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A bacterial cyclic dinucleotide activates the cytosolic surveillance pathway and mediates innate resistance to tuberculosis

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Abstract

Detection of cyclic-di-adenosine monophosphate (c-di-AMP), a bacterial second messenger, by the host cytoplasmic surveillance pathway (CSP) is known to elicit Type I interferon responses critical for antimicrobial defense[1–3]. However, the mechanisms and role of c-di-AMP signaling in *Mycobacterium tuberculosis* virulence remain unclear. Here we show that resistance to tuberculosis (TB) requires CSP-mediated detection of c-di-AMP produced by *M. tuberculosis* and that levels of c-di-AMP modulate the fate of infection. We found that a di-adenylate cyclase (*disA* or *dacA*)[4] over-expressing *M. tuberculosis* strain that secretes excess c-di-AMP activates the interferon regulatory factor (IRF) pathway with enhanced levels of IFN-β, elicits increased macrophage autophagy, and exhibits significant attenuation in mice. We show that c-di-AMP-mediated IFN-β induction during *M. tuberculosis* infection requires stimulator of interferon genes (STING)[5]-signaling. We observed that c-di-AMP induction of IFN-β is independent of the cytosolic nucleic acid receptor cyclic-GMP-AMP (cGAMP) synthase (cGAS)[6–7], but cGAS nevertheless contributes substantially to the overall IFN-β response to *M. tuberculosis* infection. In sum, our results reveal c-di-AMP to be a key mycobacterial pathogen associated molecular pattern (PAMP) driving host Type I IFN responses and autophagy. These findings suggest that modulating the levels of this small molecule may lead to novel immunotherapeutic strategies against TB.

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AUTHOR CONTRIBUTIONS

B.D. R.J.D. and W.R.B. designed the research. B.D., R.J.D and L.S.C. performed the experiments. H.G. contributed to mouse experiments. S.P. contributed to mouse, BMDM and DMDC experiments. J.H.L. contributed to LC-MS analysis. B.D., R.J.D. and W.R.B. analyzed the data and wrote the paper. W.R.B. provided overall supervision of the study.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

SUPPLEMENTARY MATERIALS

Supplementary Figures 1 to 10.

Supplementary Tables 1 to 4.

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The innate immune system is crucial for the early detection of invading pathogens and functions by recognizing specific PAMPs via germ line-encoded PRRs[8]. Downstream signaling following PRR and ligand binding leads to elicitation of innate immune responses and subsequent modulation of the adaptive responses thereby orchestrating a complex network of host defenses[8]. Cyclic di-nucleotides (CDNs), which are either produced by invading bacterial pathogens (such as, c-di-AMP and c-di-GMP) or produced endogenously in response to foreign DNA (cyclic GMP-AMP, cGAMP) represent one such category of small molecules that, during infection, act as triggers for innate immune responses leading to induction of Type I interferons (IFN-α/β)[2,6,9]. CDNs triggers Type I IFN induction through TLR-independent pathways[9] that include direct interaction with endoplasmic reticulum (ER) membrane protein STING or the cytosolic receptor DDX41, which complexes with STING followed by signal activation via phosphorylation of Tank binding kinase 1 (TBK1) and Interferon Regulatory Factor 3 (IRF3)[5,10]. While the potency of cytosolic signaling induced by CDNs and dsDNA appears comparable[9], the sensors for these molecules may be unique with minimal redundancy[10–11]. Of the several putative mammalian cytosolic DNA sensors that signal through STING[12], cyclic GMP-AMP synthase (cGAS) has recently been shown to be essential for DNA-mediated immune responses irrespective of cell type and nucleic acid sequence[11]. On detection of foreign nucleic acid, cGAS synthesizes a non-canonical CDN, cGAMP, which subsequently binds to STING leading to induction of Type I IFNs via the same signaling axis as that used by bacterial CDNs[11]. The production of a host-generated CDN in response to foreign nucleic acid may account for the apparent overlap of the responses to the structurally distinct ligands.

Like several pathogenic bacteria, the *M. tuberculosis* genome encodes a di-adenylate cyclase enzyme (*disA* or *dacA, Rv3586, MT3692*) which synthesizes c-di-AMP from ATP or ADP[4],. Orthologues of *disA* exist in all mycobacterial genomes with the exception of *M. leprae*. However, the role of c-di-AMP in *M. tuberculosis* physiology and mechanism of its interaction with the host immune system is poorly understood[13–14]. Evidence from other intracellular bacterial infections strongly suggests that both microbial DNA and CDNs stimulate the secretion of Type I IFNs[2,15]. However, the existing model for *M. tuberculosis* infection hypothesizes that extracellular mycobacterial DNA is the only ligand for CSP activation within macrophages, which leads to increased autophagy and bacterial clearance in an Esx-1 secretion system dependent manner excluding any role of bacterial CDNs in CSP activation[16–17]. Here we report that c-di-AMP, not cytosolic DNA alone, is a critical ligand for CSP activation leading to induction of Type I IFNs and autophagy during *M. tuberculosis* infection.

To confirm that *M. tuberculosis* produces and secretes c-di-AMP, we first detected and quantified bacterial c-di-AMP production in 7H9 broth culture and found considerable levels in both the intracellular and extracellular compartments by employing highly sensitive LC-MS/MS MRM methods (Supplementary Fig. 1a, b). We also observed that intracellular c-di-AMP levels increase during late-log and stationary phases of growth of *M. tuberculosis* compared to early log phase growth (Supplementary Fig. 1c). Further *M. tuberculosis*-produced c-di-AMP was also detected in the infected macrophage cytosol (Supplementary Fig. 1d). Next, we constructed a recombinant *M. tuberculosis* strain over-expressing its

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endogenous di-adenylate cyclase gene, *disA* (Mtb-OE) (Supplementary Fig. 2a, b). Analysis of the transcriptome by mRNA sequencing revealed a 95-fold over-expression of *disA* in the Mtb-OE strain compared to the *M. tuberculosis* CDC1551 wild type (WT) control strain (Supplementary Fig. 2c) with a resultant increase in the production of c-di-AMP by ~20 fold (Supplementary Fig. 2d). The absence of any c-di-AMP in the mutant bacterial strain (Supplementary Fig. 3) with a transposon insertion disrupting the di-adenylate cyclase domain of *disA* (JHU-3586, http://webhost.nts.jhu.edu/target/) confirms that c-di-AMP is produced by a single di-adenylate cyclase in *M. tuberculosis*. Complementation of the mutant bacteria with its endogenous *disA* gene and native promoter reconstituted c-di-AMP production (Mtb-COMP) (Supplementary Fig. 4).

To investigate whether perturbations of c-di-AMP levels in *M. tuberculosis* influence host Type I IFN responses, we infected J774.1 mouse macrophage cells with *M. tuberculosis* strains expressing different c-di-AMP levels, and measured IFN-β levels by ELISA at 24 hours post-infection. As shown in Fig. 1a, b, infection with the Mtb-*disA*-KO strain resulted in a significant reduction in IFN-β induction by J774.1 cells compared to infection with the Mtb-CDC1551. Conversely, infection with the Mtb-OE strain resulted in an enhanced induction of IFN-β by both resting and activated J774.1 cells. Notably, Mtb-OE infected cells also secreted significantly higher levels of TNF-α compared to the Mtb-WT infected cells, whereas Mtb-*disA*-KO infected cells produced lower TNF-α levels compared to other groups (Fig. 1c, d). Moreover, the patterns of IRF pathway activation in THP1-human monocyte cells (Supplementary Fig. 5) and IFN-β responses in mouse primary bone marrow derived macrophages (BMDM) and dendritic cells (BMDC) (Fig. 1e, f) were comparable further confirming our hypothesis. However, we noticed that mouse BMDCs are a comparatively better IFN-β producer than BMDMs in response to *M. tuberculosis* infection, which has also been reported by others[18]. Induction of IFN-β was further confirmed by real time RT-PCR of the BMDC cells infected with various *M. tuberculosis* strains (Fig. 1g). We also observed induction of significantly higher levels of pro-inflammatory cytokines including IL-1α, IL-6 and TNF-α by both BMDMs and BMDCs following infection with the c-di-AMP over-expressing *M. tuberculosis* strain (Supplementary Fig. 6). These observations suggest that perturbation of c-di-AMP levels in *M. tuberculosis* not only influences the CSP mediated Type I IFN response but also plays a critical role in modulating the pro-inflammatory cytokine signature of the infected cells.

Taking into account the ambiguous role of the Type I IFN response in host control of TB, we monitored the growth patterns of these *M. tuberculosis* strains in resting and IFN-γ/LPS activated J774.1 cells. While all *M. tuberculosis* WT and recombinant strains exhibited identical growth rates in 7H9 broth culture (Supplementary Fig. 7), the Mtb-OE strain exhibited significantly diminished intracellular growth compared with the other *M. tuberculosis* strains (Fig. 1h, i). We did not notice any growth attenuation of the Mtb-*disA*-KO strain. These observations reveal that over-expression of c-di-AMP by *M. tuberculosis* results in significant attenuation of the intracellular growth of the Mtb-OE strain.

In light of reports indicating that CDNs are potent autophagy inducers[16,19], we investigated whether enhanced macrophage autophagy might account for the attenuation of the c-di-AMP over-expressing *M. tuberculosis* strain by examining the auto-phagosome membrane

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specific marker LC3 in *M. tuberculosis* infected J774.1 cells. Fluorescence confocal imaging demonstrated a considerably higher percentage of cells (~15%) exhibiting LC3 puncta formation in the case of Mtb-OE infection compared to Mtb-CDC1551 (~10%) and Mtb-*disA*-KO infections (~6%) (Fig. 2a, b). In addition, Western blot analysis of endogenous LC3 revealed an increase in conversion of LC3-I to LC3-II in the Mtb-OE infected cells, indicating hyper-activation of autophagy (Fig. 2c). We also observed a considerably higher percentage of cells exhibiting pTBK1 positivity suggesting activation of IRF pathway[5,10] in the Mtb-OE infected J774.1 cells (Supplementary Fig. 8). These observations strongly suggest that hyper-induction of autophagy by macrophages may be one of the contributing factors that restricts the intracellular growth of Mtb-OE strain.

Next we examined the virulence and pathogenicity of the *M. tuberculosis* strains in mouse aerosol infection models. Remarkably, compared to WT infection (median time to death [MTD] of 150 days), we observed a significant increase in the survival of Mtb-OE infected mice (MTD 321 days). In contrast, the Mtb-*disA*-KO strain which is c-di-AMP deficient showed reduced survival with an MTD of 77 days (Fig. 3a). Concomitantly, the Mtb-OE strain also exhibited growth attenuation as evidenced by significantly reduced lung and spleen bacillary loads (Fig. 3b, c). Gross and histo-pathological findings of mouse lungs and spleens correlated well with the bacterial organ burden observations (Fig. 3d, e). The lungs of Mtb-OE infected-mice showed significantly fewer and smaller tubercle-like lesions compared to other groups. Concordantly, while Mtb-CDC1551, Mtb-*disA*-KO, and Mtb-COMP strain-infected mice exhibited considerable splenomegaly, spleens of the Mtb-OE infected mice appeared normal in size (Fig. 3d). Altogether, these observations clearly demonstrate attenuation of virulence in the c-di-AMP over-expressing *M. tuberculosis* strain.

Next, we compared the mouse serum cytokine levels between these groups at an early stage of disease: 2 weeks post-infection. Consistent with our *in vitro* studies in mouse and human cells, we observed increased IFN-β levels in the serum of Mtb-OE infected mice compared to the Mtb-CDC1551 and Mtb-*disA*-KO group (Fig. 3f). In addition, the Mtb-OE group exhibited significantly higher serum levels of TNF-α (Fig. 3g). Since Type I IFN (IFN-β) is known to counter-regulate Type II IFN (IFN-γ) responses[20], we measured IFN-γ levels in the serum of infected mice (Fig. 3h). We observed a strong inverse relationship between IFN-β and IFN-γ in these mice corresponding to the ability of the *M. tuberculosis*-strains to produce c-di-AMP.

Our studies reveal that bacterial c-di-AMP levels are strongly associated with the immunopathological outcome of *M. tuberculosis* infection in mice. Hence, we next examined the host cytosolic sensors that may detect *M. tuberculosis*-derived c-di-AMP starting with helicase DDX41, a cytosolic DNA and CDN receptor that signals via STING[10,21]. We performed shRNA-mediated knocked down of DDX41 using RAW-Blue™ ISG cells (InvivoGen) that allow colorimetric measurement of the induction of the IRF pathway. Knockdown of DDX41 caused a significant defect in activation of the IRF pathway and reduced IFN-β induction following infection with all the *M. tuberculosis* strains (Supplementary Fig. 9a, b, c). We also observed a significantly reduced TNF-α production by the DDX41 knock-down cells (Supplementary Fig. 9d). These results suggest

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that DDX41 is a key pattern recognition receptor for both DNA and c-di-AMP, and that DDX41 regulates the induction of Type I IFNs as well as TNF-α following *M. tuberculosis* infection.

We then investigated the contribution of STING to the c-di-AMP-mediated IFN-β response during *M. tuberculosis* infection. A partial knock-down of STING in human THP1 cells showed considerably lower IFN-β induction than control cells (Supplementary Fig. 10). Moreover, all *M. tuberculosis* strains failed to activate the IRF pathway or induce IFN-β in mouse RAW 264.7 macrophage IRF reporter cells lacking STING (STING-KO) (Fig. 4a, b, c, d). However, LPS, which stimulates Type I IFN through STING-independent pathways, induced elevated IFN-β response even in STING-KO cells (Fig. 4a, b). These results confirm that, in addition to its role in bacterial DNA mediated responses, STING is an essential component for c-di-AMP-mediated activation of the IRF pathway during *M. tuberculosis* infection[17]. Furthermore, infection of macrophages with *M. bovis* bacille Calmette–Guérin (BCG), which is known to lack the Esx-1 secretion system, also showed activation of the IRF pathway at levels 20–60% of those seen following infection with either the Mtb-CDC1551 or the Erdman strain, a WT *M. tuberculosis* strain considered to be highly virulent (Fig. 4a, b). Importantly, the c-di-AMP over-expressing *M. bovis* BCG strain (BCG-OE) produced a significantly higher IRF and IFN-β response than *M. bovis* BCG itself, thus strongly suggesting that bacterial-derived c-di-AMP gains access to the host cell cytosol despite the absence of an Esx-1 secretion system (Fig. 4a, b). These experiments indicate that, while contributory to overall type I IFN response, Esx-1 may not be essential for c-di-AMP-triggered IRF pathway activation. However, further studies with ESX-1 deleted *M. tuberculosis* strains may provide direct evidence for contribution of ESX-1 secretion system in c-di-AMP mediated responses during *M. tuberculosis* infection.

Next we evaluated the role of cGAS in the detection of bacterial c-di-AMP. We infected primary BMDMs (Fig. 4e) and BMDCs (Fig. 4f) from WT and cGAS-KO mice[22] with these mycobacterial strains and measured IFN-β protein levels. While loss of cGAS resulted in a considerably reduced IFN-β response compared to cells with intact cGAS (WT), all c-di-AMP overproducing strains continued to show significantly higher induction of IFN-β in cGAS-KO cells compared to their respective WT mycobacterial strains (Fig. 4e, f). Further, both WT and cGAS-KO BMDMs produced comparable levels of IFN-β following stimulation with synthetic c-di-AMP (Fig. 4g). Real time RT-PCR for IFN-b in BMDCs further confirmed these results (Fig. 4h). These experiments show that while c-di-AMP is a key ligand for IFN-β induction irrespective of cGAS, a significant part of the overall IFN-b response during *M. tuberculosis* infection is cGAS dependent and hence is probably due to bacterial DNA.

Our data thus reveal the involvement of c-di-AMP as an *M. tuberculosis* PAMP that triggers host cell IFN-β secretion and autophagy. These observations expand upon earlier studies which suggested that mycobacterial DNA is the exclusive ligand for inducing the Type I IFN response and that the bacterial Esx-1 secretion apparatus is required for CSP activation[17]. Our findings, which employed 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

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CSP (STING, DDX41, and cGAS), consistently demonstrated that c-di-AMP, not bacterial DNA alone, is a key mediator of Type I IFN responses. Supplementary Table 1 lists major differences in our methods compared with those of earlier studies and reveals that strain, host cell, and methodological differences may have allowed the importance of c-di-AMP to have been overlooked in earlier studies.

We considered the possibility that c-di-AMP-mediated changes in other bacterial antigens might affect Type I IFN response through NOD signaling as reported by Pandey *et al.*[23]. However, a recent study demonstrated that the enhanced Type I IFN response caused by accumulation of c-di-AMP in a c-di-AMP phosphodiesterase mutant of *M. tuberculosis* is independent of NOD signaling[24]. This report thus further supports our observations and highlights induction of Type I IFN response by c-di-AMP. Moreover, several publications have also demonstrated that NOD signaling is dispensable for IRF activation during *M. tuberculosis* infection[17]. Thus, we feel that indirect effects of c-di-AMP on other bacterial PAMPs are unlikely to account for the IFN-β responses we observe. Moreover, absence of any difference in the intra-bacterial cAMP level amongst different *M. tuberculosis* strains further ruled out any off target effect of perturbed *disA* expression or c-di-AMP levels on cAMP-a known inducer of TNF-α[25].

The enhanced Type I IFN response consequent to over-production of c-di-AMP observed in this study significantly extends correlative observations in several studies with laboratory as well as clinical isolates of pathogenic bacteria[24,26–27]. While Type I IFNs are absolutely critical for resistance to viruses, there are conflicting reports as to whether the IFN-α/β response is beneficial or detrimental to the host during tuberculosis[28–29]. For example, loss of the IFN-α receptor in knockout mice confers resistance to tuberculosis suggesting that Type I IFN responses are counterproductive in TB[30]. In contrast, IFNα/β responses have been shown to enhance antigen cross-presentation in DCs and activation of cytolytic CD8 T cells, which are critical for *M. tuberculosis* clearance[31–32], and the Type I IFN response has also been shown to limit lung infectivity of *M. tuberculosis* in animals devoid of Type II IFN[33]. Importantly, human TB transcriptome analysis of peripheral blood also revealed upregulation of both Type I and Type II IFN gene profiles suggesting overlapping and dynamic roles of both types of IFN in TB pathogenesis[34]. Beyond induction of Type I IFN, our study also found enhanced induction of several pro-inflammatory cytokines including IL1-α, TNF-α and IL-6 which are believed to play protective roles during *M. tuberculosis* infection[35].

Autophagy is increasingly appreciated as a key mechanism by which macrophages kill intracellular *M. tuberculosis*[36], and autophagy inducers such as Vitamin D have also been identified as potential candidates for the treatment of HIV-TB co-infection[37]. Indeed, certain first-line TB drugs, isoniazid (INH) and pyrazinamide (PZA), may act in part by stimulating autophagy[38]. Mounting evidence also suggests that mycobacterial DNA[17], bacterial CDNs such as c-di-AMP[16,19], and induction of the Type I IFN[39] response itself are inducers of autophagy. Our observation of CDN-activation of IRF signaling and autophagy is consistent with the emerging appreciation of crosstalk between these two pathways.

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While altered virulence and antibiotic resistance has been associated with mutation in c-di-AMP cyclase and phosphodiesterase genes of several bacterial pathogens[28], modulation of bacterial c-di-AMP levels has yet to be linked to variables in the clinical presentation of human TB. However, differences in the induction of IFN-β by clinical isolates of *M. tuberculosis*[40] and the presence of mutations in the adenylate cyclase (Rv3586) as well as phosphodiesterase (Rv2837c) genes in clinical strains of *M. tuberculosis* (http://www.tbdb.org/) suggest a potential association of c-di-AMP levels with forms of human TB disease. This study revealing that c-di-AMP produced by *M. tuberculosis* is a PAMP that modulates Type I IFN response during infection may prompt future correlative human studies.

Our findings highlight the importance of c-di-AMP detection in the host's innate resistance to TB and reveal a previously unappreciated mechanism of CSP activation by bacterial CDNs during *M. tuberculosis* infection. The ability of a small molecule, c-di-AMP, to govern the outcome of intracellular infection suggests novel approaches for the treatment and prevention of TB as well as related diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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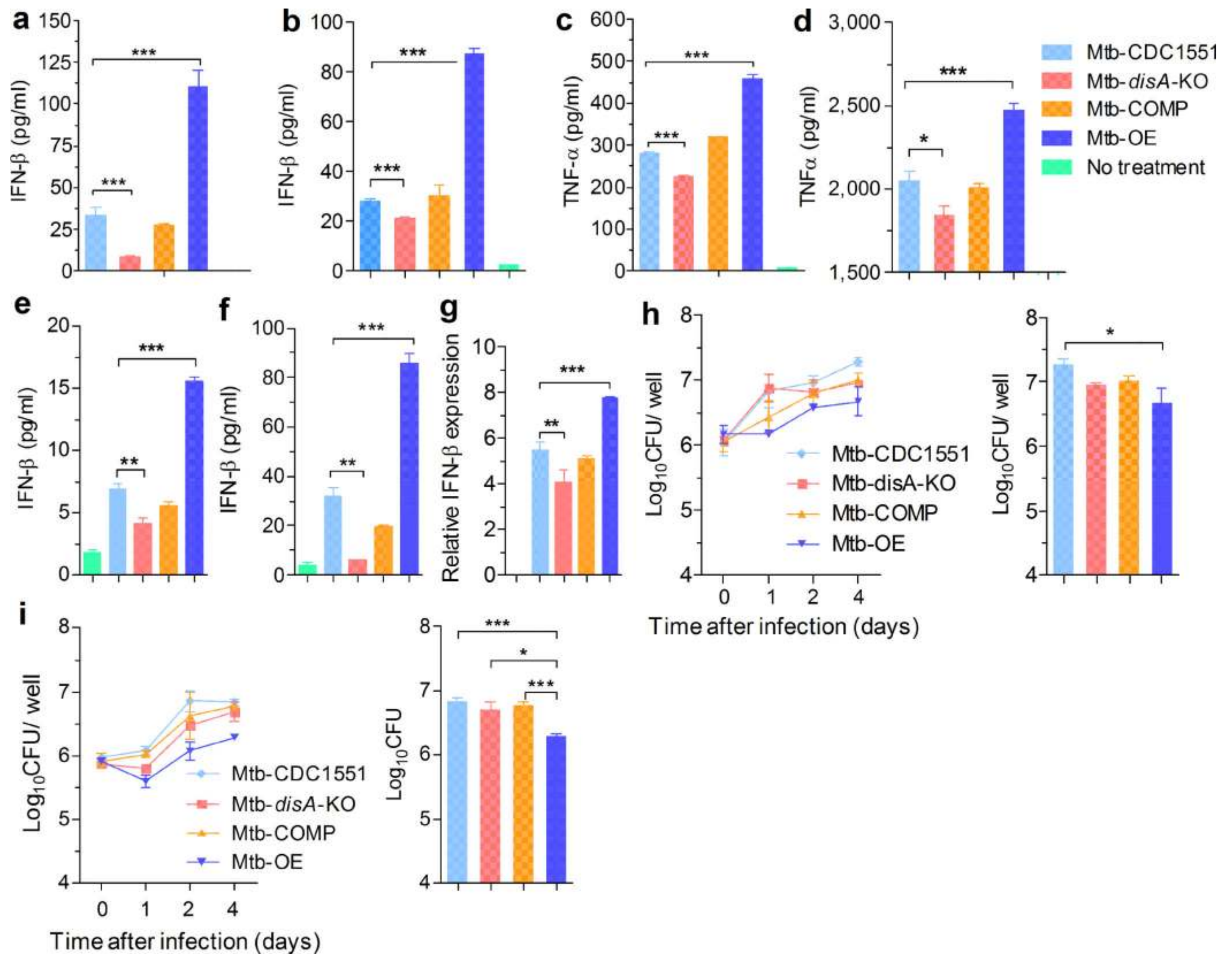

Figure 1. Modulation of host cytokine response and intracellular growth of *M. tuberculosis* by c-di-AMP

(a) Levels of IFN-β in culture media at 24h post-infection from resting and (b) IFN-γ/LPS activated J774.1 cells infected with various Mtb-strains at an MOI of 1:20. (c) Levels of TNF-α in culture media at indicated time points from resting and (d) IFN-γ/LPS activated J774.1 cells infected with various Mtb-strains at an MOI of 1:20. (e) Levels of IFN-β in culture media at 24h post-infection from BMDM and (f) BMDC cells infected with various Mtb-strains at an MOI of 1:10. ELISA Data are mean ± SE of at the least three experiments (n = 3). *, $p < 0.05$; **, $p < 0.01$ and ***, $p < 0.001$ by One-way ANOVA with Tukey's post test. (g) IFN-β mRNA were assessed by qRT-PCR in BMDCs infected with various Mtb-strains at 24h post-infection; data are mean ± SD (n = 3) and representative of two experiments. **, $p < 0.01$ and ***, $p < 0.001$ by One-way ANOVA with Tukey's post test. (h) Growth kinetics of various Mtb-strains in resting and (i) IFN-γ/LPS activated J774.1 cells. Data are Mean CFUs ± SD at each time point (n = 3) and representative of two experiments. Bar diagrams (right panels in h and i) represent Mean CFUs ± SD at Day 4. *, $p < 0.05$ and ***, $p < 0.001$ by Student's t-test (2-tailed).

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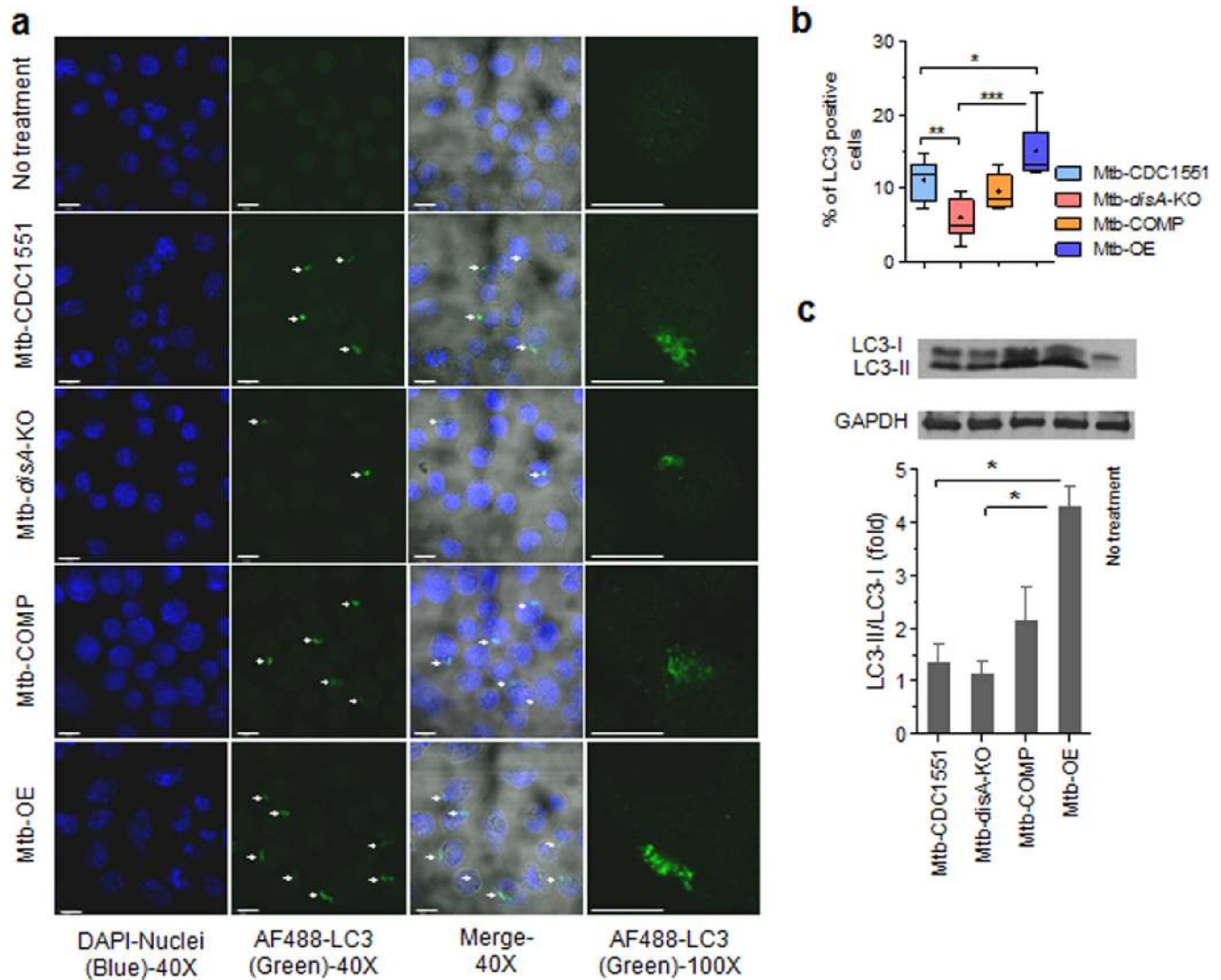

Figure 2. c-di-AMP produced by *M. tuberculosis* induces autophagy in macrophage cells

(a) Fluorescence confocal images of J774.1 cells, fixed after 6 hr of infection with various *M. tuberculosis* strains and stained with anti-LC3 antibody; Nuclei-Blue (DAPI), LC3b-Green (AF488). Scale bars depicts 20 μm for 40× images and 10 μm for 100× images. (b) Quantitative analysis of LC3 positive J774.1 cells showing puncta formation. Only those cells were considered as positive and included for quantification, which exhibited formation of large LC3 aggregates occupying area > 1μm, Percentage of LC3-II positive cells were calculated and data are depicted by box plot indicating Mean (+), Median (−) with quartiles (box margins) and ranges (bars) (n = 9). *, p < 0.05; **, p < 0.01 and ***, p < 0.001 by One-way ANOVA with Tukey's post test. (c) Western blot analysis of LC3-I and LC3-II and GAPDH (loading control) of J774.1 cells at 6 hr after infection along with bar diagram depicting densitometric ratios of normalized LC3-II/LC3-I levels. Data are mean ± SD (n = 2) from two experiments.*, p < 0.05 by Student's t-test (2-tailed).

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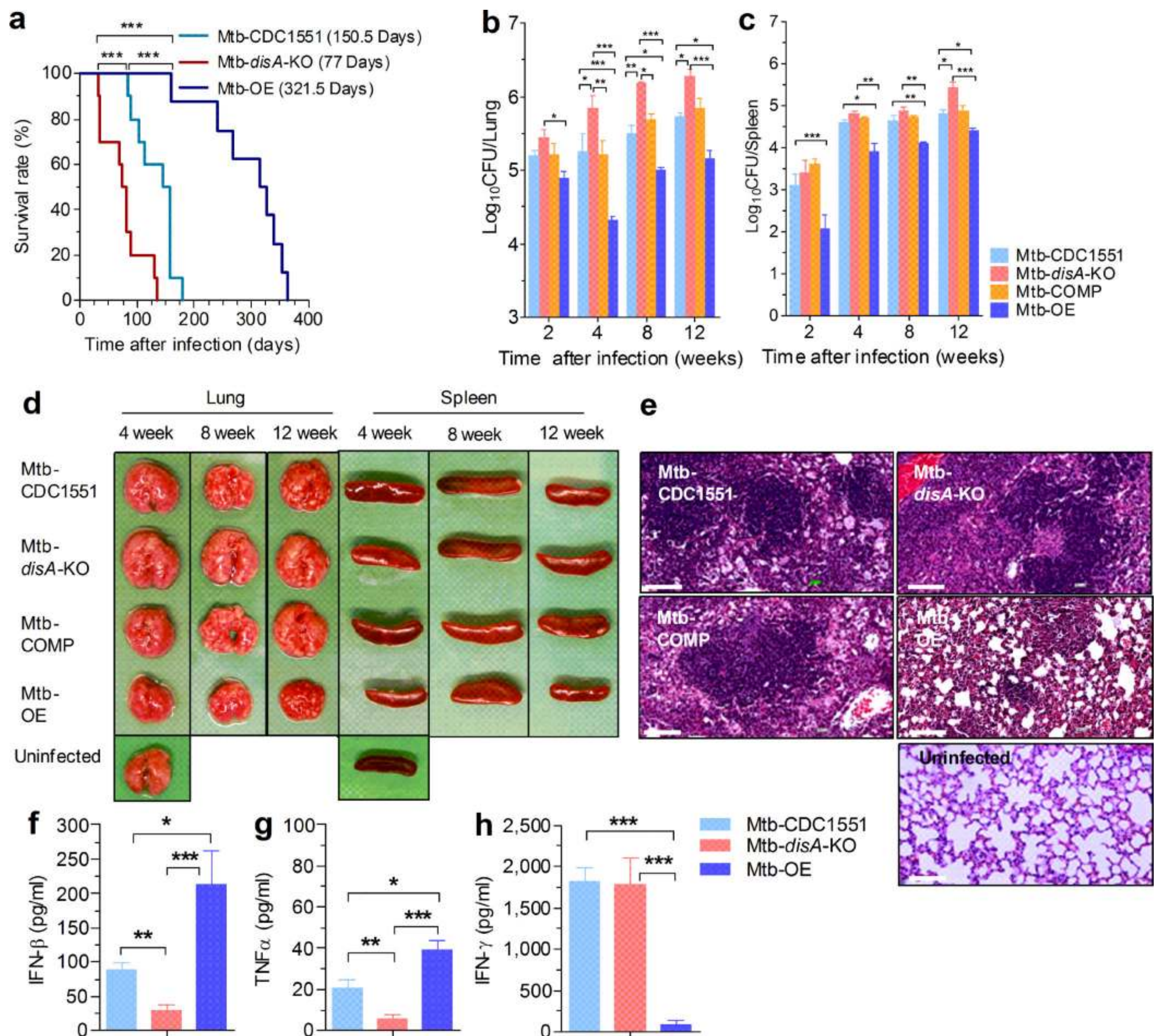

Figure 3. Attenuation of virulence and pathogenicity in c-di-AMP over-producing *M. tuberculosis* strain

(a) Survival of mice (n = 10) following infection with various Mtb-strains. ***, $p < 0.001$ by Log-rank (Mantel-Cox) test. (b) Growth kinetics of various *M. tuberculosis*-strains in mouse lungs and (c) spleen after aerosol infection. Data are mean ± SE (n = 4). *, $p < 0.05$; **, $p < 0.01$ and ***, $p < 0.001$ by Two-way ANOVA with Bonferroni post-test. (d) Gross and (e) histo-pathological features of lungs and spleen of mouse infected with various *M. tuberculosis*-strains. Scale bar is 100 μm. (f) Levels of IFN-β, (g) TNF-α and (h) IFN-γ in the serum of mice infected with *M. tuberculosis*-strains possessing varied ability to produce c-di-AMP. Data are mean ± SE (n = 4). *, $p < 0.05$; **, $p < 0.01$ and ***, $p < 0.001$ by Student's t-test (2-tailed).

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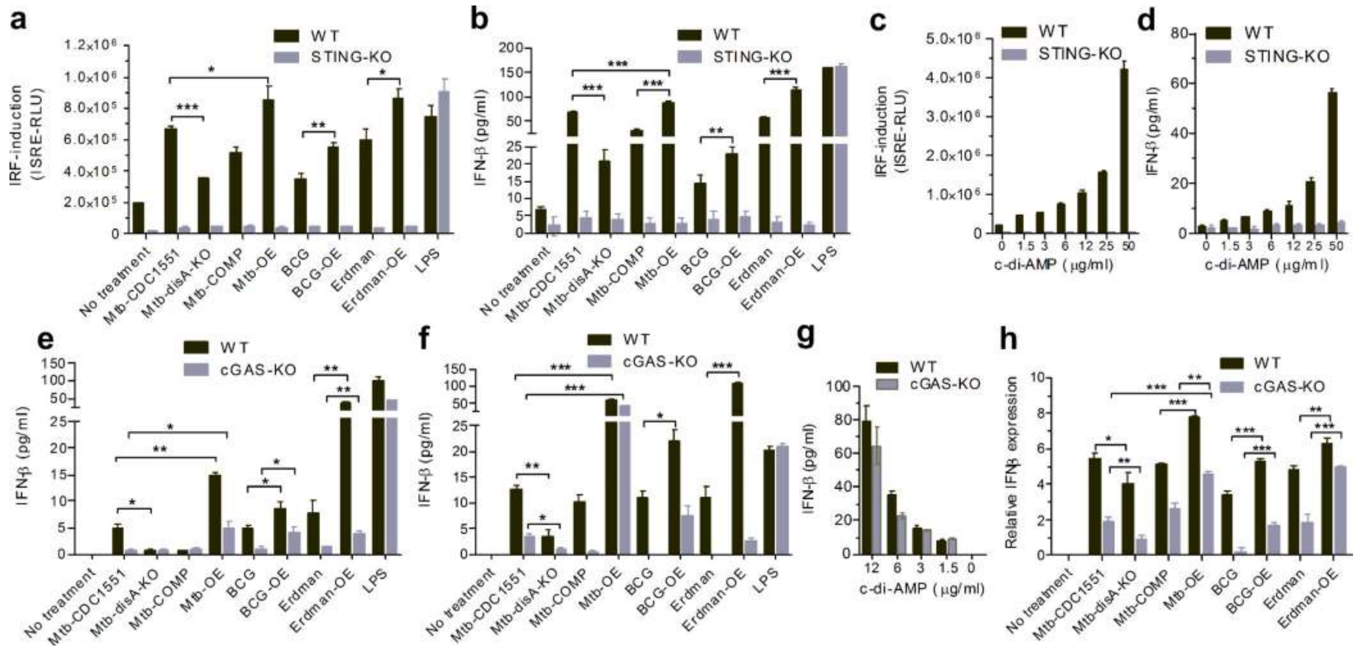

Figure 4. Contribution of STING and cytosolic DNA receptor cGAS to c-di-AMP mediated activation of IFN-β during *M. tuberculosis* infection

(a, c) IRF pathway activation as measured by luciferase reporter assay and (b, d) IFN-β levels in the 18h post-infection (MOI = 1:5) and post-stimulation culture supernatants of mouse RAW264.7 derived STING ablated [STING-KO] and control [WT] macrophage IRF reporter cells. (e) IFN-β induction in BMDMs and (f) BMDCs from control [WT] and cGAS ablated [cGAS-KO] mouse following infection (MOI = 1:10) with various *Mycobacterium* strains. (g) c-di-AMP concentration dependent induction of IFN-β in mouse BMDMs. Data are mean ± SE of at the least three experiments (n = 4 in a, b; n = 3 in c, d, e, f, g).*, $p < 0.05$; **, $p < 0.01$ and ***, $p < 0.001$ by Student's t-test (2-tailed). (h) Levels of *IFN*-β mRNA were determined by *real-time RT-PCR* in BMDCs derived from wild type cGAS sufficient [WT] and cGAS ablated [cGAS-KO] mouse following infection (MOI = 1:10) with various *Mycobacterium* strains. The IFN-β mRNA expression levels were normalized to β-actin expression and are represented relative to those of untreated cells. Data are mean ± SD (n = 3) and is representative of two experiments. *, $p < 0.05$; **, $p < 0.01$ and ***, $p < 0.001$ by Student's t-test (2-tailed).