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Autophagy stimulation by rapamycin suppresses lung inflammation and infection by *Burkholderia cenocepacia* in a model of cystic fibrosis

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Cystic fibrosis (CF) is the most common inherited lethal disease in Caucasians which results in multiorgan dysfunction. However, 85% of the deaths are due to pulmonary infections. Infection by *Burkholderia cenocepacia* (*B. cepacia*) is a particularly lethal threat to CF patients because it causes severe and persistent lung inflammation and is resistant to nearly all available antibiotics. In *CFTR* Δ F508 (Δ F508) mouse macrophages, *B. cepacia* persists in vacuoles that do not fuse with the lysosomes and mediates increased production of IL-1 β . It is believed that intracellular bacterial survival contributes to the persistence of the bacterium. Here we show for the first time that in wild-type but not in Δ F508 macrophages, many *B. cepacia* reside in autophagosomes that fuse with lysosomes at later stages of infection. Accordingly, association and intracellular survival of *B. cepacia* are higher in *CFTR*- Δ F508 macrophages than in WT macrophages. An autophagosome is a compartment that engulfs nonfunctional organelles and parts of the cytoplasm then delivers them to the lysosome for degradation to produce nutrients during periods of starvation or stress. Furthermore, we show that *B. cepacia* downregulates autophagy genes in WT and Δ F508 macrophages. However, autophagy dysfunction is more pronounced in Δ F508 macrophages since they already have compromised autophagy activity. We demonstrate that the autophagy-stimulating agent, rapamycin markedly decreases *B. cepacia* infection in vitro by enhancing the clearance of *B. cepacia* via induced autophagy. In vivo, rapamycin decreases bacterial burden in the lungs of CF mice and drastically reduces signs of lung inflammation. Together, our studies reveal that if efficiently activated, autophagy can control *B. cepacia* infection and ameliorate the associated inflammation. Therefore, autophagy is a novel target for new drug development for CF patients to control *B. cepacia* infection and accompanying inflammation.

Introduction

Cystic fibrosis (CF) is the most common inherited lethal disease in Caucasians. It is caused by mutations in the cystic fibrosis transmembrane conductance regulator encoded by the *CFTR* gene encoding a membrane chloride transporter.¹⁻³ The pathogenic factors in CF airway disease include defective innate antimicrobial activity, altered mucociliary clearance, abnormal submucosal gland function and overproduction of reactive oxygen species (ROS).⁴⁻⁶ Chronic inflammation is most central to CF pathogenesis as a consequence to pulmonary infections and leads to lung damage resulting in 85% of the deaths.⁷⁻¹⁰ Human and mouse CF airway epithelia are autophagy deficient and exhibit highly reduced autophagosome formation.^{11,12} Autophagy is a conserved physiological process that eliminates nonfunctional organelles and recycles cytosolic components for the generation of nutrients during periods of stress or starvation.^{13,14}

Autophagy targets cytosolic long-lived proteins and organelles for lysosomal degradation in eukaryotic cells and plays a role in innate immunity.¹⁵ Autophagy has been linked to a variety of disease states, including cancer, myopathies, neurodegeneration, Crohn disease, infection and inflammation.^{13,16-18} Formation of autophagosomes depends on a lipid kinase signaling complex containing class III PI3K and two ubiquitin-like conjugation pathways that activates expansion of the pre-autophagosomal membrane.^{19,20} The Atg12-Atg5-Atg16L complex is attached to the nascent autophagosome and recruits Atg8—microtubule-associated protein 1 light chain 3 (LC3)—which is expressed initially as an unprocessed form. Then, pro-LC3 is cleaved by Atg4 to generate an active form, LC3-I.²¹ LC3-I interacts with phosphatidylethanolamine (PE), yielding LC3-II.^{15,22,23} Therefore the transformation of LC3I to LC3II denotes autophagy stimulation and autophagosome formation. Subsequently, the Atg12-Atg5-Atg16L complex detaches from the formed autophagosome.^{23,24}

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This uncoating event enables the autophagosome to fuse with the lysosome. A small GTP binding protein Rab7 and the lysosomal associated membrane proteins 1 and 2 (LAMP1, LAMP2) are needed for this process.^{20,25-27} Many pharmacological agents have been reported to induce autophagy, such as rapamycin, an inhibitor of the mTOR pathway (mammalian target of rapamycin). The mTOR pathway is active in the presence of nutrients and negatively regulated by starvation or rapamycin and, under these conditions, autophagy is activated.²⁸

Autophagy contributes to the control of a variety of bacterial and viral infections. For example, Group A Streptococcus that escapes from the endosome is targeted to the autophagosome, and Atg5 deletion delays its clearance.^{29,30}

Similarly, during *Listeria monocytogenes* infection, bacterial listeriolysin-O toxin-mediated escape from phagosomes induces autophagy.³⁰⁻³² Furthermore, a subset of *Salmonella enterica*-containing vacuoles is targeted to autophagosomes, and Atg5 deletion also allows for more bacterial survival.³³ Interestingly, Mycobacteria-containing vesicles are targeted to autophagosomes, which decreases mycobacterial survival.³⁴ The antimicrobial role of autophagy has been extended to viral infections such as human immunodeficiency virus (HIV).^{35,36}

Burkholderia cenocepacia (*B. cepacia*) is an opportunistic, multidrug-resistant bacterium that infects CF patients leading to severe inflammation followed by destruction of the lung tissue, sometimes resulting in necrotizing pneumonia leading to patient death.^{37,38} Unfortunately, *B. cepacia* is resistant to essentially all antibiotics and thus impossible to treat. *B. cepacia* adopts an extracellular or intracellular lifestyle.^{39,40} The bacterium can survive within a variety of eukaryotic cells such as amoebae, epithelial cells and human macrophages.⁴¹⁻⁴⁶

The *B. cepacia*-containing vacuole within macrophages delays acidification, does not assemble the NADPH-oxidase complex and fails to activate Rab7.^{43,47-49} These phenotypes are further exaggerated in CFTR-defective macrophages. However, little is known regarding the nature of the compartment harboring *B. cepacia* and the mechanism by which the bacteria delay its delivery to the lysosome for degradation.

During *B. cepacia* infection, abundant inflammatory cytokines such as IL-1 β are detected in the bronchoalveolar lavage (BAL) of CF patients.⁵⁰⁻⁵⁸ IL-1 β is primarily expressed as a precursor inactive molecule, which is later cleaved by caspase-1 to yield active 17-kDa IL-1 β .⁵⁹ The biological activities of IL-1 β include promoting inflammatory responses and leukocyte infiltration.

Here we show that in WT macrophages a moderate number of *B. cepacia*-containing vacuoles are labeled with the specific autophagy marker LC3 within 2 h post-infection. *B. cepacia* containing vacuoles delay the fusion with the lysosome for several hours. Notably, *B. cepacia* decreases the expression of essential autophagy molecules. This *B. cepacia*-mediated effect is exacerbated in Δ F508 cells which are intrinsically defective in autophagy activity.^{11,12} In Δ F508 macrophages, *B. cepacia*-containing vacuoles do not fuse with the lysosomes and do not have autophagosome characteristics. We demonstrate that this defect is reversible since stimulation of autophagy with rapamycin decreases the bacterial burden in vitro and in vivo by accelerating the delivery of

B. cepacia to the lysosome for degradation. Rapamycin treatment also dramatically decreases the recruitment of inflammatory cells to the lungs of infected CF mice. Taken together, our data provide a preponderance of evidence that *B. cepacia* exploits the already defective autophagy pathway in Δ F508 macrophages to establish infection. Stimulating autophagy activity with rapamycin restores the ability of Δ F508 macrophages to control *B. cepacia* infection and the associated inflammation. Therefore, our studies support the notion that pharmacological stimulation of autophagy will be beneficial for CF patients to prevent *B. cepacia* infection and thwart the detrimental inflammatory response within the lungs of CF patients.

Results

Macrophages harboring the CFTR Δ F508 mutation support increased *B. cepacia* survival and produce more IL-1 β than WT macrophages. We examined whether *B. cepacia* had a survival advantage in primary murine macrophages expressing the CFTR protein harboring the Δ F508 mutation, which is the most common mutation in CF patients.⁶⁰⁻⁶² WT and CFTR Δ F508 (Δ F508) macrophages were infected with the *B. cepacia* clinical isolate K56-2 and colony-forming units (CFU) were determined from lysed infected macrophages at 30 min (Fig. S1) and at 24 h post-infection (Fig. 1A). We found that more *B. cepacia* was recovered from Δ F508 macrophages than WT cells after 24 h of infection (Fig. 1A), whereas, the initial uptake was similar in both cells (Fig. S1). We next examined the number of *B. cepacia* associated with WT and Δ F508 macrophages by confocal microscopy. WT and Δ F508 macrophages were infected with red fluorescent protein (mRFP)-expressing *B. cepacia* for 30 min and 2 h and the number of *B. cepacia* associated with 100 macrophages was evaluated. At an early time point (30 min post-infection) similar numbers of *B. cepacia* were associated with WT and Δ F508 macrophages (Fig. S2). In contrast, at 2 h there were <200 *B. cepacia* associated with 100 WT macrophages (Fig. 1B and C), whereas there were >300 *B. cepacia* associated with 100 Δ F508 macrophages (Fig. 1B and C). Thus, these data are consistent with the CFU data suggesting more growth of *B. cepacia* in Δ F508 macrophages than in WT macrophages.

Since IL-1 β is an essential pro-inflammatory cytokine that affects CF patients,⁵⁰⁻⁵⁸ we next determined the levels of active IL-1 β in culture supernatants and found that Δ F508 macrophages produced significantly more IL-1 β when infected with *B. cepacia* compared with WT cells (Fig. 1D). Yet, the mechanism is unclear. To rule out the role of macrophage survival in IL-1 β production differences, the release of lactate dehydrogenase (LDH) was evaluated in WT and Δ F508 macrophages infected with *B. cepacia*. The Δ F508 mutation did not alter macrophage survival in response to *B. cepacia* (Fig. S3). Therefore, primary macrophages expressing the Δ F508 mutation support increased *B. cepacia* intracellular survival and bacterial replication and produce more IL-1 β during *B. cepacia* infection.

The *B. cepacia*-containing intracellular vacuole acquires autophagy characteristics in WT macrophages but not in Δ F508 macrophages. Since autophagy activity is compromised

in CF epithelial cells we examined whether macrophages have the same defect by observing their autophagy response during the *B. cepacia* infection.^{11,12} WT and $\Delta F508$ macrophages were infected with mRFP-expressing *B. cepacia* for 2 h. The acquisition of endogenous LC3 by the *B. cepacia*-containing vacuole was assessed with specific antibodies by confocal microscopy. In WT macrophages, 20% *B. cepacia*-containing vacuoles were labeled with the specific autophagy marker LC3 within 2 h infection (Fig. 2A and C). Several LC3-labeled vacuoles (puncta) were identified in WT macrophages (Fig. 2A; white arrows). In contrast, *B. cepacia*-containing vacuoles in $\Delta F508$ macrophages had rare LC3-labeled structures (puncta) in response to *B. cepacia* compared with WT macrophages and only 10% of mRFP-expressing *B. cepacia*-containing vacuoles showed co-localization with LC3 (Fig. 2B and C). Notably, as shown earlier, greater numbers of *B. cepacia* were visualized in $\Delta F508$ macrophages than in WT cells at 2 h post-infection (Fig. 2A and B). To further confirm the identity of the *B. cepacia*-containing compartment, *B. cepacia*-infected WT and $\Delta F508$ macrophages were examined by transmission electron microscopy. Infected WT macrophages contained few *B. cepacia* and they were surrounded by several multilamellar membranes (Fig. 2D; black arrows) similar to autophagosomes and showed signs of bacterial degradation. In contrast, in $\Delta F508$ macrophages, several intact *B. cepacia* were associated with the macrophage (Fig. 2D; white arrow heads), and they lacked the autophagosome-like structure. Immunofluorescence quantification of puncta within infected macrophages also confirmed that the autophagy response is compromised in $\Delta F508$ macrophages during *B. cepacia* infection (Fig. 2E). Together, these data indicate that the autophagy response of $\Delta F508$ macrophages to *B. cepacia* is significantly less than that of WT macrophages and hence more *B. cepacia* are enclosed in autophagosome-like vacuoles within WT macrophages.

***B. cepacia* downregulates autophagy genes during infection of WT and $\Delta F508$ macrophages.** *B. cepacia* resides within LC3 labeled compartments reminiscent of autophagosomes in WT macrophages with the presence of puncta in several macrophages (Fig. 2). However, compared with WT macrophages infected with other organisms such as Salmonella, there were many fewer puncta in WT macrophages infected with *B. cepacia* (Fig. 2E and data not shown).^{13,14,63} Nevertheless, significantly fewer puncta were detected in $\Delta F508$ macrophages when compared with WT macrophages infected with *B. cepacia* (Fig. 2E). The cause of this observation is unknown. To examine the effect of *B. cepacia* on the autophagy pathway, we performed an array analysis for a part of autophagy genes in *B. cepacia*-infected WT and $\Delta F508$ macrophages. *B. cepacia* infection led to a significant downregulation of several autophagy genes such as *Atg9b*, *Atg5*, *Atg12* (Fig. 3A) and *Atg8* (Fig. 3B) in $\Delta F508$ and in WT macrophages (Fig. S4A and B). Quantitative PCR (qPCR) and protein gel blot analysis confirmed the downregulation of autophagy genes and their corresponding protein in both cell types (Figs. 3A, C–E and 2E and data not shown). The low number of puncta in $\Delta F508$ macrophages compared with

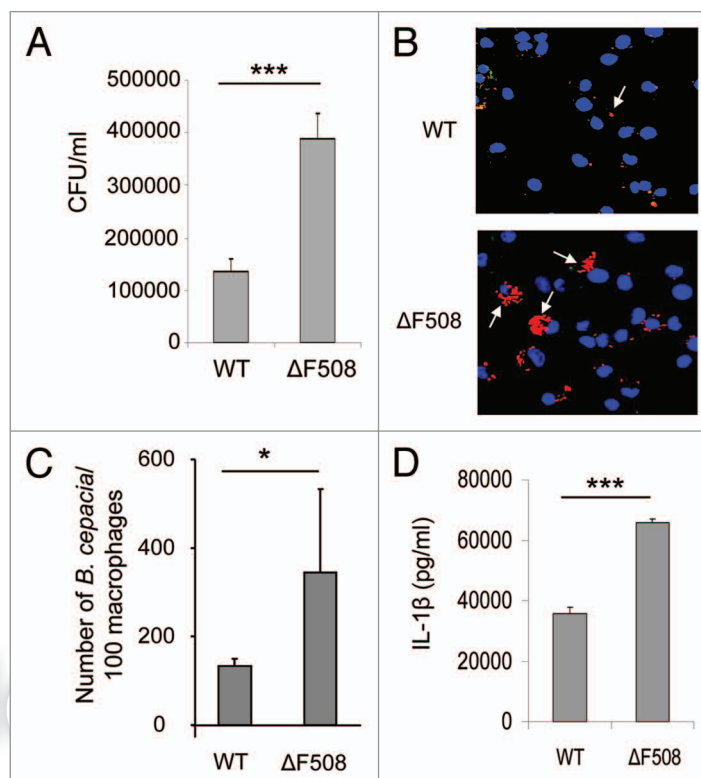


Figure 1. Macrophages harboring $\Delta F508$ mutation show more *B. cepacia* recovery and enhanced cytokine production from macrophages. (A) Wild-type (WT) or *CFTR* $\Delta F508$ ($\Delta F508$) murine bone marrow-derived macrophages (BMDM) were infected with *B. cepacia* and colony-forming units (CFUs) were enumerated at 24 h post-infection. (B) Confocal microscopy of WT or $\Delta F508$ BMDM after 2 h of infection with *B. cepacia*. Nuclei are stained blue with DAPI and *B. cepacia* expresses red fluorescent protein. White arrows indicate the sites of *B. cepacia*. (C) Scoring of the number of *B. cepacia* associated with 100 BMDMs in confocal images. (D) 24 h post-infection, supernatants from BMDM from WT or $\Delta F508$ mice were analyzed for IL-1 β . Data are representative of 3 independent experiments and presented as the means \pm SD. Asterisks in (C and D) indicate significant differences (* p < 0.05 and *** p < 0.0001).

WT macrophages further confirmed that *B. cepacia* downregulates autophagy and that this effect is more profound in $\Delta F508$ macrophages (Fig. 2E). On the other hand, other autophagy-interacting bacteria such as Salmonella induce the expression of several autophagy molecules during infection (data not shown). Therefore, it appears that the downregulation of essential autophagy molecules is a strategy employed by *B. cepacia* to delay the maturation of autophagosomes and hence avoid clearance by the host cell. The *B. cepacia*-mediated downregulation of autophagy genes is similar in WT and $\Delta F508$ macrophages but the effect is more pronounced in the latter cells suggesting that they harbor an inefficient autophagy system as described in $\Delta F508$ epithelial cells.^{11,12}

Depletion of LC3 from WT macrophages allows for more *B. cepacia* recovery and increased IL-1 β production. *B. cepacia* infection downregulates autophagy genes in both WT and $\Delta F508$ macrophages, but the effect on autophagy activity is more pronounced in $\Delta F508$ macrophages, suggesting a possible

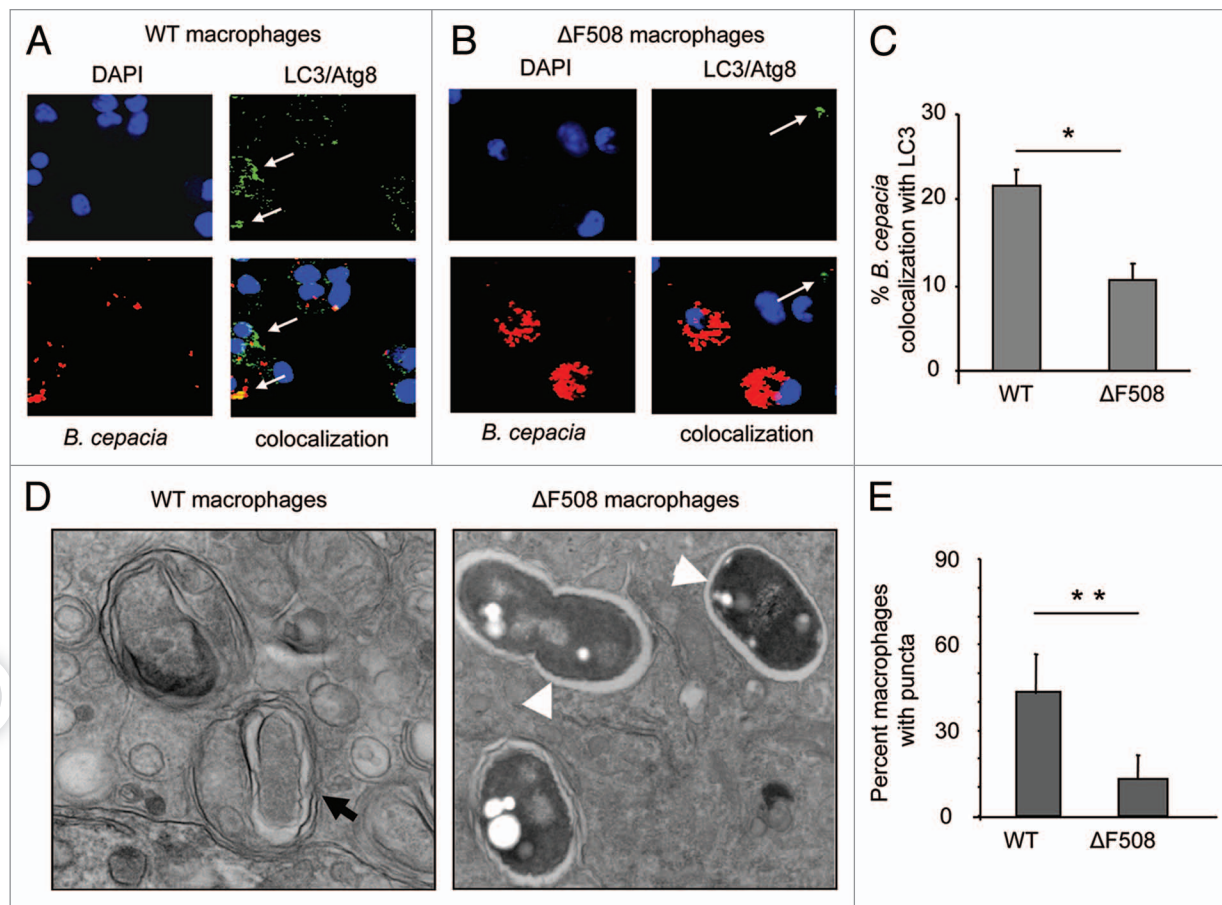


Figure 2. *B. cepacia* resides in an autophagosome-like compartment in macrophages. (A and B) Confocal microscopy for wild-type (WT) murine BMDM (A) and BMDM harboring Δ F508 mutation (Δ F508) (B) representing the colocalization of *B. cepacia* with LC3/Atg8l after 2 h infection. Nuclei are stained with DAPI, *B. cepacia* expresses red fluorescent protein and LC3/Atg8 is stained green with specific antibodies. White arrows indicate the presence of LC3/Atg8. (C) The percentage of colocalization of *B. cepacia* with LC3/Atg8 was scored. (D) Transmission electron microscopy of murine BMDM expressing WT or mutated Δ F508 CFTR protein after 2 h infection with *B. cepacia*. Black arrow indicates a multilamellar autophagosome-like vacuole. White arrow heads indicate vacuoles containing intact bacteria within single-membrane vacuoles. (E) The scores for the percentage of puncta in 100 WT and Δ F508 macrophages after 4 h infection with *B. cepacia*. Data are representative of 3 independent experiments and presented as the means \pm SD. Asterisks in (C and E) indicate significant differences (* $p < 0.05$; ** $p < 0.01$).

mechanism allowing for increased *B. cepacia* survival. This could be due to defective autophagy activity in macrophages harboring mutant CFTR protein similar to epithelial cells expressing the same mutation.^{11,12} Therefore, we examined if compromising autophagy in WT macrophages would result in a similar phenotype to Δ F508 macrophages. We reduced autophagy activity in WT macrophages by depleting LC3, an essential autophagy molecule. The depletion of LC3 was achieved with specific small interfering RNA (siRNA) to LC3 and confirmed by protein gel blots using specific antibody that recognizes LC3I and LC3II forms (Fig. S5A). Initial uptake of *B. cepacia* was similar in WT and Δ F508 macrophages pretreated with siRNA-LC3 or siRNA-control (CT) (data not shown). Depletion of LC3 in WT macrophages by specific siRNA was accompanied by an increased recovery of *B. cepacia* (Fig. 4A). Depletion of LC3 in Δ F508 macrophages was accompanied by an increase in *B. cepacia* recovery although less significant (Fig. 4B). The decrease in LC3 expression was associated with increased secretion of the active form of IL-1 β from *B. cepacia*-infected WT and Δ F508 macrophages

(Fig. 4C and D). Therefore, compromising autophagy in WT macrophages with specific siRNA produced a Δ F508-like phenotype, such as increased *B. cepacia* recovery and enhanced IL-1 β release.

Once activated, caspase-1 cleaves pro-IL-1 β to its active form, which is then released into culture supernatants. Therefore, to understand why IL-1 β release is increased in Δ F508 macrophages during *B. cepacia* infection, we compared the levels of IL-1 β pro-form in Δ F508 and WT macrophages upon *B. cepacia* infection. The depletion of LC3 with specific siRNA in WT and Δ F508 macrophages increased the levels of pro-IL-1 β in macrophages in response to *B. cepacia* infection (Fig. S5B) and led to more active IL-1 β released into culture supernatants (Fig. 4C and D). Therefore, decreasing autophagy activity resulted in increased pro-IL-1 β accumulation, activation and release of active IL-1 β in response to *B. cepacia* infection.

Stimulation of autophagy by rapamycin decreases *B. cepacia* recovery and IL-1 β production in vitro. Macrophages harboring Δ F508 mutation show increased intracellular survival

and growth of *B. cepacia* (Fig. 1A and B). To investigate whether this phenotype is due to reduced autophagy activity in Δ F508 macrophages, we examined the effect of autophagy stimulation by rapamycin on *B. cepacia*-infected Δ F508 macrophages. Rapamycin is an inhibitor of the mammalian target of rapamycin (mTor) that induces autophagy.^{64,65} Macrophages were treated with rapamycin or with the drug vehicle DMSO before and after *B. cepacia* infection. The number of *B. cepacia* recovered was evaluated at 2, 4 and 6 h post infection and expressed as CFUs/ml. Rapamycin significantly decreased the number *B. cepacia* recovered from WT and Δ F508 macrophages (Fig. 5A and B). However, the effect was observed in Δ F508 macrophages as early as 2 h post-infection despite equal initial uptake (Fig. 5B and data not shown). Adding rapamycin directly to bacterial cultures in the absence of macrophages did not alter *B. cepacia* survival (data not shown). By confocal microscopy, the number of *B. cepacia* associated with 100 macrophages at 2 h post infection was significantly lower in Δ F508 macrophages in the presence of rapamycin, thus confirming the CFU data (Fig. 5C). In contrast, the reduction in *B. cepacia* numbers recovered from infected WT macrophages was evident at later stages of infection (Fig. 5A).

We next investigated if rapamycin affects the levels of active IL-1 β released in culture supernatants.⁶⁶ In contrast to DMSO, rapamycin treatment of Δ F508 macrophages markedly decreased IL-1 β production in response to *B. cepacia* infection (Figs. 5D and S6A). Rapamycin also decreased IL-6 production (Fig. S6B and C). The levels of other cytokines such as TNF α were comparable in the presence or absence of rapamycin at 24 h post-infection (Fig. S6D and E). Therefore, stimulation of autophagy by rapamycin decreases the production of IL-1 β and not all pro-inflammatory cytokines in response to *B. cepacia* infection in vitro.

Rapamycin treatment decreases the bacterial burden and reduces the signs of inflammation in the lungs of Δ F508 mice. Since *B. cepacia* infects the lungs of CF patients we used the Δ F508 mouse model for CF to examine if rapamycin treatment decreases the bacterial burden in vivo and if it alleviates inflammatory findings in the lungs of infected mice. WT and Δ F508 mice were pretreated with two doses of rapamycin or DMSO by intra-peritoneal injections. Mice were then infected intra-tracheally with *B. cepacia* followed by an additional dose of rapamycin or DMSO. On the second day post-infection, mice were sacrificed, lungs were collected and the bacterial burden was determined. Rapamycin treatment did not affect *B. cepacia* recovery from WT lungs but reduced it drastically from Δ F508 lungs (Fig.

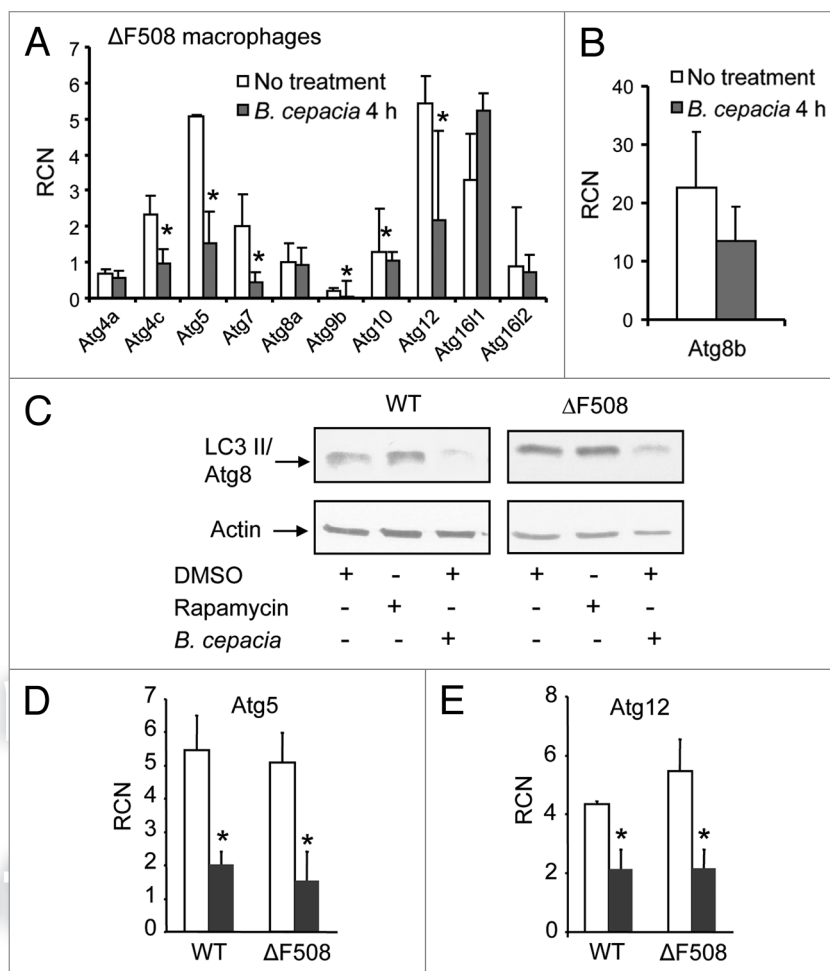


Figure 3. *B. cepacia* downregulates the expression of autophagy genes in murine macrophages. (A and B) Bone marrow-derived macrophages (BMDMs) harboring the Δ F508 mutation were either uninfected (No treatment) or infected with *B. cepacia* for 4 h and then the cells were lysed in TRIzol. RNA was extracted and SA Biosciences autophagy array analysis was performed for several autophagy genes (A) and for LC3/Atg8 (B). (C) Immunoblotting for WT and Δ F508 murine macrophages showing the expression level of LC3/Atg8 before and after 4 h infection with *B. cepacia* in the presence of rapamycin or DMSO. (D and E) q-PCR results for autophagy regulating genes 5 and 12 (Atg5, Atg12) in WT and Δ F508 BMDMs non infected (white bars) or infected (gray bars) with *B. cepacia* for 4 h. Data in (A, D and E) are expressed as relative copy numbers (RCN) and presented as means of 3 independent experiments \pm SD. Asterisks indicate significant differences from the uninfected cells at the indicated time point (* p < 0.05).

6A and B). Therefore, rapamycin treatment stimulates autophagy and decreases the *B. cepacia* burden in vivo in Δ F508 mice.

Next, we determined the effect of rapamycin treatment on the inflammatory response of lung tissue of *B. cepacia*-infected mice. Histological examination of H&E stained lung sections of WT mice infected with *B. cepacia* revealed the preservation of most of the lung tissue with patchy areas of accumulation of inflammatory cells (Fig. 6C and left upper part). Lung sections of Δ F508 mice demonstrated significantly more pronounced and generalized recruitment of inflammatory cells (arrow head) throughout the lung tissue with peribronchiolar and perivascular infiltrates and hemorrhage (arrow) (Fig. 6C and left middle part). Higher magnification of Δ F508 lung sections closely showed that *B. cepacia*

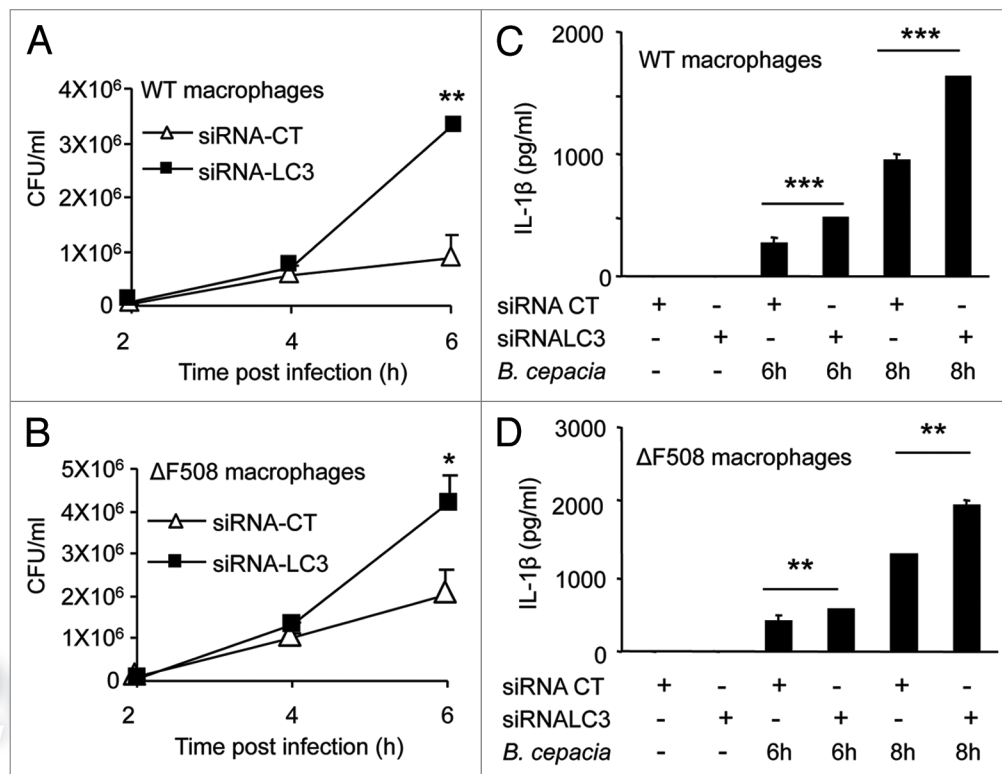


Figure 4. Depletion of LC3 with specific siRNA increases *B. cepacia* multiplication and Interleukin-1 β (IL-1 β) secretion in macrophages. (A and B) Bone-marrow derived macrophages (BMDMs) from WT and Δ F508 mutant mice were nucleofected with siRNA against LC3/Atg8 (siRNA-LC3) or control siRNA (siRNA-CT) for 48 h and then infected with *B. cepacia* for 2, 4 and 6 h. Colony forming units (CFUs) were enumerated. (C and D) Both types of macrophages were then infected for 6 and 8 h and supernatants were analyzed for IL-1 β production. Data are representative of three different experiments and presented as the means \pm SD. Asterisks indicate significant differences from the (siRNA-CT) at the respective time points (* p < 0.05; ** p < 0.01; *** p < 0.001).

infection also led to the accumulation of inflammatory cells and exudates in the alveolar spaces of Δ F508 lungs (Fig. 6C and left lower part). To determine whether rapamycin treatment rescues the appearance of the Δ F508 lung tissue during *B. cepacia* infection, mice were treated with rapamycin and infected intra-tracheally with *B. cepacia* as described above. H&E stained lung sections of rapamycin-treated mice showed that rapamycin treatment drastically reduced the recruitment of inflammatory cells throughout the CF lung, prevented peribronchiolar and perivascular hemorrhage and preserved the alveolar space of infected Δ F508 mice (Fig. 6C and right middle and lower parts). Therefore, rapamycin treatment decreases inflammation and thus rescues the lung tissue in a mouse model of CF following *B. cepacia* infection.

Rapamycin treatment decreases *B. cepacia* recovery from Δ F508 macrophages by enhancing their delivery to lysosomes. Rapamycin treatment significantly decreased the recovery of *B. cepacia* from Δ F508 mice and their derived macrophages, yet the mechanism is unclear. It is possible that rapamycin-mediated induction of autophagy enhanced the maturation and the fusion of the *B. cepacia*-enclosing vacuole with the lysosomal compartment. To explore the trafficking of *B. cepacia* within macrophages, the colocalization of mRFP-expressing *B. cepacia* with the autophagy marker LC3 was determined in the presence of rapamycin. Rapamycin increased the number of *B. cepacia*-containing

vacuole that acquired LC3 in both WT and Δ F508 macrophages (Fig. 7A; white arrows and B). Since rapamycin promoted the acquisition of LC3, we examined if it enables the delivery of *B. cepacia* to the lysosome. Green lysotracker, which accumulates in lysosomes, was added to rapamycin or DMSO-treated WT and Δ F508 macrophages prior to infection with mRFP-expressing *B. cepacia*. Almost 40% and 20% of *B. cepacia* localized with the lysotracker in DMSO-treated WT and Δ F508 macrophages respectively within 2 h infection indicating that WT macrophages are more efficient in delivering *B. cepacia* to the lysosomes (Figs. 7A, B and S7). Rapamycin treatment slightly increased the number of *B. cepacia* that trafficked to the lysosome in WT macrophages. Nevertheless, rapamycin treatment significantly increased the delivery of *B. cepacia* to the lysosomes in Δ F508 macrophages up to 50% within 2 h (Figs. 7C, D and S7). Therefore, stimulation of autophagy by rapamycin drives the maturation of the *B. cepacia*-containing vacuole enabling it to fuse with the lysosome and thereby improving the clearance of the bacterium from Δ F508 macrophages.

Discussion

CF is the most common life-shortening inherited disease among the Caucasian population owing in part to the exaggerated

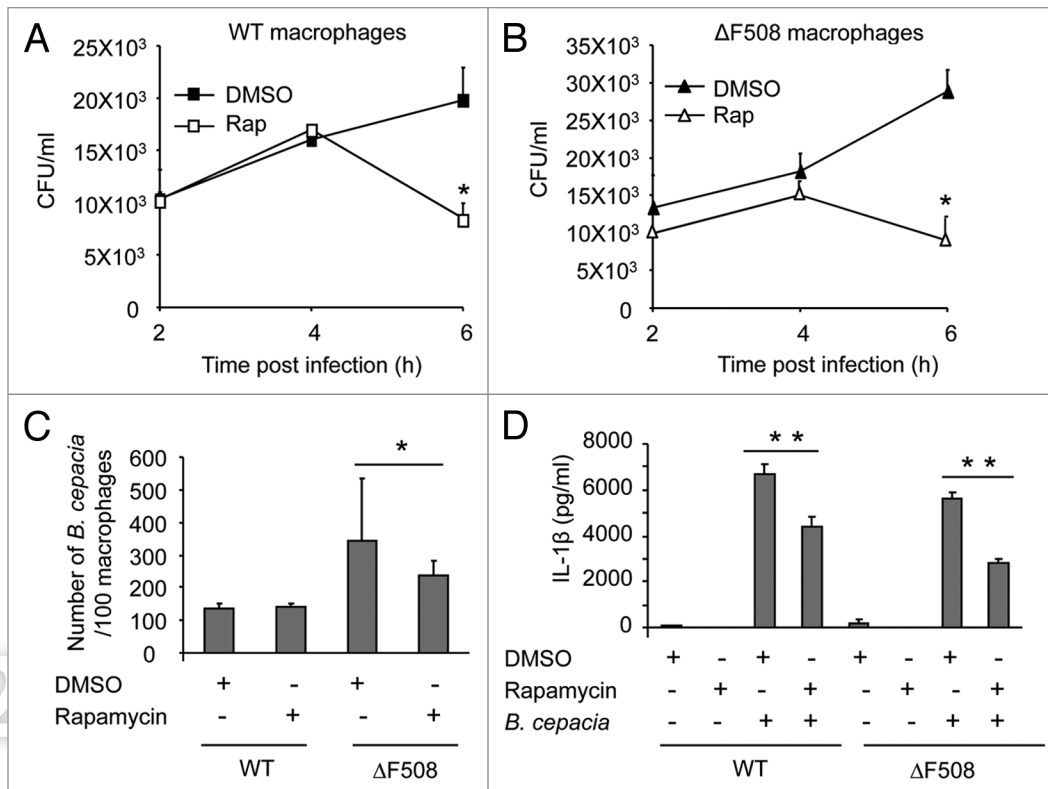


Figure 5. Rapamycin treatment decreases the recovery of *B. cepacia* and Interleukin-1 β (IL-1 β) production from infected macrophages. (A and B) Bone-marrow derived macrophages (BMDMs) from WT mice (A) and those harboring the Δ F508 mutation (B) were infected with *B. cepacia* for 2, 4 and 6 h in the presence of rapamycin or DMSO and then the number of bacteria per 100 macrophages was scored. (C) WT and Δ F508 were infected with *B. cepacia* for 2 h in the presence of rapamycin or DMSO and then the number of bacteria per 100 macrophages was scored. (D) WT and Δ F508 were infected with *B. cepacia* for 24 h in the presence of rapamycin or DMSO and the supernatants were analyzed for IL-1 β . Data are representative of 3 independent experiments and presented as means \pm SD. Asterisks indicate significant differences from the DMSO-treated cells (* p < 0.05; ** p < 0.01).

pulmonary inflammatory response in the lungs of CF patients upon infection especially with *B. cepacia*.^{59,67,68} *B. cepacia* is transmissible among CF patients and impossible to eradicate due to its inherent antibiotic resistance. Why CF patients are specifically susceptible to *B. cepacia* is unknown. Here we demonstrate that *B. cepacia* has a survival advantage in Δ F508 macrophages as the number of *B. cepacia* associated with Δ F508 macrophages is strikingly higher than those associated with WT macrophages. The results raise the possibility that *B. cepacia* uses the macrophages as a sanctuary to evade host defense.

The nature of the *B. cepacia*-containing vacuole and the mechanism halting its fusion with the lysosome were unclear. Here we show that in WT macrophages, a fraction of intracellular *B. cepacia* resides in multilayered LC3-labeled compartments consistent with autophagosomes.⁶⁹ A percentage of the *B. cepacia*-containing autophagosomes fuses with the lysosome. In contrast, in Δ F508 macrophages, LC3 is not detected around the *B. cepacia* vacuole, which rarely fuses with the lysosome (Fig. 7C). Electron microscopy data (Fig. 2D) showed that in WT macrophages, a fraction of intracellular *B. cepacia* resides in multilayered vacuoles that resemble the autophagosomes while in Δ F508 macrophages, most bacteria remained inside single-membrane vacuoles.

Autophagy stimulation with rapamycin in Δ F508 macrophages enhances the delivery of *B. cepacia* to the lysosomes.

Interestingly, the small GTP-binding protein Rab7 is inactivated by *B. cepacia*.⁴⁹ Rab7 is required for the final maturation of the late autophagic vacuole.⁷⁰ Thus, this observation is consistent with the lack of autophagy markers on the *B. cepacia* vacuole in Δ F508 macrophages.

Our data suggest that the inherently defective autophagy activity in Δ F508 cells^{11,12} in concert with the downregulation of major autophagy genes and inactivation of Rab7 by *B. cepacia* contribute to the persistence of *B. cepacia* in Δ F508 macrophages.

Why CF patients are more prone to *B. cepacia* infection and not to other autophagy-interacting pathogenic bacteria such as *Salmonella* is unknown. It seems that despite its compromised activity in Δ F508 macrophages, autophagy is still effective in clearing other autophagy-interacting bacteria. One possibility is that other intracellular bacteria like *Salmonella* do not decrease the expression levels of autophagy molecules. Thus, the downregulation of autophagy gene expression appears to give *B. cepacia* a survival advantage in Δ F508 macrophages. Suppression of autophagy has been employed by other pathogens such as *Francisella* or *Listeria*.⁷¹⁻⁷³ Yet, it seems that their strong autophagy-suppression capacity gives these pathogens a survival advantage even in WT cells, unlike *B. cepacia*.

During *B. cepacia* infection, caspase-1 is activated and pro-IL-1 β is cleaved and released from infected macrophages.⁷⁴

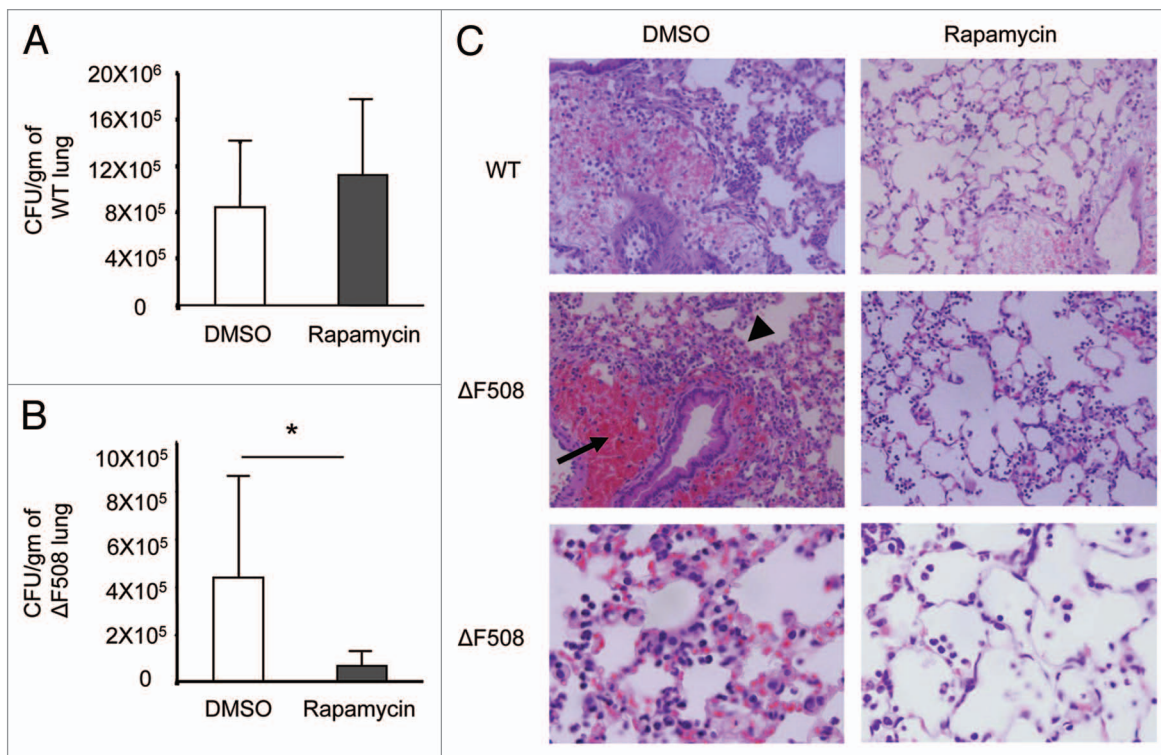


Figure 6. Treatment with rapamycin markedly decreases the recovery of *B. cepacia* from infected lungs of mice harboring $\Delta F508$ mutation in vivo. Wild-type (WT) (A) and mice harboring the $\Delta F508$ mutation ($\Delta F508$) (B) were pretreated with 2 doses of rapamycin (4 mg/kg) or with DMSO at a 24 h interval by intra-peritoneal injections. Then, mice were infected intra-tracheally with *B. cepacia* followed by a dose of rapamycin or DMSO. Colony-forming units (CFUs) recovered from homogenized lungs were enumerated and expressed as CFU per gram of lung tissue (A and B). (C) H&E staining of lung sections from WT (upper parts X40) or $\Delta F508$ mice (middle parts X40) treated as in (A and B). Lower part shows higher magnification (X100) of infected $\Delta F508$ lung sections. Data in (A and B) are represented as the means of data obtained from 3 mice \pm SD. Asterisks indicate significant differences from the DMSO treated mice (* $p < 0.05$).

More IL-1 β is produced from $\Delta F508$ macrophages than WT macrophages during *B. cepacia* infection. This could be due to increased *B. cepacia* burden in $\Delta F508$ macrophages. It is also possible that IL-1 β release is higher in $\Delta F508$ cells due to defective autophagy irrespective of the bacterial burden as suggested by a study demonstrating that autophagy regulates IL-1 β secretion in response to lipopolysaccharide (LPS) by targeting pro-IL-1 β for degradation.^{75,76} It is also plausible that both factors contribute to excess IL-1 β production in $\Delta F508$ macrophages infected with *B. cepacia*.

We found that rapamycin treatment reduces the production of inflammatory cytokines in vitro. H&E stained sections of infected lungs showed few focal regions of inflammation within WT infected lungs with the preservation of some healthy lung tissue. In contrast, stained sections of $\Delta F508$ lungs showed the accumulation of inflammatory cells in the peribronchiolar and perivascular areas. Alveolar spaces were filled with inflammatory cells and with exudates. Treatment of WT mice with rapamycin pre- and post-infection improved the preservation of healthy lung tissue. The effect of rapamycin treatment on CF lungs was most impressive because the lungs of CF mice treated with rapamycin were spared from the diffuse and intense inflammatory infiltrate observed in mice that did not receive rapamycin. Recent work has showed that human and mouse CF airway epithelia

are autophagy deficient and rescued by cystamine, an autophagy-inducing molecule, as it favored the clearance of CFTR aggregates.^{11,12} Therefore, the recognition of the role of autophagy in *B. cepacia* infection will lead to the development of a novel class of therapeutic agents that will clear CF aggregates and *B. cepacia* infection simultaneously.^{77,78} Thus, our findings have the potential for clinical application in CF patients who currently have limited options for treatment of *B. cepacia* infection and its associated deleterious inflammation.

Materials and Methods

Bone-marrow-derived macrophages. All animal experiments were performed according to protocols approved by the Animal Care Use Committee of the Ohio State University College of Medicine. Wild-type (WT) C57BL/6, $\Delta F508$ mice were obtained from Case Western University and housed in the OSU vivarium. Bone marrow-derived macrophages (BMDMs) were isolated from the femurs of 6- to 12-wk-old mice and were cultured in IMDM (GIBCO, 12440) containing 10% heat-inactivated FBS (GIBCO, 16000), 20% L cell-conditioned medium, 100 U/ml penicillin and 100 mg/ml streptomycin (GIBCO, 15140) at 37°C in a humidified atmosphere containing 5% CO₂. Macrophages were infected with *B. cepacia* K56-2 or the

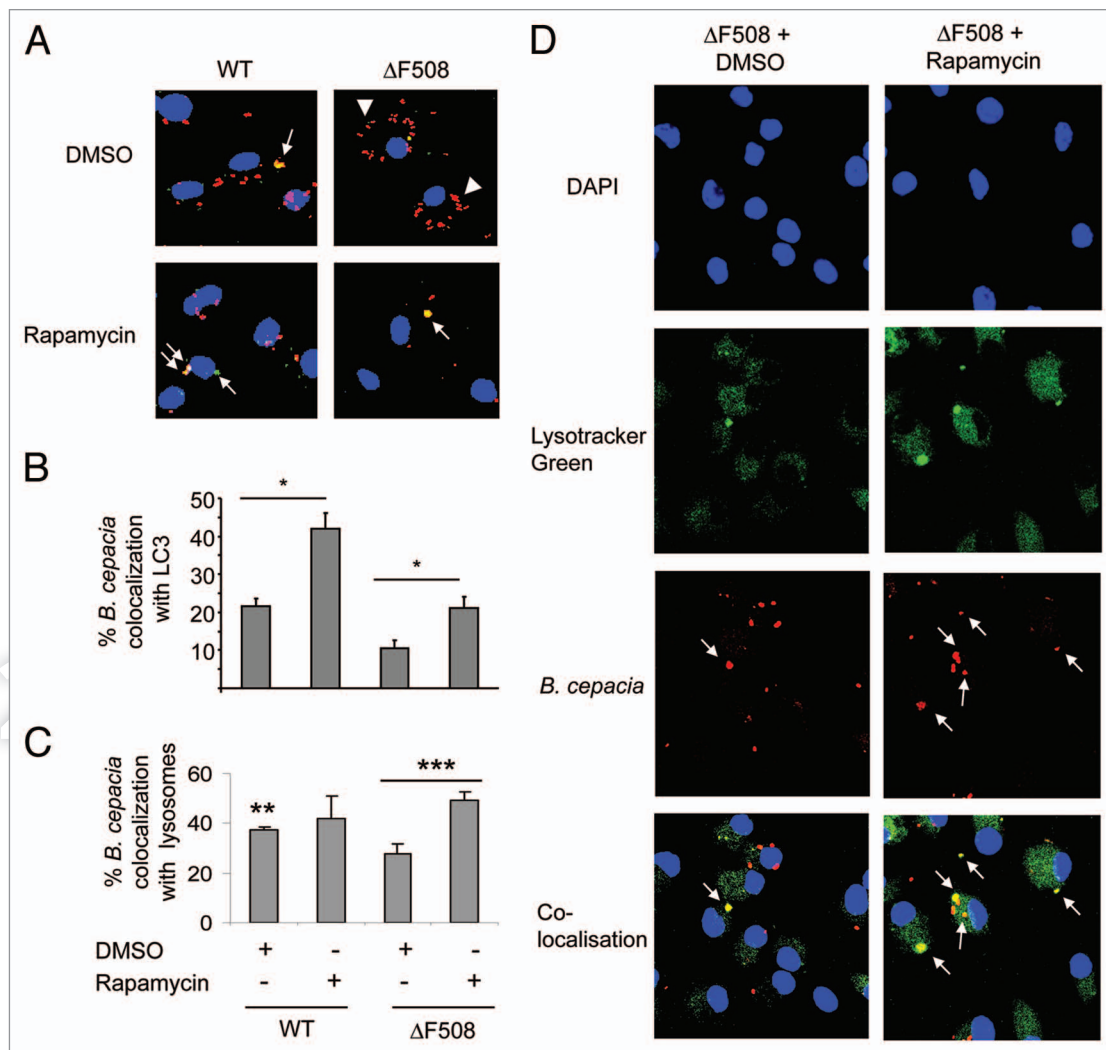


Figure 7. Treatment with rapamycin increases *B. cepacia* colocalization with LC3/Atg8 and with lysosomes. Bone marrow derived macrophages (BMDM) from wild-type (WT) and BMDM harboring $\Delta F508$ mutation ($\Delta F508$) were treated with rapamycin or DMSO for 1 h before infection, and then macrophages were infected with *B. cepacia* for 2 h. (A) Confocal microscopy demonstrates nuclei stained with DAPI, LC3 stained green and *B. cepacia* expressing monomeric red fluorescent protein (mRFP). White arrows indicate the sites of colocalization with of *B. cepacia* with LC3 with or without rapamycin. White arrows indicate colocalization, white arrow heads indicate *B. cepacia* with LC3 with or without rapamycin treatment. (B) Scoring of the percentage of colocalization of *B. cepacia* with LC3 with or without rapamycin treatment. (C) Scoring of the percentage of colocalization of *B. cepacia* with Lystracker Green with or without rapamycin. (D) Confocal microscopy showing nuclei stained with DAPI, lysosomes with Lysotracker Green and mRFP-expressing *B. cepacia*. The site of colocalization of *B. cepacia* with Lysotracker Green in $\Delta F508$ macrophages upon rapamycin ($\Delta F508$ + rapamycin) or DMSO ($\Delta F508$ + DMSO) are indicated with white arrows. Data are representative of 3 independent experiments and presented as the means \pm SD. Asterisks in (B and C) indicate significant differences from the DMSO-treated cells (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

corresponding gentamicin sensitive strain MHK1 at a multiplicity of infection (MOI) of 10.

Bacterial strains and culture. *Burkholderia cepacia* strain K56-2 was isolated from a CF patient. All bacterial strains were grown in Luria-Bertani (LB) broth at 37°C overnight with high amplitude shaking. The *B. cepacia* MHK1 strain has a mutation in an antibiotic efflux pump that confers gentamicin sensitivity but does not alter the trafficking of the mutant in macrophages.⁷⁹ To kill extracellular bacteria, Iscove's media (GIBCO, 12440) containing 10% heat-inactivated FBS (GIBCO, 16000) containing 50 $\mu\text{g/ml}$ gentamicin (GIBCO, 3564) was added for 30 min as previously described in reference 79. To

enumerate intracellular bacteria, infected macrophages were lysed using ice cold PBS (GIBCO, 14190) and physical disruption. Macrophages from monolayers were scrapped and pipetted repeatedly against the walls and bottom of the well. Recovered bacteria were quantified by plating serial dilutions on LB agar plates and counting colonies using the Acolyte Colony Counter, 5710/SYN.

Mouse in vivo infection. WT C57BL/6 and $\Delta F508$ mice were infected intra-tracheally with 10×10^6 WT bacteria with rapamycin (Sigma-Aldrich, R0395) or DMSO (Sigma-Aldrich, D2650) treatment ($n = 3$). Rapamycin was used in vivo at 4 mg/kg by intra-peritoneal injections. Mice were pretreated with two

doses of rapamycin for 2 d (24 h interval), and then infected with *B. cepacia* 2 h after the second dose of rapamycin. On the third day, mice treated with a final dose of rapamycin. The mice were sacrificed 2 h later, and the number of bacteria in the lungs was determined at second day post-infection.¹⁴ All animal experiments were performed according to animal protocols approved by the Animal Care Use Committee of the Ohio State University College of Medicine.

In vitro rapamycin treatment. Rapamycin (Sigma-Aldrich, R0395) was dissolved in DMSO (Sigma-Aldrich, D2650) at 1 mg/ml. Rapamycin was used at concentration 5 µg/ml, DMSO alone was used as a diluent control.

Immunoblotting. Macrophages were stimulated with *B. cepacia* K56-2 (MHK1) and the culture supernatant was removed. The cells were washed twice with PBS (GIBCO, 14190) and lysed in lysis buffer solution supplemented with a protease inhibitor mixture (Roche Applied Science, 10-519-978-001). The protein concentration was adjusted to 40 µg/ml. Proteins were separated by sodium dodecyl sulfate-15% PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories, 162-0117). Membranes were immunoblotted for pro-Interleukin-1β (IL-1β) antibody (kindly provided by Dr. Mark Wewers, Ohio State University) and Atg8 (LC3) (Sigma-Aldrich, L8918) protein bands were detected with secondary antibodies conjugated to horseradish peroxidase followed by enhanced chemiluminescence reagents (Amersham ECL protein gel blotting detection reagents GE Health Care-Life Sciences, RPN2106).

Enzyme-linked immunosorbent assay (ELISA). Macrophages were infected with *B. cepacia* K56-2 (MH1K) for different time points 6, 8, 24 h. Then, culture supernatants were collected, centrifuged and stored at -20°C until assayed for cytokine content. The amounts of IL-1β, IL-6 and TNFα in the supernatant were determined by specific sandwich ELISA following the manufacturer's protocol (R&D System Inc., DY201, DY406, DY410 respectively) and as previously described in reference 80.

siRNA knockdown of LC3. siRNA treatment was performed using siRNA against LC3 (Dharmacon, J-040989-09): CUA AUA AAG GCA CAA CGA A, GGA UAU AGC UCU AAG CCG G, CAU CCU AAG UUG CCA AUA A, ACU AUG GUG CGA UCA GUA A. siRNA was nucleofected into primary murine macrophages using Lonza Nucleofection kit (VPA-1009) and Amaxa equipment (AAD-10015) as we described previously in references 81 and 82.

Real time PCR. Total RNA was isolated from cells were lysed in Trizol (Invitrogen Life Technologies, 15596-026) and submitted to SA Biosciences for autophagy array study. Gene expression was calculated as relative copy numbers (RCN), as described previously in references 81 and 83. Briefly, Ct values of every target gene were subtracted from the average Ct of five housekeeping genes, present on the autophagy array (Gusb, Hprt1, Hsp90ab1, Gapdh, Actb) and the resulted ΔCt was used in the equation: $RCN = (2^{-\Delta Ct}) \times 100$. RCN for every gene represents its expression as number of copies relative to the 100 copies of average housekeeping genes.^{81,83}

Histopathological analysis. Lungs were removed en bloc and fixed in (10% Formalin) at room temperature for 24 h then

formalin was replaced by PBS (GIBCO, 14190), and processed for paraffin embedding. Formalin-preserved sections of the lungs we processed and embedded in paraffin by standard techniques. Sections of 5 µm thick were stained with hematoxylin and eosin (H&E) and examined. Longitudinal sections of 5 µm taken at regular intervals were obtained using a microtome from the proximal, medial and distal lung regions.

Confocal microscopy. Immunofluorescence microscopy experiments were performed as previously described in reference 84. *B. cepacia* expressing monomeric Red Fluorescent Protein (mRFP) was used. Localization of markers on *B. cepacia* phagosomes was performed as previously described in references 14 and 63. Antibodies used were rabbit anti-Atg8/LC3 (Abgent, AP1805a) followed by fluorescent secondary antibodies (Molecular Probes, A11008). Nuclei were stained with the nucleic acid dye 4',6'-diamino-2-phenylindole and lysosomes were stained green with LysoTracker Green (Invitrogen, L-7526) as previously described in references 14, 63, 80 and 85. In each experiment at least 100 bacteria were scored. Experiments were performed at least three times. Samples were analyzed with (Olympus FV 1000 Spectral Confocal) at the Ohio State University Microscopy Core Facility.

Transmission electron microscopy. WT and ΔF508 primary murine macrophages cultured on Permanox (Lab-Tek) chamber slides were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer with 0.1 M sucrose. The cells were postfixed with 1% osmium tetroxide in phosphate buffer and then en bloc stained with 2% uranyl acetate in 10% ethanol, dehydrated in a graded series of ethanol and embedded in Eponate 12 epoxy resin (Ted Pella Inc., 18012). Ultrathin sections were cut on a Leica EM UC6 ultramicrotome (Leica microsystems, EM FC7), collected on copper grids, and then stained with Lead citrate and uranyl acetate. Images were acquired with an FEI Technai G₂ Spirit transmission electron microscope (FEI), and Macrofire (Optronics) digital camera and AMT image capture software.

Statistical analysis. All experiments were performed at least three independent times and yielded similar results. Comparisons of groups for statistical difference were done using Student's two-tailed t test. p value ≤ 0.05 was considered significant.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Note

This study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Our protocol number 2007A0070 has been approved by the OSU Institutional Animal Care and Use Committee (IACUC). All efforts were made to minimize suffering.

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Note

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