Materials and methods

Bone marrow macrophages. Bone marrow derived macrophages were obtained from apoE-deficient mice on the AKR and DBA/2 background. Bone marrow cells were flushed from the femur and humerus bones and re-suspended in macrophage growth medium (DMEM, 10% FBS, 20% L-cells conditioned media as a source of MSCF) as previously described[1, 2] and plated. The media was renewed twice per week. Cells were used for experiments 10 to 14 days after plating when the bone marrow cells were confluent and fully differentiated into macrophages. During some experiments, serum free DMEM media was supplemented with 10ng/mL MCSF to aid cell survival.[3]

Lipoprotein preparations and labeling. Human LDL ($1.019 < d < 1.063$ g/mL) and human HDL ($1.063 < d < 1.21$g/mL) were prepared by ultracentrifugation. LDL was acetylated as described previously.[4, 5] HDL, LDL and AcLDL were dialyzed against PBS with 100μM EDTA and 20μM BHT. Protein concentrations of lipoproteins were determined using an alkaline Lowry assay.[6] For DiI (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate) labeling, 10 mg/mL DiI stock in DMSO was diluted in PBS and added to AcLDL at a final concentration of approximately 10 μg/mg of protein. The mixture was incubated at 37°C for 25min and centrifuged for 2min to pellet any insoluble debris. The supernatant was collected and filtered sterilized before use. For [^{3}H] labeling, [^{3}H]-cholesterol in ethanol was pre-incubated with undiluted AcLDL or FBS at a final concentration of 0.5 μCi/mL for 15min at 37°C before adding to DMEM at a final concentration of 50 μg/mL AcLDL or 1% FBS, respectively.

AcLDL uptake. Cells were plated in 24-well plates and differentiated as described above. Cells were washed twice with DMEM and incubated in DMEM containing 10 ng/mL MCSF and 50 μg/mL DiI-AcLDL. Lipoprotein uptake proceeded for 30min at 37°C. The cells were washed twice with cold DMEM and detached from the plate using cellstripper™ (Cellgro). The cell suspension was then place in a 5 mL tube and wash twice using flow cytometry media (PBS, 0.5 mM EDTA, 0.5% BSA). The cells were resuspended in 200 μL of flow cytometry media and were analyzed by flow cytometry (LSRII, Becton-Dickinson). Fluorescence was gated for individual live cells and the fluorescence associated with 10,000 cells was determined for each sample. The data collected from the cells were analyzed with Flowjo software. Alternatively, cells were incubated with [^{3}H]-AcLDL for 30min, cholesterol was extracted using hexane:isopropanol (3:2; v:v) and protein was solubilized using 0.2N NaOH. The radioactivity in the cells was determined by liquid scintillation counting and results expressed as dpm/μg cell protein.

Acyl-CoA cholesterol acyl transferase (ACAT) activity assay. To investigate ACAT activity, cells were plated in 6-well (cell lysate assay) or 12-well plates (live cell assay) and differentiated as described above.

Cell lysate assay – Cells were loaded for 48h using 50 μg/mL AcLDL, harvested and lysed by sonication. The ACAT activity was determined as described before with small modification.[7] Briefly, the lysates were incubated for 15min at 37°C with cholesterol-rich phosphatidylserine liposomes and an additional 15min at 37°C after the addition of [^{14}C]-oleyl-CoA in presence of fatty acid free BSA. Cholesterol was extracted using

methanol:chloroform (2:1), and the organic phase was dried down and resuspended in hexane. CE was separated using thin layer chromatography and the radioactivity of this fraction assessed by liquid scintillation counting. Proteins were dissolved with 0.2 N NaOH and the results are normalized to the protein content, evaluated by BCA assay. *Live cell assay* – Cells were incubated overnight with DMEM, 10 ng/mL MCSF and 1% FBS or 50 μg/mL AcLDL. Then, cells were incubated for 1h with [^{3}H]cholesterol-AcLDL and the lipids were extracted in hexane:isopropanol (3:2). CE and protein were analyzed as described above to determine ACAT activity.

Cholesterol efflux. Cells were cholesterol labeled with 1% [^{3}H]-FBS or 50 μg/mL [^{3}H]-AcLDL in DMEM containing 10 ng/mL MCSF for 16h at 37^{o}C. After labeling, cells were chased for 4h at 37^{o}C in DMEM with or without acceptors (10 μg/mL apolipoprotein AI or 100 μg/mL HDL). At the end of this chase period, the radioactivity in the medium and cells was determined by liquid scintillation counting, and the percent efflux was calculated as 100 x (medium dpm)/(medium dpm + cell dpm). Finally, percent efflux to acceptors was calculated as (percent efflux to acceptors)-(percent efflux in absence of acceptors). All treatments were performed in triplicate. When indicated, the loading and the chase were done in presence of 2 μg/mL ACAT inhibitor 58035 (ACATi).

Cholesterol mass quantification. Cells were plated in 12-well plates and differentiated as described above. The cells were incubated 24h at 37^{o}C with macrophage culture media without or with varying doses of AcLDL. When indicated, the loading period was followed by a chase period in DMEM media containing 10 ng/mL MCSF, 10 μg/mL apoAI and 2 μg/mL ACATi (chase media) with or without the addition of 10 μM lalistat 1, a lysosomal acid lipase inhibitor (kind gift from Fred Maxfield and Paul Helquist).[8-10] Lipids were extracted from cells, and esterified cholesterol and protein amounts were evaluated as described previously.[11]

Fluorescence microscopy studies. For lipid droplet visualization, cells were plated on 4-chamber slides and differentiated as described above. Three wells of cells were loaded for 24h with 50 μg/mL AcLDL and unloaded cells were used as a control. Two of the loaded wells were then chased for 24h in chase media with or without the addition of 10 μM lalistat 1. Finally, cells were fixed at room temperature for 20min in 10% buffered formalin phosphate (Fisher scientific), lipid droplets were stained with Nile red (100 ng/mL), and nuclei were stained with DAPI. For EGFP and mRFP tagged LC3-II reporter transfection studies, differentiated cells were scraped from a plate and transfected with ptfLC3 (Addgene plasmid #21074) using a nucleofection kit from Lonza specifically designed for macrophages (kit #VPA-1009). Cells were re-plated, and 24h later the cells were AcLDL loaded for 16h before epifluorescent imaging on live cells. Some wells were amino acid starved by incubation in EBSS for 1h prior to imaging. To determine the relative RFP/GFP puncta fluorescence for each cell, we used Adobe Photoshop to measure the red and green intensity at the center of each punctum and calculated the average red/green ratio of all puncta per cell. Aortic root sections of formalin fixed hearts were stained with Bodipy (10 μg/ml) for 1h, washed in PBS with 0.05% Tween-20 and PBS alone, and mounted with Vectashield (Vector Labs)

containing DAPI. The area of lipid droplets was assessed using Image-Pro plus 7.0 (Media Cybernetics) and expressed as percentage of foam cell area.

Lysosomal acid lipase activity assay. Lysosomal acid lipase activity was measured as described previously with a few modifications.[12] Briefly, cell lysates were incubated with liposomes containing phosphatidylcholine, cholesterol oleate and cholesterol-[1-^{14}C]-oleate at pH 5 or 7.4. Free fatty acids were separated from CE by addition of 0.1 M oleic acid and extraction in hexane:chloroform:methanol (370:185:444, vol:vol:vol) and 0.3 M NaOH. After separation, the upper fatty acid phase was collected and [^{14}C]-counts assessed using a scintillation counter. Activity was normalized to protein levels in the cell lysates.

Western blot. Cells were plated in 12-well plates and differentiated as described above. After indicated treatment, cells were lysed directly in the plate using Laemmli buffer. The lysates were heated at 95^{o}C for 5-10min and loaded on 14% tris-glycine gels without prior normalizing for total protein content (this protocol improves LC3-II stability). After transfer, membranes were probed with antibodies against LC3 (NB100-2220, Novus Biologicals) and p62 (NBP1-48320, Novus Biologicals). GAPDH levels (antibody FL-335, Santa Cruz) were used to normalize for protein loading. When needed, membranes were stripped with Restore^{TM} western blot stripping buffer (Thermo Scientific) as described by the manufacturer. Western blot results were analyzed using the ImageQuant TL software (GE healthcare).

Statistics. Comparison of two conditions was performed by student t-test, and comparison of multiple conditions was performed by ANOVA with Newman-Keuls posttest. Statistics were performed using GraphPad Prism software.

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