotide polymorphism was shown to distinctly reduce the extent of MAVS-dependent innate immune system stimulation in cells infected with Sendai or influenza A viruses [132]. The loss-of-function C79F mutation of *MAVS* was shown not to be associated with MAVS proteolysis or dissociation from mitochondria, but rather with impaired binding of the regulatory TRAF3 protein [132], which interestingly localizes to the aforementioned proline-rich region attacked specifically by picornavirus 3Cpro.

Role of caspase-8 & FLIP in RLR signaling

RIG-I pathway-mediated type I IFN secretion is tightly controlled. The first regulatory mechanism described in the pathway was the identification that the RIG-I helicases themselves contain regulatory repressor domains [133]. Even though MAVS has been shown to interact with RIG-I in overexpression experiments by several groups, it has not been clearly demonstrated that endogenous MAVS and RIG-I or MDA5 can interact in a

virus-dependent manner. The mechanisms by which MAVS is regulated by RIG-I and how MAVS signals to downstream kinases is still quite elusive. The induction of IRF3/7 and NF- κ B is mediated and regulated via the recruitment of a heterotrimeric complex of proteins: TRADD, RIP1 and FADD (Figure 1). This complex, named the TRADDosome, is located at the intermediary region of MAVS at the mitochondrial outer membrane [134]. MAVS also directly recruits various TRAF proteins, which are also required for RIP1 ubiquitination [55,96,135]. Upon RIP1 ubiquitination the MAVS-bound TRADDosome induces the recruitment and activation of kinases belonging to the IKK family, leading to NF- κ B and IRF activation [34,136]. How the signaling mechanism of MAVS is regulated is still a subject of investigation. Although it was shown that overexpression of truncated MAVS containing only the CARD and transmembrane domain is sufficient to activate IRF3 and induce type I IFN in cells [35], recent work from the same group surprisingly shows that recombinant MAVS lacking the transmembrane domain, but containing the CARD and proline-rich region, can activate IRF3, when incubated with cytosolic extracts [101]. Even though it has been shown that MAVS can interact with TRAF6 [35,55], TRAF6-deficient cells have normal induction of type I IFN following viral infection [137]. Moreover, although MAVS was shown to interact with RIP1 and FADD, RIP1-deficient MEF cells are fully capable of inducing type I IFN following viral challenge [138]. RIP1 function on MAVS was recently shown to be regulated by caspase-8 [139], and was observed to directly bind to RIG-I and cotranslocate to the mitochondrial RIG-I complex during viral infection [140]. Interestingly, our initial data (Figure 2) show that caspase-8 also translocates to mitochondria during CVB3 infection.

Recent studies examining the impact of caspase-8 on the functions of various signaling pathways that control inflammation revealed that in several cell types, caspase-8 regulates the activation of the transcription factors NF- κ B and IRF3 by intracellular foreign RNA and DNA. The first work linking caspase-8 to innate immunity showed that caspase-8 is cleaved during dsRNA stimulation, and overexpression of a cleaved form of caspase-8 activated NF- κ B [139]. Furthermore, a knockdown of caspase-8 or -10 in human cell lines or cells derived from mice deficient in caspase-8, were shown to result in reduced inflammatory cytokine production [139]. Caspase-8 were also shown to restrict RIG-I signaling by mediating RIP1 cleavage [140]. Furthermore, caspase-8 was shown to be activated by cytosolic RIG-I-dependent signaling, and to catalyze an essential intermediate step in the ubiquitination and proteasome-mediated degradation of IRF3 [141]. Although the molecular events for this regulation remain poorly understood, caspase-8 was also indicated to play a role in TLR signaling by influencing IL-1 β processing, downstream of TLR3 and TLR4 [142], and by promoting responses to TLR4 stimulation, causing enhanced nuclear translocation of NF- κ B [143].

In addition to its role in the regulation of innate immunity via the RIG-I and TLR pathways, caspase-8 is now also considered as a key regulator of necroptosis. Caspase-8 inhibits the initiation of necroptosis by cleaving and inactivating RIP1, RIP3 [144] and deubiquitinase CYLD, which was shown to be required for necroptosis induction [145]. Induction of necroptosis due to caspase-8 deficiency leads to enhanced skin and bowel inflammation, linking the process to innate immunity [146,147]. Interestingly, infection with coxsackievirus also induces necrotic-like cell death characterized by cell swelling followed by loss of membrane integrity [148]. The role of caspase-8 in picornavirus-induced necroptosis was not well studied. The virus-triggered necrosis is considered to be a passive, uncontrolled form of cellular destruction, which in contrast to apoptotic cells, usually triggers inflammatory reactions [149].

Studies of caspase-8 activation in both cell death and growth have identified that the caspase-8 paralog, FLIP, regulates caspase-8 activity [78], suggesting that caspase-8 and

FLIP could emerge as a crucial complex in the control of the innate immune response at the mitochondria in the RIG-I-regulated pathway, as well as virus- or host-induced apoptosis, necrosis or autophagy. c-FLIP is capable of heterodimerizing with and stabilizing partial activation of caspase-8 for nondeath signaling. Two isoforms of FLIP, c-FLIP_L and c-FLIP_S are detected [150]. Although both forms can bind to caspase-8, only c-FLIP_L contains an activation loop for caspase-8. Activated caspase-8 can cleave c-FLIP_L at Asp376 to form p43FLIP, and at Asp196 to form p22FLIP. p43 FLIP was shown to bind to RIP1 and TRAF1/2 and promotes activation of the NF- κ B pathway [151,152]. p22 FLIP was shown to bind effectively to the IKK complex regulating NF- κ B [153]. Of particular interest is that several DNA viruses have acquired FLIP in their genome, but it is always the short form of FLIP rather than full-length FLIP [154]. Other studies have demonstrated a direct interaction between v-FLIP produced by Kaposi's sarcoma herpes virus and the IKK complex, which renders the NF- κ B pathway constitutively active and has been linked to Kaposi's sarcoma and other malignancies [155,156]. v-FLIP from human molluscipoxvirus activates NF κ B when it is expressed at low levels by enhancing the interaction between RIP1 and TRADD [157]. When expressed at higher levels, it inhibits both NF- κ B activation and RNA-mediated IRF signaling [158].

There are currently no reports indicating a role of c-FLIP in innate responses to viral infections. One study performed using polyinosinic:polycytidylic acid (poly[I:C]), a synthetic analog of dsRNA, showed that FLIP-deficient mouse embryo fibroblasts were more susceptible to killing by cytoplasmic poly(I:C) and had noticeably increased expression of NF- κ B- and IRF3-dependent genes [159]. On the other hand, Huber *et al.* found that T-cell specific overexpression of c-FLIP_L diminished the severity of CVB3-induced myocarditis following increased type I IFN secretion and decreased surface expression of T-cell receptor and CD8 [152,160]. Furthermore, a clear reduction in the expression of c-FLIP was shown to play a vital role in cardiomyocyte survival after stress [161], and c-FLIP-deficient mice are embryonic lethal because of a cardiac malformation [162]. This highlights that c-FLIP is likely to be important for cell processes other than just protection from Fas-induced death. Remarkably, cellular and viral FLIP short forms were shown to suppress autophagy by affecting the elongation and closure stages of autophagosomal formation [163]. Enteroviruses are known to interact with the autophagy pathway [164], and stimulation of autophagy during enterovirus infection was shown to modestly increase virus yield [165]. However, the autophagosomes seem mostly immobilized [166]. Kembell *et al.* has proposed that the immobilized autophagosomes cannot fuse with endolysosomes allowing enteroviruses to minimize the exposure of their viral RNA to intravesicular TLRs, thereby delaying activation of the intracellular innate response [30]. Intriguingly, ripoptosome formation, which leads to necroptosis as discussed above, is also regulated by different forms of c-FLIP; c-FLIP_L prevents, whereas c-FLIP_S promotes ripoptosome assembly [167]. This could indicate that different forms of FLIP, in coordination with caspase-8, could be important regulatory factors of innate immunity.

In summary, although innate responses of RIG-I, TLR, autophagy, apoptosis and necroptosis use fundamentally different molecular mechanisms to execute protective functions, they all seem to be highly interconnected and regulated by caspase-8 and the regulatory protein FLIP. It will be essential to study in detail the function of caspase-8 and FLIP during picornavirus infection.

Future perspective

Picornaviruses have developed highly specialized strategies to target the cytosolic innate immune response regulated by the RIG-I/MDA5 pathway. Although much has been learned about how picornaviruses ablate this pathway, further work is required to understand the

countermeasures used by the infected cell to overcome virus-induced suppression of innate signal pathways in the continuing war between the host and the microbial agent. Host regulatory proteins, such as caspase-8 and FLIP, have a significant role in cellular defense and may be a prime target for future therapeutic strategies aimed at re-establishing and amplifying the innate immune response during picornavirus infection.

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■ of interest

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Executive summary

The *Picornaviridae* family

- Describes the family of picornaviruses and their genome, cellular receptors, replication and infectious cycle.

The innate immune responses to picornaviruses

- Defines different branches of innate immune responses responsible for picornaviruses recognition, and in detail describes the major features of the RIG-I/MDA5 signaling pathway.

Interaction of picornaviruses with MDA5

- Describes in detail structural features of picornaviral RNA and interaction with MDA5 helicase.
- Discusses how picornaviruses ablate induction of type I IFN, while targeting the MDA5 helicase.
- Debates how type I IFN and apoptosis are connected via MDA5 signaling during infection with picornaviruses.

Interaction of picornaviruses with RIG-I

- Although RIG-I was shown to play rather limited role during infection with picornaviruses, this paragraph pinpoints the fact that RIG-I is specifically targeted by the proteases of almost all known picornaviruses.
- Examines the possible sequence and structural features of picornaviral RNA, that could mitigate RIG-I recognition.
- Discusses the possible role of RIG-I association with the cytoskeleton during infection with picornaviruses.

Role of MAVS in innate-induced responses to picornaviruses

- Characterizes the specific role of MAVS and its mitochondrial versus peroxisomal localization. Furthermore, special emphasis is given to MAVS cleavage by picornaviral proteases and its role in induction of apoptosis.

Role of caspase-8 & FLIP in RIG-I-like receptors signaling

- Characterizes specific roles of caspase-8 and FLIP during viral infections. Furthermore, newly discovered aspects of RIP1 kinase cleavage during viral infection and the possible role of the ripoptosome and its regulation by caspase-8 and FLIP during picornavirus infection are discussed.

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Figure 1. Graphic representation of the RIG-I signaling pathway during infection with picornaviruses

RIG-I and MDA5 helicases are both represented in open conformation upon recognition of intracellular viral RNA, and MDA5 is shown to be interacting with the adaptor molecule MAVS at the mitochondrial outer membrane. The initiation of downstream signaling occurs via the recruitment of the regulatory proteins TRAF2 and 6, TRADD, FADD, RIP1 and caspase-8 to MAVS. This leads to the formation of the IKK complex, which in turn is responsible for the translocation of active NF-κB dimers into the nucleus and the subsequent production and secretion of proinflammatory cytokines. The branch of downstream signaling causing the activation and nuclear translocation of IRF3 and IRF7, which promote the induction of type I IFN, is thought to be regulated by TRAF3 protein binding to MAVS. Interference of this pathway by picornaviruses is highlighted, with the name of the viral protease and virus involved in the red outlined rectangles. Virus-induced cleavage of proteins of interest is highlighted with a scissors icon (red: viral proteases; orange: host cell caspases). The scheme also depicts the possible regulatory roles of cellular FLIP isoforms on intersections of innate immune responses to picornavirus infection. We have adopted from Kembell *et al.* the hypothesis that during picornavirus infection mitochondria act as a bridge between the RIG-I pathway, the intravesicular TLR receptors and autophagy [30]. TLR3 is known to be a major player in innate responses to picornavirus infection, and has the ability to interact with TRIF, which in turn is known to recruit the ripoptosome – a cell-death inducing complex of RIP1, FADD, caspase-8 and cellular FLIP isoforms [167].

Although activation of the ripoptosome during any viral infection at TLR3 or any other organelle has yet to be shown, it is possible that the ripoptosome is connecting the innate responses with apoptosis and necroptosis [167,168]. Both caspase-8 and c-FLIP were shown to specifically regulate the formation and downstream signaling of the ripoptosome [167]. The caspase-8–c-FLIP_L complex was shown to have enough catalytic activity to cleave the RIP1 kinase, thereby destabilizing the ripoptosome and suppressing necroptosis. By contrast, c-FLIP_S, which lacks the caspase-8 activation domain, was shown to suppress the cleavage of RIP1, leading to its accumulation and the further induction of necroptosis. In the absence of any cellular FLIP, procaspase-8 homodimers within the ripoptosome could undergo activation by autoproteolysis and consequently induce apoptosis. Interestingly, c-FLIP_S was also shown to downregulate the autophagosome formation [163].

C-FLIP_L: Cellular FLIP homolog long form; C-FLIP_S: Cellular FLIP homolog short form; CV: Coxsackievirus; HAV: Hepatitis A virus; HRV: Human rhinovirus; PV: Poliovirus; TLR: Toll-like receptor.

Figure 2. Colocalization of caspase-8 with the mitochondria was determined in mock- or group B coxsackieviruses 3-infected mouse embryo fibroblasts cells

Cells were grown on poly-L-lysine-coated coverslips and fixed 24 h postinfection. Specimens were incubated with a primary rat-anticaspase-8 antibody, followed by a secondary Cy3/Alexa647-conjugated donkey-antirat antibody. Mitochondria were stained using a rabbit-anti-TOM20 antibody and a secondary donkey-antirabbit Alexa405/Alexa647 conjugate. Samples were analyzed after postfixation on a Nikon N-SIM/N-STORM system using a 63× oil-immersion lens. The images show distinct grouping of mitochondria p.i. as described by Chen's group [101] (for clarity the mitochondria aggregates during infection are outlined with a green and red dotted line). CVB: Group B coxsackieviruses; p.i.: Postinfection.