Constitutive Interferon Maintains GBP Expression Required for Release of Bacterial Components Upstream of Pyroptosis and Anti-DNA Responses

Graphical Abstract

 Highlights

- Infection-driven IFN is dispensable for pyroptosis against cytosolic Legionella
- Constitutive IFN maintains GBP expression in murine and human macrophages
- GBPs are needed to release bacterial content from cytosolic Legionella bacteria
- GBPs are required for restriction of Legionella bacterial growth in vivo

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In Brief

Guanylate-binding proteins act upstream of many cytosolic pathogen sensors. It is assumed that infection-associated IFN signaling precedes GBP induction. Liu et al. find that host-intrinsic IFN signaling maintains GBPs in naive macrophages to mediate the disruption of cytosol-accessible bacteria. The findings elucidate a crucial role of tonic cytokines in maintaining immune readiness.
Constitutive Interferon Maintains GBP Expression Required for Release of Bacterial Components Upstream of Pyroptosis and Anti-DNA Responses

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SUMMARY

Legionella pneumophila elicits caspase-11-driven macrophage pyroptosis through guanylate-binding proteins (GBPs) encoded on chromosome 3. It has been proposed that microbe-driven IFN upregulates GBPs to facilitate pathogen vacuole rupture and bacteriolysis preceding caspase-11 activation. We show here that macrophage death occurred independently of microbial-induced IFN signaling and that GBPs are dispensable for pathogen vacuole rupture. Instead, the host-intrinsic IFN status sustained sufficient GBP expression levels to drive caspase-1 and caspase-11 activation in response to cytosolic-exposed bacteria. In addition, endogenous GBP levels were sufficient for the release of DNA from cytosol-exposed bacteria, preceding the cyclic GMP-AMP synthase/stimulator of interferon genes (cGAS/STING) pathway for Ifnb induction. Mice deficient for chromosome 3 GBPs were unable to mount a rapid IL-1/chemokine (C-X-C motif) ligand 1 (CXCL1) response during Legionella-induced pneumonia, with defective bacterial clearance. Our results show that rapid GBP activity is controlled by host-intrinsic cytokine signaling and that GBP activities precede immune amplification responses, including IFN induction, inflammasome activation, and cell death.

INTRODUCTION

Cytosolic presence of bacterial lipopolysaccharide (LPS) activates caspase-11, resulting in a lytic form of macrophage death known as pyroptosis (Kayagaki et al., 2011; Meunier and Broz, 2015; Yang et al., 2015). Vacuolar pathogens such as S. typhimurium and L. pneumophila predominantly bypass caspase-11 activation by maintaining pathogen replication vacuoles that protect the bacterium from cytosolic sensing (Isberg et al., 2009; LaRock et al., 2015). However, interferon (IFN)-activated macrophages can mount a caspase-11 response to vacuole-resident bacteria (Broz et al., 2012; Case et al., 2013) in a process dependent on guanylate-binding proteins (GBPs), a family of IFN-dependent immune guanosine triphosphatases (GTPases) (Meunier et al., 2014; Pilla et al., 2014). The mechanistic steps in which GBPs function remain controversial.

GBP proteins are undetectable by standard immunoblotting procedures at a cellular resting state but are highly upregulated by IFNγ and, to a lesser extent, IFNα/β (Kim et al., 2011; Yamamoto et al., 2012). In IFN-activated macrophages, several GBPs localize to pathogen-containing vacuoles (Kim et al., 2011; Meunier et al., 2014; Yamamoto et al., 2012) and have been functionally associated with pathogen vacuole rupture, bacterial killing, or both (Meunier et al., 2014, 2015; Man et al., 2016). In addition, induction of an IFN response during infection (Broz et al., 2012; Meunier et al., 2014; Man et al., 2015) has resulted in a model postulating that infection-driven IFN signaling is required for GBP synthesis and function (Meunier and Broz, 2015).

During L. pneumophila challenge, bacterial DNA/RNA trigger cytosolic sensors to induce Ifnb transcription within 4–6 hr of infection (Monroe et al., 2009; Lippmann et al., 2011), although secreted IFNβ protein is not detected until 20 hr post-infection (Coers et al., 2007). The disconnect between Ifnb gene transcription and protein accumulation is presumably due to inhibition of host cell protein translation during Legionella infection (Fontana et al., 2011; Ivanov and Roy, 2013; Asrat et al., 2014). In line with these findings, caspase-11 activation in response to vacuole-resident L. pneumophila requires IFN priming. The need for IFN
priming can be bypassed by a *L. pneumophila* mutant lacking the SdhA protein (*JsdhA*) (Monroe et al., 2009; Aachoui et al., 2013). This mutant strain forms an unstable vacuole that exposes the bacterium to the host cytosol (Laguna et al., 2006; Creasey and Isberg, 2012). Despite the cytosolic exposure of the bacterium, caspase-11 activation remains dependent on GBPPs encoded on chromosome 3 (Pilla et al., 2014). The differential requirement of IFN priming for pyroptosis when challenged with vacuole-stable or cytosol-accessible bacteria calls for a re-evaluation of the function of GBPPs upstream of caspase-11.

In this work, we investigated the initiation of antimicrobial responses toward a cytosolic bacterium. We found that GBPPs are not involved in pathogen vacuole disruption and that cytosol-exposed *L. pneumophila* drove macrophage pyroptosis in the absence of microbial-induced IFN signaling. Constitutive IFN signaling maintains GBP expression at steady-state levels that are low but sufficient for function upstream of caspase-11 and caspase-1. We also found that chromosome 3 GBPPs are required for the release of bacterial DNA, preceding the cyclic GMP-AMP synthase/stimulator of IFN genes (cGAS/STING) induction of IFN. During *Legionella*-induced pneumonia, chromosome 3 (chr3) GBPPs are required for the interleukin (IL)-1/chemokine (C-X-C motif) ligand 1 (CXCL1) response for timely bacterial clearance.

**RESULTS**

**Infection-Driven IFN Signaling Is Dispensable for Pyroptosis**

All *L. pneumophila* strains used in the present study are deficient for flagellin (*flaA*) to bypass the Naip5/NLRC4/caspase-1 cell death pathway that occurs on the C57BL/6 genetic background (Ren et al., 2006). *L. pneumophila* deficient for the SdhA protein (*JsdhA*) becomes cytosolic exposed (Laguna et al., 2006; Creasey and Isberg, 2012) and drives macrophage pyroptosis (Aachoui et al., 2013). Maximal caspase-11-mediated macrophage pyroptosis toward the *L. pneumophila JsdhA* strain was observed by 6 hr post-infection (Figure 1A). Using *L. pneumophila*-GFP to distinguish infected and bystander cells, we confirmed that cell death occurred in the subpopulation that harbored bacteria (Figures S1A and S1B). To rule out the contribution of necroptosis, another form of necrotic cell death (Wallach et al., 2016), we used Necrostatin-1 (Nec1) to inhibit RIP1/RIP3 activity, which did not affect cell death (Figure S1C).

*L. pneumophila* infection of macrophages results in robust *Ifnb* transcription via cytosolic DNA- and RNA-sensing pathways STING and RIG-I/MDA5, respectively (Monroe et al., 2009; Lippmann et al., 2011). Because *Legionella* translocates effectors that selectively block host cell protein translation, it is unclear whether type I IFN bypass the *L. pneumophila*-elicited translational block (Fontana et al., 2011; Ivanov and Roy, 2013; Asrat et al., 2014). Transcriptional induction of *Ifnb* in response to the *L. pneumophila JsdhA* mutant was similar in magnitude to that of *LPS* (Figure S1D). However, we did not detect secretion of IFNβ protein from *Legionella*-infected cells, in contrast to cells stimulated with LPS, cytosolic cyclic [G(2’,5’)]pA[3’,5’]p (cGAMP), and cytosolic polyinosine-polycytidylic acid (polyI:C) (Figure 1B; Figure S1D). To determine whether infected macrophage populations were experiencing IFN signaling, we probed for STAT1 phosphorylation downstream of IFN-α/β receptor (IFNAR). We were unable to detect increased STAT1 phosphorylation within 6 hr of *L. pneumophila* infection, in contrast to that observed in the presence of LPS, cGAMP, and polyI:C (Figures 1C and 1D). To dismiss the possibility of missed STAT1 phosphorylation in dying cells, STAT1 phosphorylation was measured in *Casp11−/−* cells or B6 cells treated with pan-caspase inhibitor zVAD (Figure 1D). No increase in STAT1 phosphorylation were detected under these conditions (Figure 1D).

IFN feedback is proposed to drive de novo synthesis of GBPPs and caspase-11, so we investigated whether these proteins were produced before cell death. We were unable to detect accumulation of GBP2 by 4 and 6 hr post-infection (Figure 1E), although we did see clear accumulation of GBP2 protein in response to the avirulent *L. pneumophila dotA* mutant by 10 hr post-infection, consistent with its inability to block protein synthesis (Figure S1E) (Ivanov and Roy, 2013). We observed an increase of pro-caspase-11 within 6 hr of infection (Figure 1E). To determine whether pro-caspase-11 was synthesized by infected or bystander cell populations, macrophages challenged with *L. pneumophila*-GFP strains were sorted to separate infected GFP− cells from bystander GFP+ cells. Pro-caspase-11 accumulated in the GFP− bystander population, but not in the GFP+-infected population (Figure 1F). The lack of GBP2 and pro-caspase-11 accumulation in infected cells suggest that low protein abundance present in resting macrophages is sufficient for pyroptosis.

To determine whether IFN signaling concurrent with infection stimulates pyroptotic death, cells were incubated with 100 IU/mL of recombinant IFNβ at various times leading up to bacterial challenge and cell death was monitored (Figures 1G–1I). We found that despite robust IFNAR signaling in response to exogenous IFNβ (Figure 1H), IFNβ stimulation simultaneous with, or 1 hr before, infection had no effect on cell death induced by either the *JsdhA* mutant (Figure 1I, left) or wild-type (WT) (Figure S1F). IFNβ pre-treatment at least 4 hr before infection was needed to significantly increase the rate of cell death, as demonstrated by a reduction in the time to 50% of maximal cell death (Figure 1I, middle). Extensive IFN treatment did not significantly increase the maximal cell death in response to the *JsdhA* mutant (Figure 1I, right). In contrast, cell death toward the WT strain required 4 hr of IFN pre-treatment (Figure S1F). In both cases, at least 4 hr of pre-activation with IFNβ was necessary to enhance cell death, indicating that cell death depends on pre-established IFN status.

**Constitutive IFNAR Signaling Controls Macrophage Cell Death Rate**

Sub-threshold amounts of IFNα/β are thought to maintain expression of a set of IFN-stimulated genes (ISGs) required for cellular responses against infection (Gough et al., 2012). To determine the impact of constitutive IFN signaling on caspase-11 activation, we treated macrophages with an IFN receptor (IFNAR)-blocking antibody (clone MAR1-5A3) for various lengths of time (Figures 2A–2D). Blocking IFNAR 1 hr before infection did not affect cell death kinetics toward the *JsdhA* mutant (Figures 2C and 2D), despite efficient block of STAT1 phosphorylation.
in response to exogenous IFNβ (Figure 2B). However, 20-hr blockade of IFNAR before infection reduced cell death in response to the ΔsdhA mutant (Figures 2C and 2D). Macrophages from mice lacking type I IFN receptor (Ifnar−/−) also exhibited defective cell death, characterized by delayed onset with a significant increase in time to 50% maximal cell death (Figures 2E and 2F). Prolonged inhibition of IFNAR signaling did not alter cell death in Casp11−/− macrophages, indicating that cell

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Figure 2. Constitutive IFNAR Signaling Controls Macrophage Cell Death Rate

(A–D) IFN receptor blocking antibody (αIFNAR) or immunoglobulin G (IgG) control were added before or during bacterial challenge.

(A) Antibody block and infection setup.

(B) Immunoblot of macrophages in response to 1 hr of treatment with 100 IU/mL of exogenous IFN, in the presence of antibodies for 1 or 20 hr before IFN addition.

(C) L. pneumophila ΔsdhA infection-driven PI incorporation of macrophages that were treated with control IgG or αIFNAR antibody for 1 or 20 hr before infection.

(D) PI uptake by 6 hpi; n = 3.

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death in the absence of IFN signaling remained caspase-11 dependent (Figure S2A).

We next determined whether loss of constitutive IFN signaling perturbed basal levels of proteins in the caspase-11 pathway. STAT1 is an ISG whose protein abundance is sustained via constitutive IFNAR signaling (Gough et al., 2010, 2012). 20-hr IFNAR block reduced total STAT1 protein levels (Figure 2G), as well as mRNA expression for several ISGs, such as Irf7, lsg15, and Mx1 (Figure S2B). We did not observe a reduction in baseline expression of pro-caspase-11 protein or Casp11 mRNA under conditions in which constitutive IFN signaling is lost (Figures 2H and 2I). Of particular interest is that for detection of GBP2 protein was expressed in macrophages in the absence of priming (Figure S2C), with expression of Gbp2 and Gbp3 reduced in Ifnar−/− and Ifnb−/− macrophages (Figure 2J; Figure S2C). Expression of Gbp2 and Gbp3 was also significantly reduced by blocking constitutive IFNAR signaling (Figure 2J). Not all GBPs required constitutive IFNAR signaling for basal expression, such as Gbp7 on chr3, as well as Gbps encoded on the chromosome 5 locus (Figure 2J; Figure S2D).

Endogenous GBP Expression and Rate of Cell Death Are Controlled by a Narrow Window of Low-Dose IFN Signaling

Pyroptosis of Ifnb−/− macrophages exhibited delayed onset after challenge with L. pneumophila JsdhA compared to B6, consistent with reduced Gbp2 and Gbp3 expression (Figure 3A). To determine the amount of IFN needed to rescue this defect, Ifnb−/− bone marrow derived macrophages (BMDMs) were treated with increasing amounts of IFN[IFN] 20 hr before challenge with bacteria (Figure 3B). JsdhA mutant cell death was restored with an IFN[IFN] dose of 0.5 IU/mL (Figure 3B), 0.5 or 1 IU/mL of IFN[IFN] restored expression of STAT1 (Figure 3C), Gbp2, and Gbp3 (Figure 3D), as well as several other ISGs (lsg15, Mx1, and Ifr7) (Figure S3) to levels observed in B6 macrophages at steady state.

We found that 4 IU/mL of IFN[IFN] added to Ifnb−/− macrophages was sufficient to induce a cytotoxic response reminiscent of cells stimulated with 100 IU/mL of IFN[IFN], a standard dose used for IFN activation (Figure 3B). This parallels the amount necessary to mimic STAT1 phosphorylation levels in B6 macrophages (Figure 3C). Of particular interest is that for detection of GBP2 protein by immunoblotting, at least 10–50 IU/mL of IFN[IFN] stimulation was needed (Figures 3E and 3F).

GBP5 Perturb Cytosolic Bacteria after Pathogen Vacuole Disruption

The localization of GBPs around pathogen-containing vacuoles has been associated with bacterial death (Meunier et al., 2014, 2015; Man et al., 2016), although this association has not been demonstrated for restriction of either L. pneumophila or Chlamydia muridum (Pilla et al., 2014; Finethy et al., 2015). In addition, there is disagreement on the exact step or steps promoted by GBPs, with earlier studies suggesting a role for GBPs in pathogen vacuole disruption and later studies showing GBPs to function downstream of pre-destroyed vacuoles (Meunier et al., 2014, 2015; Man et al., 2015, 2016). We therefore sought to determine the role of GBPs in the disruption of Legionella-containing vacuole (LCV).

The loss of LCV integrity can be assayed by immunofluorescent probing of bacteria in the absence of membrane permeabilization with detergents (Figure 4A) (Creasey and Isberg, 2012). Loss of the JsdhA mutant vacuole integrity was unaltered in Ifnar−/−, Gbpchr3−/−, and Casp11−/− macrophages (Figure 4B). Nevertheless, when challenged with L. pneumophila, JsdhA and Gbpchr3−/− macrophages exhibited delayed and reduced maximal cell death (Figure 4C). Cytosolic L. pneumophila assumes an aberrant morphology after breakdown of the LCV, which can be detected as the normally rod-shaped organism assuming a swollen, frayed, or truncated morphology (Figure 4A) (Laguna et al., 2006; Creasey and Isberg, 2012). Within the cytosol-accessible bacterial population, we observed that cytosolic L. pneumophila retained their rod shape in Gbpchr3−/− macrophages up to 10 hr post-infection, indicating that GBP function was required for inflicting damage on cytosolic bacteria (Figures 4D and 4E). In Ifnar−/− macrophages, cytosolic bacteria retained their rod-shaped morphology at early time points post-vacuole disruption (Figures 4E and 4F) but eventually gained aberrant morphology (Figures 4D, 4E, and 4G).

WT L. pneumophila triggered pyroptotic cell death only when macrophages were pre-activated with IFNs (Figures S1F, S4A, and S4B) (Case et al., 2013). This process requires the chr3-encoded GBPs (Pilla et al., 2014). 100 IU/mL of IFN[IFN] pre-activation rapidly destabilized the vacuoles of WT L. pneumophila in macrophages sufficient or deficient for chr3 GBPs (Figure S4C). Morphologically, IFN activation resulted in the loss of rod-shaped appearance in 80%–90% of cytosol-accessible L. pneumophila within 3 hr of infection in B6 and Casp11−/− macrophages. Almost all bacteria within the cytosol-accessible subpopulation retained their rod-shaped morphology in Gbpchr3−/− macrophages (Figures S4D and S4E). We therefore conclude that IFN pre-activation drives the synthesis of factors other
than GBPs that can destabilize an intact bacterial vacuole. While GBPs were dispensable for vacuole disruption under all conditions tested, they were required for the disruption of bacteria exposed to the cytosol, a crucial event upstream of caspase-11 activation and cell death.

Human Macrophages Require JAK/STAT Signaling for Constitutive Antimicrobial Responses

Caspase-4 and caspase-5 are human orthologs of murine caspase-11 that drive activation-induced cell death in response to intracellular LPS (Shi et al., 2014; Casson et al., 2015). Similar to our observations with mouse macrophages, both human bronchoalveolar lavage (BAL) cells and human peripheral blood monocyte-derived macrophages (MDM) challenged with L. pneumophila ΔsdhA exhibited cell death in the absence of IFN priming event (Figures 5A–5D; Figure S5).

We used JAK/STAT inhibitors to block human IFNAR signaling (Zurney et al., 2007; Jackson et al., 2016; Mostafavi et al., 2016). Human MDMs were treated with JAK1/2 inhibitor ruxolitinib (JAKi) for either 40 hr before infection (pre-infection block) or exclusively during infection (co-infection block) (Figure 5E). Treatment of human MDMs with JAKi abolished all IFN-β-stimulated STAT1 phosphorylation, independent of treatment protocol (Figure 5F). Treatment with JAKi for 40 hr also led to a drastic reduction of Stat1 protein levels (Figure 5F) and hISG15 and hMX1 mRNA expression (Figure 5G), indicating that there was loss of ISG signatures. The long-term block with JAKi before infection significantly reduced L. pneumophila ΔsdhA-induced cell death in human MDMs (Figures 5H and 5I), mimicking the results we observed with antibody blockade of IFNAR in murine macrophages (Figure 2).

Examining steps upstream of cell death, we observed loss of vacuole integrity with the L. pneumophila ΔsdhA mutant (Figure 5J). Long-term Jak1/2 inhibition before infection did not reduce cytosol permeability (Figure 5J). In contrast, within the cytosol-exposed ΔsdhA population, long-term Jak1/2 inhibition before infection significantly decreased the percentage of bacteria with aberrant morphology (Figures 5K and 5L). These results point to the global importance of this host-derived constitutive signaling pathway in protecting against cytosolic Gram-negative pathogens.

GBPs Mediate the Release of DNA to Activate Cytosolic DNA-Sensing Pathways

To examine whether the observed bacterial morphological changes resulted in release of bacterial content into the macrophage cytosol, we probed for the presence of bacterial DNA in the cytosol of the host cell. Using L. pneumophila carrying the non-transferable pJB908 plasmid, the presence of pJB908 in the macrophage cytosol provides a measure of DNA released from disrupted bacteria (Ge et al., 2012). We found 2.5–3 times more plasmid in cytosolic extract of B6 macrophages challenged with L. pneumophila ΔsdhA(pJB908) than with WT infection (Figure 6A), consistent with previous reports (Ge et al., 2012). In contrast, cytosolic extracts from Ifnar−/− macrophages and Gbpchr3−/− macrophages infected with the ΔsdhA mutant...
harbored significantly lower amounts of plasmid than did similarly infected B6 macrophages (Figure 6A). During WT L. pneumophila challenge, the release of pJB908 plasmid was enhanced by IFN pre-activation in B6 and Casp11/C0/C0/C0 macrophages (Figure 6B). This enrichment was lost in Gbpchr3/C0/C0/C0 macrophages (Figure 6B).

Cytosolic DNA drives cGAS/STING activation and Ifnb induction. We assayed Ifnb mRNA upregulation and found significantly reduced Ifnb induction in Gbpchr3/C0/C0/C0 macrophages in response to L. pneumophila ΔsdhA (Figure 6C). We found significantly heightened Ifnb induction in Casp11/-/- macrophages, consistent with reports that caspase-1/11 activation curtails cGAS/STING activation (Corrales et al., 2016; Wang et al., 2017).

Casp1/-/-Casp11/-/- macrophages had a further reduction in cell death compared to Casp11/-/- macrophages, implicating a role for the canonical caspase-1 inflammasome (Figure 6D). and caspase-11 (Figure 6F, right), consistent with pyroptosis curtailing ASC oligomerization. We observed no enrichment of ASC specs in Gbpchr3/C0/C0/C0 macrophages in response to L. pneumophila ΔsdhA challenge compared to WT bacteria (Figures 6E and 6F). Lastly, L. pneumophila ΔsdhA infection resulted in the cleavage of 31-kDa pro-IL-1β into the 17-kDa mature IL-1β fragment in B6 macrophages. This cleavage event was significantly reduced in Casp11/-/- macrophages and further reduced in Gbpchr3/C0/C0/C0 macrophages (Figure 6G).

Chr3 GBPs Are Involved in Restriction of Legionella Bacterium during Pneumonia
To examine the role of GBPs during Legionella-induced pneumonia, B6 and Gbpchr3/C0/C0/C0 mice were infected with L. pneumophila ΔflaA or L. pneumophila ΔflaA ΔsdhA bacteria. We observed an acute drop in body temperature of B6 mice within...
Figure 5. Human Macrophages Require JAK/STAT Signaling for Constitutive Antimicrobial Responses

(A and B) Human bronchoalveolar lavage (BAL) cells were challenged with *L. pneumophila* WT or ΔsdhA strains.

(A) Cell death as a function of time assayed by PI uptake, compiled from 5 donors with mean ± SEM plotted.

(B) Magnitude of cell death in individual BAL samples by 4 hpi, represented as one marker per donor.

(C and D) Human peripheral blood monocyte-derived macrophages (MDM) were challenged with *L. pneumophila* WT or ΔsdhA strains with kinetics of PI incorporation in (C) and maximal cell death by 4 hr post-infection (representative of 3 experiments) in (D).

(E) Experimental setup for (F)–(L), in which human MDMs were treated with JAK1/2 inhibitor (JAKi) before or during challenge with *L. pneumophila* ΔsdhA.

(F) 200 IU/mL of recombinant human IFNβ was used to stimulate human MDMs, incubated for the indicated times with JAKi. 1 hr after IFN stimulation, lysates were collected and probed by immunoblot.

(G) Gene expression in human MDMs with or without JAKi for 40 hr.

(H and I) PI incorporation of human MDMs infected with *L. pneumophila* ΔsdhA, with simultaneous or prolonged JAK inhibition. Representative kinetics of death are in (H). Maximum cytotoxicity from 7 individual donors is in (I).

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7 hr of ΔsdhA \textit{L. pneumophila} infection (Figure 7A). Mice infected with the ΔsdhA mutant recovered their temperature by 20 hr post-infection. In contrast to B6 mice infected with the ΔsdhA mutant, \textit{Gbp^{chr3/-}} mice did not exhibit a ΔsdhA \textit{L. pneumo-}

phila}-specific temperature drop at an early time point post-infec-
tion. Temperature recovery 1 and 2 days post-infection was also
delayed in \textit{Gbp^{chr3/-}} mice when infected with either WT \textit{Legion-
ella} or ΔsdhA mutant (Figure 7A).

(L) Percentage of cytosol-accessible \textit{L. pneumophila} ΔsdhA based on antibody staining without detergent permeabilization. Data are shown from 2 individual donors.

(K and L) Representative images (K) and quantification (L) of cytosolic \textit{L. pneumophila} ΔsdhA in human MDMs in the presence or absence of 40 hr of JAKi pretreatment. Images taken with 63× lens; scale bar, 5 μm.

See also Figure S5.
Figure 7. Chromosome 3 GBPs Are Involved in Restriction of Legionella Bacterium during Pneumonia

(A) Mice were infected with $1 \times 10^7$ L. pneumophila bacteria oropharyngeally. Rectal temperature of mice at various time points post-infection. Each dot represents an animal; results are pooled from 2 experiments.

(B) Serum CXCL1 was measured by ELISA. Each dot represents an animal; results are pooled from 2 experiments in which tissues were harvested between 4 and 6 hr post-infection.

(C) Lung IL-1α and IL-1β were measured by ELISA. Each dot represents an animal; results are pooled from 2 experiments in which tissues were harvested between 12 and 18 hr post-infection.

(D and E) Legionella colony-forming units from whole-lung tissue; the geometric mean is shown. Each dot represents an animal; results are pooled from 2 experiments. Animals were infected with (D) WT or ΔsdhA bacterium and (E) WT bacterium.

(F) Whole-lung tissue was extracted from B6, Ifnar−/−, and Ifnb−/− animals in the absence of bacterial challenge. mRNA transcripts for indicated GBPs were quantified by qRT-PCR.

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Pneumonia models using flagellated *Legionella* had shown that the NLRC4/NAIP5 inflammasome elicits a robust IL-1 response in the lungs. IL-1 receptor signaling was necessary for the induction of CXCL1 to recruit neutrophils, leading to clearance of bacteria (Tateda et al., 2001; Barry et al., 2013). Pyroptotic macrophage corpses have been shown to retain bacteria at the site of infection. CXCL1-driven influx of neutrophils engulfed the macrophage carcasses, along with the trapped bacteria, to facilitate clearance (Jorgensen et al., 2016). CXCL1 thus appears to be crucial for clearance of bacteria that illicit an inflammasome activation. Systemically, B6 animals infected with the *sdhA* mutant displayed a robust, acute increase in serum CXCL1 that coincides with the dip in body temperature. This increase in serum CXCL1 was absent from *Gbpchr3* mutant (Figure 7B). Locally, we observed a *sdhA*-specific increase in IL-1α and IL-1β in the lungs of B6 animals 4–6 hr post-infection (Figure S7A). This IL-1α and IL-1β production was further amplified during the next 10 hr in B6 but was absent from the lungs of *Gbpchr3*–/- mice (Figure 7C).

To determine whether the perturbations in cytokine production affected the rate of bacterial clearance, we measured bacterial colony-forming units (CFUs) in the lungs of B6 and *Gbpchr3*–/- mice. It is well established that *Legionella* growth is restricted on a C57BL/6 genetic background. We found the restriction was more severe with *sdhA* mutant in B6 animals. In contrast, bacterial load of the *sdhA* mutant is significantly higher 1 and 2 days post-infection in *Gbpchr3*–/- mice (Figure 7D). WT *Legionella* burden was also higher in *Gbpchr3*–/- mice by day 5 (Figure 7E).

Finally, to determine whether tonic IFN signaling plays a role in the expression of GBP in vivo, we performed global RNA sequencing (GEO: GSE110678) from lungs of uninfected B6 and *Ifnar*–/- animals (Figure S7B). In whole lungs of resting B6 animals, Gbps as a gene family are skewed toward higher expression in B6 as opposed to *Ifnar*–/- animals, with Gbp1 and Gbp11 being undetectable from *Ifnar*–/- lungs (Figure S7B). Gbp2, Gbp3, and Gbp7 showed small differences in the two strains by global RNA sequencing (GEO: GSE110678), but on further interrogation by RT-PCR, they were found to be significantly reduced in the lungs of both *Ifnar*–/- and *Ifnb*–/- mice (Figure 7F). We similarly observed reduced expression of *Irf7*, *Isg15*, and *Mx1* in lungs of *Ifnar*–/- animals, although not all ISGs appear to depend on IFNγ, indicating differential roles for various IFNs in tonic signaling (Figure 7C). When subjected to *Legionella* lung infection, we surprisingly found temperature fluctuation of IFN-deﬁcient animals—*Ifnar*–/- and *Ifnb*–/- mice—to be similar to that of IFN-sufficient B6 animals (Figure 7G). We found *Ifnb*–/- mice exhibit more variability in their response; thus, further experiments are conducted using *Ifnar*–/- animals to bypass possible confounding effects is the various IFNs that may play a role in vivo. In *Ifnar*–/- animals, local production of IL-1α and IL-1β in the lungs was signiﬁcantly reduced during *sdhA* mutant challenge (Figures 7H and 7I), reminiscent of *Gbpchr3*–/- mice (Figure 7C; Figure S7A). However, *Ifnar*–/- animals remained restrictive to both WT and mutant *Legionella*, because we did not detect a difference in bacterial CFUs between *Ifnar*–/- and B6 animals toward the various *Legionella* strains at 48 hr post-infection (Figure 7J). Lastly, we found that *Ifnar*–/- animals retained the ability to induce a robust serum CXCL1 response when infected with the *sdhA* mutant (Figure 7K). The discrepancy between IL-1 and CXCL1 levels in IFN-deﬁcient animals suggest the surfacing of a compensatory pathway when IFNAR signaling is lost. Work by Schliehe et al. (2015) elucidated one such mechanism, in which reduced expression of *Setdb2* in *Ifnar*–/- mice, a repressor of CXCL1 expression, is speciﬁcally induced by type I IFN signaling. Loss of IFNAR heightened CXCL1 secretion in response to *Streptococcus pneumoniae* infection (Schliehe et al., 2015). To determine whether breakthrough of CXCL1 may be a contributing factor, we measured the local production of CXCL1 in the lungs and found a trend toward higher lung CXCL1 in *Ifnar*–/- mice when infected with the *sdhA* mutant, a response that is quiescent in B6 animals. We did not observe this enrichment in lung CXCL1 production in *Gbpchr3*–/- animals infected with the *sdhA* mutant (Figure S7D). This indicates a possibility that systemic CXCL1 response from *Ifnar*–/- mice is GBP independent but stems from a CXCL1 self-amplification loop via other cytosolic-sensing mechanisms.

**DISCUSSION**

Macrophage upregulation of an IFN response facilitates antimicrobial activities, including autophagy, pathogen recognition and attack, and host cell death (Randow et al., 2013; Schneider et al., 2014). Many studies have uncovered the mechanism of hyper-IFN-mediated protection (MacMicking, 2012; Pillia-Moffett et al., 2016). However, in the absence of disease and infection, an immunocompetent host is quiescent for cytokine responses, with intermittent spikes of IFN occurring only during infection or immune dysregulation (Bocci, 1985; Taniguchi and Takaoike, 2001; Gough et al., 2012). Little is known about the initial host-pathogen encounter at the onset of infection, before the infection-driven IFN spike. In this work, we set out to investigate the mechanisms by which naïve macrophages respond to vacuolar and cytosolic bacteria while simulating a physiologically relevant host-pathogen initial encounter.

Using the cytosol-accessible bacteria *L. pneumophila* *sdhA*, we found that caspase-11 action occurred independently of infection-driven IFN feedback. Instead, constitutive IFN signaling in the absence of infection was sufficient to maintain GBP regulation.
expression and enable GBP activity against cytosol-accessible bacteria. We propose that upon challenge with vacuole-resident bacteria, a small percentage of bacteria that are unable to maintain a stable replication vacuole engage the host cytokolic surveillance pathways as a result of GBP-mediated release of bacterial components. For Gram-negative bacteria, LPS activates caspase-11 (caspase-4/5 in humans), resulting in gasdermin D-dependent pyroptotic cell death (He et al., 2015; Kayagaki et al., 2015; Shi et al., 2015). Simultaneously, GBPs play a role in leakage of bacterial nucleic acids into the cytosol, activating nucleic acid-sensing pathways that can drive transcriptional activity, as well as the canonical caspase-1 inflammasome via AIM2. In the absence of protein translation block, a STING-dependent IFN response would in turn activate neighboring bystander cells, generating an IFN-activated phenotype in uninfected macrophages. Upon further infection, these IFN-activated macrophages would lyse pathogen vacuoles in an event that does not require GBPs encoded on chr3.

The canonical caspase-1 pathway can also contribute to pyroptosis in a GBP-dependent manner to contribute to the local IL-1 response. On a systemic level, IL-1 receptor signaling on endothelial cells amplifies the CXCL1 response to recruit neutrophils to the site of infection. Jorgensen et al. (2016) showed that pyroptotic macrophage corpses can trap cytosolic bacteria, increasing the efficiency by neutrophil internalization and degradation of the invading pathogen. In our Legionella-induced pneumonia model, we found that chr3-encoded GBPs are necessary for heightened IL-1α and IL-1β produced locally in the lungs, systemic CXCL1 response, and timely bacterial clearing.

Upstream of endogenous GBP expression, there exists a sensitive balance in which constitutive cytokine signaling primes the system for critical immune responses to occur upon infection. This concept was best represented in Ifnb−/− mice (He et al., 2015), resulting in gasdermin D-dependent pyroptotic cell death (He et al., 2015; Kayagaki et al., 2015; Shi et al., 2015). Simultaneously, GBPs play a role in leakage of bacterial nucleic acids into the cytosol, activating nucleic acid-sensing pathways that can drive transcriptional activity, as well as the canonical caspase-1 inflammasome via AIM2. In the absence of protein translation block, a STING-dependent IFN response would in turn activate neighboring bystander cells, generating an IFN-activated phenotype in uninfected macrophages. Upon further infection, these IFN-activated macrophages would lyse pathogen vacuoles in an event that does not require GBPs encoded on chr3.

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## STAR METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Anti-Caspase-11 clone 17D9 | Cell Signaling Technology | 14340 |
| Anti-GBP2            | Proteintech | 11854-1-AP |
| Anti-phospho-Stat1 Y701 58D6 | Cell Signaling technology | 9167 |
| Anti-STAT1           | Cell signaling technology | 9172 |
| Rabbit polyclonal serum against L. pneumophila | Isberg Lab | (Laguna et al., 2006; Creasey and Isberg, 2012) |
| Rat polyclonal serum against L. pneumophila | Isberg Lab | (Laguna et al., 2006; Creasey and Isberg, 2012) |
| Anti-ASC             | Adipogen | AG-25B-0006 |
| Anti-Gapdh 14C10     | Cell Signaling Technology | 2118 |
| Anti-Gapdh D4C6R     | Cell Signaling Technology | 97166 |
| Anti-IFNβ (ELISA capture antibody) | Santa Cruz | sc-57201 |
| Anti-IFNβ (ELISA detection antibody) | R&D Systems | 32400-1 |
| **Bacterial and Virus Strains** |        |            |
| L. pneumophila Lp02 ΔflaA | Isberg Lab | (Creasey and Isberg, 2012; Asrat et al., 2014) |
| L. pneumophila Lp02 ΔflaA ΔsdhA | Isberg Lab | (Creasey and Isberg, 2012) |
| L. pneumophila Lp03 (dotA-) ΔflaA | Isberg Lab | (Asrat et al., 2014) |
| L. pneumophila Lp02 ΔflaA PΔac::PΔac::Gfp | Isberg Lab | (Coers et al., 2007; O’Connor et al., 2012) |
| L. pneumophila Lp02 ΔflaA PΔac::PΔac::Gfp | Isberg Lab | (Coers et al., 2007; O’Connor et al., 2012) |
| L. pneumophila Lp02 ΔflaA pJB908 | Isberg Lab | (Laguna et al., 2006; Ge et al., 2012) |
| L. pneumophila Lp02 ΔflaA ΔsdhA pJB908 | Isberg Lab | (Laguna et al., 2006; Ge et al., 2012) |
| **Biological Samples** |        |            |
| Human Bronchoalveolar lavage cells | Tufts Medical Center, Alexander Panda, M.D. | De-identified volunteers |
| Human peripheral whole blood | NY Biologics | De-identified whole blood |
| **Chemicals, Peptides, and Recombinant Proteins** |        |            |
| Purified NA/LE Mouse anti-mouse IFNAR2/β receptor MAR1-SA3 | BD PharMingen | 561183 |
| Purified NA/LE Mouse IgG1 κ Isotype control | BD PharMingen | 553447 |
| Jak inhibitor Ruxolitinib | Cayman Chemical | 11609 |
| Recombinant mouse IFNβ | PBL | 12400-1 |
| TMB substrate | ThermoFisher | N301 |
| **Deposited Data** |        |            |
| Mi-Seq GSE110678 | This paper | N/A |
| **Experimental Models: Cell Lines** |        |            |
| Murine bone marrow derived macrophages | Mouse long bones | N/A |
| **Experimental Models: Organisms/Strains** |        |            |
| C57BL/6 | Jackson Laboratory | 000664 |
| Ifnar−/− (B6.129S2-Ifnar1tm1Agt/Mmjax) | Jackson Laboratory | 32045-JAX |
| Ifnb−/− | Dr. Stephanie Vogel | N/A |
| GBPchr3−/− | Dr. Jorn Coers; Dr. Masahiro Yamamoto | (Yamamoto et al., 2012; Pilla et al., 2014) |
| Casp11−/− | Dr. Vishva Dixit | (Kayagaki et al., 2011, 2013) |
| Casp1−/−/Casp11−/− | Jackson Laboratory | (Li et al., 1995) |
| Asc−/− | Dr. Kate Fitzgerald | (Zheng et al., 2011) |

(Continued on next page)
CONTACT FOR REAGENT AND RESOURCE SHARING

Requests for resources and reagents described in manuscript should be directed to Dr. Alexander Poltorak (alexander.poltorak@tufts.edu).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

L. pneumophila Bacterial Strains

All *L. pneumophila* strains used in the present study are deficient for flagellin (*dflaA*) to mimic growth in amoeba and human macrophages and bypass the NLRP4/Naip5/Caspase-1 cell death pathway that occurs on the C57BL/6 genetic background. *L. pneumophila* derivatives used in this study were Lp02 *dflaA* (*thyA rpsL *dflaA*; referred to as WT), Lp02 *dflaA* *ssdhA* (*thyA rpsL *dflaA* *ssdhA*; referred to as *ssdhA*), and Lp03 *dflaA* (*dotA3 thyA rpsL *dflaA*; referred to as *dotA*). All strains were derived originally from *Legionella pneumophila* Philadelphia-1 (Berger and Isberg, 1993). *L. pneumophila* was propagated on charcoal–N-(2-acetamido)-2-aminoethanesulfonic acid (ACES)–yeast extract plates with 0.1mg/mL thymidine (CYE/T) and in ACES-yeast extract broth (AYE/T) with 0.2mg/mL thymidine as previously described (Creasey and Isberg, 2012; Asrat et al., 2014).

To generate *L. pneumophila*-GFP, PCR-amplified *gfp* gene was fused to *PahpC* (Coers et al., 2007) and inserted downstream of *Ptac* in the plasmid pMMB207 *mob267* (Morales et al., 1991; O’Connor et al., 2012). Strains harboring the plasmid were maintained on CYE plates containing 0.1mg/mL thymidine and 5 μg/mL chloramphenicol. Prior to challenge of cultured cells, AYE broth cultures containing 0.2mg/mL thymidine and 2mM IPTG were grown to post-exponential phase, as described (Asrat et al., 2014). Inoculation into macrophage monolayer cultures was carried out at MOI = 10 in the presence of 2mM IPTG.

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The plasmid pJB908 was described previously (pMMB66EH oriR6K oriT td ori bla; Laguna et al., 2006). For selection, pJB908-containing strains were grown on CYE plates or in AYE broth in the absence of added thymidine.

**Murine Macrophages**

8–10-weeks-old male and female mice are used for this study. C57BL/6 and Ifnar−/− mice were obtained from Jackson Laboratory. Ifnb−/− mice and Casp11−/− mice were kind gifts from Dr. Stephanie Vogel and Dr. Vishva Dixit respectively. All animal studies were approved by the Institutional Animal Care and Use Committee of Tufts University. Bone marrow macrophages are from Gbpchr3−/− mice (Pilla et al., 2014; Yamamoto et al., 2012). Bone marrow-derived macrophages were isolated from mice and propagated for 7 days in RPMI containing 20% FBS, 30% L cell supernatant, 2% Penn/Strep on non-tissue culture treated Petri dishes (Asrat et al., 2014).

**Murine in vivo infections**

Animal infection protocols were approved by the Tufts University Medical School Animal Care and Use Committees. Male and female mice between 8–10-weeks-old are used. C57BL/6, Ifnar−/−, Gbpchr3−/−, and Ifnb−/− mice were oropharyngeally inoculated with 1x10^7^ bacteria under isoflurane anesthesia. Temperature was monitored via rectal thermometer. At time points indicated in the text and figures, mice were euthanized via CO₂ asphyxiation, and blood and lungs were harvested for downstream cytokine and bacterial load quantification.

**Human peripheral blood monocytes derived macrophages (MDM)**

De-identified human peripheral blood was obtained from New York Biologics. The use of de-identified human samples followed a protocol approved by the Tufts University School of Medicine Institutional Review Board. No information on age and sex of the donor was provided by NY Biologics. Monocytes were obtained from peripheral blood using the EasySep Direct Monocyte Isolation Kit (STEMCELL technologies). CD14^+^CD16^−^ monocytes were extracted and differentiated into CD14^+^CD16^−^CD68^+^ macrophages over the course of 7 days in RPMI containing 20% FBS, 200 IU/mL Penicillin and 200 µg/ml Streptomycin, and 100 µg/ml of human monocyte colony stimulating factor (M-CSF; PeproTech) (Davies and Gordon, 2005; Jaguin et al., 2013). Nonpolarized MDMs were cultured for 40 hr further in RPMI containing 10% FBS, 200 IU/ml Penicillin and 200 µg/ml Streptomycin, in the absence of M-CSF prior to infection. Infection was carried out at MOI = 10.

**Human bronchoalveolar lavage cells (BAL)**

Bronchoalveolar (BAL) lavage of humans was performed at Tufts Medical Center. BAL was obtained with informed consent under a protocol approved by the Tufts University School of Medicine Institutional Review Board. Subjects underwent fiberoptic bronchoscopy and BAL with 180 mL of lavage solution. With the subject under local anesthesia, a 5.5-mm O.D. fiberoptic bronchoscope was advanced through the mouth and into the trachea and was wedged into a segmental or subsegmental bronchus of the right upper lobe. The wedged lung segment was lavaged with three aliquots of 60 mL each of normal sterile saline prewarmed to 37°C; and the fluid was gently aspirated (St-Laurent et al., 2009; Freeman et al., 2015). BAL fluids were provided without information on age and sex of the donors. Cells were strained from BAL fluid using 70µm cell strainers and allowed to adhere to experimental dishes in RPMI containing 10% FBS and 200 IU/ml Penicillin and 200 µg/ml Streptomycin, at a density of 0.5x10⁶ cells per cm². Cells were allowed to attach for 2 hr at 37°C, 5% CO₂, at which point non-adherent cells and antibiotics were washed away with RPMI plus 10% FBS (Davies and Gordon, 2005). Bacteria were inoculated at an MOI = 10.

**METHOD DETAILS**

**In vitro L. pneumophila infections**

For infection of murine and human macrophages, L. pneumophila was infected at MOI = 10, in the absence of antibiotics.

**Kinetic cytotoxicity assay with propidium iodide uptake**

Cells were incubated in clear bottom 96 well microplates (Costar 3603) in media containing 10 µg/mL propidium iodide (PI; Life Technologies, P3566). A TECAN Infinite 200 Pro plate reader was used to maintain temperature at 37°C and 5% CO₂ during incubations. PI uptake was monitored every 10 min at 534 nm excitation and 617 nm emission, using bottom reading setting. For 100% cytotoxicity control, cells were treated with 0.1% Triton X-100, similar to protocols for measuring Lactate Dehydrogenase (Promega). All propidium iodide incorporation assays were performed in triplicate wells. This protocol was adapted from previous work (Case et al., 2013).

**Western blotting**

Macrophages were challenged with L. pneumophila as described (Asrat et al., 2014). At the desired time points, cells were lysed directly in 1X Laemmli Buffer with 5% β-mercaptoethanol, boiled for 10 min, and incubated on ice for 10 min prior to loading on SDS-PAGE gels and western blotting (Asrat et al., 2014). Primary antibodies used were anti-Caspase-11 clone 17D9 (Cell Signaling Technology #14340, 1:500), anti-GBP2 (Proteintech 11854-1-AP, 1:1000), Anti-phospho-STAT1(Tyr701) (58D6), anti-total STAT1,
and anti-GAPDH were obtained from Cell Signaling Technology. Western blots were imaged using LI-COR Odyssey CLx or by chemiluminescent detection on film followed by analysis using LI-COR analysis software.

IFNβ ELISA

A sandwich ELISA for IFNβ was used to measure supernatant IFNβ as International Units/ml as calculated based on a bioactive IFNβ recombinant protein standard, as previously described (Roberts et al., 2007; Rathinam et al., 2012). In brief, capture antibody (monoclonal rat anti-mouse IFNβ, Santa Cruz sc-57201) was applied at 4C overnight in 0.1M carbonate buffer. Samples are incubated overnight and further antibody steps are diluted in 10% fetal calf serum in phosphate-buffered saline. Recombinant IFNβ (PBL 12400-1) was used for protein standard. Bound protein was detected using polyclonal rabbit anti-mouse IFNβ (R&D Systems, 32400-1). ELISA was developed using Goalt anti-rabbit-HRP (Cell Signaling Technology 7074) in combination with TMB substrate (ThermoFisher N301), reaction stopped with 2N H2SO4.

Quantitative RT-PCR

Cells were lysed with TRIzol (Thermo Fisher Scientific) and mRNA extracted by chloroform/isopropanol in accordance with TRIzol manufacturer's protocol. Quantitative PCR was performed with SYBR Green (Thermo Fisher Scientific) following cDNA generation by M-MuLV Reverse Transcriptase (NEB). Primers for Gbp genes were previously published (Kim et al., 2011). Primer sequences for Casp11 were same as previously published (Kayagaki et al., 2011). Primers for mouse Ifr7: (F) 5'-CTTCAGCACTTCTTTCCGAGA-3', (R) 5'-TGTAAGTGTTGGACCCCTTG-3'; Isg15: (F) 5'-GAGCTAGAGGCTGGAGCA-3', (R) 5'-TCCTGCGGAACTCTGCCTTT-3'; Mx1: (F) 5'-TCGGAGGAGGACGAGCATG-3', (R) 5'-ACCTCTGCTGAGCTGCACGAG-3'; human Ifr7 (F) 5'-CTTGGCTCTAGGAGGGCAGA-3', (R) 5'-CGAAGGTCGGAGGGAAGA-3'; human ISG15 (F) 5'-TCCTGCGGAACTCTGCCTTT-3', (R) 5'-TTGTTATTCCTCACCAGGATGCT-3'; human Mx1 (F) 5'-GTCAATCGAGAAGGTCAGA-3', (R) 5'-TCAGAGCCCTTGAATGGT-3'; human GAPDH (F) 5'-GCTCTCTCCTGAGCTGACGTA-3', (R) 5'-ACCTTCCCACATGGTTCTGA-3'.

Immunofluorescence microscopy for bacterial morphology

Both mouse and human cells were seeded at a density of 0.5x 10⁶ cells per cm² on MatriPlate 0.17mm glass bottom plates (DOT Scientific) in RPMI containing 10% FBS. Cells were challenged with L. pneumophila-GFP or L. pneumophila at MOI = 10, in the presence of 10% FBS. At indicated time point post challenge with L. pneumophila, reaction stopped with 2N H2SO4.

ASC speck quantification

Cells were seeded at a density of 0.5x 10⁶ cells/cm² on MatriPlate 0.17mm glass bottom plates (DOT Scientific) in RPMI containing 10% FBS. At indicated time point post challenge with L. pneumophila, cells were fixed/permeabilized via ice cold methanol.
Polyclonal rat serum against *L. pneumophila* (1:5000) and rabbit anti-ASC antibody were used to recognize *L. pneumophila* and endogenous ASC. Goat anti-rat AF488 and goat anti-rabbit AF594 were used to visualize *L. pneumophila* and ASC, respectively. 25 fields at 20x magnification were captured and stitched by the Cytation3 automated microscope, generating fields of view with 3000 cells each for image quantification. Signal intensity of *L. pneumophila* and ASC were analyzed with the Gen5 software, using uninfected wells, and *Asc^-/-* macrophages as negative controls to determine the appropriate signal intensity and puncta size. Puncta sizes less than 3 μm were considered positive events.

**RNA-sequencing**

Total RNA was isolated from uninfected mouse lungs using TRIzol and used to make a directional cDNA library using TrueSeq kit. cDNA libraries were sequenced on MiSeq (Illumina) and aligned using TopHat2 and Cufflinks software. Fragments per kilobases mapped was plotted in Prism7. Colors for data visualization is described in the text and figure legends. The data are available at the National Center for Biotechnology Information Gene Expression Omnibus: GSE110678

**QUANTIFICATION AND STATISTICAL ANALYSES**

Statistical analyses on experiments using murine bone marrow derived macrophages were performed using the Student’s t test (two-tailed) using GraphPad Prism7. Experiments involving human macrophages were analyzed using paired analysis, in which each pair consists of cells from the same donor. Bacterial colony forming units from mouse lungs were compared using log-transformed values. (*) p < 0.05; (**) p < 0.01; (***) p < 0.001; (****) p < 0.0001.

**ACCESSION NUMBERS**

The accession number for the RNA sequencing data reported in this paper is NCBI SRA: GSE110678.
Supplemental Information

Constitutive Interferon Maintains GBP Expression
Required for Release of Bacterial Components
Upstream of Pyroptosis and Anti-DNA Responses

Beiyun C. Liu, Joseph Sarhan, Alexander Panda, Hayley I. Muendlein, Vladimir Ilyukha, Jörn Coers, Masahiro Yamamoto, Ralph R. Isberg, and Alexander Poltorak
(A) B6 macrophages were challenged with WT, dotA- mutant, or AsdhA mutants for the indicated times and Ifnb mRNA was measured by qRT-PCR.

(E) B6 macrophages were treated with WT, dotA-, or AsdhA strains for 10 hours. Whole cell lysates were probed by Western blot for Gbp2, pro-Casp11 and GAPDH. 10 hour LPS incubation and cGAMP transfection are displayed.

(F) B6 macrophages were pre-treated with 100U/ml of IFNβ at the indicated times points prior to, or concurrent with L. pneumophila WT challenge (see timeline in Fig 1G). Displayed is PI incorporation as a function of time (left), and maximum cell death measured at 6 hpi (right).

Figure S1. (Related to Figure 1)

(A, B) Macrophages were inoculated with L. pneumophila GFP+ and at 6 hpi (A) or 8 hpi (B), cellular GFP-associated signal and propidium iodide (PI) incorporation were measured by flow cytometry. Histograms show one representative experiment. Bar graphs show compiled data from n>3 experiments of PI incorporation in the indicated gates after challenge of either B6 or Casp11-/- macrophages.

(C) B6 and Casp11-/- macrophages were challenged with the L. pneumophila WT and ΔasdhA strains in the presence of Nec1 (RIPK1/3 inhibitor) or zVAD (pan-Caspase inhibitor). PI incorporation at 6 hpi is shown.
**Figure S2.** (Related to Figure 2)

**A** B6 and *Casp11/-* Macrophages inoculated with *L. pneumophila AsdhA* after 20 hr treatment with noted antibodies. PI incorporation used to measure cell death.

**B** qRT-PCR of *Irf7*, *Isg15*, *Mx1* expression in the presence or absence of constitutive IFN signaling.

**C** mRNA transcripts of chromosome-3 encoded GBPs

**D** mRNA transcripts of chromosome-5 encoded GBPs

(B6) qRT-PCR showing the baseline expression of noted genes in macrophages from various mouse strains. The CT values of genes of interest are normalized to sample-intrinsic *Gapdh* CT values. Dotted red line indicates background amplification from knock-out macrophages.
**Figure S3.** (Related to Figure 3)

*Ifnb-/-* macrophages were treated for 20 hours with various doses of recombinant IFNβ as in Fig 3B, C, D. Transcript levels were measured by qRT-PCR. Dotted black line indicates steady-state expression of genes of interest in B6 macrophages.
Figure S4. (Related to Figure 4)
(A) Macrophages were pre-treated for 20 hrs with 100U/ml of IFN prior to inoculation with WT L. pneumophila.
(B) Kinetics of WT L. pneumophila-induced cell death in the absence and presence of IFN pre-activation.
(C) Percentage of cytosolic-accessible L. pneumophila based on antibody staining without detergent permeabilization.
(D) Representative images of cytosolic WT bacteria in noted macrophages at 3 hpi. Images were taken using 63x lens, scale bar = 5m.
(E) Quantification of cytosolic accessible WT bacteria scored for aberrant (left) or rod-shaped (right) morphology at 3 hpi.
Figure S5 (Related to Figure 5)
Individual kinetics of *ex vivo* human bronchoalveolar lavage (BAL) cells infected with *L. pneumophila* WT or ΔsdhA, from 5 independent donors.
B6 macrophages were challenged with the indicated strains of \textit{L. pneumophila} in the presence of zVAD as indicated. ASC signal positivity is quantified within areas positive for anti-\textit{Legionella} antibody (ROI).

\textbf{Figure S6.} (Related to Figure 6)
Supporting Figure 7 (Related to Figure 7)
(A) Lung IL-1α and IL-1β were measured by ELISA. Each dot represents an animal, results are pooled from 2 experiments in which tissues were harvested between 12 and 18hrs post infection.
(B) Whole genome RNA-sequencing was performed on the indicated tissue and cellular populations. Fragments per kilobase mapped (FPKM) is plotted. Each dot is an individual gene. Gray dots represent genes where the expression difference between B6 and Ifnar-/- tissue is less than 2 fold. Yellow dots represent genes in which the expression difference between B6 and Ifnar-/- tissue is more than 2 fold. Blue dots represent the 11 Gbp genes encoded on the mouse genome, dots labeled. Magenta represent classical ISGs Stat1, Irf7, Isg15, Isg20, Mx1, Mx2.
(C) Various ISGs expression levels measured by qPCR from uninfected lungs from B6, Ifnar-/- and Ifnb-/- animals. Each dot represents an animal.
(D) Lung CXCL1 was measured by ELISA. Each dot represents an animal, results are pooled from 2 experiments in which tissues were harvested within a 4-6 hour window post infection.