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A Nonpyroptotic IFN-γ–Triggered Cell Death Mechanism in Nonphagocytic Cells Promotes *Salmonella* Clearance In Vivo

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The cytokine IFN-γ has well-established antibacterial properties against the bacterium *Salmonella enterica* in phagocytes, but less is known about the effects of IFN-γ on *Salmonella*-infected nonphagocytic cells, such as intestinal epithelial cells (IECs) and fibroblasts. In this article, we show that exposing human and murine IECs and fibroblasts to IFN-γ following infection with *Salmonella* triggers a novel form of cell death that is neither pyroptosis nor any of the major known forms of programmed cell death. Cell death required IFN-γ-signaling via STAT1-IRF1–mediated induction of guanylate binding proteins and the presence of live *Salmonella* in the cytosol. In vivo, ablating IFN-γ signaling selectively in murine IECs led to higher bacterial burden in colon contents and increased inflammation in the intestine of infected mice. Together, these results demonstrate that IFN-γ signaling triggers release of *Salmonella* from the *Salmonella*-containing vacuole into the cytosol of infected nonphagocytic cells, resulting in a form of nonpyroptotic cell death that prevents bacterial spread in the gut. ***The Journal of Immunology*****, 2018, 200: 3626–3634.**

Salmonella enterica is a facultative intracellular bacterium that causes severe foodborne illness in humans worldwide (1, 2). Even though *S. enterica* infection is more common in underdeveloped countries, it is still very prevalent in the United States, infecting millions annually, usually through handling of raw or undercooked meats (3). Of the many serovars of *S. enterica*, two (Typhi and Typhimurium) are the most common causes of *Salmonella* sp. illnesses in humans (4, 5). *S.* Typhi infection is more commonly found in countries that lack sanitary food or drinking water, and it infects via the fecal–oral route following ingestion of the bacterium (6). If untreated, *S.* Typhi infection leads to severe fever and can be fatal (7). *S.* Typhimurium also infects humans but induces a self-limiting gastroenteritis that normally does not require treatment (8). *S.* Typhi does not significantly activate the host inflammasome machinery and spreads systemically through the host, whereas *S.* Typhimurium, which induces a robust immune response, is rapidly cleared after limited spread in the intestine (9, 10). *S.* Typhi does not cause disease in mice, and therefore *S.* Typhimurium, which is pathogenic in mice, is used to model *Salmonella* infection in murine systems (11, 12).

Although the outcomes of infection are different between the two serovars, the initial stages of infection and immune mechanisms triggered are very similar. Upon ingestion, *Salmonella* first infects cells of the gut epithelium (13). *Salmonella* can invade these cells through use of an acquired pathogenicity island (SPI-1) that contains a type III secretion system (T3SS) (10, 14–16). SPI-1 has also recently been shown to prolong cell survival in nonphagocytic cells through Akt (17). After invasion, *Salmonella* induces formation of a cytosolic vacuole, called the *Salmonella*-containing vacuole (SCV), around the bacterium that allows for protection against host cytosolic antibacterial responses. For survival and replication in phagocytic cells, *Salmonella* uses a second pathogenicity island (SPI-2) that is required for survival in the low pH of these cell types (18, 19).

The host macrophage has in place a mechanism of controlling *Salmonella* infection by preventing replication in the SCV. A family of IFN-inducible GTPases, called guanylate binding proteins (GBPs), localize to the SCV postinfection and lead to the formation of pores in the vacuole, releasing *Salmonella* into the cytosol of the infected macrophage (20, 21). LPS is sensed by the NLRC4 inflammasome machinery or directly by caspase-11, triggering cleavage of caspase-1/11 and activation of gasdermin D, leading to activation of pyroptosis, a proinflammatory form of cell death (22–26). In the absence of caspase-1, *Salmonella* can induce caspase-8–dependent cellular extrusion in intestinal epithelial cells (IECs) (27). Macrophages may also undergo necroptosis upon infection with *Salmonella*, dependent on type I IFN signaling (28).

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Abbreviations used in this article: GBP, guanylate binding protein; IEC, intestinal epithelial cell; LB, Luria broth; MEF, murine embryonic fibroblast; MOI, multiplicity of infection; PMN, polymorphonuclear granulocyte; SCV, *Salmonella*-containing vacuole; T3SS, type III secretion system; WT, wild-type.

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Most studies of *Salmonella*-induced cell death have been conducted in macrophages and other phagocytes, and less is known about the role or mechanism of programmed cell death pathways in the control of *Salmonella* pathogenesis in nonphagocytic cells. As we and others have previously shown that IFNs induce necroptosis in murine embryonic fibroblasts (MEFs) and other nonphagocytic cell types (29), and as *Salmonella* was reported to trigger IFN-induced necroptosis (28), we sought to test if *Salmonella* can induce cell death in nonphagocytic cells and whether death was a result of IFN-dependent necroptosis.

In this study, we show that although *Salmonella* on its own does not induce cell death in nonphagocytic cells, exposure of infected cells to IFN-γ, but not to other cytokines tested, triggered robust cell death that could not be abrogated by preventing pyroptosis, apoptosis, necroptosis, or any of the major forms of cell death or a combination of these. This pathway requires IRF-1–mediated induction of GBPs and subsequent SCV lysis and release of *Salmonella* into the cytosol of cells. In vivo, mice selectively deficient in IFN-γ signaling in IECs had higher levels of colonic bacteria and increased intestinal inflammation. Together, these results suggest that, as in phagocytic cells, nonphagocytic cells such as IECs and fibroblasts require IFN-γ signaling to undergo cell death and clear *Salmonella* infection from the colon. But, unlike in phagocytic cells, such cell death proceeds by a mechanism that is not reliant on any of the primary modes of programmed cellular demise. In addition to the ability of IFN-γ to control bacteria via macrophage-dependent mechanisms, this study now identifies a nonpyroptotic form of IFN-γ dependent death in nonphagocytic cells.

Materials and Methods

Mice, cells, and reagents

Wild-type (WT), *ripk3*$^{-/-}$ (30), *tbk1*$^{-/-}$ (31), *gbp*$^{chr3-/-}$ (32), and *stat1*$^{-/-}$ (33) MEFs were generated in-house from E14.5 embryos and used within five passages in experiments. In some studies, immortalized MEFs generated by a 3T3 protocol (34) were used. Early passage *irf1*$^{-/-}$ (J. Pavlovic); *sting*$^{gt/gt}$, *tnfr1*$^{-/-}$ *zbp1*$^{-/-}$ (J. Upton); *ripk3*$^{-/-}$*casp8*$^{-/-}$, *fadd*$^{-/-}$*mlkl*$^{-/-}$ (D. Green); and trif$^{-/-}$ (E. Mocarski) MEFs were obtained from the indicated laboratories. All other cell lines were obtained from the American Type Culture Collection. Mice were housed in specific pathogen-free facilities at the Fox Chase Cancer Center, and experiments were conducted under protocols approved by the Committee on Use and Care of Animals at this institution. Reagents were obtained from the following sources: mIFN-γ (R&D Systems), hIFN-γ (R&D Systems), mIFN-β (PBL Assay Science), mIL-1β (R&D Systems), TNF-α (R&D Systems), mIL-6 (R&D Systems), IFN-α (R&D Systems), JAK inhibitor I (Calbiochem), RIPK3 inhibitor GSK′843 (GlaxoSmithKline), RIPK1 inhibitor GSK′963 (GlaxoSmithKline), zVAD.fmk (Bachem), 3-MA (Sigma-Aldrich), Ferrostatin (S. Dixon, Stanford University), LPS (Sigma-Aldrich), streptomycin (Sigma-Aldrich), and YVAD (Enzo Life Sciences). Abs for immunoblotting were as follows: anti-IRF1 (1:1000; Santa Cruz Biotechnology), anti-GBP2 (1:1000; Santa Cruz), and anti–β-actin (1:2000; Sigma-Aldrich). Abs for microscopy were as follows: anti-GBP2 (1:1000, gift of J. Coers; Ref. 35), anti-GFP (1:1000; Thermo Fisher Scientific), anti-IFNGR2 (1:1000, Genetex), HRP-conjugated secondary Ab (BD Biosciences), and fluorophore-conjugated secondary Abs (1:500; Abcam and Jackson ImmunoResearch)

Generation of IEC-specific IFNGR2-deficient mice

IFNGR2$^{flox/flox}$ mice were generated using targeted embryonic stem cells obtained from the Knockout Mouse Project repository and injected into C57Bl6 albino blastocysts by the Fox Chase Cancer Center Transgenic Mouse Facility. Chimeric mice were obtained and crossed to C57Bl6 albino mice, and construct germline transmission was monitored by coat color and confirmed by PCR. Frt site–flanked β-Gal and Neo cassettes were excised in vivo by crossing targeted germline transmitted mice with ACTA-FLP mice from The Jackson Laboratory. The resultant heterozygous mice had *Ifngr2* exon 3 flanked with loxP sites. To generate conditional knockout of IFNGR2 in the intestinal epithelium, IFNGR2-floxed mice were intercrossed with Villin-Cre mice (B6.Cg-Tg(Vil1-cre)997Gum/J; The Jackson Laboratory) (36). Cre^{+} and Cre^{-} littermate control mice were genotyped by standard PCR and used for subsequent experimentation. Detailed generation of mice will be reported elsewhere.

Bacterial strains

Salmonella Typhimurium strain SL1344 was used as the WT in all experiments. *Salmonella*-GFP and *Salmonella*-RFP were obtained from M. O'Riordan. *ΔsifA*, *ΔAroC*, and *ΔAroCsifA* mutants were provided by D. Holden. SPI-1 and SPI-2 mutants have been described previously (37, 38).

Infection of cells

Salmonella was grown overnight with shaking at 37°C in Luria broth (LB) containing streptomycin. One milliliter of this culture was then grown in 100 ml LB without antibiotics for an additional 3 h at 37°C until an OD of 0.700 was reached. Cells were then pelleted (4000 *g* for 10 min) and the pellet was resuspended in 50 ml serum-free DMEM. The OD_{600} of 1 ml of this suspension was measured and used to determine multiplicity of infection (MOI) (1.00 OD_{600} = 1.00 × 10^9 CFU). *Salmonella* was added to cells in serum-free DMEM for 30 min. The medium was then removed and each well was washed three times with serum-free DMEM. Complete (10% FBS) medium containing 50 μg/ml gentamicin was then added to each well. After an additional 30 min, this medium was replaced with medium containing 5 μg/ml gentamicin and IFN-γ for the remainder of the experiment. Cell viability was determined by trypan blue exclusion. To determine the proportion of infected cells by FACS, cells were infected with *Salmonella*-GFP, and GFP positivity was measured using a Becton Dickinson FACScan scatter analyzer.

Immunofluorescence. An expression vector encoding LAMP1-GFP fusion protein (Addgene) was retrovirally transduced into immortalized WT MEFs, and populations stably expressing LAMP1-GFP were obtained by selection in hygromycin. These cells were plated on four-well glass slides (Millipore). Postinfection with *Salmonella* and/or treatment with IFN-γ, the cells were fixed with 4% (w/v) paraformaldehyde, permeabilized in 0.2% (v/v) Triton X-100, and blocked with 3% (w/v) BSA in PBS containing 0.1% Triton X-100. Cells were then incubated overnight at 4°C with anti-GFP and/or anti-GBP2 Abs. After three washes in PBS, samples were incubated with fluorophore-conjugated secondary Abs for 1 h at room temperature. Following an additional three washes in PBS, cells were mounted in ProLong Gold antifade reagent (Invitrogen) and imaged by confocal microscopy on a Leica SP8 instrument.

Infection of mice

Eight- to ten-week-old sex-matched control (IFNGR2$^{fl/fl}$) and IFNGR2-ΔIEC (IFNGR2$^{ΔIEC/ΔIEC}$) mice were treated with 20 mg streptomycin by oral gavage 24 h prior to infection. Mice were inoculated intragastrically with either 0.1 ml of sterile LB (mock infection) or an equal volume of LB containing *S.* Typhimurium (10^9 CFU/mouse) grown in LB broth at 37°C overnight with shaking. Mice were sacrificed 48 h postinfection. To determine the CFUs of *S.* Typhimurium, tissue samples of liver, spleen, colon contents, and cecum were collected, weighed, and homogenized in 5 ml of sterile PBS. Bacteria were enumerated by plating 10-fold serial dilutions of tissue homogenates on LB agar plates supplemented with streptomycin (100 mg/ml in water). The cecum and segments of the colon were fixed in 10% formalin and embedded in paraffin for histopathological analysis.

Histopathological analyses

Segments of the ileum, cecum, and colon were collected and fixed in 10% phosphate-buffered formalin (or 4% formaldehyde; 37% saturated formaldehyde = 100% formalin) for 24–48 h, dehydrated, and embedded in paraffin. Paraffin blocks were cut into 5-μm sections, mounted on microscope slides, and stained with H&E. Histopathological evaluation was performed in a blinded manner using the following histopathological scoring scheme (39). 1) Submucosal edema was scored as follows: 0 = no pathological changes; 1 = mild edema (the submucosa is <0.20 mm wide and accounts for <50% of the diameter of the entire intestinal wall [tunica muscularis to epithelium]); 2 = moderate edema (the submucosa is 0.21–0.45 mm wide and accounts for 50–80% of the diameter of the entire intestinal wall); and 3 = profound edema (the submucosa is >0.46 mm wide and accounts for >80% of the diameter of the entire intestinal wall). The submucosa widths were determined by quantitative microscopy and represent the averages of 30 evenly spaced radial measurements of the distance between the tunica muscularis and the lamina mucosalis mucosae. 2) Polymorphonuclear granulocyte (PMN) infiltration into the lamina propria was scored as follows. PMN in the lamina propria were enumerated in eight high-power fields (×400 magnification), and the average number of PMN per high-power field was calculated. The scores were defined as follows: 0 = <5 PMN per high-power field; 1 = 5–20 PMN per

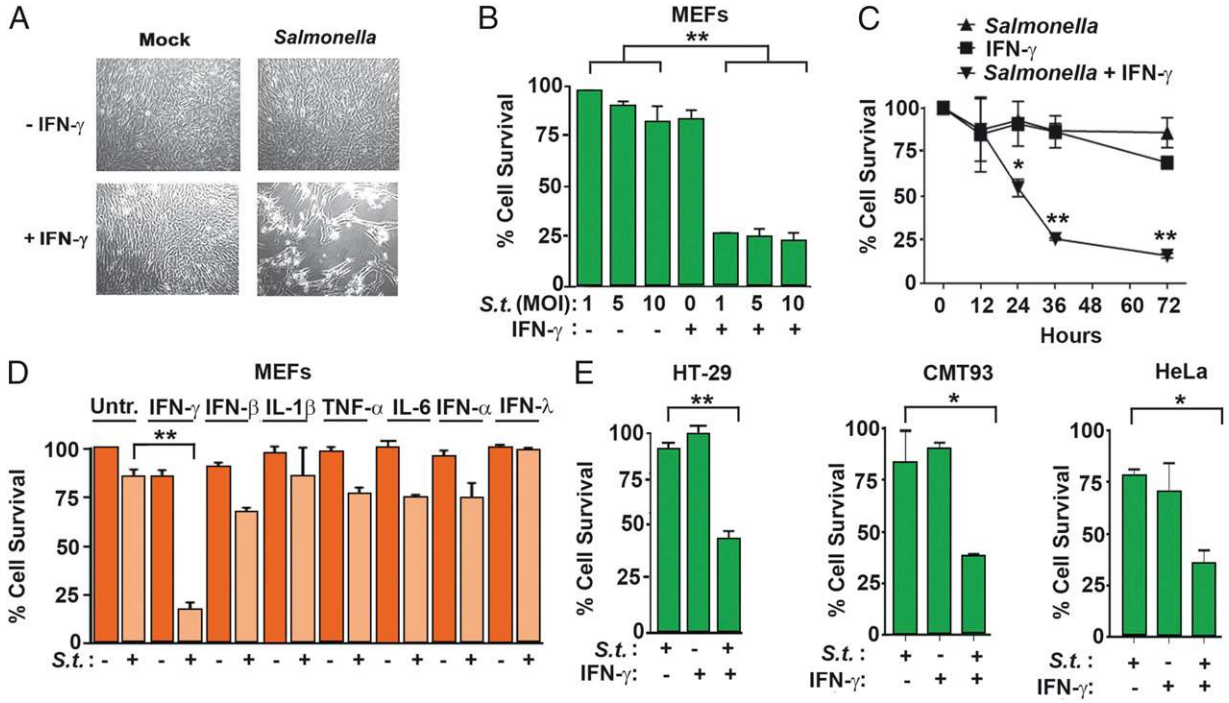

FIGURE 1. IFN-γ sensitizes nonphagocytic cells to *Salmonella*-triggered cell death. (**A**) Photomicrographs of WT MEFs either mock infected (left) or infected with *Salmonella* (MOI 10, right) and subsequently treated with IFN-γ (10 ng/ml, bottom panels). Cells were exposed to IFN-γ 1 h postinfection and photomicrographs were taken 48 h postinfection. Original magnification ×100. (**B**) Cell viability of MEFs infected with *Salmonella* (MOIs 1, 5, and 10) and subsequently exposed to IFN-γ (10 ng/ml). (**C**) Kinetics of cell death induced by *Salmonella* (MOI 10) in the presence or absence of IFN-γ (10 ng/ml). (**D**) Viability of WT MEFs infected with *Salmonella* (MOI 10) and treated with IFN-γ, IFN-β, IL-1β, TNF-α, IL-6, IFN-α (α4), and IFN-λ (λ3). All cytokines were used at 10 ng/ml. (**E**) Viability of HT-29 cells (MOI 10, left), CMT93 cells (MOI 50, center), and HeLa cells (MOI 10, right) infected with *Salmonella* in the presence or absence of human IFN-γ (10 ng/ml). Viability data shown in this figure are representative of at least three independent experiments. Error bars represent mean ± SD. $*p < 0.05$, $**p < 0.005$.

high-power field; 2 = 21–60 PMN per high-power field; 3 = 61–100 PMN per high-power field; and 4 = >100 PMN per high-power field. Transmigration of PMN into the intestinal lumen was consistently observed when the number of PMN was >60 PMN per high-power field. 3) Epithelial integrity was scored as follows: 0 = no pathological changes detectable in 10 high-power fields (×400 magnification); 1 = epithelial desquamation; 2 = erosion of the epithelial surface (gaps of 1–10 epithelial cells per lesion); and 3 = epithelial ulceration (gaps of >10 epithelial cells per lesion; at this stage, there is generally granulation tissue below the epithelium).

Statistics

Statistical significance was determined by use of Student *t* test. Significance of in vivo data was determined by a two-sided Wilcoxon rank sum test. The *p* values ≤0.05 were considered significant. Graphs were generated using GraphPad Prism 6.0 software.

Results

IFN-γ sensitizes nonphagocytic cells to Salmonella*-triggered cell death*

While examining the effects of *Salmonella* infection on MEFs, we made the unexpected observation that although *Salmonella* on its own did not cause much cell death, subsequent exposure of infected cells to IFN-γ (1 h postinfection in this and later experiments) triggered rampant cell death (Fig. 1A). To further evaluate this phenomenon, we infected MEFs with a broad dose range of *Salmonella*; from this analysis, we found that although cells that were not treated with IFN-γ remained resistant to *Salmonella*-mediated cell death up to MOIs of 25, an MOI of 1 robustly killed MEFs when IFN-γ was added to cells postinfection (Fig. 1B). Cell death was first observed ∼12 h postinfection, and most cells were dead by 36 h (Fig. 1C). Remarkably, this effect was unique to IFN-γ, as neither type I or type III IFNs (IFN-α4, IFN-β, IFN-λ3) nor the proinflammatory cytokines IL-1β, TNF-α, or IL-6 sensitized MEFs to *Salmonella*-induced cell death (Fig. 1D). Although *Salmonella* undergoes its full replication cycle in fibroblasts, it is not a cell type commonly encountered during the course of infection. We therefore also tested more physiologically relevant cell types, such as HT-29 human epithelial cells, CMT93 murine colorectal cells, and HeLa human cervical carcinoma cells (Fig. 1E), and found that these cells also succumbed to the combination of *Salmonella* and IFN-γ, but not significantly to either stimulus, when these were deployed singly. Together, these data demonstrate that although *Salmonella* does not trigger much cell death on its own in nonphagocytic cells, the addition of IFN-γ after *Salmonella* infection leads to robust cell death over a 36-h timeframe.

IFN-γ promotes a novel form of cell death in Salmonella*-infected nonphagocytic cells*

To determine if such cell death was pyroptosis, we infected MEFs from pyroptosis-deficient (caspase-1/11-null) mice with *Salmonella* before exposing them to IFN-γ. Surprisingly, these MEFs were as susceptible to *Salmonella*-triggered cell death as WT MEFs (Fig. 2A), even though macrophages from these mice were resistant to death after 90 min following *Salmonella* infection (Fig. 2B). Moreover, treatment of cells with the caspase-1 inhibitor YVAD.fmk at doses shown to block pyroptosis in macrophages (40) did not block cell death seen in MEFs (Fig. 2A), indicating that *Salmonella*-induced death in MEFs was neither canonical nor noncanonical pyroptosis. We next tested MEFs either treated with small-molecule inhibitors of other known cell death pathways or lacking essential components of major pathways of programmed cell death and/or innate signaling for

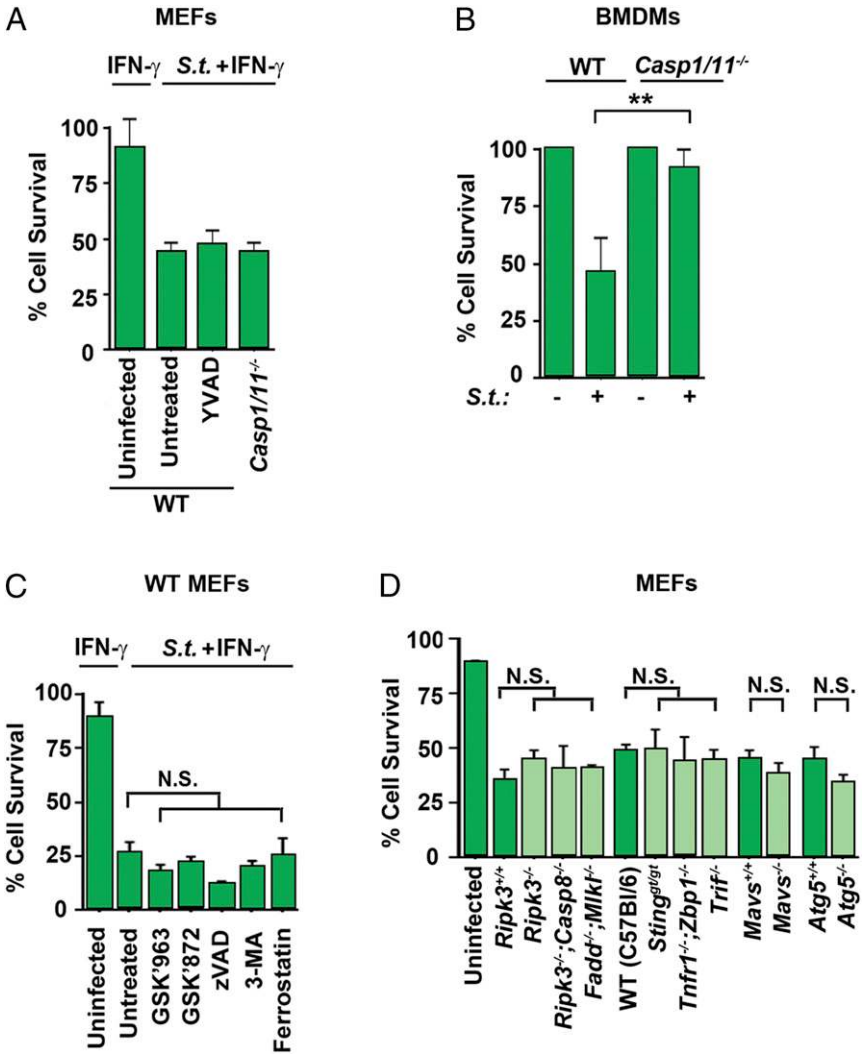

FIGURE 2. IFN-γ promotes a novel form of cell death in *Salmonella*-infected nonphagocytic cells. (**A**) WT or *caspase1*$^{-/-}$*caspase11*$^{-/-}$ double-knockout MEFs were infected with *Salmonella* (MOI 10) and exposed to IFN-γ (10 ng/ml) with or without the presence of caspase-1 inhibitor YVAD (10 μM), and cell viability was determined 48 h postinfection (p.i.). (**B**) WT and *caspase1/11* double-knockout bone marrow–derived macrophages (BMDMs) were infected with *Salmonella* (MOI 25), and cell viability was determined after 90 min. (**C**) WT MEFs were infected with *Salmonella* (MOI 10) and exposed to IFN-γ (10 ng/ml) in the presence of RIPK1 inhibitor GSK′963 (5 μM), RIPK3 inhibitor GSK′843 (5 μM), pan-caspase inhibitor zVAD (50 μM), PI3K inhibitor 3-MA (5 mM), and erstatin inhibitor Ferrostatin (2.5 μM), and cell viability was determined 48 h p.i. (**D**) *ripk3*$^{-/-}$, *ripk3*$^{-/-}$*casp8*$^{-/-}$ double-knockout; *fadd*$^{-/-}$*mlkl*$^{-/-}$ double-knockout; *sting* Goldenticket mutant; *tnfr1*$^{-/-}$*zbp1*$^{-/-}$ double-knockout; trif$^{-/-}$, *mavs*$^{-/-}$, or *atg5*$^{-/-}$ knockout MEFs; and WT controls were infected with *Salmonella* in the presence of IFN-γ (10 ng/ml), and cell viability was determined 48 h p.i. Viability data shown in this figure are representative of at least three independent experiments. Error bars represent mean ± SD. $^{**}p < 0.005$.

protection against the combination of *Salmonella* and IFN-γ. We found that treating WT MEFs with inhibitors of necroptosis (RIPK1 inhibitor GSK′963 and RIPK3 inhibitor GSK′843), apoptosis (pan-caspase inhibitor zVAD.fmk), autophagy (PI3K inhibitor 3-MA), or ferroptosis (erastin inhibitor ferrostatin) could not rescue cells from *Salmonella* and IFN-γ–mediated cell death (Fig. 2C). Similarly, MEFs lacking essential components of necroptosis (*Ripk3*$^{-/-}$) or both necroptosis and death receptor–mediated apoptosis (*Ripk3*$^{-/-}$; *Casp8*$^{-/-}$, *Fadd*$^{-/-}$; *Mlkl*$^{-/-}$) also succumbed to *Salmonella*, with kinetics and magnitude not significantly different from WT MEFs, indicating that cell death induced by *Salmonella* and IFN-γ was neither necroptosis, apoptosis, autophagy, nor ferroptosis (Fig. 2D). In agreement, neither necroptosis (as measured by examining phospho-MLKL and phospho-RIPK3 by immunoblotting), apoptosis (cleaved caspase-3), nor pyroptosis (cleaved caspase-1) was activated to any detectable extent by *Salmonella* and IFN-γ in MEFs (Supplemental Fig. 1). Moreover, MEFs lacking functional STING (Goldenticket; *Sting*$^{gt/gt}$), DAI, TNF (*tnfr1*$^{-/-}$; *zbp1*$^{-/-}$), TRIF (trif$^{-/-}$), or RLR (*mavs*$^{-/-}$) signaling succumbed to *Salmonella* and IFN-γ, indicating that none of these signaling pathways were essential for cell death induced by *Salmonella* in cells exposed to IFN-γ (Fig. 2D). Together, these findings demonstrate that *Salmonella*-induced death after exposure to IFN-γ requires GBP-induced SCV lysis and is not singly mediated by any of the known major innate pathways of programmed cell death.

IFN-γ sensitizes Salmonella*-infected nonphagocytic cells to death via Jak/STAT1-mediated induction of IRF-1 and GBPs*

IFN-γ typically mediates its effects via a Jak1/2-STAT1–mediated transcriptional program that activates the expression of hundreds of genes, called IFN-stimulated genes (41). To test if IFN-γ required Jak/STAT signaling to sensitize nonphagocytic cells to *Salmonella*-triggered cell death, we infected MEFs with *Salmonella*, following which we treated them with IFN-γ in the presence of a potent inhibitor of JAK1/2 kinase activity (JAK Inhibitor I). Cotreatment with this inhibitor in a dose-dependent manner efficiently protected cells from *Salmonella*-triggered death (Fig. 3A). Similarly, MEFs lacking STAT1 were completely protected against cell death induced by *Salmonella* and IFN-γ (Fig. 3A). Previous studies have shown that IFN-γ sensitizes macrophages to pyroptosis by induction of the IFN-stimulated genes encoding IRF1 and GBPs (4, 20, 21). In a two-step Jak/STAT-mediated process, IFN-γ first induces the rapid production of the transcription factor IRF1, which then drives induction of the genes encoding GBPs. These GBPs traffic to the SCV and promote its rupture, releasing *Salmonella* into the cytosol. The inflammasome machinery senses cytosolic *Salmonella*, resulting in pyroptosis (20, 21, 23, 42). To examine if IFN-γ promoted death of *Salmonella*-infected cells also involved IRF1 and GBPs, we determined expression levels of these proteins in MEFs. Neither IRF1 nor a representative GBP (GBP2) were expressed at significant levels in

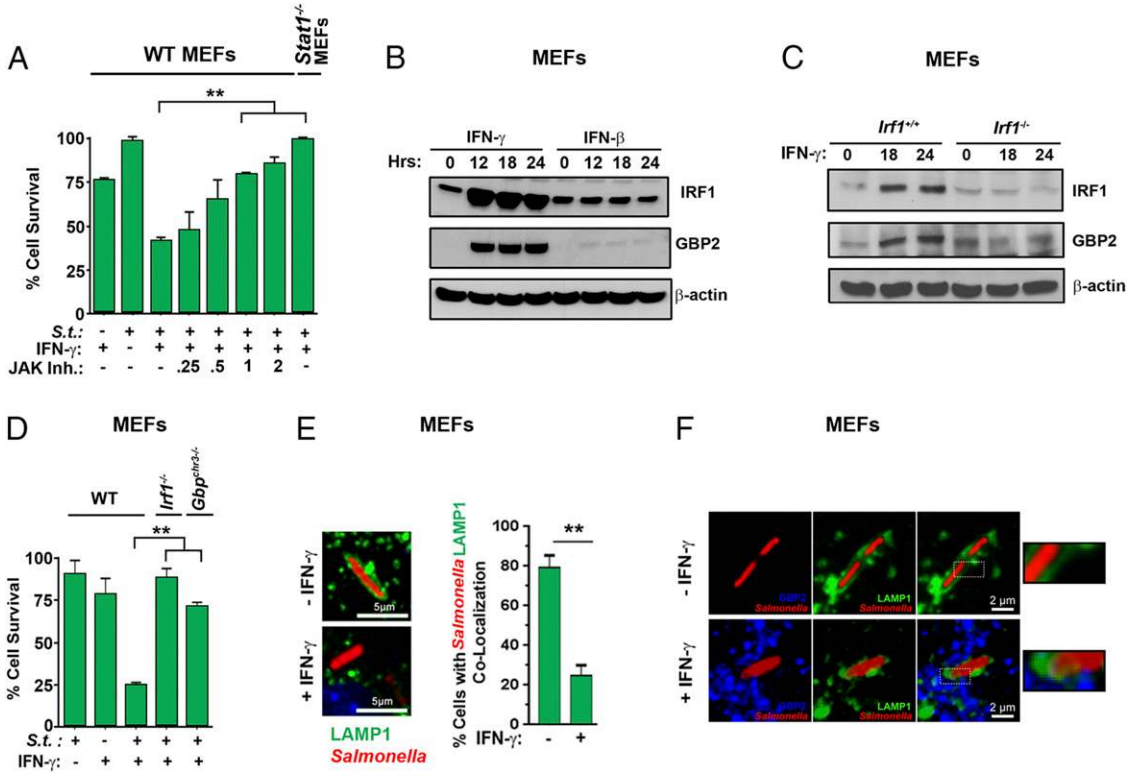

FIGURE 3. IFN-γ sensitizes *Salmonella*-infected nonphagocytic cells to death via Jak/STAT1-mediated induction of IRF1 and GBPs. (**A**) Viability of WT MEFs infected with *Salmonella* (MOI 10) and treated with IFN-γ (10 ng/ml) in the presence of increasing amounts of JAK inhibitor I. Viability of *stat1*$^{-/-}$ MEFs infected with *Salmonella* (MOI 10) and treated with IFN-γ (10 ng/ml) is also shown (rightmost bar). (**B**) WT MEFs treated with IFN-γ or IFN-β (10 ng/ml each) for the indicated times were examined for IRF1 and GBP2 by immunoblotting. β-Actin was used as a loading control. (**C**) *Irf1*$^{+/+}$ and *irf1*$^{-/-}$ MEFs were treated with IFN-γ (10 ng/ml) for the indicated times and examined for IRF1 and GBP2 by immunoblotting. (**D**) WT, *irf1*$^{-/-}$, and *gbp*$^{chr3-/-}$ MEFs were infected with *Salmonella* (MOI 10) in the presence or absence of subsequent IFN-γ treatment (10 ng/ml), and cell viability was determined 48 h postinfection. (**E**) WT MEFs stably expressing LAMP1-GFP were infected with *Salmonella*-RFP in the presence or absence of IFN-γ (10 ng/ml), and localization of LAMP1-GFP and *Salmonella*-RFP was determined by confocal microscopy. Representative images of colocalization (left) and quantification of cells showing colocalized *Salmonella* with LAMP1-GFP, indicative of intact SCVs (right), are shown. (**F**) WT MEFs stably expressing LAMP1-GFP were infected with *Salmonella*-RFP and subsequently treated with IFN-γ (10 ng/ml). Localization of *Salmonella* (red), LAMP1 (green), and GBP2 (blue) was determined by confocal microscopy. Enlarged images of boxed sections are shown to the right. Note that LAMP1-GFP encapsulates *Salmonella* in the absence of IFN-γ. Upon IFN-γ treatment, LAMP1-GFP encapsulation is lost and GBP2-LAMP1-*Salmonella* colocalization becomes evident. Viability data shown in this figure are representative of at least three independent experiments. Error bars represent mean ± SD. $^{**}p < 0.005$.

unstimulated MEFs, but both IRF1 and GBP2 were induced to high levels within 12 h by IFN-γ, but not by IFN-β in uninfected cells (Fig. 3B). Indeed, IRF1 was induced in as few as 30 min after treatment with IFN-γ (data not shown). GBP2 was not induced to any significant extent in IRF1-deficient MEFs, demonstrating that IRF1 was required for production of GBP2 by IFN-γ (Fig. 3C) Importantly, MEFs lacking *irf1* or harboring a deletion in the genetic locus on murine chromosome 3 encoding GBPs 1,2,3,5,7,2ps (*gbp*$^{Chr3-/-}$) were largely resistant to *Salmonella*-triggered death in the presence of IFN-γ (Fig. 3D). Reconstituting *gbp*$^{chr3-/-}$ MEFs by stable retroviral reintroduction of GBP2 restored susceptibility to *Salmonella* and IFN-γ–induced cell death (Supplemental Fig. 2). Thus, as in phagocytic cells, IFN signaling via a Jak/STAT1–IRF-1 axis activates GBPs that lead to eventual death of the infected fibroblast. To determine if IFN-γ–induced GBPs promote lysis of the SCV and release of *Salmonella* into the cytosol of cells, we stably expressed the SCV marker LAMP1-GFP (43) in MEFs, infected these cells with *Salmonella*-RFP, exposed them to IFN-γ (10 ng/ml), and examined integrity of their SCVs. We observed that ~80% of infected cells not treated with IFN-γ displayed intact SCVs, as measured by uniform encapsulation of *Salmonella*-RFP by LAMP1-GFP (Fig. 3E). In contrast, only ~25% of infected cells exposed to IFN-γ contained intact SCVs; the rest of these cells showed cytosolic distribution of *Salmonella*-RFP that did not localize with a GFP signal (Fig. 3E). Endogenous GBP2 was undetectable in untreated cells but was readily observed in a punctate cytosolic pattern in cells exposed to IFN-γ. In these cells, a subset of GBP2 colocalized with LAMP1 and *Salmonella* (Fig. 3F). Collectively, these findings demonstrate that IFN-γ induces GBPs to disrupt the SCV and release *Salmonella* into the host cell cytosol.

Induction of cell death requires live Salmonella *in the cytosol*

To identify *Salmonella* determinants required for induction of cell death, we first tested the requirement for live *Salmonella* in this process. We found that although live *Salmonella* + IFN-γ induced robust cell death in WT MEFs, treating these cells with heat-killed *Salmonella* or exposing MEFs to the Gram-negative bacterial cell wall component LPS did not trigger cell death in the presence of IFN-γ (Fig. 4A). Transfecting LPS into the cytosol of cells, which is capable of activating the pyroptotic machinery in macrophages (23), also did not kill cells in the presence of IFN-γ, indicating that live *Salmonella* was necessary for cell death (Fig. 4A). A mutant of *Salmonella* lacking the first of its T3SS (*invA* mutant, called ΔSPI-1 hereafter) was largely unable to kill cells, even in the presence of IFN-γ, whereas a mutant lacking the second T3SS (*ssaR* mutant, called ΔSPI-2 hereafter) was able to robustly kill cells following exposure to IFN-γ, similar to WT *Salmonella*

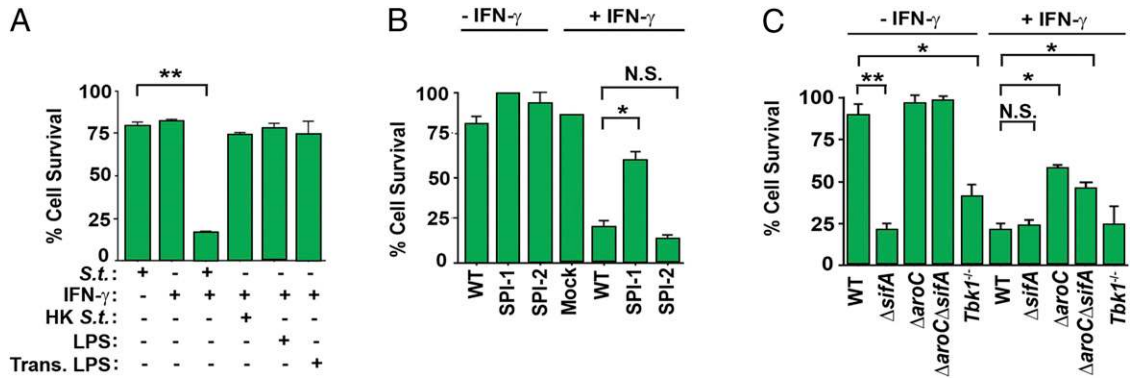

FIGURE 4. Induction of cell death requires live *Salmonella* in the cytosol. (**A**) WT MEFs were infected with *Salmonella* (MOI 10) or heat-killed *Salmonella* (HK *S.t.*) (MOI 10), treated with LPS (4 ng/ml), or transfected with LPS (4 ng/ml) in the presence or absence of IFN-γ (10 ng/ml), and cell viability was determined 48 h postinfection (p.i.). (**B**) WT MEFs infected with either WT *Salmonella* (MOI 10), *Salmonella* lacking its first pathogenicity island (ΔSPI-1), or *Salmonella* lacking its second pathogenicity island (ΔSPI-2) were exposed to IFN-γ (10 ng/ml), and cell viability was determined 48 h p.i. (**C**) WT MEFs were infected with either WT *Salmonella* (MOI 10) or *Salmonella* lacking *sifA* (Δ*sifA*) (MOI 10), *aroC* (Δ*aroC*), or both *sifA* and *aroC* (Δ*sifA*Δ*aroC*) (MOI 10) and exposed to IFN-γ (10 ng/ml), and cell viability was determined 48 h p.i. In parallel, MEFs lacking *tbk1* were infected with *Salmonella* (MOI 10) and exposed to IFN-γ (10 ng/ml); cell viability was determined 48 h p.i. Viability data shown in this figure are representative of at least three independent experiments. Error bars represent mean ± SD. $*p < 0.05$, $**p < 0.005$.

(Fig. 4B). As *Salmonella* SPI-1, but not SPI-2, is required for the invasion of MEFs (44), these findings demonstrate that *Salmonella* must invade cells to induce IFN-γ–mediated cell death. Furthermore, as ΔSPI-2 *Salmonella* behaves in this experiment as the WT bacterium does, induction of cell death in nonphagocytic cells was not dependent on a SPI-2 effector protein(s) (Fig. 4B). A mutant of *Salmonella* that can directly enter the cytosol of cells without forming the SCV (Δ*sifA*) (45) killed MEFs without the need for IFN-γ, suggesting that the primary role of IFN-γ was to lyse the SCV and release *Salmonella* into the cytosol. Notably, a *Salmonella* mutant that can invade MEFs and enter the cytosol but cannot replicate once in the cell (Δ*sifA*; Δ*aroC*) was defective in its capacity to induce cell death, demonstrating that although live *Salmonella* in the cytosol was required for death, active replication was also necessary. In line with the argument that cell death necessitated live *Salmonella* in the cytosol of infected cells, infecting MEFs lacking TBK1, a host kinase required for the formation of the SCV (46), with WT *Salmonella* triggered cell death without the need for IFN-γ (Fig. 4C).

IFN-γ does not significantly increase Salmonella *replication*

In other settings, the host cytosol has been shown to be permissive to *Salmonella* hyperreplication (47). To test if IFN-γ sensitized cells to death by licensing increased *Salmonella* infectivity or replication, we first examined if IFN-γ altered the proportion of infected MEFs compared with untreated cells. IFN-γ did not result in a significant increase in the proportion of infected cells (Fig. 5A). IFN-γ also did not significantly alter the number of intracellular *Salmonella* when examined by immunofluorescence (Fig. 5B, top row). In contrast, loss of TBK1 resulted in *Salmonella* hyperreplication, as previously reported (46, 48) Overall bacterial replication rates were largely indistinguishable between MEFs infected with *Salmonella* alone or subjected to the combination of *Salmonella* and IFN-γ, as demonstrated by direct measurement of intracellular bacterial numbers (Fig. 5C). These findings demonstrate that *Salmonella* and IFN-γ–mediated cell death requires live *Salmonella* in the cytosol of infected cells but that IFN-γ does not much alter overall replication rates of *Salmonella*; rather, it appears to increase the proportion of live *Salmonella* in the cytosol.

IFN-γ signaling in IECs is required to control Salmonella *replication in vivo*

To examine the role of IFN-γ in controlling *Salmonella* specifically in nonphagocytic cells, we generated mice selectively deficient in IFN-γ signaling in cells of the intestinal epithelium. We achieved

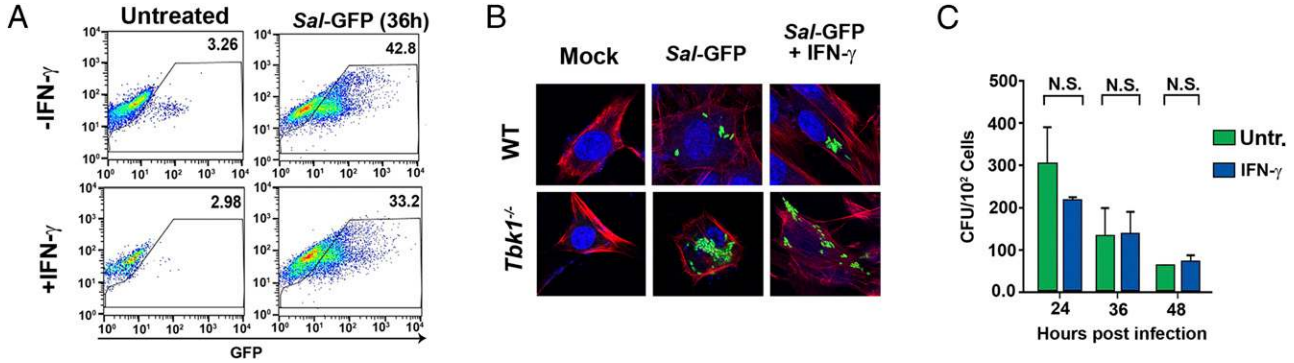

FIGURE 5. IFN-γ does not significantly increase *Salmonella* replication. (**A**) FACS analysis of WT MEFs infected with *Salmonella*-GFP (MOI 25) for 36 h. The *y*-axis shows side scatter. Numbers within each FACS panel show the percentage of GFP-positive cells. Mock-infected cells showed negligible (<2%) GFP positivity. (**B**) Immunofluorescence staining of WT or *tbk1*$^{-/-}$ MEFs infected with *Salmonella*-GFP (MOI 25) with or without the treatment of IFN-γ (10 ng/ml) 36 h postinfection. Phalloidin staining is shown in red, DAPI staining is in blue, and *Salmonella*-GFP is in green. Original magnification ×6300. (**C**) Colony counts per cell of WT MEFs infected with *Salmonella* (MOI 10), with and without IFN-γ. Colony count data shown in this figure are representative of at least three independent experiments. Error bars represent mean ± SD.

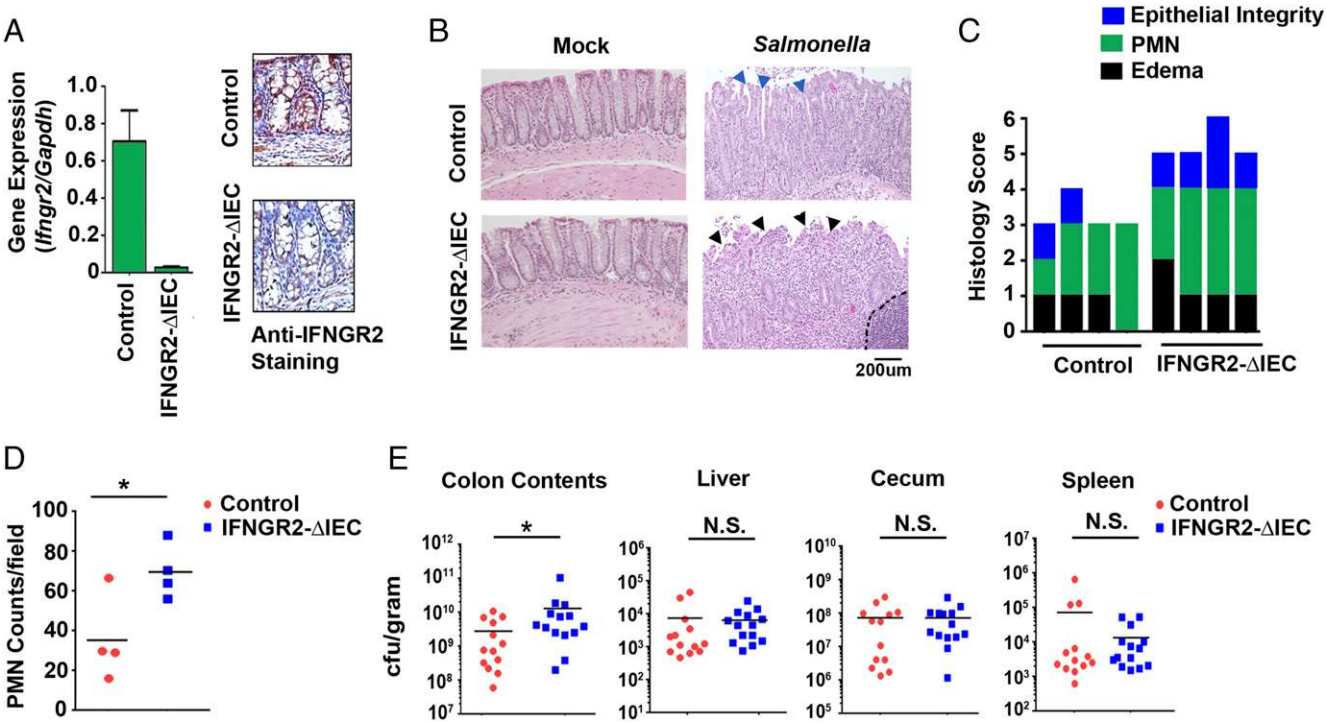

FIGURE 6. Ablating IFN-γ signaling in IECs increases *Salmonella* spread and pathology in vivo. (**A**) RT-PCR for expression of IFNGR2 gene expression in colons of IFNGR2$^{fl/fl}$ and IFNGR2$^{\Delta IEC/\Delta IEC}$ mice and histological staining of colons from IFNGR2 (IFNGR2 was stained with anti-IFNGR2, followed by HRP-conjugated secondary Ab. Brown indicates peroxidase staining for IFNGR2; blue shows counterstaining with hematoxylin.) in IFNGR2$^{fl/fl}$ and IFNGR2$^{\Delta IEC/\Delta IEC}$ mice. (**B**) Representative stained sections (H&E, original magnification ×200) of intestines from uninfected (mock) or *Salmonella*-infected (1×10^9 CFU) control and IFNGR2-ΔIEC mice 48 h postinfection. Blue arrows indicate intact intestinal lining in control mice and black arrows depict erosion of the intestinal lining in IFNGR2-ΔIEC mice. (**C**) Histological scoring of uninfected (mock), and *Salmonella*-infected (1×10^9 CFU) control and IFNGR2-ΔIEC mice after 48 h. Histological scoring consists of submucosal edema (black), PMN infiltration into the lamina propria per high-power field (PMN, green), and epithelial integrity (blue) scores of H&E-stained colonic sections. (**D**) PMN per high-power field counts in control and IFNGR2-ΔIEC mice. (**E**) Colony counts (CFU/g) of the colon contents, liver, cecum, and spleen from control and IFNGR2-ΔIEC mice infected with *Salmonella* (1×10^9 CFU) 48 h postinfection. Error bars represent mean ± SD. $^*p < 0.05$.

this by first producing IFNGR2$^{fl/fl}$ mice, which we then crossed into an IEC-specific Villin-Cre deleter strain to selectively ablate IFNGR2 expression (and IFN-γ signaling) in IECs (Fig. 6A). Histological analyses of cecum samples of *Salmonella*-infected (1×10^9 CFU by oral gavage) control (IFNGR2$^{fl/fl}$) and IFNGR2-ΔIEC (IFNGR2$^{\Delta IEC/\Delta IEC}$) mice after 48 h showed that IFNGR2-ΔIEC mice had higher levels of PMN infiltration and erosion of the intestinal lining (black arrows) compared with controls (blue arrows) (Fig. 6B). IFNGR2-ΔIEC mice infected with *Salmonella* had significantly higher overall intestinal damage (as measured by scoring epithelial integrity, PMN infiltration into the lamina propria, and edema) compared with control mice (Fig. 6C, 6D).

Importantly, *Salmonella* replicated to higher levels in IFNGR2-ΔIEC mice, as evidenced by significantly higher bacterial loads in the colon contents (Fig. 6E). However, deleting IFN-γ signaling in IECs did not notably affect bacterial dissemination beyond the colon, as similar bacterial counts were observed in the cecum, liver, and spleen in IFNGR2-ΔIEC mice versus controls (Fig. 6E). Together, these results demonstrate that IFN-γ signaling in IECs limits *Salmonella* spread and alleviates tissue damage in the infected colon.

Discussion

In this article, we show that IFN-γ sensitizes nonphagocytic cells, including IECs and fibroblasts, to death upon infection with *Salmonella* and that such cell death may help to control bacterial colonization in the gut in vivo. Mechanistically, we show that IFN-γ, via a Jak-STAT1 axis, induces IRF1 and upregulates GBPs to promote disruption of the SCV and release of *Salmonella* into the cytosol, where the bacterium triggers cell death. Distinct from macrophages, GBP-mediated release of *Salmonella* into the cytosol of nonphagocytic cells activates a form of cell death that is neither the caspase-1/11–driven pyroptosis previously shown in macrophages nor any of the major known forms of programmed cell death.

Our data suggest that, as in macrophages, the dominant role of IFN-γ in facilitating the death of *Salmonella*-infected cells is to induce expression of GBPs, which leads to the lysis of the SCV, releasing *Salmonella* into the cytosol, where the bacterium triggers cell death. A mutant of *Salmonella* (Δ*sifA*) that can directly enter the cytosol of cells without forming an SCV kills cells without the need for IFN-γ; similarly, MEFs lacking TBK1, a protein required for stabilization of the SCV, also succumb to *Salmonella*-mediated cell death (46), supporting the idea that IFN-γ-driven disruption of the SCV underlies this cytokine's ability to promote *Salmonella*-induced cell death. In agreement with the findings of Broz and colleagues (20), the cell death we observe requires live *Salmonella*, as heat-killed *Salmonella*, LPS, or cytosolic LPS does not lead to cell death. Notably, exposure to IFN-γ does not lead to an appreciable increase in bacterial replication, as evidenced by FACS, microscopy, and measurement of intracellular bacterial numbers. As suggested by Holden and colleagues (45), the cytosol of the WT fibroblast does not appear to be permissive to *Salmonella* hyperreplication.

Although the mechanism of cell death requires the release of *Salmonella* into the cytosol of cells, how cytosolic *Salmonella*

induces cell death (and whether such cell death is programmed) remains unknown. In other settings, NLRP3/caspase-11 inflammasomes detect cytosolic *Salmonella* for activation of pyroptosis (20, 23), and RLR systems sense *Salmonella* nucleic acid for production of IFN (49); as-yet unknown host innate pathway(s) similar to these may sense *Salmonella* to activate cell death in nonphagocytes exposed to IFN-γ. Alternatively, a bacterial metabolite or other product may simply toxify the host cell cytosol, resulting in unprogrammed death; these possibilities remain to be examined.

In vivo, our data suggest that IFN-mediated destruction of *Salmonella*-infected IECs is required for local control of *Salmonella* infection. Mice lacking IFN-γ signaling selectively in the intestinal epithelium have significantly higher bacterial burden in their colonic contents and manifest increased inflammation and epithelial damage in their colons. Previous reports have demonstrated that caspase-11 (caspase-4 in humans) induces epithelial cell extrusion in IECs after *Salmonella* infection, which aids in the clearance of the bacterium (50, 51). A more recent study demonstrates that caspase-8 can also induce IEC extrusion in the absence of caspase-1 (27). These processes happen quickly postinfection, are caspase-driven, and possess hallmarks of pyroptosis (27, 50, 51), whereas the mechanism of cell death reported in the current study appears to be distinct from these reports, as cell death occurs over a more delayed time course of 36–48 h, and cells lacking caspase-1/11 and caspase-8 (or cells in which caspase-dependent cell death pathways have been pharmacologically inhibited) still undergo cell death upon exposure to *Salmonella* and IFN-γ.

In conclusion, our results demonstrate that IFN-γ promotes death of *Salmonella*-infected epithelial cells and clearance of *Salmonella* in vivo. We propose a model in which *Salmonella* entry into the intestines activates a robust inflammatory response that recruits IFN-γ–producing immune cells, such as neutrophils, NK cells, and Th cells to the sites of infection. IFN-γ secreted by these cells in the vicinity of infected IECs leads to the induction of a STAT1-IRF1 signaling cascade that induces expression of GBPs, which then lyse the SCV, releasing *Salmonella* into the cytosol of epithelial cells and triggering cell death. Such cell death likely exposes *Salmonella* to an innate host defense pathway(s) that destroys the infected cell, limiting *Salmonella* spread and consequent tissue damage in the colons of infected mice.

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Disclosures

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