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Authors

Barker, Jeffrey R
Koestler, Benjamin J
Carpenter, Victoria K
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STING-Dependent Recognition of Cyclic di-AMP Mediates Type I Interferon Responses during *Chlamydia trachomatis* Infection

Jeffrey R. Barker,[a] Benjamin J. Koestler,[b] Victoria K. Carpenter,[a] Dara L. Burdette,[c] Christopher M. Waters,[b] Russell E. Vance,[c] Raphael H. Valdivia[a]

Department of Molecular Genetics and Microbiology, Center for Microbial Pathogenesis, Duke University, Durham, North Carolina, USA[a]; Department of Microbiology and Molecular Genetics, Michigan State University, East Lansing, Michigan, USA[b]; Department of Molecular and Cell Biology, University of California, Berkeley, Berkeley, California, USA[c]

ABSTRACT STING (<u>st</u>imulator of <u>in</u>terferon [IFN] <u>g</u>enes) initiates type I IFN responses in mammalian cells through the detection of microbial nucleic acids. The membrane-bound obligate intracellular bacterium *Chlamydia trachomatis* induces a STING-dependent type I IFN response in infected cells, yet the IFN-inducing ligand remains unknown. In this report, we provide evidence that *Chlamydia* synthesizes cyclic di-AMP (c-di-AMP), a nucleic acid metabolite not previously identified in Gram-negative bacteria, and that this metabolite is a prominent ligand for STING-mediated activation of IFN responses during infection. We used primary mouse lung fibroblasts and HEK293T cells to compare IFN-β responses to *Chlamydia* infection, c-di-AMP, and other type I IFN-inducing stimuli. *Chlamydia* infection and c-di-AMP treatment induced type I IFN responses in cells expressing STING but not in cells expressing STING variants that cannot sense cyclic dinucleotides but still respond to cytoplasmic DNA. The failure to induce a type I IFN response to *Chlamydia* and c-di-AMP correlated with the inability of STING to relocalize from the endoplasmic reticulum to cytoplasmic punctate signaling complexes required for IFN activation. We conclude that *Chlamydia* induces STING-mediated IFN responses through the detection of c-di-AMP in the host cell cytosol and propose that c-di-AMP is the ligand predominantly responsible for inducing such a response in *Chlamydia*-infected cells.

IMPORTANCE This study shows that the Gram-negative obligate pathogen *Chlamydia trachomatis*, a major cause of pelvic inflammatory disease and infertility, synthesizes cyclic di-AMP (c-di-AMP), a nucleic acid metabolite that thus far has been described only in Gram-positive bacteria. We further provide evidence that the host cell employs an endoplasmic reticulum (ER)-localized cytoplasmic sensor, STING (<u>st</u>imulator of <u>in</u>terferon [IFN] <u>g</u>enes), to detect c-di-AMP synthesized by *Chlamydia* and induce a protective IFN response. This detection occurs even though *Chlamydia* is confined to a membrane-bound vacuole. This raises the possibility that the ER, an organelle that innervates the entire cytoplasm, is equipped with pattern recognition receptors that can directly survey membrane-bound pathogen-containing vacuoles for leaking microbe-specific metabolites to mount type I IFN responses required to control microbial infections.

Address correspondence to Raphael H. Valdivia, raphael.valdivia@duke.edu.

The obligate intracellular pathogen *Chlamydia trachomatis* is the most common sexually transmitted bacterial infection and the leading cause of preventable blindness worldwide. As a consequence of inflammatory damage from repeated and chronic infections, severe sequelae can occur such as blinding trachoma, pelvic inflammatory disease, and infertility (1). Elementary bodies (EB), the infective form of *C. trachomatis*, attach to and invade epithelial cells of the conjunctiva and urogenital tract. After entry, the EB form transitions to the metabolically active reticulate body (RB), which replicates within a membrane-bound compartment known as the inclusion. Midway through the *Chlamydia* infectious cycle, RB replication becomes asynchronous, generating both RBs and EBs (2). EBs exit the cell through either lysis of the host cell or extrusion of the inclusion to infect neighboring cells (3).

Microbial compounds are recognized by surface-exposed and intracellular immune receptors, leading to the transcriptional activation of cytokines and other host defense genes (4). For instance, type I interferons (IFNs) are secreted in response to diverse viral and bacterial infections (5, 6). Although the antiviral properties of IFNs are fairly well characterized, the role that these cytokines play in controlling bacterial infections is less well understood, as they can both block and enhance pathogen replication *in vivo* (7–11). All cell types can initiate type I IFN responses through Toll-like receptor (TLR)-dependent and TLR-independent pathways. TLR-independent pathways include cytosolic sensing of microbial ligands such as cell wall fragments through NF-κB-activating NOD1/NOD2 receptors (12) and double-stranded DNA (dsDNA) or dsRNA-like molecules through the action of RNA polymerase III (13), RIG-I (14), MAVS (15), cGAS (16), and STING (<u>st</u>imulator of IF<u>N</u> genes; also known as Eris, MITA, and MPYS) (17–20).

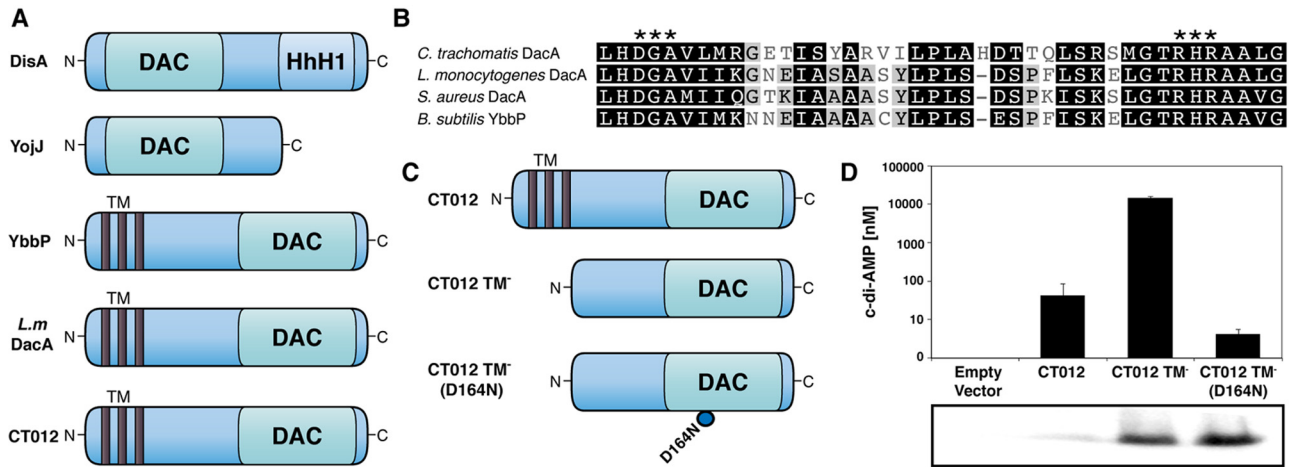*C. trachomatis* induces type I IFN responses in a variety of cell

FIG 1 The *C. trachomatis* protein DacA (CT102) displays DAC activity. (A) Domain architecture of *B. subtilis* DAC domain-containing proteins DisA, YojJ, and Ybbp with *L. monocytogenes* DacA and the *C. trachomatis* putative DAC CT012. HhH1, helix-hairpin-helix motif; TM, three TM domains). (B) Amino acid sequence alignment of conserved domain regions of *C. trachomatis* DacA and DACs from c-di-AMP-producing bacteria with conserved residues critical for c-di-AMP synthesis indicated (asterisks). One hundred percent similarity, black; 80 to 100% similarity, dark gray; 60 to 80% similarity, light gray; <60% similarity, white. (C) Schematics of three *C. trachomatis* DacA recombinant variants used in this work to monitor c-di-AMP synthesis in *E. coli*. (D) UPLC-based quantification of c-di-AMP levels from nucleotide extracts of *E. coli* expressing full-length *Chlamydia* DacA, the DAC domain of DacA, or DacA with a D164N point mutation in the putative active site with corresponding immunoblot assays showing levels of DacA in soluble protein extracts. The data shown are means ± standard deviations of two independent experiments. Commercial c-di-AMP (Biolog) was used for calibration and quantification of nucleotide extracts.

types. Previous work has provided evidence for (21) and against (22) a role for TLRs in the regulation of type I IFN responses to *Chlamydia* infection. Prantner and colleagues identified STING, a key mediator of responses to cytosolic DNA, RNA, cyclic dinucleotides (19, 23–25), as important for the induction of type I IFNs in *Chlamydia*-infected cells (22). STING is an endoplasmic reticulum (ER) integral membrane protein (26) that, upon exposure to cytoplasmic dsDNA, is redistributed to p62-positive punctate structures in the cytoplasm ("sequestosomes") that colocalize with autophagy-related proteins (26, 27). Unlike sensing of dsDNA in the cytoplasm, which requires additional cofactors, STING can directly bind to bacterium-specific cyclic dinucleotides (24) and trigger potent type I IFN responses to intracellular pathogens such as *Listeria monocytogenes* and *Legionella pneumophila* (28–30).

Diadenylate cyclases (DACs) are found in a wide variety of Gram-positive bacteria and are required for the synthesis of cyclic di-AMP (c-di-AMP) (31). A related cyclic dinucleotide, c-di-GMP, is a well-established regulator of bacterial virulence, biofilm formation, motility, and gene expression (32). In contrast, the function of c-di-AMP is less well characterized. The DNA integrity-scanning protein DisA, found in *Bacillus subtilis*, synthesizes c-di-AMP in the presence of intact DNA to activate sporulation genes (33, 34). In addition, cell wall homeostasis is regulated by c-di-AMP, as elevated levels of this cyclic dinucleotide compensate for a lack of teichoic acids in *Staphylococcus aureus* (35), and enhances resistance to β-lactam antibiotics in *B. subtilis* by increasing peptidoglycan cross-linking (36). Overall, c-di-AMP appears to be important in Gram-positive bacteria for adaptations to membrane and cell wall stress.

In this study, we provide the first experimental evidence that *C. trachomatis*, a Gram-negative bacterium, synthesizes c-di-AMP during the late stages of infection and that the CT012 gene functions as a DAC. Furthermore, we show that c-di-AMP is largely responsible for driving type I IFN responses in *Chlamydia*-

infected cells and that the sensing of this metabolite is mediated at the ER by the immune sensor STING.

RESULTS

The *Chlamydia* gene *dacA* (CT012) encodes a DAC. The host cytosolic sensor STING could directly and indirectly sense RNA, DNA, and cyclic dinucleotides (17, 19, 24, 26, 37–41) to mediate IFN responses during *Chlamydia* infection (22, 42–47). Given recent evidence that STING senses cyclic dinucleotides directly (24), we were intrigued by the presence in *C. trachomatis* of an open reading frame (CT012) with sequence homology to the *B. subtilis* DAC domain-containing protein YbbP and the *L. monocytogenes* DAC DacA (Fig. 1A and B). *B. subtilis* contains three DAC domain-containing proteins (Fig. 1A), including the DNA integrity-scanning protein DisA (34). *C. trachomatis* CT012 has an N-terminal transmembrane (TM) domain and a putative catalytic domain with the conserved amino acids Asp-Gly-Ala (DGA) and Arg-His-Arg (RHR) found in bacterial DACs (Fig. 1B). On the basis of these similarities, we hypothesized that *Chlamydia* synthesizes c-di-AMP and that IFN responses may be mediated through the sensing of this metabolite in the host cytosol.

We first determined if the putative *Chlamydia* DAC (CT012) is capable of synthesizing c-di-AMP. We expressed various hexahistidine-tagged forms of CT012 in *Escherichia coli*, which lacks any known DACs, including full-length CT012, a version lacking the TM domain (TM⁻) and a TM⁻ CT012 with the aspartate in the DGA motif mutated to asparagine (Fig. 1C). Using ultraperformance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS), we detected high levels of c-di-AMP in *E. coli* strains expressing either full-length or TM⁻ CT012 but only minor amounts in strains expressing a D164N mutant form of TM⁻ CT012 (Fig. 1D). Expression levels of these TM⁻ variants of CT012 proteins were found to be similar by Western blotting (Fig. 1D). It is possible that, on a per-protein basis, full-length DacA is more active than the truncated forms. However, without

a means to assess properly folded protein in this *E. coli* overexpression system, we cannot make any firm statement to that effect. Nonetheless, these findings indicate that CT012 has DAC activity and thus we have renamed the gene encoding this protein *dacA*.

***C. trachomatis* synthesizes c-di-AMP during infection.** To determine if *Chlamydia* synthesizes c-di-AMP during infection, nucleic acids were isolated from HeLa cells infected with *C. trachomatis* at various time points postinfection and analyzed by UPLC-MS/MS under conditions optimized for the separation and detection of cyclic dinucleotides. Synthetic c-di-AMP was used as the standard for the identification and quantification of c-di-AMP within extracts. c-di-AMP was detected in *Chlamydia*-infected cells as early as 12 h postinfection (hpi) and continued to increase throughout the infectious cycle (Fig. 2A). Peak c-di-AMP levels were observed at 36 hpi. It is unlikely that *Chlamydia* synthesizes c-di-GMP, a metabolite known to induce IFN responses, as c-di-GMP was undetectable in infected samples (data not shown) and *Chlamydia* lacks any proteins with GGDEF or EAL domains. We also generated DacA-specific antiserum that detected two immunoreactive bands consistent with the predicted DacA monomer and dimer molecular weights (Fig. 2A), consistent with recent observations with *B. subtilis* YbbP/CdaA (48), the closest homologue of *Chlamydia* DacA. The levels of DacA protein expression correlated with the abundance of c-di-AMP during infection and type I IFN responses in *Chlamydia*-infected murine embryonic fibroblasts (MEFs) (Fig. 2B).

We next compared the levels of c-di-AMP in infectious EBs with those in replicative RBs. Nucleic acids were extracted from density gradient-purified EBs and RBs and analyzed by UPLC-MS/MS. c-di-AMP was detected in EBs but not in RBs (Fig. 2C). The purity of gradient-purified EBs and RBs was assessed by their morphology (data not shown). These results are consistent with DNA microarray results (49) and proteomic data sets showing that DacA is expressed in EBs but not in RBs (50). EB lysates treated with snake venom phosphodiesterase (SVP) lost the peak corresponding to c-di-AMP, indicating that the peaks detected contain 3′-5′ phosphate linkages. Overall, these results indicate that *Chlamydia* synthesizes c-di-AMP during infection and that this metabolite accumulates primarily within EBs.

STING is required for type I IFN responses to *C. trachomatis* and cell autonomous control of bacterial replication. *Chlamydia* induces type I IFN responses in a variety of cell types, and evidence from RNA interference-mediated gene silencing suggests that this response requires STING (22). We extended these findings to primary cells derived from STING-deficient animals. First, we determined that primary mouse lung fibroblasts (MLFs) induced significant levels of type I IFN as detected by the induction of luciferase expression in a reporter cell line containing an *ISRE-luc* transgene and by measuring secreted IFN-β by enzyme-linked immunosorbent assay (ELISA) (Fig. 2B and 3A and B). These MLFs also induced type I IFN in response to the delivery of the dsRNA analogue poly(I·C), dsDNA, and c-di-AMP to the cytosol (Fig. 3A), indicating that the sensing machinery for nucleic acids in the cytoplasm is intact in these cells.

By forward genetic screening of *N*-ethyl-*N*-nitrosourea-mutagenized C57BL/6 mice, Sauer and colleagues identified a mouse strain with impaired type I IFN responses to *L. monocytogenes* (51). The loss of responsiveness in these mice (*Goldenticket*) was due to a destabilizing point mutation in STING. We isolated fibroblasts from wild-type and *Goldenticket* mouse lungs and
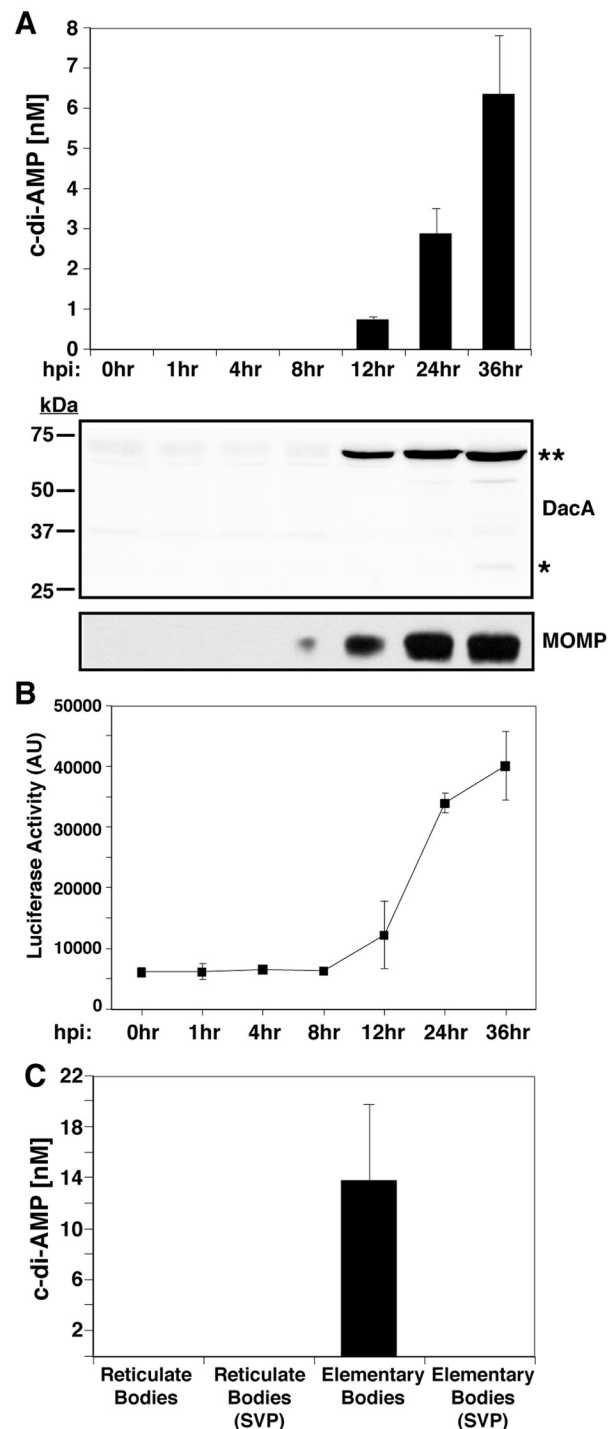

FIG 2 c-di-AMP is present in nucleic acid extracts from cells infected with *C. trachomatis*. (A) c-di-AMP levels in extracts of HeLa cells infected with *C. trachomatis* were quantified by UPLC at the indicated time points. The corresponding immunoblot shows *Chlamydia* DacA and MOMP expression levels. Asterisks indicate monomeric (*) and dimeric (**) forms of DacA. (B) MEFs induce type I IFN in response to *Chlamydia* infection. MEFs stably expressing an ISRE fused to a luciferase reporter gene were infected with *C. trachomatis* for the indicated times and lysed, and luciferase levels were assessed by an enzymatic assay. AU, arbitrary units. (C) c-di-AMP levels in lysates of density gradient-purified RBs and EBs. The signal for c-di-AMP was sensitive to SVP. The data shown are means ± standard deviations of two technical replicates.
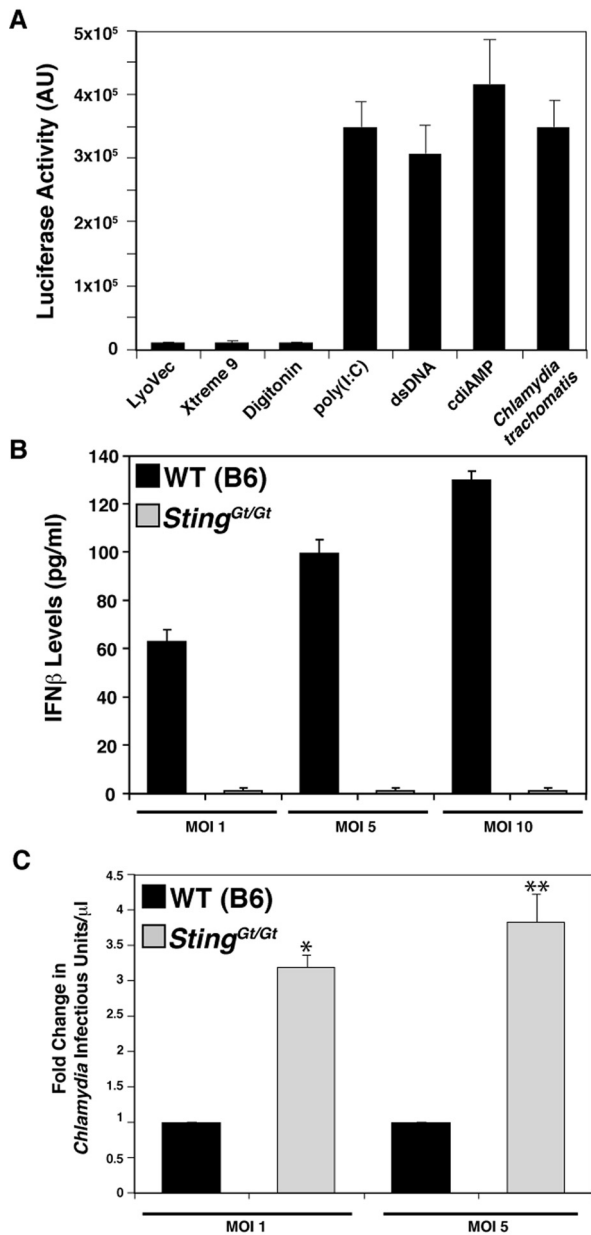
FIG 3 STING is required for the expression of type I IFN in mouse MLFs during *C. trachomatis* infection. (A) MLFs induce type I IFNs in response to various microbial-signal-like signals. MLFs were transfected with dsDNA or poly(I·C), loaded with c-di-AMP by digitonin permeabilization, or infected with *C. trachomatis* for 30 h. Cells treated with transfection (LuoVec and Xtreme9) or cell permeabilization reagents alone were included as controls. Levels of secreted type I IFNs in cell supernatants were assessed with a reporter cell line stably expressing an ISRE fused to a luciferase reporter gene and monitoring of luciferase enzymatic activity. AU, arbitrary units. (B) IFN-β responses to *C. trachomatis* infection requires STING. MLFs from wild-type (WT) and *STING^Gt/Gt* (*Goldenticket*) mice were infected with *C. trachomatis* at the indicated MOIs for 30 h, and the levels of IFN-β in cell supernatants was determined by ELISA. (C) STING-deficient (*STING^Gt/Gt*) MLFs are permissive for *C. trachomatis* replication. Wild-type and *STING^Gt/Gt* MLFs were infected with *C. trachomatis* at the indicated MOIs, and the yield of infectious units was determined at 36 hpi. Fold change represents the ratio of the number of infectious units/μl observed in *Goldenticket* fibroblasts to that observed in wild-type fibroblasts. All data are means ± standard deviations of three independent samples ×, $P < 0.001$; **, $P < 0.001$ (one-way analysis of variance and Newman-Keuls multiple-comparison test).

tested their abilities to respond to *Chlamydia* infection. *Goldenticket* MLFs were unable to mount a type I IFN response to *C. trachomatis* at increasing multiplicities of infection (MOIs) (Fig. 3B), confirming STING's essential role in mediating type I IFN responses to *Chlamydia*. Furthermore, we observed that wild-type MLFs produced fewer *Chlamydia* infectious units than did *Goldenticket* MLFs (Fig. 3C), indicating a protective role for type I IFNs in cell autonomous control of *Chlamydia* infection, as has been previously reported (46).

Chlamydia-induced type I IFN responses are mediated predominantly by c-di-AMP. Because type I IFN responses to *Chlamydia* require STING (22), we postulated that sensing of *Chlamydia* is likely due to the detection of bacterial dsDNA, RNA, or c-di-AMP in the host cytoplasm. Burdette and colleagues recently identified a mutation (R231A) that renders STING incapable of responding to cyclic dinucleotides while retaining the ability to respond to dsDNA (24). To test the contribution of c-di-AMP to the triggering of type I IFN responses during *Chlamydia* infection, we stably expressed wild-type STING-hemagglutinin (HA) or STING^R231A-HA by retrovirus-mediated transduction of expression constructs into HEK293T cells. HEK293T cells do not express detectable levels of STING (24) or TLRs (52), and exogenously expressed STING is sufficient to recapitulate IFN responses to cyclic dinucleotides but not dsDNA (24) (Fig. 4A). *Chlamydia* infection triggered the expression of type I IFNs in HEK293T cells expressing wild-type STING-HA but not c-di-AMP-blind STING^R231A-HA (Fig. 4B). At high MOIs, we observed increased cytopathic effects (data not shown), which likely explains the apparent decrease in type I IFN responses. Overall, these findings indicate that c-di-AMP sensing during *Chlamydia* infection is a relevant inducer of IFN responses.

We could not directly address the role of *Chlamydia* DNA sensing in the induction of IFN responses in these cells because HEK293T cells do not express cytoplasmic dsDNA sensors that signal through STING (16, 24, 53). To address this problem, we complemented T-antigen/telomerase-immortalized (54) *Goldenticket* MLFs with a wild-type STING-HA or STING^R231A-HA expression construct and assessed the expression of type I IFNs during infection. We observed significant variability in IFN responses among batches of primary cells (data not shown) and thus chose to complement a clonal, immortalized *Goldenticket* MLF line with STING-HA constructs to minimize cell-to-cell variation. STING^R231A-HA MLFs secreted IFN in response to transfected cytosolic poly(I·C) and dsDNA (Fig. 4C) but did not respond to c-di-AMP (Fig. 4C) or *C. trachomatis* infection (Fig. 4D; see Fig. S2 in the supplemental material), indicating that c-di-AMP is the predominant *Chlamydia*-derived molecule recognized by the host cytoplasmic sensors in fibroblasts. While the expression of wild-type STING-HA and STING^R231A-HA, as assessed by immunoblotting, was comparable among HEK293T cells, STING^R231A-HA expression was slightly elevated compared to that of wild-type STING-HA in *Goldenticket* MLFs (Fig. 4E), which likely accounts for the observed elevated type I IFN responses to poly(I·C) and dsDNA (Fig. 4C). Finally, consistent with the role of type I IFNs in cell autonomous control of intracellular pathogens (46), *C. trachomatis* replication was significantly enhanced in *Goldenticket* MLFs expressing STING^R231A-HA compared to that in MLFs expressing wild-type STING-HA (Fig. 4F).
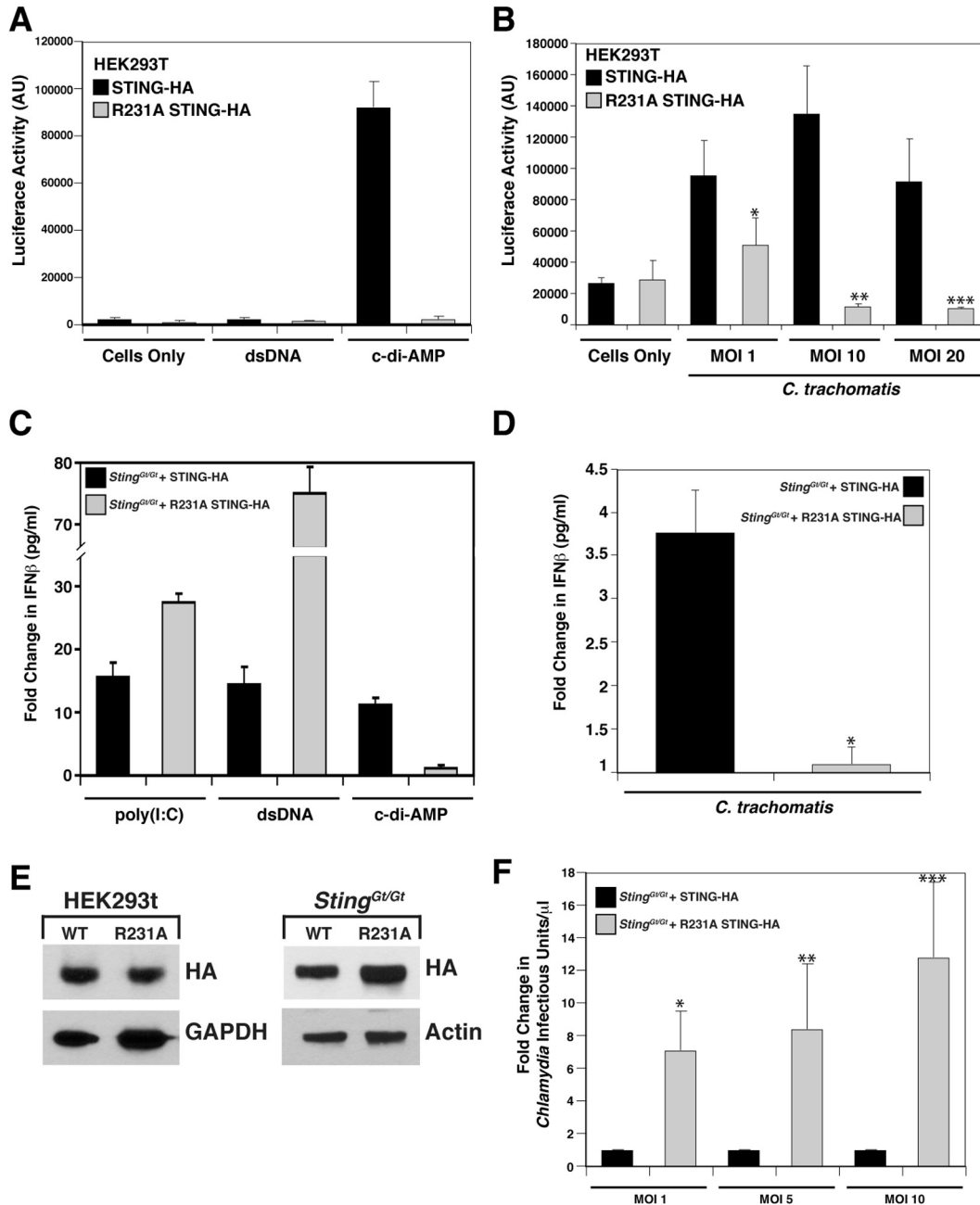
FIG 4 c-di-AMP is the main ligand responsible for *Chlamydia*-mediated type I IFN responses. (A and B) STING expression is sufficient for the induction of IFN responses during *C. trachomatis* infection. HEK293T cells stably expressing an ISRE-luciferase reporter were transduced with a wild-type STING-HA (black) or a STING[R231A]-HA (gray) retroviral vector and treated with dsDNA or c-di-AMP (A) or infected with *C. trachomatis* for 30 h at the MOIs indicated (B). $\times$, $P <$ 0.05; **, $P <$ 0.001; ***, $P <$ 0.001 (one-way analysis of variance and Newman-Keuls multiple-comparison test). Cells expressing mutant STING[R231A] cannot induce type I IFNs in response to c-di-AMP. AU, arbitrary units. (C to F) MLFs expressing STING variants that cannot signal in response to cyclic dinucleotides fail to induce type I IFNs during *C. trachomatis* infection. *Goldenticket* MLFs transduced with a wild-type STING-HA (black) or a STING[R231A]-HA (gray) construct were treated with poly(I-C), dsDNA, or c-di-AMP (C) or infected with *C. trachomatis* for 30 h (D). *, $P <$ 0.05 (one-way analysis of variance and Newman-Keuls multiple-comparison test). Supernatants were tested for IFN-β levels by ELISA. Fold changes represent the ratios of total IFN-β detected by ELISA (in pg/ml) in cells treated with stimulants to that in untreated cells (C) and that in infected cells to that in uninfected cells (D). (E) Immunoblot analysis with anti-HA (STING) antibody of protein lysates from HEK293T cells and *Goldenticket* MLFs expressing various STING constructs indicates that comparable levels of STING were expressed in these cell lines. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and actin served as loading controls. WT, wild type. (F) *Goldenticket* MLFs expressing wild-type STING-HA (black) or STING[R231A]-HA were infected with *C. trachomatis*, and infectious units were assessed as described in the legend to Fig. 3C. Note that *Goldenticket* MLF expressing the STING variant that cannot recognize ci-di-AMP did not induce IFN-β and control *C. trachomatis* replication. *, $P <$ 0.05; **, $P <$ 0.05; ***, $P <$ 0.001 (one-way analysis of variance and Newman-Keuls multiple-comparison test). Fold changes represent the ratios of infectious units/μl observed in *Goldenticket* MLF complemented with STING-HA[R231A]-HA to those in wild-type STING-HA. The data shown are means $\pm$ standard deviations of three independent samples.
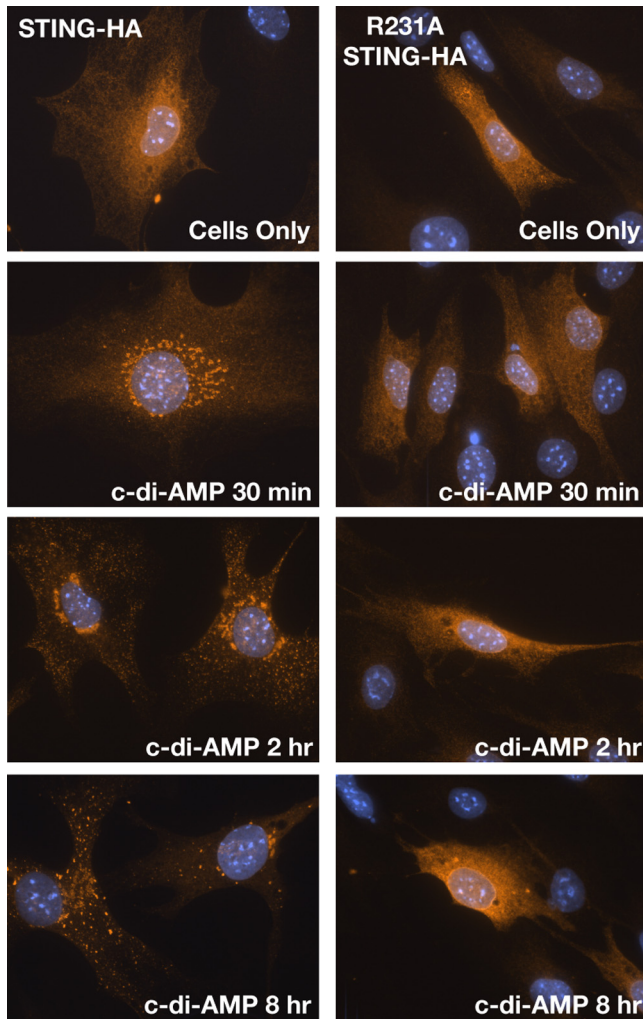
FIG 5 STING translocates to cytoplasmic structures in response to c-di-AMP. *Goldenticket* MLFs transduced with wild-type STING-HA (top) or STING[R231A]-HA (bottom) were treated with c-di-AMP for the indicated times. Cells were immunostained with anti-HA (STING) antibody and imaged by epifluorescence microscopy.

STING translocation from the ER to cytoplasmic signaling complexes upon c-di-AMP treatment correlates with the onset of type I IFN responses. Upon the recognition of dsDNA in the cytoplasm, STING is redistributed from its steady-state localization at the ER to the Golgi apparatus and p62/SQSTSM-containing cytoplasmic structures (26). Exit from the ER is required for type I IFN activation in response to dsDNA (17), leading to the recruitment of serine/threonine-protein Tank-binding kinase 1 (TBK1), which phosphorylates the transcriptional factor IRF-3 (55). We tested if c-di-AMP is sufficient to mediate the relocalization of STING. At steady state, HA-tagged wild-type STING and STING[R231A] localized to the ER-like structures in MLFs (see Fig. 6A). *Goldenticket* MLFs expressing HA-tagged wild-type STING, but not MLFs expressing STING[R231A], relocalized from the ER to punctate structures as early as 30 min posttreatment with c-di-AMP (Fig. 5). Although STING[R231A] variants bind cyclic dinucleotides (24), we failed to see any STING translocation at the time points examined. In contrast, wild-type

STING and STING[R231A] were readily translocated to p62/SQSTSM-positive puncta upon transfection with dsDNA but not poly(I-C) (Fig. 6B and C; see Fig. S3 in the supplemental material). Neither c-di-AMP treatment (Fig. 6D) nor *C. trachomatis* infection (Fig. 6E) induced the translocation of STING[R231A]. The percentages of cells containing STING-positive cytoplasmic punctate structures under all of the conditions tested are shown in Fig. 6F.

Overall, these findings strongly suggest that c-di-AMP is the most prominent microbial ligand driving type I IFN responses during *Chlamydia* infection.

DISCUSSION

The *Chlamydia*-derived ligand(s) driving type I IFN responses and the corresponding sensing mechanism(s) are unclear. In this study, we provide evidence that *Chlamydia* synthesizes c-di-AMP and that this metabolite is a prominent driver of *Chlamydia*-induced IFN responses. To our knowledge, *C. trachomatis* is the first Gram-negative pathogen shown to synthesize c-di-AMP. *Vibrio cholerae* synthesizes a hybrid cyclic di-AMP-GMP molecule (56) via a protein lacking a canonical DAC domain, but whether this nucleic acid can induce IFN responses during infection is not known.

The role type I IFNs play in the control of *Chlamydia* infections *in vivo* is unclear. Mice lacking the type I IFN receptor gene (IF-NAR^{−/−}) cleared *Chlamydia muridarum* infections earlier and displayed reduced pathology in the oviducts (8), suggesting a detrimental role for type I IFNs in the outcome of genital tract infections with *C. muridarum*. However, type I IFNs can also induce cell autonomous defense mechanism with antichlamydial activities (46).

Both murine and human cell lines sense *Chlamydia* infections and mount type I IFN responses (45, 58–61). Some studies have implicated TLR3 in *Chlamydia*-mediated IFN responses in murine oviduct epithelial cells (21, 62), while others point to a role for STING in human cells (22). Here we provide genetic evidence supporting a prominent role for STING in the sensing of *Chlamydia* ligands by murine cells. MLFs derived from STING-deficient (*Goldenticket*) mice did not induce type I IFNs in response to *C. trachomatis* infection (Fig. 3B). Importantly, this defect in IFN expression by *Goldenticket* MLFs can be complemented with a wild-type copy of STING but not with a mutant form of STING (STING[R231A]) that binds cyclic dinucleotides but cannot signal in response to them (24) (Fig. 4D). Because STING[R231A] can still initiate IFN expression in response to cytoplasmic DNA, we conclude that chlamydial dsDNA in the cytoplasm is unlikely to be a major contributor to type I IFN responses during *Chlamydia* infection. A recent study suggested that the dsDNA sensor DDX41 (40) can also directly sense cyclic dinucleotides and requires STING as an adaptor protein (63). Whether sensing of c-di-AMP occurs directly through DDX41 or STING, the lack of IFN responses in cells expressing a STING variant blind to cyclic dinucleotide-dependent signaling argues that c-di-AMP, and not dsDNA, is the major ligand driving *Chlamydia* IFN responses. Similarly, *Chlamydia*-derived RNAs are unlikely to be a major inducer of IFN, as the expression of IFN-β in *Chlamydia*-infected cells is RIG-I and MAVS independent (22).

Unlike the cytosolic pathogen *L. monocytogenes* (64), where secreted metabolites are directly accessible to immune sensors in the cytoplasm, *Chlamydia* is surrounded by a membrane vacuole that is impermeable to small charged molecules >500 Da in size
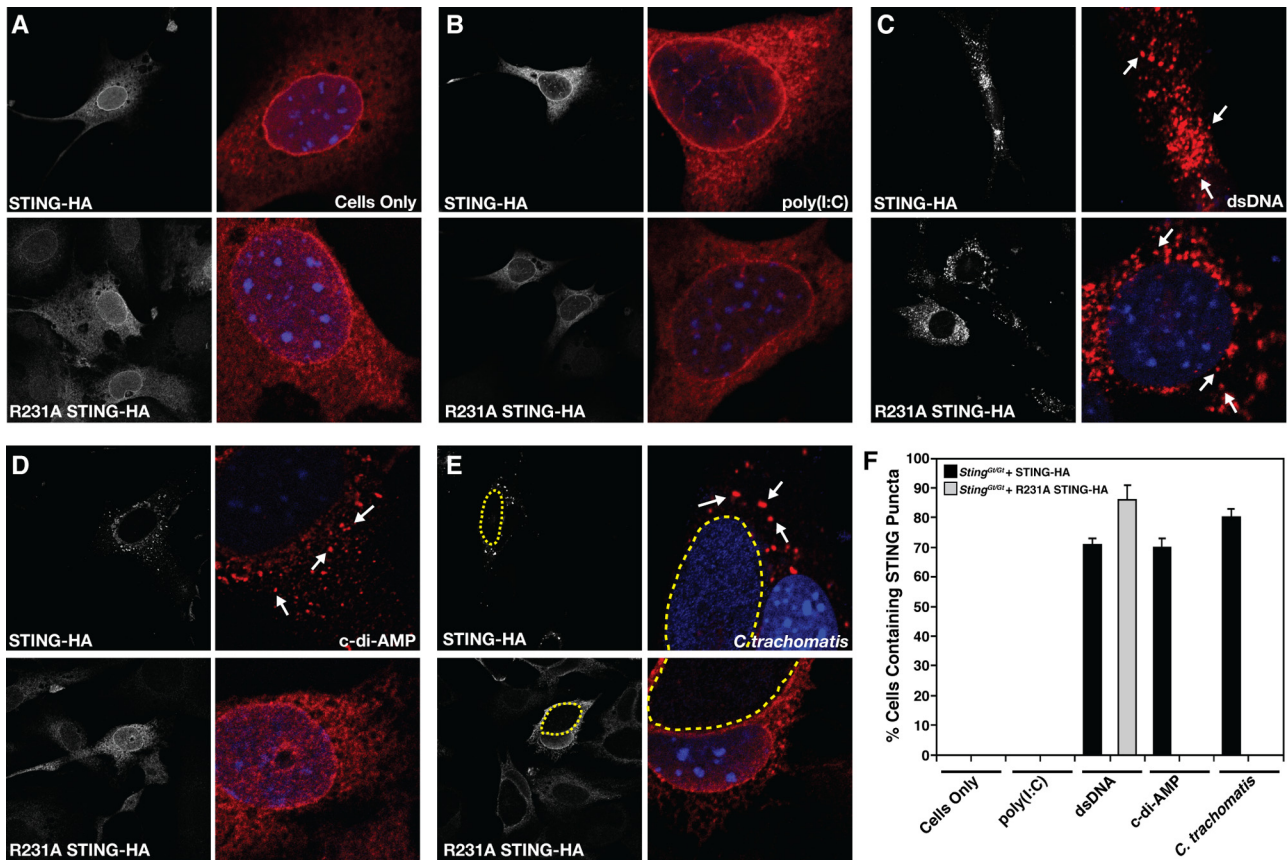
FIG 6 (A) A STING variant (STING^{R231A}) that does not signal in response to c-di-AMP fails to translocate to cytoplasmic complexes upon *Chlamydia* infection. Immortalized *Goldenticket* MLFs expressing a wild-type STING-HA or a cyclic dinucleotide-blind STING^{R231A}-HA construct were treated with digitonin alone (A), transfected with the dsRNA analogue poly(I-C) (B) or dsDNA (C) for 24 h, loaded with c-di-AMP after digitonin permeabilization for 8 h (D), or infected with *C. trachomatis* for 30 h (E). Cells were fixed and stained with anti-HA (STING) antibody and imaged by confocal laser scanning microscopy. Note that STING^{R231A} cannot translocate to cytoplasmic punctate structures (arrows) in response to *C. trachomatis* infection or c-di-AMP stimulation but is responsive to dsDNA treatment. (F) Quantification of the percentage of cells displaying STING in cytoplasmic puncta after treatment with various inducers of type I IFNs. Cells displaying puncta (arrows) were scored as positive for STING translocation (*n* = 150).

(65). This permeability barrier should limit the detection of chlamydial metabolites to microbial pattern recognition receptors. However, the inclusion is intimately associated with ER membranes, which has been interpreted as a pathogen-mediated process evolved to acquire lipids from the host (66–70). An alternative interpretation is that cells envelope large, abnormal vacuolar structures with ER membranes to increase the chance that sensors like STING can encounter microbe-derived compounds. In such a scenario, STING in the ER, an organelle that innervates the entire cytoplasm, has a greater likelihood of sensing chlamydial c-di-AMP and translocating to signaling platforms together with TBK1 and IRF3, as previously shown (29, 41, 71) (Fig. 7).

Upon sensing cytoplasmic dsDNA, STING relocalizes from the ER to the Golgi apparatus and cytoplasmic p62/SQSTSM-positive puncta (26). STING transport out of the ER upon the sensing of dsDNA is critical to the initiation of type I IFN responses (17). This intracellular transport event also occurs in response to cyclic dinucleotides, even in HEK293T cells, where cytoplasmic dsDNA sensors are not expressed (Fig. 4A) (24), and is important for immune signaling, as type I IFN induction in response to c-di-AMP is blocked by the fungal metabolite brefeldin A (unpublished data), an inhibitor of ER-to-Golgi apparatus transport (17). The

molecular mechanisms regulating STING relocalization to cytoplasmic signaling complexes are unknown. Autophagy-related proteins have been implicated in the turnover of STING-positive signaling complexes, as Atg9a knockout cells show enhanced accumulation of STING and TBK1 in punctate structures and increased IFN-β production (26). Autophagy markers such as LC3 and Atg9a also colocalize with STING, yet STING-positive structures lack double-membrane-bound morphological characteristics of autophagosomes (26).

Unlike c-di-GMP, which has well-defined roles in bacterial virulence, including motility, biofilm formation, and virulence gene expression (72), the function of c-di-AMP in bacteria is less well understood. c-di-AMP was first identified in *B. subtilis* as a second messenger involved in signaling DNA integrity to regulate sporulation (33, 34). In *S. aureus*, c-di-AMP regulates peptidoglycan cross-linking and bacterial survival in the absence of lipoteichoic acids (35). *Chlamydia* contains a single gene for the synthesis of c-di-AMP, *dacA*, and a single putative phosphodiesterase gene with homology to proteins that cleave or inactivate c-di-AMP (73). While the role of c-di-AMP in *C. trachomatis* is unknown, the timing of c-di-AMP detection during infection mimics that of conversion of RB to EB forms (~15 hpi) and a quantitative pro-
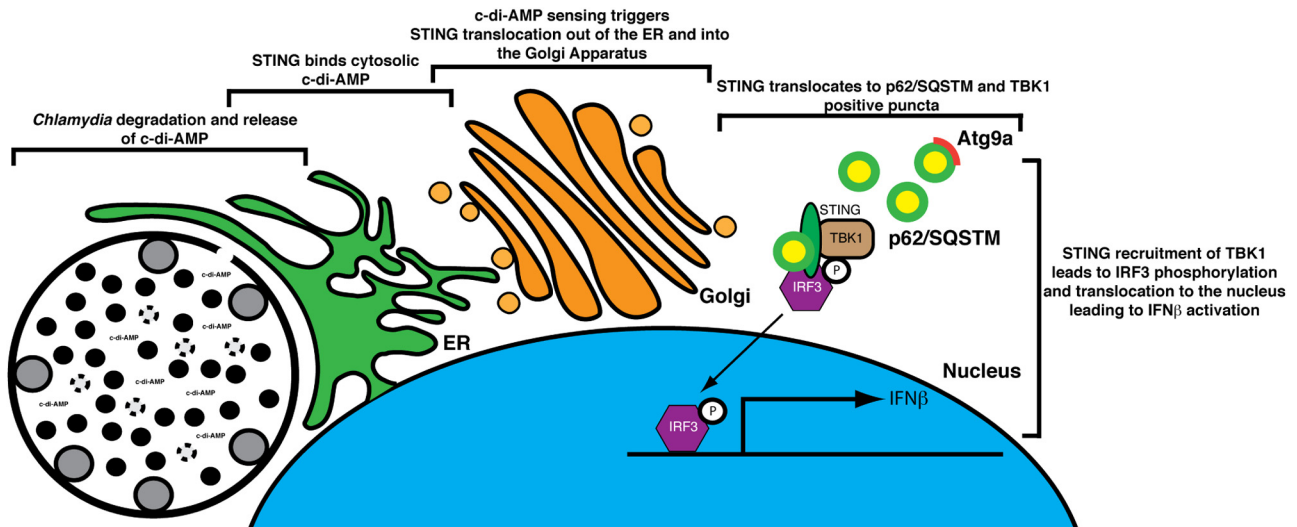
FIG 7 Model of activation of STING-dependent IFN-β responses in *Chlamydia*-infected cells. The bacterial metabolite c-di-AMP is synthesized by *Chlamydia* and sensed by STING at ER membranes enveloping the inclusion. STING is transported out of the ER (26) to the Golgi apparatus and eventually to p62/SQSTSM cytoplasmic complexes (see Fig. S3 in the supplemental material). As previously shown, the recruitment of TBK1 and IRF3 to these signaling platforms leads to IRF3 phosphorylation and translocation to the nucleus and expression of type I IFN genes (29, 71).

teomic study identified DacA only in purified EBs and not RBs (50). Given the prominent role of cyclic dinucleotides like c-di-GMP in the regulation of protein function, gene expression, and the posttranscriptional regulation of mRNAs, we speculate that c-di-AMP may have similar regulatory functions, especially during the transition from metabolically active RBs to infectious EBs.

As in other bacterial systems (29, 48), c-di-AMP may turn out to be an essential molecule in *Chlamydia*. Indeed, in a recent screen for essential *Chlamydia* genes, we have been unable to identify loss-of-function mutations in *dacA* (unpublished data). It is apt that an immune sensing mechanism would have evolved to detect conserved, essential bacterial metabolites.

MATERIALS AND METHODS

Organisms and cell culture. *C. trachomatis* serovar LGV-L2 (strain 434/Bu) was grown in HeLa cells, MLFs, or HEK293T cells maintained in Dulbecco's modified Eagle's medium plus 10% fetal bovine serum (FBS).

Primary LF isolation. Primary lung fibroblasts (LFs) were isolated from *Sting*-deficient *Goldenticket* (51) and parental wild-type C57BL/6 mouse lungs. Briefly, lung tissues were minced and transferred into a sterile beaker containing a stir bar and 10 ml of Dulbecco's modified Eagle's medium (DMEM) with 0.14 Wunsch unit/ml Liberase Blendzyme 3 (Roche) and 1× antibiotic-antimycotic (penicillin-streptomycin and amphotericin B; Gibco). Samples were incubated at 37°C with slow stirring for 60 min, washed three times with 10 ml DMEM with 15% FBS and 1× antibiotic-antimycotic. Tubes were spun at 524 × g in a swinging-bucket centrifuge for 5 min, and the cell pellet was resuspended twice in 10 ml DMEM–15% FBS–1× antibiotic-antimycotic and transferred to a 10-cm dish. Cells were incubated at 37°C in a humidified 5% CO_2 atmosphere. Primary LFs were stored in frozen aliquots or immortalized by retroviral delivery of simian virus 40 T antigen and human telomerase reverse transcriptase as previously described (54).

Plasmid constructs and virus production. STING expression constructs (24) were delivered into HEK293T cells and immortalized LFs, and positive transductants were sorted on a FACSVantage sorter (BD) on the basis of the levels of enhanced green fluorescent protein (EGFP) expression. For stable expression of STING-HA, viruses were generated in 293T cells seeded into 10-cm dishes at 1×10^6 cells/dish and transfected with

STING-HA retroviral constructs and packaging plasmid pCL10A1 at a 1:1 ratio with FuGENE6 (Promega). At 48 h posttransfection, the cell supernatant was collected, filtered over 0.44-μm filters (Pall), and added to immortalized *Goldenticket* LFs. Stable transductants were sorted on the basis of EGFP expression levels.

CT012 (*dacA*) and CT102 lacking the TM domain (minus amino acids 1 to 86) was inserted into pET28a for overexpression in BL21-CodonPlus-RIL cells (Stratagene). Mutations in the putative active site of CT012 were generated with the QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's guidelines.

Generation of anti-DacA antibodies. Hexahistidine-tagged DacA was generated by cloning a fragment of CT012 lacking the TM domain into pET28a. Recombinant protein was produced in BL21-CodonPlus-RIL cells (Stratagene) and purified by affinity chromatography on nickel-charged resin (Qiagen). Purified, recombinant DAC was used to immunize a rabbit. The antiserum was precleared of cross-reactive antibodies by incubation with crude HeLa cell lysate at 4°C for 2 h prior to use in immunoblotting.

Analysis of DacA expression. HeLa cell monolayers were infected with serovar LGV-L2 at an MOI of ~1. Infections were synchronized at 4°C, and total protein was collected at 0, 1, 4, 8, 12, 24, and 36 hpi by lysis in hot sample buffer (50 mM Tris [pH 6.8], 2% SDS, 10% glycerol, 1% β-mercaptoethanol, 0.02% bromophenol blue), followed by sonication. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad). Blots were blocked in 5% nonfat milk diluted in Tris-buffered saline (0.5 M Tris, 1.5 M NaCl, pH 7.4) with 0.1% Tween 20 (TBST) and incubated overnight with primary anti-Dac antibody diluted in 5% milk–TBST. The blots were washed in TBST and incubated with a horseradish peroxidase-linked anti-rabbit secondary antibody (GE Healthcare). Bound antibodies were detected with the SuperSignal West Dura detection kit (Thermo).

Cell stimulation. Cells were infected with serovar LGV-L2 for 30 h or transfected with 250 ng/μl poly(I-C)/LyoVec (Invivogene) or 5 μg plasmid dsDNA (Xtreme 9; Roche) for 24 h. For c-di-AMP delivery, cells were incubated with 10 μg/ml digitonin (Calbiochem) and 10 μM c-di-AMP (Biolog) in permeabilization buffer (50 mM HEPES [pH 7], 100 mM KCl, 3 mM $MgCl_2$, 85 mM sucrose, 0.2% bovine serum albumin, 0.1 mM dithiothreitol, 1 mM ATP, 0.1 mM GTP) for 30 min at 37°C in 5% CO_2. The permeabilization buffer was removed and replaced with DMEM–

10% FBS, and cells were incubated at 37°C in 5% CO_2 for 8 h. Controls included digitonin permeabilization without c-di-AMP.

Type I IFN expression. Expression of type IFNs was assessed either with reporter cell lines or by ELISA. For reporter cell line construction, lentiviruses containing an IFN-stimulated response element (ISRE)-luciferase reporter (SABiosciences) was used to stably transduce MEFs. These cells were seeded into 96-well plates in triplicate, and supernatants from cells treated with various IFN-inducing stimuli were added. Poststimulation, the medium was removed and cells were washed once with phosphate-buffered saline (PBS). Cells were lysed in 50 μl of britelite plus reagent per well (PerkinElmer), and the samples were transferred to a white OptiPlate (PerkinElmer) for measurement of luminescence on an EnSpire 2300 Multilabel Plate Reader (PerkinElmer).

For IFN-β ELISAs, cells were seeded into 96-well plates in triplicate at 1×10^4/well. Poststimulation, medium was collected and analyzed for IFN-β levels according to the manufacturer's protocol (VeriKine Mouse IFN-β ELISA kit; Pestka Biomedical Laboratories). For MLFs expressing STING-HA constructs, results were normalized to the GFP expression of adherent cells as measured by an EnSpire 2300 Multilabel Reader (PerkinElmer).

c-di-AMP extraction. *E. coli* BL21-CodonPlus-RIL (Stratagene) cells were used to overexpress *Chlamydia* DacA constructs. Overnight cultures were diluted in 100 ml minimal medium and incubated at 37°C for 3 h. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM, and cells were incubated at 37°C for 4 h (OD_{600} of 0.4). Cultures were spun at 5,100 rpm for 30 min at 4°C, and the bacterial pellets were resuspended in 250 μl extraction buffer (40:40:20 methanol-acetonitrile-H_2O plus 0.1 N formic acid). Samples were incubated at -20°C for 30 min, and then insoluble material was pelleted by centrifugation at 13,000 rpm for 5 min at 4°C. A 200-μl volume of the supernatant was collected and neutralized with NH_4HCO_3 (0.6% final concentration). Samples were stored at -80°C until UPLC analysis. For c-di-AMP extraction from infected cells, HeLa cells were seeded into six-well plates and infected with *C. trachomatis* at an MOI of 1. Infections were synchronized by centrifugation at 3,000 rpm for 30 min at 4°C and then transferred to a 37°C 5% CO_2 humidified incubator. At the indicated time points, infected cells were harvested and nucleotides were extracted from the pellet as described above.

UPLC-MS/MS analysis. c-di-AMP was quantified by UPLC-MS/MS as previously described (74). Immediately before analysis, 100 μl of each sample was centrifuged under a vacuum for 2 h to remove the extraction buffer and the remaining pellet was then resuspended in 100 μl water. A 10-μl volume was analyzed on a Quattro Premier XE mass spectrometer (Waters) coupled with an Acquity Ultra Performance LC system (Waters) alongside an eight-point standard curve of purified c-di-AMP (Biolog) ranging from 250 to 1.95 nM. The limit of detection of cyclic dinucleotides was >1 nM. c-di-AMP was detected in all infected cell lines.

Isolation of EB and RB forms of *Chlamydia*. Purification of *C. trachomatis* EBs and RBs was performed by density gradient centrifugation as previously described (50). To ensure the purity of serovar LGV-L2, stocks were plaque purified twice before generating EB and RB forms for c-di-AMP analysis. For c-di-AMP extraction, final pellets were resuspended in 250 μl extraction buffer and processed as described for *E. coli*.

Inclusion-forming unit assay. Wild-type and *Goldenticket* MLFs were seeded at 1×10^4/well into a 96-well plate. Infections were conducted in triplicate at various MOIs. At 40 hpi, cells were lysed in H_2O and SPG (3.8 mM KH_2PO_4, 7.2 mM K_2HPO_4, 4.9 mM L-glutamic acid, 218 mM sucrose, pH 7.4) was added to a final concentration of 1×. Serial dilutions were used to infect HeLa cell monolayers for 24 h, at which point, cells were fixed with methanol and processed for immunofluorescence microscopy with anti-major outer membrane protein (anti-MOMP) antibodies. The number of inclusions per well was determined with a Cellomics High Content imaging system (Thermo Scientific).

Immunofluorescence assays for STING localization. For routine immunofluorescence microscopy, cells were fixed with 3% formaldehyde–

0.025% glutaraldehyde for 20 min, permeabilized with 0.1% Triton X-100–PBS for 20 min, and then blocked with 5% bovine serum albumin in PBS. The primary antibodies used were rat anti-HA (1:500; Roche) and rabbit anti-p62/SQSTSM1 (1:1,000; Sigma) antibodies. Primary antibodies diluted in PBS were incubated with permeabilized cells at 4°C for 1 h; this was followed by staining with fluorophore-conjugated secondary antibodies (Molecular Probes) for an additional 1 h. DNA was stained with Hoechst (Molecular Probes) at a concentration of 1:10,000 in PBS. Cells were mounted on slides with Mowiol (Sigma) and stored at 4°C. Cells were imaged by confocal laser scanning microscopy on a Zeiss 510 inverted confocal microscope.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00018-13/-/DCSupplemental.

Figure S1, JPG file, 0.2 MB.
Figure S2, TIF file, 1.9 MB.
Figure S3, JPG file, 0.9 MB.

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