Different cellular traffic of LDL-cholesterol and acetylated LDL-cholesterol leads to distinct reverse cholesterol transport pathways[S]

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Abstract **Endocytosis of LDL and modified LDL represents regulated and unregulated cholesterol delivery to macrophages. 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 low density lipoprotein (AcLDL), and the cellular cholesterol traffic pathways were examined. Confocal microscopy using fluorescently labeled 3,3′-dioctyldecyloxacarbocyanine perchlorate-labeled LDL and 1,1′-dioctyldecyl-3,3,3′,3′-tetramethylindodicarbocyanine perchlorate-labeled AcLDL demonstrated their discrete traffic pathways and accumulation in distinct endosomes. ABCA1-mediated cholesterol efflux to apolipoprotein A-I (apoA-I) was much greater for AcLDL-loaded macrophages compared with LDL. Treatment with the liver X receptor ligand 22-OH increased efflux to apoA-I in AcLDL-loaded but not LDL-loaded cells. In contrast, at a level equivalent to AcLDL, LDL-derived cholesterol was preferentially effluxed to HDL, in keeping with increased ABCG1. 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 than AcLDL-derived cholesterol. This indicates a greater efficiency of HDL than lipid-poor apoA-I in interstitial fluid in controlling in vivo RCT. These assays, taken together, emphasize the importance of mediators of diffusional cholesterol efflux in RCT.**—Wang, M-D., R. S. Kiss, V. Franklin, H. M. McBride, S. C. Whitman, and Y. L. Marcel. **Different cellular traffic of LDL-cholesterol and acetylated LDL-cholesterol leads to distinct reverse cholesterol transport pathways.** ***J. Lipid Res.*** **2007.** 48: **633–645.**

Supplementary key words cholesterol efflux • ATP binding cassette transporter A1 • ATP binding cassette transporter G1 • scavenger receptor class B type I • low density lipoprotein

The development of atherosclerosis is initiated by the formation of macrophage-derived foam cells (1). As dedicated 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 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 scavenger receptor class B type I (SR-BI) (4) and the ABCG1/G4 transporter (5, 6) or the apolipoprotein A-I (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 (LDLr) (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 hand, 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 acetylated low density lipoprotein (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 AcLDL and oxidized low density lipoprotein (OxLDL) also showed that the two ligands traffic to different endosomes and accumulate in distinct lysosomal compartments (20), an

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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 macrophage receptor with collagen domain, scavenger receptor with C-like lectin, scavenger receptor type B-CD36, and LOX1 as well as scavenger receptor type A-I/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 used the modified lipoprotein 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 effluxes preferentially via the HDL-mediated pathway dependent in part on ABCG1, whereas AcLDL-derived cholesterol effluxes preferentially via the more specific lipid-poor apoA-I/ABCA1-dependent pathway. Interestingly, in vivo reverse cholesterol transport (RCT) 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 RCT.

MATERIALS AND METHODS

Animals

ABCA1$^{-/-}$ mice were a kind gift from Dr. Edward M. Rubin (Department of Energy Joint Genome Institute, Berkeley, CA). SRA$^{-/-}$ mice were transferred from Dr. T. Kodama (University of Tokyo). C57Bl6, NPC1$^{-/-}$, SR-BI$^{-/-}$, LDLr$^{-/-}$, and caveolin-1$^{-/-}$ mice were purchased from Jackson Laboratories and maintained and bred at the animal facility of the 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-^{3}H], mevalono-lactone-Rs-[5-^{3}H(N)], choline chloride-[methyl-^{3}H], and cholesteryl oleate were obtained from Perkin-Elmer Life and Analytical Sciences. 22*R*-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 was produced in our laboratory (27). Lipoproteins were prepared from the plasma of normolipidemic subjects as described by Havel, Eder, and Bragdon (28). AcLDL was prepared as described by Goldstein et al. (29). βVLDL was isolated from cholesterol-fed apoE-null mice (30).

Generation of macrophages

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

Lipid efflux

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

Lipid analysis

Cellular lipids were extracted (33) and separated by TLC using hexane-diethylether-acetic acid (105:45:1.5) as running solvent on Sil-G TLC plates (EMD Chemicals, Darmstadt, Germany). Lipid bands were detected by exposure to iodine vapors and scraped off the TLC plate, and radioactivity was measured with a scintillation counter. For total cholesterol determination, cells were washed with cold PBS, and cholesterol was 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, and cocktail protease inhibitors (Roche)], electrophoresed on a 6% SDS-polyacrylamide gel, and transferred to nitrocellulose at 125 V for 4 h. ABCA1 and ABCG1 antibodies (Novus Biologicals; 1:500 dilution) were used for detection, with an anti-rabbit secondary antibody conjugated with horseradish peroxidase (Amersham Biosciences) for detection.

RCT

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

Image analysis

Lipoproteins were labeled with 3,3′-dioctyldecyloxacarbocyanine perchlorate (DiO) and 1,1′-dioctyldecyl-3,3,3′,3′-tetramethylindodicarbocyanine perchlorate (DiD) (Molecular Probes) as reported previously (18, 32). Dansyl-cholestanol was synthesized according to the method of Wiegand et al. (35). Macrophages were loaded with LDL or AcLDL that was preequilibrated with dansyl-cholestanol. An Olympus FV1000 confocal microscope, complete with a 100× objective (numerical aperture 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 fluorescence microscope outfitted with a monochrometer from Till Photonics was used. Images were captured using the Imago charge-coupled device 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

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, whereas cells loaded with LDL displayed ~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, because mβ-CD extraction at 4°C, which measures the proportion of cholesterol present at the cell surface, showed equivalent plasma mem-

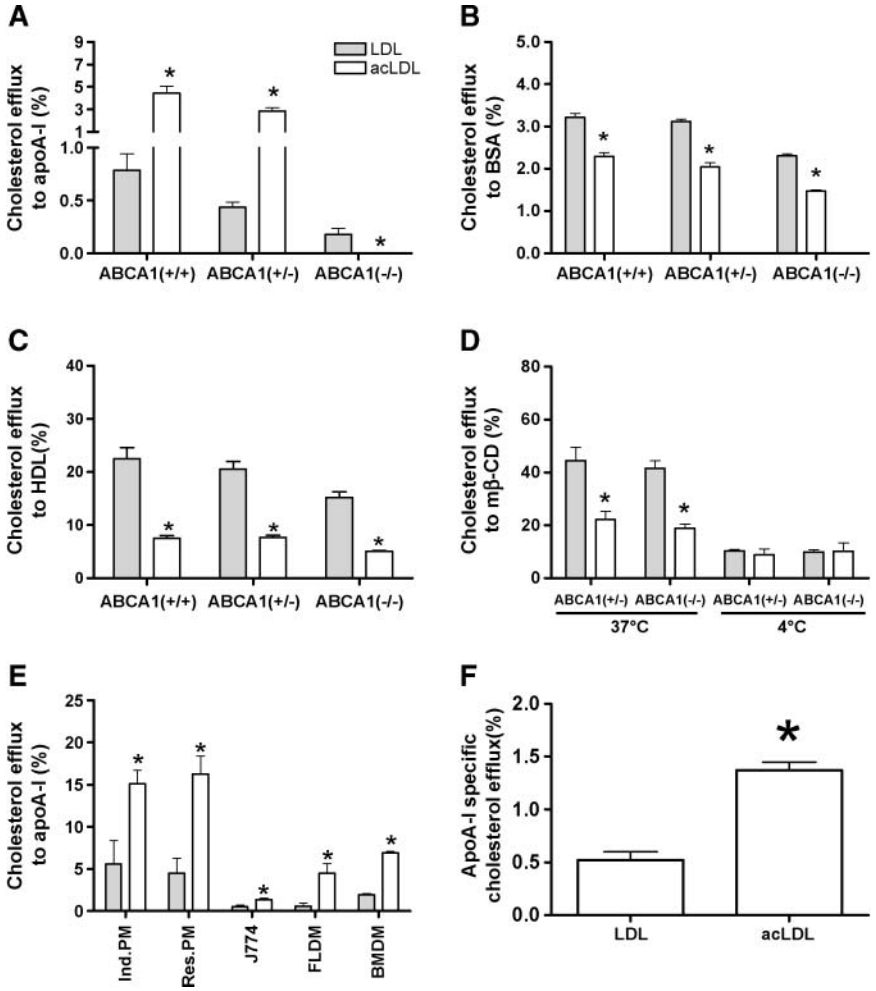

Fig. 1. Macrophages labeled with cholesterol derived from acetylated low density lipoprotein (AcLDL) or LDL efflux differentially to different acceptors. Fetal liver-derived macrophages or bone marrow-derived macrophages (BMDMs) from ABCA1 wild-type (+/+), heterozygote (+/−), or homozygote (−/−) knockout mice were labeled with cholesterol (5 μCi/ml) delivered by LDL or AcLDL (50 μg protein/ml) in 1% FBS in DMEM for 24 h, followed by overnight equilibration in DMEM with 2 mg/ml BSA. A, B: Efflux of cholesterol expressed as percentage of total cell radioactivity was measured over 5 h in the presence or absence of 50 μg of apolipoprotein A-I (apoA-I) (A) or HDL (B). C: Efflux to 2 mg/ml BSA in DMEM was measured over 16 h. D: Cholesterol efflux to 10 mM methyl-β-cyclodextrin (mβ-CD) was determined by incubation for 15 min at 4°C or 37°C. E: Efflux from peritoneal macrophages [resident (Res.PM) and elicited (Ind.PM)], J774 cells, fetal liver-derived macrophages (FLDMs), and BMDMs. F: 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 determinations and representative of two or more independent experiments. *$P < 0.001$.

brane labeling by LDL (9.9%) and AcLDL (10.2%) (Fig. 1D). However, short-term cholesterol efflux to mβ-CD at 37°C, which measures cholesterol present at the plasma membrane and the recycling compartment (36), was significantly greater from cells loaded with LDL (44%) compared with 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 37°C. This difference was not related to the use of specific differentiated murine macrophages, because 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 [^{3}H]cholesteryl oleate and preincorporated 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 ~3-fold (Fig. 2B). When cells were equally loaded with cholesteryl ester-labeled lipoproteins, the proportion of accumulated ACAT-generated cholesteryl esters was ~10-fold greater with AcLDL compared with LDL (Fig. 2C). Similar to [^{3}H]cholesterol labeling of lipoproteins, cholesterol derived from [^{3}H]cholesteryl oleate was equally accessible to mβ-CD extraction at 4°C whether delivered by LDL or AcLDL (Fig. 2D). However, mβ-CD extraction at 37°C was much higher for LDL-loaded macrophages, similar to the previous results (Fig. 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 [^{3}H]oleate in AcLDL-treated cells (data not shown). To understand whether the increased apoA-I-mediated efflux was attributable simply to increased cholesteryl ester formation, because cholesterol efflux to apoA-I is closely related to the level of cellular cholesteryl ester (see supplementary Fig. II) and in keeping with previous studies (39), macrophages were loaded for 5 or 24 h 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 (4-fold increase for AcLDL-loaded cells) (Fig. 2F). 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, administration of the ACAT inhibitor Sandoz 58-035 during the labeling period completely suppressed the cholesterol reesterification cycle (data not shown) and 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 attributable to increased loading or preferential targeting to an ACAT-accessible pool; rather, they 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 nonsaturating increase in uptake, which at 250 μg/ml doubled total cellular cholesterol and increased cholesteryl ester level to ~5% (**Fig. 3A, B**), indicating that a non-receptor-independent mechanism 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 ~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 before the 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 of 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 increased cellular cholesterol to 38.7 and 35.6 μg/mg cellular protein, respectively.

Because ABCG1 mediates efflux to HDL (5, 40) and 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 AcLDL-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 related directly only 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 LDLr and AcLDL via SRA in macrophages, we first labeled BMDMs from LDLr$^{-/-}$ and wild-type mice with [^{3}H]cholesteryl oleate-labeled LDL. Both BMDMs were pretreated with 3% lipoprotein deficient serum to upregulate LDLr by depleting cellular cho-

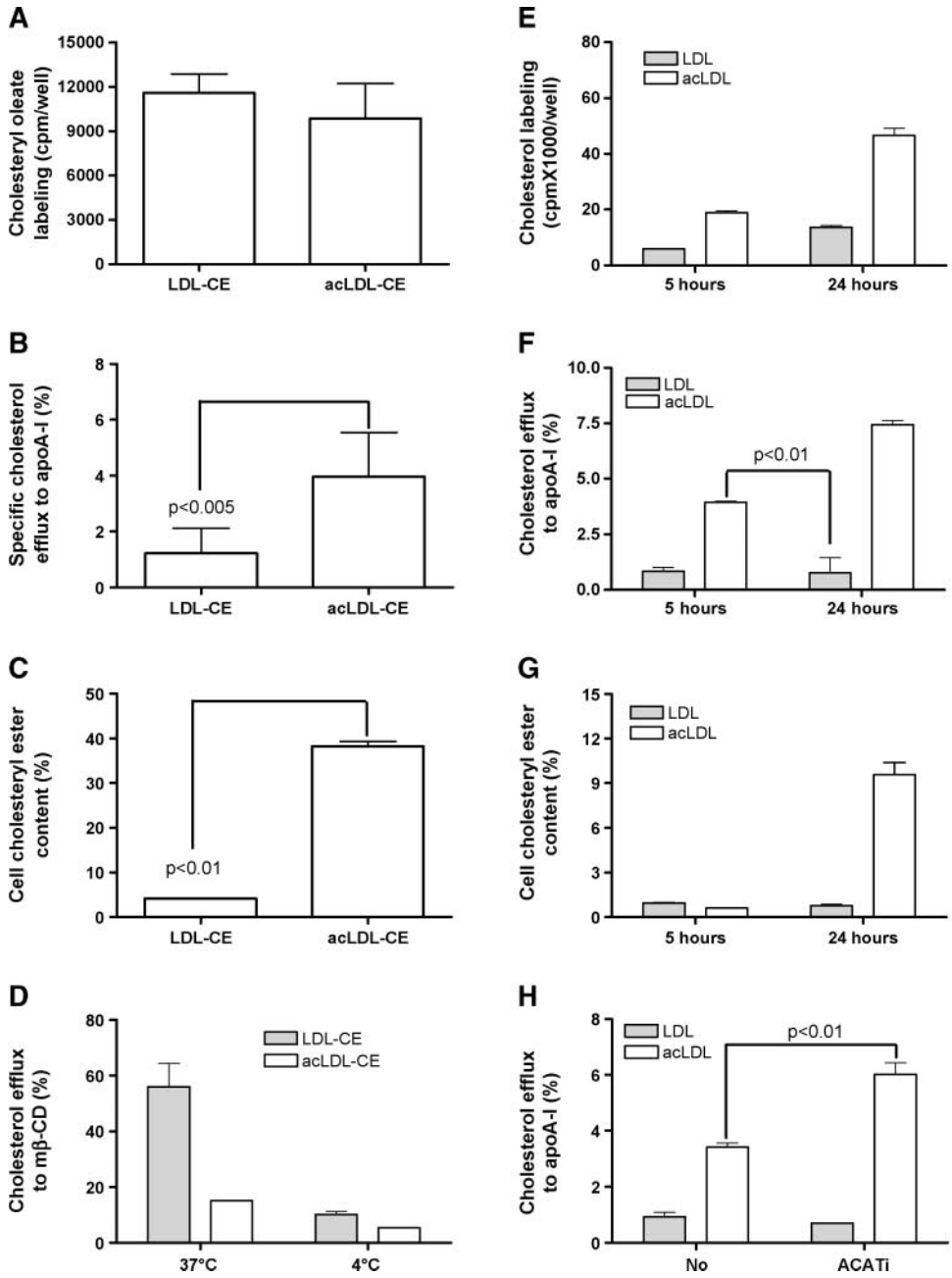

Fig. 2. Macrophages labeled with [^{3}H]cholesteryl esters or an equivalent amount of [^{3}H]cholesterol label efflux through different pathways. Fetal liver-derived macrophages were labeled for 48 h with [^{3}H]cholesteryl oleate delivered by LDL or AcLDL (A–D) or labeled for 5 or 24 h with [^{3}H]cholesterol delivered by LDL or AcLDL in 1% FBS DMEM (E–H). Total cellular labeling (A, E), apoA-I-mediated cholesterol efflux (B, F), cellular ACAT-generated cholesteryl ester content (C, G), and mβ-CD extraction at 4 or 37°C (D) were measured. For cholesteryl oleate labeling, after washing three times with plain DMEM, 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 at the same conditions as in Fig. 1. For cholesterol labeling, equilibration and efflux conditions are the same as in Fig. 1, except for the application of ACAT inhibitor (5 μM) during the labeling period (H). For each data point, the mean and SD are shown as for Fig. 1.

lesterol. Under this condition, ~70% of LDL cholesterol internalization could be inhibited by the 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 [^{3}H]cholesteryl oleate-labeled LDL or [^{3}H]cholesterol-labeled LDL without pretreatment with LPDS. Under these conditions, most LDL cholesterol internalization (~75%) was LDLr-independent (Fig. 4B), indicating that the majority of LDL cholesterol uptake

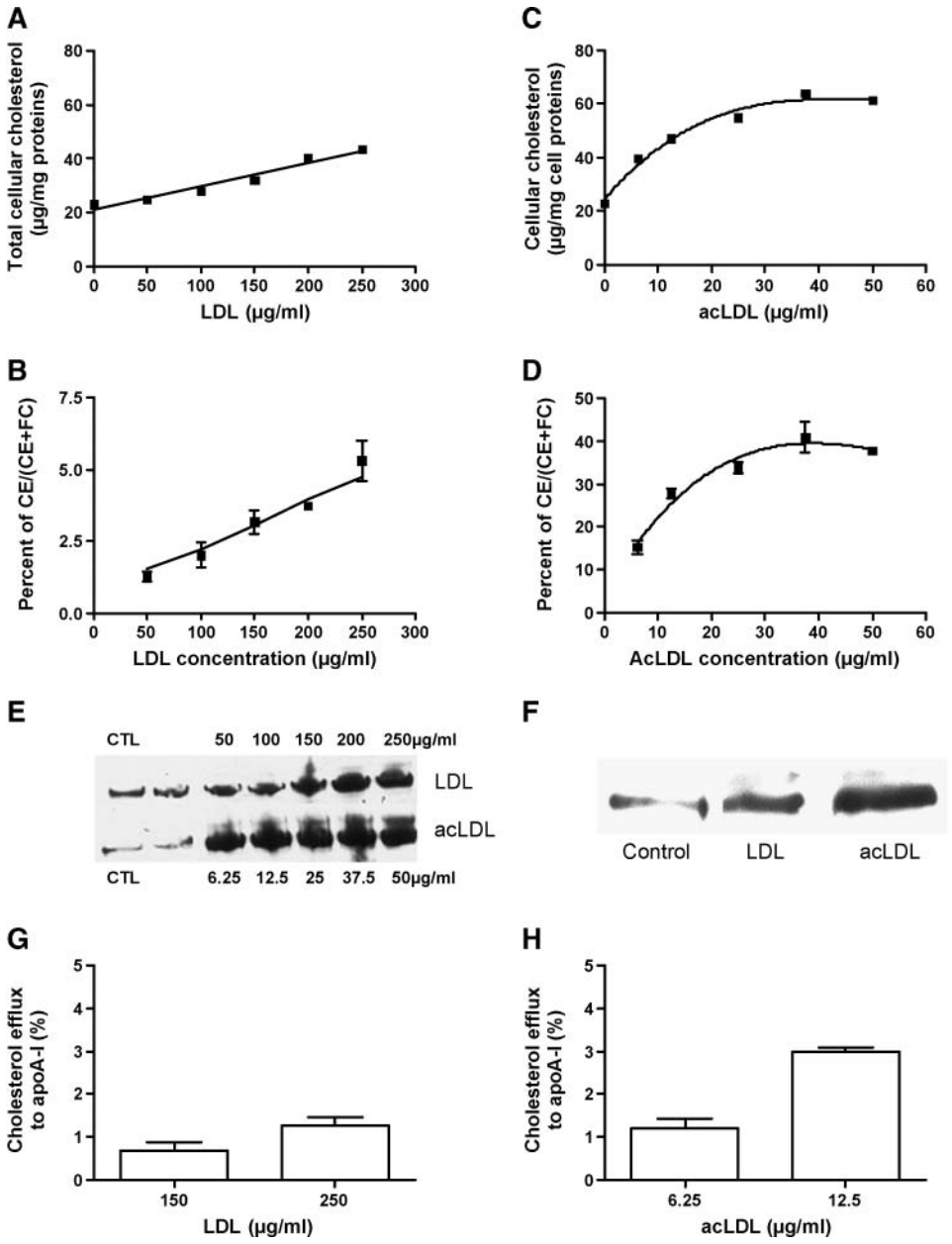

Fig. 3. Relationships of LDL and AcLDL loading of macrophages with cholesterol accumulation, regulation of ABCA1, and efflux. A, C: BMDMs from C57Bl6 mice were deprived of cholesterol by pretreatment with 5% LPDS in DMEM for 24 h. The cells were loaded with various concentrations of LDL or AcLDL for 24 h in DMEM with 5% LPDS. Total cellular cholesterol mass was then measured by colorimetric assay. B, D: Labeled cellular free cholesterol (FC) and cholesteryl ester (CE) were separated by TLC, and radioactivity was determined by scintillation counting. The results are presented as the ratio of cholesteryl ester to total cellular cholesterol label. E: ABCA1 protein was measured by Western blotting under each condition. Quantification of the bands by comparison with control levels indicated that LDL at 50, 100, 150, 200, and 250 µg/ml increased ABCA1 expression by 1.3-, 1.5-, 2.4-, 3.0-, and 2.7-fold, respectively, whereas AcLDL at 6.25, 12.5, 25, 37.5, and 50 µg/ml increased ABCA1 expression by 13.7-, 15.2-, 15.5-, 15.6-, and 16-fold, respectively. F: 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, and ABCG1 induction was 2.8- and 1.9-fold, respectively. G, H: 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, accumulating 30 and 36 µg/well cholesterol mass, respectively (G), and by loading with 6.25 and 12.5 µg AcLDL/ml, accumulating 29 and 38 µg/well cholesterol mass, respectively (H). 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 the addition of apoA-I (25 µg/well) for 5 h. Mean and SD values are as described for Fig. 1.

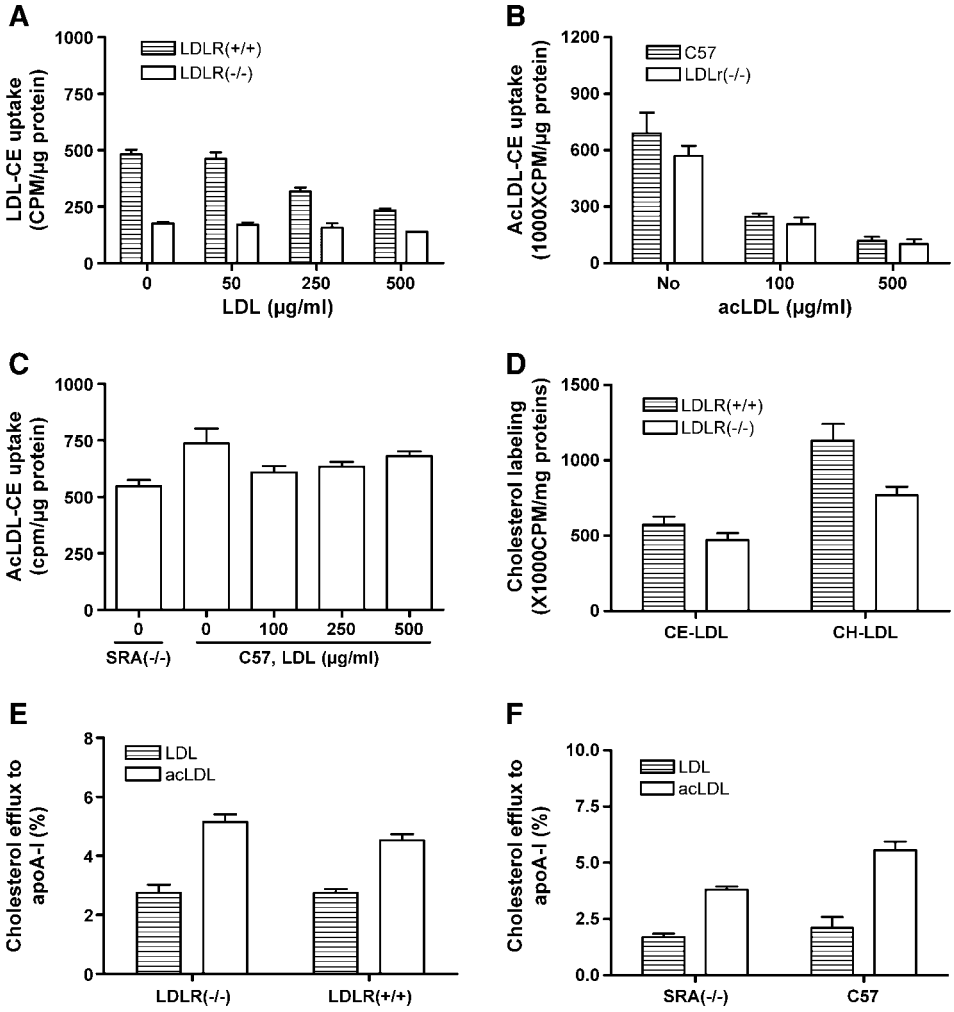

Fig. 4. Specificity of LDL and AcLDL internalization via the LDL receptor (LDLr) and SRA in macrophages. BMDMs from control or $LDLr^{-/-}$ (A–E) or $SRA^{-/-}$ (F) mice were labeled with [^{3}H]cholesteryl oleate (5 μCi/ml) delivered with LDL or AcLDL (50 μg/ml) in 5% LPDS for 24 h. Before labeling,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

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 with C57BL6 macrophages (Fig. 4C), but these cells still accumulated a large amount of cholesterol from AcLDL, suggesting a 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 (Fig. 4C), consistent with the absence of overlap between the two types of receptors in normal macrophages, as reported previously (42). Uptake of AcLDL could be almost completely inhibited (93%) by the 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 (Fig. 3C, D). Thus, the AcLDL cholesterol uptake is receptor-dependent, but LDL cholesterol could be taken up by either receptor-mediated or non-receptor-mediated pathways.

Importantly, the patterns of cholesterol efflux to apoA-I were not altered when comparing macrophages of different genetic backgrounds (i.e., wild-type, $SRA^{-/-}$, or $LDLr^{-/-}$) that were labeled with [^{3}H]cholesterol or [^{3}H]cholesteryl oleate incorporated in either LDL or AcLDL (Fig. 4E, 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 vs. AcLDL) properties.

LDL- and AcLDL-related cholesterol traffic pathways are differentially regulated

Caveolin-1, which has been shown to transport cholesterol between intracellular compartments/endoplasmic reticulum and the 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 Niemann-Pick type C1 (NPC1), which controls cholesterol traffic from late endosomes (45, 46), led to a 44% reduction in cholesterol efflux from LDL-labeled macrophages and to a 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 the 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 with control cells (Fig. 5B). Because liver X receptor 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 ABCA1 protein expression (data not shown) in both LDL- and AcLDL-labeled cells and significantly increased cholesterol efflux to apoA-I in AcLDL-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 is differentially regulated.

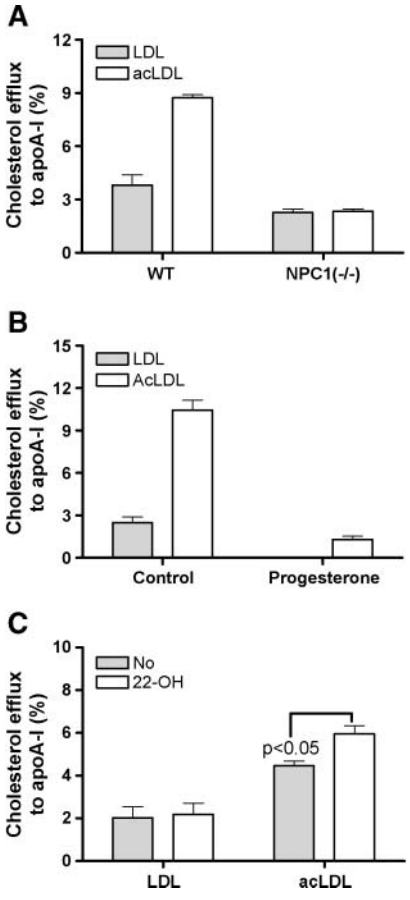

Fig. 5. Regulation of cholesterol efflux to apoA-I in macrophages loaded with LDL or AcLDL. A: Cholesterol efflux was measured in BMDMs labeled with [^{3}H]cholesterol delivered by LDL or AcLDL. We compared efflux in macrophages from Niemann-Pick type C1 (NPC1) knockout and control [wild-type (WT)] mice. B, C: We evaluated the effect of progesterone (added only during the efflux period) in control mice (B) and the response to treatment with the liver X receptor agonist 22-hydroxycholesterol (22-OH; 5 μM) in control cells (C). Mean and SD (n = 4) are as described for Fig. 1.

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 24 h (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 the 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 was separately added to wild-type or ABCA1-knockout macrophages under conditions that would achieve equal loading or labeling of [^{3}H]cholesterol (24 h of 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 (see supplementary Fig. IB). 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, whereas 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 whether the separate metabolic pathways that were shown to exist in vitro for LDL and AcLDL cholesterol could also be documented in vivo, an RCT experiment (34) was carried out. Equal cell numbers of BMDMs from normal C57BL6 mice, labeled to the same extent with [^{3}H]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. At 24 h after injection of green fluorescent protein-labeled 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 them-

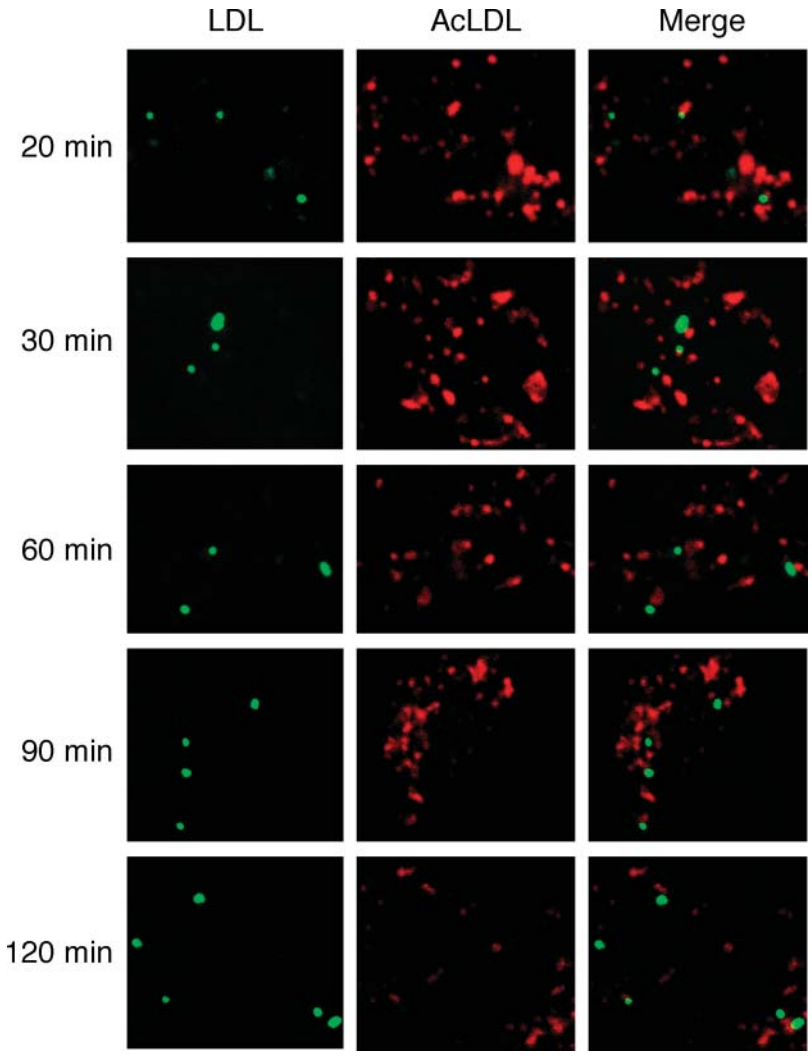

Fig. 6. LDL- and AcLDL-related cargoes traffic into different endosomes. Fluorescent hydrophobic lipid markers were incorporated into LDL and AcLDL to follow the intracellular trafficking of the lipoproteins. 3,3′-Dioctyldecyloxacarbocyanine perchlorate-labeled LDL and 1,1′-dioctyldecyl-3,3,3′,3′-tetramethylindodicarbocyanine perchlorate-labeled AcLDL were incubated with the macrophages for 5 min at 37°C, then washed and followed by fluorescence microscopy for 2 h. LDL (green) and AcLDL (red) were trafficked to distinct late endosome compartments. The results are representative of four independent experiments. Bars = 1 μm.

selves did not migrate to the liver, in agreement with the results of others (34), and thus further demonstrating that the radioactivity measured in the liver represents cholesterol transported to the liver. Agarose gel electrophoresis of the concentrated peritoneal fluid demonstrated the presence of both α-HDL- and preβ-HDL-migrating apoA-I (**Fig. 7A, B**, lane 3), with a large preponderance of preβ-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 ~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. Therefore, these results 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

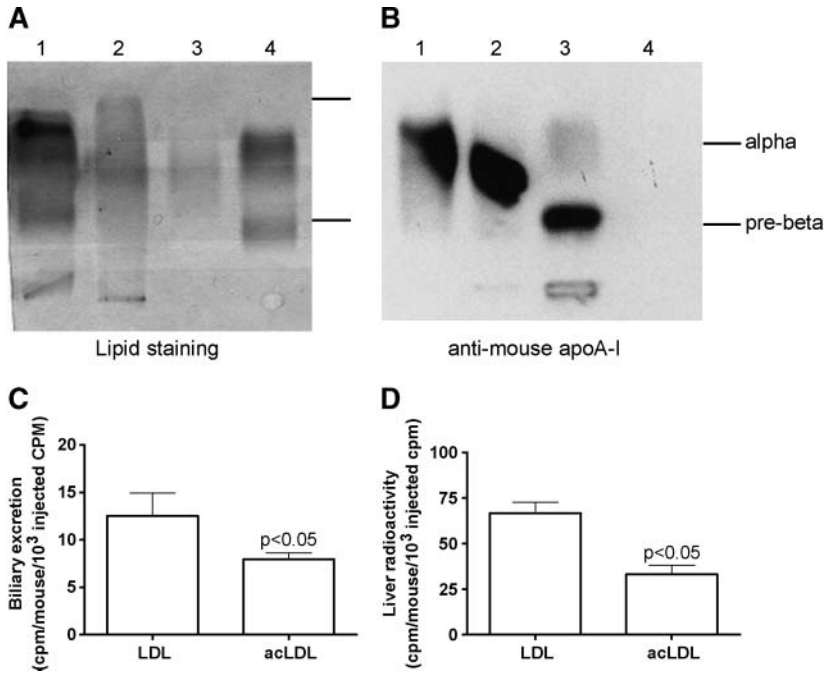

Fig. 7. Increased in vivo reverse cholesterol transport from LDL-labeled macrophages. A, B: Peritoneal fluid contains apoA-I migrating as both preβ-HDL and α-HDL. Peritoneal fluid was collected by rinsing the cavity with plain medium and concentrated 20-fold, and 2 μl of the concentrate was electrophoresed on agarose gels (lane 3). Mouse serum (lane 1), pure ascites (lane 2), and human serum (lane 4) were included as controls. A shows lipid staining with Sudan Black, and B shows a Western blot with anti-murine apoA-I. BMDMs of control C57Bl6 mice were labeled with [^{3}H]cholesterol delivered by LDL or AcLDL and then injected in the peritoneal cavity of C57Bl6 mice (six animals per condition). Gallbladders, livers, and feces were harvested 24 h later, and lipid radioactivity was counted. C, D: Radioactivity measured in bile (C) and liver (D). Similar results were obtained in four independent experiments.

into late endosomes and lysosomes and further converted to cholesteryl ester in endoplasmic reticulum-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 (OxLDL, βVLDL) was also shown to be readily accessible to apoA-I (see supplementary Fig. IIB), 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 [^{3}H]cholesterol labeling or loading (Figs. 2, 3; see supplementary Fig. IIA). All atherogenic lipoproteins are potent ACAT activators (see supplementary Fig. IIC), in agreement with previous studies (50), and the accumulated cholesterol can be mobilized primarily 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 (18, 52).

The binding of the lipoproteins to the receptors elicits the recruitment of specific sets of adaptor proteins, such as autosomal recessive hypercholesterolemia-protein for LDLr and Dab1 for LDLr-related protein (53–55), but no adaptor proteins have been 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 those mediated by ABCG1 (6, 40) and by SR-BI (56), which both deliver cholesterol to HDL rather than to 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 in both 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 compared with 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 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 downregulation of the LDLr, 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 (see supplementary Fig. II). 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/preβ-HDL, although the cavity fluid level of preβ-HDL is apparently higher than that of α-HDL (Fig. 7B). The other possibility is that the preβ-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 HDLs become effective ligands for hepatic ABCG1, which is upregulated by both LDL and AcLDL loading of macrophages (Fig. 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 downregulation of SR-BI in AcLDL-loaded macrophages (59) (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 (i.e., 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 (Fig. 7). We also recently showed that RCT from *abca1*$^{-/-}$ macrophages is decreased by ~50%, indicating the existence in vivo of a significant efflux and RCT independent of ABCA1 activity (M. D. Wang et al., unpublished data). Therefore, we must conclude that other transporters and efflux pathways operate in vivo for RCT. A recent and timely report from Rothblat and colleagues (41) highlights the heterogeneity of diffusional efflux pathways and the importance of uninhibitable or background efflux, independent of ABCA1 and SR-BI. 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 with 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 links 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 the regression of foam cells. Macrophages that have been loaded with modified lipoproteins are critically dependent on ABCA1/apoA-I-mediated cholesterol efflux for the mobilization of loaded cholesterol (i.e., in vitro lipid-poor apoA-I can efficiently promote the 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 increasing plasma HDL levels, although it is unclear whether regression is achieved by efflux and/or other antiatherogenic functions of apoA-I. In conclusion, the existence of dual pathways for macrophage cholesterol transport implies that effective intervention against atherosclerosis may require 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).

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