Aggregated LDL in Contact With Macrophages Induces Local Increases in Free Cholesterol Levels That Regulate Local Actin Polymerization

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Objective—Interaction of macrophages with aggregated matrix-anchored lipoprotein deposits is an important initial step in atherogenesis. Aggregated lipoproteins require different cellular uptake processes than those used for endocytosis of monomeric lipoproteins. In this study, we tested the hypothesis that engagement of aggregated LDL (agLDL) by macrophages could lead to local increases in free cholesterol levels and that these increases in free cholesterol regulate signals that control cellular actin.

Methods and Results—AgLDL resides for prolonged periods in surface-connected compartments. Although agLDL is still extracellular, we demonstrate that an increase in free cholesterol occurs at sites of contact between agLDL and cells because of hydrolysis of agLDL-derived cholesteryl ester. This increase in free cholesterol causes enhanced actin polymerization around the agLDL. Inhibition of cholesteryl ester hydrolysis results in decreased actin polymerization.

Conclusions—We describe a novel process that occurs during agLDL–macrophage interactions in which local release of free cholesterol causes local actin polymerization, promoting a pathological positive feedback loop for increased catabolism of agLDL and eventual foam cell formation. (Arterioscler Thromb Vasc Biol, 2009;29:00-00.)

Key Words: atherosclerosis ■ foam cells ■ surface-connected compartment ■ Rho GTPases ■ F-actin

A key step in the progression of atherosclerotic lesions is formation of lipid-loaded macrophage foam cells. Soluble lipoproteins accumulate at sites of atherosclerotic plaque formation where they undergo modification, aggregation, and anchoring to the extracellular matrix. Monoocytes migrate from the blood, differentiate into macrophages that degrade LDL, and become filled with re-esterified cholesterol droplets. These cholesteryl ester (CE)-filled foam cells acquire new biological properties, particularly loss of motility; secretion of cytokines, growth factors, and proteases; and induction of apoptosis. These processes contribute to early lesion growth and late complications leading to plaque rupture.

It has been proposed that aggregated lipoproteins (agLDL) tightly linked to the extracellular matrix play an important role in the development of atherosclerotic lesions. The interaction of macrophages with retained and aggregated lipoproteins differs significantly from the uptake of monomeric lipoproteins. For example, aggregates and associated extracellular matrix components are too large to be taken up by endocytosis or even by phagocytosis without breaking the aggregates or matrix into smaller pieces. The breakdown of the lipoproteins requires the actin cytoskeleton and activation of the Rho-family GTPases Rac1 or Cdc42.

Furthermore, for 1 to 2 hours after initial contact of a macrophage with a retained and aggregated LDL particle the agLDL remains topologically outside the macrophage even though it may be in deep plasma membrane invaginations. Interestingly, there is significant hydrolysis of CE while the agLDL remain extracellular, and this hydrolysis requires lysosomal acid lipase (LAL). To explain this, it has been suggested that selective CE uptake might deliver the CE to lysosomes, but such a process has not been observed. An alternative mechanism is that hydrolysis of CE in agLDL occurs extracellularly. Various types of macrophages and macrophage-related osteoclasts are able to form extracellular lytic compartments. Consistent with this, we have observed that macrophages can create extracellular acidic compartments on contact with agLDL, and that they can secrete lysosomal contents into these contact zones (Haka et al, unpublished data).

Herein, we test the hypothesis that macrophage interactions with agLDL lead to extracellular CE hydrolysis and a consequent increase in free cholesterol (FC) which induces local actin polymerization.

Materials and Methods
A complete description of Materials and Methods is supplied in the supplemental material (available online at http://atvb.ahajournals.org), including supplemental Figures I and II.
Lipoproteins

Human LDL was prepared and conjugated to AlexaFluor-546 (Alexa546). We also reconstituted LDL with cholesteryl oleate, cholesteryl oleoyl ether, or cholesteryl-[4-14C]-oleate.20

Interaction of agLDL With Macrophages and Fluorescent Labeling of Cells

LDL was aggregated by vortexing, and aggregates were centrifuged and resuspended in serum-free DMEM/HEPES. Approximately 50 μg/mL of agLDL was added to cells at 37°C.

Fluorescent Labeling of Cells

F-actin was labeled with Alexa488-phalloidin, free cholesterol (FC) was labeled with filipin, and the plasma membrane was labeled with Alexa488-cholera toxin subunit B (CtB).

Microscopy and Image Quantification

A Zeiss LSM510 laser scanning confocal microscope and a Leica DMI8R epi-fluorescence microscope were used for fluorescence imaging. Image analysis is described in the supplemental materials.

Transfection of siRNA

RNAi oligonucleotides (Dharmacon) were transfected using Lipofectamine for mouse peritoneal macrophages and HiPerFect for RAW 264.7 cells.

LAL Reconstitution

Recombinant Pichia-derived Human LAL (phLAL) was added to RAW 264.7 cells as described.

Hydrolysis of Cholesteryl-[4-14C]-Oleate-Containing LDL

LDL reconstituted with cholesteryl-[4-14C]-oleate was vortex-aggregated and incubated with cells in the presence of an acyl-CoA:cholesterol acyltransferase (ACAT) inhibitor followed by treatment with plasmin to release extracellular agLDL.22 Lipids were extracted, and radiolabeled FC and CE were measured by thin layer chromatography (TLC).

Results

Extracellular agLDL in Surface-Connected Compartments Leads to the Formation of F-Actin–Rich Structures

We examined the formation of F-actin in cells near sites of contact with agLDL. J774 cells were incubated with suspensions of Alexa546-agLDL followed by fixation and labeling of F-actin by Alexa488-phalloidin (Figure 1A and 1B). After 30 minutes, an enrichment of F-actin was detected near the sites of contact with agLDL (arrowheads, Figure 1A). An enlarged view (Figure 1A, inset) shows that F-actin (green) is closely associated with the agLDL (red), indicating that actin polymerization is enhanced in the immediate vicinity of the contact with agLDL. Approximately half of the aggregates touching cells had F-actin enriched structures in their immediate vicinity within 30 minutes, and the fraction of agLDL surrounded by F-actin increased further at later times (not shown). Macrophage-like RAW cells, mouse peritoneal macrophages, and mouse bone marrow–derived macrophages all exhibit similar responses to agLDL (supplemental Figure III). To investigate a more physiological model, J774 cells were plated on top of agLDL bound to a smooth-muscle cell matrix. Similar F-actin structures near contact sites were observed (supplemental Figure III). The interaction with agLDL did not induce apoptosis (assessed by annexin V binding) or cell permeabilization (assessed by propidium iodide staining).

Under the conditions used in these experiments, the sites of contact between cells and agLDL were surface-connected membrane invaginations (also called surface-connected compartments or SCCs), similar to those described previously.14,15 Cells were incubated with Alexa546-agLDL (red, Figure 1C) at 37°C and then labeled on ice with Alexa488-CtB (green, Figure 1C) to label the plasma membrane. Because the cells are not fixed or permeabilized, the macromolecular cholera toxin can only label glycolipids on the surface of the cells. Sites of contact with agLDL were labeled by CtB (Figure 1C, arrowhead), indicating that they are connected to the cell surface. An axial slice through the confocal stack at the position of the green line in C shows that the contact between the green and red channels is actually a deep SCC (Figure 1C, arrowhead). After a 2-hour incubation of J774 cells with agLDL, some SCCs containing agLDL can still be observed, but Alexa546-agLDL can also be seen in organelles that are not connected to the surface (supplemental Figure IV). Individual SCCs could persist for more than 30 minutes as confirmed by live-cell observation (supplemental Figure V).
FC Is Enriched at the Site of Contact Between agLDL and Macrophages

In a previous study it was shown that there was significant CE hydrolysis while the agLDL particles were still outside the macrophage. This hydrolysis was not seen on incubation with macrophages lacking LAL. It was hypothesized that the hydrolysis was a consequence of selective uptake of CE into cells and hydrolysis in lysosomes. To determine whether CE hydrolysis might actually take place in SCCs, we used filipin, a fluorescent sterol-binding polycrylate that can be used for quantitative measurement of FC, to detect FC.

Cells were incubated with Alexa546-agLDL, fixed, and labeled with filipin and Alexa488-phalloidin (Figure 2). Cells incubated with agLDL on ice for 30 minutes (Figure 2A and 2D) showed weak filipin staining in the region contacting agLDL (arrowheads, Figure 2A and 2D), and there was no increased F-actin near the agLDL (Figure 2D). When cells were incubated with agLDL at 37°C, there was intense filipin staining near the agLDL (arrows, Figure 2B and 2C), and these regions were enriched in F-actin (Figure 2E and 2F). The bright filipin labeling indicates that there is an increase in FC at sites of contact between agLDL and the cell. The agLDL itself contains low amounts of FC as shown by the weaker intensity of filipin labeling of regions of agLDL that are not surrounded by F-actin (arrowheads, Figure 2A through 2F).

We quantified the average filipin intensity in pixels that contained agLDL. The filipin intensity for the cells incubated on ice was similar to the filipin intensity in aggregates that did not touch cells. After 30 minutes at 37°C, the filipin labeling in pixels that contained agLDL in contact with cells was significantly higher than for cells incubated on ice, and the filipin intensity remained elevated after a 1-hour incubation (Figure 2G). These data indicate that at least a portion of the CE hydrolysis occurs while agLDL is outside the cell. After a 2-hour incubation, increased intracellular filipin labeling is seen, and an increase in staining of lipid droplets by Nile Red is observed (not shown). Using biochemical assays, it was reported that about 10% of CE associated with agLDL is still extracellular.

Under the conditions of our experiments, the majority of cell-associated cholesterol is FC. We incubated cells with cholesteryl-[4-14C]-oleate reconstituted agLDL. After 90 minutes, extracellular labeled agLDL was removed by plasmin treatment, which has been reported to release agLDL residing in the SCC. More than 60% of the cell-associated radiolabeled cholesterol was FC (supplementary Table I). As discussed below, we have also demonstrated hydrolysis of CE in the agLDL that remains extracellular.

Formation of F-Actin Structures Around agLDL and Increase of Filipin Intensity Requires Rac or CDC42 Activities but Not Rho Activity

Inhibition of all Rho family GTPases by Clostridium difficile toxin B has been shown to inhibit the degradation of matrix-retained and agLDL by >90%, whereas the selective inhibition of Rho by C3 transferase had no effect. We evaluated the role of Rho GTPases in forming the F-actin containing structures at sites of contact with agLDL and in the localized increase in FC. Cells pretreated with toxins were incubated with a suspension of Alexa546-agLDL for 30 minutes in the presence of toxins, fixed, and labeled with
filipin and Alexa488-phalloidin (Figure 2H through 2M). In control cells, filipin intensity increased, and F-actin rich compartments formed at sites of contact between agLDL and cells (Figure 2H and 2K arrows). Treatment with toxin B inhibited the increase in filipin labeling (arrowheads, Figure 2I), and the ability of cells to form an F-actin rich compartment around agLDL was blocked (arrowheads, Figure 2L). Quantitative analysis showed that the average filipin intensity in pixels containing aggregates in contact with cells was significantly lower in cells treated with toxin B than in untreated cells (Figure 2N). Treatment with C3 transferase inhibited activation of Rho as confirmed by an effector pull-down assay (not shown), but there was no effect on the increase in filipin intensity or actin polymerization around agLDL (Figure 2J, 2M, and 2N). These data indicate that actin polymerization around agLDL and the increase in FC require the activity of Rac or Cdc42 but not Rho.

Hydrolysis of CE Is Required for Actin Polymerization Around agLDL

We examined whether a local increase of FC at the sites of contact between cells and agLDL is responsible for the increased actin polymerization observed around agLDL. We incubated J774 cells with agLDL containing a nonhydrolysable analog of CE – cholesteryl-oleyl ether. LDL reconstituted with either cholesteryl-oleyl ester or cholesteryl-oleyl ether was aggregated and incubated with cells for 30 minutes. The cells were fixed and labeled with filipin (Figure 3A through 3D). Sites of contact between agLDL and cells were identified in phase-contrast microscopy images. A much greater filipin signal was observed in areas of contact with ester-containing aggregates (arrowheads, Figure 3A and 3B) as compared with ether-containing aggregates (arrowheads, Figure 3C and 3D).

To investigate the role of CE hydrolysis in the increase of F-actin near agLDL, we examined F-actin in cells incubated with either ester- or ether-containing agLDL (Figure 3E and 3F). F-actin-rich structures that formed around agLDL reconstituted with CE were prominent (Figure 3E), and their morphology was similar to that observed in cells interacting with agLDL made from native LDL. Contact with the cholesteryl ether-containing agLDL caused some actin polymerization, but the area of the F-actin structures and the brightness of the phalloidin labeling were much less than for agLDL containing CE (Figure 3F and 3G). Quantitative analysis showed that the amount of F-actin around agLDL containing cholesteryl ether is significantly lower than around CE-reconstituted agLDL. These data show that formation of FC attributable to hydrolysis of CE at sites of contact between cells and agLDL is important for inducing local actin polymerization.

Depletion of FC by treatment with methyl-β-cyclodextrin (MβCD), a cholesterol chelator, also decreased local actin polymerization during interaction of macrophages with agLDL (supplemental Figure VI).

Knockdown of LAL Reduces Actin Polymerization Around agLDL

To test whether LAL-mediated CE hydrolysis is required for stimulation of actin polymerization around agLDL, we per-formed siRNA-mediated knockdown of LAL expression in RAW cells. The amount of LAL protein in the cells was reduced by more than 50% after treatment with LAL-specific siRNAs, whereas treatment with nontargeting siRNA did not cause a significant change in LAL expression (Figure 4A). Samples were incubated with Alexa546-agLDL for 30 minutes, fixed, and labeled for F-actin (Figure 4B and 4C). Treatment with nontargeting siRNA did not affect formation of F-actin structures around agLDL, but treatment with LAL-specific siRNA significantly reduced the formation of F-actin structures, as confirmed by quantitative analysis (Figure 4D). To confirm the specificity of the siRNA-mediated effect, we added back recombinant phLAL (human LAL produced in Pichia pastoris) to LAL siRNA-treated
cells. This mannosylated pH.LAL should enter macrophage
cells via the mannose receptor and be delivered to lysosomes.
Restoration of LAL to cells reversed the effect of siRNAs on
the F-actin surrounding agLDL (Figure 4D), demonstrating
that a decrease in LAL specifically caused the decreased
F-actin accumulation in siRNA knockdown cells.
Similar results were obtained on siRNA-mediated knock-
down of LAL in mouse peritoneal macrophages (supplemen-
tal Figure VII).

Treatment With Bafilomycin A1 Inhibits FC
Production and Formation of F-Actin at Sites of
Contact Between Cells and agLDL
Proper functioning of LAL requires an acidic pH. AgLDL-
containing SCCs could be acidified by H⁺-pumping vacuolar
ATPase (V-ATPase), which resides in the plasma membrane
and in internal organelles.24 To test whether acidification of
the agLDL-containing compartment is needed for extracellu-
lar CE hydrolysis and actin polymerization around agLDL,
we treated cells with bafilomycin A1, an inhibitor of the
V-ATPase. J774 cells were incubated with Alexa546-agLDL (red in C and D) in the absence (A and C) of bafilomycin A1, fixed, and
labeled with filipin (A and B) and Alexa488-phalloidin (green in C
and D). E, Quantification of the fluorescence power of F-actin
around agLDL. nt indicates not treated; bm, bafilomycin A1
-treated. F, Quantification of the average filipin intensity in parts
of agLDL touching cells. For statistics values were compared
to nontreated cells. Error bars indicate SEM for 10 fields contain-
ing >100 cells. **P<0.01. Scale bar indicates 10 μm. Images
were taken on a wide-field fluorescence microscope.

Figure 4. siRNA-mediated knockdown of LAL reduces F-actin
around agLDL. A, LAL protein levels in control and siRNA trans-
fected cells. All lanes are from the same blot but are shown in 2
panels to remove extraneous lanes. B and C, Transfected cells
were incubated with Alexa546-agLDL (red) and labeled with
Alexa488-phalloidin (green). Arrowheads, agLDL-macrophage
contact areas. D, Fluorescence power of Alexa488-phalloidin
around agLDL was quantified for nontreated cells, cells
transfected with nontargeting siRNA, cells transfected with 2
different LAL–specific siRNAs, and cells treated with phLAL after
LAL knockdown. For statistical analysis values were compared
to nontargeting siRNA-transfected cells. Error bars indicated
SEM for 14 fields containing >150 cells. **P<0.01, n.s. indi-
cates not significant; Scale bar, 15 μm. Images are single confoc-
al slices superimposed with DIC.

Figure 5. Treatment with bafilomycin A1 reduces FC production
and decreases F-actin at agLDL contact sites. J774 cells were
incubated with Alexa546-agLDL (red in C and D) in the presence
(B and D) or absence (A and C) of bafilomycin A1, fixed, and
labeled with filipin (A and B) and Alexa488-phalloidin (green in C
and D). E, Quantification of the fluorescence power of F-actin
around agLDL. nt indicates not treated; bm, bafilomycin A1
treated. F, Quantification of the average filipin intensity in parts
of agLDL touching cells. For statistics values were compared
to nontreated cells. Error bars indicate SEM for 10 fields contain-
ing >100 cells. **P<0.01. Scale bar indicates 10 μm. Images
were taken on a wide-field fluorescence microscope.
Discussion

In previous studies we found that increasing macrophage plasma membrane cholesterol levels globally (by incubation of cells with cholesterol chelated to a carrier, MβCD) led to alterations in macrophage signaling and F-actin organization.23,25 Based on these observations, we speculated that contact of macrophages with agLDL in the vessel wall could lead to similar alteration in cellular F-actin organization as a consequence of FC transfer. In this study, we show directly that interactions of agLDL with macrophages lead to local increases in FC and that these localized increases in FC influence (and are influenced by) local changes in F-actin organization.

Although the uptake and degradation of agLDL was shown to be actin-dependent,13 the spatial relationship of F-actin and lipoprotein aggregates was not explored. We have shown that global changes in F-actin are not induced by interactions with agLDL, but rather, F-actin-rich structures form almost exclusively at sites of contact between agLDL and the macrophage surface. This is reminiscent of the actin polymerization associated with phagocytic cups.26 However, the process described here is distinct from phagocytosis because agLDL is not taken up immediately into a sealed, degradative compartment (ie, a phagosome). Instead, agLDL remains in SCCs that are still open to the extracellular space, as demonstrated by the accessibility of the compartments to CtB.

The intimate spatial association of F-actin with the agLDL suggests that specific signaling mechanisms stimulate actin polymerization at areas of contact. Based on our previous work,23,25 we hypothesize that the local transfer of FC from agLDL drives the elaboration of F-actin rich membrane structures. We show here that macrophage engagement of agLDL leads to increases in FC at sites of contact with agLDL and that these sites colocalize with local increases in F-actin (Figure 2). This process involves Rho-family GTPases, which are key regulators of many actin-dependent processes in cells.27 Inhibition of Rac and Cdc42 activation completely abolished actin polymerization around agLDL. It appears that actin assembly leads to an increase in the area of contact with agLDL. We cannot rule out the possibility that other processes (eg, stimulated secretion of lysosomal contents) are also affected.

The role of receptors in the interaction of agLDL with macrophages is not well defined. Low-density lipoprotein-receptor–related protein (LRP) has been shown to play a role in the uptake of agLDL.28 However, several other LDL-binding receptors were examined for their potential role in uptake of agLDL, and none of them were sufficient for this process.13 In studies in mice, it was found that knockout of the scavenger receptors SRA and CD36 did not significantly reduce macrophage foam cell formation in ApoE⁻/⁻ mice on a Western diet.29,30 In addition heparan sulfate proteoglycans of the syndecan family, in particular syndecan-4, were shown to mediate uptake of lipase-modified LDL.31,32

Our prior studies showed that delivery of FC to cells via MβCD (and thus without any engagement of receptors) is sufficient for the induction of actin polymerization.23 To test the necessity of FC delivery for actin polymerization after engagement of agLDL, we inhibited CE hydrolysis by replac-
Disclosures

None.

References


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Materials and Methods

Lipoproteins

Human LDL was prepared as described\(^1\). Conjugation of LDL to AlexaFluor-546 (Invitrogen) was performed according to the manufacturer’s instructions. Reconstitution of LDL with cholesteryl oleate (Sigma) or cholesteryl oleoyl ether (American Radiolabeled Chemicals, Inc.) was performed according to a published protocol\(^2\). It was shown previously that cholesteryl-ether containing LDL is taken by cells similarly to LDL reconstituted with CE\(^3\). In our experiments vortex-agLDL reconstituted with cholesteryl ester or ether were indistinguishable from agLDL prepared from native LDL in size and general morphology as observed by fluorescence microscopy. Smooth muscle anchored agLDL was prepared by a 6 hour incubation of a vortex-agLDL suspension with a monolayer of smooth muscle cells grown 3 days post-confluency.

Interaction of agLDL with macrophages and fluorescent labeling of cells.

Mouse macrophage-like J774A.1 and RAW 264.7 cells were cultured and plated for experiments as described\(^4\). LDL was aggregated by vortexing for 10 seconds at maximum speed on Vortex-Genie 2 mixer (Scientific Industries Inc., Bohemia, NY) with 3 inch rubber-covered platform, and aggregates were then centrifuged for 10 minutes at 18,000 g. The supernatant was discarded, and the pellet was resuspended in serum-free bicarbonate-free DMEM buffered with 20mM HEPES. Approximately 50μg of agLDL was added per dish of cells and incubated at 37°C for indicated time periods, followed by fixation and labeling.
Fluorescent labeling of cells.

To visualize F-actin, cells were fixed for 30 minutes with 3.3% paraformaldehyde, rinsed with PBS, and incubated with 2Unit/ml of Alexa488-phalloidin (Invitrogen) in the presence of 0.25mg/ml saponin. For co-labeling of F-actin and free cholesterol (FC), fixed cells were labeled for 1 hour with a mixture of fluorescent phalloidin and 50μg/ml filipin (Sigma). For surface labeling with fluorescent cholera toxin subunit B (CtB) (Invitrogen) cells were incubated on ice for 3 minutes in the presence of 5μg/ml Alexa488-CtB in experimental medium, rinsed with ice-cold medium and fixed with paraformaldehyde. Although the gangliosides that bind CtB are often found in detergent-resistant (raft-like) membranes, we are using the CtB as a marker for the plasma membrane because raft and non-raft membranes are indistinguishable at optical microscopy resolution without specific treatment.

To assay the effects of Rho-family GTPase inhibitors, cells were preincubated for 4 hours with either 4μg/ml of cell-permeable C3 transferase from Clostridium botulinum (Cytoskeleton, Inc.) or with 40ng/ml of Clostridium difficile toxin B (Calbiochem), followed by incubation with agLDL in the presence of inhibitors.

Microscopy and image quantification.

All two-color images of phalloidin/agLDL or CtB/agLDL were taken with a Zeiss LSM510 laser scanning confocal microscope using a 63x, 1.4 numerical aperture (NA) plan Apochromat objective, axial resolution 0.8μm, axial step size 0.45μm. Three color
images of phalloidin/agLDL/filipin were taken with a Leica DMIRB epi-fluorescence microscope equipped with a 40x, 1.25 NA plan Apochromat objective.

For image quantification, MetaMorph software (Universal Imaging Corporation) was used. All images subjected to comparative quantification were acquired on the same day using the same microscope settings. Each experiment was repeated at least twice. For every experiment >10 randomly chosen fields with a total >100 cells were imaged and subjected to quantification.

**Quantification of F-actin in the vicinity of agLDL:** The procedures used to quantify the amount of F-actin in the vicinity of agLDL are illustrated in Fig. I. We obtained stacks of confocal images for each field at wavelengths appropriate for Alexa546-agLDL (red) and Alexa488-phalloidin (green). A sum projection image for each color (sum intensity in each pixel) was generated from the confocal stack. We determined a threshold in the red channel (agLDL) that would include nearly all of the observable agLDL in the images. We then measured the total Alexa488-phalloidin fluorescence intensity (i.e., the fluorescence power) within the thresholded area for each field. To normalize for the number of cells in different fields, we divided the fluorescence power by the number of cells touching agLDL in the field. We used the same threshold level for each image within an experimental data set. By this procedure the total phalloidin fluorescence power within the thresholded regions per cell touching agLDL was measured.

**Quantification of average intensity of agLDL labeling with filipin:** The procedures used to quantify the amount of filipin fluorescence associated with agLDL are illustrated in
Fig. II. We used widefield epi-fluorescence microscopy because of the UV excitation and relatively rapid photobleaching of the filipin. We determined a threshold in the red channel (agLDL) that would include nearly all of the observable agLDL in the images. We then measured the average filipin fluorescence intensity in all of the pixels within the thresholded area for each field. These measurements would correspond to filipin labeling of the aggregates and the parts of cells that are underneath aggregates. The average filipin intensity was measured for each field. In some cases, we measured the average filipin intensity in regions of aggregates that were not in contact with cells. Those regions were selected manually by examination of the agLDL fluorescence image and the transmitted light image of the cells.

Statistical analysis was performed using GraphPad InStat software. For comparisons of more than two groups, ANOVA was used, followed by the Student-Newman-Keuls test for pairwise comparisons. All data passed the normality test.

Transfection of siRNA.

Mouse-LAL mRNA specific and non-targeting siRNA oligos were purchased from Dharmacon. Transfection was performed using Lipofectamine (Invitrogen) for mouse peritoneal macrophages and HiPerFect reagent (Quiagen) for RAW 264.7 cells according to the manufacturers’ instructions.

LAL reconstitution.

Recombinant Pichia-derived Human LAL (phLAL) was kindly provided by Drs. Hong Du and Greg Grabowski (The Children's Hospital Research Foundation, Cincinnati, OH)
and was added to RAW 264.7 cells as described\(^6\).

**Hydrolysis of cholesteryl-[4-\(^{14}\)C]-oleate-containing LDL**

LDL was reconstituted with cholesteryl-[4-\(^{14}\)C]-oleate (American Radiolabeled Chemicals, Inc.) as described\(^2\). Vortex agLDL was delivered to J774 cells in media containing 0.2% fatty-acid free BSA (to prevent non-specific protein binding), 1% ACAT inhibitor (to prevent re-esterification of hydrolyzed \(^{14}\)C]cholesteryl ester) and, where indicated, 2 \(\mu\)M bafilomycin A1. Cells were incubated with agLDL for 90 minutes followed by 15 minutes treatment with 1U/ml of plasmin (Sigma) to release extracellular agLDL\(^7\). The lipids were extracted from cells twice with 2 ml of hexane:isopropanol (3:2 v/v) for 30 minutes with shaking at room temperature, dried under argon, and re-suspended in chloroform. \(^{14}\)C]cholesteryl ester and \(^{14}\)C]free cholesterol radioactivity were quantified by thin layer chromatography (TLC). CE hydrolysis is expressed as the percentage of the sum of the signals of FC and CE.

**Supplementary material references.**


Table 1. Biochemical assay of radiolabeled CE hydrolysis during macrophage interaction with agLDL.

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<th>Bafilomycin A1</th>
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<td>FC/(FC+CE)</td>
<td>64±14%</td>
<td>7.2±3.5%</td>
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AgLDL reconstituted with cholesteryl-[4-14C]-oleate was added to a monolayer of J774 macrophages. Following a 90 minute incubation, cells were treated with plasmin to remove cell-engaged but extracellular agLDL and then assayed for cell-associated radiolabeled FC and CE. Significant CE hydrolysis and cellular accumulation of FC occurred. The generation of radiolabeled FC was decreased upon addition of bafilomycin A1, a V-ATPase inhibitor. In addition the total amount of radioactivity taken up by cells was reduced (53.8±7.5% of control value) in cells treated with bafilomycin. This decrease confirms the role of extracellular acidification in processing and uptake of cholesterol from agLDL. Error: standard deviations.
Figure I. Quantification of F-actin in areas of contact between macrophages and agLDL. Sum projections were created from confocal stacks (A-C) and corrected for background. Pixels positive for agLDL were selected by thresholding, and a binary mask was created using the thresholded area (D). The same threshold value was used for all images within each experiment. The mask was applied to each F-actin image to select those parts of the cell in contact with agLDL (i.e., those areas under the mask) (E). An additional threshold was applied to remove cellular background (asterisk) prior to quantification. Regions below the threshold intensity are colored green, and pixels with intensity above the threshold, corresponding to F-actin enriched structures around agLDL (arrows in B, C, F) are shown as grayscale values. The total fluorescence power in the gray areas was quantified on a per cell basis. (F).
Figure II. Quantification of the average filipin intensity in SCCs.

AgLDL and filipin images (A, B) were corrected for background. Pixels positive for agLDL were selected by thresholding, and a binary mask was created using the thresholded area (C). The same threshold value was used for all images within each experiment. The mask was applied to the corresponding filipin image to select those parts of the cell in contact with agLDL (D). Average fluorescence intensity per pixel in the thresholded areas was quantified. We note that this assay can include areas of agLDL that are far from the cell and would not be expected to be undergoing catabolism. To examine the filipin labeling of agLDL itself, we quantified the filipin intensity of non-cell engaged aggregates.
Figure III. Various types of macrophages form F-actin-rich structures around agLDL. Macrophage-like RAW cells (A), primary mouse peritoneal macrophages (B) or bone marrow-derived mouse macrophages (C) were incubated with Alexa546-agLDL (red), fixed, and labeled with Alexa488-phalloidin (green). Arrowheads indicate F-actin rich structures, similar to those seen in J774 cells, surrounding agLDL. As a more physiological model of the macrophage-agLDL interaction, J774 cells were plated on the top of a smooth-muscle cell generated matrix containing agLDL (D). A merged image shows the formation of F-actin-rich structures around agLDL. (A – C) maximum intensity projections, D – single confocal slice combined with DIC image. Scale bars: 10μm.

Figure IV. After a 2 hour incubation, agLDL is partially internalized. J774 cell were incubated with Alexa546-agLDL (red) for 2 hours, fixed and labeled with Alexa488-phalloidin (green in A) or labeled before fixation with Alex488-CtB (green in B). Aggregates negative for F-actin or CtB likely correspond to degraded internalized agLDL (arrows). Images are single confocal slices. Scale bars: 10μm.
**Figure V. AgLDL remains in SCC for at least 30 minutes.** J774 cells were imaged by DIC microscopy after a 30 minute incubation with Alexa546-agLDL (A). The cells were maintained on the microscope stage at 37°C for an additional 30 minutes, and then labeled for 2 minutes with Alexa488-CtB. The agLDL (red in B) and CtB (green in B,C) were imaged by confocal microscopy. A single confocal image plane is shown. Most of the agLDL remains in association with the cell surface, as indicated by CtB labeling (arrowheads in A-C). Scale bar: 10μm.

**Figure VI. Depletion of FC by methyl-β-cyclodextrin inhibits actin polymerization in macrophages contacting agLDL.** J774 cells were incubated with Alexa546-agLDL (red in A, B) for 30 minutes in the absence (A, C) or presence (B,D) of methyl-β-cyclodextrin (MβCD). The cells were fixed and labeled with Alexa488-phalloidin (green in A, B) and filipin (C, D). MβCD caused a reduction in the size of the F-actin-rich structures in macrophages contacting agLDL as compared with non-treated cells (arrowheads in A, B). MβCD treatment results in a dramatic reduction of the filipin labeling of cells and agLDL (C, D). Images were taken on a wide-field fluorescence microscope. Scale bar: 10μm.
Figure VII. siRNA-mediated knock-down of LAL in mouse peritoneal macrophages results in a decrease of actin polymerization in macrophages contacting agLDL. (A) Mouse peritoneal macrophages were transfected with LAL-specific or non-targeting siRNA oligonucleotides and incubated for 3 days. A western blot shows approximately an 80% decrease of LAL protein level by a mixture of four targeting siRNAs. (B, C) Transfected cells were incubated for 30 min with Alexa546-agLDL (red), fixed, and then labeled with Alexa488-phalloidin (green). A decrease of actin structures around agLDL was seen upon LAL knock-down (arrowheads). (D) The fluorescence power of the phalloidin signal for parts of the cells in contact with agLDL was quantified for non-transfected cells, cells transfected with non-targeting siRNA, cells transfected with 2 different LAL-specific siRNAs, or with mixture of 4 LAL-specific siRNAs. Error bars, SEM for 10 fields >100 cells. *** - p<0.001. Images are maximum intensity projection of confocal stacks. Scale bar: 10μm.