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CLINICAL IMPLICATIONS

The ins and outs of lipid efflux

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Abbreviations

ABC	ATP-binding cassette
ACAT	acetyl-CoA acetyltransferase
apoA-I	apolipoprotein A-I
apoE	apolipoprotein E
ARF	ADP-ribosylation factor
ARL7	ADP-ribosylation factor (ARF)-like 7
CE	cholesteryl esters
CEH	cholesteryl ester hydrolase
FC	free cholesterol
HDL	high-density lipoprotein(s)
LCAT	lecithin-cholesterol acyltransferase
LDL	low-density lipoprotein(s)
PL	phospholipids
SR-BI	scavenger receptor BI

Atherosclerosis has been a companion of mankind since antiquity. In recent years, the disease and its complications has advanced to become the number one killer in the world. A hallmark of atherosclerosis is the massive appearance of lipid-laden (foamy) macrophages in the subintimal space of affected arteries. These macrophage foam cells are a consequence of the retention and subsequent chemical or enzymatic modification of low-density lipoprotein (LDL) particles within the arterial wall. As the phagocytotic cells of the cellular immune system, macrophages ingest modified LDL via their scavenger receptors. In contrast to most other cells of the body, which regulate their cholesterol content by limiting its uptake via the LDL receptor pathway and by reducing its cellular synthesis by down-regulating HMG-CoA reductase, macrophages pick up large amounts of cholesterol via scavenger receptor-mediated endocytosis, a process that is not subject to negative feedback regulation by the intracellular cholesterol content. Because macrophages, like other mammalian cells, possess no mechanisms for breaking down the sterol backbone of cholesterol, they are faced with the dilemma of how to deal with the toxic unesterified cholesterol taken up; this problem is compounded by the fact that macrophages ingest substantial amounts of cholesterol by phagocytosing necrotic and apoptotic cells as well as cellular debris.

The first mechanism by which plaque macrophages deal with excess free cholesterol is by storing it mostly in the form of cholesteryl esters in cytosolic lipid droplets, the counterregulatory process protecting the cells from apoptosis and leading to the eponymous foam cell. The second means is by exporting the excess cholesterol to acceptors such as apolipoprotein A-I (apoA-I), apolipoprotein E (apoE), or high-density lipoproteins (HDL) as part of the reverse cholesterol transport that redistributes excess cholesterol from peripheral tissues to the liver. However, if these two mechanisms are overwhelmed, free cholesterol builds up to toxic levels within macrophages. This, by itself, impairs cellular metabolism and signaling processes,

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but cholesterol crystals and oxysterols forming within the cell either trigger apoptosis or kill the cell in an unregulated fashion. Whatever the reason is, the death of macrophage foam cells results in the accumulation of cholesterol and cholesteryl esters within the arterial wall. The continuous deposition of foam cell-derived lipids over the years is held responsible for the formation of the massive necrotic lipid core typical of advanced atherosclerotic lesions.

Export of cholesterol is the only way for macrophages to dispose the problem of excess cholesterol once and for all. Understanding the mechanisms underlying cholesterol efflux may help to identify new pharmacological approaches for preventing atherosclerosis and its complications by triggering the reverse cholesterol transport. Over the last years, passive and active mechanisms of cholesterol export from macrophage foam cells have been described [1]. Passive export of cholesterol involves aqueous diffusion of cholesterol from the cell surface to acceptors such as HDL, LDL, and albumin or desorption of phospholipids by apolipoproteins independently of transporters and receptors in a process termed micro-solubilization. Secretion of lipids together with endogenously produced apolipoproteins (apolipoprotein E, in particular), conversion of cholesterol into the more hydrophilic 27-hydroxycholesterol, which is then secreted, and—last but not least—transporter-mediated efflux of cholesterol from or across the plasma membrane to extracellular acceptors such as apoA-I and HDL comprise active export pathways. Recent studies identified two ABC transporters, namely ABCA1 and ABCG1, which mediate the initial steps of the latter efflux pathway and contribute to more than half of the total cholesterol efflux [1–3]. In the absence of these transporters, macrophage cholesterol efflux to the acceptor molecules apoA-I and HDL, respectively, is impaired and the cells massively accumulate cholesteryl esters. Although several cellular receptors for cholesterol acceptors and transporters involved in macrophage cholesterol efflux have been identified, it is still unclear how the cellular efflux machinery of macrophage foam cells is organized and how it works.

In this issue of the *Journal of Molecular Medicine*, Iris Lorenzi and her colleagues report on the contribution of ABCA1 and scavenger receptor BI (SR-BI) to the internalization of apoA-I by murine macrophages and the importance of apoA-I internalization for cholesterol efflux in these cells [4]. The findings support previous studies of the same group in which endothelial cells have been shown to internalize, transcytose and lipidate lipid-free apoA-I in an ABCA1-dependent fashion [5, 6]. In the present study, the authors report that macrophages of the RAW264.7 cell line bind both HDL and lipid-free apoA-I, but internalize only apoA-I. Binding of HDL is decreased by reducing SR-BI expression, whereas cell association and internalization of apoA-I correlates with expression of ABCA1. Trapping of ABCA1 at the cell surface results in reduced apoA-I internalization and inhibition of cholesterol efflux to apoA-I, but not to HDL. To sum up, the data presented by Lorenzi and colleagues provide evidence that apoA-I- but not HDL-mediated cholesterol efflux from proliferating murine macrophages may involve retroendocytosis, i.e., endocytosis of apoA-I followed by its exocytosis. These findings support a concept of ABC transporter-mediated cholesterol efflux that differs from the extracellular process depicted in Fig. 1a. According to this model, intracellular cholesteryl esters stored in lipid droplets are first hydrolyzed and the resulting free cholesterol is then transported from intracellular stores to the plasma membrane. At the plasma membrane, ABCA1 mediates the loading of phospholipids and/or free cholesterol to lipid-free apoA-I which is docked to the cell surface. Next, ABCG1 transfers cholesterol to HDL particles. Although not shown in the figure, the conversion of lipid-free apoA-I into HDL and the maturation of nascent HDL require additional steps such as cholesterol esterification by lecithin-cholesterol acyltransferase (LCAT) and integration of apolipoproteins such as apoE. In the alternative model shown in Fig. 1b, ABCA1-dependent and receptor-mediated retroendocytosis of apoA-I is necessary for cholesterol efflux to occur. According to this model, apoA-I is internalized, acquires phospholipids and free cholesterol from intracellular pools and is then resecreted. Although ABCA1 is found in endocytic vesicles, we cannot rule out that ABCA1 mediates endocytosis of apoA-I indirectly by signaling to another yet not identified endocytic receptor (see [4] for details).

Is there other experimental evidence for the concept that ABC transporter-mediated cholesterol efflux contains an intracellular step? Studies providing evidence for reversible binding of HDL at the cell surface without internalization date back to the 1980s. Later work showed that binding of HDL to its receptor stimulates the translocation of intracellular cholesterol to the plasma membrane, where it is accessible for removal by HDL [1, and references therein]. Further, cell surface retention of ABCA1 increases cholesterol efflux to apoA-I via ABCA1 from cholesterol pools of the plasma membrane [7]. Taken together, these studies provide evidence that cholesterol efflux is primarily an extracellular event but they do not exclude the possibility of an intracellular step. Endocytosis and subsequent exocytosis of HDL by human macrophages was reported in the mid 1980s, and ensuing studies revealed that internalized HDL_3 is exocytosed as apoE-containing HDL_2-like particles [8, 9]. In addition, cholesterol efflux is reduced by inhibitors of coated pit endocytosis and cyclic adenosine monophosphate (cAMP), which upregulates ABCA1 expression, increases cholesterol efflux, and binds cell association and exocytosis of apoA-I [1, and references

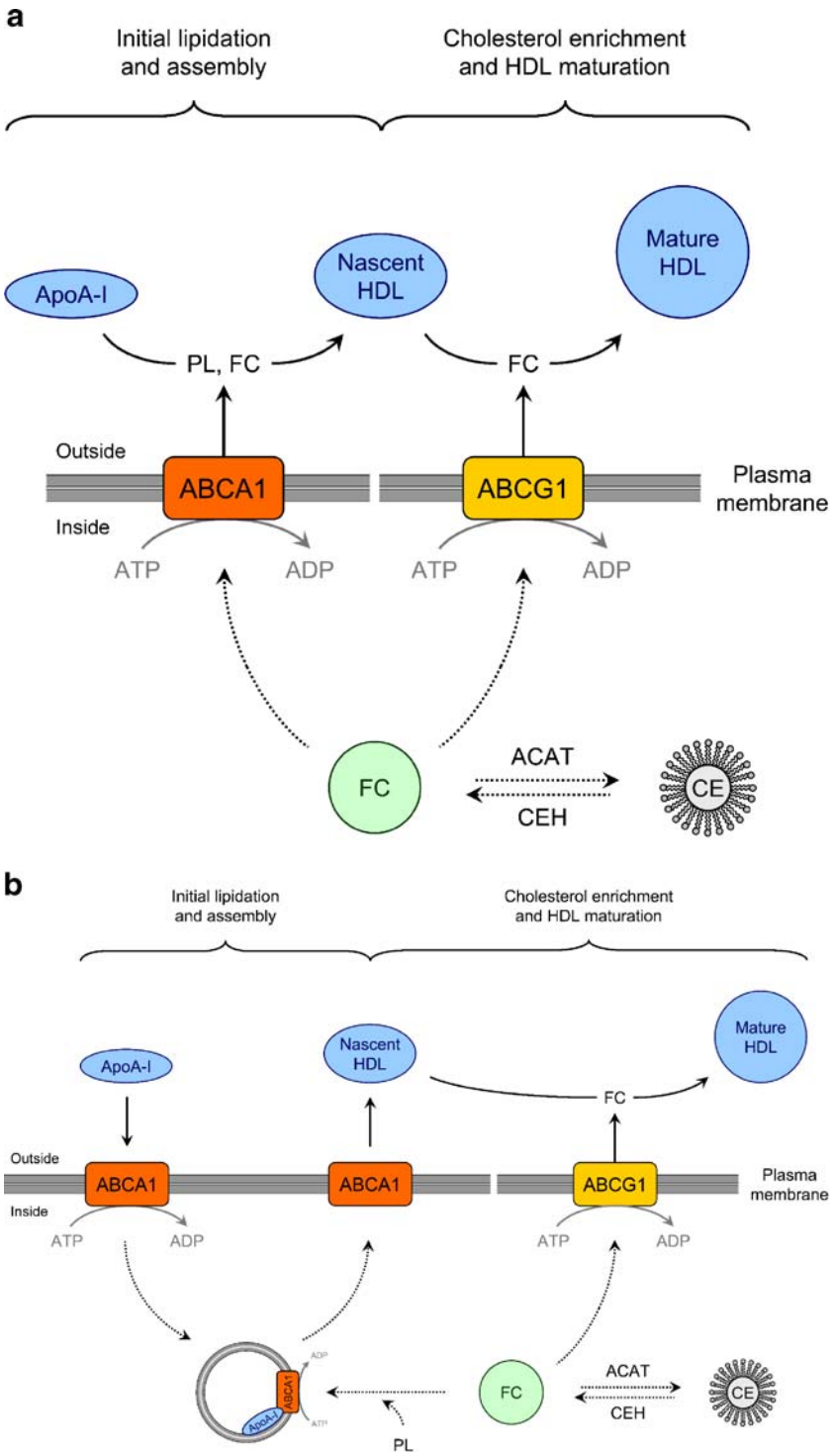

Fig. 1 Mechanisms of ABC transporter-mediated cholesterol efflux. **a** Classical concept of ABCA1- and ABCG1-mediated lipid efflux as an extracellular event. After hydrolysis of cholesteryl esters, the free cholesterol is transported from intracellular stores to the plasma membrane. ABCA1 then mediates translocation of phospholipids and/or cholesterol to lipid-free apoA-I which docks to phospholipid-rich domains of the cell surface. ABCG1 then transfers cholesterol to HDL particles. Additional steps not shown here are required for the conversion of lipid-free apoA-I into HDL and the maturation of nascent HDL (for example, cholesterol esterification by LCAT and integration of apolipoproteins other than apoA-I). **b** Alternative concept of ABCA1-mediated lipid efflux as an intracellular process involving retroendocytosis of apoA-I. ABCA1-mediates apoA-I internalization and translocation of phospholipids and free cholesterol from distinct intracellular pools to apoA-I which is resecreted as a lipidated particle. Figures were adapted from [1]

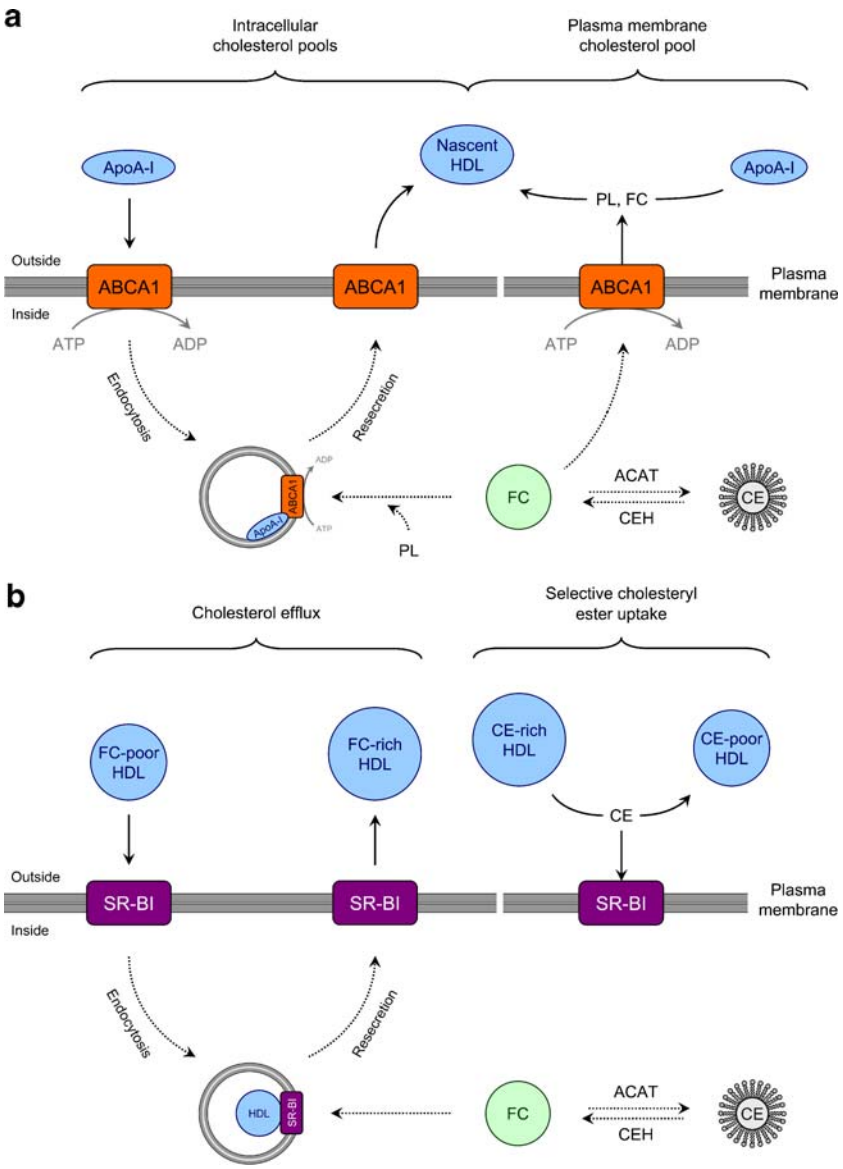

Fig. 2 Cholesterol efflux to lipid acceptors is a complex process including intracellular steps in addition to extracellular events. **a** Cholesterol efflux to apoA-I occurring independently of and in addition to apoA-I internalization. Impaired ABCA1 internalization and increased surface presence of ABCA1 results in increased efflux of surface cholesterol but reduced efflux of intracellular (lysosomal) cholesterol [8]. These findings suggest that ABCA1 acquires cholesterol for efflux simultaneously from both the external leaflet of the plasma membrane (extracellular) and from intracellular pools, possibly in order to enhance the performance of cholesterol efflux by utilizing cholesterol from different pools (see Fig. 1 for individual pathways). **b** Cholesterol efflux to HDL occurring during SR-BI-mediated retroendocytosis of HDL independently from ABCA1. SR-BI mediates the bi-directional exchange of cholesterol between cells and HDL by selective uptake of cholesteryl esters from cholesteryl ester-rich HDL particles into cells and by exporting free cholesterol from cells to free cholesterol-poor HDL. SR-BI-mediated cholesterol efflux to HDL involves retroendocytosis, whereas selective uptake of cholesteryl esters from HDL via SR-BI occurs at the cell surface [13, 17]. Although taking place, ABCG1-mediated efflux of cholesterol to HDL is not shown in this figure (see Fig. 1b for details). ABCG1 has been found in perinuclear vesicles and (recycling) endosomes [18–20], but there is no clear experimental evidence that ABCG1-mediated cholesterol efflux includes intracellular steps. However, due to recent data, we cannot rule out the possibility that ABCG1-mediated cholesterol efflux also involves intracellular steps [20]. According to current data, a complex mechanism and regulation underlies cholesterol efflux in which cells utilize retroendocytosis of either apoA-I or HDL particles at different steps of the cholesterol efflux pathway possibly depending on the cell type or on (patho)physiologic conditions in order to acquire cholesterol from different cellular pools

therein]. Further, enterocytes specifically bind, endocytose and release liposomes containing apoA-I [10]. In addition, ABCA1-mediated cholesterol efflux occurs from endosomal rather than from plasma membrane pools [1, and references therein], and ABCA1 and internalized apoA-I co-localize in endosomal compartments [11, 12]. The latter finding is corroborated by the fact that ABCA1 is involved in various endocytic processes as was reviewed in [1].

Finally, recent studies have shown that the SR-BI-mediated uptake of entire HDL particles is followed by resecretion and that retroendocytosis represents one of the pathways for cholesterol export in macrophages [13].

With these findings in mind, the question is not anymore whether ABC-transporter-mediated cholesterol efflux occurs as an extra or intracellular event. I submit that there is adequate evidence that cholesterol efflux from macrophages to acceptor particles is a multi-step process that involves retroendocytosis, possibly at different stages as outlined in Fig. 2, making the whole process of cholesterol efflux much more complex than previously thought. Further studies are necessary to elucidate the overall importance of intracellular events during cholesterol efflux and the contribution of these events to the total efflux of cholesterol. With respect to unraveling the mechanisms underlying intracellular cholesterol efflux, the following questions remain: First, why is retroendocytosis required for cholesterol efflux? Is it because retroendocytosis allows acceptor particles for accessing distinct intracellular cholesterol pools which are otherwise not accessible? Or is it just due to higher performance of cholesterol efflux? Second, is ABCA1-dependent internalization of apoA-I a general mechanism, or is this phenomenon limited to distinct (pathologic) conditions or to proliferating cell lines such as murine RAW264.7 macrophages and, in case of apoA-I transcytosis, to endothelial cells? Further studies, in particular on primary monocyte-derived (non-proliferating) macrophages, may help to answer this question. Third, is the apoA-I internalized by the macrophage really resecreted and what is its molecular composition? Recent studies have shown that ABCA1 and, to a lesser extent, ABCG1 regulate the secretion of apoE from human macrophages [14]. Thus, the question arises whether resecreted ApoA-I is lipidated only, or whether it is also complexed with other proteins such as apoE. Fourth, does cholesterol efflux to apoA-I also occur independently of apoA-I internalization suggesting that a more complex regulatory mechanism underlies this process? This implies that apoA-I internalization is required for mediating cholesterol efflux from distinct intracellular cholesterol pools in addition to utilizing cholesterol from the plasma membrane. Fifth, how can the controversial results on HDL internalization be explained? Does HDL internalization depend on the cell type or on the method used for analysis? Sixth, from which intracellular pool does apoA-I acquire lipids? In particular, is there any direct interaction, i.e., the exchange of lipids between the apoA-I-containing endosomal compartments and cytosolic lipid droplets, or are carriers used to transport lipids from lipid bodies to the site where cholesterol efflux is performed? Seventh, which other proteins and signaling pathways are involved in retroendocytosis-dependent cholesterol efflux? For example, recent studies have shown that ADP-ribosylation factor-like 7 (ARL7), which is involved in vesicular budding from the *trans*-Golgi-network, has been implicated in intracellular ABCA1-mediated translocation of lipids and in secretory lysosome trafficking [15]. In addition, ABCA1 and apoA-I have been shown to modulate different signaling cascades involving cAMP, ABCA1 autophosphorylation, JAK2, and Cdc42, and to interact with syntaxin 13 and syntrophins [1, and references therein]. Eighth, does ABCA1 modulate the formation of coated pits or endosomes, and is ABCA1 therefore mediating internalization of apoA-I bound to distinct cell surface domains at which endocytosis occurs? This would provide an explanation why ABCA1 is involved in various endocytic and phagocytic processes. Ninth, what does the exact time flow of apoA-I lipidation and HDL maturation look like? For example, when does conversion of free cholesterol into cholesteryl esters via LCAT and complexing with apoE occur during the retroendocytic process? Particularly in the case of HDL_3 internalization, we would then expect to find LCAT in endosomal vesicles. Further, does apoA-I internalization occur prior or after loading with lipids? Does loading with cholesterol appear simultaneously with or after that of phospholipids? Tenth, what are the receptors mediating internalization of apoA-I and HDL? Recent studies have shown that although HDL binds to SR-BI and SR-BI is involved in cholesterol efflux, cellular SR-BI does not contribute to net cholesterol efflux from cells to plasma HDL containing active LCAT, due to reuptake of HDL cholesteryl esters into the cells [2, 3, 16]. Last but not least, what are central factors regulating retroendocytosis of apoA-I and the accompanied cholesterol efflux, and are these regulators new molecular targets for the pharmacological modulation of cholesterol efflux which may help us to defeat atherosclerosis and its complications?

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