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The Emerging Roles of STING in Bacterial Infections

Fabio Vitarelli Marinho^{1,3,*}, Sulayman Benmerzoug^{1,2,*}, Sergio Costa Oliveira³, Bernhard Ryffel^{1,2}, and Valerie F. J. Quesniaux^{1,2,**}

¹CNRS, UMR7355, Orleans, France

²Experimental and Molecular Immunology and Neurogenetics, University of Orleans, France

³Department of Biochemistry and Immunology, Institute of Biological Sciences, Federal University of Minas Gerais, Belo Horizonte-Minas Gerais, Brazil

Abstract

The STING (Stimulator of Interferon Genes) protein connects micro-organism cytosolic sensing with effector functions of the host cell by sensing directly cyclic dinucleotides (CDNs), originating from pathogens or from the host upon DNA recognition. Although STING activation favors effective immune responses against viral infections, its role during bacterial diseases is controversial, ranging from protective to detrimental effects for the host. In this review, we summarize the important features of the STING activation pathway and recent highlights about the role of STING in bacterial infections caused by organisms from the *Chlamydia*, *Listeria*, *Francisella*, *Brucella*, *Shigella*, *Salmonella*, *Streptococcus* and *Neisseria* genera, with a special focus on *Mycobacterium* infections.

Keywords

STING; Stimulator of Interferon Genes; DNA and nucleotide sensing; bacteria; infections

Stimulator of Interferon Genes: Linking sensing to effectiveness

Innate immunity came in light in the late 1990s after the discovery of Toll-like receptors (TLRs). Intensive investigation of their activation, signaling and role in diverse pathologies followed [1]. This research field recently expanded to include a new dimension with the novel cytosolic surveillance systems. The identification of **STING** (Stimulator of Interferon Genes, see Glossary) adaptor protein [2] represented an important milestone for nucleotide sensing research. Nucleotide recognition provides a general mechanism to detect microorganisms and is involved in diverse pathological scenarios. STING connects microbial cytosolic sensing with host cell effector functions. Its role as a sensor of cyclic dinucleotides (CDNs) and as a link between DNA sensing and cell activation confers to STING a key role in host immune response. As DNA is present in most microorganisms (excepted RNA viruses) and CDNs are crucial for bacterial metabolism, these oligonucleotides are considered pathogen-associated molecular patterns (PAMPs).

**Correspondence: quesniaux@cnrs-orleans.fr (V. Quesniaux).

*The authors contributed equally to this work

Depending on the type of microorganisms, PAMPs from extracellular pathogens are mainly recognized by TLRs, while those from intracellular pathogens are predominantly sensed by NLRs (NOD-like receptors) and RLRs (RIG-I-like Receptors) [1]. STING is able to recognize CDNs or, in association with intracellular sensors, DNA from viruses [2–5], bacteria [6–9] and from host cells (thus recognizing self-DNA [10]) to control host immune responses [2, 11].

STING's function has been extensively studied during viral infections due to its major role on the induction of type I interferons (**IFNs**), as reviewed recently [12]. Furthermore, STING plays a role in the development of autoinflammatory diseases, cancer and other sterile inflammations [13]. However, less is known about the extent of STING and its related sensors implication in the immune responses to protozoa, fungi or helminthes. Concerning bacteria, the outcome of STING activation, leading to IFN β production, may be beneficial or detrimental for the host, making of STING a friend or foe to the bacterial pathogen, depending on the infection. Given the crucial role of CDNs in bacterial metabolism, such as biofilm formation and protein function [14–15], STING activation is not limited to bacteria containing secretion-systems. In this review, we present the recent findings on the role of STING during bacterial infections.

Dissecting STING signaling

STING activation

Microbial components such as lipopolysaccharide, CpG DNA and CDNs are recognized by membrane and intracellular receptors to induce the expression of multiple host defense genes [1, 8, 16]. Several studies concerning *Chlamydia muridarum*, *Streptococcus pyogenes*, *Mycobacterium tuberculosis* and other bacteria reveal STING dependent activation of type I IFN [6–9]. The CDNs are important for bacterial physiology [14–15]. These PAMPs consisting of dinucleotide monophosphate circularized by 5'3' phosphodiester bonds are recognized by STING (Fig. 1). This process leads to TANK-binding kinase 1 (**TBK1**) and Interferon Regulatory Factor 3 (**IRF3**) activation, culminating in type I IFN response [8, 17]. Mammalian 2'3'cGAMP (cGAMP) similarly triggers type I IFN production after DNA sensing [18]. Apart from microbial sensing, STING can also be activated by self-DNA [11]. Mitochondria are an important source of intracellular DNA (mtDNA) [19]. After cellular stress mtDNA is released into the cytosol and induces the expression of type I IFN genes in a STING-dependent manner [20]. Furthermore, mtDNA participates in the adjuvant mechanism of chitosan during vaccine therapy, since chitosan promotes mitochondria damage, leading to type I IFN-dependent dendritic cell (DC) activation and triggering adaptive immunity [21].

CDN binding on STING induces its migration from the endoplasmic reticulum (**ER**) to form perinuclear punctate structures. This intracellular trafficking is mediated by iRhom2/TRAP β [22]. Moreover, iRhom2 protein recruits the deubiquitinase EIF3S5 which acts to maintain the stability of STING during its trafficking [22]. STING migration from ER to perinuclear region is essential to recruit TBK1, leading to phosphorylation and activation of IRF3 [23]. Indeed, TBK1 phosphorylates the carboxy-terminal tail domain of STING that results in IRF3 recruitment and phosphorylation [24]. Following IRF3 activation, the STING-TBK1-

IRF3 complex is dissociated, and STING is readily tagged for degradation by K48-linked polyubiquitination *via* RNF5/TRIM30 α . Phosphorylated IRF3 homodimers translocate to the nucleus to activate innate immune response genes expression, such as type I IFN regulated genes [25–27]. However, it is not fully understood whether STING-TBK1 complex migrates to the perinuclear region or if STING moves alone from the ER to recruit TBK1 directly in the Golgi [28–29]. Interestingly, TBK1 also regulates IRF7 expression [30].

In addition, the STING-TBK1 axis phosphorylates I κ B, yielding NF- κ B release [31], translocation to the nucleus, and NF- κ B-dependent gene expression involved in cellular stress, tumor progression, inflammation and immunity [32]. Moreover, STING-TBK1 engagement can lead to Signal Transducer and Activator of Transcription 6 (STAT6) activation due to phosphorylation of Serine 407 by TBK1 and of Tyrosine 641 by an unidentified kinase [33]. Phosphorylated STAT6 translocates to the nucleus, inducing upregulation of chemokines such as CCL2 and CCL20. Notably, STING-induced activation of STAT6 is distinct from that induced by cytokines like IL-4 or IL-13 [33].

Besides type I IFN production, STING activation can lead to **autophagy**, a cellular degradation process where cytoplasmic constituents are degraded. It involves engulfment of cargo by double-membrane autophagosomes which then fuse with the lysosome. Autophagy core components are from the Autophagy Related Gene (Atg) proteins family, but the process also involves Bcl-2 family members and various transcription factors [34]. Autophagic elimination of bacteria is a key defense mechanism to control infection. Autophagy can target pathogens within phagosomes, in damaged vacuoles or in the cytosol [34]. Some Atg proteins can exert STING regulation, as will be discussed in the next section. However, STING can induce selective autophagy, where a specific cargo is recognized and degraded [35]. Upon STING-TBK1 activation the pathogen can be ubiquitinated by an unsolved mechanism [36–37]. This process will lead to recruitment of the ubiquitin-binding autophagy receptors p62 and NDP52 [36–37]. Both receptors interact with Atg8/LC3 that will recruit other autophagy components to create a phagophore surrounding the bacilli and then kill the pathogen [35].

STING-associated sensors and regulation

To initiate immune responses elicited by DNA, STING relies on cytosolic sensors. IFI16, a member of the PYHIN family of DNA sensors, regulates STING dimerization and phosphorylation, and subsequent TBK1/IRF3 activation [38]. IFI16 binds DNA and activates the STING-TBK1-IRF3/7 pathway leading to type I IFN response [39–40], while it also recognizes episomal dsDNA in the nucleus, resulting in assembling of a cytosolic inflammasome [41]. IFI16 seems to have a more complex role in human macrophages, where it is necessary for optimal cGAMP production and for TBK1 recruitment to STING [38]. The helicase DDX41 (a member of DEXDc family of helicases), was originally proposed to act as a sensor for c-di-AMP, c-di-GMP or double-stranded DNA (dsDNA) and associates with STING [16, 42]. However, how DDX41 helicase functions as a sensor is poorly understood. Indeed, DDX41 Tyr364 and Tyr414 are critical for DNA recognition and STING activation. The Bruton's Tyrosine Kinase (BTK) phosphorylates DDX41 Tyr414 and

interacts with DDX41 DEAD-box domain. BTK SH3/SH2 domains also interact with STING transmembrane region. This way, BTK acts as a bridge between DDX41 and STING, leading to type I IFN gene expression [43]. It remains to be showed the relevance of this model to establish DDX41 as a genuine STING-associated sensor.

Nevertheless, the best characterized STING-related sensor is the cGAMP synthase **cGAS** (also known as Mb21d1) that belongs to the nucleotidyltransferase protein family [44]. cGAS presents structural similarities to OAS (2'-5' oligoadenylate synthase) proteins that recognize dsRNA, while cGAS contains a unique zinc finger that detects B form dsDNA [45]. In the presence of DNA, cGAS catalyzes the formation of 2'3' cGAMP depending on adenosine (ATP) and guanosine triphosphate (GTP), activating STING protein [18, 46–47]. With this capacity for DNA sensing, cGAS plays a crucial role in the response against DNA viruses like Gamma herpes viruses [4]. Indeed, cGAS deficient mice and cells do not control certain viral infections due to their failure in maintaining type I IFN responses [3, 5]. Interestingly, cGAS can contribute to IFI16 stabilization in fibroblasts and keratinocytes for better DNA sensing that induces stronger innate immune responses [48]. Moreover, IFI16 contributes to enhanced cGAMP production by cGAS in human cells macrophages [38]. It is important to mention that cGAS can also be implicated in sensing RNA viruses [12]. In an intriguing study, Vermeire *et al.* reported that activated CD4⁺ T cells sense HIV-1 infection through cGAS, originally described as a DNA sensor, and promote a type I IFN response, although it remains unknown how [49]. Mankan *et al.* showed that cGAS can bind RNA:DNA hybrid to form cGAMP, with subsequent activation of STING [50]. However, the immunological importance of this hybrid recognition by cGAS needs to be investigated. Strikingly, cGAMP can be transported inside viral particles, activating innate immunity and antiviral defenses in the target cell. This may represent a defense mechanism potentially accelerating and disseminating antiviral responses to neighboring uninfected cells [51–52].

As self-DNA can trigger STING-mediated response, this pathway requires a tight regulation. To avoid excessive immune responses, the organism uses negative feedback to control STING activity. LSm14A, a member of the LSm protein family, regulates STING expression levels in a cell-specific manner, the absence of LSm14A causing a downregulation of STING protein in DCs due to impaired nuclear mRNA precursor processing [53]. The serine/threonine UNC-51-like kinase (also known as ULK1/ATG1) phosphorylates STING serine 366 to suppress IRF3 activation [26]. Atg9a, a multispinning membrane protein, restricts STING translocation from the Golgi apparatus and regulates the assembly of STING and TBK1. In absence of Atg9a there is aberrant enhanced formation of STING-TBK1 complexes, leading to CXCL10 and IFN β overexpression [23]. Further, NLRC3 (NOD-like receptor C3) associates with STING and prevents its trafficking from the ER to Golgi, resulting in reduced type I IFN response [54], while the ZDHHC1 protein, a member of the aspartate-histidine-histidine-cysteine palmitoyl acyltransferase family, associates with STING in the ER and Golgi apparatus after viral DNA stimulation and positively modulates STING activity, leading to increased TBK1-IRF3 engagement and type I IFN production [55].

Cytoplasmic DNA can also activate inflammasome pathway dependent on Absent in Melanoma 2 (**AIM2**) (reviewed in [56]). Detection of intracellular dsDNA by AIM2 results

in secretion of mature IL-1 β , IL-18 and the lytic regulated cell death pyroptosis [57–58]. This type of recognition is important during bacterial infections. However, AIM2 is completely dispensable for type I IFN production, which highlights the contrast between AIM2- and STING-dependent signaling [59]. Nevertheless, both pathways interact, in a way that is not completely clear. Recent evidence points towards an antagonism between STING and AIM2 signaling, as cells deficient for AIM2 presents enhanced cGAMP generation and STING aggregation [60–61]. However, during *Francisella tularensis* infection type I IFN production induced upon STING activation is necessary for subsequent AIM2 inflammasome activation [62–63]. One critical result of AIM2 activation is pyroptosis, that relies on the enzymatic activity of inflammatory caspases and the protein gasdermin D. This kind of cell death does not only eliminate the intracellular replication niches for pathogens but also represent a mechanism for release of bioactive IL-1 β and IL-18, been important in antibacterial immune responses (reviewed in [64]). Some pathogens may take advantage of the interplay between STING and AIM2 signaling and outcomes to promote infection.

Beyond sensing viruses: the role for STING during bacterial infection

CDNs have a great importance in bacterial metabolism. They are produced by specific dinucleotide cyclase enzymes and are degraded by phosphodiesterases [14–15]. c-di-GMP is the best characterized CDN and it is involved in bacterial virulence regulation, biofilm formation and other metabolic roles [14]. Another cyclic dinucleotide, c-di-AMP, was first discovered in *Bacillus subtilis*, and is important for regulation of sporulation [65]. This CDN is also related to cell wall homeostasis and virulence among bacteria [7, 66]. Similarly, bacterial 3'3' cGAMP is also associated with virulence, exemplified by its requirement for efficient intestinal colonization by *Vibrio cholerae* [67]. The discovery of 2'3' cGAMP production by the cGAS sensor revealed that CDNs are also important in mammalian cells. cGAS can detect DNA in the cytoplasm and catalyze the assembly of cGAMP, that in turn activates STING with high affinity [46–47]. Although CDNs contribute to the virulence of bacteria, mammalian cells use them to fight back through STING, either by direct sensing of bacterial derived CDNs or by indirect cytosolic DNA sensing. The role of STING pathway in extracellular, obligatory intracellular or facultative intracellular bacteria will be discussed in the following sections (Table 1).

Extracellular bacteria

One of the most ubiquitous and versatile human pathogen is Group A *Streptococcus pyogenes*, causing benign illness to life threatening infections [68]. Although *S. pyogenes* is generally considered as an extracellular pathogen, it can survive inside the host cell leading to type I IFN production [69–70]. Bone-marrow derived macrophages and DCs require different recognition and signaling pathways for *S. pyogenes*-induced IFN β production. DCs are fully dependent on the MyD88 adaptor and IRF5, while macrophages relies on the STING pathway and required only partially the MyD88 signaling. Both cells recognize *S. pyogenes* independently of TLR3 and TLR9. In macrophages as well as in DCs, nucleic acid sensing is probably the mechanism by which IFN β is induced [9], although the relevance of bacterial CDNs for STING activation during *S. pyogenes* infection remains to be demonstrated [71].

Group B *Streptococcus agalactiae*, which is an important cause of neonatal invasive infections, also induces a marked type I IFN response [72–73]. The immune response is mostly dependent on the TLR pathway upon macrophage exposure to heat-killed Group B *Streptococcus* whereas live bacteria elicit a cGAS-STING dependent pathway [73–74]. Interestingly, Andrade *et al.* showed that *S. agalactiae* can degrade c-di-AMP present outside the bacteria via a cell-wall anchored ectonucleotidase. This enzyme hydrolyses bacterial CDNs into the corresponding 5′ nucleoside monophosphate, which in turn is converted by another enzyme into nucleoside. Strikingly, this ectonucleotidase has no action on mammalian cGAMP. This mechanism of self-degrading extracellular CDN avoids over activation of STING and promotes bacterial virulence [74]. Identification of related strategies by other pathogens would be of great interest.

Streptococcus pneumoniae is another extracellular pathogen that induces a type I IFN response [72] likely through DNA as the principal PAMP, after reach the host cytoplasm through the pore-forming toxin pneumolysin. Two independent studies identified STING as essential for IFN β production and protection from *S. pneumoniae* infection [75–76]. However, its main upstream cytosolic sensor was not identified. Moreover, *S. pneumoniae* induced ER stress culminating in reduced STING activation and IFN β production, a phenotype exacerbated in the aged host [77]. The inhibition of STING activation was mediated by Atg9a [23]. The studies on *S. pneumoniae* infection thus shed some light on STING physiology in the elderly [77]. Indeed, enhanced ER stress in the aged host led to increased Atg9a expression, resulting in inhibition of STING activation and reduced IFN β production *in vivo* in aged mice, but also in response to cyclic dinucleotides in aged macrophages.

Unlike other extracellular pathogens, *Neisseria gonorrhoeae* can invade and survive inside the host cell cytoplasm [78] and induce marked IFN β production. However, the full type I IFN induction is achieved by the combined activation of the TLR4 and STING pathways (in response to gonococcal lipooligosaccharide (LOS) and DNA, respectively), which renders IFN β production independent of a functional type IV secretion system (T4SS). STING activation is mediated mainly by cGAS and independent of IFI16. Interestingly, type I IFN induction, which impairs *N. gonorrhoeae* killing, contributed to an increase of the host intracellular iron pool, a microenvironment beneficial for bacterium survival [79].

Obligatory intracellular pathogens

The first report showing STING activation during intracellular bacterial infection concerned *Chlamydia muridarum* [80], the murine counterpart of the human pathogen *Chlamydia trachomatis*. Chlamydiae are obligatory intracellular bacteria, with tropism for the genital tract. Prantner *et al.* showed that IFN β production induced by *C. muridarum in vitro* is dependent on STING and led to IRF3 and NF- κ B activation. Moreover, they showed that STING is recruited from the ER to be in close proximity to the chlamydial inclusion membrane [80]. *Chlamydiae* produce c-di-AMP in their elementary body form [7]. The specific expression of c-di-AMP in this life cycle could be related to the transition from the metabolically active reticulate body form to the infectious elementary bodies. *Chlamydia* DNA is also important for STING activation and IFN β production in a cGAS and T3SS

dependent manner [81]. T3SS is expressed by chlamydial reticulate bodies to maintain direct contact with the inclusion membrane, a feature necessary to inject virulence effector molecules into the host cell cytosol [82]. It is conceivable that both DNA and CDN-mediated STING activation come into play during the *Chlamydia* infectious cycle. While elementary bodies are a known source of c-di-AMP, the intense metabolism of reticulate bodies may initiate and accelerate DNA recognition, increasing IFN β expression levels.

Facultative intracellular pathogens

Listeria monocytogenes is another bacterium able to stimulate the STING pathway. *Listeria* escapes into the cytoplasm where replication occurs under more favorable conditions. *L. monocytogenes* infection is marked by type I IFN production [8, 83–84]. The expression of multidrug resistance (MDR) efflux pumps by this pathogen leads to c-di-AMP secretion into the host cell cytosol and IFN β production [8]. The overexpression of *L. monocytogenes* diadenylate cyclase or MDR efflux pump further confirmed c-di-AMP release and its role in activating cytosolic surveillance system [8, 83]. These initial results were obtained using murine cells. However, *L. monocytogenes* genomic DNA was found to be the major PAMP inducing IFN β response in human myeloid cells, in an IFI16-, cGAS- and STING-dependent manner [84]. DNA induced IFN β expression correlated with bacterial escape to the cytosol and bacteriolysis in the cytoplasm but not with expression of MDR efflux pumps [84]. Thus, it seems that cytosolic *Listeria* DNA plays a dominant role in type I IFN induction in human cells whereas both DNA and CDNs are important in activating STING in mouse macrophages. Therefore, STING is required for the type I IFN response to *L. monocytogenes* both *in vitro* [2, 16, 84] and *in vivo* [85].

Francisella tularensis is a facultative intracellular pathogen that can escape from the phagosome and replicate in the host cell cytosol. During infection by *F. tularensis*, the coordinated DNA sensing by STING- and AIM2-dependent pathways is required for activation of the immune response. Cytosolic replication is critical for *F. tularensis* virulence but during this process some bacteria are lysed, releasing bacterial PAMPs into the cytosol [62]. Among these PAMPs, DNA has a prominent role. It is correlated with the initial production of type I IFN that is required for subsequent AIM2 inflammasome activation, culminating in pyroptosis and IL-1 β release [63]. The proposed model for this cooperative interplay between STING and AIM2 pathways claims that STING activation and type I IFN production leads to guanylate-binding proteins (GBPs) expression. The GBPs will increase bacteriolysis and permit additional DNA release, allowing activation of the AIM2 inflammasome [62–63]. However, as both STING and AIM2 can be activated by DNA, it is not clear if this initial type I IFN production is achieved by direct activation of STING by bacterial CDNs or if STING pathway demands lower threshold to be activated.

STING activation occurs during the infection of another facultative intracellular bacterium, *Brucella abortus*, triggering IFN β production [86]. *B. abortus* DNA is the major bacterial PAMP inducing type I IFN response, with STING playing an important role in this process [86]. Surprisingly, elevated levels of c-di-GMP due to loss of a specific bacterial phosphodiesterase did not lead to an increased STING response [87]. However, lack of STING renders macrophages inefficient to kill *Brucella*, resulting in increased bacterial

burden [87]. Thus, *Brucella* DNA remains a major bacterial component inducing IFN β and there is no data about the importance of other bacterial CDNs in its physiology. The implication of DNA sensors upstream STING or other CDNs during *Brucella* infection is still unclear.

The investigation of STING function during *Shigella flexneri* infection shed some light on STING activation and trafficking. *S. flexneri* is a pathogen able to invade non-phagocytic cells, escape the vacuole and replicate in the host cytoplasm. *S. flexneri* T3SS effector proteins IpaJ and VirA inhibit the Golgi apparatus structure and function [88–89], indirectly interrupting STING activation. IpaJ inhibits ER-to-Golgi transport, arresting STING in the ER. This trafficking defect is due to proteolytic demyristoylation of ADP-ribosylation factor (ARF) GTPases. On the other hand, VirA does not inhibit type I IFN, but arrests STING at the ER-Golgi Intermediate Compartment (ERGIC). Thus, by using several mutated bacteria, it was shown that STING activation takes place immediately after ER exit and STING transition to the ERGIC. Indeed, in ERGIC occurs most of STING-mediated signal transduction, correlating with TBK1 recruitment and downstream signaling [29]. During a regular infection, *Shigella* limits STING signaling through IpaJ and VirA proteins to promote its growth [90].

Bacterial escape to the cytoplasm does not always lead to STING activation. *Salmonella enterica* serovar Typhimurium is a T3SS-bearing facultative intracellular pathogen that actively invades the intestinal epithelial barrier and triggers inflammation. Once this barrier is crossed, the bacterium is internalized by phagocytes. Autophagy is an important antimicrobial response that restricts the growth of *Salmonella* [91]. The close relationship between autophagy and type I IFN production was explored [92]. *Salmonella* actively suppresses autophagy in macrophages resulting in diminished IFN β production. When autophagy suppression is abrogated, there is activation of host innate immune response and IFN β production, leading to increased bacterial killing and limiting dissemination. The link between autophagy and type I IFN production is not mediated by STING, but is TLR/TRIF-dependent [92]. Nevertheless, the role of type I IFN during *Salmonella* infection needs to be further investigated [93–94].

Thus, the bacteria described here have the potential to induce STING activation, except for *S. enterica* serovar Typhimurium. Interestingly, *S. agalactiae* and *S. flexneri* can avoid STING activation either by degrading their own CDNs present outside the bacteria or by blocking STING pathway, respectively. Of note also is the fact that IFN β production, the main result of STING activation, may lead to different outcomes during bacterial infections, beneficial or not for the host, as illustrated below for *Mycobacteria*.

Focus on *Mycobacteria*

The importance of members of the *Mycobacterium* genus for human and animal health makes them the focus of many studies, including on the contribution of STING pathway to the host responses. *Mycobacterium tuberculosis*, the causative agent of tuberculosis, remains one of the leading causes of chronic infectious diseases [95]. *Mycobacteria* possesses a type VII secretion system (T7SS) and the *M. tuberculosis* ESX-1 secretion system represents a

major virulence factor [96]. ESX-1 inhibits phagosome maturation leading to the persistence of *M. tuberculosis* inside the cell upon macrophage infection. This might allow mycobacterial DNA release into the host cytoplasm together with other bacterial effectors, activating the cytosolic surveillance pathway (Fig. 2) [6, 97]. Although there is no consensus on how *M. tuberculosis* DNA is released into the cytoplasm, it is known that cGAS and IFI16 contribute to DNA recognition. After *M. tuberculosis* DNA sensing, the STING-TBK1-IRF3 pathway is activated, culminating in IFN β production [6, 37, 97–99]. Interestingly, when mycobacterial DNA engages cGAS, cGAMP is produced and then activates bystander cells via gap-junction mediated communication [99]. Thus, *M. tuberculosis* can induce type I IFN production directly from the phagosome [6].

Overexpression or genetic deletion of *M. tuberculosis* di-guanylate cyclase leads to equivalent level of IFN β production. Thus, c-di-GMP is not essential for inducing type I IFNs in response to *Mycobacteria* [6]. However, investigations of immune activation by c-di-AMP gave conflicting results. While Manzanillo *et al.* [6] found no significant alteration in bacterial phenotype by overexpression or deletion of *M. tuberculosis* di-adenylate cyclase, Dey *et al.* [98] report attenuated virulence due to the increased presence of c-di-AMP. High levels of IFN β and other proinflammatory cytokines such as IL-1 α , IL-6 and TNF- α were observed in macrophages and DCs after infection with c-di-AMP over-expressing *M. tuberculosis* strain [98]. Growth in culture broth was unaltered but high levels of c-di-AMP resulted in diminished *M. tuberculosis* growth inside macrophages. Additionally, cells infected with *M. bovis* BCG, which lacks the ESX-1 secretion system, showed small but not negligible activation of the IRF pathway. Indeed, *M. bovis* BCG overexpressing c-di-AMP induced higher IRF and IFN β responses, suggesting that CDNs enter the host cell cytosol even in the absence of ESX-1 secretion system. Thus, while contributing to the overall type I IFN response, ESX-1 is not essential for CDNs activation of IRF pathway. Overexpression of di-adenylate cyclase in *M. tuberculosis* resulted in increased host survival rate and reduced pathology in a mouse model [98]. However, the mycobacterial CDNs are not the major contributor in triggering the STING-dependent response in macrophage after *M. tuberculosis* infection [37, 97].

Autophagy is an important mechanism to control bacterial growth. Most of the data relating STING activation and autophagy came from studies regarding mycobacteria. cGAS-STING activation is crucial to target *M. tuberculosis* to ubiquitin-mediated autophagy. Upon DNA recognition and STING activation, there is recruitment of TBK1, ubiquitin, the autophagy receptors p62 and NDP52, and LC3, providing the signals necessary to trigger selective autophagy [36–37]. One key point of this process is the phagosome permeabilization elicited by the ESX-1 secretion system. Although necessary for the pathogen virulence, this phagosome breach will allow ubiquitin to gain access to the enclosed bacterium. The protein Parkin ubiquitin ligase seems to have a major role in the ubiquitin-tagging of the pathogen, mostly through K63-linkage [100]. NDP52 and p62 are ubiquitin-binding autophagy receptors that will be the bridge between the ubiquitinated cargo and LC3, that will recruit other autophagy components. TBK1 is an regulator of the whole process as it increases the affinity of p62 for ubiquitin-tagged cargos and is responsible for the autophagic maturation into autolysosomes [101]. Indeed, *M. tuberculosis* overexpression of c-di-AMP resulted in upregulation of this pathway [98]. Moreover, STING deficiency resulted in increased

bacterial survival inside macrophages [36, 97]. Lack of TBK1 or ATG5 presented similar phenotype than STING deficient-macrophages. Interestingly, a study with virulent *M. bovis* showed that AIM2 downregulates STING activation and LC3 expression, limiting autophagy. This inhibition required only AIM2 sensor, but not complete inflammasome assembly [61]. Cytosolic surveillance and inflammasome pathways may thus compete for mycobacterial DNA. Lack of ATG5 in macrophages renders mice extremely sensitive to *M. tuberculosis* infection, suggesting that bacterial elimination by autophagy plays a prominent role [36]. Although autophagy could be induced by IFN γ later during the infection [102], early bacterial restriction seems crucial for host survival.

Mycobacterium leprae also activates STING pathway during infection, with extrabacterial DNA as likely trigger, since there is no ortholog of di-adenylate cyclase in *M. leprae* genome. STING pathway engagement during leprosy leads to the expression of 2'-5' oligoadenylate synthetase-like (OASL), an IFN inducible gene. This protein is related to anti-RNA viral response in humans [103]. Surprisingly, OASL was greatly upregulated following *M. leprae* infection and its function was associated with reduced autophagy and antimicrobial response, promoting bacilli viability [104].

In addition to the aforementioned bacterial mechanisms of STING activation, *M. tuberculosis* also induces IFN β production by causing mitochondrial stress. A study comparing strains 1182 from West-Africa, H37Rv from Euro-America, and 4334 from Beijing families showed that cGAS-STING-dependent IFN β production was correlated with the accumulation of mtDNA in the cytosol. These strains induced different levels of mitochondrial stress but were otherwise similar in intracellular survival, TNF- α induction, host cytosol access or bacterial DNA release [105]. Despite STING importance for macrophage immune response against *M. tuberculosis*, absence of STING *in vivo* does not overtly alter the course of infection in mice since STING deficient mice present similar bacterial burden and pathology as wild-type control mice [97].

Thus, the studies concerning *Mycobacterium* addressed in more details the features of STING activation, particularly autophagy and bacterial-induced mitochondrial stress. It is important to highlight that *Mycobacteria* use diverse ways for inducing STING activation (DNA and c-di-AMP), while however distinct pathways compete for the same PAMPs (STING- and AIM2-dependent pathways).

Concluding remarks

Recent progress in understanding the cytosolic surveillance system provided important insights on the function of STING and the associated molecules. Interestingly, the engagement of STING-dependent pathway is not restricted to Gram-positive or negative bacteria, with or without a secretion system.

Despite the great attention recently drawn to STING-related pathways, several issues are still open (see Outstanding Questions). The recent advances in understanding the cytosolic surveillance systems point toward potential immune interventions in the future. The search for new antibiotics can focus on inhibitors of bacterial ectonucleotidases to improve the host

immune response against the bacteria. Moreover, CDNs are a safe and reliable target to explore due to their key differences between bacterial and mammalian sources. Drugs could be designed to inhibit specific features of STING activation during autoinflammatory diseases. Furthermore, new vaccine developments may include the STING pathway, depending on its role during the infection. With all these open questions, one should be reminded that honey is never far away from the sting.

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Figure 1. Schematic model for STING regulation and cytosolic DNA sensing

STING protein is activated by dsDNA or cyclic dinucleotides from pathogens (e.g. c-di-AMP and c-di-GMP). cGAS binds dsDNA to form the cyclic dinucleotide 2'3' cGAMP which in turn activates STING. Self-DNA, such as mitochondrial DNA, can also be recognized by cGAS. In parallel, other cytosolic DNA sensors like IFI16 are also able to bind dsDNA and activate STING. After this step, the full mechanism of how STING will activate IRF3 or NF- κ B is not completely understood. The first hypothesis (A) is that STING moves alone to perinuclear Golgi to bind TBK1, then phosphorylated STING is fully activated. The second postulate (B) is that TBK1 encounters STING directly in the ER, phosphorylating and activating STING at this site, and finally the complex STING-TBK1 moves together to perinuclear Golgi. These two ways lead to IRF3 and NF- κ B activation in a perinuclear punctate structure. These transcription factors migrate to the nucleus for activation of type I IFN related genes. Type I IFN response and STING protein need to be regulated to avoid an excessive response involved in several auto-inflammatory diseases (dashed lines). To do that, some regulators like NLRC3, Atg9a and ULK1/Atg1 negatively control STING while ZDHHC1 works as a positive regulator. Similarly, there is antagonism between STING and AIM2 inflammasome activation.

Figure 2. Schematic summary of STING activation during *M. tuberculosis* infection

After entering the cell, *M. tuberculosis* may elicit STING activation by diverse ways. **(A)** Mycobacterial DNA or c-di-AMP gain access to the cytosol in an ESX-1-dependent manner. *M. tuberculosis* DNA is then sensed by cGAS or IFI16, leading to STING activation. Alternatively, *M. tuberculosis* infection can cause mitochondrial stress, leading to accumulation of mitochondrial DNA in the cytosol and culminating in cGAS-dependent activation of STING pathway. cGAMP produced by cGAS can also pass to bystander cells thanks to gap junctions. Mycobacterial DNA can also activate AIM2 inflammasome, leading to IL-1 β production. **(B)** After assembly of the STING-TBK1 complex, both host detrimental IFN β production, through IRF3 activation, and autophagosome assembly, *via* the recruitment of LC3, may occur.

Table 1

STING activation and upstream DNA sensor by the different bacteria discussed in this review.

Pathogens	STING activation	Upstream DNA sensor ^a	Refs
<i>Brucella abortus</i>	Yes	N.D.	77, 78
<i>Chlamydia muridarum</i>	Yes	N.D.	69
<i>Chlamydia trachomatis</i>	Yes	cGAS	70
<i>Francisella tularensis</i>	Yes	N.D.	75, 76
<i>Listeria monocytogenes</i>	Yes	cGAS and IFI16	2, 8, 16, 72, 73, 74
<i>Mycobacterium bovis</i>	Yes	N.D.	90, 93
<i>Mycobacterium leprae</i>	Yes	N.D.	96
<i>Mycobacterium tuberculosis</i>	Yes	cGAS and IFI16	6, 88, 89, 90, 91, 92, 97
<i>Neisseria gonorrhoeae</i>	Yes	cGAS	67, 68
<i>Salmonella enterica</i> serovar Typhimurium	No	N.D.	83
<i>Shigella flexneri</i>	Yes	cGAS	81
<i>Streptococcus agalactiae</i>	Yes	cGAS	61, 62, 63
<i>Streptococcus pneumoniae</i>	Yes	N.D.	61, 64, 65, 66
<i>Streptococcus pyogenes</i>	Yes in macrophages, not in DCs	N.D.	9, 57, 58, 59, 60

^a Abbreviations: N.D., Not Determined