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Dottorato di ricerca in Farmacologia e Tossicologia Sperimentali

XXV CICLO

Role of Apolipoprotein E, Scavenger Receptor class B type I and ATP-Binding Cassette Transporter G1 in Reverse Cholesterol Transport and atherosclerosis

Tesi di Dottorato di:

DANIELA GRECO

Coordinatore del Dottorato:

Chiar.ma Prof. Elisabetta Barocelli

Tutore:

Chiar.mo Prof. Franco Bernini

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"Tell me and I forget. Teach me and I may remember. Involve me and I will learn."

B. Franklin

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Introduction

BACKGROUND

According to World Health Organization, cardiovascular diseases (CVD) are the leading causes of death in the world (Santos-Gallego, Giannarelli, and Badimón 2011). The underlying pathological process which results in coronary heart disease and cerebrovascular disease is atherosclerosis. Atherosclerosis is a complex, chronic pathology which affects the cardiovascular system. It is considered to be originated from an inflammatory process that leads to the slowly development of atherosclerotic plaque. Rupture of vulnerable plaque can results in the occlusion of the vessel, thus causing symptomatic diseases like stroke and myocardial infarction. For a long period atherosclerosis has been considered mainly caused by lipid accumulation in the arterial wall, even if epidemiological studies have identified at least two classes of risk factors, the so called behavioural (smoking, physical inactivity, unhealthy diet) and metabolic risk factors (high blood pressure, diabetes, high lipid levels, obesity). Therefore, the most common therapeutic strategy actually used to prevent CVDs aims to reduce these well known risk factors, in particular lowering lipid levels by statins drug. Despite this pharmacological approach, CVDs still remain a major and expensive health burdens. Moreover, through the past 50 years, several epidemiologic studies have strongly supported the inverse correlation between high density lipoproteins (HDL) levels and risk of CVD both in patient with high and low levels of low density lipoprotein (LDL) (Santos-Gallego, Giannarelli, and Badimón 2011). This observation has led to increased interest in HDL increasing therapy to target the so called “residual risk”.

ATHEROSCLEROSIS

During atherogenesis a series of changes occur into the arterial wall. It is generally accepted that atherosclerosis is an inflammatory disease which can be explain as "response-to-an-injury" (Ross 1993). Numerous factors such as lipids, free radicals, hypertension, can result in endothelial dysfunction. As a results of inflammatory activation, endothelial cells start to secrete chemokines leading to the recruitment of circulating monocyte, T-cells and other inflammatory cells from the circulation into the intima of the arterial wall. In particular, endothelial cells produce adhesion molecules, such as vascular cell adhesion molecule-1 (VCAM-1), intracellular adhesion molecule-1 (ICAM-1) , P-selectin and E selectin (Z. M. Dong et al. 1998) and chemotactic protein such as monocyte chemotactic protein-1 (MCP-1) (Lusis 2000). Proliferation and differentiation of macrophages are stimulated by growth factors such as macrophage colony-stimulating factor (M-CSF) (Jonathan D Smith et al. 1995).

The monocyte-derived macrophages start to accumulate modified lipids, such as oxidized LDL (OxLDL), undergoing transformation into foam cells (Cybulsky and Jongstra-Bilen 2010). Foam cells represent the hallmark of atherosclerosis and significantly contribute to the development of the early sub-endothelial lesion, called fatty steak (Walker, Reidy, and Bowyer 1986). If lipid deposition within the arterial wall persists, pro-inflammatory response continues and smooth muscle cells (SMCs) migrate from the media layer and start to proliferate. During this stage, SMCs switch from a contractile phenotype to synthetic phenotype producing extracellular matrix rich in collagen, elastin and proteoglycan. The progression of the inflammatory process allows the lesion to evolve into intermediate lesion (Lusis 2000). Successively, lesion continues to grow supported by the migration of monocyte, cell proliferation, extracellular matrix production and accumulation of extracellular lipids. The advanced lesion is characterized by a lipid-rich necrotic core, surrounded by a fibrous cup (Ross 1993). The most important clinical consequence of advance lesion is the rupture of instable plaque with thrombus development, which can result in the occlusion of artery. The symptomatic results of this occlusion are myocardial infarction and stroke, the most dangerous complications of atherosclerosis.

Macrophages play a dual role in atherogenesis as their scavenger activity initially represents a protective physiological mechanism but in the late stage contributes to support the progression of the pathology. Macrophage role in sustaining atherosclerosis progression has been supported by studies in which mice lacking macrophages show a reduces susceptibility to atherosclerosis

even in presence of a severe hypercholesterolemia (Jonathan D Smith et al. 1995). Recently, two different phenotypically subsets of monocyte, both in human and mouse, have been identified based on different surface markers (Tacke et al. 2007). Monocytes with high expression of Ly-6C^{hi} promote inflammation and selectively populate sites of experimentally induced inflammation, while the other subset with low expression of and Ly-6C^{lo} can enter tissues under homeostatic conditions in mouse (Sunderkötter et al. 2004). After activation macrophages can be differently polarize into pro-inflammatory M1 phenotype and anti-inflammatory, also known as alternatively activated, M2 macrophages (Gordon and Taylor 2005). M1 polarized macrophages produce pro-inflammatory mediators, like tumor necrosis factor α (TNF-α) and interleukin 6 (IL-6) and are activated by lipopolysaccharide (LPS) and interferon-γ (IFN-γ) (Gordon and Taylor 2005). On the other hand, incubation of macrophages with IL-4 or IL-13 induces M2 phenotype, producing anti-inflammatory cytokines like IL-10 and transforming growth factor β (TGF-β) (F. O. Martinez, Helming, and Gordon 2009). The balance of polarized M1 and M2 macrophages is generally accepted as of a great importance in atherosclerotic development.

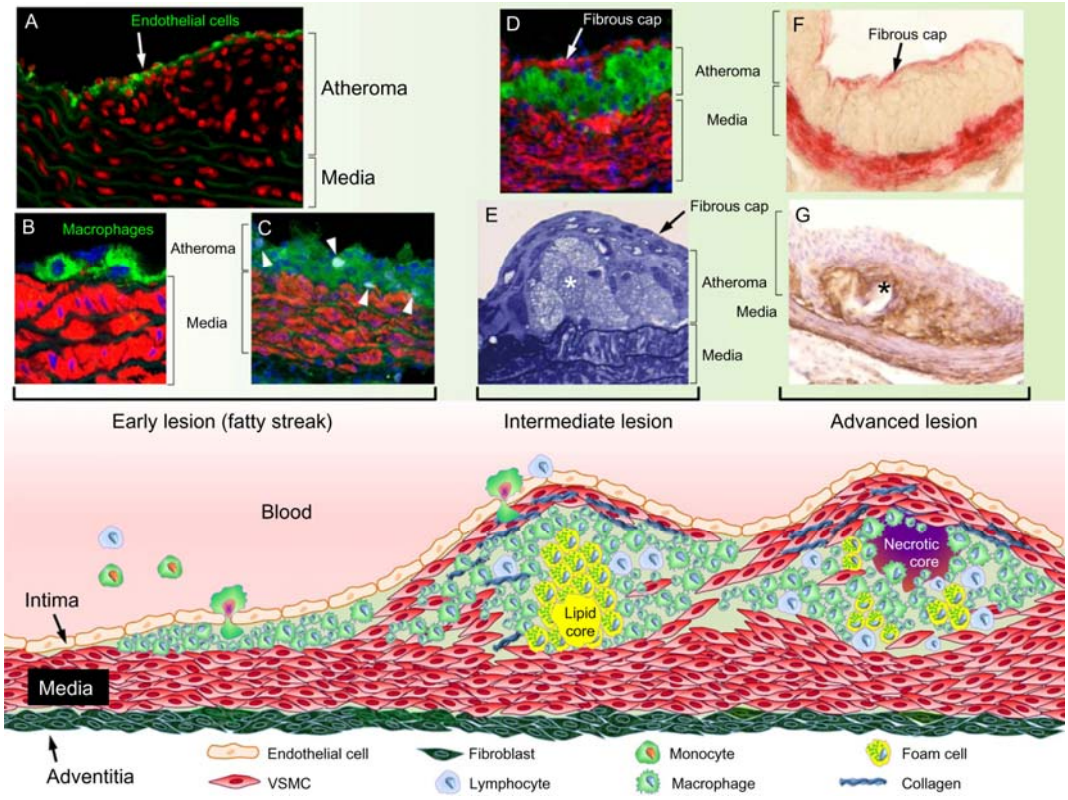

Fig.1 Different stages in the development of atherosclerosis lesion (Fuster et al. 2012)

Although macrophages account for the majority of leukocytes within the atherosclerotic plaque, others effector of the adaptive immunity may contribute to the pathogenesis of atherosclerosis. In particular, experimental models have provided evidences of a direct involvement of lymphocytes (T cells and B cells) in regulation of inflammatory during atherogenesis (Andersson, Libby, and Hansson 2010). Studies in mice suggested that B cells are a heterogeneous group in which is possible to define more than two subsets. In particular, B1 cells seem to protect from atherosclerosis as its deficiency in mice enhances the development of pathology (Caligiuri et al. 2002). More recently a study clearly linked the athero-protective property of B1 cells to the production of IgM induced by IL-5 (Lewis et al. 2009). Conversely, B2 lymphocytes are described as pro-atherogenic since the depletion of B2 with anti CD20 antibody reduces lesion size by reducing INF-γ and IL-17 production (Ait-Oufella et al. 2010). Several lines of evidences also documented the presence of lymphocytes in atheroma. In particular studies in animal models showed that T cells in atheroma are mostly Th1 CD4+ cells, which pathogenic role is mostly related to the capacity to secrete INF-γ (Gupta et al. 1997). Th2 cells in atherogenesis are normally considered to antagonize Th1 proatherogenic effect, as they oppose the differentiation of pro-atherogenic IFN-γ-producing Th1 cells (Andersson, Libby, and Hansson 2010). Regulatory T cells can result in atheroma amelioration by producing IL-10 and TGF-β, which results in inhibition of foam cells formation (J. Lin et al. 2010). Since neutrophils have not been found in a high number in atherosclerotic lesion, they have been neglected in the pathophysiology of atherosclerosis. However, despite in low frequency than monocytes and macrophages, neutrophils are present in the adventitia of mouse models of atherosclerosis, such as ApoE Knockout (KO) and LDLr Knockout (KO) mice (Rotzius et al. 2010) (Van Leeuwen et al. 2008). Those studies firmly indicated neutrophil activation during atherosclerosis and continuous recruitment of neutrophils in hyperlipidemic models (Zernecke et al. 2008). Some other experimental evidences showed the importance of neutrophils recruitment especially in the early stage of atherosclerotic development (Drechsler et al. 2010). Arterial neutrophils infiltration is triggered by E-selectin and P-selectin molecules (Eriksson et al. 2001). After capture, neutrophils roll on the endothelium where they sense chemotactic agents that mediate integrin activation, thus preparing cells for firm arrest. Granule proteins secreted by neutrophils, such as myeloperoxidase (MPO), elastase and cathepsin G affects atherosclerosis development, by aggravating endothelial dysfunction, activating macrophages and finally contributing to plaque destabilization (Soehnlein 2012).

Dysfunctional lipid homeostasis plays a pivotal role in atherogenesis. Hyperlipidemia represents an irritating stimulus which induces pro-inflammatory response of activated endothelium. This leads to increased endothelial permeability, which promotes the migration of leukocytes into the intima and a simultaneous retention of cholesterol-rich LDL. As a consequence, monocytes recruited into the sub-endothelial space differentiate toward macrophages and starts to accumulate lipid, thus becoming foam cells. Within intima, native LDL are modified and cannot be removed from circulation by the normal endocytic LDL receptor. These modified lipoproteins are recognized by macrophage scavenger receptors. Unlike classical receptors, they cannot limit the internalization of lipids. This leads to lipid engulfment, which triggers fatty steaks formation (Kzhyshkowska, Neyen, and Gordon 2012). Since macrophages lipid loading represents a key mechanism in the development of atherosclerosis, it is evident that molecular processes addressed to reduce foam cells formation, would assume an athero-protective function.

LIPOPROTEINS AND LIPID METABOLISM

In order to be transported throughout the circulatory system, cholesterol, phospholipids and triglycerides need to be associated with lipoproteins (Wasan et al. 2008). Lipoproteins are macromolecules composed by lipids and proteins (apolipoproteins) and mainly synthetized in liver and intestine. They are organized in a hydrophobic core containing triglycerides and cholesterol esters and amphipatic layer of phospholipids, free cholesterol and apolipoproteins. Apolipoproteins on the surface of macromolecules exert structural functions and act as ligands for cell receptors as well as activators for enzymes important for lipid metabolism. According to particle density upon density-gravity ultracentrifugation and protein composition, lipoproteins are assigned into four classes :

- Chylomicrons which present the lowest density and the biggest size and are synthetized in the intestine from triacylglicerols, lipids and apolipoprotein B.
- Very low density lipoproteins (VLDL) composed by triacylglycerols, free and esterified cholesterol which deliver triacylglycerols from liver to peripheral tissues.
- Intermediated density lipoproteins (IDL) derived from VLDL, they have lost most of triglycerides but retain cholesteryl esters.
- Low density lipoproteins (LDL) mostly composed by cholesterol and cholesteryl esters, they are the main transporter of cholesterol in humans (Robert W Mahley, Huang, and Weisgraber 2006).
- High density lipoprotein (HDL) are the smallest lipoproteins and transport cholesterol from peripheral tissue to the liver.

In particular, chylomicrons and VLDL are the main transporters of triglycerides, while LDL and HDL transport cholesterol. VLDL and LDL contain ApoB, whereas HDL are mostly composed by ApoAI. Plasma lipids can be metabolized through two different pathways, the exogenous and the endogenous lipid transport. The exogenous pathway is responsible of the metabolism of diet-derived lipids. In the enterocytes dietary cholesteryl esters and triglycerides are hydrolyzed and assembled into chylomicrons packaged with a variety of apolipoproteins, such as apo A-I, A-II, B-48, C-I, C-II, CIII, E. Subsequently they are secreted into the lymph and then moved to blood. In the circulation the triglycerides rich particle are hydrolyzed by lipoprotein lipase

(LPL) and the released fatty acids are taken up by peripheral tissues mostly as energy sources. In the meanwhile the chylomicron particles are remodeled, thus forming remnants molecules. The presence of ApoE on the surface of remnants chylomicrons enables the hepatic uptake through binding to LDL receptors, LDL receptor related protein-1, heparin sulphate proteoglycans and Scavenger Receptors class B Type I (R W Mahley and Ji 1999).

The endogenous pathway addressed to the metabolism of VLDL newly synthetized by liver or derived from lipoprotein uptake. VLDL are assembled by enzyme microsomal transfer protein (MTP) and contain ApoB-100, ApoE and ApoC. In the circulation triglycerides are hydrolyzed by LPL and phospholipids are transferred to HDL by phospholipid transfer protein (PLTP). This results in the formation of IDL which are partly uptaken by the liver and partly remodeled by hepatic lipase (HP) to generate LDL. Cholesterol-enriched LDL are then removed from circulation by binding to LDL receptor in the liver and in peripheral tissues. Homeostatic levels of cholesterol in tissue are under control of a feedback regulatory pathway. If the accumulation of lipids in peripheral tissue increases, the sterol regulatory element binding proteins (SREBP) modulates the transcription of genes involved in sterol and fatty acids biosynthesis.

HDL classification and metabolism

Beside endogenous and exogenous pathway, a third mechanism of cholesterol metabolism, termed reverse cholesterol transport (RCT), enables cholesterol to be moved to liver for catabolism. The principal transporter involved in RCT are HDL particles. HDL belong to a heterogeneous class of lipoproteins differing for size, density, electrophoretic mobility, lipids and lipoprotein composition (George H Rothblat and Phillips 2010). According to different structural properties, HDL can be divided into several subclasses. First, based on shape HDL can be defined discoid or spherical. Secondly, based on density HDL can be divided into two classes, HDL2 (1.063-1.1125 g/ml) and HDL3 (1.125-1.121 g/ml) (Havel, Eder, and Bragdon 1955). Each classes can be also resolved on gradient gel elettrophoresis into HDL2b, HDL2a, HDL3a, HDL3b, HDL3c in decreasing size order. In addition, HDL can be separated based on electrophoretic mobility, the so called α-HDL, which present a high negative surface charge, and the preβ-HDL which mainly contain apoAI and phospholipids (George H Rothblat and Phillips 2010). Finally, using immune-affinity methods HDL can be divided into two classes based on apolipoprotein component: LpA-I+ LpA-II and LpA-I which contains only ApoAI (Scanu and Edelstein 2008).

In plasma HDL are continuously remodeled to be interconverted into the different classes. The proteins involved in HDL metabolism are plasma enzymes which consequently have a role in regulation of RCT. The lecithin-cholesterol acyltransferase (LCAT) is a 63 kDa glycoprotein synthesized in the liver. It is a key enzyme essential for the formation of large ApoAI rich HDL, as it mediates the esterification of cholesterol on the surface of large discoidal HDL (Asztalos et al. 2007). LCAT activity enriches HDL core with cholesteryl esters leading to the formation of mature HDL3. Since RCT was first described, LCAT was believed to be an important driving force for removal of cholesterol from cells (Kunnen and Van Eck 2012). Although it is believed to be atheroprotective, LCAT deficiency in human as well as in animal models has not be associated with increased atherosclerosis (Calabresi et al. 2009) (Kunnen and Van Eck 2012). Hepatic lipase (HL) is a 65 kDa glycoprotein which hydrolyzes both triglycerides and phospholipids from all classes of lipoporteins (Vergeer et al. 2010). This enzyme promotes the conversion of lipid rich form of αHDL into smaller preβ HDL. Conversely, the endothelial lipase (EL) exhibits a higher affinity to phospholipids and the preferential substrate is represented by HDL (D J Rader and Jaye 2000). In human plasma EL inversely correlates with HDL levels and atherosclerosis. Consistent with these data, overexpression of EL in mice accelerates HDL catabolism, whereas inhibition increases HDL levels (Rosenson et al. 2012). Cholesteryl-ester transfer protein (CETP) is a 476 residues hydrophobic glycoprotein that mediates the transfer of cholesteryl esters and tryglicerides between HDL and ApoB lipoproteins. Mutation of CETP gene results in elevated HDL levels and moderates reduction in LDL and apoB levels (Matsuura et al. 2006). Phospholipid transfer protein (PLTP) belong to the same family of CETP and is involved in the moving of phospholipids among HDL and others lipoproteins (Masson et al. 2009).

REVERSE CHOLESTEROL TRANSPORT

Since majority of cells, including macrophages, cannot catabolism toxic excess of cholesterol, the only way to eliminate it is represented by efflux to extracellular acceptors. Cholesterol efflux represents the initial removal step of in the atheroprotective process of Reverse Cholesterol Transport (RCT) (Daniel J Rader et al. 2009). RCT was first described by Glomset as the physiological pathway whereby cholesterol is removed from peripherals tissue, including foam cells, delivered by lipoproteins into blood and finally uptaken by liver for disposal into feces (Glomset 1968). The major mediators of RCT are represent by HDL lipoproteins which, acting as cholesterol acceptors, transport cholesterol in the circulation.

The RCT process represents a potential target for the prevention of atherosclerosis, since it is considered of a great importance for cholesterol elimination from macrophages-derived foam cells of the arterial wall. Since now, biliary secretion has been considered the principal way to excrete cholesterol. Of note, some recent studies shown that cholesterol can be actively excreted directly from plasma to intestinal lumen, therefore suggested a novel non biliary hypothesis of RCT pathway, called Trans Intestinal Cholesterol Efflux (TICE) (Van der Velde, Brufau, and Groen 2010). In TICE pathway, which in mice account for 70% of fecal neutral sterol elimination, cholesterol is captured from plasma by intestinal cells and then excreted into feces from apical membrane by ABCG5/ABCG8 transporter.

In classical model of RCT, free cholesterol is mobilized from macrophages to lipoproteins particles such as Apo AI particles which rapidly increase the amount of lipids by being remodeled within plasma by a variety of enzymes. In particular, lipid free Apo AI, also referred as pre-β HDL, receives free cholesterol and phospholipids though ATP-Binding Cassette transporter A1 (ABCA1) transporter, giving rise to discoid HDL (a R. Tall 2008). Lecithin: cholesterol acyltransferase (LCAT) enriches nascent discoid HDL with cholesterol esters which results in forming large spherical mature HDL3 and HDL2 (Dieplinger, Zechner, and Kostner 1985). The enzyme activity contributes to expand the hydrophobic core of these particles and to maintain the gradient of free cholesterol between cells and HDL, which is necessary to drive the trafficking of cholesterol (Daniel J Rader et al. 2009). Two other macrophage transporters, ATP-Binding Cassette transporter G1 (ABCG1) and Scavenger Receptor Class B type I (SR-BI) can contribute to HDL3 and HDL2 formation by promoting cholesterol efflux from macrophages (Santos-Gallego, Giannarelli, and Badimón 2011). Within HDL, cholesterol is directly delivered back to liver where is uptaken through SR-BI, whereas small cholesterol

depleted HDL particles are released and could be recycled into plasma for lipoprotein assembly. Excess of cholesterol could be excreted via ABCB11 as bile acids (Wakabayashi, Lippincott-schwartz, and Arias 2004), which are previously synthetized by 7α-hydroxylase, or be excreted as neutral sterols by heterodimers ABCG5/ABCG8 (Yu et al. 2005).

Another pathway involved in RCT is mediated by CETP, which mediates the exchange of cholesterol esters from HDL2 to TG-rich lipoproteins, such as very low density lipoprotein (VLDL). ApoB-rich lipoproteins subsequently bind to hepatic LDLr thus completing the RCT cycle (Daniel J Rader et al. 2009).

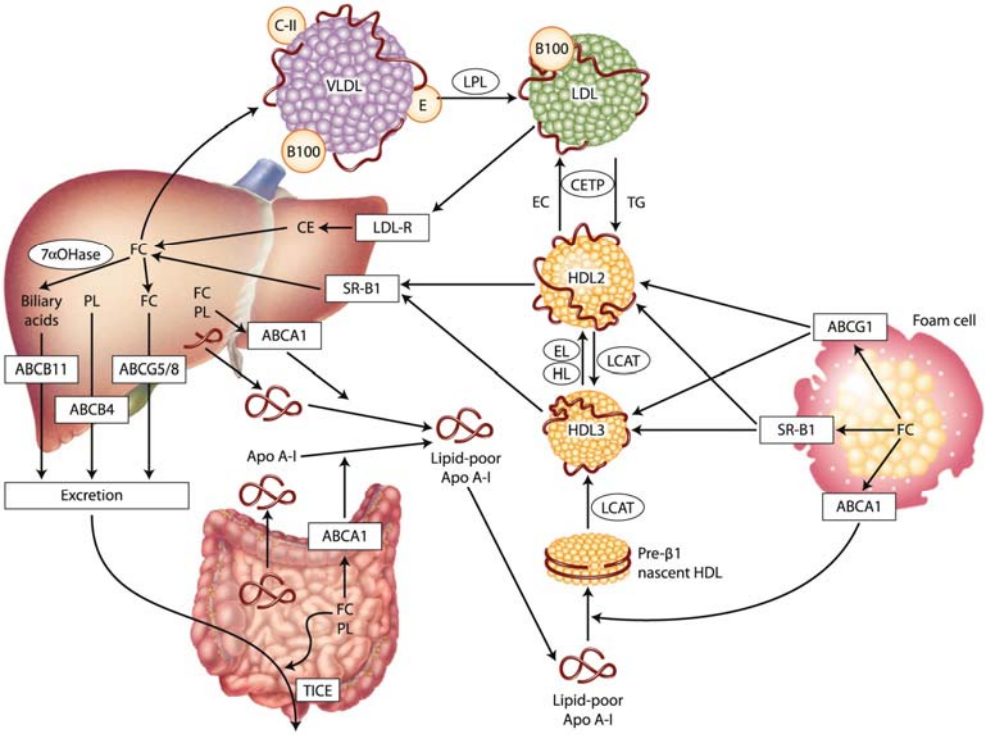

Fig.2 Reverse Cholesterol Transport (Santos-Gallego, Giannarelli, and Badimón 2011)

Several experimental evidences suggested that the rate of macrophage RCT is a stronger predictor of the progression of atherosclerosis as compared to the HDL plasma concentration in animal models. In order to measure the macrophage RCT, a radio-isotopic technique has been developed using murine models (YuZhen Zhang et al. 2003). In this method macrophages cells are radiolabeled with cholesterol and then injected into recipient mice. At established time point the macrophage-derived ^{3}H-cholesterol is directly measured in plasma, liver and feces which are the main compartments involved in RCT. Compared to the previous methods, which analyze the

total peripheral RCT, this novel technique has the advantage to specifically quantify the trafficking of pool of cholesterol directly involved in the atherogenic process. Therefore, the macrophage-derived cholesterol excreted into feces is quantified as a measure of macrophage-to-feces RCT (Daniel J Rader et al. 2009). This approach has been widely used to study the contribution to RCT of genes involved in HDL metabolism. Using this approach for example, it has been demonstrated for that the atheroprotective function of ApoAI can be attributed to the promotion of RCT, since overexpression of human ApoAI in mice enhances the macrophage RCT (YuZhen Zhang et al. 2003). Since both recipient mice and donor macrophages can be genetically manipulated this method also allows to investigate the contribution of specific macrophage and systemic genes in promoting RCT (X. Wang et al. 2007). Furthermore, several pharmacological modulations on the RCT process has been extensively investigated (Ilaria Zanotti et al. 2008) (I Zanotti et al. 2011).

CHOLESTEROL EFFLUX

HDL are key regulator of the first rate limiting step of RCT, since they are the preferential acceptors of cholesterol from lipid loaded macrophages. As it was previously stated, HDL can be considered as a marker for the efficiency of RCT process even if macrophage-derived cholesterol represents only a small part of cholesterol delivered by lipoproteins. Cholesterol can be eliminated by macrophage-derived foam cells of the arterial wall by different pathways, including passive diffusion and mechanisms mediated by membrane transporters such as the active ABC transporters, ABCA1 and ABCG1, Scavenger Receptor class B type I SR-BI, and endogenous lipid poor Apolipoprotein E.

The contribution of transporters to cholesterol efflux from macrophages has been clearly quantified in vitro. Adorni et al. demonstrated that in cholesterol loaded murine peritoneal macrophages (MPM) ABCA1 contribution is predominant and represents 35% of total efflux, ABCG1 contributes less than ABCA1 but still for a 21%; while SR-BI contribution is minimal (Adorni et al. 2007). In cholesterol loaded human macrophages ABCA1 still remains the predominant contributor to cholesterol efflux, but, differently from mice, SR-BI is relatively more important than ABCG1 (Larrede et al. 2009a).

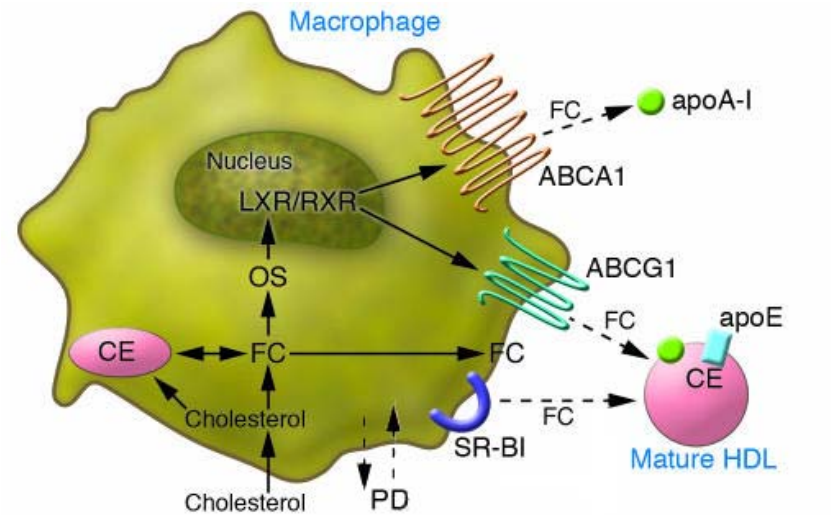

Fig.3 Cellular cholesterol efflux, adapted from (Daniel J Rader 2006)

Cholesterol efflux is dependent both from cells capacity to promote elimination of cholesterol and from the efficiency of extracellular lipoproteins to accept cholesterol. Since Fielding at al.

demonstrated that sera from different patients differ in the ability to promote cholesterol efflux (Fielding et al. 1983), cholesterol efflux capacity of serum has be suggested to be a marker of RCT efficiency. This observation led to use the strategy of measure cholesterol efflux ex vivo for large scale screening to evaluate cholesterol efflux potential. Consistent with epidemiological evidences of a positive correlation between HDL levels and reduced risk of CVD, improving cholesterol efflux represents an attractive therapeutic approach.

Aqueous diffusion

The removal of cholesterol from cells can occurs by aqueous diffusion mechanism, which mediates the bidirectional flux of cholesterol between cell and extracellular medium (George H Rothblat and Phillips 2010). Cholesterol is sufficiently water-soluble to be passively transferred to extracellular acceptors. After being desorbed from cell membrane down to a concentration gradient, it diffuses in the lipid-water interface and finally collides with acceptor molecules, thus being incorporated into phospholipid containing lipoproteins. At constant donor concentrations, the rate of passive diffusion depends of acceptor concentration and size: large acceptors are considered inefficient because they negatively affect the diffusion-collisions with cholesterol molecules (G H Rothblat et al. 1999). The movement of cholesterol is driven by concentration gradient between cells and acceptors and is not influenced by the composition of HDL particles (W.S. Davidson et al. 1995). Since rate limiting factor is the interaction between desorbing cholesterol molecule and phospholipid acceptors, factors that reduce the lipid molecular packing density tend to enhance the rate of cholesterol transfer (Patricia G Yancey et al. 2003).

ATP-Binding Cassette Transporter A1 (ABCA1)

ABCA1 (Adenosine triphosphate binding cassette transporter A1) is a 2261 amino acids protein which promotes unidirectional, net cholesterol efflux to lipid-poor helical apolipoproteins such as Apo A-I, Apo A-II, ApoA-IV and Apo C. ABCA1 is a full transporter member of the family of ABC transporters which use ATP to produce energy needed to promote free cholesterol and phospholipid efflux from cell membrane to extracellular acceptors. ABCA1 structure consists in two halves, each containing 6 helices and a nucleotide binding domain (NBD). The protein

contains two large extracellular domains linked together by cysteine residues forming cysteine bonds (John F Oram and Heinecke 2005).

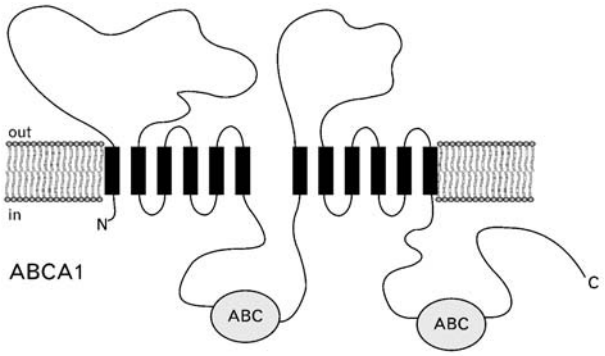

Fig.4 Structure of ATP-Binding cassette transporter A1 Adapted from (Jessup et al. 2006)

The major acknowledges on ABCA1 results from studies carried out after the description of a rare protein mutation, Tangier disease, which is characterized by almost completely absence of plasma HDL, macrophage tissue accumulation and increased atherosclerosis (Rust et al. 1999). Recently some genome-wide studies have identified some variants of ABCA1 associated with variation of HDL levels (Kathiresan et al. 2008). Taken together these observations suggested that ABCA1-mediated efflux to apo AI is the major predictor of HDL plasma levels.

The protein localizes both in plasma membrane and cytoplasmatic compartments (Neufeld et al. 2001) and even if is ubiquitously expressed, the major sites of expression are liver, brain and macrophages (Lawn et al. 1999). The expression of ABCA1 can be modulate by several factors. Briefly, as a lipid transporter, the transcription of protein is markedly induced in presence of cholesterol overloading and occurs via activation of nuclear liver X receptor (LXR) and retinoid X receptor (RXR) (Lawn et al. 1999). After being internalized, cholesterol oxidized to form oxysterols 22-hydroxycholesterol, 24-hydroxycholesterol, and 24,25-epoxycholesterol which are generated by cytochrome P-450 enzymes. This allows oxysterols to activate nuclear receptors LXR/RXR which subsequently form a heterodimer that binds to promoter motif of ABCA1 gene, thus resulting in protein transcription (Costet et al. 2000). Moreover Oram et al. showed that ABCA1 expression can be induced by analogs of cAMP (J F Oram et al. 2000). Another way to modulate ABCA1 transcription involves activators of peroxisome proliferator activating nuclear receptors (PPARs) which induce ABCA1 transcription in LXR dependent manner (John F Oram and Heinecke 2005).

Some evidences also suggested a post-transcriptional regulation of ABCA1, mainly due to the rate of protein degradation. In absences of apolipoproteins the protein turnover is rapid (1-2 hours half-life). Protein contains the so called PEST motif (proline-glutamate-serine-threonine) which is typical of the proteins undergoing rapid turnover mediated by calpain (L. O. Martinez et al. 2003). Several possibility to induce or retard protein degradation have been reviewed. In particular, binding of apoA-1 or apoE to ABCA1 was described to preserve ABCA1 from degradation and this is apparently due to reduced proteolysis (a R. Tall 2008). In contrast, unsaturated fatty acids can accelerate the protein turnover, antagonizing LXR or directly inducing protein degradation (Y. Wang, Kurdi-Haidar, and Oram 2004). Moreover, a synthetic molecule called probucol was demonstrated to inhibit the apoAI binding to ABCA1 thus reducing ABCA1-mediated cholesterol efflux from macrophages approximately up to 80% (Favari et al. 2004a).

Several experimental evidences have reported that a direct interaction between ABCA1 and Apo AI is required but not sufficient for efficient cellular cholesterol efflux. In this model ApoAI and ABCA1 form a complex with high affinity binding at the plasma membrane (Yvan-Charvet, Wang, and Tall 2010). An alternative models proposed apolipoprotein AI binding to a lipid rich membrane domain, which precedes the lipid efflux (Patricia G Yancey et al. 2003). A third hybrid models has been suggested in which apo AI interacts which a specific membrane raft generated by ABCA1 and then diffuses within the membrane to generate a complex with ABCA1 transporter (Panagotopulos et al. 2002). At least two models of ABCA1-mediated efflux have been proposed. In the “exocitosys” model cholesterol are supposed to be packaged into vesicles which are able to deliver it to membrane domain containing ABCA1. The second model proposed is the “retro-endocytosis” in which ABCA1 is supposed to be internalized to intracellular compartments where it pumps lipids into the vesicle and successively releases nascent HDL particles (John F Oram and Heinecke 2005).

The contribution of ABCA1 to HDL plasma levels and atherosclerosis has been confirmed in numerous studies. In mice, deletion of macrophage ABCA1 resulted in low levels of HDL and increased atherosclerosis (Aiello 2002), while overexpression of bone marrow ABCA1 reduces atherosclerosis in hyperlipidemic LDLr KO mice (Van Eck et al. 2006). ABCA1 is also expressed in liver where it promotes the cholesterol efflux from hepatocytes. According to the key role of ABCA1 in liver, specific hepatic deletion of ABCA1 led to increased atherosclerosis development as a results of reduced HDL (Liam R Brunham et al. 2009). Recently Rader at all. provided a new insight in the role of hepatic ABCA1 in RCT. He demonstrated that

pharmacological inhibition of hepatic ABCA1 using probucol, despite results in lowering the HDL levels, increased RCT. This study related the hepatic activity of ABCA1 with the rate of macrophage to feces RCT, demonstrating that reduce cholesterol efflux from hepatocytes to plasma via ABCA1, results in increased cholesterol biliary excretion (Yamamoto et al. 2011).

ATP-Binding Cassette Transporter G1

ABCG1 is a member of the G sub-family of ATP-binding cassette transporters and it was first identified as a mammalian homologue of the Drosophila white gene (Savary et al. 1996). It is a half-transporter composed of C-terminal membrane domain with six domains α-helice followed by an N-terminal nucleotide-binding domain (Velamakanni et al. 2007). To become functional ABCG1 requires to form a homodimer or a heterodimer with other members of ABCG family, such as ABCG4 (Nan Wang et al. 2004). ABCG1 is expressed in lung, brain and spleen, and to a lesser degree in liver. In addition, it is highly expressed in macrophages, endothelial cells and lymphocytes.

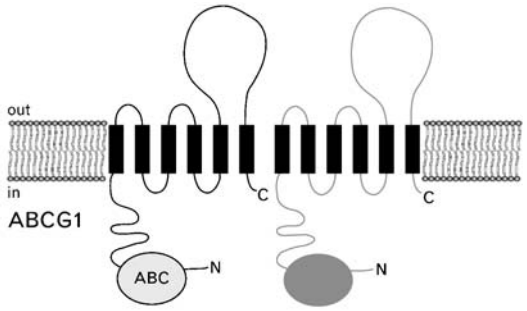

Fig.5 Structure of ATP-Binding Cassette Transporter G1 (Jessup et al. 2006)

The expression of ABCG1 in macrophages and hepatocytes is upregulated by modified LDL, such as AcLDL or OxLDL. ABCG1 is also a target gene for LXR activation (Venkateswaran et al. 2000) (Sabol, Brewer, and Santamarina-Fojo 2005). Interestingly, Wang et al showed both in murine macrophages and in human THP1 cells that upon LXR stimulation ABCG1 protein is redistributed to the cell surface (Nan Wang et al. 2006). This thus suggests that LXR not only regulates ABCG1 mRNA transcription, but also translocation of ABCG1 protein to plasma membrane. A recent study performed in cholesterol-loaded human macrophages, however

revealed that, at least in human cells, LXR stimulated efflux occurs mostly independent from ABCG1 (Larrede et al. 2009b). PPARγ activators also induce the expression of ABCG1 in macrophages (Akiyama et al. 2002). In line PPARγ agonists also stimulate ABCG1 expression in macrophages from LXR KO mice, suggesting that this activation is LXR independent (A. C. Li et al. 2004). A post transcriptional ABCG1 regulatory pathway involves AMP-activated protein kinase (AMPK) activation. Li et al showed that AMPK activation stimulates ABCG1 protein expression and results in increased cholesterol efflux from macrophage-derived foam cells (Li et al. 2010). Unsaturated fatty acids were shown to reduce ABCG1 expression (Uehara et al. 2007). Furthermore, ABCG1 degradation is regulated by lipoxygenase enzymes. In particular 12/15-lipoxygenase activity induces ABCG1 degradation in macrophages, thus resulting in reduced cholesterol efflux (Nagelin et al. 2008). Recently, some experimental evidence suggested that in peritoneal macrophages isolated from C57BL/6J ABCG1 is down regulated in the presence of high glucose levels in vitro (Mauldin et al. 2006) This was confirmed in vivo by Zhou et al. who showed that the expression of ABCG1 is reduced in monocytes from type 2 diabetic patients (Zhou et al. 2008).

ABCG1 stimulates free cholesterol efflux from macrophages to mature HDL (Nan Wang et al. 2004). In addition, ABCG1 has been demonstrated to promote efflux of phospholipids (Kobayashi et al. 2006), but the extent of its contribution in vitro has also been demonstrated to be is considerably lower as compared to ABCA1 contribution (Zanotti et al 2012). In contrast to ABCA1-mediated efflux, ABCG1-dependent efflux does not require binding of the donor cell to the extracellular acceptor (Sankaranarayanan et al. 2009). Two different models have been proposed to describe ABCG1-mediated cholesterol efflux. The first one suggests that ABCG1 allows sterol molecules to overcome the activation energy needed to pass through the hydrophobic layer. Successively, accidental collision with extracellular acceptors contributes to complete the efflux process (A. R. Tall et al. 2008). The second mechanism proposes that ABCG1 increases the availability of cholesterol in a specific membrane pool where it becomes accessible for HDL and not for lipid poor Apo AI (Vaughan and Oram 2005). This last model is consistent with the distribution of ABCG1 in the cell membrane.

ABCG1 also has a role in promoting efflux of oxysterols modified at the position 7, such as 7-ketocholesterol, from macrophages (Terasaka et al. 2007) and from endothelial cells (O'Connell, Denis, and Genest 2004). The production of modified oxysterols, the main products of oxidation of LDL, is generally induced by high fat diet feeding (Brown, Dean, and Jessup 1996). Intracellular oxysterols accumulation leads to apoptosis in endothelial cells, smooth

muscle cells, and macrophages (Müller et al. 2001). Macrophage apoptosis has been demonstrated to promote the development of advanced atherosclerotic lesion, thus contribute also to plaque instability (Tabas 2005). The loss of ABCG1 in macrophages led to a higher accumulation of 7-hydroxycholesterol, 7-ketocholesterol, 24-, 25- and 27-hydroxycholesterol than cholesterol, thus resulting in higher susceptibility to apoptosis (Terasaka et al. 2007). Due to its ability to promoting efflux of oxysterols modified at the 7 position, ABCG1 has thus a pivotal role in preventing cell death.

Several studies have established that ABCG1 alone does not markedly affect HDL levels (Out et al. 2007). However, Wiersma et al. showed that under conditions of LXR activation ABCG1 might contribute to HDL formation (Wiersma et al. 2009). The most important finding, however, is that ABCG1 plays a critical role in macrophage RCT in vivo. In particular, ABCG1 expression in macrophage induces cholesterol efflux from macrophages, whereas deletion of ABCG1 negatively influences the rate of this process (X. Wang et al. 2007).

ABCG1 has a critical role in maintaining tissue cholesterol homeostasis. Kennedy et al. demonstrated that ABCG1 KO mice do not display dyslipidemia on a chow diet. However, massive accumulation of neutral lipids in lung and liver was observed when these animals were fed with a high fat diet. Conversely, expression of ABCG1 protected from dietary fat-induced lipid accumulation (Kennedy et al. 2005). Although the role of ABCG1 in promoting cholesterol efflux from macrophages in vitro and in vivo RCT was well established, its role in the pathogenesis of atherosclerosis still remains unclear. Out et al. described that the total body ABCG1 deficiency in mice fed an atherogenic diet induced early atherosclerotic lesion development and excessive accumulation of neutral lipids in macrophage rich area of lung independent of effects on plasma lipid levels (Out et al. 2007). Conversely, overexpression of ABCG1 in LDLr KO mice increased atherosclerosis despite promoting cholesterol efflux from cells (Basso et al. 2007). A recent study by Meurs et al. showed that the effect of ABCG1 deficiency on lesion development in LDLr KO mice depends on the stage of atherogenesis. Indeed, ABCG1 is pro-atherogenic in early lesion and athero-protective in late lesion (Meurs et al. 2012). Moreover, the physiological relevance of macrophage ABCG1 for atherosclerotic lesion development has been widely investigated in several bone marrow transplantation studies. Interestingly independent groups have reported contrasting effects. In line with the anticipated atheroprotective function of ABCG1, Out et al. showed a modest increase of atherosclerosis in LDLr KO mice transplanted with ABCG1 KO bone marrow on a high cholesterol diet (15% fat, 0.25% cholesterol) (Out et al. 2006). Others, however, found that

absence of macrophage ABCG1 in LDLr KO mice fed a high fat diet (21% fat 1.25% cholesterol) protected from atherosclerosis (Baldán et al. 2006) (Ranalletta et al. 2006). In line Tarling et al. recently demonstrated that the absence of ABCG1 in all tissues or in hematopoietic cells results in protection from atherosclerosis, due to increased apoptotic macrophages (Tarling et al. 2010). A correlation analysis of seven independent studies on the role of ABCG1 deficiency on atherosclerosis susceptibility has been provided by Meurs et al.. By combining different results they shown that the effect of macrophage and total body ABCG1 deletion on atherosclerosis depends on the stage of the lesion, whereby the absence of ABCG1 leads to increased atherosclerosis at small lesion size (lesions < 167 × 103μm2), while in more advanced stages of atherosclerosis lesion progression is retarded.

Several lines of evidence suggest that ABCG1 is implicated in immune responses. Increased levels of inflammatory markers were detected in lungs of ABCG1 KO mice (Wojcik et al. 2008). The anti-inflammatory properties of ABCG1 were also elucidated by Yvan-Charvet et al. who demonstrated that ABCG1 KO macrophages have increased inflammatory gene expression (Yvan-Charvet et al. 2008) and suggested that the pro-inflammatory effect is related to the lipid accumulation in the plasma membrane. This hypothesis was confirmed by studies showing that treatment of macrophages with cyclodextrin, which induces the efflux of cholesterol independent of ABCG1, dampens the inflammatory response. Additionally, macrophages from ABCG1 KO mice are more prone to apoptosis and necrosis as a consequence of lipid accumulation into endoplasmatic reticulum (ER) which probably leads to dysfunction of intracellular signaling pathways (Baldán et al. 2006). ABCG1 is also expressed in endothelial cells where it is the predominant transporter promoting cholesterol and oxysterol efflux to HDL (Terasaka et al. 2008). By preventing cholesterol and oxysterol accumulation, ABCG1-mediated efflux promotes eNOS activity in endothelial cells (Terasaka et al. 2010). Whetzel et al. also demonstrated that aortic endothelial cells from ABCG1 KO mice produce increased amounts of cytokines and chemokines leading to enhanced monocyte adhesion (Whetzel et al. 2010).

Despite its essential role in tissue lipid homeostais, no genetic mutations in human ABCG1 has been associated with disease. Recently, a study by Shou et al. reported for the first time that a functional mutation in the ABCG1 promoter is associated with increased myocardial infarction risk in the general population (Schou et al. 2012). Another study in Japanese men showed that a ABCG1 promoter polymorphism is associated with increased severity of cardiovascular disease in the absence of relevant effects on HDL (Furuyama et al. 2009). Despite the large number of

studies conducted on ABCG1, its physiological role in development of atherosclerosis in human remains unclear to date.

Scavenger Receptor class B type I (SR-BI)

Scavenger Receptor Class B type I (SR-BI) is a member of the scavenger receptor family and it was first identified as a receptor for acetylated LDL (AcLDL) and anionic lipids (Rigotti et al. 1995). Successively, the group of Krieger discovered that it has a high affinity for HDL (Acton et al. 1996). SR-BI, also known as CD36 and lysosomal integral membrane protein-II analog I (CLA-1) in humans, is a 82 kDa cell surface glycoprotein with two trans-membrane domains, two short N-terminal and C-terminal cytoplasmatic domains and a large extracellular loop. It is expressed mostly in liver and sterodoigenic tissue but also in brain, kidney and many cell type like macrophages and platelets (Rigotti 2003). Studies using transgenic mice enabled to identify SR-BI as a multipurpose protein involved in cholesterol and steroid metabolism, as well as in platelet function.

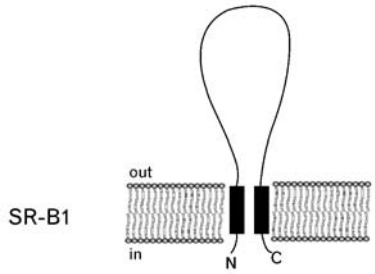

Fig.6 Structure of Scavenger Receptor Class B type I. Adapted from (Jessup et al. 2006)

Numerous endogenous molecules and synthetic compounds can modulate SR-BI mRNA transcription. SR-BI gene expression is regulated by SREBPs and steroidogenic factor-1 (SF-1) (Gene, Lopez, and Lean 1999). In addition, LXR ligands regulate hepatic SR-BI expression as well as expression of the receptor in endothelial cells in murine cell line models (Malerød et al. 2003) (Malerød et al. 2002). Within atherosclerotic plaque, the differentiation of monocytes into macrophages leads to increased expression of SR-BI, regulated by PPAR-α (Chinetti et al. 2000). Of note the SR-BI expression is modulated differently in distinct cell types. For instance, fibrate treatment decreased the expression of hepatic SR-BI (without a decrease in mRNA) in

mice (Lan and Silver 2005), whereas they upregulate SR-BI expression in macrophages but have no effect in adrenals (Chinetti et al. 2000) (Fu, Kozarsky, and Borensztajn 2003). The expression of SR-BI in liver can also be modulated both in a positive and negative manner by several exogenous compound or by endogenous factors, including insulin (Cao et al. 2004), leptin (Gruen et al. 2005), and TNFα (Khovidhunkit et al. 2001). A large number of studies have also shown that several factors can modulate the expression of SR-BI at post-transcriptional level. In hepatocytes and enterocytes of the small intestine, an adaptor protein, PDZK1, regulates the expression of SR-BI on the cell surface. In contrast, PDZK1 does not change SR-BI cell surface expression in macrophages and adrenals (Kocher et al. 2003). Interestingly, several hormones, including insulin (Shetty et al. 2006), the thyroid hormone T3 and thyromimetics ligands up-regulate SR-BI protein expression in liver (Johansson et al. 2005). Furthermore, probucol, a lipid-lowering antioxidant drug, induces SR-BI expression in human liver cells in vitro and rabbit liver in vivo, but not in mice through stabilization of the protein (Hirano et al. 2005).

SR-BI has a pivotal role in cholesterol metabolism as it facilitates the selective transfer of cholesteryl esters from circulating HDL to the liver (Rigotti et al. 1997) and also mediates bidirectional flux of free cholesterol between cells and HDL (Yong Ji et al. 1997). Several studies in murine models have supported the role of SR-BI in athero-protection. In particular, using engineered animals it was established that the total body deficiency of SR-BI is associated with increased size and levels of plasma HDL, which leads to a higher susceptibility to atherosclerosis (Rigotti et al. 1997) (Van Eck et al. 2003). Conversely, hepatic overexpression of SR-BI in mice led to reduced HDL plasma levels due to increased uptake of HDL cholesterol esters by the liver compared to non transgenic controls (Y Ji et al. 1999). The atheroprotective function of SR-BI has been primarily attributed to its essential role in macrophage reverse cholesterol transport (RCT),

The contribution of SR-BI to cholesterol efflux has been first suggested by the observation that in vitro the expression of the protein positively correlates with the release of cellular cholesterol to mature HDL (Yong Ji et al. 1997). Besides facilitating efflux, SR-BI also stimulates the influx of free cholesterol from HDL (De La Llera-Moya et al. 2001). The relevance of the interaction of SR-BI with lipoprotein in inducing lipid flux has been investigated over the years. Although SR-BI is able to bind Apo AI, it has been shown that SR-BI-mediated flux between cells and phospholipid containing acceptors is largely a consequence of changes induced by transporter in the structure of cell membrane (De la Llera-Moya et al. 1999a). Enrichment of the

extracellular acceptor with specific phospholipids influences the flux of free cholesterol via SR-BI, with phosphatidylcholine-rich acceptors being more efficient in enhancing efflux as compared to particles enriched in sphingomyelin (P G Yancey et al. 2000). The same group also demonstrated that the high affinity binding to HDL is not sufficient to promote free cholesterol flux (Connelly et al. 2003). Of note, it is assumed that SR-BI facilitates passive diffusion by increasing the amount of free cholesterol desorbed from the plasma membrane. A crucial role is attributed to the extracellular domain of SR-BI which is believed to create a polar channel for free cholesterol to cross the membrane (Patricia G Yancey et al. 2003). Despite the fact that some studies have shown that inhibition of the binding of HDL to SR-BI using a blocking antibody reduces cholesterol efflux (Xiangju Gu, Kozarsky, and Krieger 2000), consecutive studies confirmed that high affinity binding is not enough to promote efflux (Liu et al. 2002). In particular, Liu et al suggested that the formation of a "productive complex" between Apo AI in HDL and SR-BI is required to promote conformational changes both in the receptor and in the extracellular acceptor for efficient cholesterol efflux. Another mechanism of SR-BI mediated efflux that has been proposed involves retroendocytosis of HDL, i.e. whole HDL particle uptake followed by resecretion of a cholesterol-enriched HDL particle (Pagler et al. 2006). The importance of SR-BI for cholesterol efflux has, however, been under debate, as several in vitro studies demonstrated that the contribution of SR-BI to efflux is low when macrophages are cholesterol loaded with AcLDL or not loaded (Adorni et al. 2007) (Duong et al. 2006). Notably, also a possible interference between SR-BI and other proteins implicated in cholesterol efflux, including the ABCA1 and ABCG1 has been described. In particular Chen et al. suggested for the first time a possible competitive role of SR-BI and ABCA1 in mediating efflux. The study demonstrated that SR-BI inhibits cholesterol efflux to apoAI in cholesterol loaded RAW macrophages probably as a consequence of its ability to facilitate the re-uptake of cholesterol released by ABCA1 (Chen et al. 2000). More recently, a study showed that overexpression of SR-BI in Chinese Hamster Ovary (CHO) cells inhibits ABCG1-mediated cholesterol efflux (Song et al. 2012).

Through acting in two of the main steps of RCT, SR-BI is believed to positively contribute to this physiological process. In agreement, hepatic overexpression of SR-BI markedly increases the amount of macrophage-derived cholesterol excreted into feces, suggesting that hepatic expression of SR-BI is an important positive regulator of the rate of RCT (Yuzhen Zhang et al. 2005). More recently, the group of A.R. Tall showed that the deficiency of SR-BI in macrophages does not impair the process (X. Wang et al. 2007). Despite these results, two

different groups demonstrated that LDLr-/- and ApoE-/- mice which lack SR-BI in macrophages or bone marrow-derived cells develop spontaneous atherosclerosis (Covey et al. 2003) (W. Zhang et al. 2003). Another important evidence on the role of macrophage SR-BI in atherogenesis was provided by van Eck et al. who demonstrated that macrophage SR-BI could be either pro-atherogenic or anti-atherogenic, depending on the stage of atherosclerotic lesion development (Van Eck et al. 2004). Taken together these results suggest the hypothesis that atheroprotective function of macrophage SR-BI, differently from hepatic SR-BI, could be independent from effects on RCT.

SR-BI is also capable of binding apo B (X Gu, Lawrence, and Krieger 2000) and apo E (Bultel-Brienne et al. 2002). In line, several lines of in vitro and in vivo evidence suggest a role for SR-BI in the clearance of LDL and VLDL. Liver-specific overexpression of SR-BI leads to reduced levels of VLDL and LDL in the circulation (N Wang et al. 1998), while reduced expression of SR-BI leads to higher levels of LDL and VLDL and higher susceptibility to atherosclerosis in SR-BI KO (Van Eck et al. 2008), LDLr KO (Huszar et al. 2000) and apoE KO mice (Trigatti et al. 1999). SR-BI influences LDL and VLDL levels by promoting the clearance of these apoB-containing lipoproteins from the circulation (Van Eck et al. 2008). In addition, SR-BI has been implicated in the production of VLDL, as evidenced by reduced VLDL production in SR-BI knockout mice (Van Eck et al. 2008) (Wiersma et al. 2010). Moreover, Out et al. demonstrated that SR-BI plays a key role in chylomicron metabolism as hepatocytes from SR-BI KO mice showed a reduced association with chylomicron-like emulsion particles (Out et al. 2004). In vitro experiments using Caco2 cells showed that SR-BI is expressed on the membrane of intestinal cells and suggested that it might be implicated in intestinal absorption of dietary lipids in the postprandial period (Béaslas et al. 2009).

Although SR-BI is mostly recognized for its role in cholesterol metabolism, it also exerts anti-inflammatory actions. This function is mainly related to the key role of SR-BI in the delivery of cholesterol to the adrenals for the production of steroid hormones which dampen inflammation (Rigotti et al. 1997). Total body deficiency of SR-BI has been associated with impaired adrenal function, as reduced uptake of cholesterol esters from HDL in this tissue leads to depletion of the cholesterol pool needed for glucocorticoid production (Hoekstra, Van Eck, and Korporaal 2012). As a consequence SR-BI KO mice loose glucocorticoid-mediated suppression of pro-inflammatory gene expression (Hoekstra, Berkel, and Eck 2010). In line SR-BI KO mice display increased production of pro-inflammatory cytokines and reduced clearance of LPS during sepsis (Guo et al. 2009).

Beyond the role of SR-BI in lipid metabolism, it has also been shown to exert multiple functions influencing events in vascular cells (Mineo and Shaul 2012). SR-BI is expressed in endothelial cells where it plays a role in regulating the activity of endothelial NO synthase (eNOS). Several studies using endothelial cells demonstrated that SR-BI colocalizes with eNOS in cholesterol-rich caveolae in endothelial cells and that the interaction between SR-BI and HDL is essential to prevent eNOS redistribution. Importantly, HDL binding to SR-BI activates eNOS thereby inducing the production of nitric oxide, a powerful vasodilator and important anti-atherogenic factor (Shaul 2003). The signaling events induced by HDL/SR-BI in endothelial cells is well described. It was shown to include activation of Src kinase(s), PI3 kinase, Akt kinase and Erk1/2 MAPK, with Akt phosphorylation of eNOS causing enzyme activation (Mineo et al. 2003). Recently, Cai et al. demonstrated that SR-BI in macrophages regulates the inflammatory response to LPS both in vivo in mice and in isolated macrophages (Cai et al. 2012). Interestingly, this effect was independent of the cellular cholesterol content.

SR-BI also modulates platelet function and thus influences thrombosis susceptibility. In 2003, Imachi and colleagues showed that SR-BI is expressed on the surface of platelet (Imachi et al. 2003). Since then several studies have addressed the role of SR-BI in platelet activation. First, Valiyaveettil et al proved that OxHDL directly binds to platelets via SR-BI (Valiyaveettil et al. 2008). Further studies showed that also native HDL3 binds to platelets and this binding is inhibited by negative phospholipids which also bind SR-BI (Brodde et al. 2011). It has been shown that HDL binding to platelets inhibits platelet activation through inhibiting intracellular signal transduction pathways, which includes production of second messenger diacylglycerol and protein kinase C activation (Nofer et al. 1998). The groups of Krieger and Van Eck described the in vivo effects of SR-BI deletion on platelet function. SR-BI KO mice showed reduce blood platelet counts and consequently thrombocytopenia (Dole et al. 2008). More recent experimental evidence has confirmed that SR-BI KO mice have highly activated platelet and are more susceptible to thrombosis as a consequence of increased free cholesterol and total cholesterol ratio in plasma (Korporaal et al. 2011). Taken together, these results suggested that SR-BI role in maintaining lipid homeostasis is essential to maintain also normal platelet function.

Extensive studies on the effects of SR-BI deletion and overexpression have clearly shown the physiological importance of this receptor in mice. However, for long the importance of SR-BI in humans has been under debate. The role of SR-BI in humans has been clearly established in a recent study by Vergeer et al. showing the effects of a functional mutation in SR-BI (P297S)

(Vergeer et al. 2011). Heterozygous P297S carriers display higher HDL cholesterol levels and also reduced cholesterol efflux from macrophages. Moreover, the mutation was associated with impaired platelet function and adrenal steroidogenesis. Brunham et al reported two other single mutations in the extracellular loop of SR-BI that were associated with high levels of HDL (L R Brunham et al. 2011).

Apolipoprotein E

Apolipoprotein E is a multifunctional protein firstly discovered as a major component of several lipoproteins such as triglyceride-rich very low density lipoproteins (VLDL) and high density lipoprotein (HDL) (Robert W Mahley and Rall 2000). As a constituent of plasma lipoproteins, it facilitates particles hepatic uptake by binding lipid receptors. According to its role in maintaining lipid homeostasis, Apo E is considered to exert a protective action against the development of atheroma.

Apo E is a glycoprotein of 299 amino acids with a molecular mass of 34 kDa and it is synthesized as pre-apoE protein containing a 18AA extension which lately undergoes cleavage in the ER. After remodeling, it is delivered to the Golgi apparatus where it is O-glycosylated and sialylated (Kockx, Jessup, and Kritharides 2008). The hepatic production accounts for 75% of plasma ApoE. It is also produced by brain and various tissues, including tissues macrophages and other cell types (Greenow, Pearce, and Ramji 2005). Apolipoprotein E is a polymorphic protein with three common isoforms E2, E3, E4, products of three alleles at a single gene locus termed ε2, ε3, and ε4 (Zannis et al. 1982). The gene coding for three isoforms resides on chromosome 19 in human (Robert W Mahley and Rall 2000). Three homozygous (E2/E2, E3/E3, E4/ E4) and three heterozygous (E3/E2, E4/E2 and E4/E3) phenotypes are found in the general population (Anoop et al. 2010). Those three isoforms differ in primary structure at two sites, residue 112 and 158: Apo E3 has a cysteine at residue 112 and an arginine at 158, Apo E4 has arginine, while Apo E2 has a cysteine at both sides (Robert W Mahley and Rall 2000). Apo E3, the normal form, is found in more than 60% of the population. ApoE2 is associated with genetic familial hyperlipidemia disorder type III (HLP) but protects from Alzheimer's disease, while ApoE4 has been linked to 40-60% of Alzheimer's disease and is also associated with increased risk of heart disease.

Secondary structure of ApoE contains two independent folded domains linked by a protease-sensible loop. The amino terminal region consists in four amphipathic α-helix and is a functional domain which contains the binding region for low density lipoprotein receptor, LDL related protein (LRP) and a high affinity binding site for heparin-sulphate proteoglycans (HSPG) (Greenow, Pearce, and Ramji 2005). Likewise, the carboxyl-terminal region contains high affinity binding site for lipids and it appears to be important for the self-aggregation in the absence of lipids.

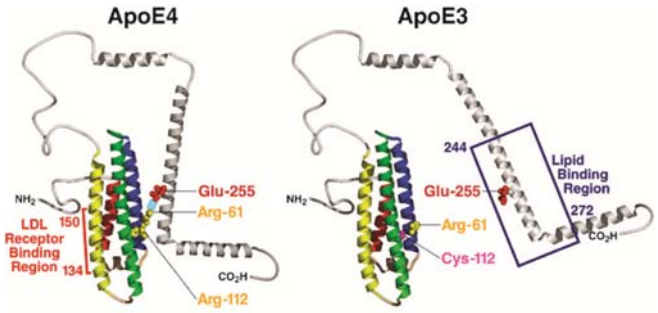

Fig.7 Structure of ApoE isoforms, domain of interaction. (Robert W Mahley, Weisgraber, and Huang 2009)

The binding to lipoprotein receptor is isoform specific: ApoE3 and ApoE4 bind with the same affinity while ApoE2 shows in vitro less affinity (Robert W Mahley and Rall 2000). Another isoform specific function is lipid binding, in particular both ApoE3 and ApoE2 preferentially bind to small phospholipid-rich high density lipoproteins (HDL) while ApoE4 preferentially binds to the larger very low density lipoprotein (VLDL). It has also been demonstrated that the binding to lipids is thereby essential for ApoE to enhance affinity to lipoprotein receptors (Saito, Lund-Katz, and Phillips 2004).

ApoE gene expression is transcriptional and post transcriptional regulated by many factors. For example, macrophage lipid accumulation induces apoE transcription, as well as the differentiation of monocytes into macrophages (Greenow, Pearce, and Ramji 2005). This lipid-induced expression of ApoE has been demonstrated to be under control of LXR pathway (Laffitte et al. 2001). A negative regulation of the expression of ApoE lipoprotein is mediated by pro-inflammatory cytokine, such as interferon γ, which was demonstrated to reduce ApoE production in macrophages (Brand, Mackman, and Curtiss 1993). Another possible modulation of the expression of macrophage ApoE is mediated by tumor necrosis factor α which promotes the expression of ApoE by inducing mRNA transcription (Curtiss and Boisvert 2000). Different

mechanisms have been suggested to explain regulation of apoE transcription. One of this suggested a modulation of the degradation of the protein, for example, pre incubation of macrophages with sterols has been shown to increase ApoE secretion by reducing protein degradation (Duan, Gu, and Mazzone 2000). In macrophages ApoE has a half-life of 22 minutes and undergoes to degradation into the Golgi compartment. Some in vitro studies suggested that the ApoE intracellular degradation is mediated by Ca2+ dependent protease (Ye, Reardon, and Getz 1993). When ApoE degradation is chemically inhibited, the secretion appeared to be inhibited too, suggesting the existence of an intracellular pool which can undergo either to degradation or secretion (Kockx, Jessup, and Kritharides 2008).

It has been also demonstrated that different cells are able to regulate the amount of secreted ApoE by a recycling pathway. In the absence of LDL receptors, Farks et al. demonstrated that ApoE undergoes recycling process (Farkas et al. 2004). The recycling pathway is observed in macrophages as well as in hepatocytes. In murine macrophages the recycling of exogenous ApoE has been shown to be promoted by HDL and ApoAI (Heeren et al. 2003). Some lines of evidences also supported the existence of two distinct secretory pathways for endogenous and recycled ApoE (Kockx, Jessup, and Kritharides 2008). The ApoE secretion from macrophages has been explained to happen both in basal and acute regulation (Kockx, Jessup, and Kritharides 2008). In the constitutive secretory pathway the rate of ApoE secretion is regulated by protein kinase A (PKA), since the inhibition of PKA leads to increased amount of intracellular ApoE. Remarkably, in this condition the inhibition of PKA does not reflect an increase in ApoE degradation, which means that the intracellular ApoE is inaccessible to lysosomal degradation (Kockx et al. 2007). This study also established that Ca2+ is required for ApoE secretion. Conversely, the stimulation of PKA as well as the increased Ca2+ levels do not enhance ApoE secretion suggesting an additional pathway to be involved. According to these findings Kockx et al. tried to further understand the mechanisms of ApoE secretion. They demonstrated that PP2B (calcineurin), a Ca2+/calmodulin-dependent protein serine/threonine phosphatase is also involved in ApoE secretion. In particular they demonstrated that the inhibition of PP2B with Cyclosporine A, a widely used immunosuppressant drug, reduces ApoE secretion in a ABCA1 independent-manner (Kockx et al. 2009).

Role of ApoE in plasma lipid metabolism and atherosclerosis

ApoE is directly involved in systemic lipid metabolism as it is associated with VLDL, chylomicron remnants and HDL. Mahley summarized the metabolic lipid function of ApoE into two main actions, the so called "endocrine–like function" and the "paracrine-like function" (Robert W Mahley and Rall 2000). The first definition was used to describe the capacity of ApoE to direct both endogenous and dietary triglycerides and cholesterol to extrahepatic cells or to the liver. On the other hand ApoE can redistribute lipids among cells within an organ or tissue, thus exerting the "paracrine-like function". The clearance of chylomicron remnants and VLDL is mediated by ApoE through binding to LDL receptor or HSPG/LDL receptor-related protein (LRP). It has also been demonstrated that ApoE directly stimulates the production of VLDL as studies in ApoE null mice have shown an impaired production of VLDL triglycerides from hepatocytes (Kuipers et al. 1997). Direct evidences of apoE role in promoting hepatic cleareance of VLDL were provided by Linton et al. who demonstrated that the LDL receptor dependent clearance of VLDL is dependent on apoE secreted by liver (Linton et al. 1998). Given its direct role in triglyceride-rich lipoprotein metabolism, it is clear that an optimal expression of ApoE is required to maintain VLDL production and clearance. Finally, a direct involvement of ApoE in lipoprotein metabolism is related to the activation of enzymes such as hepatic lipase, cholesteryl ester transfer protein and lecithin: cholesterol acyltransferase (Greenow, Pearce, and Ramji 2005).

In homeostatic condition ApoE expression is absent in vessels, but it has been demonstrated to be high in atherosclerotic plaques both in human and animals (Rosenfeld et al. 1993).

The development of gene-targeted ApoE null mice provided evidences for the central role of ApoE in maintaining lipid homeostasis and protection from atherosclerosis. The lack of ApoE in mice has been shown to result in increased lipid levels and spontaneous atherosclerotic lesion development compared to wild type counterparts (S. H. Zhang et al. 1994). These animals show high levels of VLDL cholesterol and low levels of HDL and develop fatty lesions resulting within few months in advanced fibrous lesions.

Moreover, a great attention has been paid to clarify the contribution of specific macrophage ApoE in the development of atherosclerosis. To assess the effect of macrophage apoE, Bellosta et al reconstituted the expression of human apoE in macrophage of apoE KO mice and demonstrated that the specific macrophage expression of ApoE reduces atherosclerosis even in presence of hyperlipidemia (Bellosta et al. 1995). The athero-protective effect of macrophage ApoE was confirmed in different studies, using bone marrow transplantation approach. It has

clearly demonstrated that the absence of ApoE in macrophage is associated with increased foam cells formation in C57Bl/6 mice challenged with pro-atherogenic diet (Fazio et al. 1997) (Eck et al. 2000). The increased susceptibility to atherosclerosis development in this experimental model reflects a reduced capacity of ApoE KO macrophages to prevent foam cells generation.

Apo E and RCT

The role of ApoE in macrophage reverse cholesterol transport has been widely addressed by several in vivo and in vitro studies. ApoE is involved in the overall process of RCT. Firstly its promotes cellular cholesterol efflux, the first step of RCT, to HDL thus allowing HDL to expand the cholesterol-ester rich core. Secondly, it completes the RCT process by driving the clearance of ApoE-containing lipoprotein, through facilitating the binding to hepatic receptors (Robert W Mahley and Rall 2000). Even if the macrophage ApoE represents only a small pool of circulating ApoE, it is considered of a great importance concerning athero-protection.

After being secreted from cells, ApoE can itself promote cholesterol efflux or associate with exogenous HDL particles. In line, the expression of endogenous ApoE has been established to be fundamental to promote cholesterol efflux from human monocyte-derived macrophages (W. Y. Zhang, Gaynor, and Kruth 1996). The same result was obtained from cholesterol-loaded murine peritoneal macrophages. In this study van Eck et al. showed an impaired cholesterol efflux from macrophages which do not express ApoE compared to wild type macrophages even in absence of extracellular acceptors (Eck et al. 2000).

The mechanism by which apoE mediates cholesterol efflux has been widely studied. Some lines of evidences suggested that, at least in the initial stage of atherosclerosis, apoE-mediated efflux occurs both in a ABCA1-dependent and independent way (Huang, Fitzgerald, and Mazzone 2006). Since macrophages start to overload with cholesterol, becoming foam cells, the ABCA1-dependent pathway represents the main mechanism by which for endogenous apoE–mediated efflux (Patricia G Yancey et al. 2007). Results less conclusive and contradictory on the role of ABCG1 in expression and secretion of cellular ApoE have been provided. In fact some group showed that macrophage ABCG1 deletion is associated with increased cellular ApoE levels (Ranalletta et al. 2006), whereas no effect has been associated to total body ABCG1 deficiency (Out et al. 2007).

Recently, the role of ApoE in macrophage RCT has been clarified in in vivo study by Zanotti et al. in which it has been demonstrated that the macrophage expression of ApoE is necessary to promote an efficient RCT while the absence of systemic apoE does not impact to the process (Ilaria Zanotti et al. 2011). This study also provided a direct evidence that the deficiency of ApoE in plasma does not directly influence the mobilization of macrophage derived cholesterol, as HDL fraction from ApoE null mice have the same capacity to promote the release of cholesterol from cells compared to HDL from wild type mice. It has also been demonstrated that HDL fraction from ApoE -/- mice is mostly composed by pre β-particles, which are responsible for ABCA1-mediated efflux.

Another step forward the understanding of the contribution of macrophage and systemic ApoE has been provided by a recent study. From Annema and colleagues. They showed that the hepatic overexpression of human ApoE3 in mice is associated with increased SR-BI-mediated hepatic uptake, which does not results in increase of in vivo RCT (Annema et al. 2012). This result of unchanged fecal mass sterol excretion is related to increased activity of hepatic ABCA1 which enhances the amount of cholesterol resecreted back to plasma.

Antiatherogenic functions of ApoE

The observation that ApoE expression is able to reduce atherosclerosis in ApoE deficient mice without correcting hyperlipidemia suggested additional athero-protective not lipid-related functions. Some of these pleiotropic functions, which impact on atherosclerosis, have been shown to be related to the interaction with a specific cell surface receptor, apoER2. Binding to this receptor leads to inhibition of platelet aggregation as well as vascular adhesion molecule-1 on endothelial cells, resulting in vascular protection via activation of NO production (Greenow, Pearce, and Ramji 2005).

Several lines of evidence suggested a role of ApoE in inhibition of lipid oxidation. Lipoproteins from ApoE null mice have been demonstrated in vitro to be more susceptible to oxidation compared to lipoprotein from wild type mice (Greenow, Pearce, and Ramji 2005). Apo E is also implicated in immunoregulation as it can suppress CD4+ and CD8+ lymphocyte proliferation by interfering with mitogenic signaling (Raffai 2012). It is also likely that ApoE suppresses the antigen-dependent T cells activation (Tenger and Zhou 2003). SMCs are another cell type implicated in atherogenesis which are influenced by ApoE. ApoE inhibits proliferation and

migration of SMCs within the arterial wall. In particular, ApoE suppresses the signaling transduction through activation of NO synthase, whereas the ApoE inhibition of migration has been demonstrated to be independent from NO synthase but requires activation of cAMP-dependent protein kinase A (Greenow, Pearce, and Ramji 2005). Finally, there are some evidences that ApoE plays a role also in susceptibility to bacterial infection and sepsis (Raffai 2012). Recently Baitsch et al. demonstrated that ApoE is a regulator of macrophage polarity. By binding to apoER2, it can promote the expression of M2 markers and this leads macrophage to switch to M2 anti-inflammatory phenotype (Baitsch et al. 2011). In vivo studies show that apoE null mice display hyperproliferation of hematopoietic stem and multipotential progenitor cells (HSPCs) and develop neutrophilia and monocytosis. Importantly, ApoE suppression of lipid-induced HSPC proliferation has been shown to be ABCA1 and ABCG1 dependent (Murphy et al. 2011). Although the atheroprotective function of ApoE is mostly linked to the role in promoting RCT, it is also reasonable to consider it as a result of pleiotropic properties of apoE.

ApoE polymorphism and cardiovascular disease

As previously described, there are three common ApoE isoforms which are metabolically different from each other's. The structural differences between isoforms are responsible for impaired function of less common isoforms, which lead to the associated pathologies.

The ApoE2 homozygosity associated with environmental, genetic and hormonal factors, leads to lipoprotein disorder type III HLP. Structural analysis revealed that ApoE2 isoform is defective in binding to LDL receptors because of the presence of Cys-158 residue rather than an Arg 172. This variation changes the conformation of the apolipoprotein, thus altering the binding affinity (Robert W Mahley, Weisgraber, and Huang 2009). The resulting defect in VLDL clearance induces an accumulation of remnants apolipoproteins, thus causing hyperlipidemia.

Although ApoE2 causes hyperlipidemia and is associated with type III HLP, the major cardiovascular risk is related to ApoE4 isoform. The structural difference in ApoE4 is related to the residue Arg-122 which creates a salt bridge with Glu-109. Overall this mutation results in a more compact conformation compared to ApoE3. The more compact structure leads to enhanced binding affinity for VLDL than HDL, resulting in reduced LDL clearance. In line,

epidemiological studies have reported a high level of LDLs and apoB containing lipoproteins associated with apoE4 isoforms (Robert W Mahley, Weisgraber, and Huang 2009).

It is therefore clear that the different isoforms of ApoE impact on lipid metabolism. Consequently some studies have also been performed to investigate whether these documented structural differences impact also on the ApoE capacity to promote cholesterol efflux from foam cells. In particular no significant differences have been observed among isoforms in the ability to promote cholesterol efflux in vitro from RAW264.7 cells transfected to express human ApoE isoforms (Hara 2002). The study also addressed the effect of isoforms on efflux when apoE is exogenously used as extracellular acceptors. In line with previous findings, ApoE isoforms showed a similar capacity to promote cholesterol efflux from macrophages. Notably, the study underlines the capacity of cellular heparin sulfate proteoglycans to bind with different affinity the isoforms, thus modulating the efflux potential of each ApoE isoforms. More recently the apoE4 capacity to promote cholesterol efflux has been investigated. Altenburg at al. established that cholesterol efflux from macrophages which express ApoE4 is reduced when LDLr expression is increased (Altenburg et al. 2007).

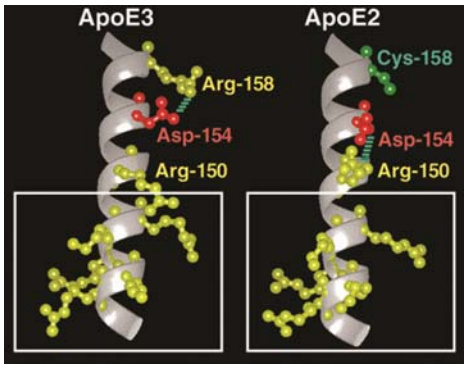

Fig.8. Structure of regions of apoE: isoform differences.(Robert W Mahley, Weisgraber, and Huang 2009)

Apo E and neuronal disorder

ApoE is the predominant apolipoprotein found in the cerebro spinal fluid (Anoop et al. 2010). Since it is directly involved in lipid transport in the brain, ApoE appears to have a critical role in neurobiological diseases. ApoE is highly produced in site of peripheral nerve injury and it was discovered to be the major gene associated with 40-60% of cases of sporadic and familial

Alzheimer's disease (Robert W Mahley and Rall 2000). ApoE effects on neurobiology appear to be isoform-specific, in particular ApoE4 has been associated with impairment in neuro-protection, whereas ApoE2 and ApoE3 protect from damage and are more effective in repairing. The functions of ApoE in central nervous system include regulation of ion-dependent receptors, modulation of neurotransmitter and protection from oxidative stress (Anoop et al. 2010). Studies showed that the ApoE expression is enhanced after injury to promote neurite outgrowth (Curtiss and Boisvert 2000).

The most important function of ApoE in brain is the capacity to regulate the production of amyloid β (Aβ) peptide which is the major component of Alzheimer's disease amyloid plaque. The interaction of ApoE with Aβ is mediated by the lipid binding region. The binding with specific ApoE receptors triggers the clearance of peptide reducing the accumulation of the peptide. It has been shown in vitro that ApoE4 enhances Aβ production more than ApoE3 but this effect can be inhibited by molecules which block the intramolecular domain interaction (L. Dong et al. 2005). Further studies performed in neuronal cells showed that treatment with exogenous ApoE4 results in increased apoptosis whereas ApoE3 appeared protective (Robert W Mahley, Weisgraber, and Huang 2009). Another detrimental effect of ApoE4 is the neurotoxicity induced by the accumulation of ApoE4 fragments. Again, the structure of ApoE4 leads this isoform to be more resistant to enzyme cleavage, resulting in translocation of apolipoprotein in mitochondria where it interferes with physiological functions (Robert W Mahley, Weisgraber, and Huang 2009).

PART I:

Role of Apolipoprotein E in cellular cholesterol flux

Aim

Apolipoprotein E is a key regulator of plasma lipid levels and its deficiency results in hyperlipidemia and increased atherosclerosis in mice (S. H. Zhang et al. 1994). Several in vivo studies have shown that the athero-protective property of apoE is totally related to its expression in macrophages and is independent from its effect on plasma lipid levels reviewed in (Greenow, Pearce, and Ramji 2005). Indeed, the apoE specifically expressed in macrophages has been found to decrease the atherosclerosis development, without affecting plasma lipid levels, in hyperlipidemic ApoE null mice (Bellosta et al. 1995). Since the expression of endogenous apoE in macrophages has been documented to enhance cellular cholesterol efflux (Eck et al. 2000), the atheroprotective role of ApoE has been generally associated to its capacity to promote macrophage RCT. More recently the role of ApoE in overall macrophage RCT in vivo has been established by Zanotti et al., who demonstrated that RCT is impaired in apoE null mice compared to WT (C57BL/6) mice. Moreover, this study distinguished for the first time between the contribution of systemic and macrophage ApoE to macrophage RCT, and clearly established that macrophage, but not systemic, ApoE is fundamental to promote RCT from foam cells (Ilaria Zanotti et al. 2011). When focused on first step of the process, this study provided the challenging observation that HDL fraction isolated from plasma of ApoE null mice promotes a similar release of cholesterol from cholesterol-loaded macrophages as compared to wild type counterparts.

The aim of this research arose from the observation that systemic ApoE deletion, despite inducing a pro-atherogenic plasma lipid profile, characterized by high concentrations of ApoB-containing lipoproteins and low HDL levels, does not affect its efficiency in mediating cholesterol release from cells. The purpose of this study is to further investigate the role of systemic and macrophage apoE on lipid exchange between cells and extracellular acceptors. In particular, this research is mainly addressed to evaluate the influence of both systemic and macrophage ApoE on foam cell formation and bidirectional cholesterol flux process. HDL represent the principal lipoprotein involved in mobilization of macrophage-derived cholesterol through RCT pathway. However, recent evidence suggested that other component of plasma, such as ApoB-containing lipoproteins, could also contribute to lipid exchange between plasma and cells (Chan et al. 2012). To provide better model of the physiological condition, whole plasma from wild type and apoE null mice is tested to analyze the influence of systemic apoE on plasma capacity to promote cholesterol efflux from cells in vitro. In this study different cell types are used to characterize different pathways of efflux.

Recently it has been demonstrated that apoE secretion from cultured macrophages could be affected by a widely used immunosuppressant drug, Cyclosporine A, (Kockx et al. 2009). Since ApoE specifically expressed in macrophage has been shown to be essential to promote RCT in vivo (Ilaria Zanotti et al. 2011), we investigated whether the treatment with CsA, by modulating the expression of endogenous ApoE, could affect RCT in vivo.

Materials and methods

ANIMALS

C57BL/6 mice (WT), and apoE-/- (B6.129P2-apoE^{tm1Unc}/Crl) mice (The Jackson Laboratory, Harbor, Me) received a standard chow diet (Mucedola) and water ad libitum. The study was performed with the approval of the Ethical Committee for Animal Experiments of the University of Parma, Parma, Italy.

EVALUATION OF PLASMA EFFLUX CAPACITY

Acetylated low density lipoproteins (acLDL) wereobtained by modification of LDL with acetic anhydride, as described previously (Bernini et al. 1997). HDL and HDL2 were kindly provided by Professor Laura Calabresi. Mice were sacrificed and blood was collected from heart puncture, recovered in plastic tubes and anticoagulated with sodium citrate 3.8%. Plasma was isolated by low speed centrifugation and stored at -80^{o}C until use. Aliquots of plasma from C57BL/6J (WT) and ApoE-/- mice were slowly defrosted in ice just before addition to cells as extracellular acceptors in cholesterol efflux experiments.

Measurement of cholesterol efflux from murine peritoneal macrophages

MPM harvested from the peritoneum of thioglycollate-treated mice, were plated and were radiolabeled with [^{3}H]-cholesterol 2μCi/mL and cholesterol enriched by incubation with 25μg/mL of AcLDL in 1% fetal calf serum (FCS) containing medium for 24h. After a 18h equilibration period in an albumin-containing medium, cholesterol efflux was promoted to plasma diluted to 0.1-0.5-1% (v/v) from either WT and ApoE-/- mice for 6h. An ACAT inhibitor (2 μg/ml, Sandoz 58035) was added during labeling and equilibration period to prevent cellular accumulation of cholesteryl ester (Zanotti et al. 2012). Plasma cholesterol capacity has been calculated as a percentage of the radioactivity released to the medium in 6h over the radioactivity incorporated by cells before addition of plasma (Time zero). The radioactivity in the medium was determined by liquid scintillation counting. To analyze cellular [^{3}H]-cholesterol content, cell monolayers were extracted by the addition of 0.6 ml of 2-propanol. The

lipid extracts were dried under a stream of N_2, resuspended in toluene, and quantified by liquid scintillation counting. Every plasma sample was analyzed in triplicate and the average and standard deviation have been obtained.

Measurement of passive diffusion and ABCA1-mediated cholesterol efflux

Efflux by passive diffusion was measured using J774 macrophages. ABCA1 mediated efflux was measured using J774 macrophage treated with cpt-cAMP to upregulate ABCA1 (Bortnick et al. 2000a). Cells were grown in DMEM with 10% FCS, incubated at 37°C in 5% CO_2, seeded in 12-well plate and utilized at 80-90% confluence. Monolayers were radiolabeled with [^{3}H]-cholesterol 2μCi/mL in medium containing 1% FCS. Following 24h labeling period, cells were washed and incubated with 0.2%BSA, with or without 0.3mM cpt-cAMP for 18h. An ACAT inhibitor Sandoz 58035) was added during labeling and equilibration period to prevent cellular accumulation of cholesteryl ester (Zanotti et al. 2012). After equilibration period cells were incubated with 2.5% (v/v) plasma isolated from WT and ApoE-/- for 4h. The radioactivity in the medium was determined by liquid scintillation counting. Cholesterol efflux was calculated as a percentage of the radioactivity released to the medium in 4h over the radioactivity incorporated by cells before addition of plasma (Time zero). To analyze cellular [^{3}H]cholesterol content, cell monolayers were extracted by the addition of 0.6 ml of 2-propanol. The lipid extracts were dried under a stream of N_2, resuspended in toluene, and quantified by liquid scintillation counting. Every plasma sample was analyzed in triplicate and the average and standard deviation have been obtained. The ABCA1-mediated cholesterol efflux was calculated as the percentage efflux from stimulated J774 macrophages minus the percentage efflux from unstimulated J774 cells.

Measurement of ABCG1-mediated cholesterol efflux

CHO-K1 cells stably expressing human ABCG1 were used to measure ABCG1-mediated cholesterol efflux and were generated as previously described (Gelissen et al. 2006). Parent and hABCG1-expressing cells were labeled for 24h with [^{3}H]-cholesterol 2μCi/mL in medium containing 10% FCS. Following 24h cells were washed, and equilibrated for 90 minutes in serum-free medium, then incubated in efflux medium containing BSA (1mg/ml) in the presence

of 1% (v/v) plasma isolated from WT and ApoE-/- mice for 6h. Cells and media were assayed for radioactivity. Cholesterol efflux was calculated as a percentage of the radioactivity released to the medium in 6h over the radioactivity incorporated by cells before addition of plasma (Time zero). To analyze cellular [^{3}H]-cholesterol content, cell monolayers were extracted by the addition of 0.6 ml of 2-propanol. The lipid extracts were dried under a stream of N_2, resuspended in toluene, and quantified by liquid scintillation counting. Every plasma sample was analyzed in triplicate and the average and standard deviation have been obtained. The ABCG1-mediated cholesterol efflux was calculated as the percentage efflux from transfected cells minus the percentage efflux from CHO-K1 cells.

Measurement of SR-BI-mediated cholesterol efflux

SR-BI-mediated efflux was tested in rat hepatoma Fu5AH cells which highly express SR-BI transporter (De la Llera-Moya et al. 1999b). Cells were seeded in 12-well plates and radiolabeled with [^{3}H]-cholesterol 2µCi/mL in DMEM medium containing 1% FCS for 24h. Cells were then equilibrated in BSA 0.2% containing medium. During labeling period and equilibration period cells were exposed to an ACAT inhibitor (2 µg/ml, Sandoz 58035) to ensure that all labeled cholesterol was present as free cholesterol. Cells were then washed and incubated with 2.5% (v/v) plasma from WT and ApoE-/- mice for 4h. The radioactivity in the medium was determined by liquid scintillation counting. Cholesterol efflux was calculated as a percentage of the radioactivity released to the medium in 4h over the radioactivity incorporated by cells before addition of plasma (Time zero). Cell monolayers of baseline (time 0) were extracted by the addition of 0.6 ml of 2-propanol. The lipid extracts were dried under a stream of N_2, resuspended in toluene, and quantified by liquid scintillation counting. Every plasma sample was analyzed in triplicate and the average and standard deviation have been obtained.

Measurement of phospholipid efflux from WT and ApoE-/- MPM

The phospholipid efflux was performed as described previously (Yancey et al., 1995). MPM harvested from the peritoneum of thioglycollate-treated WT and ApoE-/- mice, were plated and were labeled for 48 h with 4 Ci/ml [methyl-^{3}H]choline chloride 1% FCS in RPMI 1640 with AcLDL 25µg/mL, cells were equilibrated for 18 h in 0.2% BSA RPMI 1640. Efflux was promoted to plasma from WT and ApoE-/- mice diluted to 0.1-0.5-1% (v/v) After 4 hours, media were centrifuged, and the supernatant was removed and phospholipids extracted by the Bligh and Dyer method (Iverson et al., 2001).

BIDIRECTIONAL FLUX FROM MOUSE PERITONEAL MACROPHAGES

MPM harvested from the peritoneum of thioglycollate-treated WT and ApoE-/- mice, were plated and cholesterol enriched with AcLDL 25µg/mL for 24h. After an equilibration period in 0.2% BSA-containing medium, a set of cells was harvested before the incubation with cholesterol acceptors (time zero) and the cholesterol content of cell lysates was measured as described below. The remaining cells were incubated with either plasma from WT or ApoE-/- mice for 6 h. Furthermore, to prevent cholesterol esterification, 2 µg/ml of the ACAT inhibitor (Sandoz 58035) were added during labeling, equilibration, and the flux stages of the experiment.

BIDIRECTIONAL FLUX FROM FU5AH

Rat Hepatoma Fu5AH cells were seeded in 12-well plates in DMEM medium containing 10% FCS for 24h. Cells were then equilibrated in BSA 0.2% containing medium. During equilibration period cells were exposed to an ACAT inhibitor (2 µg/ml, Sandoz 58035) to ensure that all labeled cholesterol was present as free cholesterol. Cells were then washed and incubated with 2.5% (v/v) plasma from WT, ApoE-/- and LDLr-/- mice for 24h.

Cholesterol determination

The experiment was performed as described previously (Zimetti et al. 2006). At the end of the efflux period, cell monolayers were washed with PBS and lysed in 0.5 ml of a 1% sodium cholate solution in water supplemented with 10 U/ml DNase. A total of 125 µl of a reaction buffer containing 0.5% Triton X-100, 0.5 M potassium phosphate (pH 7.4), 0.25 M NaCl, and 1% sodium cholate were added to the cell lysates, and plates were shaken for 30 min at room temperature. Care must be taken to ensure complete solubilization of the cell monolayer. After heating samples at 60°C for 30 min, to inactivate enzymes that could compete with the enzymatic cholesterol assay, cholesterol was measured fluorimetrically using the Amplex Red Cholesterol Assay Kit (Molecular Probes, Eugene, OR) as described by the manufacturer. The enzymatic kit assay for cholesterol included a cholesterol esterase and allowed the measurement of all cellular cholesterol. This permitted the quantification of any CE incorporated from

lipoproteins. The amount of cholesterol in each well was measured by comparison with a cholesterol standard curve included in each experiment. An aliquot of the cell lysates was also taken to measure cell protein by a modified Lowry method (Lowry et al. 1951).

EVALUATION OF MACROPHAGE APOE CONTRIBUTION TO CHOLESTEROL EFFLUX

Measurement of SR-BI and ABCG1-mediated cholesterol efflux from murine peritoneal macrophages

MPM harvested from the peritoneum of thioglycollate-treated WT and ApoE-/-mice, were plated, treated or not with AcLDL 25µg/mL and were radiolabeled with [^{3}H]-cholesterol 2µCi/mL in 1% fetal calf serum containing medium for 24h. Cells were equilibrated for 18h in an albumin-containing medium with or without an association of 22OH-cholesterol 5µg/mL and 9cis-retinoic acid 10µM. In the equilibration period, some cells were treated with probucol (10µM) and some with BLT-1 (10µM) to inhibit ABCA1 and SR-BI respectively. After this period cholesterol efflux was promoted to HDL2 10µg/mL (v/v) or HDL 12µg/mL for 4h. An ACAT inhibitor (2 µg/ml, Sandoz 58035) was added during labeling and equilibration period to prevent cellular accumulation of cholesteryl ester (Zanotti et al. 2012). Plasma cholesterol capacity has been calculated as a percentage of the radioactivity released to the medium in 6h over the radioactivity incorporated by cells before addition of plasma (Time zero). The radioactivity in the medium was determined by liquid scintillation counting. To analyze cellular [^{3}H]-cholesterol content, cell monolayers were extracted by the addition of 0.6 ml of 2-propanol. The lipid extracts were dried under a stream of N_2, resuspended in toluene, and quantified by liquid scintillation counting. Every plasma sample was analyzed in triplicate and the average and standard deviation have been obtained.

Free cholesterol oxidation in MPM

Cholesterol oxidase experiment was performed as previously described (Llera-moya et al. 2000). MPM harvested from the peritoneum of thioglycollate-treated WT and ApoE-/-mice were plated in 12-wells plate and radiolabeled with 2 Ci/mL [^{3}H]cholesterol for 24h in RPMI medium 1% FCS with or without AcLDL 25µg/mL. Cells were then equilibrated in an albumin-containing medium for 18 hours with or without cAMP 0.3mM, or 22-OHcholesterol and 9cis retinoic acid. After this period cells were washed with PBS and Cholesterol oxidase (Sigma) was added at a concentration of 1 U/mL for 4h 37°C. At the end of oxidation period, cells were

chilled and washed with ice cold PBS. Lipids were extracted from monolayers with 2-propanol, separated by thin-layer chromatography (TLC) (mobile phase: 96 mL hexane, 15 mL methanol, 8 mL ethyl ether), and quantified by liquid scintillation counting. The amount of cholestenone was expressed as percentage of total cholesterol.

ACAT activity

MPM harvested from the peritoneum of thioglycollate-treated WT and ApoE-/-mice were plated in 12-wells plate and cholesterol loaded with AcLDL 25µg/mL for 4h in medium 0.2% BSA. Cells were then radiolabelled with [^{14}C]-oleic acid for 4h. After the incubation period, lipid were extracted from cells and separated by thin-layer chromatography (TLC) (mobile phase: 75mL isoctane, 25 mL ethyl ether, 2mL acetic acid). The experiment was performed in triplicate. The CE content was expressed on the protein content for each sample. Monolayers were extracted to measure cell protein by a modified Lowry method (Lowry et al. 1951).

QUANTIFICATION OF MACROPHAGE RCT IN VIVO

Animals and drug administration

Twelve-week-old male C57BL/6J mice were housed in a controlled environment at 25 ± 2°C with alternating 12 h light and dark cycles and received standard diet and water ad libitum. Mice were treated for 14 days by subcutaneous injection with CsA dissolved in olive oil at the dose of 50 mg·kg-1 (n=7) or vehicle (n=7), once a day. On day 14, 4h after the last drug administration, mice were sacrificed by excess anesthesia with ethyl ether. Blood was collected by cardiac puncture and recovered in plastic tubes containing sodium citrate 3.8 %. Plasma was isolated by low speed centrifugation and stored at -80°C until use, as described below. Livers were collected at the end of the treatment period and immediately frozen in liquid nitrogen. Feces were collected on day 14 of drug treatment. Samples of liver and feces were extracted by the Bligh and Dyer method (Bligh, E. G., W. J. Dyer. 1959), radioactivity in the lipid extracts was measured by liquid scintillation counting.

Animal care and experimental procedures were performed with the approval of the Ethical Committees overseeing animal experiments at University of Parma.

Evaluation of RCT in vivo

Measurement of RCT was performed as previously described (YuZhen Zhang et al. 2003). On day 11 of pharmacological treatment with CsA, thioglycollate-elicited murine peritoneal macrophages (MPM) or J774 macrophages were cholesterol-enriched with 25 μg/mL acetylated low density lipoproteins (AcLDL) and radiolabeled with 5 μCi/mL [^{3}H]-cholesterol. On day 13, cells were injected intraperitoneally into recipient mice. On day 14, mice were sacrificed and samples were collected as described above.

STATISTICAL ANALYSIS

The statistical analysis were performed with Prism 5 software. (GraphPad Software, San Diego, California). Efflux results were analyzed using Student's t test, comparisons between control and treated groups in RCT studies were made by Mann Whitney test. A level of $p<0.05$ was considered significant.

Results

ROLE OF SYSTEMIC APO E IN LIPID EFFLUX

Effect of systemic ApoE on cholesterol efflux from murine peritoneal macrophages cholesterol-loaded with AcLDL

Cholesterol efflux from cells is the rate limiting step of macrophage RCT, as it regulates the amount of cholesterol released from foam cells of the arterial wall and available to be delivered to the liver for disposal. The more physiological approach to study the variables affecting cholesterol efflux is measuring cholesterol efflux from lipid-loaded macrophages as a models representative of the foam cells of the arterial wall.

Systemic ApoE has been recently shown to not affect the efficiency of macrophage RCT in vivo (Ilaria Zanotti et al. 2011). In order to investigate the role of systemic ApoE on the first limiting step of RCT, we performed standard cholesterol efflux assay in which the capacity of plasma from C57BL/6 (WT) and ApoE-/- mice to induce cholesterol efflux was tested.

Thyoglicollate-elicited murine peritoneal macrophages (MPM) from WT mice were cholesterol-loaded with AcLDL 25 μg/mL and exposed for 6 hours to increasing concentration of plasma containing or not ApoE. Plasma from ApoE KO mice, despite a well described different lipid composition (Zhang et al 1992), showed a similar capacity to promote cholesterol release from foam cells as compared to plasma from WT mice (Fig.9).

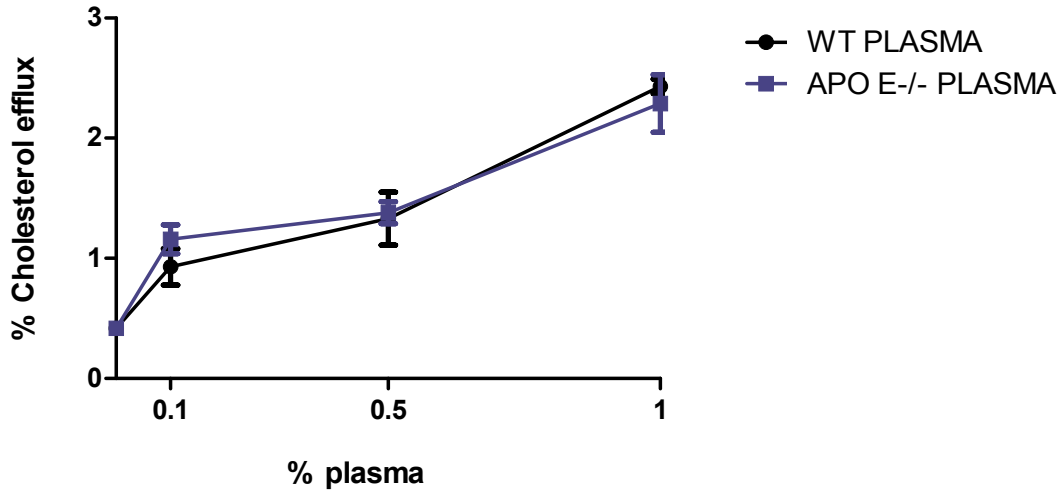

Fig.9 Cholesterol efflux from WT MPM to plasma isolated from WT and ApoE-/- mice. MPM from WT mice were cholesterol loaded with AcLDL 25µg/mL and radiolabeled with [^{3}H]-cholesterol 2µCi/mL for 24 hours in RPMI 1640 medium with 1% FCS and 2µg/mL of an ACAT inhibitor. Cells were then equilibrated in an albumin-containing medium for 18 hours, and exposed for 6 hours to increasing concentrations of plasma isolated from WT and ApoE-/- mice. The experiment was performed in triplicate. Efflux was expressed as counts per minute in medium/counts per minute of time 0x100± SD .

Effect of systemic ApoE on ABCA1-mediated efflux

ABCA1-mediated efflux has been shown to account approximately for 35% of total efflux from cholesterol-loaded macrophages (Adorni et al. 2007). The influence of systemic ApoE in promoting release of cholesterol via ABCA1 was tested in J774 macrophages in which the expression of ABCA1 was induced by treatment with cpt-AMP (Bortnick et al. 2000). Under basal conditions, when cholesterol efflux occurs mostly by passive diffusion and SR-BI, plasma from ApoE null mice showed a higher capacity to induce efflux compared to WT plasma (8.77%±0.84 and 6.41%±1.64 respectively, $p<0.01$). After treatment with cpt-AMP, which induces the expression of ABCA1 transporter, total cholesterol efflux was greater and plasma from ApoE null mice still showed a statistically significant higher percentage of cholesterol efflux in comparison to WT plasma (13.94%±2.54 vs 10.26%±1.83 respectively). The ABCA1-mediated cholesterol efflux was calculated as the difference between total efflux from cpt-AMP-stimulated cells and passive efflux from cpt-AMP-unstimulated cells. Although it failed to reach the statistical significance, plasma from ApoE null mice showed to promote a higher efflux via ABCA1.

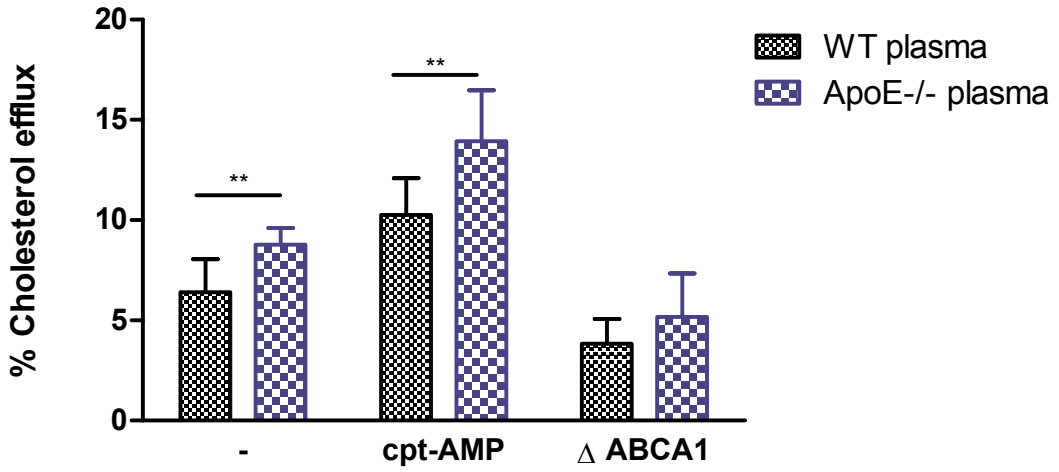

Fig.10 Cholesterol efflux from J774 macrophages to plasma isolated from WT and ApoE-/- mice. J774 macrophage were radiolabeled with [^{3}H]-cholesterol for 24 hours, in DMEM medium with 1% FCS and 2µg/mL of an ACAT inhibitor. Then, cells were incubated for 18h with 0.2% BSA in presence of 0.3mM cpt-cAMP, washed, and incubated for 4h with 2.5% plasma isolated from WT and ApoE-/-. The experiment was performed in triplicate. Efflux was expressed as counts per minute in medium/counts per minute of time 0x100± SD . ABCA1-mediated cholesterol efflux was calculated as the percentage efflux from cpt-cAMP-stimulated cells minus the percentage efflux from unstimulated cells. Statistically significant difference **p<0.01 vs WT plasma.

Effect of systemic ApoE on ABCG1-mediated efflux

Cholesterol efflux from cholesterol-loaded macrophages can occur via ABCG1, which contributes for approximately 20% of total cholesterol efflux (Adorni et al. 2007). To test the effect of systemic ApoE on ABCG1-mediated efflux, we performed efflux assay in stably transfected ABCG1-overexpressing CHO cells (Gelissen et al. 2006). The ABCG1 contribution to cholesterol efflux was then calculated as the difference between the percentage efflux from transfected cells and the percentage efflux from CHO-K1 parent cells. Non transfected cells (CHO CTRL) express little ABCG1 and release small amount of cholesterol to plasma by passive diffusion. ApoE containing plasma showed increased capacity to promote efflux from CTRL cells as compared to WT plasma (9.3%±2.2 vs 6.9%±0.7 $p<0.05$). In ABCG1 transfected cells efflux to plasma from ApoE-/- mice was higher than efflux to WT mice (20.7±2.3 vs 17.7±2.2 $p<0.05$). The ABCG1 contribution to efflux was calculated as difference between efflux from cells transfected with human ABCG1 and efflux from parent cells. As showed in

Fig.11 plasma from ApoE-/- null mice showed to induce a similar ABCG1- mediated efflux as compare to plasma from WT mice.

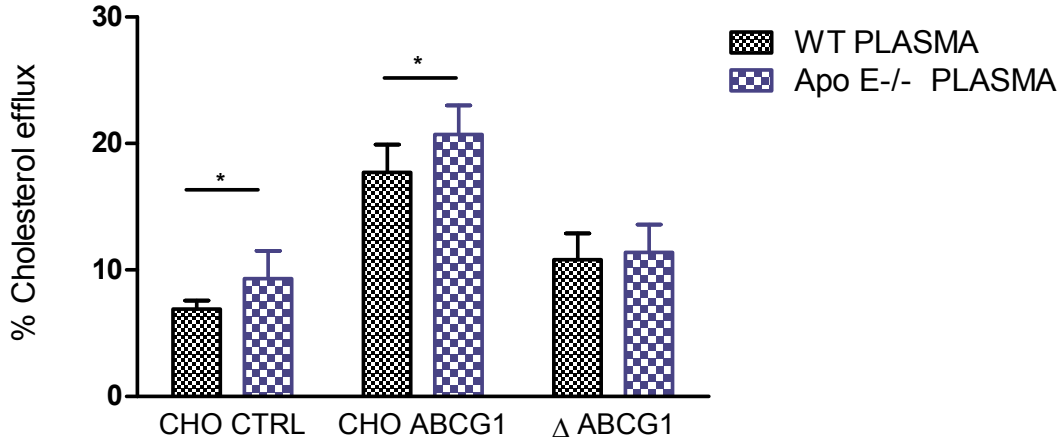

Fig.11 Cholesterol efflux from CHO cells to plasma isolated from WT and ApoE-/- mice. Wild-type and human ABCG1-overexpressing CHO cells were labeled with 1 μCi/mL [^{3}H]cholesterol in HAM's medium with10% FCS for 24 h. Then, cells were washed, equilibrated for 90 min in serum-free medium, and incubated for 6 h with 1% plasma isolated from WT and ApoE-/- mice. The experiment was performed in triplicate. Efflux was expressed as counts per minute in medium/counts per minute of time 0x100± SD . The ABCG1-mediated cholesterol efflux was then calculated as the difference between the percentage efflux from transfected cells and the percentage efflux from CHO-K1 parent cells. Statistically significant difference *$p<0.05$, vs WT plasma.

Effect of systemic ApoE on SR-BI-mediated efflux

SR-BI-mediated efflux was tested in rat hepatoma Fu5AH cells which highly express SR-BI transporter (De la Llera-Moya et al. 1999). The ApoE contribution to ability of plasma to induce release of cholesterol from cells were measured comparing efflux promoted by plasma isolated from WT and ApoE-/- mice.

Plasma from ApoE deficient mice promote a significantly greater cholesterol efflux through SR-BI compared to WT plasma (11.1±1.3 vs 7.8±1.9 $p<0.001$).

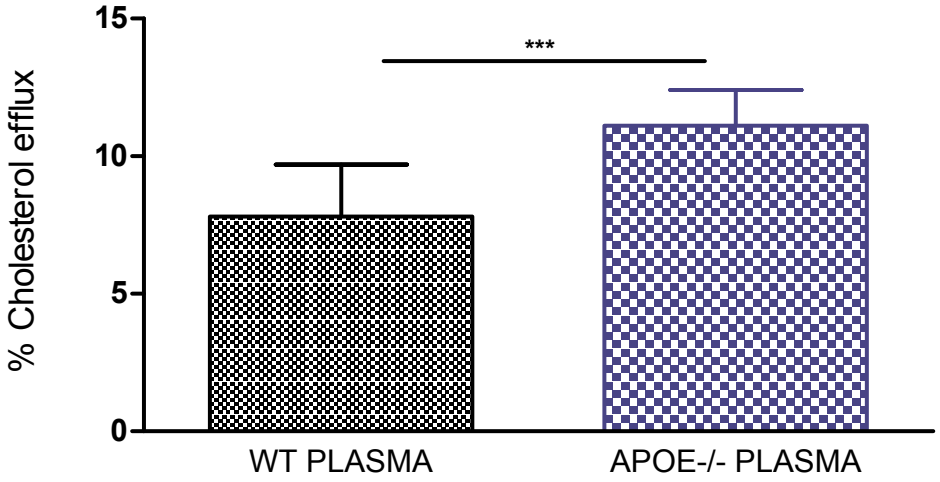

Fig.12 Cholesterol efflux from Fu5AH cells to plasma isolated from WT and ApoE-/- mice. Cells were labeled with 2μCi/ml [^{3}H]cholesterol for 24h in DMEM medium with 1% FCS and 2μg/ml of an ACAT inhibitor. Cells were then incubated for 18h with 0.2% BSA, washed and incubated for 4h with 2.5% plasma isolated from WT and ApoE-/- mice. The experiment was performed in triplicate. Efflux was expressed as counts per minute in medium/counts per minute of time 0x100± SD . Statistically significant difference ***p<0.001, vs WT plasma.

Role of systemic ApoE in phospholipid efflux from cholesterol-loaded MPM

To further evaluate the effect of systemic ApoE on lipid efflux from foam cells in the first step of RCT, we compared phospholipid efflux after exposition to increasing concentration of plasma from WT and ApoE null mice. Since this process is mainly mediated by ABCA1 (N Wang et al. 2001), we induced the expression of this transporter by treating MPM from WT mice with AcLDL 25 μg/mL. Plasma isolated from ApoE-/- mice resembles plasma from WT mice in the capacity to promote phospholipid efflux from foam cells.

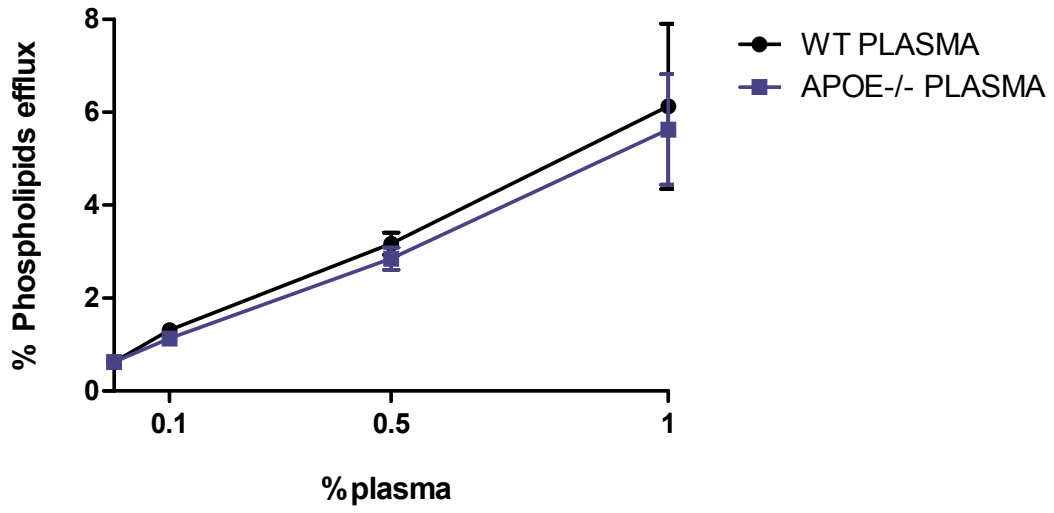

Fig.13 Phospholipid efflux from cholesterol-loaded MPM to plasma isolated from WT and ApoE-/- mice. Cells were cholesterol loaded with AcLDL 25µg/mL and radiolabeled with 4 Ci/ml [^{3}H]choline for 48h in RPMI 1640 medium 1% FCS. Cells were then incubated for 18h with 0.2% BSA, washed and incubated for 4h with increased concentration of plasma isolated from WT and ApoE-/- mice. The experiment was performed in triplicate. Efflux was expressed as counts per minute in medium/counts per minute of time 0x100± SD .

ROLE OF SYSTEMIC AND MACROPHAGE APOE ON BIDIRECTIONAL FLUX BETWEEN EXTRACELLULAR LIPOPROTEIN AND CELLS

Exposition to whole plasma leads to bidirectional flux of cholesterol between cells and extracellular acceptors. The rate of this process depends on both cell cholesterol content and plasma lipid composition. Net flux is measured as the difference between efflux and influx and can results in net accumulation, depletion or not change in the amount of intracellular cholesterol after exposition to plasma (Zimetti et al. 2006).

Net flux of cholesterol from WT and ApoE-/- MPM after exposition to WT and Apo E-/- plasma

We investigated the role of both systemic and macrophage ApoE in net exchange of cholesterol when foam cells are incubated with whole plasma.

Cholesterol enriched MPM from WT and ApoE null mice were respectively exposed to plasma from WT and ApoE null mice. Exposition to plasma from WT or ApoE-/- mice resulted in cholesterol depletion independently from the expression of ApoE. Macrophage from ApoE null mice showed less significant reduction of cellular cholesterol content as compared to WT macrophages (-3.41 ±2.72μg/mg vs -20.40±0.36 μg/mg and -5.98 ±5.03 vs -24.53±0.80).

Moreover, WT macrophages showed a higher reduction of the amount of intracellular cholesterol after incubation with plasma not expressing ApoE as compared to WT plasma. After cell exposition to both WT and apo E-/- plasma, the amount of intracellular cholesterol measured in ApoE-/- MPM decreased in a comparable manner (-3.41 ±2.72μg/mg vs -5.98 ±5.03 respectively).

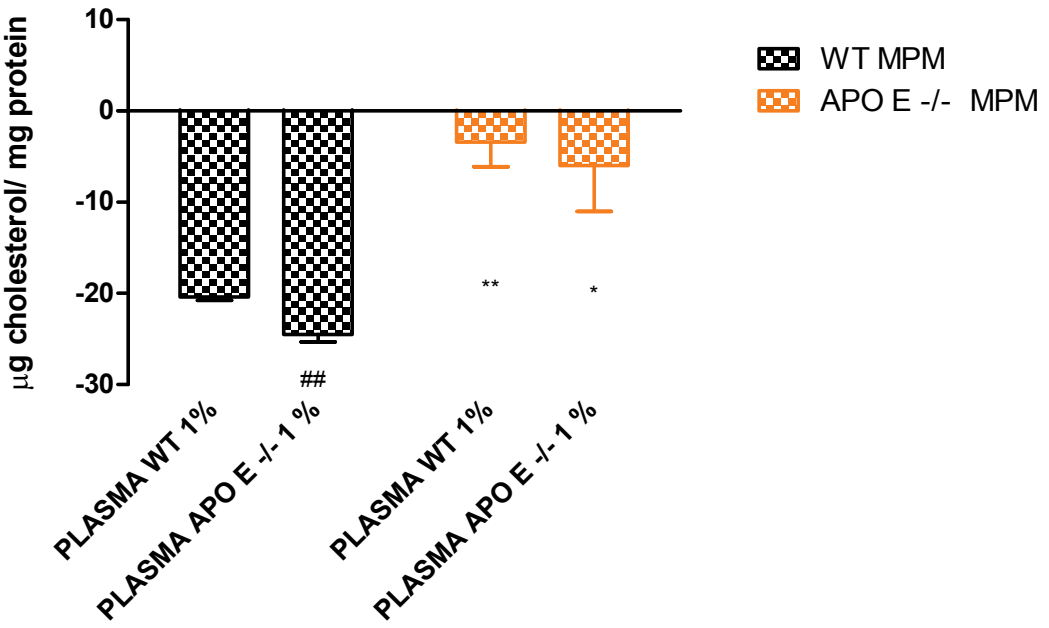

Fig.14 Net cholesterol flux in MPM from WT and ApoE-/- after exposition to plasma isolated from WT and ApoE-/- mice. MPM from WT and ApoE-/-mice were cholesterol loaded with AcLDL 25µg/mL for 24 hours in RPMI 1640 medium with 1% FCS and 2µg/mL of an ACAT inhibitor, equilibrated in an albumin-containing medium for 18 hours, and exposed for 6 hours to 1% plasma isolated from WT and ApoE-/- mice. Intracellular cholesterol content was quantified as described in method section. The experiment was performed in triplicate. Statistically significant difference *p<0.05**p<0.01, vs WT MPM; ## p<0.01 vs WT plasma.

Net flux of cholesterol from Fu5AH after exposition to WT vs Apo E-/- vs LDLr-/- plasma

In order to evaluate the influence of systemic ApoE on lipid exchange between cells and plasma in the second step of RCT, the net flux of cholesterol was evaluated in rat Fu5AH hepatoma cells after exposition to plasma from ApoE-/- mice as compared to normolipidemic plasma (WT) and hyperlipidemic plasma from LDLr-/-. LDLr -/- mice are characterized by elevated levels of VLDL and LDL, due to impaired clearance of LDL from circulation (Ishibashi et al. 1993). Incubating Fu5AH with plasma from WT and ApoE null mice resulted in a similar change of intracellular cholesterol content, indicating almost not changes in cholesterol mass (-0.74µg/mg ±1.05 vs 1.83µg/mg ±3.3 respectively).

In contrast, exposure of hepatoma cells to hyperlipidemic plasma from LDLr-/- mice resulted in net cholesterol mass accumulation as compared to exposure to ApoE-/- plasma (12.03µg/mg ± 2.49 vs 1.83µg/mg ±3.3 respectively).

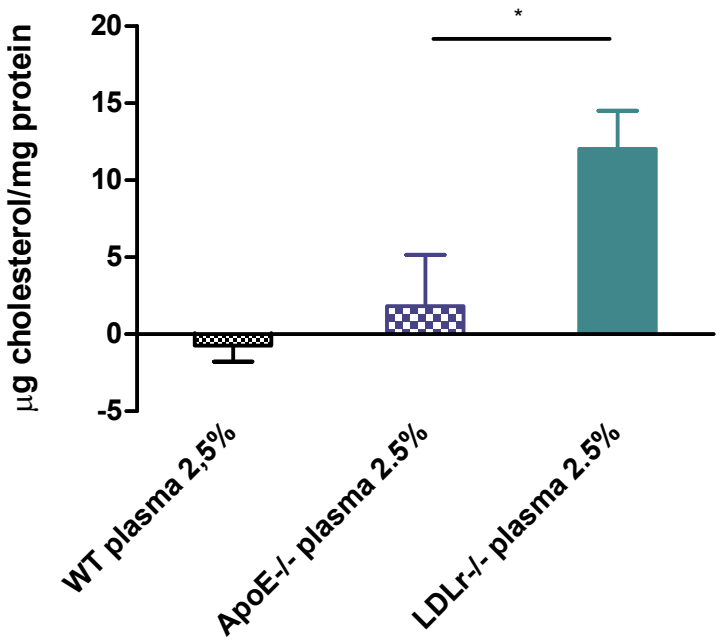

Fig.15 Net cholesterol flux in Fu5AH after incubation with plasma isolated from WT, ApoE-/- and LDLr-/- mice. Fu5AH hepatoma cells were cholesterol loaded with AcLDL 25µg/mL for 24 hours in DMEM medium with 1% FCS, equilibrated in an albumin-containing medium for 18 hours, and exposed for 24 hours to 1% plasma isolated from WT and ApoE-/- and LDLr-/- mice. Intracellular cholesterol content was quantified as described in method section. The experiment was performed in triplicate. Statistically significant difference *$p<0.05$, vs LDLr-/- plasma.

ROLE OF MACROPHAGE APOE IN LIPID EFFLUX

In cholesterol-loaded macrophages the cholesterol efflux occurs principally via passive diffusion and via ABCA1, which account for approximately 30% of total cholesterol efflux (Adorni et al. 2007). Since the interaction between ABCA1 and ApoE expressed in macrophages has been already clarified (Patricia G Yancey et al. 2007), we focused on the role of macrophage ApoE on cholesterol efflux pathways mediated by ABCG1 and SR-BI transporters.

Role of macrophage ApoE in ABCG1-mediated cholesterol efflux

Macrophage ApoE promotes cholesterol efflux to extracellular acceptors. Since ABCG1 efflux has been shown to account for approximately 20% of efflux from foam cells (Adorni et al. 2007), we investigated the effect of macrophage ApoE on specific ABCG1-mediated efflux by comparing efflux from WT and ApoE null macrophages.

To specifically evaluate ABCG1 contribution to efflux from macrophages, we induced the expression of this transporter by treating cells with LXR/RXR agonist, 22-OH cholesterol/9cis retinoic acid (Zanotti et al. 2012). Then, we promoted efflux using large phospholipid-rich HDL2 particle as cholesterol acceptors, which specifically interacts with ABCG1 (Gelissen et al. 2006). Moreover, to eliminate ABCA1 contribution to efflux, we treated cells with probucol, a specific inhibitor of ABCA1 (Favari et al. 2004). In all conditions, macrophage expressing ApoE showed an increased cholesterol efflux as compared to ApoE-/- macrophages (Fig.16).

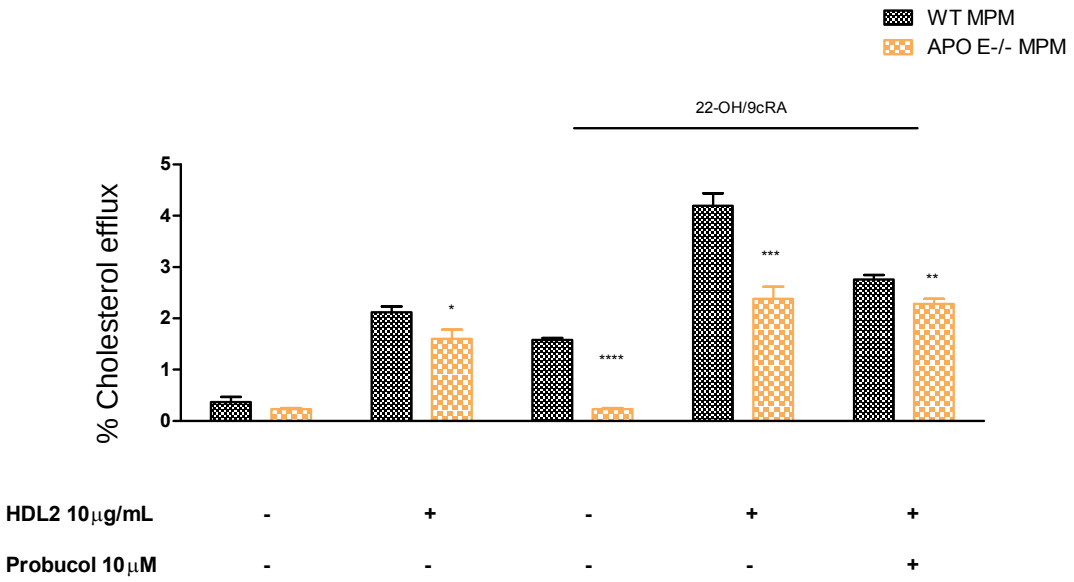

Fig.16 Cholesterol efflux from WT and ApoE-/- MPM to HDL2. MPM from WT and ApoE-/- mice were radiolabeled with [^{3}H]-cholesterol for 24 hours in RPMI 1640 medium with 1% FCS and 2µg/mL of an ACAT inhibitor, equilibrated in an albumin-containing medium for 18 hours in presence or not of 22-OHcholesterol 5µg/mL and 9cis retinoic 10µM acid. Cells were then treated or not with probucol 10 µM for 2h and exposed for 4 hours to HDL2 10µg/mL. The experiment was performed in triplicate. Efflux was expressed as counts per minute in medium/counts per minute of time 0x100± SD. Statistically significant difference *p<0.05,**p<0.01, ***p<0.001, ****p<0.0001vs WT MPM.

Role of macrophage ApoE in SR-BI-mediated cholesterol efflux from cholesterol-loaded MPM

SR-BI mediated efflux from foam cell macrophages has been reported to account for only a 9% of total cholesterol efflux in vitro (Adorni et al. 2007). We investigated the effect of the expression of ApoE in macrophage on SR-BI-mediated cholesterol efflux comparing efflux from WT and ApoE-/- macrophages. To evaluate the contribution of SR-BI to cholesterol efflux from foam cells we first induced the cholesterol loading by treating cells with AcLDL. In order to appreciate the specific SR-BI-mediated efflux we inhibited the receptor by using BLT-1 before promoting efflux to HDL acceptors.

Results showed that macrophages from ApoE-/- showed a reduced efflux to HDL as compared to WT macrophage. Moreover, treatment with BLT-1 did not induce a significant different cholesterol efflux from ApoE lacking macrophage (Fig.17).

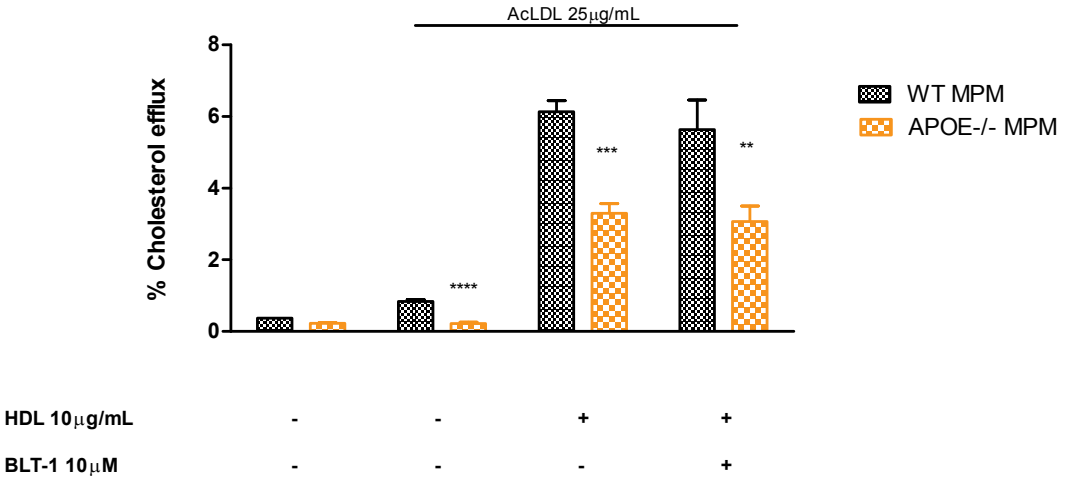

Fig.17 Cholesterol efflux from WT and ApoE-/- MPM to HDL. MPM from WT and ApoE-/- mice were cholesterol loaded or not with AcLDL 25µg/mL and radiolabeled with [^{3}H]-cholesterol for 24 hours in RPMI 1640 medium with 1% FCS and 2µg/mL of an ACAT inhibitor, equilibrated in an albumin-containing medium for 18 hours. Cells were treated with BLT-1 10 µM for 2h and then exposed for 4 hours to HDL 10µg/mL. The experiment was performed in triplicate. Efflux was expressed as counts per minute in medium/counts per minute of time 0x100± SD. Statistically significant difference **p<0.01 vs WT MPM; ***p<0.001 vs WT MPM;****p<0.0001 vs WT MPM.

Role of macrophage ApoE in phospholipid efflux from cholesterol-loaded MPM

To provide further insight into the effect of ApoE specifically expressed in macrophages we investigated its influence on phospholipid efflux from macrophage. MPM from WT and ApoE-/- mice were cholesterol enriched with AcLDL 25 µg/mL to induce the expression of ABCA1 which is the main transporter involved in phospholipid efflux (N Wang et al. 2001). Cells were successively exposed to increasing concentrations of normolipidemic plasma. As shown in Fig.18, the deletion of macrophage ApoE did not influence the rate of phospholipid efflux promoted by plasma.

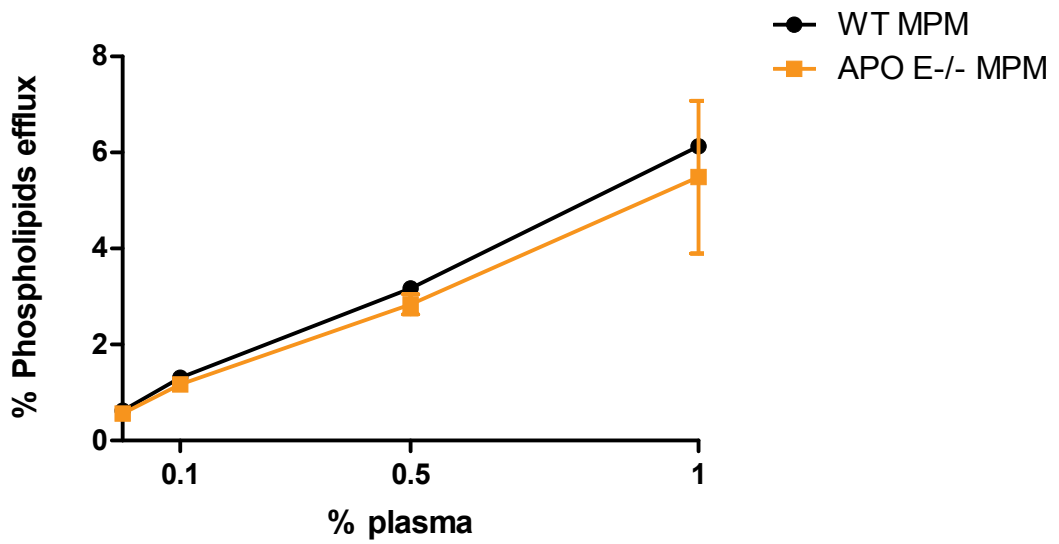

Fig.18 Phospholipid efflux from cholesterol-loaded WT and ApoE-/- MPM to plasma isolated from WT mice. Cells were cholesterol loaded with AcLDL 25µg/mL and radiolabeled with 4 Ci/ml [^{3}H]choline for 48h in RPMI 1640 medium 1% FCS. Cells were then incubated for 18h with 0.2% BSA, washed and incubated for 4h with increased concentration of plasma isolated from WT mice. The experiment was performed in triplicate. Efflux was expressed as counts per minute in medium/counts per minute of time 0x100± SD .

Role of endogenous ApoE on the sensitivity of membrane cholesterol to cholesterol oxidase

Furthermore, we investigated if the expression of ApoE in macrophages influences the distribution of cellular cholesterol to membrane microdomains, which represent the cholesterol pools available for interaction with extracellular acceptors. We measured the cholesterol oxidase sensitivity after incubating macrophages from WT and ApoE-/- mice with oxidase (Llera-moya et al. 2000). As showed in Fig.19, the percentage of the pool of cholesterol that was converted to cholestanone upon treatment with cholesterol oxidase in apoE expressing cells is greater as compared to percentage observed in ApoE-/- macrophages. After incubating with AcLDL, cpt-AMP and 22OH cholesterol-9cis retinoic acid, to induced the expression of ABCA1 transporter, the percentage of oxidized cholesterol increased both in WT and ApoE-/- mice. The lower percentage of oxidized cholesterol observed in ApoE-/- mice as compared to WT suggested that the cellular expression of ApoE influences the pool of membrane cholesterol which is available for efflux.

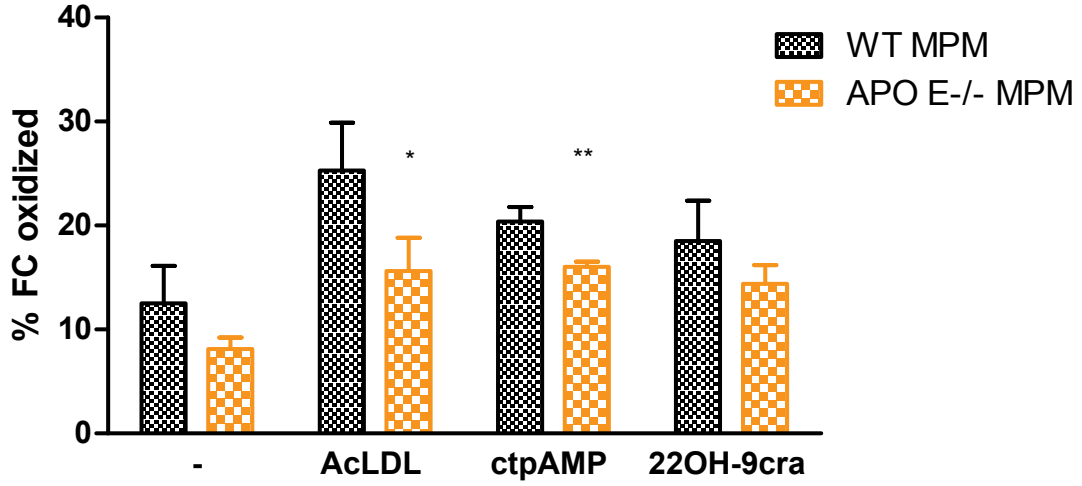

Fig.19 Free cholesterol oxidation in macrophage from WT and ApoE null mice. Cells were radiolabeled with 2 Ci/mL [^{3}H]cholesterol for 24h in RPMI 1640 medium 1% FCS ± AcLDL 25µg/mL. Cells were then equilibrated in an albumin-containing medium for 18 hours in presence of cAMP 0.3mM, or 22-OHcholesterol and 9cis retinoic acid. Cholesterol oxidase was added at a concentration of 1 U/mL for 4h 37 °C After this time, the cells were chilled and washed with ice cold PBS. Lipids were extracted from monolayers, separated by thin-layer chromatography (TLC), as described in method section, and quantitated by liquid scintillation counting. The experiment was performed in triplicate.

Role of endogenous ApoE on ACAT activity in macrophage

We investigated a possible influence of ApoE on enzymes involved in regulation of intracellular cholesterol, Acetyl Coenzyme A acyl transferase (ACAT) (Lee et al. 2000), which catalyzes the esterification of free cholesterol . The effect of ApoE on activity of intracellular ACAT enzyme was measured in MPM from WT and ApoE null mice before and after incubation with AcLDL 25 µg/mL. Upon basal condition, macrophages not expressing ApoE showed increased amount of cholesterol esters as compared to WT macrophages. After incubation with AcLDL cholesterol esters increased both in WT and ApoE-/- macrophages. Cholesterol-loading with AcLDL induced a higher fold increas in WT macrophages, as compared to ApoE-/- macrophages, indicating a greater activity of the ACAT enzyme (9.5-fold higher vs 2.5- fold ApoE-/-).

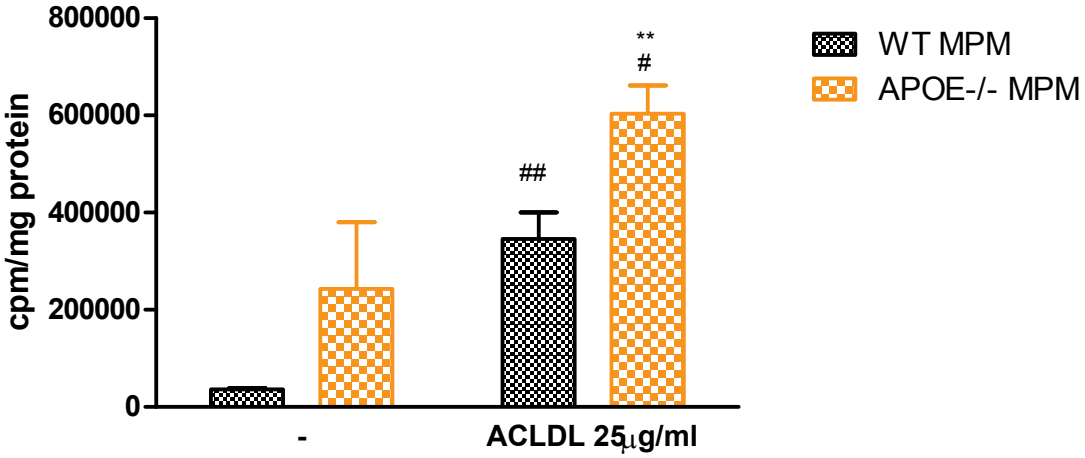

Fig.20 ACAT activity in cholesterol loaded MPM isolated from WT and ApoE-/- mice. WT and ApoE-/- MPM were cholesterol loaded with AcLDL 25µg/mL for 4h in RPMI 1640 medium 0.2% BSA. Cells were then radiolabelled with [^{14}C] oleic acid for 4h. After the incubation period, lipid were extracted from cells and separated by thin-layer chromatography (TLC) as described in method section. The experiment was performed in triplicate. The CE content was expressed on the protein content for each sample. Statistically significant difference**$p<0.01$ vs WT acLDL;# $p<0.05$ vs unloaded ApoE-/-; ##$p<0.01$ vs unloaded WT.

EFFECT OF THE EXPRESSION OF APOE ON THE CSA MODULATION OF MACROPHAGE RCT IN VIVO

Cyclosporin A is an immunosuppressant drug, which has been shown to induce dyslipidemia and increased susceptibility to atherosclerosis in mice (Kockx, Jessup, and Kritharides 2010). We evaluated if the pro-atherosclerotic effect of CsA could be related to impairment of RCT process by treating mice with short-term administration of CsA. The macrophage RCT was then quantified by a radioisotopic method which allows to trace the mobilization of radiolabeled cholesterol from macrophages injected into recipient CsA-treated mice to plasma, liver and feces (YuZhen Zhang et al. 2003).

Evaluation of CsA effect on macrophage RCT in vivo from MPM

In the first experiment we measured the macrophage RCT in C57BL/6J mice treated for 14 day with CsA 50 mg/kg/days or vehicle. At day 11 we injected animals with cholesterol-loaded MPM radiolabeled with [^{3}H]cholesterol and after 24h we measured the appearance of radiolabelled cholesterol into plasma, liver and feces. Treatment with CsA did not affect the amount of macrophage cholesterol detected in plasma and liver, but led to reduced amount of macrophage-derived cholesterol in the feces as compared to untreated mice, indicating an impairment of in vivo mobilization of cholesterol through the RCT pathway (Fig.21).

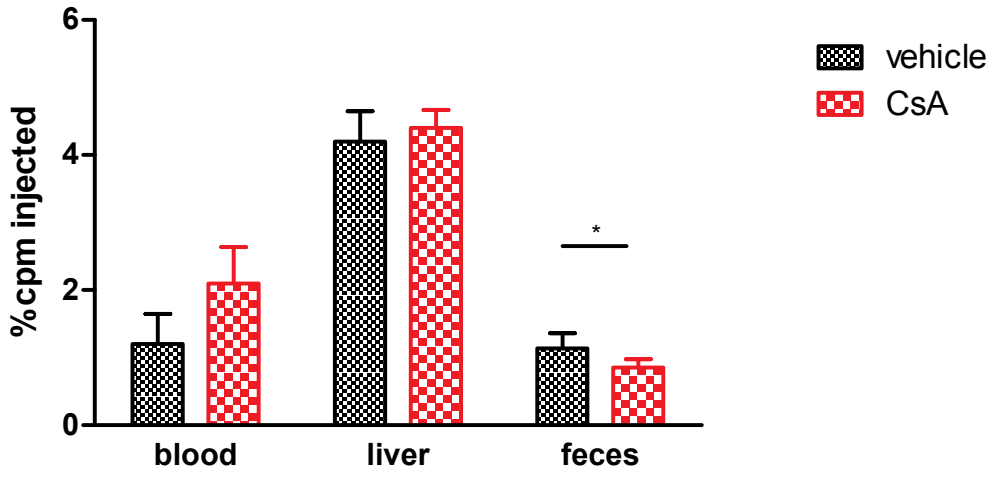

Fig.21 Effect of CsA on macrophage reverse cholesterol transport (RCT) in vivo. C57BL/6 mice were treated with CsA 50 mg·kg^{-1}·days^{-1} (red bar) or vehicle (black bar) by oral gavage for 14 days. On day 11 of drug treatment, animals were i.p. injected with [^{3}H]-cholesterol loaded MPM foam cells. After 24h from injection mice were sacrificed by excess of anaesthesia and macrophage-derived [^{3}H]-cholesterol distribution was quantified in blood, liver and feces. Blood were extracted by cardiac puncture and directly quantified in β-counter after plasma isolation. Samples of liver and feces were extracted using Bligh and Dyer method to isolate radiolabeled sterol. Results are expressed as percentage of injected [^{3}H]-dose ± SD (mean ±SD) (n=7 mice per group). Statistically significant *p<0.05vs. vehicle.

Evaluation of CsA effect on macrophage RCT in vivo from J774

Since CsA has been shown to affect ApoE secretion pathway from macrophage in vitro (Kockx et al. 2009), we evaluated if the observed effect of CsA treatment on RCT in vivo is dependent on the ApoE expression in macrophage. In the second experiment we evaluated the RCT in C57BL/6J mice treated for 14 days with CsA 50mg/kg/days and injected with radiolabelled J774, macrophages that do not express ApoE. In this experimental setting, CsA treatment impaired RCT, as it reduced the amount of macrophage-derived cholesterol mobilized to feces (Fig.22), similarly to the previous experiment.

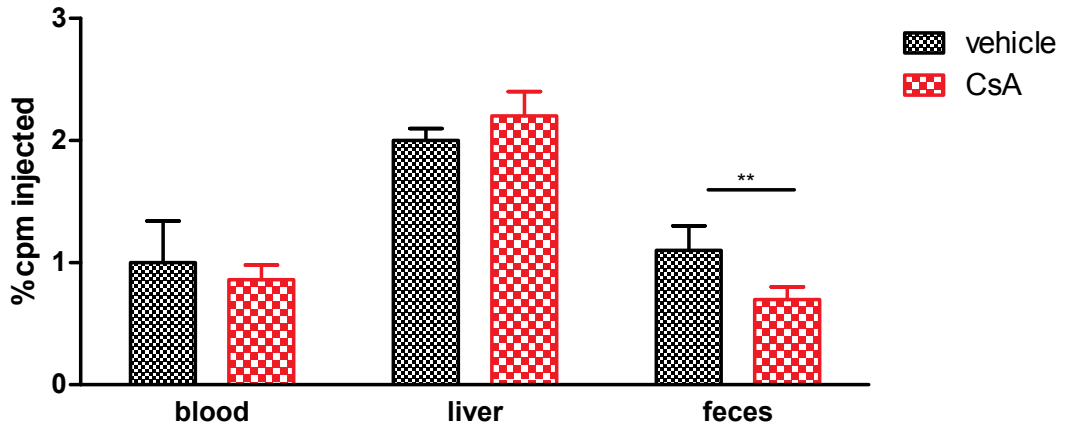

Fig.22 Effect of CsA on macrophage reverse cholesterol transport (RCT) in vivo. C57BL/6 mice were treated with CsA 50 mg·kg^{-1}·days^{-1} (red bar) or vehicle (black bar) by oral gavage for 14 days. On day 11 of drug treatment, animals were i.p. injected with [^{3}H]-cholesterol loaded J774 foam cells. After 24h from injection mice were sacrificed by excess of anaesthesia and macrophage-derived [^{3}H]-cholesterol distribution was quantified in blood, liver and feces. Blood were extracted by cardiac puncture and directly quantify in β-counter after plasma isolation. Samples of liver and feces were extracted using Bligh and Dyer method to isolate radiolabeled sterol. Results are expressed as mean ± SD (n=5 mice per group). Statistically significant **p<0.05vs. vehicle.

Discussion

In the current study we investigated the macrophage and systemic ApoE contribution to the first, rate limiting step of RCT, cholesterol efflux. The effect of endogenous macrophage apoE on cholesterol release from murine macrophages has been widely described (Eck et al. 2000), whereas the relevance of systemic expression of ApoE on plasma capacity to promote cholesterol efflux still remains unclear. The first evidence that systemic deficiency of ApoE causes a hyperlipidemic plasma profile, which does not result in reduced RCT, has been provided in vivo as well in vitro by our group. In particular, this study showed, for the first time, that HDL fraction isolated from plasma of both WT and ApoE null mice promotes a similar cholesterol efflux (Ilaria Zanotti et al. 2011). HDL represent the principal lipoproteins involved in delivery of macrophage-derived cholesterol and, as it interacts specifically with transporters involved in cholesterol efflux, it provides a useful experiment tool to evaluate specific efflux pathways. Since it does not represent the only plasma component involved in lipid exchange with cells, it has been argued that using isolated lipoprotein fraction is not completely representative of the in vivo situation. Therefore, it is evident the importance to assess the role of systemic ApoE on cholesterol efflux in condition more representative of the physiological settings. For that reason we performed cholesterol efflux assay in vitro using whole plasma from normolipidemic WT and ApoE null mice, which is characterized not only by low levels of HDL cholesterol, but also by high levels of apoB containing proteins (Zhang S.H. et al. 1992). In AcLDL-loaded elicited murine peritoneal macrophages (MPM), plasma from ApoE KO mice, despite altered lipid composition, showed a similar capacity to promote cholesterol efflux from foam cells, consistently with results using isolated HDL fraction. To investigate different contributions of single pathways to total cholesterol efflux, we used cell lines expressing specific transporters. Using J774 macrophages stimulated with cAMP analogue to upregulate ABCA1 (Bortnick et al. 2000a), we observed that plasma lacking ApoE has a similar and even slightly higher capacity to promote ABCA1-mediated efflux as compared to ApoE expressing plasma. This latter result is consistent with the electrophoresis analysis of plasma from ApoE KO mice which revealed an intact pre-β HDL particles, which is specific acceptor for ABCA1-mediated efflux (Ilaria Zanotti et al. 2011). Moreover, we measured the ABCG1 and SR-BI–mediated efflux in CHO transfected with human ABCG1 (Gelissen et al. 2006) and Fu5AH, which express high levels of SR-BI (De la Llera-Moya et al. 1999b). Strikingly, ApoE deficiency in plasma results in an even higher cholesterol efflux both via SR-BI, thus indicating a plasma lipoprotein composition which is able to interact with this receptor.

Cholesterol efflux could be influenced both from cell and extracellular acceptor capacity to induce the release of cellular cholesterol, so it is clear that the process can be analyzed from two different point of view in order to experimentally distinguish between different contributions.

The ApoE specifically expressed in macrophage foam cells has been shown to promote cholesterol efflux even in absence of extracellular acceptors (Ilaria Zanotti et al. 2011). Based on its well-established effect on total efflux, we tried to further discern the effect of endogenous ApoE on different pathways involved in cholesterol efflux from foam cells. In lipid loaded macrophage, which better represent the cell type involved in the development of atherosclerosis, the major contribution to efflux is represented by ABCA1 transporter, which accounts for 50% of total cholesterol (Adorni et al. 2007). In the same condition, ABCG1 mediates 20% of cholesterol efflux, whereas SR-BI-mediated efflux has been shown not to be relevant (Adorni et al. 2007). Yancey et al. have clearly established the importance of the interaction between ABCA1 and endogenous ApoE to promote cholesterol efflux from foam cells (Patricia G Yancey et al. 2007). In this study we tried to evaluate the role of macrophage ApoE in efflux pathway others than ABCA1. We evaluated the ABCG1-mediated efflux from macrophages by using an experimental approach which combined the stimulation of the expression of the transporter with the use of an extracellular acceptor which has been reported to specifically interact with ABCG1. Moreover, we used probucol which is a specific ABCA1 inhibitor, to rule out its contribution to efflux (Favari et al. 2004). In this condition, the expression of ApoE in macrophage has been revealed to promote efflux through ABCG1 pathway both in normal and lipid enriched MPM. Recently, van Eck group showed that ABCG1 and ApoE independently promote cholesterol efflux from macrophages to HDL but also observed that ApoE macrophages showed a significant reduction in ABCG1 mRNA expression (Lammers et al. 2011). According to these results, it is reasonable to speculate that the observed reduced ABCG1-mediated efflux in macrophages lacking ApoE is due to a reduced expression of the transporters. Further studies will be necessary to confirm this hypothesis in the same experimental setting used for efflux measurement. The SR-BI contribution to efflux from cholesterol normal macrophages has been shown not to be relevant, whereas in lipid enriched MPM it accounts for 9% of total efflux (Adorni et al. 2007). In lipid unloaded macrophages from both WT and ApoE null mice we did not appreciate efflux via SR-BI, confirming that SR-BI plays no role in this condition as it was previously stated (data not show). In cholesterol-rich macrophages not expressing ApoE, cholesterol efflux to HDL in presence of BLT-1, which specifically inhibits SR-BI (Nieland et al. 2002), was not reduced. It is conceivable to speculate

that the SR-BI contribution to efflux is too low to allow us to appreciate a pharmacological inhibition to efflux in our experimental conditions. Moreover, the reduced efflux to HDL observed in macrophages from ApoE lacking mice, is likely to be due to a reduced contribution of both passive diffusion and ABCG1 to cholesterol efflux compared to WT macrophages. In conclusion, the observed effect of macrophage ApoE on ABCG1 and SR-BI-mediated cholesterol efflux is consistent with previous study showing that endogenous ApoE enhance efflux to HDL, preferential acceptor for both SR-BI and ABCG1 transporters (Langer et al. 2000).

To further investigate the effect of systemic and macrophage ApoE on lipid efflux, we also evaluated phospholipid efflux from cholesterol loaded macrophages. Phospholipids have been reported to be released coordinately with cholesterol from cells (C. Y. Lin, Duan, and Mazzone 1999) and mostly by ABCA1 (N Wang et al. 2001). Consistently with effect on cholesterol efflux, systemic deletion of ApoE does not reduce phospholipid efflux from cholesterol loaded macrophages. Previous studies have shown that phospholipid efflux is enhanced by endogenous ApoE in macrophages stimulated with cAMP (J D Smith et al. 1996). In our experimental conditions macrophage ApoE expression does not seem to influence phospholipid efflux from AcLDL-loaded macrophages. It has been demonstrated that phospholipid and cholesterol release from macrophages are differently regulated (Kiss, Maric, and Marcel 2005). Furthermore, the same study underlined that primary and immortalized cells shown different way to regulate protein expression. It is therefore conceivable to speculate that the reasons for the different effect observed in our study compared to Smith, may rely on differences in experimental setting. In our experimental conditions, which are more representative of the in vivo physiology, the similar effect observed in macrophage expressing or not ApoE, suggested that ApoE influences the intracellular mechanism underling cholesterol, but not phospholipid efflux.

Finally, to gain better insight into the mechanisms underlying the ability of endogenous ApoE to promote cholesterol efflux, we focused on ApoE effect on intracellular enzymes involved in cholesterol metabolism.

Initially, we investigated a possible role of macrophage ApoE on ACAT activity. After loading with AcLDL, the activity of ACAT increased both in WT and ApoE null macrophages. The cholesteryl esters fold increase between AcLDL-loaded cells and basal condition is higher in WT macrophages compared to ApoE lacking macrophages. This suggested that endogenous ApoE modulates the enzyme activity, thus influencing the balance between intracellular free

cholesterol and cholesteryl esters content. Further studies will be necessary to clarify if the observed modulation of ACAT by macrophage ApoE could be related to its capacity to promote cholesterol efflux .

Afterwards, we tested the effect of endogenous presence of ApoE on oxidation susceptibility of membrane cholesterol, which is representative of a cholesterol pool available for efflux. Notably, the percentage of free cholesterol which undergoes oxidation by exogenous oxidase is higher in WT macrophage under basal condition. After treatment with different stimuli, the percentage of oxidized cholesterol increased as compared to basal condition, both in WT and ApoE-/- macrophages. However, the percentage of cholesterol oxidase sensitive pool is lower in ApoE-/-, indicating less cholesterol available to be released from cells. This result is consistent with the observed reduced efflux from cells in absence of macrophage ApoE through all the efflux pathways. Interestingly, our results suggested that the ApoE effect on cholesterol efflux could be also related to its capacity to modulate the intracellular cholesterol metabolism.

It is worth noting that incubation with whole plasma induces bidirectional movement of cholesterol between cells and lipoproteins (Zimetti et al. 2006). The difference between movement out of the cells (efflux) and movement into cells (influx) represents the net flux, that is the net change in intracellular cholesterol content. Since the absence of macrophage ApoE influences lipid efflux, we tested if it is able to alter also cholesterol mass in MPM when exposed to normolipidemic plasma. After exposition to normolipidemic murine plasma the amount of intracellular cholesterol of both WT mice and ApoE null mice decreased, indicating that efflux out of the cell is more relevant than influx. Consistently with efflux results, the net depletion of cholesterol in ApoE lacking macrophages is less significant compared to WT macrophages. This result confirmed that ApoE has a role in foam cells formation, as it act to prevent the accumulation of lipids in macrophages.

Moreover, we also tested the contribution of systemic apoE to net flux. Results showed that exposition to plasma from both WT and ApoE KO mice results in a similar effect on cholesterol net mass. According to the observation that cholesterol efflux measurement reflects changes in cholesterol mass if cells are enriched with cholesterol (Sankaranarayanan et al. 2010), these results confirmed the pivotal role of macrophage, but not systemic, ApoE on lipid efflux. Taken together these results confirmed that the absence of intracellular ApoE impairs protective mechanisms which effectively regulate homeostatic amount of intracellular cholesterol. Interestingly, we elucidated that systemic ApoE deficiency causes a modification in plasma

lipid profile which has been demonstrated to be proatherogenic, but it does not influence the atheroprotective process of RCT.

Since the composition of plasma can influence cholesterol hepatic uptake, another relevant step of RCT, we tested the effect of systemic ApoE on net flux in rat hepatoma cells Fu5AH, which naturally express high levels of SR-BI receptor. In this experiment we measured net change in cholesterol mass after exposition to plasma from ApoE null mice. Despite a well-established hyperlipidemia associated with systemic ApoE deficiency (S. H. Zhang et al. 1994), we did not observe a significantly different effect from control WT plasma. Plasma from ApoE null mice results in almost no change of cholesterol content. Moreover, we compared the effect of systemic ApoE deficiency on net flux with effect of another dyslipidemic plasma, characterized by severe hyperlipidemia, whole plasma from LDLr mice. LDLr-deficient mouse is a widely used experimental animal model with elevated plasma cholesterol levels. The dyslipidemia associated with LDL receptor deficiency is mainly due impaired removal of VLDL and LDL from circulation (Ishibashi et al. 1993). Conversely to what we observed for apoE null mice, exposition to plasma from LDLr null mice results in net accumulation of cholesterol in hepatoma cells. It has been clearly established that the hepatic ApoE facilitates the HDL presentation to hepatic SR-BI (Arai et al. 1999). Our results suggested that the altered plasma lipid profile in ApoE null mice does not impair the ability of lipoproteins to bind to hepatic receptors. Taken together these results allow us to speculate that the systemic ApoE does not influence RCT, as it does not influence the ability of plasma to promote either cholesterol efflux/influx and hepatic uptake. By comparing the effect of plasma from two different models of dyslipidemia, we confirmed that despited the modified the ratio between HDL cholesterol and non HDL cholesterol, the apoE systemic deficiency does not results in impaired lipid exchange with cells.

To sum up, our results confirmed that macrophage ApoE promotes RCT, while the systemic ApoE, despite affecting plasma lipid levels, does not influence RCT. The constitutive pathway of ApoE secretion from macrophages has been demonstrated to involve Protein kinase A (PKA) activity and intracellular Ca^{2++} (Kockx et al. 2007). The same group demonstrated that a calcineurine inhibitor, Cyclosporine A (CsA), reduces ApoE secretion from human macrophages (Kockx et al. 2009). Cyclosporine A is an immunosuppressant drug, routinely used in organ transplanted patients to reduce rejection, which is associated with side effects, such as hyperlipidemia and increased susceptibility to atherosclerosis (Kockx, Jessup, and Kritharides 2010). Consequently the mechanism of inhibition of ApoE secretion from

macrophages has been suggested to provide a reasonable mechanism underling the pro-atherosclerotic effect of Cyclosporine A (Kockx et al. 2009). Based on the inverse correlation between cardiovascular risk and the efficiency of RCT in animal models (Daniel J Rader et al. 2009), we first investigated whether CsA effect of increased susceptibility to atherosclerosis could be related to an impairment of RCT in vivo. Since macrophage ApoE has been demonstrated to be essential to promote an efficient RCT, we further evaluated if CsA treatment affects RCT by inhibiting ApoE secretion from macrophages. In the first experiment, we observed that short treatment with CsA impaired cholesterol mobilization from ApoE expressing macrophages to liver (Zanotti et al. under review). In particular, CsA treatment causes a slight decrease of macrophage derived cholesterol mobilized to feces. Furthermore, the radioisotopic methods used to measure in vivo macrophages-to feces delivery of cholesterol allow us to assess the impact of a genetic variable in macrophage to the overall process in vivo (Daniel J Rader et al. 2009). In particular, we repeated the experiment using macrophage which do not express ApoE injected into CsA treated mice. Interestingly, we observed that even in absence of macrophage ApoE, in vivo RCT is impaired and cholesterol distribution among the main compartment involved in RCT pathway is the same observed using ApoE expressing cells. In this study we showed, for the first time, that in vivo treatment with CsA impairs RCT but this effect is not related to its capacity to modulate ApoE secretion. This suggested that the pro-atherogenic effect observed in vitro does not have consequences in vivo, at least in our experimental setting. It is reasonable to speculate that compensatory mechanisms occur in vivo, such as upregulation of different efflux pathways, which may explain the discrepancy of outcomes from what has been observed in vitro.

This research provides more insight into the role of ApoE in the first, rate limiting step of RCT, cholesterol efflux from macrophage. First, we confirmed that only apoE specifically expressed in macrophage influences the rate of the process which counteract foam cell formation. Second, we demonstrated that systemic ApoE, despite causing hyperlipidemia, does not impair plasma capacity to promote the release of cholesterol through different pathways of efflux.

PART II:

Effect of ABCG1 and SR-BI total body deletion on atherosclerosis and lipid homeostasis

Aim

Several epidemiological studies established that HDL cholesterol correlates inversely with the incidence of cardiovascular events (Ross 1999). HDL mainly exerts this atheroprotective function by promoting the process of Reverse Cholesterol Transport (RCT) (Gordon et al.1977). This pathway enables cholesterol to be driven from cholesterol-loaded macrophages of the arterial wall to the liver for disposal (Daniel J Rader et al. 2009). It is thus a natural mechanism that prevents excessive macrophage cholesterol accumulation in the arterial wall protecting against the development of atherosclerosis. Numerous proteins are involved in RCT, including ABC-transporter G1 (ABCG1) and scavenger receptor BI (SR-BI). The knowledge on the physiological relevance of these transporters for the RCT process has been provided mostly by studies in mouse models in which the gene expression was disrupted, down-regulated or overexpressed.

SR-BI is the first HDL receptor described and it regulates homeostatic HDL plasma levels by promoting the selective hepatic uptake of cholesterol esters from HDL in the last step of RCT (Rigotti et al. 1997). SR-BI also acts in the first step of RCT, as it mediates bidirectional flux of free cholesterol between cells and mature HDL particles (Yong Ji et al. 1997). Deletion of SR-BI in mice leads to a specific atherosclerotic prone phenotype, characterized by the accumulation of abnormally large HDL particles both on chow and on a high-cholesterol diet (Van Eck et al. 2003). Likewise, macrophage selective deletion results in increased susceptibility to atherosclerotic lesion development. Moreover, it has been clearly established that only hepatic SR-BI contributes to RCT (X. Wang et al. 2007), whereas protective functions of SR-BI seem to be more related to its pleiotropic effects on vascular homeostasis.

Similarly to SR-BI, ABCG1 is also expressed on macrophages where it actively promotes cellular cholesterol efflux to mature HDL (Nan Wang et al. 2004). By facilitating efficient release of cholesterol from foam cells, ABCG1 promotes macrophage RCT in vivo (X. Wang et al. 2007). Although its role in macrophage lipid homeostasis has been clearly demonstrated (Kennedy et al. 2005), the physiological relevance of ABCG1 on atherosclerosis still remains unclear. Independent studies have provided contradicting results on the effect of ABCG1 on atherosclerosis development. Recently a study from Meurs et al. suggested that ABCG1 can exert different properties depending on the stage of atherosclerotic lesion development (Meurs et al. 2012). In line, macrophage specific ABCG1 expression has been reported to be pro atherogenic as well as anti-atherogenic. Several bone marrow transplantation studies demonstrated that macrophage ABCG1 deletion is associated with reduced atherosclerosis as a results of compensatory increased of ABCA1 expression (Ranalletta et al. 2006) or increased

apoptosis (Tarling et al. 2010). In contrast, Out et al. observed slightly increased atherosclerosis in mice lacking macrophage ABCG1 (Out et al. 2006).

According to the well-established role of SR-BI and ABCG1 in the different steps of RCT, it is interesting to investigate a possible synergistic effect of these two proteins on lipid metabolism. Moreover, because they have similar substrate specificity, it is reasonable to speculate that they might act sequentially to promote cholesterol efflux. In addition, studying the effect of ABCG1 in absence of compensatory proteins involved in RCT, like SR-BI, would help to clarify its contribution to lipid metabolism.

The aim of this study, performed at Division of Biopharmaceutics, Leiden Academic Center for Drug Research at Leiden University, The Netherlands, in the group directed by Miranda van Eck, is to investigate the effects of total body ABCG1 deletion in SR-BI knockout mice on plasma lipids, tissue lipid homeostasis and atherosclerosis. We generated unique SR-BI/ABCG1 double knockout (DKO) mice to analyze the potential synergistic role of these transporters in mediating cellular cholesterol homeostasis.
Gain better insight into the molecular mechanisms involved in the athero-protective process of RCT is expected to provide putative novel therapeutic targets for the development of therapies to reduce atherosclerosis, one of the major causes of death in the Western society.

Materials and methods

ANIMALS

SR-BI KO and ABCG1 KO animals were crossed to generate double heterozygous offspring, which were further intercrossed to obtain the ABCG1/SR-BI double knockout (DKO) mice, and single ABCG1 KO, SR-BI KO, and wild type (WT) littermates. At 8-10 weeks of age, animals were fed a semi synthetic high cholesterol Western-type diet (WTD), containing 15% fat and 0.25% cholesterol for 20 weeks. Animal experiments were performed at the Gorlaeus Laboratories of the Leiden Academic Center for Drug Research, Leiden University, The Netherlands. All experimental protocols were approved by Ethics Committee for Animals Experiments of Leiden University.

SERUM LIPID ANALYSES

The concentrations of total cholesterol were determined by incubation of 0.025 U/ml cholesterol oxidase (Sigma) and 0.065 U/ml peroxidase and 15 µl/ml cholesteryl esterase (Merck, USA) in reaction buffer (1.0 KPi buffer, pH 7.7 containing 0.01 M phenol, 1 mM 4-amino-amtipyrine, 1% polyoxyethylene-9-laurylether, and 7.5% methanol). Absorbance was read at 490 nm. Precipath (standardized serum Roche) was used as an internal standard. Triglycerides and phospholipids were measured enzymatically using commercial kits (Roche. Diagnostics, Mannheim, Germany; Instruchemie, The Netherlands.). The distribution of cholesterol over the different lipoproteins in serum was determined by fractionation of pooled serum using a Superose 6 column (3.2 x 300mm, Smart system; Pharmacia, Uppsala, Sweden). Individual fractions were determined for cholesterol concentrations as described above.

BLOOD AND PERITONEAL LEUKOCYTE ANALYSIS

Upon sacrifice the blood was collected by retro-orbital puncture under anesthesia. Thereafter, the peritoneal cavity of the mice was lavaged with 10 ml cold PBS to collect peritoneal leukocytes. Total white blood cells, neutrophil, lymphocyte and monocyte counts in the blood and macrophage foam cells in the peritoneal cavity were analyzed using an automated Sysmex XT-2000iV veterinary Hematology analyzer (Sysmex Corporation). Corresponding samples

were cytospun for manual confirmation and stained with Oil-red-O for detection of lipid accumulation.

FLOW CYTOMETRY

White blood cell suspensions from whole blood were prepared by lysis of red blood cells. Cell surface immunolabelling of monocytes and neutrophils was performed according to the manufacturer's instructions (eBioscience & BD Biosciences). Briefly, fluorochrome-conjugated monoclonal antibodies to CD11b (eBiosciences) and to Ly6G and Ly6C (BD Biosciences) were incubated with the white blood cell suspensions for 30 min at 4^{o}C in labeling buffer (1% mouse serum in PBS). Flow cytometeric analysis was performed with FacsCalibur and then analyzed with CellQuest software (Beckton Dickinson, San Jose), correcting for nonspecific staining with isotype antibody controls.

HISTOLOGICAL ANALYSIS OF AORTIC ROOT

On sacrifice the arterial tree was perfused in situ with PBS and the heart was excised and stored in 3.7% neutral-buffered formalin (Formal-fixx; Shandon Scientific) until use. Atherosclerotic lesion development was quantified in the aortic root from Oil-red-O/hematoxylin-stained cryostat sections using a Leica image analysis system, consisting of a Leica DMRE microscope coupled to a video camera and Leica Qwin Imaging software (Leica Ltd). Mean lesion area was calculated from 10 Oil-red-O /hematoxylin stained sections, starting from the appearance of the tricuspid valves.

HISTOLOGICAL ANALYSIS OF ORGAN LIPID ACCUMULATION

Seven micrometer cryosections of formalin-fixed lung, spleen and liver were prepared and stained for lipid accumulation with Oil-red-O. Hematoxylin was used to visualize nuclei.

ANALYSIS OF LIVER LIPID COMPOSITION

Hepatic lipids were extracted according to the method of Bligh & Dyer (Bligh, E. G., W. J. Dyer. 1959) and dissolved in 2% Triton X-100. Total cholesterol, Triglycerides and Phospholipids were determined as described above. Lipids levels in liver were normalized to their protein concentrations determined using the BCA-TM protein assay (Pierce Biotechnology, Rockford, USA).

DETERMINATION OF SERUM LEVELS OF KC

Keratinocyte chemoattractant (KC) levels in serum were assayed using an ELISA kit (Invitrogen) according to the manufacturer's protocol.

ANALYSIS OF GENE EXPRESSION BY REAL-TIME QUANTITATIVE PCR

Total RNA was extracted from liver by the guanidium thiocyanate-phenol chloroform extraction method according to Chomczynski et al (Chomczynski, P., N. Sacchi. 1987) and quantified using a UV-vis spectrophotometer (NanoDrop Technologies, Wilmington, DE USA). cDNA was synthetized from 1 μg of total RNA using RevertAidTM M-Multi Reverse Transcriptase according to manufacturer's instructions. Real-time PCR was carried out on an ABI Prism® 7700 Sequence Detection System (Applied Biosystems, Foster City, CA) using SYBR-green technology. mRNA expression levels were indicated relative to the average of the housekeeping genes ribosomal protein 36B4, β-actin and glyceraldehyde-3-phosphate dehydrogenase (GADPH).

STATISTICAL ANALYSIS

Value are expressed as mean ± SEM. Statistical analyses were performed with Prism 5 software (GraphPad Software, San Diego, California). Statistically significant differences among the means of different groups were tested using analysis of variance (ANOVA). The Student-Newman-Keuls multiple comparison test was performed after ANOVA. Two-way ANOVA was used to check possible interactions. A p value of <0.05 was considered significant.

Results

EFFECT OF COMBINED ABCG1 AND SR-BI DELETION ON PLASMA LIPID LEVELS

To determine the combined effect of ABCG1 and SR-BI deficiency on atherosclerosis DKO mice were generated. On chow, total cholesterol levels of both SR-BI KO and DKO mice were increased 2.4 and 2.8-fold respectively due to accumulation of abnormally large HDL particles, while single ABCG1 deletion did not result in any significant changes in total cholesterol compared to WT mice (64.4±3.4mg/dL vs 60.5 ±6.3 mg/dL respectively).

To induce atherosclerotic lesion formation, WT, ABCG1 KO, SR-BI KO, and DKO mice were fed Western type diet (WTD) (containing 0.25% [w/w] cholesterol and 15% [w/w] fat) for 20 weeks. Upon challenge with WTD SR-BI KO and DKO mice showed increased total cholesterol levels (345 ±72 mg/dL and 295 ±35 mg/dL respectively) compared to WT and ABCG1 KO mice (93 ±18 mg/dl and 133±28 mg/dl) (Fig.23). The distribution of these lipids over the different lipoproteins in the circulation was analyzed using FPLC. Mice with a combined deletion of SR-BI and ABCG1 were characterized by accumulation of abnormally large HDL particles and increased VLDL levels, although less extensive as compared to SR-BI KO animals. Moreover, compared to WT and single ABCG1 KO mice, SR-BI KO and DKO animals displayed a higher amount of triglycerides (3.2 and 2.7-fold higher, $p<0.001$) and phospholipids (1.5 and 1.2-fold higher, respectively, $p<0,01$ and $p>0.05$) in plasma.

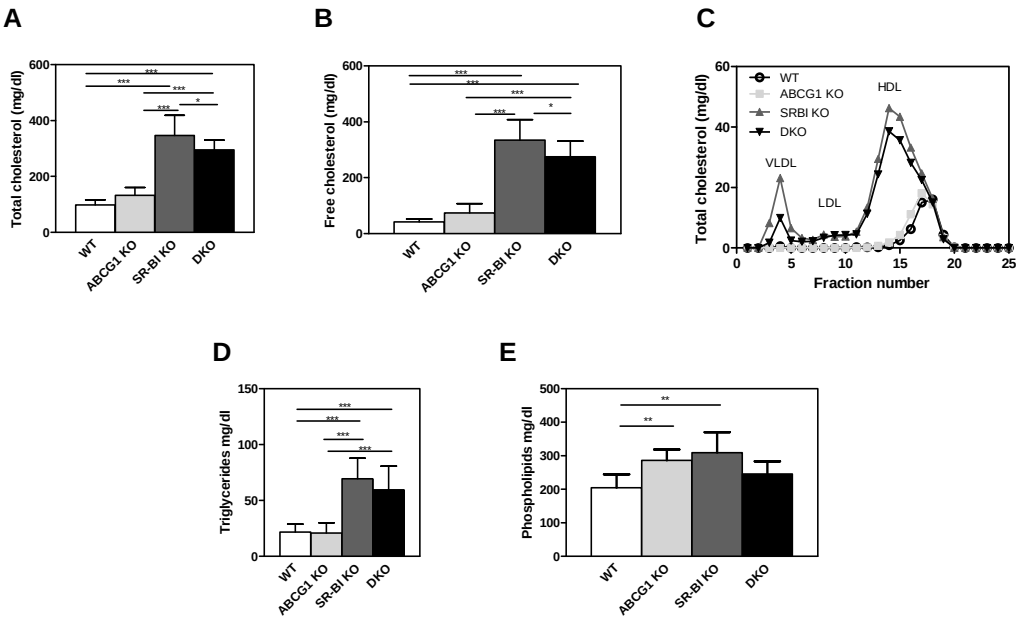

Fig.23 Effect of combined ABCG1 and SR-BI deletion on plasma lipid levels and lipoprotein cholesterol distribution on WTD. Total cholesterol (A), free cholesterol (B), lipoprotein cholesterol profile (C), triglycerides (D) and phospholipids (E) of WT (white bar), ABCG1 KO (light grey bar), SR-BI KO mice (dark grey bar) and ABCG1/SR-BI DKO mice (black bar) on WTD. Values are means ±SEM. Statistically significant difference *p<0.05, **p<0.01, ***p<0.001.

EFFECTS OF ABCG1 AND SR-BI COMBINED DEFICIENCY ON ATHEROSCLEROTIC LESION DEVELOPMENT

Atherosclerotic lesion development was quantified in cryostat sections of the aortic root of mice fed WTD for 20 weeks. ABCG1 KO mice did not show any evidence of atherosclerosis in the aortic root, while SR-BI deficiency induced atherosclerotic development compared to WT mice (46-fold increased, $p<0.001$). Importantly, no added effect of combined deletion of ABCG1 and SR-BI on lesion size was observed as compared to single deletion of SR-BI (117±18x103 µm2 vs. 108± 33x103 µm2) (Fig.24).

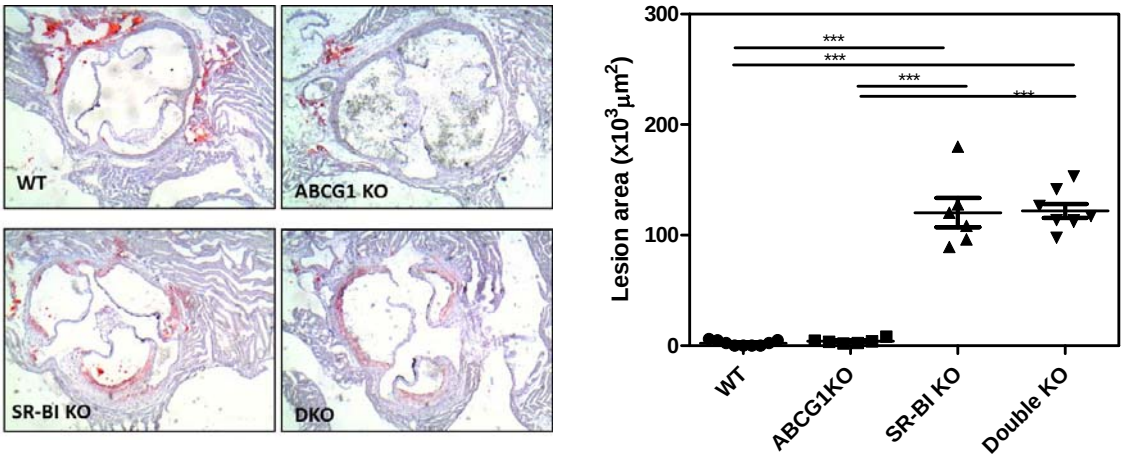

Fig.24 Effect of combined ABCG1 and SR-BI deletion on atherosclerosis development. Photomicrographs showing representative Oil red O stained sections of the aortic root (left panel, magnification 50X) and scatter dot plot of atherosclerotic lesion quantification (right panel) after 20 weeks WTD feeding. Mean lesion area of each individual mouse is shown. The horizontal lines represent the means of the groups. Statistically significant difference ***$p<0.001$.

EFFECTS OF ABCG1 AND SR-BI COMBINED DEFICIENCY ON LEUKOCYTOSIS

Circulating leukocytes were analyzed using a hematology analyzer and flow cytometry. A trend to larger amounts of white blood cells was observed in ABCG1/SR-BI DKO mice in comparison with WT and single ABCG1 KO and SR-BI KO animals (5.3±1.1 x106/mL vs WT: 3.8±1.6 x106/mL; ABCG1 KO: 3.0±0.9 x106/mL; SR-BI: 2.6±0.9 x106/mL), mainly due to increased numbers of circulating monocytes and neutrophils (Fig.25A). Deletion of ABCG1 and SR-BI resulted in enhanced monocytes counts with increased Ly6C^{high} and Ly6C^{low} subsets in DKO but not in WT and single ABCG1 KO and SR-BI KO mice (Fig.25B).

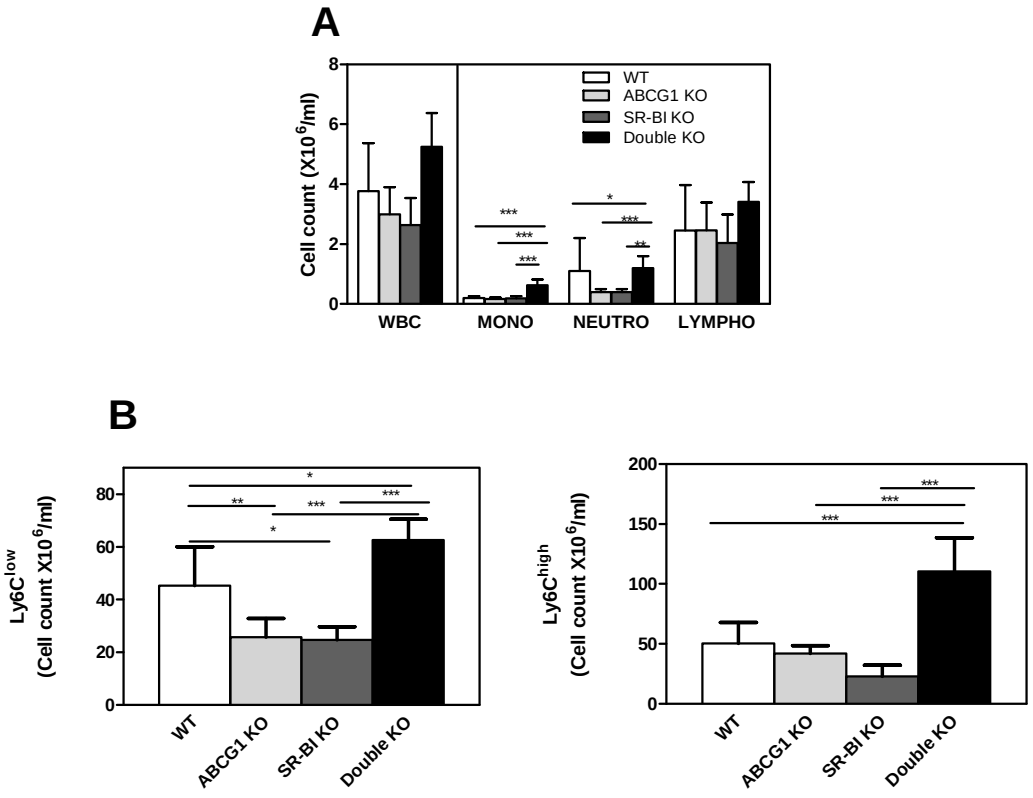

Fig.25 Effect of combined ABCG1 and SR-BI deletion on circulating white blood cells after 20 weeks on WTD. A) Circulating leukocytes in the blood were analyzed using an automated Sysmex XT-2000iV Veterinary Hematology analyzer. B) Monocyte subsets expressing different levels of Ly6C were quantified using a fluorescence-activated cell sorter (FACS). Values are mean ± SEM. Statistically significant difference *p<0.05, **p<0.01, ***p<0.001.

Peritoneal leukocyte counts were analyzed next. Upon challenge with WTD, the leukocyte count was not changed in the peritoneal cavity of ABC

G1 KO and SR-BI KO mice compared to WT mice (8.8±2.9x106/mL and 8.6±3.3x106/mL vs. WT 9.6±4.2 x106/mL), while combined ABCG1 and SR-BI deletion resulted in a slightly increased accumulation of total leukocytes in the peritoneal cavity (11.5 ±3.1x106/mL) (Fig.26).

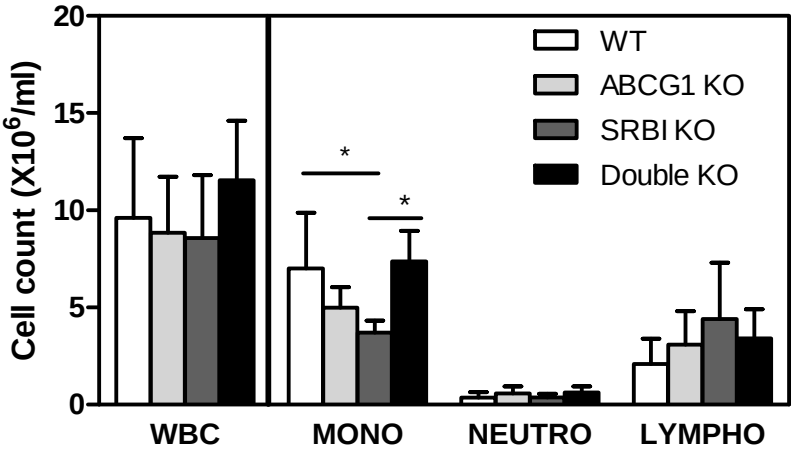

Fig.26 Effect of combined ABCG1 and SR-BI deletion on leukocyte accumulation in the peritoneal cavity after 20 weeks on WTD. Peritoneal leukocytes were analyzed using an automated Sysmex XT-2000iV Veterinary Hematology analyzer. Values are mean ± SEM. Statistically significant difference *$p<0.05$.

EFFECT OF COMBINED ABCG1 AND SR-BI DELETION ON SERUM KC (IL-8) LEVELS

Serum KC levels (murine ortholog of IL-8) were determined by ELISA in the different groups of animals. After 20 weeks on WTD an increased level of KC was observed in serum of ABCG1 KO mice as well as in DKO animals. The 2-way ANOVA analysis showed that increased KC levels observed in serum from DKO mice is statistically related to the effect of ABCG1 deletion.

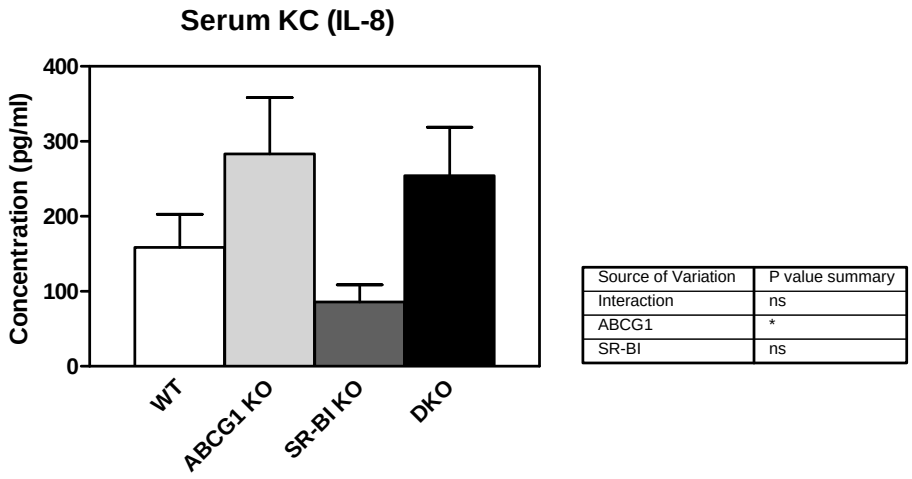

Fig.27 Effect of combined ABCG1 and SR-BI deletion on serum concentration of the pro-inflammatory cytokine KC (IL-8) after 20 weeks on WTD. Serum KC levels were determined by ELISA in WT (white bar), ABCG1 KO (light grey bar), SR-BI KO mice (dark grey bar) and ABCG1/SR-BI DKO mice (black bar) on WTD. Values are mean ± SEM. Statistically significant difference *$p<0.05$.

TISSUE LIPIDS HOMEOSTASIS IN ABCG1/SR-BI DOUBLE KNOCKOUT MICE

Cryostat sections of liver, spleen and lung of WT, ABCG1 KO, SR-BI KO, and DKO mice were prepared and stained for lipids using oil-red-O and counterstained with hematoxylin. No Oil-red-O positive staining was observed in spleens of WT, ABCG1 KO, SR-BI KO, and DKO mice (Fig.28A). Conversely, massive lipid accumulation was observed in livers of all groups of animals (Fig.28B). In agreement, hepatic lipid extraction with the Bligh and Dyer method showed no significant difference in lipid levels (WT: 27.4 ±3.2 µg/mg; ABCG1 KO: 25.2 ±4.2 µg/mg ; SR-BI KO: 22.9±7 µg/mg; DKO: 22.0±3.9 µg/mg). Furthermore, upon challenge with WTD lipid accumulation was observed in lung of ABCG1 KO mice and DKO mice, whereas no lipid accumulation was observed in WT and SR-BI KO mice as indicated by Oil-red-O staining (Fig.28C).

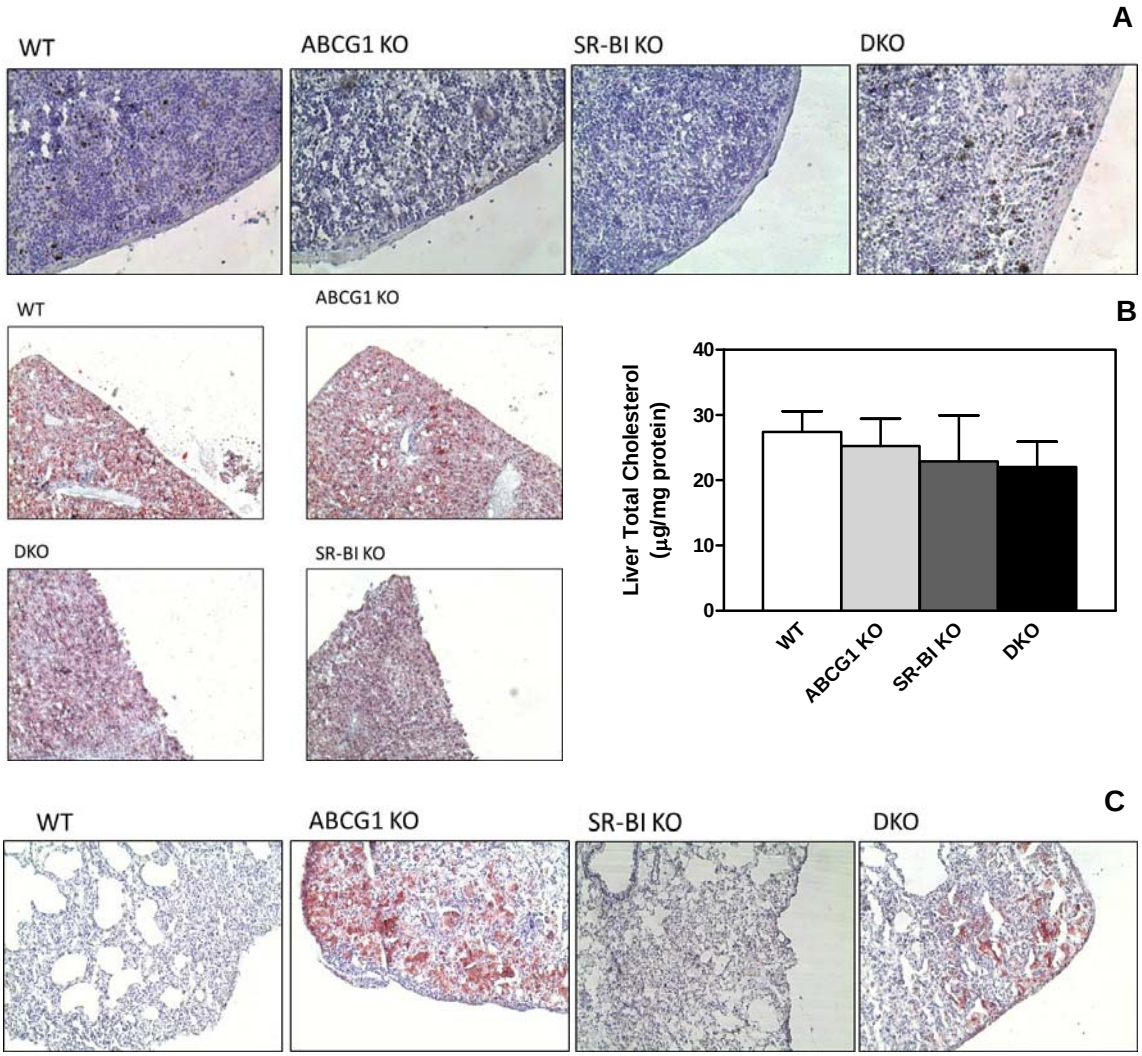

Fig.28 Effect of combined ABCG1 and SR-BI deletion on lipid accumulation in tissue. Lipid accumulation in spleen (A), liver (B) and lung (C) of WT, ABCG1 KO, SR-BI KO and DKO mice on WTD . Cryostat sections were stained with Oil red O to visualize lipid accumulation. Total cholesterol levels in liver of WT (white bar), ABCG1 KO (light grey bar), SR-BI KO mice (dark grey bar) and ABCG1/SR-BI DKO mice (black bar) on WTD extracted as described in methods section.

EFFECT OF COMBINED ABCG1 AND SR-BI DELETION ON FOAM CELL FORMATION IN THE PERITONEAL CAVITY

Upon WTD challenge no increased foam cell formation was observed in the peritoneal cavity as a result of combined ABCG1 and SR-BI deficiency compared to WT and single ABCG1 and SR-BI deletion (0.64 ±0.13 x106/mL vs WT 0.55 ±0.40 x106/mL; ABCG1 KO 0.77 ±0.11 x106/mL; SR-BI KO 0.83 ±0.29 x106/mL).

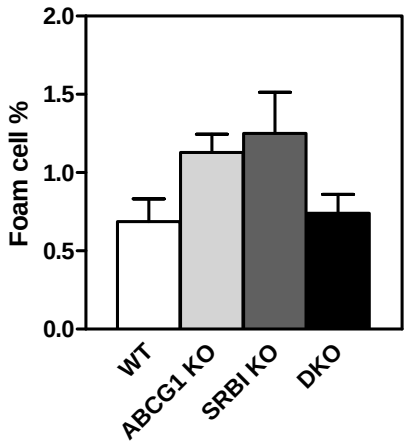

Fig.29 Effect of combined ABCG1 and SR-BI deletion on macrophage foam cell formation in peritoneal cavity on WTD. Peritoneal leukocytes were analyzed using a hematology analyzer. Macrophage foam cells in the peritoneal cavity of WT (white bar), ABCG1 KO (light grey bar), SR-BI KO mice (dark grey bar) and ABCG1/SR-BI DKO mice (black bar) at 20 weeks of WTD was quantified as percentage of the amount of total leukocytes in the peritoneal cavity. Values are means ±SEM. No significant differences were observed.

EFFECT OF COMBINED ABCG1 AND SR-BI DELETION ON HEPATIC GENE EXPRESSION

In order to further investigate the effect of ABCG1 and SR-BI double deletion on lipid metabolism and atherosclerosis the mRNA levels of genes of interest were quantified in livers of the different groups of animals. First, the hepatic expression of ABCG1, SR-BI, and genes involved in hepatic lipid homeostasis were quantified. ABCG1 deficiency did not affect the expression of hepatic SR-BI (Fig.30A). Similarly, SR-BI deletion did not influence the mRNA expression of ABCG1 (Fig.30A). Combined deletion of ABCG1 and SR-BI, as expected, ablated the expression of both ABCG1 and SR-BI. The expression of ApoE and the LDLr, which are both essentially involved in regulating the cholesterol content of the liver was not affected by combined deletion of ABCG1 and SR-BI. DKO mice, however, did show a slightly reduction in the hepatic expression of CYP7α, the enzyme responsible for bile acid production (Fig.30A). In agreement with the literature (Hoekstra et al. 2008), the expression of ApoA4 was reduced 2-fold in SR-BI KO mice compare to WT mice. In addition, single ABCG1 deletion reduced ApoA4 expression 1-fold. In ABCG1 and SR-BI DKO mice, apoA4 expression was decreased 3-fold, Secondly, the mRNA expression of antioxidant enzyme was analyzed (Fig.30A) The mRNA expression of Heme Oxygenase-1 (HO-1) was increased both in SR-BI KO mice (4.6-fold $p<0.05$) and in DKO mice (5.5-fold $p<0.05$) as compared to WT mice. In addition, the mRNA expression of inflammatory markers were analyzed (Fig.30B). DKO mice showed higher levels of Macrophage receptor with Collagenous structure (MARCO) as compared to WT and single ABCG1 KO and SR-BI KO mice. No statistical significant difference were observed among group of the expression of CD68 marker. Moreover, Monocyte chemoattractant protein-1 (MCP-1), which is the key cytokine regulating monocyte migration and infiltration, was increased in DKO mice as compared to SR-BI KO and ABCG1 KO mice.

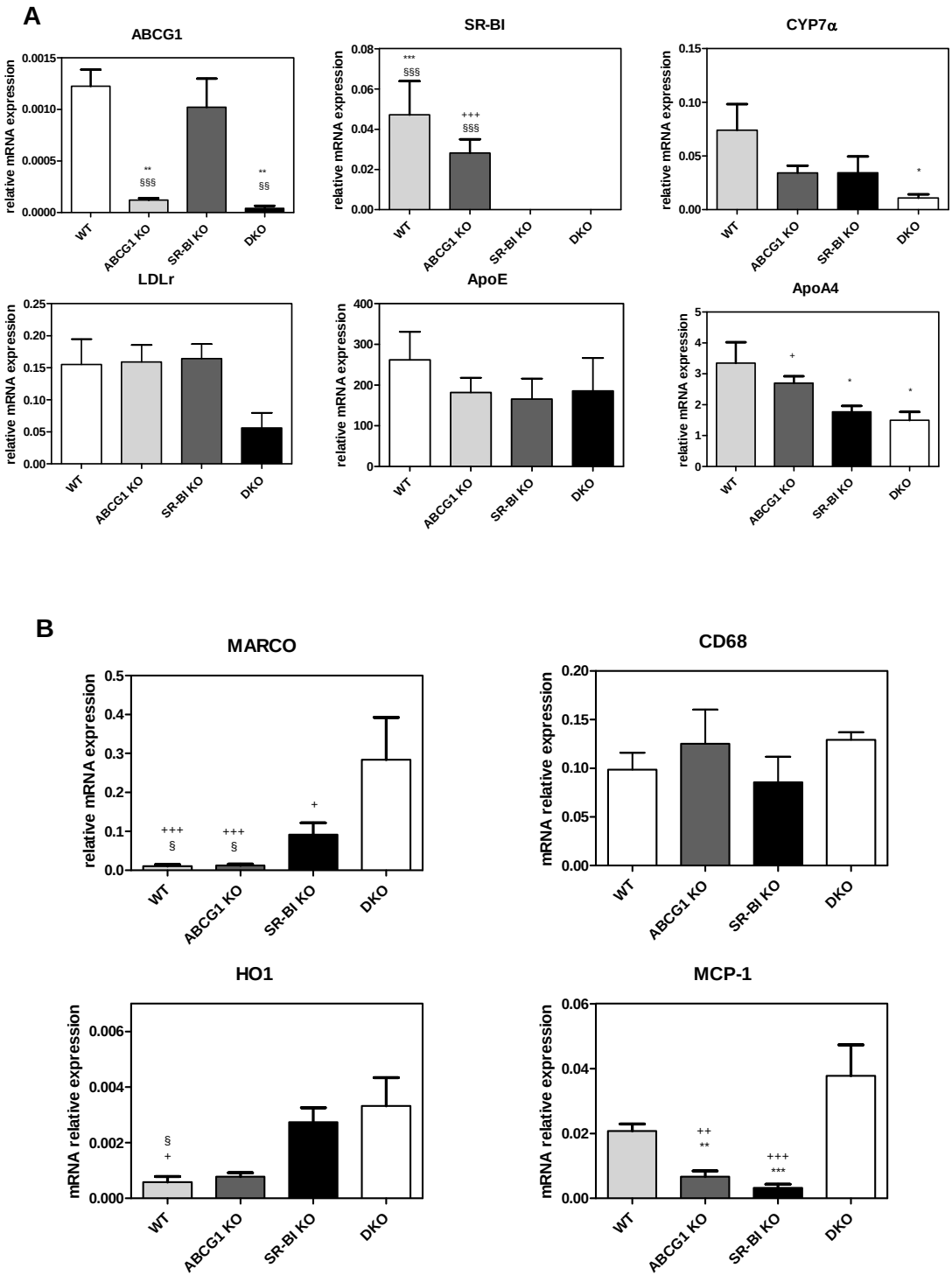

Fig.30 Effect of combined ABCG1 and SR-BI deletion on hepatic mRNA expression of different gene after 20 weeks WTD. mRNA levels of the indicated genes in livers of WT (white bar), ABCG1 KO (light grey bar), SR-BI KO mice (dark grey bar) and ABCG1/SR-BI DKO mice (black bar) were quantified using real-time PCR with SYBR-green detection. Values represent mean ± SEM of 5 mice. Statistically significant difference *p<0.05, **p<0.01, ***p<0.001 vs WT mice; §p<0.05, §§p<0.01, §§§p<0.001 vs SR-BI KO mice; $^{+}$p<0.05, $^{++}$p<0.01, $^{+++}$p<0.001 vs DKO mice.

Discussion

In the present study we investigated the effects of ABCG1 deletion in SR-BI KO mice on lipid metabolism and atherosclerosis using unique ABCG1/SR-BI DKO mice. Both receptors are implicated in lipid metabolism and are important players in the RCT process. In particular they are expressed in macrophages where they promote cholesterol efflux to the same extracellular acceptors, mature HDL particles. In addition, SR-BI is expressed in liver where it promotes the selective uptake of cholesteryl esters from HDL. Deletion of SR-BI in mice impairs the uptake of HDL cholesterol esters by the liver resulting in the accumulation of large, dysfunctional HDL particles (Rigotti et al. 1997) (Van Eck et al. 2003). On the other hand, the effect of ABCG1 on HDL metabolism still remains unclear, despite a well-established role of this ATP-binding transporter in tissue lipid homeostasis (Kennedy et al. 2005).

Combined ABCG1/SR-BI knockout mice display a distribution of lipids among the different lipoproteins similar to that observed in the single SR-BI KO mice, which is characterized by increased HDL and pro-atherogenic VLDL subfractions. Interestingly, disruption of ABCG1 in SR-BI KO mice partly alleviated the effect of SR-BI deficiency on plasma lipid levels, in particular on VLDL. To further investigate the mechanism behind the altered lipid levels observed in the DKO mice, we analyzed the expression of key liver genes involved in lipid metabolism. No significant differences were observed in the expression of apoE and LDLr, two proteins essential for the clearance of VLDL from the circulation. If anything, LDLr expression appeared slightly reduced, which is clearly not in line with the observed decreased in VLDL levels in the DKO animals as compared to single SR-BI KO mice. Furthermore, the expression of genes related to cholesterol synthesis, including HMGCoA reductase, was similar in SR-BI KO and DKO mice. In DKO mice we did observe a slight decrease in the expression of the gene coding for cholesterol 7a-hydroxylase (CYP7α), an enzyme responsible for bile acid production, as compared to WT. Furthermore, we observed a statistically significant lower expression of ApoA4 in livers of DKO mice. ApoA4 is a plasma protein, which is involved in lipid metabolism (Elshourbagy et al. 1985) and exerts antiatherosclerotic functions (Duverger N, et al 1996). However, a similar reduction in ApoA4 gene expression was observed in single SR-BI KO mice. Thus, further investigations are required to assess the mechanism underlying the reduction in serum lipids observed in DKO mice as compared to SR-BI KO mice.

In agreement with the role of ABCG1 in tissue lipid homeostasis (Kennedy et al. 2005), massive accumulation of lipids was observed in lungs of both ABCG1 KO and DKO mice compared to WT and single SR-BI KO mice on WTD. In contrast, ABCG1 disruption and SR-BI/ABCG1 double deletion did not influence lipid accumulation in spleen and liver.

The impact of SR-BI as well as ABCG1 on atherosclerosis and RCT has been widely investigated in vivo, mainly using experimental murine models. Total body deletion of SR-BI increases atherosclerosis susceptibility, indicating a protective role of SR-BI in atherosclerosis. The relevance of ABCG1 in atherogenesis has, however, not been clearly established. Over the years independent studies have reported contradicting results regarding the role of ABCG1, which has been shown to be either pro- or anti-atherosclerotic (Basso et al. 2007) (Out et al. 2007). Recently, Meurs et al. showed that the effect of ABCG1 on atherosclerosis depends on the stage of lesion development (Meurs et al. 2012). After 20 weeks of WTD feeding we did not observe any evidence of lesion development in ABCG1 KO or WT mice, which is in line with the fact that plasma lipid levels are not sufficiently high to induce atherosclerosis in these animals. Single SR-BI KO and ABCG1/SR-BI DKO mice did develop atherosclerotic lesions. However, despite the modest decrease in total serum cholesterol in the DKO mice, in comparison to single SR-BI KO animals, we could not demonstrate an effect of combined ABCG1 and SR-BI deletion on atherosclerotic lesion development. Previous studies have shown that the dysfunctional HDL particles that accumulate in SR-BI KO mice display a reduced anti-oxidant activity, leading to increased oxidative stress in these animals, potentially contributing to the proatherogenic effect of SR-BI deficiency (Van Eck et al. 2007). In line, we observed elevated mRNA levels of the anti-oxidant enzyme heme oxidase 1 (HO-1) in livers of SR-BI KO mice compared to WT animals after 20 weeks WTD feeding. Similarly, increased levels of HO-1 were observed in livers of the DKO mice, suggesting that the effect of SR-BI deficiency on HO-1 is not modulated by ABCG1 expression.

In addition to serum lipids, also leukocytes (and in particular monocytes) are an important causative factor in atherosclerotic lesion development (Galkina and Ley 2009). Double disruption of ABCG1 and SR-BI resulted in increased white blood cell counts, due to augmented levels of monocytes and neutrophils. Surprisingly, this increase in circulating immune cells was not observed in single SR-BI KO and ABCG1 KO mice. Previous data have suggested that HDL is essential to suppress hematopoietic proliferation by promoting cholesterol efflux from bone marrow derived cells (Yvan-Charvet et al. 2010). Of note, despite hyperlipidemia, no leukocytosis was observed in single SR-BI KO mice, indicating that the large HDL particles that accumulate in SR-BI KO mice are functional in preventing hematopoietic proliferation. Taken together these results suggest that the leukocytosis observed in the DKO mice is caused by a mechanism independent from hyperlipidemia.

Monocytes represent approximately 4% of the leukocyte population in mice. Two specific monocyte subsets, that vary in their capacity to infiltrate into atherosclerotic lesions, are defined based on the surface marker Ly6C (Sunderkötter et al. 2004). Upon WTD feeding, DKO mice show increased levels of both "pro-inflammatory" Ly6C^{high} and "more homeostatic" Ly6C^{low} monocytes subsets as compared to WT and single SR-BI and ABCG1 KO mice. Furthermore, a trend towards increased amounts of leukocytes was also observed in the peritoneal cavity of the DKO mice.

ABCG1 regulates inflammatory signalling pathways (Yvan-Charvet et al. 2008). The pro-inflammatory cytokine KC (the murine ortholog of IL-8), is a potent chemoattractant for neutrophils (Peveri P.et al. 1988) and its level has been shown to increase as a consequence of foam cell formation (Wang et al. 1996). Importantly, both ABCG1 and DKO mice show increased plasma levels of KC as compared to WT and SR-BI mice. Interestingly, two-way ANOVA analysis indicated that the increased levels of KC observed in DKO mice are statistically related to the absence of ABCG1. The increased inflammatory status of DKO mice was further illustrated by a large induction of the chemokine MCP-1 in liver. MARCO is a macrophage-specific membrane receptor classified as a class A scavenger receptor. In normal mice the expression of MARCO is limited to peripheral macrophages, whereas in case of infection its expression is induced in macrophages of various organs, including liver and lung (Elomaa O, et al. 1995). SR-BI and DKO mice show increased hepatic expression of MARCO compared to WT and ABCG1 KO mice. We did not observe differences in hepatic CD68 expression, a specific macrophage marker involved in uptake of OxLDL (Yamada et al.1998), among the diferent groups of mice. It is thus reasonable to speculate that the increase in MARCO expression in the DKO mice is not an effect of a general increase in the amount of macrophages, but rather the consequence of specific upregulation of MARCO in the macrophages. MARCO plays an important role in host defense. It is expressed in murine atherosclerotic lesions (Sakaguchi et al. 1998), but it does not mediate the uptake of modified lipoproteins (Elshourbagy NA. et al 2000). Hence, it is currently unknown if MARCO is a causative factor in lesion development.

In conclusion, in this study for the first time we phenotypically characterized ABCG1/SR-BI DKO mice, in regard to lipid metabolism and atherosclerosis. Our data indicate that, despite slightly lower plasma lipid levels, ABCG1 deficiency is not able to reduce the detrimental effects of the absence of SR-BI on atherosclerosis, probably as a consequence of increased leukocytosis.

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