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Detection of Microbial Infections Through Innate Immune Sensing of Nucleic Acids

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Abstract

Microbial infections are recognized by the innate immune system through germline-encoded pattern recognition receptors (PRRs). As most microbial pathogens contain DNA and/or RNA during their life cycle, nucleic acid sensing has evolved as an essential strategy for host innate immune defense. Pathogen-derived nucleic acids with distinct features are recognized by specific host PRRs localized in endolysosomes and the cytosol. Activation of these PRRs triggers signaling cascades that culminate in the production of type I interferons and proinflammatory cytokines, leading to induction of an antimicrobial state, activation of adaptive immunity, and eventual clearance of the infection. Here, we review recent progress in innate immune recognition of nucleic acids upon microbial infection, including pathways involving endosomal Toll-like receptors, cytosolic RNA sensors, and cytosolic DNA sensors. We also discuss the mechanisms by which infectious microbes counteract host nucleic acid sensing to evade immune surveillance.

Contents

INTRODUCTION 448
NUCLEIC ACID SENSING OF MICROBIAL INFECTION 450
- RNA Viruses 450
- DNA Viruses 451
- Retroviruses 451
- Bacteria 452

ENDOSOMAL NUCLEIC ACID SENSING 453
- Ligand Recognition and TLR Activation 453
- TLR Signaling 455
- TLR Trafficking and Microbial Nucleic Acid Sensing 456

CYTOSOLIC RECOGNITION OF MICROBIAL RNA 457
- RIG-I-Like Receptors Sense Microbial RNA in the Cytosol 457
- Structural Basis for Ligand Recognition by RIG-I-Like Receptors 459
- Ubiquitination Promotes Oligomerization of RIG-I Like Receptors 459
- MAVS Aggregation 460
- MAVS Signaling 460

CYTOSOLIC DNA SENSING 461
- STING Signaling in Type I Interferon Production 461
- Cyclic GMP-AMP Synthase Is a Cytosolic DNA Sensor 463
- 2′3′-cGAMP Is a Second Messenger That Activates STING 463
- STING Trafficking and Signaling 464
- Cytosolic DNA Sensing in Antimicrobial Autophagy 465
- The AIM2 Inflammasome Pathway 465

MICROBIAL EVASION OF HOST NUCLEIC ACID SENSING 466
- Degrading, Hiding, or Modifying Microbial Nucleic Acids 467
- Antagonizing Nucleic Acid Sensors 467
- Antagonizing Signaling Adaptors and Other Signaling Molecules 467

CONCLUSIONS AND FUTURE PERSPECTIVES 468

INTRODUCTION

Animals have evolved comprehensive biological systems to maintain homeostasis in response to constantly changing environments. Environmental or physiological challenges can result in various cellular stresses that are detected by stress sensors, leading to activation of stress response pathways. One special type of challenge is infection by microbial pathogens. Given the enormous diversity of microbes, vertebrates frequently face microbial invasions, which can be detrimental if not well controlled. As the first line of host defense, the innate immune system recognizes microbial pathogens and triggers both intracellular and intercellular signaling pathways to fight infections.

Microbial pathogens are identified by host germline–encoded pattern recognition receptors (PRRs) that identify unique pathogen-associated molecular patterns (PAMPs). Infections can also be detected through pathogen-induced cellular damage or stress. Early studies elucidated important roles for cell surface PRRs in pathogen detection. However, recent progress has increasingly drawn attention to intracellular pathogen recognition systems. Among various PAMPs, microbial

Table 1 Major nucleic acid–sensing pathways in microbial recognition

Subcellular localization	Nucleic acid sensing			Nucleic acid signaling			
	Pathogen detected	Ligand specificity	Nucleic acid sensor	Signaling adaptor	Signaling molecules	Transcription factor	Cytokines
Endosomal nucleic acid sensing	RNA viruses	dsRNA	TLR3	TRIF	IKK	NF-κB	Proinflammatory cytokines
					TBK1/IKKε	IRF3	IFN-β
		ssRNA	TLR7/8	MyD88	IKK	NF-κB	Proinflammatory cytokines
	Bacteria	Bacterial CpG DNA	TLR9		IRAK4, IRAK1, IKKα	IRF7	IFN-α
		Bacterial 23S rRNA	TLR13		IRAK4, IRAK2	IRF5	Proinflammatory cytokines
Cytosolic RNA sensing	RNA viruses, HBV	Short 5′-di- or triphosphate dsRNA	RIG-I	MAVS	IKK	NF-κB	Proinflammatory cytokines
		Long dsRNA	MDA5		TBK1/IKKε	IRF3	IFN-β
	RNA viruses	dsRNA	LGP2	No intrinsic signaling			
Cytosolic DNA sensing	DNA viruses, retroviruses, bacteria	dsDNA	cGAS	STING	IKK	NF-κB	Proinflammatory cytokines
					TBK1/IKKε	IRF3	IFN-β
	DNA viruses, bacteria	dsDNA	AIM2	ASC	Caspase-1	N/A	Proinflammatory cytokines (e.g., IL-1β)

nucleic acids have emerged as the major target for innate immune recognition, as all microbial pathogens depend on their DNA and/or RNA for replication.

Three categories of innate immune sensors for intracellular pathogen-derived nucleic acids have been discovered: endosomally localized transmembrane Toll-like receptors (TLRs), which detect various microbial DNA and RNA in the lumen of endolysosomes; cytosolic microbial RNA sensors; and cytosolic DNA sensors (**Table 1**). Pathogen-derived RNA in the cytosol is sensed by the retinoic acid–inducible gene I (RIG-I)-like receptors (RLRs) (211), whereas cytosolic DNA is mainly recognized by cyclic GMP-AMP (cGAMP) synthase (cGAS) (177, 201). Cytosolic DNA can also be recognized by AIM2 (absent in melanoma 2) in some immune cells (14, 35, 59, 151). Most nucleic acid–sensing TLRs are expressed predominantly in immune cells, such as macrophages, dendritic cells (DCs), and B cells; in contrast, the major cytosolic RNA and DNA sensors are expressed at various levels in most cell types.

All nucleic acid sensors directly bind to and activate their downstream signaling adaptors, except for cGAS, which signals by producing the small-molecule second messenger cGAMP, which in turn binds to and activates the endoplasmic reticulum (ER)-localized adaptor stimulator of interferon genes (STING, also known as MITA, ERIS, and MPYS) (65, 179, 219) (**Table 1**). Activation of signaling adaptors results in proinflammatory cytokine production through NF-κB and/or type I interferon secretion through interferon regulatory transcription factor 3 (IRF3) or IRF7 (76). AIM2 activates cytosolic inflammasomes that catalyze processing and release of inflammatory cytokines, such as interleukin-1β (IL-1β) and IL-18, as well as induction of pyroptosis, an inflammatory form of cell death. Type I interferons function through autocrine and paracrine

signaling by binding to and activating a heterodimer receptor complex of IFN-α receptor 1 (IFNAR1) and IFNAR2. Receptor engagement leads to activation of signaling pathways that induce the transcription of interferon-stimulated genes (ISGs) to suppress pathogen replication and assembly. Additionally, the proinflammatory cytokines and chemokines facilitate recruitment of neutrophils and activation of macrophages for pathogen clearance. Type I interferons, inflammatory cytokines, and other immune modulatory molecules further instruct and enhance activation of the adaptive immune system to clear pathogens.

In this review, we discuss how nucleic acids from microbial pathogens are recognized by endosomal and cytosolic sensors. We focus on the molecular mechanisms of nucleic acid sensing and signaling as well as countermeasures deployed by pathogens to evade the host innate immune recognition pathways.

NUCLEIC ACID SENSING OF MICROBIAL INFECTION

All microbial pathogens use DNA or RNA as their genome and generate additional nucleic acid intermediates during replication. Thus, nucleic acid sensing has become a common mechanism that recognizes a large variety of microbial pathogens. The expression and different subcellular localization of multiple nucleic acid sensors allow efficient capture of various invading pathogens. Depending on the features of their genomic nucleic acids and the replication strategies, different pathogens can expose their DNA or RNA at different subcellular compartments. Endolysosomes and the cytosol are the main sites for microbial nucleic acid detection (**Table 1**). While certain pathogens directly invade the host cytoplasm, most bacteria and viruses enter host cells through endocytosis, after which they can escape from the endosome into the cytosol or be destroyed after being delivered to the lysosome (5). Additionally, some pathogens and their replication intermediates in the cytosol are wrapped through autophagy into autophagosomes that then fuse with lysosomes (91). Therefore, the lumen of endolysosomes is a common site of microbial nucleic acid exposure and is monitored by five members of the TLR family expressed in immune cells: TLR3 [for double-stranded RNA (dsRNA), TLR7 [single-stranded RNA (ssRNA)], TLR8 (ssRNA), TLR9 [single-stranded unmethylated DNA containing the cytosine-phosphate-guanine (CpG) dideoxynucleotide motif], and TLR13 (a specific sequence in the catalytic center of bacterial 23S ribosomal RNA) (76). As each TLR recognizes distinct nucleic acid features, endosomal TLRs can detect many microbial infections in immune cells.

However, most nonimmune cells do not express nucleic acid–sensing TLRs, and pathogens can escape endosomal detection by invading the cytosol. Nevertheless, infections in these situations can still trigger a robust innate immune response due to surveillance by cytosolic PRRs, including DNA and RNA sensors (**Table 1**). The primary cytosolic sensors of pathogen-derived RNA are three members of the RLR family: RIG-I (211), MDA5 (melanoma differentiation associated gene 5) (73), and LGP2 (laboratory of genetics and physiology 2) (154). As mentioned above, the cytosolic DNA sensors include cGAS (177, 201) and, in certain immune cell types, AIM2 (14, 35, 59, 151). In this section, we discuss representative microbes and their recognition by host nucleic acid sensors.

RNA Viruses

Viruses are the most abundant form of life on earth and are divided into seven groups based on their genome and replication strategy (9). The genome of most RNA viruses belongs to one of three categories: dsRNA, positive sense ssRNA, or negative sense ssRNA. Replication of RNA viruses usually occurs in the cytoplasm, during which each group of RNA viruses may generate

both dsRNA and ssRNA intermediates. Thus, the three groups are not well discriminated by host innate immune sensors. In the endosomal pathway, TLR3 recognizes dsRNA ligands, but it can also detect viruses from the other two groups (6). In addition to the dsRNA reoviruses, TLR3 recognizes a list of (−)ssRNA viruses [such as the human respiratory syncytial virus (HRSV)], and a number of (+)ssRNA viruses [including those in the *Picornaviridae* family, such as encephalomyocarditis virus (EMCV) and coxsackie virus, and viruses in the *Flaviviridae* family, such as West Nile virus (WNV)] (5, 76, 128). In contrast, TLR7 and TLR8 prefer ssRNA ligands and are known to detect vesicular stomatitis virus (VSV) of the *Rhabdoviridae* family and influenza A virus of the *Orthomyxoviridae* family, both of which are (−)ssRNA viruses (30, 52, 107).

Most RNA viruses are also detected by the cytosolic RNA sensors RIG-I and MDA5. RIG-I preferentially binds to viral RNAs bearding di- or triphosphate at the 5′ end, whereas MDA5 prefers longer dsRNA ligands (48, 60, 143, 161, 162). Accordingly, RIG-I and MDA5 recognize both unique and common RNA virus species. Viruses preferred by RIG-I include certain (−)ssRNA viruses such as HRSV, VSV, and influenza A/B virus, and a list of (+)ssRNA viruses, such as hepatitis C virus (HCV) and Japanese encephalitis virus (JEV) in the *Flaviviridae* family and Newcastle disease virus and Sendai virus (SeV) in the *Paramyxoviridae* family (75, 102). Two members of the *Picornaviridae* family, EMCV and polio virus, are preferentially recognized by MDA5 instead of RIG-I (46). Despite such differences, RIG-I and MDA5 recognize some common viruses, including the dsRNA reoviruses and some (+)ssRNA viruses, such as dengue virus and WNV from the *Flaviviridae* family (38, 102). LGP2, the third member of the RLR family, has no intrinsic signaling activity and has been suggested to regulate viral RIG-I and MDA5 signaling. Several studies indicated positive roles for LGP2 in MDA5-mediated response to picornaviruses, such as EMCV and mengo virus (159, 190), which is further supported by recent structural studies (13, 187) and is discussed below. Interestingly, infection by two RNA viruses, WNV and dengue virus, is also sensed by the cytosolic DNA sensor cGAS, possibly through detection of cell damage–induced host DNA leakage after infection, as is the case for dengue virus, which induces mitochondrial DNA leakage into the cytosol for cGAS recognition (4, 164).

DNA Viruses

DNA viruses carry dsDNA or ssDNA as their genome, and most enter the nucleus for replication, as they require certain host replication machinery. Common examples include herpes simplex virus 1 (HSV-1), adenovirus, human papilloma virus (HPV), vaccinia virus (VACV), and cytomegalovirus (CMV), all of which are detected by cGAS, which is activated by binding to dsDNA exposed in the cytosol (87, 164, 177, 201). AIM2 was initially reported to sense infection of DNA viruses including VACV and mouse cytomegalovirus (MCMV) (148). However, cGAS has been recently found as an important CMV sensor in vivo (98). MCMV-infected mice launch two phases of interferon production, but only the first phase, which is cGAS/STING dependent, is essential for controlling viral replication (98). Thus, cGAS is a major innate immune sensor of DNA viruses.

Retroviruses

Retroviruses have either an RNA or a DNA genome. RNA retroviruses have (+)ssRNA but depend on reverse transcription for replication. Upon infection, the viral RNA is reverse-transcribed into DNA, which is inserted into the host genome, followed by normal transcription and translation for viral gene expression. Thus, in principle, an RNA retrovirus might expose its RNA, DNA, and/or RNA:DNA hybrids during infection. However, the human body contains numerous endogenous retroelements with no obvious pathological outcomes; thus, there must be mechanisms to prevent

innate immune recognition of endogenous retroviruses (191). The best example of a pathogenic RNA retrovirus is human immunodeficiency virus (HIV). HIV is protected from innate immune detection through multiple mechanisms (146, 208). When such protection is compromised, HIV infection can be quickly detected, followed by a robust antiviral response (112, 146, 208). For example, TREX1, a host cytosolic exonuclease, usually degrades reverse-transcribed DNA from retroviruses, preventing cytosolic DNA accumulation (175). In human and mouse macrophages and DCs, depletion of TREX1 in HIV-infected cells leads to accumulation of viral DNA that is sensed by a cytosolic sensor (208), which was discovered to be cGAS (42, 177, 201); additionally, reverse transcription activity is a prerequisite for cGAS activation (42, 86, 164). Upon recognition of reverse-transcribed DNA of HIV and other retroviruses, cGAS synthesizes the second messenger cGAMP to activate the downstream signaling cascade to induce type I interferons (42, 177, 201).

DNA retroviruses are a small group of viruses carrying a dsDNA genome, but they synthesize a (+)ssRNA strand called pregenomic RNA (pgRNA) in the nucleus, which is then reverse-transcribed in the cytosol for genomic DNA replication (149). The best example is hepatitis B virus (HBV), which infects hundreds of millions of people. Although HBV infection introduces both viral DNA and RNA into the host, the innate immune response to HBV is rather weak, with no apparent type I interferon induction. Nevertheless, both MDA5 and RIG-I have been recently proposed as HBV sensors (106, 157). Depletion of MDA5 causes increased HBV replication in human hepatocyte cell lines and in mouse models (106). However, another study reported that RIG-I but not MDA5 recognizes the pgRNA of HBV (157). RIG-I binding to the HBV pgRNA not only mediates the production of type III interferons, consisting of three members (IFN-λ1, 2, and 3), but also suppresses reverse transcription by interfering with recruitment of HBV polymerase P to the pgRNA (157). It is likely that HBV detection by MDA5 or RIG-I depends on the viral genotype, as the two seemingly contradictory studies used HBV of different genotypes. Indeed, many genotypes, subgenotypes, and mutations of HBV have occurred due to a lack of proofreading functions in its reverse transcriptase.

Bacteria

Bacterial pathogens enter the host cell via phagocytosis and can then be sensed by endosomal TLR9 and TLR13 (not found in humans) in immune cells. TLR9 identifies bacterial unmethylated CpG DNA (54), whereas mouse TLR13 recognizes bacterial 23S ribosomal RNA (94, 133). Bacteria can also be detected by cytosolic DNA sensors. For example, cGAS has been reported to detect a variety of intracellular bacteria, including *Chlamydia trachomatis* (217), *Listeria monocytogenes* (51), and *Francisella tularensis*. *F. tularensis* in macrophages can also activate AIM2 inflammasomes (36, 148). Although both are dsDNA sensors, cGAS and AIM2 appear to have different ligand preferences. One example involves the role of cGAS in the accessibility of *Francisella novicida*–derived DNA to AIM2. *F. novicida* infection first induces type I interferon through the cGAS pathway, leading to expression of guanylate-binding proteins (GBPs) (110). With the help of GBPs, the host factor immunity-related GTPase family member B10 (IRGB10) is recruited to the bacterial surface, inducing bacterial DNA release to activate AIM2 (111).

Recent years have witnessed significant progress in the study of mycobacterial recognition by nucleic acid sensors. Despite residing inside phagosomes after endocytosis, *Mycobacterium tuberculosis* can permeabilize phagosomal membrane through its ESX1 secretion system (6-kDa early secretory antigenic target secretion systems, also known as type VII secretion systems) (194), which facilitates cytosolic exposure of bacterial DNA (113). Earlier studies supported AIM2 as a critical sensor of *M. tuberculosis* in vivo (153). More recently, several groups independently demonstrated an essential role for cGAS in the recognition of *M. tuberculosis* and subsequent IFN-β production

(25, 193, 194). Both AIM2 and cGAS have been shown to colocalize with and be activated by *M. tuberculosis*–derived DNA in the cytosol, and the extent to which each pathway is activated is likely regulated by bacterial factors secreted through the ESX1 secretion system (193).

ENDOSOMAL NUCLEIC ACID SENSING

TLRs, as the most extensively studied PRRs, are expressed primarily in immune cells and recognize a variety of PAMPs from microbes (72). The five nucleic acid–sensing TLRs (TLR3, TLR7, TLR8, TLR9, and TLR13) are all transmembrane proteins recognizing nucleic acids in endolysosomes (**Figure 1**, **Table 1**). As most microbes enter host cells via endocytosis or phagocytosis, TLRs provide an effective layer of infection surveillance in the endosomal compartments.

Ligand Recognition and TLR Activation

Nucleic acid–sensing TLRs are single transmembrane proteins that share similar domain structures: an ectodomain that recognizes PAMPs, a transmembrane domain, and a C-terminal cytosolic Toll/IL-1 receptor (TIR) domain (76). The ectodomains bind nucleic acids with different features in the lumen of endolysosomes. Significant progress in understanding the structural basis for their ligand recognition and activation has been made in recent years.

TLR3 recognizes dsRNA. The ectodomain of TLR3 comprises a leucine-rich repeat (LRR) N-terminal motif followed by 23 LRRs and a C-terminal capping domain. It adopts a horseshoe-shaped solenoid structure. Upon ligand binding, the ectodomain:dsRNA complex contains two ectodomain molecules that sandwich one dsRNA molecule in a typical right-handed A-DNA-like structure (99). Despite being heavily glycosylated, each ectodomain has a glycan-free side that contains two RNA-binding sites interacting with the sugar-phosphate backbone of the ligand, indicating sequence-independent RNA recognition. At mildly acidic pH, as in endolysosomes, protonation of the imidazole groups of essential histidine residues provides positive charges necessary for binding to negatively charged dsRNA (99).

TLR7/8 recognizes ssRNA. The ectodomains of TLR7/8/9 share high homology, each containing 26 LRRs with an insertion called a Z-loop in the middle. Different from TLR3, the ectodomain of TLR7/8 adopts a donut-like structure, with the N and C termini in direct contact (218). Inactive TLR7 ectodomain is a monomer; ligand binding induces the formation of an M-shaped symmetrical dimer with their C termini in the center. Each dimer binds two small molecules, such as guanosine or resiquimod (R848), and two ssRNA (UUU) ligands (218). Each small molecule is completely embedded in the dimerization interface, whereas each UUU binds to the concave surface of both ectodomains as well as the Z-loop. Unlike TLR7, TLR8 is a preformed dimer without ligand binding, but the C-terminal TIR domains in an inactive dimer are far from each other. Binding to uridine and GU-rich ssRNA, in a manner similar to that of TLR7, induces rearrangement of the dimer interface, bringing the two C termini to close proximity (183). For both TLR7 and TLR8, activation can be induced solely by small-molecule ligands, but not by ssRNA alone. However, ssRNA binding increases the affinity for binding of guanosine or uridine that might be derived from ssRNA.

TLR9 detects bacterial CpG DNA. Similar to TLR7, the ectodomain of TLR9 is a donut-shaped monomer in the absence of ligand (132). Binding to bacterial CpG-containing ssDNA induces the formation of a 2:2 complex, with two binding surfaces for each CpG DNA molecule

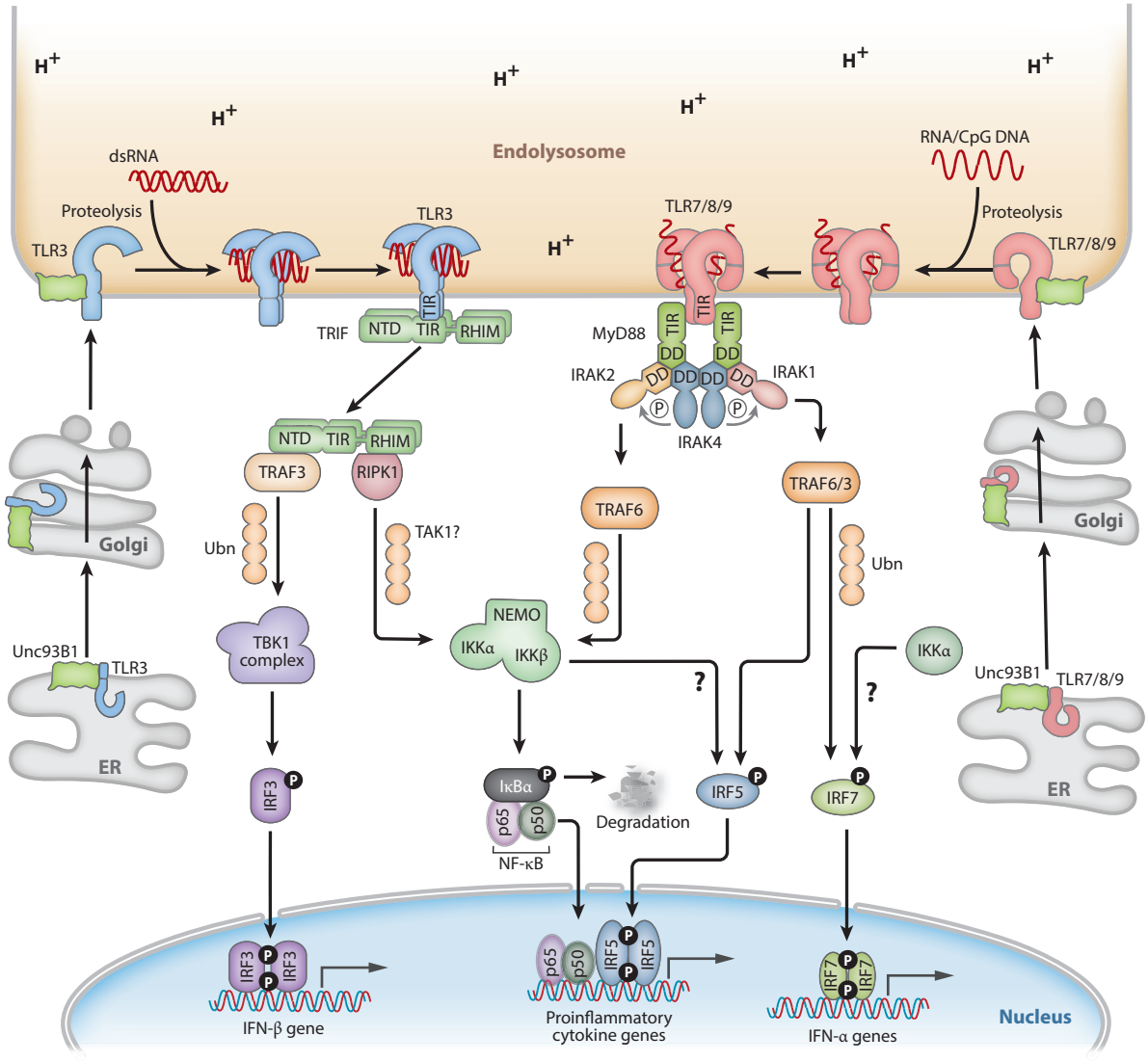

Figure 1

Endosomal nucleic acid sensing by Toll-like receptors. TLR3, 7, 8, 9, and 13 are transmembrane proteins synthesized in the ER. Unc93B1 escorts TLRs from the ER through the Golgi apparatus to endolysosomes, where TLRs are further processed by proteolysis. Binding to their specific nucleic acid ligands leads to formation of TLR dimers and oligomerization of their cytoplasmic TIR domains, which recruit signaling adaptors through TIR-TIR interaction. TLR3 recruits the adaptor protein TRIF, which in turn recruits TRAF3 to activate the TBK1/IRF3 cascade for transcriptional upregulation of type I interferons. TRIF also recruits RIPK1 to activate the IKK complex, leading to NF-κB-mediated transcription of proinflammatory cytokines. Ubiquitination controls the activation of both TBK1 and IKK. In the case of TLR7/8/9, and likely TLR13, recruitment of MyD88 leads to oligomerization of its death domain, which in turn triggers the formation of the Myddosome complex containing MyD88, IRAK4, IRAK1 and IRAK2. Formation of the Myddosome results in activation of the IRAKs and the ubiquitin E3 ligase TRAF6, which in turn activate NF-κB, IRF5, and IRF7. Abbreviations: ER, endoplasmic reticulum; DD, death domain; NEMO, NF-κB essential modulator; P, phosphoryl group; Ubn, polyubiquitin chain; RHIM, receptor-interacting protein 1 homotypic interaction motif; TIR, Toll/IL-1 receptor domain.

(132). Surface 1 comes from the N terminus of one ectodomain and interacts with the base moieties of the DNA, whereas surface 2 is from the C terminus of the other ectodomain in the dimer and interacts with the DNA backbone (132). Recently another binding site in TLR9 has been identified; it binds DNA carrying cytosine at the second position from the 5′ end (5′-xCx DNA) (131). This additional binding site corresponds to the nucleotide binding site in TLR7 and TLR8. 5′-xCx DNA binds to TLR9 in the presence of CpG DNA, forming a 2:2:2 complex, which is essential for optimal signaling of TLR9 (131).

TLR13 identifies bacterial 23S rRNA. TLR13 is expressed in mice but not in humans. TLR13 recognizes a conserved single-strand sequence, CGGAAAGACC, in the catalytic center of the bacterial 23S rRNA (94, 133). Binding to 23S rRNA induces the formation of an M-shaped 2:2 dimer containing two TLR13 ectodomains and two RNA molecules (174, 192). Each ectodomain forms an oval-shaped structure with the ssRNA ligand fitting into its inner concave. The RNA ligand forms a stem-loop-like structure required for TLR13 recognition. The 2′-hydroxyl groups of the RNA ligand mediate important RNA-specific hydrogen bonds to maintain the stem-loop-like structure, explaining how TLR13 distinguishes RNA from DNA.

TLR Signaling

Ligand binding induces oligomerization of TLRs and their cytoplasmic TIR domain, which initiates signaling by recruiting adaptor proteins. With the exception of TLR3, which recruits the adaptor protein TRIF (TIR-domain-containing adapter-inducing interferon-β), also known as TICAM1 (TIR-domain-containing adaptor molecule-1), all TLRs recruit MyD88 (myeloid differentiation primary response 88) (158, 207). TRIF initiates signaling cascades leading to activation of IRF3 and NF-κB, whereas MyD88 activates NF-κB, IRF5, and IRF7 (**Figure 1**, **Table 1**). Below we discuss the cellular signaling downstream of TLR3 and TLR7/9 as models to understand nucleic acid–induced TLR signaling.

TLR3 signaling. Binding of dsRNA to TLR3 in endolysosomal lumen facilitates the oligomerization of the TIR domain that in turn binds to the TIR domain of the adaptor protein TRIF (135, 207). Besides the TIR domain, TRIF also contains an N-terminal domain (NTD) and a C-terminal receptor-interacting protein 1 (RIP-1) homotypic interaction motif (RHIM). Inactive TRIF is autoinhibited by an intramolecular interaction that masks binding sites for downstream factors (184). The TIR-TIR interaction between TRIF and TLR3 triggers TRIF oligomerization and activation. Activated TRIF binds tumor necrosis factor receptor–associated factor 3 (TRAF3), a ubiquitin ligase essential for subsequent recruitment of TANK-binding kinase 1 (TBK1) and the inhibitor of kappa B kinase-ε (IKKε) complex through a ubiquitination-dependent mechanism (50, 130). The TRIF/TBK1 complex further recruits and phosphorylates IRF3, resulting in IRF3 dimerization and IFN-β production.

In addition to IRF3, TRIF also activates the NF-κB pathway. In resting cells, NF-κB is a heterodimer of p65 and p50 sequestered in the cytoplasm by its inhibitory subunit IκB. Phosphorylation of IκB by activated IKK complex, which consists of the catalytic subunits IKKα and IKKβ and the regulatory subunit NEMO, leads to IκB polyubiquitination and proteasomal degradation, releasing NF-κB for nuclear translocation and transcription of proinflammatory genes (172). The RHIM domain of TRIF binds to receptor-interacting serine/threonine-protein kinase 1 (RIPK1), which activates the transforming growth factor β (TGF-β)-activated kinase 1 (TAK1), leading to IKK activation. In *RIPK1* knockout mouse embryonic fibroblasts (MEFs),

poly(I:C)-induced NF-κB activation was impaired, but IFN-β production was intact (117), indicating that TRIF-mediated activation of IRF3 and NF-κB can be uncoupled.

TLR7/9 signaling. TLR7 and TLR9 signal through the adaptor protein MyD88 to induce IFN-α and proinflammatory cytokines (53, 163). The two pathways are unique in that they activate IRF7 instead of IRF3 for type I interferon production (58). In addition, TLR7 and TLR9 activate IRF7 by kinases other than TBK1 or IKKε. The adaptor protein MyD88 contains an N-terminal death domain, an intermediate domain, and a C-terminal TIR domain that interacts with the TIR domain of TLR7/9. Through the TIR-TIR interaction, MyD88 molecules are recruited to the receptor complex, and their death domains are oligomerized. MyD88 activation triggers the formation of the Myddosome signaling complex, consisting of MyD88 itself and the IL-1 receptor–associated kinase (IRAK) family kinases IRAK4 and IRAK1/2 (97, 121). Myddosome formation is driven by oligomerization of the death domains of MyD88 and IRAKs (97). The Myddosome then promotes IRAK4 *trans*-autophosphorylation and activation, leading to IRAK4-mediated phosphorylation and activation of IRAK1/2 (92, 97). Both MyD88 and IRAK1 can directly recruit IRF7, leading to IRAK1-mediated phosphorylation and activation of IRF7 (77). Consistently, genetic evidence indicates that the kinase activity of both IRAK4 and IRAK1 is required for IFN-α production (80, 188). Another kinase, IKKα, is also involved in IRF7 activation (61), but the functional relationship between IRAK1 and IKKα remains to be clearly delineated.

Two E3 ubiquitin ligases, TRAF6 and TRAF3, are involved in the activation of IRF7 in the TLR7/9 pathway (50, 130). How ubiquitination promotes IRF7 activation requires further investigation. TRAF6, but not TRAF3, is also required for NF-κB activation and inflammatory cytokine responses downstream of TLR7/9 signaling (47). Here the activation of NF-κB is mediated by a similar mechanism downstream of TRIF, which involves the recruitment of TAK1 and IKK complexes. TLR7 and TLR9 induction of proinflammatory cytokines, but not IFN-α, requires an additional transcription factor IRF5, which is also downstream of MyD88 and TRAF6 (181). IRF5 is phosphorylated by IKKβ at Ser462, and this phosphorylation is essential for induction of proinflammatory cytokines (103, 150). Both IRF5 and NF-κB bind to the promoter regions of these proinflammatory genes, leading to transcriptional activation.

TLR Trafficking and Microbial Nucleic Acid Sensing

As nucleic acid–sensing TLRs launch signaling cascades from endolysosomes, their trafficking from the ER to endolysosomes is a prerequisite for their functions (**Figure 1**). CpG DNA stimulation triggers robust TLR9 trafficking from the ER to endolysosomes (22), whereas TLR7 traffics in response to its agonist imiquimod as well as other stimulants that do not activate TLR7, such as CpG DNA or LPS (81). The exact mechanism by which various stimuli trigger TLR trafficking is unclear, but an ER-resident protein unc-93 homolog B1 (UNC93B1) is essential for the translocation of all endosomal TLRs (81, 90). UNC93B1 directly binds to TLRs through their transmembrane domains, escorting TLRs into ER-derived vesicles and further trafficking to the Golgi apparatus and post-Golgi endolysosomes (90). Consistently, signaling from nucleic acid–sensing TLRs is defective in mice or human patients deficient in *UNC93B1* (18, 180). TLR9-mediated pathways are further fine-tuned by post-Golgi trafficking at endolysosomal compartments (156). In plasmacytoid DCs (pDCs), TLR9 in the early endosome is capable of activating the NF-κB pathway for secretion of proinflammatory cytokines upon CpG-DNA stimulation, but activation of IRF7 for type I interferon production is dependent on adaptor protein complex 3 (AP3)-mediated TLR9 trafficking to a pool of LAMP2-positive endosomes (156). Prolonged retention of the signaling complex containing MyD88, IRAK4, IRAK1, and IRF7 on the endosomal

membrane contributes to the generation of large amounts of IFN-α in pDCs (57). Trafficking of TLRs to endolysosomes leads to proteolytic processing of TLRs in the acidic compartments; such processing has been shown to be required for activation of the endosomal TLRs, including TLR7 and TLR9 (34, 56, 114, 139).

CYTOSOLIC RECOGNITION OF MICROBIAL RNA

While TLRs can detect a plethora of microbial infections through endosomal nucleic acid sensing, this system has its own limitations due to a lack of TLR expression in most nonimmune cells and the orientation of the ligand binding domain that faces the lumen of endolysosomes. Thus, a more general mechanism to detect microbes out of the endosome is needed. During early investigations, two antiviral proteins were found to be activated by dsRNA. One is the interferon-inducible 2′5′-oligoadenylate synthetase (OAS), which upon binding to dsRNA synthesizes 2′5′-oligoadenylates to trigger viral RNA degradation by the ribonuclease RNase L (119, 173). The other is dsRNA-dependent protein kinase R (PKR), a serine/threonine kinase that, once activated by dsRNA binding, inhibits viral replication by phosphorylating the α subunit of eukaryotic initiation factor 2 and thus inhibiting the translation of viral mRNA (8). Subsequently, the cytosolic RNA-induced, TLR-independent interferon response was found to depend on the cytosolic RNA sensors RIG-I and MDA5 (211) as well as their common downstream signaling adaptor MAVS (mitochondrial antiviral-signaling protein, also known as IPS-1, VISA, and CARDIF) (78, 118, 167, 205). Recognition of cytosolic dsRNA by RIG-I and MDA5 activates MAVS signaling, leading to transcriptional expression of type I interferons and proinflammatory cytokines (**Table 1**). Type I interferons induce a battery of ISGs, including OAS and PKR, to fight viral infections.

RIG-I-Like Receptors Sense Microbial RNA in the Cytosol

The RIG-I-like receptor family includes three members: RIG-I (211), MDA5 (73), and LGP2 (154), all of which reside in the cytoplasm and recognize viral RNA structures. All RLRs have a C-terminal domain (CTD) and an intermediate RNA helicase domain that catalyzes ATP hydrolysis. Both RIG-I and MDA5, but not LGP2, contain tandem caspase recruitment domains (CARDs) in their N termini that directly interact with MAVS to activate downstream signaling. LGP2, without a CARD, cannot activate MAVS by itself, but it is thought to regulate dsRNA-stimulated RIG-I and DMA5 signaling (159, 190).

All RLRs recognize pathogen-derived dsRNA, with some differences in ligand preference. RIG-I recognizes blunt-end short dsRNA bearing 5′-triphosphate, or with lower affinity 5′-diphosphate, whereas MDA5 is thought to detect virus-derived long dsRNA, although the exact identity of the endogenous viral RNA ligand that activates MDA5 remains unknown (48, 60, 143, 161, 162) (**Figure 2**). The 5′-triphosphate is a common RNA feature for many RNA viruses, such as HCV and influenza A virus, and 5′-diphosphate is found in the genome segments of reoviruses. In contrast, cellular RNAs are usually modified at the 5′ end. For example, mRNA is modified by the 5′ cap, and tRNA is processed from its precursors such that it contains 5′-monophosphate. However, some cellular RNA such as 7SL RNA is transcribed by RNA polymerase III and remains unmodified at the 5′ end (i.e., bearing 5′ triphosphate). It is thought that such RNA is shielded from RIG-I by binding to some cellular proteins, but more work is needed to test this possibility. Another important feature that distinguishes host and viral RNA is 2′-*O* methylation of the guanine cap of cellular mRNA, which prevents it from being recognized by RIG-I and MDA5 (166, 220). As an evasion mechanism, viruses employ host or viral capping enzymes as well as 2′-*O* methyltransferase to modify viral mRNA in a way similar to host mRNA (166, 220).

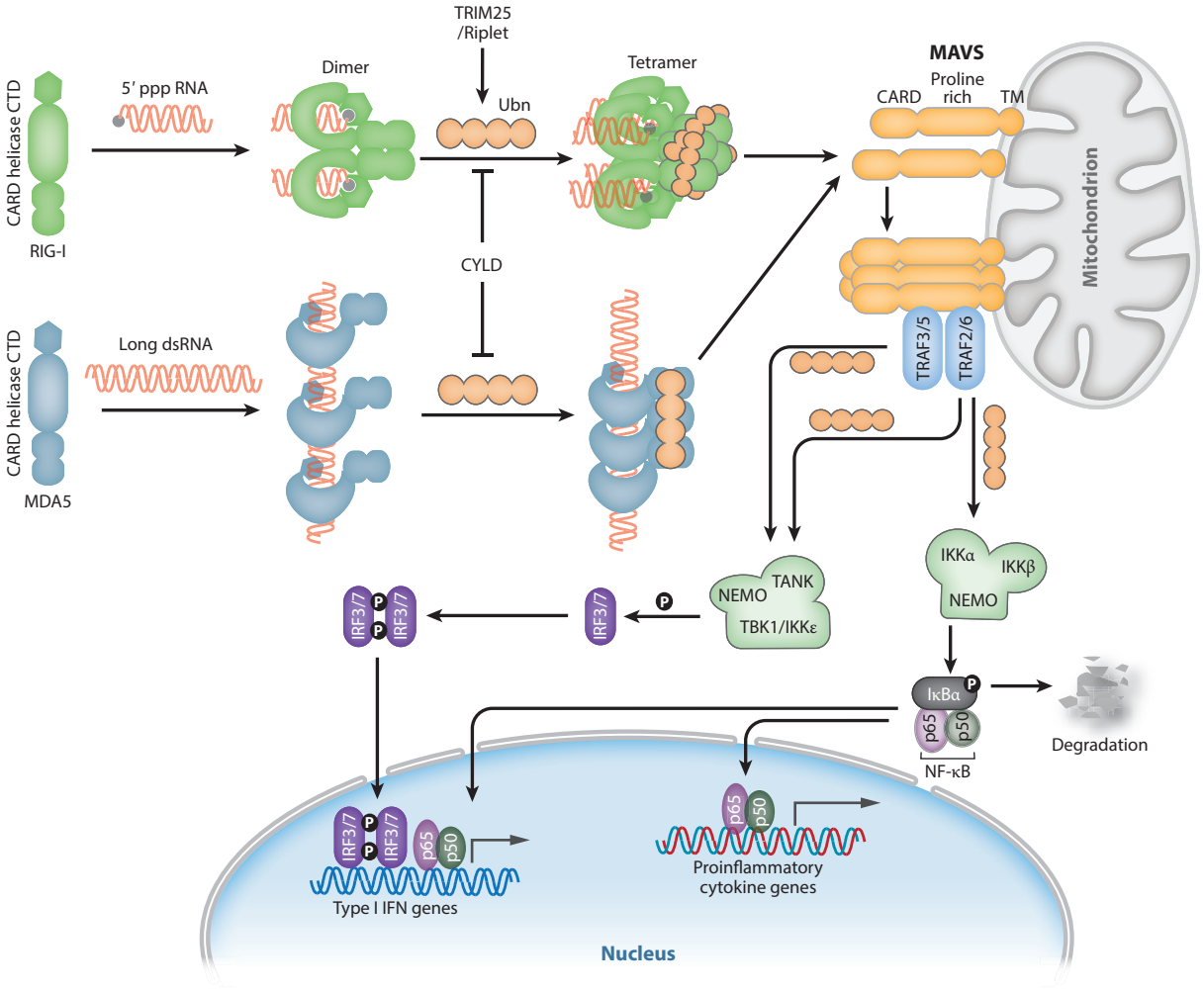

Figure 2

Cytosolic RNA-sensing pathway. Viral RNA in the cytosol is sensed by RIG-I-like receptors (RLRs), which include RIG-I, MDA5, and LGP2. All three RLRs can directly bind RNA ligands, but LGP2 does not have intrinsic signaling activity, due to a lack of CARD. LGP2 facilitates recognition of viral RNA by MDA5. RIG-I specifically recognizes dsRNA bearing 5′-triphosphate, or 5′-diphospate. RNA binding induces a conformational change of RIG-I that exposes its tandem CARDs. MDA5 recognizes long dsRNA, and multiple MDA5 molecules can be assembled along RNA, with the CARDs exposed. Binding of K63-linked polyubiquitin chains to CARDs of either RIG-I or MDA5 triggers their oligomerization, which provides oligomer templates to initiate a helical assembly of the adaptor protein MAVS on the outer membrane of mitochondria. Through recruitment of TRAF proteins, MAVS activates TBK1 and IKK through a K63 polyubiquitin-dependent mechanism. TBK1 and IKK complexes activate transcription factors IRF3 and NF-κB, respectively, and turn on type I interferon and proinflammatory cytokines. Bar-ended lines indicate inhibition. Abbreviations: P, phosphoryl group; TM, transmembrane domain; Ubn, polyubiquitin chain.

IFIT1 (interferon-induced protein with tetratricopeptide repeats 1) is an antiviral protein that also recognizes 5′-triphosphate RNA through direct binding, but it is proposed to accomplish antiviral activity by direct viral RNA sequestration in a large complex containing multiple IFIT family members (142). 2′-*O* methylation of viral mRNA cap also results in viral evasion from IFIT1-mediated suppression of viral protein translation (26).

Specific RNA features recognized by LGP2 are unclear, but it is known that LGP2 also binds to dsRNA with high affinity (125, 144). Interestingly, host recognition of EMCV requires both MDA5 and LGP2, the latter specifically recognizing an antisense RNA corresponding to the L region of the viral RNA (29). The L region of EMCV RNA is critical for the generation of LGP2-associated stimulatory RNA ligand for MDA5 and is required for EMCV detection (29).

Structural Basis for Ligand Recognition by RIG-I-Like Receptors

All RLRs have a CTD with a similar fold and a basic surface, but features of their RNA binding loops differ. RLRs can bind dsRNA through their CTDs, and only the CTD of RIG-I specifically identifies 5′-diphosphate- or 5′-triphosphate-bearing RNA (105). Without RNA ligand, RIG-I is autoinhibited, but its CTD is flexible and contains a loop critical for recognition of the 5′-diphosphate or 5′-triphosphate end of RNA. Upon ligand capture by the CTD, cooperative binding of the helicase domain to ATP and RNA remodels RIG-I into an active conformation in which the CTD and helicase domain organize into a ring around RNA and the N-terminal CARD domains are exposed (67, 83, 108). ATP hydrolysis propels RIG-I to translocate along RNA from the 5′ end, and this translocation has been shown to be important for RIG-I activation (127).

MDA5 and RIG-I share similar domain architectures, but MDA5 prefers longer dsRNA for signaling, as supported by recent structural studies (11, 140, 198). MDA5 interacts with dsRNA primarily through CTD binding to RNA phosphate backbone and 2′-hydroxyl groups, consistent with sequence-independent RNA recognition. While the CTD loop in RIG-I recognizes 5′-diphosphate or 5′-triphosphate, the counterpart loop in MDA5-CTD is not required for RNA binding. A major difference in RNA binding is that MDA5 binds dsRNA at the stem whereas RIG-I binds dsRNA at the end, which leads to the orientation of MDA5 into a C-shaped structure and RIG-I into an O-shaped ring after RNA binding (198). Binding to dsRNA triggers the assembly of MDA5 along dsRNA to form helical, filamentous oligomers (11, 140). MDA5 filaments are based on head-to-tail arrangements of monomers with extensive protein-protein interactions, which explains the high cooperativity of MDA5 in dsRNA binding and its preference for long dsRNA (198).

The LGP2 CTD is structurally similar to that of RIG-I, and it also prefers dsRNA binding but with no significant preference for 5′-triphosphate. LGP2 binds to RNA termini through its CTD with higher affinity than MDA5 (144). The structures of LGP2 from chickens and humans reveal that LGP2 binds dsRNA through both its CTD and its helicase domain, which is required for LGP2-mediated promotion of MDA5 signaling (187). LGP2 also forms filaments along dsRNA, but less efficiently than MDA5 (187). The presence of LGP2 is found to enhance the rate of MDA5 binding to RNA while attenuating MDA5 filament elongation, resulting in the formation of more and shorter MDA5 filaments, which are believed to have better signaling performance than longer filaments containing only MDA5 (13). Further studies are required to gain deeper insights into the detailed mechanisms by which LGP2 regulates innate immune signaling by RIG-I and MDA5.

Ubiquitination Promotes Oligomerization of RIG-I Like Receptors

RLRs must be oligomerized to be activated. RNA binding induces structural remodeling of RIG-I and MDA5 into active conformations, with RIG-I assembling into a tetramer and MDA5 longer filamentous oligomers (**Figure 2**). RIG-I is activated by the binding of its CARDs with free K63 polyubiquitin chains, which induces the formation of RIG-I tetramers, complexes essential for signaling activation (68, 212). RIG-I CARDs can also be conjugated by K63 polyubiquitin chains, and this may further enhance its activation (41, 136, 137). Ubiquitin ligases, such as TRIM25 and Riplet, promote RIG-I signaling by synthesizing K63 polyubiquitin chains (41, 136,

137), whereas the RIG-I-associated deubiquitination enzyme cylindromatosis (CYLD) suppresses antiviral signaling by removing K63 polyubiquitin chains (39). Free ubiquitin chains bind to the outer rim of the RIG-I helical trajectory and stabilize the tetramer complex, which may be further stabilized by covalent polyubiquitination of the CARDs (141). The RIG-I tetramer forms a left-handed, single-stranded helical architecture, providing the structural basis for MAVS recruitment and activation (see below) (199).

MDA5 oligomerization also requires binding of its CARDs to free K63 polyubiquitin chains (68). This might be accomplished by polyubiquitin-mediated stabilization of the MDA5 oligomer complex, similarly to the stabilization of RIG-I tetramer (141). The formation of MDA5 filaments brings its CARDs into close proximity, triggering CARD oligomerization outside of the main MDA5 filaments. ATP hydrolysis by the helicase domain drives conformational changes to expose CARDs, leading to its oligomerization (198). The oligomerized MDA5 CARDs then recruit and activate MAVS for downstream signaling.

MAVS Aggregation

Both RIG-I and MDA5 activate the signaling adaptor MAVS, a mitochondrial membrane protein (78, 118, 167, 205). Therefore, MAVS is critical for an effective innate immune response to infection by various RNA viruses (85, 178). MAVS contains a C-terminal transmembrane domain inserted into the outer membrane of mitochondria (170). MAVS activation requires its assembly into prion-like aggregates on the mitochondrial surface (16, 62, 203) (**Figure 2**). Formation of MAVS aggregates is mediated by the CARD in the cytosolic N terminus of MAVS that interacts with the tandem CARDs of RIG-I or MDA5 oligomers (62). The RIG-I tetramer serves as a template to recruit individual CARDs of MAVS, leading to the assembly of MAVS into active, prion-like filaments with the same helical architecture (199, 204). The helical MDA5 filaments might also function as a template to activate MAVS aggregation; structural evidence of this is needed. The prion nature of MAVS has been supported by both biochemical and genetic evidence (16, 62). One hallmark of MAVS aggregates is its self-perpetuation mechanism, similar to that of a prion. Once a small amount of MAVS is assembled into aggregates by RIG-I or MDA5, these aggregates further recruit and activate other MAVS molecules, leading to the formation of large aggregates. This is a unique mechanism for fast and robust activation of antiviral signaling. Of note, MAVS displacement from mitochondria via a site-specific cleavage near the transmembrane domain by an HCV-encoded protease disrupts its signaling (95, 118). It is still unclear how mitochondrial localization mechanistically controls MAVS signaling, but it is likely that the mitochondrial membrane supports MAVS aggregation.

MAVS Signaling

MAVS activates downstream signaling through kinases including IKK and TBK1, leading to activation of NF-κB and IRF3, which enter the nucleus to turn on the transcription of proinflammatory cytokines and interferons (**Figure 2**). K63 polyubiquitination is essential for the RNA-sensing pathway, as replacement of endogenous ubiquitin with its K63R mutant abolished IRF3 activation upon RNA virus infection (213). K63 polyubiquitination not only promotes the oligomerization of RLRs as mentioned above but also plays important roles in MAVS downstream signaling. Several E3 ubiquitin ligases including TRAF2, TRAF5, and TRAF6 are directly recruited by MAVS through distinct motifs (101). The three TRAFs are the major E3 ligases activating downstream signaling of MAVS, as concomitant knockdown of all of them results in a total loss of IRF3 and IKK activation upon RNA virus infection (101). These TRAFs redundantly

promote K63 polyubiquitination, but the ubiquitination targets important for downstream signaling remain to be identified. NEMO, the regulatory subunit of IKK and TBK1, is a ubiquitin sensor (33, 200) and is recruited to the MAVS/TRAFs complex through its ubiquitin-binding domains (101). Ubiquitin binding but not ubiquitination of NEMO is essential for MAVS signaling (101, 213). NEMO then recruits IKK and TBK1 to the MAVS complex, leading to activation of NF-κB and IRF3. IRF3 phosphorylation by TBK1 requires phosphorylation of MAVS at a specific sequence motif (pLxIS); this phosphorylation recruits IRF3 to TBK1, thereby licensing IRF3 phosphorylation by TBK1 (100, 170). The phosphorylation motif in MAVS is also conserved in STING and TRIF, explaining why signaling pathways that employ MAVS, STING, and TRIF as the adaptor proteins have the unique ability to activate IRF3 and induce type I interferons (see below) (100).

CYTOSOLIC DNA SENSING

Except for RNA viruses, all microbial pathogens depend on DNA for their replication. In normal conditions, DNA resides in the nucleus and mitochondria but not cytoplasm of cells. Thus, cytosolic DNA sensing is a powerful strategy to recognize infection by many pathogens, although it also creates the risk of autoimmunity in situations of abnormal cytoplasmic exposure of host DNA. Cytosolic DNA has been known to trigger the type I interferon pathway for over a decade (64, 176). In recent years, a large number of proteins have been proposed as cytosolic DNA sensors upstream of interferon production (189). However, among all the proposed sensors, only the knockout of cGAS completely abolished interferon production in response to cytosolic DNA. Genetic evidence also demonstrates that AIM2 activates the inflammasome pathway in response to DNA stimulation. Here we focus on cGAS and AIM2 as the DNA sensors that activate type I interferon production and the inflammasome pathway, respectively.

STING Signaling in Type I Interferon Production

It was observed more than a decade ago that in many cell lines cytosolic DNA triggered IRF3-mediated type I interferon production and that the response was independent of TLR9, the only DNA-sensing TLR (64, 176). Surprisingly, B-form dsDNA poly(dA:dT) was found to induce both type I interferon production and NF-κB activation, which depended on RIG-I and MAVS, two essential components of the RNA-sensing pathway (20, 64). This finding was resolved by subsequent studies that identified RNA polymerase III as a DNA sensor that converts poly(dA:dT) into 5′-trisphosphate RNA, the bona fide ligand for RIG-I activation (1, 21). However, RNA polymerase III only recognizes highly AT-rich DNA sequences, which does not explain the sequence-independent DNA response observed in many cell lines (176).

STING, a protein containing four transmembrane domains and localized on the ER with cytoplasmic N and C termini, was identified as a third adaptor protein, after TRIF and MAVS, that also activates IRF3 and type I interferon production (65, 179, 219) (**Figure 3**, **Table 1**). STING is essential for DNA-induced type I interferon production in multiple cell types and is critical for host defense against infection by HSV-1, a DNA virus (66). Macrophages from *STING* knockout mice or *STING*$^{gt/gt}$ mice, which have a single amino acid mutation (I199N) leading to STING degradation, cannot produce interferon in response to DNA transfection or DNA virus or bacterial infection (66, 160). These studies established STING as an important adaptor for cytosolic DNA–stimulated interferon production.

Besides responding to cytosolic DNA, STING was also found to be a direct sensor of cyclic dinucleotides (CDNs), such as cyclic di-GMP (c-di-GMP) and c-di-AMP, which are second messengers produced by bacteria (27) (**Figure 3**). CDNs were initially found to trigger type I interferon

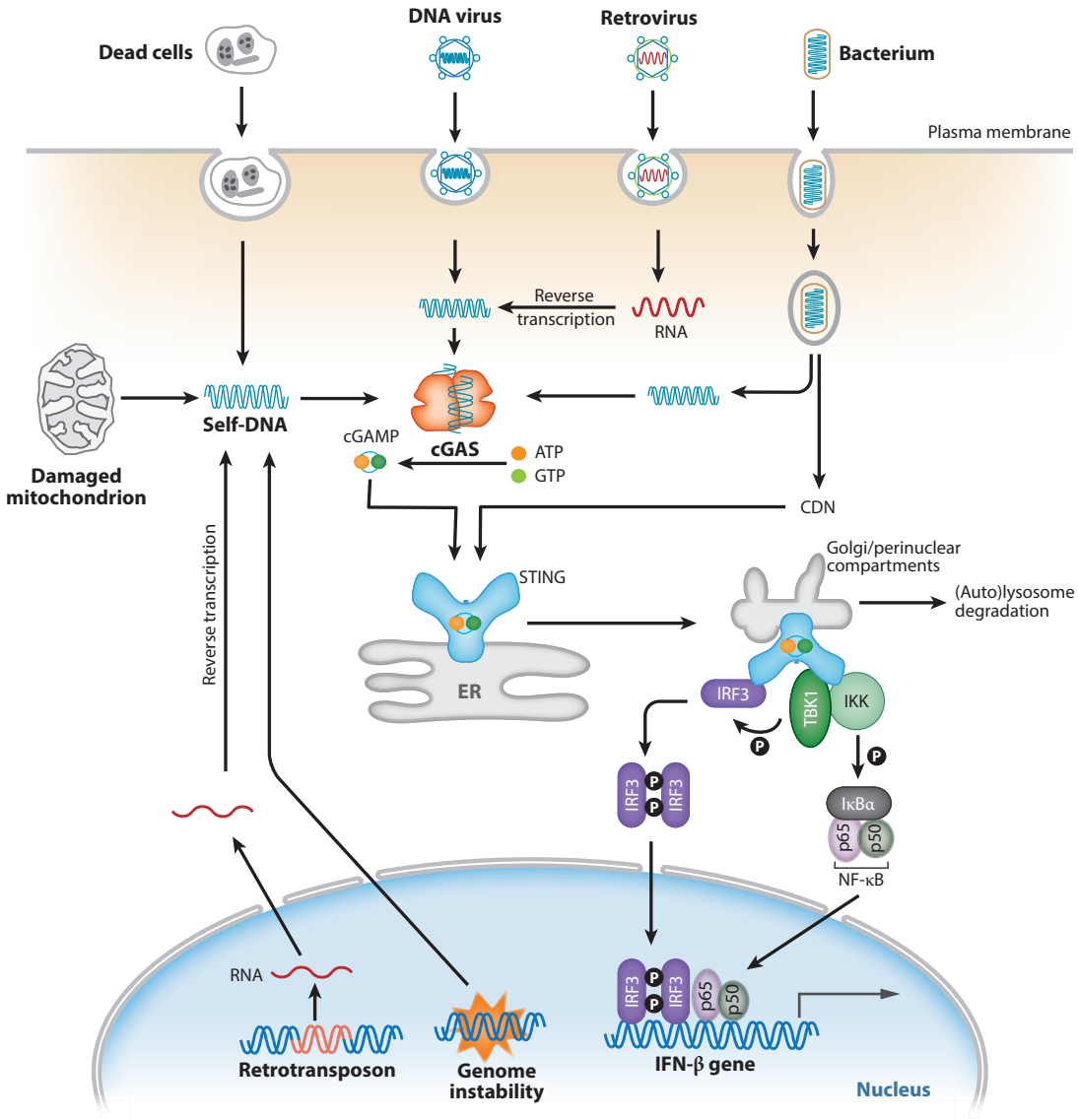

Figure 3

Cytosolic DNA–sensing pathway. cGAS binds to DNA from invading microbes, including DNA viruses, retroviruses, and bacteria, or to self-DNA generated from DNA damage, reverse transcription of endogenous retroelements, damaged mitochondria, or phagocytosed dead cells. Such binding leads to activation of cGAS, which synthesizes the second messenger 2′3′-cGAMP from ATP and GTP. cGAMP is a high-affinity ligand for the ER-localized adaptor protein STING. After binding to cGAMP, STING undergoes conformational changes and translocates to perinuclear compartments, where it activates TBK1 and the IKK complex, leading to activation of transcription factors IRF3 and NF-κB, respectively. Nuclear translocation of these active transcription factors turns on type I interferons and proinflammatory cytokines. Abbreviation: ER, endoplasmic reticulum; cGAMP, 2′3′-cyclic guanosine monophosphate–adenosine monophosphate; P, phosphoryl group.

response (116, 197) through an unknown sensor that was later identified as STING (15, 69, 160). STING directly binds c-di-GMP, with one c-di-GMP molecule fitting into the central pocket of a STING dimer (63, 138, 168, 171, 210). However, in all of these structures, the last 40 amino acids at the C-terminal tail essential for STING signaling (182) were not visible, possibly due to structural flexibility of this region.

Cyclic GMP-AMP Synthase Is a Cytosolic DNA Sensor

Although STING directly senses bacterial CDNs, it is not a DNA-binding protein. The fact that STING is essential for type I interferon production in response to cytosolic DNA suggests the existence of a general cytosolic DNA sensor upstream of STING. By establishing a biochemical assay, our group found DNA-stimulated activity in the cytosol that can activate STING (201). Through biochemical purification, this activity was found to come from a small molecule, cGAMP, the first CDN found in mammals (201). The enzyme that synthesizes cGAMP upon DNA recognition was identified as cGAS (177) (**Figure 3**). cGAS contains a nucleotidyltransferase (NTase) domain that is structurally homologous to the catalytic domain of the RNA-sensing enzyme OAS (84). Deletion of *cGAS* in various mammalian cell types results in a complete loss of type I interferon induction in response to cytosolic DNA delivery or DNA virus infection (42, 96). In addition, cGAS deficiency renders mice more susceptible to fatal HSV infection (42, 96). cGAS also senses infection by many other DNA viruses and retroviruses, including HIV (42, 86, 96, 164). These studies demonstrate that cGAS is a general cytosolic DNA sensor that generates the second messenger cGAMP to activate STING, leading to type I interferon production.

Crystal structures show that DNA binding induces cGAS dimerization and the formation of a 2:2 complex with two dsDNA molecules (93, 216). Each cGAS molecule contains two DNA-binding surfaces, each of which interacts with a dsDNA molecule. The 2:2 complex is stabilized not only by two DNA-binding surfaces, but also by the cGAS dimerization interface. Mutation analysis revealed that both the dimerization interactions and the two DNA-binding surfaces are crucial for cGAS activity in type I interferon induction (24, 43, 84, 93, 216). For both mouse and human cGAS, DNA binding triggers significant conformational changes of the highly conserved activation loop (residues 210–220), which moves inward to rearrange the active site for cGAMP synthesis (24, 43, 84, 93, 216). The activation of cGAS by dsDNA is length dependent. The cGAS complex with short dsDNA (~20 bp) is unstable, whereas longer dsDNA (>45 bp) triggers the formation of stable ladder-like networks with cGAS dimers, leading to stronger cGAMP production (7). Interestingly, short dsDNAs (12–20 bp) become highly stimulatory to cGAS when they carry unpaired guanosine extensions (Y-form DNA), which can be found in HIV1-derived ssDNA with stem-loop structures (55).

2′3′-cGAMP Is a Second Messenger That Activates STING

The discovery of cGAS and its product cGAMP explains why STING mediates type I interferon production in response to both cytosolic DNA and bacterial CDNs. Subsequent studies further revealed that the endogenous cGAMP produced by cGAS contains mixed phosphodiester linkages, with one between the 2′-hydroxyl group of GMP and the 5′-phosphate of AMP and the other between the 3′-hydroxyl group of AMP and the 5′-phosphate of GMP, resulting in the unique cyclic dinucleotide named 2′3′-cGAMP (2, 31, 43, 215). The endogenous messenger 2′3′-cGAMP has much higher binding affinity toward STING ($K_d \approx 4$ nM) than does any other cGAMP molecule containing other combinations of phosphodiester linkages (2′2′-cGAMP, 3′3′-cGAMP

and 3′2′-cGAMP) or bacterial CDNs (215). In addition, 2′3′-cGAMP is much more potent in IFN-β induction than bacterial CDNs and other cGAMP isomers (201, 215).

Ligand-free STING forms a butterfly-shaped dimer on the ER that undergoes a conformational change upon 2′3′-cGAMP binding (44, 215). In the absence of ligand, the STING dimer adopts an open conformation with the two butterfly wings far away from each other. Upon 2′3′-cGAMP binding, the two wings are pulled much closer, with the ligand sitting deep inside, which is accompanied by the concomitant formation of a lid consisting of four antiparallel β-sheet strands on top of the binding pocket, resulting in a closed conformation (44, 215). Such changes of the binding pocket rearrange outer segments of the STING dimer, which may allow recruitment of downstream signaling components of the type I interferon pathway. Of note, in most initial structural studies of STING, c-di-GMP binding did not induce significant conformational changes of the STING dimer, probably due to the use of a functionally compromised H232 variant of human STING for crystallization (63, 138, 168, 171, 210). For wild-type R232 STING, c-di-GMP can indeed induce similar structural changes comparable to 2′3′-cGAMP (63). Nevertheless, the 2′3′-cGAMP-induced, closed conformation of STING dimer is independent of this allelic variation.

In response to cytosolic DNA, 2′3′-cGAMP produced by cGAS not only initiates intracellular antimicrobial signaling, but also is transported to surrounding cells through at least three distinct routes. First, 2′3′-cGAMP produced in one cell is directly transferred to neighboring cells through gap-junctions, which are cell-cell contact–dependent channels connecting the cytoplasm of neighboring cells (3). Second, during infection by certain viruses, such as lentiviruses, HSV, MCMV, and modified vaccinia Ankara virus, 2′3′-cGAMP is packaged into new viral particles and carried to target host cells during the next round of infection (12, 45). Finally, for HIV infection, cGAMP can be transferred from donor T cells to primary macrophages through HIV envelope–induced intercellular membrane fusion (206). Intercellular 2′3′-cGAMP spreading is positioned to provide faster innate immune protection for host cells that may have impaired ability in 2′3′-cGAMP production, especially for those cells infected by the viruses that encode antagonists of cGAS (see below). Moreover, intercellular transfer of this second messenger permits neighboring uninfected cells to mount an interferon response that protects them from infection.

STING Trafficking and Signaling

The intracellular trafficking of STING plays an essential role for downstream signaling functions (**Figure 3**). Binding of cGAMP triggers robust STING trafficking from the ER to perinuclear compartments where TBK1 colocalizes with STING (66, 155). Brefeldin A or the *Shigella* effector protein IpaJ, both of which disrupt ER-to-Golgi trafficking, blocks STING downstream signaling (32, 82). Upon cGAMP binding, STING has been observed to partially colocalize with multiple organelles including the ER-Golgi intermediate compartments (ERGICs) (32), the Golgi complex (123, 155), endosomes (66), and autophagosomes (82). Each of these compartments has been proposed by different groups as the site of STING-mediated TBK1/IRF3 activation. However, a consensus has not been reached as to where STING is activated inside the cell and the mechanism for trafficking-meditated STING activation is still elusive.

Although the cell biological mechanisms for STING activation are unclear, biochemical studies have revealed that the STING C terminus is essential for scaffolding TBK1-mediated IRF3 activation (182). The extensive conformational changes of STING upon cGAMP binding reorganize the STING C tail (44, 215), which may expose essential elements required for STING trafficking, TBK1 recruitment, and other signaling events. A peptide consisting of the last 39 amino acids of STING is both necessary and sufficient to activate IRF3 phosphorylation by TBK1 (182). More recently, a common mechanism for adaptor-scaffolded, TBK1-mediated IRF3 activation was

identified (100). Specifically, a consensus pLxIS motif (p is hydrophilic, x indicates nonaromatic, and S represents serine) was identified as an essential adaptor component to support TBK1-mediated IRF3 phosphorylation (100). This motif is shared by all three innate immune adaptors upstream of IRF3: TRIF, MAVS, and STING. Upon activation of any of the three adaptors, this consensus motif is directly phosphorylated by TBK1 (Phosphor-Ser366 for human STING), and the phosphorylated motif in turn serves as a docking site for IRF3, facilitating its phosphorylation by TBK1, which leads to IRF3 dimerization and activation (100). STING phosphorylation at Ser366 by Unc-51-like kinase 1 (ULK1, Atg1 in yeasts) was proposed to suppress STING signaling (82), but this contradicts other studies showing that phosphorylation of the same site by TBK1 is essential for IRF3 activation and interferon production (100, 182, 186). In a molecular dynamics simulation model, the lid structure of STING induced by cGAMP binding provides an anchor point for the C-terminal tail of STING to adopt a suitable conformation that facilitates its phosphorylation by TBK1 (186). This model is supported by data showing that point mutants that disrupt a putative lid:C-tail interaction impair type I interferon induction (182, 186). However, it remains to be elucidated how TBK1 is recruited and activated by STING upon cGAMP binding.

Cytosolic DNA Sensing in Antimicrobial Autophagy

In addition to interferon production, substantial evidence indicates that the cGAS-STING pathway also induces autophagy. DNA stimulates the trafficking of STING through the Golgi to endosome-like compartments where STING colocalizes with not only TBK1 but also autophagy-related protein 9 (ATG9) and the microtubule-associated protein 1A/1B-light chain 3 (LC3), an autophagosome marker (155). Infection with DNA viruses, such as HSV-1 and HCMV, triggers robust LC3 lipidation in human fetal foreskin fibroblasts, and so does electroporation of DNA, but not RNA, into these cells (115). HSV-1-induced LC3 lipidation was found to depend on the genomic DNA of the virus and STING from host cells (147). Similarly, the bacterial pathogen *M. tuberculosis* also activates STING-dependent autophagy to control intracellular bacterial replication. Bacterial CDNs, such as c-di-GMP and c-di-AMP, also induce autophagy (195). Recently, cGAS was found responsible for the recognition of *M. tuberculosis*–derived DNA, which then produces cGAMP to activate STING-mediated autophagy and type I interferon induction (25, 193, 194). Thus, besides interferon production, the cGAS/STING pathway also triggers autophagy as a cell autonomous strategy to control pathogen replication and to potentially also resolve infection-induced cellular stress (120). However, it remains to be investigated how autophagy is activated by STING and how cytosolic DNA or pathogens are targeted by autophagy.

The AIM2 Inflammasome Pathway

Apart from inducing type I interferons through the cGAS-STING pathway, cytosolic dsDNA in immune cells also initiates the assembly of inflammasome, a multiprotein complex that serves as a platform for caspase-1-depedent maturation of proinflammatory cytokines such as IL-1β. The first-discovered group of inflammasome sensors, nucleotide oligomerization domain (NOD)-like receptors (NLRs), is characterized by the presence of a NACHT domain responsible for agonist sensing and self-oligomerization (185). One of the most extensively studied NLRs, NLRP3, senses a plethora of microbial and endogenous stimuli (74, 126). However, internalized bacterial, viral, and mammalian dsDNA is able to activate inflammasome independent of NLRP3 (126). The DNA sensor was identified as AIM2 (14, 35, 59, 151) (**Figure 4**). AIM2 is the founding member of a family of inflammasome sensors, AIM2-like receptors (ALRs), which possess one or more HIN-200 domains instead of the NACHT domain found in NLRs.

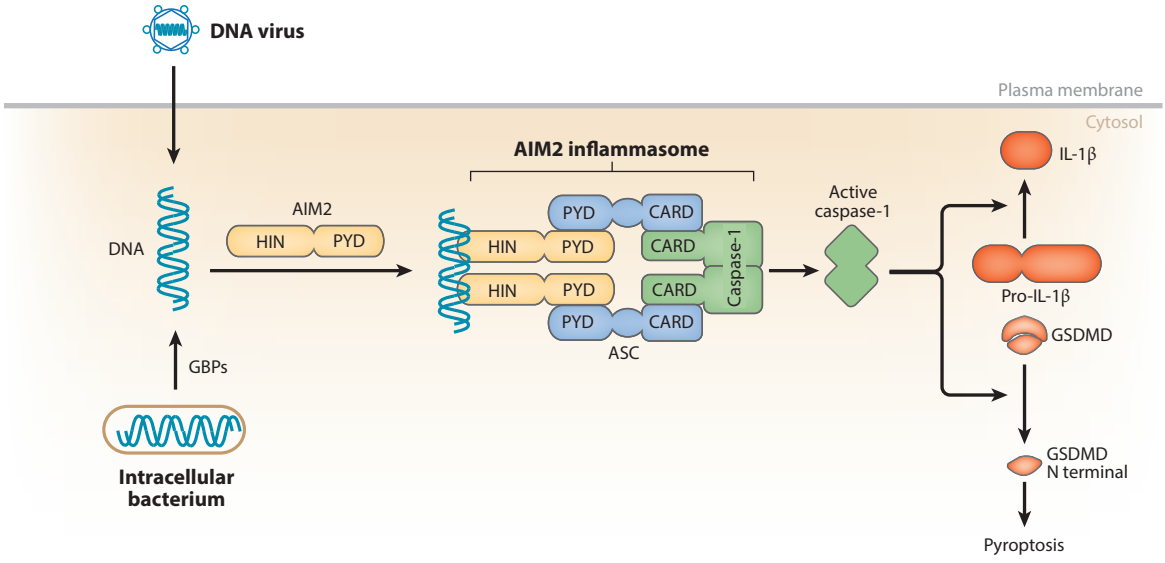

Figure 4

The AIM2 inflammasome. Binding of DNA to the HIN domain of AIM2 induces a conformational change that leads to AIM2 oligomerization. The PYD of AIM2 interacts with the PYD of ASC, resulting in a prion-like polymerization of ASC, which provides a platform for the recruitment of caspase-1 through CARD-CARD interactions. Caspase-1 is activated to cleave pro-IL-1β to generate mature IL-1β. Caspase-1 also cleaves and activates GSDMD to trigger pyroptosis. Abbreviations: GBP, guanylate-binding protein; GSDMD, gasdermin D; HIN, hematopoietic expression, interferon inducibility, and nuclear localization; PYD, pyrin domain.

Unlike NLRP3, which indirectly responds to diverse stimuli through potential intermediate signals (37), AIM2 senses dsDNA through direct binding. The C-terminal HIN-200 domain of AIM2 embraces both strands of dsDNA across both major and minor grooves (70), which explains why AIM2 responds to dsDNA but not ssDNA (151). The AIM2 interaction is mediated by the sugar-phosphate backbone of dsDNA, consistent with sequence-independent DNA recognition (70). Similar to cGAS, the lack of sequence preference may allow AIM2 to detect dsDNA from a large variety of invasive microbes.

Both NLRs and ALRs contain a pyrin domain (PYD) that mediates homotypic interaction with the N-terminal PYD of the common downstream adaptor ASC (apoptosis-associated speck-like protein containing a CARD) (35) (**Figure 4**). ASC then undergoes prion-like polymerization (16, 104) and recruits the cysteine protease caspase-1 through homotypic CARD-CARD interaction, leading to caspase-1 autocleavage and activation. Active caspase-1 promotes the maturation of IL-1β and IL-18 (88). Caspase-1 also cleaves gasdermin D, which then forms pores on cellular membranes to cause pyroptosis (79, 169).

MICROBIAL EVASION OF HOST NUCLEIC ACID SENSING

With various innate immune systems evolved in metazoans against infection, microbial pathogens also developed strategies to evade or attack host immunity. This is true for most disease-causing microbes that would have otherwise been efficiently eliminated without causing diseases in the host. Many mechanisms developed by microbes to evade different steps of nucleic acid sensing have been discovered in recent years. Below we discuss different strategies employed by representative microbial pathogens.

Degrading, Hiding, or Modifying Microbial Nucleic Acids

Since the first step of innate immunity is the recognition of various PAMPs from invading microbes, hiding their PAMPs, especially nucleic acids, has evolved as a common strategy to avoid host attack. An effective way of hiding nucleic acids from being sensed by TLRs is to evade the endosomal compartments, as do many viruses and bacteria, some of which simply bypass the endocytic process through direct cytosol invasion. A method of avoiding cytosolic sensing is to remove microbial nucleic acids by degradation. Mammalian cells encode a variety of nucleases that degrade intracellular and extracellular DNA. For example, the nuclease TREX1 degrades ssDNA or nicked dsDNA, and this enzyme is hijacked by HIV to avoid cytosolic viral DNA accumulation and evade cGAS recognition (208).

In addition to DNA degradation, viruses also physically hide their nucleic acids through various approaches. For example, besides TREX1-mediated DNA digestion, the viral capsid of HIV also recruits host factors to hide viral DNA from being exposed in the cytosol (146). Ebola virus uses its own structural protein VP35 to hide viral RNA through direct RNA binding (17). A more complex approach by dengue virus is hiding its dsRNA inside ER-derived vesicles formed after infection (196), a potential strategy to subvert RNA sensing in the cytosol.

An additional strategy is specific modifications of microbial nucleic acids to avoid recognition by host sensors. As RIG-I recognizes 5′-diphosphate or 5′-triphosphate RNAs, some RNA viruses hydrolyze the 5′ phosphates of its genome into 5′ monophosphate to avoid RIG-I detection (49). Eukaryotic mRNAs have ribose 2′-*O*-methylation at the 5′ cap as a self-signature that cannot be recognized by RIG-I, MDA5, and IFIT-1 (26, 166, 220). However, viruses also use viral or cellular enzymes to methylate their mRNA to avoid detection by the immune system (26, 166, 220).

Antagonizing Nucleic Acid Sensors

Besides strategies applied to their nucleic acids, an additional way used by microbes to interfere with the sensing process is to directly or indirectly attack host nucleic acid sensors. For example, the nonstructural protein NS1 of influenza A virus targets both RIG-I and the E3 ligase TRIM25, blocking polyubiquitination and RIG-I activation (40, 143). Similarly, the V proteins of paramyxoviruses directly bind MDA5 to block its filament formation (122). Recently, various viral strategies targeting cGAS have been described. A truncated isoform of the latency-associated nuclear antigen encoded by Kaposi sarcoma herpesvirus (KSHV) interacts with cGAS and blocks activation of downstream signaling (214). KSHV tegument protein ORF52 and its homologs in other gammaherpesviruses inhibit cGAS activity by directly binding to both cGAS and DNA (202). Dengue virus, despite successfully hiding viral RNAs in ER-derived vesicles, causes host DNA release from damaged mitochondria, which can be detected by cGAS to trigger an immune response (4). However, dengue virus also antagonizes DNA sensing through viral protein NS2B-mediated degradation of cGAS (4).

Antagonizing Signaling Adaptors and Other Signaling Molecules

Given that nucleic acid–sensing pathways activate several common adaptor proteins and similar signaling cascades, many pathogens have evolved strategies targeting signaling adaptors and downstream molecules. In many cases, host adaptors are directly destroyed by viral protease-mediated cleavage. For example, the HCV protease NS3/4A inhibits MAVS signaling by directly cleaving MAVS off the mitochondrial membrane (95, 118). Similarly, the cysteine protease 3C of coxsackievirus B and the 3C protease precursor of hepatitis A virus cleave both TRIF and MAVS to suppress host interferon production (124, 145, 209).

Some viruses inhibit adaptor functions through direct binding instead of cleavage. A poxvirus-encoded PYD-containing protein M13L interacts with ASC to suppress inflammasome activity (71). KSHV, a gammaherpesvirus, encodes six viral proteins that block IFN-β activation through the cGAS-STING pathway (109). Among them, viral interferon regulatory factor 1 (vIRF1) inhibits STING interaction with TBK1, resulting in a block of STING phosphorylation and downstream signaling. Two DNA tumor viruses, human papilloma virus (HPV) and adenovirus, also attack the cGAS-STING pathway with their oncogene product (89). Specifically, E7 from HPV and E1A from adenovirus share a common LxCxE motif, where x refers to any amino acid, that directly binds STING to suppress its antiviral signaling (89).

Many microbial pathogens interfere with signaling downstream of nucleic acid sensors and adaptors. For example, the *Shigella* effector protein IpaJ indirectly inhibits STING signaling by blocking STING trafficking from the ER, as mentioned above (32). HSV-1 ICP27 binds to the active STING-TBK1 complex to block downstream IRF3 phosphorylation (23), whereas the VACV protein K7 targets DEAD box protein 3 to suppress TBK1 signaling (165). Other examples of microbial evasion by targeting specific signaling molecules have recently been reviewed (10, 19, 134).

CONCLUSIONS AND FUTURE PERSPECTIVES

Tremendous progress has been made toward understanding the innate immune sensing of microbial nucleic acids. Cytosolic RNA sensors and DNA sensors, together with endosomal TLRs, establish a multilayer surveillance system that triggers type I interferons and proinflammatory cytokines in response to microbial nucleic acids. Biochemical, genetic, and structural studies have elucidated the molecular mechanisms underlying specific nucleic acid recognition and antimicrobial signaling in response to infection from diverse pathogens. Future investigations will need to address how multiple nucleic acid–sensing pathways are regulated and how they cooperate with each other to initiate an optimal immune response. A clearer connection has to be drawn between the natural ligands of different pathogens and the respective nucleic acid sensors that detect them. Recent development of imaging technologies should enable visualization of the direct and dynamic interactions between nucleic acid sensors and their cognate ligands from microbes in the course of infections in live cells.

More work is needed to understand the cell biological regulation of each nucleic acid–sensing pathway. For example, although both TLRs and STING undergo substantial intracellular trafficking required for signaling activation, the molecular mechanisms are still unknown. It is unclear what happens to STING beyond the Golgi complex. How is autophagy activated by STING? What are the mechanisms to turn off MAVS and STING signaling? Answers to these questions will provide a better understanding of how these pathways are regulated in cells.

Despite being initially discovered as innate immune pathways against microbial infections, nucleic acid sensing and signaling are also found to be triggered by self-DNA or -RNA in various physiological and pathological conditions. This can lead to undesirable consequences such as autoimmune and autoinflammatory diseases (152). On the other hand, detection of tumor DNA by the cGAS-STING pathway is important for antitumor immunity (129). Moreover, the cGAS pathway has recently been shown to be essential for cellular senescence and for inflammation caused by nuclear DNA damage or chromosome missegregation (28). Thus, it is critical to understand how nucleic acid sensors effectively detect pathogen infections while avoiding triggering autoimmune reactions to self–nucleic acids. Such knowledge is important for developing new therapeutics that will be safe and efficacious for the treatment of infectious diseases, autoimmune diseases, senescence-associated diseases, and cancer.

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