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Cytoplasmic DNA innate immune pathways

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Summary: The innate immune system is responsible for detecting microbial invasion of the cell and for stimulating host defense countermeasures. These anti-pathogen procedures include the transcriptional activation of powerful antiviral genes such as the type I interferons (IFNs) or the triggering of inflammatory responses through interleukin-1 (IL-1) production. Over the past decade, key cellular sensors responsible for triggering innate immune signaling pathways and host defense have started to be resolved and include the Toll-like receptor (TLR), RIG-I-like helicase, and the cytoplasmic nucleotide-binding oligermerization domain-like receptor families. These sensors recognize non-self pathogen-associated molecular patterns such as microbial lipopolysaccharides and nucleic acids. For example, TLR9 has evolved to detect CpG DNA commonly found in bacteria and viruses and to initiate the production of IFN and other cytokines. In contrast, AIM2 (absent in melanoma 2) has been shown to be essential for mediating inflammatory responses involving IL-1β following the sensing of microbial DNA. Recently, a molecule referred to as STING (stimulator of IFN genes) was demonstrated as being vital for recognizing cytoplasmic DNA and for activating the production of innate immune genes in response to a variety of DNA pathogens and even certain RNA viruses. Comprehending the mechanisms of intracellular DNA-mediated innate immune signaling may lead to the design of new adjuvant concepts that will facilitate vaccine development and may provide important information into the origins of autoimmune disease.

Keywords: *innate immunity, STING, DNA, AIM2, TLR9*

Introduction

The host innate immune system provides a primary line of defense against microbial invasion. These responses are triggered by cellular sensors that recognize pathogen molecules and switch on the production of host defense genes such as the type I interferons (IFNs) (1–4). Many of these innate immune-stimulated proteins have direct anti-pathogen activity or can stimulate adaptive immune responses involving antibody production and cytotoxic T-cell activity. Pathogen-derived nucleic acid constituting microbial RNA and DNA, referred to as PAMPs (pathogen-associated molecular patterns) or DAMPS [danger-associated molecular patterns such as reactive oxygen species (ROS)] are now known to be potent activators of innate immune signaling (1–4). A variety of

sensors have evolved in different cell types such as macrophages and dendritic cells (DCs) to detect PAMPs. Most types of fibroblasts or endothelial cell types are able to recognize microbial genomes to directly trigger innate immune gene production including type I IFN. In addition, hematopoietic cells, including macrophages and DCs, can produce IFN as well as inflammatory cytokines such interleukin-1β (IL-1β) following infection or engulfment of apoptotic cell debris that contains microbial antigen and nucleic acid (1–3). Significant progress has been made in the last few years relating to how the cell recognizes pathogen-derived RNA, although it has been less clear how the cell triggers innate immune signaling in response to cytoplasmic DNA species originating from microbes such as herpes simplex virus (HSV) or bacteria. Toll-like receptor 9 (TLR9) has been shown to be important for recognizing pathogen-derived, non-self CpG species in plasmacytoid DCs (pDCs), while a member of the nucleotide-binding oligomerization domain (Nod)-like receptor (NLR) family, referred to as absent in melanoma 2 (AIM2), has been demonstrated as being critical for cytosolic DNA-mediated, inflammasome-dependent innate signaling (5–9). Less is known, though about how cells such as macrophages, DCs, and fibroblasts recognize cytoplasmic dsDNA to trigger the production of type I IFN production. Lately, a breakthrough into the regulation of this signaling process came through discovery of a molecule referred to as STING (stimulator of IFN genes) (10). Here, we provide a brief review of innate signaling pathways stimulated by cytoplasmic DNA that have important implications for our understanding of microbial-based pathogenesis and perhaps even autoimmune disease.

The Toll-like pathway

The Toll pathway was first recognized in insects and is thus an evolutionarily conserved component of host defense (1–3). Pattern recognition receptors (PRRs) such as the TLR family are important innate immune sensors capable of detecting a variety of bacterial or viral DNA/RNA and protein (PAMPS). There are approximately 10 human TLRs, which sense microbial PAMPs such as lipopolysaccharides (TLR4) which are common on bacterial cell walls, viral double stranded RNA (dsRNA) (TLR3), viral single stranded RNA (ssRNA) (TLR7/8), as well as viral or bacterial unmethylated DNA referred to as CpG DNA (TLR9; see below) (2, 3). These PAMPs are potent inducers of innate immune gene production, which includes type I IFN as well as other cytokines. For example, TLR3, TLR7/8, and TLR9 reside within the endoplasmic reticulum (ER) and traffic to endosomal compartments, where they are able to detect pathogen-derived nucleic acid. TLR3 recruits an adapter referred to as TRIF [Toll-IL-1 receptor (TIR) domain-containing adapter inducing IFN-β], which associates with tumor necrosis factor receptor (TNFR)-associated factor 3 (TRAF3) and TRAF6 as well as receptor-interacting protein 1 (RIP1) (11–13). This signaling complex involves interaction with Fas-associated death domain (FADD)-containing proteins and the TNFR-associated death domain containing protein (TRADD) (11). Innate immune signaling processes involve the activation of nuclear factor of κ light polypeptide gene enhancer in B cells (NF-κB) (1–3). TRAF3 stimulation can also cause the activation of mitogen-activated protein kinases (MAPKs) and two inhibitor of NF-κB kinase (IKK)-related kinases referred to as Tank-binding kinase 1 (TBK1) and IKK-I, which are required for phosphorylation of IFN regulatory factor 3 (IRF3) and IRF7. IRF3 and IRF7 dimerize and, following translocation to the nucleus with NF-κB, activate IFN β promoter transcription (14, 15). Type I IFN is translated, secreted, and binds to IFN receptors (IFNARs) present on many cell types, a consequence that triggers the production of hundreds of antiviral genes through the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway (1–4). Cells treated with type I IFN are remarkably resistant to virus replication, while murine models harboring defects in type I IFN signaling pathways are sensitive to virus infection. pDCs are considered a major source of type I IFN production when exposed to RNA and DNA viruses (15, 16). In this situation, pDCs infected by an RNA virus, or that engulfs apoptotic cell debris containing viral RNA, activate TLR7, which causes association with MyD88 (myeloid differentiation primary response gene 88) and members of the IRAK (IL-1 receptor-associated kinase) family, TRAF3, and TRAF6. This signaling cascade potently triggers IRF7 phosphorylation, which as mentioned, translocates to the nucleus to activate the production of type I IFN (15).

While the TLR pathway comprises an important component of our host defense capabilities, it became evident that TLR3- or TLR7-deficient animals remained able to produce type I IFN in response to RNA virus infection (1–3). These observations stimulated investigations to find TLR-independent pathways that could explain alternate mechanisms of viral RNA-mediated innate immune signaling. A key breakthrough came with discovery of three DExD/H box RNA helicases known as retinoic acid-inducible gene I (RIG-I), melanoma differentiation-associated gene 5 (MDA5), and LGP2 (17). These events required the death domain containing proteins FADD and RIP1 (4, 18). RIG-I and MDA5 helicases turned out

to be responsible for the production of IFN in all cell types, in response to RNA virus infection, except for pDCs, which are TLR7 dependent (4, 17, 19). RIG-I recognizes 5′-triphosphate-containing RNA for example encoded by rhabdoviruses such as vesicular stomatitis virus (VSV). In contrast, MDA5 may recognize longer RNA, such as synthetic dsRNA (polyIC) and the genomes of picornaviruses such as encephalomyocarditis virus (EMCV) (4). LGP2, despite originally being portrayed as a possible negative regulator of RIG-I and MDA5 function, seems to be critical for protection against a variety of virus types such as EMCV, as shown using knockout mice models lacking this gene (20, 21). It was found that helicase interaction with viral RNA induces the recruitment of a mitochondrial molecule referred to as IPS-1 (also known as VISA, Cardif, or MAVS) as well as FADD, RIP1, and TRAF3, for the activation of mitogen-activated protein kinases (MAPKs), TBK1, and IKK-I, which enable the transcription factors activator protein-1 (AP-1), IRF3/7, and NF-κB to translocate into the nucleus and activate the IFNβ promoter (22–25).

Despite this progress, it remains to be determined how foreign DNA, derived from viruses, bacteria, fungi, and parasites, is sensed by the cell to trigger innate immune signaling. What is apparent is that TLR9 is important for triggering the production of type I IFN production in pDCs in response to non-methylated CpG DNA. Furthermore AIM2, a member of the NLR family, has recently been reported to be essential for recognizing pathogen-derived dsDNA to activate caspase-1-mediated proinflammatory cytokine production (6–9). It was unclear, though, how type I IFN production occurred in response to non-CpG-based DNA. Most cells such as fibroblasts, macrophages, and DCs respond to the presence of cytoplasmic dsDNA by activating an innate immune signaling cascade that involves the production of type I IFN. A clue into the regulation of this pathway, however, came from the discovery of STING, as discussed below (10). Here we describe some of the key pathways responsible for recognizing cytoplasmic DNA, including the TLR9 and STING-dependent pathways as well as inflammatory pathways controlled by AIM2.

TLR9 and the detection of CpG DNA

TLR9 is predominantly expressed in pDCs and B cells and recognizes CpG DNA commonly found in bacteria and viruses, not in vertebrates (5). TLR9 contains a leucine-rich repeat (LRR) motif, a Toll/IL-1R homology domain, and is a type I integral membrane glycoprotein (26–28). Inactive TLR9 is localized to the ER in unstimulated pDCs, though it traffics to endolysosomal compartments where it becomes proteolytic cleaved into an active state. The trafficking of TLR9 from the ER to endolysosomal compartments is controlled by UNC93B, a 12-membrane-spanning ER protein that directly interacts with TLR9 (29, 30). Adapter protein-3 (AP-3) is also required for TLR9 trafficking. CpG DNA, internalized via a clathrin-dependent endocytic pathways, traffics to lysosomal compartments to associate with pre-active TLR9 present in these regions (31). Upon recognition of CpG DNA in endosomes, TLR9 interacts with MyD88, which contains a TIR domain and a death domain (1–3). MyD88 interacts with IRAK-1 (IL-1R associated kinase 1), IRAK-4, and IRF-7. This event leads to recruitment of TRAF3 and TRAF6, which activates TAK1 (transforming growth factor β-activated kinase 1), MAPK, as well as NF-κB. IRAK1 directly interacts with and phosphorylates IRF7, which is required for IFNβ transcriptional activity (1–3). It is thought that TRAF3-dependent activation of IRF7 takes place in LAMP2 containing endosomes, while the activation of NF-κB occurs in different endosomes, rich in VAMP3. Thus, different endosomal compartments both containing CpG DNA and TLR9 may be responsible for either NF-κB or IRF7 activation (32).

Several studies using TLR9-deficient mice have emphasized a role for this receptor in host innate immune responses against DNA viruses such as HSV (5, 26, 27). Reports have also indicated that TLR9 may facilitate plasmid-based immunization protocols (33). Of interest is that TLR ligands have been used to improve the immunogenicity of a variety of antigens used in vaccines strategies. For example, synthetic oligodeoxynucleotide (ODN) ligands, such as CpG ODN for TLR9, have been reported to be a potent adjuvant that enhances immunization procedures (34, 35). Engagement of the TLRs can stimulate the production of a variety of costimulatory molecules as well as IFN-induced genes that can facilitate T- and B-cell activation. Aside from exhibiting strong adjuvant properties, CpG monotherapy has also been demonstrated to have prophylactic efficacy against a wide range of viral, bacterial, and parasitic pathogens (35, 36). Finally, TLR9 agonists have been utilized as treatments for hematologic malignancies, skin cancers, and glioblastoma (35, 36).

TLR9 has been also implicated in the development of autoimmune diseases such as systemic lupus erythematosus (SLE) (37). Considerable evidence indicates that self nucleic acids can act as endogenous ligands for DNA receptors, which raise serum levels of IFN and which correlate with both SLE disease activity and severity (4, 38). For example, anti-DNA antibodies, found in serum of patients with SLE, bind self-DNA and induce type I IFN via a cooperative interaction between TLR9 and CD32 in pDCs (37). Thus, unraveling the molecular

mechanisms of DNA-triggered innate immune signaling pathways may have important implications in our understanding of autoimmune disease and could lead to the development of new therapeutics to treat such disorders.

STING and intracellular DNA signaling

While TLR9 plays a key role in recognizing CpG DNA predominantly in pDCs, it was apparent that transfected dsDNA could still activate the production of type I IFN in cells that apparently lacked this TLR member (3). For example, DNA-based immunization procedures, HSV1, or intracellular bacteria such as *Listeria monocytogenes* remained able to stimulate immune responses in TLR9-deficient mice (33, 34, 39, 40). Therefore, while TLR9 signaling certainly comprises a key element of our innate immune responses, evidence suggested the existence of additional DNA sensors, especially in non-pDCs. Recently, however, a new molecule STING has been shown to be critically important for facilitating dsDNA's ability to induce innate immune gene induction, including type I IFN production in fibroblasts, macrophages, and DCs (10, 41). STING was discovered using a cDNA expression library designed to isolate activators of type I IFN signaling. STING, also known as MITA/MPYS/ERIS, turned out to be a transmembrane protein component of the ER that could potently activate the transcription factors NF-κB as well as IRF3/7 to stimulate type I IFN production (10, 41–44). STING-deficient mice were found to be viable, although preliminary studies indicated that HSV-1 or Gram-positive L. *monocytogenes*, or a variety of dsDNA species including viral or bacterial genomes or plasmids, completely failed to trigger the production of type I IFN in STING-deficient MEFs, macrophages, or conventional DCs (10, 41). STING has also been shown to be critical for the production of type I IFN in macrophages infected with a Gram-negative bacterium (*F. tularensis*) (45). The DNA pathogen *Chlamydia muridarum* has similarly been shown to activate the STING pathway (46). Our studies additionally indicated that STING was important for both cytoplasmic dsDNA and HSV-1-activated type I IFN production in pDCs (5). However, the presence of TLR9 in pDCs was found to substitute, partially, for the loss of STING in response to *Listeria* infection. STING-deficient animals succumb to lethal HSV-1 infection due to a lack of type I IFN production required to protect the host (41). The production of IL-1β in STING-deficient macrophages following HSV1 and intracellular *Listeria* infection did not appear to be affected by loss of STING, confirming that this pathway is controlled by AIM2 (see below) (5). Thus, STING functions independent of AIM2 and is not essential for caspase-1-mediated proinflammatory cytokine production.

In an attempt to identify STING-associating proteins, a yeast-two hybrid approach was adopted that revealed that STING interacted with a member of the translocon family, known as SSR2/TRAPβ (translocon associated protein β) (41). The TRAP complex is made up of four subunits (α–Δ) and interacts with the Sec61 complex, which itself is comprised of 3 subunits (SEC61α, SEC61β, and SEC61γ) (47–49). Proteins that are meant for secretion or for membrane incorporation are directed into the translocon by the signal recognition particle (SRP), which is attached to the translating ribosome. The SRP is made of several proteins and an RNA molecule (7SL) and recognizes a signal sequence at the N-terminus of the nascent peptide as it is translated (50). Peptide synthesis is halted while the SRP attaches to the signal recognition receptor (SRR), which is complexed to the translocon. As the peptide enters the translocon, the SRP and SRR separate, and translation ensues. Thus, peptide synthesis and translocation are tightly associated and important for ensuring proper protein folding, glycosylation, secretion, or delivery to the appropriate membrane compartment (49, 50). Approximately, 30% of cellular proteins are destined to be escorted into the ER via the translocon. The translocon has also been indicated to play an important role in ER-associated degradation, where misfolded or aberrantly glycosylated proteins are re-directed for destruction, following ubquitination, by the 26S proteosomal pathway (retrotranslocation) (51). It remains to be determined, however, whether STING plays a role in regulating these pathways.

It also remains to be seen whether STING itself recognizes intracellular DNA species or whether STING is downstream of a key sensor that has yet to be identified. If STING does indeed complex directly or indirectly with intracellular dsDNA, it is not apparent whether this event occurs in the ER or elsewhere in the cell, such as in endosomal compartments, similar to TLR9, as described above (1–3). It is known that ssDNA from stressed S-phase cells or derived from endogenous retroelements can accumulate in the ER. The exonuclease TREX1 is responsible for degrading such DNA before these nucleic acids inadvertently stimulate innate signaling pathways and provoke autoimmune disorders (52–55). Polyomaviruses, such as SV40, are also known to traffic to the ER compartment where viral disassembly occurs, but it is unknown whether the dsDNA genome from these viruses triggers dsDNA-dependent innate signaling pathways. SV40 entry involves caveolin-mediated endocytosis, while other viruses utilize macropinicytosis, calveolin, or clathrin-dependent entry (56). It is possible

that endosomes/lysosomes carrying viruses become associated with the ER, where pathogen-derived DNA could activate STING-dependent signaling. What is apparent is that in the presence of intracellular DNA, STING rapidly traffics from the ER region through to the Golgi to reside in a distinct perinuclear endosomes (10, 41). TBK1 was observed to similarly traffic to these regions in a STING-dependent manner. Presumably, STING escorts TBK1 to endosomal compartments to associate with and activate IRF3 and IRF7 which subsequently translocate into the nucleus to activate innate immune gene transcription. STING is also known to stimulate NF-κB signaling, although the regulatory processes are less understood. Although the mechanisms of STING trafficking remain to be fully comprehended, it has been reported that the translocon may physically associate with exocyst complexes responsible for tethering secretory vesicles to membranes and which are essential for protein synthesis and secretion (57, 58). It has been reported that TBK1 may comprise part of the exocyst complex through Sec5 association. Possibly, STING trafficking may involve the autophagy pathway as autophagy-related gene 9a (Atg9a) has been shown to facilitate STING action (59). STING is also ubiquitinated (lysine 63-linked) by IFN-inducible tripartite-motif 56 (TRIM56), perhaps as a prerequisite for association with TBK1 (60). Finally, ubiquitination and degradation of STING likely provides a mechanism to prevent STING from overstimulating innate immune gene transcription (61). Thus, intracellular dsDNA causes STING to escort TBK1 through the Golgi to endosomal compartments to activate members of the IRF family, plausibly through interactions with components of the exocyst family.

STING also plays an important role in triggering innate immune gene activation in response to infection by intracellular bacteria such as *L. monocytogenes* (10). Bacteria can enter non-phagocytic cells via the clathrin-mediated pathway, similarly indicating a key role for endocytosis. Possibly leaked bacterial DNA may be able to activate STING-dependent signaling, as described above. Recently, a study that used a forward genetic mutagenesis screen to generate mice that failed to produce type I IFN in response to *Listeria* infection identified STING as the basis for the defect. The mouse, referred to as Goldenticket, had a single point mutation (T596A) in STING that rendered this molecule inactive (62). Studies demonstrated that bacterial secreted cyclic dinucelotides are potent inducers of type I IFN production and that STING is essential for this facilitating this process. Recently, loss-of-function STING mutations have been identified in approximately 3% of Americans, and it is speculated that these individuals may have heightened susceptibility to pathogen infection (63). Thus, STING is essential for innate immune reactions to pathogen secreted second messenger cyclic dinucleotides as well as cytoplasmic dsDNA and may even be a sensor for other types of pathogen-derived danger signals such as ROS (64).

STING may also be critical for facilitating innate immune responses by negative-stranded RNA viruses such as VSV or Sendai virus (SV). MEFs lacking STING were defective in VSV and SV-dependent type I IFN production. STING-deficient mice were also extremely sensitive to VSV infection (10, 41). However, synthetic dsRNA (polyIC) was not affected in its ability to produce IFN in the absence of STING. This finding suggested that STING may play an important role in RIG-I-mediated signaling, which senses RNA viruses such as VSV and SV, but not MDA5, responsible for facilitating polyIC signaling. RIG-I signaling is also known to be dependent on IPS-1, a mitochondrial protein. Our data indicated that ER-associated STING likely associates in close proximity with mitochondria associated with the ER [mitochondria-associated membrane (MAM)] (65). Mitochondria form close contacts with the ER to obtain calcium for oxidative phosphorylation purposes. Thus, one plausible model could include that viral RNA associated with ER-attached ribosomes could activate RIG-I and subsequently IPS-1 associated with MAMs. STING, associated with the translocon, may facilitate this process (*Fig.* 1). Given the importance of the STING pathway in mediating innate immune signaling events, it is likely that viruses may target this pathway to avoid host defense responses. In this light, STING has been found to exhibit homology with flavivirus proteins (Dengue virus, yellow fever virus, and hepatitis C virus, NS4) known to reside in the ER. Thus, flaviviruses, and possibly other viruses, may target the translocon to inactivate innate immune signaling (10, 41).

DNA-dependent innate immune signaling pathways are important for plasmid DNA-based immune responses. Previous work indicated the importance of TBK1 in facilitating these processes. TBK1-dependent pathways are triggered by intracellular plasmid B-form DNA which stimulates immune responses by activating type I IFN and NF-κB-dependent pathways, similar to agonists of the TLR9 pathway (66, 67). As we observed that STING facilitates TBK1 function, we investigated whether STING was important for the adjuvant effects of plasmid DNA. This investigation confirmed that STING was indeed required for effective plasmid DNA and vaccinia virus-stimulated adaptive immune responses (41). In summary, data so far indicate that STING regulates innate immune gene regulation in many cell types except for those governing the recognition of GpG DNA and governing caspase-1-dependent

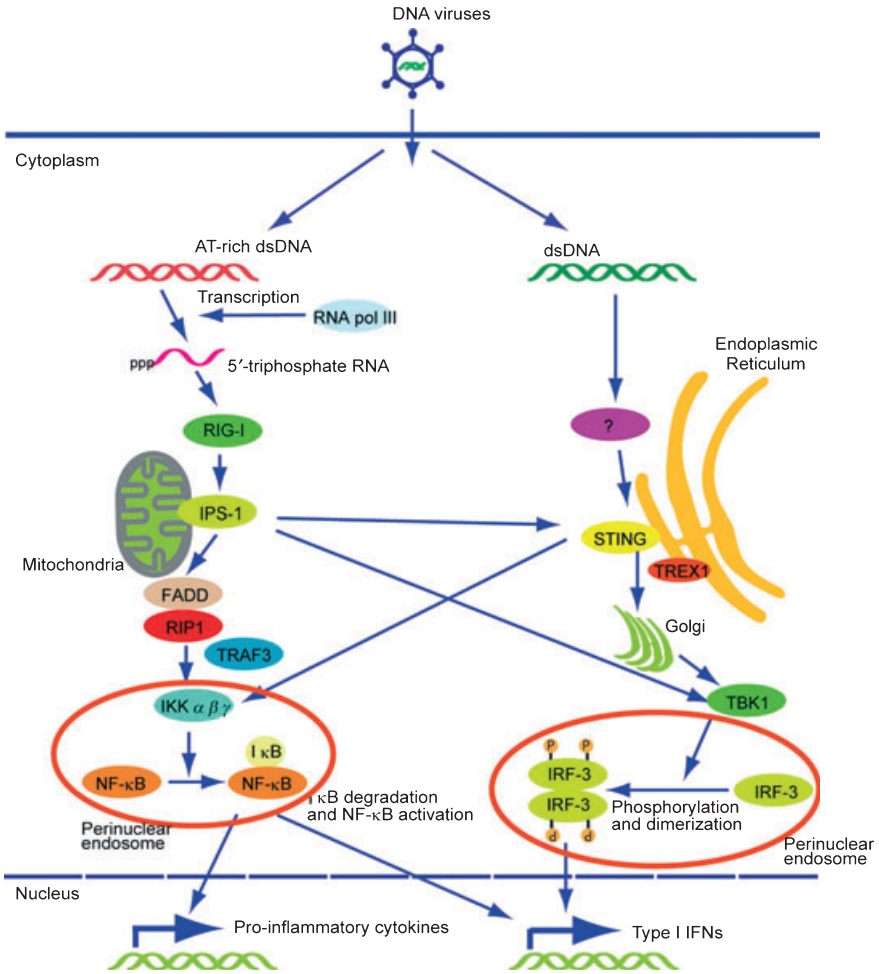

Fig. 1. DNA-mediated activation of innate immune signaling. Cytosolic AT-rich dsDNA is recognized by RNA PolIII and transcribed into 5′-triphosphate RNA, which activates RIG-I/IPS-1 signaling. IPS-1 interacts with FADD/RIP1 which activates NF-κB. IPS-1 also activates TBK1, which phosphorylates IRF-3/7 and induces type I IFN expression. AT-rich dsDNA or non-AT-rich dsDNA activates STING-dependent signaling. STING localizes in the ER with translocon complex (TRAPβ, Sec61), and escorts TBK1 to endosomal compartments to activate NF-κB and IRF3/7 and induce type I IFN.

inflammatory responses. Understanding STING regulatory pathway may lead to the development of novel, effective adjuvants that stimulate components of the innate immune response, without invoking unwanted inflammatory side effects.

Negative-regulation of cytoplasmic DNA signaling: TREX1

While the production of type I IFN and other innate immune genes are important for protecting the host against microbial infection, it is also known that the overstimulation of innate signaling pathways can facilitate autoimmune disease (68). Host cellular DNA sensors are predominantly located to the cytoplasmic compartment, where self-DNA is not normally present. Indeed, host DNA is limited to the mitochondria and nucleus, and so the inadvertent activation of pro-inflammatory cytokine pathways by host defense sensors is largely prevented. However, studies have shown that defective clearance of self-DNA from apoptotic or necrotic bodies can lead to the inappropriate activation of cytokines including type I IFN production. For example, DNase I deficiency or mutations are associated with lupus-like syndrome in mice and humans, as macrophages cannot digest the engulfed host DNA properly (4, 38, 68). This leads to the activation of cellular DNA sensors and the overproduction of cytokines which contributes towards systemic lupus-like diseases and chronic polyarthritis (69). The importance of type I IFN in this observation was emphasized by crossing animals with a defect in the DNase I gene, which are susceptible to autoimmune disease and die before birth, with mice deficient in type I IFN signaling (68). Double-defective animals were born normally, underscoring the importance of IFN production in the observed pathogene-

sis (38). Thus, a cellular DNA sensor pathway may play a role in autoimmunity by recognizing self DNA, or speculatively viral DNA arising from chronic infections.

Autoimmune disease is also known to be caused by defects to negative regulators of cytoplasmic DNA signaling pathways. For example, it is known that mutations in TREX1, a 3′-repair exonuclease 1, can cause SLE and AGS (Aicardi-Goutieres syndrome), which is an autosomal recessive encephalopathy in humans (52–55, 69–71). TREX1 deficiency in mice causes lethal autoimmune non-infectious inflammatory myocarditis via the elevated production of cytokines and auto-antibodies (71). TREX1-deficient cells show cell cycle defects and abnormal responses to γ irradiation, exhibited by the accumulation of ssDNA in the ER (52). Excessive cytokine production in TREX1-deficient mice was also found to be due to ssDNA derived from endogenous retroelements (71). Crossing TREX1-deficient mice with mice lacking IRF3 or IFNR rescued TREX1$^{-/-}$ animals from death, suggesting that type I IFN production was responsible for the observed autoimmunity (68). These observations suggest that TREX1 is required for degrading DNA activators that would otherwise trigger innate immune signaling-dependent autoimmunity (71). It is tempting to speculate that TREX1 may play a role in regulating the STING pathway. Both TREX1 and STING reside in the ER. However, while TREX1 is known to exhibit exonuclease activity on ssDNA as well as dsDNA substrates, STING is not robustly activated by ssDNA species (10). Nevertheless, a recent report indicated that HIV1 DNA used TREX1 to control STING activity that would otherwise suppress viral replication. HIV1 utilized TREX1 to rapidly digest excess viral DNA and avoid STING-dependent innate immune signaling (72). It remains to be seen whether TREX1 is indeed a *bona fide* negative regulator of the STING pathway, but genetic studies crossing animals defective in both TREX1 and STING may shed light into this possibility.

AIM2 and cytosolic DNA-mediated inflammasome-dependent innate signaling: the NLR pathway

The NLRs comprise 22 genes in humans and are segregated into three inflammasome subfamilies (NLRP1, NLRP3, and NLRC4) (73, 74). The NLR family predominantly triggers inflammasome-dependent innate immune signaling, although it is not clear how these proteins recognize PAMPs or DAMPs or environmental stresses. NLRs may provide a scaffold to recruit molecules that facilitate the activation of caspase-1-dependent processing of cytokines such as IL-1β, a key pro-inflammatory mediator that can stimulate recruitment of macrophages and DCs to sites of infection or injury (74). NLR-dependent signaling does not usually involve the production of type I IFN (6–9, 73). NLRP1 (NACHT-LRR-PYD containing protein 1) was the first described member of the NLR family and has been reported as playing a role recognizing bacteria such as *Bacillus anthracis*, although the mechanisms of its activation are unclear (74, 75). In contrast, NLRP3 has been reported to trigger the production of IL-1β in response to a variety of stimuli including viral DNA. In this situation, adenovirus infection has been reported to activate ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain)/caspase-1-mediated secretion of IL-1β (74, 75). NLRC4 (NLR family, CARD domain-containing 4) has been reported as being activated by Gram-negative bacteria and can directly associate with caspase-1 independent of ASC. However, a related pyrin domain (PYD)-containing molecule, AIM2, has been demonstrated as also being a major regulator of DNA-mediated inflammatory responses. Indeed, a number of groups identified AIM2, a HIN-200 family member, as the cytoplasmic dsDNA sensor that activates ASC/caspase-1-mediated secretion of IL-1β (6–9, 73). AIM2 is a member of the IFI20X-IFI16 (PHYIN) family. Cytoplasmic DNA species was found to directly bind to dsDNA through AIM2's HIN-200 domain. This event causes the N-terminal PYDs to recruit ASC and activate caspase-1. Importantly, AIM2 inflammasome is essential for caspase-1 activation, but completely dispensable for type I IFN production in response to cytosolic dsDNA. This observation underscores that cytosolic DNA-mediated AIM2 inflammasome-dependent signaling is distinct from type I IFN-dependent innate signaling, which is largely dependent on STING. Thus, different DNA sensors exist to trigger pro-inflammatory or type I IFN production in response to infection.

RNA polymerase III and other types of cytoplasmic DNA signaling

Studies have indicated that TLR9 is responsible for pDC-dependent innate immune signaling in response to CpG-based DNA. In addition, AIM2 is important for caspase-1-dependent IL-1β-produced inflammasome reactions. STING, in contrast, governs intracellular DNA-mediated type I IFN production and the stimulation of other innate immune genes in a variety of cell types, although the exact mechanism of sensing remains to be clarified. Other investigations have demonstrated that transfected Poly(dA:dT) (synthetic AT-rich dsDNA), but not other types of DNA including poly(dG:dC) (synthetic GC-rich dsDNA), calf thymus DNA, PCR fragments, or plasmid DNA, are able to transcribe dsRNA containing 5′-triphosphate ends that are capable of activating the RIG-I

pathway. This event was found to involve DNA-dependent RNA polymerase III (76, 77). As described, RIG-I recognizes 5′-triphosphate RNA species and is capable of manufacturing type I IFN (4, 18, 22–25). The physiological importance of these processes, however, awaits clarification.

Additional investigations have also indicated that other molecules and pathways may be involved in the sensing of intracellular DNA to trigger innate immune cascades. These include the high mobility group box (HMGB) proteins, as cells lacking HMGB1 exhibited defects in intracellular DNA as well as poly IC-mediated IFN production (78). In contrast, cells lacking HMG2 exhibited defects in IFN production only in response to intracellular DNA. Interestingly, HMGB1-3 deficiency manifested defects in AIM2-dependent IL-β production. It is thus plausible that HMBGs are required for the regulation of RIG-I-like, TLR, AIM2, and STING pathways, although the mechanisms of downstream signaling are unclear. A further protein from the same group also reported that DAI [DNA-dependent activator of IRFs, also referred to as Z DNA binding-binding protein-1 (ZBP-1)] was a sensor for intracellular dsDNA (78). The importance of DAI in innate signaling remains to be clarified, as DAI-deficient mice induced type I IFN normally in response to cytosolic DNA and elicited a normal immune response to DNA-based vaccines. Other molecules reported to play a role in sensing intracellular DNA species include IFI16, which is an IFN-inducible protein and member of the PYHIN protein family that contains a pryin domain and two DNA-binding HIN domains. RNA interference studies have indicated that IFI16 depletion reduces the induction of IFN by synthetic DNA and HSV-1, an event that may be dependent on STING. The study of IFI16-deficient mice or MEFs from mice may provide additional information on the importance of this PYHIN member in regulating cytoplasmic DNA-dependent innate immune signaling pathways. Recently, a LRR-containing protein, LRRFIP1 was also suggested to be involved in DNA sensing and the production of type I IFN in response to *Listeria* infection (79). Finally, two helicases, DHX9 and DHX36, have similarly been inferred to be involved in DNA-dependent TNFα and IFNα production (80). Thus, unraveling these DNA-dependent innate immune pathways is gaining momentum and could provide explanations for the cause of a variety of autoimmune disorders.

Concluding remark

Evidence indicates that at least TLR9, AIM2, and STING regulate pathways important for the initiating host defense mechanisms in response to intracellular DNA species. Vital issues that remain to be determined include clarifying the key sensor(s) of the STING-dependent pathway that not only may be important for host defense purposes but also be involved in the cause of autoimmunity. It will thus be of significant interest to establish the role of STING and AIM2 in autoimmune disease. Alternatively, screening for compounds which stimulate these pathways may facilitate vaccination or anti-pathogen prophylactic strategies. The regulation of many of these innate immune signaling pathways by pathogens is also just starting to be unraveled and may shed light into causes of pathogenesis. Collectively, understanding how the cell recognizes cytoplasmic DNA has profound implications for the diagnosis, prevention, and treatment of a variety of diseases and may lead to the development of new vaccine strategies.

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