TISSUE-SPECIFIC ROLE OF ATP BINDING CASSETTE TRANSPORTER A1 IN ATHEROGENESIS

By

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<tbody>
<tr>
<td>ABC</td>
<td>ATP-binding cassette transporter</td>
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<tr>
<td>TD</td>
<td>Tangier disease</td>
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<td>TPC</td>
<td>Total plasma cholesterol</td>
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<td>FC</td>
<td>Free cholesterol</td>
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<td>TG</td>
<td>Triglyceride</td>
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<td>CE</td>
<td>Cholesteryl ester</td>
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<td>PL</td>
<td>Phospholipid</td>
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<tr>
<td>ApoB Lp</td>
<td>ApoB containing lipoproteins</td>
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<tr>
<td>HSKO</td>
<td>Hepatocyte-specific ABCA1 knockout</td>
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<td>MSKO</td>
<td>Myeloid-specific ABCA1 knockout</td>
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<tr>
<td>PM</td>
<td>Peritoneal macrophages</td>
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<td>BMM</td>
<td>Bone marrow-derived macrophages</td>
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<tr>
<td>LXR</td>
<td>Liver X receptor</td>
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<tr>
<td>RXR</td>
<td>Retinoid X receptor</td>
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<tr>
<td>CVD</td>
<td>Cardiovascular disease</td>
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<td>CHD</td>
<td>Coronary heart disease</td>
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<td>RCT</td>
<td>Reverse cholesterol transport</td>
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<td>CM</td>
<td>Chylomicron</td>
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<td>VLDL</td>
<td>Very low density lipoprotein</td>
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<td>IDL</td>
<td>Intermediate density lipoprotein</td>
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<td>LDL</td>
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<td>HDL</td>
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<td>--------------</td>
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<tr>
<td>ApoE KO</td>
<td>Apolipoprotein E knockout</td>
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<td>LDLrKO</td>
<td>LDL receptor knockout</td>
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<tr>
<td>ApoA-I</td>
<td>Apolipoprotein A-I</td>
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<td>LFA-I</td>
<td>Lipid free apolipoprotein A-I</td>
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<tr>
<td>PLTP</td>
<td>Phospholipid transfer lipoprotein</td>
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<tr>
<td>LCAT</td>
<td>Lecithin: cholesterol acyltransferase</td>
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<td>SR-BI</td>
<td>Scavenger receptor class BI</td>
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<tr>
<td>CETP</td>
<td>Cholesteryl ester transfer protein</td>
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<td>HL</td>
<td>Hepatic lipase</td>
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<td>EL</td>
<td>Endothelial lipase</td>
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<td>BMT</td>
<td>Bone marrow transplantation</td>
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ABSTRACT

Bi, Xin
Tissue-specific Role of ATP Binding Cassette Transporter A1 in Atherogenesis

Dissertation under the direction of
John S. Parks, Ph.D., Professor of Pathology and Biochemistry

ATP binding cassette transporter A1 (ABCA1) facilitates cellular free cholesterol and phospholipid efflux across the plasma membrane to combine with apolipoproteins, forming nascent high density lipoproteins (HDL), the precursor of mature plasma HDL. Mutations in ABCA1 cause Tangier disease, characterized by extremely low plasma HDL cholesterol (HDL-C) levels and widespread storage of cholesterol in macrophages. We developed hepatocyte- and macrophage-specific ABCA1 knockout mice (HSKO and MSKO, respectively) and crossed them into the atherogenic low density lipoprotein receptor knockout (LDLrKO) background to study their roles in atherogenesis.

We hypothesized that hepatocyte ABCA1 is atheroprotective by maintaining plasma HDL-C concentrations. Despite a greatly diminished HDL pool, atherogenic diet-fed HSKO/LDLrKO mice were equally susceptible to atherosclerosis as LDLrKO mice due to a paradoxical decrease in atherogenic plasma apoB lipoproteins (apoB Lp) and the maintenance of reverse cholesterol transport (RCT) from macrophages to the liver for efficient fecal cholesterol excretion. Additional in vitro cholesterol efflux studies suggested that HDL-C levels, per se, were not the primary determinant of plasma efflux capacity. Our
data are also compatible with the concept that hepatic ABCA1 may normally recycle a significant proportion of plasma HDL-C taken up by the liver back into circulation for maintenance of plasma HDL-C pool.

We hypothesized that macrophage ABCA1 protects against atherosclerosis by promoting macrophage cholesterol efflux. We observed a novel role for macrophage ABCA1 in regulating hepatic apoB Lp secretion and plasma apoB Lp levels in atherogenic diet-fed LDLrKO mice, with lower plasma apoB Lp in MSKO/LDLrKO vs. LDLrKO mice offsetting increased macrophage cholesterol accumulation and inflammatory response, leading to minimal impact on atherosclerosis. When differences in apoB Lp levels were minimized (i.e., chow diet), MSKO/LDLrKO mice had significantly higher atherosclerosis compared to LDLrKO mice, suggesting macrophage ABCA1 is atheroprotective when differences in apoB Lp are minimal.

In summary, important new roles were discovered for hepatocyte and macrophage ABCA1 in regulating apoB Lp metabolism in hyperlipidemic LDLrKO mice. Our results suggest careful consideration must be given to therapeutic interventions that affect ABCA1 expression to limit heart disease as these may have paradoxical tissue-specific effects on apoB Lp metabolism that may oppose atheroprotection.
Chapter I

INTRODUCTION

Xin Bi prepared this chapter. Dr. John Parks acted in an advisory and editorial capacity.
1. Cardiovascular Disease

1.1 Prevalence of CVD

Atherosclerosis-associated cardiovascular disease (CVD), mainly coronary heart disease (CHD) and stroke, is the leading cause of death in westernized societies [1]. The overall rate of death attributable to CVD was 236.1 per 100000 in 2009. More than 33% American adults have one or more types of CVD and it is estimated that 41% of the US population will have some form of CVD by 2030 [2, 3]. Studies that lead to a greater understanding of the underlying pathogenesis of CVD will likely help with development of better strategies for treatment.

1.2. Atherosclerosis

1.2.1. Pathogenesis of atherosclerosis

Atherosclerosis is a chronic disease characterized by lipid accumulation and fibrous elements with subsequent progressive blockage and hardening of large and medium-sized arteries [4]. Typically, early lesions consist of subendothelial cholesterol-loaded macrophage foam cells, whereas more advanced plaques contain necrotic cores and fibrous caps [5]. The etiology of atherosclerosis remains unclear, but increasing knowledge has been gained with regard to its pathogenesis. Epidemiological studies and clinical trials support a positive association between hyperlipidemia and atherogenesis as well as the beneficial effects of cholesterol reduction [5, 6]. Indeed, elevated apoB lipoproteins (apoB Lp) levels are recognized as the primary risk factor for atherosclerosis and as a prerequisite for disease development [6]. Conversely, plasma HDL is inversely
associated with CVD risk, most likely by stimulating reverse cholesterol transport (RCT), a process by which cholesterol is effluxed from peripheral cells and transported back to the liver for excretion [7, 8]. Chronic inflammation is also a key feature of atherosclerosis disease, which may be instigated by arterial retention and modification of plasma apoB Lp [6]. Oxidized lipids from apoB Lp activate endothelial cells, resulting in secretion of cytokines and chemokines that attract circulating monocytes to atherosclerotic lesions. Monocytes then differentiate into macrophages that take up modified or proteoglycan-trapped apoB Lp in an unregulated manner, ultimately resulting in cholesteryl ester (CE) enriched foam cells, the hallmark of atherosclerotic plaques. If this process continues unabated, macrophage necrosis and plaque rupture can lead to an acute ischemic event [9].

1.2.2. Atherosclerotic lesion development
One of the hallmarks of early atherosclerosis is the formation of cholesterol-laden macrophage foam cells, which is the predominant cell type in lesions [5, 9]. Circulating low density lipoproteins (LDL) enter into the arterial intima and are retained through binding to proteoglycans [10]. Retained LDL undergoes modification to become minimally-modified LDL (mm LDL) or extensively oxidized LDL (ox LDL), which can activate endothelial cells to express adhesion molecules and secrete chemokines, attracting circulating monocytes to migrate and attach to the inflamed endothelium with subsequent transmigration into the intima. Once activated, monocytes undergo differentiation into macrophages.
The proteoglycan-retained lipoproteins are ingested by macrophages via scavenger receptors (SR) in an unregulated manner. This leads to lipid accumulation in the cytoplasm and activation of liver X receptor (LXR), resulting in upregulation of a group of genes involved in cholesterol efflux, including ABCA1. When uptake of modified LDL particles exceeds the ability of macrophages to efflux the cholesterol, the excess FC is esterified by ACAT1 to form CE, resulting in deposition of intracellular CE droplets that give macrophages their foamy appearance [6]. If this process continues unabated, apoptosis and/or impaired efferocytosis will occur, leading to more advanced and vulnerable plaques [11]. Macrophage apoptosis in advanced lesions is proposed to be regulated by several mechanisms such as growth factor deprivation and the presence of toxic cytokines and oxidized lipids or lipoproteins [12]. However, currently little in vivo evidence exists for these hypotheses. Increasing in vivo evidence supports the involvement of ER stress while in vitro studies suggest potential ER stressors including excess lipoprotein-derived cholesterol accumulation [11, 13]. Phagocytic clearance of apoptotic cells (i.e., efferocytosis) before they undergo secondary necrosis plays a critical role in the resolution of inflammation. In chronic atherosclerosis progression, most apoptotic cells cleared are macrophages and defective efferocytosis in advanced lesions is a major cause of necrotic core formation, which can lead to vulnerable plaque formation [11]. Eventually, shortage of blood supply to important organs and sudden rupture of vulnerable plaques with subsequent acute occlusion give rise to life-threatening clinical events, such as myocardial infarction and stroke.
1.2.3. Mouse Models of Atherosclerosis

Mice are currently the most widely used *in vivo* model for atherosclerosis research. There are three major mouse models for atherosclerosis evaluation that have been developed since chow diet-fed wild type mice do not develop significant atherosclerosis due to low levels of plasma apoB Lp. The first model developed by Beverly Paigen’s lab consists of feeding C57BL/6 mice a diet (i.e., Paigen diet) containing fat (15%), cholesterol (1.25%) and cholate (0.5% cholic acid) [14], which results in small lesions in the aortic root region of the heart, with minimal cholesterol accumulation in the aorta [15]. With the advent of gene targeting in the late 1980’s, two mouse models were developed that displayed hyperlipidemia while consuming chow. One model consists of targeted deletion of apolipoprotein E (ApoE KO), the primary apolipoprotein required for hepatic uptake of apoB Lp, particularly intestinal derived Lp particles (i.e, chylomicron and VLDL). apoE KO mice have elevated TPC concentrations (~500 mg/dl) while consuming chow that is due to plasma accumulation of chylomicron and VLDL remnants. As a result, atherosclerosis develops in chow-fed mice, with more advanced lesions developing in apoE KO mice fed a western-type diet (WTD). Another mouse model of hyperlipidemia and atherosclerosis was developed by targeted deletion of the low density lipoprotein receptor (i.e., LDLrKO), resulting in delayed clearance of plasma LDL and elevated TPC concentrations (~250 mg/dl) in chow-fed mice. Feeding LDLrKO mice a WTD results in very high plasma TPC levels (~1500 mg/dl) and considerable atherosclerosis development.
over a 16 week period. Many studies of atherosclerosis modifying genes have been conducted using the apoE and/or LDLrKO mouse backgrounds [16].

2. Lipid and Lipoprotein Metabolism

2.1. Introduction to Lipids and Lipoproteins

Plasma lipids are transported by lipoproteins, which are composed of apolipoproteins, neutral lipids and polar lipids. The main neutral lipids are triglycerides (TG) and CE. Free cholesterol (FC) and phospholipids, mainly phosphatidylcholine (PC) and sphingomyelin, comprise of the polar lipids in lipoproteins [17]. Based on the densities at which they are isolated, lipoproteins are identified as four major classes: chylomicrons (CM, d<0.93 g/ml), very low density lipoprotein (VLDL, d=0.95-1.006 g/ml), low density lipoprotein (LDL, d=1.019-1.063 g/ml), and high density lipoprotein (HDL, d=1.063-1.21 g/ml). Intermediate density lipoproteins (IDL, d=1.006-1.019 g/ml) are VLDL remnants resulting from VLDL TG hydrolysis by lipoprotein lipase (LPL) and are usually not detectable in the circulation due to rapid removal from plasma or conversion to LDL [18].

2.2. ApoB Containing Lipoprotein Metabolism

2.2.1. ApoB-containing lipoproteins

Chylomicron, VLDL, IDL, and LDL are referred to apoB LP, due to the presence of a common structural component, apolipoprotein B (apoB). ApoB, a nonexchangeable apolipoprotein, is required for the synthesis of TG-rich
lipoproteins in the liver and intestine [19]. ApoB exists in two isoforms, apoB-100 and apoB-48. ApoB-100, an essential apolipoprotein for VLDL formation in the liver, contains 4536 amino acids, whereas apoB-48, the essential apolipoprotein for CM formation, consists of the amino terminal 2152 amino acids of apoB-100, resulting from a unique APOBEC-1 mRNA-editing mechanism [20]. In humans, apoB synthesized in the intestine is exclusively apoB-48 due to expression of APOBEC-1, whereas human liver only produces apoB-100 due to the absence of APOBEC-1. Some mammals, including mice, produce hepatic apoB-48 due to expression of APOBEC-1 [21]. Plasma apoB-100 levels are an important risk factor for CVD risk [22].

2.2.2. Chylomicrons

Chylomicrons are the largest lipoproteins with sizes ranging from 100-1,000 nm. They are present in postprandial plasma and stay at the origin on paper and gel electrophoretograms due to their large diameter [18]. CM are synthesized in the intestine and secreted into lymph to transport dietary-derived lipids from the site of intestinal absorption to a variety of cells in the body. Chylomicrons are rich in TG, but also contain substantial amounts of cholesterol in both the unesterified and esterified form. LPL hydrolyzes CM TG to non-esterified fatty acids (NEFAs) at the endothelial cell surface in peripheral tissues for energy storage (adipose tissue) or utilization (muscle) [23]. The residual CE-rich, TG-depleted lipoprotein particles, referred to as chylomicron remnants, are liberated back into the plasma
compartment and rapidly cleared by the liver through chylomicron remnant receptors, LDLr and LDL receptor related protein (LRP) [24, 25].

2.2.3. VLDL, IDL and LDL

VLDL particles (30-90 nm in diameter) are assembled and secreted by hepatocytes and to a lesser extent by intestinal epithelial cells in the fasting state. VLDL are also enriched in TG and function to distribute fatty acids to different tissues through hydrolysis of TG by LPL. Gradual depletion of TG in VLDL gives rise to serial conversion to IDL and ultimately LDL (~ 20 nm in diameter), which is the major cholesterol transporting lipoprotein. LDL is catabolized via binding to LDLr on the cell surface of the liver and extrahepatic tissues [17].

2.3. High Density Lipoprotein Metabolism

2.3.1. HDL Heterogeneity

HDLs are the most dense plasma lipoproteins, are polydisperse, and composed of discrete subfractions that differ in size, density and chemical composition. Therefore, HDLs can be further classified into subpopulations based on size, density, apolipoprotein content and electrophoretic mobility. HDLs were separated by gradient gel electrophoresis into HDL_{2b}, 2a, 3a, 3b and 3c [26]. HDLs can also be fractionated based on apolipoprotein content using immunoaffinity chromatography into HDLs contain apoA-I but no apoA-II (LpA-I) and HDLs contain both apoA-I and apoA-II (LpA-I/A-II). According to agarose gel electrophoretic mobility, HDLs can also be separated into pre-β HDL and α-
migrating HDL. Pre-β HDL is a mixture of lipid-free apoA-I (LFA-I), lipid-poor apoA-I containing a few lipid molecules, and nascent discoidal HDL containing no neutral lipid core, whereas α-migrating HDL are spherical particles containing a neutral lipid core. Pre-β HDL is a minor fraction in plasma accounting for about 5-10% HDL particles [27]; however, emerging evidence suggests that pre-β HDL may serve as preferential acceptors for FC efflux [28]. Despite multiple analytical and preparative procedures to isolate and investigate HDL subfractions, the origin of the particle heterogeneity is poorly understood.

2.3.2. HDL biosynthesis

HDL biogenesis is a complex process involving the synthesis and secretion of the major HDL-associated apolipoproteins, followed by extracellular lipidation, nascent HDL (nHDL) particle formation, and subsequent maturation of nascent HDL to mature spherical plasma HDL in plasma. ApoA-I is synthesized in the liver and intestine, comprises ~70% of total protein in HDL, and is present on essentially all HDL particles [29]. ApoA-II is also synthesized in the liver, constitutes ~20% of total HDL protein, and is present on two-thirds of human plasma HDL particles [30]. Plasma HDL-C levels are reduced in mice and humans lacking apoA-I or apoA-II, suggesting both apoA-I and apoA-II are required to maintain plasma HDL levels. [30-32]. Secreted HDL apolipoproteins must undergo lipid acquisition to form HDL particles. Recent work suggests that HDL apolipoprotein lipidation occurs predominantly via ATP binding cassette transporter A1 (ABCA1) at the cell membrane surface [33]. Much of the HDL lipid
mass, particularly PL, can also be derive from other lipoproteins. Hydrolysis of TG in TG-rich lipoproteins by LPL results in release of surface PLs, which are acquired by HDL. In addition, PL transfer protein (PLTP) transfers PLs from apoB Lp to HDL [34]. After initial formation via ABCA1, nHDLs undergo maturation in plasma via lecithin: cholesterol acyltransferase (LCAT) mediated esterification of FC to CE, generating a neutral lipid core [35-38].

2.3.3. HDL catabolism
2.3.3.1. HDL cholesterol catabolism

The liver is the primary site of HDL cholesterol uptake from plasma. Scavenger receptor class BI (SR-BI)-mediated selective HDL CE uptake in the liver, without degrading HDL apolipoproteins, is the best investigated mechanism. In vitro studies show that hepatocyte SR-BI mediated internalization of whole HDL particles results in cholesterol removal and resecretion of cholesterol-depleted HDL [39]. In vivo studies using rodent models with SR-BI deletion or overexpression also suggest it regulates hepatic HDL uptake and plays a major role in controlling plasma HDL cholesterol (HDL-C) levels [40-42]. In humans and animal species expressing CE transfer protein (CETP), an alternative pathway for HDL cholesterol catabolism has been described. CETP exchanges TG from apoB LP for CE from HDL, leading to TG enrichment and CE depletion in HDL particles. Individuals with loss of function mutations in the CETP gene have high HDL-C levels, whereas in rodents, which normally lack CETP, overexpression of CETP results in significant reductions in HDL-C [43, 44].
2.3.3.2. HDL apoA-I catabolism

The importance of the liver and kidney in apoA-I catabolism was established from animal studies. More than half of plasma apoA-I is catabolized by the liver, whereas kidneys catabolize approximately one-third of plasma apoA-I [45]. These sites are also assumed to mediate apoA-I catabolism in humans, although this has never been formally investigated. The underlying mechanisms for apoA-I catabolism in the kidney are better understood than that of the liver. Cubilin, an extracellular protein localized to the apical surface of proximal renal tubular cells, binds apoA-I and HDL and interacts with a megalin coreceptor for apoA-I uptake and degradation [46-49]. Small, poorly lipidated apoA-I particles are primarily catabolized by the kidney, presumably because they are small enough to transverse the glomerular filtration system. For example, humans and mice deficient in ABCA1 have smaller plasma HDL particles that exhibit more rapid plasma decay and increased catabolism by the kidney [50, 51]. HDL modification by lipolytic enzymes is also an important determinant of apoA-I catabolism rate. Hepatic lipase (HL) is the best understood enzyme regarding HDL metabolism. It is capable of hydrolyzing both HDL TG and PL. HL plays an important role in lipolysis of TG-enriched HDL that results from CETP-mediated CE for TG exchange [29]. The resultant shedding of lipid-poor apoA-I gives rise to increased renal catabolism. Endothelial lipase (EL) has recently been shown to affect HDL concentrations. Compared to HL, EL has more PL than TG lipase activity and has a preference for HDL over apoB Lp [52]. EL overexpression and absence is associated with decreased and increased HDL-C and apoA-I levels, respectively,
in rodents and human subjects [52, 53]. A substantial amount of apoA-I degradation can be attributed to the liver in animals [45]; however, the mechanism is unclear. One proposed hepatic uptake pathway is that apoE-enriched HDL binds to LDLr and LRP through interaction with apoE on the HDL particles [54]. However, since most plasma HDL particles do not contain apoE, there must be additional pathways for HDL whole particle uptake by the liver. A potential mechanism is that binding of apoA-I to an ecto-F1-ATPase stimulate the production of extracellular ADP that activates a P2Y13-mediated HDL endocytosis [55].

2.3.4. HDL and Atherosclerosis

Epidemiological studies in the 1970’s documented an inverse relationship between HDL-C levels and coronary heart disease (CHD) [56-59]. HDL-C has been used as a risk factor by clinicians to evaluate cardiovascular disease (CVD) risk since the 1980’s. While statins have been successful in reducing CVD events by 20%-40%, there is still a large residual disease burden in the US population [7]. More recently, HDL-C levels were shown to be an independent predictor of CVD event risk in patients treated with statins, and baseline HDL levels were a strong predictor of one year morbidity and mortality of CVD events in patients on statins with stent placement for acute coronary syndromes (ACS) [60]. Infusion of recombinant HDL in humans and transgenic overexpression of apoA-I in mice leads to reduced progression or regression of atherosclerosis [61-63]. The above
observations suggest HDL as a potential therapeutical target to reduce the CVD burden in patients undergoing lipid-lowering therapies.

It is widely believed that low HDL is causally associated with increased atherogenesis based largely on animal studies, even though the question of whether low HDL-C is simply a marker of CVD risk has been under debate over years. The most popular mechanism for the inverse association between HDL-C and CVD risk is thought to be reverse cholesterol transport (RCT), a process in which HDL particles transport excess cholesterol from peripheral tissues back to the liver for secretion into bile with ultimate excretion in the feces [7, 8]. RCT is the only quantitatively important pathway for cholesterol elimination from the body. In fact, emerging evidence supports the concept that HDL cholesterol efflux capacity is a stronger predictor of CVD risk than HDL-C levels [64]. A robust inverse correlation between macrophage RCT and atherosclerosis has been documented in animal studies that is stronger than the correlation between HDL-C and atherosclerosis [65]. A group of proteins has been implicated in macrophage cholesterol efflux, the initial step in macrophage RCT, including ATP-binding cassette transporters ABCA1 and ABCG1, and scavenger receptor class B type I (SR-BI). The pivotal role of ABCA1 in facilitating cholesterol efflux to lipid-free or lipid-poor apoA1 has been shown by in vitro studies, whereas ABCG1 and SR-BI mediate cholesterol efflux to mature HDL [7, 8]. ABCA1 and ABCG1 have also been demonstrated to promote macrophage RCT in vivo and act synergistically [66]. Other proposed antiatherogenic mechanisms for HDL
include protecting plasma LDL from oxidation [67], decreasing inflammation [7], inhibiting hematopoietic stem cell proliferation [68], and improving vascular function [69].

3. ABCA1

3.1. Introduction

The molecular defect of Tangier disease (TD), a rare autosomal recessive disease first discovered in the 1960s [70, 71], became intriguing due to the near absence of plasma HDL in subjects with this disorder. Over a decade ago, several groups independently reported ABCA1 as the genetic defect in TD [72-74]. ABCA1 belongs to the ATP binding cassette transporter A family, which is one of the seven subclasses of the ABC superfamily. It is a 2261 amino acid integral membrane protein that contains two transmembrane domains each with six helices and two intracellular nucleotide binding domains. ABCA1 is predicted to have N and C termini oriented in the cytosol and two extracellular loops that are linked by one or more disulfide bonds [75].

3.2. Tissue Distribution and Regulation

ABCA1 is ubiquitously expressed throughout the body. ABCA1 mRNA was shown to be most abundant in liver, kidney, adrenal, heart, bladder, testis and brain in mice [76], whereas in humans, it is highly expressed in liver, placenta, small intestine and lung [77]. Transcription of ABCA1 is highly regulated by nuclear receptors liver X receptor (LXR)/ retinoid X receptor (RXR) heterodimers
and induced by cellular cholesterol accumulation [78]. LXRαs and RXRs bind oxysterols and retinoic acid, respectively. Binding of either or both ligands is able to activate the transcription of their target genes, which contain LXR/RXR response elements [79]. In human macrophages, the LXRα gene promoter contains a LXR response element suggesting, the positive feed-forward pathway for LXRα expression [80, 81]. The cholesterol taken up by cells accumulates and a small proportion is oxidized to LXR ligands, mainly 22-hydroxycholesterol, 24-hydroxycholesterol, and 24,25-epoxycholesterol in the liver [82, 83]. The presence of sterol 27-hydroxylase (Cyp27), an enzyme involved in oxysterol generation, in various tissues suggests 27-hydroxycholesterol as a major LXR ligand in macrophages and other peripheral cells [84]. A very recent study showed that desmosterol is a more potent LXR ligand in macrophages than oxysterols [85]. ABCA1 expression can also be regulated through non-sterol lipids, such as polyunsaturated fatty acids that antagonize oxysterol binding to LXRα response elements [86, 87]. Another nuclear hormone receptor family, peroxisome proliferator activated receptors (PPARs) also activate LXRα transcription, which in turn enhances ABCA1 transcription [88, 89]. LXR/RXR independent mechanisms also play a role in regulating ABCA1 transcription, though these pathways are not as well-studied as the LXR pathway.

ABCA1 protein expression is discordant with mRNA levels in some tissues, such as heart and kidney, in which protein levels are low despite high mRNA levels. This suggests that posttranscriptional regulation of ABCA1 expression is
important, at least in some tissues. Several studies have suggested the ABCA1 protein expression is also controlled by protein turnover. For example, ABCA1 has a short half-life (<1h) [90] and, in the absence of bound apolipoprotein, ABCA1 is phosphorylated at a specific intracellular PEST sequence, which leads to degradation by calpain protease [91, 92]. In the presence of a bound apolipoprotein, the PEST sequence is dephosphorylated and ABCA1 is stabilized at the membrane surface. Unsaturated fatty acids have also been reported to increase the turnover of ABCA1 protein and this appears to be mediated through phospholipase D mediated phosphorylation of ABCA1 by protein kinase C, resulting in ABCA1 degradation [93, 94]. These studies demonstrate regulation of ABCA1 at both the transcriptional and post-transcriptional level and emphasize the importance of investigating the physiological function of ABCA1 in a cell-specific manner.

3.3. ABCA1 and HDL Cholesterol

The seminal discovery of ABCA1 as the mutant gene in TD uncovered its pivotal role in HDL biogenesis [95]. Indeed the primary function of ABCA1 is to mediate FC and PL transport across cell membranes to lipid-free apolipoproteins. ABCA1 is a membrane protein that recycles rapidly between the plasma membrane and late endosomal/lysosomal compartments [96, 97]. LFA-I lipidation takes place at the cell membrane surface through direct binding to ABCA1 or at an area of the membrane adjacent to ABCA1 with simultaneous addition of PL and FC or addition of PC first, followed by FC [98-101]. Increased lipid acquisition and apo-
AI molecules/particle leads larger nHDL particles. Nascent pre-β HDL are formed simultaneously [102, 103], but the underlying pathways are poorly understood. The nascent HDLs, once formed by ABCA1, are poor substrates for further lipidation via ABCA1 [102, 104, 105]. In vivo mouse studies also support a significant role for ABCA1 in maintaining plasma HDL levels as global deletion of ABCA1 recapitulated the plasma lipid phenotype of TD [106, 107]. However, as discussed previously, ABCA1 is widely expressed across a variety of tissues [76], but apo-AI, the major HDL apolipoprotein, is only produced by the liver and intestine [108]. Thus, a fundamental question is whether HDL biogenesis occurs at sites of apoA-I production (liver and intestine) or in all cell according to cellular ABCA1 expression. This question was addressed with a tissue-specific gene targeting strategy. The liver has been identified as the single most important source of plasma HDL in chow-fed mice (~70-80%), whereas the intestine contributes a lesser amount (~20-30%) [51, 109]. However, macrophage ABCA1 deletion in WT mice had minimal effect on the plasma HDL pool [110-112], whereas adipocyte deletion resulted in a modest (~10-15%), but significant reduction in plasma HDL [113]. Thus, ABCA1 expression in multiple tissues produce nHDL that maintain the plasma HDL pool, but the liver is quantitatively the most important source.

3.4. ABCA1 and ApoB Containing Lipoprotein Metabolism

TD patients also have 50% reduction in plasma apoB Lp concentrations due to a two-fold increase in LDL catabolism compared to normal controls [114]. The
lower LDL concentrations in TD subject may explain why some kindreds have no increase in CHD risk despite <5% of normal HDL levels [32, 70]. A similar plasma lipid profile has been observed in animals with ABCA1 deficiency. Mice deficient in whole-body or hepatocyte ABCA1 also had a 50% reduction in apoB Lp concentration compared with their WT controls [115, 116]. Chung et al [116] demonstrated a two-fold increase in plasma $^{125}$I-LDL tracer removal rate and a two-fold increase in hepatic LDLr expression in the absence of hepatocyte ABCA1 expression, suggesting that increased hepatic LDLr expression was responsible for increased plasma decay of LDL and decrease LDL levels in these mice. Transgenic overexpression of ABCA1 in the liver resulted in increased apoB Lp concentrations and increased atherosclerosis. This study also found decreased apoB Lp turnover that presumably resulted from increased transfer of cholesterol from HDL to LDL, resulting in cholesterol-enriched LDL with a slower turnover rate [117].

3.5. ABCA1 and Inflammation

Although macrophage ABCA1 contributes minimally to the plasma HDL pool, it plays key role in cellular cholesterol homeostasis by facilitating cholesterol efflux [7]. An increasing body of evidence suggests that ABCA1 is a key regulator of macrophage inflammatory responses [112, 118-120]. For example, Koseki et al. showed that LPS-induced activation of NFκB and TNF-α was greater in macrophages from whole body ABCA1 KO mice [119]. Our group reported that macrophages from macrophage-specific ABCA1 knockout (MSKO) mice are
hypersensitive to LPS with enhanced proinflammatory responses. This response was due to a small, but significant, increase in membrane FC and lipid raft content that enhanced inflammatory signaling via Toll-like receptors (TLRs) [112, 121]. Another study by Yvan-Charvet et al. supported this idea [118]. Furthermore, ABCA1 has been implicated in phagocytic clearance of apoptotic cells [122-124]. LDLrKO mice transplanted with bone marrow (BM) cells overexpressing human ABCA1 vs. WT bone marrow had a significantly decreased number of apoptotic cells in atherosclerotic lesions after 12 or 15 weeks of atherogenic diet feeding, which may have resulted from decreased apoptosis and/or more efficient efferocytosis and may partially account for the decreased atherosclerosis in recipient mice receiving human ABCA1 expressing BM [125]. In fact, most recently, a novel inhibition of hematopoietic stem and multipotential progenitor cell (HSPC) proliferation has been uncovered involving ABCA1 and other cholesterol efflux pathways (i.e., ABCG1 and HDL) [68]. Since leukocytosis, monocyteosis and increased monocyte recruitment into lesions are seen under conditions of hypercholesterolemia and blood monocyte count is directly associated with lesion burden, defective regulation of leukocytosis, particularly monocyteosis, may serve as another link between ABCA1 and altered susceptibility to atherosclerosis [126-128]. Therefore, ABCA1 has many properties that could potentially modulate atherogenesis susceptibility at different stages.
4. ABCA1 and Atherosclerosis

4.1. Global ABCA1 expression and atherosclerosis

Given the potential atheroprotective effects of ABCA1, one would anticipate an increase in atherosclerotic CVD events in the absence of ABCA1. However, the relationship between ABCA1 expression and atherosclerosis is more complicated than originally expected. Common genetic variants in ABCA1 have been reported to influence the risk and severity of CVD [129-131]. However, low HDL caused by loss-of-function mutations in ABCA1 does not contribute to increased risk of CVD in the general population [132]. Increased CVD incidence was observed in a subgroup, but not all TD patients [133]. The less striking CVD burden in TD patients is likely due to low LDL levels, as discussed above. Controversial results also exist in animal model studies. Physiological overexpression of a full-length hABCA1 containing bacterial artificial chromosome (BAC) in apoE KO and LDLrKO mice both revealed an atheroprotective role of ABCA1 [134, 135]. Overexpression of human ABCA1 (hABCA1) in the liver and macrophages of B6 mice resulted in an anti-atherogenic lipid profile and lower aortic atherosclerosis, whereas in apoE KO mice, overexpressing hABCA1 increased atherosclerosis with minimal effect on plasma lipids [136]. The conflicting outcomes of ABCA1 transgenic studies may be explained by the use of different promoters for transgenic overexpression or altered atherogenic lipoprotein profiles. In contrast, atherosclerosis severity in LDLrKO or apoE KO mice with whole body ABCA1 deficiency was not significantly different from that of the control mice, likely due to decreased apoB Lp concentrations as discussed above [115].
4.2. Tissue specific role of ABCA1 in atherogenesis

ABCA1 has multiple roles that may impact the pathogenesis of atherosclerosis, including nascent HDL formation, macrophage reverse cholesterol transport, regulation of apoB LP metabolism, cellular inflammatory response, and HPSC proliferation. These functions vary among different cell types, making it difficult to determine the role of ABCA1 in total ABCA1 KO (TKO) mice. Indeed, tissue/cell-specific role of ABCA1 in atherosclerosis and lipid metabolism during atherogenesis is poorly understood. Hepatocyte ABCA1 could be a potential treatment target for reducing CVD risk, given its role in maintaining the plasma HDL pool. However, only two studies have addressed the role of hepatocyte ABCA1 in atherosclerosis development. Hepatic overexpression of human ABCA1 resulted in a paradoxical increase in atherosclerosis in WTD-fed LDLrKO mice compared with non-transgenic controls, likely due to the coincident increase in plasma apoB Lp levels, despite a significant increase in plasma HDL [117]. In contrast, deletion of hepatic ABCA1 accelerated early atherosclerosis development in chow-fed apoE KO mice despite a significant reduction in plasma apoB Lp levels relative to control mice [135]. However, several questions were not addressed in that study. Firstly, only early atherosclerosis was examined (i.e. 12wk chow diet, TPC 250-400 mg/dl). Therefore the effect of hepatocyte deletion of ABCA1 on more advanced atherosclerosis is unknown. Secondly, HDL cholesterol concentrations are low in apoE KO mice (~29 mg/dl). Thus, the difference between apoE KO and HSKO-apoE KO strains may be too small to differentially affect atherosclerosis. Thirdly, atheroprotective roles of apoE at the
level of the arterial wall [137] are absent in the apoE KO background. Finally, in vivo RCT studies were not performed.

While the effect of whole body and hepatocyte ABCA1 expression in atherogenesis is complex, macrophage ABCA1 appears to be a more promising target for intervention due to its critical role in mediating RCT. Several BMT studies have shown that chow or WTD-fed apoE and LDLrKO KO mice transplanted with BM from global ABCA1 KO mice had accelerated atherosclerosis compared to those with WT BM [111, 115, 138, 139]. Another study reported that LDLrKO mice transplanted with BM from human ABCA1 transgenic mice developed smaller lesions when they were fed a WTD for 12 or 15wks, but not for a shorter period of time [125]. Taken together, these studies suggest that leukocyte ABCA1 expression may be atheroprotective in a stage-specific manner, independent of plasma HDL. The fact that BM is comprised of a variety of leukocytes makes it difficult to simply interpret the results of BMT studies as specific to macrophage ABCA1 expression, especially since several of these leukocyte populations (i.e., T and B lymphocytes) have been implicated in atherogenesis [140, 141]. Thus far, only one recent study suggested a minor role for myeloid cell ABCA1 in the development of atherosclerosis [135]. However, the mice used for that study were not fully backcrossed to the C57BL/6 (B6) background and mouse strains have different susceptibility to atherogenesis [142].
Statement of research intent

ABCA1, the molecular defect in Tangier disease, is a critical transporter that mediates cellular FC and PL transport across the plasma membrane to form nascent HDL particles that can be further converted to mature HDL in plasma. The predicted atheroprotective role of ABCA1 based on plasma HDL and macrophage characteristics of TD subjects was challenged by the absence of premature CVD in kindreds of TD patients and the similar extent of atherogenesis in global ABCA1 deficient animals. Due to the ubiquitous expression of ABCA1, the tissue-specific effect of ABCA1 on atherogenesis has been difficult to determine. To date, the role of hepatocyte and macrophage ABCA1 expression in atherosclerosis progression and lipid metabolism are poorly understood. In this dissertation, we investigated the relationship between hepatocyte and macrophage ABCA1 and atherosclerosis development using atherogenic diet-fed LDLrKO mice.

In the first set of studies, we tested the hypothesis that hepatocyte ABCA1 protects against atherogenesis through its key role in maintaining plasma HDL levels, which are inversely correlated with CVD risk. We hypothesized that deletion of hepatocyte ABCA1 in atherogenic diet-fed LDLrKO mice would result in lower plasma HDL, impaired macrophage RCT, and increased atherosclerosis.

In the second set of studies, we tested the hypothesis that macrophage ABCA1 is atheroprotective due to its essential role in stimulating macrophage FC efflux,
the initial step of macrophage RCT, to combat with unregulated cholesterol uptake and foam cell formation under atherogenic conditions. We hypothesized that macrophage ABCA1 deficiency would result in a significant defect in cholesterol efflux with increased cellular cholesterol accumulation and enhanced inflammatory response, leading to accelerated atherogenesis in LDLrKO mice.

The results from this thesis fill the considerable gaps in knowledge regarding the impact of hepatocyte and macrophage ABCA1 expression on atherosclerosis and CVD risk. Additionally, these studies shed light on the potential of modulating ABCA1 expression as a target for therapeutical intervention.
Reference


Chapter II

Liver ABCA1 Deletion in LDLrKO Mice Does Not Impair Macrophage Reverse Cholesterol Transport or Exacerbate Atherogenesis

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Abstract

**Objective:** Hepatic ATP binding cassette transporter A1 (ABCA1) expression is critical for maintaining plasma HDL concentrations, but its role in macrophage reverse cholesterol transport (RCT) and atherosclerosis is not fully understood. We investigated atherosclerosis development and RCT in hepatocyte specific ABCA1 knockout (HSKO) mice in the LDL receptor knockout (LDLrKO) C57BL/6 background.

**Approach and Results:** Male and female LDLrKO and HSKO/LDLrKO mice were switched from chow at 8 wks of age to an atherogenic diet (10% palm oil, 0.2% cholesterol) for 16 wks. Chow-fed HSKO/LDLrKO mice had HDL concentrations 10-20% of LDLrKO mice, but similar VLDL and LDL concentrations. Surprisingly, HSKO/LDLrKO mice fed the atherogenic diet had significantly lower (40-60%) VLDL, LDL, and HDL concentrations (50%) compared to LDLrKO mice. Aortic surface lesion area and cholesterol content were similar for both genotypes of mice, but aortic root intimal area was significantly lower (20-40%) in HSKO/LDLrKO mice. Although macrophage $^3$H-cholesterol efflux to apoB lipoprotein-depleted plasma was 24% lower for atherogenic diet-fed HSKO/LDLrKO vs. LDLrKO mice, variation in percentage efflux among individual mice was <2-fold compared to a 10-fold variation in plasma HDL concentrations, suggesting that HDL levels, *per se*, were not the primary determinant of plasma efflux capacity. *In vivo* RCT, resident peritoneal
macrophage sterol content, biliary lipid composition, and fecal cholesterol mass were similar between both genotypes of mice.

**Conclusions:** The markedly reduced plasma HDL pool in HSKO/LDLrKO mice is sufficient to maintain macrophage RCT, which, along with reduced plasma VLDL and LDL concentrations, prevented the expected increase in atherosclerosis.
Introduction

Atherosclerosis-associated cardiovascular disease (CVD) is the leading cause of death worldwide \(^1\). The inverse relationship between plasma HDL levels and CVD risk has made raising HDL levels a popular potential therapeutic target for CVD prevention. ATP binding cassette transporter A1 (ABCA1) belongs to a large family of the ATP binding cassette transporters \(^2\). ABCA1 mediates cellular free cholesterol (FC) and phospholipid (PL) efflux to apolipoprotein A-I (apoA-I), resulting in the formation of nascent HDL that undergo subsequent maturation to become plasma HDL \(^2\). The critical role of ABCA1 in HDL formation was established when it was found to be the genetic defect in Tangier disease, a disorder in which HDL levels are <5% of normal\(^3-5\). Studies with animal models have also documented the essential function of ABCA1 in maintaining plasma HDL levels \(^6-8\).

Despite the well-established role of ABCA1 in HDL formation, its effect on atherogenesis is less clear. Premature atherosclerosis has been reported in some, but not all, people with Tangier disease \(^9\). Common genetic variants in ABCA1 have been reported to influence the risk and severity of CVD \(^10-12\); however, low HDL caused by loss-of-function mutations in ABCA1 does not contribute to increased risk of CVD in the general population \(^13\). Controversial results also exist in studies with mouse models. Overexpression of human ABCA1 (hABCA1) in the liver and macrophages of B6 mice resulted in an anti-atherogenic lipid profile and lower aortic atherosclerosis, whereas in apoE KO
mice, overexpressing hABCA1 increased atherosclerosis with minimal effect on plasma lipids\textsuperscript{14}. Physiological overexpression of a full-length hABCA1 containing bacterial artificial chromosome (BAC) in apoE KO and LDLrKO mice both revealed an atheroprotective role of ABCA1\textsuperscript{15, 16}. Significantly larger lesions occurred in ApoE or LDLrKO mice transplanted with bone marrow from mice with total body ABCA1 deficiency\textsuperscript{17-20}. In contrast, total body ABCA1 deficiency in apoE KO or LDLrKO mice did not result in increased atherosclerosis compared to control mice\textsuperscript{18}. The complex relationship between global ABCA1 expression and atherosclerosis susceptibility observed in humans and mouse models of atherosclerosis was at least partially attributed to reduction in atherogenic lipoproteins concomitant with ABCA1 deficiency, or to the use of different promoters for transgenic overexpression.

Subsequent studies with hepatocyte-specific ABCA1 knockout (HSKO) mice suggested a major role for the liver in maintaining systemic HDL levels, leading to investigation of the role of hepatic ABCA1 in atherogenesis\textsuperscript{6}. Joyce et al found that liver-specific overexpression of ABCA1 in LDLrKO mice led to increased atherosclerosis, presumably due to increased plasma concentrations of apoB-containing lipoproteins (apoB Lp) concomitant with a significant increase in plasma HDL \textsuperscript{21}. A more recent study of hepatic ABCA1 deletion in chow-fed apoE KO mice showed a significant increase in early-stage atherosclerosis\textsuperscript{16}. However, several issues were not addressed in that study. First, only early atherosclerosis was examined; mice consumed a chow diet for 12 wks, and total plasma
cholesterol (TPC) concentrations were relatively low (250-400 mg/dl). Thus, the effect of hepatocyte deletion of ABCA1 on more advanced atherosclerosis is unknown. Second, HDL cholesterol concentrations are quite low in apoE KO mice (~29 mg/dl). Therefore, the difference between apoE KO and HSKO-apoE KO strains may be too small to differentially affect atherosclerosis. Third, apoE has atheroprotective roles at the level of the arterial wall that are absent in the apoE KO background \(^{22}\). Finally, no \textit{in vivo} RCT studies were performed.

To address these gaps in knowledge, we investigated the influence of hepatic ABCA1 expression on RCT and development of more advanced atherosclerotic lesions in LDLrKO mice. Our results suggest a minimal impact of hepatic ABCA1 deletion on \textit{in vivo} macrophage RCT and atherogenesis development in atherogenic diet-fed LDLrKO mice, even though plasma HDL concentrations were markedly reduced in HSKO/LDLrKO mice compared to LDLrKO mice. This surprising outcome likely resulted from the significant reduction in atherogenic lipoproteins (i.e., VLDL and LDL) observed in diet-fed HSKO/LDLrKO mice, as well as sufficient HDL to mediate RCT.
Methods

Animals and Diet

HSKO mice in the C57BL/6 background (>99%) were generated as described previously \(^6, 23\) and crossed with LDLrKO mice in the C57Bl/6 background (Jackson Laboratories) to generate heterozygous HSKO/LDLrKO. Mice used for atherosclerosis studies were generated by crosses of heterozygous HSKO/LDLrKO mice; genotypes of offspring were determined by PCR\(^6\). Female and male HSKO/LDLrKO and LDLrKO littermate control mice were fed a chow diet until 7-9 wks of age, and then switched to an atherogenic diet containing 10% palm oil and 0.2% cholesterol for 16 wks in most experiments, unless otherwise indicated. Diet composition has been published previously \(^24\). Mice were maintained on a 12h light/dark cycle. All protocols and procedures were approved by the Institutional Animal Care and Use Committee.

Lipid and lipoprotein analysis

Plasma was collected by tail bleeding or cardiac puncture from mice fasted for 4 hr. TPC, free cholesterol (FC), and triglycerides (TG) concentrations were determined by enzymatic assays using commercial kits\(^25\). Cholesterol distribution among lipoproteins was determined after fractionation of plasma by gel filtration chromatography using a Superose 6 10/300 GL column (GE Healthcare). An aliquot of plasma containing approximately 15 µg total cholesterol was injected onto the column and eluted with 0.9% saline containing 0.01% EDTA and 0.01% sodium azide at a flow rate of 0.4 ml/min. The column effluent was mixed with a
commercially available enzymatic total cholesterol reagent (Pointe Scientific, Inc., Canton, MI) delivered at 0.125 ml/min. After passing through a knitted reaction coil maintained at 37°C, the absorbance of the reaction mixture was read at 500 nm using a UV-VIS detector. The area under the VLDL, LDL, and HDL peaks was calculated using Chromperfect Spirit (Justice Laboratory Software) chromatography software. To calculate the cholesterol concentration in each lipoprotein fraction, the ratio of the respective peak area to total peak area was multiplied by the total plasma cholesterol. Liver lipid analysis was performed by enzymatic assay of detergent-extracted liver 26.

Analysis of atherosclerotic lesions
Mice were sacrificed after 16 wks of atherogenic diet consumption. First they were anesthetized with ketamine/xylazine, and the vasculature then was perfused with cold PBS. Aortas were isolated and fixed in 10% buffered formalin. After fixation, aortas were cleaned of adventitial fat and pinned open for measurement of surface lesion areas. Images of en face aortas were analyzed using WCIF Image J software. Aortas were then lipid extracted for quantification of total and FC content using gas-liquid chromatography, as described previously 24. Aortic roots were embedded in Optimal Cutting Temperature (Tissuetek) media in a plastic mold, frozen, and cut at 8 µm intervals. Sections were collected from the aortic region moving toward the apex of the heart and sequentially placed on 8 slides, such that each slide had sections 64 µm apart. The sections were fixed in 10% buffered formalin, stained in 0.5% Oil Red O for
25 minutes and counterstained with hematoxylin. Stained sections were photographed and Image-Pro software (Media Cybernetics Inc., Rockville, Md.) was used to quantify lesion area. The lesion areas of three sections representing different regions were averaged for each mouse.

**In vivo Macrophage RCT**

Macrophage RCT assays were conducted as described by Rader and colleagues with minor modifications. J774 mouse macrophages or bone marrow-derived macrophages (BMM) from LDLrKO mice were radiolabeled and cholesterol-loaded with $^3$H-cholesterol and acetylated LDL, respectively. Cells were then injected into the peritoneal cavity of recipient mice fed the atherogenic diet. Plasma samples were collected at 6h, 24h, and 48h after injection. Feces were collected throughout the 48h study. At necropsy, tissues were harvested and $^3$H-tracer levels in plasma, liver, bile, and feces were then quantified after lipid extraction and liquid scintillation counting. Aliquots of plasma were also fractionated by FPLC and cholesterol mass, and radiolabel distribution among lipoprotein fractions was quantified.

**Real-time PCR Analysis**

At sacrifice, tissues were harvested and snap frozen in liquid nitrogen. Total RNA was isolated from livers of male mice using TRIzol (Invitrogen), and real-time RCR was performed as reported previously. Primer sequences were the same as described previously. GAPDH was used as the endogenous control.
**Cellular cholesterol efflux**

Cholesterol efflux from J774 macrophages to plasma of mice fed the atherogenic diet was measured as previously reported, with minor modifications\(^2^9\). Briefly, 350,000 J774 cells were plated into each well of a 24-well plate and incubated in labeling medium (RPMI 1640 medium containing 1% FBS and 2 µCi \(^3\)H-cholesterol) for 24 hours. Cells were then washed once and incubated with efflux medium (MEM-HEPES media containing 2.8% apoB lipoprotein-depleted plasma). Four hours later, medium was harvested and cellular lipid was extracted with isopropanol. A 100 µl aliquot of medium and cellular lipid extract was taken for scintillation counting to determine percentage cholesterol efflux during incubation. Aliquots of efflux medium were fractionated on a Superdex 200HR column (1X30 cm), and radioactivity of each fraction was determined by scintillation counting.

**Statistical analysis**

Results are reported as mean ± standard error of the mean. Data were analyzed using two-tailed Student’s t test (with Welch’s correction in case of unequal variance) using Graphpad Prism software. \(P <0.05\) was considered statistically significant.
Results

Hepatocyte ABCA1 deletion reduces plasma lipids

To induce atherosclerosis development, mice were switched from chow to an atherogenic diet at 8 wks of age for 16 wks. After 2 wks of diet feeding, TPC and FC increased for both groups of mice, but values were significantly lower in HSKO/LDLrKO mice throughout the 16-wk study (area under curve; $P<0.01$) (Figure 1A-B). A consistent trend in plasma triglyceride concentrations was not observed (Figure 1A-B). We previously reported that HDL cholesterol (HDL-C) levels in chow-fed HSKO mice were ~80% lower than WT mice $^6$, $^23$. FPLC fractionation of plasma lipoproteins showed that chow-fed HSKO/LDLrKO had significant reductions in HDL-C (1.74 mmol/L vs. 0.21 mmol/L in males; 67.3 mg/dl vs. 8.1 mg/dl in males, $P<0.0001$; 1.50 mmol/L vs. 0.28 mmol/L in females; 58.0 mg/dl vs. 10.9 mg/dl in females, $P<0.0001$), contributing to the lower TPC levels in HSKO/LDLrKO vs. LDLrKO mice (4.48 mmol/L vs. 2.68 mmol/L in males; 173.1 mg/dl vs. 103.6 mg/dl in males, $P<0.0001$; 5.17 mmol/L vs. 3.44 mmol/L in females; 199.7 mg/dl vs. 133 mg/dl in females, $P=0.0004$). VLDL-C and LDL-C concentrations were similar between genotypes (Figure 1C). The less pronounced hyperlipidemia in atherogenic diet-fed HSKO/LDLrKO mice was mainly attributed to lower VLDL-C (14.43 mmol/L vs. 6.08 mmol/L in males; 557.9 mg/dl vs. 235.0 mg/dl in males, $P=0.0050$; 16.49 mmol/L vs. 10.05 mmol/L in females; 637.7 mg/dl vs. 388.7 mg/dl in females, $P=0.0283$) and LDL-C levels (16.19 mmol/L vs. 11.65 mmol/L in males; 626.2 mg/dl vs. 450.5 mg/dl in males, $P=0.0221$; 11.80 mmol/L vs. 10.32 mmol/L in females; 456.3 mg/dl vs. 399.0
mg/dl in females, \( P=0.3441 \), whereas HDL-C concentrations remained significantly different between genotypes (2.0 mmol/L vs. 1.05 mmol/L in males; 77.4 mg/dl vs. 40.5 mg/dl in males, \( P=0.0007 \); 0.98 mmol/L vs. 0.36 mmol/L in females; 37.9 mg/dl vs. 14.0 mg/dl in females, \( P<0.0001 \)) (Figure 1D). These data document the critical role of hepatocyte ABCA1 in maintaining the plasma HDL-C pool in hyperlipidemic mice and demonstrate a potential role for hepatic ABCA1 in regulating plasma apoB-containing lipoprotein concentrations during atherogenesis.

**VLDL catabolism is increased in HSKO/LDLrKO mice**

To determine the explanation for reduced VLDL and LDL concentrations in atherogenic diet-fed HSKO/LDLrKO mice, we investigated VLDL production and catabolism. VLDL TG production was measured after *in vivo* inhibition of TG lipolysis with intravenous Triton administration to fasted mice. The rate of hepatic VLDL TG mass accumulation during Triton block of lipolysis was similar for both genotypes of mice (Supplemental Figure 1 A-B). Similarly, in a separate experiment, secretion rates of TG and radiolabeled apoB were not significantly different between groups (data not shown). We next investigated VLDL particle turnover using \( ^{125}\text{I-VLDL} \) from LDLrKO mice, which was indistinguishable in chemical composition from HSKO/LDLrKO mouse VLDL (Supplemental Table I). VLDL particles were enriched in CE (41-43%) at the expense of TG (5%), typical of \( \beta \)-VLDL \(^{30}\), likely due to the prolonged residence time in plasma in the absence of active LDLr \(^{31}\). VLDL particle turnover, measured as decay of \( ^{125}\text{I-VLDL} \) apoB
clearance from plasma, was more rapid in HSKO/LDLrKO vs. LDLrKO recipient mice (area under curve; $P<0.05$) (Supplemental Figure II), suggesting that the lower VLDL-C concentrations in HSKO/LDLrKO mice were due to increased VLDL particle catabolism. Expression of hepatic genes involved in VLDL catabolism was similar for HSKO/LDLrKO and LDLrKO mice (Supplemental Figure II C). However, plasma apoE levels were lower in atherogenic diet-fed HSKO/LDLrKO mice in addition to the anticipated reduction in apoA-I levels due to low plasma HDL concentrations (Supplemental Figure II D). Furthermore, most of the plasma apoE as well as apoA-I migrated in the HDL size range (8-10 nm) on non-denaturing gradient gels (Supplemental Figure II E and F). Given these results, we speculate that lower VLDL-C levels in HSKO/LDLrKO mice were likely due to decreased competition by apoE-containing plasma HDL for hepatic VLDL uptake, resulting in increased removal of VLDL particles from plasma in HSKO/LDLrKO vs. LDLrKO mice.

**Effect of hepatocyte ABCA1 deletion on hepatic and biliary lipids**

To address whether hepatocyte ABCA1 ablation impacts liver lipid metabolism, we measured hepatic and biliary lipid levels. Unlike our previous study in which similar hepatic lipid content was observed for chow-fed HSKO vs. WT mice, atherogenic diet-fed HSKO/LDLrKO mice had lower (significant in female mice) hepatic TC, FC, and cholesterol ester (CE) concentrations relative to LDLrKO mice (Figure 2A-B). Hepatic TG and phospholipid (PL) concentrations were similar between the two genotypes (Figure 2A-B). However, there was no
significant difference in biliary TC, PL, and bile acid (BA) concentrations or molar percentage composition between the two genotypes (Supplemental Figure III A-D) and fecal cholesterol excretion was similar (Supplemental Figure III E). To investigate whether the decreased liver cholesterol content was associated with transcriptional regulation concomitant with ABCA1 ablation, we measured expression of genes involved in hepatic lipid metabolism. SREBP1c was significantly downregulated, and several other genes (HMGCoA synthase, ACC1) showed downward trends in expression in HSKO/LDLrKO mouse liver, suggesting decreased de novo lipogenesis (Figure 2C). Liver expression of several LXR target genes was similar in HSKO/LDLrKO and LDLrKO mice (Figure 2C).

Impact of hepatic ABCA1 on atherosclerosis development

To investigate the impact of hepatocyte ABCA1 deficiency on atherosclerosis development in LDLrKO mice, three measurements of atherosclerosis were made. *En face* aortic surface lesion area (Figure 3A, C) and aortic cholesterol content (Figure 3B) were similar for HSKO/LDLrKO and LDLrKO mice, although there was a trend towards reduced aortic cholesterol content in HSKO/LDLrKO mice. Furthermore, cross-sectional analysis of oil red O stained aortic root sections revealed significantly smaller lesions in both female (0.51 mm² vs 0.40 mm², p=0.0141) and male (0.48 mm² vs 0.29 mm², p=0.0341) HSKO/LDLrKO mice vs. their LDLrKO counterparts (Figure 3D-E), suggesting that deletion of hepatocyte ABCA1 expression may actually protect against more advanced
atherosclerotic lesion development in the aortic root. Additional support for this concept was obtained with additional analysis of lesion complexity; aortic root sections stained with Masson’s trichrome showed less necrosis, acute inflammation, and adventitial inflammation in lesions of HSKO/LDLrKO vs. LDLrKO mice fed the atherogenic diet for 16 wks (Supplemental Figure IV A). In a separate experiment to evaluate very early stages of aortic atherosclerosis (i.e., 5 wks atherogenic diet feeding), we observed similar aortic cholesterol content between genotypes (Supplemental Figure IV B). Taken together, unlike previous findings in apoE KO mice\textsuperscript{16}, the absence of hepatic ABCA1 did not accelerate early-stage atherogenesis in LDLrKO mice and appeared to protect against late-stage, more advanced atherosclerosis.

**Role of hepatocyte ABCA1 in macrophage RCT in vivo**

One atheroprotective mechanism proposed for HDL is the transport of excess macrophage cholesterol to the liver for excretion (i.e., RCT)\textsuperscript{32}. To determine whether the large reduction of plasma HDL in HSKO/LDLrKO mice diminished RCT, we performed *in vivo* macrophage RCT assays. \(^{3}\text{H}-\text{cholesterol-radiolabeled J774 macrophages were injected into the peritoneal cavity of HSKO/LDLrKO or LDLrKO mice after 5 wk of atherogenic diet feeding. The plasma}^{3}\text{H}-\text{cholesterol closely tracked with lipoprotein cholesterol mass (Figure 4A-B). The amount of}^{3}\text{H tracer in plasma 48h after injection was significantly lower in HSKO/LDLrKO mice, likely reflecting the lower levels of plasma lipoproteins in these mice (Figure 4C). However, the tracer levels in the liver, bile,
and feces were similar between the two groups (Figure 4D-F), suggesting that in vivo macrophage RCT was not impaired in HSKO/LDLrKO mice, despite the much lower HDL-C in these mice. A similar outcome was obtained using radiolabeled bone marrow-derived macrophages (BMM) injected into mice fed the atherogenic diet for 16 wk (Supplemental Figure V). We also measured cholesterol content of resident peritoneal macrophages in atherogenic diet-fed mice and observed no significant difference between the two genotypes, although there was a trend toward decreased cholesterol in HSKO/LDLrKO mice (Supplemental Figure VI). Collectively, these data suggest that in vivo macrophage RCT is not impaired in HSKO/LDLrKO mice, despite significantly lower plasma steady-state HDL-C levels.

**Plasma cholesterol efflux capacity**

*In vivo* RCT results suggested that the plasma HDL pool in HSKO/LDLrKO mice was sufficient to maintain normal cholesterol efflux from macrophages for ultimate excretion into feces. One possible explanation for this outcome could be a fraction of mouse plasma HDL that is highly efficient at effluxing macrophage FC, compensating for low plasma HDL levels in RCT in HSKO/LDLrKO mice. For example, pre-1 appears to be the preferred acceptor for ABCA1-mediated FC efflux in human plasma, but its concentration is typically <10% of total HDL. To examine this possibility, we measured the ability of apoB lipoprotein-depleted plasma from atherogenic diet-fed HSKO/LDLrKO and LDLrKO mice to efflux 3H-cholesterol from cholesterol-loaded macrophages and observed a significant
reduction (24%) in HSKO/LDLrKO vs. LDLrKO plasma (10.14% vs. 7.75%, p=0.0067; Figure 5A). However, analysis of individual animal HDL-C concentrations vs. percent of efflux values (Figure 5B) demonstrated < 2-fold variation in percentage efflux values compared to a 10-fold variation in plasma HDL-C concentrations, suggesting that HDL-C concentration, per se, is not a primary determinant of plasma efflux capacity.

After the cholesterol efflux experiment, we fractionated a subset of individual plasma-containing efflux media using an FPLC column capable of separating plasma HDL particles, pre-β 1 HDL and lipid-free apoA-I from one another (Supplemental Figure VII). The distribution of 3H-cholesterol between the main HDL peak (fractions 41-50) and pre-β 1 HDL elution region (fractions 51-55) did not reveal a disproportionate amount of 3H-FC in the pre-β 1 peak in HSKO/LDLrKO vs. LDLrKO plasma (Figure 5C) and the percentage of 3H-FC in the pre-β 1 peak relative to the entire HDL elution region (i.e. fractions 41-55) was 20.9 ± 3.4% and 20.7 ± 5.2%, respectively (n=6/genotype). In addition, apoA-I Western blot analysis of plasma separated by agarose gel electrophoresis did not show an increase in pre-β 1 HDL for HSKO/LDLrKO compared to LDLrKO mice (Supplemental Figure VII D). Taken together, these results suggest that HSKO/LDLrKO mice do not compensate for reduced plasma HDL levels with an increase in amount or cholesterol efflux efficiency of pre-β 1 HDL to maintain in vivo RCT at levels comparable with LDLrKO mice.
**Discussion**

Hepatocyte ABCA1 plays a crucial role in HDL biogenesis, but its role in atherogenesis is less clear. In the current study, we addressed this gap in knowledge by performing atherosclerosis and *in vivo* macrophage RCT studies in atherogenic diet-fed HSKO mice crossed into the LDLrKO background. Compared to LDLrKO (control) mice, HSKO/LDLrKO mice had reduced TPC, primarily due to a 40-50% reduction in VLDL and LDL, and similar or reduced atherosclerosis. Furthermore, *in vivo* macrophage RCT to feces was similar for both genotypes of mice, although efflux of macrophage $^{3}$H-FC was significantly reduced in apoB lipoprotein-depleted plasma from HSKO/LDLrKO mice. In agreement with the *in vivo* RCT data, resident peritoneal macrophage cholesterol content, biliary lipid composition, and fecal cholesterol mass were similar for mice with or without hepatocyte ABCA1 expression. These results suggest that the markedly reduced plasma HDL pool in HSKO/LDLrKO mice is sufficient to maintain macrophage RCT, which, together with decreased VLDL and LDL levels, prevented the expected increase in atherosclerosis.

Atherogenic diet-fed HSKO/LDLrKO mice displayed similar *en face* aortic lesion area and cholesterol content, but reduced aortic root lesion size relative to LDLrKO mice (Figure 3). The fact that development of atherosclerosis in the aortic root precedes that in the whole aorta and is typically more advanced may suggest that hepatic ABCA1 deficiency is protective in more advanced stages of atherosclerosis, perhaps by preserving RCT during extended periods of
hyperlipidemia (see below). Only one other study has investigated the role of hepatocyte ABCA1 deficiency in atherogenesis\textsuperscript{16}. In that study, aortic lesion size and cholesterol content were significantly increased in HSKO/apoE KO vs. apoE KO mice fed chow for 12 wks, supporting an anti-atherogenic role for hepatic ABCA1. There are several differences between the studies that may explain the divergent outcomes. First, chow-fed apoE KO mice had modest hypercholesterolemia (TPC= 250-400 mg/dl) and early atherosclerotic lesions, with low amounts of aortic cholesterol \textsuperscript{16}, whereas atherogenic-diet fed LDLrKO mice in the present study had much higher TPC concentrations (~1000 mg/dl) and more aortic cholesterol accumulation (~60-fold more TC). In a follow-up study of very early atherosclerosis (5 wks atherogenic diet feeding), we again observed no difference in aortic cholesterol content between HSKO/LDLrKO and LDLrKO mice (Supplemental Figure IV B), although aortic TC levels were 4-6 fold less than mice fed the atherogenic diet for 16 wks (compare Figure 3B to Supplemental figure IV B), but 10-fold higher than aortic TC levels of HSKO/apoEKO mice fed chow for 12 wks \textsuperscript{16}. VLDL and LDL concentrations were significantly lower in the HSKO/apoEKO vs. apoEKO mice, but the magnitude of the reduction (45 mg/dl for VLDL; 60 mg/dl for LDL) was much less than HSKO/LDLrKO mice (250-300 mg/dl for VLDL-C; 60-170 mg/dl for LDL-C) compared to LDLrKO mice. Although the percentage reduction in HDL-C compared to their respective controls was similar for both studies (~50%), the absolute HDL-C values were much lower in HSKO/apoEKO mice compared with HSKO/LDLrKO mice, which may have been low enough to limit RCT; however,
macrophage RCT was not measured in the Brunham study. Finally, apoE expression is atheroprotective independent of plasma lipoprotein concentrations\textsuperscript{34-36}. One or more of these differences may explain why hepatocyte ABCA1 deletion was neutral or atheroprotective in LDLrKO mice and atherogenic in apoE KO mice.

Epidemiological studies have documented an inverse association between plasma HDL-C concentrations and coronary heart disease (CHD), supporting HDL’s role as an anti-atherogenic lipoprotein\textsuperscript{37}. The protective role of HDL in CHD is primarily attributed to RCT, but HDL also inhibits lipoprotein oxidation, inflammation, and hematopoiesis and maintains endothelial function, all of which are atheroprotective \textsuperscript{38, 39}. However, recent studies have challenged the assumption that raising HDL-C levels will uniformly translate into reductions in CHD \textsuperscript{40, 41}. Other studies have suggested the HDL particle number and size (i.e., subfraction distribution) are better predictors of CHD risk than HDL-C \textsuperscript{42-44} and that HDL function may be more important in preventing CHD than HDL-C \textsuperscript{45, 46}. Animal studies have shown a more consistent association between increased atherosclerosis and decreased macrophage RCT than with reduced HDL-C \textsuperscript{47}. In support of the concept that HDL function may be more important than HDL-C in determining CHD risk, we show that a substantial reduction of plasma HDL-C in atherogenic diet-fed HSKO/LDLrKO mice did not significantly affect aortic atherogenesis, \textit{in vivo} macrophage RCT, or fecal cholesterol excretion. These results are compatible with the idea of a small, dynamic HDL pool that efficiently
removes cholesterol from arterial macrophage foam cells and rapidly transports it
to the liver for excretion without a detectable increase in plasma HDL-C. Pre-β 1
HDL is a preferential acceptor for macrophage cholesterol efflux via ABCA1 and
may function in this regard, although it is generally <10% of total HDL mass\textsuperscript{29}.
However, a large HDL pool would not be necessary for this mechanism to be
operational, since aortic CE mass in atherogenic-diet fed HSKO/LDLrKO mice is
<1% of the steady-state plasma cholesterol pool. Overall, these observations
support the concept that HDL quality or function may be a better predictor of
atheroprotection than HDL-C, \textit{per se}.

A recent study by the Rader lab supports our results that a marked decrease in
plasma HDL did not affect macrophage RCT\textsuperscript{48}. They found that pharmacologic
inhibition of ABCA1 by probucol resulted in an 80% reduction of plasma HDL-C
in chow-fed WT mice, but macrophage RCT was unaffected, although the flux of
\textsuperscript{3}H-cholesterol from plasma HDL into the liver and feces was increased. In
addition, probucol treatment of SR-BI knockout mice reduced plasma HDL-C by
63% and stimulated macrophage RCT. Based on these combined results,
Yamamoto et al suggested that hepatocyte ABCA1 may normally function to
counterbalance RCT by mobilizing FC from hepatocytes back into plasma to help
maintain plasma HDL-C. However, probucol, an anti-oxidant drug with a long
history of clinical use, has broad pharmacological properties (including effects
relevant to whole body lipid metabolism) and is not specific for ABCA1\textsuperscript{49}. Here,
we show that specific genetic deletion of hepatocyte ABCA1 supports the
conclusions of Yamamoto and coworkers. Results from both studies suggest that the plasma HDL pool remaining after whole body pharmacological inhibition of ABCA1 or genetic deletion of hepatocyte ABCA1, though small, is quantitatively sufficient or functionally efficient to mediate macrophage cholesterol transport back to the liver for excretion. As discussed above, maintaining macrophage RCT in the face of considerable reductions in the plasma HDL pool appear to result in atheroprotection over long periods of hyperlipidemia when advanced atherosclerosis development is ongoing (Figure 3E).

The similar atherosclerosis outcome in aortas of HSKO/LDLrKO and LDLrKO mice and the aortic root atheroprotection in HSKO/LDLrKO mice may be partially ascribed to the significant reduction in plasma VLDL and LDL levels in atherogenic diet-fed HSKO/LDLrKO mice (Figure 1D). Whole body ABCA1 KO mice in LDLrKO or apoEKO backgrounds also had similar extent of atherosclerosis that was attributed to reduced plasma apoB Lp concentrations. Altered apoB Lp metabolism is also a feature of Tangier disease, in which plasma LDL levels are ~ 50% of normal due to a two-fold increase in the fractional catabolic rate (FCR) of LDL. Chow-fed HSKO mice demonstrated a 50% lower plasma LDL concentration, a two-fold increase in 125I-LDL tracer removal rate from plasma, and a two-fold increase in hepatic LDLr expression relative to WT mice, suggesting that increased hepatic LDLr expression may be responsible for the decreased plasma LDL concentrations. Crossing chow-fed HSKO mice into the LDLrKO background normalized (i.e., increased) plasma
LDL concentrations to those of chow-fed LDLrKO mice, lending additional support that increased hepatic LDLr expression is the key mediator of reduced plasma LDL in chow-fed HSKO mice (Figure 1C). However, atherogenic diet-fed HSKO/LDLrKO mice exhibited diminished VLDL-C and LDL-C relative to LDLrKO mice, which was due to increased VLDL catabolism (Supplemental Figure II) with no difference in VLDL production (Supplemental Figure I). Since these mice lack functional LDLr, increased catabolism of VLDL had to be mediated through an LDLr-independent pathway. Hepatic LDLr related protein (LRP) mRNA (Supplemental Figure II C) and protein levels (data not shown) were similar between groups, suggesting the involvement of other potential pathways, such as heparan sulfate proteoglycans. However, expression of hepatic lipoprotein catabolic genes such as SR-BI, syndecan 1, hepatic lipase, lipoprotein lipase, and apoC3 were similar between genotypes (Figure 2B). Van Eck et al have shown that HDL can effectively compete for hepatic uptake of βVLDL particles via SR-BI dependent and independent-mediated mechanisms. In the absence of functional LDLr, VLDL residence time in plasma is significantly prolonged, allowing alternate hepatic VLDL particle uptake pathways to predominate. We show that the pool of apoE-enriched HDL, a likely competitor for alternate hepatic VLDL particle uptake pathways, is diminished in atherogenic diet-fed HSKO/LDLrKO mice (Supplemental figure II F). Based on our combined results and the previous studies by Van Eck et al, we propose that the increased VLDL particle catabolism in HSKO/LDLrKO mice is mediated through decreased competition for hepatic uptake of VLDL by apoE-containing HDL.
Regardless of the exact mechanism, hepatic ABCA1 expression appears to be an important regulator of plasma apoB Lp as well as HDL levels, both of which modulate atherosclerosis progression.

In conclusion, we investigated the impact of hepatocyte ABCA1 deletion on relatively advanced atherosclerosis and macrophage RCT during disease progression. Unexpectedly, we found that hepatocyte ABCA1 deletion did not exacerbate lesion development in atherogenic diet-fed LDLrKO mice, and was atheroprotective in the aortic root, likely due to reduced apoB Lp levels and maintenance of macrophage RCT, despite a large reduction in plasma HDL-C. Our results are also compatible with the idea proposed by Rader and colleagues \(^{48}\) that hepatic ABCA1 may normally recycle a significant amount of plasma HDL-C removed by the liver back into plasma to maintain the plasma HDL-C pool. If true, these findings would result in a paradigm shift, since decreased hepatic ABCA1 expression, resulting in lower plasma HDL-C, may actually increase RCT and reduce CHD risk by reducing the recycling of hepatic cholesterol back into plasma through nHDL formation by ABCA1.
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Disclosures- None
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Figure 1

A

TPC

FC

TG

[Graphs showing data over diet weeks for TPC, FC, and TG for males and females]

B

TPC

FC

TG

[Graphs showing data over diet weeks for TPC, FC, and TG for males and females, with markers for LDLrKO and HSKO/LDLrKO]

C

Male

Female

[Bar graphs showing data for TPC, VLDL, LDL, and HDL for Chow Diet for males and females]

D

[Bar graphs showing data for TPC, VLDL, LDL, and HDL for Atherogenic Diet for LDLrKO and HSKO/LDLrKO]
Figure 1. Plasma lipid and lipoprotein concentrations during atherosclerosis progression. Fasting (4h) plasma total cholesterol (TPC), free cholesterol (FC), and triglyceride (TG) concentrations were measured by enzymatic assays in male (A) and female (B) mice of the indicated genotype during a 16 wk atherosclerosis progression phase (n=9-13). Plasma lipoprotein cholesterol distribution of mice before (C; chow-fed at 7-9 wks of age) or after (D) 16 wks of atherogenic diet consumption were determined after FPLC fractionation of plasma (n=9-19). Data expressed as mean ± SEM. * \( P<0.05 \). HSKO, hepatocyte-specific ABCA1 knockout.
Figure 2
Figure 2. Hepatic lipid content and gene expression. Lipid content was determined using detergent-based enzymatic assays of hepatic lipid extracts from 4h-fasted male (A) or female (B) mice after 16 wks of atherogenic diet consumption (n=7-12). C. Hepatic gene expression in male mice (n=7). Data was expressed in mean ± SEM. * P<0.05.
Figure 3

(A) Male: % aorta surface lesion area

(B) ug cholesterolling protein

(C) LDLrKO vs HSKO/LDLrKO

(D) LDLrKO vs HSKO/LDLrKO

(E) Male and Female Lesion Area (mm²)
Figure 3. Atherosclerosis evaluation. (A) Aorta surface lesion area was expressed as the percentage of total aorta surface area. (B) Aortas were lipid extracted for quantification of total cholesterol (TC) and free cholesterol (FC) content using gas-liquid chromatography. Cholesterol ester (CE) content was calculated as (TC-FC) x1.67. (C) Representative en face aorta from a LDLrKO (left) and HSKO/LDLrKO (right) mouse. (D) Representative LDLrKO (left) and HSKO/LDLrKO (right) mouse aortic root sections stained with Oil Red O. (E) Aortic root lesion area. Each point represents the average lesion area of 3 sections per mouse. Horizontal lines denote the mean ± SEM for each genotype. * $P<0.05$. 
Figure 4

A

B

C

D

E

F


Liver

Bile

Feces

Cholesterol Bile Acids Total Sterol

Cholesterol Bile Acids Total Sterol
Figure 4. Macrophage RCT. $^3$H-cholesterol radiolabeled, cholesterol-loaded J774 macrophages were injected into the peritoneal cavity of mice fed the atherogenic diet for 5 wks. Plasma cholesterol mass (A) and $^3$H-cholesterol (B) distribution were determined after FPLC separation of plasma. Plasma $^3$H-cholesterol radiolabel at different time points (C) and in liver (D), bile (E) and feces (F) 48 hours after injection. Data are expressed as mean ± SEM, n=7-8. * $P<0.05$. 

Figure 5. Plasma cholesterol efflux capacity. (A) Cholesterol efflux to apoB lipoprotein-depleted plasma. J774 macrophages were radiolabeled with $^3$H-cholesterol for 24 h and then incubated for 4 h with medium containing 2.8% plasma from LDLrKO or HSKO/LDLrKO mice fed an atherogenic diet (n=8). An aliquot of medium and cellular lipid extract was taken for scintillation counting to determine percentage cholesterol efflux during the 4h incubation. * $P<0.05$. (B) Percentage cholesterol efflux was plotted against each animal’s plasma HDL-C level. Open symbols=LDLrKO; closed symbols=HSKO/LDLrKO. (C) apo-B depleted plasma medium 4h after cholesterol efflux underwent FPLC separation; radioactivity of each fraction was counted by scintillation counting.
Supplemental Methods:

Biliary lipids
A measured volume of gallbladder bile collected from mice (n=5-10) was subjected to neutral lipid extraction. 5-α cholestane (5 µg) was added to each extraction tube as an internal standard. Aliquots of the bottom organic phase were used to determine total cholesterol (TC) content by gas-liquid chromatography and phospholipid (PL) content by enzymatic assay (4). Bile acid (BA) was measured as reported previously using the top phase of the lipid extract \(^1\).

Fecal cholesterol excretion
Two-day fecal collections were subjected to lipid extraction, and cholesterol content was measured as described previously\(^2\).

Macrophage cholesterol content
Peritoneal cells were harvested by lavage from 4h-fasted mice. Cells were suspended in RPMI-1640 medium containing 1% Nutridoma-SP (Roche Applied Science), 100 units/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine and cultured at 37\(^\circ\) C for 2 hours. Then, non-adherent cells were removed by washing with PBS and adherent macrophages were extracted with isopropanol at room temperature overnight. TC and FC content was determined by gas-liquid chromatography and cellular protein was measured by Lowry protein assay after NaOH digestion, as previously reported\(^3\).
In vivo VLDL TG secretion rate determination

Tyloxyapol (500 mg/kg body weight) was injected intravenously into 4h-fasted, anesthetized, atherogenic diet-fed mice (n=4-6). Blood was collected before (0 min), 30, 60, and 90 min after injection for measurement of plasma triglyceride (TG) concentration by enzymatic assay. VLDL TG secretion rate was determined by calculating the slope of the time vs. plasma TG concentration plot for each animal using linear regression analysis.

VLDL/IDL composition and size analysis

Plasma was collected from LDLrKO mice (n=4/group) fed an atherogenic diet for 16 wk after they were fasted for 4h. VLDL/IDL were isolated by ultracentrifugation at d=1.019 g/ml (100,000 rpm for 4h, Beckman Coulter TLA100.2 rotor) and chemical composition was determined by enzymatic assay and chemical assays. Lipid and protein content were expressed as percentage of total mass. Aliquots of VLDL/IDL were used to measure particle size using a Zetasizer Nano S dynamic light scattering instrument (Malvern Instruments Ltd., Worcestershire, UK). Particle sizes are reported as the major peak mean by volume analysis.

In vivo VLDL turnover

VLDL were separated from plasma of fasted LDLrKO mice fed an atherogenic diet for 16 wk by ultracentrifugation at d=1.006 g/ml. The VLDL preparation was refloated at d=1.006 g/ml and radiolabeled with $^{125}$I using the iodine monochloride method. Ninety-seven percent of the radioactivity was
trichloroacetic acid-precipitable. VLDL tracer (0.25 x 10^6 cpm) was diluted to 200 µl with saline for retro-orbital injection into 4h-fasted, anesthetized recipient mice (n=5/genotype) fed an atherogenic diet for 16 wk. Blood samples were collected at 2 and 30 min and 1, 3, 5, 8 and 24 h after injection and ^125^I radiolabel in plasma was determined by gamma radiation counting. ApoB was precipitated from plasma using isopropyl alcohol and ^125^I radiolabel in apoB was quantified by gamma radiation counting. Turnover curves were plotted as the percentage of injected tracer remaining in plasma or as the percentage of ^125^I-apoB remaining in plasma relative to the injected dose vs. time.

**Gel electrophoresis**

Plasma (1 µl) from LDLrKO or HSKO/LDLrKO mice fed an atherogenic diet for 19 wk was fractionated by 0.7% agarose gel electrophoresis. Following a 2 h capillary transfer of the fractionated plasma from the agarose gel to a 0.2 µm nitrocellulose membrane, Western blot analysis was performed using anti-mouse apoA-I (Meridian Life Science, Inc. K23600G). SDS-PAGE and 4-30% non-denaturing gradient gel electrophoresis and Western blot analysis was performed as previously described.

80
Supplemental Table I. VLDL/IDL chemical composition and size

<table>
<thead>
<tr>
<th>Genotype</th>
<th>% of total mass</th>
<th>Diameter (nm)</th>
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<tbody>
<tr>
<td></td>
<td>FC</td>
<td>CE</td>
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<tr>
<td>LDLrKO</td>
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<td>40.7±2.4</td>
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<tr>
<td>HSKO/LDLrKO</td>
<td>10.0±1.4</td>
<td>42.8±3.0</td>
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</tbody>
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VLDL/IDL were isolated from atherogenic diet-fed mice by ultracentrifugation at d=1.019 g/ml. Chemical composition was analyzed by enzymatic or chemical assays. VLDL/IDL particle size was determined by dynamic light scatter. Mean ± SEM, n=4. IDL, intermediate density lipoprotein.

Supplemental Figure Legends

Supplemental Figure I. VLDL secretion was evaluated after in vivo inhibition of lipolysis with triton. Plasma TG levels were measured by enzymatic assay before (0 min) and after (30, 60, 90, 180 min) intravenous Triton injection. (A) Plasma TG concentration was plotted for each genotype of mice (mean ± SEM; n=4-6), and the line of best fit was determined by linear regression analysis. (B) TG secretion rate during the 3h Triton block experiment was determined for each animal as the slope of the regression line of plasma TG vs. time. Results were then plotted for both genotypes of mice as mean ± SEM, n=4-6.
**Supplemental Figure II.** Plasma VLDL was isolated, radiolabeled with $^{125}$I, and injected intravenously into fasted atherogenic diet-fed mice (n=5/genotype). Periodic blood samples were taken after tracer injection, and whole plasma (A) and apoB (B) radiolabel remaining in plasma was determined. Turnover curves were plotted as percentage of injected tracer remaining in plasma (A) or percentage of $^{125}$I-apoB remaining in plasma (B) relative to the injected dose vs. time. Mean ± SEM, n=5. (C) Hepatic expression of genes involved in VLDL catabolism (n=7 male mice). Data was expressed in mean ± SEM. * $P<0.05$. (D) Plasma samples (1µl) were separated using 12% SDS-PAGE followed by Western blot analysis for mouse apoA-I and apoE. From left to right: atherogenic diet-fed LDLrKO vs. HSKO/LDLrKO mouse plasma (n=4/group). (E-F) Plasma samples (1µl) from atherogenic diet-fed mice (n=4/group) were subjected to 4-30% non-denaturing gradient gel electrophoresis (NDGGE) and Western blotted for mouse apoA-I (E) or apoE (F). Plasma from apoE knockout (apoE$^{-/-}$) and apoA-I knockout (apoA-I$^{-/-}$) mice was used as negative controls.

**Supplemental Figure III.** Gallbladder bile total cholesterol (TC), phospholipid (PL), and bile acid (BA) concentrations (A,B) and molar percentage (C,D) were determined for male (A, C) and female (B, D) mice (n=6-9). E. Two-day fecal collections were assayed for cholesterol content by gas liquid chromatography and the data were normalized to mg cholesterol/day/100g body weight (n=3-5). Data are expressed as mean ± SEM.
Supplemental Figure IV. (A) Representative Masson’s trichrome-stained aortic root sections from 16wk atherogenic diet fed LDLrKO (left) and HSKO/LDLrKO (right) mice. (B) Aortas were isolated from mice fed the atherogenic diet for 5 wks. Aortic lipid was extracted for quantification of total cholesterol (TC) and free cholesterol (FC) content using gas-liquid chromatography. Cholesterol ester (CE) content was calculated as (TC-FC) x1.67. Data are expressed as mean ± SEM, n=8 HSKO/LDLrKO, 7 LDLrKO.

Supplemental Figure V. $^{3}$H-cholesterol radiolabeled, cholesterol-loaded bone marrow-derived macrophages from LDLrKO mice were injected into the peritoneal cavity of mice fed an atherogenic diet for 16 wks. Forty-eight hr after macrophage injection, plasma $^{3}$H-cholesterol radiolabel (A) and cholesterol mass (B) distribution was determined after FPLC fractionation of plasma lipoproteins. $^{3}$H-cholesterol tracer was measured in plasma at different time points (C), and in liver (D), bile (E) and feces (F) 48 hr after macrophage injection. Data are expressed as mean ± SEM (n=3).

Supplemental Figure VI. Resident macrophages were harvested by peritoneal lavage from mice fed an atherogenic diet for 16 wk. Cells were cultured for 2h in RPMI medium containing 1% Nutridoma and non-adherent cells were removed by washing 3 times with PBS. The adherent macrophages were lipid extracted and the extract was assayed for total cholesterol (TC) and free cholesterol (FC)
content. Data are normalized for cellular protein and expressed as mean ± SEM (n=3-5).

**Supplemental Figure VII.** FPLC fractionation of plasma HDL and pre-β 1 HDL. 

$^{125}$I-apoA-I alone or plasma from an atherogenic-diet fed LDLrKO mouse with added $^{125}$I-apoA-I on ice was fractionated by FPLC (1 X 30 cm Superdex HR200, flow rate at 0.3 ml/min, 300 µl/fraction) and individual fractions were collected and counted for $^{125}$I (A) or assayed for cholesterol concentration (B). Media containing apoB-depleted plasma used for 4h macrophage FC efflux (Figure 5) study was fractionated by FPLC and $^3$H-radiolabel was quantified for each fraction by liquid scintillation spectroscopy (C). Vertical lines in panels A-C denote elution positions of VLDL/LDL, HDL, pre-β 1 HDL and apoA-I. (D) Agarose electrophoresis of plasma. Plasma samples (1 µl) were subjected to 0.7% agarose gel electrophoresis followed by Western blot analysis for mouse apoA-I. From left to right: chow-fed C57Bl/6 mouse plasma, n=1, atherogenic diet-fed LDLrKO mouse plasma, n=5, atherogenic diet-fed HSKO/LDLrKO mouse plasma, n=5. Alpha and pre-β HDL migration positions are shown for reference.
References


Supplemental Figure I

A  Plasma TG

B  TG Secretion Rate

LDLrKO  HSKO/LDLrKO

mmol/L  mmol/L/min

Time(min)
Supplemental Figure III

A

Male

B

Female

C

D

E

Fecal Cholesterol Excretion (mg/day/100g BW)

LDLrKO  HSKO/LDLrKO

0  2  4  6  8  10

mol/L bile
Supplemental Figure IV

A

LDLrKO

HSKO/LDLrKO

4X

10X

B

Aortic cholesterol content

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ug cholesterol/g protein

4X

10X
Supplemental Figure VI

Resident Peritoneal Macrophages

Male

Female

LDLrKO
HSKO/LDLrKO
Chapter III

Myeloid Cell Specific ABCA1 Deletion Has Minimal Impact on Atherogenesis in Atherogenic Diet-Fed LDL Receptor Knockout Mice

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Abstract

**Objective:** Bone marrow (BM) transplantation studies suggest that leukocyte ABCA1 protects against atherosclerosis development. However, the *in vivo* impact of macrophage ABCA1 expression on atherogenesis is not fully understood due to the presence of other leukocyte populations in BM. Myeloid-specific ABCA1 knockout (MSKO) mice in the LDL receptor knockout (LDLrKO) C57BL/6 background were developed to address this question.

**Approach and Results:** MSKO/LDLrKO (DKO) and LDLrKO (SKO) mice were fed chow or an atherogenic diet to examine different stages of atherosclerosis. Basal (i.e. chow) plasma lipid levels were similar, but atherogenic diet-fed DKO mice had reduced plasma VLDL and LDL levels compared with SKO mice resulting from decreased hepatic VLDL TG secretion. Atherogenic diet-fed DKO vs. SKO mice had significantly higher resident peritoneal macrophage cholesterol content and higher plasma proinflammatory cytokine/chemokine levels. Atherosclerosis extent was similar between genotypes at early/intermediate (i.e. 10-16wk) stages, but increased modestly in DKO mice at later stage atherosclerosis (i.e. 24wk atherogenic diet). Transplantation of DKO or SKO BM into SKO mice followed by 16wk atherogenic diet feeding also showed a similar extent of atherosclerosis and reduced plasma apoB Lp in mice receiving DKO BM. When differences in plasma VLDL/LDL concentrations were minimized by maintaining mice on chow for 24 wks, DKO mice had minimal, but significantly higher atherosclerosis compared to SKO mice.
**Conclusions:** These results suggest a novel role for myeloid cell ABCA1 in increasing hepatic VLDL TG secretion and plasma VLDL/LDL concentrations in atherogenic diet-fed LDLrKO mice that offsets its atheroprotective role in decreasing macrophage cholesterol content and inflammatory response, resulting in a minimal increase in atherosclerosis in its absence.
Introduction

ATP binding cassette transporter A1 (ABCA1) is a membrane transporter that facilitates the movement of cellular free cholesterol (FC) and phospholipid (PL) across the plasma membrane to combine with apolipoproteins, principally apoA-I, forming nascent HDL particles that are subsequently converted in plasma to mature HDL. Interest in understanding the metabolism of plasma HDL arises from studies that document an inverse association between plasma HDL cholesterol (HDL-C) concentration and cardiovascular disease (CVD) risk [1]. The apparent protective effect of elevated plasma HDL-C levels is likely related to the role of HDL in facilitating macrophage reverse cholesterol transport (RCT), a process in which excess macrophage cholesterol in atherosclerotic plaques is effluxed to HDL particles, which then transport cholesterol back to the liver for excretion [2, 3]. Recent studies suggest that plasma HDL’s capacity to efflux cholesterol is a better predictor of CVD risk than static HDL-C concentrations [4].

ABCA1 is a key transporter in facilitating macrophage RCT [5]. Individuals with Tangier disease, a rare genetic disorder caused by functional mutations in ABCA1, have <5% of normal plasma HDL and massive storage of cholesteryl ester (CE) in macrophages throughout the body. Based on the Tangier disease plasma and macrophage lipid phenotype, one would predict increased CVD in these subjects. However, CVD is paradoxically similar to controls in many Tangier subjects [6] and in animal models with global deficiency of ABCA1 [7, 8]. This paradoxical finding is likely due to the 50% decrease in plasma atherogenic
apoB lipoproteins (apoB Lp; i.e., VLDL and LDL) that accompanies whole body deficiency of ABCA1. It has been difficult to determine tissue specific roles of ABCA1 expression on atherogenesis and CVD because ABCA1 is expressed to a variable extent in nearly all tissues of the body [9]. In particular, the presumed atheroprotective role of macrophage ABCA1, based primarily on the well-accepted function of ABCA1 to stimulate macrophage FC efflux, lacks sufficient supporting evidence.

Attempts to identify and isolate the role of macrophage ABCA1 in atherosclerosis development have relied on bone marrow transplantation (BMT) experiments to date. Several BMT studies have uniformly identified an atheroprotective role for leukocyte ABCA1 [7, 10-13]. Nevertheless, bone marrow (BM) contains a variety of cell populations, many of which have been implicated in atherogenesis. For instance, T and B lymphocytes [14, 15], natural killer cells [16] and dendritic cells [17] have been reported to affect atherosclerosis, making it difficult to distinguish the specific contribution of macrophage ABCA1 expression in atherosclerosis. Therefore, the impact of macrophage ABCA1 on atherogenesis is still poorly understood and requires further exploration.

To define the role of macrophage ABCA1 expression in atherosclerosis, we generated myeloid (i.e., macrophage and neutrophil) cell-specific ABCA1 knockout (MSKO) mice [18]. Macrophages from these mice have defective FC efflux and respond to Toll-like receptor (TLR) agonists with an exaggerated
production of inflammatory cytokines [18]. An initial atherosclerosis study using these mice crossed into the LDLrKO background suggested a minimal role for myeloid cell-specific ABCA1 in atherogenesis [19], in contrast to published BMT results [7, 10-13]. However, the initial study only investigated one time frame of atherogenic diet feeding (16 wks) and was performed with MSKO/LDLrKO mice in a mixed genetic background (90% C57BL/6, 10% 129 SVEV). Since 129 mice strains are atherosclerosis resistant relative to C57BL/6 mice [20], the 10% 129 SVEV background in MSKO/LDLrKO mice may have lessened atherosclerosis development. Thus, results of our previous study did not definitively address the role of macrophage ABCA1 in atherogenesis. To address this deficiency, we have backcrossed the MSKO/LDLrKO mice to >99% in the C57BL/6 background and investigated early to late stage atherosclerosis progression. Our studies show that myeloid cell-specific deletion in atherogenic diet-fed LDLrKO mice does not result in the expected increase in atherosclerosis development relative to LDLrKO mice due to a considerable reduction in plasma apoB Lp concentrations in MSKO/LDLrKO mice, resulting from reduced hepatic VLDL TG production. However, when differences in plasma apoB Lp concentrations between genotypes of mice are minimized by maintaining mice on a chow diet, expression of myeloid cell ABCA1 is atheroprotective.
Methods

Animals and Diet

MSKO mice were generated by gene targeting as described previously [18] and backcrossed with C57BL/6-LDLrKO mice (Jackson Laboratories) until they were >99% in the C57BL/6 background [21]. LDLrKO and MSKO/LDLrKO mice used for these studies were generated from crosses of LysM Cre, ABCA1+/flox, LDLrKO X ABCA1+/flox, LDLrKO. PCR of genomic DNA was used to identify offspring of the appropriate genotype [18]. Female and male mice were fed a chow diet for 24wks or switched to an atherogenic diet containing 10% palm oil and 0.2% cholesterol at 7-9 wks of age for 10-24 wks. Mice were housed in a pathogen-free facility on a 12h light/dark cycle. All protocols and procedures were approved by the institutional animal care and use committee.

Plasma lipid and lipoprotein analysis

Plasma was collected from 4h-fasted mice by tail bleeding or cardiac puncture. Plasma total cholesterol (TPC), free cholesterol (FC) and triglycerides (TG) levels were measured by enzymatic assays using commercial kits [18]. Plasma cholesterol mass distribution among lipoproteins was determined after fast protein liquid chromatographic fractionation of plasma using a Superose 6 10/300 column (GE Healthcare). Cholesterol concentration in each lipoprotein fraction was calculated by multiplying each animal’s TPC values by the fractional distribution of cholesterol in each lipoprotein fraction.
In vivo quantification of VLDLTG secretion rate

Tyloxapol (500mg/kg body weight) was intravenously injected into 4h-fasted, anesthetized, atherogenic diet-fed mice (n=6-7) to block TG lipolysis. The accumulation of TG before (0 min), and 30, 60, 120 and 180 min after injection was determined by enzymatic assay. TG secretion rate was calculated using linear regression analysis to determine the slope of the time vs. TG concentration plot for each individual animal.

Macrophage cholesterol content

Resident peritoneal cells were harvested from mice fed either chow or the atherogenic diet after a 4h fast by flushing the peritoneal cavity with PBS. Cells were pelleted and resuspended in RPMI 1640 medium containing 1% nutridoma-SP (Roche Applied Science), 100 units/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine for a two hour incubation at 37°C. Non-adherent cells were removed by washing with PBS, leaving adherent macrophages for lipid extraction using isopropyl alcohol. Cellular TC and FC content was measured by gas-liquid chromatography (GC) and normalized to macrophage protein, determined by Lowry protein assay as described previously [18].

Analysis of atherosclerotic lesions

Mice were anesthetized with ketamine/xylazine and the vasculature was perfused with ice-cold PBS. Aortas were then isolated and fixed in 10% buffered formalin. Fixed aortas were cleaned of adventitial fat and lipid extracted to
quantify FC and TC content by gas-liquid chromatography [22]. Aortic roots were frozen in Optimal Cutting Temperature (Tissuetek) media in a plastic mold and cut at 8µm intervals. Sections were collected from the apex of the heart moving toward the aortic region and sequentially placed on 20 slides, such that each slide had sections 160 µm apart. The sections were stained with Oil Red O for aortic root lesion area determination and immunostained with CD68 for quantification of lesional macrophage content as previously described [23].

**Bone marrow transplantation (BMT)**

BM cells were harvested from cleaned femurs and tibias of male MSKO/LDLrKO and LDLrKO control mice and resuspended in serum-free RPMI 1640 medium. Female LDLrKO recipient mice (Jackson Laboratories) were fasted overnight and received a lethal dose of radiation (900 rads) 4h prior to BM injection. BM cells (7 × 10^6/ mouse) were injected into the retro-orbital venous plexus of anesthetized recipient mice. Recipient mice received autoclaved, acidified (pH 2.7) water supplemented with 100 mg/l neomycin and 10 mg/l polymyxin B sulfate 3 days before and 2 weeks after the transplantation. Mice were then given acidified water until the end of the study as described previously [24].

**Statistical analysis**

Values are shown as mean ± standard error of the mean. Data were analyzed using two-tailed student’s t test using Graphpad Prism software. A p=0.05 value was considered statistically significant and is indicated as asterisk *. 

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Results

Myeloid-specific ABCA1 deletion reduces plasma lipids in atherogenic diet-fed LDLrKO mice

To study atherosclerosis development in a stage-specific manner (i.e., early to advanced), MSKO/LDLrKO and LDLrKO mice were switched from chow at 7-9 wks of age to an atherogenic diet for 10, 16, or 24 wks with periodic measurements made of plasma total cholesterol (TPC), FC and triglyceride (TG) levels during disease progression. Chow-fed (i.e., baseline) MSKO/LDLrKO and LDLrKO mice displayed similar plasma lipid levels (Figure 1A-B; 0 wks), consistent with previous results from chow-fed MSKO and wild type mice [18]. Two weeks of atherogenic diet consumption led to increased TPC and FC in both genotypes, but the increase was significantly attenuated in MSKO/LDLrKO mice throughout the 16 wk disease progression phase (area under curve; P< 0.05) (Figure 1A-B). Plasma TG concentrations varied little during atherogenic diet feeding but were lower in MSKO/LDLrKO vs. LDLrKO mice (Figure 1A-B). FPLC fractionation of plasma showed that lipoprotein cholesterol distribution was similar for both groups of chow-fed mice (Figure 1C). However, after 16 wks of atherogenic diet feeding, plasma VLDL-C (100.7 vs. 189.3 mg/dl in males, P<0.05; 131 vs. 369 mg/dl in females, P<0.05) and LDL-C (410 vs. 508 mg/dl in males, P<0.05; 253 vs. 363 mg/dl in females, P<0.05) concentrations were significantly lower for both genders of MSKO/LDLrKO mice relative to their LDLrKO counterparts (Figure 1D). Collectively, these results suggest a novel role for myeloid cell ABCA1 in apoB Lp metabolism under atherogenic
hyperlipidemia conditions and further support its minimal contribution to the plasma HDL pool [18].

**VLDL secretion is decreased in atherogenic diet-fed MSKO/LDLrKO mice**

To determine the potential mechanism for decreased plasma apoB Lp concentrations in atherogenic diet-fed MSKO/LDLrKO mice, we measured VLDL secretion *in vivo* using the Triton block procedure. Secretion of VLDL TG mass was significantly reduced in MSKO/LDLrKO vs. LDLrKO mice (4.0 mg/dl/min vs. 3.1 mg/dl/min, P<0.05) ([Figure 2A-B](#)). In addition, hepatic TG content was significantly lower in MSKO/LDLr vs. LDLrKO mice after 16 wks of atherogenic diet feeding ([Figure 2C](#)), whereas TC, FC and CE were not ([Supplemental Figure I](#)). We also investigated food intake, fractional cholesterol absorption, and fecal neutral sterol loss and observed no significant difference between genotypes of mice ([Supplemental Figure II](#)). Overall, these data suggest a novel role for myeloid cell ABCA1 in inducing VLDL production during atherogenesis in LDLrKO mice.

**ABCA1 deletion in macrophages results in massive cellular cholesterol accumulation**

Macrophage ABCA1 is critical in preventing excess cellular cholesterol accumulation [18, 25]. To determine the degree of *in vivo* macrophage cholesterol accumulation during atherosclerosis progression, we measured cellular cholesterol content in resident peritoneal macrophages (PMs). Despite
the significant reduction of apoB Lp in MSKO/LDLrKO mice, macrophages from these mice had dramatically higher TC, FC and cholesteryl ester (CE) content after 16 wks of atherogenic diet feeding compared to LDLrKO mice (643 vs. 19 ug CE/mg protein in males, P<0.01; 732 vs. 9 ug CE/mg protein in females, P<0.01) (Figure 3A-B). A similar trend was observed for mice fed the atherogenic diet for 24 wks, with the fold difference between genotypes being even greater (1496 vs. 48 ug TC/mg protein in females, P<0.01) (Figure 3C). These data suggest that macrophage ABCA1 deficiency results in a massive accumulation of CE that is progressive in the face of continued but stable hyperlipidemia (Figure 1) and that no other macrophage cholesterol efflux system can compensate for loss of ABCA1 over an extended (10-24 wk) period of hyperlipidemia.

**ABCA1 deletion in macrophages results in elevated plasma cytokines**

Macrophages lacking ABCA1 expression secreted more proinflammatory cytokines upon TLR4 stimulation with lipopolysaccharide (LPS) compared with macrophages expressing ABCA1 [18, 25]. Dietary saturated fatty acids also activate TLR4 signaling *in vitro* and *in vivo* [26]. To determine whether atherogenic diet fed MSKO/LDLrKO mice exhibit a chronic low grade inflammation *in vivo* that is greater than that of LDLrKO mice, we examined plasma cytokine protein expression semi-quantitatively using a cytokine array. More proinflammatory cytokines and chemokines were present in the plasma of MSKO/LDLrKO vs. LDLrKO mice fed the atherogenic diet for 16wks
Supplemental Figure III), suggesting an enhanced in vivo inflammatory response in MSKO/LDLrKO mice. These data suggest that the combination of atherogenic diet and resulting hyperlipidemia is sufficient to result in an exacerbated inflammatory state in vivo in MSKO/LDLrKO vs. LDLrKO mice during atherosclerosis progression.

Myeloid-specific ABCA1 deficiency does not affect blood leukocyte distribution

Previous studies have suggested that deletion of macrophage ABC transporters (ABCA1 and ABCG1) results in blood monocytosis, neutrophilia, and increased numbers of Ly6-C^hi monocytes, likely through increased expansion of hematopoietic stem or multipotential progenitor cells [27]. In our study, the percentage distribution of blood leukocytes was similar for both genotypes of atherogenic diet-fed mice (Supplemental Figure IV). In addition, the anticipated increase in percentage of Ly6-C^hi monocytes that accompanies diet-induced hyperlipidemia was similar for MSKO/LDLrKO and LDLrKO mice (Supplemental Figure IV E).

Myeloid-specific ABCA1 deficiency enhances advanced but not early or intermediate stage atherosclerosis development

We evaluated the effect of myeloid ABCA1 expression on atherosclerosis development using several measurements. MSKO/LDLrKO and LDLrKO mice fed the atherogenic diet for 10 wks or 16 wks had similar levels of aortic total
cholesterol (TC), FC, and CE content, though FC level was slightly, but significantly lower for 10wk diet-fed MSKO/LDLrKO mice (Figure 4A-B). Advanced atherosclerosis (i.e., 24 wks atherogenic diet) was enhanced in MSKO/LDLrKO mice as aortic TC and FC levels were increased relative to LDLrKO mice and CE was higher on average (Figure 4C). Cross-sectional analysis of aortic root lesions stained with Oil red O revealed comparable lesion size for both genotypes of mice fed the atherogenic diet for 16 wks (Figure 4D-E), supporting the aortic cholesterol measurements. In addition, no significant difference was observed in lesional macrophage (Figure 4F-G), T cell (Supplemental Figure V A-B), or collagen content (Supplemental Figure V C-D), suggesting similar aortic root lesion composition. Thus, myeloid cell ABCA1 expression had minimal impact on early/intermediate atherogenesis, but mildly enhanced late stage atherosclerosis in atherogenic diet-fed LDLrKO mice.

Similar extent of atherosclerosis in LDLrKO mice transplanted with MSKO/LDLrKO or LDLrKO bone marrow

Previous BMT studies uniformly found a significant atheroprotective role of leukocyte ABCA1, and macrophage ABCA1 was assumed to be the major player contributing to this effect. However, atherogenic diet-fed MSKO/LDLrKO mice did not display accelerated atherosclerosis under similar conditions (i.e. atherogenic diet for 10 or 16 wks). To determine whether the different outcomes were due to different experimental strategies (BMT vs. non-BMT) or to more specific deletion of ABCA1 (BM vs. myeloid cell), we transplanted male MSKO/LDLrKO (DKO) or
LDLrKO (SKO) BM into lethally-irradiated female LDLrKO recipient mice and measured atherosclerosis extent after 16 wks of atherogenic diet feeding. Successful replacement of hematopoietic cells was confirmed 4 wks after transplantation (Supplemental Figure VI). The plasma phenotype of BMT mice resembled that of the non-transplanted counterparts in our previous experiment. TPC and plasma TG levels in DKO BM recipients were ~40-50% lower than those of SKO BM recipients as soon as 2 wks after initiation of the atherogenic diet, and remained lower throughout the 16 wk progression phase (Figure 5A). There was only a 5% difference in basal TPC (i.e. chow diet) with indistinguishable cholesterol distribution among lipoproteins (Figure 5B, time 0), whereas VLDL-C and LDL-C contributed to the decreased TPC in atherogenic diet-fed DKO BM recipient mice (Figure 5B, 2-16 wks). In agreement with the non-transplant 16-week study, cholesterol content of resident PMs was greatly increased (Figure 5C), but whole aorta cholesterol content was not higher in DKO vs. SKO BM recipient mice (Figure 5D). These data suggest that the difference in outcome between our study and previous BMT studies is due to the more restrictive myeloid cell ABCA1 deletion in our study.

**Chow diet-fed MSKO/LDLrKO mice are more susceptible to early stage atherogenesis**

The finding that atherogenic diet-fed MSKO/LDLrKO mice had similar early and intermediate atherosclerosis compared with LDLrKO mice, but lower plasma VLDL and LDL concentrations and higher macrophage cholesterol content led us...
to hypothesize that the deleterious effects of ABCA1 deletion in macrophages were offset by the substantial reduction of plasma atherogenic apoB Lp. To test this possibility, we fed mice chow until they were 24 wks of age to minimize the differences in plasma lipids and lipoproteins observed with atherogenic diet feeding and investigated atherogenesis. This time course was similar to the previously described 16 wk atherogenic diet study (16 wks of atherogenic diet feeding starting at 8 wks of age) using non-BMT and BMT mice. As anticipated, chow-fed MSKO/LDLrKO vs. LDLrKO mice had similar TPC and plasma TG levels over time (Figure 6A-B). Although the chow-fed MSKO/LDLrKO mice had significantly lower TPC values compared with chow-fed LDLrKO mice, the difference was minimal (~6-17%) throughout the study and likely achieved statistical significance due to the very low variability in measurements. Plasma lipoprotein cholesterol distribution of 12 wk old mice revealed very minor differences between genotypes, with LDL-C and HDL-C being slightly, but significantly, decreased in MSKO/LDLrKO mice (113 mg/dl vs. 101 mg/dl LDL-C; 43 mg/dl vs. 36 mg/dl HDL-C) (Figure 6C). Resident PMs without ABCA1 expression exhibited a 1.5-fold and 3-fold increase in cellular FC and TC content, respectively, and a striking 70-80-fold induction in CE accumulation (0 vs. 71 ug CE/mg protein in females, P<0.05; 0 vs. 85 ug CE/mg protein in males, P<0.01) under mild hyperlipidemic conditions (Figure 6D). Whole aorta chemical analysis by gas-liquid chromatography showed low, but detectable, amounts of cholesterol compared to atherogenic diet-fed mice. A significant ~70% higher aortic CE content (0.77 vs. 1.31 ug CE/ mg protein, p<0.05) in MSKO/LDLrKO
mice suggests increased very early stage atherosclerosis compared with LDLrKO mice (Figure 6E). Analysis of aortic root Oil red O-stained sections from representative animals of each group also showed a ~76% higher lesion size for MSKO/LDLrKO vs. LDLrKO mice, though the difference did not achieve statistical significance (0.0342 mm² vs. 0.0598 mm²) (Figure 6F). Collectively, these results suggest that in the absence of major differences in plasma lipid levels, deletion of myeloid cell ABCA1 enhanced early atherosclerotic lesion development, supporting the conclusion that less atherogenic lipid profiles may counterbalance the proatherogenic effects in MSKO/LDLrKO mice, preventing the expected increase in early/intermediate stage atherogenesis.
Discussion

Macrophages are a major cellular constituent of atherosclerotic lesions and are involved in the uptake and breakdown of modified lipoproteins [28]. This process can result in the formation of CE-enriched macrophages or foam cells. ABCA1 and other export proteins (e.g., ABCG1 and SR-BI) counterbalance the unregulated accumulation of macrophage CE by effluxing excess cellular cholesterol for subsequent transport by HDL particles to the liver for excretion (i.e., RCT) [29]. BMT studies using whole body ABCA1 knockout mouse BM have suggested that genetic deletion of macrophage ABCA1 uniformly increases atherosclerosis [7, 10-12]. However, the role of macrophage ABCA1 in atherosclerosis development has never been formally tested as BM used for transplantation studies contains other leukocytes that are likely important in atherosclerosis progression [30]. We addressed this question by performing atherosclerosis studies using a unique animal model with genetic deletion of ABCA1 in leukocytes of myeloid lineage crossed into the LDLrKO C57BL/6 background (i.e., MSKO/LDLrKO). We report that, contrary to expectation, atherogenic diet-fed MSKO/LDLrKO mice did not have accelerated atherosclerosis relative to LDLrKO mice at early and intermediate time points due to an unanticipated decrease in plasma VLDL and LDL concentrations. Only advanced atherosclerosis (24 wks of atherogenic diet feeding) was modestly but significantly increased in MSKO/LDLrKO mice. Similar atherosclerosis and plasma lipoprotein results were obtained for intermediate stage atherosclerosis using transplantation of SKO and DKO BM into LDLrKO recipient mice,
suggesting that our unique finding was not due to a different study design (i.e., non-transplantation vs. BMT) compared with previous BMT studies. We hypothesized that the negative impact of myeloid cell ABCA1 deletion in vivo was offset by an atheroprotective reduction in plasma VLDL and LDL, significantly delaying (16 wks to 24 wks) and minimizing the increase in atherosclerosis in MSKO/LDLrKO vs. LDLrKO mice. In support of this hypothesis, minimizing the difference in plasma VLDL and LDL by maintaining mice on a chow diet resulted in a modest, but significant increase in atherosclerosis in MSKO/LDLrKO mice. Overall, our study results suggest that myeloid ABCA1 expression is atheroprotective in similar states of hyperlipidemia and has a novel function in controlling plasma VLDL and LDL concentrations.

This is the first atherosclerosis study using MSKO/LDLrKO mice backcrossed into the C57BL/6 background. Brunham et al [19] previously reported that atherosclerosis development was similar for MSKO/LDLrKO and LDLrKO mice fed an atherogenic diet for 16 wks, in agreement with our results (Figure 4). However, mice used in that study were in a mixed genetic background (~90 C57BL/6, ~10% 129 SVEV) and since the 129 background is atherosclerosis resistant [20], it was unclear whether the similarity in atherosclerosis between MSKO/LDLrKO and LDLrKO mice was due to background effects. In addition, previous BMT studies using different study designs (i.e., diets: chow, Western, Paigen; genetic backgrounds: apoE, LDLrKO) uniformly observed a 1.4-1.8-fold increase in early to intermediate stage (10-14 wks diet feeding) atherosclerosis.
development [7, 10-13]. In two of the studies, atherosclerosis increased 40-60% despite a significant reduction of plasma cholesterol levels [10, 12]. The atheroprotective effect of ABCA1 observed in these BMT studies was mainly attributed to macrophage ABCA1. However, our results using a more restrictive deletion of ABCA1 in BM cells of myeloid origin showed no difference in atherosclerosis at early (10 wks) and intermediate (16 wks) stages of progression. The different outcomes may have arisen from distinct experimental strategies (i.e. BMT vs. myeloid cell-specific gene targeting) or the relative contribution of non-myeloid BM vs. myeloid cell ABCA1 expression in atherogenesis. The first possibility was eliminated by finding similar atherosclerosis and plasma lipoprotein outcomes as our non-transplant study after completion of a 16 wk BMT study using atherogenic diet-fed LDrKO recipient mice transplanted with MSKO/LDLrKO or LDLrKO BM. The significantly accelerated atherosclerosis development observed in earlier BMT studies is likely due to an atheroprotective impact of ABCA1 expression on non-myeloid BM cells, since more specific deletion of ABCA1 in only myeloid cells under similar experimental conditions failed to exacerbate atherogenesis over the same time frame.

Macrophages from chow-fed MSKO mice have defective FC and PL efflux [18]. Although CE accumulation is minimal in chow-fed MSKO mouse macrophages, FC is significantly elevated (~10%), leading to increased membrane lipid raft content and hyperresponsiveness to proinflammatory agents like LPS. Previous studies showed that macrophage FC efflux is facilitated by ABCA1, ABCG1, SR-
BI and aqueous diffusion [5]. In the hyperlipidemic background of LDLr deficiency, resident MSKO macrophages had massive CE accumulation that increased in severity from early to late stages of atherosclerosis development. In fact, even chow fed MSKO/LDLrKO mice had elevated resident macrophage CE content. These data document that loss of myeloid cell ABCA1 expression in atherogenic diet-fed LDLrKO mice results in progressive accumulation of macrophage CE even though plasma lipoprotein concentrations were relatively stable after two wks of atherogenic diet feeding. In addition, the results suggest that no other efflux mechanism can compensate for loss of macrophage ABCA1 in vivo to prevent massive macrophage CE accumulation.

Another novel finding from our study was that plasma VLDL and LDL concentrations were ~50% lower in atherogenic diet-fed MSKO/LDLrKO vs. LDLrKO mice, suggesting an in vivo role for macrophage ABCA1 expression in regulating plasma apoB Lp metabolism during atherogenesis. Of interest, similar observations were made in two published BMT studies after global ABCA1 knockout vs. WT BM was transplanted into LDLrKO mice [10, 12]. Although the two BMT studies did not address this finding with additional experiments, we show that reduced hepatic VLDL TG secretion can at least partially contribute to lower plasma VLDL. The liver plays a central role in regulating plasma lipid levels through synthesis, secretion and clearance of lipoproteins, particularly apoB Lp. The rate of TG synthesis is determined by substrate availability [31] and hepatic lipid analysis revealed a significant ~40% reduction in TG accumulation in
MSKO/LDLrKO vs. LDLrKO mice after 16wk atherogenic diet consumption (Figure 2C) with no change in the content of other hepatic lipids (Supplemental figure I). A similar trend (p=0.08) towards decreased hepatic TG content was observed in 16 wk atherogenic diet-fed LDLrKO recipient mice receiving MSKO/LDLrKO BM (data not shown). The decreased hepatic TG content was associated with decreased expression of some lipogenic genes, such as FAS and ACC1 (data not shown), suggesting decreased de novo lipogenesis. Since cytokines can modulate lipid metabolism [32] and macrophages are an important source of cytokines, the different plasma cytokine profiles and decreased hepatic apoB Lp production in atherogenic diet-fed MSKO/LDLrKO mice may result from macrophage cytokine production locally (i.e., Kupffer cells) or systemically. However, acute ablation of Kupffer cells did not normalize the difference in TPC and plasma TG between genotypes of mice (Supplemental Figure VII), suggesting that resident hepatic macrophages were not affecting hepatic lipogenesis and TG secretion. Moreover, Kupffer cells are replaced relatively slowly after BMT, possibly giving rise to a mixed donor and recipient Kupffer cell population [24, 33]. The fact that the plasma lipid phenotype in our BMT study recapitulated the 16wk atherogenic diet study also suggests that Kupffer cells may not be the major player in mediating altered liver lipid metabolism in this case. The underlying mediator and mechanism responsible for decreased hepatic TG production in atherogenic diet-fed MSKO/LDLrKO mice has yet to be determined.
A perplexing observation in our study was the similar aortic cholesterol content in atherogenic diet-fed MSKO/LDLrKO and LDLrKO mice, even though resident PM cholesterol content was considerably greater in MSKO/LDLrKO mice. These observations suggest that resident PMs do not have the same phenotypic characteristics as macrophages in atherosclerotic lesions or that fewer CE-enriched macrophages accumulated in the lesions of MSKO/LDLrKO mice. The latter possibility was not supported by our finding that CD68+ aortic lesion area was similar for MSKO/LDLrKO and LDLrKO mice (Figure 4F-G). Hyperlipidemic mouse models (i.e., LDLrKO and apoEKO) exhibit monocytosis and increased trafficking of monocytes into atherosclerotic lesions when fed cholesterol-containing atherogenic diets [34, 35]. We also observed an atherogenic diet-induced Ly6C\textsuperscript{hi} monocytosis that was similar for both genotypes of mice (Supplemental Figure 7), a finding that is compatible with similar aortic root lesional macrophage content for both genotypes of mice. Attempts to isolate F4/80+ cells from aortas of mice fed the atherogenic diet for 20 wks using FACS failed to yield sufficient cells for mass spectrometric quantification of FC and CE. Considering the likely distinct differences in local environment where macrophages reside (i.e. peritoneal cavity vs. atherosclerotic plaque), characterization of lesional macrophages, though difficult to approach, would be worthy of further investigation.

In conclusion, we investigated the effects of myeloid cell-specific ABCA1 deletion on early to advanced stage atherosclerosis development. Myeloid cell ABCA1
expression appears to be modestly atheroprotective in chow-fed mice, in which plasma lipid changes are minimal, but its atheroprotective effect is eliminated or significantly delayed in atherogenic diet-fed LDLrKO mice due to a paradoxical decrease in plasma VLDL and LDL concentrations, resulting from decreased hepatic production of VLDL TG. Our findings also indirectly support an atheroprotective role for ABCA1 expression in non-myeloid cells in atherogenesis given the difference in atherosclerosis outcome in BMT studies using MSKO/LDLrKO vs. global ABCA1 knockout BM. Finally, this study highlights the multifaceted impact of myeloid ABCA1 expression on atherogenesis and lipoprotein metabolism that leads to complex patterns of atherosclerosis progression.
Acknowledgments

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Disclosures- None
Reference


Figure 1. Plasma lipid and lipoprotein concentrations. Fasting (4h) plasma total cholesterol (TPC), free cholesterol (FC), and triglyceride (TG) concentrations in male (A) and female (B) mice during a 16 wk atherosclerosis progression phase were measured by enzymatic assays (n=5-11). Plasma cholesterol distribution among lipoproteins before (C; chow-fed at 7-9 wks of age) or after (D) 16 wks of atherogenic diet feeding were determined after fractionation of plasma by FPLC (n=4-14). Data expressed as mean ± SEM. * P<0.05. MSKO, Myeloid-specific ABCA1 knockout.
Figure 2

A. Plasma TG

B. TG Secretion Rate

C. Liver TG

LDLrKO

MSKO/LDLrKO

LDLrKO

MSKO/LDLrKO

chow

16 wk diet
Figure 2. VLDL secretion was determined after *in vivo* inhibition of TG lipolysis with triton administration. Plasma TG levels were measured by enzymatic assay before (0 min) and after (30, 60, 120, 180 min) Triton injection intravenously. (A) Plasma TG concentration (mean ± SEM; n=6-7) was plotted for each genotype of mice and the line of best fit was determination by linear regression analysis. (B) TG secretion rate during the 3h experiment was calculated for each animal as the slope of the regression line. Results were then plotted for both groups of mice as mean ± SEM, n=6-7. * $P<0.05$. (C) Hepatic TG content was measured by enzymatic assay and normalized to liver protein. Results are from two separate atherosclerosis studies (chow and 16 wk atherogenic diet). Data are expressed as mean ± SEM (n=6-12).
Figure 3
Figure 3. Resident peritoneal macrophage cholesterol content. Resident macrophages were isolated by peritoneal lavage from atherogenic diet-fed mice. After a 2h culture in RPMI medium containing 1% Nutridoma and removal of non-adherent cells, the adherent macrophages were lipid extracted and the cellular total cholesterol (TC) and free cholesterol (FC) content were quantified by gas-liquid chromatography (GC). CE was calculated as (TC-FC) x1.67. Resident peritoneal macrophage cholesterol content in male (A) and female (B) mice fed atherogenic diet for 16wks. (C) Cholesterol content of resident PMs from female mice fed atherogenic diet for 24wks. Data are normalized for cellular protein and expressed as mean ± SEM (n=5-11). * P<0.05.
Figure 4

Atherogenic Diet

A. 10 weeks
B. 16 weeks
C. 24 weeks

LDLrKO vs. MSKO-LDLrKO

D. ORO
E. Oil Red O

F. CD68
G. (Mcrophage)

 ug cholesterol/protein
Figure 4. Atherosclerosis in atherogenic diet-fed mice. Aortas were lipid extracted for quantification of total cholesterol (TC) and free cholesterol (FC) content by GC. Cholesterol ester (CE) content was calculated as (TC-FC) x1.67. Aortic cholesterol of mice fed atherogenic diet for 10wks (A), 16 wks (B) and 24wks (C). (D) Representative LDLrKO (left) and MSKO/LDLrKO (right) mouse aortic root sections stained with Oil Red O. (E). Aortic root lesion area. Each point represents the average lesion area of 3 sections per mouse. (F) Representative LDLrKO (left) and MSKO/LDLrKO (right) mouse aortic root sections immunostained with CD68. (G) Percentage aortic root lesional area occupied by CD68+ cells. Each point represents the average lesion area of 4 sections per mouse.
Figure 5. Bone marrow transplantation. Seven weeks after transplantation of LDLrKO (SKO) of MSKO/LDLrKO (DKO) bone marrow, LDLrKO recipient mice were switched from chow to an atherogenic diet for 16 wks. (A) Periodic TPC, plasma FC, and TG measurements were made during the atherosclerosis progression phase. (B) Pooled plasma from subgroups of mice (n=3 plasma pools from 5 mice) was fractionated by FPLC to measure lipoprotein cholesterol distribution. (C) Cholesterol content (TC, total cholesterol; FC, free cholesterol, CE, cholesteryl ester) of resident PMs from recipient mice fed atherogenic diet for 16 wks was determined by gas-liquid chromatography. (D) Aortic cholesterol content of mice fed atherogenic diet for 16 wks. Data expressed as mean ± SEM (n=15).
Figure 6

A. TPC

B. Plasma TG

C. Lipoprotein Cholesterol

D. Female

E. Aortic Cholesterol

F. Oil Red O
Figure 6. Atherosclerosis study in chow-fed mice. (A) TPC and (B) plasma TG were measured by enzymatic assays in chow-fed mice were at different ages. (C) Plasma lipoprotein cholesterol distribution was determined in chow-fed mice at 12 wks of age. (D) Resident PM TC, FC and CE content were assayed by gas-liquid chromatography. Atherosclerosis was evaluated by two measurements. (E) Aortic cholesterol content of female chow-fed mice (n=15-16). (F) Aortic root lesion area of representative animals from each genotype (n=8). Each point represents the average lesion area of 4 sections per mouse. Data expressed as mean ± SEM. * P<0.05.
Supplemental methods:

Food intake
Atherogenic diet-fed mice were separated into individual wire-bottom, no-bedding cages for daily diet consumption measurements. The weight of diet fed to the mice and the remaining diet left on the second day were measured for 4 days to evaluate daily food intake. The values were then normalized for body weight measured at the beginning of the experiment.

Fractional cholesterol absorption and fecal neutral sterol loss
Intestinal cholesterol absorption and fecal neutral sterol excretion were performed with atherogenic diet-fed mice as described previously [1].

Plasma Cytokine
Plasma from 16wk atherogenic diet-fed mice was pooled (n=4) for cytokine analysis using a commercial Proteome Profiler Mouse Cytokine Array Kit, Panel A (R&D, Minneapolis, MN).

Aortic root lesion analysis (CD3, collagen)
Aortic root sections were prepared as described under “analysis of atherosclerosis lesions” section of the methods. Aortic root cross-sections were stained for CD3 (rat anti-mouse CD3 monoclonal antibody, Serotec) and Masson’s Trichrome. The number of CD3+ cells within lesions were manually counted at 20 x magnifications or quantified as CD3+ lesion area using a color
picking method in Image Pro software. Collagen was stained blue using Masson's Trichrome. The blue color stained area within lesions was identified as collagen positive area using Image Pro software. An average of 3 sections from each animal was used for comparison between groups.

**Hematopoietic cell replacement in BMT**

To confirm the replacement of hematopoietic cells by donor BM, the presence of male DNA in hematopoietic genomic DNA was determined 4 weeks after BMT. Briefly, genomic DNA from whole blood was extracted with a Wizard Genomic DNA Purification kit (Promega). The y-chromosome associated sex-determining region Y gene (Sry) was amplified by PCR to a linear amplification phase under the following conditions: 95°C for 3 min followed by 30 cycles of 94°C for 30 s, 61°C for 1 min, and 72°C for 1 min. Acyl-CoA: cholesterol acyltransferase 2 (ACAT2) was amplified by PCR as an internal control. The PCR products were separated on 0.8% agarose gels and visualized with ethidium bromide.

**Real-time PCR Analysis**

Mice were sacrificed and tissues were harvest and snap frozen in liquid N₂. Total RNA was isolated from liver of male mice using TRIzol (invitrogen), and real-time RCR was performed as reported previously [2]. Primer sequences were the same as described previously [3]. GAPDH was used as the endogenous control.
Hepatic lipid analysis

Hepatic lipid content was performed as described previously using enzymatic assays of detergent-extracted liver [4].

GdCl₃-induced ablation of Kupffer cells

GdCl₃.6H₂O (Sigma Aldrich) powder was reconstituted to 6 mg/ml with saline and sterilized by passing through a 0.22 µm filter. Atherogenic diet-fed, anesthetized mice were injected with the GdCl₃ solution (25µg/g body weight) through the retro-orbital venous plexus. Injections were repeated every 4 days for a total of 3 injections. TPC and plasma TG levels were measured at indicated days.

Flow cytometry

Blood was harvested by cheek bleeding and samples were kept on ice throughout the procedure for leukocyte subset analysis, unless otherwise indicated. White blood cells (WBC) were isolated after lysing (GIBCO A10492) red blood cells and resuspended in staining buffer (PBS containing 3% FBS). Cells were stained with a cocktail of antibodies consisting of FITC-Ly6C, PE-Cy7-CD19, APC-CD45, APC-Cy7 CD3e (BD pharmingen), PE-CD115 and PerCP-cy5.5-Ly6G (eBioscience). CD45+CD115+Ly6G- cells were identified as monocytes and further separated into Ly6C<sup>hi</sup> and Ly6C<sup>lo</sup> subpopulations. Neutrophils, T cells and B cells were identified as CD45+Ly6G+CD115- cells, CD45+CD3e+, and CD45+CD19+, respectively. Cell fluorescence was
determined using a FACSCanto II flow cytometer (BD Biosciences) and analyzed with Diva software.

**Supplemental Figure Legends**

**Supplemental Figure I. Hepatic lipid content.** Liver TC (A), FC (B) and CE content (C), calculated as (TC-FC) x 1.67 were measured by enzymatic assay. Results are from two separate atherosclerosis studies (chow and 16 wk atherogenic diet) and were normalized to liver protein. Data are expressed as mean ± SEM (n=6-12). * P<0.05.

**Supplemental Figure II. Whole body cholesterol homeostasis.** Food intake (A), fractional cholesterol absorption (B) and fecal neutral sterol loss (C) were determined in atherogenic diet fed female LDLrKO and MSKO/LDLrKO mice. Data are expressed as mean ± SEM (n=6-10).

**Supplemental Figure III. Plasma cytokine levels.** The presence of plasma cytokines and chemokines was determined by a commercial cytokine array kit. (A) Images of expression of different cytokines in plasma of LDLrKO (top) and MSKO/LDLrKO (bottom) mice. Pixel density of different dots was quantified (B). Data are expressed as mean ± SEM.

**Supplemental Figure IV. Blood leukocyte distribution.** Before (i.e. chow) and after atherogenic diet feeding for 13wks (i.e. atherogenic), percentage distribution
of peripheral neutrophils (A), B cells (B), T cells (C), monocytes (D), as well as Ly6C hi (E) and Ly6C lo (F) subsets of monocytes were quantified by flow cytometry. Results are normalized to blood CD45+ cells. Data are expressed as mean ± SEM (n=5-9).

**Supplemental Figure V. Aortic root lesion analysis.** Aortic root sections from 16wk atherogenic diet-fed mice were stained with CD3 or Masson’s trichrome for quantification of T cell and collagen content, respectively. Representative images from sections stained with CD3 (A) and Masson’s trichrome (C). T cells per section were counted and average number of T cells per mouse was plotted (B). Percentage of blue-stained collagen+ area in total lesion area was compared between groups (D). Data are expressed as mean ± SEM (n=7-8).

**Supplemental Figure VI. Verification of transplant efficiency of hematopoietic cells after BMT.** The presence of the male sry gene in female recipient mouse blood was confirmed by PCR analysis of genomic DNA in blood. ACAT2 was used as an internal control. The PCR products were separated on a 0.8% agarose gel and visualized with ethidium bromide. Representative (n=5/donor group) LDLrKO recipient mice transplanted with BM from LDLrKO and MSKO/LDLrKO mice are shown along with male and female control samples for reference.
Supplemental Figure VII. Acute Kupffer cell ablation. TPC (A, C) and plasma TG (B, D) concentrations in female and male mice before (day 0) and after GdCl₃ injection (day 6, 12 and 18) were measured by enzymatic assays. Data are expressed as mean ± SEM (n=6-12).


Supplemental Figure I.
Supplemental Figure II.
Supplemental Figure IV.
Supplemental Figure V.
Supplemental Figure VI.
Supplemental Figure VII

**Female**

**A**

TPC

Time (day)

mg/dl

0 6 12 18

**B**

TG

Time (day)

mg/dl

0 6 12 18

**Male**

**C**

TPC

Time (day)

mg/dl

0 6 12 18

**D**

TG

Time (day)

mg/dl

0 6 12 18
Chapter IV

DISCUSSION

Xin Bi prepared this chapter. Dr. John Parks acted in an advisory and editorial capacity.
Atherosclerosis related CVD is the leading cause of death in the United States [1]. Efforts have been made to lower plasma cholesterol since hypercholesterolemia and elevated apoB Lp are well-accepted primary risk factors for atherosclerosis development [2, 3]. The beneficial effect of stains is obvious with a 20%-40% decrease in CVD events [4]. The large residual disease burden remaining in statin-treated individuals has focused attention on therapies to raise plasma HDL levels, which are inversely associated with CVD risk. The discovery of ABCA1 as the underlying molecular defect in TD, a rare disease featuring a near absence of plasma HDL and increased risk of CVD in some kindreds [5], further emphasized the importance to elucidate the relationship between ABCA1 and atherogenesis. However, conflicting results exist regarding the role of global ABCA1 expression and CVD risk and severity in humans and animals [6-13], which are thought to arise from the multifaceted effects of ABCA1 on lipid metabolism. The ubiquitous presence of ABCA1 throughout the body along with transcriptional and post-translational tissue-specific variation in expression makes it difficult to distinguish the tissue/cell specific effects in the context of atherogenesis. In this dissertation work, we used unique mouse models with genetic deletion of ABCA1 in hepatocytes or myeloid cells to explore the impact of ABCA1 expression in the liver and macrophages, respectively, on atherosclerosis and lipid metabolism.

Reverse cholesterol transport (RCT), a physiologic process originally proposed several decades ago [14], has long been hypothesized to be the main mechanism through which HDL protects against atherosclerotic CVD. In the
context of atherosclerosis development, the process initiates from intimal macrophages and hence termed macrophage RCT. The cholesterol-laden macrophage foam cell is a cardinal feature of atherosclerotic lesions. Efflux of cholesterol from lesional foam cells, the initial step of RCT involving specific cellular transporters and extracellular HDL-based acceptors, may reduce lesion progression and complication [15]. Cholesterol esterification in circulation or delivery of cholesterol back to liver may also be relevant to atherogenesis by affecting the rate of macrophage RCT. Recent studies suggest that elevated HDL-C levels do not always translate into reduced CVD events [16, 17]. In contrast, the capacity of human apoB Lp-depleted plasma to efflux cholesterol from macrophages has recently been shown to be inversely associated with CVD risk independent of HDL-C levels in humans [18]. The rate of macrophage RCT in mice with various genetic or pharmacologic manipulations correlates better with susceptibility to atherosclerosis than steady state plasma HDL-C levels [19].

ABCA1 is well-known for its essential role in facilitating FC and PL transport across cell membrane for nascent HDL formation. Studies in chow-fed tissue-specific ABCA1 knockout mice established the liver as the single most important source of plasma HDL in vivo [20]. Conversely, macrophage ABCA1 contributes minimally to maintenance of plasma HDL pool [21], but significantly promotes in vitro cholesterol efflux to lipid-poor apoA-I and in vivo macrophage RCT [22, 23]. Based on these earlier findings, we investigated the effects of hepatocyte (Chapter II) and macrophage (Chapter III) ABCA1 expression on atherogenesis, since they may protect against atherosclerosis by supporting hepatic HDL
production, thereby providing HDL particles to participate in RCT, and by facilitating cholesterol efflux from foam cells, respectively.

We monitored plasma lipid and lipoprotein metabolism, risk factors for atherosclerosis, and observed novel phenotypes for both studies. The relative contribution of various cell types to systemic HDL-C levels has been established by a series of studies performed in chow-fed mice using tissue-specific gene targeting strategy [20, 21, 24-26]. Consistent with studies in chow-fed mice, in Chapter II we demonstrated that hepatocyte ABCA1 expression plays a critical role in maintaining plasma HDL-C pool in atherogenic diet-fed LDLrKO mice. Deletion of hepatic ABCA1 in chow-fed LDLrKO mice resulted in a remarkable 80-90\% reduction in plasma HDL-C. A significant 50\% lower HDL-C in atherogenic diet-fed HSKO/LDLrKO vs. LDLrKO mice further confirmed the substantial contribution of hepatocyte ABCA1 to the plasma HDL pool under hyperlipidemic conditions. Examination of plasma HDL in MSKO/LDLrKO vs. LDLrKO mice in Chapter III revealed comparable HDL-C between genotypes before and after atherogenic diet consumption for all three study groups, strongly supporting the concept that HDL particles assembled by macrophage ABCA1 are quantitatively insignificant relative to the total plasma HDL pool. Overall, our results suggest that the liver is the major source of plasma HDL in normolipidemic and hyperlipidemic states.
Cholesterol efflux by ABCA1 is a critical initial component in macrophage RCT pathway. In Chapter III, we observed massive CE accumulation in resident peritoneal macrophages from MSKO/LDLrKO mice even when these mice were maintained on chow diet and had similar plasma HDL levels as LDLrKO mice. The severity of cholesterol accumulation increased as atherosclerosis progressed from early (10 wks) to late (24 wks) stages even though hyperlipidemia remained relatively uniform within 2 wks of initiation of atherogenic diet feeding. Besides ABCA1, several other pathways mediating macrophage FC efflux to plasma HDL particles in vitro involve active export by ABCG1 and SR-B1 in addition to passive aqueous diffusion facilitate, ridding macrophages of excess cholesterol [15]. However, optimal in vivo macrophage RCT appears dependent on ABCA1 and ABCG1, but not SR-BI [22]. Despite the presence of multiple pathways for elimination of excess cholesterol in macrophages, they were unable to compensate for loss of macrophage ABCA1 in vivo to prevent massive foam cell formation in our study. This finding is also supported by the wide-spread presence of CE-enriched macrophages in subjects with TD in the absence of hyperlipidemia [5]. These findings support the argument that ABCA1 is likely the most important pathway for macrophages to eliminate excess cellular cholesterol, at least with regard to peritoneal macrophages.

HSKO/LDLrKO mice had a predicted reduction in HDL-C, but paradoxically, no alternation in macrophage RCT compared to LDLrKO mice (Chapter II). A small
but dynamic HDL pool, such as pre-β1 HDL, may be able to transport cholesterol from macrophage foam cells to the liver without a detectable increase in HDL-C. Some studies suggested that the quality of HDL, characterized by particle number, size and function, may be more important in preventing disease progression and a better predictor of CVD risk than steady-state HDL-C levels [18, 27-29]. Moreover, the pathway of HDL-C delivery to the liver also has an impact on the rate of RCT. A well-studied example is SR-BI expression in the liver. Overexpression of hepatic SR-BI significantly decreased plasma HDL-C levels but greatly induced macrophage RCT, whereas deletion of SR-BI substantially increased HDL-C levels but impaired RCT [30]. Recently, a study by Yamamoto and colleagues supports our findings that a remarkable reduction in HDL-C did not have major impact on macrophage RCT [31]. In this study, pharmacologic inhibition of ABCA1 by probucol resulted in equivalent macrophage RCT in chow-fed WT mice despite an 80% reduction of plasma HDL-C. Additionally, SR-BI knockout mice treated with probucol had a significant 63% decrease in HDL-C and stimulated macrophage RCT [31]. A potential explanation for the maintenance of macrophage RCT in the face of 80% reductions in HDL-C is that hepatocyte ABCA1 may normally recycle a significant amount of plasma HDL-C removed by the liver back into plasma to maintain the plasma HDL-C pool. Verification of this idea will require detailed HDL metabolic studies in WT and HSKO mice. If hepatocyte ABCA1 plays an important role in braking the macrophage RCT pathway in the liver, therapeutic strategies aimed at increasing expression ABCA1 (i.e., more specific LXR agonists), particularly in
the liver, may paradoxically increase the risk of CVD and should be carefully examined for this potential untoward side effect.

The relationship between ABCA1 expression and atherosclerosis lesion development is more complicated than originally expected likely due to the multifaceted effects of ABCA1 on lipid metabolism. Contrary to our expectation, we found similar extent of atherosclerosis in whole aortas as well as smaller and less complex aortic root lesions in 16 wk atherogenic diet-fed HSKO/LDLrKO vs. LDLrKO mice (Chapter II). In a separate smaller study, we also observed similar extent of atherosclerosis between groups fed the atherogenic diet for only 5 wks. Therefore, lack of hepatic ABCA1 in atherogenic diet-fed LDLrKO mice did not enhance early atherogenesis. Atherosclerotic lesions in the aortic root develop before those in the whole aorta and usually are more advanced [32]. In the aortic root, hepatic ABCA1 deletion paradoxically protected against advanced atherosclerotic lesion development. The likely explanation for these surprising outcomes is that efficient macrophage RCT together with significantly reduced atherogenic apoB Lp levels in HSKO/LDLrKO mice prevented the anticipated increase in atherosclerosis. If hepatic ABCA1 indeed normally recycles a significant proportion of plasma HDL-C taken up by the liver back into circulation to maintain the plasma HDL-C pool as mentioned above, deletion of hepatocyte ABCA1 may reduce CVD risk by stimulating RCT. Similar to atherosclerosis studies with hepatic SR-BI overexpression or SR-BI knockout in mice [33, 34], our findings support the idea that flux of cholesterol via RCT better reflects
atheroprotective potential than steady-state plasma HDL-C levels. Other proteins that can potentially influence macrophage RCT and atherosclerosis susceptibility include apoA-I, LCAT and CETP. Macrophage RCT was promoted with apoA-I overexpression [35] and impaired with apoA-I deficiency [36], endorsing the atheroprotective role of apoA-I. Low plasma HDL-C in human LCAT deficiency has not been consistently associated with enhanced atherogenesis, presumably due to coincident low LDL-C levels as with TD. Controversial results also exist in animal models with LCAT overexpression [37, 38] or deletion [39, 40] regarding its atheroprotective role. LCAT knockout mice have almost complete preserved in vivo macrophage RCT despite extremely low HDL-C levels, similar to our results in HSKO/LDLrKO mice. The maintenance of macrophage RCT in LCAT KO mice is likely due to high levels of lipid-poor apoA-I and enhanced efflux via ABCA1 [41]. On the other hand, overexpression of LCAT modestly reduced in vivo macrophage RCT at least partly due to a reduction in lipid-poor apoA-I and cholesterol efflux via ABCA1 [41]. Again, these data suggest that factors important in maintaining HDL-C may not necessarily be critical for determining the rate of macrophage RCT and possibly CVD risk.

Studies of myeloid cell ABCA1 and atherogenesis in Chapter III also generated unexpected results. Genetic deletion of myeloid ABCA1 in LDLrKO mice followed by atherogenic diet consumption did not exacerbate early to intermediate stage atherosclerosis, although advanced atherogenesis was modestly and significantly increased. Normalizing the differences in plasma lipid levels through
feeding chow diet resulted in a 70% increase in aortic CE content and 76% larger aortic root lesion size in MSKO/LDLrKO mice, suggesting the deleterious effect of myeloid ABCA1 deletion in cholesterol efflux was offset by the less atherogenic lipoprotein profile, resulting in delayed and minimal increase in atherosclerosis. Earlier atherosclerosis studies using transplantation of BM from whole body ABCA1 knockout mice into LDLrKO recipients observed a uniform increase in atherosclerosis in the absence of BM ABCA1 expression [9, 42-44]. The possibility that different experimental designs could explain the divergent outcomes between our study and previous BMT studies was eliminated when we observed similar plasma lipids and atherosclerosis between our non-transplantation study and a BMT study using MSKO/LDLrKO vs. LDLrKO BM in LDLrKO mice. On the whole, these data suggest an atheroprotective role for myeloid ABCA1 expression in situations where a similar extent of hyperlipidemia exists. The relative importance of other proteins involved in cholesterol FC efflux in atherosclerosis development has also been investigated. ABCG1 promotes cholesterol efflux to mature HDL [15] and ABCG1 deficient macrophages have impaired ex vivo cholesterol efflux to mature HDL and in vivo RCT [22]. However, atherosclerosis was decreased in hyperlipidemic mice transplanted with ABCG1 deficient bone marrow, which was likely attributed to compensatory upregulation of other proteins (i.e., ABCA1 and apoE) or increased susceptibility of macrophages to oxidized LDL-induced apoptosis [45, 46]. ABCA1 and ABCG1 act in a synergistic way to maintain cellular cholesterol homeostasis. Loss of both transporters in macrophages resulted in a greater reduction in RCT than the
effect of either transporter alone [22]. Transplantation of ABCA1 and ABCG1
double knockout BM into LDL heterozygous recipient mice led to more severe
atherosclerosis than transplantation of either single donor BM [43]. SR-BI has
been shown to facilitate cholesterol efflux to mature HDL in vitro, but RCT from
SR-BI deficient macrophages was not impaired, suggesting a minimal role for
SR-BI in vivo. However, hematopoetic SR-BI expression appears to be
atheroprotective [47, 48], which may come from effects beyond promoting
macrophage cholesterol efflux. Additionally, combined ABCA1 and SR-BI
deletion in leukocytes significantly enhanced atherosclerosis in LDLrKO mice [42].
The above evidence suggests an overall protective role of cholesterol efflux
pathways in decreasing atherosclerosis in mice with macrophage ABCA1 likely
contributing significantly.

The atheroprotective therapeutic potential of ABCA1 has been investigated in
animals. LXR agonists reduce susceptibility to atherosclerosis in animal models
and upregulation of ABC transporters was detected in lesions of mice [49].
Macrophage LXR expression was shown to be essential in mediating actions of
LXR agonists in inhibiting atherosclerosis progression [50] and promoting
disease regression [51]. Most recently, antagonism of miR-33, an intronic
microRNA negatively regulating ABCA1 expression, was shown to promote RCT
and atherosclerotic lesion regression in LDLrKO mice [52]. In this study, the anti-
atherogenic effects were likely conferred by increased ABCA1 expression in both
macrophages and the liver as suggested by increased macrophage RCT and
elevated plasma HDL-C. Characterization of HDL from anti-miR33 treated mice showed a greater proportion of apoA-I and apoE associating with larger HDL particles enriched in CE. Enrichment of HDL apoE may facilitate cholesterol efflux partly by activating LCAT to generate a gradient for FC efflux [53]. Hence, elevated plasma HDL in these mice and increased macrophage RCT agrees with the notion that both the transporter and the nature of acceptors influence efficiency of cholesterol efflux. Whether these therapeutic strategies might be efficacious in humans is unknown.

Comparison of our BMT experiment using MSKO BM (Chapter III) with previous BMT studies using donor BM cells from total ABCA1 knockout mice indirectly supports an anti-atherogenic role for ABCA1 expression in non-myeloid cells; in our study, myeloid cell deletion of ABCA1 did not accelerate atherogenesis, whereas in previous studies, deletion of ABCA1 in all BM cells did. Many studies use BMT to test macrophage based hypotheses assuming whatever effects observed with BMT reflect effects of macrophages. However, BMT lacks specificity since all BM-derived cells including those have been implicated in atherogenesis are affected, making it difficult to distinguish a cell-specific effect. Thus, appropriate caution should be taken in interpreting results from BMT studies when the focus of the study is on macrophages. Cre-loxP strategy is an improved approach for macrophage-specific gene deletion. However, LysMCre transgenic mice have expression of Cre recombinase in macrophages and neutrophils [54], both of which are implicated in the pathogenesis of
atherosclerosis. Thus, in our study, MSKO/LDLrKO mice have deletion of ABCA1 in neutrophils and macrophages. Although we cannot conclude that lipoprotein and atherosclerosis outcomes in our study are solely dependent on macrophage ABCA1 deletion, we can at least delineate the role of ABCA1 expression in myeloid cells vs. non-myeloid cells.

Another unexpected finding in both studies is the profound impact of ABCA1 expression on apoB Lp metabolism in hyperlipidemic mice. Altered apoB Lp metabolism is also characteristic of TD subjects shown by a ~50% reduction in plasma LDL, which has been ascribed to a significant two-fold increase in the fractional catabolic rate of LDL [55]. Similarly, hepatocyte ABCA1 deletion in chow-fed mice recapitulated the phenotype of TD by exhibiting plasma LDL levels 50% of normal, significantly increased $^{125}$I-LDL tracer turnover rate, and a two-fold increase in hepatic LDLr expression [56], suggesting increased hepatic LDLr dependent LDL catabolism as the potential underlying mechanism. In agreement with this finding, the difference in plasma LDL was normalized in chow-fed mice lacking functional LDLr as shown in Chapter II (Figure 1 C). However, atherogenic diet challenge led to a significant 40-50% decrease in plasma VLDL-C and LDL-C in HSKO/LDLrKO vs. LDLrKO mice. We demonstrated that the diminished plasma apoB Lp concentrations was due to increased VLDL catabolism, not production. The absence of functional LDLr suggests the involvement of an LDLr-independent mechanism in mediating enhanced VLDL catabolism in HSKO/LDLrKO mice. Examination of alternative
pathways such as hepatic LDLr related protein (LRP) mRNA and protein expression as well as hepatic lipoprotein catabolic genes (e.g. syndecan 1) failed to show a significant difference between groups. Meanwhile, we found a diminished pool of apoE-enriched HDL, a potential competitor for alternative hepatic VLDL uptake pathways. HDL can compete for hepatic βVLDL particle uptake in SR-B1 dependent and independent pathways [57]. VLDL particles from atherogenic diet-fed HSKO/LDLrKO vs. LDLrKO are indistinguishable in chemical composition and characteristic of βVLDL with CE enrichment at the expense of TG (Supplemental Table I). Thus, less competition from apoE-enriched HDL may give rise to increased VLDL catabolism, resulting in significantly reduced VLDL in HSKO/LDLrKO mice. Decreased VLDL may also result in decreased plasma LDL concentrations in HSKO/LDLrKO mice due to less VLDL available for intravascular conversion to LDL.

The findings in Chapter III regarding apoB Lp metabolism were surprising since previous studies did not suggest a role for macrophage ABCA1 in plasma apoB Lp metabolism. Plasma apoB Lp levels were 30%-50% lower in atherogenic diet-fed MSKO/LDLrKO vs. LDLrKO mice, likely attributed to reduced hepatic VLDL TG secretion. The liver plays a central role in synthesis, secretion, and clearance of apoB Lp. Hepatic TG accumulation decreased significantly in MSKO/LDLrKO mice and was associated with a trend towards down regulation of lipogenic genes, such as FAS and ACC1, suggesting decreased de novo lipogenesis.

Macrophages are an important source of cytokines and cytokines are capable of
modulating lipid metabolism [58]. A potential explanation for the lower TPC and apoB Lp concentrations in MSKO/LDLrKO mice was local (i.e. Kupffer cells) or systemic macrophage-derived mediators that affected hepatic VLDL TG secretion. However, acute Kupffer cell ablation did not support the former possibility since depletion of Kupffer cells did not normalize the difference in plasma lipids between genotypes. The similar plasma lipid phenotype in our BMT study and 16wk atherogenic diet study also suggests the likelihood of a systemic mediator responsible for attenuated hepatic TG production and lower apoB Lp concentrations in MSKO/LDLrKO mice. Further studies are required to explore the potential macrophage mediator(s) and mechanisms. Overall, hepatocyte and macrophage ABCA1 expression appears to be important regulators of plasma apoB Lp metabolism in addition to their well-accepted roles in HDL metabolism and macrophage RCT.

In conclusion, we demonstrated novel roles for ABCA1 in regulating apoB Lp metabolism under hyperlipidemic conditions. Unexpectedly, deletion of hepatocyte or myeloid cell ABCA1 resulted in paradoxical findings in atherogenesis. These data emphasize the importance of lesional macrophage cholesterol efflux in atheroprotection and provide additional evidence to challenge the dogma that low HDL-C, per se, will result in increased CVD risk.
Reference


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**Poster Presentations:**

**Bi X**, Boudyguina E, Maeda N, Hayden MR, Parks JS. *Hepatic ABCA1 Deficiency Does not Significantly Influence Susceptibility to Atherosclerosis in C57BL/6 LDLr-/- Mice.* 2010 Arteriosclerosis, Thrombosis and Vascular Biology Scientific Sessions, San Francisco, CA

**Bi X**, Zhu X, Boudyguina E, Maeda N, Hayden MR, Parks JS. *Hepatic ABCA1 Deletion in C57BL/6 LDLr-/- Mice Does not Impair Macrophage Reverse Cholesterol Transport during the Pathogenesis of Atherosclerosis.* 2011 Arteriosclerosis, Thrombosis and Vascular Biology Scientific Sessions, Chicago, IL

**Bi X**, Boudyguina EY, Parks JS. *Macrophage ABCA1 Expression Decreases Inflammation but Increases Plasma apoB lipoproteins in Atherogenic Diet-fed LDL Receptor Knockout Mice.* 2012 Kern Lipid Conference, Vail, CO


**Publications:**

**Published Abstracts:**

**Bi X**, Boudyguina E, Maeda N, Hayden MR, Parks JS. Hepatic ABCA1 Deficiency Does not Significantly Influence Susceptibility to Atherosclerosis in C57BL/6 LDLr-/- Mice. *Arterioscler Thromb Vasc Biol.* 2010;30 e253

Articles:

Bi X, Zhu X, Duong M, Boudyguina EY, Wilson MD, Gebre AK, Parks JS. Liver ABCA1 Deletion in LDLrKO Mice Does Not Impair Macrophage Reverse Cholesterol Transport or Exacerbate Atherogenesis. (Submitted to Arteriosclerosis, Thrombosis and Vascular Biology in January 2013, revised and resubmitted in April, 2013)