cGAS and Ifi204 cooperate to produce type I interferon in response to *Francisella* infection

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

[*]Stanford University, Department of Microbiology and Immunology, 299 Campus Drive, Stanford, CA USA 94305

[†]Genentech Inc, 1 DNA Way, South San Francisco, CA USA 94080

Abstract

Type I interferon (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, 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. Additionally, we showed that dsDNA from *F. novicida* is an important type I IFN stimulating ligand. One outcome of cGAS-STING signaling is the activation of the AIM2 inflammasome in response to *F. novicida* infection. While the AIM2 inflammasome is beneficial to the host during *F. novicida* infection, type I IFN signaling by STING and IRF3 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.

Introduction

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 interferon (IFN) transcriptional response, which can prompt a broad range of additional responses to infection, including caspase-1-mediated cell death and pro-inflammatory cytokine release (2, 3). While, it is appreciated that many bacterial species, including *Francisella tularensis, Listeria monocytogenes, Salmonella typhimurium,*

[‡]Corresponding author: dmonack@stanford.edu. Phone: 650-725-4754. Fax: 650-725-7282.

Mycobacterium tuberculosis, and C*hlamydia 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 Toll-like receptor (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 phophorylates IRF-3, 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 (DAI), Lrrfip1, Ifi204 (human: IFI16), Mre11, DNA-dependent protein kinase (DNA-PK), 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 (FCV) and replicates in the cytosol (19). Cytosolic *F. novicida* trigger a pro-inflammatory 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 pro-inflammatory cytokines (IL-18 and IL-1β) 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 BMMs (bone-marrow macrophages) and RAW264.7 macrophages. Using targeted knockouts and complementation vectors, we demonstrated that cGAS and Ifi204 both contribute to STING-dependent type I IFN response to high concentrations of cytosolic dsDNA. Additionally, we show 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 knockout cell lines

Bacterial strains used in this study include wild-type *F. novicida* U112, ΔFPI (28), and ΔfopA (29) and wild-type *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 on Table S1 and cloned into MSCV2.2 retroviral expression construct upstream of an internal ribosome entry site (IRES)-green fluorescent protein (GFP). MSCV2.2 *Sting* (30) and MSCV2.2 *Sting* R231A(31) were kindly provided by R. Vance. pCherry and hCas9(32) were kindly provided by J. Carette. pCherry was created by replacing DsRed with mCherry RFP (Pubmed ID: 15558047) in pDsRed C1 (Clontech). *cGAS, Ifi204* and *Sting* knockouts were generated in RAW264.7 cells using the CRISPR/ Cas9 system (32). Target gRNA expression constructs were generated from 455bp gene blocks (IDT) containing target RNA sequences listed in Table S2 cloned into pCR-Blunt cloning vector (Invitrogen). 1×10^6 RAW264.7 macrophages were transfected with 2.5 μg hCas9, 2.5 μg Target gRNA and 0.5 μg pCherry in a 6-well tissue culture-treated plate with Targefect-RAW (Targeting Systems). Two days post-transfection, macrophages were single-cell sorted into 96-well tissue culture-treated plates and allowed to grow up ~ 2 weeks. Genomic DNA from macrophage colonies was extracted using QIAamp DNA mini kit (Qiagen). The targeted DNA sequence was amplified with primers (Table S1) flanking the mutant target site and sequenced (Elim Biopharm). qRT-PCR primers are listed in Table S1.

Cell culture, infections and immunofluorescence

Bone marrow-derived macrophages (BMMs) were isolated, differentiated, and cultured as previously described (33). Aim2−/− and ASC−/− BMMs Femurs from C57BL/6-*Tmem173*[gt] (*Sting*[gt])(30) and C57BL/6-*cGAS*^{−/−} (34) were kindly provided by R. Vance and H. Virgin, respectively. Infections with *F. novicida* were performed as previously described (20). For infections with *L. monocytogenes*, log-phase bacteria grown in Brain-Heart Infusion (BHI) 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% fetal bovine serum (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 were quantified for LAMP-1 colocalization. Antibodies 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 siRNA 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 siRNA and

TransIT-siQUEST transfection reagent according to manufacture's recommendations (Mirus). siRNAs used were: Non-targeting (NT) D-001206-13, *cGAS* D-0555608-01, *Ddx41* m-052130-00, *lrrfip1* m-047145-01, *Ifi204* m-044641-01, *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 with100 ng/mL lipopolysaccharide (LPS). dsDNA (pCherry) and poly(I:C) (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). Briefly, 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 2mM $MgCl_2$, 50mM KCl and 20mM Tris, pH 8.0. 100 µl aliquots were 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 prior to treating the lysate by heating the enzyme to 70°C for 20 min. Extracts were cleared by centrifugation and 5 µl of lysate was complexed with 1 µl targefect-RAW and transfected into RAW264.7 macrophages.

Mouse infections

Mice between seven- to nine- weeks were used for in vivo experiments comparing C57Bl/6J mice and C57Bl/6J-*Tmem173^{gt}*/J (*Sting$^{gt/gt}$*) (The Jackson Laboratory). David Schneider, with permission from Tadatsugu Taniguchi, generously provided 9 C57Bl/6 and ten *IRF3$^{-/-}$* mice (38, 39) eight- to 16-wks-old. Mice were infected by subcutaneous (s.c.) injection with a target dose of 10^5 CFU of *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 days 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). Small interfering RNAs (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 to the non-targeting (NT) control after 36 h (Fig. 1A). Upon *F. novicida* infection, siRNA knockdown of *cGAS* and *Ifi204* resulted in reduced type I IFN production, while 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 (Fig. S1A). 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). Additionally, we isolated BMMs from the recently described *cGAS*$^{-/-}$ 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 to WT B6 BMMs (Fig. 1D and Fig. S1C). 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. Additionally, the siRNA knockdown efficiency of *Ifi204* was only 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 to 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 knockouts 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 wild-type *F. novicida* quickly and efficiently escaped the FCV and entered the cytosol of both C57Bl/6 BMMs and RAW264.7 macrophages. At 8 h post-infection only 15.2% of wild-type *F. novicida* in BMMs and 14% of wild-type *F. novicida* in RAW264.7 macrophages co-localized 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, co-localized 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 to the parental strain (Fig 2C). Additionally, a *F. novicida* strain lacking FopA, a membrane protein important for maintaining cell wall stability, elicited a higher type I IFN response compared to wild-type *F. novicida* in both BMMs and RAW264.7 macrophages (Fig. 2C and Fig. 2D)(29). One notable difference was that the type I IFN response pattern in RAW264.7 macrophages was delayed compared to BMMs (Fig. S1B). We measured type I IFN levels at 24 h post-infection of RAW264.7 macrophages and 12 h post-infection of BMMs (Fig. 2C and Fig. 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 if 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 knockouts (32). Knockouts were verified by DNA sequencing (Table S2) and two clones were chosen for each gene for further study. As a proof of principle, *F. novicida* infection of *Sting* knockout (KO) RAW264.7 macrophages did not produce type I IFNs (Fig. 3A and Fig. S1D), 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 wild-type *F. novicida* strain U112 produced significantly lower amounts of type I IFN compared to the RAW264.7 parental genotype (Fig 3C, Fig 3E and Fig. S1D). 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 Fig. 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 knockout cell lines with low and high MOIs and examined two time points: 12 h post-infection, an early time point, and 24 h post-infection. 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 to *Ifi204* KO cells (Fig. 3G and Fig. 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 to the RAW264.7 parental genotype in both un-infected 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 macrophage 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 after infection (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 *Listeria monocytogenes* infection, which is another cytosolic bacterial pathogen known to trigger a robust STING-dependent type I IFN response (30, 46). While *L. monocytogenes* can directly activate STING through the secretion of c-di-AMP (11), it is unknown if *L.*

monocytogenes DNA is an important ligand during infection and whether cGAS and Ifi204 contribute to the type I IFN response. We infected wild-type, *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 interferon-stimulated genes (ISGs), including *RIG-I* (Fig. S2A and Fig. S2B). *Ifi204* is an ISG (49), and reduction of *cGAS* gene expression also resulted in lower *Ifi204* gene expression (Fig. S2A and Fig. S2B). 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 wild-type and *cGAS* KO RAW264.7 macrophages, but not *Sting* KO RAW264.7 macrophages (Fig. S2C), while *cGAS* expression levels were unchanged (Fig. S2E). Interestingly, *Sting* expression levels were lower in all macrophages during *F. novicida* infection (Fig. S2D). 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 knockout.

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 wild-type, *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 wild-type 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 wild-type and *cGAS* KO macrophages (Fig. 4D). Ectopic expression of *cGAS* or *Ifi204* in *Sting* KO macrophages infected with *F. novicida* did not restore type I IFN production (Fig. 4B and Fig. 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 and Fig. 3H). Since 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 of 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 to *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 if 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). Additionally, we examined whether cGAS or Ifi204 played a role in responding to bacterial cyclic-dinucleotides. Transfection of c-di-GMP led to similar type I IFN responses in wild-type, *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. Additionally, 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 pro-inflammatory 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 here 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 wild-type 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 to 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

While 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 PI. The bacterial loads in the spleen were significantly higher in the wild-type mice compared to 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 wild-type 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*. Interferon regulatory factor 3 (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 to wild-type mice (Fig. 7C). The median time to death for wild-type mice and *IRF3$^{-/-}$* mice was 4 d 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. While 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 show 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 demonstrate 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 to 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 orthologue of Ifi204, IFI16, interacts with STING upon DNA stimulation (13). Additionally, Ifi204 and STING were found to co-localize in the cytosol when transfected in HeLa cells (49). Future studies are required to resolve the mechanism behind the necessity of Ifi204 and determine if 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 associates with *F. novicida* DNA and ASC to activate caspase-1-mediated cell death and secretion of pro-inflammatory cytokines (23). Though 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 to 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 (Fig. S1D). In contrast, the levels of type I IFN produced by BMMs infected with the *FPI* mutant are not higher than uninfected BMMs (Fig. S1D). These results may be attributed to the higher proportion of *FPI* mutant bacteria found in the cytosol of RAW264.7 macrophages compared to 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). Herein, 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 homologue) 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 than mice deficient for the type I IFN receptor ($IFNAR1^{-/-}$) (50–52). While 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 increase 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. Since, 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 holactica live vaccine strain (LVS), and different infection route (i.p.) did not identify a difference in splenic bacterial loads between STING (MPYS)-deficient mice and wild-type mice 48 h PI (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 PI (52). Importantly, we show here that $Sting^{gt/gt}$ mice carried lower bacterial burdens in the liver and spleen 3 d PI and were significantly more resistant to *F. novicida* infection compared to wild type 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 auto-inflammatory and infectious diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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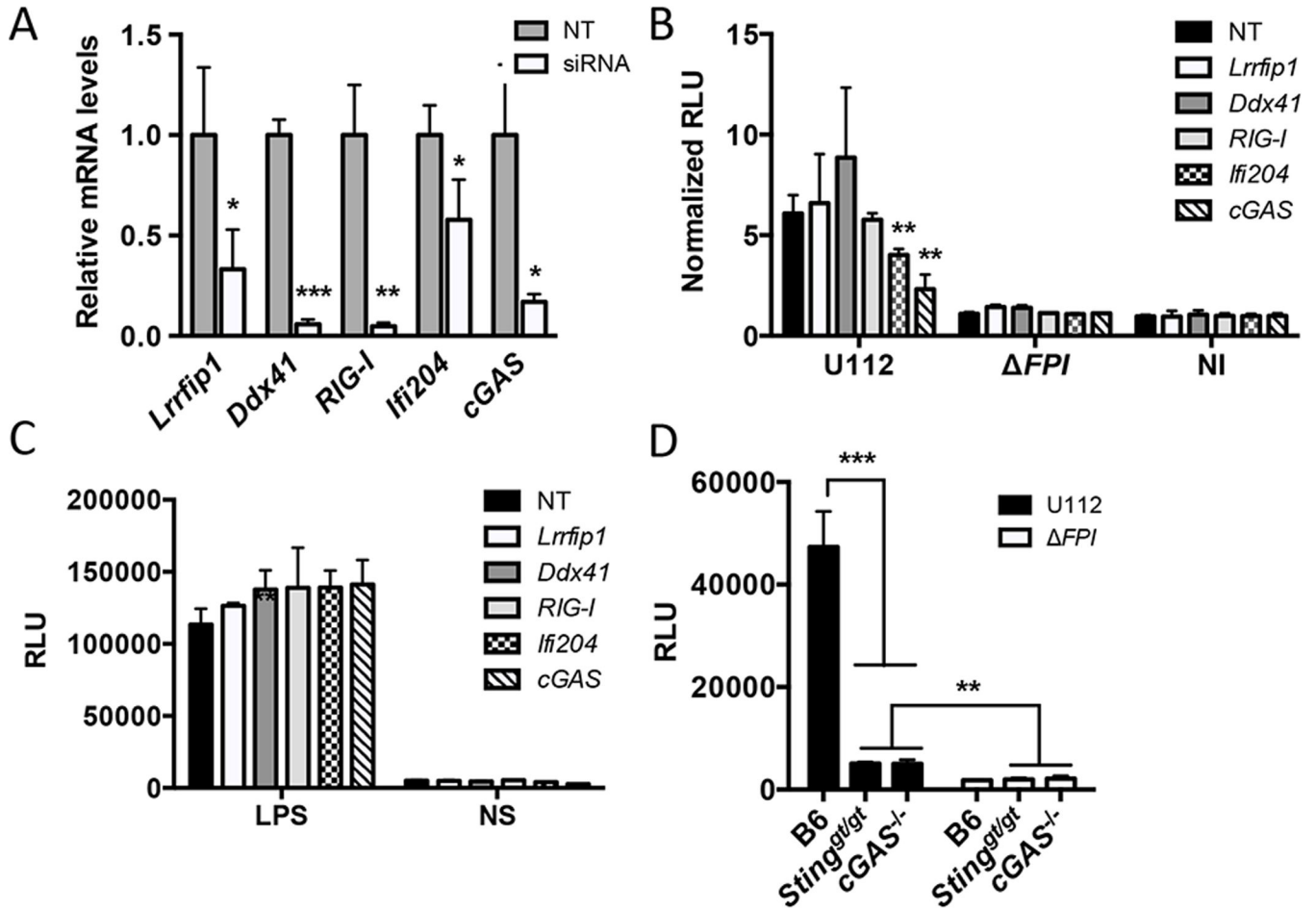
Figure 1. cGAS and Ifi204 are required for type I IFN production in response to cytosolic *F. novicida* in BMMs

siRNA targeting known cytosolic sensor genes or a non-targeting (NT) control was transfected into BMMs for 36 h (A) qRT-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 post-infection with the indicated *F. novicida* strain at an MOI of 10 or (C) 4 h post-stimulation with LPS. Results are presented as Relative Luciferase Units (RLUs). Data presented as "Normalized RLUs" are type I IFN levels normalized to the un-infected, NT control. (D) Type I IFN levels were measured from un-stimulated primary C57Bl/6 (WT), *Sting*$^{gt/gt}$ and *cGAS*$^{-/-}$ BMMs infected with *F. novicida* strains at an MOI of 10 for 12 hours. *p < 0.05, **p < 0.01, ***p < 0.001. Graphs show the mean ± standard deviation (SD) of triplicate wells and are representative of three independent experiments.
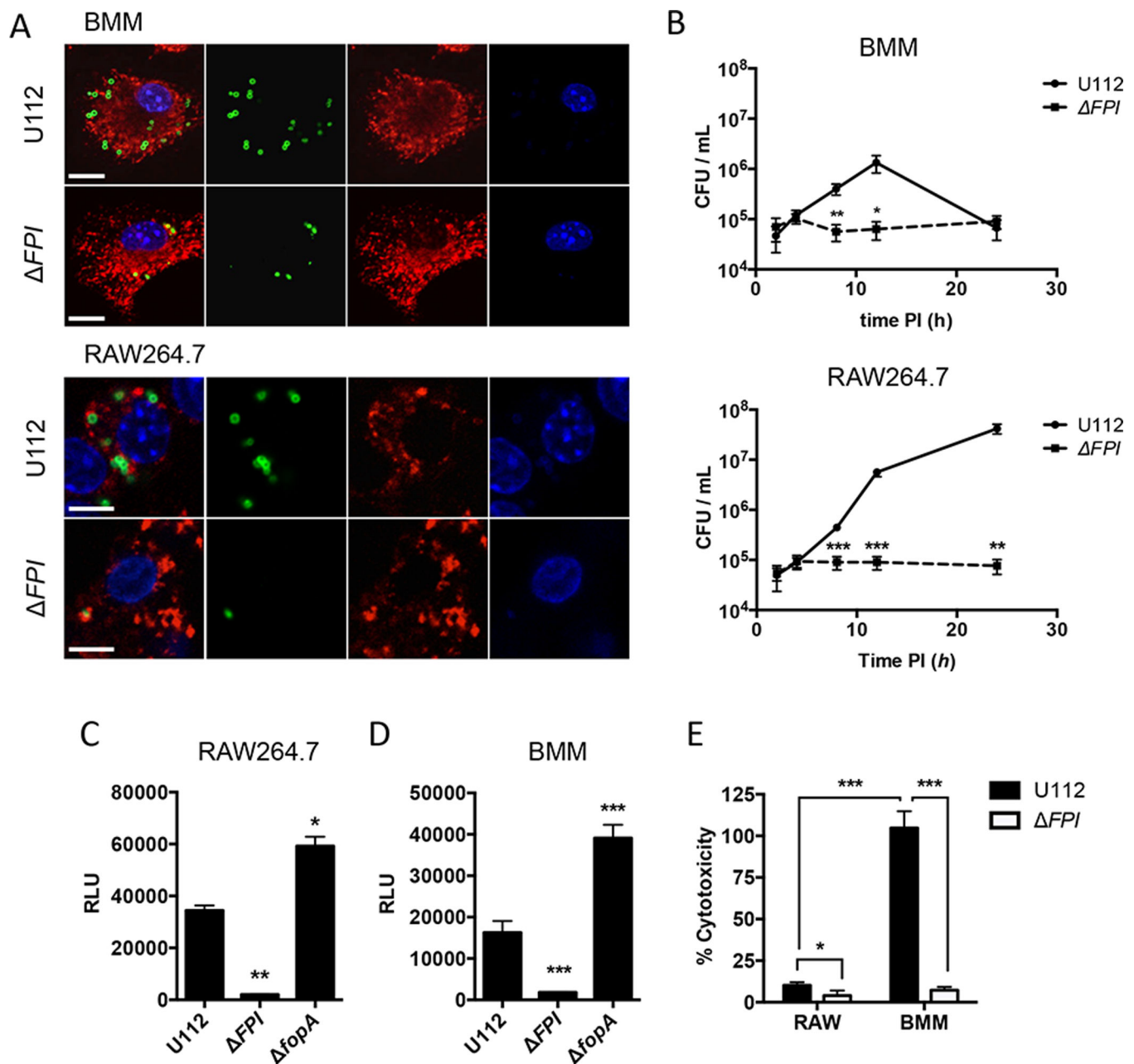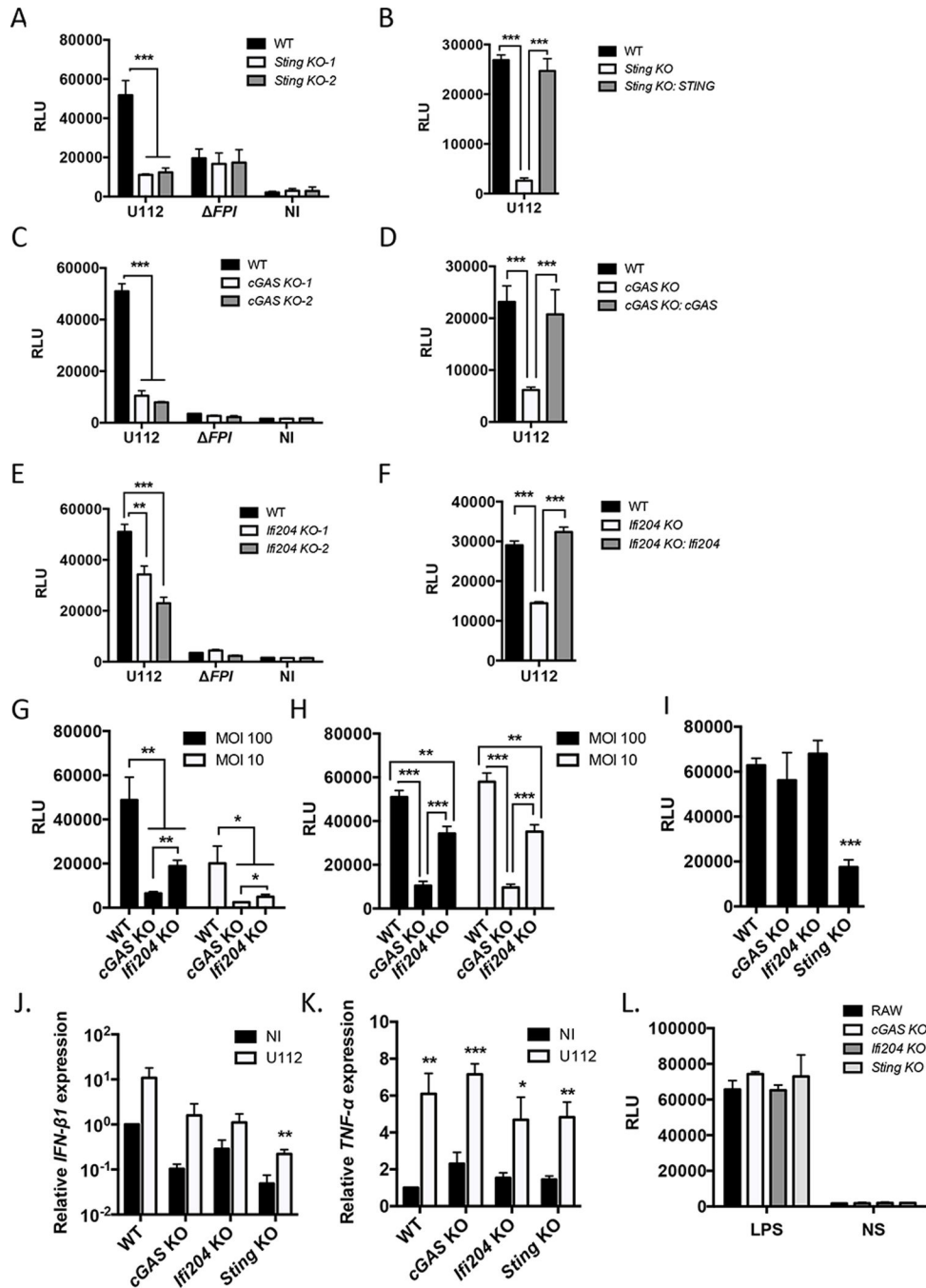
Figure 2. The type I IFN response to *F. novicida* is similar in BMM and RAW264.7 macrophages

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 hours post-infection. 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 post-infection, respectively. (E) Cytotoxicity was determined by measuring LDH release 24 h post-infection. *p < 0.05, **p < 0.01, ***p < 0.001. Graphs show the mean ± standard deviation (SD) of triplicate wells and are representative of three independent experiments.

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, E) infected with *F. novicida* strains at an MOI 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 10 for 24 h. Type I IFN levels were measured from RAW264.7 (WT) macrophages and indicated genotypes infected with *F. novicida* for12 h (G) and 24 h (H) or infected with wild-type *L. monocytogenes* for 8 h at

an MOI of 20 (I). mRNA expression levels of *IFN- βl* (J) and *TNF-α* (K) from uninfected and U112 infected macrophage cell lines at an MOI 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 *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Graphs show the mean ± standard deviation (SD) of triplicate wells and are representative of three independent experiments.
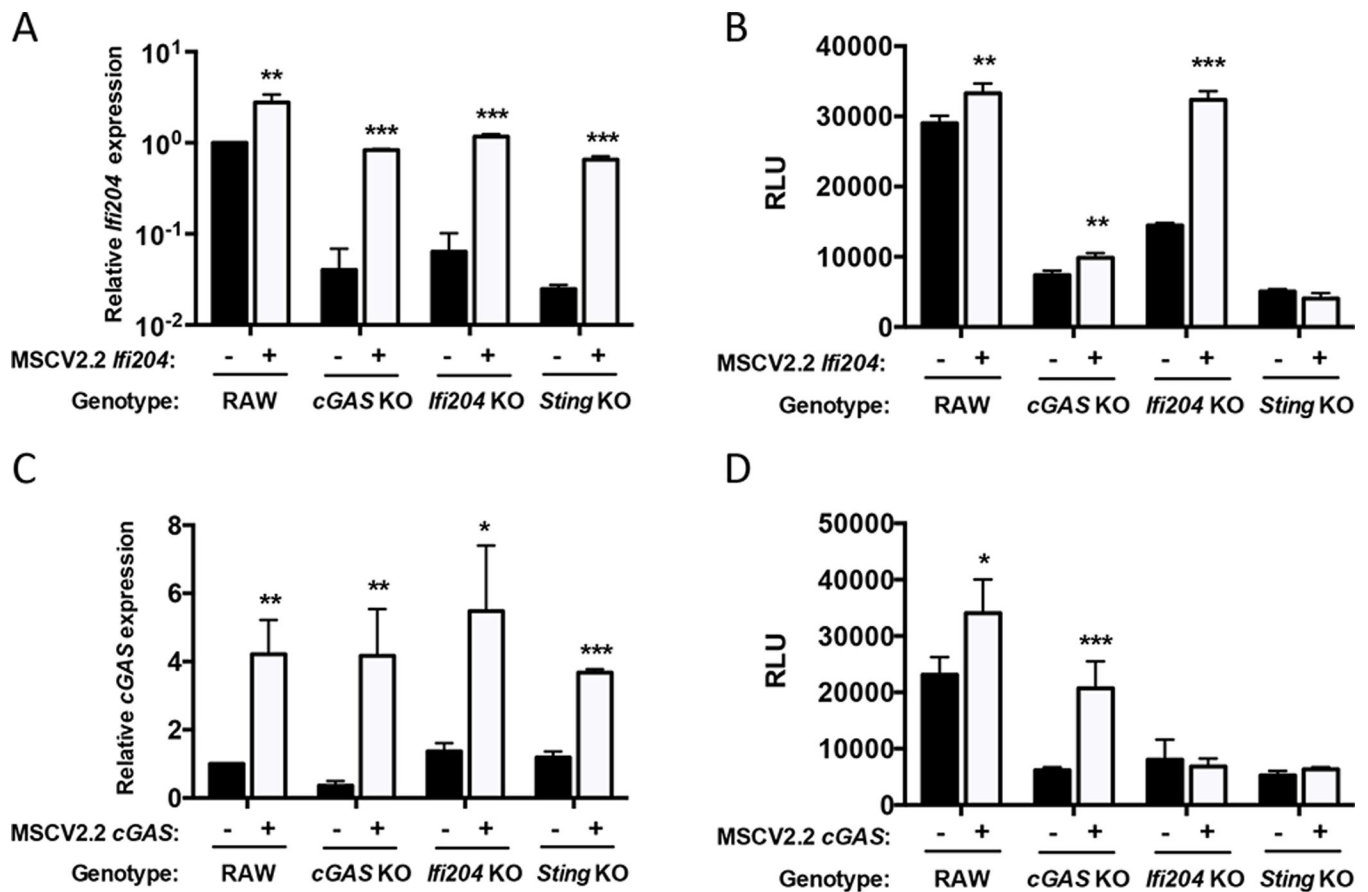
Figure 4. Both cGAS and Ifi204 are required for the full type I IFN response to *F. novicida* infection

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 wild-type (WT) RAW264.7 macrophages. Macrophages were infected with wild-type *F. novicida* at an MOI of 10 for 24 h and type I IFN levels measured (B and D, respectively). *p < 0.05, **p < 0.01, ***p < 0.001. Graphs show the mean ± standard deviation (SD) of triplicate wells and are representative of three independent experiments.
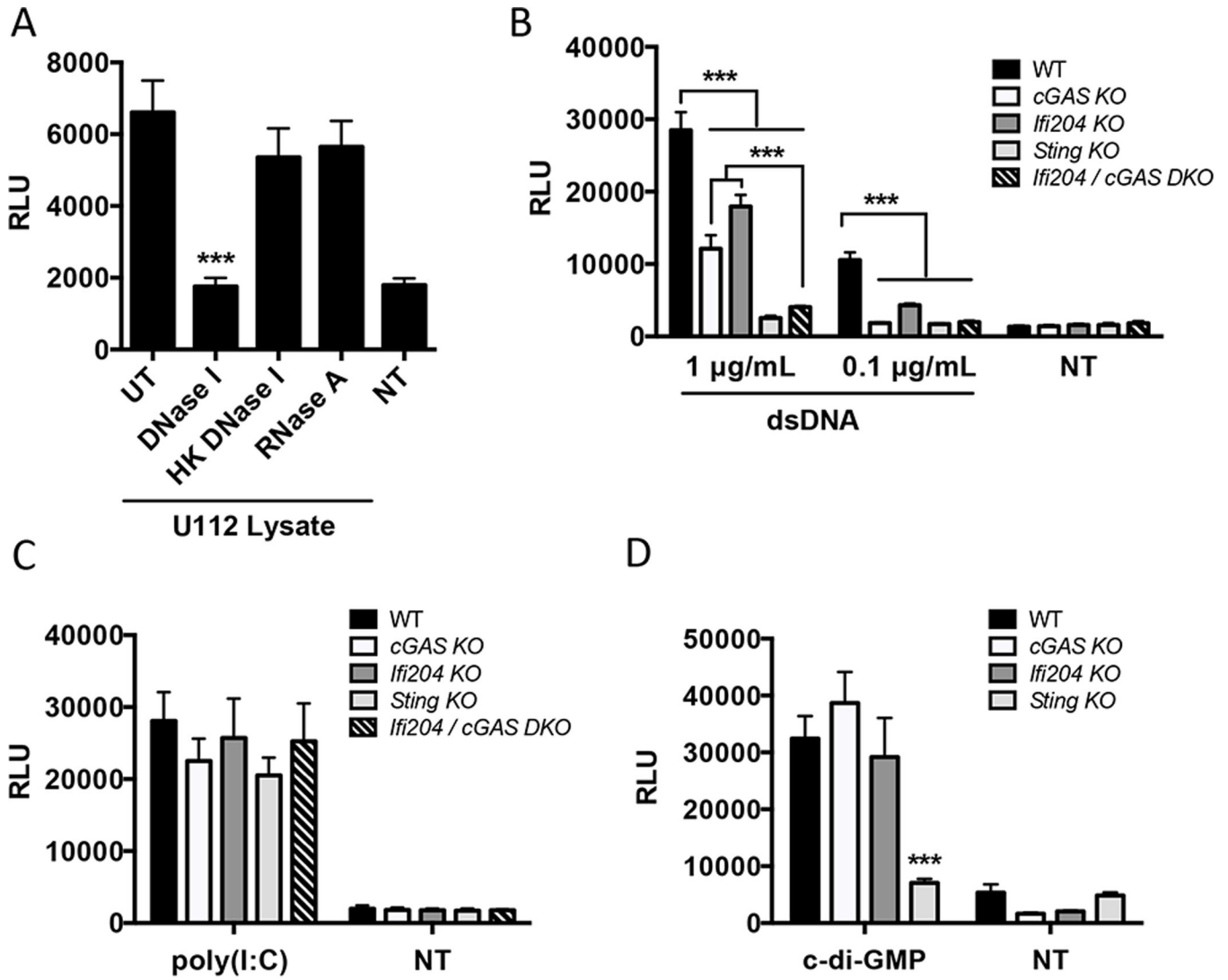
Figure 5. cGAS and Ifi204 cooperate to sense cytosolic dsDNA

(A) Type I IFN levels were measured from wild-type RAW264.7 macrophages transfected for 24 h with U112 lysates either un-treated (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 Poly(I:C) or (D) 10 μg/mL c-di-GMP NT: not transfected. *p < 0.05, **p < 0.01, ***p < 0.001. Graphs show the mean ± standard deviation (SD) of triplicate wells and are representative of three independent experiments.
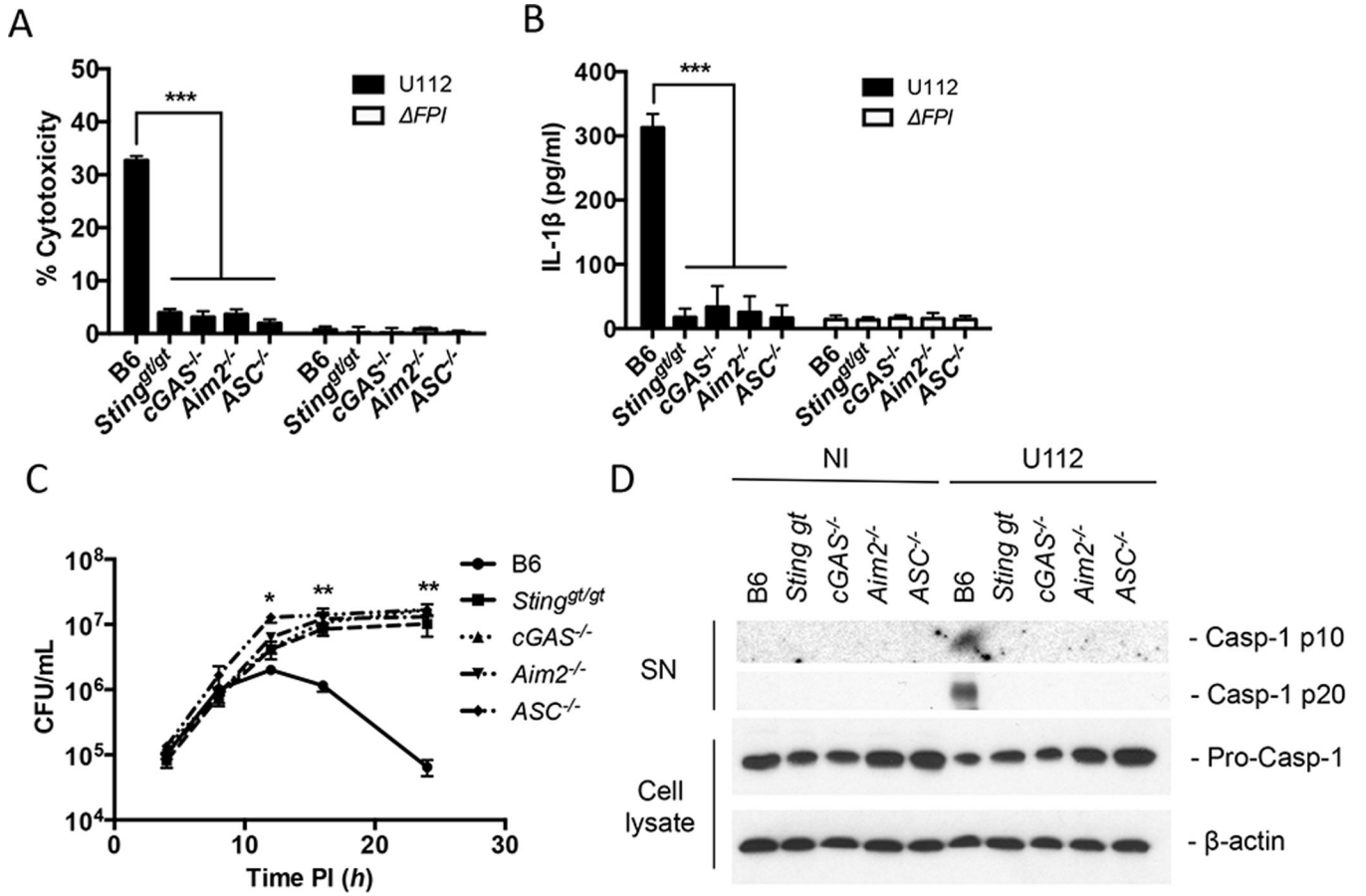
Figure 6. cGAS increases inflammasome activity in BMMs

(A) cytotoxicity, (B) IL-1β secretion and (C) intracellular survival were measured from un-stimulated primary C57Bl/6 (WT), $Sting^{gt/gt}$, $cGAS^{-/-}$, $Aim2^{-/-}$ and $ASC^{-/-}$ BMMs infected with *F. novicida* strains at an MOI of 10 for 12 hours 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 wild-type *F. novicida* at an MOI of 100 for 12 hours. Corresponding cell lysates were probed for pro-caspase-1 and β-actin. *p < 0.05, **p < 0.01, ***p < 0.001. Graphs show the mean ± standard deviation (SD) of triplicate wells and are representative of three independent experiments.

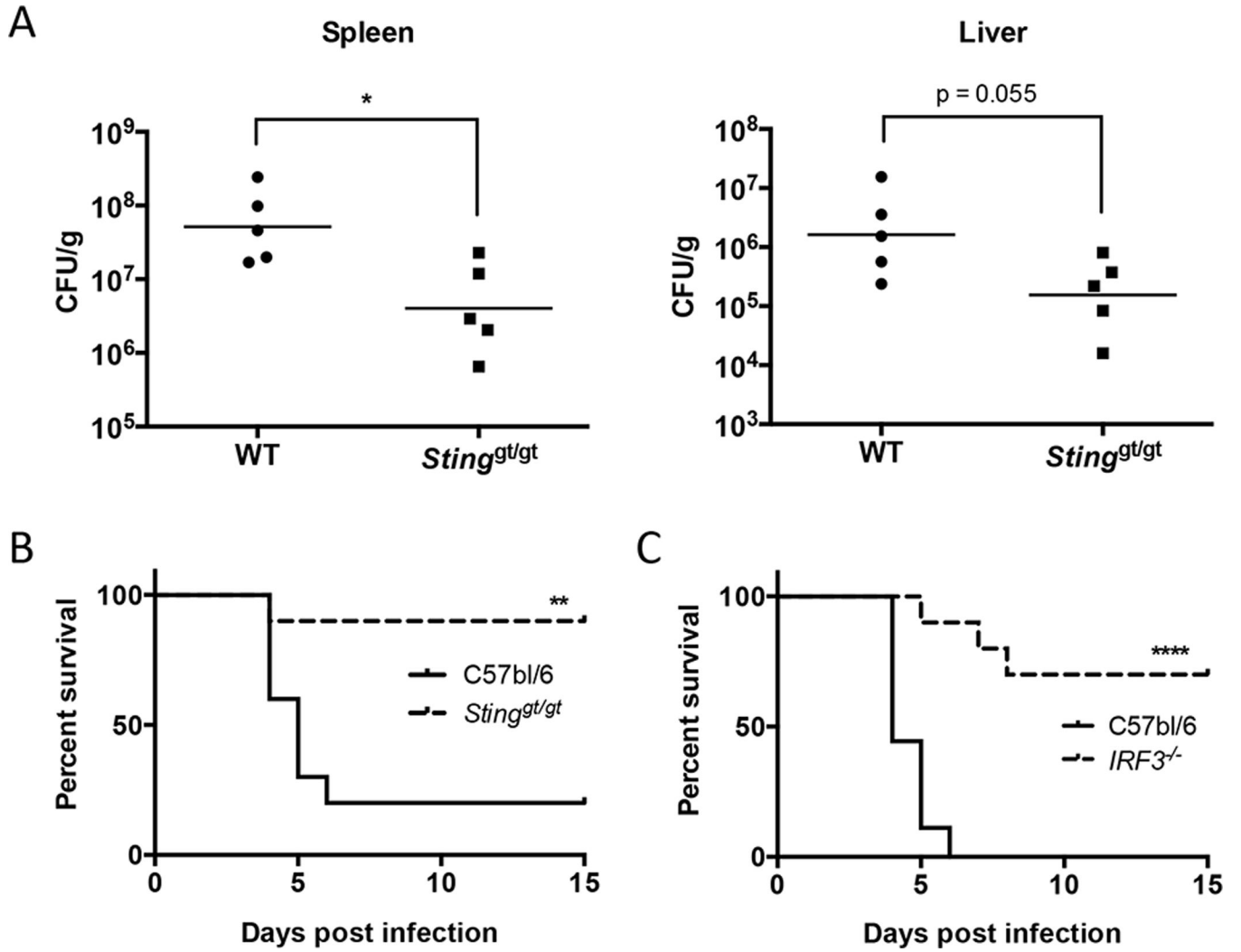**Figure 7. STING and IRF3 are detrimental to host survival during *F. novicida* infection**
WT and *Sting*$^{gt/gt}$ mice were infected s.c. with 10^5 CFU of U112. (A) The spleen and liver from five infected WT and *Sting*$^{gt/gt}$ mice were harvested and plated for CFU/g 3 d post-infection, geometric mean shown. (B) 10 WT and 10 *Sting*$^{gt/gt}$ mice were monitored twice daily over 15 d for survival. (C) 9 WT and 10 *IRF3*$^{-/-}$ mice were infected s.c. with 2×10^5 CFU of U112 and were monitored twice daily over 15 d for survival. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.