Reverse cholesterol transport : a potential therapeutic target for atherosclerosis

Zhao, Y.

CHAPTER 4

Relative roles of various efflux pathways in net cholesterol efflux from macrophage foam cells in atherosclerotic lesions

Ying Zhao, Theo J.C. Van Berkel, and Miranda Van Eck

Division of Biopharmaceutics, Leiden/Amsterdam Center for Drug Research, University of Leiden, Leiden, The Netherlands

Purpose of this review
Cholesterol efflux mechanisms are essential for macrophage cholesterol homeostasis. HDL, an important cholesterol efflux acceptor, comprises a class of heterogeneous particles that induce cholesterol efflux via distinct pathways. This review focuses on the understanding of the different cholesterol efflux pathways and physiological acceptors involved, and their regulation in atherosclerotic lesions.

Recent findings
The synergistic interactions of ATP-binding cassette A1 (ABCA1) and G1 (ABCG1) as well as ABCA1 and scavenger receptor class B type I (SR-BI) are essential for cellular cholesterol efflux and the prevention of macrophage foam cell formation. However, the importance of aqueous diffusion should also not be underestimated. Significant progress has been made in understanding the mechanisms underlying ABCA1-mediated cholesterol efflux and regulation of its expression and trafficking. Conditions locally in the atherosclerotic lesion, e.g. lipids, cytokines, oxidative stress, and hypoxia, as well as systemic factors, including inflammation and diabetes, critically influence the expression of cholesterol transporters on macrophage foam cells. Furthermore, HDL modification and remodeling in atherosclerosis, inflammation, and diabetes impairs its function as an acceptor for cellular cholesterol.

Summary
Recent advances in the understanding of the regulation of cholesterol transporters and their acceptors in atherosclerotic lesions indicate that HDL-based therapies should aim to enhance the activity of cholesterol transporters and improve both the quantity and quality of HDL.

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Introduction

Cholesterol efflux pathways are pivotal for prevention of macrophage foam cell formation as macrophages cannot limit the uptake of excessive cholesterol. The efficiency of cholesterol efflux from macrophage foam cells in atherosclerotic lesions is determined by the quantity and quality of the extracellular acceptors, the activity of cholesterol transporters, and the availability of cholesterol for efflux. Significant progress has been made in the understanding of the mechanisms involved in cellular cholesterol efflux from macrophage foam cells in atherosclerotic lesions. In this review we will focus on the current view of the various cellular cholesterol efflux pathways and the physiological acceptors involved, and discuss their relative contributions to the prevention of macrophage foam cell formation in atherosclerotic lesions.

Macrophage cholesterol efflux pathways and their physiological acceptors

An important cholesterol efflux acceptor and facilitator of reverse cholesterol transport (RCT) from macrophage foam cells in the arterial wall to liver is high-density lipoprotein (HDL) [1]. Transport of HDL across the endothelium is essential for removal of the excess cholesterol from macrophage foam cells in atherosclerotic lesions. Two studies from the group of Von Eckardstein recently showed that transcytosis of apoAI through the endothelial layer occurs via ABC-transporter A1 (ABCA1), while ABC-transporter G1 (ABCG1) and scavenger receptor class B type I (SR-BI) mediate the transcytosis of HDL [2,3•].

HDL comprises a heterogeneous class of particles with a density (d) higher than 1.063 g/mL. HDL heterogeneity has recently been carefully reviewed by Rothblat and Phillips [4]. Generally, HDL can be classified into lipid-poor discoidal nascent pre-β HDL and lipid-rich spherical mature α-HDL, based on their difference in electrophoretic mobility [5,6]. Mature α-HDL functions as an acceptor for free cholesterol (FC) that dissociates from the cell membrane by aqueous diffusion [7,8]. Aqueous diffusion is a passive process driven by the FC gradient between cells and acceptors. It is thus thought that aqueous diffusion is inefficient. In contrast, scavenger receptor class B type I (SR-BI) and ATP-binding cassette G1 (ABCG1) facilitate FC efflux to mature α-HDL [9,10]. Furthermore, pre-β HDL induces phospholipid (PL) and FC efflux via ATP-binding cassette A1 (ABCA1) [11].

SR-BI facilitates passive transport of cholesterol to mature α-HDL

SR-BI is a multi-functional cell surface receptor [12,13]. One of its functions is to stimulate the bi-directional flux of FC between cells and α-HDL down the concentration gradient [10]. Interestingly, SR-BI is also found in the late endo/lysosomes and SR-BI deficiency is associated with accumulation of lysosomal cholesterol [14]. Thus, SR-BI may be involved in intracellular cholesterol trafficking from late endo/lysosomes.

Although not proven, it is often assumed that SR-BI accelerates aqueous diffusion of FC between cells and α-HDL. Binding to SR-BI will tether α-HDL in close proximity to the plasma membrane, thereby probably facilitating aqueous diffusion [15]. However, high-affinity binding alone is not sufficient to stimulate FC flux [16]. Moreover, SR-BI increases the fraction of membrane cholesterol available for efflux to α-HDL but not lipid-

free apoAI, although apoAI can bind to SR-BI [16,17]. Thus, the efficiency of SR-BI-mediated cholesterol efflux is related to its ability to enrich the membrane with cholesterol for efflux rather than to its binding to α-HDL[18]. In addition, SR-BI mediated HDL retro-endocytosis, i.e. the uptake of HDL whole particles followed by re-secretion of CE-poor HDL, has been implicated in SR-BI mediated cholesterol efflux [19,20]. This process appears independent of cholesterol transport out of the lysosome [20]. Currently, however, the importance for mass cholesterol efflux and prevention of macrophage foam cell formation is unclear.

Regulation of SR-BI expression in macrophages

SR-BI is undetectable in human monoytes, but its expression increases upon differentiation into macrophages [21,22]. Furthermore, the expression of SR-BI in macrophages is under control of peroxisome proliferator-activated receptor (PPAR) α and γ [23]. In liver, SR-BI stability and expression are posttranscriptionally regulated by PDZK1, a multi-PDZ domain containing adaptor protein [24]. Interestingly, targeted deletion of PDZK1 in mice reduced SR-BI expression in liver by 95%, but did not affect SR-BI expression in macrophages [25], indicating that macrophages have different posttranscriptional regulatory mechanisms.

Regulation of SR-BI expression in atherosclerosis

SR-BI is expressed in macrophages of atherosclerotic lesions [21,23,26] and regulated by lesional factors such as lipid, oxidative stress, and hypoxia (Figure 1). Cholesterol loading of macrophages reduces the mRNA levels of SR-BI through mechanisms independent of liver-X-receptor (LXR) and sterol regulatory element binding proteins (SREBPs) [22]. Interestingly, induction of atherosclerotic lesion regression by transplantation of plaque-containing arterial segments from apoE knockout (KO) mice into WT animals is associated with increased SR-BI expression in macrophages of atherosclerotic lesions [27]. Moreover, there is evidence that oxidative stress dampens the expression of SR-BI. Depletion of paraoxonase 1 (PON1), a major HDL-associated anti-oxidant enzyme, reduces mRNA and protein levels of SR-BI in peritoneal macrophages [28•]. SR-BI transcription is also suppressed by 7-ketocholesterol (7-KC), a major oxysterol in atherosclerotic lesions [29]. In addition, exposure of macrophages to hypoxic conditions decreased SR-BI transcription [26].

ABCG1 actively transports sterols to mature α-HDL

ABCG1 is an ABC half-transporter that actively transports sterols, including FC and 7-KC to α-HDL [9,30,31]. In non-cholesterol loaded macrophages, ABCG1 locates intracellularly [32]. Cholesterol loading or activation of LXR leads to the trafficking of ABCG1 from intracellular organelles, principally the Golgi, to the plasma membrane [32]. This ABCG1 redistribution is associated with increased cholesterol efflux to HDL.

Several lines of evidence have suggested that aqueous diffusion is involved in ABCG1-mediated cholesterol efflux. First, binding of HDL to ABCG1 is not required for induction of cholesterol efflux [30,33••]. Second, induction of ABCG1-mediated cholesterol efflux is linked to its capacity to enrich the membrane cholesterol pool for removal [34]. Cholesterol in ABCG1 generated lipid domains can only be removed by HDL but not lipid-free apoAI [34]. Third, only minimal amounts of PL are exported to

HDL [33••], which is in line with the fact that PL has a lower aqueous solubility as compared to FC. Some studies have suggested that ABCG1-mediated cholesterol efflux is

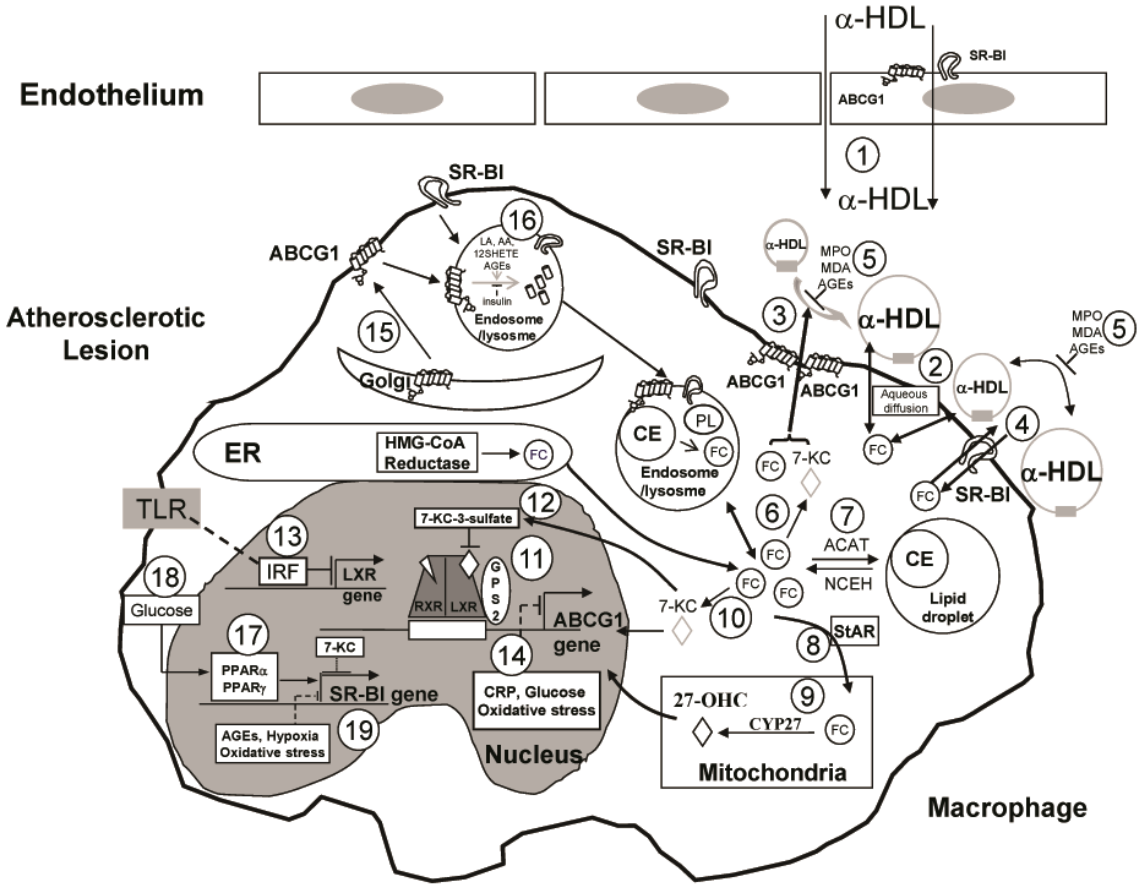

Figure1. Regulation of cholesterol efflux from macrophage foam cells to α-HDL in atherosclerotic lesions. (1) Two pathways for the infiltration of α-HDL into subendothelial space can be distinguished: paracellular transfer and ABCG1- and SR-BI-mediated transcytosis. Subsequently, α-HDL can induce efflux of free cholesterol (FC) from macrophages by aqueous diffusion (2) as well as via ABCG1 (3) and SR-BI (4). (5) In atherosclerotic lesions, MPO (myeloperoxidase)- and MDA (malondialdehyde)-mediated oxidation as well as AGEs (advanced glycation end products)-mediated glycation of HDL decrease its acceptor capacities to remove cholesterol by all these pathways. (6) Cholesterol traffics among different organelles. (7) FC can be esterified into cholesteryl ester (CE) by acyl-CoA:cholesterol acyltransferase (ACAT) while neutral cholesterol ester hydrolase (NCEH) hydrolyzes CE into FC. (8) Steroidogenic acute regulatory protein (StAR) transfers FC into mitochondrion where (9) FC is converted into 27-hydroxycholesterol (27-HOC) by sterol 27-hydroxylase (Cyp27). (10) 7-ketocholesterol (7-KC) is generated from FC and becomes the main sterol in atherosclerotic lesions. ABCG1 also exports 7-KC to HDL (3). The expression of ABCG1 and SR-BI in macrophages in lesions is regulated by different factors, thereby influencing their capacity to facilitate FC efflux. (11) ABCG1 transcription is induced by 27-HOC-mediated activation of liver-X-receptor (LXR) and recruitment of G protein suppressor 2 (GPS2). LXR heterodimerises with the retinoid-X-receptor (RXR) and induces transcription. (12) 7-KC-3-sulfate derived from 7-KC in lesions is an antagonist of LXR. (13) Activation of Toll-like receptor (TLR) can inhibit the expression of LXR through interferon regulatory factor (IRF) and indirectly decrease the ABCG1 expression. (14) In addition, the expression of ABCG1 can be downregulated by C-reactive peptide (CRP), glucose and oxidative stress. (15) After translation, ABCG1 is transported to the plasma membrane. (16) Fatty acids LA (linoleate acid) and AA (arachidonic acid) as well as 12SHETE (12S-hydroxyeicosatetranoic acid) and AGEs promote ABCG1 degradation, while insulin might increase the protein levels of ABCG1 by inhibition its degradation. (17) SR-BI transcription is under control of peroxisome proliferator-activated receptor alpha and gamma (PPARα and PPARγ). (18) Glucose might upregulate SR-BI expression through PPAR. (19) The transcription of SR-BI can be decreased by AGEs, hypoxia, and oxidative stress.

sphingomyelin-dependent [35,36]. Reduced cellular sphingomyelin levels due to a mutation of the ceramide transfer protein (CERT) are associated with a reduction of cholesterol efflux via ABCG1 [36]. Conversely, overexpression of CERT enhances ABCG1-mediated cholesterol efflux [36]. Different from SR-BI, ABCG1-mediated cholesterol transport is uni-directional from cells to HDL [33••].

Regulation of ABCG1 expression in macrophages

ABCG1 expression is highly regulated by LXR [37,38]. 27-hydroxycholesterol (27-HOC), an endogenous LXR ligand in macrophages, increases the expression of ABCG1 [39]. Activation of LXR by GW3965, an LXR agonist, triggers the recruitment of G protein suppressor 2 (GPS2), LXR, and the coactivator complex to the promoter for induction of ABCG1 transcription in THP-1 cells [40••]. Furthermore, knockdown of GPS2 by siRNA specifically decreases GW3965 induced ABCG1 expression. In addition, superoxide anions in macrophages, induced by C-reactive peptide (CRP), decrease the expression of ABCG1 in THP-1 cells, which can be abolished by the anti-oxidant selenon-L-methionine [45].

Regulation of ABCG1 expression in atherosclerotic lesions

In atherosclerotic lesions, ABCG1 co-localizes with macrophage foam cells [41]. Sterols, fatty acids (FAs), and oxidative stress regulate ABCG1 expression in macrophages (Figure 1). Cholesterol loading increases the accumulation of oxysterols, which activate LXR and induce the transcription of ABCG1 [37,39]. In contrast, the unsaturated FAs (uFAs) linoleic and arachidonic acid that accumulated in lesions promote the degradation of ABCG1 [42].

Oxidative stress in atherosclerotic lesions is also a negative regulator for ABCG1 expression. 12/15-lipoxygenase, highly expressed in macrophages of early atherosclerotic lesions [43], destabilizes ABCG1 through generation of an uFA metabolite named 12S-hydroxyeicosatetranoic acid (12SHETE) [42,44•]. 12SHETE promotes the serine phosphorylation of ABCG1 through p38- and JNK2-dependent pathways, thereby increasing proteasome-mediated ABCG1 degradation [44•].

Interestingly, ABCG1 is undetectable in macrophages from patients with diabetes, leading to a 30% decrease in cholesterol efflux to HDL [46]. High glucose levels in these patients reduce the mRNA and protein levels of ABCG1 in macrophages [47]. Importantly, insulin treatment increases the protein but not mRNA levels of ABCG1 [47].

ABCA1 mediated phospholipid and cholesterol efflux to lipid-free/poor apoAI

ABCA1 is an ABC full transporter that facilitates the efflux of PL and FC to lipid-free/poor apolipoproteins [11,48,49]. ABCA1 localizes to the plasma membrane and intracellular compartments, including the Golgi apparatus and endo/lysosomes [50]. Deletion of the C-terminal VFVNFA and PDZ motifs of ABCA1 inhibits the exit of ABCA1 from the endoplasmic reticulum (ER) [51], while palmitoylation of the cysteines of ABCA1 promotes its trafficking to the plasma membrane [52••]. Interestingly, in macrophages, SPTLC1, a subunit of the serine palmitoyltransferase enzyme, can bind to ABCA1 and trap it in the ER [51]. In addition, Rab8, a Rab GTPase was recently suggested to regulate ABCA1 trafficking to the plasma membrane in macrophages from human atherosclerotic lesions [53••]. Depletion of Rab8 keeps ABCA1 in recycling

organelles containing β1 integrin and transferrin, while overexpression of Rab8 increases ABCA1 levels at the cellular membrane and reduces cholesterol deposition in foam cells [53••].

Several different models for ABCA1-mediated cholesterol efflux have been suggested. According to the simplest model, ABCA1 acts as an apoAI receptor that induces the transfer of PL and FC upon binding [54,55]. ABCA1 expression, however, changes the cellular membrane lipid microenvironment [56-58]. Furthermore, the ATPase activity of ABCA1 is necessary for apoAI binding to the cell surface [59]. Therefore, another view is that apoAI interacts with specialized lipid domains in the plasma membrane formed by ABCA1 and subsequently induces lipid removal. In addition, a hybrid model has been proposed [60-62]. ApoAI first interacts with ABCA1 to activate janus kinase 2 (JAK2), which in turn increases binding of apoAI to ABCA1. This latter binding facilitates the interaction of apoAI to the lipid domains generated by ABCA1, thereby promoting apoAI lipidation. By using various ABCA1 mutants, Vaughan et al. recently further revealed the interdependency between JAK2 activation, apoAI binding and lipid export via ABCA1 [63•].

ApoAI binding to ABCA1 can lead to internalization of the ABCA1/apoAI complex [64]. It has been proposed that apoAI enters the cell and is re-secreted as a lipidated particle during ABCA1-mediated cholesterol efflux from macrophages [64,65]. However, other groups showed that apoAI lipidation occurs on the cell surface and that apoAI is degraded in the lysosome after internalization [66,67]. Recently, Lu et al. provided evidence that binding of apoAI to ABCA1 prevents ABCA1 degradation after internalization and promotes rapid recycling of ABCA1 to the cell surface in non-cholesterol loaded THP-1 cells [68]. Under this condition, blockade of clathrin-mediated ABCA1 endocytosis by cytochalasin D increased ABCA1 expression on the cell surface and subsequently enhanced apoAI lipidation, indicating that endocytic recycling of ABCA1/apoAI is not required for lipid efflux to apoAI. In contrast, blockade of clathrin-mediated endocytosis by monodansyl cadaverine leads to increased ABCA1 expression on the cell surface of acetylated LDL (acLDL) loaded THP-1 cells, but reduces cholesterol efflux to apoAI, indicating that intracellular trafficking of ABCA1/apoAI is important for cholesterol efflux under conditions that macrophages have accumulated excess cholesterol [69••].

In human serum, pre-β1 HDL generated by ABCA1 is characterized as a 67 kDa subspecies of HDL, containing two apoAI molecules with several molecules of PL and FC [70]. Similar to lipid-free apoAI, preβ1 HDL can interact with ABCA1 for further cholesterol efflux. Although pre-β1 HDL only comprises around 5% of the amount of apoAI in the circulation, it is suggested that the levels and function of pre-β1 HDL are important for prevention of atherosclerosis. Llera-Moya et al. recently demonstrated that the concentration of pre-β1 HDL in serum of healthy individuals significantly correlates to the serum efflux capacity via ABCA1, and is negatively associated with the carotid intima thickness [71••].

Regulation of ABCA1 in macrophages

ABCA1 is preferentially expressed on ly6C^{low} tissue macrophages [72•]. The transcription of ABCA1 is upregulated through 27-HOC-induced activation of LXR [39,73]. In line, increasing 27-HOC by enhancement of cholesterol delivery to mitochondrial sterol 27-hydroxylase (Cyp27) by steroidogenic acute regulatory protein (StAR) increases the expression of ABCA1 [74•]. LXR has two isoforms, namely LXRα and LXRβ. While

LXRα is primarily involved in the transcriptional regulation of ABCA1, LXRβ regulates ABCA1 protein expression posttranslationally [75••]. In absence of cholesterol loading, LXRβ binds to the C-terminus of ABCA1 and stabilizes it on the cellular membrane. Interestingly, this stabilization is associated with reduced ABCA1-mediated cholesterol efflux, thereby preventing excessive elimination of cholesterol. Upon cholesterol loading, increased oxysterols bind to LXRβ, leading to dissociation of LXRβ from ABCA1 and a rapid restoration of ABCA1 activity in cholesterol efflux. This interaction between ABCA1 and LXRβ thus provides a novel regulatory mechanism for cells to quickly respond to cholesterol loading.

ApoAI stabilizes ABCA1 by inhibition of thiol protease-mediated and calpain-mediated proteolysis [76-78]. Phosphorylation of a PEST sequence, rich in proline, glutamic acid, serine, and threonine at the cytoplasmic region of ABCA1 directs calpain-mediated degradation [77]. Iwamoto et al. recently demonstrated that calmodulin directly binds to a motif of ABCA1 near the PEST sequence in a Ca2+ dependent manner and protects ABCA1 from calpain-mediated degradation [79•]. Since binding of apoAI to ABCA1 quickly induces Ca2+ influx [80], it is conceivable that Ca2+/calmodulin contributes to the apoAI-increased ABCA1 stability. In addition, activation of Ras homolog family member A (RhoA) is involved in the apoAI-induced ABCA1 stabilization [81•]. The binding of apoAI to ABCA1 transiently activates RhoA, whereas inhibition of RhoA activation by pharmaceutical inhibitors or expression of a negative RhoA mutant block the ability of apoAI to stabilize ABCA1.

Regulation of ABCA1 in atherosclerotic lesions

In atherosclerotic lesions, ABCA1 co-localizes with macrophage foam cells [82,83]. Below important factors, known to regulate the expression of ABCA1 in atherosclerotic lesions and thus are expected to influence the net transfer of cholesterol out of lesions, are described (Figure 2).

Sterols and fatty acid

ABCA1 expression in atherosclerotic tissue correlates with LXRα mRNA levels [83]. 7-KC, a highly toxic oxysterol found in atherosclerotic lesions [84], induces ABCA1 transcription by activation of LXR [85]. In agreement, overexpression of LXRα in macrophages increases ABCA1-mediated cholesterol efflux to apoAI and prevents the development of atherosclerosis [86]. In advanced atherosclerotic lesions, accumulation of FC in macrophages decreases the expression of ABCA1 protein by proteasome-mediated degradation [87]. Moreover, uFA linoleic acid decreases the transcription of ABCA1 [47]. Like ABCG1, ABCA1 is destabilized by unsaturated fatty acids (uFAs) that accumulate in atherosclerotic lesions [88-90]. In line, deletion of macrophage fatty acid synthase increases cellular cholesterol efflux to apoAI [91•].

Inflammation

Atherosclerotic lesions are inflammatory sites containing various cytokines [92,93]. The balance between pro- and anti-inflammatory cytokines in atherosclerotic lesions is an important determinant for macrophage foam cell formation by regulation of ABCA1 expression. The pro-inflammatory cytokines IFNγ and TNFα downregulate ABCA1 transcription in macrophage foam cells [94,95]. Interestingly, the anti-inflammatory cytokines IL-10 and TGFβ can reverse the TNFα and IFNγ induced downregulation of

ABCA1, respectively [95,96]. In agreement, overexpression of macrophage IL-10 reduces CE accumulation in atherosclerotic lesions and inhibits atherogenesis [97].

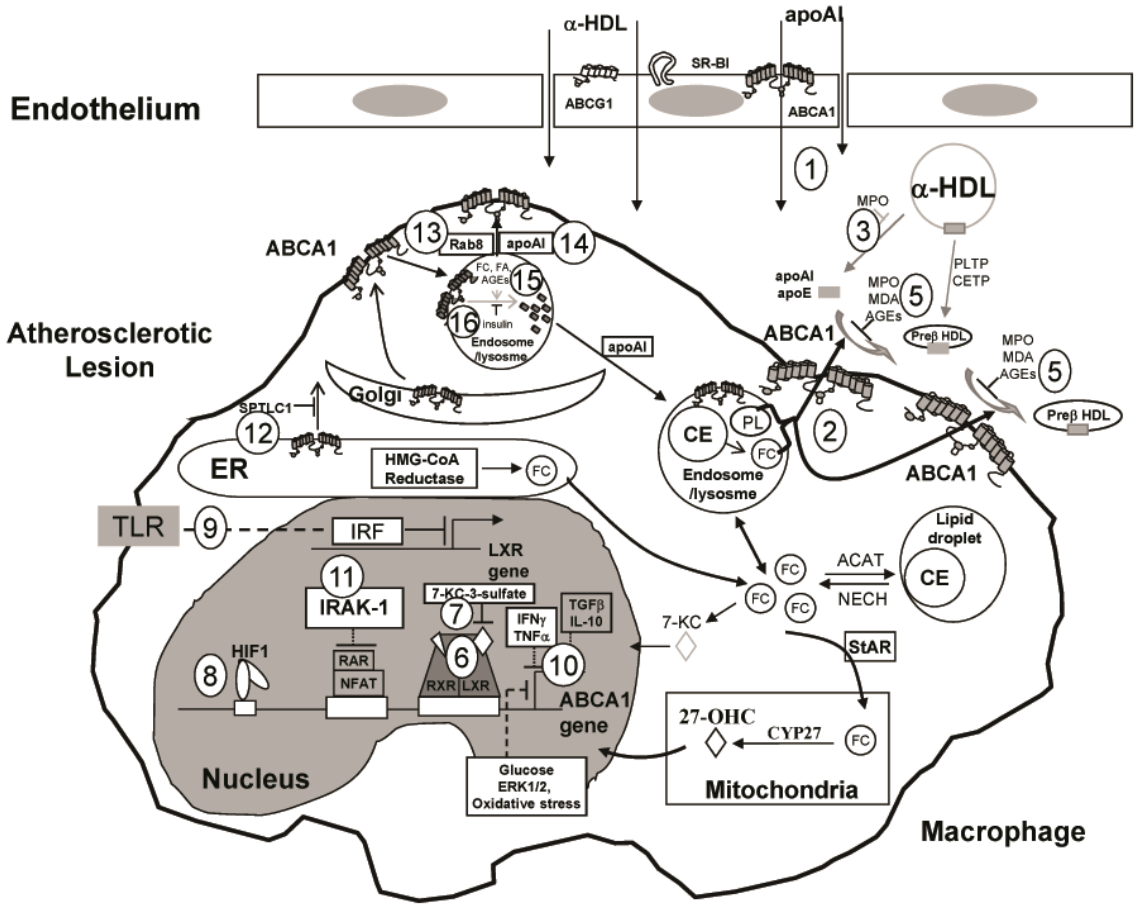

Figure 2. Regulation of cholesterol efflux from macrophage foam cells to lipid-free/poor apolipoproteins in atherosclerotic lesions. (1) Macrophages do not produce apoAI. Therefore, transport of apoAI across endothelium is pre-requisite for apoAI-mediated removal of cholesterol from macrophage foam cells in lesions. ABCA1 on endothelial cells is involved in transcytosis of apoAI. (2) ApoAI and macrophage-derived apoE induce ABCA1-mediated PL and FC efflux to form preβ HDL, which can further remove phospholipid (PL) and FC via ABCA1. (3) MPO inhibits the dissociation of apoAI from α-HDL. (4) Phospholipid and cholesteryl ester transfer protein (PLTP and CETP) can generate preβ HDL from α-HDL. (5) MPO- and MDA-mediated oxidation as well as AGEs-mediated glycation of lipid-free/poor apoAI impair its function as cholesterol acceptor. The expression of ABCA1 can be regulated by several factors locally in the atherosclerotic lesion. (6) LXR activation by the oxysterols 27-HOC and 7-KC induce the transcription of ABCA1. (7) In atherosclerotic lesions, 7-KC can be converted into 7-KC-3-sulfate, which decrease the transcription of ABCA1 by inhibiting the activation of LXR. (8) In hypoxic regions of lesions, hypoxia-inducible factor 1 (HIF1) induces the expression of ABCA1. (9) Activation of TLR during inflammation decreases the gene expression of LXR through IRF, thereby indirectly decreasing the expression of ABCA1. Inflammation can also downregulate the expression of ABCA1 through (10) the proinflammatory cytokines IFNγ and TNFα as well as (11) a key innate immunity signaling kinase (IRAK-1). IRAK-1 reduces the levels of nuclear factor of activated T-cells (NFAT) as well as retinoid acid receptor (RAR), thereby downregulating the expression of ABCA1. The trafficking of ABCA1 to the plasma membrane is important for its function. (12) SPTLC1 (a subunit of the serine palmitoyltransferase enzyme) binds ABCA1 and inhibits the exit of ABCA1 from the endoplasmic reticulum (ER), while (13) Rab8 participates in the trafficking of ABCA1 from recycling organelles to the plasma membrane. (14) Binding of apoAI to ABCA1 inhibits ABCA1 degradation and facilitates the recycling of ABCA1 back to the plasma membrane. Furthermore, (15) FC, FA (fatty acid), and AGEs promote the protein degradation of ABCA1. (16) Insulin increases the protein levels of ABCA1 probably by increasing the protein stability of ABCA1.

Viral and bacterial pathogens have long been suspected to be modulators of atherosclerosis. Microbial ligands activate toll-like receptor (TLR) signaling, which blocks LXR activation by interferon regulatory factors, thereby decreasing the expression of ABCA1 and increasing lipid accumulation in macrophages [98,99]. Conversely, LXR activation reduces TLR-mediated macrophage foam cell formation [99,100]. Importantly, Maitra et al. recently provided a novel connection between innate immunity signaling and the regulation of ABCA1 expression in macrophages [101•]. This study identified binding sites for retinoic acid receptor (RAR) and nuclear factor of activated T-cells (NFAT) in the promoter of ABCA1, and revealed that IRAK-1, a key innate immunity signaling kinase, suppressed ABCA1 transcription by downregulation of RARα and NFATc2.

In addition, CRP, an acute phase (AP) protein of inflammation, inhibits ABCA1 expression and cholesterol efflux in macrophage foam cells by activation of extracellular signal regulated kinase 1 and 2 (ERK1/2) [45]. In line, Zhou et al. demonstrated that inhibition of ERK1/2 increases the mRNA and protein levels of ABCA1 in macrophages independent of LXR [102•]. Interestingly, inhibition of ERK1/2 and activation of LXR synergistically induce ABCA1 expression and macrophage cholesterol efflux [102•]. Thus, combined targeting of ERK1/2 and LXR might be a potent therapeutic strategy for inhibition of atherosclerosis.

Oxidative stress

Induction of oxidative stress by iron/ascorbate or CRP in THP-1 cells decreases the mRNA and protein expression of ABCA1 [45,103]. Importantly, anti-oxidant treatment can rescue the oxidative stress-induced attenuation of ABCA1 expression and cholesterol efflux [45,103].

Hypoxia

Hypoxia-inducible factor 1 (HIF1), activated under hypoxic conditions [104,105], is a heterodimer composed of HIF1α and HIF1β [106]. Importantly, in macrophages isolated from human atherosclerotic lesions, ABCA1 expression showed a strong correlation with HIF1β expression [107••], indicating that HIF1β is a major regulator of macrophage ABCA1 transcription in atherosclerotic lesions. However, hypoxia also promotes the secretion of inflammatory mediators, fatty acid biosynthesis, ATP depletion and lipid droplet formation [108]. Thus, hypoxia is associated with increased macrophage foam cell formation despite HIF1-mediated induction of macrophage ABCA1 transcription.

Diabetes

In diabetes, high levels of glucose decrease the mRNA and protein levels of ABCA1 in macrophages [47]. In line, macrophages in diabetic mice show a reduced expression of ABCA1 [109]. Moreover, glyoxal and glycoaldehyde acutely and severely destabilize the ABCA1 protein [110].

Interestingly, ABCA1 expression on beta-cells of pancreatic islets influences insulin secretion and glucose homeostasis [111, 112•]. Like ABCG1, the protein levels of ABCA1 are elevated by insulin treatment [47]. Macrophages have a functional insulin receptor signaling pathway, and downregulation of this pathway due to insulin resistance has been implicated in the progression of advanced atherosclerotic lesions [113]. Thus, macrophage insulin resistance might favor increased foam cell formation in atherosclerosis by inhibiting insulin-mediated ABCA1 and ABCG1 induction.

The relative role of each pathway for net cholesterol efflux from macrophage foam cells in atherosclerotic lesions

The relative contribution of aqueous diffusion and the ABCA1-, ABCG1-, and SR-BI-mediated pathways for the net cholesterol efflux from macrophage foam cells to serum has been evaluated *in vitro* by using cholesterol-loaded macrophages isolated from ABCA1, ABCG1, and SR-BI KO mice [114,115]. The studies indicate that ABCA1, ABCG1 and SR-BI are responsible for approximately 35%, 21%, and 9%, respectively of the efflux capacity of the cells. Interestingly, a staggering 35% of the cholesterol is effluxed simply via aqueous diffusion. Moreover, data from *in vivo* RCT assays, in which *in vitro* ^{3}H-cholesterol/acLDL laden macrophages are injected into the peritoneal cavity of WT mice and the excretion of the ^{3}H-label into the feces is followed, suggest a similar contribution of the different pathways [114,115]. However, it must be noted that the following limitations in the experimental setups of these studies might impede an accurate evaluation of the relative roles of the different cholesterol efflux pathways. 1) *In vitro* cell culture might change the expression of the transporters studied; 2) Most studies use acLDL, which is not an endogenous modified lipoprotein, to induce macrophage foam cell formation. In relation to this it is important to be aware that acLDL-laden macrophages exhibit a different phenotype from oxidized LDL-laden cells [116]; Moreover, acLDL loading increases the cellular cholesterol poor for efflux to apoAI via the ABCA pathway while cells loaded with native LDL preferentially efflux cholesterol to HDL [117]; 13) Nonlipidemic serum as acceptor used for *in vitro* cholesterol efflux studies and WT mice used as recipients for the macrophage foam cells in the *in vivo* RCT assays are not likely to accurately reflect the conditions in atherosclerotic lesions *in vivo*.

The net cholesterol efflux from macrophage foam cells in atherosclerotic lesions is not simply a sum of the amount cholesterol removed via each individual pathway. Competitive and synergistic interactions of different efflux pathways can result in more or less net cholesterol efflux. Lipidation of apoAI via ABCA1 generates the substrates for ABCG1-mediated cholesterol efflux, suggesting that ABCA1 and ABCG1 work in sequence and in synergy to export cholesterol [118]. Combined deletion of ABCA1 and ABCG1 on macrophages results in a dramatic decrease in net cholesterol mass efflux to HDL *in vitro* and largely reduced macrophage RCT *in vivo* [115,119-121]. Accordingly, massive macrophage foam cell accumulation was observed in the peritoneal cavity as well as various organs such as spleen, lymph nodes, liver, Peyer's patches, and lung in total body ABCA1/ABCG1 double KO (dKO) mice on chow [119]. In contrast, single ABCA1 KO and ABCG1 KO mice only displayed a relatively small number of foam cells in the lung. In bone marrow transplanted LDLr KO mice on either chow or Western-type diet (WTD), single ABCA1 deficiency leads to a slight increase in the percentage of foam cells in the peritoneal cavity. Also a small number of foam cells were observed in the lung of ABCG1 KO transplanted animals. In contrast, combined deletion of ABCA1 and ABCG1 leads to massive foam cell accumulation in various tissues and the peritoneal cavity [120,121] (Out R, unpublished data), further illustrating the importance of ABCA1 and ABCG1 in preventing macrophage foam cell formation. As ABCA1/ABCG1 dKO mice do express SR-BI, the ABCA1- and ABCG1-mediated pathways have been thought to account for the majority of the cholesterol effluxed from macrophage foam cells *in vivo*, while SR-BI seems to be only a minor contributor. In line, SR-BI KO macrophages exhibit a small decrease in cholesterol efflux to HDL *in vitro* as compared to WT macrophages [114,122]. Also, SR-BI deficiency in macrophages does not influence *in vivo* RCT in WT mice [115]. SR-BI is a multifunctional receptor that can influence macrophage foam cell formation via different mechanisms. First, SR-BI facilitates cholesterol efflux from macrophages to α-

HDL, which will limit foam cell formation. Second, SR-BI promotes the uptake of atherogenic ligands like βVLDL and oxidized LDL [12,13,122], thereby promoting macrophage foam cell formation. Third, SR-BI mediates selective uptake of CE from HDL will promote CE accumulation in macrophages [123], but also may generate CE-depleted/PL-rich HDL particles that are efficient acceptors for cholesterol efflux. Thus, the net effect of SR-BI on macrophage foam cell formation *in vivo* is difficult to predict and may vary with conditions studied. Consistently, SR-BI on macrophages accelerates the development of small fatty streak lesions in LDLr KO mice [122], whereas the development of advanced atherosclerotic lesions in LDLr KO and apoE KO mice is inhibited by macrophage SR-BI [122,124,125]. In line, Yancey et al. showed dramatically increased FC and CE accumulation in peritoneal macrophages of apoE/SR-BI dKO mice as compared to those of apoE KO mice [126]. Interestingly, Cuchel et al. recently showed that FC mobilization *in vivo* in response to rHDL infusion is primarily mediated by SR-BI but not ABCA1 or ABCG1 [127••]. Moreover, Yvan-Charvet et al. demonstrated that overexpression of apoAI in heterozygous LDLr KO mice can protect against atherosclerosis in the absence of macrophage ABCG1 and ABCA1 [128••]. Thus, SR-BI might be more important for controlling cellular cholesterol homeostasis *in vivo* than was initially anticipated. In agreement, we found that combined deletion of macrophage ABCA1 and SR-BI resulted in massive foam cell formation in the peritoneal cavity and spleen of LDLr KO mice on WTD (Zhao Y, unpublished data). These data indicate that, at least under severe hypercholesterolemia, the synergistic action of ABCA1 and SR-BI-mediated cholesterol efflux from macrophage foam cells, similarly as the combined function of ABCA1 and ABCG1, is important for preventing excessive macrophage foam cell formation *in vivo*.

Importantly, a recent study demonstrated that in human cholesterol-loaded macrophages, ABCA1 and Cla-1, the human ortholog of SR-BI, rather than ABCG1 are key mediators in cholesterol efflux to HDL [129••], suggesting distinguished differences in the relative roles of the ABCG1 and SR-BI pathway in net cholesterol efflux from human and mouse macrophages.

Aqueous diffusion might be underestimated [114,115]. Since mature α-HDL is an important acceptor for cholesterol released from macrophages via aqueous diffusion as well as for ABCG1 and SR-BI mediated cholesterol efflux, combined deletion of ABCG1 and SR-BI will be essential for determination of the relative importance of aqueous diffusion for the prevention of macrophage foam cell formation *in vivo*. In addition, ABCG1/SR-BI dKO mice will be a useful tool for studying the interaction between macrophage ABCG1 and SR-BI and the subsequent effects on macrophage foam cell formation *in vivo*.

HDL modification and remodeling in atherosclerosis

Epidemiological studies have consistently shown that high levels of HDL cholesterol (HDL-C) are associated with reduced susceptibility to cardiovascular disease (CVD) [130,131]. However, increasing evidence indicates that HDL functionality rather than HDL-C level is a good predictor of cardiovascular risk [131]. HDL has multiple functions, including cholesterol efflux induction, anti-inflammation, anti-oxidation, anti-thrombosis, and enhancement of endothelial function [131,132]. Many of these functions are linked to various proteins associated with HDL particles [131,133•]. Changes in the protein and/or lipid composition of HDL might lead to the formation of a dysfunctional HDL particle that is no longer protective against atherosclerosis. Several pathological states, including oxidative stress [134,135], inflammation [136,137], and diabetes [138-140], modify and

remodel the HDL, leading to a compromised function as a cellular lipid acceptor (Figure 1 & 2).

MPO, a potent oxidizing enzyme secreted by macrophages and neutrophils in atherosclerotic lesions [141,142], is regarded as a good *in vivo* biomarker, associated with dysfunctional HDL. MPO binds to apoAI and uses hydrogen peroxide to generate chlorinated and nitrated apoAI [143,144] that shows a reduced ABCA1-binding capacity and an impaired ability to promote cholesterol efflux [145•]. Importantly, human atherosclerotic lesions are enriched in chlorinated and nitrated apoAI and patients with CVD have higher circulating levels of chlorinated and nitrated apoAI [143,144]. Also MPO-modified HDL is no longer capable of interacting with SR-BI [146•]. Recently, Cavigiolio et al. provided evidence that MPO-mediated crosslinking of apoAI retards the spontaneous dissociation of apoAI from HDL, thereby limiting the availability of lipid-free apoAI for efflux [147••]. Oxidation of methionine in apoAI by MPO also inhibits its ability to activate LCAT, a critical step in HDL metabolism and RCT [148]. In line, overexpression of MPO impairs *in vivo* reverse cholesterol transport [149]. In addition, MPO-modified HDL is proinflammatory as these particles can activate NF-kappaB (nuclear factor kappa-light-chain-enhancer of activated B) and increase the expression of adhesion molecules in aortic endothelial cells [146•].

Malondialdehyde (MDA) is a product of lipid peroxidation [150,151]. In human atherosclerotic lesions, MDA-apoAI adducts are elevated [152•]. This modification is suggested to hinder the interaction of apoAI with lipids and ABCA1, thereby reducing cholesterol efflux via ABCA1.

In addition, *in-vitro* experiments have shown that lipid-free apoAI and pre-β–HDL are sensitive to proteolytic degradation by matrix metalloproteinases [153], cathepsins [154], and the mast cell-derived enzymes chymase and tryptase [155, 156], which are present in atherosclerotic lesions. Interestingly, in fatty streaks, mast cells are localized close to foam cells [157]. Thus, chymase and tryptase secreted by mast cells in the atherosclerotic lesions could promote macrophage foam cell formation by reducing the availability of extracellular acceptors for ABCA1-mediated cholesterol efflux. In line, systemic activation of mast cells by compound 48/80 results in an impaired capacity of serum and intraperitoneal fluid to promote cellular cholesterol efflux from macrophage foam cells in culture [158●].

Conclusion

The relative importance of the different pathways for cholesterol efflux from macrophage foam cells in atherosclerotic lesions is attributed to the activity of SR-BI, ABCA1, and ABCG1, the interaction between these transporters, as well as the quantity and quality of their respective acceptors. Conditions locally inside the atherosclerotic lesions, including the presence of lipids, cytokines, hypoxia, and oxidative stress as well as systemic factors (e.g. diabetes and inflammation) modulate cholesterol efflux from macrophages by influencing the activity of the transporters involved and modification of their acceptors (Figure 1 & 2). Thus, improvement of HDL quality as well as quantity will be an important clinical goal for atherosclerosis treatment. Hereto, international consensus is required on standardized methodologies for HDL isolation and protocols for evaluation of HDL function. HDL proteome analysis may lead to a more comprehensive understanding of the differential functions of individual HDL subclasses. Importantly, increased understanding of the various cholesterol efflux pathways and HDL functionality will promote the development of new HDL-based therapies.

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