Guanylate binding proteins promote caspase-11–dependent pyroptosis in response to cytoplasmic LPS

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IFN receptor signaling induces cell-autonomous immunity to infections with intracellular bacterial pathogens. Here, we demonstrate that IFN-inducible guanylate binding protein (Gbp) proteins stimulate caspase-11–dependent, cell-autonomous immunity in response to cytoplasmic LPS. Caspase-11–dependent pyroptosis is triggered in IFN-activated macrophages infected with the Gram-negative bacterial pathogen *Legionella pneumophila*. The rapid induction of pyroptosis in IFN-activated macrophages required a cluster of IFN-inducible Gbp proteins encoded on mouse chromosome 3 (Gbp^{chr3}). Induction of pyroptosis in naive macrophages by infections with the cytosol-invading Δ*sdhA L. pneumophila* mutant was similarly dependent on Gbp^{chr3}, suggesting that these Gbp proteins play a role in the detection of bacteria accessing the cytosol. Cytoplasmic LPS derived from *Salmonella* ssp. or *Escherichia coli* has recently been shown to trigger caspase-11 activation and pyroptosis, but the cytoplasmic sensor for LPS and components of the caspase-11 inflammasome are not yet defined. We found that the induction of caspase-11–dependent pyroptosis by cytoplasmic *L. pneumophila*-derived LPS required Gbp^{chr3} proteins. Similarly, pyroptosis induced by cytoplasmic LPS isolated from *Salmonella* was diminished in Gbp^{chr3}-deficient macrophages. These data suggest a role for Gbp^{chr3} proteins in the detection of cytoplasmic LPS and the activation of the noncanonical inflammasome.

interferon | cell death | immunity-related GTPases | Nos2

The Gram-negative bacterium *Legionella pneumophila* resides and replicates inside free-living amoeba in the aqueous environment. When aerosolized and inhaled, *L. pneumophila* can infect alveolar macrophages and cause pneumonia in humans and animal models (1). Clearance of these pulmonary infections requires IFN-mediated immune responses (1). IFNs are proinflammatory cytokines produced by professional immune cells as well as infected nonimmune cells. Type I IFNs include IFNα and IFNβ, whereas type II IFN is IFNγ. Although type I and II IFNs sometimes have distinct effects on cells, many of the induced transcriptional responses overlap. IFN receptors are expressed on the surface of virtually every mammalian cell, including macrophages (2). Engagement of IFN receptors induces the expression of numerous host genes implicated in cell-autonomous resistance to *L. pneumophila* infections. Among these resistance factors are Irgm1, a member of the family of immunity-related GTPases (IRGs), and the nitric oxide synthase Nos2 (3, 4). These IFN-inducible host proteins mediate resistance through the modification of membrane trafficking events and the production of highly reactive oxidants (2).

Macrophage immunity to *L. pneumophila* infections is also achieved through the activation of multiprotein inflammasome complexes that trigger pyroptotic cell death. *L. pneumophila* can trigger pyroptosis through the inadvertent leakage of bacterial flagellin into the host cytosol (5, 6). The NAIP5-NLRC4 inflammasome senses cytoplasmic flagellin and triggers caspase-1 activation (7). Active caspase-1 proteolytically processes the proforms of IL-1β and IL-18, and additionally promotes the rapid formation of death-inducing plasma membrane pores (8, 9).

In addition to the canonical inflammasome pathway induced by cytoplasmic flagellin, *L. pneumophila* infections can trigger a flagellin-independent, noncanonical inflammasome pathway defined by the activation of caspase-11 (10, 11). Activated caspase-11 promotes rapid cell death independent of caspase-1 and independent of any known components of canonical inflammasomes (12, 13). Whereas the composition of the noncanonical inflammasome complex is unknown, the microbial trigger for caspase-11 activation was recently identified as LPS released from Gram-negative bacteria accessing the cytosol (14, 15). Accordingly, an *L. pneumophila* mutant that aberrantly enters the cytosol, Δ*sdhA*, was shown to initiate a rapid caspase-11 response in naive macrophages (12). Rapid, flagellin-independent activation of caspase-11 is also observed in macrophages infected with *sdhA*$^{+}$ *L. pneumophila* strains, but only if macrophages were primed with stimuli, such as external LPS (10). The caspase-11 response observed in LPS-primed macrophages infected with flagellin-deficient (Δ*flaA*), *sdhA*$^{+}$ *L. pneumophila* requires cofactors that are IFN-inducible (10). In this study, we show that IFN-inducible guanylate binding protein (Gbp) proteins function as critical cofactors for the activation of the noncanonical

Significance

A major component of the cell envelope of Gram-negative bacteria is LPS, also known as endotoxin. LPS produced during bacterial infections triggers inflammation, which can lead to septic shock and death. Our immune system can recognize LPS both outside and inside of cells. The recognition of extracellular and vacuolar LPS by LPS binding proteins is well described, but little is known about the recognition of cytoplasmic LPS. Here, we show that cytoplasmic LPS derived from the intracellular bacterial pathogen *Legionella* activated a proinflammatory immune response. We further identified host guanylate binding proteins as critical mediators of immunity triggered by cytoplasmic LPS. These findings are likely to advance our understanding of how cells can sense intracellular LPS.

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inflammasome by cytoplasmic LPS derived from *L. pneumophila*. We further demonstrate that activation of the caspase-11 pathway by a cytosol-invading *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) mutant also requires Gbp expression. Therefore, our studies identify Gbp proteins as critical mediators of caspase-11–driven, cell-autonomous immunity directed against Gram-negative bacteria accessing the cytosol.

Results

IFNγ-Activated Macrophages Reduce Bacterial Burden Through Nos2-, Nox2-, and Irgm1/m3-Dependent and -Independent Mechanisms. Type II IFNγ is produced by professional immune cells and acts as a potent inducer of macrophage immunity. Among the most abundantly expressed IFNγ–inducible proteins are GTPases (2). One class of IFNγ–inducible GTPases previously implicated in resistance to *L. pneumophila* infections comprises IRG proteins, specifically Irgm1 (4). In support of previous observations, we found that IFNγ–activated bone marrow-derived macrophages (BMMs) lacking the paralogous genes *Irgm1* and/or *Irgm3* were moderately deficient in restricting intracellular growth of *L. pneumophila* Δ*flaA* (Fig. 1 *A* and *B*). The concomitant removal of the antimicrobial enzyme Nos2 and the NADPH oxidase Nox2 similarly diminished the ability of IFNγ–primed BMMs to restrict intracellular growth of *L. pneumophila* Δ*flaA* (Fig. 1*B*). To determine whether additional host resistance factors and pathways existed that could provide IFNγ–inducible macrophage immunity to *L. pneumophila*, we generated quadruple knockout (QKO) mice deficient in *Irgm1*, *Irgm3*, *Nos2*, and *Nox2*. IFNγ–activated BMMs derived from QKO mice allowed for significantly greater bacterial replication than IFNγ–activated BMMs deficient in only subsets of these four genes (Fig. 1*B*). However, IFNγ–activated QKO BMMs maintained the ability to reduce bacterial burden substantially relative to unstimulated controls (Fig. 1*B*), demonstrating the existence of additional IFNγ–inducible resistance pathways.

Pyroptosis Is Activated Independent of Nos2, Nox2, and IRGM Proteins in IFNγ–Primed Macrophages. To account for the residual resistance observed in IFNγ–stimulated QKO BMMs, we considered two cell-autonomous host defense pathways: antimicrobial autophagy (also known as xenophagy) and pyroptosis. We were able to exclude xenophagy as a defense pathway active against *L. pneumophila* because QKO BMMs deficient for expression of the essential autophagy factor Atg5 were as restrictive for *L. pneumophila* growth as Atg5-expressing QKO cells (Fig. 1*C* and Fig. S1). Because IFNγ–treated QKO, QKO $Atg5^{-/-}$, and WT BMMs alike underwent cell lysis upon infection with *L. pneumophila* (Fig. 1*D* and Fig. S2), we hypothesized that IFNγ–treated QKO BMMs instead mediated resistance to *L. pneumophila* infections through the induction of pyroptosis. In support of this hypothesis, we observed that the treatment of IFNγ–activated QKO BMMs with the pan-caspase inhibitor z-Val-Ala-Asp-fluoromethylketone both suppressed *Legionella*-induced cell death (Fig. 1*D*) and enhanced bacterial burden relative to DMSO-treated control cells (Fig. 1*E*). Similarly, *L. pneumophila* burden was increased in IFNγ–stimulated $Casp11^{-/-}$ or $Casp1^{-/-}Casp11^{-/-}$ BMMs relative to IFNγ–stimulated WT BMMs (Fig. 1*F*). Together, these data show that caspase-11–dependent immunity limits *L. pneumophila* replication in IFNγ–stimulated BMMs and that caspase-11–mediated resistance operates independent of *Irgm1*, *Irgm3*, *Nos2*, and *Nox2*.

Gbp Proteins Promote Caspase-11–Dependent Pyroptosis. In LPS-stimulated macrophages, *L. pneumophila* triggers rapid caspase-11–mediated pyroptosis independent of bacterial flagellin and the canonical inflammasome components apoptosis speck-like protein (ASC) and NLRC4 (10). We found that IFNγ treatment similarly predisposed BMMs to infection-induced, caspase-11–mediated cell death, which occurred independent of flagellin and the canonical inflammasome components ASC, NLRC4, and NLRP3 (Fig. 2*A*). We hypothesized that cell priming with either LPS or IFNγ resulted in the enhanced expression of one or more host proteins critical for the activation of the noncanonical inflammasome. Although LPS

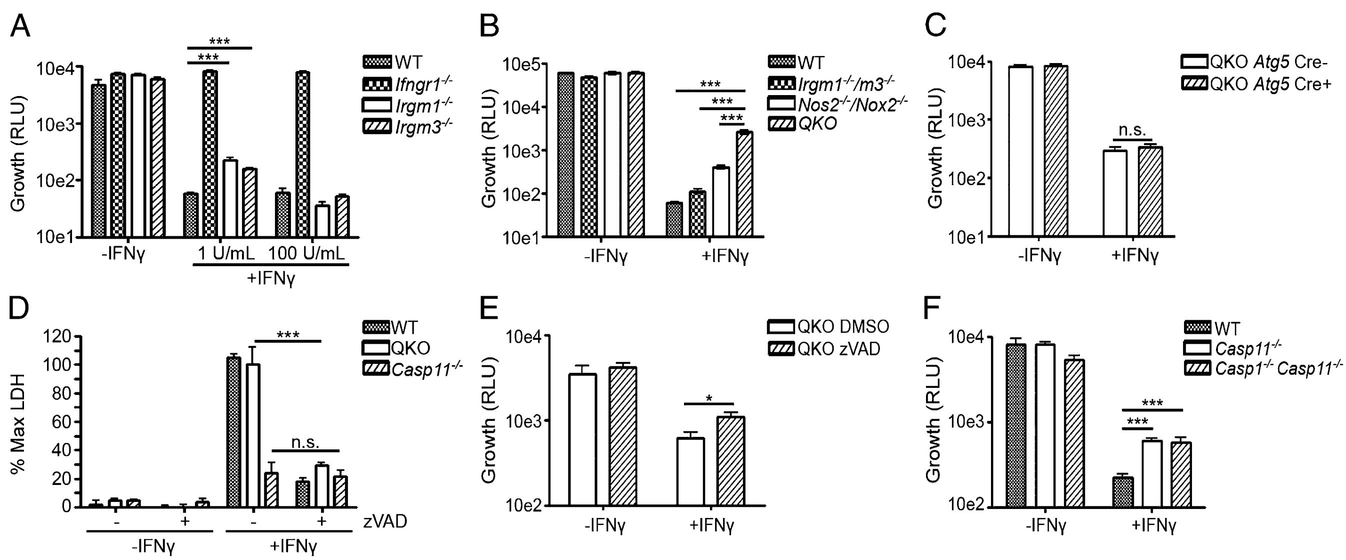

Fig. 1. Multiple IFN-induced responses restrict the growth of *L. pneumophila* in murine macrophages. (*A*–*C*) Naive and IFNγ–primed BMMs were infected with luminescent Δ*flaA L. pneumophila* at an MOI of 1, and bacterial growth was measured over 24 h. Data are shown as averages ± SD of three independent wells at 24 hpi. Unless otherwise indicated, cells were induced with 100 U/mL IFNγ overnight. (*D*) Naive and IFNγ–primed BMMs were treated with the caspase inhibitor z-Val-Ala-Asp-fluoromethylketone (zVAD) where indicated. Cells were infected with *L. pneumophila* at an MOI of 10, and LDH release was measured at 3 hpi. (*E*) *L. pneumophila* burden in QKO BMMs treated with zVAD compared with DMSO vehicle control at 24 hpi. (*F*) Bacterial burden in BMMs from the indicated mouse strains at 24 hpi. All values are averages ± SD from three independent wells. Statistical significance was calculated using the unpaired Student *t* test. Max, maximum; n.s., not significant; RLU, relative light units.

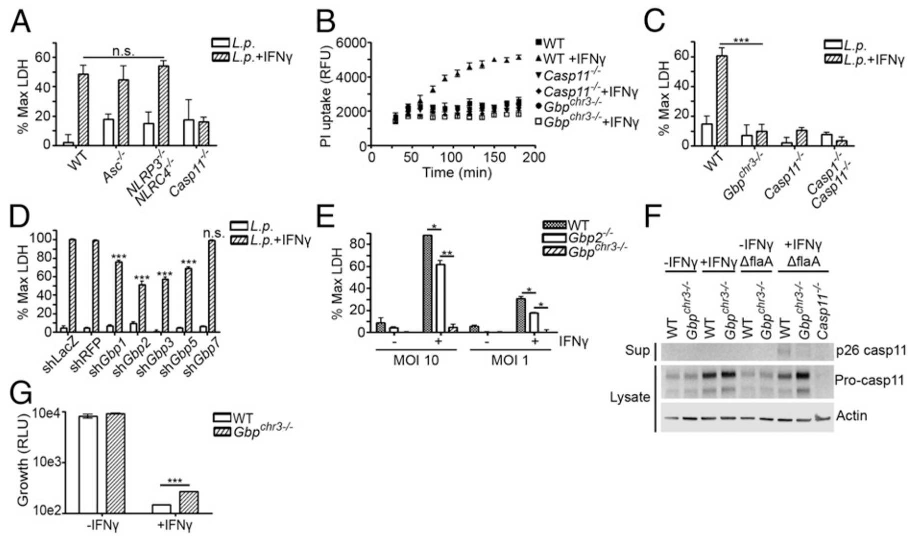

Fig. 2. Pyroptosis triggered by *L. pneumophila* (*L.p.*) is dependent on Gbp^{chr3}. (*A*) BMMs from the indicated mouse strains were left untreated or activated with 100 U/mL IFNγ overnight. Cells were infected with *L.p.* at an MOI of 10 for 3 h, and LDH release was measured. (*B*) Fluorometric plots show propidium iodide (PI) uptake at 3 hpi. (*C*) Cell death was measured at 3 hpi in BMMs primed with IFNγ (100 U/mL) and infected with *L.p.* at an MOI of 10. (*D*) WT immortalized BMMs (iBMMs) were transduced with shRNAs against the indicated Gbp^{chr3} targets and infected with *L.p.* at an MOI of 1, and LDH was measured at 3 hpi. shLacZ and shRFP were used as negative controls. (*E*) Cell death was measured in primary $Gbp2^{-/-}$ BMMs infected with *L.p.* at MOIs of 10 and 1 at 3 hpi. (*F*) Cell lysates and supernatants (Sup) from WT and $Gbp^{chr3-/-}$ BMMs were probed for caspase-11 by Western blot. Infections with *L.p.* were performed at an MOI of 10 for 3 h. (*G*) Growth of *L.p.* in naive or primed BMMs (100 U/mL IFNγ) infected at an MOI of 1 at 24 hpi. Values represent averages ± SD from three independent wells. Statistical significance was calculated using the unpaired Student *t* test (*A*–*D*, *F*, and *G*) and one-way ANOVA (*E*).

and IFNγ both induce the expression of caspase-11 (16), strong evidence exists that additional IFN-inducible host factors other than caspase-11 are needed for the execution of the noncanonical inflammasome pathway (10, 14, 15, 17). To identify IFN- and LPS-inducible factors required for the execution of pyroptosis via caspase-11, we pursued a candidate approach.

Robust activation of the NLRP3 inflammasome by pathogenic bacteria requires expression of Gbp5, a member of the Gbp family of IFN-inducible GTPases (18). We therefore hypothesized that one or more Gbp proteins could similarly regulate the activation of the noncanonical inflammasome. To test this hypothesis, we monitored the ability of BMMs deficient for a cluster of five *Gbp* genes (*Gbp1*–*Gbp3*, *Gbp5*, and *Gbp7*) on mouse chromosome 3 (Gbp^{chr3}) to undergo rapid *Legionella*-induced, caspase-11–dependent pyroptosis. Similar to IFNγ–activated $Casp11^{-/-}$ and $Casp1^{-/-}Casp11^{-/-}$ BMMs, we found that IFNγ–activated $Gbp^{chr3-/-}$ BMMs were resistant to *Legionella*-induced cell death, as determined by monitoring the incorporation of propidium iodide into the host cell nuclei (Fig. 2*B*) at 3 h postinfection (hpi) and the release of cytoplasmic lactate dehydrogenase (LDH) into the cell culture supernatant at 3 hpi (Fig. 2*C*) as well as at 24 hpi (Fig. S3). $Gbp^{chr3-/-}$ BMMs were similarly resistant to caspase-11–dependent cell death when primed with IFNβ (Fig. S4).

To determine which Gbp^{chr3} proteins promote the execution of caspase-11–dependent cell death, we ablated expression of individual Gbp^{chr3} proteins through the use of shRNAs. Interference with the expression of Gbp1, Gbp2, Gbp3, and Gbp5 led to a moderate reduction in *Legionella*-induced cell death (Fig. 2*D*). Further corroborating these results, we found *Legionella*-induced pyroptosis to be modestly reduced in $Gbp2^{-/-}$ BMMs (Fig. 2*E*). However, $Gbp2^{-/-}$ BMMs remained significantly more susceptible to *Legionella*-induced death than $Gbp^{chr3-/-}$ BMMs (Fig. 2*E*). These data indicate that maximal activation of the noncanonical inflammasome pathway is mediated by a network of Gbp^{chr3} proteins rather than by one single Gbp^{chr3} protein.

Gbp Proteins Promote Caspase-11 Activation and Cell-Autonomous Resistance. IFN-activated $Gbp^{chr3-/-}$ BMMs failed to undergo pyroptosis following *L. pneumophila* infection despite normal caspase-11 protein expression (Fig. 2*F*), suggesting a potential role for Gbp^{chr3} proteins in caspase-11 activation rather than in the regulation of caspase-11 expression. In support of this model, processed caspase-11 p26 was detected in the supernatants of IFN-primed, *Legionella*-infected WT BMMs but not $Gbp^{chr3-/-}$ BMMs (Fig. 2*F*). Although the importance of caspase-11 cleavage for the execution of cell death remains in doubt (14), these results hint at a possible role for Gbp proteins in promoting proteolytic processing of caspase-11.

Because caspase-11 promotes cell-autonomous immunity to *L. pneumophila* in IFNγ–primed BMMs (Fig. 1), we hypothesized that Gbp^{chr3} proteins were similarly required to restrict *L. pneumophila* growth inside BMMs. In support of our hypothesis, we observed increased bacterial burden in IFNγ–activated $Gbp^{chr3-/-}$ BMMs relative to littermate control cells across a range of multiplicities of infection (MOIs) (Fig. 2*G* and Fig. S3). Collectively, these data indicate that Gbp proteins promote rapid pyroptosis and cell-autonomous immunity to *L. pneumophila* infections via caspase-11 activation in IFN-primed macrophages.

Cell Death Mediated by Infection with the *L. pneumophila* ΔsdhA Mutant Requires Gbp^{chr3} Proteins. Caspase-11 activation occurs when Gram-negative bacteria enter the cytosol (12). *L. pneumophila* normally resides within an intracellular vacuole and fails to induce a rapid caspase-11 response in naive macrophages (10). In IFN-primed BMMs, however, *L. pneumophila* infections activate the noncanonical inflammasome pathway (Figs. 1 and 2). We considered two models to account for enhanced cell death via caspase-11 in IFN-activated cells: IFN-primed BMMs could either promote the disruption of *Legionella*-containing vacuoles (LCVs) to expel bacteria into the cytosol or increase their sensitivity for cytoplasmic LPS. We first monitored the effects of IFN on LCV integrity by measuring the localization of a galectin-3–YFP fusion protein to intracellular bacteria. The cytoplasmic galectin-3 protein recognizes disrupted vacuoles by binding to glycosylated proteins confined to the luminal side of vacuoles and only accessible from the cytosol once loss of membrane integrity has occurred (19). As previously reported (20), the *L. pneumophila* Δ*sdhA*Δ*flaA* mutant defective for maintenance of LCV membrane integrity associated with galectin-3 more frequently than Δ*flaA* bacteria did (Fig. 3*A* and Fig. S5). However, IFN treatment failed to increase galectin-3 localization to Δ*flaA* bacteria (Fig. 3*A* and Fig. S5), suggesting that LCV membrane integrity is not dramatically altered in IFN-activated BMMs.

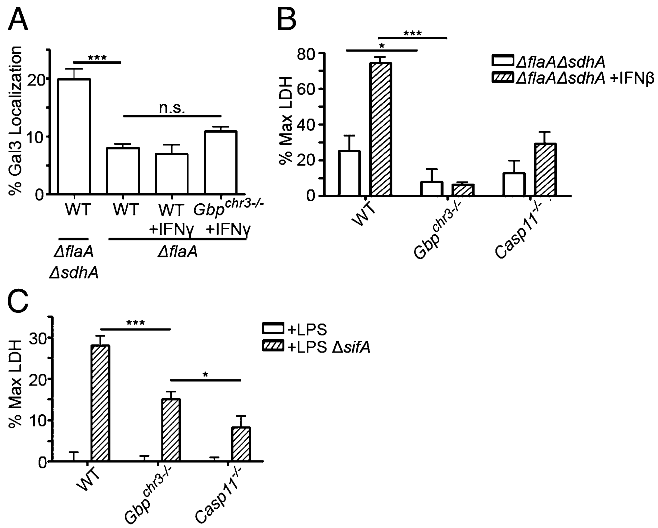

Fig. 3. Gbp^{chr3} proteins are important for recognizing cytosolic bacteria. (*A*) iBMMs expressing galectin-3 (Gal3)–YFP either uninduced or primed with 100 U/mL IFNγ were infected with Δ*sdhA*Δ*flaA* and Δ*flaA L.p.* at an MOI of 2. Localization of Gal3-YFP to LCVs was quantified at 4 hpi. Values are shown as averages ± SEM from a total of 800 infected cells from two independent experiments. Significance between samples was calculated using one-way ANOVA. (*B*) Cell death at 3 hpi in naive or IFNγ–primed BMMs (100 U/mL) from the indicated mouse strains infected with Δ*sdhA*Δ*flaA L.p.* at an MOI of 10. (*C*) Cell death at 3 hpi in LPS-activated BMMs (50 ng/mL) infected with Δ*sifA S.* Typhimurium at an MOI of 50. All values shown are averages ± SD of three independent wells. Statistical significance was calculated using one-way ANOVA (*A* and *C*) and the unpaired Student *t* test (*B*).

Because IFN treatment appeared to have little or no effect on the integrity of LCVs, we hypothesized that Gbp^{chr3} proteins function downstream of LCV disintegration. To test this hypothesis, we first set out to determine whether Gbp^{chr3} proteins are required for the induction of cell death by the *L. pneumophila* Δ*sdhA*Δ*flaA* mutant known to enter the cytosol aberrantly. As previously observed (12), Δ*sdhA*Δ*flaA* induced caspase-11–dependent cell death in naive BMMs (Fig. 3*B*). We found that priming of BMMs with IFN further exacerbated Δ*sdhA*Δ*flaA*-induced pyroptosis (Fig. 3*B*). Cell death induced by Δ*sdhA*Δ*flaA* in either naive or IFN-primed BMMs required Gbp^{chr3} expression (Fig. 3*B*). Together with the observation that Gbp^{chr3} proteins fail to promote LCV destabilization (Fig. 3*A*), these data suggest that Gbp^{chr3} proteins promote the activation of the caspase-11 pathway following cytosolic invasion by *L. pneumophila*.

Pyroptosis Induced by Infections with *S.* Typhimurium Δ*sifA* Is Diminished in *Gbp*$^{chr3-/-}$ Macrophages. *L. pneumophila* injects the effector protein sdhA into the host cytosol to maintain LCV integrity (20). Similar to *L. pneumophila*, *S.* Typhimurium secretes its effector protein sifA into the host cell to preserve the integrity of the *Salmonella*-containing vacuole (SCV) (21). Loss of sifA expression in a Δ*sifA S.* Typhimurium mutant results in bacterial expulsion from the SCV into the cytosol and activation of the caspase-11 pathway (12). We observed that *Gbp*$^{chr3-/-}$ BMMs primed with LPS were more resistant to Δ*sifA*-induced pyroptosis than LPS-primed WT BMMs (Fig. 3*C*). These data show that distinct Gram-negative bacterial pathogens with access to the host cell cytosol promote caspase-11–dependent cell death through a Gbp^{chr3}-dependent pathway. However, we also observed that Δ*sifA*-induced pyroptosis was more pronounced in *Gbp*$^{chr3-/-}$ BMMs compared with *Casp11*$^{-/-}$ BMMs (Fig. 3*C*), suggesting that *S.* Typhimurium infections trigger noncanonical inflammasome activation in both Gbp^{chr3}-dependent and Gbp^{chr3}-independent manners.

Gbp^{chr3} Proteins Promote the Induction of Caspase-11 Pyroptosis by Cytoplasmic LPS. Recently, it was shown that translocation of *Escherichia coli*, *S.* Typhimurium lipid A, or LPS into the cytoplasm of primed macrophages triggers the noncanonical inflammasome pathway (14, 15). To define the *Legionella*-derived molecule responsible for the activation of the noncanonical inflammasome, we first transfected BMMs with *L. pneumophila* lysates. Transfection of *L. pneumophila* lysates into IFNγ–primed BMMs triggered caspase-11–dependent pyroptosis (Fig. 4*A*). Next, we prepared *L. pneumophila* LPS from postexponential cultures using two distinct purification methods as described in *SI Materials and Methods* and transfected these LPS preparations into BMMs. Cytoplasmic *L. pneumophila* LPS induced significant cell death in IFNγ–activated BMMs (Fig. 4*B* and Fig. S6), indicating that cytoplasmic LPS was responsible for the induction of pyroptosis in cells infected with Δ*flaA L. pneumophila*.

Cell death induced by *L. pneumophila* LPS transfection required both caspase-11 and Gbp^{chr3} expression (Fig. 4 *A* and *B*), demonstrating that Gbp^{chr3} proteins promote caspase-11–dependent pyroptosis in response to cytoplasmic LPS. To test these findings further, we delivered *L. pneumophila* LPS to the cytoplasm by an alternative method, as previously described (14). In this approach, we infected BMMs with the Gram-positive, cytosol-invading pathogen *Listeria monocytogenes* in the absence or presence of *L. pneumophila* LPS. This pathogen enters the cytosol by forming pores in phagosomes with the toxin, Listeriolysin O (LLO) (22). We found that the codelivery of *L. pneumophila* LPS with WT *L. monocytogenes* increased *L. monocytogenes*-induced cell death in WT but not *Gbp*$^{chr3-/-}$ BMMs (Fig. 4*C*). This response required invasion of the cytoplasm, because incubation of *L. pneumophila* LPS with an LLO mutant (Δ*hly)* did not cause cell death in BMMs. These data further support a role for Gbp^{chr3} proteins in the pyroptotic response to cytoplasmic LPS downstream of vacuolar disruption.

L. pneumophila LPS is characterized by several unique structural features that include the presence of fatty acid chains twice the length of the corresponding chains found in enterobacterial LPS (23). To determine whether Gbp^{chr3} proteins were also required for the induction of pyroptosis triggered by cytoplasmic LPS derived from Enterobacteriaceae, we monitored the effect

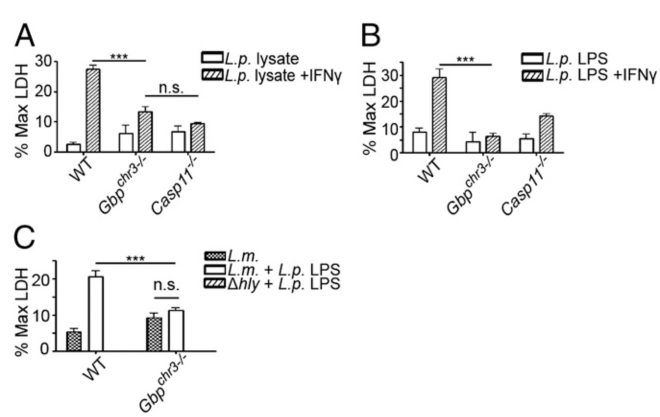

Fig. 4. Gbp^{chr3} promotes pyroptosis in response to the cytoplasmic delivery of *Legionella* LPS. Naive and activated BMMs were transfected with *Legionella* lysates (*A*), *Legionella* LPS (*B*), and LDH measured 3 h posttransfection (*C*). Naive and primed BMMs were infected with *L. monocytogenes* (MOI of 5) in the presence or absence of *Legionella* LPS. Data are shown as the average ± SD of three independent wells. Statistical significance between samples was measured using one-way ANOVA (*A* and *B*) and the unpaired Student *t* test (*C*).

of cytoplasmic *E. coli* LPS O111:B4 delivered by *L. monocytogenes* or cholera toxin B (CTB) on cell viability in WT and *Gbp*$^{chr3-/-}$ BMMs. We found that the delivery of O111:B4 into the host cytoplasm resulted in only moderately reduced rates of cell death in *Gbp*$^{chr3-/-}$ BMMs relative to WT BMMs (Fig. 5 *A* and *B*). Similarly, pyroptosis induced by cytoplasmic *Salmonella* LPS was reduced in *Gbp*$^{chr3-/-}$ BMMs (Fig. 5 *C* and *D*), although not to the same extent as in *Casp11*$^{-/-}$ BMMs (Fig. 5*D*). Together, these data demonstrate that Gbp^{chr3} promotes the execution of pyroptosis triggered by distinct LPS variants exposed to the cytosol. Additionally, our data suggest that cytoplasmic, enterobacterial LPS induces additional Gbp^{chr3}-independent mechanisms of caspase-11 activation.

Discussion

IFN-activated macrophages use multiple defense pathways to restrict intracellular microbial growth (2). IFNs induce macrophage immunity predominantly through the induction of gene expression. Among the gene products most highly expressed in IFN-activated cells are members of the IRG and Gbp families of IFN-inducible GTPases (2). Here, we demonstrate that members of both GTPase families provide macrophage immunity to *L. pneumophila* infections. Although the mechanism of IRG-mediated resistance to *L. pneumophila* remains unexplored, we show that Gbp proteins are essential for the activation of caspase-11–dependent pyroptosis in response to infections with *L. pneumophila*.

Caspase-11–dependent pyroptosis is induced by infections with various Gram-negative bacteria but not Gram-positive bacteria (13, 14, 24). The molecule common to Gram-negative bacteria and responsible for caspase-11 activation was recently identified as LPS (14, 15). Whereas extracellular LPS failed to promote cell death, it was shown that the direct injection of enterobacterial LPS into the cytoplasm through cell transfection and other methods was sufficient to trigger caspase-11–dependent pyroptosis (14, 15). These observations demonstrate that macrophages must possess one or more cytoplasmic LPS sensing pathways.

Here, we show that cytoplasmic LPS derived from *L. pneumophila* also triggers caspase-11–dependent pyroptosis. Because *L. pneumophila* takes up residence within a pathogen-controlled vacuole, only limiting amounts of *L. pneumophila* LPS are likely to enter the cytosol, thus explaining the delayed caspase-11 response in *L. pneumophila*-infected, naive macrophages (11). In contrast to naive macrophages, we show that IFN-activated macrophages undergo rapid caspase-11–dependent pyroptosis in response to *L. pneumophila* infections. The stimulatory effect of IFN treatment on caspase-11–dependent pyroptosis could potentially be explained with either of these two, not mutually exclusive, cellular activities: (*i*) IFN treatment could result in increased release of vacuolar bacteria, and thus LPS, into the cytosol, or (*ii*) IFN treatment could increase the sensitivity of the cytoplasmic LPS detection pathway. As outlined below, several lines of evidence argue that Gbp proteins predominantly mediate the latter activity to induce *Legionella*-triggered cell death.

Previous studies demonstrated a role for Irgm3 and other IRG proteins in the disruption of parasitophorous vacuoles surrounding the protozoan pathogen *Toxoplasma gondii* (25, 26). We show here that Irgm3 is dispensable for the caspase-11 response to *L. pneumophila* infections, thereby demonstrating that Irgm3-mediated vacuolar disruption is not required for caspase-11 activation. Additionally, we failed to observe a change in the number of disrupted LCVs upon IFN activation or a decrease in the number of disrupted LCVs in *Gbp*$^{chr3-/-}$ macrophages, collectively arguing against a prominent role for Gbp^{chr3} proteins in the breakdown of LCVs. Instead, we observed that Gbp^{chr3} protein expression was required for the full induction of pyroptosis by LPS delivered to the cytoplasm independent of an infection. Similarly, cell death induced by the cytosol-invading *L. pneumophila* Δ*sdhA* mutant required Gbp^{chr3} expression. Collectively, these data argue that Gbp^{chr3} proteins play a role in the detection of cytoplasmic LPS and/or the subsequent activation of the noncanonical inflammasome leading to pyroptosis.

Whereas the induction of pyroptosis by cytoplasmic *L. pneumophila* LPS appears to be strictly dependent on Gbp^{chr3} proteins, cytoplasmic LPS derived from Enterobacteriaceae can trigger pyroptosis in the absence of Gbp^{chr3} proteins, albeit with diminished efficiency. These observations may potentially be explained by structural differences in the lipid A moiety of the LPS variants derived from these distinct bacterial species: Whereas *Legionella* lipid A is characterized by long fatty acid chains (27–28 carbons in length), LPS derived from *E. coli* and *S*. Typhimurium contains shorter chains (12–14 carbons) (27, 28). These structural differences are already known to determine the specificity with which LPS variants engage Toll-like receptors (TLRs): Whereas enterobacterial LPS activates TLR4 signaling, *Legionella* LPS triggers the TLR2 signaling pathway (28, 29). Analogously, the detection of distinct LPS variants in the cytoplasm may require distinct cytoplasmic sensors. Albeit speculative, the differential requirements for Gbp^{chr3} proteins in the activation of the noncanonical inflammasome by structurally distinct LPS variants imply a role for Gbp^{chr3} proteins in LPS detection rather than in the execution of pyroptosis. Alternatively, Gbp proteins may function as signal amplifiers for those LPS species that are low-affinity substrates for the putative LPS sensor. Future studies will need to address whether or not one or more members of the Gbp family are directly involved in sensing cytoplasmic LPS.

How could Gbp proteins promote the activation of the noncanonical inflammasome? A hint at an answer to this question comes from studies on how Gbp proteins regulate the canonical inflammasome response. Activation of the canonical inflammasome is driven by the formation of multimers of the adaptor protein ASC (8, 9). ASC multimers provide a central platform

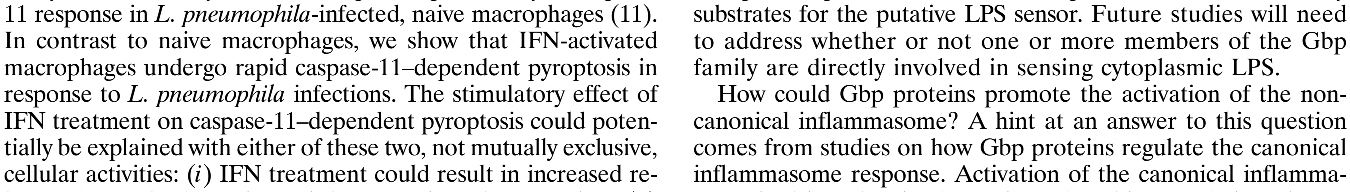

Fig. 5. Gbp^{chr3} promotes pyroptosis in response to cytoplasmic LPS derived from *E. coli* and *Salmonella*. Naive and primed iBMMs were infected with *L. monocytogenes* (MOI of 5) in the presence or absence of *E. coli* O111:B4 for 2 h (*A*) or incubated with the indicated combinations of CTB (20 μg/mL) and O111:B4 (1 μg/mL) (*B*). Naive and primed BMMs were infected with *L. monocytogenes* (MOI of 5) in the presence or absence of *S. minnesota* LPS (*C*) or transfected with *S. minnesota* LPS (*D*) and assessed for cell viability at 4 h posttransfection. Data are shown as the average ± SD of three independent wells. Statistical significance between samples was measured using the unpaired Student *t* test (*A*–*C*) and one-way ANOVA (*D*).

for the formation of several types of inflammasomes, including the NLRP3 inflammasome. Similar to ASC, IFN-inducible GTPases can form protein multimers (30). It was recently shown that tetrameric Gbp5 binds to NLRP3 and thereby promotes the assembly of an ASC–caspase-1 multimer (18). Because the noncanonical inflammasome lacks ASC and NLRP3, Gbp^{chr3} proteins must promote the activation of the noncanonical inflammasome by a different mechanism. A detailed understanding of this mechanism will require the identification of specific components of the noncanonical inflammasome, which may include Gbp^{chr3} protein complexes serving as platforms for the oligomerization of caspase-11.

Materials and Methods

Mice and Cell Culturing. C57BL/6J, LysMCre, and *Nox2*(*p47phox*)$^{-/-}$ mice were purchased from Jackson Laboratory. The *Nox2* allele was crossed onto the previously described *Nos2*$^{-/-}$*Irgm1*$^{-/-}$*Irgm3*$^{-/-}$ (triple KO) mouse (31) to generate the QKO strain. *Casp1*$^{-/-}$*Casp11*$^{-/-}$, *Casp11*$^{-/-}$, *Asc*$^{-/-}$, and *Nlrp3*$^{-/-}$*Nlrc4*$^{-/-}$ mice were previously described (14). The *Gbp*$^{chr3-/-}$ strain (32) and the *Gbp2*$^{-/-}$ strain (33) were previously described. All mice were housed in pathogen-free facilities. Animal protocols were approved by the Institutional Animal Care and Use Committees at Duke University and the University of North Carolina, Chapel Hill. Bone marrow was collected from femurs as described (34). Details of macrophage differentiation procedures can be found in *SI Materials and Methods*.

Bacterial Strains. Bioluminescent *L. pneumophila* strains were used to measure bacterial growth. As previously described (34), bioluminescence is linearly proportional to bacterial counts, and a reading of 200 relative light units equals ~10^3 cfus. Mutants in *flaA* and *sdhA* and coisogenic parental strains were described previously (6, 20). WT *S.* Typhimurium and a Δ*sifA* mutant were cultured and used as described (12). Full details can be found in *SI Materials and Methods*.

Growth Curve and Cytotoxicity Assays. BMMs were seeded at a density of 5 × 10^4 cells per well in a 96-well plate. Cells were infected at an MOI of 1 for *L. pneumophila* growth curves and at an MOI of 10 for cell death assays. Cytotoxicity assays using Δ*sifA S.* Typhimurium were performed at an MOI of 50. LDH was measured using the CytoTox One Homogeneous Membrane Integrity Assay (Promega). All values represent the percentage of LDH release compared with a maximum lysis control. Pore formation was assessed by measuring propidium iodide uptake. Full details are described in *SI Materials and Methods*.

Cytoplasmic Delivery of Bacterial Lysates and LPS. *L. pneumophila* lysates were transfected using Lipofectamine LTX with Plus Reagent (Invitrogen). *L. pneumophila* LPS was purified as previously described (23) and as described in *SI Materials and Methods*. *Salmonella minnesota* LPS was purchased from List Biologicals, and *E. coli* LPS O111:B4 was purchased from Invivogen. LPS transfections were performed using DOTAP liposomal transfection reagent (Sigma). Delivery of LPS through *L. monocytogenes* infections or CTB coincubations was performed essentially as described (14). Full details are described in *SI Materials and Methods*.

Statistical Analysis. Several key experiments (e.g., LPS transfection, *S.* Typhimurium infections) were reproduced by two independent investigators (D.M.P. and J.A.H.) at two different laboratories. Levels of statistical significance were comparable for all repeat experiments. Significance for LDH assays and growth curves was calculated using the unpaired Student *t* test and one-way ANOVA where designated (***$P \leq 0.001$; **$P \leq 0.01$; *$P \leq 0.05$).

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