MATERIALS AND METHODS

Cells and Cell Culture. J774a.1 macrophage-like cells (American Type Culture Collection, Manassas, VA) were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 50 units/ml penicillin and 50 μg/ml streptomycin in a humidified atmosphere (5% CO_2) at 37°C. Bone marrow derived macrophages (BMMs) isolated from C57BL/6 mice were differentiated for 7 days by culture in the same media supplemented with 20% L-929 cell conditioned media. Human monocytes (Life Line Cell Technology, Frederick, MD) were differentiated into macrophages *in vitro* by incubation in RPMI containing 10% heat-inactivated FBS and 10 ng/ml macrophage colony stimulating factor (R&D Systems, Minneapolis, MN) for 5 days. For all live cell imaging experiments, media was changed to DMEM containing 25 mM 4-(2-hydroxyethyl)-1-pipierazine ethane sulphonic acid without phenol red or sodium bicarbonate.

Lipoproteins and Reagents. Human LDL was prepared from donor plasma as described[1]. LDL was labeled using succinimidyl esters of AlexaFluor-546 (Alexa546) and Alexa488 (Invitrogen, Carlsbad, CA), fluorescein isothiocyanate (FITC), biotin (Sigma-Aldrich, St. Louis, MO) or CypHer 5E Mono N-hydroxysuccinimide ester (CypHer5E) (GE Healthcare, Chalfont St. Giles, U.K.). LDL was aggregated by vigorous vortexing for 10 sec[2]. Alexa546-biocytin, Alexa488-cholera toxin subunit B (CtB), LipidTOX Green, 2'7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM) and latrunculin A (LatA) were purchased from Invitrogen. Filipin, 2-(N-mopholino)ethanesulfonic acid (MES), streptavidin and acyl-coenzyme A cholesterol acyltransferase (ACAT) inhibitor 58035, were purchased from Sigma-Aldrich.

Plasmin and Plasminogen Treatment. Plasmin from human plasma was purchased from Sigma-Aldrich. Cells were incubated for the indicated time periods with aggregated LDL (agLDL) to allow attachment and formation of the lysosomal synapse, followed by treatment with 1 U/ml plasmin for 15 min. Plasminogen and α_2-antiplasmin were purchased from Calbiochem (Darmstadt, Germany). Plasminogen was tested for the absence of residual plasmin activity with a chromogenic substrate. To assay for foam cell formation, BMMs were incubated with agLDL for 4 hr in presence or absence of 2 μM plasminogen with or without 150 μg α_2-antiplasmin.

Plasminogen activator activity measurements. J774 cells were incubated with or without agLDL for indicated time periods in phenol red free DMEM containing 10% FBS. After incubation, cells were rinsed 3 times with serum-free medium and incubated in serum-free medium containing 2 μM plasminogen for 30 min. Medium was centrifuged at 14000 rpm for 5 min to remove cell debris and unattached agLDL. Plasmin activity in supernatants was assessed by addition of chromogenic substrate CS 41(03) (Aniara, West Chester, OH) according to the manufacturer's protocol, and the reaction stopped after 5 min by addition of 5% (w/v) citric acid. Absorbance was measured at 405 nm using a SpectraMAX M2 spectrophotometer (Molecular Devices, Sunnyvale, CA). To calculate relative increases in plasminogen activator activity, the optical density at 405 nm measured in medium conditioned by cells incubated with agLDL was divided by the optical density at 405 nm measured in medium conditioned by cells incubated without agLDL for the same time period.

Cell labeling and Microscopy. Labeling with LipidTOX Green was performed according to the manufacturer's protocol. To determine surface urokinase-type plasminogen activator receptor (uPAR) expression, J774 cells were incubated with agLDL for various times, fixed without permeabilization in 3% paraformaldehyde (PFA) and labeled with an antibody against uPAR at 1:50 dilution for 60 min (rabbit polyclonal against full length uPAR, FL-290, Santa Cruz Biotechnology, Santa Cruz, CA). Cell labeling with Alexa488-CtB and filipin and was performed as described[3]. Labeling of extracellular streptavidin-

conjugated agLDL was accomplished with a 2 min pulse of 200 μM Alexa546-biocytin. For cytoplasmic loading with pH sensitive dye, J774 cells were incubated with 40 μM BCECF-AM in medium 2 and 0.2% (w/v) glucose containing 250 μM sulfinpyrazone for 1 hr. For actin de-polymerization, J774 cells were incubated with agLDL for 45 min without plasmin or incubated with agLDL for 30 min and then treated with 1 U/ml plasmin during the last 15 min of aggregate incubation. 5 μM LatA was added during the last 15 min of incubation to both plasmin treated and untreated cells.

For imaging, cells were plated on Poly-D-lysine coated glass-coverslip bottom dishes. Images were acquired with a Leica DMIRB widefield microscope equipped with a 40x, 1.25 numerical aperture (NA) plan Apochromat objective, or a Zeiss LSM510 laser scanning confocal microscope using a 63x, 1.4 NA plan Apochromat objective. For time-lapse data sets, a 40x 0.8 NA objective was employed, and pinholes on the confocal microscope were opened (so laser power could be lowered to reduce photobleaching), resulting in an axial resolution of 14 μm. Cell temperature was maintained at 37°C with a heated stage and objective heater. Where indicated, plasmin was added to dishes on the microscope stage.

Electron Microscopy. J774 cells were incubated with colloidal gold-labeled agLDL[4] for 1 hr with or without the addition of plasmin for the last 15 min. Following incubation with agLDL the cells were fixed with a modified Karnovsky's solution containing 2.5% glutaraldehyde, 4% PFA and 0.02% picric acid, postfixed with 1% Osmium tetroxide, 1.5% Potassium ferricyanide , treated with uranyl acetate, dehydrated through a graded ethanol series and embedded in LX112 resin. En face serial sections were cut at 70 nm thickness and picked up on formvar-coated, 4-slot copper grids. Sections were further contrasted with uranyl acetate and lead citrate. Images were acquired at Weill Cornell Medical College on a JEOL JEM 100CX-II electron microscope operating at 80kV at a set magnification of 10,000X.

pH Measurements. J774 macrophages were incubated for 60 min with CypHer 5E (a pH sensitive fluorophore) and Alexa488 (a pH insensitive fluorophore) dual labeled agLDL. The pH value within each pixel was assessed quantitatively by comparison with ratio images obtained in calibration buffers of varying pH as described previously[5]. Live cells were imaged on the confocal microscope using a 63x 1.4 NA objective.

All data were analyzed with MetaMorph image analysis software, Molecular Devices Corporation (Downington, PA). A binary mask was created using the Alexa488 signal intensity and applied to both channels to remove background noise. Images were convolved with a 7x7 pixel Gaussian filter, and ratio images were generated.

Radiolabeled CE hydrolysis Measurement. LDL was reconstituted with cholesteryl-[4-^{14}C]-oleate (American Radiolabeled Chemicals, St. Louis, MO) as described[6]. Cells were incubated with radiolabeled reconstituted agLDL for 90 min in the presence of 30 μg/ml ACAT inhibitor (to prevent re-esterification of hydrolyzed [^{14}C]cholesteryl ester)[7], left untreated or treated with plasmin for the last 15 min of incubation. Cell associated lipids were extracted, and the amount of cholesteryl ester and free cholesterol was quantified as described[5].

Statistics. Statistical analysis was performed using Excel. For comparisons of two groups, student's t test was used. For comparisons of more than two groups, two-way ANOVA was used.

SUPPLEMENTARY MATERIAL REFERENCES

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