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Interferon–γ in *Salmonella* Pathogenesis: New Tricks for an Old Dog

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Abstract

Salmonella enterica is a facultative intracellular bacterium that is the leading cause of food borne illnesses in humans. The cytokine IFN-γ has well-established antibacterial properties against *Salmonella* and other intracellular microbes, for example its capacity to activate macrophages, promote phagocytosis, and destroy phagocytosed microbes by free radical-driven toxification of phagosomes. But IFN-γ induces the expression of hundreds of uncharacterized genes, suggesting that this cytokine deploys additional antimicrobial strategies that await discovery. Recently, one such mechanism, mediated by a family of IFN-inducible small GTPases called Guanylate Binding Proteins (GBPs) has been uncovered. GBPs were shown to facilitate the pyroptotic clearance of *Salmonella* from infected macrophages by rupturing the protective intracellular vacuole this microbe forms around itself. Once this protective vacuole is lost, exposed *Salmonella* activates pyroptosis, which destroys the infected cell. In this review, we summarize such emerging roles for IFN-γ in restricting *Salmonella* pathogenesis.

Keywords

Interferon-γ; *Salmonella*; GBPs; pyroptosis; inflammasomes

Introduction

Salmonella enterica is a facultative intracellular bacterium implicated in a variety of illnesses, from gastric disorders to cancer, and is the leading cause of food borne illnesses in humans[12]. *S. enterica* infects over 90 million people worldwide, including 1.2 million annually in the United States[3]. There are many serovars of *S. enterica*, but this review will primarily focus on Typhi and Typhimurium, as these are the best-characterized in terms of their interactions with host cells in culture in and in vivo. Both serovars infect epithelial cells

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of the small intestine but differ in the nature and magnitude of illnesses they cause. *S*. Typhi is a major health concern in developing countries, where it infects over 21 million people each year, primarily via the fecal-oral route following ingestion of contaminated food and, more commonly, drinking water[4, 5]. *S*. Typhi is efficient in evading the host immune response, and causes a systemic severe disease, called typhoid fever, that is often fatal[6]. *S*. Typhimurium is also spread through contaminated food and drinking water, but is efficiently controlled by host immunity and typically only causes self-limiting gastroenteritis in humans[7]. Notably, *S*. Typhimurium is pathogenic in mice, where infection leads to systemic disease, and promotes many of the same symptoms as *S*. Typhi infection does in humans[8]. In contrast, *S*. Typhi is unable to infect or cause disease in mice, for which reason *S*. Typhimurium is often used as an experimental model of systemic salmonellosis[910]. *S*. Typhimurium can also be used as a model to study *Salmonella*-induced gastroenteritis, if mice are first treated with streptomycin to alter their gut microbiota[11]. Thus, infecting mice with *S*. Typhimurium can either induce a typhoid fever-like disease or colitis-like disease depending on the mouse model used, which allows for broad study of *Salmonella* pathogenesis. In this review, we will summarize new insights into innate host defense against *S*. Typhimurium induced fever or colitis, with a focus on emerging roles for IFN-γ in controlling *Salmonella* pathogenesis.

The life cycle of *Salmonella* in mammalian hosts

Upon ingestion, *Salmonella* travels through the stomach into the small intestine, where it begins its pathogenesis cycle. The initial cells targeted by *Salmonella* are those of the intestinal epithelium[12]. Invasion of intestinal epithelial cells is mediated by a chromosomal genetic island called *Salmonella* Pathogenicity-Island 1 (SPI-1)[13–16]. SPI-1 encodes a Type III secretion system (T3SS) that injects dozens of effector proteins into the cytosol of epithelial cells[17]. A subset of these effectors, most notably the Sop proteins SopB, SopE, and SopE2, remodel host actin, allowing ingress of *Salmonella* into the host cytosol[18–21]. In parallel, SipA and SipC inhibit actin depolymerization, thus maintaining the sustained polymerization required to move plasma membrane around the bacterium until it is fully engulfed[22–24]. Once *Salmonella* is engulfed, another SPI-1 protein, SptP, deactivates the actin polymerization proteins Cdc42 and Rac1, allowing for the closure of the membrane “ruffle” and formation of a vacuole around the bacterium. This vacuole is called the *Salmonella* containing vacuole, or SCV[25].

Although most *Salmonella* remain enclosed in the SCV, occasional bacteria are able to escape and replicate freely in the cytosol of non-phagocytic cells, eventually disseminating through the intestinal lining into the underlying lamina propina, which houses macrophages, dendritic cells and polymorphonuclear cells that can phagocytose the bacterium[26]. *Salmonella* can also invade and disseminate through M cells into Peyer’s patches, where it is phagocytosed by dendritic cells (DCs). DCs then carry the bacterium to mesenteric lymph nodes, allowing spread to distal organs such as the liver and spleen, via lymphatic ducts. Alternatively, *Salmonella* can be phagocytosed by $CD18^+$ monocytes in the intestinal lumen and spread to distal organs through the bloodstream without having to first travel to mesenteric lymph nodes[27].

Normally, the acidic environment of the phagocyte is toxic to bacteria, but *Salmonella* has evolved to combat this environment through the use of another plasmid-encoded pathogenicity island, SPI-2[28]. SPI-2 induces the manipulation of vesicle trafficking to prevent the phagocyte SCV from rupturing or fusing with the lysosome[29]. SPI-2 encoded proteins of the Sif family are essential for maintenance of SCV integrity. Of these, SifA is best studied, and interacts with host adaptor proteins Pleckstrin homology domain containing, family M member 1 (PLEKHM1), Rab7, and Homotypic fusion and protein sorting protein (HOPS)[30]. These proteins normally act to stabilize the interaction between autophagosomes and lysosomes to help degrade damaged proteins or pathogens in mitophagy and xenophagy, respectively[31]. However, *Salmonella* has co-opted these proteins to stabilize the SCV by preventing lysosomal fusion as well as by maintaining SCV pH balance and optimal membrane dynamics[30, 31]. Like SifA, the T3SS protein SseJ also cooperates in these activities[32]. SseJ and SifA also interact with an adaptor protein called SifA kinesin interacting protein (SKIP), which binds to microtubule-based motor kinesin-1, recruiting it to the vacuole, and promoting efficient interaction between the SCV and the microtubule network of the cell[32]. SCV forming proteins aid retention of early endosome markers, including Rab5, transferrin receptor (TnfR) and early endosome associated antigen 1 (EEA1) on the SCV, which prevent the SCV from fusing with lysosomes[33].

These and other immune-evasion mechanisms enable the SCV in phagocytes to serve not only as a replicative niche for *Salmonella*, but also as a protective barrier against cytosolic anti-microbial effector mechanisms[29].

Salmonella clearance in non-phagocytic cells

Within the SCV, *Salmonella* remains well-contained[12], but *Salmonella* that escape the SCV and enter the cytosol of non-phagocytic cells, replicate well in this environment and eventually lyse the infected cell, allowing spread to surrounding cells[34]. The SCV in non-phagocytic cells thus protects the host by limiting the spread of *Salmonella* to uninfected neighboring cells, and such cells have in place mechanisms that maintain the integrity of the SCV[34, 35].

A key mechanism by which the non-phagocytic cell maintains SCV integrity involves the host kinase TBK1. Although TBK1 is best known for its role in antiviral innate immunity, where it functions as an IRF3/7 kinase and is a pivotal player in the induction of type I IFNs[36], recent studies have revealed a distinct function for this protein in regulating the SCV[37]. TBK1 regulates levels of aquaporin-1 (AQP1), which controls the osmotic balance of the SCV and prevents it from expanding or collapsing[38]. TBK1 deficient MEFs, which cannot form a stable SCV, are thus hypersensitive to *Salmonella* replication, as are cells overexpressing AQP1[39].

In cases where *Salmonella* is able to escape the SCV and enter the cytosol, it can be ubiquitinated and targeted for degradation, via the host cell's autophagy machinery. Ubiquitination of *Salmonella* is mediated by an E3 ubiquitin ligase called Leucine Rich Repeat and Sterile Alpha Motif Containing 1 (LRSAM1)[40]. Nuclear dot protein 52 kDa (NDP52) and its paralog TAX1BP1 recognize ubiquitinated *Salmonella* and bind to myosin

VI, allowing for autophagosome formation around the bacterium[39, 41]. Like NDP52, the adaptor proteins optineurin and p62 (also called SQSTM1), which are activated by TBK1-mediated phosphorylation, can recognize poly-ubiquitinated *Salmonella*[42]. Upon activation, these proteins bind to the autophagosome marker LC3, linking the autophagosome machinery to ubiquitinated *Salmonella* and targeting the bacterium for degradation[43]. Like LRSAM1, Parkin can also ubiquitinate *Salmonella*, leading to autophagic clearance, although how LRSAM1- and Parkin-driven ubiquitination mechanisms differ from each other await elucidation[44]. Autophagosomes engulf and subsequently degrade the bacterium, controlling, to some extent, *Salmonella* infection in the cytosol of cells. It is noteworthy, however, that cytosolic mechanisms for control of *Salmonella* in non-phagocytic cells may not be very efficient, as TBK1-deficent cells, or cells infected with mutant SifA *Salmonella* that cannot form an SCV and directly enter the cytosol of cells, display increased *Salmonella* replication compared to controls[45]. Interestingly, while phagocytic cells produce high levels of antibacterial proteins, such as defensins, that combat *Salmonella*, these proteins are produced at much lower levels in non-phagocytic cells, providing an explanation for the differences in cytosolic replication between the two cell types[27].

IFN-γ and control of *Salmonella* in the phagocyte SCV

When *Salmonella* infects a macrophage, it “hides” in SCVs. This allows the bacterium to both survive the harsh environment of the macrophage and replicate without interference from cytosolic antimicrobial factors[46]. However, at least two mechanisms are in place to eliminate *Salmonella* from the macrophage, both of which require IFN-γ at crucial steps to mediate bacterial clearance. First, IFN-γ activates transcription of a group of proteins called Guanylate Binding Proteins (GBPs) that localize to the SCV, inducing its lysis and subsequently releasing *Salmonella* into the cytosol[47]. Second, IFN-γ “primes” the macrophage through induction of reactive oxygen species, which activates the NLRP3 inflammasome, leading to pro-inflammatory cell death and eventual clearance of the bacterium[48]. Each of these anti-Salmonella strategies will be discussed next.

Recently, an IFN-γ-driven mechanism has come to light that ruptures the macrophage SCV, preventing *Salmonella* from hiding within the protective confines of this vacuole. The drivers of release of *Salmonella* into the cytosol are a group of small interferon-inducible GTPases called Guanylate Binding Proteins (GBPs)[49]. There are 11 genes encoding GBPs in mice and seven in humans, of which, at least five (GBPs 1,2,3,5,7) are critical for *Salmonella* clearance[50]. GBPs are diffusely cytosolic proteins that are recruited to the SCV in infected macrophages. Recruitment of GBPs to the SCV requires dimerization and subsequent prenylation of these proteins, the latter step of which is mediated by the interferon-stimulated gene Immunity-related GTPase family M protein 1 (IRGM1), a GTPase[51]. Upon sensing ubiquitinated bacterial structures, IRGM1 activates farnesyltransferase-I and geranylgeranyltransferase-I that prenylate GBPs on conserved CaaX sequences in their C-termini[52]. This prenylation step induces their localization to the *Salmonella*-containing vacuole[53]. Once at the SCV, GBPs insert into and puncture the integrity of the vacuolar membrane, rupturing the SCV and releasing *Salmonella* into the cytosol[54]. GBPs also participate in directly activating the NLRP3 inflammasome in response to both *Salmonella* and *Chlamydia*[55, 56]. Further, GBPs recruit the interferon-inducible

protein IRGB10 to intracellular bacteria, which then mediates the release of bacterial ligands for detection by inflammasome sensors[57].

IFN-γ is critical to the function of GBPs at multiple distinct steps in this process. First, IFN-γ is required for the induction of GBP-encoding genes. IFN-γ induces expression of GBPs in a two-step mechanism: IFN-γ-mediated JAK/STAT1 signaling first induces rapid expression of the transcription factor IRF-1, which then transactivates GBP-encoding genes[58, 59]. Second, IFN-γ is necessary for activating GBPs in the cytoplasm, as well as for localizing these GTPases to the SCV[60]. Details of how IFN-γ promotes the activation of GBPs and how IRGM1 mediated prenylation sites recruit GBPs to the SCV are currently unclear. Notably, simply overexpressing GBPs does not mimic their induction by IFN-γ, demonstrating the requirement for IFN-γ signaling at additional post-transcriptional steps in the biology of these intriguing enzymes[61].

Besides facilitating the rupture of SCVs, IFN-γ promotes the destruction of *Salmonella* and other intracellular microbes by inducing the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in phagocytes[62]. ROS, besides being directly toxic to *Salmonella*, can also promote *Salmonella* clearance by other means. For example, ROS promotes activation of NF-κB, a transcription factor that induces expression of a variety of anti-bacterial immune factors, including NLRP3[63]. ROS can also initiate autophagy to control *Salmonella* in phagocytic cells. This process is mediated by the enzyme NOX2, which recruits LC3 to phagosomes by sensing ROS mediated cellular damage[64].

Pyroptosis and clearance of *Salmonella*-infected cells

Upon rupture of the SCV, *Salmonella* is released into the cytosol of macrophages, which can trigger pyroptosis, a rapid pro-inflammatory form of cell death. The rupturing of membranes and the release of IL-1β from the cell, producing robust inflammation, characterize pyroptosis[65]. Activation of caspase-1 (the driver of IL-1β release) and secretion of IL-1β are mediated by cytoplasmic signaling complexes called inflammasomes[66]. All canonical inflammasomes contain Nod-like (NLR) proteins, but differ in the pathogen-associated molecular patterns (PAMPs) they recognize[67]. *Salmonella* is detected by two canonical inflammasomes, NLRC4 and NLRP3[6869]. Notably, NLRC4 and NLRP3 interact with each other and may co-operate to activate caspase-1 in response to *Salmonella* infection[70]. The NLRC4 inflammasome senses the PAMP flagellin, as well as components of the *Salmonella* T3SS, specifically, the SPI-1-encoded needle proteins PrgJ and PrgI, to activate caspase-1 and trigger pyroptosis[71]. What triggers NLRP3 inflammasome activation during *Salmonella* infection is less known, although extracellular ATP and citrate are required for this process[72, 7374].

Pyroptosis can be induced non-canonically, through a mechanism reliant on recognition of Salmonella LPS by caspase-11. In this pathway, caspase-11 directly senses cytosolic LPS as a signal to release IL-1α, mediating cell death without need for NLRC4 and NLRP3[75]. Notably, caspase-11 is also needed for caspase-1 induced cleavage of IL-1β by canonical inflammasomes[76]. Once activated, caspase-1/11 then cleaves a protein called gasdermin D.

Upon cleavage, the N-terminus of gasdermin D translocates to the membrane, triggering rupture of the cell and release of IL-1β[77–79].

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Outstanding Questions

Although much is known about how *Salmonella* induces disease, there are still multiple questions that remain to be answered. First, the molecular underpinnings of GBP activation leading to rupture of the SCV are unclear. Second, the role of IFN-γ in clearance of *Salmonella* in non-phagocytic cells is relatively unexplored. Recent studies demonstrate that epithelial cells infected with *Salmonella* manifest caspase-11 (caspase-4 in humans) cleavage and extrusion of infected cells from the intestinal epithelial monolayer[35]. Whether IFN-γ-mediated induction of GBPs is responsible for such clearance, or whether other ISGs are required to lyse the SCV and trigger caspase-11 activation remains to be seen. Finally, mechanisms by which inflammasomes are negatively-regulated to prevent their inopportune activation, or to terminate the inflammatory response, remain relatively unexplored.

Highlights

- IFN-γ is important in controlling *Salmonella* replication and spread in phagocytes
- IFN-γ induces expression of Guanylate Binding Proteins (GBPs)
- GBPs promote *Salmonella* clearance by rupturing its protective intracellular vacuole
- *Salmonella* thus exposed triggers pyroptosis of the host phagocyte

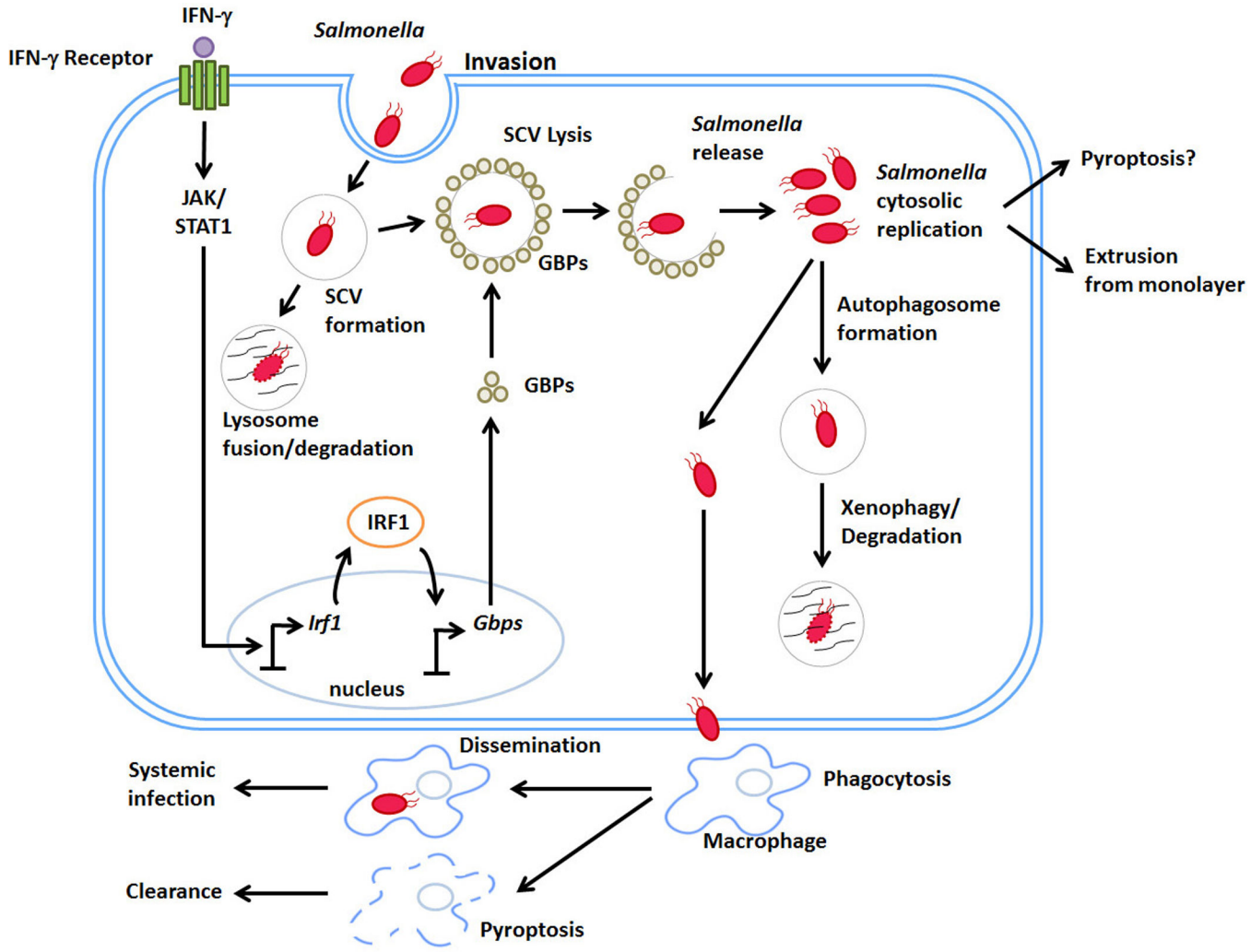

Figure 1. IFN-γ control of *Salmonella* life cycle in non-phagocytic cells

Upon ingestion, *Salmonella* travels to the small intestine where it invades non-phagocytic cells through use of a TTSS encoded by the pathogenicity island SPI-1. After invasion, a *Salmonella*-containing vacuole (SCV) forms around the bacterium, from which is eventually cleared by fusion with lysosomes. Following exposure to IFN-γ, a family IFN-inducible GTPases called GBPs are transcribed by a two-step mechanism involving JAK/STAT1 dependent induction of the transcription factor IRF1. IRF1 then drives GBP expression. Once synthesized, GBPs lyse the SCV, releasing *Salmonella* into the cytosol of the cell. *Salmonella* can replicate in the host cytosol, and is often cleared by autophagosome-mediated mechanisms. In some cases where *Salmonella* hyper-replicates in the cytosol, the infected cell is extruded from the monolayer. A few bacteria can escape these processes and disseminate into the underlying lamina propina, where they are phagocytosed by macrophages.

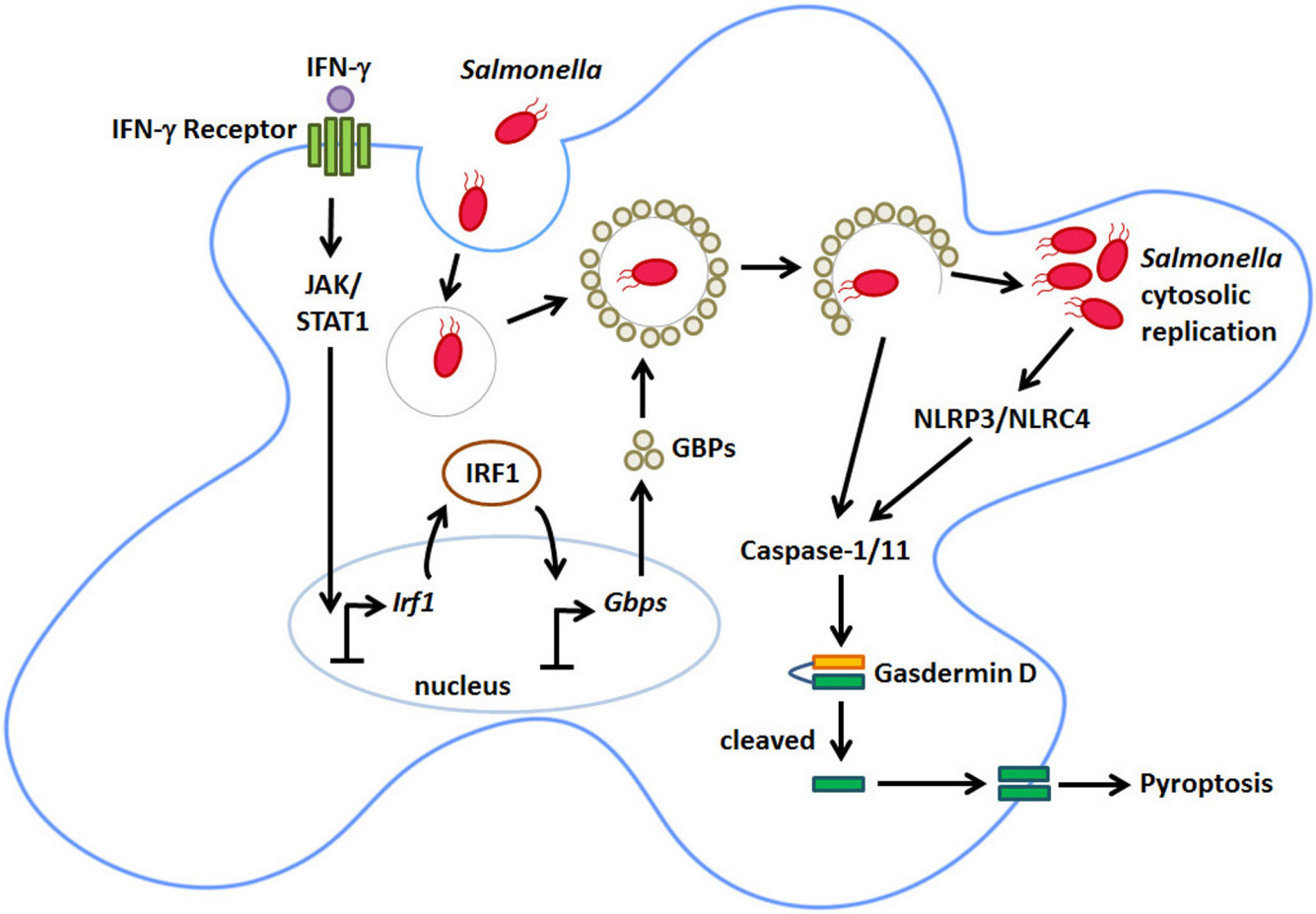

Figure 2. IFN-γ control of *Salmonella* lifecycle in phagocytic cells

Salmonella is phagocytosed by the macrophage and immediately forms an SCV to survive in the unfavorable acidic environment of the cell. IFN-γ leads to JAK/STAT1 dependent IRF1 induction and transcription of GBPs, which localize to the SCV, lysing it. Upon lysis, *Salmonella* is released into the cytosol of the macrophage where the bacterium is recognized by the inflammasome sensors NLRC4 and NLRP3, which activate caspase-1/11. In parallel, cytosolic LPS can be directly sensed by caspase-11. Once activated, these caspases cleave Gasdermin D. The N-terminal cleavage product of Gasdermin D then localizes to and punctures holes in the plasma membrane, triggering pyroptotic death.