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

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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 response to *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: 3236–3245.**

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-1–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-β1* (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, Lrrfip1, 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-18 and IL-1β) and

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Abbreviations used in this article: AIM2, absent in melanoma 2; ALR, AIM2-like receptor; BMM, bone marrow–derived macrophage; cGAMP, cyclic GMP-AMP; cGAS, cyclic GMP-AMP synthase; FPI, *Francisella* pathogenicity island; IRF, IFN regulatory factor; ISG, IFN-stimulated gene; KO, knockout; MOI, multiplicity of infection; NT, nontargeting; PAMP, pathogen-associated molecular pattern; RLU, relative luciferase unit; siRNA, small interfering RNA; STING, stimulator of IFN genes; WT, wild-type.

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*FPI* (28), and Δ*fopA* (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 created by replacing 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 blocks (IDT) containing target RNA sequences listed in Supplemental Table II cloned into pCR-Blunt cloning vector (Invitrogen). A total of 1×10^6 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 Targefect-RAW (Targeting Systems). Two days posttransfection, macrophages were single-cell sorted into 96-well tissue culture–treated plates and allowed to grow up ∼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 (Elim Biopharm). 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$^{-/-}$ BMM femurs from C57BL/6-*Tmem173*^{gt} (*Sting*^{gt}) (30) and C57BL/6-*cGAS*$^{-/-}$ (34) were kindly provided by R. Vance (University of California, Berkeley) 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 1×10^5 macrophages per well of a 96-well tissue culture–treated plate or 2.5×10^5 macrophages per well of a 24-well tissue culture–treated plate and allowed to adhere overnight at 37°C, 5% CO_2. 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–*F. novicida* (Monack 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 recommendations (Mirus). siRNAs used were nontargeting (NT) D-001206-13, *cGAS* D-0555608-01, *Ddx41 m*-052130-00, *lrrfip1 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 specified, RAW264.7 macrophages were stimulated with 100 ng/ml LPS. dsDNA (pCherry) and polyinosinic-polycytidylic acid (Invivogen) were transfected into RAW264.7 macrophages using Targefect-Raw (Targeting Systems). c-di-GMP (Invivogen) 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 resuspended in 1 ml PBS supplemented with 1 mg/ml lysozyme. The cells were lysed using multiple freeze–thaw cycles. Remaining debris was removed by centrifugation, and cleared extracts were adjusted to contain 2 mM $MgCl_2$, 50 mM KCl, 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 Targefect-Raw and transfected into RAW264.7 macrophages.

Mouse infections

Mice between 7 and 9 wk were used for in vivo experiments comparing C57BL/6J mice and C57BL/6J-*Tmem173*^{gt}/J (*Sting*$^{gt/gt}$) (The Jackson Laboratory). D. Schneider (Stanford University), with permission from T. Taniguchi (University of Tokyo, Tokyo, Japan), generously provided 9 C57BL/6 and 10 *IRF3*$^{-/-}$ mice (38, 39) 8–16 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 *Lrrfip1*, *RIG-I*, *Ddx41*, *Ifi204*, and *cGAS* and transfected 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 *Lrrfip1*, *RIG-I*, and *Ddx41* did not influence the type I IFN response (Fig. 1B). The type I IFN response was measured using the L929-ISRE 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*$^{gt/gt}$) 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 only moderately effective, reducing *Ifi204* mRNA levels ∼50% (Fig. 1A).

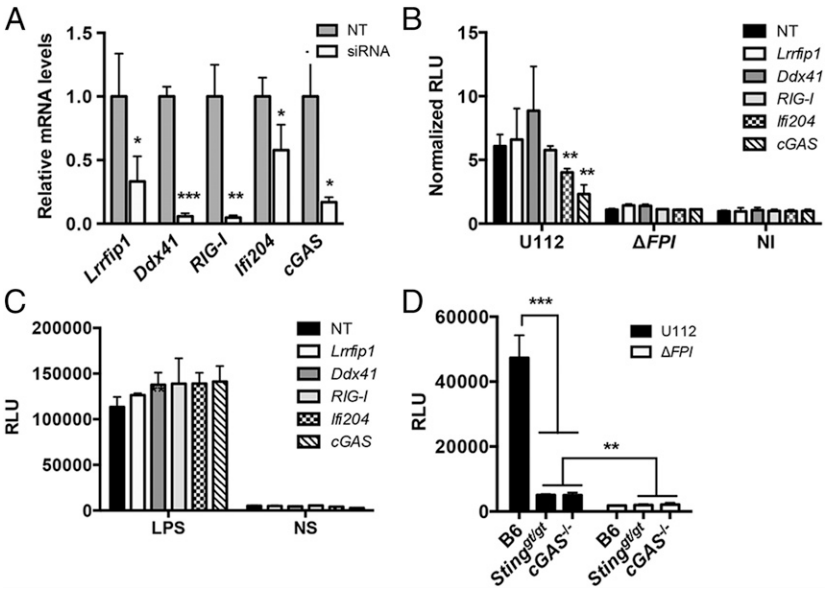

FIGURE 1. cGAS and Ifi204 are required for type I IFN production in response to cytosolic *F. novicida* in BMMs. siRNA targeting known cytosolic sensor genes or an NT control was transfected into BMMs for 36 h. (**A**) Quantitative RT-PCR measured mRNA levels for each targeted gene in uninfected BMMs. Gene expression was normalized to GAPDH and the NT control. Type I IFN levels were measured (**B**) 9 h postinfection with the indicated *F. novicida* strain at an MOI of 10 or (**C**) 4 h poststimulation with LPS. Results are presented as RLUs. Data presented as normalized RLUs are type I IFN levels normalized to the uninfected, NT control. (**D**) Type I IFN levels were measured from unstimulated primary C57BL/6 (WT), *Sting*$^{gt/gt}$, and *cGAS*$^{-/-}$ BMMs infected with *F. novicida* strains at an MOI of 10 for 12 h. Graphs show the mean ± SD of triplicate wells and are representative of three independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

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 BMMs (41). We confirmed that WT *F. novicida* quickly and efficiently escaped the *Francisella*-containing vacuole and entered 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-

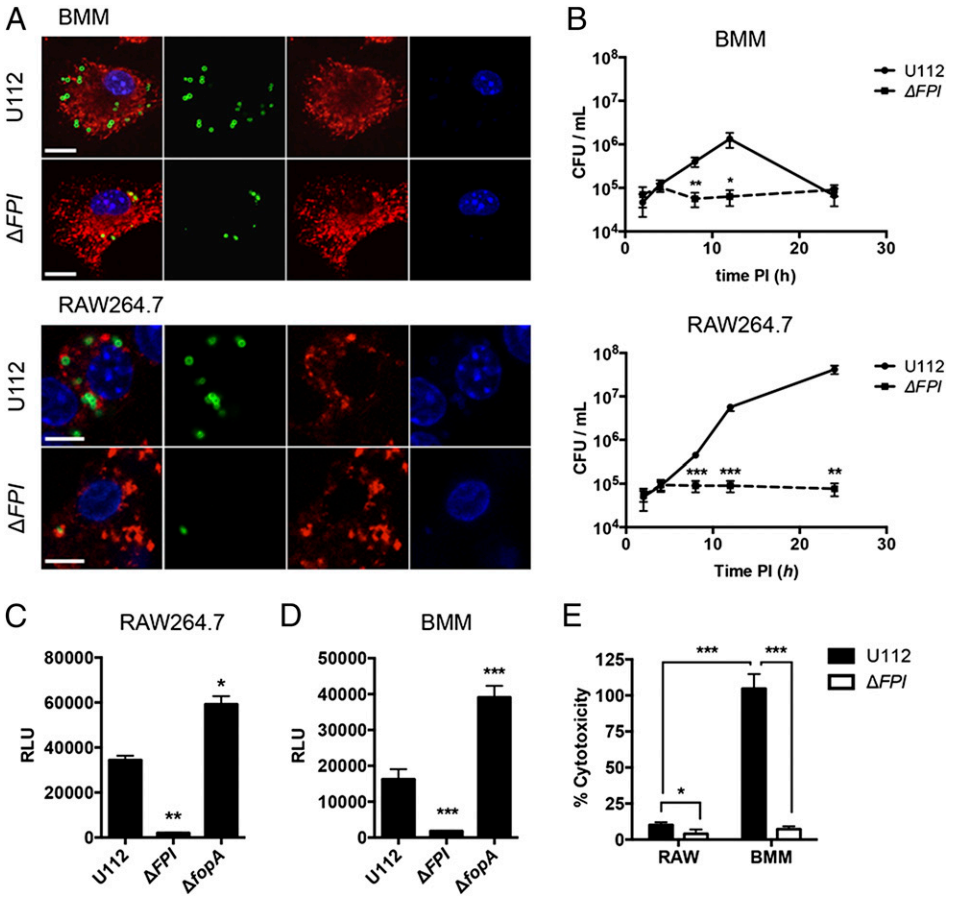

FIGURE 2. The type I IFN response to *F. novicida* is similar in BMMs and RAW264.7 macrophages. BMMs and RAW264.7 macrophages were infected with the indicated *F. novicida* strains at an MOI of 10. (**A**) Immunofluorescence microscopy of BMMs and RAW264.7 macrophages stained for *F. novicida* (green), 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. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

rophage cell line expressed similar levels of *TNF-α* before infection, and there were no differences in the levels of *TNF-α* mRNA between the cell lines postinfection (Fig. 3K). We also stimulated type I IFN production through STING-independent mechanisms (44, 45). TLR4-dependent type I IFN production via LPS stimulation resulted in comparable type I IFN levels for all macrophage cell lines (Fig. 3L). Thus, cGAS, Ifi204, and STING specifically influence type I IFNs in response to *F. novicida* infection.

We next tested whether cGAS and Ifi204 are important for type I IFN signaling during *L. monocytogenes* infection, which is another cytosolic bacterial pathogen known to trigger a robust STING-dependent type I IFN response (30, 46). Although *L. monocytogenes* can directly activate STING through the secretion of c-di-AMP (11), it is unknown whether *L. monocytogenes* DNA is an important ligand during infection and whether cGAS and Ifi204 contribute to the type I IFN response. We infected WT, *cGAS*, *Ifi204*, and *Sting* KO macrophages with *L. monocytogenes* and found that only the *Sting* KO macrophages had reduced type I IFN levels (Fig. 3I). These results suggest that cGAS and Ifi204 are important for triggering the type I IFN response to *F. novicida*, but not *L. monocytogenes*, in RAW264.7 macrophages.

cGAS regulates Ifi204 expression

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). Not surprisingly, we found that reducing either *cGAS* or *Ifi204* expression levels resulted in lower endogenous *IFN-β1* gene expression and other IFN-stimulated genes (ISGs), including *RIG-I* (Supplemental Fig. 2A, 2B). *Ifi204* is an ISG (49), and reduction of *cGAS* gene 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 response in *cGAS* KO (Fig. 4B). From these data, we hypothesized that cGAS was epistatic to Ifi204. To test this, we ectopically expressed *cGAS* in an *Ifi204* KO and measured type I IFN levels after *F. novicida* infection. Surprisingly, ectopic expression of *cGAS* in *Ifi204* KO macrophages did not rescue the ability of *Ifi204* KO macrophages to secrete type I IFN (Fig. 4D). Importantly, ectopic expression of *cGAS* increased *cGAS* mRNA levels in all macrophages (Fig. 4C) and type I IFN production in WT and *cGAS* KO macrophages (Fig. 4D). Ectopic expression of *cGAS* or *Ifi204* in

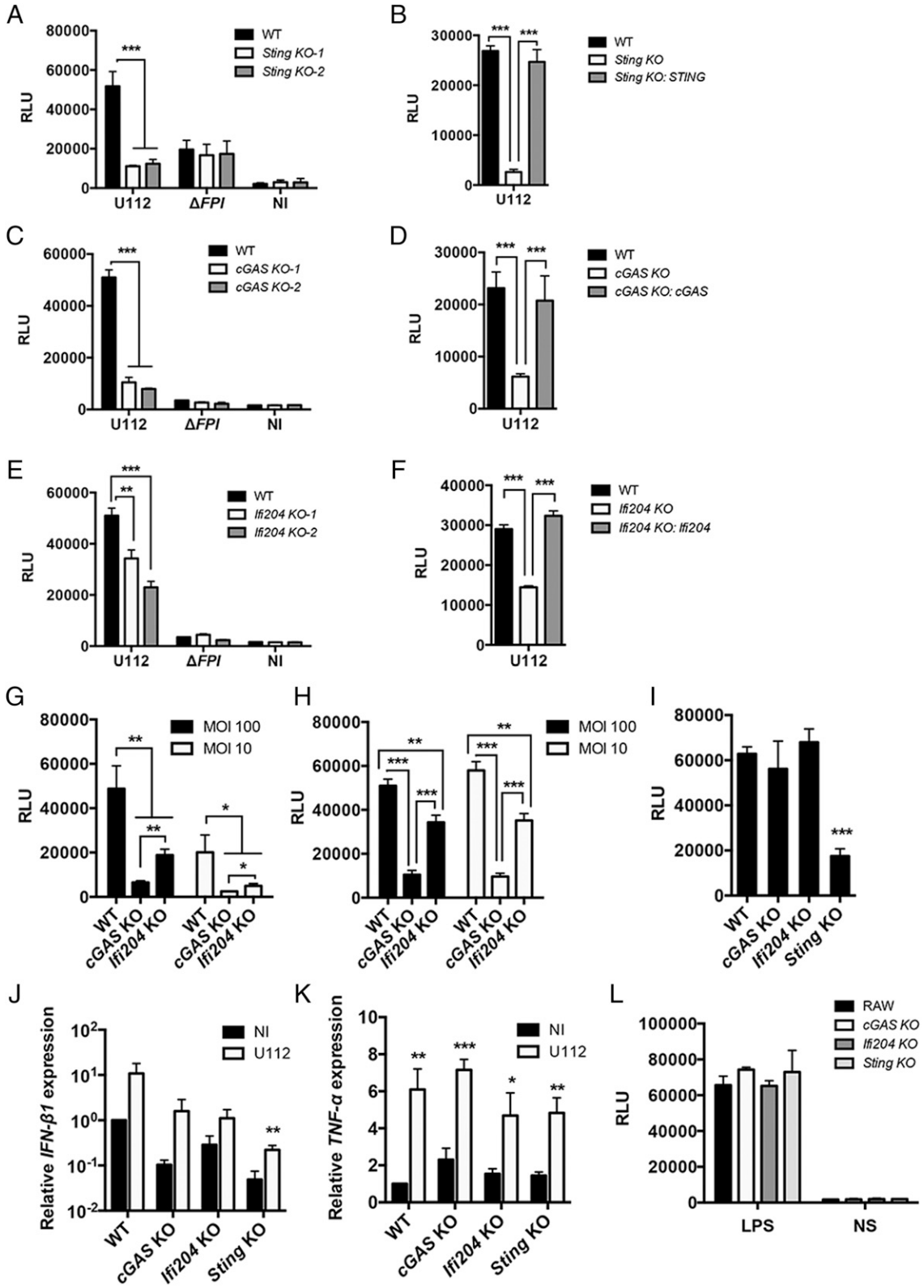

FIGURE 3. The type I IFN response to cytosolic *F. novicida* requires cGAS, Ifi204, and STING in RAW264.7 macrophages. Type I IFN levels were measured from RAW264.7 (WT) macrophages and the indicated genotypes (**A**, **C**, and **E**) infected with *F. novicida* strains at an MOI of 10 for 24 h or not 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-β1* (**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. Graphs show the mean ± SD of triplicate wells and are representative of three independent experiments. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. NS, not stimulated.

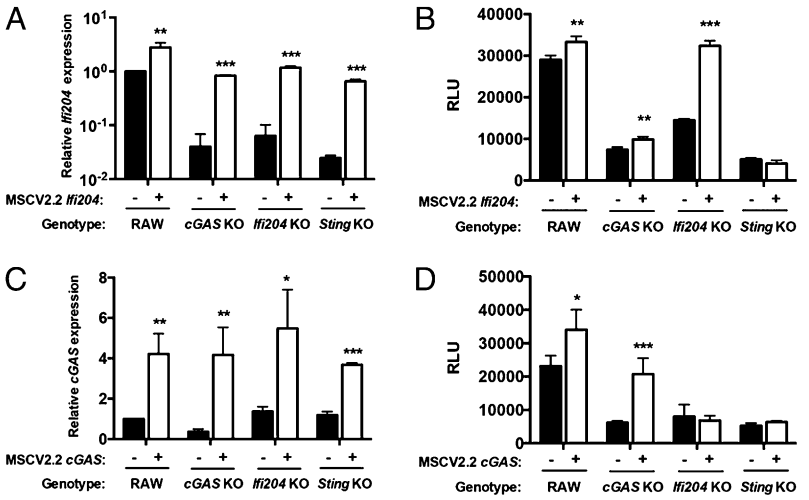

FIGURE 4. Both cGAS and Ifi204 are required for the full type I IFN response to *F. novicida* infection. mRNA expression levels were measured in uninfected RAW264.7 (WT) cell lines stably expressing *Ifi204* (**A**) or *cGAS* (**C**). mRNA expression was normalized to *GAPDH* and WT RAW264.7 macrophages. Macrophages were infected with WT *F. novicida* at an MOI of 10 for 24 h and type I IFN levels measured (**B** and **D**, respectively). 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

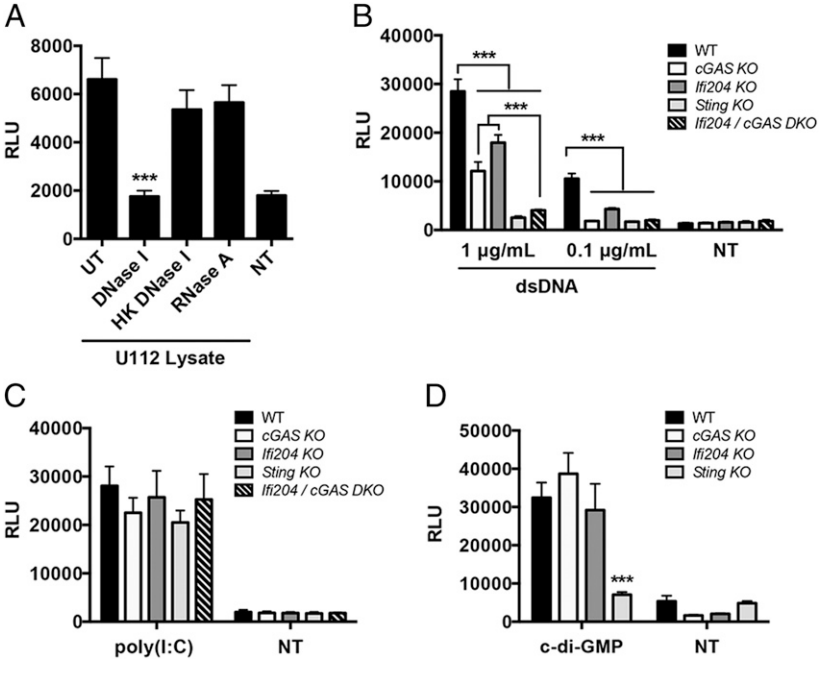

FIGURE 5. cGAS and Ifi204 cooperate to sense cytosolic dsDNA. (**A**) Type I IFN levels were measured from WT RAW264.7 macrophages transfected for 24 h with U112 lysates either untreated (UT) or treated with DNase I, heat-killed (HK) DNase I or RNase A. Type I IFN levels were measured from RAW264.7 macrophages transfected for 24 h with (**B**) endo-free plasmid DNA (pCherry), (**C**) 1 μg/ml polyinosinic-polycytidylic acid [poly(I:C)], or (**D**) 10 μg/ml c-di-GMP NT, not transfected. Graphs show the mean ± SD of triplicate wells and are representative of three independent experiments. $***p < 0.001$.

cyclic-dinucleotides. Transfection of c-di-GMP led to similar type I IFN responses in WT, *cGAS* KO, and *Ifi204* KO macrophages (Fig. 5D). In contrast, *Sting* KO macrophages were defective for signaling in response to c-di-GMP (Fig. 5D), which is consistent with previous studies (31). Our results demonstrate that cGAS and Ifi204 cooperate to signal through the STING-dependent type I IFN pathway in response to cytosolic dsDNA. In addition, these data support dsDNA as the primary *F. novicida* PAMP that triggers the type I IFN response.

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, $Sting^{gt/gt}$, $cGAS^{-/-}$, $Aim2^{-/-}$, and $ASC^{-/-}$ 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 $Sting^{gt/gt}$, $cGAS^{-/-}$, $Aim2^{-/-}$, and $ASC^{-/-}$ 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 $Sting^{gt/gt}$ mice with *F. novicida*. Mice were infected s.c. with 10^5 *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 $Sting^{gt/gt}$ 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 $Sting^{gt/gt}$ 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 $Sting^{gt/gt}$ mice, $IRF3^{-/-}$ mice infected s.c. with 2×10^5 *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 $IRF3^{-/-}$ 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

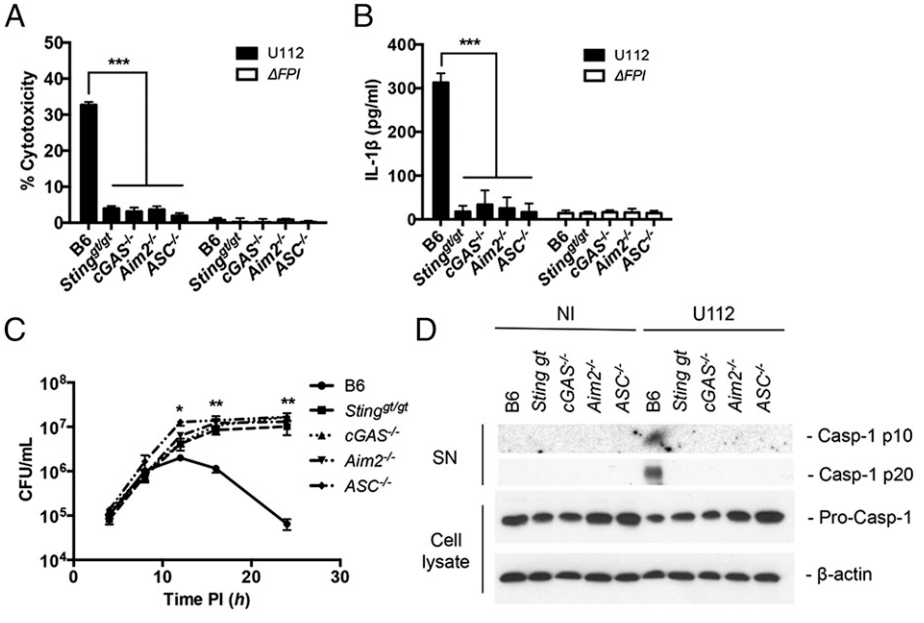

FIGURE 6. cGAS increases inflammasome activity in BMMs. (**A**) Cytotoxicity, (**B**) IL-1β secretion, and (**C**) intracellular survival were measured from unstimulated primary C57BL/6 (WT), $Sting^{gt/gt}$, $cGAS^{-/-}$, $Aim2^{-/-}$, and $ASC^{-/-}$ BMMs infected with *F. novicida* strains at an MOI of 10 for 12 h unless otherwise indicated. (**D**) Release of processed caspase-1 (casp-1 p10 and p20) into the supernatants (SN) was measured by Western blot from cells either not infected (NI) or infected with WT *F. novicida* at an MOI of 100 for 12 h. Corresponding cell lysates were probed for procaspase-1 and β-actin. Graphs show the mean ± SD of triplicate wells and are representative of three independent experiments. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

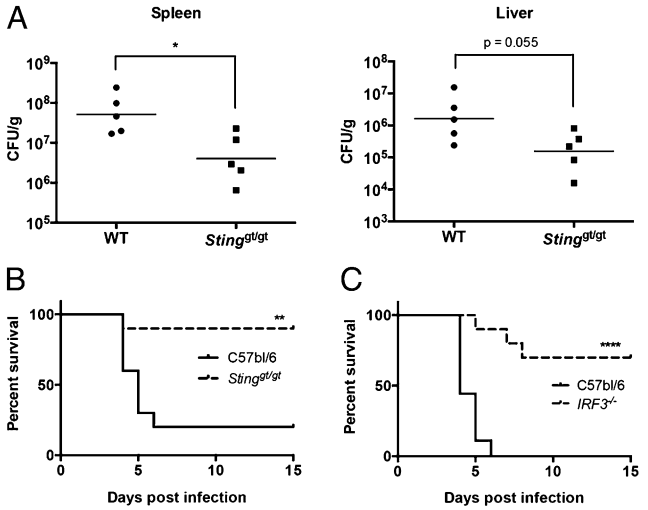

FIGURE 7. STING and IRF3 are detrimental to host survival during *F. novicida* infection. WT and $Sting^{gt/gt}$ mice were infected s.c. with 10^5 CFU U112. (**A**) The spleen and liver from five infected WT and $Sting^{gt/gt}$ mice were harvested and plated for CFU/g 3 d postinfection; geometric mean is shown. (**B**) Ten WT and 10 $Sting^{gt/gt}$ mice were monitored twice daily over 15 d for survival. (**C**) Nine WT and 10 $IRF3^{-/-}$ mice were infected s.c. with 2×10^5 CFU U112 and were monitored twice daily over 15 d for survival. $*p < 0.05$, $**p < 0.01$, $****p < 0.0001$.

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 Targefect-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 stimulated 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 signaling, we sought to evaluate *Sting*$^{gt/gt}$ 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 *Sting*$^{gt/gt}$ mice carried lower bacterial burdens in the liver and spleen 3 d postinfection and were significantly more resistant to *F. novicida* infection compared with WT mice (Fig. 7A). Furthermore, we show that mice deficient for the downstream transcription factor, IRF3, were also significantly more susceptible to *F. novicida* infection. We postulate that both *Sting*$^{gt/gt}$ mice and *Irf3*$^{-/-}$ mice are more resistant to infection due to similar mechanisms described for *IFNAR1*$^{-/-}$ mice (52). Despite lower bacterial burdens found in *Sting*$^{gt/gt}$ mice and *IFNAR1*$^{-/-}$ mice, the type I IFN response is important in activating specific host responses important for bacterial control, including the AIM2 inflammasome during *F. novicida* 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

The authors have no financial conflicts of interest.

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