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Crosstalk between *Mycobacterium tuberculosis* and the host cell

Bappaditya Dey and **William R. Bishai***
Department of Medicine, Johns Hopkins University, School of Medicine, Baltimore, MD, USA.
Howard Hughes Medical Institute, Chevy Chase, MD, USA

Abstract

The successful establishment and maintenance of a bacterial infection depends on the pathogen's ability to subvert the host cell's defense response and successfully survive, proliferate, or persist within the infected cell. To circumvent host defense systems, bacterial pathogens produce a variety of virulence factors that potentiate bacterial adherence and invasion and usurp host cell signaling cascades that regulate intracellular microbial survival and trafficking. *Mycobacterium tuberculosis*, probably one of the most successful pathogens on earth, has coexisted with humanity for centuries, and this intimate and persistent connection between these two organisms suggests that the pathogen has evolved extensive mechanisms to evade the human immune system at multiple levels. While some of these mechanisms are mediated by factors released by *M. tuberculosis*, others rely on host components that are hijacked to prevent the generation of an effective immune response thus benefiting the survival of *M. tuberculosis* within the host cell. Here, we describe several of these mechanisms, with an emphasis on the cyclic nucleotide signaling and subversion of host responses that occur at the intracellular level when tubercle bacilli encounter macrophages, a cell that becomes a safe-house for *M. tuberculosis* although it is specialized to kill most microbes.

Keywords

Mycobacterium tuberculosis; Macrophage; Immunity; Interferon; Cyclic AMP; Cyclic di-AMP

1. Introduction

The unique crosstalk between microbial pathogens and their hosts reflects the co-evolutionary balance that the host and pathogen must reach in order to secure their survival. *Mycobacterium tuberculosis*- an intracellular and primarily vacuolar pathogen-has evolved a plethora of virulence factors which subvert a range of host physiological responses to allow propagation of the bacilli in one of the most inhospitable cells in the body, the macrophage.

*Corresponding author: William R. Bishai, MD, PhD, 1550 Orleans St. Room 108, Baltimore, MD 21231-1001, wbishai@jhmi.edu.

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However, this interaction of *M. tuberculosis* with macrophages is by no means unidirectional; they engage in a true two-way biochemical interaction pivoting on dedicated proteins, small molecules and secretion systems which export bacterial molecules into the host cell cytoplasm. Recognition of these pathogen-associated molecular patterns (PAMPs), during bacterial internalization as well as when *M. tuberculosis* either resides inside a membrane bound phagosomal compartment or translocates into the cytoplasm, by host pattern recognition receptors (PRRs) that activate myriad of signaling cascades. Signaling events initiated by PAMP and PRR interaction are critical components of the host defense arsenal and allows the host to mount immunoresponses against *M. tuberculosis* [1–4].

While much remains to be learned, research over the past few decades is beginning to reveal how *M. tuberculosis* manages to withstand the hostile environment inside the macrophages or manipulate host responses in order to replicate and persist. Many of these strategies are uniquely employed by pathogenic mycobacteria compared to other intracellular pathogens (recently reviewed by Jayachandran et al. [3]). While some overlap exists, most pathogens have evolved specific ways to interfere with and circumvent host immune responses; this may be due to either the discrete intracellular niches that different pathogens occupy or the exclusivity of various pathogens with regard to their physiological necessities.

2. Subversion of host responses from beginning to the end: phagocytosis, phagosomal trafficking and maturation

Tuberculosis (TB) is primarily an airborne respiratory infection which is transmitted by aerosolized *M. tuberculosis* from patients with active TB. The establishment of a primary focus of infection depends on the activation status of the resident alveolar macrophages (AM) that phagocytose the inhaled bacilli as well as the virulence of the bacilli [5–7]. As the first line of cellular defense against inhaled bacilli, AMs express a broad range of immune receptors, including Fcγ receptors (FcγRs), complement receptors (CRs), toll-like receptors (TLRs), PRRs such as C-type lectin mannose receptors (MR), dectin-1, and scavenger receptors (SRs) that mediate phagocytosis (recently reviewed by Kleinnijenhuis et al. [8]). In addition, the dendritic cell (DC)-specific intracellular adhesion molecule (ICAM)-3-grabbing nonintegrin (DC-SIGN) receptor also plays a critical role in *M. tuberculosis* internalization by the DCs [9–10]. An array of biosynthetically related mannosylated lipoglycoconjugates within the mycobacterial cell envelope (e.g., phosphatidyl-myo-inositol mannosides (PIMs), lipomannan (LM) and mannose-capped lipoarabinomannan (ManLAM)) [11], glycoproteins (e.g. the 38-kDa and 19-kDa protein antigens), and mycobacterial heat shock proteins 60/65 have been shown to bind to the receptors on macrophages, DCs and other phagocytic cells to facilitate receptor-mediated internalization of *M. tuberculosis* and bacterial entry to the phagosome. Details on receptor mediated internalization of *M. tuberculosis* have recently been reviewed [8, 12].

Recognition and internalization of the bacteria by specific receptors trigger diverse host intracellular signaling pathways, which initiate the development of the unique phagosome biochemistry, characteristic of *M. tuberculosis* infection, and determine the nature of the early inflammatory response. For example, Fc receptor-mediated internalization results in a respiratory burst [13], whereas CR3-mediated uptake of *M. tuberculosis* prevents the

activation of the macrophages and leads to the cholesterol-dependent prevention of phagosome-lysosome fusion [14–15]. Further, activation through TLRs and dectin-1 initiates what is essentially a pro-inflammatory response by signaling through the DC-SIGN. The primary function of MRs and CRs is to modulate internalization, without necessarily triggering a pro-inflammatory response. Interestingly, although MRs and DC-SIGN are both C-type lectins that recognize *M. tuberculosis* ManLAM, they regulate phagosomal trafficking differently. While the former is involved in efficient phagocytosis, endocytosis and endosomal sorting, the latter targets *M. tuberculosis* to lysosomes. This may explain why *M. tuberculosis* prefers macrophages, which possess high surface MR levels, for its major intracellular niche over DCs, which possess high surface levels of DC-SIGN and rapidly shuttle bacteria to the destructive environment of the lysosomes. Employing several proteins and lipid molecules, *M. tuberculosis* interferes with the phagosomal maturation pathway thereby blocking its transfer to lysosomes [12]. The *M. tuberculosis* lipid phosphatase SapM dephosphorylates phosphatidylinositol 3-phosphate (PI3P) [16–17]; the tyrosine phosphatase PtpA dephosphorylates and inactivates the host vacuolar protein sorting- VPS33B which in turn regulates membrane fusion and arrests phagosome maturation [18]. Additionally, ManLAM inhibits the Ca/Calmodulin and Rab5-dependent recruitment of PI3K resulting in reduced PI3P formation on the phagosomal membrane [19–20]. Phagosomes containing *M. tuberculosis* also fail to acidify due to bacterial interference with the recruitment of the vesicular proton ATPase pump and failure to acquire late endocytic markers such as Rab7 [12].

Another host pathway that *M. tuberculosis* regulates coronin-1-dependent cytosolic calcium influx and activation of calcineurin via its lipoamide dehydrogenase [21–22]. During phagocytosis, coronin-1 (a phagosomal coat protein) is released from the cytosolic surface of the maturing phagosome resulting in fusion of the phagosome with the lysosome and transfer of its internal contents for degradation. *M. tuberculosis* is known to prevent or delay coronin-1 release, thereby blocking phagosomal fusion with lysosomes [22–23]. *M. tuberculosis* also possesses at least 11 eukaryotic-like protein kinases which have been shown to regulate mycobacterial signal transduction pathways, morphology, and cell division [24–25]. An example is PknD which has been shown to play an active role in the invasion of the central nervous system by *M. tuberculosis* [26] in addition to its role in regulation of gene expression by phosphorylation of alternative sigma factor regulators [27]. Another kinase, PknG, has been shown to be released from pathogenic mycobacteria inside the macrophage cytosol where it prevents lysosomal delivery and degradation [28–29]. It has also been proposed that *M. tuberculosis* interferes with phagolysosome biogenesis by a putative Zn^{2+}-dependent metalloproteinase (Zmp1) that interferes with caspase-1 dependent activation and secretion of IL-1β [30]. Recently, several groups have reported that mycobacterial ‘enhanced intracellular survival protein’ (Eis) may inhibit JNK-dependent ROI production thereby inhibiting TNF-α production and preventing macrophage activation, inflammation, and autophagy [31–33].

There is considerable evidence that *M. tuberculosis* cell wall lipids act as virulence factors during infection [4, 34–37]. While there is a scarcity of information regarding the nature of molecular interactions of mycobacterial lipids with the host cells, *M. tuberculosis* lipids

have been observed to intercalate into host membranes leading to decreased membrane fluidity and increased passive permeability [38]. The ability of mycobacterial phthiocerol dimycocerosates (PDIMs) and trehalose-6, 6′-dimycolates (TDMs) to alter host membrane fluidity may influence the process of phagocytosis as well as subsequent trafficking. *M. tuberculosis* lipids are abundantly produced during macrophage infection and have been shown to be actively trafficked out of the phagosome [39–42] and finally exocytosed from infected macrophages where they are taken up by neighboring macrophages [43–44]. This process could potentially influence CD1-mediated lipid antigen presentation by resident DCs and subsequent immune responses [42, 45]. Consequently, host lipids are important regulators of inflammatory signaling pathways in bacteria as well, since several host lipids are the primary building blocks of the mycobacterial lipid load [46]. Recent reports of nuclear receptor-peroxisome proliferator-activated receptor (PPAR)-mediated sensing of mycobacterial fatty acid derived eicosanoids and modulation of cytokine responses and its effect on virulence and pathogenicity of *M. tuberculosis* further highlights the complexity and importance of lipids in host-pathogen crosstalk [47–48].

While *M. tuberculosis* employs several strategies to prevent phagolysosome-mediated early killing by the host, how the bacilli manipulate other macrophage functions while residing within the phagosome remains an intriguing question. Several studies have demonstrated that *M. tuberculosis* successfully accesses and/or translocates into the macrophage cytoplasm from the phagosome [49–51]. ESAT-6, a member of the region of difference- 1 (RD-1) gene cluster, plays a key role in this process [51–52]. The *M. bovis*-derived vaccine strain Bacille Calmette-Guérin (BCG), which lacks the RD-1 region, is unable to translocate to the cytosol and is avirulent [52]. Therefore, cytosolic escape could be a potential mechanism of virulence exerted by the proteins encoded within the RD-1 region. The ESAT-6 is a member of the ESX-1 specialized secretion system that not only allows bacterial proteins to be secreted, but also damages the phagosomal membrane thereby permitting mixing of luminal contents with the cytoplasm of the host cell [1, 53–54]. The mixing of phagosomal and cytoplasmic contents allows for recognition of mycobacterial components, including bacterial chromosomal DNA, CpG motifs, peptidoglycan fragments, dsRNA, and nucleotides by a range of host cytosolic receptors such as nucleotide-binding oligomerization domain (NOD) proteins [55–56], nucleic acid receptors [1, 57–64]. Once engaged, these cystosolic receptors activate inflammatory response pathways including Type-1 IFN, the inflammasome, and autophagy [1–2, 65–67]. Interestingly, a recent study demonstrated that an ESAT-6-deficient *M. tuberculosis* laboratory strain of H37Rv and *M. bovis* BCG could translocate into the cytosol in the absence of TLR signaling, indicating that host TLR signaling plays a decisive role in preventing mycobacterial translocation into the host cytosol [68]. Since several mycobacterial components are known to regulate TLR signaling, this study suggests that *M. tuberculosis* may regulate its translocation into the cytosol by modulating TLR signaling. Thus, it is apparent that *M. tuberculosis* occupies several intracellular niches based on host cell immunity and activation status coupled with temporal requirements at particular phases of infection. Table 1 lists some of the prominent host- receptors, which are involved in recognition of *M. tuberculosis* and its components and initiation of immune responses. For details of receptor mediated signaling and cytokine

responses refer to the chapter ‘Pro- and anti-inflammatory cytokines in TB’ by Eliana Coccia.

3. Host-pathogen crosstalk via nucleotide second messengers

While there is no doubt that the role of ESX-1-based specialized secretion, phagosomal permeabilization, and cytosolic translocation during *M. tuberculosis* infection are important determinants of host-pathogen molecular exchange and crosstalk, there is a vast array of small, soluble, signaling molecules from the bacteria that enter the host cell by known or unknown mechanisms [69–71]. These small molecules have been shown to control host gene expression or relay information to effector molecules within the host cell [72–74]. Classical second messengers include diverse molecules such as cyclic nucleotides (cAMP and cGMP), guanosine pentaphosphate or tetraphosphate [(p)ppGpp], Ca^{2+}, inositol trisphosphate, and diacylglycerol (DAG) [75–78]. Recent additions to this list include cyclic-di-nucleotide molecules such as cyclic di-guanylate (c-di-GMP) [79–80], cyclic di-adenylate (c-di-AMP) [81], and cyclic-GMP-AMP (c-GAMP) [82–85]. Nucleotide polymers are perhaps the oldest molecules of life, and the cyclized form of the energy building block ATP, 3′, 5′-cyclic adenosine monophosphate (cAMP), is a carrier of sensory information in all domains of life While the roles of cAMP and its sister molecule 3′, 5′-cyclic guanosine monophosphate (cGMP) have been studied for over five decades [69–70], the cyclic dinucleotides c-di-GMP [79–80], c-di-AMP [81], and c-GAMP [82–85] were more recently identified as important signaling molecules in both prokaryotes and eukaryotes. In this review, we aim to provide a perspective on how cAMP and c-di-nucleotides participate in the crosstalk between *M. tuberculosis* and the macrophage, and how these molecules facilitate *M. tuberculosis* survival and pathogenesis.

3.1 Modulation of cAMP homeostasis and subversion of host immunity

3.1.1 cAMP signaling in bacteria and the host: an overview—Although the basic module of cyclic nucleotide signaling is conserved from bacteria to complex eukaryotes, the molecular players that are involved in the signaling pathways are often markedly different [69, 86–87]. The initiation of signaling cascades is triggered by the first messenger (external stimuli or stress such as alterations in levels of ambient biochemicals including nutrients, hormones, or neurotransmitters) leading to activation of adenylyl (ACs) and guanylyl cyclases (GCs) which catalyze the formation of cyclic nucleotides (the second messenger) from either ATP or GTP. This is followed by binding of the cyclic nucleotides to corresponding receptors leading to downstream regulatory effector functions and, finally, culminating in the hydrolysis of the cyclic nucleotide by corresponding phosphodiesterases (PDEs) [88]. In prokaryotes and eukaryotes the primary effector function of cyclic nucleotide signaling is regulation of gene expression. As cyclic nucleotide signaling governs functions unique to both the host and the bacterial pathogen, there are numerous bacterial virulence strategies that interfere with host cyclic nucleotide signaling. Similarly, there are host defense strategies for counter-regulation.

In bacteria, glucose depletion induces AC activition in some but not all species [69, 89–90]. The use of the cAMP receptor protein (CRP) family of transcription factors, which bind upstream of specific promoters to stimulate transcription, is a common means of cAMP

message relay. The effects of cAMP are further amplified in some cases by cAMP-mediated co-regulation of other global regulators [74, 91–92]. In *Escherichia coli* alone, CRP is known to activate transcription from more than 100 different promoters, suggesting a wide range of cAMP-mediated regulatory effects in bacteria [93]. In eukaryotes, binding of the first messenger to G-protein coupled receptors (GPCRs) leads to activation of cellular ACs [86, 94–96]. The resulting elevated cAMP levels lead to protein kinase A (PKA) activation by promoting release of its catalytic subunit. PKA-mediated phosphorylation of target proteins, including the cAMP response element binding protein (CREB), finally results in differential expression of target genes. AC activity is also regulated by an inhibitory G-protein α-subunit (Gαi) that reduces AC activity following binding to ligands which include several chemokines and leukotrienes [94]. To date, among the existing six classes of ACs, four are restricted to prokaryotes [71]. The most widely distributed ACs are the class III enzymes which are metal-dependent [71]. Class III ACs are found in mycobacteria and are also represented by the eukaryotic, GPCR-activated ACs. The activity of all three classes of cAMP-hydrolyzing PDEs is also metal dependent. Class I PDE enzymes are present exclusively in eukaryotes; while low affinity, class II PDEs have been identified in yeast, *Vibrio* species, and *Dictyostelium*. Class III PDEs were first described in *E. coli*. In eukaryotes, 10 AC isoforms and 11 PDE families are known to be expressed in a tissue-specific manner [71]. The structural and functional classifications of the cAMP ACs and PDEs are described in detail by a recent review [71].

Literature spanning several decades has described a multitude of bacterial signaling pathways that are regulated by cAMP. These include catabolite repression in enteric bacteria, regulation of competence, chromosomal replication by binding to DnaA, phototaxis and heterocyst formation in cyanobacteria, secondary metabolite production and germination in *Streptomycetes*, regulation of virulence regulons in *Pseudomonas*, and biofilm formation in *Vibrio cholerae* [69, 90, 97–98]. In higher eukaryotes, cAMP and cGMP are important second messengers that mediate effects of light, nitric oxide, hormones, and other signals to regulate vision, muscle contraction, vasodilatory effects, sleep, memory, and various other functions [99]. cAMP mediates the regulation of ion channels such as the hyperpolarization-activated cyclic nucleotide-gated (HCN) channels, which have also recently been reported to be co-regulated by c-di-AMP [100]. PKA-independent functions of cAMP include activation of calcium channels and binding of cAMP to exchange protein activated (Epac) family proteins. These proteins activate small Ras-like GTPases such as Ras-proximate-1 (Rap1) which is predominantly involved in cell adhesion and cell junction formation during cell proliferation [101–103]. cAMP is also known to enhance the phosphorylation of extracellular-signal-regulated kinases-1/2 (ERK-1/2) via a mitochondria-derived, reactive oxygen species (ROS)-dependent activation of Ras [104].

It is evident from the above discussion that cAMP plays important regulatory roles in many cellular processes in both bacteria and host cells. Not surprisingly, there are also highly efficient, bacterial systems that target host ACs and PDEs to subvert host cAMP-governed processes. Elevated levels of cAMP can suppress innate immune functions by modulating the expression of inflammatory mediators, inhibiting phagocytic responses, and reducing intracellular killing of ingested pathogens [69–70]. Increased intracellular cAMP is also

known to interfere with the phagosomal actin assembly cascade and phagosome maturation in macrophages [105]. Bacterial pathogens are known to elevate host intracellular cAMP levels via several distinct mechanisms: (i) by direct injection of bacterial ACs into the host cell (ii) by regulating the activity of host ACs through GPCR, (iii) by secretion of cAMP directly into the host cell, and (iv) by exploiting alternative host signaling pathways to indirectly trigger cAMP production (see reviews by Ahuja *et al*. [106] Agarwal and Bishai. [70], McDonough and Rodriguez. [69]). A rapid increase of cAMP levels in the host cell following infection with pathogenic bacteria results in impaired chemotaxis, phagocytosis, ROI and RNI responses, induction of apoptosis, and reduced bactericidal activity of macrophages and neutrophils [107–114].

3.1.2. Subversion of cAMP signaling during *M. tuberculosis* infection—

Adenylate cyclase activity was first reported in mycobacterial cell-free extracts close to four decades ago [115–116]. To date, 16 AC-like proteins, ten of which possess confirmed AC activity, have been reported in *M. tuberculosis* [71, 117]. In contrast, only a single protein (Rv0805) with cAMP PDE activity has been identified [118]. In addition to the AC catalytic core region, many *M. tuberculosis* ACs contain other domains such as receptor binding domains, DNA-binding elements, and HAMP (histidine kinases, ACs, methyl-binding proteins, and phosphatases) domains suggesting that their AC activity is linked to a regulatory function [71]. Furthermore, sub-cellular localization studies of mycobacterial ACs reveals multiple cellular locations suggesting the presence of soluble, membrane-bound, and membrane-integral subclasses [71]. The presence of multiple AC genes in the *M. tuberculosis* genome is also intriguing as the mammalian genome encodes multiple ACs with tissue-specific expression. Since *M. tuberculosis* can infect a range of cell types, whether mycobacterial ACs have host cell-specific expression remains an interesting puzzle.

Unlike *E. coli*, exogenous glucose does not have a prominent effect on *M. tuberculosis* cAMP levels [116, 119]. However, a range of *in vitro* conditions that mimic infection conditions, such as pH, fatty acids, carbon dioxide (CO_2), hypoxia and starvation, directly alter expression of *M. tuberculosis* ACs [74]. Several studies have reported an increase in cAMP level in the macrophage cytosol following mycobacterial infection [119–120]. Agarwal et al. reported the key of role of an *M. tuberculosis* AC, Rv0386, in delivering bacterial-derived cAMP into the host cytoplasm [120]. In addition, that study highlighted the role of the bacterial cAMP-specific PDE, Rv0805, which, when over-expressed in *M. tuberculosis*, significantly reduced intra-bacterial and intra-macrophage cAMP concentrations with consequent reductions in CREB phosphorylation and TNF-α production in murine macrophages (Figure 1). Agarwal et al. also demonstrated that a dysregulated host inflammatory response following the *M. tuberculosis*-mediated, cAMP intoxication of the macrophage cytoplasm favors bacterial survival. This confirms cAMP as a mycobacterial virulence mediator and suggests that there may be therapeutic value in manipulating mycobacterial cAMP or PDE activity [121].

Because of the functional redundancies of multiple ACs, several investigators targeted downstream signaling molecules to study the effect of modulation of cAMP in *M. tuberculosis. M. tuberculosis* possesses ten putative cAMP-binding proteins [71, 74, 122], two bona fide CRP-family transcription factors (Crp and Cmr), and a cAMP-responsive

protein lysine acetylase [123–125]. Of the two transcription factors, Crp, controls a regulon of >100 genes in *M. tuberculosis* [124], while Cmr, has been shown to control the expression of a different set of genes in response to cAMP levels and macrophage passage [126]. Deletion of the *crp* gene (Rv3676) results in a mutant strain with impaired *in vitro* and *in vivo* growth and attenuated virulence in mice [123]. The functions of the cAMP-responsive lysine acetylase and the remaining putative cAMP-binding proteins remain unknown.

Recently, another exciting novel function of cAMP in *M. tuberculosis* has recently been reported by Pelly et al., wherein they have identified a small, non-coding RNA (sRNA) *ncrMT1302* in a locus involved in cAMP metabolism that is responsive to changes in pH and cAMP concentration [127]. The differential expression of *ncrMT1302* observed in wild-type *M. tuberculosis* during growth is abolished in a strain lacking *MT1302 (Rv1264)*, an AC-encoding gene. They also report that *ncrMT1302* is expressed in *M. tuberculosis* residing in the lungs of mice during active infection. As cAMP contributes to virulence and a pH stress response is vital for the survival of the bacillus, this study demonstrates a key link between cAMP-mediated responses and sRNA-regulated transcriptional regulation.

3. 2. Modulation of cyclic di-nucleotide signaling and subversion of host immunity

3.2.1. Cyclic di-nucleotide signaling: an overview—With the exception of c-di-GMP, which was discovered more than two and a half decades ago as an allosteric activator of cellulose synthase in the fruit-degrading bacterium *Gluconacetobacter xylinus* [128], both c-di-AMP and the cyclic AMP-GMP hybrid dinucleotide c-GAMP (both bacterial and eukaryotic) are the newest additions to the growing list of second messenger molecules that are involved in host-pathogen crosstalk (see [77, 79, 129–130] for recent reviews). c-di-GMP and c-di-AMP synthesizing and degrading enzymes are found in a number of bacterial species [130–131], but to date such enzymes have not been found in mammalian cells. These two signaling, cyclic dinucleotides (CDNs) share several common features. Both c-di-AMP and c-di-GMP are synthesized by domains that are usually part of multi-domain proteins, such that more than one input signal may affect their enzymatic activity. Both molecules regulate a variety of similar physiological processes, including cell wall metabolism, antibiotic resistance, biofilm formation, cell differentiation, morphology, and motility [130–131]. And several of the regulatory functions exerted by these two CDNs have direct effects on bacterial virulence mechanisms [130–131]. For example, c-di-GMP plays a central role in the switch from the motile to sessile state within multicellular biofilms in several bacterial pathogens [132]. Likewise, deletion mutants for the cyclase gene or gene domain for either of these CDNs exhibit compromised virulence in these pathogens [130, 132–133].

While the precise mechanism of c-di-AMP-mediated regulation in bacteria remains unknown, the basic signaling modules are similar for both c-di-AMP and c-di-GMP. Following an external or internal signal, condensation of two nucleotide triphosphates (GTP or ATP) by CDN cyclases generates either c-di-GMP or c-di-AMP. The CDNs then bind to target proteins and elicit allosteric changes which alter effector protein function thereby regulating specific cellular pathways [78, 131]. Finally, CDNs are degraded by specific

CDN-PDEs. Both of these CDNs have also been shown to bind specific riboswitches that are known to regulate transcription and translation of downstream sequences [134–136].

The hybrid CDN of bacterial origin, 3′-5′-c-GMP-AMP (cGAMP), is a canonical nucleotide formed by 3′-5′ linkages between the guanosine and the adenosine residues, as is also the case with c-di-AMP and c-di-GMP [64, 83]. To date, 3′-5′-cGAMP has only been reported in *Vibrio cholerae* where it is required for efficient intestinal colonization [137]. The mammalian cGAMP is a 2′3′-CDN produced in mammalian cells by cGAMP synthase (cGAS) in response to double-stranded DNA detected in the cytoplasm [64, 85, 129]. 2′3′-cGAMP is also referred to as 'non-canonical' cGAMP due to the presence of the atypical 2′-3′ phosphodiester linkages between the nucleotide residues. A fascinating phenomenon is that despite structural and source differences between the bacterial and host-derived CDNs, both the 3′5′-cGAMP of bacterial origin and 2′3′-cGAMP of mammalian origin (as well as c-di-AMP and c-di-GMP) are detected by a common detector protein in the host cell cytoplasm - the stimulator of interferon gene signaling (STING) protein. STING activates the TBK1-IRF3-dependent Type-I IFN signaling pathway leading to IFN-β production [82–84]. However, recent studies have revealed mechanisms that may differentiate these two sets of CDNs. Certain variants of STING are able to distinguish between the non-canonical and canonical cGAMP [138–139]. Further, cGAMP is more potent in activating the Type I IFN response than c-di-AMP or c-di-GMP, bacterial-derived cyclic dinucleotides that also bind STING [140]. Moreover, while physiologically relevant levels of c-di-AMP and c-di-GMP trigger robust secretion of IL-1β in an NLRP3 inflammasome-dependent manner [141], the host-derived cGAMP does not stimulate IL-1β release [142]. Thus, host cells appear capable of recognizing the presence of bacterial CDNs in the cytoplasm and launching an immune response that is qualitatively different from the response generated by self-derived cGAMP, though there is a certain degree of overlap with respect to the Type-I IFN responses. Given the presence of cytosolic DNA receptors, such as DDX41, which recognize both bacterial DNA (bDNA) and bacterial CDNs and the fact that some host receptors are regulated by bacterial CDNs (e.g., IFI16 and p202) [143], it may be difficult to assess the relative contribution of bDNA versus CDNs to simulating IFN-β induction during bacterial infection. Further evidence of overlap between the bDNA- and CDN-based responses is in the induction and regulation of autophagy- a key mechanism by which macrophages kill intracellular bacteria [1–2, 144]. Induction of autophagy has been reported in murine macrophage cells following exogenous stimulation with synthetic, bacterial CDNs [2]. Further, direct interaction between cGAS and the autophagy protein Beclin-1 not only suppresses cGAMP synthesis to halt IFN-β production upon dsDNA stimulation, but also enhances autophagy-mediated degradation of cytosolic pathogen DNA to prevent excessive cGAS activation and persistent immune stimulation [145]. Thus, the cGAS-Beclin-1 interaction governs innate host defense strategies by regulating both cGAMP production and autophagy induction.

The Type-I IFN response is a well-characterized and critical antiviral host response. In the case of bacterial infections, Type-I IFNs appear to exert both beneficial and detrimental effects on the host [146–148]. An enhanced Type I IFN response as a consequence of elevated c-di-AMP levels has been observed in several studies with laboratory as well as

clinical isolates of pathogenic bacteria [149–151]. A c-di-AMP over-secreting *Listeria monocytogenes* strain which induces a host IFN-β response was found to be attenuated in a mouse model of infection [149]. Similarly, the deletion of a c-di-GMP cyclase gene, *cgsB,* in *Brucella melitensis* produced hypervirulence, while deletion of the PDE genes, *bpdA* and *bpdB,* resulted in attenuation of virulence [152]. Likewise, deletion of CDN PDEs from other pathogenic bacteria has resulted in attenuation of virulence in animal models of infection [133, 153]. In contrast, mutation of the c-di-AMP cyclase, *dacA*, reduces fitness in some strains of *Staphylococcus aureus* and *L. monocytogenes* [154–155].

3.2.2. Cyclic dinucleotides and modulation of host responses during *M. tuberculosis* infection—While most of the bacterial species possess multiple CDN-cyclases and CDN-PDEs, the *M. tuberculosis* genome encodes only one cyclase and one PDE for c-di-GMP and similarly one cyclase and one PDE for c-di-AMP (Table 2) [156–158]. However, the diguanylate cyclase (DGC) domain-containing protein in *M. tuberculosis* (Rv1354c) is a bi-functional protein possessing both GGDEF and EAL domains which possess cyclase and phosphodiesterase activities, respectively [157]. Studies with *M. smegmatis* c-di-GMP null and overexpression mutants demonstrate that while neither of these defects affect growth or biofilm formation, they do affect long-term survival under conditions of nutritional starvation [158]. Furthermore, Hong et al. reported that while a diguanylate cyclase (DGC) deletion mutant of the *M. tuberculosis* Rv1354c gene exhibited an increased dormancy phenotype, the c-di-GMP PDE (Rv1357c) deletion strain exhibited a reduced dormancy phenotype [159]. They also reported that the c-di-GMP PDE deletion strain was attenuated for virulence and pathogenicity in both human THP-1 derived macrophages as well as in a mouse model. However, none of these studies convincingly demonstrated whether the attenuation phenotype is due to the effects of altered c-di-GMP levels on the bacteria alone, the host responses, or both.

In *M. tuberculosis*, Rv3586 (*disA*, also referred as *dacA*) encodes a diadenylate cyclase [156]. Orthologues of *dacA* exist in all mycobacterial genomes with the exception of *M. leprae*. It has been reported that synthesis of c-di-AMP by a DisA homologue in *M. smegmatis* is inhibited by RadA (Rv3585), encoded by the adjacent gene, through a physical interaction with the cyclase [160]. Furthermore, a c-di-AMP binding transcription factor, DarR, was identified in *M. smegmatis*, and this transcription factor represses the expression of several genes associated with fatty acid metabolism and transportation [161]. However, a DarR orthologue has not been identified in *M. tuberculosis.*

Dey et al. recently showed that c-di-AMP is produced by *M. tuberculosis* and is secreted into the host cytosol during infection. This leads to STING-dependent induction of the cytoplasmic surveillance pathway (CSP) and consequently, induction of the Type I IFN pathway, autophagy, and increased secretion of a number of pro-inflammatory cytokines such as IL-1α, IL-6 and TNF-α (Dey, B *et al.*, 2014, unpublished data). They further found that a di-adenylate cyclase (*dacA*) over-expressing *M. tuberculosis* strain that secretes excess c-di-AMP into the macrophage cytoplasm displayed attenuated virulence in mice compared to the *dacA* deficient and wild type strains. c-di-AMP-mediated IFN-β induction during *M. tuberculosis* infection was also found to be dependent on STING-signaling with

contributions from DDX41 (Dey, B *et al.*, 2014, unpublished data). In addition, cGAMP synthase (cGAS) [82, 84, 162], while contributing to the overall Type I IFN response, was not strictly required for c-di-AMP mediated responses since c-di-AMP could activate the Type I IFN pathway even in the absence of this cytosolic DNA receptor (Dey, B *et al.*, 2014, unpublished data).

These observations by Dey et al. expand upon earlier studies which suggested that mycobacterial DNA is the exclusive ligand for inducing the host Type I IFN response, and that the bacterial Esx-1 secretion apparatus is required for signaling [1]. By employing multiple bacterial strains (the *M. tuberculosis* CDC1551 and Erdman strains, and *M. bovis* BCG) each modified to overexpress c-di-AMP and a variety of host phagocytic cells, including those defective in important mediators of the CSP (STING, DDX41, and cGAS), Dey et al. consistently demonstrated that c-di-AMP, not bacterial DNA alone, is a key mediator of Type I IFN responses (Figure 2) (Dey, B *et al.*, 2014, unpublished data). They also showed that bacterial-derived c-di-AMP activates Type I IFN in the absence of an Esx-1 secretion system and, while contributory, Esx-1 is not required for c-di-AMP-triggered IRF pathway activation. Along similar lines, Bishai and colleagues and others also found that a c-di-AMP-PDE deletion strain (Rv2837c) of *M. tuberculosis* induced a heightened Type-I IFN response and that this mutant was also attenuated in the murine model of TB (Bishai and colleagues, unpublished data)[150].

Cyclic di-AMP mediated enhanced Type I IFN response during *M. tuberculosis* infection observed by Dey et al. extends correlative observations by several studies with laboratory as well as clinical isolates of pathogenic bacteria [149–151]. While Type I IFNs are critical for resistance to viruses, there are reports as to whether the IFN-α/β response is beneficial or detrimental to the host during TB [146–148]. For example, loss of the IFN-α/β receptor knockout mice confers resistance to *M. tuberculosis* infection, suggesting that Type I IFN responses are counterproductive in TB [163]. In contrast, IFNα/β promote antigen cross-presentation in DCs and activation of cytolytic CD8 T cells, which are crucial for *M. tuberculosis* clearance [164–165]. Furthermore, in Type II IFN deleted mutant mice, the Type I IFN response has also been shown to limit lung infectivity of *M. tuberculosis* [166]. Importantly, a human transcriptome analysis of peripheral blood in patients with TB also revealed high levels of Type I and Type II IFN inducible genes, suggesting an overlapping and dynamic role of both types of IFN in TB pathogenesis [167].

It is evident from the above discussion that CDNs play an important regulatory role in many cellular processes in both the virulence and pathogenicity of *M. tuberculosis* and host-pathogen crosstalk during infection. Thus, it is not surprising that both the host and pathogen employ strategies to regulate intracellular CDN levels to establish supremacy over the other. Future research in these areas is warranted to uncover the exquisite virulence mechanism based on CDNs that *M. tuberculosis* utilizes to manipulate host defense machinery.

4. Summary and future perspectives

The enduring co-evolution of *M. tuberculosis* with its hosts has enabled the pathogen to develop a number of strategies to thwart the host defense for its survival, especially within

macrophages. These tactics range from interfering with phagosomal acidification and trafficking, blocking autophagy and apoptosis-mediated killing, perturbing calcium signaling, and inhibiting inflammasome activation in order to modulate the host cytokine responses and quench the reactive oxygen and nitrogen species produced by activated macrophages. Manipulation of these host pathways is achieved by employing a plethora of bacterial components including cell wall lipids, serine threonine kinases, phosphatases and proteases, and actively using specialized secretion systems. In this review, we have focused on a relatively recently described virulence strategy involving perturbation of nucleotide-based second messenger signaling in the host.

Subversion of host signaling molecules, particularly nucleotide second messengers, has emerged as a common evolutionary strategy of pathogens to counteract the host's innate responses [168–171]. For instance, a number of pathogenic viruses such as Murine hepatitis virus (MHV) and Group A rotavirus (RVA) efficiently counteract innate immunity by degrading host derived oligoadenylates employing specific phosphodiesterase thus inhibiting activation of ribonuclease L (RNase L), which constitute important components of the host antiviral pathway [172–173]. Moreover, recent research has demonstrated that cGAS is an important innate sensor of retroviral DNA such that infection with human immunodeficiency viruses (HIV), simian immunodeficiency viruses (SIV), or murine leukemia virus (MLV) activates cGAS to produce cGAMP, thus stimulating the cGAMP/STING/IFN axis [82]. These viruses are well-known to obviate CSP responses by concealing viral nucleic acids within capsid structures and/or limiting the accumulation of cytosolic viral DNA by co-opting host factors such as TREX1 and SAMHD1 [174]. TREX1 is a cytosolic exonuclease, which inhibits the host CSP or Interferon stimulatory DNA response by degrading the cytosolic DNA derived from HIV. TREX1 has also been shown to play an important role in *M. tuberculosis*-induced Type I IFN response. Knocking out the TREX1 gene substantially increased the cellular innate response while its over-expression resulted in a reduced host response to *M. tuberculosis* infection [1]. Such observations may establish important links in the co-pathogenesis of HIV and *M. tuberculosis*, each of which is known to exacerbate the other in co-infected humans. It is interesting to speculate presence of specific bacterial and viral mechanisms to manipulate CDN second messenger signaling to facilitate TB-HIV co-infection. Box 1 summarizes some of the key issues related to cyclic nucleotide signaling in *M. tuberculosis* infection.

Box 1

Key issues on cyclic nucleotide signaling in *M. tuberculosis* infection

- How does *M. tuberculosis* secrete cyclic mono- and di-nucleotides?
- Do mycobacteria secrete cyclic nucleotide cyclases and phosphodiesterases into the host cell to utilize host substrate molecule as well as to subvert host signaling pathways?
- How do both the bacilli and the host regulate the balance and interconnected functions of multiple cyclic nucleotides?

- How does the host differentiate seemingly similar self and non-self cyclic nucleotides to initiate specific signaling cascades?
- Like the host, does *M. tuberculosis* expresses different cyclic nucleotide cyclases and phosphodiesterases in a tissue- or organ-specific manner during infection?
- Does cyclic-nucleotide signaling plays any role in TB-HIV co-infection?
- Does small molecule targeting of the cyclic nucleotide signaling pathways have potential for developing therapeutics or immunotherapeutics against TB?

The past decade has revealed numerous mechanisms which contribute to the virulence of *M. tuberculosis.* Similarly there are host mechanisms in place to counteract these *M. tuberculosis* virulence strategies. Considering the overlapping and interconnected nature of the host-microbe crosstalk in tuberculosis, future research to characterize this interplay promises to unravel new vistas of therapeutic and prophylactic possibilities against this stubborn pathogen.

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Abbreviations

AC	Adenylyl Cyclase
AKAP7	A-Kinase Anchor Protein 7 isoform gamma
AM	Alveolar Macrophage
ATP	Adenosine Triphosphate
BCG	Bacille Calmette–Guérin
cAMP	Cyclic Adenosine Monophosphate
CD1	Cluster of Differentiation 1
c-di-AMP	Cyclic-di-Adenosine Monophosphate
c-di-GMP	Cyclic-di-Guanosine Monophosphate
CDN	Cyclic Dinucleotide
cGAMP	Cyclic- Guanosine Monophosphate - Adenosine Monophosphate
cGAS	Cyclic GMP-AMP synthase
cGMP	Cyclic Guanosine Monophosphate
CR	Complement Receptor
CREB	cAMP Response Element-Binding Protein

CSP	Cytoplasmic Surveillance Pathway
DAG	Diacyl-Glycerol
DC	Dendritic Cell
DC-SIGN	Dendritic Cell-Specific Intercellular Adhesion Molecule-3-Grabbing Non-integrin
DGC	Diguanylate Cyclase
disA	DNA Integrity Scanning Protein-A
Eis	Enhanced Intracellular Survival protein
ESAT-6	Early Secretory Antigenic Target - 6
ESX-1	ESAT-6 secretion system
FcγR	Complement Factor- γ Receptor
GC	Guanylyl Cyclase
GTP	Guanosine Triphosphate
HCN	Hyperpolarization-Activated Cyclic Nucleotide-Gated Channels
HIV	Human Immunodeficiency Virus
ICAM	Intercellular Adhesion Molecule 1
IFN	interferon
IL	interleukin
LM	Lipomannan
M. tuberculosis	*Mycobacterium tuberculosis*
ManLAM	Mannose-Capped Lipoarabinomannan
MHV	Murine Hepatitis Virus
RNasL	Ribonuclease L
MLV	Murine Leukemia Virus
MR	Mannose Receptors
MyD88	Myeloid Differentiation; Primary Response Gene 88
NLRP3	NLR Family Pyrin Domain Containing 3
NOD	Nucleotide-Binding Oligomerization Domain
PAMP	Pathogen Associated Molecular Pattern
PDE	Phosphodiesterase
PDIM	Phthiocerol Dimycocerosates
PI3P	Phosphatidylinositol 3-Phosphate

PIM	phosphatidyl-myo-inositol mannosides
PKA	Protein Kinase A
(p)ppGpp	Guanosine Pentaphosphate or Tetraphosphate
PRR	Pattern Recognition Receptor
SAMHD1	SAM Domain and HD Domain-Containing Protein 1
SR	Scavenger Receptors
STING	Stimulator of Interferon Gene
TB	Tuberculosis
TDM	Trehalose Dimycolates
TLR	Toll-Like Receptor
TNF	Tumor Necrosis Factor
TREX1	Three Prime Repair Exonuclease 1
VPS33B	Vacuolar Protein Sorting-Associated Protein 33b

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Highlights

- The macrophage cytosolic surveillance pathway detects foreign DNA and nucleotides.
- *M.tb* delivers pathogen-derived cAMP into the host cell eliciting hyperinflammation.
- c-di-AMP and c-di-GMP are bacterial derived nucleotides detected by host receptors.
- Bacterial DNA and *M.tb*- derived c-di-AMP trigger IFN-β secretion and autophagy.
- Manipulation of the cytosolic surveillance pathway may enable therapeutics for TB.

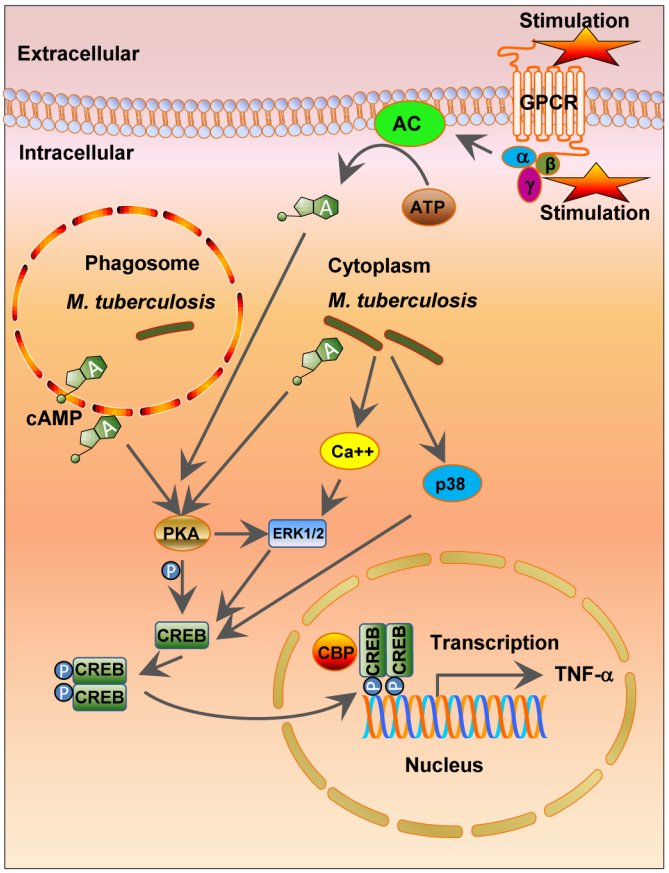


Figure 1. cAMP-mediated signaling in *M. tuberculosis* infection

Macrophages produce intracellular cAMP through G-protein-coupled receptor (GPCR)-adenylate cyclases (ACs). Increased cAMP stimulates protein kinase A (PKA) leading to the phosphorylation of cAMP response-element-binding protein (CREB), and subsequent transcriptional changes including modulation of cytokine expression. *M. tuberculosis* secretes cAMP directly into host macrophages leading to increased intracellular cAMP levels following infection. The *M. tuberculosis* infection-induced cAMP burst activates the PKA–CREB pathway leading to production of TNF-α, one of the key cytokines for TB granuloma formation. P = phosphate, TNF-a = tumor necrosis factor-a, CBP = CREB-binding protein, ERK1/2 = extracellular-signal-regulated kinase-1/2, p38 = p38 mitogen-activated protein (MAP) kinase.

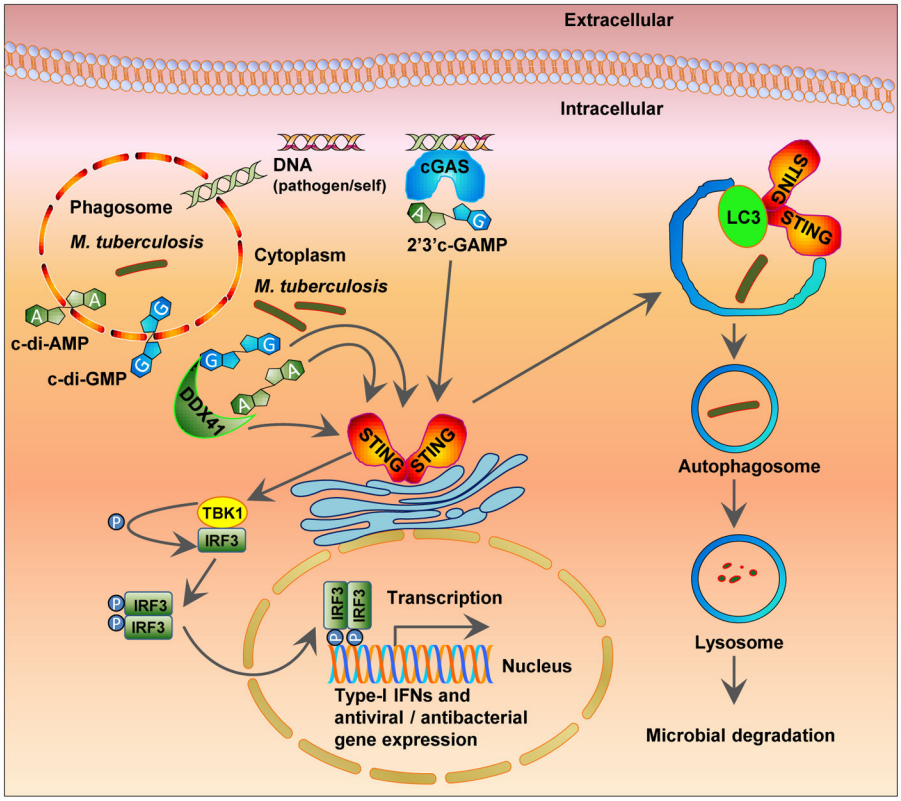


Figure 2. Cyclic dinucleotide signaling in *M. tuberculosis* infection

Cyclic dinucleotides (c-di-AMP and c-di-GMP) secreted by either phagosomal or cytoplasmic *M. tuberculosis* are detected by host cytoplasmic receptors DDX41 and STING. Receptor-bound DDX41 also interacts with and activates STING. Activated STING subsequently interacts with and activates kinase TBK1 leading to phosphorylation and dimerization of IRF3 which translocates into the nucleus and stimulates transcription of Type-1 IFN response genes. Activated STING also co-localizes with LC3, an autophagosome membrane component, and initiates autophagosome formation that ultimately leads to bacterial degradation. Correspondingly, bacterial DNA gains access to the cytosolic compartment and binds to the dsDNA receptor cyclic-GAMP synthase (cGAS) stimulating the synthesis of 2′,3′-cGAMP. Host-produced cGAMP binds to STING and stimulates a signaling cascade similar to that of the bacterial cyclic dinucleotides. P = phosphate, IFN = interferon, STING = stimulator of interferon genes, TBK1 = TANK-binding kinase 1, IRF3 = interferon regulatory factor 3.

Table 1

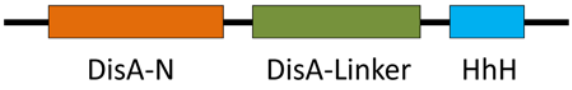
Host immune receptors involved in recognition of *M. tuberculosis* components

Host receptors[a]	*M. tuberculosis* component	Type of immune response/function	Reference
Cell surface receptors			
TLRs	LM, lipoprotein, PIMs	Pro-inflammatory	8, 12
CRs	C3-opsonized/non-opsonized bacteria, PIMs	Low levels of inflammation	12, 14, 15
MR	ManLAM, PIMs	Anti-inflammatory	8, 11, 12
DC-SIGN	ManLAM, LM, PIMs	Anti-inflammatory	8, 9, 10
Dectin, Mincle	Glycolipids, TDM	Anti-/Pro-inflammation	8, 12
Fc γRs	IgG-opsonzed bacilli	Phagolysosome fusion	8, 13
SR-A, CD14	non-opsonized bacteria	Anti-/Pro-inflammation	8, 12
Cytosolic receptors			
NOD	Peptidoglycan	Pro-inflammatory	55, 56
TLR9	CpG DNA	Pro-inflammatory	57, 58
DAI, cGAS	dsDNA	Type 1 IFN, pro- inflammatory	59, 64
AIM2, NLRP3	dsDNA	Inflammasome activation	65, 66
IFI16	ssDNA, dsDNA	Type 1 IFN, pro- inflammatory	60
STING	c-di-GMP, c-di-AMP, cGAMP	Type 1 IFN, pro- inflammatory	1, 2, 62, 67
DDX41	dsDNA, c-di-GMP, c-di-AMP	Type 1 IFN, pro- inflammatory	62, 63
RNA Pol III	AT rich dsDNA	Type 1 IFN, pro- inflammatory	61

[a]DAI, DNA-dependent activator of IFN-regulatory factors; AIM2, Absent in melanoma 2; IFI16, Interferon-inducible protein-16. See Abbreviations for other definitions.

Table 2

Cyclic di-nucleotide cyclases and phosphodiesterase of *M. tuberculosis*

Cyclic di-GMP		
Gene number/name	**Function**	**Domain composition**
Rv1354c	Cyclase- phosphodiesterase (bi- functional protein)	
Rv1357	Phosphodiesterase	
Cyclic di-AMP		
Rv3586 disA dacA	Cyclase	
Rv2837c NrnA cnpB	Phosphodiesterase	

Domain composition of cyclic di-nucleotide cyclases and phosphodiesterases are shown as predicted using the SSDB Motif (http://www.kegg.jp/kegg/ssdb/) and conserved domain (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi?db+cdd) databases. Gene nomenclature is according to the *M. tuberculosis* H37Rv genome annotation. Major functional domains: GGDEF, c-di-GMP cyclase; EAL, c-di-GMP phosphodiesterase; DisA-N, c-di-AMP cyclase; DHH, c-di-AMP phosphodiesterase.