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Autophagy Is a Defense Mechanism Inhibiting BCG and *Mycobacterium tuberculosis* Survival in Infected Macrophages

Maximiliano G. Gutierrez,[1,2] Sharon S. Master,[2] Sudha B. Singh,[2] Gregory A. Taylor,[3] Maria I. Colombo,[1,*] and Vojo Deretic[2,4,*]
[1]Instituto de Histologia y Embriologia
Facultad de Ciencias Medicas
Universidad Nacional de Cuyo-CONICET
Mendoza 5500
Argentina
[2]Department of Molecular Genetics
and Microbiology
University of New Mexico School of Medicine
Albuquerque, New Mexico 87131
[3]Departments of Medicine and Immunology
Duke University Medical Center
Durham, North Carolina 27710
[4]Department of Cell Biology and Physiology
University of New Mexico School of Medicine
Albuquerque, New Mexico 87131

Summary

***Mycobacterium tuberculosis* is an intracellular pathogen persisting within phagosomes through interference with phagolysosome biogenesis. Here we show that stimulation of autophagic pathways in macrophages causes mycobacterial phagosomes to mature into phagolysosomes. Physiological induction of autophagy or its pharmacological stimulation by rapamycin resulted in mycobacterial phagosome colocalization with the autophagy effector LC3, an elongation factor in autophagosome formation. Autophagy stimulation increased phagosomal colocalization with Beclin-1, a subunit of the phosphatidylinositol 3-kinase hVPS34, necessary for autophagy and a target for mycobacterial phagosome maturation arrest. Induction of autophagy suppressed intracellular survival of mycobacteria. IFN-γ induced autophagy in macrophages, and so did transfection with LRG-47, an effector of IFN-γ required for antimycobacterial action. These findings demonstrate that autophagic pathways can overcome the trafficking block imposed by *M. tuberculosis*. Autophagy, which is a hormonally, developmentally, and, as shown here, immunologically regulated process, represents an underappreciated innate defense mechanism for control of intracellular pathogens.**

Introduction

The notoriety of the facultative intracellular pathogen *Mycobacterium tuberculosis*, as a hard-to-eradicate human pathogen infecting over a billion people worldwide (Dye et al., 1999), is based on its ability to parasitize host macrophages (Russell et al., 2002). In host phagocytic cells, *M. tuberculosis* interferes with the phagosome maturation pathway (Vergne et al., 2004), a phenomenon referred to in the classical literature as the inhibition of phagosome-lysosome fusion (Armstrong and Hart, 1971). *M. tuberculosis* phagosomal compartments exhibit diminished acidification due to the paucity of H^+-ATPase (Sturgill-Koszycki et al., 1994), attributed to mycobacterial disruption of the delivery of V_o H^+ATPase subunits and lysosomal hydrolases via a TGN-to-phagosome pathway dependent on the phosphatidylinositol 3-kinase (PI3K) hVPS34 (Fratti et al., 2003). *M. tuberculosis* produces lipids that mimic mammalian phosphatidylinositols and inhibit phosphatidylinositol 3-phosphate (PI3P)-dependent trafficking pathways in the infected macrophage (Vergne et al., 2004).

The production of PI3P via hVPS34 is essential for autophagy, a major intracellular pathway for programmed turnover of long-lived cytoplasmic macromolecules and organelles (Levine and Klionsky, 2004) in response to starvation (Takeshige et al., 1992). Autophagy can also be stimulated or inhibited by hormones, as, for example, it is induced by insulin limitation (Saeki et al., 2003) or glucagon presence (Deter and De Duve, 1967). An onset of autophagy can be induced by pharmacological means using rapamycin, an inhibitor of the Ser/Thr kinase Tor (*t*arget *o*f *r*apamycin) (Noda and Ohsumi, 1998), which controls cell growth in response to nutrients (Jacinto and Hall, 2003). The autophagy trafficking pathway has been originally described as a cellular adaptation to starvation, whereby bulk cytoplasmic ingredients are engulfed by a membrane of still-unknown origin and degraded for reprocessing to sustain cellular anabolic needs. During macroautophagy, cytoplasmic components are sequestered by a double membrane sac (a phagophore or isolation membrane) to form an autophagosome, which in mammalian cells eventually acquires endosomal and lysosomal characteristics (Dunn, 1990). At the molecular level, a series of factors conserved from yeast to man are involved in sequential steps of autophagy (Levine and Klionsky, 2004). Some of the best-defined markers of autophagy are the Apg5 (Atg5, according to the recently standardized nomenclature [Klionsky et al., 2003]), which is associated with the nascent isolation membrane (Mizushima et al., 2001), and the microtubule-associated protein 1 (MAP1) light chain 3 (LC3) (Kabeya et al., 2000). LC3, the mammalian equivalent of Aut7/Apg8(Atg8), undergoes complex C-terminal proteolytic and lipid (i.e., addition of phosphatidylethanolamine) modifications, upon which it translocates from the cytosol to the autophagosomal membrane (Kabeya et al., 2000). LC3 is currently the only highly specific marker of the autophagosome (Yoshimori, 2004).

The signaling pathways governing autophagy remain to be fully delineated, but some critical points have been uncovered: (1) the starvation-initiated signaling results in the inhibition of Tor, which, by still unknown downstream mechanisms, induces autophagy; and (2) the progression of autophagy is sensitive to the PI3K inhibitors wortmannin and 3-methyladenine (3MA), with the target being the type III PI3K hVPS34 (Petiot et al., 2000). In yeast, VPS34 (the equivalent of mammalian hVPS34) forms a lipid kinase complex with additional autophagy-

*Correspondence: mcolombo@fcm.uncu.edu.ar (M.I.C.); vderetic@salud.unm.edu (V.D.)

specific subunits, Atg14 and Atg6 (Kihara et al., 2001b), which is necessary for the downstream recruitment and proper localization of critical Atg complexes: Atg5 (conjugated to Atg12) on preautophagosomal isolation membrane, followed by the proper translocation of Atg8 (the yeast equivalent of LC3) to the autophagosomal membrane (Suzuki et al., 2001). In mammalian cells, the equivalent of Atg6, Beclin 1 (Liang et al., 1999), in a complex with the PI3 kinase hVPS34, apparently originating from the trans-Golgi network (Kihara et al., 2001a), is specifically activated upon amino acid starvation (Tassa et al., 2003). Moreover, the product of hVPS34, PI3P, promotes autophagic proteolysis in amino acid-starved cells (Tassa et al., 2003).

Based on the critical role of hVPS34 and PI3P in phagosomal maturation (Fratti et al., 2001; Vieira et al., 2001) and autophagic pathways (Petiot et al., 2000), we wondered whether physiological or pharmacological induction of autophagy may influence the mycobacterial phagosome maturation block. In this work, we show that induction of autophagy promotes maturation of mycobacterial phagosomes in a PI3K-dependent fashion and concomitantly suppresses mycobacterial survival. We also show that autophagy is induced by relevant immunological signals with known antituberculosis action. These findings indicate that autophagy represents a previously unrecognized innate defense mechanism against intraphagosomal microbes. This process, when appropriately activated, can boost innate immunity to control intracellular pathogens.

Results

Induction of Autophagy Results in Increased Acidification of Mycobacterial Phagosomes

We tested whether starvation, a conventional inducer of autophagy, can alter the mycobacterial phagosome acidification defect using GFP-labeled *Mycobacterium tuberculosis* variant *bovis* BCG (BCG) and the murine macrophage cell line RAW 264.7. Cells were subjected to the standard starvation protocol (amino acid/serum-deprived medium), and acidification of mycobacterial phagosomes was examined using LysoTracker, an acidotropic fluorescent dye that accumulates in acidic organelles (Via et al., 1998). Starved cells showed 51.4% $\pm$ 8.3% colocalization with LysoTracker, versus 26.5% $\pm$ 6.3% in control samples ($p < 0.01$; ANOVA) (Figures 1A and 1B). These findings show that exposure of infected macrophages to starvation conditions, routinely used to induce autophagy, renders mycobacterial phagosomes susceptible to acidification. Next, we tested whether the starvation effect on enhanced acidification of mycobacterial phagosomes could be counteracted by treatment with the classical inhibitor of autophagy 3MA (Blommaart et al., 1997). Both 3MA and wortmannin, inhibitors of PI3 kinases typically used to block the earliest stages of autophagosome formation, abrogated starvation-promoted acidification of mycobacterial phagosomes (Figures 1A and 1B).

Rapamycin Enhances Mycobacterial Phagosome Acidification

The observations with starvation and inhibitors of autophagy suggest that stimulation of autophagic pathways may be responsible for the improved acidification of the mycobacteria-containing phagosomes. To confirm this, we next induced autophagy by a different mechanism, employing pharmacological means. Tor kinase plays a central role in the amino acid pool-sensing mechanism: Tor is inhibited in response to starvation, resulting in the induction of autophagy (Jacinto and Hall, 2003). Since Tor can be inhibited by rapamycin, this compound is routinely used as a pharmacologic agent capable of inducing autophagy even under nutrient-replete conditions (Noda and Ohsumi, 1998). Rapamycin treatment of infected macrophages triggered increased acidification of mycobacterial phagosomes (Figures 1A and 1B). Rapamycin-treated cells showed 41.0% $\pm$ 2.7% LysoTracker colocalization, while control cells displayed 26.5% $\pm$ 6.3% ($p < 0.01$; ANOVA) acidified mycobacterial phagosomes. These results indicate that, in cells incubated under conditions that favor autophagy, mycobacterial vacuoles no longer have the acidification defect, which is normally a hallmark of the phagosomes containing pathogenic mycobacteria (Crowle et al., 1991; Oh and Starubinger, 1996; Sturgill-Koszycki et al., 1994).

Induction of Autophagy Promotes Maturation of Mycobacterial Phagosomes

The major defining properties of the *M. tuberculosis* phagosome are the restricted acquisition of the vacuolar H^+-ATPase (Sturgill-Koszycki et al., 1994) and paucity of mature lysosomal hydrolases such as cathepsin D (Sturgill-Koszycki et al., 1996). To characterize further the compartment occupied by mycobacteria after induction of autophagy, we analyzed the distribution of markers of late endocytic/lysosomal compartments: cathepsin D, LAMP-1, the vacuolar H^+-ATPase, and the late endosomal lipid lysobisphosphatidic acid (LBPA). As shown in Figure 2, starvation induced a strong increase in colocalization of mycobacterial phagosomes with late endosome/lysosome markers indicating enhanced mycobacterium phagosome maturation. Starvation resulted in increased presence of cathepsin D (40.6% in starved cells versus 11.3% in control cells; $p < 0.01$), LAMP-1 (24.3% in starved cells versus 14.4% in control cells; $p < 0.01$), $V_o H^+$ATPase (55.6% in starved cells versus 7.9% in control cells; $p < 0.01$), and LBPA (48.1% in starved cells versus 16.9% in control cells; $p < 0.01$).

Autophagic Markers on Mycobacterial Phagosomes in Cells Induced for Autophagy

To characterize further the compartment occupied by mycobacteria in macrophages subjected to starvation or treated with rapamycin, we examined whether mycobacterial phagosomes colocalize with proteins involved in the autophagic pathway. LC3 is a mammalian homolog of the yeast Atg8, which is a specific constituent of the autophagosomal membrane (Kabeya et al., 2000). It has been shown that LC3 exists in two forms (Kabeya et al., 2000), the 18 kDa cytosolic form (LC3-I) and the 16 kDa processed form (LC3-II) located on the autophagosomal membrane. The LC3 C-terminal penultimate residue, glycine, becomes exposed upon a proteolytic removal of the C-terminal arginine and is conjugated

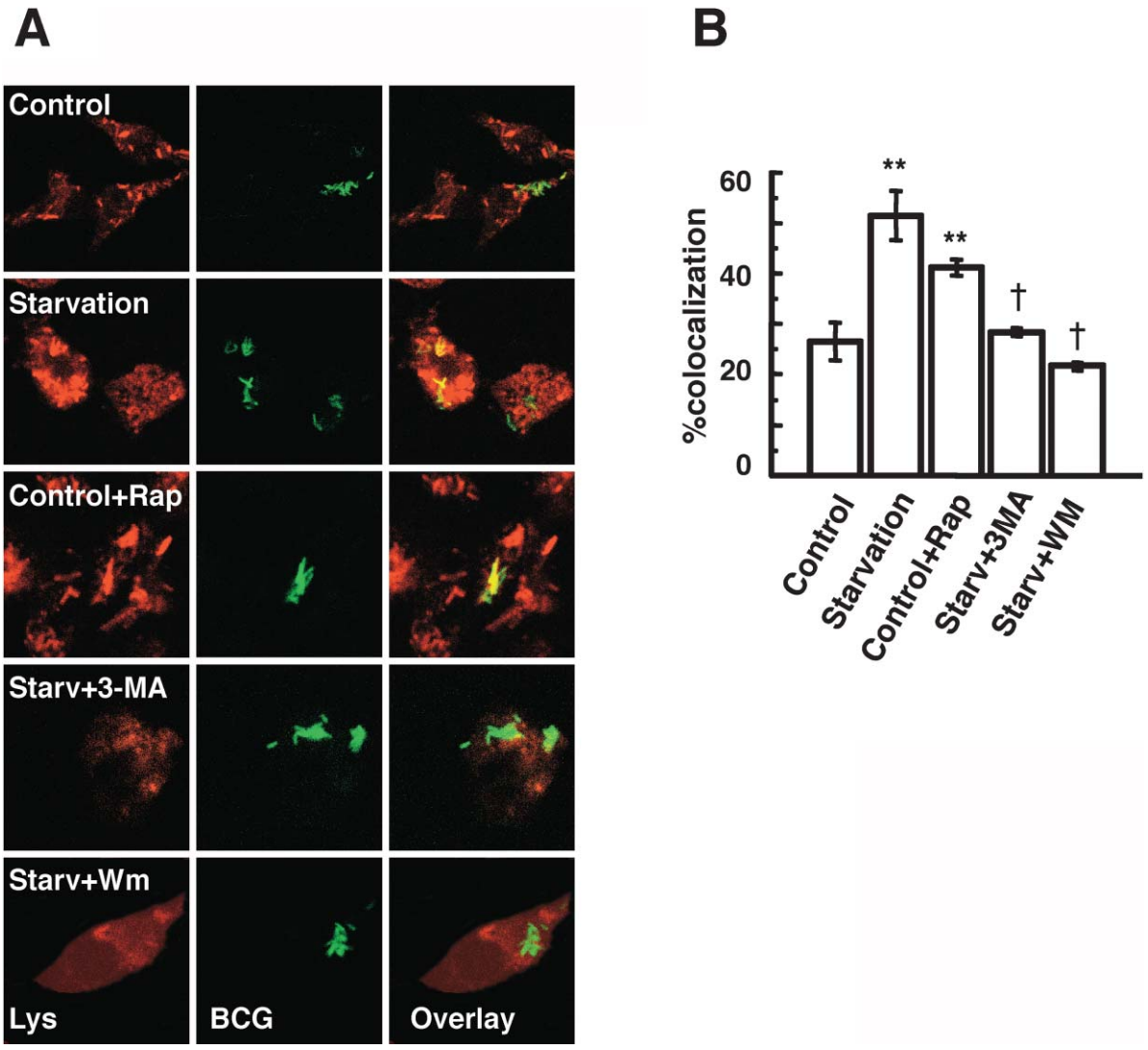


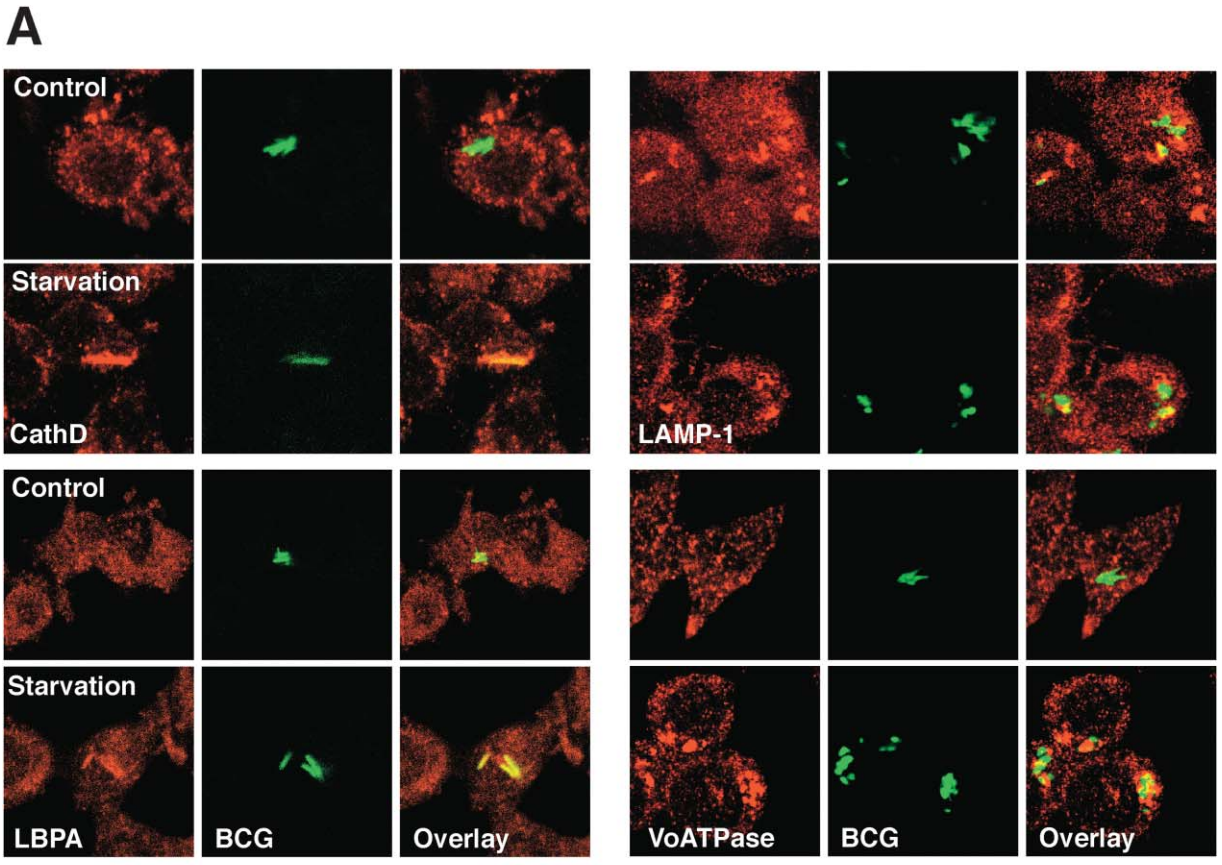
Figure 1. Induction of Autophagy Results in Increased Acidification of Mycobacterial Phagosomes

RAW 264.7 macrophages were infected with BCG-expressing GFP for 1 hr. Cells were incubated in full medium with 50 μg/ml rapamycin (Control+Rap) and without treatment (Control), or in starvation media (Starv) in the presence or absence of 10 mM 3-methyl adenine (3-MA) or 100 nM wortmannin (Wm). (A) Confocal microscopy images of the acidotropic dye LysoTracker (Lys) (red channel) and mycobacteria (green channel). (B) Fraction (%) of mycobacterium-containing phagosomes colocalizing with the acidotropic dye LysoTracker. Data, means ± SEM from three independent experiments ($n = 100$ phagosomes). **$p \leq 0.01$, †$p \geq 0.05$.

to phosphatidylethanolamine. The lipid conjugation is essential for association of LC3 with the autophagic membrane, where it plays a role in elongation/enlargement of the autophagosome (Reggiori and Klionsky, 2002). We examined the distribution of LC3 relative to mycobacterial phagosomes. RAW macrophages were transiently transfected with GFP-LC3, and localization relative to the mycobacterial phagosomes was determined by confocal microscopy (Figure 3A) in cells incubated under nutrient-replete conditions versus cells induced for autophagy by starvation. A marked increase in the colocalization between GFP-LC3 and mycobacterial phagosomes was observed under starvation (33.0% versus 4.5%; $p < 0.01$) (Figure 3B). In contrast, no colocalization was observed with the LC3 G120A mutant, as it has alanine in place of the critical penultimate glycine required for the normal processing of LC3 (Figures 3C and 3D). Similar negative results were observed in cells transfected with another LC3 mutant truncated for the last 22 residues (Figures 3E and 3F). These observations indicate that starvation promotes recruitment of critical autophagy effectors to mycobacterial phagosomes, as exemplified by the membrane-associated LC3, a marker deemed to be the gold standard for autophagic organelles (Yoshimori, 2004).

Mycobacterial Vacuoles Colocalize with Beclin 1, a Subunit of the Type III PI3K Associated with Autophagy

Beclin 1 is the mammalian equivalent of the yeast autophagy protein Atg6 (also known as Vps30) (Liang et al., 1999). It has been shown that Beclin 1 represents a



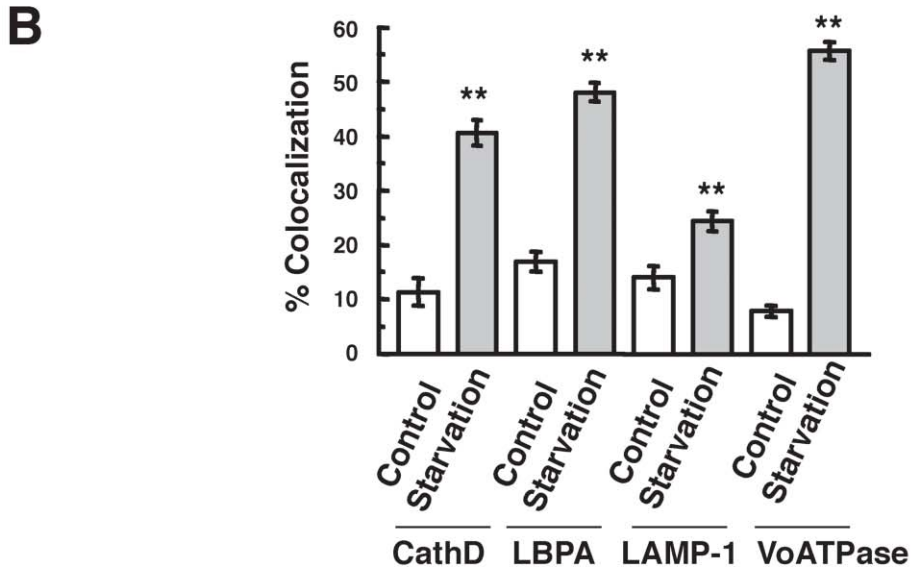


Figure 2. Induction of Autophagy Favors the Acquisition of Late Endosomal/Lysosomal Markers by Mycobacterium-Containing Phagosomes

Macrophages were infected with BCG-expressing GFP for 1 hr. After phagocytosis, cells were incubated for 2 hr under full nutrient conditions (control) or in starvation media (starvation). Cells were fixed, permeabilized, and incubated with antibodies against cathepsin D, LBPA, LAMP-1, and the V_o H^+-ATPase proteolipid subunit, followed by Alexa-568 conjugated secondary antibodies. (A) Confocal images showing different markers (red) and mycobacteria expressing GFP (green). (B) Quantitative analysis of marker colocalization with mycobacteria. Data, means ± SEM from triplicate experiments. **$p \leq 0.01$.

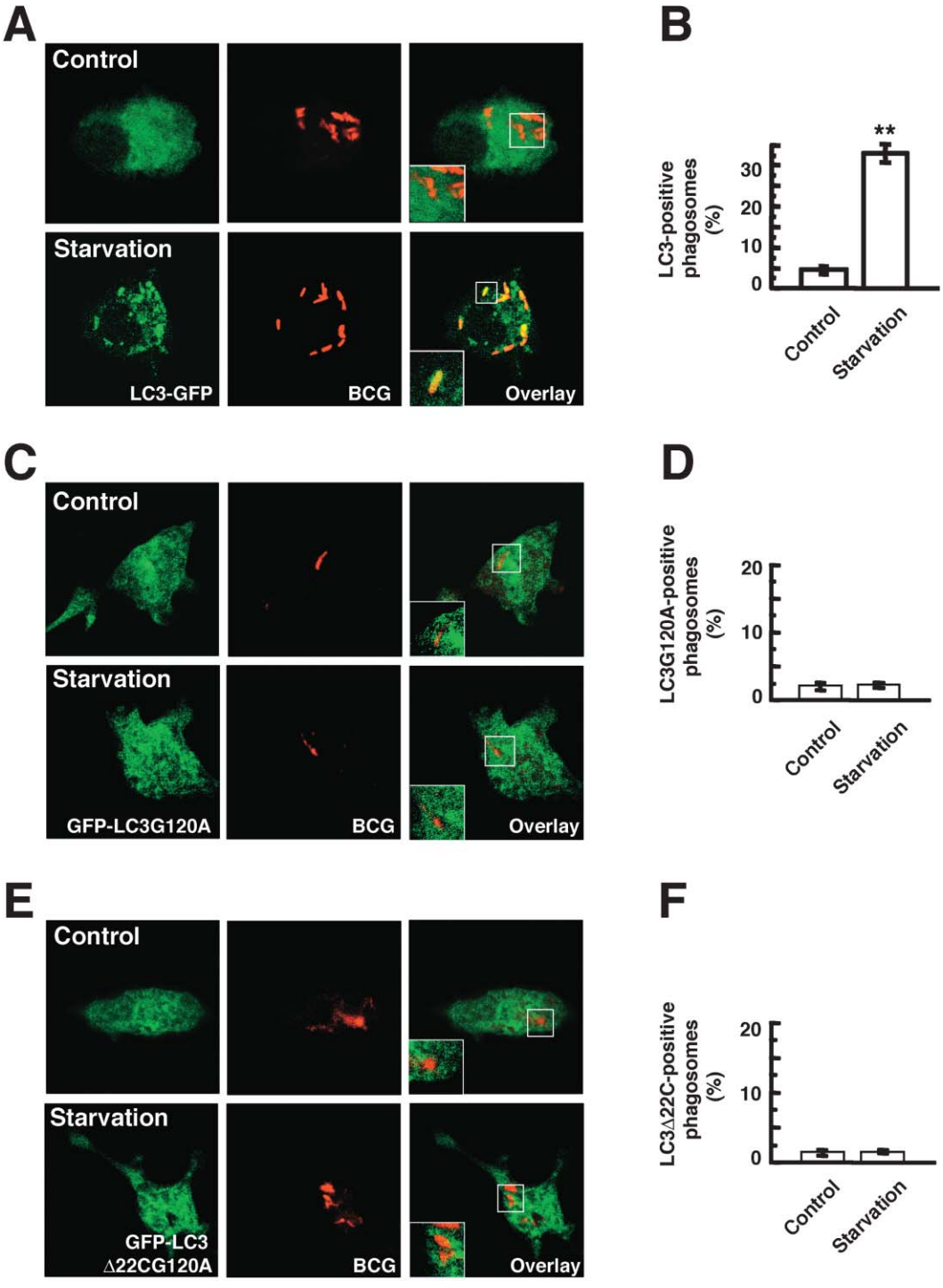


Figure 3. Mycobacterial Phagosomes Colocalize with the Autophagosome-Specific Marker LC3 upon Induction of Autophagy

RAW 264.7 cells were transiently transfected with GFP-LC3 wild-type (A) or the mutant LC3 constructs GFP-LC3G120A (C) and GFP-LC3G120AΔ22C (E) and then infected with BCG-expressing DsRed for 1 hr. Cells were incubated under control or starvation conditions and analyzed by confocal microscopy. (B, D, and F) Quantitive analysis of colocalization between mycobacterial phagosomes and LC3-GFP fusion proteins in cells incubated under control or starvation conditions. Data, means ± SEM from three independent experiments (LC3 wild-type, n = 100 phagosomes; LC3 mutants, n = 25 phagosomes). **p ≤ 0.01.

subunit of the PI3K hVPS34 complex participating in autophagy (Kihara et al., 2001a, 2001b; Liang et al., 1999; Tassa et al., 2003). We analyzed whether mycobacterial phagosomes acquired Beclin 1 upon induction of autophagy. Macrophages were transiently transfected with FLAG epitope-tagged human Beclin 1 and incubated both under nutrient-replete and starvation conditions. Colocalization of Beclin 1 with mycobacterial

phagosomes increased from 37.6% to 57.9% ($p < 0.01$) upon amino acid deprivation (see Supplemental Figure S1 at http://www.cell.com/cgi/content/full/119/6/753/DC1/). Under full nutrient conditions, almost 40% of mycobacterium-containing phagosomes colocalized with Beclin 1. The previous report showing that Beclin 1 gene transfer in MCF7 cells increases basal level of autophagy in transfected cells (Liang et al., 1999) explains the high basal level (40%) of colocalization with Beclin 1. The increase in colocalization between Beclin 1 and mycobacterial phagosomes upon full induction of autophagy is consistent with our observations using other phagosomal maturation or autophagy markers.

Induction of Autophagy Inhibits Mycobacterial Survival in Infected Macrophages

Our results indicate that induction of autophagy can override the mycobacterial block of phagolysosome biogenesis and that mycobacterial phagosomes acquire critical proteins involved in the autophagic pathway. The next logical step was to test whether induction of autophagy can affect mycobacterial survival in infected macrophages. The effects of stimulation of autophagy on mycobacterial viability were monitored by two methods: (1) direct scoring for bacterial colony-forming units (CFU) on bacteriological media (Figure 4) and (2) Alamar Blue assay, based on oxidation-reduction dye as an indicator of *M. tuberculosis* growth (Yajko et al., 1995) (see Supplemental Figure S2 on the *Cell* web site).

RAW cells were infected with BCG for 1 hr at 37°C and incubated for 2 hr in nutrient-replete (control) or in amino acid/serum-deprived medium (induction of autophagy). Starvation diminished mycobacterial viability in RAW macrophages (Figure 4A and Supplemental Figure S2). Mycobacterial survival was also examined in cells upon induction of autophagy by rapamycin. Rapamycin treatment reduced mycobacterial viability (Figure 4A) in a dose-dependent manner (Supplemental Figure S2B). No effect was observed with another immunosuppressant, cyclosporin A (Supplemental Figure S2B). Upon addition of autophagy inhibitors 3MA or wortmannin, the starvation- or rapamycin-induced suppression of mycobacterial viability was abrogated (Figure 4A). To exclude the possibility that apoptosis, a process coinciding in some instances with autophagy (Clarke, 1990) and known to inhibit intracellular mycobacteria (Lammas et al., 1997; Molloy et al., 1994), was responsible for suppression of mycobacterial viability in macrophages induced for autophagy, we employed a pan-caspase inhibitor, N-benzyloxycarbonyl-Val-Ala-Asp(OMe)-fluoromethylketone (z-VAD-fmk), which blocks apoptosis. Addition of z-VAD-fmk was not capable of counteracting the antimycobacterial effects of starvation or rapamycin assessed by CFU (Figure 4B) and Alamar Blue assays (Supplemental Figure S2C). Differences in mycobacterial survival could not be attributed to release of bacteria into the supernatant (Figure 4H) or differential macrophage detachment or viability (Figure 4I).

Autophagy Inhibits Mycobacteria in Primary Macrophages and Is Effective against Virulent *M. tuberculosis*

The antimycobacterial effects of autophagy were validated in primary cells, using murine bone marrow-derived macrophages (BMM) and human peripheral blood monocyte-derived macrophages (MDM). Upon induction of autophagy, BCG showed reduced survival in BMM (Figure 4C) and MDM (Figure 4E and Supplemental Figures S2D–S2I). This effect was inhibited by 3MA and wortmannin (Figures 4C and 4E; Supplemental Figures S2D and S2G), albeit there was some residual suppression of mycobacterial growth in MDM (Figure 4E; Supplemental Figure S2G). Pan-caspase (apoptosis) inhibitor z-VAD-fmk did not counteract inhibition of mycobacterial growth in BMM or MDM (Figures 4D and 4F). This excludes apoptosis as a mechanism of mycobacterial killing in our experiments, as z-VAD-fmk specifically inhibits apoptosis and can only promote, as recently shown, authophagic cell death (Yu et al., 2004). All findings were confirmed in Alamar Blue assay (Supplemental Figure S2). Controls for possible detachment of macrophages showed that only 4%–5% of total input cells were detached, irrespective of treatment, and that live mycobacteria were not detectable in culture supernatants (Figures 4H and 4I).

Autophagy also inhibited viability of virulent *M. tuberculosis*. Induction of autophagy in infected macrophages, by starvation or by rapamycin, inhibited virulent *M. tuberculosis* H37Rv; this effect was abrogated by traditional inhibitors of autophagy (Figure 4G). Thus, virulent *M. tuberculosis* H37Rv can be inhibited by autophagy.

The effects of autophagy induction on mycobacterial phagosomes were examined at the ultrastructural level. Electron microscopy analyses showed vacuoles with bacilli and partially degraded internal membranes typical of maturing autophagosomes (Inbal et al., 2002; Mizushima et al., 2001; Talloczy et al., 2002) (Figures 5A and 5B, control, versus Figure 5C and 5D, autophagy induced by starvation). Phagosome fusion with late endosomal multivesicular bodies was also observed (Figure 5E). There was presence of onion-like structures (Figure 5F, main panel and inset) previously reported to occur during induction of autophagy (Hernandez et al., 2003) and noticed morphologically in the original studies by D'Arcy Hart and colleagues (Armstrong and Hart, 1971) as a subset of phagosomes containing damaged mycobacteria.

Macrophage Activation with Interferon-γ Induces Autophagic Processes

To examine whether physiological (e.g., immunological) stimuli induce autophagy in phagocytic cells, we treated macrophages with interferon-γ (IFN-γ), a cytokine associated with protective immunity against *M. tuberculosis* (Flynn and Chan, 2001). Activation of macrophages with IFN-γ resulted in increased proteolysis of long-lived proteins (Figure 6A), which is a marker of autophagy (Mizushima et al., 2001; Talloczy et al., 2002). IFN-γ showed effects equal to or stronger than starvation. The proteolysis induction by IFN-γ was abrogated by the conventional inhibitors of autophagy 3MA and wortmannin (Figure 6A). Activation of cells with IFN-γ resulted in characteristic monodansylcadaverine staining of autophagosomes in treated macrophages (Figures 7A and 7B). Treatment of macrophages with IFN-γ resulted in a translocation of the autophagy marker LC3 from the cytosol to endomembranes, consistent with formation

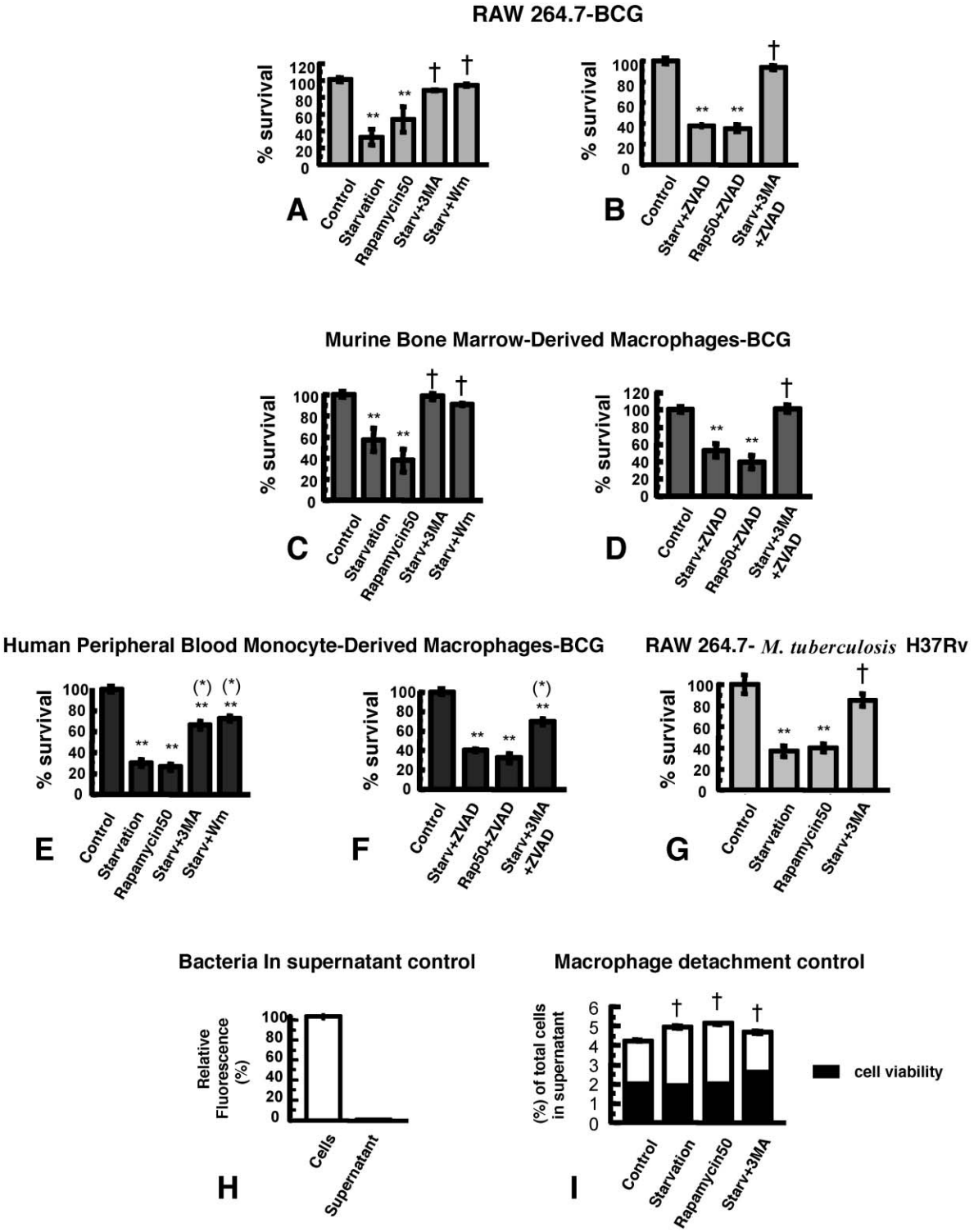


Figure 4. Autophagy Inhibits Mycobacterial Survival in Macrophages

(A and B) RAW 264.7 cells were infected with BCG for 1 hr and incubated for 2 hr in starvation medium with or without 10 mM 3-methyl adenine (3-MA) or 100 nM wortmannin (WM), added where indicated. Full nutrient medium was used for rapamycin (50 μg/ml) treatment. The pan-caspase inhibitor z-VAD-fmk (100 μM) was added where indicated (ZVAD). Cells were lysed and mycobacterial viability (CFU) determined. (C and D) Murine bone marrow-derived macrophages were infected with 2×10^6 BCG for 1 hr. Cells were washed, further incubated for 2 hr as indicated, and lysed for viability (CFU) determination (expressed relative to control). (E and F) Human peripheral blood monocyte-derived macrophages were infected with BCG for 1 hr and subjected to conditions as in (C) and (D). (G) RAW 264.7 cells were infected with virulent *M. tuberculosis* H37Rv under indicated conditions and CFU determined. (H) Cell culture supernatants were tested for presence of mycobacteria using Alamar Blue assay (see caption to Supplemental Figure S1). (I) RAW 264.7 cells were infected with BCG for 1 hr and incubated for 2 hr in starvation medium with or without 10 mM 3-MA and control media plus 50 μg/ml rapamycin. After incubation, detached cells found in the supernatants were counted; viability was assessed by trypan blue exclusion. The results are expressed as percentage of the cells compared to the total number of cells in the sample. Data, means $\pm$ SEM from three independent experiments. **$p \leq 0.01$, *$p < 0.05$, $^{\dagger}p \geq 0.05$, all relative to control. (*)$p < 0.05$, relative to single treatment samples.

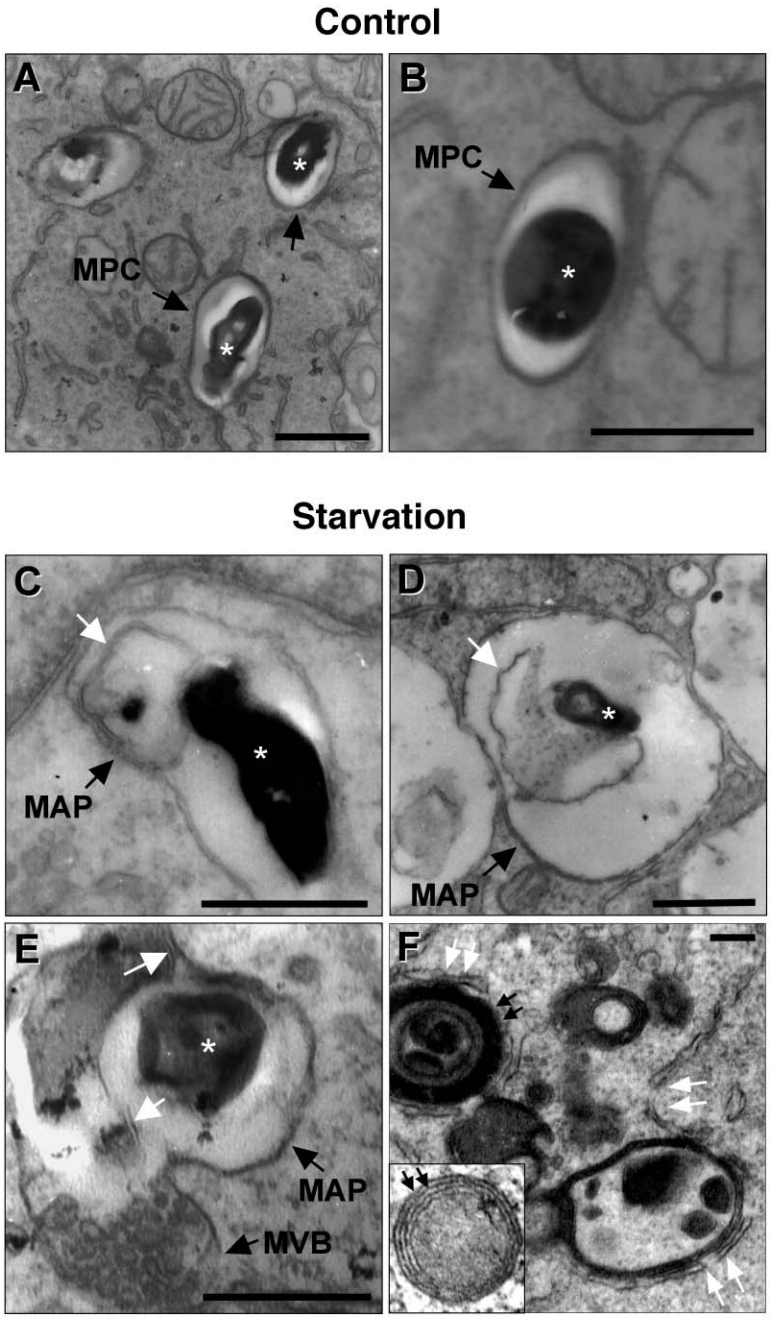


Figure 5. Ultrastructural Analysis of Mycobacterial Phagosomes upon Induction of Autophagy

RAW 264.7 cells were infected with BCG (for 1 hr), incubated in full nutrient or starvation medium (for 2 hr), and processed for electron microscopy. (A and B) Full nutrient medium control. The bacilli were found inside typical myobacterial phagosomal compartments (MPC). (C–F) Infected macrophages induced for autophagy by starvation. The bacilli were found in altered phagosomes, referred to as mycobacterial autophagosomes (MAP) with internal membranes (C and D). Fusion events between MAP and multivesicular bodies (MVB) were observed (E). Under starvation condition, numerous onion-like multilamella structures (inset) or myelin-like figures (as originally noted and termed by D'Arcy Hart and colleagues (Armstrong and Hart, 1971) containing bacterial and other debris were observed (F). Black arrows, phagosomal membrane. Asterisks, bacilli. White arrows, internal membranes within autophagosomes. Double black arrows, onion (myelin)-like multilamellar structures; double white arrows, ER-like membranes juxtaposed to autophagosomes and multilamellar structures.

of autophagosomes (Figure 6B). There was a partial colocalization of LC3 with mycobacteria in IFN-γ-activated cells (Figure 6C). The colocalization between the bacilli and LC3 in IFN-γ-activated cells was incomplete, and there were cells with LC3-GFP found in larger aggregates.

Similarly to the effects of IFN-γ, when macrophages were transfected with LRG-47, a downstream effector of IFN-γ (Taylor et al., 2004) with specific antimycobacterial action (MacMicking et al., 2003), this caused formation of monodansylcadaverine-positive organelles (Figures 7C and 7D). LRG-47 partially colocalized with smaller LC3-positive profiles (Figures 7E and 7F), but LC3 was no longer detectable on the large LRG-47-positive organelles. There was evidence of internal membranes (LBPA-positive) in the large LRG-47-induced vacuoles (Figure 7G). Transfection with LRG-47 caused increased maturation of mycobacterial phagosomes (Figure 7H), in keeping with previous reports (MacMicking et al., 2003).

Our data demonstrating that IFN-γ induces autophagy are consistent with the previously reported induction of autophagy in HeLa cells in response to IFN-γ (Inbal et al., 2002) and show that these phenomena extend to phagocytic cells of the immune system. Furthermore, a known effector of IFN-γ, LRG-47, participates in this process. These findings indicate that physiological stimuli such as activation with IFN-γ, a marquee cytokine associated with protective immunity against tuberculo-

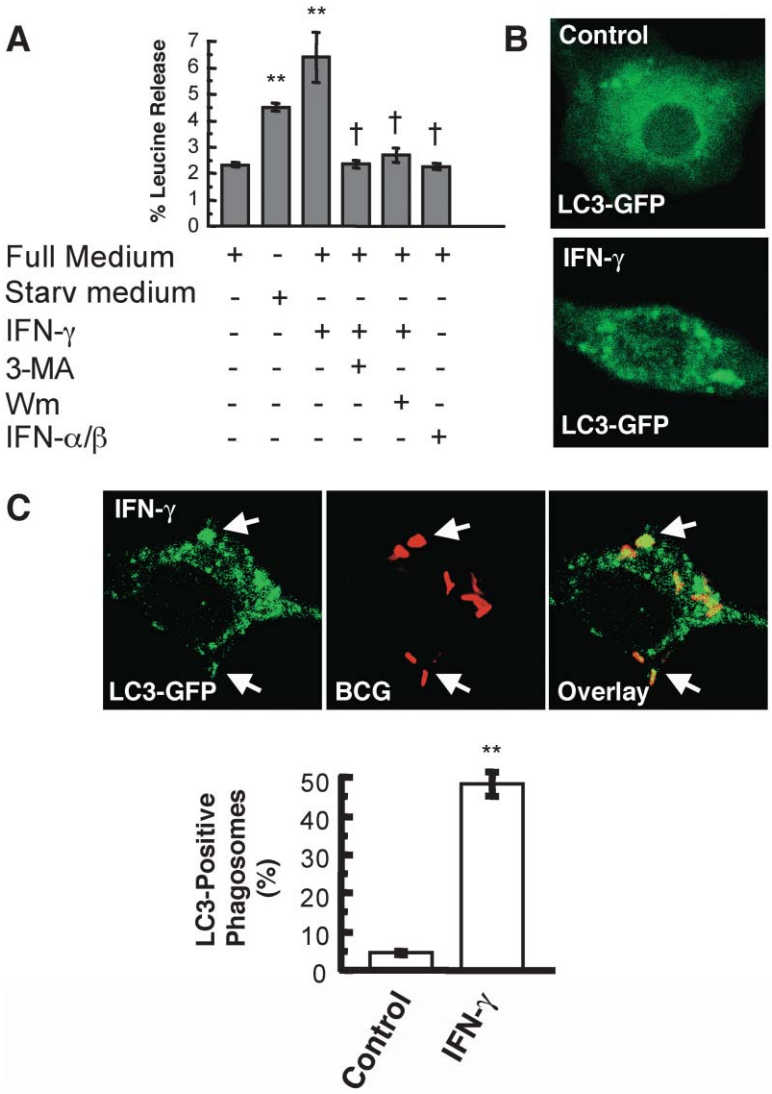


Figure 6. IFN-γ Induces Autophagy in Macrophages

(A) RAW cells were labeled for 24 hr in media containing [^{3}H] leucine. Cells were washed to remove unincorporated radiolabeled leucine and then incubated for 1 hr in complete medium (containing cold leucine) to allow degradation of short-lived proteins. Prepared cells were incubated with or without 200 U/ml IFN-γ, 10 mM 3-methyl adenine (3-MA), 100 nM wortmannin (Wm), or 500 U/ml IFN-α+IFN-β for 2 hr. Leucine release (a measure of proteolysis of stable proteins), as a marker of autophagy, was calculated from radioactivity in the TCA-soluble form relative to the total cell radioactivity. Data, means ± SEM. **$p \leq 0.01$, †$p \geq 0.05$.

(B) RAW 264.7 cells overexpressing LC3-GFP were incubated for 2 hr in full medium without (control) or with 200 U/ml IFN-γ (IFN-γ).

(C) Macrophages expressing LC3-GFP were infected with BCG for 1 hr and chased for 2 hr in presence of 200 U/ml IFN-γ. Arrows, colocalization of mycobacteria (red) with LC3-GFP (green); graph, quantification of LC3 colocalization with BCG phagosomes.

sis (Flynn and Chan, 2001), induce autophagy, which, as shown here, displays antimycobacterial action.

Discussion

The studies presented here demonstrate that stimulation of autophagy by physiological or pharmacological means promotes maturation of mycobacterial phagosomes and overcomes the *M. tuberculosis*-induced phagolysosome biogenesis block. Moreover, induction of autophagy results in a decreased viability of intracellular mycobacteria, consistent with enhanced phagosomal maturation. Since, in cells exposed to starvation, mycobacterial phagosomes acquire critical and highly specific autophagosomal marker LC3, this suggests that *M. tuberculosis* phagosomes are diverted to a compartment with autophagic characteristics, a conclusion further confirmed by electron microscopy. As the normal sequence of trafficking events within the autophagic pathway leads to fusion with lysosomes, the processes uncovered in this study suggest novel means of counteracting the phagosome-lysosome fusion block imposed by *M. tuberculosis*.

The evolutionarily perfected virulence determinants of *M. tuberculosis*, a pathogen that infects close to one third of the human population, endow this microorganism with the ability to inhibit phagosomal maturation and prevent elimination by the host macrophage (Russell et al., 2002). Only a handful of host cell processes appear to be capable of counteracting and overriding the action of the pathogen in vivo (Flynn and Chan, 2003; Vergne et al., 2004), including elevation of cytosolic Ca^{2+} (Malik et al., 2000) that leads to the downstream enhanced recruitment of hVPS34 to organellar membranes, thus promoting phagolysosome biogenesis (Vergne et al., 2003). In this work, we have uncovered a new molecular cascade beginning with the highly relevant immunological cytokine INF-γ and ending with autophagy as a newly recognized antimycobacterial innate defense mechanism.

IFN-γ has been recognized as a marquee antituberculosis immune mediator (Flynn and Chan, 2003). The antimycobacterial action of IFN-γ has implicated a dominant

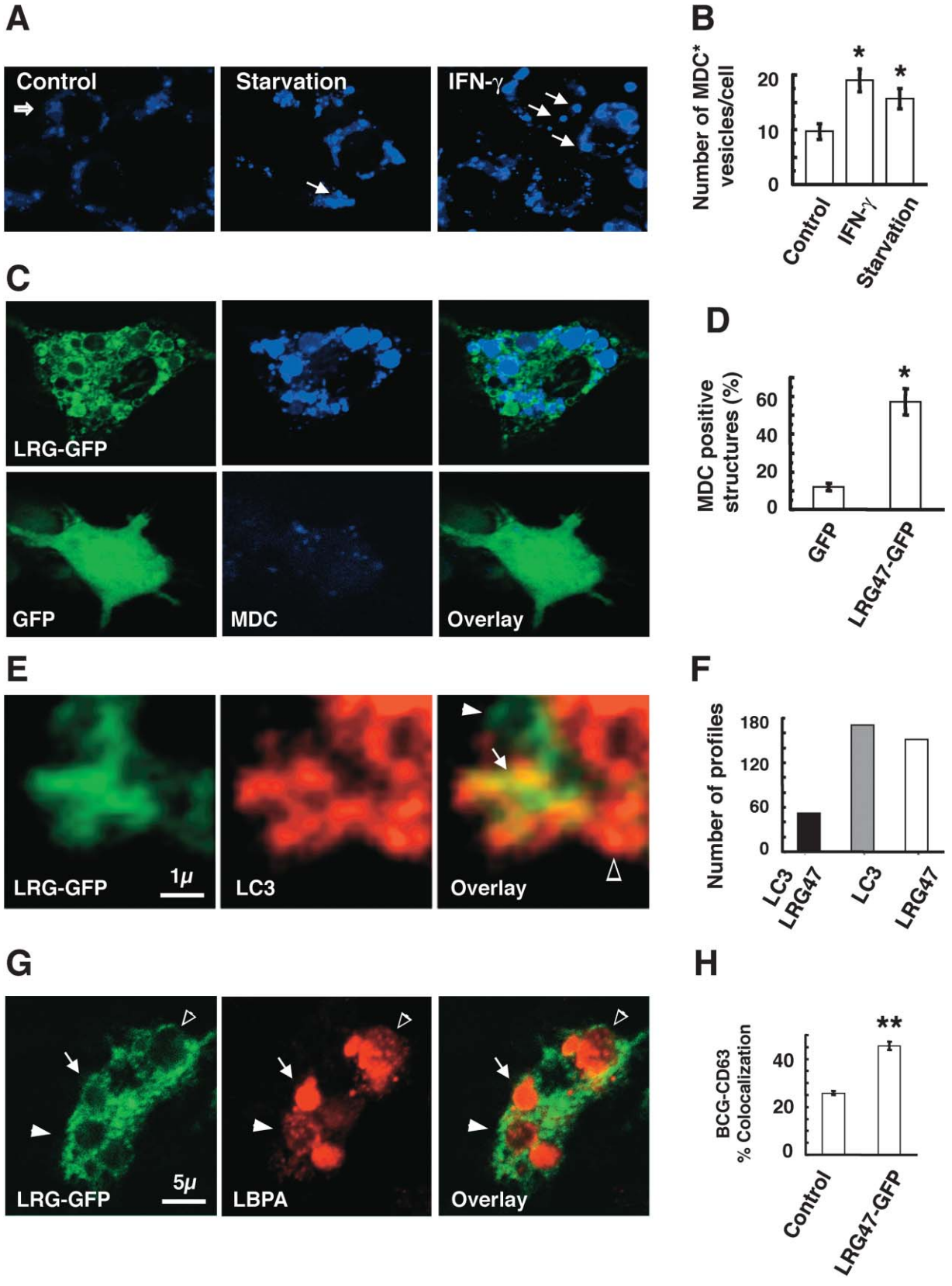


Figure 7. IFN-γ and its Effector LRG-47 Induce Monodansylcadaverine-Positive Autophagic Organelles in Macrophages

(A) Induction of monodansylcadaverine (MDC)-positive autophagic compartments in IFN-γ-activated cells. RAW 264.7 macrophages were incubated for 2 hr in full medium, starvation medium, or full medium + 200 U/ml IFN-γ, as indicated. Autophagic vacuoles were revealed by MDC staining. Control, untreated cells; Starvation, autophagy induced by starvation; IFN-γ, cells treated with IFN-γ. Filled arrows, MDC-

role for a heretofore unidentified effector of IFN-γ action besides the well-known oxidative enzymatic systems (MacMicking et al., 2003). Nitric oxide production upon IFN-γ activation can only partially explain antituberculosis action of this cytokine (MacMicking et al., 2003). Activation of cells with IFN-γ promotes phagosomal maturation by a mechanism that, until now, has remained undefined (MacMicking et al., 2003; Schaible et al., 1998; Via et al., 1998). Recently, it has been shown that IFN-γ effects occur at least in part through a novel small GTPase, LRG-47 (MacMicking et al., 2003). LRG-47 is a member of the family of p47 resistance GTPases, which are among the most potent known protective factors against intracellular parasites (Taylor et al., 2004). The p47 GTPases are strongly inducible in macrophages by IFN-γ. Within this group of cytoplasmic GTP binding proteins, LRG-47 has been singled out as the only one that is specifically active against *M. tuberculosis* (MacMicking et al., 2003). In vivo studies have demonstrated that LRG-47 knockout mice fail to control *M. tuberculosis* infection and that this effect was independent of production of reactive nitrogen and oxygen intermediates (MacMicking et al., 2003). However, the mechanism of action of LRG-47 was not previously known (Taylor et al., 2004), although involvement of membrane trafficking pathways had been implicated (MacMicking et al., 2003; Taylor et al., 2004). Our data suggest that LRG-47 may participate in IFN-γ-dependent induction of autophagy. LRG-47 induced the formation of large organelles with autophagolysosomal properties. These compartments were highly acidic and colocalized 100% with LysoTracker. They also displayed proteolytic activity based on intense BODIPY TR-X dequenching of DQ Red BSA in these organelles, which is a sign of active protein hydrolysis (data not shown). While LC3 was present on small LRG-47 profiles, it was most likely released from LRG-47 organelles as they matured into autophagolysosomes. This has been previously described and explained by either degradation of LC3 or its recycling back to the cytosol (Kabeya et al., 2000).

The work presented here identifies autophagy as a specific intracellular trafficking and organelle biogenesis process that represents the thus-far elusive reactive nitrogen- and oxygen-independent pathway of IFN-γ antimicrobial action. IFN-γ induction of autophagy has not been previously reported in immune or phagocytic cells but has been observed in HeLa cells (Inbal et al., 2002). The finding that IFN-γ, a quintessential cytokine associated with protective innate and adaptive immunity against tuberculosis (Flynn and Chan, 2001), induces, as shown here, autophagy in macrophages is an important new connection between immune mediators and protection against intracellular pathogens.

Mycobacteria-containing phagosomes colocalize with Beclin 1, an autophagy-promoting subunit of the hVPS34 PI3K complex (Kihara et al., 2001a, 2001b; Liang et al., 1999). Thus, it is likely that, under starvation conditions, mycobacterial phagosomes recruit Beclin 1 complexed with PI3-kinase. Phagosomal colocalization with Beclin 1 in cells induced for autophagy approaches 60% of all mycobacteria-containing vacuoles, matching closely the overall reduction in mycobacterial viability. This suggests the existence of a functional overlap between autophagy and phagosome maturation pathways, based on hVPS34 as a common denominator.

Two nonmutually exclusive models could explain how autophagy promotes mycobacterial phagosome maturation and associated intracellular killing of the pathogen. In the model that we favor, consistent with our electron microscopy observations, mycobacterial phagosomes are subsumed by the autophagic pathway and become subject to a merger between autophagosomes and late endosomes/lysosomes (Dunn, 1990). Consistent with participation of this process is the increased colocalization (from 5% to 30%) of mycobacterial phagosomes with the highly specific autophagic membrane marker LC3 in infected macrophages induced for autophagy. Since autophagosomes have a short half-life ($t_{1/2} = 8$ min) (Yoshimori, 2004), this could explain the observation that not all or the majority of mycobacterial phagosomes label with LC3, as, at a steady state, only a fraction of phagosomes would be positive for markers specific for a given stage along the autophagic pathway. Our ultrastructural analysis supports this model.

In an alternative scenario, stimulation of autophagic pathways may result in the generation of a more robust PI3K hVPS34 enzyme capable of overcoming *M. tuberculosis*-imposed block without necessarily invoking a complete merger with the autophagosomal pathway. Since hVPS34 is essential for both phagosomal maturation (Fratti et al., 2001; Vieira et al., 2001) and autophagy (Petiot et al., 2000), it is difficult to separate the two

stained autophagosomes; open arrow, background MDC staining.

(B) Quantification of MDC-positive autophagic organelles. Shown are mean values (number of MDC^+ vacuoles per cell) and SE. *$p < 0.05$.

(C) Transfection with LRG-47 induces MDC-positive autophagic organelles in macrophages. RAW 264.7 cells were transiently transfected with LRG-47-GFP (LRG-GFP) or with GFP only (GFP) and MDC-positive structures revealed as in (A).

(D) Quantification shows % colocalization between LRG-47-GFP and MDC-positive organelles (30 cells, three independent experiments, *$p < 0.05$).

(E) Partial colocalization of LRG-47 and small LC3-positive profiles. Shown is a portion of the cytoplasm in a cell displaying partial colocalization of LRG-47 and endogenous LC3 on organelles in LRG47-GFP-transfected macrophages. Endogenous LC3 was revealed by immunofluorescence. Arrow, LRG-47-GFP and LC3 colocalization; filled triangle, LRG-47-GFP-positive, LC3-negative profile; open triangle, LC3-positive, LRG-47-GFP-negative organelle. Scale bar, 1 μm.

(F) Graph, quantitative analysis (number of profiles) of organelles with LC3 and LRG-47 colocalization (LC3 LRG47) or positive solely for LC3 (LC3) or LRG-47 (LRG47).

(G) Internal membranes in LRG-47-positive vacuoles were revealed by LBPA staining. Arrow, homogenously LBA-stained vacuole; triangles, LRG-47 positive vacuoles with internal LBPA-positive membranes of variable shape and size. Scale bar, 5 μm.

(H) Increased maturation of mycobacterial phagosomes in macrophages transfected with LRG-47. Shown is quantification (100 phagosomes from three independent experiments, **$p < 0.01$) of CD63-positive BCG phagosomes in RAW 264.7 cells transiently transfected with LRG47-GFP relative to untransfected controls. CD63 was visualized by immunofluorescence using anti-CD63 antibody.

actions of PI3K in this context. Nevertheless, even when enhanced fusion between mycobacterial phagosomes and late endosomes was evident (Figure 5E), such events occurred with mycobacteria already localized in autophagosomes. This notion is in keeping with the known autophagosome ability to fuse with late endosomal multivesicular bodies (Dardalhon et al., 2002). Regardless of whether hVPS34 enhanced fusogenicity of mycobacterial phagosomes, in our experiments, autophagy was an essential element of antimycobacterial activity supported by the following evidence: (1) the gold standard inhibitor of autophagy 3-MA reversed all cell biological and bactericidal effects in our experiments, while inhibitors of other processes did not; (2) ultrastructural analysis showed that mycobacterial vacuoles transited into autophagosomes upon induction of autophagy; and (3) quantitative analysis with LC3, a specific marker for autophagy, showed that mycobacteria-containing organelles were autophagosomes.

The effects of autophagy on intracellular pathogens (Kirkegaard et al., 2004) are not limited to the inhibition of mycobacterial survival shown here. Recently, it has been reported that induction of autophagy can also help eliminate *Listeria monocytogenes* upon its escape from the phagosome into the cytosol (Rich et al., 2003). We propose that autophagy represents a previously underappreciated innate immunity mechanism that can be used by the host cells to eliminate intracellular pathogens. Developing means of selectively inducing autophagy in infected cells should be viewed as a new window of opportunity in dealing with hard-to-eliminate intracellular pathogens.

Experimental Procedures

Cell and Bacterial Cultures

RAW 264.7 cells were maintained in D-MEM 10% FBS (full nutrient medium). Murine BMM were isolated from the femurs of C57/BL6 mice and maintained in DME supplemented with 4 mM L-glutamine, 20% FBS, and m-CSF for 7 days. Human MDM were prepared from normal individual donors by density gradient centrifugation (400 $\times$ g for 30 min) through a Ficoll-Hypaque gradient (Pharmacia). Mononuclear cells were harvested, and 5×10^4 PBMCs were plated for 45 min. Adherent monocytes were matured into macrophages by 5 days' incubation. Cell viability was >95%. *M. tuberculosis* H37Rv and BCG, harboring phsp60-gfp or phsp60-dsRed, were grown in Middlebrook 7H9 broth or on 7H11 plates with 0.5% Tween, 0.2% glycerol, and albumin-dextrose-catalase supplement (+ oleic acid for H37Rv) and homogenized to generate a single cell suspension.

Antibodies, Cytokines, Fluorescent Dyes, and DNA Constructs

The antibody against cathepsin D (Transduction Laboratories) was used at a 1:250 dilution. A polyclonal rabbit antibody to V_0-16 kDa (16 kDa subunit of the vacuolar H^+-ATPase) was from M. Skinner (University of Guelph, Canada) and used at 1:500 dilution; the mouse polyclonal anti-LAMP-1 antibody (Development Studies Hybridoma Bank, Iowa City) was used at 1:200 dilution; the mouse monoclonal antibody to LBPA from Dr. J. Gruenberg (University of Geneva,) was used at a dilution of 1:500; and the rabbit polyclonal antibody against FLAG (Sigma) was used at 5 μg/ml. The Alexa-568- and Alexa-448-conjugated secondary antibodies were from Molecular Probes. Staining with the acidotropic dye LysoTracker Red DND-99 (Molecular Probes, Eugene, OR) was carried out as previously described (Via et al., 1998). Monodansylcadaverine, a fluorescent dye known to accumulate in autophagic organelles (Biederbick et al., 1995), was used as previously described (Gutierrez et al., 2004). The plasmid pSG5-FLAG-Beclin was from Dr. Beth Levine (Columbia University). pEGFP-LC3, pEGFP-LC3Δ22CG120A, and pEGFP-LC3G120A were from Drs. Noboru Mizushima and Tamotsu Yoshimori (National Institute for Basic Biology, Okazaki, Japan). Murine IFN-γ, IFN-α, and IFN-β were from Sigma.

Induction of Autophagy

Autophagy was induced by amino acid and serum starvation. Cells were washed three times with PBS at 37°C and incubated in 2 ml Earle's balanced salts solution (starvation medium) at 37°C for 2 hr. Alternatively, autophagy was triggered by treatment with rapamycin (25–50 ng/μl) for 2 hr in full nutrient medium (Noda and Ohsumi, 1998).

Macrophage Transfection by Nucleoporation

A procedure for efficient transient transfection of macrophages (Chua and Deretic, 2004) was applied. RAW 264.7 cells were harvested at day 2 of culture and resuspended in the electroporation buffer to a final concentration of 2×10^7 cells/ml. Plasmid DNA (10 μg) was mixed with 0.1 ml of cell suspension, transferred to an electroporation cuvette, and nucleofected with an Amaxa Nucleofector apparatus (Amaxa). After electroporation, cells were transferred to 2.0 ml of complete medium and cultured for 12 hr.

Fluorescence Confocal Microscopy

Cells were fixed with −20°C methanol (10 min) and then with −20°C acetone (1 min). Cells were permeabilized and blocked for 1 hr in PBS containing 2% BSA and 0.2% saponin and then incubated with the primary antibodies followed by secondary antibody. The coverslips were mounted in Permeafluor Aqueous mounting medium (Immunon) and examined by confocal microscopy using the Zeiss 510 Laser Scanning Microscope.

Transmission Electron Microscopy

Infection of RAW 264.7 cells with wild-type BCG was carried out as described above and then subjected to full medium or starvation media. After 2 hr, cells were fixed in 1% glutaraldehyde in 0.1 M cacodylate buffer (pH 7). Fixed monolayers were scraped; postfixed in 2% osmium tetroxide, 100 mM cacodylate buffer; dehydrated with increasing concentrations of ethanol; and gradually infiltrated with Epon resin (Pelco). Thin sections were stained with uranyl acetate and lead citrate and examined using a Zeiss EM 900 transmission electron microscope.

Colony-Forming Unit Assay

RAW 264.7 cells were plated in 12-well plates (2×10^5 cells per well). Cells were infected with 2×10^6 mycobacteria for 1 hr at 37°C, washed two times with PBS, and incubated 2 hr in full medium in the presence or absence of rapamycin (50 μg/ml) or in starvation medium. 3MA (10 mM) or wortmannin (100 nM) was added where indicated. Cells were lysed with 1 ml of water. Quantitative culturing was performed using 10-fold serial dilutions. Aliquots of 5 μl of each dilution were inoculated on Middlebrook 7H10 agar plates with OADC. Plates were incubated for 2 weeks. For each time point, counts were made from three different wells. Colonies were counted on plates with dilutions yielding 10–50 visible colonies.

Proteolysis of Long-Lived Proteins

RAW 264.7 cells were plated at 7×10^5 cells/well in a 24-well plate. Cells were labeled for 24 hr in media containing 1 μCi/ml [^{3}H] leucine (Amersham Biosciences), washed to remove unincorporated label, and pulsed for 1 hr in full medium containing cold leucine to allow degradation of short-lived proteins. Cells were incubated in either amino acid-free or full nutrient medium (+ or − IFN-γ) for 6 hr. Trichloroacetic acid (TCA)-precipitable radioactivity of the cells monolayers and the TCA-soluble radioactivity in the media were determined. Leucine release was calculated as a ratio between TCA-soluble supernatant and total cell-associated radioactivity (Mizushima et al., 2001; Talloczy et al., 2002).

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