A Pathway-Dependent on ApoE, ApoAI, and ABCA1 Determines Formation of Buoyant High-Density Lipoprotein by Macrophage Foam Cells

Patricia G. Yancey, Hong Yu, MacRae F. Linton, Sergio Fazio

Objective—ABCA1-dependent and ABCA1-independent pathways may operate in high-density lipoprotein formation by macrophages secreting apolipoprotein (apo) E. We examined the impact of ABCA1 on apoE-mediated efflux from cholesterol-enriched macrophages.

Methods and Results—Without acceptors, wild-type, ABCA1−/−, and apoE−/− macrophages released 5.7%±0.3%, 1.8%±0.1%, and 2.3%±0.2% of their cholesterol, and the LXR agonist, TO-901317, enhanced efflux by 137%, 10%, and 20%. Although similar amounts of apoE were secreted from ABCA1−/− and wild-type cells, apoE from ABCA1−/− cells was only partially phospholipidated and floated at density >1.21g/mL, whereas apoE from wild-type cells floated at density of 1.09 to 1.17g/mL and paralleled the density of cholesterol. With apoAI, LXR stimulation increased efflux by 139% and 86% from wild-type and apoE−/− cells, resulting in a large difference in efflux (29.5%±0.2% versus 17.0%±0.5%). The density of apoE and cholesterol from wild-type cells did not change with apoAI, and most apoAI floated at density ≥1.17g/mL. In apoE−/− cells, apoAI and cholesterol floated at similar density, but the peak fraction only contained 4 μg cholesterol/mg protein versus 18 in WT cells.

Conclusions—Macrophage apoE requires ABCA1 for formation of high-density lipoprotein. ApoAI facilitates association of apoE with more buoyant high-density lipoprotein, suggesting that apoE, plasma apoAI, and ABCA1 operate together to optimize mobilization of macrophage cholesterol, a process critical to limiting plaque development. (Arterioscler Thromb Vasc Biol, 2007;27:000–000-.)

Key Words: ABCA1 ■ apolipoprotein AI ■ cholesterol efflux ■ endogenous apolipoprotein E ■ LXR stimulation ■ macrophage foam cell ■ nascent high-density lipoprotein

Accumulation of cholesterol in macrophages leads to the formation of foam cells, a crucial event in the development of atherosclerotic lesions. Cholesterol efflux is a regulated process using several alternative or partially redundant pathways to counter accumulation driven by receptor uptake of lipoproteins. A number of cholesterol efflux mechanisms may be relevant, including: (1) efflux to phospholipid-containing acceptors such as high-density lipoprotein (HDL), and HDL either via aqueous diffusion, scavenger receptor class B type I, or ATP-binding cassette transporter-G1 (ABCG1); and (2) efflux to lipid-poor apoproteins via ABCA1. In addition to these mechanisms of efflux, endogenous apolipoprotein (apo) E has been proposed to mediate cholesterol mobilization from macrophages. Studies have shown that macrophage apoE expression accelerates cholesterol release both in the absence and presence of “physiological” acceptors, including HDL and lipid-free apoAI. In addition, macrophage apoE secretion is stimulated by cholesterol-enrichment and incubation with either HDL or lipid-free apoAI as cholesterol acceptors. A number of studies have shown that macrophage apoE protects against lesion development. Deletion of macrophage apoE in several different murine models accelerates atherosclerotic lesion development. Furthermore, low-level expression of macrophage apoE via retroviral transduction of bone marrow reduces susceptibility to atherosclerosis without affecting plasma lipids. These anti-atherogenic effects of macrophage apoE are likely caused, at least in part, by its role in cholesterol mobilization.

At present, the mechanism(s) by which macrophage endogenous apoE stimulates cholesterol efflux is unclear. In particular, studies are conflicting regarding the contribution of ABCA1 to lipidation of macrophage apoE. Huang et al used inhibitors of cholesterol efflux together with cAMP to enhance ABCA1 expression and found that the apoE mechanism of efflux in cholesterol-normal J774 cells is independent of ABCA1. However, more recent studies comparing cholesterol-normal wild type (WT) and ABCA1−/− macro-

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Mice

ABCA1"-/-" mice on the DBA/1J background were a generous gift from Dr Omar Francone (Pfizer Inc, Groton, Conn). ABCA1"-/-" mice were backcrossed 10 generations onto the C57BL/6 background. Macrophages were isolated from 8- to 12-week old female WT, apoE"-/-", or ABCA1"-/-" mice maintained on a chow diet. Animal care and procedures were performed in accordance with regulations of the Institutional Animal Care and Usage Committee of Vanderbilt University.

Cholesterol Efflux

Mouse macrophages were isolated by peritoneal lavage 4 days after injection of 2.5 mL of 3% thioglycollate and seeded onto 24-well plates. After 4 hours, nonadherent cells were removed by washing and the macrophages were cholesterol-enriched by incubation for 48 hours to 72 hours in DMEM containing 0.25% fetal bovine serum, [3H]cholesterol (3 to 10 μCi/mL), and either human acetylated low-density lipoprotein (70 to 100 μg protein/mL) or remnant lipoproteins (density=1.006 to 1.019 g/mL, 20 μg protein/mL) isolated from apoE"-/-" mice. The cells were incubated for 1 hour with DMEM containing 0.1% bovine serum albumin, and then washed once with 1% bovine serum albumin DMEM and once with HEPES-buffered DMEM. The cells were then incubated for 7 hours to 24 hours in DMEM alone or with apoAI (20 μg protein/mL). To upregulate apoAI synthesis, parallel incubations were performed with the LXR agonist TO-901317 (1 μmol/L). To control for differences among macrophage strains in the re-esterification of cholesterol during efflux, the ACAT inhibitor CP113,818 (2 μmol/L) was included in all efflux incubations. After filtering aliquots of media through 0.45 μmol/L multiscan filtration plates to remove floating cells, the [3H]cholesterol was measured by liquid scintillation counting. Cellular [3H]cholesterol was extracted by incubating the monolayers overnight in isopropanol. Cellular cholesterol content and proteins were measured as described.

Sucrose Density Ultracentrifugation

Density of medium apoE, [3H]cholesterol, and [3H]choline-containing phospholipids released was determined by sucrose density ultracentrifugation. [3H]cholesterol (10 μCi/mL) or [3H]choline (20 μCi/mL) was included in the cholesterol-enrichment medium. After efflux, 900 μL of sample was centrifuged at 14 000 g for 10 minutes to remove floating cells. The supernatant was underlayered with 2 mL of 1.33% sucrose solution, 1.175 mL of 35% sucrose solution, and 1.175 mL of 65% sucrose solution and centrifuged for 48 hours at 50 000 rpm using a SW55-TI rotor. Twenty-one 250-μL aliquots released were taken from the top of the tubes, and density of each fraction determined by the index of refraction. The [3H]cholesterol was measured in 50 μL of each fraction. For measurement of [3H]phospholipids, the lipids were extracted from 100 μL of the method of Bligh and Dyer, and the chloroform phase washed 3 times with methanol:H2O (10:9, v/v) to remove [3H]choline. The lipid extract was dried under N2 and the [3H]phospholipid measured. For immunoblotting of apoE and apoAI, the protein was precipitated from aliquots (volume indicated in Figure Legends) using trichloroacetic acid (10%, final concentration). The protein pellets were washed in succession with 10% trichloroacetic acid and ice-cold acetone, dissolved in 30 μL of phosphate-buffered saline containing 2% SDS, mixed with NuPage Reducing and Loading Buffer (4 μL to 8 μL), heated at 90°C for 10 minutes, and separated using NuPage 10% Bis-Tris gels. After transfer to nitrocellulose membranes, the apoE was detected using rabbit anti-serum against mouse apoE (1:1000 dilution) and goat anti-rabbit IgG (1:5000 dilution) conjugated to horseradish peroxidase. To detect apoAI, goat polyclonal anti-human apoAI (1:1000 dilution; Biodesign International) and rabbit anti-goat IgG (1:5000 dilution) were used. ApoE and apoAI were visualized by chemiluminescence (Amersham), and signal was quantitated using Bio-Rad Quantity One 1-D Analyst Software.

Results

Impact of ABCA1 Deficiency on ApoE-Mediated Cholesterol Efflux From Cholesterol-Enriched Macrophages

To examine the impact of ABCA1 on the mechanism of endogenous apoE-mediated cholesterol efflux, we compared
the efflux of [3H]cholesterol from cholesterol-enriched WT, ABCA1−/−, and apoE−/− macrophages in the absence of cholesterol acceptors (Figure 1A). After 24-hour incubation in DMEM alone, the cholesterol efflux from WT macrophages was 3.2-fold higher than efflux from ABCA1−/− cells (5.7%±0.3% versus 1.8%±0.1%), demonstrating that ABCA1 deficiency markedly impairs the apoE mechanism of efflux. This impairment in efflux may be caused in part by effects of ABCA1 deficiency on apoE secretion because the concentration of apoE secreted from WT cells was 3.1-fold higher than that of apoE from ABCA1−/− macrophages (Figure 2A and 2C). However, cholesterol efflux from apoE−/− macrophages (Figure 1A) was significantly higher than that of ABCA1−/− cells (2.3%±0.2% versus 1.8%±0.1%; P=0.008), suggesting that the impairment in cholesterol efflux was mainly caused by the absence of ABCA1 rather than by the inefficient apoE secretion seen in ABCA1−/− cells. To further determine the contribution of the ABCA1 pathway to the apoE mechanism of efflux and distinguish between effects of inefficient apoE secretion versus ABCA1 deficiency, LXR stimulation was used to enhance the expression of apoE in ABCA1−/− cells. Compared with 24-hour incubation with DMEM alone (no acceptors, Figure 1A), LXR stimulation (Figure 1B) enhanced the cholesterol efflux from WT macrophages by 2.4-fold (5.7%±0.3% versus 13.5%±1.1%). Paralleling the enhancement in cholesterol efflux, LXR stimulation increased apoE secretion from WT cells by 2.3-fold (P=0.001; Figure 2A and 2C). Compared with incubation with DMEM alone, LXR stimulation increased the cellular apoE levels by 57% (apoE/β-actin intensity ratio, 2.1±0.3 versus 3.3±0.3), suggesting that the majority of the apoE produced via LXR agonist treatment is secreted. In contrast to WT macrophages, LXR stimulation only enhanced efflux by 11% and 20% in ABCA1−/− and apoE−/− macrophages, respectively, resulting in a marked difference in cholesterol efflux between ABCA1−/− and WT cells (2.1%±0.1% versus 13.5%±1.1%). Interestingly, LXR stimulation increased the medium apoE levels from ABCA1−/− macrophages by 6.4-fold, resulting in comparable apoE secretion between WT and ABCA1−/− cells (P=0.5; Figure 2A and 2C). Thus, under conditions of efficient apoE secretion, deficiency of ABCA1 in cholesterol-enriched macrophages results in a marked impairment in the apoE mechanism of cholesterol efflux.

Because studies have shown that apoAI stimulates secretion of endogenous apoE10 and macrophage apoE facilitates cholesterol efflux to apoAI,7,8 we also examined if LXR...
stimulation altered cholesterol efflux from the cholesterol-enriched macrophages in the presence of apoAI (Figure 1C). Without LXR stimulation, and in the presence of apoAI, the WT and apoE<sup>−/−</sup> macrophages released 12.3%±0.4% and 9.1%±0.3%, of their [3H]cholesterol content, respectively. These differences in efflux to apoAI were not attributable to differences in cellular ABCA1 (please see Figure I available at http://atvb.ahajournals.org) because WT and apoE<sup>−/−</sup> cells contained similar amounts of ABCA1 when incubated with DMEM alone, and ABCA1 was similarly upregulated in the presence of apoAI. LXR stimulation increased the amount of cholesterol released to apoAI from the WT macrophages by 139% compared with only a 86% increase from apoE<sup>−/−</sup> cells, resulting in a marked difference in cholesterol efflux between the 2 cell types (29.5%±0.2% versus 17.0%±0.5%). Because LXR stimulation and incubation with apoAI similarly enhanced the expression of ABCA1 in WT versus apoE<sup>−/−</sup> macrophages (Figure 1), the differential effect of LXR stimulation on efflux to apoAI is likely caused by endogenous apoE. Compared with incubation with DMEM alone (Figure 2B and 2C), apoAI increased apoE secretion from WT cells by 1.7-fold, and similar to previous studies using ABCA1<sup>−/−</sup> human monocyte-derived macrophages, apoAI treatment also increased apoE secretion from ABCA1<sup>−/−</sup> mouse macrophages (4.1-fold; Figure 2B and 2C), resulting in nonsignificant differences (P=0.1) in medium apoE levels between the 2 cell types. Despite similar medium apoE levels, the ABCA1<sup>−/−</sup> cells only released 1.7%±0.1% of their [3H]cholesterol compared with 12.3%±0.4% from WT cells (Figure 1C). Similarly, LXR stimulation in the presence of apoAI induced similar apoE secretion (Figure 2B and 2C) between WT and ABCA1<sup>−/−</sup> macrophages, but LXR only increased efflux from ABCA1<sup>−/−</sup> cells by 16% (Figure 1C). Similar to LXR stimulation in the absence of apoAI, the WT cellular apoE levels only increased by 50%±2% in the presence of apoAI, suggesting that most of the apoE produced is secreted. Taken together, the data suggest that ABCA1 is also required for efficient cholesterol efflux by the endogenous apoE produced via the apoAI pathway.

Effect of ABCA1 Deficiency on the Density Distribution of Medium ApoE and HDL Particles From Cholesterol-Enriched Macrophages

Because the inability of the LXR agonist treatment to stimulate apoE-mediated cholesterol efflux from ABCA1<sup>−/−</sup> macrophages was not caused by impaired secretion of apoE, we next examined whether the apoE secreted from the ABCA1<sup>−/−</sup> cells was normally lipiddated. The density distribution of [3H]cholesterol released from cholesterol-enriched WT, ABCA1<sup>−/−</sup>, and apoE<sup>−/−</sup> macrophages after 24-hour incubation with DMEM in the presence of the LXR agonist is shown in Figure 3A. The peak density of the [3H]cholesterol released from WT macrophages was 1.11 to 1.17g/mL. In contrast, the peak density range of the particles produced by both ABCA1<sup>−/−</sup> and apoE<sup>−/−</sup> macrophages was 1.21 to 1.23g/mL. In addition, the peak density fraction from WT macrophages contained 5.0 μg [3H]cholesterol/mg cell protein compared with only 0.38 and 0.36 μg/mg in the peak fractions from ABCA1<sup>−/−</sup> and apoE<sup>−/−</sup> cells. Consistent with the marked differences in medium [3H]cholesterol between the cell types, large differences were observed in cellular cholesterol mass before and after the 24-hour incubation with the LXR agonist. In WT macrophages, the cholesterol mass decreased from 89±4 to 67±3 μg cholesterol/mg cell protein, whereas in both ABCA1<sup>−/−</sup> (118±4 versus 115±3 μg/mg cell protein) and apoE<sup>−/−</sup> (127±8 versus 129±3 μg/mg cell protein), there were no significant decreases in cellular cholesterol mass. The presence of cholesterol-containing particles with similar densities in medium of ABCA1<sup>−/−</sup> and apoE<sup>−/−</sup> macrophages suggests that these higher-density, cholesterol-poor particles do not require either apoE or ABCA1 for formation. Consistent with this concept, the density range of the apoE released from the ABCA1<sup>−/−</sup> macrophages did not parallel the density range of the cholesterol containing particles (Figure 3B). Instead, the bulk of the apoE (Figure 3B) was concentrated in the highest density (lipid-free) fractions (≥1.23g/mL). In contrast, the density range of apoE released from WT cells (Figure 3B) paralleled the density range of the cholesterol containing particles with the bulk of the apoE in the density range 1.09 to 1.20g/mL. A similar density distribution of cholesterol and apoE was observed when WT, apoE<sup>−/−</sup>, and ABCA1<sup>−/−</sup> cells were incubated with DMEM alone (data not shown). In addition, similar differences in both the amounts and density distribution of medium [3H]cholesterol were observed when
the 3 macrophage strains were incubated with DMEM in the absence of ACAT inhibition and LXR stimulation (Figure II A). The density distributions of cholesterol and apoE are consistent with endogenous apoE requiring ABCA1 for efficient lipidation and formation of buoyant F-containing HDL particles.

We next examined the density distribution of medium cholesterol (Figure 4A) and apoE (Figure 4B) when macrophage foam cells were incubated with both apoAI and the LXR agonist. The peak density of the cholesterol-containing particles released from WT macrophages was similar whether apoAI was present (1.12 to 1.17 g/mL, Figure 4A) or absent (1.11 to 1.17 g/mL; Figure 5A). Also, apoE \( \sim \) cells formed particles with a similar peak density (1.12 to 1.17 g/mL) except that the fraction only contained 4.1 \( \mu \)g [\(^{3}H\)cholesterol]/mg cell protein compared with 18.0 \( \mu \)g/mg for WT cells (Figure 4A). Consistent with this large difference in medium \( [\text{H}] \)FC, incubation with apoAI caused a large decrease in cholesterol mass in WT cells (50 \( \pm \) 1%) and only a modest one (16 \( \pm \) 1%) in apoE \( \sim \) macrophages. Similar to incubation with DMEM alone, the particles formed by ABCA1 \( \sim \) cells incubated with apoAI were cholesterol-poor and had a peak density >1.21 g/mL. Similar differences in both the amounts and density distribution of medium \( [\text{H}] \)cholesterol were observed when the 3 macrophage strains were incubated with apoAI in the absence of ACAT inhibition and LXR stimulation (Figure 3B). Furthermore, the apoE from ABCA1 \( \sim \) cells was concentrated in the highest-density fractions (Figure 4B). Similar results were observed for apoE secreted from ABCA1 \( \sim \) cells in the presence of apoAI without LXR stimulation (data not shown). The apoE secreted by WT macrophages in the presence of apoAI encompassed the density distribution of the cholesterol particles, with larger amounts of apoE density at 1.10 to 1.20g/mL fraction. Unlike apoE, most of the apoAI associated with denser particles (density \( \geq \)1.20 g/mL). The density distribution of apoAI was not affected by the presence or absence of apoE (Figure 4B). These results suggest that even in the presence of apoAI endogenous apoE competitively facilitates the formation of buoyant HDL particles.

We next examined the density distribution of cell-derived \( [\text{H}] \)choline-containing phospholipid(PL) to determine whether the poorly lipidated apoE that is secreted by ABCA1 \( \sim \) macrophages contains phospholipid. Similar to the density distribution of cell-derived \( [\text{H}] \)cholesterol (Figure 3A), the \( [\text{H}] \)phospholipid released from WT macrophages to either DMEM alone (Figure 5A) or apoAI (Figure 5B) appeared in a peak density of 1.12 to 1.17 g/mL. Under DMEM incubations, the majority of endogenous apoE was in particles with a \( [\text{H}] \)FC/[\(^{3}H\)PL] molar ratio of 1.9 to 1.1 (Figure 5B). In the presence of apoAI, the \( [\text{H}] \)FC/[\(^{3}H\)PL] ratio of the particles formed by WT cells was 1.7 for the apoE-enriched peak (fraction 11) and 0.74 for the apoAI-enriched peak (fraction 14; Figure 4B). This demonstrates that endogenous apoE preferentially forms cholesterol-rich particles even when competing with apoAI. The \( [\text{H}] \)PL released...
from the ABCA1−/− cells to DMEM colocalized with the [3H]FC (Figure 3B), but the [3H]FC/[3H]PL ratio of the peak density fraction was much lower compared with WT cells (0.38 versus 1.47). In addition, a significant amount of [3H]PL colocalized with the apoE. Similar results were observed when ABCA1−/− cells were incubated with apoAI (Figure 5B).

**Discussion**

Macrophage foam cell formation is a critical determinant of the development and progression of atherosclerotic lesions. During cholesterol loading, expression of numerous gene products involved in cholesterol homeostasis is enhanced via LXR, in a concerted effort to minimize foam cell formation. Two such genes with critical roles in macrophage lipid homeostasis are apoE and ABCA1. Macrophage specific deletion of either apoE11 or ABCA128 enhances atherosclerotic lesion development, whereas expression of apoE potently reduces atherosclerosis.13,29 The current studies have examined the contribution of ABCA1 to the mechanism of endogenous apoE cholesterol efflux. Our studies demonstrate that the ABCA1-dependent pathway is the main mechanism for formation of buoyant, cholesterol-containing HDL particles with apoE secreted from macrophage foam cells. We also show that that even in the presence of apoAI endogenous apoE promotes efficient formation of buoyant, cholesterol-enriched particles. Taken together, our studies suggest that endogenous apoE, plasma apoAI, and ABCA1 work in concert to activate reverse cholesterol transport in the artery wall.

Studies of Huang et al14–15 suggest that macrophage apoE-mediated efflux occurs via both ABCA1-dependent and ABCA1-independent pathways. The authors propose that both pathways could make significant contributions to cellular cholesterol losses. In addition, studies with microglial cells16 demonstrated that apoE-mediated cholesterol efflux is mostly ABCA1-independent. A number of observations in the current studies support the conclusion that, instead, the ABCA1-dependent pathway is the main mechanism for endogenous apoE-mediated cholesterol efflux and formation of FC-containing HDL particles in macrophages. Compared with WT cells, ABCA1−/− cells (which secrete apoE) had a marked decrease in cholesterol efflux (Figure 1A to 1C) either in the absence or presence of exogenous apoAI. Furthermore, LXR stimulation increased apoE secretion from ABCA1−/− macrophages but only enhanced cholesterol efflux by 10% to 16% (Figure 1A to 1C). Most convincing, however, is the observation that the apoE secreted from cholesterol-enriched ABCA1−/− cells was not associated with buoyant FC-containing HDL particles (Figures 3 to 5). A major difference between our studies and previous studies14–16 is that we used cholesterol-enriched macrophages (foam cells), whereas the other studies used cholesterol-normal cells. It is conceivable that the contribution of the ABCA1-dependent pathway is magnified under conditions of cholesterol enrichment and LXR activation where ABCA1 and apoE levels are elevated. Consistent with this concept are earlier studies of Kruth et al,30 which demonstrated that apoE is secreted lipid-poor unless the macrophages are cholesterol-enriched. This observation and our present studies suggest that the ABCA1-dependent pathway for apoE-mediated efflux is the predominant mechanism for cholesterol exit from foam cells within atherosclerotic lesions. The current studies do not rule out the possibility that the ABCA1-independent pathway could play a significant role during the initial stages of cholesterol enrichment of macrophages.

Compared with WT macrophages, basal apoE secretion was decreased in cholesterol-enriched ABCA1−/− macrophages, consistent with other studies suggesting a role for ABCA1 in apoE secretion.31 Studies of Kockx et al10 demonstrated that apoAI stimulates apoE secretion from human monocyte-derived macrophages via an ABCA1-independent pathway. The current studies demonstrated that apoAI also increases apoE secretion from mouse ABCA1−/− macrophages, consistent with the concept that both ABCA1-dependent and ABCA1-independent pathways (Figure 6A and 6B, step 1) regulate secretion of endogenous apoE. Interestingly, the current studies demonstrate that LXR stimulation induces efficient apoE secretion from ABCA1−/− macrophages, probably through activation of an autocrine feed-forward loop (Figure 6A and 6B, step 1), which is in line with data showing that exogenous apoE stimulates secretion of endogenous apoE.10 At present it is unclear if the autocrine and apoAI-dependent modes of enhanced apoE secretion operate via the same pathways. Regardless, both mechanisms are probably relevant to stimulating apoE secretion (Figure 6A and 6B, step 1) and minimizing foam cell formation in atherosclerotic lesions in which accessibility of plasma apoproteins to macrophages is limited.11,32 Possible ABCA1-independent pathways that could regulate apoE secretion include ABCG1 and PLTP because studies have demonstrated that both proteins are upregulated via LXR and their expression impacts apoE secretion.31,33

The current studies demonstrated that macrophage apoE facilitates formation of buoyant FC-containing HDL in the presence of exogenous apoAI (Figure 4). This observation is consistent with studies showing that expression of endogenous apoE facilitates cholesterol efflux to both exogenous apoE and apoAI from a number of cell types,7,34 and with studies showing that in astrocytes endogenous apoE stimulates formation of larger FC-enriched HDL compared with either exogenous apoE or apoAI.34 These and the current studies suggest a fundamental difference between exogenous apoproteins and endogenous apoE in macrophage-mediated particle formation. In contrast, an earlier report demonstrated that 85% of the particles formed from THP-1 cells contained only exogenous apoAI, with the rest containing only apoE or apoE with apoAI.35 This discrepancy is likely explained by the fact that cholesterol-enriched THP-1 cells secrete very low levels of apoE in the absence or presence of apoAI (0.1 µg apoE/mg cell protein), which is up to 70-times less than the amount secreted by cholesterol-enriched mouse peritoneal and human monocyte-derived macrophages.10,30,35

ABCA1-dependent (Figure 6A) and ABCA1-independent mechanisms (Figure 6B) can be proposed for the enhanced formation of buoyant HDL particles by macrophage apoE in the presence of apoAI. Because endogenous apoE also
The concentration of apoAI (20 \mu g/mL) used in our study is 4- to 10-fold greater than that reported to saturate ABCA1 efflux and, in addition, apoAI was easily detected in the highest-density fractions (Figure 4), suggesting that even though there was abundant availability of poorly lipidated apoAI as an acceptor for ABCA1, more buoyant apoAI-containing HDL particles were not formed. In contrast, endogenous apoE was more heavily associated with the most buoyant HDL particles, suggesting that compared with exogenous apoAI or apoE, endogenous apoE possesses unique properties that increase the effectiveness of its interaction with ABCA1 (Figure 6A, step 2 versus step 3).

Compared with exogenous apoAI or apoE, several features of endogenous apoE could influence ABCA1-mediated particle formation (Figure 6A, steps 2 versus step 3). The present studies have demonstrated that without ABCA1, secreted apoE floats at density >1.21 g/mL, yet it is partially phospholipidated. Consistent with this observation, hepatic apoAI is also phospholipidated in the absence of ABCA1, and ABCA1 deficiency minimally decreases formation of these very-high-density HDL particles. ABCA1-mediated particle formation involves interaction of apoproteins with ABCA1 and phospholipid-containing, trypsin-sensitive components of the extracellular matrix. Association of phospholipid with endogenous apoE could induce conformational changes that increase the effectiveness of its interaction with either ABCA1 or the extracellular matrix. In support of this theory, studies have shown that depletion of cell surface proteoglycans has a major impact on efflux to endogenous apoE efflux, but minimally decreases efflux to exogenous apoE. It is also worth noting that the poorly phospholipidated apoE-containing HDL in plasma are the most efficient inducers of cholesterol efflux and also float at density >1.21 g/mL. Another difference between endogenous and exogenous apoE is that endogenous apoE is almost completely sialylated, which may also affect its association with phospholipid and the extracellular matrix.

The enhanced formation of buoyant, FC-containing HDL in the presence of endogenous apoE may occur in part via mechanisms independent of ABCA1 where the endogenous apoE on HDL enhances cholesterol loading of the particles via other pathways including scavenger receptor class B type I and ABCG1 (Figure 6B, step 2 versus step 3). This possibility is consistent with studies showing that endogenous apoE enhances efflux to mature HDL particles, which are acceptors for cholesterol derived from scavenger receptor class B type I and ABCG1, and that HDL stimulates secretion of apoE, which then associates with HDL. The increased apoE content of nascent HDL would be expected to enhance association with scavenger receptor class B type I and other cell surface sites in close proximity to cholesterol domains formed via scavenger receptor class B type I or ABCG1 (Figure 6B, step 2 versus step 3). Indeed, recent studies using either cell lines or cholesterol-enriched astrocytes demonstrated that ABCG1 enriches the HDL formed via ABCA1 with cholesterol.

In summary, macrophage foam cell apoE requires ABCA1 for efficient formation of buoyant, FC-containing HDL particles. LXR stimulation enhances apoE secretion, which in turn enhances cholesterol mobilization in the absence and

![A Pathway 1](image)

![B Pathway 2](image)
presence of apoAI. In this regard, ABCA1, endogenous apoE, plasma apoAI, and possibly other efflux pathways (Figure 6A and 6B) act in a concerted effort to minimize foam cell formation in the arterial wall.

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Supplemental Figures

Figure I. Effects of LXR stimulation and/or apoAI on ABCA1 levels in WT and apoE−/− macrophages. WT or apoE−/− macrophages were cholesterol enriched by incubation with acetylated LDL and then incubated for 24h in DMEM and CP113,818 (2 µg/ml) alone or including TO-901317 (1µM) or 20µg of apoAI/ml or both TO-901317 and apoAI (A) and cellular ABCA1 was detected by western blotting and quantitated (B) as described under “Methods”. Data in Panel B are the mean ± S.D. of triplicate determinations.

Figure II. Buoyant density of [3H] cholesterol in medium from WT, apoE−/−, or ABCA1−/− macrophages. WT, apoE−/−, or ABCA1−/−macrophages were cholesterol-enriched by incubation with apoE−/− remnant lipoproteins and then incubated for 24h in DMEM alone (A) or with 20µg/ml of apoAI (B). For each fraction, the [3H]cholesterol in 50µl was measured and the density was determined as described under “Methods”. The specific activity of the [3H]cholesterol in cells prior to efflux was used to estimate the µg of cholesterol in the density fractions, and the data are expressed as µg cholesterol/mg cell protein. Data are representative of 2 independent experiments.
Figure I
Figure II