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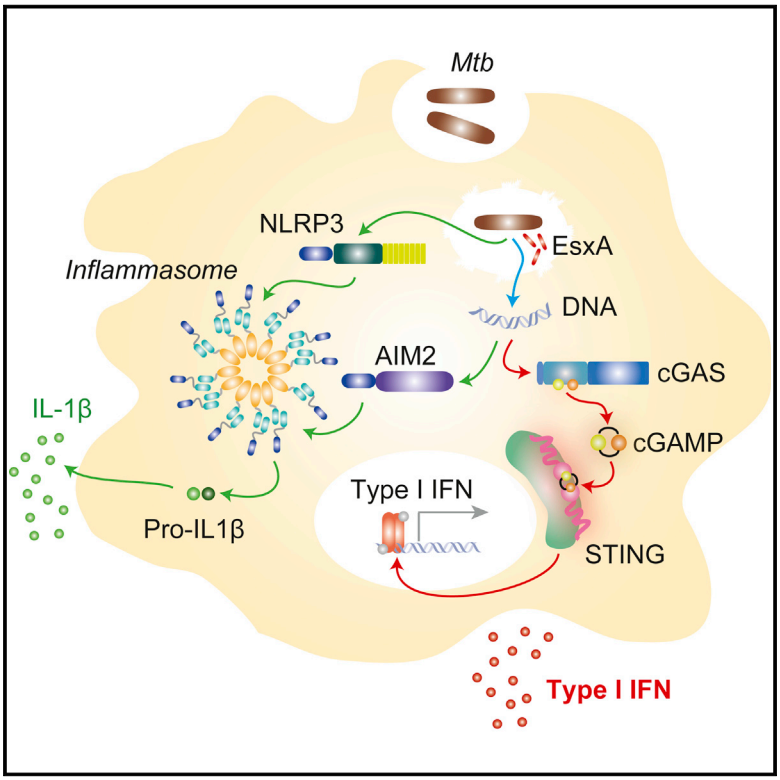
Mycobacterium tuberculosis Differentially Activates cGAS- and Inflammasome-Dependent Intracellular Immune Responses through ESX-1

Graphical Abstract



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In Brief

The receptor(s) responsible for type I IFNs activation in response to *Mycobacterium tuberculosis* (*Mtb*) infection has remained elusive. Wassermann et al. find that intracellular *Mtb* DNA sensing by the cytosolic sensor cGAS drives type I IFN induction upon infection, and mutations in the *Mtb* ESX-1 secretion system abrogate this mechanism.

Highlights

- The DNA sensor cGAS is essential for type I IFN induction in response to *Mtb* infection
- cGAS aggregates and colocalizes with DNA in the cytosol of *Mtb*-infected cells
- *Mtb* ESX-1 secretion system mutants abrogate cGAS-mediated type I IFN responses
- Modulation of the ESX-1 system uncouples IFN production from IL-1β responses to *Mtb*

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Mycobacterium tuberculosis Differentially Activates cGAS- and Inflammasome-Dependent Intracellular Immune Responses through ESX-1

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SUMMARY

Cytosolic detection of microbial products is essential for the initiation of an innate immune response against intracellular pathogens such as *Mycobacterium tuberculosis* (*Mtb*). During *Mtb* infection of macrophages, activation of cytosolic surveillance pathways is dependent on the mycobacterial ESX-1 secretion system and leads to type I interferon (IFN) and interleukin-1β (IL-1β) production. Whereas the inflammasome regulates IL-1β secretion, the receptor(s) responsible for the activation of type I IFNs has remained elusive. We demonstrate that the cytosolic DNA sensor cyclic GMP-AMP synthase (cGAS) is essential for initiating an IFN response to *Mtb* infection. cGAS associates with *Mtb* DNA in the cytosol to stimulate cyclic GAMP (cGAMP) synthesis. Notably, activation of cGAS-dependent cytosolic host responses can be uncoupled from inflammasome activation by modulating the secretion of ESX-1 substrates. Our findings identify cGAS as an innate sensor of *Mtb* and provide insight into how ESX-1 controls the activation of specific intracellular recognition pathways.

INTRODUCTION

Tuberculosis (TB) is a major cause of morbidity and mortality worldwide (Lechartier et al., 2014). Upon infection with *Mycobacterium tuberculosis* (*Mtb*), several factors contribute to the outcome of the disease, with the innate immune response representing one of the most critical determinants. To date several cytokines have been shown to participate in the innate host response against *Mtb*, where they can either function to confer host resistance or act as regulatory molecules that may exacerbate the infection (O'Garra et al., 2013). Studies have established the critical role of inflammatory cytokines, such as interleukin-1β (IL-1β), in the containment of *Mtb* by enhancing the antimicrobial function of macrophages (Fremond et al., 2007). On the contrary, animal models and studies in humans have revealed that type I interferons (IFNs) have probacterial activity and are associated with disease progression in TB (Berry et al., 2010; Manca et al., 2005; Stanley et al., 2007). The immunomodulatory effect of type I IFNs appears to be related to their anti-inflammatory properties, principally by antagonizing the production and activity of IL-1β (Mayer-Barber et al., 2011).

A key feature of pathogenic mycobacteria is the type VII secretion system ESX-1 that manipulates innate immune responses, presumably by translocating bacterial effector molecules into the host cytosol (Stoop et al., 2012). In macrophages intracellular detection of *Mtb*-associated molecular patterns is regulated via two major sensing systems that are linked to distinct signaling pathways. One pathway involves the multimeric inflammasome complex, which employs a sensor molecule, NLRP3 or AIM2, the adaptor ASC, and caspase-1 to regulate the secretion of IL-1β (Mishra et al., 2010; Saiga et al., 2012). Whereas AIM2 recognizes DNA, the NLRP3 inflammasome is stimulated by a mechanism involving K^+ efflux (Dorhoi et al., 2012). The second pathway, which is responsible for the expression of type I IFNs, relies on the cyclic dinucleotide (CDN) sensor STING (Manzanillo et al., 2012). Within this pathway, activation of STING by CDNs recruits the kinase TBK-1, which phosphorylates the transcription factor IRF-3 to promote transcription of IFN-β and interferon-stimulated genes (ISGs). Notably, during intracellular bacterial infection activation of STING can be accomplished via two differential mechanisms. First, STING can directly recognize bacterial CDNs and thus function as a primary pattern recognition receptor (PRR) (Burdette et al., 2011). Alternatively, DNA sensing via cGAS triggers the synthesis of the second messenger cGAMP, which then engages STING as a secondary receptor (Sun et al., 2013). But despite these advances in understanding the downstream part of these signaling cascades, the molecular events that lead to ESX-1-dependent activation of intracellular host receptors and the nature of the stimulatory elements remain poorly defined.

Here we report that the DNA sensor cGAS is essential for mounting type I IFN responses upon *Mtb* infection. We show that ESX-1-proficient mycobacteria trigger cGAS to form intracellular complexes that colocalize with DNA and promote

intercellular signal transduction via the production of cGAMP. Remarkably, by comparing the cytokine profile induced by distinct ESX-1 mutants, we observed that intracellular inflammasome-dependent and cGAS-mediated responses can be disconnected. Together, these data reveal the importance of cGAS in controlling interferon production upon mycobacterial infection and uncover a unique function of ESX-1 in eliciting specific innate immune responses.

RESULTS

cGAS Is Critical for IFN Responses upon *Mtb* Infection

In order to investigate the individual role of cytosolic PRRs during infection with *Mtb* in human cells, we employed human THP-1 cells, in which STING was knocked out via the CRISPR/Cas9 technology (see Figure S1A online). Similar to what had previously been shown in murine macrophages, we found that *Mtb*-triggered production of IP-10, a surrogate cytokine for type I IFN expression, was completely compromised in human THP-1 cells lacking STING (Figure S1B). The same was observed when gene expression of IFN-β was directly analyzed by qPCR (Figure S1C). We furthermore noted that transfection of *Mtb* genomic DNA regulated the induction of IP-10 via STING (Figure S1D). Recently, Rv3586 was identified as a mycobacterial diadenylate cyclase, DacA, which synthesizes cyclic di-AMP (Bai et al., 2012). Overexpression of DacA in conjunction with STING in HEK293T cells induced robust transactivation of an IFN-β reporter gene (Figure S1E). This effect was abolished when endogenous cyclic di-AMP levels were reduced by the activity of the cyclic di-AMP-specific phosphodiesterase YybT (Figure S1F) (Rao et al., 2010). In line with this, DacA was unable to activate IFN-β upregulation when coexpressed with a STING variant that cannot sense cyclic di-AMP (data not shown). Finally, purified cyclic di-AMP also triggered IP-10 production in THP-1 cells in a STING-dependent fashion (Figure S1G). Together, these data confirm STING-mediated DNA/CDN sensing in human macrophages, but they also illustrate that during *Mtb* infection both mycobacterial cyclic di-AMP and genomic DNA may serve as the molecular pattern that initiates type I IFN induction.

To definitively clarify the role of DNA- versus CDN-mediated IFN regulation in *Mtb*-infected cells, we employed THP-1 cells knocked out for cGAS (Figure 1A). Using these cell lines we examined the role of cGAS in the innate recognition of *Mtb*. As expected, cGAS knockout cells did not activate a type I IFN response upon transfection with dsDNA (Figure 1B). As controls, cGAS knockout cell lines were not compromised in their response toward the RIG-I ligand triphosphate RNA or the STING agonists cGAMP or cyclic di-AMP (Figures 1B, S1H, and S1J and data not shown). Importantly, when infected with *Mtb*, cGAS knockout cells failed to mount a detectable level of IP-10 and IFN-β production (Figures 1C and S1K). Moreover, gene expression of IFN-β or the ISG IFIT2 was also completely absent in cGAS mutant cells after *Mtb* challenge (Figure 1D). To explore the relevance of cGAS for the immune response in murine cells, we infected bone marrow-derived macrophages (BMDMs) from *cGAS*$^{-/-}$ mice and compared their type I IFN response with that of wild-type macrophages. Whereas comparable levels of IP-10 and IFN-β were induced after stimulation with triphosphate RNA or cGAMP, BMDMs derived from *cGAS*$^{-/-}$ animals were almost completely unresponsive in terms of IP-10 and type I IFN production upon *Mtb* infection (Figures 1E and S1I). To provide definitive proof of the involvement of cGAS in the innate response to *Mtb*, we reconstituted cGAS knockout cells with a human Flag-tagged cGAS construct via lentivirus-mediated transduction, whereas a Flag-tagged GFP construct served as a control. In cells transduced with the cGAS construct, cGAS protein expression was restored to a level comparable to that of wild-type cells (Figure 1F). Consistently, when transfected with dsDNA, reconstituted THP-1 cells produced comparable levels of IP-10 and displayed similar induction of IFN-β or IFIT2 as wild-type cells (Figure 1G and data not shown). Importantly, expression of cGAS-Flag but not GFP-Flag restored the ability of cGAS-deficient cells to respond to *Mtb* infection (Figure 1G). To assess the biological role of the cGAS-STING-IFN-β pathway on *Mtb* virulence, we compared the cytotoxicity caused by two different *Mtb* strains, H37Rv and HN878, on wild-type, cGAS-deficient, and STING-deficient cell lines. HN878 is a clinical isolate belonging to the East Asia (W-Beijing) family, while H37Rv is a member of the Euro-American lineage (Reed et al., 2007). Notably, HN878 has been described as capable of inducing higher levels of type I IFN in the murine model of infection (Manca et al., 2005). Testing these strains confirmed the increased production of IP-10 by wild-type cells after HN878 challenge, while deletion of cGAS or STING abolished this response (Figure S2A). We observed that cell survival was partially rescued by cGAS and STING knockout cells upon HN878 infection (Figure S2B). Together, these results demonstrate that cGAS is crucial for the innate type I IFN response in both human and murine macrophages infected with *Mtb*. In addition, these findings suggest that dsDNA but not CDNs functions as the mycobacteria-associated pattern required for IFN regulation.

cGAS Associates with DNA in the Cytosol

To further characterize the molecular mechanism of *Mtb*-driven cGAS activation, we examined the localization of cGAS in THP-1 cells by immunofluorescence microscopy. In untreated cells cGAS was distributed diffusely throughout the cytoplasm (Figure 2A). After transfection with dsDNA we observed cGAS forming punctate structures that colocalized with DNA (Figure 2A). Interestingly, the formation of these structures was specifically induced upon stimulation with DNA, but not after transfection with the endogenous cGAS enzymatic product cGAMP or triphosphate RNA (Figure S3). Strikingly, upon infection with *Mtb* we observed significant formation of cGAS aggregates, which colocalized with DNA (Figure 2A). In contrast, cells infected with an attenuated *Mtb* strain, H37RvΔRD1, which lacks IFN-stimulatory capacity due to loss of ESX-1 (see below), failed to stimulate cGAS punctate structure formation (Figure 2A). In addition, we found that pretreatment of cells with chloroquine, an inhibitor of autophagy, interfered with both DNA- and *Mtb*-triggered cGAS redistribution, suggesting the involvement of the autophagy machinery for the execution of the cGAS relocalization process (Figure 2B). In accordance with this notion and as previously described, the observed cGAS punctate structures colocalized with the autophagy-related protein Beclin-1, but not with the inflammasome complex (Figure 2C) (Liang et al.,

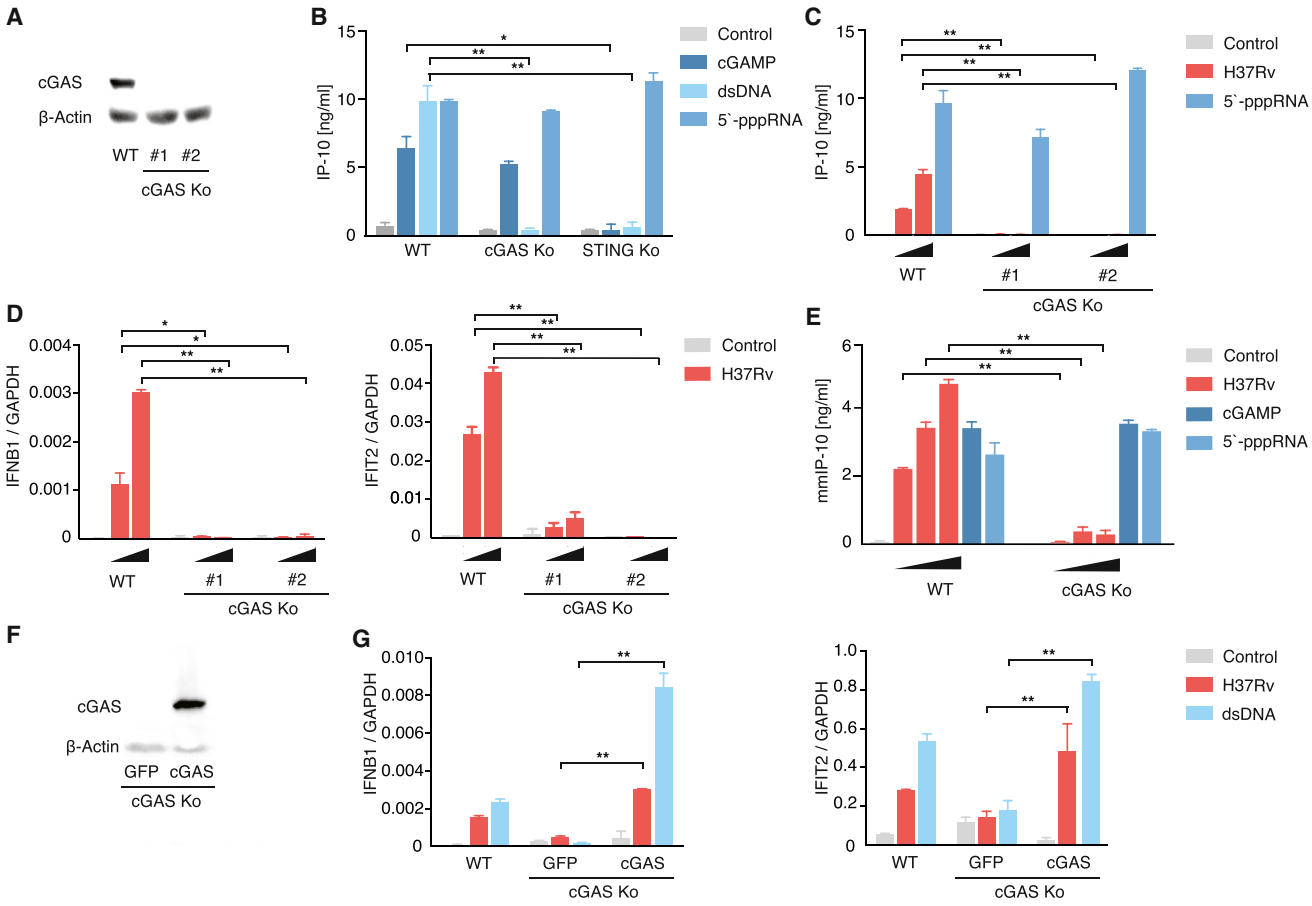

Figure 1. cGAS Is Essential for Type I IFN Responses Triggered by *Mtb*

(A) Expression of cGAS was studied in wild-type (WT) THP-1 cells and two distinct cGAS knockout cell lines by immunoblot.

(B) WT THP-1 cells, cGAS, and STING knockout cells were transfected with cGAMP, dsDNA, or 5′-triphosphate RNA (5′-pppRNA). IP-10 production was measured by ELISA 24 hr after stimulation.

(C and D) WT THP-1 cells and cGAS knockout cells were infected with H37Rv (multiplicity of infection [moi] 5, 10) or stimulated with 5′-pppRNA as indicated. After 24 hr IP-10 levels were assessed by ELISA (C) or IFN-β and IFIT2 mRNA levels were quantified by qPCR (D).

(E) ELISA measurement of IP-10 from the supernatants of BMDMs from WT mice and cGAS knockout mice after overnight culture left untreated, infected with H37Rv (moi 2.5, 5, and 10) or stimulated with cGAMP or 5′-pppRNA.

(F) Immunoblot of reconstituted cGAS-deficient cell lines (cGAS). Transduction of GFP served as a control (GFP).

(G) WT THP-1 cells and cells from (F) were left untreated, infected with H37Rv (moi 10) or transfected with dsDNA. After 24 hr mRNA levels of IFN-β and IFIT2 were measured by qPCR.

Mean + SD of duplicate measurements from one representative experiments out of four (B), three (C), or two (D, E, and G) independent experiments are depicted, or representative results from two independent experiments are shown (A and F). $p < 0.05$ (*); $p < 0.01$ (**). See also Figures S1 and S2.

2014). Together, this shows that infection of macrophages with *Mtb* leads to cytosolic association of cGAS and DNA.

Dissemination of the *Mtb*-Triggered Host Response through cGAMP-Based Intercellular Communication

Next we assessed whether *Mtb* infection leads to the synthesis of cGAMP within human macrophages. Toward this goal we took advantage of the fact that cGAMP can activate bystander cells via its horizontal transfer through gap junctions (Ablasser et al., 2013b). We hypothesized that, even though individual cGAS or STING knockout cells are unable to upregulate type I IFNs in a cell-intrinsic fashion, the combination of both would restore this response upon DNA transfection or *Mtb* infection if cGAMP were produced (Figure 3A, left, and Figure 3C, left). Indeed, when activated by transfection with dsDNA, monocultures of both cGAS- or STING-deficient cells failed to elicit type I IFN induction, yet the combination of both cell types resulted in marked upregulation of IFN-β or IFIT2 gene expression (Figure 3A, right, and data not shown). Consistent with a gap junction-dependent mode of transfer, this effect was abolished upon pretreating the mixed cocultures with the connexin inhibitor carbenoxolone (Figure 3B). We then tested whether cGAMP-dependent in *trans* signaling would also occur in the context of *Mtb* infection (Figure 3C, left). Similar to the results obtained above, coculturing of cGAS- or STING-deficient cells led to strong upregulation of IFN-β mRNA levels and of IFN-β secretion (Figure 3C, right, and Figure 3E). Again, this phenomenon was sensitive to carbenoxolone treatment

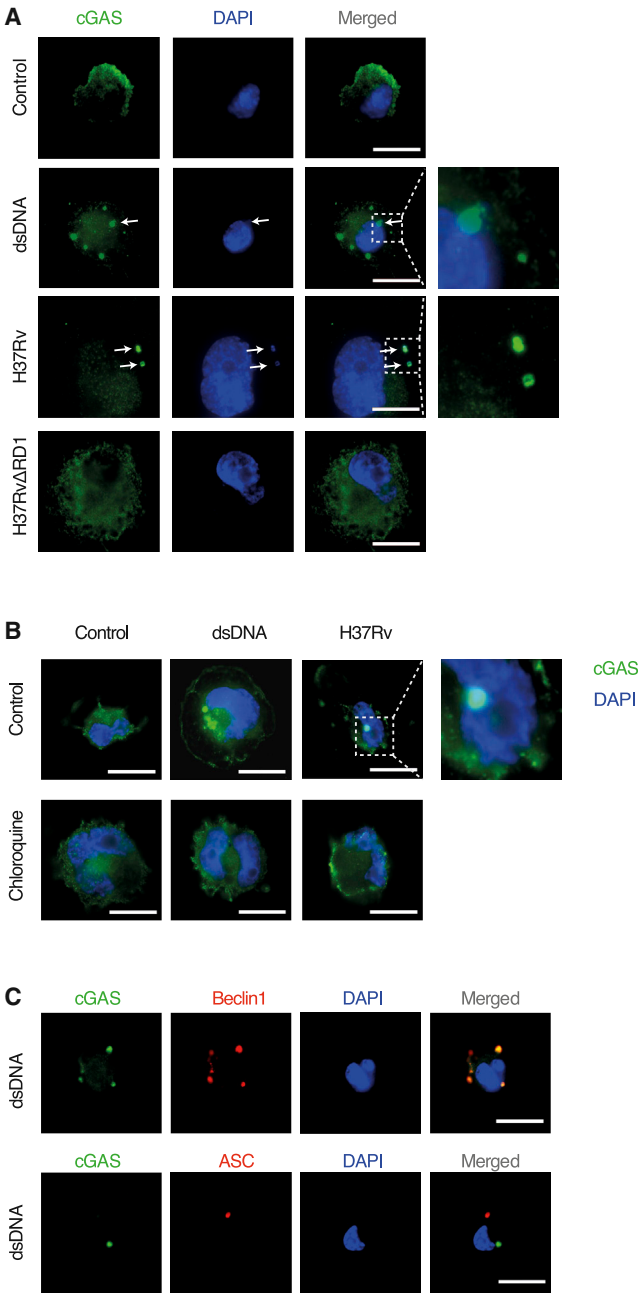

Figure 2. Upon *Mtb* Infection cGAS Forms Aggregates and Colocalizes with DNA

(A and B) THP-1 cells stably expressing cGAS-Flag were seeded on coverslips and treated as depicted. Sixteen hours after dsDNA transfection or 48 hr after *Mtb* infection in the absence (A) or presence of 50 μM chloroquine (B), cells were stained for Flag-tagged cGAS (green) and nuclei/DNA (DAPI, blue).

(C) Cells were stained for Flag-tagged cGAS (green), ASC or Beclin-1 (red), and nuclei/DNA (DAPI, blue). Scale bar, 5 μm; right, magnification of area outlined. Arrows highlight spots of DNA and cGAS colocalization.

Representative images out of three independent experiments are shown. See also Figure S3 and Table S1.

(Figure 3D). These results reveal that during *Mtb* infection macrophages produce cGAMP. Furthermore, this suggests that the detection of *Mtb* can be relayed from infected macrophages to noninfected neighboring cells via gap-junction-mediated communication.

Comparison of ESX-1- and cGAS-Regulated Cytokine Responses

We next sought to better characterize the molecular events that lead to DNA-dependent activation of cGAS. To this end we analyzed a selection of cytokines, which are known as central mediators of the in vivo response toward *Mtb* infection, including TNF-α, IL-1β, and IL-10. Besides using the wild-type *Mtb* strain H37Rv, macrophages were also infected with the attenuated vaccine strain *Mycobacterium bovis* BCG, which lacks the ESX-1 protein secretion system, known to be required for type I IFN responses (Stanley et al., 2007). As expected, macrophages only produced type I IFN and the related cytokines IP-10 and IL-10 after infection with H37Rv, but not with BCG (Figures 4A and 4C). In addition, upon analyzing the levels of IP-10 and IL-10 from wild-type, cGAS-deficient, and STING-deficient cells, we observed that expression of IP-10 and IL-10 was equally affected in the knockout cell lines (Figure 4B), thereby confirming that ESX-1- and DNA/cGAS-dependent cytokines are coregulated. On the other hand, production of the proinflammatory cytokines TNF-α and IL-6 was similarly triggered by both strains (Figure 4A and data not shown) and not altered in the mutant cell lines as compared to the wild-type cells (Figure 4B). However, these congruent patterns differed when IL-1β production was assessed. Whereas BCG failed to promote IL-1β secretion, absence of cGAS or STING did not compromise IL-1β secretion following H37Rv infection (Figures 4A and 4B). These findings can be explained by the inflammasome-mediated regulation of IL-1β, which reportedly also involves an ESX-1-dependent mechanism of activation (Kurenuma et al., 2009). In this regard it is interesting to note that the stimulation of the inflammasome by *Mtb* also appears to involve a sensing mechanism relying on the detection of intracellular DNA via AIM2 (Saiga et al., 2012). However, studies also reported NLRP3 as the main mediator of inflammasome activation through a mechanism triggered via K^+ efflux (Dorhoi et al., 2012; Kurenuma et al., 2009; Mishra et al., 2010). To elucidate the individual roles of these factors, we targeted AIM2 and the shared inflammasome adaptor molecule ASC in THP-1 cells. We found that the absence of AIM2 reduced IL-1β levels to 50% in response to *Mtb* infection, while the absence of ASC completely abrogated the IL-1β signal (Figure S4A). Type I IFN responses in both AIM2- and ASC-mutated cells were comparable to those of wild-type cells (Figure S4A). Importantly, inhibition of NLRP3 via blocking K^+ efflux by glibenclamide entirely blocked the residual secretion of IL-1β in AIM2 knockout cells, indicating that *Mtb*-triggered activation of the inflammasome complex in human macrophages is mediated by the collective actions of AIM2 and NLRP3 (Figure S4B). So far, our data suggest that during infection with virulent tubercle bacilli two intracellular DNA sensing systems—cGAS and AIM2—become activated, each inducing a complementary cytokine response—type I IFNs and IL1-β, respectively. In addition, more than one ESX-1-controlled mechanism operates in *Mtb*-infected cells to trigger IL-1β release via an inflammasome-dependent mechanism.

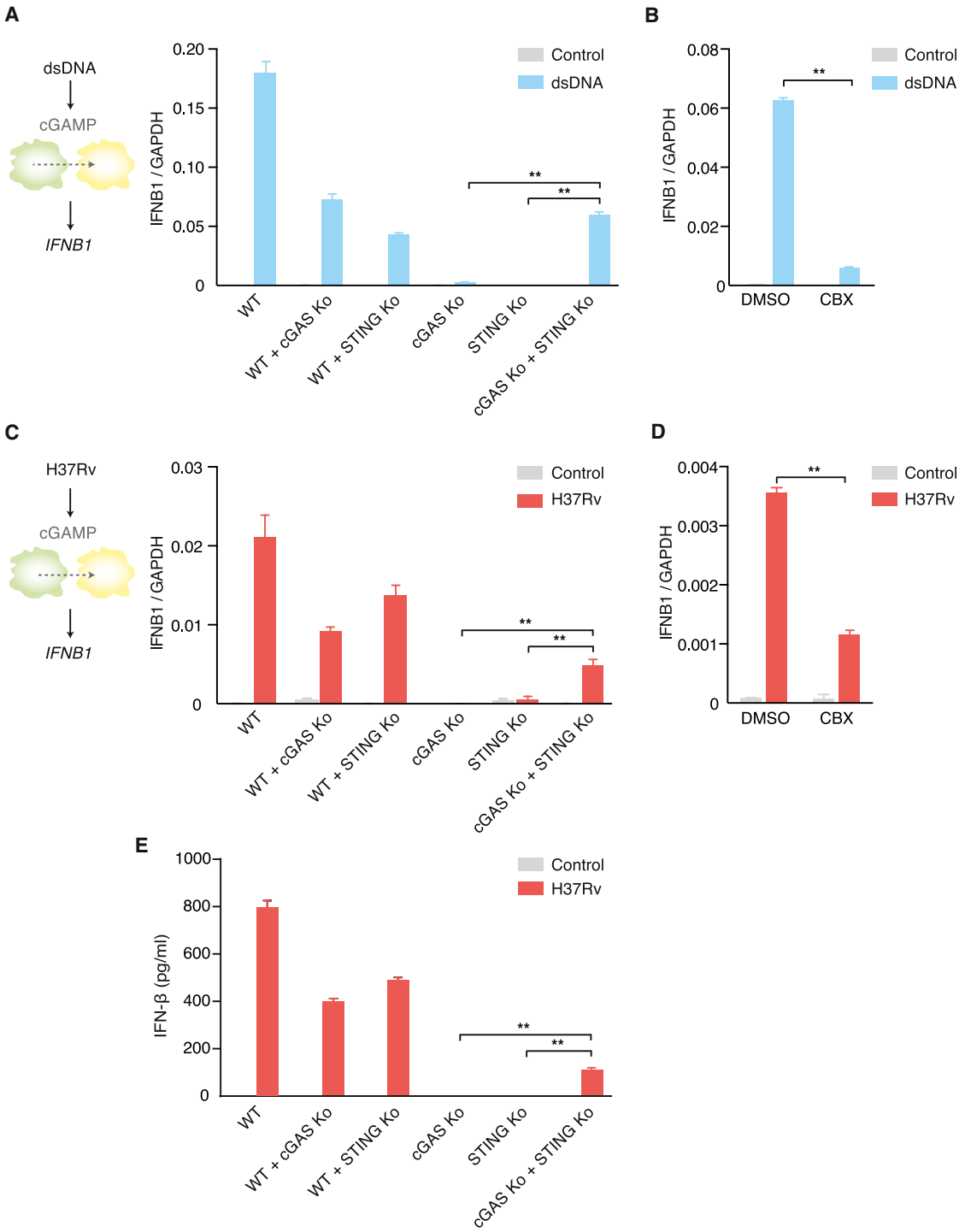

Figure 3. cGAMP Shuttling through Gap Junctions Restores Type I IFN Responses upon *Mtb* Infection in Mixed Cocultures

(A and C) (Left) Scheme depicting model of horizontal transfer of cGAMP upon DNA transfection (A) or H37Rv infection (C); (right) mono- and cocultures (as indicated) of WT THP-1 cells, cGAS-deficient cells, or STING-deficient cells, which were left untreated or stimulated via DNA transfection (A) or H37Rv infection (moi 10) (C). After 24 hr, mRNA levels of IFN-β were measured by qPCR.

(B and D) Cocultures of cGAS- and STING-deficient THP-1 cells were left untreated or transfected with DNA (B), or infected with H37Rv (moi 10) (D) in the presence or absence of carbenoxolone (CBX; 100 μM) as indicated. mRNA levels of IFN-β were assessed by qPCR after 24 hr.

(E) Type I IFN was measured 24 hr postinfection of mono- and cocultures of WT THP-1 cells, cGAS-deficient cells, and STING-deficient cells.

Mean + SD of duplicate measurements of representative results from two independent experiments is depicted. **$p < 0.01$.

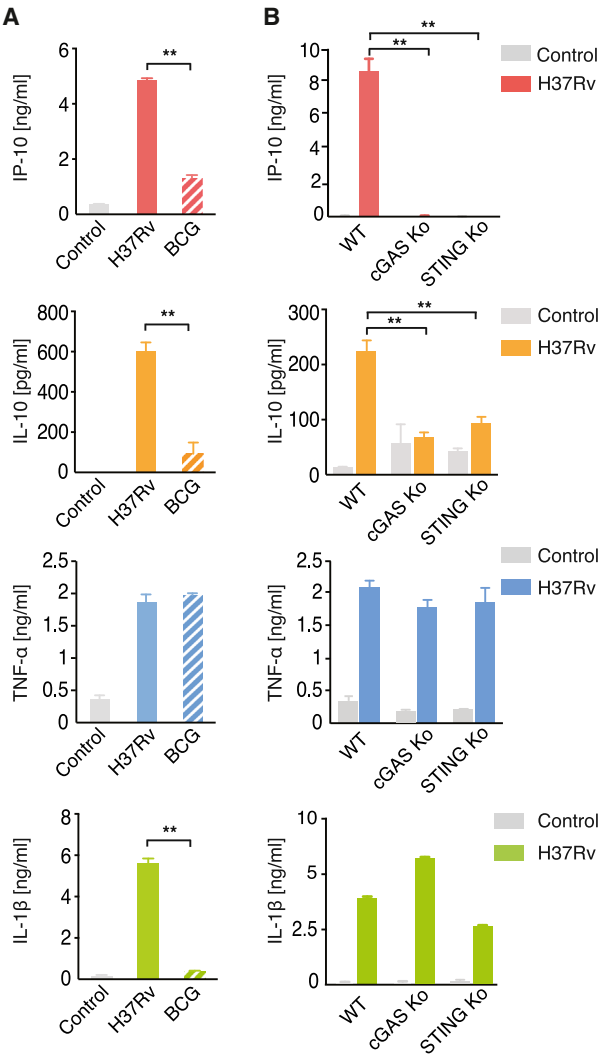

Figure 4. Virulent and Attenuated Mycobacteria Differentially Activate Innate Cytokine Responses

(A and C) THP-1 cells were infected with H37Rv and BCG (moi 10).

(B) WT THP-1 cells, cGAS-deficient cells, or STING-deficient THP-1 cells were infected with H37Rv (moi 10). After 24 hr supernatants were assessed by ELISA for production of IP-10, IL-10, TNF-α, IL-1β, or IFN-β.

Mean + SD of duplicate measurements of representative results from three independent experiments is depicted. **p < 0.01. See also Figure S4 and Table S1.

Mutations within ESX-1 Abrogate cGAS-Dependent IFN Responses

To further explore the role of ESX-1 for the stimulation of intracellular signaling pathways, we tested distinct mycobacterial strains for their capability to induce type I IFNs or IL-1β responses, which we speculated to be coregulated and used as an indirect means to monitor the functionality of the secretory system. First, we focused on the region of difference 1 (RD1) locus, given that its loss led to the attenuation of BCG (Pym et al., 2002). Next to strains harboring disruptions of the entire and extended RD1 locus, H37RvΔRD1 and H37RvΔΔRD1 (Bottai et al., 2011), respectively, we also tested the attenuated H37Ra strain of *Mtb*, which carries the S129L mutation in the transcriptional regulator *phoP* and is defective for secretion of EsxA, a major substrate of ESX-1 (Frigui et al., 2008). Analyzing cytokine levels of infected macrophages, we observed that none of the mutant strains were able to activate IP-10 production and, as expected from the experiments above, both H37RvΔRD1 and H37RvΔΔRD1 were impaired in inducing IL-1β secretion (Figure 5A). Surprisingly, infection with H37Ra still led to significant release of IL-1β, even though no IP-10 was produced (Figure 5A).

Furthermore, we found that the residual IL-1β response upon H37Ra infection was profoundly reduced upon pretreatment with glibenclamide (Figure 5B). This suggests that H37Ra can still stimulate the cytosolic sensor NLRP3, despite its inability to trigger cytosolic DNA-sensing pathways based on AIM2 and cGAS. To confirm the physiological relevance of this finding, we infected primary human macrophages with H37Ra, which consistently resulted in the secretion of IL-1β, while at the same time IP-10 production was markedly reduced (Figure 5C). These results indicate that ESX-1-dependent activation of two central yet opposite host responses can be uncoupled.

Dissecting the Activation of Intracellular Host Sensors by Manipulating ESX-1

The above results indicate that secretion of EsxA or another ESX-1 substrate is an essential feature for activating intracellular DNA-sensing pathways in macrophages. In contrast to the strains BCG or H37RvΔRD1, in which the genes encoding EsxA are deleted, the undetectable secretion of EsxA in H37Ra is achieved by an indirect mechanism involving lack of PhoP-mediated expression of the *espACD* operon, a region distal to the RD1 locus but required for full ESX-1 function (Figure 6A, right) (Stoop et al., 2012). To directly assess the contribution of the EspA/C substrates, we used the respective *espA* and *espC* mutants, with and without complementation, to analyze the host cytokine response (Chen et al., 2012). Compared to the wild-type strain or the respective complemented strains, both mutants failed to trigger IP-10 production upon infection of macrophages (Figure 6A left). In contrast, we observed significant levels of IL-1β production (Figure 6B). Consistent with the involvement of NLRP3 for this cellular response, the IL-1β signal triggered by both EspA/C-deficient strains was inhibited by glibenclamide (Figure 6C). Thus, we conclude that EsxA secretion is essential for provoking intracellular DNA sensing pathways and that reducing the EsxA levels in the secreted fraction by genetic means does not abolish the cytosolic activation of the NLRP3 inflammasome. These findings

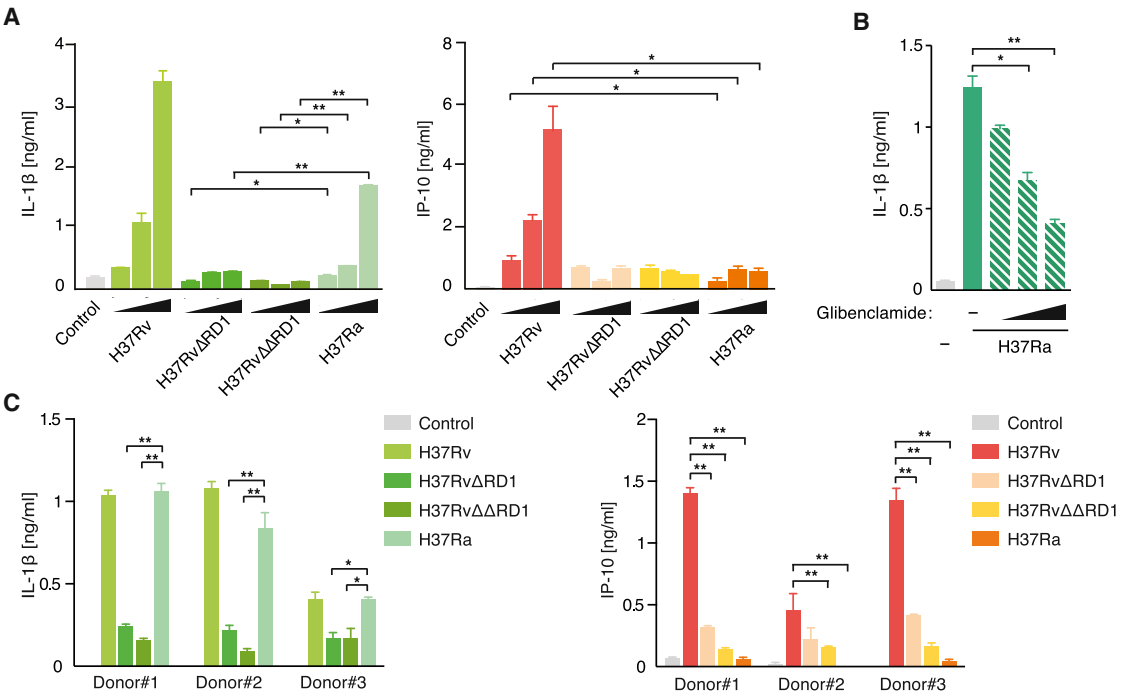

Figure 5. Intracellular Recognition Pathways Are Differentially Activated by Virulent and Attenuated *M. tuberculosis*

(A and C) THP-1 cells (A) or primary human macrophages (C) were infected with the following bacterial strains: H37Rv, H37RvΔRD1, H37RvΔΔRD1, or H37Ra (moi 2.5, 5, and 10). After 24 hr, IP-10 and IL-1β levels were assessed by ELISA.

(B) ELISA measurement of IL-1β in the supernatant of THP-1 cells after 24 hr of infection with H37Ra (moi 10) in the presence of glibenclamide (0, 25, 50, and 100 μg/ml).

Mean + SD of duplicate measurements of representative results from three independent experiments is depicted. *$p < 0.05$; **$p < 0.01$; $p > 0.05$ (n.s.). See also Table S1.

raised the possibility that a pharmacological intervention targeting ESX-1 function and hence EsxA release may be exploited to selectively inhibit cGAS-mediated type I IFN responses while leaving NLRP3-triggered IL-1β production intact. To prove this hypothesis, we tested two small compounds, the benzothiophene inhibitor BTP15 and the benzyloxybenzylidene-hydrazine compound BBH7, which we have recently identified as potent inhibitors of EsxA secretion in *Mtb* (Figure S5A) (Rybniker et al., 2014). Treatment of *Mtb* with both BTP15 and BBH7 resulted in significantly lower amounts of IP-10 production from infected THP-1 cells (Figure 6D). In contrast, neither compound affected the release of IL-1β (Figure 6D). Of note, a similar pattern was observed in *Mtb*-infected primary human macrophages, where a decrease in IP-10 was measured, while IL-1β was unaltered (Figure 6E). In a control experiment, upon stimulation with DNA, IL-1β responses were not altered in the presence of BTP15 and BBH7, thereby confirming that both compounds inhibit *Mtb* and not host functions (Figure S5B). Thus, we conclude that pharmacological manipulation of the ESX-1 secretion system can sway the *Mtb*-triggered immune cytokine pattern in favor of host-protective IL-1β production by accentuating intracellular sensing via the inflammasome complex (Figure S6).

DISCUSSION

Our study demonstrated that in *Mtb*-infected cells cGAS is the sole sensor driving the synthesis of type I IFNs and ISGs. We revealed that infection of macrophages with virulent and attenuated *Mtb* can engage distinct cytosolic PRR systems, namely the cGAS-IFN-axis and the inflammasome-IL-1β-pathway, and that the decision as to which pathway is triggered is determined by the relative abundance of EsxA and/or by additional ESX-1/EsxA-dependent effectors. Our results disclose an as-yet-unnoticed interconnection between the mycobacterial virulence factors and the cytokine pattern of mammalian cells.

The host protein STING has been identified as a central signaling molecule in the innate immune response to various pathogens, including DNA viruses, retroviruses, and intracellular bacteria (Barber, 2014). During DNA virus or retrovirus infection STING seems to carry out this function by being part of the intracellular, cGAS-based DNA sensing pathway and by detecting host-derived cGAMP (Sun et al., 2013; Gao et al., 2013). However, when considered in the context of intracellular bacterial infection, such as with *Mtb*, the role of STING and cGAS/cGAMP is less consistent. Some authors have proposed that mycobacterial CDNs can be secreted into the cytosol and bind directly to STING (Bai et al., 2012; Woodward et al., 2010). Another report has proposed IFI204 as a putative DNA sensor for *Mtb* in murine BMDMs, where shRNA silencing of IFI204 resulted in reduced expression levels of IFIT1 and IFN-β upon *Mtb* infection (Manzanillo et al., 2012). Using both human and murine cGAS-deficient cells, we clearly demonstrated that cGAS is critical for the induction of IFN-β after infection with *Mtb*. We found that cGAS and intracellular DNA come together in the cytosol, supporting its

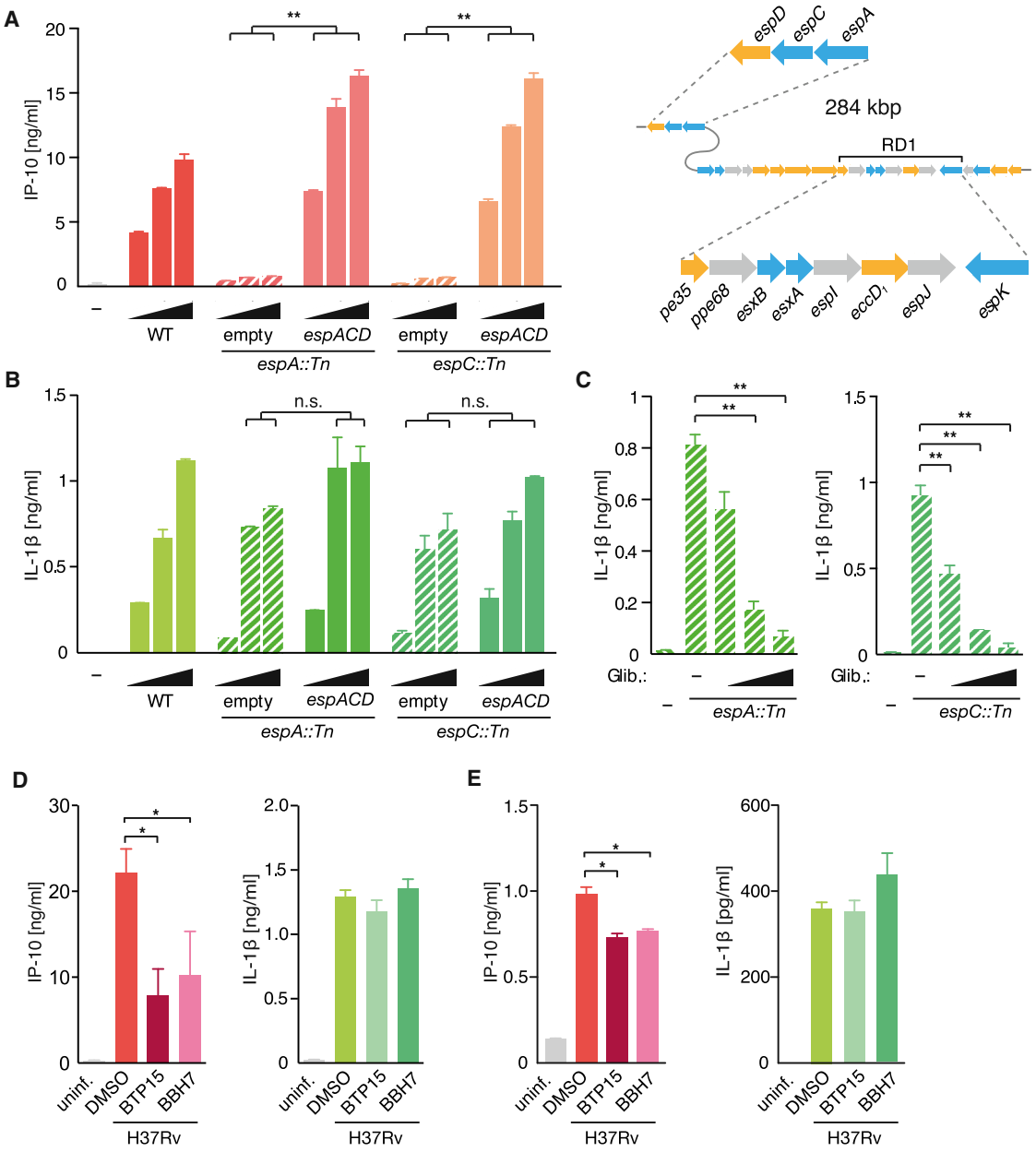

Figure 6. Selective Disruption of the ESX-1 Secretion System Uncouples Intracellular Recognition Pathways of *Mtb*

(A and B) THP-1 cells were infected with the following bacterial strains: Erdman (WT), Erdman *espA*::Tn/pMD31 ("empty" in figure), Erdman *espA*::Tn/pMD*espACD*, Erdman *espC*::Tn/pMD31, and Erdman *espC*::Tn/pMD*espACD* (moi 2.5, 5, and 10). After 24 hr IP-10 (A) and IL-1β (B) levels of the supernatants were assessed by ELISA. (Upper right panel) Scheme of the *espACD* operon and the ESX-1 locus of the *Mtb* genome is depicted; RD1, region of difference 1.

(C) ELISA measurement of IL-1β in the supernatant of THP-1 cells after 24 hr of infection with the strains Erdman *espA*::Tn/pMD31 and Erdman *espC*::Tn/pMD31 (moi 10) in the presence of glibenclamide (Glib., 0, 25, 50, and 100 μg/ml).

(D) H37Rv was incubated with BTP15 (10 μM) or BBH7 (10 μM) for 24 hr. THP-1 cells were infected with the inhibitor-treated H37Rv (moi 10) as indicated or left untreated (uninf.). After 24 hr, IP-10 and IL-1β production was quantified by ELISA.

(E) H37Rv was incubated with BTP15 (10 μM) or BBH7 (10 μM) for 24 hr. Primary human macrophages were infected with the inhibitor-treated H37Rv (moi 10) as indicated or left untreated (uninf.). After 24 hr, IP-10 and IL-1β production was quantified by ELISA.

Representative results out of three (A–D) and two (E) independent experiments are depicted, whereas data are presented as mean + SD of duplicate measurements. *$p < 0.05$; **$p < 0.01$; $p > 0.05$ (n.s.). See also Figure S5 and Table S1.

role as a DNA receptor. As such, our study provides genetic proof that cGAS is necessary to confer responsiveness to an intracellular bacterial pathogen and indicates that at least in an ex vivo setting mycobacterial-derived CDNs play a minor role, if any, in triggering IFN-β gene upregulation. Notably, in parallel to our own work, two independent studies by Chen, Shiloh,

and colleagues and Cox and colleagues, in this issue of *Cell Host & Microbe*, report the same finding, that cGAS is essential for the induction of type I IFN in *Mtb*-infected macrophages (Collins et al., 2015; Watson et al., 2015).

Interestingly, we observed that *Mtb* can trigger the expression of type I IFNs even in the absence of an intact, cell-intrinsic cGAS-cGAMP-STING signaling pathway. We explain this phenomenon by the existence of an intercellular second-messenger-based signaling network that exploits gap junctions to deliver cGAMP into neighboring cells and thus complements the cGAS or STING deficiency when mutant cell lines are cocultured (Ablasser et al., 2013b). Indeed, the sensitivity of this phenomenon to carbenoxolone treatment shown in the present work confirms the gap junction dependency. In the lungs of infected mice, macrophages have been reported to communicate immunomodulatory signals via Ca^{2+} waves using gap junctions as the conducting pathway (Westphalen et al., 2014). Future studies are needed to elucidate whether in vivo cGAMP-based intercommunication might also participate in modulating the immune response to *Mtb*.

It is interesting to note that cells infected by the HN878 strain, belonging to the W-Beijing lineage, but not by the H37Rv strain, benefited from the cGAS-STING knockout when analyzed for cell survival. This finding agrees with the literature, where a correlation between IFN-β production and the virulence of W-Beijing strains has been established (Manca et al., 2001; 2005). Moreover, a recent investigation of the association between type I IFN production and bacterial virulence demonstrated a role for type I IFN in inducing the immunosuppressive cytokine IL-10 and in repressing the cytoprotective effect of IFN-γ in *Mtb*-infected macrophages (McNab et al., 2014). These results are consistent with our data, which show both strongly suppressed production of IL-10 and enhanced macrophage survival in cGAS knockout and STING knockout cells.

Mtb also elicits inflammatory responses, an event that is not mediated by cGAS or STING but by the intracellular inflammasome complex. Interestingly, the inflammasome-mediated host response also relies on the presence of cytosolic DNA, which is sensed by AIM2 (Saiga et al., 2012; Hornung et al., 2009). We proved that deletion of AIM2 causes reduction of IL-1β production but that a significant inflammatory IL-1β signal is maintained via a functional NLRP3-dependent inflammasome system (Dorhoi et al., 2012).

When characterizing the cytokine signature of several mutated mycobacterial strains, we noticed that expression of type I IFNs correlated with the secretion of the ESX-1 substrate EsxA. Surprisingly, mutations compromising EsxA secretion (as in H37Ra) did not affect inflammasome activation in the same way, but instead shifted the *Mtb*-induced host response toward the selective production of IL-1β. It has been generally assumed that activation of the cGAS-STING-IFN-pathway and the inflammasome-IL-1β-pathway in response to virulent mycobacteria is coregulated and triggered by a common (unknown) mechanism mediated by ESX-1. Our work has now revealed that intracellular host recognition pathways can be stimulated differentially according to the level of EsxA secreted. Based on the results obtained, we propose the following molecular explanation. Strains H37RvΔRD1 and H37RvΔΔRD1 lack the genes for most of the ESX-1 secretion apparatus and its substrate EsxA, and therefore they generate no measurable intracellular host response. EsxA is still produced by the *espA* and *espC* mutants and by H37Ra, but its secretion is not detectable (Chen et al., 2013; Fortune et al., 2005; Frigui et al., 2008). However, a small amount of EsxA may be released intracellularly by these bacteria, for instance by cell lysis, and this suffices to stimulate production of IL-1β. In other words, EsxA levels shift the host response either toward the detrimental cytokines (type I IFN and downstream effectors) or toward the beneficial IL-1β. In this sense, Solans et al. demonstrated that the reported degree of virulence of various *Mtb* strains in guinea pigs correlates with different levels of EsxA expression and secretion (Solans et al., 2014) (Palanisamy et al., 2008). In accordance with this explanation, W-Beijing strains, which secrete more EsxA (Solans et al., 2014), elicit higher levels of IP-10, as demonstrated in this work. This interpretation predicts that the specific expression of distinct cytokine classes can be achieved by manipulating the ESX-1 system. Indeed, we found that blocking EsxA secretion by pharmacological means significantly downregulates IFN expression yet still allows for robust production of IL-1β, as further discussed below.

Insight into the mechanism of ESX-1-regulated innate immune response is highly relevant for the development of vaccine adjuvants or immunotherapeutics. Effective live vaccines have to balance "harmlessness" with eliciting powerful and specific immune responses. In this respect it is interesting to note that the attenuated *Mtb ΔphoPR* mutant, which serves as the candidate TB vaccine, MTBVAC (Arbues et al., 2013), displays greatly reduced secretion of EsxA (Frigui et al., 2008). One plausible reason for the advantageous efficacy of MTBVAC involves its ability to selectively trigger inflammatory cytokines and hence to bypass the pathological consequences of the type I IFN system. Alternatively, manipulating the IFN-β-IL-1 regulatory cytokine network may represent a potential approach for the treatment of established *Mtb* infection (Mayer-Barber et al., 2014). Our finding that coregulation of the inflammasome-IL-1β-pathway and the cGAS-IFN-β-pathway can be disconnected via manipulation of the mycobacterial ESX-1 secretory system could guide the development of a novel class of anti-TB drugs for use in conjunction with conventional chemotherapy. As proof of concept, our ex vivo data indicate that selective EsxA secretion inhibitors (BTP15 or BBH7) can shift the innate immune cytokine profile effectively toward the host-beneficial, antimicrobial IL-1β direction. The experimental design, which involved pretreatment of the bacilli with BTP15 or BBH7 and thus preventive depletion of EsxA, did not allow any reduction of IL-1β to be observed, despite this being partly AIM2/DNA dependent. We reason that intracellular *Mtb* multiplication was associated with de novo secretion of EsxA. However, the levels of EsxA did not reach those of the DMSO-treated bacteria, and we therefore observed a decrease in IP-10 while IL-1β remained unaffected. This confirms that wild-type levels of EsxA stimulate both type I IFN and IL-1β, whereas a decrease in EsxA secretion mainly affects type I IFN production while leaving the host-protective IL-1β response virtually intact. This is corroborated by knocking down EsxA secretion by genetic means in the *espA* and *espC* mutants. In the latter cases, the amount of secreted EsxA is undetectable in vitro, and this is reflected in an even greater impact on IP-10 and IL-1β as compared to chemical inhibition. Importantly, the data obtained with BTP15 and BBH7 directly correlate

with their level of protection of *Mtb*-infected fibroblasts, as demonstrated recently (Rybniker et al., 2014). Future investigations will be required to see whether such a host-directed anti-TB treatment may also prove to be successful in an in vivo infection model.

EXPERIMENTAL PROCEDURES

Reagents and Plasmids

DNA oligonucleotides corresponding to the 90 bp long dsDNA (sense sequence, 5′-TACAGATCTACTAGTGATCTATGACTGATCTGTACATGATC TACATACAGATCTACTAGTGATCTATGACTGATCTGTACATGATCTACA-3′) were obtained from Metabion and annealed in PBS. 5′-triphosphate RNA was prepared as previously described (Schlee et al., 2009). PMA (Phorbol 12-myristate 13-acetate), coelenterazine, chloroquine, carbenoxolone, and DAPI were obtained from Sigma-Aldrich. Cyclic di-AMP and cGAMP (2′-5′) were obtained from Biolog. Expression plasmids for Ybt and DacA (Rv3586) were kind gifts from Professor Zhao-Xun Liang (School of Biological Sciences, Nanyang Technological University) and Professor Guangchun Bai (Center for Immunology and Microbial Disease, Albany Medical College), respectively. An expression plasmid coding for human GFP-tagged cGAS was previously described (Ablasser et al., 2013b). Expression plasmids coding for murine STING and human STING are based on pEFBOS (Ablasser et al., 2013a).

CRISPR/CAS9-Mediated Knockout Cell Line Generation

THP-1 cells were coelectroporated with a gRNA- and a mCherry-Cas9-expression plasmid as described (Schmid-Burgk et al., 2014). The gRNA target sequences used were GAACTTTCCCGCCTTAGGCAGGG (cGAS), TCC ATCCATCCCGTGTCCCAGGG (STING), GCTGGAGAACCTGACCGCCGAGG (ASC), and TTTGGCAAAACGTCTTCAGGAGG (AIM2). After FACS enrichment of mCherry-Cas9-positive cells, monoclones were grown out for 2 weeks and were genotyped using deep sequencing as described. For each target gene, two clones bearing all-allelic frameshift mutations were expanded for further study.

Cell Line Generation by Lentiviral Transduction

The method for lentivirus-based expression of cGAS-Flag and GFP-Flag in cGAS mutated THP-1 cells was performed according to the standard protocol (Barde et al., 2010). Stable cGAS- or GFP-expressing cell lines were generated under puromycin selection.

Cell Culture

HEK293T cells were cultured in DMEM and human macrophages ($CD14^+$ cells differentiated with GM-CSF [20 ng/ml]), and PMA-activated THP-1 cells were cultured in RPMI1640, both supplemented with 10% (v/v) FCS, sodium pyruvate (all Life Technologies), and Ciprofloxacin (Bayer Schering Pharma). BMDMs were generated using L929-cell-conditioned medium (LCCM) as a source of granulocyte/macrophage colony stimulating factor. *cGAS*$^{-/-}$ mice were generated in the laboratory of Charles M. Rice (Schoggins et al., 2014). For the generation of human macrophages, PBMCs were obtained by Ficoll–Hypaque density gradient centrifugation, and $CD14^+$ cells were isolated by MACS using the $CD14^+$ cell isolation kit (Miltenyi Biotech) according to the manufacturer's instructions. Buffy coats were obtained from the blood transfusion center (Centre de Transfusion Interrégionale, Croix Rouge Suisse), and the experiments were approved by the Office Ethical Committee (Commission Cantonale D'éthique de la Recherche) with the authorization number 107/15.

Cell Stimulation and Transient Transfection

Murine BMDMs (1×10^6/ml), THP-1 cells (1×10^6/ml), and human macrophages (1×10^6/ml) were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. HEK293T cells (2×10^5/ml) were transfected using GeneJuice (Novagen) according to the manufacturer's instructions. DsDNA and 5′-triphosphate RNA were transfected at a final concentration of 1.33 μg/ml. CDNs were transfected at a final concentration of 2 μg/ml.

Bacterial Strains and Culture Conditions

Mtb strains (described in Table S1) were grown at 37°C in 7H9 medium (Difco) supplemented with 0.2% glycerol, 0.05% Tween 80, and 10% albumin-dextrose-catalase (ADC, Middlebrook) or on 7H10 plates supplemented with 0.5% glycerol and 10% oleic acid-albumin-dextrose-catalase (OADC, Middlebrook). Kanamycin (20 μg/ml) and hygromycin (50 μg/ml) were used when necessary.

Preparation of *Mycobacterium tuberculosis* Cultures for Cell Infection

Bacteria were grown to exponential phase (optical density at 600 nm, OD600, between 0.4 and 0.8), washed once in 7H9 medium, resuspended in 7H9 to an OD_{600} of 1, equivalent to 3×10^8 bacteria/ml. The required volume of *Mtb* bacterial suspension was then added to RPMI1640 or DMEM for infection of human THP-1 cells or mouse BMDM, respectively, at the multiplicity of infection (moi) reported in the text. Plates were sealed with gas-permeable sealing film and incubated at 37°C under 5% CO_2.

Cell Viability Assay

CellTiter-Blue Assay (Promega) was performed according to the manufacturer's instructions.

Immunofluorescence Microscopy

THP-1 cells were activated with 100 ng/ml PMA and seeded on coverslips in 24-well plates. Cells were then either transfected with the indicated nucleic acids, CDNs, or infected with *Mtb* for indicated time periods. After incubation cells were fixed with 4% formaldehyde in PBS for 20 min at 25°C. Cells were then blocked and permeabilized in blocking buffer (2% BSA, 0.2% Triton X-100 in PBS) for 30 min. Cells were stained with anti-Flag (Sigma), anti-Beclin-1 (Cell Signaling), anti-ASC (Santa Cruz), Texas Red anti-rabbit, and Alexa Fluor 488 anti-mouse antibodies (Life Technologies) for 1 hr at 25°C. Imaging was performed using the Zeiss Axioplan fluorescence microscope.

Immunoblotting

Cells were lysed in 1× Laemmli buffer and denatured at 95 °C for 5 min. Cell lysates were separated by 10% SDS-PAGE and transferred onto nitrocellulose membranes. Blots were incubated with anti-STING (Cell Signaling), anti-MB21D1 (anti-cGAS, Sigma-Aldrich), anti-β-actin-HRP, and anti-rabbit-IgG-HRP (both Santa Cruz Biotechnology).

Luciferase Reporter Assays

HEK293T cells (2×10^4 cells in each well of a 96-well plate) were transiently transfected with 25 ng IFN-β promoter reporter plasmid (pIFN-B-Gluc) in conjunction with expression vectors as indicated using GeneJuice (Novagen). cGAS was used to induce cGAMP production, and DacA was used to induce cyclic di-AMP production within HEK293T cells upon transient overexpression. Luciferase activity was assessed in the supernatants 14–20 hr posttransfection with coelenterazine (2.2 μM) as substrate.

ELISA

Cell culture supernatants were removed from infected or transfected cells after 24 hr. In the case of infection, supernatants were filtered through NANOSEP centrifugal devices (Life Sciences) and assayed for mouse IP-10 (R&D Systems), human IP-10, IL-1β, TNF-α, IL-10 (BD Biosciences), and IFN-β (PBL Interferon Source) according to the manufacturer's instructions.

Analysis of Murine Type I IFN Production via Bioassay

Mouse type I IFN levels were determined by incubating LL171 cells with supernatants from infected BMDMs for 4 hr. Luciferase activity was measured, and type I IFN levels were calculated using a dilution series of the standard.

qPCR

RNA from cells was reverse transcribed using the RevertAid First Strand cDNA Synthesis kit (Fermentas), and quantitative PCR analysis was performed on an ABI 7900HT. All gene expression data are presented as relative expression to GAPDH. The sequences were as follows: GAPDH forward, 5′-GAGTCAACG GATTTGGTCGT-3′; GAPDH reverse, 5′-GACAAGCTTCCCGTTCTCAG-3′;

IFNB1 forward, 5′-CAGCATCTGCTGGTTGAAGA-3′; IFNB1 reverse, 5′-CATTACCTGAAGGCCAAGGA-3′; IFIT2 forward, 5′-GCGTGAAGAAGGTGAAGAGG-3′; and IFIT2 reverse, 5′-GCAGGTAGGCATTGTTTGGT-3′.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and one table and can be found with this article at http://dx.doi.org/10.1016/j.chom.2015.05.003.

AUTHOR CONTRIBUTIONS

R.W., M.F.G., C.S., S.G.P., Y.L., J.R., and A.A. performed experiments. R.W., M.F.G., C.S., Y.L., J.R., A.A., and S.T.C. designed experiments and analyzed the data. J.L.S.-B., T.S., and V.H. developed the CRISPR/Cas9 targeting strategy. R.W., M.F.G., C.S., S.T.C., and A.A. wrote the manuscript with input and approval from all authors. S.T.C. and A.A. supervised the project.

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