

Delgado et al. Supplemental Materials.

Methods

Transient transfection by nucleoporation

RAW 264.7 cells were harvested at day 2 of culture and re-suspended in the appropriate electroporation buffer (Amaxa Biosystems, MD). 5-10 µg plasmid DNA and/or 1.5 µg siRNA were mixed with 0.1 ml of cell suspension, transferred to an electroporation cuvette, and nucleofected with Amaxa Nucleofector apparatus (Amaxa Biosystems, MD). After electroporation, cells were transferred to 0.5 ml of complete media, diluted as it was convenient and cultured in 6-wells plates or over coverslips in 24-well plates for 24 h before any stimulation. HeLa cells were transfected with 1.5 µg siRNA using the Amaxa Nucleofector apparatus.

TLR ligand concentrations

The concentrations of TLR ligands used were based on the reported working concentrations in cognate TLR stimulation assays (Alexopoulou et al., 2001; Flo et al., 2002; Grabiec et al., 2004; Honda et al., 2004; Mansson et al., 2006; Pestka and Zhou, 2006; Tallant et al., 2004).

Fluorescence confocal microscopy

Cells were cultured on glass coverslips, fixed with paraformaldehyde for 10 min at room temperature, and then washed with PBS. The coverslips were mounted onto glass slides with Permafluor Aqueous mounting medium (Thermo Scientific, MA) and analyzed on a Zeiss LSM510 META laser scanning confocal microscope.

Transmission electron microscopy

RAW 264.7 macrophages were harvested at day 2 of culture and cultured over coverslips in 6-well for 24 h before any stimulation. Where indicated, cells were transfected with siRNA 46 h before stimulation. Cells were stimulated as indicated, washed with PBS and fixed for 1 h at room temperature with 3% formaldehyde and 2% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4. Cells were washed with 0.1 M sodium cacodylate buffer, postfixed for 1 h in 1% osmium tetroxide and 0.5% potassium ferricyanide in cacodylate buffer, washed with cacodylate buffer, dehydrated with increasing concentrations of ethanol and gradually infiltrated with Epon-Araldite resin. Embedded in straight resin, cured at 80°C for 24 h, and examined using a Zeiss EM 900 transmission electron microscope.

Colony-Forming units assay

RAW 264.7 macrophages cells were plated in 12-well plates (3×10^5 cells per well). Cells were infected with 3×10^6 mycobacteria for 1 h at 37°C, washed with

PBS, and incubated for 4 h with full media alone or with imiquimod (10 µg/ml) or ssRNA (10 µg/ml) or with starvation medium. When cells were transfected with siRNA, 5 h following transfection, cells were plated at 4×10^5 cells per well and infected 24 h later (Beclin 1, Atg5 and MyD88 knockdowns), or 8×10^4 cells per well for infection 48 h later (TLR7 knockdown). Cells were lysed with ice-cold water, and quantitative culturing performed using serial dilutions. Aliquots of 5 µl of each dilution were inoculated on Middlebrook 7H11 agar plates with ADC. Plates were incubated for 12-14 days at 37°C and colonies were counted on plates with dilutions yielding 1-50 visible colonies.

Proteolysis of long-lived proteins

RAW 264.7 macrophages cells were plated in 12-well plates (4×10^4 cells per well). Cells were labeled the next day for 24 h in media containing 1 µCi/ml ^3H -leucine, washed to remove unincorporated label, and pulsed for 24 h in full medium containing cold leucine to allow degradation of short-lived proteins. Cells were incubated in full media alone or in the presence of TLR ligands for 24 h. For starvation control, cells were incubated in starvation media for the last 4 h. When indicated, an 1 h preincubation with 10 µg/ml E-64d and 10 µg/ml pepstatin A was carried out and the inhibitors were maintained during the stimulation. Trichloroacetic acid (TCA)-precipitable radioactivity of the cells monolayers and the TCA-soluble radioactivity in the media were determined. Leucine release (a measure of proteolysis of stable proteins) was calculated as a ratio between TCA-soluble supernatant and total cell-associated radioactivity.

Western blotting

Cells were plated in 6-well plates, incubated overnight (J774 and RAW 264.7) or for 3 days (BMM), and stimulated for 30 min - 4 h. Where indicated, 100 nM Bafilomycin A1 or 10 µg/ml E-64d and 10 µg/ml Pepstatin A were added along with the stimulus. Cells were washed and lysed. Fractions (50 µg proteins) of cellular extract proteins were subjected to SDS-polyacrylamide gel electrophoresis using a Tris/glycine buffer system based on the method of Laemmli. After electrophoresis, proteins were transferred to a nitrocellulose transfer membrane. Blots were blocked and then incubated with primary antibodies using the manufacturer's protocol followed by the appropriate horseradish peroxidase-conjugated secondary antibody. Staining was revealed with SuperSignal West Dura Extended Duration Substrate (Pierce, Rockford, IL). Immunostained proteins were visualized on X-ray film.

GFP-LC3 puncta assay in bone marrow macrophages

Bone marrow-derived macrophages from transgenic mice expressing GFP-LC3 transgene (Mizushima et al., 2004) were seeded on coverslips in 24-well plates, and 24 h later incubated in the indicated conditions. Cells were washed with PBS, fixed with 2% paraformaldehyde for 10 min, washed with PBS, permeabilized with 0.2% saponin for 5 min, washed with PBS, blocked for 30 min, and incubated with rabbit polyclonal antibodies against GFP overnight, to visualize

the GFP-LC3 fusion protein (GFP fluorescence in BMMs isolated from these transgenic mice is sporadic and GFP antibody is routinely used to visualize GFP-LC3). Slides were washed with PBS, incubated with a secondary anti-rabbit Alexa 488-conjugated antibody, and finally washed and mounted onto glass slides with Permafluor Aqueous mounting medium (Thermo Scientific, MA) and analyzed on a Zeiss LSM510 META laser scanning confocal microscope.

HIV infection

HIV (NL4-3 Δ env) pseudotyping with vesicular stomatitis virus glycoprotein (VSV-G) and infections were done as previously described (Olivetta and Federico, 2006). HeLa cells were allowed to adsorb virus for 6 h, washed, and infections allowed to go for 48 h. Cells were then lysed for Western blots.

NF- κ B: luciferase reporter assay

RAW 264.7 macrophages were transfected in a proportion of 9:1 with a NF- κ B-responsive luciferase reporter plasmid PathDetect[®] NF- κ B cis-Reporting System and a β -galactosidase construct (pEF1-Bos) for normalization. 24 h after transfection, cells were stimulated for 4 or 24 h. Luciferase activity was assayed using the luciferase reagent (Promega Corporation, Madison, WI), and β -galactosidase activity was assayed using the Galacto-Star luminescence system (Tropix, Bedford, MA). Transfection efficiency was controlled by standardizing luciferase activity to constitutive β -galactosidase production.

IFN- β detection

To analyze IFN- β secretion by RAW 264.7 macrophages, supernatants from GFP-LC3 transfected cells after stimulation for 4 h were analyzed by using using an enzyme-linked immunosorbent assay (ELISA) kit for mouse IFN- β (R&D Systems, Minneapolis, MN) according to the manufacturer's protocol.

Statistical analysis

All statistical analyses were performed using Fisher's protected least significant difference post hoc test analysis of variance (ANOVA; Super-ANOVA version 1.11; Abacus Concepts Inc., CA), with the exception of Fig. 5B where paired t test was applied. Data are presented as means \pm standard errors. A P value of <0.05 was considered significant relative to the untreated control.

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Supplementary figures and movies

Figure S1. **Quantification of GFP-LC3 puncta ($\geq 1 \mu\text{m}$) in RAW 264.7 macrophages transfected with GFP-LC3 following treatment with a PAMP panel.** After 24 h cells were incubated in the presence of 10 $\mu\text{g/ml}$ E-64d and 10 $\mu\text{g/ml}$ Pepstatin A for 2 h in starvation media or 4 h in full media alone or in the presence of 1 $\mu\text{g/ml}$ Pam₃CSK₄ (TLR1/TLR2), 100 ng/ml Pam₂CSK₄ (TLR2/TLR6), 25 $\mu\text{g/ml}$ poly(I:C) (TLR3), 500 ng/ml LPS (TLR4), 1 $\mu\text{g/ml}$ *S. typhimurium* flagellin (TLR5), 10 $\mu\text{g/ml}$ ssRNA (TLR7), or 3 μM CpG oligonucleotide 1826 (TLR9). Data are means \pm s.e.m. (n = 3); ** P<0.01, * P<0.05, † P \geq 0.05 (ANOVA).

Figure S2. **Macrophage response to TLR ligands. (A)** RAW 264.7 macrophages were cotransfected with NF- κ B:luciferase and β -galactosidase constructs. 24 h later, cells were incubated 4 h with full media alone (Control) or in the presence of TLR stimulus: 1 $\mu\text{g/ml}$ Pam₃CSK₄ (TLR1/2), 100 ng/ml Pam₂CSK₄ (TLR2/6), 25 $\mu\text{g/ml}$ poly(I:C) (TLR3), 500 ng/ml LPS (TLR4), 1 $\mu\text{g/ml}$ *S. typhimurium* flagellin (TLR5), 10 $\mu\text{g/ml}$ Imiquimod (TLR7), 10 $\mu\text{g/ml}$ ssRNA (TLR7) or 3 μM CpG ODN 1826 (TLR9). Data are means \pm s.e.m. (n = 3); ** P<0.01, * P<0.05, † P \geq 0.05 (ANOVA). **(B)** IFN- β secretion of RAW 264.7 macrophages incubated for 4 h with full media alone (Control) or in the presence of 25 $\mu\text{g/ml}$ poly(I:C), 500 ng/ml LPS or 10 $\mu\text{g/ml}$ ssRNA. Data are means \pm s.e.m. (n = 3); ** P<0.01, † P \geq 0.05 (ANOVA). **(C)** RAW 264.7 macrophages were lysed and analyzed by Western blotting using anti-TLR5 antibodies. **(D and E)** RAW 264.7 macrophages were incubated for 30 min or 4 h in full media alone (C), or in the presence of TLR ligands (as in A). Cells were lysed and analyzed by Western blotting using I κ B- α , phosphoJNK (P-JNK), JNK and actin antibodies.

Figure S3. **Complex PAMP can induce autophagy in RAW 264.7 macrophages. (A)** Quantification of big puncta ($\geq 1 \mu\text{m}$) of GFP-LC3 in RAW 264.7 macrophages transiently transfected with GFP-LC3 and incubated for 2 h with starvation media or 4 h with full media alone (Control) or in the presence of 1 $\mu\text{g/ml}$ Pam₃CSK₄ (Pam3), 100 ng/ml Pam₂CSK₄ (Pam2) or 10 $\mu\text{g/ml}$ zymosan. Data are means \pm s.e.m. (n \geq 3); ** P<0.01, † P \geq 0.05 (ANOVA). **(B)** RAW 264.7 macrophages were cotransfected with NF- κ B:luciferase and β -galactosidase constructs. 24 h later, cells were incubated 4 h with full media alone (Control) or in the presence of 10 $\mu\text{g/ml}$ zymosan. Data are means \pm s.e.m. (n = 3); ** P<0.01 (ANOVA).

Figure S4. **TLR7 ligands affect LC3-I to LC3-II conversion. A)** RAW 264.7 macrophages were transfected with LC3B siRNA (LC3B) or not transfected (none) and after 24 h cells were incubated for 4 h with full media alone (C), or in the presence of 50 $\mu\text{g/ml}$ rapamycin (Rap) or 10 $\mu\text{g/ml}$ ssRNA. Cells were lysed and analyzed by Western blotting using LC3 or GAPDH antibodies. **(B)** RAW

264.7 macrophages were incubated for 4 h with full media alone (C), or in the presence of 50 µg/ml rapamycin (Rap), 500 U/ml IFN-γ or 10 µg/ml Imiquimod (Imiq). Cells were lysed and analyzed by Western blotting using LC3 or GAPDH antibodies.

Figure S5. Autophagic vacuoles formation upon stimulation with ssRNA depends on TLR7. RAW 264.7 macrophages were transfected with control scrambled siRNA (**A and B**) or TLR7 siRNA (**C**). After 48 h, cells were incubated in full media alone or in the presence of ssRNA for 2 (A) or 4 h (B and C). **A'** and **B'**, enlarged areas outlined in A and B, respectively. Arrowheads, autophagic vacuoles containing electron dense material typical of Av(d) (Eskalinen, 2008). **(D)** Quantification of surface density in a volume (S_v) (Weibel and Bolender, 1973) for the autophagic vacuoles in cells transfected with scrambled siRNA (sc) or with TLR7 siRNA and stimulated 2 h and 4 h with ssRNA (n=10 for scrambled, n=8 for siTLR7). * P<0.05 (ANOVA).

Figure S6. Proteolysis of long-lived proteins by TLR ligands. Proteolysis of long-lived proteins was measured in RAW 264.7 cells labeled for 24 h in media containing ^3H -leucine. Cells were washed, incubated for 24 h in complete medium (containing cold leucine), and incubated with starvation media for 4 h or with full media alone (Control) or in the presence of TLR stimulus: 1 µg/ml Pam₃CSK₄, 100 ng/ml Pam₂CSK₄, 10 µg/ml zymosan, 25 µg/ml poly(I:C), 500 ng/ml LPS, 1 µg/ml *S. typhimurium* flagellin or or 3 µM CpG ODN 1826 for 24 h. Leucine release, was calculated from radioactivity in the TCA-soluble form relative to the total cell radioactivity. Data are means ± s.e.m. (n = 9); ** P<0.01, † P≥0.05 (ANOVA).

Figure S7. LC3-II conversion upon stimulation with Imiquimod or ssRNA is TLR7 dependent. **(A)** RAW 264.7 macrophages cells were transfected with control scrambled siRNA (sc) or TLR7 siRNA. After 46 h cells were incubated for 4 h in the presence of 100 nM Bafilomycin A in full media alone (Control) or in the presence of 10 µg/ml ssRNA. Cells were lysed and analyzed by Western blotting using anti-LC3 or anti-actin antibodies. The LC3-II/actin ratios are shown. **(B)** RAW 264.7 macrophages were transfected with control scrambled siRNA (sc) or TLR7 siRNA and after 46 h cells were incubated for 4 h in full media with 10 µg/ml Imiquimod. Cells were lysed and analyzed by Western blotting using anti-TLR7 or anti-LC3 antibodies.

Figure S8. LC3-II conversion stimulated by TLR7-ligands is MyD88 dependent. RAW 264.7 macrophages cells were transfected with control scrambled siRNA (sc) or MyD88 siRNA. After 22 h cells were incubated for 4 h in the presence of 10 µg/ml E-64d and 10 µg/ml Pepstatin A in full media alone (C) or in the presence of 10 µg/ml Imiquimod (Imiq) or 10 µg/ml ssRNA. Cells were lysed and analyzed by Western blotting using anti-MyD88, anti-LC3 or anti-actin antibodies. The LC3-II/actin ratios are shown.

Figure S9: **Ligands stimulating TLR but not inducing autophagy do not inhibit intracellular mycobacteria.** RAW 264.7 macrophages cells were infected with BCG for 1 h, washed and incubated for 4 h in full media alone (C) or in the presence of 100 ng/ml Pam₂CSK₄, 3 μM CpG ODN 1826, or in starvation medium (Starv). Cells were lysed to quantify bacterial survival by counting cfu. Data are means ± s.e.m. (n = 6); ** P<0.01, † P≥0.05 (ANOVA). See Fig. S2 for IκB-α degradation, NF-κB activation, and JNK phosphorylation upon stimulation with the corresponding PAMP.

Figure S10. **HIV infection stimulates LC3-I to LC3-II conversion in HeLa cells.** (A) HeLa cells were infected (+) with HIV (VSV-G pseudotyped HIVΔenv), or not infected (-). After 48 h, cells were lysed and analyzed by Western blotting using anti-LC3 or anti-actin antibodies. (B) HeLa cells were transfected with control scrambled siRNA (sc) or TLR8 siRNA, allowed to adhere for 2 h and infected (+) with HIV (VSV-G pseudotyped HIVΔenv) or not infected (-). After 48 h, cells were lysed and analyzed by Western blotting using anti-TLR8, anti-LC3 or anti-actin antibodies. The LC3-II/actin ratios are shown.

Movie 1. **Four-dimensional live confocal microscopy analysis of imiquimod induction of LC3 puncta.** RAW264.7 macrophages were transfected with tdTomato-LC3 (Bjorkoy et al., 2005) and analyzed by live confocal microscopy in a 5LIVE Zeiss microscope. Imiquimod was added as for LC3 puncta assay with fixed cells, and z-stacks collected at 3 min intervals for a total of 45 min. The collected images were processed to generate a maximum projection (collapsing a 3D image into an x-y projection) for each time point, as previously described for 4-D live confocal imaging (Chua and Deretic, 2004; Kyei et al., 2006; Roberts et al., 2006) .

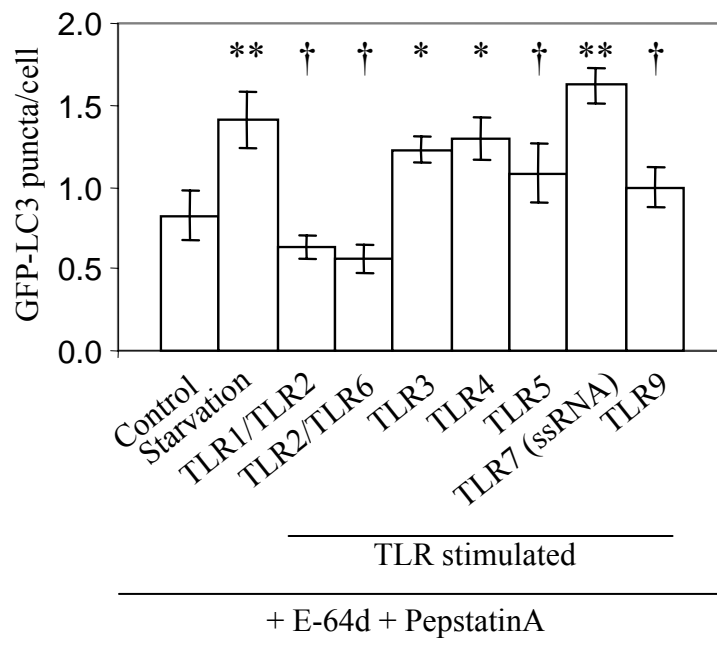


Fig. S1

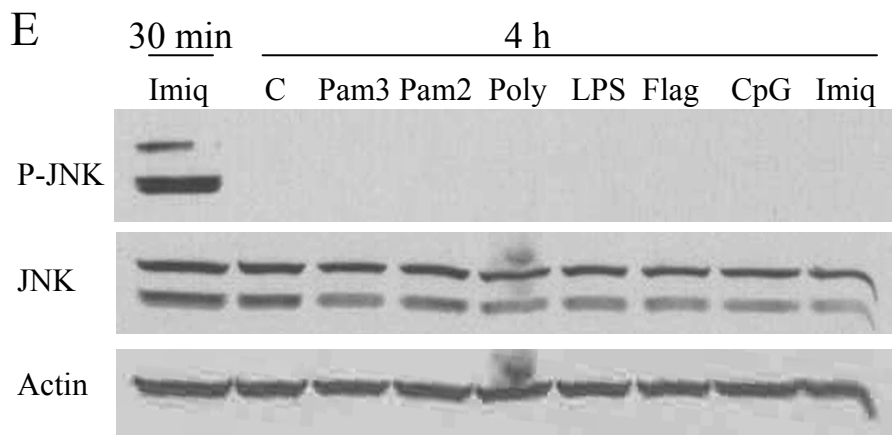
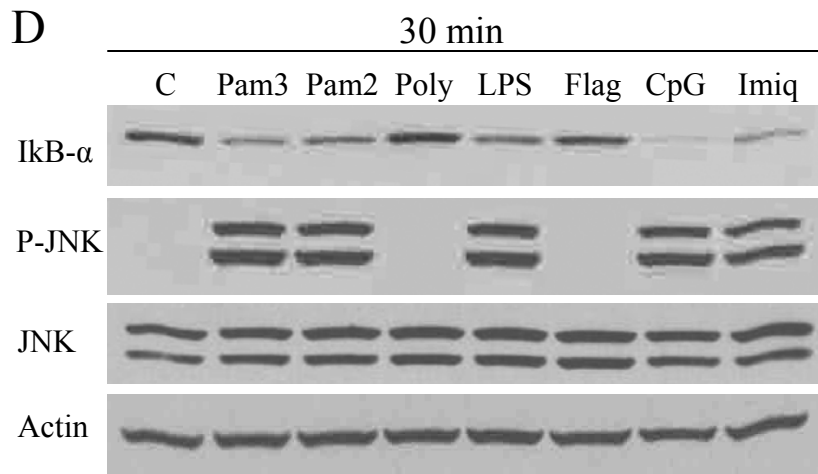
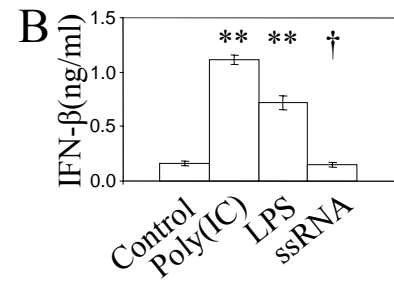
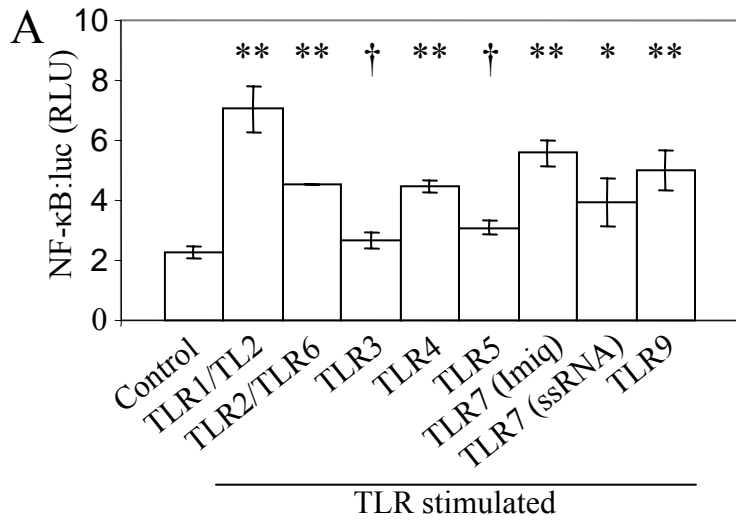


Fig. S2

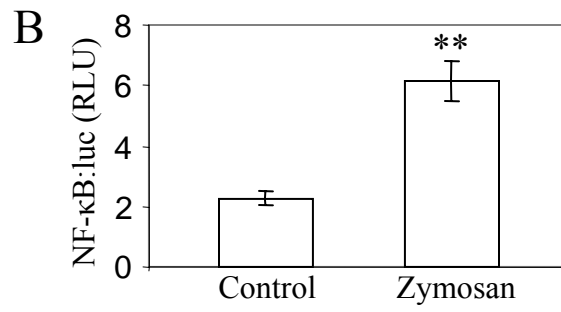
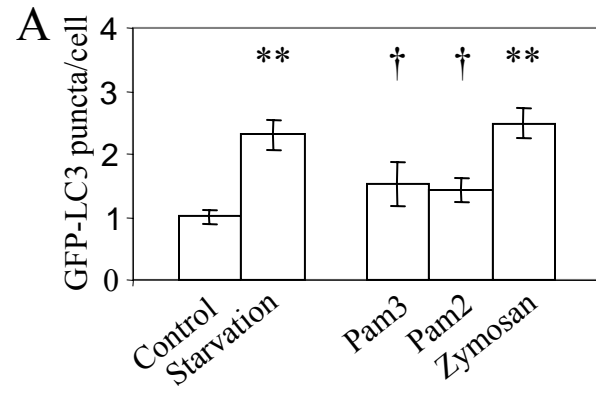


Fig. S3

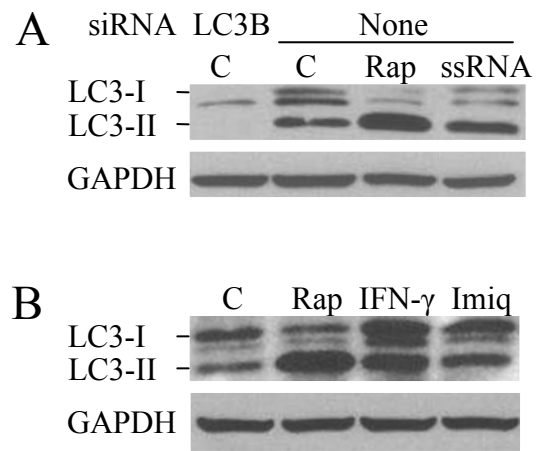
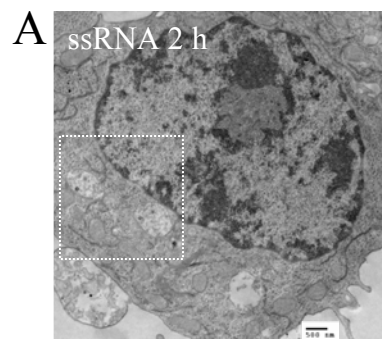
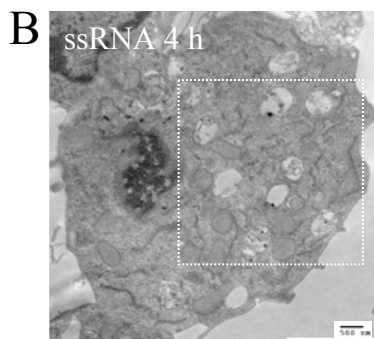


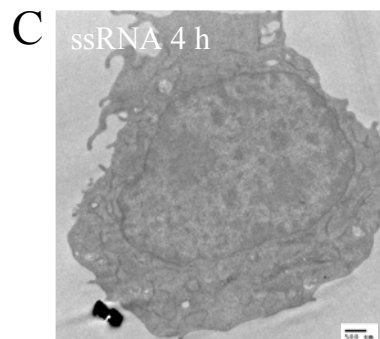
Fig. S4



Scrambled siRNA



Scrambled siRNA



TLR7 siRNA

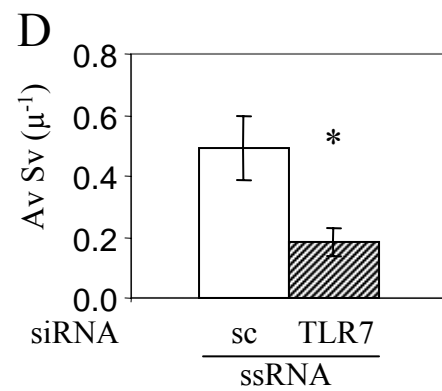
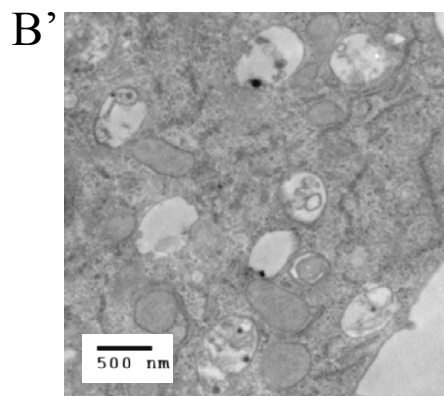
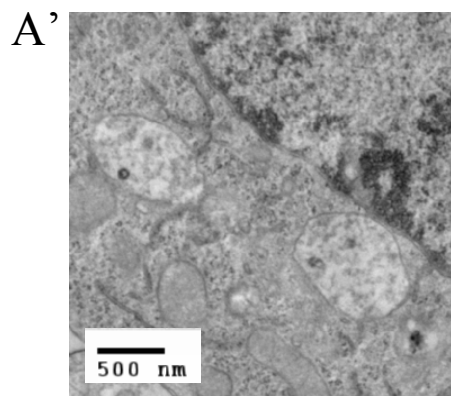


Fig. S5

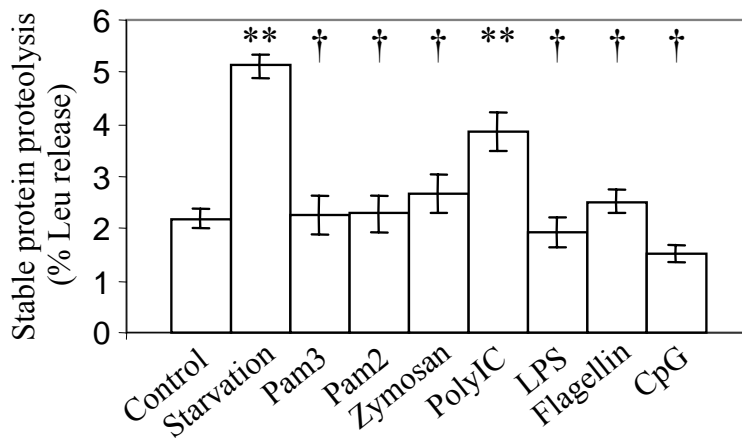


Fig. S6

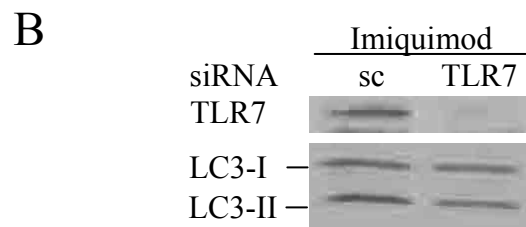
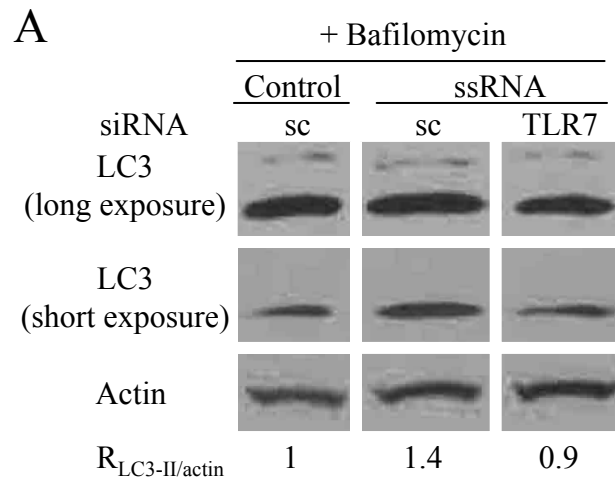


Fig. S7

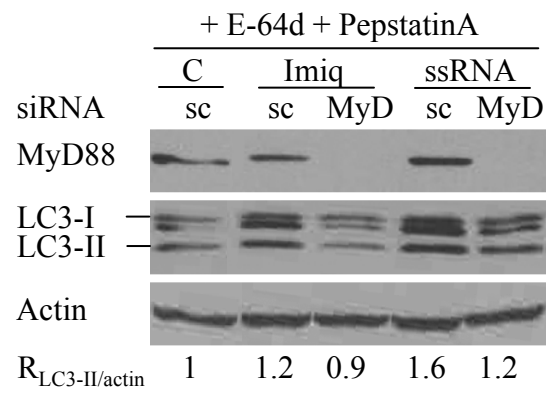


Fig. S8

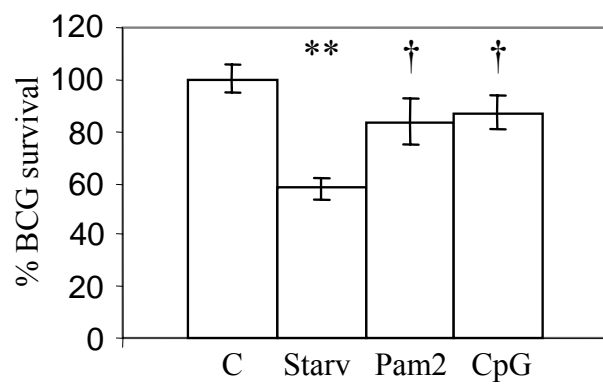


Fig. S9

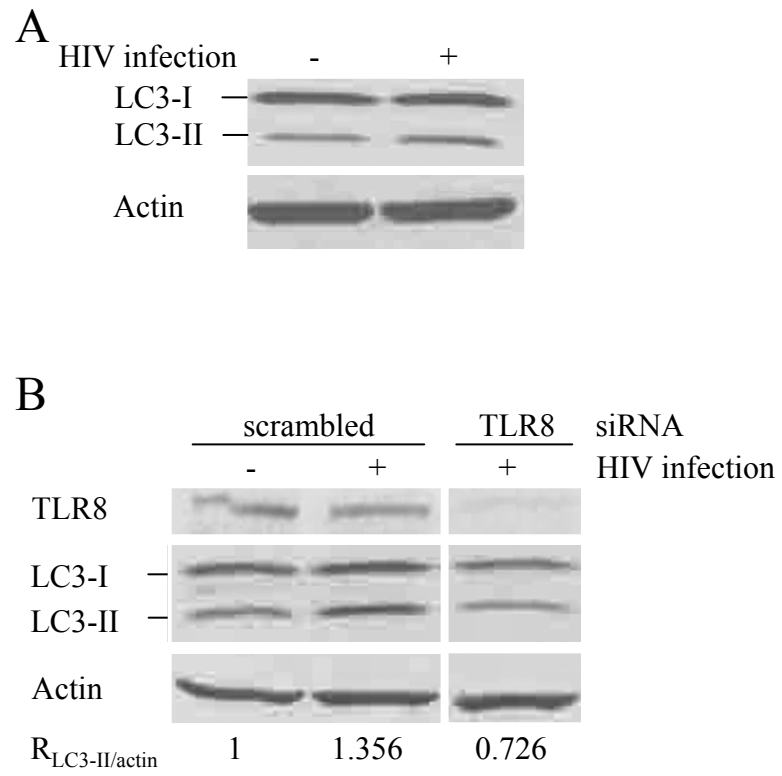


Fig. S10