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Supplemental Information

Autophagy Regulates Cholesterol Efflux from Macrophage Foam Cells via Lysosomal Acid Lipase

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Inventory of Supplemental Information

1) Figure S1, related to Figure 1. AcLDL-derived cholesterol is esterified by ER-resident ACAT and accumulates as neutral lipid in LDs; accumulated CE in AcLDL-loaded macrophages can subsequently be removed by the cholesterol acceptor apoA-I.

2) Figure S2, related to Figure 3. The addition of Lalistat 1 during AcLDL loading prevents LD formation whereas Lalistat 1 treatment in AcLDL-loaded cells decreases LD cholesterol efflux.

3) Figure S3, related to Figures 4. The T0901317 LXR agonist increases cholesterol efflux in normal macrophages but not when autophagy-mediated efflux is inhibited.

4) Movie S1, Lipid droplet motility in lipid-loaded macrophages, related to Figure 1.

5) Movie S2, LDs in lysosomes, related to Figure 1.

6) Supplemental Experimental Procedures.

7) Supplemental References**.**

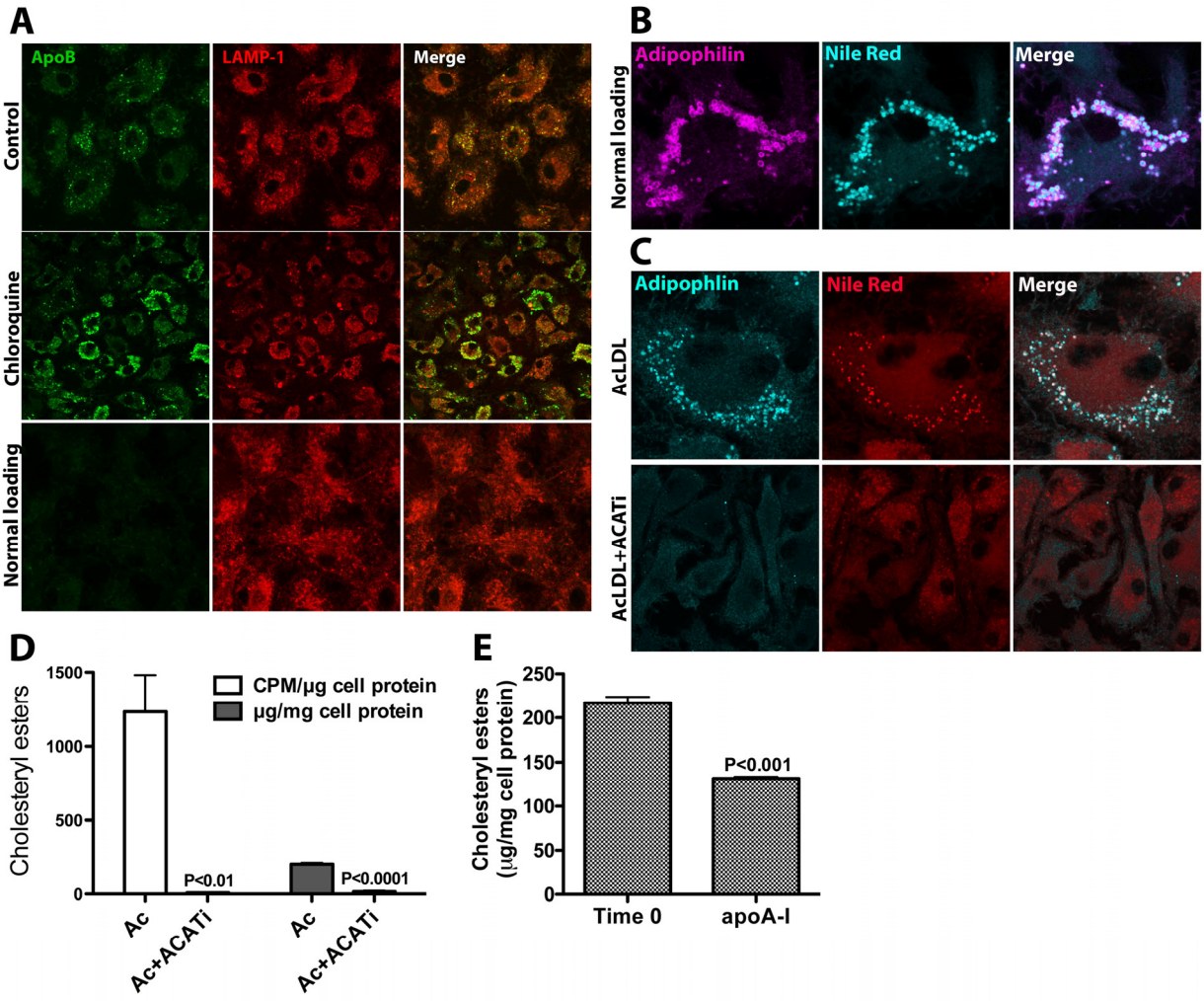


Figure S1, related to Figure 1. AcLDL-derived cholesterol is esterified by ER-resident ACAT and accumulates as neutral lipid in LDs; accumulated CE in AcLDL-loaded macrophages can subsequently be removed by the cholesterol acceptor apoA-I.

(A and B) Macrophages were loaded with AcLDL, and equilibrated O/N in BSA media (normal loading) or otherwise cells were loaded with AcLDL for 6h in the presence or absence of chloroquine. IF was performed to visualize apoB (A) or adipophilin (B) and neutral lipids. Clearly, there is no apoB in cells with the normal loading, indicating that there is no lysosomal accumulation of undigested AcLDL (A), and all neutral lipids are in adipophilin-coated LDs (B). (C) AcLDL-derived cholesterol is unesterified in the presence of an ACAT inhibitor (ACATi) and consequently there is no LD biogenesis. Here macrophages were loaded with AcLDL for 6h in the presence or absence of ACATi after which cells were fixed and stained for microscopy.

(D) Macrophages were loaded with AcLDL in the presence or absence of 10μM ACATi (normal loading = 30h AcLDL, wash, equilibration in BSA). Cholesteryl esters were quantified by TLC or using the Cholesterol Quantitation Kit.

(E) Following cholesterol loading (time0), efflux to apoA-I was carried out for 24h after which CE was quantified using the Cholesterol Quantitation Kit.

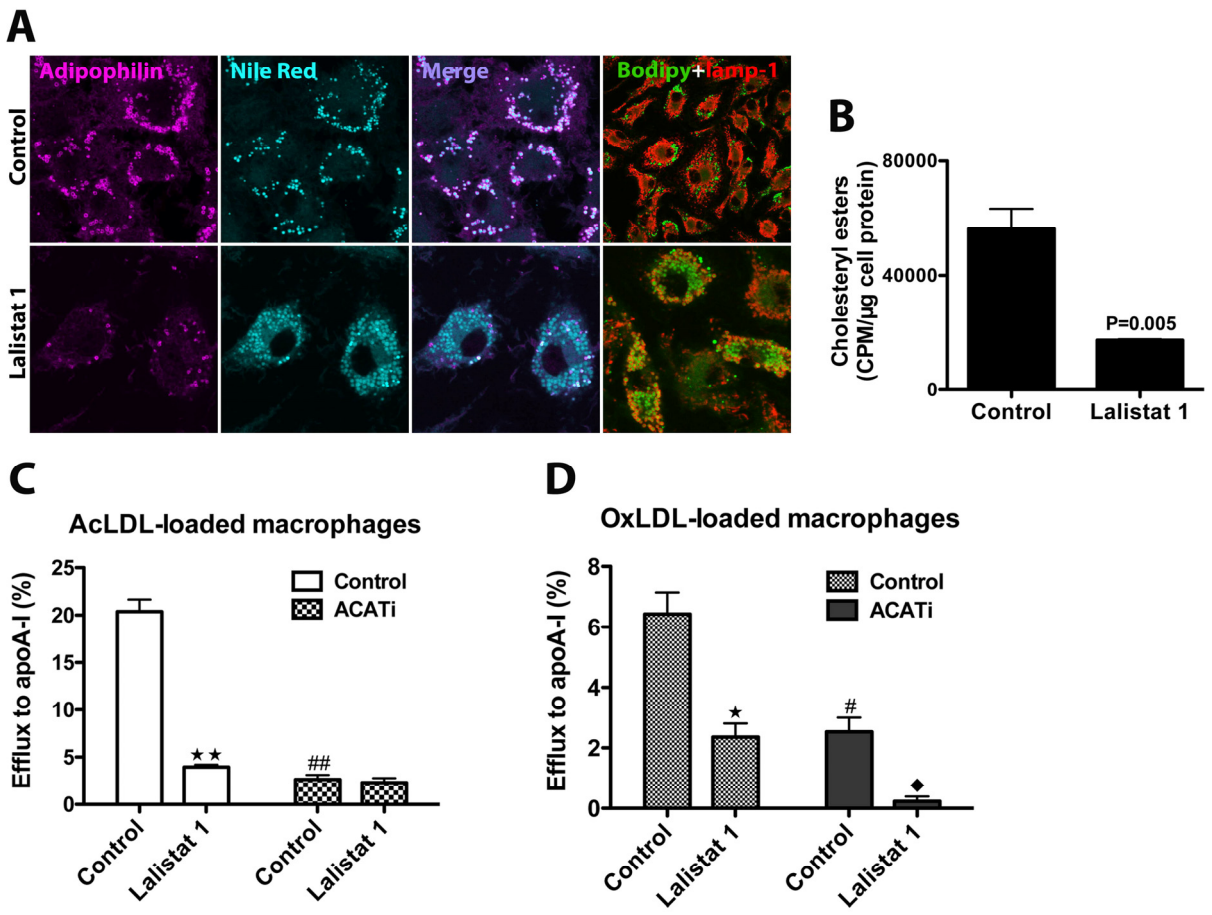


Figure S2, related to Figure 3. The addition of Lalistat 1 during AcLDL loading prevents LD formation whereas Lalistat 1 treatment in AcLDL-loaded cells decreases LD cholesterol efflux.

(A) Reduced LDs in cells loaded with AcLDL in the presence of the Lalistat 1. Macrophages were loaded with AcLDL in the presence or absence of 10µM Lalistat 1 for 24h, after which cells were fixed, permeabilized and stained for microscopy. In the absence of Lalistat 1, AcLDL-associated CE is hydrolyzed by LAL and excess cholesterol is esterified and accumulates in LDs (all neutral lipid inclusions are surrounded by the LD coat protein adipophilin, whereas there is no overlap between neutral lipids and the lysosomal marker LAMP-1). In the presence of Lalistat 1, LAL is inhibited and AcLDL-associated CE is not hydrolyzed (neutral lipids accumulate in lysosomes, as evidenced by the extensive co-localization of neutral lipids and LAMP-1-positive lysosomes, whereas there are scarce LDs).

(B) Reduced incorporation of ^{3}H-Oleate into CE in cells loaded with AcLDL in the presence of Lalistat 1. Macrophages were loaded with AcLDL in the presence or absence of 10µCi/mL of

^{3}H-Oleic acid and 10μM Lalistat 1 for 30h, after which total lipids were extracted, separated by TLC and CE was quantified.

(C and D) LAL inhibition reduces LD cholesterol efflux in AcLDL-loaded cells (C), whereas it reduces both LD and lysosomal cholesterol efflux in OxLDL-loaded cells (D). Macrophages were loaded AcLDL or OxLDL, after which efflux to apoA-I was measured for 24h, in the presence or absence of 10μM Lalistat 1 and / or ACATi (note that for ACATi-treated cells, the ACATi was present during the entire experiment). $P<0.05$ (★) or <0.001 (★★) for Lalistat 1-treated cells as compared to control, $P<0.01$ (#) or <0.001 (##) for ACATi-treated cells as compared to control, or $P<0.05$ (♦) for ACATi- and Lalistat 1-treated cells as compared to ACATi-treated cells.

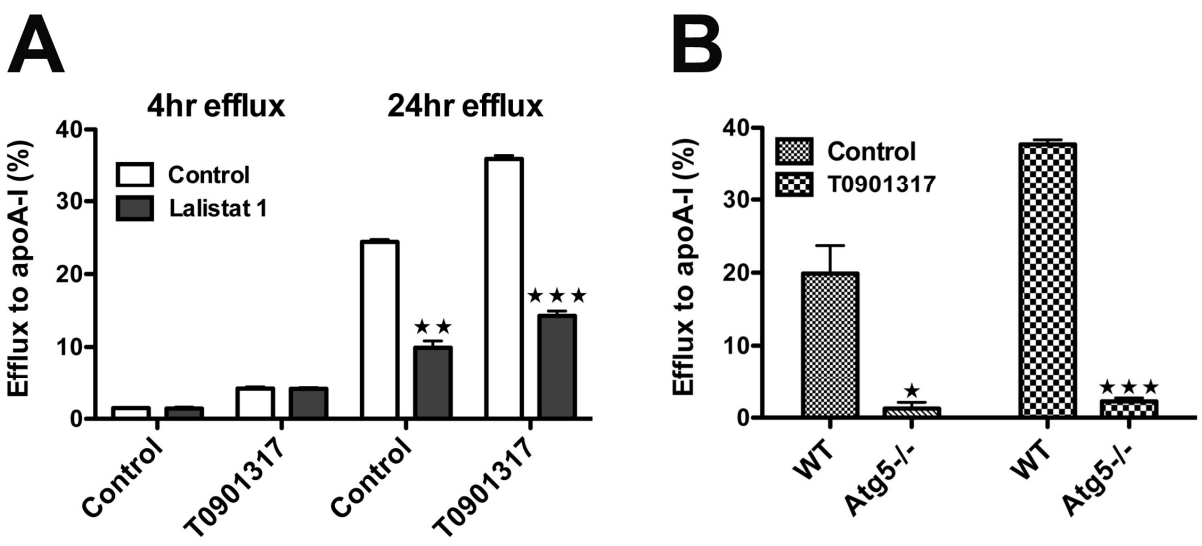
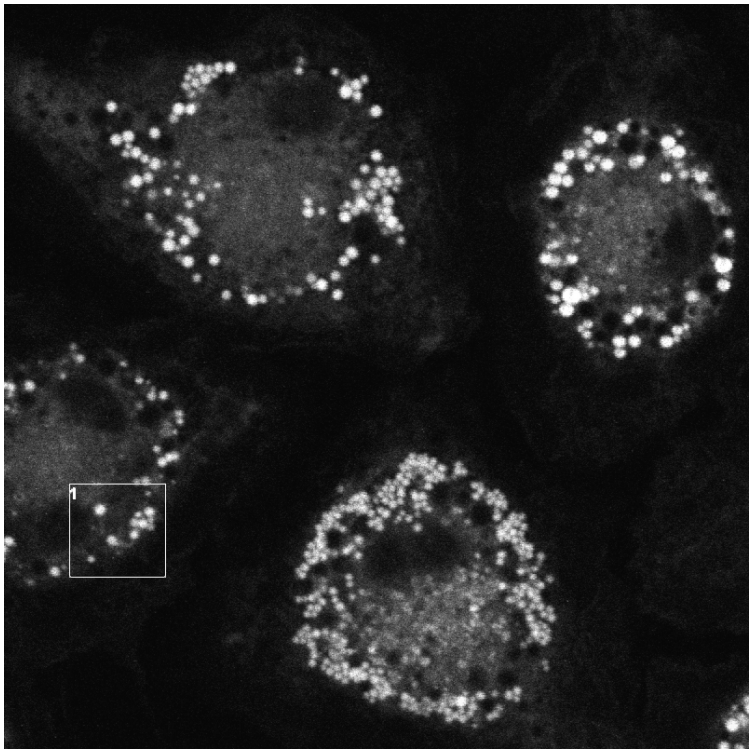


Figure S3, related to Figure 4. The T0901317 LXR agonist increases cholesterol efflux in normal macrophages but not when autophagy-mediated efflux is inhibited.

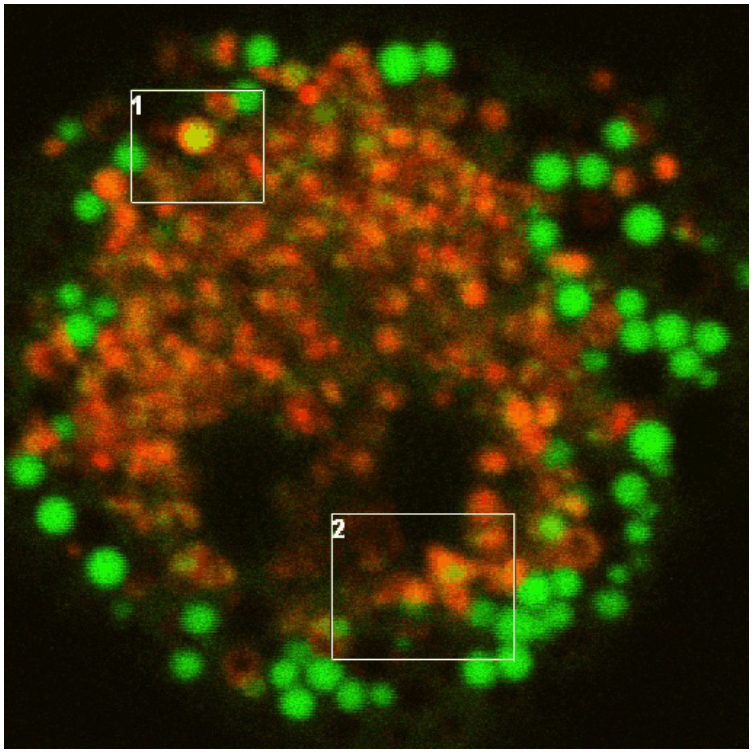
(A) Cholesterol efflux is stimulated by T0901317 in control cells, but not in Lalistat 1-treated cells. Cholesterol efflux to apoA-I in AcLDL-loaded macrophages was measured for 4h and 24h. In agreement with Figure 3, LAL inhibition by Lalistat 1 reduces cholesterol efflux to apoA-I (no effect is observed at a short time point). Maximal ABCA1 activation by addition of T0901317 increases the magnitude efflux to apoA-I. Because Lalistat1 prevents hydrolysis of LD CE over time, there is no such increase of cholesterol efflux in response to T0901317 treatment in the presence of Lalistat 1 for 24h.

(B) Cholesterol efflux is stimulated by T0901317 in WT, but not in *Atg5-/-* macrophages. Activation of ABCA1 by T0901317 treatment increases efflux to apoA-I in WT macrophages. Despite the increase in ABCA1 activity upon T0901317 treatment, efflux is not increased in *Atg5-/-* macrophages because delivery of LD cholesterol to ABCA1 is impaired by the lack of autophagy.

(A and B) $P<0.01$ (★) , <0.001 (★★) or <0.0001 (★★★) for Lalistat 1-treated cells compared to control (A) or for *Atg5-/-* cells as compared to WT (B).



Movie S1, Lipid droplet motility in lipid-loaded macrophages, related to Figure 1. Confocal video analysis of Bodipy-labeled neutral lipids in AcLDL-loaded bone marrow-derived macrophages. LDs are seen surrounding a circular organelle, and the formation of a 'ring' comprised of neutral lipids emerges from the LDs to surround this unknown organelle.



Movie S2, LDs in lysosomes, related to Figure 1. Confocal video analysis of Bodipy-labeled neutral lipids and LysoTracker Red-labeled acidic compartements in AcLDL-loaded BMDMS. Here, note that LD-derived lipid rings overlap with acid rings, and some LDs are found within lysosomes (fusion between the LD and lysosome is ongoing).

Supplemental Experimental Procedures

Cell culture. Bone marrow derived-macrophages: bone marrow cells were flushed from the femurs of C57BL/6 mice (Jackson Laboratories) or from *Atg5-/-*/*LDLR-/-* and *LDLR-/-* mice on a C57BL/6 background and differentiated into macrophages by incubation in DMEM media supplemented with 10% FBS, 1% P/S, and 15% L929-conditioned media for 7 days. Peritoneal macrophages: peritoneal macrophages from *Atg5-/-*/*LDLR-/-* and *LDLR-/-* were harvested 4 days after i.p. injection of methyl-BSA in mice previously immunized with this antigen (Cook et al., 2003), as previously described (Devries-Seimon et al., 2005). For *in vivo* macrophage cholesterol loading, male mice 8 months of age (*apoe-/-* or wild-type on a C57BL/6 background) were placed on a Western diet (Harlan-Teklad) for 2 weeks after which peritoneal macrophages were harvested 3 days after thioglycolate injection, as previously described (Gallily and Feldman, 1967). We confirmed that neutral lipids were increased in macrophages harvested from *apoe-/-* mice as compared to wild-type mice (CE was of 94±4μg/mg cell protein as compared to 9±4μg/mg cell protein, respectively). All experiments performed were in accordance with protocols approved by the University of Ottawa Animal Care Committee.

Lipoprotein preparation. VLDL and LDL were isolated by sequential density ultracentrifugation (Havel et al., 1955). Modification of LDL: LDL was either acetylated by repetitive additions of acetic anhydride (Goldstein et al., 1979), aggregated by vortexing (Otero-Vinas et al., 2007), or oxidized by incubation with 5μmol/L $CuSO_4$ at room temperature for 24h, adapted from Kunjathoor et al (Kunjathoor et al., 2002). HDL was purified by density gradient ultracentrifugation (Sattler et al., 1994). VLDL was isolated from a pool of plasma obtained from untreated patients with severe mixed hyperlipidemia recruited from the Lipid Clinic, UOHI. The protocol was approved by the Human Research Ethics Committee and written informed consent was obtained from all subjects. ^{3}H-Cholesteryl oleate lipoproteins were prepared as described by others (Brown et al., 1975). Briefly, dried ^{3}H-cholesteryl oleate was re-suspended in 0.5mL of a 0.15M NaCL, 0.3mM EDTA solution, and incubated 15min at 37ºC. 300-400mg of lipoprotein (AcLDL or OxLDL) was added and the mixture was incubated for an additional 4h at 37ºC, after which it was dialyzed overnight. The supernatant was collected after a brief centrifugation and lipoproteins were used at a 50μg/mL final concentration.

Cholesterol efflux. Cells were labelled similarly to that previously described (Wang et al., 2007b): macrophages were incubated for 30h in 10% FBS media containing 50μg/mL of

lipoproteins that were pre-incubated with ^{3}H-cholesterol (5μCi/mL). Cells were washed, and incubated in equilibration media (2 mg/mL BSA, 1% P/S) overnight (O/N). After equilibration, cholesterol efflux was determined in the presence or absence of human recombinant apoA-I (50μg/mL) prepared as previously described (Bergeron et al., 1997) or HDL (50μg/mL) in serum-free media (2 mg/mL BSA, 1% P/S) with the indicated reagent (paraoxon 100μM, chloroquine 30μM, vinblastine 30μM, bafilomycin 10nM, ACATi 10μg/mL, Lalistat 1 10μM) for 18 to 24h (unless otherwise specified). The supernatant was then removed and briefly centrifuged to remove non-adherent cells, and the remaining cells were dissolved in 0.5N NaOH. The radioactivity within aliquots of supernatants and dissolved cells was measured by scintillation counting. Cholesterol efflux is expressed as a percentage of ^{3}H-cholesterol in medium/(^{3}H-cholesterol in medium+^{3}H-cholesterol in cells)x100%. Efflux to apoA-I or HDL was calculated by subtracting effluxes of the wells without apoA-I or HDL from those containing apoA-I or HDL.

ACAT inhibitor studies: the Sandoz 58-035 Acyl-CoA : cholesterol acyltransferase (ACAT) inhibitor (ACATi) was administered at a final concentration of 10μM where indicated.

Stimulated cholesterol efflux. Cells were labelled with ^{3}H-cholesterol-AcLDL as described above, and equilibrated O/N in the presence of 10μM T0901317 (Biomol) to maximally activate the ABCA1 transporter. Efflux to apoA-I was then measured as above.

***In vivo* RCT studies.** Macrophage RCT experiments were carried out similarly to that previously described (Wang et al., 2007a). Bone marrow-derived macrophages from wild-type or *Atg5-/-* mice were loaded with ^{3}H-cholesterol-AcLDL as described above for cholesterol effluxes, and 200μL of cell preparations (~5x10^{6} cells containing ~2x10^{6}CPM) were injected subfascially in the lumbar region of C57BL/6 mice (n=10 per group for the first two experiments, n=13 for the third experiment – pooled data represents a total of n=33 mice per group). The initial proportion of cellular cholesterol that was esterified was equivalent in wild-type and *Atg5-/-* macrophages in all independent RCT experiments (42±1.1% and 42±1.3% for WT and *Atg5-/-* cells, respectively). Blood was collected at 24h via the saphenous vein and at 48h via cardiac puncture of anesthetized mice. Plasma was used for liquid scintillation counting. Following cardiac puncture of anaesthetized mice, gallbladders were emptied, and livers were removed for scintillation counting. Feces were collected over a 48h period and total feces radioactivity (of equivalent wet weight) was measured. All ^{3}H-tracer measurements are

expressed relative to the injected amount. All experiments performed were in accordance with protocols approved by the University of Ottawa Animal Care Committee.

Lipid measurements. The Biovision Cholesterol Quantitation Kit was used to determine cellular mass of cholesterol (CH) and cholesteryl esters (CE). Briefly, isopropanol (1% Triton X-100) was added to the cells and left for 2h at room temperature, subsequently transferred to glass vials, from which the solvent was removed under a constant nitrogen flow. The lipids were re-dissolved in cholesterol reaction buffer and measured by the fluorometric method, as per the manufacturer's instructions. Variations in CE are expressed as % hydrolysis or as fold-change relative to control, calculated as follows: % hydrolysis = $(CE_i - CE_f)/(CE_i)*100$, where CE_i represents the CE mass (μg/mg cell protein) immediately after AcLDL loading, and CE_f represents the CE mass (μg/mg cell protein) after the cells were incubated for 24h with apoA-I; fold-change = (% hydrolysis sample / % hydrolysis control).

CE quantification by TLC: total lipids were extracted (Bligh and Dyer, 1959) and separated by thin layer chromatography (TLC) on silica gel plates using a nonpolar solvent system (hexane/diethyl ether/acetic acid, 70:30:1, v/v) for separation of cholesterol and CE. The bands corresponding to cholesterol and CE were excised and counted for radioactivity.

^{3}H-cholesterol and ^{14}C-oleic acid esterification. In specific experiments, sodium ^{14}C-oleate-albumin complex was prepared as described previously (Goldstein et al., 1983) and used at a final concentration of 0.1mM. Macrophages were incubated with ^{3}H-cholesterol-AcLDL in the presence of 0.1mM ^{14}C-Oleate for 30h, in the presence or absence of ACATi, after which total lipids were extracted and the ^{3}H and ^{14}C labels were quantified in the CE fraction after TLC.

Protein quantification. Total cellular protein levels were determined using the Markwell Lowry assay (Markwell et al., 1978).

Western blotting. Cells were washed twice with ice-cold PBS, scrapped in Laemmli sample buffer (Bio-Rad). Total protein samples (25-30μg/well) were electrophoresed on precast 4-20% or 18% SDS-polyacrylamide gels (Invitrogen) and transferred to nitrocellulose or PVDF membranes at 125V for 2h. Membranes were probed with anti-β-Actin (1:500, BioLegend), anti-LC3 (1:500, MBL international), anti-adipophilin (1:2000, RDI), anti-beclin (1:500, Novus Biologicals), etc. Proteins were detected using an enhanced HRP-based chemiluminescence detection system (HRP-conjugated secondary antibodies from Amersham Biosciences and

SuperSignal West Femto Maximum Sensitivity Substrate from Pierce) and analyzed using a FluorChem Imager (Alpha Innotech).

Lipid droplet isolation. LDs were isolated from lipid-loaded macrophages by density gradient centrifugation using a previously described method (Brasaemle and Wolins, 2006), which the exception that whole LD fractions were used for SDS-PAGE (fractions were not delipidated prior to Western Blotting – fractional proteins were precipitated using ice-cold acetone and the pellet was re-dissolved in 25µL of loading buffer prior to SDS-PAGE).

Fluorescence microscopy. All live cell imaging was carried out in Hepes-buffered media, and the cells were visualized by confocal microscopy in a 37˚C heat chamber. Neutral lipids were stained using Bodipy 493/503 or Nile Red, as previously described (Listenberger LL, 2007). AcLDL-loaded macrophages were incubated with Bodipy (10µg/mL) with or without LysoTracker Red (50nM) for 30min prior to visualization, or with 10µg/mL of the BSA conjugate 4h prior to labeling with Bodipy. For immunofluorescence (IF), cells were fixed in 4% PFA for 30min at room temperature, and blocked/permeabilized in 2.5% BSA/0.1% TritonX-100 dissolved in 1X PBS. Cells were incubated with anti-adipophilin (1:500) anti LAMP-1 (1:800) and anti-LC3 (1:100, Cell Signaling) for 1h at 37ºC. Fluorophore-conjugated secondary antibodies (1:500, Molecular Probes) were incubated in the presence of Nile Red (50ng/mL) to stain for neutral lipids. Confocal images and movies were obtained using a 100 3 NA 1.4 objective on an Olympus IX80 FV1000 confocal microscope with appropriate lasers.

Electron microscopy (EM): Cells were cultured on monolayers, loaded with AcLDL for 30h, washed and equilibrated in BSA media O/N, then lifted in 5mM PBS-EDTA for 20min and fixed in 1.6% glutaraldehyde prior to postfixation in osmium tetroxide and uranyl acetate en bloc staining. Samples were then processed and embedded in Spurr epoxy resin, thin sectioned, and counterstained with lead citrate. Digital images were obtained with a JEOL 1230 TEM at 60kV adapted with a 2000 3 2000 pixel bottom mount CCD digital camera and AMT software.

LC3 Immunogold labeling: A pre-embedding method followed by silver enhancement was used for immunoelectron microscopy. AcLDL-loaded macrophages were fixed in 4% paraformaldehyde and 0.1% glutaraldehyde in PBS. Cells were permeabilized and blocked in PBS containing 0.1% saponin and 5% bovine serum albumin (BSA) for 30min. Cells were immunolabeled with anti-LC3 (1:100, Cell Signaling #2775) in the wash solution overnight (1% BSA, 0.05% saponin in PBS). The cells were then washed, and incubated with anti-rabbit IgG that was conjugated to colloidal gold (1.4 nm diameter) for 1h (Nanoprobes). Cells were washed

with PBS, and post-fixed with 1% glutaraldehyde in PBS for 10 min. The gold was intensified using the HQ Silver Enhancement Kit (Nanoprobes) according to manufacturer's directions (staining was done for 8min). Cells were then washed thoroughly with distilled water to stop the enhancement process, following which the cells were postfixed in 0.5% OsO4 for 90 min at 4°C, washed with distilled water, incubated with 50% ethanol for 10 min, and stained with 2% uranyl acetate in 70% ethanol for 2 h. The cells were further dehydrated with a graded series of ethanol and then embedded, counterstained and imaged as described above.

Co-localization quantification: blind counting by 4 individuals was averaged for LC3 immunogold labeling.

Statistical analysis: Experiments were run in triplicates, and all presented values are mean ± SEM. The statistical significance of the differences between groups was determined using the two-tailed unpaired Student's t-test of the means with GraphPad InStat v3.1a software (GraphPad Software Inc).

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