Romao et al., http://www.jcb.org/cgi/content/full/jcb.201308173/DC1

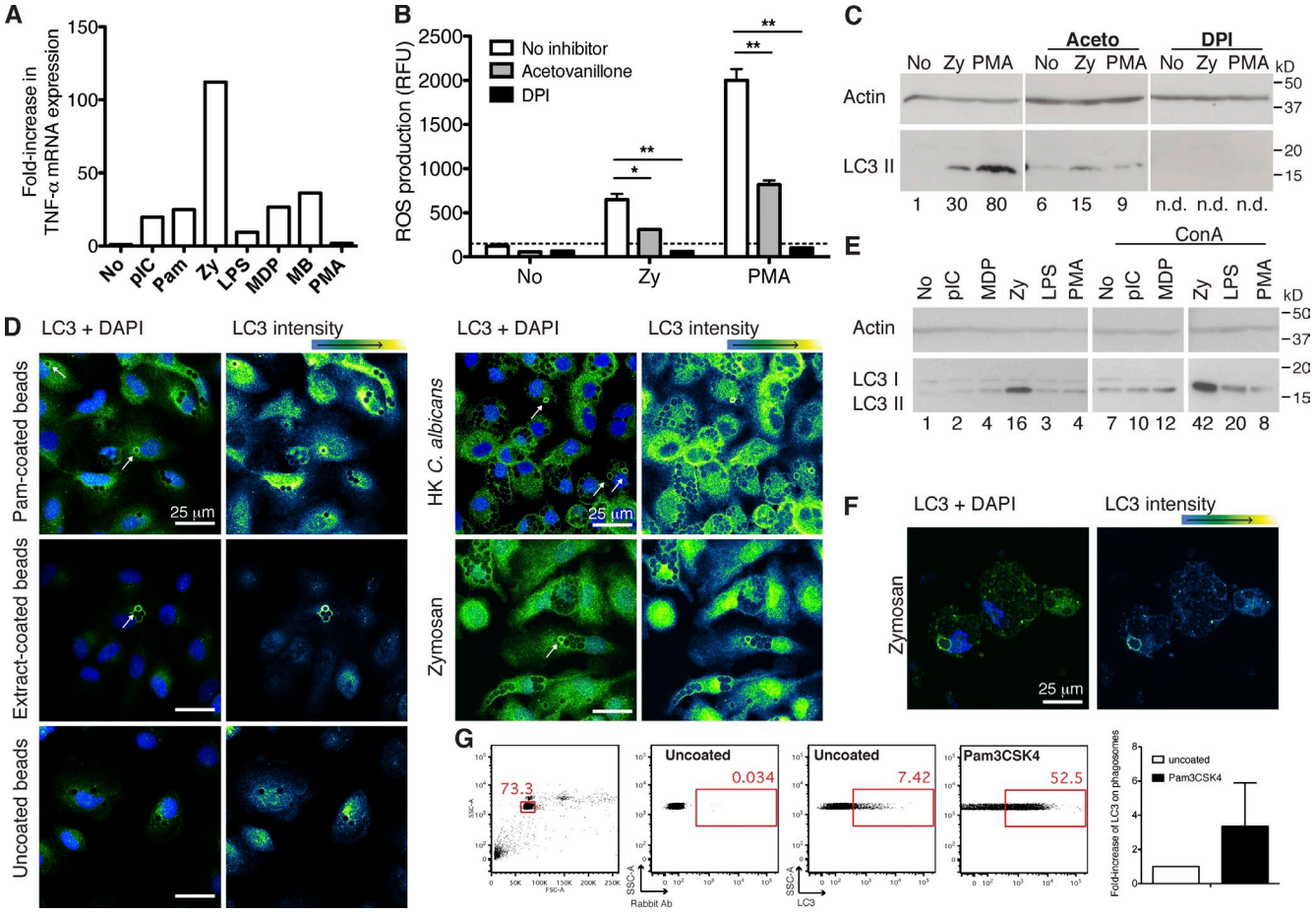


Figure S1. **Atg8/LC3 binding to the phagosomal membrane depends on PRR signaling.** (A) Fold increase of mRNA levels of TNF, as compared with GAPDH mRNA, upon treatment with the indicated stimuli for 2 h (No, medium alone; pIC, polyinosinic/polycytidylic acid; Pam, Pam3CSK4; Zy, zymosan; LPS, lipopolysaccharide; MDP, muramyl dipeptide; MB, murabutide; and PMA). Graph shows data pooled from three independent assays. (B) ROS production was determined after 3 h of zymosan stimulation or 30 min of PMA, by the increase in fluorescence of the cell-permeable probe Amplex UltraRed measured by a fluorometer. Stimuli were tested in the presence or absence of the NADPH oxidase inhibitors acetovanillone (Aceto) and diphenylene iodonium (DPI). Data are depicted as the means ± SD; paired *t* test; *, $P < 0.5$; **, $P < 0.01$. RFU, relative fluorescent units. (C) LC3-II protein levels decrease in the presence of acetovanillone and diphenylene iodonium. Numbers below the blot represent the fold increase of LC3-II band compared with the nonstimulated/noninhibited control cells after normalization to the corresponding actin levels. n.d., not detectable. (D) Macrophages were stained for Atg8/LC3 and nuclear content (DAPI) after 6 h of treatment with uncoated beads, beads coated with Pam3CSK4, or with *C. albicans* protein extract, heat-killed (HK) *C. albicans*, or zymosan. Right images represent the intensity of Atg8/LC3 signal of the corresponding confocal pictures on the left. Arrows point to Atg8/LC3-positive phagosomes. (E) Immature human monocyte-derived DCs were treated with several stimuli (medium alone, polyinosinic/polycytidylic acid, muramyl dipeptide, zymosan, lipopolysaccharide, and PMA) for 8 h in the presence or absence of concanamycin A (ConA), and LC3-II levels were assessed by WB. Numbers below the blot represent the fold increase of LC3-II compared with the nonstimulated control cells after normalization to the corresponding actin levels. (F) Immature DCs were stained for Atg8/LC3 and DAPI 6 h after engulfment of zymosan. Right image represents the LC3 signal intensity in the same confocal picture. (G) Phagosomes containing uncoated or Pam3CSK4-coated beads were isolated from macrophages and analyzed by flow cytometry. An example of a forward scatter/side scatter (SSC) plot obtained when acquiring phagosomes containing beads (population highlighted with red squares) is shown. The presence of Atg8/LC3 on these phagosomes was detected using anti-Atg8/LC3 antibody and anti–rabbit Alexa Fluor 488 as a secondary antibody. Control plot without primary antibody staining is also shown. Bar graph displays the increase in Atg8/LC3 fluorescence on phagosomes containing the TLR2 agonist. Each bar represents the means ± SD of data pooled from four independent experiments.

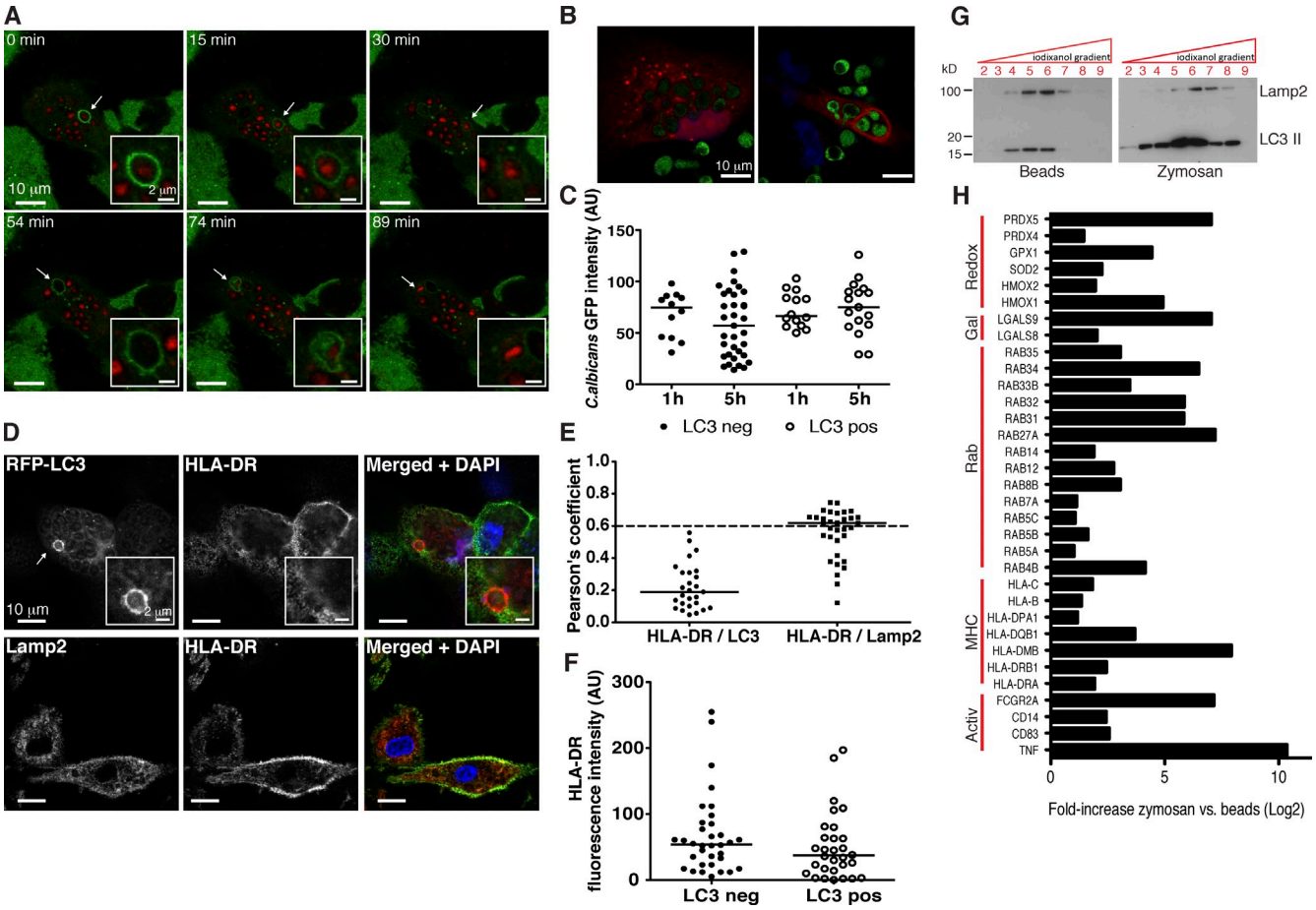


Figure S2. **Characterization of Atg8/LC3-positive phagosomes.** (A) GFP-Atg8/LC3–expressing macrophages were preloaded with LysoTracker (red), and zymosan-containing phagosomes were followed over time. Acidification of phagosomes was assessed by the intensity of the lysosomal dye. Arrows point to Atg8/LC3 uncoupling from phagosomes and subsequent uptake of lysosomal marker (see Video 4). Insets show a zoomed-in view of the phagosomes of interest. (B) RFP-Atg8/LC3–expressing macrophages were incubated with UV-killed GFP–*C. albicans* yeast cells. After 1 and 5 h, cells were fixed and stained with DAPI. Example confocal pictures are shown displaying *C. albicans*–engulfing phagosomes with (right) and without (left) Atg8/LC3 recruitment. (C) Quantification of GFP intensity inside Atg8/LC3-negative (LC3 neg) and -positive (LC3 pos) phagosomes after 1 and 5 h of engulfment of UV-killed GFP–*C. albicans*. Median values are represented as lines. Minimum of 15 phagosomes was analyzed. (D, top) Macrophages transduced with RFP-Atg8/LC3 lentiviral construct were stained for MHC-II and DAPI. Arrow points to an Atg8/LC3-positive phagosome. Insets show zoomed-in views of the phagosomes of interest. (bottom) Macrophages were costained for Lamp2 (Alexa Fluor 647) and HLA-DR (Alexa Fluor 488). One representative picture out of at least three independent experiments is shown. (E) To determine colocalization, Pearson's correlation coefficient values were calculated for the region surrounding Atg8/LC3-positive phagosomes ($n \geq 25$) or the cytosolic region, in the case of HLA-DR/Lamp2 colocalization experiments ($n \geq 30$). Median values are represented as lines. A Pearson score >0.6 is indicated in each graph as a dashed line. (F) HLA-DR signal intensity was compared in LC3-positive versus -negative phagosomes ($n \geq 25$). Median values are represented as lines. (G) Fractions resulting from the iodixanol gradient were blotted for LAMP2 and Atg8/LC3 to identify the phagosome-enriched ones. Fractions 5 and 6 were pooled for MS analysis. (H) Graphical representation of 33 out of 266 protein hits found enriched in zymosan compared with uncoated beads containing phagosomes. Values were log2 transformed and represent at least two-fold enrichment in at least two out of three independent assays performed. Proteins are grouped into activation markers (Activ), MHC molecules (MHC), Rab proteins (Rab), galectins (Gal), and regulators in cell redox control (redox). AU, arbitrary unit.

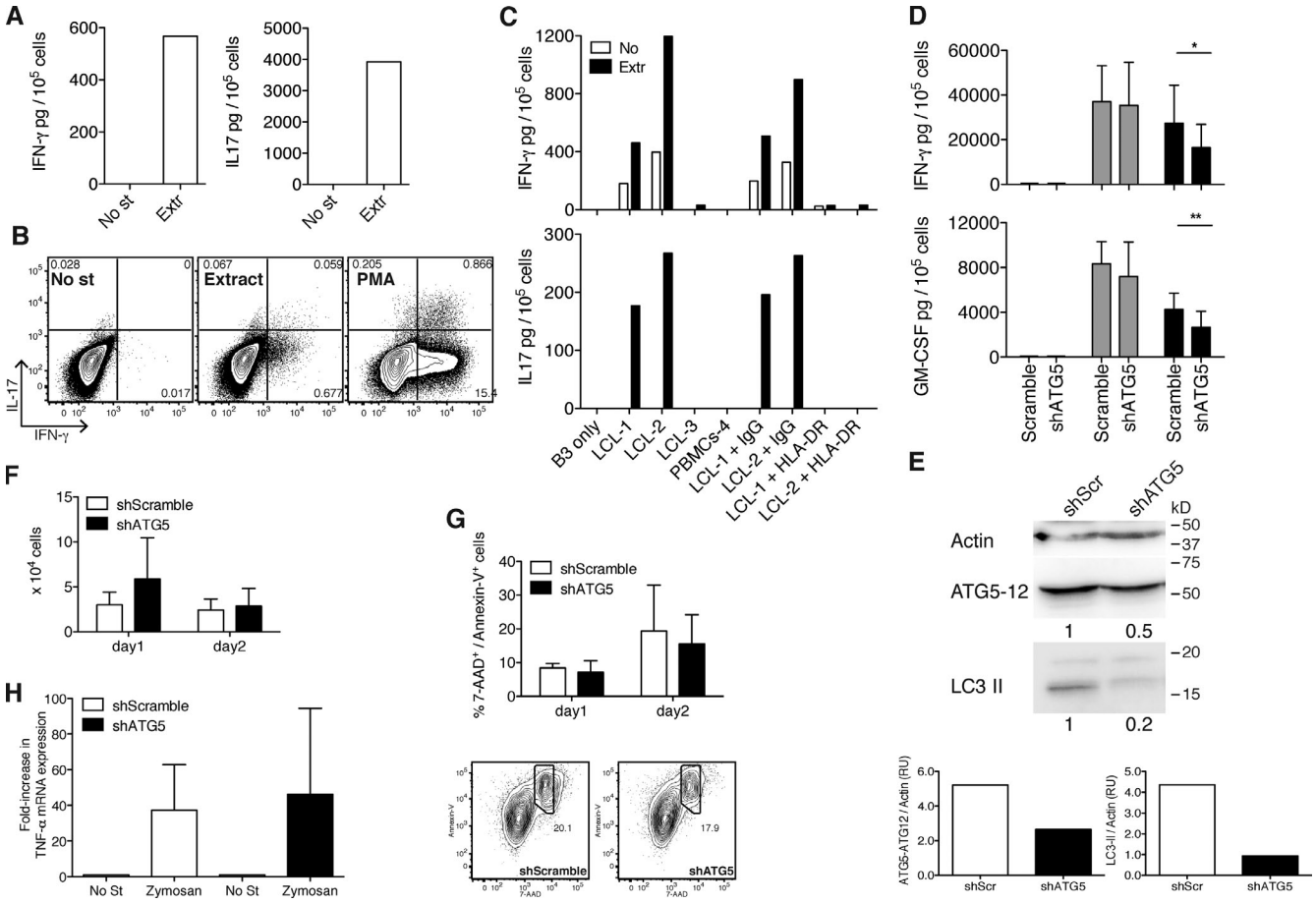
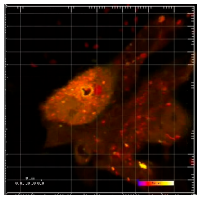
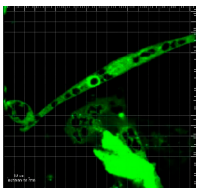


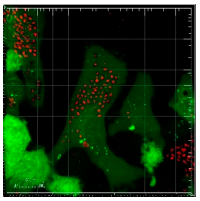
Figure S3. **Generation and characterization of *C. albicans*–specific T cells, antigen presentation assay after silencing of autophagy-related genes, and survival and activation patterns of macrophages with *atg5* silencing.** (A) IFN-γ and IL-17A ELISAs on supernatants of the *C. albicans*–specific T cell clone B3, co-cultured with cognate macrophages for 18 h, in the presence (extract [Extr]) or absence (no stimulus [No st]) of *C. albicans* protein extract. One representative experiment out of at least three performed is shown. (B) IFN-γ and IL-17A intracellular staining on the *C. albicans*–specific T cell clone B3 in the presence (extract) or absence (no stimulus) of *C. albicans* protein extract or PMA. Numbers inside flow cytometer plots represent the percentage of cells within the gates. One representative experiment out of at least three performed is shown. (C) IFN-γ and IL-17A ELISAs on supernatants of the *C. albicans*–specific T cell clone B3, treated with HLA-DR–blocking antibody or isotype control (IgG), and co-cultured with different lymphoblastoid cell lines or PBMCs for 18 h, in the presence (extract) or absence (No) of *C. albicans* protein extract. LCL-1 (HLA-DRB1*0401), LCL-2 (HLA-DRB1*0401), LCL-3 (HLA-DRB1*03), and PBMC-4 (HLA-DRB1*0445) are shown. One representative experiment out of at least two performed is shown. (D) IFN-γ and GM-CSF ELISAs on supernatants of the *C. albicans*–specific T cell clone B3, co-cultured with cognate DCs transduced with *atg5* shRNA or control shRNA, and pulsed or not pulsed with *C. albicans* extract (gray bars) or *C. albicans*–coated beads (black bars). After 4 h of pulsing, cells were washed and incubated in new medium for an additional 38 h before the T cells were added. *, $P < 0.5$; **, $P < 0.01$. (E) *atg5* silencing levels on DCs were controlled by WB for LC3-II on day 5 after transduction and quantified by densitometry. Numbers below the blot represent the fold increase of LC3-II compared with the control cells after normalization to the corresponding actin levels. One representative WB out of at least three performed is shown. RU, relative units; shScr, scrambled shRNA. (F and G) Survival and viability of macrophages transduced with *atg5*-specific shRNA or scramble shRNA was analyzed on day 3 after transduction by cell counting (F) or staining for Annexin V and 7-AAD (G). Example of flow cytometric plots, showing necrotic cells double stained for 7-AAD and Annexin 5 shown beside the bar graph. One representative assay is shown out of at least two performed. (H) Fold increase of mRNA levels of TNF, as compared with GAPDH mRNA, in macrophages transduced with Atg5 shRNA or control shRNA lentiviruses treated or not treated with zymosan for 2 h. Error bars show SDs.



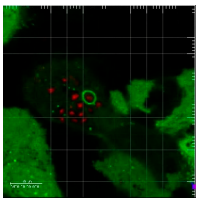
Video 1. **Green and red fluorescence of the LC3 tandem reporter construct associate and dissociate with phagosomes.** Macrophages transduced with tandem-fluorescent (green/red) Atg8/LC3 lentiviral construct stimulated with zymosan. Images were analyzed by time-lapse confocal microscopy using a laser-scanning confocal microscope (SP5; Leica). Frames were taken every 3 min for 1 h.



Video 2. **LC3-associated phagosomes persist for prolonged time periods.** GFP-Atg8/LC3 (green)–expressing macrophages showing Atg8/LC3 translocation to zymosan-containing phagosomes. Images were analyzed by time-lapse confocal microscopy using a laser-scanning confocal microscope (SP5; Leica). Frames were taken every 8 min for 4 h.



Video 3. **LC3-associated phagosomes are less acidified than LC3-negative phagosomes.** GFP-Atg8/LC3 (green)–expressing macrophages were preloaded with LysoTracker (red), and zymosan-containing phagosomes were followed over time. In general, Atg8/LC3-positive phagosomes contain less lysosomal dye than negative ones. Images were analyzed by time-lapse confocal microscopy using a laser-scanning confocal microscope (SP5; Leica). Frames were taken every 5 min for 2 h. Representative frames of this video are shown in Fig. 3.



Video 4. **LC3 dissociates from phagosomes before fusion with lysosomes.** GFP-Atg8/LC3 (green)–expressing macrophages were preloaded with LysoTracker (red), and zymosan-containing phagosomes were followed over time. Intensity of the lysosomal dye increases in Atg8/LC3-positive phagosomes after uncoupling of the macroautophagy protein. Images were analyzed by time-lapse confocal microscopy using a laser-scanning confocal microscope (SP5; Leica). Frames were taken every 5 min for 2 h. Representative frames of this video are shown in Fig. S3.