THE ROLE OF CHOLESTEROL EFFLUX IN MACROPHAGE CHOLESTEROL HOMEOSTASIS

By

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To my teachers and mentors.

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5-LO	5-lipoxygenase
ABC	ATP-binding cassette
ACAT	acyl-coenzyme A: cholesterol acyltransferase
acLDL	acetylated LDL
aP2	adipocyte fatty acid binding protein
apo	apolipoprotein
apoER2	apoE receptor 2
BSA	bovine serum albumin
CCR2	CC-chemokine receptor 2
CD	cluster of differentiation antigen
CE	cholesteryl ester
CETP	cholesteryl ester transfer protein
COX-2	cyclooxygenase 2
cpm	counts per minute
DGAT	acyl-coenzyme A: diacylglycerol acyltransferase
DMEM	Dulbecco's modified Eagle medium
DMSO	dimethylsulfoxide
EEA1	early endosome antigen 1
FBS	fetal bovine serum
FC	free cholesterol
h	hours
h-apoAI	human apolipoprotein AI transgene
HDL	high density lipoprotein
HL	hepatic lipase
HMG-CoA reductase	hydroxymethylglutaryl-coenzyme A reductase
HSPG	heparan sulfate proteoglycans
ICAM	intercellular adhesion molecule
IDL	intermediate density lipoproteins
Ig	immunoglobulin
IKK2	IκB kinase 2
IL	interleukin
keV	kilo-electron-volts
KO	knockout
LAMP1	lysosome associated membrane protein 1
LCAT	lecithin: cholesterol acyltransferase
LDL	low density lipoprotein
LDLR	low density lipoprotein receptor
LPL	lipoprotein lipase
LRP	LDLR related protein
LXR	liver X receptor
MBCD	methyl-ß-cyclodextrin
MCP	monocyte chemoattractant protein
M-CSF	macrophage-colony stimulating factor
MHC	major histocompatibility complex
MMP	matrix metalloproteinase
NCEH	neutral cholesteryl ester hydrolase
NF-κB	nuclear factor-κB

nm	nanometers
NPC	Niemann-Pick type C
oxLDL	oxidized LDL
PAI-1	plasminogen activator inhibitor 1
PLA_2	phospholipase A_2
PLTP	phospholipid transfer protein
PPAR	peroxisome proliferator-activated receptor
PSGL	P-selectin glycoprotein ligand
RCT	reverse cholesterol transport
RXR	retinoid X receptor
SR	scavenger receptor
SREBP	sterol regulatory element binding protein
TNF	tumor necrosis factor
tng	transgenic
UCP2	uncoupling protein 2
VCAM	vascular cell adhesion molecule
VLA	very late antigen
VLDL	very low density lipoproteins
WT	wildtype

CHAPTER I

INTRODUCTION

Objective

The risk for atherosclerosis is influenced greatly by genetic or pharmacologic disruptions of cellular cholesterol homeostasis. For example, the expression of apolipoprotein (apo) E protects macrophages from accumulating cholesterol and lowers atherosclerosis risk. Another example is the statin drugs, which reduce intracellular cholesterol synthesis and have become the most common intervention for risk reduction. Cholesterol homeostasis in macrophages is of critical importance because these cells have a pivotal function in the vessel wall and in the development of atherosclerotic lesions. Macrophages scavenge modified lipoproteins in the arterial wall and transform into foam cells as cholesterol accumulates intracellularly. Processes that influence cellular cholesterol balance and foam cell transformation include cholesterol synthesis, lipoprotein uptake, cholesterol ester storage, and cholesterol efflux. Research on apoE, apoAI, and on the ATP-Binding Cassette (ABC) A1 transporter suggests that cholesterol efflux has a role in macrophage cholesterol homeostasis that directly affects atherosclerosis risk. Decreases in cholesterol efflux can be a causative mechanism for foam cell formation, while increases in cholesterol efflux may represent compensation for dysregulation of cholesterol synthesis, uptake, or storage. Few studies have been done to characterize the interactions between efflux and the other processes of cholesterol balance in macrophages of atherosclerosis models. We hypothesize that macrophages with genotypes that are known to affect the progression of atherosclerosis, will have changes in cholesterol efflux and cellular cholesterol homeostasis. Our studies emphasize the ability for cholesterol efflux to change in response to cholesterol imbalances. We studied the cholesterol imbalance created by the cholesterol storage deficit that results from the deletion of the cholesterol esterifying enzyme, acyl-coenzyme A: cholesterol acyltransferase (ACAT). Our studies also emphasize the capacity for cholesterol acceptors to stimulate cholesterol efflux from macrophages that endogenously synthesize apoE or transgenic apoAI.

Specific Aims

We hypothesize that cholesterol efflux is an important mechanism of cholesterol homeostasis that is affected by deficits in cholesterol storage and the synthesis of cholesterol acceptors. This hypothesis was addressed by the following aims:

Aim 1. To develop experimental protocols and data analysis software for assaying cholesterol efflux *in vitro* (Chapter III).

Aim 2. To characterize the effects of cholesterol ester storage deficits on cholesterol efflux and cholesterol homeostasis (Chapter IV and V).

Aim 3. To examine the effects of endogenously synthesized cholesterol acceptors on cholesterol efflux (Chapter VI, VII, and VIII).

CHAPTER II

BACKGROUND AND SIGNIFICANCE

Pathogenesis of Atherosclerosis

Atherosclerosis is a complex disease resulting from a coordinated series of events including endothelial dysfunction, lipid accumulation, inflammation, oxidative stress, cell proliferation, and cell death. These events are the consequence of risk factors and genetic predisposition and are a part of the development of fatty streaks, complex lesions, and clinically relevant complications.

Risk Factors. Atherosclerosis is the major cause of morbidity and mortality in the United States, with coronary heart disease and stroke being its two most common expressions (Gordon and Rifkind, 1989; Gordon et al., 1977). The progression of this disease involves multiple steps that span decades of human life. Numerous risk factors for atherosclerosis have been identified: age, male sex, family history, high levels of "bad cholesterol" (LDL), low levels of "good cholesterol" (HDL), high plasma triglycerides, hypertension, sedentary lifestyle, tobacco smoking, obesity, and diabetes mellitus (ATPIII, 2002; Fruchart et al., 2004; Grundy et al., 1999; Pearson et al., 2002). These factors modify the progression of the disease from normal vessels, to fatty streaks, to complicated plaques, and finally to clinical manifestations. Processes that characterize atherogenesis within the vessel wall are inflammation, immune response, and lipid homeostasis. Within the vessel wall, macrophages are a central player in all of these processes.

Systemic Cholesterol Homeostasis. Cholesterol in the body comes from two sources, dietary uptake and peripheral synthesis. Cholesterol is incorporated into particles called lipoproteins, which contain apolipoproteins and lipids. Apolipoproteins help to make lipoproteins soluble in aqueous solution, act as ligands for receptor-mediated uptake, and activate enzymes involved in lipoprotein processing. Lipoprotein assembly occurs primarily in the liver and intestine. Each type of lipoprotein has distinct profiles of

apolipoproteins and lipids. Lipoproteins like LDL, that lead to the deposition of cholesterol in peripheral tissues, are considered pro-atherogenic. Lipoproteins like HDL, that promote the return of cholesterol from peripheral tissue, are considered anti-atherogenic. This is the reason that LDL is referred to as "bad cholesterol" and HDL is referred to as "good cholesterol". There is an anti-atherogenic cascade of events that removes cholesterol from peripheral tissues, transports it through blood plasma and lymphatics back to the liver, and then excretes the cholesterol as bile. This system is called the reverse cholesterol transport (RCT) system. The deposition of cholesterol in the walls of vessels and the reverse transport of cholesterol significantly modify the progression of atherosclerosis.

Foam cells and Fatty Streaks. The earliest events of atherosclerosis involve endothelial damage, subendothelial lipoprotein retention, and oxidative stress (Kovanen and Tabas, 2001). Lipoproteins deposited in the subendothelial space result in the recruitment of macrophages. As macrophages accumulate cholesteryl esters, they transform into foam cells, which have a characteristic foamy appearance upon microscopic analysis. Foam cells become constituents of fatty streaks in the walls of medium and large arteries. In humans, fatty streak formation begins in the first decade of life (Burke et al., 1986; Restrepo and Tracy, 1975). Lipid accumulation, inflammation, oxidative stress, cell proliferation, and cell death influence the formation and progression of fatty streaks. Foam cell lesions mature into plaques that are more complex in structure and can cause clinically significant complications.

Complicated Plaques. The maturation of fatty streaks into complex plaques involves multiple cell types and many pathological processes (Forrester, 2002; Libby et al., 1996). Early lesions mature as more macrophages are recruited and transform into foam cells. Concurrently, smooth muscle cells migrate into the intima of the vessel wall and proliferate (Fazio et al., 2005). More cholesterol accumulates in the extracellular space and an acellular lipid core forms that is surrounded by cells undergoing apoptosis and necrosis. In very complex lesions, a fibrous cap forms on the luminal side of the

plaque. The plaque can undergo calcification, rupture, and promote thrombosis. Rupture and thrombosis lead to lumen occlusion and tissue infarction.

The Multiple Roles of Macrophages in Atherosclerosis

Atherosclerosis involves many cell types, but macrophages are arguably the most important and play a central role during every stage of disease progression. Lipid-laden foam cell macrophages are the defining characteristic of the atheroma. Gene deletion and transgene expression have been used to dissect the contributions of single genes to foam cell formation and atherosclerosis. Focusing specifically on the physiology of macrophages within the vessel wall has proven to be difficult because of the systemic effects of the genetic manipulation (e.g. decreased animal viability or changes in plasma lipid profile). While *in vivo* experiments with genetically engineered animals are good for studying systemic effects on atherosclerosis, *in vitro* experiments are better at isolating specific effects on macrophage physiology. Bone marrow transplantation has been used to study the specific effects of macrophage physiology on the progression of atherosclerosis, *in vivo* (Fazio and Linton, 2001; Linton et al., 1995; Linton and Fazio, 1999). In bone marrow transplantation studies, recipient mice are lethally irradiated to kill their bone marrow cells. The irradiated recipient mice are reconstituted with bone marrow from healthy donor mice of a different genotype. Macrophages and other types of blood cells are derived from bone marrow progenitor cells. A transplanted recipient mouse is chimeric and will produce new macrophages of the donor's genotype. The advantage of this type of study over systemic deletion of genes is the focus on macrophage physiology and the expression of genes by macrophage locally in the vessel wall. These studies isolate the contributions of macrophages to atherosclerosis. Bone marrow transplantation studies have been a powerful tool for understanding the roles of macrophages in the progression of atherosclerosis. Bone marrow transplantation and other types of studies have elucidated many roles for macrophages in the vessel wall: an inflammatory cell, an immune cell, a scavenger cell, and a cell that affects vascular lipid metabolism (Figure 1).

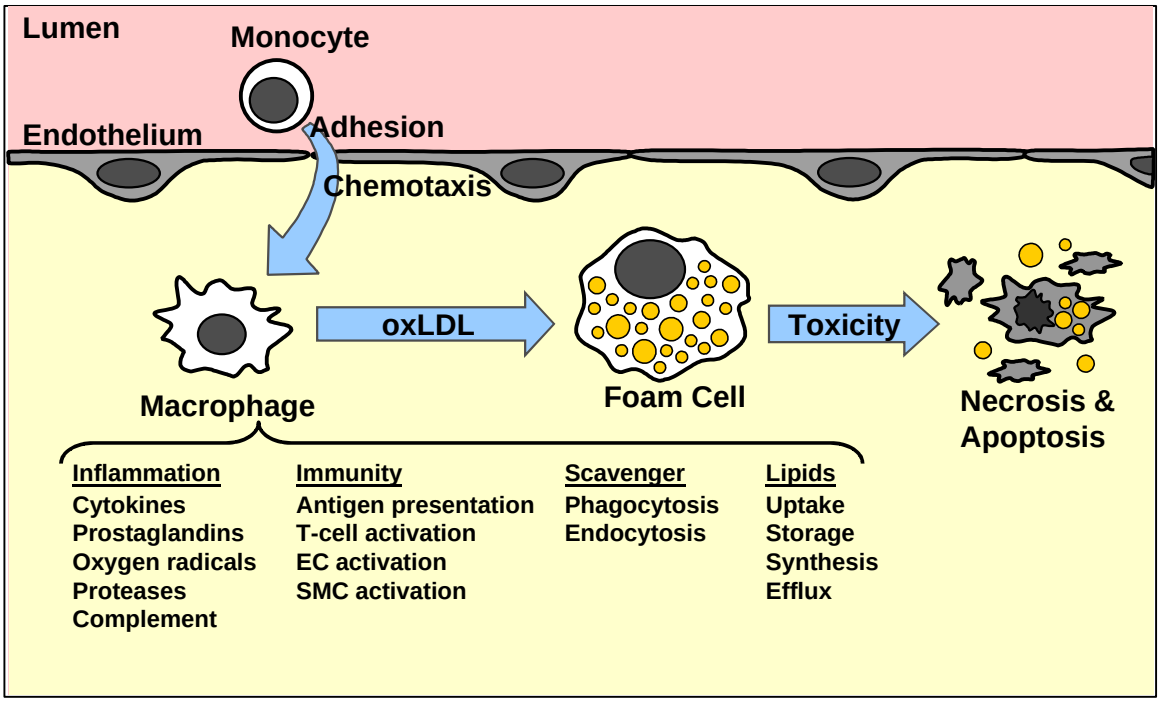


Figure 1. The Multiple Roles of Macrophages in Atherosclerosis. Macrophages in the vessel wall have many roles that affect the development of atherosclerosis. Macrophages act as inflammatory cells, an immune cells, scavenger cells, and cells that affects vascular lipid metabolism. Each of these roles influence macrophage cholesterol homeostasis and foam cell formation.

Role as an Inflammatory Cell

Atherosclerosis is not just a disease of lipid imbalance, it also has the characteristics of an inflammatory disease (Ross, 1999). Like other inflammatory diseases, atherosclerosis involves the initial injury, leukocyte attraction, release of chemical mediators, proteolytic degradation, and tissue repair.

Injury, Activation, Adhesion, and Chemoattraction. The initial injury in atherosclerosis is in many instances perpetuated by lipoproteins from the plasma. This injury triggers the activation of endothelial cells which up-regulate adhesion molecules. Leukocytes that are circulating in the plasma, including monocytes, interact with the endothelial adhesion molecules. Activated vascular endothelial cells express vascular cell adhesion molecule (VCAM)-1, intercellular adhesion molecule (ICAM)-1, P-selectin, and E-selectin. Monocyte/macrophages express cell adhesion molecules including very

late antigen (VLA)-1 integrin, β_2 integrins, P-selectin glycoprotein ligand (PSGL)-1, and sialyl Lewis^x (Osterud and Bjorklid, 2003). Adhesion molecules in the monocyte are up-regulated, leading to firm attachment and migration into the vascular wall. Chemoattraction is mediated by chemokines and other molecules including monocyte chemoattractant protein (MCP)-1 and interleukin (IL)-8 (Boisvert, 2004; Nelken et al., 1991). Maturation of monocytes into macrophages also occurs by stimulation with macrophage-colony stimulating factor (M-CSF) (Clinton et al., 1992; Rosenfeld et al., 1992).

Macrophage Activity. Macrophages orchestrate inflammation within the vessel wall. They do so by secreting cytokines, prostaglandins, oxygen radicals, proteases, and complement factors (Linton and Fazio, 2003). Macrophages also secrete matrix metalloproteinases (MMP) that digest structural proteins in the extracellular matrix, such as collagen (Galis et al., 1995; Galis et al., 1994). These MMPs can be grouped into gelatinases (MMP-2 and 9), interstitial collagenase (MMP-1), and stromelysin (MMP-3) (Galis et al., 1994). MMP activity leads to tissue remodeling, which is a characteristic of acute and chronic inflammation. Together, these activities put macrophages at the center of the changes that occur during vascular inflammation.

Inflammation and Macrophage-Induced Atherosclerosis. As an inflammatory cell, the macrophage has direct effects on atherogenesis. Studies designed to isolate the contributions of macrophages to atherosclerosis have focused on many mediators of inflammation (Table 1). These mediators, which are expressed by macrophages, have a wide range of inflammatory functions. For example, phospholipase A_2 (PLA_2) liberates fatty acids for the production of bioactive lipids like prostaglandins and leukotrienes (Webb et al., 2003). Cyclooxygenase 2 (COX-2) is an inducible enzyme that is rate limiting in the production of prostaglandins from arachidonic acid (Burleigh et al., 2002). 5-Lipoxygenase (5-LO) is the rate limiting enzyme in the production of leukotrienes (Mehrabian et al., 2002). Uncoupling protein 2 (UCP2) produces reactive oxygen species in cells (Blanc et al., 2003). CC-chemokine receptor 2 (CCR2) mediates signal transduction in response to the chemotactic cytokine, MCP-1 (Guo et al., 2003).

Plasminogen activator inhibitor-1 (PAI-1) inhibits fibrinolysis and may promote cell migration, cell proliferation, and matrix remodeling (Luttun et al., 2002). The growth suppressor p27 inhibits cell proliferation and migration (Diez-Juan et al., 2004). The tumor suppressor p53 inhibits cell proliferation and promotes cell death (Merched et al., 2003; van Vlijmen et al., 2001). Bax is a pro-apoptotic member of the Bcl-2 family of cell death regulators (Liu et al., 2004). Expression of each of these genes by macrophages affects the cellular processes involved in inflammation and changes the course of atherosclerosis.

Role as an Immune Cell

There is overlap between the role of macrophages as immune cells and their role as inflammatory cells. Macrophages participate in innate immunity by recognizing and phagocytizing pathogens (Gough and Gordon, 2000; Li and Glass, 2002). Macrophages also participate in acquired immunity by functioning as antigen-presenting cells (Gordon et al., 1995). In atherosclerosis, it is hypothesized that modified lipoproteins can be an immunologic stimulus (Boyd et al., 1989; Major et al., 2002). The role of the macrophages as an immune cell also involves the orchestration of responses from T-cells, endothelial cells, and smooth muscle cells.

T-lymphocyte Activation. Macrophages express major histocompatibility complex (MHC) class II, which is involved in antigen presentation and activation of T-lymphocytes via the T-cell antigen receptor (Libby et al., 1996). Activated T-lymphocytes secrete lymphokines that stimulate macrophages to release the cytokines IL-1 and tumor necrosis factor (TNF)-α.

Endothelial Cell Activation. Cytokines released by macrophages can activate endothelial cells (Libby et al., 1996). The endothelial cells secrete tissue factor when they are activated, which promotes coagulation. Activated endothelial cells also secrete plasminogen activator inhibitor, which blocks the fibrinolysis of clots. Cell adhesion molecules are at increased levels on activated endothelial cells (O'Brien et al., 1993).

Smooth Muscle Cell Activation. Cytokines released by macrophages can activate smooth muscle cells (Libby et al., 1996). This activation increases the secretion of enzymes that degrade the extracellular matrix and decreases the secretion of collagen from smooth muscle cells. Together, these changes contribute to tissue remodeling and make plaques less stable.

Table 1. Macrophage-Induced Atherosclerosis as Studied by Bone Marrow Transplantation Experiments

Bone Marrow Genotype	Aortic Plaques	Serum Cholesterol	(Ref.)
INFLAMMATORY FUNCTIONS			
Secretory PLA_2 (tng)	↑ 75%	↔	(Webb et al., 2003)
COX2(-/-)	↓ 35%	↔	(Burleigh et al., 2002) *
5-LO(+/-)	↓ 95%	↔	(Mehrabian et al., 2002)
UCP2(-/-)	↑ 95%	↔	(Blanc et al., 2003)
CCR2(-/-)	↓ 85%	↔	(Guo et al., 2003)
PAI-1(-/-)	↑ 20%	↔	(Luttun et al., 2002)
p27(-/-)	↑ 85%	↔	(Diez-Juan et al., 2004)
p53(-/-)	↑125%	↔	(van Vlijmen et al., 2001)
"	↑ 80%	↔	(Merched et al., 2003)
Bax(-/-)	↑ 50%	↔	(Liu et al., 2004)
IMMUNE FUNCTIONS			
IL-5(-/-)	↑ 20%	↔	(Binder et al., 2004)
CD11b(-/-)	↔	↔	(Kubo et al., 2000)
CXCR2(-/-)	↓ 60%	↓ 30%	(Boisvert et al., 1998)
IKK2(-/-)	↑ 60%	↔	(Kanters et al., 2003)
SCAVENGER FUNCTIONS			
SR-A (overexpression)	↓ 20% (n.s.)	↓ 20%	(Van Eck et al., 2000a)
"	↓ 20% (n.s.)	↑ 20%	(Herijgers et al., 2000a)
SR-BI(-/-)	↑ 85%	↔	(Zhang et al., 2003a)
"	↑ 70%	↔	(Covey et al., 2003)
LIPID METABOLISM			
apoE(-/-)	↑ 900%	↔	(Fazio et al., 1997)

Bone Marrow Genotype	Aortic Plaques	Serum Cholesterol	(Ref.)
"	↑ 270%	↔	(Van Eck et al., 2000b)
apoE(+/+)	↓ 98%	↓ 70%	(Linton et al., 1995)
"	↓ (qual.)	↓ 80%	(Boisvert et al., 1995)
"	↓ (qual.)	↓ 80%	(Van Eck et al., 1997)
"	↓ 95%	↓ 90	(Van Eck et al., 2000b)
apoE(+/+) LDLR(-/-)	↓ 60%	↔	(Fazio et al., 2002)
apoAI(tng) apoE(-/-)	↓ 95%	↔	(Major et al., 2001)
"	↓ 95%	↔	(Ishiguro et al., 2001) #
"	↓ 30%	↔	(Su et al., 2003)
apoE3-(tng) apoE(-/-)	↓ 40%	↔	(Yoshida et al., 2001) #
apoE3-Leiden(tng) apoE(-/-)	↓ 30% (n.s.)	↓ 50% (tr.)	(Van Eck et al., 2000b)
apoE2(tng) apoE(-/-)	↓ 30% (n.s.)	↓ 50% (tr.)	(Van Eck et al., 2000b)
"	↔	↔	(Yoshida et al., 2001) #
apoEcys142(tng) apoE(-/-)	↑ 60%	↔	(Yoshida et al., 2001) #
LDLR(-/-)	↓ 65%	↔	(Herijgers et al., 2000b)
	↓ 65%	↔	(Linton et al., 1999)
LDLR(+/+)	↔	↔	(Boisvert et al., 1997)
"	↔	↔	(Linton et al., 1999)
"	↔	↔	(Herijgers et al., 1997)
ACAT1(-/-)	↑ 120%	↔	(Fazio et al., 2001)
ABCA1(-/-)	↑ 50%	↔	(Aiello et al., 2002)
"	X	↔	(Haghpassand et al., 2001)
PPARγ(-/-)	↑ 35%	↔	(Chawla et al., 2001)
aP2(-/-)	↓ 65%	↔	(Makowski et al., 2001)
LPL(-/-)	↓ 35%	↔	(Babaev et al., 2000)
"	↓ 55%	↔	(Babaev et al., 1999)
"	↓ 50%	↓ 50%	(Van Eck et al., 2000c)
HL(-/-)	↓ 65%	↔	(Nong et al., 2003)

Bone marrow transplantation and the genes that have been studied by this method are briefly described in the text. Bone marrow genotypes are compared to appropriate controls. For example, recipients of (-/-) marrow were compared with recipients of (+/+) marrow, while recipients of (+/+) marrow were compared with recipients of (-/-) marrow. Data is reported as percentage change relative to the appropriate control group: increase (↑), decrease (↓), no difference (↔), not reported (X), not significant (n.s.), transient change (tr.), and qualitative assessment (qual.). Changes were statistically significant unless otherwise noted. Genes can also be introduced by viral transduction (#) of bone marrow cells before transplantation. Fetal liver cell transplant (*) is another type of hematopoietic cell transplant.

Immunity and Macrophage-Induced Atherosclerosis. The role of the macrophage as an immune cell affects atherogenesis. Studies designed to isolate the contributions of macrophages to atherosclerosis have focused on many mediators of immunity (Table 1). These mediators, which are expressed by macrophages, function as ligands, receptors, and transcription factors in the signaling pathways for immunity. For example, IκB kinase 2 (IKK2) is an activator of NF-κB, an important pathway in innate and adaptive immune responses (Kanters et al., 2003). IL-5 stimulates B-cells to secrete immunoglobulin (Ig) M (Binder et al., 2004). CD11b is a subunit of the β_2-integrin, Mac-1, which mediates adherence and extravasation of leukocytes (Kubo et al., 2000). CXCR2 is the receptor for IL-8, which is a chemokine that is a mediator of acute inflammation (Boisvert et al., 1998). Expression of each of these genes by macrophages affects immunity and inflammation in the vascular wall.

Role as a Scavenger Cell

Macrophages are commonly referred to as scavenger cells because of their role in the clearance of lipoproteins, cells, bacteria, and tissue debris. Endocytosis is the process by which macrophages engulf and then degrade material. The different types of endocytosis include pinocytosis, phagocytosis, and patocytosis. Clearance of LDL and modified LDL is considered the major factor that contributes to the accumulation of intracellular cholesterol and foam cell transformation (Kruth, 2001; Kruth, 2002). Pinocytosis of LDL and modified LDL occurs by the receptor-mediated entry of monomeric lipoproteins into vesicles. Phagocytosis occurs with larger LDL aggregates and cellular debris. Patocytosis is a very distinctive type of endocytosis where very large aggregates of LDL are taken into an intracellular membrane labyrinth that remains connected to the surface. Receptors such as SR-A, SR-BI, and CD36 are important in the recognition of modified lipoproteins and many other ligands that macrophages scavenge. In addition to being scavengers of lipoproteins, macrophages have a role in the clearance of apoptotic and necrotic cells (Fadok et al., 2001). Apoptotic cells present specific phospholipids like phosphatidyl serine, oxidized phospholipids, and certain vitronectins,

that lead to receptor-mediated recognition and clearance by macrophages. Not only can scavenger receptors bind these apoptotic ligands, but they can also interact with receptors that have higher specificity for apoptotic ligands (Savill and Fadok, 2000).

Scavenger Functions and Macrophage-Induced Atherosclerosis. Studies designed to isolate the contributions of macrophages to atherosclerosis have focused on some macrophage scavenger receptors that are involved in binding and endocytosis of modified lipoproteins and cellular debris (Table 1). Each scavenger receptor is expressed by macrophages and has a wide ligand specificity. For example, SR-A is an oxLDL receptor but it also binds many other ligands (Herijgers et al., 2000a; Horiuchi et al., 2003; Van Eck et al., 2000a). SR-BI is an HDL receptor, but it also binds LDL, oxLDL and a wide range of other ligands, as well (Covey et al., 2003; Horiuchi et al., 2003; Zhang et al., 2003a).

Role in Vascular Lipid Metabolism

In the vascular wall, macrophages maintain cholesterol homeostasis by balancing lipoprotein uptake, cholesterol storage, cholesterol synthesis, and cholesterol efflux. Pathways of macrophage cholesterol homeostasis are discussed in detail in subsequent sections.

Vascular Cholesterol Metabolism and Macrophage-Induced Atherosclerosis. Studies designed to isolate the contributions of macrophages to atherosclerosis have focused on many mediators of cholesterol and lipoprotein metabolism (Table 1). Most of these mediators have a role in cholesterol transport. For example, both apoE and apoAI play important roles in lipoprotein metabolism with structural functions, ligand functions, and cholesterol acceptor functions (Fazio et al., 1997; Ishiguro et al., 2001; Major et al., 2001; Van Eck et al., 2000b). LDLR binds and mediates the uptake of LDL, IDL, and VLDL (Boisvert et al., 1997; Herijgers et al., 1997; Herijgers et al., 2000b; Linton et al., 1999). ACAT1 is the enzyme that esterifies cellular cholesterol so that it can be stored in lipid droplets (Buhman et al., 2000a; Fazio et al., 2001). ATP-Binding Cassette (ABC)-A1 is responsible for phospholipid and cholesterol efflux to apoAI (Aiello et al., 2002;

Haghpassand et al., 2001). Peroxisome proliferator-activated receptors (PPAR) are nuclear hormone receptors that are involved in cholesterol metabolism and inflammation (Chawla et al., 2001; Moore et al., 2001). The fatty acid binding protein aP2 is involved in the cellular metabolism of fatty acids (Makowski et al., 2001). Lipoprotein lipase (LPL) is involved in the de-esterification of triglycerides and cholesterol esters from lipoproteins (Babaev et al., 1999; Babaev et al., 2000; Van Eck et al., 2000c). Hepatic lipase (HL) hydrolyzes triglycerides and phospholipids in chylomicron remnants, IDL, and HDL (Nong et al., 2003). Each of these genes directly or indirectly affects cellular cholesterol homeostasis in macrophages.

The Pathways of Macrophage Cholesterol Homeostasis

The studies of cholesterol homeostasis presented here focus mainly on cholesterol efflux because this process is both a marker and a mediator of cholesterol balance (Rothblat et al., 1999). Three other processes that influence cellular cholesterol balance are cholesterol ester storage, cholesterol synthesis, and lipoprotein uptake. Cholesterol homeostasis in macrophages is of critical importance because these cells have a pivotal function in the vessel wall and in the development of atherosclerotic lesions. Disruption of one process of cholesterol balance results in compensation by others to maintain homeostasis (Figure 2). As the initial step of the reverse cholesterol transport (RCT) system, cholesterol efflux is a homeostatic process with great potential to affect atherosclerosis risk. Overburdening the compensatory processes leads to total disruption of homeostasis, foam cell formation, and apoptosis (Mitchinson et al., 1996).

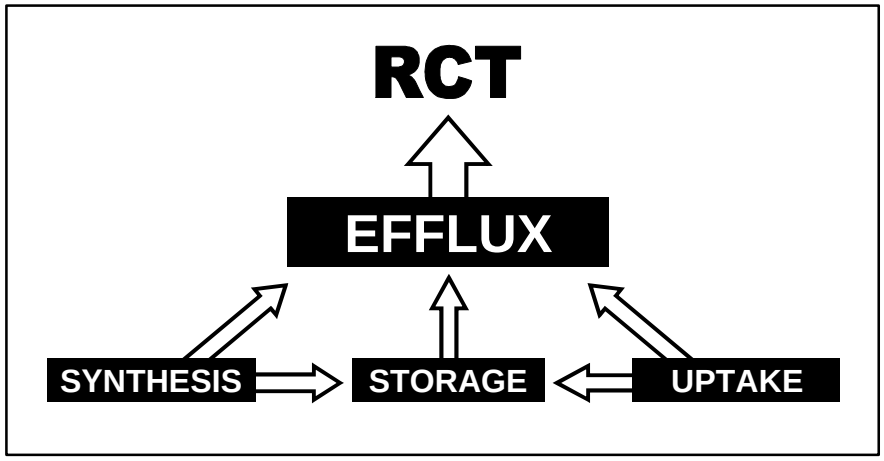


Figure 2. Cellular Cholesterol Homeostasis and the Reverse Cholesterol Transport System.

Cholesterol Storage

Free Cholesterol. Most cellular free cholesterol is incorporated into membranes but crystallization can occur when concentrations are too high (Kellner-Weibel et al., 1998; Kellner-Weibel et al., 1999). Regulation of the amount of cellular free cholesterol is accomplished by mediators such as sterol regulatory element binding protein (SREBP) and HMG-CoA reductase. Because free cholesterol is the substrate for ACAT, this enzyme lowers the concentration of free cholesterol in the cell. Free cholesterol is available for efflux, which also lowers cellular cholesterol concentrations.

Cholesterol Esterification and ACAT. Cholesterol is stored by ACAT enzymes in neutral lipid droplets (Buhman et al., 2000a; Buhman et al., 2001). By the enzymatic action of ACAT, free cholesterol is esterified with fatty acids. The sterol specificity of ACAT includes cholesterol and oxysterols (Zhang et al., 2003b). The specificity of ACAT for bile acid derivatives is relatively low (Cho et al., 2003). ACAT is allosterically activated by cholesterol and some oxysterols (Cases et al., 1998a; Cho et al., 2003). ACAT has broad specificity for long-chain fatty acids but the highest specificity is for oleic acid (Cases et al., 1998a).

ACAT has two isozymes, ACAT1 and ACAT2. In mice, the ACAT1 isozyme is found at high levels in macrophages and steroidogenic tissues, where it is involved in intracellular storage of cholesterol. ACAT2 is expressed in the liver and the small

intestines, where it is involved in absorption of dietary cholesterol and in lipoprotein assembly. The distribution is similar in humans, except that hepatocytes express both ACAT1 and ACAT2 (Buhman et al., 2000b; Chang et al., 2000; Lee et al., 2000).

The regulation of ACAT expression differs from other genes that are involved in cholesterol homeostasis. ACAT1, unlike many other mediators of sterol metabolism, does not have a promoter element for regulation by sterol regulatory element binding protein (SREBP) (Li et al., 1999). However, expression is increased by monocyte-macrophage differentiation, 1,25-dihydroxyvitamin D_3, and 9-*cis*-retinoic acid (Buhman et al., 2001; Maung et al., 2001).

ACAT Inhibition and ACAT Deletion. The identification of cholesterol storage within macrophages as one of the initial steps in atherosclerosis has prompted studies on pharmacologic inhibition of the enzymes involved in this process. Many studies have reported increased cellular cholesterol efflux with pharmacologic inhibition of ACAT activity (Kellner-Weibel et al., 1998; Mazzone and Reardon, 1994; Rodriguez et al., 1999; Warner et al., 1995; Zhang et al., 1996). However, there are many conflicting reports of both increased and decreased atherosclerosis in animal models following the administration of ACAT inhibitors (Buhman et al., 2000a). A study from our laboratory showed that hyperlipidemic mice reconstituted with *ACAT1(-/-)* macrophages have increased atherosclerosis (Fazio et al., 2001). The mechanisms behind this increase are currently under investigation, both in the studies presented here and in other studies within our laboratory. The effects of decreased cholesterol storage on macrophage cholesterol efflux and cholesterol homeostasis have only been studied with pharmacologic inhibitors of ACAT. Understanding the cellular consequences of the total absence of cholesterol storage by ACAT1 gene deletion has great significance because it may provide explanations for discrepancies between the effects of ACAT1 gene deletion and the effects of ACAT inhibition on atherogenesis.

Cholesterol Synthesis

Cholesterol synthesis occurs in many tissues and is tightly regulated (Hampton et al., 1996). HMG-CoA reductase is the rate-limiting enzyme in cholesterol synthesis. This

enzyme is regulated by feedback inhibition by cholesterol and other mevalonate metabolites, by hormonal sensitivity to insulin and glucagon, by sterol-mediated transcriptional regulation, and by competitive inhibitors of HMG-CoA reductase. Although most cholesterol in foam cells is likely to be derived from lipoprotein uptake, *de novo* cholesterol synthesis may contribute to internal cholesterol pools that lead to foam cell formation (Johnson et al., 1995; Mendez et al., 1991). Newly synthesized cholesterol is available for efflux to cholesterol acceptors (Johnson et al., 1995).

Cholesterol Uptake

Uptake of cholesterol occurs via the binding and uptake of native or modified lipoproteins by lipoprotein receptors including LDLR, SR-BI, cluster of differentiation antigen (CD)36, SR-A, heparan sulfate proteoglycans, and LRP (Herz and Strickland, 2001; Krieger, 2001; Linton and Fazio, 2001; Nicholson et al., 2001). The constitutive nature of SR-A expression and the feed-forward regulation of CD36 in macrophages lead to unregulated uptake of modified LDL and foam cell formation (Kita et al., 2001). The delivery of modified LDL to lysosomes following receptor-mediated uptake results in the hydrolysis of cholesterol esters and the processing of the free cholesterol. Accumulation of free cholesterol results in dysfunction of hydrolysis, which leads to lysosomal accumulation of both cholesterol esters and free cholesterol. Cholesterol in loaded lysosomes is resistant to efflux (Yancey and Jerome, 2001).

Scavenger Receptors. Macrophages are scavenger cells that take up and degrade lipoproteins and cellular debris in the walls of vessels. Research into scavenger receptors began with the study of a putative receptor for modified lipoproteins, later identified as SR-A (Goldstein et al., 1979). One characteristic of scavenger receptors is that they bind multiple ligands. Lipoprotein uptake is just one of the physiological functions of scavenger receptors (Krieger, 1997). This class of receptors includes SR-A, CD36, and SR-BI (Herz and Hui, 2004; Horiuchi et al., 2003).

LDL Receptor Family. LDLR is an essential mediator in cholesterol metabolism because it takes up apoB containing lipoproteins, like LDL, from plasma (Brown and

Goldstein, 1986; Goldstein and Brown, 1974; Wilkinson, 1950). LDLR binds apoE and apoB on LDL, IDL, and VLDL. Mutations in LDLR have been shown to be the cause of familial hypercholesterolemia (Goldstein and Brown, 1974). LDLR is down-regulated by cellular cholesterol via SREBP (Wang et al., 1993). The LDLR family is a group of receptors that share homology with LDLR. The receptor family has a broad range of functions and many members: LDLR, LRP, VLDLR, megalin, and apoE receptor 2 (apoER2) (Takahashi et al., 2003).

Proteoglycans. Heparan sulfate proteoglycans (HSPG) are high capacity, low affinity receptors on hepatocytes, macrophages, and other peripheral cells (Libeu et al., 2001; Saito et al., 2003; Wilsie and Orlando, 2003). HSPG binds apoE-containing lipoproteins including VLDL, IDL, and LDL as well as enzymes like lipoprotein lipase (Mahley, 1988). The uptake of lipoproteins is often a multi-step process. These steps include transfers of lipoproteins between HSPG and receptors like LDLR and LRP, followed by uptake and degradation.

Lipoprotein Internalization and Degradation. Lipoproteins are internalized by endocytosis after binding to receptors associated with clathrin-coated pits on microvilli or uncoated regions on the membrane ruffles (Kruth, 2001). Lipoproteins are taken into endosomes and then trafficked to lysosomes where degradation occurs. Lysosomal acid lipase hydrolyzes the cholesteryl esters on lipoproteins (Du and Grabowski, 2004; Werb and Cohn, 1972). In contrast to endocytosis, selective uptake of cholesteryl esters can occur by docking to receptors or uptake into surface-connected tubules (Rinninger et al., 1995; Rinninger and Greten, 1990).

Cholesterol Trafficking. Once free cholesterol dissociates from lipoproteins and cholesteryl esters have been hydrolyzed, lipoprotein-derived cholesterol is trafficked by Neiman-Pick protein to the plasma membrane (Reid et al., 2003; Soccio and Breslow, 2004). From the plasma membrane, cholesterol is trafficked to the endoplasmic reticulum where it can be esterified by ACAT. Cholesteryl esters are stored within neutral lipid droplets and are subsequently hydrolyzed by neutral cholesteryl ester hydrolase (NCEH).

In addition to these pathways, cellular cholesterol trafficking involves many sterol carrier proteins (Ioannou, 2001; Schroeder et al., 2001).

Cholesterol Efflux and Reverse Cholesterol Transport

Cholesterol homeostasis in macrophages is of critical importance in atherosclerosis research because these cells perform crucial functions in the vessel wall. One process by which macrophages maintain cholesterol balance is cholesterol efflux. As the initial step of the reverse cholesterol transport (RCT) system, cholesterol efflux is a homeostatic process with great potential to affect atherosclerosis risk. The importance of RCT in preventing atherosclerosis and the high degree of connectivity between the four processes of cholesterol homeostasis make cholesterol efflux the sentinel measurement of cholesterol balance in macrophages (Figure 2). Besides the notable exceptions of apoE deficiency and ABCA1 deficiency, very few studies have been performed to test the correlation of lesion development with cholesterol efflux from macrophages of genetically engineered atherosclerosis models. Bone marrow and fetal liver cell transplantation studies have led to the identification of many macrophage-specific factors that contribute to atherogenesis (Table 1). While much attention is given to the plasma components of the RCT system, most characterizations of these models fall short of characterizing cholesterol efflux from macrophages.

Cholesterol Efflux: Mechanisms and Mediators

Cholesterol efflux can occur by many mechanisms including diffusion, membrane microsolubilization, ABCA1-mediated efflux, receptor-mediated efflux, and lipoprotein assembly (Figure 3). Early investigations of cellular cholesterol metabolism by Rothblat and of the low density lipoprotein receptor (LDLR) by Brown and Goldstein have driven the study of cholesterol efflux (Ho et al., 1980; Rothblat and Phillips, 1986).

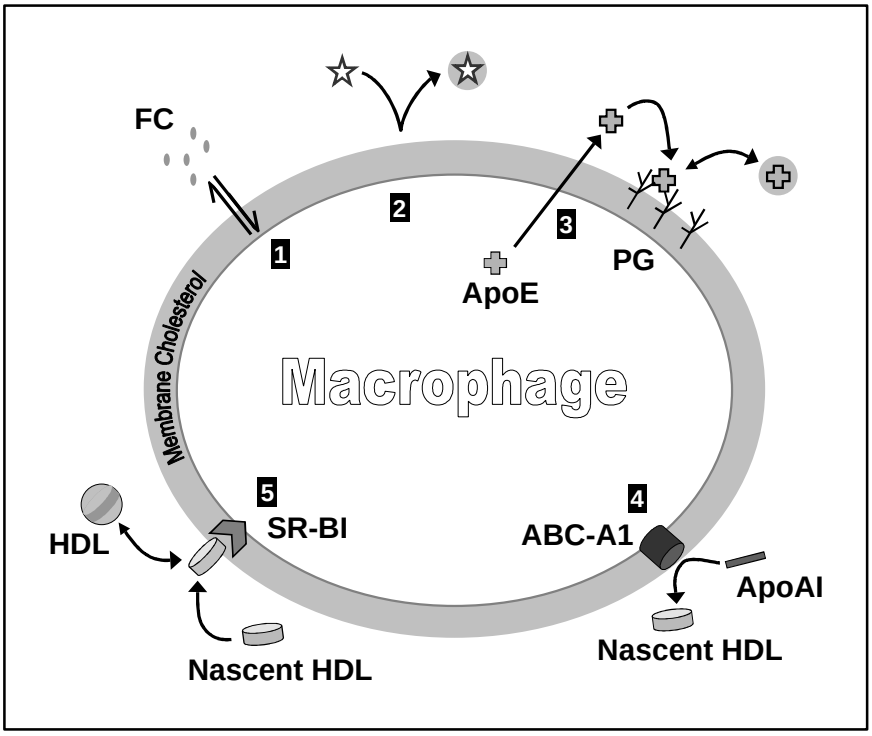


Figure 3. Mechanisms of Macrophage Cholesterol Efflux.
(1) Diffusion of Free Cholesterol, **(2)** Cholesterol Acceptors, **(3)** ApoE and Proteoglycans, **(4)** ApoAI and ABCA1 and **(5)** HDL and SR-BI.

Diffusion

Membrane cholesterol spontaneously enters the aqueous phase by a process known as desorption or diffusion (Rothblat and Phillips, 1982). Diffusion of membrane cholesterol into the aqueous extracellular space follows first-order kinetics and is reversible. Diffusion is thought to be the first step of efflux to most cholesterol acceptors.

Cholesterol Acceptors

Cholesterol efflux is stimulated by cholesterol acceptors, which provide a hydrophobic environment for cholesterol while remaining aqueous. The common structural organization of cholesterol acceptors is a hydrophobic core or pocket surrounded by a hydrophilic shell. This is the structural organization of phospholipid vesicles, apolipoproteins, lipoproteins, cyclodextrins, and albumin. Receptor binding, microsolubilization of membranes, and diffusion are mechanisms of interaction between acceptors and the cholesterol in cellular membranes (Rothblat et al., 1999). Important

work with apolipoproteins, phospholipid vesicles, and cyclodextrins determined that the relationship between acceptor concentration and cholesterol efflux is linear but saturable (Rothblat and Phillips, 1986; Yancey et al., 1996).

Apolipoproteins

Research in the field of cholesterol efflux has focused on two cholesterol acceptors that are important in the prevention of atherosclerosis, apoE and apoAI. Previous studies to investigate the protective roles of macrophage-derived apolipoproteins have not characterized the effects on macrophage cholesterol efflux or macrophage cholesterol homeostasis. The association of certain lipoproteins with either increased or decreased atherosclerosis risk has led to research on the functions, structure, and metabolism of apolipoproteins. One important class of apolipoproteins, known as exchangeable apolipoproteins, has important anti-atherogenic properties. These exchangeable apolipoproteins, including apoE and apoAI, have common secondary structural elements that facilitate common functional characteristics (Hara and Yokoyama, 1991; Segrest et al., 1992). Targeted mutagenesis and other types of studies have shown that amphipathic alpha helices are necessary for the cholesterol acceptor functionality of these apolipoproteins (Hara et al., 1992). Understanding the nature of the protective functions of apolipoproteins as locally synthesized cholesterol acceptors has great significance because it will provide validation for therapeutic efforts to deliver apolipoproteins to lesions through drug therapy, viral gene therapy, and bone marrow transplantation.

Apolipoprotein E. Apolipoprotein E is a cholesterol acceptor that is a protein constituent of very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL), and high density lipoproteins (HDL) (Mahley, 1988; Mahley and Rall, 2000). Receptors with specificity for apoE-containing particles include LDLR, proteoglycans, and LDLR related protein (LRP). Hepatocytes, adipocytes, and macrophages synthesize apoE (Basu et al., 1981; Jeejeebhoy et al., 1975). Macrophage apoE has been shown to protect against atherosclerosis (Fazio et al., 1997; Linton et al., 1995). Endogenous synthesis of apoE in J774 cells, a macrophage-like cell line, results in increased

cholesterol efflux (Lin et al., 1999). These studies also determined that endogenous apoE resulted in more efflux from the cells than similar concentrations of exogenous apoE. Furthermore, endogenous apoE synthesis increases cholesterol efflux to exogenous acceptors such as phospholipid vesicles, cyclodextrins, and albumin. Other studies show that interactions with proteoglycans are necessary for the endogenous apoE-mediated efflux (Lin et al., 2001).

Apolipoprotein AI. Apolipoprotein AI is a cholesterol acceptor that is the main protein constituent of HDL (Atmeh et al., 1983). ApoAI is synthesized by hepatocytes, but in contrast to apoE, it is not synthesized by macrophages (Basu et al., 1981). Plasma apoAI and HDL have been shown to protect against atherosclerosis (Gordon and Rifkind, 1989). There is an inverse relationship between plasma HDL and risk for coronary artery disease (Wilson et al., 1988). Like macrophage apoE, macrophage expression of transgenic apoAI has been shown to be protective (Ishiguro et al., 2001; Major et al., 2001; Su et al., 2003).

Exchangeable Apolipoproteins. Although apoE and apoAI are the main mediators of cholesterol efflux from macrophages in the vessel wall, many other apolipoproteins act as cholesterol acceptors. The exchangeable apolipoproteins have similar structural and functional characteristics (Saito et al., 2004). This group includes apoE, apoAI, apoAII, apoAIV, apoAV, apoCI, apoCII, and apoCIII. These apolipoproteins have amphipathic helices that are able to solubilize phospholipids and cholesterol. All of these apolipoproteins are substrates for ABCA1-mediated cholesterol efflux, and some accept cholesterol independently of the ABCA1 pathway (Bortnick et al., 2000; Remaley et al., 2001). Efflux to apoE, for example has been reported to occur by mechanisms that are independent of ABCA1 (Huang et al., 2001).

HDL, ATP Binding Cassette-A1, and Tangier Disease

Studies of ABCA1 suggest that it adds phospholipids to apoAI (Fielding et al., 2000; Wang et al., 2001). ApoAI binding studies suggest that ABCA1 is a phospholipid flippase that changes the local phospholipid content of the outer membrane leaflet in a

manner that favors the association of apoAI with the membrane (Chambenoit et al., 2001). Although apoAI is thought to be the physiological acceptor for ABCA1, other apolipoproteins and amphipathic peptides can accept cholesterol from ABCA1 (Bortnick et al., 2000). The local importance of ABCA1 in the vessel wall is not fully understood, but systemically, ABCA1 is a critical part of the RCT system (Aiello et al., 2002; McNeish et al., 2000). Tangier disease is a classic example of how decreased HDL and apoAI can dramatically increase atherosclerosis in humans (Assmann et al., 1977; Henderson et al., 1978; Mautner et al., 1992; Schmitz et al., 2000). Mutations in the *ABCA1* gene cause this inherited disease. Tangier patients lack the ability to add phospholipids to apoAI. Lipid-free apoAI is quickly cleared from plasma by the kidneys. Low plasma apoAI concentrations are associated with increased atherosclerosis (Gordon and Rifkind, 1989; Gordon et al., 1977). Not only is phospholipid-associated apoAI resistant to clearance, it is a very efficient acceptor of cellular cholesterol.

With increased association of cholesterol and phospholipids, apoAI progresses to nascent HDL, to pre-beta HDL, and then to alpha HDL. Nascent HDL has two apoAI proteins surrounding a small unilamellar phospholipid bilayer and has a discoidal shape (Segrest et al., 1999). Spherical alpha HDL has two to four apoAI proteins and a more complex lipid composition. The maturation of HDL structure and composition is mediated by ABCA1 which adds more cellular lipids and SR-BI which docks HDL to cells for cholesterol and cholesteryl ester exchange. HDL is also modified by lecithin: cholesterol acyltransferase (LCAT) which esterifies HDL cholesterol and phospholipid transfer protein (PLTP) which transfers lipids between HDL and other lipoproteins (Segrest et al., 2000).

Receptor-Mediated Docking

Receptor-mediated docking allows for the transfer of lipids between cells and lipoproteins without uptake and degradation. Scavenger receptor (SR)-BI and proteoglycans mediate this type of interaction. SR-BI allows HDL to dock at the plasma membrane and exchange cholesterol along a concentration gradient. Docking facilitates bi-directional exchange of cholesterol between the cell and HDL particles (Yancey et al., 2000). Selective uptake of cholesteryl esters also occurs via interactions between SR-BI

and HDL (Connelly et al., 2001). Docking of apoE to proteoglycans facilitates cholesterol efflux by preventing escape from the cell surface (Lin et al., 2001). Proteoglycans are low-affinity receptors for apoE but have a high capacity on the cell surface.

Oxysterols and Liver X Receptor

Liver X receptor (LXR) is a transcription factor that up-regulates many cholesterol homeostasis genes including *apoE* and *ABCA1* (Millatt et al., 2003). Oxysterols are the endogenous ligands for LXR. LXR is a transcription factor that binds oxysterols and forms heterodimers with retinoid X receptor (RXR) to regulate genes with LXR response elements. Genes under the control of LXR perform functions that include cholesterol efflux (e.g. ABCA1 and apoE), cholesterol ester metabolism (e.g. cholesterol ester transfer protein), cholesterol synthesis (e.g. SREBP-2 and HMG-CoA synthase), fatty acid synthesis (e.g. SREBP-1c), and inflammation (e.g. TNFα). Together, oxysterols from modified lipoproteins and LXR control a coordinated response that includes apoE-mediated and ABCA1-mediated cholesterol efflux.

Summary

Atherosclerosis is characterized by the failure of cholesterol homeostasis in macrophages within vessel wall lesions (Figure 4). Cholesterol-filled macrophage foam cells are the hallmark of this disease. Cholesterol efflux is an important marker and mediator of macrophage cholesterol homeostasis because it is the first step of the reverse cholesterol transport system. We hypothesize that macrophages with genotypes that are known to affect the progression of atherosclerosis, will have changes in cholesterol efflux and cellular cholesterol homeostasis. The studies presented here investigate whether cholesterol storage deficits cause changes in cholesterol efflux. We also investigate the mechanisms by which endogenously synthesized cholesterol acceptors produce increases in macrophage cholesterol efflux.

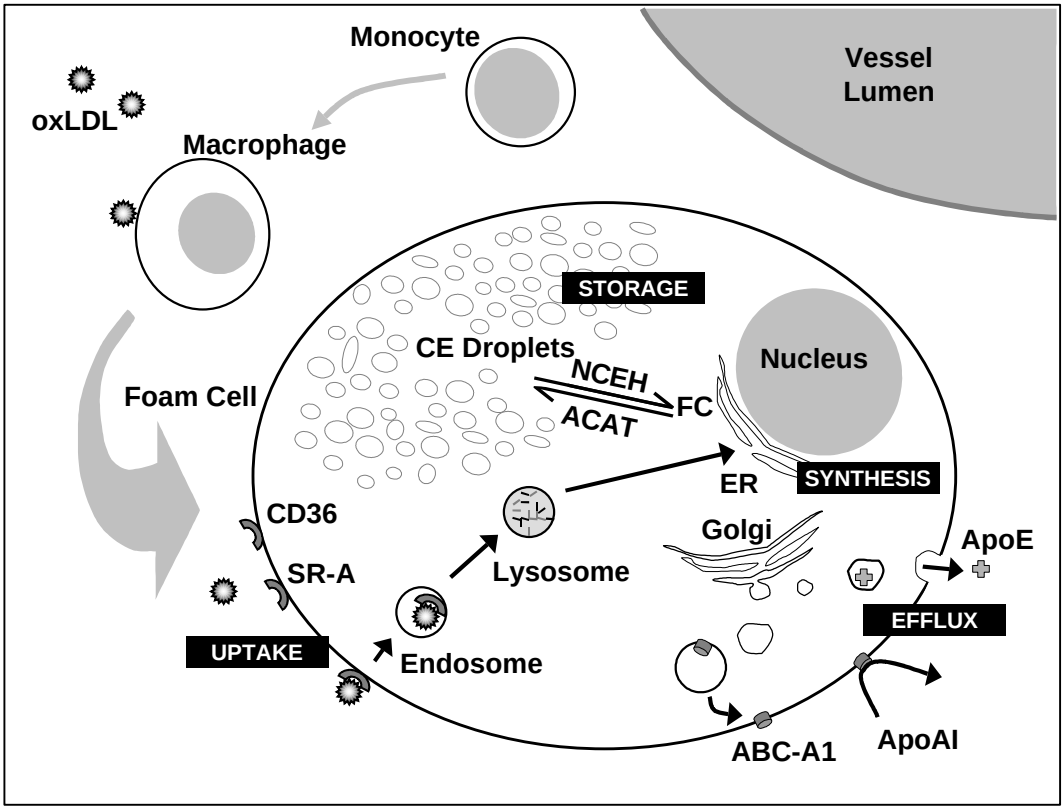


Figure 4. Overview of Macrophage Cholesterol Homeostasis: Cholesterol Uptake, Storage, Synthesis, and Efflux.

CHAPTER III

DEVELOPMENT OF EXPERIMENTAL PROTOCOLS AND DATA ANALYSIS SOFTWARE FOR ASSAYING CHOLESTEROL EFFLUX

Abstract

Objective. The study of cholesterol efflux began in the 1960's and 1970's with studies of cellular cholesterol metabolism and macrophage foam cell formation. The efflux of cholesterol to cholesterol acceptors is the first step in the reverse cholesterol transport system and is considered to be anti-atherogenic. The central role of macrophages in the pathophysiology of atherosclerosis has made this cell type the primary focus of most studies of cholesterol efflux. The objective of the current study was to develop experimental protocols for assaying cholesterol efflux from macrophages. The aim was to apply these cholesterol efflux protocols to the study of cholesterol storage deficits and the study of endogenously synthesized cholesterol acceptors.

Developments. Through reviews of the literature and further development, we have designed original protocols for assaying cholesterol efflux with ^{3}H-cholesterol as a tracer. Cultured macrophages are loaded with modified lipoproteins and labeled with ^{3}H-cholesterol. After extensive washes, cholesterol efflux is initiated by the addition of various cholesterol acceptors. Derivations of this type of protocol were made in order to facilitate larger experiments, to study the autocrine and paracrine actions of cholesterol acceptors, and to study the effects of extracellular fluid mixing and kinetic energy on cholesterol efflux.

Conclusions. These protocols are efficient tools with which to probe the role of cholesterol efflux in macrophage cholesterol homeostasis. Progress and future developments are discussed.

Introduction

The central role of macrophages in the pathophysiology of atherosclerosis has made macrophages the primary focus of most studies of cholesterol efflux. As

macrophages take up modified lipoproteins, they transform into lipid-filled foam cells (Brown et al., 1979). Following efflux, cholesterol from peripheral cells is carried through the plasma on apolipoproteins (e.g. apoE and apoAI) and on lipoproteins (e.g. HDL) (Ho et al., 1980). Cholesterol is taken up by the liver and secreted as bile (Schwartz et al., 1978). This pathway is referred to as the reverse cholesterol transport (RCT) system. The efflux of cholesterol to cholesterol acceptors is the first step in the reverse cholesterol transport system and is, therefore, considered to be an anti-atherogenic event.

Literature on cholesterol efflux reveals that assaying cholesterol efflux with ^{3}H-cholesterol as a tracer has been the historical standard (Rothblat et al., 2002). A reproducible assay for cholesterol efflux and efficient data analysis techniques are necessary for accurate measurements of macrophage cholesterol efflux. The objective of the current study was to develop experimental protocols for assaying cholesterol efflux from macrophages. Through reviews of the literature on cholesterol efflux, we have designed original protocols for assaying cholesterol efflux with ^{3}H-cholesterol as a tracer. Derivations of this type of protocol were developed to facilitate larger experiments, to study the autocrine and paracrine actions of cholesterol acceptors, and to study the effects of extracellular fluid mixing and kinetic energy on cholesterol efflux. These protocols are efficient tools with which to probe the role of cholesterol efflux in macrophage cholesterol homeostasis.

Protocol Development: Results and Discussion

General ^{3}H-Cholesterol Efflux Protocols

Cholesterol efflux protocols from murine peritoneal macrophages are based on modifications to a general protocol from the laboratory of T. Mazzone (Table 2) (Lin et al., 1999). These modifications are based on published methodologies designed to test cholesterol efflux mediated by the ATP-Binding Cassette (ABC)-A1 transporter, diffusional efflux, efflux mediated by endogenously synthesized cholesterol acceptors, receptor-mediated efflux to acceptors, and pharmacologically induced changes in cholesterol efflux. The validity of these protocols has been tested by comparing results to

previously published results from other laboratories (Figure 5) (Bortnick et al., 2000; Lin et al., 1999). The protocols and techniques have been utilized and refined in our laboratory. Experiments with six samples (n=6) per group result in statistically interpretable data. Group means have relative standard deviations of 5-10%. These experiments are able to show that efflux changes of less than 1% of cellular ^{3}H-cholesterol counts are statistically significant by Student's t-test or ANOVA. This sample size (n=6) was calculated to detect a relative effect of 10-20% with an alpha value of 0.05 and a power of 0.8. Trends that are present in experimental data are reproducible in subsequent replicate experiments.

Table 2. Summary of a General Cholesterol Efflux Protocol

1. Labeling/Loading	2. Rinses & Equilibration	3. Cholesterol Efflux
^{3}H-Cholesterol	Rinse (3x)	Initiation of Efflux to Acceptors
acLDL	Equilibration (24 h)	Sampling (0,3, or 6 h)
Serum	Rinse (1x)	Analysis

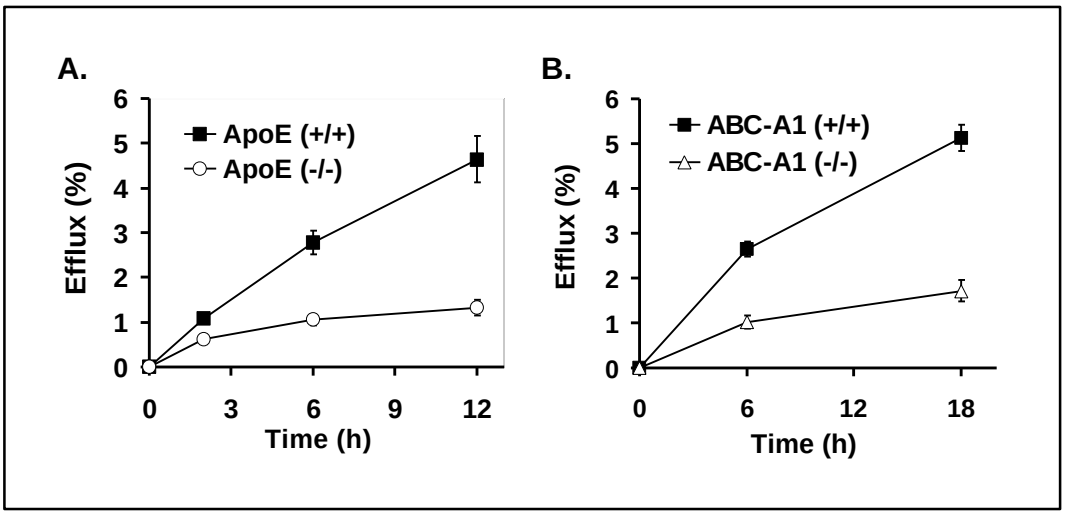


Figure 5. Macrophage Cholesterol Efflux Mediated by Endogenous ApoE and ABCA1.
Macrophages were treated for 36 hours with 1.5 μCi/ml of ^{3}H-FC and 70 μg/ml acLDL in DMEM/2% FBS to label cellular cholesterol. Cells were then cultured in efflux media. Bars and error bars represent the mean (n=6) and standard deviation of samples. **(A)** Effects of endogenous apoE synthesis on macrophage cholesterol efflux to bovine serum albumin (0.1%). **(B)** Effects of ABCA1 expression on macrophage cholesterol efflux to apoAI (10 μg/ml).

Protocol Summary. Elicited murine peritoneal macrophages are harvested and cultured on 24-well plates. To cholesterol-load cultured macrophages, culture media is replaced with loading media consisting of Dulbecco's Modified Eagle Medium (DMEM)/2% fetal bovine serum (FBS) containing 1.5-2.0 μCi/ml of ^{3}H-cholesterol and 70 μg/ml acLDL for 36 hours. After washing, monolayers are equilibrated for 4 hours and rinsed once. The efflux period is initiated by the addition of efflux media. A) *Diffusional Efflux*. To measure diffusional efflux, serum-free DMEM with 0.1% BSA is added to designated wells. B) *ABCA1-Mediated Efflux*. To measure efflux mediated by ABCA1, serum-free DMEM with 10 μg/ml human apoAI is added to designated wells. C) *Endogenous Acceptor-Mediated Efflux*. To measure efflux mediated by endogenously synthesized cholesterol acceptors, serum-free DMEM with no acceptors is added to designated wells.

During efflux experiments, samples are removed from each well at appropriate time intervals depending on the macrophage genotype, acceptor type, and acceptor concentration. ^{3}H-cholesterol counts are detected with a Beckman LS 6000IC scintillation counter after the removal of cell debris by centrifugation. Total cellular ^{3}H-cholesterol counts and total cellular protein masses are determined by rinsing and lysing labeled monolayers. Cholesterol efflux is calculated from the total supernatant counts (media) and is expressed as a percentage of the total cell counts (media and lysate). Experiments are performed with six samples for each unique experimental condition. Efflux data is analyzed with *Efflux 4.4* software.

96-Well Microtiter Plate Efflux Protocol

Basic modifications to the general efflux protocols (Table 2) were made in order to use 96-well culture plates for efflux assays. 96-well plates have 8 rows and 12 columns and wells are 6.4 mm in diameter with 0.32 cm^2 area per well. These plates support culture volumes from 50 to 250 μl per well. Plates can accommodate between $5x10^4$ and $1.5x10^5$ cells per well. The advantages of a 96-well efflux assay are that a greater number of conditions can be tested using fewer cells, less ^{3}H-cholesterol, and less modified LDL. Also, the use of multi-channel pipetors can increase manual efficiency. The disadvantages include greater variability among samples in each group, higher

concentrations of ^{3}H-cholesterol during labeling, and insufficient lipid mass for thin layer chromatography or gas chromatography analyses. Since the 96-well efflux assay allows for experiments with a larger number of groups, analysis software was expanded to process, compare, and summarize data from up to 48 experimental groups. This system has proven to be efficient and applicable to many studies (Chapter VI).

A typical experiment in a 96-well system compared unloaded macrophages to macrophages treated with 100 μg/ml acLDL. The 96-well system used approximately 70% fewer cells, 70% less acLDL, and 30% less ^{3}H-cholesterol compared to the same experiment performed in a 24-well system (Figure 6). 96-well efflux assays using eight samples per unique condition have relative standard deviations of 10-15% and yield reproducible results. This sample size (n=8) was calculated to detect a relative effect of 15-25% with an alpha value of 0.05 and a power of 0.8.

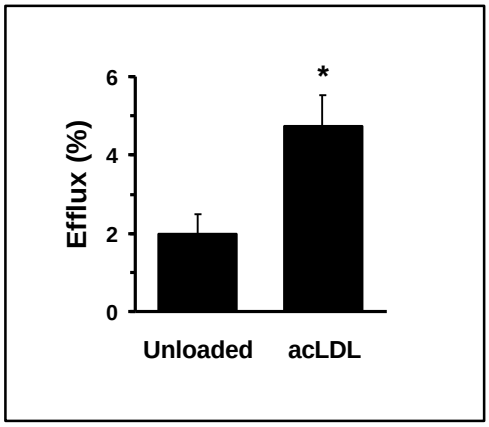


Figure 6. Macrophage Cholesterol Efflux in a 96-well Culture System. Experiments with 96-well system studying the effects of cholesterol loading used approximately 70% fewer cells, 70% less acLDL, and 30% less ^{3}H-cholesterol compared to the same experiment performed in a 24-well system. Macrophages ($7x10^{4}$ cells/well) were treated for 36 hours with 3.0 μCi/ml of ^{3}H-FC and 0 ("Unloaded") or 100 μg/ml acLDL ("acLDL") in DMEM/1% FBS to label cellular cholesterol. Cells were then cultured in efflux media with 0.1 % BSA for 6 hours. Bars and error bars represent the mean (n=8) and standard deviation of samples. Asterisk (*) denotes a statistically significant difference ($p<0.05$) compared to the unloaded group as determined by Student's *t*-test.

Efflux 4.4 Data Analysis Spreadsheet

Software, in the form of a Microsoft Excel™ spreadsheet, was designed in our laboratory to analyze, summarize, and present efflux data. The software includes data processing selections and sheets for computation, results and statistical summaries, graphs, and conclusions (Appendix, Figure 29). All results and graphs update automatically upon the entry of new data or with changes in data processing methodology. *Efflux 4.4* is augmented by macros, which are automated routines written using the Visual Basic for Applications language (Simon, 2002). This software is flexible enough to process data from most cholesterol efflux protocols and other types of kinetic experiments.

Volume-Dependence Efflux Protocol

A novel technique was developed with the aim of separating the autocrine and paracrine effects of cholesterol acceptors. For example, a single apoE protein can accept cholesterol from the macrophage that originally secreted it (i.e. autocrine effect) and then accept cholesterol from surrounding macrophages (i.e. paracrine effect). The terms "autocrine" and "paracrine" are traditionally applied to signaling peptides and hormones, but they have also been used to describe the actions of mediators with a broad range of functions besides signal transduction, including apoE (Kockx et al., 2004; Lafarga et al., 1994; Shimano et al., 1991). Understanding which of these mechanisms is more important for macrophages will give insight into the physiological importance of macrophage apoE. The fact that the phrase “autocrine and/or paracrine” can be found in so many papers emphasizes the point that traditional methodologies have not been very successful at separating these effects. The objective of the novel methodology presented here was to separate the autocrine and paracrine effects of macrophage apoE (Chapter VII).

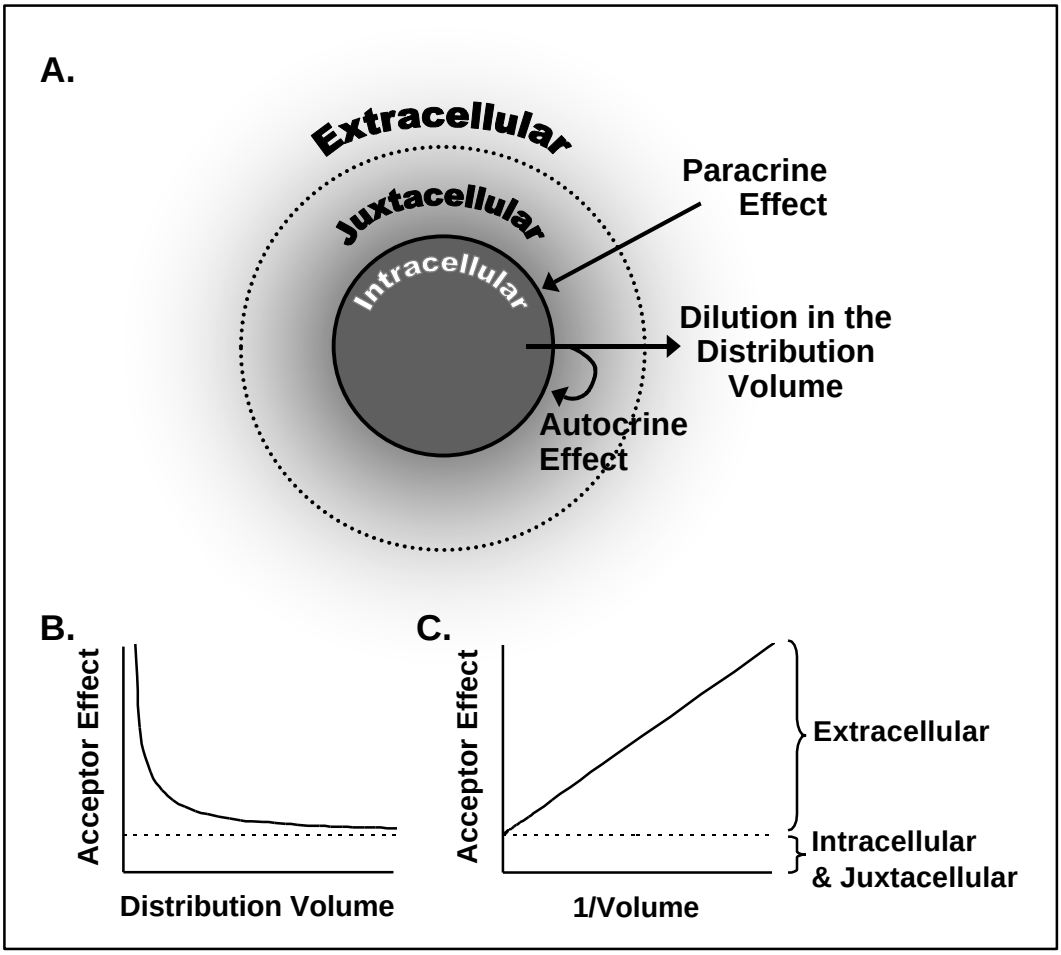


Figure 7. Theoretical Separation of the Autocrine and Paracrine Effects of Cholesterol Acceptors.
(A) Effects of an endogenous cholesterol acceptor that occur solely in the intracellular/juxtacellular space (i.e. autocrine effect) are independent of the extracellular distribution volume. Once an acceptor enters the extracellular space it is diluted in the distribution volume before it can affect another cell (i.e. paracrine effect). **(B)** The effect of an endogenous acceptor depends on the distribution volume. **(C)** The effect of an endogenous acceptor depends on its relative concentration (reciprocal volume).

Protocol Summary. We used the following strategy to create a dose-response curve for endogenously synthesized apoE. Increasing the distribution volume of an endogenously synthesized acceptor decreases the concentration and decreases acceptor-mediated efflux. The trend of this volume-dependent effect on efflux can be extrapolated to an infinitely large distribution volume where the acceptor is so dilute that the concentration is negligible. When the extracellular concentration of an acceptor is extremely low or when the distribution volume for the acceptor is extremely large, the

acceptor-mediated efflux is due only to intracellular or juxtacellular interactions of the acceptor with cellular cholesterol. Theoretically, the extracellular acceptor mechanisms of an endogenously synthesized cholesterol acceptor can be mathematically separated from the intracellular/juxtacellular mechanisms (Figure 7). Efflux experiments to measure volume-dependence have varying extracellular volumes of media with no exogenous acceptors (e.g. 0.3, 0.5, 0.75, 1.0, 1.5, or 2.0 ml for $4x10^5$ macrophages/well in a 24-well plate). The acceptor-specific effects are calculated as the difference between efflux from macrophages synthesizing the acceptor and efflux from acceptor deficient macrophages. Acceptor-specific efflux is plotted versus the reciprocal of the distribution volume. Since reciprocal volume is proportional to concentration, this plot is a relative concentration curve. Linear regression through the linear portion of the plot produces a line with a y-intercept that represents the intracellular/juxtacellular contribution of the acceptor. This methodology is described in more detail in Chapter VII.

Flow-Dependence Efflux Protocol

Mixing cultured cells causes the culture media to flow and creates kinetic energy that can stimulate cholesterol efflux (Yancey et al., 1996). The term 'mixing' as it is used here takes into account multiple processes including diffusion of acceptors through extracellular fluid, the flow of extracellular fluid, and turbulence within flowing extracellular fluid. Together diffusion, flow rate, and turbulence affect the efficiency of extracellular acceptors. A sigmoidal mixing trend created by increasing volumes of fluid in a cell culture dish (Figure 8A) was also present as a sigmoidal anomaly in cholesterol efflux data (Figure 8B). These data are indirect evidence of a correlation between mixing within the extracellular space and cholesterol efflux.

Protocol Summary. Colorimetric mixing assays were used to demonstrate the mixing parameters of the culture plates used for cholesterol efflux assays. These cell-free mixing assays consisted of 1 µl of glycerol-based loading dye (bromophenol blue and xylene cyanole) placed at the bottom of increasing volumes of fluid in 24-well culture plates (duplicates of 0.3, 0.5, 1.0, 1.5, and 2.0 ml of distilled water). Plates were oscillated on a Thermolyne™ Roto Mix orbital mixer at a constant speed for 2 minutes.

Mixing of the dye with the fluid was measured spectrophotometrically from samples of the fluid (540 nm absorption). Mixing was expressed as the percentage of the absorbance after 2 minutes oscillation compared to the absorbance after equilibrium is reached. Although the speed of the oscillating plates were the same for all of the fluid volumes, a sigmoidal trend in mixing was produced by differences in the fluid dynamics and the fluid velocities of columns of fluid with increasing heights.

Cholesterol efflux from macrophages was measured in experiments that are analogous to the cell-free mixing assays. Cholesterol efflux was measured from unloaded peritoneal macrophages in increasing volumes of serum-free media with 0.1% BSA (quadruplets of 0.3, 0.5, 1.0, 1.5, and 2.0 ml). Plates were mixed on an oscillator at a constant speed for 1 minute every 10 minutes over a total period of 6 hours. Efflux was measured according to the general cholesterol efflux protocol.

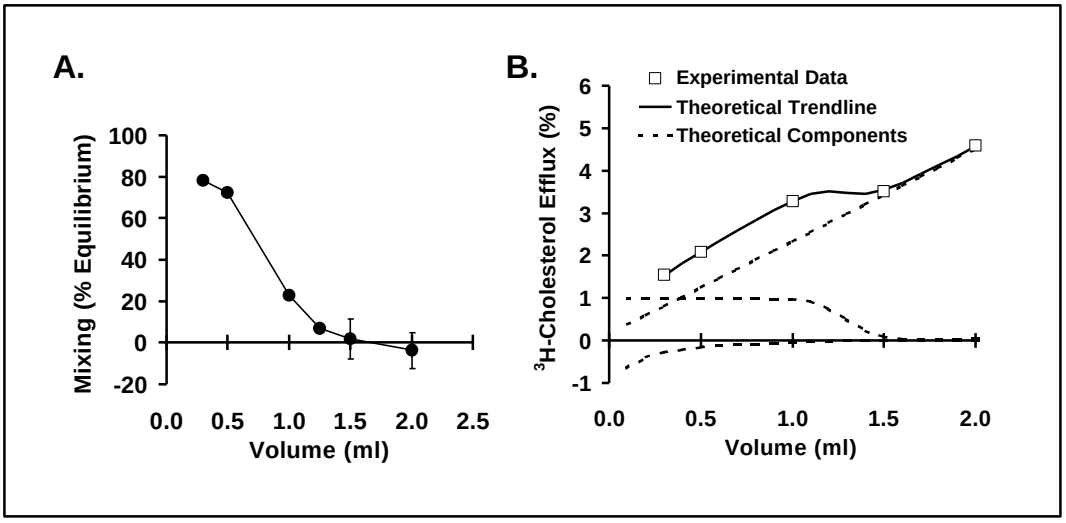


Figure 8. Mixing in the Extracellular Space and Macrophage Cholesterol Efflux.
(A) Effects of culture volume on mixing in a colorimetric cell-free mixing assay. Loading dye (1 µl) was place in the bottom of culture plates with orbital mixing. The mixing of the fluid were measured spectrophotometrically. Bars and error bars represent the mean (n=2) and standard deviation of samples. **(B)** Effects of culture volume on cholesterol efflux from macrophages with mixing. Macrophages were treated for 36 hours with 1.5 µCi/ml of ^{3}H-FC and 70 µg/ml acLDL in DMEM/2% FBS to label cellular cholesterol. Cells were then cultured in efflux media (0.1% BSA) with orbital mixing. Bars and error bars represent the mean (n=4) and standard deviation of samples.

Summary

In summary, we describe protocols for assays for macrophage cholesterol efflux. These protocols were further modified to focus on larger experimental capacity and greater experimental efficiency. Other developments include software for data analysis, protocols to study the autocrine and paracrine effects of cholesterol acceptors, and protocols to study the effects of extracellular fluid mixing on cholesterol efflux.

CHAPTER IV

THE EFFECTS OF CHOLESTEROL STORAGE DEFICITS ON CHOLESTEROL EFFLUX

Abstract

Objective. Acyl-coenzyme A: cholesterol acyltransferase (ACAT) converts intracellular free cholesterol (FC) into cholesteryl esters (CE) for storage in lipid droplets. Recent studies in our laboratory have shown that the deletion of the macrophage *ACAT1* gene results in apoptosis and increased atherosclerotic lesion area in the aortas of hyperlipidemic mice. The objective of the current study was to elucidate the mechanism of the increased atherosclerosis.

Methods and Results. CE storage and FC efflux were studied in *ACAT1(-/-)* peritoneal macrophages that were treated with acetylated low density lipoprotein (acLDL). Our results show that efflux of cellular cholesterol was reduced by 25% in *ACAT1(-/-)* cells compared to wildtype controls. This decrease occurred despite the up-regulated expression of ABCA1, an important mediator of cholesterol efflux. In contrast, ACAT1 deficiency increased efflux of the cholesterol derived from acLDL by 32%. *ACAT1(-/-)* macrophages also showed a 26% increase in the accumulation of FC derived from acLDL, which was associated with a 75% increase in the number of intracellular vesicles.

Conclusions. Together, these data show that macrophage ACAT1 influences the efflux of both cellular and lipoprotein-derived cholesterol and propose a pathway for the pro-atherogenic transformation of *ACAT1(-/-)* macrophages (Dove et al., 2005b).

Introduction

The development of atherosclerosis is influenced by abnormalities in cellular cholesterol homeostasis. Acyl-coenzyme A: cholesterol acyltransferase (ACAT) is responsible for the storage of cholesteryl ester (CE) within neutral lipid droplets in macrophage foam cells (Buhman et al., 2000a). The ACAT1 isoform is found at high

levels in macrophages and steroidogenic tissues. The ACAT2 isoform is expressed only in the liver and the small intestine and is involved both in the absorption of dietary cholesterol and in the assembly of lipoproteins. Inhibition of cholesterol esterification in macrophages is expected to slow down foam cell formation and decrease lesion size by blocking the storage of cholesterol and facilitating cholesterol efflux. However, recent studies in our laboratory have shown that the deletion of the *ACAT1* gene in macrophages results in an increase in atherosclerotic lesion area in the aortas of hyperlipidemic mice (Fazio et al., 2001).

Apolipoproteins (apo) E and AI, and the ATP-Binding Cassette (ABC) A1 are the main modulators of cholesterol efflux in macrophages (Aiello et al., 2002; Linton et al., 1995; Major et al., 2001). ABCA1 is responsible for the movement of phospholipids and cholesterol to apoAI, which results in the formation of nascent HDL particles (Fielding et al., 2000; Wang et al., 2001). Although the local importance of ABCA1 specifically within the vessel wall is not completely understood, systemically ABCA1 is a critical part of the reverse cholesterol transport (RCT) system (Aiello et al., 2002; McNeish et al., 2000). Tangier disease, caused by mutations in the ABCA1 gene, results in severely decreased HDL and apoAI levels, and accelerates atherosclerosis (Schmitz et al., 2000).

Both CE storage and FC efflux are important physiologic markers of cholesterol balance in macrophages. Pharmacologic inhibition of ACAT has been shown to increase cholesterol efflux in many studies (Kellner-Weibel et al., 1998; Mazzone and Reardon, 1994; Rodriguez et al., 1999; Warner et al., 1995; Zhang et al., 1996). However, there are conflicting reports of both increased and decreased atherosclerosis in animal models following the administration of ACAT inhibitors (Buhman et al., 2000a). In our previous study, the worsening effect of *ACAT1* gene deletion in hyperlipidemic mice may have been related to increased macrophage apoptosis induced by FC accumulation (Fazio et al., 2001; Kellner-Weibel et al., 1999; Warner et al., 1995). In the current studies, we labeled cellular cholesterol to measure the effects of ACAT1 deficiency on CE formation and FC efflux from macrophages. The mild cholesterol loading conditions used in these studies are capable of perturbing cholesterol homeostasis without inducing toxicity. The goal of this investigation was to determine whether ACAT1 deficiency in macrophages was associated with changes in cholesterol efflux, cholesterol storage, or cellular

morphology that would be consistent with increased susceptibility to atherosclerosis. Our results suggest that ACAT1 deficiency disrupts the efflux of cholesterol and affects cholesterol homeostasis prior to any toxic cellular effects (Dove et al., 2005b).

Methods

Primary Culture of Peritoneal Macrophages. Murine peritoneal macrophages were elicited by intraperitoneal injection of 3% thioglycollate. Macrophages were harvested 3-4 days after injection by peritoneal lavage with ice-cold Dulbecco's Modified Eagle Medium (DMEM). Macrophages were washed, counted, and plated in DMEM with 10% fetal bovine serum (FBS) at 37°C. After 2-6 hours of culture to allow adherence to culture plates, non-adherent cells were aspirated and macrophage monolayers were culture in DMEM with 4% FBS at 37°C.

Lipoprotein Preparation, ^{3}H-Cholesteryl Oleate Association, and Chemical Modification. Low density lipoproteins (LDL, density = 1.019 g/ml to 1.063 g/ml) were isolated from human plasma by sequential centrifugation. Briefly, human plasma from normolipemic adults were adjusted to a density of 1.019 g/ml with sodium chloride. The solution was centrifuged at 100,000 RPM for 3-4.5 hours in a Beckman TLN 100 rotor with a Beckman-Coulter Optima™ TLX ultracentrifuge. Lipoproteins with density greater than 1.019 g/ml were isolated and this solution was adjusted to 1.063 g/ml with sodium chloride for another round of centrifugation. The LDL between the density of 1.019 and 1.063 g/ml was dialyzed in "lipoprotein buffer" (0.1 M sodium chloride and 0.3 mM EDTA). Incorporation of ^{3}H-cholesteryl oleate into LDL was performed by incubating LDL with ^{3}H-cholesteryl oleate in a dimethylsulfoxide solution (Brown et al., 1975; Johnson et al., 1990). Acetyl-LDL (acLDL) was prepared by repeated addition of acetic anhydride to LDL in a sodium acetate solution (Basu et al., 1976). All lipoproteins were dialyzed in lipoprotein buffer prior to use in cell culture experiments. Lipoprotein species were confirmed by electrophoretic mobility. Protein concentrations were determined by a modified Lowry assay (Lowry et al., 1951).

^{14}C-Adenine Release Assay for Cellular Toxicity. Cholesterol-induced toxicity in macrophages was assayed by measuring the leakage of ^{14}C-adenine into media (Warner et al., 1995). Murine peritoneal macrophages were elicited with thioglycollate and were harvested. Cells were labeled in DMEM with 0.4 μCi/ml ^{14}C-adenine (Amersham) for 3 hours. Cells were rinsed three times with DMEM. The release period was initiated by the addition of loading media with DMEM/1% FBS and 70 μg/ml acLDL. Loading media was removed following release periods of 24 or 48 hours and cell debris was removed by centrifugation. Remaining cellular ^{14}C-adenine was harvested by rinsing cells with PBS and then lysing cells with 1.0 ml of 0.1N sodium hydroxide. Sample aliquots were loaded into Ecolite™ scintillation fluid (ICN, Costa Mesa, CA) and ^{14}C-Adenine counts were detected with a Beckman LS 6000IC scintillation counter. Adenine release was calculated from the media ^{14}C-adenine counts and expressed as a percentage of the total counts (lysate plus media).

Quantitation of Sterol Mass in Cultured Macrophages. Cellular lipids were extracted by the Bligh-Dyer method and dried under nitrogen (Bligh and Dyer, 1959). Cholesterol mass was determined by gas chromatography (Fazio et al., 2001).

Efflux of Cholesterol Mass. Macrophages were cultured for 36 hours in DMEM/1% serum with 70 μg/ml acLDL. Cells were rinsed and efflux was initiated by the addition of 10 μg/ml lipid-free human apoAI in DMEM. After 18 hours of efflux, cellular lipids were extracted with isopropanol overnight and the total cholesterol mass was determined by gas chromatography with cholesterol methyl ester as an internal standard (Yancey and Jerome, 2001). This procedure was performed as described by Ishikawa et al. (Ishikawa et al., 1974) and as modified by Klansek et al. (Klansek et al., 1995). The cholesterol mass remaining after the efflux period was expressed as a percentage of the mass of parallel samples harvested before the efflux period.

Efflux of Radiolabeled Cellular or Lipoprotein-Derived Cholesterol. Macrophages were labeled by two different methods of sterol delivery, either to preferentially label cellular (membrane) cholesterol or to label lipoprotein-derived

cholesterol (Chen et al., 2001). [1,2-^{3}H(N)]cholesterol (^{3}H-FC) and [1,2,6,7-^{3}H(N)]cholesteryl oleate (^{3}H-CE) were obtained from Perkin Elmer Life Sciences.

Cellular cholesterol pools, including the plasma membrane and recycling endosomes, were targeted by incubation with 1.5 μCi/ml of ^{3}H-FC in DMEM/2% FBS for 36 hours (Lin et al., 1999; Mukherjee et al., 1998). The addition of 70 μg/ml unlabeled acLDL during the labeling period promotes cholesterol loading.

Lipoprotein-derived cholesterol contributed to lysosomes and late endosomes were targeted by labeling/loading with 70 μg/ml ^{3}H-CE/acLDL in DMEM/2% FBS for 36 hours (Yancey and Jerome, 2001). The uptake of ^{3}H-CE/acLDL by macrophages was calculated from ^{3}H-sterol counts and the specific activity of ^{3}H-CE/acLDL (cpm/mg acLDL). The turnover of ^{3}H-CE/acLDL by macrophages was measured by the appearance of ^{3}H-FC in loading media. Cholesterol storage as ^{3}H-FC and ^{3}H-CE was measured in lysate.

Labeled macrophages were rinsed and the efflux period was initiated by the addition of efflux media with 10 μg/ml lipid-free human apoAI in DMEM. Efflux media was removed following the efflux period and cell debris was removed by centrifugation. Remaining ^{3}H-sterol and cellular protein were harvested by rinsing and lysing cells with 0.1N sodium hydroxide. ^{3}H-Sterol counts in media samples and lysate samples were detected by scintillation. Cholesterol efflux in media was expressed as a percentage of the total counts (lysate plus media).

Separation of Cholesterol by Thin Layer Chromatography. The samples were extracted by the Bligh-Dyer method (Bligh and Dyer, 1959), dried, and spotted on silica G thin layer chromatography plates (Alltech Associates). Plates were developed in 90:10:1 petroleum ether/ ethyl ether/ acetic acid and visualized with iodine vapor (Sigma Chemical Co.). FC and CE bands were scraped and counted by liquid scintillation.

Quantitation of ABCA1 mRNA and ABCA1 Protein. Cells were cultured for 36 hours in DMEM/1% FBS media with 70 μg/ml acLDL. Total RNA was isolated using Trizol reagent (Invitrogen). Relative quantification of ABCA1 mRNA was performed using a FAM-labeled TaqMan™ probe with the TaqMan™ One-Step RT-PCR Master

Mix reagent kit (Applied Biosystems) on an ABI Prism 7700 sequence detection system (Applied Biosystems) according to the method developed by Su et al. (Su et al., 2002). Relative quantification of ABCA1 was normalized with 18S ribosomal RNA as an internal control. The data were analyzed using the comparative C_T method.

For Western blot analysis of ABCA1 protein, cell extracts were separated by 3-8% NuPAGE™ Tris-Acetate gels (Novex) and transferred to nitrocellulose membranes. Murine ABCA1 was detected with a primary antibody (Novus Biological), visualized by a chemiluminescent ECL Plus™ (Amersham Pharmacia Biotech) according to Bortnick et al. (Bortnick et al., 2000), and quantified by densitometric analysis.

Transmission Electron Microscopy and Image Analysis. Macrophages were treated with DMEM/1% FBS for 48 hours with 0 or 70 μg/ml acLDL. Cellular morphology was characterized by transmission electron microscopy as described (Yancey and Jerome, 1998). Sections (80 nm) of Spurr-embedded macrophages where stained with uranyl acetate and lead citrate and visualized by a Philips CM12 transmission electron microscope operated at 80 keV. Twenty macrophages per group were randomly selected. The percentages of total cellular volume occupied by vesicles were determined by using point counting sterologic techniques (Weibel et al., 1966). Volume percentages were calculated from the number of points on vesicles as a percentage of total points on macrophages.

Immunofluorescence and Cytochemistry for Endosomes and Lysosomes. Macrophages were treated with 0 or 70 μg/ml acLDL in DMEM/1% FBS for 36 hours and then used for immunofluorescence or cytochemistry experiments. For immunofluorescence, macrophages were fixed, permeabilized, and treated with primary antibodies against the endosomal marker, EEA1 (Affinity BioReagents), or the lysosomal marker, LAMP1 (BioDesign Pharmingen). Cells were labeled with TRITC-conjugated secondary antibody (BioDesign Pharmingen). Fluorescence was visualized with a Zeiss Axioplan Imaging™ fluorescence microscope with digital camera and analyzed with MetaMorph™ 5.0 imaging software (Universal Imaging Corp.). The threshold was set to display the brightest 30% of positive pixels for EEA1 fluorescence and brightest 45% of

positive pixels for LAMP1 fluorescence. The area of fluorescence from five random fields was expressed as pixels per cell.

Cytochemistry for acid phosphatase in lysosomes was performed using Naphthol ASBI phosphate (Vaughan et al., 1971). Cells were fixed, washed with cacodylate buffer. Cells were stained with naphthol ASBI phosphate (0.2 mg/ml) and fast red violet LB salt (0.6 mg/ml) in sodium acetate buffer (0.1 M). The reaction precipitate from acid phosphatase activity was visualized by light microscopy with a Zeiss Axioplan Imaging™ microscope.

Results

Cholesterol Mass

To confirm that ACAT1 deficiency resulted in a functional deficit in the esterification of cholesterol, sterol mass in macrophages was measured by gas chromatography. Macrophages were treated for 28 hours with 50 μg/ml acLDL in DMEM/10% FBS. *ACAT1(-/-)* macrophages treated with acLDL showed an 88% decrease in CE mass compared to wildtype macrophages (3.6 ± 2.0 vs. 29.5 ± 0.7 μg CE/mg cell protein, $p<0.001$, $n=3$). Under these mild cholesterol-loading conditions, the mass of FC was not significantly different between genotypes (24.4 ± 1.7 vs. 25.1 ± 1.6 μg FC/mg cell protein, $p=0.68$, $n=3$).

Cholesterol-Induced Cytotoxicity

Mild cholesterol loading conditions were necessary because the goal of the study was to examine changes in cholesterol homeostasis that may precede cholesterol-induced toxicity. To determine whether the loading conditions could stress cholesterol homeostasis in *ACAT1(-/-)* macrophages without inducing toxicity, the release of ^{14}C-adenine was measured during treatment with modified lipoproteins. As shown in Table 3, there was no significant difference in toxicity between *ACAT1(-/-)* macrophages and wildtype macrophages treated with acLDL.

Table 3. Cholesterol-Induced Cytotoxicity in Macrophages Treated with acLDL

	Wildtype	ACAT1(-/-)	p-value
24 Hours of ^{14}C-Adenine Release (%)	32.70 ± 0.46	32.23 ± 0.62	0.16
48 Hours of ^{14}C-Adenine Release (%)	41.62 ± 1.33	41.81 ± 0.55	0.76

Macrophages were treated with 70 μg/ml acLDL in DMEM/1% FBS for 24 and 48 hours. Cytotoxicity was measured by the release of ^{14}C-adenine into the media. Data are means (n=6) and standard deviations of samples from *ACAT1(+/+)* (Wildtype) or *ACAT1(-/-)* macrophages as compared by Student's *t*-test.

Efflux of Cellular Cholesterol versus Lipoprotein-Derived Cholesterol

Because the efflux of both cellular and lipoprotein-derived sterols directly affect foam cell formation, efflux was measured from macrophages labeled by two different methods of sterol delivery, either to preferentially label cellular (membrane) cholesterol or lipoprotein-derived cholesterol pools. Time-course experiments were performed for these isotopic experiments.

The method to label cellular cholesterol pools resulted in decreased ^{3}H-cholesterol efflux from *ACAT1(-/-)* macrophages compared to wildtype macrophages (Figure 9A). The method to label lipoprotein-derived cholesterol pools resulted in increased ^{3}H-cholesterol efflux from *ACAT1(-/-)* macrophages compared to wildtype macrophages (Figure 9B).

^{3}H-cholesterol efflux was compared to changes in total cholesterol mass during the efflux period. Regardless of the labeling method, the cholesterol mass remaining after the efflux period showed that *ACAT1(-/-)* macrophages retain more cholesterol mass compared to wildtype macrophages (Figure 9A and Figure 9B). Cholesterol esters were less than 1% of the cholesterol mass in either cell type, indicating very mild loading conditions.

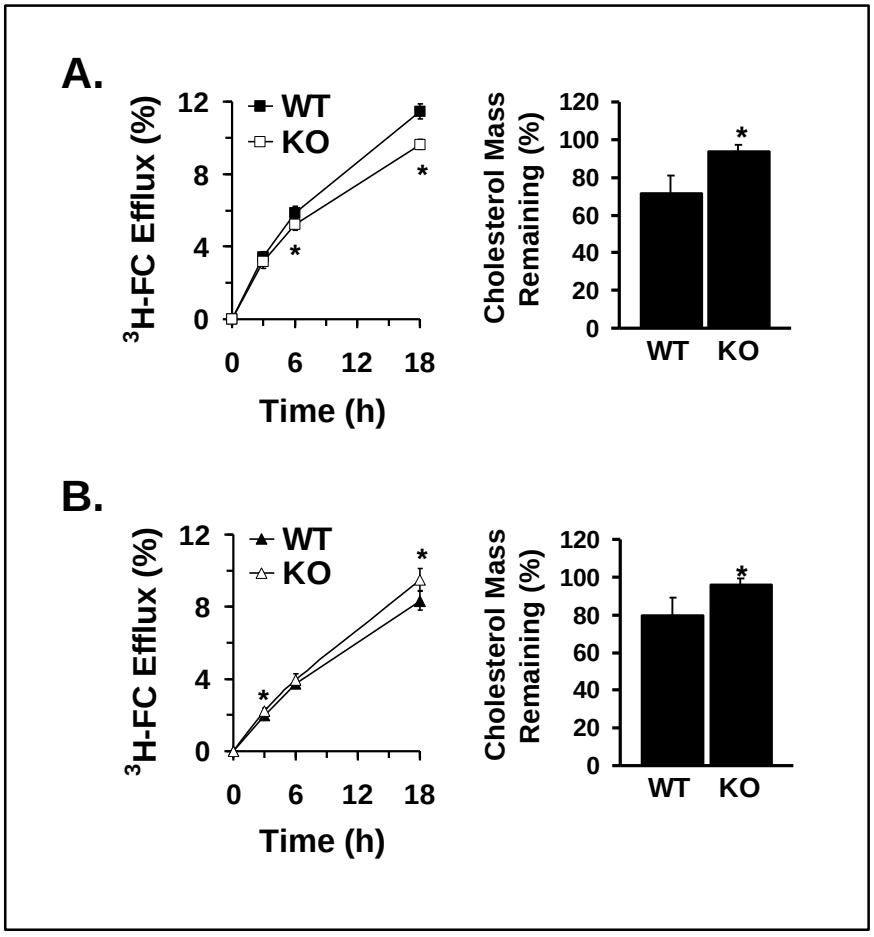


Figure 9. ACAT1 Deficient Macrophages Have Decreased Cellular Cholesterol Efflux and Increased Lipoprotein-Derived Cholesterol Efflux. Macrophages were treated for 36 hours with 1.5 μCi/ml of ^{3}H-FC and 70 μg/ml acLDL in DMEM/2% FBS to label cellular cholesterol or 70 μg/ml ^{3}H-CE/acLDL in DMEM/2% FBS to label lipoprotein-derived cholesterol pools. Cells were then cultured in efflux media with 10 μg/ml human apoAI for 18 hours. Bars and error bars represent the mean (n=4) and standard deviation of samples from *ACAT1(+/+)* (WT) or *ACAT1(-/-)* (KO) macrophages. Asterisks (*) denote a statistically significant difference ($p<0.05$) compared to the appropriate *ACAT1(+/+)* group as determined by Student's *t*-test. **(A)** Efflux of cellular cholesterol to apoAI for 18 hours. The wildtype macrophages in this experiment contained 37.9 ± 1.8 μg cholesterol/mg cell protein and 14701.14 ± 1373.92 cpm/μg cholesterol. The *ACAT1(-/-)* macrophages in this experiment contained 36.0 ± 4.5 μg cholesterol/mg cell protein and 13868.64 ± 1288.55 cpm/μg cholesterol. **(B)** Efflux of lipoprotein-derived cholesterol to apoAI for 18 hours. The wildtype macrophages in this experiment contained 51.9 ± 2.8 μg cholesterol/mg cell protein and 44146.84 ± 3213.66 cpm/μg cholesterol. The *ACAT1(-/-)* macrophages in this experiment contained 44.5 ± 5.3 μg cholesterol/mg cell protein and 40448.52 ± 6638.60 cpm/μg cholesterol.

Cholesterol Efflux and ABCA1 Expression

The efflux of cellular and lipoprotein-derived cholesterol were measured in experiments where ABCA1 was also measured. Cellular cholesterol was labeled with ^{3}H-FC while macrophages were treated with acLDL. Efflux of cellular ^{3}H-FC to apoAI was decreased by 25% in *ACAT1(-/-)* macrophages compared to wildtype macrophages ($p<0.005$, n=4) (Figure 10A). Lipoprotein-derived cholesterol was labeled by the delivery of ^{3}H-CE to lysosomes via acLDL. Efflux of lipoprotein-derived ^{3}H-FC to apoAI was increased by 32% in *ACAT1(-/-)* macrophages compared to wildtype macrophages ($p<0.05$, n=4) (Figure 10A).

In light of the decreased efflux of cellular cholesterol in *ACAT1(-/-)* macrophages, we measured the expression of ABCA1, which is responsible for most of the cholesterol efflux to apoAI. Unexpectedly, *ACAT1(-/-)* macrophages had a 236% increase in ABCA1 mRNA compared to wildtype macrophages (Figure 10B). The large increase in expression was associated with a minor increase of ABCA1 protein compared to wildtype macrophages (Figure 10C).

Uptake, Turnover, and Storage of Lipoprotein-Derived Cholesterol

In addition to changes in cholesterol efflux, other aspects of cholesterol homeostasis can be affected by a defective cholesterol esterification process. Because the uptake, turnover, and storage of lipoprotein-derived sterols directly affect foam cell formation, these processes were measured following the delivery of ^{3}H-CE to lysosomes via acLDL.

Uptake of ^{3}H-CE/acLDL was increased by 38% in *ACAT1(-/-)* macrophages compared to wildtype macrophages ($p<0.005$, n=4) (Figure 10D).

Turnover of lipoprotein-derived cholesterol, which is the cumulative function of many processes, is indicated by the appearance of ^{3}H-FC in the growth media of macrophages during incubation with lipoprotein associated ^{3}H-CE. The turnover of lipoprotein-associated CE by macrophages is the combination of lipoprotein uptake, trafficking of endosomes, CE hydrolysis within lysosomes, trafficking of FC, FC esterification, CE de-esterification, and finally, efflux of FC to extracellular cholesterol

acceptors. *ACAT1(-/-)* macrophages treated with ^{3}H-CE/acLDL released increased amounts of ^{3}H-FC into the loading media compared to wildtype macrophages (Figure 10E). Media ^{3}H-FC was increased by 78% in *ACAT1(-/-)* macrophages ($p<0.005$, n=4).

Storage of intracellular ^{3}H-CE in *ACAT1(-/-)* macrophages was decreased by 81% in *ACAT1(-/-)* macrophages ($p<0.005$, n=4) (Figure 10F). However, storage of intracellular ^{3}H-FC was increased by 26% in *ACAT1(-/-)* macrophages ($p<0.005$, n=4) (Figure 10F).

Macrophage Morphology

Electron microscopy analyses were performed to determine whether changes in cholesterol homeostasis caused by ACAT1 deficiency were associated with morphological changes. *ACAT1(-/-)* macrophages appeared to have increased surface activity as characterized by cytoplasmic extensions and intracellular vesicles (Figure 11B). In untreated macrophages, total vesicle volume was 74% larger in *ACAT1(-/-)* macrophages compared to wildtype macrophages ($p<0.01$, n=20). A similar trend was measured in macrophages treated with acLDL, where total vesicle volume was 28% larger in *ACAT1(-/-)* macrophages compared to wildtype macrophages but this difference did not reach statistical significance. In these measurements, all membrane-limited vesicles were counted, including endosomes, lysosomes, and secretory vesicles.

Immunofluorescence of EEA1 revealed that *ACAT1(-/-)* macrophages have significantly more endosomes than wildtype macrophages (Figure 12A). Treatment with acLDL significantly increased EEA1 fluorescence in both cell types. Immunofluorescence of LAMP1 revealed that *ACAT1(-/-)* macrophages have significantly more lysosomes than wildtype macrophages (Figure 12B). Treatment with acLDL increased LAMP1 fluorescence in wildtype cells but that levels in *ACAT1(-/-)* macrophages stayed at the same high level. Naphthol staining for lysosomal acid phosphatase revealed that wildtype macrophages (Figure 12C) had less activity than *ACAT1(-/-)* macrophages (Figure 12D).

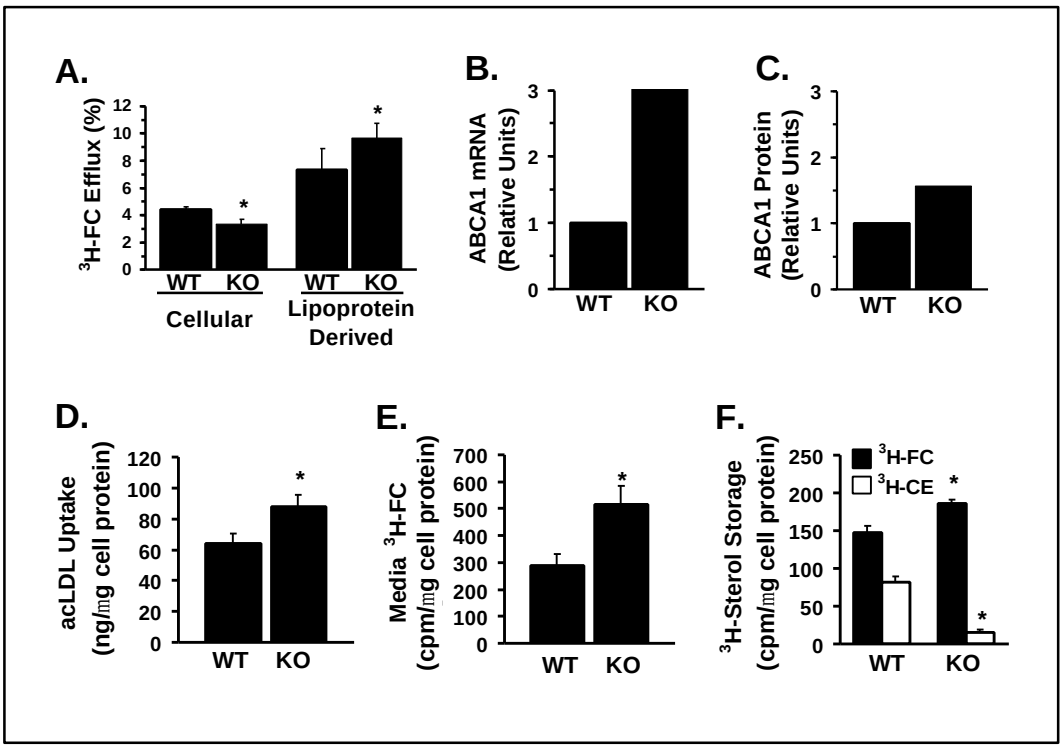


Figure 10. ACAT1 Deficient Macrophages Have Increased Efflux, Uptake, Turnover, and Storage of Lipoprotein-Derived Cholesterol.
Macrophages were treated for 36 hours with 1.5 μCi/ml of ^{3}H-FC and 70 μg/ml acLDL in DMEM/2% FBS to label cellular cholesterol (Cellular) or 70 μg/ml ^{3}H-CE/acLDL in DMEM/2% FBS to label lipoprotein-derived cholesterol pools (Lipoprotein Derived). **(A)** Cholesterol efflux was measured from macrophages to media with 10 μg/ml human apoAI for 6 hours. Bars and error bars represent the mean (n=4) and standard deviation of samples from *ACAT1(+/+)* (WT) or *ACAT1(-/-)* (KO) macrophages. Asterisks (*) denote a statistically significant difference ($p<0.05$) compared to the appropriate *ACAT1(+/+)* group as determined by Student's *t*-test. **(B and C)** The relative quantity of ABCA1 mRNA was determined by real time quantitative RT-PCR. The relative quantity of ABCA1 protein was measured by Western blot analysis. Values are expressed relative to *ACAT1(+/+)* (WT) macrophages. Bars represent the value of pooled samples (n=3, $2x10^6$ cells/sample) and are representative of results from similar experiments. **(D, E, and F)** The uptake, turnover, and storage of ^{3}H-CE/acLDL by macrophages was calculated after 36 hours. The turnover of ^{3}H-CE by macrophages was measured by the appearance of ^{3}H-FC in the culture media during the treatment period. After treatment with ^{3}H-CE/acLDL, cholesterol storage as ^{3}H-FC (solid bars) and ^{3}H-CE (empty bars) was measured in cellular lysate. Bars and error bars represent the mean (n=4) and standard deviation of samples from *ACAT1(+/+)* (WT) or *ACAT1(-/-)* (KO) macrophages. Asterisks (*) denote a statistically significant difference ($p<0.05$) compared to the appropriate *ACAT1(+/+)* group as determined by Student's *t*-test.

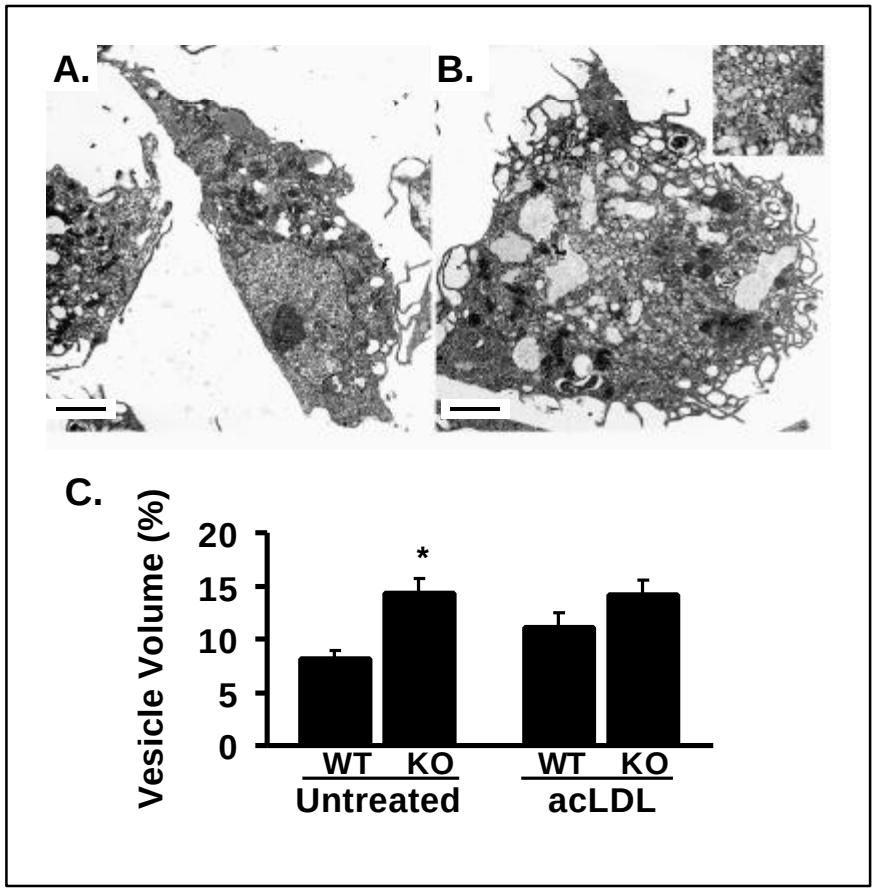


Figure 11. ACAT1 Deficient Macrophages have Altered Morphology and Increased Cellular Vesicle Volume.
Macrophages were treated with 0 or 70 μg/ml acLDL in DMEM/1% FBS for 48 hours. Macrophages were fixed and visualized by transmission electron microscopy (5600X magnification). Electron micrographs of **(A)** an *ACAT1(+/+)* macrophage and **(B)** an *ACAT1(-/-)* macrophage treated with acLDL with a magnified insert in the upper right to show smaller vesicles. The black scale bars represent 2 μm. **(C)** Intracellular vesicle volumes were determined from electron micrographs by point counting and is expressed as a percentage of total cellular volume. Bars and error bars represent the mean (n=20) and standard error of *ACAT1(+/+)* (WT) or *ACAT1(-/-)* (KO) macrophages treated with control or acLDL media. Asterisk (*) denotes a statistically significant difference ($p<0.05$) compared to the appropriate *ACAT1(+/+)* group as determined by two-way ANOVA with Bonferroni post-test.

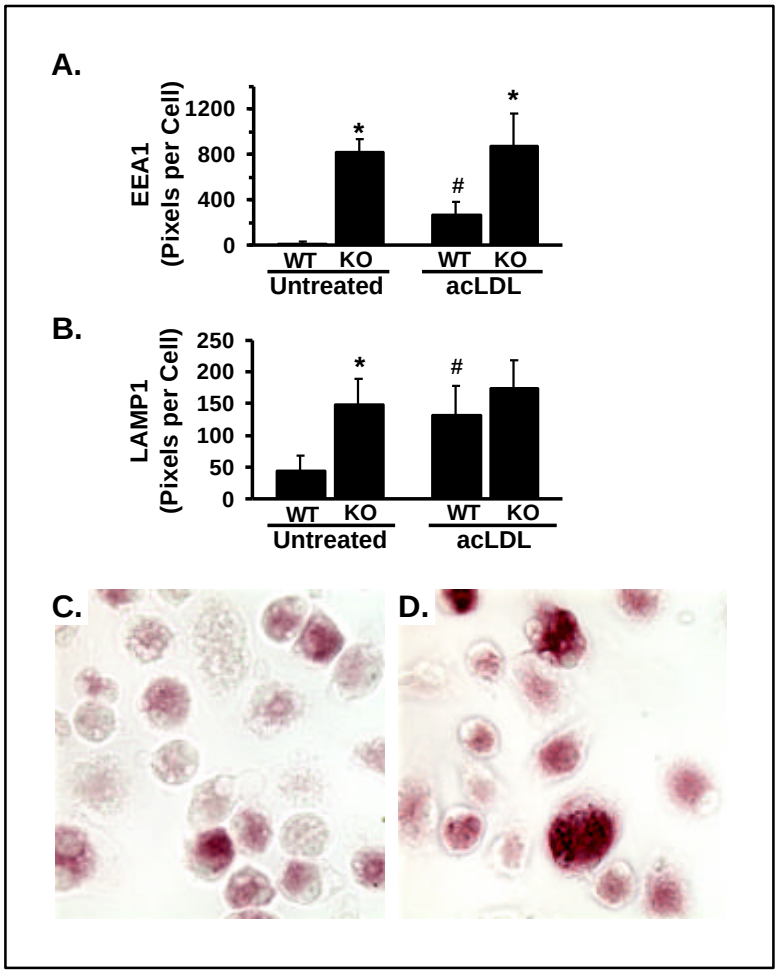


Figure 12. ACAT1 Deficient Macrophages have More Endosomes and Lysosomes.

Macrophages were treated with 0 or 70 μg/ml acLDL in DMEM/1% FBS for 48 hours. **(A and B)** Immunofluorescence microscopy was performed on macrophages treated with primary antibodies against the endosomal marker, EEA1, or the lysosomal marker, LAMP1 with TRITC-conjugated secondary antibody. The area of fluorescence is expressed as pixels per cell. Bars and error bars represent the mean (n=5 random fields) and standard deviation of *ACAT1(+/+)* (WT) or *ACAT1(-/-)* (KO) macrophages. Asterisks (*) denote a statistically significant difference ($p<0.05$) compared to the appropriate *ACAT1(+/+)* group and pound signs (#) denote a statistically significant difference ($p<0.05$) compared to untreated groups as determined by two-way ANOVA with Bonferroni post-test. Light micrographs with naphthol staining for acid phosphatase activity in **(C)** an *ACAT1(+/+)* macrophage and **(D)** an *ACAT1(-/-)* macrophage treated with acLDL.

Discussion

To address the question of whether the absence of ACAT1 results in altered cholesterol homeostasis, we studied cholesteryl ester formation and free cholesterol efflux in *ACAT1(-/-)* macrophages. We found that *ACAT1(-/-)* macrophages have decreased efflux of the cellular cholesterol and increased efflux of the lipoprotein-derived cholesterol. These changes were associated with an accumulation of lipoprotein-derived free cholesterol and with an expanded pool of intracellular vesicles, but were not a consequence of the toxic effects of free cholesterol.

ACAT1 Deficiency and Cholesterol Efflux. For lipoprotein-derived cholesterol, ACAT deficiency increased efflux to apoAI, which is the main cholesterol acceptor for ABCA1-mediated efflux (Figure 10A). This is in agreement with the increased ABCA1 expression (Figure 10B and Figure 10C), since ABCA1 preferentially mediates cholesterol mobilization and efflux from late endosomes/lysosomes (Chen et al., 2001).

In contrast to lipoprotein-derived cholesterol, absence of ACAT1 was associated with decreased efflux of cellular cholesterol to apoAI (Figure 10A). Warner et al. reported decreased cholesterol efflux with ACAT inhibition under heavy loading conditions, where FC accumulation induces cytotoxicity (Warner et al., 1995). In our study, we deliberately used mild cholesterol loading conditions that did not induce cytotoxicity. However, efflux was decreased in *ACAT1(-/-)* macrophages, indicating that cellular toxicity is not the reason why cholesterol efflux is affected. Another possibility is that under our mild loading conditions, cholesterol was sequestered in a non-cytotoxic pool (Kellner-Weibel et al., 2001).

The primary reason for using mild cholesterol loading conditions was to avoid the changes that are secondary to cholesterol toxicity (Tabas, 2002). Shiratori et al. have described the appearance of phospholipid whorls within the cytoplasm of heavily FC-loaded macrophages (Shiratori et al., 1994). Under the mild cholesterol loading conditions used in the current study, however, there was no morphological evidence of phospholipid whorls in *ACAT1(-/-)* macrophages (Figure 11B). Another characteristic of heavily FC-loaded macrophages, as reported by Feng et al., is decreased ABCA1 levels due to increased degradation of the protein (Feng and Tabas, 2002). In contrast, the

increased expression of ABCA1 mRNA and protein in *ACAT1(-/-)* macrophages in our studies supports our contention that we were indeed successful in inducing only mild cholesterol loading (Figure 10B and Figure 10C). However, the minor increase in protein relative to the large increase in mRNA may indicate reduced stability of ABCA1 protein. It is interesting to note that macrophages treated with the ACAT inhibitor, MCC-147, have increases in ABCA1 expression that are similar to what we report for *ACAT1(-/-)* macrophages (Sugimoto et al., 2004).

ACAT Inhibitors and Cholesterol Efflux. Atherosclerosis studies with hyperlipidemic mice show that macrophage ACAT1 deficiency increases necrosis and apoptosis in lesions (Fazio et al., 2001). The rationale for treating atherosclerosis with inhibitors of ACAT1 is to stop intracellular CE formation and prevent foam-cell formation. The current studies confirm that the deficiency of ACAT1 increases the turnover of lipoprotein-derived cholesterol in macrophages by blocking cholesterol storage. This is viewed as an anti-atherogenic mechanism. However, the decrease in the efflux of cellular cholesterol may represent a critical change in cholesterol homeostasis in macrophages.

The current studies add to previous reports in which ACAT activity was reduced by means of pharmacologic inhibitors. Rodriguez et al. reported increased efflux of lipoprotein-derived cholesterol during treatment with the ACAT inhibitor 58-035 in human monocyte-derived macrophages (Rodriguez et al., 1999). ACAT inhibition has been reported to increase the efflux of cholesterol to various cholesterol acceptors (Kellner-Weibel et al., 1998; Mazzone and Reardon, 1994; Warner et al., 1995; Zhang et al., 1996). In our studies, *ACAT1(-/-)* macrophages had increased turnover of lipoprotein-derived CE as measured by the reappearance of processed CE in the media as FC (Figure 10E). However, ACAT1 deficiency reduced the efflux of cellular cholesterol from macrophages (Figure 10A). Although ACAT1 deficiency resulted in increased cholesterol turnover during acLDL treatment (Figure 10E), this increase was not sufficient to prevent the accumulation of FC (Figure 10F). The increase in FC may be related to the increase in membrane-bound vesicles in *ACAT1(-/-)* macrophages (Figure 11), as these membranes represent a potential pool for FC.

ACAT Inhibition and ACAT1 Deficient Macrophages. In additional experiments not presented here, ACAT inhibition was compared with the effects ACAT1 deficiency on lipoprotein uptake, cholesterol turnover to acceptors in media, and cholesterol esterification. Both ACAT1 deficiency and ACAT inhibition increased the turnover of lipoprotein-derived cholesterol and decreased the esterification of labeled cholesterol (Appendix, Figure 30C). One notable difference was that ACAT1 deficiency increased FC accumulation while ACAT inhibition did not change intracellular FC when compared to untreated wildtype macrophages. This may be due to residual ACAT activity or to the acuteness of pharmacologic inhibition. ACAT1 deficiency may result in structurally and functionally abnormal macrophages due either to chronic compensatory mechanisms or to direct effects of the absence of ACAT1. For example, the accumulation of membrane-bound vesicles with ACAT1 deficiency (Figure 11) may represent compensatory changes in vesicular transport and cholesterol trafficking in macrophages. A second notable difference was that ACAT1 deficiency increased the efflux of lipoprotein-derived cholesterol while ACAT inhibition reduced efflux compared to untreated wildtype macrophages. This may be due to the differences in FC accumulation between ACAT inhibition and ACAT1 deficiency.

Cholesterol Trafficking and Vesicles in ACAT1 Deficient Macrophages. What is clear from our studies is that cholesterol efflux is not the simple consequence of free cholesterol availability. Complex interconnected processes like the trafficking and sequestration of intracellular cholesterol are intimately associated with cholesterol efflux. Mechanistic insight into the disruptions in cholesterol efflux and morphological changes can be gained by comparing *ACAT1(-/-)* macrophages with *NPC1(-/-)* cells. Niemann-Pick type C1 (NPC1) traffics cholesterol from lysosomes to the plasma membrane and endoplasmic reticulum. The phenotype of *NPC1(-/-)* cells includes the accumulation of cholesterol in lysosomes, decreased cholesterol efflux, decreased cholesterol esterification, increased cholesterol synthesis, and decreased lipoprotein uptake (Ory, 2004). Like the *NPC1(-/-)* cells, *ACAT1(-/-)* macrophages have accumulation of lipoprotein derived cholesterol (Figure 10F), disrupted cholesterol efflux (Figure 9A),

decreased cholesterol esterification, increased cholesterol synthesis (Chapter V), and increased lipoprotein uptake (Figure 10D). The phenotype of disrupted cholesterol efflux in *ACAT1(-/-)* macrophages and the increased number of intracellular vesicles suggests that these macrophages have a disruption in cholesterol trafficking that goes beyond decreased esterification. Decreased efflux of cellular cholesterol (Figure 9A) may indicate sequestration of cholesterol. Based on these data, one could speculate that the cholesterol esterification cycle is an essential loop in the cholesterol trafficking route, even under conditions where cholesterol esters do not accumulate.

The intracellular vesicles that accumulate in *ACAT1(-/-)* macrophages are an interesting finding. Previous studies with ACAT inhibitors have described similar accumulations of vesicles (Robenek and Schmitz, 1988). In these studies, Robenek and Schmitz described foamy organelles and lamellar bodies that form in macrophages treated with acLDL and an ACAT inhibitor. These vesicles were stored in a cytoplasmic compartment and interacted with HDL to promote cholesterol efflux. In other studies, similar vesicles were observed in macrophages that were treated with acLDL (Sakashita et al., 2000). These vesicles resulted from vesiculation of the endoplasmic reticulum and co-localized with a significant portion of the total cellular ACAT1. Accumulation of these types of vesicles, in both intracellular and extracellular spaces, is associated with the progression of fatty streaks into more complicated plaques (Guyton and Klemp, 1989; Guyton and Klemp, 1994). These vesicles may be analogous to those seen in the *ACAT1(-/-)* macrophages in our studies. The proliferation of intracellular vesicles in *ACAT1(-/-)* macrophages provides a potential source for the extracellular cholesterol in atherosclerotic lesions and thus a potential mechanism for increased atherosclerosis. In addition to the vesicular changes associated with ACAT inhibition, Robenek and Schmitz described vesicular changes that were associated with disrupted calcium homeostasis (Robenek and Schmitz, 1988). Free cholesterol loading has been shown to deplete endoplasmic reticulum calcium stores and lead to apoptosis (Feng et al., 2003). The proliferation of intracellular vesicles in *ACAT1(-/-)* macrophages may be the first sign of a cholesterol-mediated disruption of calcium homeostasis and apoptosis.

Summary. In summary, these data show that the deficiency in macrophage ACAT1 disrupts the efflux of cellular cholesterol despite increased expression of ABCA1. The increased efflux of lipoprotein-derived cholesterol cannot counter-balance the accumulation rate of FC. These alterations in cholesterol homeostasis are associated with changes in cellular morphology, precede cholesterol-induced cytotoxicity, and may represent the basis for accelerated macrophage apoptosis in the growing plaque.

CHAPTER V

THE EFFECTS OF CHOLESTEROL STORAGE DEFICITS ON CHOLESTEROL SYNTHESIS AND ESTERIFICATION

Abstract

Objective. Acyl-coenzyme A: cholesterol acyltransferase (ACAT) esterifies free cholesterol and stores cholesteryl esters in lipid droplets. Our laboratory has shown that macrophage ACAT1 deficiency results in increased atherosclerotic lesion area in hyperlipidemic mice. These atherogenic macrophages have disrupted cholesterol efflux, increased lipoprotein uptake, and accumulation of intracellular vesicles. The objective of the current study was to determine whether lipid synthesis in macrophages is affected by ACAT1.

Methods and Results. The synthesis, esterification, and efflux of new cholesterol and new phospholipids were measured in peritoneal macrophages from *ACAT1(-/-)* mice. Cholesterol synthesis was increased by 134% ($p<0.001$) in *ACAT1(-/-)* macrophages compared to wildtype macrophages. Increased synthesis resulted in a proportional increase in the efflux of the newly synthesized cholesterol. Although the esterification of new cholesterol was reduced by 93% ($p<0.001$), *ACAT1(-/-)* macrophages still contained trace amounts of newly synthesized cholesteryl esters. In contrast to cholesterol synthesis, there were no differences in the synthesis or efflux of phospholipids in *ACAT1(-/-)* macrophages. Because fatty acids are substrates of ACAT1 and regulators of cholesterol synthesis, the fatty acid content of membrane phospholipids was also studied. Saturated/monounsaturated fatty acids were not altered in *ACAT1(-/-)* macrophages and were not affected by treatment with modified LDL. However, for macrophages treated with modified LDL, polyunsaturated fatty acids were increased 26% ($p=0.036$) in *ACAT1(-/-)* macrophages compared to wildtype macrophages.

Conclusions. Together, these data show that macrophage ACAT1 influences the synthesis of cholesterol but not phospholipids. Increased cholesterol synthesis in *ACAT1(-/-)* macrophages suggests that ACAT1 contributes to a regulatory pool of

cholesterol in macrophages. This change in cholesterol homeostasis may contribute to the atherogenic potential of *ACAT1(-/-)* macrophages.

Introduction

Atherosclerosis is characterized by the accumulation of cholesteryl esters in lipid droplets in arterial macrophages. The enzyme responsible for cholesterol esterification in macrophages is acyl-coenzyme A: cholesterol acyltransferase 1 (ACAT1) (Tabas et al., 1986). Recent studies in our laboratory have shown that *ACAT1(-/-)* macrophages promote the formation of atherosclerosis in hyperlipidemic mice (Fazio et al., 2001). ACAT1 deficiency has a variety of effects on cholesterol homeostasis that may contribute to the paradoxical formation of foam cell lesions in the absence of cholesterol esterification. These changes include decreased efflux of cellular cholesterol, increased uptake of modified lipoproteins, the accumulation of intracellular membrane bound vesicles, and the accumulation of free cholesterol despite higher turnover of lipoprotein-derived cholesterol (Chapter IV). However, little is known about the relationship between cholesterol synthesis and the cholesterol esterification cycle. The contribution of *de novo* cholesterol synthesis to foam cell formation may be physiologically relevant, especially if synthesis is not down-regulated when the cholesterol esterification cycle is not functional. Previous studies have reported that cholesterol efflux results in increased cholesterol synthesis because a regulatory pool of cholesterol is being depleted (Johnson et al., 1995; Mendez et al., 1991). This regulatory pool of cholesterol is also diminished by the esterification activity of ACAT1 (Tabas et al., 1988; Tabas et al., 1986). It is unknown if ACAT1 can modulate cholesterol synthesis by contributing cholesterol to a regulatory cholesterol pool. It is also unknown if ACAT1 can affect cholesterol synthesis indirectly by affecting the fatty acid substrates. Studies suggest that polyunsaturated fatty acids, which are substrates for ACAT1, affect cholesterol homeostasis by altering the amount of cholesterol in a regulatory cholesterol pool (Worgall et al., 1998).

In the current study, we measured ^{3}H-acetate incorporation into free cholesterol, cholesteryl esters, and phospholipids in peritoneal macrophages from *ACAT1(-/-)* mice. We show that cholesterol synthesis is increased in *ACAT1(-/-)* macrophages compared to wildtype macrophages. Despite unchanged phospholipid synthesis, polyunsaturated fatty

acids accumulate in *ACAT1(-/-)* macrophages. These data suggest that increased cholesterol synthesis and changes in membrane phospholipids contribute to the atherogenic potential of *ACAT1(-/-)* macrophages.

Methods

Lipoprotein Preparation and Modification. Low density lipoproteins (LDL) (density = 1.019 to 1.063 g/ml) and lipoprotein deficient serum (LPDS) (density > 1.210 g/ml) were isolated from human blood by sequential centrifugation as described in Chapter IV. Acetyl-LDL (acLDL) was prepared by repeated addition of acetic anhydride to LDL (Basu et al., 1976). Oxidized LDL (oxLDL) was prepared by copper oxidation (Yancey and Jerome, 2001). Lipoprotein species and modifications were confirmed by electrophoretic mobility on agarose gels. Lipoprotein oxidation was also confirmed spectrophotometrically by measuring absorbance at 234 nm (Lopes-Virella et al., 2000).

^{3}H-Acetate Incorporation. Cholesterol synthesis was measured by the incorporation of ^{3}H-acetate into cholesterol (Lin et al., 2001). Peritoneal macrophages were thioglycollate-elicited as described in Chapter IV. Macrophages were cultured for 36 hours in DMEM/5% LPDS. The incorporation period (24 h) was initiated by the addition of 40 μCi/ml ^{3}H-acetate in DMEM/2% FCS. During this incorporation period macrophages where stimulated with 0 or 50 μg/ml oxLDL. Cells were rinsed and lysed in 0.1 N sodium hydroxide. Protein concentration in cell lysates was determined by a modified Lowry assay (Lowry et al., 1951). Lipids were extracted, separated by TLC, and counted as described in Chapter IV. Lipid synthesis was expressed as counts per unit cell protein. Efflux of new lipids was expressed as counts in media per unit cell protein or counts in media as a percentage of the total counts (media plus lysate) for that lipid.

^{14}C-Oleate Incorporation. The incorporation of [1-^{14}C]-oleate into cholesteryl esters and phospholipids were measured. Macrophages were cultured for 48 hours in DMEM/2% FCS with 0 or 70 μg/ml acLDL. The incorporation period (3 h) was initiated by the addition of oleate and ^{14}C-oleate complexed with BSA (0.25 μCi/ml, 0.66% BSA, molar ratio of 1:1 oleate/BSA, molar ratio of 1:20 ^{14}C-oleate/oleate) in DMEM (Brown et

al., 1979; Kritharides et al., 1998). Cells were rinsed and scraped in PBS. Protein concentration in cell lysates was determined by a modified Lowry assay (Lowry et al., 1951). Lipids were extracted, separated by TLC, and counted as described in Chapter IV. Incorporation of oleate into lipids was expressed as counts per unit cell protein.

Fatty Acid Profile of Phospholipids. Macrophages were cultured for 48 hours in DMEM/2% FCS with 0 or 70 μg/ml acLDL. Cells were rinsed and scraped in PBS. Protein concentration in cell lysates was determined by a modified Lowry assay (Lowry et al., 1951). To measure phospholipid fatty acids, sample extracts were transmethylated and the total fatty acid composition was quantified using gas chromatography with flame ionization detection (Fazio et al., 2001). The profile was separated into saturated/monounsaturated (14:0, 16:0, 16:1, 18:0, and 18:1) and polyunsaturated fatty acids (18:2, 20:4, 20:5, 22:4, 22:5, 22:6).

Results

Synthesis and Efflux of New Cholesterol and New Phospholipid

To determine whether ACAT1 deficiency causes changes in cholesterol synthesis, ^{3}H-acetate incorporation was measured in untreated or oxLDL-treated macrophages. In untreated macrophages, synthesis of ^{3}H-cholesterol was increased by 33% ($p<0.001$) in *ACAT1(-/-)* macrophages compared to wildtype (C57Bl/6) macrophages (Figure 13A). After treatment with oxLDL, cholesterol synthesis was 134% higher ($p<0.001$) in *ACAT1(-/-)* macrophages than in C57BL/6 wildtype macrophages. The down-regulation of cholesterol synthesis that resulted from treatment with oxLDL was present in both types of macrophages but was somewhat attenuated in the *ACAT1(-/-)* macrophages (decreased 52%, $p<0.001$) compared to wildtype macrophages (decreased 73%, $p<0.001$).

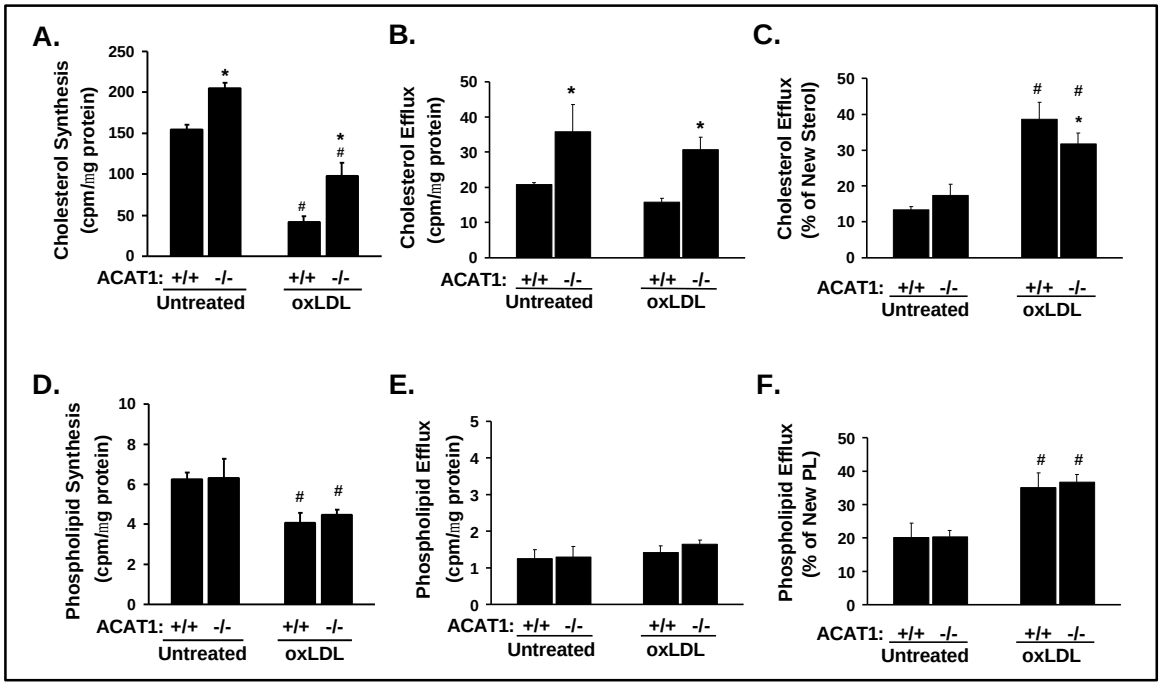


Figure 13. ACAT1 Deficiency Increases the Synthesis and Efflux of New Cholesterol but Does not Affect New Phospholipids in Macrophages.
(A) The synthesis of ^{3}H-Cholesterol, **(B and C)** the efflux of newly synthesized ^{3}H-Cholesterol, **(D)** the synthesis of ^{3}H-Phospholipid, and **(E and F)** the efflux of newly synthesized ^{3}H-Phospholipid were measured in *ACAT1(+/+)* or *ACAT1(-/-)* macrophages. Macrophages were cultured with 0 or 50 μg/ml oxLDL during incorporation of ^{3}H-Acetate. Bars and error bars represent the mean (n=4) and standard deviation of samples. Asterisks (*) denote a statistically significant difference ($p<0.05$) between *ACAT1(+/+)* and *ACAT1(-/-)* groups as determined by two-way ANOVA with Bonferroni post-test. Pounds (#) denote a statistically significant difference ($p<0.05$) between untreated and oxLDL groups as determined by two-way ANOVA with Bonferroni post-test.

^{3}H-cholesterol efflux was measured during ^{3}H-acetate incorporation because the efflux of newly synthesized cholesterol is a marker of the removal of internal cholesterol pools (Johnson et al., 1995). In untreated macrophages, efflux of newly synthesized ^{3}H-cholesterol was increased by 73% ($p<0.001$) in *ACAT1(-/-)* macrophages compared to wildtype macrophages (Figure 13B). In oxLDL-treated macrophages, efflux was increased by 94% ($p<0.001$) in *ACAT1(-/-)* macrophages. However, when efflux was expressed as a percentage of the ^{3}H-cholesterol synthesized during the incorporation period, there was no increase between wildtype and *ACAT1(-/-)* macrophages (Figure 13C), indicating that the proportional increase in efflux was linked with or possibly due to increased synthesis.

To determine whether ACAT1 deficiency causes changes in phospholipid synthesis, ^{3}H-acetate incorporation was measured in untreated or oxLDL-treated macrophages. In untreated macrophages, synthesis of ^{3}H-phospholipid was not different in *ACAT1(-/-)* macrophages compared to wildtype macrophages (Figure 13D). Similarly, in oxLDL-treated macrophages, there were no differences in phospholipid synthesis. Treatment with oxLDL down-regulated phospholipid synthesis in wildtype and *ACAT1(-/-)* macrophages by 35% ($p<0.001$) and 29% ($p<0.001$), respectively. The absence of ACAT1 did not affect the efflux of newly synthesized ^{3}H-phospholipid in untreated or in oxLDL-treated macrophages (Figure 13E and Figure 13F).

Esterification of New Cholesterol

To determine if newly synthesized cholesterol was available for esterification, ^{3}H-cholesteryl esters were measured after ^{3}H-acetate incorporation. ^{3}H-cholesteryl esters represented a small percentage (<2%) of the ^{3}H-cholesterol synthesized during the incorporation period. It is likely that hydrolysis of new ^{3}H-cholesteryl esters prevents their accumulation. In wildtype macrophages, the amount of ^{3}H-cholesteryl esters increased 182% ($p<0.001$) when treated with oxLDL (Figure 14A). Unexpectedly, *ACAT1(-/-)* macrophages had measurable amounts of ^{3}H-cholesteryl esters. *ACAT1(-/-)* macrophages had less than 8% ($p<0.001$) of the ^{3}H-cholesteryl esters that were found in wildtype macrophages (Figure 14B). The results were similar when macrophages were treated with oxLDL.

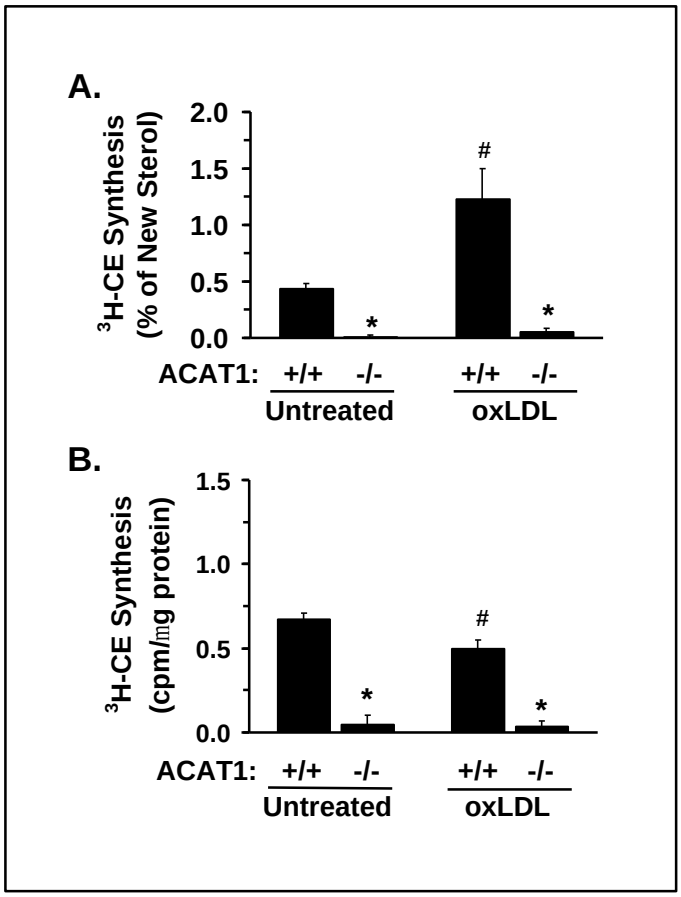


Figure 14. ACAT1 Deficiency Decreases but does not Eliminate the Esterification of Newly Synthesized Cholesterol.
Esterification of newly synthesized ^{3}H-Cholesteryl ester was measured in *ACAT1(+/+)* or *ACAT1(-/-)* macrophages. Macrophages were cultured with 0 or 50 μg/ml oxLDL during incorporation of ^{3}H-Acetate. The amount of esterified cholesterol is expressed **(A)** as a percentage of all the cholesterol synthesized during the incorporation period and **(B)** as counts normalized by cellular protein. Bars and error bars represent the mean (n=4) and standard deviation of samples. Asterisks (*) denote a statistically significant difference ($p<0.05$) between *ACAT1(+/+)* and *ACAT1(-/-)* groups as determined by two-way ANOVA with Bonferroni post-test. Pounds (#) denote a statistically significant difference ($p<0.05$) between untreated and oxLDL groups as determined by two-way ANOVA with Bonferroni post-test.

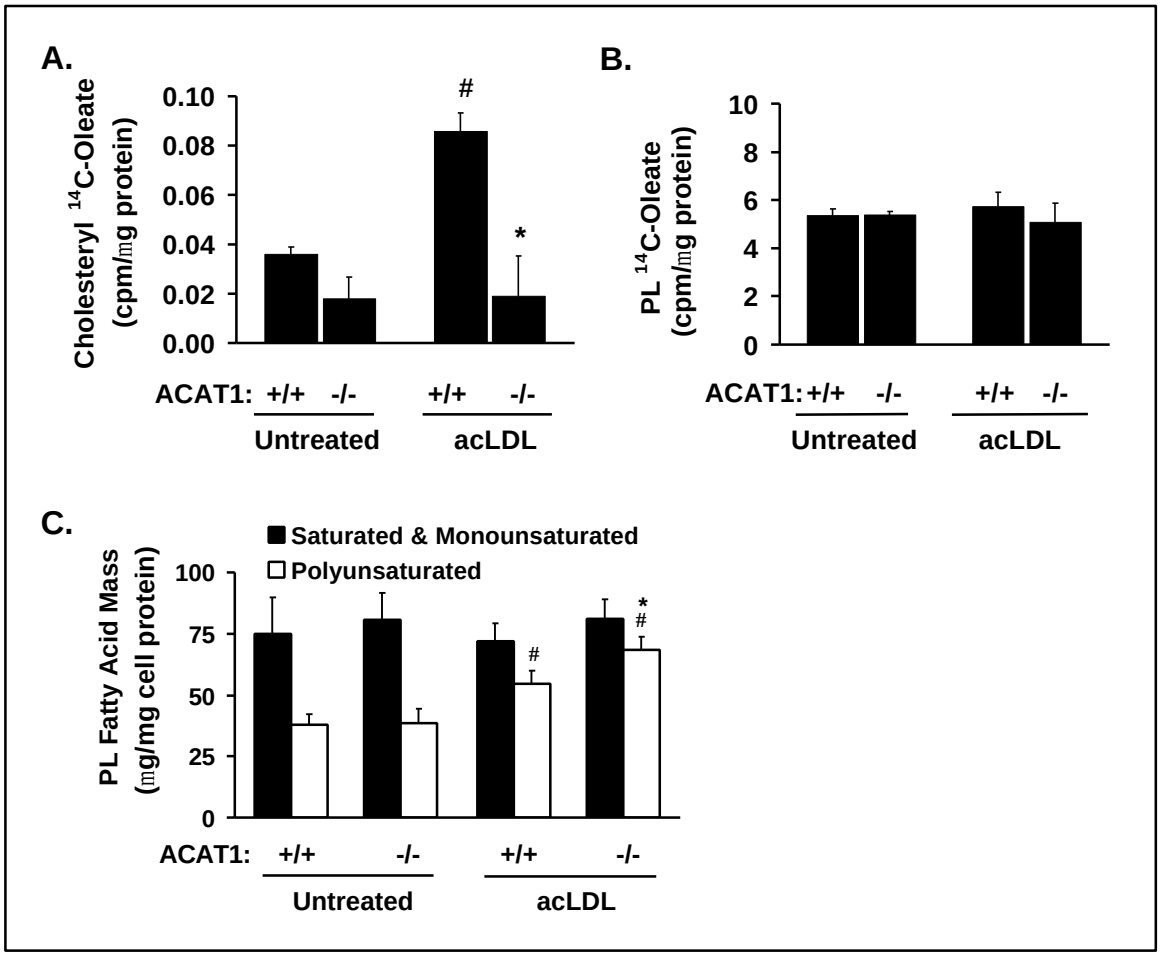


Figure 15. ACAT1 Deficiency Increases the Polyunsaturated Fatty Acid Content of Membrane Phospholipids.
The fatty acid content of phospholipids was measured in *ACAT1(+/+)* or *ACAT1(-/-)* macrophages by gas chromatography. Macrophages were cultured with 0 or 70 μg/ml acLDL. Synthesis of **(A)** cholesteryl esters and **(B)** phospholipids was measured by the incorporation of ^{14}C-oleate. **(C)** The fatty acid masses are divided into unsaturated/monounsaturated fatty acids (solid bars) and polyunsaturated fatty acids (empty bars) and were normalized by cellular protein. Bars and error bars represent the mean (n=3) and standard deviation of samples. Asterisks (*) denote a statistically significant difference ($p<0.05$) between *ACAT1(+/+)* and *ACAT1(-/-)* groups as determined by two-way ANOVA with Bonferroni post-test. Pounds (#) denote a statistically significant difference ($p<0.05$) between untreated and acLDL groups as determined by two-way ANOVA with Bonferroni post-test.

Oleate Incorporation and the Fatty Acid Content of Membrane Phospholipids

Fatty acids modulate cholesterol synthesis and ACAT1 activity (Rumsey et al., 1995; Seo et al., 2001; Worgall et al., 1998). Because fatty acids are hydrolyzed from membrane phospholipids for utilization by ACAT1 (Akiba et al., 2003), the effects of ACAT1 deficiency on the incorporation of ^{14}C-oleate into cellular lipids and on the fatty acid profile of phospholipids were studied. Similar to the residual esterification activity observed with newly synthesized cholesterol, *ACAT1(-/-)* macrophages pulsed with ^{14}C-oleate had measurable amounts of cholesteryl ^{14}C-oleate. Relative to wildtype macrophages, the amount cholesteryl ^{14}C-oleate for untreated and acLDL-treated *ACAT1(-/-)* macrophages was 50% (not significant) and 22% ($p<0.001$), respectively (Figure 15A). In wildtype macrophages, the amount of cholesteryl ^{14}C-oleate increased 140% ($p<0.001$) when cells were treated with acLDL. In *ACAT1(-/-)* macrophages, the amount of cholesteryl ^{14}C-oleate was not changed ($p=0.90$) when cells were treated with acLDL. In untreated macrophages pulsed with ^{14}C-oleate, incorporation into phospholipid was not different in *ACAT1(-/-)* macrophages compared to wildtype macrophages (Figure 15B). Similarly, in acLDL-treated macrophages, there was no difference in the incorporation of ^{14}C-oleate into phospholipids. Treatment with acLDL did not affect the incorporation of ^{14}C-oleate into phospholipids.

In addition, there was no difference in the mass of unsaturated and monounsaturated fatty acids in phospholipids of the two macrophage types. However, polyunsaturated fatty acids increased 26% ($p<0.05$) in *ACAT1(-/-)* macrophages treated with acLDL (Figure 15C) compared to wildtype macrophages.

Discussion

The purpose of this study was to determine the contributions of cholesterol and phospholipid synthesis to the disrupted cholesterol homeostasis and the atherogenic potential of *ACAT1(-/-)* macrophages. We measured lipid synthesis in peritoneal macrophages and found that ACAT1 deficiency increases cholesterol synthesis without affecting phospholipid synthesis. This change in cholesterol homeostasis in *ACAT1(-/-)* macrophages may contribute to foam cell formation and atherosclerosis.

ACAT1 Deficiency and Cholesterol Synthesis. The down-regulation of cholesterol synthesis by modified LDL was attenuated in *ACAT1(-/-)* macrophages (Figure 13A). Although most cellular cholesterol is likely to be derived from lipoprotein uptake, *de novo* cholesterol synthesis may contribute to internal cholesterol pools that lead to foam cell formation (Johnson et al., 1995; Mendez et al., 1991). The increased cholesterol synthesis in *ACAT1(-/-)* macrophages may play a role in the accelerated progression of these cells to the stage of foam cell. Efflux of newly synthesized cholesterol is a marker of the removal of internal cholesterol pools. Although efflux of newly synthesized cholesterol was increased in *ACAT1(-/-)* macrophages (Figure 13B), the increase was proportional to the increase in cholesterol synthesis (Figure 13C). This indicates that the proportional increase in efflux was linked with or possibly due to increased synthesis. In contrast, the efflux of cellular cholesterol was decreased whereas the efflux of lipoprotein-derived cholesterol was increased in *ACAT1(-/-)* macrophages (Chapter IV). Efflux of newly synthesized ^{3}H-phospholipid in untreated or in oxLDL-treated macrophages was not affected by the absence of ACAT1 (Figure 13E and Figure 13F). This indicates that the effects of ACAT are limited to cholesterol synthesis, and suggests that phospholipid synthesis is not responsible for the accumulation of membrane-bound intracellular vesicles in *ACAT1(-/-)* macrophages (Chapter IV).

Esterification Despite ACAT1 Deficiency. *ACAT1(-/-)* macrophages had measurable amounts of ^{3}H-cholesteryl esters (Figure 14A and Figure 14B). The physiologic importance of this residual esterification activity in *ACAT1(-/-)* macrophages is unknown. This activity could represent physiologic redundancy (i.e. another ACAT isozyme) or broad substrate specificity of other acyl-transferases (e.g. DGAT). Although ACAT2 has not been detected in resident murine peritoneal macrophages, ACAT2 mRNA and protein is present in thioglycollate-elicited mouse peritoneal macrophages and in atherosclerotic lesions from mice (Anderson et al., 1998; Cases et al., 1998a; Meiner et al., 1996; Sakashita et al., 2003). Functional studies characterizing ACAT2 activity in macrophages have not been reported. Interestingly, the esterification of ^{3}H-cholesterol in *ACAT1(-/-)* macrophages was not totally eliminated by the ACAT inhibitor cp113-818 (Appendix, Figure 30C). This ACAT inhibitor is non-selective (Lada et al.,

2004), so these results suggest that ACAT2 activity is not responsible for the residual esterification activity. The specificity of DGAT for fatty acids has been well characterized, but cholesterol is not a substrate for DGAT (Cases et al., 1998b). Also, there may be other uncharacterized ACAT isozymes and acyl-transferases contributing to the residual cholesterol esterification activity in *ACAT1(-/-)* macrophages. The reversible reactions catalyzed by lipases could also contribute to esterification of newly synthesized cholesterol with fatty acids (Bhat and Brockman, 1981).

ACAT1 Deficiency and Phospholipids. There was no difference in the incorporation of ^{14}C-oleate into phospholipids in *ACAT1(-/-)* macrophages (Figure 15B). While unsaturated and monounsaturated fatty acids were unchanged, polyunsaturated fatty acids increased in *ACAT1(-/-)* macrophages (Figure 15C) compared to wildtype macrophages. The increase of the polyunsaturated fatty acids in phospholipids may represent an attempt to solubilize excess or aberrantly trafficked cholesterol resulting from the disruption of the cholesterol esterification cycle (Spector and Yorek, 1985a; Stubbs and Smith, 1984). Since polyunsaturated fatty acids have an inhibitory effect on cholesterol synthesis (Worgall et al., 1998), sequestration of these fatty acids in phospholipid membranes could explain the increased cholesterol synthesis in *ACAT1(-/-)* macrophages treated with acLDL. However, untreated macrophages had increased cholesterol synthesis without any changes in phospholipid fatty acids, suggesting that these changes were not directly related. These changes in the fatty acid content of membranes may be associated with the accumulation of intracellular membrane bound vesicles and the accumulation of free cholesterol observed in other studies (Chapter IV).

Cholesterol Trafficking and Cholesterol Synthesis in ACAT1 Deficient Macrophages. Mechanistic insight into the cholesterol homeostasis phenotype of *ACAT1(-/-)* macrophages can be gained by considering similarities with the Niemann-Pick type C1 (NPC1) phenotype and similarities with cells treated with drugs like the U18666A compound or progesterone. Increased cholesterol synthesis is an important part of the phenotypic change in *ACAT1(-/-)* macrophages. Other changes in *ACAT1(-/-)* macrophages include decreased cholesterol esterification, the accumulation of

lipoprotein-derived cholesterol, disrupted cholesterol efflux, and increased lipoprotein uptake (Chapter IV). The similarity of these changes with the NPC1 phenotype suggests that cholesterol trafficking is disrupted. NPC1 protein plays a key role in cholesterol trafficking between lysosomes, the plasma membrane, and the endoplasmic reticulum. The phenotype of *NPC1(-/-)* cells includes increased cholesterol synthesis, decreased cholesterol esterification, the accumulation of cholesterol in lysosomes, and decreased cholesterol efflux (Ory, 2004). In contrast to *ACAT1(-/-)* macrophages, *NPC1(-/-)* cells have decreased lipoprotein uptake. By mechanisms that are not entirely clear, progesterone and U18666A disrupt cholesterol trafficking and result in the same phenotype as *NPC1(-/-)* cells (Soccio and Breslow, 2004). Decreased delivery of cholesterol to regulatory or signaling pools may result in this common phenotype. For macrophages, the cholesterol esterification cycle may be an essential pathway in cholesterol trafficking.

Summary. In summary, these data show that the absence of ACAT1 in macrophages has effects on lipid homeostasis that go beyond the decrease in cholesteryl esters. ACAT1 deficiency in macrophages increases cholesterol synthesis without affecting phospholipid synthesis. This increase may represent dysregulation of the cholesterol sensing mechanisms in macrophages and suggests that *de novo* synthesis of cholesterol may contribute to the atherogenic potential of *ACAT1(-/-)* macrophages.

CHAPTER VI

THE EFFECTS OF ENDOGENOUSLY SYNTHESIZED APOLIPOPROTEIN E ON CHOLESTEROL EFFLUX

Abstract

Objective. Apolipoprotein (apo) E is a cholesterol acceptor that drives cholesterol efflux from macrophages and prevents foam cell transformation. Our laboratory has shown that secretion of apoE by macrophages decreases atherosclerotic lesion area in hyperlipidemic mice. The ATP-Binding Cassette (ABC)-A1 transporter is another major pathway for cholesterol efflux. Both of these pathways are under the transcriptional regulation of oxysterols via the Liver X Receptor (LXR). The objective of the current study was to measure the effects of endogenous apoE synthesis on ABCA1-mediated cholesterol efflux.

Methods and Results. Cholesterol efflux mediated by apoE or ABCA1 was measured with peritoneal macrophages from *apoE(+/+)* and *apoE(-/-)* mice. As a cholesterol acceptor, macrophage apoE increased cholesterol efflux by 164% ($p<0.001$) in *apoE(+/+)* macrophages compared to *apoE(-/-)* macrophages. In addition to its role as an acceptor, endogenous apoE had a modest stimulatory effect on ABCA1-mediated cholesterol efflux, which was increased by 9% ($p=0.003$) in *apoE(+/+)* macrophages compared to *apoE(-/-)* macrophages. Stimulation of *apoE(+/+)* macrophages with 22(R)-hydroxycholesterol, an LXR agonist, increased ABCA1-mediated cholesterol efflux by 59% ($p<0.001$) and increased apoE-mediated cholesterol efflux by 108% ($p<0.001$). To test for functional cooperation between ABCA1 and apoE, experiments were also performed with *apoE(+/+)* and *apoE(-/-)* macrophages that were *ABCA1(-/+)* or *ABCA1(-/+)*. When stimulated with an LXR agonist, ABCA1 mediates efflux to apoAI independently of endogenous apoE. However, a small fraction of apoE-mediated efflux depends on ABCA1 ($p=0.0311$).

Conclusions. Together, these data show that endogenous apoE is an important mediator of cholesterol efflux. Under certain experimental conditions, macrophage apoE

and macrophage ABCA1 can cooperate to stimulate both apoE-mediated and ABCA1-mediated cholesterol efflux. Although statistically significant, the cooperation between apoE and ABCA1 was very small, suggesting that apoE-mediated efflux and ABCA1-mediated efflux are parallel pathways.

Introduction

Cholesterol efflux from macrophages and reverse cholesterol transport prevents foam cell formation and atherosclerosis. The two primary mediators of cholesterol efflux that are endogenously synthesized by macrophages are apolipoprotein (apo) E and ATP-Binding Cassette (ABC)-A1. ApoE is secreted locally by macrophages in the walls of vessels. Although apoE synthesized by the liver plays a major role in cholesterol transport, previous studies from our laboratory have demonstrated that apoE synthesized by macrophages has a greater role in the prevention of atherosclerosis (Fazio et al., 1997; Linton et al., 1995). ApoE-mediated cholesterol efflux is thought to be the mechanism of this protective effect (Kinoshita et al., 2000; Laffitte et al., 2001; Lin et al., 1999).

ABCA1 is a membrane protein that facilitates the movement of cholesterol and phospholipids to cholesterol acceptors. While apoAI is the main acceptor for ABCA1, apoE and other apolipoproteins interact with ABCA1 and accept lipids (Krimbou et al., 2004; Remaley et al., 2001). Both apoE and ABCA1 are up-regulated by oxysterols through the LXR/RXR system (Laffitte et al., 2001; Schwartz et al., 2000). It is not known if the coordinated expression of apoE and ABCA1 promotes cooperation between these two efflux pathways. The objective of the current study was to determine if apoE synthesis can stimulate ABCA1-mediated cholesterol efflux. Peritoneal macrophages from *apoE(+/+)*, *apoE(-/-)*, *ABCA1(+/-)* and *apoE(-/-)ABCA1(+/-)* mice were used in cholesterol efflux studies. Under certain experimental conditions, endogenous apoE synthesis stimulated ABCA1-mediated cholesterol efflux. Conversely, ABCA1 stimulated apoE-mediated cholesterol efflux. This study found that endogenous apoE functions as an acceptor in a cholesterol efflux pathway but can also act as a stimulator of a separate efflux pathway that is mediated by ABCA1 and apoAI.

Methods

ApoE-Mediated and ABCA1-Mediated Cholesterol Efflux. Murine peritoneal macrophages were elicited with thioglycollate (3%) as described in Chapter IV and cultured in 24-well or 96-well plates. Macrophages were treated as specified in the Results. To cholesterol load cultured macrophages, culture media was replaced with media containing 2-3 μCi/ml of ^{3}H-cholesterol and 70-100 μg/ml acLDL for 24-48 hours. Acetyl-LDL (acLDL) was prepared as described in Chapter IV. Monolayers were treated with 1 μM 22(R)-hydroxycholesterol when specified in the Results. The efflux period was initiated by the addition of efflux media with no acceptors or apoAI. To measure apoE-mediated efflux, serum-free DMEM with 0-0.1% BSA was added to designated wells. To measure ABCA1-mediated efflux, serum-free DMEM with 10-15 μg/ml human apoAI was added to designated wells. Efflux media was sampled at multiple time points and cell debris was removed by centrifugation. Remaining cellular ^{3}H-cholesterol were harvested by rinsing cells with PBS and then lysing cells with 1.0 ml of 0.1N sodium hydroxide. Counts (cpm) in media and lysate were detected with a Beckman LS 6000IC scintillation counter using Ecolite™ scintillation fluid (ICN, Costa Mesa, CA). Cholesterol efflux was calculated from the counts in the media as a percentage of the total counts (media plus lysate).

ApoE and ABCA1 mRNA Expression. For mRNA quantification, total RNA was isolated from cultured peritoneal macrophages using Trizol™ reagent (Invitrogen). The relative quantities of apoE or ABCA1 message were measured by real time RT-PCR according to the method described by Su et al. (Su et al., 2002). TaqMan™ one-step RT-PCR master mix reagent kit (Applied Biosystems) was used for RT-PCR. Relative quantification of apoE or ABCA1 was normalized with 18S ribosomal RNA.

Western Blot Analysis for ApoE or ABCA1 Protein. For Western blot analysis of apoE or ABCA1, proteins were separated on 3-8% NuPAGE™ Tris-Acetate gels (Novex) and transferred to nitrocellulose membranes. Murine apoE in growth media was measure by Western analysis with rabbit anti-mouse apoE antiserum. Murine ABCA1 from cell extracts was detected with a primary antibody (Novus Biological). Bands were visualized

by a chemiluminescent ECL Plus™ (Amersham Pharmacia Biotech) according to Bortnick et al. (Bortnick et al., 2000). Bands were quantified by densitometric analysis.

Results

Endogenously Synthesized ApoE Mediates Cholesterol Efflux

In order to measure the effects of apoE on cholesterol efflux, peritoneal macrophages were labeled with ^{3}H-cholesterol and then incubated in serum-free efflux media containing only BSA. ApoE-mediated cholesterol efflux after 6 hours was increased by 164% ($p<0.001$) in *apoE(+/+)* macrophages compared to *apoE(-/-)* macrophages (Figure 16).

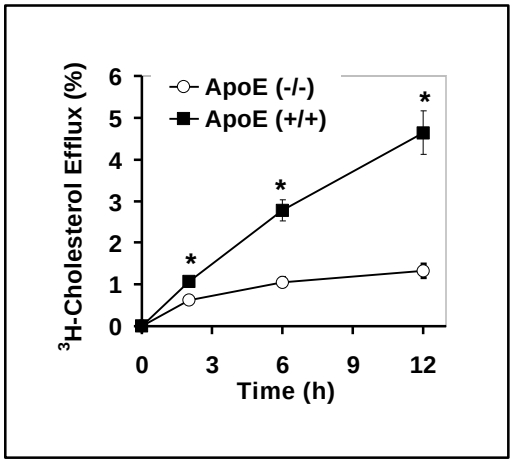


Figure 16. Endogenous ApoE Synthesis Increases Cholesterol Efflux from Macrophages.
Macrophages were treated for 36 hours with 2.0 μCi/ml of ^{3}H-FC and 70 μg/ml acLDL in DMEM/2% FBS to label cellular cholesterol. Cells were then cultured in serum-free efflux media with bovine serum albumin (0.1%). Bars and error bars represent the mean (n=6) and standard deviation of samples. Asterisks (*) denote a statistically significant difference ($p<0.05$) compared to the *apoE(-/-)* group as determined by Student's *t*-test.

Endogenous ApoE Stimulates ABCA1-Mediated Cholesterol Efflux

To determine the effects of apoE on ABCA1-mediated cholesterol efflux, macrophages labeled with ^{3}H-cholesterol were incubated with serum-free efflux media containing only apoAI. ABCA1-mediated cholesterol efflux was increased by 9%

(p=0.003) in *apoE(+/+)* macrophages compared to *apoE(-/-)* macrophages (Figure 17A). ABCA1 mRNA expression was not changed in *apoE(+/+)* macrophages (Figure 17B).

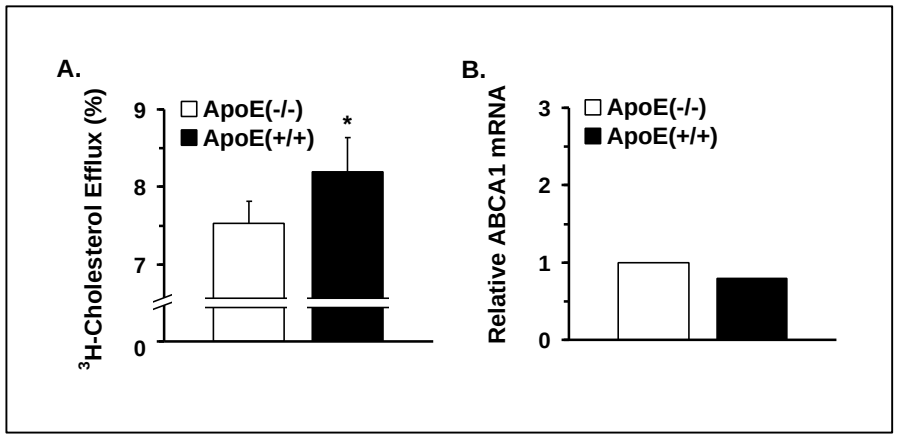


Figure 17. Endogenous ApoE Stimulates ABCA1-Mediated Cholesterol Efflux from Macrophages.
ApoE(-/-) (empty bars) and *apoE(+/+)* (solid bars) macrophages were treated for 24 hours with 70 μg/ml acLDL and 1.5 μCi/ml ^{3}H-cholesterol in serum-free DMEM/RPMI (1:1) with 1% Nutridoma™ media supplement. **(A)** ABCA1-mediated cholesterol efflux was measured from macrophages cultured in serum-free efflux media with excess apoAI (15 μg/ml) for 4.5 hours. Bars and error bars represent the mean (n=8) and standard deviation of samples. Asterisks (*) denote a statistically significant difference ($p<0.05$) compared to the *apoE(-/-)* group as determined by Student's *t*-test. **(B)** The relative quantity of ABCA1 mRNA was measured by real time RT-PCR. Each bar represents the measurement of a pooled sample from two mice.

LXR Agonism Stimulates ABCA1 and ApoE Cholesterol Efflux Pathways

Because LXR up-regulates both apoE and ABCA1 expression, the effects of the LXR agonist 22(R)-hydroxycholesterol on cholesterol efflux were measured. Stimulation with 22(R)-hydroxycholesterol increased ABCA1 mRNA 248% in *apoE(+/+)* macrophages and by 146% ($p<0.001$) in *apoE(-/-)* macrophages (Figure 18A). Stimulation with 22(R)-hydroxycholesterol increased ABCA1-mediated cholesterol efflux by 59% ($p<0.001$) in *apoE(+/+)* macrophages and by 60% ($p<0.001$) in *apoE(-/-)* macrophages (Figure 18B). Stimulation with 22(R)-hydroxycholesterol increased apoE-mediated cholesterol efflux to media with BSA by 108% ($p<0.001$) in *apoE(+/+)* macrophages, but in *apoE(-/-)* macrophages efflux to media with BSA was only increased by 72% ($p<0.001$) (Figure 18C).

ABCA1 Stimulates Endogenous ApoE-Mediated Cholesterol Efflux

To determine the effects of ABCA1 on apoE-mediated cholesterol efflux, experiments were performed with *ABCA1(+/-)* and *ABCA1(+/+)* macrophages that were also either *apoE(-/-)* or *apoE(+/+)*. These macrophages were stimulated with 22(R)-hydroxycholesterol to stimulate both efflux pathways.

On the background of *apoE(-/-)* macrophages, homozygous *ABCA1(+/+)* expression increased ABCA1-mediated cholesterol efflux by 54% ($p<0.001$) compared to heterozygous *ABCA1(+/-)* expression (Figure 19A). On the background of *apoE(+/+)* macrophages, homozygous *ABCA1(+/+)* expression increased ABCA1-mediated cholesterol efflux by 48% ($p<0.001$) compared to heterozygous *ABCA1(+/-)* expression (Figure 19A).

On the background of *apoE(-/-)* macrophages, homozygous *ABCA1(+/+)* expression did not change apoE-mediated cholesterol efflux compared to heterozygous *ABCA1(+/-)* expression (Figure 19B). On the background of *apoE(+/+)* macrophages, homozygous *ABCA1(+/+)* expression increased apoE-mediated cholesterol efflux by 13% ($p<0.001$) compared to heterozygous *ABCA1(+/-)* expression (Figure 19B).

ABCA1 protein was drastically increased in *apoE(+/+)ABCA1(+/+)* macrophages compared to *apoE(+/+)ABCA1(+/-)* macrophages (Figure 19C). This increase was not associated with increased ABCA1 mRNA in these macrophages. ApoE protein was similar in *apoE(+/+)ABCA1(+/+)* macrophages compared to *apoE(+/+)ABCA1(+/-)* macrophages (Figure 19C). There was increased apoE mRNA in *apoE(+/+)ABCA1(+/-)* macrophages (Figure 19D).

Two-way ANOVA with Bonferroni post-test was used to determine if apoE and ABCA1 cooperate to mediate cholesterol efflux. For ABCA1-mediated cholesterol efflux, *ABCA1(+/+)* homozygosity had a significant effect ($p<0.0001$) but endogenous apoE expression did not (Table 4). There was no interaction between ABCA1 and endogenous apoE for ABCA1-mediated cholesterol efflux. For apoE-mediated cholesterol efflux, both *ABCA1(+/+)* homozygosity ($p=0.0311$) and endogenous apoE ($p<0.0001$) had a significant effect (Table 4). There was a small but significant interaction ($p=0.0311$) between ABCA1 and endogenous apoE for ABCA1-mediated cholesterol efflux.

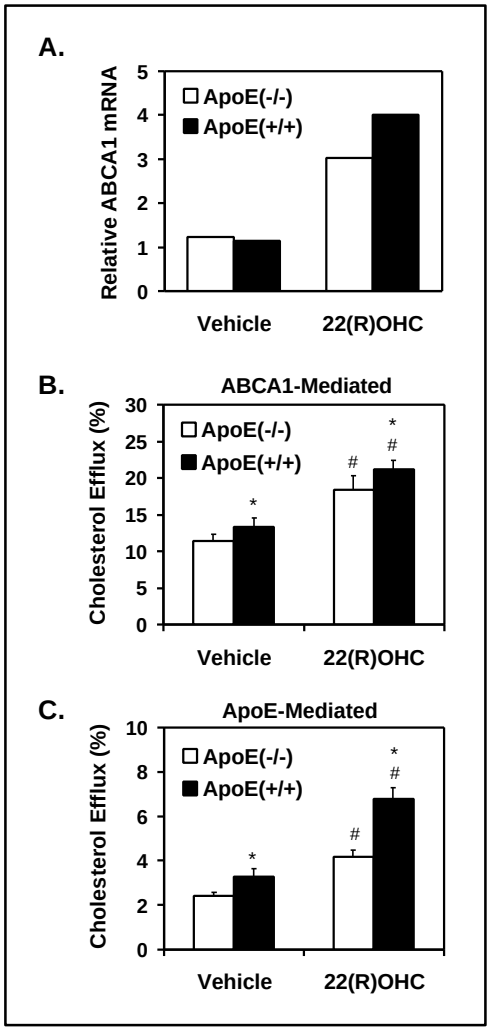


Figure 18. LXR Agonism Increases ApoE-Mediated and ABCA1-Mediated Cholesterol Efflux from Macrophages.
ApoE(-/-) (empty bars) and *apoE(+/+)* (solid bars) macrophages were treated for 48 hours with 3.0 μCi/ml of ^{3}H-FC and 100 μg/ml acLDL in DMEM/1% FBS to label cellular cholesterol. Macrophages were treated with 1 μM 22(R)-hydroxycholesterol for 18 hours. **(A)** The relative quantity of ABCA1 mRNA was measured by real time RT-PCR. Each bar represents the measurement of a pooled sample. **(B)** ABCA1-mediated cholesterol efflux was measured from macrophages cultured for 6 hours in serum-free efflux media with 10 μg/ml apoAI and 1 μM 22(R)-hydroxycholesterol. Bars and error bars represent the mean (n=8) and standard deviation of samples. Asterisks (*) denote a statistically significant difference ($p<0.05$) compared to the *apoE(-/-)* group as determined by Student's *t*-test. **(C)** ApoE-mediated cholesterol efflux was measured from macrophages cultured 6 hours in serum-free efflux media with bovine serum albumin (0.1%) and 1 μM 22(R)-hydroxycholesterol. Bars and error bars represent the mean (n=8) and standard deviation of samples. Asterisks (*) denote a statistically significant difference ($p<0.05$) compared to the *apoE(-/-)* group as determined by Student's *t*-test.

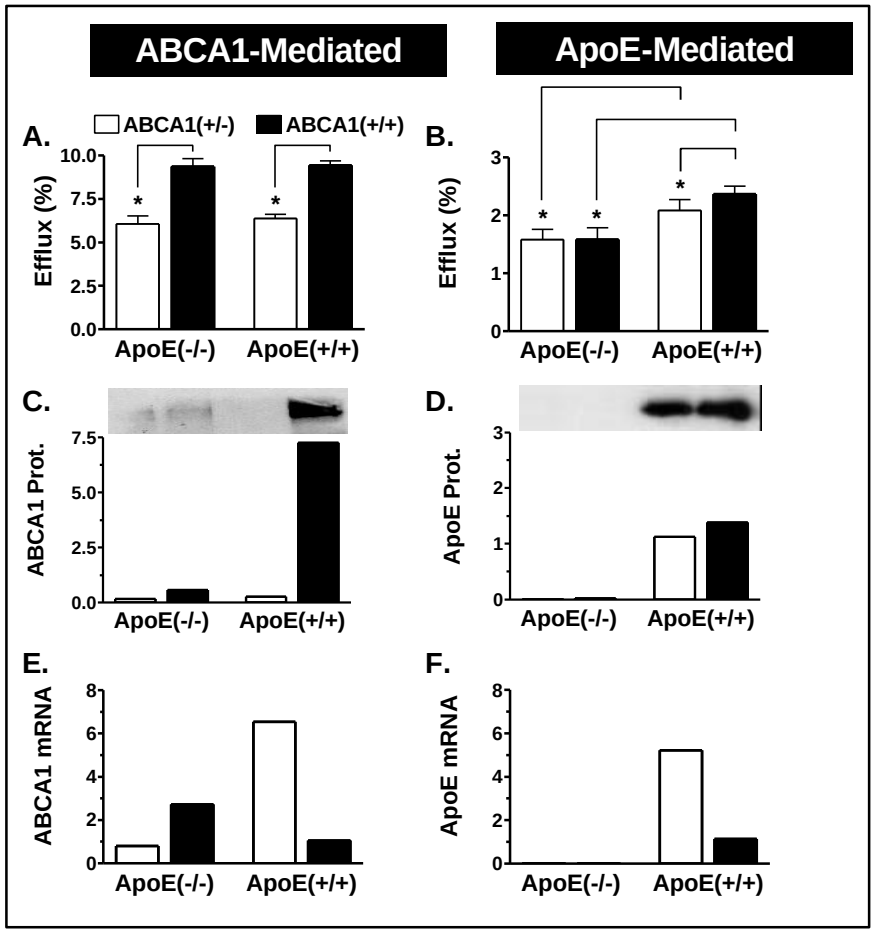


Figure 19. ABCA1 Stimulates Endogenous ApoE-Mediated Cholesterol Efflux from Macrophages.

ABCA1(+/-) (empty bars) and *ABCA1(+/+)* (solid bars) macrophages were treated for 48 hours with 3.0 μCi/ml of ^{3}H-FC and 100 μg/ml acLDL in DMEM/1% FBS to label cellular cholesterol. Macrophages were treated with 1 μM 22(R)-hydroxycholesterol for 18 hours. **(A)** ABCA1-mediated cholesterol efflux was measured from macrophages cultured for 6 hours in serum-free efflux media with 10 μg/ml apoAI and 1 μM 22(R)-hydroxycholesterol. Bars and error bars represent the mean (n=8) and standard deviation of samples. Statistical significance is determined by two-way ANOVA with Bonferroni post-test (*$p<0.05$). **(B)** ApoE-mediated cholesterol efflux was measured from macrophages cultured 6 hours in serum-free efflux media with 1 μM 22(R)-hydroxycholesterol. Bars and error bars represent the mean (n=8) and standard deviation of samples. Statistical significance is determined by two-way ANOVA with Bonferroni post-test (*$p<0.05$). **(C and E)** The relative quantity of ABCA1 protein and ABCA1 mRNA. Each bar represents the measurement of a pooled sample. **(D and F)** The relative quantity of apoE protein in media and apoE mRNA. Each bar represents the measurement of a pooled sample.

Table 4. Functional Cooperation between ApoE and ABCA1 Cholesterol Efflux Pathways.

ABCA1-Mediated Efflux (Figure 19A)			ApoE-Mediated Efflux (Figure 19B)		
ABCA1 Effect?	Yes	$p<0.0001$	ABCA1 Effect?	Yes	$p<0.0311$
ApoE Effect?	No		ApoE Effect?	Yes	$p<0.0001$
Interaction?	No		Interaction?	Yes	$p<0.0311$

Statistical significance ($p<0.05$) was calculated by two-way ANOVA.

Discussion

To determine if endogenous apoE affects ABCA1-mediated cholesterol efflux, we studied cholesterol efflux from *apoE(+/+)* and *apoE(-/-)* macrophages. We found that as a cholesterol acceptor, endogenous apoE increased cholesterol efflux from macrophages. Endogenous apoE also stimulated ABCA1-mediated cholesterol efflux to the cholesterol acceptor apoAI. Conversely, ABCA1 stimulated apoE-mediated cholesterol efflux. The cooperation between these two pathways of cholesterol efflux may be a mechanism by which the synthesis of endogenous apoE by macrophages maintains cholesterol homeostasis.

Interactions Between Cholesterol Efflux Pathways. Endogenous apoE synthesis caused an increase in ABCA1-mediated cholesterol efflux to apoAI (Figure 17). The current study and previous reports from other laboratories show only very small increases in cholesterol efflux from *apoE(+/+)* macrophages compared to *apoE(-/-)* controls when apoAI, the acceptor for ABCA1-mediated cholesterol efflux, is the exogenous acceptor (Langer et al., 2000). These data show the important role of endogenous apoE as a mediator of cholesterol efflux to certain acceptors but also support the idea that apoE and ABCA1-mediated cholesterol efflux are only minimally synergistic.

Both apoE and ABCA1 were able to mediate cholesterol efflux but there was a small amount of cooperative interaction between the pathways. Under certain experimental conditions, apoE stimulated the ABCA1-mediated cholesterol efflux (Figure 17A) and ABCA1 stimulated apoE-mediated cholesterol efflux (Figure 19B). The magnitude of these interactions were relatively small suggesting that these two pathways of efflux are parallel. Previous studies that use high concentrations of exogenous apoE show that apoE can bind and accept lipids from ABCA1 (Krimbou et al., 2004; Remaley

et al., 2001). The current study focused on endogenously synthesized apoE because of the physiologic delivery and concentrations achieved.

In the current study (Figure 18) and in previous reports from other laboratories, there were large increases in cholesterol efflux when macrophages are treated with LXR agonists (Chawla et al., 2001; Schwartz et al., 2000; Sparrow et al., 2002). However, there have been no reports of the effects of LXR agonist on apoE deficient macrophages. There was an increase in efflux to BSA even in the absence of apoE secretion (Figure 18C). This may be the result of LXR-induced changes in cholesterol trafficking within the cell that allows for greater diffusion of cholesterol from the plasma membrane or the secretion of other apolipoproteins (Mak et al., 2002). Alternatively, this increase may actually represent ABCA1-mediated cholesterol efflux to apolipoprotein contaminants in the BSA.

Cholesterol Acceptors and Atherosclerosis. ApoE-mediated cholesterol efflux was increased in *apoE(+/+)* macrophages compared to *apoE(-/-)* macrophages (Figure 16). These data are in agreement with previous reports that show that apoE secretion increases cholesterol efflux in macrophage-like cells and in mouse macrophages (Langer et al., 2000; Lin et al., 1999). The relationship between cholesterol efflux and protection from atherosclerosis are upheld in studies of apoE, ABCA1, HDL, and other mediators of the RCT system (Fazio et al., 1997; Ishiguro et al., 2001; Lin et al., 1999; Linton et al., 1995; Major et al., 2001; Su et al., 2003; Zhang et al., 2003a).

Summary. In summary, these data show that apoE is a mediator of cholesterol efflux, both as a cholesterol acceptor in the apoE efflux pathway and as a mild stimulator of the ABCA1 efflux pathway. In the apoE-mediated efflux pathway, ABCA1 has a modest stimulatory effect on efflux to apoE. These data suggest that although there is interaction and mutual stimulation of apoE-mediated and ABCA1-mediated cholesterol efflux, these two efflux pathways are largely independent.

CHAPTER VII

THE AUTOCRINE AND PARACRINE EFFECTS OF ENDOGENOUSLY SYNTHESIZED APOLIPOPROTEIN E ON CHOLESTEROL EFFLUX

Abstract

Objective. Macrophages in the vessel wall secrete high levels of apolipoprotein (apo) E. Cholesterol efflux from macrophages to apoE has been shown to decrease foam cell formation and prevent atherosclerosis. An apoE molecule can mediate cholesterol efflux from the macrophage that originally secreted it (i.e. autocrine effect) or from surrounding macrophages (i.e. paracrine effect). Traditional methodologies have not been able to separate these serial effects. The novel methodology presented here was developed to separate autocrine and paracrine effects by using a simple mathematical model to interpret the effects of dilution on apoE-mediated cholesterol efflux.

Results. Our results show that at very dilute concentrations, the paracrine effect of apoE is not evident and that the autocrine effect becomes the dominant mediator of efflux. However, at saturating concentrations, paracrine apoE causes 80 to 90% of the apoE-mediated cholesterol efflux, while autocrine apoE is responsible for the remaining 10 to 20%.

Conclusions. These results suggest that the relative importance of autocrine and paracrine apoE depends on the size of the local distribution volume, a factor not considered in previous *in vitro* studies regarding apoE function. Furthermore, autocrine effects of apoE could be critical in the prevention of foam cell formation, *in vivo*. This novel methodology may be applicable to other types of mixed autocrine/paracrine systems, such as signal transduction systems (Dove et al., 2005a).

Introduction

An important challenge in atherosclerosis research is the characterization of the effects of locally synthesized apolipoprotein (apo) E within the vessel wall. Endogenous synthesis and secretion of apoE by macrophages in the vessel wall has been shown to

protect against atherosclerosis (Linton et al., 1995). Arterial macrophages participate in inflammation, tissue remodeling, and lipid metabolism. ApoE, which is synthesized by hepatocytes, adipocytes and macrophages, mediates lipoprotein metabolism and affects cellular cholesterol homeostasis. ApoE from macrophages accepts cholesterol from cells in the vessel wall and transports it back to the liver where the cholesterol can be excreted as bile (Basu et al., 1983; Dory, 1989). This pathway is called the reverse cholesterol transport (RCT) system. The effect of apoE can be due to its cellular or extracellular positioning, and therefore an apoE molecule can mediate cholesterol efflux from the macrophage that originally secreted it (i.e. autocrine effect) or from surrounding macrophages (i.e. paracrine effect) (Figure 20). The terms "autocrine" and "paracrine", traditionally applied to signaling peptides and hormones, have also been used to describe the actions of mediators with a broad range of functions besides signal transduction (Balboa et al., 2003; Coussens et al., 2000; Moran et al., 2002). It can be assumed that for the sake of cholesterol efflux an individual macrophage cannot distinguish the apoE that it secretes from the apoE derived from neighboring cells. However, since the endogenous synthesis of apoE causes high spatial proximity, autocrine apoE is at an advantage compared to paracrine apoE. The spatial proximity advantage of endogenous apoE results in a temporal sequence of autocrine effects occurring before paracrine effects and a concentration gradient from an autocrine compartment (high concentration) to a paracrine compartment (low concentration).

Making comparisons between the autocrine and paracrine effects of apoE synthesized by macrophages has proven difficult. For *in vitro* experiments with cultured macrophages, exogenously applied apoE has been used to approximate paracrine or endocrine apoE (Ho et al., 2000; Lin et al., 1999). Endogenously synthesized apoE has been used to approximate autocrine apoE (Huang et al., 2001; Lin et al., 1999; Lin et al., 2001; Lin et al., 1998). These approximations do not consider that a single molecule of an endogenously synthesized mediator can have a series of interactions that employ a combination of autocrine and paracrine mechanisms (Batsilas et al., 2003).

The novel methodology discussed here was developed to separate autocrine and paracrine mechanisms by using a simple mathematical model to interpret mediator-induced biological effects. This study shows that while macrophage apoE has both

autocrine and paracrine effects on cholesterol efflux, autocrine apoE has smaller but more consistent effects than paracrine apoE (Dove et al., 2005a). Whereas macrophage apoE is used to demonstrate this novel methodology, the concepts have applications for separating the autocrine/paracrine effects of many other secreted biological mediators including growth factors, cytokines, and carrier proteins.

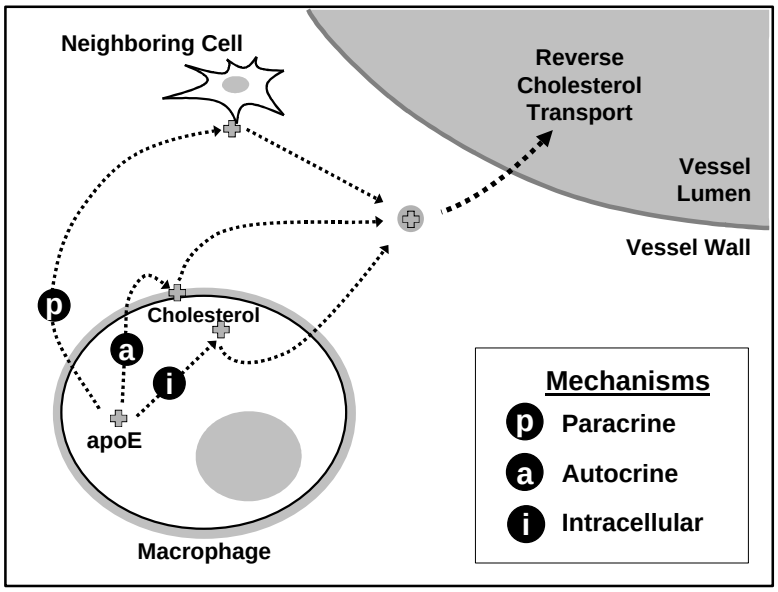


Figure 20. Potential Mechanisms of ApoE-Mediated Cholesterol Efflux. Within the walls of vessels, endogenously synthesized apoE can mediate cholesterol efflux from the macrophage that originally secreted it (i.e. autocrine effect) or from surrounding macrophages (i.e. paracrine effect). Regardless of mechanism, apoE-mediated cholesterol efflux removes cholesterol and engages the reverse cholesterol transport system.

Glossary

t	time (hours)
E_{WT}	cholesterol efflux (% efflux) from *apoE(+/+)* macrophages
E_{KO}	cholesterol efflux (% efflux) from *apoE(-/-)* macrophages
DE	apoE-mediated cholesterol efflux (% efflux)
V	volume of the extracellular space (ml)
V^{-1}	relative concentration (ml^{-1}) of apoE in the extracellular space
m	slope (% efflux / (ml^{-1})) of linear DE vs. V^{-1} curve

b — y-int. (% efflux) of the linear DE vs. V^{-1} curve

C — effective concentration (ml^{-1}) of apoE in the juxtacellular space

n — slope (ml^{-1} / 24h) of the b/t vs. m curve

f — y-int. (% efflux / 24h) of the b/t vs. m curve

k_E — coefficient (ml^{-1}) for apoE-mediated cholesterol efflux

k_I — coefficient (ml^{-1}) for apoE-mediated cholesterol influx

Methods

Primary Culture of Peritoneal Macrophages. Murine peritoneal macrophages were elicited by intraperitoneal injection of 3% thioglycollate as described in Chapter IV. Macrophages were plated at 4 x 10^5 macrophages/well on 24-well plates. Macrophages were treated with 100 μg/ml acLDL in DMEM/2% FBS for 48 hours to load with cholesterol and to stimulate apoE secretion (Mazzone et al., 1987). Acetyl-LDL (acLDL) was prepared as described in Chapter IV.

Efflux of Cellular Cholesterol. Macrophages were labeled with 2 μCi/ml [1,2-^{3}H(N)]cholesterol (Perkin Elmer Life Sciences, Boston, MA) in DMEM for 12 hours (Lin et al., 1999; Mukherjee et al., 1998). *ApoE(+/+)* and *apoE(-/-)* macrophages, respectively, had 64.1 ± 5.0 and 98.0 ± 12.3 μg total cholesterol/mg cell protein. *ApoE(+/+)* and *apoE(-/-)* macrophages, respectively, had 786.66 ± 133.36 versus 782.8 ± 98.93 μg cpm/mg cell protein. Labeled macrophages were rinsed three times with DMEM/0.2% BSA. The efflux period is initiated by the addition of DMEM with no acceptors (0.3, 0.5, 0.75, 1.0, 1.5, or 2.0 ml). Efflux media was removed following efflux periods of 8, 24, 48, or 72 hours and cell debris was removed by centrifugation. Remaining cellular ^{3}H-cholesterol were harvested by rinsing cells with PBS and then lysing cells with 1.0 ml of 0.1N sodium hydroxide. Counts (cpm) in media and lysate were detected with a Beckman LS 6000IC scintillation counter using Ecolite™ scintillation fluid (ICN, Costa Mesa, CA). Cholesterol efflux was calculated from the counts in the media as a percentage of the total counts (media plus lysate). The difference in cholesterol efflux (E) between *apoE(+/+)* (WT) and *apoE(-/-)* (KO) macrophages is

considered to be the cholesterol efflux that is specifically mediated by apoE. ApoE-mediated efflux (*DE*) is calculated as

$$\Delta E = E_{WT} - E_{KO} \quad . \tag{1}$$

Western Blot Analysis of Secreted ApoE. Macrophages were incubated in DMEM with no acceptors (0.3, 0.5, 0.75, 1.0, 1.5, or 2.0 ml) for 18 hours and cell debris was removed by centrifugation. After bringing the total culture volumes to 2.0 ml with fresh DMEM, ApoE was extracted with Liposorb™ gel (Calbiochem, San Diego, CA). Western blot analysis of secreted apoE was performed as described in Chapter VI.

^{14}C-Adenine Release Assay for Cellular Toxicity. As described in Chapter IV, cholesterol-induced toxicity in macrophages was assayed by measuring the leakage of ^{14}C-adenine into media (Warner et al., 1995). After labeling, the release period was initiated by the addition of DMEM. Media was removed after a release period of 24 hours and counted by scintillation.

Volume-Dependence Theory. In order to manipulate the extracellular concentration of an endogenously synthesized biological mediator, this theory takes advantage of the implicit relationship between concentration and distribution volume. The concentration of a secreted mediator such as apoE is proportional to the reciprocal of the extracellular distribution volume. Increasing the distribution volume (*V*) causes a decrease in the relative concentration (V^{-1}) of the secreted mediator. Varying the extracellular volume of a given number of cells (Figure 21A) allows for the juxtacellular and extracellular mechanisms of the synthesized mediator to be mathematically separated and characterized. This theory can be illustrated graphically as follows. For a plot of the biological effect (*DE*) versus extracellular volume (*V*), as the extracellular volume is increased, the effect decreases asymptotically to a plateau that represents the portion of the total effect that is independent of the extracellular volume (Figure 21B). The value of this plateau is the *y*-intercept (*b*) of a plot of the biological effect (*DE*) versus reciprocal volume (V^{-1}) (Figure 21C). This dose-response curve is linear at low relative concentrations (V^{-1}) and fits the linear equation,

$$\Delta E = mV^{-1} + b\,. \qquad (2)$$

Data Analysis. Data were expressed as mean ± standard deviation. Means were compared by Student's *t*-test. Curves were analyzed by linear regression.

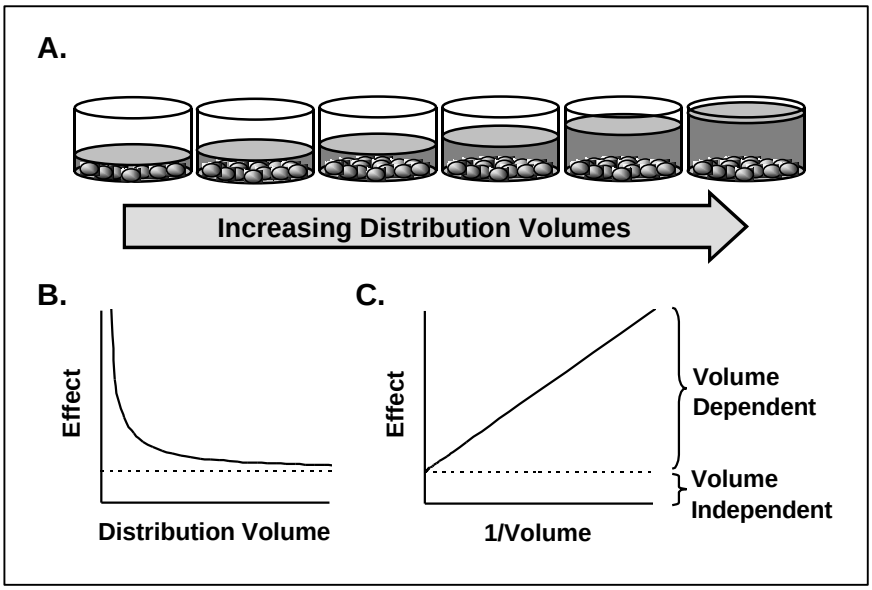


Figure 21. Theoretical Basis for the Separation of Autocrine and Paracrine Effects of ApoE on Cholesterol Efflux.
(A) The experimental design that changes the relative concentrations of apoE by increasing the extracellular distribution volume. **(B)** Dependence of the effect of apoE on the distribution volume. **(C)** Dependence of the effect of apoE on the reciprocal volume. Effects of apoE that depend on the relative concentration in the extracellular distribution volume are paracrine. Effects that occur solely in the juxtacellular space are independent of the extracellular distribution volume and are autocrine.

Results

Volume-Dependence of ApoE-Mediated Cholesterol Efflux

In *apoE(+/+)* macrophages, efflux decreased as the distribution volume increased (Figure 22A). ApoE-mediated efflux appeared to be saturated at smaller distribution volumes and then fell to a plateau (*b*) as the volume increased. At 48 hours, the dependence of apoE-mediated efflux on reciprocal volume was initially linear (m = 2.95 ± 0.63 % efflux / (ml^{-1}), b = 0.85 ± 0.58 % efflux, R^2 = 0.92) as determined by linear

regression analysis (Figure 22B and Figure 22C). Similar trends were seen at 8, 24, and 72 hours. At least 10 to 20% (*b* expressed as a percentage of the maximum efflux) of the apoE-mediated cholesterol efflux was independent of the reciprocal volume (Table 5). The remaining 80 to 90% of the apoE-mediated cholesterol efflux was linearly dependent on the reciprocal volume (Table 5). The volume-independent contributions to efflux become dominant as extracellular apoE becomes more dilute. A descriptive equation for apoE-mediated cholesterol efflux can be generated from these data.

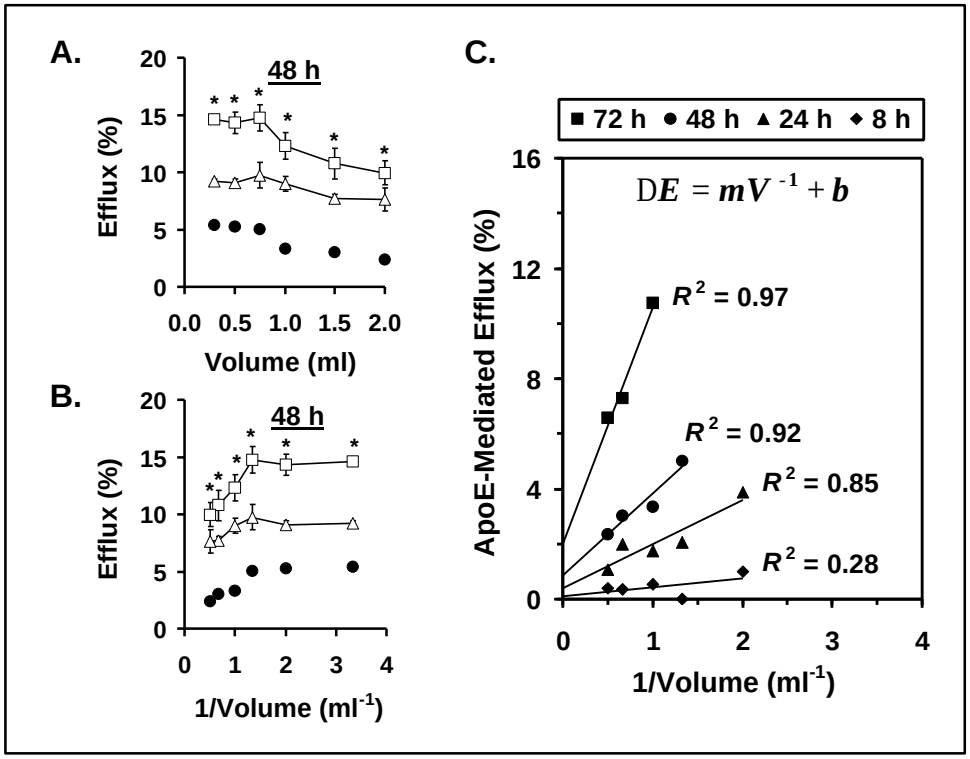


Figure 22. Volume-Dependence of ApoE-Mediated Cholesterol Efflux.
The **(A)** volume-dependence and the **(B)** relative concentration-dependence of cholesterol efflux from *apoE(+/+)* macrophages (squares) *apoE(-/-)* macrophages (triangles) and the apoE-mediated efflux (filled circles). Values are expressed as mean (4 samples) ± SD of efflux from *apoE(+/+)* and *apoE(-/-)* macrophages with asterisks (*) denoting a statistically significant difference ($p<0.05$) by Student's *t*-test. **(C)** Linear fit of the apoE-mediated efflux versus reciprocal volume for 8 h (diamonds), 24 h (triangles), 48 h (circles), and 72 h (squares).

Table 5. Autocrine and Paracrine ApoE Contributes to Cholesterol Efflux.

Time (h)	Maximum *DE* (% efflux)	Volume-Independent: *b*		Volume-Dependent: mV^{-1} (% efflux)
		(% efflux)	(% of max. *DE*)	
72	~10.74	1.98	~18	8.61 V^{-1}
48	~5.41	0.85	~16	2.95 V^{-1}
24	~3.87	0.38	~10	1.61 V^{-1}
8	~0.99	0.10	~11	0.32 V^{-1}

ApoE-mediated efflux (*DE*) data from experiments measuring dependence on distribution volume (*V*) was fit to Equation 2 ($DE = mV^{-1} + b$) to determine *y*-intercepts (*b*) and slopes (*m*). The maximum *DE* for each time-point was estimated from the highest *DE* value beyond the linear portion of the curve. The volume-independent (autocrine) effects of apoE correspond to the *y*-intercept (*b*). The volume-dependent (paracrine) effects of apoE correspond to the product of the slope and the reciprocal volume (mV^{-1}).

Volume-Dependence of ApoE Secretion

Western blot analysis was performed to determine if changes in apoE secretion were related to trends in efflux. In order to determine if macrophages secreted equal masses of apoE, the apoE was extracted from the culture media. By densitometric analysis, macrophages cultured in higher media volumes (0.75, 1.0, 1.5, and 2.0 ml) secreted similar amounts of apoE per cell culture suggesting that the size of the distribution volume had no effect on apoE secretion (Figure 23A). However, macrophages cultured in smaller volumes (0.3 and 0.5 ml) secreted more apoE per cell culture.

Volume-Dependence of Cellular Viability

^{14}C-adenine release studies were performed to determine if changes in viability were related to trends in efflux or apoE secretion. Viability was increased in smaller distribution volumes as indicated by higher amounts of ^{14}C-adenine remaining (Figure 23B). Viability increased in a log-linear fashion ($R^2 = 0.99$) with increasing reciprocal volume (Figure 23C). This log-linear trend in viability is different from the linear trend in efflux.

Model for ApoE-Mediated Cholesterol Efflux

With a limited number of assumptions, a mathematical model can be generated that suggests a physical model for the autocrine and paracrine effects of apoE on cholesterol efflux. The calculation of apoE-mediated efflux (*DE*) in Equation 1 assumes that any difference is a primary effect of apoE and not a secondary effect of changes in cellular cholesterol homeostasis (i.e. changes in viability or changes in membrane fluidity). The data in Figure 22, Table 5, and Figure 23 assume that evaporation of the extracellular distribution volume is negligible. However evaporation was determined to be ~0.03 ml/ 24 hours for this system. The evaporation rate affects the calculation of apoE-mediated efflux (*DE*) and the calculation of reciprocal volume (V^{-1}). Factoring in the evaporation rate does not change the trends in the data. However, the evaporation corrections are necessary to estimate the parameters in the mathematical model.

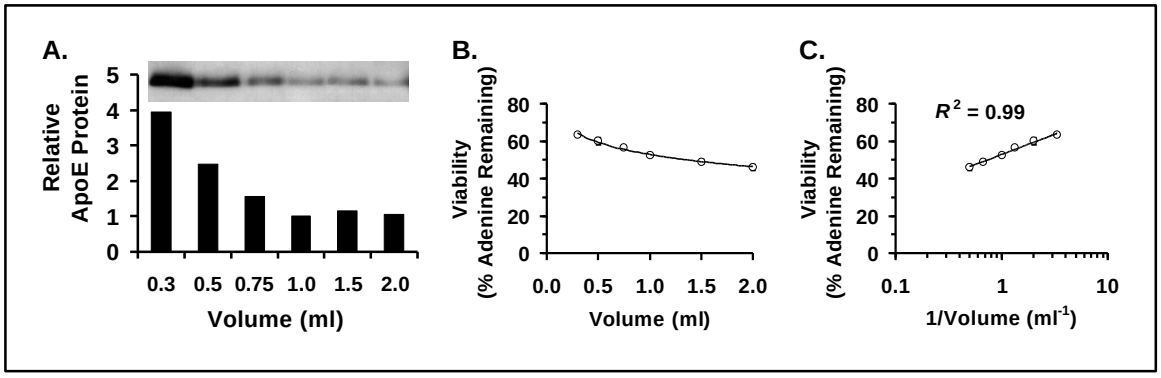


Figure 23. Volume-Dependence of ApoE Secretion and Macrophage Viability.
The **(A)** secretion of apoE per cell culture and the **(B and C)** viability of *apoE(+/+)* macrophages in varying reciprocal volumes. Values are expressed as mean (4 samples) ± SD of adenine release from *apoE(+/+)* macrophages.

From an extrapolation of the concentration-dependence lines, a common *x*-intercept is observed and is assigned a value of negative *C* (-0.270 ± 0.047 ml^{-1}) (Figure 24A). The common *x*-intercept (-*C*, 0) for all the lines that fit Equation 2 yields the following equation:

$$b/C = m \ . \tag{3}$$

This reveals that the concentration-dependence lines can be combined into one equation that is a function of time rather than four unrelated equations. This would be expected because the secretion of apoE is a function of time and, therefore, apoE-mediated effects would also be a function of time. If the *y*-intercept represents an autocrine effect that results from a constant rate of secretion of apoE then normalizing the *y*-intercept for time would reveal a constant (*f*) where

$$b/t = f \ . \tag{4}$$

Normalized *y*-intercepts (*b*/*t*), however, are not constant for each time point: 0.66, 0.43, 0.39 and 0.33 % efflux/24h (Figure 24B). A plot of normalized *y*-intercepts (*b*/*t*) versus the corresponding slope (*m*) reveals an unexpected relationship that is linear (*n* = 0.0411 ± 0.0009 ml^{-1}/24h, *f* = 0.305 ± 0.004 % efflux/24h, R^2 = 1.00) as determined by linear regression analysis and can be expressed as

$$b/t = nm + f \ . \tag{5}$$

Equations 2, 3, and 5 are combined in order to generate a function that can describe the effects of apoE on cholesterol efflux and that can be interpreted in a mechanistically compartmentalized manner:

$$\Delta E = ft\left(\frac{V^{-1} + C}{C - nt}\right). \tag{6}$$

Equation 6 fits the experimental data (Figure 24C and Figure 24D). In this form, Equation 6 expresses apoE-mediated cholesterol efflux as the product of the capacity (*ft*) of the system and the ratio of two coefficients: an efflux coefficient ($k_E = V^{-1} + C$) and an influx coefficient ($k_I = C - nt$). Based on this mathematical model, a biological model is proposed (Figure 25).

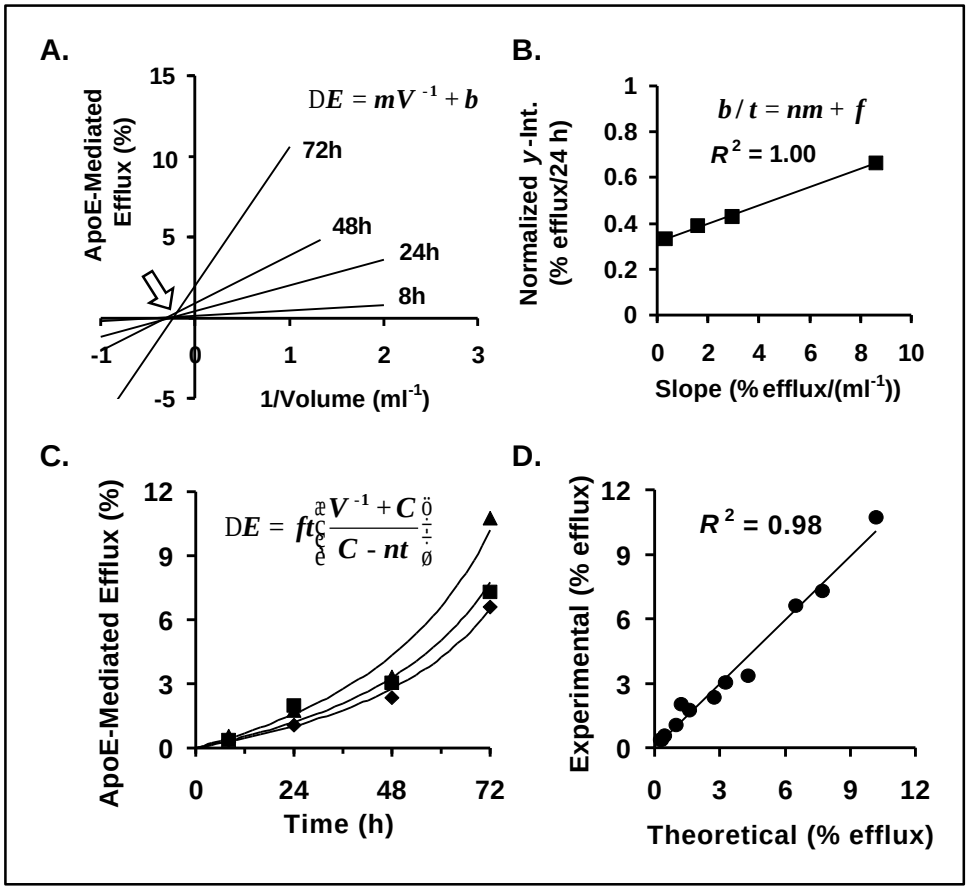


Figure 24. Mathematical Trends in ApoE-Mediated Cholesterol Efflux Data. **(A)** The common *x*-intercept (-*C*, 0) (arrow) of lines from apoE-mediated efflux versus reciprocal volume plots. **(B)** The linear relationship between the normalized y-intercept (b/t) and the slope (m) of lines from apoE-mediated efflux versus reciprocal volume plots. **(C)** The kinetics of apoE-mediated efflux at distribution volumes of 1.0 ml (triangles), 1.5 ml (squares), and 2.0 ml (diamonds). The experimental data were fit to Equation 6 with experimentally estimated values for the parameters *C*, *n*, and *f*. **(D)** Correlation of experimental and theoretical values for the proposed model for apoE-mediated efflux (Equation 6).

Discussion

To determine if the autocrine and paracrine actions of apolipoprotein E can be separated and quantified we measured cholesterol efflux from macrophages cultured in varying distribution volumes. We found that the volume-independent effects of apoE can be identified by using a simple mathematical model (Equation 2). We theorize that these volume-independent effects correspond to the autocrine actions of apoE. The basis of this theory is that the range of autocrine signals approximates the width of the secretion layer,

which separates the cell from the bulk media. Studies by Oehrtman et al. (Oehrtman et al., 1998) and Shvartsman et al. (Shvartsman et al., 2001) suggest that two phenomena occur when a mediator goes beyond the autocrine range: 1) the mediator is no longer autocrine, and 2) the mediator becomes diluted in the bulk media. Our data suggests that the relative importance of the autocrine and paracrine effects of apoE depends on the size of the local distribution volume, with the autocrine effects remaining constant even when the paracrine effects are diminished by apoE dilution. The descriptive equation generated from these data (Equation 6) suggests that autocrine and paracrine apoE work in parallel to mediate cholesterol efflux from macrophages.

Autocrine and Paracrine Effects of ApoE. Our study suggests that autocrine apoE has advantages that are biologically relevant. Even when the extracellular space is saturated with apoE, at least 10 to 20% of the apoE-mediated cholesterol efflux is the result of volume-independent interactions of endogenous apoE (Table 5). Lin et al. found that with apoE deficient cells, the exogenous application of apoE at ten times the concentration of normal endogenous apoE secretion did not increase cholesterol efflux as much as normal endogenous apoE secretion by apoE-producing cells (Lin et al., 1999). The fundamental importance of these media transfer studies are that they show that exogenous apoE (paracrine) is not equivalent to endogenous apoE (autocrine and paracrine). For a macrophage with endogenously synthesized apoE, the advantage of autocrine apoE may become critical if homeostasis is challenged by cholesterol loading, if the conditioned space is large (making apoE diluted), if the turnover of conditioned space is high because of interstitial flow, or if a macrophage is isolated from other macrophages. The interstitial concentration, distribution volume, and turnover of macrophage apoE in the vessel wall are not known. These factors are critical for the interpretation of data from *in vitro* cell culture systems and the generalization of these data to the biology of macrophages *in vivo*. For example, the trend in viability may be related to changes in apoE secretion observed at very low volumes (Figure 23A). The log-linear trend in viability (Figure 23C) may be due to the relative concentrations of confounding mediators like growth factors and culture gases (e.g. carbon dioxide) in varying distribution volumes. Since apoE is not the only mediator being secreted in our

system, it was necessary to subtract the effects of confounding mediators. This is why *apoE(+/+)* macrophages were compared to *apoE(-/-)* macrophages and why the specific apoE effect was calculated as apoE-mediated cholesterol efflux (ΔE). The effects of confounding mediators on cholesterol efflux were minimal (Figure 22A and Figure 22B). Although the major role of apoE is as an extracellular cholesterol acceptor, it is also possible that a portion of the effect of apoE on cholesterol efflux is the consequence of other actions of apoE, such as receptor or proteoglycan binding (Ji et al., 1998), intracellular cholesterol routing (Deng et al., 1995), or even stabilization of the cytoskeleton (Brecht et al., 2004). Irrespective of which ultimate mechanisms or combination of effects may be responsible for the modulation of cholesterol efflux by apoE, these can only occur through either autocrine or paracrine events, and are therefore addressed as a whole in our study.

Although the volume-dependence of apoE secretion and viability could not explain the volume-dependent trends of apoE-mediated efflux (Figure 22C), these data definitely underscore the sensitivity of biological processes to *in vitro* culture volumes. Some of the higher order characteristics of macrophage tissue could be overlooked by *in vitro* studies with macrophages. For example, an aggregate of macrophages may become a tissue that works with autocrine efficiency instead of being just the sum of its autocrine/paracrine cellular units. The spatial range of autocrine effect (Shvartsman et al., 2001) is very important because if another cell is within this range there is the possibility of autocrine cooperation. This possibility could be explored by *in vitro* or *in vivo* studies on the effects of the geometry and the density of cell aggregates on apoE-mediated cholesterol efflux. Compared to exogenous lipoprotein-bound apoE of hepatic origin, local secretion of lipid free apoE by macrophages results in decreased atherosclerosis due to a small amount of apoE in a critical location (Fazio et al., 1997; Linton et al., 1995). In the study by Fazio et al., wildtype mice were transplanted with *apoE(-/-)* bone marrow. These chimeric mice had normal plasma apoE but no macrophage apoE production in the vessel walls. Although immunocytochemical staining of lesions with wildtype macrophages reveals high apoE levels, these chimeric mice with only plasma apoE had little to no apoE in the lesions. The lack of staining for plasma-derived apoE within the artery wall suggests that penetration into clusters of macrophages is limited. The

macrophage, not plasma-derived apoE, is the primary source of apoE for reverse cholesterol transport (Blum et al., 1980; Fazio et al., 1997; Linton et al., 1995). Along these same lines, paracrine apoE may not be able to fulfill the critical actions of the small amount autocrine apoE in the juxtacellular space or possibly even the intracellular space. ApoE recycling, the internalization and re-secretion of apoE (Farkas et al., 2003; Heeren et al., 2003; Swift et al., 2001), has been reported to mediate cholesterol efflux in hepatocytes and in macrophages (Heeren et al., 2003). The methodology in this study is unable to resolve the "internal autocrine" (Sporn and Roberts, 1992) or "intracrine" (Re, 1988; Re, 2002) mechanism that could be mediated by intracellular apoE (Figure 20).

Proposed Model for Autocrine/Paracrine ApoE. We propose a model from Equation 6 in which macrophages secrete apoE to create an extracellular sink for cellular cholesterol. The capacity, concentration and compartmentalization of the sink affects cholesterol efflux (Figure 25). The sink has a capacity (*ft*) that expands as apoE is secreted. The sink can be separated into two compartments that are a consequence of the location of apoE in juxtacellular or extracellular space, also described as the secretion layer and bulk layer, respectively (Lauffenburger et al., 1998; Oehrtman et al., 1998). The dilution of apoE as it moves to the extracellular space diminishes its efficacy. ApoE-mediated efflux (*DE*) is a balance between the efflux and influx of cholesterol. Efflux depends on the sink capacity (*ft*), the concentration of extracellular apoE (V^{-1}), and the functional concentration of juxtacellular apoE (*C*). Influx depends on the cholesterol in the apoE sink (*DE*) and the functional concentration of juxtacellular apoE (k_I). Decreases in the functional concentration of apoE that contribute to influx (*nt*) may represent changes in the capacitance of apoE due to accumulation of phospholipids that stabilize cholesterol or due to proteolytic alteration of apoE. It is also possible that *n* is an artifact of slight changes in viability or apoE secretion.

Mixed Autocrine/Paracrine Systems. In many of the documents that are found by a literature search for the term "autocrine", the phrase "autocrine and/or paracrine" describes the proposed mechanism of action. It is difficult to quantitatively separate these two mechanisms experimentally. Traditional approaches to this problem have limitations

and rely on simple qualitative assessments. Traditional methodologies that have been used to study macrophage apoE include characterizing the tissue distribution of apoE (Fazio et al., 2002), measuring the effects of the endogenous synthesis of apoE versus its complete absence (Fazio et al., 2002), and measuring the effects of the endogenous synthesis versus exogenous application of apoE (Ho et al., 2001; Lin et al., 1999). These methods are useful in comparative assessments but they are unable to simultaneously quantify the serial effects mediated by apoE or other secreted biological mediators.

In the current study, we have performed experiments based on the theory that the portion of the endogenous acceptor that mediates autocrine effects cannot be diluted. As introduced in Chapter III, the protocol for these experiments relies on dilution of extracellular mediators by increasing the distribution volumes in which cells are cultured. Previous studies have utilized experimental strategies similar to the one used in our study. Steck et al. have performed volume-dependence experiments to study whether efflux to exogenous acceptors is proceeded by aqueous diffusion of cholesterol or by acceptor-membrane collisions (Steck et al., 1988). They found that for certain types of acceptors, efflux approached a "volume-insensitive plateau" and they proposed that these types of acceptors may have "sites for efficient collisional transfer" (Steck et al., 1988). Our studies with endogenously synthesized acceptors suggest that these sites may be a combination of binding sites on the membrane and an enriched aqueous layer around the membrane (i.e. the juxtacellular space). Oehrtman et al. used a theoretical model to study the escape of autocrine signaling ligands into the extracellular space. They found in a model of a ligand-receptor signaling system that "varying volume heights shows little effect on ligand concentrations" (Oehrtman et al., 1998). It remains to be seen if the dilution methodology discussed here can change mediator concentrations over the large dynamic range (many orders of magnitude) of a log-linear dose-response curve such as those seen for ligand-receptor signaling pathways.

Summary. In summary, this study separates and quantifies the autocrine and paracrine effects of apoE on cholesterol efflux from macrophages. These data suggest that the relative importance of autocrine and paracrine apoE depends on the size of the

local distribution volume, with the autocrine effects remaining constant even when the paracrine effects are diminished by the dilution of apoE.

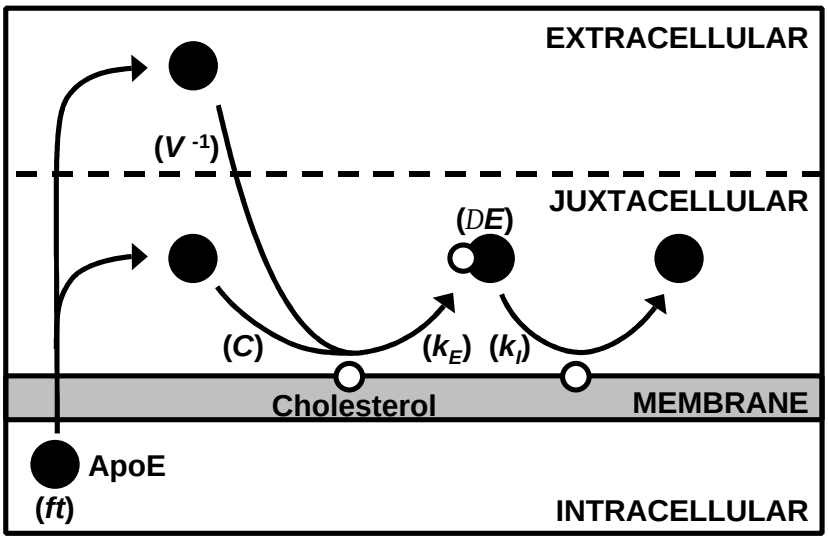


Figure 25. Proposed Model of ApoE-Mediated Cholesterol Efflux: Autocrine and Paracrine Effects.
There are two compartments in which secreted apoE (•) is distributed: the juxtacellular space and the extracellular space. All endogenously synthesized apoE is initially distributed in the juxtacellular space where it accepts cholesterol (o) in an autocrine fashion. ApoE is eventually distributed into the extracellular space where it accepts cholesterol in a paracrine fashion that is sensitive to the volume in which the cells are cultured. Determining the volume-independent effects on cholesterol efflux can distinguish the autocrine actions of apoE from the paracrine actions.

CHAPTER VIII

THE EFFECTS OF ENDOGENOUSLY SYNTHESIZED HUMAN-APOLIPOPROTEIN AI ON CHOLESTEROL EFFLUX

Abstract

Objective. Apolipoprotein (apo) AI is a strong acceptor of cellular cholesterol and is the major protein in HDL. The ability of apoAI to mediate cholesterol efflux from macrophages makes apoAI a critical part of the reverse cholesterol transport system. While apoE is synthesized locally in vessel walls by macrophages, apoAI is synthesized in the liver. ATP-Binding Cassette (ABC)-A1 facilitates cholesterol efflux and has been shown to use apoAI as an acceptor. The objectives of the current study were to measure whether the endogenous production of transgenic apoAI by macrophages increases cholesterol efflux and to compare the effects of endogenously synthesized apoAI with exogenously applied apoAI.

Methods and Results. Cholesterol efflux was measured from peritoneal macrophages that express transgenic (tng) human-apoAI (h-apoAI). *H-apoAI(tng)apoE(-/-)* macrophages showed about a 40% increase in efflux compared to *apoE(-/-)* macrophages. Efflux was further increased when macrophages were treated with a low concentration (<1 mM) of cyclodextrin in order to facilitate the exchange of cholesterol between cells and extracellular acceptors. Furthermore, endogenous synthesis of apoAI stimulated the efflux of cholesterol to exogenously applied apoAI by increasing the expression of ABCA1.

Conclusions. These results suggest that increased cholesterol efflux and increased ABCA1 expression are the mechanisms by which h-apoAI is anti-atherogenic. The ability of transgenic h-apoAI to substitute for the efflux functions of apoE emphasizes the importance of endogenous cholesterol acceptors.

Introduction

Apolipoprotein (apo) AI is the main protein component of HDL (Atmeh et al., 1983). ApoAI and HDL plasma levels are inversely correlated with atherosclerosis risk (Gordon and Rifkind, 1989). Like apoE, apoAI from plasma mediates an anti-atherogenic effect by acting as an acceptor of cholesterol and inducing efflux from macrophages (Moore et al., 2003; Zhang et al., 2003c). Unlike apoE, however, apoAI is not naturally expressed by macrophages (Basu et al., 1981). ATP-binding cassette (ABC)-A1 is a mediator of cholesterol and phospholipid efflux. Both apoAI and apoE have been shown to interact with ABCA1 (Krimbou et al., 2004; Remaley et al., 2001). The exogenous application of apoAI has been shown to stabilize and prevent the turnover of ABCA1 (Arakawa and Yokoyama, 2002). It remains unknown if endogenous apoAI has the potential to perform the functions of endogenous apoE. Other studies from our laboratory have shown that expression of h-apoAI by macrophages prevents atherosclerosis in hyperlipidemic mice (Ishiguro et al., 2001; Major et al., 2001; Su et al., 2003). The mechanism of this protection has not been determined. Many studies suggest that the expression of apoE by macrophages prevents atherosclerosis by increasing cholesterol efflux from macrophages in the vessel wall (Bellosta et al., 1995; Kinoshita et al., 2000; Laffitte et al., 2001; Lin et al., 1999; Linton et al., 1995). The objective of the current study was to determine whether apoAI production by macrophages can mediate cholesterol efflux from macrophages. *In vitro* studies with peritoneal macrophages from transgenic h-apoAI mice were performed. The expression of apoAI by *h-apoAI(tng)apoE(-/-)* macrophages increased cholesterol efflux and ABCA1 expression compared to *apoE(-/-)* macrophages. The substitution of the efflux functions of apoE by transgenic h-apoAI emphasizes the importance of the endogenous synthesis of cholesterol acceptors in order to maintain macrophage cholesterol homeostasis and prevent atherosclerosis.

Methods

Human-ApoAI Transgenic Mice. As described by Major et al., a transgenic mouse model was generated from a construct with human apoAI driven by the scavenger receptor A enhancer/promoter (Major et al., 2001). This promoter is macrophage-specific

(Horvai et al., 1995). Briefly, a construct with the macrophage-specific scavenger receptor A enhancer/promoter region 5` to the human apoAI cDNA and human growth hormone splicing and polyadenylation sites on the 3` end were microinjected into fertilized oocytes. A founder line was confirmed by southern blot. Expression and secretion of h-apoAI by peritoneal macrophages was confirmed by immunocytochemistry with an anti-h-apoAI monoclonal antibody.

Cholesterol Efflux to Endogenously Synthesized Acceptors. Cholesterol efflux in peritoneal macrophages was determined by a modified procedure from Lin et al. (Lin et al., 1999). Peritoneal macrophages were elicited with thioglycollate (3%) as described in Chapter IV and plated in 24-well plates. After 24 hours, media were removed and replaced with loading media containing 2.0 µCi/ml of ^{3}H-cholesterol (Perkin Elmer Life Sciences) and 70 µg/ml acLDL. Acetyl-LDL (acLDL) was prepared as described in Chapter IV. Cells were loaded for 48 hours, after which the monolayers were washed and equilibrated for 2 hours in DMEM/0.5% BSA at 37°C and 5% CO_2. Cells were incubated in efflux media with DMEM/0.1% BSA at 37°C, 5% CO_2, and 100 µL samples were removed from each well at 0, 2, and 6 hours. Methyl-ß-cyclodextrin (MBCD) treatment groups were incubated in efflux media with 0.2 mM MBCD (Sigma). Low concentrations (<1 mM) of cyclodextrins like MBCD facilitates the exchange of cholesterol between cells and extracellular acceptors (Atger et al., 1997). Cholesterol efflux was calculated from the total counts in supernatant and is expressed as a percentage of the total counts (media and lysate).

Effects on Endogenous and Exogenous ApoAI on ABCA1-Mediated Cholesterol Efflux. Elicited peritoneal macrophages were plated on 24-well plates in DMEM/10% FBS. In order to wean cells from serum, cells were cultured in DMEM/RPMI (1/1) with 1% FBS and 1% Nutridoma™ (Roche) for 24 hours prior to experiments. In a loading period, cells were incubated with 70 µg/ml acLDL and 1.5 µCi/ml ^{3}H-cholesterol (Perkin Elmer Life Sciences) in serum-free DMEM/RPMI (1/1) with 1% Nutridoma™ for 24 hours. During the loading period, macrophages were pre-treated with 0, 0.5, or 2 µg/ml exogenous human apoAI (Calbiochem). After the loading/pre-treatment period, cells

were rinsed and 15 μg/ml human apoAI was added exogenously to measure cholesterol efflux mediated by the ABCA1 transporter. Media was collected after 4.5 h, centrifuged, and counted by scintillation. Cells were lysed with 0.1 N sodium hydroxide and counted by scintillation. Cholesterol efflux was calculated from the counts in media expressed as a percentage of the total counts (medium and lysate).

Real-time quantitative RT-PCR for ABCA1. Relative quantification of ABCA1 mRNA was performed as described in Chapter IV. ABCA1 mRNA expression levels in different macrophage types were expressed relative to the expression in *apoE(-/-)* macrophages.

Results

Endogenous h-ApoAI Increases Cholesterol Efflux

To determine if the expression of h-apoAI by macrophages affects cholesterol efflux, peritoneal macrophages were labeled with ^{3}H-cholesterol and then incubated in serum-free efflux media containing only BSA. In time course experiments, cholesterol efflux from *h-apoAI(tng)apoE(-/-)* macrophages was increased compared to *apoE(-/-)* controls (Figure 26). At 6 hours, there was a 40% increase in cholesterol efflux from *h-apoAI(tng)apoE(-/-)* macrophages compared to *apoE(-/-)* controls (Figure 27).

To facilitate cholesterol efflux to unsaturated acceptors in growth media, macrophages were treated with MBCD. At low concentrations (<1 mM), MBCD acts as a cholesterol shuttle between cells and high-capacity cholesterol acceptors such as apoAI (Atger et al., 1997). In the presence of MBCD, we observed increased cholesterol efflux from all groups of macrophages. Interestingly, cholesterol efflux in *h-apoAI(tng)apoE(-/-)* macrophages increased to levels equal to that of *apoE(+/+)* macrophages (Figure 27). The MBCD-facilitated increase in efflux (represented as Δ Efflux, Figure 27 inset) was highest for the *h-apoAI(tng)apoE(-/-)* macrophages.

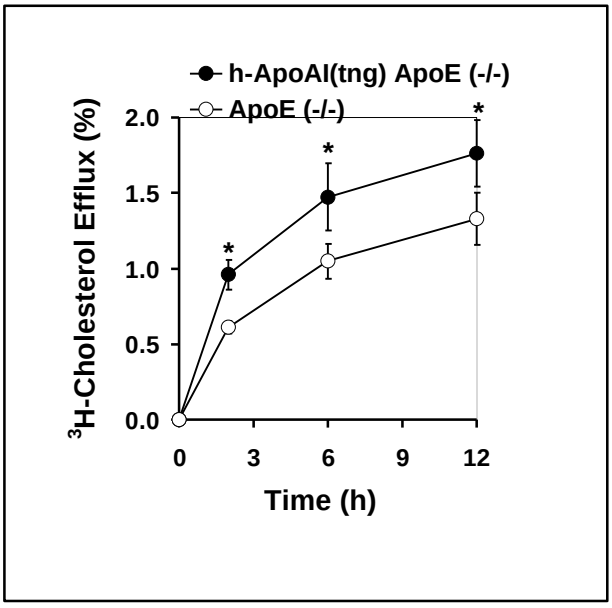


Figure 26. Endogenous Transgenic h-ApoAI Synthesis Increases Cholesterol Efflux from Macrophages.
Macrophages were treated for 36 hours with 2.0 μCi/ml of ^{3}H-FC and 70 μg/ml acLDL in DMEM/2% FBS to label cellular cholesterol. Cells were then cultured in serum-free efflux media with bovine serum albumin (0.1%). Bars and error bars represent the mean (n=6) and standard deviation of samples. Asterisks (*) denote a statistically significant difference ($p<0.05$) compared to the *apoE(-/-)* group as determined by Student's *t*-test.

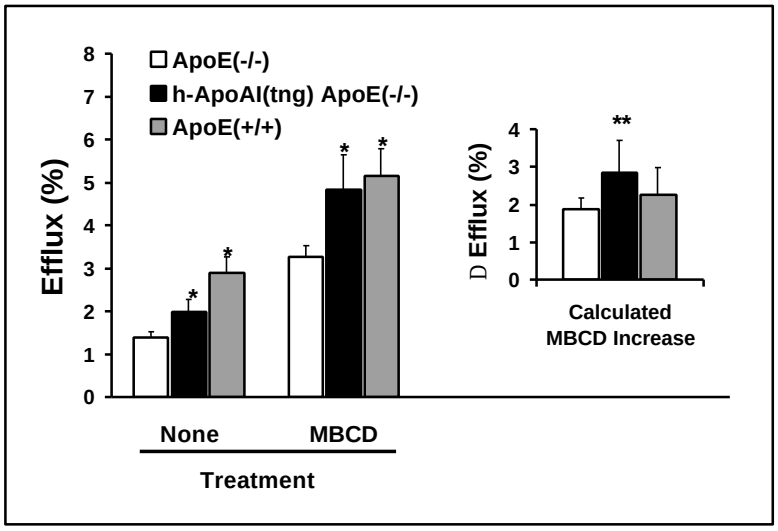


Figure 27. Cholesterol Shuttles Facilitate Cholesterol Efflux to Transgenic h-ApoAI.
Macrophages were treated for 36 hours with 2.0 μCi/ml of ^{3}H-FC and 70 μg/ml acLDL in DMEM/2% FBS to label cellular cholesterol. Macrophages were then cultured in serum-free efflux media with bovine serum albumin (0.1%). During the efflux period, macrophages were treated with 0 or 0.2 mM of the cholesterol shuttle, MBCD. Bars and error bars represent the mean (n=6) and standard deviation of samples from *apoE(-/-)* macrophages (open bars), *h-apoAI(tng)apoE(-/-)* macrophages (solid bars), and *apoE(+/+)* macrophages (hatched bars). The inset illustrates the change in cholesterol efflux that was facilitated by MBCD (MBCD-treated minus control). Asterisks (*p<0.01 or **p<0.05) denote a statistically significant difference compared to the *apoE(-/-)* group as determined by Student's *t*-test.

Endogenous h-ApoAI Stimulates ABCA1-Mediated Cholesterol Efflux and Up-regulates ABCA1

To determine whether endogenous transgenic h-apoAI can stimulate cholesterol transport to exogenous apoAI, we measured efflux to culture media with an excess of exogenously applied apoAI. Efflux to exogenous apoAI was higher for *h-apoAI(tng)apoE(-/-)* macrophages compared to *apoE(-/-)* macrophages (Figure 28A). For comparison, we also pre-treated *apoE(-/-)* macrophages with low concentrations of exogenous apoAI (0.5 to 2.0 μg/ml) which are comparable to the apoAI produced by the transgenic h-apoAI-expressing macrophages. This pre-treatment with exogenous apoAI resulted in a dose-dependent and significant increase in ABCA1-mediated cholesterol efflux from *apoE(-/-)* cells.

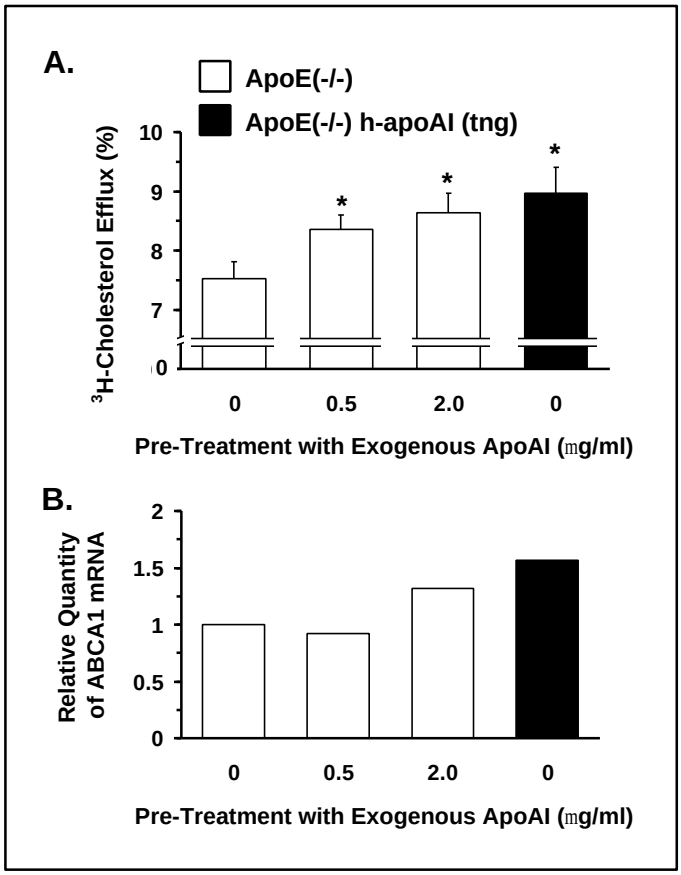


Figure 28. Endogenous h-ApoAI Stimulates ABCA1-Mediated Cholesterol Efflux to Exogenous ApoAI.
ApoE(-/-) (empty bars) and *h-apoAI(tng)apoE(-/-)* (solid bars) macrophages were treated for 24 hours with 70 μg/ml acLDL and 1.5 μCi/ml ^{3}H-cholesterol in serum-free DMEM/RPMI (1:1) with 1% Nutridoma™ media supplement. During the loading period, cells were pre-treated with small amounts of exogenous apoAI to compare the effects of endogenous and exogenous apoAI on ABCA1. **(A)** ABCA1-mediated cholesterol efflux was measured from macrophages cultured in serum-free efflux media with excess apoAI (15 μg/ml) for 4.5 hours. Bars and error bars represent the mean (n=8) and standard deviation of samples. Asterisks (*) denote a statistically significant difference ($p<0.05$) compared to the *apoE(-/-)* group as determined by Student's *t*-test. **(B)** The relative quantity of ABCA1 mRNA was measured by real time RT-PCR. Each bar represents the measurement of a pooled sample from two mice.

Expression of ABCA1 mRNA was increased by the transgenic expression of human apoAI in *apoE(-/-)* macrophages. The pre-treatment of *apoE(-/-)* macrophages with low concentrations of exogenous apoAI also increased ABCA1 expression (Figure 28B). In similar experiments with *apoE(-/-)* macrophages, Su et al. found by Western analysis that ABCA1 protein was increased by endogenous expression or exogenous application of human apoAI (Su et al., 2003).

Discussion

To determine if endogenously synthesized human apoAI can act as a cholesterol acceptor, we studied cholesterol efflux from *h-apoAI(tng)apoE(-/-)* and *apoE(-/-)* macrophages. We found that the endogenous expression of h-apoAI increased cholesterol efflux from cultured macrophages. Furthermore, endogenous h-apoAI stimulated ABCA1-mediated cholesterol efflux and increased the expression of the ABCA1 transporter. The increase in efflux and increase in ABCA1 expression may be the mechanism by which endogenous synthesis of apoAI protects against foam cell formation and atherosclerosis.

Transgenic Macrophage ApoAI and Cholesterol Efflux. The present studies determine the functional equivalence of endogenous acceptors by genetically substituting endogenous apoE with transgenic h-apoAI. Because lipid-free apoAI can enhance cellular cholesterol efflux (Hara and Yokoyama, 1992), the possibility exists that the beneficial effect of an activated reverse cholesterol transport system may be evident even through more subtle modifications that do not affect plasma or serum HDL cholesterol levels. When analyzed for the ability to efflux cholesterol, macrophages from *h-apoAI(tng)apoE(-/-)* mice showed significant increases compared to *apoE(-/-)* macrophages. In the absence of exogenously added extracellular acceptors (e.g. HDL, apoAI, or apoE), the efflux of cholesterol was higher from macrophages expressing h-apoAI than from controls (Figure 26).

Treatment with low concentrations of cyclodextrin facilitated a significantly greater increase in efflux from *h-apoAI(tng)apoE(-/-)* macrophages than from *apoE(-/-)* or wildtype *apoE(+/+)* macrophages (Figure 27). Low concentrations of cyclodextrins

have previously been shown to be catalysts of cholesterol efflux by acting as cholesterol shuttles and allowing for a more rapid equilibration of cholesterol between cells and extracellular acceptors (Atger et al., 1997). The greater cyclodextrin-facilitated increase suggests that media conditioned by *h-apoAI(tng)apoE(-/-)* macrophages may have a greater capacity for accepting cholesterol than unconditioned media. This suggests that upon secretion from macrophages, apoAI is not saturated with cholesterol. These data support a model in which the production of apoAI may confer an autocrine or paracrine property to the macrophage, probably mediated by the accumulation of secreted apoAI outside the plasma membrane, where it activates cholesterol efflux. Such an anti-atherogenic property related to increased cellular cholesterol efflux has been described for macrophage-derived apoE (Lin et al., 1999; Mazzone and Reardon, 1994). ApoE and apoAI are thought to be anti-atherogenic by several mechanisms. Because enhancement of cholesterol efflux is a shared function of apoAI and apoE, these findings emphasize the protective effect of reverse cholesterol transport in the developing atherosclerotic plaque.

Transgenic Macrophage ApoAI and ABCA1 Stimulation. Synergy between endogenous and exogenous acceptors was observed for ABCA1-mediated efflux from *h-apoAI(tng)apoE(-/-)* macrophages (Figure 28A). The ability of apolipoproteins to stabilize ABCA1 and stimulate ABCA1 expression are well characterized (Arakawa et al., 2004; Natarajan et al., 2004). These data suggest that functionally, endogenous apoAI is more effective than pre-treatment with exogenous apoAI (2 μg/ml). Based on the secretion rate determined by Major et al., we estimate that the cells in the current study conditioned the media with h-apoAI to a concentration of approximately 0.2 μg/ml. This suggests that despite very low concentrations, endogenous apoAI was able to be more effective than exogenous apoAI at stimulating ABCA1-mediated cholesterol efflux.

One potential mechanism for increased ABCA1 expression in cells treated with apoAI is the ability of apoAI to induce cAMP signaling. The current study showed increased ABCA1 expression upon treatment with endogenous or exogenous apoAI (Figure 28B). ApoAI has been shown to trigger phosphorylation of ABCA1 and subsequent rises in cellular cAMP (Haidar et al., 2004). Because cAMP is a known

stimulator of ABCA1 expression (Oram et al., 2000), this potential signaling mechanism warrants further investigation.

Macrophage ApoAI and Atherosclerosis. These *in vitro* results in the current study correlate with decreases in atherosclerosis shown by other studies from our laboratory. We have previously shown that the lack of apoE expression by macrophages results in a 10-fold increase in atherosclerosis without affecting serum lipids (Fazio et al., 1997). Since macrophages do not naturally express apoAI, we hypothesized that apoAI production by macrophages could substitute for the anti-atherogenic effects of apoE. Macrophage expression of human apoAI reduces aortic lesions in mice susceptible to spontaneous atherogenesis with no differences in serum lipid levels or lipoprotein distribution (Major et al., 2001). In the bone marrow transplantation study by Major et al., apoE deficient mice were the recipients of bone marrow. The donors were *apoE(-/-)*, *h-apoAI(tng)apoE(-/-)*, and wildtype *apoE(+/+)* mice. The lesion area in the proximal aorta of the *h-apoAI(tng)apoE(-/-)*, bone marrow recipients was about 60% less than the *apoE(-/-)* bone marrow recipients.

Experiments with *h-apoAI(tng)apoE(-/-)* macrophages showed that endogenous human apoAI has an effect on cholesterol efflux that is similar to that of exogenous apoAI on *apoE(-/-)* macrophages. These results suggest that the local effects of human apoAI produced by macrophages can promote removal of excess cholesterol in the plaque. In normolipidemic human plasma, lipid-free apoAI levels are around 5–10% of the total plasma apoAI (Neary and Gowland, 1987). The lipid-free apoAI particles are able to enter the subendothelial space and promote the removal of excess cholesterol from peripheral cells. If the lipid-free human apoAI produced by macrophages in the subendothelial space can effectively promote cholesterol efflux, high levels of apoAI in plasma may not represent a necessary condition to obtain beneficial effects on the vascular wall. Modulating atherogenesis by increasing the apoAI concentration locally in the vascular wall may represent a viable therapeutic approach.

Summary. In summary, macrophage-specific expression of human apoAI increased cholesterol efflux from loaded apoE deficient macrophages and increased the

expression of ABCA1. Similar effects were seen with endogenous apoE synthesis (Chapter VI), suggesting that the endogenously synthesized cholesterol acceptors like apoE or apoAI have overlapping functions in macrophages and may be interchangeable. These results also suggest that cholesterol efflux is a mechanism by which the apoAI produced by macrophages protects against atherosclerosis.

CHAPTER IX

CONCLUSION: CHOLESTEROL EFFLUX AFFECTS CHOLESTEROL HOMEOSTASIS AND THE ATHEROGENIC POTENTIAL OF MACROPHAGES

Overview

Atherosclerosis and foam cell formation are the consequence of altered cholesterol homeostasis in macrophages. Cholesterol efflux, the first step of the reverse cholesterol transport system, is associated with protection from atherosclerosis. To determine the role of cholesterol efflux in macrophage cholesterol homeostasis, we developed protocols to measure cholesterol efflux, and performed studies to measure cholesterol efflux from macrophages with cholesterol storage deficits and from macrophages that secrete cholesterol acceptors. In previous *in vivo* studies with mice, our laboratory has shown that cholesterol storage deficits in macrophages and cholesterol acceptor secretion from macrophages affect foam cell transformation and atherosclerosis (Fazio et al., 2001; Ishiguro et al., 2001; Linton et al., 1995; Major et al., 2001; Su et al., 2003). The current studies examine cholesterol efflux from these macrophages, *in vitro*. We show that *ACAT1(-/-)* macrophages, which lack the enzyme that stores cholesterol, are characterized by disruptions in cholesterol efflux, altered cellular morphology, increased lipoprotein uptake, and increased cholesterol synthesis. We show that the secretion of cholesterol acceptors, like apolipoprotein (apo) E or transgenic human apoAI increased cholesterol efflux by multiple mechanistic pathways. These studies show that cholesterol efflux is an integral part of cholesterol balance in macrophages. In combination with previous *in vivo* studies, these *in vitro* studies suggest that cholesterol efflux is a mechanism that affects the atherogenic potential of macrophages.

General Discussion

Macrophage cholesterol homeostasis is a central focus in understanding the biology of foam cell formation and atherosclerosis. Atherosclerosis is a complex disease process that leads to complications such as coronary heart disease and stroke. Macrophages are a critical part of the progression of this disease because macrophages accumulate cholesterol and transform into foam cells. The uptake, storage, synthesis, and

efflux of cholesterol are processes that maintain cholesterol homeostasis in macrophages. Because cholesterol efflux is the initial step of the reverse cholesterol transport system, it has a critical role in protecting macrophages from foam cell transformation. The aims of our studies were to develop protocols to measure cholesterol efflux, to study the effects of cholesterol storage deficits on cholesterol efflux, and to study the effects of cholesterol acceptors on cholesterol efflux. These aims were addressed by experiments with peritoneal mouse macrophages that were elicited from genetically engineered mice. We show that cholesterol storage deficits disrupted cholesterol efflux while endogenous synthesis of cholesterol acceptors stimulated cholesterol efflux. These findings will be discussed by their relevance to cholesterol efflux, cholesterol homeostasis, technical developments, potential therapeutics, and atherosclerosis.

Cholesterol Efflux

Cholesterol efflux from macrophages is associated with the reverse cholesterol transport system and with protection from foam cell formation. In the current studies we studied cholesterol efflux in the contexts of macrophages with a cholesterol storage deficit and macrophages that secrete cholesterol acceptors. The findings from both of these systems have helped to elucidate the role of cholesterol efflux in maintaining cholesterol homeostasis in macrophages.

ACAT1 Deficiency and ABCA1-Mediated Cholesterol Efflux. Acyl-coenzyme A: cholesterol acyltransferase (ACAT) is the enzyme that esterifies cholesterol with fatty acids. Esterified cholesterol is the storage form of cholesterol that is abundant in foam cells. In Chapter IV, we showed that *ACAT1(-/-)* macrophages, which lack this enzyme, had major alterations in cholesterol efflux mediated by the ATP-Binding Cassette (ABC)-A1 transporter. *ACAT1(-/-)* macrophages have decreased efflux of cellular cholesterol and increased efflux of lipoprotein-derived cholesterol. The net result, under relatively mild cholesterol loading conditions, was the accumulation of lipoprotein-derived free cholesterol and the accumulation of intracellular vesicles. These changes in cholesterol homeostasis and cellular morphology were not the result of cholesterol-induced toxicity. These changes show that cholesterol esterification is a crucial part of the cellular

cholesterol cycle and suggest that the absence of cholesterol esterification makes *ACAT1(-/-)* macrophages pro-atherogenic.

Cholesterol Acceptors and ACAT1 Deficient Macrophages. The disruptions in cholesterol efflux from *ACAT1(-/-)* macrophages were not limited to the ABCA1/apoAI efflux pathway. In additional experiments, cholesterol efflux from *ACAT1(-/-)* macrophages to other cholesterol acceptors was measured. ApoE-mediated cholesterol efflux was decreased from *ACAT1(-/-)* macrophages compared to wildtype macrophages (Appendix, Figure 31A). Cholesterol efflux mediated by HDL was also decreased from *ACAT1(-/-)* macrophages (Appendix, Figure 31B). Together with the finding of decreased ABCA1-mediated efflux, these data suggest that the disruptions in *ACAT1(-/-)* macrophages may be due to a global change in cellular cholesterol homeostasis, like cholesterol trafficking or vesicular trafficking.

General defects in protein secretory pathways could affect apoE secretion and apoE-mediated cholesterol efflux from macrophages. The endogenous synthesis of apoE by macrophages stimulated cholesterol efflux, as discussed in Chapter VI. For *ACAT1(-/-)* macrophages, it is unclear if the morphological changes and the accumulation of intracellular vesicles (Chapter IV) affect apoE secretion. In additional experiments, the secretion of apoE from *ACAT1(-/-)* macrophages was measured by Western blot analysis. Endogenous apoE secretion was not changed by ACAT1 deficiency (Appendix, Figure 32A). Despite normal apoE secretion, apoE-mediated cholesterol efflux was decreased in *ACAT1(-/-)* macrophages (Figure 31A). To determine if the effects of ACAT1 deficiency on ABCA1-mediated cholesterol efflux are affected by endogenous synthesis of apoE, we measured cholesterol efflux from *ACAT1(-/-)apoE(-/-)* macrophages. Cholesterol efflux to apoAI from *ACAT1(-/-)apoE(-/-)* macrophages was not different from *ACAT1(-/-)* macrophages but was significantly reduced compared to either wildtype or *apoE(-/-)* (Appendix, Figure 32B). These additional data suggest that the endogenous apoE secretion from *ACAT1(-/-)* is not responsible for the decreases in apoE-mediated or ABCA1-mediated efflux of cellular cholesterol.

Interactions Between Cholesterol Efflux Pathways. The presence of multiple efflux pathways suggests that cholesterol efflux has a high physiological importance. Cholesterol-loaded macrophages in the vascular wall benefit from the multiple effects that apoAI, apoE, and HDL have on cholesterol homeostasis. In addition to their direct effects as cholesterol acceptors, apoAI, apoE, and HDL affect cholesterol homeostasis by other mechanisms. ApoAI is the major cholesterol acceptor for the ABCA1 efflux pathway, a pathway that is disrupted in Tangier disease. ApoAI also has a stabilizing effect on ABCA1 proteins, which further stimulates cholesterol efflux (Feng and Tabas, 2002). In Chapter VIII and in other studies from our laboratory, we showed that transgenic expression of apoAI in macrophages stimulated cholesterol efflux by stimulating the expression of ABCA1 (Su et al., 2003). Other laboratories have shown that both apoAI and ABCA1 have a positive effect on the secretion of apoE, the acceptor for a separate cholesterol efflux pathway (Kockx et al., 2004; Von Eckardstein et al., 2001). As apoAI accepts cellular lipids it matures into HDL. ApoE can also be constituents of HDL particles (Weisgraber et al., 1977). HDL accepts cholesterol by interacting with the SR-BI receptor and by acting as an extracellular sink for cholesterol. Studies have shown that cholesterol efflux to HDL is increased by endogenous synthesis of apoE in macrophages (Lin et al., 1998). Together, apoAI, HDL, and apoE found locally in the vascular wall can protect macrophages from foam cell transformation through multiple mechanisms that increase cholesterol efflux and maintain cholesterol homeostasis.

In Chapter VI, we showed that apoE increased cholesterol efflux by acting as a cholesterol acceptor in the apoE efflux pathway and as a mild stimulator of the ABCA1 efflux pathway. In the apoE-mediated efflux pathway, ABCA1 had a modest stimulatory effect on efflux to apoE. These data suggest that although there is interaction and mutual stimulation of apoE-mediated and ABCA1-mediated cholesterol efflux, these two efflux pathways are largely independent.

The ABCA1 transporter is a mediator of cholesterol efflux that can tie together efflux pathways involving apoAI and apoE. One way that ABCA1 ties these pathways together is its specificity for the class of apolipoproteins known as exchangeable apolipoproteins (Bortnick et al., 2000; Krimbou et al., 2004; Remaley et al., 2001).

Krimbou et al. found that apoE can bind ABCA1. However, when compared to apoAI, the affinity of apoE for ABCA1 was not as specific and cholesterol efflux was not as much. In these studies, Krimbou et al. applied relatively large amounts of exogenous apoE to fibroblasts. In contrast, we were able to detect this interaction between apoE and ABCA1 with the endogenously synthesized apoE that was secreted from macrophages (Chapter VI). Our study used macrophages that were *ABCA1(+/-)* and *ABCA1(+/+)* to explore interactions of ABCA1 and apoE at the functional level of cholesterol efflux. As discussed in Chapter VIII, endogenous transgenic h-apoAI also had a functional interaction with ABCA1. These studies highlight the ability for two different cholesterol acceptors to interact with ABCA1 when synthesized endogenously by macrophages.

Transgenic Macrophage ApoAI and Cholesterol Efflux. In Chapter VIII, we showed that macrophage-specific expression of transgenic human apoAI increased cholesterol efflux from loaded apoE deficient macrophages and increased the expression of ABCA1. Similar effects were present with endogenous apoE synthesis (Chapter VI), suggesting that the endogenously synthesized cholesterol acceptors like apoE or apoAI have overlapping functions in macrophages and may be interchangeable. These results also suggest that cholesterol efflux is a mechanism by which the transgenic apoAI produced by macrophages could protect against atherosclerosis.

The ability of apolipoproteins to stabilize ABCA1 and stimulate ABCA1 expression are well characterized (Arakawa et al., 2004; Natarajan et al., 2004). In Chapter VIII, we observed synergy between endogenous and exogenous acceptors for ABCA1-mediated efflux. For stimulation the ABCA1 efflux pathway, endogenous apoAI was more effective than treatment with ten times more exogenous apoAI. This suggests that despite very low concentrations, endogenous apoAI has an advantage. Differences between endogenous and exogenous cholesterol acceptors were reported in previous studies. Lin et al. showed that endogenously synthesized apoE was able to increase cholesterol efflux more than exogenously applied apoE that was ten times the concentration (Lin et al., 1999). The advantages of endogenous cholesterol acceptors, even at relatively low levels compared to exogenous cholesterol acceptors, may be related to intracellular events or to the proximity of the acceptors to point of action (i.e.

cellular membranes). As discussed in Chapter VII, the effects of endogenous apoE on cholesterol efflux were separated into autocrine effects and paracrine effects.

Autocrine and Paracrine Effects of Cholesterol Acceptors. In Chapter VII, we separated and quantified the autocrine and paracrine effects of apolipoprotein E on cholesterol efflux from macrophages. These data show that the relative importance of autocrine and paracrine apoE depends on the size of the local distribution volume. The autocrine effects of apoE remain constant even when the paracrine effects are diminished by the dilution of apoE. This suggests that paracrine apoE may not be able to fulfill the critical actions of a small amount of autocrine apoE in the juxtacellular space. Together, these findings suggest that the apoE that is endogenously synthesized by macrophages uses autocrine and paracrine mechanisms to protect against atherosclerosis.

The effect of apoE secretion rate on apoE-mediated cholesterol efflux is not known. In additional experiments, apoE-mediated cholesterol efflux was measured using heterozygous *apoE(+/-)* macrophages as a "low-secretor" cell model. Even though *apoE(+/-)* macrophages were expected to have apoE expression levels of about 50% of the apoE that is secreted by wildtype *apoE(+/+)* macrophages, they only secreted approximately 10% of the apoE (Appendix, Figure 33). Although apoE was available in the extracellular space, the heterozygous *apoE(+/-)* macrophages did not efficiently engage it to mediate cholesterol efflux. This data suggests that a certain threshold level of apoE secretion may be necessary for the autocrine action of apoE. "High-secretor" cell models, with apoE over-expression and secretion that is higher than physiologic levels, have not been examined. Future experiments will characterize the effects of different levels of apoE over-expression on the autocrine and paracrine mechanisms of apoE-mediated cholesterol efflux.

The autocrine and paracrine actions of endogenous acceptors were further characterized in additional experiments with macrophage that secrete transgenic human apoAI instead of apoE. As discussed in Chapter VIII, endogenous apoAI stimulated cholesterol efflux by stimulating ABCA1-mediated cholesterol efflux. These studies showed that endogenous apoAI, like endogenous apoE, increased cholesterol efflux compared to macrophages that do not synthesize cholesterol acceptors (Appendix, Figure

34). The positive effect on cholesterol efflux was diminished by increasing the distribution volumes in which the cells were cultured. These results suggest that this methodology may be applicable to other cholesterol acceptors.

Cholesterol Homeostasis

Cholesterol efflux works in concert with many other cellular processes to maintain cholesterol homeostasis. The uptake, storage, synthesis, or trafficking of cholesterol has the potential to affect cholesterol efflux. In the current studies, we explored the association between cholesterol efflux and other aspects of cholesterol homeostasis in the context of macrophages with a cholesterol storage deficit. The findings from this system suggest that a specific disruption in a single process of cholesterol homeostasis has global cellular consequences.

ACAT1 Deficiency and Cholesterol Synthesis. In Chapter V, we showed that the absence of ACAT1 in macrophages had effects on lipid homeostasis that go beyond decreased cholesteryl esters. ACAT1 deficiency in macrophages increases cholesterol synthesis without affecting phospholipid synthesis. This increase may represent dysregulation of the cholesterol sensing mechanisms in macrophages and suggests that *de novo* synthesis of cholesterol may contribute to the atherogenic potential of *ACAT1(-/-)* macrophages.

Cholesterol Trafficking in ACAT1 Deficient Macrophages. As discussed in Chapter IV and Chapter V, the similarities between ACAT1 deficiency, Niemann-Pick type C1 (NPC1) deficiency, U18666A treatment, and progesterone treatment suggests that *ACAT1(-/-)* macrophages may suffer from aberrant cholesterol trafficking. Although these treatments and genotypes have phenotypic similarities, they represent an array of mechanisms. ACAT1 esterifies cholesterol. NPC1 traffics cholesterol from lysosomes to the plasma membrane and endoplasmic reticulum. U18666A is an amphiphile that inhibits intracellular cholesterol trafficking by sequestering cholesterol in vesicles (Kellner-Weibel et al., 1998; Sparrow et al., 1999). Progesterone and imipramine, which are also amphiphiles, have similar effects. It is interesting that NPC1 is necessary for the

actions of amphiphile compounds on cholesterol trafficking and that the resulting phenotype resembles NPC1 deficiency (Lange et al., 2000). In a study of these amphiphiles, Lange et al. suggest that disruption of cholesterol trafficking results in the accumulation of vesicles that are "modified for lipid storage" (Lange et al., 1998). Vesicular accumulation in *ACAT1(-/-)* macrophages was described in Chapter IV. The association of vesicular accumulation, disrupted cholesterol trafficking, and altered cholesterol homeostasis supports the idea that ACAT1 deficiency disrupts normal cholesterol trafficking. The cycle of cholesterol esterification and de-esterification may be a critical part of cholesterol trafficking and cholesterol homeostasis.

Characterization of Vesicles in *ACAT1(-/-)* Macrophages. *ACAT1(-/-)* macrophages have increased surface activity, more cytoplasmic extensions, and increased intracellular vesicles (Chapter IV). These vesicles were identified as endosomes and lysosomes by the presence of specific protein markers. In future experiments, time-lapse microscopy of these macrophages *in vitro* could identify differences in vesicle formation and movement. This would provide important clues about potential changes in cholesterol trafficking that result from ACAT1 deficiency.

Studies with ACAT inhibitors have described accumulations of intracellular vesicles and extrusion of the vesicles into the extracellular space (Robenek and Schmitz, 1988). In future studies, we will explore the possibility that *ACAT1(-/-)* macrophages extrude vesicles into the interstitial space of atherosclerotic lesions *in vivo* or into culture media *in vitro*. Extrusion of vesicles into the extracellular space could be a pro-atherogenic event, especially if they are enriched in cholesterol. Accumulation of these types of vesicles, in both intracellular and extracellular spaces, is associated with the progression of fatty streaks into more complicated plaques (Guyton and Klemp, 1989; Guyton and Klemp, 1994).

Vesicles in *ACAT1(-/-)* macrophages appear to be heterogeneous. Identification of vesicles as both endosomes and lysosomes explains some of the morphological differences in vesicles. In future experiments, it would be interesting to correlate the functional differences (i.e. endosome, lysosome, secretory vesicle, trafficking vesicles) with structural differences in the vesicles (i.e. size, density, lipid composition). Cellular

vesicles can be isolated by density gradient centrifugation (Galloway et al., 1983). Protein markers and lipid constituents on isolated vesicles could also be characterized is these experiments. Isolated vesicle fractions can be visualized by electron microscopy to characterize size and structure. Density gradient separation would facilitate characterization of the different types of vesicles and may suggest functional differences in cholesterol trafficking between the wildtype and *ACAT1(-/-)* macrophages.

Technical Developments

Protocol Developments. In Chapter III, we described protocols for assaying cholesterol efflux from macrophages. These protocols were further modified to focus on larger experimental capacity and greater experimental efficiency. Other developments include software for data analysis (Appendix, Figure 29) and protocols to study the autocrine and paracrine effects of cholesterol acceptors (Chapter VII).

Development of data analysis software for efflux experiments (Appendix, Figure 29) will continue with a strategy of making the current software user-friendly. The strategy to improve this software will include automating many analysis steps (e.g. data input, analysis decisions, and report output). Improvements will be achieved by constructing user input forms, which will guide the user through data entry and analysis decisions while protecting the user from interfacing the data at the level of the Microsoft Excel™ spreadsheet. Input forms will be very simple interfaces with yes/no answer boxes, brief explanations of available choices, and entry boxes for data input. Visual Basic™ for Applications, which is a Microsoft Windows™-based programming language, will be used along with the built-in capabilities of Microsoft Excel™ to achieve a user-friendly analysis tool for cholesterol efflux data (Tack et al., 1995).

Targeted Cholesterol Labeling and ACAT1 Deficient Macrophages. In Chapter III, we described protocols for targeted labeling of cholesterol pools that were used to study the effects of ACAT1 deficiency on cholesterol efflux. In Chapter IV, *ACAT1(-/-)* macrophages treated with the “lipoprotein-derived” labeling methodology had greater efflux of the labeled lipoprotein-derived cholesterol but less efflux of cholesterol mass (Figure 1B). This does not represent a discrepancy, but rather demonstrates that the

cholesterol pool targeted with ^{3}H-cholesterol in this experiment is distinct from the bulk of the cellular cholesterol. The ^{3}H-cholesterol in the lipoprotein-derived pool does not reach equilibrium with the rest of the cholesterol mass, and does not behave like the bulk of cellular cholesterol. The efflux measurements from the labeled pool and the mass measurements from the bulk cholesterol were performed on same set of cells in this study. These data suggest that even though the *ACAT1(-/-)* macrophages efflux less of their total cholesterol mass, they are selectively increasing the efflux of the labeled cholesterol that comes from lipoproteins, possibly due to changes in trafficking.

96-Well Efflux System and ApoE/ABCA1 Interaction Studies. In Chapter III, we summarized the protocols for cholesterol efflux in 96-well cell culture plates. These protocols were used in Chapter VI to study the effects of ABCA1 heterozygosity. The 96-well protocol was useful in this set of experiments because of the large number of experimental groups and the scarcity of experimental animals. In these experiments, individual experimental groups included combinations of four different genotypes, two agonist treatment conditions, and three cholesterol acceptors. This generates a total of 24 experimental groups. Experiments of this magnitude would be cumbersome in 24-well or 6-well culture systems because of the large number of mice needed to produce enough peritoneal macrophages. The cost and availability of reagents such as radiolabeled cholesterol and modified lipoproteins are another issue.

Future developments in this protocol will partially automate the washes by using a Tecan™ Microplate Washer. This plate washer is equipped with an 8-channel manifold that allows the simultaneous wash of an entire column of wells in a standard 96-well plate. The plate washer has dispense, aspirate, and continuous wash functions. Sequences of these functions can be stored as programmed protocols. This cell culture system has great potential for more efficient studies of cholesterol homeostasis.

Modeling the Effects of Autocrine/Paracrine ApoE. As discussed in Chapter VII, by using a basic mathematical model, the effects of endogenous apoE on cholesterol efflux were separated into autocrine effects and paracrine effects. Modeling of this type has been used in previous studies of cholesterol efflux. Rothblat et al. found that

cholesterol efflux increased linearly with cholesterol acceptor concentration in a saturable manner (Rothblat and Phillips, 1986; Yancey et al., 1996). Yancey et al. found that efflux to cyclodextrins, very strong acceptors of cholesterol, occurs from two kinetic pools of cholesterol (Yancey et al., 1996). Yancey et al. describe these two pools with a bi-exponential model of cholesterol efflux. More complex mathematical computer models have been used to study autocrine and paracrine actions of endogenously synthesized ligands (Oehrtman et al., 1998; Shvartsman et al., 2001). Together, these studies demonstrate that mathematical modeling is a viable method for generating testable hypothesis regarding cholesterol efflux and autocrine/paracrine mechanisms. In Chapter VII, a simple model of the relationship between distribution volume and cholesterol efflux generated data that contained more complex mathematical trends. From these mathematical trends a biological model was proposed. However, it remains to be seen if the proposed model correlates with actual biological mechanisms. Future studies will test the proposed model for autocrine/paracrine apoE.

Effects of Lesion Geometry on Cholesterol Efflux from Macrophages. As discussed in Chapter VI and Chapter VIII, endogenous synthesis of cholesterol acceptors increased cholesterol efflux. Of the two major cholesterol efflux pathway, one depends on an endogenous cholesterol acceptor (i.e. macrophage apoE) and the other depends on an exogenous cholesterol acceptor (i.e. hepatic apoAI). The relative physiological relevance of these two pathways is not known. In the vessel wall, apoE of macrophage origin may be more anti-atherogenic than apoAI of hepatic origin. Studies from our laboratory have shown that macrophages that endogenously synthesize transgenic human apoAI can protect against atherosclerosis, even when ample amounts of hepatic apoAI are present in the plasma compartment (Ishiguro et al., 2001). Two factors that could contribute to a higher efficacy of endogenous acceptors are the geometry of the lesion and the proximity of acceptors to their point of action. While the macrophage monolayer is both the most commonly used and the most simplistic *in vitro* model of macrophage physiology, monolayers are a poor representation of the atherosclerotic lesion. For example, this model cannot account for the effects of lesion geometry on the progression of macrophages into foam cells. For certain cell types, three-dimensional culture

techniques have been successful at revealing cell characteristics that cultured monolayers do not demonstrate (Wang and Tarbell, 2000). Likewise, three-dimensional macrophage aggregates may be a more effective model to study how lesion geometry affects the functions of apoE and apoAI. Future directions in the development of cholesterol efflux protocols will include macrophage aggregates and macrophage culture in three-dimensional extracellular matrices.

Potential Therapeutics

The current studies establish a basis for potential therapeutic interventions that could aid cholesterol efflux, block foam cell formation, and prevent atherosclerosis. These potential therapeutics include bone marrow transplantation to deliver genetically engineered macrophages and pharmacologic manipulation of individual processes of cholesterol homeostasis. In the current studies we studied cholesterol efflux in the contexts of macrophages with a cholesterol storage deficit and macrophages that secrete cholesterol acceptors. Together, these systems suggest that cholesterol acceptors and LXR agonist may have therapeutic value but that abolishing cholesterol esterification may have undo consequences.

ACAT Inhibitors and Cholesterol Efflux. ACAT inhibition has been reported to increase the efflux of cholesterol to various cholesterol acceptors (Kellner-Weibel et al., 1998; Mazzone and Reardon, 1994; Warner et al., 1995; Zhang et al., 1996). There are methodological considerations that are important for the interpretation of previous studies with ACAT inhibitors. A common experimental setup found in many studies is that cells are cholesterol loaded until a large cholesteryl ester pool exists. Immediately following the loading period, cells are treated with ACAT inhibitor. This acute blockage of the esterification cycle in the presence of a large cholesteryl ester pool, increased cholesterol efflux by liberating free cholesterol. A second experimental setup is that cells are loaded with lipoproteins that contain radiolabeled cholesteryl esters. Cells are concurrently treated with ACAT inhibitor. As lipoproteins are degraded and lipids are hydrolyzed in lysosomes, the radiolabeled cholesterol is blocked from entering the esterified cholesterol pool. Thus, turnover of the radiolabeled cholesterol occurs as the cholesterol is

hydrolyzed and effluxed to acceptors in the media. A good example of this setup comes from Rodriguez et al., who reported increased efflux of lipoprotein-derived cholesterol during treatment with the ACAT inhibitor 58-035 in human monocyte-derived macrophages (Rodriguez et al., 1999).

The experimental setup of the current studies differs from those of previous studies in one fundamental way. In our experiments, it was necessary to compare genetic ACAT1 deficiency with treatment with ACAT inhibitor. To mimic the conditions of ACAT1 deficiency, ACAT inhibitors must be continuously applied. Instead of establishing a cholesteryl ester pool before the application of the inhibitor, the cells were continuously inhibited throughout the cholesterol loading and cholesterol efflux periods. Like Rodriguez et al., our experimental setup was able to test the turnover of lipoprotein-derived cholesterol during the loading period. Subsequently, ACAT inhibitor treatment continued as ABCA1-mediated cholesterol efflux was measured. Our findings are similar to previous studies with ACAT inhibitors. Our studies have an extra dimension because we measure the effects of deficiency or inhibition independently of an established cholesteryl ester pool. Chronic therapy with ACAT inhibitors to block the establishment of atherosclerotic lesions is likely to mimic these experimental conditions.

Pharmacologic Inhibition of ACAT versus Genetic ACAT1 Deficiency. In additional experiments, both ACAT1 deficiency and ACAT inhibition increased the turnover of lipoprotein-derived cholesterol and decreased the esterification of labeled cholesterol (Appendix, Figure 30). A notable difference, however, was that ACAT1 deficiency increased FC accumulation while ACAT inhibition did not change intracellular FC when compared to untreated wildtype macrophages. This may be due to residual ACAT activity or to the abrupt onset of pharmacologic inhibition. ACAT1 deficiency may result in structurally and functionally abnormal macrophages due either to chronic compensatory mechanisms or to direct effects of the absence of ACAT1.

ACAT1-Independent Effects of ACAT Inhibitors. ACAT1-independent effects from ACAT inhibitors have not been reported previously. The decrease in the efflux of lipoprotein-derived cholesterol in *ACAT1(-/-)* macrophages with ACAT inhibitor

compared to vehicle would suggest secondary effects of the inhibitor that may affect cholesterol homeostasis (Appendix, Figure 30). The concentration of ACAT inhibitor used did not induce toxicity in our studies or in previous ACAT inhibitor studies (Warner et al., 1995) so it is unlikely that toxicity is responsible for the secondary effects seen in *ACAT1(-/-)* macrophages.

The residual esterification activity in *ACAT1(-/-)* macrophages raises the question of whether ACAT2 can compensate functionally for a loss of ACAT1. Cholesterol esterification experiments showed that *ACAT1(-/-)* macrophages were able to esterify cholesterol even when treated with an inhibitor of both ACAT1 and ACAT2 (Appendix, Figure 30C). Since the cp113-818 ACAT inhibitor is non-selective (Lada et al., 2004), the experiments suggest that ACAT2 activity is not responsible for the residual esterification activity in *ACAT1(-/-)* macrophages.

Because of the apparent effects of a non-selective ACAT inhibitor on *ACAT1(-/-)* macrophages, more detailed studies are warranted. Non-selective inhibitors for ACAT include 58-035 (Sandoz), cp113-818 (Pfizer), PD-138142-15 (Parke-Davis), PD-156759 (Parke-Davis), XD-793-11 (DuPont), and DuP128 (DuPont) (Lada et al., 2004). Lada et al., also found that derivatives of a Werner-Lambert compound are selective for ACAT1. Pyripyropene A is selective for ACAT2. Different non-selective ACAT inhibitors, as well as ACAT1-selective and ACAT2-selective inhibitors should be used in future studies with *ACAT1(-/-)* macrophages. Also, the expression of ACAT2 will be measured in future experiments to further exclude ACAT2 activity in *ACAT1(-/-)* macrophages.

Transgenic ApoAI and ABCA1 Deficiency. In Chapter VI, we showed that endogenous apoE and ABCA1 cooperated to mediate cholesterol efflux. We also showed that endogenous transgenic h-apoAI stimulated cholesterol efflux (Chapter VIII). While stimulation of ABCA1 was a mechanism of the increased cholesterol efflux, there may also be ABCA1-independent mechanisms. In future studies, it will be essential to determine if transgenic h-apoAI can still protect against atherosclerosis and stimulate efflux even when ABCA1 is not expressed by macrophages (i.e. *h-apoAI(tng)ABCA1(-/-)* macrophages).

LXR Agonists and ACAT1 Deficient Macrophages. The phenotype of *ACAT1(-/-)* macrophages resembles the changes that occur in *NPC1(-/-)* cells. The similarities include the accumulation of cholesterol in lysosomes, decreased cholesterol efflux, decreased cholesterol esterification, increased cholesterol synthesis, and decreased lipoprotein uptake (Ory, 2004). In *NPC1(-/-)* cells, this phenotype and cholesterol trafficking problems can be corrected by treating cells with LXR agonists (Frolov et al., 2003). *ACAT1(-/-)* macrophages also respond to LXR agonists by increasing cholesterol efflux (Appendix, Figure 35). oxLDL contains oxysterols that stimulate LXR. *ACAT1(-/-)* macrophages respond to oxLDL with decreased cholesterol synthesis (Figure 13A). However, oxLDL-treated wildtype cells still have higher cholesterol synthesis than oxLDL-treated *ACAT1(-/-)* macrophages. Together, these data suggest that LXR agonism does not correct the underlying defect that causes disruptions in cholesterol efflux from *ACAT1(-/-)* macrophages. Regardless, the positive effects of LXR agonism on cholesterol efflux and cholesterol synthesis still have the potential to protect *ACAT1(-/-)* macrophages from foam cell formation. In future studies, the effects of LXR agonists on cholesterol uptake, cholesterol storage, and on the morphology of *ACAT1(-/-)* macrophages should be determined.

Atherosclerosis and Other Diseases

The many functions of macrophages in the vascular wall can directly or indirectly affect cellular cholesterol balance. The transformation from macrophage into foam cell represents more than a shift in lipid metabolism. Foam cell transformation is associated with changes in the inflammatory functions, immune functions, and scavenger functions of macrophages (Table 1). With such complex pathogenesis, it is likely that lipid burden can either lead to or result from these other changes in macrophage function. Because macrophage cholesterol homeostasis is at the center of this complex disease, cholesterol efflux has the potential to be protective or to promote atherogenesis.

Macrophage ApoE and Atherosclerosis. ApoE is a lipoprotein structural protein, a ligand for lipoprotein receptors, a cholesterol acceptor, has anti-inflammatory and anti-oxidant properties, and can act as a growth factor (Bocksch et al., 2001). Each of these

functions can modify atherogenesis. Atherosclerosis studies with macrophages that secrete different isoforms of apoE allow for a direct comparison of how subtle changes in cholesterol efflux and cholesterol homeostasis affect the development of atherosclerosis (Table 1). Bone marrow replacement studies in mice have shown that mouse apoE from macrophages protects while a lack of macrophage apoE promotes atherosclerosis (Fazio et al., 1997; Linton et al., 1995). Studies with human apoE show that some isoforms are protective and others are atherogenic. ApoE3 is the most common isoform. ApoE2 has reduced LDLR binding compared to apoE3, but apoE4 binds LDLR with an affinity similar to apoE3 (Weisgraber et al., 1982). The apoEcys142 mutant has reduced HSPG binding (Ji et al., 1994). The differences in these isoforms are associated with differences in cholesterol efflux and cholesterol loading. ApoE3 and apoE2 prevent macrophages from becoming loaded with cholesterol by stimulating cholesterol efflux (Cullen et al., 1998). However, apoE4 does not stimulate cholesterol efflux due to enhanced surface binding and re-uptake. In atherosclerosis studies apoE3 significantly decreases lesions size, while apoE2 does not have significant effects. Expression of apoEcys142 increases atherosclerosis. This increase in atherosclerosis can be explained by the critical role of HSPG binding in apoE-mediated cholesterol efflux (Lin et al., 2001). Interestingly, apoE can bind and accept cholesterol from ABCA1 (Krimbou et al., 2004). ApoE2, apoE3, and apoE4 isoforms are functionally equivalent in this regard. In Chapter VII, the effects of endogenous apoE on cholesterol efflux were separated into autocrine effects and paracrine effects that may depend on interactions of apoE with surface receptors. Because the human apoE isoforms have different receptor binding affinities, macrophages that express these human apoE proteins will be useful for determining the nature of proteoglycans and LDLR in autocrine and paracrine cholesterol efflux mechanisms. Endogenous interactions of these human apoE proteins with ABCA1 will also be tested in future experiments.

Macrophage ApoAI and Atherosclerosis. Plasma apoAI and HDL protect against atherosclerosis (Gordon and Rifkind, 1989). ApoAI is synthesized by hepatocytes, but in contrast to apoE, it is not synthesized by macrophages (Basu et al., 1981). Previous studies from our laboratory express transgenic human apoAI (h-apoAI) regulated by a

macrophage-specific promoter. Major et al. show that in the absence of macrophage apoE expression, macrophage h-apoAI protects hyperlipidemic mice from atherosclerosis without altering plasma lipid profiles (Major et al., 2001). Ishiguro et al. found that apoE deficient macrophages expressing h-apoAI as a result of viral transduction, decrease lesion size in hyperlipidemic mice (Ishiguro et al., 2001). Ishiguro et al. also found that macrophage h-apoAI can protect against atherosclerosis, even in a model of hepatic over-expression of transgenic apoAI, were ample amounts of hepatic apoAI are present in the plasma compartment. Recently, Su et al. found increased expression of ABCA1 in macrophages that express h-apoAI (Su et al., 2003). In chapter VIII, we showed that endogenous macrophage h-apoAI stimulated ABCA1-mediated cholesterol efflux to exogenous apoAI. Together these studies provide evidence supporting cholesterol efflux as a mechanism of protection from atherosclerosis in hyperlipidemic mice.

Macrophage ACAT1 and Atherosclerosis. While blocking the esterification of cholesterol is a strategy for blocking foam cell formation, Fazio et al. found that hyperlipidemic mice that were reconstituted with *ACAT1(-/-)* macrophages had increased atherosclerosis and cellular death within lesions (Fazio et al., 2001). In Chapter IV and Chapter V, we showed that *ACAT1(-/-)* macrophages had severe changes in cholesterol homeostasis. While Fazio et al. reported that free cholesterol accumulates in the vascular wall, the mechanism of this accumulation remains unclear. Because the availability of free cholesterol to extracellular acceptors is thought to be protective, cholesterol toxicity or decreased efflux to available acceptors may counteract any advantages that would result from free cholesterol availability. The current *in vitro* studies show that even under mild cholesterol-loading conditions macrophage cholesterol homeostasis assumes a phenotype that is similar to that found with disruptions in cholesterol trafficking. Under the severe conditions present in an atherosclerotic lesion *in vivo*, these macrophages with compromised cholesterol homeostasis are likely to have a high susceptibility to free cholesterol accumulation and toxicity.

Cholesterol Efflux in Atherosclerosis and Other Diseases. Inflammation, oxidative stress, and cell death are common pathological elements in atherosclerosis,

diabetes, inflammatory diseases, and neurodegenerative diseases. Inflammation and oxidative stress occurs in each of these diseases (Aiello et al., 2003; Andersen, 2004; Linton and Fazio, 2003; Schmitz et al., 1999). Cell death and tissue damage occurs in each of these diseases, making phagocytic cells and the removal of cellular membrane debris a common element. Whether the phagocytes involved in each disease are macrophages or other cell types, intracellular lipid accumulation is a possibility.

While evidence for connections between cholesterol efflux and atherosclerosis are substantial, the connections between cholesterol efflux and diseases like diabetes and Alzheimer's disease require more investigation. The importance of HDL and apoE in preventing atherosclerosis is partially due to their effects on cholesterol efflux. HDL and apoE also have a protective role in other diseases including diabetes (Rohrer et al., 2004; von Eckardstein et al., 2000) and Alzheimer's disease (Rebeck, 2004). Since HDL and apoE have effects not directly related to choletserol efflux, it is necessary to study cellular cholesterol homeostasis in the context of these other diseases. Other mediators of lipid homeostasis such as peroxisome proliferator-activated receptors (PPAR) (Moore et al., 2001; Torra et al., 2001), scavenger receptors (Miyazaki et al., 2002), and unsaturated fatty acids (Wang and Oram, 2002) provide additional links between cholesterol efflux, atherosclerosis, and other diseases, especially diabetes.

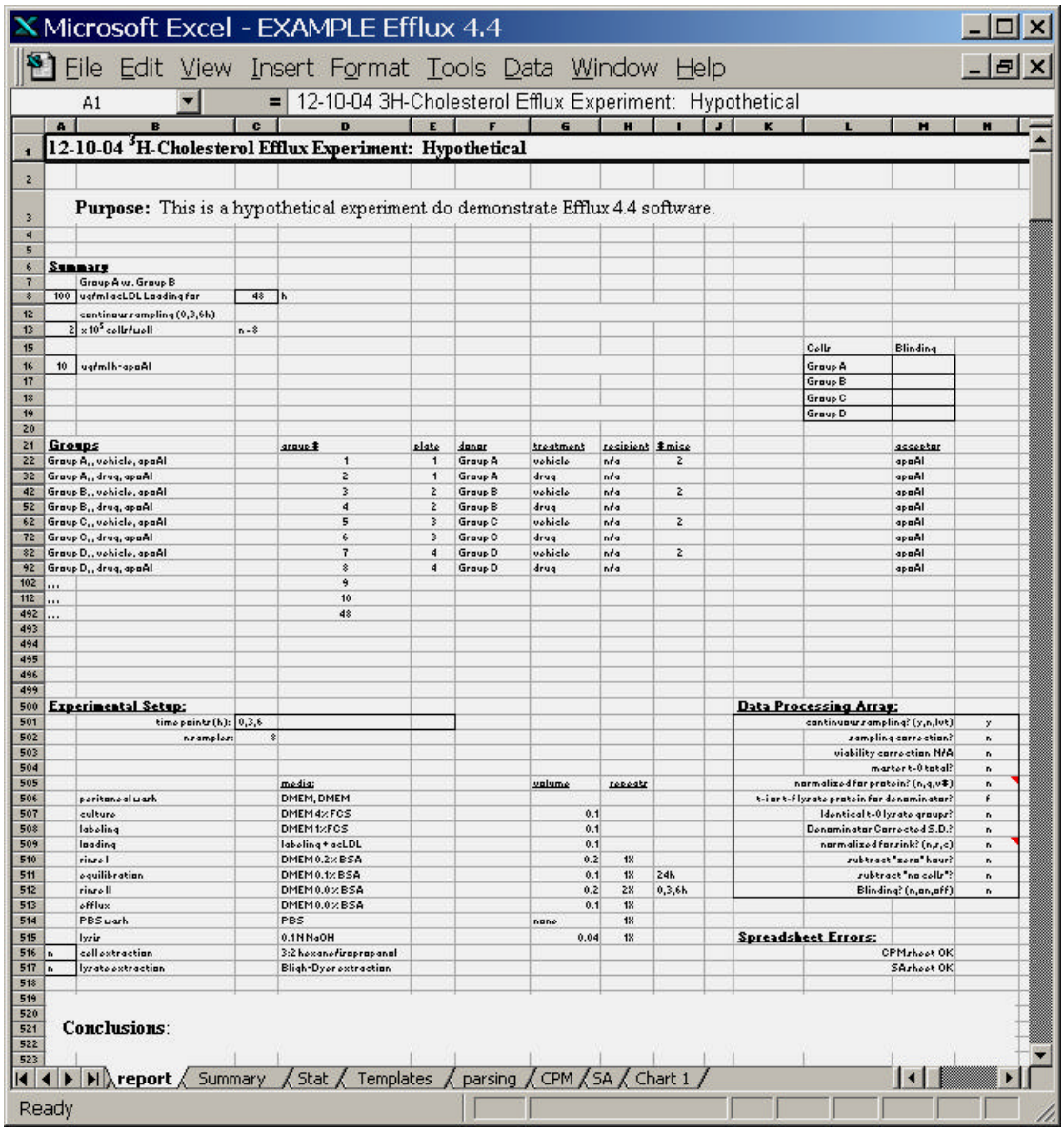
Changes that accompany any disease process may also affect the function of mediators of cholesterol efflux. For example, glycosylation of cholesterol acceptors or lipid transporters could affect cholesterol efflux in diabetes. Sequestration of apoE in amyloid plaques could affect choletserol efflux in Alzheimer's disease. Membrane function and surface receptors are critical aspects of cellular viability. The importance of cholesterol for membrane fluidity and the presence of cholesterol in specific membrane domains suggests mechanisms by which cholesterol efflux could affect the progression of diseases (Incardona and Eaton, 2000; Spector and Yorek, 1985b). Because cholesterol homeostasis is important in all cells, there is a potential role for cholesterol efflux in the pathogenesis of atherosclerosis and many other important diseases.

General Conclusion

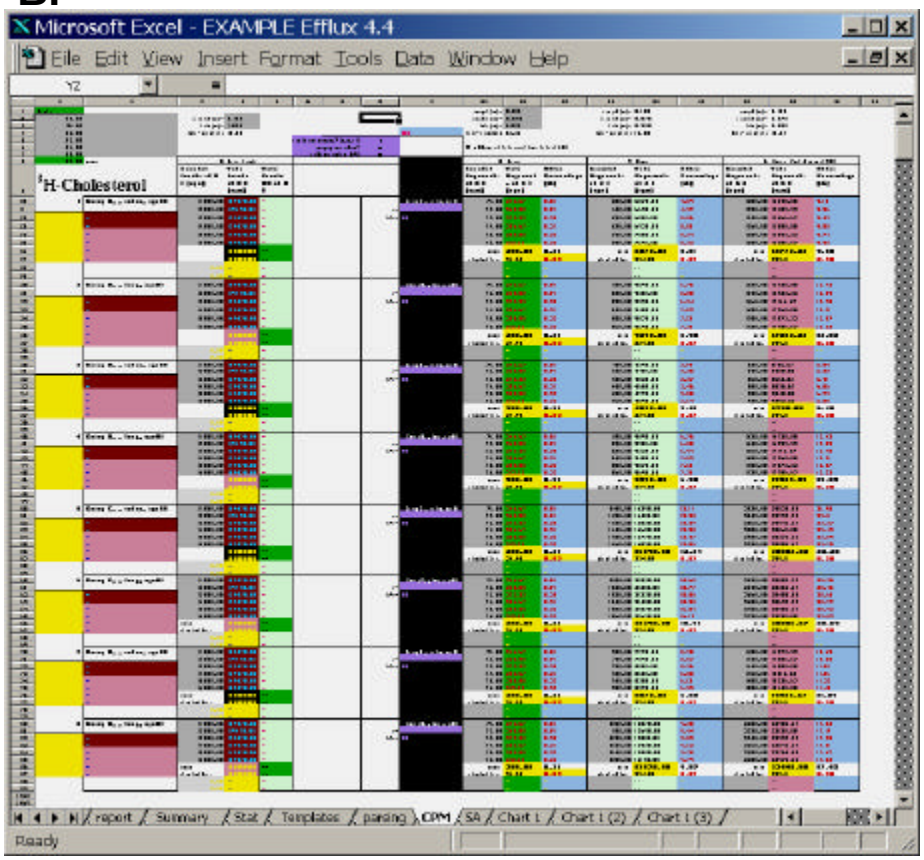
With the current studies, we have focused on the role of cholesterol efflux in macrophage cholesterol homeostasis. We have approached this problem from two angles. We have studied macrophages with cholesterol storage deficits and macrophages that secrete cholesterol acceptors. Studying cholesterol efflux by each of these approaches has provided mechanistic detail about cholesterol homeostasis in macrophages. The degree of overlap between these two approaches highlights the connections between the mechanisms of macrophage cholesterol homeostasis. In macrophages, a cholesterol storage deficiency precipitated disruptions in cholesterol efflux and was associated with a constellation of cellular changes including altered morphology, aberrant cholesterol trafficking, and apparent changes in cholesterol sensing. There were connections between different pathways of cholesterol efflux in macrophages that secrete cholesterol acceptors. The presence of multiple efflux pathways and the degree to which these pathways interact suggests a high biological importance for macrophage cholesterol efflux. As an addition to previous *in vivo* studies of atherosclerosis, the current *in vitro* studies converge on the idea that cholesterol efflux helps to maintain cholesterol homeostasis in macrophages. These studies suggest that cholesterol efflux is a mechanism used by macrophages to protect against foam cell formation and atherosclerosis.

Figure 29. Sample Screens from the *Efflux 4.4* Data Analysis Spreadsheet. As discussed in Chapter III, *Efflux 4.4* is a data analysis spreadsheet that **(A)** describes experiments, **(B)** analyzes data, **(C)** executes automated routines, **(D)** displays graphics, and **(E)** summarizes and statistically compares data.

A.



B.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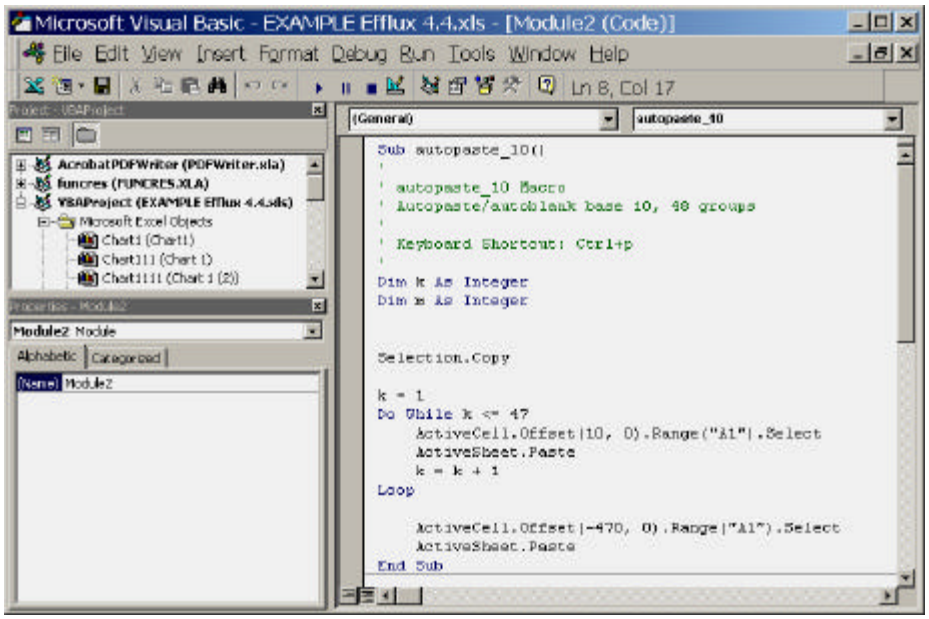
C.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Ln 8, Col 17
AcrobatPDFWriter (PDFWriter.xla)
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VBAProject (EXAMPLE Efflux 4.4.xls)
Microsoft Excel Objects
Chart1 (Chart1)
Chart111 (Chart 1)
Chart1111 (Chart 1 (2))
Module2 Module
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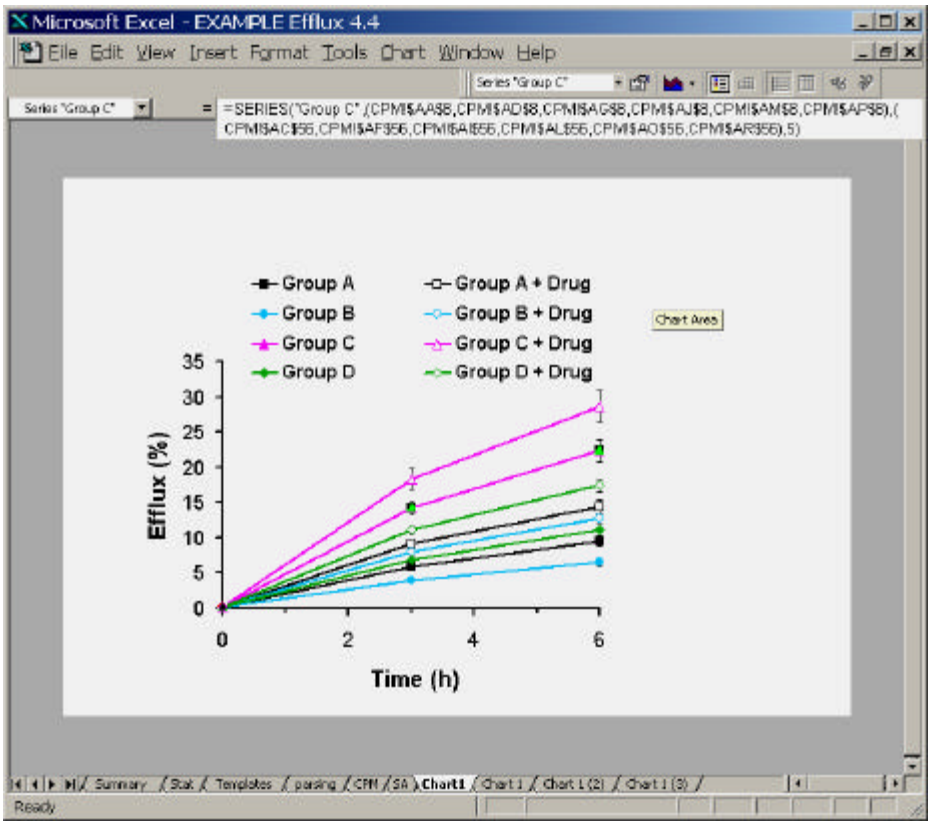
(Name) Module2
(General)
autopaste_10
Sub autopaste_10()
' autopaste_10 Macro
' Autopaste/autoblank base 10, 40 groups
' Keyboard Shortcut: Ctrl+p
Dim k As Integer
Dim m As Integer
Selection.Copy
k = 1
Do While k <= 47
ActiveCell.Offset(10, 0).Range("A1").Select
ActiveSheet.Paste
k = k + 1
Loop
ActiveCell.Offset(-470, 0).Range("A1").Select
ActiveSheet.Paste
End Sub

D.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Group A
Group B
Group C
Group D
Group A + Drug
Group B + Drug
Group C + Drug
Group D + Drug
Chart Area
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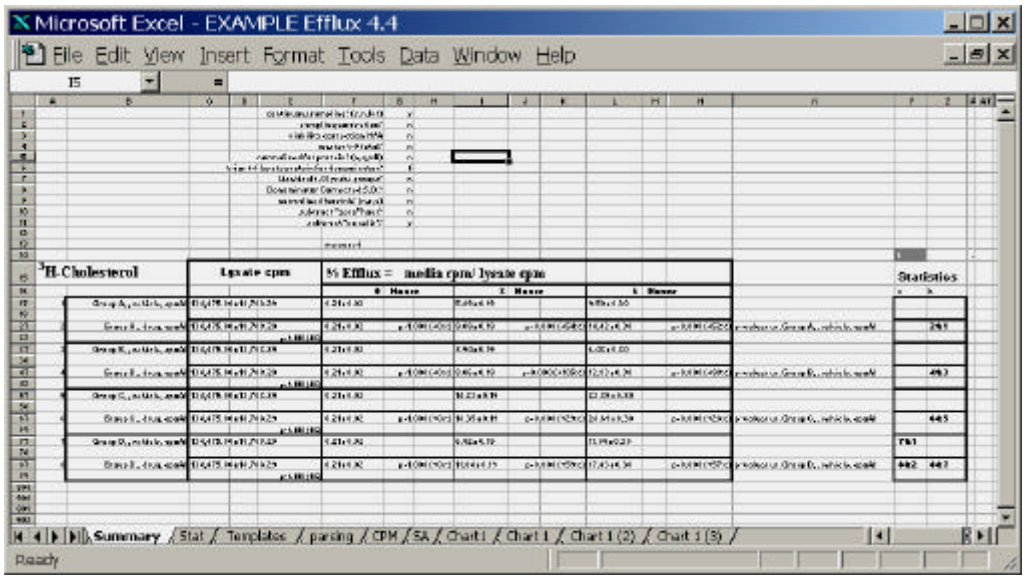
E.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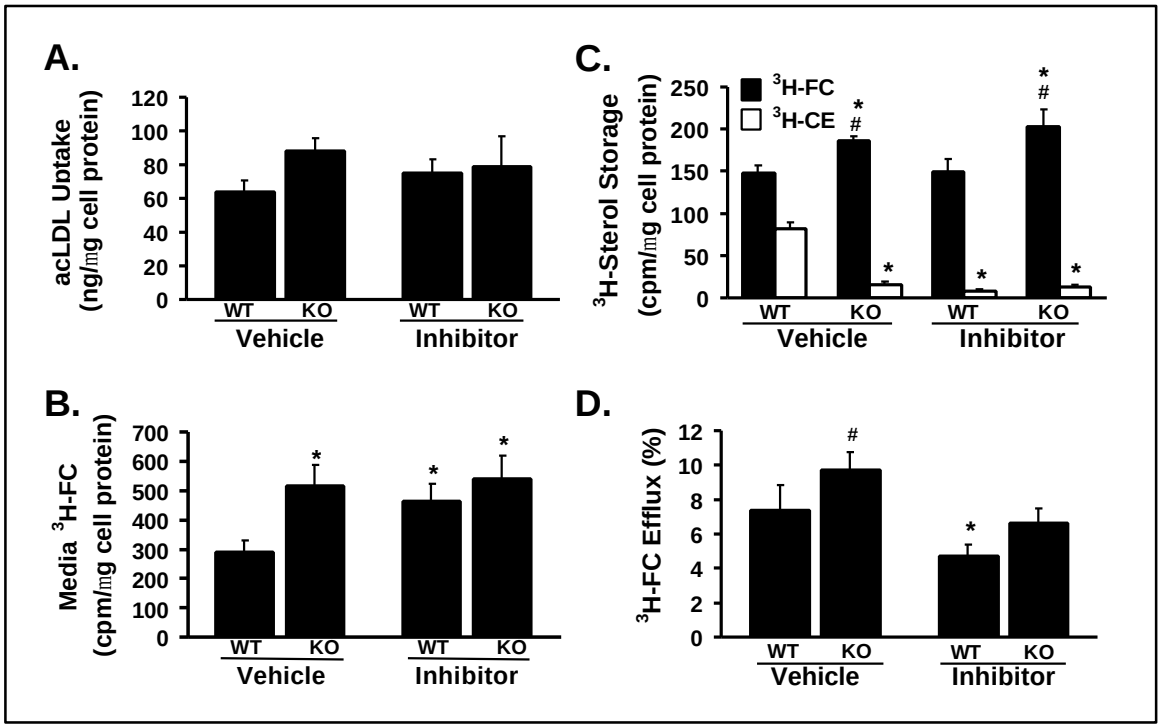

Figure 30. Effects of ACAT1 Deficiency or Inhibition on Lipoprotein-derived Cholesterol in Macrophages.
As described in Chapter IV, *ACAT1(+/+)* (WT) or *ACAT1(-/-)* (KO) macrophages were treated with 70 μg/ml ^{3}H-CE/acLDL in DMEM/2% FBS for 36 hours to label lipoprotein-derived cholesterol pools. Macrophages where treated with vehicle or 2 μg/ml cp113-818 ACAT inhibitor. Bars and error bars represent the mean (n=4) and standard deviation of samples from *ACAT1(+/+)* or *ACAT1(-/-)* macrophages treated with vehicle or 2 μg/ml cp113-818 ACAT inhibitor. Asterisks (*) denote a statistically significant difference ($p<0.05$) compared to the *ACAT1(+/+)*/Vehicle group and pound signs (#) denote a statistically significant difference ($p<0.05$) compared to the *ACAT1(+/+)*/Inhibitor group as determined by ANOVA with Bonferroni post-test. **(A)** The uptake of ^{3}H-CE/acLDL by macrophages was calculated by adding the counts from the ^{3}H-FC and ^{3}H-CE stored within the cell and the ^{3}H-FC that appeared in the culture media during loading. The sum of these counts was normalized for cellular protein and then converted to mass by dividing by the counts of ^{3}H-CE per mass acLDL. **(B)** The turnover of ^{3}H-CE by macrophages was measured by the appearance of ^{3}H-FC in the culture media over 36 hours and was normalized for cellular protein. **(C)** Cholesterol storage as ^{3}H-FC (solid bars) and ^{3}H-CE (empty bars) was measured in cellular lysate and was normalized for cellular protein. **(D)** Cholesterol efflux was measured from macrophages to media with 10 μg/ml human apoAI for 6 hours.

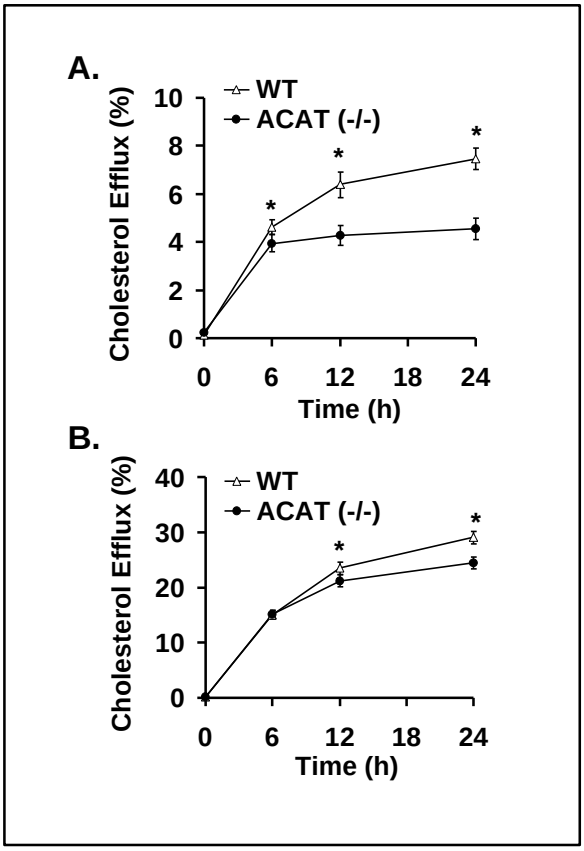

Figure 31. ApoE-Mediated and HDL-Mediated Cholesterol Efflux from ACAT1 Deficient Macrophages.

Macrophages were treated for 24 hours with 2 μCi/ml of ^{3}H-cholesterol and 50 μg/ml acLDL in DMEM/4% FBS. Cholesterol efflux was then measured for 24 hours. Bars and error bars represent the mean (n=6) and standard deviation of samples from *ACAT1(+/+)* (WT) or *ACAT1(-/-)* macrophages. Asterisks (*) denote a statistically significant difference ($p<0.05$) compared to the *ACAT1(-/-)* group as determined by Student's *t*-test. **(A)** To measure apoE-mediated cholesterol efflux, cells were cultured in efflux media with 0.1% BSA. **(B)** To measure HDL-mediated cholesterol efflux, cells were cultured in efflux media with 25 μg/ml HDL_3 (density = 1.125 to 1.210 mg/ml).

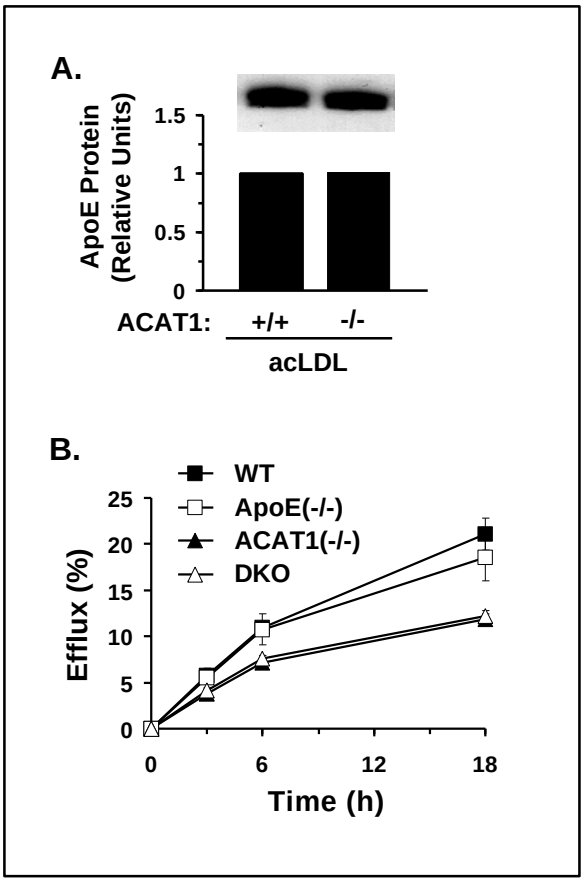

Figure 32. ABCA1-Mediated Cholesterol Efflux from ACAT1/ApoE Deficient Macrophages.

Macrophages were treated for 24 hours with 2 μCi/ml of ^{3}H-cholesterol and 50 μg/ml acLDL in DMEM/4% FBS. **(A)** ApoE secretion was measured for 18 hours from *ACAT1(+/+)* or *ACAT1(-/-)* macrophages by Western analysis as described in Chapter VI. **(B)** ABCA1-mediated cholesterol efflux was measured to media with 10 μg/ml human apoAI for 18 hours. Bars and error bars represent the mean (n=6) and standard deviation of samples from *ACAT1(+/+)* (WT), *apoE(-/-)*, *ACAT1(-/-)*, or *apoE(-/-)ACAT1(-/-)* (DKO) macrophages.

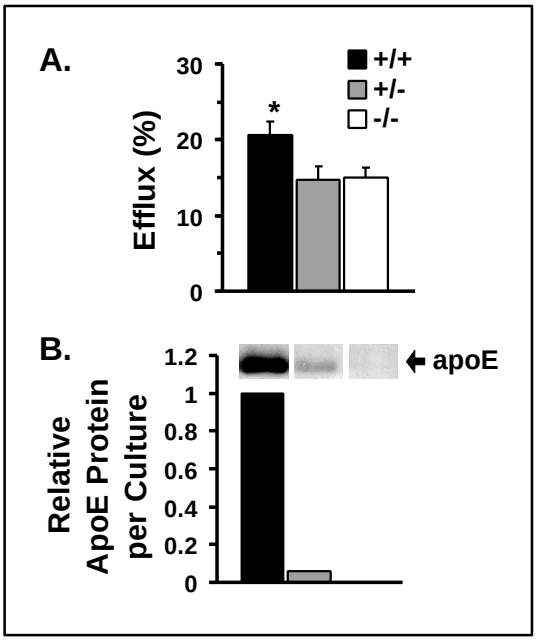

Figure 33. ApoE-Mediated Cholesterol Efflux from ApoE Heterozygous Macrophages.
ApoE(+/+), *apoE(+/-)*, and *apoE(-/-)* macrophages in 24-well plates were stimulated with modified LDL, labeled with ^{3}H-cholesterol, and then cultured in 1.0 ml of media with no acceptors for 48 h. **(A)** Efflux was measured by counts in the culture media. Bars and error bars represent the mean (n=4) and standard deviation of samples. Asterisks (*) denote a statistically significant difference ($p<0.05$) compared to the *apoE(-/-)* group as determined by Student's *t*-test. **(B)** ApoE secretion was measured by Western analysis as described in Chapter VI.

Cholesterol efflux from heterozygous *apoE(+/-)* macrophages was significantly less ($p<0.001$) compared to efflux from wildtype *apoE(+/+)* macrophages (Appendix, Figure 33A). However, there was no significant increase in cholesterol efflux from heterozygous *apoE(+/-)* macrophages when compared to knockout *apoE(-/-)* macrophages. Even though these cells were expected to have apoE expression levels of about 50% of normal, they only secreted approximately 10% of the apoE compared to wildtype *apoE(+/+)* macrophages (Appendix, Figure 33B).

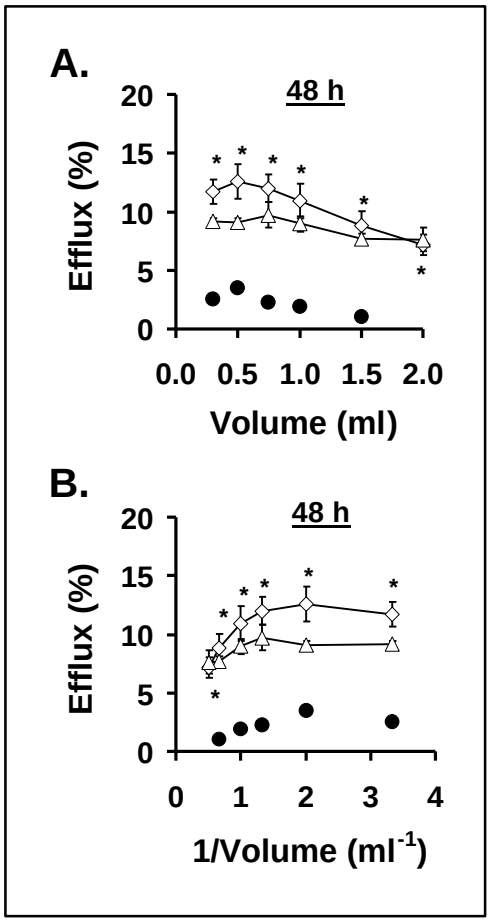

Figure 34. Volume-Dependence of h-ApoAI-Mediated Cholesterol Efflux. The **(A)** volume-dependence and the **(B)** relative concentration-dependence of cholesterol efflux from *h-apoAI(tng)apoE(-/-)* macrophages (diamonds) *apoE(-/-)* macrophages (triangles) and the h-apoAI-mediated efflux (filled circles). Values are expressed as mean (4 samples) ± SD of efflux from *h-apoAI(tng)apoE(-/-)* and *apoE(-/-)* macrophages with asterisks (*) denoting a statistically significant difference ($p<0.05$) by Student's *t*-test. Methodology is described in Chapter VII.

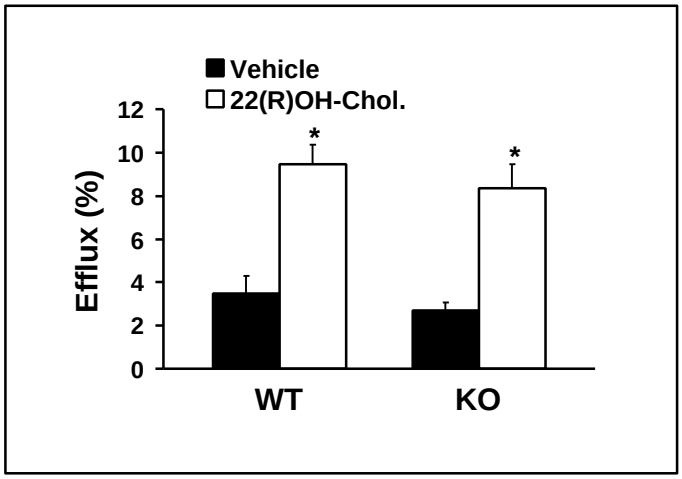

Figure 35. LXR Agonism Increases ABCA1-Mediated Cholesterol Efflux from ACAT1 Deficient Macrophages.
ACAT1(+/+) (WT) and *ACAT1(-/-)* (KO) macrophages were treated for 48 hours with 3.0 μCi/ml of ^{3}H-FC and 100 μg/ml acLDL in DMEM/1% FBS to label cellular cholesterol. Macrophages were treated with 1 μM 22(R)-hydroxycholesterol for 18 hours. Methods are described in Chapter VI. ABCA1-mediated cholesterol efflux was measured from macrophages cultured for 6 hours in serum-free efflux media with 10 μg/ml apoAI and 1 μM 22(R)-hydroxycholesterol. Bars and error bars represent the mean (n=8) and standard deviation of samples. Asterix (*) denote a statistically significant difference ($p<0.05$) compared to the vehicle group as determined by two-way ANOVA with Bonferroni post-test. Pounds (#) denote a statistically significant difference ($p<0.05$) compared to the *ACAT1(+/+)* (WT) group as determined by two-way ANOVA with Bonferroni post-test.

BIBLIOGRAPHY

Aiello, R. J., Brees, D., Bourassa, P. A., Royer, L., Lindsey, S., Coskran, T., Haghpassand, M., and Francone, O. L. (2002) Increased atherosclerosis in hyperlipidemic mice with inactivation of ABCA1 in macrophages. *Arterioscler Thromb Vasc Biol*, **22**, 630-7.

Aiello, R. J., Brees, D., and Francone, O. L. (2003) ABCA1-deficient mice: insights into the role of monocyte lipid efflux in HDL formation and inflammation. *Arterioscler Thromb Vasc Biol*, **23**, 972-80.

Akiba, S., Yoneda, Y., Ohno, S., Nemoto, M., and Sato, T. (2003) Oxidized LDL activates phospholipase A2 to supply fatty acids required for cholesterol esterification. *J Lipid Res*, **44**, 1676-85. Epub 2003 Jun 1.

Andersen, J. K. (2004) Oxidative stress in neurodegeneration: cause or consequence? *Nat Med*, **10 Suppl**, S18-25.

Anderson, R. A., Joyce, C., Davis, M., Reagan, J. W., Clark, M., Shelness, G. S., and Rudel, L. L. (1998) Identification of a form of acyl-CoA:cholesterol acyltransferase specific to liver and intestine in nonhuman primates. *J Biol Chem*, **273**, 26747-54.

Arakawa, R., Hayashi, M., Remaley, A. T., Brewer, B. H., Yamauchi, Y., and Yokoyama, S. (2004) Phosphorylation and stabilization of ATP binding cassette transporter A1 by synthetic amphiphilic helical peptides. *J Biol Chem*, **279**, 6217-20. Epub 2003 Dec 29.

Arakawa, R., and Yokoyama, S. (2002) Helical Apolipoproteins Stabilize ATP-binding Cassette Transporter A1 by Protecting It from Thiol Protease-mediated Degradation. *J Biol Chem*, **277**, 22426-9.

Assmann, G., Herbert, P. N., Fredrickson, D. S., and Forte, T. (1977) Isolation and characterization of an abnormal high density lipoprotein in Tangier Diesase. *J Clin Invest*, **60**, 242-52.

Atger, V. M., de la Llera Moya, M., Stoudt, G. W., Rodrigueza, W. V., Phillips, M. C., and Rothblat, G. H. (1997) Cyclodextrins as catalysts for the removal of cholesterol from macrophage foam cells. *J Clin Invest*, **99**, 773-80.

Atmeh, R. F., Shepherd, J., and Packard, C. J. (1983) Subpopulations of apolipoprotein A-I in human high-density lipoproteins. Their metabolic properties and response to drug therapy. *Biochim Biophys Acta*, **751**, 175-88.

ATPIII (2002) Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III) final report. *Circulation*, **106**, 3143-421.

Babaev, V. R., Fazio, S., Gleaves, L. A., Carter, K. J., Semenkovich, C. F., and Linton, M. F. (1999) Macrophage lipoprotein lipase promotes foam cell formation and atherosclerosis in vivo. *J Clin Invest*, **103**, 1697-705.

Babaev, V. R., Patel, M. B., Semenkovich, C. F., Fazio, S., and Linton, M. F. (2000) Macrophage lipoprotein lipase promotes foam cell formation and atherosclerosis in low density lipoprotein receptor-deficient mice. *J Biol Chem*, **275**, 26293-9.

Balboa, M. A., Perez, R., and Balsinde, J. (2003) Amplification mechanisms of inflammation: paracrine stimulation of arachidonic acid mobilization by secreted phospholipase A2 is regulated by cytosolic phospholipase A2-derived hydroperoxyeicosatetraenoic acid. *J Immunol*, **171**, 989-94.

Basu, S. K., Brown, M. S., Ho, Y. K., Havel, R. J., and Goldstein, J. L. (1981) Mouse macrophages synthesize and secrete a protein resembling apolipoprotein E. *Proc Natl Acad Sci U S A*, **78**, 7545-9.

Basu, S. K., Goldstein, J. L., Anderson, G. W., and Brown, M. S. (1976) Degradation of cationized low density lipoprotein and regulation of cholesterol metabolism in homozygous familial hypercholesterolemia fibroblasts. *Proc Natl Acad Sci U S A*, **73**, 3178-82.

Basu, S. K., Goldstein, J. L., and Brown, M. S. (1983) Independent pathways for secretion of cholesterol and apolipoprotein E by macrophages. *Science*, **219**, 871-3.

Batsilas, L., Berezhkovskii, A. M., and Shvartsman, S. Y. (2003) Stochastic model of autocrine and paracrine signals in cell culture assays. *Biophys J*, **85**, 3659-65.

Bellosta, S., Mahley, R. W., Sanan, D. A., Murata, J., Newland, D. L., Taylor, J. M., and Pitas, R. E. (1995) Macrophage-specific expression of human apolipoprotein E reduces atherosclerosis in hypercholesterolemic apolipoprotein E-null mice. *J Clin Invest*, **96**, 2170-9.

Bhat, S. G., and Brockman, H. L. (1981) Enzymatic synthesis/hydrolysis of cholesteryl oleate in surface films. Inhibition by lecithin and its reversal by bile salts. *J Biol Chem*, **256**, 3017-23.

Binder, C. J., Hartvigsen, K., Chang, M. K., Miller, M., Broide, D., Palinski, W., Curtiss, L. K., Corr, M., and Witztum, J. L. (2004) IL-5 links adaptive and natural immunity specific for epitopes of oxidized LDL and protects from atherosclerosis. *J Clin Invest*, **114**, 427-37.

Blanc, J., Alves-Guerra, M. C., Esposito, B., Rousset, S., Gourdy, P., Ricquier, D., Tedgui, A., Miroux, B., and Mallat, Z. (2003) Protective role of uncoupling protein 2 in atherosclerosis. *Circulation*, **107**, 388-90.

Bligh, E., and Dyer, W. (1959) A rapid method of total lipid extraction and purification. *Can J Biochem Phys*, **37**, 911-7.

Blum, C. B., Aron, L., and Sciacca, R. (1980) Radioimmunoassay studies of human apolipoprotein E. *J Clin Invest*, **66**, 1240-50.

Bocksch, L., Stephens, T., Lucas, A., and Singh, B. (2001) Apolipoprotein E: possible therapeutic target for atherosclerosis. *Curr Drug Targets Cardiovasc Haematol Disord*, **1**, 93-106.

Boisvert, W. A. (2004) Modulation of atherogenesis by chemokines. *Trends Cardiovasc Med*, **14**, 161-5.

Boisvert, W. A., Santiago, R., Curtiss, L. K., and Terkeltaub, R. A. (1998) A leukocyte homologue of the IL-8 receptor CXCR-2 mediates the accumulation of macrophages in atherosclerotic lesions of LDL receptor-deficient mice. *J Clin Invest*, **101**, 353-63.

Boisvert, W. A., Spangenberg, J., and Curtiss, L. K. (1995) Treatment of severe hypercholesterolemia in apolipoprotein E-deficient mice by bone marrow transplantation. *J Clin Invest*, **96**, 1118-24.

Boisvert, W. A., Spangenberg, J., and Curtiss, L. K. (1997) Role of leukocyte-specific LDL receptors on plasma lipoprotein cholesterol and atherosclerosis in mice. *Arterioscler Thromb Vasc Biol*, **17**, 340-7.

Bortnick, A. E., Rothblat, G. H., Stoudt, G., Hoppe, K. L., Royer, L. J., McNeish, J., and Francone, O. L. (2000) The correlation of ATP-binding cassette 1 mRNA levels with cholesterol efflux from various cell lines. *J Biol Chem*, **275**, 28634-40.

Boyd, H. C., Gown, A. M., Wolfbauer, G., and Chait, A. (1989) Direct evidence for a protein recognized by a monoclonal antibody against oxidatively modified LDL in atherosclerotic lesions from a Watanabe heritable hyperlipidemic rabbit. *Am J Pathol*, **135**, 815-25.

Brecht, W. J., Harris, F. M., Chang, S., Tesseur, I., Yu, G. Q., Xu, Q., Dee Fish, J., Wyss-Coray, T., Buttini, M., Mucke, L., Mahley, R. W., and Huang, Y. (2004) Neuron-specific apolipoprotein e4 proteolysis is associated with increased tau phosphorylation in brains of transgenic mice. *J Neurosci*, **24**, 2527-34.

Brown, M. S., Dana, S. E., and Goldstein, J. L. (1975) Receptor-dependent hydrolysis of cholesteryl esters contained in plasma low density lipoprotein. *Proc Natl Acad Sci U S A*, **72**, 2925-9.

Brown, M. S., and Goldstein, J. L. (1986) A receptor-mediated pathway for cholesterol homeostasis. *Science*, **232**, 34-47.

Brown, M. S., Goldstein, J. L., Krieger, M., Ho, Y. K., and Anderson, R. G. (1979) Reversible accumulation of cholesteryl esters in macrophages incubated with acetylated lipoproteins. *J Cell Biol*, **82**, 597-613.

Buhman, K. F., Accad, M., and Farese, R. V. (2000a) Mammalian acyl-CoA:cholesterol acyltransferases. *Biochim Biophys Acta*, **1529**, 142-54.

Buhman, K. K., Accad, M., Novak, S., Choi, R. S., Wong, J. S., Hamilton, R. L., Turley, S., and Farese, R. V., Jr. (2000b) Resistance to diet-induced hypercholesterolemia and gallstone formation in ACAT2-deficient mice. *Nat Med*, **6**, 1341-7.

Buhman, K. K., Chen, H. C., and Farese, R. V., Jr. (2001) The enzymes of neutral lipid synthesis. *J Biol Chem*, **276**, 40369-72. Epub 2001 Sep 5.

Burke, G. L., Cresanta, J. L., Shear, C. L., Miner, M. H., and Berenson, G. S. (1986) Cardiovascular risk factors and their modification in children. *Cardiol Clin*, **4**, 33-46.

Burleigh, M. E., Babaev, V. R., Oates, J. A., Harris, R. C., Gautam, S., Riendeau, D., Marnett, L. J., Morrow, J. D., Fazio, S., and Linton, M. F. (2002) Cyclooxygenase-2 promotes early atherosclerotic lesion formation in LDL receptor-deficient mice. *Circulation*, **105**, 1816-23.

Cases, S., Novak, S., Zheng, Y. W., Myers, H. M., Lear, S. R., Sande, E., Welch, C. B., Lusis, A. J., Spencer, T. A., Krause, B. R., Erickson, S. K., and Farese, R. V., Jr. (1998a) ACAT-2, a second mammalian acyl-CoA:cholesterol acyltransferase. Its cloning, expression, and characterization. *J Biol Chem*, **273**, 26755-64.

Cases, S., Smith, S. J., Zheng, Y. W., Myers, H. M., Lear, S. R., Sande, E., Novak, S., Collins, C., Welch, C. B., Lusis, A. J., Erickson, S. K., and Farese, R. V., Jr. (1998b) Identification of a gene encoding an acyl CoA:diacylglycerol acyltransferase, a key enzyme in triacylglycerol synthesis. *Proc Natl Acad Sci U S A*, **95**, 13018-23.

Chambenoit, O., Hamon, Y., Marguet, D., Rigneault, H., Rosseneu, M., and Chimini, G. (2001) Specific docking of apolipoprotein A-I at the cell surface requires a functional ABCA1 transporter. *J Biol Chem*, **276**, 9955-60.

Chang, C. C., Sakashita, N., Ornvold, K., Lee, O., Chang, E. T., Dong, R., Lin, S., Lee, C. Y., Strom, S. C., Kashyap, R., Fung, J. J., Farese, R. V., Jr., Patoiseau, J. F., Delhon, A., and Chang, T. Y. (2000) Immunological quantitation and localization of ACAT-1 and ACAT-2 in human liver and small intestine. *J Biol Chem*, **275**, 28083-92.

Chawla, A., Boisvert, W. A., Lee, C. H., Laffitte, B. A., Barak, Y., Joseph, S. B., Liao, D., Nagy, L., Edwards, P. A., Curtiss, L. K., Evans, R. M., and Tontonoz, P. (2001) A PPAR gamma-LXR-ABCA1 pathway in macrophages is involved in cholesterol efflux and atherogenesis. *Mol Cell*, **7**, 161-71.

Chen, W., Sun, Y., Welch, C., Gorelik, A., Leventhal, A. R., Tabas, I., and Tall, A. R. (2001) Preferential ATP-binding cassette transporter A1-mediated cholesterol efflux from late endosomes/lysosomes. *J Biol Chem*, **276**, 43564-9.

Cho, K. H., An, S., Lee, W. S., Paik, Y. K., Kim, Y. K., and Jeong, T. S. (2003) Mass-production of human ACAT-1 and ACAT-2 to screen isoform-specific inhibitor: a different substrate specificity and inhibitory regulation. *Biochem Biophys Res Commun*, **309**, 864-72.

Clinton, S. K., Underwood, R., Hayes, L., Sherman, M. L., Kufe, D. W., and Libby, P. (1992) Macrophage colony-stimulating factor gene expression in vascular cells and in experimental and human atherosclerosis. *Am J Pathol*, **140**, 301-16.

Connelly, M. A., de la Llera-Moya, M., Monzo, P., Yancey, P. G., Drazul, D., Stoudt, G., Fournier, N., Klein, S. M., Rothblat, G. H., and Williams, D. L. (2001) Analysis of chimeric receptors shows that multiple distinct functional activities of scavenger receptor, class B, type I (SR-BI), are localized to the extracellular receptor domain. *Biochemistry*, **40**, 5249-59.

Coussens, L. M., Tinkle, C. L., Hanahan, D., and Werb, Z. (2000) MMP-9 supplied by bone marrow-derived cells contributes to skin carcinogenesis. *Cell*, **103**, 481-90.

Covey, S. D., Krieger, M., Wang, W., Penman, M., and Trigatti, B. L. (2003) Scavenger receptor class B type I-mediated protection against atherosclerosis in LDL receptor-negative mice involves its expression in bone marrow-derived cells. *Arterioscler Thromb Vasc Biol*, **23**, 1589-94. Epub 2003 Jun 26.

Cullen, P., Cignarella, A., Brennhausen, B., Mohr, S., Assmann, G., and von Eckardstein, A. (1998) Phenotype-dependent differences in apolipoprotein E metabolism and in cholesterol homeostasis in human monocyte-derived macrophages. *J Clin Invest*, **101**, 1670-7.

Deng, J., Rudick, V., and Dory, L. (1995) Lysosomal degradation and sorting of apolipoprotein E in macrophages. *J Lipid Res*, **36**, 2129-40.

Diez-Juan, A., Perez, P., Aracil, M., Sancho, D., Bernad, A., Sanchez-Madrid, F., and Andres, V. (2004) Selective inactivation of p27(Kip1) in hematopoietic progenitor cells increases neointimal macrophage proliferation and accelerates atherosclerosis. *Blood*, **103**, 158-61. Epub 2003 Sep 22.

Dory, L. (1989) Synthesis and secretion of apoE in thioglycolate-elicited mouse peritoneal macrophages: effect of cholesterol efflux. *J Lipid Res*, **30**, 809-16.

Dove, D. E., Linton, M. F., and Fazio, S. (2005a) ApoE-Mediated Cholesterol Efflux from Macrophages: Separation of Autocrine and Paracrine Effects. *Am J Physiol Cell Physiol*.

Dove, D. E., Su, Y. R., Zhang, W., Jerome, W. G., Swift, L. L., Linton, M. F., and Fazio, S. (2005b) ACAT1 Deficiency Disrupts Cholesterol Efflux and Alters Cellular Morphology in Macrophages. *Arterioscler Thromb Vasc Biol*, **25**, 128-34.

Du, H., and Grabowski, G. A. (2004) Lysosomal acid lipase and atherosclerosis. *Curr Opin Lipidol*, **15**, 539-44.

Fadok, V. A., Bratton, D. L., and Henson, P. M. (2001) Phagocyte receptors for apoptotic cells: recognition, uptake, and consequences. *J Clin Invest*, **108**, 957-62.

Farkas, M. H., Swift, L. L., Hasty, A. H., Linton, M. F., and Fazio, S. (2003) The recycling of apolipoprotein E in primary cultures of mouse hepatocytes. Evidence for a physiologic connection to high density lipoprotein metabolism. *J Biol Chem*, **278**, 9412-7. Epub 2003 Jan 10.

Fazio, S., Babaev, V. R., Burleigh, M. E., Major, A. S., Hasty, A. H., and Linton, M. F. (2002) Physiological expression of macrophage apoE in the artery wall reduces atherosclerosis in severely hyperlipidemic mice. *J Lipid Res*, **43**, 1602-9.

Fazio, S., Babaev, V. R., Murray, A. B., Hasty, A. H., Carter, K. J., Gleaves, L. A., Atkinson, J. B., and Linton, M. F. (1997) Increased atherosclerosis in mice reconstituted with apolipoprotein E null macrophages. *Proc Natl Acad Sci U S A*, **94**, 4647-52.

Fazio, S., Dove, D. E., and Linton, M. F. (2005) ACAT Inhibition: Bad for Macrophages, Good for Smooth Muscle Cells? *Arterioscler Thromb Vasc Biol*, **25**, 7-9.

Fazio, S., and Linton, M. F. (2001) Mouse models of hyperlipidemia and atherosclerosis. *Front Biosci*, **6**, D515-25.

Fazio, S., Major, A. S., Swift, L. L., Gleaves, L. A., Accad, M., Linton, M. F., and Farese, R. V., Jr. (2001) Increased atherosclerosis in LDL receptor-null mice lacking ACAT1 in macrophages. *J Clin Invest*, **107**, 163-71.

Feng, B., and Tabas, I. (2002) ABCA1-mediated cholesterol efflux is defective in free cholesterol- loaded macrophages. Mechanism involves enhanced ABCA1 degradation in a process requiring full NPC1 activity. *J Biol Chem*, **277**, 43271-80.

Feng, B., Yao, P. M., Li, Y., Devlin, C. M., Zhang, D., Harding, H. P., Sweeney, M., Rong, J. X., Kuriakose, G., Fisher, E. A., Marks, A. R., Ron, D., and Tabas, I. (2003) The endoplasmic reticulum is the site of cholesterol-induced cytotoxicity in macrophages. *Nat Cell Biol*, **5**, 781-92. Epub 2003 Aug 10.

Fielding, P. E., Nagao, K., Hakamata, H., Chimini, G., and Fielding, C. J. (2000) A two-step mechanism for free cholesterol and phospholipid efflux from human vascular cells to apolipoprotein A-1. *Biochemistry*, **39**, 14113-20.

Forrester, J. S. (2002) Prevention of plaque rupture: a new paradigm of therapy. *Ann Intern Med*, **137**, 823-33.

Frolov, A., Zielinski, S. E., Crowley, J. R., Dudley-Rucker, N., Schaffer, J. E., and Ory, D. S. (2003) NPC1 and NPC2 regulate cellular cholesterol homeostasis through generation of low density lipoprotein cholesterol-derived oxysterols. *J Biol Chem*, **278**, 25517-25. Epub 2003 Apr 28.

Fruchart, J. C., Nierman, M. C., Stroes, E. S., Kastelein, J. J., and Duriez, P. (2004) New risk factors for atherosclerosis and patient risk assessment. *Circulation*, **109**, III15-9.

Galis, Z. S., Sukhova, G. K., Kranzhofer, R., Clark, S., and Libby, P. (1995) Macrophage foam cells from experimental atheroma constitutively produce matrix-degrading proteinases. *Proc Natl Acad Sci U S A*, **92**, 402-6.

Galis, Z. S., Sukhova, G. K., Lark, M. W., and Libby, P. (1994) Increased expression of matrix metalloproteinases and matrix degrading activity in vulnerable regions of human atherosclerotic plaques. *J Clin Invest*, **94**, 2493-503.

Galloway, C. J., Dean, G. E., Marsh, M., Rudnick, G., and Mellman, I. (1983) Acidification of macrophage and fibroblast endocytic vesicles in vitro. *Proc Natl Acad Sci U S A*, **80**, 3334-8.

Goldstein, J. L., and Brown, M. S. (1974) Binding and degradation of low density lipoproteins by cultured human fibroblasts. Comparison of cells from a normal subject and from a patient with homozygous familial hypercholesterolemia. *J Biol Chem*, **249**, 5153-62.

Goldstein, J. L., Ho, Y. K., Basu, S. K., and Brown, M. S. (1979) Binding site on macrophages that mediates uptake and degradation of acetylated low density lipoprotein, producing massive cholesterol deposition. *Proc Natl Acad Sci U S A*, **76**, 333-7.

Gordon, D. J., and Rifkind, B. M. (1989) High-density lipoprotein--the clinical implications of recent studies. *N Engl J Med*, **321**, 1311-6.

Gordon, S., Clarke, S., Greaves, D., and Doyle, A. (1995) Molecular immunobiology of macrophages: recent progress. *Curr Opin Immunol*, **7**, 24-33.

Gordon, T., Castelli, W. P., Hjortland, M. C., Kannel, W. B., and Dawber, R. T. (1977) High density lipoprotein as a protective factor against coronary heart disease. The Framingham study. *Am J Med*, **62**, 707-14.

Gough, P. J., and Gordon, S. (2000) The role of scavenger receptors in the innate immune system. *Microbes Infect*, **2**, 305-11.

Grundy, S. M., Pasternak, R., Greenland, P., Smith, S., Jr., and Fuster, V. (1999) Assessment of cardiovascular risk by use of multiple-risk-factor assessment equations: a statement for healthcare professionals from the American Heart Association and the American College of Cardiology. *Circulation*, **100**, 1481-92.

Guo, J., Van Eck, M., Twisk, J., Maeda, N., Benson, G. M., Groot, P. H., and Van Berkel, T. J. (2003) Transplantation of monocyte CC-chemokine receptor 2-deficient bone marrow into ApoE3-Leiden mice inhibits atherogenesis. *Arterioscler Thromb Vasc Biol*, **23**, 447-53. Epub 2003 Jan 30.

Guyton, J. R., and Klemp, K. F. (1989) The lipid-rich core region of human atherosclerotic fibrous plaques. Prevalence of small lipid droplets and vesicles by electron microscopy. *Am J Pathol*, **134**, 705-17.

Guyton, J. R., and Klemp, K. F. (1994) Development of the atherosclerotic core region. Chemical and ultrastructural analysis of microdissected atherosclerotic lesions from human aorta. *Arterioscler Thromb*, **14**, 1305-14.

Haghpassand, M., Bourassa, P. A., Francone, O. L., and Aiello, R. J. (2001) Monocyte/macrophage expression of ABCA1 has minimal contribution to plasma HDL levels. *J Clin Invest*, **108**, 1315-20.

Haidar, B., Denis, M., Marcil, M., Krimbou, L., and Genest, J., Jr. (2004) Apolipoprotein A-I activates cellular cAMP signaling through the ABCA1 transporter. *J Biol Chem*, **279**, 9963-9. Epub 2003 Dec 29.

Hampton, R., Dimster-Denk, D., and Rine, J. (1996) The biology of HMG-CoA reductase: the pros of contra-regulation. *Trends Biochem Sci*, **21**, 140-5.

Hara, H., Komaba, A., and Yokoyama, S. (1992) Alpha-helical requirements for free apolipoproteins to generate HDL and to induce cellular lipid efflux. *Lipids*, **27**, 302-4.

Hara, H., and Yokoyama, S. (1991) Interaction of free apolipoproteins with macrophages. Formation of high density lipoprotein-like lipoproteins and reduction of cellular cholesterol. *J Biol Chem*, **266**, 3080-6.

Hara, H., and Yokoyama, S. (1992) Role of apolipoproteins in cholesterol efflux from macrophages to lipid microemulsion: proposal of a putative model for the pre-beta high-density lipoprotein pathway. *Biochemistry*, **31**, 2040-6.

Heeren, J., Grewal, T., Laatsch, A., Rottke, D., Rinninger, F., Enrich, C., and Beisiegel, U. (2003) Recycling of apoprotein E is associated with cholesterol efflux and high density lipoprotein internalization. *J Biol Chem*, **278**, 14370-8. Epub 2003 Feb 12.

Henderson, L. O., Herbert, P. N., Fredrickson, D. S., Heinen, R. J., and Easterling, J. C. (1978) Abnormal concentration and anomalous distribution of apolipoprotein A-I in Tangier disease. *Metabolism*, **27**, 165-74.

Herijgers, N., de Winther, M. P., Van Eck, M., Havekes, L. M., Hofker, M. H., Hoogerbrugge, P. M., and Van Berkel, T. J. (2000a) Effect of human scavenger receptor class A overexpression in bone marrow-derived cells on lipoprotein metabolism and atherosclerosis in low density lipoprotein receptor knockout mice. *J Lipid Res*, **41**, 1402-9.

Herijgers, N., Van Eck, M., Groot, P. H., Hoogerbrugge, P. M., and Van Berkel, T. J. (1997) Effect of bone marrow transplantation on lipoprotein metabolism and atherosclerosis in LDL receptor-knockout mice. *Arterioscler Thromb Vasc Biol*, **17**, 1995-2003.

Herijgers, N., Van Eck, M., Groot, P. H., Hoogerbrugge, P. M., and Van Berkel, T. J. (2000b) Low density lipoprotein receptor of macrophages facilitates atherosclerotic lesion formation in C57Bl/6 mice. *Arterioscler Thromb Vasc Biol*, **20**, 1961-7.

Herz, J., and Hui, D. Y. (2004) Lipoprotein receptors in the vascular wall. *Curr Opin Lipidol*, **15**, 175-81.

Herz, J., and Strickland, D. K. (2001) LRP: a multifunctional scavenger and signaling receptor. *J Clin Invest*, **108**, 779-84.

Ho, Y. K., Brown, M. S., and Goldstein, J. L. (1980) Hydrolysis and excretion of cytoplasmic cholesteryl esters by macrophages: stimulation by high density lipoprotein and other agents. *J Lipid Res*, **21**, 391-8.

Ho, Y. Y., Al-Haideri, M., Mazzone, T., Vogel, T., Presley, J. F., Sturley, S. L., and Deckelbaum, R. J. (2000) Endogenously expressed apolipoprotein E has different effects on cell lipid metabolism as compared to exogenous apolipoprotein E carried on triglyceride-rich particles. *Biochemistry*, **39**, 4746-54.

Ho, Y. Y., Deckelbaum, R. J., Chen, Y., Vogel, T., and Talmage, D. A. (2001) Apolipoprotein E inhibits serum-stimulated cell proliferation and enhances serum-independent cell proliferation. *J Biol Chem*, **276**, 43455-62. Epub 2001 Sep 10.

Horiuchi, S., Sakamoto, Y., and Sakai, M. (2003) Scavenger receptors for oxidized and glycated proteins. *Amino Acids*, **25**, 283-92. Epub 2003 Aug 21.

Horvai, A., Palinski, W., Wu, H., Moulton, K. S., Kalla, K., and Glass, C. K. (1995) Scavenger receptor A gene regulatory elements target gene expression to macrophages and to foam cells of atherosclerotic lesions. *Proc Natl Acad Sci U S A*, **92**, 5391-5.

Huang, Z. H., Lin, C. Y., Oram, J. F., and Mazzone, T. (2001) Sterol efflux mediated by endogenous macrophage ApoE expression is independent of ABCA1. *Arterioscler Thromb Vasc Biol*, **21**, 2019-25.

Incardona, J. P., and Eaton, S. (2000) Cholesterol in signal transduction. *Curr Opin Cell Biol*, **12**, 193-203.

Ioannou, Y. A. (2001) Multidrug permeases and subcellular cholesterol transport. *Nat Rev Mol Cell Biol*, **2**, 657-68.

Ishiguro, H., Yoshida, H., Major, A. S., Zhu, T., Babaev, V. R., Linton, M. F., and Fazio, S. (2001) Retrovirus-mediated expression of apolipoprotein A-I in the macrophage protects against atherosclerosis in vivo. *J Biol Chem*, **276**, 36742-8.

Ishikawa, T. T., MacGee, J., Morrison, J. A., and Glueck, C. J. (1974) Quantitative analysis of cholesterol in 5 to 20 microliter of plasma. *J Lipid Res*, **15**, 286-91.

Jeejeebhoy, K. N., Ho, J., Breckenridge, C., Bruce-Robertson, A., Steiner, G., and Jeejeebhoy, J. (1975) Synthesis of VLDL by isolated rat hepatocytes in suspension. *Biochem Biophys Res Commun*, **66**, 1147-53.

Ji, Z. S., Fazio, S., and Mahley, R. W. (1994) Variable heparan sulfate proteoglycan binding of apolipoprotein E variants may modulate the expression of type III hyperlipoproteinemia. *J Biol Chem*, **269**, 13421-8.

Ji, Z. S., Pitas, R. E., and Mahley, R. W. (1998) Differential cellular accumulation/retention of apolipoprotein E mediated by cell surface heparan sulfate proteoglycans. Apolipoproteins E3 and E2 greater than e4. *J Biol Chem*, **273**, 13452-60.

Johnson, W. J., Chacko, G. K., Phillips, M. C., and Rothblat, G. H. (1990) The efflux of lysosomal cholesterol from cells. *J Biol Chem*, **265**, 5546-53.

Johnson, W. J., Fischer, R. T., Phillips, M. C., and Rothblat, G. H. (1995) Efflux of newly synthesized cholesterol and biosynthetic sterol intermediates from cells. Dependence on acceptor type and on enrichment of cells with cholesterol. *J Biol Chem*, **270**, 25037-46.

Kanters, E., Pasparakis, M., Gijbels, M. J., Vergouwe, M. N., Partouns-Hendriks, I., Fijneman, R. J., Clausen, B. E., Forster, I., Kockx, M. M., Rajewsky, K., Kraal, G., Hofker, M. H., and de Winther, M. P. (2003) Inhibition of NF-kappaB activation in macrophages increases atherosclerosis in LDL receptor-deficient mice. *J Clin Invest*, **112**, 1176-85.

Kellner-Weibel, G., Jerome, W. G., Small, D. M., Warner, G. J., Stoltenborg, J. K., Kearney, M. A., Corjay, M. H., Phillips, M. C., and Rothblat, G. H. (1998) Effects of intracellular free cholesterol accumulation on macrophage viability: a model for foam cell death. *Arterioscler Thromb Vasc Biol*, **18**, 423-31.

Kellner-Weibel, G., McHendry-Rinde, B., Haynes, M. P., and Adelman, S. (2001) Evidence that newly synthesized esterified cholesterol is deposited in existing cytoplasmic lipid inclusions. *J Lipid Res*, **42**, 768-77.

Kellner-Weibel, G., Yancey, P. G., Jerome, W. G., Walser, T., Mason, R. P., Phillips, M. C., and Rothblat, G. H. (1999) Crystallization of free cholesterol in model macrophage foam cells. *Arterioscler Thromb Vasc Biol*, **19**, 1891-8.

Kinoshita, M., Kawamura, M., Maeda, T., Fujimaki, Y., Fujita, M., Kojima, K., and Teramoto, T. (2000) Apolipoprotein E accelerates the efflux of cholesterol from macrophages: mechanism of xanthoma formation in apolipoprotein E deficiency. *J Atheroscler Thromb*, **6**, 22-7.

Kita, T., Kume, N., Minami, M., Hayashida, K., Murayama, T., Sano, H., Moriwaki, H., Kataoka, H., Nishi, E., Horiuchi, H., Arai, H., and Yokode, M. (2001) Role of oxidized LDL in atherosclerosis. *Ann N Y Acad Sci*, **947**, 199-205; discussion -6.

Klansek, J. J., Yancey, P., St Clair, R. W., Fischer, R. T., Johnson, W. J., and Glick, J. M. (1995) Cholesterol quantitation by GLC: artifactual formation of short-chain steryl esters. *J Lipid Res*, **36**, 2261-6.

Kockx, M., Rye, K. A., Gaus, K., Quinn, C. M., Wright, J., Sloane, T., Sviridov, D., Fu, Y., Sullivan, D., Burnett, J. R., Rust, S., Assmann, G., Anantharamaiah, G. M., Palgunachari, M. N., Katz, S. L., Phillips, M. C., Dean, R. T., Jessup, W., and Kritharides, L. (2004) Apolipoprotein A-I-stimulated apolipoprotein E secretion from human macrophages is independent of cholesterol efflux. *J Biol Chem*, **279**, 25966-77. Epub 2004 Apr 1.

Kovanen, P. T., and Tabas, I. (2001) In search of a starting point. *Curr Opin Lipidol*, **12**, 475-6.

Krieger, M. (1997) The other side of scavenger receptors: pattern recognition for host defense. *Curr Opin Lipidol*, **8**, 275-80.

Krieger, M. (2001) Scavenger receptor class B type I is a multiligand HDL receptor that influences diverse physiologic systems. *J Clin Invest*, **108**, 793-7.

Krimbou, L., Denis, M., Haidar, B., Carrier, M., Marcil, M., and Genest, J., Jr. (2004) Molecular interactions between apoE and ABCA1: impact on apoE lipidation. *J Lipid Res*, **45**, 839-48. Epub 2004 Feb 1.

Kritharides, L., Christian, A., Stoudt, G., Morel, D., and Rothblat, G. H. (1998) Cholesterol metabolism and efflux in human THP-1 macrophages. *Arterioscler Thromb Vasc Biol*, **18**, 1589-99.

Kruth, H. S. (2001) Macrophage foam cells and atherosclerosis. *Front Biosci*, **6**, D429-55. Print 2001 Mar 1.

Kruth, H. S. (2002) Sequestration of aggregated low-density lipoproteins by macrophages. *Curr Opin Lipidol*, **13**, 483-8.

Kubo, N., Boisvert, W. A., Ballantyne, C. M., and Curtiss, L. K. (2000) Leukocyte CD11b expression is not essential for the development of atherosclerosis in mice. *J Lipid Res*, **41**, 1060-6.

Lada, A. T., Davis, M., Kent, C., Chapman, J., Tomoda, H., Omura, S., and Rudel, L. L. (2004) Identification of ACAT1- and ACAT2-specific inhibitors using a novel, cell-based fluorescence assay: individual ACAT uniqueness. *J Lipid Res*, **45**, 378-86. Epub 2003 Nov 16.

Lafarga, M., Crespo, P., Berciano, M. T., Andres, M. A., and Leon, J. (1994) Apolipoprotein E expression in the cerebellum of normal and hypercholesterolemic rabbits. *Brain Res Mol Brain Res*, **21**, 115-23.

Laffitte, B. A., Repa, J. J., Joseph, S. B., Wilpitz, D. C., Kast, H. R., Mangelsdorf, D. J., and Tontonoz, P. (2001) LXRs control lipid-inducible expression of the apolipoprotein E gene in macrophages and adipocytes. *Proc Natl Acad Sci U S A*, **98**, 507-12.

Lange, Y., Ye, J., Rigney, M., and Steck, T. (2000) Cholesterol movement in Niemann-Pick type C cells and in cells treated with amphiphiles. *J Biol Chem*, **275**, 17468-75.

Lange, Y., Ye, J., and Steck, T. L. (1998) Circulation of cholesterol between lysosomes and the plasma membrane. *J Biol Chem*, **273**, 18915-22.

Langer, C., Huang, Y., Cullen, P., Wiesenhutter, B., Mahley, R. W., Assmann, G., and von Eckardstein, A. (2000) Endogenous apolipoprotein E modulates cholesterol efflux and cholesteryl ester hydrolysis mediated by high-density lipoprotein-3 and lipid-free apolipoproteins in mouse peritoneal macrophages. *J Mol Med*, **78**, 217-27.

Lauffenburger, D. A., Oehrtman, G. T., Walker, L., and Wiley, H. S. (1998) Real-time quantitative measurement of autocrine ligand binding indicates that autocrine loops are spatially localized. *Proc Natl Acad Sci U S A*, **95**, 15368-73.

Lee, R. G., Willingham, M. C., Davis, M. A., Skinner, K. A., and Rudel, L. L. (2000) Differential expression of ACAT1 and ACAT2 among cells within liver, intestine, kidney, and adrenal of nonhuman primates. *J Lipid Res*, **41**, 1991-2001.

Li, A. C., and Glass, C. K. (2002) The macrophage foam cell as a target for therapeutic intervention. *Nat Med*, **8**, 1235-42.

Li, B. L., Li, X. L., Duan, Z. J., Lee, O., Lin, S., Ma, Z. M., Chang, C. C., Yang, X. Y., Park, J. P., Mohandas, T. K., Noll, W., Chan, L., and Chang, T. Y. (1999) Human acyl-CoA:cholesterol acyltransferase-1 (ACAT-1) gene organization and evidence

that the 4.3-kilobase ACAT-1 mRNA is produced from two different chromosomes. *J Biol Chem*, **274**, 11060-71.

Libby, P., Geng, Y. J., Aikawa, M., Schoenbeck, U., Mach, F., Clinton, S. K., Sukhova, G. K., and Lee, R. T. (1996) Macrophages and atherosclerotic plaque stability. *Curr Opin Lipidol*, **7**, 330-5.

Libeu, C. P., Lund-Katz, S., Phillips, M. C., Wehrli, S., Hernaiz, M. J., Capila, I., Linhardt, R. J., Raffai, R. L., Newhouse, Y. M., Zhou, F., and Weisgraber, K. H. (2001) New insights into the heparan sulfate proteoglycan-binding activity of apolipoprotein E. *J Biol Chem*, **276**, 39138-44. Epub 2001 Aug 10.

Lin, C. Y., Duan, H., and Mazzone, T. (1999) Apolipoprotein E-dependent cholesterol efflux from macrophages: kinetic study and divergent mechanisms for endogenous versus exogenous apolipoprotein E. *J Lipid Res*, **40**, 1618-27.

Lin, C. Y., Huang, Z. H., and Mazzone, T. (2001) Interaction with proteoglycans enhances the sterol efflux produced by endogenous expression of macrophage apoE. *J Lipid Res*, **42**, 1125-33.

Lin, C. Y., Lucas, M., and Mazzone, T. (1998) Endogenous apoE expression modulates HDL3 binding to macrophages. *J Lipid Res*, **39**, 293-301.

Linton, M. F., Atkinson, J. B., and Fazio, S. (1995) Prevention of atherosclerosis in apolipoprotein E-deficient mice by bone marrow transplantation. *Science*, **267**, 1034-7.

Linton, M. F., Babaev, V. R., Gleaves, L. A., and Fazio, S. (1999) A direct role for the macrophage low density lipoprotein receptor in atherosclerotic lesion formation. *J Biol Chem*, **274**, 19204-10.

Linton, M. F., and Fazio, S. (1999) Macrophages, lipoprotein metabolism, and atherosclerosis: insights from murine bone marrow transplantation studies. *Curr Opin Lipidol*, **10**, 97-105.

Linton, M. F., and Fazio, S. (2001) Class A scavenger receptors, macrophages, and atherosclerosis. *Curr Opin Lipidol*, **12**, 489-95.

Linton, M. F., and Fazio, S. (2003) Macrophages, inflammation, and atherosclerosis. *Int J Obes Relat Metab Disord*, **27**, S35-40.

Liu, J., Thewke, D. P., Su, Y. R., Linton, M. F., Fazio, S., and Sinensky, M. S. (2004) Reduced Macrophage Apoptosis Is Associated With Accelerated Atherosclerosis in Low-Density Lipoprotein Receptor-Null Mice. *Arterioscler Thromb Vasc Biol*, **21**, 21.

Lopes-Virella, M. F., Koskinen, S., Mironova, M., Horne, D., Klein, R., Chassereau, C., Enockson, C., and Virella, G. (2000) The preparation of copper-oxidized LDL for

the measurement of oxidized LDL antibodies by EIA. *Atherosclerosis*, **152**, 107-15.

Lowry, O. H., Rosenbrough, N. J., Farr, A. L., and Randall, R. J. (1951) Protein Measurment with Folin Phenol Reagent. *J Biol Chem*, **193**, 265-75.

Luttun, A., Lupu, F., Storkebaum, E., Hoylaerts, M. F., Moons, L., Crawley, J., Bono, F., Poole, A. R., Tipping, P., Herbert, J. M., Collen, D., and Carmeliet, P. (2002) Lack of plasminogen activator inhibitor-1 promotes growth and abnormal matrix remodeling of advanced atherosclerotic plaques in apolipoprotein E-deficient mice. *Arterioscler Thromb Vasc Biol*, **22**, 499-505.

Mahley, R. W. (1988) Apolipoprotein E: cholesterol transport protein with expanding role in cell biology. *Science*, **240**, 622-30.

Mahley, R. W., and Rall, S. C., Jr. (2000) Apolipoprotein E: far more than a lipid transport protein. *Annu Rev Genomics Hum Genet*, **1**, 507-37.

Major, A. S., Dove, D. E., Ishiguro, H., Su, Y. R., Brown, A. M., Liu, L., Carter, K. J., Linton, M. F., and Fazio, S. (2001) Increased cholesterol efflux in apolipoprotein AI (ApoAI)-producing macrophages as a mechanism for reduced atherosclerosis in ApoAI((-/-)) mice. *Arterioscler Thromb Vasc Biol*, **21**, 1790-5.

Major, A. S., Fazio, S., and Linton, M. F. (2002) B-lymphocyte deficiency increases atherosclerosis in LDL receptor-null mice. *Arterioscler Thromb Vasc Biol*, **22**, 1892-8.

Mak, P. A., Laffitte, B. A., Desrumaux, C., Joseph, S. B., Curtiss, L. K., Mangelsdorf, D. J., Tontonoz, P., and Edwards, P. A. (2002) Regulated expression of the apolipoprotein E/C-I/C-IV/C-II gene cluster in murine and human macrophages. A critical role for nuclear liver X receptors alpha and beta. *J Biol Chem*, **277**, 31900-8. Epub 2002 May 24.

Makowski, L., Boord, J. B., Maeda, K., Babaev, V. R., Uysal, K. T., Morgan, M. A., Parker, R. A., Suttles, J., Fazio, S., Hotamisligil, G. S., and Linton, M. F. (2001) Lack of macrophage fatty-acid-binding protein aP2 protects mice deficient in apolipoprotein E against atherosclerosis. *Nat Med*, **7**, 699-705.

Maung, K., Miyazaki, A., Nomiyama, H., Chang, C. C., Chang, T. Y., and Horiuchi, S. (2001) Induction of acyl-coenzyme A:cholesterol acyltransferase-1 by 1,25-dihydroxyvitamin D(3) or 9-cis-retinoic acid in undifferentiated THP-1 cells. *J Lipid Res*, **42**, 181-7.

Mautner, S. L., Sanchez, J. A., Rader, D. J., Mautner, G. C., Ferrans, V. J., Fredrickson, D. S., Brewer, H. B., Jr., and Roberts, W. C. (1992) The heart in Tangier disease. Severe coronary atherosclerosis with near absence of high-density lipoprotein cholesterol. *Am J Clin Pathol*, **98**, 191-8.

Mazzone, T., Gump, H., Diller, P., and Getz, G. S. (1987) Macrophage free cholesterol content regulates apolipoprotein E synthesis. *J Biol Chem*, **262**, 11657-62.

Mazzone, T., and Reardon, C. (1994) Expression of heterologous human apolipoprotein E by J774 macrophages enhances cholesterol efflux to HDL3. *J Lipid Res*, **35**, 1345-53.

McNeish, J., Aiello, R. J., Guyot, D., Turi, T., Gabel, C., Aldinger, C., Hoppe, K. L., Roach, M. L., Royer, L. J., de Wet, J., Broccardo, C., Chimini, G., and Francone, O. L. (2000) High density lipoprotein deficiency and foam cell accumulation in mice with targeted disruption of ATP-binding cassette transporter-1. *Proc Natl Acad Sci U S A*, **97**, 4245-50.

Mehrabian, M., Allayee, H., Wong, J., Shi, W., Wang, X. P., Shaposhnik, Z., Funk, C. D., Lusis, A. J., and Shih, W. (2002) Identification of 5-lipoxygenase as a major gene contributing to atherosclerosis susceptibility in mice. *Circ Res*, **91**, 120-6.

Meiner, V. L., Cases, S., Myers, H. M., Sande, E. R., Bellosta, S., Schambelan, M., Pitas, R. E., McGuire, J., Herz, J., and Farese, R. V., Jr. (1996) Disruption of the acyl-CoA:cholesterol acyltransferase gene in mice: evidence suggesting multiple cholesterol esterification enzymes in mammals. *Proc Natl Acad Sci U S A*, **93**, 14041-6.

Mendez, A. J., Oram, J. F., and Bierman, E. L. (1991) Protein kinase C as a mediator of high density lipoprotein receptor-dependent efflux of intracellular cholesterol. *J Biol Chem*, **266**, 10104-11.

Merched, A. J., Williams, E., and Chan, L. (2003) Macrophage-specific p53 expression plays a crucial role in atherosclerosis development and plaque remodeling. *Arterioscler Thromb Vasc Biol*, **23**, 1608-14. Epub 2003 Jul 3.

Millatt, L. J., Bocher, V., Fruchart, J. C., and Staels, B. (2003) Liver X receptors and the control of cholesterol homeostasis: potential therapeutic targets for the treatment of atherosclerosis. *Biochim Biophys Acta*, **1631**, 107-18.

Mitchinson, M. J., Hardwick, S. J., and Bennett, M. R. (1996) Cell death in atherosclerotic plaques. *Curr Opin Lipidol*, **7**, 324-9.

Miyazaki, I., Asanuma, M., Higashi, Y., Sogawa, C. A., Tanaka, K., and Ogawa, N. (2002) Age-related changes in expression of metallothionein-III in rat brain. *Neurosci Res*, **43**, 323-33.

Moore, K. J., Fitzgerald, M. L., and Freeman, M. W. (2001) Peroxisome proliferator-activated receptors in macrophage biology: friend or foe? *Curr Opin Lipidol*, **12**, 519-27.

Moore, R. E., Kawashiri, M. A., Kitajima, K., Secreto, A., Millar, J. S., Pratico, D., and Rader, D. J. (2003) Apolipoprotein A-I deficiency results in markedly increased

atherosclerosis in mice lacking the LDL receptor. *Arterioscler Thromb Vasc Biol*, **23**, 1914-20. Epub 2003 Aug 21.

Moran, E. C., Kamiguti, A. S., Cawley, J. C., and Pettitt, A. R. (2002) Cytoprotective antioxidant activity of serum albumin and autocrine catalase in chronic lymphocytic leukaemia. *Br J Haematol*, **116**, 316-28.

Mukherjee, S., Zha, X., Tabas, I., and Maxfield, F. R. (1998) Cholesterol distribution in living cells: fluorescence imaging using dehydroergosterol as a fluorescent cholesterol analog. *Biophys J*, **75**, 1915-25.

Natarajan, P., Forte, T. M., Chu, B., Phillips, M. C., Oram, J. F., and Bielicki, J. K. (2004) Identification of an apolipoproteinA-I structural element that mediates cellular cholesterol efflux and stabilizes ABCA1. *J Biol Chem*, **29**, 29.

Neary, R. H., and Gowland, E. (1987) Stability of free apolipoprotein A-1 concentration in serum, and its measurement in normal and hyperlipidemic subjects. *Clin Chem*, **33**, 1163-9.

Nelken, N. A., Coughlin, S. R., Gordon, D., and Wilcox, J. N. (1991) Monocyte chemoattractant protein-1 in human atheromatous plaques. *J Clin Invest*, **88**, 1121-7.

Nicholson, A. C., Han, J., Febbraio, M., Silversterin, R. L., and Hajjar, D. P. (2001) Role of CD36, the macrophage class B scavenger receptor, in atherosclerosis. *Ann N Y Acad Sci*, **947**, 224-8.

Nong, Z., Gonzalez-Navarro, H., Amar, M., Freeman, L., Knapper, C., Neufeld, E. B., Paigen, B. J., Hoyt, R. F., Fruchart-Najib, J., and Santamarina-Fojo, S. (2003) Hepatic lipase expression in macrophages contributes to atherosclerosis in apoE-deficient and LCAT-transgenic mice. *J Clin Invest*, **112**, 367-78.

O'Brien, K. D., Allen, M. D., McDonald, T. O., Chait, A., Harlan, J. M., Fishbein, D., McCarty, J., Ferguson, M., Hudkins, K., Benjamin, C. D., and et al. (1993) Vascular cell adhesion molecule-1 is expressed in human coronary atherosclerotic plaques. Implications for the mode of progression of advanced coronary atherosclerosis. *J Clin Invest*, **92**, 945-51.

Oehrtman, G. T., Wiley, H. S., and Lauffenburger, D. A. (1998) Escape of autocrine ligands into extracellular medium: experimental test of theoretical model predictions. *Biotechnol Bioeng*, **57**, 571-82.

Oram, J. F., Lawn, R. M., Garvin, M. R., and Wade, D. P. (2000) ABCA1 is the cAMP-inducible apolipoprotein receptor that mediates cholesterol secretion from macrophages. *J Biol Chem*, **275**, 34508-11.

Ory, D. S. (2004) The niemann-pick disease genes; regulators of cellular cholesterol homeostasis. *Trends Cardiovasc Med*, **14**, 66-72.

Osterud, B., and Bjorklid, E. (2003) Role of monocytes in atherogenesis. *Physiol Rev*, **83**, 1069-112.

Pearson, T. A., Blair, S. N., Daniels, S. R., Eckel, R. H., Fair, J. M., Fortmann, S. P., Franklin, B. A., Goldstein, L. B., Greenland, P., Grundy, S. M., Hong, Y., Miller, N. H., Lauer, R. M., Ockene, I. S., Sacco, R. L., Sallis, J. F., Jr., Smith, S. C., Jr., Stone, N. J., and Taubert, K. A. (2002) AHA Guidelines for Primary Prevention of Cardiovascular Disease and Stroke: 2002 Update: Consensus Panel Guide to Comprehensive Risk Reduction for Adult Patients Without Coronary or Other Atherosclerotic Vascular Diseases. American Heart Association Science Advisory and Coordinating Committee. *Circulation*, **106**, 388-91.

Re, R. N. (1988) Emerging issues in the cellular biology of the cardiovascular system. *Am J Cardiol*, **62**, 7G-12G.

Re, R. N. (2002) The origins of intracrine hormone action. *Am J Med Sci*, **323**, 43-8.

Rebeck, G. W. (2004) Cholesterol efflux as a critical component of Alzheimer's disease pathogenesis. *J Mol Neurosci*, **23**, 219-24.

Reid, P. C., Sugii, S., and Chang, T. Y. (2003) Trafficking defects in endogenously synthesized cholesterol in fibroblasts, macrophages, hepatocytes, and glial cells from Niemann-Pick type C1 mice. *J Lipid Res*, **44**, 1010-9. Epub 2003 Mar 1.

Remaley, A. T., Stonik, J. A., Demosky, S. J., Neufeld, E. B., Bocharov, A. V., Vishnyakova, T. G., Eggerman, T. L., Patterson, A. P., Duverger, N. J., Santamarina-Fojo, S., and Brewer, H. B., Jr. (2001) Apolipoprotein specificity for lipid efflux by the human ABCAI transporter. *Biochem Biophys Res Commun*, **280**, 818-23.

Restrepo, C., and Tracy, R. E. (1975) Variations in human aortic fatty streaks among geographic locations. *Atherosclerosis*, **21**, 179-93.

Rinninger, F., Brundert, M., Jackle, S., Kaiser, T., and Greten, H. (1995) Selective uptake of low-density lipoprotein-associated cholesteryl esters by human fibroblasts, human HepG2 hepatoma cells and J774 macrophages in culture. *Biochim Biophys Acta*, **1255**, 141-53.

Rinninger, F., and Greten, H. (1990) High-density lipoprotein particle uptake and selective uptake of high-density lipoprotein-associated cholesteryl esters by J774 macrophages. *Biochim Biophys Acta*, **1043**, 318-26.

Robenek, H., and Schmitz, G. (1988) Ca++ antagonists and ACAT inhibitors promote cholesterol efflux from macrophages by different mechanisms. II. Characterization of intracellular morphologic changes. *Arteriosclerosis*, **8**, 57-67.

Rodriguez, A., Bachorik, P. S., and Wee, S. B. (1999) Novel effects of the acyl-coenzyme A:Cholesterol acyltransferase inhibitor 58-035 on foam cell

development in primary human monocyte-derived macrophages. *Arterioscler Thromb Vasc Biol*, **19**, 2199-206.

Rohrer, L., Hersberger, M., and von Eckardstein, A. (2004) High density lipoproteins in the intersection of diabetes mellitus, inflammation and cardiovascular disease. *Curr Opin Lipidol*, **15**, 269-78.

Rosenfeld, M. E., Yla-Herttuala, S., Lipton, B. A., Ord, V. A., Witztum, J. L., and Steinberg, D. (1992) Macrophage colony-stimulating factor mRNA and protein in atherosclerotic lesions of rabbits and humans. *Am J Pathol*, **140**, 291-300.

Ross, R. (1999) Atherosclerosis--an inflammatory disease. *N Engl J Med*, **340**, 115-26.

Rothblat, G. H., de la Llera-Moya, M., Atger, V., Kellner-Weibel, G., Williams, D. L., and Phillips, M. C. (1999) Cell cholesterol efflux: integration of old and new observations provides new insights. *J Lipid Res*, **40**, 781-96.

Rothblat, G. H., de la Llera-Moya, M., Favari, E., Yancey, P. G., and Kellner-Weibel, G. (2002) Cellular cholesterol flux studies: methodological considerations. *Atherosclerosis*, **163**, 1-8.

Rothblat, G. H., and Phillips, M. C. (1982) Mechanism of cholesterol efflux from cells. Effects of acceptor structure and concentration. *J Biol Chem*, **257**, 4775-82.

Rothblat, G. H., and Phillips, M. C. (1986) Cholesterol efflux: mechanism and regulation. *Adv Exp Med Biol*, **201**, 195-204.

Rumsey, S. C., Galeano, N. F., Lipschitz, B., and Deckelbaum, R. J. (1995) Oleate and other long chain fatty acids stimulate low density lipoprotein receptor activity by enhancing acyl coenzyme A:cholesterol acyltransferase activity and altering intracellular regulatory cholesterol pools in cultured cells. *J Biol Chem*, **270**, 10008-16.

Saito, H., Dhanasekaran, P., Nguyen, D., Baldwin, F., Weisgraber, K. H., Wehrli, S., Phillips, M. C., and Lund-Katz, S. (2003) Characterization of the heparin binding sites in human apolipoprotein E. *J Biol Chem*, **278**, 14782-7. Epub 2003 Feb 14.

Saito, H., Lund-Katz, S., and Phillips, M. C. (2004) Contributions of domain structure and lipid interaction to the functionality of exchangeable human apolipoproteins. *Prog Lipid Res*, **43**, 350-80.

Sakashita, N., Miyazaki, A., Chang, C. C., Chang, T. Y., Kiyota, E., Satoh, M., Komohara, Y., Morganelli, P. M., Horiuchi, S., and Takeya, M. (2003) Acyl-coenzyme A:cholesterol acyltransferase 2 (ACAT2) is induced in monocyte-derived macrophages: in vivo and in vitro studies. *Lab Invest*, **83**, 1569-81.

Sakashita, N., Miyazaki, A., Takeya, M., Horiuchi, S., Chang, C. C., Chang, T. Y., and Takahashi, K. (2000) Localization of human acyl-coenzyme A: cholesterol

acyltransferase-1 (ACAT-1) in macrophages and in various tissues. *Am J Pathol*, **156**, 227-36.

Savill, J., and Fadok, V. (2000) Corpse clearance defines the meaning of cell death. *Nature*, **407**, 784-8.

Schmitz, G., Kaminski, W. E., and Orso, E. (2000) ABC transporters in cellular lipid trafficking. *Curr Opin Lipidol*, **11**, 493-501.

Schmitz, G., Kaminski, W. E., Porsch-Ozcurumez, M., Klucken, J., Orso, E., Bodzioch, M., Buchler, C., and Drobnik, W. (1999) ATP-binding cassette transporter A1 (ABCA1) in macrophages: a dual function in inflammation and lipid metabolism? *Pathobiology*, **67**, 236-40.

Schroeder, F., Gallegos, A. M., Atshaves, B. P., Storey, S. M., McIntosh, A. L., Petrescu, A. D., Huang, H., Starodub, O., Chao, H., Yang, H., Frolov, A., and Kier, A. B. (2001) Recent advances in membrane microdomains: rafts, caveolae, and intracellular cholesterol trafficking. *Exp Biol Med (Maywood)*, **226**, 873-90.

Schwartz, C. C., Halloran, L. G., Vlahcevic, Z. R., Gregory, D. H., and Swell, L. (1978) Preferential utilization of free cholesterol from high-density lipoproteins for biliary cholesterol secretion in man. *Science*, **200**, 62-4.

Schwartz, K., Lawn, R. M., and Wade, D. P. (2000) ABC1 gene expression and ApoA-I-mediated cholesterol efflux are regulated by LXR. *Biochem Biophys Res Commun*, **274**, 794-802.

Segrest, J. P., Jones, M. K., De Loof, H., Brouillette, C. G., Venkatachalapathi, Y. V., and Anantharamaiah, G. M. (1992) The amphipathic helix in the exchangeable apolipoproteins: a review of secondary structure and function. *J Lipid Res*, **33**, 141-66.

Segrest, J. P., Jones, M. K., Klon, A. E., Sheldahl, C. J., Hellinger, M., De Loof, H., and Harvey, S. C. (1999) A detailed molecular belt model for apolipoprotein A-I in discoidal high density lipoprotein. *J Biol Chem*, **274**, 31755-8.

Segrest, J. P., Li, L., Anantharamaiah, G. M., Harvey, S. C., Liadaki, K. N., and Zannis, V. (2000) Structure and function of apolipoprotein A-I and high-density lipoprotein. *Curr Opin Lipidol*, **11**, 105-15.

Seo, T., Oelkers, P. M., Giattina, M. R., Worgall, T. S., Sturley, S. L., and Deckelbaum, R. J. (2001) Differential modulation of ACAT1 and ACAT2 transcription and activity by long chain free fatty acids in cultured cells. *Biochemistry*, **40**, 4756-62.

Shimano, H., Fukazawa, C., Shibasaki, Y., Mori, N., Gotoda, T., Harada, K., Shimada, M., Yamada, N., Yazaki, Y., and Takaku, F. (1991) The effect of apo E secretion on lipoprotein uptake in transfected cells. *Biochim Biophys Acta*, **1086**, 245-54.

Shiratori, Y., Okwu, A. K., and Tabas, I. (1994) Free cholesterol loading of macrophages stimulates phosphatidylcholine biosynthesis and up-regulation of CTP: phosphocholine cytidylyltransferase. *J Biol Chem*, **269**, 11337-48.

Shvartsman, S. Y., Wiley, H. S., Deen, W. M., and Lauffenburger, D. A. (2001) Spatial range of autocrine signaling: modeling and computational analysis. *Biophys J*, **81**, 1854-67.

Simon, J. (2002) "Excel Programming: Your visual blueprint for creating interactive spreadsheets" (New York, Hungry Minds, Inc.).

Soccio, R. E., and Breslow, J. L. (2004) Intracellular cholesterol transport. *Arterioscler Thromb Vasc Biol*, **24**, 1150-60. Epub 2004 May 6.

Sparrow, C. P., Baffic, J., Lam, M. H., Lund, E. G., Adams, A. D., Fu, X., Hayes, N., Jones, A. B., Macnaul, K. L., Ondeyka, J., Singh, S., Wang, J., Zhou, G., Moller, D. E., Wright, S. D., and Menke, J. G. (2002) A potent synthetic LXR agonist is more effective than cholesterol loading at inducing ABCA1 mRNA and stimulating cholesterol efflux. *J Biol Chem*, **277**, 10021-7.

Sparrow, S. M., Carter, J. M., Ridgway, N. D., Cook, H. W., and Byers, D. M. (1999) U18666A inhibits intracellular cholesterol transport and neurotransmitter release in human neuroblastoma cells. *Neurochem Res*, **24**, 69-77.

Spector, A. A., and Yorek, M. A. (1985a) Membrane lipid composition and cellular function. *J Lipid Res*, **26**, 1015-35.

Spector, A. A., and Yorek, M. A. (1985b) Membrane lipid composition and cellular function. *J Lipid Res*, **26**, 1015-35.

Sporn, M. B., and Roberts, A. B. (1992) Autocrine secretion--10 years later. *Ann Intern Med*, **117**, 408-14.

Steck, T. L., Kezdy, F. J., and Lange, Y. (1988) An activation-collision mechanism for cholesterol transfer between membranes. *J Biol Chem*, **263**, 13023-31.

Stubbs, C. D., and Smith, A. D. (1984) The modification of mammalian membrane polyunsaturated fatty acid composition in relation to membrane fluidity and function. *Biochim Biophys Acta*, **779**, 89-137.

Su, Y. R., Ishiguro, H., Major, A. S., Dove, D. E., Zhang, W., Hasty, A. H., Babaev, V. R., Linton, M. F., and Fazio, S. (2003) Macrophage apolipoprotein A-I expression protects against atherosclerosis in ApoE-deficient mice and up-regulates ABC transporters. *Mol Ther*, **8**, 576-83.

Su, Y. R., Linton, M. F., and Fazio, S. (2002) Rapid quantification of murine ABC mRNAs by real time reverse transcriptase-polymerase chain reaction. *J Lipid Res*, **43**, 2180-7.

Sugimoto, K., Tsujita, M., Wu, C. A., Suzuki, K., and Yokoyama, S. (2004) An inhibitor of acylCoA: cholesterol acyltransferase increases expression of ATP-binding cassette transporter A1 and thereby enhances the ApoA-I-mediated release of cholesterol from macrophages. *Biochim Biophys Acta*, **1636**, 69-76.

Swift, L. L., Farkas, M. H., Major, A. S., Valyi-Nagy, K., Linton, M. F., and Fazio, S. (2001) A recycling pathway for resecretion of internalized apolipoprotein E in liver cells. *J Biol Chem*, **276**, 22965-70. Epub 2001 Apr 13.

Tabas, I. (2002) Consequences of cellular cholesterol accumulation: basic concepts and physiological implications. *J Clin Invest*, **110**, 905-11.

Tabas, I., Rosoff, W. J., and Boykow, G. C. (1988) Acyl coenzyme A:cholesterol acyl transferase in macrophages utilizes a cellular pool of cholesterol oxidase-accessible cholesterol as substrate. *J Biol Chem*, **263**, 1266-72.

Tabas, I., Weiland, D. A., and Tall, A. R. (1986) Inhibition of acyl coenzyme A:cholesterol acyl transferase in J774 macrophages enhances down-regulation of the low density lipoprotein receptor and 3-hydroxy-3-methylglutaryl-coenzyme A reductase and prevents low density lipoprotein-induced cholesterol accumulation. *J Biol Chem*, **261**, 3147-55.

Tack, G., Roselli, R. J., Overholser, K. A., and Harris, T. R. (1995) The use of microsoft excel as a user interface for biological simulations. *Comput Biomed Res*, **28**, 24-37.

Takahashi, S., Sakai, J., Fujino, T., Miyamori, I., and Yamamoto, T. T. (2003) The very low density lipoprotein (VLDL) receptor--a peripheral lipoprotein receptor for remnant lipoproteins into fatty acid active tissues. *Mol Cell Biochem*, **248**, 121-7.

Torra, I. P., Chinetti, G., Duval, C., Fruchart, J. C., and Staels, B. (2001) Peroxisome proliferator-activated receptors: from transcriptional control to clinical practice. *Curr Opin Lipidol*, **12**, 245-54.

Van Eck, M., De Winther, M. P., Herijgers, N., Havekes, L. M., Hofker, M. H., Groot, P. H., and Van Berkel, T. J. (2000a) Effect of human scavenger receptor class A overexpression in bone marrow-derived cells on cholesterol levels and atherosclerosis in ApoE-deficient mice. *Arterioscler Thromb Vasc Biol*, **20**, 2600-6.

Van Eck, M., Herijgers, N., Van Dijk, K. W., Havekes, L. M., Hofker, M. H., Groot, P. H., and Van Berkel, T. J. (2000b) Effect of macrophage-derived mouse ApoE, human ApoE3-Leiden, and human ApoE2 (Arg158-->Cys) on cholesterol levels and atherosclerosis in ApoE-deficient mice. *Arterioscler Thromb Vasc Biol*, **20**, 119-27.

Van Eck, M., Herijgers, N., Yates, J., Pearce, N. J., Hoogerbrugge, P. M., Groot, P. H., and Van Berkel, T. J. (1997) Bone marrow transplantation in apolipoprotein E-

deficient mice. Effect of ApoE gene dosage on serum lipid concentrations, (beta)VLDL catabolism, and atherosclerosis. *Arterioscler Thromb Vasc Biol*, **17**, 3117-26.

Van Eck, M., Zimmermann, R., Groot, P. H., Zechner, R., and Van Berkel, T. J. (2000c) Role of macrophage-derived lipoprotein lipase in lipoprotein metabolism and atherosclerosis. *Arterioscler Thromb Vasc Biol*, **20**, E53-62.

van Vlijmen, B. J., Gerritsen, G., Franken, A. L., Boesten, L. S., Kockx, M. M., Gijbels, M. J., Vierboom, M. P., van Eck, M., van De Water, B., van Berkel, T. J., and Havekes, L. M. (2001) Macrophage p53 deficiency leads to enhanced atherosclerosis in APOE*3-Leiden transgenic mice. *Circ Res*, **88**, 780-6.

Vaughan, A., Guilbault, G. G., and Hackney, D. (1971) Fluorometric methods for analysis of acid and alkaline phosphatase. *Anal Chem*, **43**, 721-4.

Von Eckardstein, A., Langer, C., Engel, T., Schaukal, I., Cignarella, A., Reinhardt, J., Lorkowski, S., Li, Z., Zhou, X., Cullen, P., and Assmann, G. (2001) ATP binding cassette transporter ABCA1 modulates the secretion of apolipoprotein E from human monocyte-derived macrophages. *Faseb J*, **15**, 1555-61.

von Eckardstein, A., Schulte, H., and Assmann, G. (2000) Risk for diabetes mellitus in middle-aged Caucasian male participants of the PROCAM study: implications for the definition of impaired fasting glucose by the American Diabetes Association. Prospective Cardiovascular Munster. *J Clin Endocrinol Metab*, **85**, 3101-8.

Wang, N., Silver, D. L., Thiele, C., and Tall, A. R. (2001) ATP-binding cassette transporter A1 (ABCA1) functions as a cholesterol efflux regulatory protein. *J Biol Chem*, **276**, 23742-7.

Wang, S., and Tarbell, J. M. (2000) Effect of fluid flow on smooth muscle cells in a 3-dimensional collagen gel model. *Arterioscler Thromb Vasc Biol*, **20**, 2220-5.

Wang, X., Briggs, M. R., Hua, X., Yokoyama, C., Goldstein, J. L., and Brown, M. S. (1993) Nuclear protein that binds sterol regulatory element of low density lipoprotein receptor promoter. II. Purification and characterization. *J Biol Chem*, **268**, 14497-504.

Wang, Y., and Oram, J. F. (2002) Unsaturated fatty acids inhibit cholesterol efflux from macrophages by increasing degradation of ATP-binding cassette transporter A1. *J Biol Chem*, **277**, 5692-7.

Warner, G. J., Stoudt, G., Bamberger, M., Johnson, W. J., and Rothblat, G. H. (1995) Cell toxicity induced by inhibition of acyl coenzyme A:cholesterol acyltransferase and accumulation of unesterified cholesterol. *J Biol Chem*, **270**, 5772-8.

Webb, N. R., Bostrom, M. A., Szilvassy, S. J., van der Westhuyzen, D. R., Daugherty, A., and de Beer, F. C. (2003) Macrophage-expressed group IIA secretory

phospholipase A2 increases atherosclerotic lesion formation in LDL receptor-deficient mice. *Arterioscler Thromb Vasc Biol*, **23**, 263-8.

Weibel, E. R., Kistler, G. S., and Scherle, W. F. (1966) Practical stereological methods for morphometric cytology. *J Cell Biol*, **30**, 23-38.

Weisgraber, K. H., Innerarity, T. L., and Mahley, R. W. (1982) Abnormal lipoprotein receptor-binding activity of the human E apoprotein due to cysteine-arginine interchange at a single site. *J Biol Chem*, **257**, 2518-21.

Weisgraber, K. H., Mahley, R. W., and Assmann, G. (1977) The rat arginine-rich apoprotein and its redistribution following injection of iodinated lipoproteins into normal and hypercholesterolemic rats. *Atherosclerosis*, **28**, 121-40.

Werb, Z., and Cohn, Z. A. (1972) Cholesterol metabolism in the macrophage. 3. Ingestion and intracellular fate of cholesterol and cholesterol esters. *J Exp Med*, **135**, 21-44.

Wilkinson, C. F., Jr. (1950) Essential familial hypercholesterolemia: cutaneous, metabolic and hereditary aspects. *Bull N Y Acad Med*, **26**, 670-85.

Wilsie, L. C., and Orlando, R. A. (2003) The low density lipoprotein receptor-related protein complexes with cell surface heparan sulfate proteoglycans to regulate proteoglycan-mediated lipoprotein catabolism. *J Biol Chem*, **278**, 15758-64. Epub 2003 Feb 21.

Wilson, P. W., Abbott, R. D., and Castelli, W. P. (1988) High density lipoprotein cholesterol and mortality. The Framingham Heart Study. *Arteriosclerosis*, **8**, 737-41.

Worgall, T. S., Sturley, S. L., Seo, T., Osborne, T. F., and Deckelbaum, R. J. (1998) Polyunsaturated fatty acids decrease expression of promoters with sterol regulatory elements by decreasing levels of mature sterol regulatory element-binding protein. *J Biol Chem*, **273**, 25537-40.

Yancey, P. G., de la Llera-Moya, M., Swarnakar, S., Monzo, P., Klein, S. M., Connelly, M. A., Johnson, W. J., Williams, D. L., and Rothblat, G. H. (2000) High density lipoprotein phospholipid composition is a major determinant of the bi-directional flux and net movement of cellular free cholesterol mediated by scavenger receptor BI. *J Biol Chem*, **275**, 36596-604.

Yancey, P. G., and Jerome, W. G. (1998) Lysosomal sequestration of free and esterified cholesterol from oxidized low density lipoprotein in macrophages of different species. *J Lipid Res*, **39**, 1349-61.

Yancey, P. G., and Jerome, W. G. (2001) Lysosomal cholesterol derived from mildly oxidized low density lipoprotein is resistant to efflux. *J Lipid Res*, **42**, 317-27.

Yancey, P. G., Rodrigueza, W. V., Kilsdonk, E. P., Stoudt, G. W., Johnson, W. J., Phillips, M. C., and Rothblat, G. H. (1996) Cellular cholesterol efflux mediated by cyclodextrins. Demonstration Of kinetic pools and mechanism of efflux. *J Biol Chem*, **271**, 16026-34.

Yoshida, H., Hasty, A. H., Major, A. S., Ishiguro, H., Su, Y. R., Gleaves, L. A., Babaev, V. R., Linton, M. F., and Fazio, S. (2001) Isoform-specific effects of apolipoprotein E on atherogenesis: gene transduction studies in mice. *Circulation*, **104**, 2820-5.

Zhang, W., Yancey, P. G., Su, Y. R., Babaev, V. R., Zhang, Y., Fazio, S., and Linton, M. F. (2003a) Inactivation of macrophage scavenger receptor class B type I promotes atherosclerotic lesion development in apolipoprotein E-deficient mice. *Circulation*, **108**, 2258-63. Epub 003 Oct 27.

Zhang, W. Y., Gaynor, P. M., and Kruth, H. S. (1996) Apolipoprotein E produced by human monocyte-derived macrophages mediates cholesterol efflux that occurs in the absence of added cholesterol acceptors. *J Biol Chem*, **271**, 28641-6.

Zhang, Y., Yu, C., Liu, J., Spencer, T. A., Chang, C. C., and Chang, T. Y. (2003b) Cholesterol is superior to 7-ketocholesterol or 7 alpha-hydroxycholesterol as an allosteric activator for acyl-coenzyme A:cholesterol acyltransferase 1. *J Biol Chem*, **278**, 11642-7. Epub 2003 Jan 17.

Zhang, Y., Zanotti, I., Reilly, M. P., Glick, J. M., Rothblat, G. H., and Rader, D. J. (2003c) Overexpression of apolipoprotein A-I promotes reverse transport of cholesterol from macrophages to feces in vivo. *Circulation*, **108**, 661-3. Epub 2003 Aug 4.