Supplementary Methods

Constructs.

GFP-LC3 construct was kindly provided by Annete Khaled (USF). PX-mCherry was obtained by cloning the PX domain of mouse p40(PHOX) to the N-terminus of mCherry that was previously cloned into pBabe-puro. The p40PX domain was cloned from RAW cDNA by standard RT-PCR. The primers used contained suitable restriction enzyme sites and the sequences 5'-ATGGCCCTGGCCCAGCAGC-3' (sense) and 5'-TCTGCGGAGTGCCTGGGG-3' (anti-sense) to amplify the desired domain. mCherry, and pBabe-puro were kindly provided by Drs. RY Tsien (UCSD, San Diego, CA), and C. Benedict (LIAI, La Jolla, CA). LZRS eGFP-LC3 ires Zeo was generated by cloning of GFP and rat LC3 into EcoRI/Not I sites. The construct was sequence verified and used generating 293T cells expressing GFP-LC3. Amphotropic retroviral particles encoding GFP-LC3 were produced by transfecting 293T cells with packaging plasmids and the LZRS-vector in which GFP-LC3 was subcloned. 293T cells were transduced by standard protocols using these retroviral particles. During the course of two weeks the cells were sorted twice for GFP to create a stable cell line. mRFP1 was expressed in E.coli using pRSETB (Invitrogen, Carlsbad, CA).

Mice and cell lines.

All mice were housed pathogen-free. $TLR2^{-/-}$ mice were purchased from Jackson Laboratories. $MyD88^{-/-}$ mice were kindly provided by Shizuo Akira (Osaka University, Japan). To generate $ATG7^{+/-}$ mice, male $ATG7^{flox/flox}$ mice[1] were bred to female *EIIaCre* mice[2].

$ATG7^{-/-}$ and $ATG7^{+/+}$ littermates were used to generate E15.5 fetal liver stem cells that were differentiated into macrophages by culturing in 20ng/ml mGM-CSF (Preprotech, Rocky Hill, NJ) for 10 days. Unattached cells were removed at day 3 and day 6. Mouse bone marrow-derived macrophages were generated from bone marrow progenitors. Freshly prepared bone marrow cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 10 mM HEPES buffer, 50 μg/ml penicillin, and non-essential amino acids in the presence of GM-CSF (20 ng/ml). Unattached cells were removed at day 3, 6, and 9.

RAW cells were grown in complete Dulbecco's Modified Eagle Medium (DMEM, GIBCO BRL). For starvation, the cells were washed twice and then incubated in Earle's buffered saline solution (EBSS, GIBCO BRL). RAW cells stably expressing GFP-LC3 (RAW-GFP-LC3) or GFP-LC3 plus PX-mCherry (RAW-LC3/PX) were generated by electroporation (Amaxa Biosystems).

Time-Lapse Imaging and Microscopy.

Confocal microscopy was performed using the following systems:

-Spinning Disk Confocal Microscopy (SDC):

SDC on live cells was performed with a Marianas SDC imaging system (Intelligent Imaging Innovations/3i, Denver, CO) consisting of a CSU22 confocal head (Yokogowa Electric Corporation, Japan), DPSS lasers (CrystaLaser, Reno, NV) with wavelengths of 445nm, 473nm, 523nm, 561nm, and 658 nm, and a Carl Zeiss 200M motorized inverted microscope (Carl Zeiss MicroImaging, Thornwood, New York), equipped with spherical aberration correction optics (3i). Temperature was maintained at ~37°C and 5% CO_2 using an environmental control chamber (Solent Scientific, UK). Images were acquired

with a Zeiss Plan-Neofluar 40x 1.3 NA DIC objective on a CascadeII 512 EMCCD (Photometrics, Tucson, AZ), using SlideBook 4.2 software (3i) (Figure 2b, 2c, 3c, 3g).

-Laser Scanning Confocal Microscopy (LSCM):

LSCM on live cells was performed with a Nikon TE2000-E inverted microscope equipped with a C1Si confocal system, (Nikon, Melville, NY), an argon ion laser at 488nm and DPSS lasers at 404nm and 561 nm (Melles Griot, Carlsbad, CA). Temperature was maintained at ~37°C and 5% CO_2 using an environmental control chamber (InVivo Scientific, St Louis, MO). Images were taken at the intervals indicated in the figure legends using an oil-immersion Nikon Plan Fluor 40X 1.3 NA objective with phase contrast optics (Figure 1c, 1e-g, 3a, 3e, 4a-c, S1).

LSCM on fixed tissues was performed with a Zeiss Axioplan 2 upright microscope equipped with an LSM 510 NLO confocal system (Carl Zeiss MicroImaging, Thornwood, New York), a Mira 900 Ti Sapphire laser (Coherent Inc., Santa Clara, CA), an argon ion laser at 488nm, and a HeNe laser at 543nm. Images were acquired with a Zeiss Plan-Neofluar 40x 1.3 NA objective. Cells were stimulated as indicated in the figure legends and subsequently fixed with 4% paraformaldehyde in PBS, washed twice with PBS and mounted using DAPI containing Vectashield solution (Vector Laboratories, Burlingame, CA) (Figure 1a, 1d, 2a).

For time-lapse microscopic imaging of phagocytosis, macrophages were plated on collagen coated glass bottom dishes (MatTek, Ashland, MA). GFP-LC3 translocation to the phagosome was quantified by acquiring a time lapse movie over at least 2 hours and counting the number of GFP-LC3 positive phagosomes out of the total number of internalized yeasts for that period. For each condition, three independent experiments

were performed and the mean with range error bars was represented. Phagocytosis uses the activity of PI3-Kinase for the initial internalization[3] and the rate of internalization was particularly decreased on cells treated with wortmannin. Therefore confocal images of living macrophages were obtained every 3 minutes allowing them to internalize Alexa Fluor® 594 labeled zymosan (Invitrogen, Carlsbad, CA). After that, but before translocation of GFP-LC3 to the phagosome, wortmannin (1μM) was added and the capture of time-lapse images from the same field was resumed. Beads used for internalization were prepared by conjugating Alexa Fluor 555 labeled BSA (Invitrogen, Carlsbad, CA) (Red-BSA) or PAM3CSK4 (Invivogen, San Diego, CA) to 6 micron latex microspheres as described[4]. Sonicated Biotinylated-LPS (0.5mg/ml) (Invivogen, San Diego, CA) was conjugated to SPHERO™ Streptavidin-Polystyrene beads (Lybertiville, IL). Zymosan particles were added at a ratio 8:1 (particle:cell), while beads were added at a 10:1 ratio (bead:cell). Zymosan was fed for 2 hours to 293T that express GFP-LC3 and that were previously transfected transiently with hTLR2 using lipofectamine 2000 (Invitrogen, Carlsbad, CA). Cells were then fixed in paraformaldehyde. At the end of each experiment, we confirmed that particles were completely internalized by differential focusing.

Electron Microscopy.

Macrophages grown on coverslips were prefixed in glutaraldehyde (2.5%) dissolved in 0.1 M Tousimis phosphate sodium buffer, pH 7.35 (Tousimis, Rockville, MD), for 1 h at room temperature. The coverslips were then washed twice with 0.1 M sodium cacodylate buffer, treated with 1% tannic acid in 0.1 M sodium cacodylate buffer for 60 min, and

then submerged in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer for 60 min. After dehydration in a graded ethanol series, the cells were cleared with propylene oxide for 10 min and dried overnight. The next day the samples were embedded and pictures were taken with a JEOL 1200 EX-II transmission electron microscope with a digitalized Gatan camera (ES500W).

siRNA gene silencing of Atg5.

Knock down of mouse ATG5 was achieved by using complementary sense and antisense oligonucleotides corresponding to nucleotides 780-798, of mouse Atg5 (sense, 5'-CCCAGAUAACUUUCUUCAUTT-3', and antisense, 5'-AUGAAGAAAGUUAUCUGGGTT-3'). As a control, a nontargeting sequence comprising 19 nucleotides was used (sense, 5'- GCUGAGGGUAGGAAUCGUATT-3', and antisense 5'- CGGAUAUAGUUUCACCAATUU-3'). Oligonucleotides were annealed using standard recombinant DNA methodology. RAW cells were transfected with 1 mg of each duplex by electroporation according to the manufacturer's recommendations (Amaxa Biosystems).

Real-time RT-PCR.

Total RNA was isolated from cultured RAW cells 24 h. posttransfection using TRIzol (Gibco BRL, Rockville, MD) according to the manufacturer's instructions. First strand synthesis was performed using M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA). Real time PCR™ was performed using SYBR GREEN PCR master mix (Applied Biosystems (Forster City, CA), in a Applied Biosystems 7900HT thermocycler using SyBr Green detection protocol as outlined by the manufacturer using the following PCR

conditions: 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min. Atg5 mRNA expression was normalized against cyclophilin, L32, 18S and HPRT, allowing comparison of mRNA levels. The following primers were used: mouse ATG5 (forward primer: 5- AACTGAAAGAGAAGCAGAACCA-3; reverse primer: 5-TGTCTCATAACCTTCTGAAAGTGC-3), mouse L32 (forward primer: 5-GAAACTGGCGGAAACCCAX-3; reverse primer: 5-GGATCTGGCCCTTGAACCTT-3).

Yeast viability assay.

Saccharomyces cerevisiae (EGY48) was cultured in YPD medium (BD Biosciences, Palo Alto, CA), washed twice in PBS and added to macrophages (10 yeast cells per macrophage cell). After 1 hr of internalization the plate was washed with PBS to remove free yeast cells. Macrophages were then trypsinized and replated in 6 well plates. After 0h, 4h, and 8h yeast cells were extracted from macrophages by applying 1 ml of distilled water and the lysate was plated on YPD agar medium (BD Biosciences). Yeast colony forming units were counted and normalized to the number of colonies obtained from the 0h time point.

Cell lysis and immunoblotting.

Cells were lysed in RIPA buffer for 30 min on ice (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.5% DOC, 0.1% SDS, protease inhibitor tablet [Roche], 1 mM NaF, 1 mM Na_3VO_4, and 1 mM PMSF). After centrifugation (20,000 *g*, 10 min, 4°C), supernatants were analyzed by SDS-PAGE. LC3-antibody was kindly provided by John

Cleveland (The Scripps Research Institute, FL). Anti-Actin (C4) was from MP Biomedicals (Solon, OH)

References:

1. Komatsu, M. et al. Impairment of starvation-induced and constitutive autophagy in Atg7-deficient mice. J Cell Biol 169, 425-34 (2005).
2. Williams-Simons, L. & Westphal, H. EIIaCre -- utility of a general deleter strain. Transgenic Res 8, 53-4 (1999).
3. Gillooly, D. J., Simonsen, A. & Stenmark, H. Phosphoinositides and phagocytosis. J Cell Biol 155, 15-7 (2001).
4. Avrameas, S. & Ternynck, T. The cross-linking of proteins with glutaraldehyde and its use for the preparation of immunoadsorbents. Immunochemistry 6, 53-66 (1969).

Figure-S1

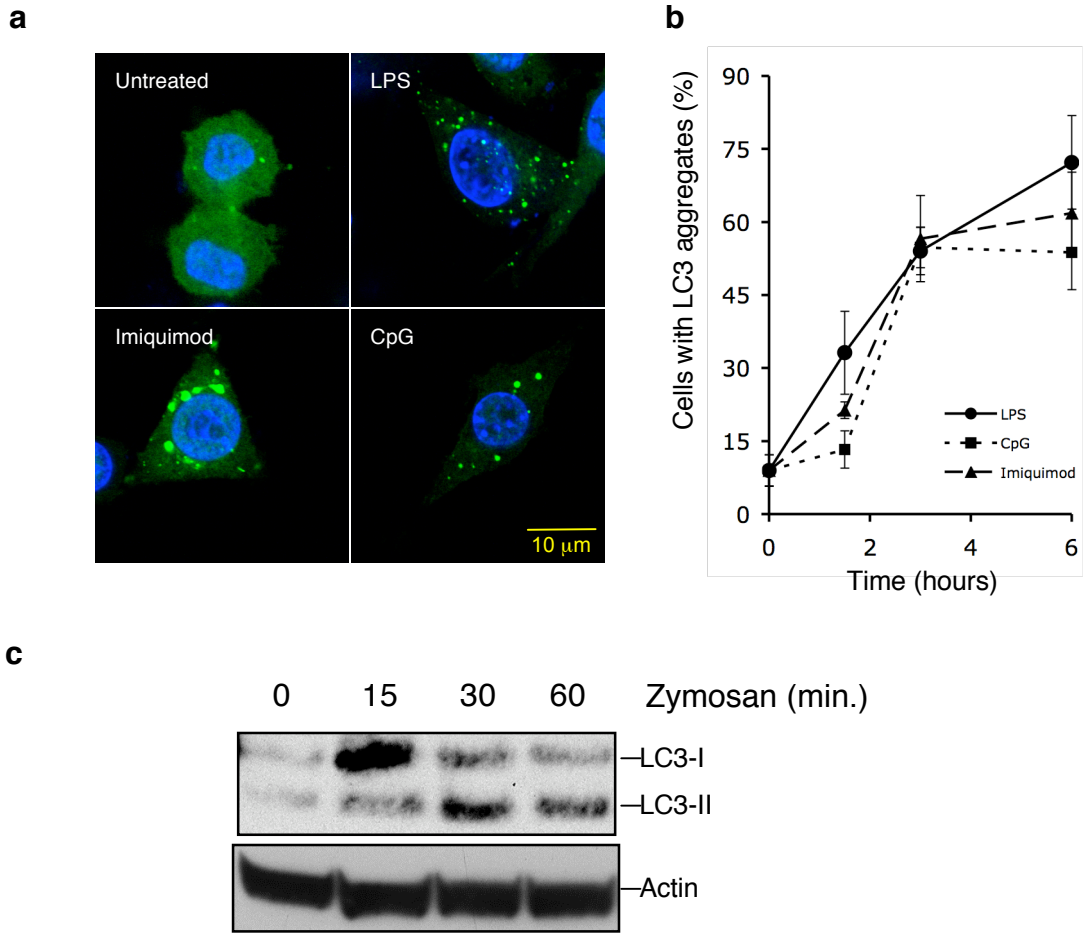

Figure S1. **TLR signaling induces components of the autophagic pathway.** (**a**) RAW cells expressing GFP-LC3 (RAW-LC3) were challenged with the TLR agonists LPS (TLR4), imiquimod (TLR7), and CpG (TLR9) for 3 hours. After fixation, nuclei were stained with DAPI (blue) and confocal images were taken. (**b**) TLR induced LC3-aggregates were quantified (means ± S.D.) by counting 4 different fields at 2h, 4h, and 6h after adding LPS (solid line), Imiquimod (dashed line), or CpG (dotted line). Cells showing a marked pattern of aggregation were counted as positive (n≥100 cells/group). (**c**) RAW cells were fed with Zymosan for 15, 30, and 60 minutes. Cell lysates were prepared and the levels of LC3-I, and the active conjugated form LC3-II were followed by western blot. As a loading control, the same membrane was re-stained for actin.

Figure-S2

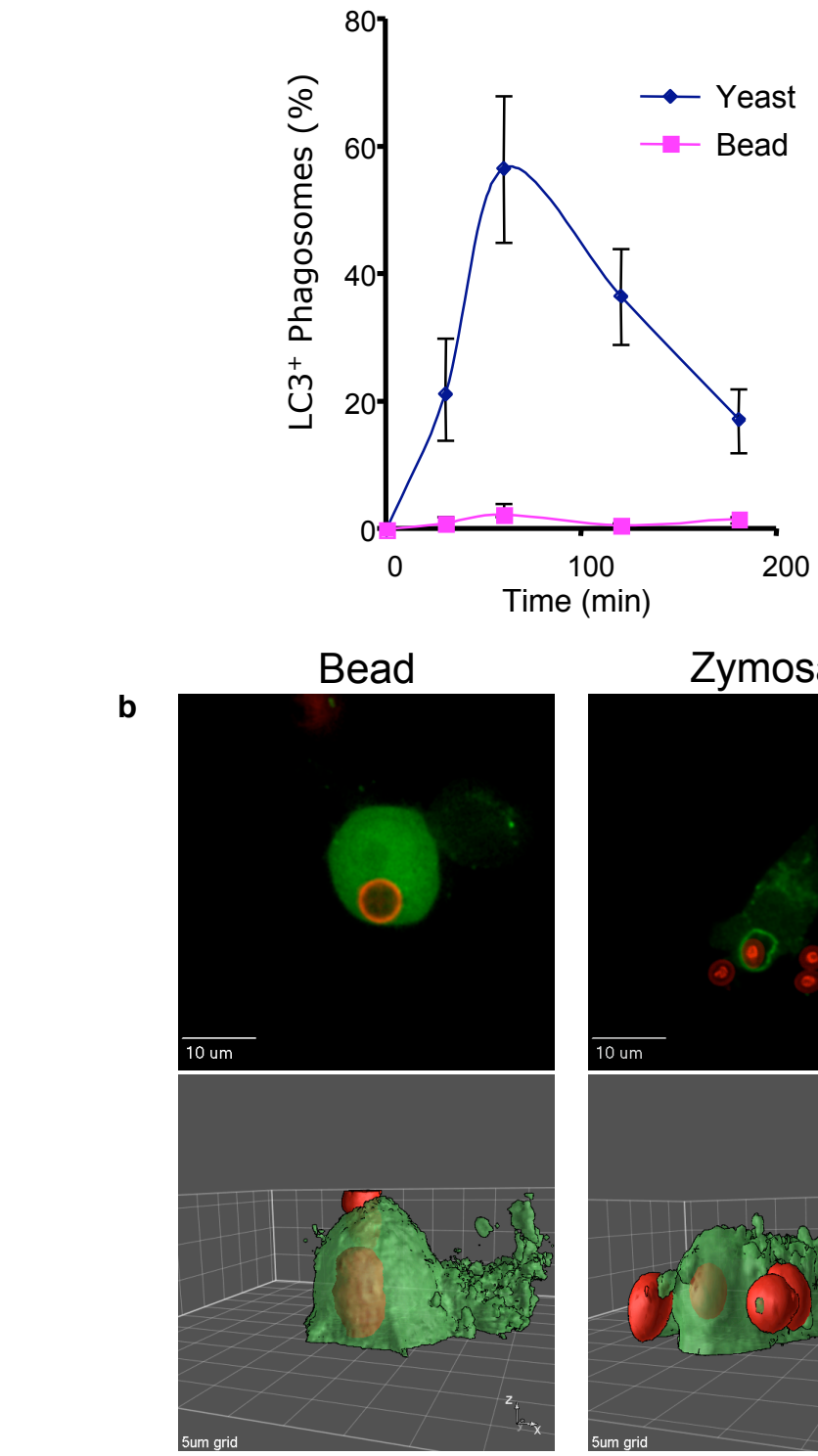

Figure S2. **Internalization of beads and zymosan particles.** RAW cells expressing GFP-LC3 were fed with either latex beads covered with red-BSA or zymosan. **(a)** Time course of the appearance of $LC3^+$ phagosomes. Means and ranges from three experiments are shown. **(b)** In the upper panels, representative confocal sections were chosen at the center of the internalized particle (after 1.5 hours). The lower panels show corresponding 3D isosurface renderings composed from 0.1 μm voxels, with 15 and 17 μm z-series, respectively.

Figure-S3

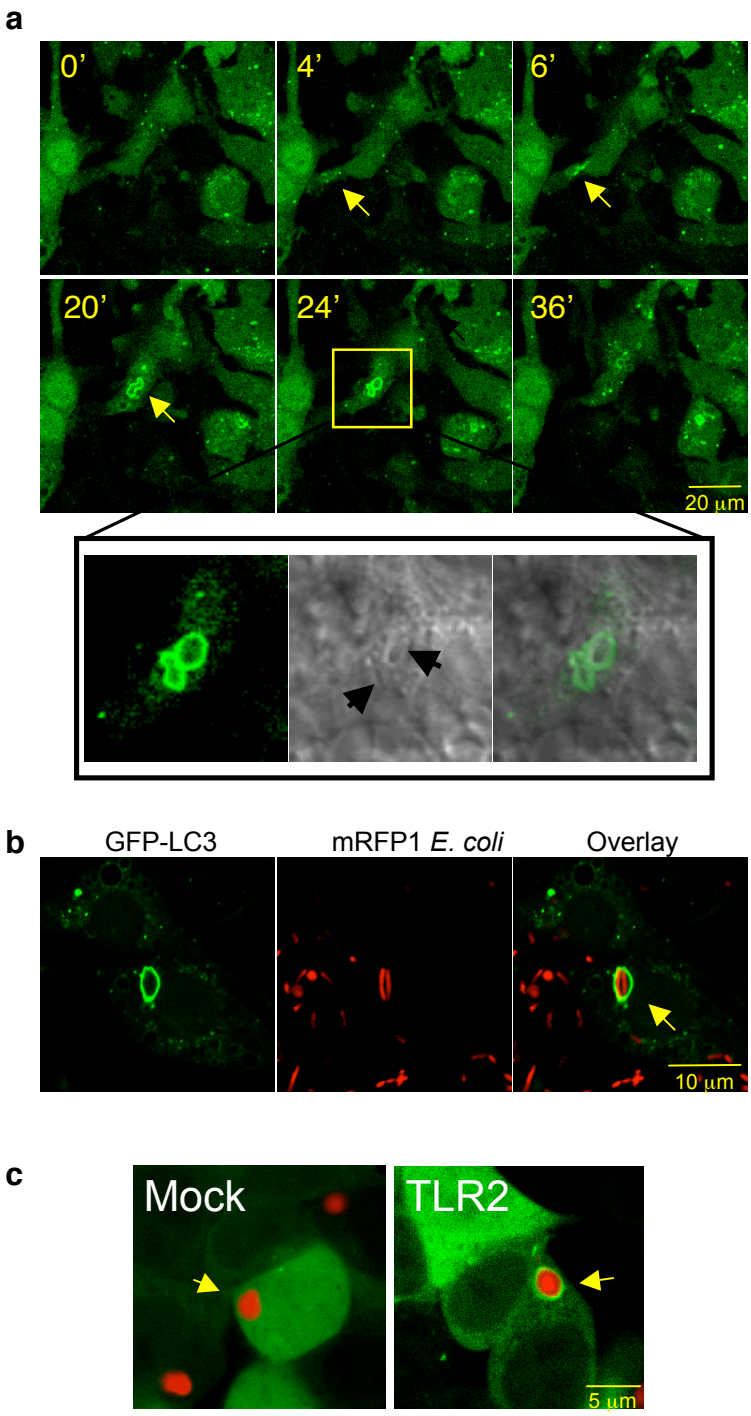

Figure S3. ***E. coli* engulfment involves translocation of LC3 to the phagosome.** (**a**) RAW cells expressing GFP-LC3 were fed with *E. coli*. Confocal images were taken every minute. Frames from the time-lapse movie (supplemental movie 1) are shown in the upper panel. The insert in the lower panel shows details of a bacterium inside a phagosome at 24 minutes. (**b**) RAW cells expressing GFP-LC3 were fed with *E. coli* expressing mRFP1 and 45 mins later confocal images were obtained depicting red bacteria inside a phagosome. (c) 293T cells stably expressing GFP-LC3 were transiently transfected with empty plasmid (Mock) or TLR2. After 36 hours cells were fed with zymosan (100:1 ratio zymosan:cell) and images were obtained 2 hours later.

Figure-S4

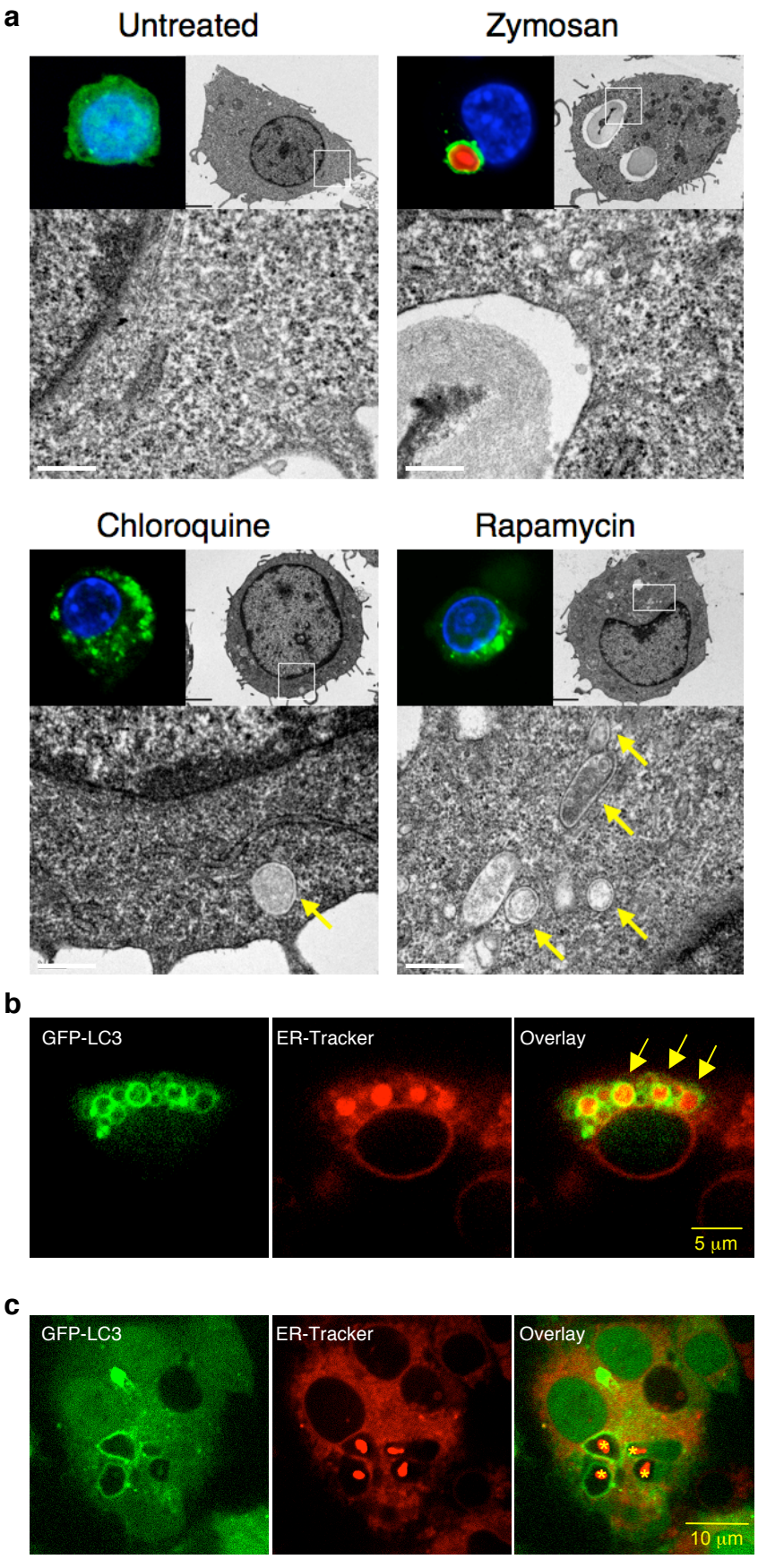
a
Untreated
Zymosan
Chloroquine
Rapamycin
b
GFP-LC3
ER-Tracker
Overlay
5 μm
c
GFP-LC3
ER-Tracker
Overlay
10 μm

Figure-S4

Figure S4. **TLR-induced LC3 localization to the phagosome appears to be distinct from autophagy.** (**a**) Accumulation of autophagic vacuoles was pharmacologically induced in RAW-LC3 cells with chloroquine (50μM; 3 hours) or rapamycin (6 hours). Alternatively, cells were exposed to zymosan (red) for 1 hour. The panels show confocal images of cells after fixing them in paraformaldehyde (nuclei were stained with DAPI (blue). Transmission electron microscopy pictures of similarly treated cells (see methods for details) are also shown. Chloroquine and rapamycin induced the accumulation of double-membrane vesicles (yellow arrows) that were absent in untreated cells. Although GFP-LC3 translocated to phagosome-containing zymosan particles, a double membrane was never detected surrounding the yeast in any of the cells (scale bars equal 0.4 μm). (**b**) RAW cells expressing GFP-LC3 were treated with chloroquine for 5 hours. Chloroquine induced the accumulation of large autophagosomes that were positive for the endoplasmic reticulum marker ER-Tracker (red). The image shown is a middle frame of a z-stack (see Supplemental Movie 3 for full z-stack). (**c**) RAW cells expressing GFP-LC3, were stained with ER-Tracker (red) and exposed to zymosan. The phagosomes containing yeast particles did not contain any ER as determined by ER-Tracker (the small area in the center of the yeast (yellow asterisks) was already red before uptake in the phagosome, see Supplemental Movie 4 for details).

Figure-S5

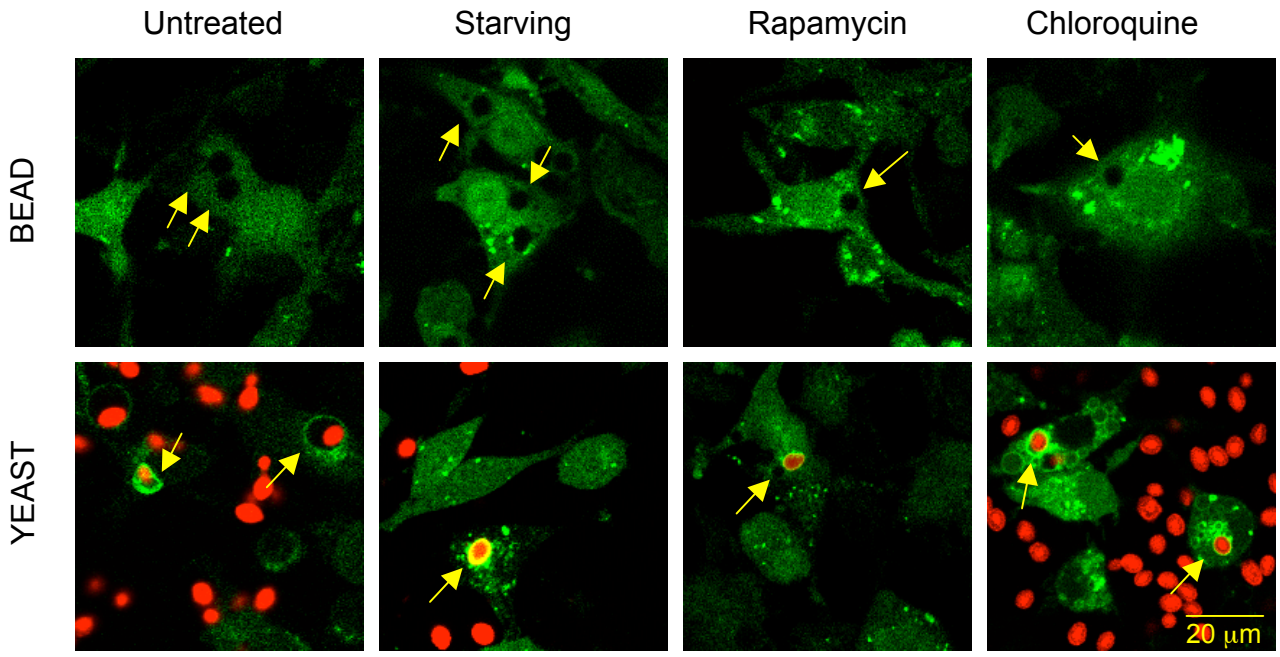

Figure S5. **TLR-induced LC3 localization to the phagosome appears to occur independently of autophagy.** RAW-GFP-LC3 cells were pretreated with chloroquine (50μM), starvation media, or rapamycin (200 nM) for 3 hours. Cells were then fed with beads (upper panel) or zymosan (lower panel) and followed by time-lapse video at 3 min. intervals for 3 hours. Representative frames (time 60 min.) are shown. Quantification is shown in Figure 2b.

Figure-S6

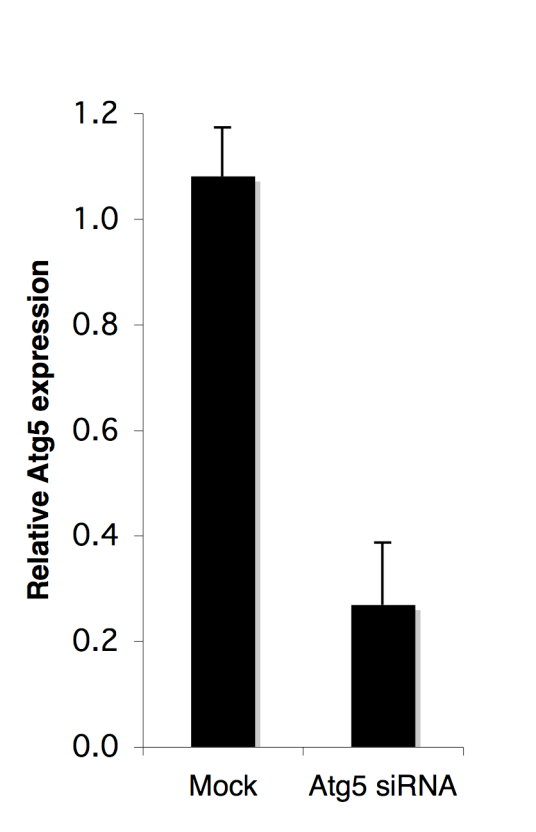

Figure S6. **Knockdown of ATG5.** RAW cells were electroporated with control or Atg5 siRNA oligonucleotides. After 24 hours the level of Atg5 mRNA was quantified by real-time PCR.

Figure-S7

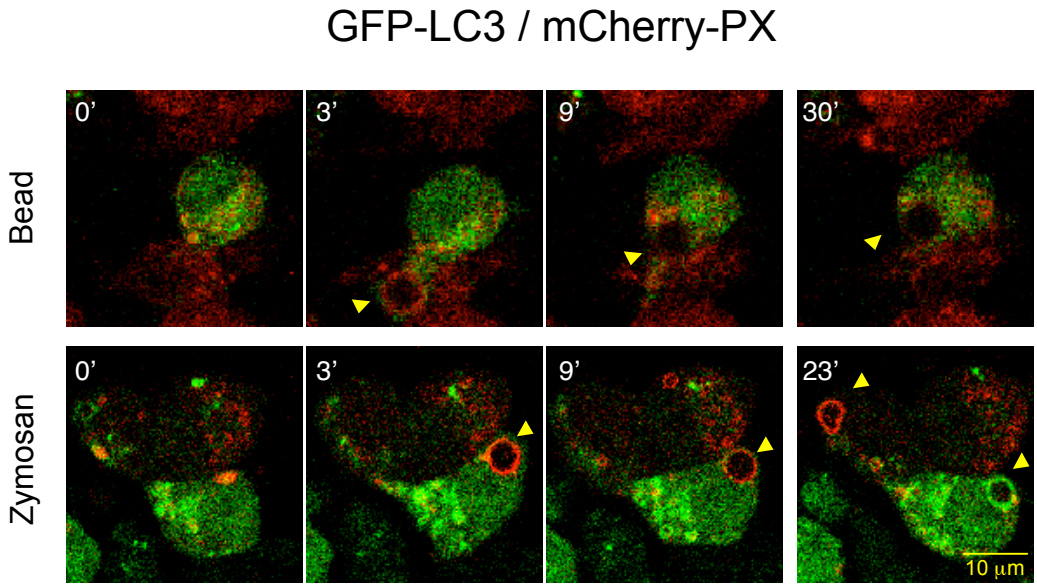

Figure S7. **Zymosan induces a durable accumulation of PtdIns3P at the phagosome.** RAW cells expressing GFP-LC3 and the PX-domain of p40(phox) fused to mCherry (PX-mCherry, red) were fed with latex beads or zymosan (yellow arrows). Zymosan internalization was monitored by capturing time-lapse confocal images. The images provided are representative frames of supplemental videos 6 and 7.

Figure-S8

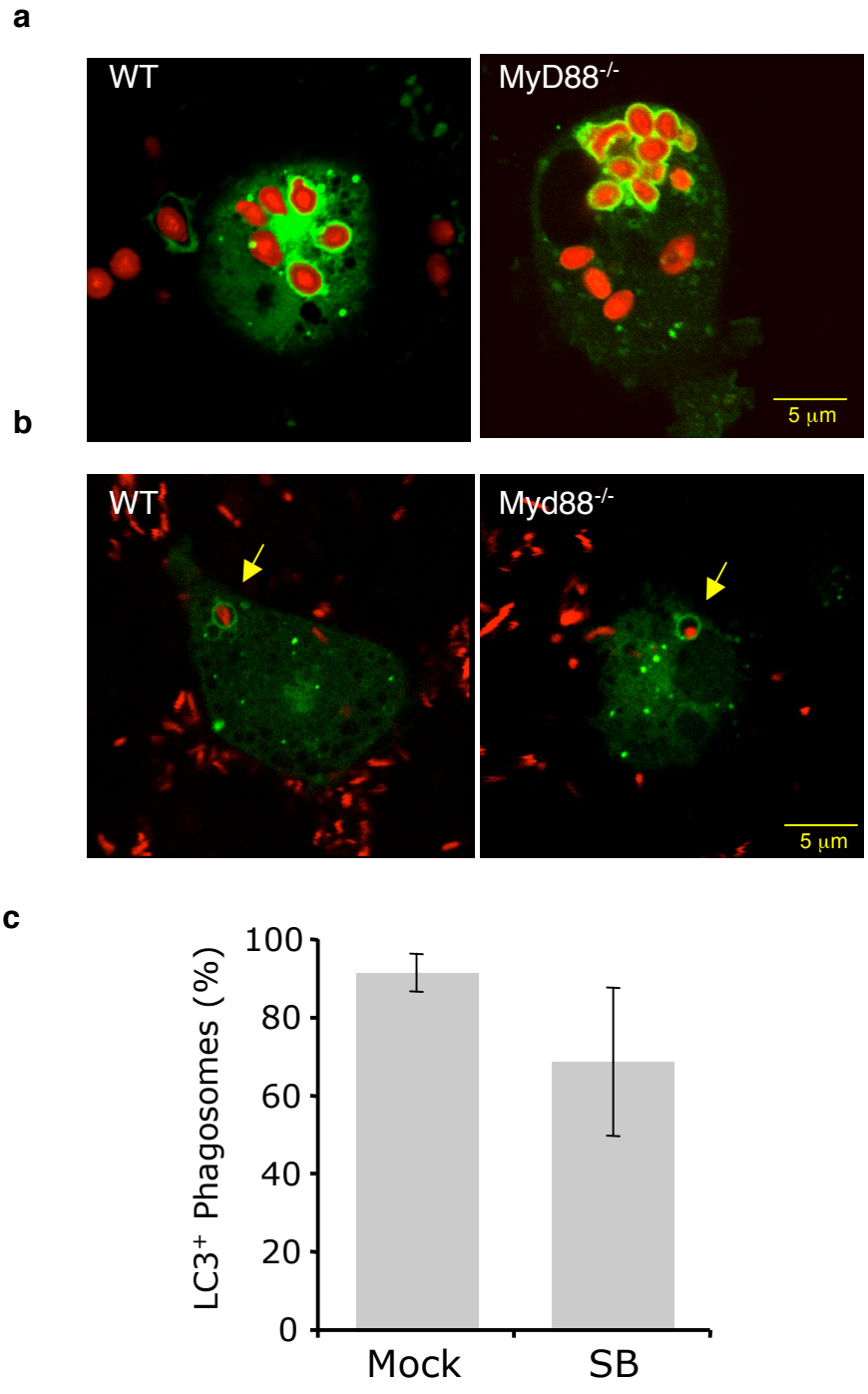

Figure S8. **LC3 translocation to the phagosome is independent of Myd88 and p38**. (**a**) Bone marrow-derived macrophages from wt and MyD88$^{-/-}$ mice were transiently transfected with GFP-LC3. At 24 h. post-transfection, Zymosan (**a**), or mRFP1 expressing *E. coli* (**b**), were added for 45 min. upon which confocal images were captured of cells that had internalized the particles. (**c**) Zymosan was fed to RAW cells that were pretreated with the p38 inhibitor SB202190 (SB) (1 μ g/ml) for 10 minutes. (**f**) The % of GFP-LC3-associated phagosomes (n≥100/group) were assessed by time lapse video using 3 independent experiments. Means and ranges are shown.