Macrophage lipid genes in cardiovascular disease

Lammers, B.

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BART LAMMERS

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Imagination is more important than knowledge

Albert Einstein

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Bart Lammers

Division of Biopharmaceutics, LACDR, Leiden University, the Netherlands

Chapter 1

General introduction

Bart Lammers

Division of Biopharmaceutics, LACDR, Leiden University, the Netherlands

Cardiovascular diseases (CVD) are still a leading cause of death in the Western society. Atherosclerosis is the underlying cause of most cardiovascular diseases. Lipid accumulation in the arterial wall leads to an inflammatory condition clinically causing occlusive vascular disease, myocardial infarction (MI), and stroke.[1] In 2010, 39735 people were documented to have died of the consequences of CVD in the Netherlands, accounting for 29% of all reported deaths.[2] This clearly illustrates the CVD problem in the Dutch society. In the last decades, several risk factors for atherosclerosis have been identified including smoking, hyperlipidemia, hypertension, physical inactivity, and being male.[3] To date, treatments are mostly based on the elimination of these identified risk factors and mainly consist of lowering of serum lipid levels by statins, reduction of blood pressure, and decreasing blood thrombosis. However, these interventions cannot prevent that CVD still remain a leading cause of death in the Western society. Therefore, there is an urge to develop new therapies targeting the different molecular pathways and stages of atherogenesis.

Myocardial infarction (MI), commonly known as a heart attack, results from the interruption of blood supply to a part of the heart. This commonly occurs when an atherosclerotic plaque slowly builds up in the inner lining of a coronary artery. Rupture of these plaques may result in thrombus formation, leading to occlusion of a coronary artery and preventing blood flow downstream. The resulting ischemia can cause damage or death of myocardial tissue. Although cases of ischemic heart disease have been described for two centuries,[4] the factors responsible for the sudden thrombotic occlusion of a coronary artery have only been elucidated in the last two decades.[5–7] In at least 70% to 80% of cases, acute myocardial infarction is attributable to rupture of a vulnerable plaque, resulting in local thrombus formation and acute disruption in the coronary blood supply in the affected area.[8] Many important predictors of early clinical outcome in myocardial infarction are independent of treatment. Most of the early mortality is explained by factors such as the age of the patient, initial heart rate and blood pressure. However, the time to administration of reperfusion therapy is a critical adjustable determinant. Several studies have revealed that patients with myocardial infarction treated most rapidly have a lower mortality and, among survivors, reduced infarct size.[9] Another important risk factor is the composition of the atherosclerotic lesion. Independent of its severity, vulnerable plaques are more prone to rupture, thereby inducing thrombus formation.

Atherosclerosis

Pathogenesis of atherosclerosis

Processes involved in the initiation of atherosclerosis have been debated for many years and several hypotheses have been proposed. The "response-to-injury" hypothesis was one of the first to state that endothelial injury leads to an inflammatory response as part of a healing process in the arterial wall.[10] Under normal conditions, the healthy endothelium is able to respond to physical and chemical signals by the production of a wide range of factors that regulate vascular tone, cellular adhesion, thrombus resistance, smooth muscle cell proliferation, and vessel wall inflammation.[11] Endothelial activation caused by hyperlipidemia, hypertension, diabetes, and smoking, results in an increased migration of monocytes and T-lymphocytes from the circulation into the intima of the arterial wall. The attracted leukocytes transmigrate through the endothelial layer in a multi-step process in which the following stages can be identified; rolling on the endothelium, firm adhesion, and transmigration into the sub-endothelial space.[12,13] Under hypercholesterolemic conditions, the transmigrated monocyte-derived macrophages start to accumulate lipids, including cholesterol, and transform into macrophage foam cells.[14] The accumulation of these macrophage foam cells is the hallmark of the development of the so called fatty streak: the first step in the pathogenesis of atherosclerosis (Fig. 1).[15,16] The atherosclerotic plaque is a dynamic tissue, where increases in cell number (driven by cell proliferation and migration) and decreases in cell number (driven by cell death and possibly emigration) are continuous processes.[17]

Although macrophages are able to release their cholesterol through efflux (see also chapter 2), they cannot limit the uptake of cholesterol. Therefore, atherosclerotic lesion development will progress with time. In more advanced atherosclerotic lesions, smooth muscle cells (SMC) contribute to foam cell formation by also taking up lipids. In addition, SMC can synthesize large amounts of collagen, elastin, and proteoglycans, resulting in the fatty streak to evolve into an intermediate (fibrofatty) lesion. SMC cover the core of these lesions, which are constituted of extracellular lipids, foam cells, and T-lymphocytes, surrounded by a poorly developed matrix of connective tissue.[3] Further progression of the lesion severity subsequently results in the formation of an advanced fibrous lesion, characterized by a fibrous cap covering a core of extracellular lipid and necrotic debris, together with macrophages, T-lymphocytes, SMC and sometimes calcification.[18,19] Activated macrophages play a key role in atherosclerotic plaque stability and thrombotic complications by producing several proteases, including matrix metalloproteinases (MMP).[20] These MMP can degrade extracellular matrix, which strengthens the fibrous cap of the plaque. In addition, macrophages can secrete collagenases and

induce apoptosis of vascular SMC, thereby negatively influencing the collagen production and thus plaque stability.[21–23] Subsequent plaque rupture can result in secondary hemorrhage and thrombosis. This in turn leads to occlusion of the artery, and may lead to clinical symptoms such as MI and cerebral stroke.[24–26]

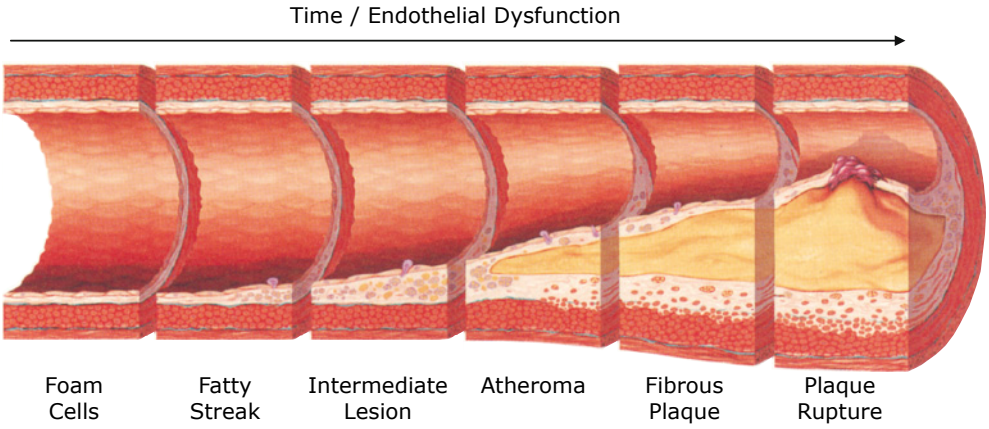

Figure 1. Atherosclerotic lesion progression over time. See text for explanation. Adapted from Pepine CJ.[193]

Monocytes, macrophages and atherogenesis

It is generally accepted that macrophages play an essential role in all stages of atherosclerotic lesion development. The fatty streak is predominantly composed of macrophages, whereas more advanced atherosclerotic lesions consist of clusters of macrophages surrounding a core of lipid and necrotic material.[27] In addition, mice lacking macrophages (due to macrophage colony-stimulating factor (M-CSF) deficiency) exhibit a marked reduction in atherosclerotic lesion development, even despite severe hypercholesterolemia, underlining the important function of macrophages in atherosclerotic lesion development.[28,29]

Monocytes, derived from pluripotent hematopoietic stem cells in the bone marrow, migrate into the blood circulation within 24 hours after production in the bone marrow.[30] Subsequently, they either remain in the blood stream or migrate from the circulation into tissues and body cavities. Monocytes can then, upon stimulation with M-CSF, differentiate into macrophages and become foam cells after excessive accumulation of lipids, which results in the initiation of atherosclerosis. The accumulation of monocyte-derived macrophages is progressive and correlates to initial atherosclerotic lesion formation.[31] In addition to influx of monocyte-derived macrophages, some studies have suggested that macrophages in the arterial wall also originate from local proliferation of monocytes/macrophages.[32] Moreover,

renewal of resident macrophages in body cavities and tissues have been shown to depend on macrophage proliferation.[33]

The "response-to-injury" hypothesis suggests the importance of local endothelial injury for the pathogenesis of atherosclerosis. Damaged, and thus activated endothelial cells produce chemokines, cytokines and adhesion molecules designed to interact with leukocytes and platelets to direct these inflammatory cells to specific tissues in response to vascular injury.[34] Activated endothelial cells express adhesion molecules, such as vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), P-selectin and E-selectin.[14,35] Leukocytes express counter receptors for these adhesion molecules and decelerate in the bloodstream via interaction with P- and E-selectin. Once slowed down, a more firm adhesion is facilitated via VCAM-1 and ICAM-1.[36,37] When firm adhesion is established, leukocytes migrate through the interendothelial junction into the subendothelial space (diapedesis). This process is facilitated by additional adhesion molecules, like platelet/endothelial cell adhesion molecule-1 (PECAM-1) and junctional adhesion molecule-1 (JAM-1).[38,39] Specific blockade of the interaction of monocytes with these adhesion molecules completely abolishes the accumulation of monocytes in the subendothelial space, demonstrating the importance of these adhesion molecules in monocyte migration and atherogenesis.[40,41]

Furthermore, activated endothelial cells produce several chemokines and interleukins (IL), which enhance diapedesis,[42] and the recruitment of leukocytes into the lesion. The deficiency of monocyte chemoattractant protein-1 (MCP-1),[43] or its receptor CC-chemokine receptor 2 (CCR2)[44] results in dramatic protection from monocyte recruitment and atherosclerotic lesion formation. Different environmental signals, like microbial products and cytokines activate macrophages diversely, leading to macrophage heterogeneity. In the mouse, one subset of monocytes with high expression of Ly6C promotes inflammation while the other subset with low expression of Ly6C attenuates inflammation and promotes angiogenesis in models of tissue injury.[45] Moreover, upon activation, macrophages can either turn into a pro-inflammatory phenotype (M1), producing TNF-α and IL-6,[46] or an anti-inflammatory phenotype (M2), secreting IL-10 and TGF-β.[47] Besides the amount of monocyte-derived macrophages, it is the phenotype of the macrophages that can greatly affect atherosclerotic lesion development.[48–50]

Macrophages play an important role in a wide variety of inflammatory reactions and diseases. They scavenge debris and subsequently migrate away from the site of injury into the blood circulation, thereby removing tissue debris. In atherosclerosis, however, the turnover of macrophages seems to be impaired as macrophages remain in the intima of the arterial wall for a prolonged period of time, resulting in a prolonged inflammatory response, and thus an increased severity of the atherosclerotic lesion. Although it is demonstrated that macrophage foam cells are able to leave the intima of the arterial wall and return to the blood

circulation,[51] most macrophages remain in the atherosclerotic lesion until they die.[52] This might be the result of the formation of a fibrous cap which makes it more difficult for a macrophage foam cell to leave the atherosclerotic lesion. On the other hand, the presence of a fibrous cap also results in an attenuated recruitment of monocytes to the atherosclerotic lesion.[53,54]

Large atherosclerotic plaques are often associated with increased risk for clinical manifestations. However, the major cause of these manifestations is rupture of the atherosclerotic plaque and the subsequent development of thrombosis. The statistic possibility for an atherosclerotic plaque to rupture highly correlates with its cellular composition. In general, plaques can be defined as stable or unstable. The stability of the plaque is mainly determined by the integrity of the fibrous cap overlying the lesion. A thick fibrous cap with low amounts of macrophages is highly stable. Stability of the fibrous cap is achieved by SMC that synthesize extracellular matrix.

On the contrary, large amounts of macrophage foam cells, together with a thin fibrous cap overlying a large lipid pool are known to be unstable.[55,56] Macrophage-derived MMP and collagenases can degrade the extracellular matrix and collagen, thereby reducing the stability of the atherosclerotic plaque.[21–23] Although the evolution of an atherosclerotic plaque is a complicated and multifactorial process, macrophages have differential adverse effects on the pathology, which may ultimately lead to a clinical event.

Besides their primary scavenging role, macrophages also produce many different proteins, which are potentially involved in the initiation and progression of atherosclerotic lesion formation. Important machophage-derived proteins in lipoprotein metabolism relevant to this thesis will be discussed in more detail below. Specific details on macrophage-derived apolipoprotein E (apoE) and adipose triglyceride lipase (ATGL) are also described in this introduction, whereas ABCA1, ABCG1, and macrophage reverse cholesterol transport are defined in chapter 2.

Lipids, lipoproteins and lipid metabolism

Although elevated plasma lipid levels are strongly correlated with an increased susceptibility to atherosclerosis, they also play an important role in healthy individuals. Plasma lipids are essential for every cell in order to maintain membrane fluidity. The major lipids in human plasma are cholesterol, cholesteryl esters (CE), triglycerides (TG), and phospholipids (PL). Cholesterol is of critical importance for all eukaryotic organisms, as it modulates membrane fluidity and is essential for steroid hormone synthesis, whereas TG are used as an energy source for muscles and storage in adipose tissue. PL are a major structural component of all the different lipoproteins.

Lipoproteins

Since lipids are insoluble in blood, they are transported by lipoproteins. Lipoproteins are water-soluble protein-lipid complexes, which consist of a hydrophobic core of neutral lipids (CE and TG), surrounded by a monolayer of PL, unesterified cholesterol and specific apolipoproteins (apo).[57] Apolipoproteins are required for the assembly, structure, and function of lipoproteins. Furthermore, they activate enzymes important in lipid metabolism and act as ligands for cell surface receptors. Several different lipoproteins can be distinguished based upon their lipid and apolipoprotein composition and size: chylomicrons (CM), very low-density lipoproteins (VLDL), intermediate-density lipoproteins (IDL), low-density lipoproteins (LDL), and high-density lipoproteins (HDL)(Table 1).[57] In more detail, CM and VLDL are the major carriers of TG in the blood, while plasma cholesterol is mainly transported as CE in LDL and HDL. Furthermore, CM, VLDL and LDL have apoB as their primary apolipoprotein, comprising approximately 30, 60, and 95% of these lipoproteins, respectively, whereas apoA-I is the principal apolipoprotein of HDL, comprising 75% of the protein mass.[58,59] HDL is a universal plasma acceptor for cholesterol efflux and comprise a complex lipoprotein class containing different subclasses of HDL varying in size, density, and lipid composition. Generally, HDL can be classified into lipid-poor/lipid-free discoidal nascent pre-β HDL and lipid-rich spherical mature HDL, based on their difference in electrophoretic mobility.[60] Pre-β HDL mainly contains apoA-I and PL with small amounts of cholesterol, whereas mature HDL encompasses two main density classes: Large CE-rich HDL_2 and small CE-poor HDL_3.[61,62] The heterogeneity in lipoprotein size and composition induces

Table 1. Physical properties and composition of human plasma lipoproteins.[194-196]

	Diameter	Density	Mw	Lipid (%)*			Apolipoproteins
	(nm)	(g/mL)	(*10^6 Da)	TG	CHOL	PL	
Chylo-microns	75-1200	0.95	400	80-95	2-7	3-9	A-I, A-II, A-IV; B48; C-I, C-II, C-III; E
VLDL	30-80	0.95-1.006	10-80	55-80	5-15	10-20	B100; C-I, C-II, C-III; E
IDL	25-35	1.006-1.019	5-10	20-50	20-40	15-40	B100; C-I, C-II, C-III; E
LDL	18-25	1.019-1.063	2.3	5-15	40-50	20-50	B100
HDL	5-12	1.063-1.21	0.16-0.41	1-5	15-35	15-35	A-I, A-II, A-IV; C-I, C-II, C-III; E
HDL_{2b}	10.4	1.099	0.41	4	33	30	"
HDL_{2a}	10.3	1.107	0.4	4	30	33	"
HDL_{3a}	9.9	1.123	0.36	3	27	29	"
HDL_{3b}	8.0	1.155	0.2	2	24	24	"
HDL_{3c}	7.3	1.186	0.16	1	17	16	"

* Values are expressed as percentage of total weight

changes in the interaction with different tissues, thereby influencing lipoprotein metabolism.

Lipoprotein metabolism

The plasma lipid levels depend on the integrated balance of the exogenous and endogenous pathways of lipoprotein metabolism, in which the liver plays an essential central role.

Exogenous lipid metabolism

The exogenous pathway describes the metabolic route of dietary lipids after their absorption by the intestine (Fig. 2). The amount of cholesterol and TG absorbed from the diet is a major contributor to lipid levels in the blood circulation. Dietary TG and CE are hydrolyzed in the proximal small intestine and subsequently assembled into CM. These large TG-rich CM are transported from the lymph to the blood circulation. Upon entering the circulation, the TG in the core of CM are hydrolyzed by lipoprotein lipase (LPL).[63,64] The released free fatty acids (FFA) are taken up by peripheral tissues such as adipose tissue (for storage as TG), skeletal muscle and heart (as energy source), and the liver (for storage or generation of lipoproteins).[65] Progressive hydrolysis of TG from CM is accompanied by exchange of TG for CE, mediated by cholesteryl ester transfer protein (CETP).[66,67] As a result, CM shrink in size and turn into CM remnants. These CM remnants, relatively enriched in CE and apoE, are rapidly removed from the circulation by the liver via the LDL receptor (LDLr), LDL receptor related protein-1 (LRP-1), remnant receptors, heparin sulphate proteoglycans, and scavenger receptor class B type I (SR-BI),[68–70] finally resulting in the uptake of dietary cholesterol by the liver.

Endogenous lipid metabolism

The endogenous pathway describes the distribution of lipids from the liver to peripheral tissues (Fig. 2). VLDL is synthesized in the liver from cholesterol and TG derived from de novo synthesis or lipoprotein uptake.[71,72] Human nascent VLDL contains a single copy of apoB100 as well as newly synthesized apoE and apoC's. ApoE is of particular importance for VLDL and CM remnants since it plays a role in the secretion,[73,74] as well as in the metabolic fate of these particles, the latter due to its interaction with specific receptors.[75] Upon its secretion into the circulation, TG-rich VLDL particles acquire additional apoE and apoC. The TG in the core of VLDL are subject to lipolysis by LPL,[63] leading to the formation of VLDL remnants or IDL, which is partly cleared by the liver, mediated by apoE.[76] The remaining IDL undergoes further lipolysis via LPL and hepatic lipase (HL) to become cholesterol-rich LDL with apoB100 as its sole apolipoprotein, which is recognized by the LDLr on the liver and peripheral tissues.[77] The liver thus serves as the major site for both cholesterol synthesis and catabolism of apoB-containing lipoproteins.

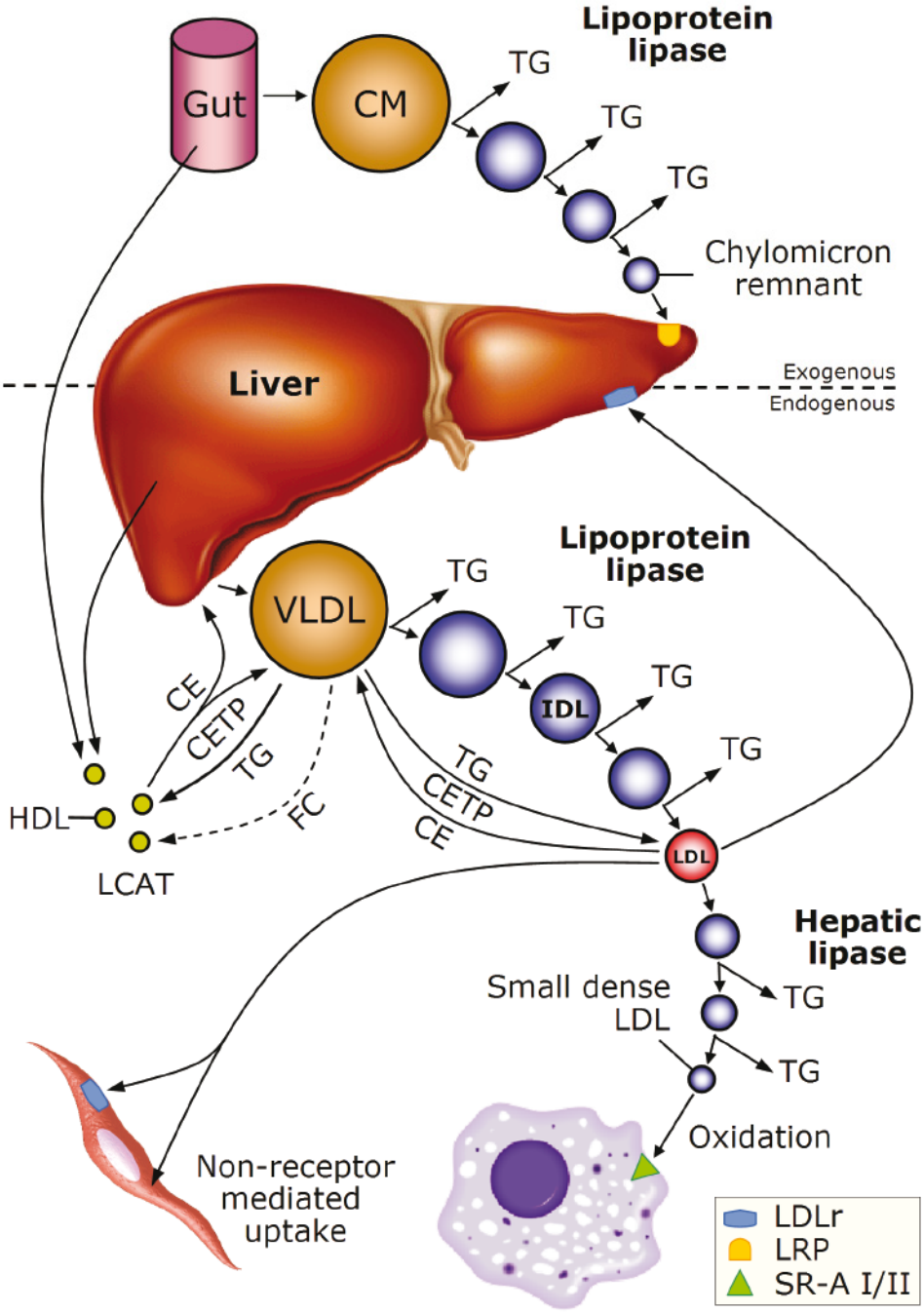

Figure 2. Exogenous and endogenous lipid transport. See text for explanation. CM, chylomicrons; apo, apolipoprotein; VLDL; very low-density lipoprotein; LDL, low-density lipoprotein; IDL, intermediate-density lipoprotein; HDL, high-density lipoprotein; FC, free cholesterol; TG, triglycerides; CE, cholesteryl esters; CETP, cholesteryl ester transfer protein; LCAT, lecithin: cholesterol acyltransferase; SR, scavenger receptor; LDLr, low-density lipoprotein receptor; LRP, low-density lipoprotein related protein. Modified from Durrington PN.[197]

Reverse cholesterol transport (RCT)

All nucleated cells synthesize cholesterol. However, only hepatocytes and enterocytes are able to efficiently excrete cholesterol from the body into feces. RCT is a pathway by which excess of cholesterol from peripheral tissues, including macrophages, is transported back to the liver for excretion, and thus of potential interest for the prevention of atherosclerosis (Fig. 3). In the liver, cholesterol can be recycled or irreversibly converted to degradation products like bile acids and subsequently excreted from the body. Although cellular RCT is not a major contributor in the mass excretion of cholesterol into the feces, it does play a pivotal role in the cholesterol homeostasis in arterial macrophages. The removal of cholesterol via hepatobiliary secretion was considered as the sole route in the RCT process. However, a novel non-biliary RCT pathway called transintestinal cholesterol efflux (TICE), has been recently identified.[78]

The classical process of RCT is mediated by HDL.[79] Small discoidal HDL particles are synthesized and secreted by the intestine and the liver.[80] These nascent HDL particles are potent acceptors of excess cholesterol from peripheral cells. The ATP-binding cassette transporter ABCA1 specifically mediates the lipidation of nascent HDL particles with PL and FC, leading to the generation of pre-β HDL particles.[81,82] The subsequent esterification of FC into CE in pre-β HDL by lecithin:cholesterol acyltransferase (LCAT) generates spherical mature HDL.[83] The esterification of cholesterol inside the core of the HDL particle creates an unesterified cholesterol concentration gradient between HDL and cell membranes, allowing the HDL_3 particle to accept more FC. This process is considered critical for efficient cholesterol efflux from cells to HDL.[84] Another ABC transporter, ABCG1, as well as SR-BI, are able to efflux cellular cholesterol to these larger HDL_3 particles (Fig. 3). In combination with additional LCAT activity, this leads to the formation of large, CE-rich HDL, converting HDL_3 to larger HDL_2.[85,86]

HDL_1 is the largest HDL particle found in serum, and is enriched in apoE. Since apoE is a ligand for the LDLr, HDL_1 is able to transport its cholesterol to the liver via the LDLr.[87] Whereas the LDLr engulfs the whole HDL particle, SR-BI mediates the selective uptake of CE from HDL, without taking up the HDL particle.[88] Alternatively, CETP can transfer CE to TG-rich lipoproteins like VLDL in exchange for TG,[89] which can be taken up by the liver through their lipoprotein receptors, such as the LDLr and LRP-1.[90] Importantly, except for hamsters, CETP is not expressed in rodents. Upon delivery of HDL-cholesterol to the liver, CE are hydrolyzed and reused for lipoprotein assembly. Excess cholesterol can also be secreted into the bile, either as neutral sterols via ABCG5/8 or as bile salts via ABCB11.[91] Overall, HDL plays a crucial role in the transport of excess cholesterol from peripheral tissues back to the liver. The RCT pathway may be of great importance with respect to atherosclerotic lesion development. Targeting its key mediators can hopefully contribute to the development of new therapies protecting against CVD. More recent advances

regarding RCT, as well as the structure, expression, and regulation of ABCA1 and ABCG1 will be discussed in chapter 2 of this thesis.

Apolipoprotein E

Apolipoprotein E (apoE) is an apolipoprotein that coats the surface and mediate the metabolism of lipoprotein particles.[75] ApoE serves as a ligand for the LDLr and LRP-1 and, through its interaction with these receptors, participates in the transport of cholesterol and other lipids among various cells of the body, including macrophages.[75,92] ApoE principally resides on VLDL, IDL, and large HDL, as well as on CM and their remnants.[75]

Considerable interest in understanding the role of apoE in lipoprotein metabolism came from the observation of Havel and Kane that apoE enriched VLDL accumulates in the plasma of patients with type III hyperlipoproteinemia, a genetic disorder characterized by elevated plasma cholesterol levels and accelerated coronary artery disease.[93] An isoform of apoE (apoE2) that is defective in binding to the LDLr is associated with type III hyperlipoproteinemia.[75,94] This affects about 1 in 5000 individuals.[95]

Structure, expression and regulation

In humans, the three major isoforms of apoE, referred to as apoE2, E3, and E4,[96] are products of three alleles (ε2, ε3, ε4) at a single gene locus. Three homozygous phenotypes (apoE2/2, E3/3 and E4/4) and three heterozygous phenotypes (apoE3/2, E4/3, and E4/2) arise from the expression of any two of the three alleles.[75] Population studies have shown that this allelic variation causes small but significant changes in plasma LDL levels.[97] The most common phenotype is apoE3/3 and the most common allele is ε3. Therefore, apoE3 is considered to be the parent form of the protein, and apoE4 and E2 are variants.[92,98] ApoE2 differs from apoE3 by having a cysteine at position 158 instead of an arginine. This amino acid substitution in the LDLr binding region of apoE reduces the LDLr binding ability of apoE2 to less than 2% relative to that of apoE3.[95] Approximately 1% of the population are E2/2 homozygotes. The complexity of the pathogenesis of hyperlipoproteinemia is, however, illustrated by the fact that only about 2% of the E2/2 individuals develop hyperlipoproteinemia. The majority of individuals with this genotype have, in fact, lower than normal plasma cholesterol levels. Thus, other factors are necessary for the development of type III hyperlipoproteinemia associated with apoE2.[99,100]

The human apoE gene is 3.7 kilobytes in length, contains four exons and is found on chromosome 19.[101,102] The promoter sequence TATAATT occurs approximately 30 base pairs (bp) upstream from the transcriptional initiation site.[103] The apoE

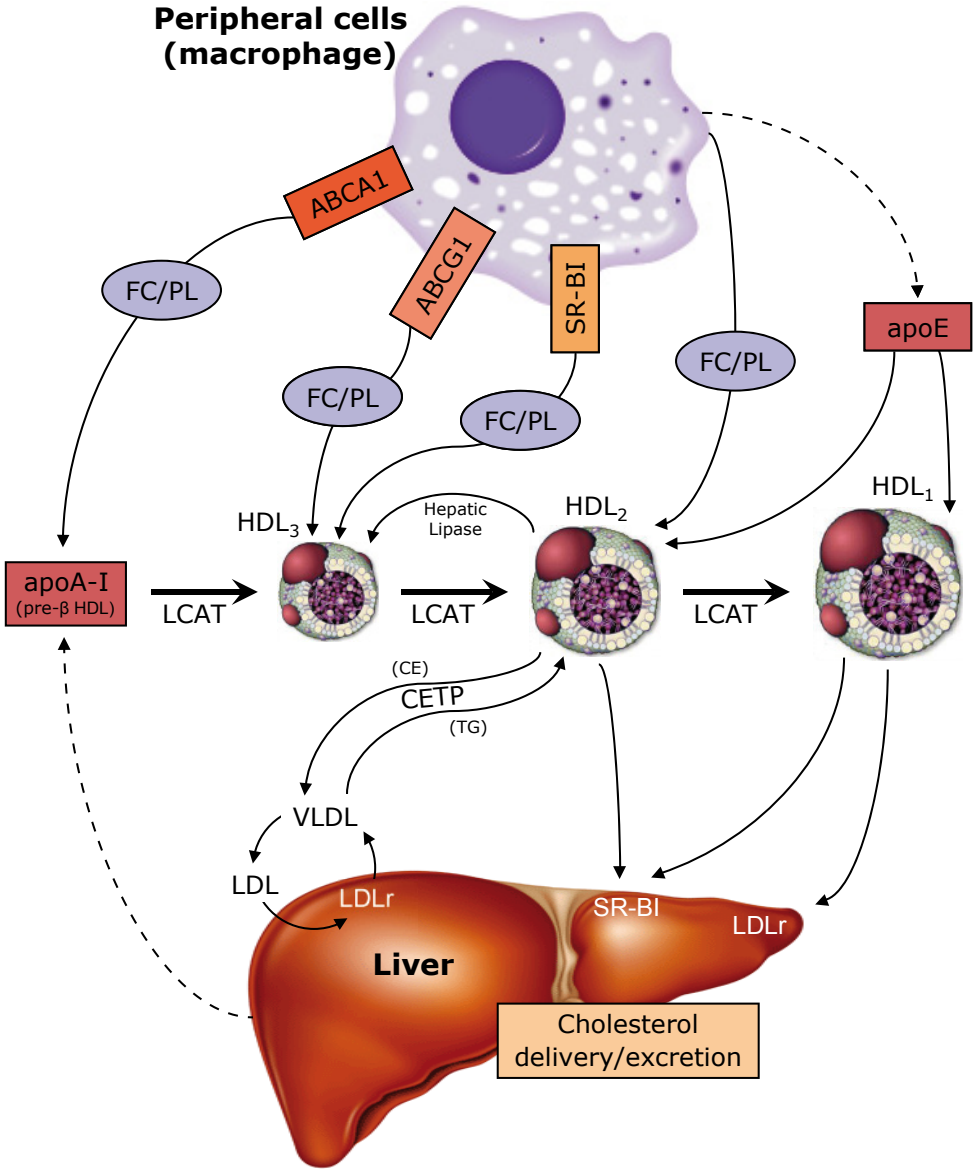

Figure 3. Reverse cholesterol transport. See text for explanation. ABCA1 / G1, ATP-binding cassette transporter A1 / G1; SR-BI; scavenger receptor class B type I; apoE / A-I, apolipoprotein E / A-I; FC, free cholesterol; PL, phospholipid; LCAT, lecithin: cholesterol acyltransferase; HDL, high-density lipoprotein; LDL, low-density lipoprotein; VLDL, very low-density lipoprotein; LDLr, low-density lipoprotein receptor; CE, cholesteryl ester; TG, triglyceride; CETP, cholesteryl ester transfer protein. Modified from Mahley RW.[198]

messenger RNA (mRNA) is 1163 bp in length.[104,105] The primary translation product is composed of 317 amino acids, with the 18 amino-terminal amino acids serving as a signal peptide. The mature apoE is secreted as a 299-amino acid protein with a Mr of 34,200.[75]

ApoE is primarily synthesized by hepatocytes in the liver, which probably accounts for two-thirds to three-fourths of the plasma apoE, although synthesis has been documented in virtually every tissue in both humans and mice.[106,107] In addition, the second largest site of apoE synthesis is the brain, where it is solely responsible for the formation of lipoproteins in the brain.[108] It is also present in high concentrations in interstitial fluid, where it appears to participate in cholesterol redistribution from cells with excess cholesterol to those requiring cholesterol.[109,110] The participation of several cell types in the widespread production of apoE indicates the importance of apoE in lipid transport and possibly in roles unrelated to lipid transport.[75,107]

ApoE plays an essential role in systemic lipoprotein metabolism,[63] as well as in lipid metabolism at the level of the individual cell.[111] The various metabolic pathways involving apoE can be divided into three different categories:

1. Redistribution of lipids among cells of different organs.
    a. Transport of dietary lipids from the intestine to the liver and peripheral tissues.
    b. Transport of lipids from the liver to peripheral tissues.
    c. Transport of lipids from peripheral tissues to the liver.
2. Redistribution of lipids among cells within an organ or tissue.
    a. Macrophages or other cells capable of storing and releasing lipids to acceptors in the interstitial fluid (apoE-lipid complexes or HDL) play a key role in this redistribution.
3. Functions of apolipoprotein E unrelated to lipid transport.
    a. ApoE produced by smooth muscle cells may be involved in modulating cell proliferation and differentiation.[75]
    b. Regulation of hematopoietic stem cell proliferation.[112]

ApoE deficiency and atherosclerosis

ApoE deficiency causes severe hyperlipidemia and atherosclerosis in humans and in gene-targeted mice.[113] Individuals deficient for apoE have type III hyperlipoproteinemia with elevated cholesterol but near normal triglyceride levels.[95] Several groups created apoE-deficient mice by inactivating the apoE gene by targeting.[114] ApoE-deficient mice have markedly increased total plasma cholesterol levels which are five times higher than those of normal littermates.[95,114] At 10 weeks of age, apoE-deficient mice have already developed spontaneous atherosclerotic lesions in the aorta and coronary and pulmonary arteries, demonstrating that a simple lack of apoE is sufficient to initiate atherogenesis.[95]

Macrophage-derived apoE in atherogenesis

The anti-atherogenic role of macrophage apoE can be mainly attributed to its function in the removal of excess cholesterol from macrophage foam cells[111] as well as its anti-inflammatory,[115] antiproliferative,[116] and immunomodulatory properties.[117,118] Macrophages isolated from the peritoneal cavity of mice and human blood monocytes produce large quantities of apoE.[119–121] ApoE represents up to 8-10% of protein constitutively secreted from macrophages,[122] and its synthesis and secretion can be highly induced via liver X receptor (LXR), upon loading of mouse peritoneal macrophages with cholesterol.[119] LXR is a sterol-responsive nuclear receptor that regulates the expression of genes involved in cholesterol metabolism and homeostasis, including apoE.[123] In agreement, expression of macrophage apoE is transcriptionally modulated by cholesterol and cytokines.[124,125] Interestingly, although the pathways stimulating apoE secretion and cholesterol efflux are disctinct,[126,127] apoE, whether added to cells or during its secretion from cells, can itself mediate cholesterol efflux.

In the developing atherosclerotic lesion, macrophages are a significant source of apoE. Physiological concentrations of apoE in the arterial wall have been demonstrated to be atheroprotective,[128] since it is able to modulate lipid and lipoprotein metabolism in arterial wall cells.[129] At sites of atherosclerotic lesion development, apoE has predominantly been found on the surface of macrophages and in the matrix surrounding macrophages. ApoE expression in the vessel wall is important for modulating vessel wall cholesterol homeostasis.[130–132] In fact, macrophage-specific expression of apoE in apoE-null mice protects against atherosclerotic lesion development even in the presence of high levels of circulating atherogenic lipoproteins.[113] Macrophage-derived apoE has thus been shown to be anti-atherogenic and anti-inflammatory in several ways. Yet, both pro- and anti-atherogenic functions for macrophage apoE in atherosclerosis have been described.[133–136]

We[133] and others[136] have shown that mice transplanted with apoE KO bone marrow developed 4- to 10-fold larger atherosclerotic lesions as compared to WT transplanted animals. In contrast, a significant decrease in lesion development is also described.[134,135]

Chapters 3 and 4 contain more detailed information about the role of macrophage apoE in atherosclerosis.

Adipose triglyceride lipase (ATGL)

Triglyceride stores are the most important energy reserve in vertebrates. Dysfunctional lipolysis affects energy homeostasis and may contribute to the pathogenesis of diseases like obesity and insulin resistance. Upon fasting, TG in fat

are hydrolyzed to sustain energy levels within the body. Hydrolysis of TG leading to the release of FFA requires the enzymatic activity of lipases. The first step in TG hydrolysis, resulting in the generation of diacylglycerols (DG) and FFA is facilitated by adipose triglyceride lipase (ATGL).[137,138] ATGL is mainly expressed in adipose tissue. Mutations in the gene for ATGL lead to neutral lipid storage disease (NLSD) in humans. NLSD is a rare, autosomal genetic disorder characterized by excessive accumulation of TG in all tissues of the body.[139,140] Similarly, mice deficient for ATGL suffer from massive TG accumulation in organs and tissues including the heart, resulting in cardiac dysfunction and subsequent premature death.[138] The hydrolyzed TG by ATGL can be further hydrolyzed to monoglycerides (MG) by hormone-sensitive lipase (HSL).[141] The final conversion of a monoglyceride into FFA and glycerol is facilitated by monoglyceride lipase (MGL).[142]

Structure, expression and regulation

The murine ATGL gene is approximately 6kb in length, contains 9 exons, and is found on chromosome 7. The 2kb mRNA codes for a 486-amino-acid protein with a molecular mass of 54 kDa, whereas the human gene encodes a 504-amino-acid protein that shares 86% homology with the murine protein.[137] Surprisingly, ATGL and classical lipases like LPL, HL, and endothelial lipase (EL) have no clear sequence similarity and differ with regard to catalytic mechanisms,[143] since the N-terminal region of the ATGL gene contains a "patatin" domain. These proteins have been shown to have acyl-hydrolase activity on phospholipid, monoacylglycerol and diacylglycerols substrates.[144]

In mice and humans, ATGL is predominantly expressed in white and brown adipose tissue. In addition, progressively decreasing amounts of ATGL are found in the testis, cardiac muscle, and skeletal muscle.[137,145–147] ATGL expression is highly increased during adipocyte differentiation,[148] reaching maximal levels when the cells accumulate visible lipid droplets.[137,149,150] Interestingly, ATGL is also expressed in macrophages and macrophage foam cells in atherosclerotic lesions.[151]

ATGL mRNA content is strongly controlled by nutritional factors, being increased during fasting and decreased by re-feeding in adipose tissue[145,147] and liver.[146] Insulin appears to be a potent regulator of ATGL, since it dose-dependently inhibits ATGL transcription in vitro, and enhances ATGL mRNA expression in murine models of insulin deficiency and insulin resistance.[145,152,153]

Unlike HSL, relatively little is known about the molecular pathways leading to the activation of ATGL activity. ATGL has been shown to reside in the cytoplasm and on lipid droplets (LD) in the basal state,[137,154] and protein kinase A (PKA) activation only elicits minor translocation of ATGL to LD.[154] In addition to possible regulation of activity by phosphorylation, ATGL activity is highly dependent on several biochemical events for efficient lipolysis. First by association with an activating protein, CGI-58,[155,156] and secondly by phosphorylation of perilipin A.[157,158]

Macrophage-derived ATGL

Besides its important role in adipocytes, ATGL also has hydrolytic capacities regarding macrophage TG stores. Chandak et al. demonstrated that macrophages lacking ATGL fail to efficiently hydrolyze macrophage TG stores, which led to decreased cellular FFA concentrations and concomitant accumulation of lipid droplets.[151] As a consequence, cellular ATP concentrations decreased. Importantly, both macrophage RCT (largely mediated via ABCA1 and ABCG1), as well as its phagocytosis capacities are ATP driven. Indeed, phagocytosis was shown to be decreased in vivo when ATGL deficient mice were challenged with bacterial particles.[151] In addition, macrophages lacking ATGL were shown to favor an anti-inflammatory M2-like phenotype,[159] implying a possible role for macrophage ATGL in inflammation and atherosclerosis.

ATGL deficiency and atherosclerosis

ATGL deficiency in both mice and humans leads to massive TG accumulation in organs. However, not much is known about the specific role of ATGL in atherosclerosis. This is primarily the result of the fact that mice lacking ATGL die prematurely (50% dies at 16-20 weeks) from cardiac dysfunction.[138] With such an acute phenotype, ATGL deficient mice are unable to develop sufficient atherosclerosis. However, to investigate the importance of macrophage-produced ATGL in the pathogenesis of atherosclerosis, the bone marrow transplantation technique can be used.

Bone marrow transplantation (BMT)

Monocyte-derived macrophages play a significant role in the pathogenesis of atherosclerosis. Through the expression of proteins such as apoE and several of the receptors involved in lipoprotein uptake including the LDLr, macrophages can impact the growth of the atherosclerotic plaque both by affecting systemic lipoprotein metabolism and locally by mediating cholesterol accumulation.[160]

Gene targeting studies in mice provide a powerful approach to examine the contribution of a given gene to atherogenesis. A number of the genes of interest in lipoprotein metabolism and atherosclerosis are expressed both by the liver and by macrophages. The expression of a gene by the liver may have different effects on the process of atherosclerosis as compared to its expression by macrophages. Bone marrow transplantation experiments using knock-out or knock-in mice have proven to be a useful approach to dissect out the contribution of macrophage gene expression to atherogenesis in vivo.[161]

The transport, repair, and defense functions of peripheral blood cells are well known. Their diversity in function is reflected by an extreme variation in shape

and size. Common to the different blood cells is a relatively short life-span: an average human produces daily about 10^{11} new blood cells. This process, termed hematopoiesis, is maintained from a small population of bone marrow stem cells.[162] Transplantation of the hematopoietic system offers a unique opportunity to replace genes in cells from hematopoietic origin, including macrophages. In humans, bone marrow, mobilized peripheral blood stem cells, or cord blood can be used as stem cell source for transplantation. In mice, fetal liver cells[163] and bone marrow[164] are generally used as donor sources for transplantation of the hematopoietic system. In both cases, transplantation of the hematopoietic system will result in a gradual replacement of the cells of the recipient by cells from donor origin, including monocytes and tissue macrophages.[165]

The bone marrow transplantation procedure involves four different stages:

- conditioning of the recipient
- isolation of the donor bone marrow
- transplantation of the donor bone marrow cells into the recipient
- repopulation of the recipients bone marrow by the donor cells

The primary purpose of conditioning of the recipient prior to infusion of donor bone marrow is to create space for the donor cells in the bone marrow by eradication of the endogenous bone marrow cells of the recipient and immunosuppression to prevent immunological reactions. The most active single conditioning agent in mammals to eradicate endogenous bone marrow is total body irradiation (TBI). The recovery time after lethal irradiation and bone marrow transplantation is 4-8 weeks.[166–168] Without donor bone marrow cell transplantation after lethal total body irradiation, the recipient will eventually die within two weeks as peripheral hematopoietic cells die in the two weeks following TBI. In the second week after irradiation (without transplantation) depletion of thrombocytes and leukocytes causes haemorrhages and septicaemia, leading to the death of the animal. Recovery of the hematopoietic cells in the irradiated recipient can be obtained by bone marrow cell infusion. Bone marrow is isolated by either bone marrow puncture in large animals or flushing femurs and tibias with saline in small animals, including mice. Repopulation of the bone marrow starts at four days after administration.[169–171]

Following successful bone marrow transplantation, the bone marrow cell population is generally completely restored at the seventh day after the transplantation. The peripheral blood cell count is returned to normal values at 8 weeks after transplantation.[54] Finally, a stable hematopoietic system is formed in which hematopoiesis is derived from small numbers of hematopoietic pluripotent stem cells of donor origin.[172] This bone marrow transplantation technique provides a unique opportunity to create an animal model in which the effect of certain genes

in cells of hematopoietic origin, including macrophages, can be studied against a specified, for example hyperlipidemic or atherosclerotic, background.

Animal models in atherosclerosis

Atherosclerosis is a complex, multifactorial, chronic inflammatory disease. The environmental and genetic factors make it particularly difficult to study the function of specific genes on the pathogenesis of atherosclerosis in man. To overcome this, suitable animal models, which possess an identical genetic background and can be kept in controlled environmental conditions, are widely used to study the mechanisms affecting atherosclerotic lesion development.[173] An animal model frequently used to study lipoprotein metabolism and atherogenesis is the mouse. With the development of genetic models of atherosclerosis, the mouse has become a very accessible model, especially since a very large genetic database about this species in relation to human biology has become available.[174] However, mouse lipoprotein metabolism differs from that in humans in several respects, thereby influencing the development of atherosclerosis.

The predominant lipoprotein in mice is HDL, whereas LDL is particularly abundant in humans.[175,176] Moreover, mice do not express CETP, which transfers CE from HDL to LDL and VLDL. Notably, compared to humans, mice are highly resistant to atherosclerosis (Table 2). Feeding mice a high cholesterol cholic acid containing diet, however, results in dramatic changes in their lipoprotein profiles, with the majority of cholesterol now in VLDL and LDL.[177,178] Consequently, feeding this diet for several months will result in fatty streak formation in the C57BL/6J mouse strain.[179] In addition to feeding high cholesterol diets, transgenesis and gene targeting are frequently used methods to alter the murine genome in order to study the function of specific genes regarding lipoprotein metabolism and atherogenesis.[180–182] To overcome the natural lack of atherosclerosis susceptibility in mice, several mouse strains were developed with genetic alterations, resulting in increased susceptibility to atherosclerotic lesion development.

The LDLr KO mouse model is among the most widely used models for characterization of atherosclerotic lesion development. Due to the absence of LDL receptors, VLDL and LDL removal from the circulation is impaired,[183] which subsequently results in

Table 2. Differences in lipid metabolism and atherosclerosis between humans and mice.

Parameter	Human	Mouse
HDL	50 mg/dL	50 mg/dL
LDL	150 mg/dL	10 mg/dL
CETP	Present	Absent
Atherosclerosis	Susceptible	Resistant

elevated serum cholesterol levels. Upon feeding a high-cholesterol diet, LDLr KO mice show strongly elevated serum cholesterol levels and thus rapid development of atherosclerosis.[184]

Another model to study atherosclerotic lesion development is the earlier mentioned apoE KO mouse model. Absence of apoE results in severe hypercholesterolemia. As a result, apoE KO mice spontaneously develop atherosclerosis, which can be strongly accelerated by a high-cholesterol diet.[185] Although murine atherosclerotic plaques resemble human plaques in several manners, they are not prone to rupture. As a result, atherosclerotic mouse models do not usually develop MI due to advanced atherosclerotic lesion development. Therefore, in order to study the consequences of MI, specific animal models need to be used. The predominant models of MI/ischemia are the Langendorff perfusion system (ex vivo; isolated heart),[186–189] and left anterior descending (LAD) occlusion (in vivo; Fig 4).[190,191]

In 1895, Langendorff for the first time described a method for perfusion of an isolated heart.[192] The modernized Langendorff setup is an ex vivo model used to study cardiac physiology, acute global or regional ischemia reperfusion, or the efficacy of drug or cell therapy on the heart.[187] The aorta is cannulated above the aortic valves, and perfused with a physiologic Krebs and Henseleit solution, which replaces the blood. The supply is reversed in the aorta so that the coronary

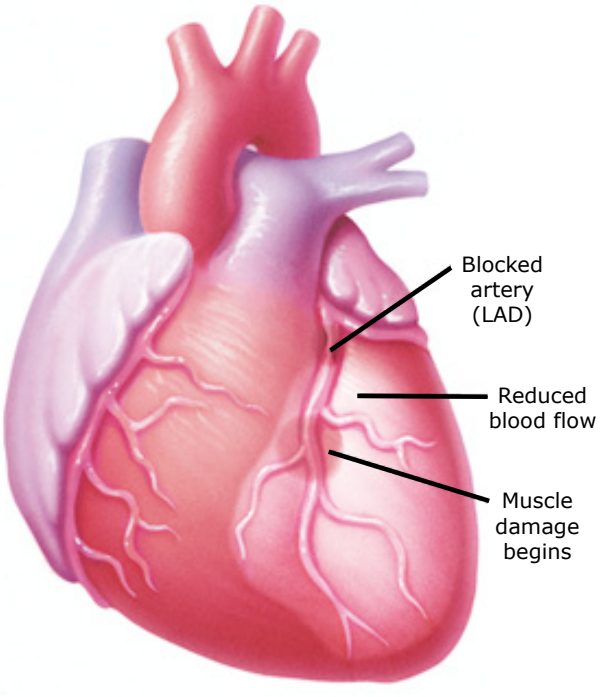

Figure 4. Myocardial infarction. See text for explanation. LAD, left anterior descending coronary artery.

arteries are filled with the perfusion fluid and the aortic valve is closed by the perfusion pressure. The venous effluent can then easily be collected via openings of the caval veins or the pulmonary artery. Global ischemia is caused by clamping the aortic flow, whereas regional ischemia can be induced by ligation of a coronary artery.

Unlike the Langendorff model, which studies the heart outside the systemic influences from the body, the LAD occlusion model provides insight into the systemic influences on the heart after MI.[190,191] Occlusion of the LAD is generally performed through a left thoracotomy. After exposing the LAD, a suture is used to either temporarily occlude or permanently ligate the LAD or a branch of the LAD.[186] The use of an LAD occlusion model is most suitable for investigating the effects of MI over time, as these mice can be followed for several weeks after intervention without complicating factors such as anesthesia or artificial ventilation.

Thesis outline

In this thesis, the roles of several key genes in relation to reverse cholesterol transport and cardiovascular disease are described. **Chapter 2** provides a comprehensive review of the importance of ABCA1 and ABCG1 in HDL metabolism, macrophage cholesterol efflux, and atherogenesis. **Chapter 3** focuses on the combined deletion of macrophage ABCA1 and apoE in atherosclerosis susceptible LDLr KO mice. ABCA1/apoE dKO transplanted mice were shown to have elevated serum cholesterol levels as compared to single ABCA1 KO transplanted mice. Importantly, these dKO transplanted mice also suffered from enhanced systemic and hepatic inflammation. Together with an observed defect in cholesterol efflux, this led to augmented atherosclerotic lesion development. **Chapter 4** delineates the combined effect of macrophage ABCG1 and apoE has been studied by means of bone marrow transplantation. Whereas single deletion of macrophage ABCG1 or apoE led to a moderate increase in atherosclerotic lesion development, combined deletion induced a more dramatic increase in atherosclerosis. Moreover, gene- and protein expression data revealed independent roles for both genes in atherogenesis. Since differential effects of (macrophage) ABCG1 deficiency were observed in different laboratories, in **chapter 5**, the effect of ABCG1 deficiency in LDLr KO mice has been studied at different stages of atherosclerotic lesion development. In **chapter 6**, the role of macrophage ATGL with respect to atherosclerotic lesion development has been investigated. Despite the observed TG accumulation in ATGL deficient macrophages, LDLr KO mice transplanted with ATGL KO bone marrow showed attenuated atherosclerotic lesion formation as compared to controls. Decreased infiltration of less inflammatory macrophages into the arterial wall, and increased macrophage apoptosis most likely caused this

effect. In **chapter 7**, the importance of macrophage ABCA1 in atherosclerosis in the absence or presence of the spleen has been investigated. Although splenic alterations were observed upon macrophage ABCA1 deficiency, this had no effects on atherosclerotic lesion development. **Chapter 8** describes the role of ABCA1 in case of acute myocardial infarction. ABCA1 deficiency was shown to attenuate cardiac ischemia after a myocardial infarction, despite its protective role in atherosclerosis. The presence of ABCA1 in isolated hearts did not contribute to the observed effects, indicating that the reduced myocardial infarction has been caused by secondary, ABCA1-mediated effects. In **chapter 9**, the results obtained in this thesis are summarized and discussed.

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Chapter 2

ABC transporters A1 and G1, HDL metabolism, cholesterol efflux, and inflammation: important targets for the treatment of atherosclerosis

Curr Drug Targets.
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Dan Ye
Bart Lammers
Ying Zhao
Illiana Meurs
Theo J.C. van Berkel
Miranda van Eck

Division of Biopharmaceutics, LACDR, Leiden University, the Netherlands

Abstract

Atherosclerosis has been characterized as a chronic inflammatory response to cholesterol deposition in arteries. Plasma high density lipoprotein (HDL) levels bear a strong independent inverse relationship with atherosclerotic cardiovascular disease. One central antiatherogenic role of HDL is believed to be its ability to remove excessive peripheral cholesterol back to the liver for subsequent catabolism and excretion, a physiologic process termed reverse cholesterol transport (RCT). Cholesterol efflux from macrophage foam cells, the initial step of RCT is the most relevant step with respect to atherosclerosis. The ATP-binding cassette (ABC) transporters ABCA1 and ABCG1 play crucial roles in the efflux of cellular cholesterol to HDL and its apolipoproteins. Moreover, ABCA1 and ABCG1 affect cellular inflammatory cytokine secretion by modulating cholesterol content in the plasma membrane and within intracellular compartments. In humans, ABCA1 mutations can cause a severe HDL-deficiency syndrome characterized by cholesterol deposition in tissue macrophages and prevalent atherosclerosis. Disrupting ABCA1 or ABCG1 in mice promotes accumulation of excessive cholesterol in macrophages, and physiological manipulation of ABCA1 expression affects atherogenesis.

Here we review recent advances in the role of ABCA1 and ABCG1 in HDL metabolism, macrophage cholesterol efflux, inflammation, and atherogenesis. Next, we summarize the structure, expression, and regulation of ABCA1 and ABCG1. Finally, we give an update on the progress and pitfalls of therapeutic approaches that target ABCA1 and ABCG1 to stimulate the flux of lipids through the RCT pathway.

Introduction

Plasma levels of high density lipoprotein (HDL) are inversely proportional to the risk of atherosclerotic cardiovascular disease (CVD) in humans.[1] The key mechanism underlying the atheroprotective properties of HDL is thought to be its role in reverse cholesterol transport (RCT), a physiologic process by which excess peripheral cholesterol is transported by HDL to the liver for excretion in the bile and feces.[2] In addition, HDL can prevent oxidation of low density lipoprotein (LDL),[3] act as an anticoagulant,[4] and have anti-inflammatory properties,[5] all of which may contribute to the antiatherogenic effects of HDL. This presumption leads to an assertion that raising HDL cholesterol (HDL-C) levels alone would improve RCT and protect against the development of atherosclerosis. However, high HDL-C concentration appears not to be always synonymous with an efficient RCT. Therefore, improving the HDL functionality may be more attractive to potentially stimulate RCT. The initial step of RCT is thought to be dependent upon extracellular lipid acceptors, including HDL and/or lipid-poor apolipoproteins. Studies have indicated that the ATP-binding cassette (ABC) transporter ABCA1 is required for the maintenance of normal plasma HDL-C levels, and is important for the first step of RCT, i.e., transfer of cholesterol to lipid-poor HDL apolipoproteins (e.g., apoA-I).[6] ABCG1 is also of interest, since it participates in an intermediate RCT step, i.e. transfer of cholesterol to mature HDL particles (e.g., HDL_2 and HDL_3).[7] Moreover, ABCA1 and ABCG1 export lipids to either cell surface-bound or internalized apolipoproteins.[8,9] Minor changes in cholesterol content in the plasma membrane or within intracellular vesicular compartments can significantly impact cell function via influencing cellular signaling events.[10,11] Thus, the traditional role of ABCA1 and ABCG1 in lipid transport is mechanistically linked to their potential role in suppressing inflammation.

Here we review recent advances in the role of ABCA1 and ABCG1 in HDL metabolism, macrophage cholesterol efflux, inflammation, and atherogenesis. Next, we summarize the structure, expression, and regulation of ABCA1 and ABCG1. Finally, we attempt to integrate this information and discuss the progress and pitfalls of therapeutic approaches that target ABCA1 and ABCG1 to stimulate the flux of lipids through the RCT pathway.

ABCA1 and ABCG1 in HDL metabolism and consequences for atherosclerosis

ABCA1, HDL, and atherosclerosis

ABCA1, first known as ABC1, was cloned in 1994 by Luciani and colleagues,[12] as a homolog of yeast ced-7, and is a member of the large superfamily of ABC

transporters that use ATP as an energy source to transport lipids and other molecules across membranes.[13] Understanding of the role of ABCA1 in HDL biogenesis came with the discovery that mutations in the ABCA1 gene result in the extreme HDL deficiency syndrome Tangier disease (TD). In 1999, the mutation in TD was mapped to chromosome 9q31 in the ABCA1 gene.[14-17] Since then, an enormous amount of research has been set off to investigate the mechanism of action, regulation, and suitability of ABCA1 as a target to increase HDL formation therapeutically for the treatment and prevention of atherosclerosis.

The defective cholesterol and phospholipid efflux to apoA-I from fibroblasts derived from TD patients suggested that the removal of cellular lipids to apoA-I is a major predictor of plasma HDL-C levels.[18,19] Although the structure and synthetic rates of apoA-I are normal in TD, the hypercatabolism of circulating apoA-I may result in the severe HDL deficiency syndrome in this disease.[20] As a result, homozygous TD is associated with extremely low levels of apoA-I (<1% normal) and HDL-C (<5% normal). In accord, several recent genome-wide association studies have identified common variants in ABCA1 as a significant source of variation in plasma HDL-C levels across multiple ethnic groups,[21-23] establishing ABCA1 as a major gene influencing HDL levels in humans. However, the impact of ABCA1 on atherosclerosis remains controversial. An early systematic survey of TD patients indicated homozygotes over the age of 30 display a 6-fold higher incidence of CVD than normolipidemic control subjects.[24] Of note, the increased risk of atherosclerosis in TD patients does not appear to be as dramatic as one would expect in individuals with an almost complete absence of HDL. One possible explanation is that TD homozygotes in addition to having low HDL-C also have reduced levels of VLDL and LDL cholesterol (VLDL-C and LDL-C), and thus the atherogenic stimulus may be diminished. Comparison of the phenotypes of TD homozygotes with heterozygotes who have decreased HDL-C (≈40% normal) but normal VLDL-C or LDL-C levels confirmed that ABCA1 is a rate-limiting factor in HDL biosynthesis, while one functional ABCA1 allele is sufficient to maintain normal LDL levels. Moreover, heterozygotes do not display the excessive accumulation of lipid-laden tissue macrophages as observed in homozygotes,[25] implying that one functional ABCA1 allele is sufficient to prevent excessive cholesterol accumulation in macrophages. Interestingly, heterozygotes also showed significant increases in CVD incidence and carotid artery intima media thickness as compared to healthy controls.[26] One study of the Copenhagen City Heart cohort revealed that genetic variations in ABCA1 affect CVD in the general population.[27] However, a follow up study of the same cohort concluded that low HDL caused by loss-of-function mutations in ABCA1 does not contribute to the risk of CVD.[28] It was also reported that both common and rare ABCA1 variations are associated with increased CVD despite normal HDL levels.[27,29,30] Collectively, these studies support the idea that impaired ABCA1 function increases atherosclerosis

in humans, although not in all cases by mechanisms that reflect reduced plasma HDL-C levels.

Studies in mice where the ABCA1 locus is deleted or human ABCA1 is overexpressed generally support the hypothesis that ABCA1 by maintaining circulating HDL-C levels and cellular cholesterol efflux significantly prevents atherosclerosis. Total-body ABCA1-deficient (ABCA1 KO) mice exhibit low plasma HDL-C levels and cholesterol accumulation in peripheral macrophages, a phenotype similar to that of TD patients.[31] However, atherosclerotic lesions were not increased when ABCA1 KO mice were fed chow or a high-fat, high-cholesterol (HF/HC) diet.[31] Upon cross-breeding of ABCA1 KO mice with hypercholesterolemic mouse models (apoE KO or LDLr KO mice), the accumulation of lipid-laden foam cells in peripheral tissues was especially pronounced.[32] However, compared with the control mice, lesion size was not increased in either ABCA1 KO apoE KO or ABCA1 KO LDLr KO double knockout mice, which is probably caused by a less atherogenic lipid profile in these animals. Recent studies have used tissue-specific

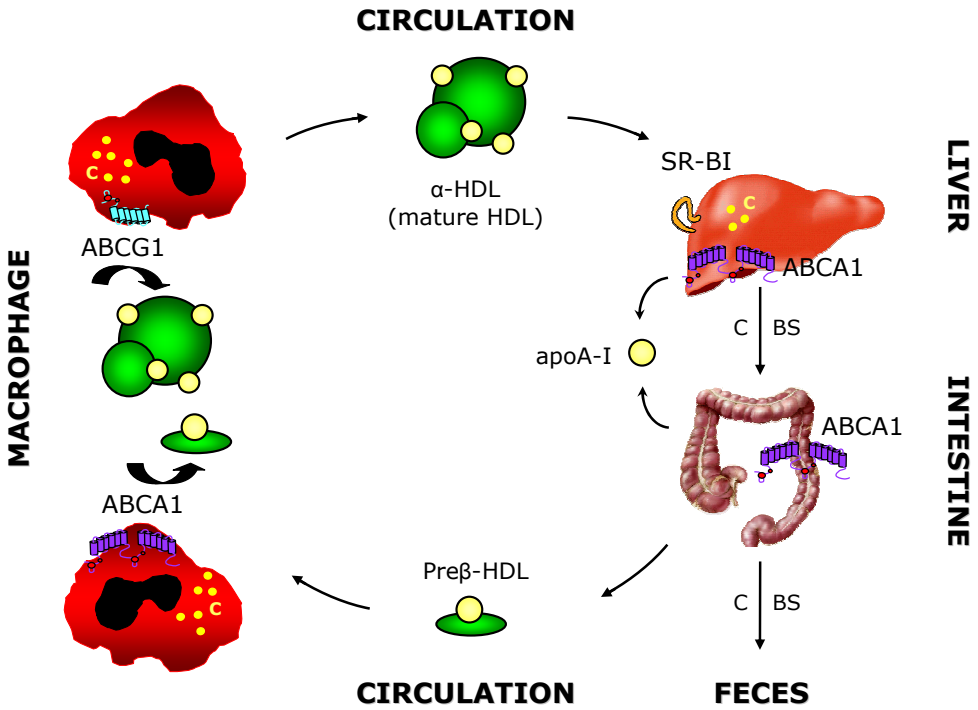

Figure 1. Schematic overview of the role of ABCA1 and ABCG1 in the reverse cholesterol transport (RCT). ApoA-I is secreted by liver and intestine and loaded with cholesterol (C) and phospholipids by ABCA1. The thus formed preβ-HDL picks up cholesterol and phospholipids from ABCA1 in macrophages and peripheral cells and is converted to α-HDL. These mature HDL particles can be further loaded with cholesterol at least in part by ABCG1 in macrophages and delivers in turn its cargo to SR-BI in the liver. Subsequently, cholesterol can be secreted into the bile either in the free form or after conversion as bile salt (BS). After transport via the bile into the intestine, cholesterol and bile salts are reabsorbed or excreted in the feces.

knockout or knockdown of the ABCA1 locus to assess the respective role of liver, intestine, and macrophage ABCA1 in HDL metabolism and atherogenesis. Liver-specific ABCA1 KO mice have a ≈80% reduction in HDL-C levels,[33] and liver-specific partial gene knockdown of ABCA1 significantly reduced HDL-C levels by ≈40%.[34] Subsequent studies reported that intestine-specific ABCA1 KO mice have a ≈30% reduction in HDL-C levels.[35] Thus, the liver and intestine that synthesize apoA-I,[36,37] are primarily responsible for lipidating newly secreted lipid-poor apoA-I via ABCA1-mediated lipid efflux. More recently, Brunham et al. elegantly showed the tissue-specific role of ABCA1 in influencing susceptibility to atherosclerosis.[38] Their results indicated that physiologically global ABCA1 overexpression reduced atherosclerosis. Deletion of hepatic ABCA1 significantly accelerated atherosclerosis, indicating that the liver is an important site at which ABCA1 plays an antiatherogenic role. Surprisingly, selective deletion of macrophage was not found to have a strong impact on lesion burden. Moreover, previous studies have shown that, independently of changes in plasma HDL-C levels, deletion of ABCA1 in bone marrow-derived hematopoietic cells increases atherosclerosis,[32,39] whereas overexpression of bone marrow ABCA1 reduces atherosclerosis.[40] Whether ABCA1 expression in other hematopoietic cells (e.g., dendritic cells, lymphocytes, and endothelial progenitor cells) prevents atherosclerosis via modulating immunologic responses, including the secretion of inflammatory cytokines and T cell proliferation, remains to be further investigated. Together, these studies demonstrate that ABCA1 is a rate-limiting factor in HDL biosynthesis. In liver and intestine ABCA1 is essential for maintaining plasma HDL-C levels, while macrophage ABCA1 only has a minor effect on circulating HDL but is important as a mediator of cellular lipid efflux to exogenous lipid-poor HDL apolipoproteins (Fig. 1). Physiological deletion or expression of ABCA1 modulates susceptibility to atherosclerosis in mice, and hepatic ABCA1 may be an appropriate therapeutic target for raising HDL levels and reducing atherosclerosis in humans.

ABCG1, HDL, and atherosclerosis

ABCG1, also known as ABC8 or human white gene, exhibits high homology with the Drosophila white gene, which acts as a regulator of tryptophan and guanine uptake in conjunction with the scarlet and brown genes.[41,42] To date, no genetic variations in human ABCG1 have been identified that link it to any inheritable disease. Nevertheless, Mauldin et al. reported that ABCG1 expression and function are significantly reduced in patients with type 2 diabetes mellitus, potentially contributing to the formation of lipid-laden macrophages and accelerated atherosclerosis in these patients.[43] Schou et al. demonstrated that a functional variant in the ABCG1 promoter, ABCG1 -376C>T located in a putative SP1 binding site, reproducibly predicts an increased incidence of

ischemic heart disease in the general population.[44] In addition, Furuyama et al. reported that another ABCG1 -257T>G promoter polymorphism confers an appreciable increase in the risk of atherosclerotic disease severity in Japanese men.[45] In the latter two studies, plasma HDL-C levels are relatively normal in subjects with ABCG1 mutations.[44,45] However, Furuyama et al. suggested that ABCG1 may influence the lipid composition in HDL particles, affect the turnover of HDL metabolism and atherogenesis in humans.[45] In an attempt to determine the function for human ABCG1, Klucken et al. treated human macrophages with antisense oligonucleotides to ABCG1, and this treatment resulted in decreased efflux of cholesterol and phospholipids to HDL_3.[7] However, the exact cellular and physiological function of ABCG1 remained unclear.

Studies evaluating the physiological relevance of ABCG1 transport activity have relied mainly on mouse models where the endogenous ABCG1 locus is deleted or human ABCG1 is overexpressed. Kennedy et al. first reported that ABCG1 KO animals did not display an overt dyslipidemia when maintained on a chow diet.[46] However, these null animals accumulated massive both neutral lipids and phospholipids in hepatocytes and in macrophages within multiple tissues following administration of a HF/HC diet. Conversely, overexpression of human ABCG1 protected murine tissues from dietary fat-induced lipid accumulation. Despite these observations, when administered a HF/HC diet, loss of total body ABCG1 transport did not significantly accelerate atherosclerosis, and loss of macrophage ABCG1 in atherosclerotic mouse models (apoE KO or LDLr KO mice) has been variously reported to be either pro- or antiatherosclerotic.[47-50] Moreover, total body human ABCG1 overexpression resulted in either no effect,[51] or aggravation,[52] of atherosclerosis. These results indicate that manipulating ABCG1 alone does not markedly impact atherosclerosis in mice.

ABCA1 converts lipid-poor/free apoA-I to partially lipidated "nascent" HDL, and these particles in turn may serve as substrates for ABCG1-mediated cholesterol export.[53] This raises the interesting possibility that ABCA1 and ABCG1 may coordinate the removal of excessive cellular cholesterol. Recently, ABCA1 and ABCG1 double knockout (ABCA1/ABCG1 dKO) mice were generated by several independent groups to investigate the potential synergistic relationship between ABCA1 and ABCG1 in modulating macrophage cholesterol homeostasis and atherogenesis. Out et al. reported that combined ABCA1 and ABCG1 deficiency resulted in a completely abolished cholesterol mass efflux from macrophages to HDL.[54] Similar studies by Yvan-Charvet et al. showed that ABCA1/ABCG1 dKO macrophages displayed a more than 2-fold decrease in the ability to promote cholesterol efflux to HDL or serum as compared to ABCA1 KO, ABCG1 KO and wild-type macrophages.[55] In vivo studies using a macrophage-specific RCT assay also indicated that cholesterol efflux from ABCA1/ABCG1 dKO macrophages to plasma, liver, and feces was highly reduced as compared to animals that received

macrophages from single knockout or wild-type mice.[56] Defective cholesterol efflux due to the combined deficiency of ABCA1 and ABCG1 was associated with markedly increased cholesterol content, primarily in the form of cholesteryl ester (CE), in resident macrophages of the peritoneal cavity, lung, liver, spleen, Peyer's patches, and lymph nodes in chow-fed ABCA1/ABCG1 dKO mice.[54] However, these mice did not show lipid accumulation in the arterial wall. This is most likely caused by the severe hypocholesterolemia in these mice, which is unable to provide the stimulus to attract macrophages to infiltrate into the arterial wall. Furthermore, Yvan-Charvet et al. showed that LDLr heterozygous mice receiving ABCA1/ABCG1 dKO transplants had substantially greater atherosclerosis as compared to recipients receiving single knockout or wild-type bone marrow, following administration of a HF/HC bile salt diet (1.25% cholesterol, 7.5% cocoa butter and 0.5% cholic acid).[55] Out et al. reported that reconstitution of LDLr KO mice with ABCA1/ABCG1 dKO bone marrow cells resulted in a prominent accumulation of lipids in the liver, lung and spleen as compared to recipients receiving single knockout or wild-type bone marrow, following a Western-type diet (0.15% cholesterol and 15% fat).[57] However, in the context of LDLr KO recipients, loss of ABCG1 did not significantly worsen atherosclerosis beyond that caused by the loss of only ABCA1, which is possibly reflecting an unexpected decreased in plasma VLDL/LDL levels in the ABCA1/ABCG1 dKO transplanted mice. The study of Out et al. was interpreted as showing a disproportionate increase in atherosclerosis given the degree of VLDL/LDL lowering.

Taken together, these studies indicate that ABCG1 plays a critical role in lipid homeostasis by controlling tissue lipid levels and the efflux of cellular cholesterol to HDL (Fig. 1). Unlike ABCA1, ABCG1 does not have a rate-limiting role in maintaining plasma HDL-C levels. Likewise, loss of ABCG1 alone does not markedly accelerate atherosclerosis. In spite of having a varied effect on atherosclerosis, ABCG1 in concert with ABCA1 does make a major contribution to cellular cholesterol export and prevent macrophage foam cell formation.

ABCA1 and ABCG1 in inflammation, independent of changes in circulating HDL levels

Atherosclerosis is a chronic inflammatory disease in which macrophages and endothelial cells (EC) play important roles. Macrophages are a primary cell type involved in innate immunity. Lipopolysaccharide (LPS), a major outer membrane constituent of Gram-negative bacteria, is a potent endotoxin that, through the activation of cellular immunity, induces a cytokine-mediated systemic inflammatory response in the host.[58,59] A series of studies have shown that deficiency of ABCA1 and/or ABCG1 in macrophages leads to LPS

hypersensitivity,[55,60,68,78] implying a potential role of these two transporters in suppressing macrophage inflammation. In addition to macrophages, aortic EC also express ABCA1 and ABCG1 and export cholesterol to HDL.[61] EC in the artery wall become activated in many cases by oxidized lipids in early atherogenesis.[62] Activated EC secrete pro-inflammatory chemokines that recruit monocytes to the activated endothelium.[63] Several studies have shown that incubation of EC with HDL reduces the endothelial inflammatory response.[64-66] It is thus possible that ABCA1 and ABCG1 modulate inflammatory responses in vascular EC at least in part by facilitating cholesterol efflux to HDL in these cells.

Anti-inflammatory properties of ABCA1

Francone et al. reported that LPS-induced septic shock was exacerbated in ABCA1/LDLr KO mice compared with LDLr KO mice,[59] suggesting a potential role of ABCA1 in suppressing inflammation. Subsequent studies by Aiello et al. showed that macrophages from ABCA1 KO/LDLr KO mice were enriched in CE content (80-fold higher), and plasma HDL concentrations in these mice were extremely low.[67] However, it is not clear whether the observed heightened response to LPS in macrophages from ABCA1 KO/LDLr KO mice is due to the massive cellular CE accumulation, the low plasma HDL-C levels, or some other alterations. Recently, macrophage-specific ABCA1 KO mice were generated by Zhu et al.,[68] and it was demonstrated that macrophages lacking ABCA1 are hypersensitive to LPS stimulation, independently of low plasma HDL-C levels, requiring the expression of MyD88 which is an adaptor protein that mediates LPS signaling initiated by the Toll-like receptor 4 (TLR4). Koseki et al. showed that cultured ABCA1 KO macrophages secreted greater amounts of pro-inflammatory tumor necrosis factor-α (TNF-α) in response to LPS, and that this response was associated with increased levels of free cholesterol (FC) in lipid rafts on the cell surface.[69] These data support the concept that ABCA1 modulates cell surface cholesterol levels and inhibits its partitioning into lipid rafts, which may explain the hypersensitivity to LPS in ABCA1 KO macrophages. Moreover, Sun et al. reported that FC accumulation in the endosomal compartment increased the inflammatory response in a TLR-dependent fashion, with TLR3 playing the major role.[70] Combined, it is plausible that the anti-inflammatory properties of ABCA1 are mediated by its ability to modulate cholesterol content both on the cell surface and within intracellular compartments (Fig. 2).

However, recent work by Tang et al. suggested that not all of the anti-inflammatory properties of ABCA1 are a consequence of its lipid transport activity.[71] It was reported that the interaction of apoA-I and its mimetic peptides with ABCA1 promotes cholesterol removal and activates signaling molecules, such as Janus kinase 2 (JAK2), which optimize the lipid export activity of ABCA1.[72,73] JAK2 activation, however, also stimulates signaling pathways that activate

transcription factors called STATs.[74] One of the STATs, STAT3, plays a major role in suppressing inflammatory cytokine production by macrophages.[74,75] As a result, incubation of apoA-I with activated ABCA1-expressing macrophages suppressed the production of inflammatory cytokines TNF-α, IL-1β, and IL-6 by

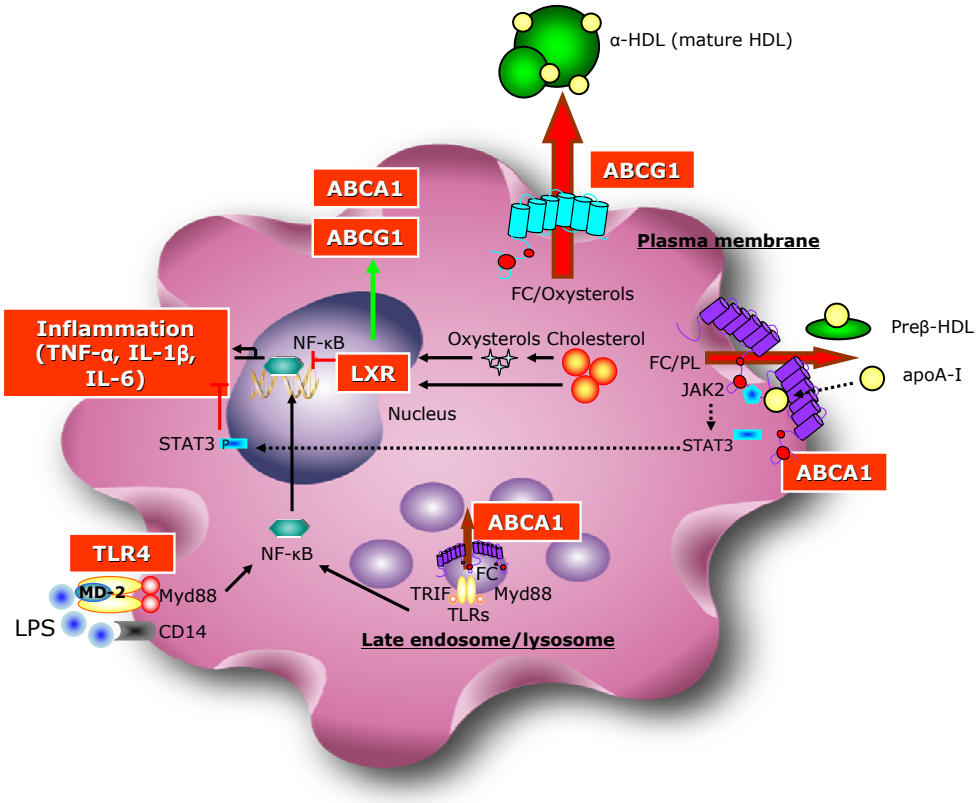

Figure 2. Protective effects of ABCA1 and ABCG1 on macrophage cholesterol efflux and inflammation. The ABC transporters ABCA1 and ABCG1 coordinate to facilitate macrophage cholesterol efflux to HDL and its apolipoproteins. ABCA1 transports phospholipids (PL) and cholesterol, while ABCG1 is largely a cholesterol transporter. Cholesterol efflux to apoA-I specifically requires ABCA1, whereas efflux to HDL requires ABCG1. Unlike ABCA1, ABCG1 has a specific role in promoting cellular efflux of oxysterols modified at the 7 position, such as 7-ketocholesterol- and 7β-hydroxycholesterol. Activation of LXR by oxysterols robustly induces the transcriptional expression of ABCA1 and ABCG1 in macrophages, leading to enhanced cholesterol efflux and resolution of the cholesterol overload in these cells. ABCA1 and ABCG1 modulate the fluidity of the plasma membrane as well lipid raft formation. This, in turn, modulates the Toll-like receptor 4 (TLR4) expression and signaling upon activation via the LPS/MD-2 complex formed after binding of LPS to CD14. Subsequently, downstream signaling molecules such as Myd88 will control the activation of NF-κB and the inflammatory gene expression response. Activation of LXR can also modulate the inflammatory response through suppression of NF-κB, but this mechanism appears to be independent of ABCA1 or ABCG1 activity. Free cholesterol (FC) accumulation in the late endosome/lysosome can also modulate the inflammatory response by spontaneous activation of TLRs, such as TRIF-dependent TLR3, present in these intracellular compartments. By promoting cholesterol efflux from the late endosomal compartment, ABCA1 thus has a unique role in dampening inflammation. In addition, binding of apoA-I to ABCA1 stimulates the activation of signaling molecules, such as Janus kinase 2 (JAK2). The activated JAK2 both enhances the interaction of apoA-I with ABCA1 and stimulates the transcription factor STAT3. The phosphorylated STAT3 is translocated to the nucleus where it suppresses LPS-mediated production of inflammatory cytokines, without inhibiting LPS-induced NF-κB activation.

a STAT3-dependent process.[75] This novel role of ABCA1 was further confirmed by results showing that apoA-I lost its anti-inflammatory activity in ABCA1 KO macrophages.[75] The generation of mutant forms of ABCA1 that lack the ability to activate STAT3 but retain lipid export activity is worth exploiting, and is expected to provide important mechanistic insight into the anti-inflammatory properties of ABCA1 (Fig. 2).

Anti-inflammatory properties of ABCG1

Wojcik et al. reported that ABCG1 KO mice had increased pro-inflammatory cytokine levels in their lungs, leading to the recruitment of neutrophils, eosinophils, B cells, T cells, and dendritic cells.[76] They also showed that macrophages are the primary cell type involved in the onset of both pulmonary lipidosis and inflammation in ABCG1 KO mice. Likewise, Yvan-Charvet et al. found that macrophages lacking either ABCG1, or both ABCA1 and ABCG1 secreted significantly more cytokines and chemokines.[55] Both ABCA1 and ABCG1 modulate cell surface cholesterol levels and inhibit its partitioning into lipid rafts. Given lipid rafts may provide platforms for innate immune receptors to respond to inflammatory signals,[77] it follows that loss of ABCA1 and ABCG1 by increasing raft content will increase signaling through these receptors. In agreement, basal and LPS-stimulated thioglycollate-elicited peritoneal macrophages showed increased inflammatory gene expression in the order ABCA1/ABCG1 dKO>ABCG1 KO>ABCA1 KO>wild-type, and TLR4 cell surface concentration was increased in ABCA1/ABCG1 dKO>ABCG1 KO>ABCA1 KO>wild-type macrophages.[78] Interestingly, replenishment or removal of cholesterol using cyclodextrin reduced the inflammatory response of ABCA1 KO and ABCG1 KO macrophages,[78,79] supporting the hypothesis that the increased inflammatory response is attributable to cholesterol accumulation in the plasma membrane of ABCA1 KO and ABCG1 KO macrophages. ABCG1 appears to have a more potent role in modulating macrophage inflammation than ABCA1, which perhaps reflects a predominant role of this transporter in modulating cholesterol content in lipid rafts.[80]

Both apoptosis and necrosis was increased in ABCG1 KO alveolar macrophages,[76] which may also contribute to or dampen pulmonary inflammation. In agreement, Baldán et al. showed that ABCG1 KO peritoneal macrophages were more apoptotic.[47] When FC accumulates in the macrophage, the ratio of FC to phospholipids is disturbed in the endoplasmic reticulum (ER) membrane.[81] The "stiffening" of the ER membrane bilayer leads to ER protein dysfunction, ER stress, and the unfolded protein response, resulting in apoptosis of the cell.[81] Furthermore, oxysterols that are formed within macrophages as a consequence of cholesterol accumulation may cause cells to be more prone to apoptosis.[82,83] Unlike ABCA1, ABCG1 has a specific role in promoting cellular efflux of oxysterols

modified at the 7 position, such as 7-ketocholesterol- and 7β-hydroxycholesterol, to prevent macrophages from oxysterol-induced cell death.[84,85] Thus, it is possible that ABCG1 KO macrophages are more prone to apoptosis due to the toxic effects of FC and oxysterol accumulation.

More recently, Whetzel et al. reported that reductions in ABCG1 expression in endothelium promote a pro-inflammatory endothelial phenotype.[86] Aortic EC from ABCG1 KO mice displayed increased production of chemokines and increased surface expression of the adhesion molecules that promote monocyte adhesion and interactions. The regulation of monocyte-endothelial interactions by ABCG1 appeared to be independent of HDL binding. Furthermore, the pro-inflammatory phenotype of ABCG1 KO EC was not mediated by NFκB, a key molecule involved in the regulation of the inflammatory response to both infection and tissue damage. Instead, the IL-6-IL-6 receptor axis was found to be induced in ABCG1 KO EC, and this pathway, through STAT3 signaling, regulates monocyte-endothelial interactions in ABCG1 KO mice. Thus, in addition to suppressing macrophage inflammation, ABCG1 may also be important for regulating the early inflammatory response in EC in the vessel wall. Further studies examining whether the effect of ABCG1 deficiency on the total cholesterol content and/or the intracellular distribution of cholesterol in EC as well as macrophages and the consequence for cellular signaling are needed to fully understand the anti-inflammatory properties of ABCG1.

Structure, expression and regulation of ABCA1 and ABCG1

The structure, expression, and regulation of ABCA1

ABCA1 is a 2261 amino acid integral membrane protein. It is a full transporter, comprising two halves of similar structure. Each half has a transmembrane domain containing six helices and a nucleotide binding domain (NBD) containing two conserved peptide motifs known as Walker A and Walker B, which are present in many proteins that use ATP in energizing their transport activity, and a Walker C signature unique to ABC transporters. ABCA1 is predicted to have an N terminus oriented into the cytosol and two large extracellular loops that are highly glycosylated and linked by one or more cysteine bonds.[87,88]

ABCA1 is expressed ubiquitously, but with the highest concentrations in the liver, brain, adrenal glands, and macrophage foam cells.[89-91] Experiments using biotinylation,[92] anti-FLAG antibody in FLAG-ABCA1 transfected cells,[93] green fluorescent protein (GFP)-ABCA1 transfected cells,[94,95] and a polyclonal antibody,[96] have suggested that ABCA1 localizes to the plasma membrane and to intracellular compartments (e.g., early and late endosomes and lysosomes).

Although still controversial, it is suggested that ABCA1 may form a channel in the plasma membrane that promotes flipping of lipids from the inner to outer membrane leaflet by an ATPase-dependent process.[97] Some data support a direct efflux mechanism wherein ABCA1 first binds apoA-I, resulting in subsequent transfer of lipid to the bound apoA-I, which is then released from the cell, with the latter steps presumably still occurring within the context of the high affinity complex.[98,99] Other more indirect models have also proposed that ABCA1 activity creates lipid raft membrane domains in the absence of apoA-I, and that these lipid domains may represent a larger capacity but lower affinity cellular binding site for apoA-I that is not closely associated with ABCA1.[100,101] Subsequently, cellular phospholipids and cholesterol are transported simultaneously or phospholipid efflux precedes cholesterol efflux.[102,103] These partially lipidated apolipoproteins can then acquire additional cholesterol by other processes and mature into larger spherical HDL particles (HDL_2 and HDL_3). The selective uptake of HDL by the liver is mediated by scavenger receptor class B type I (SR-BI), which has a high affinity for lipid-rich HDL particles, but low affinity for lipid-poor HDL apolipoproteins.[104]

In addition to mediating lipid efflux on the cell surface, ABCA1 may also be internalized along with apoA-I in vesicles to intracellular compartments where ABCA1 pumps lipids into the vesicles for association with apoA-I, and subsequent release of nascent HDL particles upon fusion with the plasma membrane (retroendocytosis).[105,106] Smith et al. reported the uptake and resecretion of labeled apoA-I,[107] and colocalization of GFP-ABCA1 and apoA-I in intracellular compartments.[108] In agreement, Hassan et al. showed that cell binding of apoA-I specifically mediates the continuous endocytic recycling of ABCA1, and that this pathway plays a central role in the genesis of nascent HDL.[109] Moreover, Chen et al. found impaired ABCA1 internalization in cells expressing a mutant form of ABCA1 (ABCA1delPEST), and the failure of the ABCA1-PEST mutant to reach late endosomes was associated with less ability to export endosome/lysosomal cholesterol to apoA-I, but not cell surface cholesterol.[110] They also reported that trafficking of ABCA1, and possibly apoA-I, to the late endosome/lysosome compartment is responsible for a quantitatively significant percentage of total ABCA1-dependent cholesterol efflux.[110] Thus, it is conceivable that internalization of ABCA1 is necessary for the mobilization of cellular cholesterol, at least during transit through the late endosome/lysosome compartment.

As expected for a transporter that mediates excess cellular cholesterol export, transcription of ABCA1 is markedly induced by overloading cells with cholesterol.[89,90] ABCA1 expression is regulated at multiple levels, including the nuclear receptors liver X receptor (LXR) and retinoid X receptor (RXR) binding to the promoter region of the ABCA1 gene, with LXR being activated by elevated levels of oxysterols in cholesterol-loaded cells.[111,112] Several additional modes of transcriptional regulation of ABCA1 by the transcription factors Sp1/3, upstream

stimulatory factor 1/2 (USF1/2), hepatocyte nuclear factor 1a (HNF-1a), cyclic AMP (cAMP), and Zinc finger protein 202 (ZNF202) have been reviewed.[113] Although much emphasis has been placed on the transcriptional regulation of ABCA1, new studies are beginning to reveal its post-transcriptional regulation. Under basal conditions in cultured cells, ABCA1 has a relatively rapid turnover rate, as evidenced by the fact that ABCA1 protein is highly unstable in absence of inducers (half-life of 1 to 2 hours).[114-116] ABCA1 is stabilized by apoA-I binding to cells, through apoA-I-dependent inhibition of calpain-mediated proteolysis of ABCA1.[115] Multiple constitutive and apoA-I-stimulated phosphorylation events have been described that both enhance,[72,117-121] or inhibit,[122] ABCA1 activity. In addition, Arakawa and Yokoyama showed that apoA-I itself stabilizes ABCA1 by retarding its degradation.[123] In their study, A,N-acetyl-leucine-leucyl-norleucinal aldehyde (ALLN), but not lactacystin, blocked the turnover of ABCA1 in the absence of apoA-I, and these authors speculated that a non-proteasomal thiol protease was involved. In another study by Feng et al., the decrease in ABCA1 protein in FC-loaded macrophages was blocked to similar degrees by ALLN and lactacystin, suggesting an important role for proteasomal degradation in this process.[124] An important area of future investigation will be to determine the molecular mechanisms linking FC loading to proteasomal degradation of ABCA1, including the possibility that FC loading triggers ubiquitination of ABCA1. Moreover, Wang and Oram reported that unsaturated fatty acids (FAs) also accelerate the degradation of ABCA1 and thus decrease apoA-I-mediated efflux.[116] Unsaturated FAs not only act on the transcriptional regulation of ABCA1 (as antagonists of oxysterols in LXR activation), but also induce ABCA1 protein degradation (via a signaling pathway involving activation of phospholipase D2 and phosphorylation of ABCA1 serines).[125]

The structure, expression, and regulation of ABCG1

ABCG1 is a half-transporter containing only one 6-helix transmembrane domain and a single nucleotide binding fold. As an active transporter which needs two NBD and two transmembrane bundles, ABCG1 needs to form a homodimer or a heterodimer with another ABC transporter to become active.[126,127]

In general, high expression of ABCG1 mRNA was noted in spleen, lung, thymus, and brain, whereas expression in kidney, liver, and heart was low or undetectable.[127,128] Subsequent studies using ABCG1-null/LacZ knock-in mice demonstrated that ABCG1 is predominantly expressed in macrophages, EC, and lymphocytes.[46] The mammalian ABCG1 cDNA was originally identified from studies using degenerate PCR and RNA from either a murine macrophage cell line,[127] a murine pre-B cell library,[128] or a human Jurkat T-cell line,[128] or after using exon-trapping and a human chromosome 21 cDNA library.[129] Both the human and murine ABCG1 genes utilize multiple promoters and alternative splicing to

produce a diverse array of mRNAs that encode multiple putative protein isoforms that range from 64-79 kDa.[130-132] It is not known if these isoforms, which vary only at the amino terminus upstream of the Walker A motif, form distinct dimers and/or have different functions in vivo. One interesting human isoform variation is predicted to arise due to alternative splicing in a section of the cytoplasmic domain of ABCG1 between the transmembrane regions and the ATP cassette. Splicing in this region leads to either the presence or absence of a 12 amino acid (AA) internal segment in the ABCG1 protein, termed as ABCG1(+12) and ABCG1(−12).[128,133] The two ABCG1 isoforms are expressed at both RNA and protein levels in human macrophages, with ABCG1(−12) tending to be the more abundant.[134] Of note, ABCG1(+12) is not expressed in mice,[135] while mouse models are widely used to elucidate the function of ABCG1, further investigations into the importance of this human ABCG1 isoform are warranted.

ABCG1 promotes cholesterol efflux from cells to mature HDL but not to lipid-free apoA-I.[7,136,137] In contrast to ABCA1-dependent lipid export, which requires apolipoprotein binding, ABCG1-dependent cholesterol efflux does not appear to directly bind HDL.[138] Different models have been proposed to explain how ABCG1 acts to increase the availability of plasma membrane cholesterol to its acceptors. One suggested that ABCG1 helps cholesterol molecules to overcome the energy barrier for entry into the hydrophilic water layer, perhaps by using ATP to promote protrusion of the cholesterol molecule into water, followed by a transient collision with an acceptor.[138] A second model pointed to a function of ABCG1 as a phospholipid floppase, promoting changes in the organization of plasma membrane phospholipids and subsequent attraction of cholesterol to the outer leaflet for diffusional efflux.[139] Although expression of ABCG1 does not increase cellular binding of HDL, it does increase the oxidation sensitivity of cellular cholesterol in the absence of HDL,[100,137] suggesting that ABCG1 (like ABCA1) can modify plasma membrane cholesterol domains such that they are more accessible to cholesterol oxidase. Whether the cholesterol in the ABCG1 modified membrane domains is spatially different from those generated by ABCA1 remains to be investigated.

ABCG1 predominantly localizes to the intracellular compartments in basal macrophages, and it can be redistributed to the plasma membrane after LXR activation.[140] Thus, the LXR system performs a dual function of inducing ABCG1 transcription and its translocation to the plasma membrane. However, one recent study by Larrede et al. demonstrated that stimulation of cholesterol efflux to HDL by LXR activation in human foam cells involves an ATP-dependent transport mechanism solely mediated by ABCA1 that it appears to be independent of ABCG1 expression.[141] In addition to LXR activation, ABCG1 expression can also be induced by agonist of peroxisome proliferator-activated receptor gamma (PPARγ) paralleling with increased LXR.[142] In agreement, Akiyama et al. showed that

hepatic expression of ABCG1 was reduced in mice following conditional disruption of hepatic PPARγ.[143] Moreover, PPARγ agonists were reported to induce ABCG1 in LXR α/β-null macrophages and to exert antiatherogenic effects.[144] These results suggest that the expression of macrophage ABCG1 could be increased by PPARγ agonists, which is associated with or independent of LXR activation.

Like ABCA1, ABCG1 expression can also be post-transcriptionally regulated by diverse factors. Uehara et al. demonstrated that unsaturated FAs significantly suppress the stimulatory effects of oxysterols and retinoids on the mRNA and protein expression of ABCG1 as well as the activity of the wild-type human ABCG1 promoter.[145] In addition, lipoxygenases comprise a family of enzymes capable of mediating selective lipid oxidation. One of them, called 12/15-lipoxygenase (12/15LO) catalyzes the conversion of free FAs to produce eicosanoids.[146] Nagelin et al. previously showed that 12/15LO activity in macrophages enhances the degradation of ABCG1 protein and thus reduced cholesterol efflux.[147] Correlating with the increased turnover of ABCG1, the 12/15LO eicosanoid product 12S-hydroxyeicosatetranoic acid (12SHETE) enhances the serine phosphorylation of ABCG1.[147] More recently, the mechanism by which 12/15LO regulates ABCG1 was further elucidated by the same group, and it was suggested that 12/15LO activates c-Jun NH_2-terminal kinase 2 (JNK2) and p38 mitogen-activated protein kinase (MAPK) pathways to enhance the phosphorylation and degradation of ABCG1.[148] Furthermore, Mauldin et al. demonstrated that ABCG1 expression was decreased in peritoneal macrophages isolated from Type 2 diabetic mice.[149] Similarly, down-regulation of ABCG1 was observed by using C57BL/6J peritoneal macrophages cultured in the presence of elevated glucose levels. These results suggest that ABCG1 expression in diabetic macrophages is regulated by chronic exposure to elevated glucose. Zhou et al. recently reported that the expression of ABCG1, but not ABCA1, was reduced in monocytes from Type 2 diabetic patients, which is partly related to the increased exposure to serum advanced glycation end (AGE) products.[150] The effect of AGEs on ABCG1 expression is mediated by the receptor for AGE (RAGE) through a LXR-independent pathway. However, the exact mechanism remains unknown.[151]

Therapeutic targeting ABCA1 and ABCG1

As described above ABCA1, but not ABCG1, is a rate-limiting factor in HDL biosynthesis. Both ABCA1 and ABCG1 play critical roles in lipid homeostasis by controlling tissue lipid levels and cellular cholesterol efflux. Cholesterol efflux to apoA-I specifically requires ABCA1, whereas efflux to HDL requires ABCG1. In addition, ABCA1 and ABCG1 have also anti-inflammatory properties which are independent of changes in plasma HDL-C levels. The beneficial effects of ABCA1

and ABCG1 have made them important new therapeutic targets for preventing atherosclerosis. The expression and activity of ABCA1 and ABCG1 is regulated transcriptionally and post-transcriptionally by multiple processes. Nowadays, various programs have been initiated by the pharmaceutical industry to target these two ABC transporters.

Target transcription of ABCA1 and ABCG1

Both ABCA1 and ABCG1 are strongly induced in human macrophages on LXR/RXR activation, and ABCG1 appears to be a preferential target of LXR ligands in foam cells.[141] Thus, upregulation of macrophage ABCA1 and ABCG1 by LXR agonists may constitute an effective pharmacological approach to attenuate foam cell formation, and thereby to prevent arterial lipid accumulation and lesion progression in dyslipidemic patients. In addition to ABCA1 and ABCG1, LXR agonists are also known to induce the transcription of other molecules involved in the RCT pathway, including apoE,[152] which promotes cholesterol efflux from macrophages by both ABCA1-independent and -dependent mechanisms; liver and intestinal ABCG5 and ABCG8,[153] which promote excretion of liver and dietary cholesterol into the bile; lipid transfer proteins, such as cholesteryl ester transfer protein (CETP),[154] and phospholipid transfer protein (PLTP),[155] which remodel extracellular HDL particles to regenerate lipid-free apolipoproteins. On the other hand, Joseph et al. reported that LXR is important for macrophage survival and regulation of the innate immune response, but this mechanism does not seem to be controlled by ABCA1 or ABCG1 activity.[156] In agreement, Valledor et al. demonstrated that LXR and RXR coordinately regulate the network of genes that control programmed cell death, resulting in protection of macrophages from bacterial-induced apoptosis.[157]

Initial studies using two non-steroidal synthetic LXR agonists (TO901317 and GW3965) in mice suggested they were able to increase plasma HDL-C levels and reduce atherosclerosis.[158-161] Recently, Verschuren et al. demonstrated that TO901317 strongly suppressed atherosclerotic lesion evolution and promoted lesion regression in apoE*3Leiden mice, a model for atherosclerosis with predictive value for the human situation.[162] Remarkably, the effect of TO901317 on plaque regression occurred even in the presence of proatherogenic plasma cholesterol and triglyceride profiles. The ability of TO901317 to induce atherosclerosis regression is mechanistically linked to (1) enhanced cholesterol efflux from macrophages in the aortic lesions (via upregulation of ABCA1, ABCG1, and apoE); (2) suppressed endothelial monocyte adhesion (at least in part via upregulation of ABCG1); (3) reduced lesional macrophage content by apoptosis; and (4) increased expression of the chemokine receptor CCR7 in foam cells, which is essential for the emigration of mature dendritic cells to lymph nodes during regression. The exciting possibilities for LXR agonists as new therapeutics are,

however, counterbalanced by one significant side effect: the induction of liver de novo lipogenesis and elevation of plasma triglyceride levels.[163-165] This liability is, at least in part, mediated by LXR-induced transcription of sterol regulatory element binding protein-1c (SREBP-1c), which induces some of the key enzymes involved in fatty acid synthesis, such as fatty acid synthase (FAS) and acetyl-coenzyme A carboxylase (ACC).[163,164]

Clearly, a major challenge for LXR agonists is to achieve a favorable balance between the beneficial effects of induction of cholesterol exporters (e.g., ABCA1 and ABCG1) and the detrimental effects of induction of SREBP-1c. A few possible strategies have been proposed to minimize the side effects of LXR agonist treatment. Both LXR isoforms (i.e., LXRα and LXRβ) respond to the same natural and synthetic ligands, but they show different tissue distribution. LXRα predominates in tissues that synthesize triglycerides, such as the liver, whereas LXRβ has a ubiquitous tissue distribution.[166,167] It is thus possible that LXR subtypes can control different genes due to their different tissue distribution. Interestingly, peritoneal macrophages from LXRβ knockout mice, but not LXRα knockout mice, show altered basal expression of ABCA1 mRNA,[166] suggesting that LXRβ is the subtype more responsible for controlling ABCA1 transcription in macrophages. Furthermore, unlike LXRα-selective agonists, LXRβ-selective agonists may not induce hepatic lipogenesis.[167,168] However, the crystal structures of LXRα and β are very similar in the ligand binding domains, and it may be a challenge to develop subtype-selective ligands.[169,170]

Another approach is the identification of target gene-selective or tissue-selective LXR agonists. Encouragingly, several natural or synthetic steroidal LXR agonists have been shown to selectively activate gene expression with minimal SREBP-1c gene activation in the liver and strongly induce ABCA1 expression in macrophages.[171,172] More recently, a novel synthetic steroidal LXR agonist ATI-829 was reported in mice to selectively activate LXR target gene expression in the intestine and macrophages rather than in the liver, without inducing hypertriglyceridemia and liver steatosis.[173] Given the variety of factors that modulate atherosclerosis in mice, the development of LXR agonists that are LXRβ, gene, or tissue selective, which can raise circulating HDL-C and stimulate macrophage cholesterol efflux without causing liver and plasma triglyceride accumulation is critical for the development of LXR-based agonists as therapeutically useful agents.

Target post-transcription of ABCA1 and ABCG1

The possibility of targeting posttranscriptional regulation of ABCA1 and ABCG1 has received less attention. A series of studies have indicated that both ABCA1 and ABCG1 are highly unstable proteins, and that they may be post-transcriptionally regulated in vivo by metabolic factors associated with common

disorders, such as diabetes. It is also likely that transcription of ABCA1 and ABCG1 in highly cholesterol-loaded cells may already be maximally stimulated. Under these conditions, stimulating transcriptional process alone may have a limited contribution to enhance the overall activity of ABCA1 and ABCG1.

ABCA1 is degraded by calpain,[115] and it appears to be one of the major mechanisms for regulation of its cellular level, and helical apolipoproteins protect ABCA1 against this degradation.[123] Helical apolipoproteins interact with ABCA1 and generate new HDL by removing cellular lipid. ABCA1 is phosphorylated and stabilized by a mechanism involving protein kinase C that is presumably activated by diacylglycerol generated by replenishment reaction for the removal of sphingomyeline.[120] To date, several synthetic amphipathic α-helical peptides have been produced. One of them, called D4-F because it contains 4 phenylalanines and only D-amino acids, can be absorbed orally into the blood stream with a low rate of turnover in mice.[174] Administration of this peptide in drinking water has been shown to markedly reduce atherosclerosis in mouse models.[174,175] D-4F protects against atherosclerosis by several possible mechanisms including interactions with ABCA1. Another advantage of synthetic amphipathic α-helical peptides is that they may be relatively resistant to the oxidative damage which has been reported to impair the interaction of apoA-I with ABCA1.[176] However, a disadvantage of these peptides is that they have non-specific detergent effects on membrane lipids and can accumulate in tissues and damage cells.[177,178] Further investigation is needed to develop novel synthetic peptides that mimic amphiphilic helical segments of apolipoproteins to specifically remove cellular lipids via the ABCA1 pathway without having detergent-like effects.

Other potential therapeutic targets

Another potential strategy under investigation is to increase the concentration of acceptors for ABCA1 and ABCG1. ABCA1 transports phospholipids to lipid-poor apoA-I to generate partially lipidated "nascent" HDL, called preβ-HDL. These preβ-HDL particles can acquire additional cholesterol by other processes and then convert into spherical larger HDL, called α-HDL. These mature HDL particles are effective substrates for ABCG1-mediated cholesterol transport.[53,179,180] In this regard, intravenous infusion of apoA-I or apoA-I mimetic peptides are promising approaches. Infusion of recombinant apoA-IMilano/phospholipid complexes in apoE KO mice reduced the foamcell content of arterial lesions within 48h post infusion.[181] A weekly infusion of recombinant apoA-IMilano/phospholipid complexes in humans for 5 weeks appeared to induce regression of coronary atherosclerosis in a small study.[182] Currently, one intravenously administered apoA-I mimetic peptide, known as the RLT peptide (ETC-642, Esperion Therapeutics Inc., Ann Arbor, Michigan), is being studied in clinical trials.[183] More recently, Sacks et al. developed a novel selective delipidation technique by which

large HDL in human or monkey plasma was converted into cholesterol-depleted HDL, resembling preβ-HDL and small spherical HDL_3 that are active in removing excessive cholesterol from cells.[184] Importantly, treatment with the delipidated plasma tended to reduce diet-induced aortic atherosclerosis in monkeys, suggesting that the selective HDL delipidation could be an attractive therapy for atherosclerosis.

Conclusions

Studies of human disorders and mouse models show that the ABC cholesterol transporters ABCA1 and ABCG1 have a major impact on whole-body lipid homeostasis by a process termed RCT. ABCA1 and ABCG1 work synergistically to remove excess cholesterol from cells (particularly macrophages), and they also play important roles in suppressing inflammation through multiple mechanisms. In humans, ABCA1 mutations can cause a severe HDL-deficiency syndrome characterized by cholesterol deposition in tissue macrophages and prevalent atherosclerosis. Disrupting ABCA1 or ABCG1 in mice promotes accumulation of excessive cholesterol in macrophages, and physiological manipulation of ABCA1 expression affects atherogenesis in mouse models. ABCA1 and ABCG1 have complex cellular and regulatory pathways that are far from being completely understood. At present, a number of approaches have been utilized to target ABCA1 and ABCG1 in efforts to raise circulating HDL levels and stimulate lipid flux through the RCT pathway. However, the efficacy and safety of these therapies remains an open question. As we learn more, there will undoubtedly be additional novel therapeutic targets that offer the best opportunity to treat atherosclerosis.

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Chapter 6

Augmented atherogenesis in LDL receptor deficient mice lacking both macrophage ABCA1 and apoE

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Bart Lammers
Ying Zhao
Menno Hoekstra
Reeni B. Hildebrand
Dan Ye
Illiana Meurs
Theo J.C. van Berkel
Miranda van Eck

Division of Biopharmaceutics, LACDR, Leiden University, the Netherlands

Abstract

Objective: ABCA1 protects against atherosclerosis by facilitating cholesterol efflux from macrophage foam cells in the arterial wall to extracellular apolipoprotein (apo) A-I. In contrast to apoA-I, apoE is secreted by macrophages and can, like apoA-I, induce ABCA1-mediated cholesterol efflux. Yet, the combined effect of macrophage ABCA1 and apoE on lesion development is unexplored.

Methods: LDL receptor knockout (KO) mice were transplanted with bone marrow from ABCA1/apoE double KO (dKO) mice, their respective single knockouts, and wild-type (WT) controls and were challenged with a high-fat/high-cholesterol diet for 9 weeks.

Results: In vitro cholesterol efflux experiments showed no differences between ABCA1 KO and dKO macrophages. The serum non-HDL/HDL ratio in dKO transplanted mice was 1.7-fold and 2.4-fold ($p<0.01$) increased compared to WT and ABCA1 KO transplanted mice, respectively. The atherosclerotic lesion area in dKO transplanted animals ($650\pm94\times10^3$ μm^2), however, was 1.9-fold ($p<0.01$) and 1.6-fold ($p<0.01$) increased compared to single knockouts (ABCA1 KO: $341\pm20\times10^3$ μm^2; apoE KO: $402\pm78\times10^3$ μm^2, respectively) and 3.1-fold increased ($p<0.001$) compared to WT ($211\pm20\times10^3$ μm^2). When normalized for serum cholesterol exposure, macrophage ABCA1 and apoE independently protected against atherosclerotic lesion development ($p<0.001$). Moreover, hepatic expression levels of TNFα and IL-6 were highly induced in dKO transplanted animals (3.0-fold; $p<0.05$, and 4.3-fold; $p<0.001$, respectively). In agreement, serum IL-6 levels were also enhanced in ABCA1 KO transplanted mice ($p<0.05$) and even further enhanced in dKO transplanted animals (3.1-fold as compared to ABCA1 KO transplanted animals; $p<0.05$).

Conclusions: Combined deletion of macrophage ABCA1 and apoE results in a defect in cholesterol efflux and, compared to ABCA1 KO transplanted mice, elevated serum total cholesterol levels. Importantly, these mice also suffer from enhanced systemic and hepatic inflammation, together resulting in the observed augmented atherosclerotic lesion development.

Introduction

Accumulation of cholesterol in macrophages leads to the formation of foam cells, a crucial event in the development of atherosclerotic lesions. Because macrophages are incapable of limiting the uptake of lipoproteins, these cells rely on reverse cholesterol transport (RCT) for maintaining cellular cholesterol homeostasis.[1,2] We have shown that apolipoprotein (apo) E as well as the ATP-binding cassette (ABC) transporters A1 and G1 are key players in the efflux of cholesterol from macrophages, the first step in RCT and protection against atherosclerosis.[3-5] ABCA1 facilitates cholesterol efflux to lipid-poor apolipoproteins like apoA-I,[6] resulting in the formation of lipidated HDL. Subsequently, ABCG1 mediates the efflux of cellular cholesterol to these mature HDL particles.[7,8] In addition, ABCA1 can modulate the secretion of apoE.[9] Secreted apoE by macrophages facilitates cellular cholesterol efflux[10] both in the presence and absence of extracellular cholesterol acceptors.[11] Although both pro- and anti-atherogenic functions for macrophage apoE have been described,[12-15] we recently showed that macrophage apoE protects against atherosclerotic lesion development, independent of ABCG1,[5] in LDL receptor knockout (LDLr KO) mice. Moreover, very recently, Zanotti et al. showed that expression of apoE only in macrophages is sufficient to promote RCT, emphasizing the pivotal anti-atherogenic role for macrophage apoE.[16] Importantly, macrophage apoE-mediated cholesterol efflux was shown to be independent of ABCA1 in apoE expressing J774 macrophages,[17] suggesting that the atheroprotective effects of macrophage ABCA1 are independent of apoE production by macrophages. However, in primary human monocyte-derived macrophages and THP-1 cells it was shown that apoE secretion from macrophages is promoted by ABCA1.[9]

To investigate the possible interaction between ABCA1 and apoE in promoting macrophage cholesterol efflux and their combined roles in atherogenesis, we generated mice deficient for both ABCA1 and apoE and performed a bone marrow transplantation (BMT) experiment in LDLr KO mice. Our results evidently show that transplantation of LDLr KO mice with either ABCA1 KO or apoE KO bone marrow resulted in a moderate increase in atherosclerotic lesion development, while combined deletion of ABCA1 and apoE in bone marrow-derived cells led to a more dramatic increase in atherosclerosis.

Methods

Animals and bone marrow transplantation

ABCA1 KO[18] and apoE KO (The Jackson Laboratory, Bar Harbor, ME, USA) mice (both more than 7 times backcrossed onto a C57BL/6J background) were mated to generate F1 heterozygotes. Heterozygote F1 animals were crossbred to obtain ABCA1 KO/apoE WT (ABCA1 KO), ABCA1 WT/apoE KO (apoE KO),

ABCA1 KO/apoE KO (dKO), and ABCA1 WT/apoE WT (WT)mice, which were used as donors for the bone marrow transplantation. These donor mice were anaesthetized subcutaneously with a mix of 70 mg/kg body weight xylazine, 1.8 mg/kg bodyweight atropine and 350 mg/kg body weight ketamine. Animals were subsequently sacrificed by cervical dislocation. Bone marrow cells were then isolated from the femurs and tibias from these mice. Homozygous C57BL/6J LDL receptor knockout (LDLr KO) mice were obtained from The Jackson Laboratory as mating pairs and bred at the Gorlaeus Laboratories, Leiden, The Netherlands. Bone marrow transplantations to male LDLr KO mice were performed as described.[19] Briefly, irradiated recipients (≥11 per group) received 5×10^6 bone marrow cells by intravenous injection into the tail vein. After a recovery period of 8 weeks, the animals were challenged with a high-fat/high-cholesterol (HF/HC; 1.25% cholesterol, 21% fat (Abdiets, Woerden, the Netherlands)) diet for 9 weeks to induce atherosclerotic lesion development. At 17 weeks after transplantation, mice were anaesthetized subcutaneously with a mix of 70 mg/kg body weight xylazine, 1.8 mg/kg bodyweight atropine and 350 mg/kg body weight ketamine. Animals were subsequently sacrificed by cervical dislocation. The hematologic chimerism of the transplanted LDLr KO mice was confirmed in genomic DNA from bone marrow by PCR analysis (Fig. 1). Animal experiments were approved by the Ethics Committee for Animal Experiments of Leiden University (permit number 08015) and performed at the Gorlaeus Laboratories of the Leiden/Amsterdam Center for Drug Research in accordance with the National Laws and the Directive 2010/63/EU of the European Parliament.

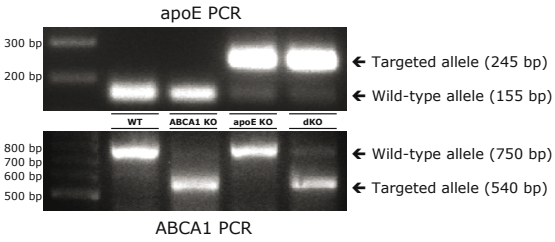

Figure 1. Successful disruption of macrophage ABCA1 and apoE by bone marrow transplantation. RT-PCR on genomic DNA of bone marrow isolated from ABCA1 and/or apoE KO transplanted LDLr KO mice and their WT transplanted controls at 17 weeks after transplantation.

Histological analysis of the aortic root, aortic arch, and spleen

To analyze the development of atherosclerosis at the aortic root and aortic arch, transplanted LDLr KO mice were euthanized after 9 weeks of feeding the HF/HC diet. The arterial tree was perfused in situ with PBS (100 mm Hg) for 10 min via a cannula in the left ventricular apex. The heart plus aortic root and the aortic arch were excised and stored in 3.7% neutral-buffered formalin (Formal-fixx; Shandon Scientific Ltd, Runcorn, UK). Serial sections (10 μm) of the aortic root were cut using a Leica CM3050S cryostat. The atherosclerotic lesion areas in oil red-O-stained cryostat sections of the aortic root and whole aortic arches were quantified using the Leica image analysis system, consisting of a Leica DMRE microscope coupled to a video camera and Leica Qwin Imaging software (Leica Ltd, Cambridge, UK). Mean lesion area (in μm^2) was calculated from 10 consecutive oil red-O-stained sections of the aortic root, starting at the appearance of the tricuspid valves. Sections were stained immunohistochemically for the presence of macrophages using a rat MoMa-2 antibody, dilution 1:50 (Serotec Ltd, Oxford, UK). Goat anti-rat coupled to horse radish peroxidase (HRP) (1:100) (Dako, Glostrup, Denmark) was used as a secondary antibody and Nova red substrate (Vector Laboratories, Burlingame, CA, USA) was used for visualization of HRP. Collagen content of the plaques was determined after Masson Trichrome staining (Sigma Diagnostics, St Louis, MO, USA). Relative plaque area in the aortic arch was quantified en face calculating the ratio of atherosclerotic lesions to the surface of the entire aortic arch. In addition, 7 μm cryosections of formalin-fixed spleen from the transplanted LDLr KO mice were prepared and stained for lipid accumulation using oil red-O and counterstained with hematoxylin.

Lipid analyses

Eight weeks after bone marrow transplantation, 100 µL of blood was drawn from each individual mouse by tail bleeding after an overnight fasting period. Upon sacrifice, 17 weeks after bone marrow transplantation, blood was collected by retro-orbital venous plexus puncture after an overnight fasting period. Lipid analyses were performed as described.[19] Lipoprotein profiles from all mice were determined by fractionation of 50 µL samples of serum, pooled from 2 mice per genotype, using a Superose 6 column (3.2×300 mm, Smart-System; Pharmacia, Uppsala, Sweden). Total cholesterol (TC) content of the effluent was determined as described,[19] and calculated as a percentage of serum TC. Splenic free cholesterol (FC), cholesteryl ester (CE), and triglyceride (TG) content were determined as described[19] after extraction according to Bligh and Dyer,[20] and dissolving the lipids in 2% Triton X-100. ApoE Western blot analysis of pooled VLDL and LDL fractions was performed by running equal amounts of total cholesterol per lipoprotein (VLDL: 3.8 mg, LDL: 4.6 mg) on a 15% SDS-PAGE gel. ApoE was detected using a rabbit-anti-mouse apoE polyclonal antibody (SB Rabbit 67-AH). As a secondary antibody a goat-anti-rabbit IgG-HRP (Jackson ImmunoResearch, Suffolk, UK) was used.

Cellular cholesterol efflux

For isolation of bone marrow-derived macrophages (BMDM), femurs and tibias were lavaged with PBS. Bone marrow cells were plated in DMEM / 20% FCS / 1% penicillin / 1% streptomycin and differentiated into macrophages by addition of 30% L929 cell-conditioned media (as a source of M-CSF) for 7 days. Subsequently, BMDM were incubated with 0.5 µCi/mL 3H-cholesterol in DMEM / 0.2% fatty acid free BSA and 30 µg/mL non-labeled cholesterol for 24h at 37°C in 24 well tissue culture plates (Greiner Bio-One). Cholesterol efflux was determined after incubating the cells in DMEM / 0.2% fatty acid free BSA in the absence or presence of 10 µg/mL apoA-I (Calbiochem, La Jolla, CA, USA) or 50 µg/mL human HDL, isolated according to Redgrave et al.[21] Radioactivity in the medium and the cells was determined by scintillation counting after 24h of incubation. Cholesterol efflux is expressed as the percentage of total cell ^{3}H-cholesterol present in the medium after 24h. Basal efflux to BSA (in the absence of apoA-I and HDL) has been subtracted from the data shown.

mRNA expression analysis by real time PCR

Total RNA from BMDM was isolated using the guanidinium thiocyanate (GTC) method[22] and reverse transcribed using a RevertAid M-MuLV enzyme (Fermentas, Burlington, Canada). Relative mRNA expression was measured from the following genes: scavenger receptor class B type I (SR-BI), LDL receptor-related protein 1 (LRP1), 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMG-CoA reductase), microsomal triglyceride transfer protein (MTP), ATP-binding cassette transporter sub-family G member 1 (ABCG1), apolipoprotein (apo) B and E, lipoprotein lipase (LPL), fatty acid synthase (FAS), interleukin 6 (IL-6), tumor necrosis factor α (TNFα), and diacylglycerol acyltransferase-1 (DGAT-1). The mRNA expression levels were assessed by real time PCR (ABI PRISM 7500; Applied Biosystems, Foster City, CA, USA) using SYBR Green technology (Applied Biosystems). Housekeeping genes RPS13, 36B4, and HPRT were used as a control. Primer sequences are available upon request.

Determination of serum cytokine concentrations

Murine IL-6 and IFNγ serum levels were assayed using an instant ELISA kit (eBioscience, Hatfield, UK) according to the manufacturer's protocol. Murine IL-10 and TNFα serum levels were assayed using an instant ELISA kit (BD Biosciences, Erembodegem, Belgium) according to the manufacturer's protocol.

Statistical analysis

Statistically significant differences among the means of the different populations were tested using

analysis of variance (ANOVA) and when specifically indicated the unpaired Student's t-test (GraphPad InStat and Prism software). The Student-Newman-Keuls multiple comparison test was performed after ANOVA. Two-way ANOVA was used to check possible interactions. The probability level (alpha) for statistical significance was set at 0.05. Results are expressed as an average ± SEM.

Results

Shift toward a more pro-atherogenic lipoprotein profile in ABCA1/apoE dKO transplanted LDLr KO mice

Cholesterol levels were measured at 8 weeks (chow diet) and at 17 weeks (HF/HC diet, containing 1.25% cholesterol and 21% fat) after BMT (Table 1). On chow diet, free cholesterol (FC) levels were moderately increased in apoE KO transplanted mice (15%, $p<0.01$). However, no effect of ABCA1 and/or apoE deletion in bone marrow-derived cells was observed on serum total cholesterol (TC) levels. After 9 weeks of feeding the HF/HC diet, serum cholesterol levels of the control mice and the apoE KO transplanted mice increased ≈3-fold. However, in agreement with previous studies,[3,23] deletion of ABCA1 in bone marrow cells resulted in an attenuated 1.7-fold increase in plasma TC levels upon feeding an atherogenic diet compared to WT controls ($p<0.001$). Importantly, although ABCA1/apoE dKO transplanted mice still exhibited an attenuated rise in TC levels compared to WT controls (2.4-fold; $p<0.01$), these levels were significantly higher compared to levels of ABCA1 KO transplanted mice (1.4-fold; $p<0.05$).

The distribution of TC among serum lipoproteins was analyzed by liquid chromatography (Fig. 2). The observed decrease in cholesterol levels of ABCA1

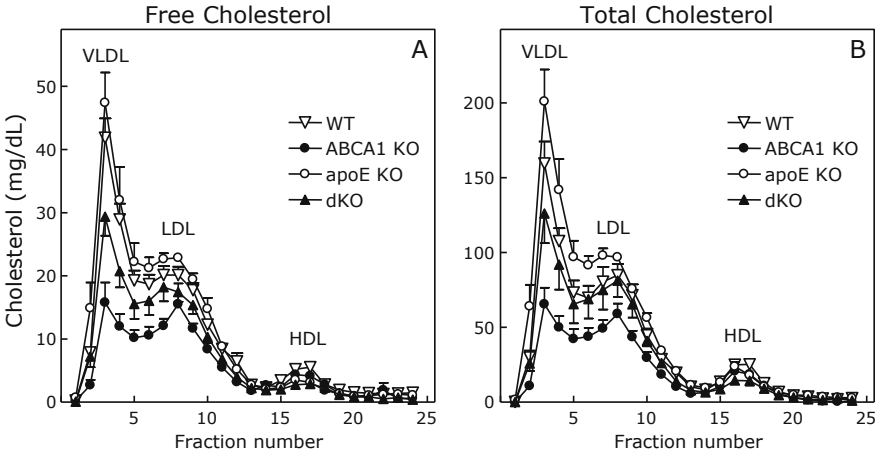

Figure 2. Lipoprotein profiles from ABCA1 and/or apoE KO transplanted LDLr KO mice and their WT transplanted controls. Fifty mL samples of serum, pooled from 2 mice per genotype, collected after 9 weeks of HF/HC feeding, were subjected to FPLC fractionation. Distribution of serum FC and TC over the fractions (25 mL) was determined enzymatically. Values represent the means of n≥5 samples per group. 1 sample corresponds to pooled serum of 2 mice.

KO transplanted mice upon feeding the HF/HC diet was mainly caused by a 54% decrease in cholesterol associated with VLDL and LDL (non-HDL-C; $p<0.001$; Table 1). However, HDL-C levels in these mice were only reduced by 32%, which resulted in a decreased non-HDL/HDL ratio (Table 1). In contrast, the non-HDL/HDL ratio of apoE KO transplanted mice was increased compared to WT controls (1.7-fold; Table 1), since HDL-C was reduced by 34% ($p<0.05$; Table 1), whereas non-HDL-C levels remained stably high. In agreement with ABCA1 KO transplanted mice, non-HDL-C of apoE/ABCA1 dKO transplanted animals was reduced compared to WT controls (30%; $p<0.05$; Table 1). However, also a dramatic 52% reduction was observed in HDL-C levels of these mice ($p<0.001$; Table 1), which resulted in an increased non-HDL/HDL ratio (2.4-fold; $p<0.01$ compared to ABCA1 KO; Table 1), suggesting a more pro-atherogenic index.

Table 1. Serum lipid and lipoprotein levels were measured at 8 weeks (chow diet) and 17 weeks (HF/HC diet) after transplantation.

Donor BM	Time (wks)	Diet	FC (mg/dL)	TC (mg/dL)	Non-HDL-C (mg/dL)	HDL-C (mg/dL)	Non-HDL/HDL ratio
WT	8	Chow	81±2	31±15	n.d	n.d	n.d
	17	HF/HC	267±14	1021±75	929±89	92±11	10±1
ABCA1 KO	8	Chow	75±5	294±15	n.d	n.d	n.d
	17	HF/HC	148±13***	493±41***	430±56***	63±5**	7±1
apoE KO	8	Chow	93±3**	323±14	n.d	n.d	n.d
	17	HF/HC	303±17	1030±84	969±67	61±5*	17±3$^{\#\#}$
dKO	8	Chow	83±3	294±13	n.d.	n.d	n.d
	17	HF/HC	216±18$^{*\#\#}$	691±66$^{**\#}$	647±106*	44±7***	17±4$^{\#\#}$

Data represent mean ± SEM of n≥10 mice. *$p<0.05$, **$p<0.01$, ***$p<0.001$ compared to WT; $^{\#}p<0.05$, $^{\#\#}p<0.01$ compared to ABCA1 KO; n.d. = not determined.

No differences in serum VLDL/LDL apoE concentrations after HF/HC diet feeding

ApoE is essential for clearance of lipoproteins from the circulation via the LDLr or LDL receptor-related protein-1 (LRP1). To investigate if normalization of the HF/HC diet-induced increase in VLDL/LDL is the consequence of a lower apoE content on these particles, apoE was determined in the lipoprotein fractions by Western blotting and normalized for cholesterol in the fractions (Fig. 3).

No apparent differences were observed in the apoE content of VLDL and LDL between the different groups of mice, indicating that disruption of apoE production by bone marrow-derived cells does not impair the availability of apoE on the particles for clearance. No apoE was visible in the HDL fraction of the different groups.

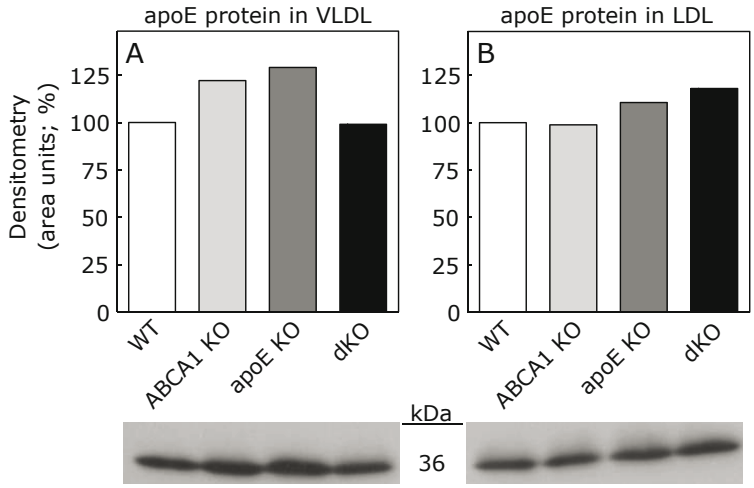

Figure 3. ApoE protein content in VLDL and LDL fractions from ABCA1 and/or apoE KO transplanted LDLr KO mice and their WT transplanted controls. Western blots showing apoE protein expression in VLDL (A) and LDL (B) isolated from ABCA1 KO and/or apoE KO transplanted LDLr KO animals and WT transplanted controls after feeding the HF/HC diet for 9 weeks. Samples were adjusted for cholesterol content. The density of the bands was determined using quantification software (Image J). n≥5 samples per group (pooled). WT values were set at 100%.

Increased lipoprotein lipase mRNA expression in liver

In order to further investigate the possible mechanisms regarding the observed differences in serum TC levels, we quantified mRNA expression levels of key liver enzymes in fatty acid and triglyceride synthesis (FAS and DGAT-1, respectively), cholesterol efflux (ABCG1) and synthesis (HMG-CoA reductase), apolipoproteins (apo) B and E, and genes involved in lipoprotein assembly (MTP) and uptake (SR-BI, LRP1), but found no differences. However, hepatic lipoprotein lipase (LPL) expression was 1.9-fold increased in ABCA1 KO transplanted animals as compared to dKO transplanted animals ($p<0.05$). LPL is essential for the lipolysis of lipoproteins. Induction of LPL leads to rapid clearance of VLDL and chylomicrons. Therefore, the observed attenuation of LPL expression in the dKO transplanted mice might have contributed to the increased TC levels as compared to ABCA1 KO transplanted animals.

Enlarged, cholesteryl ester enriched spleens in ABCA1/apoE dKO transplanted mice

Upon sacrifice (17 weeks after BMT; 9 weeks HF/HC diet feeding), spleens from all mice were examined. While both single KO transplanted groups showed no differences in spleen weight compared to WT controls, spleens from dKO transplanted animals were enlarged by 39% ($p<0.001$; Fig. 4). To further investigate this, spleen sections were stained with oil red-O to visualize lipid accumulation. No apparent staining was visible in spleens from WT controls and

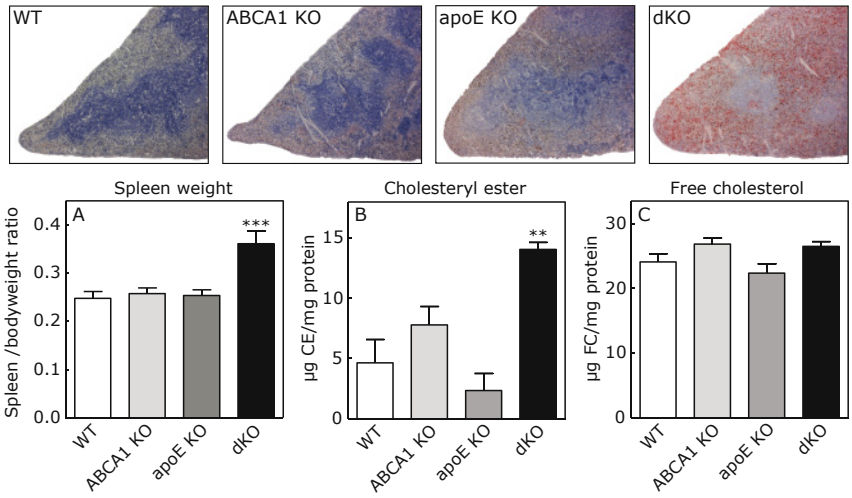

Figure 4. Increased CE content in enlarged spleens from ABCA1/apoE dKO transplanted LDLr KO mice. Representative cross sections of spleens from the bone marrow transplanted LDLr KO mice stained with oil red-O for the detection of neutral lipids and counterstained with hematoxylin (magnification 50x; upper panels). Spleen weight to bodyweight ratio is increased in ABCA1/apoE dKO transplanted LDLr KO mice (A). Values represent the means of n≥11 mice ± SEM per group. Quantification of cholesteryl ester (CE) and free cholesterol (FC) content of spleens (B and C, respectively). Values represent the means of n≥5 mice ± SEM per group. **p<0.01, ***p<0.001 as compared to WT transplanted controls.

apoE KO transplanted mice, while spleens from ABCA1 KO transplanted mice stained mildly for lipids (Fig. 4). In contrast, mice transplanted with ABCA1/apoE dKO bone marrow exhibited a heavily lipidated spleen, predominantly in the red pulp, explaining the large increase in size. To further investigate the splenic lipid composition, we isolated the lipid content and measured FC, CE and TG levels. In agreement with the lipid accumulation visualized by oil red-O staining, spleens

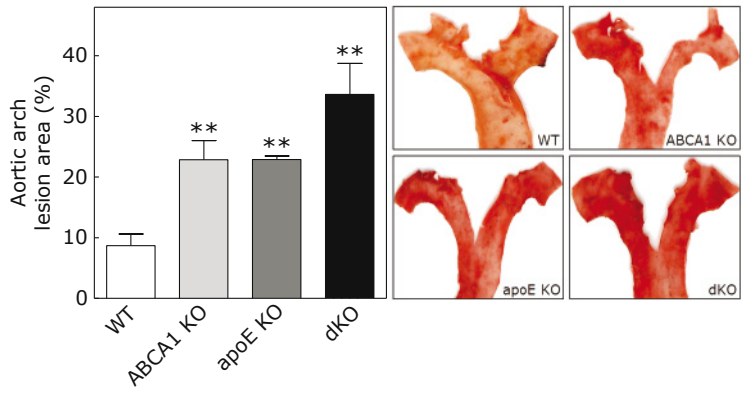

Figure 5. Analysis of atherosclerosis in the aortic arch of bone marrow transplanted LDLr KO mice after 9 weeks of HF/HC diet feeding. Relative plaque area in the aortic arch was quantified en face calculating the ratio of atherosclerotic lesions to the surface of the entire aortic arch (left panel). Representative pictures (right panels). **P<0.01 as compared to WT transplanted controls.

from dKO transplanted mice revealed a 3.0-fold ($p<0.01$; Fig. 4B) increase in CE levels. FC and TG content of these spleens were not significantly different. No lipid accumulation was observed in other tissues.

Increased atherosclerosis in ABCA1/apoE dKO transplanted LDLr KO mice

After feeding the HF/HC diet for 9 weeks, lesion development in the aortic root and the aortic arch of the transplanted animals was analyzed. En face quantification of the aortic arch lesions revealed a 2.5-fold increased lesion area in single KO transplanted mice compared to WT controls ($p<0.01$; WT: 9±2%, ABCA1 KO: 23±3%, apoE KO: 22±1%) and a striking 3.8-fold increase in dKO transplanted animals ($p<0.01$; dKO: 34±5%; Fig. 5). In agreement, quantification of the lesion sizes in oil red-O-stained sections of the aortic root showed that single deletion of macrophage ABCA1 or apoE led to a 1.6- or 1.9-fold increase in the mean atherosclerotic lesion size, respectively, compared to lesions in WT transplanted animals (341±20×10³ μm² in mice reconstituted with ABCA1 KO bone marrow and 402±78×10³ μm² in animals transplanted with apoE KO bone marrow, compared to 211±20×10³ μm² in mice reconstituted with WT

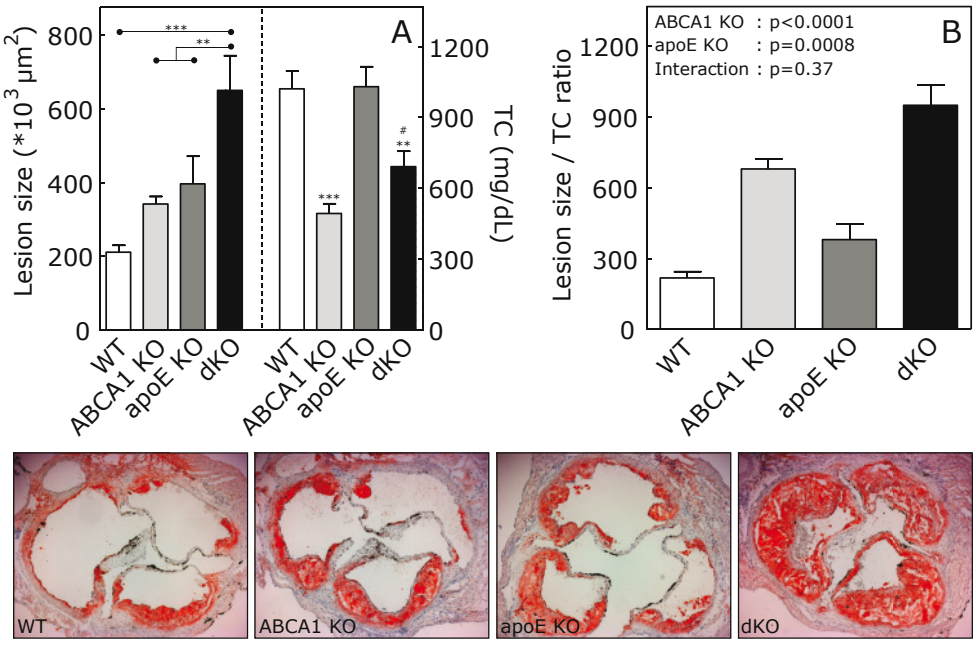

Figure 6. Analysis of atherosclerosis in the aortic root of bone marrow transplanted LDLr KO mice after 9 weeks of HF/HC diet feeding. Quantification of atherosclerotic lesion sizes in the aortic root and TC levels in bone marrow transplanted LDLr KO mice after 9 weeks of HF/HC diet feeding (A). Lesion size divided by TC levels show cholesterol independent effects on lesion development (B). Representative cross-sections, stained with oil red-O (magnification 50×; lower panels). Values are the means of 10 aortic root sections of individual mice ($n\geq11$ mice ± SEM per group. **$p<0.01$, ***$p<0.001$ as compared to WT transplanted controls, #$p<0.05$ as compared to ABCA1 KO transplanted animals.

bone marrow; Fig. 6A). This however failed to reach statistical significance by ANOVA. As the current study consisted of four groups, ANOVA is the appropriate test to check for significant differences. Previous studies,[3,12,13] however, used two groups (WT vs. KO), which only required a t-test. When the same t-test is used, both ABCA1 KO and apoE KO transplanted animals showed a significant increase in atherosclerotic lesion size compared to mice reconstituted with WT bone marrow ($p<0.001$ and $p=0.042$, respectively). Combined deletion of both ABCA1 and apoE in bone marrow-derived cells in LDLr KO mice induced a significant 3.1-fold ($p<0.001$; ANOVA) increase in the mean atherosclerotic lesion size compared to WT transplanted animals ($650\pm94\times10^3$ μm^2 compared to $211\pm20\times10^3$ μm^2). In addition, the lesion size in the dKO transplanted animals was also significantly larger compared to mice transplanted with single ABCA1 KO or apoE KO bone marrow (1.9- and 1.6-fold, respectively; $p<0.01$; ANOVA).

No differences in macrophage content in the aortic root plaques were observed (WT: $143\pm19\times10^3$ μm^2, ABCA1 KO: $136\pm19\times10^3$ μm^2, apoE KO: $198\pm27\times10^3$ μm^2, dKO: $139\pm21\times10^3$ μm^2). As expected, the collagen content was significantly increased in plaques from dKO transplanted mice, indicating more advanced lesions ($p<0.01$ to single KO transplanted mice and WT controls (WT: $3.3\pm1.2\times10^3$ μm^2, ABCA1 KO: $6.8\pm1.8\times10^3$ μm^2, apoE KO: $3.6\pm0.8\times10^3$ μm^2, dKO: $26\pm9.3\times10^3$ μm^2).

Interestingly, although single deletion of macrophage ABCA1 or apoE showed a comparable increase in atherosclerotic lesion development, TC levels between these groups were dramatically different (Fig. 6A). In humans, TC levels are correlated with atherosclerosis and are therefore a widely accepted risk factor for atherosclerotic disease.[24] To adjust for cholesterol exposure, we divided the atherosclerotic lesion size by TC concentrations (Fig. 6B). As a result, the corrected atherosclerotic lesion size of ABCA1 transplanted mice was 3.1-fold increased compared to WT controls, underscoring the important anti-atherogenic role for macrophage ABCA1 in atherosclerotic lesion development. Furthermore, since TC levels did not differ between apoE KO transplanted mice and WT controls, the corrected lesion size also showed a similar anti-atherogenic role for macrophage apoE. Importantly, dKO transplanted mice displayed a 4.3-fold increased lesion development compared to WT controls after TC adjustment.

To examine the relative contribution of both genes to atherosclerotic lesion development, independent of effects on TC levels, two-way ANOVA was used on the corrected atherosclerosis data to analyze possible interactions between macrophage ABCA1 and apoE expression. The regular P value was used to express the significance of the effect of apoE deletion on ABCA1 and vice versa, whereas the interaction P value shows the independent effects of both genes on atherosclerotic lesion development. This test showed highly independent effects of ABCA1 or apoE deletion in bone marrow-derived cells on atherosclerotic

lesion formation (Fig. 6B). The apoE gene accounted for 10% of the variation (p=0.0008), whereas the ABCA1 gene contributed 59% to the total variation (p<0.0001). In agreement, no interaction (p=0.37) was observed, suggesting that single deletion of ABCA1 or apoE induces atherosclerosis, independent of cholesterol levels. Moreover, atherosclerotic lesion development in dKO transplanted mice is an additive, independent effect of the single deletions of ABCA1 and apoE.

In vitro cellular cholesterol efflux

Combined deletion of macrophage ABCA1 and apoE resulted in a significant increase in atherosclerotic lesion development. Moreover, spleens of these mice were significantly enlarged and enriched in CE content. To determine whether these effects were caused by a defect in the ability to transport cholesterol out of the cells to exogenous lipid acceptors, an in vitro cholesterol efflux assay to BSA, apoA-I, and HDL was performed. In line with previous results,[5] deletion of macrophage apoE resulted in an impaired cholesterol efflux to BSA (41%, p<0.001; Fig. 7A) emphasizing its ability to promote the efflux of cellular cholesterol in the absence of extracellular acceptors. Similarly, a 35% decreased cholesterol efflux to BSA was observed with ABCA1 KO and dKO cells (p<0.001). As expected, cholesterol efflux to apoA-I was completely inhibited when macrophages lacking ABCA1 were used (p<0.001; Fig. 7B). ABCA1 KO macrophages also exhibited a 47% decreased efflux capacity to HDL (p<0.001; Fig. 7C). In agreement with previous data,[5,12] deficiency of apoE in macrophages resulted in a small but significant decrease in cholesterol efflux to both apoA-I (16%, p<0.001), and HDL (21%; p<0.001). However, surprisingly, no additional effect of apoE deficiency was observed on cholesterol efflux to HDL in the absence of macrophage ABCA1, suggesting

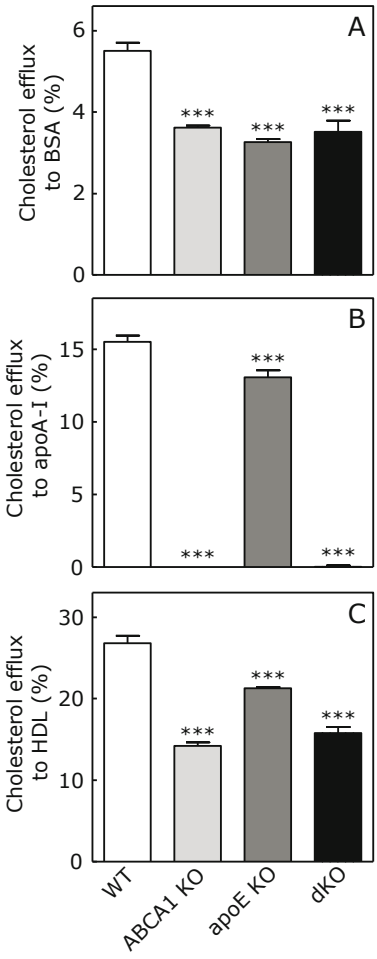

Figure 7. In vitro cellular cholesterol efflux. Cholesterol efflux from bone marrow-derived macrophages (BMDM) to BSA (A), apoA-I (10 mg/mL; B) and HDL (50 mg/mL; C) was determined after 24h. Data are expressed as the percentage of radioactivity released into the medium. Values represent the means of n≥3 mice ± SEM per group. ***p<0.001 as compared to WT transplanted controls.

that efflux induced by endogenously produced apoE requires ABCA1 expression. It also indicates that the enlarged lipidated spleen and the enhanced atherosclerotic lesion development specifically observed in dKO transplanted mice cannot be explained by additional effects on the cholesterol efflux mechanisms.

Increased expression of hepatic and systemic pro-inflammatory cytokines

In order to further investigate the possible mechanisms regarding the increased atherosclerotic lesion development in ABCA1/apoE dKO transplanted mice, we quantified mRNA expression levels of the inflammatory molecules TNFα and IL-6 in the liver. Surprisingly, we found highly increased TNFα (3.0-fold; $p<0.05$; Fig. 8A) and IL-6 (4.3-fold; $p<0.001$; Fig. 8B) expression levels, indicating an enhanced inflammatory status of these animals. In addition, we analyzed both pro- and anti-inflammatory cytokines in the circulation, including TNFα, IFNγ, IL-6 and IL-10. Whereas TNFα, IFNγ, and IL-10 concentrations were too low to be detected in the serum of these animals, a major increase in the secretion of the pro-inflammatory cytokine IL-6 in ABCA1 KO transplanted animals was observed ($p<0.05$; Fig. 8C), which was even further enhanced in dKO transplanted animals (3.1-fold as compared to ABCA1 KO transplanted animals; $p<0.05$). Serum IL-6 is generally considered a systemic inflammatory marker. As a result, the observed elevated IL-6 levels indicate an intensified inflammatory environment which, at least partly, contributed to the observed increase in atherosclerotic lesion development.

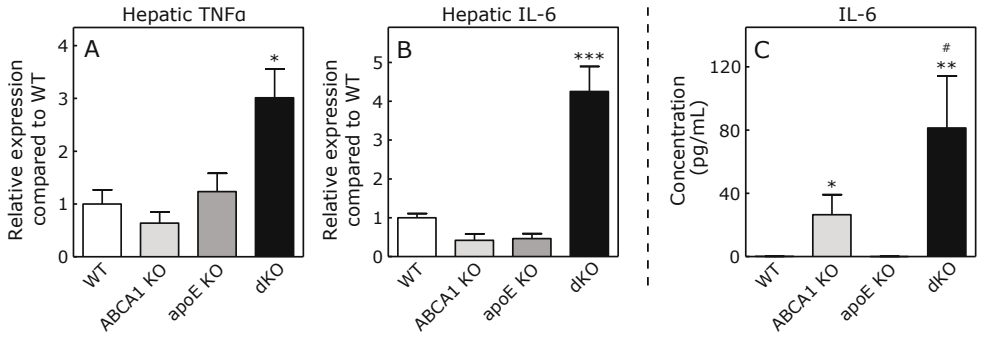

Figure 8. Hepatic gene expression levels and circulating IL-6 levels. mRNA expression of TNFα (A), and IL-6 (B) in liver from bone marrow transplanted LDLr KO mice. Gene expression analysis was performed by RT-PCR. Values are relative to the average expression of housekeeping genes and expressed as fold induction compared to WT transplanted controls. Values are mean ± SEM, n≥3 mice per group as compared to WT. Increased circulating IL-6 levels in LDLr KO mice transplanted with ABCA1/apoE dKO bone marrow (C). Serum from the transplanted LDLr KO mice was collected at 17 weeks after transplantation including 9 weeks of WTD feeding. Values are mean ± SEM, n≥8 mice per group. *$p<0.05$, **$p<0.01$, ***$p<0.001$ as compared to WT transplanted controls. #$p<0.05$ as compared to ABCA1 KO transplanted animals.

Discussion

Although the functions of macrophage ABCA1 and apoE in the development of atherosclerosis have been studied extensively,[3-5,12-15] their potential combined role in atherosclerotic lesion development is still unexplored. In the present study, we show that macrophage ABCA1 and apoE independently protect against atherosclerotic lesion formation, when normalized for serum cholesterol exposure. ABCA1 is known to facilitate cholesterol efflux to lipid-poor apolipoproteins.[6] As a result, cholesterol efflux to apoA-I was completely abolished upon deletion of ABCA1 in macrophages. Macrophages do not produce apoA-I, but, apoE secreted by macrophages[10] is able to induce efflux of cholesterol both in the presence and absence of extracellular cholesterol acceptors (e.g. apoA-I or HDL),[11] which is confirmed in this study. Interestingly, no added effect on cholesterol efflux was observed in dKO macrophages compared to ABCA1 deficient macrophages, indicating that endogenously produced apoE requires ABCA1 to facilitate cholesterol efflux.

Nonetheless, spleens of ABCA1/apoE dKO transplanted mice were enlarged due to severe CE accumulation. In agreement with the current study, several publications have shown increased lesion development in ABCA1 KO transplanted mice, despite a clear reduction in serum cholesterol levels.[3,23,25] Interestingly, TC levels in serum were markedly increased upon combined deletion of macrophage ABCA1 and apoE as compared to single deletion of ABCA1 in macrophages. This thus leads to ABCA1 deficiency - a defect in cholesterol efflux - combined with elevated serum total cholesterol levels. Therefore, the observed enlargement and CE accumulation of spleens from dKO transplanted mice are most likely the result of enhanced lipid uptake due to the increased TC pressure from the circulation. In agreement with this hypothesis, less lipid accumulation and no changes in spleen size were observed in single KO transplanted animals.

In the current study, transplanted LDLr KO mice were fed a HF/HC diet for 9 weeks to induce increased serum cholesterol levels. A minimum serum cholesterol concentration of >300 mg/dL is required for the development of experimental atherosclerosis in LDLr KO mice.[26] Single deletion of macrophage ABCA1 or apoE led to a comparable increase of atherosclerotic lesion development. However, circulating TC levels were over 2-fold higher in apoE KO transplanted animals compared to ABCA1 KO transplanted mice. Accordingly, although the absolute lesion size did not differ between both genotypes, the lesions adjusted for serum total cholesterol exposure showed the high relative importance of ABCA1 deficiency for determining atherosclerosis susceptibility. Strikingly, despite the fact that single macrophage apoE deficiency did not alter serum TC concentrations, dKO transplanted mice had significantly higher TC levels than

single macrophage ABCA1 KO transplanted animals.

We previously found that circulating apoE levels can be influenced by macrophage ABCA1 deficiency.[3] In addition, ABCA1 was shown to promote macrophage apoE secretion in primary human monocyte-derived macrophages and THP-1 cells.[9] However, in the present study, the amount of apoE on VLDL and LDL particles was not reduced upon transplantation of apoE or ABCA1 deficient bone marrow into LDLr KO mice, indicating no direct quantitative effects of macrophage ABCA1 deficiency on apoE availability.

To get insight into the mechanisms behind the altered TC levels, key genes involved in lipoprotein metabolism were analysed in liver. Of all genes analysed only LPL was found to be differentially expressed in liver. Therefore, it is tempting to speculate that the lower serum TC levels in ABCA1 KO transplanted animals might be the result of the observed increase in hepatic LPL expression levels. LPL is essential for the lipolysis of lipoproteins. Induction of LPL leads to rapid clearance of VLDL and chylomicrons.[27] Therefore, the observed attenuation of LPL expression in the dKO transplanted mice as compared to ABCA1 KO transplanted animals might have contributed to the increased TC levels.

Besides the general significance of serum TC levels, the distribution over the different lipoproteins in the circulation can vary greatly, thereby changing its impact on atherosclerotic lesion development. Non-HDL is considered to have a pro-atherogenic function, whereas HDL - involved in RCT acts as an anti-atherogenic lipoprotein.[2,28,29] Serum lipoprotein distribution analysis revealed that dKO transplanted mice not only showed an increase in the pro-atherogenic non-HDL cholesterol levels, but also exhibited a decrease in anti-atherogenic HDL cholesterol which will probably also have contributed to the enhanced lesion development in the dKO transplanted animals.

Two-way ANOVA analysis showed that the effects of ABCA1 and apoE on lesion development, when normalized for serum TC levels, were independent of each other. This is surprising since apoE can induce cholesterol efflux via ABCA1 and our in vitro studies using the single and dKO macrophages clearly showed that ABCA1 is required to induce efflux via endogenously produced apoE.

Importantly, ABCA1/apoE dKO transplanted mice exhibited a large induction of the pro-inflammatory cytokines IL-6 and TNFα in the liver. In addition, serum IL-6 levels were also highly increased in these animals, indicating an enhanced inflammatory status of these animals. The pro-inflammatory cytokine IL-6 is reported to promote endothelial dysfunction, smooth muscle cell proliferation and migration as well as recruitment and activation of inflammatory cells.[30] Moreover, it induces the formation of macrophage-derived foam cells; a key feature of early and advanced atherosclerotic lesion formation.[31]

In conclusion, LDLr KO mice transplanted with either ABCA1 or apoE KO bone marrow exhibited a distinct increase in atherosclerosis compared to WT

transplanted animals. Importantly, combined deletion of macrophage ABCA1 and apoE results in a defect in cholesterol efflux and, compared to ABCA1 KO transplanted mice, elevated serum total cholesterol levels, and enhanced systemic and hepatic inflammation, together resulting in the observed augmented atherosclerotic lesion development.

Acknowledgements

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Chapter 4

Independent protective roles for macrophage ABCG1 and apoE in the atherosclerotic lesion development

Atherosclerosis.
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*Bart Lammers[1]
*Ruud Out[1]
Reeni B. Hildebrand[1]
Carmel M. Quinn[2]
David Williamson[2]
Menno Hoekstra[1]
Illiana Meurs[1]
Theo J.C. van Berkel[1]
Wendy Jessup[2]
Miranda van Eck[1]

1: Division of Biopharmaceutics, LACDR, Leiden University, the Netherlands
2: Centre for Vascular Research, University of New South Wales, Sydney, Australia
*Equal contribution

Abstract

Objective: ATP-binding cassette transporter G1 (ABCG1) and apolipoprotein E (apoE) play a role in macrophage cholesterol efflux and consequently the development of atherosclerosis. A possible interaction between ABCG1 and apoE in cholesterol efflux was postulated, but the potential combined action of these proteins on atherosclerotic lesion formation is unclear.

Methods: LDL receptor knockout (KO) mice were transplanted with bone marrow from ABCG1/apoE double KO (dKO) mice, their respective single knockouts, and wild-type (WT) controls and challenged with a high-fat/high-cholesterol diet for 6 weeks to induce atherosclerosis.

Results: No differences were found in serum lipid levels. The mean atherosclerotic lesion area in dKO transplanted animals ($187\pm18\times10^3$ μm^2) was 1.4-fold ($p<0.01$) increased compared to single knockouts (ABCG1 KO: $138\pm5\times10^3$ μm^2; apoE KO: $131\pm7\times10^3$ μm^2) and 1.9-fold ($p<0.001$) as compared to WT controls ($97\pm15\times10^3$ μm^2). In vitro cholesterol efflux experiments established that combined deletion of ABCG1 and apoE leads to a larger attenuation of macrophage cholesterol efflux to HDL as compared to single knockouts.

Conclusions: Single deletion of macrophage ABCG1 or apoE does lead to a moderate non-significant increase in atherosclerotic lesion development as tested by ANOVA, while combined deletion of ABCG1 and apoE induces a more dramatic and significant increase in atherosclerosis. Our results indicate an additive, independent effect for both macrophage ABCG1 and apoE in the prevention of atherosclerosis.

Introduction

Reverse cholesterol transport (RCT) is a pathway by which excess cholesterol is transported from macrophages in the vessel wall to the liver for excretion, thereby preventing atherosclerosis.[1,2] We have shown that the ATP-binding cassette (ABC) transporters A1 and G1 are key players in the efflux of cholesterol from macrophages, the first step in RCT and the protection against atherosclerosis.[3-6] ABCA1 was shown to facilitate cholesterol efflux to lipid-poor apolipoproteins,[7] while ABCG1 mediates the efflux of cellular cholesterol to mature high-density lipoproteins (HDL).[8,9]

In addition to ABCA1 and ABCG1, apoE secretion by macrophages facilitates cellular cholesterol efflux.[10] Interestingly, apoE enhances cholesterol efflux both in the presence and absence of extracellular cholesterol acceptors.[11] Yet, both pro- and anti-atherogenic functions for macrophage apoE in atherosclerosis have been described.[12-15] We[12] and others[13] showed that mice transplanted with apoE KO bone marrow developed 4- to 10-fold larger atherosclerotic lesions as compared to WT transplanted animals. In contrast, a significant decrease in lesion development is also described.[14,15]

Von Eckardstein et al. observed that apoE secretion from macrophages is promoted by ABCA1 and ABCG1.[16] Whereas ABCA1 is well known for its anti-atherogenic function, the putative role of macrophage ABCG1, like macrophage apoE, is under debate. Recently, we showed that specific deletion of ABCG1 in bone marrow-derived cells in LDL receptor knockout (LDLr KO) mice resulted in increased atherosclerosis after feeding a Western-type diet.[17] In contrast to our findings, Baldán et al. and Ranalletta et al. unexpectedly showed a decrease in lesion size in LDLr KO mice transplanted with ABCG1 KO bone marrow upon feeding a high-fat/high-cholesterol diet. Despite a comparable setup of the latter studies, Baldán et al. proposed accelerated apoptosis of ABCG1 KO macrophages to explain the decrease in the atherosclerosis.[18] Ranalletta et al., however, observed a compensatory upregulation of apoE secretion by ABCG1 deficient macrophages. This latter study thus suggests a protective role for macrophage apoE in the absence of macrophage ABCG1.[19]

To investigate the possible interaction between ABCG1 and apoE in macrophage cholesterol efflux and their combined roles in atherogenesis, we generated mice deficient for both ABCG1 and apoE and performed a bone marrow transplantation (BMT) experiment in LDLr KO mice. Our results evidently show that transplantation of LDLr KO mice with either ABCG1 KO or apoE KO bone marrow resulted in a moderate increase in atherosclerotic lesion development, while combined deletion of ABCG1 and apoE in bone marrow-derived cells led to an additional more dramatic increase in atherosclerosis. These results indicate an

additive, independent protective effect for both ABCG1 and apoE in macrophage cholesterol efflux and atherosclerotic lesion development.

Methods

Animals and bone marrow transplantation

ABCG1 KO (Deltagen Inc., San Carlos, CA, USA) and apoE KO (The Jackson Laboratory, Bar Harbor, ME, USA) mice (both more than seven times backcrossed onto a C57BL/6J background) were mated to generate F1 heterozygotes. Genotyping for ABCG1 and apoE was performed according to the protocol from Deltagen and The Jackson Laboratory, respectively. Heterozygote F1 animals were crossbred to obtain the ABCG1 KO/apoE WT (ABCG1 KO), ABCG1 WT/apoE KO (apoE KO), ABCG1 KO/apoE KO (dKO), and ABCG1 WT/apoE WT (WT) mice, which were used as donors for the bone marrow transplantation. Homozygous LDL receptor knockout (LDLr KO) mice were obtained from The Jackson Laboratory as mating pairs and bred at the Gorlaeus Laboratories, Leiden, The Netherlands. Bone marrow transplantations to male LDLr KO mice were performed as described.[17] Briefly, irradiated recipients received 5×10^6 bone marrow cells by intravenous injection into the tail vein. After a recovery period of 8 weeks, the animals were challenged with a high-fat/high-cholesterol (HF/HC; 1.25% cholesterol, 21% fat) diet for 6 weeks to induce atherosclerotic lesion development. This diet has previously been used in experiments concerning the role of macrophage ABCG1.[18] During the experiment, serum lipid analysis was performed as described previously.[17] Upon sacrifice, at 14 weeks after transplantation, the hematologic chimerism of the transplanted LDLr KO mice was determined in genomic DNA from bone marrow by PCR analysis as described above (Fig. 1). Animal experiments were approved by the Ethics Committee for Animal Experiments of Leiden University and performed at the Gorlaeus Laboratories of the Leiden/Amsterdam Center for Drug Research in accordance with the National Laws.

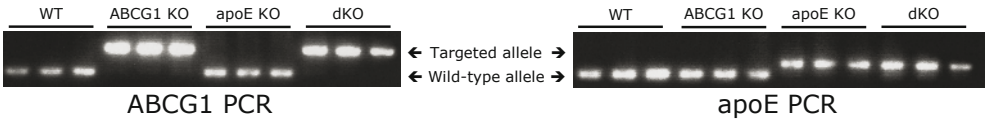

Figure 1. Verification of success of bone marrow transplantation. To verify whether reconstitution with donor hematopoietic cells was successful, PCR amplification of both ABCG1 (left) and apoE (right) wild-type and targeted alleles was performed using genomic DNA isolated from bone marrow at 14 weeks after transplantation.

Histological analysis of the aortic root and other organs

To analyze the development of atherosclerosis at the aortic root, transplanted LDLr KO mice were euthanized after 6 weeks of feeding the HF/HC diet. Mean lesion area in the aortic root was calculated as described.[17] Sections were stained immunohistochemically for the presence of macrophages using a rat MoMa-2 antibody, dilution 1:50 (Serotec Ltd., Oxford, UK). Masson Trichrome-staining (Sigma Diagnostics, USA) was used to determine the collagen content of the plaque. Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) staining of lesions was performed to determine the rate of apoptosis of macrophages in the atherosclerotic lesions using the In Situ Cell Death Detection Kit (Roche Diagnostics). In addition, 7 µm cryosections of formalin-fixed lung, liver, and spleen from the transplanted LDLr KO mice were prepared and stained for lipid accumulation using oil red-O.

Table 1. Sequences of the PCR primers used for the bone marrow-derived macrophage real-time PCR.

Gene	Forward Primer	Reverse Primer
ABCA1	5'-AAAACCGCAGACATCCTTCAG-3'	5'-CATACCGAAACTCGTTCACCC-3'
ABCG1	5'-CGAGAGGGCATGTGTGACG-3'	5'-CCGAGAAGCTATGGCAACC-3'
apoE	5'-CTGACAGGATGCCTAGCCG-3'	5'-CGCAGGTAATCCCAGAAGC-3'
GAPDH	5'-AGGTCGGTGTGAACGGATTTG-3'	5'-TGTAGACCATGTAGTTGAGGTCA-3'

In vitro studies using bone marrow-derived macrophages

Cellular cholesterol efflux from acetylated LDL-loaded bone marrow-derived macrophages, stimulated with 22-hydroxycholesterol (10 µmol/L) and 9-cis retinoic acid (1 µmol/L) was performed as described.[6] Cholesterol efflux is expressed as the percentage of total cell cholesterol present in the medium after 24h. Total RNA was isolated from the bone marrow-derived macrophages using the guanidinium thiocyanate (GTC) method.[20] The resulting cDNA was used in the real time PCR assay to perform gene expression analysis[21] (Table 1). Protein expression was measured by Western blot analysis as described.[6]

Statistical analysis

Statistically significant differences among the means of the different populations were tested using analysis of variance (ANOVA) and the unpaired Student's t-test (GraphPad InStat and Prism software). The Student-Newman-Keuls multiple comparison test was performed after ANOVA. Two-way ANOVA was used to check possible interactions. The probability level (alpha) for statistical significance was set at 0.05. Results are expressed as an average ± SEM.

Results

Effect of macrophage ABCG1 and apoE disruption on plasma lipid levels in LDLr KO mice

Cholesterol levels were measured at 8 weeks (chow diet) and at 14 weeks (HF/HC diet, containing 1.25% cholesterol and 21% fat) after BMT (Table 2). On a chow diet, no effect of ABCG1 or ABCG1/apoE deletion in bone marrow-derived

Table 2. Serum lipid levels were measured at 8 weeks (chow diet) and 14 weeks (HF/HC diet) after transplantation.

Donor BM	Time (wks)	Diet	FC (mg/dL)	TC (mg/dL)	TG (mg/dL)	PL (mg/dL)	HDL (mg/dL)	non-HDL (mg/dL)
WT	8	Chow	86±3	285±8	n.d.	n.d.	114±11	146±10
	14	HF/HC	228±23	1018±104	92±12	755±65	89±15	864±73
ABCG1 KO	8	Chow	80±3	269±5	n.d.	n.d.	100±7	154±7
	14	HF/HC	239±19	1031±77	99±8	717±42	76±6	1064±114
apoE KO	8	Chow	74±3	243±8*	n.d.	n.d.	101±9	140±6
	14	HF/HC	197±15	881±71	120±11	731±27	60±10	865±107
dKO	8	Chow	85±4	273±11	n.d.	n.d.	96±7	173±6
	14	HF/HC	219±12	960±58	129±12	724±26	63±8	1128±97

Data represent mean ± SEM of ≥12 mice. Non-HDL indicates the total of VLDL and LDL concentrations. n.d., not determined. *p<0.05 compared to WT.

cells was observed on serum total cholesterol (TC) and free cholesterol (FC) levels. Deletion of apoE in bone marrow-derived cells, however, resulted in a 14% decrease of TC ($p<0.05$, compared to WT), whereas FC levels were not affected.
After 6 weeks of feeding the HF/HC diet, the serum TC levels in both the control and the experimental groups increased ≈3-fold, but no significant differences were observed between the groups. Also no significant differences were visible in triglyceride (TG) and phospholipid (PL) levels both before and after feeding the HF/HC diet (Table 2).
The distribution of TC among serum lipoproteins was analyzed by liquid chromatography. The lipoprotein profiles of the different groups showed no significant differences on both chow diet and the HF/HC diet (Fig. 2). After the switch to the HF/HC diet, non-HDL cholesterol (VLDL and LDL cholesterol) increased ≈6-fold in all groups, while there was no significant change of HDL cholesterol. Thus, no significant effect of transplantation of ABCG1 KO and apoE KO bone marrow to LDLr KO mice on the cholesterol distribution among the different lipoproteins was observed.

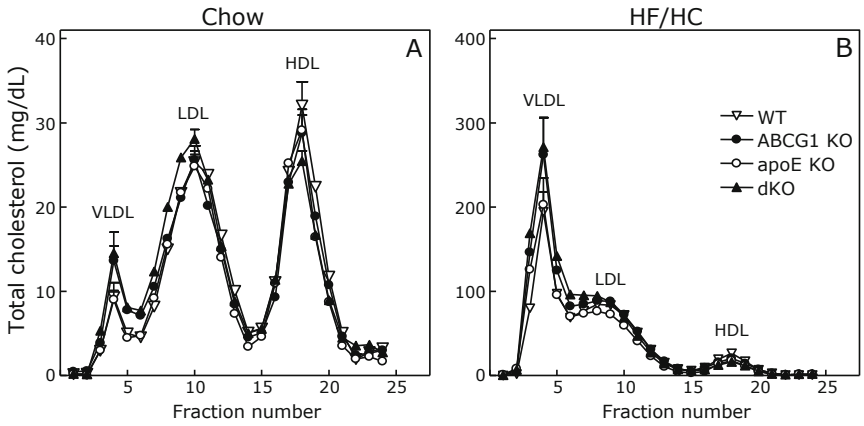

Figure 2. Distribution of serum cholesterol over the different lipoprotein fractions on (A) chow diet and after 6 weeks on (B) the HF/HC diet. Values are the means of ≥6 samples for each group. No significant differences in the distribution of serum cholesterol were observed between the groups.

Increased atherosclerosis in ABCG1/apoE dKO transplanted LDLr KO mice

After feeding the HF/HC diet for 6 weeks, lipid accumulation in different organs and lesion development in the aortic root (Fig. 3) of the transplanted animals were analyzed. dKO transplanted mice and, in agreement with our previous study,[17] ABCG1 KO transplanted animals showed accumulation of large amounts of lipids in the lungs as compared to WT transplanted mice. No differences between the groups were seen in oil red-O-stained sections of the spleen and liver (Fig. 4).

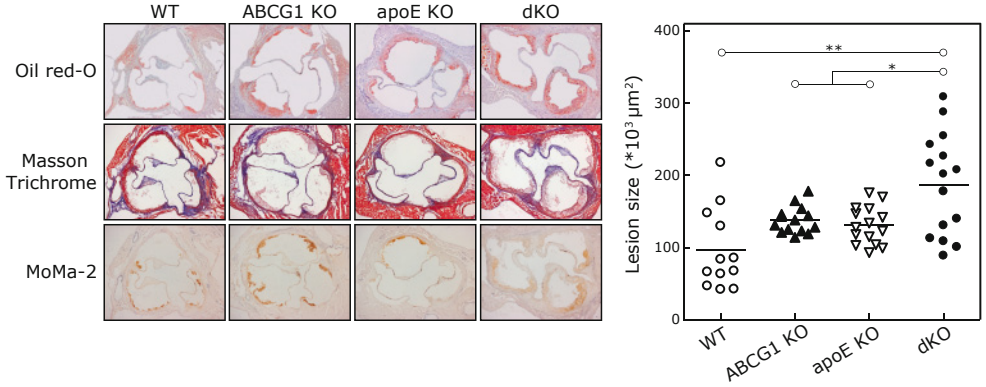

Figure 3. Analysis of atherosclerosis in cross-sections at the aortic root after 6 weeks on the HF/HC diet. Left: representative cross-sections, stained with oil red-O, Masson Trichrome, and MoMa-2 for detection of lipids, collagen, and macrophages, respectively (magnification 50×). Right: quantification of atherosclerotic lesion sizes. Values are the means of 10 aortic root sections of individual mice (≥12 animals per group). *p<0.01, **p<0.001.

Quantification of the lesion sizes in oil red-O-stained sections of the aortic root showed that single deletion of macrophage ABCG1 or apoE led to a 1.4-fold increase in the mean atherosclerotic lesion size as compared to lesions in WT transplanted animals, which however failed to reach statistical significance by ANOVA ($138\pm5\times10^3$ µm^2 in mice reconstituted with ABCG1 KO bone marrow and $131\pm7\times10^3$ µm^2 in animals transplanted with apoE KO bone marrow, compared to $97\pm15\times10^3$ µm^2 in mice reconstituted with WT bone marrow) (Fig. 3). As the current study consisted of four groups, ANOVA is the appropriate test to check for significant differences. Previous studies,[12,13,17-19] however, used

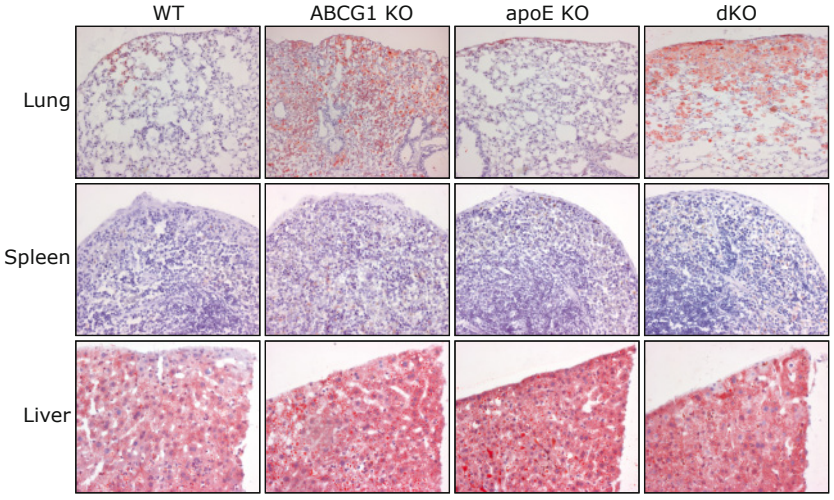

Figure 4. Oil red-O-stained cryostat sections of lung, liver, and spleen of LDLr KO mice transplanted with bone marrow of WT, ABCG1 KO, apoE KO, and dKO mice after 6 weeks on HF/HC diet (magnification 50×).

two groups (WT vs. KO), which only required a t-test. When the same t-test is used, both ABCG1 KO and apoE KO transplanted animals showed a significant increase in atherosclerotic lesion size as compared to mice reconstituted with WT bone marrow ($p=0.016$ and $p=0.046$, respectively). Combined deletion of both ABCG1 and apoE in bone marrow-derived cells in LDLr KO mice induced a significant 1.9-fold ($p<0.001$; ANOVA) increase in the mean atherosclerotic lesion size as compared to WT transplanted animals ($187\pm18\times10^3$ μm^2 compared to $97\pm15\times10^3$ μm^2). In addition, the lesion size in the dKO transplanted animals was also significantly larger as compared to mice transplanted with single ABCG1 KO or apoE KO bone marrow (both 1.4-fold; $p<0.01$; ANOVA). Two-way ANOVA was used to check possible interactions between macrophage ABCG1 and apoE in atherosclerotic lesion development. The p-value for was used to express the significance of the effect of apoE deletion on ABCG1 and vice versa, whereas the interaction p-value was considered to explore the independent effects of both genes on atherosclerotic lesion development. This test showed highly

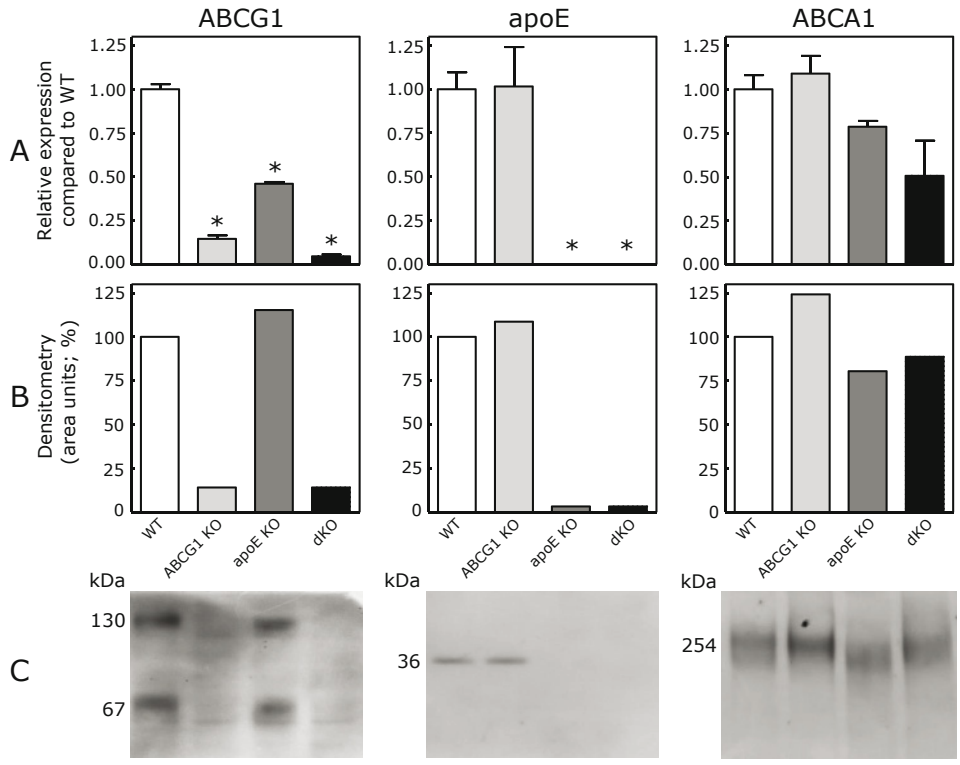

Figure 5. mRNA (A) and protein expression (B) of ABCG1, apoE and ABCA1 in bone marrow-derived macrophages of transplanted animals. Gene expression analysis was performed by RT-PCR. Values are relative to the average expression of the housekeeping gene GAPDH and expressed as fold induction compared to WT transplanted animals. Western blots (C) were used for protein quantification. Values are mean ± SEM, n≥3 mice per group for mRNA expression. n=4 mice per group (pooled) for protein expression. *p<0.001.

independent effects of ABCG1 (p=0.0004) or apoE (p=0.0019) deletion in bone marrow-derived cells on atherosclerotic lesion formation. Moreover, no interaction (p=0.55) was observed, suggesting that the effect of combined deletion of ABCG1 and apoE on atherosclerotic lesion development is an additive, independent effect of the single deletions of ABCG1 and apoE.

Masson Trichrome staining showed no collagen accumulation in the plaques which is indicative for early lesions. In agreement, MoMa-2-stained sections showed that the lesions fully consisted of macrophages. No difference was observed between lesions of the different groups of mice. Furthermore, TUNEL-staining revealed no differences in apoptosis (WT: 4.4±1.0%, ABCG1 KO: 3.2±0.3%, apoE KO: 5.0±0.5% and dKO: 5.7±1.5%, n=9 per group). Deletion of ABCG1 or apoE in bone marrow-derived cells thus did not affect the composition of the lesions.

In vitro cellular cholesterol efflux and mRNA and protein expression in macrophages lacking ABCG1 and apoE

Combined deletion of macrophage ABCG1 and apoE resulted in a significant increase in atherosclerotic lesion development. To determine whether the enhanced atherosclerosis susceptibility of animals transplanted with ABCG1 KO, apoE KO, or dKO bone marrow was due to a (combined) defect in the transport of cholesterol out of the cells to exogenous lipid acceptors, a cholesterol efflux assay to apoA-I and HDL was performed. To investigate if deletion of ABCG1 and/or apoE in macrophages leads to compensatory upregulation of other cholesterol efflux mechanisms, we also determined the expression of apoE, ABCG1 and ABCA1 in bone marrow-derived macrophages from the different groups of transplanted animals.

Deletion of macrophage apoE resulted in a complete absence of apoE mRNA (p<0.001) and protein expression (Fig. 5). In addition, cells of ABCG1 KO transplanted animals

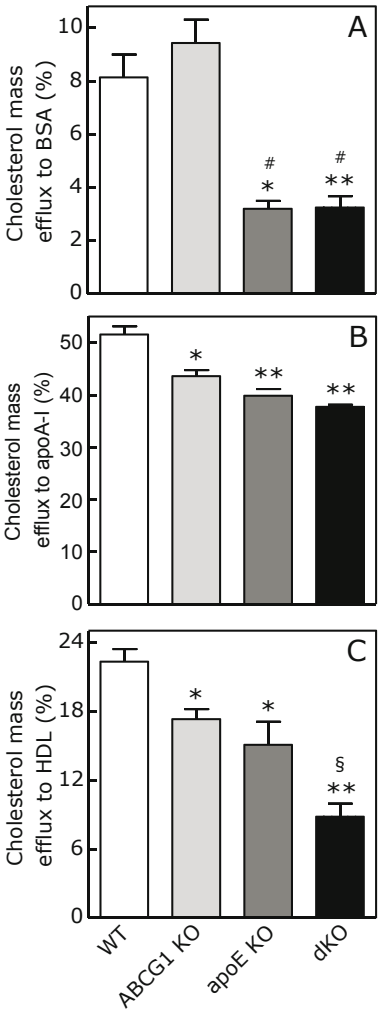

Figure 6. In vitro cholesterol mass efflux from BMDM to 0,1% BSA (A), 10µg/mL apoA-I (B), and 10 µg/mL HDL (C) for 24h. n≥3 mice per group. *p<0.01, **p<0.001, #p<0.001 compared to ABCG1 KO, §p<0.05 compared to single knockouts.

revealed a major decrease of ABCG1 mRNA ($p<0.01$) and protein expression. Remarkably, in contrast to apoE, ABCG1 mRNA was not completely absent. The observed low level of ABCG1 mRNA expression measured in the bone marrow-derived macrophages of mice transplanted with single ABCG1 KO bone marrow is probably just due to technical variations. This is also strengthened by the fact that in cells from mice transplanted with dKO bone marrow no residual apoE or ABCG1 mRNA and protein expression was observed (Fig. 5). Alternatively, although unlikely, during the culture period of the bone marrow-derived cells a positive selection of low numbers of ABCG1 positive cells, that could not be detected by the PCR on the genomic bone marrow, might have occurred.

ApoE KO transplanted animals showed a significant decrease of ABCG1 mRNA ($p<0.001$), whereas ABCG1 protein expression was not affected. In agreement, apoE mRNA and protein expressions were not affected upon deletion of ABCG1.

No differences between the groups were observed in ABCA1 mRNA and protein levels. Moreover, both single and combined deletion of ABCG1 and apoE on macrophages resulted in a slight decrease on apoA-I-mediated cholesterol efflux as compared to WT macrophages (Fig. 6B). However, no differences were observed between single KO and dKO transplanted macrophages.

ApoE is known to facilitate cholesterol efflux in the absence of extracellular cholesterol acceptors.[11] In agreement, cholesterol efflux to BSA is highly decreased in apoE deficient macrophages compared to WT macrophages ($p<0.001$; Fig. 6A). Macrophages of both ABCG1 KO and apoE KO mice did show a significant decrease ($p<0.01$) in HDL-mediated efflux (22% and 33%, respectively, compared to WT; Fig. 6C). Furthermore, a striking 61% decrease ($p<0.001$) in HDL-mediated efflux was observed upon combined deletion of ABCG1 and apoE in macrophages. Two-way ANOVA analysis showed clear independent effects of ABCG1 ($p=0.0032$) and of apoE ($p=0.0004$). Moreover, no interaction ($p=0.65$) between both genes was observed, suggesting independent roles for ABCG1 and apoE in cellular cholesterol efflux to HDL.

Interestingly, a clear inverse relationship is apparent when the HDL-mediated cholesterol efflux percentages of the different groups are plotted against the atherosclerotic plaque size measured at the aortic root ($R^2=0.94$, $p=0.0298$; Fig. 7), suggesting that HDL efflux is an important determinant of lesion size.

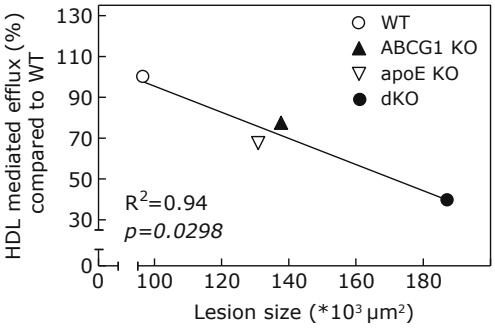

Figure 7. Lower panels: HDL-mediated cholesterol efflux plotted against atherosclerotic plaque size resulted in a clear inverse correlation. n≥3 mice per group for cholesterol efflux towards HDL, n≥12 mice per group for atherosclerotic lesion size.

Discussion

Both ABCG1 and apoE have been implicated in cholesterol efflux and the development of atherosclerosis.[22,23] The importance of macrophage apoE secretion for the pro- or anti-atherogenic effect of macrophage ABCG1, however, was still poorly understood. In the current study, we, for the first time provide evidence for an additive, independent role of ABCG1 and apoE in macrophage cholesterol efflux and atherosclerosis.

ABCG1 exports cellular cholesterol to mature HDL.[23] As a result, ABCG1 deletion results in a decreased cholesterol efflux from macrophages to HDL. In contrast, apoE facilitates cholesterol efflux both in the presence and absence of extracellular sterol acceptors (e.g. HDL or apoA-I),[11] which is underlined by this study. Cholesterol efflux to BSA was highly impaired in macrophages lacking apoE. Furthermore, apoE KO macrophages showed a clear, significant reduction in cholesterol efflux from macrophages to HDL. Macrophages lacking apoE showed a significant reduction in ABCG1 mRNA expression. We cannot completely exclude that this might have partially contributed to observed reduced efflux to HDL. However, reduced ABCG1 expression was not observed on protein level. Moreover, upon combined deletion of ABCG1 and apoE on macrophages, a larger attenuation of cholesterol efflux to HDL was observed, indicating that both ABCG1 and apoE stimulate cholesterol efflux to HDL. Two-way ANOVA analysis indicated that the effects of ABCG1 and apoE on HDL-mediated efflux were independent of each other, in accordance with the independent additive role of macrophage ABCG1 and apoE in the protection against atherosclerosis.

Despite the clear role of ABCG1 in HDL-induced macrophage cholesterol efflux, the effect of single ABCG1 deletion on atherosclerotic lesion development has been under debate.[24] In a previous study using a mild Western-type diet, we showed that macrophage ABCG1 deficiency led to a 1.4-fold increase in atherosclerosis in hyperlipidemic LDLr KO mice compared to WT transplanted mice.[17] In contrast to this finding, Baldán et al. found a decrease in atherosclerotic lesion development upon disruption of macrophage ABCG1 in LDLr KO mice fed a more extreme HF/HC diet, containing 21% fat and 1.25% cholesterol.[18] This diet induced a rise in plasma cholesterol levels to >1000 mg/dL. The observed decrease in lesion size in ABCG1 KO transplanted animals was, at least partly, attributed to accelerated apoptosis of macrophages in the absence of ABCG1. Despite the use of a similar HF/HC diet in the current study and corresponding plasma cholesterol levels (>1000 mg/dL), again a 1.4-fold increase in atherosclerotic lesion development was observed in the ABCG1 KO transplanted animals, suggesting that differences in the diet cannot fully explain the differential outcome of the studies. Furthermore, we observed no differences in apoptosis

in lesions of the ABCG1 KO transplanted animals. In agreement with the current study and our earlier observations, we[5] and others[25] also recently showed a distinct atheroprotective role for ABCG1 by transplanting ABCA1/ABCG1 dKO bone marrow into LDLr deficient mice.

In addition to the observed moderate increase in atherosclerotic lesion development upon disruption of macrophage ABCG1, transplantation of apoE KO bone marrow to LDLr KO mice also led to a slight increase in atherosclerotic lesion development, compared to WT transplanted animals. This is in agreement with previous studies from our group[12] and Fazio et al.[13] that showed a 4- or 10-fold increase, respectively, in atherosclerotic lesion development in C57BL/6J mice upon disruption of macrophage apoE.

Most importantly, we show for the first time that transplantation of bone marrow deficient for both ABCG1 and apoE into LDLr KO mice resulted in an additive, independent increase in atherosclerotic lesion development, compared to single ABCG1 KO or apoE KO transplanted mice. Despite a significant decrease of ABCG1 mRNA in apoE KO macrophages, no compensatory upregulations of apoE and ABCG1 protein expression were observed in bone marrow-derived macrophages from single ABCG1 KO and apoE KO transplanted animals, respectively, indicating independent cholesterol efflux pathways for both ABCG1 and apoE.

The observed significant increase in lesion size in the dKO transplanted animals is thus likely a specific effect of the combined deletion of these two macrophage cholesterol efflux genes. Indeed, in the in vitro cholesterol efflux studies also the largest significant reduction in cholesterol efflux to HDL was observed in dKO macrophages. Furthermore, a clear significant inverse relation between HDL-mediated efflux and the size of atherosclerotic plaques was apparent.

In conclusion, a moderate increase in atherosclerotic lesion development is observed in LDLr KO mice transplanted with either ABCG1 KO or apoE KO bone marrow compared to WT transplanted animals, while combined deletion of ABCG1 and apoE in bone marrow leads to a more dramatic increase in atherosclerosis. These results indicate an additive, independent effect for both ABCG1 and apoE in macrophage cholesterol efflux and atherosclerotic lesion development.

Acknowledgements

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Chapter 5

The effect of ABCG1 deficiency on atherosclerotic lesion development in LDL receptor knockout mice depends on the stage of atherogenesis

Atherosclerosis.
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Illiana Meurs
Bart Lammers
Ying Zhao
Ruud Out
Reeni B. Hildebrand
Menno Hoekstra
Theo J.C. van Berkel
Miranda van Eck

Division of Biopharmaceutics, LACDR, Leiden University, the Netherlands

Abstract

Objective: As ABCG1 plays a role in cholesterol efflux, macrophage ABCG1 expression has been suggested to protect against atherosclerosis. However, we and others observed varying effects of ABCG1 deficiency on atherosclerotic lesion size. The objective of this study was to define the effect of ABCG1 deficiency during atherosclerotic lesion progression in LDL receptor knockout (LDLr KO) mice.

Methods: ABCG1/LDLr dKO and LDLr KO littermates were fed a Western-type diet for 10 and 12 weeks in order to study the effect of ABCG1 deficiency in the exponential phase of atherosclerotic lesion formation.

Results: At 10 weeks of diet feeding, a significant 1.5-fold increase in early atherosclerotic lesion size ($130\pm12x10^3$ μm^2) was observed in ABCG1/LDLr KO mice compared to LDLr KO mice ($88\pm11x10^3$ μm^2; $p<0.05$). Interestingly, in more advanced lesions, induced by 12 weeks of WTD feeding, ABCG1/LDLr dKO mice showed a significant 1.7-fold decrease in atherosclerotic lesion size ($160\pm20x10^3$ μm^2 vs $273\pm19x10^3$ μm^2 in control mice; $p<0.01$), indicating that in the ABCG1/LDLr dKO mice progression of lesion formation is retarded as compared to LDLr KO mice. In addition, correlation analysis performed on published studies and the current study showed a high correlation when the fold increase/decrease in atherosclerotic lesion size of ABCG1 KO mice compared to ABCG1 WT mice is plotted against the atherosclerotic lesion size of ABCG1 WT mice.

Conclusions: It appears that the effect of ABCG1 deficiency on lesion development in LDLr KO mice depends on the stage of atherogenesis, whereby the absence of ABCG1 leads to increased lesions at sizes $<167x10^3$ μm^2 while in more advanced stages of atherosclerosis enhanced apoptosis and/or compensatory mechanisms lead to retarded lesion progression.

Introduction

Reverse cholesterol transport (RCT), defined as the transport of excess cholesterol from peripheral tissues back to the liver for biliary excretion, plays an important protective role in the development of atherosclerosis.[1,2] Previously, the ATP-binding cassette (ABC) transporter A1 has been reported to play an important role in the prevention of atherosclerosis by facilitating cholesterol and phospholipid efflux from macrophages to lipid-poor or lipid-free apolipoprotein A-I (apoA-I).[3-6] Similar to ABCA1, ABCG1 has been implicated in macrophage lipid homeostasis by actively effluxing cellular cholesterol to mature HDL.[7,8] As cholesterol efflux from macrophages is an important protective mechanism to prevent excessive cellular lipid accumulation, macrophage ABCG1 expression was expected to protect against atherosclerosis. However, independent groups have shown that ABCG1 might be pro-atherogenic as well as anti-atherogenic.

Previously, we reported that both total body and macrophage ABCG1 deficiency led either to a significantly increased susceptibility to atherosclerotic lesion development,[9,10] or to no change in lesion size.[11,12] In contrast, the group of Edwards et al.[13,14] and Tall et al.[15] reported decreased atherosclerosis in LDL receptor knockout (LDLr KO) mice transplanted with ABCG1 KO bone marrow cells, which was explained by accelerated apoptosis of ABCG1 KO macrophages or compensatory upregulation of ABCA1 expression and apoE secretion in macrophages lacking ABCG1. Thus, the role of macrophage ABCG1 in the development of atherosclerosis still remains uncertain.

The aim of this study was to assess the effect of ABCG1 deficiency on different stages of atherosclerotic lesion development and especially during the exponential phase of lesion formation in order to unravel the mechanism by which ABCG1 deficiency affects atherogenesis. Upon atherogenic diet feeding, total body ABCG1 KO mice develop only modest atherosclerotic lesions. Therefore, we generated ABCG1/LDLr double knockout (ABCG1/LDLr dKO) mice to perform this lesion stage dependent study.

Methods

Animals

ABCG1 heterozygous mice, obtained from Deltagen Inc., San Carlos, CA, USA were cross-bred with single LDLr KO mice to generated ABCG1/LDLr dKO mice on a C57BL/6J background (8 generations). Genotyping for ABCG1 and LDLr was performed according to the protocol from Deltagen and The Jackson Laboratory, respectively. Male ABCG1/LDLr dKO and LDLr KO mice were maintained on sterilized regular chow diet containing 4.3% (w/w) fat and no cholesterol (RM3, Special Diet Services, Witham, UK) and water ad libitum. At 10 weeks of age, the diet was switched to a high-fat, high-cholesterol Western-type diet (WTD), containing 15% (w/w) cocoa butter and 0.25% (w/w) cholesterol (Diet W; Abdiets, Woerden, The Netherlands) to induce atherosclerotic lesion development. After 10 and 12 weeks of diet

feeding, mice were sacrificed after an overnight fasting period. Animal experiments were performed at the Gorlaeus Laboratories of the Leiden/Amsterdam Center for Drug Research in accordance with the National Laws. All experimental protocols were approved by the Ethics Committee for Animal Experiments of Leiden University.

Serum lipid analyses

Serum concentrations of free cholesterol were determined by enzymatic colorimetric assays with 0.048 U/mL cholesterol oxidase (Sigma) and 0.065 U/mL peroxidase (Roche Diagnostics, Mannheim, Germany) in reaction buffer (1.0 KPi buffer, pH=7.7 containing 0.01 M phenol, 1 mM 4-amino-antipyrine, 1% polyoxyethylene-9-laurylether, and 7.5% methanol). For the determination of total cholesterol, 0.03 U/mL cholesteryl esterase (Seikagaku, Tokyo, Japan) was added to the reaction solution. Absorbance was read at 490 nm. Precipath (standardized serum; Roche Diagnostics) was used as internal standard. The distribution of cholesterol over the different lipoproteins in serum was determined by fractionation of 25 µl of serum of each mouse using a Superose 6 column (3.2 x 300 mm, Smart-System; Pharmacia, Uppsala, Sweden). Total cholesterol content of the effluent was determined as described above.

ApoE western blot

The circulating apoE levels were measured by analyzing 0.1µl of serum on a 10% SDS-page gel. ApoE was detected using a rabbit-anti-mouse apoE polyclonal antibody (Abcam) as primary antibody and goat-anti-rabbit IgG (Jackson ImmunoResearch) as a secondary antibody.

Histological analysis of the aortic root and other organs

To analyze lipid accumulation in the different tissues, mice were sacrificed after 10 and 12 weeks of WTD feeding. After in situ perfusion, organs were excised and stored in 3.7% neutral-buffered formalin (Formal-fixx; Shandon Scientific Ltd, UK). Atherosclerotic lesion development was quantified in oil red-O-stained cryosections of the aortic root of LDLr KO and ABCG1/LDLr dKO mice using the Leica image analysis system, consisting of a Leica DMRE microscope coupled to a video camera and Leica Qwin Imaging software (Leica Ltd., Cambridge, UK). Mean lesion area (in μm^2) was calculated from ten sections at 20 µm intervals, starting at the appearance of the tricuspid valves. Sections were stained immunohistochemically for the presence of macrophages using a rat MoMa-2 antibody, dilution 1:50 (Serotec Ltd., Oxford, UK). The percentage area of the lesion that is composed of macrophages was calculated as a ratio of the macrophage-stained area and the lesion area of one heart section per mouse of 7-8 mice in total. Masson Trichrome-staining (Sigma Diagnostics, USA) was used to determine the collagen content of the plaque. Apoptotic macrophages in atherosclerotic lesions were identified by terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) staining using the In Situ Cell Death Detection Kit (Roche Diagnostics, Mannheim, Germany). The percentage area of the lesion that is composed of necrotic core was calculated as a ratio of the necrotic-stained area (TUNEL-staining positive and lack of nuclei) and the lesion area of one heart section per mouse of 7-8 mice in total. Corresponding sections of atherosclerotic lesions were stained immunohistochemically with a primary antibody specific for murine ABCA1 (Santa Cruz, Santa Cruz, CA, USA). In addition, seven µm cryosections of formalin-fixed lung and spleen from LDLr KO and ABCG1/LDLr dKO mice were prepared and stained for lipid accumulation using oil red-O staining. Hematoxylin (Sigma) was used to stain the nuclei in the different organs.

Cellular cholesterol efflux

Bone marrow cells, isolated from LDLr KO and ABCG1/LDLr dKO mice, were cultured for 7 days in complete RPMI medium supplemented with 20% fetal calf serum (FCS) and 30% L929 cell-conditioned medium, as the source of macrophage colony-stimulating factor (M-CSF), to generate bone marrow-

derived macrophages. Bone marrow-derived macrophages were subsequently incubated with 0.5 µCi/mL ^{3}H-cholesterol in DMEM / 0.2% bovine serum albumin (BSA) for 24 hours at 37°C in 24 well tissue culture plates (Greiner Bio-One). To determine cholesterol loading, cells were washed 3 times with washing buffer (50 mmol/L Tris containing 0.9% NaCl, 1 mmol/L EDTA, and 5 mmol/L $CaCl_2$, pH 7.4), lysed in 0.1 mol/L NaOH, and the radioactivity was determined by liquid scintillation counting. Cholesterol efflux was studied by incubation of the cells with DMEM / 0.2% BSA alone or supplemented with either 10 µg/mL apoA-I (Calbiochem) or 50 µg/mL human HDL (density 1.063 to 1.21 g/mL), isolated according to Redgrave et al.[15] Radioactivity in the medium and the cells was determined by scintillation counting after 24 hours of incubation. The cholesterol efflux percentages are calculated as the amount of radioactivity in the medium compared to the total amount of radioactivity measured in the medium plus the cells. Basal efflux to BSA (in the absence of added acceptors) has been subtracted from the data shown.

Statistical analysis

Statistical analyses were performed using GraphPad Instat software. The probability level (alpha) for statistical significance was set at 0.05. Results are expressed as an average ± SEM.

Results

Effect of ABCG1 deficiency on serum lipid levels and lipid homeostasis in tissues

On regular chow diet, containing 4.3% fat and no added cholesterol, no significant difference in total serum cholesterol levels were observed between LDLr KO and ABCG1/LDLr dKO mice (Table 1). Fractionation of serum lipoproteins, however, showed a moderate shift of HDL cholesterol to the LDL and VLDL fraction in

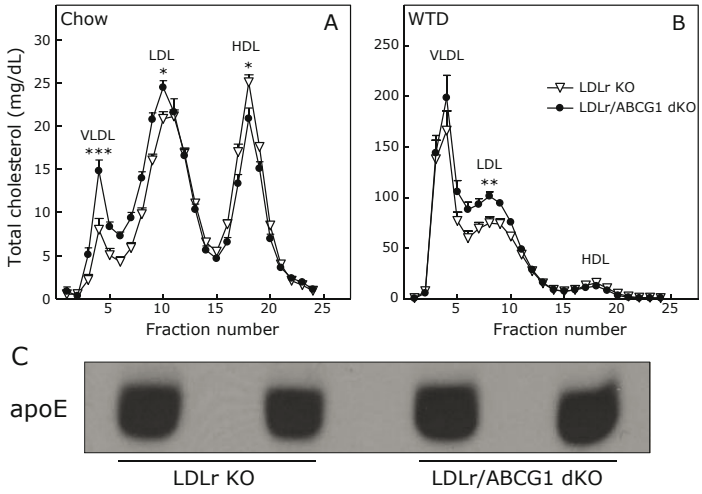

Figure 1. Blood samples were drawn after an overnight fasting period while feeding a regular chow diet (A) and after 12 weeks on WTD (B). Serum samples from individual mice were loaded onto a Superose 6 column, and fractions were collected. The distribution of cholesterol over the different lipoproteins is shown. Values represent the mean ± SEM of 8 mice per group. A representative immunoblot of apoE in serum of LDLr KO and ABCG1/LDLr dKO mice after 12 weeks of WTD feeding (C). *p<0.05, **p<0.01, ***p<0.001.

Table 1. Serum lipid levels in ABCG1/LDLr dKO and LDLr KO control mice on chow and WTD.

Mice	Time (wks)	Diet	FC (mg/dL)	TC (mg/dL)	VLDL-C (mg/dL)	LDL-C (mg/dL)	HDL-C (mg/dL)
LDLr KO	0	Chow	96±4	230±12	19±3	108±4	90±3
	10	WTD	289±32	1030±36	361±82	369±42	58±4
	12	WTD	289±16	1010±82	441±53	374±24	65±6
ABCG1/LDLr dKO	0	Chow	91±4	233±9	36±3***	123±5*	75±4*
	10	WTD	292±23	1206±107	492±81	451±33	58±6
	12	WTD	332±20	1047±111	536±57	467±19**	54±5

Data represent the means ± SEM of 8 mice. *p<0.05, **p<0.01, ***p<0.001.

ABCG1/LDLr dKO mice (HDL: 75±4 compared to 90±3 mg/dL for LDLr KO mice, p<0.05; VLDL: 36±3 compared to 19±3 mg/dL, p<0.001; and LDL: 123±5 compared to 108±4 mg/dL for LDLr KO mice; p<0.05; Table 1, Fig. 1A). To induce atherosclerotic lesion development, ABCG1/LDLr dKO and control mice were fed a WTD for 10 and 12 weeks, which induced approximately a 5-fold increase in serum cholesterol concentrations in both LDLr KO and ABCG1/LDLr dKO mice. Both after 10 weeks and 12 weeks on the WTD, total serum cholesterol levels did not differ between the groups. Lipoprotein profiles of mice fed the WTD for 10 and 12 weeks were essentially identical. Therefore, the representative 12 weeks WTD profile of ABCG1/LDLr dKO and control mice on WTD is shown in Figure 1B. A moderate increase in VLDL (~25%) and LDL (~23%) cholesterol levels was observed in ABCG1/LDLr dKO mice compared to control animals, which only reached significance for LDL after 12 weeks WTD feeding (p<0.01; Table 1). This increase in VLDL and LDL was associated with a moderate non-significant decrease in HDL (~16%) levels in ABCG1/LDLr dKO. Furthermore, during the course of the experiment, the weight gain curve did not show significant differences between ABCG1/LDLr dKO and LDLr KO mice both after 10 or 12 weeks on WTD (data not shown).

ABCG1 deficiency has been shown to coincide with increased secretion of apoE by macrophages and elevated serum apoE levels.[16] Immunoblotting for apoE was performed to analyse the association of ABCG1 deficiency with serum apoE levels of the LDLr KO mice fed WTD for 10 and 12 weeks. No significant effect of ABCG1 deficiency was observed on serum apoE levels between the ABCG1/LDLr dKO and LDLr KO mice fed WTD (Fig. 1B). In addition, ABCG1 deficiency in macrophages has been shown to be correlated with an induction of ABCA1 expresssion.[15] However, immunohistochemical staining of aortic root cryosections showed no apparent increase in ABCA1 expression in macrophages located in atherosclerotic lesions of ABCG1/LDLr dKO mice, neither after 10 nor 12 weeks WTD feeding (data not shown).

Abnormal lung morphology was observed in ABCG1/LDLr dKO mice compared

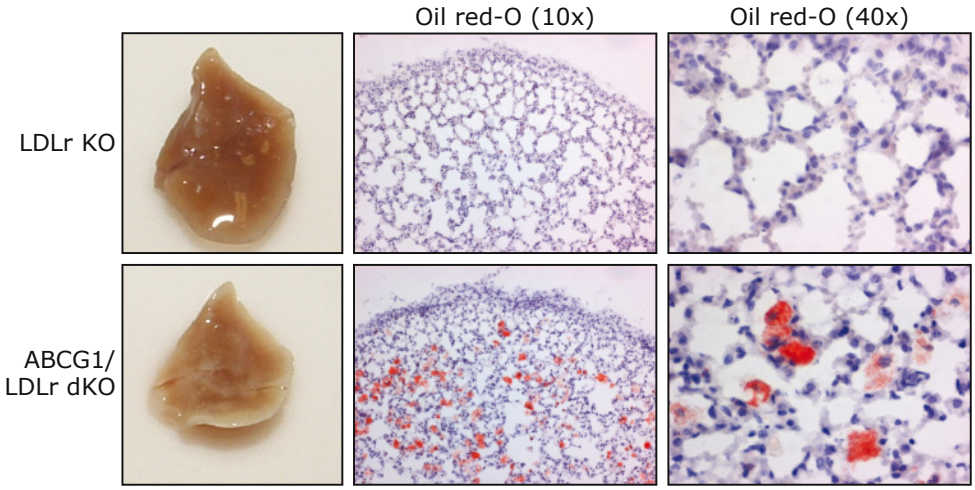

Figure 2. Representative photomicrographs of oil red-O-stained lung sections of LDLr KO and ABCG1/LDLr dKO mice after 10 weeks of WTD feeding at different magnifications (10x and 40x).

with control mice. Both after 10 and 12 weeks of diet feeding, ABCG1 deficiency resulted in accumulation of large amounts of lipids in the subpleural regions of the lungs (Fig. 2). In addition, spleens of ABCG1/LDLr dKO showed lipid accumulation in the red pulp regions, while no accumulation was observed in spleens of control mice (Fig. 3).

Effect of ABCG1 disruption on atherosclerotic lesion formation

To define the role of ABCG1 in the exponential phase of atherogenesis, atherosclerotic lesion development was analyzed in the aortic root of LDLr KO and ABCG1/LDLr dKO mice after 10 and 12 weeks of WTD feeding. Representative photomicrographs of the aortic root of control mice and mice deficient for ABCG1 are shown in Figure 4A. After 10 weeks WTD feeding, a significant 1.5-fold

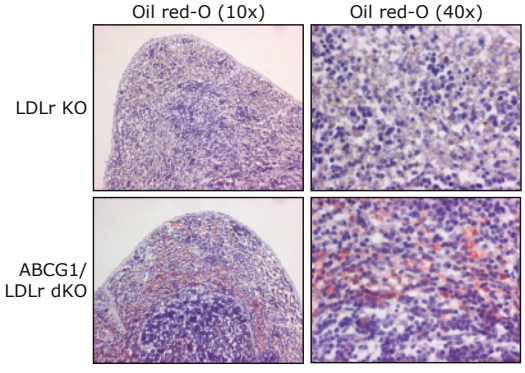

Figure 3. Representative photomicrographs of oil red-O-stained spleen sections of LDLr KO and ABCG1/LDLr dKO mice after 10 weeks of WTD feeding at different magnifications (10x and 40x).

increase in atherosclerotic lesion size was observed in the aortic root of ABCG1/LDLr dKO mice (130±12x10^3 μm^2 (n=8) compared to 88±11x10^3 μm^2 (n=7 for LDLr KO mice; p<0.05). In vitro studies using bone marrow-derived macrophages of LDLr KO and ABCG1/LDLr dKO mice showed that disruption of ABCG1 results in a 15% decrease in cholesterol efflux to HDL (p<0.001), whereas the cholesterol efflux to apoA-I was unaffected (Fig. 4B). Additional 2 weeks of WTD feeding resulted in rapid progression of atherosclerotic lesion development in control mice (3.1-fold; Fig. 4C). Interestingly, atherogenesis in the ABCG1-deficient mice

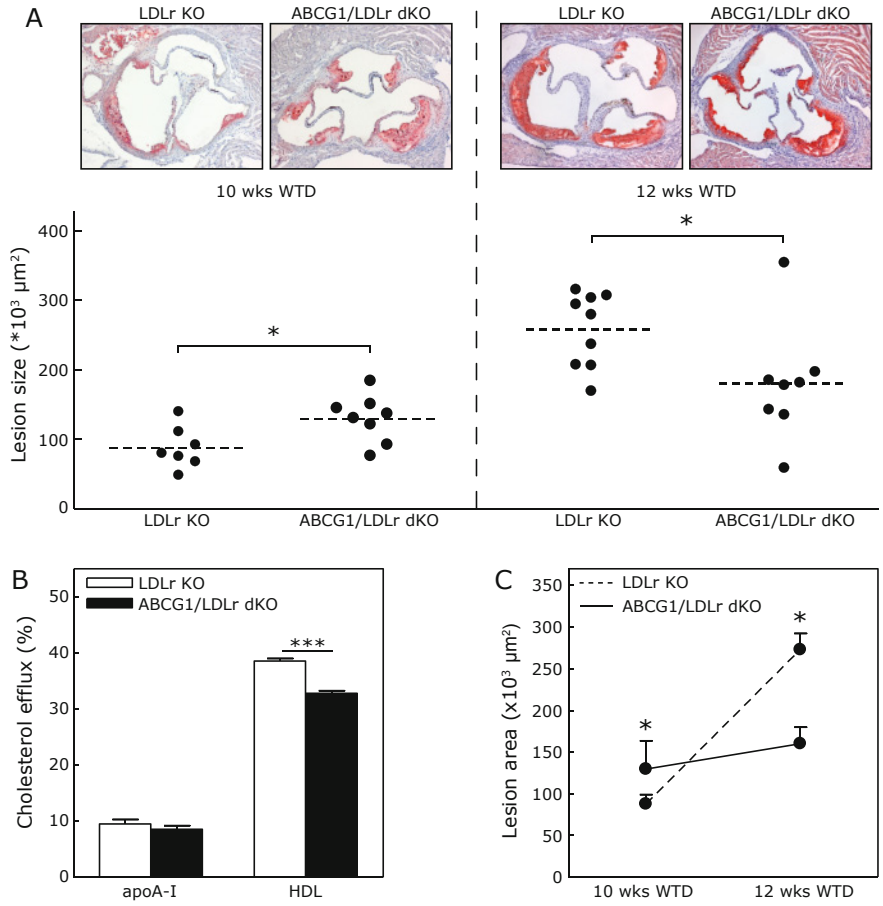

Figure 4. Atherosclerotic lesion formation was determined in the aortic root at the level of the tricuspid valves of LDLr KO and ABCG1/LDLr dKO mice fed a WTD for 10 and 12 weeks (separated by the dotted line). Mean lesion area of each individual mouse is shown (A). Representative photomicrographs of oil red-O-stained lesions are shown (magnification 50x). Cholesterol efflux to HDL is impaired in ABCG1-deficient macrophages. ApoA-I (10 μg/mL) and HDL (50 μg/mL) induced cellular cholesterol efflux from ^{3}H-cholesterol-labeled bone marrow-derived macrophages of LDLr KO and ABCG1/LDLr dKO mice (B). Basal efflux to BSA (in the absence of added acceptors) has been subtracted from the data shown. Values represent the mean ± SEM of 4 animals. Progression of atherosclerotic lesions LDLr KO and ABCG1/LDLr dKO mice from 10 to 12 weeks WTD feeding (C). Values represent the mean ± SEM of 7 to 9 mice. *p<0.05, ***p<0.001.

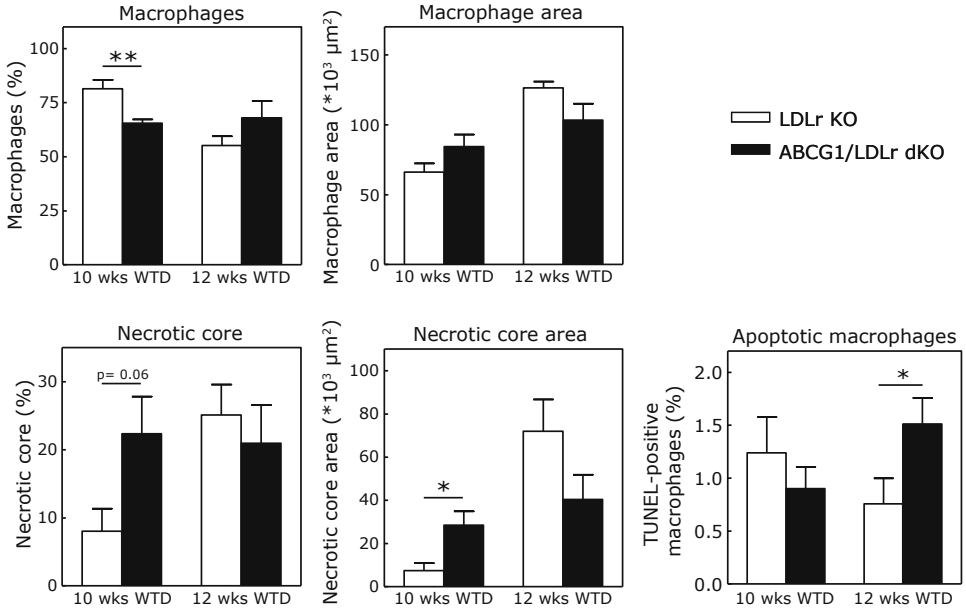

Figure 5. Quantification of lesion macrophages and necrotic core in LDLr KO and ABCG1/LDLr dKO mice after 10 and 12 weeks of WTD feeding. The macrophage lesion area, necrotic core area and apoptotic macrophages were histochemically quantified and expressed as % area of the lesions that is composed of macrophages or necrotic core and/or expressed as absolute area (μm^2). *p<0.05 and **p<0.01.

appeared to be attenuated from 10 till 12 weeks and only a 1.2-fold increase in lesion size is noticed over this period. As a result, ABCG1-deficient mice showed a 1.7-fold smaller atherosclerotic lesions as compared to control mice after 12 weeks WTD feeding ($160\pm20x10^3$ μm^2 (n=8) compared to $273\pm19x10^3$ μm^2 (n=9); p<0.01, respectively; Fig. 4A).

Disruption of ABCG1 in LDLr KO mice also affected the composition of atherosclerotic lesions. Immunostaining for macrophages showed less staining in atherosclerotic lesions of ABCG1/LDLr dKO mice fed WTD for 10 weeks (65±2% of atherosclerotic area compared with 81±4% in control mice; p<0.01; Fig. 5A). The lower macrophage content, coincided with an almost significantly larger percentual necrotic core area after 10 weeks on WTD (22±5% of atherosclerotic area compared with 8±3% in control mice; p=0.06). Additional 2 weeks of WTD feeding resulted in an increase in absolute macrophage area and necrotic core area (Fig. 5A). However, no significant differences could be observed between lesions of LDLr KO and ABCG1/LDLr dKO mice. Furthermore, Masson Trichrome-staining showed no significant differences in collagen accumulation in the atherosclerotic plaques between the ABCG1-deficient mice and control mice after 10 weeks and 12 weeks of WTD feeding (data not shown).

As in vitro studies have recently demonstrated that macrophage ABCG1 deficiency

is associated with increased susceptibility to apoptosis in response to the altered cellular lipid homeostasis,[13] we examined the apoptotic macrophage content of the lesions by TUNEL staining. Lesions of ABCG1-deficient mice fed the WTD for 10 weeks showed no differences in TUNEL-positive macrophages compared with lesions of control mice (Fig. 5B). After 12 weeks of WTD feeding, a 2.5-fold increase in TUNEL-positive macrophages was observed in lesions of ABCG1/LDLr dKO mice compared with LDLr KO animals ($p<0.05$, n=7-8). The decrease in atherosclerotic lesion size observed in ABCG1/LDLr dKO mice fed WTD for 12 weeks, might therefore be a result of increased susceptibility to apoptosis of ABCG1-deficient macrophages inside the lesions.

Overall, these findings indicate that the effect of ABCG1 deficiency on atherosclerotic lesion development depends on the stage of atherogenesis. ABCG1 expression protects against early atherosclerotic lesion development, by facilitating cholesterol efflux from macrophages to HDL. In the more advanced lesions, however, accumulation of cholesterol due to impaired cholesterol efflux from ABCG1-deficient macrophages, eventually, will lead to increased macrophage apoptosis and a reduced further progression of atherogenesis.

Discussion

Several pathways are involved in the efflux of cholesterol from macrophages, including aqueous diffusion, SR-BI mediated cholesterol efflux, efflux dependent on macrophage apoE secretion, and active cholesterol efflux mediated by ABCA1.[17-19] Also ABCG1 has been implicated in cellular lipid homeostasis by its property to actively efflux cholesterol to mature HDL.[8,20] Studies using genetically engineered mice have established the physiological importance of ABCG1. Targeted disruption of ABCG1 in mice resulted in age-related progressive pulmonary lipidosis when fed a regular chow diet.[21-23] In addition, overexpression of ABCG1 protected against diet-induced lipid deposition within multiple tissues.[8] These findings imply a critical role for ABCG1 in maintaining normal lipid metabolism in the lung, thereby preventing inflammatory responses triggered by massive cholesterol and/or cholesterol metabolite accumulation.

Although the critical role of ABCG1 in lung lipid homeostasis is clearly established, contradictory findings on the role of macrophage ABCG1 in the development of atherosclerosis have been reported by different groups/laboratories.[9-14,16,24-26]

Transgenic mice overexpressing human ABCG1 showed either no effect,[25] or increased atherosclerosis.[26] In contrast, Westerterp et al.[27] recently reported an atheroprotective role of vascular ABCG1, which is likely related to its role in the preservation of endothelial NO synthase activity. Furthermore, independent studies using ABCG1-deficient mice or LDLr KO mice transplanted with bone

marrow cells of ABCG1-deficient mice, have shown that macrophage ABCG1 might be proatherogenic,[9,10,12] as well as antiatherogenic.[13-15] In the present study, we show that ABCG1 deletion in LDLr KO mice can both induce and attenuate atherosclerotic lesion development. ABCG1 deficiency led to a significant 48% increase in atherosclerotic lesion size after only 10 wks Western-type diet feeding, while a significant 32% decrease in lesion size was observed after 12 wks WTD feeding. These data imply that the effect of ABCG1 deficiency on atherosclerotic lesion development in LDLr KO mice depends on the stage of atherogenesis.

The reduced atherosclerosis in LDLr KO mice transplanted with ABCG1 KO bone marrow was suggested to be a result of compensatory induction of apoE secretion and ABCA1 expression in ABCG1-deficient macrophages.[16] In this study, however, both at 10 weeks and 12 weeks of WTD feeding, ABCG1/LDLr dKO mice showed no compensatory increase in serum apoE levels, although ABCG1/LDLr dKO mice did exhibit a decrease in atherosclerotic lesion development at 12 weeks of WTD feeding. These findings are in agreement with our previous studies showing that apoE mRNA and protein expressions were not affected upon deletion of ABCG1.[10,12] In addition, no apparent increase in ABCA1 expression was observed in ABCG1-deficient macrophages in atherosclerotic lesions of LDLr KO. Furthermore, accelerated apoptosis was proposed as a mechanism for the reduced atherosclerosis susceptibility of LDLr KO mice lacking ABCG1 in macrophages.[13,14] In agreement, in the present study, the more advanced atherosclerotic lesions of ABCG1/LDLr dKO mice fed WTD for 12 weeks were decreased in size and showed a significant increase in TUNEL-positive macrophages as compared to control mice. Macrophage apoptosis is an important

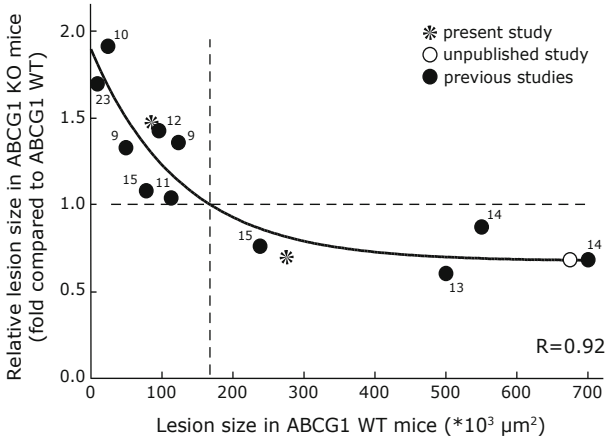

Figure 6. Data from bone marrow transplantation studies in LDLr KO mice and total body studies by different groups, the present study, and unpublished studies are included (experimental details represented in Table 2). In early atherosclerotic lesions (lesions <167x10³ µm²), ABCG1 deficiency causes an increase in atherosclerotic lesion development (ratio>1.0), while at atherosclerotic lesion sizes above 167x10³ µm², enhanced apoptosis and/or compensatory mechanisms lead to retarded lesion progression. The numbers given in the graph represent the reference numbers of the different studies.

feature of atherosclerotic plaque development and occurs during all stages of atherosclerosis with increasing frequencies as the plaque develops.[28] Research directed at understanding the functional consequences of macrophage death in atherosclerosis has revealed opposing roles for apoptosis in atherosclerotic plaque progression. Under normal physiologic conditions, apoptotic cells are rapidly cleared by phagocytes, a process called efferocytosis. In early atherosclerotic lesions, macrophage apoptosis, followed by efferocytosis limits lesion cellularity and suppresses plaque progression.[29,30] In advanced lesions, efferocytosis is defective and under these conditions macrophage apoptosis thus promotes the development of the necrotic core.[31,32]

We show that lesions of ABCG1/LDLr dKO mice fed WTD for 10 weeks exhibited an increase in necrotic core in the absence of an increase in TUNEL-positive macrophages. In addition, in atherosclerotic lesions induced by 12 weeks WTD feeding, ABCG1 deficiency did not affect necrotic core size, despite a significant increase in macrophage apoptosis. These findings suggest that analysis of macrophage apoptosis is a snapshot and that there may be additional determinants of the lesional necrotic core area. Necrosis can be either secondary to apoptosis (secondary necrosis) or a primary process.[33] In particular, necrotic cores are formed by multiple processes, including accumulation of both intracellular and extracellular lipid.[34,35] Several studies have reported that ABCG1 KO macrophages are indeed more susceptible to oxLDL-induced apoptosis as compared to ABCG1-expressing cells.[13,14,36] Efflux of 7-ketocholesterol, the main oxysterol present in oxLDL, is completely dependent on expression of ABCG1 and not on the expression of ABCA1.[36] Therefore, ABCG1-deficient macrophages show increased accumulation of 7-ketocholesterol upon oxLDL loading, which is cytotoxic to the cell and induces accelerated apoptosis,[14,36] indicating that ABCG1 is essential for the prevention of oxLDL-induced apoptosis.

Our findings suggest that in addition to accelerated apoptosis, ABCG1 deficiency also leads to increased necrotic core formation. Under normal physiological conditions, necrotic core formation becomes more prominent in advanced lesions. In absence of ABCG1, however, necrotic core formation is already evident in early atherosclerotic lesions.

The current study indicates that the effect of ABCG1 deficiency on atherosclerotic lesion size depends on the stage of lesion development. To investigate the differential effects found on atherosclerosis susceptibility upon disruption of ABCG1, correlation analysis was performed on published studies of the independent groups, the current study, and one other unpublished study of our group. Experimental details of these studies are represented in Table 2. Although different diets and protocols were used in the individual studies, a high correlation (R=0.92) can be found when the fold increase/decrease in atherosclerotic lesion size of ABCG1 KO mice compared to ABCG1 WT mice is

plotted against the atherosclerotic lesion size of ABCG1 WT mice (Fig. 6). Based on this clear correlation, we propose that the effect of ABCG1 deficiency on lesion development depends on the stage of atherogenesis. In early atherosclerotic lesions (lesions $<167x10^3$ μm^2), ABCG1 deficiency causes an increase in atherosclerotic lesion development (ratio>1.0), most likely as a direct result of the impaired cholesterol efflux to HDL in ABCG1-deficient macrophages. This indicates that ABCG1 expression is protective in early atherosclerotic lesion development. Interestingly, at atherosclerotic lesion sizes above $167x10^3$ μm^2, the role of ABCG1 in atherogenesis switches from anti-atherosclerotic to pro-atherosclerotic. In more advanced lesions, the persistent impaired cholesterol efflux from ABCG1-deficient macrophages is likely to induce accumulation of (oxy)sterols, which leads to enhanced apoptosis/compensatory mechanisms and, subsequently, decreased atherosclerotic lesion size (ratio<1.0). Previously, we[10] also have reported a highly significant correlation when the fold increase/decrease in atherosclerotic lesion size of ABCG1 KO as compared with ABCG1 WT is plotted against total serum cholesterol whereby at about 900 mg/dL serum cholesterol a switch from ABCG1's protective function to lesion formation was noticed. When recent published studies of the independent groups, the current study, and two other unpublished studies of our group are included, again a high correlation between the fold increase/decrease in atherosclerotic lesion size and total serum cholesterol is observed with a switch at 1000 mg/dL serum cholesterol from an atheroprotective function of ABCG1 to a proatherogenic function ($p=0.0075$; $R=0.73$). Therefore, under normal physiological levels of cholesterol, the role of ABCG1 in atherogenesis is likely to be protective. Furthermore, since higher serum cholesterol levels are associated with a more rapid development of atherosclerotic lesions, this correlation is probably also a direct effect of the stage of atherosclerotic lesion development.

In conclusion, our results indicate that the effect of ABCG1 on lesion development depends on the stage atherogenesis, whereby the absence of ABCG1 leads to increased lesions at sizes $<167x10^3$ μm^2 while in more advanced stages of atherosclerosis enhanced apoptosis and/or compensatory mechanisms lead to retarded lesion progression.

Acknowledgements

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Table 2. Overview of experimental details of the different studies on the role of ABCG1 in atherosclerosis

Author	Baldan *et al.* [13]	Lammers *et al.* unpublished	Tarling *et al.* [14]	Meurs *et al.* present study	Ranalletta *et al.* [16]	Tarling *et al.* [14]	Out *et al.* [11]	Ranalletta *et al.* [16]	Out *et al.* [9]	Out *et al.* [9]	Lammers *et al.* [12]	Meurs *et al.* present study	Yvan-Charvet *et al.* [24]	Out *et al.* [10]
Total body (TB) or macrophage deficiency (Mφ)	Mφ	Mφ	TB	TB	Mφ	Mφ	Mφ	Mφ	Mφ	Mφ	Mφ	TB	Mφ	TB
Diet (C=cholesterol) (CA=cholic acid)	21% fat 1.25% C	21% fat 1.25% C	21% fat 0.2% C	15% fat 0.25% C	21.2% fat 0.2% C	21% fat 0.2% C	15% fat 0.25% C	21.2% fat 0.2% C	15% fat 0.25% C	15% fat 0.25% C	21% fat 1.25% C	15% fat 0.25% C	7.5% fat 1.25% C 0.5% CA	15% fat 1% C 0.5% cholate
Diet (wks)	16	12	16	12	11	12	8	7	6	12	6	10	12	12
ABCG1 WT lesion area (μm^2)	500000	674600	700000	272600	238100	550000	113000	77900	49000	123000	96000	87800	9100	24000
ABCG1 KO lesion area (μm^2)	300000	459800	475000	187000	180000	480000	118000	84000	65000	167000	137000	129600	15400	46000
ABCG1 WT TC (mg/dL)	1083	1326	1065	1011	1182	1105	760	911	676	632	1018	1030	241	210
ABCG1 KO TC (mg/dL)	1043	1176	935	1047	1172	1132	800	829	511	670	1031	1206	215	220
Lesion area relative to ABCG1 WT	0.60	0.68	0.68	0.69	0.76	0.87	1.04	1.08	1.33	1.36	1.43	1.48	1.70	1.92

Abbreviations: Mφ= macrophage; TB= total body; C= cholesterol; CA= cholic acid; TC= total cholesterol.

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Chapter 9

Macrophage adipose triglyceride lipase deficiency attenuates atherosclerotic lesion development in LDL receptor knockout mice

Arterioscler Thromb Vasc Biol.
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*Bart Lammers[1]
*Prakash G. Chandak[2]
Elma Aflaki[2]
Gijs H.M. van Puijvelde[1]
Branislav Radovic[2]
Reeni B. Hildebrand[1]
Illiana Meurs[1]
Ruud Out[1]
Johan Kuiper[1]
Theo J.C. van Berkel[1]
Dagmar Kolb[2,3]
Guenter Haemmerle[3]
Rudolf Zechner[3]
Sanja Levak-Frank[2]
Miranda van Eck[1]
Dagmar Kratky[2]

1: Division of Biopharmaceutics, LACDR, Leiden University, the Netherlands
2: Institute of Molecular Biology and Biochemistry, Medical University of Graz, Austria
3: Institute of Molecular Biosciences, University of Graz, Austria
*Equal contribution

Abstract

Objective: The consequences of macrophage triglyceride (TG) accumulation on atherosclerosis have not been studied in detail so far. Adipose triglyceride lipase (ATGL) is the rate-limiting enzyme for the initial step in TG hydrolysis. Because ATGL knockout (KO) mice exhibit massive TG accumulation in macrophages, we used ATGL KO mice to study the effects of macrophage TG accumulation on atherogenesis.

Methods: Low-density lipoprotein receptor (LDLr) KO mice were transplanted with bone marrow from ATGL KO or wild-type (WT) mice and challenged with a Western-type diet for 9 weeks.

Results: Despite TG accumulation in ATGL KO macrophages, atherosclerosis in ATGL KO transplanted mice was 43% reduced associated with decreased plasma monocyte chemoattractant protein-1 (MCP-1) and macrophage interleukin-6 concentrations. This coincided with a reduced amount of macrophages, possibly because of a 39% increase in intraplaque apoptosis and a decreased migratory capacity of ATGL KO macrophages. The reduced number of white blood cells might be due to a 36% decreased Lin^{-} $Sca\text{-}1^{+}$ $cKit^{+}$ hematopoietic stem cell population.

Conclusions: We conclude that the attenuation of atherogenesis in ATGL KO transplanted mice is due to decreased infiltration of less inflammatory macrophages into the arterial wall and increased macrophage apoptosis.

Introduction

In most mammalian cells, excess neutral lipids form lipid droplets as storage organelles. Macrophage-derived foam cells are a hallmark of atherogenesis. Generally, foam cells in early atherosclerotic lesions accumulate cholesteryl esters (CE), whereas advanced lesions have increased concentrations of free cholesterol (FC), leading to macrophage apoptosis and an increased risk for plaque rupture.[1-4] Macrophages may also transform into triglyceride (TG)-rich foam cells on incubation with very-low-density lipoproteins.[5-7]

However, the (patho)physiological consequences of TG accumulation in macrophages on atherogenesis have not been studied in detail so far. Unesterified fatty acids (NEFA) in macrophages are taken up from the plasma after hydrolysis of TG-rich lipoproteins by lipoprotein lipase or by absorption of albumin-bound fatty acids (FA). Inside the cells, FA are esterified within TG and CE, which are less harmful to the cells than NEFA,[8,9] or routed to mitochondrial FA β-oxidation. Thus, CE and TG accumulation within lipid droplets might be seen as a buffer to detoxify excess NEFA and FC.

Both FA and FC are released from lipid droplets by intracellular lipases. Adipose triglyceride lipase (ATGL) (also named patatin-like phospholipase domaincontaining protein-2, PNPLA2, desnutrin, and phospholipase A2ζ) plays a critical role in cellular lipid homeostasis because it specifically catalyzes the initial step in TG hydrolysis.[10-12] Mice lacking ATGL exhibit massive TG accumulation in many tissues and cells as a consequence of defective TG hydrolysis and die from heart failure at an early age.[13]

The importance of ATGL in macrophage TG metabolism and whether its activity contributes to atherogenesis in mice has not been investigated so far. Consistent with the hypothesis that lipase-mediated modulation of FA concentrations affects atherogenesis, it was reported that atherosclerosis susceptibility is increased in mice with high expression levels of lipoprotein lipase in macrophages and reduced in mice that lack lipoprotein lipase.[14-16]

The present study was designed to address the role of ATGL in macrophages and to examine the consequences of macrophage TG accumulation for atherogenesis. We used bone marrow transfer to investigate mechanisms by which the absence of macrophage ATGL may modulate atherosclerotic susceptibility. Evidence is provided that ATGL deficiency in macrophages and the concomitant TG accumulation resulted in reduced atherosclerosis in low-density lipoprotein receptor (LDLr) knockout (KO) mice. Downregulation of ATGL in macrophages might thus be an interesting and prospective strategy to attenuate early atherogenesis.

Methods

Animals and bone marrow transplantation

Female ATGL KO mice and wild-type (WT) littermates (on a C57BL/6J background) were housed and bred at the Medical University of Graz, Austria. Bone marrow of these mice was transplanted into female homozygous LDL receptor knockout (LDLr KO) mice (The Jackson Laboratory, Bar Harbor, ME,USA) as described previously.[17] Briefly, irradiated recipients received $5x10^6$ bone marrow cells by intravenous injection into the tail vein. The recipient mice were housed in sterilized filter-top cages and given unlimited access to food and water. They were maintained for 8 weeks on a sterilized regular chow, containing 4.3% (w/w) fat with no added cholesterol (RM3; Special Diet Services, Witham, UK). After a recovery period of 8 weeks, the animals were challenged with a Western-type diet (WTD; 0.25% cholesterol and 15% cocoa butter; Special Diet Services, Witham, Essex, UK) for 9 weeks to induce atherosclerotic lesion development. Seventeen weeks after transplantation, the hematologic chimerism of the transplanted LDLr KO mice was determined in genomic DNA from bone marrow by PCR analysis (ATGL-fwd: 5'-AGAGAGAGAAGCTGAAGCCTGG-3'; ATGL-rev: 5'-GCCAGCGAATGAGATGTTCC-3'; ATGL-neo: 5'-CTGCGTGCAATCCATCTTGT-3'). These experiments were performed at the Gorlaeus Laboratories of the Leiden/Amsterdam Center for Drug Research in accordance with the National Laws. All experimental protocols were approved by the Austrian Federal Ministry of Science and Research, Division of Genetic Engineering and Animal Experiments, Austria, and the Leiden University, the Netherlands.

Peritoneal and bone marrow-derived macrophage isolation and culturing

Peritoneal macrophages were obtained 3 days after i.p. injection of 3 ml 3% thioglycollate. Macrophages were cultured for 2h in DMEM / 10% LPDS / 1% penicillin / 1% streptomycin and subsequently used for measuring lipid parameters. To achieve foam cell formation of WT cells, macrophages were incubated with acetylated (ac)LDL for 24h in 24 well tissue culture plates (Greiner Bio-One). LDL was isolated from human plasma by density gradient ultracentrifugation and LDL was acetylated as described.[18] For isolation of bone marrow-derived macrophages (BMDM), femurs and tibias were lavaged with phosphate-buffered saline (PBS). Bone marrow cells were plated in DMEM / 20% FCS / 1% penicillin / 1% streptomycin and differentiated into macrophages by addition of 30% L929 cell-conditioned media (as a source of M-CSF) for 7 days. Macrophages were incubated for 24h in the absence or presence of 50 µg/mL β-VLDL isolated according to Van Eck et al.[19] and subsequently lysed for mRNA extraction or fixed and stained with oil red-O to visualize neutral lipid accumulation.

Electron microscopy

Cells were plated on a Melinex Film (Groepl, Tulln, Austria). Cells were fixed in 0.06 M phosphate buffer (pH 7.2) containing 2.5% glutardialdehyde for 90 min. Thereafter, cells were rinsed twice in 0.06 M phosphate buffer for 10 min and post-fixed in 1% osmium tetroxide in the same buffer for 1h. Then the cells were rinsed 4 times for 10 min in 0.06 M phosphate buffer and dehydrated in 50%, 70%, 90%, and 100% cold acetone for 20 min each. Thereafter, cells were infiltrated by 2:1, 1:1, 1:2 mixtures of 100% aceton and agar 100 epoxy resin (Groepl) and pure agar 100 epoxy resin for 4h. The cells were then placed in agar 100 epoxy resin at RT for 8h, transferred into embedding molds and polymerized at 60°C for 48h. Sections (75 nm) were cut with a Reichert Ultracut S Ultramicrotome and stained with lead citrate (5 min) and uranyl acetate (15 min). Images were taken on a Zeiss transmission electron microscope equipped with a plate camera system.

Lipid analyses

Eight weeks after bone marrow transplantation, 100 µL of blood was drawn from each individual

mouse by tail vein bleeding after an overnight fasting period. Upon sacrifice, 17 weeks after bone marrow transplantation, blood was collected by retro-orbital venous plexus puncture after an overnight fasting period. Lipid analyses were performed as described.[20] NEFA concentrations were assayed enzymatically (Diasys, Holzheim, Germany) according to the manufacturer's protocol. Lipoprotein profiles were determined by fractionation of 50 µL of pooled serum from two mice by fast protein liquid chromatography (FPLC) using a Superose 6 column (3.2 × 300 mm, Smart-System; Pharmacia, Uppsala, Sweden). Total cholesterol (TC) and TG content of the effluent was determined as described.[20]

Histological analysis of the aortic root

To analyze the development of atherosclerosis at the aortic root, transplanted LDLr KO mice were euthanized after 9 weeks of WTD feeding. The arterial tree was perfused in situ with PBS (100 mm Hg) for 10 min via a cannula in the left ventricular apex. The heart plus aortic root and descending aorta were excised and stored in 3.7% neutral-buffered formalin (Formal-fixx; Shandon Scientific Ltd, Runcorn, UK). Serial sections (7 µm) of the aortic root were cut using a Leica CM3050S cryostat. The atherosclerotic lesion areas in oil red-O-stained cryostat sections of the aortic root were quantified using the Leica image analysis system, consisting of a Leica DMRE microscope coupled to a video camera and Leica Qwin Imaging software (Leica Ltd, Cambridge, UK). Mean lesion area (in μm^2) was calculated from 10 consecutive oil red-O-stained sections, starting at the appearance of the tricuspid valves. Sections were stained immunohistochemically for the presence of macrophages using a rat MoMa-2 antibody, dilution 1:50 (Serotec Ltd, Oxford, UK). Goat anti-rat coupled to horse radish peroxidase (HRP) (1:100) (Dako, Glostrup, Denmark) was used as a secondary antibody and Nova red substrate (Vector Laboratories, Burlingame, CA, USA) was used for HRP visualization. Collagen content of the lesions was determined after Masson Trichrome staining (Sigma diagnostics, St Louis, MO, USA). Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) staining of lesions was performed to determine the rate of apoptosis of macrophages in the atherosclerotic lesions using the In Situ Cell Death Detection Kit (Roche Diagnostics, Mannheim, Germany). TUNEL-positive nuclei were visualized with Nova Red (Vector Laboratories), and sections were counterstained with 0.3% methylgreen. All quantifications were performed blinded by computer-aided morphometric analysis using the Leica image analysis system.

Western blotting analysis

Control and acLDL-loaded peritoneal macrophages were washed twice with Ripa buffer (50 mM Tris-HCl pH 8, 150 mM NaCl, 1% Triton X-100, 50% sodium deoxycholate) in the presence of 1 µg protease inhibitor cocktail (Sigma, Vienna, Austria). Forty µg of protein were separated on a 12.5% SDS polyacrylamide gel by electrophoresis and blotted onto nitrocellulose protran BA85 membranes (Whatman, Vienna, Austria). The blot was blocked with 5% BSA plus 0.1% Tween-20 and incubated with an anti-rabbit PARP antibody (1:800) (Cell Signaling, Vienna, Austria) and a monoclonal anti-mouse β-actin (1:1000) (SantaCruz, Heidelberg, Germany). The blot was incubated overnight at 4°C. The horseradish peroxidase-conjugated goat anti-rabbit (1:5000) (Santa Cruz) and rabbit anti-mouse antibodies (1:1000) (Dako, Glostrup, Denmark) were visualized by enhanced chemiluminescence detection (ECL Plus, Amersham Biosciences, Piscataway, NJ, USA).

Cellular cholesterol efflux

BMDM were incubated with 0.5 µCi/mL ^{3}H-cholesterol in DMEM / 0.2% fatty acid-free BSA and 30 µg/mL non-labeled cholesterol for 24h at 37°C in 24 well tissue culture plates (Greiner Bio-One). Cholesterol efflux was determined after incubating the cells in DMEM / 0.2% fatty acid-free BSA in the absence or presence of 10 µg/mL human apoAI (Calbiochem, La Jolla, CA, USA) or 50 µg/mL human HDL, isolated according to Redgrave et al.[21] Radioactivity in the medium and in the cells was determined by

scintillation counting after 24h of incubation. Cholesterol efflux is expressed as the percentage of total cell ^{3}H-cholesterol present in the medium after 24h. Basal efflux to BSA (in the absence of acceptors) has been subtracted from the data shown.

mRNA expression analysis by real time PCR

Total RNA from BMDM was isolated using the guanidinium thiocyanate (GTC) method[22] and reverse transcribed using a RevertAid M-MuLV enzyme (Fermentas, Burlington, Canada). Relative mRNA expression was measured from the following genes: Scavenger receptor A (SR-A), scavenger receptor class B type 1 (SR-BI), CD36 receptor (CD36), LDLr, acyl-CoA:cholesterol acyltransferase 1 (ACAT-1), ATP-binding cassette sub-family A member 1 (ABCA1), ABCG1, apolipoprotein E (apoE), hormone sensitive lipase (HSL), lipoprotein lipase (LPL), lysosomal acid lipase (LAL) and diacylglycerol acyltransferase 1 (DGAT1). The mRNA expression levels were assessed by real time PCR (ABI PRISM 7500; Applied Biosystems, Foster City, CA, USA) using SYBR Green technology (Applied Biosystems). Housekeeping genes were used as a control. Primer sequences are available upon request.

Determination of cytokine concentrations

Murine monocyte chemoattractant protein 1 (MCP-1) serum levels were assayed using an MCP-1 instant ELISA kit (eBioscience, Hatfield, UK) according to the manufacturer's protocol. IL-6 concentrations (R&D Systems Europe Ltd, Abingdon, UK) were determined in supernatants of peritoneal macrophages cultured in 6-well plates in 300 µL DMEM / 10% LPDS for 24h in the absence or presence of LPS (10 ng/mL).

White blood cell content andturnover analyses

White blood cell (WBC) repopulation and content was analyzed using an automated Sysmex XT-2000iV Veterinary Haematology analyzer (Sysmex Corporation, Kobe, Japan). To determine the life span of WBC in the circulation, blood cells were biotinylated by tail vein injection of 3 mg EZ-Link Sulfo-NHS-biotin (Pierce, Rockford, IL, USA) dissolved in 0.2 mL PBS. After 2.5h circulation, 20 µL of blood was collected in ethylene diaminetetraacetic acid (EDTA)-coated tubes (Sarstedt, Numbrecht, Germany) by tail bleeding for 100% biotinylated WBC determination. Twenty µL blood was drawn at set timepoints for turnover analysis. Blood samples were washed twice with erythrocyte lysis buffer to eliminate all red blood cells and WBC were subsequently labeled with phycoerythrin-conjugated streptavidin (1:500 in PBS) (Molecular Probes; Eugene, OR, USA) and analyzed by flow cytometry (FACS Canto II; BD Biosciences, Mountain View, CA, USA). WBC survival was determined by assessment of the number of biotinylated (streptavidin-labeled) WBC relative to the starting level, which was 100% biotinylated.

Flow cytometry

Using sterile procedures, bone marrow cells were obtained by flushing femoral and tibial bones from donor mice with PBS. Subsequently, erythrocytes were lysed and the cells were analyzed on a FACS Canto II (BD Biosciences, Mountain View, CA). As a control, total bone marrow cells were gated for the life-gate, based on forward scatter/side scatter characteristics. The Lin^{-}, $Sca\text{-}1^{+}$, $cKit^{+}$ (LSK) population within the bone marrow was assessed by flow cytometry. Bone marrow cells were stained with FITC-labeled anti-CD3, anti-CD4, anti-CD8, anti-CD11b, anti-CD19, anti-GR-1, anti-NK1.1, and anti-TER119 (all obtained from eBioscience, Vienna, Austria). These markers are further designated as Lin^{-}. The LSK population is assigned as the Lin^{-} population, double positive for PE-labeled anti-LY6A/E (Sca-1) and APC-labeled anti-CD117 (c-Kit) (both obtained from eBioscience).

Cell migration

Cell migration assays were performed using 24-well transwell plates (5 µm pore size; Corning, Vienna,

Austria). Two million peritoneal macrophages were added into the upper wells. The lower chambers were filled with DMEM / 10% LPDS and 50 ng/mL MCP-1. After 4h at 37°C, macrophages were analyzed by FACS (BD Biosciences, San Jose, CA, USA) after staining with anti-F4/80 antibody (eBioscience, Vienna, Austria).

Statistical analysis

Statistically significant differences among the means of different populations were tested using the unpaired Student's t-test (GraphPad InStat and GraphPad Prism software). The probability level (alpha) for statistical significance was set at 0.05. Results are expressed as an average ± SEM.

Results

Increased lipid accumulation in ATGL KO macrophages

First, we determined the presence of ATGL in bone marrow-derived macrophages (BMDM) and foam cells by real-time polymerase chain reaction. ATGL was highly expressed in both BMDM and β-very low-density lipoprotein-loaded macrophage foam cells (ATGL cycle threshold: 22.1±0.31 and 22.5±0.51 respectively; β-actin cycle threshold: 17.0±0.55 and 17.0±0.23 respectively). No significant difference in ATGL expression was found between non-loaded BMDM and β-very low-density lipoprotein-loaded foam cells, indicating that ATGL expression was not regulated by lipid loading.

Next, we visualized lipid droplets in peritoneal macrophages of WT and ATGL KO mice by electron microscopy. For comparison, acetylated low-density lipoprotein (ac-LDL)-laden macrophage foam cells were included. As expected,

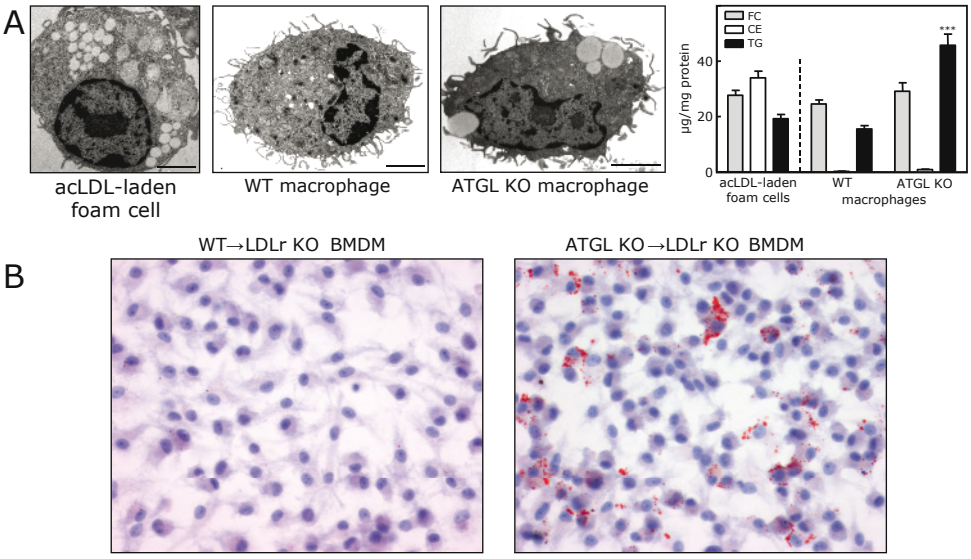

Figure 1. ATGL deficiency induces lipid droplet accumulation in macrophages. Electron micrographs (scale bar, 2 µm) and lipid parameters of isolated peritoneal macrophages (A). BMDM from transplanted mice (B). Images show representative oil red-O-stained BMDM. Magnification 50×. ***p<0.001.

ac-LDL-laden macrophage foam cells showed extensive lipid droplet accumulation and a drastic increase in cellular CE concentrations (Fig. 1A). ATGL KO BMDM accumulated lipid droplets even in the absence of exogenous lipid loading, whereas this was not observed in the majority of the WT macrophages (Fig. 1A). Lipid droplet formation in the ATGL KO macrophages was the consequence of a 3-fold ($p<0.001$) increase in cellular TG concentrations compared with WT macrophages (Fig. 1A). FC and CE concentrations were comparable in macrophages from both groups.

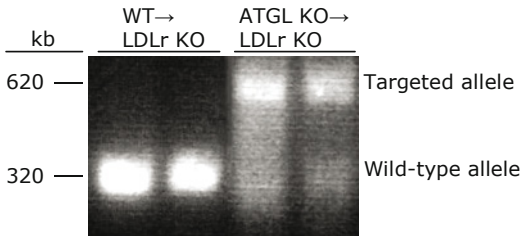

Figure 2. Successful disruption of ATGL by bone marrow transplantation. RT-PCR on genomic DNA of bone marrow from ATGL KO→LDLr KO and WT→LDLr KO mice.

To investigate the consequences of macrophage ATGL deficiency on atherosclerotic lesion formation, we performed a transplantation of ATGL KO and WT bone marrow into LDLr KO mice. Successful hematologic chimerism of transplanted LDLr KO mice was confirmed by polymerase chain reaction analysis of genomic DNA from bone marrow isolated 17 weeks after BMT (Fig. 2). As expected, BMDM from LDLr KO mice transplanted with WT bone marrow (WT→LDLr KO mice) exhibited no lipid accumulation. In contrast, BMDM from ATGL KO→LDLr KO mice accumulated cytoplasmic lipid droplets (Fig. 1B), indicative of the reduced TG hydrolytic capacity and TG accumulation in ATGL KO macrophages.

Serum lipid levels are comparable in ATGL KO→LDLr KO and WT→LDLr KO mice

After BMT, mice were first fed a chow diet for 8 weeks followed by WTD feeding for another 9 weeks. Lipid parameters in WT→LDLr KO and ATGL KO→LDLr KO mice were measured at the end of each diet regimen. No significant differences in plasma TG and NEFA concentrations were observed (Table 1). WTD feeding

Table 1. Plasma lipid parameters of overnight fasted ATGL KO→LDLr KO and WT→LDLr KO mice. After BMT, mice were first fed chow diet for 8 weeks (wks) followed by WTD feeding for another 9 weeks. The data represents mean ± SEM of ≥ 9 mice.

Donor BM	Time (wks)	Diet	FC (mg/dL)	TC (mg/dL)	TG (mg/dL)	NEFA (mM)
WT	8	Chow	63±2	31±9	131±9	n.d.
	17	WTD	274±16	1516±96	104±10	1.07±0.03
ATGL KO	8	Chow	78±5*	357±22	161±16	n.d.
	17	WTD	315±15	1711±87	133±18	1.06±0.03

n.d. not determined; *$p<0.05$.

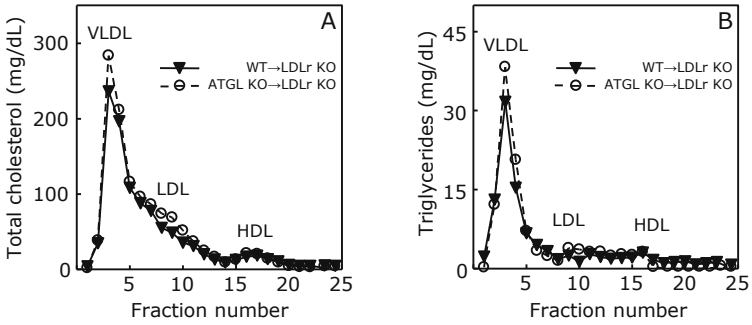

Figure 3. Unchanged lipoprotein profiles in ATGL KO→LDLr KO mice. Fifty µl pools of 2 mice per genotype after 9 weeks of WTD feeding were subjected to FPLC. Distribution of serum TC and TG concentrations in 25 µl fractions was determined enzymatically. Values represent the means of n≥5 samples per group.

resulted in a general ≈5-fold increase in cholesterol levels in both groups. The distribution of total cholesterol (TC) and TG within serum lipoproteins was analyzed by fast protein liquid chromatography. Lipoprotein profiles of ATGL KO→LDLr KO and WT→LDLr KO mice were similar on both chow diet (data not shown) and WTD (Fig. 3). WTD feeding led to ≈6-fold increased non-high-density lipoprotein cholesterol (very low-density lipoprotein and low-density lipoprotein cholesterol) in both groups, whereas no significant changes in high-density

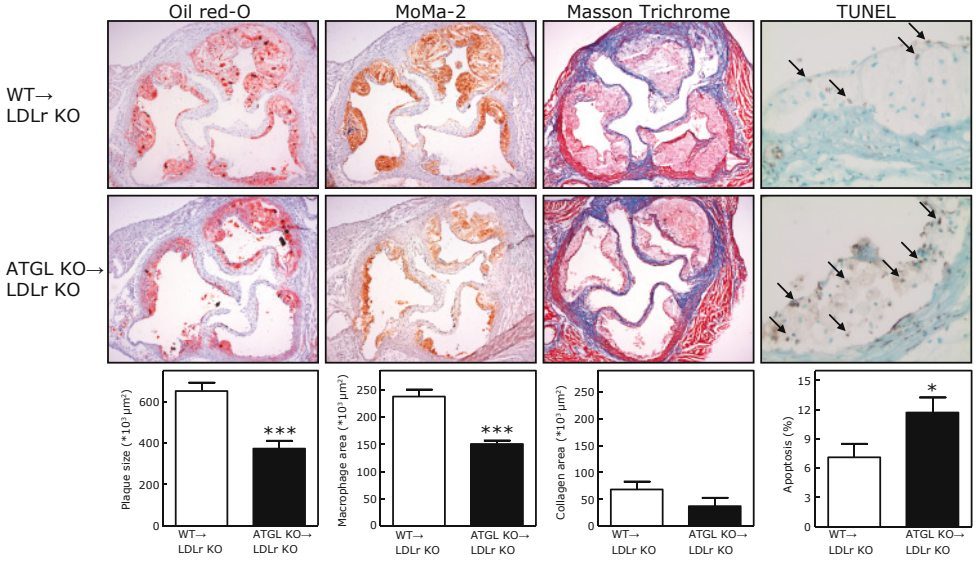

Figure 4. Decreased lesion formation and increased apoptosis in aortic root sections of ATGL KO→LDLr KO mice. Representative slides were stained with oil red-O, MoMa-2, Masson trichrome, and terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) for the detection of lipids, macrophages, collagen, and apoptosis, respectively. Arrows indicate apoptotic nuclei. Oil red-O and lesion composition data represent the means of 10 or 5 aortic root sections of n≥9 or n≥5 animals ± SEM, respectively. Magnification 5× (apoptosis: 40×). *p<0.05; ***p<0.001.

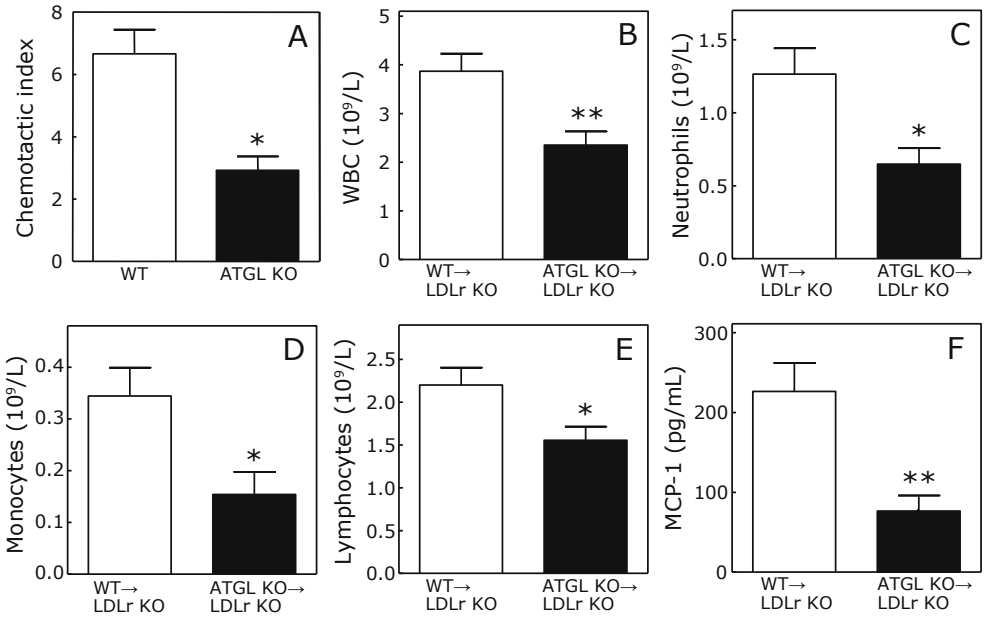

Figure 5. Reduced WBC and MCP-1 concentration in ATGL KO→LDLr KO mice. Macrophage migration assays were performed using transwell plates (A). Values represent the means of n=3 ± SEM. WBC (B), neutrophils (C), monocytes (D), and lymphocytes (E) in plasma were determined with a hematology analyzer. MCP-1 concentrations were determined by ELISA (F). Values represent the means of n≥9 ± SEM. *p<0.05; **p<0.01.

lipoprotein cholesterol concentrations were observed. In addition, lipoprotein-associated TG distribution was similar in both groups.

6

Decreased atherosclerosis in ATGL KO→LDLr KO mice

Visual inspection of oil red-O-stained aortic root sections showed a clear difference in lesion formation between WT→LDLr KO and ATGL KO→LDLr KO animals (Fig. 4). Quantitative analysis of lesion areas revealed a 43% reduction in the mean atherosclerotic lesion size in ATGL KO→LDLr KO mice compared with WT controls ($373\pm37\times10^3$ μm² and $652\pm40\times10^3$ μm², respectively; $p<0.001$).

Masson Trichrome staining, which was used to identify collagen in the lesion, showed no differences between WT→LDLr KO and ATGL KO→LDLr KO mice ($68\pm15\times10^3$ μm² and $37\pm16\times10^3$ μm², respectively; Fig. 4). To visualize macrophages inside the lesions, sections were stained with the monocyte/macrophage-specific MoMa-2 antibody (Fig. 4). In agreement with the observed attenuation in lesion size, quantification of the macrophage area revealed a 37% decrease in macrophage content between lesions of WT→LDLr KO ($238\pm36\times10^3$ μm²) and ATGL KO→LDLr KO mice ($151\pm17\times10^3$ μm²; $p<0.001$; Fig. 4). Next, we examined whether the migration ability of the macrophages might be influenced

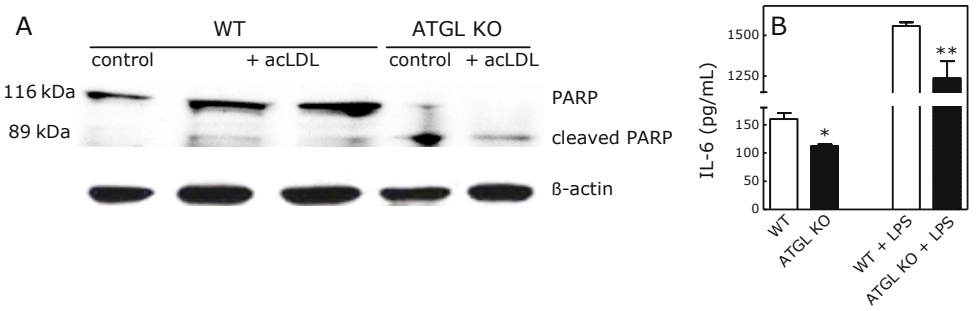

Figure 6. Decreased IL-6 production in ATGL KO macrophages. A, PARP protein expression in peritoneal macrophages of ATGL KO and WT mice. B, IL-6 concentrations were determined by ELISA in supernatants of peritoneal macrophages. Values represent the means of n≥3 ± SEM. *p<0.05; **p<0.01.

by ATGL deficiency. As shown in Fig. 5A, ATGL KO macrophages had a 56% (p<0.05) reduced migration rate compared with WT macrophages. In addition, terminal deoxynucleotidyl transferase dUTP nick-end labeling staining showed a marked 39% increase in intraplaque apoptosis in ATGL KO→LDLr KO compared with WT→LDLr KO animals (11.7±1.5% and 7.1±1.4%, respectively; p<0.05; Fig. 4).

To further investigate the observed increase in intraplaque apoptosis, we measured poly(ADP-ribose) polymerase (PARP) protein expression in ATGL KO macrophages. During apoptosis, full-length PARP (116 kDa) is cleaved by caspase-3, and possibly other caspases, into an 89-kDa fragment.[23] In agreement with the observed increase in intraplaque apoptosis, a clear shift toward cleaved PARP was detectable in ATGL KO macrophages, which is not the case in WT macrophages (Fig. 6). This finding indicates that macrophages deficient for ATGL are more prone to apoptosis.

Cellular cholesterol efflux and mRNA levels of genes implicated in lipid homeostasis in ATGL KO macrophages

To study the effect of ATGL deficiency on the cholesterol transport from macrophages to exogenous lipid acceptors, we measured cholesterol efflux to apolipoprotein A-I and high-density lipoprotein. Cholesterol efflux toward apolipoprotein A-I and high-density lipoprotein was comparable in BMDM isolated from both genotypes (Fig. 7). Next, we performed real-time polymerase

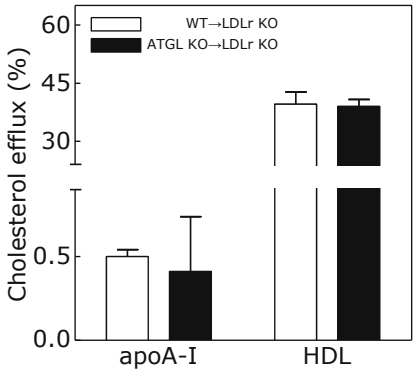

Figure 7. Decreased IL-6 production in ATGL KO macrophages. PARP protein expression in peritoneal macrophages of ATGL KO and WT mice (A). IL-6 concentrations were determined by ELISA in supernatants of peritoneal macrophages (B). Values represent the means of n≥3 ± SEM. *p<0.05; **p<0.01.

chain reaction to investigate whether lack of ATGL in macrophages resulted in compensatory upregulation of genes involved in TG hydrolysis (HSL, LPL, LAL) and biosynthesis (DGAT-1) or cholesterol uptake (SR-A, SR-BI, CD36, LDLr), esterification (ACAT-1), and efflux (ABCA1, ABCG1, SR-BI, apoE). We found a 1.6-fold upregulation of CD36 ($p<0.05$) but no significant differences in mRNA expression of other genes (data not shown), indicating that ATGL deficiency does not affect macrophage cholesterol homeostasis, TG synthesis, and expression of other lipases.

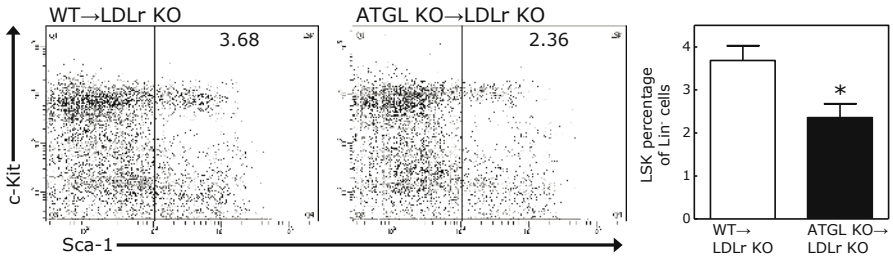

Figure 8. Decreased LSK population in ATGL KO→LDLr KO mice. Dot plots show representative examples of the LSK population within the bone marrow (Lin^- cells, double positive for anti-LY6A/E (Sca-1) and anti-CD117 (c-Kit)). The right panel shows the percentage of LSK cells within the Lin^- population. Values represent the means of n=5 ± SEM. *$p<0.05$.

Attenuated production of the proinflammatory cytokine interleukin-6 in ATGL KO macrophages

Because inflammation is a critical process in atherogenesis, we measured interleukin-6 (IL-6) concentrations in the supernatant of peritoneal macrophages cultured in the absence or presence of lipopolysaccharide (Fig. 6B). Basal and lipopolysaccharide-stimulated IL-6 concentrations were significantly lower in ATGL KO macrophages compared with WT macrophages (30% ($p<0.05$) and 20% ($p<0.01$), respectively).

Reduced circulating number of WBC and plasma MCP-1 levels in ATGL KO→LDLr KO mice challenged with WTD

On a regular chow diet, no differences in WBC counts were observed. After 9 weeks of WTD feeding, the amount of circulating WBC, however, was 39% lower in ATGL KO→LDLr KO mice ($2.35\pm0.24\times10^9$/L) compared with WT→LDLr KO animals ($3.87\pm0.33\times10^9$/L; $p<0.01$; Fig. 5B). Challenging the mice with WTD is thus essential for the observed reduction in WBC counts on disruption of ATGL in bone marrow-derived cells. This decrease was caused by a 49% decrease in neutrophils (WT, $1.26\pm0.18\times10^9$/L; ATGL KO, $0.65\pm0.11\times10^9$/L; $p<0.05$; Fig. 5C) and a 55%

decrease in monocytes (WT, 0.34±0.05×10^9/L; ATGL KO, 0.15±0.04×10^9/L; $p<0.05$; Fig. 5D). We also observed a significant 29% reduction in lymphocytes (WT, 2.20±0.20×10^9/L; ATGL KO, 1.56±0.16×10^9/L; $p<0.05$; Fig. 5E). In addition, monocyte chemoattractant protein-1 (MCP-1) concentrations were 66% lower in ATGL KO→LDLr KO mice (74±19 pg/mL) compared with WT→LDLr KO mice (220±35 pg/mL; $p<0.01$; Fig. 5F).

Unchanged WBC half-life in the circulation and decreased LSK population in bone marrow of ATGL KO→LDLr KO mice

To further investigate the apparent decrease in WBC in ATGL KO→LDLr KO mice, circulating WBC were biotinylated to measure their half-life. WBC of ATGL KO→LDLr KO mice had a half-life of 96±2.2 hours compared with 99±3.9 hours for WT→LDLr KO animals. These data indicate that the WBC half-life cannot account for the observed decrease in the total number of WBC in ATGL KO→LDLr KO mice.

Therefore, next, we measured the LSK population representing hematopoietic stem and multipotential progenitor cells in the bone marrow. Hematopoietic stem and multipotential progenitor cells are capable of extensive self-renewal and are responsible for the production of WBC.[24-26] No differences were observed in the Lin^- population between groups. However, ATGL KO→LDLr KO mice exhibited a significant 36% decrease in LSK cells within the Lin^- population as compared with WT→LDLr KO mice ($p<0.05$; Fig. 8), indicating a decreased WBC production from the bone marrow.

Discussion

ATGL is expressed at high levels in macrophages and foam cells, raising the possibility that it might affect atherosclerosis. Similar to other tissues, the absence of ATGL in macrophages resulted in increased intracellular TG concentrations. Generally, macrophage-derived foam cells are filled with CE, which are hydrolyzed by the action of a neutral CE hydrolase resulting in the release of FC. ATGL KO macrophages, however, accumulated TG even in the absence of exogenous lipid loading, resulting in a cell morphology resembling macrophage foam cells.[27] CE and FC concentrations were unchanged compared with control cells. Therefore, ATGL KO macrophages represent a perfect tool to elucidate the in vivo consequences of TG accumulation in macrophages on atherosclerosis. Thus, we created mice chimeric for macrophage ATGL expression by transplanting LDLr KO mice with bone marrow from ATGL KO and WT littermates. BMT results in the replacement of tissue macrophages, including those of the arterial wall that are involved in fatty streak formation.[28]

In contrast to total body ATGL KO mice,[13] no differences were found in plasma

TG, TC, or NEFA concentrations between ATGL KO→LDLr KO and WT→LDLr KO mice after feeding the WTD. Thus, lack of ATGL in bone marrow-derived cells has no significant influence on serum lipid levels. In line with biochemical data determined in peritoneal ATGL KO macrophages, distinct neutral lipid loading was observed in BMDM of ATGL KO→LDLr KO and WT→LDLr KO mice. Despite increased macrophage TG concentrations, atherosclerotic lesion formation in the aortic root was highly attenuated in ATGL KO→LDLr KO mice. The integrity of the fibrous cap overlying lipid-rich cores fundamentally determines the stability of an atherosclerotic lesion.[29] Quantification of the collagen content in the lesions revealed no differences between the groups, indicating that the plaque stability was unchanged. However, the observed reduction in atherosclerotic lesion formation coincided with increased apoptosis, which might limit lesion cellularity and the progression of early lesions in these animals.[1,30] During atherosclerotic lesion development, macrophages inside the lesion undergo a steady rate of apoptosis. In early lesions, apoptotic macrophages are rapidly cleared by other macrophages, thereby limiting lesion progression.[31,32] In advanced atherosclerotic lesions, however, impaired clearance of apoptotic macrophages leads to secondary necrosis and the formation of a necrotic core.[33] Depending on the stage of lesion development, increased apoptosis can thus either enhance or suppress lesion development. The increased apoptosis rate observed in lesions of mice lacking macrophage ATGL might have prevented the formation of an advanced lesion and, at least in part, might have contributed to the observed attenuation of lesion development. In isolated ATGL KO macrophages, a shift toward cleaved PARP (a marker for apoptosis) was evident, indicating that macrophages lacking ATGL are more susceptible to apoptosis, which explains the observed increase in intraplaque apoptosis.

Leukocytes play an essential role in all stages of atherosclerotic lesion development.[34-36] In humans, increased levels of neutrophils and monocytes induce the progression of atherosclerosis.[37,38] Conversely, a reduced number of circulating monocytes inhibits the initiation and progression of atherosclerotic lesions in mice lacking endogenous macrophage colony-stimulating factor production.[39,40] Importantly, we found a greatly decreased number of circulating WBC in WTD-fed ATGL KO→LDLr KO mice. No differences were observed in the half-life of WBC of ATGL KO→LDLr KO mice compared with WT→LDLr KO controls, suggesting that the reduced number of circulating WBC is merely the consequence of impaired cellular recruitment from the bone marrow.

WBC are produced in the bone marrow by hematopoietic stem and multipotential progenitor cells. Virtually all hematopoietic stem and multipotential progenitor cell activity is contained within the LSK population in the bone marrow.[24-26] Remarkably, the LSK population from ATGL KO→LDLr KO mice is decreased compared with WT→LDLr KO controls, indicating a reduction in WBC-producing

cells. This phenomenon most likely contributed to the observed attenuation in circulating WBC.

Furthermore, the production of the proinflammatory cytokine IL-6 was markedly reduced in ATGL KO macrophages. IL-6 is one of the most important mediators of inflammation because it promotes migration as well as recruitment and activation of inflammatory cells. Although ATGL KO macrophages exhibited a decreased IL-6 production on lipopolysaccharide stimulation, basal IL-6 production was already mitigated in ATGL KO macrophages, indicating an impaired proinflammatory IL-6 signaling. It is generally accepted that inflammatory responses contribute to the development of atherosclerosis. Interestingly, recently Koliwad et al. also found that overexpression of macrophage diacylglycerol acyltransferase-1, which catalyzes the final step in TG biosynthesis, protects against free FA-induced inflammatory activation of the macrophages during diet-induced obesity.[41] Thus, increasing macrophage TG accumulation by either ATGL deletion or DGAT-1 overexpression impairs macrophage proinflammatory signaling.

Moreover, plasma levels of MCP-1, an important chemoattractant for mononuclear cells, were drastically reduced in ATGL KO→LDLr KO mice. Deletion of chemokine (C-C motif) receptor 2, the receptor for MCP-1, inhibits the egress of monocytes from bone marrow into the blood circulation.[42] Decreased concentrations of MCP-1 in the circulation might thus also have affected the amount of circulating mononuclear cells. Mice lacking MCP-1 or its receptor chemokine receptor 2 develop fewer and smaller atherosclerotic lesions than control mice, as a consequence of the reduced ability to recruit monocytes to sites in the arterial wall prone to atherosclerotic lesion development.[43-45] In addition to the reduction in plasma MCP-1 levels, we also observed a reduced migration rate of ATGL KO macrophages compared with control cells in response to MCP-1. The highly attenuated lesion size of ATGL KO→LDLr KO mice coincided with a decrease in the macrophage area of the lesion, indicating that the total number of macrophages that had infiltrated into the arterial wall is decreased. These findings suggest that ATGL-dependent lipolysis of TG and the mobilization of NEFA as energy substrate is necessary for efficient migration.

In summary, our data indicate that macrophage ATGL plays a significant role in atherogenesis independent of its expression in other tissues. The absence of ATGL in macrophages resulted in reduced susceptibility to diet-induced atherosclerosis in LDLr KO mice because of decreased infiltration of less inflammatory macrophages. Therefore, we hypothesize that increasing intracellular TG content in macrophages is protective against lesion development and that macrophage ATGL is a novel target to attenuate early atherogenesis.

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Chapter 7

Leukocyte ABCA1 remains atheroprotective in splenectomized LDL receptor knockout mice

Submitted for publication

*Bart Lammers
*Ying Zhao
Amanda C. Foks
Reeni B. Hildebrand
Johan Kuiper
Theo J.C. van Berkel
Miranda van Eck

Division of Biopharmaceutics, LACDR, Leiden University, the Netherlands
*Equal contribution

Abstract

Objective: ATP-binding cassette transporter A1 (ABCA1) is an important mediator of macrophage cholesterol efflux. It mediates the efflux of cellular cholesterol to lipid-poor apolipoprotein A-I. LDL receptor (LDLr) knockout (KO) mice deficient for leukocyte ABCA1 show increased atherosclerosis and splenic lipid accumulation despite largely attenuated serum cholesterol levels. In the present study, we aimed to explore the role of the spleen in atherosclerotic lesion development.

Methods: LDLr KO mice were transplanted with bone marrow from ABCA1 KO mice or wild-type (WT) controls. After 8 weeks recovery, mice were either splenectomized (SP-x) or underwent a sham operation, and were subsequently challenged with a Western-type diet (WTD) for 8 weeks.

Results: In agreement with previous studies, the atherosclerotic lesion area in leukocyte ABCA1 KO transplanted sham animals ($655\pm82\times10^3$ μm^2) was 1.4-fold ($p=0.03$) larger compared to sham WT controls ($459\pm33\times10^3$ μm^2) after 8 weeks WTD feeding, despite 1.7-fold ($p<0.001$) lower serum cholesterol levels. Interestingly, deletion of ABCA1 in leukocytes led to 1.6-fold higher neutrophil content in the spleen in absence of differences in circulating neutrophils. Levels of KC, an important chemoattractant for neutrophils, in serum, however, were increased 2.9-fold ($p=0.07$) in LDLr KO mice transplanted with ABCA1 KO bone marrow.

SP-x induced blood neutrophilia as compared to WT controls (1.9-fold; $p<0.05$), but did not evoke differences in serum cholesterol and anti-oxLDL antibody levels. Atherosclerotic lesion development, however, was 1.3-fold induced both in the presence and absence of leukocyte ABCA1 (WT: $614\pm106\times10^3$ μm^2, ABCA1 KO: $786\pm44\times10^3$ μm^2). Two-way ANOVA revealed independent effects on atherosclerosis for both leukocyte ABCA1 deficiency and SP-x ($p<0.05$).

Conclusions: The observed splenic alterations induced by leukocyte ABCA1 deficiency do not play a significant role in the anti-atherogenic effects of leukocyte ABCA1 on lesion development.

Introduction

Reverse cholesterol transport (RCT) is an important mechanism by which HDL and its major apolipoprotein A-I (apoA-I) protect against atherosclerosis.[1] In this process, the cellular cholesterol efflux machinery is essential to maintain cellular lipid homeostasis in macrophages and to prevent pathological foam cell formation, a hallmark of atherosclerosis. A key regulator of macrophage cholesterol efflux is ATP-binding cassette (ABC) transporter ABCA1, which facilitates cholesterol efflux to lipid-poor apolipoproteins like apoA-I,[2] thereby initiating the generation of HDL.[3,4] Deficiency of leukocyte ABCA1 on the LDLr KO background led to increased atherosclerosis, despite largely attenuated cholesterol levels.[5] Interestingly, these mice also showed elevated leukocyte counts in the circulation,[6] and accumulation of macrophages in the peritoneal cavity, liver, and spleen.[5] This indicates that leukocyte ABCA1, in addition to its role in cholesterol efflux, exerts regulatory functions in the recruitment of inflammatory cells to the periphery.

The spleen is the largest lymphoid organ in the body with important immunological functions. It produces antibodies, facilitates phagocytosis and is capable of eliminating foreign antigens.[7,8] However, it also serves as a blood filter by removing old and abnormal red blood cells,[9] and functions as an important monocyte reservoir.[10] Since atherosclerosis is believed to result from a combination of dyslipidemia and vascular inflammation,[11] the role of the spleen with respect to atherosclerosis and serum lipid levels has been thoroughly investigated.[12–16] It has been previously reported that total cholesterol (TC) levels increase after splenectomy.[12,13] However, Western-type diet fed, splenectomized apoE KO mice display increased atherosclerosis as compared to sham-operated controls, without changes in TC levels.[15,17]

To investigate the possible interplay between the spleen and leukocyte ABCA1 with respect to the development of atherosclerosis, we transplanted bone marrow from ABCA1 deficient mice into LDLr deficient recipient mice, which were subsequently either splenectomized or underwent a sham operation. Our results evidently show that leukocyte ABCA1 deficiency resulted in decreased TC levels, increased inflammation, and lipid and neutrophil accumulation in the spleen. However, the observed splenic alterations induced by leukocyte ABCA1 deficiency did not alter anti-oxLDL antibody levels, nor played a significant role in atherosclerotic lesion development as evidenced by splenectomy.

Methods

Animals, bone marrow transplantation, and splenectomy

Animal experiments were approved by the Ethics Committee for Animal Experiments of Leiden University (permit number 08015) and performed at the Gorlaeus Laboratories of the Leiden/Amsterdam Center

for Drug Research in accordance with the National Laws and the Directive 2010/63/EU of the European Parliament.

C57BL/6J mice and ABCA1 KO[18] mice (more than 7 times backcrossed onto a C57BL/6J background) were used as donors for the bone marrow transplantation. These donor mice were anaesthetized subcutaneously with a mix of 70 mg/kg body weight xylazine, 1.8 mg/kg bodyweight atropine and 350 mg/kg body weight ketamine. Animals were subsequently sacrificed by cervical dislocation. Homozygous C57BL/6J LDL receptor knockout (LDLr KO) mice were obtained from The Jackson Laboratory as mating pairs and bred at the Gorlaeus Laboratories, Leiden, The Netherlands. Bone marrow transplantations into LDLr KO mice were performed as described.[19] Briefly, irradiated recipients (≥11 per group) received 5×10^6 bone marrow cells by intravenous injection into the tail vein. After 8 weeks, mice were either splenectomized (SP-x) or underwent a sham operation. Mice were anesthetized with isoflurane inhalation. After anesthesia, mice were surgically prepared by first shaving the incision site, followed by preparing the incision site with alcohol. A small incision was made in the left subcostal abdominal wall, through which the spleen was exteriorized. Splenectomy was performed by placing ligatures around the splenic vasculature and subsequently removing the spleen. The incision was closed in two layers using surgical sutures. Mice were monitored for recovery from anesthesia and kept at 37°C until wakeup. Control mice underwent a sham operation and were maintained in the same conditions.

After a recovery period of 2 weeks, the animals were challenged with a Western-type diet (WTD; 0.25% cholesterol and 15% cocoa butter; Special Diet Services, Witham, Essex, UK) for 8 weeks to induce atherosclerotic lesion development. At 18 weeks after transplantation, mice were anaesthetized subcutaneously with a mix of 70 mg/kg body weight xylazine, 1.8 mg/kg bodyweight atropine and 350 mg/kg body weight ketamine. Animals were subsequently sacrificed by cervical dislocation.

Histological analysis of the aortic root and spleen

To analyze the development of atherosclerosis at the aortic root, transplanted LDLr KO mice were euthanized 18 weeks after bone marrow transplantation. The arterial tree was perfused in situ with PBS (100 mm Hg) for 10 min via a cannula in the left ventricular apex. The heart plus aortic root and the aortic arch were excised and stored in 3.7% neutral-buffered formalin (Formal-fixx; Shandon Scientific Ltd, Runcorn, UK). Serial sections (10 µm) of the aortic root were cut using a Leica CM3050S cryostat. The atherosclerotic lesion areas in oil red-O-stained cryostat sections of the aortic root were quantified using the Leica image analysis system, consisting of a Leica DMRE microscope coupled to a video camera and Leica Qwin Imaging software (Leica Ltd, Cambridge, UK). Mean lesion area (in μm^2) was calculated from 10 consecutive oil red-O-stained sections of the aortic root, starting at the appearance of the tricuspid valves. Sections were stained immunohistochemically for the presence of neutrophils and macrophages using a rat Ly6G antibody (monoclonal rat IgG_{2b}, dilution 1:100; eBioscience, San Diego, CA, USA), and a MoMa-2 antibody (dilution 1:50; Serotec Ltd, Oxford, UK), respectively. Goat anti-rat coupled to horse radish peroxidase (HRP) (1:100) (Dako, Glostrup, Denmark) was used as a secondary antibody and Nova red substrate (Vector Laboratories, Burlingame, CA, USA) was used for visualization of HRP. In addition, 7 µm cryosections of formalin-fixed spleen from the transplanted sham-operated LDLr KO mice were prepared and stained for neutrophils using a rat Ly6G antibody (monoclonal rat IgG_{2b}, dilution 1:100; eBioscience, San Diego, CA, USA), and counterstained with hematoxylin.

Lipid analysis

At eight weeks after bone marrow transplantation, 100 µL of blood was drawn from each individual mouse by tail bleeding after an overnight fasting period. Upon sacrifice, 18 weeks after bone marrow transplantation, blood was collected by retro-orbital venous plexus puncture after an overnight fasting period. Total cholesterol and triglyceride analyses were performed as described.[19]

Serum antibody detection

Murine monocyte chemoattractant protein-1 (MCP-1; BD Biosciences, Erembodegem, Belgium) and keratinocyte chemoattractant (KC; Biosource) serum levels were assayed using an ELISA kit according to the manufacturer's protocol. IgM and IgG_{2a} levels against oxLDL were detected in serum using antibodies recognizing mouse IgM/IgG_{2a} and HRP-labeled goat anti-rat Ig (BD Pharmingen). OxLDL (5 µg/mL) was dissolved in $NaHCO_3$/Na_2CO_3 buffer (pH 9.6) and was coated overnight onto a flat-bottom 96-well high binding plate (Corning, New York, USA). Absorbance was detected at 450 nm.

Flow cytometry

Upon sacrifice, blood was collected from the transplanted animals. Erythrocytes were lysed using erythrocyte lysis buffer (0.15 M NH_4Cl, 10 mM $NaHCO_3$, 0.1 mM EDTA, pH 7.3). For the detection of $CD11b^+GR\text{-}1^+$ neutrophils, the blood cells were stained with the surface markers CD11b and GR-1 (0.25 µg Ab/200,000 cells). Antibodies were purchased from eBioscience, Vienna, Austria). Fluorescent activated cell sorting (FACS) analyses were performed on a FACS Canto II (BD Biosciences, Mountain View, CA, USA). Data were analyzed using FACSDiva software (BD Biosciences).

Statistical analysis

Statistically significant differences among the means of the different populations were tested using analysis of variance (ANOVA) and when specifically indicated the unpaired Student's t-test (GraphPad InStat and Prism software). The Student-Newman-Keuls multiple comparison test was performed after ANOVA. Two-way ANOVA was used to check possible interactions. The probability level (alpha) for statistical significance was set at 0.05. Results are expressed as an average ± SEM.

Results

Increased splenic and systemic inflammation in leukocyte ABCA1 KO mice

Deficiency of leukocyte ABCA1 has been shown to induce the number of leukocytes in the circulation. In addition, the spleens of these mice exhibited increased macrophage accumulation.[6] In the current study, we observed induced concentrations of the proinflammatory cytokines MCP-1 (2.2-fold; $p<0.05$), and

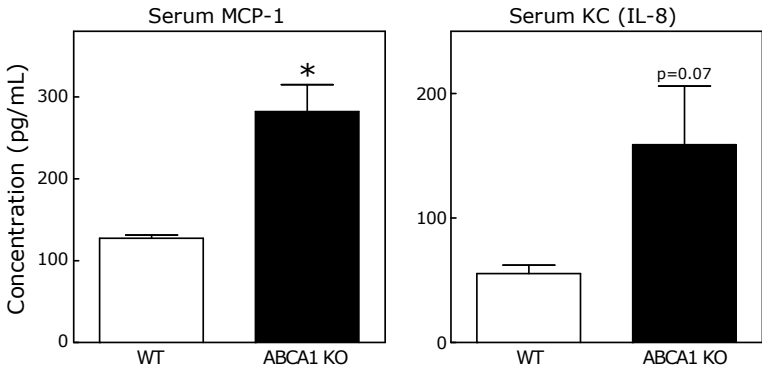

Figure 1. Highly induced concentrations of the proinflammatory cytokines MCP-1 and KC (murine IL-8) in serum of ABCA1 KO transplanted LDLr KO mice. Serum MCP-1 and KC levels were determined by ELISA. n≥4 mice ± SEM per group. *p<0.05.

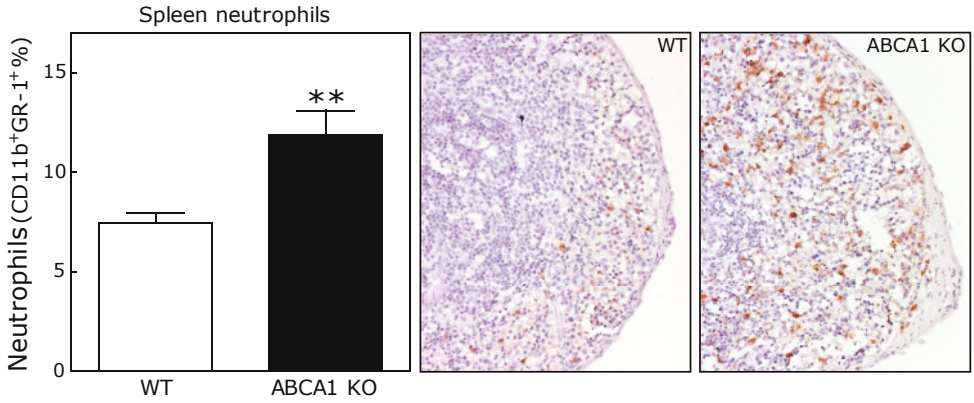

Figure 2. Increased splenic neutrophil content in leukocyte ABCA1 KO mice. Splenic neutrophil content ($CD11b^+GR\text{-}1^+$ cells) was analyzed by FACS. Representative spleen sections were stained with an anti-Ly6G antibody (original magnification 100x; right panels). Values are the percentage of total spleen cells. n≥6 mice ± SEM per group. **p<0.01.

KC (murine IL-8; 2.9-fold; p=0.07) in serum of leukocyte ABCA1 KO mice (Fig. 1). Interestingly, IL-8 is one of the most potent chemoattractants for neutrophils.[20,21] In agreement, spleen sections stained for Ly6G revealed increased neutrophil presence in spleens from leukocyte ABCA1 KO mice. This observation was confirmed by FACS analysis on the spleen, which showed that the splenic neutrophil content was increased 1.6-fold (p<0.01) in ABCA1 KO transplanted mice compared to WT transplanted controls (Fig. 2). In order to establish the importance of the spleen for the atheroprotective effects of leukocyte ABCA1, we subjected WT and KO mice to splenectomy.

Table 1. Serum TC, TG and anti-oxLDL antibody levels were measured at 8 and/or 18 weeks (wks) after BMT. Data represent mean ± SEM of ≥ 10 mice. *p<0.05, **p<0.01, compared to WT.

Donor bone marrow	Time (wks)	Diet	Body weight (g)	Total cholesterol (mg/dL)	Trigly-cerides (mg/dL)	α-oxLDL IgM (OD)	α-oxLDL IgG_{2a} (OD)
WT	8	Chow	n.d.	271±33	n.d.	n.d.	n.d.
	18	WTD	27±0.5	1137±71	68±9	1.10±0.38	0.25±0.01
ABCA1 KO	8	Chow	n.d.	231±31	n.d.	n.d.	n.d.
	18	WTD	29±1.3	676±30**	62±11	0.85±0.47	0.17±0.02
WT SP-x	8	Chow	n.d.	272±19	n.d.	n.d.	n.d.
	18	WTD	27±0.8	1144±177	74±6	1.26±0.15	0.27±0.05
ABCA1 KO SP-x	8	Chow	n.d.	239±19	n.d.	n.d.	n.d.
	18	WTD	28±1.4	713±54*	100±26	0.88±0.07	0.21±0.08

No effects of splenectomy on serum total cholesterol, triglyceride and anti-oxLDL levels

Cholesterol levels were measured at 8 weeks (chow diet) and at 18 weeks (WTD, containing 0.25% cholesterol and 15% cocoa butter) after bone marrow transplantation (BMT; Table 1). On chow diet, total cholesterol (TC) levels were not different between the groups. As expected, after feeding WTD, serum TC levels of the WT transplanted mice increased ≈4.5-fold. However, in agreement with previous studies,[6,22,23] deletion of ABCA1 in bone marrow cells resulted in an attenuated increase in plasma TC levels upon feeding the atherogenic diet (≈2.9-fold as compared to both SP-x ($p<0.05$) and sham operated controls ($p<0.01$)). No differences were observed between splenectomized animals and sham operated controls. In addition, triglyceride (TG) levels were unaffected in all experimental groups (Table 1). Splenectomized mice did not show altered antibody titers of anti-oxLDL IgM and IgG_{2a} (Table 1). Leukocyte ABCA1 deficiency resulted in slightly decreased anti-oxLDL antibody titers as compared to WT transplanted controls. However, these values did not reach statistical significance. These data indicate that splenectomy did not induce significant changes in TC, TG, and anti-oxLDL antibody response.

Increased neutrophil levels in splenectomized leukocyte ABCA1 KO mice

Upon sacrifice after 8 weeks of WTD feeding, FACS analysis was performed to measure neutrophil content in the blood. Although ABCA1 KO transplanted mice exhibited increased splenic neutrophil content, no differences in neutrophil levels were observed between WT and leukocyte ABCA1 KO sham operated animals. Upon splenectomy, however, leukocyte ABCA1 KO mice displayed a 1.9-fold increase in neutrophil levels in blood compared to splenectomized WT controls ($p<0.05$; Fig. 3).

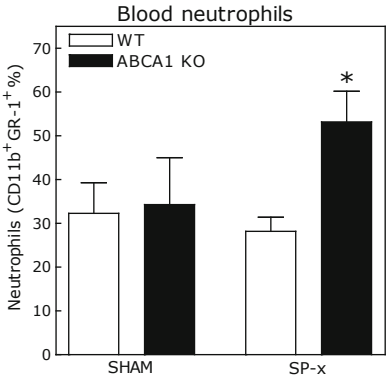

Figure 3. Neutrophil content ($CD11b^+$ $GR\text{-}1^+$ cells) of whole blood was analyzed by FACS. Values are the percentage of total white blood cell counts. n≥3 mice ± SEM per group. *$p<0.05$ compared to splenectomized controls.

Increased atherosclerosis in splenectomized transplanted LDLr KO mice

Atherosclerotic lesion size and composition in the aortic root of the transplanted animals were analyzed after feeding the mice a WTD for 8 weeks. As expected, quantification of the lesion sizes in oil red-O-stained sections of the aortic root of sham-operated animals showed that deletion of leukocyte ABCA1 led to a 1.4-fold increase (t-test; $p=0.03$) in the mean atherosclerotic lesion size compared to lesions from WT transplanted animals ($655\pm82\times10^3$ μm^2 and $459\pm33\times10^3$

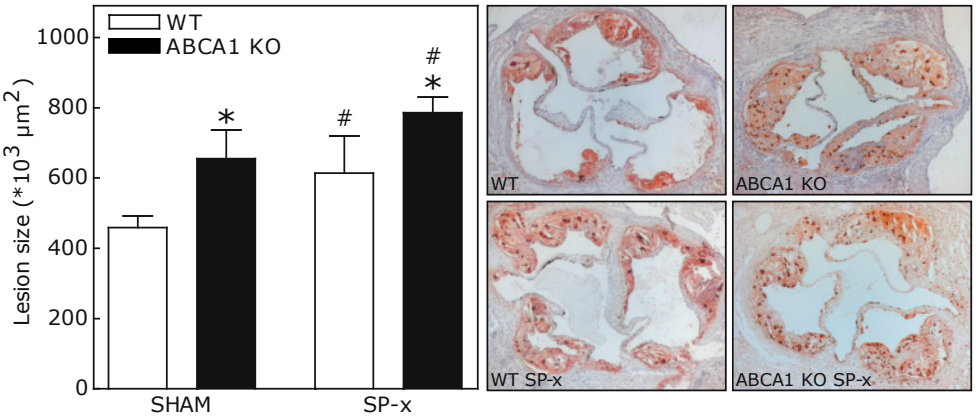

Figure 4. Independent effects on atherosclerosis for both leukocyte ABCA1 deficiency and splenectomy. Quantification of atherosclerotic lesion size after 8 weeks of WTD feeding (left panel). Representative oil red-O-stained cross-sections (original magnification 50x; right panels). Values represent the means of 10 consecutive aortic root sections of individual mice. n≥8 mice ± SEM per group. *p<0.05 compared to respective WT controls; #p<0.05 compared to sham operated controls.

µm², respectively; Fig. 4). Splenectomy induced a 1.2- and 1.3-fold increase in atherosclerotic lesion formation in ABCA1 KO transplanted animals (786±44×10³ µm²) and their WT transplanted controls (614±106×10³ µm²), respectively (Fig. 4). In agreement with the observed increased lesion development in ABCA1 KO transplanted mice, macrophage content of lesions in these mice was attenuated (p<0.05; Fig. 5). Neutrophil content of the lesions, however, was not affected. Two-way ANOVA was used to verify the independent effects of leukocyte ABCA1 deficiency and splenectomy on atherosclerotic lesion development. This test showed significant, independent contributions of both leukocyte ABCA1 deficiency (p=0.012) and splenectomy (p=0.048) to the observed increases in atherosclerotic lesion development, demonstrating the particular importance of both leukocyte ABCA1 and the spleen with respect to atherosclerosis.

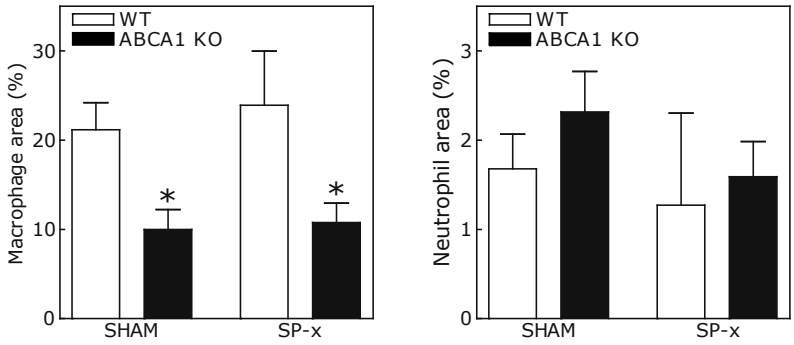

Figure 5. Quantification of macrophage (left panel) and neutrophil (right panel) content after 8 weeks of WTD feeding. Values represent the means of 5 consecutive aortic root sections of individual mice. n≥7 mice ± SEM per group. *p<0.05 compared to respective WT controls.

Discussion

The importance of ABCA1 in cellular cholesterol transport became clear when mutations in the ABCA1 gene were discovered to cause Tangier disease.[24] Additional research revealed a critical role for ABCA1 in leukocytes, as it was shown to be responsible for macrophage lipid metabolism.[25] As a consequence, deficiency of ABCA1 in leukocytes resulted in increased atherosclerotic lesion development.[6,26] However, more recent findings also suggested an anti-inflammatory role for leukocyte ABCA1 in atherosclerosis.[27–29] In agreement, the current study showed highly induced concentrations of MCP-1 and KC (murine IL-8) in the serum of leukocyte ABCA1 KO mice fed a WTD. MCP-1 is an important chemoattractant for mononuclear cells. Mice deficient for MCP-1 or its receptor chemokine receptor 2 (CCR-2) develop fewer and smaller atherosclerotic lesions than control mice, as a consequence of the reduced ability to recruit monocytes to sites in the arterial wall prone to atherosclerotic lesion development.[30–32] KC (murine IL-8) triggers monocyte arrest on early atherosclerotic endothelium,[33] and plays a central role in macrophage accumulation in established fatty streak lesions.[34] Interestingly, IL-8 is also one of the most potent chemoattractants for neutrophils.[20,21] Neutrophils are short-lived phagocytic cells that serve as essential early cellular effectors of innate immunity and constitute the "first line of defense." Accordingly, leukocyte ABCA1 KO mice exhibited increased neutrophil presence in the spleen. Upon splenectomy, ABCA1 KO transplanted animals exhibited blood neutrophilia as compared to WT splenectomized controls. Surprisingly, leukocyte ABCA1 deficieny or splenectomy alone did not alter serum neutrophil concentrations. In response to inflammatory processes, neutrophils are rapidly mobilized from the bone marrow, creating a blood neutrophilia.[35] Following their accumulation at sites of inflammation, neutrophils become apoptotic and are efficiently cleared, primarily by the liver and the spleen,[36] in order to prevent excessive tissue damage. The observed blood neutrophilia in splenectomized ABCA1 KO transplanted animals might be the combined result of a chronic advanced inflammatory status because of the lack of macrophage ABCA1, and absence of splenic neutrophil clearance.

The spleen is also associated with systemic immune responses in which it is the principal organ responding to antigens such as oxLDL.[17] OxLDL deposits in the arterial wall are believed to be involved in the initiation of atherosclerosis by damaging the vascular endothelium and engulfment by macrophages.[37] Accordingly, anti-oxLDL antibody serum titers have been suggested to play an anti-atherogenic role.[17] Anti-oxLDL antibody production, however, might also be increased as a result of enhanced inflammation. No differences in anti-oxLDL antibody serum titers were observed upon splenectomy. Unexpectedly, deficiency

of leukocyte ABCA1 resulted in a moderate decrease in anti-oxLDL antibody serum titers. This might be the direct result of the attenuated serum TC levels in ABCA1 KO transplanted animals, since serum TC levels have been positively correlated to serum titers of anti-oxLDL antibodies.[38]

The importance of the spleen in lipid metabolism has been investigated previously.[12–14] However, differential results were obtained regarding serum TC and TG concentrations after splenectomy. Splenectomized apoE KO mice displayed increased atherosclerotic lesion development as compared to their sham operated littermates, in absence of changes in serum TC levels.[15] As expected, the current study revealed attenuated TC levels in leukocyte ABCA1 KO mice. However, no differences in TC levels were observed as a result of splenectomy.

Despite the fact that splenectomy did not reveal differences in either serum TC and TG levels, or anti-oxLDL antibody titers, splenectomized mice did show enhanced atherosclerotic lesion development. As expected, deficiency of leukocyte ABCA1 also resulted in an increased atherosclerotic lesion formation. Moreover, splenectomized ABCA1 KO transplanted animals exhibited an additional increased lesion development. These results suggest that the observed splenic alterations induced by leukocyte ABCA1 deficiency do not play a significant role in the anti-atherogenic effects of leukocyte ABCA1. Leukocyte ABCA1 deficiency, as well as splenectomy independently induce atherosclerotic lesion development, demonstrating the particular importance of both leukocyte ABCA1 and the spleen with respect to atherosclerosis.

Acknowledgements

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Chapter 8

ABCA1 deficiency protects the heart against injury following myocardial infarction

Manuscript in preparation

*Bart Lammers[1]
*Mieke Louwe[2]
Miguel A. Frias[3]
Amanda C. Foks[1]
Reeni B. Hildebrand[1]
Johan Kuiper[1]
Johannes W.A. Smit[2]
Theo J.C. van Berkel[1]
Richard W. James[3]
Patrick C.N. Rensen[2]
Miranda van Eck[1]

1: Division of Biopharmaceutics, LACDR, Leiden University, the Netherlands
2: Department of Endocrinology and Metabolic Diseases, LUMC, the Netherlands
3: Division of Endocrinology, Diabetology and Nutrition, University of Geneva, Switzerland
*Equal contribution

Abstract

Objective: ATP-binding cassette transporter A1 (ABCA1) exerts important anti-atherogenic functions in the pathogenesis of atherosclerosis. In the present study, we aimed to explore the role of ABCA1 in heart injury after acute myocardial infarct (MI) induction.

Methods: MI was induced in ABCA1 knockout (KO) mice and wildtype (WT) controls by ligation of the Left Anterior Descending (LAD) coronary artery in vivo, after which the mice were allowed to recover for two weeks. In addition, isolated hearts from ABCA1 KO and WT mice were subjected to MI induction in a Langendorff perfusion system ex vivo.

Results: ABCA1 KO mice exhibited a substantial 59% reduction in MI size as compared to WT mice ($p=0.03$) after LAD coronary artery ligation in vivo. Ex vivo MI induction by Langendorff perfusion had no effect on infarct size in hearts from ABCA1 KO mice. The smaller infarct size after in vivo coronary artery ligation in ABCA1 KO mice is thus likely not due to a direct effect of ABCA1 deficiency on myocyte function. Interestingly, after in vivo MI induction, a 2.9-fold increase ($p=0.01$) in circulating leukocyte numbers was observed in ABCA1 KO mice as compared to WT controls, which was primarily the result of higher levels of $CD19^+$ B-lymphocytes (3.0-fold, $p=0.02$) and $CD3^+$ T-lymphocytes (4.2-fold, $p=0.002$). Both $CD4^+$ T-helper lymphocytes (4.6-fold increase; $p=0.002$) and $CD8^+$ cytotoxic T-lymphocytes (3.7-fold increase; $p=0.002$) contributed to the observed increase in $CD3^+$ T-lymphocytes. No differences were found in circulating $F4/80^+$ monocytes/macrophages and $CD11c^+$ dendritic cells.

Conclusions: Although ABCA1 has an important protective role in atherosclerosis, it exerts detrimental effects on cardiac function after MI, possibly as a result of decreased tissue repair due to lower numbers of circulating lymphocytes.

Introduction

Tangier Disease (TD), resulting in extreme HDL deficiency, is caused by detrimental mutations in the ABCA1 gene.[1–3] Since HDL plays a key protective role in atherosclerosis, by exerting several cardioprotective functions, including anti-oxidative, anti-inflammatory and vasomotor activities,[4] the mechanism of action of ABCA1 and its regulation have been investigated extensively.[5–8] Mice deficient for ABCA1 exhibit low plasma HDL levels as well as cholesterol accumulation in peripheral macrophages, a phenotype similar to that of TD patients.[9] Although atherosclerosis-prone mouse models deficient for ABCA1 do display impaired cellular cholesterol efflux,[10] atherosclerotic lesion development does not increase in these mice,[11] probably caused by a less atherogenic lipid profile despite the almost complete absence of protective HDL. Deficiency of ABCA1 in leukocytes, however, did increase atherosclerotic lesion development, coinciding with increased numbers of peritoneal foam cells and highly impaired cholesterol efflux from macrophages towards HDL, indicating a pronounced anti-atherosclerotic effect of leukocyte ABCA1.[12,13] In humans, low HDL levels have been correlated to an increased risk of myocardial infarction (MI); an acute cardiovascular event, often resulting from rupture of advanced atherosclerotic lesions and superimposed thrombus formation.[14]

Inflammatory responses are a critical factor in the balance between adverse ventricular remodeling induced by MI on the one hand,[15,16] and cardiac repair on the other hand.[17] Ischemia induces the Janus kinase 2/signal transducer and activator of transcription 3 (JAK2/STAT3) pathway, an important regulator of cytokine signaling which plays a vital role in cardioprotection by inducing cytoprotective and survival signals in infarcted hearts.[18] Interestingly, ABCA1 acts as an anti-inflammatory mediator in baby hamster kidney (BHK) cells by inducing signaling through the JAK2/STAT3 pathway in response to binding lipid poor apoA-I.[19] Furthermore, ABCA1 exerts important anti-inflammatory properties, due to its key role in modulating cholesterol content both on the plasma membrane as well as within intracellular compartments.[20,21] ABCA1 is thus anticipated to be cardioprotective during MI, indirectly by generating HDL as well as directly by its anti-inflammatory effects through JAK2/STAT3 signaling. The actual role of ABCA1 during MI, however, is currently unknown.

In order to investigate the importance of ABCA1 with respect to MI, we performed permanent coronary artery ligation in ABCA1 KO and wil-dtype control mice. Our results evidently show that ABCA1 has unfavorable cardiac effects after MI.

Methods

Animals

Female C57BL/6J mice and ABCA1 KO[22] mice (kindly provided by Dr. G. Chimini, Centre d'Immunologie de Marseille-Luminy; more than 7 times backcrossed onto a C57BL/6J background), between 15 and 26 weeks of age, were housed in a temperature and humidity-controlled room with ad libitum access to food and water. Body weights were measured weekly. At the end of each experiment the mice were sacrificed and hearts and/or blood were isolated for further examination. Animal experiments were approved by the Ethics Committee for Animal Experiments of Leiden University and performed at the Gorlaeus Laboratories of the Leiden/Amsterdam Center for Drug Research in accordance with the National Laws.

Induction of myocardial infarctions

Mice were anesthetized by intraperitoneal injection of a mixture of dormicum (0.7 mg/kg b.w.), domitor (7.2 mg/kg b.w.), and fentanyl (0.07 mg/kg b.w.) (DDF). Body temperature was maintained at 37°C with an automatic heating pad. Mice were artificially ventilated using a dedicated rodent ventilator (model 845, Harvard Apparatus, Holliston, MA, USA). The left anterior descending (LAD) coronary artery was ligated with a 7-0 Ethilon suture (Johnson and Johnson, New Brunswick, NJ, USA), just distal to the left atrial appendix. Ischemia was confirmed by bleaching of the left ventricle (LV). The thorax was closed and the mice received a subcutaneous injection of Anexate (0.5 mg/kg b.w.), Antisedan (2.5 mg/kg b.w.), Naloxon (1.2 mg/kg b.w.), and 50 µL Temgesic/PBS (1.5 µg/50 mL PBS). Thereafter the mice were allowed to recover on a heating pad. Mice received 50 µL Temgesic/PBS (1.5 µg/50 mL PBS) at 24h after operation.

Infarct size and immunohistochemistry

At 2 weeks after induction of myocardial infarction (MI), mice were sacrificed and the arterial tree was perfused in situ with PBS (100 mm Hg) for 10 min via a cannula in the left ventricular (LV) apex. Subsequently, hearts were isolated and cut into four equal 1-2 mm thick slices, perpendicular to the long axis of the heart. The two lower slices, from the middle of the heart to the apex, represent the infarcted area, and were used for infarct quantification. These slices were flat-embedded and serial sections (10 µm) were cut using a Leica CM3050S cryostat. To delineate the LV area and infarct area, sections were stained immunohistochemically with Sirius red for collagen. Total LV wall area (including septum) and infarct area were measured with cell^D imaging software (Olympus Soft Imaging Solutions). Infarct areas were normalized to total left ventricular areas and averaged for individual hearts.

Leukocyte content and flow cytometry

Upon sacrifice, 2 weeks after MI, blood was collected by retro-orbital venous plexus puncture. Leukocyte content was analyzed using an automated Sysmex XT-2000iV Veterinary Hematology analyzer (Sysmex Corporation, Kobe, Japan). For fluorescent activated cell sorting (FACS) analysis, erythrocytes were lysed using erythrocyte lysis buffer (0.15 M NH_4Cl, 10 mM $NaHCO_3$, 0.1 mM EDTA, pH 7.3). Blood cells were subsequent stained (0.25 µg Ab/200,000 cells) for T-lymphocytes (CD3, CD4, and CD8), B-lymphocytes (CD19), monocytes/macrophages (F4/80) and dendritic cells (DC; CD11c). Antibodies were purchased from eBioscience, (Vienna, Austria). FACS analyses were performed on a FACS Canto II (BD Biosciences, Mountain View, CA, USA). Data were analyzed using FACSDiva software (BD Biosciences).

Ex vivo langendorff perfusion

To properly isolate the heart, the chest was opened excising the sternum and attached costal cartilages to give adequate access to the mediastinum. The heart was rapidly removed and placed in ice cold (4°C)

Krebs–Henseleit buffer and the aorta cannulated. Hearts were then perfused with a Krebs–Henseleit buffer (118.0 mM NaCl; 24.0 mM $NaHCO_3$; 4.0 mM KCl; 1.0 mM NaH_2PO_4; 2.5 mM $CaCl_2$; 1.2 mM $MgCl_2$; 0.5 mM EDTA.Na_2; 10 mM glucose, pH 7.4; gassed with 95% O_2 / 5% CO_2 at 37°C) in a retrograde fashion with a constant pressure of 110 cm H_2O. The coronary flow rate was measured by timed collection of the perfusate. The hearts were stabilized for 20 min and subsequently exposed to 35 min of no-flow global ischemia followed by 45 min of reperfusion. At the end of the reperfusion period, the heart was immediately frozen. Frozen hearts were cut into 6-7 slices, perpendicular to the long axis of the heart, and incubated with triphenyltetrazolium chloride (TTC) to stain viable myocardium. Total myocardium and infarcted areas were measured from computed images using NIH Image software.

Statistical analysis

Statistically significant differences among the means of the different populations were tested using the unpaired Student's t-test (GraphPad InStat and Prism software). The probability level (alpha) for statistical significance was set at 0.05. Results are expressed as average ± SEM.

Results

Increased myocardial infarction following coronary artery ligation in ABCA1 KO mice in vivo

To investigate the effects of ABCA1 deficiency on MI in vivo, we subjected ABCA1 KO and WT mice to LAD coronary artery ligation. Two weeks after induction of MI, infarct size was quantified in ABCA1 KO mice and WT controls. Surprisingly, despite the anticipated cardioprotective functions of ABCA1, ABCA1 KO mice displayed a substantial 59% reduction in myocardial infarct size as compared to WT mice (p=0.03; Fig. 1, left panel). No differences in total LV wall area were found, indicating that the observed reduction in MI did not result from alterations in LV size (Fig. 1, right panel).

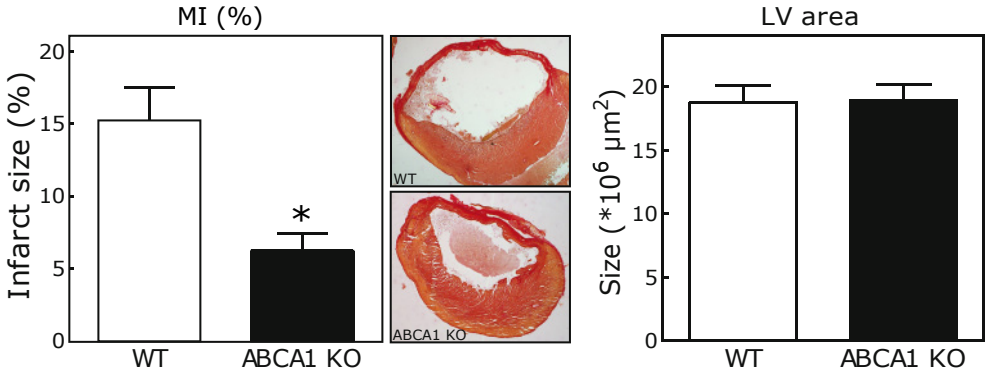

Figure 1. Reduced myocardial infarct size in ABCA1 KO mice after coronary artery ligation in vivo. Two weeks after induction of MI by ligation of the LAD coronary artery, infarct size was determined (left panel). Representative cross sections are shown, stained with Sirius red to visualize the collagen-rich infarcted area (middle panels). Total left ventricle wall area was measured to determine heart size (right panel). Values are means ± SEM (n≥4 per group). *p<0.05.

Higher circulating white blood cell and lymphocyte numbers in ABCA1 KO mice after myocardial infaction in vivo

Before MI induction, no differences in leukocyte subsets were observed between ABCA1 KO and WT mice (data not shown). Two weeks after MI, however, total leukocyte counts were 2.9-fold higher in ABCA1 KO mice (p=0.01; Fig. 2), which was primarily the result of augmented numbers of circulating lymphocytes (4.6-fold; p=0.002). To further investigate the increased lymphocyte population in the circulation of ABCA1 KO mice after MI, blood cells were subjected to FACS analysis (Fig. 3). Before MI, no differences in T-lymphocytes ($CD3^+$, $CD4^+$, and $CD8^+$), B-lymphocytes ($CD19^+$), dendritic cells ($CD11c^+$) or monocytes/macrophages ($F4/80^+$) were observed between both genotypes (Fig. 3A). After MI, however, $CD3^+$ T-lymphocytes increased a striking 4.2-fold (p=0.002; Fig. 3B). $CD4^+$ T-helper lymphocytes (4.6-fold increase; p=0.002) and $CD8^+$ cytotoxic T-lymphocytes (3.7-fold increase; p=0.002) both contributed to this phenomenon. In addition, ABCA1 KO mice displayed a clear 3.0-fold increase (p=0.02) in $CD19^+$ B-lymphocytes after MI. In contrast, monocytes/macrophages ($F4/80^+$) and dendritic cells ($CD11c^+$) did not change between both genotypes upon MI. This indicates that the induction of MI in ABCA1

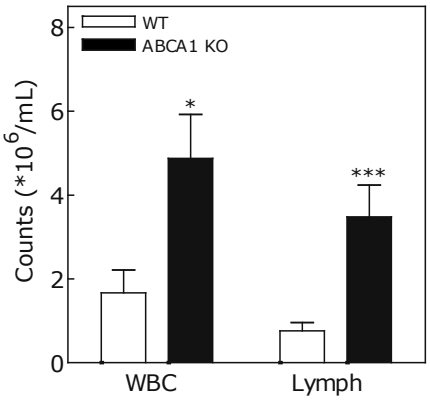

Figure 2. Increased white blood cells and lymphocytes in ABCA1 KO mice after MI induction by coronary artery ligation in vivo. Two weeks after induction of MI, total WBC and lymphocytes in plasma were determined with a hematology analyzer. Values are means ± SEM (n≥4 mice per group). *p<0.05, ***p<0.001.

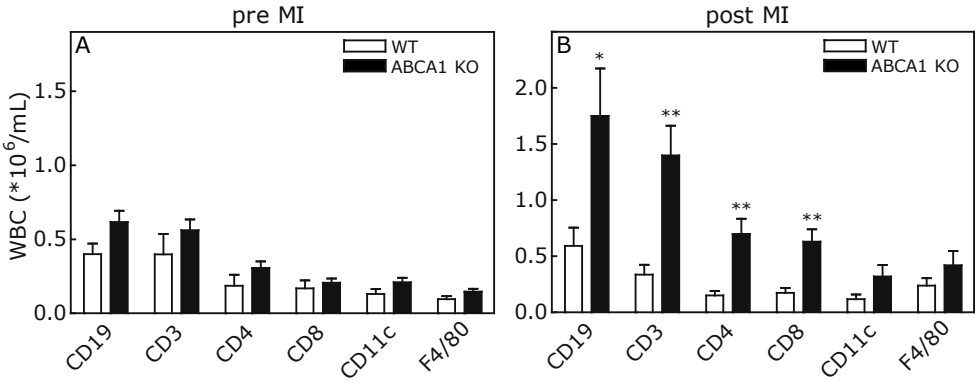

Figure 3. Increased circulating T- and B-cells in ABCA1 KO mice after MI induction by coronary artery ligation in vivo. Isolated WBC were stained for T-cells ($CD3^+$, $CD4^+$, and $CD8^+$), B-cells ($CD19^+$), monocytes/macrophages ($F4/80^+$) and dendritic cells ($CD11c^+$) and analyzed by flow cytometry before (A) and after MI (B). Values are means ± SEM (n≥4 mice per group). *p<0.05, **p<0.01.

KO mice primarily induced common lymphoid progenitor (CLP)-derived cells, such as T- and B-lymphocytes, rather than common myeloid progenitor (CMP)-derived cells including monocytes/macrophages and dendritic cells.

Unaltered myocardial infarction in a langendorff perfusion system in ABCA1-deficient hearts ex vivo

ABCA1 KO mice have been shown to develop cardiomegaly.[23] To determine the direct effects of ABCA1 deficiency on myocyte function during ischemia, MI was induced ex vivo in isolated hearts from ABCA1 KO mice and WT controls using the Langendorff perfusion method. After 35 minutes of no-flow global ischemia followed by 45 minutes of reperfusion, only a tendency towards a 15% decrease (p=0.47; Fig. 4) in infarct size was observed in hearts from ABCA1 KO mice.

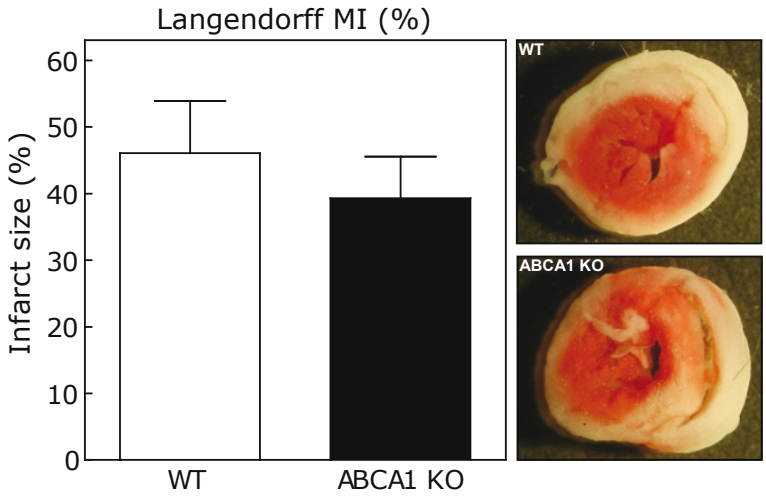

Figure 4. Unaltered infarcted area of isolated hearts from ABCA1 KO mice subjected to ischemia/reperfusion ex vivo. Isolated hearts were stabilized for 20 minutes in a Langendorff perfusion system, followed by 35 minutes of no-flow global ischemia, and 45 minutes of reperfusion. Infarct size was measured with cell^D imaging software. Infarct areas were normalized to total left ventricular areas and averaged for individual hearts (left panel). Values are means ± SEM (n≥5 mice per group). Representative cross sections are shown, stained with TTC to determine viable myocardium (red staining; right panels).

Discussion

In the current study we show for the first time that mice lacking ABCA1, thereby having reduced HDL levels, are protected against cardiac damage after permanent coronary artery ligation. At first sight, this is remarkable as several studies have revealed protective effects of HDL after MI.[24,25] HDL protects against

MI by inhibiting ischemia-induced cardiomyocyte apoptosis and by reducing the recruitment of inflammatory neutrophils into the infarcted area.[24] In addition, intravenous injection of apoA-I before the onset of reperfusion after MI reduced TNF-α and IL-6 expression in the heart, as well as suppressed ICAM-1 expression in the heart, thereby diminishing neutrophil adherence and subsequent reduced myocyte injury.[25] On the other hand, mice lacking the HDL receptor scavenger receptor BI (SR-BI), have high levels of HDL but spontaneously develop MI.[26,27] Deficiency of ABCA1 not only results in the lack of HDL, but also highly attenuates total cholesterol levels.[28] Although cholesterol levels are positively correlated with MI risk in humans,[29] differential results have been obtained in murine MI models.[30–32]

Interestingly, induction of MI in ABCA1 KO mice resulted in a substantial increase in circulating leukocytes due to higher numbers of circulating B- and T-lymphocytes. No differences in leukocyte numbers or subsets were found prior to coronary artery ligation. Therefore, the increased inflammatory environment, caused by the induction of MI, might have attributed to induce this phenotype in ABCA1 KO mice. Proliferation of LSK stem cells and committed CMPs is regulated by cholesterol efflux mechanisms, whereby HDL suppresses proliferation by facilitating cellular cholesterol efflux via the ABC-transporters ABCA1 and ABCG1.[33] However, mice lacking ABCA1 and ABCG1 did not show an increase in CLP cells, that give rise to B-and T-lymphocytes.[33]

Importantly, we have previously shown that deficiency of leukocyte ABCA1 increases circulating lymphocytes upon induction of atherosclerosis,[13] similarly as observed in the current study upon coronary artery ligation. Since the infarct size in ABCA1 KO mice was drastically attenuated, the higher number of circulating lymphocytes might have been mediating tissue repair. This hypothesis is strengthened by the fact that isolated hearts from ABCA1 KO and WT mice did not display differences in infarct size, induced ex vivo using the Langendorff perfusion system. Since these hearts were perfused with a designated buffer instead of whole blood, no secondary ABCA1-mediated effects could interfere.

T-lymphocytes rapidly accumulate in the heart after ischemia/reperfusion injury. Specifically, $CD4^+$ T-lymphocytes were identified as protective mediators of myocardial perfusion injury after MI.[33,34] Recently, Wara et al. showed that LSK-derived CMPs, but not CLPs, can differentiate into proangiogenic cells, thereby promoting neovascularization.[34] Instead, $CD4^+$ lymphocytes modulate the influx of among others monocytes, which is a prerequisite for proper myocardial wound healing.[35,36] Moreover, intramyocardial injection of B-lymphocytes into early post-ischemic myocardium has been shown to preserve cardiac function,[37] emphasizing the protective roles of B- and T-lymphocytes upon MI.

The most pronounced lipid phenotype of ABCA1 deficiency is the near absence of HDL cholesterol in the circulation.[23] It is therefore plausible to suggest that

the absence of HDL, at least in part, promoted the observed secondary effects on lymphocyte numbers upon MI. In agreement, Wilhelm et al.[38] observed increased circulating lymphocytes in Western-type diet fed LDLr KO mice lacking apoA-I. ApoA-I KO mice, like ABCA1 KO mice, have virtually no circulating HDL. To provide definitive proof for the distinct importance of ABCA1 and HDL, MI should be induced in mice lacking apoA-I, which express ABCA1, but have virtually no HDL. In addition, bone marrow transplantation experiments might be of interest to establish the importance of leukocyte-derived ABCA1 with respect to MI.

In conclusion, despite its protective effects regarding the development of atherosclerosis, ABCA1 has adverse effects on cardiac function after MI, probably by attenuating the increase in cardioprotective B- and T-lymphocytes. ABCA1 is considered to be an important therapeutic target to treat atherosclerosis based on its protective effects. However, it is of great importance to further identify the possible adverse effects of ABCA1 upregulation on cardiac recovery after MI.

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Chapter 6

General discussion and considerations

Bart Lammers

Division of Biopharmaceutics, LACDR, Leiden University, the Netherlands

Introduction

Atherosclerosis, the underlying cause of most cardiovascular diseases, is the consequence of lipid deposition in the arterial wall, mainly as a result of high serum cholesterol levels. Currently, the main therapeutic strategy to prevent the progression of atherosclerosis is to reduce serum cholesterol levels by lipid lowering medication, such as statins. Despite impressive progress in the treatment of cardiovascular diseases, atherosclerosis associated clinical events are still a major cause of death in the Western society.[1] This clearly indicates the need for new therapies. Numerous epidemiological studies have established high HDL cholesterol levels to be protective for the development of atherosclerosis. Besides its anti-oxidative and anti-inflammatory functions, HDL plays a crucial role in facilitation of reverse cholesterol transport from macrophages in the arterial wall to the liver and subsequently to the feces.[2]

In this thesis, the bone marrow transplantation technique was used to specifically investigate the importance of macrophage-derived proteins, associated with the removal of lipoproteins and triglycerides, with respect to atherosclerosis. In addition, a myocardial infarction model was used to establish the role of ABCA1 in an acute cardiovascular event.

The role of key reverse cholesterol transport genes in macrophage cholesterol homeostasis and atherosclerotic lesion development

Reverse cholesterol transport is a pathway by which excess cholesterol is transported from macrophages in the arterial wall to the liver for excretion, thereby preventing atherosclerosis.[2,3] The first step in this process involves the efflux of cholesterol from macrophages. This can be facilitated by several macrophage proteins including ABCA1, ABCG1 and apoE.[4,5] As a transmembrane protein, ABCA1 pumps a wide variety of substrates across the cell membrane. It plays an essential role in the maintenance of cellular lipid homeostasis by facilitating the transport of cholesterol and phospholipids towards lipid-poor apoA-I.[6,7] In addition, ABCG1 plays an important role in the efflux of cholesterol from macrophages. ABCA1-mediated cholesterol efflux results in the lipidation of apoA-I, and thus the formation of mature HDL particles, that subsequently act as substrate for ABCG1-mediated transport of cholesterol and phospholipids.[8–10] As a result, combined deletion of macrophage ABCA1 and ABCG1 in mice results in massive foam cell formation in peripheral tissues, coinciding with an attenuated increase in Western-type diet-induced plasma cholesterol levels.[11]

ApoE, secreted by macrophages can also induce cellular cholesterol efflux.[12] Interestingly, apoE enhances the efflux of cholesterol both in the presence and absence of extracellular cholesterol acceptors.[13] In addition, apoE can act as a substrate for ABCA1-mediated cholesterol efflux.[14] Therefore, in this thesis, several studies were performed in order to investigate the (combined) importance of these macrophage-derived cholesterol-related proteins on atherosclerotic lesion development.

In **chapter 3**, LDLr KO mice were transplanted with bone marrow from ABCA1/apoE double KO mice, their respective single knockouts, or WT controls, and challenged with a high-fat/high-cholesterol diet for 6 weeks to induce atherosclerotic lesion development. Macrophage-derived apoE induces efflux of cholesterol, independent of extracellular acceptors.[13] However, no added effect on cholesterol efflux was observed in dKO macrophages compared to ABCA1 deficient macrophages, suggesting that endogenously produced apoE requires ABCA1 to facilitate macrophage cholesterol efflux. In agreement with previous studies,[11,15,16] macrophage ABCA1 deficiency resulted in increased atherosclerotic lesion development, despite a clear reduction in serum cholesterol levels. Single deletion of macrophage apoE led to a comparable increase in atherosclerotic lesion development as single macrophage ABCA1 deletion. Circulating TC levels, however, were over 2-fold higher in apoE KO transplanted animals compared to ABCA1 KO transplanted animals. When adjusted for serum total cholesterol exposure, the pro-atherogenic effects of macrophage ABCA1 deficiency became even more evident. Surprisingly, TC levels in serum were markedly higher upon combined deletion of macrophage ABCA1 and apoE as compared to single

deletion of ABCA1 in macrophages. This thus leads to ABCA1 deficiency – a defect in cholesterol efflux – combined with elevated serum total cholesterol levels. Interestingly, hepatic LPL expression was attenuated in dKO transplanted mice. Since LPL is essential for the lipolysis of TG in lipoproteins, LPL facilitates the clearance of VLDL and chylomicrons.[17] In addition, LPL can also promote LDL[18] and VLDL remnant uptake,[19] independent of apoE-recognizing receptors. The attenuated LPL expression might thus have contributed to the observed increase in serum TC levels compared to ABCA1 KO transplanted animals.

Importantly, ABCA1/apoE dKO transplanted mice exhibited a large induction of the pro-inflammatory cytokines IL-6 and TNFα in the liver. In addition, IL-6 levels in the circulation were highly increased in these animals, indicating an enhanced inflammatory status. All together, combined deletion of macrophage ABCA1 and apoE resulted in a defect in cholesterol efflux and, compared to ABCA1 KO transplanted mice, elevated serum total cholesterol levels, and enhanced systemic and hepatic inflammation, together resulting in the observed augmented atherosclerotic lesion development. Despite the beneficial function of ABCG1 in cellular cholesterol efflux, differential results have been published on the role of macrophage ABCG1 in atherosclerosis.[20–22] In agreement with its protective role in cellular cholesterol efflux, macrophage ABCG1 has been reported to play an anti-atherogenic role.[21,23] In contrast, studies showing a pro-atherogenic role for macrophage ABCG1 suggested that ABCG1 KO macrophages are either more prone to undergo apoptosis,[20] or exhibit increased ABCA1 expression and apoE secretion.[22] The effect of combined deficiency of macrophage ABCG1 and apoE on atherosclerotic lesion development is described in **chapter 4**.

Deletion of macrophage ABCG1 or apoE resulted in a comparable effect on cholesterol efflux towards HDL, whereas a larger attenuation of cholesterol efflux to HDL was observed in dKO macrophages. Two-way ANOVA analysis indicated that the effects of macrophage ABCG1 and apoE on HDL-mediated efflux were independent of each other. Similar results were obtained with respect to atherosclerotic lesion development in these mice. Single ABCG1 or apoE KO transplanted mice showed a 1.4-fold increase in atherosclerotic lesion development as compared to WT transplanted controls, whereas their dKO transplanted littermates revealed a 1.9-fold increase. Furthermore, a clear significant inverse relation between HDL-mediated efflux and the size of atherosclerotic plaques was apparent. The independent effects of both genes on atherosclerotic lesion development was further strengthened by the fact that no compensatory upregulations of apoE or ABCG1 protein expression were observed in bone marrow-derived macrophages from single ABCG1 KO and apoE KO transplanted animals, respectively. The specific role of ABCG1 in atherosclerosis was further defined in **chapter 5**. Total body ABCG1/LDLr dKO mice were used to assess the effect of ABCG1 deficiency on different stages of atherosclerotic

lesion development. In this study, ABCG1 was shown to be able to induce, as well as attenuate atherosclerotic lesion development. Feeding ABCG1/LDLr dKO mice a Western-type diet for 10 weeks resulted in a significant increase in early atherosclerotic lesion size. However, a significant reduction in more advanced lesions was observed after 12 weeks of Western-type diet feeding, indicating that the effect of ABCG1 deficiency on atherosclerotic lesion development in LDLr KO mice depends on the stage of atherogenesis.

In order to validate this observation, all studies on the role of ABCG1 in atherosclerotic lesion development were compared using correlation analysis, including data from eight published studies, the current study, and one unpublished study of our group. The correlation analysis revealed that the fold increase/decrease in atherosclerotic lesion size of ABCG1 KO mice compared to ABCG1 WT mice is highly correlated (R=0.92) with the atherosclerotic lesion size. Based on these data, ABCG1 is primarily protective in early atherosclerotic lesions as its deficiency causes an impaired cholesterol efflux to HDL, resulting in increased atherosclerotic lesion development. In more advanced atherosclerotic lesions, however, the role of ABCG1 in atherogenesis switches from anti- to pro-atherosclerotic, since the persistent impaired cholesterol efflux from ABCG1-deficient macrophages is likely to induce accumulation of (oxy)sterols. This leads to enhanced apoptosis and/or other compensatory mechanisms, and subsequently decreased atherosclerotic lesion size. A previous study on the role of ABCG1 in atherosclerosis suggested a correlation between serum cholesterol levels and the fold increase/decrease in atherosclerotic lesion size compared to ABCG1 WT mice, whereby at about 900 mg/dL serum cholesterol a switch from the protective function of ABCG1 to lesion formation was noticed.[21] Upon inclusion of recent studies, as well as the studies described in this thesis, again a high correlation between the fold increase/decrease in atherosclerotic lesion size and total serum cholesterol is observed.

Therefore, under normal physiological levels of cholesterol, the role of ABCG1 in atherogenesis is likely to be protective. Furthermore, since higher serum cholesterol levels are associated with a more rapid development of atherosclerotic lesions, this correlation is probably also a direct effect of the stage of atherosclerotic lesion development.

Role of macrophage ATGL in atherosclerosis

Triglyceride stores are the most important energy reserves in vertebrates. Dysfunctional lipolysis affects energy homeostasis and may contribute to the pathogenesis of diseases like obesity and insulin resistance. Adipose triglyceride lipase (ATGL) is mainly expressed in adipose tissue.[24] However, ATGL is also expressed at high levels in macrophages and foam cells in atherosclerotic lesions,[25] raising the possibility that it might impact atherosclerosis.

In **chapter 6**, the specific role of ATGL in macrophages, as well as the consequences of macrophage TG accumulation for atherogenesis was examined. Hereto, LDLr KO mice were transplanted with bone marrow from ATGL KO mice or WT littermates.

Although lack of ATGL in bone marrow-derived cells had no significant influence on serum lipid levels, distinct TG loading was observed in BMDM of ATGL KO transplanted mice. Surprisingly, despite increased macrophage TG concentrations, atherosclerotic lesion formation in the aortic root was highly attenuated in ATGL KO transplanted mice. The observed reduction in atherosclerotic lesion formation coincided with increased apoptosis, which might limit lesion cellularity and the progression of early lesions in these animals.[26,27] Depending on the stage of lesion development, increased apoptosis can either enhance,[28] or suppress[29,30] atherosclerotic lesion development. In agreement, ATGL deficiency in macrophages was recently shown to result in enhanced apoptosis involving mitochondrial dysfunction and activation of the mitochondrial apoptosis pathway.[31] Leukocytes play an essential role in all stages of atherosclerotic lesion development,[32–34] whereby the amount of predominantly neutrophils and monocytes are positively correlated with atherosclerotic lesion development.[35,36] Importantly, we found highly decreased numbers of circulating leukocytes in Western-type diet fed ATGL KO transplanted LDLr KO mice. Remarkably, the LSK stem cell population in bone marrow from ATGL KO transplanted mice was decreased compared to WT controls. LSK cells are the most primitive hematopoietic stem cells, which can give rise to all mature cell types found in the circulation. The attenuated LSK population might therefore have resulted in a reduction of leukocyte-producing progenitor cells. In addition, plasma MCP-1 levels were drastically reduced in ATGL KO transplanted animals. Since MCP-1 is an important chemoattractant for mononuclear cells,[37] the observed reduction most likely attributed to the observed attenuation in circulating leukocytes.

Altogether, the absence of ATGL in macrophages resulted in reduced susceptibility to diet-induced atherosclerosis in LDLr KO mice due to decreased infiltration of less inflammatory macrophages. Based on these data, macrophage ATGL plays a significant role in atherogenesis independent of its expression in other tissues. A high intracellular TG content in macrophages is therefore, at least under these conditions, protective against lesion development, making macrophage ATGL a putative novel target to attenuate early atherogenesis when the removal of apoptotic cells is still functional.

Further understanding of the role of ABCA1 in cardiovascular disease

Deficiency of leukocyte ABCA1 on the LDLr KO background leads to increased atherosclerosis, despite largely attenuated cholesterol levels.[38] Interestingly,

these mice also showed elevated leukocyte counts in the circulation,[39] and accumulation of macrophages in the peritoneal cavity, liver, and spleen.[38] This indicates that leukocyte ABCA1, in addition to its role in cholesterol efflux, exerts regulatory functions in the recruitment of inflammatory cells to the periphery. The spleen is the largest lymphoid organ in the body, producing antibodies, facilitating phagocytosis and being capable of eliminating foreign antigens.[40,41] Moreover, it functions as an important monocyte reservoir.[42] In order to investigate the possible interplay between the spleen and leukocyte ABCA1 with respect to the development of atherosclerosis, in **chapter 7**, ABCA1 KO bone marrow was transplanted into LDLr deficient recipient mice, which were subsequently either splenectomized or underwent a sham operation. Leukocyte ABCA1 KO mice fed WTD showed highly induced concentrations of MCP-1 and KC (murine IL-8) in the serum. IL-8 is one of the most potent chemoattractants for neutrophils.[43,44] Accordingly, leukocyte ABCA1 KO mice exhibited increased neutrophil presence in the spleen. Upon splenectomy, ABCA1 KO transplanted animals exhibited blood neutrophilia as compared to WT splenectomized controls. Surprisingly, leukocyte ABCA1 deficieny or splenectomy alone did not alter circulating neutrophil concentrations.

The spleen is also associated with systemic immune responses in which it is the principal organ responding to antigens such as oxLDL. In addition, the titers of anti-oxLDL antibodies in serum have been suggested to play an anti-atherogenic role.[45] However, no differences in anti-oxLDL antibody serum titers were observed upon splenectomy. Moreover, whereas leukocyte ABCA1 KO mice displayed attenuated serum TC levels, TC concentrations remained unaffected after splenectomy. In contrast, splenectomized mice did show enhanced atherosclerotic lesion development as compared to sham operated controls. Moreover, splenectomized ABCA1 KO transplanted animals exhibited an additional increased lesion development. These results evidently show that leukocyte ABCA1 deficiency resulted in decreased TC levels, increased inflammation, and lipid and neutrophil accumulation in the spleen. However, the observed splenic alterations induced by leukocyte ABCA1 deficiency did not alter anti-oxLDL antibody levels, nor played a significant role in atherosclerotic lesion development as evidenced by splenectomy.

The importance of ABCA1 in cellular cholesterol transport became clear when mutations in the ABCA1 gene were discovered to cause Tangier disease; a rare inherited disorder characterized by a severe reduction in HDL.[46] HDL has been proposed to exert multiple anti-atherogenic and anti-inflammatory functions beyond cellular cholesterol efflux, including the modulation of lymphocyte activity,[47] endothelial function,[48] and anti-oxidative mechanisms.[48] Interestingly, low plasma HDL is associated with a long-term unfavorable prognosis in patients who have recovered from a MI, suggesting detrimental effects of low plasma HDL

on post ischemic myocardial function. To investigate the effects of low plasma HDL after MI, in **chapter 8**, ABCA1 KO mice were subjected to MI by ligation of the left anterior descending coronary artery. To our surprise, ABCA1 deficiency attenuated cardiac ischemia after a myocardial infarction. Thus, although ABCA1 has an important protective role in atherosclerosis, it has detrimental effects on cardiac function after MI. The presence of ABCA1 in isolated hearts did not contribute to the observed effects, indicating that the smaller myocardial infarction area was caused by secondary, ABCA1-mediated effects. Although MI did not affect leukocyte numbers in control mice, ABCA1 KO mice displayed an induction of circulating leukocytes after MI. Most leukocyte subtypes are generally associated with increased inflammation and subsequent cardiovascular disease. B- and T-lymphocytes, however, exhibit protective effects upon acute cardiovascular events,[49–51] thereby attenuating adverse effects on cardiac function after MI.
ABCA1 is considered to be an important therapeutic target to treat atherosclerosis based on its protective effects. However, it is of great importance to further identify the possible adverse effects of ABCA1 upregulation on cardiac recovery after MI.

Considerations

Cardiovascular disease in humans develops over decades, and clinical manifestations often only occur as a result of ruptured, advanced atherosclerotic plaques. Although the use of animal experiments enables us to induce and investigate atherosclerotic lesion development in for example mice and rats, it has proven to be challenging to induce ruptures in established atherosclerotic lesions in these animal models.
The lack of these so-called "plaque rupture animal models" makes it difficult to extrapolate findings to the human situation. On the other hand, the possibility of controlled induction of atherosclerotic lesion formation in (genetically engineered) mice has resulted in a powerful tool to identify key proteins and mechanisms behind the onset and progression of atherosclerosis. Over the last decades, these efforts have resulted in an extensive amount of data, which have increased our insight in the mechanism of atherosclerotic lesion development, and more importantly also provided numerous promising targets for therapeutic intervention.
One of the earliest success stories resulting from basic cardiovascular research is the development of statins, which inhibit HMG-CoA reductase, the first step in cholesterol synthesis.[52] By their action on the liver, statins lower circulating cholesterol levels and thereby reduce the risk of clinical events from cardiovascular disease. More recently, CETP was believed to be a new promising target. Inhibiting the action of CETP in genetically engineered CETP-expressing mouse models (mice naturally do not express CETP) resulted in highly favorable

changes in lipid profile, including increased HDL levels and decreased VLDL/LDL levels. The subsequent phase III clinical trial, however, was prematurely stopped because patients treated with the CETP inhibitor torcetrapib had a 25% increased chance to suffer from major cardiovascular events and a 40% increased chance to die from cardiovascular causes as compared to patients treated with placebo.[53] Despite the favorable changes in lipid profile, torcetrapib also caused severe off-target effects. In addition, very recently, a phase III clinical trial with another CETP inhibitor, dalcetrapib, was stopped due to a lack of clinically meaningful efficacy.[54] These results indicate that the current CETP inhibitors do not result in additional lowering of cardiovascular risk on top of statins. Importantly, it also demonstrates that the pathways of the underlying basic research have to be studied extensively and interpreted with great care.

The research described in this thesis mainly focuses on the role of important lipid-related genes on macrophages. Macrophages play a crucial role in the onset and progression of atherosclerotic lesions in the arterial wall by engulfing pro-atherogenic lipoproteins. The subsequent transformation of macrophages into lipid-rich foam cells - considered the initial step in the development of atherosclerosis - underlines the importance of macrophage lipid homeostasis.

Macrophage reverse cholesterol transport is a pathway by which excess cholesterol from peripheral tissues, including macrophages, is transported back to the liver for excretion, and thus of potential interest for the prevention of atherosclerosis.[2,3] Induction of cholesterol efflux from macrophage foam cells in atherosclerotic lesions, the first step in reverse cholesterol transport, is therefore considered an attractive approach to prevent lesion development or even evoke regression of atherosclerotic lesions. ABCA1, ABCG1 and apoE play pivotal roles in macrophage reverse cholesterol transport.[5,55] Interestingly, expression of these proteins can be enhanced upon pharmacological LXR activation.[56] Of note, systemic application of LXR agonists also induces off-target effects in liver, including increased lipogenesis and production of TG.[57] However, specific targeting of LXR agonists to macrophages inside the atherosclerotic lesion might be a potential new therapeutic strategy for inhibiting atherosclerotic lesion progression of induction of regression of established lesions.

Another, somewhat underrated part of macrophage lipid homeostasis is the storage and metabolism of TG. The rate limiting enzyme in this process is ATGL, responsible for the catabolism of TG into DG and FFA. Disruption of ATGL in mice results in premature death.[58] However, mice lacking ATGL in all tissues except cardiac muscle have a normal life expectancy,[59] indicating that TG accumulation specifically in cardiac muscle has detrimental effects. Surprisingly, accumulation of TG in macrophages causes apoptosis and attenuates atherosclerotic lesion development in leukocyte ATGL KO mice. In early atherosclerotic lesions, macrophage apoptosis results in attenuated progression or even regression of

the lesion.[29,60] On the other hand, apoptosis of macrophages in advanced lesions can lead to increased inflammation and vulnerable atherosclerotic lesions.[61,62] In addition, differential stages of atherosclerotic lesion are present throughout the arterial tree.[63] It is therefore difficult to regard macrophage apoptosis as a possible target against atherosclerosis.

In conclusion, modulating macrophage lipid homeostasis may provide an attractive possibility for future drug design in the field of atherosclerosis. However, due to the complexity of the disease and difficulties concerning the extrapolation of advances in basic cardiovascular research, further investigations are necessary before these potential new drug candidates can be applied in clinical research.

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Nederlandse samenvatting

Bart Lammers

Division of Biopharmaceutics, LACDR, Leiden University, the Netherlands

Introductie

Hart- en vaatziekten zijn de meest voorkomende doodsoorzaken in de huidige westerse maatschappij. Een belangrijk kenmerk bij de ontwikkeling van hart- en vaatziekten is de vorming van vernauwingen in de bloedvaten. Deze vernauwingen zijn het gevolg van een chronische ontsteking in het bloedvat wat resulteert in plaatselijke ophoping van vetten. Dit proces staat bekend als slagaderverkalking, ook wel atherosclerose genoemd.

Atherosclerose ontwikkelt zich al tijdens de adolescentie en manifesteert zich door de afzetting van cholesterol in de vaatwand: de zogenoemde "fatty streak". Dit leidt echter niet tot klinische symptomen, omdat deze fatty streaks de bloedtoevoer niet geheel blokkeren. In de loop der jaren kunnen de fatty streaks veranderen in bloedvatvernauwende atherosclerotische laesies. Of en wanneer dit leidt tot klinische symptomen is in grote mate afhankelijk van de blootstelling aan risicofactoren. Hierbij kan onderscheid worden gemaakt tussen erfelijke factoren, zoals een hoge bloeddruk, hoog cholesterol en diabetes, en tussen risicofactoren ten gevolge van een ongezonde levensstijl, zoals een vetrijk en ongevarieerd dieet, roken, overgewicht en te weinig lichaamsbeweging.

De voornaamste behandelmethoden richten zich op het verminderen en verlagen van de risicofactoren. Veel voorgeschreven medicijnen zijn statines, die niet alleen in staat zijn om het cholesterol niveau in het bloed te verlagen, maar ook een ontstekingsremmende werking hebben. In geval van acute symptomen wordt veel gebruik gemaakt van chirurgische technieken zoals een bypass operatie of het plaatsen van stents, om zo de bloeddoorstroming te herstellen. Ondanks deze behandelingen blijft het sterftecijfer dat gerelateerd is aan hart- en vaatziekten erg hoog. Het ontwikkelen van nieuwe behandelmethoden is daarom noodzakelijk.

Een hoog cholesterol gehalte in het bloed wordt, zoals eerder vermeld, gezien als een risicofactor voor atherosclerose. Desondanks is cholesterol één van de belangrijkste vetten in het menselijk lichaam, en zorgt het onder andere voor de elasticiteit van de huid. Daarnaast kan er onderscheid worden gemaakt tussen het zogenaamde "goede" en "slechte" cholesterol. Cholesterol is niet oplosbaar in bloed en wordt daarom door het lichaam getransporteerd in lipoproteïnen. Op basis van grootte, samenstelling en dichtheid kunnen ze in vier verschillende categorieën worden ingedeeld: chylomicronen, zeer lage dichtheids lipoproteïnen (VLDL), lage dichtheids lipoproteïnen (LDL), en hoge dichtheids lipoproteïnen (HDL). Terwijl het "slechte" LDL cholesterol een belangrijke risicofactor is voor het ontstaan van hart- en vaatziekten, speelt het "goede" HDL cholesterol juist een beschermende rol in de ontwikkeling van atherosclerose. Tezamen zijn deze lipoproteïnen verantwoordelijk voor zowel het cholesterol transport naar de ontvangende cellen en organen, als ook het transport van overtollig cholesterol naar de lever, vanwaar het wordt hergebruikt of uitgescheiden via de darm in de ontlasting. Indien het cholesterolgehalte in het lichaam stijgt, door bijvoorbeeld een defect in het verwijderingsmechanisme of een vetrijk dieet, kunnen ook macrofagen – de opruimcellen van het lichaam – de cholesterolrijke lipoproteïnen opnemen. Overmatige opname transformeert deze macrofagen vervolgens in zogenaamde "foamcellen". De aanwezigheid van foamcellen in de slagader markeren het ontstaan van een atherosclerotische laesie.

Omdat macrofagen een cruciale rol spelen in de ontwikkeling van atherosclerose, is het belangrijk om te bepalen welke eiwitten in of op deze macrofagen verantwoordelijk zijn voor de opname en afgifte van (overtollig) cholesterol. Macrofagen worden geproduceerd uit stamcellen in het beenmerg. Door gebruik te maken van beenmergtransplantaties is het mogelijk om de aanwezige macrofagen te vervangen door macrofagen met een andere genetische samenstelling.

De beenmergtransplantatie bestaat uit verschillende stappen: 1) isolatie van beenmerg uit de donor, dat bijvoorbeeld een bepaald eiwit mist; 2) uitschakeling van het eigen beenmerg in de ontvanger door middel van een hoge dosis straling; 3) transplantatie van het donor beenmerg naar de ontvanger. Na deze handelingen zal het beenmerg – en dus ook de macrofagen - van de ontvanger vervangen worden door beenmerg van de donor. Op deze wijze is het dus mogelijk om het effect van de afwezigheid van een bepaald macrofaag eiwit op de ontwikkeling van hart- en vaatziekten te onderzoeken.

In tegenstelling tot mensen zijn muizen relatief ongevoelig voor de ontwikkeling van hart- en vaatziekten, onder andere omdat ze veel meer cholesterol in het goede HDL vervoeren. Met behulp van genetische modificatie zijn muizen gegenereerd die wel gevoelig zijn voor de ontwikkeling van atherosclerose. Deze muizen missen de receptor voor LDL (LDL receptor), wat zorgt voor een toename

van het LDL cholesterol gehalte in het bloed. LDL receptor-deficiënte muizen vormen een goed model om de ontwikkeling van atherosclerose te bestuderen.
In dit proefschrift staat onderzoek beschreven waarin de rol van verschillende macrofaag-eiwitten is bestudeerd met behulp van beenmergtransplantatie in atherosclerose gevoelige muizen. Daarnaast zijn experimenten beschreven over de rol van bepaalde macrofaag-eiwitten bij een acute vorm van hart- en vaatziekten zoals een hartaanval.

Evenals vele andere celtypen bezitten macrofagen eiwitten die verantwoordelijk zijn voor de opname van lipoproteïnen. Dit kan leiden tot stapeling van grote hoeveelheden cholesterol in deze cellen. Omdat macrofagen de opname van cholesterol niet kunnen beperken is de verwijdering van cholesterol uit macrofagen een belangrijk proces om de vorming van foamcellen en dus de ontwikkeling van atherosclerose tegen te gaan. Verwijdering van cholesterol uit macrofagen is voor een groot deel afhankelijk van zogenaamde ABC (ATP-binding cassette) transporters. Deze eiwitten zijn betrokken bij het transport van een groot aantal substraten – waaronder ook vetten (cholesterol) – in en uit de cel. Eerdere studies hebben aangetoond dat ABCA1 een belangrijke rol speelt bij de verwijdering van cholesterol uit macrofagen. De hoeveelheid ABCA1 in macrofagen neemt toe in cholesterolrijke macrofagen, waarmee het overmatige cholesterol uit de macrofaag kan worden getransporteerd.
Een andere belangrijke groep eiwitten zijn de zogenaamde apolipoproteïnen. Deze eiwitten bevinden zich onder andere op macrofagen en lipoproteïnen en dienen voornamelijk als herkenningseenheden voor receptoren op cellen. Met behulp van apolipoproteïnen zijn lipoproteïnen als LDL en HDL in staat cholesterol op te nemen (influx) of af te geven (efflux). Apolipoproteïne E (apoE) is essentieel voor de verwijdering van lipoproteïnen uit het bloed via de lever. Daarnaast speelt apoE een belangrijke rol bij de verwijdering van cholesterol uit macrofagen, en mogelijk stimuleert het ook de ABCA1 afhankelijke cholesterol effux. In **hoofdstuk 3** van dit proefschrift is de potentiële interactie tussen ABCA1 en apoE in macrofagen bestudeerd met betrekking tot de ontwikkeling van atherosclerose. Met behulp van de beenmerg transplantatie techniek is beenmerg van muizen – deficiënt voor ABCA1, apoE, of beide – getransplanteerd naar LDL receptor deficiënte muizen. In overeenstemming met eerdere studies bleek dat deficiëntie van ABCA1 of apoE in macrofagen zorgde voor een verhoging van de atherosclerotische laesie formatie, wat suggereert dat beide genen een beschermende functie hebben met betrekking tot atherosclerose. LDL receptor deficiënte muizen, getransplanteerd met beenmerg wat deficiënt is voor zowel ABCA1 als apoE, ontwikkelden nog grotere atherosclerotische laesies. Daarnaast vertoonden deze muizen ook verhoogde cholesterol concentraties in het bloed en meer ontstekingsmarkers in het bloed (IL-6) en in de lever (IL-6 en TNFα). In vitro experimenten bewezen

echter dat de capaciteit van ABCA1/apoE dubbel deficiënte macrofagen om cholesterol te effluxen naar onder andere het "goede" HDL niet verschilde van ABCA1 deficiënte macrofagen. Dit suggereert dat deficiëntie van ABCA1 en apoE in macrofagen via verschillende mechanismen een beschermende werking hebben met betrekking tot atherosclerose, en dat ze gezamenlijk ontstekingsremmend werken.

Naast ABCA1 speelt ook een andere ABC transporter, ABCG1, een belangrijke rol in de verwijdering van cholesterol uit macrofagen. Eerdere studies hebben aangetoond dat ABCA1 en ABCG1 in macrofagen een cruciale gezamenlijke beschermende rol spelen in de verwijdering van cholesterol uit macrofagen. Daarnaast is ook een gecombineerde rol voor ABCG1 en apoE in macrofagen met betrekking tot cholesterol efflux en atherosclerose verondersteld. In **hoofdstuk 4** is beenmerg van muizen – deficiënt voor ABCG1, apoE, of beide – getransplanteerd naar LDL receptor deficiënte muizen. Deficiëntie van ABCG1 of apoE in macrofagen resulteerde in de formatie van meer atherosclerotische laesies. Daarnaast bleek dat LDL receptor deficiënte muizen, getransplanteerd met beenmerg wat deficiënt is voor zowel ABCG1 als apoE, nog grotere atherosclerotische laesies ontwikkelden. Omdat zowel in de gecombineerde ABCG1/apoE als in de enkelvoudige deficiënte muizen geen regulatie van compensatoire mechanismen of genen geobserveerd werd, kan uit deze studie geconcludeerd worden dat ABCG1 en apoE in macrofagen geheel onafhankelijk van elkaar een beschermende rol spelen in de ontwikkeling van atherosclerose.

Aangezien de precieze rol van ABCG1 (in macrofagen) met betrekking tot atherosclerose nog ter discussie staat, is in **hoofdstuk 5** onderzocht wat de gevolgen zijn van ABCG1 deficiëntie tijdens verschillende stadia van atherosclerotische laesie formatie. Het effect van ABCG1 deficiëntie blijkt afhankelijk van het stadium van atherosclerose ontwikkeling: in vroege stadia van atherosclerose werkt ABCG1 beschermend, waarschijnlijk door een verminderde capaciteit van de macrofagen om het cholesterol uit te scheiden. In latere stadia daarentegen stimuleert ABCG1 de ontwikkeling van atherosclerose, waarschijnlijk als resultaat van verhoogde apoptose (celdood) van deze macrofagen of andere compenserende mechanismen. Deze studie toont aan dat ABCG1 een belangrijke rol speelt in de ontwikkeling van atherosclerose.

Naast de opname en het transport van vetten speelt ook de afbraak een belangrijke rol ter voorkoming van een verstoorde balans in het lichaam. ATGL (adipose triglyceride lipase) is een cruciaal eiwit dat verantwoordelijk is voor de afbraak van opgeslagen vetten naar vetzuren die belangrijk zijn voor de energievoorziening van de spieren. Zonder ATGL is afbraak van triglyceriden onmogelijk, ontstaat er overmatige vetstapeling in het lichaam en is er verminderde spierarbeid. Muizen die dit eiwit missen sterven dan ook vroegtijdig aan complicaties. Omdat ATGL ook in hoge mate wordt geproduceerd

door macrofagen, is in **hoofdstuk 6** het effect van ATGL in macrofagen met betrekking tot de ontwikkeling van atherosclerose bestudeerd. Verrassenderwijs leidde de afwezigheid van ATGL in macrofagen tot een vermindering van de atherosclerotische laesie formatie. Dit effect bleek samen te vallen met een verhoogde apoptose in de laesie en een verminderde concentratie witte bloedcellen (leukocyten) in het bloed. Aanvullend onderzoek liet zien dat een verlaagde hoeveelheid witte bloedcel producerende stamcellen (LSK cellen) hieraan ten grondslag lag. Een verhoging van de hoeveelheid leukocyten in het bloed is meestal een indicatie voor een ontstekings- of afweerreactie. Minder leukocyten zou dus duiden op een verminderde ontstekingsreactie. Dit zou, in combinatie met de waargenomen apoptose in de laesie kunnen verklaren waarom ATGL deficiëntie in macrofagen resulteert in een verlaagde atherosclerotische laesie formatie. Ondanks de negatieve gevolgen van ATGL deficiëntie voor belangrijke lichaamsfuncties, kan ATGL in macrofagen als belangrijke target ter preventie van atherosclerose gezien worden.

In het tweede deel van dit proefschrift wordt de belangrijke rol van ABCA1 bestudeerd. Naast de cruciale rol in atherosclerose, heeft macrofaag ABCA1 ook effecten op de milt. De milt speelt een belangrijke rol in de productie van bepaalde witte bloedcellen (monocyten) en de afbraak van rode bloedcellen. Daarnaast is de milt onderdeel van het lymfesysteem, en resulteert de verwijdering van de milt (splenectomie) in een verhoging van atherosclerose. Macrofaag ABCA1 deficiëntie in muizen resulteert in cholesterol stapeling en een verhoging van het aantal neutrofielen in de milt. In **hoofdstuk 7** is daarom gekeken naar de rol van macrofaag ABCA1 in atherosclerose in de aan- of afwezigheid van de milt. Zoals verwacht resulteerde splenectomie in een verhoging van atherosclerose. Daarnaast bevatte de milt meer neutrofielen in de afwezigheid van macrofaag ABCA1 in vergelijking met controle dieren. Verrassenderwijs speelde de absentie van macrofaag ABCA1 geen rol in de ontwikkeling van atherosclerose in afwezigheid van de milt. Hieruit valt te concluderen dat de veranderingen in de milt, veroorzaakt door macrofaag ABCA1 deficiëntie, geen belangrijke rol spelen bij de beschermende effecten van macrofaag ABCA1 met betrekking tot de ontwikkeling van atherosclerose.
Vergevorderde atherosclerose leidt in veel gevallen tot levensbedreigende complicaties. Omdat (macrofaag) ABCA1 een belangrijke beschermende werking heeft met betrekking tot atherosclerose, is in **hoofdstuk 8** de rol van ABCA1 in een acuut hartinfarct model bestudeerd. Ondanks de beschermende rol van ABCA1 bij de ontwikkeling van atherosclerose waren de gevolgen twee weken na een hartinfarct veel milder in muizen die ABCA1 deficiënt waren, dan in controle dieren. Echter, additionele resultaten wezen uit dat de specifieke aanwezigheid van ABCA1 in het hart geen effect had op de gevolgen van een hartinfarct. Dit

suggereert dat de waargenomen resultaten veroorzaakt zijn door secundaire, ABCA1-gemedieerde effecten op andere plaatsen in het lichaam. Terwijl geen zichtbare veranderingen optraden in de witte bloedcel aantallen in de controle dieren, was er sprake van een opmerkelijke verhoging van het aantal witte bloedcellen in ABCA1 deficiënte muizen na het infarct. Hoewel de meeste witte bloedcel subtypen geassocieerd worden met verhoogde inflammatie en kans op atherosclerose, zouden deze in een acute situatie een meer beschermende rol kunnen spelen.

De afgelopen decennia is veel vooruitgang geboekt betreffende inzichten, operatieve ingrepen, en medicijngebruik bij hart- en vaatziekten. Desondanks krijgen nog steeds veel mensen te maken met de gevolgen van bijvoorbeeld trombose of zelfs een hartinfarct. Het is daarom van belang de zoektocht naar nieuwe targets voor medicijnen voort te zetten.
De in dit proefschrift beschreven studies ontvouwen nieuwe inzichten aangaande cholesterol- en vet gereguleerde eiwitten in macrofagen, die als potentieel interessante targets gezien kunnen worden om hart- en vaatziekten tegen te gaan. Waar huidige medicijnen zich vooral richten op het verlagen van het cholesterolniveau in het lichaam, lijken macrofagen een veelbelovend potentieel doelwit voor de ontwikkeling van nieuwe therapieën en medicijnen. Dit zal in de toekomst hopelijk leiden tot de genezing en zelfs voorkoming van hart- en vaatziekten.

Curriculum Vitae

Bart Lammers

Division of Biopharmaceutics, LACDR, Leiden University, the Netherlands

Bart Lammers werd geboren op 21 december 1983 te Hengelo. In mei 2003 behaalde hij zijn HAVO diploma aan het Twickel College te Hengelo. In datzelfde jaar begon hij met de studie Biotechnologie aan de hogeschool Van Hall Larenstein te Leeuwarden, waar hij in juni 2004 het propedeutisch examen behaalde. Als afstudeerrichting werd medische biotechnologie gekozen. De hoofdstage werd gelopen bij de vakgroep Biofarmacie van het Leiden/Amsterdam Center for Drug Research (LACDR) aan de Universiteit Leiden, onder begeleiding van Ing. R.B. Hildebrand en dr. M. van Eck. Deze stage werd afgesloten met een verslaglegging getiteld: "Combined deletion of macrophage ABCG1 and apoE in LDL receptor knockout mice results in increased atherosclerosis compared to single macrophage ABCG1 or apoE deletion". Het ingenieursdiploma werd behaald in augustus 2007, waarna hij op dezelfde afdeling als analist werd aangesteld op het project getiteld: "Role of the key mediators of HDL metabolism ABCA1 and SR-BI in the prevention of thrombosis". Van september 2008 tot september 2012 was hij als promovendus werkzaam bij de vakgroep Biofarmacie van het LACDR aan de Universiteit Leiden. Het onderzoek, beschreven in dit proefschrift, werd verricht onder begeleiding van Dr. M. van Eck en Prof. dr. Th.J.C. van Berkel, en werd gefinancierd door de Nederlandse Hartstichting. Tijdens zijn periode als promovendus ontving hij in mei 2010 een travel grant van de "6th IAS-sponsored workshop on HDL". In maart 2011 ontving hij een prijs voor de beste poster presentatie tijdens de "1^{e} Cardiovasculaire Conferentie". Daarnaast ontving hij in maart 2012 een "Early Career Poster Award" tijdens de "XVI International Symposium on Atherosclerosis". Om naast wetenschappelijke inzichten ook een flinke dosis levenservaring op te doen en de wereld te ontdekken, is hij na zijn promotie voor onbepaalde tijd op reis gegaan.

Awards

Poster Award - FIGON Medicines Days	(2007, Lunteren)
Travel Grant - 6th IAS-sponsored workshop on HDL	(2010, Newport, RI, USA)
Poster Award - 1st Cardiovascular Conference	(2011, Noordwijkerhout)
Early Career Poster Award - ISA	(2012, Sydney, AUS)

Oral presentations

FIGON Medicines Days	(2008, Lunteren)
XV International Symposium on Atherosclerosis	(2009, Boston, MA, USA)
Dutch Lipoprotein Club (NLC)	(2009, Leiden)
Scandinavian Society for Atherosclerosis Research	(2011, Humlebæck, DK)
LACDR Spring Symposium	(2011, Amsterdam)
XVI International Symposium on Atherosclerosis	(2012, Sydney, AUS)

Poster presentations

11th Dutch Atherosclerosis Society Meeting	(2008, Ermelo)
Leiden Vascular Medicine Meeting	(2008, Leiden)
LACDR Spring Symposium	(2009, Amsterdam)
HDL Satellite Symposium of XV ISA	(2009, Newport, RI, USA)
Dutch Heart Foundation PhD Meeting	(2009, Papendal)
LACDR Spring Symposium	(2010, Amsterdam)
6th IAS-Sponsored Workshop on HDL	(2010, Whistler, CA)
American Heart Association Scientific Sessions	(2010, Chicago, IL, USA)
ATVB Early Career Reception	(2010, Chicago, IL, USA)
1st Rembrandt Symposium	(2010, Leiden)
1st Cardiovascular Conference	(2011, Noordwijkerhout)
LACDR Spring Symposium	(2011, Amsterdam)
Gordon Conference on Atherosclerosis	(2011, Newport, RI, USA)
10th ULLA Summerschool	(2011, Parma, IT)
2nd Rembrandt Symposium	(2011, Amsterdam)
XVI International Symposium on Atherosclerosis	(2012, Sydney, AUS)
HDL Satellite Symposium of XVI ISA	(2012, Cairns, AUS)

Other conferences attended

Dutch Lipoprotein Club (NLC)	(2008, Leiden)
Ernst Klenk Symposium	(2011, Keulen, DE)
NHS Masterclass	(2011, Utrecht)

Courses

Scientific Writing Course	(2008, Leiden)
Write-it-Right and Presentation Course	(2008, Leiden)
Presentation Skills	(2008, Oegstgeest)
LACDR PhD Introductory Course	(2009, Noordwijkerhout)
International Atherosclerosis Research School	(2010, Hamburg, DE)
Scientific Writing Course – Academic Writing Plus	(2011, Leiden)

Publications

Bart Lammers

Division of Biopharmaceutics, LACDR, Leiden University, the Netherlands

Published abstracts

Lammers B, Chandak PG, Radovic B, Aflaki E, Buchebner M, Haemmerle G, Zechner R, Van Eck M, Kratky D. Adipose triglyceride lipase deficiency affects macrophage morphology and function and significantly reduces atherosclerosis in LDL receptor knockout mice.
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Meurs I, Lammers B, Out R, Hildebrand RB, Hoekstra M, van Berkel TJC, van Eck M. The effect of total body ABCG1-deficiency on lesion development depends on the stage of atherogenesis.
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Hoekstra M, Lammers B, Out R, Li Z, Van Eck M, Van Berkel TJC. Activation of the nuclear receptor PXR decreases plasma LDL-cholesterol levels and induces hepatic steatosis in LDL receptor knockout mice.
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Hildebrand RB, Lammers B, de Haan W, Meurs I, Van Berkel TJC, Rensen PCN, Van Eck M. Normalization of HDL cholesterol levels by CETP expression in SR-BI knockout mice does not reduce atherosclerosis.
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Full papers

Lammers B, Louwe MC, Frias MA, Foks AC, Hildebrand RB, Kuiper J, Smit JWA, Van Berkel TJC, James JW, Rensen PCN, Van Eck M. ABCA1 deficiency protects the heart against injury following myocardial infarction. *Manuscript in preparation.*

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Ye D, Lammers B, Zhao Y, Meurs I, Van Berkel TJC, Van Eck M. ATP-binding cassette transporters A1 and G1, HDL metabolism, cholesterol efflux, and inflammation: implications for the treatment of atherosclerosis. *Curr. Drug Targets. 2011;12(5):647-650.*

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