Brucella abortus intracellular survival and intercellular trafficking

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Brucella abortus intracellular survival and intercellular trafficking

by

Jennifer A. Ritchie

A thesis submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

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LIST OF ABBREVIATIONS

CDC- United State Centers for Disease Control and Prevention  
S19- *Brucella abortus* strain 19  
EEA1- Early endosomal antigen 1  
RILP- Rab7 interacting lysosomal protein  
NF-κB- Nuclear factor kappa-light-chain-enhancer of activated B cells  
IFN-γ- Interferon-gamma  
GTPase- Guanosine triphosphate-binding proteins  
Atg- Autophagy-related protein  
PE- Phosphotidylethanolamine  
V-ATPase- Vacuolar H⁺-ATPase  
mTOR- Mammalian-target of rapamycin protein  
3MA- 3-methyladenine  
PI3K- Phosphotidylinositol-3’ kinase  
MHC- Major histocompatibility complex  
ROI- Reactive oxygen intermediate  
NO- Nitric oxide  
iNOS- Inducible nitric oxide synthase  
IL- Interleukin  
PGE2- Prostaglandin E2  
LPS- Lipopolysaccharide  
IRG- Immunity-Related p47 GTPases  
TGN- Trans-golgi network  
WT- Wild-type  
KO-Knockout
MTT- 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
LC3- Microtubule-associated protein light chain 3
LAMP1- Lysosome associated membrane protein 1
PI3K- Phosphotidylinositol-3’ kinases
FBS- Fetal bovine serum
SDS- Sodium dodecyl sulfate
SLCM- Scanning laser confocal microscopy
BA- Trypticase Soy agar supplemented with 5% bovine blood
BSP- Bovine serum albumin/PBS solution containing a final concentration of 0.1% saponin
Ig- Immunoglobulin
SNAREs- Soluble N-ethylmaleimide-sensitive factor attachment protein receptors
ABSTRACT

*Brucella* spp. are host specific facultative intracellular pathogens. *Brucella abortus* is responsible for causing abortions in cattle and is also able to cause disease in humans. *Brucella* internalization and intracellular trafficking varies depending on whether the bacterium was opsonized or non-opsonized with serum immunoglobulin. Interferon-γ has been shown to be critical for the control of *B. abortus* infection *in vivo* and *in vitro*. A family of host Immunity-Related p47 guanosine triphosphate-binding proteins (GTPase) induced by IFN-γ has been identified that are important for host defense against many intracellular pathogens such as *M. tuberculosis*, *T. gondii*, and *L. monocytogenes*. In these studies it was found that LRG-47 is required for *Brucella* survival and replication. Autophagy is a process in the cell whereby damaged proteins and organelles are broken down so their contents can be reused. In the following studies, an autophagosome-like extracellular organelle has been identified that is released by RAW264.7 murine macrophages in normal growth conditions and in conditions to induce autophagy. We have observed *B. abortus* using the natural intercellular trafficking pathway of the autophagosome-like extracellular organelle to infect a new host cell while evading the host immune system.
CHAPTER 1. GENERAL INTRODUCTION

Introduction

*Brucella* spp. are host specific intracellular pathogens that are able to cause severe disease in both mammals and humans. *Brucella abortus* is the causative agent of brucellosis in cattle which results in abortions or the birth of weak calves. In humans, chronic *Brucella* infection can lead to reproductive defects as well as undulant fever. The mechanism of entry and intracellular trafficking varies based on the opsonization status of the *Brucella*. Immunoglobulin-opsonized *Brucella* enter a macrophage via Fc receptors, whereas non-opsonized *Brucella* enter by attachment to lipid rafts on the macrophage surface. Phagocytosed bacteria traffic through a modified early and late endosome at which point opsonized bacteria stay and replicate. Non-opsonized *Brucella* replicate in a compartment formed by the endoplasmic reticulum. The host interferon induced p47 GTPase LRG-47 is important in host cell defense against a number of pathogens including *M. tuberculosis*, *T. gondii*, and *C. trachomatis*. The following studies have demonstrated a requirement for LRG-47 for *Brucella* survival and replication. Further, the differences between opsonized and non-opsonized *B. abortus* intracellular trafficking as well as the activation state of the macrophage contribute to the complex requirement for LRG-47. Though the intracellular trafficking pathway for *B. abortus* has been well described, the mechanism this bacterium uses to persist within the host remains unclear. These studies have identified an extracellular organelle which can be exploited by *B. abortus* to traffic from one host cell to the next while evading the host immune system. This knowledge will contribute to the bacterial pathogenesis field by possibly providing a mechanism for the trafficking of many
intracellular pathogens associated with the autophagy pathway that has, to this point, been unable to be explained.

Dissertation Organization

This thesis is organized into 5 chapters; a general review of the literature pertaining to my studies, three chapters to be submitted for publication, and a final concluding chapter including suggestions for future research. The first chapter covers bacterial characteristics, host protein and process information as well as a justification for the studies. Chapter 2 describes a host protein, LRG-47, required for *Brucella* survival and replication inside the host cell. A novel autophagosome-like extracellular organelle is identified and examined in detail in Chapter 3. In Chapter 4 the mechanism for intercellular transport of *Brucella* within the host via the extracellular organelles identified in Chapter 3 is described. The thesis concludes with a chapter providing a general conclusion and recommendations for future experiments.
LITERATURE REVIEW

**Brucella**

**Brucella overview**

*Brucella* is a gram negative, non-spore forming, coccobacillus which lives in a microaerophilic environment inside its host. It is a partially acidfast bacterium that does not contain a native plasmid or express a capsule (1). This small bacterium, approximately 0.6-1.5 μm in length and 0.5-0.7 μm in diameter, is part of the α2-Proteobacteriacea family of bacteria (1). The *Brucella* species contains many subtypes which include *Brucella abortus* (2), *Brucella melitensis* (2), *Brucella suis*, *Brucella ovis* (3), *Brucella canis* (4), *Brucella neotomae* (5), *Brucella ceti* (6, 7), *Brucella pinnipedialis* (6, 7), and the latest addition, *Brucella microti* (8). Each of these species is host specific with *B. abortus* infecting cattle, *B. canis* infecting dogs, *B. suis* infecting swine (9). All of these species are known to infect humans with *B. melitensis* being the most virulent (8). *Brucella* species are extremely virulent pathogens. It takes as few as $10^3$ *B. abortus* organisms to cause disease in a human which is why this pathogen has been listed as a category B biowarfare agent by the United State Centers for Disease Control and Prevention (CDC) (9).

**Brucella in humans**

Humans are commonly exposed to the bacterium through contact with infected animals, inhalation of infected fecal material, or consumption of unpasteurized dairy products and undercooked meat products (1). *B. abortus* transmission also occurs through mucosal membranes and skin abrasions, through the handling of aborted animal tissues, or by human to human transmission and accidental laboratory exposures (1). Symptoms are generally consistent with those of influenza such as fever, chills, headache, sweats, fatigue, malaise, arthralgia, and weight loss (1), which commonly begin anywhere from 2 weeks to 3 months after the initial infection (10). Brucellosis is also referred to as “undulant fever” due to the
cyclic nature of the fever experienced by chronically infected individuals. Other symptoms reported with disease are involuntary limb movement, burning feet, low back ache, and ischemic heart attacks (1). The symptoms of human brucellosis also involve non-specific symptomology such as those of gastrointestinal, respiratory, dermal, or neurological detriments (11), and are commonly misdiagnosed as diseases such as enteric fever, malaria, autoimmune disease, tuberculosis, rheumatic fever, thrombophlebitis, fungal infection, tumors, or cholecystitis (1). Clinical manifestations of human brucellosis often include those common to many systemic bacterial infections such as liver, spleen, and lymph node enlargement (1, 10). Patients often suffer from spondylitis (10), or joint inflammation, which occurs in up to 40% of cases (1). Infective endocarditis also be caused by *B. abortus* infection in humans, though this is rare, but deadly (10). Reproductive complications are frequently reported in men and women (1). The treatment for brucellosis, as recommended by the CDC and the World Health Organization, is the combined daily dose of 600 mg of rifampin and 2.0 g doxycycline for 6 weeks (12, 13).

**Brucella in cattle**

*Brucella abortus* is the primary cause of brucellosis or Bang’s disease in cattle, bison, and elk all over the world (8). *B. abortus* was found to cause varying levels of disease from acute to chronic placentitis depending on the route of infection, amount of innoculum, virulence of the infecting strain, age and immunological status of the host, reproductive status of the host, and natural resistance of the host (8). Brucellosis is contracted by a number of different routes including exposure to infected uterine secretions, aborted fetuses, consumption of contaminated milk, venereal transmission, inhalation of infected fecal material, or infection through the skin, conjunctiva, or gastrointestinal tract (8). The most detrimental to the cattle industry is the asymptomatic carrier, who is able to give birth to an infected calf who will shed the bacterium throughout its lifetime (8). The end, and most concerning, result of brucellosis in cattle is abortion (14). Within two weeks of infection,
bacteria are isolated from the spleen of the animal, and two weeks later are found within uterine tissue (14). Initial infection of the placental tissue of the pregnant female happens in the erythro-phagocytic trophoblasts (15). \textit{B. abortus} has a natural tropism for uterine tissue in the last trimester of pregnancy (8). This tropism is thought to be due to the hormones produced in the uterus during this stage of pregnancy (8), as well as the erythritol present in the placental tissue of the cow (15). \textit{B. abortus} is able to use erythritol as a sole carbon and energy source (15). Interestingly, erythritol was not found in the placentas of humans, mice, rabbits, rats, or guinea-pigs, all animals which are infrequently found to have abortive issues caused by \textit{B. abortus} infection (15). \textit{B. abortus} infection in cattle causes the calves to be weak and sometimes infertile (8). It also causes the adult animal to be leaner with lower milk production (8).

\textit{Brucella vaccination}

The most common way to control brucellosis in cattle herds is by preventing it through vaccination of calves and adult cows. In the United States there are two attenuated live vaccines that are approved for the use in cattle, RB51 and strain 19 (S19) (16). Both RB51 and S19 are attenuated derivatives of the virulent \textit{B. abortus} 2308 (16). Much like the virulent strain of \textit{B. abortus}, S19 has a smooth lipopolysaccharide determined by the length of the polysaccharide O-side chain on the cell surface (16). It is thought that the smooth cell surface contributes to the bacteria’s virulence, making it better able to enter the host cell (8). The S19 vaccine induces an antibody response to long O-side chain (16). This strong antibody response is the reason for this vaccine’s effectiveness but also causes a problem as the antibodies produced will create a false positive in a serological test for \textit{Brucella} infection (17). RB51 was created by repeated passage of \textit{B. abortus} 2308 in media containing rifampicin (16). The resulting mutant lacks the O-side chain modification resulting in attenuation of RB51 in animals and cell culture pathogenesis assays (18). Strain 19 vaccine has strong evidence showing that it causes abortions in pregnant cows (16). \textit{B. abortus} S19
and RB51 cause infection in humans and do not afford protective immunity. Currently, no human vaccine exists against any species of *Brucella*. Reducing exposure for humans relies solely on the limiting the numbers of infected animals that can serve as a reservoir of the zoonosis (17).

**Intracellular lifestyle of non-opsonized Brucella**

*Brucella* is a facultative intracellular pathogen of resident tissue monocytes and macrophages. Internalization of *Brucella* by these cells occurs either by the bacteria interacting directly with the surface of the macrophage or indirectly through the attachment of serum opsonins to the bacterial surface. Non-opsonized *Brucella* are bound by the long O-chain of their LPS to lipid rafts found occasionally on the surface of the macrophage (19). It has also been suggested that class A scavenger receptors in the lipid rafts bind the bacterial LPS facilitating uptake into the host cell (20). Lipid raft microdomains are comprised of cholesterol, GM1 gangliosides, and glycosylphosphatidylinositol-anchored proteins (19).

One hour post infection, the *Brucella*-containing compartment interacts with the endoplasmic reticulum subdomains of the early secretory pathway called ER exit sites (20) (Figure 1.1). This interaction of the *Brucella*-containing vacuole with the ER induces loss of LAMP1 from the surface of the vacuole (20). It is at this point that the pH of the *Brucella*-containing compartment increases to become more permissive to *Brucella* replication as a pH above 5.5-6 is required for this to occur (21). Between 10-12 hours post infection *B. abortus* finally begins to replicate in the endoplasmic reticulum-like replicative niche (22) that is rich in nutrients and amino acids (23). Over a 48 hour period, *Brucella* will replicate in the ER-type vesicles until the cytoplasm of the cell is filled (21). *Brucella* have the ability to replicate extensively without disrupting the cellular processes of the host cell while preventing its apoptosis (20). *Brucella* will replicate in this cell until space is no longer available, or nutrients become depleted, at which time they will lyse the cell and infect a new cell by an unknown mechanism (21).
Figure 1.1. Opsonized and non-opsonized *Brucella* internalization and trafficking in a macrophage. Once internalized, *Brucella* are trafficked through an vesicle sharing markers with an early endosome. Later the *Brucella* are found in a compartment resembling a late endosome where a majority of the bacteria will be killed. The surviving bacteria will replicate in a vesicle sharing markers with the endoplasmic reticulum.
**Intracellular lifestyle of opsonized Brucella**

Immunoglobulin opsonized *Brucella* are internalized by macrophages through crosslinking of surface receptors (24). Anti-Brucella antibodies arise rapidly and are easily detected by the end of the first week of infection. The presence of antibodies not only facilitates antibody mediated complement killing of bacteria in the sera, but also increases internalization of the bacteria by monocytes and macrophages through Fc receptors (25). The intracellular pathways utilized by *B. abortus* for survival and replication vary depending on the method used for internalization into the host cell (Figure 1.1). As a result, the intracellular trafficking of opsonized *Brucella* differs from that observed for non-opsonized bacteria. Within minutes after internalization, opsonized *Brucella* are found within the early endosomal compartment (similar to that observed for non-opsonized) (26) defined by the presence of the small GTPase Rab5, early endosomal antigen 1 (EEA1) and the transferrin receptor (24). The pH of *Brucella* containing vesicles changes from ~5.5 of the early endosome to pH < 4 by 2 hours post infection with the acquisition of the regulator Rab7 that recruits the late endosomal effectors LAMP1, cathepsins (27), vacuolar H⁺-ATPase and rab7 interacting lysosomal protein (RILP) (28). At this stage, 60% of the bacteria are degraded (29).

**Brucella virulence, intracellular survival, and replication**

The low pH of the late endosomal compartment is critical for up-regulation of the *virB* type IV secretion system (22). VirB is required for non-opsonized *Brucella* that survive within the phagolysosome to form a successful intracellular replicative compartment (21). In addition to VirB, other *B. abortus* virulence factors help the pathogen resist degradation inside the phagolysosome. One of these mechanisms is the production of periplasmic cyclic β-1,2-glucans (21) that disrupt lipid rafts and prevent fusion of *Brucella* containing vesicles with lysosomes (23). Superoxide dismutases *sodC* and *sodA* are produced to detoxify $O_2^-$, and catalases AhpC and KatE protect against $H_2O_2$ inside the phagosome (30). This
bacterium also employs a urease and a proton symporter, GadC, to resist killing in extremely acidic conditions (23).

The reason *Brucella* is such a powerful intracellular infectious bacterium is due to its ability to evade the host immune system. One such way that *Brucella* is able to do so is by inhibiting the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), which usually is responsible for the activation of cytokine production as an inflammatory response (31). In addition, *Brucella* lipoprotein is able to down regulate the surface expression of CD64 via TLR2 on the infected host cell (32). This decrease in CD64 expression restricts *Brucella* uptake via the FCγRI receptor possibly favoring uptake through lipid rafts, which seems to lead to a higher survival rate of the bacteria (32). In addition to enabling the invasion of *Brucella* into the host cell, the LPS has little to no endotoxicity and is able to down regulate antigen presentation by major histocompatibility complex (MHC) class II molecules (21). It does this by blebbing off replicating *Brucella* in large quantities, and then is trafficked through late endosomes, and loaded onto MHC class II molecules and displayed on the surface of the host cell (21). The MHC class II molecules displaying the *Brucella* LPS on the surface of the cell form large microdomains called ‘lipid mega rafts’ which remain on the surface of the host cell for up to 3 months (21). These mega rafts cause a down regulation in antigen presentation via MHC class II resulting in a decrease in activation of CD4+ T cells (21). CD4+ T cells are instructed to become a variety of different T helper cells by the presence of different cytokines. T helper-1 cells produce interferon-gamma (IFN-γ) in response to *B. abortus* infection (8). IFN-γ is a type II interferon initially identified in 1965 for its production in response to viral infections by lymphocytes (33). It is produced primarily by natural killer cells and T lymphocytes, and its production is controlled by the cytokine IL-12 produced by myeloid cells (33). IFN-γ was shown to be essential for
protective immunity against many pathogens (33), and it is the primary activator of
macrophages (8).

Interferon mediated anti-Brucella activity

Interferon gamma is essential for resistance to facultative intracellular bacterial pathogens (34). It has been shown to be critical for the control of *B. abortus* infection *in vivo* and *in vitro* (8). Aside from CD4+ T cells producing IFN-γ within the host, CD8+ T cells are also able to secrete this cytokine, and it is these cells that are critical for the IFN-γ mediated immunity against *B. abortus* infection (8). It was found that pretreatment of mice with a dose of IFN-γ prior to infection and two doses of the cytokine post infection greatly decrease the bacterial load in the spleen of the infected animals (35).

**LRG-47**

LRG-47 and intracellular defenses

Interferon gamma induces expression of nearly 1300 genes in the macrophage (36). Among this collection of genes is a family of Immunity-Related p47 guanosine triphosphate-binding proteins (GTPase) comprised of 6 members divided into 2 groups (37). Group 1 of the p47 GTPases contains GTPI (38), IGTP (39), and LRG-47 (37) and is identified as the GMS subgroup. Group 2, the GKS subgroup, consists of IRG-47 (40), TGTP/Mg21 (41), and IIIGP (38). The LRG-47 protein is comprised of 409 amino acids with a predicted molecular weight of 46.5 kDa. Expression of LRG-47 is induced by LPS, IFN-γ and IFN-α/β (37). Induction of LRG-47 initiates within 2 hours of IFN-γ stimulation and peaks at 8-12 hours (37, 42).

Induction of this family of proteins provides additional protection against intracellular infections (41). Interestingly, protection afforded by individual p47 GTPases is pathogen specific, whereby a p47 GTPase will inhibit some pathogenic infections but not others. IGTP reduces intracellular *Toxoplasma gondii* parasite burden, however, does not alter the course
of murine cytomegalovirus or *Listeria monocytogenes* infection (43). Vesicular stomatitis virus infection is inhibited by TGTP whereas herpes virus infection and replication are not affected. A similar pathogen specific relationship exists for LRG-47. Infection of LRG-47 knockout mice with *M. tuberculosis* (44), *L. monocytogenes*, and *T. gondii* led to a higher burden of pathogen, however, murine cytomegalovirus infections in the knockout did not (45). Pathogen specific protection can also vary between pathogens belonging to the same genus. Among pathogenic *Chlamydia* which share >99% of their predicted open reading frames, *C. trachomatis* infection is controlled by LRG-47 and *C. muridarum* is not (46).

**LRG-47 mechanism**

LRG-47 has a number of important regulatory functions inside the host cell. The different functions of the protein are determined by the different types of structures or organelles that have acquired the LRG-47 protein. Association of LRG-47 with cortical, polymerized actin coordinately regulates cellular adhesion and motility, as well as phagocytosis of external particles, following stimulation of the macrophage with IFN-γ (47). Disruption of membrane organization leads to altered vesicle/membrane trafficking within cells (47). LRG-47 localizes to the *cis*-Golgi in a nucleotide independent manner, and is recruited to the plasma membrane during a phagocytic event that requires GTP binding (42). LRG-47 associates with the autophagosome where it stays associated for the entire maturation process and eventually colocalizes with LAMP1 (42) (Figure 1.2). This intracellular trafficking is said to be directed by an amphipathic helix on the C-terminus of LRG-47, as well as by its G domain sequence (42). More LRG-47 is rapidly released by the Golgi and possibly the endoplasmic reticulum, and trafficked quickly to the plasma membrane (42). It was later found that LRG-47 also colocalizes to the *trans*-Golgi network and is found on acidic vesicles (48). Transferrin labeled with a fluorochrome was used to show that LRG-47 was barely detectable in early or recycling endosomes (48).
Figure 1.2. Autophagy and LRG-47. The process of autophagy from initial generation of the phagophore, to complete degradation of the autophagosomal contents. The source of the phagophore membrane can be from the nuclear membrane, the golgi apparatus, the endoplasmic reticulum, or the mitochondrial membrane as indicated by the arrows. Other proteins involved in the elongation of the phagophore membrane not listed on the figure include Atg12-Atg5 complex, Atg7-Atg10 complex, and Atg16. Diagram also shows LRG-47 intracellular localization and involvement in the process of autophagy inside the cell.
Humans possess a single p47 GTPase, IRGM1, that is homologous to the p47 GTPases found in mice (49). Humans express 5 distinct isoforms of the IRGM protein through splice variation of the encoding transcript (50). Splice variation is hypothesized to provide variable functions to IRGM within the cell without encoding for separate p47 GTPases.

The link between autophagy and LRG-47 was strengthened by the confirmatory findings of Gutierrez et al. and their work with the murine monocyte cell line, RAW264.7 (51). Upon transfecting RAW264.7 cells with LRG-47, this group observed the formation of autophagosomes inside the cells, an effect similar to that seen when macrophages are treated with the cytokine IFN-γ (51). Interestingly, LRG-47 positive vesicles became larger, and their association with the autophagosome marker LC3 rapidly decreased, though it was clear that these organelles were autophagosomes by their 100% colocalization with the acidity indicator LysoTracker (51).

**Autophagy**

*Introduction*

Autophagy is the process of programmed self-digestion in a cell that recycles amino acids, enzyme cofactors, lipids and nucleotides from damaged proteins and organelles. There are three types of autophagy that occur in a cell, microautophagy, chaperone-mediated autophagy, and macroautophagy (52). Macroautophagy, more commonly known as autophagy, is important for degrading protein aggregates, influencing cell death (52), and clearing intracellular pathogens (53, 54). Research to describe the autophagy pathway in detail has been performed primarily on the yeast *Saccharomyces cerevisiae* (55), but the pathway is well conserved in mammalian cells (49). The term ‘autophagy’ was coined by Christian de Duve and Robert Wattiaux in their paper describing an intracellular process where smooth ER membrane encircled cytoplasmic contents and acquired lysosomal hydrolases (56). Other sources of membrane that can incorporate into the autophagosome
include the outer mitochondrial membrane (57), the outer nuclear membrane (58), or the
golgi apparatus (59) (Figure 1.2). Autophagy initiates with a portion of membrane, called the
phagophore, which expands through the assembly of the multimer complex of autophagy
related (Atg) proteins Atg 5, 7, 10, and 12 (60, 61). This complex recruits Atg16 and LC3, a
microtubule-associated protein light chain 3 (initially identified as Atg8) (62). Within the
cytosol, nascent LC3 polypeptide (pro-LC3) is cleaved by a specific protease into LC3A (aka
LC3-I) (62-64). LC3A is converted into LC3B (aka LC3-II), the membrane bound lipidated
form, through the covalent attachment of phosphotidyl-ethanolamine (PE) (65). LC3B then
localizes to the phagophore membrane where it directs development of the phagophore into
an early autophagosome, an enclosed structure that acquires the early endosomal regulator
Rab5 (60). Maturation of the early autophagosome into a late autophagosome is carried out
by Rab5 recruiting and being replaced by Rab7 (66, 67). Rab7 and the recruited effector
protein, RILP, mediate the subsequent fusion of the late autophagome with lysosomes in a
microtubule dependent manner to generate the autolysosome (68, 69). With the fusion of
lysosomes, constituents of the autophagosome including the inner (70) and outer membranes
(60, 65) are degraded through the combined activity of the vacuolar H⁺-ATPase (V-ATPase),
cathepsins D, H, and S, and acid hydrolases (71, 72). The contents of the autolysosome are
released into the cytoplasm of the cell, where they are reused in many cellular processes.

**Autophagy activation**

Autophagy is induced in mammalian cells by stress, damage (52), serum starvation (56)
and amino acid starvation (73). The mammalian-target of rapamycin protein (mTOR), is a
PI3K-related kinase that signals cell health and promotes cell division (74). Inhibition of
mTOR by rapamycin (51), simulates amino acid starvation even under nutrient replete
conditions thereby inducing autophagy. Assembly of the autolysosome is disrupted by the
PI3K inhibitors, 3-methyladenine (3MA) (75) and wortmannin (76). Both 3MA and
wortmannin act by inhibiting type III phosphotidylinositol-3’ kinases (PI3K), cytosolic
enzymes required for the early stages of autophagosome development (77). Acidity of autolysosomes can be neutralized by nanomolar concentrations of the V-ATPase specific inhibitor, bafilomycin A1 (78). Loss of the proton gradient mutes the degradative activity of the autolysosome and prevents additional lysosomes from fusing with the organelle (79).

Autophagy in macrophages results from the general cell stressors mentioned above, and can also be stimulated by exposure to IFN-γ (80). Induction by the pro-inflammatory cytokine elevates cellular defenses to protect against intracellular parasitism during infection. Activation of autophagy both by IFN-γ and rapamycin is evident by the increase in cellular amounts of LC3B (51, 75, 81). In macrophages, autophagy also performs a critical role in host innate immunity by sequestering intracellular pathogens that are either free within the cytosol or in a membrane bound compartment into an autophagosome (81). Pathogens sequestered in this manner will not only be degraded, but the microbial peptides will be directed into antigen processing and for MHC class I and class II presentation (82). Should the autolysosome disintegrate releasing microbial peptides into the cytosol, the peptides can act as targets for the cytosolic proteasome after which they are transported by TAP-1 and -2 into the ER and loaded onto MHC class I molecules for cross presentation (82). In contrast, should the autophagosome/autolysosome modify or fuse with constituents of the MHC class II loading compartment, the degraded peptides will serve as a source of antigen for MHC class II loading (83). It is in this way that the autophagosomal pathway contributes to antigen presentation via MHC molecules.

*Autophagy and disease*

Dysregulation of autophagosomal processes within the cell can be detrimental to the health of the host causing a plethora of problems including uncontrolled intracellular pathogen infections, a number of neurodegenerative diseases, Huntington’s disease, diabetes, aging, muscle atrophy, lipid metabolism, antioxidant response, and different types of cancer
Of the different types of neurodegenerative diseases caused by the dysregulation of autophagy are Alzheimer’s disease and Parkinson’s disease. Alzheimer’s disease is caused by a build-up of autophagosomes inside neuronal cells of the brain that contain undigested proteins (85). These neurons also contain amyloid-β peptide and extracellular β-amyloid deposits in extensive amounts, which is due to the autophagic defect in protein clearance (85). Parkinson’s disease, the second most common human neurodegenerative disease, is also due to a dysregulation of autophagy processes (86). Parkinson’s disease is caused by an accumulation of protein aggregates in the cytoplasm of neuronal tissue cells causing an eventual loss of dopaminergic neurons in the brain (86, 87). One major protein that makes up the protein aggregates is α-synuclein which was found to inhibit the early stages of autophagosome formation, explaining the build-up of protein aggregates inside these tissues (86). It was also said that α-synuclein may play a role in lysosome malfunction, causing autophagosomes containing undigested protein to build up inside the cell (87). This was evidenced by an accumulation of LC3-II protein and an increase in LC3 gene expression in the brain tissue of Parkinson’s disease patients (87). As seen by its importance in preventing neurological disorders, the process of autophagy is critical for much more than controlling intracellular infection.
References


CHAPTER 2. HOST INTERFERON-γ INDUCIBLE PROTEIN CONTRIBUTES TO

BRUCELLA SURVIVAL

A paper to be submitted

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Abstract

Brucella spp. are highly adapted intracellular parasites of mammals that cause chronic infections while surviving and replicating in host monocytes and macrophages. Although monocytes are normally susceptible to infection, a newly discovered host defense mechanism combats infection by altering the intracellular replication of pathogens persisting within cells following stimulation of host cells with pro-inflammatory cytokine interferon-γ (IFN-γ). We examined the contribution of the IFN-γ inducible GTPase, LRG-47, to Brucella abortus 2308 infection in in vitro and in vivo murine models. Infecting macrophages harvested from LRG-47⁻/⁻ mice revealed that loss of this host protein negatively affected the intracellular survival and replication of IgG opsonized B. abortus while the viability of non-opsonized bacteria was similar to levels seen with similarly treated C57/B6 macrophages. This opsonization dependent effect was reversed in LRG-47 cells by pre-stimulating LRG-47⁻/⁻ macrophages with IFN-γ as observed by a severe defect in Brucella survival of non-opsonized bacteria and
a stimulation of *Brucella* replication of IgG opsonized bacteria. The requirement for LRG-47 for *B. abortus* survival and replication in murine macrophages is unlike any other pathogen studied and provides interesting insight into the complex interaction between *Brucella* and their host.

**Introduction**

*Brucella* spp. are highly infectious intracellular pathogens that establish chronic infections by surviving and replicating within host monocytes and macrophages. Production of pro-inflammatory cytokines by the host stimulates phagocytosis and killing of pathogenic microbes. *In vivo* and *in vitro* experiments demonstrate that the most potent inducer of anti-*Brucella* activity in monocytes and macrophages is type II interferon (IFN-γ) (1). IFN-γ stimulation of monocytes and macrophages increases production of reactive oxygen and nitrogen species. In the case of *Brucella abortus*, reactive oxygen intermediate (ROI) production was found to play a larger role in intracellular resistance to the bacteria than nitric oxide (NO) (2), while *Brucella suis* infected monocytes reduced bacterial load primarily through inducible nitric oxide synthase (iNOS) activity (3). Interestingly, pretreatment of monocytes with the cytokine IFN-γ yielded the strongest anti-*Brucella* activity. This activity was characterized as a bacteriostatic mechanism that prevented intracellular replication of the *Brucella* spp. rather than simply increasing killing (2).

Human T-cells respond to *Brucella* spp. by stimulating interleukin (IL)-2 production, whereby increasing IFN-γ release (4). It was shown by Stevens *et al.* that intraperitoneal injection of recombinant murine IFN-γ into mice significantly lowered the number of *B. abortus* isolated from the spleen at day 7 (IFN-γ was delivered 1 day prior and 2 and 4 days post infection). This increase in resistance to *B. abortus* was amplified by co-administration with the cyclooxygenase inhibitor, indomethacin, which blocks prostaglandin E2 (PGE2), thereby increasing IL-12 and IFN-γ production (5). 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 (6). 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 (7). 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 (8) by the cytokine IFN-γ (9). This 47 to 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 (8, 10). 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 IIGP (Irga6) (10). These two groups of proteins do not act independently but interdependently to control intracellular and extracellular pathogen invasion (10). The GMS proteins function as regulators of the GKS proteins’ GTPase cycle, acting as attenuators by preventing premature activation of the GKS proteins (11, 12). These IRG proteins have pathogen specific behavior; each protein is involved in controlling a specific pathogen (8, 10, 13). 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 LRG-47 is essential in macrophage resistance to a variety of intracellular pathogens such as *Toxoplasma cruzi* (14), *Toxoplasma gondii* (13, 15), *Listeria monocytogenes* (15), *Mycobacterium avium* (16), *Mycobacterium tuberculosis* (17), and *Chlamydia trachomatis* (18). LRG-47 is normally associated with the cis- and medial-golgi network inside a macrophage (8, 13, 19). Upon actin remodeling and phagocytosis, LRG-47 is recruited to
phagocytic cups on the plasma membrane, where it will stay for the full life cycle of the phagosomal compartment (8). 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 (8, 19), and the acidic vesicle stain LysoTracker® inside macrophages (19). Zhao et al. speculated that the golgi-localized LRG-47 is the GDP bound form (inactive protein), where as the lysosome-associated LRG-47 is the GTP bound form (active protein) (19). 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 (12, 20) 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 a wild-type mouse (36). The susceptibility was traced to a deficiency in phagosome maturation; stimulated macrophages from LRG-47 knockout mice failed to acidify *Mycobacterium*-containing phagosomes (17). 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 (21). 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 nonopsonized 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**

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 in prior studies (22) by introducing the plasmid pBBR1MCS6-Y encoding GFP expression downstream of the constitutively active promoter for *aph3A*-derived from pBlueKS+Kan (Stratagene) (23). 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 four days. All chemicals were obtained from Sigma-Aldrich unless otherwise stated.

**Culture of murine 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 pre-filled 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 hrs at 37°C with 5% CO₂. Non-adherent 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.
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 x 10^5 cells/ml 24 hrs prior to infection. Cell culture viability was monitored in suspension by hemocytometer-trypan blue dye exclusion.

**Assessing intracellular survival and replication of *B. abortus***

Bacterial suspensions were generated by scraping 48 hour cultures of the *B. abortus* strains grown on BA into screw cap microfuge tubes containing phosphate buffered saline (PBS). *Escherichia coli* cultures were grown on TSA media for 24 hrs prior to harvesting. 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 monocyte monolayers treated as described above but plated into 96 well plates 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 nonopsonized bacteria, synchronized internalization was achieved by cooling infected cultures to 4ºC 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 pre-warmed media after which cells were incubated for 20 min. at 37ºC / 5% CO₂. After incubation, cultures were washed 3 times with cold PBS and fresh media containing 100 µg/ml gentamicin was added to cells for 1 hour to kill adherent but not internalized bacteria. After one hour 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 (24). Percent survival of bacteria at 24 and 48 hrs were calculated based on the number of internalized bacteria detected at 1 hour post infection which represents 100% of internalized bacteria. Statistical comparisons were made using the Student’s t-test.

**Antibodies and reagents for fluorescence microscopy**

Infected monolayers were prepared for immunofluorescence microscopy at indicated times post infection using methods described previously (22). Infected monolayers were fixed by incubating coverslips for 20 min. with 4% paraformaldehyde/PBS solution at room temperature. Samples were washed with PBS and monolayers were permeabilized and stained with antibody in 0.25% bovine serum albumin/PBS solution containing a final concentration of 0.1% saponin (BSP). Incubations with primary and secondary antibodies were at room temperature on a shaking platform for 1 hour. Primary antibodies were diluted in BSP and incubated with fixed Brucella infected cells at the following concentrations: 1/50 mouse anti-LAMP 1 monoclonal (ID4B- Iowa Hybridoma), 1/5 anti-mannose-6-phosphate receptor (Iowa Hybridoma); 1/100 mouse anti-EEA1, anti-calnexin, anti-BiP/GRP74, anti-p115, anti-p230, anti-SRP54, anti-Rab5 (Transduction Laboratories), mouse anti-transferrin receptor (Molecular Probes), rabbit and mouse anti-Cathepsin D (Oncogene). Coverslips were washed 3 times with cold BSP and affixed to glass slides with ProLong® Gold Antifade reagent plus DAPI stain mounting solution (Invitrogen). Acidic vesicles were visualized by incubating cultures with 1/1000 Lysotracker® DND-99 for 10 min. 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).

**Results**

**Expression and localization of LRG-47 to Brucella-containing vesicles**

To determine if LRG-47 is found on *Brucella*-containing phagosomes in IFN-γ activated monocytes, RAW264.7 cells pre-treated with IFN-γ and infected with opsonized GFP-expressing-*B. abortus* were fixed at 18 hrs post infection and immunostained for LRG-47. This revealed that individual *Brucella* within these cells did colocalize with LRG-47 (Figure 2.1). Similar results were also obtained with IFN-γ activated peritoneal macrophages (data not shown).

![Figure 2.1](image)

Figure 2.1. *Brucella abortus* containing phagosomes acquire the IFN-γ inducible protein LRG-47. Monolayers of RAW264.7 cells were stimulated with IFN-γ, infected with opsonized-*B. abortus* 2308 fixed 18 hrs post infection and stained to visualize the spatial relationship of the LRG-47 with the host cell (red). *Brucella* were observed almost exclusively as isolated bacteria within phagosomes positive for LRG-47 (arrow heads). Panel of images shown are representative of results from 3 separate experiments (bar = 5 µm).
**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. The distribution pattern of the LRG-47-Flag protein in non-IFN-γ activated cells was indistinguishable from that of endogenous LRG-47 in nontransformed IFN-γ treated RAW264.7 cells (data not shown). 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 hrs post infection. CFU’s recovered from 2, 12, 24 and 48 hrs post infection revealed that intracellular survival of *B. abortus* was not affected by constitutive expression of LRG-47 while significant reduction in intracellular viability was observed in IFN-γ treated RAW264.7 cells (Figure 2.2). The results clearly show that IFN-γ induced anti-*Brucella* activity was not evoked within cells simply overexpressing LRG-47. Although long term intracellular survival was not reduced by LRG-47 overexpression, the level of *Brucella* in these cells was more similar to IFN-γ treated cells than non-IFN-γ treated controls up to 24 hrs post infection. These observations suggest a possible role for LRG-47 in opsonized *Brucella* internalization and early survival; however, LRG-47 does not limit bacterial replication by itself.

**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 nonstimulated macrophages would behave similarly to nonactivated 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 2.3). 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 hrs, the number of bacteria recovered from IFN-γ treated LRG47−/− cultures increased from 24 hour values, suggesting that some degree of intracellular bacterial replication does occur. By contrast, both *Brucella* survival (CFU at 24 hr) and replication (CFU at 48 hr) were significantly reduced in nonstimulated LRG-47−/− cells, suggesting that the IFN-γ inducible protein contributed to intracellular bacterial survival. Repeat experiments with nonstimulated peritoneal and BMDM macrophages showed similar reductions in replication of opsonized *Brucella* within monocytes from LRG-47−/− mice.

![Figure 2.2. Constitutive expression of LRG-47 aids in bacteria replication and survival.](image)

RAW264.7 murine macrophages stably transfected with Flag-tagged LRG-47 (Raw264.7 LRG) were infected with virulent *B. abortus*. Colony forming units/ml were enumerated at 0.5, 1, 24, and 48 hrs post infection and compared with the CFU/ml values for non-activated RAW264.7 cells (Raw264.7) and IFN-γ activated RAW264.7 cells (Raw264.7 IFN) both of which are not overexpressing the LRG-47 protein. (Student’s T test *p* values * < 0.05, ** < 0.01).
Figure 2.3. Intracellular viability of *Brucella* in peritoneal macrophages harvested from C57/Black6 and LRG47 knockout mice. IFN-γ was added 24 hrs prior to infection with immunoglobulin opsonized *B. abortus* 2308. Means and standard deviations were calculated from triplicate samples (** $p \leq 0.01$ Student’s t-Test). Results shown are representative of 3 independent experiments demonstrating similar significant differences.

**Survival and replication of Brucella within RAW264.7 cells and IFN-γ activated cells**

Bacteria opsonized by serum components are internalized faster with greater efficiency a non-opsonized bacteria. Cell surface receptors that mediate internalization of opsonized particles influence the intracellular trafficking of the new phagosomes through their cytoplasmic domains (25). Previous studies determined that immunoglobulin opsonized *B. abortus* localized to a replicative compartment that lacked ER components (22). In contrast,
non-opsonized *B. abortus* did localize to an ER positive compartment as others have reported (26). Thus, the intracellular fate of *Brucella* depends on the context by which the bacteria are presented to monocytes. To determine if the mode of *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. abortus* 2308 employing increasing MOIs. Numbers of intracellular *Brucella* recovered at 24 and 48 hrs were largely in proportion to the number of bacteria internalized at the beginning of the experiment (Figure S2.1). 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, the calculation of survival of *Brucella* at 48 hrs as a percentage of the number of bacteria initially internalized revealed that the replication of 20:1 opsonized (1236.4% ± 15.6%) was double that of 100:1 non-opsonized (600.0% ± 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-γ treatment was equally effective for opsonized and non-opsonized bacteria, intracellular viability experiments were performed with RAW264.7 cells stimulated 24 hrs prior to infection with 50 U/ml of recombinant IFN-γ (IFN-γ). Similar degrees of IFN-γ anti-*Brucella* activity were observed at 24 hrs and 48 hrs post infection and did not differ among opsonized or non-opsonized bacteria (Figure S2.2). Treatment of monocytes with IFN-γ resulted in an approximate 2 log reduction in both opsonized and non-opsonized bacteria by 24 hrs, and reduced the number of *Brucella* recovered at 48 hrs down to the limit of detection. The calculation of percent survival did not uncover any additional significant findings. It is of note that pretreatment of monocytes with IFN-γ 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-γ treated cells.
Effect of opsonization on *Brucella* survival

An earlier report by our laboratory showed that intracellular localization of *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 *Brucella* trafficking through autophagosomes before reaching their replicative compartment (27). 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 *Brucella* varied in LRG-47<sup>−/−</sup> cells in response to varying the method of bacterial internalization. Comparisons between non-stimulated (resting) and IFN-γ stimulated (activated) cells isolated from either B6 or LRG-47<sup>−/−</sup> mice were carried out using optimal MOI’s of 20:1 for Ig-opsonized and 100:1 for non-opsonized, yielding equivalent numbers of internalized bacteria within RAW264.7 cells. Infected peritoneal macrophages from wild type C57/B6 mice treated with IFN-γ exhibited significant and equal anti-*Brucella* activity against both opsonized and non-opsonized *Brucella*. In contrast, *Brucella* viability in LRG-47<sup>−/−</sup> cells was dependent on the whether the bacteria were opsonized, and on the presence of IFN-γ activation (Table 2.1). Resting LRG-47 deficient macrophages harbored significantly more non-opsonized *Brucella* at 48 hrs (137.2 % ± 12.1%) than Ig-opsonized (79.4% ± 15%). In addition, Ig-opsonized *Brucella* survival in LRG-47 KO was significantly lower than was seen in resting B6 isolated macrophages (114% ± 2.7%). An inverse relationship was seen between *Brucella* survival and the affects of opsonization in IFN-γ stimulated-LRG-47 KO macrophages (Figure S2.3). Percent survival of non-opsonized *Brucella* dropped dramatically in IFN-γ activated LRG47<sup>−/−</sup> cells to 32.6% while Ig- opsonized *Brucella* survived as well as, or better than, the opsonized bacteria in the resting LRG-47<sup>−/−</sup> or B6 macrophages. Post-hoc analysis revealed that averaging the opsonized and non-opsonized
LRG-47/− macrophage results together based on IFN-γ activated and non-activated status yielded values similar to those recorded from the corresponding IFN-γ treated B6 macrophage cultures.

Table 2.1. Summary of B. abortus percent intracellular viability results within primary non-elicited peritoneal macrophages from B6 and LRG-47/− KO mice.

<table>
<thead>
<tr>
<th>Mouse Line</th>
<th>Opsonin</th>
<th>IFN-γ</th>
<th>48hrs % Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>B6</td>
<td>IgG</td>
<td>-</td>
<td>114.4 ± 2.7</td>
</tr>
<tr>
<td>B6</td>
<td>None</td>
<td>-</td>
<td>123.9 ± 6.9</td>
</tr>
<tr>
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<td>83.8 ± 13.0</td>
</tr>
<tr>
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<tr>
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<td>IgG</td>
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<td>79.4 ± 15.0 (^1)</td>
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</tr>
<tr>
<td>LRG47</td>
<td>None</td>
<td>+</td>
<td>32.6 ± 0.0 (^2)</td>
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\(^1\) \(p < 0.01\) (C57/B6 vs. LRG47 of same condition)
\(^2\) \(p < 0.001\) (C57/B6 vs. LRG47 of same condition)

\(\#\) \(p < 0.01\) IgG opsonized vs. non-opsonized

**Brucella survival in LRG-47/− unaffected by autophagy inhibitor**

Reduced survival of M. tuberculosis in IFN-γ activated macrophages has been attributed to the ability of LRG-47 to redirect resident intracellular bacteria towards lysosomes by repackaging intracellular pathogens within autophagosomes (12, 20). 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 50 µM 3-methyladenine (3-MA), a pharmacologic agent known to inhibit autophagosome formation. Within B6 cells, the addition of 3-MA reduced Brucella CFUs to 5.8 ± 0.16 log10 for untreated, and 4.6 ± 0.09 log10. CFUs equivalent to those recovered from B6 IFN-γ activated cultures with a value of 4.3 ± 0.56 log10 (Figure 2.4). Interestingly, the reduced levels of Brucella recovered from nonactivated LRG47−/− cells observed earlier remained unchanged by administering 3-MA or IFN-γ supplementation. Co-administration of these agents had a synergistic effect that significantly decreased the number of intracellular bacteria in both the wild-type (WT) and LRG-47 deficient mouse macrophages. A more dramatic drop in bacterial survival was seen within LRG-47 KO macrophages (B6: 3.2 ± 0.06 log10; LRG-47−/−: 0.6 ± 0.8 log10) than in wildtype. 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 (data not shown).

**LRG-47 contributes to stability of chronic Brucellosis in mice.**

To determine the overall contribution of LRG-47 to host resistance during chronic brucellosis, the number of *B. abortus* residing within the spleens of experimentally infected B6 and LRG-47−/− mice were followed for 9 weeks post infection. The LRG-47 mutation was introduced into the L129 background for initial experiments (data not shown). Both C57/B6 and L129 F-2 hybrids were used as controls for *Brucella* infection. Time points at 2 and 6 weeks post infection showed no significant difference between any of the mouse lines, including the controls (data not shown). We determined the chronic brucellosis models in C57/B6 and L129 mice were equivalent and subsequent mouse experiments were performed in C57/B6 mice. It is standard that *Brucella* are routinely recovered from the spleens and livers of C57 mice up to and beyond 12 weeks. The splenic colonization study was repeated
with C57/B6 and LRG-47<sup>-/-</sup> mice and no differences in colonization of <i>B. abortus</i> were seen between the two models. Continuing this second study out to 9 weeks post infection revealed a significant decrease in the amount of bacteria recovered from the spleens of LRG-47<sup>-/-</sup> mice (Figure 2.5). A semi-quantitative assessment for the presence of anti-<i>Brucella</i> antibodies revealed that sera from LRG-47<sup>-/-</sup> mice was less able to agglutinate heat killed bacteria, suggesting a possible deficiency in the production of anti-<i>Brucella</i> antibodies (data not shown).

Figure 2.4. <i>Brucella</i> survival in LRG47<sup>-/-</sup> macrophages is unaffected by the autophagy inhibitor 3-methyladenine (3-MA). Peritoneal macrophages were collected from C57/B6 and LRG-47<sup>-/-</sup> mice and infected with Ig-opsonized virulent <i>B. abortus</i>. Cells were stimulated with IFN-γ and treated with 50 μM 3-MA. Colony forming units were enumerated at 1hr and 48 hrs post infection. Loss of LRG-47 in non-activated cells had a similar effect as treatment with 3-MA or IFN-γ in B6 cells on <i>Brucella</i> survival after 48 hrs. The inhibition of autophagy plus IFN-γ activation greatly decreased the number of viable <i>Brucella</i> after 48 hrs in LRG-47<sup>-/-</sup> cells.
Figure 2.5. Murine model of chronic brucellosis. Transgenic LRG-47 \(-/-\) mice and C57/B6 mice were infected intraperitoneally with $1 \times 10^5$ CFU of *B. abortus* 2308. Values represent the mean and standard deviation of CFU’s enumerated from a minimum of 5 mice per group (** $p \leq 0.01$). Separate experiments that included 129/B6 F2 mice yielded similar results of no defect spleen colonization before 4 weeks post infection for any group tested, including LRG-47 KO mice.

**Discussion**

Pro-inflammatory cytokines elicit anti-microbial responses in monocytes and macrophages that serve as a critical defense against intracellular pathogens. Recently, attention has been focused on the ability of IFN-\(\gamma\) to stimulate monocytes to alter normal intracellular vesicle and organelle trafficking to regain control over intracellular pathogens. Many p47 GTPases induced by IFN-\(\gamma\) 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. 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 hrs 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 hrs post infection in the LRG-47−/− cells without stimulation. This suggests a requirement for LRG-47 for *Brucella* survival in the early stages after entry into the host cell. To determine the intracellular localization of LRG-47 in regards to *Brucella*, RAW264.7 murine macrophages with and without IFN-γ stimulation were infected with opsonized *Brucella* and stained for LRG-47. We observed a clear colocalization of individual *Brucella* with LRG-47 inside stimulated cells. 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.

Over expression 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 are needed to 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. A plausible explanation is that *Brucella* must orchestrate autophagosome biogenesis to maintain an intracellular niche. Bacterial control over autophagosome formation becomes even more critical when anti-*Brucella* activity in macrophages is increased by IFN-γ activation. Loss of LRG-47 was similar in effectiveness to 3-MA in inhibiting *Brucella* replication in nonstimulated macrophages, suggesting a similar means of interference in *Brucella* pathogenesis for both LRG-47 and 3-MA. 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. It should be noted that *Brucella* did not survive as well in LRG-47 KO cells grown under nonstimulating 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 (22). This difference in bacteria trafficking of opsonized versus 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 hrs of infection, suggesting a requirement for LRG-47 in the survival and replication of opsonized *Brucella* in unstimulated macrophages. Opsonized *Brucella* encounter conditions similar to this in the first stages of infection of a host before an immune response has been induced. LRG-47 is required at this stage of
infection to provide *Brucella* with its appropriate trafficking compartment through which it will access its replicative late endosome-like vesicle. A low percent of non-opsonized *Brucella* were found to survive after 48 hrs of infection in a stimulated LRG47 KO cell. This data suggests 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 cell that occur during IFN-γ 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. By performing *in vivo* experiments with an LRG-47−/− mouse we were able to confirm the significant role LRG-47 has in *Brucella* pathogenesis. A significant decrease in viable *Brucella* was recovered from the spleen of LRG-47−/− mice after 9 weeks of infection confirming a requirement for LRG-47 in *Brucella* survival and replication inside the host.

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 towards 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 *B. abortus* did not differ in the magnitude or timing of splenic colonization compared to B6 mice. Considering that IFN-γ levels and antibody production increase over this time span, the stable number of
Brucella detected in vivo during the chronic phase is likely the product of mixed populations of variably opsonized bacteria together with fluctuating IFN-γ activation of host monocytes. In fact, recalculating percent survival of Brucella in LRG-47 cells (Table 2.1) by averaging the opsonized and non-opsonized values together for IFN-γ treated and non-treated equal the percent survival of Brucella recovered from similarly treated B6 macrophages.

Past research regarding LRG-47’s affect on pathogenic infection has determined a requirement for LRG-47 to reduce the replicative capacity of infectious agents such as T. gondii, Mycobacterium tuberculosis (17), and L. monocytogenes (15). B. 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 C. trachomatis and C. muridarum with C. trachomatis infection being kept at bay by LRG-47 but not C. muridarum (18). In the future it will be important to perform similar experiments using B. melitensis to determine the role of LRG-47 in this related subtype’s ability to survive and replicate inside the host. Similarly, trafficking, survival, and replication experiments with Ig-opsonized and non-opsonized B. melitensis in IFN-γ activated and non-activated cells will be important to report any differences in behavior of this closely related subtype. Though the mouse has proven to be a good model for Brucella infection, it is important to look further into the impact of LRG-47 on B. abortus infection in human monocytes in the future.

References


Figure S2.1. 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 represent means ± standard deviation of samples in triplicate, and were confirmed by separate independent experiments. T=0, 0.5 hours post infection, T=1, 24 hours post infection, T=2, 48 hours post infection.
Figure S2.2. Effect of IFN-γ treatment on *Brucella* infected Raw264.7 cells. Pretreatment of Raw264.7 cells with 50 μg/μl of IFN-γ 24 hours prior to infection with *B. abortus* induces an anti-bacterial response that increases *Brucella* killing at 24 hrs post infection (T=1), continuing through 48 hrs post infection (T=2), T=0 represents 0.5 hours post infection. Magnitude of anti-*Brucella* effect was equal among opsonized and non-opsonized *Brucella*. Similarly, bacterial survival at 24 hrs post infection, and replication detected at 48 hrs were the same between opsonized and non-opsonized bacteria infected at normalized MOI’s. Results represent the means and standard deviation from 6 replicate wells for each condition and time point. Statistical analysis was performed using non-paired Student’s *t*-test.
Figure S2.3. 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 minutes, 2 hrs, 24 hrs, and 48 hrs post infection and percent survival was calculated compared to CFU/ml 30 minutes 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 hours whereas in the non-activated cells there is a sharp increase in percent survival between 24 and 48 hours post infection.
CHAPTER 3. IDENTIFICATION AND INTRACELLULAR TRANSPORT OF AN AUTOPHAGOSOME-LIKE EXTRACELLULAR ORGANELLE

A paper submitted to *Autophagy*

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Abstract

Autophagy is a well conserved and highly orchestrated cellular mechanism that degrades unwanted cytosolic material, including damaged organelles and aggregated proteins, and recycles core molecules and substrates for future use by the cell. Starving cells of amino acids or serum increases autophagy activity as does stimulating cells with proinflammatory cytokines such as interferon-\(\gamma\). While examining autophagy specific responses to IFN-\(\gamma\) stimulation by monocytes, we observed an accumulation of discreet, extracellular structures in spent media that are significantly larger than exosomes (0.2 um avg.) averaging 2 µm in size. These repeatedly observed structures were positive for LC3, LAMP1, Rab7 and V-ATPase cellular markers frequently found within autophagosomes inside cells. Accumulation of the autophagosome-like vesicles in spent culture media was enhanced by stimulating autophagy with rapamycin, serum and amino acid starvation. In contrast, extracellular accumulation was not reduced in the presence of the pharmacologic inhibitor of autophagy, 3-methyladenine. The extracellular autophagosome-like structures were readily internalized by cells cultured separately by co-incubating with either spent tissue culture media containing these extracellular vesicles or with concentrated extracts produced by size exclusion columns and centrifugation. Cell to cell transfer and intracellular trafficking of
Macroautophagy, commonly known as autophagy, is a regular occurring process inside many cell types. Autophagy acts as the garbage processing center of the cell, where damaged proteins and organelles are broken down so their contents can be reused by the cells. Along with damaged proteins, the autophagy processes are important for degrading protein aggregates, influencing cell death, and clearing intracellular pathogens (1, 2). Research to describe the autophagy pathway in detail has been performed primarily on the yeast *Saccharomyces cerevisiae*, but the pathway is well conserved in mammalian cells (3). The generation of the intracellular double membrane bound vesicle begins with a portion of the rough endoplasmic reticulum membrane(4), called the phagophore, extending in length via assistance of the autophagy related (Atg) proteins, Atg12 and Atg5 (5). Class III phosphatidylinositol-3-phosphate (PI3P) is also a crucial phospholipid involved in the elongation and expansion of the phagophore membrane (6). The Atg12-Atg5 complex is responsible for the recruitment of the protein Atg16, and the microtubule-associated protein light chain 3 (LC3)/Atg8. Prior to its proteolytic cleavage by Atg4 protease, LC3 is found in the cytosol as pro-LC3 (7). After its proteolytic cleavage, pro-LC3 is converted to the
cytosolic form, LC3-I. LC3 is covalently linked to phosphatidylethanolamine (PE) molecules after its ubiquitination, converting it from the LC3-I glycosylated form to the active LC3-II form (8). The LC3-II bound PE is incorporated into both the exterior and interior sides of the phagophore membrane and is responsible for the curvature and eventual sealing of both ends of the phagophore membrane (5) forming the early autophagosome. The Rab GTPase (small molecular weight GTP-binding proteins) Rab5, which is present on the early autophagosome membrane, is required for trafficking and further development of the autophagosome (6).

The GTPase Rab7 is recruited to the membrane removing Rab5 and transitioning the vesicle into a late autophagosome (9, 10). Rab7 is required for trafficking of the late autophagosome to fuse with the lysosome (6), creating an autolysosome that averages about 1 μm in diameter (8). The presence of many lysosomal proteins is maintained in/on the newly formed autolysosome, such as vacuolar H⁺-ATPase (V-ATPase), cathepsins, acid hydrolases, and the lysosome associated membrane protein 1 (LAMP1)(4, 11). V-ATPases on the membrane acidify the autolysosome and the contents are subsequently degraded, followed later by the inner and outer membranes (5, 8). The contents of the autolysosome are released into the cytoplasm of the cell, where they are reused in many cellular processes.

Autophagy can be induced in mammalian cells through serum starvation, or through treatment with Rapamycin, an inhibitor of the mammalian protein mTOR (12). Autophagy can also be induced in macrophages via activating cells through treatment with Interferon-γ (IFN-γ) (13). Both IFN-γ and Rapamycin treatment of cells is shown to upregulate the ubiquitination of LC3-I to LC3-II (12, 14). This process is inhibited by treatment of the cells with 3-methyladenine (3MA) (14), and wortmannin. Both 3MA and wortmannin work through inhibiting phosphotidylinositol-3’ kinases (PI3K) that are responsible for the formation of PI3P. The final degradative step of autophagy can be inhibited by nanomolar concentrations of bafilomycin A1 which inhibits the cellular V-ATPase (15).
In RAW264.7 murine macrophages a novel extracellular organelle has been identified in these studies that contains many characteristics of the autophagy pathway. The organelle identified in these studies differs from the described autophagy process in the literature, in that, these organelles are being released by the cell and taken into neighboring cells to complete the autophagy process. We have observed these autophagosome-like extracellular organelles produced by one cell, are taken up by a neighboring cell, suggesting this organelle could provide a means for antigen transport between cells, a mechanism for a cell to export access proteins that it cannot process and degrade efficiently, or a pathway for intercellular trafficking of intracellular pathogens giving them the ability to evade the host immune system. In the current study we isolate and characterize a novel extracellular organelle and distinguish it from an autophagosome by its response to pharmacologic agents and by western blot and scanning laser confocal microscopy.

**Materials and Methods**

**Cell culture and transient transfection**

RAW264.7 murine monocytes were passaged every 3 days in DMEM with 10% fetal bovine serum (FBS) and incubated at 37°C/5% CO₂. 2.0x10⁶ per reaction of RAW264.7 cells were transiently transfected with 2 μg total LC3-GFP and/or Rab7WT-RFP (wild-type form), Rab7TN-RFP (dominant negative form), or Rab7QL-RFP (constitutively active form) plasmid constructs using Amaza Nucleofector® kit V for RAW264.7 cells. Cells were allowed to recover after nucleofection for 6 hours prior to autophagosome induction at 37°C/5% CO₂.

**Autophagosome-like vesicle generation, staining, and harvesting**

Autophagy was induced by three different treatments; serum starvation by treatment with Opti-MEM, 20 μM Rapamycin, or 200 U/ml recombinant human IFN-γ (Sigma) in Opti-MEM for 24 hours at 37°C/5% CO₂. Control cells were treated with Opti-MEM
supplemented with 10% FBS. Pharmacologic treatments were given at time of autophagy induction for each treatment group at concentrations of 10 mM or 40 mM 3-methyl adenine (3MA), 100 nM or 400 nM wortmannin, 25 nM, 50 nM, or 100 nM Bafilomycin A1 and 30 μM Cytochalasin D for 24 hours at 37°C/5% CO₂ while inducing autophagosome generation. LysoTracker® DND-99 (Invitrogen) was used at a ratio of 1:500 for 10 minutes at 4°C and then the cells were fixed for 10 minutes at -20°C with 100% acetone. When vesicles were not collected for use in a consecutive experiment, cells were centrifuged at 90 x g for 10 minutes at 25°C and fixed with either 100% acetone or 4% paraformaldehyde depending on staining requirements.

**Autophagosome-like vesicle isolation**

RAW264.7 cells were nucleofected with 1.923 μg of siRNA for LC3α/β (sc-156052 Santa Cruz Biotechnologies, Santa Cruz, CA) as per the Amaxa Nucleofector® kit V protocol and transferred to prewarmed DMEM+10% FBS at a final concentration of 5.0 x 10⁵ cells/ml. Five ml of cells were seeded into tissue culture treated T-25 flasks and incubated for 24 hours at 37°C/5% CO₂. Control flasks were seeded with RAW264.7 cells that had been nucleofected without DNA, added to pre warmed media to a final concentration of 5.0 x 10⁵ cells/ml, and 5ml of cells were seeded into tissue culture treated T-25 flasks. After 24 hours, the media was removed from the flasks and cell monolayers were washed with phosphate-buffered saline (PBS) and 5ml of fresh DMEM+10% FBS, Opti-MEM, DMEM+10% FBS+20uM Rapamycin, or DMEM+10% FBS+200U/ml IFN-γ containing pharmacologic inhibitors such as 10 mM 3MA, 100 nM Wortmannin, or 25 nM Bafilomycin A1 was added. Flasks were incubated for 24 hours at 37°C/5% CO₂. After 24 hours, supernatants were removed from flasks and centrifuged at 90 x g for 10 minutes at 4°C. Cell monolayers in each flask were washed with PBS and incubated for 10 minutes at room temperature in 2X sodium dodecyl sulfate (SDS) laemmli sample buffer. After 10 minutes, whole cell monolayer lysates were collected and frozen at -80°C. After supernatants were centrifuged,
Post-nuclear supernatants were decanted into an Amicon Ultra-15 ultracentrifugal filter of NMWL 100,000 (Millipore, Billerica, MA). Dead cell pellets remaining in the bottom of the centrifuged supernatants were lysed in RIPA lysis Buffer (150mM sodium chloride, 1.0% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate, 50mM Tris, pH 8.0) for 45 minutes at 4°C and mixed with 6X SDS laemmli sample buffer and 100X protease inhibitor cocktail (Thermo Scientific) and frozen at -80°C. Post-nuclear supernatants were centrifuged at 4,000 x g for 60 minutes at 4°C. After centrifugation, protease inhibitor cocktail was added to the concentrated supernatants and the filter run through. A TCA precipitation was performed on both the concentrated supernatants and the filter run through. A solution of 10% TCA (500g trichloroacetic acid in 227 ml of dH2O) was added to the filtrates, and incubated on ice for 45 minutes, after which time the samples were centrifuged at 10,000 x g for 45 minutes at 4°C. Supernatants were removed from the pellet, and the pellet was resuspended in 1.0 ml of 100% acetone and centrifuged at 10,000 x g for 45 minutes at 4°C. This was repeated once more, and the protein pellets were dried in a vacufuge at 30°C for 5 minutes. Protein samples were resuspended in 8M urea containing 1x protease inhibitor cocktail and A280 was measured using the nanodrop.

**Western blotting**

Protein samples collected directly into laemmli sample buffer were loaded equally by volume at 14ul of each sample per well. Whole cell lysate samples were heated for 10 minutes in 100°C heating block and cooled before loading onto a 12% TGX Tris-glycine gel (Biorad, Hercules, CA). All other protein samples were measured on the nanodrop and loaded equally by protein concentration. Samples collected in Opti-MEM media were loaded equally to each other but not equally to those samples containing FBS. Samples collected from media containing FBS were loaded comparably in mg of protein per well. All protein samples were aliquoted for equal mass of protein and mixed with 6X SDS laemmli sample buffer and heated to 100°C heating block for 2 minutes. Samples were centrifuged for 2
minutes at 13,000 rpm and loaded into a 12% TGX Tris-glycine gel. Protein gels were run at 200V for 45 minutes and transferred to a 0.2 μm nitrocellulose membrane at 100V for 1 hour. Membranes were blocked overnight at 4ºC in Tween 20 blocking Buffer (2.42 g Tris, 8.0 g sodium chloride, 1.0 g gelatin, 1.0 ml Tween-20 in 1 L d H2O, pH 7.5). Membranes were incubated in 1/200 Rabbit anti-LC3A (Sigma L8793) overnight at 4ºC. Membranes were washed and incubated in 1/1000 Alkaline Phosphatase-conjugated AffiniPure F(ab’)2 Fragment Goat Anti-Rabbit IgG (Jackson ImmunoResearch, West Grov, PA) for 1 hour at room temperature. Membranes were washed again and incubated in Fast Red (Sigma, St. Louis, MO) chromogenic substrate for 30 minutes at room temperature and air dried.

**Live cell time-lapse imaging**

For live cell imaging and membrane fusion experiments before autophagy induction, transiently transfected cells were stained with a general membrane stain PKH26 (Invitrogen, Carlsbad, CA) at 10⁻⁶ M in 1.0 ml Diluent C for 5 minutes at room temperature after which time 1.0 ml 100% FBS was added to the stain solution and incubated for 1 minute at room temperature. Cells were washed 3 times with DMEM+ 10% FBS and treated for autophagy induction as stated above. Autophagosome-like vesicles were harvested by collecting the supernatant from the induced cells and kept at 4ºC for up to 8 hours post harvest. RAW264.7 cells were seeded into a zero-glass bottom 96-well plate at a density of 2.0 x 10⁶ cells per well and incubated for 10 minutes at 37ºC/5% CO₂. Spent media was removed completely and 200 μl of harvested autophagosomes were added to the well. Three color scanning laser confocal microscopy (SLCM) was used to obtain images for separate fluorophore channels by strobe emission/collection every 10 seconds to create a movie of autophagosome uptake by RAW264.7 cells in real time.

**Autophagosome-like vesicle trafficking and uptake**

For autophagosome-like vesicle trafficking experiments, vesicles were produced and harvested as stated previously and coincubated with target cells. Target cells were seeded in
wells 10 minutes prior to coincubation with harvested autophagosome-like vesicles. At the
time of target cell seeding, cells were stained with a ratio of 1:1000 Bodipy® FL C5
(Invitrogen) in Opti-MEM for 2 hours at 37°C/5% CO₂. After incubation with stain, spent
media was removed and replaced with either Opti-MEM supplemented with 10% FBS, Opti-
MEM, Opti-MEM+20 μM Rapamycin, or Opti-MEM+200 U/ml recombinant human IFN-γ
(Sigma). Cells were coincubated with equal volumes of media containing harvested
bariphores for 24 hours at 37°C/5% CO₂. Blue fluorescent 2.0 μm FluoSpheres®
carboxylate-modified microspheres (365/415, 2% solids) (Invitrogen) were used as a control
for cellular uptake experiments. Beads were opsonized using dog gamma globulin at a ratio
of 1:500 in Opti-MEM for 30 minutes at room temperature. Opsonized and non-opsonized
beads were added to each well containing a monolayer of RAW264.7 cells at a ratio of
1:1000 and centrifuged immediately at 120 rpm for 10 minutes at 4°C. Cells were incubated
with beads for 24 hours at 37°C/5% CO₂.

**Immunofluorescence microscopy**

Rat anti-1D4B (anti-LAMP1) (Hybridoma Bank, University of Iowa, Iowa City, IA) used
at a concentration of 1/25 diluted in BSP (50 ml PBS, 50 mg saponin, 125 mg BSA) +0.5%
donkey serum for 1 hour at room temperature. Goat polyclonal IgG anti-Rab7 (Santa Cruz
Biotechnologies) was used at a ratio of 1:500. All secondary antibodies were purchased from
Jackson ImmunoResearch, conjugated with a fluorescent molecule, and used at
concentrations of 1/250 diluted in BSP and stained for 1 hour at room temperature.
Coverslips were mounted using ProLong® Gold Antifade reagent with DAPI (Invitrogen).
Three color laser scanning confocal microscopy (SLCM) was used to obtain images for
separate fluorophore channels by strobe emission/collection. Pearson’s coefficients were
calculated using Olympus Fluoview software ver. 2.1b. Post acquisition image processing
was performed with ImageJ v1.33g (http://rsb.info.nih.gov/ij/index.html).
Results

Identification of extracellular vesicles released from RAW264.7 cells

While characterizing the antimicrobial responses of monocytes, extracellular vesicles approximately 2 µm were frequently seen in cell culture supernatants and extracellular space. Initially, the origin of the vesicles was attributed to cell disruption during cell culture manipulation. Upon closer examination of monocytes cultured in media with variable amounts of nutrients, it appeared that cultures grown with nutrient poor media produced more extracellular vesicles than cells grown in rich media supplemented with FBS. Given that serum starvation is a potent inducer of autophagy (12), we explored the potential link between the autophagosomal pathway and the formation of extracellular vesicles.

RAW264.7 cells were transiently transfected with an LC3-GFP construct to visualize autophagosome formation. Transformed cells were sub-cultured in different Opti-MEM based media to provoke amino acid starvation, Opti-MEM supplemented with 10% FBS (A.A. starve only), Opti-MEM only (A.A. starve + serum starve), Opti-MEM+Rapamycin (A.A. starve induction of autophagy), or Opti-MEM+IFN-γ (A.A. starve with pro-inflammatory cytokine) for 24 hours. Acidic lysosomes were labeled by incubating cultures with LysoTracker® DND-99 (Invitrogen) prior to fixation. The cells were centrifuged before fixing to collect all secreted vesicles onto the monolayer for 10 minutes at 90 x g, 4°C. Cells were stained with rat anti-LAMP1 antibody, mounted and imaged using laser scanning confocal microscopy (SLCM). Extracellular LC3-GFP positive vesicles were seen in all treatment groups. The extracellular LC3 positive vesicles were found to be acidic, and all vesicles co-localized with LAMP1 stain (average Pearson’s coefficient of 0.418 ±0.228 and 0.724 ± 0.146, respectively). Extracellular LC3 positive vesicles were also observed to be internalized by non-LC3-GFP expressing cells in all treatment groups (Figure 3.1a). The extracellular and internalized LC3 positive vesicles were found to be an average of 2.0 µm in diameter, and more than one internalized LC3 positive vesicle per cell in all treatment groups.
was never observed (Figure 3.1a). Consistent observations were noted over 5 separate experiments. This same set of test conditions was duplicated, and cells were stained with goat anti-Rab7 antibody. The Rab7 staining was found to be co-localized with both extracellular and internalized vesicles. Two replicates of this set of experiments were performed, and the same results were observed in each replicate.
Figure 3.1. RAW264.7 cells transiently transfected with LC3-GFP and/or Rab7WT-RFP constructs. Cells grown in Opti-MEM+ 10% FBS, Opti-MEM only, Opti-MEM+20 µM rapamycin, or Opti-MEM+1/1000 IFN-γ to induce autophagy for 24 hours at 37°C/5% CO₂. Cells were fixed with acetone and stained using anti-LAMP1 antibodies. A.) Prior to fixing, cells were stained with LysoTracker DND-99, fixed, and stained with anti-LAMP1 antibodies. Autophagosome-like extracellular organelles (Bariphore) seen extracellularly in all treatment groups after 24 hours. It was observed, in all treatment groups, that the LC3-GFP expressing bariphores were secreted and taken up by neighboring cells. These LC3-GFP expressing bariphores were colocalized with both LAMP1 and LysoTracker stains. Arrows indicate LC3+ extracellular autophagosome-like vesicles colocalized with LysoTracker DND-99 and LAMP1 that have been internalized by a non-LC3-GFP expressing cell. B.) Cells co-transfected with Rab7WT-RFP and LC3-GFP produced GFP fluorescing bariphores that were also RFP labeled. These LC3-GFP-Rab7WT-RFP labeled bariphores colocalized with LAMP1 staining and were secreted as well as taken up by neighboring cells. Arrows indicate an LC3+ autophagosome-like extracellular organelle colocalized with Rab7 and LAMP1 found extracellularly.

RAW264.7 cells were transiently co-transfected with LC3-GFP and Rab7WT-RFP constructs and treated for autophagy induction as stated earlier. Cells were stained with anti-LAMP1. Extracellular LC3-GFP positive vesicles were also expressing the Rab7WT-RFP construct. These dually expressing extracellular vesicles also co-localized with the LAMP1 stain in all treatment groups. These experiments were performed a minimum of three times, each revealing the same results (Figure 3.1b).
**LC3 positive autophagosome-like extracellular vesicles can be internalized by neighboring cells via actin-independent membrane fusion**

RAW264.7 cells transfected with the LC3-GFP construct and stained with PKH26 general membrane stain (Invitrogen) were treated with rapamycin to induce autophagy. Fluospheres® 2 μm in diameter, were added to supernatants containing LC3-GFP positive extracellular vesicles that were harvested and chilled to 4°C. Supernatants were co-incubated with pre-chilled RAW264.7 cells that were pretreated with cytochalasin D; one set of control cells was not treated with the inhibitor. Cells were fixed after 30 minutes of incubation at 4°C. A control set of cells with the same treatment was incubated at 37°C for 30 minutes and fixed. Using scanning laser confocal microscopy (SLCM) we observed that LC3+ autophagosome-like extracellular vesicles were internalized in all treatment groups both at 4°C and 37°C. Fluospheres® in the same samples were not internalized by either the treated or the untreated cells at both test temperatures. Puncta of the PKH26 membrane stain were observed on the plasma membrane of target cells that internalized an LC3+ autophagosome-like extracellular vesicle, while the LC3-GFP fluorescing vesicle was still membrane bound inside the target cell (Figure 3.2a).

LC3 positive extracellular vesicles were generated in the same way as mentioned above, and their uptake by live cells was captured by video. Target cells for video were untreated and unstained RAW264.7 cells. In 10 minutes of footage, we observed a PKH26 stained LC3-GFP expressing extracellular vesicle fuse with the target cell plasma membrane and was slowly internalized by the target cell. After internalization was complete, the PKH26 stain of the extracellular vesicle membrane was integrated into the plasma membrane of the target cell, and the contents of the LC3-GFP extracellular vesicle were observed to be membrane bound in the cytoplasm of the target cell (Figure 3.2b).
A.)

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<th>Condition</th>
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<th>LC3-GFP (green)</th>
<th>PKH26 (red)</th>
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B.)

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Figure 3.2. Autophagosome-like vesicles taken up by actin-independent membrane fusion. RAW264.7 cells stained with PKH26 were transiently transfected with and LC3-GFP construct and grown in either Opti-MEM+ 10% FBS, Opti-MEM only, Opti-MEM+20 μM rapamycin, or Opti-MEM+1/1000 IFN-γ to induce autophagy for 24 hours at 37°C/5% CO₂. Supernatants were harvested. A.) Harvested supernatants were cooled to 4°C, combined with blue Fluospheres®, and co-incubated with a monolayer of RAW264.7 cells treated with cytochalasin D. Cells were incubated for 30 minutes at 4°C and 37°C, fixed with 4% paraformaldehyde, and visualized using scanning laser confocal microscopy. In all temperature and treatment groups, Fluospheres® were not taken in by the macrophages. In all treatment conditions, LC3-GFP and PKH26 fluorescing bariphores were taken up by neighboring cells. Arrows indicate polystyrene beads, or LC3+, PKH26 stained autophagosome-like vesicles that have been internalized by the target cell after 30 minutes incubation. B.) Harvested bariphores were coin incubated with a RAW264.7 cell monolayer and a video was captured for 45 minutes. The first frame of the video (t=0) shows the PKH26 stained bariphore that is also LC3-GFP expressing sitting on the surface of the cell. After 35 minutes (t=35), it is evident that the PKH26 stained membrane of the bariphore has incorporated into the membrane of the RAW264.7 cell, and the LC3-GFP is membrane bound and was delivered to the inside of the cell. Arrows indicate PKH26 lipid stain incorporating into the membrane of the cell that took up the LC3+ vesicle, while the LC3-GFP expressing contents are internalized.

**Release of LC3 positive autophagosome-like extracellular vesicles not inhibited by known autophagy inhibitors**

RAW264.7 cells expressing LC3-GFP were treated for autophagy induction either by: Opti-MEM supplemented with 10% FBS, Opti-MEM only, Opti-MEM+Rapamycin, or Opti-MEM+IFN-γ. At the same time as induction began, the cells were all treated with 10 mM
3MA and incubated for 24 hours. Cells were stained with LysoTracker DND-99, fixed, and stained with anti-LAMP1. The 3MA treatment of the cells inhibited LAMP1 and LC3-GFP co-localization within the cells in all treatment groups, as expected (average Pearson’s coefficient of 0.596 ± 0.117). Although LAMP1 was found to loosely co-localize with LC3 inside cells, LC3+ autophagosome-like extracellular vesicle production, acidification, secretion and internalization by target cells was not inhibited by treatment with 3MA in all test conditions (Figure 3.3). This set of experiments was repeated treating cells with 40 mM 3MA, and no change in acidification, production, secretion, or internalization of LC3+ autophagosome-like extracellular vesicles was observed.

A similar set of experiments was performed with LC3-GFP expressing RAW264.7 cells induced for autophagy and treated with 100 nM wortmannin. Cells were stained with LysoTracker® DND-99, fixed, and stained for LAMP1 and observed using scanning laser confocal microscopy. We observed that the wortmannin treatment inhibited the co-localization of LAMP1 and LC3 inside the cells (average Pearson’s coefficient of 0.558 ± 0.108). Although autophagy was inhibited in the cells, the wortmannin treatment did not inhibit production or release of LC3+ autophagosome-like extracellular vesicles. The extracellular LC3 positive vesicles co-localized with LAMP1 and the LysoTracker® stain. This set of experiments was repeated with a higher concentration of wortmannin (400 nM) and the same results were observed.

RAW264.7 cells expressing the LC3-GFP construct were grown in Opti-MEM supplemented with 10% FBS, Opti-MEM only, Opti-MEM+ Rapamycin, or Opti-MEM+IFN-γ and all cells were treated with 30 μM cytochalasin D for 24 hours. Control cells were grown in the four types of growth conditions but were not subject to treatment with cytochalasin D. LC3-GFP was observed in all treatment groups to be haphazardly distributed in the cells in a very diffuse pattern compared to the control cells. This effect was most pronounced in those cells treated with IFN-γ. Despite the inhibition of actin
polymerization in the cells, LC3+ autophagosome-like extracellular vesicles were observed to be excreted and internalized by cells in all treatment conditions just as was observed in the control cells (data not shown).
Figure 3.3. 3-methyladenine does not inhibit autophagosome-like vesicle production or secretion. RAW264.7 cells were transiently transfected with LC3-GFP and grown in either Opti-MEM+10% FBS, Opti-MEM only, Opti-MEM+20 μM rapamycin, or Opti-MEM+1/1000 IFN-γ to induce autophagy for 24 hours at 37°C/5% CO₂. At time of autophagy induction, cells were treated with 10 mM (not shown) and 40 mM 3MA- a known autophagy inhibitor. Before fixing, cells were stained with LysoTracker DND-99, fixed with acetone, and stained with anti-LAMP1 antibodies and visualized using scanning laser confocal microscopy. It is apparent that 3MA inhibits autophagy inside the cells but fails to inhibit the secretion and uptake of LC3-GFP expressing bariphores in all treatment groups. Similar experiments were performed using 100 nM and 400 nM wortamannin. Arrows indicate LC3+ autophagosome-like vesicles colocalized with LAMP1 and LysoTracker DND-99 internalized by non-LC3-GFP expressing cells.

**Inhibiting V-ATPases with bafilomycin A₁ does not inhibit acidification of LC3 positive autophagosome-like extracellular vesicles**

RAW264.7 cells expressing LC3-GFP were treated with rapamycin, IFN-γ, serum starvation, or serum supplemented Opti-MEM to induce autophagy. Cells in all growth media were treated with 25, 50 or 100 nM bafilomycin A₁ and incubated for 24 hours. Cells were stained with LysoTracker® DND-99, fixed, and stained with anti-LAMP1. Bafilomycin A₁ treatment at 50 and 100 nM concentrations successfully inhibited the acidification of lysosomes inside cells in all treatment groups. However, all concentrations of bafilomycin A₁ used were unsuccessful in inhibiting the acidification of extracellular and internalized LC3 positive vesicles (Figure 3.4). The LC3-GFP expressed in the extracellular as well as the internalized autophagosome-like vesicles was co-localized with LAMP1 and LysoTracker with an average Pearson’s coefficient of 0.700 ± 0.184 and 0.774 ± 0.092, respectively.
Figure 3.4. Bafilomycin A₁ does not inhibit acidification of autophagosome-like extracellular vesicles. RAW264.7 cells were transiently transfected with a LC3-GFP construct, and grown in Opti-MEM+10% FBS, Opti-MEM only, Opti-MEM+20 μM rapamycin, or Opti-MEM+1/1000 IFN-γ to induce autophagy for 24 hours at 37°C/5% CO₂. At time of autophagy induction, cells were treated with 25, 50, or 100nM bafilomycin A₁. Before fixing, cells were stained with LysoTracker DND-99, fixed with acetone, and stained with anti-Vacuolar-ATPase antibody and visualized using SLCM. At all concentrations, bafilomycin A₁ inhibited acidification of the RAW264.7 cells but failed to inhibit acidification of the
LC3-GFP expressing bariphores occurring both extracellularly and internalized by neighboring cells. In all treatment groups, LysoTracker, LC3-GFP, and V-ATPase stains were colocalized both on internalized and extracellular bariphores. Arrows indicate extracellular LC3+ autophagosome-like vesicles colocalized with V-ATPase and LysoTracker DND-99.

**Secretion of LC3 positive autophagosome-like vesicles by RAW264.7 cells is Rab7 dependent**

RAW264.7 cells co-transfected with LC3-GFP and Rab7DN-RFP constructs were grown in different growth media to stimulate autophagy: Opti-MEM supplemented with 10% FBS, Opti-MEM only, Opti-MEM+Rapamycin, or Opti-MEM+IFN-γ. Cells were fixed and stained with anti-LAMP1. Cells expressing Rab7TN-RFP that had been grown in Opti-MEM+IFN-γ were found to contain large vesicles with LC3 and LAMP1 on their periphery (data not shown). In all other treatment groups, Rab7TN-RFP expressing cells contained smaller LC3 positive vesicles that did not co-localize with LAMP1 (average Pearson’s coefficient of 0.404 ± 0.102). Secretion of LC3+ autophagosome-like extracellular vesicles was inhibited with the expression of the Rab7TN-RFP construct in all treatment groups.

**LC3 positive autophagosome-like vesicles colocalized with LC3 of target cell**

RAW264.7 cells nucleofected with an LC3-GFP construct as noted above were combined with RAW264.7 cells that had been nucleofected with an LC3-RFP construct in the same way. Cells were grown in different growth media to stimulate autophagy: Opti-MEM supplemented with 10% FBS, Opti-MEM only, Opti-MEM+Rapamycin, or Opti-MEM+IFN-γ. After 24 hours, cells were fixed, mounted, and analyzed using SLCM. LC3-GFP+ autophagosome-like extracellular vesicles taken up by non-GFP expressing cells were found to colocalize with the LC3-RFP+ autophagosome-like vesicles found within the LC3-RFP expressing RAW264.7 cells. The same phenomenon was found with LC3-RFP+
autophagosome-like extracellular organelles taken up into LC3-GFP expressing cells. The average Pearson’s coefficient for colocalization of the LC3 of the autophagosome-like extracellular organelle and the LC3 of the target cell was found to be 0.443 ± 0.282. RAW cells expressing the LC3-GFP construct were co-incubated with opsonized and non-opsonized 2.0 μm and 0.2 μm Fluospheres® (Invitrogen) for 24 hours while inducing autophagy via treatment of cells with either: Opti-MEM supplemented with 10% FBS, Opti-MEM only, Opti-MEM+Rapamycin, or Opti-MEM+IFN-γ. Non-opsonized spheres of both sizes were not found to be internalized by autophagosome-like vesicles inside the cells of all treatment groups. These non-opsonized spheres were only found extracellularly inside apoptotic blebs or floating unpackaged in the extracellular fluid. When internalized, these spheres were not packaged inside the cell but were left unaffected. Like the non-opsonized spheres, the opsonized spheres were not found to be packaged inside autophagosome-like extracellular vesicles. When taken into a target cell, the opsonized spheres were colocalized with the lysosomal marker LAMP1 (data not shown). Opsonized spheres were frequently observed in small apoptotic blebs that did not contain LC3 but were acidic and colocalized with LAMP1 (data not shown).

**Lipidated LC3-II on autophagosome-like extracellular vesicles isolated from supernatant of RAW264.7 cells**

To determine the LC3-II content of extracellular vesicles, supernatants were removed from RAW264.7 cells grown in media to induce autophagy and were treated with autophagy inhibitors. Autophagosome-like extracellular organelles were isolated from the supernatant by running the supernatant over a centrifugal size exclusion column allowing particles under 100,000 kDa to pass through, retaining the vesicles of interest. A TCA precipitation was performed on the concentrated sample off the filter. Samples were equally loaded among like types (see methods) into a 12% SDS-PAGE gel, and a western blot was performed on the proteins using an anti-LC3 antibody (Figure 3.5). Lipidated LC3-II was found in the
supernatants of most treatment groups. A large amount of LC3-II was seen in the supernatants from RAW264.7 cells treated with autophagy inhibitors. The most notable increase in lipidated LC3-II seen in the supernatants was from the cells treated with bafilomycin A1. These supernatants also contained pro-LC3 which was not seen in any of the other treatment groups (Figure 3.5). The cytoplasmic form, LC3-I was seen in the control supernatants taken from RAW264.7 cells grown in DMEM+10% FBS+Rapamycin and DMEM+10% FBS+IFN-γ as well as in the supernatants taken from cells grown in DMEM+10% FBS and Opti-MEM (Figure 3.5).

**Autophagosome-like extracellular vesicle production inhibited by siRNA for LC3α/β**

RAW264.7 cells were nucleofected with siRNA for LC3α/β and incubated for 24 hours, after which time they were given fresh media to induce autophagy, either DMEM+10% FBS, Opti-MEM, or DMEM+10% FBS+IFN-γ. Cells were also treated with bafilomycin A1. Non-transfected cells were used as controls as well as non-treated cells. Supernatants were harvested and processed as explained above and a TCA precipitation was performed on the concentrated vesicles. Proteins were analyzed by western blot using an anti-LC3 antibody. Transfection with siRNA specific for LC3 α/β was able to greatly decrease the release of autophagosome-like extracellular vesicles as indicated by a decrease in lipidated LC3-II in the supernatants from the siRNA treated RAW264.7 cells in comparison with the supernatants from the control cells (Figure 3.6). Whole cell lysates are shown to confirm accuracy as well as success of the siRNA.
Figure 3.5. Lipidated LC3 present in supernatants collected from RAW264.7 cells induced for autophagy. Supernatants were isolated from RAW264.7 cells grown in DMEM+10%FBS, Opti-MEM, DMEM+10%FBS+rapamycin, or DMEM+10%FBS+IFN-γ and treated with either 10 mM 3MA, 100 nM wortmannin, or 25 nM bafilomycin A₁ for 24 hours. Supernatants were run over a centrifugal size exclusion column isolating any proteins or vesicles larger than 100,000 kDa. A TCA precipitation was performed on these isolated proteins. Proteins were equally loaded into a 12% Tris-glycine gel (Biorad) with all proteins isolated from samples containing FBS loaded at 47.6 mg/well, and all Opti-MEM samples loaded at 4.2 mg/well. Whole cell lysates were loaded at an equal volume of 14 μl/well. Samples were transferred to a 0.2 μm nitrocellulose membrane and labeled with an anti-LC3A antibody (Sigma). Arrows indicate pro-LC3 (~30kDa), cytosolic LC3-I (18kDa), and lipidated LC3-II (15kDa).
Figure 3.6. LC3 required for release of autophagosome-like vesicles from RAW264.7 cells. Supernatants were isolated from RAW264.7 cells nucleofected with siRNA for LC3α/β (Santa Cruz Biotechnologies) and grown in either DMEM+10%FBS, Opti-MEM, or DMEM+10%FBS+IFN-γ. Cells were treated with 25nM bafilomycin A₁ for 24 hours. Control cells were nucleofected without siRNA and were untreated. Supernatants were run over a centrifugal size exclusion column isolating any proteins or vesicles larger than 100,000 kDa. A TCA precipitation was performed on the isolated proteins. Proteins were equally loaded into a 12% Tris-glycine gel (Biorad) with all proteins isolated from samples containing FBS were loaded at 44.52 mg/well, and all Opti-MEM samples loaded at 1.92 mg/well. Whole cell lysates were loaded at an equal volume of 14 μl/well. Samples were transferred to a 0.2 μm nitrocellulose membrane and labeled with an anti-LC3A antibody (Sigma). Arrows indicate pro-LC3 (~30kDa), cytosolic LC3-I (18kDa), and lipidated LC3-II (15kDa). 1=DMEM+10%FBS, 2= Opti-MEM, 3=DMEM+10%FBS+1/1000 IFN-γ.

Discussion

The LC3 positive autophagosome-like extracellular organelles are naturally produced by RAW264.7 cells during normal growth conditions. The production and secretion of these extracellular organelles is increased during serum starvation and with rapamycin or
interferon-γ treatment. It is unclear whether treatment with rapamycin, serum starvation, or IFN-γ increases the frequency of ingestion by neighboring cells or if the relative frequency of ingestion of LC3+ autophagosome-like extracellular organelles is simply a result of increased secretion causing an increase in the occurrence of a fusion events between a target cell and an autophagosome-like vesicle. Treatment of RAW264.7 cells with wortmannin did not inhibit secretion or production of LC3+ autophagosome-like extracellular organelles, despite the inhibition of autophagy. Likewise, treatment of these cells with high concentrations of 3-methyladenine did not inhibit the production or secretion of LC3+ autophagosome-like extracellular organelles in any of the treatment conditions tested, despite the fact that the 3MA treatment successfully inhibited autophagosome maturation in these cells. Secretion, production and, most importantly, uptake, are not deterred by inhibiting the actions of actin inside the cell with cytochalasin D treatment. It is clear that LC3+ autophagosome-like extracellular organelles are taken into neighboring cells (target cells) by membrane fusion in less than 30 minutes, as was clearly shown by our live cell LC3+ autophagosome-like extracellular vesicle uptake video where the vesicle membrane was fused with the target cell membrane releasing the vesicular contents into the cytoplasm of the accepting cell.

LC3+ autophagosome-like extracellular organelles contain many autophagy markers such as LC3, LAMP1, V-ATPase, and Rab7. They are differentiated from autophagosomes, however, by their size and the inability of nanomolar concentrations of bafilomycin A1 to inhibit acidification both extracellularly and inside a non-acidic target cell.

Although it is probable that this novel extracellular organelle is a product of the autophagy pathway, the data does not confirm it. To avoid further confusion, we will from this point forward refer to the LC3+ autophagosome-like extracellular organelle as a “Bariphore”.

The release of bariphores is dependent on the GTPase Rab7 as the secretion of these vesicles is inhibited by transfection of cells with the Rab7TN construct. It is clear from the
results of the western blot of supernatants collected from cells transfected with siRNA for LC3, that LC3 is also essential for release of the bariphore into the extracellular environment. As we can see by the blot, little to no bariphores are seen extracellularly in the supernatants collected from siRNA transfected cells (Figure 3.6).

From the information that has been gathered thus far, it is proposed that this organelle may be secreted from the cell at the time of late autophagosome fusion with the lysosome but before the degradative process begins. It is unclear what proton pump these extracellular organelles have on their surface that allows them to maintain acidity in the extracellular environment or inside a non-acidic target cell, as well as the origin of the acidifying agent. We are confident that these extracellular organelles are not exosomes or multivesicular bodies. It was shown by Fader et al. that multivesicular bodies are directed to the autophagic pathway and exosome secretion is inhibited under conditions that stimulate autophagy (16). The average size of an exosome is about 100-300 nm (17), which is also relatively small compared to our bariphores which are an average of 2 μm in diameter. Although it was found that bafilomycin A1 treatment of the mouse epithelial cell line AtT-20 generated mixed organelles that shared many markers with autophagosomes, it was never shown that these mixed organelles were secreted from the cells. We are confident that the extracellular organelle that we have identified is not the same as the mixed organelle identified by Sobota et al. (15) because our bariphores are appearing in all treatment groups including those that are serum starved or treated with rapamycin or IFN-γ. Most importantly, bariphore secretion and internalization occurs in control cells grown in Opti-MEM supplemented with serum.

At this time it is unknown whether there are other cell types that are able to produce and secrete these extracellular organelles. Bariphore production, secretion, and uptake by neighboring cells may be critical in long term antigen persistence in the host. The production and secretion of bariphores by macrophages, in general, may play an important role in antigen transport to neighboring cells providing a more efficient and rapid display of
antigen by cells that have never encountered the pathogen. By this mechanism, the bariphore is allowing the first cell that phagocytoses the pathogen to export pathogen antigens for MHC class II loading to a variety of different cells throughout the body in a very rapid manner.

The bariphore may also function as a way for cells to export proteins that they may not have the ability or the capacity to efficiently dispose of. This hypothesis comes from the observation of the pro-LC3 found in bariphores isolated from the supernatants of the bafilomycin A1 treated cells. Treating the cells with bafilomycin A1 inhibits degradation of unwanted proteins in the cell which allows for these proteins to build up. The RAW264.7 cells may be using the bariphores as a way to export unwanted proteins, which they may not need at the time. Other cells may take up this exported waste if they are in need of amino acids or other resources which may be found within these exported bariphores. This may explain why the bariphores are trafficked into the autophagy pathway of the target cell once they are taken up.

Though opsonized and non opsonized beads of two different sizes were not found to be inside bariphores in any of the treatment groups, it is possible that intracellular pathogens such as Mycobacterium tuberculosis, Legionella pneumophila, Coxiella brunetii, Francisella tularensis, or Brucella spp. may be able to manipulate the bariphore formation pathway enabling them to be packaged into a bariphore. It is also a possibility that bariphore trafficking between different host cell types may be used by a variety of intracellular pathogens to infect large numbers of host cells while evading a response by the host immune system. This would provide the pathogen the ability to persist inside the host where the bariphore could protect them from antibiotics, complement proteins, or antibodies.

Conclusion

Autophagy is a naturally occurring process inside all host cells as a way to degrade unwanted proteins and organelles, as well as pathogens. The formation of the phagosome
membrane is dependent upon many autophagy related proteins (Atg) as well as PI3P and Rab GTPases (5). Autophagy can be induced by serum starvation as well as treatment with rapamycin (12) and IFN-γ (13). There are a number of pharmacologic agents known to inhibit autophagy such as 3-methyladamine, wortmannin, and bafilomycin A1. Bariphores were found to be excreted by RAW264.7 when grown in conditions to induce autophagy. These acidic extracellular vesicles carry a number of autophagosomal markers such as Rab7, LC3, LAMP1, and V-ATPase and their release from the cell is LC3 and Rab7 dependent. The release of bariphores from RAW264.7 cells cannot be inhibited treatment with 3MA, wortmannin, or bafilomycin A1. The uptake of these vesicles is not actin dependent and occurs rapidly through a membrane fusion event. Once taken into a target cell, the bariphore is trafficked with the LC3+ bariphores inside the cell that has taken it up as if it was part of that cell. This naturally occurring process may serve as antigen delivery vesicles for quick and efficient antigen presentation by cells that has never encountered the pathogen as well as providing a means of antigen preservation within a host. Extracellular release of bariphores may also be used by the cell as a way to dispose of unwanted proteins and waste, while other cells may take up bariphores as a way to acquire much needed amino acids and nutrients. Bariphore trafficking between host cell types may also serve as a means for intracellular pathogens to infect large numbers of host cells while evading the host immune system.

References

CHAPTER 4. PROTECTED CELL TO CELL SPREAD OF *BRUCELLA ABORTUS*

A paper to be submitted

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**Abstract**

*Brucella* species are facultative intracellular pathogens that are able to cause severe disease in animals and humans. *Brucella* are internalized and trafficked differently inside the macrophage based on whether the bacterium was opsonized or non-opsonized with serum immunoglobulin. Once internalized, *Brucella* traffic through the early and late endosome where opsonized *Brucella* will replicate. Non-opsonized *Brucella* replicate in a vesicle derived from the endoplasmic reticulum. Autophagy is a process in the cell whereby damaged proteins and organelles are broken down so their contents can be reused. Recently, an autophagosome like extracellular organelle has been identified that is released by RAW264.7 murine macrophages in normal growth conditions and in conditions to induce autophagy. We have observed *Brucella abortus* using the natural intercellular trafficking pathway of the autophagosome-like extracellular organelle to infect a new host cell while evading the host immune system.
Introduction

*Brucella* is a gram negative, non-spore forming, coccobacillus capable of living in microaerophilic environments (1). The species *Brucella* contains many sub-species which include *Brucella abortus* (2), *Brucella melitensis* (2), *Brucella suis*, *Brucella ovis* (3), *Brucella canis* (4), *Brucella neotomae* (5), *Brucella ceti* (6, 7), *Brucella pinnipedialis* (6, 7), and the latest addition, *Brucella microti* (1). Each of these species is host specific with *B. abortus* infecting cattle, *B. canis* infecting dogs, and *B. suis* infecting swine (8). *Brucella abortus* is the primary cause of brucellosis in cattle, bison, and elk all over the world (1). Brucellosis primarily causes abortions in pregnant cattle (9) or the birth of weak cows with a lower milk output (1). In humans, *B. abortus* infections result in undulant fever (10) and endocarditis (11).

*Brucella* is a facultative intracellular pathogen found primarily in the macrophage of its host (10). *Brucella* is internalized by a different mechanism depending on whether the surface of the bacterium is opsonized by serum antibodies against *Brucella* LPS or non-opsonized. Opsonized *Brucella* are internalized by Fc receptors on the surface of the macrophage, which bind the constant region of the antibody bound to the surface of the bacterium (12). Non-opsonized *Brucella* are bound by the long O-chain of their LPS to lipid rafts found occasionally on the surface of the macrophage (13). Within 10 minutes, the internalized *Brucella*, whether opsonized or non-opsonized, is found within a compartment that shares many markers of an early endosome (14) such as the early endosomal antigen 1 (EEA1), the small GTPase Rab5 and the transferrin receptor (12). Along with these early endosome markers, this *B. abortus*-containing compartment is also highly acidic, which is thought to be due to the large amount of vacuolar H⁺-ATPase this compartment carries on its surface (15). Once a high acidity is reach in the *Brucella*-containing compartment, the early endosomal markers are lost from the vesicle and late endosomal markers LAMP1 and
cathepsin D (16) as is the small GTPase Rab7 and its effector Rab-interacting lysosomal protein (RILP) (17). At this stage, 60% of the bacteria are degraded (18). One hour post infection, the Brucella-containing compartment interacts with the endoplasmic reticulum subdomains of the early secretory pathway called ER exit sites at which point LAMP1 is lost from the vacuole surface (19). The pH of the Brucella-containing compartment increases to become more permissive to Brucella replication as a pH above 5.5-6 is required for this to occur (20). Between 10-12 hours post infection, B. abortus finally begin to replicate in their endoplasmic reticulum-like replicative niche (21). The Brucella will replicate in this cell until space is no longer available, or nutrients become depleted, at which time they will lyse the cell and infect a new cell by an unknown mechanism (20).

Interferon gamma is essential for resistance to facultative intracellular bacterial pathogens (22). It has been shown to be critical for the control of B. abortus infection in vivo and in vitro (1).

Macroautophagy, more commonly known as autophagy, is a regular occurring process inside many cell types where damaged proteins and organelles are broken down so their contents can be reused by the cells. Along with damaged proteins, the autophagy processes are important for degrading protein aggregates, influencing cell death (23), and clearing intracellular pathogens (24, 25). The autophagosome membrane begins as the phagophore and is believed to bleb off from the golgi apparatus (26), the outer nuclear membrane (27), the outer mitochondrial membrane (28), or from the endoplasmic reticulum (29). The phagophore membrane extends in length via assistance of the autophagy related (Atg) proteins, Atg12, which is ubiquitin-like, and Atg5 (30). Atg12 and Atg5 are attached to each other by complexing with Atg 10 and Atg 7 (31). The Atg12-Atg5 complex is responsible for the recruitment of the protein Atg16, and the microtubule-associated protein light chain 3 (LC3)/Atg8 (32). LC3 is covalently linked to phosphotidylethanolamine (PE) molecules after
its ubiquitination from the LC3-I glycosylated form to the active LC3-II form (33). The LC3-II bound PE is incorporated into both the exterior and interior sides of the phagophore membrane and is responsible for the curvature and eventual sealing of both ends of the phagophore membrane (30) forming the early autophagosome. The Rab GTPase (small molecular weight GTP-binding proteins) Rab5, which is present on the early autophagosome membrane, is required for trafficking and further development of the autophagosome (34). The GTPase Rab7 is recruited to the membrane, removing Rab5, transitioning the vesicle into a late autophagosome (35, 36). Rab7 is required for trafficking of the late autophagosome to fuse with the lysosome (34), creating an autolysosome that averages about 1 μm in diameter (33). The presence of many lysosomal proteins is maintained in/on the newly formed autolysosome, such as vacuolar H⁺-ATPase (V-ATPase), cathepsins, acid hydrolases, and the lysosome associated membrane protein 1 (LAMP1) (37, 38). V-ATPases on the membrane acidify the autolysosome and the contents are subsequently degraded, followed later by the inner and outer membranes (30, 33). The contents of the autolysosome are released into the cytoplasm of the cell, where they are reused in many cellular processes.

Recently an autophagosome like extracellular organelle has been found that is released by the murine macrophage cell line, RAW264.7 in normal growth conditions and under conditions that induce autophagy (Ritchie et al., unpublished data). This extracellular organelle, termed a ‘bariphore’, shares many characteristics of an autophagosome such as colocalization with LAMP1, the GTPase Rab7, V-H⁺-ATPase, as well as colocalizing with the acidotropic stain LysoTracker® (Ritchie et al., unpublished data). These bariphores are taken up into neighboring cells and trafficked into the target cell’s preexisting autophagy pathway (Ritchie et al., unpublished data). Previously we showed that LC3 is required for the release of the bariphores into the extracellular milieu (Ritchie et al., unpublished data). Though the bariphore shares many characteristics with the autophagosomal pathway, its
production and release is not inhibited by 3-methyladenine, wortmannin, cytochalasin D, or bafilomycin A1, all common inhibitors of autophagy (Ritchie et al., unpublished data).

We hypothesize that *B. abortus* within bariphores would be able to infect new cells without exposing the bacteria to antimicrobial molecules present in the serum or standard, soluble administered antibiotics. Evidence for bariphore mediated transmission of *B. abortus* would demonstrate an effective means for persistent infection to be maintained despite antimicrobial intervention and rapid development of anti-*Brucella* immunoglobulin. Using fluorescence confocal microscopy, live *B. abortus* were readily observed within extracellular bariphores. Transfer of culture supernatants containing *B. abortus* laden bariphores to uninfected monocyte monolayers efficiently transmitted the disease to the new monolayer. Infection of the monolayer was not reduced or inhibited by the presence of bactericidal concentrations of soluble gentimicin. We propose that bariphore mediated transmission of *Brucella* is a significant contributor to *Brucella* persistence.

**Materials and Methods**

**Bacteria culture**

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 in prior studies (16) by introducing the plasmid pBBR1MCS6-Y encoding GFP expression downstream of the constitutively active promoter for *aph3A*-derived from pBlueKS+Kan (Stratagene) (39). GFP positive *Brucella* were maintained in culture using a concentration of 6 µg/ml chloramphenicol. All chemicals were obtained from Sigma-Aldrich unless otherwise stated.
**Cell culture and transient transfection**

The murine monocytic cell line RAW264.7 (ATCC # TIB-71) murine monocytes were passaged every 3 days in DMEM with 10% fetal bovine serum (FBS) and incubated at 37°C under 5% CO₂. RAW264.7 cells from a fresh split were seeded into 24 well plates, 0.5 ml/well, each well contained an acid washed circular coverslip. After 24 hours, media was removed from all cells, and cells were washed gently with PBS and given fresh prewarmed media with or without 200 U/ml recombinant interferon gamma (IFN-γ). Cells were transiently transfected by packaging 1 μg of an LC3-RFP construct in lipofectamine 2000 (Invitrogen) as per the product protocol. Cells were returned to incubator for 24 hours at 37°C/5% CO₂.

**Intracellular viability of *B. abortus***

Cell culture monolayers were infected with IgG opsonized *B. abortus* 2308 harboring the GFP encoding plasmid pBBR1MCS6Y as described (16). Supernatants were collected from cells infected as explained above that had incubated for 48 hours with bacteria. Supernatants from identical experimental groups were combined and used to infect monolayers of RAW264.7 cells that had been seeded into a 24 well plate with coverslips at 5.0 x10⁵/ml 24 hours previously in DMEM+10% FBS. After addition of the supernatants to each well, 500 μl/well, plates were centrifuged at 90 x g at 4°C for 10 minutes to bring vesicles from the supernatant down to the RAW264.7 cell monolayer. Cells were co-incubated with supernatants for 2 hours at 37°C / 5% CO₂. After 2 hours, media was washed off cells with PBS, and cells were given fresh DMEM+10% FBS+10 μg/ml gentamicin and returned to the incubator at 37°C / 5% CO₂. Coverslips were fixed 72 hours post infection with the bacteria laden supernatants and stained for examination by microscopy.
**Immunofluorescence microscopy**

Coverslips harboring the adherent and infected macrophages were fixed with freshly prepared 4% paraformaldehyde in PBS (pH 7.4) at room temperature for 20 minutes. Monolayers were routinely permeabilized with 0.25% bovine serum albumin/PBS solution containing a final concentration of 0.1% saponin (BSP). Incubations with primary and secondary antibodies were at room temperature on a shaking platform for 1 hour. Primary antibody was diluted in BSP and incubated with fixed *Brucella* infected cells at 1/25 mouse anti-LAMP 1 monoclonal (ID4B- Iowa Hybridoma). Secondary antibodies was diluted in BSP and used at 1/500 Cy®5-conjugated AffiniPure F(ab’)_2 Fragment Rabbit Anti-Mouse IgG (Jackson ImmunoResearch). Confocal microscopy was performed with an Olympus FV1000 Fluoview laser scanning microscope equipped with 405, 488, 563 and 635 laser lines and 4 PMTs. Post-acquisition image processing was performed with ImageJ v1.43m (http://rsb.info.nih.gov/ij/index.html).

**Results**

*Brucella* in LC3+, LAMP1+ vesicles inside cells

To determine if *Brucella* may be associated with the trafficking pathway of a bariphore we infected RAW264.7 murine macrophages transfected with an LC3-RFP construct with virulent *Brucella abortus* that had either been opsonized with serum immunoglobulin (Ig) or non-opsonized. Infected cells were activated with IFN-γ or non-activated 24 hours prior to infection. Samples were fixed 48 hours post infection and scanning laser confocal microscopy was performed after staining for LAMP1. Ig-opsonized *Brucella* was observed in LC3 positive vesicles that colocalized with LAMP1 in non-activated cells. Non-opsonized *Brucella* from IFN-γ activated cells were seen directly adjacent to LC3 positive vesicles but were not found to occupy the same space (Figure 4.1). In the non-activated cells, non-opsonized *Brucella* were in LAMP1 positive vesicles but were not found in close proximity
to LC3 positive vesicles (data not shown). The same was true for the opsonized *Brucella* in IFN-γ activated macrophages (data not shown).

Figure 4.1. *Brucella* are found in LC3 positive and LAMP1 positive vesicles inside RAW264.7 cells. Opsonized *Brucella* in non-activated macrophages colocalize with LC3 positive vesicles and non-opsonized *Brucella* from IFN-γ activated cells are tightly adjacent to LC3 positive vesicles. RAW264.7 cells transfected with an LC3-RFP construct were activated with IFN-γ, or non-activated, and were infected with Ig-opsonized or non-opsonized *B. abortus* for 48 hours. Samples were fixed, stained for LAMP1, and images were taken using scanning laser confocal microscopy. Non-opsonized *Brucella* in non-activated cells were not found to be associated with LC3 vesicles as was true for opsonized *Brucella* in the IFN-γ activated cells. Though each treatment group of bacteria and macrophage activation state interacted differently with LC3 positive vesicles, all *Brucella* were found in LAMP1 positive vesicles. Arrowheads indicate individual *Brucella*. Bar=5μm.
Brucella traffics to a new cells monolayer via bariphores

In order to determine if Brucella is able to enter into the bariphore trafficking pathway and use this pathway to infect a new monolayer of cells, we collected supernatants from an infected monolayer and used them to infect new cells. RAW264.7 macrophages transfected with an LC3-RFP construct were activated with IFN-γ or non-activated, and infected with Ig-opsonized or non-opsonized virulent B. abortus for 48 hours. After 48 hours, supernatants of like types (opsonized +IFN-γ, opsonized without IFN-γ, non-opsonized +IFN-γ, non-ops without IFN-γ) were collected and used to infect a fresh monolayer of non-activated RAW264.7 cells. After 72 hours of infection, coverslips were fixed and stained for LAMP1 and analyzed using scanning laser confocal microscopy. Opsonized Brucella were observed in LC3 positive vesicles heavily surrounded by LAMP1 in IFN-γ stimulated cells (Figure 4.2). This same phenomenon was observed for all other treatment groups; opsonized Brucella from non-activated cells, and non-opsonized Brucella from both activated and non-activated cells (data not shown).

Figure 4.2. Brucella traffic in bariphores to infect new monolayer of macrophages. Supernatants were collected from RAW264.7 cells non-activated or treated with IFN-γ, transfected with LC3-RFP, and infected for 48 hours with opsonized or non-opsonized virulent B. abortus. Supernatants were treated with gentamycin and used to infect a new non-activated, untransfected monolayer of RAW264.7 cells. Cells were incubated with bacteria-laden supernatants for 96 hours, fixed, and stained for LAMP1. Scanning laser confocal
microscopy was used to collect these images. Arrowheads indicate individual *B. abortus* inside LC3 positive, LAMP1 positive bariphores that have been taken in to a RAW264.7 cell. Bar=5μm.

**Discussion**

Ig-opsonized and non-opsonized *Brucella* are internalized into macrophages by different mechanisms and as a result are trafficked differently once they get into the cell. Once internalized, *Brucella* pass through the early and late endosome. Opsonized *Brucella* replicate in a late endosome-like compartment, whereas, non-opsonized *Brucella* replicate in a vesicle of endoplasmic reticulum origin. It has previously been shown in our lab that there are mechanistic differences in trafficking between opsonized and non-opsonized *B. abortus* in activated versus non activated murine macrophages (Ritchie *et al.*, unpublished data). After 48 hours of infection of IFN-γ activated or non-activated RAW264.7 murine macrophages with virulent *B. abortus* 2308, we observed a colocalization of opsonized *Brucella* with LC3+ vesicles in non-activated cells. Vesicles containing non-opsonized *B. abortus* in activated cells were found closely adjacent to LC3+ vesicles appearing as if the two vesicles were docked but the contents had not mixed. Both opsonized and non-opsonized bacteria were in LAMP1+ vesicles. Supernatants were isolated from *B. abortus* infected LC3-RFP transfected cells and used to infect fresh monolayers of RAW264.7 cells. After 72 hours of infection, opsonized and non-opsonized *Brucella* in both activated and non-activated cells were observed in LC3+ vesicles heavily surrounded by LAMP1 inside the infected RAW264.7 cell. The origin of the LC3 in these vesicles could have only come from the cells originally transfected with the LC3-RFP construct, the cell monolayer originally infected. The LC3+, LAMP1+ vesicles containing *B. abortus* are believed to be bariphores originally described in our lab as they share many characteristics. *Brucella* is able to invade this regularly occurring mechanism of bariphoire production and release inside the
macrophage and use it to transport itself to a new host cell. We have shown that the bariphore protects Brucella from gentamycin treatment, suggesting that this vesicle can provide the bacterium protection from innate immune mechanisms such as complement and antibody opsonization. This mechanism may provide an explanation for the previously unknown mechanism by which Brucella is able to evade the host immune system allowing the infection to persist. Knowing that Brucella is able to use the bariphore to traffic to new host cells provides insight for a possible persistence mechanism for other intracellular pathogens also associated with the autophagy pathway. With this knowledge we can now work to develop new drug targets inhibiting the production or release of the bariphore whereby inhibiting the persistence of Brucella infection.

References


CHAPTER 5. GENERAL CONCLUSIONS

General Discussion

Brucella spp. are intracellular pathogens that survive and replicate inside a late autolysosome-like intracellular vesicle if they are opsonized, and an endoplasmic reticulum generated vesicle if they are non-opsonized. These studies have shown that Brucella spp. require LRG-47 for their replication and intracellular survival inside a macrophage. LRG-47 is a p47 GTPase whose activities are induced by the cytokine IFN-γ (1). LRG-47 is important in controlling many intracellular pathogen infections such as Mycobacterium tuberculosis (2), Salmonella typhimurium (3), Toxoplasma gondii (4), and Trypanosoma cruzi (5). It has never before been shown that a pathogen requires the presence of LRG-47 for its survival, as has been shown with Brucella abortus. LRG-47 colocalizes to the autophagosome membrane and stays on the membrane for the lifespan of the vesicle. Due to LRG-47’s colocalization at the membrane of the autophagosome, this p47 GTPase has the ability to control vesicular transport inside the cell (3). It was thought that it was through LRG-47’s control of vesicular transport that the cell was able to control pathogenic intracellular infection (3). In previous studies, there was no significant difference seen in viable Brucella recovered from C57/B6 mice and LRG-47 knockout mice treated with the autophagy inhibitor, 3-methyladenine (3MA). 3MA is a phosphotidylinositol-3’ kinase (PI3K) inhibitor which acts through inhibiting GTPases. Since LRG-47 is a GTPase, I am proposing that LRG-47 is a target for 3MA. This would explain why there is no heightened Brucella killing seen when 3MA is used in addition to the knockout of LRG-47 in B. abortus infected cells. Also in previous studies involving the autophagy pathway, an autophagosome-like extracellular organelle which was termed a ‘bariphore’ was observed. It was determined that the bariphore shares many characteristics with the autophagy pathway. Using siRNA and dominant negative protein expression as well as confocal microscopy, I was able to determine that bariphores are released from the cell as an autolysosome before degradation of vesicular contents has
occurred. In addition, it was observed that *B. abortus* is able to exploit the autophagy pathway to traffic intercellularly via the bariphore, and possibly using this as a persistence mechanism.

It is reasonable to say that by in LRG-47/− cells the production or appropriate vesicular trafficking of bariphores containing *Brucella* may have been inhibited. Without the appropriate release of the *Brucella*-containing bariphores, the infection was unable to persist inside the host. It isn’t necessarily that *Brucella* spp. require LRG-47 for their survival, but they require the appropriate vesicular trafficking which is regulated by LRG-47. Moreover, it is possible that *Brucella* spp. require LRG-47 to appropriately regulate the bariphore trafficking and release from the host cell, allowing the *Brucella* population to persist.

**Recommendations for Future Research**

Now that it has been determined that LRG-47 is vital for the survival and replication of *B. abortus* in murine macrophages, it is important to determine if the same is true for other subtypes of *Brucella*. 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 *Chlamydia trachomatis* and *C. muridarum* with *C. trachomatis* infection being kept at bay by LRG-47, but not *C. muridarum* (6). In the future it will be important to perform similar experiments using *B. melitensis* to determine the role of LRG-47 in this related subtype’s ability to survive and replicate inside the host. Similarly, trafficking, survival, and replication experiments with Ig-opsonized and non-opsonized *B. melitensis* in IFN-γ activated and non-activated cells will be important to report any differences in behavior of this closely related subtype. Though the mouse has proven to be a good model for *Brucella* infection, it is important to look further into the impact of LRG-47 on *B. abortus* infection in human monocytes in the future.
Through the previous studies involving the autophagy pathway, much information was collected regarding the characterization and intra- and intercellular trafficking of the bariphore. There is still much more research to be done to detail the mechanism of release and uptake of the bariphore. Vesicle fusion events in exocytosis and endocytosis are controlled by membrane proteins called soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) of which there are several classes (7). The previous studies determined that bariphores are taken into a target cell by a membrane fusion event. In order to determine if SNAREs are involved in bariphore uptake, it would be interesting to treat bariphore producing cells with anti-SNARE antibodies in an attempt to inhibit the uptake of the bariphore into the target cell. It has been speculated that vacuolar H\(^{+}\)-ATPase (V-ATPase) in the autolysosome membrane can also assist in vesicle docking and membrane fusion (8). This can also be tested by treating the cells with antibodies specific for V-ATPase to inhibit uptake of bariphores by target cells.

In the previous studies we were able to determine that *B. abortus* uses bariphores to traffic from one host cell to another while evading the host immune system. Recognizing this important mechanism of persistence for the bacterium within the host has provided a new opportunity to discover drug targets to inhibit chronic *Brucella* infection. One possible drug target may be the GTPase, Rab7. In the previous studies we were able to show that Rab7 is required for the production and release of bariphores in RAW264.7 cells. By infecting cells transfected with a dominant negative Rab7 construct and performing the supernatant transfer experiments as well as intracellular viability experiments explained in these studies it could be determined if Rab7 is important for *Brucella* transport in bariphores. It was also shown in these studies that LC3\(\alpha\) and \(\beta\) are important in bariphore release. LC3\(\alpha/\beta\) siRNA could be used to determine if LC3\(\alpha\) and \(\beta\) are important in *Brucella* intercellular trafficking and infection persistence in a host. This could also be done using knockout mice, though other
complications would have to be considered when inhibiting the process of autophagy in both cells and an animal model. Finally, it was discussed earlier the importance of LRG-47 in vesicular trafficking and the impact this may have had on bariphore formation and *Brucella* survival. In order to determine if LRG-47 plays any role in bariphore maturation or release it will be important to knockdown LRG-47 using siRNA and observe its effect on bariphore production and release. Secondly, these same experiments involving siRNA for LRG-47 will have to be repeated and the cells infected with virulent *Brucella*. Supernatant transfer experiments will need to be performed to determine if LRG-47 is important in *Brucella* intercellular trafficking in bariphores.

**References**


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