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Depletion of the ubiquitin binding adaptor molecule SQSTM1/p62 from macrophages harboring *cftr* ΔF508 mutation improves the delivery of *Burkholderia cenocepacia* to the autophagic machinery

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Running title: p62 dictates the fate of *B. cepacia* in murine macrophages

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Background: Cystic fibrosis is characterized by defective autophagy and increased *Burkholderia cenocepacia* infection.
Result: The depletion of SQSTM1/p62 from ΔF508 macrophages improves bacterial clearance via autophagy.
Conclusion: p62 expression level determines the fate of *B. cepacia* infection in ΔF508 macrophages.
Significance: Our study reveals the role of p62 in diseases characterized by protein aggregates that compromise autophagy by consuming essential autophagy molecules.

SUMMARY

Cystic fibrosis (CF) is the most common inherited lethal disease in Caucasians. It is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) of which the *cftr* ΔF508 mutation is the most common. ΔF508 macrophages are intrinsically defective in autophagy due to the sequestration of essential autophagy molecules within unprocessed CFTR aggregates. Defective autophagy allows *Burkholderia cenocepacia* (*B. cepacia*) to survive and replicate in ΔF508 macrophages. Infection by *B. cepacia* poses great risk to CF patients because it causes accelerated lung inflammation and in some cases a lethal necrotizing pneumonia. Autophagy is a cell survival mechanism whereby an autophagosome engulfs non-functional organelles and delivers them to the lysosome for degradation. The ubiquitin binding adaptor protein SQSTM1/p62 is required for the delivery of several ubiquitinated cargos to the autophagosome. In wild-type (WT) macrophages, p62

depletion and overexpression lead to increased and decreased bacterial intracellular survival, respectively. In contrast, depletion of p62 in ΔF508 macrophages results in decreased bacterial survival, whereas overexpression of p62 leads to increased *B. cepacia* intracellular growth. Interestingly, the depletion of p62 from ΔF508 macrophages results in the release of the autophagy molecule beclin1 (BECN1), from the mutant CFTR aggregates and allows its redistribution and recruitment to the *B. cepacia* vacuole, mediating the acquisition of the autophagy marker LC3 and bacterial clearance via autophagy. These data demonstrate that p62 differentially dictates the fate of *B. cepacia* infection in WT and ΔF508 macrophages.

Cystic fibrosis (CF) is the most common inherited lethal disease among Caucasians, which is caused by mutations in the *cftr* gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR). The most common CFTR mutation results in a deletion of phenylalanine at position 508 (ΔF508), which affects the processing of the CFTR protein in such way that it cannot reach the epithelial cell surface. This mutation results in an aggresome-prone protein that forms intracellular aggregates (1-4).

Autophagy is a conserved physiological process that eliminates non-functional organelles and recycles cytosolic components to generate nutrients during periods of stress or starvation (5,6). Autophagy also targets cytosolic long-lived proteins and organelles for lysosomal degradation in eukaryotic cells and plays a role in innate immunity (7). Loss of autophagy in murine tissues is accompanied by accumulation of protein aggregates and disordered organelles leading to life-threatening diseases (8). Autophagy plays a key role in protecting the cytosol from bacterial infection. The mechanisms of bacterial recognition by this pathway are starting to be elucidated. Some cellular cargos are marked for autophagy by acquiring adaptor proteins such as Calcoco2 (also known as NDP52) and Neighbor of BRCA1 gene product (NBR1) (9-14). In addition, SQSTM1 (also known as p62) is required for targeting *Salmonella enterica* serovar Typhimurium (*S. typhimurium*), intracytosolic *Shigella* and *Listeria* to the autophagic pathway (9,10).

The adaptor molecule p62 is a ubiquitously expressed cellular protein that is conserved in metazoa but not in plants or fungi (15,16). The quantity of p62 is critical for cell viability and is strictly controlled (17). p62 has multiple protein-protein interaction domains, including the ubiquitin-associated domain for ubiquitinated cargo binding and the LC3 interaction region for binding LC3 (10). Accordingly, impaired autophagy is accompanied by accumulation of p62 followed by formation of aggregates containing p62 and ubiquitinated proteins. This accumulation occurs due to the nature of both self-oligomerization and ubiquitin-binding of p62 (18,19).

Burkholderia cenocepacia (*B. cepacia*) is an opportunistic Gram-negative bacterium that infects CF patients and leads to severe lung inflammation and lung tissue destruction. Occasionally, this infection results in a lethal necrotizing pneumonia (20-22). Unfortunately, *B. cepacia* is resistant to most known antibiotics and thus is nearly impossible to treat. *B. cepacia* adopts an extracellular or intracellular lifestyle (23,24). This bacterium can survive within a variety of eukaryotic cells such as amoebae, epithelial cells and macrophages (25-28).

We have previously demonstrated that in WT macrophages the majority of *B. cepacia*-containing vacuoles slowly acquire the specific autophagy marker LC3 within 2 h of infection. Subsequently, these vacuoles fuse with the lysosomes and the bacterium is degraded. In ΔF508 macrophages, *B. cepacia*-containing vacuoles do not acquire autophagosome markers and do not fuse with the lysosomes.

Here, we demonstrate that in WT macrophages, p62 is required for targeting *B. cepacia* to the autophagosome. Upon p62 down regulation, bacterial growth increases,

while the over expression of p62 results in a significant decrease in *B. cepacia* replication. On the contrary, down regulation of p62 in ΔF508 macrophages is associated with decreased bacterial growth and p62 over expression results in increased *B. cepacia* replication. p62 down regulation in ΔF508 macrophages releases the trapped BECN1 from CFTR aggregates, allowing its recruitment to the *B. cepacia* vacuole. BECN1 acquired by the *B. cepacia*-containing vacuole subsequently attracts LC3, thereby mediating the fusion of the maturing autophagosome containing *B. cepacia* with the lysosome via the autophagic machinery. These data provide mechanistic insight on how *B. cepacia* persists in ΔF508 macrophages. This report also suggests that p62 may be an attractive drug target to improve *B. cepacia* clearance by autophagic machinery.

EXPERIMENTAL PROCEDURES

Bone-marrow-derived macrophages. 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 were purchased from Jackson. ΔF508 mice on a C57BL/6 background were obtained from Case Western University and housed in the OSU vivarium. Bone marrow-derived macrophages were isolated from the femurs of 6 to 12 week 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% CO_2. Macrophages were infected with *B. cepacia* K56-2 expressing m-RFP or the corresponding gentamicin sensitive strain MHK1 at a multiplicity of infection (MOI) of 10.

Bacterial Strains and Culture. Burkholderia cenocepacia strain K56-2 is a clinical isolate from a CF patient. The corresponding gentamicin sensitive strain MHK1 strain was previously described (29). All bacterial strains were grown in Luria-Bertani (LB) broth at 37°C overnight with high amplitude shaking. To kill extracellular bacteria, Iscove's media (GIBCO, 12440) plus FBS (GIBCO, 16000) containing 50 μg/ml gentamicin (GIBCO, 3564) were added for 0.5 h as previously described.(29) To enumerate intracellular bacteria, infected macrophages were lysed with ice cold PBS (GIBCO, 14190) at designated times. Recovered bacteria were quantified by plating serial dilutions on LB agar plates and counting colonies using Acolyte Colony Counter, 5710/SYN.

Immuno-blotting. Macrophages were stimulated with *B. cepacia* and the culture supernatant was removed. Cells were lysed in lysis buffer solution supplemented with a protease inhibitor mixture (Roche Applied Science, 10-519-978-001). The protein concentration was adjusted to 30 μg/ml. Proteins were separated on a sodium dodecyl sulfate 15% polyacrylamide gel and transferred to polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories, 1p62-0117). Membranes were Immuno-blotted for p62 (Sigma-Aldrich, P0067), LC3 (Sigma-Aldrich, L8918), Calreticulin (stressgen, SPA600), BECN1 (Abcam, ab55878), NDP52 (Millipore, MAB4386), NBR1 (Santa Cruz, SC-130380), and Actin (Abcam, ab8299). Protein bands were detected with secondary antibodies conjugated to horseradish peroxidase followed by enhanced chemiluminescence reagents (Amersham ECL Western blotting detection reagents GE Health care-Life sciences, RPN2106).

siRNA Treatment and plasmid transfection. siRNA treatment was performed using siRNA against p62 (Dharmacon-18412) ACAGAUGCCAGAAUCGGAA, CUGCUCAGGAGGAGACGAU, GAACAGAUGGAGUCGGGAA, CCAUGGGUUUCUCGGAUGA, siRNA against NDP52 (Dharmacon-76815) CAACACAGAGGGUCAAUAA, CAGAAGAGGACAUCCGGAU, CCAAGGAUGUGGAGCGCUA, GAGUUGAGGUGUCCGUGUA, siRNA against NBR1 (Dharmacon-17966) GAAAUGGGAUUCUGCGACA, AGUCCGUGGAAGCGAGUAA, CAAGCAAAGCUGACGAUUU, ACAGGAGGCAUUCGGGUUA. siRNA against Atg7 (Dharmacon-49953)

CAUCAUCUUUGAAGUGAAA, GCUAGAGACGUGACACAUA, AGCGAAAGCUGGUCAUCAA, GGUCGUGUCUGUCAAGUGC. siRNA was nucleofected into primary macrophages 48 h before infection using Lonza Nucleofection kit and Amaxa equipment as previously described(30,31). Successful knockdown was confirmed by Immuno-blot for each experiment. DsRed-p62 plasmid was obtained from addgene (32) and was nucleofected into primary macrophages using Lonza Nucleofection kit and Amaxa equipment. The plasmid was nucleofected 24 h before the infection. Successful p62 over expression was confirmed by Immuno-blotting.

Real Time PCR. Total RNA was isolated from cells lysed in Trizol (Invitrogen Life Technologies, 15596-026) and then, converted to cDNA. Gene expression was calculated as relative copy numbers (RCN), as described previously (30,33). Briefly, C_t values of p62 gene were subtracted from the average Ct of two housekeeping genes, (GADPH, CAP1) and the resulted ΔC_t was used in the equation: RCN= $(2^{-\Delta Ct})$ 100. The relative copy number (RCN) of a gene is represented as number of copies relative to the 100 copies of average housekeeping genes (30,33).

Confocal Microscopy. Immuno-fluorescence experiments for colocalization with autophagy markers were performed as previously described (34,6). Rabbit anti-LC3 (Abgent, AP1805a), mouse anti-p62 (BD Bioscience, 610832), FK2 mAb (Enzo Bioscience, BML-PW8810), and rabbit anti-BECN1 (Abcam, ab55878) were used followed by fluorescent secondary antibodies (Molecular Probes, A11008). Nuclei were stained with the nucleic acid dye 4′,6′-diamino-2-phenylindole (DAPI) (6,35). Samples were analyzed with (Olympus Flouview FV10i Confocal) at The Ohio State University, Department of Microbial Infection and Immunity.

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

Ethics statement. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institute of health and The Ohio State University. The Institutional Animal Care and Use Committee (IACUC) have approved our protocol number 2007A0070. All efforts were made to minimize suffering.

RESULTS

More B. cepacia colocalized with p62 in WT macrophages than in ΔF508 macrophages. We previously demonstrated that *B. cepacia* is cleared by the autophagy machinery in WT macrophages but not in their ΔF508 counterparts. To determine why the *B. cepacia* vacuole is not efficiently recognized by the autophagy machinery in ΔF508 macrophages, we followed their trafficking within WT and ΔF508 macrophages. Recent studies showed that p62 is required for targeting *S. typhimurium, Shigella,* and *Listeria* to the autophagic pathway (9,10). Therefore, we examined the colocalization of *B. cepacia* with p62 in WT and ΔF508 macrophages. The time course for infection was 0.5, 1.5, 2 and 4 h. In WT macrophages, a significant percentage of *B. cepacia* colocalized with p62 at 1.5 h post infection. Colocalization then declined at latter time points (Fig. 1A and B). However, *B. cepacia* vacuoles in ΔF508 macrophages did not colocalize with p62 at any time point throughout infection (Fig. 1A and B). Together, these data shows that p62 labels *B. cepacia* vacuole in WT but not in ΔF508 macrophages.

The B. cepacia vacuole efficiently acquires ubiquitin in ΔF508 macrophages. Autophagy recognizes cargo for uptake and degradation when it becomes ubiquitinated and bound to an autophagy adaptor molecule (10). The lack of p62 acquisition by the *B. cepacia* vacuole in ΔF508 macrophages could be due to defective ubiquitination of the *B. cepacia*-containing vacuole or due to lack of p62 expression in ΔF508 macrophages. To differentiate between these possibilities, first

we infected WT and ΔF508 macrophages with *B. cepacia* expressing m-RFP for 0.5 h or 2 h and examined the colocalization of *B. cepacia* with ubiquitin. There was no significant difference in the colocalization of *B. cepacia* with ubiquitin between WT and ΔF508 macrophages (Fig. 2A and B). These data demonstrate that equivalent numbers of *B. cepacia* vacuoles acquired ubiquitin in WT and ΔF508 macrophages. Therefore, the lack of colocalization of *B. cepacia* with autophagosomes in ΔF508 macrophages is not due to the absence of ubiquitin around the *B. cepacia* vacuole.

Next, to determine if the failure of the autophagy machinery to target the *B. cepacia* vacuole is due to the lack of p62 expression in ΔF508 macrophages, we examined the level of p62 within WT and ΔF508 macrophages. Immuno-blot using an antibody against p62 revealed that murine macrophages harboring the ΔF508 mutation exhibited a higher level of p62 compared to WT macrophages (Fig. 3A). Quantitative PCR (q-PCR) was performed to determine whether the increase in p62 protein level in ΔF508 macrophages, compared to WT macrophages, is due to regulation of gene expression or accumulation of the p62 protein. There was no significant difference in the p62 mRNA level in both types of macrophages (Fig. 3B). Together, these data show that the increase in p62 level in ΔF508 macrophages is due to accumulation of the protein inside the cell, suggesting defective autophagy activity.

B. cepacia infection elevates p62 expression within WT and ΔF508 macrophages. p62 is well expressed in ΔF508 macrophages, however *B. cepacia* infection down regulates autophagy in both WT and ΔF508 macrophages. Thus, it is possible that *B. cepacia* infection is accompanied by depletion of p62 from ΔF508 macrophages upon infection. To examine this possibility, we examined the effect of *B. cepacia* on p62 expression upon infection in WT and ΔF508 macrophages by q-PCR and Immuno-blot. At 4 h post infection, q-PCR analysis demonstrated increased expression of p62 gene level in both WT and ΔF508 macrophages compared to non-infected macrophages (Fig. 3C). Similarly, Immuno-blotting showed a higher p62 level in both types of macrophages (Fig. 3D). Together, these data show that *B. cepacia* infection increases the expression level of p62 in WT and ΔF508 macrophages.

Over expression of p62 conversely affects B. cepacia replication in WT and ΔF508 macrophages. To determine the role of p62 in *B. cepacia* replication in WT and ΔF508 macrophages, we examined *B. cepacia* survival in the presence of ectopically expressed p62. WT and ΔF508 macrophages were nucleofected with p62 plasmid or vector control and after 24 h, cells were infected with *B. cepacia* for 2, 4, and 6 h (Fig. 4G). In WT macrophages harboring the p62 plasmid, recovered *B. cepacia* CFUs decreased at 6 h post-infection compared to the cells harboring the vector alone (Fig. 4A). Confocal microscopy revealed significantly less bacterial accumulation upon over expression of p62 (Fig. 4B and C). In contrast, ΔF508 macrophages harboring the p62 plasmid allowed significantly increased *B. cepacia* accumulation after 6 h post infection (Fig. 4D). Confocal microscopy confirmed increased bacterial accumulation (Fig. 4E and F). Together, these results demonstrate that the availability of p62 differentially determines the fate of *B. cepacia* in WT and ΔF508 macrophages.

Down regulation of p62 decreases the growth of B. cepacia in ΔF508 macrophages. To determine if p62 targets *B. cepacia* vacuoles to autophagosomes for degradation, we nucleofected WT and ΔF508 macrophages with p62 siRNA or scrambled siRNA (Fig. 5G). After 48 h, cells were infected with *B. cepacia* for 2, 4, and 6 h. In WT macrophages, *B. cepacia* colony forming units (CFU) significantly increased upon down regulation of p62 (Fig. 5A). In addition, confocal microscopic analysis demonstrated significantly increased bacterial numbers at 2 h post-infection (Fig. 5B and C). In contrast, ΔF508 macrophages showed decreased *B. cepacia* CFUs upon down regulation of p62 (Fig. 5D). Furthermore, confocal microscopy revealed significantly low bacterial accumulation 2 h after *B. cepacia* infection upon down regulation of p62 (Fig. 5E and F).

Therefore, these data demonstrate that p62 controls *B. cepacia* infection in WT macrophages, but not in ΔF508 macrophages. The details of this differential role are not clear.

Decreased p62 expression promotes LC3 acquisition by B. cepacia vacuole in ΔF508 macrophages. LC3 is the main marker for autophagosomes. The conversion of LC3-I to LC3-II denotes autophagy stimulation and autophagosome formation (7,36). We have previously demonstrated that *B. cepacia* colocalization with LC3 is markedly decreased in ΔF508 macrophages compared to WT macrophages (37,38). To determine the underlying mechanism, WT and ΔF508 macrophages were nucleofected with either siRNA against p62 to down regulate p62 or with scrambled siRNA and after 48 h, nucleofected macrophages were infected with *B. cepacia* expressing m-RFP for 0.5 h and 2 h. Confocal microscopy showed that in WT macrophages, *B. cepacia* colocalization with LC3 decreased significantly when p62 was down regulated compared to the siRNA control treated cells (Fig. 6A and C). In contrast, ΔF508 macrophages allowed significantly more *B. cepacia* colocalization with LC3 after the down regulation of p62 compared to the siRNA control treated cells (Fig. 6B and D). Together, these data suggest that p62 is required for the delivery of *B. cepacia* to the autophagosomes in WT macrophages, a role that is compromised for unknown reasons in ΔF508 macrophages.

Depletion of p62 liberates BECN1 allowing its redistribution and recruitment by the B. cepacia vacuole in ΔF508 macrophages. A growing body of evidence indicates that BECN1 is sequestered within the mutant CFTR aggresomes (1,2). BECN1/Atg6 is a member of the class III PI3K complex and is essential for the early stages of autophagosome formation (5,39). Thus its unavailability leads to defective autophagic activity (1,2). Mutant CFTR aggregates sequester autophagy molecules such as BECN1 depleting them from their storage areas leading to defective autophagy. We examined the colocalization of *B. cepacia* with BECN1 in WT and ΔF508 macrophages. Confocal microscopy showed that in WT macrophages, high numbers of *B. cepacia* colocalized with BECN1 compared to ΔF508 macrophages (Fig. 7A and B, arrows). In WT macrophages BECN1 was distributed throughout the cytosol, while in ΔF508 macrophages, BECN1 was condensed in patches (Fig. 7 A, arrow heads).

Since the sequestration of BECN1 in CFTR aggregates requires p62 (1,2), we examined the effect of p62 depletion on BECN1 distribution within the cytosol and around the *B. cepacia* vacuole inside ΔF508 macrophages. ΔF508 macrophages nucleofected with p62 siRNA showed significantly more colocalization of *B. cepacia* with BECN1 compared to siRNA control (Fig. 7C, arrows and 7D). Additionally, within the ΔF508 macrophages nucleofected with siRNA against p62, BECN1 was redistributed within the cytosol with the disappearance of BECN1-containing patches (Fig. 7C) after down regulation of p62 (Fig. 7B). Notably, our immuno-blot using antibody specific to BECN1 showed equal amounts of the total BECN1 in the ΔF508 macrophages before and after p62 depletion (Fig. 7E). Together, these data show that depletion of p62 from ΔF508 macrophages allows the redistribution of BECN1 throughout the cell and increases its availability for the *B. cepacia*-containing vacuole.

Together, these data suggest that depletion of p62 from ΔF508 macrophages mediates *B. cepacia* clearance via recuperated autophagy. To confirm this conclusion, ΔF508 macrophages were depleted of p62 and Atg7 (an essential autophagy molecule) to disrupt the autophagy machinery, then, infected with *B. cepacia*. Depletion of p62 alone from ΔF508 macrophages improved *B. cepacia* clearance, yet concomitant depletion of Atg7 hindered bacterial clearance (Fig. 7F and G). Thus, improved bacterial clearance upon depletion of p62 from ΔF508 macrophages is mediated by autophagy.

NBR1 and NDP52 contribute to the delivery of B. cepacia to autophagosomes after down regulation of p62 in ΔF508 macrophages. Thus, down regulation of p62 in ΔF508 macrophages improves *B. cepacia*

clearance by restoring autophagy activity. However, what targets the *B. cepacia* vacuole for autophagy in the absence of adequate amounts of p62 is unknown. Recently, it has been shown that p62 and NDP52 act cooperatively to drive efficient antibacterial autophagy of *Salmonella*, *Shigella* and *Listeria* (9,41). Furthermore, it was revealed that NBR1 and p62 mark ubiquitinated cargo for autophagy (11,42). To determine if NDP52 or NBR1 contribute to the delivery of *B. cepacia* to autophagosomes, we nucleofected WT and ΔF508 macrophages with siRNA against NDP52 or NBR1 prior to infection with *B. cepacia*. Our results showed that in WT macrophages, down regulation of NDP52 did not affect *B. cepacia* recovered CFUs while that of NBR1 resulted in a significant increase in *B. cepacia* growth. In ΔF508 macrophages, the down regulation of either NDP52 or NBR1 resulted in an increase in *B. cepacia* growth. This result demonstrates that both NDP52 and NBR1 facilitate the delivery of *B. cepacia* to autophagosomes in ΔF508 macrophages. In WT macrophages, however, only NBR1 contributes to the delivery of *B. cepacia* to autophagosomes (Fig. 8 A, B and D). To determine if NDP52 and NBR1 mark the *B. cepacia* vacuole for autophagy uptake in p62 depleted ΔF508 macrophages, we nucleofected ΔF508 macrophages with siRNA against p62 alone or in combination with either NDP52 or NBR1. Simultaneous down regulation of p62 and NDP52 or p62 and NBR1 in ΔF508 macrophages resulted in a significant increase in *B. cepacia* growth (Fig. 8C and E). Together, our data suggest that both NDP52 and NBR1 contribute to labeling the *B. cepacia* vacuole for autophagy uptake when p62 is unavailable.

DISSCUSION

Human and mouse CF macrophages and airway epithelial cells exhibit impaired autophagy that is associated with formation of aggregates including p62 and ubiquitinated mutant CFTR protein. This is due to both self-oligomerization and ubiquitin-binding nature of p62 (1,2,10,37,38). Similar aggregates have been identified in various neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis and cancer (1,2,43,44). Impaired turnover of p62 is a major cause of the pathogenic changes seen in the autophagy-deficient mice as the loss of Atg7 in mouse livers results in severe p62 accumulation. Loss of p62 greatly attenuates liver injury resulting from autophagy deficiency (18).

Here, we found that ΔF508 macrophages express more p62 than WT macrophages. This could be due to p62 accumulation because of reduced recycling in ΔF508 macrophages as a consequence of compromised autophagosome formation and maturation. Alternatively, the accumulation of p62 could stimulate the formation of more ΔF508 CFTR aggregates. This later possibility agrees with the observation that depletion of p62 from ΔF508 macrophages improves autophagy and decreases the BECN1 positive aggregates. Also, a previous study using CF epithelial cells showed that p62 promotes aggresome accumulation of misfolded or modified proteins (43,45). Recently, it has reported that reducing the levels of p62 can rescue ΔF508-CFTR trafficking to the plasma membrane of CF airway epithelial cells (1,2,46).

The presence of intracellular bacteria such as *B. cepacia* increases the level of p62 expression in both WT and ΔF508 macrophages. It is possible that p62 over expression upon infection worsens the biology of ΔF508 macrophages, providing an explanation for the deterioration of lung function and innate immune responses in the infected CF lung. There are several mechanisms by which *B. cepacia* may lead to the accumulation of p62. It is plausible that *B. cepacia* increases p62 accumulation by inhibiting autophagy in ΔF508 macrophages as we have previously published (37,38). Notably, *B. cepacia* infection increases p62 mRNA. Regardless of the mechanism of p62 accumulation, the p62 aggregates sequester essential autophagy molecules, such as BECN1, making them unavailable for efficient autophagosome formation (48).

The sequestration of BECN1 occurs via Transglutaminase-2 (TG2)-mediated crosslinking in aggresomes because BECN1

protein sequence contains QP and QXXP motifs, which are specific target sites for TG2 activity (49) and TG2 is an autophagy inhibitor in pancreatic adenocarcinoma cells (50). Increased reactive oxygen species (ROS) in CF epithelia sustains high TG2 levels through TG2 SUMOylation (48). Thus, BECN1, and not all autophagy molecules, is specifically recruited to aggresomes in CF cells.

Examining the sequential acquisition of autophagy molecules by the *B. cepacia* vacuole revealed that although ubiquitination is efficient in both WT and ΔF508 macrophages, BECN1 acquisition is defective only in ΔF508 macrophages. BECN1, also known as autophagy-related gene product 6 (Atg6) and its binding partner class III phosphoinositide 3-kinase (PI3K; also named Vps34), are required for the initiation of the autophagosome formation (47). Thus, supplementation of p62 alone to ΔF508 macrophages will not improve the targeting of *B. cepacia* to autophagosomes. This conclusion is supported by the over expression experiment of p62 in ΔF508 macrophages which actually lead to more bacterial growth. Therefore, to correct the trafficking defect of *B. cepacia* in ΔF508 macrophages, "free" BECN1 is required, which is achieved by depletion of p62.

p62 targets several pathogens such as *S. typhimurium, Shigella* and *Listeria* to the autophagosome (9,10). Similarly, p62 associates with the *B. cepacia* vacuole in WT macrophages. However, depletion of p62 from ΔF508 macrophages promotes *B. cepacia* uptake by autophagosomes and decreases bacterial burden. It is possible that another adaptor molecule such as NBR1 compensates for the loss of p62. The structure of NBR1 resembles that of p62, it can bind both LC3 and ubiquitinated proteins through the LC3 interaction region and ubiquitin-associated domain, respectively (11,14). NDP52 is another cargo marker that drives certain bacteria to the autophagy machinery (9,41). In this study, we found that NDP52, facilitates autophagy uptake of *B. cepacia* in ΔF508 macrophages but not in WT cells. NBR1, however, appears to contribute to the delivery of *B. cepacia* to the autophagy machinery in both WT and ΔF508 macrophages. To our knowledge, this is the first demonstration of a role for NDP52 or NBR1 in bacterial targeting by autophagy.

We previously showed (52) that autophagy stimulation by rapamycin can overcome the down regulating effect of *B. cepacia* on the ATG genes and can control the *B. cepacia* infection in ΔF508 mouse model both *in vivo* and *in vitro*. In the current work, we demonstrate that p62 depletion from ΔF508 mouse macrophages is another approach to improve autophagic control on *B. cepacia* infection.

Together, these data provide a molecular framework to better understand the emerging complexity of diseases related to autophagic defect such as CF and the ability of macrophages to defend against the bacterial infection. This study also indentifies p62 as a promising drug target for improving *B. cepacia* clearance in CF macrophages.

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FIGURE LEGENDS:

FIGURE 1. More *B. cepacia* vacuoles colocalize with p62 in wild-type (WT) macrophages than in ΔF508 macrophages. (A) Confocal microscopy for WT and ΔF508 macrophages infected with *B. cepacia* expressing m-RFP for 0.5 or 1.5 h. p62 stained green while nuclei were stained with DAPI. (B) The percentage of colocalization of *B. cepacia* with p62 was quantified at the indicated time points. More than 200 cells were scored. White arrows indicate the sites of colocalization. Data in (B) are presented as means ± S.D. Asterisk indicate significant difference (*$P< 0.05$).

FIGURE 2. The ubiquitination of *B. cepacia* vacuole is similar in wild-type (WT) and ΔF508 macrophages. (A) Confocal microscopy for WT and ΔF508 macrophages infected with m-RFP-expressing *B. cepacia* for 0.5 or 2 h. Ubiquitin stained green and nuclei were stained with DAPI (white arrow indicates the sites of colocalization). (B) The percentage of colocalization of *B. cepacia* with ubiquitin was quantified (More than 200 cells were scored).

FIGURE 3. Murine bone marrow derived macrophages harboring ΔF508 mutation have a higher level of p62 than wild-type (WT) macrophages (A) Upper panel, immuno-blot of WT and ΔF508 macrophage lysates showing the expression level of LC3 (I/II) and p62, respectively. Lower panel, densitometry of p62 protein level. (B) q-PCR expression profile of p62 in WT and ΔF508 macrophages. (C) q-PCR expression profile of p62 in WT and ΔF508 macrophages non-infected (NT) or infected with *B. cepacia* for 4h (4h BC) (D) Upper panel, immuno-blot for WT and ΔF508 macrophages using p62 antibody prior and at 4 h post-infection with *B. cepacia*. Lower panel, densitometry of p62 protein level. Data in (B and C) are expressed as relative copy numbers (RCN) and shown as means of three independent experiments ± S.D. Asterisks in (C) indicate significant differences from the uninfected cells at the indicated time point (**$P< 0.01$ and ***$P< 0.001$).

FIGURE 4. p62 over expression decreases growth of *B. cepacia* in wild-type (WT) macrophages while increases bacterial growth in ΔF508 macrophages. (A and D) WT and ΔF508 macrophages were nucleofected with p62 plasmid or vector control 24 h prior to infection and then infected with *B. cepacia* for 2, 4 and 6 h. Colony forming units (CFU) were enumerated. (B and E) Confocal microscopy for WT and ΔF508 macrophages overexpressing p62 at 0.5 or 2 h post-infection. White arrows indicate *B. cepacia* stained with DAPI. (C and F) Both types of macrophages overexpressing p62 were infected for 0.5, 2 and 4 h and the number of bacteria per 100 cells was quantified (more than 200 cells were scored). (G) Immuno-blot for WT macrophages after 24 h nucleofection with DsRed-p62 plasmid showing the over expression of p62. Data are representative of three different experiments and presented as the means ± S.D. Asterisks in (A, C, D and F) indicate significant differences from the vector at the respective time points (*$P< 0.05$; **$P<0.01$).

FIGURE 5. Down regulation of p62 results in increased growth of *B. cepacia* in wild-type (WT) murine macrophages while in ΔF508 macrophages leads to decreased growth. (A and D) WT and ΔF508 macrophages were nucleofected with siRNA against p62 (siRNA-p62) or control siRNA (siRNA-CT) 48 h prior to infection and then infected with *B. cepacia* for 2, 4 and 6 h. Colony forming units (CFU) were enumerated. (B and E) Confocal microscopy of p62-depleted WT and ΔF508 macrophages infected with *B. cepacia* for 0.5 h or 2 h. White arrows show *B. cepacia* stained with DAPI. (C and F) Both types of macrophages were infected for 0.5 and 2 h after depletion of p62. The number of bacteria per 100 cells was quantified (more than 200 cells were

scored). (G) Upper Panel, immuno-blot for WT and ΔF508 macrophages after 48 h nucleofection with siRNA against p62 (si-p62) or control siRNA (si-CT). Lower panel, densitometry of p62 protein level. Data are representative of three different experiments and presented as the means ± S.D. Asterisks in (A, C, D and F) indicate significant differences from the (siRNA-CT) at the respective time points (**P*< 0.05; ***P*<0.01; ***<0.001).

FIGURE 6. Down regulation of p62 decreases *B. cepacia* colocalization with LC3 in wild-type (WT) macrophages but increases the colocalization in ΔF508 macrophages. (A and B) Confocal microscopy for WT macrophages and ΔF508 macrophages infected with *B. cepacia* expressing m-RFP for 0.5 or 2 h. LC3 stained green while nuclei were stained with DAPI. The percentage of colocalization of *B. cepacia* with LC3 at the indicated time points was scored in both WT and ΔF508 macrophages, respectively (C and D) (more than 200 cells were scored). Data in (C and D) are presented as means ± S.D. Asterisks indicate significant difference (**P*< 0.05 and ***P*< 0.01).

FIGURE 7. Colocalization of *B. cepacia* with BECN1 is increased in ΔF508 macrophages upon depletion of p62. (A) Confocal microscopy for WT and ΔF508 macrophages infected with *B. cepacia* expressing m-RFP for 2 h. BECN1 stained green and nuclei were stained with DAPI. White arrows indicate *B. cepacia* while arrow heads indicate BECN1 aggregates. (B) The percentage of colocalization of *B. cepacia* with BECN1 was scored by examining more than 400 cells. (C) Confocal microscopy for ΔF508 macrophages nucleofected with siRNA against p62 (Si-p62) or scrambled siRNA control (si-CT) 48 h prior to infection. Nucleofected macrophages were infected with *B. cepacia* expressing m-RFP for 2 h. BECN1 stained green while nuclei were stained with DAPI. Arrows indicate *B. cepacia* while arrow heads indicate BECN1 aggregates. (D) The percentage of colocalization of *B. cepacia* with BECN1 at the assigned time point was scored (More than 400 bacteria were scored). (E) Immuno-blot for ΔF508 macrophages nucleofected with siRNA against p62 (si-p62) or control siRNA (si-CT) for 48h, antibodies against p62 and BECN1 have been used. (F) ΔF508 macrophages nucleofected with siRNA against p62 (si-p62) or control siRNA (si-CT) or siRNA against p62 and Atg7 together (si-p62+si-Atg7) for 48h and then infected with *B. cepacia* for 2, 4 and 6h. Colony forming units (CFU) were enumerated. (G) Immuno-blot for ΔF508 macrophages nucleofected with control siRNA (si-CT), siRNA against p62 (si-p62), siRNA against Atg7 (si-Atg7) or siRNA against p62 and Atg7 together (si-p62+si-Atg7) for 48h, antibodies specific to p62 And Atg7 have been used to detect the down regulation. Data in B, D and F are presented as means of three different experiments ± S.D. Asterisks in (B and D) indicate significant differences between both types of macrophages at the designated time point (***P*< 0.01, ****P*<0.001).

FIGURE 8. Depletion of p62 in ΔF508 macrophages improves clearance of *B. cepacia* by autophagosomes via NDP52 and NBR1. (A, B and C) WT (A) and ΔF508 (B and C) macrophages were nucleofected with siRNA against NDP52 (si-NDP52), NBR1 (si-NBR1) or control siRNA (si-CT) (A and B). (C) ΔF508 macrophages nucleofected with siRNA against p62 (si-p62) or si-p62+si-NDP52 or si-p62+si-NBR1. Macrophages in A, B and C were then infected with *B. cepacia* for 2, 4 and 6 h. Colony forming units (CFU) were quantified. (D and E) Western blot analysis of macrophages treated as in A and B respectively with specific antibodies to NDP52, NBR1, p62 or actin. Data in A, B and C are presented as the means ± S.D. Asterisks in A, C, D, E indicate significant differences at the respective time points (**P*<0.05), (***P*<0.01), (****P*<0.001).

Figure 1

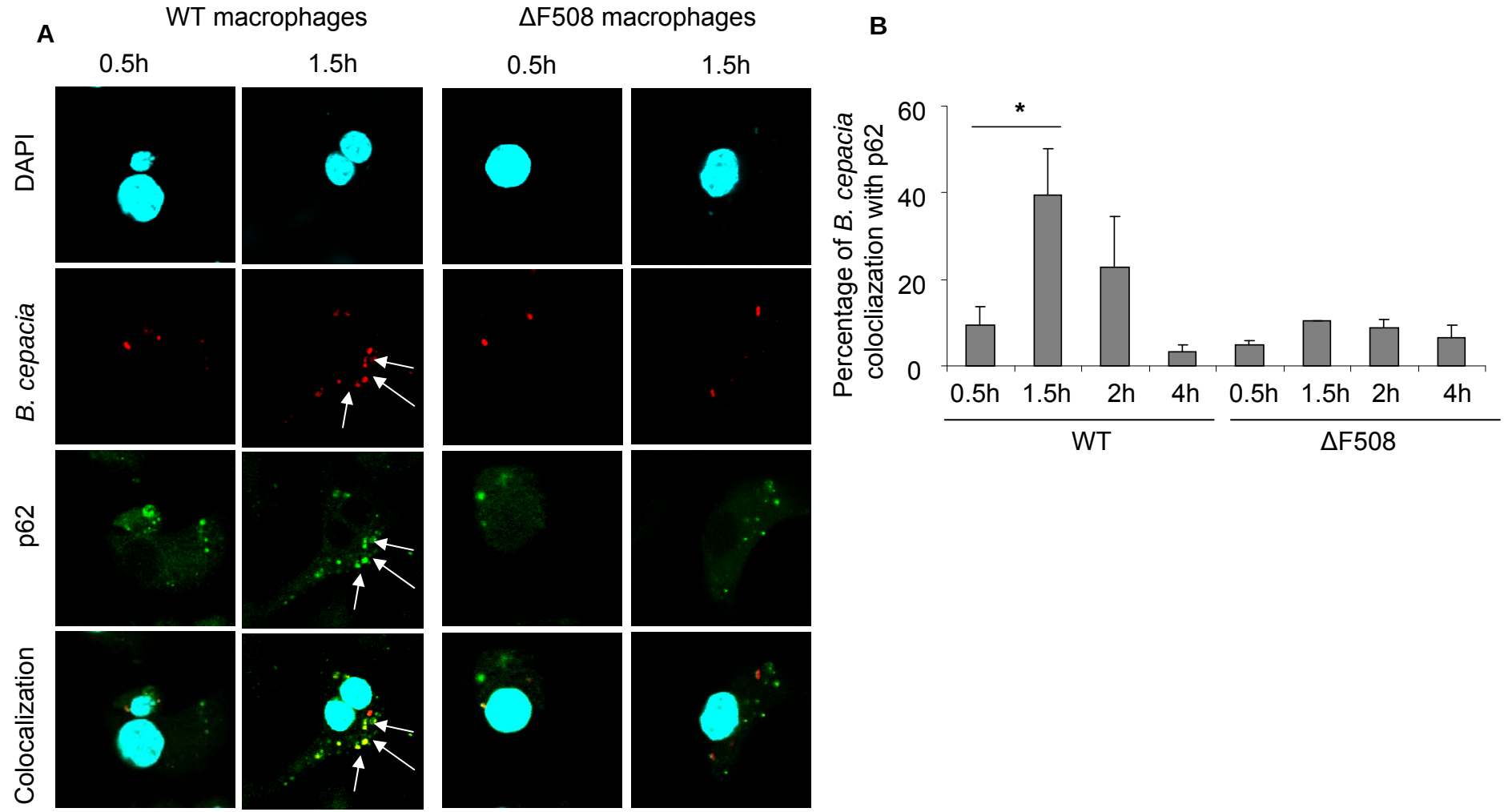
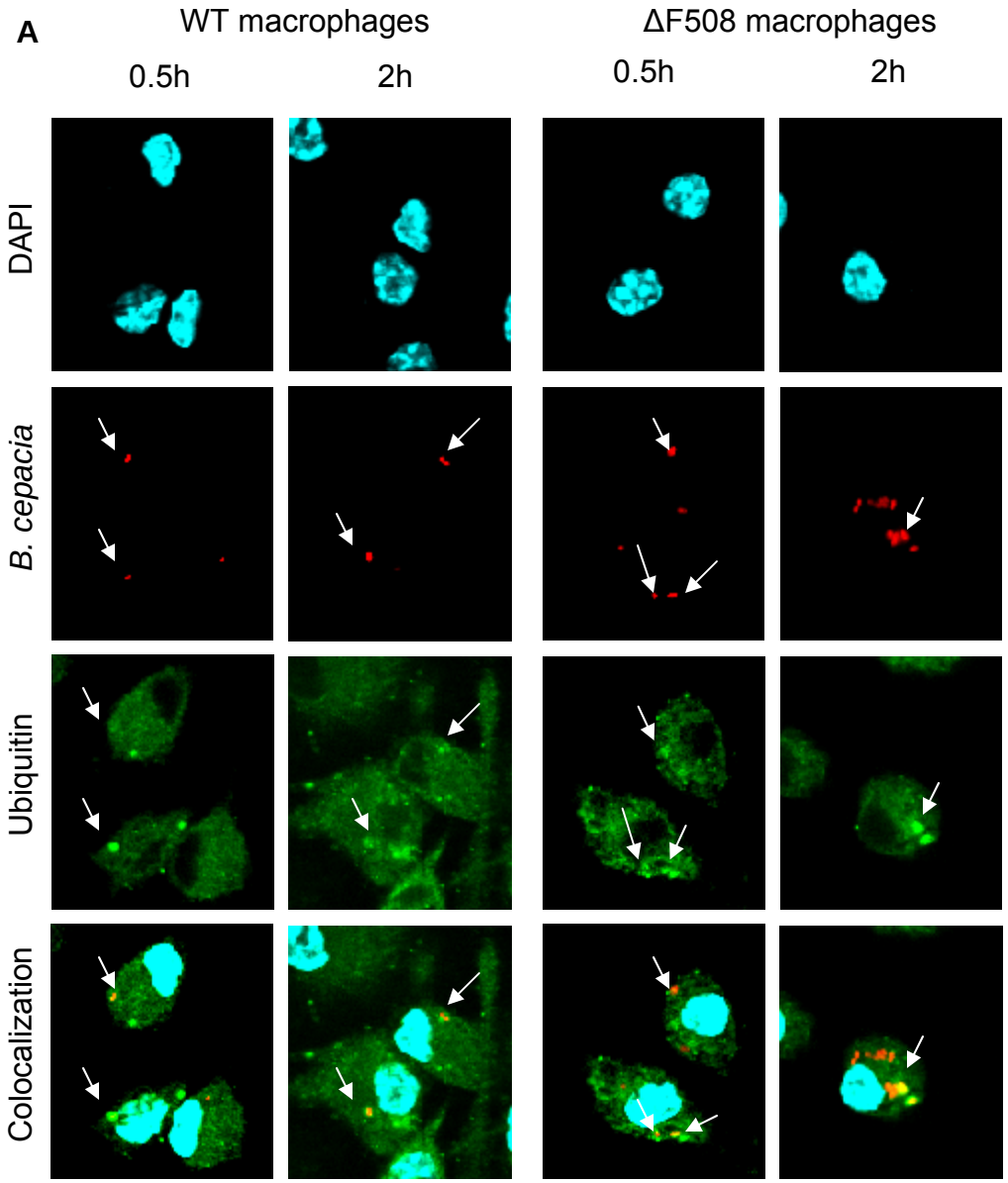


Figure 2

p62 dictates the fate of B. cepacia in murine macrophages



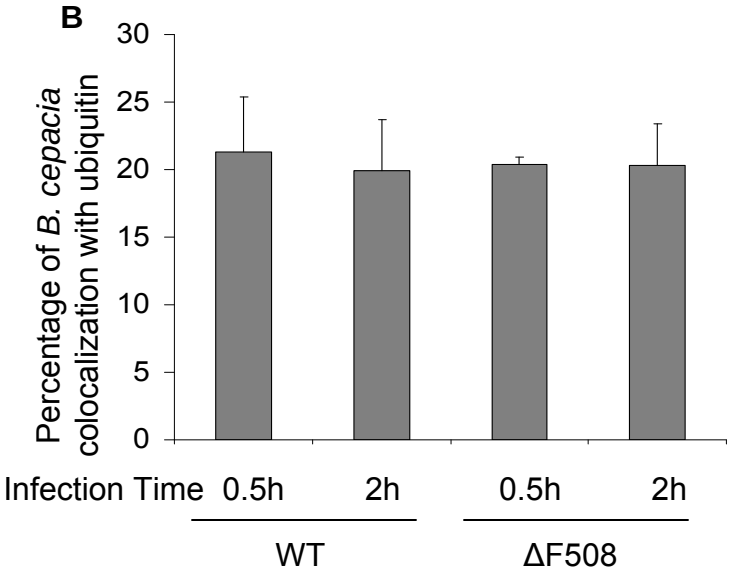


Figure 3

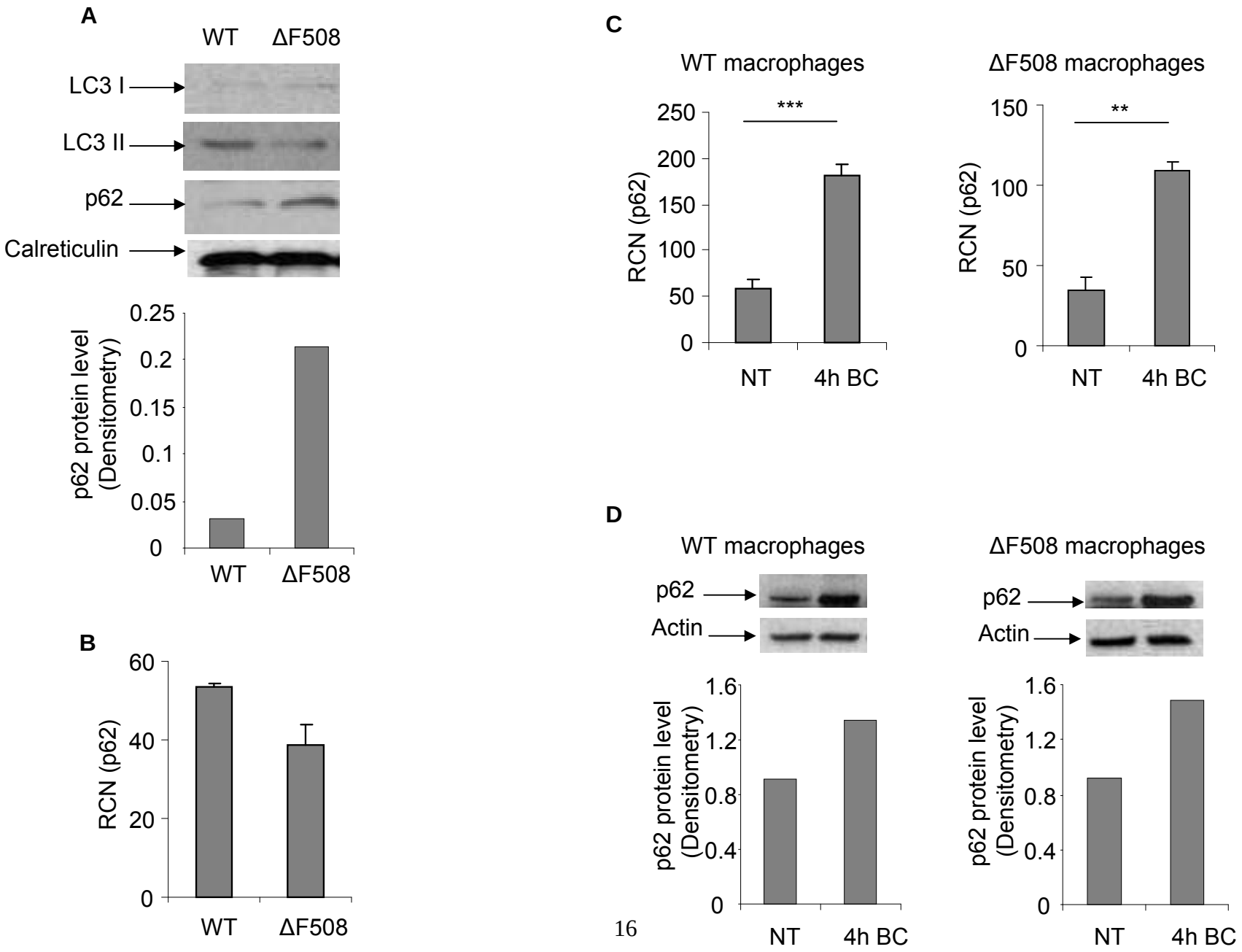
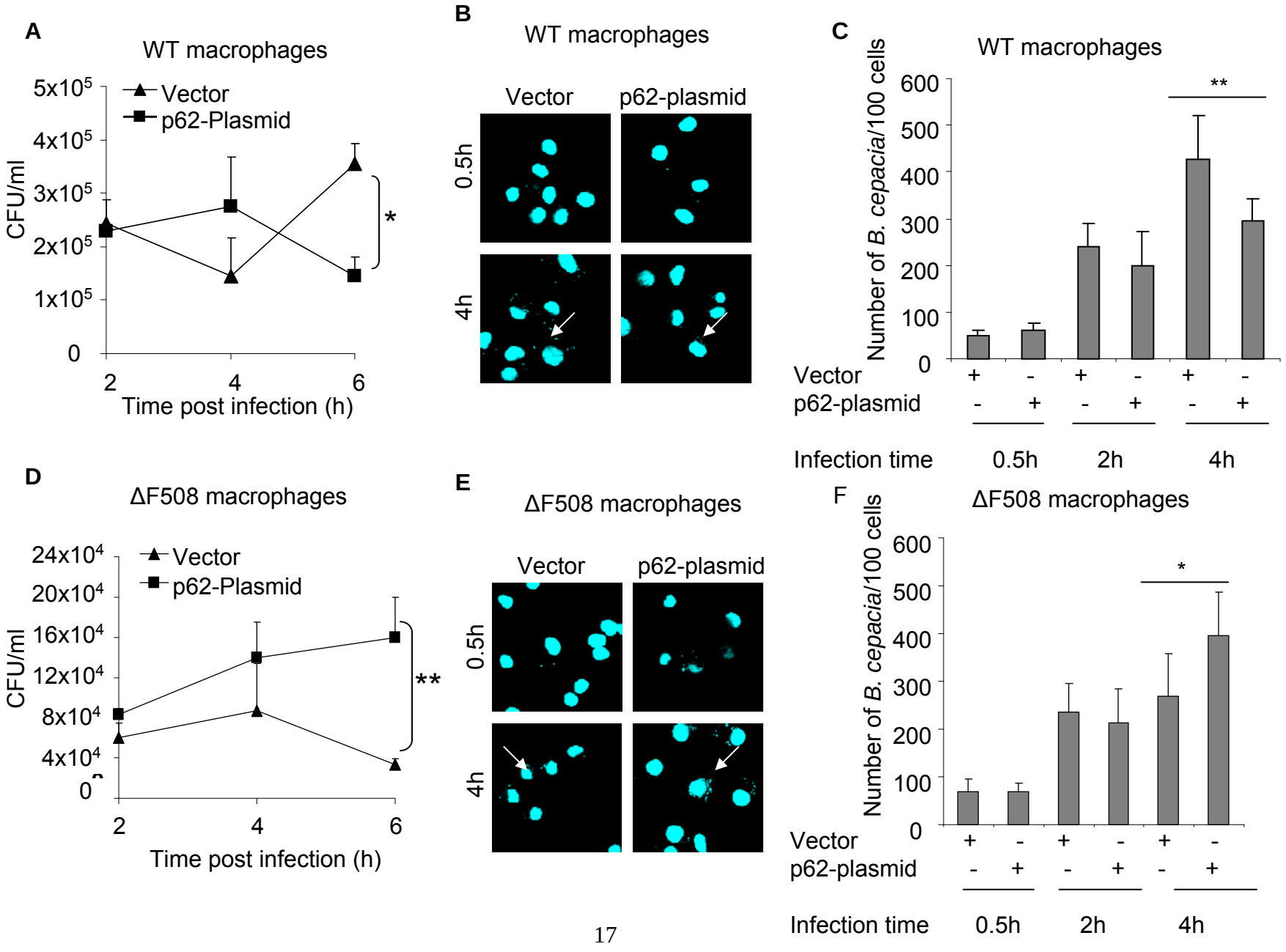


Figure 4

p62 dictates the fate of B. cepacia in murine macrophages



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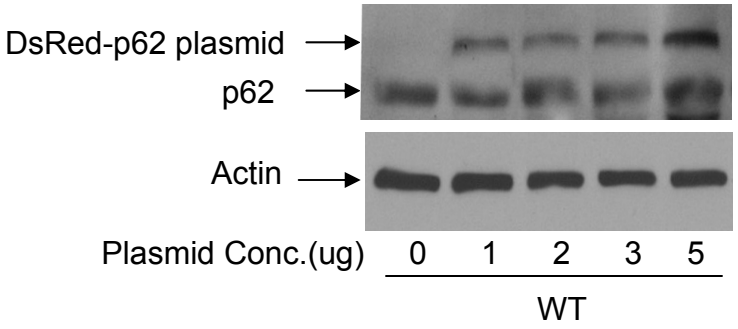
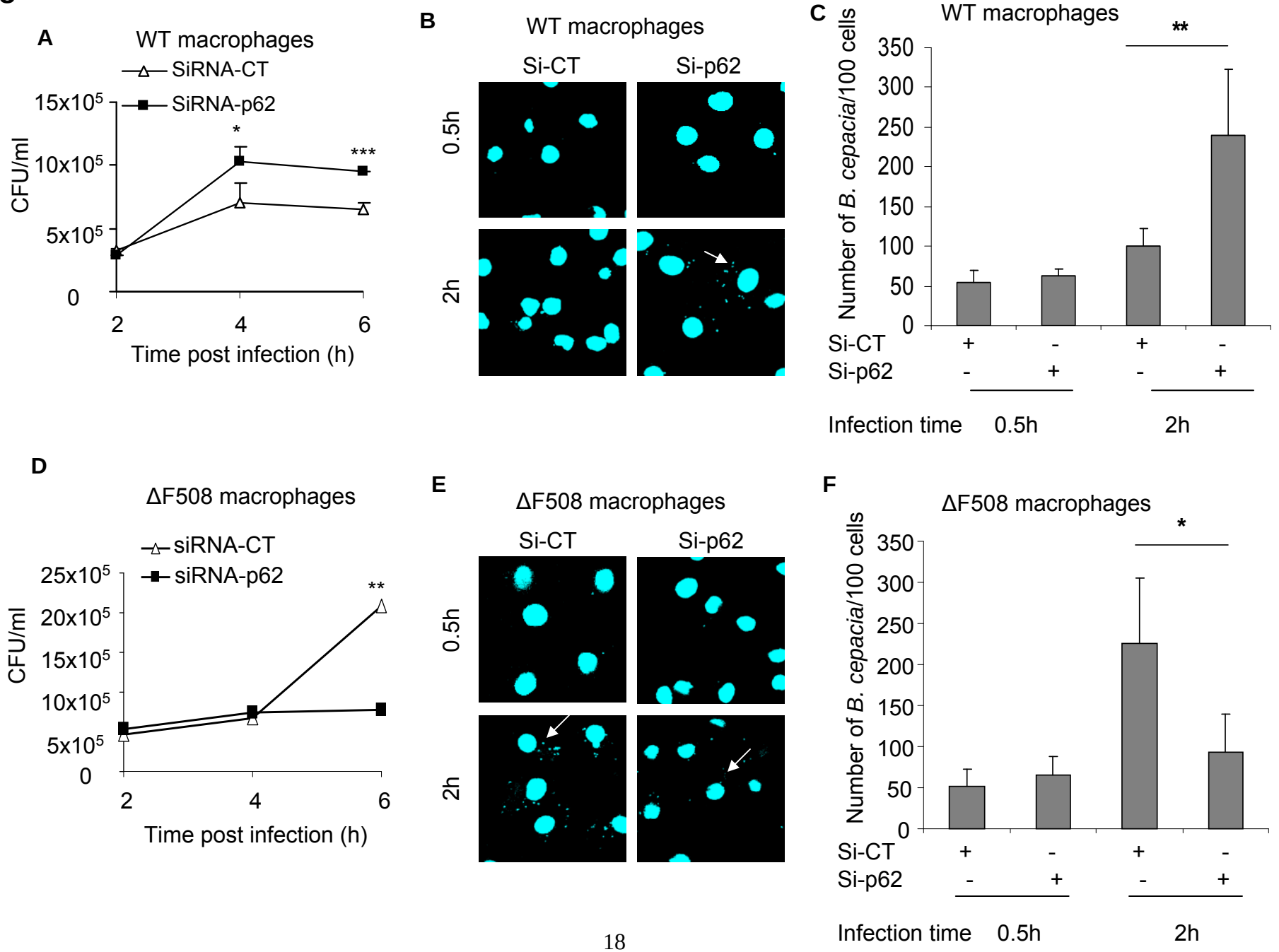


Figure 5



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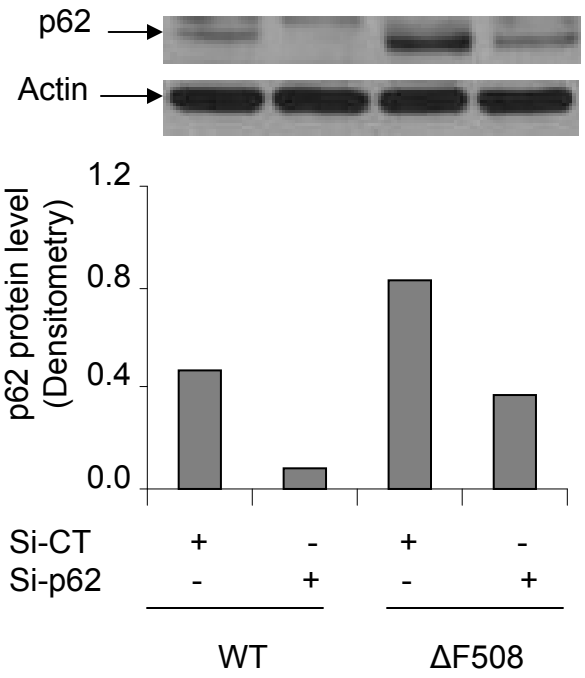


Figure 6

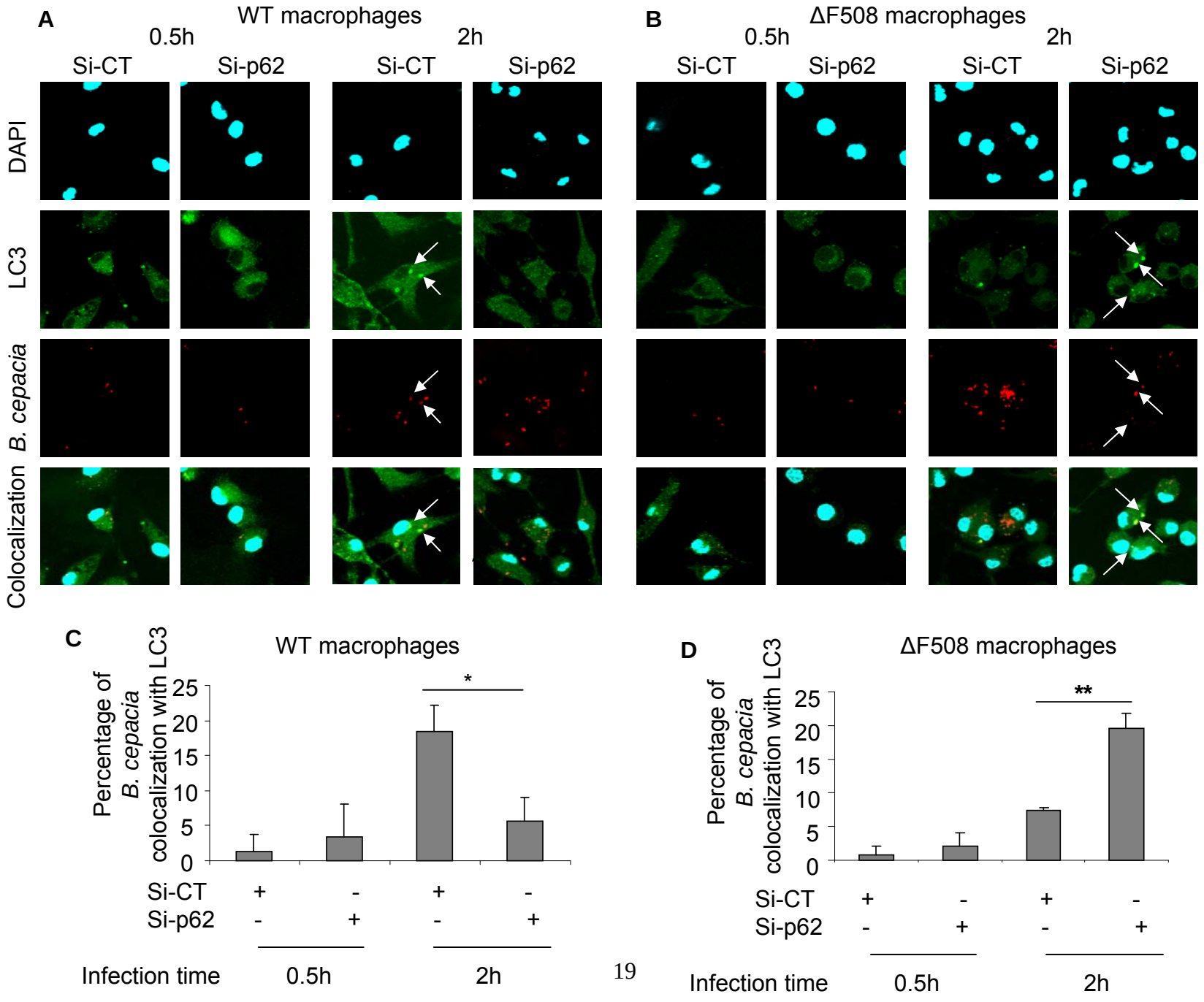


Figure 7

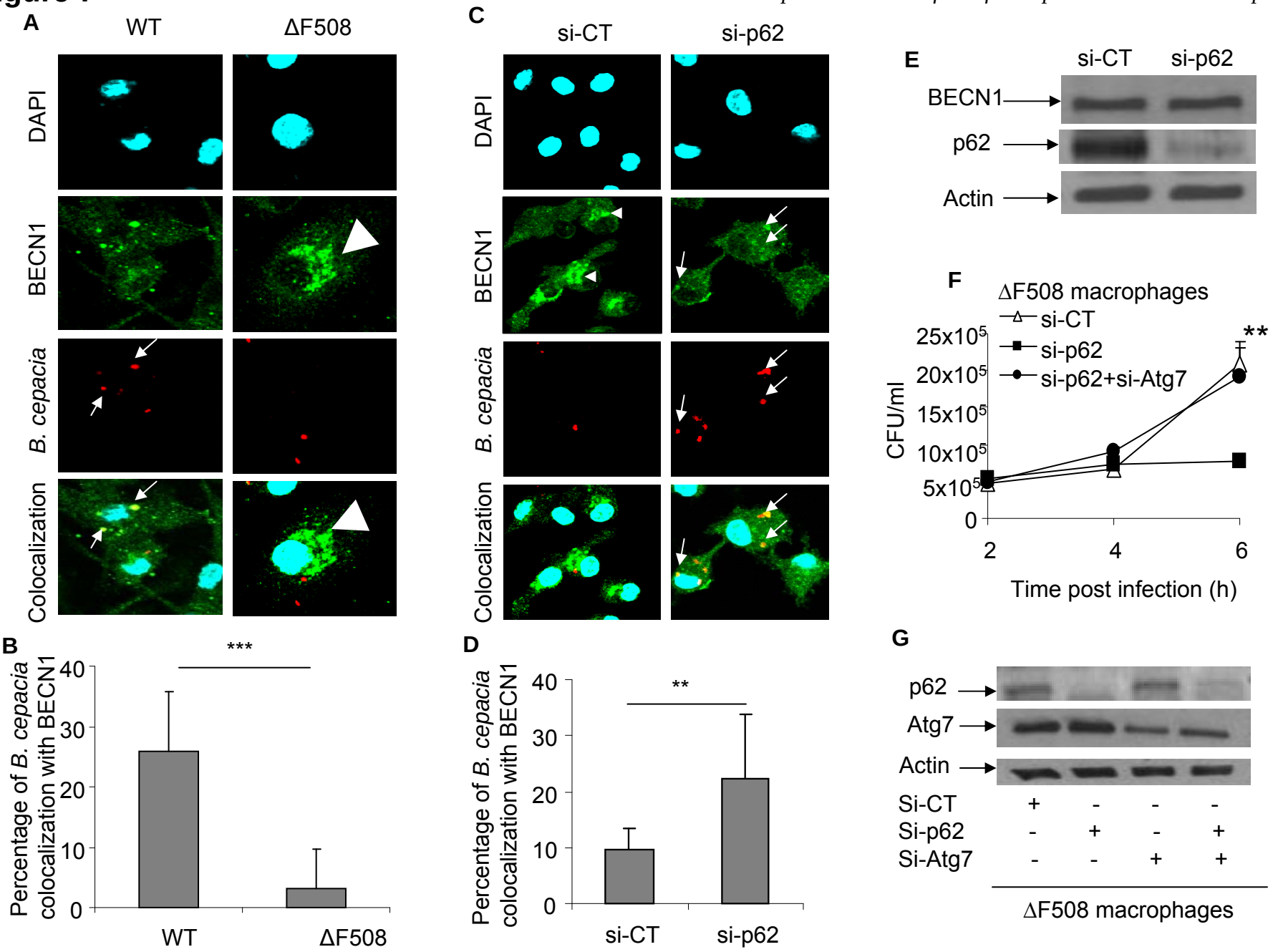
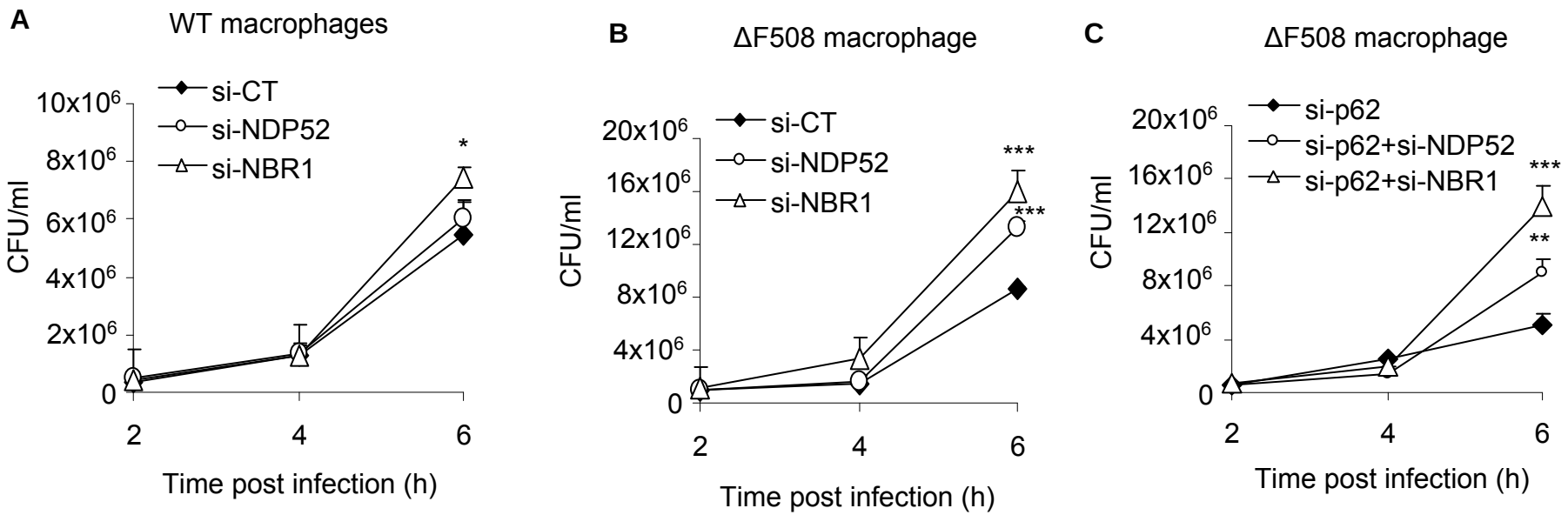
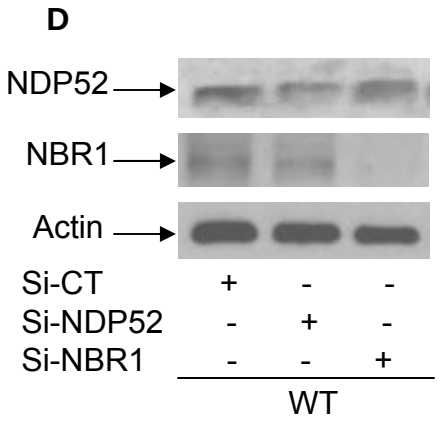
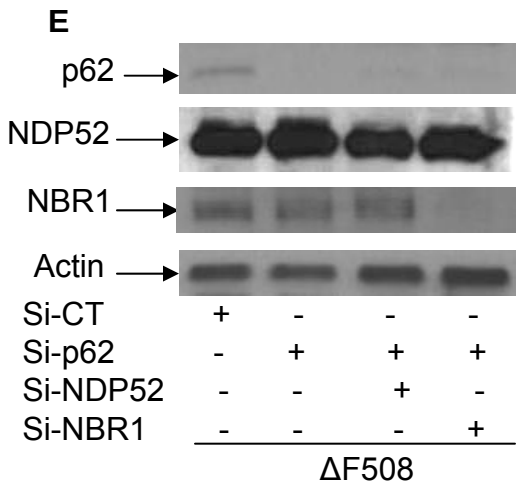


Figure 8







Depletion of the ubiquitin binding adaptor molecule SQSTM1/p62 from macrophages harboring cftr ΔF508 mutation improves the delivery of Burkholderia cenocepacia to the autophagic machinery

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