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Supplemental Information

Noncanonical Autophagy Is Required for Type I Interferon Secretion in Response to DNA-Immune Complexes

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Supplemental Inventory

Figure S1 (Related to Figure 1)

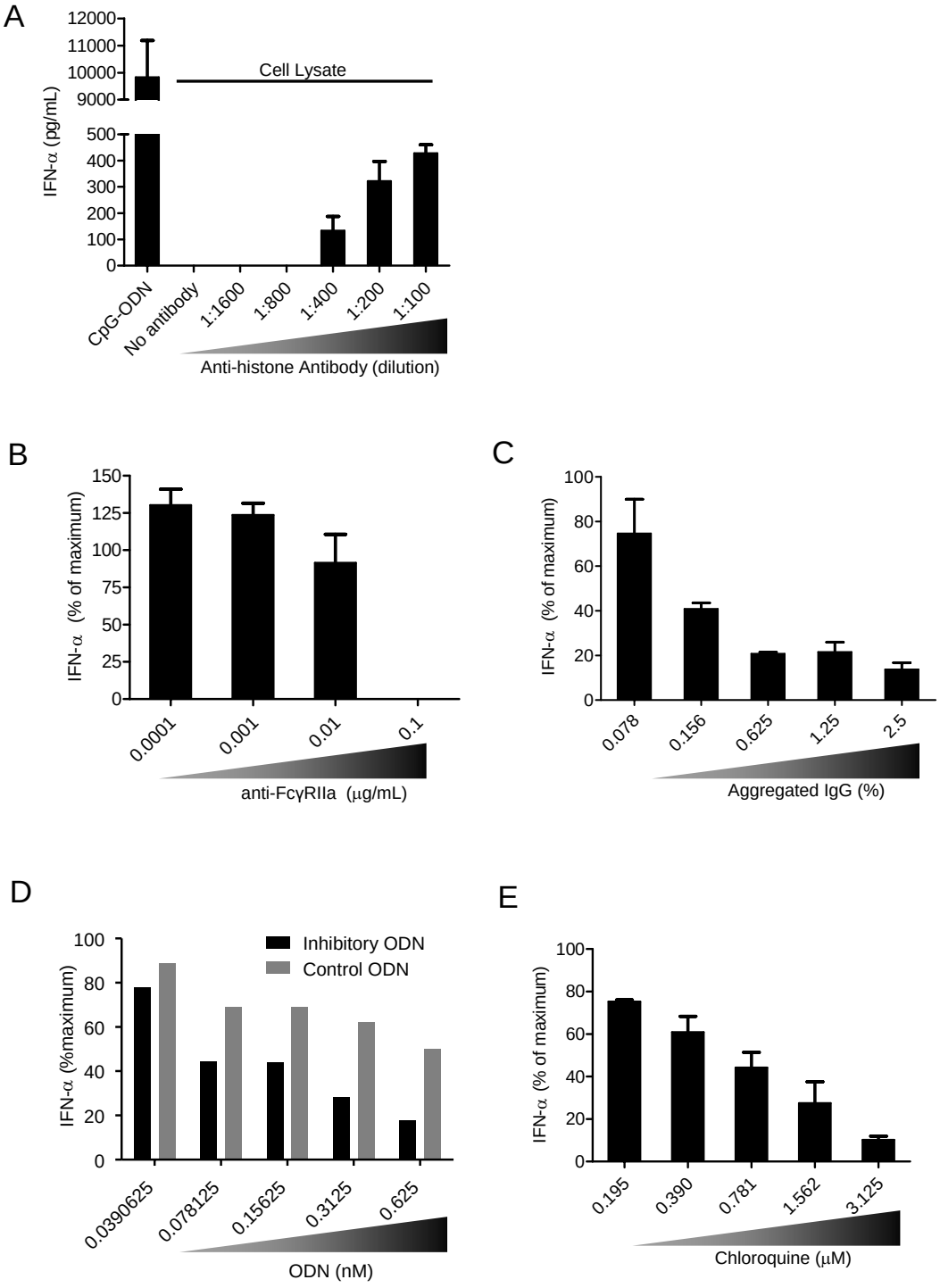


Figure S1. DNA-IgG-Mediated Production of IFN-α Requires Both FcγR and TLR9 in Human PBMCs, Related to Figure 1

(A) Human PBMCs were challenged for 18 hours with ODN 2216 (CpG-ODN) or with serial dilutions of anti-histone antibodies in the presence of Jurkat cell lysate to form DNA-containing immune complexes (DNA-IgG), as described in experimental procedures. IFN-α production in supernatant was measured by ELISA.

(B-E) Human PBMCs were stimulated with the Jurkat lysates and a 1:50 dilution of anti-histone antibodies for 18 hours. Cells were pretreated for 30 min. with serial dilutions of antagonistic anti-FcγRIIa (B), heat aggregated human IgG (C), the TLR9 antagonist ODN TTAGGG (inhibitory ODN) and control ODN TTAGGG (D) or chloroquine (E)**.** Supernatants were collected and IFN-α was quantitated by ELISA. Data are representative of three independent experiments. Data are presented as mean ± s.d.

Figure S2 (Related to Figure 2)

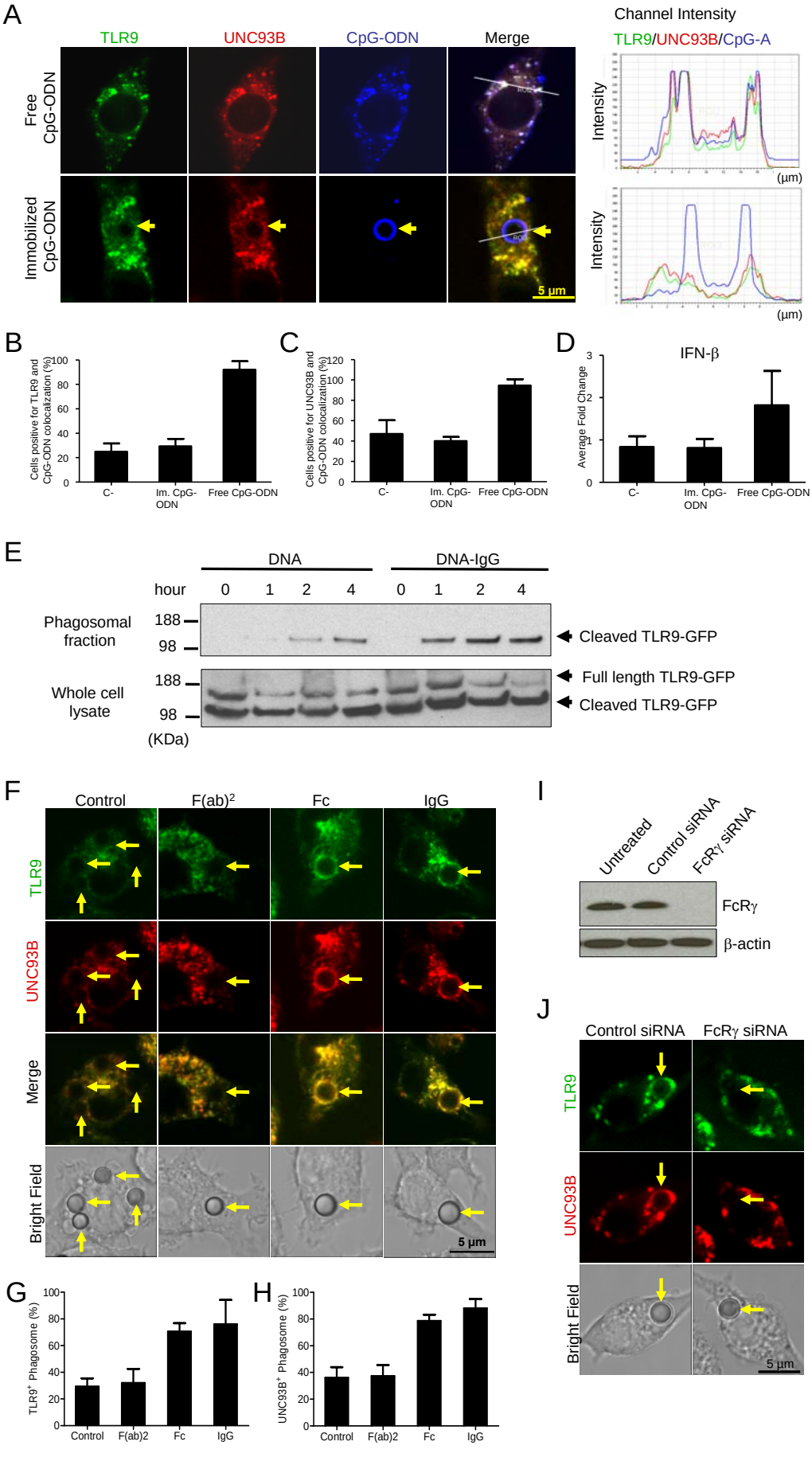


Figure S2. DNA-IC-Mediated Enhancement of TLR9 and UNC93B Trafficking to Phagosomes Depends on FcγR, Related to Figure 2

(A-C) Mouse macrophages expressing TLR9-GFP and UNC93B-mCherry were stimulated for 60 min with either 3.0 μM of free biotin-ODN 2216 (Free CpG-ODN)) or biotin-ODN 2216 immobilized on beads (Immobilized CpG-ODN). Cells were then fixed, permeabilized and incubated with Alexa Fluor 647-labeled streptavidin. Intracellular localization of TLR9-GFP, UNC93B-mCherry and biotin-ODN 2216 (CpG-ODN) was assessed by confocal microscopy. Yellow arrows point to internalized particles. Representative images and signal intensity profiles for TLR9 (green), UNC93B (red) and CpG-ODN (blue) are shown (A). The percentage of cells positive for TLR9/CpG-ODN colocalization (B) and UNC93B/CpG-ODN colocalization (C) were quantified for three independent experiments ($n \geq 75$ / group). Data are presented as mean $\pm$ s.d. Scale bar, 5μm.

(D) Mouse macrophages were fed with 3.0 μM of free or immobilized CpG-ODN, as in (A), for 4 hours. Cellular mRNA levels for IFN-β were assessed by quantitative RT-PCR. Data are presented as mean $\pm$ s.d. of three independent experiments.

(E) Immunoblot analysis of phagosome proteins. Raw 264.7 cells expressing TLR9-GFP were incubated with CG50 plasmid DNA-coated magnetic beads (DNA) or magnetic beads coated with a combination of both CG50 plasmid DNA and DNA antibody (DNA-IgG) for 0, 1, 2 or 4 hours. Phagosomes were purified using magnet as described in experimental procedures. Phagosome proteins or control whole cell lysates were solubilized in SDS-PAGE and TLR9-GFP was detected using anti-GFP antibodies by immunoblotting. As previously described, both full length and the cleaved form of TLR9 were detected in whole cell lysates, while only the cleaved form was detected in the phagosome fraction (Ewald et al., 2008). The results presented are representative of three independent experiments.

(F-H) Mouse macrophages expressing TLR9-GFP and UNC93B-mCherry were incubated with uncoated beads (Control) or beads covered with mouse $F(ab)^2$, Fc or IgG. Engulfed particles were visualized by time lapse confocal acquisition. Representative frames acquired 30 min after particle internalization are shown (F). Yellow arrows point to internalized particles. The percentage of particles recruiting TLR9-GFP (G) and UNC93B-mCherry (H) were quantified for three independent experiments ($n \geq 75$ / group). Data are presented as mean $\pm$ s.d. Scale bar, 5μm.

(I-K) Mouse macrophages expressing TLR9-GFP and UNC93B-mCherry were treated with siRNA oligonucleotides targeting FcRγ or control siRNA. FcRγ protein expression in cells was assessed by immunoblotting 48 hours after siRNA oligonucleotide treatment (I). siRNA-treated cells were then fed beads coated with a combination of CG50 plasmid DNA and DNA antibody and recruitment of TLR9-GFP and UNC93B-mCherry to the phagosomes was visualized by confocal microscopy. Representative images taken 60 min after bead internalization are shown (J). Yellow arrows point to internalized particles. The percentage of beads recruiting TLR9 and UNC93B-mCherry were quantified for three independent experiments ($n \geq 75$ / group) (K). Data are presented as mean $\pm$ s.d. Scale bar, 5μm.

Figure S3 (Related to Figure 4)

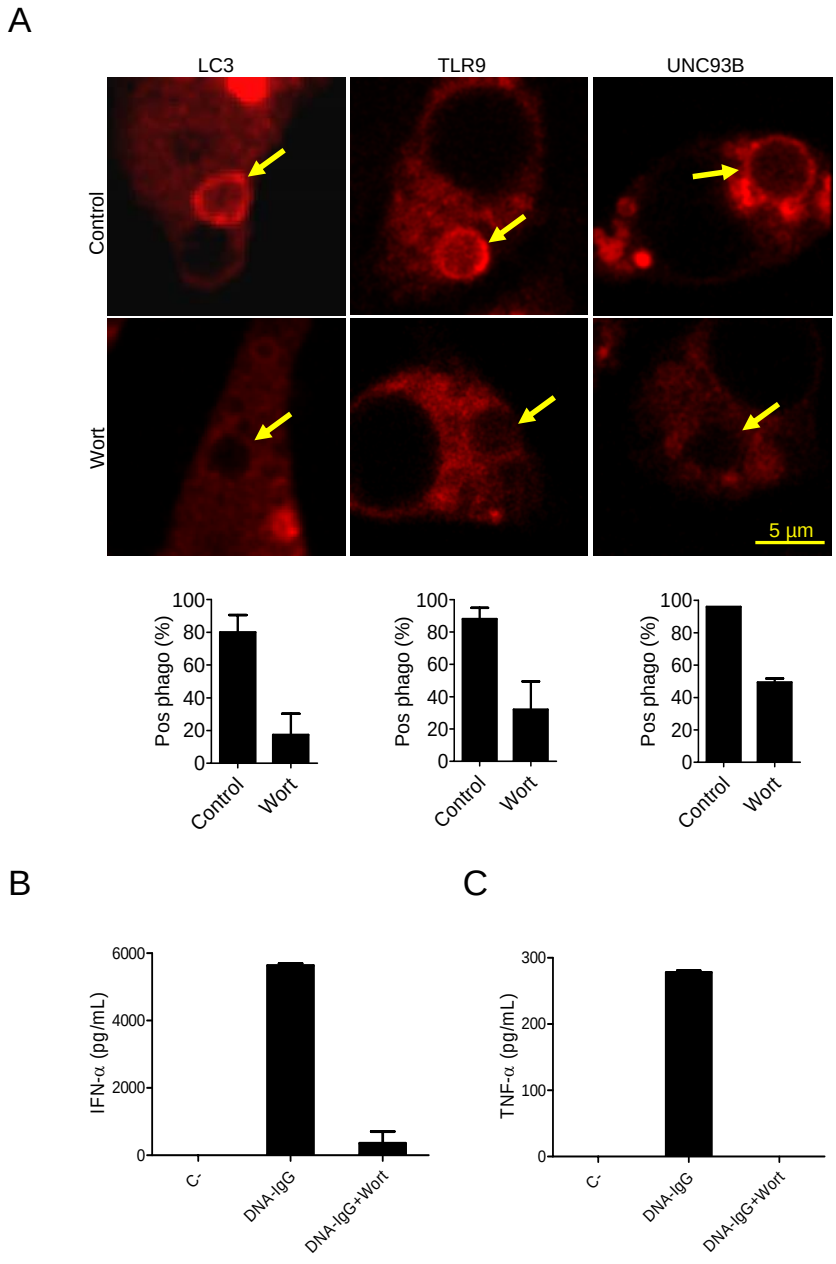


Figure S3. Wortmannin Blocks the Recruitment of LC3, TLR9 and UNC93B to the Phagosome, Related to Figure 4

(A) RAW 264.7 cells expressing LC3-mCherry, TLR9-mCherry or UNC93B-mCherry were incubated with IgG-opsonized RBC particles in the presence or absence of 1.0 μM PI3K inhibitor Wortmannin (Wort). Representative images taken 15 to 30 min after particle internalization are shown. The percentage of particles recruiting LC3, TLR9 and UNC93B-mCherry were quantified for three independent experiments ($n \geq 75$ / group). Yellow arrows point to internalized particles. Data are presented as mean $\pm$ s.d. Scale bar, 5μm.

(B) Freshly isolated human pDCs were stimulated with immune complexes made by combining CG50 plasmid DNA and DNA antibody, in presence or absence of 1.0 μM Wort. IFN-α and TNF-α in supernatant were assessed by ELISA after 16 hours. Data are presented as mean $\pm$ s.d. of two independent experiments.

Figure S4 (Related to Figure 6)

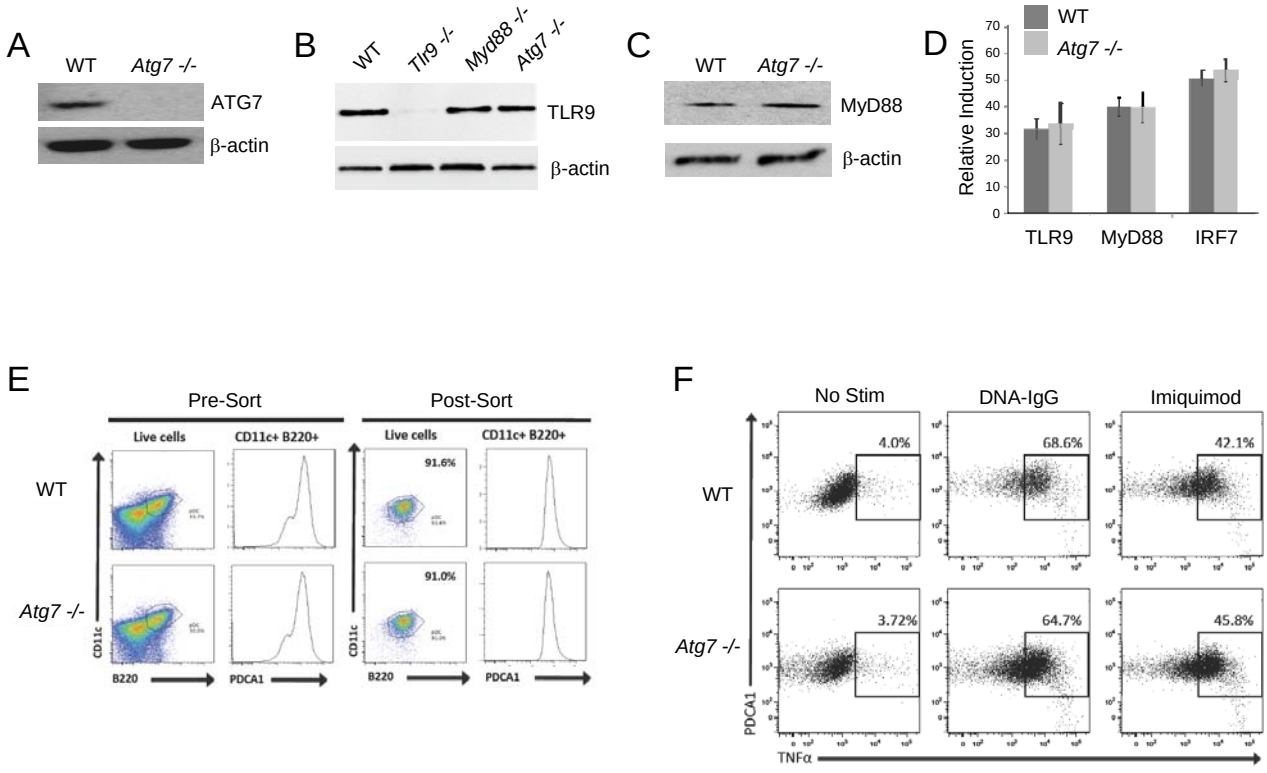


Figure S4. ATG7 and ATG5 Are Not Required for TLR9 Expression and DNA-IC-Mediated Mobilization to the Phagosome, Related to Figure 6

(A-F) Fetal liver-derived pDCs from WT, *Atg7*$^{-/-}$, *Tlr9*$^{-/-}$ and *Myd88*$^{-/-}$ mice were prepared as described in experimental procedures.

(A-C) Protein expression of ATG7 (A), TLR9 (B) and MyD88 (C) was assessed in pDCs by immunoblotting. Data are representative of at least two independent experiments.

(D) TLR9, MyD88 and IRF7 mRNA transcript expression in pDCs was determined by quantitative RT-PCR. Data are presented as mean $\pm$ s.d.

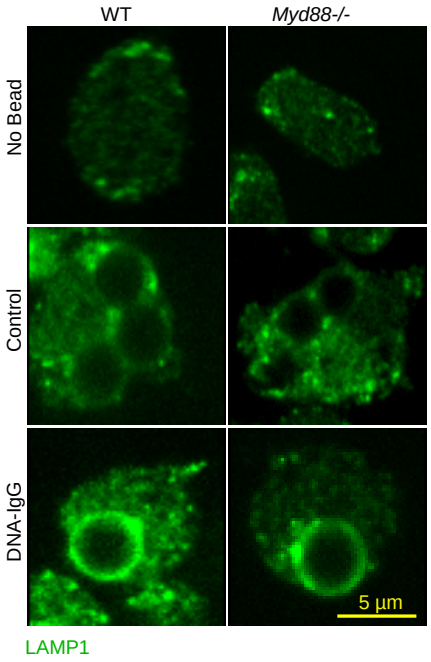
(E) Percentage of CD11c^{+} B220^{+} cells in fetal-liver-derived preparations from WT and *Atg7*$^{-/-}$ mice was assessed by flow cytometry Pre and Post-sort. PDCA1 expression in CD11c^{+} B220^{+} was also assessed.

(F) Sorted CD11c^{+} B220^{+} pDCs were stimulated with a combination of CG50 plasmid DNA and DNA antibody (DNA-IgG) as described in experimental procedures or with imiquimod for 16 hours. Expression of PDCA1 and intracellular production of TNF-α was assessed by flow cytometry. Data presented are representative of at least two independent experiments.

(G-H) Mouse macrophages expressing both TLR9-GFP and UNC93B-mCherry (G) or RAW 264.7 cells expressing LC3-GFP (H) were treated with non-targeting control or ATG5 siRNA oligonucleotides and then incubated with uncoated beads or mouse IgG-coated beads. Engulfed particles were followed by time lapse confocal microscopy and representative frames taken between 15 to 30 min after particle internalization are shown. Yellow arrows point to internalized particles. The percentages of phagosomes that were positive for TLR9 and UNC93B (G) and for LC3 (H), upon particle ingestion, were quantified for three independent experiments ($n \geq 75$ phagosomes per group). Data are presented as mean $\pm$ s.d. Scale bar, 5µm.

Figure S5 (Related to Figure 7)

A



B

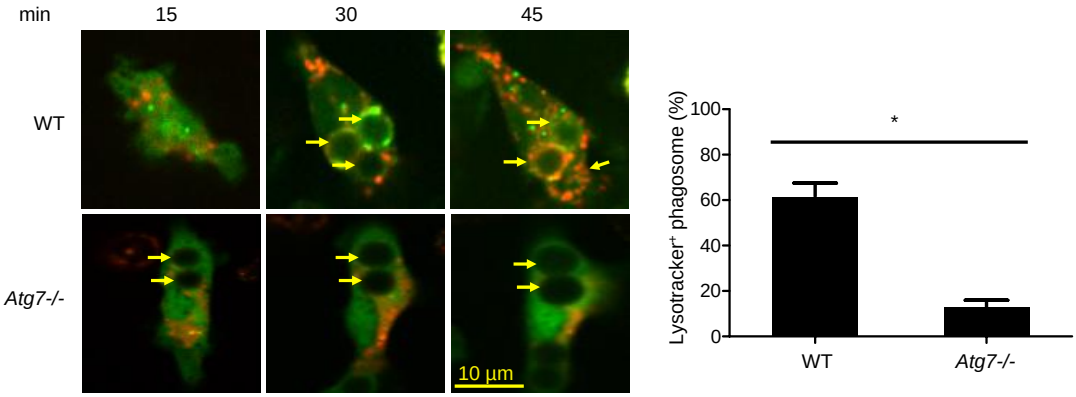


Figure S5. **Phagosome Acidification Is Compromised in *Atg7*$^{-/-}$ pDCs, Related to Figure 7**

(A) WT and *Myd88*$^{-/-}$ fetal liver-derived pDCs were fed with uncoated beads (Control) or beads coated with a combination of CG50 plasmid DNA and DNA antibody (DNA-IgG) for 4 hours ($n \geq 50$ phagosomes per group). Cells were then fixed and LAMP1 was detected by immunofluorescence. Confocal images were obtained and representative images shown. Scale bar, 5μm.

(B) Fetal liver-derived pDCs from WT and *Atg7*$^{-/-}$ mice expressing LC3-GFP (green) were allowed to phagocytose DNA-IgG-coated beads, as in (A), for the indicated time. Phagosome acidification was visualized with Lysotracker (red). Representative images are shown. Yellow arrows point to internalized particles. The percentage of particles recruiting Lysotracker (red) were quantified for three independent experiments ($n \geq 75$ per / group). Data are presented as mean $\pm$ s.d. (*$p < 0.05$). Scale bar, 10μm.

SUPPLEMENTARY EXPERIMENTAL PROCEDURES

Antibodies and Reagents

Recombinant mouse IFN-γ was from PeproTech, recombinant human IFN-β was from PBL Biomedical, recombinant mouse IFN-β was from R&D Systems and recombinant human Flt-3L was from Origene Inc. Anti-CD32a (clone IV.3) was from Stemcell Technologies, Alexa-Fluor-647 conjugated anti-LAMP1 (clone eBio1D4B) was from eBioscience, APC-conjugated anti-B220 (clone R3-6B2) was from Biolegend, PE-conjugated anti-CD11c (clone HL3) was from BD Pharmigen and anti-TNF-α (clone MP6-XT22) was from eBioscience. Mouse IgG, Fc and $F(ab)^2$ were from Jackson Immunoresearch. Wortmannin (W1628) was from Sigma, Chloroquine diphosphate (L10382), Lipofectamine 2000 and Lysotracker Red DND-99 were from Invitrogen. ODN1585, ODN1826, Biotin ODN2216, ODN2216, TLR9 inhibitory ODN (ODN TTAGGG), ODN TTAGGG control and Imiquimod were from Invivogen.

Differentiation of pDCs from Mouse Bone Marrow and Fetal Liver Cells

Bone marrow cells from the femur and tibia of adult C57BL/6 mice and fetal livers from pups at day E14 were harvested and differentiated into pDCs as previously described (Brawand et al., 2002; Zhang et al., 2000). Briefly, harvested cells were resuspended in blood cell lysis buffer (Sigma) to remove RBCs. Cells were then re-suspended in RPMI 1640 supplemented with 10% (v/v) FBS, 100U/ml penicillin, 100 µg/ml streptomycin and 2-Mercaptoethanol (2-ME, 1x) at a concentration of 1-1.5 x 10^6 cell/ml. Cells were then kept in culture for 7 days in the presence of 100 ng/ml of recombinant human Flt-3L. Cells were harvested on day 8 and $PDCA1^+$ $B220^+$ $CD11c^+$ cells (pDCs) were sorted. The purity of $PDCA1^+$ $B220^+$ $CD11c^+$ cells was consistently above 90%. Transient expression of PH-TRAF3 and TLR9-GFP in mouse pDCs was performed using Lipofectamine 2000 according to manufacturer's protocol.

Constructs

PX-GFP was obtained by cloning the PX domain of mouse p40 (PHOX) to the N-terminus of eGFP using a 6 amino acid linker. The p40PX domain was cloned from RAW 264.7 cDNA by standard RT-PCR. The primers used to amplify the p40PX domain-eGfp fusion were 5'-TTAATTGCGGCCGCCTAGGTTCGAAACCATGGCCC TGGCCCAG-3' and 5'-TTAATTCTCGAGATCTGAATTCTTACTTGTACAGC TCGTCCATGCCG 3'. The PCR product was digested with AvrII/XhoI and cloned into the NheI/XhoI sites of pMSCVNeo. A murine TLR9-eGfp fusion construct (muTLR Genbank#NM_031178.2; 6 amino acid linker between genes) was codon optimized and synthesized by GeneArt. This cassette was cloned into the pMSCVPuro retroviral backbone (Clontech). Mouse UNC93B-mCherry-wtpMSCVNeo and UNC93B-Gfp-wtpMSCVNeo plasmids were generated as previously described (Kim et al., 2008). TLR9a-mCherry was obtained by fusing hTLR9a, from pUNO1-hTLR09a (InvivoGen) to the N-terminus of mCherry, using a 6 amino acid linker. Both hTLR9a and mCherry were PCR amplified, from their respective parental plasmids, and subsequently fused using SOEing PCR. The primers, 5'-TTAATTGCTAGCACCATGGGTTTCTGCCGCAGCGC-3', and 5'-TTAATTCTCGAGTTACTTGTACAGCTCGTCCA-3' were used to amplify, and add NheI/XhoI sites to hTLR9a-mCherry. This PCR product was cloned into the AvrII/XhoI sites of a modified pMSCV-Neo vector. GFP-LC3B was gene optimized, synthesized, and sequenced by GeneArt. The fusion gene was cloned into the BglII/AvrII sites of the pMSCV-Neo vector to generate pMSCV-Gfp-LC3B-Neo plasmid. mCherry-LC3B was generated by fusing mCherry to the N-terminus of the LC3B of the pMA-Gfp-LC3B-RQ plasmid. Both mCherry and LC3B were PCR amplified, from their respective parental plasmids, and subsequently fused using SOEing PCR. The primers,

5'-TTAATTGCTAGCACCATGGTGAGCAAGGGCGAGGA-3', and 5'TTAATTCTCGAGTCACACGCTCAGCTTCATGC-3'were used to amplify, and add NheI/XhoI sites to mCherry-LC3B. Next, the fusion gene was digested with appropriate enzymes and cloned into AvrII/XhoI sites of pMSCV-Neo vector to generate pMSCV-mCherry-LC3B-Neo plasmid. PH-TRAF3 construct was generated as previously described (Ikonomov et al., 2006; Sasai et al., 2010).

The fusion plasmids were used to transfect the pT67 packaging cell line (Clontech), or the Gryphon amphotropic packaging cells (Allele Biotech), using the standard transfection protocol. Amphotropic retroviral particles encoding the desired fusion protein were produced from the selected cell populations, and these viral particles were collected and filtered prior to their use for transductions.

ELISA Cytokine Measurements

Human PBMCs: DNA-immune complexes used to stimulate human PBMCs were generated by combining human anti-histone polyclonal antibody (clone MD-14-0357, RayBiotech) or human polyclonal isotype control antibody (clone 7112H, Meridian Life Science) with Jurkat cell lysate. Antibodies were used at a 1:50 (v/v) final dilution unless otherwise indicated in the figure legend. Antibodies were mixed with 1.25% Jurkat cell lysates for 30 min. Jurkat cells lysates were prepared by pelleting cells and resuspending in 100μL sterile dH_20 per 40 x 10^6 cells. Cells were sonicated using a 550 Sonic Dismembrator sonicator (Fisher Scientific), cell debris were removed and supernatant volume was adjusted to 1mL RPMI with 10% (v/v) FBS. Human PBMCs were pre-treated with 500IU/mL of recombinant IFN-β for 30 min prior to stimulation. Following IFN-β pre-incubation, immune complexes were added to the cells and incubated for 20-22 hours at 37°C/5% CO_2. For pharmalogical inhibition of IFN-α and TNF-α production, serial dilutions of pharmacological inhibitors were prepared in RPMI 1640 supplemented with 10% (v/v) FBS and stored at 4 ºC until use. Heat aggregated human IgGs were generated by heating 12 mg/mL of human IgG (Talecris Biotherapeutics) for 30 minutes at 63ºC. IFN-β pre-treated human PBMCs were incubated with the inhibitors for 20 minutes at 37ºC/5% CO_2. DNA Immune complexes were then added for 20-22 hours at 37ºC/5% CO_2. IFN-α and TNF-α levels in harvested supernatants were evaluated with a multisubtype IFN-α ELISA kit (PBL Biomedical) or TNF-α human ELISA kit (Invitrogen) according to the manufacturer's protocol. Mouse pDCs: CG50 plasmid was made by cloning a previously described sequence that contains 50 optimal CpG motifs (Viglianti et al., 2003) into a pMK-RQ vector. The DNA antibody clone E11 was generated by phage library screening. Clone E11 showed high affinity binding to plate-bound DNA. Unless mentioned otherwise in the figure legend, DNA-immune complexes (DNA-IgGs) used to stimulate mouse pDCs were generated by combining 50 μg/ml of E11 DNA antibody and 0.5 μg/ml of CG50 plasmid DNA. Formation of DNA-immune complexes was achieved by incubating both components for 30 min on a plate shaker. Flt-3L-differentiated pDCs from bone marrow or fetal livers were resuspended at a concentration of 5 x 10^6/ml in fresh RPMI 1640 supplemented with 10% (v/v) FBS, 100U/ml penicillin, 100 μg/ml streptomycin and 2-Mercaptoethanol (2-ME, 1x). Recombinant mouse IFN-β (100 u/ml) was added to the cells 1 h prior to stimulation. CpG-ODN, E11 antibody and CG50 DNA were added to the cells at the indicated concentrations for 24 h and IFN-α and TNF-α levels were measure in supernatant by ELISA (R&D Systems).

siRNA Gene Silencing

Silencing of mouse ATG5, FIP200, ATG13, and FcRγ chain was achieved using ON-TARGET plus siRNA oligonucleotides from Dharmacon. siRNA oligonucleotides were delivered in cells using lipofectamine RNAi Max (Invitrogen) following manufacturer's recommendations.

Quantitative RT-PCR

Mouse macrophages: Total RNA was isolated from macrophages using RNeasy Plus Mini Kit (Qiagen) according to the manufacturer's recommendations. First strand cDNA synthesis was completed using the SuperScript III First Strand Synthesis (Invitrogen) and RT-PCR was completed with TaqMan Fast Universal PCR mix in a 7900HT thermocycler (Applied Biosystems) using the following PCR parameters: 40 cycles of 95°C for 21 seconds and 60°C for 20 seconds. mRNA expression was normalized against murine 18s allowing for comparison of mRNA levels. The following Applied Biosystems primers were utilized: mouse 18s (Mm03928990_g1), mouse TNFα (Mm00443260_g1), mouse IL-6 (Mm00446190_m1), and mouse IFNβ (Mm00439552_s1). Mouse pDCs: Total RNA was isolated from pDC cells using TRIzol (Gibco) according to the manufacturer's instructions. First strand synthesis was performed using M-MLV reverse transcriptase (Invitrogen). Real time PCR™ was performed using SYBR GREEN PCR master mix (Applied Biosystems), 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. mRNA expression was normalized against L32, allowing comparison of mRNA levels. The following primers were used: mouse TLR9 (forward primer: 5- GTACCCTGCCTGCCTTCCTAC-3; reverse primer: 5- GAACAGCCAATTGCAGTCCA-3), mouse MyD88 (forward primer: 5-CATGGTGGTGGTTGTTTCTGAC-3; reverse primer: 5-TGGAGACAGGCTGAGTGCAA– 3), mouse IRF7 (forward primer: 5- CACCCCCATCTTCGACTTCA-3; reverse primer: 5- CCAAAACCCAGGTAGATGGTGTA-3), and mouse L32 (forward primer: 5-

GAAACTGGCGGAAACCCA-3; reverse primer: 5-GGATCTGGCCCTTGAACCTT- 3). mRNA expression was normalized against murine actin allowing for comparison of mRNA levels.

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 (13.2k rpm, 15 minutes, 4°C), supernatants were analyzed by SDS-PAGE. Anti-TLR9 antibody was from Abcam, anti-MyD88 antibody was from Cell Signaling, anti-actin antibody (clone C4) was from MP Biomedicals and anti-ATG7, anti-LC3B, and anti-Beclin1 antibodies were from Cell Signaling. Anti-VAMP3 was from Synaptic Solutions. Anti-LAMP2 was from eBioscience. Anti-GFP antibodies were from Santa Cruz and Clontech.

Quantification of Phagocytosis and Fusion-Protein Translocation

The percentage of cells positive for phagocytosis was calculated by dividing the number of cells that had fully engulfed a bead by the total number of cells in the field of vision. Calculation is an average of four independent time-lapse movies, with more than 50 total cells counted per frame.

Quantification of fusion-protein translocation to the bead-containing phagosome was performed by acquiring 2-18 hour time-lapse movies and counting the number of fusion-protein-positive bead-containing phagosomes out of the total number of engulfed beads for that period. For each condition, three independent experiments were performed, with at least 25 phagosomes counted per experiment. At the end of each experiment, we confirmed that particles were completely internalized by differential focusing.

Supplemental References

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