The concept of “reverse cholesterol transport” (RCT) was first introduced in 1968 by Glomset\(^1\) to describe the process by which extracellular (peripheral) cholesterol is returned to the liver for excretion in the bile and ultimately the feces. The physiological need for this process is clear, as nonhepatic cells acquire cholesterol through uptake of lipoproteins and de novo synthesis and yet (with the exception of steroidogenic tissues that convert cholesterol to steroid hormones) are unable to catabolize it. Excess unesterified cholesterol (UC) is toxic to cells, and therefore, cells have developed several ways to protect themselves against cholesterol toxicity. One key pathway is the efflux of cholesterol to extracellular “acceptors.” The return of this “peripheral” cholesterol to the liver is necessary to balance cholesterol intake and de novo synthesis and thus to maintain whole-body steady-state cholesterol metabolism.

The relationship of RCT to atherosclerosis was first suggested by Ross and Glomset,\(^2\) who hypothesized that atherosclerotic lesions develop when an imbalance occurs between the deposition and removal of arterial cholesterol after endothelial injury. This concept was further developed by Miller and Miller,\(^3\) who suggested that on the basis of the inverse relation between HDL cholesterol (HDL-C) and cardiovascular disease, emphasis should be placed on increasing HDL as a way to increase clearance of cholesterol from the arterial wall to prevent cardiovascular disease. Despite 3 decades of work, the relationship of RCT to atherosclerosis remains more of a hypothesis than an established fact. Because the physiological process of RCT clearly occurs from all peripheral tissues, it has often been measured and discussed as a general peripheral process. However, in atherosclerotic lesions, the primary cell type that is overloaded with cholesterol is the macrophage, and therefore, it makes more sense to conceptualize and measure RCT as a macrophage-specific phenomenon when it comes to atherosclerosis.\(^4\) Indeed, we support the use of the more specific term “macrophage RCT” when discussing this process as it relates to atherosclerosis. Here we review recent developments in the understanding of the molecular regulation of macrophage RCT, the challenges in measuring macrophage RCT in animal models and humans, the evidence linking macrophage RCT to the prevention or regression of atherosclerosis, and the additional work that must be performed in this important area of research.

**Cholesterol Efflux From Nonmacrophage Tissues: Critical for Maintaining Normal HDL Biosynthesis and Metabolism**

A discussion of RCT should begin with a discussion of the recent developments in our understanding of the biosynthesis of HDL (Figure). It has been known for quite some time that the liver and intestine are both capable of synthesizing and secreting apolipoprotein (apo) A-I, the major HDL apolipoprotein that is required for normal HDL metabolism.\(^5\) The relative contribution of these 2 organs to the plasma apoA-I pool in humans remains unknown. The nature of “nascent” HDL has been debated, but recent studies provide important insight into this issue. In 1999, the molecular basis of Tangier disease, a rare genetic disorder, was found to be loss-of-function mutations in both alleles for the gene encoding the ATP binding cassette transporter A1 (ABCA1).\(^7\)–\(^9\) Tangier disease is associated with virtually undetectable levels of HDL-C and very low levels of apoA-I that are confined to pre-\(β\)-HDL, as well as with cholesterol accumulation in peripheral macrophage-enriched tissues. ABCA1 is a ubiquitously expressed cellular lipid transport protein that promotes efflux of phospholipids and UC from cells to lipid-poor apoA-I, a form of “pre-\(β\)-HDL.” ABCA1 is required for normal “lipidation” of lipid-poor apoA-I, and in its functional absence, apoA-I is rapidly catabolized.\(^10\) However, the specific tissues mainly responsible for lipidation of lipid-poor apoA-I via ABCA1 were not known until recently. ABCA1-knockout (KO) mice have a phenotype similar to that of Tangier disease patients.\(^11\) Transplantation of normal wild-type bone marrow into ABCA1-KO mice did not substantially increase HDL-C levels, indicating that macrophages and other hematopoietically derived cells are not responsible for the bulk lipidation of apoA-I via ABCA1.\(^12\) In contrast, liver-specific ABCA1-KO mice have HDL-C levels that are markedly reduced by \(\sim 80\%\),\(^13\) and liver-specific partial gene knockdown of ABCA1 significantly reduced HDL-C levels by 40\%,\(^14\) indicating that the liver is quantitatively the more important organ for lipidation of lipid-poor apoA-I via ABCA1. Subsequent studies have indicated that the intestine-
specific ABCA1-KO mouse has an \(\approx 30\%\) reduction in HDL-C.\(^{15}\) Thus, the 2 organs that synthesize apoA-I, the liver and intestine, are also primarily responsible for lipidating newly secreted lipid-poor apoA-I via ABCA1-mediated lipid efflux (the Figure).

Importantly, although liver and intestine ABCA1 may be the most critical for lipidating newly synthesized lipid-free apoA-I, substantial additional cholesterol efflux to HDL occurs from other tissues. Studies in mice of “peripheral” cholesterol efflux suggest that \(\approx 90 \, \text{mg} \cdot \text{d}^{-1} \cdot \text{kg}^{-1}\) body weight of cholesterol is effluxed from peripheral tissues.\(^{16}\) However, the tissues that contribute to the greatest extent to the mass of cholesterol in HDL are unknown, and the pathways by which they efflux cholesterol, whether by ABCA1 and/or other pathways, such as the ATP binding cassette transporter G1 (ABCG1) or the scavenger receptor class B type I (SR-BI) (see following sections), remain unknown. For example, when expressed per unit of protein or organ mass, adipose tissue contains more cholesterol than do most other organs, including the liver,\(^{17}\) and adipocytes in culture have the ability to efflux cholesterol to HDL acceptors.\(^{18}\) Thus, an important quantitative source of HDL-C mass could be adipose tissue. Although peripheral nonmacrophage cholesterol efflux may not be directly relevant to atherosclerosis per se, it could be important in contributing to the overall pool of HDL-C mass and therefore to HDL-C levels, and thus, it may indirectly influence cardiovascular risk. More investigation regarding the quantitative contribution of peripheral tissues to HDL-C and the mechanisms of efflux by these tissues is required.

### Macrophage Cholesterol Efflux: the First and Potentially Most Critical Step in Macrophage RCT

The discovery that the tissues mainly responsible for lipidation of lipid-poor apoA-I via ABCA1 are the liver and intestine has altered the concept of RCT and makes clear that a general focus on “total-body” cholesterol efflux and RCT can be misleading. Not all cholesterol efflux from tissues (such as the liver or intestine) is part of the classic RCT model or is directly relevant to atherosclerosis. In reality, it is cholesterol efflux from cells in the arterial wall that have the potential to transform into foam cells—primarily macrophages—that is most directly relevant to atherosclerosis. However, the mass of cholesterol derived from macrophages is only a tiny fraction of the overall efflux of cholesterol from peripheral tissues. Macrophages are “professional phagocytes,” taking up dead cells (which contain much cholesterol), modified lipoproteins, and other extracellular debris (which can include aggregated lipoproteins).\(^{19}\) Thus, macrophages probably take up more cholesterol per cell than does any cell type other than hepatocytes and enterocytes and, possibly, steroidogenic cells. “Free” (unesterified) cholesterol is toxic to macrophages and can lead to activation of the unfolded protein response and ultimately to apoptosis.\(^{20}\) The first line of defense against cholesterol toxicity in macrophages is the esterification of cholesterol to cholesteryl ester (CE) by the enzyme acyl:coenzyme A cholesterol O-acyltransferase-I (ACAT1).\(^{21}\) CE is hydrophobic and is stored in lipid droplets within the cytoplasm; it is the accumulation of CE that leads to the formation of the foam cell. Regression of atherosclerosis might be expected to be
accompanied by a loss of CE mass from foam cells, which would require hydrolysis of the CE to UC.

A second line of defense against cholesterol toxicity in the macrophage is cholesterol efflux, and extensive work has been done to characterize the molecular pathways and regulation of cholesterol efflux in macrophages (the Figure). At one time, it was believed that passive diffusion was the most important mode of cellular cholesterol efflux from macrophages. However, the discovery of ABCA1 as an active cholesterol efflux pathway in macrophages led to major interest in facilitated pathways of efflux. Macrophages from ABCA1-KO mice have substantially reduced cholesterol efflux to lipid-poor apoA-I as an acceptor.12,22 The potential physiological importance of this finding was suggested by studies in which mice that were transplanted with bone marrow from ABCA1-KO mice were found to develop accelerated atherosclerosis.23 However, the mechanism of increased atherosclerosis was not proven to be due to impaired macrophage cholesterol efflux and could have other explanations. For example, in vitro studies showed that ABCA1-KO macrophages have an increased response to chemotactic factors.24 Furthermore, ABCA1 may promote the efflux of other proatherogenic lipids, such as oxidized phospholipids, from vascular cells.25 Interestingly, ABCA1-KO mice have normal rates of excretion of cholesterol into the bile.26 Thus, although it is clear that ABCA1 is critical for lipidation of newly secreted apoA-I by the liver and intestine and thus for normal HDL biosynthesis and metabolism, the quantitative role of ABCA1 in macrophage cholesterol efflux and RCT in vivo has yet to be definitively established.

Importantly, macrophages lacking ABCA1 still efflux considerable amounts of cholesterol to mature HDL and to whole serum, indicating that macrophages have additional pathways by which they are capable of effluxing cholesterol. Recently, ABCG1 was identified as promoting an alternative cholesterol efflux pathway from macrophages.27,28 In contrast to ABCA1, ABCG1 promotes macrophage efflux to mature HDL particles, which represent a much larger proportion of the HDL and apoA-I found in the plasma than the small pool of lipid-poor apoA-I. ABCG1-KO mice demonstrate macrophage lipid accumulation, and their macrophages have impaired cholesterol efflux to mature HDL.28 The importance of ABCG1 in macrophage RCT in vivo and atherosclerosis in mice, as well as the physiological relevance of ABCG1 in humans, remains to be determined.

ABCA1 and ABCG1 are both regulated by the nuclear receptors liver X receptor (LXR)-α and LXR-β.29 The endogenous ligands for LXRs are oxysterols that are generated through intracellular enzymatic action on cholesterol.30 Thus, excess cellular cholesterol generates formation of the oxysterol ligand(s) for LXRs that then upregulate major cholesterol efflux pathways (ABCA1 and ABCG1), an elegant homoeostatic mechanism that presumably evolved to protect cells against cholesterol toxicity. Indeed, mice deficient in LXR-α/β have tissue lipid accumulation, including within macrophages, and increased atherosclerosis.31 In light of these findings, LXRs are currently viewed as the master regulators of macrophage cholesterol efflux. Interestingly, peroxisome proliferator-activated receptor (PPAR)-α and PPAR-γ agonists have also been shown to promote macrophage cholesterol efflux, possibly in part through upregulation of LXRs.32–35

Finally, SR-BI is expressed in macrophages and can promote cholesterol efflux to mature HDL.36 Because SR-BI can also promote selective uptake of HDL-C by cells, its role in promoting net removal of cholesterol mass from macrophages has been questioned. However, SR-BI–deficient mice fed a Western diet have increased lipid deposition and atherosclerosis in the aorta.37 Furthermore, in mice, SR-BI deficiency on the background of apoE deficiency results in increased early atherosclerosis38 and markedly accelerated atherosclerosis and mortality,39 and on the background of LDL receptor deficiency and high-fat diet results in increased atherosclerosis.40 More relevant to the role of macrophage SR-BI, bone marrow transplantation from SR-BI–deficient mice into LDL receptor–deficient40 or apoE-deficient41 mice results in increased atherosclerosis, consistent with a protective role of macrophage SR-BI, although the effect of macrophage SR-BI deficiency may depend on the stage of lesion development.42 Whether macrophage SR-BI contributes in a meaningful way to macrophage cholesterol efflux and RCT in vivo has not been resolved.

**Cholesterol Esterification by LCAT: an Essential Step in Macrophage RCT?**

The cholesterol that effluxes from cells is unesterified (or “free”) cholesterol. Once associated with HDL in the plasma, it may become esterified to CE by the action of lecithin:cholesterol acyltransferase (LCAT).1–3 (the Figure). CE is more hydrophobic than UC and moves to the core of the lipoprotein particle, allowing the formation of mature HDL. LCAT is critical for normal HDL metabolism, because its absence results in the inability to generate mature HDL particles with normal CE cores. LCAT-deficient mice44,45 and humans46 have extremely low HDL-C levels and also have low apoA-I levels due to rapid catabolism of apoA-I.47

It was originally hypothesized by Glomset1 that LCAT-mediated cholesterol esterification was important for RCT because it maintained a gradient of UC from the cell to HDL acceptors, which helped drive cholesterol efflux. This view was prominent at a time when passive diffusion was thought to be the primary mechanism for cholesterol efflux. Now that much cholesterol efflux is believed to occur via active transporters, the importance of LCAT-mediated cholesterol esterification for driving cholesterol efflux and RCT is less certain. (However, it has been suggested that ABCG1 may act to increase the availability of cholesterol to different acceptors at the level of the plasma membrane,27 and thus, the “passive diffusion” model and role of LCAT may be applicable to ABCG1.) Furthermore, whereas the original focus of the RCT pathway was on the uptake of CE by the liver, it has become clear that UC can be efficiently taken up by the liver as well.48,49 Thus, the importance of LCAT for RCT in general, and for macrophage RCT in particular, is unknown. Data in animals with regard to the impact of LCAT on atherosclerosis are conflicting. Studies on LCAT overexpression in rabbits resulted in increased HDL-C levels50 and reduced atherosclerosis.51 LCAT overexpression in mice increases HDL-C levels,52–54 but its effect on atherosclerosis...
is conflicting, as atherosclerosis is either unaffected or increased; this discrepancy may be due to the absence of CE transfer protein (CETP) in mice, as atherosclerosis is reduced in mice when CETP is coexpressed with LCAT. LCAT-KO mice have been reported to have increased atherosclerosis; this discrepancy has not been satisfactorily resolved. Data in humans are scarce, given the rarity of LCAT deficiency syndromes. However, a study in subjects heterozygous for LCAT gene mutations found increased carotid intima-media thickness in heterozygotes compared with family controls, suggesting that a reduction in LCAT activity may be proatherogenic. Although LCAT is clearly important for normal HDL metabolism, more studies are required to determine the effect of LCAT activity on the rate of macrophage RCT and on atherosclerosis in animals and humans.

Transfer of HDL-C to the Liver and Targeting Cholesterol to Biliary Excretion

In the classic RCT model, HDL-C is ultimately transported to and taken up by the liver; hence, the mechanisms by which HDL-C is taken up by the liver have been the topic of substantial investigation. The most direct pathway is that of selective uptake of HDL-C by the hepatic HDL receptor SR-BI (the Figure). SR-BI promotes “selective uptake,” meaning that cholesterol is taken up, but that the HDL proteins, such as apoA-I, are not. Most discussion of SR-BI-mediated selective uptake of HDL-C has focused on CE, but as mentioned before, SR-BI is also capable of mediating selective uptake of HDL UC. Overexpression of SR-BI in the liver reduces plasma HDL-C levels due to increased hepatic uptake, whereas the SR-BI–KO mouse has increased plasma HDL-C levels due to reduced hepatic uptake. Recently, we showed that hepatic SR-BI expression in mice is a positive regulator of macrophage RCT, as hepatic overexpression of SR-BI resulted in increased macrophage RCT (despite reduced plasma HDL-C levels), and the SR-BI deficiency was associated with reduced macrophage RCT (despite increased HDL-C levels). Thus, the inverse relation of hepatic SR-BI expression to atherosclerosis may be related to its effect in promoting macrophage RCT. It is clear that hepatic SR-BI is critically important to HDL metabolism and RCT in rodents. However, its physiological importance in humans has yet to be established. Studies in healthy normolipidemic humans have suggested that relatively little HDL-CE is directly taken up by the liver and targeted to the bile. No SR-BI–deficient patients have been reported to date, but extrapolation from mouse studies would suggest that SR-BI deficiency may be associated with high HDL-C but increased cardiovascular risk, a phenotype that does exist.

Cholesterol from HDL can also be transferred to apoB-containing lipoproteins within the plasma compartment (the Figure). UC can transfer relatively easily among lipoproteins, and LCAT is present on apoB-containing lipoproteins and is responsible for cholesterol esterification there as well. HDL-CE can be transferred to apoB-containing lipoproteins in exchange for triglyceride by CETP. CETP-deficient patients have extremely high levels of HDL-C and slow turnover of apoA-I. Rodents lack CETP, but introduction of CETP expression in mice results in reduced HDL-C levels. Thus, CETP expression has a major effect on plasma HDL metabolism and levels; however, its role in RCT remains uncertain. A human study showed that after injection of HDL labeled with a CE tracer, most of the tracer that was ultimately found in the bile arrived there after transfer to apoB-containing lipoproteins, suggesting that CETP plays an important role in the transfer of HDL-CE to the liver and biliary excretion. Conversely, in similar studies when HDL was labeled with a UC tracer and injected, the majority of HDL-UC that appeared in the bile arrived there directly without transfer to apoB-containing lipoproteins, suggesting some role for a hepatic process in the direct uptake of HDL-UC (perhaps SR-BI or alternatively, a novel pathway). Thus, the role of CETP in RCT and the potential metabolic divergence of HDL-CE versus HDL-UC have yet to be resolved and have implications for the effects of CETP inhibition on macrophage RCT and atherosclerosis (see following sections).

The classic RCT pathway involves the targeting of HDL-C that has been taken up by the liver to a biliary excretion pathway. Although cholesterol excretion in the bile and ultimately in the feces may not be required for the antiatherosclerotic effect of macrophage RCT, there is nevertheless substantial interest in understanding the mechanisms that regulate excretion of cholesterol into the bile, especially after the discovery of several transporters involved in this process. A body of evidence suggests that HDL-derived cholesterol may be more directly shunted toward the bile than other pools of hepatic cholesterol. CETP expression in mice results in reduced HDL-C levels. HDL-UC can be directly excreted into the bile or converted to bile acids (the rate-limiting enzyme for bile acid synthesis is 7α-hydroxylase) before biliary excretion. HDL-CE requires hydrolysis to UC before being able to be excreted or converted to bile acids. ABCG5 and ABCG8 are half-transporters that work together as heterodimers at the apical membranes of hepatocytes to promote the transport of cholesterol (and other sterols, such as plant sterols) into the bile. Of note, hepatic ABCG5 and ABCG8 are also upregulated by LXR. Overexpression of ABCG5 and ABCG8 in mice promotes biliary cholesterol secretion. A genetic deficiency of ABCG5 or ABCG8 causes sitosterolemia, which is characterized by reduced biliary sterol excretion and elevated plasma and tissue cholesterol and plant sterol levels. In an analogous fashion, ABCB11 (also known as the bile salt export pump) transports bile acids from the hepatocyte apical membrane into the bile. Thus, these hepatic transporters could be considered a part of the RCT pathway (the Figure). However, their effects on the overall rate of RCT, and specifically macrophage RCT, are unknown.

The Intestine and RCT: More Important Than Previously Appreciated?

It is well established that bile acids are avidly reabsorbed in the terminal ileum via the intestinal bile acid transporter. Biliary cholesterol can also be reabsorbed from the intestinal lumen, and between 50% and 80% of luminal cholesterol is reabsorbed. The mechanisms of cholesterol absorption are still being worked out, aided in part by the discovery of ezetimibe, a small molecule that specifically inhibits intestinal cholesterol absorption. Recent data suggest that a key
molecule in intestinal cholesterol absorption is Niemann-Pick C1-like 183,84 and that this molecule is the direct target of ezetimibe.85 However, other molecules may also be involved in cholesterol absorption, and the detailed molecular mechanisms have yet to be fully clarified. Once imported from the intestinal lumen into the enterocyte, cholesterol may be packaged into chylomicrons or, as noted earlier, effluxed via ABCA1 to lipid-free apoA-I. Importantly, ABCG5 and ABCG8 are also expressed by intestinal epithelial cells and promote the apical transport of enterocyte cholesterol back into the intestinal lumen, thus directly influencing the efficiency of cholesterol absorption. Overexpression of ABCG5 and ABCG8 in mice reduces intestinal cholesterol absorption.79 Thus, ABCG5 and ABCG8 influence the RCT pathway not only in the liver but also in the intestine.

As mentioned earlier, the classic RCT pathway includes the delivery of HDL-C to the liver with excretion into the bile. However, studies in rodents suggest that the intestine may be responsible for net cholesterol secretion.82,86 Furthermore, recent data suggest that HDL may directly transfer cholesterol from the plasma compartment to the intestine, with the potential for direct excretion of this cholesterol into the intestinal lumen.82,87 The direct transfer of HDL-C to the intestinal lumen via the intestine, thus bypassing the liver, would be a major revision of the classic RCT paradigm that “forces” all RCT through the liver into the bile.

Integrated Measures of Macrophage RCT In Vivo

Attempts have been made to measure integrated RCT in animal models. Methods have included administration of tritiated water to measure the rate of peripheral cholesterol synthesis and to infer, in steady-state, peripheral cholesterol efflux, as well as the quantification of bile and fecal sterol excretion. These studies have been performed in animal models that have been selectively engineered in single steps of the RCT pathway. For example, studies in mice in which the expression of apoA-I, ABCA1, LCAT, CETP, SR-BI, and 7α-hydroxylase have been genetically altered have generally failed to demonstrate effects on net “reverse cholesterol” flux from the periphery to the liver or on fecal sterol excretion.26,88–90 Only acute injection of reconstituted HDL particles containing apoA-I and phospholipids was shown to result in increased efflux of cholesterol from peripheral tissues (but not increased fecal sterol excretion).90 In humans, a single infusion of pro-apoA-I into 4 patients acutely increased fecal sterol excretion by 30%,91 but inhibition of CETP for 4 weeks, although it raised HDL-C levels, had no effect on macrophage cholesterol efflux in vitro,100,101 increases macrophage RCT in vivo,95 and reduces atherosclerosis in mice.102 Although some LXR agonists have resulted in hepatic steatosis and increased plasma triglyceride and LDL-C in animal models,103,104 there is still hope that this approach will be tested in humans and may prove ultimately safe and effective. In addition, there have been reports that synthetic agonists of PPAR-α, PPAR-γ, and possibly PPAR-β/δ may promote macrophage cholesterol efflux,32,33,105 and thus, existing drugs (fibrates, thiazolidinediones) and new compounds under development in this area may be another way to promote macrophage RCT. Whether inhibition of CETP71,106 or promotion of LCAT activity or hepatic SR-BI
expression will be viable therapeutic approaches to increase macrophage RCT and retard or regress atherosclerosis has yet to be determined. As we learn more about the molecular regulation of macrophage cholesterol efflux and RCT, there will undoubtedly be additional targets for the development of new therapies. Thus, although there have been many twists and turns in the understanding of the RCT pathway as originally proposed by Glomset, it remains a tantalizing target for the development of novel therapies that potentially may afford the best opportunity to regress atherosclerosis.

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