Supplemental Material

Methods

Culture of Human Monocyte-Derived Macrophages

Human monocytes were purified by counterflow centrifugal elutriation of mononuclear cells from normal human donors. Cells were plated at a density of 2 X 10^5 monocytes/cm^2 in RPMI 1640 (MediaTech) containing 10% FBS (Gibco) in 6-well Cellbind plates (Corning). After a 2 h incubation at 37°C with 5% CO_2/95% air, cells were rinsed three times with RPMI 1640 and cultured for 5 days with complete medium: RPMI 1640 containing 10% FBS, 50 ng/ml M-CSF (Peprotech) and 25 ng/ml IL-10 (Peprotech).[1] Cells were then rinsed three times with RPMI 1640 and cultured further with complete medium. After 7 days in culture, experiments were performed with serum-free RPMI, 50 ng/ml M-CSF, 25 ng/ml IL-10, and the indicated addition. For experiments assessing the effect of SU6656 (EMD) and toxin B (EMD) on LDL uptake and cholesterol accumulation, macrophages were pretreated 5 h with the indicated agent. To assess LDL uptake in the presence of SU6656 and/or bafilomycin A1 (EMD), macrophages were pretreated 1 h with the indicated agent. For experiments assessing the effect of PP1 (Enzo), PP2 (EMD) and SU6656 on LDL uptake, macrophages were not pretreated with the indicated agent prior to incubation with LDL.

Dialysis of Lipoproteins

Unlabeled LDL (Intracel) and ^{125}I-LDL (BTI) were dialyzed separately in a 10 kDa Slide-A-Lyzer Cassette (Pierce) against 1 liter of RPMI 1640 with one medium change.

After 24 h of dialysis, LDL was removed from cassettes and filtered through sterile Acrodisc low protein-binding 0.45 μm filters (Pall). For experiments with ^{125}I-labeled LDL, specific activity was adjusted to 2.25 X 10^{-5} μCi/ng.

Analysis of ^{125}I –LDL Cell Association and Degradation

Macrophage cell association and degradation of ^{125}I-LDL was determined according to the method of Goldstein *et al* .[2] Culture media samples were centrifuged at 15,000 X *g* for 10 min and trichloroacetic acid-soluble organic iodide radioactivity was measured to quantify lipoprotein degradation.

To measure cell-associated ^{125}I-LDL, macrophages were rinsed three times with Dulbecco's phosphate-buffered saline (DPBS) containing Ca^{2+}, Mg^{2+}, and 0.2% bovine serum albumin (BSA), followed by three rinses with DPBS containing Ca^{2+} and Mg^{2+} all at 4°C. Macrophages were dissolved overnight in 0.1 N NaOH at 37°C and then assayed for ^{125}I radioactivity with a γ counter. ^{125}I radioactivity values for wells incubated with ^{125}I-LDL without macrophages were subtracted from ^{125}I radioactivity values obtained for macrophages incubated with ^{125}I-LDL. Values were <1% of cell-associated for ^{125}I-LDL. For protein quantification, a small aliquot of cell lysate was measured using the Lowry method[3] with a BSA standard. ^{125}I-LDL uptake is presented as the sum of cell-associated ^{125}I-LDL and degraded ^{125}I-LDL.

Quantification of Macrophage Cholesterol

Cells were rinsed three time in DPBS containing Mg^{2+} and Ca^{2+}, lysed with 1 ml of ultrapure water per well, and then detached with a cell scraper. Lipid was isolated using the Folch method,[4] and cholesterol was quantified as previously described by Gamble *et al.*[5] For protein quantification, a small aliquot of cell lysate was measured using the Lowry method[3] with a BSA standard.

Western Blot Analysis

Macrophages were lysed in radioimmunoprecipitation (RIPA) buffer (Pierce). Lysates were diluted with Laemmli Sample Buffer and loaded onto 4-15% Tris-HCl polyacrylamide gradient gels (BioRad). After SDS-PAGE, protein was transferred to polyvinylidene fluoride membranes and then incubated overnight at 4°C in Tris Buffered Saline containing 0.1% Tween-20, 5% w/v nonfat dry milk, and primary antibody. The following primary antibodies were from Cell Signaling: rabbit anti-Fyn (catalog number 4023), rabbit anti-Blk (catalog number 3262), rabbit anti-Fgr (catalog number 2755), and rabbit anti-Yes (catalog number 2734) (all diluted 1:1000). Mouse anti-Rak (catalog number sc-166478; diluted 1:100), mouse anti-Lyn (catalog number sc-7274; diluted 1:200), rabbit anti-Hck (catalog number sc-72; diluted 1:200), rabbit anti-Brk (catalog number sc-1188; diluted 1:200), mouse anti-Lck (catalog number sc-433; diluted 1:200), and mouse anti-c-Src (catalog number sc-5266; diluted 1:200) were all from Santa Cruz Biotechnology. After incubation with primary antibody, membranes were washed an incubated overnight with either 0.08 μg/ml goat anti-rabbit IgG-HRP (Santa Cruz Biotechnology) or 0.2 μg/ml goat anti-mouse IgG-HRP (Santa Cruz Biotechnology).

Detection of proteins was determined by chemiluminescence using Western Blotting Luminol Reagent (Santa Cruz Biotechnology). Positive control cell lysates were as follows: 293T cells (Fyn), Namalwa cells (Blk), U-87 MG (Yes), human Rak-transfected cells (Rak), HeLa Cells (Lyn), SW480 cells (Brk), CCRF-HSB-2 cells (Lck), WEHI-231 cells (c-Src), and HL-60 (Hck), Raji Cells (Fgr). Raji Cell lysate was from Genescript. All other control lysates were from Santa Cruz Biotechnology.

Quantification of Fluorescent Micropinocytosis Cholera Toxin Tracer

Macrophage cultures were pretreated in complete medium containing the indicated agent for 1 h. Then, macrophages were incubated with 10 μg/ml Alexa Fluor 488® conjugated-cholera toxin subunit B (Invitrogen) in the presence of the indicated agent for 5 h. Incubation of macrophages containing the indicated agent without cholera toxin was used to control for cell and drug fluorescence. Samples were lysed with 1 ml of ultrapure water per well, and then detached with a cell scraper. Fluorescence was measured with a spectrofluorometer using an excitation wavelength of 488 nm and an emission wavelength of 515 nm. The amount of cholera toxin taken up by macrophages was determined using a standard curve to relate cholera toxin fluorescence and concentration. Cellular protein quantification was determined using the Lowry method[3] with a BSA standard.

Time-lapse Microscopy

Macrophages cultures were observed by time-lapse phase-contrast digital video microscopy with a 20X long working distance panfluor objective lense (0.3 N.A.)

mounted on an Olympus L70 inverted microscope. Cultures were maintained in an enclosed LiveCell™ chamber (Pathology Devices) containing 5% CO_2/95% hydrated air at 37°C. Images were aquired every 10 s for 30 min. The acquired 180 images were converted into digital movies created using IP Lab software (Becton Dickonson). When viewed at standard rates (*i.e.* 10 frames/s), movies are 100X real time.

Microscopic Analysis of Fluid-phase Pinocytosis Pathways

Macrophage cultures were pretreated with complete medium containing the indicated agent for 4.5 h. Medium was then replaced with DMEM containing 10 mM HEPES (Sigma) and 2 mg/ml mannan (to saturate the mannose receptor)[6, 7] (Sigma), with or without inhibitor and then incubated 30 min. Next, macrophages were incubated 10 min with the fluid-phase tracer horseradish peroxidase (HRP) (1 mg/ml) (Sigma) or HRP-conjugated cholera toxin (10 μg/ml) (Invitrogen) in the same medium. These procedures were adapted from previously described methods.[8, 9] All incubations were performed at 37°C with 5% CO_2/95% air. Subsequently, macrophages were rinsed three times with cold (4°C) DPBS containing Ca^{2+} and Mg^{2+}, then fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) overnight at 4°C. After fixation, macrophages were rinsed with 0.1 M cacodylate buffer (pH 5.1) and incubated 30 min with 2 mg/ml diaminobenzidine (Sigma) in the same buffer, and then incubated 60 min with 2 mg/ml diaminobenzidine containing 0.01% H_2O_2. Then, macrophages were rinsed with 0.1 M cacodylate buffer (pH 5.1) and removed from culture dishes by scraping with cacodylate buffer (pH 5.1). Macrophages were pelleted (1,500 *g* for 5 min, 4°C), then fixed 1 h with 2% OsO_4. After each subsequent step (up to embedding), macrophages were pelleted by

centrifugation. Macrophages were then progressively dehydrated with 70%, 95%, and 100% ethanol. Samples were infiltrated sequentially with 1:1 ethanol and Spurr mixture for 2 h, 1:3 ethanol Spurr mixture for 2 h, and then with pure Spurr mixture overnight. Macrophage pellets were then embedded and polymerized at 70°C. Thick and thin sections without counter-stain were analyzed using a Zeiss Axiophot light microsope or a Joel 1200EX electron microscope, respectively.

To directly visualize LDL entering macrophages by fluid-phase pinocytosis, macrophages were incubated with 5 mg/ml LDL, fixed, embedded in LR White resin, and prepared for immunogold labeling of LDL as described previously[10] except that 1% dry skim milk instead of 1% BSA was used to block nonspecific staining. Thin sections were labeled with 10 μg/ml affinity-purified rabbit anti-human LDL antibody (catolog number BT-905; Biomedical Technologies, Inc.), and then with a 1:10 dilution of 10 nm gold-conjugated goat $F(ab)_2$ anti-rabbit IgG (BBInternational). As a control, macrophages incubated with 5 mg/ml LDL were labeled with 10 μg/ml affinity-purified rabbit anti-GFP antibody (catalog number 14-6774; eBioscience). Alternatively, macrophages were incubated without LDL and subsequently labeled with the anti-human LDL antibody. All sections were counter-stained with lead and analyzed with a Joel 1200EX electron microscope.

Statistical Analysis

Data are presented as the mean ± standard error of the mean. Means were representative of three replicate wells. A Student's *t* test was utilized for statistical comparisons, with a *p*-value of less than 0.05 considered significant.

References

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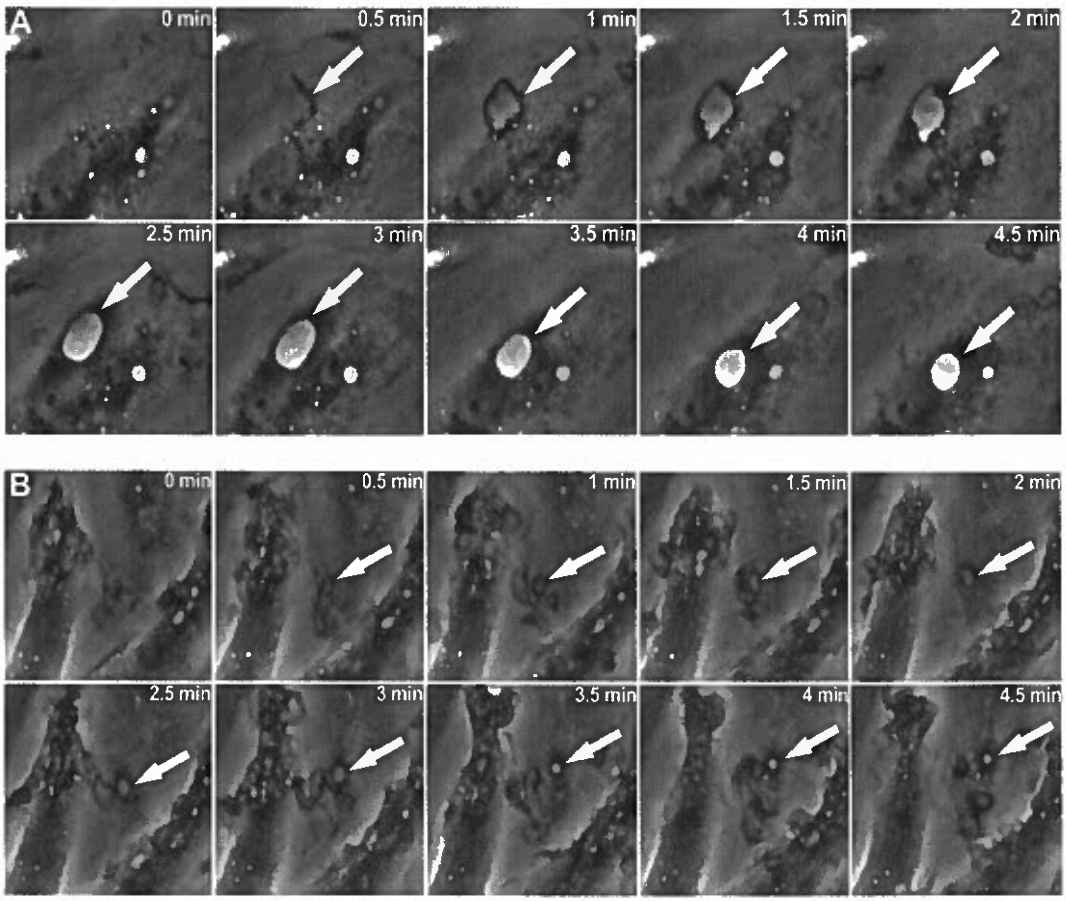
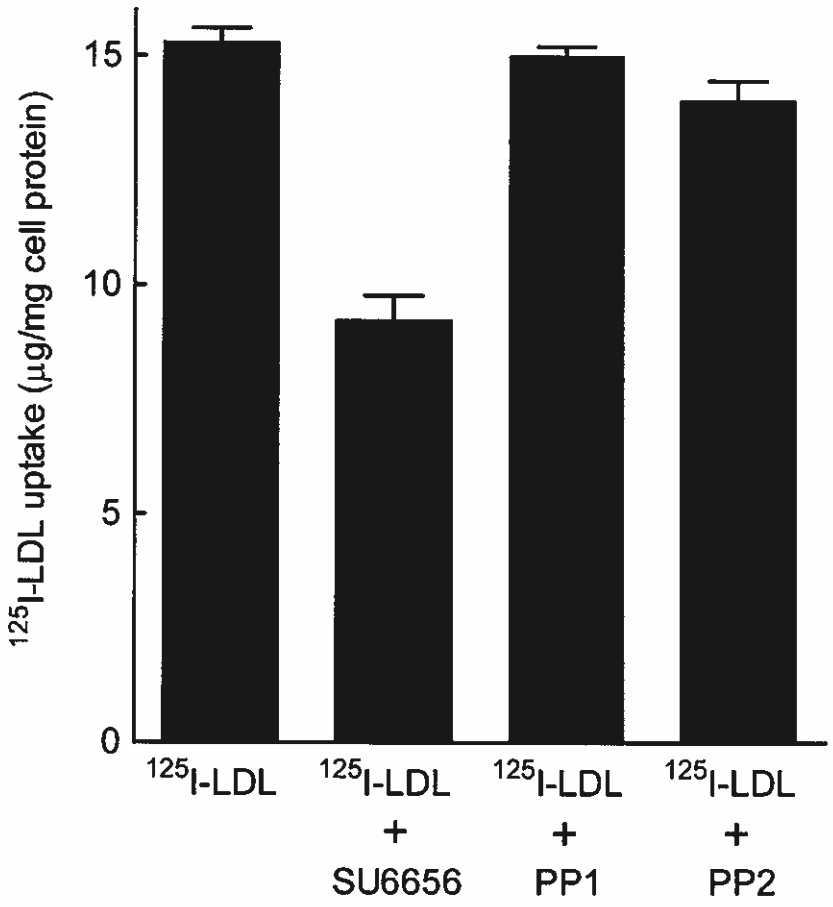
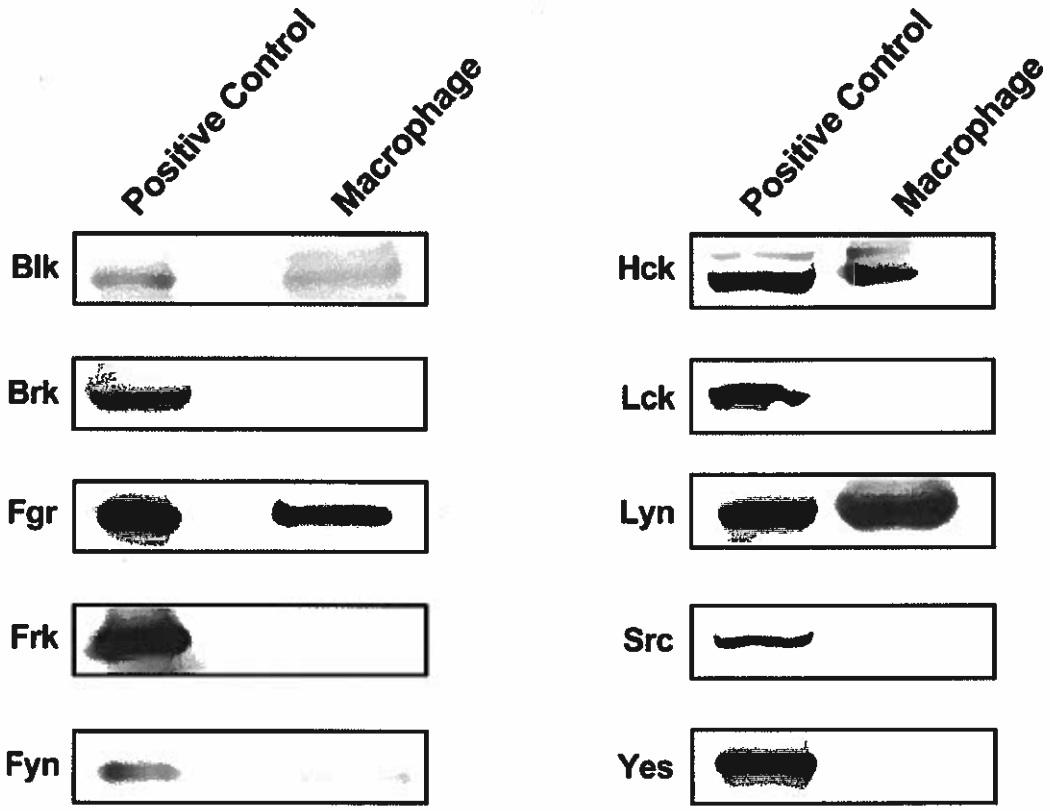


Supplemental Figure I. Time-lapse phase-contrast digital microscopy of macrophage macropinocytosis. Two fields (A and B) were observed for macropinosome formation. Arrows indicate macropinosome formation. The width of each frame is 50 μm.

Supplemental Figure II. SU6656 inhibits macrophage macropinocytosis. Macrophages were treated for 4 h with (A) vehicle or (B) 20 μM SU6656 and then observed by time-lapse digital microscopy for 30 min with the indicated addition. Movies are 100X real-time when viewed at standard rates (10 frames/s). The entire field is 270 μm. Please see link for movie.



Supplemental Figure III. The Src Family Kinase inhibitors PP1 and PP2 do not inhibit macrophage uptake of LDL. Macrophages were incubated 24 h with 250 μg/ml ^{125}I-LDL and 5 μM SU6656, or 1 μM PP1, or 1 μM PP2, or no drug addition. ^{125}I-LDL uptake is the sum of cell-associated and degraded ^{125}I-LDL.

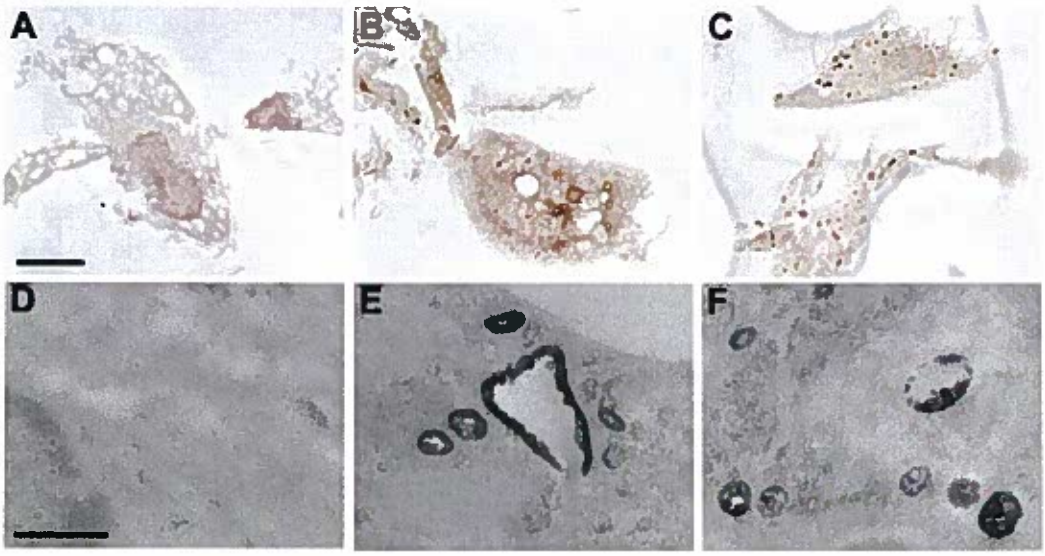


Supplemental Figure IV. Macrophage expression of Src Family Kinases. Src Family Kinase protein expression of macrophage lysates was assessed by Western blot analysis. Cell lysates known to express the indicated Src Family Kinase were used as positive controls.

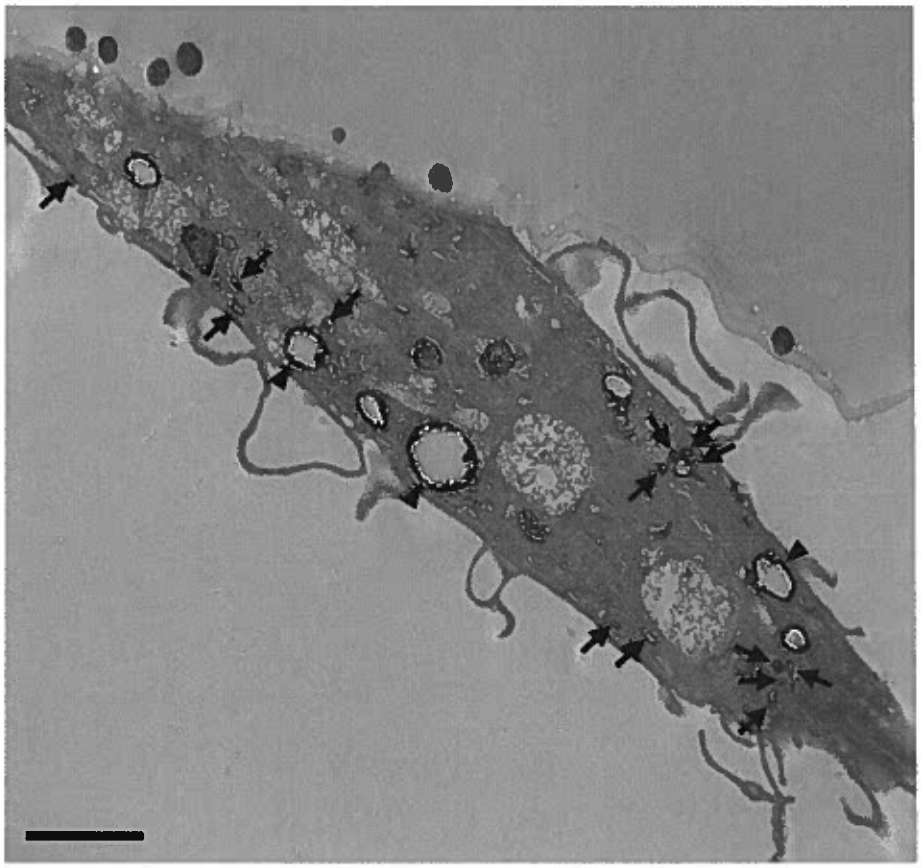
Supplemental Figure V. Inhibition of Syk does not affect macrophage macropinocytosis. Macrophages were treated for 4 h with (A) vehicle or (B) 1 μg/ml Syk inhibitor IV and then observed by time-lapse digital microscopy for 30 min with the indicated addition. Movies are 100X real-time when viewed at standard rates (10 frames/s). The entire field is 270 μm. Please see link for movie.

Supplemental Figure VI. Toxin B inhibits macrophage macropinocytosis. Macrophages were treated for 4 h with (A) vehicle or (B) 100 ng/ml toxin B and then observed by time-lapse digital microscopy for 30 min with the indicated addition. Movies are 100X real-time when viewed at standard rates (10 frames/s). The entire field is 270 μm. Please see link for movie.

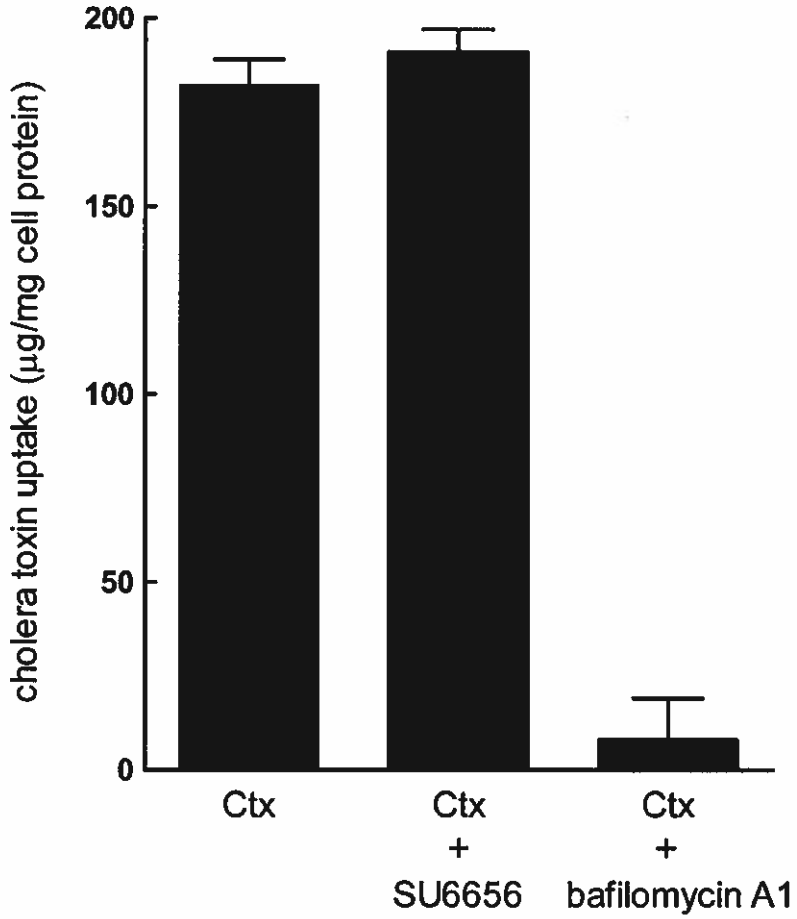
Supplemental Figure VII. Bafilomycin A1 does not affect macrophage macropinocytosis. Macrophages were treated for 4 h with (A) vehicle or (B) 500 nM bafilomycin A1 and then observed by time-lapse digital microscopy for 30 min with the indicated addition. Movies are 100X real-time when viewed at standard rates (10 frames/s). The entire field is 270 μm. Please see link for movie.



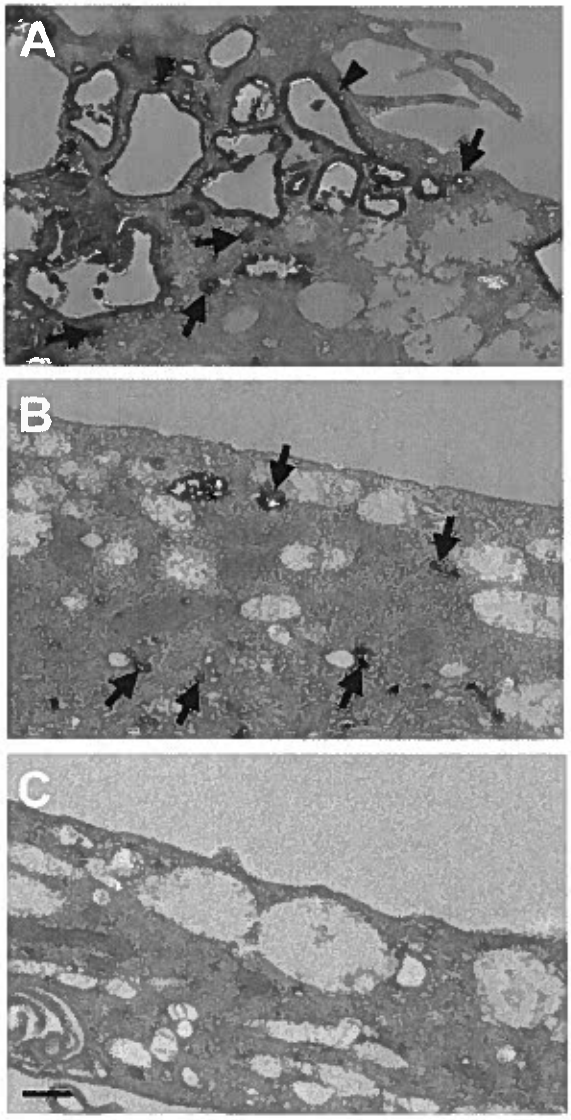
Supplemental Figure VIII. Toxin B inhibits HRP uptake by macropinocytosis. Macrophages were incubated 10 min (A and D) without HRP, or with 1 mg/ml HRP (B and E) without inhibitor addition or with (C and F) 100 ng/ml toxin B. The scale bar for (A-C) light microscopic and (D-F) electron microscopic observation of macrophages is 10 μm and 0.5 μm, respectively.



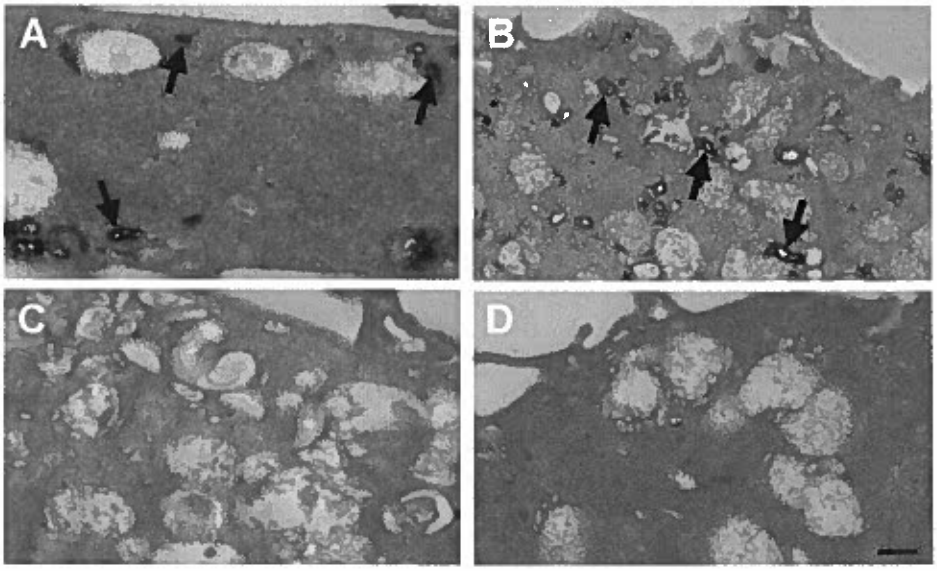
Supplemental Figure IX. Low magnification electron micrograph of macrophage uptake of the fluid-phase pinocytosis tracer HRP within macropinosomes and micropinosomes. Macrophages incubated without inhibitor addition were assessed for macropinosome and micropinosome uptake of HRP. Arrows and arrowheads indicate micropinosomes and macropinosomes, respectively. Scale bar = 2 μm.



Supplemental Figure X. Bafilomycin A1 inhibits macrophage uptake of the micropinocytosis tracer cholera toxin. Macrophages were incubated 5 h in medium containing 10 µg/ml Alexa Fluor 488® conjugated-fluorescent cholera toxin (Ctx) and the indicated inhibitor.



Supplemental Figure XI. Cholera toxin uptake identifies macrophage micropinosomes. Macrophages were incubated 10 min in medium containing (A) 1 mg/ml HRP, (B) 10 μg/ml HRP-conjugated cholera toxin, or (C) no addition. Arrows and arrowheads indicate micropinosomes and macropinosomes, respectively. Scale bar = 0.5 μm.



Supplemental Figure XII. The micropinocytosis tracer cholera toxin is not taken up by bafilomycin A1-treated macrophages. Macrophages were incubated 10 min in medium containing (A) 10 μg/ml HRP-conjugated cholera toxin, (B) 10 μg/ml HRP-conjugated cholera toxin and 20 μM SU6656, (C) 10 μg/ml HRP-conjugated cholera toxin and 500 nM bafilomycin A1, or (D) no addition. Arrows indicate micropinosomes. Scale bar = 0.5 μm.