Regulation of Lipid Droplet Cholesterol Efflux from Macrophage Foam Cells: a Role for Oxysterols and Autophagy

MIREILLE OUIMET

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Department of Biochemistry, Microbiology and Immunology

Faculty of Science

University of Ottawa

Abstract

Macrophage foam cells are the major culprits in atherosclerotic lesions, having a prominent role in both lesion initiation and progression. With atherosclerosis being the main factor underlying cardiovascular complications, there is a long-standing interest on finding ways to reverse lipid buildup in plaques. Studies have shown that promoting reverse cholesterol transport (RCT) from macrophage foam cells is anti-atherogenic because it alleviates the cholesterol burden of the plaques. The goal of this thesis was to gain insight into the mechanisms that govern cholesterol efflux from macrophage foam cells. The first part of this study looked at the ability of different oxysterols to promote cholesterol efflux in unloaded as compared to lipid-loaded macrophages, and our major finding here is that epoxycholesterol decreases efflux in lipid-loaded macrophages. It appears that epoxycholesterol does so by impairing the release cholesterol from its cellular storage site, the lipid droplet (LD), where it accumulates in the form of cholesteryl esters (CE). These results highlighted the importance of cholesterol release from LDs for efflux; indeed, this process is increasingly being recognized as the rate-limiting step for RCT *in vivo*. Subsequent experiments aimed at elucidating the mechanisms that govern LD CE hydrolysis in macrophage foam cells lead to the discovery of a novel pathway involved in cholesterol efflux. Macrophage CE hydrolysis is classically defined as being entirely dependent on neutral CE hydrolases. In the second part of this study, we demonstrate that in addition to the canonical CE hydrolases, which mediate neutral lipid hydrolysis, lysosomal acid lipase (LAL) also participates in the hydrolysis of cytoplasmic CE. Autophagy is specifically triggered in macrophages by atherogenic lipoproteins and delivers LD CE to LAL in lysosomes, thus generating free cholesterol for efflux. This autophagy-mediated cholesterol efflux is a process that is primarily dependant on the ABCA1 transporter and, importantly, is important for whole-body RCT. Overall, the studies presented in this thesis support that macrophage LD CE hydrolysis is rate-limiting for cholesterol efflux and shed light on the mechanisms of cholesterol mobilization for efflux in macrophage foam cells.

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Contents

List of Abbreviations

ABCA1	ATP-binding cassette A-1
ABCG1	ATP-binding cassette G-1
ACAT	acetyl-coenzyme A acyltransferase
ACATi	ACAT inhibitor
AcLDL	acetylated LDL
AgLDL	aggregated LDL
AP-1	activator protein-1
Apo	apolipoprotein
Arg	arginase 1
Atg	autophagy-related protein
BMDM	bone marrow-derived macrophage
CE	cholesteryl ester
CESD	cholesteryl ester storage disease
CHD	coronary heart disease
CVD	cardiovascular disease
DR	direct repeat
Epoxy	24(S),25-epoxycholesterol
ER	endoplasmic reticulum
HC	hydroxycholesterol
HDL	high density lipoprotein
HSL	hormone sensitive lipase
Idol	inducible degrader of the LDL receptor
IL	interleukin
INSIG	insulin-induced gene
LAL	lysosomal acid lipase
LD	lipid droplet
LDL	low density lipoprotein
LpL	lipoprotein lipase
LXR	liver X receptor
LXRE	LXR response element
mTOR	mammalian target of rapamycin
LPS	lipopolysaccharide
LXR	liver X receptor
MCP-1	monocyte chemoattractant protein-1
M-CSF	macrophage colony-stimulating factor
MTP	microsomal triglyceride transfer protein
NCEH	neutral cholesteryl ester hydrolase
Nceh1	neutral cholesterol ester hydrolase 1
NCoR	nuclear receptor co-repressor
NFκB	nuclear factor-κB
NPC	Niemann Pick type C
OxLDL	oxidized LDL

OSC	oxidosqualene:lanosterol cyclase
PLA_2	phospholipase A_2
PPAR	peroxisome proliferator-activated receptor (PPAR)
PPRE	PPAR response element
PCSK9	proprotein convertase subtilisin/kexin type 9
RCT	reverse cholesterol transport
RXR	retinoid X receptor
SCAP	SREBP cleavage-activating protein
SMase	sphingomyelinase
SRA	scavenger receptor A
SRE	sterol regulatory element
SREBP	sterol regulatory element binding protein
TLR	toll-like receptor
TG	tryglyceride
TGH	triglyceride hydrolase
TGF	transforming growth factor
TNFα	tumor necrosis factor alpha
VCAM-1	vascular cell adhesion molecule-1
VLDL	very low density lipoprotein
WD	Wolman disease
WT	wild-type

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1 Introduction

Heart disease, a leading cause of death worldwide, is particularly prevalent in westernized countries, which suffer from an obesity epidemic. Atherosclerosis is the major underlying factor leading to cardiovascular events such as heart attacks and stokes, and therefore understanding the mechanisms that govern atherosclerotic lesion initiation and progression are important in order to devise interventions to prevent ensuing cardiovascular complications. It is well established that lipid-laden macrophages, or macrophage foam cells, are at the centre stage of the atherosclerotic plaque. There is strong evidence to support that enhancing macrophage reverse cholesterol transport is a good anti-atherogenic strategy, and in recent years how cholesterol is mobilized from the lipid droplet – the rate-limiting step in this process – has received a great deal of attention. A better understanding of this process and the molecular mechanisms of atherosclerosis will enable the development of treatment and prevention strategies to improve the health and quality of life of individuals globally.

1.1 Atherosclerosis and Heart disease

1.1.1 Etiology of Atherosclerosis

Lipids and lipoprotein metabolism: triglycerides and cholesteryl esters are shuttled in the circulation surrounded by a polar coat of free cholesterol and phospholipids, in association with apolipoproteins, and together these comprise lipoproteins. In addition to binding and transporting plasma lipids, apolipoproteins are ligands for receptor-mediated lipoprotein uptake. There are 5 classes of lipoproteins, which differ in their protein and lipid composition and, consequently, in their density: chylomicrons, very low density lipoprotein (VLDL), intermediate density lipoprotein (IDL), low density lipoprotein (LDL) and high density lipoprotein (HDL). Importantly, these lipoproteins supply the peripheral tissues with triglycerides (TG) for energy production and cholesterol, which is an important constituent of cellular membranes and a precursor for steroid hormones, vitamin D and bile acids. Chylomicrons are produced in the intestine and supply muscle and adipose tissues with dietary lipids, after which chylomicron remnants return to the liver, which produces its own lipoprotein to transport newly synthesized lipids: VLDL. The later continues to supply peripheral tissues with lipids between meals. As the VLDL-associated TG is depleted during

lipolysis to generate free fatty acids that cells of the peripheral tissues use for energy production, there is progressive conversion of the VLDL particle to IDL, and LDL.

Dyslipidemia: high concentrations of triglyceride-rich lipoproteins and low HDL are strong and independent risk factors for the development of cardiovascular disease (CVD). Epidemiological studies reveal the correlation between high plasma cholesterol levels and 1) atherosclerotic lesions, 2) coronary heart disease (CHD).[1] Dyslipidemia is complex and can arise from environmental factors or genetic predisposition, or both. For instance, a number of genetic defects impairing binding, uptake, or degradation of LDL and resulting in elevated plasma LDL have been associated with premature atherosclerosis.[2] Tangier disease patients, who have loss-of-function mutations in the ATP-binding cassette A1 (ABCA1) critical for HDL genesis, on average have a 5-fold higher incidence of cardiovascular disease as compared to unaffected individuals.[3] Some environmental considerations include diet, exercise, etc. According to estimates of the adequate plasma LDL level to supply cholesterol to cells of the body, the appropriate plasma LDL-cholesterol level in man should be about 25mg/dl, whereas in the average 'Western man', it is about 5-fold higher than that,[2] making the western diet of industrialized countries such as Canada and the United States an important environmental risk factor.

Theories of atherogenesis: atherosclerosis has plagued mankind for millennia – lesions have even been observed in the aorta, coronary, and peripheral arteries of Egyptian mummies dating back to the XVIIIth Dynasty (1580 B.C.).[4,5] First noticed and characterized by the medical field in the mid-1700's, atherosclerosis was subsequently studied in greater detail in the beginning of the 19th century. At this time, Rokitansky and Virchow's theories on atherosclerosis prevailed. Whereas Rokitansky believed the intimal thickening to be derived from surface deposits, Virchow demonstrated that this thickening was located to the subendothelial layer and considered that this was an inflammatory process. Early in the 20th century, Aschoff noted that the fatty deposits in atherosclerotic lesions constituted cholesteryl esters. Not long after, Anitschkow's seminal work showed that feeding rabbits a cholesterol-rich diet generated arterial lipid deposits, giving rise to the lipid hypothesis. Finally, Russell Ross unified the current concepts of atherogenesis in the response-to-injury

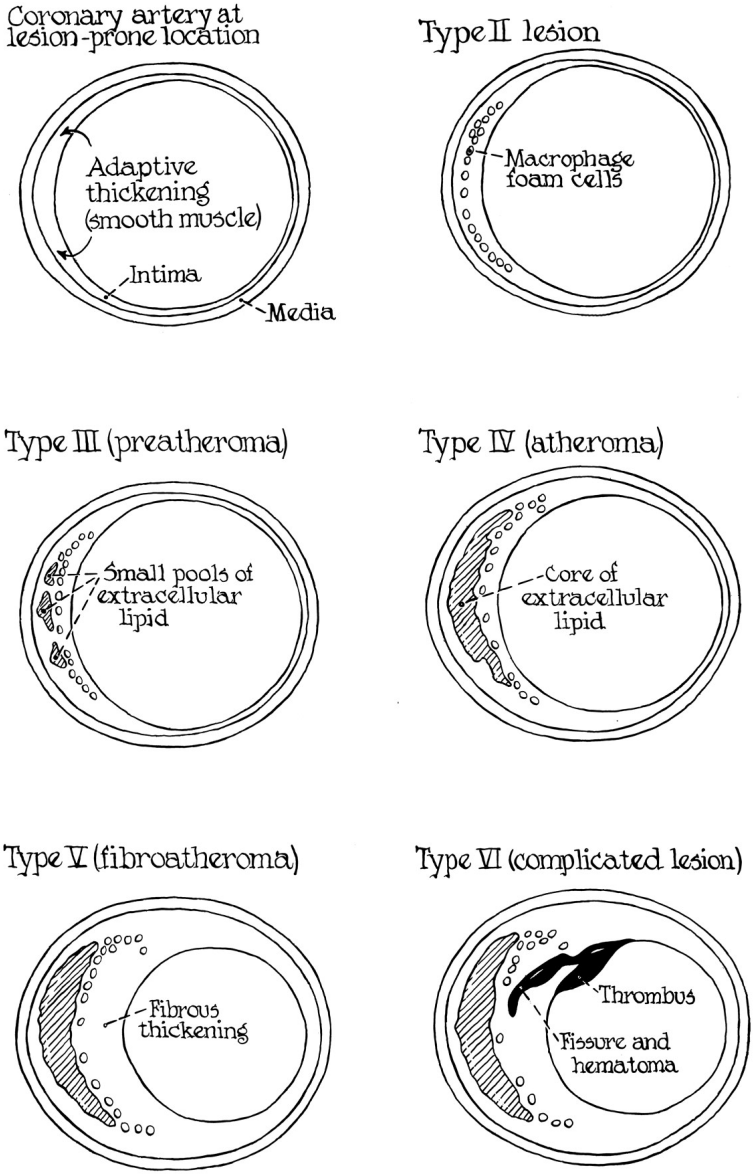
theory and put emphasis on the role of oxidative modification in the causation of atherosclerosis, ultimately declaring the latter an inflammatory disease. The progression of atherosclerosis is accompanied by characteristic histological changes in the artery, and the American Heart Association has classified the distinct features of human atherosclerotic lesions observed throughout disease progression into six morphologically characteristic types (**Figure 1.1**).[1,6]

Early lesions: type I to type III atherosclerotic lesions, the precursors of advanced lesions, are referred to as 'early lesions' or 'fatty streaks' and are mainly comprised of accumulated lipoproteins and cholesteryl esters, which largely accumulate within macrophage lipid droplets.[1] The geometry of the blood vessels dictates the athero-susceptible regions of the arteries: atherosclerosis tends to develop in regions of non-laminar or turbulent flow.[7] The endothelial integrity is compromised in these regions of turbulent flow, and LDL accumulates in the inner layer of the artery wall, where it becomes modified. Macrophages internalize the modified LDL, via scavenger receptors, leading to massive intracellular cholesterol accumulation as cholesteryl esters (CE) in cytosolic lipid droplets (LDs) and foam cell formation.

The type I lesion is the earliest, most frequently seen in infants and children; small, isolated macrophage foam cells are present.[8] Type II lesions are comprised of macrophage foam cells arranged in layers, as opposed to the isolated macrophages of type I. Additionally, there is increased macrophage cellularity, as well as smooth muscle foam cells. As for the type III lesions, continued lipid accumulation in macrophages and among smooth muscle cells disrupts smooth muscle connectivity, bridging the early and late lesions. The type I-III lesions are not yet clinically relevant; they are 'silent precursors of possible future disease'[1] and may progress towards more advanced (type IV-VI) lesions throughout an individual's lifetime. The most abundant cell type in the early lesions is the macrophage; macrophage foam cells are a hallmark of early lesions and are at the center stage of atherosclerosis disease initiation and progression.

Advanced lesions: whereas the architectural integrity of the artery wall is maintained in type I-III lesions, it is compromised in the more advanced type IV-VI lesions. The type IV lesion,

Figure 1.1: **Classification of human atherosclerotic lesions** Cross sections of descending coronary arteries, describing the progression of atherosclerosis from early (types I-III) to late (types IV-VI) lesions.



Stary et al., Circulation, 1995; 92(5):1355-74.
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also known as atheroma, is considered the first advanced lesion because of the intimal disorganization and arterial deformity that bias the lesion toward further progression and sudden ischemic events. The disruption of the artery integrity at this stage is due to the lipid core – extracellular lipid accumulation –, which disperses smooth muscle cells and the intercellular matrix of the intima. These events immediately precede the accumulation of fibrous material (consisting mainly of collagen and smooth muscle cells) over the lipid core, leading to the type V lesion that substantially narrows the arterial lumen. Type VI lesions, or complicated lesions, consist of type IV or V lesions with surface disruptions, hematoma, and thrombosis – independently or altogether – that trigger the clinical events that ensue from partial or complete vessel occlusion.

1.1.2 Macrophage Foam Cells

Macrophages play a crucial role in atherogenesis, through the uptake of modified LDL and secretion of inflammatory modulators, cytokines and matrix-degrading enzymes.[9] The primary cellular event of lesion initiation is the formation of macrophage foam cells, resulting from internalization of various forms of modified LDL that accumulate in the artery. Clearance of the arterial cholesterol deposits by macrophages is beneficial during the early stages of atherogenesis, but as the macrophage cholesterol homeostasis machinery becomes overwhelmed, they become foam cells that contribute to disease progression and the establishment of chronic inflammation.[10]

Modified LDL: the 'response to retention' hypothesis proposes that subendothelial retention of atherogenic lipoproteins is the central pathogenic event that initiates atherosclerosis.[11] Proteoglycans in the extracellular matrix retain LDL in the intima, where lipolytic enzymes (lipoprotein lipase (LpL), phospholipase A_2 (PLA_2) and sphingomyelinase (SMase)) induce LDL aggregation.[12] Lipoprotein oxidation occurs within the microenvironment of the arterial wall, as a consequence of lipoprotein trapping, due to the sequestration of the lipoproteins from the protective elements in plasma and the action of reactive oxygen species, myeloperoxidases and lipoxygenases.[10,11]

Modified lipoprotein particles that accumulate in the arterial intima create a local pro-inflammatory microenvironment at the site of lesion formation; TNF-α and IL-1β

found in atherosclerotic lesions induce VCAM-1 expression in endothelial cells.[13] In turn, VCAM-1 recruits circulating monocytes to the nascent atherosclerotic plaque. Oxidized LDL (OxLDL) induces monocyte chemotaxis activity in endothelial and smooth muscle cells, and can also directly recruit monocytes, smooth muscle cells and T cells to the site of apoB-containing atherogenic lipoprotein retention.[11]

Scavenger receptors: having infiltrated the endothelial layer into the arterial intima, the monocyte acquires characteristics of the tissue macrophage, expressing scavenger receptors that bind and internalize modified lipoproteins. Unlike cholesterol uptake via the LDL receptor (LDLR), which is feedback inhibited, the various classes of scavenger receptors that internalize distinct forms of modified LDL do so in an unregulated fashion, leading to foam cell formation. There are now eight subclasses of scavenger receptors that bind to modified LDL and are expressed by macrophages in atherosclerotic lesions.[10] Amongst those expressed by macrophages, SRA and CD36 largely contribute to the formation of foam cells *in vitro* and these receptors exert both pro- and anti-atherogenic forces *in vivo*.[10] Both of them recognize polyanionic macromolecules, such as acetylated LDL (AcLDL), and OxLDL. Mechanisms for foam cell formation independent of scavenger receptors have been also been identified, such as patocytosis of aggregated LDL,[14] receptor-mediated internalization of VLDL and VLDL remnants,[15] and pinocytosis of native LDL.[16]

M1 versus M2 macrophages: the foam cell within the arterial intima secretes a number of cytokines that can amplify the local inflammation, and the functional polarization of macrophages has a big impact on inflammation and atherosclerosis progression. Macrophages are phenotypically classified into classically activated (M1) or alternatively activated (M2) macrophages: activated M1 macrophages secrete proinflammatory cytokines (TNFα, IL-6, IL-12) whereas M2 macrophages secrete anti-inflammatory cytokines (IL-10, TGF-β).[17]

1.1.3 Reverse Cholesterol Transport

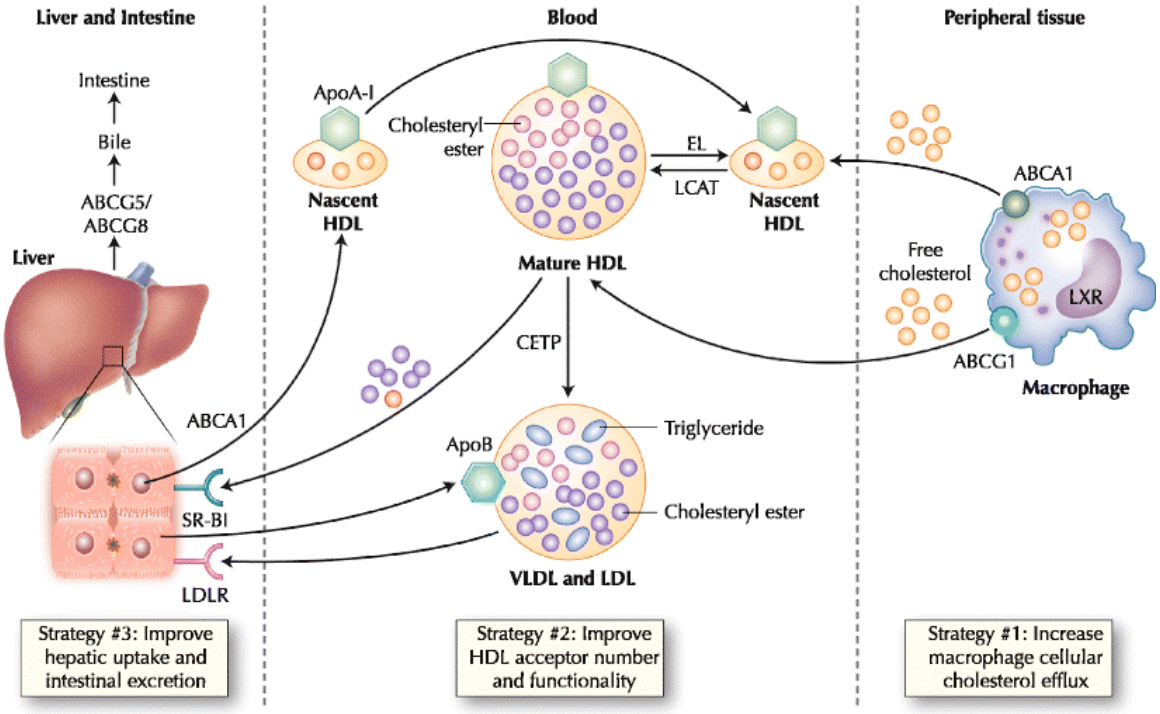
The two main conceptual approaches to therapy for atherosclerosis presently are 1) intervening at the level of inflammatory pathways active in the development of

atherosclerotic plaques and 2) targeting aspects of lipoprotein metabolism.[18] Modulation of lipoprotein metabolism on a therapeutic level has been geared towards lowering LDL or raising HDL. Statins, which inhibit cholesterol synthesis and accelerate plasma LDL clearance, have been highly effective in reducing the progression of atherosclerosis and the incidence of coronary and cerebrovascular events. More recent targets for modulating LDL levels include the proprotein convertase subtilisin/kexin type 9 (PCSK9), an important mediator of LDLR degradation and consequently of plasma LDL cholesterol. In addition, reducing the hepatic production rates of the metabolic LDL precursor VLDL - for instance by inhibition of the microsomal triglyceride transfer protein (MTP) which is required for VLDL assembly – is in clinical development.[18]

HDL is highly atheroprotective; in fact, plasma HDL cholesterol levels are inversely correlated to coronary heart disease (CHD).[19] As such, there is considerable interest in HDL-raising therapeutic approaches. A highly promising approach is the use of cholesteryl ester transfer protein (CETP) inhibitors to increase circulating HDL levels, but the phase III clinical trials of the first CETP inhibitor torcetrapib were prematurely terminated due to a higher mortality rate and cardiovascular complications in individuals receiving the inhibitor.[20] Since this deceptive human trial, the focus has been on enhancing HDL function as opposed to simply raising plasma HDL concentrations. HDL has antioxidant, antithrombotic and vasodilatory capabilities, which contribute to its ability to prevent CHD.[21] Another important way that HDL achieves its cardioprotective effect is to facilitate the reverse cholesterol transport (RCT) pathway, which involves the uptake of peripheral cholesterol and its transport to the liver for clearance into the bile and ultimately the feces (**Figure 1.2**).[22] Specifically relevant to atherosclerosis and regression of atherosclerosis is macrophage RCT.

Cholesterol efflux from macrophages is the first and potentially most important step in macrophage RCT, and accordingly the macrophage ATP binding cassette (ABC) transporters ABCA1 and ABCG1 have been identified as being critical for this process.[23] Using an *in vivo* RCT assay to track the appearance of a ^{3}H-tracer from peripheral ^{3}H-cholesterol-loaded macrophages into the plasma, liver and feces of mice, it was shown that ABCA1 and ABCG1 coordinate the removal of macrophage cholesterol for its clearance from the body.[24] Clearly, ABCA1 and ABCG1 activity in arterial macrophages has

Figure 1.2: **Macrophage reverse cholesterol transport** Lipid-poor apoA-I is synthesized by the liver and intestine, released in the circulation, and lipidated with phospholipids and cholesterol via hepatic ABCA1 to generate nascent HDL. These nascent HDL particles acquire additional cholesterol and other lipids from peripheral cells (such as macrophages), and the acquired cholesterol is esterified by lecithin cholesterol acyltransferase (LCAT) to cholesteryl esters, giving rise to mature HDL. The later is the preferred cholesterol acceptor of the ABCG1 transporter, which mediates cholesterol efflux from cells of the periphery to these larger HDL particles. Remodeling of the HDL particle can occur through the hydrolysis of HDL triglycerides and phospholipids, mediated by hepatic lipase and endothelial lipase, respectively. Cholesteryl esters in the HDL core can be transferred to triglyceride-rich lipoproteins for elimination via hepatic clearance in the liver through the low-density lipoprotein receptor (LDLR), or they may be selectively taken up via the hepatic scavenger receptor class B type 1 (SR-B1) receptor. In the liver, the ABCG5 and ABCG8 transporters mediate the secretion of HDL-derived cholesterol into the bile for excretion into the feces. Three conceptual approaches to enhancing macrophage RCT have been proposed: 1) improve macrophage cholesterol efflux; 2) improve HDL functionality; and 3) improve hepatic cholesterol uptake and biliary/intestinal excretion.



Khera and Rader, Curr Atheroscler Rep, 2010; 12(1):73-81.
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important implications for atherogenesis, given that the rate of macrophage RCT is an important determinant in HDL's antiatherogenic effects.[25] SR-BI expressed on hepatocyes mediates the selective uptake of HDL cholesterol by the liver, and the last step of RCT is cholesterol excretion into the feces.[25]

1.2 Cholesterol Homeostasis

Because excess free cholesterol (FC) can disrupt membranes and is cytotoxic, cellular cholesterol levels are tightly regulated. The cell has evolved complex mechanisms to control its abundance and distribution within cells.[26] For instance, under conditions of sterol depletion, there are cellular mechanisms in place to increase cholesterol uptake and *de novo* synthesis, and accordingly decrease its efflux. In contrast, under conditions of abundant cholesterol, feedback mechanisms to reduce cholesterol uptake and synthesis, and increase cholesterol efflux are in place. If these compensatory mechanisms fail to reduce free cholesterol levels appropriately, excess FC will be esterified and stored as CE (non-cytotoxic) to maintain the proper membrane FC concentration required for normal cellular functions to proceed. Nuclear receptors and the SREBP transcription factors control the expression of numerous genes implicated in lipid metabolism and are in this way intimately linked to cholesterol homeostasis, both at the cellular and whole-body level.

1.2.1 Nuclear Receptors

PPARs: the peroxisome proliferator-activated receptor (PPAR) subfamily includes PPARα, PPAPδ/β and PPARγ, and after activation by fatty acid metabolites, the PPARs heterodimerize with the retinoid X receptor (RXR) to bind to PPAR response elements (PPREs) in target genes.[27] Target genes in macrophages include diacylglycerol transferase 1 (DGAT1) to store TGs,[28] and the liver X receptor α (LXRα), which itself induces the expression of genes involved in cholesterol efflux.[29] Of all the PPARs, PPARγ seems to be the most relevant to atherosclerosis, and synthetic ligands for this receptor have been identified. While macrophage-specific deletion of PPARγ increases lesion formation in atherosclerosis-prone mice, a PPARγ-specific agonist inhibits atherosclerosis.[30] An important role attributed to the PPARs is the repression of inflammatory gene expression

through transrepression. In fact, PPAR signaling is connected to macrophage phenotypic (M1 or M2) development and atherosclerosis, whereby PPARγ activation is positively correlated with the expression of M2 markers in human atherosclerotic lesions.[31]

LXRs: liver X receptors LXRα and LXRβ bind to LXR response elements (LXREs) in target genes, which are comprised of two hexamer sequence direct repeats (DRs) AGGTCA separated by 4 base pairs (DR-4), and activate gene expression.[27] LXRβ is ubiquitously expressed, while LXRα is expressed in the liver, intestine, kidney, adrenal glands, adipocytes and macrophages. Both LXRs regulate genes involved in cholesterol metabolism as heterodimers with RXR, similarly to the PPARs. LXR activation induces cholesterol trafficking genes, such as Niemann Pick type C (NPC) 1 and 2, a number of genes involved in RCT, such as macrophage ABCA1, ABCG1, apoE, and genes implicated in bile synthesis in the liver. In addition, LXR suppresses LDL uptake through induction of Idol (inducible degrader of the LDLR), which targets the LDLR for degradation.[32] LXR activation also inhibits the expression of inflammatory molecules, such as TNFα, IL-β, via transrepression.[33]

Activated LXRs decrease intestinal cholesterol absorption and increase hepatic cholesterol excretion by inducing ABCG5 and ABCG8 expression in the intestine and liver, respectively. Because of the beneficial effect that LXR agonists have on inducing many genes involved in RCT and their inhibitory effect on inflammatory gene expression, they are predicted to be anti-atherogenic. Indeed, this avers to be the case: 1) macrophage-specific LXRα or LXRβ deletion in mice fed a hypercholesterolemic diet or LXRαβ double knockout mice fed a chow diet all exhibit accelerated atherosclerosis, 2) the systemic administration of synthetic LXR ligands protects against lesion development in mice and 3) synthetic LXR ligands have been shown to actually reverse pre-existing atherosclerotic lesions.[34] But, unfortunately, LXR agonists also induce hepatic lipogenesis, via SREBP1c activation, resulting in elevated plasma TG in mice.[35] Thus, LXR agonists specifically active in macrophages but not in the liver may provide the optimal therapeutic outcomes.

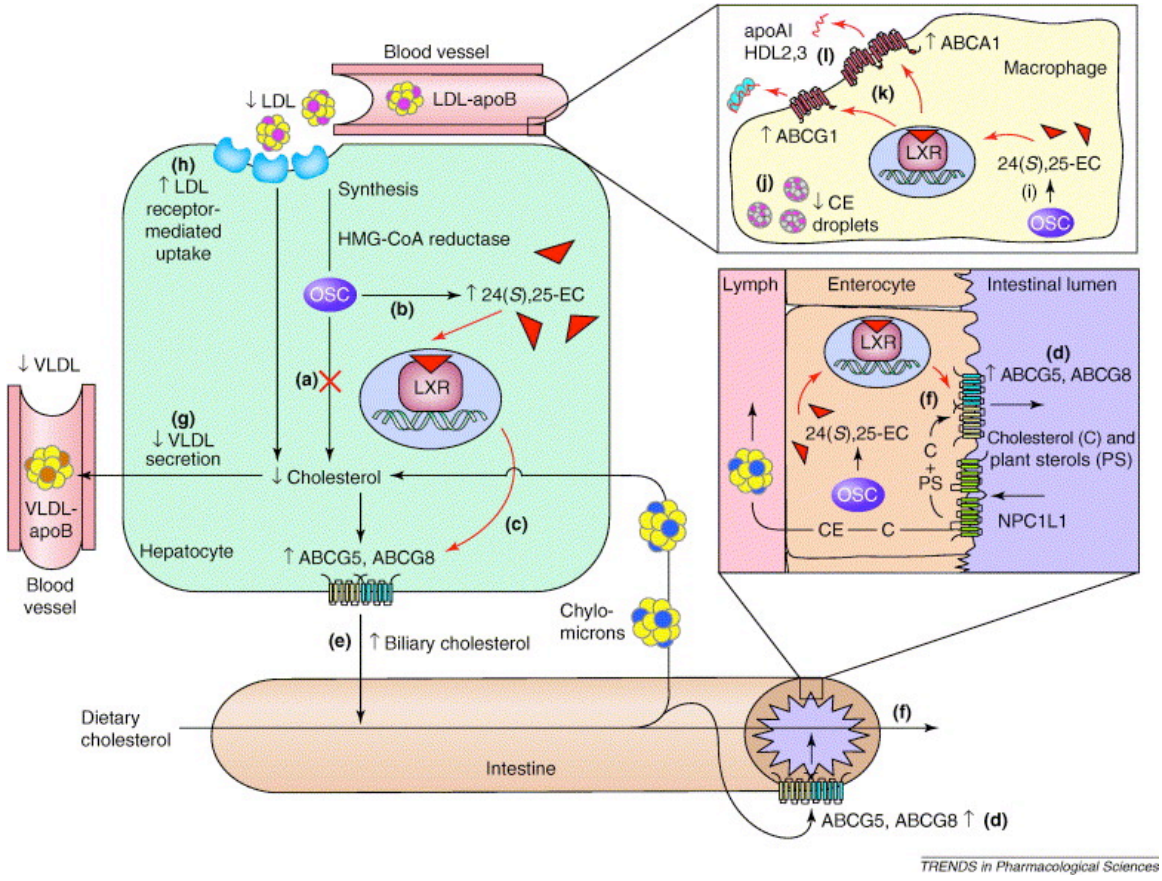
Oxysterols, produced by enzymatic or nonenzymatic oxidation of the cholesterol side chain, are natural LXR ligands.[36] Some of the important oxysterols produced by

mitochondrial P450 enzymes include 7α-hydroxycholesterol and 27-hydroxycholesterol (intermediates in bile acid synthesis), 24(S)-hydroxycholesterol and 22(R)-hydroxycholesterol (22-HC). Uniquely, 24(S),25-epoxycholesterol (epoxycholesterol) is produced by a shunt in the cholesterol biosynthesis pathway, where oxidosqualene:lanosterol cyclase (OSC) catalyzes the formation of epoxycholesterol while catalyzing the formation of the initial four-ringed cholesterol precursor.[37] In addition to arising from bile acid and cholesterol biosynthesis, oxysterols are generated during steroid hormone synthesis, and they can also come from exogenous sources such as OxLDL. In fact, multiple studies document the presence of oxysterols in atherosclerotic plaques (27-HC, 7-ketocholesterol, 7-β- and α-HC are the most abundant), and it is believed that they contribute to lesion progression due to their cytotoxicity; they can trigger apoptosis of lesional macrophages.[36]

Intriguingly, distinct oxysterols have differing abilities to modulate gene expression and repression. For instance, 22-HC, 24-HC and epoxycholesterol all suppress inflammatory responses through transrepression, whereas 25-HC and 27-HC lack this ability.[38] Additionally, endogenous synthesis of epoxycholesterol selectively activates LXR-responsive cholesterol efflux genes without a concomitant increase in fatty acid and TG synthesis that typically occurs through SREBP1 activation by synthetic LXR agonists.[39] This oxysterol-specificity makes epoxycholesterol particularly attractive as a selective LXR ligand to increase RCT, because the treatment of CVD with LXR agonists will require the selection of specific LXR ligands that are able to stimulate cholesterol efflux, inhibit inflammation, but do not increase plasma TG levels. OSC inhibition, which enhances epoxycholesterol synthesis, has been proposed as an attractive target for inhibition of cholesterol synthesis.[37] Similarly to statins, partial OSC inhibition lowers cholesterol synthesis by favoring epoxycholesterol synthesis, and the rise in epoxycholesterol enhances the expression of LXR-regulated lipid metabolism genes, without affecting liver triglyceride expression (**Figure 1.3**).

Transrepression: the negative regulation of gene expression by PPARs and LXRs occurs through transrepression.[27] Nuclear factor kappa B (NF-κB) and activator protein-1 (AP-1), key inflammatory transcription factors, are bound to inactive promoters of target genes in association with the nuclear receptor co-repressor (NCoR) complex to sustain basal

Figure 1.3: **Partial OSC inhibition reduces circulating apoB-containing lipoproteins and blocks macrophage foam cell formation** OSC inhibition prevents cholesterol biosynthesis in the liver (a), and enhances 24(S),25-epoxycholesterol (EC) synthesis (b). By activating LXR, epoxycholesterol induces ABCG5 and ABCG8 expression in the liver (c) and intestine (d), promoting cholesterol clearance into the bile (e) and limiting net cholesterol in the intestine (f) and consequently the amount of cholesterol reaching the liver for VLDL production (g). Reduced hepatic cholesterol levels result in enhanced LDL-apoB clearance (h). Increases in epoxycholesterol levels in macrophages inhibits cholesteryl ester (CE) deposition (i) by upregulation of ABCA1 and ABCG1 (ii) which increases efflux to apoA-I and HDL (iii). Overall, increasing epoxycholesterol levels by OSC inhibition is predicted to enhance macrophage RCT and to reduce hypercholesterolemia.



Huff and Telford, TRENDS in Pharmacological Sciences, 2005; 26(7): 335-340
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repression. Ligand-activated nuclear receptors become SUMOylated, and SUMOylated PPAR and LXR receptors bind to NFκB and AP1 sites in the promoter regions of inflammatory genes and retain NCoR corepressor complexes to these sites and repress gene activation.

1.2.2 SREBP transcription factors

The sterol regulatory element-binding proteins (SREBPs) are a family of membrane-bound transcription factors that regulate lipid homeostasis.[40] SREBPs activate genes involved in the synthesis and uptake of cholesterol, fatty acids, TGs and phospholipids, and they regulate hepatic lipid production for export into the plasma or into the bile. There are three mammalian SREBP isoforms: SREBP-1a and SREBP-1c (the predominant isoform expressed in the liver and adipose tissue), which are the products of alternative splicing of the SREBP1 gene, and SREBP2 (ubiquitously expressed) that is encoded by a different gene.[41] SREBPs reside in the endoplasmic reticulum (ER) in their precursor form, and they must undergo proteolytic cleavage and nuclear translocation to exert transcriptional activity.

SREBPs interact with the SREBP cleavage-activating protein (SCAP), an escort protein that shuttles SREBPs from the ER to the Golgi for the proteolytic processing that is required to convert precursor membrane-bound SREBPs into their soluble active forms. INSIGs (insulin-induced genes) are ER-resident proteins that bind to SCAP and prevent the translocation of the SCAP-SREBP complex from ER to Golgi. Together, the INSIG-SREBP-SCAP complex regulates the expression of genes containing SREs (sterol response elements). During cholesterol depletion, SCAP undergoes a conformational change causing its dissociation from INSIG, enabling the movement of the SCAP-SREBP cargo to the Golgi where site 1 and 2 proteases cleave SREBP, generating the active N-terminal fragment that translocates to the nucleus and binds to SREs in target genes.

The SREBP1 transcription factor preferentially activates genes involved in fatty acid metabolism. Target genes of the fatty acid biosynthetic pathway include numerous genes that modulate oleate synthesis, the major fatty acid esterified to glycerol to form TGs, as well as the first committed enzyme in TG and phospholipid synthesis (glycerol-3-phosphate acyltransferase).[40] In turn, SREPB2 primarily activates cholesterol metabolism genes.

SREBP2-responsive genes involved in cholesterologenesis include hydroxymethyl-glutaryl-coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme in cholesterol synthesis and the target of cholesterol-lowering statins, HMG-CoA synthase, and squalene synthase.[40] It also modulates LDL cholesterol uptake by controlling the expression of the LDLR. Finally, SREBP2 exerts control over the expression of ABCA1, ABCG1 and NPC1 via miR-33, an intronic microRNA within the SREBP2 gene that is co-transcribed along with SREBP2 during states of cholesterol depletion to limit cholesterol export.[42]

In addition to being potent LXR agonists, oxysterols also regulate the SREBP pathway. INSIGs function as oxysterols sensors,[43] and SCAP detects cellular cholesterol levels.[44] When oxysterols are bound to INSIG, the INSIG-SREBP-SCAP complex is trapped at the ER, and thus oxysterols are negative modulators of cholesterol uptake and *de novo* synthesis.

1.2.3 The Lipid Droplet

LDs are recognized as metabolically active but atypical intracellular organelles, constituted of a hydrophobic core of triacylglycerides (TG) and cholesteryl esters (CE) which are surrounded by a phospholipid monolayer coated with specific proteins.[45] Several proteomic studies of LD proteins have identified signature coat proteins, including the PAT family of proteins (perilipin, adipophilin, TIP47). Many of these proteins are exchangeable and stable either in their lipid-bound form or free in the cytosol, a property that may be structurally linked to plasma exchangeable apolipoproteins. In many aspects, the LD resembles a lipoprotein, comprised of a neutral lipid core surrounded by cholesterol, phospholipids, and finally proteins that coat the phospholipid monolayer. In keeping with the postulated ER origin of the LD, several enzymes of lipid synthetic pathways that reside in the ER have also been found in association with this organelle, including ACAT.[46,47]

The cholesterol content and nature of LDs differ among cell types, reflecting the specialized nature of lipid storage. The adipocyte LD is primarily enriched in TG, and its cholesterol store is unique because of its high FC and very low CE content. In contrast, macrophages primarily accumulate CE in lipid droplets upon incubation with CE-enriched lipoproteins, such as acetylated or aggregated LDL, whereas they accumulate TG upon

exposure to TG-rich lipoproteins, such as VLDL and remnant lipoproteins. Neutral lipid storage in macrophages occurs in mixed CE and TG droplets; interestingly, the cholesterol from CE in mixed droplets in the liquid or isotropic state is effluxed more effectively than from droplets in the liquid crystalline or anisotropic state resulting from LD TG depletion or CE enrichment.[48] Independently of the nature of the neutral lipid accumulating in the macrophage LD, lipoprotein-loading leads to increased adipophilin expression.[49] The influence of adipophilin, the major macrophage LD coat protein, on atherogenesis was recently uncovered. Whole-body as well as macrophage-specific adipophilin ablation inhibits foam cell formation and protects against atherosclerosis development.[50] Importantly, other macrophage PAT family proteins do not undergo compensatory upregulation in the absence of adipophilin, suggesting that adipophilin may represent a new direct target for antiatherosclerosis therapy.[50]

1.2.4 The Cholesteryl Ester Futile Cycle

Newly synthesized cholesterol as well as lipoprotein-derived cholesterol can be incorporated into LDs in the ER where the ER-resident protein, acyl-CoA:cholesterol acyl transferase (ACAT), catalyzes the esterification of excess cholesterol for storage in LDs. Cholesterol in the LD undergoes constitutive cycles of esterification-hydrolysis, which controls cholesterol availability for cell membranes and for efflux, whereby unesterified cholesterol released from the LD by CE hydrolysis can be effluxed to a cholesterol acceptor if one is present – which results in net CE hydrolysis - or otherwise re-esterified by ACAT.[51,52] The original studies that characterized this 'futile cycle' concluded that cytoplasmic CE hydrolysis in macrophage foam cells was mediated by extralysosomal, cytoplasmic neutral CE hydrolases.[51,53]

1.2.5 Neutral Cholesteryl Ester Hydrolases

Although the mechanisms for cholesterol traffic from LDs to an efflux compartment remain to be fully elucidated, it is becoming clear that LD-associated CE hydrolysis is a limiting factor in cholesterol efflux. Evidence for this is provided by studies demonstrating that overexpression of CE hydrolases markedly increases cholesterol efflux from macrophages, whereas impaired CE hydrolase activity contributes to lipid accumulation and reduced macrophage RCT. The human homolog of the murine triglyceride hydrolase (TGH),

carboxylesterase 1 (CES1)[54] also termed CEH, was cloned from human macrophages[55] and from human livers[56] and has CE hydrolase activity. CEH overexpression in macrophages reduces CE accumulation and enhances ABCA1- and ABCG1-mediated cholesterol efflux[57], while macrophage-specific CEH expression *in vivo* reduces atherosclerosis.[58] Enhanced RCT from CEH-overexpressing macrophages was documented in *Ldlr(-/-)/Ceh*-transgenic mice, demonstrating the physiological importance of the CE hydrolase and its limiting activity in macrophages.[58]

Another candidate enzyme for CE hydrolysis in macrophages is hormone sensitive lipase (HSL), encoded within the LIPE gene and consequently also known as LIPE, which has a well-defined role in adipocyte lipolysis.[59] Macrophage-specific transgenic expression of rat HSL and apoA-IV in mice challenged with an atherogenic diet has been demonstrated to reduce the size of aortic lesions,[60] similarly to what is observed when macrophage CE hydrolase activity is increased by CEH overexpression. A third neutral CE hydrolase reported to mediate macrophage CE hydrolysis is neutral cholesterol ester hydrolase 1 (Nceh1), with KIAA1363 being its human ortholog. Absence of this enzyme in macrophages challenged with AcLDL results in increased cellular CE content and decreased cholesterol efflux.[61] Together, knock down of Nceh1 and HSL in macrophages nearly completely abolishes all CE hydrolysis in cell-free assays.[61] *In vivo*, macrophage-specific ablation of Nceh1 increases atherosclerosis plaque development, as does macrophage-specific HSL ablation, and together a compound lack of macrophage Nceh1 and HSL has an additive effect on promoting atherogenesis.[61] A contradictory report stating that whereas HSL plays a role in macrophage CE hydrolysis Nceh1 exhibits no CE hydrolase activity emphasizes that macrophage neutral CE hydrolases are still a matter of debate.[62,63] Although the exact identity of macrophage-specific CE hydrolases remains to be clarified, a common point to all of these studies is that enhancing LD-associated CE hydrolysis increases cholesterol efflux and is antiatherogenic. Understanding how cholesterol is mobilized from LDs offers new steps for modulating cholesterol efflux.

1.2.6 Lysosomal Acid Lipase

Following receptor-mediated endocytosis, lipoprotein-associated CEs and TGs are hydrolyzed by an active cholesterol esterase with optimal activity in the acid pH of the lysosomal lumen. Using chloroquine to block the hydrolytic ativity of this enzyme during lipoprotein endocytosis results in the accumulation of lipoprotein-associated neutral lipids in lysosomes. During the early characterization of the CE cycle, studies of cytoplasmic CE hydrolysis in which lysosomal cholesterase activity was inhibited by using chloroquine post lipoprotein-labeling ruled out a role for lysosomal hydrolysis in neutral CE breakdown.[51,53]

Lysosomal acid lipase (LAL) is the critical enzyme for the hydrolysis of neutral lipids delivered to lysosomes, and mutations in the gene encoding this enzyme lead to cholesterol storage disorders. LAL deficiency in humans leads to two phenotypes: cholesteryl ester storage disease (CESD) and Wolman disease (WD).[64] An infantile onset disorder, WD has a more severe phenotype, with death usually occurring within the first year of life because of massive CE and TG accumulation in the liver and small intestine, resulting in cachexia due to malabsorption. CESD is a milder, later onset disorder, characterized by CE accumulation in visceral tissues; higher residual LAL activity in patients affected by CESD as compared to WD may be the basis for the milder phenotype of CESD.[65]

1.3 Cholesterol Efflux

1.3.1 Efflux to Lipid-poor apoA-I

Apolipoprotein A-I (apoA-I) is a major HDL apolipoprotein, constituting approximately 80% of HDL protein.[66] ABCA1 has been shown to transfer phospholipids and cholesterol to HDL apolipoproteins that are associated with very little or no lipid,[67] and ABCA1-mediated apoA-I lipidation occurs exclusively at the plasma membrane.[68] The exact mechanisms of ABCA1-mediated lipidation of apoA-I are unclear, but it is believed that ABCA1 forms a membrane channel that flips lipid from the inner to the outer membrane leaflet, a process that is ATP-dependent.[67] ApoA-I binding to ABCA1 would first induce phospholipid flipping to the exofacial leaflet of the plasma membrane, causing strain due to asymmetrical packing of the bilayer, and consequently exovesiculation of lipid domains from which lipid is released to apoA-I to generate discoidal particles.[69]

Whereas ABCA1 translocates phospholipids at the plasma membrane to form specialized membrane domains with which apoA-I specifically interacts, it is apparent that it also acquires lipid from intracellular sites, such as the late endosomal/lysosomal compartments. ABCA1 traffics between the cell surface and late endocytic vesicles,[70] and preferentially mobilizes cholesterol deposited in late endosomes/lysosomes to stimulate cholesterol efflux.[71] Specifically, the internalization and shuttling of ABCA1 is functionally important for the efflux of cholesterol out of endosomal compartments.[72] This is corroborated by the observation that in NPC1-deficient cells, where cholesterol accumulates in late endosomes/lysosomes, enhancing ABCA1 expression is sufficient to overcome the impaired flux of cholesterol out of these compartments.[73] Shuttling of the ABCA1 transporter from the plasma membrane to late endosomal / lysosomal compartments is increased in lipid-loaded cells as compared to unloaded cells, highlighting the importance of this pathway in cells that have accumulated excess lipoprotein-derived cholesterol in endosomes.[74]

1.3.2 Efflux to HDL

Plasma HDL constitutes a heterogeneous group of particles of different size, density, electrophoretic mobility, lipid composition and protein content.[75] Two main HDL fractions are obtained when human HDL is separated by ultracentrifugation: HDL_2 (1.0063-1.125 g/mL) and HDL_3 (1.125-1.21 g/mL).[76] Alternatively, HDL gives rise to two main subpopulations when separated by electrophoresis: α-HDL is the major subfraction (most of the HDL in plasma is α-HDL) characterized by a high negative surface charge density, and preβ-HDL is the minor subfraction (preβ-HDL represents only 5% of the total apoA-I in the plasma) which contains mainly apoA-I and phospholipids along with small amounts of cholesterol.[25] *In vivo*, HDL continually undergoes remodeling events, and the lipid-free population of apoA-I (~5-10%) is maintained as a balance of its lipidation by ABCA1 to form preβ-HDL, its incorporation into HDL, and its release from HDL, all of which have been proposed to occur in the intima.[77]

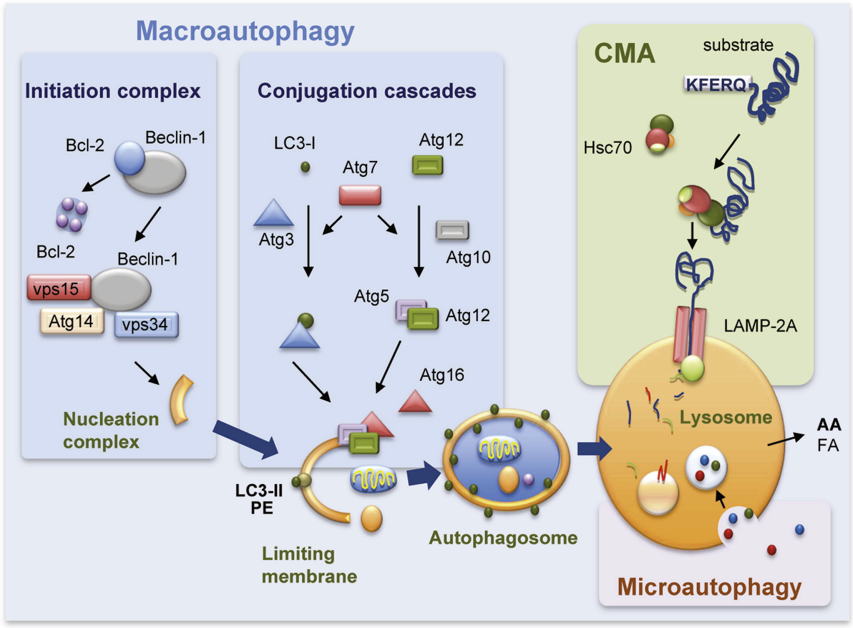
Efflux to HDL involves passive diffusion of cholesterol as well as active cholesterol transfer, and there are three main transporters (ABCA1, ABCG1 and SR-BI) that partake

in lipid transfer to HDL particles. First, the aqueous diffusion pathway is passive, does not require acceptor binding, and mediates the bidirectional flux of cholesterol between the plasma membrane and HDL with cholesterol diffusing down its gradient of chemical potential.[78] SR-BI facilitates the diffusion of cholesterol from the plasma membrane to HDL particles by forming a hydrophobic channel along which cholesterol molecules can diffuse.[25] Macrophage ABCG1 expression has been shown to specifically stimulate net cholesterol efflux to HDL, but not to lipid-poor apoA-I,[79] and a critical role for ABCG1 in preventing macrophage lipid accumulation *in vivo* has been established.[80] ABCG1 promotes cholesterol efflux by redistributing plasma membrane cholesterol to cell-surface domains where it becomes available for removal by HDL.[81] Finally, ABCA1 mediates the efflux of free cholesterol and phospholipids to preβ-HDL to generate discoidal nascent HDL particles.

1.4 Autophagy

Autophagy is an ancient, evolutionary conserved process that plays a major role in the degradation of cellular components. Essentially, autophagy is a trafficking pathway that delivers cytosolic constituents to the cell's lytic compartments. Whereas the ubiquitin-proteasome system is involved in the turnover of short-lived proteins, autophagy recycles stable cytosolic macromolecules to supply nutrients and maintain essential cellular functions under starvation conditions. In addition, autophagy has an important housekeeping function in the clearance of damaged proteins and organelles, long-lived proteins and protein aggregates, as well as serving to combat infection by a number of pathogens.[82] There exist different forms of autophagy: 1) chaperone-mediated autophagy, where single proteins are recognized by cytosolic chaperone complexes and delivered to lysosomes for internalization via a membrane translocation complex; 2) microautophagy, where cellular components are taken into lysosomes by invagination and pinching of the lysosomal membrane into the lumen; and 3) macroautophagy, where cargo is sequestered in *de novo* formed vesicles called autophagosomes, which subsequently fuse with lysosomes.[83] A scheme of the three types of autophagy at play in mammalian cells is depicted in **Figure 1.4**. Macroautophagy (referred to as autophagy hereafter), the subject of this thesis, can sequester cytosol 'in bulk' or selectively; in the later, autophagosomes surround specific cargo, such as mitochondria or

Figure 1.4: Components of the autophagic pathways in mammalian Cells
Macroautophagy is the autophagic pathway that involves *de novo* formation of a limiting membrane that engulfs cytosolic constituents and seals to generate an autophagosome. Fusion of autophagosomes with lysosomes leads to degradation of the autophagic body. The class III PI3K kinase complex initiates autophagy by recruiting components of the conjugation cascades (LC3/PE and Atg5/12) to the limiting membrane. Chaperone-mediated autophagy (CMA) involves the cytosolic chaperone Hsc70 that recognizes a targeting motif in cytosolic proteins and accompanies them to the lysosomal membrane, where they cross into the lysosomal lumen. Finally, microautophagy describes trapping and internalization of cytosolic cargo via invaginations at the lysosomal membrane that pinch off into the lysosomal lumen.



Singh and Cuervo, Cell Metabolism Review, 2011; 13(5):495-504.
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ribosomes, using cargo-recognizing proteins that link the autophagic machinery to the target cargo.

1.4.1 Overview of the Autophagy Process

Even though autophagy was originally described in the 1960s, the first proteins contributing to this process – autophagy-related proteins or Atgs - were only identified in 2008, making autophagy a relatively new and thriving field of study. There are now over 30 Atg proteins identified; these organize into functional complexes that oversee the autophagic process, first concentrating on single lipid bilayer membranes ('limiting membranes' or 'phagophores') that bud from pre-existing organelles such as ER, and then modulating membrane elongation to form cup-shaped structures that engulf the cytoplasm and fuse at the mouth, giving rise to spherical autophagosomes.[84] Subsequent autophagosome fusion with lysosomes releases the 'autophagic body' into the lysosome lumen where it is degraded.

The best-characterized autophagy modulator is mTOR (mammalian target of rapamycin), a nutrient-sensing kinase that becomes inactivated in response to nutrient limitation. mTOR is a negative regulator of autophagy; under normal nutrient conditions, mTOR phosphorylates ULK1, sequestering the ULK1-Atg13-FIP200 complex in an inactive state at the mTOR complex 1 (mTORC1).[83] During starvation, AMPK inhibits mTOR activity, which reduces ULK1 phosphorylation and consequently favors the release of ULK1-Atg13-FIP200 complex from mTORC1 to the site of autophagosome formation, where it is required for autophagic induction. mTOR itself is regulated by class I phosphoinositide 3-kinases (PI3K): ligand binding to a cell surface receptor (such as the insulin receptor) stimulates class I PI3 kinases, resulting in mTOR activation and inhibition of autophagy.[85]

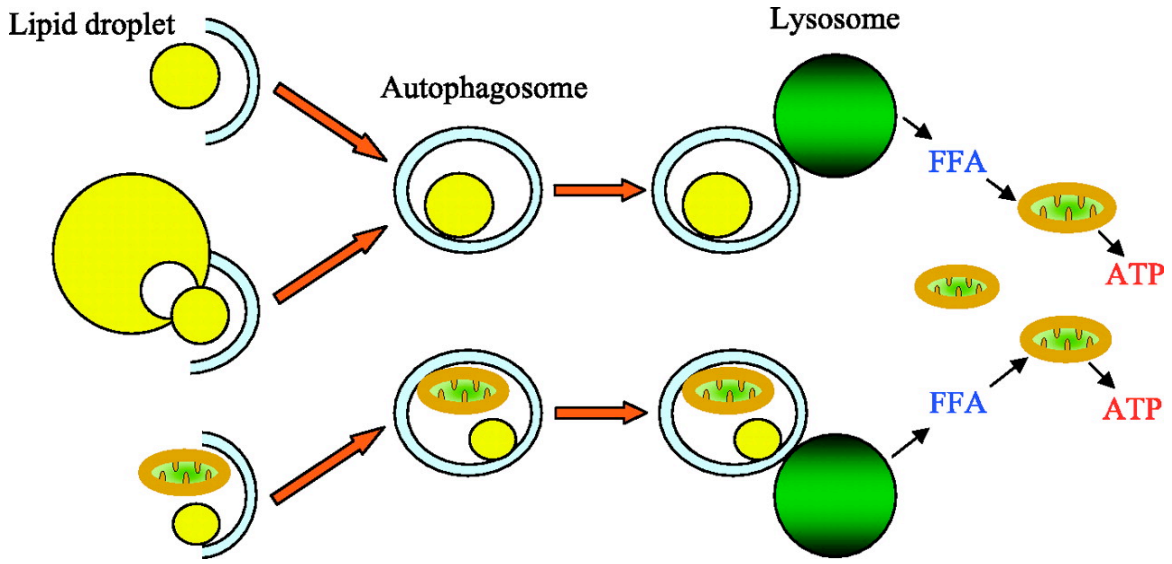
Beclin-1, a class III PI3K, forms a lipid kinase complex with vps15, vps34 and Atg14 which phosphorylates phosphatidylinositol (PI) to form PI3P and is essential for autophagy.[84] Local accumulation of PI3P in specific sub-domains of the ER increases membrane curvature at the sites of autophagosome formation, and components of the LC3/PE and Atg5/12 conjugation cascades are recruited to limiting membranes at these sites.[83,84] Atg12 and the microtubule-associated protein light chain 3 (LC3) are two

ubiquitin-like (Ubl) proteins that mediate the elongation and expansion of the phagophore.[86] Atg12 is conjugated to Atg5 by the action of Atg7 (E1-like enzyme) and Atg10 (E2-like enzyme), after which Atg16 multimerizes to form the Atg12-Atg5-Atg16 tetrameric complex. Atg4 cleaves soluble LC3-I at the C-terminus, exposing the glycine residue that is conjugated to phosphatidylethanolamine (PE) by Atg7 and Atg3 (E2-like), thus giving rise to the membrane-bound LC3-II. Both of these Ubl systems are required for elongation and closure of the phagophore.

1.4.2 Autophagy and lipid metabolism

Autophagy's contribution to lipid metabolism was recently elucidated through the discovery of macrolipophagy, which constitutes the engulfment of neutral LDs by the autophagic machinery and their delivery to lysosomal lipases for breakdown in the lysosomal lumen.[87] In addition, that autophagy regulates lipoprotein metabolism and cholesterol homeostasis is an emerging concept that has important implications in regards to atherosclerosis and cardiovascular disease. During hepatic VLDL assembly, autophagy plays an important role in the degradation of apoB aggregates that accumulate on LDs,[88] suggesting that manipulating autophagy may influence VLDL circulating levels. Indeed, TGs secreted as VLDL are significantly decreased in mice with a hepatocyte-specific knockout of the autophagy gene Atg7 along with increased liver lipid content, providing direct evidence of the involvement of autophagy in the regulation of hepatic lipids *in vivo*.[87] Whereas lipolysis has been largely viewed as a process regulated by cytosolic lipases, a seminal report documenting the incorporation of LDs into autophagosomes through a process termed macrolipophagy and its requirement for TG breakdown forces us to reconsider this traditional view (**Figure 1.5**). Singh *et al.* show that blockage of autophagy in hepatocytes leads to increased LD size and number under basal conditions, in response to starvation and under high fat feeding conditions.[87] Thus, macrolipophagy is critical because the lysosomal neutral lipid hydrolysis that ensues once lysosomes have fused with LD-containing autophagosomes is important for normal lipid homeostasis. These observations raise important questions as to the identity of the lysosomal lipase (s) responsible for the breakdown of neutral lipids that enter the lysosomal compartment via autophagy.

Figure 1.5: **Hepatic autophagy and lipid metabolism** Lipid droplets are engulfed into autophagosomes on their own or in combination with other components such as mitochondria; portions of larger lipid droplets are sequestered in autophagosomes. Fusion of lipid droplet-containing autophagosomes with lysosomes delivers the cargo for degradation; lysosomal lipases mediate the hydrolysis of lipid droplet triglycerides to yield free fatty acids (FFA) that undergo mitochondrial β-oxidation for ATP production.



Czaja, Am J Physiol Cell Physiol, 2010; 298: 973-978.
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In addition to the role attributed to lipophagy in regulating hepatic TG levels, this process has significant implications in regards to cellular cholesterol; impaired autophagy in the liver leads to abnormally high levels of hepatic cholesterol along with aberrant TG deposition, due to the defective clearance of LDs.[87] Sterol depletion has directly been shown to induce autophagy in human fibroblasts, in a process involving PI3K activation and mTOR inactivation, although the exact mechanism by which cholesterol depletion initiates autophagy was not clear.[89] Recently, SREBP2 was linked to activation of the autophagy gene network in response to starvation. Using a chromatin immunoprecipitation (ChIP)-deep sequencing approach, the autophagy genes LC3B, ATG4B and ATG4D were identified as SREBP2 targets.[90] Increased LC3 association with LDs and cellular TG mobilization after starvation was shown to be dependent on SREBP2 induction. Thus, the LD can be viewed, in addition to *de novo* cholesterol synthesis and LDL receptor-mediated uptake, as a third source of cellular cholesterol regulated by SREBP2.[90]

1.5 Summary

To summarize, lipid deposition in arterial macrophages is a central process in the pathogenesis of atherosclerosis, the underlying cause of CAD. Intracellular lipid accumulates in LDs in macrophage foam cells, and the mechanisms that regulate LD lipolysis to liberate cholesterol from these sites of lipid buildup for efflux are unclear. The purpose of this doctoral thesis is to gain insights into the mechanisms that regulate this important pathway. Better understanding of LD-cholesterol mobilization could lead to the identification of new targets to positively modulate cholesterol efflux and macrophage RCT, and ultimately aid in the prevention and treatment of CAD.

1.6 Research Objectives

LXR agonists, such as the naturally occurring oxysterols, are interesting compounds for the treatment of atherosclerosis. Whereas oxysterols have been shown to be beneficial for enhancing cholesterol efflux and preventing foam cell formation, it was not established whether they are similarly effective when macrophages become cholesterol-loaded. In the first part of this study, our objective was to compare the ability of different oxysterols to increase cholesterol efflux from macrophage foam cells. We hypothesized that

epoxycholesterol would be the most efficient in reversing cholesterol accumulation in these cells, since it has been shown to be the most potent LXR activator. In addition, epoxycholesterol is particularly interesting in that it is the only oxysterol that does not promote TG synthesis, which is the main adverse side effect of LXR agonists in the treatment of atherosclerosis in murine models.

In the second part of the study, our objective was to better understand the mechanisms that control LD-associated CE hydrolysis in macrophage foam cells. This process is surprisingly poorly understood despite being a rate-limiting step in RCT. In studying LD motility in macrophage foam cells, we observed a curious event that involved neutral lipid transfer from LDs to a recipient organelle that we presumed to be the lysosome. Subsequent localization of LDs within lysosomes led us to hypothesize that lysosomal LD CE hydrolysis, in addition to the well-documented neutral CE hydrolysis mediated by neutral CE hydrolases, was also contributing to the release of LD cholesterol for efflux from macrophage foam cells. Thus the objective of the latter part of this study was to determine whether, and by what mechanism, lysosomes were contributing to LD breakdown in macrophage foam cells.

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2 Epoxycholesterol impairs cholesteryl ester hydrolysis in macrophage foam cells, resulting in decreased cholesterol efflux

Authors: Mireille Ouimet, Ming-Dong Wang, Natalie Cadotte, Kenneth Ho and Yves L. Marcel

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2.1 Significance of this Manuscript

The research presented in this manuscript uncovers a dual role for epoxycholesterol in lipid-loaded macrophages as compared to unloaded macrophages. Whereas 22-hydroxycholesterol enhances cholesterol efflux from macrophage foam cells, epoxycholesterol impairs cholesteryl ester hydrolysis and consequently cholesterol efflux from these cells, despite upregulating the cholesterol efflux transporters. Our results emphasize the varying efficacy of distinct LXR ligands in modulating cholesterol homeostasis and indicate that measuring the expression levels of the efflux transporters is not sufficient to screen for LXR agonists that enhance cholesterol efflux. Additionally, our findings highlight the importance of assessing the effectiveness of LXR ligands of therapeutic interest in macrophage foam cells, the diseased arterial macrophage, since following lipid-loading their appeal may be diminished.

2.2 Author Contributions

The experiments were planned by Yves and myself. As first author, I conducted the majority of the experiments pertaining to the study. Ming-Dong, second author and research associate in the lab at the time, helped me in the experimental design and conceptualization of this project, and also taught me the majority of the techniques that I used to carry out the experiments. I wrote a first draft of the manuscript. Together, Yves and I went through multiple editing rounds, eliciting the advice of others, until a final draft was submitted to the journal. During the first and second revisions of the manuscript, our technician Natalie Cadotte helped me carry out the required additional experiments.

2.3 Abstract

Strategies to inhibit or reverse cholesterol accumulation in macrophages have been shown to be atheroprotective. Notably, the administration of LXR agonists up-regulates key players in the reverse cholesterol transport pathway, including the ABCA1 and ABCG1 transporters. However, the effects of natural LXR activators, oxysterols, on lipid-laden macrophages remains elusive. **Methods and Results:** We assessed the ability of two oxysterols, 22(R)-hydroxycholesterol (22-OH) and 24(S),25-epoxycholesterol (epoxycholesterol), to promote cholesterol efflux to apoA-I from LDL- and modified LDL-labelled and loaded macrophages and thus rescue the phenotype associated with the accumulation of cellular cholesterol in these cells. **Results:** In macrophages labelled with LDL-derived cholesterol, epoxycholesterol treatment enhances ABCA1-mediated cholesterol efflux. In contrast, in AcLDL-loaded macrophages, epoxycholesterol treatment decreases cholesterol efflux to apoA-I, despite a dramatic increase in the expression of ABCA1 in response to epoxycholesterol treatment. We show that the decreased efflux is due to impaired cholesterol mobilization from lipid droplets, resulting from decreased cholesteryl ester hydrolase activity. **Conclusion:** Epoxycholesterol impairs cholesteryl ester hydrolysis activity in macrophage foam cells, thus reducing the availability of cholesterol for efflux to cholesterol acceptors.

2.4 Introduction

Whereas low density lipoprotein (LDL) endocytosis is a regulated process, the uptake of modified LDL, via macrophage scavenger receptors[1] or via macropinocytosis[2], lacks feedback inhibition by intracellular cholesterol accumulation. The internalization of modified LDL, such as oxidized LDL (OxLDL), acetylated LDL (AcLDL), or aggregated LDL (AgLDL), leads to the formation of foam cells,[3,4] which are a major component of atherosclerotic lesions.

The liver X receptors (LXRs) α and β, members of the nuclear receptor family of transcription factors, are key regulators of whole body lipid homeostasis. LXRβ is expressed ubiquitously whereas LXRa is predominantly expressed in tissues important in lipid metabolism including liver, adipose tissue, and macrophages.[5,6] The naturally occurring oxysterols 22(R)-hydroxycholesterol (22-HC) and 24(S),25-epoxycholesterol

(epoxycholesterol) are endogenous LXR ligands and have been shown to be potent physiological activators of both LXRs.[7,8] LXRs function by forming an obligate heterodimer with the retinoid X receptor (RXR), and this complex drives LXR-dependent transactivation of multiple genes involved in cellular cholesterol homeostasis, including ABCA1, ABCG1, and apoE in the lipid efflux pathway, which normally prevent cholesterol accumulation as intracellular lipid droplets.[9]

Given that ABCA1-mediated cholesterol efflux to lipid-poor apolipoprotein A-I (apoA-I) is the preferred efflux pathway of the macrophage foam cell,[10] we sought to promote this pathway in cholesterol-loaded cells using 22-HC and epoxycholesterol. Oxysterols have been shown to be beneficial for enhancing cholesterol efflux[6,11-13] and preventing foam cell formation[14], but it is not established whether they are similarly effective when macrophages become cholesterol-loaded. Large quantities of excess lipoprotein-derived cholesterol are esterified by the ER-resident protein, acyl-CoA:cholesterol acyl transferase (ACAT) and stored as cholesteryl esters (CE) in cytoplasmic lipid droplets.[15] Unesterified cholesterol can be released from the lipid droplets via CE hydrolysis, and subsequently effluxed to a cholesterol acceptor or re-esterified by ACAT.[16]

Unexpectedly, we found that while 22-HC enhanced cholesterol efflux from AcLDL-loaded macrophages, epoxycholesterol promoted cholesterol efflux only in unloaded or LDL-labelled macrophages. Despite inducing a dramatic increase in ABCA1 protein, epoxycholesterol impaired CE hydrolysis in lipid droplets of macrophage foam cells and reduced efflux to apoA-I, resulting in a net increase of CE and exacerbation of the foam cell phenotype.

2.5 Experimental Procedures

Materials. Oxysterols: 22(R)-hydroxycholesterol, 22(S)-hydroxycholesterol, and 24(S),25-epoxycholesterol (Steraloids Inc.). DMEM and RPMI 1640 medium (Invitrogen/Gibco), and Penicillin/Streptomycin (P/S) (Cambrex Bio Science). Fetal bovine serum (FBS), bovine serum albumin (BSA), and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma. Radioactive compounds: [1,2-^{3}H]-Cholesterol, [5-^{3}H(N)]mevalono-lactone-Rs, [^{3}H]-Oleate, and [^{3}H]-Acetic acid (PerkinElmer Life Sciences, Boston, MA). Human

recombinant apoA-I was produced as previously described.[17] The Sandoz 58-035 ACAT inhibitor was a kind gift from Novartis.

Cell culture. Bone marrow-derived macrophages (BMDM): hematopoietic stem cells were flushed from the femurs of C57Bl6 mice (Jackson Laboratories), and differentiated into mature macrophages by incubation in DMEM media supplemented with 10% FBS 1% P/S, and 15% L929-conditioned medium for 7 days. Monocyte-derived macrophages (MDM): mononuclear cells were isolated from the blood of normolipemic volunteers, and differentiated to macrophages, as previously described.[18] THP-1 human monocytes (ATCC) were cultured in complete growth medium (RPMI 1640 supplemented with 10% FBS, 0.05mM 2-mercaptoethanol, and 1% P/S), and were differentiated by treatment with PMA (100nM) for 7 days.

Lipoprotein preparation. Plasma was collected from normolipemic volunteers. LDL and HDL were isolated by sequential density ultracentrifugation, as previously described.[19,20] Modification of LDL: LDL was acetylated by repetitive additions of acetic anhydride[21], aggregated by vortexing[22], or oxidized by incubation with 5μM $CuSO_4$ at room temperature for 24h, adapted from Kunjathoor *et al*.[23]

Efflux of exogenously delivered cholesterol. Differentiated macrophages were incubated for 24h in medium (1% FBS, 1% P/S DMEM) containing LDL or AcLDL (50μg/ml) that were pre-incubated with ^{3}H-cholesterol (5μCi/ml). Cells were washed, and incubated in equilibration media (2mg/ml BSA, 1% P/S DMEM) containing oxysterols (10μM, unless otherwise specified). Cells were washed, and cholesterol efflux was determined in the presence or absence of human apoA-I (50μg/mL) in serum-free medium (2mg/mL BSA, 1% P/S DMEM). After 5h, the supernatant was removed and briefly centrifuged to remove non-adherent cells. Macrophages were dissolved in 0.5N NaOH. Radioactivity of aliquots of both supernatants and dissolved cells was measured by scintillation counting. Cholesterol efflux is expressed as a percentage of ^{3}H-cholesterol in medium / (^{3}H-cholesterol in medium + ^{3}H-cholesterol in cells) x 100%. The radioactivity of apoA-I-free media was subtracted from that of the apoA-I-containing media.

Efflux of newly synthesized cholesterol. Macrophages were incubated in medium (1% FBS, 1% P/S DMEM) containing ^{3}H-mevalonate (20μCi/ml) and oxysterols (10μM) for 48h.

Cells were washed, and cholesterol efflux was measured over 24 h, in the presence or absence of human apoA-I in serum-free medium (2mg/mL BSA, 1% P/S DMEM). ApoA-I in media aliquots was immunoprecipitated under native conditions with a polyclonal anti-human apoA-1 rabbit anti-serum (Calbiochem) and Protein G Sepharose (Amersham Biosciences). The immunoprecipitates were collected following centrifugation and washed four times with phosphate-buffered saline and resuspended in a final volume of 0.5ml for scintillation counting. The radioactivity of the apoA-I-free media was subtracted from that of the apoA-I-containing media. Total cellular protein levels were determined using the Markwell Lowry assay.[24]

Intracellular cholesterol distribution. Lipids were extracted from the cell lysates,[25] and separated by thin layer chromatography on silica gel plates using a non polar solvent system (hexane/diethyl ether/acetic acid, 70:30:1) for separation of cholesterol and CE. The bands corresponding to cholesterol and CE were excised and counted for radioactivity. Alternatively, cellular cholesterol and CE were extracted and measured using a fluorometric assay (Cholesterol/Cholesteryl Ester Quantitation Kit, BioVision, Mountain View, CA), according to the manufacturer's instructions.

Western Blot Analysis. Cells were incubated with or without LDL or AcLDL (50μg/mL) for 24h, then treated with oxysterols overnight. Cells were washed with PBS, scrapped with lysis buffer (Tris-EDTA-EGTA + Complete™ protease inhibitor (Roche)) and mechanically homogenized. Total protein samples (25μg/well) were electrophoresed on a pre-cast 8% SDS-polyacrylamide gel (Invitrogen), and transferred to nitrocellulose membranes at 125V for 4h. Membranes were incubated overnight with anti-ABCA1 (1:500, Novus Biologicals), anti-ABCG1 (1:2500, Novus Biologicals), anti-ß-Actin (1:500, BioLegend) or anti Heat Shock Protein 60 (HSP60) (1:500, Sigma). An anti-rabbit secondary antibody conjugated with horseradish peroxidase (Amersham Biosciences), and SuperSignal West Pico Chemiluminescent Substrate (Pierce) were used for detection.

Statistical analysis. Results are shown as mean ± SEM and all experiments were run in triplicates. The statistical significance of the differences between groups was determined using Student's *t* test with or without Welsh correction or one-way ANOVA with Tukey

post test using GraphPad InStat v.3.06 statistical analysis software (GraphPad Software Inc.).

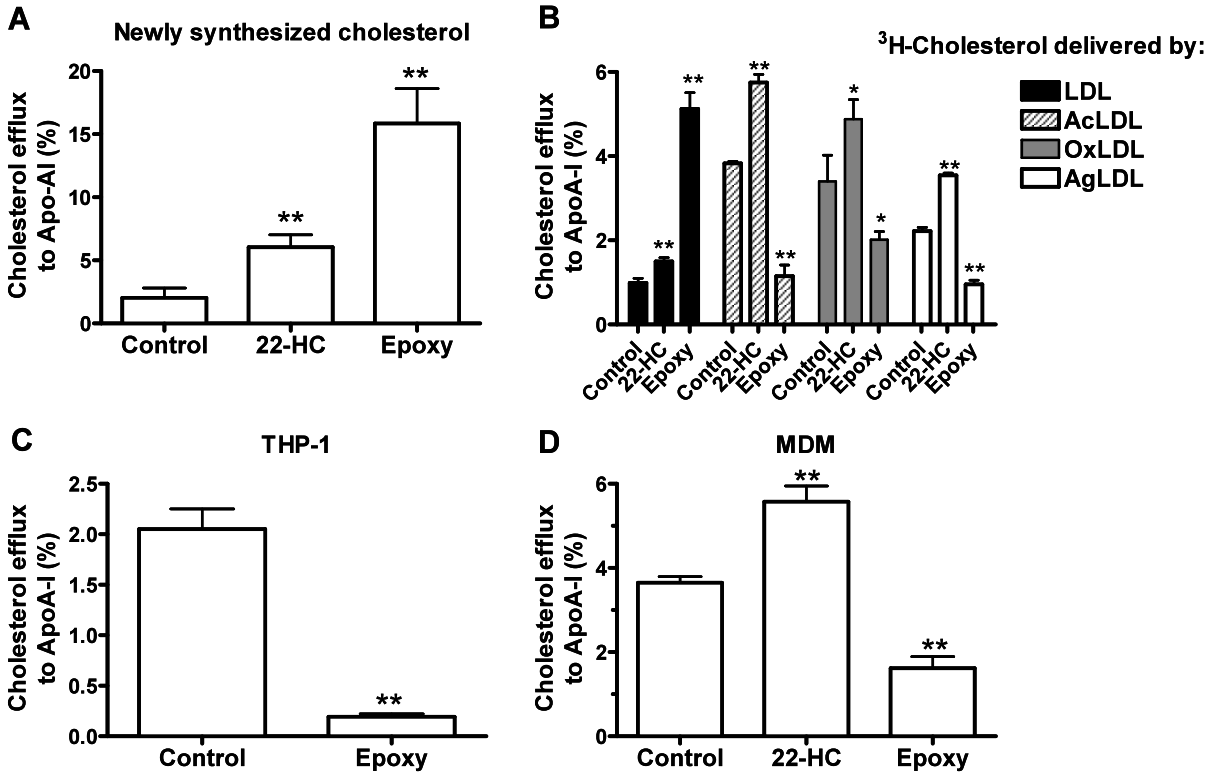
2.6 Results

Epoxycholesterol promotes cholesterol efflux to lipid-poor apoA-I in LDL-treated but not in AcLDL-loaded macrophages

Bone marrow-derived macrophages (BMDM) were labelled with ^{3}H-mevalonate in the presence of oxysterols for 48h, following which efflux of *de novo* synthesized cholesterol to apoA-I was measured. In unloaded macrophages (not preincubated with lipoproteins), both 22-HC and epoxycholesterol increased efflux of newly synthesized cholesterol (**Figure 2.1A**). Epoxycholesterol, which has a higher affinity for LXR than 22-HC,[26] was a stronger inducer of efflux of newly synthesized cholesterol to apoA-I. The observed increase in cholesterol efflux was not due to an increase of cholesterol synthesis in response to treatment with oxysterols, since total cholesterol synthesis was decreased in cells treated with oxysterols (data not shown). This is in agreement with the well-documented down-regulation of HMG-CoA reductase by oxysterols.[27-29]

The effect of oxysterols on cholesterol efflux in macrophage foam cells has not been well characterized. Here in BMDM foam cells generated by incubating with AcLDL, the net cellular cholesterol level (μg/mg cell protein) was 8.7-fold higher compared to unloaded macrophages (488±18 versus 56±5). As expected, due to the feedback inhibition of the LDL receptor, the cholesterol content of LDL-labelled macrophages was similar to that of unloaded macrophages (63±9 compared to 56±3). Surprisingly, epoxycholesterol decreased cholesterol efflux to apoA-I in AcLDL-loaded foam cells, whereas 22-HC had a stimulatory effect (**Figure 2.1B**). This efflux reduction was also observed in macrophages treated with oxLDL and agLDL, two other well-known foam cell inducers, but not in LDL-treated macrophages. Epoxycholesterol not only decreased cholesterol efflux in murine macrophage foam cells, but also in human THP-1 macrophages (**Figure 2.1C**) and in primary human monocyte-derived macrophages (MDM) (**Figure 2.1D**) loaded with AcLDL-derived cholesterol.

Figure 2.1: Epoxycholesterol promotes cholesterol efflux in unloaded, but not in lipid-laden macrophages. Efflux of newly synthesized cholesterol (A), and lipoprotein-derived cholesterol (B, C, D) to lipid poor apoA-I in murine (A, B) and human (C, D) macrophages, in response to treatment with oxysterols. P<0.0001 (**), <0.001 (*) compared to control.


A
Newly synthesized cholesterol
Cholesterol efflux to Apo-AI (%)
20
15
10
5
0
**
**
Control
22-HC
Epoxy
B
3H-Cholesterol delivered by:
LDL
AcLDL
OxLDL
AgLDL
Cholesterol efflux to ApoA-I (%)
6
4
2
0
**
**
**
*
*
**
**
Control
22-HC
Epoxy
C
THP-1
Cholesterol efflux to ApoA-I (%)
2.5
2.0
1.5
1.0
0.5
0.0
**
Control
Epoxy
D
MDM
Cholesterol efflux to ApoA-I (%)
6
4
2
0
**
**
Control
22-HC
Epoxy

In unloaded or LDL-treated macrophages, epoxycholesterol stimulated cholesterol efflux, suggesting that the reduction of efflux in response to epoxycholesterol is specific to the macrophage foam cell (**Figure 2.1B**). Indeed, when macrophages were loaded with increasing amounts of AcLDL-derived cholesterol (**Figure 2.2A**), epoxycholesterol concomitantly reduced cholesterol efflux to apoA-I proportionally to the amount of AcLDL internalized into the cell (**Figure 2.2B**).

Induction of ABCA1 and ABCG1 expression by epoxycholesterol treatment in AcLDL-loaded macrophages is not associated with enhanced ABCA1- and ABCG1-mediated cholesterol efflux

In AcLDL-loaded BMDM, we observed a dose-dependent decrease of cholesterol efflux to lipid-poor apoA-I in response to epoxycholesterol treatment (**Figure 2.3A**). Another member of the ATP-binding cassette (ABC) family, ABCG1, has previously been described as a LXR-responsive gene,[13,30] and has an established role in the efflux of cholesterol to high density lipoprotein (HDL) acceptors.[31,32] Interestingly, increasing doses of epoxycholesterol also correlated with a decrease in cholesterol efflux to HDL in AcLDL-loaded BMDM (**Figure 2.3B**), indicating that epoxycholesterol does not uniquely affect the ABCA1-mediated pathway. Rather, epoxycholesterol seems to impair cholesterol efflux regardless of the cholesterol acceptor.

The decline of cholesterol efflux occurred in spite of the up-regulation of the transporters implicated in these processes. Expression of both ABCA1 (**Figure 2.3C**) and ABCG1 (**Figure 2.3D**) was induced by epoxycholesterol, in a dose-dependent fashion, and translocation of the transporters to the plasma membrane was not hindered (data not shown).

Epoxycholesterol impairs the mobilization of cholesterol

Excess cellular cholesterol is stored as CE, which can be mobilized for efflux by CE hydrolase activity. We therefore evaluated the effects of 22-HC and epoxycholesterol on CE stores. In unloaded BMDM, epoxycholesterol dramatically increased the proportion of newly synthesized cholesterol in CE pools **(Figure 2.4A)**. Epoxycholesterol also promoted the accumulation of CE in BMDM incubated with LDL, AcLDL, OxLDL, and AgLDL

Figure 2.2: Epoxycholesterol-mediated decrease in cholesterol efflux is inversely proportional to the increasing macrophage cholesterol load. Increasing amounts of intracellular cholesterol (A) leads to inversely proportional decreases in cholesterol efflux (B). Results are expressed as a fold-change of the efflux of the epoxycholesterol-treated cells relative to controls. $P<0.0001$ (one-way ANOVA).

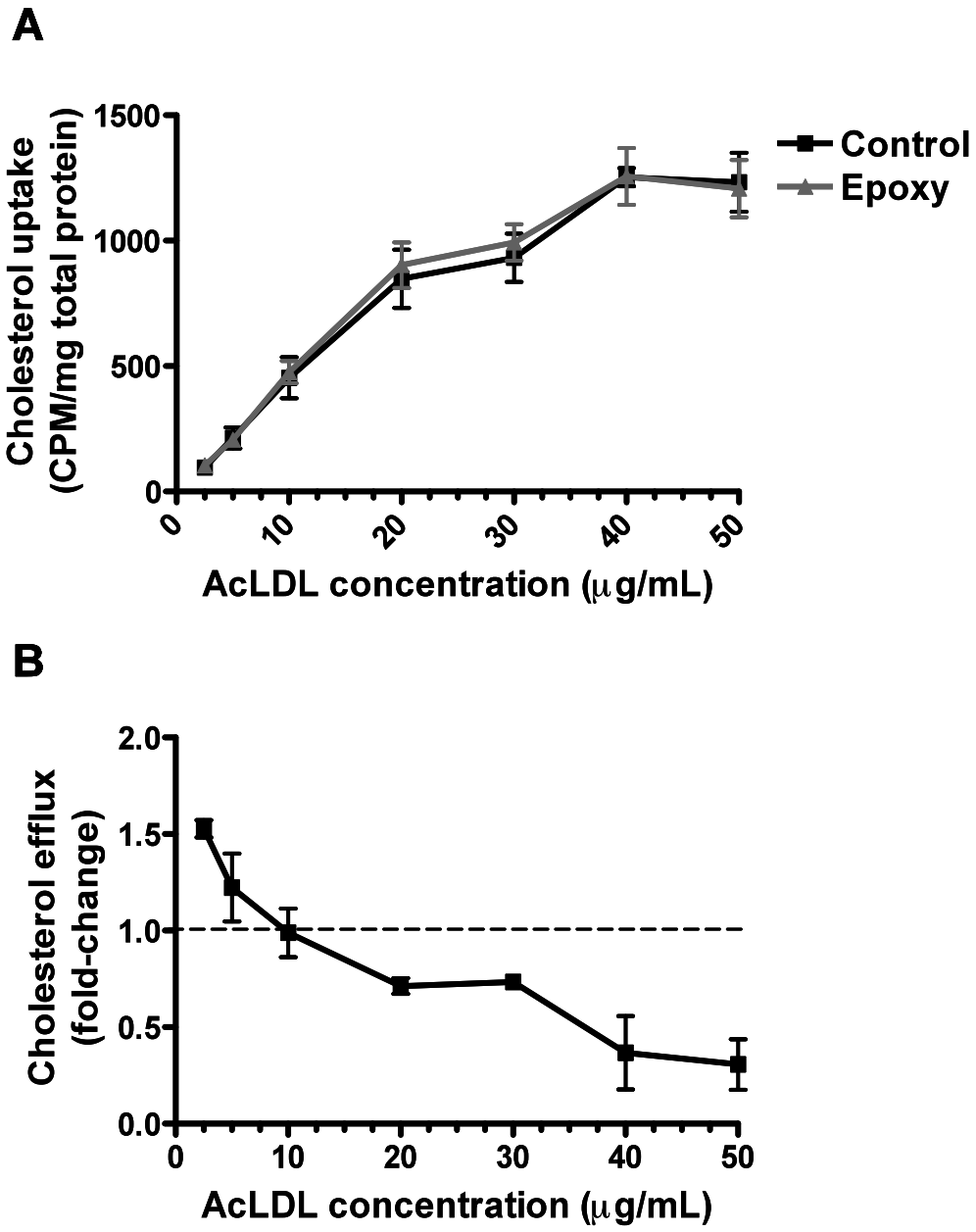

A
Cholesterol uptake
(CPM/mg total protein)
1500
1000
500
0
0
10
20
30
40
50
AcLDL concentration (μg/mL)
Control
Epoxy
B
Cholesterol efflux
(fold-change)
2.0
1.5
1.0
0.5
0.0
0
10
20
30
40
50
AcLDL concentration (μg/mL)

Figure 2.3: Epoxycholesterol decreases cholesterol efflux in a dose-dependent manner, despite inducing ABCA1 and ABCG1 expression. Cholesterol efflux to apoA-I (A) or human HDL (B), and expression of ABCA1 (C) and ABCG1 (D) in unloaded and AcLDL-loaded BMDM treated with increasing amounts of epoxycholesterol. $P<0.0001$ (one-way ANOVA), $P<0.001$ (**) or <0.01 (*) compared to control.

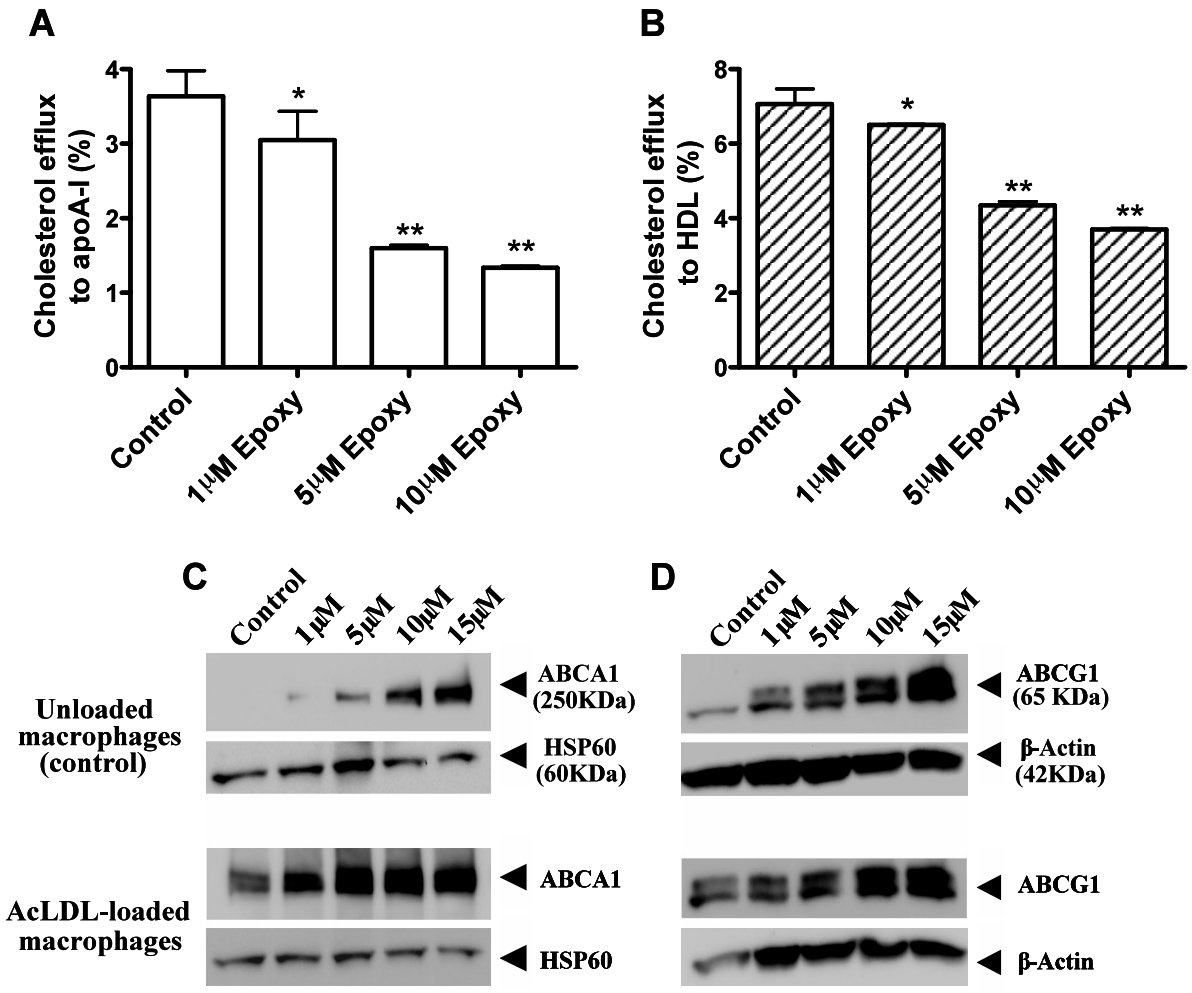
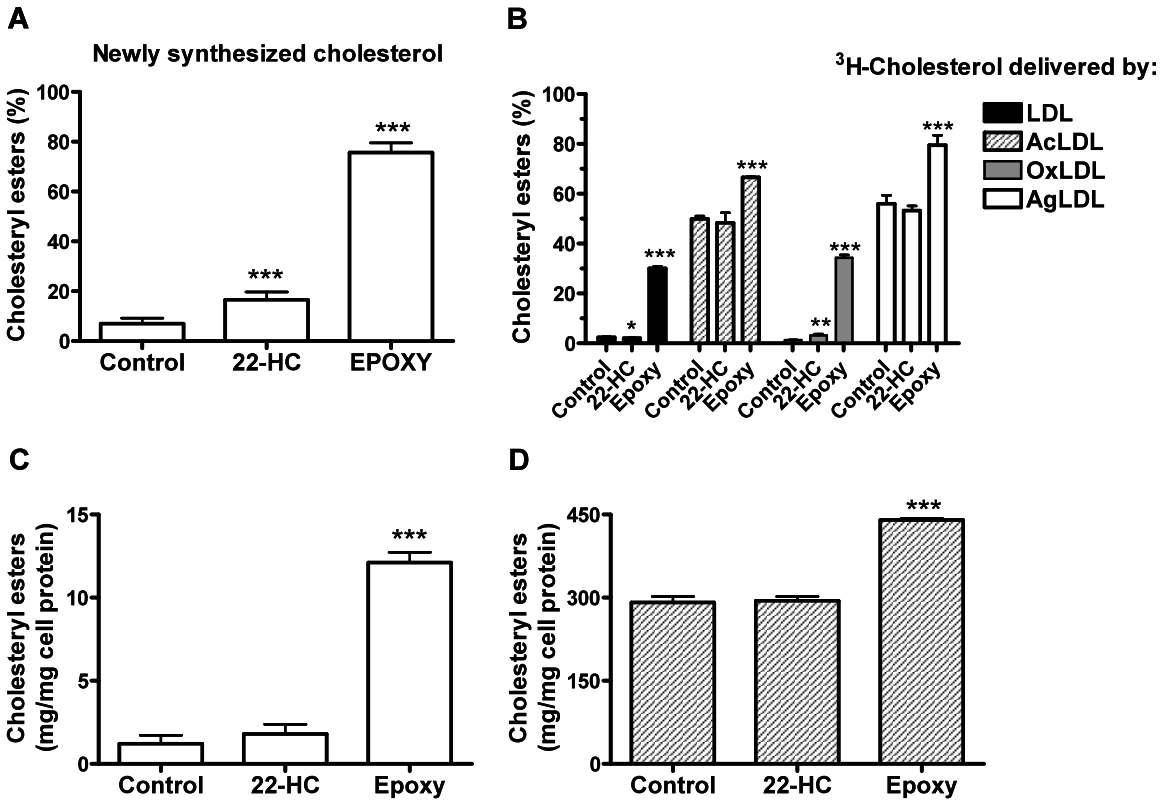

A
Cholesterol efflux to apoA-I (%)
0
1
2
3
4
*
**
**
Control
1μM Epoxy
5μM Epoxy
10μM Epoxy
B
Cholesterol efflux to HDL (%)
0
2
4
6
8
*
**
**
Control
1μM Epoxy
5μM Epoxy
10μM Epoxy
C
Control
1μM
5μM
10μM
15μM
Unloaded macrophages (control)
ABCA1 (250KDa)
HSP60 (60KDa)
AcLDL-loaded macrophages
ABCA1
HSP60
D
Control
1μM
5μM
10μM
15μM
ABCG1 (65 KDa)
β-Actin (42KDa)
ABCG1
β-Actin

Figure 2.4. Epoxycholesterol promotes the accumulation of cholesteryl esters. Relative levels of newly synthesized cholesterol (A) or lipoprotein-derived cholesterol (B) stored as CE measured by TLC. Quantification of CE by fluorometric assay is presented for unloaded (C) and AcLDL-loaded macrophages (D). P<0.0001 (***), <0.001 (**), or <0.05 (*) compared to control.


A
Newly synthesized cholesterol
Cholesteryl esters (%)
100
80
60
40
20
0
***
***
Control
22-HC
EPOXY
B
3H-Cholesterol delivered by:
LDL
AcLDL
OxLDL
AgLDL
Cholesteryl esters (%)
*
***
***
**
***
***
Control
22-HC
Epoxy
C
Cholesteryl esters (mg/mg cell protein)
15
10
5
0
***
Control
22-HC
Epoxy
D
Cholesteryl esters (mg/mg cell protein)
450
300
150
0
***
Control
22-HC
Epoxy

(**Figure 2.4B**). In contrast, treatment with 22-HC did not induce a comparable rise in cellular CE. The relative changes in CE levels shown in **Figure 2.4A, 2.4B** were indeed representative of net CE accumulation, as confirmed by direct measurement of esterified cholesterol cellular content (**Figure 2.4C, 2.4D**). Additionally, epoxycholesterol enhanced CE mass in THP-1 macrophages in both unloaded and AcLDL-loaded cells (**Table 2.1**).

Since the accumulation of CE was specifically attributable to epoxycholesterol treatment, we speculated that this might be implicated in the impairment of cholesterol efflux from epoxycholesterol-treated macrophage foam cells. One of two scenarios (or perhaps a combination of both) could be proposed: Epoxycholesterol promotes the esterification of cholesterol via stimulation of ACAT, and/or epoxycholesterol decreases CE hydrolysis. In both, availability of free cholesterol for efflux to apoA-I would be decreased. To distinguish between the two, we performed a series of experiments in the presence of an ACAT inhibitor, which was added at key times during the experiments.

First, the ACAT inhibitor was added during treatment with epoxycholesterol, after the cells had been incubated with ^{3}H-cholesterol-AcLDL for 24h. Subsequently, efflux to apoA-I was measured for 5h. Whereas the ACAT inhibitor-treated cells displayed increased cholesterol efflux, the addition of epoxycholesterol increased but failed to restore efflux to apoA-I (**Figure 2.5A**), indicating that the ability of epoxycholesterol to reduce cholesterol efflux in macrophage foam cells is largely independent of cholesterol esterification.

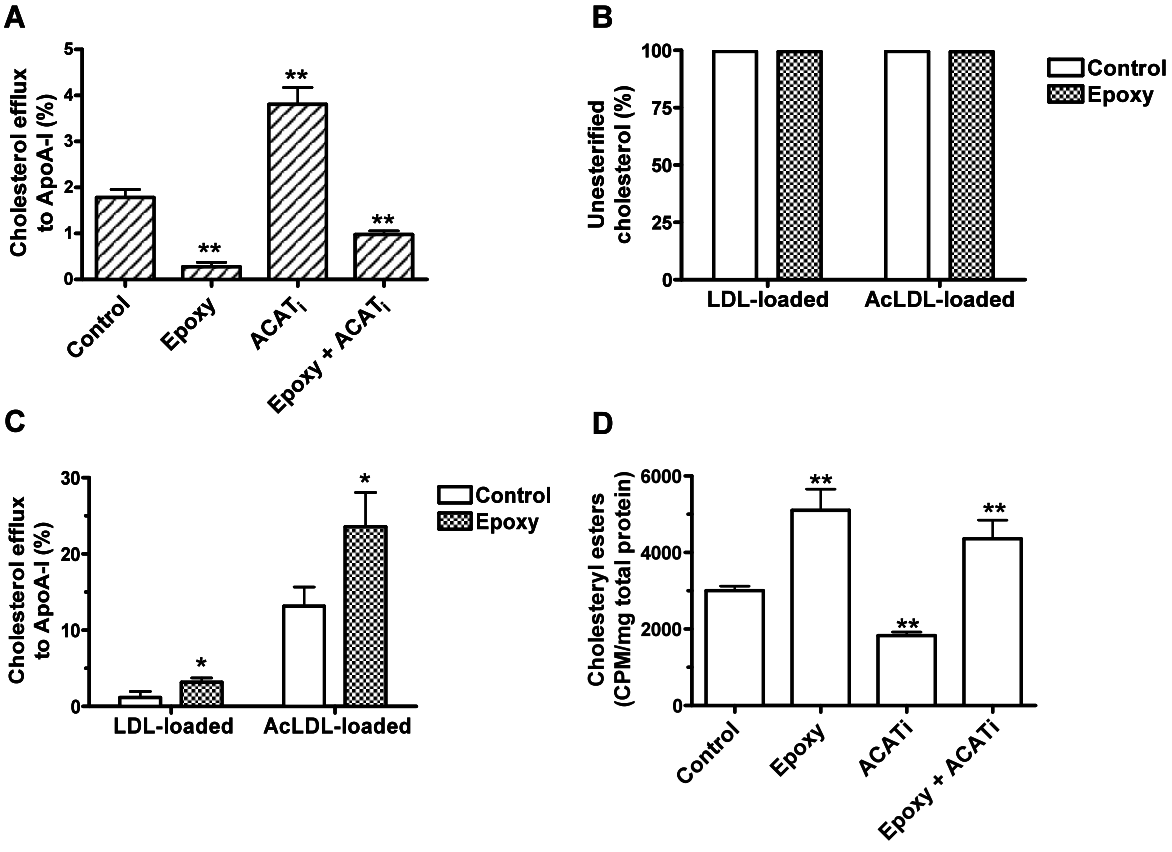
Next, the ACAT inhibitor was administered during labelling of BMDM with LDL- or AcLDL-derived ^{3}H-cholesterol for 6h, as well as during treatment with epoxycholesterol (12h) and efflux to apoA-I (5h). Under these conditions, cholesterol esterification in the ER for storage in the lipid droplet was inhibited, and essentially all of the lipoprotein-derived ^{3}H-cholesterol remained unesterified (**Figure 2.5B**). We observed an increase in both LDL- and AcLDL-derived cholesterol efflux in response to epoxycholesterol treatment (**Figure 2.5C**), demonstrating that epoxycholesterol does not hinder efflux of unesterified cholesterol, and mediates its effect downstream of cholesterol esterification in the ER.

Finally, to directly assess the degree of CE hydrolysis in response to epoxycholesterol treatment, BMDM were incubated with unlabelled AcLDL in the presence of ^{3}H-oleate for 24 h, such that AcLDL-derived cholesterol could be esterified to the radio-labelled oleate.

Table 2.1: Cholesterol and Cholesteryl Ester levels in human THP-1 macrophages. THP-1 macrophages were incubated for 24h in medium containing AcLDL (50µg/mL) or not. Cells were treated with 22(R)-hydroxycholesterol or 24(S),25-epoxycholesterol (10µM) for 12h, after which cellular cholesterol mass was measured by fluorometric assay. P <0,05 (*), <0,01 (**),<0.005 (***), or < 0.0001 (****).

	Total Cholesterol (μg/mg cell protein)	Cholesteryl Ester (μg/mg cell protein)
Unloaded		
Control	102 ± 6	3 ± 1
22-HC	97 ± 5	5 ± 2
Epoxy	132 ± 3***	49 ± 2****
AcLDL-loaded		
Control	386 ± 23	138 ± 7
22-HC	384 ± 24	139 ± 4
Epoxy	450 ± 24*	177 ± 11**

Figure 2.5: Epoxycholesterol impairs the mobilization of cholesterol from cholesteryl esters in lipid droplets. Efflux of cholesterol following treatment with epoxycholesterol in the presence of an ACAT inhibitor (ACATi) (A), efflux of non-esterified cholesterol (C), and cellular CE (B, C). $P<0.0001$ (**) or <0.0005 (*) compared to control.


A
Cholesterol efflux to ApoA-I (%)
0
1
2
3
4
5
**
**
**
Control
Epoxy
ACAT$_i$
Epoxy + ACAT$_i$
B
Unesterified cholesterol (%)
0
25
50
75
100
LDL-loaded
AcLDL-loaded
Control
Epoxy
C
Cholesterol efflux to ApoA-I (%)
0
10
20
30
*
*
LDL-loaded
AcLDL-loaded
Control
Epoxy
D
Cholesteryl esters (CPM/mg total protein)
0
2000
4000
6000
**
**
**
Control
Epoxy
ACATi
Epoxy + ACATi

Subsequent treatment with epoxycholesterol in the presence of an ACAT inhibitor and apoA-I resulted in equivalent CE cellular content as epoxycholesterol treatment alone (**Figure 2.5D**), therefore allowing us to conclude that epoxycholesterol treatment impairs mobilization of cholesterol from the CE pools. Specifically, epoxycholesterol decreases CE hydrolysis from the lipid droplet, and reduces the availability of free cholesterol for efflux to lipid-poor apoA-I or HDL.

2.7 Discussion

Because activated LXRs induce the expression of numerous genes involved in the reverse cholesterol transport pathway, notably ABCA1 and ABCG1, they have become attractive targets for pharmacological treatment of atherosclerosis.[33] Whereas the therapeutic potential of LXR activation has been demonstrated by the targeted disruption of LXR in macrophages in athero-susceptible mice,[34] systemic LXR activation is less favourable because of an associated rise in plasma triglycerides attributed to LXR activation of sterol regulatory element binding protein 1c (SREBP-1c), which induces genes involved in fatty acid and triglyceride synthesis.[35-38]

The naturally occurring oxysterols, 22-HC and epoxycholesterol, are potent LXR ligands.[8] Interestingly, increased epoxycholesterol production in macrophages was recently reported to selectively up-regulate cholesterol efflux genes without a simultaneous increase in genes that promote triglyceride synthesis, in contrast to a nonsteroidal synthetic LXR agonist.[14,39] Given that epoxycholesterol has a higher affinity for LXR than 22-HC,[26] and does not promote triglyceride synthesis,[39] we hypothesized that epoxycholesterol should be the most effective oxysterol to induce cholesterol efflux in lipid-loaded macrophages and decrease the foam cell phenotype.

While epoxycholesterol enhanced cholesterol efflux in unloaded or LDL-treated and labelled macrophages in keeping with earlier reports of the effects of oxysterols,[6,11-13] it unexpectedly decreased cholesterol efflux in lipid-laden primary murine macrophages generated by incubation with AcLDL, OxLDL, or AgLDL (**Figure 2.1A, 2.1B**). Moreover, epoxycholesterol also decreased cholesterol efflux in human THP-1 and monocyte-derived macrophages loaded with AcLDL-derived cholesterol (**Figure 2.1C, 2.1D**). In lipid-loaded

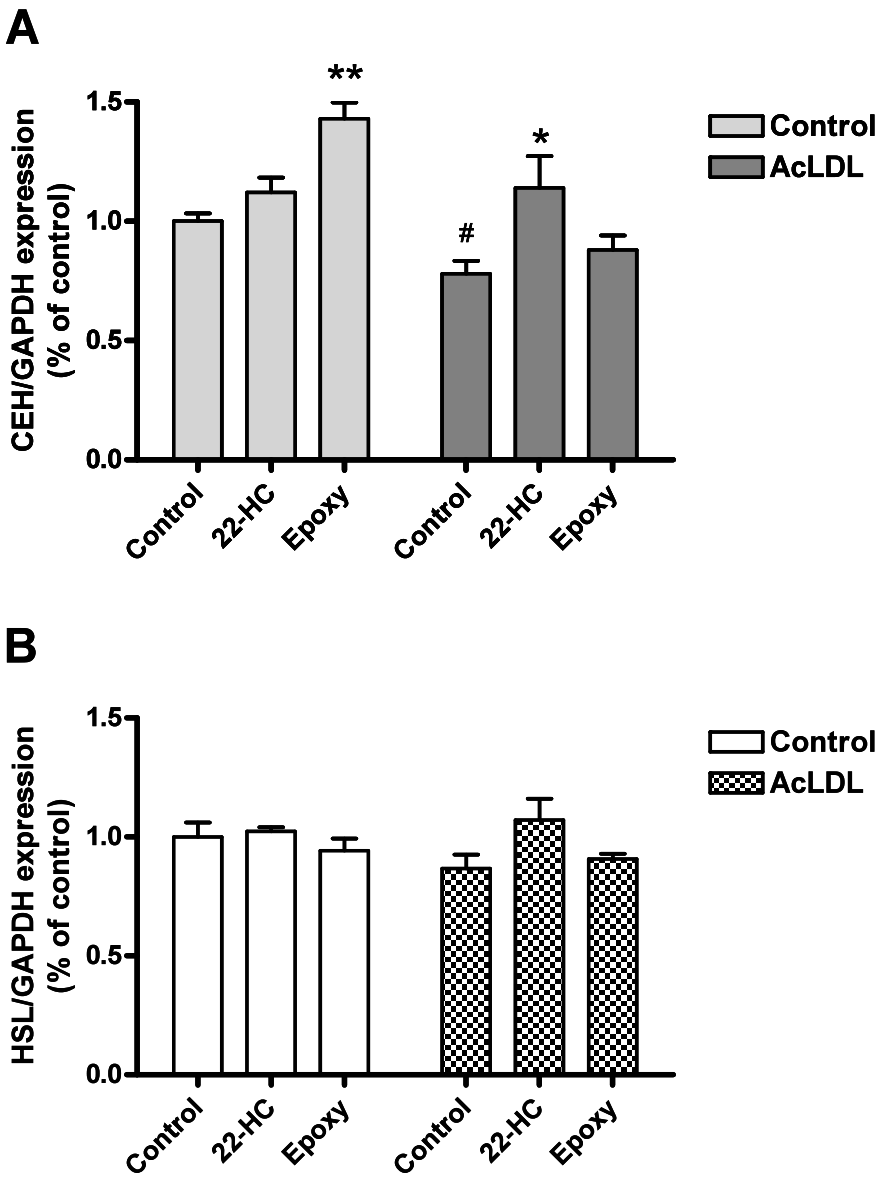
macrophages, epoxycholesterol impaired both ABCA1-mediated cholesterol efflux to lipid-poor apoA-I and ABCG1-mediated cholesterol efflux to HDL (**Figure 2.3A, 2.3B**). The decrease in cholesterol efflux upon epoxycholesterol treatment in macrophage foam cells occurred despite the significant up-regulation of the ABCA1 and ABCG1 transporters (**Figure 2.3C, 2.3D**), in agreement with earlier reports of oxysterol stimulation of ABCA1[11,13] and ABCG1[32,40] expression. Interestingly, it has previously been shown that in macrophage foam cells 7-ketocholesterol impairs cholesterol efflux to apoA-I, compared with macrophages loaded with AcLDL alone, thus indicating that this oxysterol also inhibits reverse cholesterol transport in AcLDL-loaded macrophages.[41]

CE stored in cytoplasmic lipid droplets must be mobilized by enzymes with CE hydrolase activities before export of cholesterol from the cell via ABCA1- and ABCG1-mediated efflux. We have found that epoxycholesterol reduces cholesterol efflux downstream of the ER-resident enzyme responsible for the esterification of cholesterol (**Figure 2.5A-C**). When esterification of cholesterol for storage in the lipid droplets was inhibited via the administration of an ACAT inhibitor, epoxycholesterol enhanced efflux of both LDL- and AcLDL-derived cholesterol. In contrast, epoxycholesterol treatment in the presence of the ACAT inhibitor, following macrophage lipid-loading, leads to a reduction of cholesterol efflux and accumulation of CE in lipid droplets. We thus conclude that epoxycholesterol reduces cholesterol availability for efflux via down-regulation of CE hydrolysis.

The exact identity of the enzymes that catalyze CE lipolysis in macrophages remains ambiguous. Whereas the presence of the hormone-sensitive lipase (HSL) in human macrophages is controversial,[42] a neutral CEH gene was cloned from THP-1 and PBMC libraries,[43] the over expression of which enhanced both ABCA1- and ABCG1-mediated efflux in THP-1 cells.[44] We have investigated whether epoxycholesterol regulates the expression of this human CEH in THP-1 macrophages. Our results indicate that epoxycholesterol does not decrease cholesterol efflux via transcriptional regulation of CEH expression in human macrophages (**Figure 2.6A**).

Interestingly, we were able to detect HSL mRNA in THP-1 macrophages using the primers designed by Reue *and al.*[45] However, we did not observe any significant change in HSL mRNA in our treated versus untreated cells (**Figure 2.6B**). Hence, changes in HSL

Figure 2.6: **CEH and HSL mRNA levels in THP-1 macrophages.** THP-1 macrophages were incubated for 24h in medium (1% FBS, 1% P/S, RPMI 1640) containing AcLDL (50μg/mL) or not. Cells were washed, and equilibrated in 2mg/mL BSA RPMI containing 22(R)-hydroxycholesterol or 24(S),25-epoxycholesterol (10μM) for 12h. Cells were washed twice with PBS, and mRNA was extracted and measured by quantitative real-time PCR. Expression of the human macrophage cholesteryl ester hydrolase (CEH) and hormone sensitive lipase (HSL) were normalised to that of the internal control GAPDH. The two-tailed p-value from student's *t* test from oxysterol-treated cells compared to their respective controls (unloaded and AcLDL-loaded cells) is <0.0001 (**) or <0.05 (*), or from AcLDL-loaded oxysterol-treated cells versus unloaded control cells <0.005 (#).

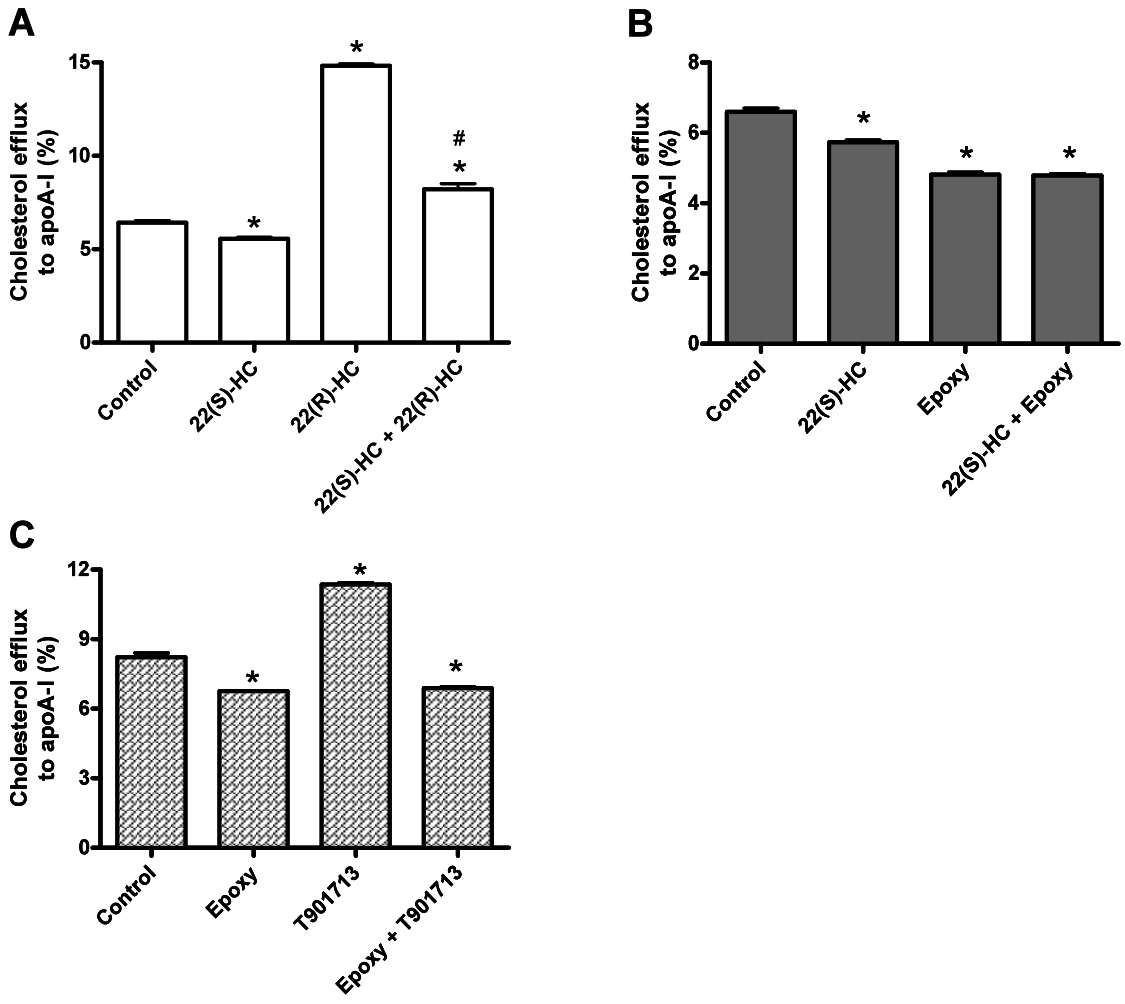

A
CEH/GAPDH expression
(% of control)
1.5
1.0
0.5
0.0
**
*
#
Control
AcLDL
Control
22-HC
Epoxy
Control
22-HC
Epoxy
B
HSL/GAPDH expression
(% of control)
1.5
1.0
0.5
0.0
Control
AcLDL
Control
22-HC
Epoxy
Control
22-HC
Epoxy

expression at the transcriptional level cannot explain the decrease in CE hydrolysis measured in epoxycholesterol-treated cells. It is, however, unlikely that HSL plays a major role in the CE hydrolysis in these THP-1 macrophages given that its mRNA levels were strikingly lower, by as much as 20 000-fold, compared to CEH mRNA. Conversely, as one early study has shown, for certain hydrolases there is little correlation between mRNA levels and lipolytic activity.[46]

It remains to be determined how epoxycholesterol decreases CE hydrolysis in lipid-loaded macrophages. We do know that this occurs independently of LXR activation, since the simultaneous treatment of lipid-loaded THP-1 cells with epoxycholesterol and an LXR antagonist does not increase cholesterol efflux, and the addition of an LXR synthetic agonist in conjunction with epoxycholesterol fails to rescue the efflux defect (**Figure 2.7**). Although epoxycholesterol does not reduce CE lipolysis in lipid-loaded macrophages via transcriptional down-regulation of CEH or HSL, the possibility of altered post-translational regulation of these enzymes remains to be investigated, as well as the contribution of other candidate enzymes for CE hydrolysis in these macrophages. It is worth to note that other lipid droplet associated proteins, such as perilipin and adipocyte differentiation-related protein (ADRP), were reported to influence lipid hydrolysis by regulating the accessibility of the hydrolytic enzymes to lipids within the droplet core. The examination of the effects of epoxycholesterol on these proteins is underway.

Potential LXR agonists for therapeutic intervention in atherosclerosis need to be selected with extreme caution. As shown here, an increase in the expression of ABCA1 and ABCG1 transporters does not necessarily translate into enhancement of cholesterol efflux from the macrophage. We have observed that once the macrophage becomes cholesterol-loaded, its response to oxysterols differs than that observed in unloaded or LDL-loaded cells. Our results indicate that 22-HC is better capable of promoting cholesterol efflux in lipid-laden macrophages while epoxycholesterol appears to impair this process. In conclusion, epoxycholesterol elicits cholesterol efflux from non-cholesterol loaded cells but aggravates the phenotype of the macrophage foam cell. Hence, epoxycholesterol may have quite different effects on the prophylaxis versus regression of atherosclerosis.

Figure 2.7: **Administration of an LXR antagonist does not affect cholesterol efflux in epoxycholesterol-treated macrophages.** Human THP-1 macrophages were incubated for 24h with AcLDL (50μg/mL) containing ^{3}H-cholesterol. Cells were washed, and treated overnight with 22(R)-hydroxycholesterol (10μM), 22(S)-hydroxycholesterol (10μM), or both (10μM each) (A), 24(S),25-epoxycholesterol (10μM), 22(S)-hydroxycholesterol (10μM) or both (10μM each) (B), or 24(S),25-epoxycholesterol (10μM), T901713 (5μM) or both (10μM Epoxy, 5μM T901713) (C). Cholesterol efflux was measured in the presence or absence of human apoA-I (50μg/mL). Efflux was calculated as a percentage of total medium count relative to the total amount of radioactivity incorporated by the macrophages for the control and epoxycholesterol-treated cells, as described under Methods. The two-tailed p-value from student's *t* test from treated cells compared to control is <0.0001 (*), or oxysterol alone compared to oxysterol + 22(S)-hydroxycholesterol (or oxysterol + T901713) is <0.001 (#).


A
Cholesterol efflux to apoA-I (%)
0
5
10
15
*
*
*
#
Control
22(S)-HC
22(R)-HC
22(S)-HC + 22(R)-HC
B
Cholesterol efflux to apoA-I (%)
0
2
4
6
8
*
*
*
Control
22(S)-HC
Epoxy
22(S)-HC + Epoxy
C
Cholesterol efflux to apoA-I (%)
0
3
6
9
12
*
*
*
Control
Epoxy
T901713
Epoxy + T901713

2.8 Acknowledgements

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3 Autophagy Regulates Cholesterol Efflux from Macrophage Foam Cells via Lysosomal Acid Lipase

Authors: Mireille Ouimet, Vivian Franklin, Esther Mak, Xianghai Liao, Ira Tabas, and Yves L. Marcel

Cell Metabolism

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3.1 Significance of this Manuscript

Before the results in this manuscript were published, the paradigm for lipid droplet cholesteryl ester hydrolysis in macrophage foam cells was that it was entirely dependent on neutral cholesteryl ester hydrolases. In this work, we show a novel role for autophagy and lysosomal acid lipase in the hydrolysis of lipid droplet breakdown for cholesterol efflux from macrophage foam cells. Importantly, we show that this pathway contributes to macrophage reverse cholesterol transport, making this pathway a potential new target that can be modulated to enhance this anti-atherogenic process to reduce cholesteryl ester accumulation in arterial macrophages.

3.2 Author Contributions

The experiments were planned by Yves and myself. As first author, I performed the majority of the experiments for this paper. Vivian's help was instrumental during the animal studies, and these experiments wouldn't have taken shape without her. Esther performed many LC3 western blots and we had to troubleshoot these for quite a while before getting good blots, so I thank her for her patience and help with this, as well as during our perfecting of the lipid droplet isolation protocol. Some of the experiments included in the manuscript were performed at Columbia University, in collaboration with the laboratory of Ira Tabas. I was in Ira's lab for two weeks, and during this time Xianghai actively participated with the *Atg5-/-* macrophages experiments. For this paper, I wrote a preliminary draft of the manuscript after which Yves and I went through multiple rounds of editing, specially after each series of revisions (there were two sets of revisions).

3.3 Abstract

The lipid droplet (LD) is the major site of cholesterol storage in macrophage foam cells and is a potential therapeutic target for the treatment of atherosclerosis. Cholesterol, stored as cholesteryl esters (CE) is liberated from this organelle and delivered to cholesterol acceptors. The current paradigm attributes all cytoplasmic CE hydrolysis to the action of neutral CE hydrolases. Here, we demonstrate an important role for lysosomes in LD CE hydrolysis in cholesterol-loaded macrophages, in addition to that mediated by neutral hydrolases. Furthermore, we demonstrate that LDs are delivered to lysosomes via autophagy, where lysosomal acid lipase (LAL) acts to hydrolyze LD CE to generate free cholesterol mainly for ABCA1-dependent efflux; this process is specifically induced upon macrophage cholesterol loading. We conclude that, in macrophage foam cells, lysosomal hydrolysis contributes to the mobilization of LD-associated cholesterol for reverse cholesterol transport.

3.4 Introduction

Macrophage foam cells are a key component of atherosclerotic lesions. Because enhancing cholesterol efflux from these cells is an attractive means to reverse plaque lipid buildup, there has been continued investigation on ways to promote net cholesterol flux from peripheral tissues to the liver for excretion via the bile, a process referred to as macrophage reverse cholesterol transport (RCT).[1] The first step in RCT is the release of cholesterol from lipid droplets (LDs). Although classically defined as the cellular cholesterol 'storage' organelle, current research highlights the dynamic nature of LDs.[2] LDs are comprised of a neutral lipid core delineated by a phospholipid monolayer, which is coated by proteins. Adipophilin is the macrophage LD coat protein and its levels are directly correlated to cellular neutral lipid content and, recently, to atherosclerosis.[3] It is important to understand how cholesteryl esters (CE) in LDs are hydrolyzed and mobilized for efflux given that cytoplasmic CE hydrolysis is increasingly becoming recognized as the rate-limiting step for cholesterol efflux and whole-body RCT.[4]

Macroautophagy (hereafter referred to as autophagy) has been shown to participate in glucose, protein and, recently, lipid metabolism.[5] The autophagic process involves the formation of double membrane autophagosomes that sequester cytoplasmic contents and

subsequently fuse with lysosomes, thus delivering the autophagic body into the lysosomal lumen for degradation.[6] The initiating event in autophagy is nucleation of the membrane that will form the autophagosome,[7] after which the microtubule-associated protein 1A/1B light chain 3 (LC3) conjugation system acts in membrane elongation and autophagosome formation.[8] Following autophagosome fusion with the lysosome, cytosolic components sequestered in the autophagosome are degraded by acid proteases and hydrolases in the autolysosome.

The idea that autophagy is simply a means for bulk degradation of cytoplasmic constituents is changing as examples of preferential targeting of cargo for autophagic degradation emerge, revealing the selectivity of the process.[9] In addition to the forms of selective autophagy previously described, such as xenophagy (selective delivery of microorganisms to lysosomes), pexophagy (selective peroxisome cargo), mitophagy, ribophagy, ERphagy, etc., each of which possess unique protein requirements,[9] a type of autophagy describing the selective delivery of LDs for lysosomal degradation was recently described and has been termed 'lipophagy'.[10] Here, we report that in atherogenic pathophysiological conditions (exposure to modified low density lipoprotein [LDL]) autophagy is activated in macrophages, and this process contributes to intracellular lipid breakdown. Cytoplasmic LD-associated CE delivered to lysosomes by way of autophagy undergoes lysosomal acid lipase (LAL)-dependent lipolysis, thereby generating free cholesterol for efflux to a cholesterol acceptor. Consequently, cholesterol efflux from lipid-loaded macrophages is dependent upon LD catabolism by autophagy and the importance of this process in whole-body RCT is highlighted by the impaired ability of *Atg5-/-* macrophages to clear accumulated ^{3}H-cholesterol *in vivo*.

3.5 Experimental procedures

Cell culture. Bone marrow derived-macrophages: bone marrow cells were flushed from the femurs of C57BL/6 mice (Jackson Laboratories) or from *Atg5-/-/LDLR-/-* and *LDLR-/-* mice on a C57BL/6 background and differentiated into macrophages by incubation in DMEM media supplemented with 10% FBS, 1% P/S, and 15% L929-conditioned media for 7 days. Peritoneal macrophages: peritoneal macrophages from *Atg5-/-/LDLR-/-* and

LDLR-/- were harvested 4 days after i.p. injection of methyl-BSA in mice previously immunized with this antigen,[11] as previously described.[12] For *in vivo* macrophage cholesterol loading, male mice 8 months of age (*apoe-/-* or wild-type on a C57BL/6 background) were placed on a Western diet (Harlan-Teklad) for 2 weeks after which peritoneal macrophages were harvested 3 days after thioglycolate injection, as previously described.[13] We confirmed that neutral lipids were increased in macrophages harvested from *apoe-/-* mice as compared to wild-type mice (CE was of 94±4μg/mg cell protein as compared to 9±4μg/mg cell protein, respectively). All experiments performed were in accordance with protocols approved by the University of Ottawa Animal Care Committee.

Lipoprotein preparation. VLDL and LDL were isolated by sequential density ultracentrifugation.[14] Modification of LDL: LDL was either acetylated by repetitive additions of acetic anhydride,[15] aggregated by vortexing,[16] or oxidized by incubation with 5μmol/L $CuSO_4$ at room temperature for 24h, adapted from Kunjathoor et al.[17] HDL was purified by density gradient ultracentrifugation.[18] VLDL was isolated from a pool of plasma obtained from untreated patients with severe mixed hyperlipidemia recruited from the Lipid Clinic, UOHI. The protocol was approved by the Human Research Ethics Committee and written informed consent was obtained from all subjects. ^{3}H-Cholesteryl oleate lipoproteins were prepared as described by others.[19] Briefly, dried ^{3}H-cholesteryl oleate was re-suspended in 0.5mL of a 0.15M NaCL, 0.3mM EDTA solution, and incubated 15min at 37°C. 300-400mg of lipoprotein (AcLDL or OxLDL) was added and the mixture was incubated for an additional 4h at 37°C, after which it was dialyzed overnight. The supernatant was collected after a brief centrifugation and lipoproteins were used at a 50μg/mL final concentration.

Cholesterol efflux. Cells were labelled similarly to that previously described[20]: macrophages were incubated for 30h in 10% FBS media containing 50μg/mL of lipoproteins that were pre-incubated with ^{3}H-cholesterol (5μCi/mL). Cells were washed, and incubated in equilibration media (2 mg/mL BSA, 1% P/S) overnight (O/N). After equilibration, cholesterol efflux was determined in the presence or absence of human recombinant apoA-I (50μg/mL) prepared as previously described [21] or HDL (50μg/mL) in serum-free media (2 mg/mL BSA, 1% P/S) with the indicated reagent (paraoxon 100μM, chloroquine 30μM, vinblastine 30μM, bafilomycin 10nM, ACATi 10μg/mL, Lalistat 1 10μM) for 18 to 24h

(unless otherwise specified). The supernatant was then removed and briefly centrifuged to remove non-adherent cells, and the remaining cells were dissolved in 0.5N NaOH. The radioactivity within aliquots of supernatants and dissolved cells was measured by scintillation counting. Cholesterol efflux is expressed as a percentage of ^{3}H-cholesterol in medium/(^{3}H-cholesterol in medium+^{3}H-cholesterol in cells)x100%. Efflux to apoA-I or HDL was calculated by subtracting effluxes of the wells without apoA-I or HDL from those containing apoA-I or HDL.

ACAT inhibitor studies: the Sandoz 58-035 Acyl-CoA : cholesterol acyltransferase (ACAT) inhibitor (ACATi) was administered at a final concentration of 10μM where indicated.

Stimulated cholesterol efflux. Cells were labelled with ^{3}H-cholesterol-AcLDL as described above, and equilibrated O/N in the presence of 10μM T0901317 (Biomol) to maximally activate the ABCA1 transporter. Efflux to apoA-I was then measured as above.

In vivo **RCT studies.** Macrophage RCT experiments were carried out similarly to that previously described [22]. Bone marrow-derived macrophages from wild-type or *Atg5-/-* mice were loaded with ^{3}H-cholesterol-AcLDL as described above for cholesterol effluxes, and 200μL of cell preparations (~5x10^{6} cells containing ~2x10^{6}CPM) were injected subfascially in the lumbar region of C57BL/6 mice (n=10 per group for the first two experiments, n=13 for the third experiment – pooled data represents a total of n=33 mice per group). The initial proportion of cellular cholesterol that was esterified was equivalent in wild-type and *Atg5-/-* macrophages in all independent RCT experiments (42±1.1% and 42±1.3% for WT and *Atg5-/-* cells, respectively). Blood was collected at 24h via the saphenous vein and at 48h via cardiac puncture of anesthetized mice. Plasma was used for liquid scintillation counting. Following cardiac puncture of anaesthetized mice, gallbladders were emptied, and livers were removed for scintillation counting. Feces were collected over a 48h period and total feces radioactivity (of equivalent wet weight) was measured. All ^{3}H-tracer measurements are expressed relative to the injected amount. All experiments performed were in accordance with protocols approved by the University of Ottawa Animal Care Committee.

Lipid measurements. The Biovision Cholesterol Quantitation Kit was used to determine cellular mass of cholesterol (CH) and cholesteryl esters (CE). Briefly, isopropanol (1%

Triton X-100) was added to the cells and left for 2h at room temperature, subsequently transferred to glass vials, from which the solvent was removed under a constant nitrogen flow. The lipids were re-dissolved in cholesterol reaction buffer and measured by the fluorometric method, as per the manufacturer's instructions. Variations in CE are expressed as % hydrolysis or as fold-change relative to control, calculated as follows: % hydrolysis = $(CE_i - CE_f)/(CE_i)*100$, where CE_i represents the CE mass (μg/mg cell protein) immediately after AcLDL loading, and CE_f represents the CE mass (μg/mg cell protein) after the cells were incubated for 24h with apoA-I; fold-change = (% hydrolysis sample / % hydrolysis control).

CE quantification by TLC: total lipids were extracted[23] and separated by thin layer chromatography (TLC) on silica gel plates using a nonpolar solvent system (hexane/diethyl ether/acetic acid, 70:30:1, v/v) for separation of cholesterol and CE. The bands corresponding to cholesterol and CE were excised and counted for radioactivity.

^{3}H-cholesterol and ^{14}C-oleic acid esterification. In specific experiments, sodium ^{14}C-oleate-albumin complex was prepared as described previously[24] and used at a final concentration of 0.1mM. Macrophages were incubated with ^{3}H-cholesterol-AcLDL in the presence of 0.1mM ^{14}C-Oleate for 30h, in the presence or absence of ACATi, after which total lipids were extracted and the ^{3}H and ^{14}C labels were quantified in the CE fraction after TLC.

Protein quantification. Total cellular protein levels were determined using the Markwell Lowry assay.[25]

Western blotting. Cells were washed twice with ice-cold PBS, scrapped in Laemmli sample buffer (Bio-Rad). Total protein samples (25-30μg/well) were electrophoresed on precast 4-20% or 18% SDS-polyacrylamide gels (Invitrogen) and transferred to nitrocellulose or PVDF membranes at 125V for 2h. Membranes were probed with anti-β-Actin (1:500, BioLegend), anti-LC3 (1:500, MBL international), anti-adipophilin (1:2000, RDI), anti-beclin (1:500, Novus Biologicals), etc. Proteins were detected using an enhanced HRP-based chemiluminescence detection system (HRP-conjugated secondary antibodies from Amersham Biosciences and SuperSignal West Femto Maximum Sensitivity Substrate from Pierce) and analyzed using a FluorChem Imager (Alpha Innotech).

Lipid droplet isolation. LDs were isolated from lipid-loaded macrophages by density gradient centrifugation using a previously described method,[26] which the exception that whole LD fractions were used for SDS-PAGE (fractions were not delipidated prior to Western Blotting – fractional proteins were precipitated using ice-cold acetone and the pellet was re-dissolved in 25μL of loading buffer prior to SDS-PAGE).

Fluorescence microscopy. All live cell imaging was carried out in Hepes-buffered media, and the cells were visualized by confocal microscopy in a 37°C heat chamber. Neutral lipids were stained using Bodipy 493/503 or Nile Red, as previously described.[27] AcLDL-loaded macrophages were incubated with Bodipy (10μg/mL) with or without LysoTracker Red (50nM) for 30min prior to visualization, or with 10μg/mL of the BSA conjugate 4h prior to labeling with Bodipy. For immunofluorescence (IF), cells were fixed in 4% PFA for 30min at room temperature, and blocked/permeabilized in 2.5% BSA/0.1% TritonX-100 dissolved in 1X PBS. Cells were incubated with anti-adipophilin (1:500) anti LAMP-1 (1:800) and anti-LC3 (1:100, Cell Signaling) for 1h at 37°C. Fluorophore-conjugated secondary antibodies (1:500, Molecular Probes) were incubated in the presence of Nile Red (50ng/mL) to stain for neutral lipids. Confocal images and movies were obtained using a 100 3 NA 1.4 objective on an Olympus IX80 FV1000 confocal microscope with appropriate lasers.

Electron microscopy (EM). Cells were cultured on monolayers, loaded with AcLDL for 30h, washed and equilibrated in BSA media O/N, then lifted in 5mM PBS-EDTA for 20min and fixed in 1.6% glutaraldehyde prior to postfixation in osmium tetroxide and uranyl acetate en bloc staining. Samples were then processed and embedded in Spurr epoxy resin, thin sectioned, and counterstained with lead citrate. Digital images were obtained with a JEOL 1230 TEM at 60kV adapted with a 2000 3 2000 pixel bottom mount CCD digital camera and AMT software.

LC3 Immunogold labeling. A pre-embedding method followed by silver enhancement was used for immunoelectron microscopy. AcLDL-loaded macrophages were fixed in 4% paraformaldehyde and 0.1% glutaraldehyde in PBS. Cells were permeabilized and blocked in PBS containing 0.1% saponin and 5% bovine serum albumin (BSA) for 30min. Cells were immunolabeled with anti-LC3 (1:100, Cell Signaling #2775) in the wash solution overnight (1% BSA, 0.05% saponin in PBS). The cells were then washed, and incubated with anti-

rabbit IgG that was conjugated to colloidal gold (1.4 nm diameter) for 1h (Nanoprobes). Cells were washed with PBS, and post-fixed with 1% glutaraldehyde in PBS for 10 min. The gold was intensified using the HQ Silver Enhancement Kit (Nanoprobes) according to manufacturer's directions (staining was done for 8min). Cells were then washed thoroughly with distilled water to stop the enhancement process, following which the cells were postfixed in 0.5% OsO4 for 90 min at 4°C, washed with distilled water, incubated with 50% ethanol for 10 min, and stained with 2% uranyl acetate in 70% ethanol for 2 h. The cells were further dehydrated with a graded series of ethanol and then embedded, counterstained and imaged as described above.

Co-localization quantification. blind counting by 4 individuals was averaged for LC3 immunogold labeling.

Statistical analysis: Experiments were run in triplicates, and all presented values are mean ± SEM. The statistical significance of the differences between groups was determined using the two-tailed unpaired Student's t-test of the means with GraphPad InStat v3.1a software.

3.6 Results

Lysosomes contribute to lipid breakdown in macrophage foam cells

Because the internalization of modified LDL is an unregulated process that leads to foam cell formation,[15,28] we used acetylated LDL (AcLDL) to elevate macrophage intracellular cholesterol levels. Bone marrow-derived macrophages were incubated with AcLDL for 30h, after which the cells were washed and the lipoproteins were chased during an overnight equilibration. At this time, all of the AcLDL was degraded and excess cholesterol was stored as neutral lipids in cytoplasmic LDs (**Figure 3.1**). Whereas oxidized LDL (OxLDL) tends to become trapped within the endolysosomal compartment,[29] AcLDL is known to be quite rapidly processed in the lysosome and the majority of its associated cholesterol is stored in cytoplasmic LDs.[30-34] Bodipy 493/503 is commonly used to fluorescently stain neutral lipids.[27] To visualize LDs in real-time, neutral lipids were stained with Bodipy and the cells were observed by confocal microscopy in a 37°C heat chamber. Macrophage LDs could be seen surrounding an unidentified circular organelle, and over time the formation of a 'ring' stained for neutral lipids could be observed around this unknown organelle (**Figure 3.2A**).

Figure 3.1: **AcLDL-derived cholesterol is esterified ACAT and accumulates as neutral lipid in LDs; accumulated CE in AcLDL-loaded macrophages can subsequently be removed by the cholesterol acceptor apoA-I.** Macrophages were loaded with AcLDL, and equilibrated O/N in BSA media (normal loading) or otherwise cells were loaded with AcLDL for 6h in the presence or absence of chloroquine (A and B). IF was performed to visualize apoB (A) or adipophilin (B) and neutral lipids. Clearly, there is no apoB in cells with the normal loading, indicating that there is no lysosomal accumulation of undigested AcLDL (A), and all neutral lipids are in adipophilin-coated LDs (B). AcLDL-derived cholesterol is unesterified in the presence of an ACAT inhibitor (ACATi) and consequently there is no LD biogenesis (C). Here, macrophages were loaded with AcLDL for 6h in the presence or absence of ACATi after which cells were fixed and stained for microscopy (C). Macrophages were loaded with AcLDL in the presence or absence of 10μM ACATi (normal loading = 30h AcLDL, wash, equilibration in BSA), and CE was quantified by TLC or using the Cholesterol Quantitation Kit (D). Following cholesterol loading (time0), efflux to apoA-I was carried out for 24h, and CE was quantified using the Cholesterol Quantitation Kit (E).

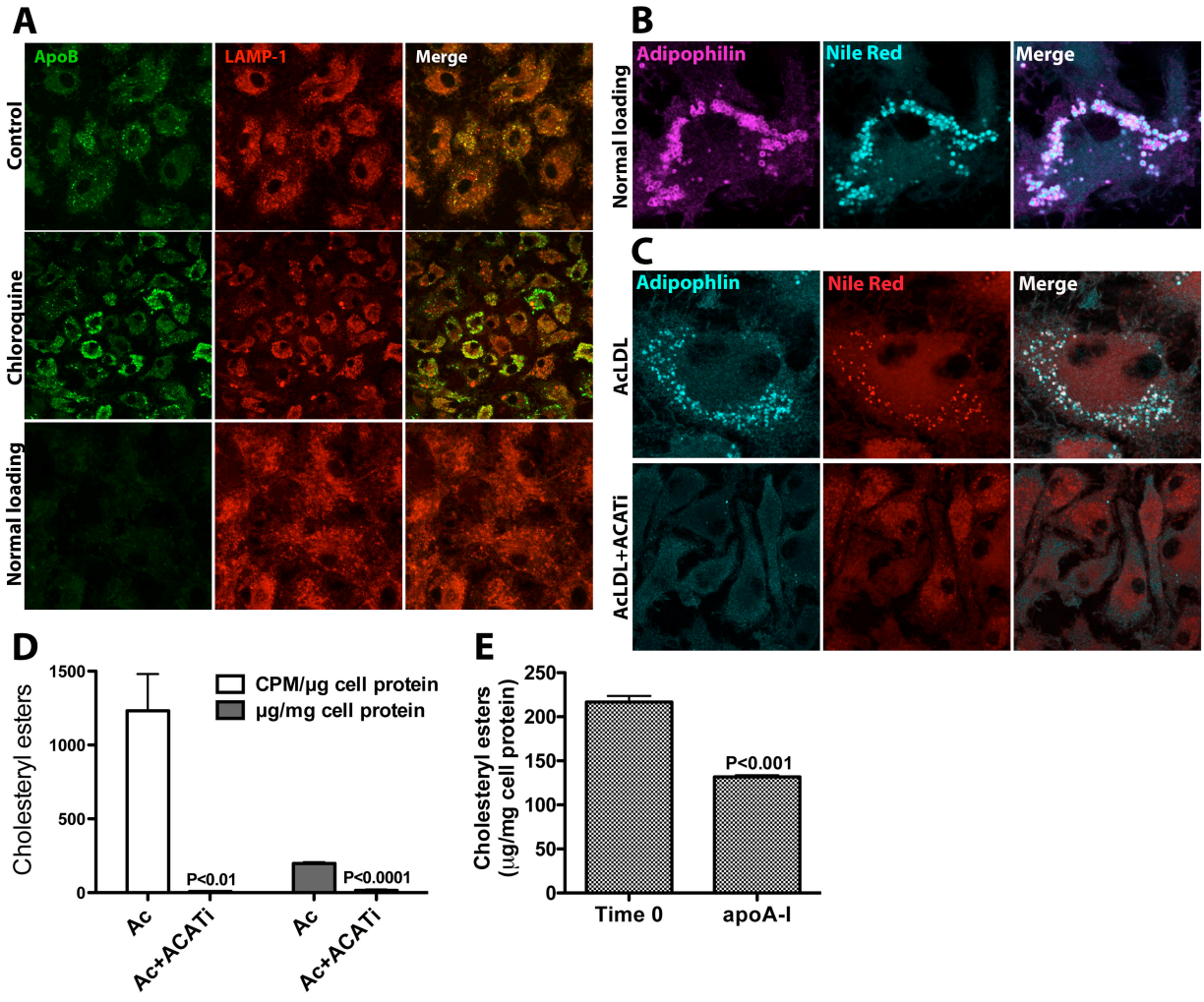
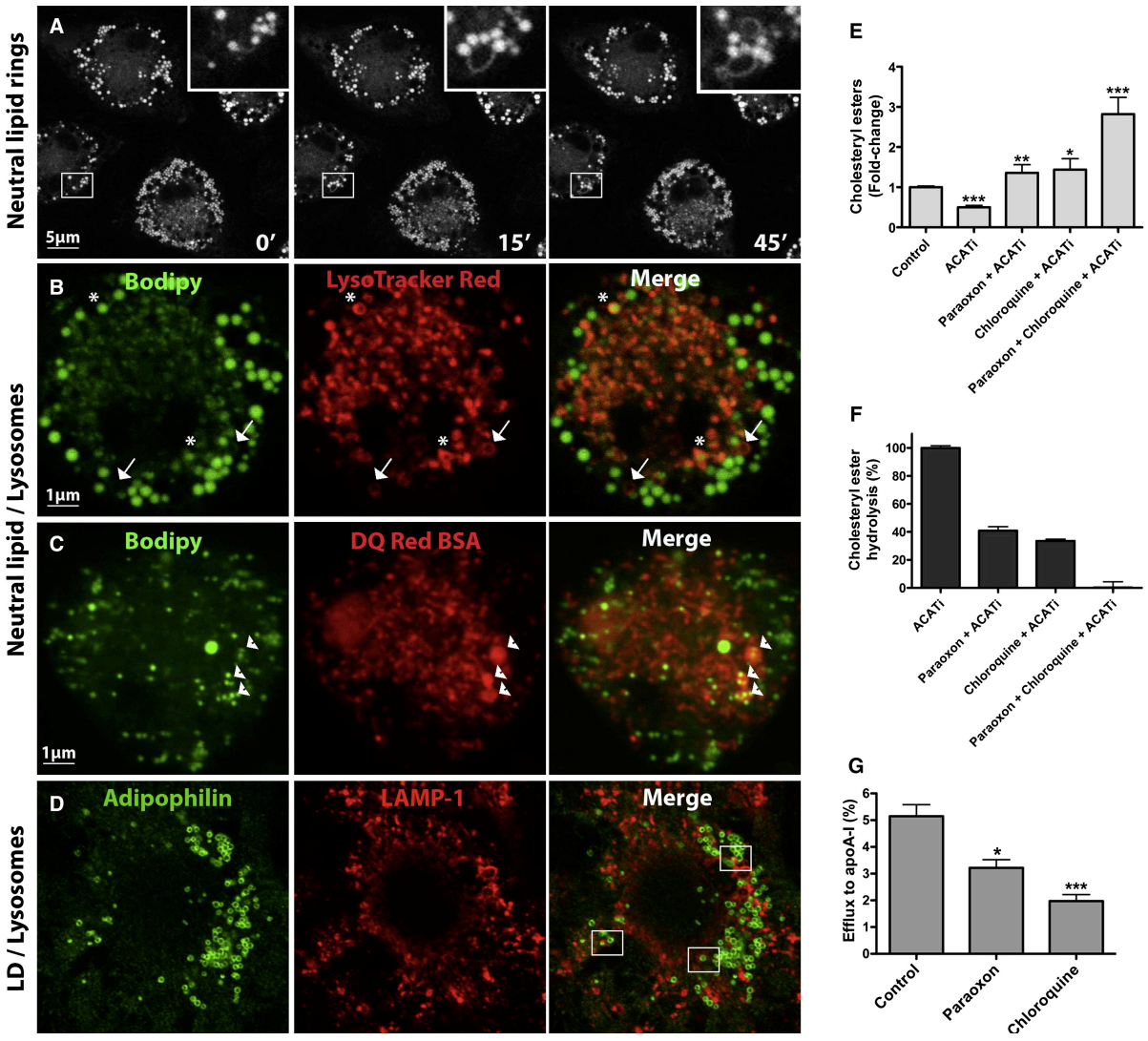

A
ApoB
LAMP-1
Merge
Control
Chloroquine
Normal loading
B
Adipophilin
Nile Red
Merge
Normal loading
C
Adipophlin
Nile Red
Merge
AcLDL
AcLDL+ACATi
D
Cholesteryl esters
1500
1000
500
0
CPM/µg cell protein
µg/mg cell protein
P<0.01
P<0.0001
Ac
Ac+ACATi
Ac
Ac+ACATi
E
Cholesteryl esters (µg/mg cell protein)
250
200
150
100
50
0
P<0.001
Time 0
apoA-I

Figure 3.2: LDs surround neutral lipid rings and co-localize with lysosomes; inhibition of lysosomal function reduces CE hydrolysis and cholesterol efflux. BMDMs were loaded with AcLDL-derived cholesterol 30h, equilibrated overnight in BSA media, and then incubated with media containing Bodipy (10μg/mL) with or without LysoTracker Red (50nM) for 30min prior to visualization (A and B). Cells were cholesterol-loaded as above (A and B), and then incubated with a BSA conjugate prior to labeling with Bodipy (C). Co-localization between the macrophage LD coat protein adipophilin and LAMP-1-positive lysosomes in AcLDL-loaded macrophages (D). Cellular CE (E) and CE hydrolysis (F) were measured in AcLDL-loaded cells treated with paraoxon or chloroquine for 24h in the presence of apoA-I, with or without ACATi. Variations in CE are expressed as fold-change relative to control (E) or as a percent CE hydrolysed in 24h (F). $P<0.0001$ (***), <0.001 (**) or $P<0.005$ (*) compared to ACATi, and ACATi was compared to control. BMDMs were loaded with ^{3}H-cholesterol-AcLDL for 30h, equilibrated overnight, and efflux to apoA-I was measured 24h in the presence or absence of paraoxon or chloroquine (G).


Neutral lipid rings
A
5µm
0′
15′
45′
Neutral lipid / Lysosomes
B
Bodipy
LysoTracker Red
Merge
1µm
C
Bodipy
DQ Red BSA
Merge
1µm
LD / Lysosomes
D
Adipophilin
LAMP-1
Merge
E
Cholesteryl esters (Fold-change)
Control
ACATi
Paraoxon + ACATi
Chloroquine + ACATi
Paraoxon + Chloroquine + ACATi
F
Cholesteryl ester hydrolysis (%)
ACATi
Paraoxon + ACATi
Chloroquine + ACATi
Paraoxon + Chloroquine + ACATi
G
Efflux to apoA-I (%)
Control
Paraoxon
Chloroquine

Recently, it was suggested that LDs could undergo transient heterotypic fusion events with bilayered organelles, whereby lipids transferred from the LDs to the space between the leaflets of the phospholipid bilayer surrounding the recipient organelle could subsequently be hydrolyzed and incorporated into the recipient organelle.[35] To investigate whether such an event was occurring in our cells, LDs were observed in the presence of LysoTracker Red, which stains acidic organelles. Interestingly, the neutral lipid rings that seem to originate from LDs overlapped with acidic rings (arrows, **Figure 3.2B**) and even more intriguing, some LDs were entirely surrounded by acidic rings (asterisks, **Figure 3.2B**), suggesting that they had fused with acidic organelles. To determine whether the acidic organelle within which LDs could be observed was the lysosome, we incubated the cells with DQ Red BSA, which selectively fluoresces in the degradative environment of the lysosome. Indeed, LDs were observed within lysosomes (arrowheads, **Figure 3.2C**). The proportion of LD neutral lipids within lysosomes was 15±5%. Because of the co-localization between the macrophage LD coat protein adipophilin and lysosomal-associated membrane protein 1 (LAMP-1)-positive lysosomes (**Figure 3.2D**), we concluded that the neutral lipid observed within lysosomes was LD-associated CE, and not simply CE from undigested AcLDL. The frequency at which adipophilin was found within lysosomes (9±3%) was similar although slightly lower than that observed for neutral lipids. Together, these observations led us to postulate that lysosomes may contribute to LD-associated CE hydrolysis in macrophage foam cells.

While the importance of mobilizing cholesterol from LDs is acknowledged, it remains a poorly understood process. Many candidate neutral CE hydrolases have been identified, none of which has been unambiguously shown to be responsible for all CE hydrolytic activity in macrophages; individual contributions of the various CE hydrolases have yet to be defined.[4] Paraoxon or E600, which binds irreversibly to the active site of lipases such as carboxylesterases,[36] is commonly used as a general lipase inhibitor.[37] To determine the importance of neutral and potentially acid hydrolases in macrophage CE hydrolysis, we added paraoxon (to inhibit neutral CE hydrolysis) or chlororoquine, a lysosomotropic agent that interferes with vesicular acidification (to inhibit lysosomal CE hydrolysis), during incubation of lipid-loaded macrophages with lipid-poor apoA-I. Because

macrophage CE undergoes a continual cycle of hydrolysis and re-esterification,[38] we included an ACAT inhibitor during this period to prevent the re-esterification of hydrolyzed CE. We found that macrophage CE mass increased in both chloroquine- and paraoxon-treated cells, their compound effect being cumulative (**Figure 3.2E**). The independent inhibition of neutral and lysosomal CE hydrolysis resulted in a substantial decrease in CE hydrolysis, whereas inhibition of both pathways abolished CE hydrolysis entirely (**Figure 3.2F**).

To evaluate whether lysosomal LD-associated CE hydrolysis is implicated in mobilization of cholesterol for efflux, macrophages were loaded with AcLDL containing ^{3}H-cholesterol, and efflux of the labeled cholesterol to lipid-poor apoA-I was measured. AcLDL-derived ^{3}H-cholesterol is esterified in the ER and accumulates in LDs (**Figure 3.1**). The movement of ^{3}H-cholesterol from lipid-loaded macrophages to apoA-I is unidirectional and is an accurate parameter of the flux of cholesterol mass out of the cell (**Figure 3.1** and [39]). Paraoxon diminished but did not abolish cholesterol efflux (**Figure 3.2G**), implying that an additional mechanism for cholesterol mobilization for efflux in lipid-loaded macrophages exists. In agreement with a role for lysosomal hydrolysis in regulating cytoplasmic CE, the addition of chloroquine also resulted in decreased cholesterol efflux (**Figure 3.2G**). Together, these results point to a role for both neutral and acidic CE hydrolysis in mobilizing cholesterol from cytoplasmic CE stores.

Autophagic flux modulates foam cell lipolysis

Having established a role for lysosomal function in LD CE hydrolysis and efflux, how LDs became localized within lysosomes was an enigma, particularly since complete fusion between monolayered and bilayered organelles is not possible. A timely report by Singh and colleagues,[10] documenting the sequestration of LDs by the autophagic machinery and their subsequent delivery to lysosomes in triglyceride-enriched hepatocytes provided a plausible explanation for our own observations in CE-enriched macrophages. To assess whether autophagy was involved in LD CE processing in macrophage foam cells, we first searched for evidence of autophagic vacuoles in lipid-loaded macrophages and, if present, whether these were associated with LDs.

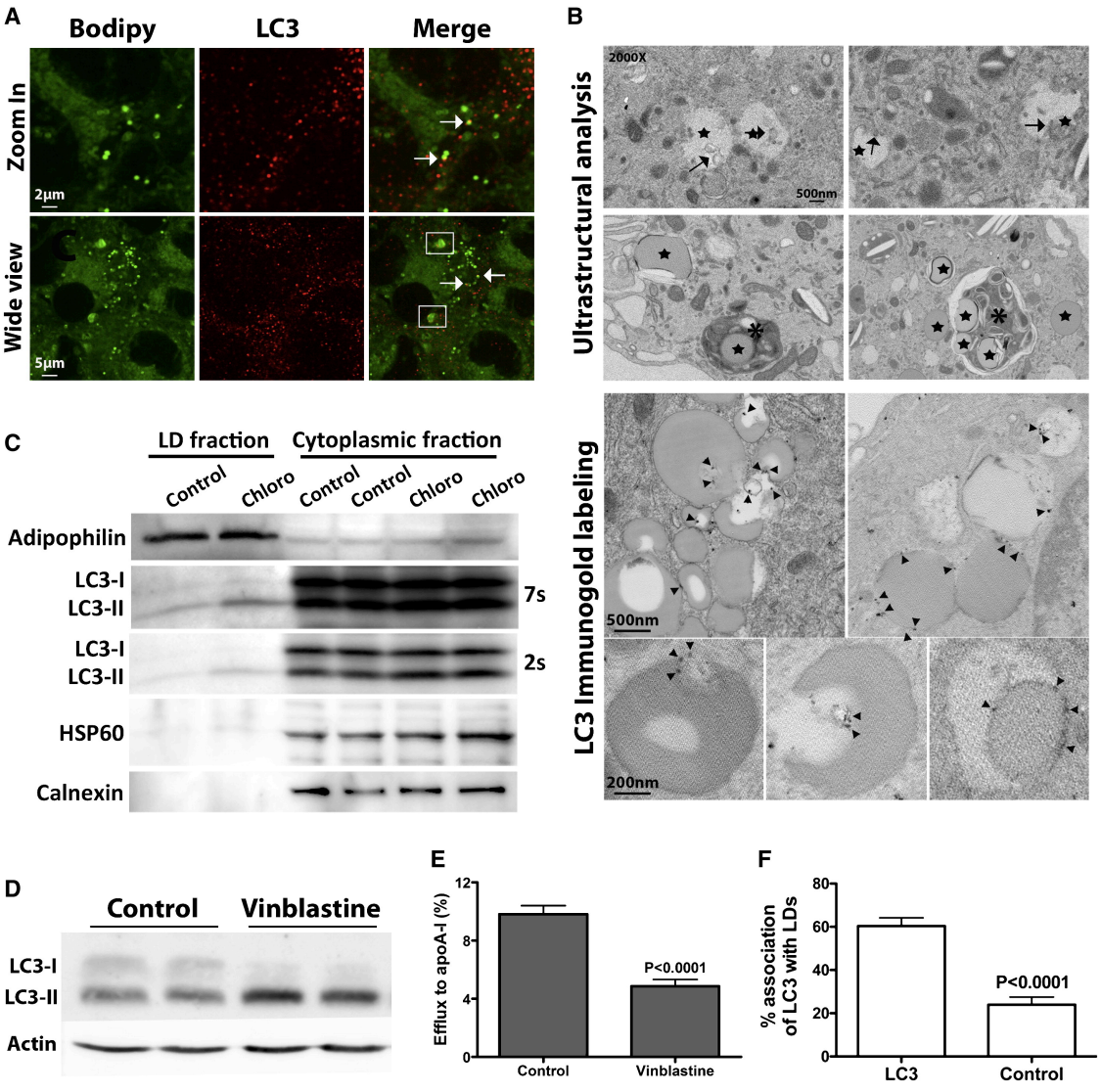
The LC3 conjugation system is essential for autophagy.[8] Cytosolic LC3 (LC3-I) is modified to its membrane-bound form (LC3-II), which is located on preautophagosomes and autophagosomes and thus commonly used as an autophagosomal marker.[40] A direct association between LDs and LC3 was observed by immunofluorescence (arrows, **Figure 3.3A**). In some instances, multiple LC3 puncta were observed on Bodipy-positive structures larger than the LDs (boxed, **Figure 3.3A**) that could represent lysosomes or multivesicular bodies containing significant amounts of neutral lipids (perhaps multiple LDs). Previously described morphological criteria[10] were used to further define LD sequestration by autophagic vesicles by electron microscopy. LDs (stars, **Figure 3.3B**) were easily recognized as circular electron-transparent organelles that were not surrounded by the double membrane characteristic of other organelles. Electron microscopy of lipid-loaded macrophages revealed double-membrane vesicles analogous to autophagosomes in and around LDs (arrows, **Figure 3.3B**) and degradative structures enriched in LDs (asterisks, **Figure 3.3B**). The autophagosomal nature of the double membrane vesicles at the LD periphery was confirmed by LC3 immunogold label (open arrow heads, Figure 2B). Blind counts revealed that 40% of LDs were associated with LC3 (**Figure 3.3F**).

Another line of evidence for the association of autophagosomes and LDs was the occurrence of LC3-II protein in the LD fractions of foam cells (**Figure 3.3C**). Chloroquine treatment inhibits LC3 degradation, and thus there was increased LC3-II in both the cytoplasmic and LD pools – best seen in 2s and 7s exposures, respectively. Finally, we evaluated whether a pharmacological modulator of autophagy alters cholesterol efflux. Vinblastine, which inhibits the fusion between autophagosomes and lysosomes, leads to the accumulation of autophagosomes and their associated protein marker, LC3-II (**Figure 3.3D**). Inhibition of autophagy by vinblastine in lipid-loaded macrophages reduced cholesterol efflux (**Figure 3.3E**). Collectively, these results support a role for autophagy in the degradation of cytoplasmic LDs.

Activation of the autophagic machinery in response to lipid loading

Because all cytoplasmic CE hydrolysis has previously been attributed to the action of extra-lysosomal, neutral CE hydrolases,[38,41] we were compelled to investigate whether the

Figure 3.3: **Autophagy is implicated in cytoplasmic LD degradation.** In lipid-loaded BMDMs, direct association of autophagomes with LDs is observed by immunofluorescence (A), electron microscopy (B) and in an isolated LD fraction (C). Vinblastine treatment inhibits autophagosome degradation, as shown by elevated LC3-II (D). Inhibition of autophagy by vinblastine treatment during cholesterol efflux decreases efflux to apoA-I (E). Quantification of LDs containing gold particles (cells immunostained with LC3 are compared to the secondary antibody alone negative control) (F).


A
Bodipy
LC3
Merge
Zoom In
Wide view
2μm
5μm
B
Ultrastructural analysis
2000X
500nm
LC3 Immunogold labeling
500nm
200nm
C
LD fraction
Cytoplasmic fraction
Control
Chloro
Control
Control
Chloro
Chloro
Adipophilin
LC3-I
LC3-II
7s
LC3-I
LC3-II
2s
HSP60
Calnexin
D
Control
Vinblastine
LC3-I
LC3-II
Actin
E
Efflux to apoA-I (%)
12
8
4
0
P<0.0001
Control
Vinblastine
F
% association of LC3 with LDs
80
60
40
20
0
P<0.0001
LC3
Control

autophagy-dependent mobilization of cholesterol for efflux observed in our foam cell model was specific to lipid-loaded cells and sensitive to the length of treatment. We found chloroquine to be effective in the attenuation of cholesterol efflux from lipid-loaded cells, but not in LDL-treated, unloaded macrophages (**Figure 3.4A**). Chloroquine treatment had no significant effect on AcLDL-loaded cells when included during a 4h cholesterol efflux experiment, as previously reported,[38] but a significant inhibition could be seen at 18h (**Figure 3.4B**). Thus, lysosomal cytosolic CE hydrolysis is required for cholesterol efflux in lipid-loaded cells; LD-associated CE hydrolysis in macrophage foam cells is not exclusively dependent upon neutral CE hydrolases.

To investigate whether autophagy is specifically induced in macrophage foam cells, we probed protein samples for LC3, and found LC3-II to be elevated in response to lipid loading (**Figure 3.4C**). To ensure that the rise in LC3-II was not simply due to defective autophagosome clearance resulting in cellular autophagosome accumulation, chloroquine was added to the media 2h prior to protein isolation. A further increase in LC3-II protein upon lysosomal inhibition was observed, indicative of a functional autophagic flux in these cells, as cellular LC3 levels per se are not a good marker for autophagy.[42] Thus, autophagy is expressly triggered in macrophages in response to an expanded cytoplasmic cholesterol pool.
Lysosomal acid lipase (LAL) is the enzyme that hydrolyzes neutral lipids delivered to lysosomes by receptor-mediated endocytosis.[43] Because the lysosome population within a cell is comprised of a heterogeneous pool of lysosomal subgroups with distinct roles in autophagy,[44] and since it has been proposed that autophagy-competent lysosomes could utilize Atg15, an Atg (autophagy-related genes) family member predicted to hydrolyze neutral lipids,[5] we next sought to determine which acid lipase is implicated in autophagy-mediated LD CE hydrolysis. We used a potent and specific LAL inhibitor, compound 13 or 3a2,[45,46] herein referred to as Lalistat 1, to determine whether LAL is implicated in LD breakdown in our foam cell model. As previously shown,[46] the administration of Lalistat 1 during AcLDL loading prevents LD formation because Lalistat 1 effectively blocks LAL-mediated AcLDL lipid hydrolysis (**Figure 3.5**).

The addition of Lalistat 1 to lipid-loaded cells during cholesterol efflux reduced efflux and resulted in a concomitant rise in cellular CE mass (**Figures 3.4D and 3.4E**). To

Figure 3.4: Autophagy is induced in response to lipid loading and LAL mediates LD catabolism. Chloroquine inhibits cholesterol efflux in macrophage foam cells, but not in unloaded cells (A). Chloroquine has no effect on cholesterol efflux at an early time point, but impairs cholesterol efflux upon prolonged inhibition of lysosomal function (B). Autophagic flux is induced in lipid-loaded macrophages (C). Effect of lysosomal acid lipase (LAL) inhibition on cholesterol efflux (D) and cellular CE mass (E) in unloaded versus lipid-loaded macrophages.

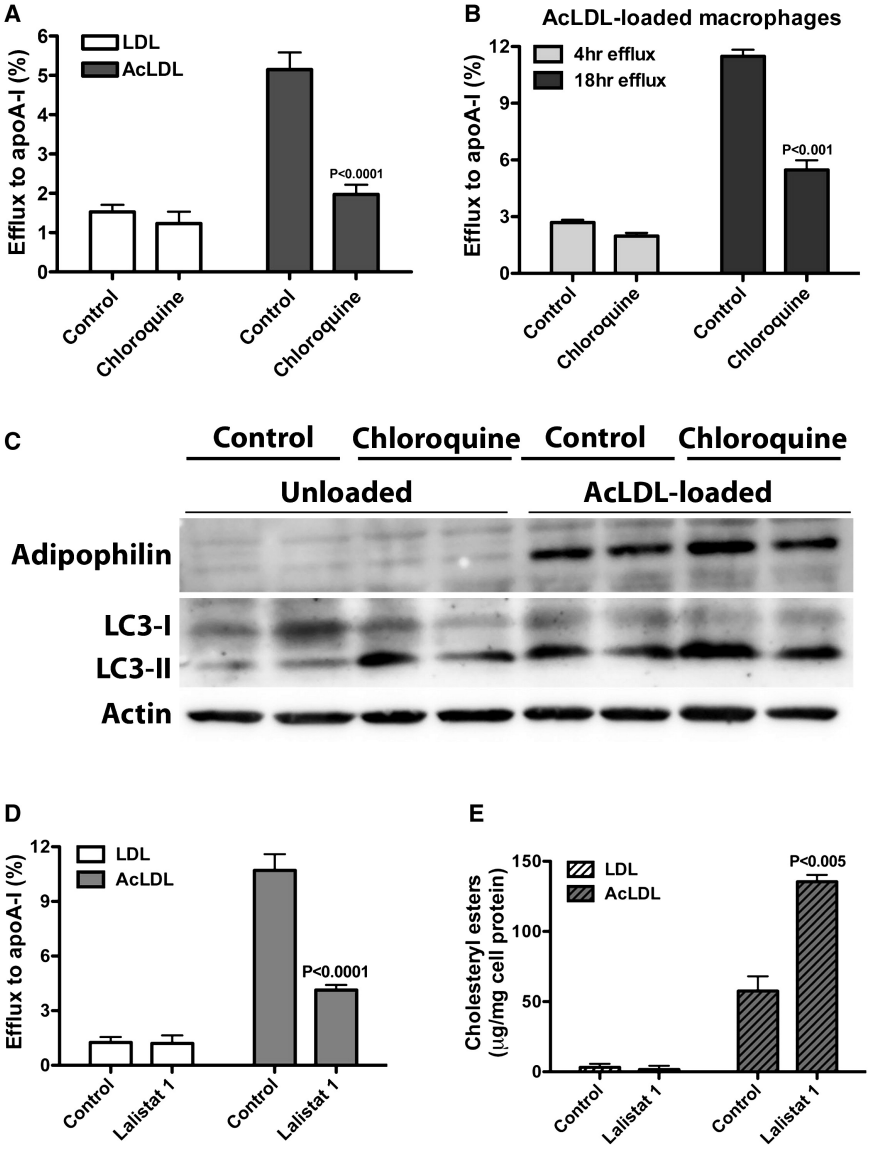
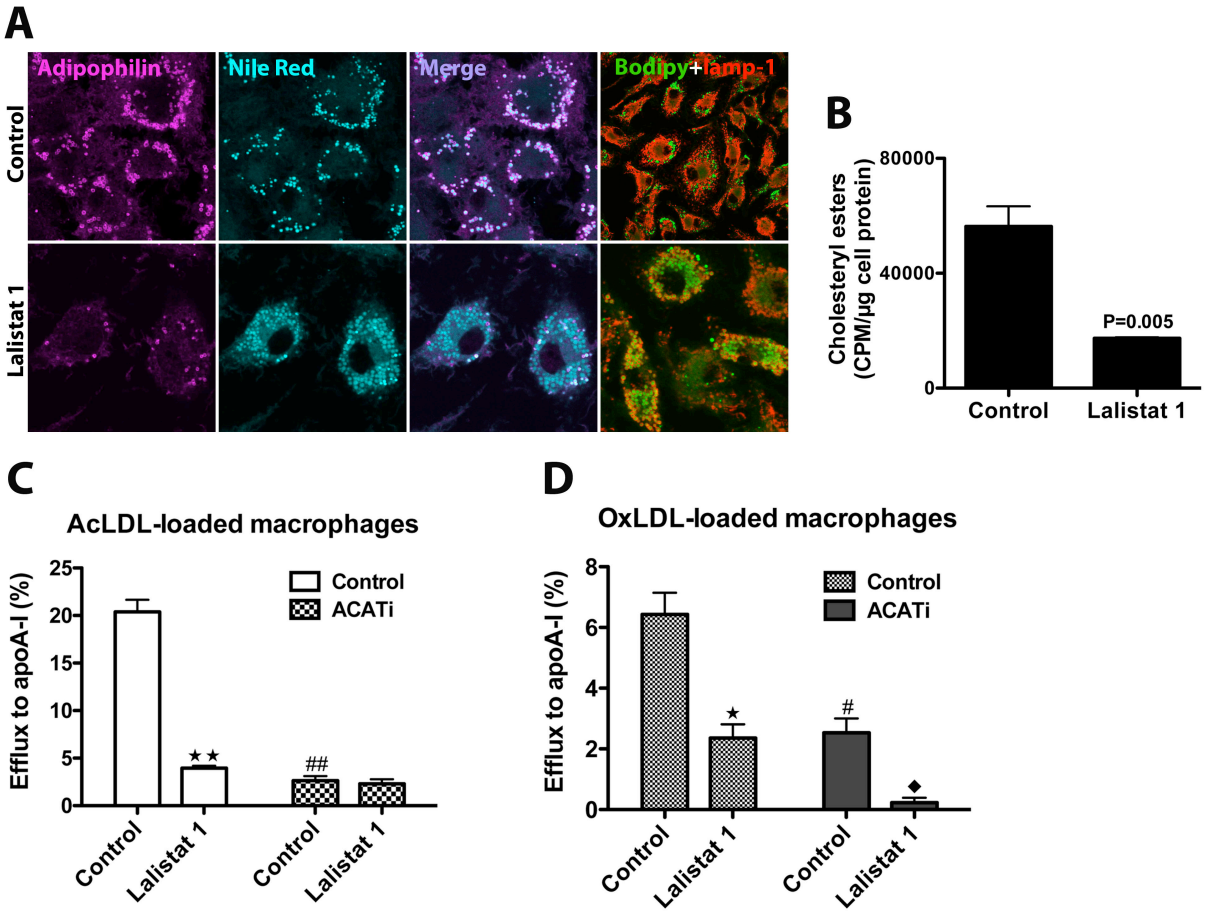

A
LDL
AcLDL
Efflux to apoA-I (%)
P<0.0001
Control
Chloroquine
Control
Chloroquine
B
AcLDL-loaded macrophages
4hr efflux
18hr efflux
Efflux to apoA-I (%)
P<0.001
Control
Chloroquine
Control
Chloroquine
C
Control
Chloroquine
Control
Chloroquine
Unloaded
AcLDL-loaded
Adipophilin
LC3-I
LC3-II
Actin
D
LDL
AcLDL
Efflux to apoA-I (%)
P<0.0001
Control
Lalistat 1
Control
Lalistat 1
E
LDL
AcLDL
Cholesteryl esters (μg/mg cell protein)
P<0.005
Control
Lalistat 1
Control
Lalistat 1

Figure 3.5: **The addition of Lalistat 1 during AcLDL loading prevents LD formation whereas Lalistat 1 treatment in AcLDL-loaded cells decreases LD cholesterol efflux.** Reduced LDs in cells loaded with AcLDL in the presence of the Lalistat 1 (A). Macrophages were loaded with AcLDL in the presence or absence of 10μM Lalistat 1 for 24h, after which cells were fixed, permeabilized and stained for microscopy. In the absence of Lalistat 1, AcLDL-associated CE is hydrolyzed by LAL and excess cholesterol is esterified and accumulates in LDs (all neutral lipid inclusions are surrounded by the LD coat protein adipophilin, whereas there is no overlap between neutral lipids and the lysosomal marker LAMP-1). In the presence of Lalistat 1, LAL is inhibited and AcLDL-associated CE is not hydrolyzed (neutral lipids accumulate in lysosomes, as evidenced by the extensive co-localization of neutral lipids and LAMP-1-positive lysosomes, whereas there are scarce LDs) (A). Reduced incorporation of ^{3}H-Oleate into CE in cells loaded with AcLDL in the presence of Lalistat 1 (B). Here, macrophages were loaded with AcLDL in the presence or absence of 10μCi/mL of ^{3}H-Oleic acid and 10μM Lalistat 1 for 30h, after which total lipids were extracted, separated by TLC and CE was quantified. LAL inhibition reduces LD cholesterol efflux in AcLDL-loaded cells (C), whereas it reduces both LD and lysosomal cholesterol efflux in OxLDL-loaded cells (D). Macrophages were loaded AcLDL or OxLDL, after which efflux to apoA-I was measured for 24h, in the presence or absence of 10μM Lalistat 1 and / or ACATi (note that for ACATi-treated cells, the ACATi was present during the entire experiment). $P<0.05$ (★) or <0.001 (★★) for Lalistat 1-treated cells as compared to control, $P<0.01$ (#) or <0.001 (##) for ACATi-treated cells as compared to control, or $P<0.05$ (♦) for ACATi- and Lalistat 1-treated cells as compared to ACATi-treated cells.


A
Adipophilin
Nile Red
Merge
Bodipy+lamp-1
Control
Lalistat 1
B
Cholesteryl esters (CPM/µg cell protein)
80000
40000
0
P=0.005
Control
Lalistat 1
C
AcLDL-loaded macrophages
Efflux to apoA-I (%)
25
20
15
10
5
0
Control
ACATi
★★
##
Control
Lalistat 1
Control
Lalistat 1
D
OxLDL-loaded macrophages
Efflux to apoA-I (%)
8
6
4
2
0
Control
ACATi
★
#
◆
Control
Lalistat 1
Control
Lalistat 1

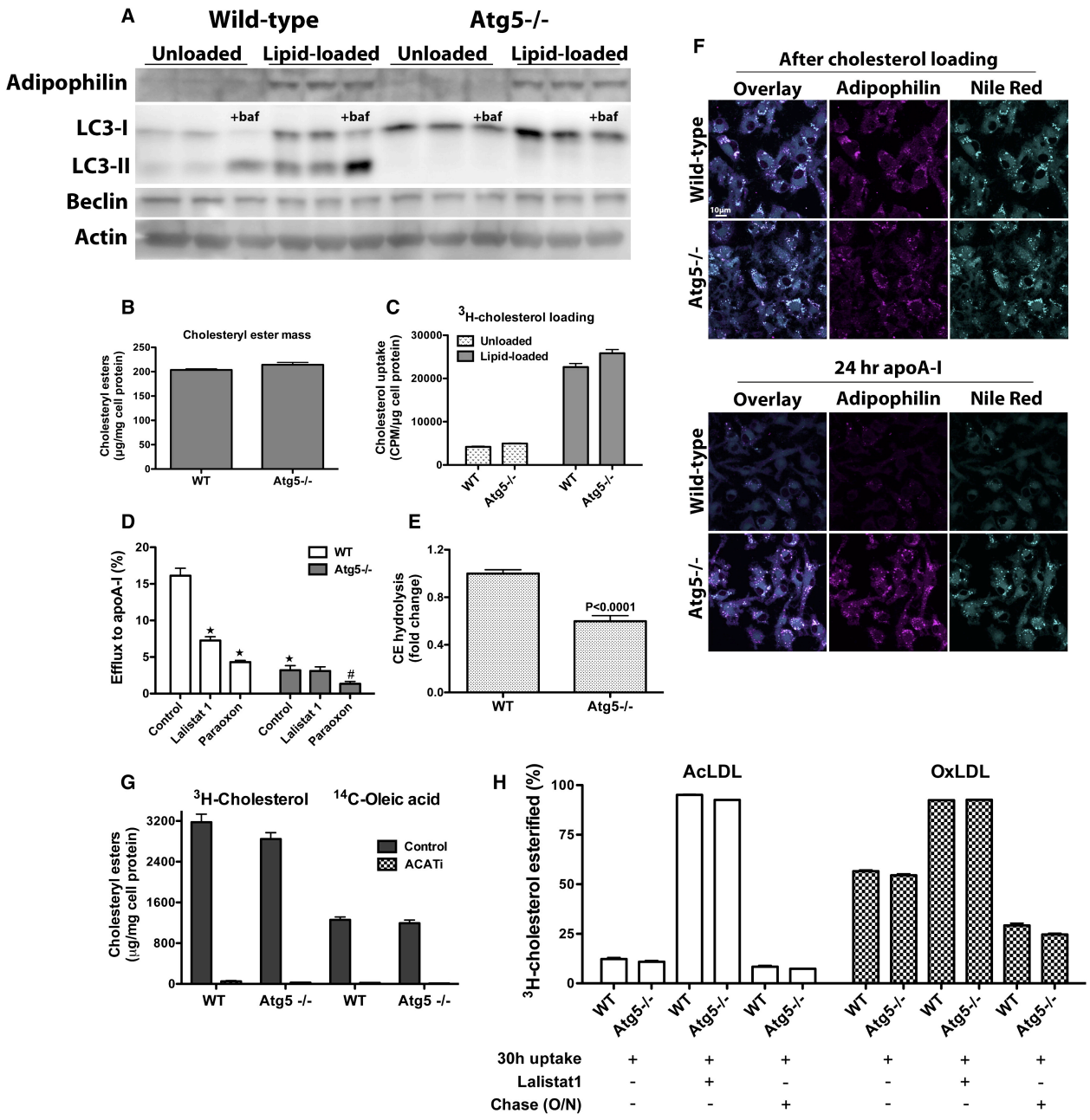
ensure that the Lalistat 1-inhibitable efflux from AcLDL-loaded macrophages indeed represents LD-associated cholesterol, and not cholesterol associated with AcLDL trapped in lysosomes, we used an ACAT inhibitor to prevent LD formation and measured efflux in the presence of Lalistat 1. We found that in the absence of LDs, inhibition of LAL had no effect on cholesterol efflux, indicating that the Lalistat 1-inhibitable efflux in AcLDL-loaded macrophages is LD cholesterol (**Figure 3.5**). Together, these data indicate that autophagy is selectively induced in lipid-loaded cells and mediates the delivery of cytoplasmic LDs to lysosomes, where LD-associated CE is hydrolyzed by LAL to liberate cholesterol for efflux.

Impaired LD catabolism in *Atg5-/-* macrophage foam cells

Next, we sought to measure cholesterol efflux in macrophages isolated from *Atg5-/-* mice as compared to wild-type (WT) controls. In cells lacking Atg5, the modification of LC3-I to LC3-II is impaired[47] – corroborated here by the complete absence of LC3-II in these cells (**Figure 3.6A**) - and autophagy cannot ensue. Again, autophagosome formation was induced in macrophages in response to lipid loading (**Figure 3.6A**). Autophagosomes are initiated by repression of the mammalian target of rapamycin (mTOR) or by the class III phosphoinositide 3-kinase (PI3K) / Beclin-1 complex. We have thus far failed to observe changes in Beclin-1 protein levels in lipid-loaded macrophages (**Figure 3.6A**), making mTOR a likely candidate for modulating autophagy in foam cells, although an alternative pathway independent of mTOR and class III PI3K/Beclin-1 for triggering of autophagy has also been described.[48]

Given that autophagy is required for LD genesis and lipid storage in adipose tissue,[49-51] along with additional findings that the Atg conjugation system is involved in LD formation in hepatocytes and cardiomyocytes,[52] we assessed the ability of autophagy-defective macrophages to accumulate neutral lipids in LDs when exposed to AcLDL. Neutral lipids in AcLDL-loaded *Atg5-/-* macrophages effectively accumulated in LDs, since all cytoplasmic lipid inclusions were surrounded by adipophilin (**Figure 3.6F**, after cholesterol loading). Thus, LD formation in lipid-loaded *Atg5-/-* macrophages occurs normally. Moreover, the extent of cholesterol loading was comparable in WT and *Atg5-/-* macrophages (**Figure 3.6B and 3.6C**). Importantly, cholesterol efflux from lipid-loaded

Figure 3.6: **Ablation of autophagy impairs LD delivery to lysosomes for LAL-mediated CE hydrolysis in macrophage foam cells.** *Atg5* deletion in macrophages impairs LC3-I maturation to LC3-II and autophagy cannot ensue (A). Cholesterol loading is similar in WT and *Atg5-/-* macrophages, as recorded by mass measurements (B) and ^{3}H-cholesterol uptake (C). Cholesterol efflux to apoA-I is impaired in *Atg5-/-* lipid-loaded macrophages; LAL inhibition reduces cholesterol efflux in WT, but not *Atg5-/-* macrophages (D). $P<0.0001$ (★) relative to WT control, and $P<0.05$ (#) relative to *Atg5-/-* control. CE hydrolysis in lipid-loaded WT and *Atg5-/-* macrophages over 24h in the presence of apoA-I, expressed as a fold-change relative to the WT control (cellular CE mass was measured before and after cholesterol efflux, and resulting % hydrolysis was normalized to that of WT) (E). Autophagy-deficient macrophages exhibit impaired LD catabolism (F). Lipid-loaded WT or *Atg5-/-* macrophages were immediately fixed for fluorescence microscopy after AcLDL loading or after a 24h incubation with media containing lipid-poor apoA-I in the presence of an ACAT inhibitor, and neutral lipids were stained with Nile Red. AcLDL-derived ^{3}H-cholesterol is esterified to ^{14}C-oleic acid to the same extent in WT and *Atg5-/-* macrophages (G). In the absence of the ACATi, esterification of the ^{3}H and ^{14}C labels parallel each other and is equivalent in WT and *Atg5-/-* cells. When ACATi inhibits ACAT, esterification of both the ^{3}H and ^{14}C labels is abolished. Degradation of lipoprotein ^{3}H-cholesteryl oleate occurs at the same rate in WT and *Atg5-/-* macrophages (H). Macrophages were loaded with AcLDL or OxLDL containing ^{3}H-cholesteryl oleate in the presence or absence of Lalistat 1 to inhibit LAL, followed by an O/N equilibration or not. All experiments were performed in the presence of the ACATi to prevent re-esterification of the liberated ^{3}H-cholesterol.


A
Wild-type
Atg5-/-
Unloaded
Lipid-loaded
Unloaded
Lipid-loaded
Adipophilin
LC3-I
LC3-II
Beclin
Actin
+baf
+baf
+baf
+baf
B
Cholesteryl ester mass
Cholesteryl esters (µg/mg cell protein)
WT
Atg5-/-
C
3H-cholesterol loading
Cholesterol uptake (CPM/µg cell protein)
Unloaded
Lipid-loaded
WT
Atg5-/-
WT
Atg5-/-
D
Efflux to apoA-I (%)
WT
Atg5-/-
Control
Lalistat 1
Paraoxon
Control
Lalistat 1
Paraoxon
E
CE hydrolysis (fold change)
P<0.0001
WT
Atg5-/-
F
After cholesterol loading
Overlay
Adipophilin
Nile Red
Wild-type
Atg5-/-
10µm
24 hr apoA-I
Overlay
Adipophilin
Nile Red
Wild-type
Atg5-/-
G
3H-Cholesterol
14C-Oleic acid
Cholesteryl esters (µg/mg cell protein)
Control
ACATi
WT
Atg5 -/-
WT
Atg5 -/-
H
AcLDL
OxLDL
3H-cholesterol esterified (%)
WT
Atg5-/-
30h uptake
Lalistat1
Chase (O/N)

Atg5-/- macrophages was significantly reduced as compared to WT macrophages (**Figure 3.6D**), likely due to decreased CE hydrolysis in *Atg5-/-* macrophages as compared to WT (**Figure 3.6E**).

Our hypothesis that autophagy plays a key role in the efflux of LD cholesterol was further corroborated by microscopic observations showing that accumulated cytoplasmic LDs dissipate after incubation with apoA-I in WT, but not in *Atg5-/-* macrophages (**Figure 3.6F**). Adipophilin, whose levels are directly correlated to cellular LD content, also visibly dissipates in WT cells after efflux, but not in *Atg5-/-* cells. The observation that Lalistat 1 treatment impairs cholesterol efflux to apoA-I in WT, but not *Atg5-/-* macrophages (**Figure 3.6D**), firmly establishes that autophagy is the means by which LDs are delivered to LAL for hydrolysis. The observation that paraoxon is effective at reducing efflux in both WT and *Atg5-/-* macrophages substantiates that CE hydrolysis by neutral CE hydrolases and autophagy are mutually exclusive pathways. Notably, inhibition of both pathways nearly abolishes all efflux to apoA-I.

To rule out that the CE accumulation observed in *Atg5-/-* macrophages is due to enhanced esterification of cholesterol or decreased lysosomal hydrolysis of lipoprotein CE in *Atg5-/-* cells compared to WT we directly measured both of these parameters in WT and *Atg5-/-* macrophages. We found that esterification of lipoprotein ^{3}H-cholesterol to ^{14}C-oleic acid did not differ between WT and *Atg5-/-* macrophages (**Figure 3.6G**). Consistent with the well established role for ACAT in mediating cholesterol esterification in the ER, the esterification of both radiolabels was abolished in the presence of the ACAT inhibitor.
Degradation of lipoprotein-associated ^{3}H-cholesteryl oleate was equivalent in WT and *Atg5-/-* macrophages (**Figure 3.6H**), indicating that the observed decrease in cholesterol efflux from *Atg5-/-* cells does not result from augmented lipoprotein CE retention in *Atg5-/-* cells as compared to WT. Most importantly, these results clearly demonstrate that AcLDL CE is processed very rapidly in lysosomes as compared to OxLDL CE. During this experiment, only 10% of AcLDL CE remained after a 30h incubation. This small amount of residual AcLDL CE minimally decreased during an O/N chase (to 7-8%) and is likely to represent a background amount that could not account for the relative decrease in cholesterol efflux observed in *Atg5-/-* cells.

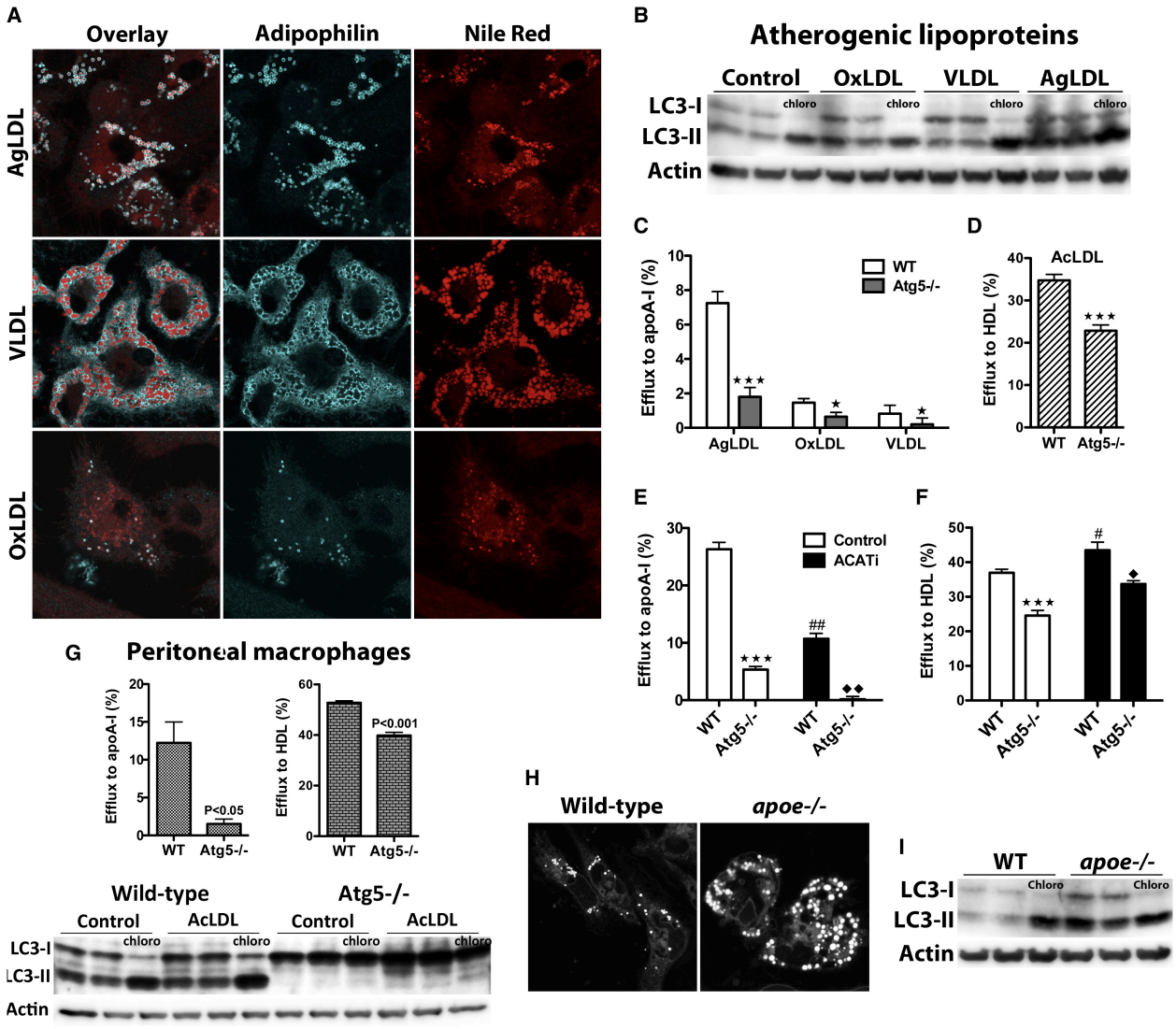
Autophagy is induced by atherogenic lipoproteins and by cholesterol loading *in vivo*

To further characterize the physiological relevance of this pathway, we tested whether autophagy was also induced in macrophages incubated with pathophysiological forms of modified LDL, such as OxLDL and aggregated LDL (AgLDL), and with very low density lipoprotein (VLDL). We found that, similarly to AcLDL, incubation of macrophages with AgLDL or VLDL resulted in the accumulation of cytoplasmic neutral lipids, whereas mildly OxLDL generated few LDs (consistent with its documented lysosomal retention) (**Figure 3.7A**). Importantly, all of these lipoproteins increased autophagic flux in macrophages (**Figure 3.7B**), and autophagy depletion in these cells resulted in diminished cholesterol efflux to apoA-I (**Figure 3.7C**).

Efflux assays to lipid-free apoA-I and HDL probe two distinct pathways of unidirectional and bidirectional cholesterol transfers, respectively.[53] Here, we assessed whether both of these efflux pathways were dependent on autophagy. Interestingly, whereas virtually all efflux to apoA-I was attributable to autophagy in AcLDL-loaded cells, approximately 30% of HDL-mediated efflux was dependent on this process (**Figure 3.7D**). It should be noted that the efflux rates measured here are similar to those in the literature, where efflux to HDL is greater than that to apoA-I.[54,55] To assess the proportion of autophagy-mediated efflux that derives from LD cholesterol in cells incubated with apoA-I or HDL, we performed efflux experiments in the presence of an ACAT inhibitor. ACATi was included for the entire duration of these experiments, preventing cholesterol esterification and LD biogenesis (**Figure 3.1**), which results in endosomal and lysosomal cholesterol accumulation.[56] Unexpectedly, the complete absence of LDs impaired efflux to apoA-I quite significantly (60% decrease) whereas efflux to HDL was slightly increased (**Figure 3.7E and 3.7F**), indicating that the major source of cholesterol for apoA-I-mediated efflux is the LD. Additionally, we found that lack of autophagy reduced cholesterol efflux even in the absence of LDs, suggesting a role for this pathway in enhancing efflux of lysosomal cholesterol independently of its ability to transfer LD CE to these organelles for LAL-mediated hydrolysis.

We next assessed whether our findings also apply to peritoneal macrophages. Similar to bone marrow-derived macrophages, autophagy was induced in peritoneal macrophages

Figure 3.7: **Enhanced autophagy in response to various atherogenic lipoproteins and in peritoneal macrophages from hypercholesterolemic mice.** LD genesis in bone marrow-derived macrophages exposed to AgLDL, VLDL and OxLDL (A). Elevated autophagic flux in response to atherogenic lipoproteins (B). Impaired efflux to apoA-I in *atg5-/-* macrophages loaded with AgLDL, VLDL, and OxLDL (C). Atg5 ablation reduces cholesterol efflux to HDL in AcLDL-loaded macrophages (D). Efflux to apoA-I (E) and HDL (F) in WT and *Atg5-/-* macrophages with (control) or without (ACATi) cytoplasmic LDs. P<0.0001 (★★★) or <0.05 (★) relative to WT control, and P<0.001 (##) or <0.05 (#) for ACATi-treated WT cells relative to WT control, and P<0.001 (♦♦) or <0.05 (♦) for ACATi-treated *Atg5-/-* cells relative to ACATi-treated WT cells (C-F). Autophagy mediates cholesterol efflux in peritoneal macrophages (G). Peritoneal macrophages isolated from hypercholesterolemic *apoe-/-* mice contain more LDs than their WT counterparts (H). Autophagy levels are elevated in peritoneal macrophages lipid-loaded *in vivo* (I).


A
Overlay
Adipophilin
Nile Red
AgLDL
VLDL
OxLDL
B
Atherogenic lipoproteins
Control
OxLDL
VLDL
AgLDL
LC3-I
LC3-II
Actin
chloro
C
Efflux to apoA-I (%)
WT
Atg5-/-
AgLDL
OxLDL
VLDL
D
AcLDL
Efflux to HDL (%)
WT
Atg5-/-
E
Efflux to apoA-I (%)
Control
ACATi
F
Efflux to HDL (%)
G
Peritoneal macrophages
Efflux to apoA-I (%)
P<0.05
Efflux to HDL (%)
P<0.001
Wild-type
Atg5-/-
Control
AcLDL
H
Wild-type
apoe-/-
I
WT
apoe-/-
Chloro

upon AcLDL loading and *Atg5* deficiency reduced cholesterol efflux to apoA-I and HDL in these cells (**Figure 3.7G**). Moreover, we found that autophagy was induced in peritoneal macrophages isolated from hypercholesterolemic *apoe-/-* mice as compared to WT cells (**Figure 3.7I**). Cellular LDs were more numerous in the cytoplasm of *apoe-/-* macrophages as compared to control mice (**Figure 3.7H**), confirming the *in vivo* loading of these macrophages. Thus, our findings in macrophages lipid-loaded *in vitro* appear generalizeable to *in vivo* macrophage loading conditions.

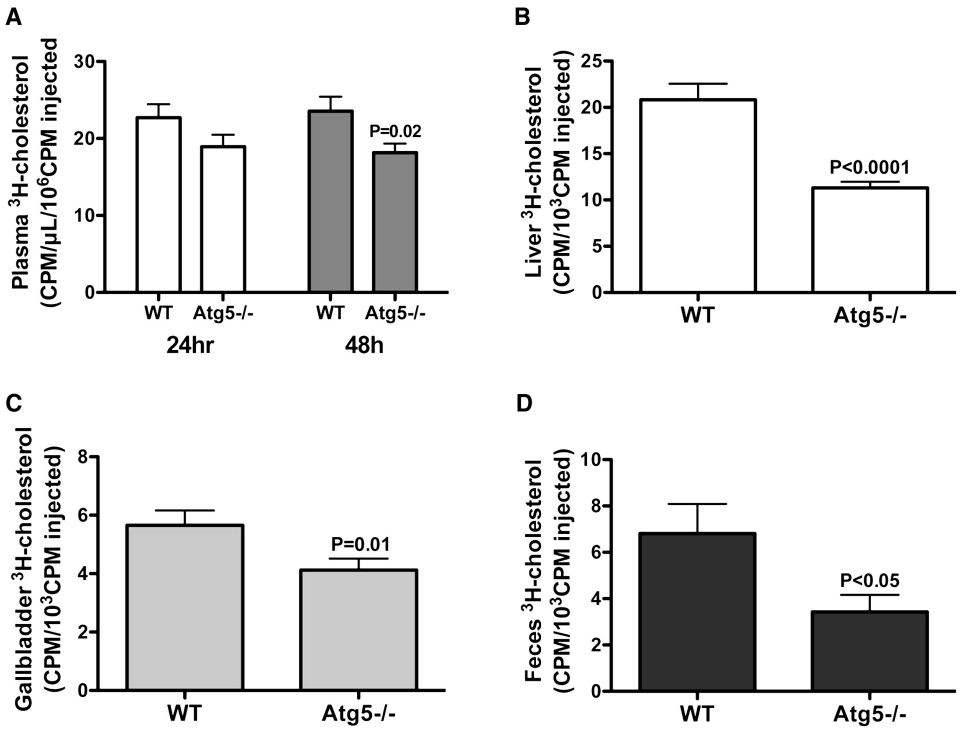
Lipophagy contributes to RCT *in vivo*

Because the mobilization of cholesterol from LDs is the first step in the RCT pathway, defined as the flux of cholesterol from macrophages in peripheral tissues to the liver for excretion, we reasoned that impaired efflux from autophagy-deficient macrophages would result in ineffective whole-body clearance of accumulated macrophage cholesterol. To measure RCT *in vivo*, we used a method developed to quantify ^{3}H-cholesterol movement from macrophages into plasma, liver, gallbladder and feces[22,55] over a two day period. Following the injection of WT or *Atg5-/-* macrophages loaded with AcLDL-derived ^{3}H-cholesterol into C57BL/6 mice, we found that the clearance of the macrophage ^{3}H-tracer was significantly reduced in mice receiving *Atg5-/-* macrophages as compared to WT macrophages (**Figure 3.8 A-D**). Thus, macrophage-specific impairment of autophagy is clearly detrimental to *in vivo* RCT, a process that plays a critical atheroprotective role.

3.7 Discussion

Here we demonstrate an important role for lysosomes in cytoplasmic CE hydrolysis in macrophage foam cells. Furthermore, we demonstrate that LDs are delivered to lysosomes via autophagy, where lysosomal acid lipase (LAL) acts to hydrolyze LD-associated CE to generate free cholesterol for efflux, a process that is specifically induced upon cholesterol loading in macrophages. Lysosomal lipolytic activity has been well studied, but it is only now realized that the source of lipids undergoing lipolysis in this compartment is not limited to extracellular lipoprotein-associated lipids reaching lysosomes via endocytosis but also extends to cytoplasmic LDs.[57] CE-enriched macrophage foam cells have been shown (puzzlingly) to divert some of their cytoplasmic neutral lipids to lysosomes for hydrolysis.[58] We corroborate

Figure 3.8: **Macrophage-specific deletion of *Atg5* reduces RCT *in vivo*.** (A) ^{3}H-tracer in plasma at 24h and 48h post injection (A). ^{3}H-tracer in livers at 48h post injection (B). ^{3}H-tracer in gallbladders at 48h post injection (C). ^{3}H-tracer in feces at 48h post injection (D).

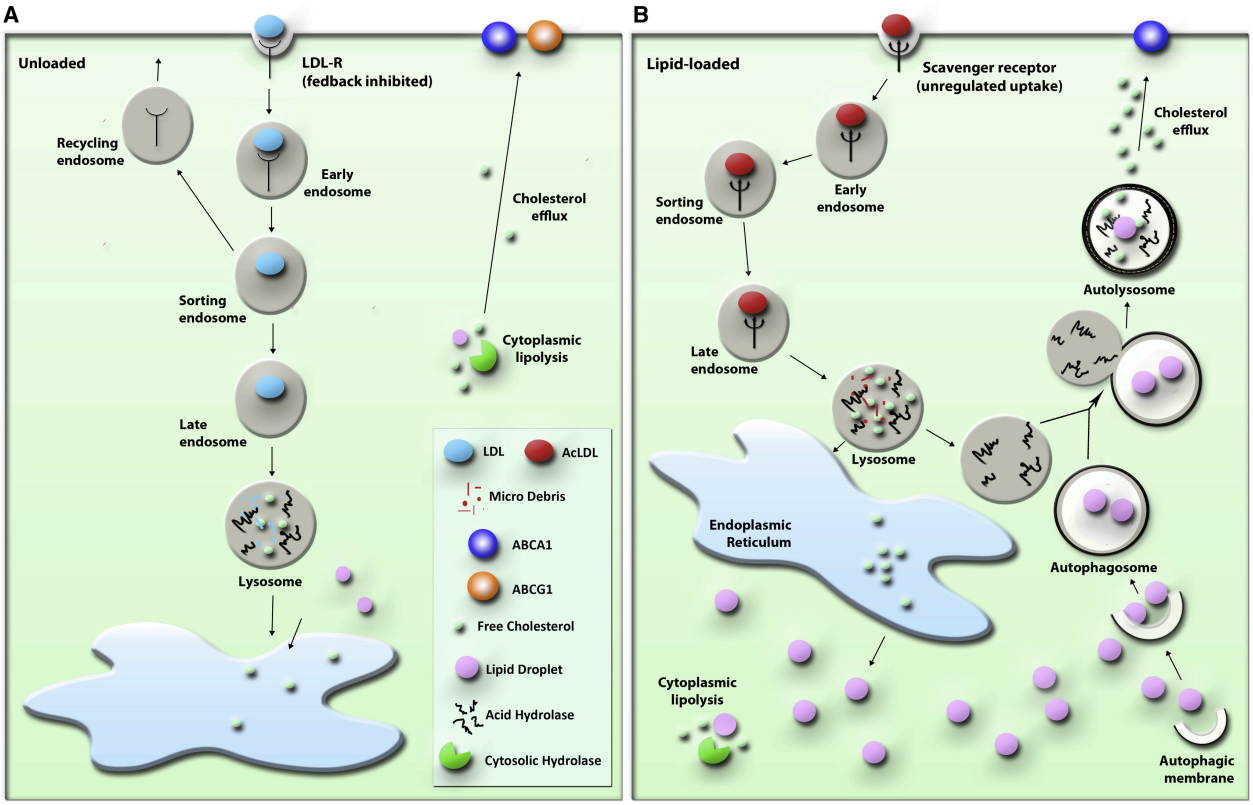

A
Plasma ^{3}H-cholesterol (CPM/μL/10^{6}CPM injected
30
20
10
0
P=0.02
WT
Atg5-/-
WT
Atg5-/-
24hr
48h
B
Liver ^{3}H-cholesterol (CPM/10^{3}CPM injected)
25
20
15
10
5
0
P<0.0001
WT
Atg5-/-
C
Gallbladder ^{3}H-cholesterol (CPM/10^{3}CPM injected)
8
6
4
2
0
P=0.01
WT
Atg5-/-
D
Feces ^{3}H-cholesterol (CPM/10^{3}CPM injected)
10
8
6
4
2
0
P<0.05
WT
Atg5-/-

and extend these earlier observations: sequestration of LDs by autophagosomes delivers LD CE to lysosomes where it undergoes LAL-mediated hydrolysis to generate free cholesterol for efflux (**Figure 3.9**).

There are many models for foam cell formation *in vitro*, such as incubation of macrophages with AcLDL (a model modified-LDL) or other atherogenic lipoproteins such as OxLDL, AgLDL and VLDL (all of which are formed and may accumulate in pathological conditions *in vivo*). The AcLDL model has been extensively characterized since the late 1970s[15,59] and the cytoplasmic accumulation of AcLDL-derived cholesterol as CE in LDs is well-documented.[30-34] Multiple pieces of evidence support the premise that LDs are processed in lysosomes in the AcLDL-loaded macrophages used in our study. Firstly, we could not detect any undigested AcLDL in lysosomes and all of the Bodipy- or Nile Red-stained neutral lipids were surrounded by the LD coat protein, adipophilin (**Figure 3.1**). Also, adipophilin itself was localized to lysosomes (**Figure 3.2**). Furthermore, using an ACAT inhibitor during AcLDL uptake to prevent cholesterol esterification and the formation of LDs (**Figure 3.1** confirms the lack of esterification and the lack of cytoplasmic LDs under these conditions), we found that the Lalistat 1-inhibitable efflux was abolished in the absence of LDs (**Figure 3.5**). In addition, there was diminished dissipation of adipophilin-coated neutral lipids after a 24h incubation of AcLDL-loaded macrophages with apoA-I in *Atg5-/-* macrophages relative to WT cells (**Figure 3.6F**). Moreover, efflux to apoA-I, which was nearly abolished in *Atg5-/-* macrophages (**Figure 3.7E**), was primarily dependent on LDs as a cholesterol source (**Figure 3.7E**, there is a 60% reduction in efflux to apoA-I in WT cells loaded with AcLDL in the presence of the ACATi as compared to WT cells loaded without ACATi in which LDs form). Finally, we show that AcLDL CE is processed very rapidly in lysosomes, and that lipoprotein-associated CE hydrolysis was equivalent in WT and *Atg5-/-* macrophages (**Figure 3.6H**). Thus, the decrease in cholesterol efflux from *Atg5-/-* cells could not result from enhanced lipoprotein CE retention in these cells as compared to WT. In any case, residual lysosomal CE after AcLDL loading is quite minimal and could not account for the decrease in efflux observed in *Atg5-/-* macrophages.

While cytosolic LDs are the site for CE accumulation in macrophages incubated with AcLDL, other modified lipoproteins (oxLDL, agLDL, enzymatically modified LDL)

Figure 3.9: Model of autophagy in LD-associated CE hydrolysis in macrophage foam cells. Cholesterol homeostasis in 'normal', unloaded macrophages; a model for CE hydrolysis that mainly involves the action of neutral CE hydrolases is presented (A). Autophagy is induced in early macrophage foam cells and delivery of LDs to lysosomes enhances LD-associated CE hydrolysis and cholesterol efflux (B).


A
Unloaded
LDL-R
(fedback inhibited)
Recycling
endosome
Early
endosome
Cholesterol
efflux
Sorting
endosome
Cytoplasmic
lipolysis
Late
endosome
LDL
AcLDL
Micro Debris
ABCA1
ABCG1
Free Cholesterol
Lipid Droplet
Acid Hydrolase
Cytosolic Hydrolase
Lysosome
B
Lipid-loaded
Scavenger receptor
(unregulated uptake)
Cholesterol
efflux
Early
endosome
Sorting
endosome
Autolysosome
Late
endosome
Lysosome
Endoplasmic
Reticulum
Autophagosome
Cytoplasmic
lipolysis
Autophagic
membrane

show some degree of accumulation in endolysosomal structures.[29,60] Macrophage foam cells in human atherosclerotic lesions also display similar lipid accumulation. In early lesions, macrophage foam cells accumulate mostly cytosolic LDs[61] whereas in foam cells of advanced atherosclerotic plaques much of the cholesterol is trapped in lysosomes.[62] The evidence that autophagy is induced with lipid loading and actively mobilizes LD CE indicates that autophagy and LAL may be particularly relevant to the reversal of early lesions. Our results also support a possible role in the regression of advanced lesions. As seen from **Figure 3.7E**, even in the absence of any cytoplasmic LDs, there is an autophagy-attributable efflux to apoA-I.

Here, we clearly establish that autophagy-mediated efflux is closely linked to the ATP-binding cassette (ABC) transporter ABCA1, which itself is linked to the endosomal/lysosomal cholesterol pools. In the absence of LDs and autophagy, efflux to apoA-I is nearly completely abolished, whereas this is not the case for efflux to HDL (**Figure 3.7E and 3.7F**). This implies that autophagy-mediated efflux is primarily ABCA1-dependent, since efflux to lipid-poor apoA-I is completely dependent on ABCA1 whereas this transporter contributes to a small proportion of HDL-mediated efflux.[54] Whereas ABCA1 mediates a unidirectional transport, mostly to apoA-I,[54] HDL-mediated efflux is a bidirectional, diffusional process that involves multiple transporters[53] and represents a complex pathway for which the contribution of autophagy remains to be studied in greater detail. Macrophage ABCA1 is responsible for approximately 50% of total RCT *in vivo* [22] but its contribution to efflux to HDL is low *in vitro*.[54] In agreement with these findings, we observed a modest decrease in efflux to HDL in *Atg5-/-* macrophages *in vitro* (**Figure 3.7D and 3.7F**), and a more pronounced effect of macrophage-specific autophagy deletion on excretion of macrophage ^{3}H-cholesterol *in vivo* (**Figure 3.8**).

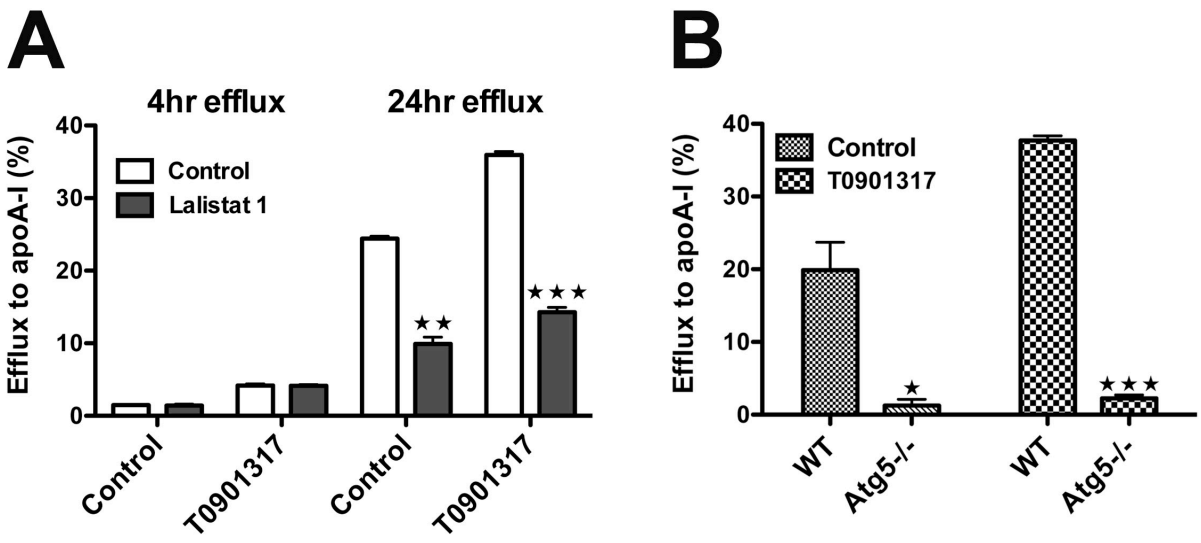
Autophagy is markedly elevated in macrophages in response to lipid-loading (**Figure 3.4**). Fittingly, under lipid-loaded conditions, apoA-I/ABCA1 retroendocytosis (a process by which apoA-I acquires lipid from late endosomal/lysosomal compartments) contributes to efficient cholesterol efflux, whereas in the absence of cholesterol loading, lipidation of apoA-I occurs primarily at the plasma membrane.[63] Additionally, ABCA1 expression is increased in response to cholesterol loading, both transcriptionally via the liver X receptor (LXR)[64] and

post-transcriptionally by miR-33.[65] Using an LXR activator (T0901317) to maximally stimulate ABCA1 activity dramatically enhances cholesterol efflux to apoA-I; in Lalistat 1-treated and *Atg5-/-* macrophages, however, T0901317 fails to increase efflux to apoA-I given that cholesterol delivery to ABCA1 under these conditions is seemingly limiting (**Figure 3.10**).

Our observation that the expansion of intracellular cholesterol pools triggers autophagy seems contradictory to previous reports linking the depletion of cellular cholesterol levels to autophagy induction in fibroblasts and in hepatocytes.[41,66] However, increased LD delivery to lysosomes has been shown to occur in response to starvation as well as triglyceride loading in hepatocytes.[10] Importantly, the prolonged presence of lipogenic stimuli ultimately results in inhibition of autophagosome clearance by lysosomes. Whereas a moderate cholesterol increase was shown to increase autophagic flow, the fusogenic ability of the autophagic / lysosomal compartments is attenuated in conditions of chronic lipid exposure.[67] It will be interesting to determine whether autophagy becomes defective in macrophages derived from advanced atherosclerotic lesions as compared to early foam cells. Impairment of autophagy-mediated cholesterol clearance in advanced foam cells would be predicted to exacerbate lipid accumulation in these lesions, given that RCT from *Atg5-/-* macrophages is significantly reduced *in vivo* (**Figure 3.8**).

In summary, we demonstrate an important role for autophagy in cholesterol efflux from macrophage foam cells (**Figure 3.9**). We demonstrate that this pathway is important for macrophage LD clearance in primary bone marrow-derived and peritoneal macrophages. Autophagy-dependent macrophage cholesterol efflux is an ABCA1-mediated process that is enhanced by the uptake of various atherogenic lipoproteins such as AcLDL, OxLDL, AgLDL and VLDL *in vitro*. In addition, we report that the autophagy pathway is induced in peritoneal macrophages loaded *in vivo*. Based on the finding that macrophage-specific autophagy deficiency is detrimental to RCT, impairment of macrophage autophagy would be expected to promote atherosclerotic lipid accumulation. Activators of autophagy, which trigger autophagy via mTOR inhibition, are emerging as promising agents to treat coronary heart disease.[68] The systemic administration of mTOR inhibitors has been shown to prevent development of atherosclerosis, attenuate plaque progression and reduce cholesterol content

Figure 3.10: The T0901317 LXR agonist increases cholesterol efflux in normal macrophages but not when autophagy-mediated efflux is inhibited. Cholesterol efflux is stimulated by T0901317 in control cells, but not in Lalistat 1-treated cells (A). Cholesterol efflux to apoA-I in AcLDL-loaded macrophages was measured for 4h and 24h. In agreement with Figure 3, LAL inhibition by Lalistat 1 reduces cholesterol efflux to apoA-I (no effect is observed at a short time point). Maximal ABCA1 activation by addition of T0901317 increases the magnitude efflux to apoA-I. Because Lalistat1 prevents hydrolysis of LD CE over time, there is no such increase of cholesterol efflux in response to T0901317 treatment in the presence of Lalistat 1 for 24h. Cholesterol efflux is stimulated by T0901317 in WT, but not in *Atg5-/-* macrophages (B). Activation of ABCA1 by T0901317 treatment increases efflux to apoA-I in WT macrophages. Despite the increase in ABCA1 activity upon T0901317 treatment, efflux is not increased in Atg5-/- macrophages because delivery of LD cholesterol to ABCA1 is impaired by the lack of autophagy. $P<0.01$ (★), <0.001 (★★) or <0.0001 (★★★) for Lalistat 1-treated cells compared to control (A) or for *Atg5-/-* cells as compared to WT (B).


A
4hr efflux
24hr efflux
Efflux to apoA-I (%)
40
30
20
10
0
Control
Lalistat 1
★★
★★★
Control
T0901317
Control
T0901317
B
Efflux to apoA-I (%)
40
30
20
10
0
Control
T0901317
★
★★★
WT
Atg5-/-
WT
Atg5-/-

in the aortic arch in atherogenic mouse models.[69-71] Although further studies are required to establish a correlation between the regulation of autophagy in macrophages and atherosclerosis, our results suggest that the controlled stimulation of autophagy may provide therapeutic potential to enhance macrophage cholesterol efflux and promote RCT.

3.8 Acknowledgements

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4 Discussion

This thesis provides new insights on the regulation of cytoplasmic CE hydrolysis for cholesterol efflux from macrophage foam cells. Because the LD represents the major source of cholesterol available to the efflux pathway in foam cells, maintenance of normal cellular cholesterol homeostasis must entail mechanisms for the rapid mobilization and re-distribution of cholesterol from LDs to efflux-competent compartments. A major finding presented herein is that the mobilization of LD CE for efflux requires the activity of both neutral and acid lipases. Our work showing that epoxycholesterol impairs the mobilization of cholesterol from LD CE in cholesterol-loaded macrophages emphasizes that the hydrolysis of CE in LDs is critically required upstream of cholesterol efflux. Importantly, we have uncovered a novel role for LAL and autophagy in cholesterol efflux and whole-body RCT, both of which represent new targets for the prevention or reversal of macrophage cholesterol accumulation. This work highlights that LD lipolysis in macrophage foam cells constitutes a biological process with an inherent property for regulation; first evidenced by the specific activation of the autophagy machinery in response to an expanded macrophage cholesterol pool, and secondly given that the rate of neutral hydrolysis can be modulated by oxysterols. The implications of these findings in the context of our current understanding of foam cell biology as well as the future of LD-associated cholesterol efflux in heart disease will be discussed below.

4.1 LXR ligands and Cholesterol Efflux

4.1.1 Epoxycholesterol and Neutral Lipid Hydrolysis

Because only free cholesterol can be excreted from macrophages, LD-associated CE hydrolysis is the obligatory, rate-limiting first step in cholesterol efflux from foam cells. In macrophage foam cells, addition of the LXR ligand, epoxycholesterol, markedly enhanced ABCA1 and ABCG1 expression but failed to increase cholesterol efflux to both HDL and apoA-I (**Figure 2.3**). This LXR agonist impaired the mobilization of cholesteryl esters from LDs in cholesterol-loaded macrophages, thus indicating that hydrolysis of CE in LDs is critically required upstream of cholesterol efflux. The exact mechanism by which epoxycholesterol decreased cholesterol efflux was unclear, but we concluded that it involved

the inhibition of neutral CE hydrolysis (**Figure 2.5**) in an LXR-independent fashion (**Figure 2.7**). It was later confirmed that epoxycholesterol indeed reduces cholesterol efflux by inhibiting the activity of neutral hydrolases (**Appendix 1**).

Our observation that epoxycholesterol decreases LD CE hydrolysis is one that has subsequently been corroborated,[1] and a mechanistic explanation for the inhibition of neutral lipolysis by epoxycholesterol has been proposed.[2] In human macrophages, for which CEH is the best candidate enzyme that mediates the neutral CE hydrolase activity, epoxycholesterol was shown to be a powerful allosteric effector of CEH enzyme activity.[2] In their investigation of CEH regulation by cholesterol and its derivatives, Crow *et al.* discovered that while cholesterol, 22-HC, 24-HC and 25-HC had little impact on CEH activity, both epoxycholesterol and 27-HC had strong inhibitory effects on CEH activity, with 27-HC being the most potent. Of note, 27-HC is the predominant oxysterol that accumulates in atherosclerotic plaques,[3,4] suggesting that LXR-independent downregulation of LD lipolysis by oxysterols may contribute to lipid buildup in the arterial wall.

Epoxycholesterol was found to inhibit CEH activity through a partially noncompetitive mode of inhibition, indicating that it interacts with a binding site distinct from the active site to modulate CEH activity.[2] While epoxycholesterol increases the efflux of free cholesterol from macrophages, the CE pool remains unaltered despite a decrease in free cholesterol levels.[2] In contrast, 22-HC, that does not inhibit CEH, was shown to reduce cellular CE levels.[2] In light of these findings, epoxycholesterol would be expected to increase cholesterol efflux from unloaded cells where the majority of the cholesterol is unesterified, whereas under lipid-laden conditions where the majority of cholesterol is esterified, epoxycholesterol-mediated inhibition of CEH activity would impair CE hydrolysis and consequently impair cholesterol efflux from this cellular pool. This is precisely what we observed for lipid-loaded as compared to unloaded macrophages treated with epoxycholesterol (**Figure 2.1**).

4.2 Insights into Macrophage Foam Cell Lipolysis and Cholesterol Efflux

4.2.1 Neutral Lipolysis

Understanding the mechanisms that govern cytoplasmic CE hydrolysis in macrophage foam cells has been of longstanding interest in the cardiovascular research community, and it is an issue of great importance. Since the macrophage foam cell CE cycle was first characterized in the 70-80s by Brown and Goldstein, numerous candidate neutral hydrolases have been proposed, but the only consensus in this field of study is that increasing neutral lipolysis has tremendous potential to promote cholesterol efflux. While promoting LD lipolysis is certainly a promising approach to prevent or reverse atherosclerosis and CAD, progress in this undertaking has been hindered by the eternal quest to identify THE macrophage-specific neutral CE hydrolase. However, given the large number of putative candidates, the matter has been difficult to resolve. Furthermore, macrophage lipolysis cannot be entirely abolished by inhibiting those candidate enzymes, since specific inhibition is difficult to achieve in a biological context (see below). Despite general agreement that LD lipolysis is a critical step in the regulation of foam cell formation and atherosclerosis, significant advancements in our understanding of this process have been impeded by the controversies surrounding the exact identity of the macrophage neutral hydrolase.[5]

An important limitation in understanding the pathways that mediate foam cell lipolysis stems from the popular use of cell-free enzymatic assays, which can be problematic if not performed with rigor. When neutral hydrolases come into contact with their substrate, LDs, they undergo a conformational change upon adsorption at the water/lipid interface.[5] The interfacial microenvironment is thus an important factor for enzymatic activity, and surfactants or detergents can expel interfacial enzymes from the surface of LDs and reduce their activity.[5] In a number of cell-free assays, detergents are included, which can affect the overall lipolytic activity of these interfacial enzymes and impede accurate measurements of hydrolysis. The ideal way to characterize CE hydrolytic enzymes in cell-free assays is thus to present the substrate in its physiological form (as droplets), a methodology that has unfortunately not widely been employed.[5]

Because researchers have concentrated their efforts on measuring candidate CE hydrolase enzymatic activity in cell-free assays, it is not surprising that potential endocytic

pathways that contribute to this process - requiring cytoskeletal integrity to transpire – were not discovered. Evidently, inhibition of neutral hydrolases in a cell-free assay would abolish all hydrolysis and lead to the conclusion that all cytoplasmic lipolysis can be attributed to neutral lipases, given that autophagy or other trafficking pathways under these conditions would not be operational. A better way to characterize the hydrolytic arm of the CE cycle would be to incubate cells in the presence of a cholesterol acceptor and an ACAT inhibitor (to prevent cholesterol re-esterification following hydrolysis), and to measure the amount of CE remaining as compared to the initial amount of cellular CE; in this way, dissipation of CE can be expressed as % hydrolysis and the contribution of various enzymes / pathways to this process can be measured in a biologically relevant context, using LDs as substrate. Using this method, we were surprised to find that paraoxon - an inhibitor of all neutral lipases - did not abolish all lipolysis in macrophage foam cells (**Figure 3.2**). This led to our discovery that lysosomes can hydrolyze LDs CE in CEH-independent pathways.

4.2.2 Acid Lipolysis

How did we overlook the contribution of the lysosome to LD-cholesterol efflux for so long? The fact is that evidence for a role of the lysosomal pathway for LD neutral hydrolysis exists throughout the literature. Using electron microscopy to characterize the CE cycle in murine macrophage foam cells, lamellar arrangements adjacent to LDs, projecting into the matrix of the droplets, often appearing in continuity with ER juxtaposed to LDs, were frequently observed (approximately 20% of LDs contained these specialized membrane structures).[6] Could these represent nascent autophagosomes at the LD periphery? In their study using electron microscopy to describe CE accumulation in macrophages, Brown and Goldstein reported that although membrane-bound LDs were occasionally observed, most of them were not surrounded by a classical membrane.[7] Could these occasional membrane-bound LDs represent LDs sequestered in autophagosomes? In 1999, Avart *et al.* reported that the hydrolysis of cytoplasmic CE in CE-enriched macrophages occurs both in the cytoplasm and in lysosomes.[8] In this report, the authors had no explanation for these 'puzzling' observations, and stated that 'these data raise the question of why cytoplasmically synthesized lipid droplets should find their way to lysosomes at all'.[8]

When we first observed LDs within lysosomes by confocal microscopy, our first concern was that we had perhaps omitted the equilibration step after cholesterol loading, i.e. that the AcLDL was not chased from the lysosome. However, despite increasing periods of chase in BSA media following lipoprotein loading, there was always a fraction of lysosomes that stained positive for neutral lipids. In perfect accordance with the report from Avart *et al.*, when we tested the functional importance of this observation, we found that a fraction of the cytoplasmic CE hydrolysis in CE-enriched macrophages could be attributed to neutral lipases while the other was mediated by a chloroquine-sensitive lysosomal function (**Figure 3.2**). Similarly to what Avart *et al.* described in their study, we also observed a portion of the cytoplasmic LD pool in lysosomes by electron microscopy (**Figure 3.3**).

Our observations were in sharp contradiction with the conclusions of the important research conducted by Nobel Laureates and a very well established dogma in lipid research. When Brown and Goldstein first demonstrated that CE hydrolysis in macrophage foam cells is extra lysosomal and requires a neutral CE hydrolase, they set the stage for the discovery of the numerous candidate enzymes that have since been proposed to fulfill this role. We reproduced the experimental conditions used in Brown and Goldstein's studies, and have been able to replicate their findings in the early time points of efflux which they used. When cholesterol efflux is carried out in LDL- or AcLDL-loaded macrophages for 4hrs, chloroquine does not inhibit cholesterol efflux (**Figure 3.4**). It is only during longer efflux periods that chloroquine and LAL inhibition reduces cholesterol efflux, and only in AcLDL-loaded macrophages (**Figure 3.4**). We have found autophagy to be specifically induced in response to atherogenic lipoprotein loading (**Figure 3.7**), and thus the autophagy-LAL pathway for LD CE hydrolysis is one that is explicitly triggered in foam cells.

At any given time in the macrophage foam cell, only a small proportion of LDs are transported to lysosomes (**Figure 3.2**), but cumulatively this results in a significant contribution to LD breakdown and cholesterol efflux. The export of cholesterol mobilized by autophagy reveals itself to be an ABCA1-dependent process (**Figure 3.7**); perhaps the trafficking of ABCA1 from the plasma membrane to endolysosomal compartments to acquire free cholesterol released from LDs through the enzymatic action of LAL explains why this efflux proceeds at low speed. Additionnally, the sequence of events leading to LD

delivery to LAL by autophagy is a lengthy process compared to cytoplasmic hydrolysis. It requires sequestration of LDs in autophagosomes, fusion of LD-containing autophagosomes with endosomes or lysosomes, acidification of the newly fused endocytic compartments, degradation of the autophagosomal membrane to release the cargo in the lysosomal lumen for degradation. Alternatively, cholesterol efflux may begin with free cholesterol at the plasma membrane and in the endosomal recycling compartment (ERC) that is readily available as a primary source, forming a cholesterol gradient in the membranes of these organelles and following which efflux from LAL-derived LD cholesterol in the endolysosomal network ensues.

A new role for LAL in the hydrolysis of LD cholesterol for efflux suggests that LAL activity in macrophage foam cells of atherosclerotic lesions could promote macrophage RCT and exhibit antiatherogenic properties. The exact role of LAL in atherosclerosis remains to be determined. Whereas low plasma HDL cholesterol and premature atherosclerosis often occur in CESD patients,[9,10] two recent GWAS report links a gain-of-function LAL mutation with enhanced susceptibility to CAD.[11,12] Elevated LAL expression, presumably leading to increased LAL activity, was associated with lower HDL-cholesterol levels.[12] Follow-up functional studies are required to clarify how either low or high LAL activity are causative of premature CAD, and to evaluate how macrophage-specific LAL activity influences atherosclerotic lesions, but it is clear that LAL plays an important role in the pathogenesis of atherosclerosis and CAD.

4.2.3 Lipoprotein-induced Autophagy

Autophagy induction by OxLDL has previously been reported in human vascular endothelial cells, and autophagy was proposed to play a role in OxLDL processing upon its uptake by these cells.[13] Conversely, we did not uncover a requirement for autophagy in OxLDL or AcLDL degradation, since lipoprotein-CE was hydrolyzed and excess lipoprotein-derived cholesterol was esterified to accumulate as cytoplasmic CE to a similar extent in WT and *Atg5-/-* macrophages (**Figure 3.6**). However, we did observe increased autophagy upon atherogenic lipoprotein loading (**Figure 3.7**); therefore a common conclusion to both studies is that atherogenic lipoproteins trigger autophagy. At this time, the mechanisms by which

atherogenic lipoproteins elicit autophagy in macrophages remain unclear. We are currently exploring whether central molecular components of the canonical autophagy machinery, such as mTOR, are involved in the activation of lipoprotein-induced autophagy. Alternatively, autophagy may be initiated through other non-canonical autophagy inducing pathways. It can be speculated that autophagy ensues as part of an innate immune response to atherogenic lipoproteins; in fact, toll-like receptor (TLR) activation has recently been linked to the induction of autophagy,[14-16] a finding that may be extendable to scavenger receptors, or otherwise attributable to scavenger receptor / TLR crosstalk.

Because autophagy and cholesterol homeostasis are both ancient cellular pathways, it seems fitting that both mechanisms have co-evolved to share common regulatory elements. Indeed, there is an intimate connection between regulators of autophagy and master regulators of cholesterol homeostasis. The PI3K/Akt/mTORC1 pathway – which controls cellular autophagy activities - regulates the SREBP/SCAP pathway, although the exact regulatory mechanism of SREBPs by mTORC1 is unclear.[17-19] Several autophagy genes have been identified as SREBP targets, and it was shown that these SREBP target genes are activated by SREBP induction during sterol depletion, suggesting that the LD is a new lipid source regulated by SREBPs.[20] Whereas it is becoming increasingly apparent that cholesterol depletion induces autophagy,[20,21] the role of autophagy under conditions of intracellular lipid accumulation, which is particularly relevant to many diseased states such as atherosclerosis, remains uncertain.

4.2.4 Autophagy and Cholesterol Efflux

We have demonstrated that disruption of autophagy in macrophages impairs cholesterol efflux from macrophage foam cells. From a clinical standpoint, the question that now arises is whether autophagy can be stimulated in macrophage foam cells to enhance cholesterol clearance from lesional macrophages. mTOR inhibition, using the small-molecule inhibitor Torin1, activates autophagy.[22] In lipid-loaded macrophages, we find that the increased autophagic flux resulting from mTOR inhibition significantly enhances cholesterol efflux out of these cells (**Appendix II**). We have recently obtained validated antisense oligonuclotides (ASOs) targeting mTOR from Isis (**ISIS** Pharmaceuticals Inc, California, USA).

Experiments are currently underway to test whether these ASOs, similarly to Torin1, stimulate cholesterol efflux out of macrophage foam cells. If so, these antisense drugs will be suitable for future *in vivo* experimentation whereby the ability of macrophage-specific mTOR silencing to increase macrophage RCT will be evaluated.

4.3 Atherosclerotic Plaque Regression

Since the 1950's, the list of interventional studies demonstrating substantial shrinkage of atherosclerotic lesions has grown.[23] The atherosclerosis research community is entering an era where the characterization of the cellular and molecular features of the regressing plaque is now possible with the advent of numerous mouse models suitable for such studies; the fact that atherosclerotic lesions at all stages of development can regress affirms that this is a worthy therapeutic pursuit. A rapid way to robustly improve the plaque environment and trigger lesion remodeling is to transplant a segment of plaque-containing aorta from a hypercholesterolemic mouse to a normolipidemic mouse.[24] Alternatively, pre-existing plaques in atherogenic-susceptible apoE-/- mice can be regressed by the reversal of hyperlipidemia via MTP inactivation in 'Reversa' mice.[25] These regression experiments reveal that reducing plasma apoB-lipoprotein levels initiate plaque regression. Enhancing reverse lipid transport is also a good approach to lesion regression, as exemplified by the ability of HDL-like particles to rapidly induce plaque remodeling.[26] Collectively, these studies indicate that 'plaque regression requires robust improvements in the plaque environment, specifically large reductions in plasma concentrations of apoB-lipoproteins and large increases in the 'reverse' transport of lipids out of the plaque for disposal'.[23]

In addition to halting plaque progression, LXR agonists have been shown to induce the regression and stabilization of established atherosclerotic plaques.[27,28] A recent study reported that LXRα is activated in macrophages of regressing lesions, and that LXRα-dependent arginase 1 (Arg1) expression – which is inversely correlated with atherosclerosis – was enhanced in regressing as opposed to progressing lesions.[29] The success of LXR agonists as anti-atherogenic agents is dampened by their adverse effect on liver triglycerides. The challenge now lies in the development of new LXR agonists that do not stimulate SREPB1 processing and do not exert undesirable LXR-independent side effects such as

epoxycholesterol's nefast downregulation of CEH activity. Novel LXR agonists with therapeutic potential will require functional testing in macrophage foam cells to ensure that they indeed promote cholesterol efflux from these cells, an important prerequisite for their consideration as plaque regression enhancing agents.

Based on our findings that autophagy contributes to macrophage RCT *in vivo*, defective autophagy in arterial macrophages is expected to favor LD accumulation and promote atherogenesis, whereas increasing macrophage RCT by stimulating autophagy is predicted to help resolve cholesterol buildup in the arteries and promote plaque regression. On the first point, efforts in our and others' laboratories are focused on assessing whether macrophage autophagy contributes to the pathogenesis of atherosclerosis. Two research groups have already developed macrophage-specific *Atg5-/-* mouse strains on a hypercholesterolemic background to address whether macrophage autophagy is pro- or anti-atherogenic. Our own lab has observed elevated levels of the autophagy marker LC3 in human atherosclerotic lesions (**Appendix III**). It is yet unclear whether the elevated LC3 levels in the lesions of these human subjects correlates with functional autophagy, but it is interesting to note that the LC3 staining is accentuated in macrophages located in the 'shoulder region', or the rupture-prone site of vulnerable plaques that is characteristically located to the side (shoulder) of the lesions. Interestingly, it was reported that autophagy is induced in response to lipogenic conditions in hepatocytes, while chronic exposure to lipogenic stimuli ultimately leads to slighted autophagy in these cells.[30] In light of these findings, we hypothesize that defective autophagy in arterial macrophages faced with a prolonged lipogenic challenge may contribute to atherosclerotic plaque progression because of impaired autophagic clereance of LDs.

To establish a potential role for macrophage autophagy in lesion regression, our lab is taking two approaches. The first is to determine whether the lack of arterial macrophage autophagy would impair lesion regression in Reversa mice. For this, we have generated tamoxifen-inducible cre / Atg5-floxed mice. Bone marrow transplants will be performed to reconstitute the bone marrow of irradiated Reversa mice with progenitor cells from the tamoxifen-inducible cre / Atg5-floxed mice. Atherosclerotic lesions will be established by placing the reconstituted Reversa mice on an atherogenic diet, after which lesion regression

will be initiated by reversal of hyperlipidemia via stimulated MTP cleavage, and at this time tamoxifen will be administered (or not) to abolish autophagy in macrophage foam cells of established plaques. By comparing the reversal of plaques comprised of autophagy-deficient and autophagy-competent macrophages, we will evaluate whether macrophage autophagy is implicated in plaque regression. The second approach that the lab is taking to address whether macrophage autophagy can participate in plaque regression is to stimulate autophagy and evaluate whether it promotes macrophage RCT. For this, ^{3}H-AcLDL-loaded macrophages will be treated with mTOR-targeting ASOs, and the classical RCT assay will be performed. Together, the outcome of the two studies will provide knowledge as to whether autophagy indeed can promote macrophage RCT and whether this process impacts on the ability of established atherosclerotic lesions to resolve.

4.4 Autophagy and CAD

Activators of autophagy, namely rapamycin and its analogues (everolimus, sirolimus) which trigger autophagy via mTOR inhibition, are emerging as promising agents to treat coronary heart disease.[31] These mTOR inhibitors have potent immunosuppressive and antiproliferative properties and are routinely used in drug eluting stents for the treatment of cardiovascular disease. The systemic administration of mTOR inhibitors is reported to be atheroprotective. Not only were these inhibitors shown to prevent development of atherosclerosis and attenuate plaque progression in atherogenic mouse models,[32,33] they were also shown to specifically reduce the cholesterol content of the aortic arch.[34] Most of the atheroprotective effects of rapamycin are currently attributed to the selective clearance of lesional macrophages.[35]

While cellular autophagic flux has not directly been quantified in any of the *in vivo* studies conducted using mTOR inhibitors, it is likely that some of the observed effects of these compounds are attributable to this pathway. For instance, our results demonstrate that autophagy is important for macrophage RCT. Thus, enhancing autophagy in these cells would be expected to reduce the cholesterol content in atherosclerotic lesions, perhaps accounting for the previously observed rise in apoA-I and HDL-C levels of patients receiving sirolimus[36] and the reduced lesional cholesterol in hypercholesterolemic mice

receiving everolimus.[34] In turn, reduced macrophage cholesterol content would be expected to attenuate inflammation and inhibit monocyte chemotaxis, as has been proposed to occur in response to rapamycin.[33] Although further studies are required to establish a correlation between the regulation of autophagy in macrophages and CAD, our results suggest that the controlled stimulation of autophagy by rapamycin or its analogs is a potentially interesting target to enhance macrophage cholesterol efflux and promote RCT.

4.5 The Future of Lipid Droplet Cholesterol Efflux and Heart Disease

LDs can no longer be considered as mere passive blobs of fat that store cholesterol. Our own live imaging portrays macrophage LDs as dynamic, highly motile organelles that frequently come into contact with vesicles of the endolysosomal network. The routes of cholesterol traffic from LDs to cell surface cholesterol acceptors, and whether this involves vesicular or non-vesicular transport, are unclear. This remains an active area of investigation; with future discoveries potentially leading to the identification of novel targets to stimulate cholesterol efflux. We have long understood that the macrophage LD CE pool undergoes a continual cycle of hydrolysis and re-esterification, and that cellular cholesterol levels regulate the esterification arm of the cycle. Following receptor-mediated endocytosis, lipoprotein-derived cholesterol stimulates ACAT activity to store excess cholesterol in the cytoplasm as CE droplets.[37] Whereas the presence of a cholesterol acceptor interrupts the CE cycle, promoting net CE hydrolysis and cholesterol excretion, net hydrolysis is achieved primarily via a reduced rate of ACAT-mediated cholesterol re-esterification rather than an increase in the rate of hydrolysis per se.[7] Evidence for regulation of the hydrolysis arm of the CE cycle has thus far been lacking.

The work presented in this thesis provides the first evidence that macrophage LD CE hydrolysis is not exclusively a constitutive process – its inherent plasticity provides exciting new ways to manipulate this pathway to promote the flux of cholesterol out of macrophage foam cells. This is a major advancement in the field of foam cell biology, whereby autophagy and LAL represent new targets for the regulation of macrophage RCT. Both cytoplasmic and lysosomal LD lipolysis require further investigation, and unraveling the precise molecular mechanisms that underlie cholesterol mobilization from LDs in

macrophage foam cells could have tremendous impact on the development of new therapeutics for the treatment of atherosclerosis.

4.6 Conclusion

Understanding the pathways that regulate the generation of free cholesterol from macrophage LDs is critical to develop therapeutic strategies that promote arterial macrophage RCT and reduce the lipid burden in atherosclerotic plaques. Both neutral and lysosomal lipolysis are critical for the efficient removal of cholesterol from macrophage foam cells. Future development of clinical strategies for enhancing macrophage RCT should target both pathways to maximize our chances of success. Additionally, the advent of new mouse models for the study of atherosclerotic lesion regression will help decipher the contribution of distinct macrophage RCT pathways to plaque regression. Autophagy and LAL are novel mediators of cholesterol efflux from macrophage foam cells, and future studies to elucidate their role in foam cell biology will certainly provide new insights into atherogenesis, CAD and potential therapeutic avenues to treat them.

4.7 Reference List

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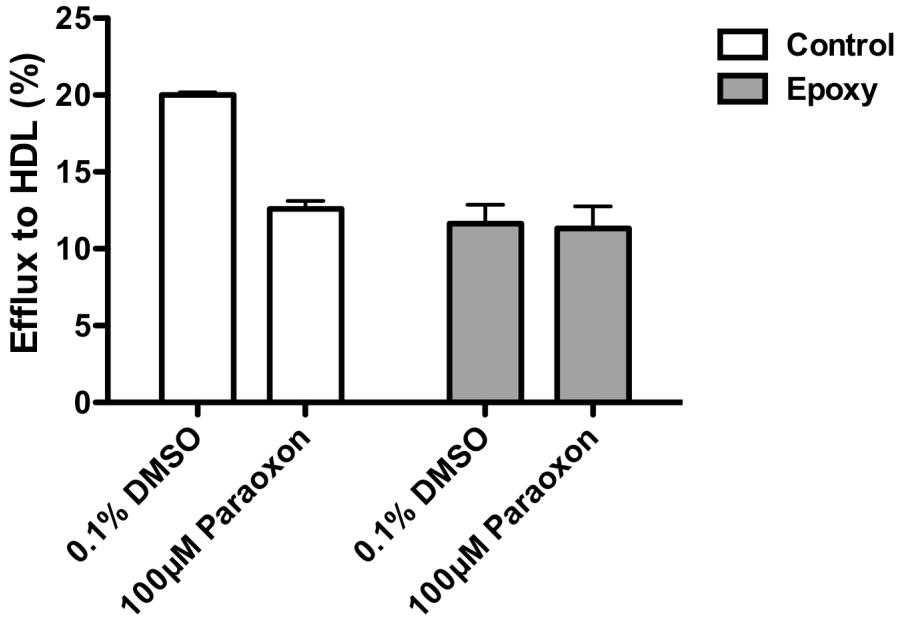
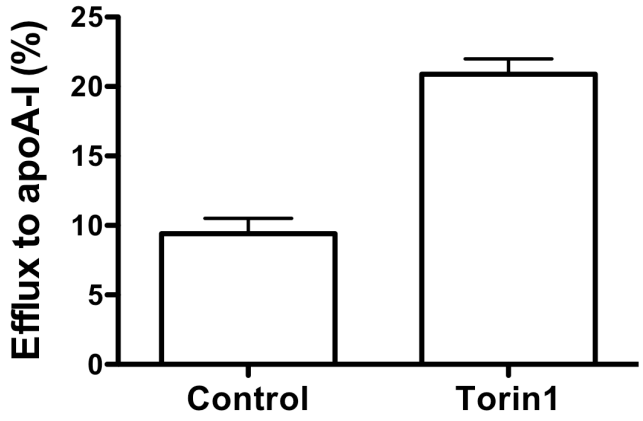
Appendix I



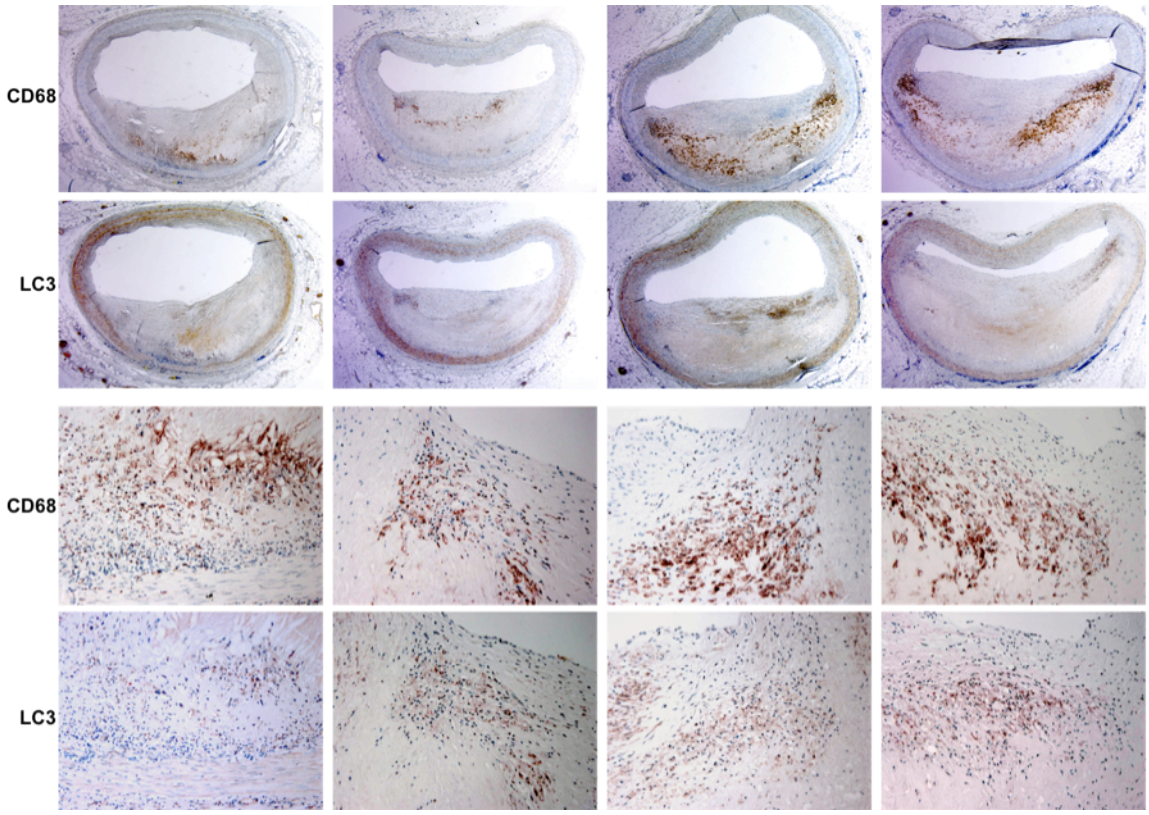
Epoxycholesterol decreases efflux by inhibiting neutral cholesterol hydrolases. Murine bone marrow-derived macrophages were loaded with ^{3}H-cholesterol-AcLDL for 30h, after which cells were washed and equilibrated overnight (2mg/mL BSA media) in the presence or absence of epoxycholesterol (10μM). Efflux to HDL was carried out for 5hrs in the presence or absence of an inhibitor of neutral lipolysis, paraoxon (100μM). Inhibition of neutral lipid hydrolysis using paraoxon reduces cholesterol efflux to the same extent as epoxycholesterol treatment, and epoxycholesterol along with paraoxon treatment inhibits cholesterol efflux to the same extent, indicating that epoxycholesterol reduces cholesterol efflux via inhibition of neutral lipolysis.

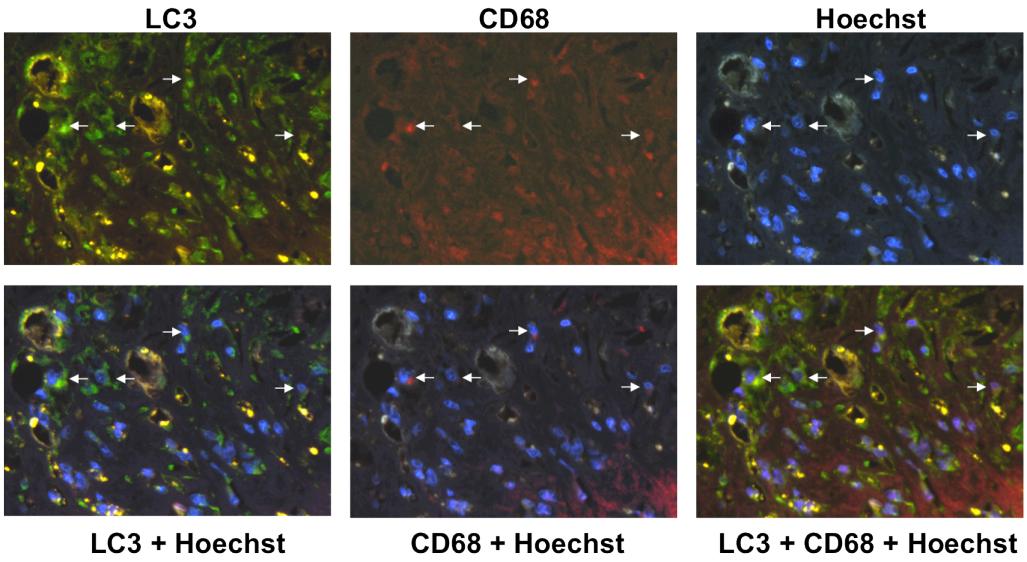
Appendix II



mTOR inhibition increases cholesterol efflux from macrophage foam cells. Murine bone marrow-derived macrophages were loaded with ^{3}H-cholesterol-AcLDL for 30h, after which cells were washed and equilibrated overnight (2mg/mL BSA media). Efflux to apoA-I was carried out for 24hrs in the presence or absence of Torin1 (250nM), which inhibits mTOR signaling and activates autophagy.

Appendix III





LC3 staining in human atherosclerotic lesions. In diseased human atherosclerotic lesions, LC3 is primarily expressed in macrophages, as shown by the extensive co-localization of LC3 and the macrophage marker, CD68, as shown by immunofluorescence. LC3 expression is particularly concentrated in the shoulder region of human atherosclerotic arteries, as shown by immunohistochemistry. Staining performed by Yong-Xian Chen.

Mireille Ouimet

Education

- **PhD in Biochemistry (2005-present)**
  University of Ottawa Heart Institute, Ottawa, ON, Canada

- **Bachelor of Science (Hons) in Biochemistry (2001-2005)**
  University of Ottawa, Ottawa, ON, Canada

Distinctions

- **Dean's Honour List**
  University of Ottawa, Canada (2001-2005 inclusive)

- **Graduation with Honorable Mention, Magna Cum Laude**
  University of Ottawa , Canada (B.Sc. 2001-2005)

Scholarships and Awards

- **Vanier Canada Graduate Scholarship (2009-present)**
  Doctoral Award

- **Heart and Stroke Foundation of Ontario (2005-2007)**
  Master's Award

- **University of Ottawa Excellence Scholarship (2005-present)**
  Tuition scholarship

- **University of Ottawa Entrance Scholarship (2001-2005)**
  Tuition Scholarship

Teaching Experience

- **Teaching Assistant, University of Ottawa (2006-2009)**
  Department of Biochemistry
  Biochemistry and Molecular Biology laboratories

Conferences

- Gordon Research Conference on Atherosclerosis (June 19-24 2011)
  Newport, Rhodes Island, USA
  Oral abstract presentation and poster presentation (poster award, one of the top 10 ranked posters)

- University of Ottawa Heart Institute Research Day (May 2001)
  Ottawa, Ontario, Canada
  Oral abstract presentation (1st prize, Basic Science Category)

- Arteriosclerosis, Thrombosis and Vascular Biology (April 28-30 2011) Chicago, Ilinois, USA
  Poster presentation

- Canadian Lipoprotein Conference (Oct 21-24 2010)
  Niagara-on-the-Lake, Ontario, Canada
  Oral abstract presentation (Graduate Student Oral Abstract Presentation Award)

- Gordon Research Conference on Lipoprotein Metabolism (June 22-27 2008)
  Waterville Valley, New Hampshire, USA
  Poster presentation (Outstanding Poster Presentation Award)

- Canadian Lipoprotein Conference (Oct 11-14 2007)
  Quebec, Quebec, Canada
  Poster presentation

- Arteriosclerosis, Thrombosis and Vascular Biology (April 19-21 2007), Chicago, Illinois, USA
  Poster presentation

Scholarly Publications

Published refereed papers

1. **Ouimet M**, Franklin V, Mak E, Liao X, Tabas I, Marcel YL. 'Autophagy Regulates Cholesterol Efflux from Macrophage Foam Cells via Lysosomal Acid Lipase.' Cell Metabolism. Jun 8;13(6):655-67. PMID: 21641547.

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3. Marcel YL, **Ouimet M**, Wang MD. Regulation of cholesterol efflux from macrophages. Curr Opin Lipidol. 2008 Oct;19(5):455-61. PMID: 18769226.

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1. Marcel YL, **Ouimet M**, Wang MD. (2009). Chapter 'Cellular Lipid Traffic and Lipid Transporters: Regulation of Efflux and HDL Formation'. From the book 'Cellular Lipid Metabolism' published by Springer Berlin Heidelberg.

Published Abstracts

1. **Ouimet M**, Franklin V, Mak E, Liao X, Tabas I, Marcel YL. (2011). Autophagy and Lysosomal Acid Lipase Regulate Macrophage Reverse Cholesterol Transport. Arteriosclerosis, Thrombosis, and Vascular Biology.

2. **Ouimet M**, Wang MD, Ho K, Franklin V, Marcel YL. (2007). Epoxycholesterol Treatment Increases ABCA1-Mediated Cholesterol Efflux in LDL- but not Acetylated LDL-loaded Macrophages. Arteriosclerosis, Thrombosis, and Vascular Biology.27:e61-e61.