DIFFERENT INTRACELLULAR TRAFFICKING OF LDL- AND ACETYLATED LDL-CHOLESTEROL LEAD TO DISTINCT REVERSE CHOLESTEROL TRANSPORT MECHANISMS

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Running footnote: Cholesterol traffic in macrophages.

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Abstract. Endocytosis of low density lipoprotein (LDL) and modified LDL is classically described as regulated and unregulated cholesterol delivery to macrophages, respectively. To elucidate the mechanisms of cellular cholesterol transport and egress under both conditions, various primary macrophages were labeled and loaded with cholesterol or cholesteryl ester from LDL or acetylated-LDL (acLDL), and the intracellular cholesterol trafficking pathways were examined. Confocal microscopy using fluorescently labeled DiO-LDL and DiD-acLDL, clearly demonstrated their discrete intracellular traffic pathways leading to accumulation in distinct endosome compartments. ABCA1-mediated cholesterol efflux to apoA-I was much greater for acLDL-loaded macrophages as compared to LDL. Treatment of LXR ligand 22-OH failed to significantly increase cholesterol efflux to apoA-I in LDL treated cells, but significantly increase efflux in acLDL loaded cells. In contrast, at equivalent levels of cellular cholesterol loading, LDL-derived cholesterol was preferentially effluxed by diffusional pathways, as indicated by increased ABCG1 expression and specificity for HDL. In vivo studies of reverse cholesterol transport (RCT) from cholesterol-labeled macrophages injected intraperitoneally demonstrated that LDL-derived cholesterol was more efficiently transported to the liver and secreted into bile as compared to acLDL-derived cholesterol. This contrasts with in vitro cholesterol efflux to apoA-I but is consistent with in vitro cholesterol efflux to HDL, indicating a greater efficiency of HDL than lipid-poor apoA-I in interstitial fluid in controlling the in vivo RCT. These assays, taken together, emphasize the importance of mediators of diffusional cholesterol efflux in reverse cholesterol transport and the role of ABCA1-mediated efflux in foam cell regression.

Supplementary keywords: Cholesterol efflux, ABCA1, ABCG1, SR-BI.

Abbreviations: AcLDL, acetylated LDL; BMDM, bone marrow derived macrophage; mβ-CD, methyl β-cyclodextrin; RCT, reverse cholesterol transport;
Development of atherosclerosis is initiated by the formation of macrophage-derived foam cells (1). As professional scavenger and sentinel cells, macrophages actively take up and process apoptotic and necrotic cells (2) as well as excess plasma and tissue LDL and modified LDL (1), which under pathological conditions lead to the accumulation of large amounts of cholesterol. To maintain cellular cholesterol homeostasis, the macrophage distributes and transports the excess cholesterol into specific cellular compartments and converts it into nontoxic cholesteryl ester (CE) for storage (1,3). The cell can also export excess cholesterol to appropriate extracellular acceptors by transfer mechanisms through cholesterol gradients that involve mostly HDL, mediated via SR-BI (4) and the ABCG1/G4 transporter (5,6) or the apoA-I-mediated pathway that operates through ABCA1 (7-10). In addition to the canonical receptor pathway for regulated LDL uptake by the LDL-receptor (11,12), the macrophage can take up LDL via receptor independent pathways, such as macropinocytosis, which, in a dose dependent manner, can lead to macrophage foam cell formation under certain conditions in vitro (13-15). On the other side, the unregulated internalization via a number of scavenger receptors and other unknown receptors (16) accounts for up to 95% of uptake of modified lipoproteins. Receptor mediated uptake of LDL and acLDL is mostly via clathrin-coated pits (17). Whereas LDL is delivered to centrally located vesicles, βVLDL or acLDL is observed in peripherally distributed vesicles where its catabolism is slower (18,19). Comparison of the uptake of acetylated LDL (acLDL) and oxidized LDL (oxLDL) also showed that the two ligands traffic to different endosomes and accumulate in distinct lysosomal compartments (20), an observation compatible with the reported morphological and functional heterogeneity of the endocytic compartment in macrophages (21-24). However, the cellular uptake of oxLDL is complex and mediated by a variety of receptors that include MARCO, SRCL, CD36, and LOX1 as well as SR-AI/II (16). In addition, oxLDL contains both oxidized proteins and lipids, which have multiple effects, including impaired degradation (25) and enhanced inflammatory stimulation (26). To further explore how the differential internalization mechanisms of native and modified lipoproteins affect cholesterol transport and homeostasis in macrophages and avoid the complications of multiple effects of oxLDL, we have used the modified lipoprotein, acetylated LDL (acLDL), in comparison with native LDL. Here we demonstrate that LDL and acLDL traffic to discrete endosomal compartments, and that cholesterol derived from each of these lipoproteins enters different cellular pools and is effluxed in vitro through largely distinct pathways: LDL-derived cholesterol efflux preferentially via the HDL-mediated pathway dependent in part on ABCG1, whereas acLDL-derived cholesterol efflux preferentially via the more specific lipid-poor apoA-I/ABCA1-dependent pathway. Interestingly, in vivo reverse cholesterol transport to the liver and bile is shown to be more significant for the LDL-derived cholesterol reflecting the importance of the available mediators of diffusional cholesterol efflux in reverse cholesterol transport.
Materials and Methods:

Animals:
ABCA1 (-/-) mice were a kind gift from Dr. Edward M Rubin, DOE Joint Genome Institute, Berkeley, CA. SRA(-/-) mice were transferred from Dr. T. Kodama (University of Tokyo, Japan). C57Bl6 (C57), NPC1 (-/-), SR-BI (-/-), LDLr (-/-) and Caveolin-1 (-/-) mice were purchased from Jackson Lab, and maintained and bred in the animal facility of Ottawa Heart Institute. All protocols were approved by the University of Ottawa Animal Care Committee.

Reagents:
The ACAT inhibitor, Sandoz 58-035, was a gift from Novartis. Simvastatin was kindly given by Merck. Cholesterol-[1, 2-3H], mevalono-lactone-Rs-[5-3 H(N)], choline chloride-[Methyl-3 H], cholesteryl oleate were obtained from PerkinElmer Life and Analytical Sciences. 22R-hydroxy cholesterol and progesterone were purchased from Gibco. Methyl-β-cyclodextrin (mβ-CD) was obtained from Cerestar (Cargill, Inc., Minneapolis, MI). Recombinant human apoA-I is produced in our laboratory (27). Lipoproteins were prepared from the plasma of normolipidemic subjects as described by Havel et al. (28). Acetylated-LDL (acLDL) was prepared as described by Goldstein et al. (29). βVLDL were isolated from cholesterol-fed apoE-null mice (30).

Generation of macrophages:
Fetal liver-derived macrophages (FLDM) were harvested from pregnant mice before delivery (31). Bone marrow-derived macrophages (BMDM) were flushed from mouse femurs. Macrophages were generated by incubating fetal liver cells (2x10^6 cells/ml) or bone marrow cells (10^6 cells/ml) with DMEM of 10% FBS complemented with 15% L929 conditioned medium for 7 d.

Lipid efflux:
Unless indicated in the legends, labeling conditions are as follows. Macrophages were washed three times with plain DMEM medium and then labeled with LDL or acLDL (50μg protein/ml) that had been preincubated with 5μCi 3H-cholesterol in 1% FBS of DMEM for 24 h. The cells were equilibrated with 2mg/ml BSA overnight. For labeling with 3H-mevalonate (10μCi/ml), 3H-acetate (20μCi/ml), 3H-cholesteryl oleate (5μCi/ml) or 3H-choline (5μCi/ml), the cells were incubated for 40 h in DMEM with 1% FBS. Incorporation of 3H-cholesteryl oleate into LDL and acLDL was carried out as described before (32). Efflux to apoA-I (50μg in 2mg/ml BSA medium) was monitored for 3-5 h. Efflux to BSA (2mg/ml BSA of DMEM medium) was allowed to proceed for 16 h. Efflux to mβ-CD (10mM in 2mg/ml BSA DMEM) was carried out for 15 min at 37°C or 4°C.

Lipid analysis:
Cellular lipids were extracted (33), separated by thin layer chromatography (TLC) using hexane:diethylether:acetic acid (105:45:1.5) as running solvent on Sil-G TLC plates (EMD Chemicals,
Darmstadt). Lipid bands were detected by exposure to iodine vapors, scraped off the TLC plate and radioactivity measured with a scintillation counter. For total cholesterol determination, cells were washed with cold PBS, cholesterol extracted by isopropanol and measured by colorimetric assay (Wako Chemicals, Richmond, VA).

**Western Blotting:**
Cellular proteins were solubilized in RIPA buffer (20 mM Tris-HCl, 150 mM NaCl, 0.1% SDS, 1% deoxycholic acid, 1% Triton X-100, cocktail protease inhibitors (Roche)), electrophoresed on a 6% SDS-polyacrylamide gel, and transferred to nitrocellulose at 125V for 4 h. An ABCA1 and ABCG1 antibody (Novus Biologicals, 1:500 dilution) were used for detection, with an anti-rabbit secondary antibody conjugated with horseradish peroxidase (Amersham Biosciences) for detection.

**Reverse cholesterol transport:**
Macrophages either from ABCA1-/-, +/- or +/+ mice were labeled with cholesterol delivered by LDL or acLDL for 24 h. Cells are removed with 5mM EDTA PBS, and injected intraperitoneally into C57BL6 mice (34). Gallbladders, livers and feces were harvested 24 h later. Tissues and feces were treated with 0.5N NaOH and lipid radioactivity counted.

**Image analysis:**
Lipoproteins were labeled with 3,3’-dioctyldecyloxycarbocyanine perchlorate (DiO) and 1,1’-dioctyldecyl-3,3,3’,3’-tetramethylindodicarbocyanine perchlorate (DiD) (Molecular Probes) as previously reported (18,32). Dansyl-cholestanol was synthesized according to the method of Wiegand et al. (35). Macrophages were loaded with LDL or acLDL that was pre-equilibrated with dansyl-cholestanol. An Olympus FV1000 confocal microscope, complete with a 100X objective (NA 1.4), a 488 Argon-ion laser, and a 633 Helium/Neon laser, was used for DiO and DiD fluorescence microscopy. For dansyl-cholestanol imaging, an Olympus IX70 inverted fluorescent microscope outfitted with a monochrometer from Till Photonics was used. Images were captured using the Imago CCD camera and processed using TILLvisION software.

**Statistics:**
Student’s T test was applied to evaluate significant differences.

**Results**

**Macrophages labeled and loaded with acLDL-derived cholesterol preferentially efflux through ABCA1 and macrophages labeled and loaded with LDL-derived cholesterol preferentially efflux by a diffusional pathway.**

Bone marrow derived macrophages (BMDMs) were labeled and loaded with \( ^3 \)H-cholesterol delivered by either acLDL or LDL. AcLDL-loaded macrophages effluxed cholesterol to apoA-I at a rate nearly 7-fold higher than
that of the macrophages loaded with LDL (Fig. 1A). Furthermore, apoA-I-mediated-cholesterol efflux from the cells loaded with acLDL was totally dependent on ABCA1 expression, while cells loaded with LDL displayed about 25% residual apoA-I-specific cholesterol efflux in ABCA1(-/-) macrophages (Fig. 1A). In contrast, macrophages loaded with LDL effluxed cholesterol to HDL and BSA at a significantly greater rate than those loaded with acLDL (Fig. 1B,C). This difference was not related to the preferential plasma membrane cholesterol labeling by LDL cholesterol, since methyl β-cyclodextrin (mβ−CD) extraction at 4ºC, which measures the proportion of cholesterol present at the cell surface, showed equivalent plasma membrane labeling by LDL (9.9%) and by acLDL (10.2%, Fig. 1D). However, short-term cholesterol efflux to mβ-CD at 37ºC, which measures cholesterol present at the plasma membrane and the recycling compartment (36), was significantly greater from cells loaded with LDL (44%) compared to acLDL (22%), regardless of ABCA1 expression level (Fig. 1D). Therefore cholesterol derived from LDL preferentially loaded the recycling compartment, a conclusion supported by the greater efflux to HDL and to mβ-CD at 37ºC. This difference was not related to the use of specific differentiated murine macrophages, since the same efflux specificity existed in all tested murine macrophages (Fig. 1E). The same specificity was also observed for efflux to immunoprecipitated apoA-I (Fig. 1F).

To further document the differential trafficking of LDL- and acLDL-derived cholesterol and its independence of the labeling efficiency of cell surface compartments, macrophages were labeled to an equivalent level with 3H-cholesteryl oleate, pre-incorporated into LDL or acLDL to the same specific activity (Fig. 2A). Under these conditions, cholesterol efflux to apoA-I from macrophages loaded with acLDL remained significantly greater (p<0.004) than that from cells loaded with LDL by about 3-fold (Fig. 2B). When cells were equally loaded with cholesteryl ester-labeled lipoproteins, the proportion of accumulated acylCoA:cholesterol acyltransferase (ACAT) generated cholesteryl esters was about 10-fold greater with acLDL as compared to LDL (Fig. 2C). Similar to 3H-cholesterol labeling of lipoproteins, cholesterol derived from 3H-cholesteryl oleate was equally accessible to mβ-CD extraction at 4ºC whether delivered by LDL or acLDL (Fig. 2D). However mβ-CD extraction at 37ºC was much higher for LDL-loaded macrophages, similar to the previous results (Figure 1D) and confirming a preferential labeling by LDL of the recycling compartment.

It is well known that acLDL is a potent ACAT activator (37,38) and our own data showed that treatment with the same amount of lipoprotein (either LDL or acLDL) in macrophages causes significantly more cholesterol esterification by labeling with 3H-oleate in acLDL treated cells (data not shown). To understand if the increased apoA-I-mediated efflux was simply due to increased cholesteryl ester formation, since cholesterol efflux to apoA-I is closely related to the level of cellular cholesteryl ester (Supplementary Fig. 2) and in keeping with previous studies (39), macrophages were loaded for 5h or 24h with acLDL or LDL to achieve similar levels of radioactive labeling (Fig. 2E) and cholesteryl ester formation (Fig. 2G). Under these conditions, a clear difference in the amount of apoA-I mediated cholesterol efflux was evident (Fig. 2F; 4-fold increase for acLDL
Increased efflux to apoA-I might reflect preferential cholesterol mobilization from the ACAT accessible pool, however, ACAT inhibition during the labeling period increased apoA-I-mediated cholesterol efflux from the macrophages labeled with acLDL but not LDL (Fig. 2H). Increased efflux to apoA-I might reflect preferential cholesterol mobilization from the ACAT accessible pool (cholesteryl ester droplets). However, ACAT inhibitor Sandoz 58-035 administration during the labeling period completely suppressed the cholesterol re-esterification cycle (data not shown), increased apoA-I-mediated cholesterol efflux from the macrophages labeled with acLDL but not LDL (Fig. 2H). These results demonstrate that the observed differences of LDL and acLDL loaded macrophages are not simply due to increased loading or preferential targeting to an ACAT-accessible pool, but rather further demonstrate that lipoprotein-derived cholesterol, which internalizes through distinct mechanisms (see below), remains in two functionally distinct pools.

**Level of cholesterol loading with LDL or AcLDL does not affect efflux specificity.**

Next, we showed that the specificity of the pathways is independent of the net uptake of lipoprotein cholesterol. Increasing LDL concentration resulted in a linear and non-saturating increase in uptake, which at 250 μg/ml doubled total cellular cholesterol and increased cholesteryl ester level to about 5% (Fig. 3A,B), indicating that a non-receptor-independent mechanism was involved in contributed to macrophage LDL uptake as reported by others (13,14). In contrast, increasing acLDL concentration up to 50 μg/ml rapidly increased cellular cholesterol, which plateaued at a 3-fold increase, and was accompanied by an increase in cholesteryl ester to about 40% (Fig. 3C, D) as well as a greater upregulation of ABCA1 (Fig. 3E). Under conditions that achieve equivalent cholesterol loading with LDL and acLDL, we measured the total cellular cholesterol level prior to addition of apoA-I and then carried out an efflux assay (Fig. 3G, H). Thus, acLDL at 6.25 μg/ml and LDL at 150 μg/ml loaded the cells to the same level, 30.2 and 29 μg total cholesterol mass respectively (SD<15%), but acLDL elicited twice as much ABCA1-mediated efflux. The same difference in efflux was maintained with 12.5 μg/ml acLDL and 250 μg/ml LDL, which raised cellular cholesterol to 38.7 and 35.6 μg/mg cellular protein, respectively.

Since ABCG1 mediates efflux to HDL (5,40) and since LDL derived cholesterol is effluxed to HDL at a higher rate than acLDL-derived cholesterol (Fig. 1B), we also measured ABCG1 protein level in LDL and ac-LDL treated cells (Fig. 3F). Clearly, ABCG1 protein expression was increased in LDL treated cells, but not as much as in acLDL treated cells, suggesting that the robust cholesterol efflux to HDL in LDL treated cells was not only directly related to ABCG1 expression but also to a specific targeting of cholesterol and possibly of the transporter, to the recycling compartment. Alternatively, this discrepancy may reflect the contribution of another, yet, uncharacterized mediator of the diffusional pathway (41).

**Specificity of LDL and AcLDL cholesterol internalization.**

To determine the specificity of LDL cholesterol internalization via the LDL receptor (LDLr) and acLDL via SRA in macrophages, we first labeled BMDM from LDLr(-/-) and WT mice with 3H-cholesteryl oleate-LDL.
Both BMDM were pretreated with 3%LPDS in order to upregulate LDLr by depleting cellular cholesterol. Under this condition about 70% of LDL cholesterol internalization could be inhibited by addition of cold LDL, indicating its dependence on LDLr (Fig. 4A), whereas the fraction of LDL cholesterol uptake, which could not be inhibited by cold LDL, may be mediated by macropinocytosis (13-15). Under in vivo physiological conditions, there is no cholesterol deprivation. Therefore we labeled LDLr(-/-) macrophages with 3H-cholesteryl oleate -LDL or 3H-cholesterol-LDL without pretreatment with LPDS. Under these conditions, most LDL cholesterol internalization (about 75%) was LDLr independent (Fig. 4B), indicating that the majority of LDL cholesterol uptake in vivo was dependent on macropinocytosis or another uncharacterized mechanism. Next, we tested the specificity of acLDL cholesterol internalization via SRA. The uptake of acLDL was decreased in SRA(-/-) macrophages compared to C57BL6 macrophages (Fig. 4C), but these cells still accumulated a large amount of cholesterol from acLDL, suggesting contribution of other scavenger receptors in normal and SRA(-/-) macrophages. The uptake of acLDL cholesterol by C57BL6 macrophages could not be competed by the addition of cold LDL (Figure 4C), consistent with the absence of overlap between the two types of receptors in normal macrophages, as previously reported (42). Uptake of acLDL could be almost completely inhibited (93%) by addition of a 50 fold excess of cold acLDL in C57BL6 macrophages (Fig. 4D) and was independent of the expression of LDLr, indicating that acLDL uptake was receptor dependent (see Fig. 3C,D). Taken together, the acLDL cholesterol uptake is receptor dependent, but LDL cholesterol could be taken up by either receptor or non-receptor mediated pathways.

Importantly, the patterns of cholesterol efflux to apoA-I were not altered when comparing macrophages of different genetic background (ie. wildtype, SRA(-/-) or LDLR(-/-)) that were labeled with 3H-cholesterol or -cholesteryl oleate incorporated in either LDL or acLDL, (Fig. 4 E,F). Similarly, the specificity of efflux to HDL or mβ-CD was not altered by LDLr or SRA deficiencies (data not shown), but reflected the ligand (i.e., LDL versus acLDL) properties.

**LDL- and acLDL-related cholesterol traffic pathways are differentially regulated.**

Caveolin-1, which has been shown to transport cholesterol between intracellular compartments/ER and plasma membrane (43,44), had no effect on apoA-I-mediated cholesterol efflux from macrophages labeled with LDL or acLDL cholesterol (data not shown). On the other hand, deficiency in NPC1, which controls cholesterol traffic from late endosomes (45,46), led to a 44% reduction in cholesterol efflux from LDL labeled macrophages, and 72% reduction from acLDL labeled cells (Fig. 5A). A similar difference in cholesterol traffic from late endosomes was also observed upon treatment with progesterone, which blocks transport from late endosomes to plasma membrane (36,47,48). Progesterone abrogated cholesterol efflux to apoA-I in LDL labeled cells, whereas a small but significant (16%) cholesterol efflux remained in acLDL labeled cells compared to control (Fig. 5B). Since LXR ligands, such as hydroxycholesterol, are potent ABCA1 inducers, we treated the cholesterol-loaded cells with 22-hydroxycholesterol overnight during the equilibration time (before incubation...
with apoA-I), in an attempt to bypass the differential induction of ABCA1 by the two lipoproteins. As expected, 22-hydroxycholesterol significantly increased the ABCA1 protein expression (data not shown) in both LDL and acLDL labeled cells and significantly increased cholesterol efflux to apoA-I in ac-LDL labeled cells, but cholesterol efflux to apoA-I in LDL labeled cells was not altered (Fig. 5C). These results further indicate that trafficking of LDL-derived and –acLDL-derived cholesterol are differentially regulated.

**Imaging of LDL- and acLDL-related traffic.**

Dual fluorescent label experiments were performed in which DiO-labeled LDL and DiD-labeled acLDL were jointly incubated with macrophages. The fluorescently labeled lipoproteins trafficked to distinct endosomes which only partly overlapped at early time points but remained separated at later time points (over a period from 20 min to 2 h; Fig. 6). We followed the fluorescence for up to 24h (the conditions that were used for loading) and saw no colocalization at any point (data not shown). These compartments were lysotracker positive (data not shown), suggesting that the DiO-labeled LDL and DiD-labeled acLDL were trafficked to distinct late endosomes/lysosomes in agreement with earlier observations of others (19,20). To specifically follow cholesterol movement, we also labeled LDL and acLDL with dansyl-cholestanol, a fluorescent analog of cholesterol. Dansyl-cholestanol-labeled LDL or acLDL were separately added to WT or ABCA1-KO macrophages under conditions that would achieve equal loading or labeling of 3H-cholesterol (24h incubation), and examined by fluorescence microscopy. Dansyl-cholestanol delivered by LDL showed diffuse fluorescence throughout the cell with some punctate structures. On the other hand, dansyl-cholestanol delivered by acLDL was present in multiple bright punctate structures (Supplementary Fig. 1b). In the absence of ABCA1, both LDL and acLDL-delivered dansyl-cholestanol loaded macrophages showed increased total cellular fluorescence (data not shown). The fluorescence patterns observed here suggest that, over time, acLDL-delivered dansyl-cholestanol tends to accumulate in the late endosomes, while LDL-delivered dansyl-cholestanol is transferred to other cellular membrane compartments.

**LDL- and acLDL-derived cholesterol returns to the liver at different rates.**

To test if the separate metabolic pathways that were shown to exist in vitro for LDL and acLDL cholesterol could also be documented in vivo, a reverse cholesterol transport (RCT) experiment (34) was carried out. Equal cell numbers of BMDM from normal C57BL6 mice, labeled to the same extent with 3H-cholesterol delivered either by LDL or acLDL, were injected intraperitoneally into normal C57BL6 mice. After 24 h, the animals were killed (after a 5 h fast) and the cholesterol radioactivity transported from the intraperitoneal site of injection to the liver, gallbladder, or feces was measured. After 24 h post-injection of fluorescent GFP-macrophages, we estimated that the majority of injected macrophages remained in the peritoneal cavity. Histological examination of liver sections failed to detect any fluorescent macrophages (data not shown), suggesting that the injected macrophages themselves did not migrate to the liver in agreement with the results of
others (34) and thus further demonstrated that the radioactivity measured in the liver represent cholesterol transported to the liver. Agarose gel electrophoresis of the concentrated peritoneal fluid demonstrated the presence of both α-HDL and pre-β-HDL migrating apoA-I (Fig. 7A and B; Lane 3), with a large preponderance of the pre-β-HDL, which can mediate cholesterol efflux in an ABCA1 dependent manner. The return of cholesterol from LDL labeled macrophages to the liver was significantly greater than that from acLDL labeled macrophages by about 50% (Fig. 7D). Similar results were obtained for RCT to the bile, which was 35% higher for LDL derived cholesterol (Fig. 7C) and to feces (results not shown). As established above, the in vitro efflux of cholesterol from macrophages follows distinct pathways that are faster for acLDL derived cholesterol; however, in vivo efflux and RCT are dependent on the concentration of HDL and apoA-I present in the peritoneal fluid. The results therefore reflect the respective in vivo contributions of efflux mediated by apoA-I and ABCA1 versus HDL and ABCG1 or SR-BI or other pathways and the peritoneal fluid concentration of their acceptors.

Discussion

We have demonstrated that the intracellular transport pathways for acLDL- and LDL-derived cholesterol are different and that cholesterol derived from these lipoproteins segregates into two mostly distinct cellular pools. AcLDL delivered cholesterol is preferentially transported into late endosomes and lysosomes, and further converted to cholesteryl ester in ER-associated lipid droplets, whose pools are readily accessible to efflux to apoA-I by an ABCA1 dependent pathway in agreement with earlier reports (7,49). On the other hand, the pool of LDL-derived cholesterol is preferentially transported to a recycling compartment, where it is apparently more accessible to efflux mediated by HDL, BSA and mβ-CD. These specific efflux phenotypes for acLDL and LDL derived cholesterol were consistently observed in all murine macrophages tested (Fig. 1E). In addition, cholesterol from other types of modified or pathological lipoproteins (ox-LDL, βVLDL) was also shown to be readily accessible to apoA-I (Supplementary Fig. 2B), whereas cholesterol derived from native physiological lipoproteins (HDL, LDL) as well as from engulfment of apoptotic cells was not. The traffic through the apoA-I-mediated efflux pathway is clearly independent of the level of 3H-cholesterol labeling or loading (Fig. 2, 3, Supplementary Fig. 2A). All atherogenic lipoproteins are potent ACAT activators (Supplementary Fig. 2C) in agreement with earlier studies (50), and the accumulated cholesterol can be primarily mobilized by ABCA1-mediated efflux. The dichotomy of cholesterol traffic is dictated by the specificity of entry (different receptors, adaptors, and receptor-independent uptake, such as macropinocytosis), transport, accumulation in different endosomes and mobilization for efflux. The specificity of entry starts with the retention of acLDL in the cell periphery in protrusions, such as microvilli, in contrast with the rapid delivery of LDL to late endosomes (19). The initial association/retention of acLDL at the cell surface appears to take place in large cell surface structures different from the classical clathrin-coated-pit pathway (19,51), a site of initial retention also shared by βVLDL.
The binding of the lipoproteins to the receptors elicits the recruitment of specific sets of adaptor proteins, such as ARH for LDL receptor and Dab1 for LDL-receptor related protein (53-55), but no adaptor proteins have been yet identified for SRA. Presumably, distinct adaptors and their cognate receptors control the specific targeting of acLDL and LDL into distinct lysosomes with little overlap (Fig. 6). The segregation of the ligands is not only morphological but also functionally distinct and results in the formation of two differently accessible cholesterol pools. The mobilization of cholesterol from these pools proceeds through different pathways with partial overlap: one operates through apoA-I and ABCA1 and the other through diffusional efflux across cholesterol gradients including that mediated by ABCG1 (6,40) and by SR-BI (56), which both deliver cholesterol to HDL rather than lipid-poor apoA-I.

The regulation of diffusional cholesterol efflux through cholesterol gradients from cellular membranes to lipoprotein acceptors is apparently dependent on the transport from various cellular pools both in the plasma membrane and intracellular membranes that differ in their accessibility to exogenous acceptors. Cells have been shown to have slow and fast cholesterol pools in terms of mβ-CD accessibility (57,58). Here we have shown that LDL-derived cholesterol is more rapidly removed by mβ-CD as compared to acLDL-derived cholesterol, indicating that acLDL preferentially labels a slow pool in terms of accessibility for diffusional efflux. This slow pool may be in late endosomes, given its preferential mobilization by ABCA1. In contrast, LDL, like HDL, preferentially labels cholesterol pools with rapid access to diffusional efflux (50), which includes the recycling compartment as shown here (Fig. 1D) and in a recent report (36).

Our observation that LDL-derived cholesterol stored in macrophages can return to the liver of wild type mice and be secreted through the bile more efficiently than acLDL-derived cholesterol indicates that diffusional efflux through cholesterol gradients, including ABCG1 and SR-BI mediated efflux, is an efficient pathway. Although the net amount of LDL cholesterol taken up by macrophages is limited by the down-regulation of the LDL receptor, the efficient labeling and delivery with lipoproteins and phagocytosis of apoptotic cells requires an efflux mechanism, which we know is less dependent on apoA-I (Suppl. Fig. 2). Therefore, the greater efficiency of in vivo RCT from LDL loaded versus acLDL loaded macrophages in the control mice implies that HDL and other lipoproteins mediating diffusional efflux are more effective in RCT than lipid-poor apoA-I/pre-β-HDL, although the cavity fluid level of pre-β-HDL is apparently higher than α-HDL (Fig. 7B). The other possibility is that the pre-β-HDL becomes α-HDL via lipid efflux in an ABCA1 dependent manner, which then further quickly removes lipids from the macrophage foam cells. These interstitial HDL become effective ligands for hepatic ABCG1, which is upregulated by both LDL and acLDL loading of macrophages (Figure 3F). It was recently shown to play a major role in the control of tissue lipid levels and, although its deficiency does not affect HDL levels, it has been suggested to contribute to in vivo RCT via HDL (40). Although the down regulation of SR-BI in acLDL loaded macrophages (59 and data not shown) would argue against a major role for this transporter in efflux and in RCT at the step of efflux, we observed slight increases in SR-BI protein in
LDL-loaded macrophages (data not shown) as reported by others (60). In vitro, macrophages loaded with acLDL (ie. foam cells) are exquisitely dependent on ABCA1-mediated, apoA-I-dependent efflux in agreement with reports in the literature (7,61,62). However, in vivo RCT from acLDL loaded macrophages is clearly less effective than that from LDL loaded macrophages (Figure 7). We have also shown recently that RCT from abca1(-/-) macrophages is decreased by about 50%, indicating the existence in vivo of a significant efflux and RCT independent of ABCA1 activity (M.D. Wang et al, manuscript submitted for publication). We must therefore conclude that other transporters and efflux pathways operate in vivo for RCT. A recent and timely report from Rothblat and colleagues highlights the heterogeneity of diffusional efflux pathways and the importance of uninhibitable or background efflux, independent of ABCA1 and SR-BI (41). Such pathways may contribute to RCT from LDL-labeled macrophages. Peripheral lymph and interstitial fluid have been shown to contain all plasma lipoproteins (63-67), albeit at reduced concentrations and with significantly increased free cholesterol compared to plasma counterparts. This increased free cholesterol in interstitial lipoproteins may reflect the reduced lymph LCAT activity (63,66) and the active cholesterol efflux to interstitial lipoproteins. More studies of these lipoproteins and their relevance to RCT are clearly needed. Because it is a bidirectional process, diffusional efflux has never been recognized as a significant contributor to RCT. The in vivo evidence presented here that link in vitro diffusional efflux to irreversible transport to the liver and bile demonstrates its physiological importance.

Finally, in mitigating against atherosclerosis, it is critical that appropriate acceptors (i.e. HDL or lipid poor apoA-I) be present to promote regression of foam cells. Macrophages that have been loaded with modified lipoproteins are critically dependent on ABCA1/apoA-I-mediated cholesterol efflux for mobilization of loaded cholesterol (ie. in vitro lipid-poor apoA-I can efficiently promote regression of foam cells). Treatments such as apoA-I Milano or apoA-I mimetic peptide infusion (68-70) appear to promote foam cell regression without raising plasma HDL levels, although it is unclear whether regression is achieved by efflux and/or other anti-atherogenic functions of apoA-I. In conclusion, the existence of dual pathways for macrophage cholesterol transport implies that effective intervention against atherosclerosis may require both LDL cholesterol lowering therapy with statins in combination with specific agonists to increase the expression and function of both ABCA1 and ABCG1 (40,71) as well as SR-BI (72,73).

Acknowledgements: We would like to thank Dr. Tony Durst, Chemistry Department, Faculty of Sciences, University of Ottawa, for synthesizing dansyl-cholestanol, and Dr. Ruth McPherson, University of Ottawa Heart Institute, for advice and critical reading of the manuscript. S. C. Whitman is the recipient of a Great-West Life & London Life New Investigator Award from the Heart and Stroke Foundation of Canada.
Figure Legends.

**Fig. 1:** Macrophages labeled with cholesterol derived from acLDL or LDL efflux differentially to different acceptors. FLDM or BMDM from ABCA1 wild type, heterozygote or homozygote knockout mice were labeled with cholesterol (5 Ci/ml) delivered by LDL or acLDL (50 μg protein/ml) in 1% FBS of DMEM medium for 24 h, followed by overnight equilibration in DMEM with 2 mg/ml BSA. Efflux of cholesterol expressed as percentage of total cell radioactivity was measured over 5 h in the presence or absence of 50 μg of apoA-I (Panel A) or HDL (Panel B). Efflux to 2 mg/ml BSA in DMEM was measured over 16 h (Panel C). Cholesterol efflux to 10 mM mβ-CD was determined by incubation for 15 min at 4 or 37°C (Panel D). Panel E shows the efflux from peritoneal macrophages (resident and elicited), J774 cells, FLDM and BMDM. Panel F shows specific apoA-I mediated cholesterol efflux from J774 cells measured by immunoprecipitation of efflux medium with anti-apoA-I antibody. Each data point is the mean and SD of triplicate determination and representative of two or more independent experiments.

**Fig. 2:** Macrophages labeled with 3H-cholesteryl esters or an equivalent amount of 3H-cholesterol label efflux through different pathways. FLDM were labeled for 48 h with 3H-cholesteryl oleate delivered by LDL or acLDL (Panels A-D), or labeled for 5 or 24 h with 3H-cholesterol delivered by LDL or acLDL in 1% FBS DMEM (Panels E-H). Total cellular labeling (Panel A, E), apoA-I-mediated cholesterol efflux (Panel B, F), cellular ACAT-generated cholesteryl ester content (Panel C, G) and m-CD extraction at 4 or 37°C (Panel D) were measured. For cholesteryl oleate labeling, after washing three times with plain DMEM medium, cholesterol efflux to apoA-I was determined by incubation with 2 mg/ml BSA DMEM for 4 h. Cholesterol efflux to mβ-CD (D) was carried out as the same condition in Figure 1. For cholesterol labeling, equilibration and efflux condition are same as in Figure 1, except the application of ACAT inhibitor (5 μM) during labeling period (H). For each data point, the mean and SD are present as for Fig. 1.

**Fig. 3:** Relationships of LDL and acLDL loading of macrophages with cholesterol accumulation, regulation of ABCA1 and efflux. BMDM from C57 mice were deprived of cholesterol by pre-treatment with 5% LPDS in DMEM for 24 h. The cells were loaded with various concentrations of LDL or acLDL for 24 h in DMEM with 5% LPDS. Total cellular cholesterol mass was then measured by colorimetric assay (Panels A, C). Labeled cellular free cholesterol and cholesteryl ester were separated by TLC and radioactivity determined by scintillation counting. The results are presented as the ratio of cholesteryl ester to total cellular cholesterol label (Panels B, D). ABCA1 protein was measured by western blotting under each condition (Panel E). Quantification of the bands by comparison with control levels indicated that LDL at 50, 100, 150, 200, and 250 μg/ml increased ABCA1 expression to 1.3, 1.5, 2.4, 3.0, 2.7 fold, respectively, whereas acLDL at 6.25, 12.5, 25, 37.5, and 50 μg/ml increased ABCA1 expression to 13.7, 15.2, 15.5, 15.6, 16 fold, respectively. ABCG1 protein was measured by western blot under conditions that achieved equal cholesterol loading, acLDL (12.5 μg/ml) and LDL (250 μg/ml) loaded cells (Panel F), and ABCG1 induction is 2.8 and 1.9 fold respectively. Conditions that achieve similar cholesterol loading were selected for an efflux assay. Equivalent total cholesterol was obtained by loading with 150 and 250 μg LDL/ml (panels G, H), accumulating 30 and 36 μg/well cholesterol mass, respectively, and by loading with 6.25 and 12.5 μg AcLDL/ml, accumulating 29 and 38 μg/well cholesterol mass, respectively. The cells, thus labeled and cholesterol loaded, were equilibrated with 2 mg BSA/ml in lipid free medium overnight and efflux to apoA-I was started with addition of apoA-I (25 μg/well) for 5 h. Mean and SD values are as for Fig. 1.

**Fig. 4:** Specificity of LDL and acLDL internalization via LDLr and SRA in macrophages. Bone marrow derived macrophages from control or LDLr(-/-)[A,B,C,D,E], or SRA(-/-)[F] mice were labeled with 3H-cholesteryl oleate (5 μCi/ml) delivered with LDL or acLDL(50 μg/ml) in 5% LPDS for 24 h. Before the labeling,
all the cells (except B) were pretreated with 3%LPDS for 24 h in order to upregulate LDLr by depriving cellular cholesterol. After labeling, cells were washed twice with DMEM. The cells were then lysed by addition of 0.5N NaOH and incubation at room temperature overnight. Cell count was measured. Cholesterol efflux to apoA-I is presented in E and F.

**Fig. 5: Regulation of cholesterol efflux to apoA-I in macrophages loaded with LDL or acLDL.** Cholesterol efflux was measured in BMDM labeled with 3H-cholesterol delivered by LDL or AcLDL. We compared efflux in macrophages from NPC1 knockout and control mice (A). We evaluated the effect of progesterone (added only during the efflux period) in control mice (B) and the response to treatment with the LXR agonist 22-hydroxycholesterol (22-OH; 5μM) in control cells (C). Mean and SD, n=4; as for Fig. 1.

**Fig. 6: LDL and acLDL-related cargoes traffic into different endosomes.** Fluorescent hydrophobic lipid markers were incorporated into LDL and acLDL to follow intracellular trafficking of the lipoproteins. DiO-labeled LDL and DiD-labeled acLDL were incubated with the macrophages for 5 min at 37ºC, then washed and then followed by fluorescence microscopy for 2 h. LDL (green) and acLDL (red) were trafficked to distinct late endosome compartments. Scale bar represents 1 μm. The results are representative of 4 independent of experiments.

**Fig. 7: Increased in vivo reverse cholesterol transport from LDL-labeled macrophages.** Panels A and B, peritoneal fluid contains apoA-I migrating as both pre-β-HDL and α-HDL. Peritoneal fluid was collected by rinsing the cavity with plain medium, concentrated 20 folds and 2ul of the concentrate was electrophoresed on agarose gel (Lane 3). Mouse serum (lane 1), pure ascites (lane 2), and human serum (lane 4) are included as controls. Panel A shows the lipid staining with Sudan Black. Panel B shows the western blot with anti-murine apoA-I. BMDM of control C57 mice were labeled with 3H-cholesterol delivered by LDL or acLDL and then injected in the peritoneal cavity of C57 mice (6 animals per condition). Gallbladders, livers, and feces were harvested 24 h later and lipid radioactivity counted. Panels C and D represent radioactivity measured in bile and liver respectively. Similar results were obtained in 4 independent experiments.

**Supplementary Figure 1: Cholesterol from acLDL accumulates in large vesicles.** BMDM were incubated with dansyl cholesterol-LDL or dansyl cholesterol–acLDL under the conditions used for equivalent loading and labeling (6.25 μg acLDL, 250 μg LDL; 24h incubation). Left panel shows a diffused distribution of dansyl cholesterol delivered by LDL, but right panel shows an accumulation of dansyl cholesterol delivered by ac-LDL into endosomal structures.

**Supplementary Figure 2: Cholesterol efflux to apoA-I is determined by the respective receptors and their ligands.** FLDM were incubated with 3H-cholesterol pre incorporated in different lipoproteins or apoptotic cells (LDL, HDL, ox-LDL, βVLDL, acLDL, 50μg protein/ml each) in 1%FBS for 24 h. For phagocytosis of apoptotic cells (74), the incubation was only for 8 h. Then cells were washed three times with DMEM to remove free apoptotic cells. Equilibration and efflux periods were the same as in Fig. 1. Total cellular labeling (A), cholesterol efflux to apoA-I (B) or accumulation of ACAT generated cholesteryl ester (C) were measured.
REFERENCES


Figure 1

A

Cholesterol efflux to apoA-I (%)

ABCA1(+/+)
ABCA1(+-)
ABCA1(-/-)

Cholesterol efflux to HDL (%)

0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0

* *

B

Cholesterol efflux to BSA (%)

ABCA1(+/+)
ABCA1(+-)
ABCA1(-/-)

0.0
1.0
2.0
3.0
4.0

* *

C

Cholesterol efflux to mβ-CD (%)

ABCA1(+/+)
ABCA1(+-)
ABCA1(-/-)

37°C
4 °C

* *

D

Cholesterol efflux to apoA-I (%)

ALDL
acLDL

0.0
0.5
1.0
1.5
2.0

* *

E

Cholesterol efflux to apoA-I (%)

Ind.PM
Res.PM
J774
FLDM
BMDM

0.0
5
10
15
20

* *

F

ApoA-I specific cholesterol efflux (%)

LDL
acLDL

0.0
0.5
1.0
1.5
2.0

*
Figure 2

A

Cholesterol labeling (cpm/well)

LDL-CE

acLDL-CE

5 hours

24 hours

B

Specific cholesterol efflux to apoA-I (%)

p<0.005

LDL-CE

acLDL-CE

C

Cell cholesteryl ester content (%)

p<0.01

LDL-CE

acLDL-CE

D

Cholesterol efflux to mβ-CD (%)

p<0.01

37°C

4°C

E

Cholesterol labeling (cpmX1000/well)

LDL

acLDL

5 hours

24 hours

F

Cholesterol efflux to apoA-I (%)

p<0.01

LDL

acLDL

5 hours

24 hours

G

Cell cholesteryl ester content (%)

LDL

acLDL

5 hours

24 hours

H

Cholesterol efflux to apoA-I (%)

p<0.01

No

ACATi
Figure 3

(A) Total cellular cholesterol (µg/mg protein) vs. LDL concentration (µg/ml)

(B) Percent of CE/(CE+FC) vs. LDL concentration (µg/ml)

(E) Western blot analysis of LDL and acLDL in CTL samples

(G) Cholesterol efflux to apoA-I (%) vs. LDL concentration (µg/ml)

(H) Cholesterol efflux to apoA-I (%) vs. acLDL concentration (µg/ml)
Figure 4

A

LDL-CE uptake (CPM/ug protein)

LDLR(+/+)
LDLR(-/-)

LDL (ug/ml)

B

AcLDL-CE uptake (1000XCPM/ug protein)

No
100
500

acLDL (ug/ml)

C

AcLDL-CE uptake (cpm/ug protein)

SRA(-/-)
C57, LDL (ug/ml)

D

Cholesterol labeling (X1000CPM/mg proteins)

CE-LDL
CH-LDL

E

Cholesterol efflux to apoA-I (%)

LDLR(-/-)
LDLR(+/+)

F

Cholesterol efflux to apoA-I (%)

SRA(-/-)
C57
Figure 5

A

Cholesterol efflux to apoA-I (%)

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<th>acLDL</th>
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B

Cholesterol efflux to apoA-I (%)

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C

Cholesterol efflux to apoA-I (%)

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p < 0.05
Figure 6

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Figure 7

A

Lipid staining

B

anti-mouse apoA-I

C

D

Liver radioactivity (cpm/mouse/10^3 injected cpm)

Biliary excretion (cpm/mouse/10^3 injected CPM)

alpha

pre-beta

LDL  acLDL

p<0.05

LDL  acLDL

p<0.05
Supplementary Figure 1

Dansyl cholestanol LDL  Dansyl cholestanol acLDL