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“Who’s on First”: Determining the Roster for the Key Players in the Reverse Cholesterol Transport Pathway

A.T. Remaley^{1,2}

¹Lipoprotein Metabolism Section, Cardiopulmonary Branch, NHLBI, National Institutes of Health, Bethesda MD

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In 1938, the comedy duo Abbot and Costello performed for the first time their now classic skit “Who’s on First”, by amusing and confusing their audience about the roster of a baseball team. In this issue of *Atherosclerosis* (see page ?), Ying Zhao and colleagues from the laboratory of Dr. Miranda Van Eck describe an interesting study that thankfully does not confuse but instead begins to clarify the roster of the key players in the Reverse Cholesterol Transport (RCT) Pathway, as cholesterol goes around the bases on its journey from the peripheral cell to the liver and eventually the stool.

The key findings of the study are summarized in Figure 1. Using a series of elegant assays and methods developed over the years by researchers in the HDL field, they interrogate the role of the ATP-binding cassette transporter A1 (ABCA1) and the scavenger receptor class B type I receptor (SR-BI) in the various steps of the RCT pathway (1). This was done by comparing 4 lines of mice, namely C57BL/6 wild type mice, ABCA1 Ko mice, SR-BI Ko mice, and double Ko (DKo) (ABCA1 Ko × SR-BI Ko) mice. As previously described, ABCA1 Ko mice had extremely low levels of HDL-C, because of the role of ABCA1 in HDL biogenesis (2). In contrast, SR-BI Ko mice had increased HDL-C, because of its role in the hepatic uptake of cholesterol from HDL in one of the last steps of the RCT pathway (3,4). Not surprisingly, DKO mice had levels of HDL-C similar to those of ABCA1 Ko mice, showing that the absence of ABCA1 trumps the effect from missing SR-BI on HDL-C levels. A similar pattern was observed with non-HDL-C. Both ABCA1 Ko and DKO mice had very low levels of non-HDL-C, whereas it was increased in SR-BI Ko mice. This is again consistent with the known role of SR-BI in clearing plasma lipids not only from HDL but also other lipoproteins (5). ABCA1 Ko mice have decreased non-HDL-C, because a significant fraction of cholesterol on these apoB-containing lipoproteins is originally transferred from HDL (6). This is supported by the observation, in this study, that the absence of ABCA1 actually increased the production of non-HDL lipoproteins, possibly by a mechanism related to cell signaling by pre-beta HDL that is not fully understood (7, 8). Hepatic receptors for apoB-containing lipoproteins were largely unchanged at the protein

²Corresponding Author: Alan T. Remaley, MD, PhD, National Institutes of Health, Bldg. 10, Room 2C-433, 10 Center Drive, Bethesda, MD 20892-1508, USA, Phone: +1-301-402-9796, Fax: +1-301-402-1885, aremaleyl@cc.nih.gov.

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level in the single Ko mice but in the DKO mice, LDL-receptor was decreased and LRP-1 was increased.

As expected from previous studies (9), the plasma clearance of radiolabeled cholesteryl ether on HDL was considerably delayed in SR-BI Ko mice (Fig. 1), but interestingly it was accelerated in ABCA1 Ko mice, although much of the uptake was into extra-hepatic tissue. The two opposing effects were nearly cancelled out in the DKO mice, which had only a slight decrease in HDL associated cholesteryl ether clearance compared to WT mice. Despite the large differences in hepatic cholesterol uptake among the different lines of mice, there was no significant difference in the rate of biliary secretion of cholesterol or bile salts or in the mass of fecal excretion of cholesterol and bile salts in the different mice. This was especially surprising given the results of the macrophage-RCT assay performed with radiolabeled WT macrophages introduced into the peritoneal space of the 3 different Ko mice. All the mice showed a similar 25% decrease when compared to WT mice in the amount of the radiotracer excreted into the stool (Fig. 1). Interestingly, fecal cholesterol excretion was not more affected, particularly in the DKO mice with low HDL and absent SR-BI, which suggests that alternative cholesterol acceptors, such as non-HDL and alternative pathways, such as the Transintestinal Cholesterol Excretion (TICE) pathway (10), can also promote the fecal excretion of sterols. Studies involving the ligation and or diversion of the bile duct in different genetic mouse models have confirmed the existence of this liver bypass pathway for cholesterol excretion (11), but the relative importance of TICE versus biliary secretion in cholesterol balance is not known. The discordance between the mass assays for the fecal loss of sterols and the results for the radiotracer in the stool from the macrophage-RCT assay also indicates that the global flux of cholesterol may not necessarily correlate with the flux from specific cell types, such as macrophages, which represent only a relatively small pool size of cholesterol. It is also important to note that although conditions that favor an increase in the loss of fecal sterols most likely correlate with atheroprotection, in some cases a redistribution of cholesterol with no net loss of fecal cholesterol may still result in decrease plaque formation or regression given the relatively small pool size of cholesterol in atherosclerotic lesions.

Perhaps the most interesting part of this study was the effect of ABCA1 and SR-BI deletion on tissue cholesterol accumulation. No major differences were observed in the lipid content of all the major organs and tissues tested. This also includes the aorta in which no increase in lipid accumulation or atherosclerosis was observed in any of the 3 Ko mice lines when compared to WT mice. The only increase in tissue lipids was in the lung and in Peyer's patches of the intestine where macrophages were found to contain abundant Oil Red-O positive lipid droplets, thus resembling foam cells found in atherosclerotic plaques. No lipid accumulation was found in macrophages from SR-BI Ko mice, but macrophages from the lung and Peyer's patches of DKO mice had more lipid accumulation than ABCA1 Ko mice. Although SR-BI is not thought to play a major role in cholesterol efflux, it is expressed on some peripheral cells, such as macrophages and is thought to promote the bidirectional exchange of cholesterol (1). Defects in the human ABCA1 gene results in Tangier disease in which increased neutral lipids, such as cholesteryl esters, accumulate in macrophages in various organs of the body but predominantly in lymphoid tissue (6). These patients may have some slight increased risk of atherosclerosis (6), but this is an inconsistent finding unlike patients with Familial Hypercholesterolemia and defective LDL-receptors. Interestingly, Tangier patients also do not typically develop xanthomas, which like atherosclerosis is largely driven by the deposition of excess lipid from non-HDL but into the macrophages of the skin instead of the vessel wall. Besides having low levels of HDL-C, Tangier disease patients often also have low non-HDL-C like the mice in this study. This suggests that although HDL is may be beneficial in preventing atherosclerosis by promoting RCT and in some of its other actions, atherosclerosis, however, will not likely develop in

patients with low HDL unless the threshold of LDL-C that is needed to initiate atherosclerosis is exceeded. A similar argument has been made for explaining why LCAT-deficient patients with low HDL-C and low LDL-C also do not appear to be at a greatly increased risk for cardiovascular disease (6).

In not from excess LDL, what then is the source of the cholesterol that accumulates in the macrophages of Tangier disease patients and in the ABCA1 Ko and DKo mice in this study? One possibility that was investigated in this study was that the phagocytosis of cellular membranes by macrophages could be the source. Recently, it has been shown that dyslipidemia, particularly when associated with an increase in free cholesterol (12), leads to the enrichment of cholesterol on various types of cells in the blood, such as red blood cells and platelets (Fig. 1). This interferes with their function and accelerates their clearance (12, 13), particularly by macrophages in the spleen, leading to lipid accumulation and also extramedullary hematopoiesis, which accounts for the splenomegaly observed in SR-BI Ko mice. Although the ABCA1 Ko and DKo mice also had increased spleen size, they did not have extramedullary hematopoiesis and had a normal red cell production rate based on reticulocyte counts. The fact that DKo had very low plasma cholesterol levels like the single ABCA1 Ko mice probably prevented the accumulation of cholesterol on blood cells even in the presence of low HDL. Another possibility not explored in this paper is that the decrease in cholesterol efflux from the low HDL in the ABCA1 Ko and DKo mice altered the rate of cellular apoptosis or proliferation of lymphocytes or monocytes and thus the turnover of cell membranes in lymphoid tissue. It was recently shown in an interesting study from the laboratory of Dr. Alan Tall that ABCA1 Ko mice crossed with mice defective in the ABCG1 transporter, another key transporter for cellular efflux (14), have a generalized leukocytosis because of proliferation from increased stimulation of the GM-CSF and IL-3 receptors (15). The Beta-subunit of the GM-CSF receptor, which is also shared with the IL-3 receptor, was found to be enriched in the plasma membrane of cholesterol loaded cells missing the ABCA1 and ABCG1 transporters, which could account for the increased sensitivity of these cells to GM-CSF and IL-3. In addition, it has been shown that deficiency of ABCA1 and ABCG1 also leads to an increase in the apoptosis of macrophages, during efferocytosis of cell membranes, which could also contribute to lipid accumulation in the surviving macrophages in lymphoid tissue (16). Thus, although there appears to be insufficient levels of Non-HDL-C to infiltrate into the vessel wall and initiate atherosclerosis in the ABCA1 Ko $\times$ SR-BI Ko mice in this study, an increase in the uptake of exogenous cholesterol from the turnover of cell membranes in lymphoid tissue could possibly explain the conundrum of foam cells in these mice without atherosclerosis. Pulmonary macrophages may also be particularly predisposed to lipid accumulation, because of their role in the uptake of lipids in surfactants. This has been proposed to be one of the causes of lipid accumulation in Pulmonary Alveolar Proteinosis, which can be caused by autoantibodies to GM-CSF, leading to decreased ABCG1 expression in pulmonary macrophages (17, 18). Consistent with this hypothesis, one of the main features of ABCG1 Ko mice is the massive accumulation of neutral lipids in lung macrophages (14).

One of the most entertaining aspects of the “Who’s on First” skit by Abbot and Costello is that they did it differently almost every time, because it was so confusing, but it invariably ends the same way when the exasperated Costello says, “I don’t give a darn”. Thanks to the findings in this paper and other recent work on the RCT pathway, the exasperated researchers in the HDL field are finally starting to understand the key players in the RCT pathway, which will hopefully lead someday to new therapeutic and diagnostic interventions for cardiovascular disease.

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Abbreviations

HDL	High density lipoproteins
LDL	Low density lipoproteins
ABCA1	ATP Binding Cassette Transporter A1
SR-BI	Scavenger Receptor B1

Figure 1. Diagram of RCT pathway and effect of ABCA1 and SR-BI

Arrows indicate relative magnitude and direction for the effect observed for the indicated lines of mice when compared to control WT mice.