Innate Immune Sensing and Signaling of Cytosolic Nucleic Acids

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Abstract

The innate immune system utilizes pattern-recognition receptors (PRRs) to detect the invasion of pathogens and initiate host antimicrobial responses such as the production of type I interferons and proinflammatory cytokines. Nucleic acids, which are essential genetic information carriers for all living organisms including viral, bacterial, and eukaryotic pathogens, are major structures detected by the innate immune system. However, inappropriate detection of self nucleic acids can result in autoimmune diseases. PRRs that recognize nucleic acids in cells include several endosomal members of the Toll-like receptor family and several cytosolic sensors for DNA and RNA. Here, we review the recent advances in understanding the mechanism of nucleic acid sensing and signaling in the cytosol of mammalian cells as well as the emerging role of cytosolic nucleic acids in autoimmunity.

INTRODUCTION

In vertebrates, two complementary systems have evolved to detect and fight against microbial pathogens: the innate and adaptive immune systems. As the first line of host defense, the innate immune system deploys a limited number of germline-encoded receptors called pattern-recognition receptors (PRRs) to detect and respond to the presence of pathogens. PRRs recognize conserved molecular structures known as pathogen-associated molecular patterns (PAMPs) that are essential for the life cycle of the pathogen (1). Many PAMPs, such as lipopolysaccharides, peptidoglycans, and flagellin, are found in microbes but not in the host, allowing the host to distinguish nonself from self through PRRs. One apparent exception is the detection of pathogen-derived nucleic acids. The fact that nucleic acids could serve as PAMPs greatly expands the repertoire of microorganisms detectable by the host immune system. In principle, all microbes use DNA and/or RNA as genetic information carriers in their life cycle and could therefore potentially activate host nucleic acid sensors. However, this also introduces the risk of self nucleic acid recognition by innate immune sensors, an event that causes several autoimmune and autoinflammatory diseases (2).

Innate immune sensors for nucleic acids can be generally divided into two groups on the basis of their subcellular localization and expression pattern. The first group includes several members of the Toll-like receptor (TLR) family that function mostly in immune cells, such as dendritic cells (DCs), macrophages, and B cells. These TLRs localize on the endosomal membrane and monitor the lumen of endosomes and lysosomes to detect various forms of nucleic acids from bacteria and viruses. The second group of receptors, which have not been well characterized until recently, detects nucleic acids in the cytoplasm of almost all cell types. Cytosolic nucleic acid sensors include proteins that detect cytoplasmic DNA as well as the RIG-I-like receptor (RLR) family members that detect pathogen-derived RNA in the cytosol.

Upon recognition of nucleic acids, the endosomal TLRs and cytosolic sensors activate a signaling cascade that culminates in production of type I interferons (IFNs), which primarily include numerous subtypes of IFN-α and a single IFN-β, as well as proinflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β). Type I IFNs then induce a large array of antiviral genes through the activation of the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway (3). The induction of type I IFNs is regulated by the transcription factors nuclear factor κ-light-chain enhancer of activated B cells (NF-κB), and the IFN regulatory factors IRF3 and IRF7. The activation of NF-κB requires phosphorylation of its inhibitor IκB by the IκB kinase (IKK) complex. The phosphorylation of IκB targets this inhibitor for polyubiquitination and subsequent proteasome-dependent degradation, releasing NF-κB to the nucleus to regulate downstream genes (4). Activation of IRF3 and IRF7 requires phosphorylation by two IKK-related kinases, TBK1 and IKKε (5, 6). After phosphorylation, these two IRFs undergo homodimerization and translocate into the nucleus, where they form an enhanceosome complex together with NF-κB and other transcription factors to turn on the transcription of type I IFN genes (7).

In this review, we briefly introduce the TLRs involved in nucleic acid sensing and then focus on describing the recent advances in the molecular understanding of cytosolic sensing and signaling of RNA and DNA that lead to the production of type I IFNs. In addition, we discuss how aberrant recognition of self-DNA in the cytosol contributes to autoimmunity.

TOLL-LIKE RECEPTORS SENSE NUCLEIC ACIDS IN THE ENDOSOME

TLRs are among the best-studied groups of PRRs (1). TLRs are single transmembrane proteins with ectodomains containing leucine-rich repeats for PAMP recognition, a transmembrane

domain, and a cytosolic Toll/IL-1 receptor (TIR) domain responsible for transducing signals to downstream adaptors including TRIF and MyD88 (8). Among the 10 and 13 identified TLRs in human and mouse, respectively, 5 are involved in nucleic acid sensing: TLR3, TLR7, TLR8, TLR9, and TLR13. These receptors monitor the endolysosomal lumen for pathogen-derived nucleic acids and function via two signaling pathways: TLR3 activates TRIF, whereas TLR7, TLR8, TLR9, and TLR13 activate MyD88. Both adaptor proteins lead to the activation of NF-κB, whereas IRF3 is activated by the TRIF pathway and IRF7 by the MyD88 pathway (8).

Using gene-targeted mice, studies with synthetic ligands and microbes have shed light on the ligand specificity of these nucleic acid–sensing TLRs. TLR3 was originally identified as a sensor for double-stranded RNA (dsRNA) using synthetic analog polyinosinic-polycytidylic acid (poly[I:C]) as the stimulus (9). Later studies showed that TLR3 was also involved in the host response to respiratory syncytial virus, encephalomyocarditis virus (EMCV), West Nile virus (WNV), and coxsackievirus (8, 10, 11). The two phylogenetically close TLRs, TLR7 and TLR8, share a general ligand specificity for single-stranded RNA (ssRNA) derived from RNA viruses (12, 13) and imidazoquinoline derivatives such as resiquimod (R848) (14). However, ssRNA activates only TLR8 and not TLR7 in humans, and mice lack TLR8 but respond normally to these agonists. Thus, TLR7 and TLR8 appear to have distinct functions in different species (8, 11). TLR9 recognizes unmethylated cytosine-guanosine (CpG) DNA motifs that are common for bacterial but rare in mammalian genomes (8, 15). Recently, our group and Kirschning's group independently identified murine TLR13 as the sensor for bacterial 23S ribosomal RNA (16, 17). A sequence containing 13 nucleotides near the active site of 23S rRNA ribozyme is both necessary and sufficient to activate TLR13.

SENSING NUCLEIC ACIDS IN THE CYTOSOL

The ligand-binding domain of the nucleic acid–sensing TLRs faces the lumen of the endosome, rendering these TLRs "blind" to microbes that have successfully invaded the host cytoplasm and replicated inside the cells (18). In addition, most nonimmune cells, such as epithelial cells and fibroblasts that normally line the mucosal surface and thus are most susceptible to infections, do not express the nucleic acid–sensing TLRs but can nevertheless mount effective innate immune responses against microbial infections. Thus, a cell-intrinsic, cytoplasmic surveillance system must exist to defend against microbes that invade both immune and nonimmune cells. The sensing and signaling mechanisms of this cytosolic surveillance system are being extensively studied, and much progress has been made. In the following section, we discuss recent advances in elucidating the cytosolic nucleic acid–sensing pathways.

Recognition of Cytosolic RNA

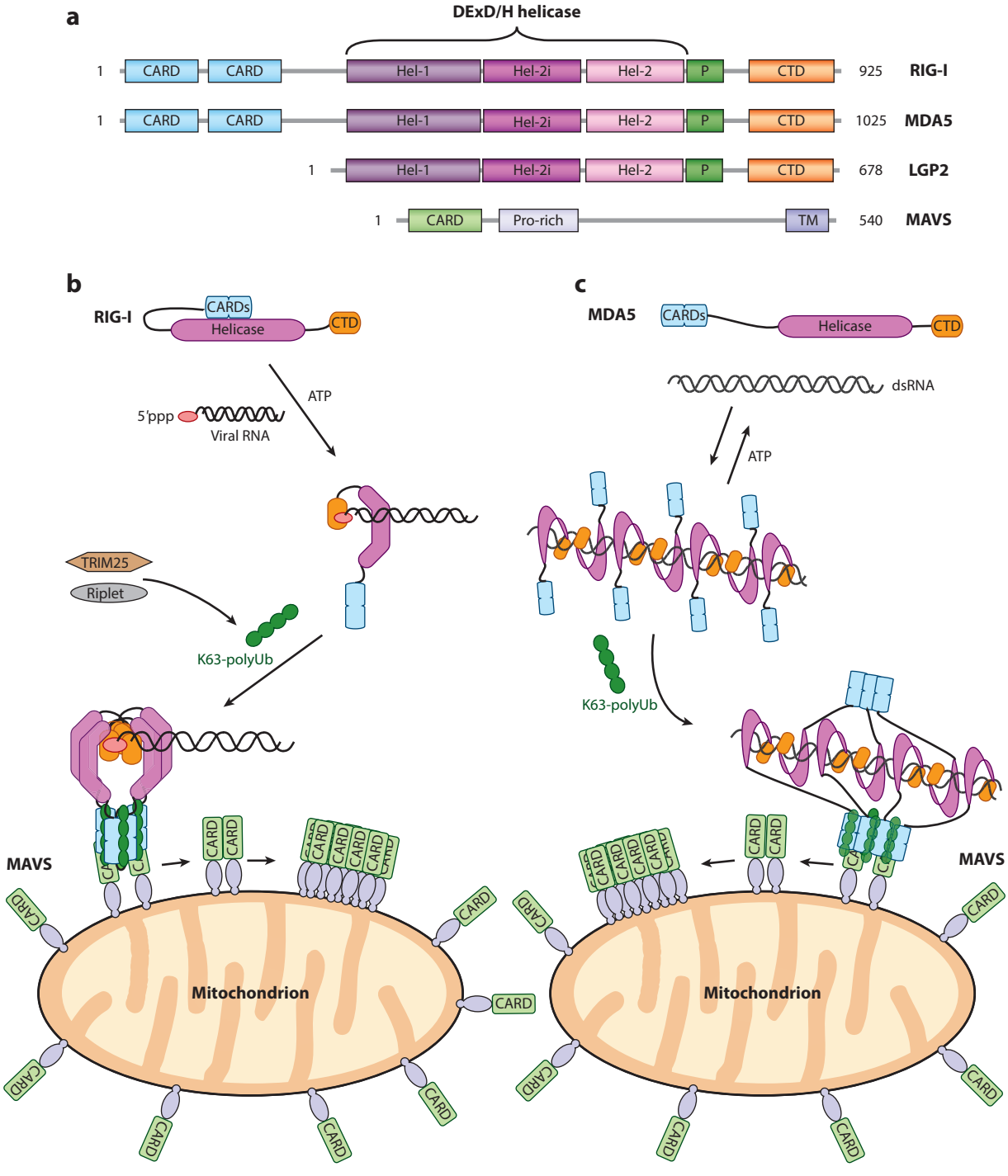
Infection by RNA viruses such as influenza and hepatitis C virus (HCV) triggers a strong production of type I IFNs. The major PAMP from RNA viruses that induce type I IFNs is viral RNA, which is delivered to the cytosol in the form of incoming viral genomes or is generated through viral RNA replication. Pioneering studies have shown that IFN induction by the viral RNA is independent of TLRs (19). In 2004, retinoic acid inducible gene-I (RIG-I) and its homolog melanoma differentiation associated gene 5 (MDA5) were identified as the sensors of cytosolic viral RNA (20). These landmark discoveries set off a decade of intense study that has yielded a much better understanding of the signaling pathways and mechanisms by which viral RNA in the cytosol triggers the production of type I IFNs.

RIG-I-like receptors. Initially identified as a DExD/H-box-containing protein required for intracellular dsRNA-induced IFN production (20), RIG-I is the founding member of the RIG-I-like receptor (RLR) family of cytosolic RNA sensors. The other two members are MDA5 and LGP2 (laboratory of genetics and physiology 2) (21) (**Figure 1*a***). All three RLRs share highly conserved domain structures, including a central DExD/H-box helicase core composed of two helicase domains (Hel-1 and Hel-2) with a specific insertion within Hel-2 (referred to as Hel-2i) and a C-terminal domain (CTD) that confers part of the ligand specificity. The CTD is connected to Hel-2 by a unique, elbow-shaped helical extension, referred to as pincer domain (22, 23). In the N termini of RIG-I and MDA5, but not that of LGP2, two tandemly linked caspase activation and recruitment domains (CARDs) mediate signaling to downstream adaptor proteins (18, 24, 25) (**Figure 1*a***). Due to the lack of a CARD, LGP2 was considered an inhibitory factor for the RLR signaling pathway (21). However, later studies suggested that LGP2 plays a positive role in MDA5 signaling (26, 27). RIG-I can recognize viruses in the Paramyxoviridae family, such as Sendai virus and Newcastle disease virus; viruses in the Flaviviridae family, such as Japanese encephalitis virus and HCV; and viruses in the Rhabdoviridae family, such as vesicular stomatitis virus. MDA5 detects viruses in the Picornaviridae family, such as Polio virus and EMCV (28, 29). Some viruses, including Reovirus, Dengue virus, and WNV, are recognized by both RIG-I and MDA5 (30, 31).

Ligands for RLRs. The differential recognition of viruses by RIG-I and MDA5 is attributed to their distinct preference for RNA ligands. Both RIG-I and MDA5 respond to the synthetic dsRNA analog poly(I:C), but with different length dependency. Long fragments (>4 kb) are preferred by MDA5, whereas shorter fragments generated by enzyme digestion (~300 bp) are recognized by RIG-I (32). Using different approaches, several groups discovered that the most important molecular feature of RNA for RIG-I recognition is a triphosphate group at the 5′ end (33, 34), a structure that is lacking in host mRNAs. This serves as a mechanism for self/nonself discrimination by RIG-I. Two later studies further showed that 5′-triphosphate RNA needs additional base-paired structures to activate RIG-I (35, 36). This kind of 5′-triphosphate-bearing panhandle structure is predicted to be present in the genomes of some negative-strand ssRNA viruses such as influenza A virus (flu) and may function as the in vivo ligand for RIG-I. In addition, another category of RNA was reported to activate RIG-I (37, 38). 5′-hydroxyl (5′-OH) and 3′-monophosphoryl short RNA molecules with double-stranded stems generated by RNase L (see details below) are also RIG-I ligands. Thus, the physiological agonist for RIG-I in virus-infected cells could potentially be viral genomes, viral replication intermediates, viral transcripts, or RNA cleaved by RNase L. To explore the relative contribution of each of these RNA species to RIG-I activation and IFN induction, one study (39), using a viral reconstitution system, demonstrated that it is the full-length viral genome of flu or Sendai virus (which bears 5′-triphosphates) that

Figure 1

Model for cytosolic RNA-induced activation of RLR-MAVS pathway. (*a*) Domain organization of RLRs and their adaptor MAVS. (*b*) Binding to 5′ppp-containing viral RNA induces a structural rearrangement in RIG-I that liberates its CARDs for subsequent association with unanchored K63-linked ubiquitin chains to form oligomers. (*c*) MDA5 stacks along dsRNA in a head-to-tail fashion to form helical filaments, which facilitate the formation of discrete CARD oligomers along the filaments. Oligomerized CARD domains of RIG-I and MDA5 interact with the CARD domain of MAVS. This interaction promotes the polymerization of MAVS CARD, which in turn serves as a "seed" to trigger the prion-like conversion of other MAVS on the mitochondrial outer membrane. (Abbreviations: CARD, caspase activation and recruitment domain; CTD, C-terminal domain; dsRNA, double-stranded RNA; Hel-1 and Hel-2: helicase domain 1 and 2; Hel-2i: helicase insertion domain; MAVS, mitochondrial antiviral signaling; MDA5, melanoma differentiation associated gene 5; P, pincer domain; pro-rich, proline rich; RLR, RIG-I-like receptor; TM, transmembrane domain.)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DExD/H helicase
1
CARD
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RIG-I
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LGP2
Pro-rich
TM
540
MAVS
b
RIG-I
CARDs
Helicase
ATP
5′ppp
Viral RNA
TRIM25
Riplet
K63-polyUb
Mitochondrion
c
MDA5
dsRNA

has RIG-I-stimulatory activity; short replication intermediates do not contribute substantially to RIG-I activation. However, by isolating RIG-I-bound RNA after viral infection and using next-generation sequencing technology, another study (40) found that RIG-I preferentially associates with shorter 5′-triphosphate-containing viral RNA segments in cells infected with flu and Sendai virus. RIG-I may react to different forms of natural RNA in different cell types under different circumstances.

Compared with RIG-I agonists, the ligand for MDA5 is less well characterized. MDA5 was thought to function as a sensor for long dsRNA, as it could be activated by long poly(I:C) (32). In support of this notion, a recent study identified the picornavirus 7.5-kb replicative-form dsRNA as a physiological ligand of MDA5 (41). However, an earlier study argued that viruses such as EMCV and vaccinia virus could produce high-molecular-weight RNA during replication/transcription. High-molecular-weight RNA consists of both ssRNA and dsRNA and forms web-like structures. This RNA, rather than dsRNA, has MDA5-dependent IFN-stimulatory activity (42). Very little is known about the nature of RNAs that serve as ligands for LGP2. A better understanding of the molecular features of RNA that can bind to LGP2 may help to resolve how LGP2 positively regulates the MDA5 pathway.

Structural basis for RLR activation. Early studies showed that overexpression of a RIG-I N-terminal fragment containing only the CARD domains constitutively induced IFN-β production, leading to the hypothesis that CARDs are sequestered by intramolecular interactions with other domains of the protein until its release by RNA-induced conformational change (20). Recent structural studies have shed light on how RNA activates RIG-I to trigger downstream signaling that results in the production of type I IFNs. In the absence of RNA ligand, RIG-I adopts an autorepressed conformation that prevents the N-terminal CARDs from signaling (**Figure 1*b***). In this state, tandem CARDs form a rigid, head-to-tail unit where the N terminus of CARD2 directly connects to the C terminus of CARD1 (43). Binding of CARD2 to Hel-2i blocks dsRNA binding to the helicase. This also prevents the CARDs from binding to polyubiquitin or being modified by ubiquitination enzymes. The CTD is linked to the pincer motif and is free for RNA ligand binding (22, 44). Upon viral infection, binding of dsRNA to the CTD brings Hel-2i in contact with dsRNA, resulting in the reorientation of the pincer domain. ATP hydrolysis further drives a conformational change that releases the CARD domains for signaling (43, 45–47). The CARDs are then available for modification of K63 polyubiquitin chains by the E3 ligases tripartite motif protein 25 (TRIM25) and Riplet (also termed RNF135 or REUL) (48–50). Recently, by reconstituting the RIG-I pathway from an RNA ligand to activation of the transcription factor IRF3 in a cell-free system, we found that the RIG-I CARDs bind to unanchored K63 polyubiquitin chains and that this binding is essential for RIG-I activation (51). We further showed that polyubiquitin binding induces the formation of an oligomeric complex consisting of four RIG-I and four ubiquitin chains, which is highly potent in activating downstream signaling cascades (24) (**Figure 1*b***).

Significant progress has also been made in understanding the structural mechanism of dsRNA recognition by MDA5. We now know that MDA5 assembles into a polar filamentous oligomer along dsRNA (52–54) (**Figure 1*c***). The stability of the MDA5 filament is regulated by ATP hydrolysis, which triggers dissociation of MDA5 from dsRNA (52). The crystal structure of MDA5 (without CARDs) bound to dsRNA also shows a global domain organization similar to that of RIG-I. However, instead of forming an O-ring structure with dsRNA, as in the case of RIG-I, MDA5 uses an open, C-shaped structure to bind dsRNA. This is caused by a unique orientation of the CTD which is approximately 20° rotated relative to the RIG-I CTD (55). The tandem CARD domains, which stretch outside the core MDA5 filament, may be prone to oligomerize into a structure capable of activating the downstream signaling pathway (**Figure 1*c***). Unanchored

K63 polyubiquitin chains also bind to the tandem CARD domains of MDA5. This binding is important for signaling by MDA5, as mutations of conserved residues in MDA5 that disrupt its ubiquitin binding also abrogate its ability to activate IRF3 (24).

Mechanism of MAVS activation. Signaling downstream of RIG-I depends on the essential adaptor protein MAVS (mitochondrial antiviral signaling; also known as IPS-1, CARDIF, and VISA) (56–59). MAVS comprises a CARD domain at its N terminus, followed by a proline-rich domain, and a short transmembrane domain at its C terminus, which localizes MAVS to the mitochondrial outer membrane. The homotypic interaction of the CARD domain of MAVS and the CARD domains of RIG-I and MDA5 is essential for signaling (**Figure 1*b*,*c***), as is the mitochondrial localization of MAVS. The in vivo role of MAVS in antiviral immunity has been confirmed by knockout studies (60, 61). Loss of MAVS abolished the induction of type I IFNs and proinflammatory cytokines in response to RNA viruses in multiple cell types. Compared with their wild-type littermates, MAVS knockout mice are highly susceptible to lethal infection by vesicular stomatitis virus (60).

Recent biochemical studies have provided new insights into the mechanism of MAVS activation. Virus infection leads to massive aggregation of MAVS on the mitochondrial membrane (62) (**Figure 1*b*,*c***). When isolated from the cells, the MAVS aggregates potently activate IRF3 in the cytosol. These MAVS aggregates exhibit the hallmarks of prions in several ways: They are resistant to harsh detergent and proteinase K treatment; they form fiber-like polymers that can be visualized by electron microscopy; and most strikingly, they are self-perpetuating, because aggregated MAVS can convert endogenous MAVS on the mitochondrial membrane into functional aggregates. In virus-infected cells, RIG-I is activated by viral RNA and K63 polyubiquitin chains. The released CARD domains interact with the CARD domain of MAVS, converting a small fraction of MAVS molecules into prion-like aggregates. These "infected" MAVS then function as a "seed" to recruit other MAVS molecules to form large aggregates. Such an aggregation event allows a rapid and robust amplification of RIG-I signaling, which is consistent with the observation that a tiny amount of viral RNA could trigger robust IRF3 activation in cells (51).

Despite significant advances in understanding the mechanism of MAVS activation, it is still not clear why the mitochondrial localization of MAVS is required for its function. HCV utilizes its protease NS3/4A to cleave MAVS at a position (Cys-508) adjacent to the mitochondrial transmembrane domain, dislocating it from mitochondria and thus preventing the induction of IFN-β (58, 63) (**Figure 2**). This finding further underscores the importance of mitochondrial localization in the function of MAVS. Several studies have implicated mitochondrial dynamics in the regulation of MAVS signaling. Two groups reported that MAVS interacts with mitofusin 1 (MFN1), a protein of the mitochondrial fusion machinery, and that mitochondrial fusion is required for efficient MAVS signaling, as knockdown of either MFN1 or optic atrophy type 1 inhibits virus-induced IRF3 activation and IFN production (64, 65). However, a different study showed that MFN2, another mediator of mitochondrial fusion, functions as an inhibitor of MAVS signaling (66). In addition, a recent study indicated that the mitochondrial membrane potential ($\Delta\Psi m$) is important for antiviral signaling mediated by MAVS (67). When cells were treated with carbonyl cyanide *m*-chlorophenylhydrazone, a protonophore that dissipates $\Delta\Psi m$ by increasing membrane permeability to protons, IFN production in response to viruses was abolished. It is possible that mitochondrial dynamics and/or $\Delta\Psi m$ are essential for the formation of MAVS aggregates and/or recruitment of downstream signaling proteins.

Signaling downstream of MAVS. RLRs and MAVS induce IFNs and cytokines through two cytosolic kinases, IKK and TBK1. Several studies have suggested an important role for

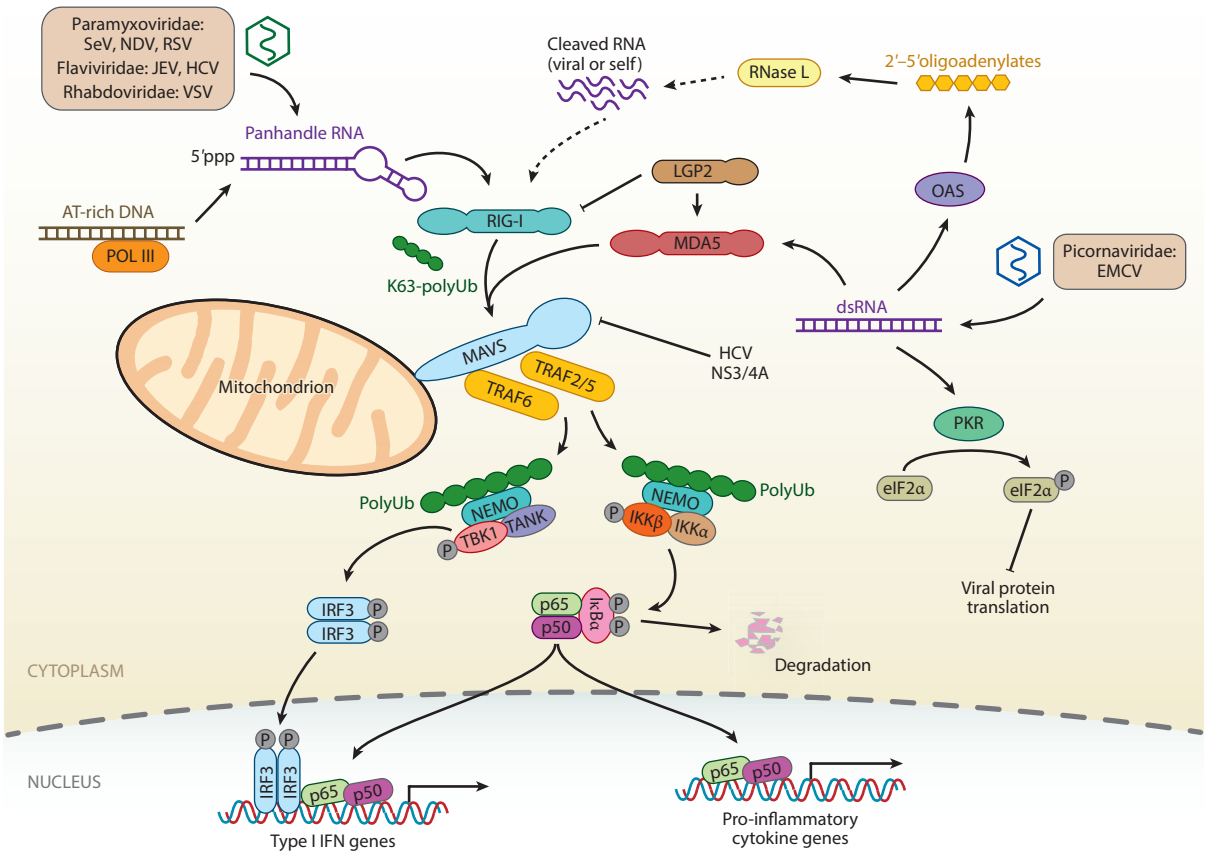


Figure 2

Cytosolic RNA recognition system. RIG-I and MDA5 play nonredundant roles in cytosolic RNA sensing by recognizing different groups of viral RNAs. Whereas RIG-I detects viral RNA containing 5′ppp and panhandle-like secondary structures, MDA5 recognizes long dsRNA in the viral genome. RNAs generated by RNase L cleavage may also serve as ligands for RIG-I. LGP2 may function as a regulator to modulate the activity of RIG-I and MDA5. RIG-I and MDA5 activation induce the prion-like polymerization of MAVS, which in turn recruits and activates E3 ligases TRAF2, TRAF3, TRAF5, and TRAF6. These E3 ligases then synthesize polyubiquitin chains that are sensed by NEMO through its ubiquitin-binding domains. NEMO then recruits IKK and TBK1 complexes to the MAVS polymer, where the kinases phosphorylate IκBα and IRF3, respectively, leading to the induction of type I interferons and other cytokines. AT-rich DNA is recognized by RNA polymerase III, which transcribes the DNA into 5′ppp-RNA that triggers the RIG-I pathway. In addition, dsRNA can also activate the OAS/RNase L pathway as well as PKR to trigger antiviral responses. (Abbreviations: dsRNA, double-stranded RNA; eIF2, eukaryotic initiation factor 2; HCV, hepatitis C virus; IKK, IκB kinase; IRF3, interferon regulatory factor 3; JEV, Japanese encephalitis virus; MAVS, mitochondrial antiviral signaling; MDA5, melanoma differentiation associated gene 5; NDV, Newcastle disease virus; NEMO, NF-κB essential modulator; OAS, oligoadenylate synthetase; PKR, protein kinase R; polyUb, polyubiquitination; RSV, respiratory syncytial virus; SeV, Sendai virus; TRAF, tumor necrosis factor receptor associated factor; VSV, vesicular stomatitis virus.)

ubiquitination in the RLR pathway. NEMO, the regulatory subunit of IKK and TBK1, functions as a ubiquitin sensor (68, 69). NEMO contains two ubiquitin binding domains, which are essential for the activation of IKK and TBK1 in response to virus infection (70). K63 polyubiquitination is required for virus-induced activation of TBK1 and IRF3 (70). Additionally, several E3 ligases including TRAF3, TRAF5, cIAP1/2, and MIB1/2 were proposed to regulate NF-κB and IRF3 activation downstream of MAVS (71–75).

Recently, we showed that MAVS polymers recruit multiple TRAF proteins, including TRAF2, TRAF5, and TRAF6, through distinct TRAF-binding motifs (76). TRAF2 and TRAF5 act redundantly with TRAF6 to promote ubiquitination reactions that recruit NEMO to the MAVS signaling complex, which then turns on IKK and TBK1, leading to the activation of NF-κB and IRF3 (76) (**Figure 2**). In support of this model, mutations of both TRAF2/5 and TRAF6 binding sites of MAVS, but not each alone, abolished IRF3 and IKK activation by viral infection. In addition, IRF3 activation was completely abolished in cells lacking TRAF2, TRAF5, and TRAF6, but not each alone (76). Future work is needed to identify the ubiquitination target(s) of these TRAF proteins as well as other components in the MAVS signaling complex.

Regulators of RLRs. RLR-mediated innate immune signaling is tightly regulated to effectively control infections while minimizing autoimmune damage. Ubiquitin ligases and deubiquitination enzymes regulate the RLR pathway through synthesizing or removing polyubiquitin chains (77). As mentioned above, TRIM25 and Riplet are positive regulators of RIG-I and function by synthesizing K63 polyubiquitin chains (48, 49, 51). The functions for TRIM25 and Riplet seem nonredundant, as deficiency in either leads to a severe defect in IFN induction. It is possible that they play sequential or cooperative roles in ubiquitination events that eventually lead to RIG-I activation. Two other ubiquitin ligases, ring-finger protein 125 (RNF125) and c-Cbl, are negative regulators of RIG-I and mediate K48-linked polyubiquitination of RIG-I, leading to its degradation by the proteasome (78, 79). In addition, RNF125 could also conjugate ubiquitin to MDA5 and MAVS (78). Deubiquitination enzymes such as cylindromatosis (CYLD) and ubiquitin-specific protease 4 (USP4) are also involved in the regulation of RIG-I. A microarray study showed that CYLD has an expression profile that correlates with that of RIG-I (80). CYLD also physically interacts with RIG-I and removes K63-linked polyubiquitin chains from RIG-I to attenuate antiviral responses (80). As opposed to CYLD, USP4 functions as a positive regulator through deubiquitinating K48-linked ubiquitin chains and stabilizing RIG-I (81).

Other posttranslational modifications have been implicated in regulating RLRs. Phosphorylation at Ser-8 and Thr-170 on the CARDs of RIG-I negatively regulates the activity of RIG-I (82, 83). MDA5 phosphorylation at Ser-88 also inhibits its activity (84). Using an RNAi screen, a study further identified PP1α and PP1γ as the primary phosphatases responsible for MDA5 and RIG-I dephosphorylation, which is important for their activation (84). Covalent modification of RIG-I by the ubiquitin-like protein ISG15 and SUMO may also play a role in regulating RIG-I activity (85, 86).

Apart from covalent modifications, several proteins regulate RLR signaling through other mechanisms. FAK, a focal adhesion kinase that transmits signals between the extracellular matrix and the cytoplasm, functions as an RLR signaling component by facilitating MAVS activation (87). ZAPS, the shorter isoform of poly(ADP-ribose) polymerase 13 (PARP-13), associates with RIG-I to promote its oligomerization and ATPase activity (88). The dsRNA-binding protein PACT physically binds to the CTD of RIG-I and augments RIG-I-induced type I IFN production (89). Ribonucleoprotein PTB-binding 1 (RAVER1) specifically interacts with and activates MDA5 without affecting RIG-I activity (90). Several additional proteins, such as mitochondrial-targeting chaperone 14-3-3ε (91), ankyrin repeat protein ankrd17 (92), and receptor for globular head

domain of complement component C1q (gC1qR) (93), also contribute to the increasing complexity of RLR regulation. In most cases, however, the proposed role of these proteins in the RLR pathway requires validation by genetic experiments.

Other cytosolic RNA sensors. Even before the discovery of RLRs, several proteins were reported to recognize dsRNA and regulate antiviral responses. These include IFN-inducible 2′–5′-oligoadenylate synthetase (OAS) and dsRNA-dependent protein kinase R (PKR) (94) (**Figure 2**). When activated by binding to dsRNA (95), OAS catalyzes the conversion of ATP to 2′–5′-linked oligoadenylates, which in turn bind to and activate the latent ribonuclease RNase L. This activity then degrades ssRNAs of viral and cellular origin (96). RNase L cleavage products further induce type I IFN production through the RIG-I pathway (37, 38). PKR is a serine/threonine kinase that is activated by binding to dsRNA. Activated PKR suppresses translation initiation by phosphorylating the α-subunit of eukaryotic initiation factor 2 (97). Although PKR is involved primarily in inhibiting viral replication (97, 98), it has also been implicated in type I IFN induction by dsRNA in some cell types (19, 99).

Cytosolic Sensing of DNA

DNA was known to trigger immune responses more than a century ago, but the mechanism was not understood until recently. Although CpG DNA can induce type I IFNs through TLR9 in plasmacytoid dendritic cells (pDCs), many cells that do not express TLR9 can robustly produce type I IFNs when exogenous DNA is delivered into the cytoplasm (100, 101). This TLR9-independent cytosolic pathway, which detects intracellular DNA in a sequence-independent manner, functions in a broad range of cell types including mouse embryonic fibroblasts (MEFs), pDCs, conventional dendritic cells, and bone marrow–derived macrophages (100). Research in the past few years has led to significant progress toward understanding the mechanism of cytosolic DNA sensing and signaling, culminating in the recent discoveries of the cytosolic DNA sensor, adaptor, and a new second messenger that mediates signal transduction in this pathway.

RNA polymerase III/RIG-I pathway. In one of the initial studies, B-form dsDNA poly(dA:dT) was shown to activate type I IFNs and NF-κB in a MAVS-dependent manner (101). Subsequently, a report found that both RIG-I and MAVS were required for poly(dA:dT)-induced IFN-β promoter activation in a human hepatoma cell line (Huh-7) (102). In addition, overexpression of RLRs could sensitize cells to stimulation by B-DNA, and RIG-I was shown to co-immunoprecipitate with B-DNA (103). This is surprising because genetic studies have shown that cytosolic DNA can induce IFN production in mouse cells lacking RIG-I or MAVS, which indicates that the DNA signaling pathway is distinct from the RIG-I-MAVS pathway (60, 101). Thus, the RIG-I/MAVS-dependent induction of IFN-β by poly(dA:dT) in certain human cell lines raises the question of how DNA may activate the RNA sensor RIG-I.

This conundrum was resolved by subsequent studies showing that transfected poly(dA:dT) could be converted into RNA that bears 5′-triphosphate and double-stranded secondary structures (104, 105). As described above, this form of RNA serves as the bona fide ligand for RIG-I to trigger type I IFN production through MAVS (**Figure 2**). Biochemical fractionation of cytosolic extracts led to the identification of DNA-dependent RNA polymerase III (Pol-III) as the enzyme responsible for synthesizing 5′-triphosphate RNA from the poly(dA:dT) template (104). The employment of Pol-III as a DNA sensor may allow the host to take advantage of the RIG-I-MAVS pathway to defend against certain bacteria and DNA viruses. However, the strict dependence of Pol-III on AT-rich sequence and the fact that DNA induces IFN production in a

sequence-independent manner suggest the existence of another more general cytoplasmic DNA-sensing pathway.

STING is the adaptor for cytosolic DNA signaling. In complementary DNA (cDNA) expression screens to identify genes that could activate the IFN-β promoter, several groups independently identified stimulator of IFN genes (STING, also known as MITA, MPYS, ERIS, and TMEM173) as a crucial signaling adaptor for type I IFN induction (106–108). STING is a predominantly endoplasmic reticulum–localized protein that was predicted to contain five transmembrane helices (TM1–TM5) and a large cytosolic domain (amino acids 173–379 in humans) (106) (**Figure 3**). However, recent structural studies revealed that the previously assigned TM5 is not a transmembrane domain but a folded, soluble domain that mediates dimerization (109, 110). RNA blot analyses showed that STING has a broad tissue distribution (107). Overexpression of STING in HEK293T cells, which do not express detectable endogenous STING, strongly activated transcription factors IRF3 and NF-κB and robustly induced IFN-β production. Conversely, STING deficiency in various cell types, such as MEFs, macrophages, and DCs, abolished IFN-β production after dsDNA stimulation or DNA virus infection (111). STING knockout mice are highly susceptible to lethal infection by herpes simplex virus 1 (HSV-1), demonstrating that STING is essential for host defense against DNA virus in vivo (111). STING was also reported to mediate type I IFN induction by cytosolic RNA (107, 108). However, later studies did not support this model (112).

Once activated by cytosolic DNA signaling, STING undergoes a dramatic relocalization from the endoplasmic reticulum to the Golgi complex and assembles into punctate structures that contain the kinase TBK1 (111, 113). This process may somehow stimulate TBK1, resulting in the phosphorylation of IRF3. In support of this, we recently showed that the C-terminal tail (CTT) of human STING containing only 39 amino acid residues (341–379) was necessary and sufficient to activate TBK1 and recruit IRF3 to TBK1 (114). The signal-dependent recruitment of TBK1 and IRF3 by STING was further confirmed in DNA-stimulated L929 cells. Thus, STING CTT may be sequestered in the absence of stimulation, whereas DNA-induced upstream signaling leads to the release of CTT (115). Further structural studies are needed to provide direct evidence for CTT rearrangement in response to upstream signaling. STING was also reported to recruit STAT6 for its phosphorylation of Ser407 by TBK1. This leads to the induction of STAT6-dependent chemokines, including CCL2, CCL20, and CCL26 (116).

STING directly senses bacterial cyclic dinucleotides. Cyclic dinucleotides (CDNs), traditionally including cyclic (3′–5′) diguanylate (c-di-GMP) and cyclic (3′–5′) diadenylate (c-di-AMP), are bacterial second messengers with a regulatory role in several processes, such as biofilm formation, virulence, and DNA integrity surveillance (117, 118). Enzymes responsible for producing these cyclic dinucleotides are found widely distributed in most bacterial genomes. Specifically, cyclases harboring the GGDEF domains and DUF147 domains generate c-di-GMP and c-di-AMP, respectively (117, 118). Recently, a hybrid molecule called cyclic (3′,5′)AMP-(3′,5′)GMP was identified in *Vibrio cholerae* and shown to be important for its virulence (119).

The widespread distribution and essential roles in bacterial life cycles make CDNs ideal PAMPs for host detection of bacterial infection. Indeed, the presence of CDNs in the cytosol of mammalian cells leads to TBK1 and IRF3 activation and type I IFN production (120, 121). The gene expression profile induced by CDNs is indistinguishable from that of DNA-stimulated cells (112). Genetic studies demonstrated that STING is required for the type I IFN response to both c-di-GMP and c-di-AMP (112, 122) (**Figure 3**). An important recent study has demonstrated that STING is the sensor for CDNs (123). STING was shown to directly bind to

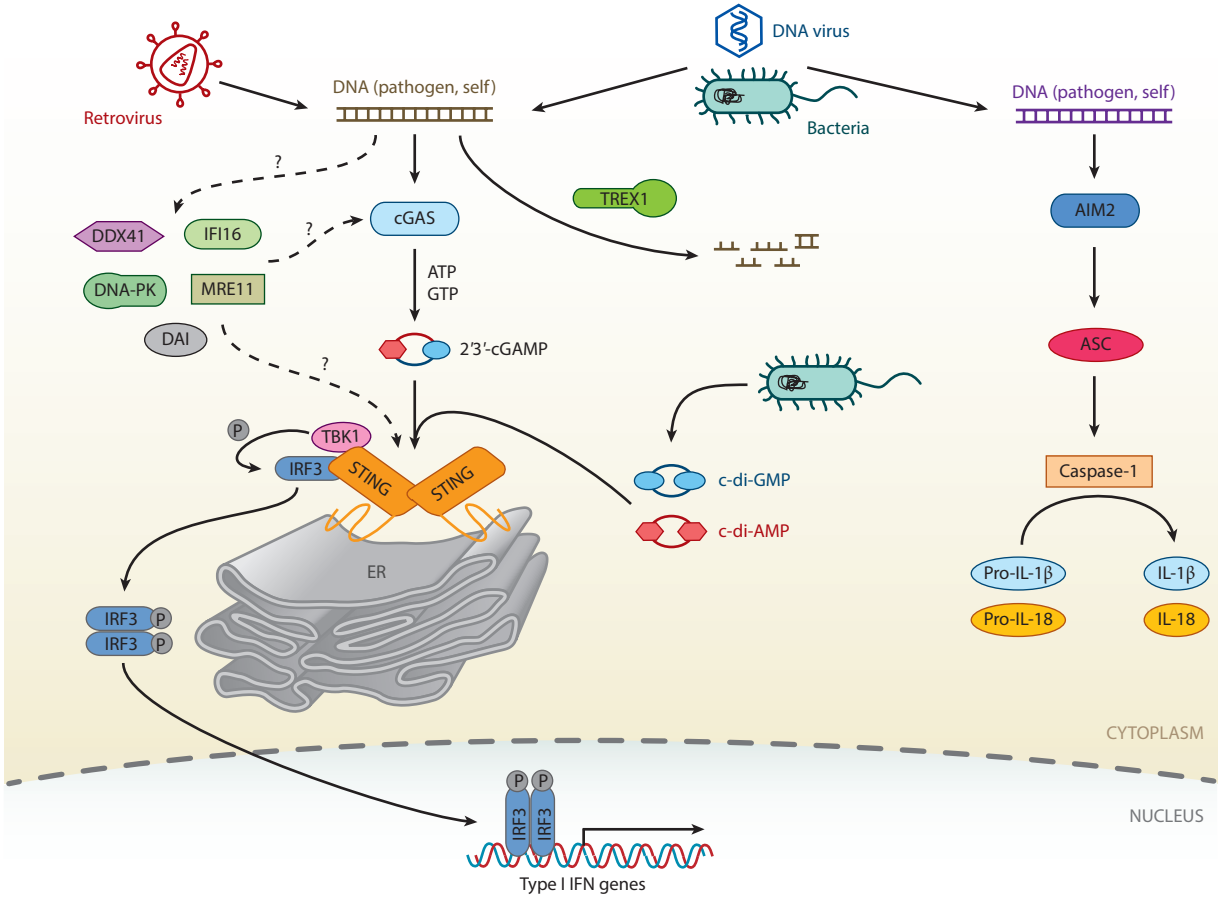


Figure 3

Cytosolic DNA–sensing system. Cytoplasmic DNA of self or microbial origin could activate cGAS and potentially other putative DNA sensors, which are all proposed to transduce signals to the ER-localized adaptor protein STING. STING recruits and activates the kinase TBK1, which then activates the transcription factor IRF3 to induce type I IFNs. Bacterial second messenger c-di-GMP and c-di-AMP are directly sensed by STING. Endogenous DNase, such as Trex1, could degrade self-DNA to prevent the aberrant activation of the DNA-sensing pathway. In addition to inducing type I IFNs, cytosolic DNA could also activate the AIM2 inflammasome, leading to caspase-1 activation and IL-1β maturation. (Abbreviations: AIM2: absent in melanoma 2; c-di-AMP, cyclic diadenylate; c-di-GMP, cyclic diguanylate; cGAMP, cyclic GMP-AMP; cGAS, cyclic GMP-AMP synthase; DAI, DNA-dependent activator of IRFs; DDX41, DEAD box polypeptide 41; DNA-PK, DNA-dependent protein kinase; ER, endoplasmic reticulum; IFI16, interferon gamma-inducible protein 16; IFN, interferon; MRE11, meiotic recombination 11; STING, stimulator of interferon genes; Trex1, three prime repair exonuclease 1.)

c-di-GMP with a K_d of 4.9 μM. Expression of STING was sufficient to restore c-di-GMP responses in HEK293T cells, which do not express endogenous STING. A series of subsequent studies solved the crystal structure of the cytosolic c-di-GMP binding domain (CBD) of human STING both alone and in complex with c-di-GMP (109, 110, 124–126). The CBD exhibits an α + β fold and forms a dimer in the crystal and in solution. A c-di-GMP molecule sits in the central crevice of the STING dimeric interface, using a set of π-π stacking and hydrogen-bonding

interactions (110). However, in these structures, the binding of c-di-GMP to STING did not cause an obvious conformational change that could be responsible for activating downstream signaling. This may be in part due to the use of a rare H232 variant of human STING, which was later shown to be functionally compromised (127), for crystallization in most of these studies. In addition, the CTT of STING is not visible in all of these structures, even though it is included in the protein fragments (amino acids 139–379) used for crystallization. Although further work is needed to understand how STING is activated by binding to CDNs, the structural studies provide strong supporting evidence for the direct binding between STING and CDNs (**Figure 3**).

Cyclic GMP-AMP synthase. Although it is clear that STING is a direct sensor of CDNs and an important adaptor for type I IFN induction by cytosolic DNA, the identity of the cytosolic DNA sensor remained unresolved until recently. Using biochemical purification and quantitative mass spectrometry, cyclic GMP-AMP synthase (cGAS) has been identified as a sensor for cytosolic DNA (128, 129) (**Figures 3** and **4**). cGAS belongs to the nucleotidyltransferase (NTase) family and exhibits structural and sequence homology to the catalytic domain of OAS. Sequence alignment revealed that the C-terminal NTase and Mab21 domains are highly conserved in vertebrates, whereas the N-terminal sequence is less conserved (128). When activated by DNA through direct binding, cGAS catalyzes the production of cyclic GMP-AMP (cGAMP) from ATP and GTP. cGAMP in turn functions as an endogenous second messenger to activate STING (129) (**Figures 3** and **4**). This discovery unified our understanding of the role of STING in cytosolic response to DNA and CDNs (130). Overexpression of cGAS activated the transcription factor IRF3 and induced IFN-β in a STING-dependent manner. Depletion of cGAS by RNAi or the TALEN technology in mammalian cell lines abolished both IRF3 activation and IFN-β production induced by DNA transfection or DNA virus infection (131). Furthermore, various cell types, including macrophages, fibroblasts, and DCs, from cGAS-deficient mice showed a complete loss of type I IFN production in response to cytosolic DNA (132), demonstrating that cGAS functions as a nonredundant cytosolic DNA sensor in the type I IFN pathway. Consistently, cGAS knockout mice were more sensitive to HSV-1 killing than their wild-type littermates (132). Interestingly, a subsequent study suggested that cGAS may also contribute to the innate control of RNA virus, as cGAS knockout mice were more vulnerable to lethal WNV infection (133). It is possible that RNA virus–induced tissue damage leads to the release of host DNA, which activates cGAS-STING pathway to potentiate host defense responses. Very recently, cGAS was also shown to mediate the innate immune response to HIV and other retroviruses by detecting reverse-transcribed viral cDNA (131, 134, 135).

Structural studies of cGAS. Several groups solved the crystal structure of cGAS alone or in complex with dsDNA (136–138, 180, 181) (see **Figure 4*a*,*b*** and the Note Added in Proof), which elucidated the structural mechanism of how DNA binding leads to the activation of cGAS. Upon dsDNA binding, cGAS undergoes a significant conformational change, making the catalytic pocket accessible for generation of cGAMP (**Figure 4*c***). The catalytic reaction proceeds in a two-step manner through initial formation of the intermediate pppGpA, followed by cyclization to cGAMP (136). The binding affinity of cGAS to dsDNA and ssDNA was determined to be K_d ~87.6 nM and K_d ~1.5 μM, respectively (137). Mutations in the DNA-binding or catalytic residues of cGAS led to reduction or loss of IFN-β-inducing activity. The structure also revealed a unique but conserved zinc ribbon domain insertion in the C termini of all vertebrate cGAS, which is essential for its activation (137, 138).

2′3′-cGAMP is an endogenous second messenger. Although the initial report unambiguously identified cGAMP as the endogenous second messenger produced by cGAS in

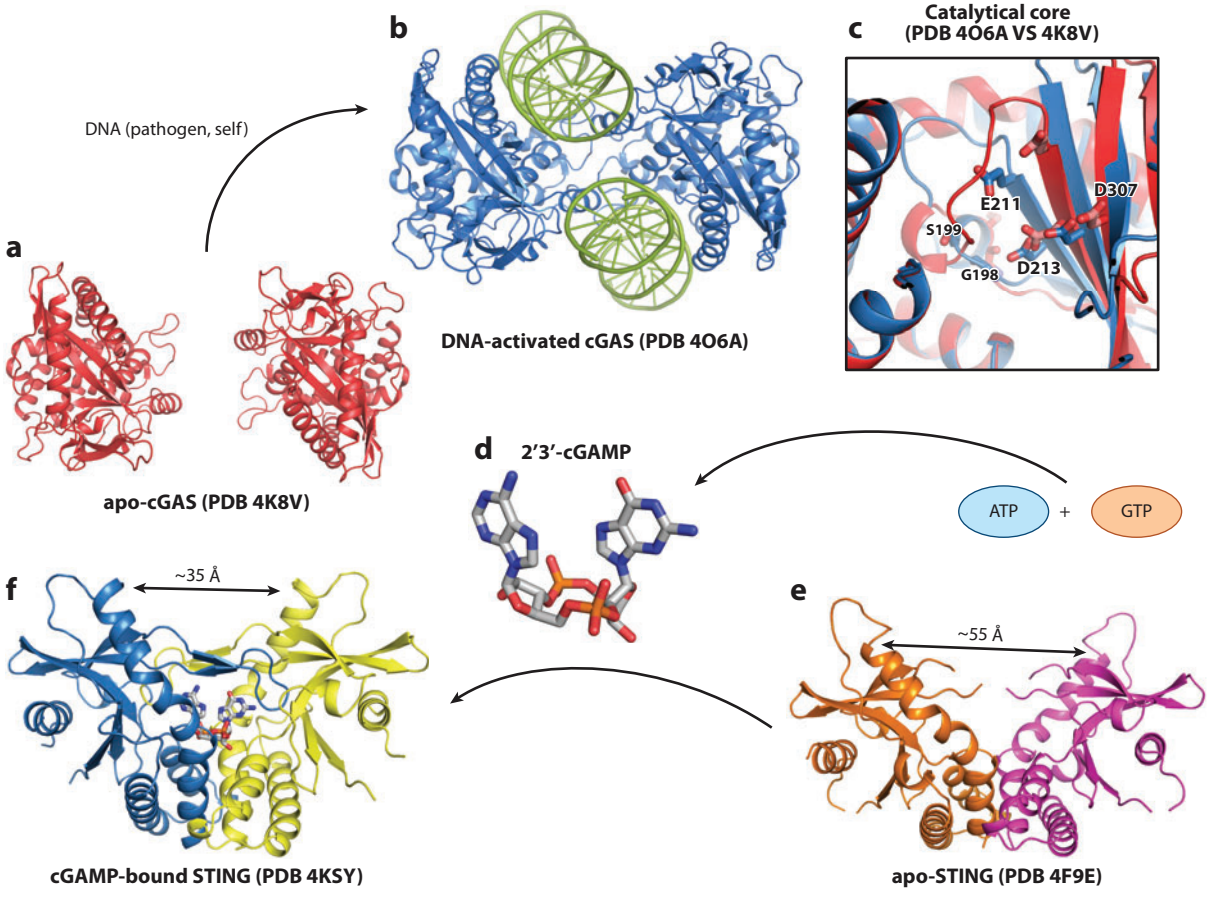


Figure 4

Structural basis for cGAS-cGAMP-STING pathway. (*a–c*) Binding of cytosolic DNA to two distinct positively charged surfaces of cGAS induces dimerization and conformational rearrangement of cGAS active site. (*d*) This results in the relocation of the catalytic residues and formation of a highly accessible nucleotide-binding pocket for the generation of 2′3′-cGAMP. (*e*,*f*) 2′3′-cGAMP then binds STING and triggers a conformational change that may underlie its activation. (Abbreviations: cGAMP, cyclic GMP-AMP; cGAS, cyclic GMP-AMP synthase; STING, stimulator of interferon genes.)

DNA-stimulated cells, the exact nature of the internal phosphodiester linkages between GMP and AMP in cGAMP remained to be determined (129). In a series of subsequent studies, we and others independently addressed this question using different approaches (127, 136, 139, 140). These studies led to the same conclusion that the cGAS product contained mixed phosphodiester linkages, with one between 2′-OH of GMP and 5′-phosphate of AMP and the other between 3′-OH of AMP and 5′-phosphate of GMP (**Figure 4*d***). This endogenous second messenger, abbreviated as 2′3′-cGAMP, is unique in that it binds to STING with a K_d of ~4 nM, which is several hundred–fold lower than that of bacterial c-di-GMP (127). Consistently, 2′3′-cGAMP is much more potent in inducing IFN-β than are bacterial CDNs (127, 129). In addition, 2′3′-cGAMP could activate mouse STING R231A mutant, whereas other CDNs did not (139), explaining a previous observation that mouse STING R231A could support IFN-β induction by DNA but not by CDNs (123). The potent induction of type I IFNs and other cytokines by 2′3′-cGAMP suggests that this molecule could be used as a vaccine adjuvant. Indeed, we have recently shown

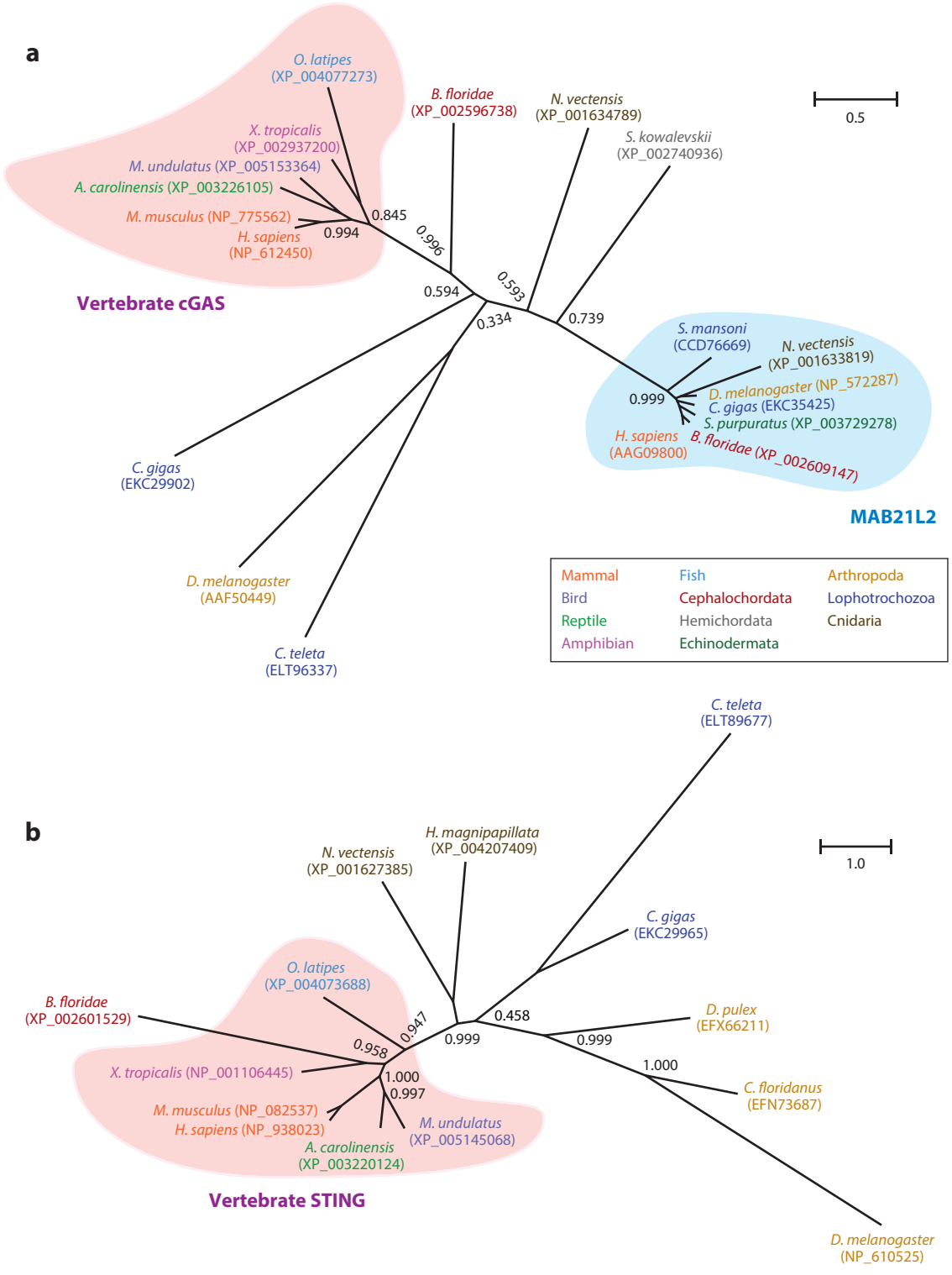
that 2′3′-cGAMP boosts antigen-specific T cell activation and antibody production in mice (132). As a second messenger, 2′3′-cGAMP can also function nonautonomously by spreading from producing cells to neighboring cells through gap junctions (141). This intercellular communication through the second messenger may provide a rapid response mechanism that protects neighboring cells from virus infection, especially when some viruses produce antagonists that prevent the production of type I IFNs in the virus-infected cells.

A recent study suggested that cGAMP can also act on adenosine monophosphate–activated protein kinase (AMPK) and Unc51-like autophagy activating kinase 1 (ULK1) to trigger a negative-feedback control of STING activity (142). It was proposed that AMPK normally phosphorylates and inhibits ULK1 and that cGAMP induces dephosphorylation of AMPK at Thr-172, which inactivates AMPK. Inactivation of AMPK relieves its inhibition of ULK1, allowing ULK1 to phosphorylate STING at Ser-366, which was suggested to suppress STING activity. However, this model contradicts previous studies showing that ULK1 is activated (rather than inhibited) by AMPK-mediated phosphorylation (143, 144). In addition, the mutation of Ser-366 to either alanine or aspartic acid inactivates STING (114, 142), which indicates that Ser-366 is critical but leaves open the question of whether phosphorylation at this residue positively or negatively regulates the activity of STING.

Structures of STING bound to cGAMP. The crystal structure of 2′3′-cGAMP in complex with human STING CBD revealed several remarkable differences from the structures of STING-CBD in its apo form and in complex with c-di-GMP (110, 127). First, 2′3′-cGAMP sits ∼2.5 Å deeper than does c-di-GMP in the crevice of the STING dimer. Second, the two wings of the butterfly-shaped STING dimer move closer by ∼20 Å (**Figure 4*e*,*f***). Third, the cGAMP binding site is covered by a lid of four antiparallel β sheet strands, a segment disordered in the apo form of STING or c-di-GMP-bound STING (127) (**Figure 4*f***). This dramatic conformational change of human STING induced by 2′3′-cGAMP binding, which may underlie the mechanism of its activation, was further confirmed by a later study that solved the structure of 2′3′-cGAMP-bound human STING H232 variant and mouse STING (145). Additionally, one of the STING-c-di-GMP structures using wild-type STING also revealed a similar conformational change of STING induced by c-di-GMP binding (124).

Evolution of cGAS and STING. Detection of foreign DNA by cGAS and the resulting production of endogenous cGAMP vastly expand the repertoire of pathogens detectable by the innate immune system, from CDN-producing bacteria to potentially any microorganism that carries or generates DNA in its life cycle. It remains to be determined whether STING evolved first to detect bacterial CDNs or endogenous 2′3′-cGAMP produced as a result of cGAS activation by DNA from different microbes. The fact that endogenous 2′3′-cGAMP binds to STING with a much higher affinity and serves as a more potent activator of IFN seems to suggest that the cGAS-cGAMP-STING pathway has a selective advantage through evolution.

To gain more insights into the evolution of cGAS and STING, we search individual genomes of major animal phyla for homologs of human cGAS and STING proteins using BlastP. Whereas in vertebrates cGAS homologs are confidently identified and all contain the conserved residues involved in catalysis and DNA binding, the invertebrate cGASs are more orthologous to human protein MAB21L2 (**Figure 5*a***). Interestingly, cGAS from the cephalochordate *Branchiostoma floridae* also contains key catalytic residues and is in close proximity to the highly conserved vertebrate cGAS cluster in the phylogenetic tree (**Figure 5*a***). This suggests that cGAS originated during the transition from invertebrates to vertebrates. In contrast, STING proteins are found in almost all phyla, indicating a disparate evolution pattern to cGAS. However, further phylogenetic


a
O. latipes (XP_004077273)
B. floridae (XP_002596738)
N. vectensis (XP_001634789)
S. kowalevskii (XP_002740936)
0.5
X. tropicalis (XP_002937200)
M. undulatus (XP_005153364)
A. carolinensis (XP_003226105)
M. musculus (NP_775562)
H. sapiens (NP_612450)
0.845
0.994
0.996
0.593
0.594
0.334
0.739
Vertebrate cGAS
S. mansoni (CCD76669)
N. vectensis (XP_001633819)
D. melanogaster (NP_572287)
C. gigas (EKC35425)
S. purpuratus (XP_003729278)
B. floridae (XP_002609147)
H. sapiens (AAG09800)
0.999
MAB21L2
C. gigas (EKC29902)
D. melanogaster (AAF50449)
C. teleta (ELT96337)
Mammal
Bird
Reptile
Amphibian
Fish
Cephalochordata
Hemichordata
Echinodermata
Arthropoda
Lophotrochozoa
Cnidaria
b
C. teleta (ELT89677)
H. magnipapillata (XP_004207409)
N. vectensis (XP_001627385)
1.0
C. gigas (EKC29965)
O. latipes (XP_004073688)
B. floridae (XP_002601529)
0.947
0.958
0.458
0.999
0.999
D. pulex (EFX66211)
1.000
X. tropicalis (NP_001106445)
1.000
0.997
C. floridanus (EFN73687)
M. musculus (NP_082537)
H. sapiens (NP_938023)
M. undulatus (XP_005145068)
A. carolinensis (XP_003220124)
Vertebrate STING
D. melanogaster (NP_610525)

analysis of STING proteins from representative species reveals that cephalochordate and vertebrate STINGs form a group that is distant from those in invertebrates (**Figure 5*b***). Thus, it remains to be determined if these invertebrate STINGs are functional sensors for CDNs. It is still possible that both full-fledged cGAS and STING emerge from the vertebrate lineage, functioning cooperatively to detect pathogen infections.

Other putative cytoplasmic DNA sensors. Before the discovery of cGAS as a nonredundant and general cytosolic DNA sensor that activates STING, several other proteins were suggested as candidates for cytosolic DNA sensors. These proteins have been extensively reviewed elsewhere (115, 146), so they are discussed only briefly below.

DAI. DNA-dependent activator of IRFs (DAI; also known as ZBP1 or DLM1) was the first putative DNA sensor suggested to be involved in type I IFN induction (147) (**Figure 3**). DAI contains a Z-α and a Z-β domain at the N terminus, which are defined as Z-DNA-binding domains. The D3 domain that follows Z-β largely contributes to its B-DNA binding. Overexpression of DAI enhanced the DNA-mediated induction of type I IFNs, whereas RNA interference of DAI in L929 cells inhibited IFN induction. DAI could associate with TBK1 and IRF3, and this association was enhanced upon DNA stimulation. Although these data suggest that DAI functions as a cytosolic DNA sensor, later studies using DAI knockout mice showed that DAI-deficient MEFs and mice mount normal type I IFN responses to DNA stimulation (148). Thus, DAI may not function as an indispensable or nonredundant sensor for cytosolic DNA. Whether it plays other regulatory roles in this pathway requires additional studies.

IFI16/p204. IFI16 was identified by dsDNA affinity pull-down using cytosolic extracts from human THP-1 monocytes (149) (**Figure 3**). IFI16 and its mouse ortholog p204 are members of the PYHIN protein family that contain a pyrin domain and two DNA-binding HIN domains. siRNA-mediated knockdown of IFI16 or p204 partially inhibited DNA or HSV-1-induced IRF3 activation and IFN-β production. Although predominantly a nuclear protein, a small fraction of IFI16 could be detected in the cytoplasm, which colocalizes with transfected DNA. The recruitment of STING to IFI16 could also occur upon DNA transfection (149). In addition, IFI16 senses HSV-1 or Kaposi's sarcoma–associated herpes virus (KHSV)-derived DNA in the nucleus (150, 151). However, some groups failed to detect any effect of IFI16/p204 knockdown on IFN-β induction by cytoplasmic DNA (128, 152, 153). Thus, the exact role of IFI16 in DNA sensing requires further investigation, preferably in p204 knockout mice or in IFI16 knockout human cells.

DDX41. In an RNAi screen of 59 members of the DExD/H family of helicases, DDX41 was found to be required for induction of type I IFNs by cytosolic DNA in a mouse DC line (D2SC cells)

Figure 5

Phylogenetic analyses of (*a*) cyclic GMP-AMP synthase (cGAS) and (*b*) stimulator of interferon genes (STING). cGAS and STING proteins from representative species were obtained by NCBI BlastP search. STING homologs were found throughout metazoa. Although cGAS homologs were identified with certainty in vertebrates (*red*), the invertebrate cGASs are likely to be orthologs of human protein MAB21L2 (*blue*). Multiple sequence alignments of cGAS and STING from different species were generated using ClustalW. Alignments were adjusted manually before loading to the PhyML program for phylogenetic analysis. The evolutionary tree was built with LG as the substitution model, four discrete rate categories for the rate heterogeneity among sites, Nearest Neighbor Interchange for the tree improvement, and SH-like approximate likelihood-ratio test for estimating the branch support. Bootstrap support values are shown for major branches. Trees were drawn using Dendroscope software.

and human THP-1 cells (154) (**Figure 3**). Knockdown of DDX41 inhibited the induction of type I IFNs by cytosolic DNA and HSV-1. DDX41 directly binds to various DNA through its DEADc domain. In addition, DDX41 colocalizes with STING in the cytoplasm when overexpressed in HEK293T cells. Two recent reports from the same group further extended the role of DDX41 in STING-dependent immune responses: One showed that DDX41 can also bind to and function as a director sensor for c-di-GMP and c-di-AMP (155), and the other reported that the E3 ubiquitin ligase TRIM21 could target DDX41 for degradation, thereby negatively regulating DNA-induced innate immunity (156). Although these data suggest that DDX41 plays a role in recognizing DNA and CDNs, other groups found that depletion of DDX41 by RNAi had little effect on IFN-β induction by DNA stimulation or DNA virus infection (128, 153, 157, 158). Genetic studies using DDX41 knockout mice are needed to clarify the role of this protein in vivo.

DNA-PK and MRE11. Several proteins, well known to be involved in the DNA damage response, have also been implicated in cytosolic DNA sensing. DNA-dependent protein kinase (DNA-PK) is a heterotrimeric complex composed of three subunits: Ku70, Ku80, and the catalytic subunit DNA-PKcs. This complex was identified as a potential DNA sensor in affinity pull-down experiments using biotinylated DNA in HEK293T cells (**Figure 3**). Further studies using DNA-PKcs-deficient MEFs or mice showed attenuated but not abolished cytokine production in response to DNA or DNA virus stimulation (159). Interestingly, Ku70, another member of the complex, is also involved in cytosolic DNA–induced IFN-λ1 production in HEK293 cells (160). Recently, the DNA damage sensor meiotic recombination 11 homolog A (MRE11) was reported to be a cytosolic dsDNA sensor that activates the STING pathway (**Figure 3**). Taking advantage of cells derived from a patient with ataxia-telangiectasia-like disorder, in which the MRE11 protein is truncated and destabilized owing to a genetic mutation, Kondo et al. (161) found that MRE11 is required for type I IFN induction following cytosolic DNA stimulation but not HSV-1 infection. This finding suggests a possible link between the DNA damage response and DNA-induced immune response.

STING. A recent study suggested that STING could function as a direct sensor for DNA (153) (**Figure 3**). The C terminus of STING (animo acids 181–379) was found to associate with dsDNA without the requirement of other proteins. However, the binding affinity of STING to dsDNA ($K_d \sim 200$–300 μM) was several orders of magnitude lower than that of cGAS ($K_d \sim 88$ nM). Moreover, reconstitution of STING in HEK293T cells, which do not express endogenous STING, could restore IFN-β induction in response to CDNs but not to dsDNA, indicating that STING is insufficient to confer DNA sensing in cells (123, 129).

Cytosolic DNA-induced inflammasome activation. All the pathways discussed above culminate in the transcriptional regulation of type I IFNs and other inflammatory cytokines, yet the presence of DNA in the cytosol of macrophages also triggers the activation of the inflammasome, an intracellular multiprotein complex that mediates the activation of the proteolytic enzyme caspase-1 and the maturation of IL-1β (162). Several groups identified AIM2 (absent in melanoma 2) as the receptor for cytosolic DNA in the inflammasome pathway (163–166) (**Figure 3**). As a member of the PYHIN protein family (see IFI16 section above), AIM2 also contains an N-terminal pyrin domain (PYD) but only one C-terminal HIN200 domain. Upon DNA binding via its HIN200 domain, AIM2 associates with the adaptor protein ASC (apoptosis-associated speck-like protein containing a CARD) through a homotypic PYD:PYD interaction. ASC, which contains a CARD domain, further recruits and activates caspase-1 through a homotypic CARD:CARD interaction. Caspase-1 in turn processes the inactive precursors of IL-1β and IL-18 into mature cytokines

(**Figure 3**). The activation of caspase-1 can also result in a rapid inflammatory form of cell death called pyroptosis. RNAi of AIM2 impaired DNA-induced maturation of IL-1β in murine macrophages and human THP-1 cells. Reconstitution of unresponsive HEK293 cells with components of the pathway could restore AIM2-induced IL-1β maturation (165). Later studies using AIM2 gene–deleted mice not only confirmed the role of AIM2 as the DNA sensor for inflammasome activation but also revealed the importance of AIM2 in host defense against pathogens such as *Francisella tularensis* (167–169). Recently, the putative DNA sensor IFI16 was also shown to interact with ASC and procaspase-1 to form an inflammasome in the nucleus during KHSV infection of endothelial cells (151).

Cytosolic DNA sensing and autoimmunity. Although sensing foreign DNA in the cytosol allows the host to effectively detect a broad range of pathogens, this strategy is built on the potential recognition of self-DNA, which can lead to to autoimmunity. Three major mechanisms have been developed by the host to avoid inappropriate detection of endogenous DNA. First, the host receptor may preferentially recognize structures or modifications that are only present in the pathogen genome. Second, the distribution of the receptor may be restricted to certain subcellular compartments that are free of self-DNA. Third, the deployment of several endogenous nucleases may keep the level of self-DNA under the threshold of receptor activation. Although the first two mechanisms are well represented in the case of TLR9, which detects bacterial unmethylated CpG DNA in the lumen of endosomes, accumulating evidence has revealed the importance of the third mechanism in preventing self-DNA detection in the cytosol.

3′ repair exonuclease 1 (Trex1; also known as DNase III) is the major 3′ DNA exonuclease in mammalian cells. Mutations in the human Trex1 gene can cause Aicardi-Goutières syndrome, an encephalopathy exhibiting phenotypic overlaps with the autoimmune disease systemic lupus erythematosus (170). Trex1 knockout mice display inflammatory myocarditis that leads to dramatically reduced survival (171). Mechanistically, lack of Trex1 results in cytosolic accumulation of DNA derived from endogenous retroelements or replication debris (172, 173) (**Figure 3**). These endogenous DNA substrates further activate the STING-dependent cytosolic DNA-sensing pathway, triggering type I IFN–dependent autoimmune diseases. Remarkably, when the Trex1 gene–deleted mice were crossed with mice deficient in STING, the resultant double-knockout mice were completely rescued from mortality and autoimmune tissue destruction (174). In accordance with its role in digesting reverse-transcribed DNA from endogenous retroelements, Trex1 could also degrade retroviral (e.g., HIV) cDNA to prevent its recognition by cGAS, thus suppressing the host IFN response against HIV (131, 175).

DNase II, a lysosome-localized endonuclease that degrades DNA from apoptotic cells and expelled nuclei of erythroid precursors, also contributes to protecting the host from DNA-induced autoimmunity (176). Mice lacking DNase II die during embryonic development, owing to the overproduction of type I IFNs induced by undigested DNA accumulated in macrophages. The embryonic lethality is rescued by type I IFN receptor deficiency, but mice develop a chronic polyarthritis resembling human rheumatoid arthritis by 2 months after birth because of the production of other inflammatory cytokines such as TNF-α (176, 177). However, when DNase II knockout mice were crossed to mice deficient in TLR9, IFN-β was still produced in the fetal liver, suggesting that TLR9 signaling may not be responsible for the innate immune response induced by endogenous undigested DNA (178). Strikingly, a recent study found that mice deficient in both DNase II and STING were rescued from not only embryonic lethality but also polyarthritis (179). This discovery further highlights the crucial role of cytosolic DNA sensing in the pathogenesis of autoimmune diseases.

CONCLUSIONS AND PERSPECTIVES

The past decade has witnessed tremendous progress in the understanding of innate recognition of pathogen-derived nucleic acids and their central role in initiating host defense responses. Research in the field of cytosolic nucleic acid sensing has been very fruitful, as represented by the discovery of the RLR-MAVS pathway for cytoplasmic RNA sensing and the cGAS-cGAMP-STING pathway for cytosolic DNA recognition. Extensive studies using diverse approaches in biochemistry, genetics, and structural biology have revealed the details of ligand recognition by the receptors and signal-transducing events in these pathways. These studies also reveal new signaling mechanisms in innate immunity, such as the recognition of 5′-triphosphorylated RNA by RIG-I, the prion-like polymerization of MAVS and the generation of a second messenger (i.e., 2′3′-cGAMP). Future work is needed to uncover new components and regulatory mechanisms of these pathways. In addition, it will also be important to gain insights into some unresolved questions, such as the mechanism of MDA5 activation, the role of STING translocation, and the mechanisms by which the RIG-I and cGAS signaling pathways are turned off.

As a sequence-independent DNA sensor, cGAS is in principle capable of detecting all microorganisms that harbor or generate DNA in their life cycles. Indeed, genetics studies have already shown that cGAS is indispensable for triggering innate immune responses against several DNA viruses and retroviruses. It remains to be seen whether cGAS is responsible for detecting DNA from various bacteria, parasites, and fungi. However, because these microorganisms likely generate multiple PAMPs (e.g., many bacteria produce CDNs as well as TLR ligands), considerable effort is needed to tease apart the contributions of different pathways to the overall innate and adaptive immune responses.

Although cytosolic detection of microbial nucleic acids has a fundamental role in host defense, it is also becoming clear that loss of negative regulation of cytosolic DNA sensing will lead to the aberrant recognition of self-DNA, which is strongly associated with the pathogenesis of autoimmune diseases such as lupus. Our increasing understanding of cytosolic nucleic acid sensing and of the self/nonself-discrimination mechanism at the molecular level should greatly facilitate the development of therapies against autoimmunity. The discovery of cGAMP as an endogenous second messenger that potently stimulates innate immune responses in human cells also suggests that this small molecule may be formulated as an adjuvant for developing new vaccines for the prevention and treatment of various diseases, including those that have so far been refractory to vaccine development, such as HIV, tuberculosis, and malaria.

NOTE ADDED IN PROOF

Two recent studies provide new insights into the structural mechanism of DNA-induced cGAS activation (180, 181) (**Figure 4*a*,*b***). These studies showed that cGAS forms a 2:2 complex with dsDNA instead of the 1:1 complex as described before (136, 138). In support of this new model, functional analyses of cGAS mutants demonstrate that both the dimer interface and two DNA binding surfaces are critical for cGAS activation.

DISCLOSURE STATEMENT

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