Review

STING-dependent cytosolic DNA sensing pathways

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STING (STimulator of INterferon Genes) has recently been identified as being essential for controlling host defense countermeasures triggered by microbial cytosolic DNA and subsequently cyclic dinucleotides (CDNs). However, chronic STING activation may also be responsible for initiating certain inflammatory diseases manifested by self DNA. Recent studies have also revealed a key role for cyclic GMP–AMP synthase (cGAS) in STING activation. Although a full understanding of the mechanisms of STING activation requires further studies, new insights into STING function afford the opportunity of designing novel compounds aimed at facilitating vaccine development or new therapies for the treatment of inflammatory disease.

DNA-sensing innate immune pathways

Much progress has been made in understanding how RNA viruses trigger host defense countermeasures [1–3]. Conversely, less is known relating to the sensing of DNA-based pathogens, which can include not only DNA viruses, but also bacteria and even parasites. Toll-like receptor (TLR)9 was discovered just over a decade ago and was found to recognize CpG (cytidine–phosphate–guanosine) DNA, which is typically generated by microbes such as bacteria and viruses [1,4]. This recognition event leads to the production of type I interferon (IFN) and other cytokines. However, TLR9 is mainly expressed in plasmacytoid dendritic cells (pDCs) and B cells, and DNA-triggered innate immune signaling pathways controlling IFN production remain largely intact in cells lacking TLR9 such as fibroblasts, indicating the existence of alternate DNA sensing mechanisms [5]. Subsequently, a pathway that was found to control the production of proinflammatory cytokines such as interleukin (IL)-1β, following infection with select DNA pathogens, was shown to be dependent on a protein referred to as absent in melanoma (AIM)2, a member of the Pyhin (Ifi202/IFI16) family [6–10]. Despite this progress and the known requirement of the transcription factor IFN regulatory factor (IRF)3, the key pathways considered to control TLR-independent DNA-mediated innate signaling remained elusive until the discovery of a cellular protein referred to as STING [11].

In this review, we highlight recent studies that have provided new insights into STING activation by CDNs, and discuss the potential implications of these findings to our understanding of STING function; both in productive immune responses and in autoinflammatory pathologies. We place these findings in the context of key earlier work, including studies that identified STING and provided initial insights into its relevance in controlling signaling response to cytoplasmic DNA. We conclude with a discussion of important questions for future research on STING.

STING controls TLR-independent cytosolic DNA signaling

STING was discovered by high-throughput screening of a cDNA expression library that enabled the isolation of molecules that could activate the IFNβ promoter when transfected into 293T cells [11,12]. STING, a 398 and 378 amino acid protein in human and mouse cells, respectively, is anchored in the endoplasmic reticulum (ER) of unstimulated cells through several transmembrane regions residing in its N-terminal region. STING appears to exist as a dimer, with its C-terminal tail residing in the cytosolic region of the cell. Studies indicate that STING, also referred to as transmembrane protein 173 (TMEM173), mediator of interferon regulatory factor 3 activation (MITA), MPYS or endoplasmic reticulum interferon stimulator (ERIS), is predominantly expressed in macrophages, T cells, a variety of DCs, endothelial cells, and select fibroblasts and epithelial cells [11–15]. Mice lacking STING are viable though prone to lethal infection following exposure to a variety of microbes.

Aside from CpG DNA-mediated activation of TLR9, little was known relating to how DNA species stimulated the production of type I IFN or other cytokines. A key breakthrough came when it was observed that cells lacking STING were unresponsive to cytokine production following exposure to a wide array of different types of DNA (viral DNA, plasmid DNA, genomic DNA, apoptotic DNA and necrotic DNA) or DNA pathogens [herpes simples virus (HSV)1 or the Gram-positive bacterium *Listeria monocytogenes*] [11,12]. Moreover, mice lacking STING were unable to mount a robust type I IFN response following infection with HSV1 and rapidly succumbed to lethal disease. It was further observed that STING-deficient mice could not generate vigorous adaptive immune responses in response to plasmid DNA-based vaccines [12]. Taken

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together, it was evident STING controlled a novel, TLR-independent, cytosolic DNA-mediated innate immune signaling pathway.

Activation of STING by CDNs

Following the transfection of double stranded (ds)DNA species greater than 45–90 bp into normal cells containing STING (or infection with DNA pathogens such as HSV1), numerous cytokines are transcribed in addition to type I IFN, including members of the IFN-induced protein with tetratricopeptide repeats (IFIT) family and chemokines such as chemokine CXC ligand (CXCL)9 and 10 [16]. These products presumably exert direct anti-pathogen effects as well as stimulate adaptive immune responses. However, it is unclear whether STING is directly involved in the sensing of DNA species, or whether upstream molecules facilitate this purpose. Recent evidence indicates that STING associates with dsDNA directly [16]. In addition, STING has also been shown to associate directly with second messengers referred to as CDNs, which are secreted by intracellular bacteria such as *L. monocytogenes*. These activators can take the form of canonical cyclic di-GMP (c[G(3′-5′)pG(3′-5′)p] or cyclic di-AMP or cyclic GAMP (cGMP–AMP). Woodward *et al*. showed that bacteria that enter nonphagocytic cells can leak CDNs, triggering type I IFN production by the host cell [17]. Subsequently, a study using a forward genetic mutagenesis screen to generate mice that failed to produce type I IFN in response to *Listeria* identified STING as the basis for the defect [18]. The mouse, referred to as Goldenticket, has a single point mutation (T596A) in the *Sting* gene, which renders STING insensitive to CDNs [19]. In addition to these observations, studies in mice revealed that several type I IFN-inducing compounds such as 5,6-dimethylxanthenone-4-acetic acid (DMXAA) or 10-carboxymethyl-9-acridanone (CMA) were also found to function by binding to and triggering STING activity in a manner similar to CDNs [20,21]. However, these compounds were shown to be unable to bind human STING and failed to activate innate immune signaling, explaining why they were not effective drugs in humans. Thus, depending on the species, STING has varying ability to become activated by drugs such as DMXAA and CMA [22].

During this period, an IFN-inducible protein molecule referred to as cGAS, also referred to as male abnormal 21 domain containing 1 (Mab-21 domain containing1/MB21D) or C6orf150 was also shown to be involved in generating cyclic GMP–AMP or cGAMP) [22,23]. cGAS is 522 amino acids long in humans and encodes a protein of ~60 kDa. cGAS was reported to interact with DNA species directly, and to utilize GTP and ATP to generate cGAMP capable of STING activation [23]. It became evident that the dinucleotide ligands generated by cGAS *in vitro* and likely *in vivo* are predominantly non canonical 2′-5′- linked structures (c[G(2′-5′)pA(3′-5′)p] [16,24–29]. cGAS harbors a highly conserved DNA-stimulated nucleotidyl transferase (NTase) domain and is structurally similar to the antiviral IFN-inducible oligoadenylate synthase (OAS)1, which synthesizes 2′-5′-linked oligoadenylates (2-5As) from ATP following association with dsRNA [28,30]. These 2-5As then bind to inactive monomeric molecules of ribonuclease (RNase) L, causing dimerization and activation. The stimulation of RNase L leads to degradation of cellular as well as viral RNA, resulting in the inhibition of protein synthesis and the suppression of viral replication. Thus, cGAS and OAS1 NTases sense dsDNA and dsRNA species, respectively, via similar mechanisms, illustrating a conserved role for these enzymes in anti-pathogen activity. Possibly, STING evolved in mammals to detect cyclic di-GMP from bacteria. A similar sensing mechanism evolved to detect cytosolic DNA via cGAS, which generated noncanonical CDNs that were also able to stimulate STING activity. Therefore, STING is able to recognize various dinucleotide moieties; an event that is capable of triggering innate immune signaling processes.

A number of groups have now determined the crystal structure of CDN binding to the C-terminal region of STING [31–37]. These studies confirmed that STING exists as a dimer, in a V-shaped structure, in an inactive state. CDNs bind at the groove between two STING molecules, and this binding event likely results in a conformational change that triggers STING activation. Amino acid variation between human and mouse STING has been reported to explain why compounds such as DMXAA or certain CDN-like compounds associate with one species of STING and not another [24–28]. In addition, it is evident that different polymorphisms of STING exist and respond differently to activators. For example, human STING harboring the amino acid arginine at position 232 (R232) can be stimulated by bacterial CDNs whereas alternate STING alleles (H232) are poorly responsive. Recently, the crystal structure of cGAS in complex with ATP, GTP, and DNA has been reported and shown to contain a unique zinc thumb that is able to distinguish B-form DNA [28]. Suprisingly, cGAS was also found to bind RNA; however, this binding is unable to produce STING-activating dinucleotide ligands.

STING signaling and autophagy

It is plausible that after cellular invasion, DNA from the microbe leaks out of endosomal compartments into the cytosol and encounters molecules such as cGAS residing in the cytosol and/or STING residing in the ER. Alternatively, DNA viruses such as the polyomavirus SV40 have been reported to traffic to the ER compartment where viral disassembly occurs [38]. Although some virus entry involves caveolin-mediated endocytosis, other viruses utilize macropinocytosis or clathrin-dependent entry [38]. Evidence indicates that STING is likely associated with the ER translocon system, where is attaches to translocon-associated protein (TRAP)β. The TRAP complex is made up of four subunits (α–Δ) and is tightly linked with the Sec61 membrane pore complex; itself made up of three subunits (SEC61α, SEC61β, and SEC61γ) [39]. Proteins intended for secretion or for membrane incorporation are moved into the translocon by the signal recognition particle (SRP) associated with translating ribosomes [40]. Although the regulation of STING within the ER remains to be unraveled, it is apparent that intracellular DNA causes STING to traffic rapidly from the ER region through to the Golgi, to reside in distinct perinuclear endosomes by mechanisms that almost certainly involve autophagy [12]. For example, autophagy-related gene (Atg)9a has been

reported to be required for STING motility [41]. The process of utilizing autophagy is unique in innate signaling processes and is not thought to govern the control of TLR signaling. Evidence indicates that STING escorts tank binding kinase 1 (TBK1) to endosomal compartments to associate with and activate IRF3 and IRF7, which translocate into the nucleus to stimulate innate immune gene transcription [12]. STING is also known to trigger nuclear factor (NF)-κB signaling, although the mechanisms underlying this process remain to determined [11]. STING is subsequently post-translationally modified by ubiquitination and is rapidly degraded after endosomal delivery of TBK1, almost certainly to prevent the overstimulation of innate immune gene transcription, as discussed below [42–44].

Recently, some insight into the mechanisms of how STING is negatively controlled has come to light [45]. Following activation, STING reportedly traffics via autophagy that is dependent on VPS34 (class III phosphatidylinositol 3-kinase) and as mentioned, ATG9a [41]. Subsequently, STING is phosphorylated at several serine residues; one of which is S366 in humans and S365 in murine cells [46]. This event inhibits the ability of STING to facilitate IRF3 translocation. As a result, IRF3-dependent gene transcription, including type I IFN production is prevented. STING is then degraded. The kinase responsible for phosphorylating STING on S366 was found to be serine/threonine UNC-51-like kinase (ULK1/ATG1), first identified as being important in promoting autophagy in yeast under starvation conditions [47]. However, ULK1 was not found to be required for STING-dependent autophagy. Further analysis indicated that ULK1 activity was controlled by AMP-activated protein kinase (AMPK), rather than the other key regulator of ULK1, mammalian target of rapamycin (mTOR). Surprisingly, the control of AMPK turned out to be dependent on CDNs generated by cGAS. These findings suggest that CDNs, while initially facilitating STING function, also trigger a negative feedback loop involving AMPK disassociation from ULK1; the latter of which is then able to phosphorylate STING to inhibit IRF3-dependent gene transcription.

In summary, the collective evidence from studies discussed above delineate the following model of STING activation and downstream signaling (Figure 1). Intracellular dsDNA induces autophagy and the trafficking of STING/TBK1 through the Golgi to endosomal compartments that harbor members of the IRF and NF-κB family. These transcription factors become activated and numerous immune-related genes are induced such as type I IFN and a variety of cytokines and chemokines [16]. STING is then phosphorylated and degraded and the signaling process halted. These events ensure the transient production of host defense genes required for direct antimicrobial effects as well as stimulating the adaptive immune response. Eventual suppression of STING function also ensures that the chronic production of cytokines is prevented, thus avoiding the consequences of inflammatory disease.

Relevance of STING in antimicrobial host defense

The importance of STING in host defense is illustrated by STING-deficient mice, which are viable but sensitive to infection by several microbes [11,12]. For example, HSV1, Gram-positive *L. monocytogenes*, or a variety of dsDNA species including viral or bacterial genomes or plasmids, fail to trigger the production of type I IFN in STING-deficient murine embryonic fibroblasts (MEFs), macrophages, or conventional DCs [11,12]. cGAS and STING have also been shown to be important for host defense against retroviruses [48,49]. The DNA pathogens *Chlamydia muridarum* and *Chlamydia trachomatis* have similarly been shown to activate the STING pathway [50,51]. *Mycobacterium tuberculosis* activates STING-dependent autophagy, which leads to the lysosomal destruction of these microbes [52,53].

STING may not only be important for dsDNA-dependent or CDN-dependent innate immune signaling, but may also be critical for facilitating innate immune responses by negative-stranded and positive-stranded RNA viruses such as vesicular stomatitis virus (VSV), Sendai Virus (SV) or dengue virus [11,12,54,55]. MEFs or mice lacking STING are extremely sensitive to VSV and SV infection. However, synthetic dsRNA (polyIC) is not affected in its ability to produce type I IFN, in the absence of STING. Neither do RNA pathogens robustly trigger STING-dependent autophagy or innate immune gene activation, in contrast to dsDNA. Thus, the anti-pathogen mechanisms exerted by STING to thwart RNA-virus replication may be different to those involved in impeding DNA virus replication [12]. It is conceivable that STING may play an important role in retinoic acid-induced gene I (RIG-I)-mediated signaling, which senses RNA viruses such as VSV and SV, but not melanoma differentiation-associated protein 5 (MDA5), responsible for facilitating poly IC signaling. Collectively, the mechanism by which STING regulates the replication of RNA viruses remains to be determined. However, STING may exhibit multiple functions in the cell, speculatively including the control of translocon-associated tasks, which may affect viral protein post-translational modifications.

STING and inflammatory disease

Type I IFN is an essential factor for host defense against invading pathogens, but inappropriate production of type I IFN leads to autoimmune diseases such as systemic lupus erythematosus (SLE) [56,57]. In eukaryotes, localization of self-DNA is restricted to the cell nucleus and mitochondria, thereby sequestering self-DNA- from cytoplasmic-DNA-sensing mechanisms that may activate proinflammatory cytokine pathways. Cellular DNases eliminate aberrant self-DNA found in apoptotic bodies, extracellular space, cytosol, and endosomes [57]. Several studies have shown that defective clearance of self-DNA leads to inappropriate activation of type I IFN production through a TLR-independent innate immune signaling, which is tightly linked to autoimmune diseases [58,59]. For example, DNase I deficiency or mutations are associated with lupus-like syndrome in mice and humans [60]. In addition, DNase-II-deficient mice suffer from the accumulation of incompletely digested DNA, which causes TLR-independent type I IFN production and embryonic lethality [59]. Crossing susceptible mice with mice deficient in type I IFN signaling enabled DNase-II-deficient animals to be born, indicating

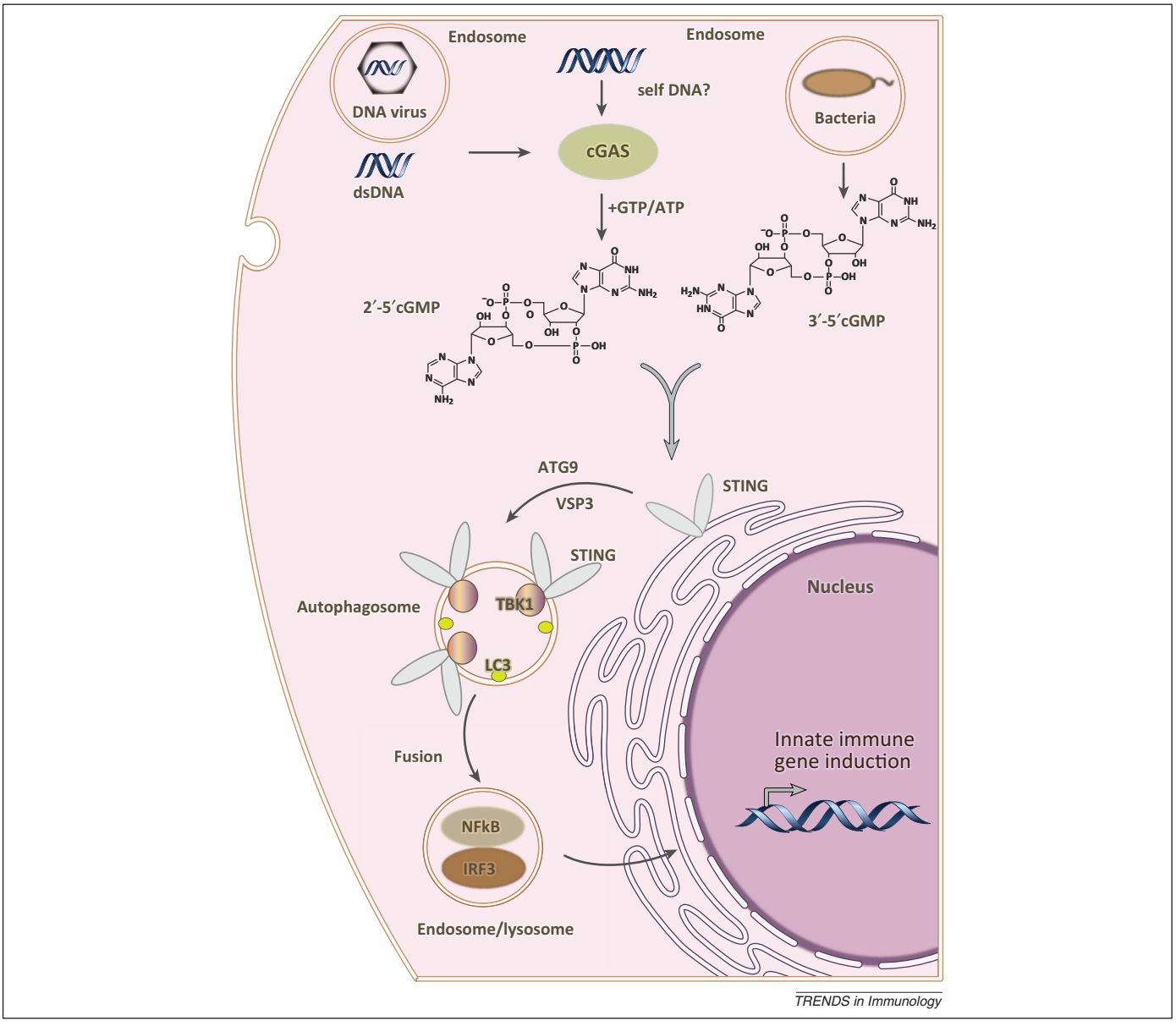

Figure 1. STING (STimulator of INterferon Genes)-dependent innate immune signaling. Bacteria can produce canonical 3′-5′ cyclic dinucleotides (CDNs) that directly bind to STING, to activate innate immune signaling. In addition, DNA from DNA pathogens such as herpes simplex virus can associate with cyclic GMP–AMP synthase (cGAS), which utilizes GTP/ATP to generate noncanonical 2′-3′ CDNs that subsequently bind to STING to activate innate immune signaling. STING may also bind DNA directly to facilitate this process. These events induce autophagy where STING traffics with tank binding kinase (TBK1) to endosomes/lysosomes carrying interferon regulatory factor (IRF)3 and nuclear factor (NF)-κB and innate immune gene transcription ensues. The STING-dependent signaling pathway needs to be controlled, however, to avoid lethal inflammatory disease which can be manifested by self-DNA.

the importance of excessive IFN production in the observed pathogenesis. However, such mice developed chronic polyarthritis due to high levels of proinflammatory cytokines such as tumor necrosis factor (TNF)α being produced as a result to innate immune response to the undigested DNA [59]. Significantly, crossing STING-deficient mice with DNase II heterozygote mice generated comparatively healthy STING–DNase-II-deficient mice that were subsequently found to be completely devoid of any forms of polyarthritis [56]. Thus, the STING pathway appears responsible for lethal DNAse-II-mediated embryonic lethal disease and the development of inflammatory disease.

Several studies have also reported that Trex1, 3′-5′ repair exonuclease 1 (DNase III) is also linked to autoimmune diseases. For example, mutations in the human Trex1 gene are related to SLE and Aicardi–Goutieres syndrome (AGS) [61,62]. Trex1-deficient mice develop lethal inflammatory disease after ~8 weeks via elevated production of type I IFNs and autoantibodies. It was known that genetic ablation of IRF3 or the type I IFN receptor (IFNR) rescued Trex1-deficient mice from early death, suggesting IRF3-dependent IFN production was linked to autoimmune symptoms in Trex1-deficient animals [63]. These data suggest that Trex1 may be responsible for degrading some form of self-DNA, either intrinsically or from phagocytosed dying cells [64,65]. Accumulated DNA, usually degraded by Trex1 could plausibly trigger cellular DNA sensors and innate immune gene activation. Given this, it was feasible to consider whether STING played a role in the chronic production of type I

IFN, and furthermore, whether Trex1 may negatively regulate STING activity. For example, it was reported that reverse transcribed HIV1 DNA could activate the STING pathway, to suppress viral replication [48]. HIV was reported to use TREX1 to digest rapidly excess viral DNA in an effort to avoid STING activation. Consequently, the crossing of STING-deficient mice with TREX1-deficient mice did indeed eliminate TREX1-mediated lethal inflammatory disease [66].

Thus, remarkably, the STING pathway appears to regulate inflammatory disorders manifested through at least two pathways, namely those involving DNase II and Trex1 (DNase III). It is tempting to speculate on the role of cGAS in facilitating these diseases, and perhaps the generation of cGAS-deficient mice may facilitate such studies.

In addition to cGAS, a variety of alternate molecules have also been reported to influence STING activity, including the DNA damage sensor meiotic recombination 11 homolog A (MRE11). In this situation, MRE11 and associating protein DNA repair protein 50 (RAD50) may facilitate STING-dependent innate immune gene induction in response to DNA damage [67]. Moreover, DEAD-box helicase family 41 (DDX41) and IFI16, among others, have also been reported as recognizing CDNs and/or DNA to trigger STING activity [68,69]. No doubt, *in vivo* studies using knockout mice will shed light on the importance of these proteins in cytosolic DNA innate immune signaling.

Concluding remarks

The studies discussed in this review illustrate the rapid progress being made in our emerging understanding of the multifaceted roles of STING. Recent studies have shown a role for CDNs in STING activation, and have further provided insight into the biochemical pathway responsible for the production of these molecules. Indeed, the generation of CDNs in response to pathogen-related RNA or DNA, by synthetases such as OAS or cGAS, respectively, appears to be a prevalent method of galvanizing antiviral host defense. Advances in our understanding of the cell biology of STING function also afford opportunities to unravel processes governing the autophagy pathway. The requirement for autophagy to facilitate STING-dependent innate immune signaling seems relatively unique compared to other innate immune signaling pathways such as those controlled by RIGI/MDA5. Finally, *in vivo* studies have confirmed the importance of STING in innate immune responses to microbial infection, and have in addition, revealed that STING may play a key role in autoinflammatory disease manifested by aberrant self-DNA.

Future questions that remain to be addressed include clarifying the mechanisms of STING activation in response to dsDNA or CDNs. This includes understanding how STING escorts TBK1 through the autophagy process to associate with IRF3. Little is also known relating to how STING activates the NF-κB pathway. STING is further known to be important in host defense against RNA virus infection through mechanisms that seem to be dissimilar from those used to thwart DNA virus infection. Finally, it is intriguing to consider what other types of inflammatory disease are manifested through the STING pathway. Understanding the control of innate signaling processes may lead to a new generation of therapeutics that may help to avert inflammatory/autoimmune disease. Conversely, innovative compounds that transiently stimulate the STING pathway may be useful as adjuvants in vaccine strategies that target malignant and infectious disease.

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