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cGAS and Ifi204 Cooperate To Produce Type I IFNs in Response to Francisella Infection

Kelly M. Storek,* Nina A. Gertsvolf,* Maikke B. Ohlson,† and Denise M. Monack*

Type I IFN production is an important host immune response against viral and bacterial infections. However, little is known about the ligands and corresponding host receptors that trigger type I IFN production during bacterial infections. We used a model intracellular pathogen, Francisella novicida, to begin characterizing the type I IFN response to bacterial pathogens. F. novicida replicates in the cytosol of host cells and elicits a robust type I IFN response that is largely TLR independent, but is dependent on the adapter molecule STING, suggesting that the type I IFN stimulus during F. novicida infection is cytosolic. In this study, we report that the cytosolic DNA sensors, cyclic GMP-AMP synthase (cGAS) and Ifi204, are both required for the STING-dependent type I IFN response to F. novicida infection in both primary and immortalized murine macrophages. We created cGAS, Ifi204, and Sting functional knockouts in RAW264.7 macrophages and demonstrated that cGAS and Ifi204 cooperate to sense dsDNA and activate the STING-dependent type I IFN pathway. In addition, we show that dsDNA from F. novicida is an important type I IFN stimulating ligand. One outcome of cGAS–STING signaling is the activation of the absent in melanoma 2 inflammasome in F. novicida infection. Whereas the absent in melanoma 2 inflammasome is beneficial to the host during F. novicida infection, type I IFN signaling by STING and IFN regulatory factor 3 is detrimental to the host during F. novicida infection. Collectively, our studies indicate that cGAS and Ifi204 cooperate to sense cytosolic dsDNA and F. novicida infection to produce a strong type I IFN response. The Journal of Immunology, 2015, 194: 000–000.

The innate immune system plays a key role in the early recognition and elimination of invading pathogens. Many recognition systems are in place to detect conserved pathogen-associated molecular patterns (PAMPs), such as nucleic acids and cell-wall components (1). Upon PAMP recognition, immune cells initiate signal transduction cascades that trigger a type I IFN transcriptional response, which can prompt a broad range of additional responses to infection, including caspase–mediated cell death and proinflammatory cytokine release (2, 3). Although it is appreciated that many bacterial species, including Francisella tularensis, Listeria monocytogenes, Salmonella typhimurium, Mycobacterium tuberculosis, and Chlamydia trachomatis, can initiate a type I IFN response, the mechanism of host recognition remains largely undetermined (4, 5).

Microbes can stimulate the type I IFN response either by activating members of the TLR family that signal through the endosomal adapter TRIF or by activating cytosolic receptors that activate a type I IFN transcriptional response (6, 7). One important class of cytosolic receptors includes DNA sensors that activate the endoplasmic reticulum membrane protein stimulator of IFN genes (STING, also known as MITA, MPSY, ERIS, and TMEM173). STING activation leads to the recruitment of the kinase TBK1, which phosphorylates IFN regulatory factor 3 (IRF3), a transcription factor required for the induction of IFN-β (8, 9). Recent studies have demonstrated that the host-derived second-messenger cyclic GMP-AMP (cGAMP) synthesized from the DNA sensor cyclic GMP-AMP synthase (cGAS) or bacterial-derived cyclic dinucleotides can directly activate STING (8, 10, 11). In addition to cGAS, a number of cytosolic sensors were identified to bind DNA and trigger the type I IFN response. They include RNA polymerase III, DNA-dependent activator of IFN-regulatory factors, Lrfrp1, Ifi204 (human: IFI16), Mre11, DNA-dependent protein kinase, and Ddx41 (7, 12–17). Although many cytosolic DNA sensors have been identified, the role of these sensors during bacterial infections remains unclear.

Francisella novicida is a model organism used to study the cytosolic responses of immune cells to intracellular bacteria (18). Upon phagocytosis by host macrophages, F. novicida rapidly escapes the Francisella-containing vacuole and replicates in the cytosol (19). Cytosolic F. novicida trigger a proinflammatory response characterized by the production of type I IFNs followed by pyroptotic cell death (20, 21). The type I IFN response to F. novicida infection is largely TLR independent, but STING dependent, making F. novicida an ideal organism to study the cytosolic responses in macrophages to an intracellular bacterial pathogen (20, 22, 23). To date, the Francisella ligand(s) and corresponding host sensor(s) have not been identified. Ultimately, production of type I IFNs increases protein levels of the DNA sensor, absent in melanoma 2 (AIM2), a protein that binds cytosolic DNA and engages the adaptor protein ASC to form a caspase–1 inflammasome complex (24–27). An active AIM2 inflammasome leads to the secretion of proinflammatory cytokines (IL-1β and IL-18) and...
caspase-1-dependent cell death (20), which are required for a protective innate immune response in mice (23).

In this study, we identified cGAS and Ifi204 as two important host factors involved in type I IFN signaling in response to *F. novicida* infection in both bone marrow–derived macrophages (BMMs) and RAW264.7 macrophages. Using targeted knockouts (KOs) and complementation vectors, we demonstrated that cGAS and Ifi204 both contribute to STING-dependent type I IFN response to high concentrations of cytosolic dsDNA. In addition, we showed that dsDNA is the primary molecule found in *F. novicida* lysates that stimulate cGAS- and Ifi204-dependent type I IFN production. Taken together, our results suggest that cGAS and Ifi204 sense dsDNA during a *F. novicida* infection to elicit STING activation and the type I IFN response.

**Materials and Methods**

**Bacteria, plasmids, primers, and generation of RAW264.7 KO cell lines**

Bacterial strains used in this study include wild-type (WT) *F. novicida* U112, Δ*FP1* (28), and Δ*AFP* (29) and WT *L. monocytogenes* strain 10403S. The cDNA from cGAS (Clone ID: 40130956; Thermo Scientific) and Ifi204 (Clone ID: 4018506; Thermo Scientific) were amplified with primers listed in Supplemental Table I and cloned into MSCV2.2 retroviral expression construct upstream of an internal ribosome entry site-GFP. MSCV2.2 *Sting* (30) and MSCV2.2 *Sting R231A* (31) were kindly provided by R. Vance (University of California, Berkeley, Berkeley, CA), pCherry and hCas9 (32) were kindly provided by J. Carette (Stanford University). pCherry was replaced by DsRed with mCherry RFP (PubMed ID 15558047) in pDsRed C1 (Clontech). cGAS, *Ifi204*, and Sting KOs were generated in RAW264.7 cells using the CRISPR/Cas9 system (32). Target guide RNA expression constructs were generated from 455-bp gene-specific target RNA sequences listed in Supplemental Table I and cloned into pCR-Blunt cloning vector (Invitrogen). A total of $1 \times 10^6$ RAW264.7 macrophages was transfected with 2.5 μg hCas9, 2.5 μg Target guide RNA, and 0.5 μg pCherry in a six-well tissue culture–treated plate with Targetfect-RAW (Targeting Systems). Two days posttransfection, macrophages were single-cell sorted into 96-well tissue culture–treated plates and allowed to grow up to 2 wk. Genomic DNA from macrophage colonies was extracted using QiAamp DNA mini kit (Qiagen). The targeted DNA sequence was amplified with primers (Supplemental Table I) flanking the mutant target site and sequenced (Eliopharm). Quantitative RT-PCR primers are listed in Supplemental Table I.

**Cell culture, infections, and immunofluorescence**

BMMs were isolated, differentiated, and cultured as previously described (33). Aim2- and ASC-/- BMMs from C57BL/6-*Tmef173* (Sting-/-) (30) and C57BL/6-*cGAS*–/- (34) were kindly provided by R. Vance (University of California, Berkeley, Berkeley, CA) and H. Virgin (Washington University, St. Louis, MO), respectively. Infections with *F. novicida* were performed as previously described (20). For infections with *L. monocytogenes*, log-phase bacteria grown in Brain-Heart Infusion broth at 37°C, shaking, were washed twice with PBS and infected similarly to *F. novicida*. RAW264.7 macrophages and constructed KOs were cultured in DMEM 10% FBS. RAW264.7 macrophages were seeded at a density of $1 \times 10^5$ macrophages per well of a 96-well tissue culture–treated plate or $2.5 \times 10^5$ macrophages per well of a 24-well tissue culture–treated plate and allowed to adhere overnight at 37°C, 5% CO2. Infections were conducted as described for BMMs (20). Immunofluorescence microscopy and Western blots were performed as previously described (23, 35). A minimum of 50 bacterial cells was quantified for LAMP-1 colocalization. Abs used include caspase-1 p10 (sc514; Santa Cruz Biotech), β-actin (M-2; Santa Cruz Biotech), anti-Δ–*novicida* (Monak Laboratory), and anti-LAMP-1 (1D4B; Abcam).

**Retroviral transductions and small interfering RNA knockdown**

Retroviral constructs were transduced into RAW264.7 macrophages using vesicular stomatitis pseudotyped virus packaged in 293FT cells and sorted by FACS to select for GFP+ macrophages. Gene expression in BMMs was knocked down using small interfering RNA (siRNA) and TransIT®-SiQUEST transfection reagent according to manufacturer’s recommen- dations (Mirus). siRNAs used were nontargeting (NT) D0-01206-13, cGAS D-0555608-01, Ddx41 m-052130-00, lrp1p1 m-047145-01, Ifi204 m-044641-01, and RIG-I m-0655328-01 (Dharmacon, GE Healthcare).

**Cytotoxicity, cytokine measurement, and cell stimulations**

Secreted type I IFNs were measured using the ISRE-L929 reporter cells as previously described (29, 36). IL-1β was measured by ELISA (R&D Systems). Cytotoxicity was measured via lactate dehydrogenase release using CytoTox 96 (Non-Radioactive Cytotoxicity Assay; Promega). When the bacteria were RAW264.7 macrophages were stimulated with 100 ng/ml LPS, dsDNA (pCherry) and polyinosinic-polyricydilic acid (Invivogen) were transduced into RAW264.7 macrophages using Targefect-Raw (Targeting Systems). c-di-GMP (Invigene) was transfected into RAW264.7 macrophages using Lipofectamine 2000 (Life Technologies) as previously described (22). *F. novicida* lysates were prepared similarly to previously described protocols (37). In brief, a 3-ml overnight culture was pelleted and resus- pended in 1 ml PBS supplemented with 1 mg/ml lysosome. The cells were lysed using multiple freeze–thaw cycles. Remaining debris was removed by centrifugation, and cleared extracts were adjusted to contain 2 mM MgCl₂, 50 mM KCI, and 20 mM Tris, pH 8.0. A total of 100 μl aliquots was treated with 100 U/ml Dnase I (Invitrogen) or 100 μg/ml RNase A (Qiagen) for 45 min at 37°C. EDTA was added to 2.5 mM, and samples were heated to 70°C for 10 min. DNase I was heat inactivated before treating the lysate by heating the enzyme to 70°C for 20 min. Extracts were cleared by centrifugation, and 5 μl lysate was complexed with 1 μl Targetfect-Raw and transfected into RAW264.7 macrophages.

**Mouse infections**

Mice between 7 and 9 wk were used for in vivo experiments comparing C57BL/6 (28) and C57BL/6-∗Tmef173*+/– (Sting+/–) (The Jackson Laboratory). D. Schneider (Stanford University), with permission from T. Taniguchi (University of Tokyo, Tokyo, Japan), generously provided C57BL/6 and 10 *IFR3*–/- mice (38, 39) 5–16 wk old. Mice were infected by s.c. injection with a target dose of $10^5$ CFU *F. novicida* strain U112. After 72 h, spleens and livers were harvested, weighed, and ground in PBS for CFU determination. For survival experiments, mice were monitored twice daily for 15 d for survival.

**Results**

F. novicida induces a type I IFN response in macrophages that is dependent on cGAS, Ifi204, and STING

Previous studies showed that *F. novicida* releases dsDNA into the macrophage cytosol during infection and elicits a type I IFN response that is dependent on STING (22, 23, 29). We hypothesized that a DNA sensor is necessary to mediate the STING-dependent type I IFN response. To test this notion, we knocked down the expression of known DNA sensors that are important for triggering a type I IFN response (10, 13, 14, 17, 40). siRNAs were generated for Lrfp1, RIG-I, Ddx41, Ifi204, and cGAS and trans- fected into BMMs. Gene expression levels for each siRNA-targeted gene were reduced compared with the NT control after 36 h (Fig. 1A). Upon *F. novicida* infection, siRNA knockdown of cGAS and Ifi204 resulted in reduced type I IFN production, whereas siRNA knockdown of Lrpfp1, RIG-I, and Ddx41 did not influence the type I IFN response (Fig. 1B). The type I IFN response was measured using the L929-ISR E reporter cell line (29, 36). A representative standard curve for purified IFN-β and relative luciferase units (RLUs) is shown (Supplemental Fig. 1A). To confirm that these siRNAs were not indirectly affecting other type I IFN pathways, we stimulated the siRNA knockdowns with LPS and observed similar type I IFN responses across all knockdowns (Fig. 1C). In addition, we isolated BMMs from the recently described *cGAS*–/- (34) mice and examined the type I IFN response to *F. novicida* infection. As expected, the type I IFN response to *F. novicida* infection was dampened in Sting-deficient (Sting–/-) and *cGAS*–/- BMMs compared with WT B6 BMMs (Fig. 1D and Supplemental Fig. 1C). Collectively, these results indicate that cGAS and Ifi204 contribute to the type I IFN response to cytosolic *F. novicida* in BMMs.

To our knowledge, Ifi204-deficient mice are not available. In addition, the siRNA knockdown efficiency of Ifi204 was moderately effective, reducing Ifi204 mRNA levels ∼50% (Fig. 1A),
Despite this modest siRNA knockdown, type I IFN production was significantly reduced upon infection with *F. novicida* compared with the NT control (Fig. 1B). To further dissect the roles of Ifi204 and cGAS in the generation of type I IFNs during *F. novicida* infection of macrophages, we created gene KOs in immortalized RAW264.7 murine macrophages using the newly described CRISPR/Cas9 system (32). However, we first needed to determine whether *F. novicida* infection in RAW264.7 macrophages induces type I IFN responses that are similar to infections of BMMs. It has been previously published that Francisella escapes the initial phagosome and replicates in the cytosol of both C57BL/6 BMMs and RAW264.7 macrophages. At 8 h postinfection only 15.2% of WT *F. novicida* in BMMs and 14% of WT *F. novicida* in RAW264.7 macrophages colocalized with the lysosomal marker LAMP-1 (Fig. 2A). These levels are similar to previous studies (41). Moreover, *F. novicida* replicated to high levels in the cytosol of RAW264.7 macrophages, similar to the first 12 h of infected BMMs (Fig. 2B). In contrast, a *F. novicida* strain lacking the Francisella pathogenicity island (FPI), a predicted type 6 secretion system that is important for escaping the phagosome, colocalized with LAMP-1 at a higher percentage (78% in BMMs and 61% in RAW264.7 macrophages) and did not replicate (Fig. 2A, 2B). The FPI mutant also failed to stimulate type I IFNs in BMMs (Fig. 2D) and dramatically reduced type I IFN stimulation in RAW264.7 macrophages compared with the parental strain (Fig. 2C). In addition, a *F. novicida* strain lacking FopA, a membrane protein important for maintaining cell-wall stability, elicited a higher type I IFN response compared with WT *F. novicida* in both BMMs and RAW264.7 macrophages (Fig. 2C, 2D) (29). One notable difference was that the type I IFN response pattern in RAW264.7 macrophages was delayed compared with BMMs (Supplemental Fig. 1B). We measured type I IFN levels at 24 h postinfection of RAW264.7 macrophages and 12 h postinfection of BMMs (Fig. 2C, 2D). We, and others, have shown that, *F. novicida* infection of BMMs results in cell death, which requires AIM2, ASC, and caspase-1 (23, 24). In contrast, *F. novicida* infection of RAW264.7 macrophages did not result in high levels of cell death (Fig. 2E), which is consistent with the observation that this cell line does not express ASC (42, 43). Moreover, the induction of cell death in BMMs mirrored a reduction in bacterial CFUs in our intracellular replication assays likely due to bacterial exposure to the extracellular antibiotic gentamicin (Fig. 2B). These results indicate that RAW264.7 macrophages are suitable to study mechanisms of type I IFN production during *F. novicida* infections.

To determine whether cGAS, Ifi204, and STING are important factors in mediating the type I IFN response to *F. novicida* in RAW264.7 macrophages, we created out-of-frame biallelic indel mutations in each gene using the CRISPR/Cas9 genome editing system, resulting in functional KOs (32). KOs were verified by DNA sequencing (Supplemental Table II), and two clones were chosen for each gene for further study. As a proof of principle, *F. novicida* infection of Sting KO RAW264.7 macrophages did not produce type I IFNs (Fig. 3A and Supplemental Fig. 1D), similar to infections with Sting-deficient BMMs (Fig. 1D) (22, 23). To verify that the altered type I IFN response was not due to off-target effects of the CRISPR/Cas9 system, we stably expressed STING in Sting KO cells and complemented the type I IFN response to *F. novicida* infection (Fig. 3B). cGAS KO macrophages and Ifi204 KO macrophages infected with WT *F. novicida* strain U112 produced significantly lower amounts of type I IFN compared with the RAW264.7 parental genotype (Fig. 3C, 3E, and Supplemental Fig. 1D). Stable expression of cGAS in cGAS KO macrophages complemented the type I IFN response to *F. novicida* infection, as did stable expression of Ifi204 in Ifi204 KO macrophages (Fig. 3D and 3F, respectively). To further evaluate the contribution of cGAS and Ifi204 to the type I IFN response during a *F. novicida* infection, we infected the KO cell lines with low and high multiplicity of infection (MOIs) and examined two time points: 12 h postinfection, an early time point, and 24 h postinfection. Similar to the siRNA knockdown experiments (Fig. 1B), cGAS deficiency resulted in a more pronounced reduction in type I IFN response to *F. novicida* infection compared with Ifi204 KO cells (Fig. 3G, 3H).

To corroborate our type I IFN data as measured by the L929-ISRE assay, we also measured *IFN-β1* gene expression in each RAW264.7 macrophage cell line 8 h after *F. novicida* infection. *IFN-β1* gene expression increased, albeit to different levels, upon *F. novicida* infection in all macrophage cell lines, but was reduced in expression compared with the RAW264.7 parental genotype in both uninfected and infected conditions (Fig. 3J). To confirm that these cell lines are not altered in type I IFN–independent responses, we measured mRNA expression of *TNF-α*. Each mac-
IFN signaling during *L. monocytogenes* and *F. novicida* in influence type I IFNs in response to infection resulted in comparable type I IFN levels for all macrophage cell lines postinfection (44, 45). TLR4-dependent type I IFN production via LPS stimulation resulted in comparable type I IFN levels for all macrophage cell lines (Fig. 3K). We also stimulated TNF-α and measured 24 h and 12 h postinfection, respectively. (E) Cytotoxicity was determined by measuring LDH release 24 h postinfection. Graphs show the mean ± SD of triplicate wells and are representative of three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001.

FIGURE 2. The type I IFN response to *F. novicida* is similar in BMMs and RAW264.7 macrophages. BMMs and RAW264.7 macrophages were infected with the indicated *F. novicida* strains at an MOI of 10. (A) Immunofluorescence microscopy of BMMs and RAW264.7 macrophages stained for *F. novicida* (green), LAMP-1 (red), and DAPI (blue) 8 h postinfection. Scale bars, 10 μm. (B) Intracellular survival was assessed by CFU plating. Type I IFN levels from (C) RAW264.7 macrophages or (D) C57BL/6 BMMs were measured 24 h and 12 h postinfection, respectively. (E) Cytotoxicity was determined by measuring LDH release 24 h postinfection. Graphs show the mean ± SD of triplicate wells and are representative of three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001.

In addition to viruses and bacteria releasing nucleic acids in the cytosol of infected cells, endogenous nucleic acids, such as retroelements, can also trigger a robust type I IFN response (47, 48). cGAS expression also resulted in lower *Ifi204* gene expression (Supplemental Fig. 2A, 2B). In contrast, reduction of *Ifi204* did not alter cGAS gene expression, and reduced cGAS or *Ifi204* levels did not alter Sting or Ddx41 gene expression. We also examined gene expression changes during an infection. *Ifi204* gene expression increased in WT and cGAS KO RAW264.7 macrophages, but not Sting KO RAW264.7 macrophages (Supplemental Fig. 2C), whereas cGAS expression levels were unchanged (Supplemental Fig. 2E). Interestingly, Sting expression levels were lower in all macrophages during *F. novicida* infection (Supplemental Fig. 2D). These data demonstrate that *Ifi204* and cGAS are important in regulating type I IFN levels in the absence of infection and indicate that a cGAS KO may, in effect, function as a cGAS and *Ifi204* double KO.

**Signaling through both cGAS and *Ifi204* is required for the full type I IFN response to *F. novicida* infection.**

To determine whether the reduced type I IFN response to *F. novicida* in cGAS KO macrophages was due to lower expression of the *Ifi204* gene, we stably expressed *Ifi204* in WT, cGAS KO, *Ifi204* KO, and Sting KO RAW264.7 macrophages. Ectopic expression of *Ifi204* led to increased *Ifi204* mRNA expression in all cells (Fig. 4A). As expected, expression of *Ifi204* in WT and *Ifi204* KO macrophages complemented the type I IFN phenotype in response to *F. novicida* infection, but only modestly increased the type I IFN responses in WT and *Ifi204* KO macrophages (Fig. 4D). Ectopic expression of cGAS or *Ifi204* in...
FIGURE 3. The type I IFN response to cytosolic *F. novicida* requires cGAS, Ifi204, and STING in RAW264.7 macrophages. Type I IFN levels were measured from RAW264.7 (WT) macrophages and the indicated genotypes (A, C, and E) infected with *F. novicida* strains at an MOI of 10 for 24 h or not infected (NI). Type I IFN levels were measured from deficient macrophages stably expressing Sting (B), cGAS (D), or Ifi204 (F) infected with U112 at an MOI of 10 for 24 h. Type I IFN levels were measured from RAW264.7 (WT) macrophages and indicated genotypes infected with *F. novicida* for 12 (G) and 24 h (H) or infected with WT *L. monocytogenes* for 8 h at an MOI of 20 (I). mRNA expression levels of IFN-β (J) and TNF-α (K) from uninfected and U112-infected macrophage cell lines at an MOI of 10 for 8 h. mRNA levels were normalized to GAPDH and the WT RAW264.7 macrophages. (L) Type I IFN levels were measured from macrophages stimulated with LPS for 8 h. NS, not stimulated. Graphs show the mean ± SD of triplicate wells and are representative of three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001.
Sting KO macrophages infected with *F. novicida* did not restore type I IFN production (Fig. 4B, 4D). These data are consistent with previous studies showing cGAS and Ifi204 signal through STING to facilitate the type I IFN response (10, 13). Collectively, our results suggest that Ifi204 and cGAS are both necessary to fully engage STING-dependent type I IFN responses upon *F. novicida* infection.

**Type I IFN induction by cytosolic dsDNA and dsDNA from *F. novicida* lysates is dependent on cGAS and Ifi204**

We showed that cGAS and Ifi204 are key factors in the type I IFN response to *F. novicida* infection (Fig. 3G, 3H). Because cGAS and Ifi204 have both been implicated in cytosolic DNA sensing, we hypothesized that the type I IFN–stimulating PAMP during *F. novicida* infection is DNA (7). To test this, we transfected RAW264.7 macrophages with untreated *F. novicida* lysates or with bacterial lysates treated with DNase or RNase. *F. novicida* lysates stimulated a robust type I IFN response that was abolished when the lysates were treated with DNase I, but not with heat-inactivated DNase I or with RNase A (Fig. 5A). These results suggest that DNA from *F. novicida* lysates is the primary type I IFN stimulus. We next examined the contribution of cGAS, Ifi204, and STING to type I IFN signaling in response to purified dsDNA. Each macrophage cell line was transfected with two different concentrations of dsDNA. Transfection with 0.1 μg/ml dsDNA resulted in a type I IFN response that was dampened in Ifi204 KO and undetectable in cGAS KO, Sting KO, and cGAS/Ifi204 DKO macrophages (Fig. 5B). Transfecting a higher concentration of dsDNA (1 μg/ml) produced an intermediate type I IFN response for both cGAS KO and Ifi204 KO compared with Sting KO macrophages, which produced a nearly undetectable response (Fig. 5B). Surprisingly, the cGAS/Ifi204 DKO macrophages were equally defective for type I IFN signaling as the Sting KO macrophages (Fig. 5B). These results suggest that cGAS and Ifi204 independently contribute to the cytosolic dsDNA type I IFN response.

To determine whether cGAS and Ifi204 are solely involved in the STING-dependent type I IFN signaling pathway, we stimulated type I IFN production through STING-independent mechanisms in the cytosol (44, 45). MDA5-mediated type I IFN production in response to cytosolic dsRNA stimulation also resulted in comparable type I IFN levels (Fig. 5C). In addition, we examined whether cGAS or Ifi204 played a role in responding to bacterial DNA.
cGAS increases inflammasome activity in BMMs

*F. novicida* infection triggers the activation of the AIM2 inflammasome, a host response pathway that is critical for the defense against *F. novicida*, resulting in the secretion of proinflammatory cytokines (IL-1β and IL-18) and caspase-1–dependent pyroptotic cell death (23, 24). Previous work demonstrated AIM2 protein levels are regulated by type I IFNs, and in *Sting*-deficient BMMs, the AIM2 inflammasome is not activated during *F. novicida* infection (23, 24). Thus, we hypothesized that the AIM2 inflammasome response to *F. novicida* infection would also be dampened in the absence of either of the DNA sensors that we showed in this study stimulate STING (cGAS or Ifi204). Using the recently described cGAS−/− mice (34), we examined inflammasome activation by measuring cell death, IL-1β secretion, and caspase-1 processing in response to *F. novicida* infection. As predicted, when WT, *Sting*<sup>−/−</sup>, cGAS<sup>−/−</sup>, Aim2<sup>−/−</sup>, and ASC<sup>−/−</sup> BMMs were infected with *F. novicida*, only WT cells showed appreciable levels of cell death (Fig. 6A), secreted IL-1β (Fig. 6B), and processed caspase-1 (Fig. 6D). Moreover, *F. novicida* replicated to higher levels in *Sting*<sup>−/−</sup>, cGAS<sup>−/−</sup>, Aim2<sup>−/−</sup>, and ASC<sup>−/−</sup> BMMs compared with WT BMMs, consistent with the increased cell death of WT BMMs limiting the intracellular niche (Fig. 6C). Collectively, these results demonstrate that cGAS is important for activating the AIM2 inflammasome during *F. novicida* infection.

**STING and IRF3 deficiency enhances host survival during *F. novicida* infection**

Although type I IFN production during viral infections can lead to increased host survival, there are many examples in which type I IFN production in the context of a bacterial infection is associated with decreased host survival (50–52). Indeed, we have previously shown that mice deficient in the type I IFN signaling receptor, IFNAR, are more resistant to *F. novicida* infection (20, 52). We have shown in this study that both cGAS and Ifi204, which signal through STING, are required to produce the maximum type I IFN levels in response to *F. novicida* infection. Thus, to test the role of the cGAS/Ifi204/STING axis in mice, we infected *Sting*<sup>−/−</sup> mice with *F. novicida*. Mice were infected s.c. with 10<sup>7</sup> *F. novicida* CFU and evaluated for bacterial burdens in the spleen and liver 3 d postinfection. The bacterial loads in the spleen were significantly higher in the WT mice compared with the *Sting*<sup>−/−</sup> mice and similar trends occurred in the liver (Fig. 7A). Mice were also evaluated for their relative susceptibility to *F. novicida* as assessed in a survival experiment (Fig. 7B). The median time to death of WT mice was 5 d, and 80% of this group did not survive the *F. novicida* challenge. In contrast, the median time to death of *Sting*<sup>−/−</sup> mice was >15 d, and 90% of this group survived the infection. To corroborate our findings that type I IFN–dependent STING signaling was detrimental to the host during *F. novicida* infection, we also infected IRF3-deficient mice with *F. novicida*. IRF3 is the transcription factor activated by STING to induce the transcription of IFN-β and other ISGs (9, 53). Similar to *Sting*<sup>−/−</sup> mice, *IRF3*<sup>−/−</sup> mice infected s.c. with 2 × 10<sup>7</sup> *F. novicida* CFU were significantly more resistant to *F. novicida* infection compared with WT mice (Fig. 7C). The median time to death for WT mice and *IRF3*<sup>−/−</sup> mice was 4 and >15 d, respectively. These results demonstrate that STING- and IRF3-dependent signaling is detrimental to the host during a *F. novicida* infection.

**Discussion**

Recognition of microbial pathogens is essential to initiate an effective immune response. Host cells have developed numerous strategies to identify infection and tissue injury. One mode of detection is surveying the cytosol for the presence of nucleic acids. Although the importance of recognizing and responding to nucleic acids in the cytosol is well appreciated for viral infections, the role of nucleic acids triggering the type I IFN response during bacterial infections is not well understood. To complicate matters, recent studies have identified numerous cytosolic receptors important for type I IFN signaling in response to dsDNA. We were interested in identifying the host sensors important for type I IFN signaling in response to bacterial infections. In this study, we showed that...
during infection with the cytosolic bacterium, *F. novicida*, the primary type I IFN response is likely dependent on the presence of *F. novicida* DNA in the cytosol. Moreover, we demonstrated that two proposed host DNA sensors, cGAS and Ifi204, are required to generate type I IFNs during *F. novicida* infection and in response to cytosolic dsDNA in murine macrophages.

We observed that during a *F. novicida* infection, both cGAS and Ifi204 were necessary to mediate a full type I IFN response (Fig. 4). In addition, we show that both cGAS and Ifi204 need to be eliminated (e.g., cGAS/Ifi204 DKO in RAW264.7 cells) to prevent a type I IFN response to high concentrations of transfected DNA in RAW264.7 macrophages. This result was surprising given that cGAS alone was previously demonstrated to bind cytosolic DNA and generate the STING-activating second messenger, cGAMP, resulting in type I IFN production (8, 10, 54, 55). This discrepancy in findings may be attributed to fundamental variances in cell types, including differences in transfection efficiency and/or alterations in the Ifi204 expression signature. In particular, we observe that transfecting RAW264.7 macrophages using standard transfection reagents (i.e., Lipofectamine 2000 and Targefet-Raw) results in a considerably higher percentage of cells being transfected compared with BMMs and much lower cytotoxicity (data not shown). These factors may permit detection of intermediate to low type I IFN responses that are not seen in cGAS−/− BMMs (54). However, further studies in a variety of cell types are needed to assess the role and/or expression of Ifi204 in the absence of cGAS. It also remains to be determined how Ifi204, a member of AIM2-like receptors (ALRs) (49), fits into the cGAS/STING/type I IFN pathway. To date, 13 ALR genes have been identified within the mouse genome (49). They are all encoded within a single continuous locus and contain a Pyrin and/or HIN domain. One possible model for the requirement of Ifi204 in the cGAS/STING/type I IFN pathway is that Ifi204, either due to higher affinity for dsDNA or to appropriate subcellular localization, may initially recognize *F. novicida* DNA and transport the DNA ligand to cGAS. This would result in the production of cGAMP and subsequent activation of STING to trigger type I IFN production. Evidence for this model is supported by previous studies, which demonstrate that the human ortholog of Ifi204, IFI16, interacts with STING upon DNA stimulation (13). In addition, Ifi204 and STING were found to colocalize in the cytosol when transfected in HeLa cells (49). Future studies are required to resolve the mechanism behind the necessity of Ifi204 and to determine whether other ALR proteins are important in facilitating the cGAS-dependent signaling response to intracellular bacteria and potentially other stimuli.

Access of *F. novicida* DNA to the host cytosol is a requirement to activate two independent host responses. First, *F. novicida* DNA is likely recognized by cGAS and Ifi204 to trigger a STING-dependent type I IFN response. Through autocrine and paracrine type I IFN signaling, AIM2 protein levels increase and subsequently associate with *F. novicida* DNA and ASC to activate caspase-1–mediated cell death and secretion of proinflammatory cytokines (23). Although the mechanism of DNA release by *F. novicida* into the host cytosol is not fully known, *F. novicida* mutants that are prone to increased bacterial lysis trigger higher type I IFNs and inflammasome responses compared with the parental strain (29). This observation suggests that one mechanism of facilitating access of the DNA sensors to *F. novicida* DNA is a low level of bacterial lysis in the cytosol. One notable difference between the two cell types we used in this study is that the FPI mutant elicits a type I IFN response that is higher than the levels produced by uninfected RAW264.7 macrophages (Supplemental Fig. 1D). In contrast, the levels of type I IFN produced by BMMs infected with the FPI mutant are not higher than uninfected BMMs (Supplemental Fig. 1D). These results may be attributed to the higher proportion of FPI mutant bacteria found in the cytosol of RAW264.7 macrophages compared with BMMs. These studies underscore the importance of DNA recognition during a bacterial infection. This is particularly important for a bacterial pathogen like Francisella that is a stealth invader and does not elicit a TLR-mediated type I IFN response (20).

Many intracellular bacterial pathogens elicit a type I IFN host response, and several of these have been demonstrated to require STING, including *L. monocytogenes*, *M. tuberculosis*, and *C. trachomatis* (5, 11, 56). In addition to STING, the type I IFN response in immortalized BMMs to *M. tuberculosis* infection was also demonstrated to require Ifi204 (56). In this article, we demonstrated that the STING-dependent type I IFN response elicited by *L. monocytogenes* did not require the DNA sensors, cGAS or Ifi204 (Fig. 3I). These results are consistent with previous findings demonstrating that *L. monocytogenes* secretes cyclic-di-AMP to directly stimulate STING in murine cells (11). Notably, a recent study showed *L. monocytogenes* infection in human macrophages induced the type I IFN response that is dependent on cGAS, IFI16 (Ifi204 homolog), and STING (57). Further studies are needed to elucidate the roles of cGAS and Ifi204/IFI16 in recognizing other intracellular bacterial pathogens and potential differences between human and mouse type I IFN signaling pathways.

The role of type I IFNs in controlling bacterial infections is complex. Wild-type mice infected with *L. monocytogenes*, *F. novicida*, and *M. tuberculosis* support increased bacterial burdens compared with mice deficient for the type I IFN receptor (*Ifnar1−−*) (50–52). Although these studies were initially surprising, it is now known that type I IFN production leads to the transcription of hundreds of ISGs, which modulate a variety of factors in both innate and adaptive immunity (3). In the case of a *F. novicida* infection, *Ifnar1−−* mice are more resistant to infection largely due to an increased expansion of IL-17A+ γδ T cells and increased splenic neutrophils (52). The role of cGAS and Ifi204 during a *F. novicida* infection in vivo is unknown.
Because cGAS and Ifi204 both require STING for type I IFN production, we sought to evaluate STING-deficient mice for their susceptibility to F. novicida infection. Previous studies using a different Francisella subspecies, F. tularensis subspecies holarctica live vaccine strain, and different infection route (i.p.) did not identify a difference in splenic bacterial loads between STING (MPYS)-deficient mice and WT mice 48 h postinfection (22). This finding is similar to our previous findings using IFNAR−/− mice, in which there is no difference in bacterial burdens 1 and 2 d postinfection (52). Importantly, we show in this article that there is no difference in bacterial burdens 1 and 2 d postinfection postulate that both F. novicida infection compared with WT mice (Fig. 7A). Furthermore, we show that mice deficient for the downstream transcription factor, IRF3, were ried lower bacterial burdens in the liver and spleen 3 d postin-

mediated type I IFN production may discover unique signaling involved may aid in our therapeutic efforts to prevent and treat infections (23).

Our results begin to illuminate the mechanisms involved in type I IFN production during intracellular bacterial infections. Our data strongly support the hypothesis that F. novicida DNA is sensed by cGAS and Ifi204 to trigger the STING-dependent type I IFN response. Future experiments investigating the cross talk between TLR-mediated type I IFN production and cytosolic DNA-mediated type I IFN production may discover unique signaling signatures depending on the host receptor activated. Understanding how type I IFN production is regulated and what factors are involved may aid in our therapeutic efforts to prevent and treat autoinflammatory and infectious diseases.

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Disclosures

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