Host interferon-γ inducible protein contributes to Brucella survival

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INTRODUCTION

Brucella spp. are highly adapted intracellular pathogens of mammals that cause chronic infections while surviving and replicating in host monocytes and macrophages. Although monocytes are normally susceptible to infection, pretreatment with pro-inflammatory cytokine interferon-γ (IFN-γ) activates cellular defense mechanisms that increase intracellular killing of Brucella and prevents bacterial replication. We examined the contribution of the IFN-γ inducible GTPase, LRG-47, to Brucella and their host. Brucella abortus, which blocks prostaglandin E2 (PGE2), whereby increasing IFN-γ production (Stevens et al., 1992). Murine macrophages infected with B. abortus secrete IFN-γ, and inactive forms of IL-12, while B. abortus lipopolysaccharide (LPS) treated monocytes produce IFN-γ and active IL-12 (Fernandez-Lago et al., 1999). Hoover et al. demonstrated that IFN-γ treatment did not stimulate Brucella killing, although intracellular replication of opsonized and non-opsonized B. abortus was significantly reduced in cultures treated with the cytokine (Eze et al., 2000).
As confirmed by the literature, the restriction in intracellular replication of *Brucella* involves more than the NO and ROI generating mechanisms of the host cell.

Recently, a family of Immunity-Related p47 GTPases (IRG) has been identified whose activities are strongly induced from undetectably low resting levels (Martens et al., 2004), by the cytokine IFN-γ (Henry et al., 2009). This 47–48 kDa family of proteins, which was first identified in the mouse, is divided into two groups based on sequence homology of their G1 motif (Martens et al., 2004; Chen and He, 2009). Group I p47s (GMS) consists of interferon gamma-induced GTPase (IGTP) (Irgm3), GTPI (Irgm2) and LRG-47 (Irgm1). Group II (GKS) includes IRG-47 (Irgd), TGTP/Mg21 (Irgb6) and IIIGP (Irga6) (Chen et al., 2010). These two groups of proteins do not act independently, but interdependently to control intracellular and extracellular pathogen invasion (Chen and He, 2009). The GMS proteins function as regulators of the GKS proteins’ GTPase cycle acting as attenuators by preventing premature activation of the GKS proteins (Hunn et al., 2008; Hunn and Howard, 2010).

These IRG proteins have pathogen specific behavior; each protein is involved in controlling specific pathogens (Martens et al., 2004; Butcher et al., 2005; Chen and He, 2009). The recent discovery and characterization of these IFN-γ inducible proteins reveals that the intracellular environment within monocytes is substantially altered in response to IFN-γ to defend against intracellular pathogens. Recent data indicated that, specifically, LRG-47 is essential in macrophase resistance to a variety of intracellular pathogens such as *Toxoplasma cruzi* (Santiago et al., 2005), *Toxoplasma gondii* (Collazo et al., 2001; Butcher et al., 2005), *Listeria monocytogenes* (Collazo et al., 2001), *Mycobacterium avium* (Taylor et al., 2004), *Mycobacterium tuberculosis* (MacMicking et al., 2003) and *Chlamydia trachomatis* (Coers et al., 2008). LRG-47 is normally associated with the cis- and medial-golgi network inside a macrophage (Martens et al., 2004; Butcher et al., 2005; Zhao et al., 2010a). Following phagocytosis of a particle, LRG-47 is recruited to the maturing phagosome, where it will stay for the full life cycle of the phagosomal compartment (Martens et al., 2004). Since this IRG protein remains associated with the phagosomal membrane upon association with a lysosome, LRG-47 is found to colocalize with the lysosome-associated membrane protein LAMP1 (Martens et al., 2004; Zhao et al., 2010b), and the acidic vesicle stain LysoTracker® inside macrophages (Zhao et al., 2010b). Zhao et al. speculates that the Golgi-localized LRG-47 is the GDP-bound form (inactive protein), whereas, the lysosome-associated LRG-47 is the GTP bound form (active protein). It is noted by Hunn et al. and MacMicking et al. that LRG-47 binds *Mycobacterium* containing vacuoles where it accelerates fusion with the lysosome, whereby controlling pathogen replication and survival. Pathogen load in the host cell can also be controlled by enhancement of the formation of autophagosomes (Gutierrez et al., 2004; Hunn and Howard, 2010), and by promotion of phagosome acidification by LRG-47. LRG-47 deficient mice infected with *M. tuberculosis* have an increased bacterial burden and tissue damage within their lung tissue as compared to wild-type mice. The susceptibility was traced to a deficiency in phagosome maturation; stimulated macrophages from LRG-47 knockout mice failed to acidify *Mycobacterium* containing phagosomes (MacMicking et al., 2003). It has been hypothesized that LRG-47 regulates an alternative pathway for delivery of proteins from the trans-Golgi network (TGN) This pathway may aid the host cell in overcoming attempts by pathogens to inhibit phagosome-lysosome fusion (Martens et al., 2004). To our knowledge, there have been no studies relating the changes in *Brucella* trafficking to the decrease in intracellular viability induced by IFN-γ stimulation.

Prior work in our laboratory has revealed that the type of intracellular replicative niche that *Brucella* occupies depends on how the bacteria were internalized. In the absence of opsonin, *Brucella* are internalized into vesicles through a process that is dependent on lipid raft aggregation at the plasma membrane prior to uptake. In contrast, opsonization of *Brucella* with specific immunoglobulin directs internalization through an Fc receptor-dependent process. Opsonized bacteria were found to replicate within modified endosomes that were non-acidic, and indicated a lack of fusion with a lysosome. Since the activity of individual IFN-γ induced proteins is associated with their intracellular localization within the cell, both opsonized and non-opsonized bacteria were examined in the following experiments to determine whether different intracellular niches were uniquely sensitive to specific IFN-γ induced anti-*Brucella* activities.

**MATERIALS AND METHODS**

**BACTERIAL CULTURE**

All chemicals were obtained from Sigma-Aldrich unless otherwise stated. All research with live *B. abortus* were conducted in University, state and Federally approved BSL3 facilities at either Louisiana State University Health Sciences Center-Shreveport or Iowa State University College of Veterinary Medicine. Virulent *B. abortus* laboratory strain 2308 cultures were grown on Trypticase Soy agar (Difco) supplemented with 5% bovine blood (BA) (Gemini Bioproducts) under 5% CO₂ at 37°C. A GFP-expressing derivative of *B. abortus* 2308 was constructed by introducing the plasmid pBBR1MCS6-Y encoding GFP expression downstream of the constitutively active promoter for aph3A-derived from pBlueKS+ (Stratagene) (Murphy et al., 2002). GFP positive *Brucella* were maintained in culture using a concentration of 6 μg/ml chloramphenicol. Heat killed *B. abortus* cells were prepared by incubating cell suspensions at 70°C for 30 min. Loss of viability was confirmed by plating portions of the heated cell suspension on BA and subsequent incubation at 37°C for 4 days.

**CULTURE OF MURINE PERITONEAL, BONE MARROW DERIVED MONOCYTES AND RAW264.7 MONOCYTIC CELLS**

Mice were euthanized by halothane overdose prior to removal of the long bones of the legs. Marrow was flushed out using a prefilled 5 cc syringe with 5 ml DMEM and a 26-G needle. Cells were pelleted from marrow wash solution by centrifugation and resuspended in DMEM + 10% FBS supplemented with 1000 units/ml M-CSF and incubated for 24 h at 37°C with 5% CO₂. At which time, nonadherent cells were transferred to a 75 cm² tissue culture flask with 10 ml DMEM and 1000 units/ml M-CSF. After 7 days of culture cells were plated for experimental use. Isolation of non-elicited peritoneal macrophages were harvested by first removing adherent tissue cultures. Through centrifugation and resuspension, 10 million cells were obtained from each mouse. Adherent peritoneal macrophages were harvested by first removing adherent tissue cultures. Through centrifugation and resuspension, 10 million cells were obtained from each mouse.
cavity of euthanized mice as previously described (Robertson and Roop, 1999). Low passage RAW 264.7 (ATCC #TIB-71) were routinely cultured in RPMI 1640 medium with 2 mM glutamine supplemented with 1.5 g/ml sodium bicarbonate (Mediatech) and 10% fetal bovine serum (Gemini Bioproducts). Cells were seeded into both 24 and 96 well plates at a density of $1 \times 10^5$ cells/ml, 24 h prior to infection. Cell culture viability was monitored in suspension by hemocytometer-trypan blue dye exclusion. Transient transfection of RAW264.7 cells employed the Nucleofector device using the manufacturers recommended protocols and solution reagents (Amaza-Lanza Biologics).

**ASSESSING INTRACELLULAR SURVIVAL AND REPLICATION OF B. abortus**

Bacterial suspensions were generated by scraping 48 h cultures of the B. abortus strains grown on BA into screw cap microfuge tubes containing PBS. Pellets of bacteria were resuspended by vigorous vortexing and numbers of bacteria present in the suspensions were determined by OD$_{600}$ measurements. Bacterial suspensions were diluted to desired concentrations in complete RPMI 1640 medium and split into two aliquots where one received nonagglutinating concentrations of 1/2500–1/5000 of anti-Brucella IgG antibody (Difco) to opsonize bacteria (opsonized), and the other received the same volume of PBS to serve as a mock control (non-opsonized). Bacterial cell suspensions and antisera were incubated at room temperature for 30 min followed by brief vortexing. Bacteria were added to monocytes monolayers generally at a multiplicity of infection (bacteria to monocyte ratio) of 20:1 for opsonized and 100:1 for non-opsonized Brucella. Due to anticipated differences for internalization kinetics of opsonized and non-opsonized bacteria, synchronized internalization was achieved by cooling infected cultures to 4°C followed by 10 min centrifugation at 270 x g. Monolayers were washed gently with cold PBS to remove non-adherent bacteria. Phagocytosis of adherent bacteria was initiated by the addition of fresh prewarmed media after which, cells were incubated for 20 min at 37°C/5% CO$_2$. After incubation, cultures were washed 3 times with cold PBS and fresh media containing 100 μg/ml gentamicin was added to cells for 1 h to kill adherent but not internalized bacteria. After 1 h antibiotic treatment, media was removed and replaced with fresh media containing 10 μg/ml gentamicin. Cells remained in this medium for the duration of the experiment. Viability of intracellular Brucella was determined by lysing monocytes with 0.1% deoxycholate, diluting suspensions in PBS, and plating aliquots in triplicate on BA medium (Robertson and Roop, 1999). Percent survival of bacteria at 24 and 48 h were calculated based on the number of internalized bacteria detected at 1 h post infection which represents 100% of internalized bacteria. Statistical comparisons were made using Student’s t-test.

**FLUORESCENCE MICROSCOPY**

Infected monolayers were prepared for immunofluorescence microscopy at indicated times post infection using methods described previously (Bellaire et al., 2005). Goat anti-LRG-47 primary antibody (clone P-20) was purchased from SantaCruz Biotechnology (Santa Cruz, CA). Coverslips were washed three times with cold BSP and affixed to glass slide with Pro-long with DAPI mounting solution (Molecular Probes/Invitrogen). Secondary antibodies conjugated with fluorochromes indicated in text were purchased from Jackson ImmunoResearch (WestGrove, PA). Immunofluorescence microscopy was performed using an Olympus IX-71 epifluorescence microscope with appropriate DAPI/GFP/TRITC filter sets and image processing was performed with ImageJ v1.33g (http://rsb.info.nih.gov/ij/index.html).

**ANIMAL EXPERIMENTS**

Animal facilities and protocols were ALAAC approved and all B. abortus experiments were performed using appropriate ABSL3 protocols and procedures. Five- to seven-week-old female BALB/c mice were infected with $1 \times 10^5$ B. abortus 2308 through the intraperitoneal route using previously reported protocols. At the indicated times post infection, mice were humanely euthanized to harvest spleens and livers. Organs were homogenized in PBS and tissue lysates were serially diluted and then plated onto TSA blood agar to enumerate bacteria (Bellaire et al., 1999). A mouse colony was established and maintained in AALAC accredited facilities for LRG-47$^{-/-}$ mice using breeding pairs generously gifted from Gregory Taylor (Collazo et al., 2001). Averages and standard deviations were calculated from CFU data collected from a minimum of 5 mice for each group at each time point. Statistical significance was determined using a Student’s two-way t-test.

**RESULTS**

**EXPRESSION AND LOCALIZATION OF LRG-47 TO Brucella-CONTAINING VESICLES**

To determine if LRG-47 can be found on Brucella-containing phagosomes in IFN-γ activated monocytes, RAW264.7 cells were pre-treated with IFN-γ, infected with opsonized GFP-expressing B. abortus, and fixed at 18 h post infection. Immunostaining for LRG-47 revealed that some but not all Brucella colocalized with LRG-47 (Figure 1). Similar results were also obtained with IFN-γ activated peritoneal macrophages (data not shown).

**CONSTITUTIVE EXPRESSION OF LRG-47 DOES NOT ALTER THE COURSE OF in vitro Brucella INFECTION**

Since LRG-47 is induced by IFN-γ and can be localized to Brucella-containing phagosomes in these cells, we postulated that...
LRG-47 had a role in limiting the intracellular growth of the bacteria. This notion was examined by generating stable RAW264.7 cells that constitutively overexpressed a FLAG-tagged LRG-47 fusion protein. Constitutive overexpression was monitored by immunofluorescence microscopy using antibodies directed either toward LRG-47 or the FLAG tag (DNS). The distribution pattern of the LRG-47-Flag protein in non-IFN-γ activated cells was indistinguishable from that of endogenous LRG-47 in non-transformed IFN-γ treated RAW264.7 cells. Overexpressing cells were infected with opsonized Brucella in the absence of exogenous IFN-γ treatment and the intracellular viability of the bacteria was followed over 48 h post infection. CFU’s recovered at 0.5, 1, and 24 post infection were equivalent among infected cells either pretreated with IFN-γ or constitutively expressing LRG-47. While the IFN-γ treated monocytes further reduced the number of viable Brucella at 48 h, bacterial replication occurred in the RAW264.7 LRG-47 expressers that was equivalent to the non-IFN-γ treated cultures (Figure 2). The results show that overexpressing of LRG-47 alone does not evoke IFN-γ induced anti-Brucella activity. Although long term intracellular survival was not reduced by LRG-47 overexpression, the level of Brucella in these cells were more similar to IFN-γ treated cells than non-IFN-γ treated controls up to 24 h post infection. These observations suggest a possible role for LRG-47 in opsonized Brucella internalization and early survival; however, LRG-47 by itself does not limit bacterial replication.

LRG-47 HAS A POSITIVE ROLE IN Brucella’s RESIDENCE IN MURINE MONOCYTES

Since the overexpression of LRG-47 alone did not affect the viability of Brucella within monocytes, we wanted to examine if the loss of this host protein would upset the ability of IFN-γ to induce an anti-Brucella activity by monocytes. It was anticipated that macrophages from LRG-47−/− mice would be defective to some degree in their IFN-γ induced anti-Brucella activity while non-stimulated macrophages would behave similarly to non-activated cells from normal mice. Intracellular viability of Ig-opsonized Brucella in peritoneal macrophages isolated from C57/B6 and LRG47−/− were compared in the presence or absence of IFN-γ stimulation (Figure 3). Interestingly, significantly fewer viable Brucella were recovered from LRG47−/− cells compared to similarly treated C57/B6 cultures, including non-IFN-γ treated cultures. At 48 h, the number of bacteria recovered from IFN-γ treated LRG47−/− cultures increased from 24 h values, suggesting that some degree of intracellular bacterial replication does occur. By contrast, both Brucella survival (CFU at 24 h) and replication (CFU at 48 h) were significantly reduced in non-stimulated LRG-47−/− cells, suggesting that the IFN-γ inducible protein contributed to intracellular bacterial survival. Repeat experiments with non-stimulated peritoneal macrophages showed similar reductions in replication of opsonized Brucella within monocytes from LRG-47−/− mice (Figure A3).

SURVIVAL AND REPLICATION OF Brucella WITHIN RAW264.7 CELLS AND IFNγ ACTIVATED CELLS

Bacteria opsonized by serum components are internalized faster with greater efficiency than non-opsonized bacteria. Cell surface receptors that mediate internalization of opsonized particles influence the intracellular trafficking of the new phagosomes through their cytoplasmic domains (Gyles, 2010). Previous studies determined that immunoglobulin opsonized B. abortus localized to a replicative compartment that lacked ER components.
In contrast, non-opsonized \( B. \) \textit{abortus} did localize to an ER positive compartment as others have reported (Pizarro-Cerda et al., 1998). Thus, intracellular fate of \textit{Brucella} depends on the context by which the bacteria are presented to monocytes. To determine if the mode of \textit{Brucella} uptake altered the subsequent fate of these bacteria, we performed an intracellular viability experiment with RAW264.7 cells infected with either IgG opsonized or non-opsonized virulent \( B. \) \textit{abortus} 2308 employing increasing MOIs. Numbers of intracellular \textit{Brucella} recovered at 24 and 48 h were largely in proportion to the number of bacteria internalized at the beginning of the experiment (Figure A1). Comparing the groups receiving opsonized bacteria, an identical number of internalized bacteria were seen with MOI’s of 20:1 for opsonized and 100:1 for non-opsonized. Interestingly, by calculating the survival of \textit{Brucella} at 48 h as a percentage of the number of bacteria initially internalized revealed that the replication of 20:1 opsonized (1236.4\% \pm 15.6\%) was double that of 100:1 non-opsonized (600.0\% \pm 1.3\%). Considering these results, future experiments maintained MOI’s of 20:1 for opsonized and 100:1 for non-opsonized to maintain consistency in the number of internalized bacteria.

To determine if IFN-\( \gamma \) treatment was equally effective for opsonized and non-opsonized bacteria, intracellular viability experiments were performed with RAW264.7 cells stimulated 24 h prior to infection with 50 U/ml of recombinant IFN-\( \gamma \) (rIFN-\( \gamma \)). Similar degrees of IFN-\( \gamma \) anti-\textit{Brucella} activity were observed at 24 h and 48 h post infection and did not differ among opsonized or non-opsonized bacteria (Figure A2). Treatment of monocytes with IFN-\( \gamma \) resulted in, roughly, a 2 log reduction in both opsonized and non-opsonized bacteria by 24 h, and reduced the number of \textit{Brucella} recovered at 48 h down to the limit of detection. Calculating percent survival did not uncover any additional significant findings. It is of note that pretreatment of monocytes with IFN-\( \gamma \) did increase the number of non-opsonized bacteria internalized 2.5-fold over any other group examined, including the 20:1 opsonized bacteria in IFN-\( \gamma \) treated cells.

**EFFECT OF OPSONIZATION ON \textit{Brucella} SURVIVAL**

An earlier report by our laboratory showed that intracellular localization of \textit{Brucella} depended on how the bacteria were initially internalized by the host cell. Those results revealed that non-opsonized bacteria replicated in human cells in an intracellular compartment resembling the endoplasmic reticulum. Others have demonstrated \textit{Brucella} trafficking through autophagosomes before reaching their replicative compartment (Starr et al., 2008). By contrast, Ig opsonized bacteria replicated in modified late-endosomes and do not traffic through an autophagosomal compartment. Since opsonized and non-opsonized bacteria differ in their interactions with autophagosomes, we wanted to explore whether the survival of \textit{Brucella} varied in LRG-47\( ^{-/-} \) cells in response to varying the method of bacterial internalization. Comparisons between non-stimulated (resting) and IFN-\( \gamma \) stimulated (activated) cells isolated from either C57/B6 or LRG-47\( ^{-/-} \) mice were carried out using optimal MOI’s of 20:1 for Ig-opsonized and 100:1 non-opsonized (yielding equivalent numbers of internalized bacteria within RAW264.7 cells). Infected peritoneal macrophages from wild-type C57/B6 mice treated with IFN-\( \gamma \) exhibited significant and equal anti-\textit{Brucella} activity against both opsonized and non-opsonized \textit{Brucella}. In contrast, \textit{Brucella} viability in LRG-47\( ^{-/-} \) cells was dependent on the whether the bacteria were opsonized, and on the presence of IFN-\( \gamma \) activation (Table 1). Resting LRG-47 deficient macrophages harbored significantly more non-opsonized \textit{Brucella} at 48 h (137.2\% \pm 12.1\%) than Ig-opsonized (79.4\% \pm 15\%). In addition, Ig-opsonized \textit{Brucella} survival in LRG-47 KO was significantly lower than was seen in resting C57/B6 isolated macrophages (114\% \pm 2.7\%). An inverse relationship was seen between \textit{Brucella} survival and the affects of opsonization in IFN-\( \gamma \) stimulated-LRG-47 KO macrophages (Figure A3). Percent survival of non-opsonized \textit{Brucella} dropped dramatically in IFN-\( \gamma \) activated LRG47\( ^{-/-} \) cells, to 32.6\%, while Ig-opsonized \textit{Brucella} survived as well as, or better than, the opsonized bacteria in the resting LRG-47 KO or C57/B6 macrophages. Post hoc analysis revealed that averaging the opsonized and non-opsonized LRG-47 KO results together based on IFN-\( \gamma \) activated and non-activated status yielded values similar to those recorded from the corresponding IFN-\( \gamma \) treated C57/B6 macrophage cultures.

**\textit{Brucella} SURVIVAL IN LRG-47\( ^{-/-} \) UNAFFECTED BY AUTOPHAGY INHIBITOR**

Reduced survival of \textit{M. tuberculosis} in IFN-\( \gamma \) activated macrophages has been attributed to the ability of LRG-47 to redirect resident intracellular bacteria toward lysosomes by repackaging intracellular pathogens within autophagosomes (Gutierrez et al., 2004; Hunn and Howard, 2010).

| Mouse line | Opsonin | IFN-\( \gamma \) | 48 hours % survival |
|------------|---------|-----------------|---------------------|
| B6         | IgG     | –               | 114.4\% \pm 2.7\%   |
| B6         | None    | –               | 123.9\% \pm 6.9\%   |
| B6         | IgG     | +               | 83.8\% \pm 13.0\%   |
| B6         | None    | +               | 76.8\% \pm 15.1\%   |
| LRG47      | IgG     | –               | 79.4\% \pm 15.0\%** |
| LRG47      | None    | –               | 1372\% \pm 12.1\%** |
| LRG47      | IgG     | +               | 95.7\% \pm 13.2\%   |
| LRG47      | None    | +               | 32.6\% \pm 0.00\%***|

Summary of \( B. \) \textit{abortus}, percent intracellular viability results within primary non-elicited peritoneal macrophages from C57/B6 and LRG-47\( ^{-/-} \) KO mice. Results shown are the culmination of three replicates calculated from log CFU/ml values recovered from peritoneal macrophages treated as indicated with cytokine, and infected with either IgG opsonized or non-opsonized bacteria. For C57/B6 macrophages, replication of \textit{Brucella} in non-IFN\( \gamma \) treated and anti-\textit{Brucella} activity induced by IFN\( \gamma \) were equivalent among the different opsonin groups. Parallel experiments with LRG47 macrophages, however, showed a striking difference between the different opsinin groups. In the absence of LRG-47, replication of IgG opsonized bacteria was significantly reduced compared to non-opsonized \( B. \) \textit{abortus} in quiescent cells.

**p < 0.01 (C57/B6 vs. LRG47 of same condition).**

**p < 0.001 (C57/B6 vs. LRG47 of same condition).**

**p < 0.01 IgG opsonized vs. non-opsonized.**
The autophagosomal pathway could provide a safe haven for the intracellular bacteria enabling them to survive and replicate while evading the host immune response, which seems counterintuitive for the host. It is plausible that IFN-γ induction of autophagosome biogenesis could either drive the elimination of *Brucella*, or accelerate its survival and intracellular replication. To determine which of these mechanisms has the dominant role in *Brucella*'s intracellular viability, we treated infected peritoneal C57/B6 and LRG47−/− macrophages with a 50 μM of 3-methyladenine (3-MA), an inhibitor of autophagosome formation. Treatment of C57/B6 cells with the inhibitor reduced *Brucella* CFUs (4.6 ± 0.09 log_{10}) compared to non-treated cultures (5.8 ± 0.16 log_{10}). CFUs from 3-MA treated cultures were equivalent to the number of bacteria recovered from C57/B6 IFN-γ activated cultures (4.3 ± 0.56 log_{10}) (Figure 4). Addition of 3-MA acted synergistically with IFN-γ stimulation to further decrease bacterial viability in C57/B6 macrophage cultures (3.2 ± 0.06 log_{10}). For LRG-47 KO macrophages, 3-MA treatment alone did not alter intracellular bacterial viability in non-IFN-γ stimulated cells. In contrast, stimulation of these monocytes with IFN-γ a dramatic decrease in the ability of intracellular *B. abortus* to survive within these cells (0.6 ± 0.8 log_{10}). The relative health of infected macrophages was monitored for gross morphological changes by phase microscopy, and enzymatically by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assays. Incubating cells with 3-MA and IFN-γ at the levels tested was found to have no detrimental effect on macrophage health.

**DISCUSSION**

Pro-inflammatory cytokines elicit antimicrobial responses in monocytes and macrophages that serve as a critical defense against intracellular pathogens. Recently, attention has been
focused on the ability of IFN-γ to stimulate monocytes to alter normal intracellular vesicle and organelle trafficking to regain control over intracellular pathogens. Many p47 GTPases induced by IFN-γ localize to specific intracellular compartments or structures, and most have been shown to play a role in limiting intracellular parasitism by bacterial or protozoan pathogens. Discovery of the different defense systems of individual intracellular niches led to the idea that each of these proteins may patrol a different intracellular region within the cell thus providing defense against a variety of intracellular pathogens (Collazo et al., 2001). For Brucella, production of ROI and NO was found to play a relatively minor role in the potent antimicrobial response induced by IFN-γ stimulation in macrophages. The most significant effect of IFN-γ activation is the dramatic inhibition of Brucella replication that is found to occur between 24 and 48 h in non-activated monocytes. Given that IFN-γ production by the host is critically important to restriction of Brucella replication in the host, we examined the ability of one of the IFN-γ inducible proteins, LRG-47, to inhibit Brucella’s ability to replicate intracellularly.

Peritoneal macrophages collected from LRG-47−/− mice were infected with opsonized Brucella with and without IFN-γ stimulation. We observed a significant reduction in the number of bacteria present at 24 and 48 h post infection in the LG-47−/− cells without stimulation. This suggests a requirement for LRG-47 for Brucella survival in the early stages after entry into the host cell. Within RAW264.7 cells expressing the Flag-tagged LRG-47 protein, the overexpressed LRG-47 colocalized with Brucella phagosomes in an identical pattern to native LRG-47 using IFN-γ activated primary and RAW264.7 macrophages. Intracellular replication of bacteria in these cells was only inhibited when monocytes were stimulated with IFN-γ. Overexpression experiments demonstrate that LRG-47 alone is not sufficient to fully support intracellular replication of these bacteria. It is likely that additional cellular factors stimulated by IFN-γ inhibit Brucella replication; however, these data show that LRG-47 is, in fact, required by Brucella for replication and/or survival inside a host macrophage.

Intracellular viability of Brucella requires the biogenesis of autophagosomes for replication and/or survival (Starr et al., 2008). A plausible explanation is that Brucella must orchestrate autophagosome biogenesis to maintain an intracellular niche. Recently, Starr et al. described that the activity of autophagy-initiation proteins (ULK1, Beclin 1, ATG14L and PI3-kinase), but not autophagy-elongating proteins (ATG5, ATG4B, ATG16L1 and LC3B) are required for the autophagic B. abortus-containing vacuole (aBCV) formation in non-stimulated monocytes (Starr et al., 2012). Treating infected cells with the PI3-kinase inhibitor 3-MA, these authors observed a decrease in aBCV formation. Changes in aBCV formation reported by these authors coincide with the dramatic decrease in intracellular viability that we observed when treating either WT or LRG-47 KO IFN-γ activated monocytes with 3-MA (Figure 4). Given the importance of aBCV formation on the intracellular pathogenesis of Brucella, bacterial control over autophagosome formation becomes even more critical when antimicrobial activity in macrophages is increased by IFN-γ activation (Starr et al., 2012). Loss of LRG-47 was similar in effectiveness to 3-MA in inhibiting Brucella replication in non-stimulated macrophages, it is likely that process of LRG-47 and 3-MA interference in Brucella pathogenesis involve similar disruptions in critical autophagy dependent mechanisms. Moreover, blocking autophagosome formation in LRG-47−/− cells revealed that this host protein likely supports Brucella viability when host cells are activated with IFN-γ. This support of bacterial replication is likely in the form of stimulating autophagosome formation (Starr et al., 2012). It should be noted that Brucella did not survive as well in LRG-47 KO cells grown under non-stimulating conditions suggesting that Brucella possibly requires not only LRG-47, but also a low amount of IFN-γ present in the host cell to effectively replicate and survive.

Brucella are trafficked into different replicative compartments based on whether they were internalized by opsonized or non-opsonized means. Opsonized Brucella replicate in a modified late endosome, whereas, non-opsonized bacteria replicate in a vesicle sharing markers with the endoplasmic reticulum (Bellaire et al., 2005). This difference in bacteria trafficking of opsonized vs. non-opsonized Brucella translates to a difference in a LRG-47 requirement for survival and replication in stimulated and unstimulated RAW264.7 macrophages. We observed a significant decrease in the percent survival of opsonized Brucella in non-stimulated LRG-47 KO cells after 48 h of infection, suggesting a requirement for LRG-47 in the survival and replication of opsonized Brucella in unstimulated macrophages. LRG-47 would be required to provide Brucella with access to a replicative late endosome-like vesicle. A low percentage of non-opsonized Brucella was found to survive after 48 h of infection in a stimulated LRG47 KO cell. These data suggest that LRG-47 plays a protective role for non-opsonized Brucella in an IFN-γ stimulated cell. LRG-47 may “rescue” the non-opsonized Brucella and provide it with a niche protecting it from anti-bacterial actions of the host cell that occur during IFN activation.

It is clear by in vitro experiments that LRG-47 supports replication of Ig-opsonized Brucella in non-activated cells and the survival of non-opsonized Brucella in IFN-γ activated cells. Given that ample anti-Brucella antibody is maintained throughout infection, we would anticipate any change in Brucella tissue survival to appear at times post infection when IFN-γ levels decrease at 4–6 weeks (Baldwin and Goenka, 2006). For our in vivo experiments using LRG-47−/− mice, changes in the ability of the bacteria to persist were not seen until week 9 post infection when IFN-γ levels would be predicted to decrease. This remains speculative as we did not attempt to measure IFN-γ levels within the tissues of these mice during infection.

Survival of Brucella within monocytes is the single most important aspect of pathogenesis contributing to persistence of the bacteria in host tissues. The previous in vitro data suggests that the role LRG-47 plays during Brucella infection is conditional based on IFN-γ stimulation and the opsonization status of the bacteria prior to internalization. High IFN-γ levels during the chronic phase are attributed to limiting the numbers of Brucella within the spleens of infected mice. Anti-Brucella immunoglobulin undergoes rapid class switching to high affinity IgG2a in an IFN-γ dependent manner, coincidently, aiding in the establishment of the chronic plateau phase of Brucella occurring at 2 weeks.
post infection. Internalization would likely transition from mostly non-opsonized within the first few days toward immunoglobulin-opsonization that would last throughout the chronic phase. It is difficult to predict which set of conditions would predominate within the host at any particular time during infection. Infection of LRG-47 KO mice with \textit{B. abortus} did not differ in the magnitude or timing of splenic colonization compared to C57/B6 mice. Considering that IFN-\(\gamma\) levels and antibody production increase over this time span, the stable number of \textit{Brucella} detected in \textit{vivo} during the chronic phase is likely the product of mixed populations of variably opsonized bacteria together with fluctuating IFN-\(\gamma\) activation of host monocytes. In fact, recalculating percent survival of \textit{Brucella} in LRG-47 cells (Table 1) by averaging the opsonized and non-opsonized values together for IFN-\(\gamma\) treated and non-treated equal the percent survival of \textit{Brucella} recovered from similarly treated C57/B6 macrophages.

Past research regarding LRG-47's effect on pathogenic infection has determined a requirement for LRG-47 to reduce the replicative capacity of infectious agents such as \textit{Toxoplasma gondii}, \textit{Mycobacterium tuberculosis} (MacMicking et al., 2003) and \textit{Listeria monocytogenes} (Collazo et al., 2001). \textit{Brucella abortus} is unique from other intracellular pathogens in that LRG-47 does not inhibit its survival and replication, but aids it. LRG-47 and other members of the p47 GTPase family are pathogen specific with the ability to decipher between two pathogens that share >99\% of their open reading frames as in \textit{C. trachomatis} and \textit{C. muridarum} with \textit{C. trachomatis} infection being kept at bay by LRG-47, but not \textit{C. muridarum} (Coers et al., 2008). In the future it will be important to perform similar experiments using \textit{B. melitensis} to determine the role of LRG-47 in this related subtype's ability to survive and replicate inside the host. Similarly, examination of changes in trafficking, survival, and replication experiments among Ig-opsonized and non-opsonized \textit{B. melitensis} in IFN-\(\gamma\) activated and non-activated cells will be important to understand the mechanism host defense proteins have in controlling the fate of \textit{Brucella}.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.
FIGURE A1 | Internalization and intracellular viability of IgG opsonized and non-opsonized *B. abortus*. Monolayers of Raw264.7 cells were infected with increasing concentrations of IgG-opsonized or non-opsonized *B. abortus*. The average CFU per time point illustrates the correlation between the amount of *Brucella* internalized following infection, and the rate of *Brucella* replication and survival. To compare results from opsonized and non-opsonized *Brucella*, remaining experiments were normalized to numbers of *Brucella* taken up by cells using MOI’s of 20:1 for ops and 100:1 for non-ops. Results presented were calculated from samples performed in triplicate and confirmed by separate independent experiments.

FIGURE A2 | Effect of IFN-γ treatment on *Brucella* infected Raw264.7 cells. Pretreatment of Raw264.7 cells with 50 ug/ul of IFN-γ 24 h prior to infection with *B. abortus* induces an anti-bacterial response that increases *Brucella* killing at 24 h p.i. (*T* = 1), continuing through 48 h p.i. (*T* = 2). Magnitude of anti-*Brucella* effect was equal among opsonized and non-opsonized *Brucella*. Similarly, bacterial survival at 24 h p.i., and replication detected at 48 h were the same between opsonized and non-opsonized bacteria infected at normalized MOI’s (Figure 1). Results represent the averages and standard deviation from six replicate wells for each condition and time point. Statistical analysis was performed using non-paired Student’s *t*-test.
FIGURE A3 | Effects of opsonization on *Brucella* survival in LRG-47 knockout peritoneal macrophages. Peritoneal macrophages were collected from LRG-47−/− mice and infected with Ig-opsonized or non-opsonized virulent *Brucella abortus*. Colony forming units (CFU) were enumerate at 30 min, 2 h, 24 h, and 48 h post infection and percent survival was calculated compared to CFU/ml 30 min post infection. Ig-opsonized *Brucella* have a lower percent survival in the non-activated cells than they do in the activated cells suggesting a requirement for LRG-47 in opsonized *Brucella* survival in non-activated macrophages. In IFN-γ stimulated cells, the percent survival of the *Brucella* steadily decreases over the duration of 48 h whereas in the non-activated cells there is a sharp increase in percent survival between 24 and 48 h post infection. (**p ≤ 0.01 Student’s t-Test vs. opsonized for each stimulation group).