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Supplemental Information

SWAP70 Organizes the Actin Cytoskeleton and Is Essential for Phagocytosis

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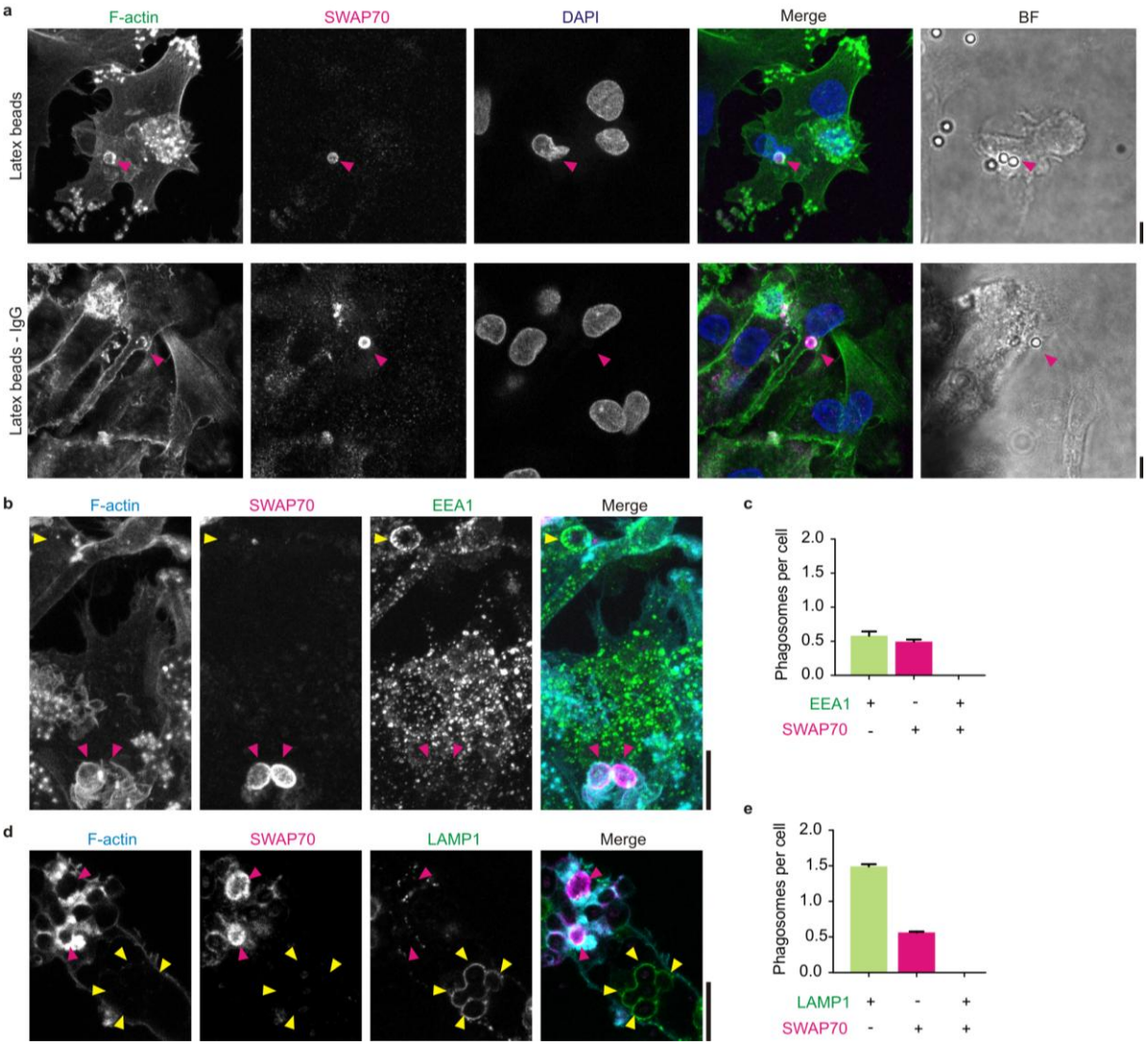

Figure S1; related to Figure 2. Phagosomal SWAP70 recruitment occurs prior to EEA1 and is independent of the type of antigen. (a) Confocal micrographs of dendritic cells pulsed with uncoated latex beads (top panels) or IgG-opsonized latex beads (bottom) immunostained for SWAP70 (magenta in merge) and F-actin (green). Pink arrowheads indicate double-positive phagosomes. Blue: DAPI. BF: bright field. (b–c) Dendritic cells pulsed with zymosan and immunostained for SWAP70 (magenta), EEA1 (green) and F-actin (cyan) (b) and quantification (c; >40 cells/condition for 3 donors; mean ± SEM). Arrowheads indicate SWAP70 (pink) and EEA1 (yellow) positive phagosomes. (d–e) Same as panels b–c but now for LAMP1 (green). No double-positive phagosomes containing both SWAP70 and EEA1 or LAMP1 were observed. Scale bars: 10 µm.

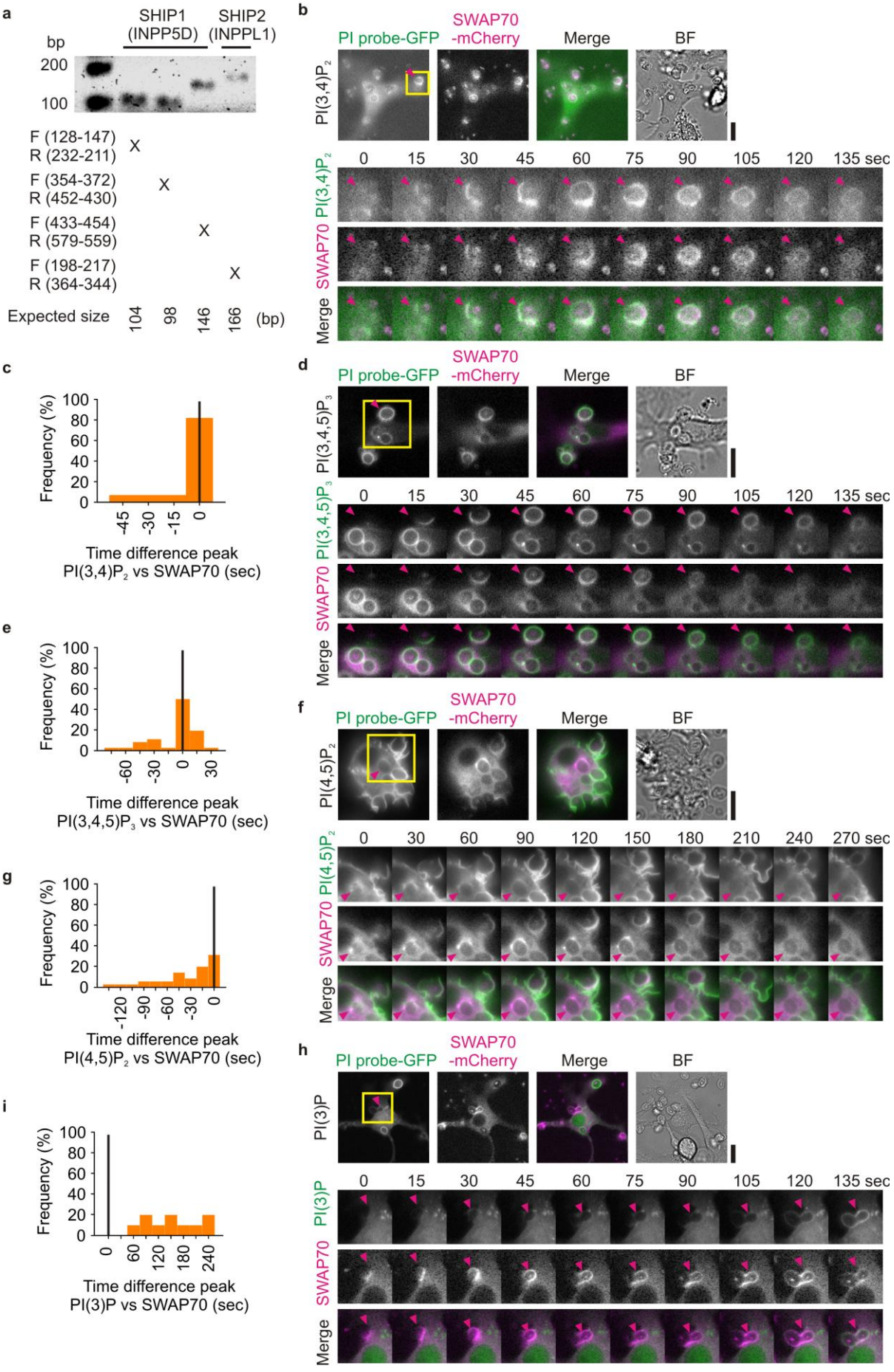
a
SHIP1 (INPP5D)
SHIP2 (INPPL1)
bp
200
100
F (128-147)
R (232-211)
F (354-372)
R (452-430)
F (433-454)
R (579-559)
F (198-217)
R (364-344)
Expected size
104
98
146
166
(bp)
b
PI probe-GFP
SWAP70 -mCherry
Merge
BF
PI(3,4)P2
0 15 30 45 60 75 90 105 120 135 sec
SWAP70
c
Frequency (%)
Time difference peak PI(3,4)P2 vs SWAP70 (sec)
d
PI(3,4,5)P3
e
Time difference peak PI(3,4,5)P3 vs SWAP70 (sec)
f
PI(4,5)P2
0 30 60 90 120 150 180 210 240 270 sec
g
Time difference peak PI(4,5)P2 vs SWAP70 (sec)
h
PI(3)P
i
Time difference peak PI(3)P vs SWAP70 (sec)

Figure S2; related to Figure 3. PI(3,4)P_2 mediates SWAP70 recruitment to the phagosome. (a) SHIP1 (INPP5D) and 2 (INPPL1) expression by PCR on cDNA from dendritic cells. The position of the forward (F) and reverse (R) primers as well as the expected fragment sizes are indicated. (b) Live cell imaging of dendritic cells pulsed with zymosan (arrowhead) and expressing a GFP-tagged PI(3,4)P_2-binding probe (the PH-domain of TAPP2 (PLEKHA2); green in merge) and SWAP70-mCherry (magenta). The inset shows a time series during zymosan uptake. See also Movie S10. BF: bright field. (c) Quantification from b. The histogram shows the time difference of peak recruitment of SWAP70-mCherry and the PI probe based on fluorescence intensities (>10 phagosomes). Negative values indicate that the PI probe was recruited prior to and positive values later than SWAP70-mCherry. (d–e) Same as b–c, but now for a PI(3,4,5)P_3-binding probe (PH-domain of AKT). See also Movie S11. (f–g) Same as b–c, but now for a PI(4,5)P_2-binding probe (PH-domain of PLCδ1 (PLCD1)). See also Movie S12. (h–i) Same as b–c, but now for a PI(3)P-binding probe (PX-domain of NCF4 (p40phox)). See also Movie S13. Scale bars: 10 µm.

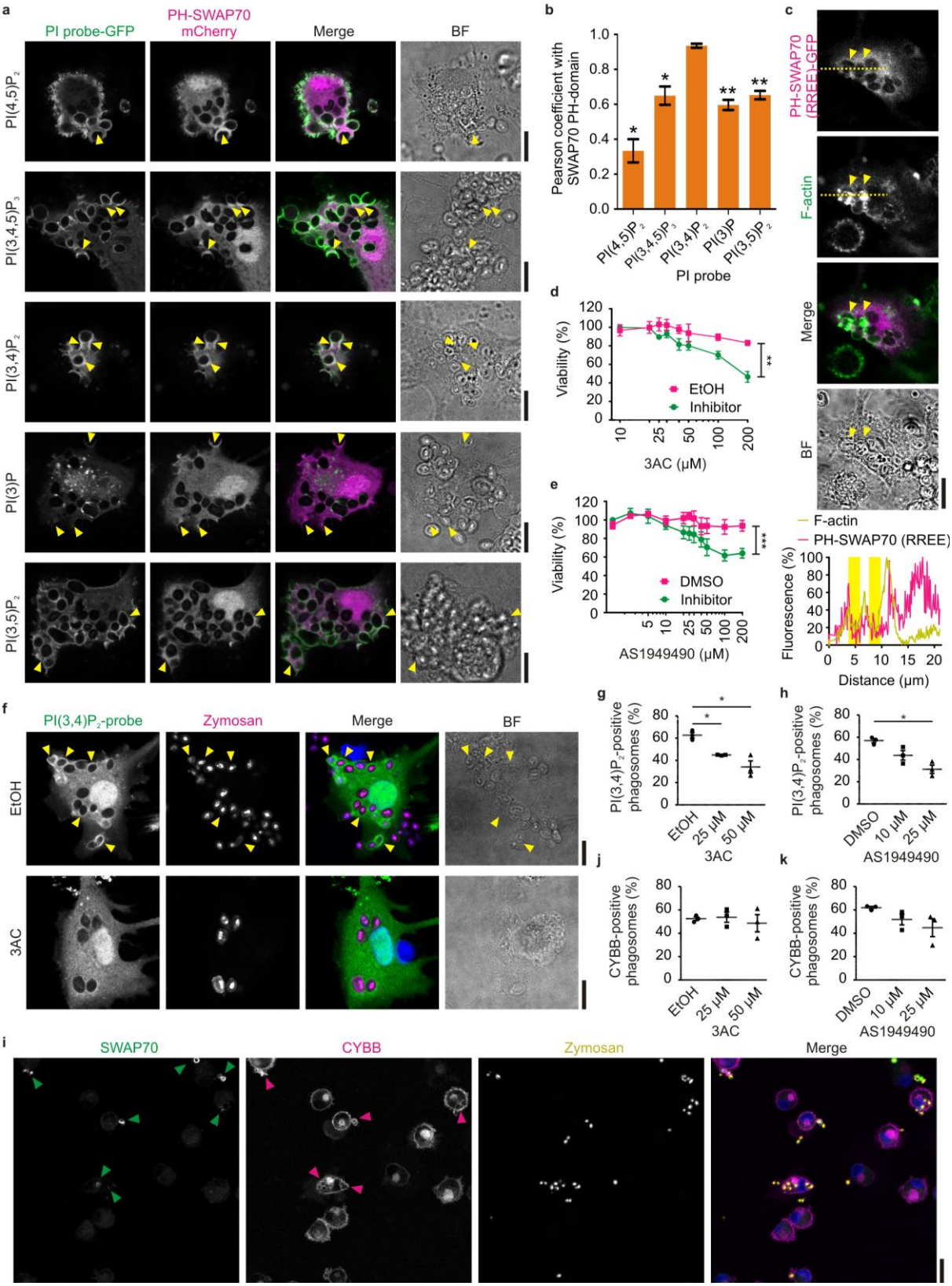

Figure S3; related to Figure 3. PH-domain mediates SWAP70 recruitment to the phagosome by PI(3,4)P_2. (a) Confocal images of zymosan-pulsed dendritic cells co-expressing GFP-tagged phosphoinositide probes (PI probe; green in merge) with the mCherry tagged PH-domain of SWAP70 (PH-SWAP70; magenta). Yellow arrowheads indicate PH-SWAP70-positive phagosomes. BF: bright field. (b) Pearson correlation coefficients for the PI probes and PH-SWAP70 from panel a (mean ± SEM; >25 phagosomes/condition for at least 3 donors). (c) Confocal image of zymosan-pulsed dendritic cells expressing GFP-tagged PH-SWAP70 (magenta) carrying

mutations R223E and R224E. Arrowheads: phagosomes positive for F-actin (green). No recruitment of mutant PH-SWAP70 to phagosomes was observed. (d) Cell viability of dendritic cells by the MTT assay for different concentrations of the SHIP1 (INPP5D) inhibitor 3AC (mean ± SEM for 3 donors). EtOH: ethanol solvent control. (e) Same as panel d, but now for the SHIP2 (INPPL1) inhibitor AS1949490. DMSO: solvent control. (f) Confocal image of zymosan-pulsed (magenta) dendritic cells expressing the $PI(3,4)P_2$ probe (green) in presence or absence of 25 µM 3AC. DAPI is blue in merge. (g) Quantification of phagosomes positive for the $PI(3,4)P_2$ probe for the 3AC concentrations indicated (individual donors shown; >30 phagosomes/donor/condition; mean ± SEM). (h) Same as panel g, but now for AS1949490. (i) Confocal image of dendritic cells pulsed with zymosan (yellow) and immunolabeled for SWAP70 (green) and CYBB (gp91phox; magenta). Cells were treated with 10 µM AS1949490. DAPI is blue in merge. (j) Quantification of gp91phox-positive phagosomes for the 3AC concentrations indicated (individual donors shown; >80 phagosomes/donor/condition; mean ± SEM). (k) Same as panel j, but now for AS1949490. Scale bars: 10 µm (a, c, f) and 20 µm (i).

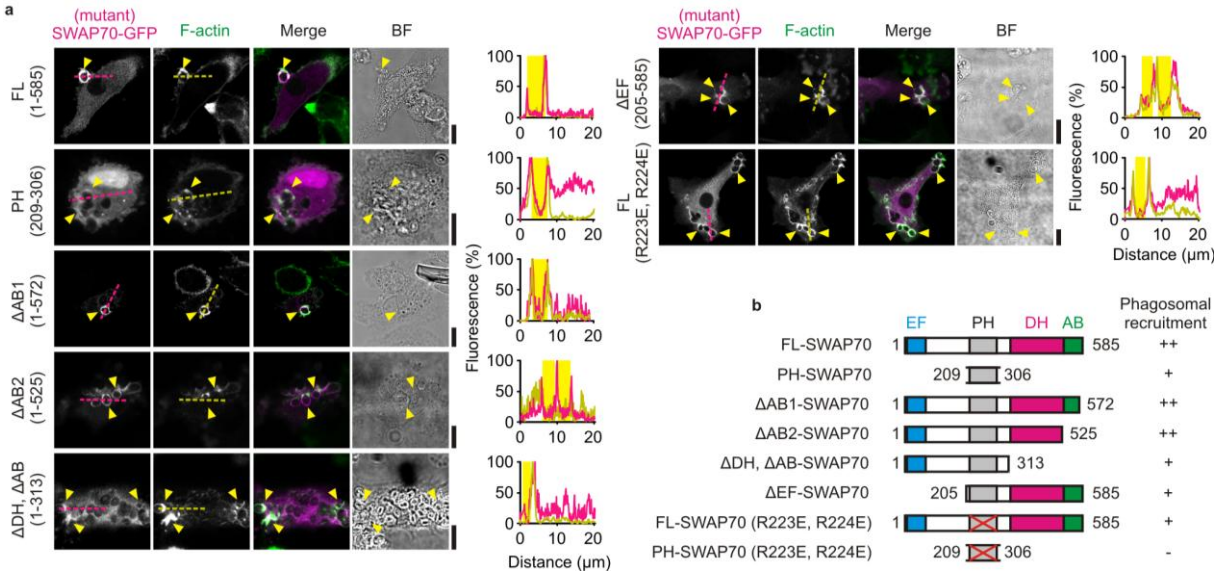

Figure S4; related to Figure 3; Phagosomal SWAP70 recruitment by phosphoinositides and RAC1but not or less by F-actin. (a) Representative confocal micrographs of zymosan-pulsed dendritic cells overexpressing full-length or mutant SWAP70 N-terminally fused to GFP (magenta in merge). F-actin was labeled with phalloidin (green). Yellow arrowheads indicate F-actin-rich phagosomes. The graphs show the intensity plot profiles as indicated. The position of phagosomes is indicated by the yellow shaded areas. FL: full-length SWAP70 (residues 1–585); PH: PH-domain of SWAP70 (209–306); ΔAB1: C-terminal truncation mutant of SWAP70 with part of its actin-binding (AB) domain removed (1–572); ΔAB2: C-terminal truncation mutant of SWAP70 with its entire AB domain removed (1–525); ΔDH, ΔAB: C-terminal truncation mutant of SWAP70 with its putative Dbl-homology (DH) domain (Shinohara, et al. (2002) Nature. *416*, 759-763) and AB domain removed (1–313); ΔEF: N-terminal truncation mutant of SWAP70 with its EF-hand motif removed (205–585); FL (R223E, R224E): full-length SWAP70 carrying mutations R223E and R224E. BF: bright field. Scale bars: 10 μm (b) Domain topologies of the tested mutants and summary of their phagosomal recruitment as judged from the confocal images. Representative images for the PH-domain of SWAP70 (PH-SWAP70) carrying mutations R223E and R224E are in figure S3c. –: no association; +: some association; ++: regular association.

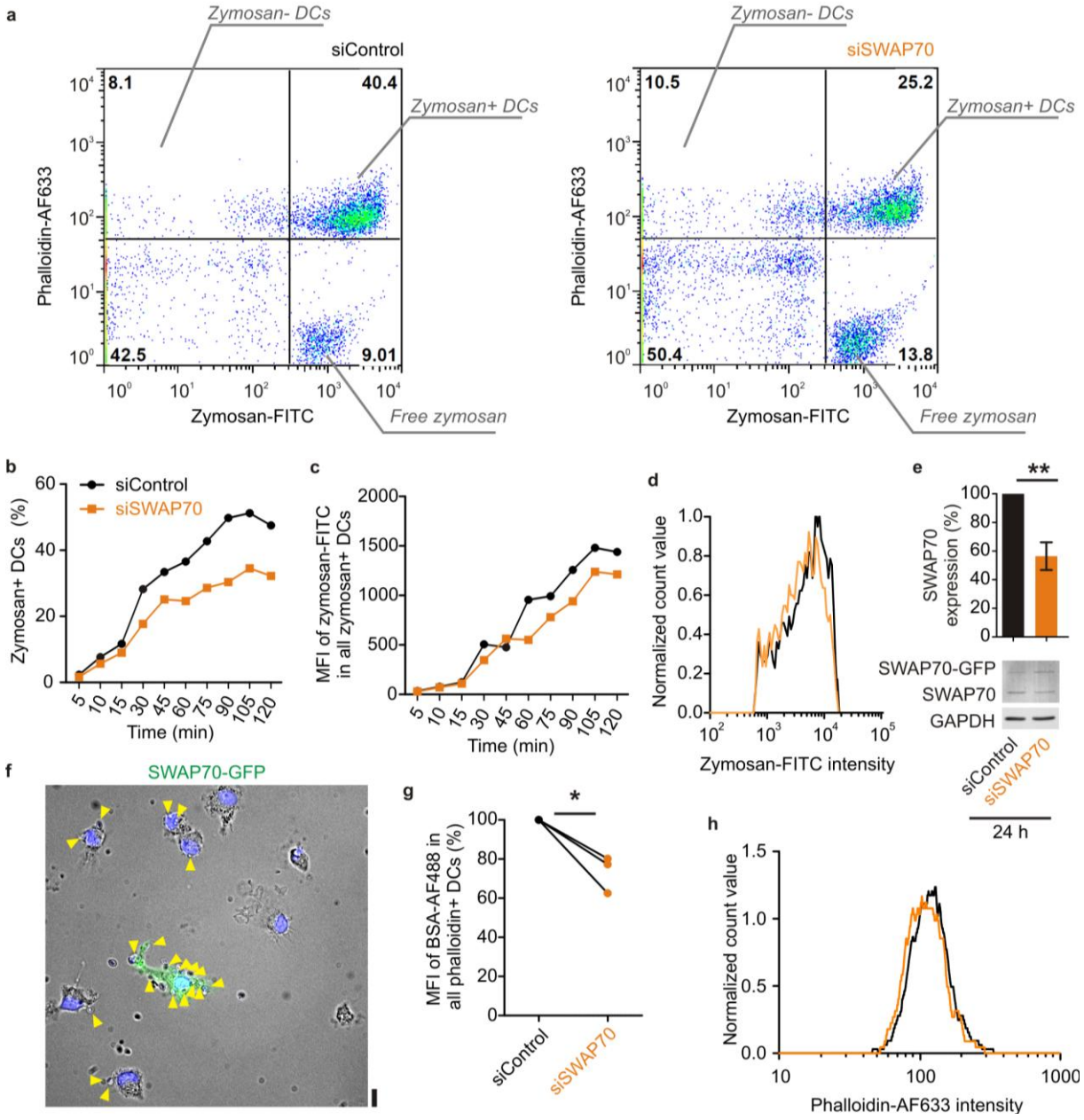

Figure S5; related to Figure 4. SWAP70 regulates phagocytosis via F-actin. (a) FACS plot of dendritic cells transfected with control (siControl) or SWAP70 siRNA (siSWAP70). Cells were pulsed with FITC-labeled zymosan (zymosan-FITC) for 60 min, fixed and stained with phalloidin conjugated to Alexa Fluor 633 (phalloidin-AF633). (b) Percentage of zymosan-phagocytosing cells relative to total cells for a representative donor with siControl (black) or siSWAP70 (orange). (c) Mean fluorescence intensity (MFI) of zymosan-FITC for the zymosan-positive cells from panel b. (d) Fluorescence distribution of the zymosan-FITC signal of zymosan-positive cells for a representative donor 60 min after uptake. (e) SWAP70 knockdown efficiency for the rescue experiments. siRNA was transfected simultaneously with plasmid encoding SWAP70-GFP and knock-down levels were determined 24 h post-transfection by Western blot (mean ± SEM for 3 donors). GAPDH: loading control. (f) Epi-fluorescence microscopy image of rescue experiment. Note that the cell expressing SWAP70-GFP (green) contains more zymosan particles (arrowheads) than the surrounding cells without rescue. Scale bar, 10 µm. (g) Uptake of BSA labeled with Alexa Fluor 488 (BSA-AF488) by FACS with siControl or siSWAP70 for 3 different donors. Cells were pulsed with BSA for 60 min. (h) Fluorescence distribution of phalloidin-AF633 staining in siControl or siSWAP70 for a representative donor 15 min after zymosan uptake.

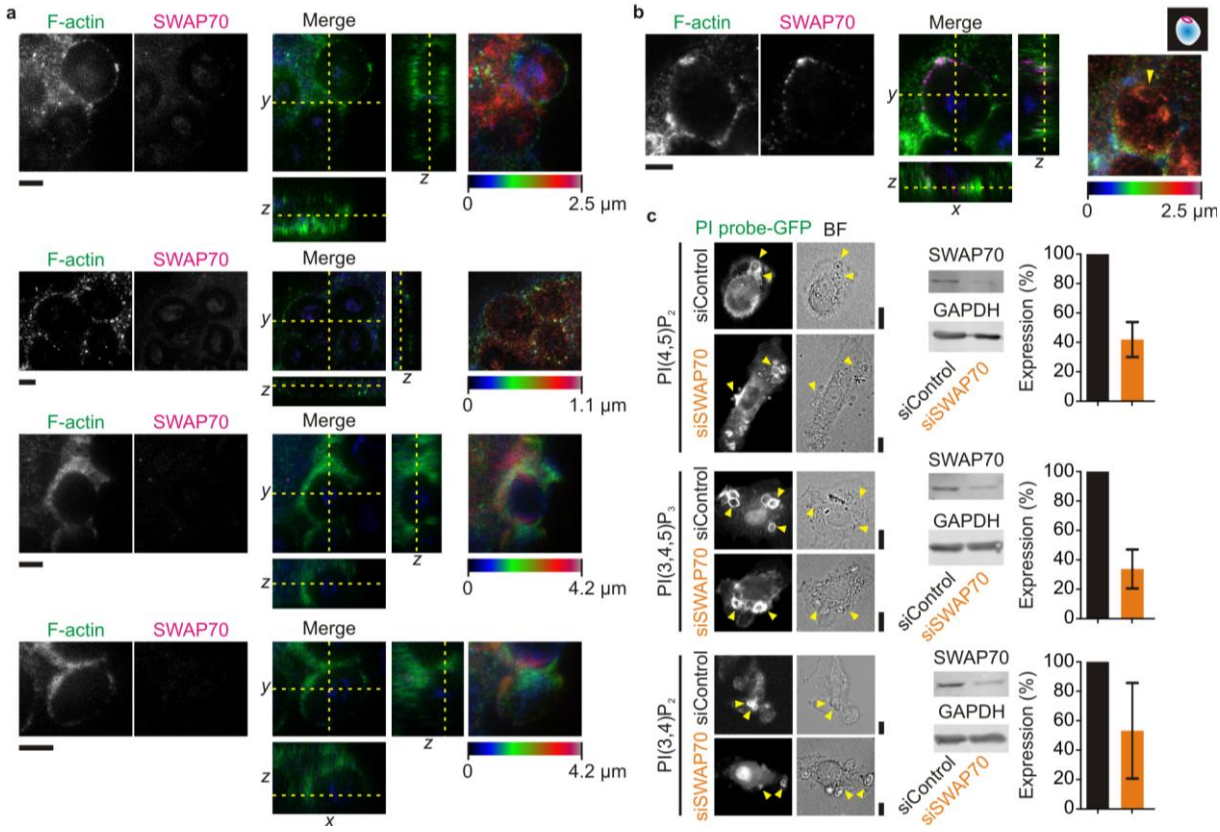

Figure S6; related to Figure 4. SWAP70 knockdown affects the phagosomal F-actin cage, phosphoinositides are still present. (a) Super-resolution multicolor 3D-STED microscopy of dendritic cells with siRNA knockdown of SWAP70. Cells were pulsed with zymosan (blue in merge) and immunostained for F-actin (green) and SWAP70 (magenta; the SWAP70 signal is absent due to the knockdown). Shown are cross-sections (left), orthogonal sections (middle; indicated by the dashed yellow lines) and a depth-encoded maximum intensity height map of the F-actin (right) for 4 representative phagosomes. Note the absence of parallel arches or ring-like structures of F-actin on the surface of the phagosomes. (b) Same as a, but now with non-targeting siRNA control. Arrowhead: ring-like structure of F-actin on the surface of the phagosome (depicted in the inset). (c) GFP-tagged phosphoinositide probes (PI probe-GFP; green in merge) were co-expressed with non-targeting siRNA (siControl) or SWAP70 siRNA (siSWAP70) in zymosan-pulsed dendritic cells. The PI probes for $PI(4,5)P_2$ (PH-domain of PLCδ1 (PLCD1)), $PI(3,4,5)P_3$ (PH-domain of AKT) and $PI(3,4)P_2$ (PH-domain of TAPP2 (PLEKHA2)) were used. Left: representative confocal images (from 3–5 donors/condition). Arrowheads: PI probe-positive phagosomes. BF: bright field. Right: representative Western blots and quantifications of knockdown efficiencies (mean ± SEM). GAPDH: loading control. Scale bars: 2 µm (a, b), 10 µm (c).

Legends to Supplemental Movies

Movie S1; related to Figure 1. Live cell imaging of SWAP70-GFP and LifeAct-RFP recruitment to the phagosome. Time-lapse epi-fluorescence imaging of dendritic cells transiently expressing SWAP70-GFP (green) and LifeAct-RFP (magenta) and pulsed with IgG-opsonized zymosan. Images were acquired every 20 sec. BF: bright field. Scale bar, 5 µm.

Movies S2–6; related to Figure 1. Ultrastructure of SWAP70 on the phagosomal surface by multicolor 3D-STED microscopy. Dendritic cells were pulsed with zymosan (blue; autofluorescent core) and immunostained for SWAP70 (magenta). Total membranes were labeled with the fixable endocytic marker mCLING (green; Revelo, et al., 2014). The bright mCLING-labeled structures surrounding the phagosome are endo/lysosomal compartments. The movies show 3D-rotations of single SWAP70-positive phagosomes. Note the alignment of SWAP70 in concentric fibers (movies 2–4) and rings (movies 5–6) on the surface of the phagosomes. The ventral membrane is positioned on the surface of the microscope cover glass; the dorsal membrane is facing away from it. Scale bars, approximately 2 µm.

Movie S7; related to Figure 1. SWAP70 and F-actin alignment on the phagosomal surface by multicolor 3D-STED microscopy. Dendritic cells were pulsed with zymosan (blue; autofluorescent core) and immunostained for SWAP70 (magenta) and actin (green). The movie shows a 3D-rotation of a single SWAP70-positive phagosome. Note the alignment of SWAP70 and F-actin on the surface of the phagosomes. Yellow lines indicate the orientation of F-actin and SWAP70-positive filamentous structures. Scale bar, approximately 2 µm.

Movie S8; related to Figure 2. Polarized recruitment of SWAP70 to the nascent phagosomal cup. 3D-confocal stack of a nascent phagosome. FITC-labeled zymosan (green) was phagocytosed by dendritic cells. Uninternalized zymosan and nascent cups were labeled with an antibody directed against FITC (blue). SWAP70 was immunolabeled (magenta). The movie shows a 3D-rotation of a single SWAP70-positive nascent phagosomal cup. Scale bar, approximately 2 µm.

Movie S9; related to Figure 2. Live cell imaging of SWAP70-GFP and RAB5A-RFP recruitment to the phagosome. Time-lapse epi-fluorescence imaging of dendritic cells transiently expressing SWAP70-GFP (green) and RAB5A-RFP (magenta) and pulsed with IgG-opsonized zymosan. Images were acquired every 15 sec. BF: bright field. Scale bar, 5 µm.

Movie S10; related to Figure 3. Live cell imaging of the phosphoinositide probe for PI(3,4)P_2 and SWAP70-mCherry recruitment to the phagosome. Time-lapse epi-fluorescence imaging of dendritic cells transiently expressing the GFP-tagged PH-domain of TAPP2 (PLEKHA2) (binds to PI(3,4)P_2; green) and SWAP70-mCherry (magenta) and pulsed with IgG-opsonized zymosan. Images were acquired every 15 sec. BF: bright field. Scale bar, 5 µm.

Movie S11; related to Figure 3. Live cell imaging of the phosphoinositide probe for PI(3,4,5)P_3 and SWAP70-mCherry recruitment to the phagosome. Time-lapse epi-fluorescence imaging of dendritic cells transiently expressing the GFP-tagged PH-domain of AKT (binds to PI(3,4,5)P_3; green) and SWAP70-mCherry (magenta) and pulsed with IgG-opsonized zymosan. Images were acquired every 15 sec. BF: bright field. Scale bar, 5 µm.

Movie S12; related to Figure 3. Live cell imaging of the phosphoinositide probe for PI(4,5)P_2 and SWAP70-mCherry recruitment to the phagosome. Time-lapse epi-fluorescence imaging of dendritic cells transiently expressing the GFP-tagged PH-domain of PLCδ1 (PLCD1; binds to PI(4,5)P_2; green) and SWAP70-mCherry (magenta) and pulsed with IgG-opsonized zymosan. Images were acquired every 15 sec. BF: bright field. Scale bar, 5 µm.

Movie S13; related to Figure 3. Live cell imaging of the phosphoinositide probe for PI(3)P and SWAP70-mCherry recruitment to the phagosome. Time-lapse epi-fluorescence imaging of dendritic cells transiently expressing the GFP-tagged PX-domain of NCF4 (p40phox; binds to PI(3)P; green) and SWAP70-mCherry (magenta) and pulsed with IgG-opsonized zymosan. Images were acquired every 15 sec. BF: bright field. Scale bar, 5 µm.

Movie S14; related to Figure 5. Live cell imaging of SWAP70-GFP and RAC1-RFP recruitment to the phagosome. Time-lapse epi-fluorescence imaging of dendritic cells transiently expressing SWAP70-GFP (green) and RAC1-RFP (magenta) and pulsed with IgG-opsonized zymosan. Images were acquired every 15 sec. BF: bright field. Scale bar, 5 µm.

Movie S15; related to Figure 5; SWAP70 and RAC1 alignment on the phagosomal surface by multicolor 3D-STED microscopy. Dendritic cells were pulsed with zymosan (blue; autofluorescent core) and immunostained for SWAP70 (magenta) and RAC1 (green). The movie shows a 3D-rotation of a single SWAP70-positive phagosome. Note the alignment of SWAP70 and RAC1on the surface of the phagosomes. Yellow lines indicate the orientation of SWAP70 and RAC1-positive filamentous structures. Scale bar, approximately 2 µm.

Movie S16; related to Figure 5; RAC1and F-actin alignment on the phagosomal surface by multicolor 3D-STED microscopy. Dendritic cells were pulsed with zymosan (blue; autofluorescent core) and immunostained for RAC1 (magenta) and actin (green). The movie shows a 3D-rotation of a single SWAP70-positive phagosome. Note the alignment of RAC1and F-actin on the surface of the phagosomes. Yellow lines indicate the orientation of RAC1and F-actin-positive filamentous structures. Scale bar, approximately 2 µm.

Supplemental Experimental Procedures

Particle preparation and phagocytosis assays

IgG-coated latex beads were produced by incubating 0.3% streptavidin bead suspension with 1.3 mg ml^{-1} biotin-SP (long spacer) Chrompure human IgG (Jackson ImmunoResearch; 009-060-003) for 30 min at 37°C followed by extensive washing. The beads were added at a 1:10 cell-to-beads ratio. IgG opsonized zymosan particles were produced by incubating 20 mg/ml zymosan suspension with an equal volume of Opsonizing solution from ThermoFisher (Z2850) for 60 min at 37°C followed by extensive washing. FITC-labeled zymosan was purchased from ThermoFisher (Z2841). Unlabeled zymosan was from Sigma (Z4250-1G). The same zymosan was labeled with Alexa fluor 633 maleimide (Life Technologies) by incubating 16.4 mg ml^{-1} with 77 µM in 0.2 M Na-carbonate at pH 9.0 for 1 hour followed by vigorous washing. BSA Alexa fluor 488 conjugate was from ThermoFisher (cat. No. A13100). For the SHIP1 (INPP5D) or SHIP2 (INPPL1) inhibition, cells were incubated with zymosan at 1:10 cell-to-particles and either with 3AC (Echelon; B-0341; 10–200 µM in ethanol) or AS1949490 (Echelon; B-0342; 1.25–200 µM in DMSO).

Cell viability assay

For cell viability, 0.66 mg/ml MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma M2128-1G) was applied to the cells together with 3AC or AS1949490 for 2 h. The MTT reaction was stopped with lysis buffer containing 90% isopropanol, 0.0125% SDS and 0.04 M HCl. Absorbance was measured at 595 nm to determine cell death.

SiRNA knock-down

Knockdown in human dendritic cells was performed as described (Dingjan et al., 2016), except that 3 day differentiated monocytes were used for transfection and samples were used 24-72 h post-transfection. A mix of 3 siRNAs was used (Life Technologies): GCCUU CAGAC UCAAG UGGAA CUUCA, AAAGA AGCUG GAGAU GGCAA CUAAU, and CAGAA GAGAU UGAAU ACCUG CUUAA. Control samples were transfected with irrelevant ON-TARGET plus non-targeting (NT) siRNA (Dharmacon). For the rescue experiments and the combination of siRNA with phosphoinositide probes, day 6 differentiated monocytes were simultaneously transfected with siRNA and plasmid DNA and used 24 h post-transfection. For all experiments, only samples with knockdown levels above 70% (72 h post-transfection) or 40% (24 h) were used.

Immunofluorescence

For immunofluorescence, cells were incubated with zymosan at 1:10 cell-to-zymosan ratio in serum-free medium for 15 to 60 minutes. The cells were subsequently fixed with 4% PFA in PBS and permeabilized in S-PBA (0.1% Saponin, 0.5% BSA and 0.01% NaN_3 in PBS) for 5 min. Primary and secondary antibodies were incubated in S-PBA. Cells were embedded in DAPI containing mounting medium containing 0.01% (v/v) 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid and 68% (v/v) glycerol in 200 mM sodium phosphate buffer at pH 7.5. In order to label the phagocytic cups, FITC-labeled zymosan was used and samples were labeled with an antibody directed against FITC prior to permeabilization.

PCR

The total mRNA from day 6 differentiated monocytes was isolated with the Quick-RNA MiniPrep kit (Zymo Research, R1055) and reversely transcribed into cDNA. SHIP1 (INPP5D) mRNA was amplified by PCR with 3 forward primers (nucleotides 128–147: GCGTG CTGTA TCGGA ATTGC; 354–372: AAGTG TCGTG TCTCC ACCC; 433–454: TTTTC AAACG AGAAT CCCCG AG) and 3 reverse primers (232–211: TGGTG AAGAA CCTCA TGGAG AC; 452–430: CGGGG ATTCT CGTTT GAAAA AGG; 579–559: GGCGA GCTGA GTGCT TAAAT A). SHIP2 (INPPL1) mRNA was amplified with one forward primer (198–217: GCACA CGTAT CGCAT TCTGC) and reverse primer (364–344: CTCGC TCACC CTCTA CAGGA A).

Supplemental References

Dingjan, I., D.R. Verboogen, L.M. Paardekooper, N.H. Revelo, S.P. Sittig, L.J. Visser, G.F. Mollard, S.S. Henriet, C.G. Figdor, M. Ter Beest, and G. van den Bogaart. (2016) Lipid peroxidation causes endosomal antigen release for cross-presentation. Sci. Rep. *6*, 22064.

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