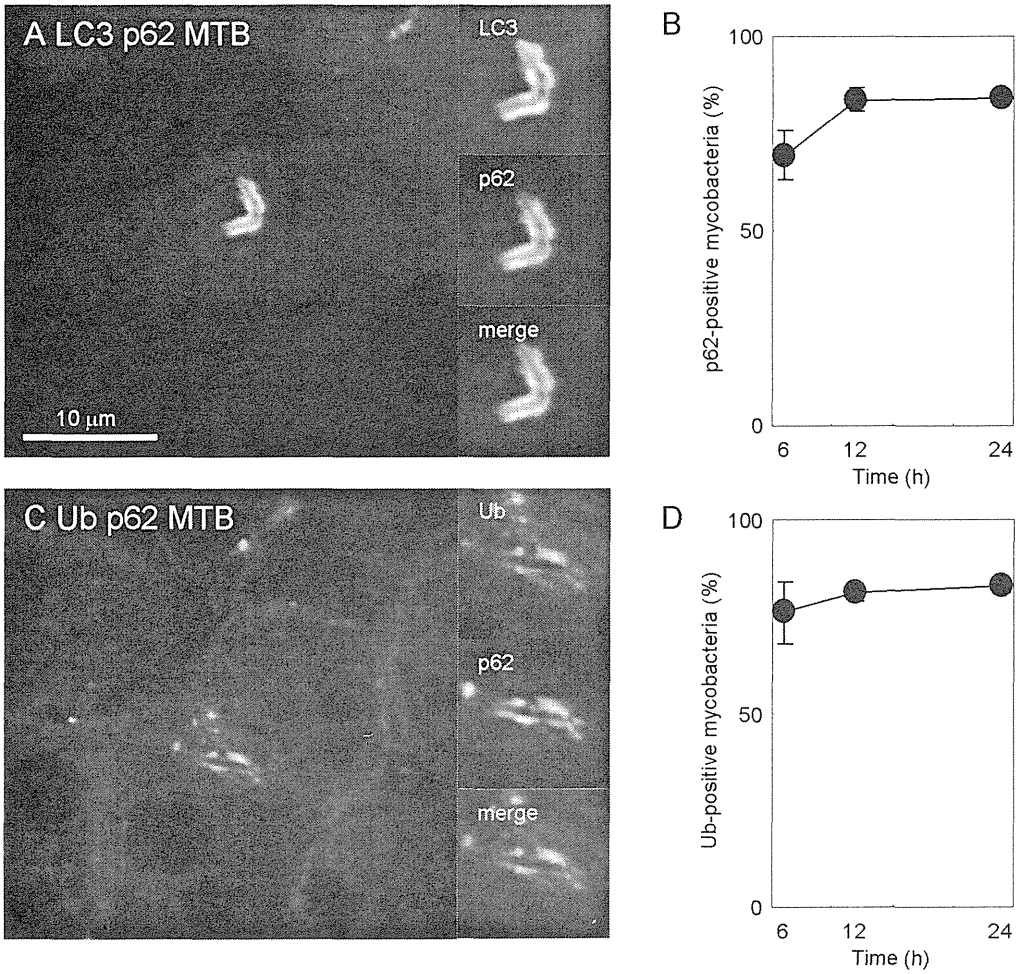

Figure 3. Maturation of mycobacterial autophagosomes in DC2.4 cells. (A) Recruitment of p62 to LC3-positive mycobacteria. DC2.4 cells were infected with Alexa Fluor 405-labeled *M. tuberculosis* (blue) for 24 h and immunostained with anti-LC3 (green) and anti-p62 antibodies (red). (B) The proportion of p62 localization to LC3-positive mycobacteria in DC2.4 cells.
(C) Recruitment of ubiquitin to p62-positive mycobacteria. DC2.4 cells were infected with Alexa Fluor 405-labeled *M. tuberculosis* (blue) for 24 h and immunostained with anti-p62 (red) and anti-ubiquitin antibodies (green). (D) The proportion of ubiquitin localization to p62-positive mycobacteria in DC2.4 cells. Data represent the mean and SD of three independent experiments.
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mycobacteria are ubiquitinated in a p62-dependent manner in DC.

Atg5 functions in autolysosome biogenesis

To further investigate the function of Atg5 in autophagosome formation to mycobacteria in DC, we observed the ultrastructures of *M. tuberculosis*-infected DC2.4 transfected with p62 or Atg5 siRNA. Thin-section electron microscopy revealed that depletion of p62 or Atg5 inhibits autophagosome formation (Figure 7A-D), suggesting that Atg5 is also important for autophagosome formation to mycobacteria in DC.

Watson et al. demonstrated that Atg5 functions in autolysosome biogenesis to ubiquitinated *M. tuberculosis* in macrophages [36]. To determine the function of Atg5 in autophagosome formation to mycobacteria in DC, we examined the localization of LAMP1 or MHC II to ubiquitinated mycobacteria in JAWSII cells transfected with Atg5 siRNA (Figure 7E, F). Atg5 depletion decreased the proportion of LAMP1-positive or MHC II-positive ubiquitinated mycobacteria, suggesting that Atg5 functions in the fusion of lysosomes with mycobacterial autophagosomes in DC.

Discussion

The induction of autophagy can eliminate *M. tuberculosis* in phagocytic cells [37,38], but the precise mechanism by which mycobacterial infection induces autophagy in phagocytic cells is not fully understood. Previous reports demonstrated that *M. tuberculosis* infection itself did not induce autophagy in infected macrophages [16,26,39]. However, other studies demonstrated

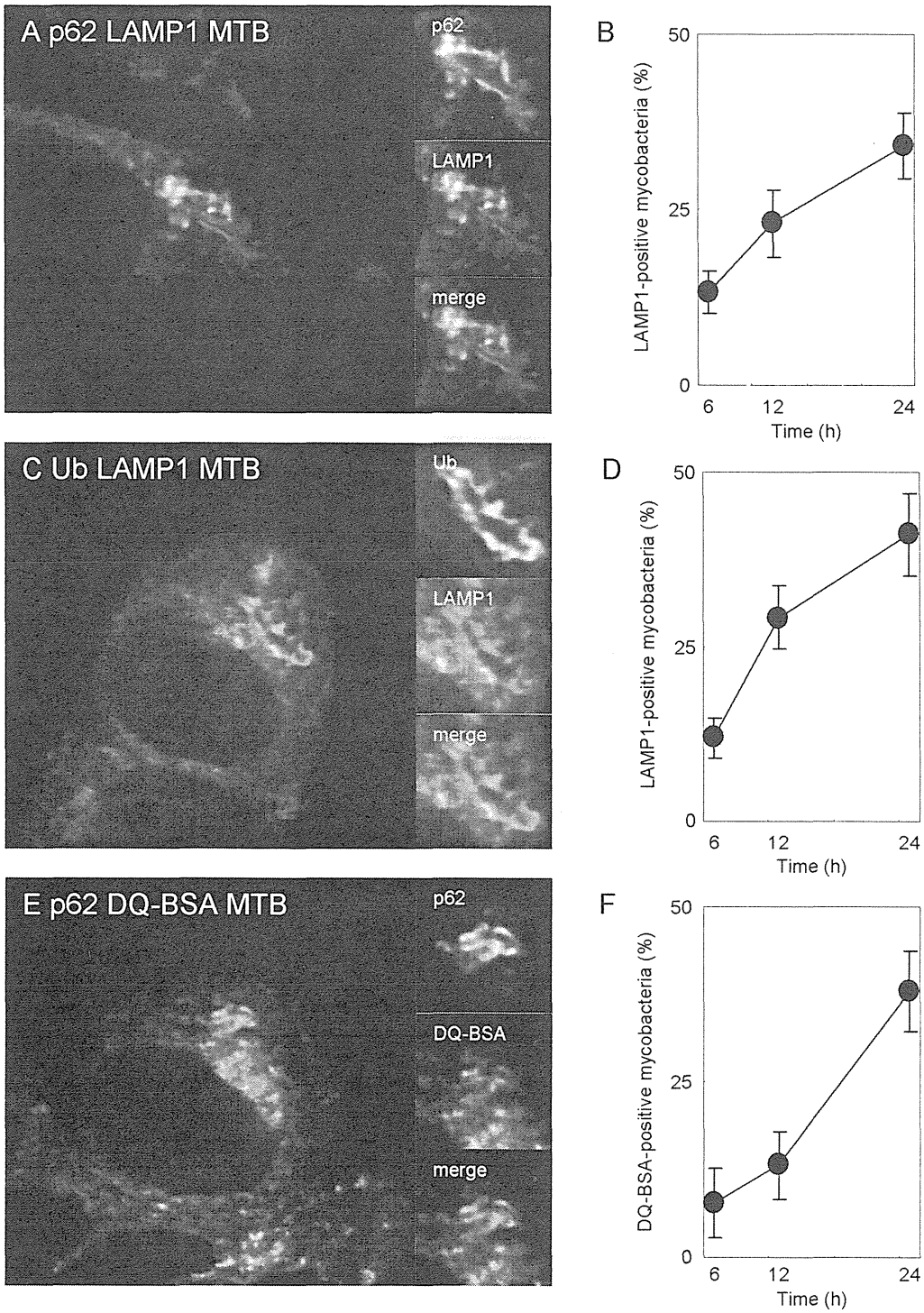

Figure 4. Mycobacterial autolysosome biogenesis. (A, C) Localization of LAMP1 to p62-positive of ubiquitin-positive mycobacteria in DC2.4 cells. DC2.4 cells were infected with Alexa Fluor 405-labeled *M. tuberculosis* (blue) for 24 h and immunostained with anti-LAMP1 (red) and anti-p62 antibodies (green) (A) or anti-LAMP1 (red) and anti-ubiquitin antibodies (green) (C). (B, D) The proportion of LAMP1-localized p62-positive (B) or ubiquitin-positive (D) mycobacteria. (E) Localization of DQ-BSA to mycobacterial autophagosomes. DC2.4 cells were preloaded with DQ-BSA to label degradative vesicles. DC were infected with Alexa Fluor 405-labeled mycobacteria for 24 h and immunostained with anti-p62 antibody. (F) The proportion of DQ-BSA-labeled p62-positive mycobacteria in DC2.4 cells. Data represent the mean and SD of three independent experiments.

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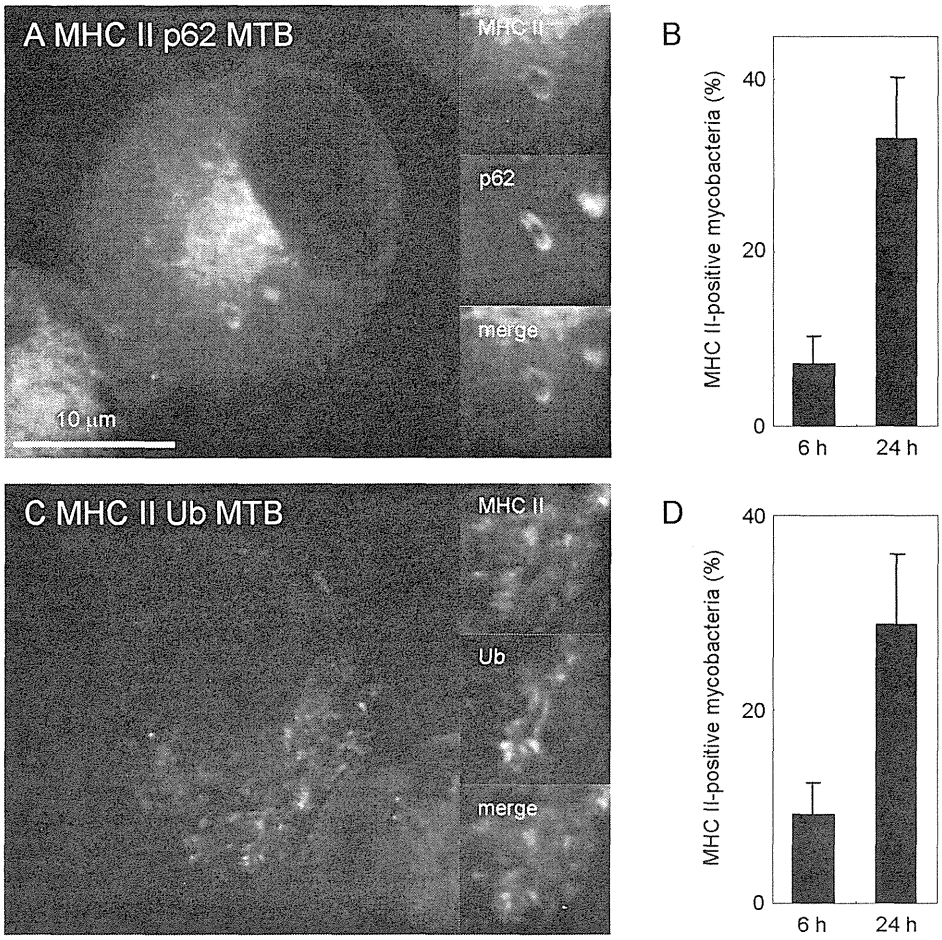

Figure 5. Localization of MHC class II to mycobacterial autophagosomes in DC. (A, C) Localization of MHC class II to p62-positive or ubiquitin-positive mycobacteria in JAWSII cells. JAWSII cells were infected with Alexa Fluor 405-labeled *M. tuberculosis* (blue) for 24 h and immunostained with anti-MHC class II (green) and anti-p62 antibodies (red) (A) or anti-MHC class II (green) and anti-ubiquitin antibodies (red) (C). (B, D) The proportion of MHCII-localized p62-positive (B) orubiquitin-positive (D) mycobacteria in JAWSII cells. Data represent the mean and SD of three independent experiments.

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that autophagosomes were formed in response to mycobacteria infection in macrophages [36]. Recently, it was demonstrated that *M. tuberculosis* infection induced autophagy but impaired the autophagic flux in human primary macrophages and DC [20,40]. In this study, we demonstrated that autophagosome markers localized to *M. tuberculosis* in BMDC but not in BMM (Figure 1). In DC cell lines, we also showed that autophagosomes are formed in response to *M. tuberculosis* infection (Figure 2), and found that lysosomal vesicles fuse with mycobacterial autophagosomes in DC (Figure 4).

Since autophagy is thought to be involved in antigen presentation via MHC II [22], it is assumed that autophagy promotes the presentation of mycobacterial antigens in DC. In this study, we demonstrated that *M. tuberculosis* infection induced selective autophagy in DC and that mycobacterial autophagosomes fuse with lysosomes followed by the recruitment of MHC II. These results suggest that the autophagosome formation in DC promotes degradation of infected mycobacteria and the resulting degradative peptides are then loaded onto MHC II, which are recruited to mycobacterial autophagosomes, leading the antigen presentation of mycobacterial peptides to $CD4^+$ T lymphocytes via MHC II.

What is the initial event that triggers the induction of autophagosome formation to mycobacteria in phagocytic cells? The ubiquitination of bacteria or bacterial phagosomes is important for selective autophagy because ubiquitinated substrates are associated with the autophagy adaptor proteins which recruit LC3 and autophagosome membranes [41]. In *Mycobacterium marinum*, the ubiquitination of this bacterium is dependent upon the ESAT-6 and ESX-1 secretion system [42]. ESAT-6 of *M. marinum* disrupts the phagosomal membranes to assist bacilli escape from phagosomes to the cytosol [43-46].

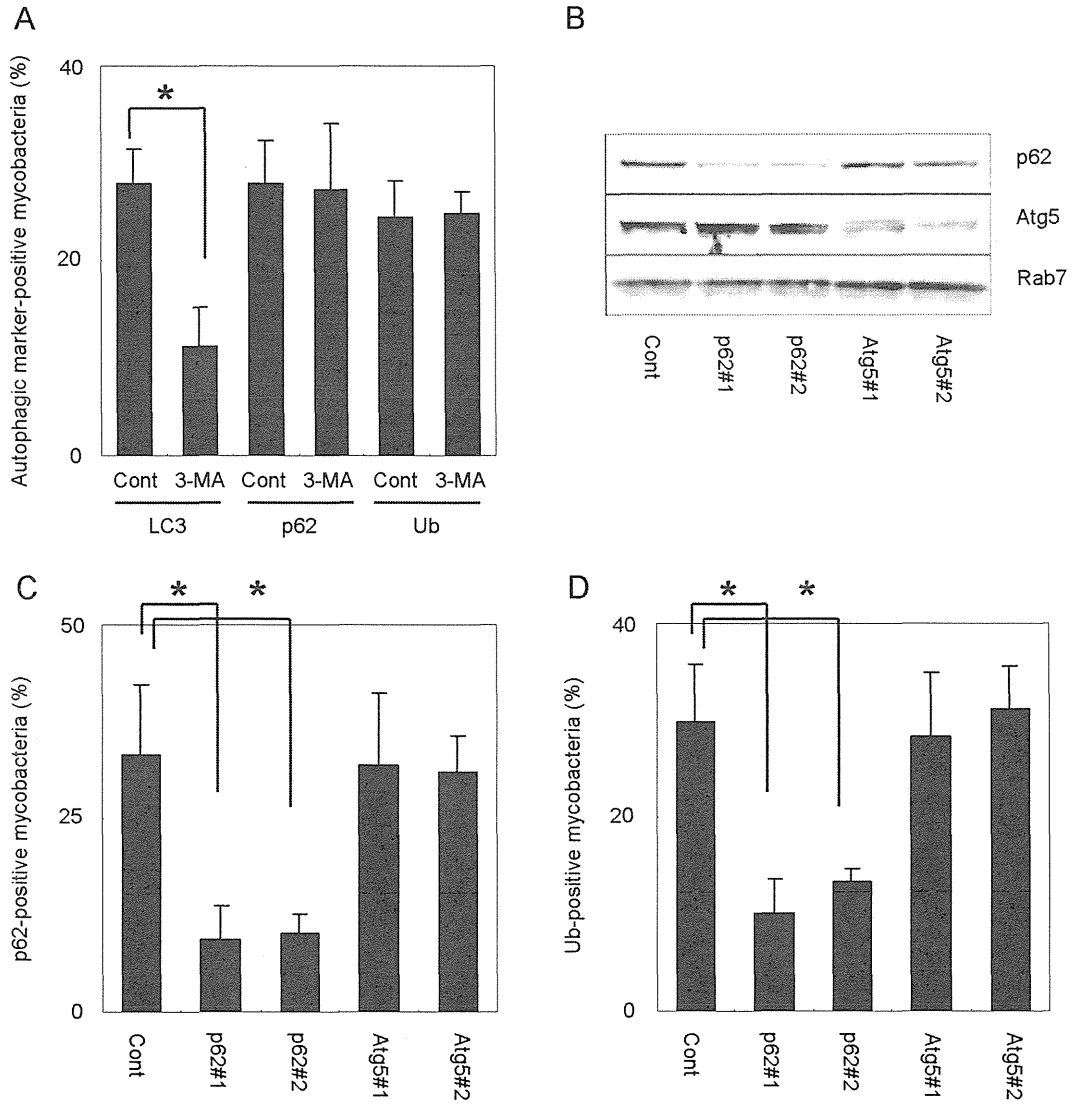

Figure 6. p62-dependent ubiquitination of mycobacteria in DC. (A) The proportion of LC3, p62 or ubiquitin recruitment to mycobacteria in DC treated with 3-MA. DC2.4 were infected with DsRed-expressing *M. tuberculosis* for 24 h with or without 3-MA and immunostained with anti-LC3, anti-p62 or anti-ubiquitin antibodies. Data represent the mean and SD of three or four independent experiments. *$p < 0.05$ (unpaired Student's *t*-test). (B) Immunoblot analysis on the silencing effects of p62 and Atg5. DC2.4 cells were transfected with siRNA for p62 or Atg5 for 48 h and subjected to immunoblot analysis using indicated antibodies. (C, D) The proportion of p62 or ubiquitin recruitment to mycobacteria in DC. DC2.4 cells transfected with siRNA for p62 or Atg5 were infected with DsRed-expressing *M. tuberculosis* for 12 h and immunostained with anti-p62 (C) or anti-ubiquitin (D) antibody. Data represent the mean and SD of three independent experiments. *$p < 0.05$ (unpaired Student's *t*-test).

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M. tuberculosis can also damage the phagosome membrane and translocate from phagosomes to the cytosol in a mechanism that is dependent upon the ESAT-6 and ESX-1 secretion system [47,48]. In macrophages, the permeabilization of the phagosomal membrane signals the ubiquitination of *M. tuberculosis* [36,49,50]. Our results suggest that the initial step for ubiquitination of mycobacteria in DC is also triggered by the permeabilization of phagosomal membrane by ESAT-6 because BCG bacilli were not ubiquitinated in DC (data not shown). Recently, an E3 ligase was identified that interacts with NDP52 and is responsible for ubiquitination of *Salmonella* [51]. Manzanillo et al. demonstrated that a ubiquitin ligase, Parkin mediates the ubiquitination of intracellular bacteria in macrophages [50]. Results from the current study suggest the possibility that p62 mediates the recruitment of an E3 ligase for ubiquitination of *M. tuberculosis* bacilli or bacilli-containing phagosomes in DC.

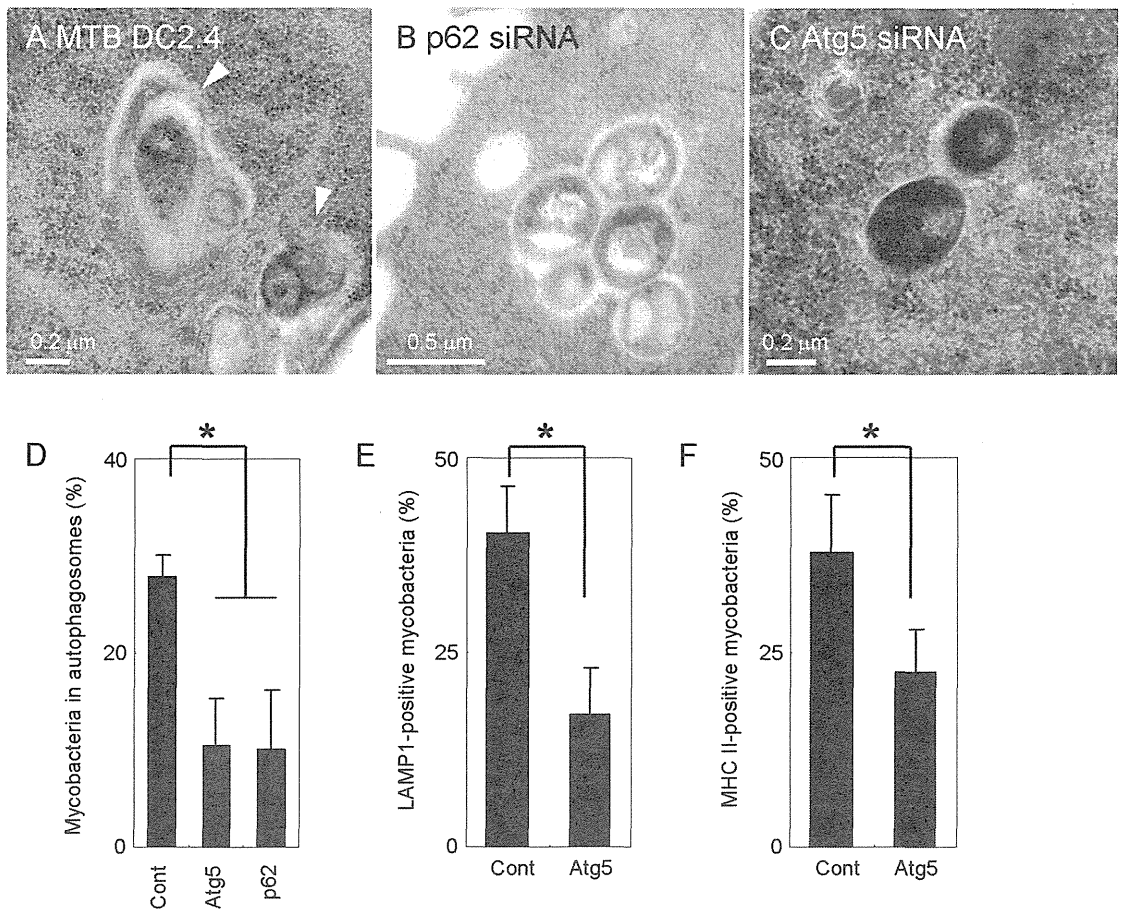

Figure 7. Atg5-dependent localization of LAMP1 and MHC class II to mycobacterial autophagosomes. (A-C) Thin-section electron micrograph of *M. tuberculosis* bacilli in p62- or Atg5-knockdown DC. DC2.4 cells transfected with siRNA for control (A), p62 (B) or Atg5 (C) were infected with *M. tuberculosis* for 24 h and observed by thin-section electron microscopy. Autophagosomes are indicated by arrowheads. (D) Proportion of mycobacteria in multi-membrane structures in DC2.4 transfected with siRNA for p62 or Atg5. (E, F) The proportion of LAMP1 (E) or MHC class II (F) localization to ubiquitin-positive mycobacteria is shown. JAWSII cells transfected with control or Atg5 siRNA for 24 h were infected with Alexa Fluor 405-labeled *M. tuberculosis* and immunostained with anti-LAMP1 and anti-ubiquitin antibodies or anti-MHC class II and anti-ubiquitin antibodies. Data represent the mean and SD of three independent experiments. $*p < 0.05$ (unpaired Student's *t*-test).

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Atg5 is involved in the elongation of isolation membrane in autophagosome formation [52]. We did not observe autophagosome membrane structures around infecting mycobacteria in DC by thin-section electron microscopy, however p62 and ubiquitin were recruited to mycobacteria in Atg5-knockdown DC (Figures 6 and 7). These results suggest that p62 and ubiquitin are recruited to infecting mycobacteria followed by the formation of autophagosome membrane depending on the function of Atg5. This hypothesis is consistent with the results that lysosomal markers do not localize to ubiqutinated mycobacteria in Atg5-knockdown DC (Figure 7). Atg16L is another autophagic-related protein involved in elongation of autophagic isolation membrane by forming an Atg5-Atg12-Atg16L complex [53]. We found that ubiquitin is recruited to *M. tuberculosis* but that LAMP1 does not localize to ubiquitinated mycobacteria in Atg16L-knockdown DC (Figure S5). These results suggest that ubiquitination of infected mycobacteria in DC is followed by formation of the autophagosome membrane and the autolysosome.

We found that autophagosomal markers did not localize to infected mycobacteria in macrophages, but did so in DC (Figure 1). These results imply that phagosomal membranes of DC are more susceptible to ESAT-6 and/or other secreted proteins from *M. tuberculosis* than those of macrophages. We have previously demonstrated that depletion of Coronin-1a leads the autophagosome formation to infecting *M. tuberculosis* in macrophages [26]. Coronin-1a associates with F-actin and localizes to mycobacterial phagosomes [54,55], suggesting that Coronin-1a supports the phagosomal membranes in

macrophages but not in DC. However, proteomic analysis revealed that Coronin-1a localizes to mycobacterial phagosomes in both macrophages and DC [56], suggesting that other proteins localizing to mycobacterial phagosomes in macrophages but not in DC would contribute the autophagosome formation to infecting mycobacteria.

In conclusion, *M. tuberculosis* infection in DC induced autophagosome formation followed by the fusion with lysosomes and MHC II recruitment. p62 and Atg5 function in the initiation and progression of autophagosome formation to *M. tuberculosis*, respectively. Thus, p62 mediates the ubiquitination of *M. tuberculosis* and Atg5 is involved in the fusion of lysosomes with mycobacterial autophagosomes. These results imply that autophagosome formation in *M. tuberculosis*-infected DC contributes to activate $CD4^+$ T lymphocytes via MHC II antigen presentation.

Supporting Information

Figure S1. Verification of co-localization between mycobacteria and autophagic proteins. (i) Split the image into channels. (ii) Make the binary image for each channel. (iii) Merge the binary images to verify the co-localization.
(TIF)

Figure S2. Quantification of band intensity for LC3-II. The quantification of band intensity for LC3-II in Figure 2E was shown. The ratio of band intensity for LC3-II/tubulin at each condition to that of control DC2.4 cells is shown. Data represent the mean and SD of three independent experiments. $^*p < 0.05$ (paired Student's *t*-test).
(TIF)

Figure S3. The proportion of LC3-positive (A), p62-positive (B) or ubiquitin-positive (C) *M. tuberculosis* in DC2.4 cells. Data represent the mean and SD of three independent experiments.
(TIF)

Figure S4. Ubiqutination of mycobacteria in JAWSII cells. (A) Immunoblot analysis of JAWSII cells transfected with siRNA for autophagy-related genes. JAWSII cells transfected with siRNA for p62 or Atg5 genes for 48 h were subjected to immunoblot analysis using the indicated antibodies. (B) The proportion of ubiquitinated mycobacteria in JAWSII cells. JAWSII cells transfected with siRNA for p62 or Atg5 were infected with DsRed-expressing *M. tuberculosis* for 24 h and immunostained with anti-ubiquitin antibody. Data represent the mean and SD of three independent experiments. $^*p < 0.05$ (unpaired Student's *t*-test).
(TIF)

Figure S5. Ubiquitination of mycobacteria in Atg16L-knockdown DC. (A) Immunoblot analysis of DC2.4 cells transfected with siRNA for Atg16L. DC2.4 cells were transfected with Atg16L siRNA for 48 h and subjected to immunoblot analysis using anti-Atg16L antibody. (B) The proportion of ubiquitinated mycobacteria in Atg16L-knockdown DC. DC2.4 cells transfected with siRNA for Atg16 were infected with DsRed-expressing *M. tuberculosis* for 24 h and immunostained with anti-ubiquitin antibody. (C) The proportion of LAMP1 localization to ubiqutinated mycobacteria in Atg16L-knockdown DC. DC2.4 cells transfected with control or Atg16 siRNA for 24 h were infected with Alexa Fluor 405-labeled *M. tuberculosis* and immunostained with anti-LAMP1 and anti-ubiquitin antibodies. Data represent the mean and SD of three independent experiments. $^*p < 0.05$ (unpaired Student's *t*-test).
(TIF)

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Author Contributions

Conceived and designed the experiments: SS KT YK. Performed the experiments: SS. Analyzed the data: SS KT. Contributed reagents/materials/analysis tools: TH YK. Wrote the manuscript: SS KT.

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