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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 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. 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-β (8, 9). Recent studies have demonstrated that the host-derived second-messenger cyclic GMP-AMP (cGAMP) synthesized from the DNA sensor cyclic GMP-AMP synthase (cGAS) or bacterial-derived cyclic dinucleotides can directly activate STING (8, 10, 11). In addition to cGAS, a number of cytosolic sensors were identified to bind DNA and trigger the type I IFN response. They include RNA polymerase III, DNA-dependent activator of IFN-regulatory factors, Lrrip1, Ifi204 (human: IFI16), Mre11, DNA-dependent protein kinase, and Ddx41 (7, 12–17). Although many cytosolic DNA sensors have been identified, the role of these sensors during bacterial infections remains unclear.

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

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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.

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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, ΔΔFPI (28), and ΔΔfopA (29) and WT L. monocytogenes strain 10403S. The cDNA from cGAS (Clone ID: 4013056; 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, CA). pcCherry and hCas9 (32) were kindly provided by J. Carette (Stanford University). pcCherry 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 I and cloned into Supplemental Table I flanking the mutant target site and sequenced (Eliim 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−/− BMF mummus from C57BL/6-Tnem1737+/− (Sting−/−) (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 generated KOs were cultured in DMEM 10% FBS. RAW264.7 macrophages were seeded at a density of 1 × 10^4 macrophages per well of a 96-well tissue culture–treated plate or 2.5 × 10^4 macrophages per well of a 24-well tissue culture–treated plate and allowed to adhere overnight at 37˚C, 5% CO2. Infections were conducted as described for BMMs (20). Immunofluorescence microscopy and Western blots were performed as previously described (23, 35). A minimum of 50 bacterial cells was quantified for LAMP-1 colocalization. Abs used include caspase-1 p10 (M-2; Santa Cruz Biotech), LAMP-1 (M-2; Santa Cruz Biotech), anti-F-actin (Monak laboratory), and anti–LAMP-1 (1D8; 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-TQ (TransIT Technologies). siRNAs used were nontargeting (NT) (IDT) of 100 nM each. siRNAs were designed against the mRNA of cGAS, Ifi204, and Sting as previously described (20, 32). siRNAs used were nontargeting (NT) (IDT) of 100 nM each. siRNAs were administered to RAW264.7 cells using Lipofectamine 2000 (Life Technologies) 72 h before infection with F. novicida. Bacterial strains used in this study include wild-type (WT) F. novicida U112, ΔΔFPI (28), and ΔΔfopA (29) and WT L. monocytogenes strain 10403S. The cDNA from cGAS (Clone ID: 4013056; 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, CA). pcCherry and hCas9 (32) were kindly provided by J. Carette (Stanford University). pcCherry 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 I and cloned into Supplemental Table I flanking the mutant target site and sequenced (Eliim Biopharm). Quantitative RT-PCR primers are listed in Supplemental Table I.

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 Lrfrp1, 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 6 h (Fig. 1A). Upon F. novicida infection, siRNA knockdown of cGAS and Ifi204 resulted in reduced type I IFN production, whereas siRNA knockdown of Lrfrp1, 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−/−) 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).
Despite the 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 macrophages using the newly described CRISPR/Cas9 system (32). However, we first needed to determine whether murine macrophages using the newly described CRISPR/Cas9 system (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, *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 genome editing system, we stably expressed *Sting* and complemented the type I IFN response to *F. novicida* infection (Fig. 3B). *cGAS* KO macrophages infected with *F. novicida* strain U112 produced significantly lower amounts of type I IFNs than the 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 (M0Is) 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 cell lines (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 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*Δex6, and cGAS+/− BMMs infected with *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.

**A** Relative mRNA levels of Lrnf1, *Ddx41*, *RIG-I*, and *cGAS* normalized to GAPDH and the NT control. **B** Type I IFN levels 4 h poststimulation with LPS. **C** Type I IFN levels normalized to the uninfected, NT control. Type I IFN levels were measured (A) 4 h poststimulation with LPS. **D** Type I IFN levels normalized to the uninfected, NT control.
IIFN signaling during L. monocytogenes cell lines (Fig. 3L). Thus, cGAS, Ifi204, and STING specifically resulted in comparable type I IFN levels for all macrophage (44, 45). TLR4-dependent type I IFN production via LPS stimulation between the cell lines postinfection (Fig. 3K). We also stimulated the type I IFN response to 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.

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.

The type I IFN response to the type I IFN response. We infected WT, cGAS KO macrophages to secrete type I IFN (Fig. 4D). Importantly, ectopic expression of cGAS in WT and Ifi204 KO macrophages did not rescue the ability of Ifi204 KO macrophages to secrete type I IFN (Fig. 4D). Ectopic expression of cGAS or Ifi204 in macrophages 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.
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 4.](http://www.jimmunol.org.) 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.

![FIGURE 5.](http://www.jimmunol.org.) 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−/−, 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. 6C). Moreover, F. novicida replicated to higher levels in Sting−/−, 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−/− mice with F. novicida. Mice were infected s.c. with 10^6 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−/− 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−/− 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−/− mice, IRF3−/− mice infected s.c. with 2 × 10^6 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...
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 present during infection to activate two independent host responses. First, *F. novicida* DNA is likely recognized by cGAS and Ifi204 to trigger a STING-dependent type I IFN response. Through autocrine and paracrine type I IFN signaling, AIM2 protein levels increase and subsequently associate with *F. novicida* DNA and ASC to activate caspase-1–mediated cell death and secretion of proinflammatory cytokines (23). Although the mechanism of DNA release by *F. novicida* into the host cytosol is not fully known, *F. novicida* mutants that are prone to increased bacterial lysis trigger higher type I IFNs and inflammasome responses compared with the parental strain (29). This observation suggests that one mechanism of facilitating access of the DNA sensors to *F. novicida* DNA is a low level of bacterial lysis in the cytosol. One notable difference between the two cell types we used in this study is that the FPI mutant elicits a type I IFN response that is higher than the levels produced by uninfected RAW264.7 macrophages (Supplemental Fig. 1D). In contrast, the levels of type I IFN produced by BMMs infected with the FPI mutant are not higher than uninfected BMMs (Supplemental Fig. 1D). These results may be attributed to the higher proportion of FPI mutant bacteria found in the cytosol of RAW264.7 macrophages compared with BMMs. These studies underscore the importance of DNA recognition during a bacterial infection. This is particularly important for a bacterial pathogen like *Francisella* that is a stealth invader and does not elicit a TLR-mediated type I IFN response (20).

Many intracellular bacterial pathogens elicit a type I IFN host response, and several of these have been demonstrated to require STING, including *L. monocytogenes*, *M. tuberculosis*, and *C. trachomatis* (5, 11, 56). In addition to STING, the type I IFN response in immortalized BMMs to *M. tuberculosis* infection was also demonstrated to require Ifi204 (56). In this article, we demonstrated that the STING-dependent type I IFN response elicited by *L. monocytogenes* did not require the DNA sensors, cGAS or Ifi204 (Fig. 3I). These results are consistent with previous findings demonstrating that *L. monocytogenes* secretes cyclic-di-AMP to directly stimulate STING in murine cells (11). Notably, a recent study showed that *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 1 IFN signaling, we sought to evaluate Sting−/− 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 (25). Importantly, we show in this article that Sting−/− 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−/− mice and Irf3−/− mice are more resistant to infection due to similar mechanisms described for IFNAR−/− mice (52). Despite lower bacterial burdens found in Sting−/− mice and IFNAR−/− 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.

References


Figure S1. Type I IFN response profiles in primary and immortalized macrophages. (A) A standard curve for the L929-ISRE reporter assay using purified IFN-β is shown. (B) Type I IFN levels were measured from C57Bl/6 BMMs and RAW264.7 macrophages infected with indicated F. novicida strains at an MOI 10 and monitored overtime. Macrophages were infected with the indicated F. novicida strain at an MOI 10. Type I IFN activity was measured from C57Bl/6 BMM 12 h post-infection (C) or from RAW264.7 macrophages 24 h post-infection (D). Graphs show the mean ± standard deviation (SD) of triplicate wells and are representative of three independent experiments.
Figure S2. mRNA expression profiles of uninfected and *F. novicida*-infected RAW264.7 mutant cell lines. mRNA expression levels for the indicated gene were measured by qRT-PCR from uninfected C57Bl/6 BMMs (A) transfected with siRNA for 36 h or (B) uninfected RAW264.7 (WT) macrophage cell lines. mRNA expression levels of (C) *Ifi204*, (D), *Sting* (E), and *cGAS* were measured from uninfected and U112 infected macrophage cell lines at an MOI 10 for 8 h. mRNA levels were normalized to GAPDH and either the NT control in BMMs or WT RAW264.7 macrophages. *p < 0.05, **p < 0.01, ***p < 0.001. Graphs show the mean ± standard deviation (SD) of three independent wells.
Table S1. Primers used for this study.

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