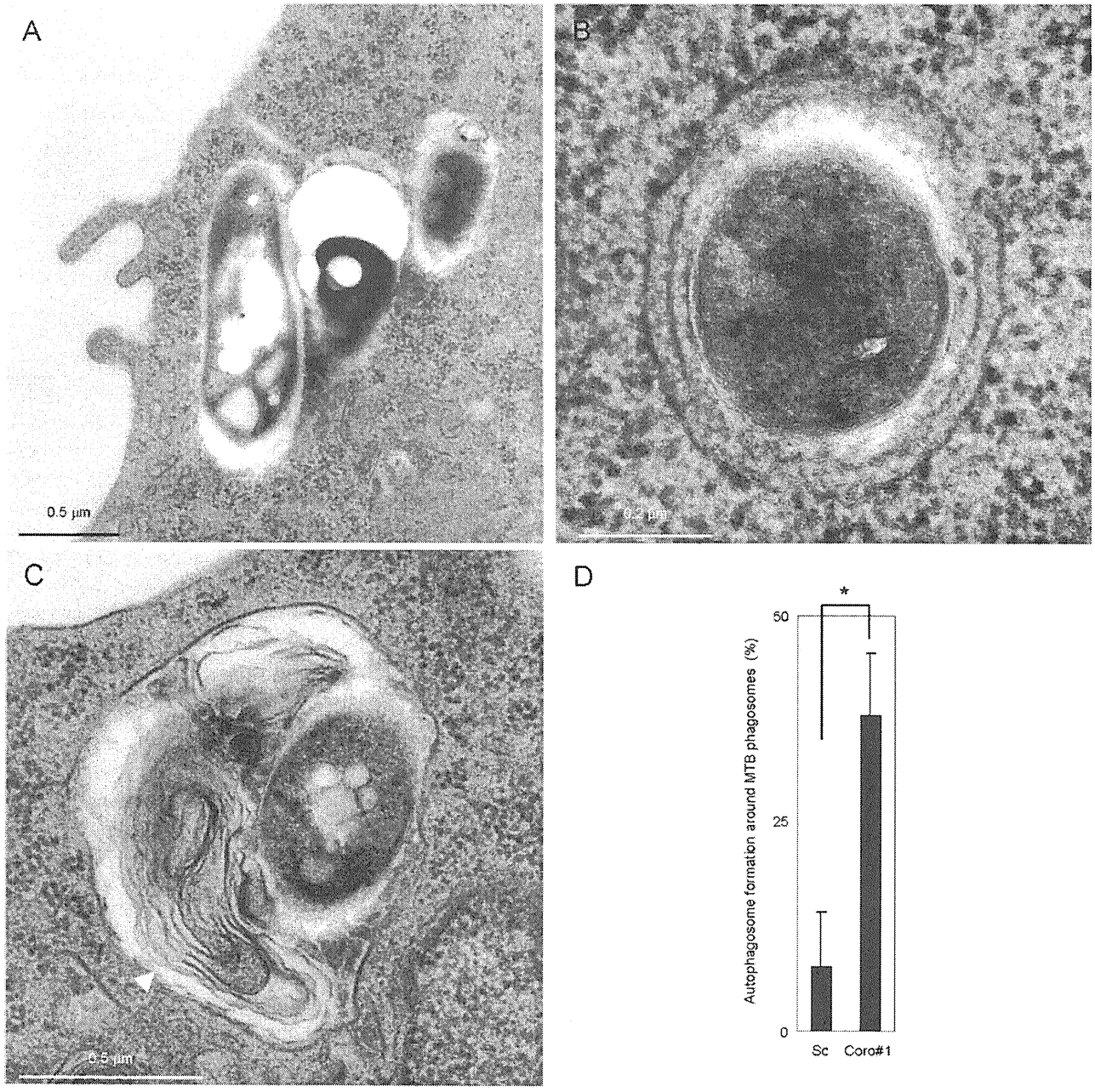

Fig. 5. Thin-section electron micrographs of Coro1a KD macrophages infected with *M. tuberculosis*.
A–C. Macrophages were transfected with scrambled (A) or Coro1a-specific (B, C) siRNA and then infected with *M. tuberculosis* for 6 h. Infected macrophages were fixed and observed with thin-section electron microscopy. An arrowhead indicates the internal membrane in the mycobacterial phagosome.
D. The proportion of *M. tuberculosis*-containing phagosomes associated with multiple membrane structures in Coro1a KD macrophages. Macrophages transfected with Coro1a-specific or scrambled siRNA were infected with *M. tuberculosis* for 6 h. Cells were fixed and observed with thin-section electron microscopy. The number of *M. tuberculosis*-containing phagosomes with multiple membrane structures was counted. Data represent the mean and SD of three independent experiments in which more than 50 phagosomes were counted for each condition. $^*P < 0.05$ (unpaired Student's *t*-test). Sc, scrambled; Coro, Coro1a; MTB, *M. tuberculosis*.

increased in Coro1a KD AM (Fig. 10B). Quantitative analysis revealed that the proportions of LC3-positive mycobacterial phagosomes were > 25% and < 5% in Coro1a KD and control AM respectively (Fig. 10C). We also examined the localization of LC3 to mycobacterial phagosomes in bone marrow-derived macrophages (BMDM) transfected with Coro1a-specific or scrambled siRNA (Fig. 10A). The depletion of Coro1a also induced the recruitment of LC3 to *M. tuberculosis*-containing phagosomes in BMDM (Fig. 10D). Quantitative analysis

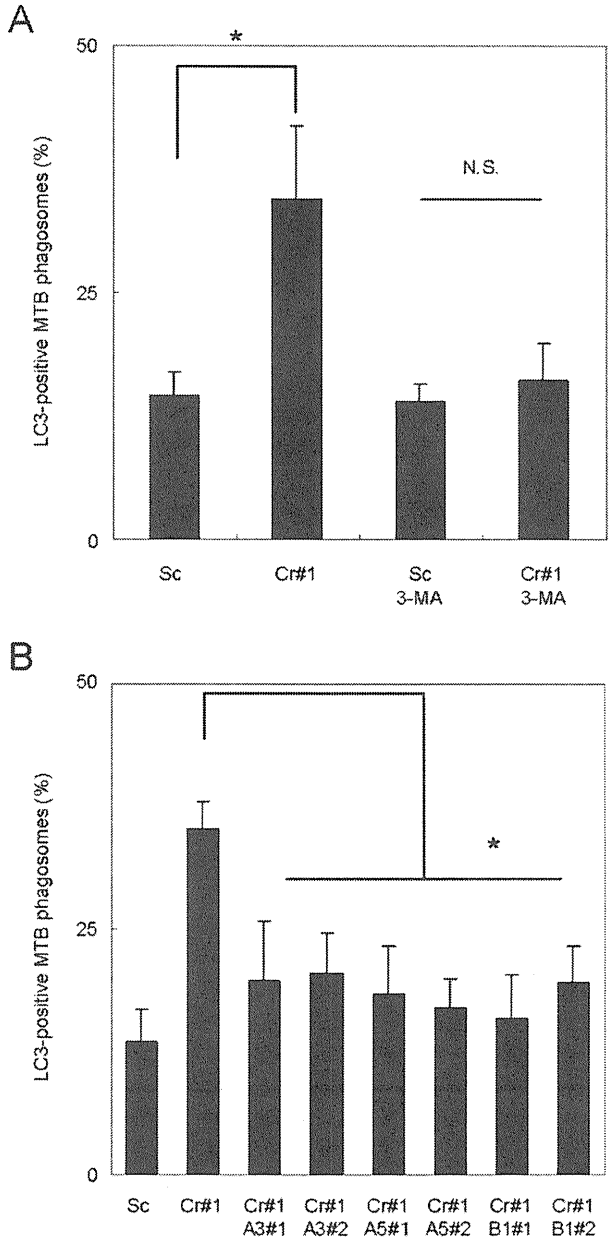

Fig. 6. LC3 recruitment to *M. tuberculosis*-containing phagosomes in Coro1a KD macrophages treated with 3-MA or siRNA for autophagy-related genes. The proportion of LC3-positive mycobacterial phagosomes in Coro1a KD macrophages treated with 3-MA at 10 mM (A) or transfected with siRNA for autophagy-related genes (B). Data represent the mean and SD of three independent experiments in which more than 200 phagosomes were counted for each condition. $^*P < 0.05$; N.S., not significant (unpaired Student's *t*-test). Sc, scrambled; Cr, Coro1a; A3, Atg3; A5, Atg5; B1, Beclin1.

revealed that approximately 10% and 2% of mycobacterial phagosomes were LC3 positive in Coro1a KD and control BMDM respectively (Fig. 10E). Treatment with 3-MA reduced the proportion of LC3-positive mycobacterial phagosomes in Coro1a KD macrophages (Fig. 10F). These results suggest that autophagosome formation around *M. tuberculosis*-containing phagosomes is also induced in AM and BMDM as a consequence of Coro1a depletion.

Discussion

Coro1a was initially reported being retained on phagosomes containing live mycobacteria, while being rapidly released from phagosomes containing inactive mycobacteria (Ferrari *et al.*, 1999). Genetic depletion or RNA interference-mediated gene silencing of Coro1a was later reported inhibiting the survival of mycobacteria within macrophages (Jayachandran *et al.*, 2007; 2008; Kumar *et al.*, 2010). In this study, we confirmed that the survival of *M. tuberculosis* was inhibited in Coro1a KD macrophages (Fig. 1). However, the infection rate of *M. tuberculosis* with Coro1a KD macrophages possibly affects its proliferation within infected macrophages, because a previous study demonstrated that the expression of a dominant-negative form of Coro1a or transfection of Coro1a siRNA decreased the activity of phagocytosis (Yan *et al.*, 2005). To address this possibility, we examined the phagocytosis rate of latex beads and the infection rate of *M. tuberculosis* in Coro1a KD macrophages but found no differences in these events between Coro1a KD and control macrophages (Fig. S2). Previous studies demonstrated that the phagolysosome biogenesis of mycobacterial phagosomes occurred by the depletion of Coro1a in macrophages (Jayachandran *et al.*, 2007; 2008). We also found that the acidification and the fusion of lysosomes with mycobacterial phagosomes were promoted in Coro1a KD macrophages (Fig. 2). However, there has been no direct evidence that the inhibition of mycobacterial proliferation in Coro1a KD macrophages is caused by the promotion of phagolysosome biogenesis.

We hypothesized that autophagy is induced in Coro1a KD macrophages and inhibits *M. tuberculosis* survival. This is because the inhibition of autophagy by 3-MA or gene silencing of autophagy-related genes restores the mycobacterial survival in Coro1a KD macrophages (Fig. 3). To verify this hypothesis, we examined the localization of LC3 and found that LC3 was recruited to *M. tuberculosis*-containing phagosomes in Coro1a KD macrophages (Fig. 4). Thin-section electron microscopy revealed that *M. tuberculosis*-containing phagosomes were surrounded by characteristic autophagic membrane structures in Coro1a KD macrophages (Fig. 5). Treatment with 3-MA or silencing of autophagy-related genes inhibited the recruitment of LC3 to *M. tuberculosis*-containing phagosomes in Coro1a KD macrophages (Fig. 6). It is reported that the delivery of anti-bactericidal protein and/or peptides to mycobacterial phagosomes depended on the induction of autophagy (Alonso *et al.*, 2007; Yuk

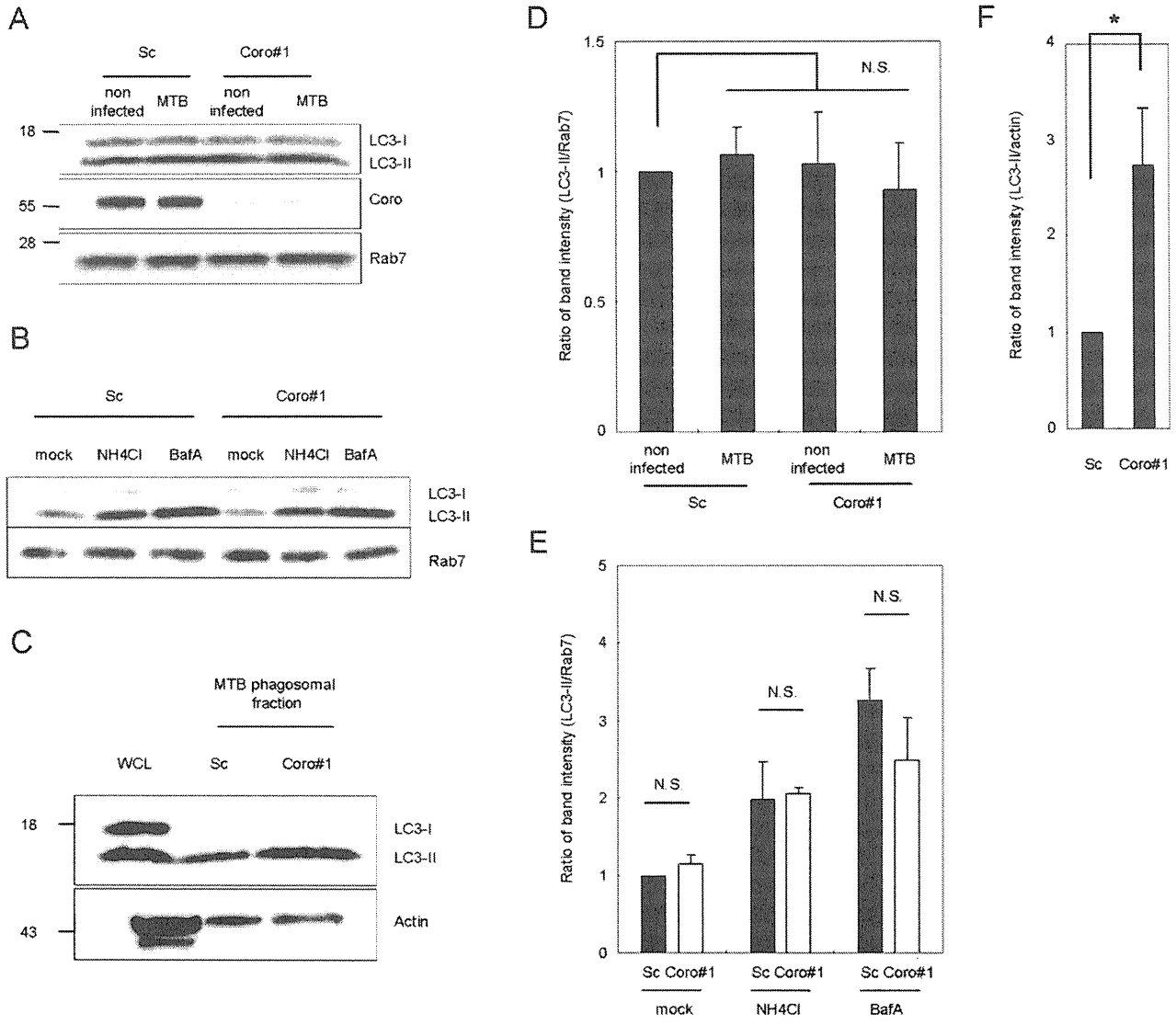

Fig. 7. Immunoblot analysis of LC3 in Coro1a KD macrophages infected with *M. tuberculosis*.
A. Monitoring LC3 processing in Coro1a KD macrophages infected with *M. tuberculosis*. Macrophages transfected with Coro1a-specific or scrambled siRNA were infected with *M. tuberculosis* for 6 h. Whole-cell lysates from non-infected or infected macrophages were subjected to SDS-PAGE, followed by immunoblot analysis using the indicated antibodies.
B. Autophagic flux in Coro1a KD macrophages infected with *M. tuberculosis*. Macrophages transfected with Coro1a-specific or scrambled siRNA were infected with *M. tuberculosis* for 6 h. Infected macrophages were then treated with NH_4Cl (10 mM) or Bafilomycin A1 (10 nM) for 2 h. Whole-cell lysates were subjected to SDS-PAGE, followed by immunoblot analysis using the indicated antibodies.
C. LC3 recruitment to isolated mycobacterial phagosomes. Macrophages transfected with Coro1a or scrambled siRNA were infected with *M. tuberculosis* for 6 h, and phagosomal fractions were isolated as previously described (Beatty *et al.*, 2002; Seto *et al.*, 2011). Whole-cell lysates and phagosomal fractions were subjected to SDS-PAGE, followed by immunoblot analysis using the indicated antibodies.
D–F. Quantification of band intensity for LC3-II. The quantification of band intensity for LC3-II in (A), (B) and (C) was shown in (D), (E) and (F) respectively. The ratio of the band intensity for LC3-II/Rab7 or actin at each condition to that in the macrophage transfected with scrambled siRNA is shown. The data represent the mean and SD of three independent experiments.
*$P < 0.05$; N.S., not significant (paired Student's *t*-test). MTB, *M. tuberculosis*; Sc, scrambled; Coro, Coro1a; NH4Cl, ammonium chloride (NH_4Cl); BafA, Bafilomycin A1.

et al., 2009; Ponpuak *et al.*, 2011). We also showed that the proportion of LC3-positive mycobacterial phagosomes colocalized with p62, ubiquitin and LAMP1 increased in Coro1a KD macrophages up to 24 h p.i., suggesting the involvement of the ubiquitin system and autophagic degradation. Combined, these results suggest that the inhibition of mycobacterial proliferation in Coro1a KD macrophages is caused by the autophagosome formation around mycobacterial phagosomes and subsequent bactericidal effector mechanisms.

In the present study, we sought key events for the induction of autophagosome formation around

M. tuberculosis-containing phagosomes induced by Coro1a depletion. Immunoblot analysis using whole-cell lysates revealed that the *M. tuberculosis* infection itself did not stimulate whole-cell LC3 processing in Coro1a KD macrophages (Fig. 7 and Fig. S1), because there was no difference in autophagic flux between control and Coro1a KD macrophages infected with *M. tuberculosis* (Fig. 7). Immunofluorescence microscopy also demonstrated that *M. tuberculosis* infection did not induce the formation of punctuated LC3 structures in Coro1a KD macrophages (Fig. 4). In addition, *M. tuberculosis* is thought to prevent the induction of autophagy by inhibiting PI3-kinase activation via the bacterial cell wall component, lipoarabinomannan or a secreted phosphatase (Vergne *et al.*, 2003; 2004; Deretic *et al.*, 2004; 2006). Present results suggest that *M. tuberculosis* infection itself cannot induce autophagy within the cytosol of Coro1a KD macrophages unlike nutrient starvation or pharmacological autophagy inducers.

It is reported that Coro1a regulated the activity of calcineurin and that the calcineurin inhibitors stimulated the fusion of lysosomes with mycobacterial phagosomes (Jayachandran *et al.*, 2007). In *Caenorhabditis elegans*, a loss-of-function or null mutation of calcineurin induces the autophagosome formation (Dwivedi *et al.*, 2009). These results imply that autophagosome formation around mycobacterial phagosomes is caused by the inhibition of calcineurin activity in Coro1a KD macrophages. We examined whether the inhibition of calcineurin activity induced the autophagosome formation around *M. tuberculosis*-containing phagosomes but found no induction of LC3 recruitment to the phagosomes in macrophages treated with FK506 or cyclosporine A (Fig. S3). Bcl-2 is a member of the anti-apoptotic proteins and interacts with Beclin1 to inhibit the induction of autophagy (Pattingre *et al.*, 2005). The expression of Bcl-2 is reduced in naïve T cells from Coro1a-deficient mice (Mueller *et al.*, 2011). We therefore addressed whether autophagosome formation around *M. tuberculosis*-containing phagosomes by Coro1a depletion is accompanied by the downregulation of Bcl-2 and found no significant change in Bcl-2 expression between control and Coro1a KD macrophages (Fig. S4). It is also reported that the transcription of Coro1a is downregulated by the combination of vitamin D3 and retinoic acid in human macrophages (Anand and Kaul, 2003). Vitamin D3 is also reported inducing autophagy in monocyte, resulting in the elimination of infected mycobacteria (Yuk *et al.*, 2009). These reports imply that vitamin D3 decreases the expression of Coro1a in mycobacteria-infected macrophages, leading the autophagosome formation and elimination of infected mycobacteria.

A recent report demonstrated that LC3 is recruited to *Mycobacterium marinum*-containing phagosomes depending on the function of ESX-1 (Lerena and Colombo, 2011). ESAT-6 homologue of *M. marinum* has a pore formation activity in phagosomal membranes and assists the bacilli to escape from phagosomes to cytosol and move by actin-based motility (Stamm *et al.*, 2003; Gao *et al.*, 2004; Smith *et al.*, 2008). *M. tuberculosis* is also reported to translocate from its containing phagosomes to cytosols in infected monocytes depending on ESX-1 secretion system (van der Wel *et al.*, 2007), suggesting that the secreted proteins including ESAT-6 by ESX-1 system damage the phagosomal membranes. Since Coro1a interacts with F-actin to stabilize the structure (Galkin *et al.*, 2008), it is likely that Coro1a localization to mycobacterial phagosomes (Ferrari *et al.*, 1999) supports the phagosomal membranes and that the depletion of Coro1a increases the susceptibility of the phagosomal membranes to ESAT-6 secreted by *M. tuberculosis*. The damage on the membrane of *M. tuberculosis*-containing phagosome could induce the autophagosome formation (Lerena *et al.*, 2010) in Coro1a KD macrophages.

We examined the activation of MAPK signalling pathways involved in autophagosome formation around mycobacterial phagosomes in Coro1a KD macrophages (Fig. 9), as they are involved in autophagy induction (Esclatine *et al.*, 2009). Activation of p38 is indispensable for the induction of autophagy via Toll-like receptor signalling pathways in innate immunity (Xu *et al.*, 2007). JNK signalling pathway was previously reported being involved in the induction of autophagy in macrophages infected with the *eis*-deletion mutant of *M. tuberculosis* (Shin *et al.*, 2010). We assessed the phosphorylation of three MAPKs (ERK-1/2, JNK and p38) and found that only the p38 pathway was specifically activated by *M. tuberculosis* infection in Coro1a KD macrophages (Fig. 9). These results suggest that Coro1a blocks the signal(s) for p38 MAPK activation in response to *M. tuberculosis* infection.

AM are the first defence line of the lung against *M. tuberculosis* infection (Russell, 2001; 2007). We found that the depletion of Coro1a induced autophagosome formation surrounding *M. tuberculosis*-containing phagosomes also in AM and BMDM (Fig. 10). These results suggest that the inhibition of autophagosome formation by Coro1a occurs in various types of macrophages. In conclusion, this study demonstrates that Coro1a regulates the autophagosome formation around *M. tuberculosis*-containing phagosomes and assists the survival of infected mycobacteria in macrophages.

Experimental procedures

Cell and bacterial cultures

Raw264.7 and MH-S macrophage cell lines were obtained from the American Type Culture Collection and maintained at 37°C under a humidified condition with 5% CO_2 in Dulbecco's modified

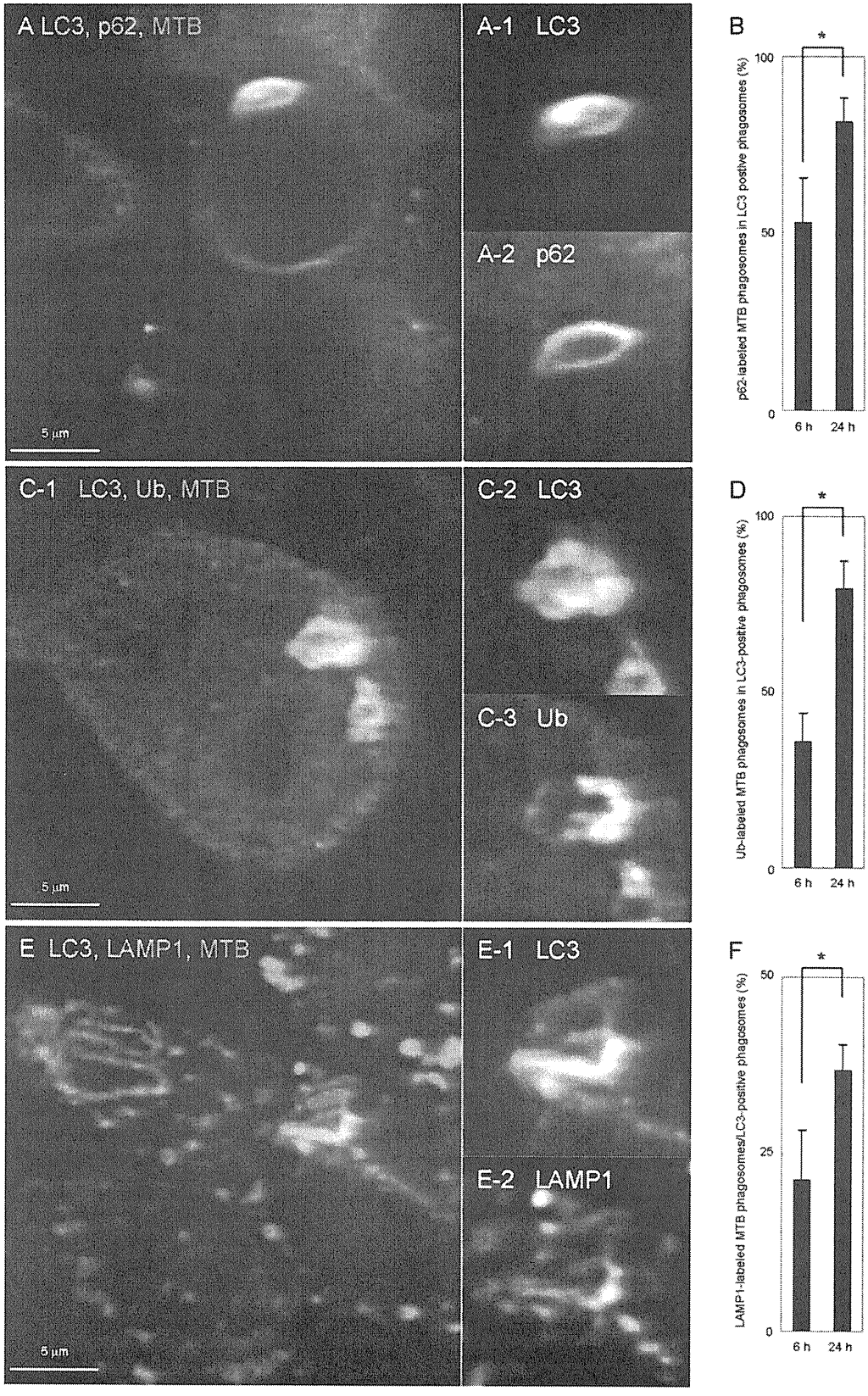

Fig. 8. Recruitment of p62, ubiquitin or LAMP1 to LC3-positive *M. tuberculosis*-containing phagosomes in Coro1a KD macrophages.
A, C and E. Raw264.7 macrophages stably expressing EGFP-LC3 were transfected with Coro1a-specific siRNA for 48 h. Transfected macrophages were infected with Alexa405-labelled *M. tuberculosis* for 24 h and then stained with anti-p62 (A), anti-ubiquitin (C) or anti-LAMP1 (E) antibodies. Enlarged images of A-1, C-1 and E-1 are represented in A-2 and A-3, B-2 and B-3, and C-2 and C-3 respectively.
B, D and F. The proportion of mycobacterial phagosomes labelled with p62 (B), ubiquitin (C) or LAMP1 (F) to the total LC3-positive ones in Coro1a KD macrophages. Macrophages stably expressing EGFP-LC3 were transfected with Coro1a siRNA, and infected with Alexa405-labelled *M. tuberculosis* for 6 or 24 h. Infected macrophages were stained with anti-p62 (B), anti-ubiquitin (D) or anti-LAMP1 (F) antibodies. The numbers of LC3-positive mycobacterial phagosomes labelled with these markers were counted. Data represent the mean and SD of three independent experiments in which more than 100 phagosomes were counted for each condition. $*P < 0.05$ (unpaired Student's *t*-test). Sc, scrambled; Coro, Coro1a; MTB, *M. tuberculosis*; Ub, ubiquitin.

Eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA), 25 μg ml^{-1} penicillin G and 25 μg ml^{-1} streptomycin. BMDM were differentiated from BALB/c mice bone marrow for 7 days in DMEM supplemented with 20% L929-conditioned medium, 10% FBS and antibiotics. *M. tuberculosis* Erdman was obtained from the Japan Research Institute of Tuberculosis, Tokyo, Japan, and grown to mid-logarithmic phase in 7H9 medium supplemented with 10% Middlebrook ADC (BD Biosciences, San Jose, CA), 0.5% glycerol and 0.05% Tween 80 (*Mycobacterium* complete medium) at 37°C. Mycobacteria transformed with a plasmid encoding DsRed were grown in *Mycobacterium* complete medium containing 25 μg ml^{-1} kanamycin.

RNA interference

siRNA duplexes were synthesized by Sigma-Aldrich according to the following sequences: Coro1a#1, sense 5′-GACUGGA CGAGUAGACAAGTT-3′, antisense 5′-CUUGUCUACUCGUCC AGUCTT-3′ (Jayachandran *et al.*, 2008); Coro1a#2 sense 5′-GCAAGACUGGACGAGUAGATT-3′, antisense 5′-UCUACU CGUCCAGUCUUGCTT-3′; Atg3#1, sense 5′-GGUGUAAACA GAUGGAGUATT-3′, antisense 5′-UACUCCAUCUGUUUACACC TT-3′; Atg3#2, sense 5′-GCAUAUCUUCCGACAGACATT-3′, antisense 5′-UGUCUGUCGGAAGAUAUGCTT-3′; Atg5#1, sense 5′-GCUUUACUCUCUAUCAGGATT-3′, antisense 5′-UC CUGAUAGAGAGUAAAGCTT-3′; Atg5#2, sense 5′-GAGACAA GAAGAUGUUAGUTT-3′, antisense 5′-ACUAACAUCUUCUUG UCUCTT-3′; Beclin1#1, sense 5′-GAAAGAUGCUUUAA AUUAATT-3′, antisense 5′-UUAAUUUAAAGCAUCUUUCTT-3′. Beclin1#2, sense 5′-CUGAGAAUGAAUGUCAGAATT-3′, antisense 5′-UUCUGACAUUCAUUCUCAGTT-3′; Mission siRNA universal negative control (Sigma-Aldrich) was used as scrambled siRNA. Transfection of macrophages with siRNA duplexes was performed using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions.

Colony-forming unit (cfu) assay

Macrophages transfected with siRNA were grown in 24-well plates at 1×10^5 cells for 24 h, and subsequently infected with *M. tuberculosis* at an moi of 10 for 4 h. Infected macrophages were washed with DMEM three times to remove non-infected mycobacteria and then incubated with DMEM and 10% FBS. At 4 and 72 h p.i., infected macrophages were lysed with 1% IGEPAL in phosphate-buffed saline (PBS), serially diluted with *Mycobacterium* complete medium, and inoculated onto 7H10 agar medium supplemented with 10% Middlebrook OADC (BD Biosciences) and 0.5% glycerol. Colony-forming unit was determined as the mean of four plates at each time point.

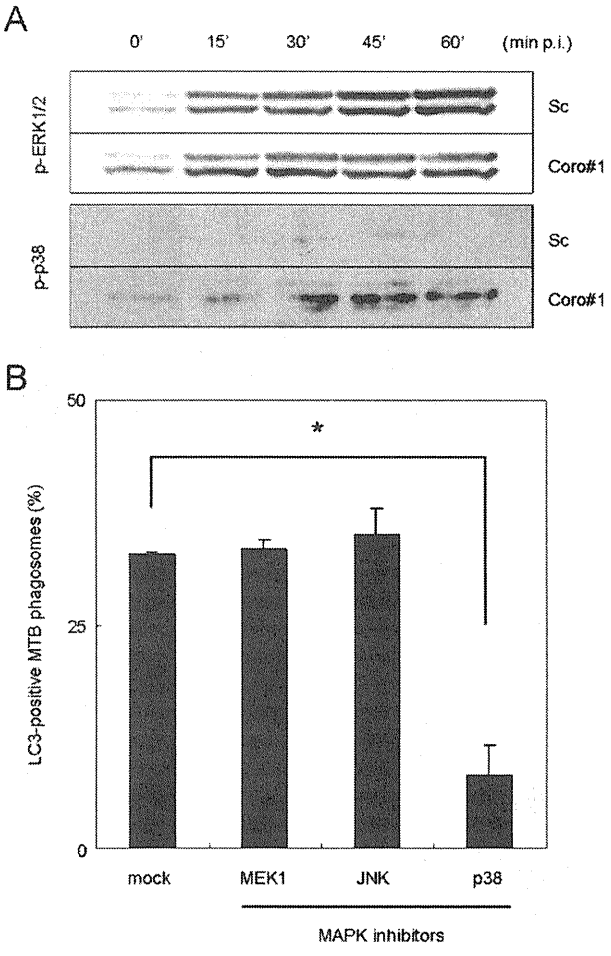

Fig. 9. Differential contribution of MAPK to autophagosome formation around *M. tuberculosis*-containing phagosomes in Coro1a KD macrophages.
A. Phosphorylation of p38 MAPK in Coro1a KD macrophages infected with *M. tuberculosis*. Macrophages transfected with Coro1a-specific or scrambled siRNA were infected with *M. tuberculosis* for the indicated time periods. Whole-cell lysates were subjected to SDS-PAGE, followed by immunoblot analysis using the indicated antibodies.
B. The proportion of LC3-positive *M. tuberculosis*-containing phagosomes in Coro1a KD macrophages treated with MAPK inhibitors. Coro1a KD macrophages expressing EGFP-LC3 were infected with *M. tuberculosis* in the presence of MAPK inhibitors (20 μM) for 6 h. PD98059, SP600125 and SB203580 were used as inhibitors for MEK1, JNK and p38 respectively. Data represent the mean and SD of three independent experiments in which more than 200 phagosomes were counted for each condition. $*P < 0.05$ (unpaired Student's *t*-test). Sc, scrambled; Coro, Coro1a; Mock, solvent control for MAPK inhibitors (0.1% DMSO).

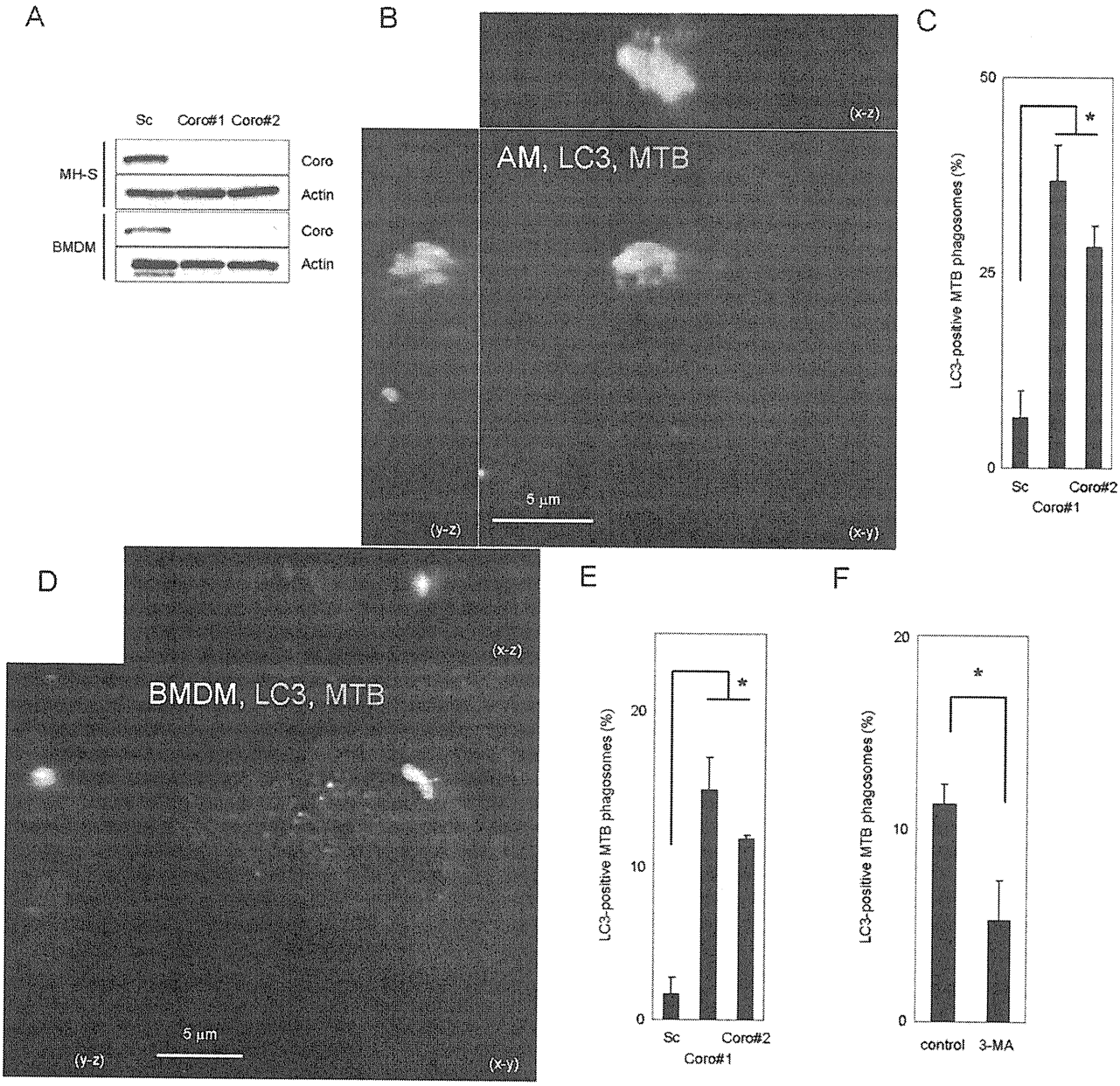

Fig. 10. LC3 recruitment to *M. tuberculosis*-containing phagosomes in the MH-S alveolar macrophage cell line and bone marrow-derived macrophage induced by Coro1a depletion.

A. Immunoblot analysis of MH-S alveolar macrophages (AM) or bone marrow-derived macrophage (BMDM) transfected with Coro1a siRNA. Whole-cell lysates of macrophages transfected with Coro1a or scrambled siRNA were subjected to SDS-PAGE, followed by immunoblot analysis using the indicated antibodies.

B and D. Analysis of LC3 recruitment to *M. tuberculosis*-containing phagosomes in Coro1a KD AM or BMDM. AM (B) or BMDM (D) transfected with Coro1a siRNA were infected with DsRed-expressing *M. tuberculosis* for 6 h. Infected macrophages were fixed and stained with anti-LC3 antibody. Infected macrophages were then observed using LSCM. Projections of focal planes with y–z and x–z side views are represented.

C and E. The proportion of mycobacterial phagosomes labelled with anti-LC3 antibody in AM or BMDM. AM (C) or BMDM (E) were transfected with Coro1a or scrambled siRNA. Transfected macrophages were infected with DsRed-expressing *M. tuberculosis* for 6 h, and then stained with anti-LC3 antibody. LC3-positive phagosomes were counted.

F. 3-MA inhibits the recruitment of LC3 to mycobacterial phagosomes in Coro1a KD BMDM. BMDM were transfected with Coro1a KD siRNA and then treated with or without 3-MA at 10 mM. Macrophages were infected with DsRed-expressing *M. tuberculosis* for 6 h, and then stained with anti-LC3 antibody. The LC3-positive mycobacterial phagosomes were counted. Data represent the mean and SD of three independent experiments in which more than 200 phagosomes were counted for each condition. $*P < 0.05$ (unpaired Student's *t*-test). Sc, scrambled; Coro, Coro1a; MTB, *M. tuberculosis*; AM, alveolar macrophage cell line MH-S; BMDM, bone marrow-derived macrophage.

Antibodies

Rabbit anti-Coro1a polyclonal antibody (Sigma-Aldrich), mouse anti-actin monoclonal antibody (Sigma-Aldrich), rat anti-mouse LAMP1 monoclonal antibody (SouthernBiotech, Birmingham, AL), mouse anti-LC3 monoclonal antibody (MBL, Nagoya, Japan), rabbit anti-LC3 polyclonal antibody (Sigma-Aldrich), rabbit anti-Atg3 polyclonal antibody (Sigma-Aldrich), rabbit anti-Atg5 polyclonal antibody (Sigma-Aldrich), rabbit anti-Beclin1 polyclonal antibody (Sigma-Aldrich), rabbit anti-p62 polyclonal antibody (MBL), mouse anti-ubiquitin monoclonal antibody (FK2, MBL), mouse anti-Rab7 monoclonal antibody (Abcam, Cambridge, UK), rabbit anti-phospho-ERK1/2 antibody (CST, Denver, MA), rabbit anti-phospho-p38 antibody (CST), rabbit anti-phospho-JNK antibody (CST) and mouse anti-Bcl-2 monoclonal antibody (BD Biosciences) were used for experiments. Alexa488- and Alexa546-conjugated anti-IgG antibodies (Invitrogen) and horseradish peroxidase-conjugated anti-IgG antibodies (Dako, Glostrup, Denmark) were also commercially purchased.

Immunoblot analysis and fluorescence microscopy

Transfected macrophages were extracted by the cell lysis buffer containing 25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 100 μM vanadate and protease inhibitor cocktail (Roche, Mannheim, Germany). For immunoblot analysis, cell lysates were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and then subjected to immunoblot analysis using anti-Coro1a antibody (1:500 v/v), anti-actin antibody (1:1000 v/v), rabbit anti-LC3 polyclonal antibody (1:250 v/v), anti-Atg3 antibody (1:200 v/v), anti-Atg5 antibody (1:300 v/v), anti-Beclin1 antibody (1:100 v/v), anti-Rab7 antibody (1:300 v/v), phospho-ERK1/2 antibody (1:100 v/v), phospho-p38 antibody (1:100 v/v), phospho-JNK antibody (1:100 v/v) or anti-Bcl-2 antibody (1:100 v/v). Band intensity from three independent experiments was quantified using ImageJ (http://rsbweb.nih.gov/ij/). To label lysosomal vesicles with fluorescent dextran, macrophages were incubated with Alexa488-dextran (Invitrogen) at 100 μg ml^{-1} for 12 h. Labelled cells were washed and chased in fluorescent dextran free DMEM with 10% FBS for 6 h.

Immunofluorescence microscopic analysis was performed as previously described (Seto *et al.*, 2009). Macrophages were stained with anti-LAMP1 antibody (1:10 v/v), mouse anti-LC3 monoclonal antibody (1:10 v/v), anti-p62 antibody (1:10 v/v) or anti-ubiquitin antibody (1:10 v/v). Fluorescence microscopy was performed using a LS-1 laser scanning confocal microscope (LSCM; Yokogawa, Tokyo, Japan).

Transfection of macrophages with plasmid

pEGFP-LC3 plasmid was generously provided by Dr Tamotsu Yoshimori (Osaka University, Suita, Japan) and used to transfect Raw264.7 macrophages using an MP-100 electroporator (Digital Bio Technology, Seoul, Korea), according to the manufacturer's instructions. Transfected macrophages were incubated in DMEM with 10% FBS for 24 h prior to the experiments.

Infection of mycobacteria

Transfected macrophages with siRNA grown for 48 h were scraped and grown on round coverslips in 12-well plates for further 12 h. Mycobacteria were washed three times with PBS containing 0.05% Tween 80 and then suspended in DMEM with 10% FBS at a multiplicity of infection (moi) of 30. Aliquots of bacterial suspension were added to 3×10^5 cells of Raw264.7 macrophages on coverslips in 12-well plates, followed by centrifugation at 150 *g* for 5 min and incubation for 10 min at 37°C. Infected cells on coverslips were washed three times with DMEM to remove non-phagocytosed bacteria and then incubated with DMEM containing 10% FBS. At the indicated time points, infected cells were fixed with 3% paraformaldehyde in PBS. For immunoblot analysis to detect the phosphorylation of MAPK, macrophages transfected with siRNA grown for 48 h in six-well plates were infected with *M. tuberculosis* at an moi of 30, and then centrifuged for 5 min and incubated for 10 min at 37°C. Infected cells were washed with DMEM to remove non-infected bacteria and then incubated with DMEM containing 10% of FBS. At the indicated time points, infected cells were washed three times with PBS and extracted with the cell lysis buffer.

Thin-section electron microscopy

Raw264.7 macrophages transfected with siRNA in six-well plates were infected with *M. tuberculosis* at an moi of 30 for 2 h, washed three times with DMEM to remove non-infected bacteria, and further incubated in DMEM with 10% FBS for 4 h. Infected macrophages were fixed with 1% glutaraldehyde in 0.1 M cacodylic acid buffer. Fixed macrophages were incubated with 0.1% (w/v) osmium tetroxide. Cells were dehydrated with a series of ethanol washes and treated with propylene oxide. Samples were embedded in Qetol812 resin (OKEN, Tokyo, Japan) according to the manufacturer's protocol. Thin sections were cut with diamond knives and mounted on copper grids. Samples on grids were counter stained with 2% (w/v) uranyl acetate, and then observed with a JEM-1220 electron microscope (JEOL, Tokyo, Japan).

Isolation of M. tuberculosis*-containing phagosomes*

Six 15 cm dishes of Raw264.7 macrophages were used for each condition. Transfection of macrophages with Coro1a or scrambled siRNA was performed using an MP-100 electroporator according to the manufacturer's instructions. Briefly, 6×10^6 Raw264.7 macrophages were transfected with 1.2 nmol of siRNA per plate. Transfected macrophages were incubated in DMEM with 10% FBS for 48 h prior to the experiments. Raw264.7 macrophages transfected with siRNA were infected with mycobacteria at an moi of 30 for 2 h, washed with DMEM three times to remove non-infected mycobacteria, and further incubated in DMEM with 10% FBS for 4 h. Preparation of isolated mycobacterial phagosomes was performed as described previously (Beatty *et al.*, 2002; Seto *et al.*, 2011).

Statistics

Paired or unpaired two-sided Student's *t*-tests was used to assess the statistical significance of differences between the two groups. Three or four independent experiments were conducted to assess mycobacterial growth in macrophages, and the number of viable bacteria was determined from the means of four plates. Three independent experiments were conducted to assess the proportions of fluorescence-positive phagosomes.

Acknowledgements

We thank Drs Toshi Nagata and Masato Uchijima (Hamamatsu University School of Medicine, Hamamatsu, Japan) for their helpful discussions. We also thank Ms Keiko Sugaya and Ms Yumiko Suzuki (Hamamatsu University School of Medicine) for their excellent assistance. This work was supported in part by Grants-in-Aid for Young Scientists (B), Scientific Research (B) and Scientific Research (C) from the Japan Society for the Promotion of Science; Scientific Research on Priority Areas from the Ministry of Education, Culture, Sports, Science and Technology of Japan; the Health and Labour Science Research Grants for Research into Emerging and Reemerging Infectious Diseases from the Ministry of Health, Labour and Welfare of Japan; and the United States-Japan Cooperative Medical Science Committee.

References

Alonso, S., Pethe, K., Russell, D.G., and Purdy, G.E. (2007) Lysosomal killing of Mycobacterium mediated by ubiquitin-derived peptides is enhanced by autophagy. *Proc Natl Acad Sci USA* **104:** 6031–6036.

Anand, P.K., and Kaul, D. (2003) Vitamin D3-dependent pathway regulates TACO gene transcription. *Biochem Biophys Res Commun* **310:** 876–877.

Armstrong, J.A., and Hart, P.D. (1971) Response of cultured macrophages to *Mycobacterium tuberculosis*, with observations on fusion of lysosomes with phagosomes. *J Exp Med* **134:** 713–740.

Beatty, W.L., Rhoades, E.R., Hsu, D.K., Liu, F.T., and Russell, D.G. (2002) Association of a macrophage galactoside-binding protein with *Mycobacterium*-containing phagosomes. *Cell Microbiol* **4:** 167–176.

Clemens, D.L., and Horwitz, M.A. (1995) Characterization of the *Mycobacterium tuberculosis* phagosome and evidence that phagosomal maturation is inhibited. *J Exp Med* **181:** 257–270.

Deretic, V., and Levine, B. (2009) Autophagy, immunity, and microbial adaptations. *Cell Host Microbe* **5:** 527–549.

Deretic, V., Vergne, I., Chua, J., Master, S., Singh, S.B., Fazio, J.A., and Kyei, G. (2004) Endosomal membrane traffic: convergence point targeted by *Mycobacterium tuberculosis* and HIV. *Cell Microbiol* **6:** 999–1009.

Deretic, V., Singh, S., Master, S., Harris, J., Roberts, E., Kyei, G., *et al.* (2006) *Mycobacterium tuberculosis* inhibition of phagolysosome biogenesis and autophagy as a host defence mechanism. *Cell Microbiol* **8:** 719–727.

Deretic, V., Delgado, M., Vergne, I., Master, S., De Haro, S., Ponpuak, M., and Singh, S. (2009) Autophagy in immunity against mycobacterium tuberculosis: a model system to dissect immunological roles of autophagy. *Curr Top Microbiol Immunol* **335:** 169–188.

Dupont, N., Lacas-Gervais, S., Bertout, J., Paz, I., Freche, B., Van Nhieu, G.T., *et al.* (2009) Shigella phagocytic vacuolar membrane remnants participate in the cellular response to pathogen invasion and are regulated by autophagy. *Cell Host Microbe* **6:** 137–149.

Dwivedi, M., Song, H.O., and Ahnn, J. (2009) Autophagy genes mediate the effect of calcineurin on life span in *C. elegans*. *Autophagy* **5:** 604–607.

Esclatine, A., Chaumorcel, M., and Codogno, P. (2009) Macroautophagy signaling and regulation. In *Autophagy in Infection and Immunity*. Levine, B., Yoshimori, T., and Deretic, V. (eds). Berlin: Springer, pp. 33–70.

Eskelinen, E.L. (2005) Maturation of autophagic vacuoles in mammalian cells. *Autophagy* **1:** 1–10.

Ferrari, G., Langen, H., Naito, M., and Pieters, J. (1999) A coat protein on phagosomes involved in the intracellular survival of mycobacteria. *Cell* **97:** 435–447.

Galkin, V.E., Orlova, A., Brieher, W., Kueh, H.Y., Mitchison, T.J., and Egelman, E.H. (2008) Coronin-1A stabilizes F-actin by bridging adjacent actin protomers and stapling opposite strands of the actin filament. *J Mol Biol* **376:** 607–613.

Gao, L.Y., Guo, S., McLaughlin, B., Morisaki, H., Engel, J.N., and Brown, E.J. (2004) A mycobacterial virulence gene cluster extending RD1 is required for cytolysis, bacterial spreading and ESAT-6 secretion. *Mol Microbiol* **53:** 1677–1693.

Gutierrez, M.G., Master, S.S., Singh, S.B., Taylor, G.A., Colombo, M.I., and Deretic, V. (2004) Autophagy is a defense mechanism inhibiting BCG and *Mycobacterium tuberculosis* survival in infected macrophages. *Cell* **119:** 753–766.

Hingley-Wilson, S.M., Sambandamurthy, V.K., and Jacobs, W.R., Jr (2003) Survival perspectives from the world's most successful pathogen, *Mycobacterium tuberculosis*. *Nat Immunol* **4:** 949–955.

de Hostos, E.L. (1999) The coronin family of actin-associated proteins. *Trends Cell Biol* **9:** 345–350.

Jagannath, C., Lindsey, D.R., Dhandayuthapani, S., Xu, Y., Hunter, R.L., Jr, and Eissa, N.T. (2009) Autophagy enhances the efficacy of BCG vaccine by increasing peptide presentation in mouse dendritic cells. *Nat Med* **15:** 267–276.

Jayachandran, R., Sundaramurthy, V., Combaluzier, B., Mueller, P., Korf, H., Huygen, K., *et al.* (2007) Survival of mycobacteria in macrophages is mediated by coronin 1-dependent activation of calcineurin. *Cell* **130:** 37–50.

Jayachandran, R., Gatfield, J., Massner, J., Albrecht, I., Zanolari, B., and Pieters, J. (2008) RNA interference in J774 macrophages reveals a role for coronin 1 in mycobacterial trafficking but not in actin-dependent processes. *Mol Biol Cell* **19:** 1241–1251.

Jo, E.K. (2010) Innate immunity to mycobacteria: vitamin D and autophagy. *Cell Microbiol* **12:** 1026–1035.

Kabeya, Y., Mizushima, N., Ueno, T., Yamamoto, A., Kirisako, T., Noda, T., *et al.* (2000) LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. *EMBO J* **19:** 5720–5728.

Kumar, D., Nath, L., Kamal, M.A., Varshney, A., Jain, A., Singh, S., and Rao, K.V. (2010) Genome-wide analysis of the host intracellular network that regulates survival of *Mycobacterium tuberculosis*. *Cell* **140:** 731–743.

Lerena, M.C., and Colombo, M.I. (2011) *Mycobacterium marinum* induces a marked LC3 recruitment to its containing phagosome that depends on a functional ESX-1 secretion system. *Cell Microbiol* **13:** 814–835.

Lerena, M.C., Vazquez, C.L., and Colombo, M.I. (2010) Bacterial pathogens and the autophagic response. *Cell Microbiol* **12:** 10–18.

Levine, B. (2005) Eating oneself and uninvited guests: autophagy-related pathways in cellular defense. *Cell* **120:** 159–162.

Mbawuike, I.N., and Herscowitz, H.B. (1989) MH-S, a murine alveolar macrophage cell line: morphological, cytochemical, and functional characteristics. *J Leukoc Biol* **46:** 119–127.

Mizushima, N., and Levine, B. (2010) Autophagy in mammalian development and differentiation. *Nat Cell Biol* **12:** 823–830.

Mueller, P., Liu, X., and Pieters, J. (2011) Migration and homeostasis of naive T cells depends on coronin 1-mediated prosurvival signals and not on coronin 1-dependent filamentous actin modulation. *J Immunol* **186:** 4039–4050.

Pattingre, S., Tassa, A., Qu, X., Garuti, R., Liang, X.H., Mizushima, N., *et al.* (2005) Bcl-2 antiapoptotic proteins inhibit Beclin 1-dependent autophagy. *Cell* **122:** 927–939.

Perrin, A.J., Jiang, X., Birmingham, C.L., So, N.S., and Brumell, J.H. (2004) Recognition of bacteria in the cytosol of mammalian cells by the ubiquitin system. *Curr Biol* **14:** 806–811.

Pethe, K., Swenson, D.L., Alonso, S., Anderson, J., Wang, C., and Russell, D.G. (2004) Isolation of *Mycobacterium tuberculosis* mutants defective in the arrest of phagosome maturation. *Proc Natl Acad Sci USA* **101:** 13642–13647.

Pieters, J. (2008) Coronin 1 in innate immunity. *Subcell Biochem* **48:** 116–123.

Ponpuak, M., Davis, A.S., Roberts, E.A., Delgado, M.A., Dinkins, C., Zhao, Z., *et al.* (2011) Delivery of cytosolic components by autophagic adaptor protein p62 endows autophagosomes with unique antimicrobial properties. *Immunity* **32:** 329–341.

Russell, D.G. (2001) *Mycobacterium tuberculosis*: here today, and here tomorrow. *Nat Rev Mol Cell Biol* **2:** 569–577.

Russell, D.G. (2007) Who puts the tubercle in tuberculosis? *Nat Rev Microbiol* **5:** 39–47.

Seglen, P.O., and Gordon, P.B. (1982) 3-Methyladenine: specific inhibitor of autophagic/lysosomal protein degradation in isolated rat hepatocytes. *Proc Natl Acad Sci USA* **79:** 1889–1892.

Seto, S., Matsumoto, S., Ohta, I., Tsujimura, K., and Koide, Y. (2009) Dissection of Rab7 localization on *Mycobacterium tuberculosis* phagosome. *Biochem Biophys Res Commun* **387:** 272–277.

Seto, S., Matsumoto, S., Tsujimura, K., and Koide, Y. (2010) Differential recruitment of CD63 and Rab7-interacting-lysosomal-protein to phagosomes containing *Mycobacterium tuberculosis* in macrophages. *Microbiol Immunol* **54:** 170–174.

Seto, S., Tsujimura, K., and Koide, Y. (2011) Rab GTPases regulating phagosome maturation are differentially recruited to mycobacterial phagosomes. *Traffic* **12:** 407–420.

Shin, D.M., Jeon, B.Y., Lee, H.M., Jin, H.S., Yuk, J.M., Song, C.H., *et al.* (2010) *Mycobacterium tuberculosis* eis regulates autophagy, inflammation, and cell death through redox-dependent signaling. *PLoS Pathog* **6:** e1001230.

Singh, S.B., Davis, A.S., Taylor, G.A., and Deretic, V. (2006) Human IRGM induces autophagy to eliminate intracellular mycobacteria. *Science* **313:** 1438–1441.

Sirakova, T.D., Dubey, V.S., Kim, H.J., Cynamon, M.H., and Kolattukudy, P.E. (2003) The largest open reading frame (pks12) in the *Mycobacterium tuberculosis* genome is involved in pathogenesis and dimycocerosyl phthiocerol synthesis. *Infect Immun* **71:** 3794–3801.

Smith, J., Manoranjan, J., Pan, M., Bohsali, A., Xu, J., Liu, J., *et al.* (2008) Evidence for pore formation in host cell membranes by ESX-1-secreted ESAT-6 and its role in *Mycobacterium marinum* escape from the vacuole. *Infect Immun* **76:** 5478–5487.

Stamm, L.M., Morisaki, J.H., Gao, L.Y., Jeng, R.L., McDonald, K.L., Roth, R., *et al.* (2003) *Mycobacterium marinum* escapes from phagosomes and is propelled by actin-based motility. *J Exp Med* **198:** 1361–1368.

Sugaya, K., Seto, S., Tsujimura, K., and Koide, Y. (2011) Mobility of late endosomal and lysosomal markers on phagosomes analyzed by fluorescence recovery after photobleaching. *Biochem Biophys Res Commun* **410:** 371–375.

Vergne, I., Chua, J., and Deretic, V. (2003) *Mycobacterium tuberculosis* phagosome maturation arrest: selective targeting of PI3P-dependent membrane trafficking. *Traffic* **4:** 600–606.

Vergne, I., Chua, J., Singh, S.B., and Deretic, V. (2004) Cell biology of *Mycobacterium tuberculosis* phagosome. *Annu Rev Cell Dev Biol* **20:** 367–394.

van der Wel, N., Hava, D., Houben, D., Fluitsma, D., van Zon, M., Pierson, J., *et al.* (2007) *M. tuberculosis* and *M. leprae* translocate from the phagolysosome to the cytosol in myeloid cells. *Cell* **129:** 1287–1298.

World Health Organization (2010) *WHO Report 2010 Global Tuberculosis Control.* Geneva: WHO [WWW document]. http://www.who.int/tb/publications/global_report/2010/en/index.html.

Xu, Y., Jagannath, C., Liu, X.D., Sharafkhaneh, A., Kolodziejska, K.E., and Eissa, N.T. (2007) Toll-like receptor 4 is a sensor for autophagy associated with innate immunity. *Immunity* **27:** 135–144.

Yan, M., Collins, R.F., Grinstein, S., and Trimble, W.S. (2005) Coronin-1 function is required for phagosome formation. *Mol Biol Cell* **16:** 3077–3087.

Yuk, J.M., Shin, D.M., Lee, H.M., Yang, C.S., Jin, H.S., Kim, K.K., *et al.* (2009) Vitamin D3 induces autophagy in human monocytes/macrophages via cathelicidin. *Cell Host Microbe* **6:** 231–243.

Zheng, Y.T., Shahnazari, S., Brech, A., Lamark, T., Johansen, T., and Brumell, J.H. (2009) The adaptor protein p62/SQSTM1 targets invading bacteria to the autophagy pathway. *J Immunol* **183:** 5909–5916.

Supporting information

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

Fig. S1. Immunoblot analysis of LC3 in macrophages infected with *M. tuberculosis* at deferent moi.

A. Raw264.7 macrophages were transfected with Coro1a-specific or scrambled siRNA. Transfected macrophages were infected with *M. tuberculosis* at different moi for 6 h. Whole-cell lysates were subjected to SDS-PAGE, followed by immunoblot analysis using the indicated antibodies.
B. The band intensity for LC3-II per Rab7 at each condition to that in the macrophage without infection is shown. The data represent the mean and SD of three independent experiments. N.S., not significant (paired Student's *t*-test); MTB, *M. tuberculosis*; Sc, scrambled; Coro, Coro1a.

Fig. S2. Phagocytosis of latex beads and infection by *M. tuberculosis* in Coro1a KD macrophage. Raw264.7 macrophages were transfected with Coro1a-specific or scrambled siRNA for 48 h. Transfected macrophages were phagocytosed by FITC-labelled latex beads or DsRed-expressing *M. tuberculosis*. The rate of phagocytosed or infected macrophages were analysed by flow cytometry or fluorescent microscopy respectively. The data represent the mean and SD of three independent experiments. N.S., not significant (paired Student's *t*-test); MTB, *M. tuberculosis*; Sc, scrambled; Coro, Coro1a.

Fig. S3. LC3 recruitment to mycobacterial phagosomes in macrophages treated with calcineurin inhibitors. Macrophages stably expressing EGFP-LC3 were treated with FK506 (0.5 μM) or cyclosporine A (0.1 μM) for 1 h, and then infected with DsRed-expressing *M. tuberculosis* for 6 h. Cells were fixed and observed with LSCM. The number of LC3-positive *M. tuberculosis* phagosomes was counted. Data represent the mean and SD of three independent experiments in which more than 200 phagosomes were counted for each condition. N.S., not significant (unpaired Student's *t*-test); FK, FK506; Cyc, cyclosporine A; MTB, *M. tuberculosis*.

Fig. S4. Bcl-2 expression in Coro1a KD macrophages. Raw264.7 macrophages were transfected with Coro1a-specific siRNA for 48 h. Whole-cell lysates were subjected to SDS-PAGE, followed by immunoblot analysis using anti-Bcl-2 or anti-Coro1a antibodies. Bec1, Beclin1; Sc, scrambled; Coro, Coro1a.

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II. 結核感染成立要因の基礎研究

結核菌の細胞内寄生メカニズム

瀬戸真太郎[1] 辻村邦夫[1] 小出幸夫[1,2]

Mechanism of intracellular parasitism by *Mycobacterium tuberculosis*

[1]Shintaro Seto, [1]Kunio Tsujimura, [1,2]Yukio Koide
[1]Department of Infectious Diseases,
[2]Executive Director, Hamamatsu University School of Medicine

Abstract

Mycobacterium tuberculosis is an intracellular bacterium that can replicate within infected macrophages. The intracellular parasitism by *M. tuberculosis* results from arresting phagosome maturation and inhibiting phagolysosome biogenesis in infected macrophages. It has been thought that *M. tuberculosis* arrests the maturation of its phagosome at the early stage. Several reports attended to the localization of Rab GTPases on mycobacterial phagosomes. Rab GTPases regulate membrane trafficking, but details of how Rab GTPases regulate phagosome maturation and how *M. tuberculosis* modulates their activities during inhibiting phagolysosome biogenesis remains elusive. Here, we introduce the new findings that *M. tuberculosis* alters the localization of Rab GTPases regulating phagosome maturation during inhibiting phagolysosome biogenesis.

Key words: *Mycobacterium tuberculosis*, macrophage, phagosome, phagolysosome biogenesis, Rab GTPase

はじめに

マクロファージは生体に侵入した細菌，ウイルス，真菌などの微生物を貪食する．貪食された微生物はマクロファージ内で殺菌，分解される．マクロファージによる貪食作用は感染初期における強力な生体防御機構である．また，細胞性免疫においてもマクロファージは機能する．すなわち，ヘルパーT細胞から産生されるIFN-γによってマクロファージは活性化されて，貪食作用が強化される．マクロファージは微生物を貪食して，細胞内で食胞(ファゴソーム)に取り込む．ファゴソームは初期エンドソーム，後期エンドソームと次々に融合することによって，ファゴソーム熟成を行う[1]．ファゴソーム熟成中に，vacuolar ATPase(v-ATPase)がファゴソームに局在する．v-ATPaseによってプロトンがファゴソーム内に流れ込み，ファゴソーム内のpHが低下する．また，NADPHオキシダーゼはファゴソーム膜状で複合体形成を行い，活性化される．NADPHオキシダーゼによって活性酸素が合成される．活性酸素によって一般的な微生物は殺菌される．ファゴソーム熟成が進行すると，ファゴソームはリソソーム

[1]浜松医科大学医学部 感染症学講座 [2]同 理事

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と融合してファゴリソソーム形成を行う．ファゴリソソーム形成によって，リソソーム由来の酸性ホスファターゼやカテプシンなどのタンパク質分解酵素がファゴソーム内に流入する．これらの加水分解酵素によって多くの微生物は殺菌，分解される．しかし，細胞内寄生性細菌は様々な戦略によって，マクロファージによる貪食，殺菌作用から回避することができる．

1. 結核菌によるファゴリソソーム形成阻害機構と細胞内寄生性

結核菌は典型的な細胞内寄生性細菌である[2]．すなわち，結核菌はヒト肺に感染したとき，肺胞マクロファージによって貪食されても殺菌，分解されずに，マクロファージ内で増殖することができる．結核菌はウレアーゼやスーパーオキシドジムスターゼ，カタラーゼなどの酵素を産生することによって，ファゴソーム内のpH低下を阻害したり，活性酸素による殺菌作用から回避している．更に，ファゴソーム熟成やファゴリソソーム形成を阻害することによって，リソソームの殺菌作用からも回避する．すなわち，結核菌はマクロファージに貪食されても，ファゴソーム内を増殖に適したニッチへと変化させることができる．

近年，結核菌もリステリアや赤痢菌と同様にファゴソーム膜を溶解して細胞質へ移行していることが観察された[3]．結核菌の近縁種である*Mycobacterium marinum*もリステリアや赤痢菌と同様に，ファゴソームから脱出して細胞質に移行することが知られている[4]．更に細胞質に移行した*M. marinum*は，宿主細胞のアクチンを重合させることによって細胞質内を移動することができる[4]．結核菌もファゴソームから脱出した後にアクチン重合によって細胞質内を移動しているかもしれない．しかし，結核菌や*M. marinum*がファゴソームから脱出して細胞質内に移行するのは，感染3日後になってようやく起こる．また，結核菌は感染後期において，感染マクロファージにネクローシスやアポトーシスを引き起こす．このことは，結核菌の細胞内寄生性を保証する初期段階の戦略としては，ファゴリソソーム形成阻害機構が優位であることを示す．

2. 結核菌はファゴソーム熟成を阻害する

結核菌のファゴソーム熟成過程は，初期ファゴソームの段階で停止しているといわれている[5]．Deretic らの研究グループは，生化学的手法および細胞生物学的手法によって結核菌ファゴソームには初期エンドソームマーカーであるRab5が局在した状態であり，後期エンドソームマーカーであるRab7は局在しないことを示している[6]．また，結核菌の細胞壁構成糖脂質が結核菌ファゴソームと初期エンドソームの融合を促進すること[7]，ファゴソーム熟成に必要であるホスファチジルイノシトール3リン酸(PI3P)の合成が結核菌ファゴソームでは抑制されることを示している[8]．更に結核菌はPI3Pホスファターゼである SapM を合成，分泌することによって，結核菌ファゴソームに局在するPI3Pを分解することができる[9]．以上の結果は，結核菌はファゴソーム熟成を阻害するために，PI3Pを標的にしていることを示す．

また，Deretic らはRab5，Rab7以外のRab GTPaseに注目して，Rab14とRab22aの結核菌ファゴソームにおける局在とファゴソーム熟成における機能を明らかにした[10,11]．Rab14やRab22aは初期エンドソームに局在するRab GTPaseであるが，ファゴソーム熟成における機能は明らかになっていなかった．Rab14やRab22aはラテックスビーズファゴソームや不活性化した結核菌のファゴソームには局在しないが，結核菌ファゴソームにおいて局在することを明らかにした．不活性化型Rab遺伝子発現やRNA干渉法によるノックダウンによって，結核菌ファゴソームの熟成が進行することを示した．また，恒常活性化型Rab遺伝子を発現するマクロファージでは，ラテックスビーズファゴソームの熟成が進行しないことを示した．以上の結果は，ファゴソームにRab14やRab22aを局在させることによって，結核菌はファゴソーム熟成の進行を阻害することを示唆する．

近年，Rab7が結核菌ファゴソームに局在し

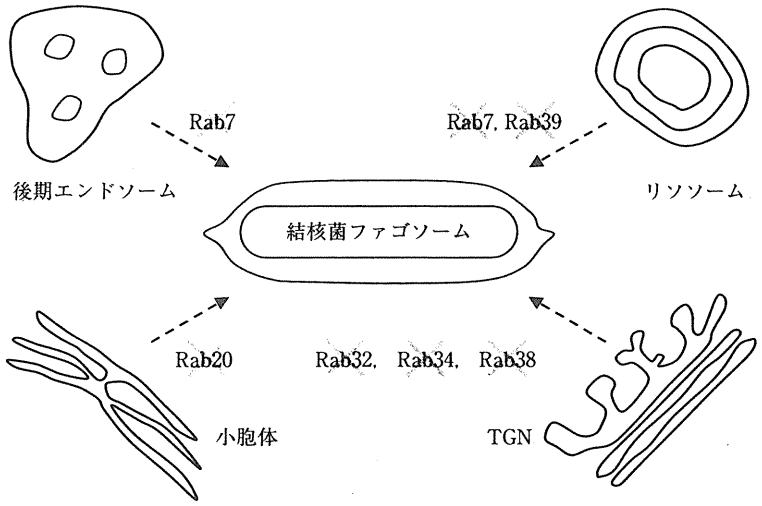

図1 結核菌ファゴソームに局在しないRab GTPaseと小胞輸送
黄色ブドウ球菌ファゴソームには局在するが，結核菌ファゴソームには局在しないRab GTPaseを示した．これらのRab GTPaseはファゴソーム内の酸性化やファゴソームへのカテプシンDの局在化に関与する．

ないことに関して，反対の観察研究が報告されている．Hmamaらの研究グループは，結核菌ファゴソームにRab7が局在することを示した[12]．また，著者らは，Rab7は一度結核菌ファゴソームに局在して，その後，結核菌ファゴソームからかい離することを示している[13]．

3. 結核菌ファゴソームにおけるRab GTPaseの局在

Rab GTPaseはRas superfamilyに属するGTPaseであり，60以上のファミリー遺伝子が属している[14]．これまで，エンドサイトーシスやエキソサイトーシスの小胞輸送におけるRab GTPaseの機能は明らかにされてきた．しかし，ファゴソーム熟成やファゴリソソーム形成におけるRab GTPaseの機能に関しては明らかになっていなかった．著者らは，結核菌ファゴソームにおける小胞輸送を理解するため，黄色ブドウ球菌ファゴソームと結核菌ファゴソームにおけるRab GTPaseの局在を網羅的に比較した[15]．黄色ブドウ球菌ファゴソームには22のRab GTPaseが局在するが，そのうちの17遺伝子が結核菌ファゴソームからかい離する，もしくは局在しないことを明らかにした．次に，ファゴソーム熟成，特にファゴソーム内の酸性化とファゴソームへのカテプシンDの輸送に関与するRab GTPaseを，不活性化型Rab遺伝子を発現するマクロファージを用いて調べた．ファゴソーム内の酸性化にはRab7，Rab20，Rab39が関与すること，ファゴソームへのカテプシンDの局在化にはRab7，Rab20，Rab32，Rab34，Rab38が関与することを明らかにした（**図1**）．Rab7はファゴソーム内の酸性化とカテプシンDのファゴソームへの局在化に関与することが，これまでに明らかになっていた．Rab20は小胞体に局在するRab GTPaseであり，ファゴソーム熟成における機能は明らかになっていなかった．興味深いことに，Rab39の局在はリソソームであり，Rab39のファゴソームへの局在は貪食後期に始まる．このことは，Rab39は貪食後期におけるファゴソーム内の酸性化に機能していることを示唆する．Rab32，Rab34，Rab38はトランスゴルジ網（TGN）に局在することが明らかになっている[14]．また，カテプシンDのファゴソームへの輸送は，後期エンドソームやリソソームのほかにも，TGNから直接輸送されていることが知られている[16]．以上の結果は，結核菌はTGNから結核菌ファ

ゴソームへのカテプシンDの輸送を阻害するため，これらのRab GTPaseを結核菌ファゴソームから排除していることを示唆する．Russellらの研究グループは，リソソームにおける結核菌殺菌因子を探索した結果，カテプシンDによって加水分解されたユビキチン分解ペプチドを同定した[17]．以上の結果は，結核菌はファゴソーム内のpH低下を阻害することによって，カテプシンDなどの加水分解酵素をファゴソーム内で活性化させない，更に後期エンドソーム，リソソーム，TGNからのカテプシンDの結核菌ファゴソームへの輸送を阻害することによって，ファゴソーム内での結核菌殺菌因子の産生を回避していることを示唆する．

おわりに

結核菌の細胞内寄生戦略におけるファゴリソソーム形成阻害機構について述べた．しかし，本当に結核菌ファゴソームは，リソソームと全く融合していないのであろうか．これまでの結核菌によるファゴリソソーム形成阻害機構の研究において，LAMP1/2やCD63などの古典的リソソームマーカーの結核菌ファゴソームへの局在が調べられてきた．しかし，近年，van der Welらはクライオ電子顕微鏡法によって，LAMP1/2やCD63が結核菌ファゴソームに局在することを示した[3]．Leeらは生化学的に単離した結核菌ファゴソームのプロテオミクスによって，これらの古典的リソソームマーカーが局在することを明らかにした[18]．また，著者らもイメージ解析によって，結核菌ファゴソームにはこれらのリソソームマーカーが局在することを示した[13,19]．ノックアウトマウスの研究結果は，LAMP1/2のファゴソームへの局在ヒエラルキーはRab7よりも上位にあることを示している[20]．以上の研究結果から著者らは，マクロファージにおいて古典的リソソームマーカーが結核菌ファゴソームにおけるリソソームマーカーとして機能しているかを明らかにする必要があるのではないかと考えている．

文献

1) Vieira OV, et al: Phagosome maturation: aging gracefully. Biochem J **366**: 689-704, 2002.
2) Russell DG: *Mycobacterium tuberculosis*: here today, and here tomorrow. Nat Rev Mol Cell Biol **2**: 569-577, 2001.
3) van der Wel N, et al: *M. tuberculosis* and *M. leprae* translocate from the phagolysosome to the cytosol in myeloid cells. Cell **129**: 1287-1298, 2007.
4) Stamm LM, et al: *Mycobacterium marinum* escapes from phagosomes and is propelled by actin-based motility. J Exp Med **198**: 1361-1368, 2003.
5) Vergne I, et al: Cell biology of *Mycobacterium tuberculosis* phagosome. Annu Rev Cell Dev Biol **20**: 367-394, 2004.
6) Via LE, et al: Arrest of mycobacterial phagosome maturation is caused by a block in vesicle fusion between stages controlled by rab5 and rab7. J Biol Chem **272**: 13326-13331, 1997.
7) Vergne I, et al: *Mycobacterium tuberculosis* phagosome maturation arrest: mycobacterial phosphatidylinositol analog phosphatidylinositol mannoside stimulates early endosomal fusion. Mol Biol Cell **15**: 751-760, 2004.
8) Chua J, Deretic V: *Mycobacterium tuberculosis* reprograms waves of phosphatidylinositol 3-phosphate on phagosomal organelles. J Biol Chem **279**: 36982-36992, 2004.
9) Vergne I, et al: Mechanism of phagolysosome biogenesis block by viable *Mycobacterium tuberculosis*. Proc Natl Acad Sci USA **102**: 4033-4038, 2005.
10) Kyei GB, et al: Rab14 is critical for maintenance of *Mycobacterium tuberculosis* phagosome maturation arrest. EMBO J **25**: 5250-5259, 2006.
11) Roberts EA, et al: Higher order Rab programming in phagolysosome biogenesis. J Cell Biol **174**: 923-929, 2006.
12) Sun J, et al: *Mycobacterium bovis* BCG disrupts the interaction of Rab7 with RILP contributing to

inhibition of phagosome maturation. J Leukoc Biol **82**: 1437-1445, 2007.
13) Seto S, et al: Dissection of Rab7 localization on *Mycobacterium tuberculosis* phagosome. Biochem Biophys Res Commun **387**: 272-277, 2009.
14) Schwartz SL, et al: Rab GTPases at a glance. J Cell Sci **120**: 3905-3910, 2007.
15) Seto S, et al: Rab GTPases regulating phagosome maturation are differentially recruited to mycobacterial phagosomes. Traffic, 2011. (in press)
16) Ullrich HJ, et al: Direct delivery of procathepsin D to phagosomes: implications for phagosome biogenesis and parasitism by *Mycobacterium*. Eur J Cell Biol **78**: 739-748, 1999.
17) Alonso S, et al: Lysosomal killing of *Mycobacterium* mediated by ubiquitin-derived peptides is enhanced by autophagy. Proc Natl Acad Sci USA **104**: 6031-6036, 2007.
18) Lee BY, et al: The *Mycobacterium bovis* bacille Calmette-Guerin phagosome proteome. Mol Cell Proteomics **9**: 32-53, 2010.
19) Seto S, et al: Differential recruitment of CD63 and Rab7-interacting-lysosomal-protein to phagosomes containing *Mycobacterium tuberculosis* in macrophages. Microbiol Immunol **54**: 170-174, 2010.
20) Huynh KK, et al: LAMP proteins are required for fusion of lysosomes with phagosomes. EMBO J **26**: 313-324, 2007.

特集 結核菌と結核を巡る新たな知見

6．結核菌ファゴソームの成熟阻害機構

瀬戸 真太郎*[1]　辻村 邦夫*[2]　小出 幸夫**

結核菌は細胞内寄生性細菌であり，貪食されたマクロファージ内で増殖することができる。結核菌の細胞内増殖能はファゴリソソーム形成を阻害することによって獲得している。これまで，結核菌ファゴソームには後期ファゴソームマーカーであるRab7が局在しないため，結核菌ファゴソームの成熟は進行しない，その結果，ファゴリソソーム形成が阻害されていると考えられてきた。しかし，近年この研究モデルと矛盾した研究結果が発表されている。ここでは，結核菌ファゴソームにおけるRab GTPaseの網羅的局在解析から明らかになった結核菌ファゴソームの成熟阻害機構について説明する。

Key Words：細胞内寄生性／ファゴソーム成熟／ファゴリソソーム形成／Rab GTPase／ファゴソームの酸性化／カテプシンD

I　はじめに

マクロファージは感染初期における強力な生体防御機構である。生体内に侵入した細菌，ウイルス，真菌などの微生物はマクロファージによって貪食される。貪食された微生物は殺菌，分解される。細胞性免疫においてもマクロファージは機能する。ヘルパーT細胞から産生されるIFN-γ（インターフェロンγ）によってマクロファージは活性化され，マクロファージの貪食，殺菌作用が強化される。貪食された微生物は食胞（ファゴソーム）に包みこまれる（図1）。ファゴソームと初期エンドソーム，後期エンドソームが次々に融合することによってファゴソーム成熟が進行する。

ファゴソーム成熟の過程においてファゴソームにvacuolar ATPase (v-ATPase)が局在する。v-ATPaseによってプロトンがファゴソーム内に汲みこまれてファゴソーム内のpH（水素イオン指数）は低下する。また，NADPH（ニコチンアミドアデニンジヌクレオチドリン酸）オキシダーゼの複合体形成がファゴソーム膜状で行われて活性化する。NADPHオキシダーゼによって合成された活性酸素は一般的な微生物を殺菌することができる。

成熟したファゴソームはリソソームと融合してファゴリソソーム形成を行う。ファゴリソソーム形成によってリソソーム由来の酸性フォスファターゼやタンパク質分解酵素がファゴソーム内に流入する。これらの加水分解酵素によって一般的な微生物は殺菌，分解される。しかし，細胞内寄生性細菌はさまざまな戦略によってマクロファージによる貪食，殺菌作用から回避することができる。

II　結核菌の細胞内寄生性戦略

結核菌は典型的な細胞内寄生性細菌である[1]。すなわち，結核菌はヒト肺に感染した時に肺胞マクロファージに貪食されても，殺菌されずにマク

Inhibitory mechanism of phagosome maturation by *Mycobacterium tuberculosis*

*浜松医科大学感染症学講座　[1]助教　Shintaro Seto　[2]准教授　Kunio Tsujimura
**浜松医科大学 理事　Yukio Koide

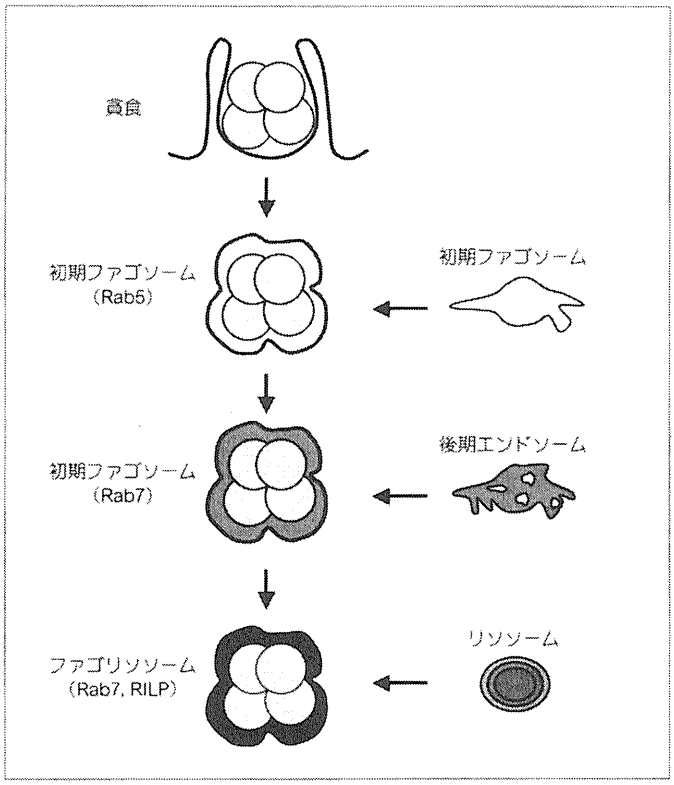

図1　ファゴソーム成熟とファゴリソソーム形成

マクロファージは一般的な微生物を貪食するとファゴソームに包み込む。ファゴソームには，初期エンドソーム，後期エンドソームが融合してファゴソーム成熟が行われる。最終的にファゴソームはリソソームと融合してファゴリソソーム形成が行われる。初期ファゴソーム，後期ファゴソーム，ファゴリソソームのマーカータンパク質も示す。

（文献6より筆者作成）

ロファージ内で増殖することができる。結核菌はウレアーゼやスーパーオキサイドジムスターゼ，カタラーゼなどの酵素を産生することによってファゴソーム内のpH低下を阻害したり，活性酸素による殺菌作用から回避する。さらに，ファゴリソソーム形成を阻害することによってリソソームの殺菌作用から回避することができる。このようにして，結核菌はマクロファージに貪食されてもファゴソーム内を結核菌の増殖に適したニッチへと変化させる。

近年，リステリアや赤痢菌と同様に，マクロファージに貪食された結核菌はファゴソーム膜を溶解して細胞質へ移行することが観察された[2]。結核菌の近縁種である *Mycobacterium marinum* もマクロファージへの感染後，ファゴソーム膜を溶解して細胞質に移行する[3]。さらに，*M. marinum* はリステリアや赤痢菌と同様に，アクチンを重合させることによって細胞質内を移動することがで

IFN-γ（インターフェロンγ）　　pH（水素イオン指数）
NADPH（ニコチンアミドアデニンジヌクレオチドリン酸）

きる[5]。結核菌もファゴソームから細胞質へ脱出した後，アクチン重合によって細胞質内を移動しているかもしれない。細胞内寄生性細菌における細胞質内での移動はオートファジー誘導の回避に関与している。オートファジーは飢餓時に誘導される細胞内タンパク質分解機構であるが，細胞質内に感染した微生物をオートファジーによって殺菌，分解することができる。

しかし，アクチン重合に機能するリステリアのActAや赤痢菌のVirGはオートファジー誘導の回避にも関与している[4,5]。結核菌や*M. marinum*におけるアクチン重合タンパク質はこれらの細胞内寄生性細菌と同様に，オートファジー誘導回避にも関与している可能性がある。

しかしながら，結核菌や*M. marinum*がファゴソームから脱出して細胞質内への移行が起こるのは感染後期（3日後）になってようやくである。同時期に，結核菌は感染マクロファージにネクローシスやアポトーシスを引き起こす。このことは，結核菌の細胞内寄生性を保証する初期段階の戦略として，ファゴソーム成熟阻害機構やファゴリソーム形成阻害機構が優先的に機能していることを示す。

Ⅲ 結核菌によるファゴソーム成熟阻害機構

1．ファゴソーム成熟におけるRab5とRab7

ファゴソーム成熟過程においてさまざまな分子がファゴソームに局在して，かい離する。ファゴソームの成熟過程を示すマーカーとしてRab5とRab7があげられる[6]。Rab GTPaseはRas superfamilyに属するGTPaseであり，Rab5とRab7を含む60以上のファミリー遺伝子が属している[7]。Rab5とRab7は初期エンドソーム，後期エンドソームのマーカーであるが，ファゴソームにおいても初期ファゴソーム，後期ファゴソームのマーカーとして機能している。

Rab5は貪食直後のファゴソームに局在する。ファゴソーム膜上で活性化されたRab5はそのエフェクター分子であるEEA1やフォスファチジルイノシトール3リン酸キナーゼ（**PI3K**）と会合する。EEA1はプラットフォームとして機能し，さまざまな分子をファゴソーム膜上に会合させる。PI3Kはファゴソーム膜上でフォスファチジルイノシトール3リン酸（**PI3P**）の生成を行い，ファゴソーム膜上でのNADPHオキシダーゼの複合体形成を促進する。次に初期ファゴソームから後期ファゴソームへと移行する。すなわち，Rab5が不活性化されてファゴソームからかい離してRab7が局在する。

Rab7はファゴソーム膜上で**RILP**（Rab7-Interacting-Lysosomal-Protein）と会合する。RILPはダイニンと複合体形成を行う。そして，ファゴソームを微小管に沿ってリソソームが集積している**MTOC**（microtubule organizing center）へ輸送する。MTOCへ輸送されたファゴソームはリソソームと融合してファゴリソソーム形成が行われる。

2．結核菌ファゴソームにおける成熟阻害

結核菌のファゴソーム成熟過程は初期ファゴソームの段階で停止していると言われている。Deretic等の研究グループは，結核菌ファゴソームではRab conversionが起こらない，すなわち，結核菌ファゴソームにはRab5が局在した状態であり，Rab7は局在しないことを示した[8]。また，ファゴソーム成熟に必要であるPI3Pの合成が結核菌ファゴソームでは抑制されることを示している[9]。さらに，結核菌はPI3PフォスファターゼであるsapMを合成，分泌することによって結核菌ファゴソームにあるPI3Pを分解していることを明らかにした[10]。

以上の結果は，結核菌はファゴソーム成熟を阻害するためにPI3Pを標的にしていることを示す。しかし，Rab7が結核菌ファゴソームに局在

PI3K（フォスファチジルイノシトール3リン酸キナーゼ）
PI3P（フォスファチジルイノシトール3リン酸）　RILP（Rab7-Interacting-Lysosomal-Protein）
MTOC（microtubule organizing center）

しないことに関して反対の観察研究が報告されている。Hmama 等の研究グループは結核菌ファゴソームに Rab7 が局在することを明らかにした[11]。我々は Rab7 は一度，結核菌ファゴソームに局在して，その後，結核菌ファゴソームからかい離することを示している[12]。

Deretic 等は Rab5, Rab7 以外の Rab GTPase に注目して，Rab14 と Rab22a の結核菌ファゴソームにおける局在とファゴソーム成熟における機能を明らかにした[13][14]。Rab14 や Rab22a は初期エンドソームに局在する Rab GTPase であるが，ファゴソーム成熟における機能は明らかになっていなかった。Rab14 や Rab22a はラテックスビーズファゴソームには局在しないが，結核菌ファゴソームには局在した。不活性化型 Rab 遺伝子発現や RNA 干渉法によるノックダウンによって結核菌ファゴソームの成熟が促進されること，恒常活性化型 Rab 遺伝子を発現するマクロファージではファゴソーム成熟が進行しないことを示した。

以上の結果は，結核菌ファゴソームに Rab14 や Rab22a が局在することによってファゴソーム成熟が阻害されていることを示した。しかし，結核菌ファゴソームにおける Rab14 や Rab22a の局在機構はまったく明らかになっていない。

Ⅳ　結核菌ファゴソームにおける Rab GTPase の局在

これまで，結核菌ファゴソームにおける Rab GTPase の網羅的局在解析は行われていなかった。また，ファゴソーム成熟やファゴリソソーム形成における Rab GTPase の機能は Rab5 と Rab7 以外ほとんど明らかになっていない。我々は，ファゴソーム成熟に機能する Rab GTPase は黄色ブドウ球菌ファゴソームに局在する，そして，これらの Rab GTPase が結核菌ファゴソームからかい離する，もしくは局在しないために結核菌ファゴソームではファゴソーム成熟が阻害され

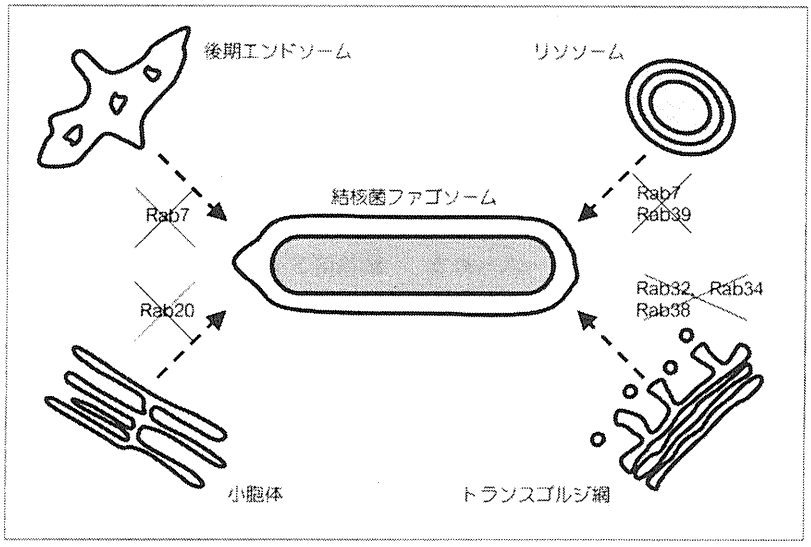

図2　結核菌ファゴソームの成熟阻害機構

黄色ブドウ球菌ファゴソームには局在するが，結核菌ファゴソームからはかい離する，もしくは局在しない Rab GTPase を示す。これらの Rab GTPase を結核菌ファゴソームに局在させないことによって，後期エンドソーム，リソソーム，トランスゴルジ網，小胞体からの小胞輸送を阻害する。その結果，結核菌ファゴソームの酸性化やカテプシン D の局在化が阻害される。

（文献 15 より筆者作成）