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# Protect this house: cytosolic sensing of viruses

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The ability to recognize invading viral pathogens and to distinguish their components from those of the host cell is critical to initiate the innate immune response. The efficiency of this detection is an important factor in determining the susceptibility of the cell to viral infection. Innate sensing of viruses is, therefore, an indispensable step in the line of defense for cells and organisms. Recent discoveries have uncovered novel sensors of viral components and hallmarks of infection, as well as mechanisms by which cells discriminate between self and non-self. This review highlights the mechanisms used by cells to detect viral pathogens in the cytosol, and recent advances in the field of cytosolic sensing of viruses.

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## Introduction

Human pathogenic viruses are a major global health concern, often leading to serious illness or death. Viral infection represents a significant challenge to host cells, as the ability to detect infection and inhibit viral replication is one of the key factors determining host susceptibility to infection. Many human pathogenic viruses have evolved strategies to avoid detection by the host cell, or to inhibit other antiviral factors, demonstrating the importance of antiviral innate immunity to protect against viral infection [1,2].

Pattern recognition receptors (PRRs) act as sensors for the products of viral infection, which are known as pathogen-associated molecular patterns (PAMPs). Viral PAMPs can include viral proteins or nucleic acids that are sensed by PRRs as non-self to elicit antiviral innate immune responses, primarily driven by type I and III interferons (IFN). This review will focus on the PRRs that sense viral nucleic acid PAMPs within the cytosol of the cell generated during RNA and DNA virus infection. We will describe how the cytosolic nucleic acid sensing PRRs, the RIG-I-like receptors (RLRs) and DNA sensors, discriminate between self and non-self to activate antiviral immune responses (Figure 1).

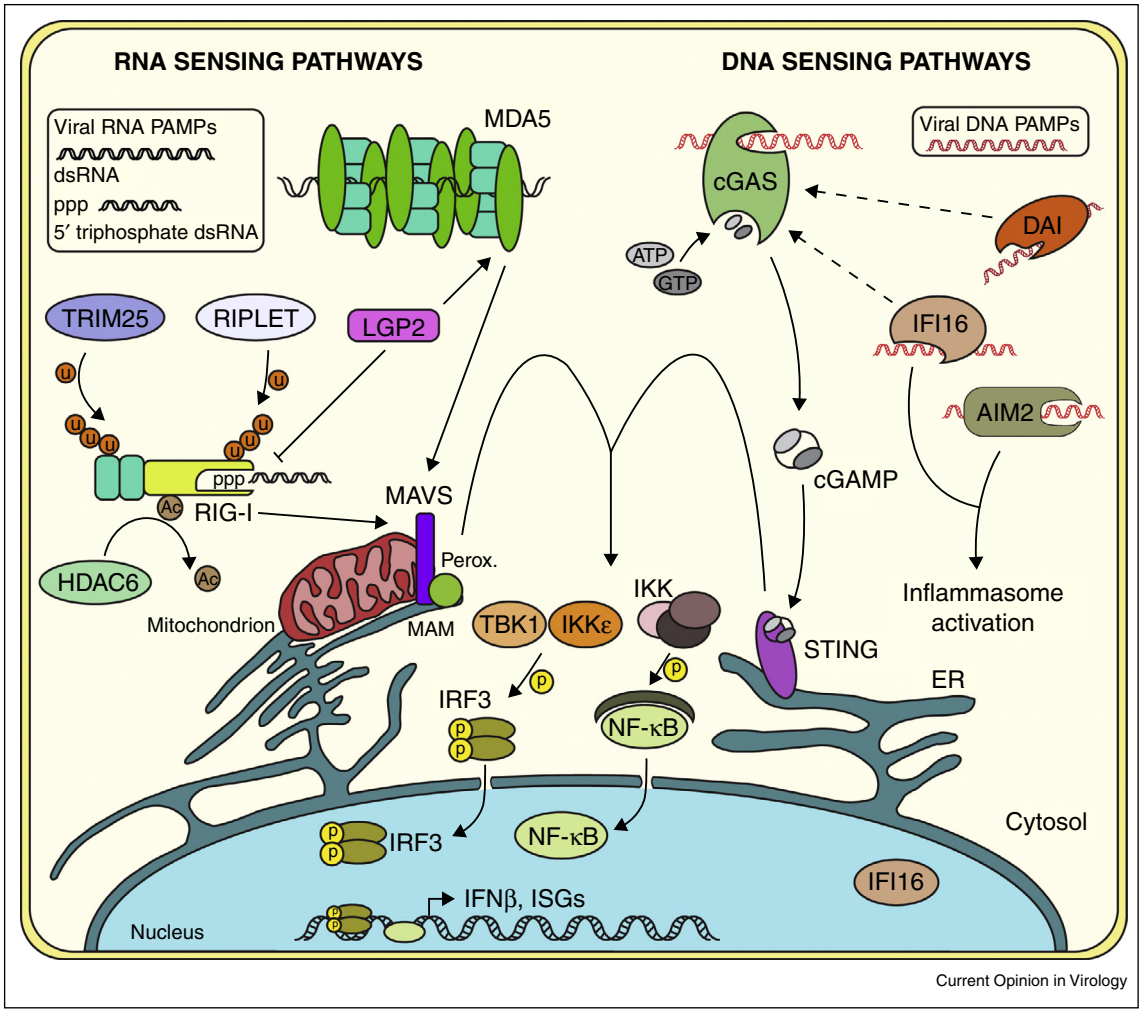
## RLRs sense cytosolic viral RNA and activate antiviral responses

The RLRs, members of the DExD/H box family of helicases, include retinoic acid-inducible gene I (RIG-I), melanoma differentiation factor 5 (MDA5), and laboratory of genetics and physiology 2 (LGP2). This family of cytosolic viral sensors is crucial for recognition of a large number of RNA viruses [3]. These sensors distinguish virus-associated RNAs from cellular RNAs to activate downstream signaling of antiviral innate immunity driven by mitochondrial antiviral signaling protein (MAVS), which aggregates into filaments following activation by PRRs [4]. Filamentous MAVS serves as a platform for interaction with other proteins involved in the signaling cascade, such as tumor necrosis factor receptor-associated factor (TRAF) proteins, which are important for MAVS signaling through TANK-binding kinase 1 (TBK1) and IκB-kinase-ε (IKKε), and the IκB kinase complex (IKK) [5]. TBK1/IKKε and IKK phosphorylate IRF3/IRF7 and the inhibitory subunit of NF-κB (IκBα), respectively. The transcription factors IRF3, IRF7, and NF-κB then translocate to the nucleus to induce transcription of type I IFNs (IFN-α and IFN-β). Type I IFN production drives autocrine and paracrine responses through the IFN-α/β receptor, which activates the JAK/STAT signaling pathway to ultimately induce the transcription of hundreds of IFN-stimulated genes (ISGs). These include many antiviral factors, which can inhibit viral replication in various ways [6–8].

### RIG-I

RIG-I senses a number of RNA viruses including flaviviruses, alphaviruses, coronaviruses, reoviruses, paramyxoviruses, orthomyxoviruses, rhabdoviruses, arenaviruses, and bunyaviruses [9,10]. RIG-I recognizes PAMPs, including short double-stranded RNA (dsRNA) containing either a 5′ triphosphate or 5′ diphosphate moiety that are generally unique to viral RNA [11,12,13•]. Interestingly, a recent report identified RIG-I as a PRR for Crimean-Congo hemorrhagic fever virus (CCHFV), whose RNA genome is 5′-monophosphorylated, implicating an additional ability

**Figure 1**




Major pattern recognition receptors (PRRs) that sense RNA and DNA virus pathogen associated molecular patterns (PAMPs) in the cytosol. Following RIG-I sensing of short dsRNA, this sensor is further activated through K63-linked ubiquitination by TRIM25 and RIPLET, as well as through lysine deacetylation by HDAC6. Following sensing of long dsRNA, MDA5 oligomerizes along the dsRNA. Both RIG-I and MDA5 activate signaling through the adaptor MAVS located on mitochondria, mitochondrial-associated ER membranes (MAM), and peroxisomes (perox.), leading to the activation and nuclear translocation of IRF3 and NF-κB and the production of type I IFN and ISGs. Viral DNA PAMPs are sensed by cGAS which catalyzes the production of cGAMP after binding to DNA. cGAMP then signals through the adaptor STING, located on ER membranes to activate IRF3 and NF-κB. Other viral DNA sensors in the cytosol such as DAI and IFI16 are also postulated to function through this pathway. Activation of IFI16 or AIM2 following viral DNA detection leads to inflammasome activation.

of RIG-I to sense 5′-monophosphate-containing viral RNAs [14•]. Therefore, while canonical ligands of RIG-I have been identified, future research may uncover additional features of ligands, such as post-transcriptional RNA modifications, that allow RIG-I to distinguish self from non-self. Indeed, self-RNAs are distinguished from foreign RNAs by post-transcriptional modifications of their 5′ triphosphate ends, which contain the RNA cap structures. These include cap0: 7-methylguanosine addition to the gamma phosphate on the 5′ end of mRNAs; cap1: identical to cap0, with 2′-O-methylation of the first nucleotide following the 5′ triphosphate; and cap2: identical to cap1, with an additional 2′-O-methyl group on the second nucleotide. The 2′-O-methylation present in cap1 is crucial for avoiding recognition by RIG-I [15••,16••]. In addition, 2′-O-methylation protects host mRNAs from sequestration by IFN-induced proteins with tetratricopeptide repeats (IFITs), which would otherwise inhibit the translation of these proteins [17]. In fact, certain viruses including flaviviruses, coronaviruses, and alphaviruses have co-opted cellular RNA capping strategies, likely to evade detection by RLRs [18,19].

RIG-I contains several functional domains that regulate sensing of PAMPs and its subsequent activation. It is comprised of two N-terminal caspase activation and

recruitment domains (CARDs), followed by two tandem helicase domains (Hel1, Hel2) separated by an insertion domain (Hel2i), as well as a C-terminal repressor domain (RD) [11,20,21]. In resting cells, Hel2i interacts with the second CARD, keeping RIG-I in an auto-inhibited state [22,23]. The RD inhibits self-association of RIG-I, preventing its interaction with MAVS [20]. Upon sensing viral RNA, RIG-I undergoes a conformational change, in which the RD interacts with viral RNA and the helicase domains, breaking the interaction of Hel2i with the second CARD to release the CARDs for interaction with MAVS, which also contains a CARD motif [21,22]. Following this conformational change, both the RD and CARDs are subject to post-translational modifications (PTMs). First, the RD of RIG-I is subject to Lysine (K) 63-linked poly-ubiquitination by the E3 ubiquitin ligase RIPLET [24,25]. This promotes tripartite motif-containing protein 25 (TRIM25) K63-linked ubiquitination of the RIG-I CARDs [26]. Mex-3 RNA Binding Family Member C (MEX3C) was recently identified as an additional essential E3 ubiquitin ligase that mediates K63-linked ubiquitination on RIG-I CARDs [27], and TRIM4 was found to enhance RIG-I signaling via K63-linked ubiquitination that is redundant with TRIM25 and RIPLET [28]. Recent structural work has demonstrated that K63-linked ubiquitination of RIG-I stabilizes assembly of its CARDs into a helical tetramer. This tetramer of RIG-I CARDs facilitates interaction with MAVS, and nucleates MAVS filament formation [29]. Removal of specific PTMs, including lysine acetylation by histone deactylase 6 (HDAC6) and dephosphorylation of RIG-I CARDs by protein phosphatase 1 (PP1), is required for full RIG-I activation [30–32]. In addition to regulation by its structure and by PTMs, RIG-I is regulated at the cell biological level. In resting cells, RIG-I is localized to the cytoplasm where it can detect PAMPs of invading viruses. However, upon activation, RIG-I interacts with various proteins, including TRIM25 and 14-3-3ε, to form a translocon that facilitates RIG-I re-localization into intracellular membranes for interaction with MAVS [33,34]. Taken together, these complex regulatory mechanisms prevent aberrant activation of RIG-I.

### MDA5

While some overlap exists between the viruses sensed by RIG-I and MDA5 — such as flaviviruses, alphaviruses, coronaviruses, reoviruses, and paramyxoviruses — MDA5 plays an indispensable role in detection of picornaviruses and caliciviruses [35]. MDA5 has similar structural domains to RIG-I (CARDs-helicases-RD) and also signals downstream through MAVS. However, MDA5 senses long dsRNA, which are viral replication intermediates [36,37]. Similar to RIG-I, 2′-O-methylation at the 5′ end of RNAs prevents MDA5 sensing [38]. While RIG-I recognizes the terminal end of dsRNA, MDA5 recognizes the internal duplex structure of dsRNA in a length-dependent fashion [37]. The crystal structure of MDA5 bound to dsRNA reveals that MDA5 stacks along dsRNA, forming filamentous structures, in a head-to-tail arrangement for signaling [39]. The ATP hydrolysis activity of the helicase domains is required for MDA5 filament formation [39,40]. These filaments expose the CARDs of MDA5 for interaction with the CARD motif of MAVS [39]. PTMs also regulate MDA5 function. Like RIG-I, PP1 dephosphorylates MDA5 CARDs, leading to MDA5 activation [32]. However, unlike RIG-I, little is known about other PTMs that may regulate MDA5 activation, such as K63-linked ubiquitination [3]. In addition, little is known about the cell biology that regulates MDA5 activation. Future studies to address these aspects of the regulation of MDA5 will be of interest.

### LGP2

LGP2 is less well characterized than RIG-I and MDA5, but increasing evidence suggests that LGP2 may act as a negative regulator of RIG-I-directed signaling and as an enhancer of MDA5-directed signaling [41–44]. LGP2 has similar domains to RIG-I and MDA5 (Helicase-RD), however it lacks the N-terminal CARDs required for interaction with MAVS. While it has been shown that LGP2 recognizes both dsRNA and single-stranded RNA (ssRNA), with a preference for RNA with a 5′ triphosphate, LGP2 is not able to independently activate downstream signaling to MAVS because it lacks N-terminal CARDs required for this interaction. In addition, multiple conflicting functions have been attributed to LGP2, including negative regulation of RIG-I [41,42], as well as negative regulation of MDA5 [45], and positive regulation of MDA5 [43•,45]. Recently, LGP2 was found to regulate the binding of MDA5 to RNA and regulate MDA5 filament assembly for enhanced signaling activity [43•]. Therefore, it seems LGP2 may serve multiple, diverse functions in response to different viruses. Further research will be required to appreciate the role and function of LGP2 in regulation of RNA virus sensing and downstream signaling.

## Cytosolic DNA sensors activate antiviral responses

The presence of DNA in the cytosol is an indicator of pathogen infection or of cellular damage. Considering the multitude of intracellular pathogens capable of replicating in the cytosol, the detection of their nucleic acids is imperative for cellular defense against not only viruses, but also bacterial and eukaryotic pathogens. While sensors and pathways related to detection of RNA viruses are well defined, many sensors of viral DNA have only recently been identified. The roles of many of these proteins in initiating innate immune responses to DNA are not fully understood [46]. In addition, the signaling pathways leading to the production of IFN following detection of viral DNA PAMPs are less well defined than pathways activated by detection of RNA PAMPs. Nonetheless, the past decade has produced numerous

discoveries uncovering key factors in DNA-sensing pathways, referred to as the IFN-stimulatory DNA (ISD) pathway [47,48]. A number of cytosolic DNA sensors activate this pathway to signal through stimulator of interferon genes (STING). STING binds to and is activated by cyclic dinucleotides, such as cyclic GMP-AMP (cGAMP) [49]. Activated STING then translocates from the endoplasmic reticulum (ER) to perinuclear compartments, such as the Golgi, endosomes, and autophagy-related compartments [50,51], which leads to its palmitoylation for signaling [52•]. It then recruits kinases that phosphorylate IRF3 and activate signaling in a fashion similar to MAVS [53••]. Interestingly, STING can also activate STAT6 for transcriptional induction of ISGs [54]. Ultimately, activation of STING signaling elicits type I IFN induction and similar antiviral response strategies as those seen in MAVS signaling. Thus, while cells use different sensors to detect RNA and DNA viruses, the signaling pathways ultimately converge in similar antiviral response strategies.

### cGAS

Cyclic GMP-AMP synthase (cGAS) is the primary protein required for type I IFN induction in response to cytosolic DNA. cGAS detects DNA in the cytosol as a result of DNA virus infection or DNA transfection and synthesizes the second messenger cGAMP [55]. cGAMP subsequently binds to STING, leading to its activation [56]. While cGAS was only recently identified, it is already recognized as the primary cytosolic DNA sensor. Indeed, recent reports suggest that cGAS may mediate the ability of other DNA sensors to activate STING and the ISD pathway [57•]. cGAS senses DNA viruses such as herpesviruses, human papillomavirus, adenovirus, and hepatitis B virus, as well as retroviruses such as human immunodeficiency virus-1 (HIV-1), simian immunodeficiency virus, and murine leukemia virus [58,59]. Additionally, cGAS plays a role in the innate immune response to a number of positive-sense RNA viruses, although it is not known whether cGAS can act as a true RNA sensor [60,61].

Structural studies on cGAS have provided important insights into how it recognizes DNA and synthesizes the second messenger cGAMP. cGAS is composed of an N-terminal unstructured region, followed by a nucleotidyl transferase domain, and a C-terminal male abnormal 21 domain [55]. Resting cGAS exists in a bilobal conformation, with a zinc thumb located between the lobes. cGAS binds to dsDNA via this zinc thumb, which induces a conformational change in cGAS. The catalytic pocket of cGAS is then accessible for synthesis of cGAMP [59,62,63]. This synthesis generates 2′3′-cGAMP, an endogenous cGAMP, which contains two unique phosphodiester bonds [59,64–66]. Interestingly, 2′3′-cGAMP binds STING with much higher affinity than cGAMP molecules with different phosphodiester linkages, demonstrating the importance of the specific product of cGAS to activate innate immunity [66]. This production of cGAMP is essential for STING activation by cGAS, as catalytically inactive cGAS does not induce type I IFN, despite its ability to bind DNA [55]. cGAMP activates STING by inducing a conformational change after which STING dimerizes and is subject to K63-linked ubiquitination by TRIM56 and TRIM32 [67,68]. Further mechanisms governing cGAS activity, such as regulation by other PTMs, remain uncharacterized. Given its widespread or ubiquitous expression and the requirement of cGAS in the ISD pathway, future studies to uncover these regulatory mechanisms will be of importance.

### DAI

DNA-dependent activator of IFN-regulatory factors (DAI) was the first cytosolic DNA sensor of antiviral innate immunity to be discovered [69]. DAI binds to cytosolic DNA derived from both viruses and host cells [69,70]. Viruses sensed by DAI include herpes simplex virus-1 (HSV-1), human cytomegalovirus (HCMV), and mouse cytomegalovirus (MCMV) [69–71]. Interestingly, the ability of DAI to sense self-DNAs in the cytosol may play a role in the development of autoimmune disease. For example, DAI expression is upregulated in people with systemic lupus erythematosus (SLE), and in SLE mouse models [72]. The role of DAI in initiating the IFN response to cytosolic DNA appears to be either cell type-specific, or redundant, as DAI-deficient mice and cells derived from these mice elicited normal IFN responses to both DNA virus infection and synthetic DNA [73,74].

The mechanism by which DAI senses cytosolic DNA is not well understood. A recent study used *in vitro* pull-down assays to show that DAI binds to DNA in a sequence-independent, but length-dependent manner [74]. Interestingly, this study also shows that DNA may serve as a scaffold upon which DAI can aggregate and that artificial dimerization of DAI induced type I IFN expression in the absence of DNA, suggesting that dimerization or oligomerization of DAI drives downstream signaling [74]. Further research will be required to gain a better understanding of the mechanisms by which DAI senses DNA. While STING is known as the major adaptor protein in DNA sensing pathways, it has not been clearly demonstrated that DAI signals through STING [75]. Though DAI may be dispensable for the innate immune response to viral DNA, future efforts should focus on dissecting the DAI pathway, given its possible roles in cell type-specific antiviral responses, and its contribution to autoimmune diseases.

### AIM2-like receptors (ALRs)

The ALRs are a family of proteins that have also been suggested to acts as sensors of the ISD pathway [76,77]. They are also known to be required for inflammasome

activation in response to multiple pathogens [78–80]. The inflammasome is an inflammatory response induced following transcriptional activation of caspase-1, which then cleaves IL-1 cytokines into IL-1β and IL-18 to promote inflammation [81]. The ALR family consists of five members in humans: absent in melanoma 2 (AIM2), gamma-interferon-inducible protein 16 (IFI16), pyrin and HIN domain family member 1 (PYHIN1), myeloid cell nuclear differentiation antigen (MNDA), and pyrin domain-only protein 3 (POP3). AIM2 and IFI16 are the two most prominent members of the ALR family and are members of the pyrin and HIN domain (PYHIN) family. Both AIM2 and IFI16 have been shown to be required for inflammasome activation after DNA virus sensing. AIM2 activates inflammasomes in response to herpes simplex virus-1 (HSV-1), vaccinia virus, and mouse cytomegalovirus [80], and IFI16 activates inflammasomes in response to HSV-1 [82].

In addition to its role in inflammasome activation, IFI16 activates the ISD pathway by sensing non-self DNA in both the nucleus and cytosol [76,77]. Depletion of IFI16 has been shown to dampen the IFN response to viruses such as the retrovirus HIV-1, HSV-1, and human cytomegalovirus (HCMV) [83–85]. While these studies have implicated IFI16 as an important sensor of both cytosolic and nuclear foreign DNA, a recent study demonstrated that in mice, ALRs are not required for the ISD response [86••]. Further, by using genetic knockouts, human IFI16 was shown to be non-essential for the IFN response to HCMV infection. Alternatively, cGAS was essential for the ISD response in both mice and human cells [86••]. Thus, similar to DAI, ALRs such as IFI16 either serve a cell type-specific role in initiating the ISD pathway, or have a redundant role with other factors. These recent findings highlight the uncertainty surrounding many putative DNA sensors, and the clear requirement of the recently discovered cGAS as the PRR of the ISD pathway.

## Conclusion

Cytosolic sensing of foreign nucleic acids by PRRs is essential for an infected cell to mount antiviral responses to inhibit replication of invading viruses and prime an effective adaptive immune response [87]. Recent findings described here have contributed to our understanding of the mechanisms used by cells to recognize infection. New discoveries in the field of cytosolic nucleic acid sensing could yield important knowledge regarding how autoimmune diseases, in which aberrant sensing leads to inflammation and self-damage, are triggered. Additionally, future research will likely elucidate novel sensors of nucleic acids, regulatory factors in antiviral signaling pathways, and strategies used by viruses to antagonize these processes. These efforts could lead to novel treatment strategies for both autoimmune diseases and viral infections.

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