Mycobacterium marinum induces a marked LC3 recruitment to its containing phagosome that depends on a functional ESX-1 secretion system

María Cecilia Lerena and María Isabel Colombo*
Laboratorio de Biología Celular y Molecular – Instituto de Histología y Embriología (IHEM), Facultad de Ciencias Médicas, Universidad Nacional de Cuyo–CONICET, Mendoza, Argentina.

Summary
Autophagy has been implicated as part of the innate immune system against different intracellular microorganisms. Mycobacterium marinum is the causative agent of the fish-tank granuloma and has been widely used as an alternative model to study pathogenic mycobacteria. In this report, we show an active interaction of M. marinum with the autophagic protein LC3, an event that requires pathogen viability and bacterial protein synthesis. Interestingly, M. marinum lacking the region of difference 1 (RD1) is unable to recruit LC3, indicating that a functional ESX-1 secretion system is an absolute requirement for this process. In addition, phagocytosis of the bacteria is also a condition for the LC3 rearrangement induced by M. marinum. We present evidence that this pathogen resides temporarily in a LC3-decorated compartment with late endocytic features but mostly devoid of lysosomal enzymes or degradative properties. In addition our results indicate that autophagy induction by rapamycin treatment leads to maturation of the M. marinum-containing compartment.

Introduction
Mycobacterium tuberculosis, one of the most challenging threats affecting human health worldwide, infects one-third of the world population and is responsible for around 1.7 million deaths every year (WHO, 2008). Innate immunity is the first barrier that M. tuberculosis comes across, and it is represented by alveolar macrophages that phagocyte the pathogen in an attempt to control the infection. However, M. tuberculosis is able to manipulate the fate of its containing phagosome; it blocks phagosome maturation, avoiding the fusion with lysosomes resulting in a non-hostile environment where the bacterium survives and replicates (Deretic et al., 2006; Russell, 2007).

Mycobacterium marinum is a natural pathogen of frogs and fishes that can eventually infect humans. Similarly to M. tuberculosis, after infecting host macrophages, M. marinum causes a tuberculosis-like disease in ectotherms. M. marinum is closely related to M. tuberculosis, with more than 85% nucleotide identity (Stinear et al., 2008). As human infections are limited to cool surfaces, such as skin and extremities, M. marinum can be easily manipulated in a BSL2 laboratory (Tobin and Ramakrishnan, 2008). All these features have made M. marinum a widely accepted model to study mycobacterial infections.

Autophagy is a self-degradative process that acts as a housekeeping mechanism in charge of getting rid of unwanted material such as old/damaged organelles from the cell’s cytoplasm. Under nutrient deprivation conditions, autophagosomes recycle cellular components in order to obtain the necessary nutrients (Yoshimori, 2004). A double-membrane structure (i.e. isolation membrane) surrounds and sequesters the material to be removed, forming an autophagosome that finally fuses with lysosomes to degrade the incorporated material. Autophagy has an important role in innate immunity, detecting and eliminating intracellular pathogens. However, the interplay between intracellular pathogens and the autophagy pathway is frequently very complex (Levine and Deretic, 2007; Campoy and Colombo, 2009). For instance, some pathogens surrender to autophagy and are degraded when this pathway is activated, such is the case of M. tuberculosis and Streptococcus pyogenes (Gutierrez et al., 2004; Nakagawa et al., 2004). In contrast, for some other microorganisms autophagy induction can benefit pathogen survival inside the cell, as it has been demonstrated for Staphylococcus aureus and Coxiella burnetii (Gutierrez et al., 2005; Schnait et al., 2007; Vazquez and Colombo, 2009b; Mestre et al., 2010).

The studies on the interplay between mycobacteria and autophagy were initiated in 2004 when our group actively participated in demonstrating the role of the autophagic

Received 22 September, 2010; revised 3 January, 2011; accepted 19 January, 2011. *For correspondence. E-mail mcolombo@fcm.uncu.edu.ar; Tel. (+54) 261 4494143 Ext. 2690; Fax (+54) 261 4494117.

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pathway in the elimination of the invading intracellular *M. tuberculosis* and *M. bovis* BCG as part of the innate immune system (Gutierrez et al., 2004). As mentioned before, *M. marinum* has been extensively used as an alternative model to study mycobacteria, thus we sought to determine the behaviour of *M. marinum* with regard to autophagy. One of the most intriguing hallmarks of *M. marinum* infection is that it is able to escape from its containing phagosome and to develop actin tails, freely moving in the host’s cytoplasm (Stamm et al., 2003; Collins et al., 2009). Noteworthy, it was demonstrated that *M. tuberculosis* is also able to escape from the phagosome after 24 h of infection (van der Wel et al., 2007), although no evidence on the development of actin tails by this pathogen has been presented. The first approximation on the study of the interaction of *M. marinum* and autophagy was performed by Collins et al. This recent publication by Eric Brown and collaborators has demonstrated that after escaping from its containing phagosome, a proportion of cytosolic *M. marinum* is ubiquitinated in the cytoplasm and reincorporated into membrane bound compartments, in a reuptake process independent of Atg5, an essential autophagic protein (Collins et al., 2009). In the present report, we have studied in detail the characteristics of the interaction of *M. marinum* with the autophagic pathway. We have observed that a substantial fraction of *M. marinum*-containing phagosomes actively recruits the autophagic protein LC3, regardless of the nutritional conditions of the culture media. These LC3-positive compartments have features of late endocytic compartments, but do not acquire lysosomal enzymes or degradative properties. Therefore, *M. marinum* is able to block autophagosomal as well as phagosomal maturation, even in cells in which the autophagic pathway has been activated by starvation. Of note LC3 recruitment depends on bacterial viability and a functional ESX-1 secretion system.

**Results**

**GFP-LC3 is recruited to *M. marinum*-containing phagosomes**

As previously demonstrated, *M. tuberculosis*, as well as *M. bovis* BCG, does not normally colocalize with LC3. However, autophagy induction by rapamycin treatment or starvation leads to the maturation of mycobacterium-containing phagosomes and the acquisition of autophagic features, with the concomitant decrease in bacterial survival (Gutierrez et al., 2004). Therefore, we first asked if, similar to *M. tuberculosis*, there was interplay between *M. marinum* and the autophagic pathway. For this purpose, Raw 264.7 macrophages (Raw cells) were infected for 1 h, and subsequently incubated for 2 h under different experimental conditions (i.e. incubation in full medium, Ctrl; or starvation conditions: amino acid-serum-deprived medium, Stv), and the distribution of endogenous LC3 was detected by indirect immunofluorescence. A clear recruitment of endogenous LC3 to the **M. marinum**-containing phagosome was observed (Fig. 1A). The fluorescence intensity measured across a line scan on a **M. marinum**-containing phagosome (panel h) showed a noticeable increase in fluorescence at the periphery of the compartment (Fig. 1B). Surprisingly, LC3 recruitment was similar in both Ctrl and Stv media, suggesting that *M. marinum* was somehow stimulating the autophagic response even in control conditions.

We next evaluated if overexpressed GFP-LC3 protein behaved similar to its endogenous partner. In this case, Raw macrophages stably transfected with GFP-LC3 were infected with *M. marinum* expressing red fluorescent protein (M. marinum-RFP), following the same experimental procedure as indicated above. Cells were subjected to control (Ctrl), starvation (Stv), starvation medium in the presence of wortmannin (Wm), a well-established inhibitor of autophagy (Stv + Wm); or full medium with the addition of Rapamycin, a pharmacological inducer of autophagy (Ctrl + Rp). As depicted in Fig. 1C, a remarkable association of GFP-LC3 with the membrane of the *M. marinum*-containing phagosome was observed. Similar to the results observed with endogenous LC3, in the control condition the association level was comparable to that of the starvation condition (see quantification in Fig. 1D). In the presence of wortmannin the level of this interaction was markedly decreased, whereas upon autophagy induction with rapamycin this association reached the highest levels (Fig. 1D). As expected, no association with the GFP vector alone, used as control, was observed (Fig. 1F).

We next addressed the kinetics of LC3 recruitment to the bacteria-containing phagosomes. For this purpose, Raw-GFP-LC3 macrophages were infected with *M. marinum*-RFP for different periods of time (15–270 min). As shown in Fig. 1E, no association was observed at 15 min of infection; however, by 30 min LC3 was strongly recruited to *M. marinum* phagosomes, suggesting that a fast response is induced at that post-infection time. The association with GFP-LC3 reached the highest level between 60 and 90 min, and gradually decreased towards 270 min of infection. These results indicate that a substantial fraction of the **M. marinum**-containing phagosomes strongly recruit GFP-LC3 as well as the endogenous LC3 protein at relatively early post-infection times.

**M. marinum resides transiently in single-membrane phagosomes**

Given the clear association between *M. marinum*-containing phagosomes and LC3, we wondered if the
bacteria were harboured within double-membrane structures, a typical feature of autophagosomes. Thus, we analysed by electron microscopy Raw cells infected with *M. marinum* at different time points. We observed that at 90 min of infection the majority of the infecting bacteria were inside single-membrane phagosomes (91.1 ± 0.06%, data not shown) (Fig. 2A). In the same picture, a typical double-membrane autophagosome is shown next to the bacterium-containing phagosome (white arrow, Fig. 2A). As indicated, most bacteria were inside typical phagosomal structures, with a clear halo surrounding the bacteria, followed by a unique bilayer. Nonetheless, it is important to consider that as autophagosomes mature and fuse with other compartments they turn into single-membrane structures (e.g. amphisomes). In order to confirm the association of the autophagic pathway with *M. marinum*-containing phagosomes we analysed this process by live cell imaging. Raw GFP-LC3 macrophages were infected with *M. marinum* for 1 h (uptake) and followed by 2 h incubation in full medium conditions (chase). Afterwards, cells were analysed using time-lapse imaging. We were able to occasionally visualize a direct fusion of an autophagosome with a phagosome harbouring *M. marinum*, which confirmed for the first time a clear interaction between phagosomes containing *M. marinum* and LC3-labelled autophagic structures (Fig. S1 and Movie S1).

The electron microscopy studies also showed that at 2 h of infection a subset (approximately 20%) of the infecting bacteria was free in the cytoplasm (black arrow in Fig. 2B). Exceptionally, a few bacteria were observed surrounded by double membranes by 4 h of infection (Fig. 2C). This compartment appears to have cytoplasmic material within, suggesting a possible attempt of the cell to engulf a cytoplasmic pathogen (Fig. 2C, white arrowheads). In addition, some bacteria free in the cytoplasm can be seen at the same post-infection time (Fig. 2C, black arrows). The quantification of the number of bacteria free in the cytoplasm is shown in Fig. 2D. By 24 h the large majority of the bacteria were free in the cytoplasm. Altogether, these results suggest that *M. marinum* transiently resides in single-membrane compartments and, as early as 2 h after infection, it starts escaping to the cytoplasm.

In order to confirm that by 3 h of infection (1 h uptake plus 2 h chase) most bacteria have not yet reached the cytoplasm, and to discard that the pathogen has been reincorporated into membrane-bound compartments, in an autophagy-dependent reuptake process, we assessed the ubiquitination of intracellular *M. marinum*. It has been suggested that many bacteria that gain access to the cytosol can be detected by the ubiquitin system (Perrin *et al*., 2004; Collins and Brown, 2010). Indeed, in a recent publication, Collins *et al.* showed that a fraction of cytosolic *M. marinum* is tagged by the ubiquitin system, and 96% of the ubiquitinated bacteria reside in the cytosol (Collins *et al*., 2009). To determine the presence of ubiquitin, Raw GFP-LC3 cells were infected with *M. marinum* for 1 h, followed by 2 h of chase in full medium, and ubiquitin was detected with an appropriate antibody (Fig. S2). Interestingly, in regard to ubiquitin labelling of GFP-LC3-decorated bacteria, we observed three different situations: (i) bacteria surrounded by GFP-LC3 with no labelling of the pathogen with ubiquitin; which was the most frequent observation (near 70%), (ii) bacteria surrounded by GFP-LC3 with a strong ubiquitin labelling of the pathogen, which was quite unusual (only 4%), and (iii) bacteria surrounded by GFP-LC3 decorated phagosome but absent inside that compartment (25%). Thus, as indicated above, only a tiny percentage (near 4%) of bacteria surrounded with GFP-LC3 were labelled with ubiquitin, confirming that most intracellular bacteria lack ubiquitin labelling at the analysed post-infection time (i.e. 3 h). This would indirectly suggest that the GFP-LC3 membrane-bound compartments contain *M. marinum* that had not previously escaped to the cytoplasm and therefore, was not accessible to the ubiquitin system.
M. marinum resides transiently in a GFP-LC3-labelled compartment with late endosomal features

Given the evident interaction between M. marinum and LC3, we next characterized in detail the LC3-positive compartments sheltering bacteria. For this purpose, Raw macrophages overexpressing GFP-Rab5 (an early endo/phagosomal marker) or GFP-Rab7 (late endo/phagosomal marker) were infected with M. marinum-RFP for 1 or 2 h in full medium. As shown in Fig. 3A and D, no association with GFP-Rab5 was observed, suggesting that M. marinum does not reside in an early phagosomal compartment at these infection times. In contrast, almost 80% of the bacteria were found within compartments clearly labelled by Rab7 (Fig. 3A and D). In addition, Raw GFP-LC3 macrophages were infected with M. marinum and the late endosomal marker LAMP-2 was detected by immunofluorescence. As depicted in Fig. 3B, in cells incubated under full nutrient medium with or without rapamycin M. marinum-containing phagosomes were strongly

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M. marinum avoids fusion with lysosomes

The late endosomal characteristics of the large GFP-LC3-positive compartment containing M. marinum led us to ask if it had also lysosomal features. For this purpose, we detected the lysosomal enzyme cathepsin D in Raw-GFP-LC3 macrophages infected with M. marinum for 1 h, followed by a 2 h chase in full medium. We observed that most GFP-LC3 structures harbouring bacteria acquired the acidotrophic marker, suggesting that those were acidic compartments (Fig. 3C). Therefore, in consistence with the observations obtained using Rab7, LAMP-2 and Lysotracker red, M. marinum appears to reside in a compartment with features of a late phagosomal compartment at the times observed.

To confirm that those phagosomes sheltering M. marinum did not fuse with lysosomes, samples were analysed by electron microscopy. Lysosomes were labelled with colloidal-gold particles coated with mannose-BSA (BSA-Au), which were incorporated by Raw macrophages through endocytosis. Lysosomes were easily identified as they were loaded with the colloidal-gold particles (black arrowheads Fig. S3A) and we observed that most bacteria-containing phagosomes (90 min post infection) did not contain colloidal gold (Fig. S3A and B). Exceptionally, we observed phagosomes containing M. marinum in the process of fusion with lysosomes (Fig. S3C and D).

We next addressed whether conditions that modulate autophagy may affect the localization of M. marinum. Cells were infected following the same approach used in Fig. 1B (1 h uptake + 2 h chase), and Cathepsin D was detected by immunofluorescence (Fig. 4B and C). Surprisingly, very limited colocalization with Cathepsin D was observed in cells subjected to Stv (black bar), indicating that autophagy induction by starvation is unable to alter the typical features of the M. marinum-containing phagosomes. In striking contrast, in cells incubated with rapamycin a remarkable increase in the colocalization with Cathepsin D was observed (Fig. 4C, black bar). All together, these results suggest that M. marinum normally resides in a late phagocytic compartment labelled by GFP-LC3, but that is devoid of lysosomal enzymes. Strikingly, this localization pattern is not modified by starvation-induced autophagy but is remarkably affected by rapamycin treatment.

In order to confirm that the GFP-LC3-positive compartment containing M. marinum was devoid of lysosomal degradative capacity, the self-quenched red Bodipy dye conjugated to BSA (DQ-BSA) was used. This probe is incorporated by cells through endocytosis and it only fluoresces upon proteolytic degradation within lysosomal structures (Vazquez and Colombo, 2009a). Cells were next infected with live Alexa-633-labelled M. marinum for 60 min in full medium in the absence or presence of rapamycin. Live images are depicted in Fig. 5 and in agreement with the above results showing the absence of Cathepsin D, we observed that only a fraction (around 30%) of those GFP-LC3 compartments containing live bacteria had DQ-BSA labelling (Fig. 5). Thus, M. marinum would be modulating the fate of its own GFP-LC3-labelled phagosome probably by hampering fusion with lysosomes. However, when macrophages were infected with bacteria in the presence of rapamycin, most GFP-LC3 structures containing bacteria acquired proteolytic capacity (Fig. 5A and B). This suggests that upon autophagy induction with rapamycin, the blockage of the fusion with lysosomes imposed by M. marinum is overridden.

All these data suggest that a substantial fraction of the intracellular population of M. marinum resides in a GFP-LC3-decorated compartment that, despite its acidic and late-endosomal features, has a limited degradative capacity.

In order to address whether the rapamycin effect was indeed due to its capacity to act as a potent autophagy inducer we used the following approach. We performed an assay labelling with Lysotracker at 3 or 9 h of infection (1 h uptake +2 or 8 h chase). We have observed that in Raw macrophages overexpressing GFP-LC3, by 3 h of infection most M. marinum resides in a compartment labelled with Lysotracker indicating that the pH was acidic (Fig. 3C). In agreement with the observations of Brown and collaborators (Collins et al., 2009), we have also observed that as early as 2 h of infection a small proportion of M. marinum is able to escape from the phagosome. It is likely that before escaping to the cytoplasm, bacteria must damage the phagosomal membrane (e.g., piercing the membrane) altering ion gradients in the phagosome. Therefore, if a phagosome is ‘damaged’ its content would have a pH similar to the cytosol, and should no longer be labelled with Lysotracker. Therefore, we quantified bacteria colocalizing with Lysotracker at 2 or 8 h post infection (chase), using live M. marinum; heat-inactivated bacteria (HK); live M. marinum adding rapamycin during the chase; or live M. marinum adding

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both rapamycin and the autophagy inhibitor wortmannin during the chase. Bacteria were observed by phase contrast since it was practically impossible to visualize Alexa-633-labelled bacteria by 8 h of chase (the dye seems to be lost by that post-infection time). As expected a decrease in Lysotracker labelling at 8 h of infection was observed for live *M. marinum*, as these bacteria were able to escape from their phagosome (Fig. S4A a–d and quantification in B). In contrast in the case of HK *M. marinum*, no variation on the rate of colocalization was observed between 2 and 8 h of chase. This was an expected result as dead bacteria are unable to damage the phagosomal membrane (Fig. S4A e–h and quantification in B). With regard to rapamycin treatment no changes in Lysotracker labelling were observed between 2 and 8 h of chase, suggesting no phagosomal damage in cells treated with rapamycin (Fig. S4A i–l and quantification in B). Interestingly, the addition of wortmannin partially prevented the effect of rapamycin (Fig. S4A m–p and quantification in B). This partial but consistent decrease in Lysotracker labelling by 8 h of infection suggests that the effect caused by rapamycin is likely due to autophagy induction.

In order to confirm these observations we also assessed the colocalization with GFP-LC3 as well as Cathepsin D. Raw macrophages were infected with *M. marinum* for 1 h (uptake) followed by 2 h chase in control (Ctrl), in the presence of rapamycin (Rp) or in the presence of rapamycin plus wortmannin (Fig. S4C and quantification in D). A significant decrease in colocalization of the bacteria with GFP-LC3 and Cathepsin D was observed when both drugs were added together during the chase period (black bars). This result confirms that the rapamycin effect on phagosomal maturation and LC3 acquisition occurs via autophagy induction, since it was partially inhibited by blocking PI3K activity with wortmannin.

**LC3 recruitment to the *M. marinum*-containing phagosome depends on bacterial viability**

As indicated earlier, autophagy has been associated with the innate immune response acting as a defence mechanism to control intracellular bacterial infection. Thus, we wondered whether this targeting of LC3 to the bacteria-containing phagosomes was a cellular response to the invading pathogen, in an attempt to control or restrict bacterial infection. We postulated that perhaps LC3-decorated compartments may contain dying or damaged bacteria targeted by autophagy. To test this hypothesis we infected Raw-GFP-LC3 macrophages with heat-inactivated bacteria (HK) following the same protocol performed with live bacteria. Surprisingly, a significant decrease in the recruitment of GFP-LC3 was observed in Stv as well as in Ctrl conditions, similar to that observed in the cells treated with wortmannin, suggesting that live bacterium may induce a cellular response leading to the recruitment of GFP-LC3 to its phagosome (Fig. 6A and B). However, upon rapamycin stimulus, the recruitment of GFP-LC3 to HK *M. marinum*-containing phagosomes reached similar levels to the ones detected for live bacteria.

Next, we wondered whether bacterial protein synthesis was required. To address this question, we used two antibiotics, chloramphenicol (CMP) and rifampicin (RIF), which are inhibitors of protein synthesis. Bacteria were incubated overnight in the presence of 100 μg ml⁻¹ CMP or 40 μg ml⁻¹ RIF. Raw GFP-LC3 macrophages were infected with both sets of bacteria following the same experimental procedure as indicated in Fig. 1. As shown in Fig. 6C (RIF) and D (CMP) a marked decrease in the percentage of phagosomes decorated with GFP-LC3 in both Ctrl and Stv was observed. In contrast, similar to the results obtained with HK *M. marinum*, in the presence of rapamycin a large percentage of antibiotic-treated bacteria localized in LC3-decorated phagosomes. Taken together, these results indicate that live bacteria are an absolute requirement for the localization of *M. marinum* in a LC3-labelled compartment. In contrast, rapamycin treatment, acting as a potent autophagy inducer, leads to a marked LC3 recruitment to the phagosomal membranes independently of bacteria viability.

Given the strong effect caused by the infection with *M. marinum* on GFP-LC3 distribution, we wondered whether the infection with the bacteria was able to activate autophagy and hence induce the processing of LC3. Upon autophagy activation LC3 is processed from a cytosolic
Fig. 4. *M. marinum*-containing phagosomes do not acquire the lysosomal enzyme Cathepsin D.

A and B. Raw GFP-LC3 cells were infected with *M. marinum*-RFP for 1 h (A) or 1 h of uptake followed by 2 h of chase (B) in Ctrl (a–e), Stv (f–j), Stv + Wm (k–o) or Ctrl + Rp (p–t). Endogenous Cathepsin D was detected by immunofluorescence (blue) as described in Experimental procedures. Scale bars: 5 μm.

C. Quantification showing the percentage of bacteria associated with GFP-LC3, Cathepsin D or GFP-LC3 + Cathepsin D. Data are mean ± SEM of two independent experiments. Significantly different from the control: *P < 0.05; **P < 0.01; ***P < 0.001.

Fig. 5. The degradative properties of *M. marinum*-containing phagosomes increase upon rapamycin treatment.

A. Raw GFP-LC3 cells were loaded with DQ-BSA, as described in Experimental procedures, in order to label degradative compartments. Cells were infected with live *M. marinum* for 60 min in Ctrl (a–d) or Rp (e–h). Scale bars: 10 μm.

B. Quantification of the percentage of bacteria contained in GFP-LC3 + DQ-BSA-positive compartment in Ctrl (black bars) or in the presence of rapamycin (grey bars). Data are mean ± SEM of two independent experiments. Significantly different from the control: *P < 0.05.

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form (LC3-I) to a membrane-bound form (LC3-II) (Kabeya et al., 2000). Consequently, autophagy can be monitored by the conversion of GFP-LC3-I to GFP-LC3-II where the level of LC3-II in cell lysates is a way to measure autophagy activity (Rubinsztein et al., 2009). Therefore, we performed a Western blot assay in order to detect GFP-LC3 in its two forms: LC3-I and LC3-II which can be easily identified due to their different electrophoretic motility. Raw GFP-LC3 macrophages were incubated in Ctrl or Stv conditions, in the presence or absence of bafilomycin (BAF) in order to inhibit autophagosome acidification. BAF causes accumulation of LC3-II by preventing autolys-
The region of difference 1 (RD1) is a virulence gene cluster present in \textit{M. marinum} as well as in \textit{M. tuberculosis} and other virulent mycobacteria, but it is absent in the attenuated strain \textit{M. bovis} BCG (Pym et al., 2002; Lewis et al., 2003). RD1 codifies for the ESX-1 secretion system (Gao et al., 2004; Cosma et al., 2008). Thus, \textit{M. marinum} lacking the RD1 locus does not have a functional ESX-1 secretion system and hence, is unable to produce the secreted proteins ESAT-6 and CFP-10, which have a direct role in cytolyis and cytotoxicity (Smith et al., 2008). Moreover, \textit{M. marinum} ∆RD1 is not able to escape from its containing phagosome (Smith et al., 2008).

In our study, we have used a \textit{M. marinum} strain obtained from a clinical isolate, we also tested an ATCC \textit{M. marinum} strain, which is the one that has been used worldwide to study \textit{M. marinum} behaviour and to generate the defective mutant \textit{M. marinum} ∆RD1. Raw GFP-LC3 cells were infected with both sets of bacteria following the same approach as the one used in Fig. 1 (1 h of uptake + 2 h of chase). As shown in Fig. 7, the wild-type ATCC strain of \textit{M. marinum} behaved in the same way as the strain obtained from a clinical isolate. Near 35% of intracellular bacteria were found residing in a GFP-LC3-decorated compartment (Fig. 7A and C). When the infection was continued in the presence of rapamycin, this percentage reached almost 65%, similar to our previous observations with the local strain. However, when \textit{M. marinum} ∆RD1 was used, a dramatic decrease in the proportion of intracellular bacteria decorated with LC3 was observed; indeed, we hardly found GFP-LC3-surrounded bacteria (Fig. 7B and C). In contrast, when cells infected with mutant bacteria were chased in the presence of rapamycin, we observed a significant increase in the percentage of bacteria decorated with GFP-LC3. This result is consistent with our previous observation using HK or antibiotic-treated bacteria, in which rapamycin treatment also increased the level of GFP-LC3 recruiting bacteria, despite bacterial status.

We next assessed the ability of these strains to induce LC3 processing; thus, we performed a Western blot assay in order to detect endogenous LC3 in Raw macrophages infected with \textit{M. marinum} wild type (wt), \textit{M. marinum} ∆RD1, heat-killed \textit{M. marinum} or \textit{M. bovis} BCG (Fig. S5). As expected, we only observed a moderate although consistent increase in LC3-II levels in cells infected with \textit{M. marinum} wt, whereas no effect was observed in cells infected with \textit{M. marinum} ∆RD1, HK \textit{M. marinum} or BCG (Fig. S5A and B). In addition, we infected GFP-LC3 macrophages with BCG and found no recruitment or colocal-
This is in agreement with our previous work indicating no association of this strain with the autophagic protein LC3 in cells incubated under full nutrient conditions (Gutierrez et al., 2004).

Altogether, these results suggest that the RD1 is strictly necessary for *M. marinum* to induce GFP-LC3 recruitment towards its phagosome, and also that rapamycin treatment is able to bypass this requirement.

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**Fig. 7.** *M. marinum* (Mm) lacking the virulence cluster RD1 is unable to recruit LC3.

A. Raw GFP-LC3 cells were infected for 1 h using the ATCC strain of *M. marinum* wt previously labelled with rhodamine. The infection was chased for 2 h in Ctrl (a–c) or Ctrl + Rp (d–f). LC3 was seen decorating a proportion of *M. marinum*, whereas this percentage increased in the presence of rapamycin.

B. Raw GFP-LC3 cells were infected with the defective mutant *M. marinum* ΔRD1 in Ctrl (a–c) or Ctrl + Rp (d–f). No association of the mutant bacteria with LC3 is observed in control conditions. In contrast, *M. marinum* ΔRD1 is clearly decorated with LC3 upon rapamycin treatment.

C. Quantification showing the percentage of the local strain Mm RFP, as well as both ATCC strains: wt and ΔRD1 associated with GFP-LC3 in Ctrl or Ctrl + Rp conditions. Data represent the mean ± SEM of two independent experiments. Significantly different from the control: *P < 0.05; **P < 0.01; ***P < 0.001. Scale bars: 10 μm.

**Rapamycin induces LC3 recruitment to latex beads-containing phagosomes**

The fact that rapamycin induced LC3 recruitment to both live and HK bacteria-containing compartments suggests that this drug may lead to the delivery of LC3 to phagosomes regardless of its content. Therefore, we wondered if phagosomes containing inert latex beads...
could also acquire LC3 upon rapamycin treatment. Raw GFP-LC3 macrophages were allowed to phagocytose 3 μm latex beads coated with rabbit IgG for 30 min (uptake) and subsequently incubated for 90 min in Ctrl, Stv or Ctrl + Rp (Fig. 8). To differentiate internalized from extracellular beads, we incubated the slides with anti-rabbit Cy5 without previous permeabilization to label only the extracellular beads. We next evaluated the percentage of phagosomes recruiting GFP-LC3 in different experimental conditions. As shown in Fig. 8A, no recruitment of LC3, either in Ctrl or in Stv conditions, was observed. However, when rapamycin was added, phagosomes containing latex beads were strongly decorated with LC3. We also measured the fluorescence intensity across a line scan on the phagosome. No increment in the fluorescence of the boundaries of the phagosomal membrane was observed in Ctrl or Stv conditions, with respect to the basal cytoplasmic fluorescence. In contrast, a strong increment in the fluorescence intensity of LC3 in the phagosomal membrane was observed after rapamycin treatment (Fig. 8B).

Triggering of host cell autophagy by M. marinum depends on the phagocytosis of the bacterium

One possibility to explain the effect of M. marinum on LC3 distribution is that this pathogen may be using one of its type VII secretion systems to inject effectors from outside the cell (without the need of a phagocytic event) to induce the autophagic response. Therefore, we wondered if phagocytosis of the bacterium was indeed essential for the marked LC3 rearrangement observed in infected cells. In order to address this point different experimental approaches were used. First, the bacteria were grown in an appropriate axenic medium and then the filtered supernatant was incubated for 60 or 90 min with Raw GFP-LC3 macrophages. As another approach, we incubated a subset of cells with supernatant from infected cells. In order to determine if there was a factor released into the supernatant of infected cells capable of inducing the LC3 rearrangement, we performed the experiments at 17°C, which allows bacterial binding to the cell surface but blocks its internalization (data not shown).

The findings described above suggest that in order to trigger autophagy M. marinum must be phagocytosed by the host cell. Therefore, we next addressed if beads phagocytosed by macrophages infected at the same time with M. marinum could acquire GFP-LC3. For that purpose, Raw GFP-LC3 macrophages were allowed to phagocytose latex beads together with M. marinum for 30 min, followed by a chase of 90 min in Ctrl, Stv or in the presence of rapamycin. As shown in Fig. 8C and D, in Ctrl or Stv only those intracellular beads in close proximity to phagocytosed M. marinum recruited GFP-LC3. Interestingly, the phagosomes containing latex beads present in the same infected cell, but detached from M. marinum-containing phagosomes, did not recruit GFP-LC3. As expected, after rapamycin treatment all the latex-beads phagosomes were labelled with this protein independently of the presence of the internalized bacteria. Quantification of these observations is depicted in Fig. 8E showing a significant increase in the percentage of latex-beads phagosomes decorated with GFP-LC3 in cells infected with M. marinum, compared with latex beads alone in the absence of the pathogen. It is important to mention that this rapamycin effect was indeed dependent on the autophagic protein Atg5, as it was not observed when analysed in Atg5-deficient MEF cells (data not shown).

Cumulative data have shown that different innate immune pathogen recognition receptors (PPRs), such as toll-like receptors (TLRs), and cytoplasmic receptors like nod and nod-like receptors (NLRs), and RIG-I and RIG-I-like receptors (RLRs) are in charge of detecting the presence of intracellular bacteria, recognizing pathogen-associated molecular patterns ‘PAMPs’ (Medzhitov, 2007). PPRs are able to trigger signals through PAMPs recognition that finally lead to induction of autophagy (Delgado et al., 2008). Thus, it is likely that M. marinum could be detected intracellularly by PPRs, generating signals that are able to activate the autophagic pathway. Many PAMPs have been identified in mycobacteria, and the glycolipid lipoarabinomannan (LAM) is one of them (Means et al., 1999). Thus, we allowed GFP-LC3 cells to phagocytose LAM-coated latex beads for 1 h. We observed no recruitment of LC3 to those beads when chased in control medium (Fig. 8F and G). However, as expected, when rapamycin was used, we observed a clear association of LC3 with the beads, which is consistent with the results obtained in Fig. 8A and B.

Taken together, our results indicate that rapamycin treatment is sufficient to induce the recruitment of GFP-LC3 to phagosomes, independently of carrying live, dead bacteria or simply an inert particle such as a latex bead. Interestingly, phagocytosis of M. marinum is an absolute requirement to trigger the autophagic response in the host cell. Furthermore, M. marinum infection is able to induce LC3 recruitment to latex beads-containing phagosomes, even in control conditions, only if their
A

Beads

GFP-LC3    DIC

Ctrl

Stv

Ctrl+Rp

B

Fluorescence intensity (A.U.)

Position

Stv

Ctrl

Ctrl+Rp

C

Beads + M. marinum-RFP

GFP-LC3    M. marinum-RFP

Ctrl

DIC

Merge

D

Fluorescence intensity (A.U.)

Position

Beads+M. marinum (Ctrl)

E

% of beads associated to LC3

Ctrl

Stv

Ctrl+Rp

F

LAM-coated beads

Ctrl

Ctrl+Rp

G

Fluorescence intensity (A.U.)

Position

Ctrl

Ctrl+Rp

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phagosomes have already fused or if they are attached to each other.

Discussion

Since autophagy has been described as a key player of the innate immune system (Gutierrez et al., 2004; Nagawa et al., 2004) many studies have been performed with different pathogens in order to determine how the microorganisms are affected by the autophagic pathway (Levine and Deretic, 2007; Lerena et al., 2010). Regarding Mycobacterium tuberculosis and Mycobacterium bovis BCG, autophagy behaves as an effective component of the innate immune system, promoting the maturation of mycobacterium-containing phagosomes and leading to pathogen killing (Gutierrez et al., 2004). One of the major characteristics of mycobacterial intracellular life is its ability to evade the immune system by hampering the maturation of its containing phagosome. We have previously demonstrated that autophagy is able, at least in part, to override the phagosome maturation blockage imposed by Mycobacterium bovis BCG and Mycobacterium tuberculosis.

In the present report, we have studied in detail the early events of Mycobacterium marinum intracellular life with respect to the autophagic pathway. We have shown that early after infection, phagosomes sheltering Mycobacterium marinum are clearly decorated by the autophagic protein LC3 and that the viability of the bacteria was essential for this LC3 rearrangement. These results suggest that the bacteria itself exerts a stimulus that promotes the recruitment of LC3 towards the compartments sheltering Mycobacterium marinum. Consistently, Mycobacterium marinum ΔRD1 was unable to recruit LC3 to its containing phagosome, which suggests that bacteria devoid of the ESX-1 secretion system were incapable of recruiting LC3. However, when rapamycin was used as an inductor of autophagy, LC3 was recruited to both HK Mycobacterium marinum and Mycobacterium marinum ΔRD1-containing phagosomes. These observations suggest that the rapamycin stimulus is strong enough to cause a marked LC3 redistribution, independently of the bacteria status. Indeed, when we evaluated the LC3 distribution in latex-beads phagosomes, we only observed recruitment of LC3 to these inert particles after rapamycin treatment. However, it is important to mention that this rapamycin effect was dependent on certain components of the autophagy machinery.

With regard to Mycobacterium marinum little is known about how it is affected by autophagy. As mentioned in the introduction a recent publication by Eric Brown and collaborators has demonstrated that after escaping from its containing phagosome, a small fraction of cytosolic Mycobacterium marinum is ubiquitinated in the cytoplasm and reincorporated into membrane bound compartments, in a reuptake process independent of the autophagic protein Atg5 in MEF cells (Collins et al., 2009). By 3.5 h post infection these authors observed that almost 43% of the bacteria reside in the cytoplasm when analysed by electron microscopy. This percentage is higher than the one we observed in our study (near 25%). On the other hand, by 24 h post infection we have found that the majority of the intracellular bacteria reside in the cytoplasm, whereas in the publication by Collins et al. the amount of cytosolic bacteria is considerably lower. Although the tendency is the same, it is likely that these quantitative differences might be due to the different strains used in both studies. In addition, in our study we have found that by 2 h of infection most bacteria contained in GFP-LC3 structures were devoid of ubiquitin labelling. Since the ubiquitination system is only able to detect cytosolic bacteria, our results clearly sustain the idea that Mycobacterium marinum was not re-captured by the autophagosomal system after escaping towards the cytoplasm. Interestingly, in near 20% of GFP-LC3 structures containing Mycobacterium marinum, ubiquitin was observed colocalizing with the limiting GFP-LC3 labelled phagosomal membrane, but not with bacteria residing inside. This was not surprising, as there are proteins such as Nbr1 that function as a link between ubiquitin and LC3 (Waters et al., 2009). Another point to analyse is that in contrast to
the strong LC3 recruitment observed in our experiments no autophagy induction upon *M. marinum* infection has been reported before. However, it is important to take into account that in our kinetic assay we observed that the strongest induction of the autophagic response occurs at early post-infection times (30–120 min), markedly decaying by 3 h. In contrast, in the study by Collins *et al.* they evaluated the activation of autophagy at later times (i.e. 6 and 24 h post infection), therefore it is likely that the observed differences are due to the distinct time points used in each study.

Our results indicate that in spite of being decorated by the autophagic protein LC3 the majority of the bacterial containing phagosomes were surrounded by a single bilayer. The presence of LC3 on phagosomal membranes (i.e. single membranes) is consistent with the publication of Sanjuan *et al.* showing that LC3 is recruited to zymosan-containing phagosomes internalized via TLR receptor (Sanjuan *et al.*, 2007). However, they did not observe any recruitment of LC3 to latex beads in the absence of TLR signalling. In contrast, and in agreement with our results, Brummel and collaborators have shown that latex-beads particles coated with IgG trigger signals that lead to the recruitment of LC3 to their phagosomes (Huang *et al.*, 2009). Moreover, Shui *et al.*, through proteomics analysis of phagosomes, also demonstrated the presence of LC3 on phagosomal membranes as well as an increase in its levels upon autophagy induction (Shui *et al.*, 2008). One possibility concerning LC3 recruitment was that *M. marinum* PAMPs were a signal detected intracellularly by PPRs. As LC3 is a well-characterized mycobacterial PAMP, we assessed the phagocytosis of LAM-coated beads. We observed that these beads were unable to recruit LC3 in full medium whereas upon rapamycin treatment LAM-coated beads became clearly decorated with LC3. This was in a way an expected result as LAM is a glycolipid that is also part of the cell wall of BCG and *M. marinum* ΔRD1, strains that were not able to activate the autophagic rearrangement induced by *M. marinum*.

Interestingly, in the present report we have also observed that in macrophages that have incorporated both latex beads and *M. marinum*, only those latex beads-containing phagosomes in close proximity to *M. marinum* phagosomes acquire the LC3 labelling. These observations suggest that intracellular *M. marinum* is able to induce a cellular response in charge of delivering LC3 to adjacent phagosomes. It is important to point out that the remarkable LC3 recruitment observed at early post-infection times (by 2 h post infection) coincides with the beginning of bacterial escape. As mentioned above, pathogenic mycobacteria possess a secretion system ESX-1, encoded in the RD1 cluster, which also codifies for the secretion proteins ESAT-6 and CFP-10. In 2008, Gao and collaborators showed that ESX-1 secretion system along with ESAT-6 play a significant role in bacterial escape. It is likely that before bacterial escape the phagosomal membrane is damaged, and ESAT-6 has been specifically pointed out as a factor responsible for this damage in *M. marinum* infection (Smith *et al.*, 2008). The authors demonstrated that ESAT-6 has a direct haemolytic role as well as a pore formation activity, necessary for the escape of *M. marinum* from its phagosome. Membrane damage has been already described in several studies involving an autophagic response (Gutierrez *et al.*, 2007; Mestre *et al.*, 2010; Meyer-Morse *et al.*, 2010). Indeed, numerous bacteria gain access to the cytosol thanks to the action of pore-forming toxins, which generate holes in the phagosomal membrane containing the bacteria, altering phagosomal-membrane integrity, a critical step prior to the pathogen escape. Noteworthy, this membrane injury induces a strong autophagic response (Gutierrez *et al.*, 2007; Mestre *et al.*, 2010; Meyer-Morse *et al.*, 2010). Therefore it is likely that early after infection, when several *M. marinum* phagosomes are being affected by the contained bacteria, this damage might be the signal for LC3 recruitment. We have shown that mutant *M. marinum* lacking RD1 are absolutely unable to induce an autophagic response. The data are also consistent with the membrane-damage hypothesis as these mutant bacteria (i.e. *M. marinum* ΔRD1) are devoid of ESX-1 secretion system as well as the haemolytic protein ESAT-6.

As mentioned before, a very important hallmark of pathogenic mycobacterial infections is its ability to block phagosomal maturation. In spite of its strong decoration with LC3, Rab7 and LAMP-2 the *M. marinum* phagosomes were practically devoid of the lysosomal enzyme Cathepsin D. Moreover, only a low proportion of bacteria-containing compartments decorated by GFP-LC3 presented degradative properties (i.e. DQ-BSA labelling). These data resulted somehow controversial as it has long been believed that targeting of autophagic proteins to membranous structures might be a tag for degradation. However, in this case, *M. marinum* was able to control the fate of its LC3-decorated phagosome, arresting maturation at a late endosomal stage. A similar situation has been observed for *Helicobacter pylori* (Terebiznik *et al.*, 2006). This bacterium, resides in a compartment with late endocytic features, positive for Rab7, LAMP-1, Lysotracker, although devoid of Cathepsin D and DQ-BSA labelling. In a later publication the same group demonstrated that *H. pylori*-containing vacuole was also strongly decorated by LC3 (Terebiznik *et al.*, 2009). *Salmonella enterica* serovar Typhimurium is another bacterium that behaves similarly, acquiring Rab 7, LAMP-1 or acidic properties, and being devoid of lysosomal hydrolases such as Cathepsin D and DQ-BSA labelling (Garcia-del *et al.*, 1993; Garcia-del and Finlay,
1995; Steele-Mortimer et al., 1999; Garvis et al., 2001; Bakowski et al., 2007).

One striking observation is that throughout our study we hardly found differences between control and autophagy induction by starvation, when analysing LC3 recruitment as well as maturation of the bacterium-containing compartment. In contrast, significant differences were found when rapamycin was used, as the phagosomal compartments acquired the lysosomal enzyme Cathepsin D, as well as degradative capacity. These differences could be attributed to a stronger autophagy inducing capacity of rapamycin. It is important to emphasize that the rapamycin effect was partially prevented by wortmannin indicating that its effect was indeed via an autophagic-dependent mechanism. Consistently, the rapamycin-induced LC3 recruitment to latex-beads phagosomes was completely abolished in Atg5-deficient MEF cells (not shown).

To summarize, our data indicate that M. marinum resides, at least temporally, in a LC3-positive compartment whose full maturation is blocked by the bacterium. Of note, this interaction with LC3 requires of bacterial viability, and more importantly, depends on a functional ESX-1 secretion system. To the best of our knowledge this is the first report showing that these pathogenic mycobacteria are able to actively recruit LC3 in the absence of additional autophagic stimulus.

**Experimental procedures**

**Bacterial strains and cell culture**

Raw 264.7 macrophages cell line was maintained in D-MEM 10% FBS (full nutrient medium) at 37°C in a 5% CO₂ atmosphere. D-MEM medium was obtained from Gibco (Invitrogen, Buenos Aires, Argentina) and fetal bovine serum (FBS) from Natocor (Argentina). Wild-type M. marinum was obtained from a human isolate kindly provided by the Institute 'Carlos G. Malbrán', a national disease institute in Buenos Aires, Argentina. M. marinum wt was then transformed with GFP or RFP plasmids, kindly provided by Dr Eric Brown (University of California, San Francisco, CA, USA). The M. marinum ATCC wt strain, as well as the M. marinum ΔRD1 generated by Dr Lalita Ramakrishnan, was kindly provided by Dr Eric Brown. Wild-type bacteria, GFP-M. marinum and RFP-M. marinum, were cultured in Middlebrook 7H9 (DIFCO) supplemented with 0.2% glycerol, 0.05% Tween 80 and 10% ADC enrichment. GFP and RFP M. marinum and M. marinum ΔRD1 strains were cultured in the presence of kanamycin 50 μg ml⁻¹. M. marinum was grown in 7H9 to mid- to late-log phase and then washed twice with phosphate-buffered saline (PBS) prior to infection. To disrupt clumps, bacterial suspensions were passed through a 27G needle 5–10 times, and then subjected to low speed centrifugation at 1500 r.p.m. for 1 min. Heat-killed bacteria (HK) were prepared by incubating a bacterial suspension for 30 min at 65°C and the loss of viability was confirmed by the lack of growth in 7H10 medium (not shown). HK bacteria were stained for 30 min at 37°C with 6-carboxyfluoroscein diacetate succinimidyl ester from SIGMA (Buenos Aires, Argentina) to a final concentration of 5 μg ml⁻¹.

For other experiments bacteria were labelled using Alexa fluor 633 carboxylic acid, succinimidyl ester, from Molecular Probes. Stained bacteria were washed twice with PBS to eliminate the remaining dye. For the experiments with chloramphenicol (CMP) and rifampicin (RIF) bacteria were pre-treated with the antibiotics the previous day. Bacteria were grown in 7H9 medium and we added the antibiotics the previous day to the appropriate concentration: 100 μg ml⁻¹ for CMP and 40 μg ml⁻¹ for RIF. The next day, and prior to infection, bacteria were washed three times with PBS, resuspended in D-MEM and clumps were eliminated as described above.

**Antibodies and reagents**

The primary antibodies used in this work were: rat polyclonal anti-LAMP-2 (ABL-93) from Developmental Studies Hybridoma Bank, Iowa City, USA. This antibody was used at a final dilution of 1:200 in PBS for 1 h at room temperature. Mouse monoclonal anti-LC3 was developed in our laboratory. Rabbit anti-Cathepsin D antibody was kindly provided by Dr Maximiliano Gutierrez (Helmholtz Centre for Infection Research, Germany). Rabbit polyclonal anti-ubiquitin was from DAKO and was used at a final dilution of 1:50 in PBS for 1 h. The secondary antibodies were: donkey anti-rat Cy3-conjugated, donkey anti-rabbit Cy5-conjugated, donkey anti-rabbit Cy3-conjugated and a donkey anti-mouse Cy3-conjugated were from Jackson Immunoresearch (USA), and were used at a final dilution of 1:800 for 45 min at room temperature.

**Macrophages transfection**

The plasmid pE mower-LC3, generously provided by Dr Noboru Mizushima (Tokyo Medical and Dental University, Tokyo, Japan) and Dr Tamotsu Yoshimori (Osaka University, Suita, Osaka, Japan), was used to transfect Raw macrophages. GFP-Rab 5 was provided by Dr Philip D. Stahl (Washington University, St Louis, USA) and GFP-Rab7 was kindly provided by Dr Bo van Deurs (University of Copenhagen, Denmark). Transfection was achieved using LipofectAMINE 2000 (Invitrogen, Buenos Aires, Argentina) following the instructions indicated in the manufacturer’s protocols. A stable cell line expressing EGFP-LC3 was generated and maintained in D-MEM 10% FBS with geneticin (SIGMA, Buenos Aires, Argentina) at a final concentration of 0.5 μg ml⁻¹.

**Macrophages infection**

Raw GFP-LC3 macrophages were plated at a confluence of 70–80%, the day before the experiment. Infecting inocula was prepared by washing bacteria twice with PBS, and resuspending them in D-MEM medium. Clumps were eliminated, as described before. Plated Raw GFP-LC3 cells were washed three times with PBS, and infected with the bacterial suspension in D-MEM at a multiplicity of infection (moi) of 20. Infection was allowed to proceed for different periods of time according to the experiment. For quantifications, at least 100 bacteria were scored in at least three independent experiments.

**Autophagy induction**

Autophagy induction was achieved by incubating cells in starvation medium (i.e. serum and amino acids free medium). Plated...
cells were washed three times with PBS, starvation medium was added, and cells were incubated for 2 h at 37°C in a 5% CO₂ atmosphere. Alternatively, autophagy was induced pharmacologically using rapamycin (LC Laboratories, USA) at a final concentration of 50 μg ml⁻¹ for 2 h in full nutrient medium.

Indirect immunofluorescence

Raw GFP-LC3 cells were fixed for 10 min with 3% paraformaldehyde solution in PBS, washed with PBS and quenched with NH₄Cl 50 mM for 20 min. Cells were then permeabilized for 15 min using 1% saponin in PBS buffer containing 1% BSA as blocking buffer. All steps were performed at room temperature. Cells were then incubated with the indicated primary antibody for 1 h at room temperature. Subsequently, cells were washed three times with the saponin-BSA buffer and incubated with the appropriate fluorophore-conjugated secondary antibody for 45 min. Finally, slides were washed three times with PBS, mounted on glass slides using Mowiol, or Mowiol-Hoechst, and analysed using confocal microscopy.

Fluorescence microscopy

Raw cells transfected with the different plasmids, or previously subjected to immunofluorescence, were analysed by confocal microscopy using a Nikon C1 Confocal Microscope System (Nikon, Japan) or a FV100 Olympus Confocal Microscope (Olympus, Japan). Images were processed using Adobe CS version 8.0 (Adobe Systems), ImageJ software, EZ-C1 program (Nikon, Japan) and FV10-ASW program (Olympus, Japan).

Test of lysosomal function by degradation of chromogenic BSA

Lysosomal degradative capacity was assessed by using the self-quenched red Bodipy dye conjugated to BSA (DQ-BSA; Molecular Probes). This probe is highly labelled with a BODIPY dye in a way that it is self-quenched. Digestion results in dequenching, therefore upon proteolytic degradation the released protein fragments that contain the fluorophores become brightly fluorescent. The appearance of red fluorescent dots within cells correlates with degradative structures, which can be monitored by confocal microscopy. Raw GFP-LC3 cells were incubated for 4 h at 37°C with 10 μg ml⁻¹ DQ-BSA in order to allow the probe to reach the lysosomal compartment. Cells were then washed three times with PBS and infected with either live or heat-killed M. marinum labelled with 0.25 μg ml⁻¹ Alexa 633 (Alexa fluor 633 carboxylic acid, succinimidyl ester, from Molecular Probes) for 60 min. Thereafter, cells were incubated under different experimental conditions and finally analysed by confocal microscopy.

Transmission electron microscopy and mannose-BSA-Au labelling of lysosomes

Raw cells were seeded in a six-well plate at a confluence of 80%, using two wells per experimental condition. Macrophages were incubated with 10 nm colloidal gold particles coated with mannose-6-phosphate-BSA, in D-MEM medium for 6 h. Cells were washed three times with PBS and incubated overnight in D-MEM containing 10% of fetal bovine serum, to ensure the labelling of the lysosomal structures. Cells were then infected with M. marinum for 15, 30 or 60 min at 37°C using a moi of 20–30. Samples were fixed with 2% glutaraldehyde for 1 h at room temperature, and then overnight with 1% glutaraldehyde at 4°C. Fixed monolayers were scraped, post-fixed in 1% osmium tetroxide in 100 mM phosphate buffer solution, dehydrated using increasing concentrations of acetone, and gradually infiltrated with Epon resin (Pelco, USA). Thin sections of each sample were stained with uranyl acetate and lead citrate and analysed in a Zeiss EM 900 transmission electron microscopy.

Western blot analysis

Raw GFP-LC3 cells were subjected to different experimental conditions: full medium (Ctrl), starvation medium (Stv) or starvation medium with the addition of 0.1 μM bafilomycin (BAF). Also, M. marinum-infected macrophages were incubated for 90 min in full medium, or in full medium plus 0.1 μM bafilomycin. Another subset of cells was infected with M. marinum previously heat-inactivated (HK). Bafilomycin was purchased from SIGMA (Buenos Aires, Argentina) and it was used in order to block lysosomal degradation. In this case, cells were pre-incubated with bafilomycin for 1 h and 30 min prior to the appropriate treatment: starvation or M. marinum infection. Subsequently, cells were washed twice, scraped and resuspended in sample buffer containing 1% 2-mercaptoethanol, and sonicated for 10 min at 4°C. Samples were frozen at -80°C until its use. For Western blot analysis, protein extracts were subjected to electrophoresis in 12.5% SDS-PAGE gels, transferred to a Hybond-enhanced chemiluminescence nitrocellulose membrane (Amersham) (GE, Buenos Aires, Argentina). Membranes were blocked using blotto (PBS with 10% non-fat milk 0.1% Tween-20) for 1 h at room temperature. Next, membranes were washed twice with PBS Tween-20 0.1% and incubated with the primary antibody. To detect LC3, membranes were incubated overnight at 4°C with rabbit anti-LC3 1:1000 (SIGMA) for 3 h at room temperature, washed and incubated with HRP-conjugated anti-rabbit antibody (Jackson Immunoresearch Laboratories, USA), at a final dilution of 1:10 000. Equal protein loadings were confirmed by incubating membranes with mouse anti-beta tubulin (1:300) for 1 h at room temperature (mouse anti-beta tubulin was developed by Michael Klymkowski and was obtained from Developmental Studies Hybridoma Bank). The bands were visualized using the ECL reagent from GE Healthcare, Buckinghamshire, UK. Images of the bands were obtained using a Luminescent Image Analyser LAS-4000 (Fujifilm).

Phagocytosis assays using latex beads

Raw GFP-LC3 macrophages were plated at a confluence of 70–80% the day before the experiment. Cells were allowed to phagocytose for 30 min 3 μm latex beads coated with rabbit IgG at a moi of 10. Cells were then washed three times with PBS and the appropriated medium was added for 90 more minutes: full nutrient medium (Ctrl), starvation medium (Stv) or full medium plus rapamycin (Ctrl + Rp). In another set of experiments, macrophages were allowed to phagocytose both latex beads and M. marinum-RFP for 30 min and incubated for 90 additional minutes.

Phagocytosis assays using latex beads

Raw GFP-LC3 macrophages were plated at a confluence of 70–80% the day before the experiment. Cells were allowed to phagocytose for 30 min 3 μm latex beads coated with rabbit IgG at a moi of 10. Cells were then washed three times with PBS and the appropriated medium was added for 90 more minutes: full nutrient medium (Ctrl), starvation medium (Stv) or full medium plus rapamycin (Ctrl + Rp). In another set of experiments, macrophages were allowed to phagocytose both latex beads and M. marinum-RFP for 30 min and incubated for 90 additional minutes.

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in full nutrient medium. In all cases cells were fixed with 3% paraformaldehyde for 10 min and quenched with 50 mM NH₄Cl for 20 min. In order to differentiate internalized from extra-
cellular beads, we incubated the slides with anti-rabbit Cy5, without previous permeabilization; consequently, only extracellular 
beads were labelled with this fluorophore. Finally, cells were 
mounted on glass slides using Mowiol and analysed by confocal microscopy.

Labelling with Lysotracker
Acidic compartments were labelled with Lysotracker red (Molecu-
lar Probes, Invitrogen, Buenos Aires, Argentina). Raw GFP-LC3 
cells previously infected with M. marinum were incubated with 
1 µM Lysotracker at 37°C for 40 min, and were immediately observed 
in vivo, using confocal microscopy.

Statistical analysis
The results are represented as the mean ± SEM from two 
or three different experiments. The comparisons were performed 
using ANOVA in conjunction with Tuckey’s or Dunnett’s tests. 
The significant differences were *P < 0.05; **P < 0.01 and 
***P < 0.001.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Autophagosomes fuse with M. marinum-containing compartment. Raw GFP-LC3 infected with M. marinum RFP for 1 h (uptake) followed by 2 h chase was analysed by live cell imaging. Images of the different time points are depicted in this figure. A phagosome harbouring M. marinum fuses with an autophagosome (AP, white arrow) in close proximity. Scale bars: 5 μm.

Fig. S2. M. marinum sheltered in GFP-LC3 structures lacks ubiquitin labelling. A. Raw GFP-LC3 cells were infected with M. marinum as described in Fig. 1 and ubiquitin was detected by immunofluorescence using an appropriate antibody. Three situations were observed: (i) bacteria surrounded by GFP-LC3 with no ubiquitin labelling (a–e), (ii) bacteria surrounded by GFP-LC3 with a strong ubiquitin labelling of the pathogen (f–j), and (ii) bacteria surrounded by GFP-LC3 with some ubiquitin labelling detected at the limiting membrane of the GFP-LC3-decorated phagosome but absent inside that compartment (k–o).
B. Quantification of the frequency of the observed situations described in (A) is depicted in this panel.
Scale bars: 10 μm.

**Fig. S3.** *M. marinum*-containing phagosomes do not fuse with lysosomes. (A) Macrophage lysosomes were labelled with colloidal-gold particles coated with mannose-BSA (BSA-Au) which were previously incorporated by Raw cells (see Experimental procedures). Typical single-membrane phagosomes harbouring a few bacteria devoid of BSA-Au are shown. Lysosomes are easily recognized as they are loaded with gold particles (black arrowheads, A, B and C). Occasionally a few bacteria phagosomes that fused with lysosomes were observed (C and D).

**Fig. S4.** Phagosome labelling with Lysotracker markedly decreased at 8 h of chase.
A. Raw GFP-LC3 cells were infected with live Mm RFP for 1 h, and chased for 2 h or 8 h (A) in full medium (A, a–d), full medium in the presence of rapamycin (A, i–l), or full medium in the presence of rapamycin plus wortmannin (A, m–p). As a control we also infected Raw GFP-LC3 cells with HK Mm (A, e–h). Cells were labelled with Lysotracker as indicated in Experimental procedures. Scale bars: 10 μm.

B. Quantification showing the percentage of phagosomes labelled by Lysotracker at 2 (black bars) and 8 h (white bars). A clear decrease between 2 and 8 h is observed for live Mm, suggesting phagosomal membrane damage. No changes on Lysotracker colocalization between 2 and 8 h were observed for HK Mm. Likewise, in the presence of Rp no decrease on Lysotracker labelling between 2 and 8 h of chase was observed. In contrast, when the Rp effect was inhibited with Wm, we observe almost a 50% decrease on bacteria colocalization at 8 h, respect to the 2 h chase. Data are representative of two independent experiments.

C. Raw GFP-LC3 macrophages were infected with *M. bovis* BCG for 1 h and chased for 2 h in full medium conditions. No interaction with LC3 is observed.
Scale bars: 10 μm.

**Movie S1.** Autophagosomes fuse with *M. marinum*-containing phagosomes. Raw GFP-LC3 macrophages infected with *M. marinum* RFP (Mm) for 1 h (uptake), followed by 2 h chase, were analysed by live cell imaging. An autophagosome (AP) is observed approaching, docking and finally fusing with the Mm-containing phagosome, leading to the formation of a typical structure decorated with LC3. First both channels (GFP and RFP) are shown in the movie. In the second part of the movie only the GFP channel is shown in order to have a better visualization of the fusion event and the formation of the LC3-decorated phagosome.

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