PKA Signaling in ABCA1 Function: A Role in Modulation of Cholesterol Efflux and Macrophage Inflammation

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Abstract

Formation of lipid-laden macrophage foam cells and inflammation are the central components in the initiation and progression of atherosclerosis. ABCA1 is well established as an anti-atherogenic factor that facilitates cellular cholesterol and phospholipid efflux, promotes reverse cholesterol transport, and suppresses pro-inflammatory cytokine secretion. Through these functions, ABCA1 is capable of reducing the lipid burden in atherosclerotic plaque. PKA signaling is an integral factor in promoting many anti-atherogenic functions of ABCA1; however, mechanistic aspects of PKA signaling associated with ABCA1 remain poorly defined. Thus, the first part of this study investigates the involvement of spatially regulated PKA signaling in ABCA1 activities through the use of st-Ht31, a PKA de-anchoring peptide. It appears that de-anchoring PKA robustly increases ABCA1-mediated microparticle release, one of the cholesterol efflux pathways of ABCA1, and reverses macrophage foam cell formation. These results highlight the significance of subcellular compartmentalization of PKA signaling in ABCA1 functions and present PKA de-anchoring as a potential therapeutic strategy for atherosclerotic lesion regression. The second part of this study provides evidence that ABCA1 activates PKA and promotes the secretion of anti-inflammatory IL-10, a cytokine crucial for inflammation resolution. Furthermore, we provide evidence that this elevated PKA activity is the underlying mechanism in which macrophage ABCA1 promotes M2-like inflammatory response. Our results also suggest that ABCA1 activates PKA by regulating cholesterol, which poises macrophages towards an anti-inflammatory or M2-activated phenotype. Collectively, we demonstrate that PKA signaling plays a crucial multifactorial role in anti-atherogenic functions of ABCA1.

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List of Abbreviations

ABC	ATP-binding cassette
AC	adenylyl cyclase
ACAT	acyl-coenzyme A:cholesterol acyltransferase
AKAP	A-kinase anchoring protein
AP-1	activator protein-1
apo	apolipoprotein
apoA-I	apolipoprotein A-I
ATP	adenosine triphosphosphate
BHK	baby hamster kidney
BIG2	brefeldin A-inhibited guanine nucleotide exchange protein 2
BMDM	bone marrow-derived macrophage
Ca^{2+}	calcium
cAMP	cyclic adenosine monophosphate
CBP	CREB-binding protein
CE	cholesterol ester
CETP	cholesterol ester transfer protein
CFP	cyan fluorescent protein
CHD	coronary heart disease
CK2	casein kinase 2
CRE	cAMP response element
CREB	cAMP-response element-binding protein
CVD	cardiovascular disease
D/D	docking and dimerization
eATP	extracellular ATP
Epac	exchange protein activated by cAMP
ER	endoplasmic reticulum
FC	free cholesterol
FHD	familial HDL deficiency
HCBS	high capacity binding site
HDL	high density lipoprotein
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A
IDL	intermediate density lipoprotein
IFN-γ	interferon-γ
IL	interleukin
JAK2	janus kinase 2
LBP	LPS-binding protein
LCAT	lecithin:cholesterol acyltransferase
LD	lipid droplet
LDL	low density lipoprotein
LRP	LDL receptor related protein
LPS	lipopolysaccharide
LXR	liver X receptor
MCD	methyl-β-cyclodextrin

MCP-1	monocyte chemoattractant protein-1
MyD88	myeloid differentiation factor 88
oxLDL	oxidized LDL
NBD	nucleotide binding domain
NCoR	nuclear receptor co-repressor
NF-κB	nuclear factor-κB
NPC1	Niemann Pick C1
PAF-AH	platelet-activating factor acetyl hydrolase
PDE	phosphodiesterase
PL	phospholipids
PLTP	phospholipid transfer protein
PKA	protein kinase A
PKA-C	PKA catalytic subunit
PKA-R	PKA regulatory subunit
PKCα	protein kinase Cα
PKI	protein kinase inhibitor
PON-1	paraoxonase-1
PPAR	peroxisome proliferator-activated receptor
PS	phosphatidylserine
RCT	reverse cholesterol transport
RIAD	RI-Anchoring Disruptor
RXR	retinoic X receptor
SCAP	SREBP cleavage-activating protein
Ser	serine
SRA	scavenger receptor A
SMC	smooth muscle cell
SOCS3	suppressor of cytokine signaling 3
SR-BI	scavenger receptor protein BI
SRE	sterol response element
SREBP	sterol regulatory element-binding protein
st-Ht31	stearated-Ht31
STAT	signal transducer and activator of transcription
TG	triglycerides
Thr	threonine
TLR	toll-like receptor
TMD	transmembrane domains
TNF-α	tumour necrosis factor-α
VLDL	very low density lipoprotein
YFP	yellow fluorescent protein

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Chapter 1: Introduction

Cardiovascular disease (CVD) is currently the leading cause of mortality worldwide, particularly in industrialized countries. According to the latest data available (2008), approximately 30% of total deaths around the world were due to CVD, and the projected statistics indicate that the rate of mortality is only expected to rise by 2030 (1). The majority of these deaths are due to coronary heart disease (CHD) and stroke. Therefore, understanding the mechanisms governing the development of atherosclerosis and regulating cholesterol homeostasis are fundamental for developing therapeutic strategies to reduce the burden of CHD. Accumulation of lipids within macrophages is well known to be a critical cellular component of atherosclerotic lesions. There is an increasing body of evidence that supports the promotion of macrophage cholesterol efflux and reverse cholesterol transport as feasible anti-atherogenic strategies. Hence, intense research has been focused on the mechanisms governing cholesterol efflux from macrophages. A better knowledge of this process will facilitate the development of therapeutic interventions to treat and prevent the progression and complications of atherosclerotic vascular disease.

1.1 Atherosclerosis and Cholesterol Metabolism

In the early years of scientific and medical research on atherosclerosis, the etiology of atherosclerosis was highly debated upon, which at the time was considered to be simply a consequence of aging. In 1913, Anitschkow was the first to introduce the “lipid hypothesis”, which suggested a causal role for high cholesterol levels in atherosclerosis. In his ground-breaking study using a rabbit model, he demonstrated high-cholesterol feeding indeed induced vascular lesions that resembled those of human atherosclerosis (2). Despite this compelling evidence and a few decades’ worth of emerging studies (3), there was still strong scepticism about the “lipid hypothesis” at the time when the American Heart Association accepted the causal relationship in 1961 and recommended people at high risk to reduce cholesterol intake. Such scepticism remained until 1984, when the National Institutes of Health published the data from the Coronary Primary Prevention Trial, which, for the first time, clearly showed significant decrease in cardiovascular end points (i.e. CHD death and/or nonfatal heart attack) due to reduction in cholesterol levels (3). Since then, extensive

research has established the fundamentals of atherosclerosis, which indeed pinpoints cholesterol as a key regulatory component.

1.1.1 Cholesterol Metabolism

The normal cholesterol metabolism within the body can be characterized as a complex network of processes, which must be understood in order to assess the pathology of atherosclerosis. Cholesterol, a sterol, is an essential structural component in vertebrate cell membranes. Its planar structure and amphipathic nature enables it to fit perfectly in between phospholipids to stabilize the lateral interactions and, in turn, regulate the rigidity of the membrane bilayers and the assembly of lateral microdomains (4). Indeed, unesterified or free cholesterol (FC) is the main driving force for the formation of ordered microdomains such as lipid rafts, which is speculated to serve as platforms for cell signaling and membrane protein trafficking (5). For instance, these cholesterol-rich microdomains on the plasma membrane are critical in receptor-mediated endocytosis during the budding of clathrin-coated pits (6). The importance of cholesterol can be seen beyond the membrane, as it is also use as a precursor to many important metabolites, such as steroid hormones, vitamin D, and bile acids (7).

The body obtains cholesterol from two sources; *de novo* synthesis and absorption from the diet (8). While dietary absorption occurs in the gastrointestinal tract, essentially all cell types in the body are capable of synthesizing cholesterol with the majority being synthesized in extrahepatic organs (9). The liver contributes only about 10% of whole body cholesterol synthesis (10), and the rate of synthesis is dependent upon dietary cholesterol levels. Although most cells can satisfy their cholesterol requirements by endogenous cholesterol synthesis, cell types with higher demand for cholesterol, including hepatocytes and steroidogenic cells, have acquired mechanisms to take up dietary sources of cholesterol (11). Thus, dietary cholesterol absorbed from the intestine is needed to be transported to peripheral tissues and the liver via the circulation. As a highly lipophilic molecule, cholesterol is transported in the circulation by carriers, mostly lipoproteins that are composed of neutral lipid cores surrounded by a monolayer of free cholesterol and phospholipids and associated with apolipoproteins (apo).

Plasma lipoproteins can be divided into 5 classes: chylomicron, very low density lipoprotein (VLDL), intermediate density lipoprotein (IDL), low density lipoprotein (LDL),

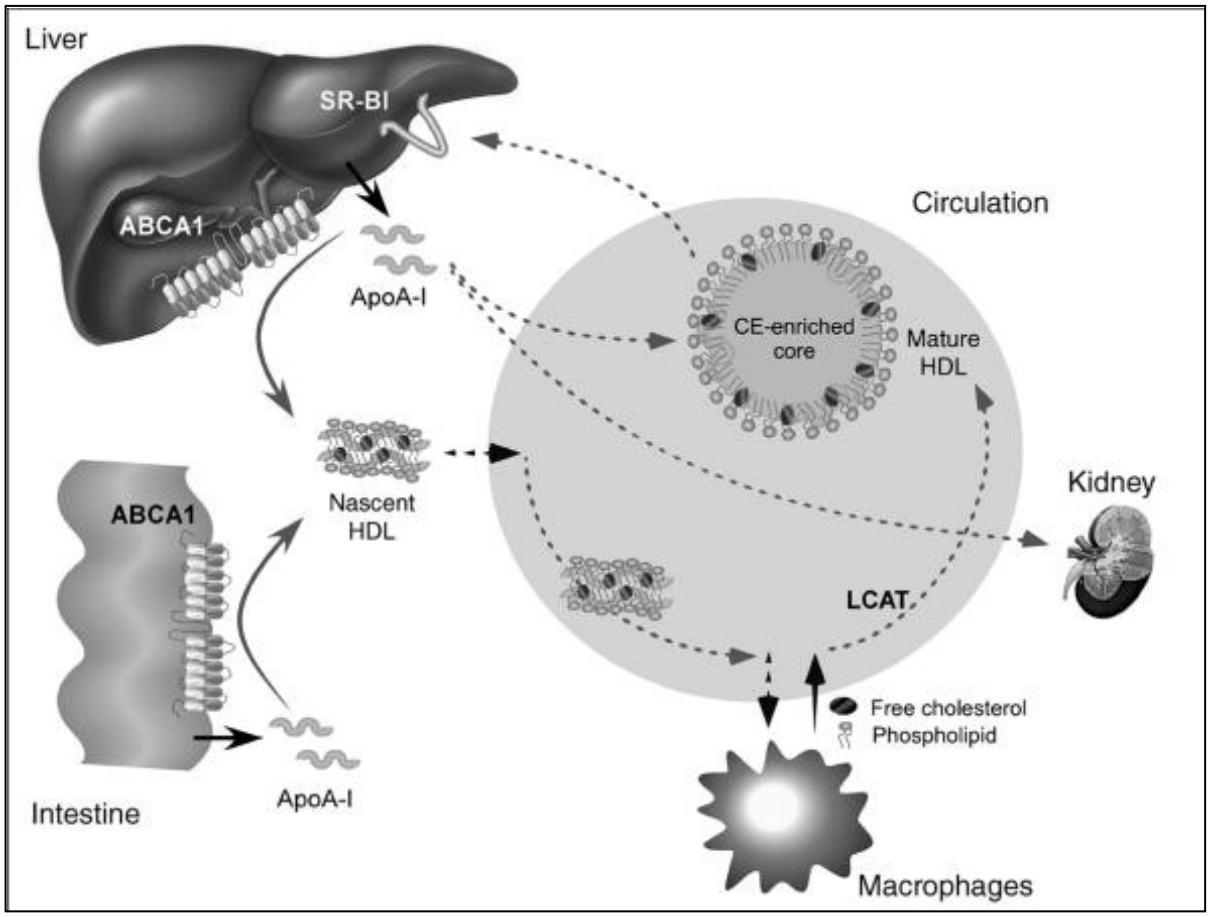
and high density lipoprotein (HDL). Each of these types of lipoproteins differs in lipid and protein composition and performs distinct functions. Chylomicrons supply muscle and adipose tissue with dietary triglycerides (TG) and cholesterol. The chylomicron remnants eventually end up in the liver. Simultaneously, the liver produces VLDL to deliver endogenously-synthesized lipids as well as lipids from chylomicron remnants to peripheral tissues, which provides a lipid supply between meals. As VLDL-associated TG is gradually depleted from the particles, VLDL converts to IDL, and then LDL, which is a major source of cholesterol for peripheral cells. Cells acquire cholesterol from these cholesterol-rich lipoproteins through receptor-mediated endocytosis involving the LDL receptor, apoE receptors, or scavenger receptors (12).

As mentioned above, cholesterol can be endogenously synthesized within most cells of the body, beginning with sequential condensation reactions of two-carbon acetyl-CoA molecules. Cholesterol biosynthesis is a complex pathway involving approximately 30 enzymatic reactions. The rate-limiting step is the conversion of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) to mevalonate, catalyzed by HMG-CoA reductase. This enzyme is currently a popular therapeutic target for treatment of hypercholesterolemia by a family of pharmacological agents termed statins. Statins competitively bind to the HMG-binding domain of the enzyme, thereby preventing HMG-CoA binding and mevalonate production. Highly-recognized work by Brown and Goldstein demonstrated that HMG-CoA reductase and other enzymes in this pathway are subject to a feedback regulation by cellular cholesterol content, which ensures proper levels of endogenous cholesterol synthesis, particularly when exogenous sources are scarce (13).

1.1.2 Reverse Cholesterol Transport

Unlike phospholipids (PLs) that are constantly turning over, cholesterol is relatively stable in most cells. Only a few cell types, such as those in the liver and steroidogenic tissues, are capable of metabolizing cholesterol. To maintain homeostasis, the body transports excess cholesterol from peripheral tissues back to the liver using the multi-step reverse cholesterol transport (RCT) process (**Figure 1.1**), which was first reported in 1968 by Dr. John Glomset (14). Briefly, the RCT process begins with HDL biosynthesis, in which apolipoprotein A-I (apoA-I), the major protein component on HDL, is synthesized by the liver and small intestine and secreted to the circulation as a lipid-free or lipid-poor form.

Figure 1.1: HDL formation and reverse cholesterol transport. ApoA-I is initially synthesized by the liver and small intestine in lipid-poor form and further acquire cholesterol and phospholipids from peripheral tissues (ie. macrophages) through ABCA1-mediated lipid efflux, generating nascent HDL particles. Lecithin:cholesterol acyltransferase (LCAT) converts free cholesterol within the HDL particles into neutral cholesterol esters, generating mature spherical HDL particles. Ultimately, mature HDLs deliver cholesterol to the liver via scavenger receptor SR-BI specific uptake for elimination as bile. Lipid free apoA-I in the circulation can associate with other HDLs or can be catabolized by the kidney. (Permission was obtained from publisher.)



Lee, Curr Opin Lipidol. 2005;16(1):19-25.

ApoA-I then acquires PLs and cholesterol from hepatocytes to generate minimally lipidated apoA-I or nascent HDL, in a process dependent on ATP-binding cassette (ABC) transporter A1 (ABCA1) (15). Once in the circulation, nascent HDL particles further acquire cholesterol and PLs from peripheral tissues including macrophages through cholesterol efflux facilitated by ABCA1. HDL-associated cholesterol is then esterified by lecithin:cholesterol acyltransferase (LCAT) to generate mature spherical HDL with a neutral lipid core of cholesterol esters (CEs) and TG. By doing so, spherical HDL can maintain lower concentrations of FC and PC than that of cell plasma membranes, increasing its capacity to accept lipids through concentration gradients. In addition, HDL particles can undergo remodelling by transferring PLs from apoB-containing lipoproteins to HDLs through PL transfer protein (PLTP) (17). Also, CE from can be exchanged between HDL and LDL by CE transfer protein (CETP) (18). Subsequently, clearance of cholesterol occurs in the liver by selective uptake of HDL cholesterol/phospholipid by scavenger receptor protein BI (SR-BI) or by LDL receptor-mediated uptake of LDL. Cholesterol in the liver is transported by bile in the form of bile acids and cholesterol, which can be eliminated in the feces, a process facilitated by ABCG5 and ABCG8 (19).

1.1.3 Pathology of Atherosclerosis

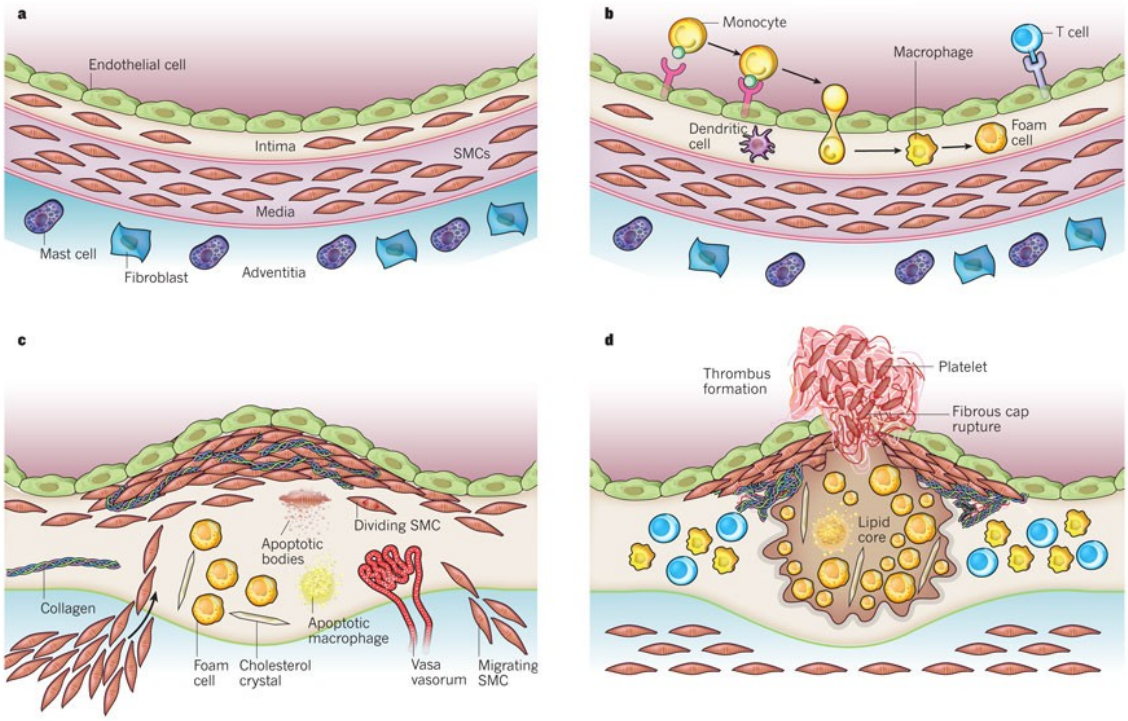
Archaeological studies have provided evidence demonstrating that ancient human populations had predominantly herbivorous diets. Many physical characteristics of a vegetarian organism, including teeth and jaw structures, and length of intestine, still remain with modern humans (7). As such, lipid metabolism is probably evolved to handle limited amounts of dietary cholesterol whereas endogenous cholesterol synthesis is fully capable of providing the body with adequate levels of cholesterol. Thus, modern humans, particularly in industrialized countries, have not yet biologically adapted to changes taken place in their diet and lifestyle. This maladaptation to the modern lifestyle has resulted in various chronic diseases, such as obesity, diabetes, and CHD. The average level of plasma LDL-cholesterol in populations on the Western diet is 5-fold higher than the postulated “appropriate” level (25 mg/dl) for humans (20). Genetic factors, including genetic mutations that impair various aspects of cholesterol metabolism, further increase the susceptibility to CHD. For instance, dysfunctional ABCA1 mutations can lead to a 6-fold higher than normal incidence of

cardiovascular disease (21). Epidemiological studies reveal an association between high plasma cholesterol levels, development of lesions, and severity of CHD (22).

The consequences of continually elevated body cholesterol have presented themselves with various health issues, including cholesterol gallstones and xanthomas, with the most severe case being atherosclerotic vascular disease (7). When plasma cholesterol levels, particularly the cholesterol associated with TG-rich lipoproteins, are persistently high, atherogenic lipoproteins gradually accumulate in the subendothelial regions of arterial walls. This retention of cholesterol-rich lipoproteins is a key initial event in early atherogenesis. Though rare, the existence of atherosclerosis can be traced back to civilizations (i.e. Egyptians) as early as 1580 B.C. (23). In the mid 19th century, Rokitansky and Virchow each proposed two opposing theories on the pathology of atherosclerosis, describing cellular inflammatory changes in atherosclerotic vessel walls (24). While Rokitansky viewed inflammation as secondary in intimal thickening derived from surface deposits, Virchow considered these inflammatory changes to have primary roles in atherogenesis. As the lipid hypothesis continued to evolve, a relationship between cholesterol levels and atherosclerosis developed, and Ross integrated the concepts into the response-to-injury hypothesis (25). According to this theory, oxidative modification and other factors may contribute to injury leading to endothelial dysfunction in the initial stages, which in turn increases permeability to lipoproteins and elicit an inflammatory response.

The development of atherosclerotic lesions occurs preferentially at arterial branch points and curvatures (26). These regions are associated with turbulent blood flow and irregular distribution of low shear stress, which impacts on endothelial integrity, resulting in accumulation of LDL within the intima. Extracellular proteoglycans facilitates LDL retention within the artery walls, whereas lipolytic enzymes (lipoprotein lipase, phospholipase A2, and sphingomyelinase) and oxidative agents (reactive oxygen species, myeloperoxidases, and lipoxygenases) induce lipoprotein modifications (27, 28). This, in turn, triggers an inflammatory response, leading to the recruitment of monocyte-derived macrophages that ingest modified LDL via a scavenger receptor pathway. As macrophages ingest large amounts of modified lipoproteins, they acquire a foam-like appearance, commonly referred to as foam cells, owning to the accumulation of cholesterol ester (CE)-rich lipid droplets. Early stages of lesions (**Figure 1.2**), referred to as fatty streaks, consist of abnormal

Figure 1.2: Stages of atherosclerotic lesion development. (a) The onset of atherosclerotic lesions can develop in regions of the arterial system where endothelial integrity is compromised, increasing permeability and retention of LDL within the intima. (b) Exposure of lipolytic and oxidative agents within the subendothelial layer generates modified LDL, which triggers inflammatory response and recruitment of immune cells, including monocytes. In early lesions, monocyte-derived macrophages internalize accumulated lipoproteins within the intima and develop into lipid-laden foam cells. (c) As atherosclerotic lesions progress to intermediate stages, macrophage foam cells undergo apoptosis and extracellular lipids accumulate in the lesions. The artery wall layers become disorganized with smooth muscle cells migrating into the intima and appearance of collagen and extracellular matrix components to form a fibrous cap. (d) In advanced lesions, substantial lipid deposits are present, forming a lipid core, and thinning of the fibrous cap can result in its rupture, which can cause complications, including thrombosis. (Permission was obtained from publisher.)



Libby et al., Nature, 2011; 473, 317–325.

accumulations of lipoproteins and cholesterol esters, most of which build up within lipid droplet filled-macrophage foam cells (22).

Initial changes in the intima frequently occur as early as infancy, with the presence of small isolated groups of macrophage foam cells. Early lesions can regress but can also continue to progress with increasing recruitment of cells, mainly macrophages but also smooth muscle cells (SMCs). As lesions transition from early to intermediate lesions, extracellular lipid droplets form among the layers of SMCs accompanied with intimal thickening. Beyond this stage is the development of advanced lesions, referred to as atheromas, which are characterized by a disorganized and deformed intima as a result of extracellular lipid accumulation, forming the lipid core (28). Subsequent progression involves formation of new fibrous tissue containing SMCs, collagen, and extracellular matrix components. This forms a fibrous cap over the lipid core that narrows the lumen of the artery. In late stages of these advanced lesions, complications can arise due to rupturing of the fibrous cap, which result in hematoma and thrombotic deposits, and in turn, trigger clinical events of CHD.

1.1.4 Macrophage Foam Cells

Macrophages play a critical role in all phases of atherosclerosis, from the initial stages of fatty streak development to processes that contribute to plaque rupture (29). Early lesions are composed primarily of macrophages, most of which contain large numbers of lipid droplets due to internalization of modified LDL. Modified LDL particles, mainly aggregated and oxidized LDL (oxLDL), trigger a cascade of proinflammatory events within the intima, including the release of pro-inflammatory factors such as monocyte chemoattractant protein-1 (MCP-1) and tumour necrosis factor (TNF)-α. Inflammatory mediators play central roles in promoting monocyte recruitment, production of oxidative agents linked to LDL oxidation, and increase production of matrix metalloproteinases that weaken the fibrous cap (29).

Macrophages are recruited to atherosclerotic lesions on the purpose of removing cholesterol deposits and thus alleviating the lipid burden in the intima. However, under persistent high plasma cholesterol in the circulation, macrophages become overwhelmed by the accumulation of lipid droplets and develop into foam cells. This leads to disease progression instead. Although lipoprotein uptake can occur through phagocytosis or

receptor-mediated uptake of native lipoproteins, the capacity of macrophages to accumulate large amounts of lipid is largely due to scavenger receptors. Lesional macrophages express more than six structurally distinct scavenger receptors that bind and internalize modified lipoproteins in an unregulated manner, with scavenger receptor A (SRA) and CD36 responsible for the majority of cholesterol uptake (30). Homeostatic mechanisms are also disrupted in foam cells, such as dampening cellular cholesterol export by ABCA1, thus further promoting cholesterol accumulation (31). As atherosclerotic lesions become more advanced, the FC:CE ratio increases in foam cells, most likely due to compromised abilities to esterify cholesterol as well as efflux cholesterol. This triggers programmed cell death via the induction of endoplasmic reticulum (ER) stress-UPR-CHOP-induced apoptosis, which in turn contributes to rupture-vulnerable atherosclerotic plaques (32).

1.2 Cholesterol Homeostasis

Although critically important, cholesterol in excessive amounts is harmful to the body as seen in atherosclerosis, and its cellular accumulation also generates cytotoxic effects in cells. This is partially due to the fact that, unlike phospholipids, cholesterol is not readily metabolized in most cell types other than the liver and steroidogenic tissues. On the cellular level, excess membrane cholesterol may result in toxic effects due to the loss of membrane fluidity, disruption of membrane domains, intracellular crystallization of cholesterol, and the induction of apoptosis (11). Cholesterol homeostasis hence must be tightly maintained. Thus, cells have acquired intricate mechanisms to regulate the abundance and distribution of cellular cholesterol (33). Sterol regulatory element-binding proteins (SREBPs) and nuclear receptor transcription factors plays a central role in maintaining cholesterol homeostasis by regulating the expression of several genes implicated in cholesterol metabolism.

1.2.1 SREBP transcription factors

The SREBPs belong to the basic helix-loop-helix–leucine zipper family of transcription factors that regulate the activation of genes involved in lipogenesis, and cholesterol and phospholipid metabolism (13). There are two human SREBP genes that generate three distinct isoforms, SREBP-1a, -1c, and 2. SREBP-1a and SREBP-1c are produced through alternative splicing of a single gene, *SREBF1*, resulting in distinct N-terminal domains. SREBP-1c is highly expressed in the liver, adipose tissue, skeletal muscle, and adrenal

glands, whereas SREBP-1a is predominantly expressed in highly proliferative tissues, such as spleen and intestine. SREBP-2, ubiquitously expressed, is derived from *SREBF2* gene and shares only 45% homology with SREBP-1a. SREBPs are synthesized in their inactive precursor forms bound to the membranes of the ER, and they require proteolytic processing to activate their transcription activity.

SREBPs are tightly associated with the SREBP cleavage-activating protein (SCAP), which escorts SREBP from the ER to Golgi where it undergoes proteolytic cleavage. The processing of SREBP is regulated by cellular cholesterol content. When cellular cholesterol is low, SCAP escorts SREBP from the ER to the Golgi, and SREBP is subsequently cleaved by site-1 and site-2 proteases. This releases the N-terminal soluble mature SREBP to translocate to the nucleus, bind sterol response elements (SREs) present on promoters of target genes involved in cholesterol synthesis and uptake and lipogenesis, and activate their transcription. When cellular cholesterol is high, cholesterol binds to the sterol-sensing domain of SCAP and alters the conformation of SCAP, enabling it to interact with ER resident proteins, Insig1 and Insig2. This retains SCAP/SREBP complex in the ER, thus preventing SREBP processing and lipogenesis (34).

SREBP-1a and SREBP-1c primarily induce lipogenic genes involved in the regulation of fatty acid synthesis, including acetyl-CoA carboxylase, fatty acid synthase, and stearoyl-CoA desaturase-1. In contrast, SREBP-2 preferentially activates genes involved in cholesterol synthesis, such as HMG-CoA reductase, HMG-CoA synthase, and squalene synthase. In addition to the regulation of cholesterol synthesis, SREBP-2 also controls cholesterol uptake by regulating LDL receptor expression (13). Moreover, SREBP-2 is simultaneously transcribed with the intronic microRNA, miR-33, encoded within the *SREBF2* gene, which modulate the expression of cellular cholesterol export machinery, including ABCA1, ABCG1, and Niemann Pick C1 (NPC1) (35).

1.2.2 Nuclear Receptors: PPAR and LXR

Nuclear receptors also have crucial functions in the regulation of lipid metabolism, particularly peroxisome proliferator-activated receptor (PPAR) and liver X receptor (LXR) subfamilies. PPARs and LXRs are ligand-activated transcription factors that, upon ligand binding, heterodimerize with retinoic X receptors (RXRs) and bind to their corresponding response elements in enhancer regions of their target genes (36). There are three PPAR

subtypes: PPARα, PPARβ/δ, and PPARγ, which can be activated by fatty acid metabolites and synthetic ligands, such as fibrates (used to treat dyslipidemia) for PPARα and thiazolidinediones (an anti-diabetic drug) for PPARγ. PPARs are expressed in all major cell types of atherosclerotic lesions; however, PPARα and PPARγ are primarily implicated in the development of atherosclerosis (37). PPARα- and PPARγ-specific agonists significantly inhibit lesion development in LDL receptor deficient mice on a high cholesterol diet. Recently, PPARβ/δ agonists have been demonstrated to attenuate VLDL-stimulated macrophage foam cell formation and suppress pro-inflammatory response (38). Both PPARs and LXRs can negatively regulate inflammatory genes through a ligand-induced SUMOylation-dependent manner (36). This leads to nuclear receptor association with the nuclear receptor co-repressor (NCoR) complex and binding to nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) sites on the promoters, which transrepress pro-inflammatory target genes.

Similar to PPARs, both LXR isoforms, LXRα and LXRβ, modulate lipid metabolism and inflammation. Each isoform has distinct tissue distributions: LXRβ is ubiquitously expressed, whereas LXRα is restricted to the intestine, fat tissue, lung, kidney, macrophage, and most abundantly in the liver (39). LXRs play an important physiological role as a sensor of elevated cellular cholesterol. They activate genes involved in reverse cholesterol transport (i.e. ABCA1, ABCG1, apoE) and bile acid synthesis (cholesterol 7α-hydroxylase), indicative of an anti-atherogenic role. While double knockout of LXRα/β in mice accelerate atherosclerosis, synthetic LXR agonists leads to regression of atherosclerotic lesions (39). Synthetic LXR agonists have been proposed as potential treatment of atherosclerosis; however, promoting LXR activation is accompanied by adverse effects of hepatic and plasma TG accumulation due to induction of SREBP-1c (13). Therefore, developing tissue selective LXR agonists lacking LXR stimulation in the liver is required to minimize their undesirable effects.

Although originally identified as an orphan receptor, the physiological LXR ligands have been determined to be specific oxysterols and sterols (40). Several oxysterols, such as 7α-hydroxycholesterol, 27-hydroxycholesterol, and 22(R)-hydroxycholesterol, arise from cytochrome P-450-mediated oxygenations of cholesterol and are involved in bile acid biosynthesis and steroid hormone synthesis (40). In contrast, 24(S),25-epoxycholesterol is

produced through a shunt pathway in cholesterol biosynthesis. More recently, desmosterol, the last intermediate in the Bloch pathway of cholesterol biosynthesis, is also a major LXR ligand (41). Their capacity to activate LXRs varies. For instance, the binding affinity of desmosterol to LXR is one-fifth that of 24(S),25-epoxycholesterol, indicating that 24(S),25-epoxycholesterol is a more potent LXR ligand. Furthermore, 22(R)-hydroxycholesterol induces lipogenesis via increasing SREBP-1c activation, whereas TG accumulation was not observed with 24(S),25-epoxycholesterol (42). Thus, enhancing 24(S),25-epoxycholesterol to upregulate LXR-responsive genes involved in RCT without stimulating lipogenesis provides a promising therapeutic strategy against atherosclerosis.

1.2.3 Cellular Cholesterol Esterification and Hydrolysis (Cholesterol Ester Cycle)

In addition to modulating cellular cholesterol synthesis, uptake and removal, most vertebrate cells also have limited capacity to buffer excess cholesterol as protection against cholesterol toxicity. Most commonly, excess FC in cells is esterified into CE by an ER-resident protein, acyl-coenzyme A:cholesterol acyltransferase (ACAT). In mammals, there are two isoenzymes of ACAT, ACAT1 and ACAT2, encoded by separate genes. ACAT1 is ubiquitously expressed in most tissues, while ACAT2 is the major isoenzyme in the liver and intestine (43). ACAT can catalyze both endogenously synthesized FC and lipoprotein-derived FC and, subsequently, the resultant CE is stored in lipid droplets (LDs). LDs are comprised of a neutral lipid core of CE and TG surrounded by a single layer of phospholipids associated with specific LD coat proteins (44).

FC can also be produced from LDs through CE hydrolysis, through processes mediated by cytoplasmic neutral CE hydrolases (such as carboxylesterase 1 and neutral cholesterol ester hydrolase 1) and lysosomal acid lipase, respectively (45). Overall, cholesterol is continually undergoing cycles of esterification and hydrolysis to maintain a dynamic reservoir of cytosolic pool of cholesterol (46). This relatively mobile pool of cholesterol is normally maintained at a steady state with membrane-associated cholesterol but can readily be used for steroid synthesis in steroidogenic tissues and for cholesterol efflux in macrophages. For cholesterol efflux, one of the rate-limiting factors is the hydrolysis of LD-associated CE (45). In the presence of extracellular cholesterol acceptors, such as apoA-I, the cytosolic pool of cholesterol is rapidly depleted due to efflux. This reduces the cholesterol available for ACAT and decreases CE production. As CE hydrolysis

is unaltered by the presence of apoA-I, there is a net increase in CE hydrolysis and hence the eventual elimination of LD. Thus, reagents that promote cholesterol mobilization from LDs to favour cholesterol efflux in macrophage foam cells can potentially serve as novel therapeutic strategies to reduce lipid burden in atherosclerotic plaques.

1.2.4 Therapeutic Approaches to Atherosclerosis

Although CHD remains as the leading cause of death worldwide, the currently available drug therapies are still unable to prevent approximately 70% of CHD-related events including strokes and myocardial infarctions, which highlight the urgency of advancements in treating clinical atherosclerosis (31). Several different aspects of cholesterol metabolism, especially plasma LDL and HDL, have been the main focuses of intervention for preventing and treating CHD (47). HMG-CoA reductase inhibitors (statins) are currently the main drugs of choice to treat elevated plasma LDL concentrations. Reduction of LDL concentrations continue to be an attractive strategy, as seen with other approaches including minimizing LDL receptor degradation, inhibiting lipolysis in adipose tissue to reduce apoB-containing lipoproteins (niacin), and lowering bile acid concentration (bile acid-binding resins) (47).

In contrast to LDL cholesterol, HDL cholesterol is inversely correlated with atherosclerosis (48). HDL is considered an important atheroprotective factor, making it an attractive therapeutic target. However, raising HDL by treatment with the CETP inhibitor torcetrapib resulted in the unexpected and premature termination of phase III clinical trials (48). This was due to adverse effects unrelated to CETP inhibition that increased mortality rates. Thus, other approaches to increase HDL, such as promoting macrophage cholesterol efflux and RCT, have received more attention. LXR agonists are currently under development as an approach to accelerate RCT as well as elicit anti-inflammatory effects to treat atherosclerosis. Interestingly, HDL particles also have anti-inflammatory effects that contribute to their antiatherogenic properties (18). This is partly due to the antioxidant enzymes, including paraoxonase-1 (PON-1), and platelet-activating factor acetyl hydrolase (PAF-AH), carried by HDL that remove proinflammatory lipids, such as oxidized phospholipids, from lipoproteins and lesional cells. Another promising therapeutic approach is the use of apoA-I mimetic peptides (49). ApoA-I mimetic peptides contain similar biological properties as native apoA-I of promoting cellular cholesterol efflux and hence

RCT, as well as anti-inflammatory effects. However, both apoA-I and mimetic apoA-I peptides require ABCA1 function to achieve their beneficial effects.

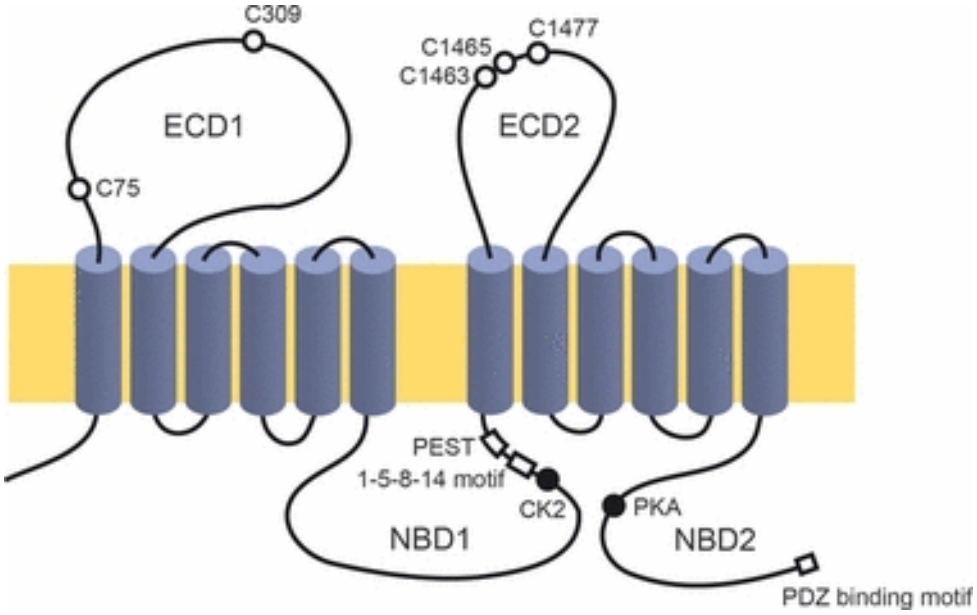
1.3 ATP Binding Cassette Transporter A1 – ABCA1

Cellular cholesterol efflux is an essential component in the RCT process, whereby ABCA1 facilitates the transfer of cholesterol and phospholipids from peripheral cells to lipid-poor apoA-I. The importance of ABCA1 can be seen in Tangier disease, a consequence of dysfunctional mutations in both alleles of *abca1* gene. Tangier patients have massive accumulation of cellular cholesterol esters residing in various tissues, including tonsils, lymph node, spleen, and liver, and have much elevated predisposition for cardiovascular disease (50). Tangier disease is also characterized by the near absence of plasma HDL and apoA-I due to increased rates of apoA-I catabolism. It took approximately 30 years after the initial discovery of Tangier disease to assign the locus for the genetic defect to chromosome 9q31, and subsequently identify the gene to be *abca1* (50). Homozygous mutations of ABCA1 cause Tangier disease, while the much more common familial HDL deficiency (FHD) is caused by heterozygous mutations of ABCA1 that also reduces HDL and apoA-I levels (51). Although only about 100 cases of Tangier disease have been reported worldwide, this disease has contributed significantly to our current knowledge on lipoproteins and atherosclerosis.

1.3.1 ABC Transporters and ABCA1

ABCA1 is a member of a large family of integral membrane proteins, consisting of 49 members in human that hydrolyze ATP to drive the transport of substrates across cellular membranes. ABCA1, similar to many other ATP transporters, are highly conserved between species, sharing 94% amino acid homology between human and murine species, supporting a critical role of ABCA1 in these species (52). Although ABCA1 may shuttle between intracellular endocytic compartments and plasma membrane of cells, it mainly localises in the plasma membrane (53). ABCA1 is a 'full-transporter' (**Figure 1.3**) with 2 transmembrane domains (TMD) and 2 nucleotide binding domains (NBDs), which contain highly conserved Walker A and B motifs that are involved in stabilizing ATP interactions and catalyzing its hydrolysis. ABC proteins are generally known to actively transport metal

Figure 1.3: The structural model of ABCA1. ABCA1 protein encompasses the components to form a full ABC transporter, including two transmembrane domains and two nucleotide binding domains (NBDs). Each transmembrane domain consists of six amphipathic α-helical structures, making ABCA1 a transmembrane protein that is mainly expressed on the plasma membrane of cells. ABCA1 also contains two large extracellular domains (ECDs) that is essential for apoA-I interactions. Other important domains are present within the cytoplasmic regions of ABCA1, including a major PKA phosphorylation site involved in apoA-I lipidation. (Permission was obtained from publisher.)



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ions, peptides, sugars, and lipids across the plasma membrane and intracellular membranes (54). However, the exact substrate for ABCA1 remains to be defined.

Several ABC transporters, in addition to ABCA1, have functions associated with lipid trafficking (55). For instance, translocation of phospholipids across cell membranes is facilitated by multidrug resistance transporters ABCB1 and ABCB4 (54). Also, ABCA4 in rod photoreceptors of the retina has phosphatidylethanolamine (PE) flippase activity, presumably to prevent deleterious retinal PE accumulation (55). Furthermore, various ABC transporters participate RCT process at different sites; the initial steps of lipid-poor apoA-I lipidation is mediated by ABCA1, further HDL lipidation is facilitated by ABCG1, and lipid elimination in bile salts is promoted by ABCB11, ABCG5 and ABCG8, respectively (56).

1.3.2 Regulation and Tissue Expression of ABCA1

ABCA1 is highly expressed in several tissues, including the lung, spleen, small intestine, and liver (57). Increasing evidence has suggested that ABCA1 plays distinct tissue-specific roles. For example, hepatic and intestinal ABCA1 is estimated to contribute 70-80% and 15-20% of circulating HDL, respectively, highlighting their importance in body lipid homeostasis (58, 59). Conversely, macrophage ABCA1 has no significant impact on plasma HDL levels, but is critically important in reversing lipid accumulation in foam cells and limiting inflammatory effects (60). Not surprisingly, macrophage-specific deletion of ABCA1 exacerbates atherosclerosis, without producing any change in plasma HDL levels (61). Thus, specific agonists that enhance macrophage ABCA1 expression could be a promising approach to treat atherosclerosis, independent of plasma cholesterol lowering.

ABCA1 expression is tightly regulated in a cell specific manner at the transcriptional level. Cholesterol loading enhances ABCA1 expression in peripheral cells, such as macrophages and fibroblasts (62). This is mediated by activation of LXR by oxidized cholesterol, which in turn interacts with LXR response element on the ABCA1 promoter. LXR-mediated upregulation of macrophage ABCA1 expression may also be activated through PPAR activation, which supports atheroprotective roles of these nuclear receptors (36). In contrast, cholesterol feeding is unable to significantly increase hepatic ABCA1 expression, which may be the consequence of a hepatic-specific promoter for ABCA1 under the control of both SREBP-2 and LXR (62). Cooperative regulation by both SREBP and LXR may serve to balance ABCA1 expression under fluctuating cholesterol conditions.

Different alternate transcripts of ABCA1, which are the products of alternative splicing of the ABCA1 gene, are specifically expressed at different levels in the liver and macrophages in response to an atherogenic diet, implying alternative transcripts contributes to distinct tissue-specific roles in lipid metabolism (63). More recently, miR-33, an intronic microRNA located within the *SREBF2* gene and co-transcribed with SREBP2, has been identified as a factor that represses both hepatic and macrophage ABCA1 expression at the post-transcriptional level (64). Other factors independent of cholesterol status can also modulate ABCA1 gene expression, including cyclic adenosine monophosphate (cAMP) zinc finger proteins Sp1 and Sp3, and factors associated with inflammation such as interferon (IFN)-γ, lipopolysaccharide (LPS), TNF-α, and interleukin (IL)-10 (65).

Post-translational regulation also contributes significantly to ABCA1 expression. Since cholesterol is a critical cellular component and needs to be regulated in a timely manner, cells have acquired molecular machinery to rapidly degrade ABCA1 (half-life 1-2 hours) to avoid excessive elimination of cholesterol (62). The degradation of ABCA1 occurs via several pathways: ABCA1 can be endocytosed and delivered to lysosomes for degradation; calpain, a calcium (Ca^{2+})-dependent thiol protease, can degrade ABCA1 particularly in the absence of apoA-I; and ABCA1 can also be degraded via the ubiquitin-proteasome pathway. ABCA1 protein turnover can also be enhanced by unsaturated fatty acids (linoleate, oleate, and eicosapentaenoic acid) (66). Alternatively, ABCA1 can be stabilized through interacting with proteins including apoA-I, calmodulin, α1-syntrophin, and β1-syntrophin. ApoA-I protects ABCA1 from calpain-mediated proteolysis through inducing protein kinase Cα (PKCα) phosphorylation of ABCA1 and dephosphorylation of the PEST sequence (a sequence rich in proline, glutamic acid, serine, and threonine) within the cytoplasmic domain of ABCA1. ABCA1 also consists of a calmodulin binding sequence, 1-5-8-14 motif, near the PEST sequence, where interaction with Ca^{2+}/calmodulin protects it from calpain-mediated degradation (67).

1.3.3 ABCA1-mediated ApoA-I Lipidation

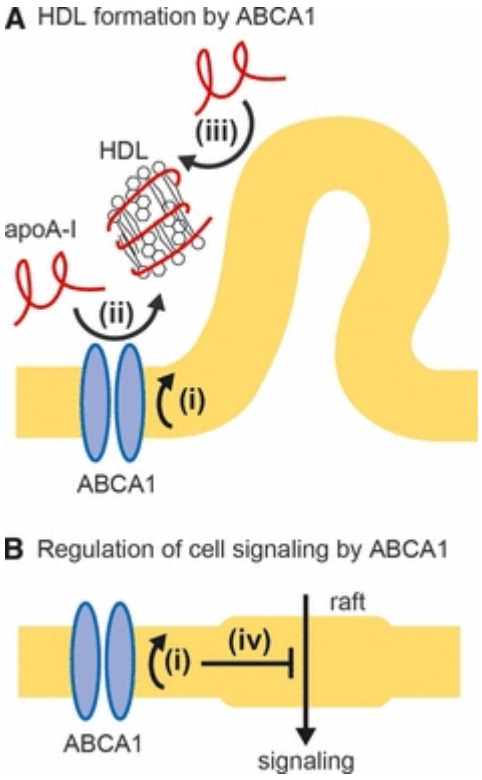
ABCA1 is responsible for the transfer of cellular phospholipid and cholesterol to lipid-free or lipid-poor apoA-I at the plasma membrane, promoting the initial steps of RCT. Although the exact mechanisms of ABCA1-mediated apoA-I lipidation is not fully elucidated, two different models have been proposed: a one-step model with ABCA1 mediating

phospholipids and cholesterol concurrently, and a two-step or 'sequential' model in which the transfer of phospholipids to apoA-I precedes the efflux of cholesterol (68, 69). The latter model is based on observations that phospholipid efflux to apoA-I is ABCA1-dependent, while phospholipid-enriched apoA-I can promote cholesterol efflux independent of ABCA1, suggesting phospholipid and cholesterol efflux are two distinct pathways. In addition, ABCA1 on the cell surface is known to modify the packing of cholesterol and phospholipids within the plasma membrane, which is believed to establish an optimal microenvironment for apoA-I docking and lipidation (70).

It has been demonstrated that ABCA1 activity generates two distinct apoA-I binding sites on the surface of the cells (**Figure 1.4**): a high affinity but low capacity site for direct apoA-I/ABCA1 binding, and a high capacity binding site (HCBS), which is phosphatidylcholine-rich and most likely associated with non-raft microdomains, for apoA-I/lipid interaction (71). Approximately 10% of lipid-poor apoA-I on the cell surface forms high affinity molecular interactions with the large extracellular domains of oligomeric ABCA1 complexes, whereas the rest of apoA-I is associated with membrane (71, 72, 73). Plasma membrane ABCA1 was first postulated to promote the flipping of phospholipids, mainly phosphatidylserine (PS), from the inner to the outer membrane leaflet, owning to ATP-dependent phospholipid flippase activity (74, 75). Membrane domains in which apoA-I binds, such as HCBS domains, may form exovesiculated domains that induces strain on the lipid bilayer packing (76). This facilitates the release of phospholipids to apoA-I, which then develop into small discoidal particles that can efficiently accept cholesterol when exposed to cholesterol-rich membrane domains to generate larger, cholesterol-rich nascent particles (77). Thus, this model of the initial docking and interactions of apoA-I to the cell surface is consistent with the 'two-step' model of apoA-I lipidation, although definitive experimental evidence remains elusive.

The plasma membrane is a dynamically organized structure, consisting of various nano-scale (10–100 nm) lateral domains, such as lipid rafts. These domains are thought to be involved in signal transduction and other cellular functions (62). ABCA1 is involved in reorganizing membrane domains (**Figure 1.4**), largely independent of apoA-I and potentially due to phospholipid and cholesterol flippase activity of ABCA1. This results in the redistribution of cholesterol and sphingomyelin away from Triton X-100-resistant membrane

Figure 1.4: Functions of ABCA1 on the plasma membrane. (A) ABCA1 promotes phospholipid transfer from the inner to outer membrane leaflets, which is suggested to form high capacity-binding sites (HCBS) for apoA-I binding on the plasma membrane. It is believed that (i) apoA-I initially docks on the extracellular loops of ABCA1 and (ii) subsequently interacts with HCBS domains on the membrane to (iii) acquire phospholipids and cholesterol. (B) The intrinsic ability for ABCA1 to induce phospholipid flippase activity may also result in disruption of lipid raft microdomains and, in turn, (iv) modulate cell signaling. (Permission was obtained from publisher.)



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domains (78). Consistent with lipid raft disruption, ABCA1 also redistributes caveolin-1, a major structural component of caveolae and a well-established resident raft protein, such that caveolin-1 is no longer concentrated in punctate caveolae-like structures. In addition, ABCA1 increases the amount of cholesterol accessible by cholesterol-oxidase, likely representing non-lipid raft domains. Interestingly, such cholesterol-oxidase accessible pool is preferred for apoA-I lipidation (79). Furthermore, re-organizing raft domains by ABCA1 is also reported to simultaneously enhance membrane fluidity, a state that is suggested to favour apoA-I lipidation (80). ApoA-I also suppresses inflammatory response in macrophages through binding to ABCA1 and activation of signal transducer and activator of transcription (STAT) 3 (81).

In addition to modulating the plasma membrane to generate specialized apoA-I binding domains, ABCA1 also influences membrane trafficking. For example, ABCA1 is able to mobilize cholesterol from the late endosomes/lysosomes and from the Golgi to lipidate apoA-I (82, 83). Consistent with this, ABCA1 deficiency leads to cholesterol accumulation in late endocytic compartments, whereas ABCA1 overexpression removes lysosomal cholesterol accumulation caused by Niemann-Pick type C1 deficiency (84, 85). Furthermore, ABCA1 mobilizes LDs for cholesterol efflux through autophagy (45).

1.3.4 ABCA1-mediated Microparticle Formation

Besides its well-established role in lipid efflux to apoA-I, ABCA1 also releases microparticles independent of apoA-I. These spherical particles are 50-200 nm in diameter, rich in phospholipids and cholesterol, and contain raft proteins, such as CD14 and ganglioside (86). Although the molecular mechanism through which microparticles are generated is still unclear, it has been suggested that they may originate from plasma membrane raft domains. Microparticles can be generated from several different cell origins, including endothelial cells, erythrocytes, platelets, and monocytes, which are implicated in various biological processes. Some functions ascribed to microparticlees include pro- and anti-coagulant effects, inflammation and fibrinolytic activities (16). The release of microparticles requires protein kinase A (PKA) activity and plasma membrane fluidity created by ABCA1, both of which, interestingly, are similarly required for apoA-I lipidation (80). The cholesterol released with microparticles is about 30% of the total cholesterol efflux,

and the rest (70%) is acquired by apoA-I (80), suggesting microparticles as an integral part of cholesterol homeostasis.

1.3.5 Signaling Pathways Regulating ABCA1 Function

In addition to PKA, several signal transduction pathways are closely associated with ABCA1 activities, including Rho family G protein Cdc42, Janus kinase 2 (JAK2) and Ca^{2+}/calmodulin. Many of these signal transduction processes are initiated by the interaction between apoA-I and ABCA1, promoting either ABCA1 lipid efflux activity or anti-inflammatory effects. For instance, apoA-I triggers ABCA1-mediated lipid efflux by activating Cdc42, likely by influencing actin dynamics and hence membrane trafficking to supply cholesterol for efflux (87). Also, apoA-I activates JAK2 to enhance apoA-I binding activity of ABCA1 and optimize lipid export to apoA-I. JAK2 activity is also known to activate STAT proteins that are involved in inflammatory signaling. Indeed, apoA-I activates STAT3 through JAK2, and the direct interaction between ABCA1 and STAT3 is necessary for apoA-I to elicit its effect on cytokine secretion (75).

Secondary messengers, i.e. Ca^{2+}, extracellular ATP (eATP), and cAMP, are also employed by ABCA1 to elicit additional regulation on cholesterol efflux. Intracellular Ca^{2+} at resting states is maintained at a significantly lower concentration (100 nM) than extracellular concentrations (1-2 mM). ApoA-I/ABCA1 interactions trigger extracellular Ca^{2+} influx and hence activate the calmodulin/calcineurin signaling cascade to facilitate apoA-I lipidation (88). Interestingly, ABCA1-expressing cells maintain a higher concentration of extracellular ATP (eATP). This elevated ATP level is necessary for ABCA1-mediated lipid efflux to apoA-I, perhaps signaling in an autocrine or paracrine manner (89). Contrary to Ca^{2+}, eATP levels are usually low (approximately 10 nM), whereas cytosolic ATP levels are much higher (3-10 mM), which raises the possibility that ABCA1 may function as an ATP channel. The mechanism of ABCA1-induced eATP signaling remains elusive, although it has been suggested that eATP may be interconnected with Ca^{2+} signaling.

In addition, ABCA1 activities are influenced by serine/threonine (Ser/Thr) protein kinases. For example, apoA-I activates PKCα to phosphorylate and stabilize ABCA1, protecting ABCA1 against calpain-mediated proteolysis (90). Moreover, ABCA1 is phosphorylated by casein kinase 2 (CK2), which is suggested to down-regulate ABCA1

function (91). Besides PKC and CK2, PKA is perhaps the most significant kinase that regulates multiple aspects of ABCA1 function.

1.4 Protein kinase A (PKA)

In 1968, cAMP-dependent protein kinase or PKA, a multisubstrate protein kinase that plays an integral role in cAMP-dependent signal transduction, was one of the first protein kinases identified (92). This cAMP/PKA signaling pathway regulates numerous enzymes and regulatory proteins, which collectively is involved in diverse cellular processes including nutritional metabolism, inflammatory response, cardiac muscle contraction, and cholesterol metabolism (93).

1.4.1 Overview of cAMP/PKA Signal Transduction

cAMP/PKA signaling pathway begins with generating local pools of cAMP, which is catalyzed by several isoforms of membrane-bound adenylyl cyclase (AC). ACs can be regulated by neurotransmitters, hormones, and other regulatory molecules via binding to G-coupled receptors (94). PKA resides in its inactive state as a tetrameric holoenzyme complex, consisting of two catalytic (PKA-C) subunits and two regulatory (PKA-R) subunits. Upon cAMP binding to PKA-R subunits, PKA-C subunits are released and hence activated. PKA phosphorylates specific Ser and Thr residues located within a RRX(S/T) consensus motif that is present in a wide range of proteins, including ABC transporters, cytoskeletal components, and transcription factors (95).

There are four isoforms of the regulatory subunits (RIα, RIβ, RIIα, RIIβ) and three isoforms of the catalytic subunits (Cα, Cβ, and Cγ). The isoforms of PKA-R and C subunits have distinct tissue distribution, likely reflecting their functional specificity (96). All three isoforms of PKA-C subunits contain two major subdomains: 1) an ATP-binding site near the N-terminal region, and 2) a large C-terminal domain consisting of a peptide-recognition site and a highly conserved catalytic core. These domains of PKA-C subunits promote close interactions between the Ser/Thr residues of the substrate and the γ-phosphates of ATP, facilitating phosphoryl group transfer.

The dynamics of PKA activity is governed by several factors, including phosphatases that dephosphorylate PKA substrates, phosphodiesterases (PDEs) that degrade cAMP, and A-kinase anchoring proteins (AKAPs) that dictate the subcellular localization of PKA (93).

1.4.2 A-Kinase Anchoring Proteins (AKAPs)

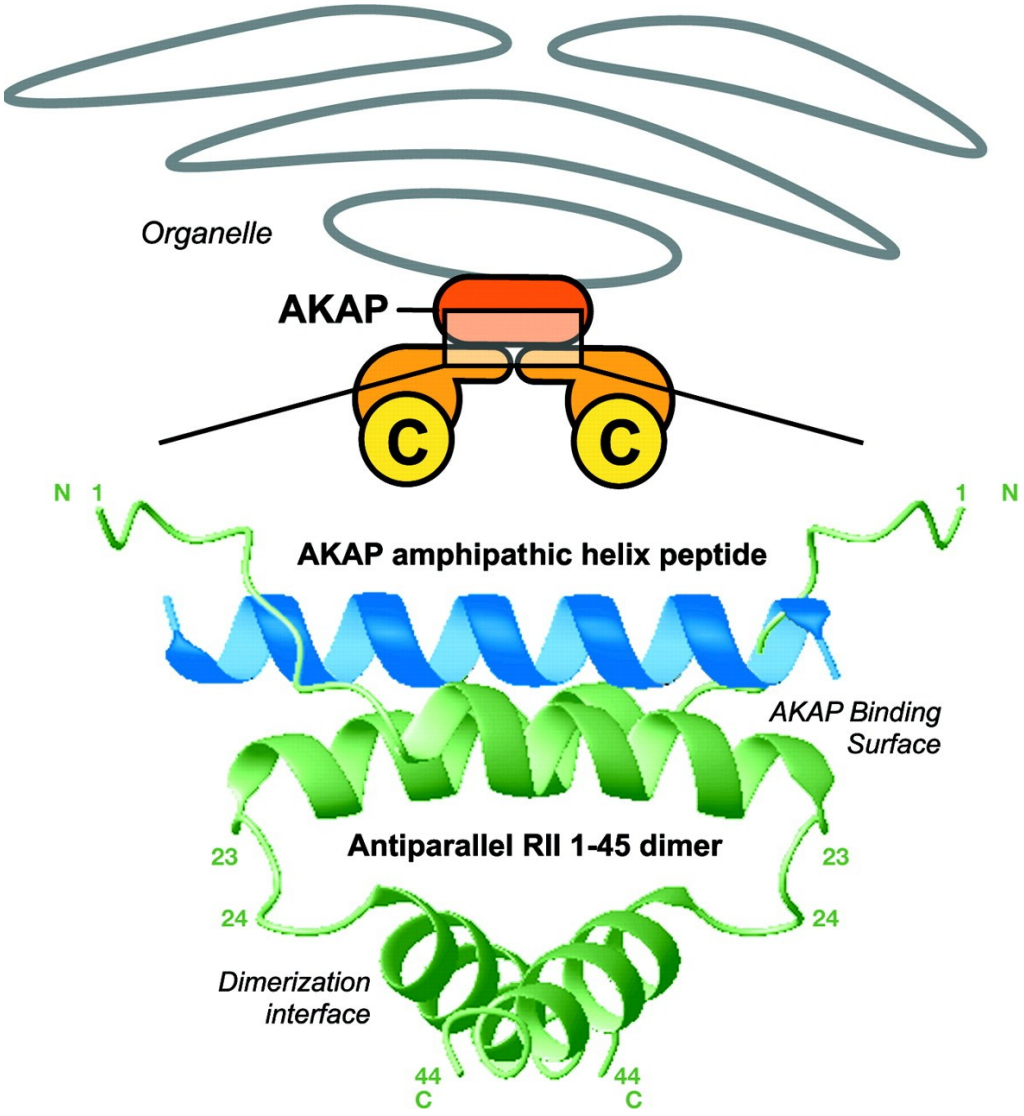
AKAPs are a structurally diverse family of scaffolding proteins, consisting of more than fifty members to date. As the name implies, AKAPs function to anchor and thus compartmentalize PKA. Particularly, the targeting motifs of AKAP determine their specific subcellular locations, which could be at the plasma membrane, internal organelles, or cytoskeleton structures (97). Understandably, a PKA-binding domain is also the common feature within all AKAPs, consisting of a 14-18 residue amphipathic helical structure. This PKA-binding domain of AKAP binds to the hydrophobic groove of the docking and dimerization (D/D) domain on the N-terminal domain of PKA-R subunit dimers (**Figure 1.5**). As PKA phosphorylates a wide spectrum of substrates, anchoring proteins provides the lateral regulation of PKA activity. For example, AKAPs can place PKA to specific cellular sites, allowing PKA to sense local cAMP concentrations and phosphorylate PKA substrates in or near the location (**Figure 1.6**). Perhaps equally importantly, AKAPs are also capable of binding other components of the cAMP/PKA pathway, such as ACs, PDEs, and phosphatases. This allows local initiation and termination of PKA. Thus, AKAP enables these signaling complexes to provide a spatially and temporally regulated cAMP/PKA signaling cascade.

Although the different isoforms of the PKA-C subunit have similar kinetic and physiochemical characteristics, the subtypes of PKA-R subunits display distinct properties that contribute to the differences observed between type I and type II PKA. Type I PKA holoenzymes are biochemically soluble and believed to be mainly cytoplasmic, while type II PKA holoenzymes are typically particulate and predominantly confined to organelles and other subcellular structures (93). These differences in intracellular localization are due to selectivity and distinct affinities of AKAPs for PKA-RI and -RII; the K_d for majority of RII-AKAP interactions are in nanomolar range, while RI-AKAP binding affinity is much lower (micromolar). AKAPs can selectively bind either PKA-RII or -RI, but dual-specific AKAPs that bind both types of R subunits have also been identified, such as dual-function anchoring protein D-AKAP2 (98).

1.4.3 PKA Deanchoring Peptides

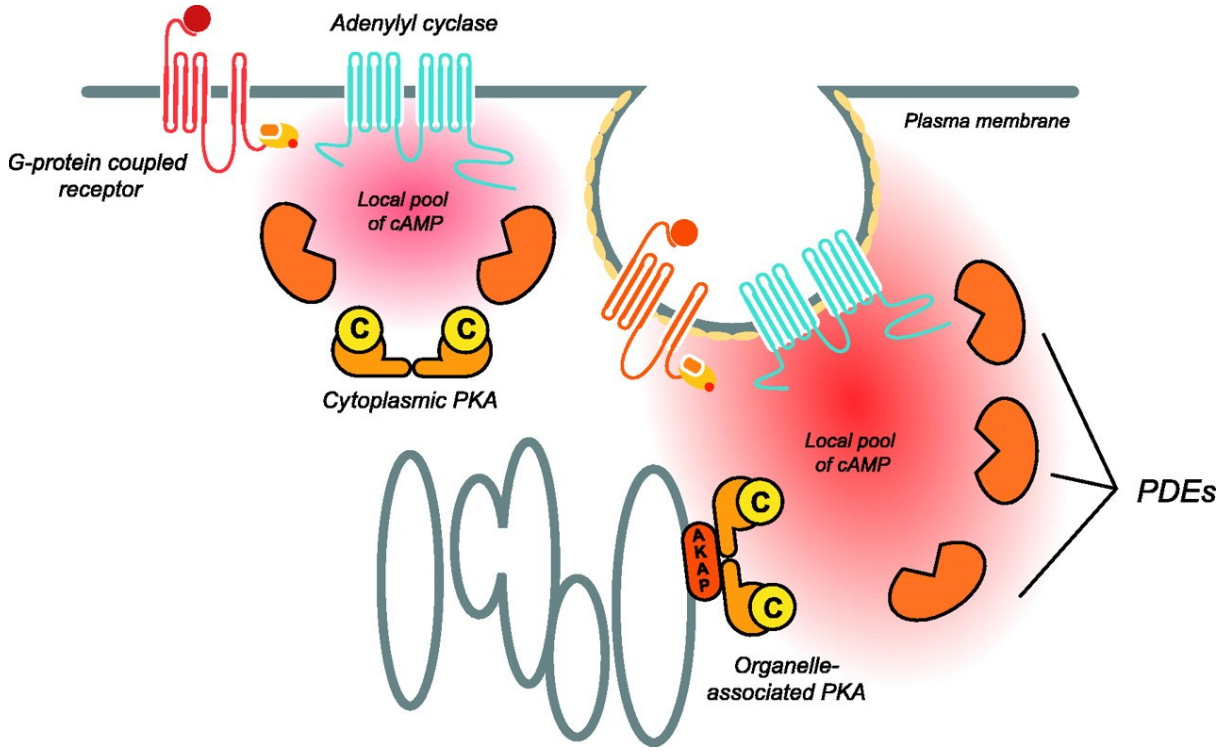
Since PKA is involved in wide range of cellular processes and its activity is regulated in part by AKAPs, peptides that mimic PKA-binding domains of AKAPs have been designed and

Figure 1.5: PKA binding domain of AKAP. Each A-kinase anchoring protein (AKAP) consists of at least two functional domains: a PKA binding domain and a targeting domain that directs the complex to specific subcellular locations. The PKA binding domain of AKAP is a 14-18 amino acid sequence that forms an amphipathic helical structure that interacts with the first 50 residues of PKA-R subunit dimers at the N-terminal D/D domain. (Permission was obtained from publisher.)



Taskén and Aandahl, Physiol Rev, 2004; 84(1):137-67.

Figure 1.6: Compartmentalized cAMP signaling. cAMP levels are unevenly distributed throughout the cell, generating local cAMP gradients. The variations in local levels of cAMP are governed by two types of enzymes: adenylyl cyclases that are regulated by G-protein coupled receptors and phosphodiesterases (PDEs), which hydrolyze cAMP. AKAPs function to anchor PKA to particular local subcellular cAMP gradients. (Permission was obtained from publisher.)



Taskén and Aandahl, Physiol Rev, 2004; 84(1):137-67.

widely used to investigate the localized PKA functions. The initial investigations of AKAP and PKA interactions utilized a 24 amino acid peptide, Ht31, consisting of the amphipathic helical PKA-binding motif of AKAP-Lbc (99). Ht31 was shown to be a global PKA de-anchoring agent, effectively disrupting both AKAP-RI and -RII interactions with slightly higher preference towards RII subunit. Modifications in the amino acid sequence of de-anchoring peptides have optimized peptides to acquire higher affinity and increase binding specificity (98). Particularly, PKA-anchoring disruptor peptides have been created to be highly specific to either RI or RII subunits. For instance, the AKAP18δ peptide binds RII subunits with high affinity and the RI-Anchoring Disruptor (RIAD) selectively disrupts AKAP-RI interactions (100, 101). Additional modifications of peptide disruptors, including coupling to fluorescent dyes and cell penetrating tags, can be used to further optimize these peptides for visualization and for cell permeation. Many of these PKA-de-anchoring peptides are conjugated to lipid moieties, such as stearic acid. For instance, stearated (st)-Ht31 and -AKAP18δ peptides have been widely used to render them cell-permeable, enhancing cellular uptake of these peptides (98).

1.4.4 cAMP/PKA Signaling and ABCA1

As mentioned above, among the various kinases, PKA is the most significant in regulating ABCA1 activities. For examples, apoA-I/ABCA1 interactions increase cAMP concentrations and consequently activate PKA, resulting in direct phosphorylation of ABCA1 at major PKA phosphorylation sites (102, 103). Sequence analysis identified at least two within the cytosolic NBDs of ABCA1; one of which is Ser-2054 and found to be a crucial regulator of apoA-I-dependent phospholipid efflux (102). While ABCA1 is a constitutively PKA phosphorylated protein, apoA-I-induced PKA phosphorylation at Ser-2054 has been suggested to influence the conformation of ABCA1 to a more active state. Consistent with this notion, cAMP analog is able to increase phosphorylation of ABCA1 and enhance apoA-I-dependent cholesterol efflux, although the particular site of ABCA1 phosphorylated by PKA in this case was not identified (103). PKA activity was reported to be necessary for anionic flux activity of ABCA1, microparticle release, and the redistribution of plasma membrane cholesterol by ABCA1 (104, 88, 105). Although the precise molecular mechanism by which PKA influences ABCA1 function remains to be elucidated,

experimental evidence collectively highlight the importance of PKA signaling in ABCA1 function.

1.4.5 cAMP/PKA Signaling and Inflammatory Signaling

cAMP/PKA possesses broad immunosuppressive functions in signaling pathways within a variety of immune cell types, including basophils, T and B lymphocytes, and macrophages (106). Exposure to stimili that elevate cellular cAMP concentrations, such as β-adrenergic catecholamine and E type prostaglandins, in basophils and mast cells, suppresses immediate hypersensitive inflammatory responses. In addition, PKA inhibits activation and proliferation of T and B cells, thereby suppressing adaptive immune functions (107). In monocytes and macrophages, production of inflammatory mediators, such as cytokines and chemokines, are also modulated by cAMP/PKA signaling (108). cAMP/PKA suppresses pro-inflammatory cytokines, (TNF-α, IL-12, etc.) and chemokines (macrophage inflammatory protein-1, etc.). On the contrary, PKA can also enhance the production of the anti-inflammatory cytokine IL-10 (109). Although exchange proteins activated by cAMP (Epac), another cAMP target, has been implicated to influence TLR-mediated cytokine release from macrophages, it was recently reported that the macrophage immunomodulatory function is primarily through PKA activation (108). As such, the cAMP/PKA signaling pathway plays an important immune modulation function, which is crucial to maintain balance during an immune response to prevent host injury from exaggerated inflammation.

One of the best characterized substrates of PKA is CREB, a transcription factor that binds to cAMP response elements (CREs) in the promoter regions of their target genes (110). CREB transcription activity regulates a variety of cellular responses, ranging from proliferation, survival, to immune responses. CREB was originally identified to be phosphorylated at Ser-133 by PKA; however, CREB can also be the target for other kinases, including PKC and Ca^{2+}/calmodulin kinases. Phosphorylating Ser-133 on CREB mediates the recruitment of coactivators CREB-binding protein (CBP) and p300, which in turn, activates transcription. However, other studies have concluded the recruitment of CBP to CREB is sufficient to activate transcription and suggested PKA kinase activity further augments CREB signaling via phosphorylation events downstream of CBP recruitment (111). More than 300 different stimuli have been reported to induce CREB phosphorylation, including growth factors, steroid hormones, and inflammatory signaling molecules.

Several immune-related genes, such as IL-6, IL-10, and TNF-α, are regulated by CREB. RelA, a subunit of the NF-κB transcription factor, directly binds to CBP/p300. It is proposed that activated CREB inhibits NF-κB activity through competitively interacting with CBP/p300, dampening the pro-inflammatory response (112). In macrophages, CREB couples with AP-1 transcription factor to subsequently bind to the IL-10 promoter and induce the production of IL-10, a potent anti-inflammatory cytokine (113). Furthermore, CREB transcriptional activity can be targeted as mechanisms to modulate immune inhibitory responses. In particular, IFN-γ potently enhances macrophage activation by inhibiting CREB activity and consequently, suppressing IL-10 production (114). These anti-inflammatory responses of CREB may provide a mechanism to protect against excessive inflammation, autoimmune responses, or tissue damage. Although CREB is involved in a diverse number of processes in immune cells, additional research is necessary to determine the signaling pathways and protein partners involved in its specific pro- and anti-inflammatory functions.

1.5 Inflammatory Signaling in Macrophages

Macrophages are derived from monocytes that initially arise from myloid progenitor cells in the bone marrow. Macrophages play important roles in the innate immune system to regulate the host tissue response upon tissue injury and infection. Macrophages achieve this by phagocytosis and removal of apoptotic cells and cellular debris (115). As well, macrophages respond to infectious diseases by producing various inflammatory and immunosuppressive cytokines (115). One of the most remarkable characteristics of macrophages is their diversity and plasticity (116); they can rapidly shift their phenotype in response to microenvironmental signals elicited by injury and infection.

1.5.1 Macrophage Activation and Inflammatory Signaling

When exposed to microenvironmental cues, macrophages acquire distinct functional phenotypes, broadly classified into a spectrum between two opposing extremes: classically activated pro-inflammatory (M1) or alternatively activated anti-inflammatory (M2) macrophages. Various factors can shift macrophages towards a certain activated phenotype. For instance, TLR agonists and IFN-γ drives M1 polarization that primarily supports the production of pro-inflammatory factors (116). However, excessive pro-inflammatory responses can cause host tissue damage. Thus, as an opposing mechanism, macrophages can

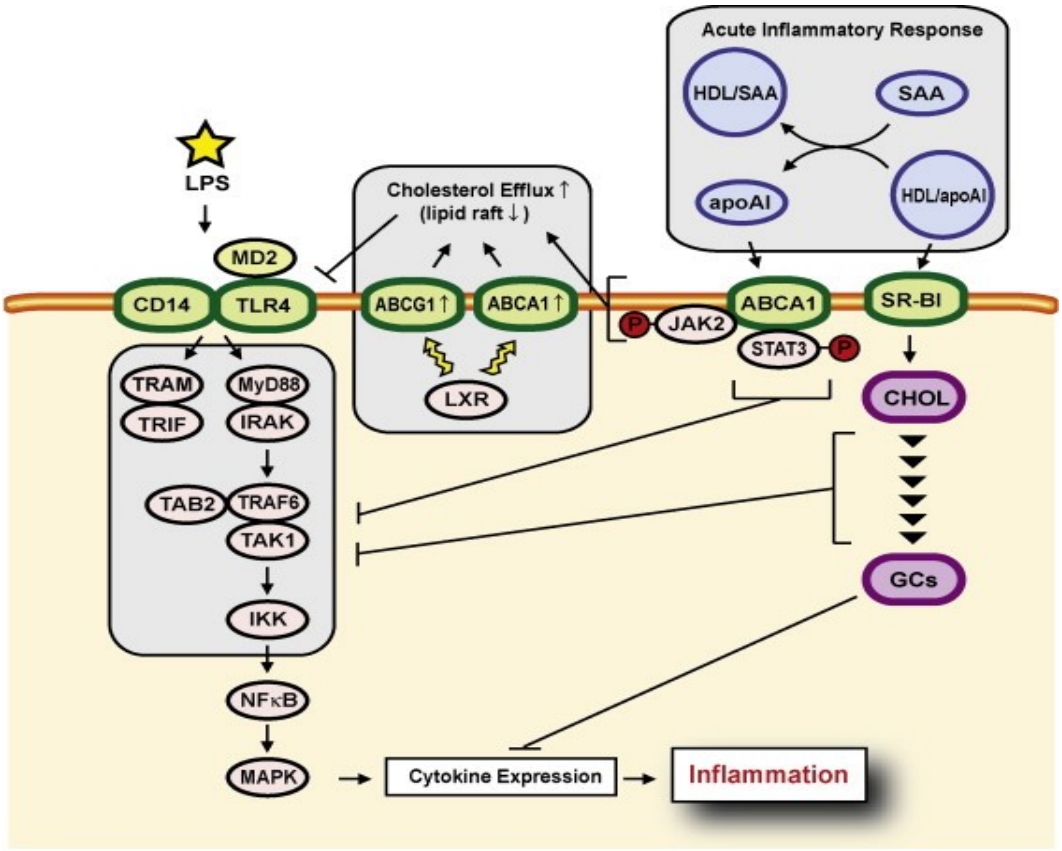
also be alternative activated, upon exposure to factors such as IL-4/IL-13, PPARs, and sphingosine-1-phosphate, shifting towards M2 polarization. M2 activation, in general, promotes inflammation resolution through anti-inflammatory IL-10 production and tissue repair (32).

Macrophages sense their surroundings with cell surface pattern recognition receptors, such as TLRs, which generate a cascade of signaling events to direct the macrophage response. The TLR family currently consists of 14 members and recognizes pathogen-associated molecules, such as LPS from the cell wall of gram negative bacteria (117). LPS initially interacts with the soluble LPS-binding protein (LBP), which enables LPS to transfer to lipid raft-associated CD14 and subsequently bind with TLR4/MD2 complex on the cell surface (**Figure 1.7**). Further downstream signaling of TLR4 is mediated by several adaptor proteins, including myeloid differentiation factor 88 (MyD88), which activates the IκB/NF-κB to trigger the expression of pro-inflammatory cytokines (118). Autocrine positive feedback on NF-κB activity can also be established by TNF-α as a secondary response. IL-10 is the major mediator of anti-inflammatory signaling events that includes the repression of pro-inflammatory cytokine production. Gene expression studies indicate that IL-10 elicits anti-inflammatory activities via STAT3 and suppressor of cytokine signaling 3 (SOCS3) (119). Since cytokines are critical in the differentiation of macrophage M1 and M2 phenotype, failure to provide the balance of pro- and anti-inflammatory cytokines may lead to pathogenesis, such as atherosclerosis.

1.5.2 Macrophage Inflammatory Signaling and Atherosclerosis

Studies have observed macrophage heterogeneity (both M1 and M2 macrophages) within atherosclerotic lesions (120). Although both M1 and M2 macrophages are present, deleterious effects contributing to disease progression is likely primarily due to M1 macrophages. Indeed, deletion of IFN-γ or its corresponding receptor in mouse model reduced atherosclerosis, whereas increasing M2 macrophages by activating PPARγ or sphingosine-1-phosphate signaling inhibited atherosclerosis (121). Several cytokines are expressed in atherosclerotic lesions, including IFN-γ, TNF-α, and IL-12. IFN-γ, TNF-α, and other pro-inflammatory cytokines have been implicated in promoting macrophage foam cell formation by enhancing scavenger receptor (SR-A and CD36) expression and reducing ABCA1/ABCG1 expression, resulting in accelerated lesion progression (31). In contrast,

Figure 1.7: Immunomodulatory properties of ABCA1 and apoA-I. Cellular cholesterol exporters, including ABCA1 and ABCG1, can reduce lipid raft microdomains on the plasma membrane through their lipid transport functions, which in turn, repress TLR4-mediated inflammatory signaling cascades. It is speculated to be a consequence of a decrease in CD14/MD2/TLR4 complex formation, a lipid raft dependent process. In addition, lipid-poor apoA-I binding with ABCA1 activates the JAK2/STAT3 signaling pathway that can suppress pro-inflammatory cytokine production. Furthermore, apoA-I can be displaced from HDL particles by serum amyloid A (SAA) during acute inflammatory response, whereby this liberated apoA-I may interact with ABCA1. SR-BI also modulates inflammatory signaling via providing cholesterol for glucocorticoid (GC) production. (Permission was obtained from publisher.)



Fitzgerald et al, Atherosclerosis, 2010; 211(2):361-70.

anti-inflammatory cytokines, such as TGF-β, IL-10, and IL-33, inhibited foam cell formation and atherosclerosis progression, through down-regulating expression of scavenger receptors and increasing expression of cholesterol exporters (31). From this perspective, therapies targeting cytokine responses in atherosclerosis, including administration of anti-inflammatory cytokines that reduce foam cell formation, could prove to be clinically beneficial in human atherosclerosis.

1.5.3 Macrophage Inflammatory Signaling and ABCA1

Apart from lipid trafficking-associated functions, there is increasing evidence that ABCA1, particularly macrophage ABCA1, plays a crucial role as an anti-inflammatory mediator. For instance, ABCA1-deficient macrophages have hypersensitive inflammatory responses to TLR ligands and chemotactic factors, resulting in elevated secretion of pro-inflammatory cytokines and chemokines (122, 123). In addition, ABCA1 is expressed only in a subset of leukocytes that correspond to non-inflammatory tissue-resident macrophages, enriched in markers of M2 or alternative activated phenotype (124). Currently, there are two potential mechanisms through which macrophage ABCA1 dampens the inflammatory response (**Figure 1.7**): 1) via modulation of cellular cholesterol status and redistribution of plasma membrane cholesterol (independent of apoA-I), and 2) through ABCA1/apoA-I interactions that activates the JAK2/STAT3 signaling pathway (125). It was speculated that, by modulating cellular cholesterol content through efflux or redistribution, ABCA1 suppresses TLR4 trafficking to lipid rafts, thus impairing TLR4 signal transduction (60). In support of this view, reducing cellular content independent of ABCA1, such as extraction by methyl-β-cyclodextrin (MCD), also exert similar anti-inflammatory effects (122). Although the mechanism remains speculative, it is clear that ABCA1 is part of the molecular machinery that associates cholesterol homeostasis with inflammation.

1.6 Summary

In summary, accumulation of lipid-laden macrophages and its pro-inflammatory response in arterial lesions are fundamental components of the pathogenesis of atherosclerosis. Lipid efflux pathways promoted by ABCA1 are capable of alleviating the lipid burden and dampen inflammation in foam cells; however, the underlying mechanisms regulating ABCA1 functions are still incompletely understood. The purpose of this doctoral thesis is to provide

insight into the underlying mechanisms in which ABCA1 achieves these functions. With more knowledge of ABCA1, ABCA1 function could be enhanced as a therapeutic approach in treatment of cardiovascular disease.

1.7 Research Objectives

ABCA1 is a vital component in modulating cellular cholesterol export and inflammatory response, both of which are crucial factors involved in the progression of atherosclerosis. PKA signaling is integrated extensively in ABCA1 function to promote anti-atherogenic functions; however, mechanistic aspects of PKA signaling associated with ABCA1 are not well established. The first objective of this study was to investigate the spatial regulation of PKA signaling in cholesterol efflux, particularly in reverse foam cell formation. To achieve this, we first studied the role of PKA anchoring in ABCA1-mediated cholesterol efflux through the use of st-Ht31 PKA de-anchoring peptide. We also used FRET-based biosensor probes to measure changes in localized PKA activity to investigate the effect of st-Ht31 on PKA activity.

In the second half of this study, our objective was to understand the mechanism by which ABCA1 regulates macrophage inflammatory response. Although ABCA1 is known to suppress inflammatory response in macrophages, the underlying factors responsible for this process remain unclear. Through studying PKA activity on various cell models, we observed that ABCA1 activated PKA. This led us to subsequently investigate the contribution of PKA activity on ABCA1-regulated inflammatory response in macrophages. Furthermore, since regulating macrophage inflammatory response by ABCA1 is suggested to be through modulating cellular cholesterol, we modulated cellular cholesterol to determine whether it recuperates ABCA1 phenotype.

1.8 Hypothesis

Thus, we hypothesize that PKA signaling is an essential factor in ABCA1 function. ABCA1 activate PKA by regulating cholesterol. This allows cells to utilize compartmentalized PKA activities to export cholesterol either to apoA-I or as microparticles. ABCA1-activated PKA is also a major contributor for immune modulatory function of ABCA1. Thus, through its

capacity of regulating cellular cholesterol, ABCA1 poises macrophages towards a more anti-inflammatory M2-like phenotype.

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Chapter 2: Ht31, a Protein Kinase A Anchoring Inhibitor, Induces Robust Cholesterol Efflux and Reverses Macrophage Foam Cell Formation through ATP-binding Cassette Transporter A1

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2.1 Significance of this Manuscript

The research provided in this manuscript reveals the role of compartmentalized PKA signaling in ABCA1-mediated cholesterol efflux with the use of Ht31 peptide. Treatment with Ht31 peptide impairs PKA anchoring to AKAPs, which in turn, elevates cytoplasmic PKA activity. Our findings highlight the importance of cytoplasmic PKA activity in modulating apoA-I independent cholesterol efflux, as Ht31 substantially enhanced ABCA1-mediated microparticle release. In addition, our results indicate that enhancement of cholesterol efflux of Ht31 peptide reverses macrophage foam cell formation and emphasize the therapeutic potential of PKA deanchoring.

2.2 Author Contributions

The experiments in this paper were planned by Dr. Zha and I. As the first author, I performed the majority of the experiments. The second author, Dr. Fumin Dong, the research associate in the lab, performed the phospho-CREB western blot. Dr. Maxime Denis and Ying Feng helped with the preliminary cholesterol efflux experiments. Ming-Dong Wang provided us with murine BMDM cells and helped with optimizing the foam cell formation protocol. I wrote a preliminary draft of the manuscript. The final draft was submitted to the journal after Dr. Zha and I both went through several rounds of editing, with the assistance and advice from others. I performed additional experiments for the revision of the manuscript before being accepted.

2.3 Abstract

Macrophage foam cell is the predominant cell type in atherosclerotic lesions. Removal of excess cholesterol from macrophages thus offers effective protection against atherosclerosis. Here we report that a protein kinase A (PKA)-anchoring inhibitor, st-Ht31, induces robust cholesterol/phospholipid efflux, and ATP-binding cassette transporter A1 (ABCA1) greatly facilitates this process. Remarkably, we found that st-Ht31 completely reverses foam cell formation, and this process is ABCA1-dependent. The reversal is also accompanied by the restoration of well modulated inflammatory response to LPS. There is no detectable toxicity associated with st-Ht31, even when cells export up to 20% cellular cholesterol per hour. Using FRET-based PKA biosensors in live cells, we provide evidence that st-Ht31 drives cholesterol efflux by elevating PKA activity specifically in the cytoplasm. Furthermore, ABCA1 facilitates st-Ht31 uptake. This allows st-Ht31 to effectively remove cholesterol from ABCA1-expressing cells. We speculate that de-anchoring of PKA offers a novel therapeutic strategy to remove excess cholesterol from lipid-laden lesion macrophages.

2.4 Introduction

Cholesterol export from peripheral cells is an essential process in maintaining cholesterol homeostasis and normal cell function. ATP-binding cassette transporter A1 (ABCA1) plays a key role in this cholesterol export. Dysfunctional mutations of ABCA1 in human result in Tangier disease, a disorder characterized by elevated risk of cardiovascular disease (1–4). ABCA1 is highly expressed in lipid-laden macrophages where it facilitates the removal of excess cholesterol. This prevents the formation of foam cells, the predominant cell type in atherosclerotic lesions.

At the molecular level, ABCA1 is best characterized as an essential protein for exporting cellular cholesterol/phospholipids to extracellular acceptors, such as apolipoprotein A-I (apoA-I), leading to the formation of HDL. In addition, ABCA1 exports cholesterol and phospholipids in the form of non-HDL microparticles (5, 6). This microparticle release relies on protein kinase A (PKA) activity. ABCA1 itself can be phosphorylated by PKA (7); however, the precise consequence of this phosphorylation is not clear. A study that mutated two of the most probable PKA phosphorylation sites on ABCA1 reported no defect on cholesterol export (8). This suggests that PKA may target molecules downstream of ABCA1.

PKA is a broad spectrum Ser/Thr kinase that regulates a wide range of cellular processes. To modulate such a diverse spectrum of events with specificity, PKA has to be activated at precise cellular locations and at specific times. This spatial-temporal regulation is conveyed in part through PKA interaction with protein kinase A-anchoring proteins (AKAPs). Although structurally diverse, all AKAPs contain a PKA-anchoring domain and a specific targeting motif that dictates their subcellular localizations. AKAPs are also scaffolding proteins that sequester not only protein kinases but also phosphatases to coordinate phosphorylation dynamics (9). For example, besides PKA, most of the AKAPs also anchor phosphodiesterases that degrade cAMP, the major PKA activator. This allows AKAP to locally regulate the amplitude and duration of PKA activation. Presently, it is not known whether this spatially governed PKA activity relates to ABCA1 function or cholesterol export.

In this study, we report that st-Ht31, a membrane-permeable peptide inhibitor of PKA anchoring, increases cytosolic PKA activity and robustly exports cholesterol as microparticles. Remarkably, st-Ht31 is able to effectively reverse foam cell formation and restores metabolic health in these otherwise dysfunctional macrophages. ABCA1 greatly facilitates this process.

2.5 Experimental Procedures

Materials and Reagents: Baby hamster kidney (BHK) cells stably transfected with a mifepristone-inducible vector containing an insert encoding ABCA1 or without insert (MOCK) were from Drs. Vaughan and Oram (University of Washington). RAW 264.7 macrophages were purchased from ATCC. Bone marrow-derived macrophages were kindly provided by Dr. Marcel (Ottawa University Heart Institute). Cell culture media and reagents were from Invitrogen, and mifepristone was from Sigma. Polyclonal antibody against ABCA1 was purchased from Novus Biological Inc. (Littleton, CO), and fluorescent secondary antibodies were from Molecular Probes (Eugene, OR). st-Ht31 and st-Ht31-p were purchased from Promega (Madison, WI). Ht31 was synthesized by Dr. Basak (Ottawa Hospital Research Institute). AKAR-3 and PM-AKAR-3 probes were generous gifts from Dr. Jin Zhang (John Hopkins University).

Cell Culture: BHK cells and RAW 264.7 macrophage were cultured in DMEM plus 10% fetal calf serum and penicillin/streptomycin. To induce expression of ABCA1, BHK cells were incubated with 5 nm mifepristone in DMEM containing 1 mg/ml BSA, and RAW macrophages were induced with 0.3 mM Br-cAMP for 18–20 h. Bone marrow cells were obtained by flushing the femurs of ABCA1+/+ and ABCA1−/− C57 mice, respectively. Macrophages were generated by incubating bone marrow cells (10^6 cells/ml) with DMEM of 10% FBS complemented with 15% L929 conditioned medium for 7 days.

Cholesterol Efflux: BHK or RAW macrophage cells were incubated in growth medium containing 1 μCi/ml [3H]cholesterol for 1–2 days. The medium was replaced by fresh DMEM containing 1 mg/ml BSA plus mifepristone or Br-cAMP. The cells were then incubated with 5 μg/ml human apoA-I or st-Ht31 for 2 h at 37 °C. The amount of [3H]cholesterol in the medium and in cells was counted by scintillation. Efflux was expressed as the percentage of cholesterol in the medium over total cholesterol (medium and cell) (23). The results are presented as the averages of triplicate wells with standard deviation.

FRET-based PKA Activity Assay: BHK cells were grown in 35-mm glass coverslip bottom microscope dishes and transfected with AKAR3 or pm-AKAR3 constructs using Lipofectamine 2000. 20–24 h after transfection, CFP, YFP, and CFPex/YFPem fluorescent images were taken with a 60×/1.4NA objective on an inverted Nikon fluorescent microscope (TE2000-E) equipped with a CCD camera and MetaMorph software (for details on filters, see Ref. 10). At least 10 fields were taken for each condition. After background and cross-talk correction, the sensitized YFP (CFPex/YFPem) image was ratioed to the correspondent CFP image from the same field to produce FRET. To analyze FRET changes, fluorescent images were taken before and after intervention, i.e. addition of st-Ht31. Changes in FRET efficiency in individual cells caused by intervention were then calculated and presented as averages of 50–100 cells and standard errors of the mean. Each experiment was repeated at least twice.

Microparticle Preparation and Characterization: The medium from BHK (ABCA1 and Mock) and RAW macrophages (induced and noninduced) was collected and centrifuged (4000 × g for 15 min and 10,000 × g for 30 min) to remove cell debris. The supernatant was then passed through a 0.2-μm filter. We have previous shown that microparticles are smaller

than 0.2 μm but could not pass through a 100-kDa molecular weight cutoff filter (6). The filtrate was then washed and concentrated with a 100-kDa filter before Western blotting.

st-Ht31 Cellular Uptake Assay: st-Ht31 was fluorescently labeled with Cy2 fluorophore according to the manufacturer's instructions and added to cells for 2 h. After removing Cy2-st-Ht31-containing medium, the cells were imaged using the fluorescent microscope equipped with a CCD camera. Images from ABCA1 and Mock BHK cells were taken and presented under identical conditions.

Oil Red O Staining and Quantification: ABCA1+/+ and ABCA1−/− BMDM cells were incubated in the growth medium containing acetylated LDL (100 μg/ml) for 2 days to allow them to develop into foam cells. The medium was replaced with fresh DMEM containing 1 mg/ml BSA with or without 10 μm st-Ht31 and incubated for 24 h. The cells were then fixed with 10% formaldehyde for 1 h and stained with Oil Red O dye at 4 °C for 2 days. After removing dye, the cells were washed 6–10 times with PBS and imaged using a fluorescent microscope equipped with a cooled CCD camera. Phase contrast pictures of Oil Red O-stained cells were taken from 10 random fields under identical setting, and representative images were shown. Fluorescent images of Oil Red O were obtained with 550-nm excitation and 580-nm emission also from 10 random fields. The fluorescent intensities from each field were then quantified and divided by the number of cells in the field (400–500/field).

2.6 Results

st-Ht31 Induces Robust Microparticle Release in the Absence of Extracellular Acceptor

We reported previously (6) that ABCA1-expressing cells constitutively release cholesterol-rich microparticles, and this release is completely abolished by PKA inhibitor PKI, a six-residue peptide that binds PKA catalytic domain with high affinity (11). To further elaborate on this observation, we tested whether removing PKA from its anchoring sites also influences microparticle release. Ht31 is a 24-residue peptide composed of a PKA-anchoring domain of AKAP and thus binds to PKA regulatory subunit RII with high affinity (12). This prevents PKA from interacting with AKAPs. Ht31 has been frequently used to complement PKI by removing PKA from AKAP, thereby abolishing localized PKA activity (13). When we treated cells with 5 μm st-Ht31, a stearated and thus membrane-permeable form, we were surprised to see that both BHK cells and RAW macrophages robustly released

microparticles, indicated by the presence of cholesterol in the medium, or cholesterol efflux, in the absence of extracellular acceptors (**Figure 2.1, A and C**). This cholesterol release by st-Ht31 predominantly occurs in ABCA1-expressing cells. Furthermore, phospholipid efflux is similarly stimulated (**Figure 2.1B**). Both ABCA1-expressing BHK cells and RAW macrophages responded to st-Ht31 in a dose-dependent manner (**Figure 2.2, A and B**). At 50 μm st-Ht31 (a concentration frequently used in the literature), ABCA1-expressing BHK cells was able to export close to 40% cellular cholesterol in 2 h (**Figure 2.2C**).

The effect of st-Ht31 is acute, because cholesterol efflux stops immediately after removal of st-Ht31 from the medium (**Figure 2.3A, second group of bars from the left**), similar to the efflux induced by apoA-I (**Figure 2.3A, third group**). Neither ABCA1 expression nor distribution was altered by st-Ht31 (not shown). Consistent with this, when the cells were treated with st-Ht31 for the first hour, washed, and then incubated with apoA-I for the second hour, the cells were able to efficiently export cholesterol to apoA-I (**Figure 2.3A, fourth group**). The slightly lower cholesterol efflux to apoA-I in the second hour here, compared with cholesterol efflux to apoA-I in untreated cells (**Figure 2.3A, third group from left**), likely reflects the cholesterol depletion by st-Ht31 during the first hour. Furthermore, we found that st-Ht31 mainly stimulates the release of microparticles, similar to the ones we reported earlier by FPLC analysis (6). ABCA1 phosphorylation by PKA was not significantly altered, as probed by an antibody recognizing phosphorylated PKA substrates (**Figure 2.3B**). Perhaps most importantly, st-Ht31-treated cells remained perfectly viable in a wide range of st-Ht31 concentrations up to 50 μm, shown by a methylthiazol tetrazolium test (**Figure 2.3C**), a well established metabolic assay for cell viability (14). This demonstrates that the robust cholesterol export by st-Ht31 is not due to cell damage or membrane fragmentation.

st-Ht31 Increases PKA Activity Specifically in the Cytoplasm

To explore the mechanism by which st-Ht31 exports cholesterol, we next analyzed spatially resolved PKA activities in st-Ht31-treated cells. Unlike PKI, st-Ht31 interferes with PKA anchoring without directly affecting PKA catalytic capacity. We reasoned that preventing PKA from anchoring should increase PKA in the cytoplasm, which in turn could elevate PKA activity specifically in the cytoplasm. Hence, we employed FRET-based biosensors to analyze compartmentalized PKA activities in live cells. The main component

Figure 2.1 **st-Ht31 enhances cholesterol/phospholipid efflux. A.** BHK cells were incubated in growth medium containing 1 μCi/ml [3H]cholesterol for 1–2 days and then induced with mifepristone overnight. Cholesterol efflux was analyzed by quantifying the percentage of cell-associated cholesterol appeared in the medium during a 2-h period with or without st-Ht31. **B.** BHK cells prelabeled with [3H]choline chloride were treated as in A. Phospholipids containing [3H]choline were extracted from both medium and cells to calculate the percentage of [3H]choline containing phospholipids in the medium. **C.** RAW macrophages were treated identically as BHK cells in A, except that they were induced with 8-Br-cAMP. The bars represent the means ± standard deviations of triplicate wells.

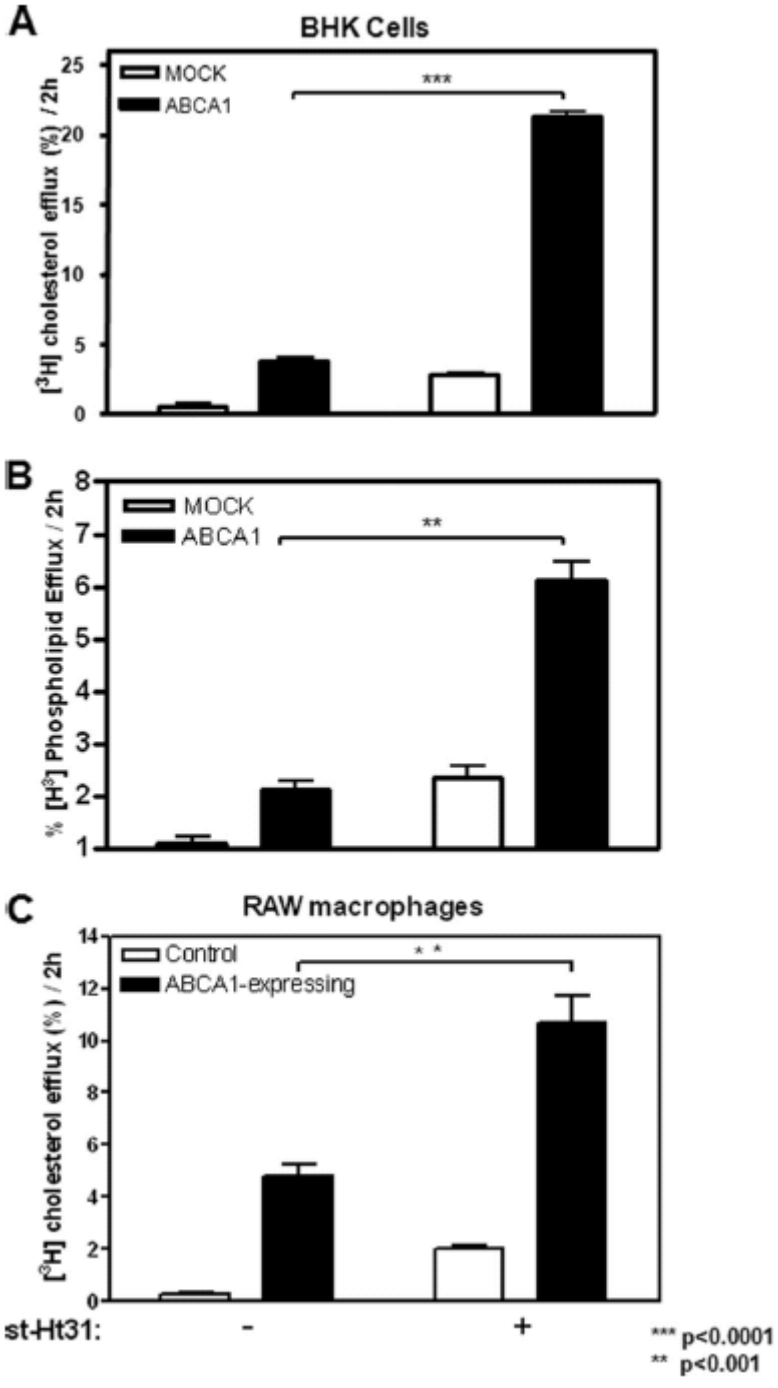

A
BHK Cells
MOCK
ABCA1
***
[3H] cholesterol efflux (%) / 2h
25
20
15
10
5
0
B
MOCK
ABCA1
**
% [H3] Phospholipid Efflux / 2h
8
7
6
5
4
3
2
1
C
RAW macrophages
Control
ABCA1-expressing
* *
[3H] cholesterol efflux (%) / 2h
14
12
10
8
6
4
2
0
st-Ht31:
-
+
*** p<0.0001
** p<0.001

Figure 2.2 **Dose dependent increase of cholesterol efflux by st-Ht31. A.** RAW macrophages were incubated in growth medium containing 1 μCi/ml [3H]-cholesterol for 1-2 days and then induced with 8-Br-cAMP overnight. Cholesterol efflux was analyzed by quantifying the percentage of cell-associated cholesterol appeared in the medium during 2 hour period with 1 μM, 3 μM, or 5 μM st-Ht31. **B.** ABCA1-BHK cells were treated as in A except induced with mifepristone overnight. **C.** Similarly induced ABCA1-BHK cells were incubated 50 μM st-Ht31 or without.

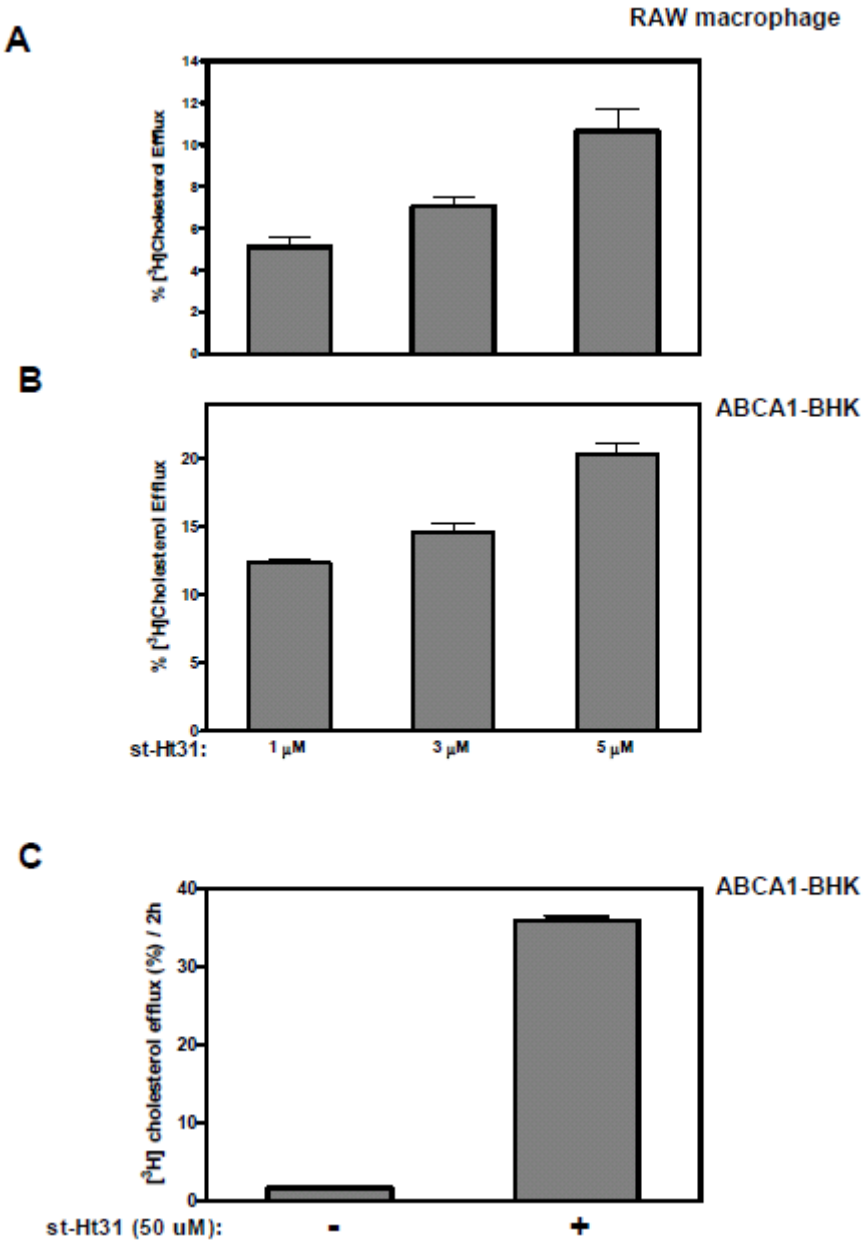
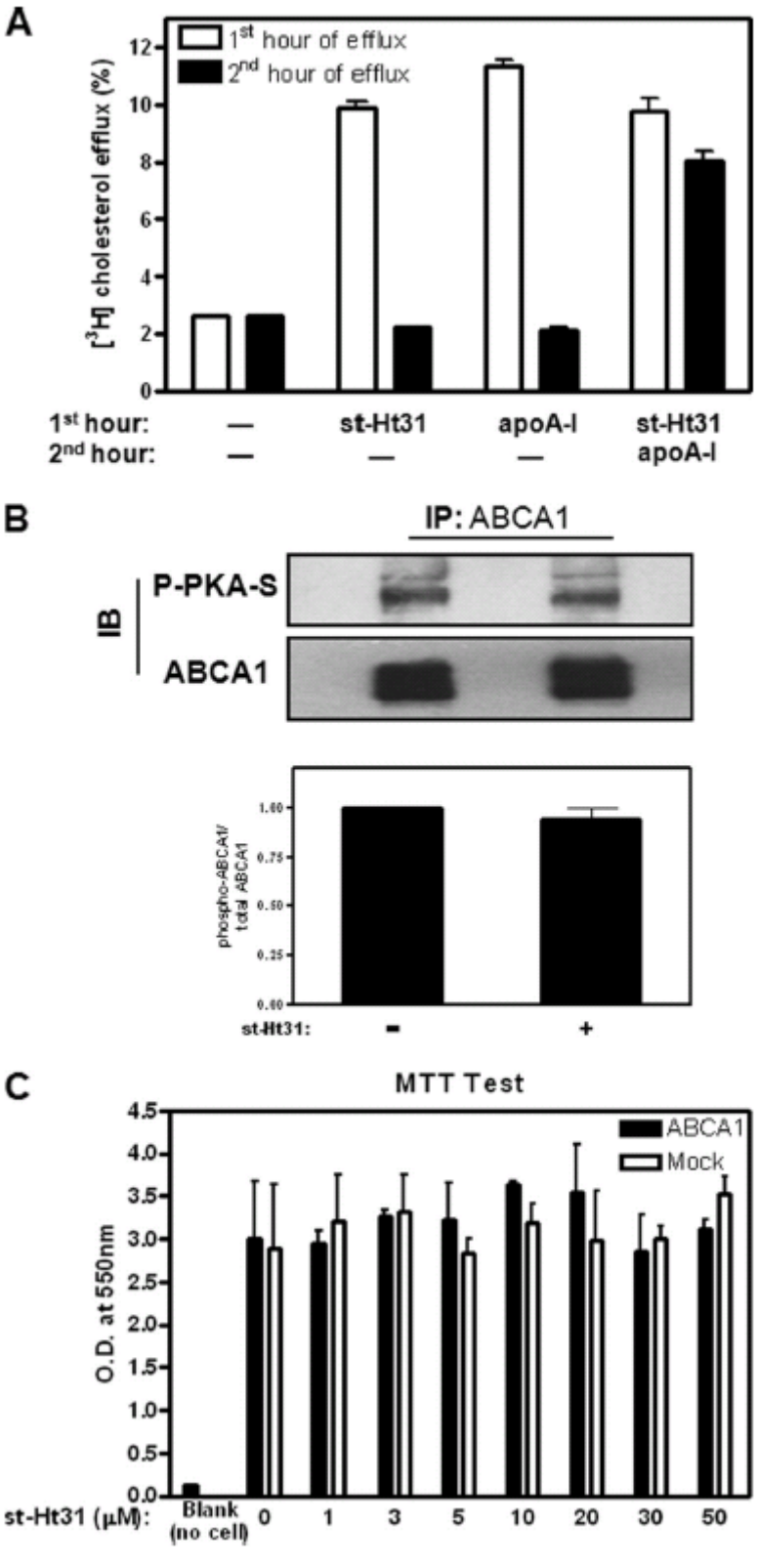

A
RAW macrophage
% [3H]Cholesterol Efflux
0
2
4
6
8
10
12
14
B
ABCA1-BHK
% [3H]Cholesterol Efflux
0
5
10
15
20
st-Ht31:
1 μM
3 μM
5 μM
C
ABCA1-BHK
[3H] cholesterol efflux (%) / 2h
0
10
20
30
40
st-Ht31 (50 uM):
-
+

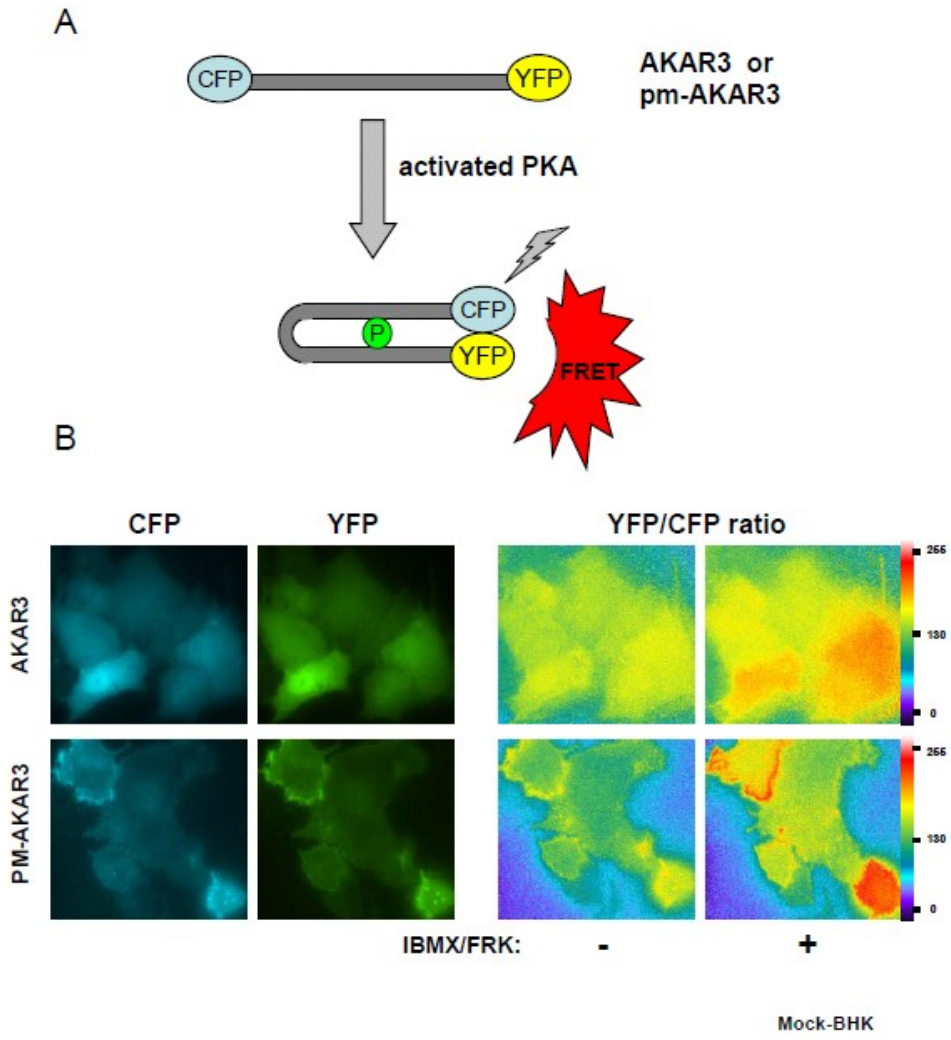
Figure 2.3 **st-Ht31 exerts no detectable toxicity and no significant influence on ABCA1 phosphorylation. A.** ABCA1-BHK cells prelabeled with 1 μCi/ml [^{3}H]cholesterol were incubated with either apoA-I (5 μg/ml) or st-Ht31 (5 μM) for the first hour. The cells were then washed and switched to DMEM or medium containing human apoA-I (5 μg/ml), as indicated, for the second hour. The percentages of cholesterol appeared in the medium during the first hour (*open bar*) or second hour (*filled bar*) were analyzed. **B.** induced ABCA1-BHK cells were treated with st-Ht31 (5 μM) for 2 h. ABCA1 was immunoprecipitated and blotted using ABCA1 and antibody phospho-PKA substrate antibody, respectively. Representative blots from three independent experiments were shown. The *bars* represent the means ± standard deviations. **C.** induced BHK cells were treated with st-Ht31 at indicated concentration for 2 h. Methylthiazol tetrazolium toxicity tests were carried out. The results are presented as O.D. (550 nm) readings from formazan, and the *bars* represent the means ± standard deviation of triplicate wells. *IP*, immunoprecipitation; *IB*, immunoblot.


A
1st hour of efflux
2nd hour of efflux
[3H] cholesterol efflux (%)
0
2
4
6
8
10
12
1st hour:
2nd hour:
—
—
st-Ht31
—
apoA-I
—
st-Ht31
apoA-I
B
IP: ABCA1
IB
P-PKA-S
ABCA1
phospho-ABCA1/ total ABCA1
0.00
0.25
0.50
0.75
1.00
st-Ht31:
-
+
C
MTT Test
ABCA1
Mock
O.D. at 550nm
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
st-Ht31 (μM):
Blank (no cell)
0
1
3
5
10
20
30
50

of the sensor, termed PKA kinase activity reporter 3 (AKAR3), is composed of a PKA substrate peptide and a phosphorylation binding domain sandwiched between cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP). Upon phosphorylation by PKA, the peptide undergoes a conformational change that brings CFP in close proximity to YFP. This generates sensitized YFP or FRET signal (10) (**Figure 2.4A**). AKAR3 can also be targeted to specific subcellular sites through additional localization motifs, which enable them to report PKA activities in their immediate environment in live cells. We were particularly interested in PKA activity in the cytoplasm as well as at the plasma membrane, because many known AKAPs are located at the plasma membrane.

AKAR3 and pm-AKAR3, a cytosolic form and a plasma membrane targeted variance, respectively (10), were expressed in ABCA1- and Mock-BHK cells. As expected, pm-AKAR3 mainly decorates the plasma membrane, whereas AKAR3 is diffusely distributed in the cytoplasm (**Figure 2.5A**). To measure FRET, a pair of CFP and sensitized YFP images was taken from the same field of the cells before and after st-Ht31 addition (the pair taken before adding st-Ht31 is shown). Each pair of images was then processed to produce a ratio image, indicative of FRET intensity (**Figure 2.5B**). FRET intensities before and after st-Ht31 addition were then compared and presented as percentages of change in individual cells in response to st-Ht31. The results from individual cells were then pooled to give FRET efficiency for the cell population. As shown in Fig. 3C, st-Ht31 significantly increased PKA activity in the cytoplasm of ABCA1-expressing BHK cells, as indicated by the boost in FRET change of AKAR3. The plasma membrane PKA activity, probed by pm-AKAR, did not increase by st-Ht31. Instead, there was a drop in plasma membrane PKA activity upon st-Ht31 addition, perhaps reflecting PKA de-anchoring from the plasma membrane. The response from similarly treated Mock-BHK cells was minimal (**Figure 2.5C**). To validate our experimental protocol, we stimulated Mock-BHK cells with forskolin/isobutylmethylxanthine, which artificially activate PKA, and detected elevated FRET signals from both biosensors (**Figure 2.4, B and C**). In addition, we probed phosphorylation of CREB, a PKA substrate. We found that st-Ht31 increases CREB phosphorylation in induced and hence ABCA1-expressing RAW macrophages, whereas this phosphorylation is minimally altered in noninduced macrophages (**Figure 2.5D**). Together, we conclude that st-Ht31 activates PKA, and this activation is predominantly in the

Figure 2.4 **AKAR3 and pm-AKAR3 detects activation of PKA by forskolin/IBMX in Mock-BHK cells. A.** Model diagram of AKAR3 and pm-AKAR3 FRET-based biosensors. **B.** CFP and sensitized YFP images were taken before and after Frk/IBMX addition (10 min). After background and cross-talk correction, a pair of ratio images (YFP/CFP) was produced and displayed under identical setting. **C.** FRET intensities (YFP/CFP) was calculated for each individual cell and presented as percentage change before and after forskolin /IBMX addition. Data represents the average from 40-100 cells and standard error of the mean.


A
CFP
YFP
AKAR3 or
pm-AKAR3
activated PKA
CFP
P
YFP
FRET
B
CFP
YFP
YFP/CFP ratio
AKAR3
PM-AKAR3
255
130
0
IBMX/FRK:
-
+
Mock-BHK

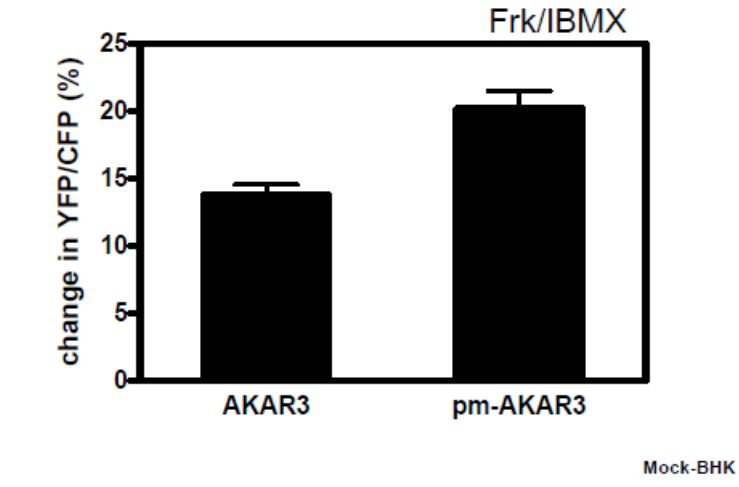
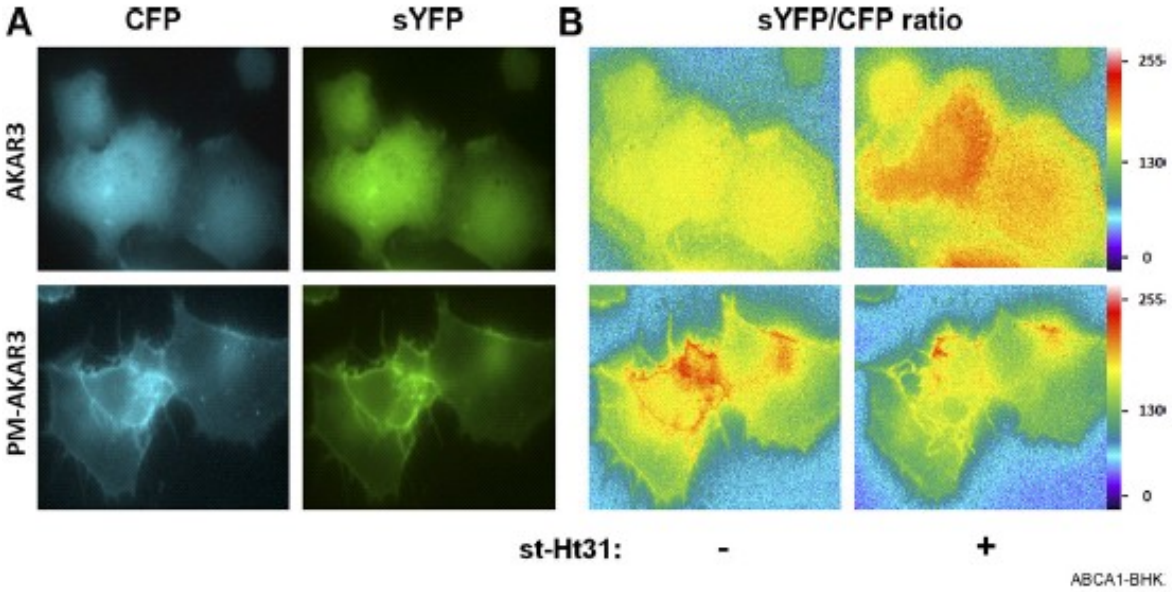
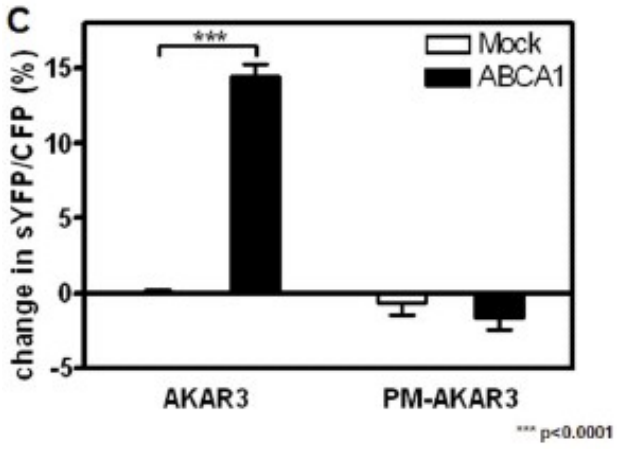
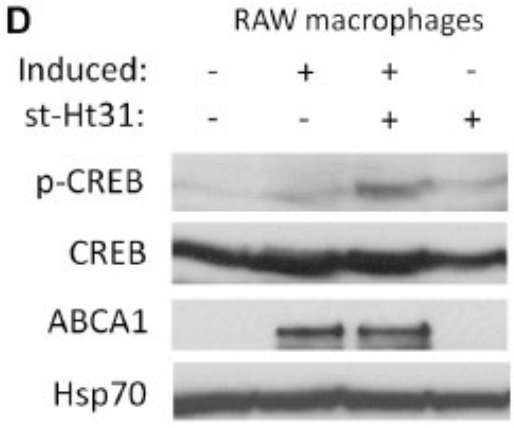

C
Frk/IBMX
change in YFP/CFP (%)
25
20
15
10
5
0
AKAR3
pm-AKAR3
Mock-BHK

Figure 2.5 **st-Ht31 specifically increases PKA activity in the cytoplasm. A.** ABCA1-BHK cells were transfected with either AKAR3 or pm-AKAR3 and induced overnight with mifepristone. Representative images from CFP and sensitized YFP channels were shown. **B.** CFP and sensitized YFP images were taken before and after st-Ht31 addition (10 min). After background and cross-talk correction, a pair of ratio images (YFP/CFP) was produced and displayed under identical setting. **C.** FRET intensities (YFP/CFP) were calculated for each individual cell and presented as percentages of change before and after st-Ht31 addition. The data represent the averages from 50–100 cells and standard errors of the mean. **D.** PKA activity was also analyzed by Western blotting using antibodies against CREB and phospho-CREB. ABCA1 was probed by a monoclonal antibody. Hsp70 was used as loading control.


A
CFP
sYFP
B
sYFP/CFP ratio
AKAR3
PM-AKAR3
255
130
0
st-Ht31:
-
+
ABCA1-BHK


C
change in sYFP/CFP (%)
15
10
5
0
-5
***
Mock
ABCA1
AKAR3
PM-AKAR3
*** p<0.0001


D
RAW macrophages
Induced:
- + + -
st-Ht31:
- - + +
p-CREB
CREB
ABCA1
Hsp70

cytoplasm.

PKA Activity Is Required for st-Ht31-induced Cholesterol Efflux

We next examined whether this elevated PKA activity is the cause of cholesterol efflux triggered by st-Ht31. If so, the cholesterol efflux stimulated by st-Ht31 should be sensitive to PKA inhibition. Indeed, we found that PKI effectively abolished this process (**Figure 2.6A**), consistent with cytosolic PKA activation by st-Ht31.

Furthermore, Ht31 is known to form an amphipathic helix (12), a functional characteristic also shared by apoA-I. We have established previously that apoA-I primarily acquires cholesterol from the plasma membrane without the need of endocytosis (15). To test the possibility that st-Ht31 might mimic apoA-I and mediate cholesterol export by lipidating itself at the plasma membrane without entering cells, we incubated cells with Ht31, a nonstearated and thus nonpermeate form. As shown in **Figure 2.6B**, Ht31 at the similar concentration did not induce cholesterol efflux. This demonstrates that although Ht31 forms amphipathic helix, such a structure motif alone is not sufficient to acquire membrane cholesterol. Ht31 therefore cannot mimic apoA-I.

We also tested a control peptide st-Ht31-p, which differs from st-Ht31 by isoleucine-to-proline substitutions and consequently can no longer bind to PKA (12). st-Ht31-p had no effect on cholesterol efflux (**Figure 2.6B**). Together, these experiments demonstrate that st-Ht31 exerts its influence on cholesterol efflux intracellularly and through its capacity of releasing and activating PKA.

ABCA1 Facilitates st-Ht31 Uptake to Efficiently Stimulate Cholesterol Efflux

We noted that st-Ht31 promotes cholesterol efflux primarily from ABCA1-expressing cells (**Figure 2.1**). PKA activation by st-Ht31, as measured with FRET, is also only seen in ABCA1-expressing BHK cells (**Figure 2.5**). To explore the mechanism by which ABCA1 accentuates the activity of st-Ht31, we tested whether ABCA1 may facilitate st-Ht31 uptake. Because st-Ht31 has to reach its target, PKA, inside of cells to be effective, an efficient uptake would be necessary for its function. We therefore analyzed the uptake of a fluorescently tagged st-Ht31. Cy2-st-Ht31 functioned equally well in promoting cholesterol export as untagged st-Ht31 (not shown). When BHK cells were incubated with Cy2-st-Ht31 on ice for 2 h, we observed much more st-Ht31 taken up by ABCA1-expressing cells than by Mock cells (**Figure 2.7A**). This suggests that st-Ht31 is

Figure 2.6 **st-Ht31 relies on its capacity to activate PKA, de-anchor PKA, and permeate into cells to enhance cholesterol export. A.** BHK cells prelabeled with 1 μCi/ml [3H]cholesterol were analyzed for their capacity to efflux cholesterol in the presence of st-Ht31 (5 μM) with or without PKI (50 μM) for 2 h. **B.** cells were also analyzed for their capacity to efflux cholesterol in the presence of Ht31, st-Ht31-P, or st-Ht31 for 2 h. The bars represent the means ± standard deviations of triplicate wells.

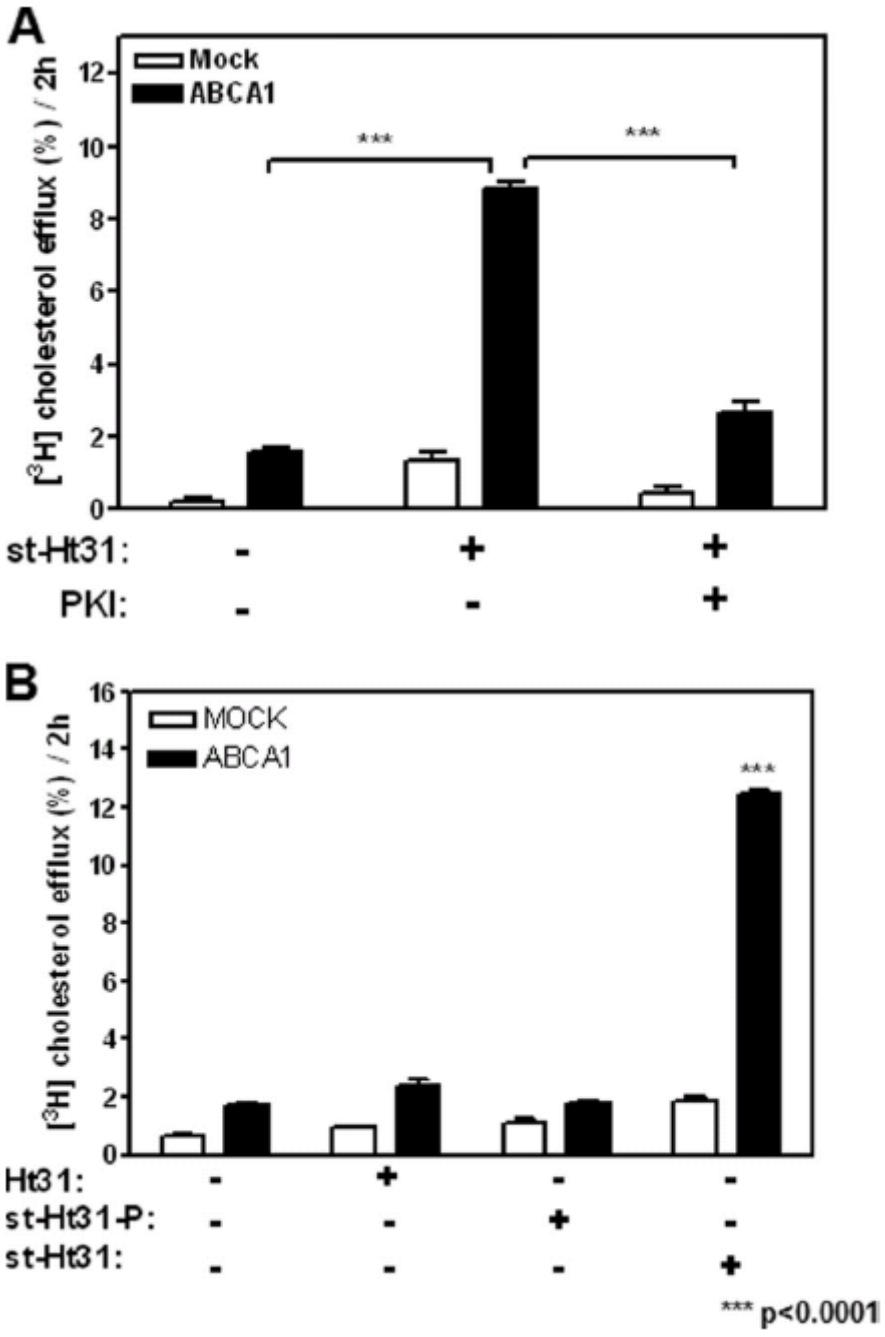
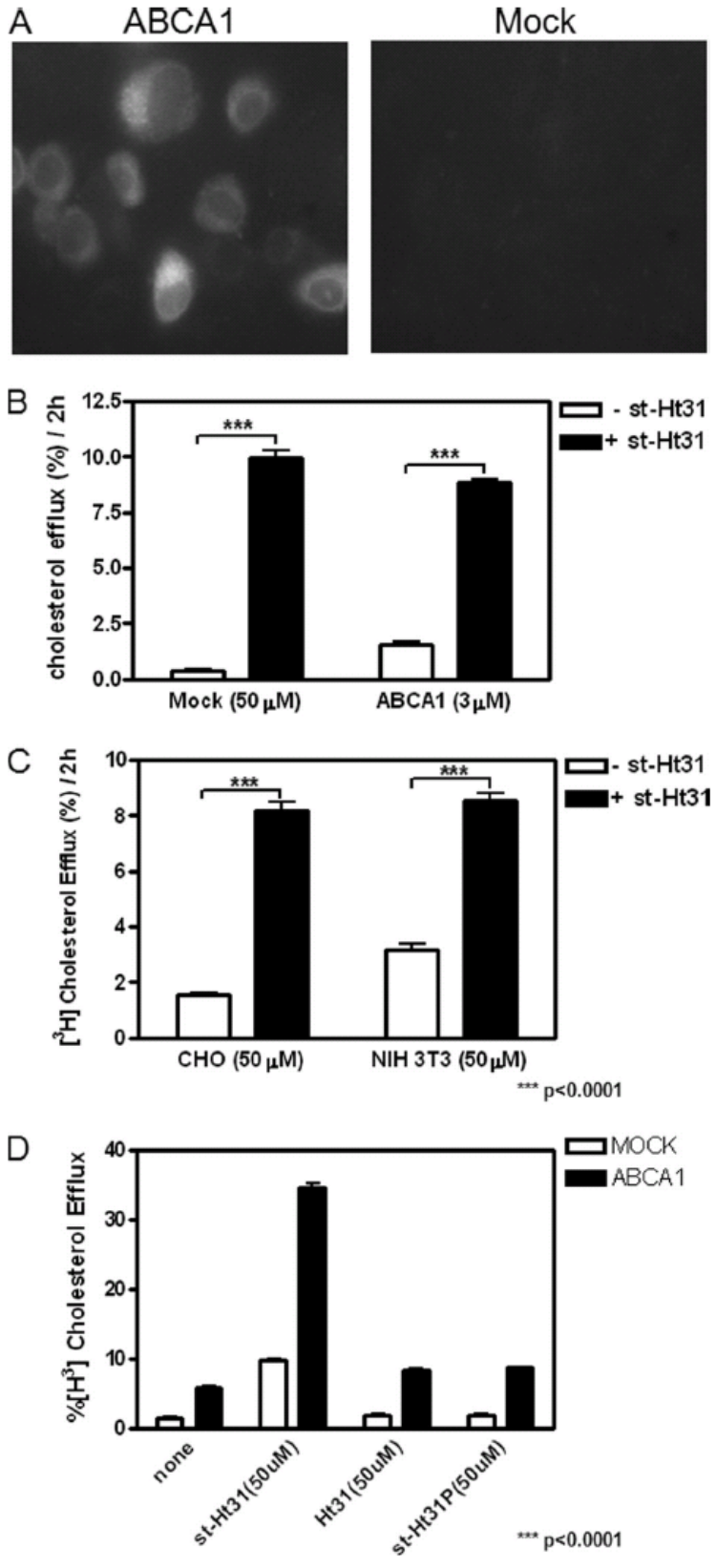

A
Mock
ABCA1
[3H] cholesterol efflux (%) / 2h
***
***
st-Ht31:
PKI:
B
MOCK
ABCA1
[3H] cholesterol efflux (%) / 2h
***
Ht31:
st-Ht31-P:
st-Ht31:
*** p<0.0001

Figure 2.7 **ABCA1 expression increases permeability of st-Ht31. A.** BHK cells were incubated with mifepristone overnight and then treated with Cy2-st-Ht31 (10 μm) for 2 h on ice. Images from ABCA1-BHK or Mock-BHK cells were taken under identical conditions and displayed identically. **B.** BHK cells were induced with mifepristone overnight. Cholesterol efflux was analyzed by quantifying the percentage of cell-associated cholesterol that appeared in the medium during a 2-h period with 50 μm (Mock) or 3 μm (ABCA1) st-Ht31. **C.** CHO and NIH 3T3 cells were incubated in growth medium containing 1 μCi/ml [3H]cholesterol for 1–2 days. Cholesterol efflux was analyzed by quantifying the percentage of cell-associated cholesterol appeared in the medium during a 2-h period with 50 μm st-Ht31. **D.** BHK cells were similarly treated as in C. The cells were then incubated with 50 μm Ht-31, st-Ht31-P, or st-Ht31, respectively, for 2 h, before cholesterol efflux analysis. The bars represent the means ± standard deviations of triplicate wells.


A
ABCA1
Mock
B
cholesterol efflux (%) / 2h
12.5
10.0
7.5
5.0
2.5
0.0
***
***
- st-Ht31
+ st-Ht31
Mock (50 μM)
ABCA1 (3 μM)
C
[3H] Cholesterol Efflux (%) / 2h
10
8
6
4
2
0
***
***
- st-Ht31
+ st-Ht31
CHO (50 μM)
NIH 3T3 (50 μM)
*** p<0.0001
D
%[H3] Cholesterol Efflux
40
30
20
10
0
MOCK
ABCA1
none
st-Ht31(50uM)
Ht31(50uM)
st-Ht31P(50uM)
*** p<0.0001

able to function much more effectively in ABCA1-expressing cells, at least partially because of enhanced st-Ht31 uptake through ABCA1.

If st-Ht31 uptake is the key event downstream of ABCA1, we should be able to induce ABCA1-null cells to export cholesterol by forcing more st-Ht31 into these cells. We therefore treated ABCA1-null cells with 50 μm st-Ht31, a concentration more than 10 times higher than we normally used. We indeed observed a moderate cholesterol efflux from Mock-BHK cells, comparable with that from ABCA1-BHK cells treated with 3 μm st-Ht31 (**Figure 2.7B**). Cholesterol release can also be moderately stimulated by 50 μm st-Ht31 in CHO cells and NIH 3T3 fibroblasts that express little ABCA1 (**Figure 2.7C**). 50 μm st-Ht31, when applied to ABCA1-expressing BHK cells, was able to export large quantity of cholesterol (~40% in 2 h; **Figure 2.2C**) without any apparent toxicity (**Figure 2.3**). Importantly, even at 50 μm, neither nonstearated Ht31 nor st-Ht31p, the defective mutant, was able to promote cholesterol export from ABCA1- or Mock-BHK cells (**Figure 2.7D**). This once again supports the notion that intracellular PKA de-anchoring is essential for st-Ht31 to promote cholesterol export. Taken together, we conclude that st-Ht31 stimulates cholesterol export preferentially from ABCA1-expressing cells. This is likely due to efficient st-Ht31 uptake by ABCA1, which in turn activates PKA in the cytoplasm to promote cholesterol export.

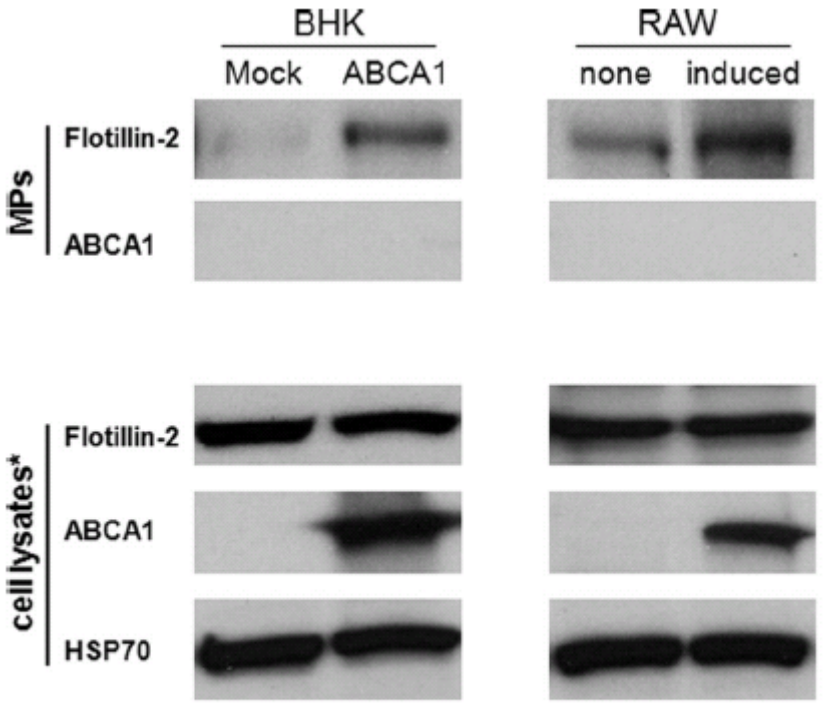
ABCA1 Likely Exports Cholesterol through Exosome Pathway

We have so far established that ABCA1-expressing cells constitutively export cholesterol because microparticles and st-Ht31 further promote this process. However, the origin of the microparticles remains elusive. It was recently reported that oligo-dentroglial precursor cells export cholesterol by exosome pathway (16). We therefore asked whether microparticle release is also through a similar mechanism. We indeed found flotillin-2, an exosome marker (17), in microparticle preparations from the medium of ABCA1-expressing BHK cells and RAW macrophages, respectively (**Figure 2.8**). The expression of flotillin-2 is not altered by ABCA1. In addition, ABCA1 itself, a plasma membrane protein, did not appear in the microparticle preparations. Together, our observations suggest that ABCA1-expressing cells release microparticles through a mechanism similar to the exosome pathway.

st-Ht31 Reverses Foam Cell Formation and Restores Metabolic Health of Macrophage

Perhaps most intriguingly, this ABCA1-facilitated cholesterol export by st-Ht31 may

Figure 2.8 **Flotillin 2 is preferentially associated with microparticles released from ABCA1-expressing cells.** BHK were induced with mifepristone, and RAW macrophages were either induced with 8-Br-cAMP (induced) or without (none) overnight. The medium was collected from equal numbers of cells, and microparticles (MPs) were prepared by a series centrifugation and filtration before being concentrated with a 100-kDa filter. Cell lysates (1/30 of total) and microparticle preparations were then analyzed by Western blotting using antibodies against flotillin 2, ABCA1, and HSP70, respectively.



*loading: 1/30 of total cell lysates

provide a unique opportunity to specifically relieve cholesterol burden from foam cells. ABCA1 is highly expressed in these macrophages as a consequence of excess cholesterol (18). st-Ht31 may preferentially remove cholesterol from foam cells, without affecting macrophages that do not express ABCA1. To test this, we treated BMDMs from ABCA1+/+ and ABCA1−/− mice with acetylated LDL for 2 days. This produces foam cells. We then removed acetylated LDL and added 10 μm st-Ht31 or medium alone for another 24 h. The cells were then stained with Oil Red O to visualize neutral lipids. Without st-Ht31, both ABCA1+/+ and ABCA1−/− BMDM remained as typical foam cells with abundant lipid droplets and heavily stained by Oil Red O (**Figure 2.9, A and B**). With st-Ht31, however, ABCA1+/+ BMDM were almost void of neutral lipids, evidenced by greatly diminished lipid droplets and Oil Red O staining (**Figure 2.9C**). BMDM are now restored to a much healthier morphology. In contrast, st-Ht31 had little effect on ABCA1−/− BMDM (**Figure 2.9D**). This is consistent with our observations that, without ABCA1 to take up st-Ht31 in Mock-BHK cells or uninduced RAW macrophages, low concentrations of st-Ht31 ($\ll$ 50 μm) were unable to induce significant cholesterol efflux (**Figure 2.1**). We quantified Oil Red O by its fluorescent intensity (19) (**Figure 2.10**) and found that st-Ht31 removed ~90% neutral lipids from ABCA1+/+ BMDM foam cells but not at all from ABCA1−/− foam cells (**Figure 2.9E**). Interestingly, the effect of st-Ht31 is reminiscent of that of apoA-I. apoA-I at 10 μg/ml was also able to fully reverse foam cell formation (**Figure 2.10A**).

Macrophages with heavy cholesterol load are known to be susceptible to excessive pro-inflammatory activation (20). We therefore tested whether st-Ht31 could rescue foam cells from such heightened inflammatory response. As shown in **Figure 2.9F**, cholesterol loading by acetylated LDL greatly augmented LPS-induced TNF-α secretion in both $ABCA1^{+/+}$ and $ABCA1^{-/-}$ BMDM. However, st-Ht31 was able to completely suppress TNF-α secretion in $ABCA1^{+/+}$ BMDM without affecting $ABCA1^{-/-}$ BMDM. Once again, the suppression of TNF-α secretion by st-Ht31 is comparable with that by apoA-I (**Figure 2.10B**), consistent with their similar capacity of removing cholesterol. Together, we conclude that st-Ht31 is capable of reversing foam cell formation in an ABCA1-dependent fashion. Perhaps more importantly, st-Ht31 is able to restore metabolic homeostasis in these

Figure 2.9 **st-Ht31 reverses foam cell formation in ABCA1+/+ BMDM, but not ABCA1−/− BMDM. A–D.** BMDM from ABCA1+/+ and ABCA1−/− mice were incubated with acetyl-LDL (acLDL, 100 μg/ml) for 2 days, washed, and changed into the fresh medium with (C and D) or without (A and B) st-Ht31 (10 μg/ml) for another day (day 3). The cells were then fixed and stained with Oil Red O. **E.** Oil Red O fluorescence was recorded and quantified from 10 random fields and expressed as fluorescent intensity/cell. The bars are the means ± S.E. of the mean. **F.** similarly treated BMDM as in A–D were challenged after day 3 with LPS (100 ng/ml). The medium was collected and analyzed by ELISA for secreted TNF-α. The data are presented as the means of triplicates ± standard deviation.

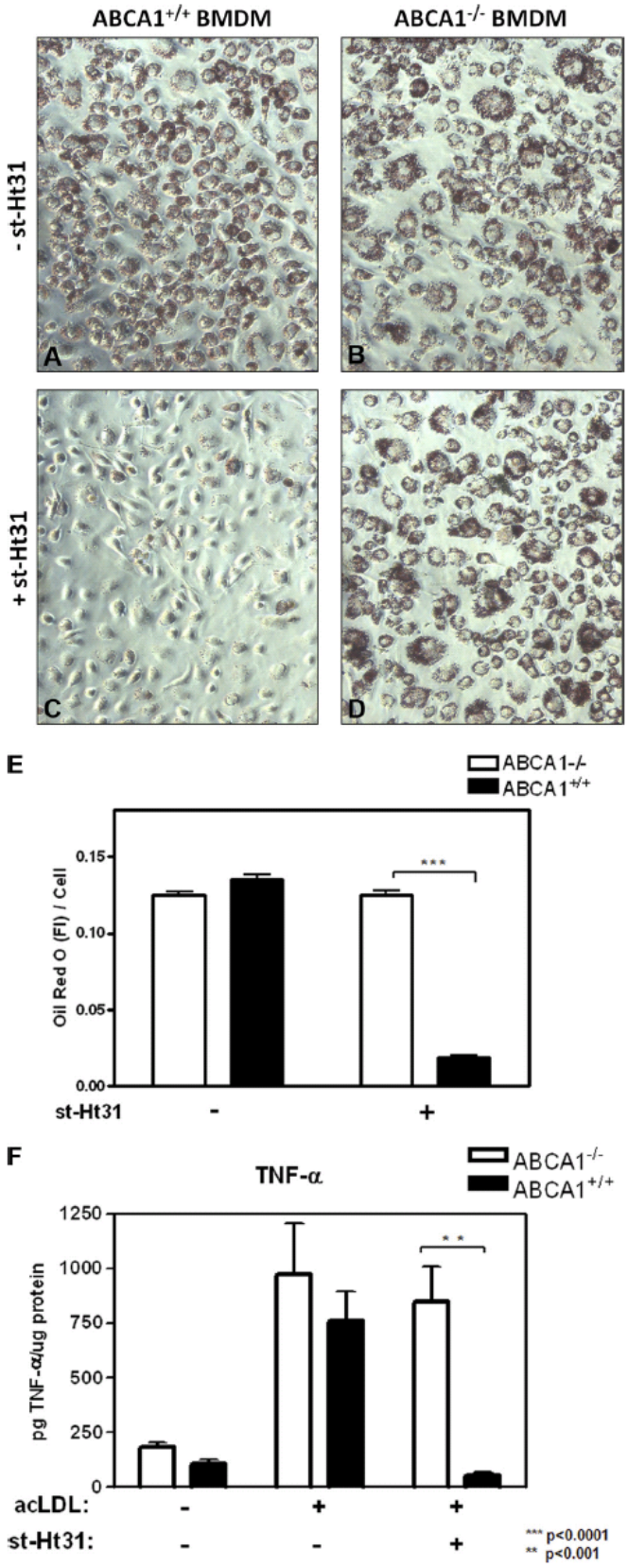
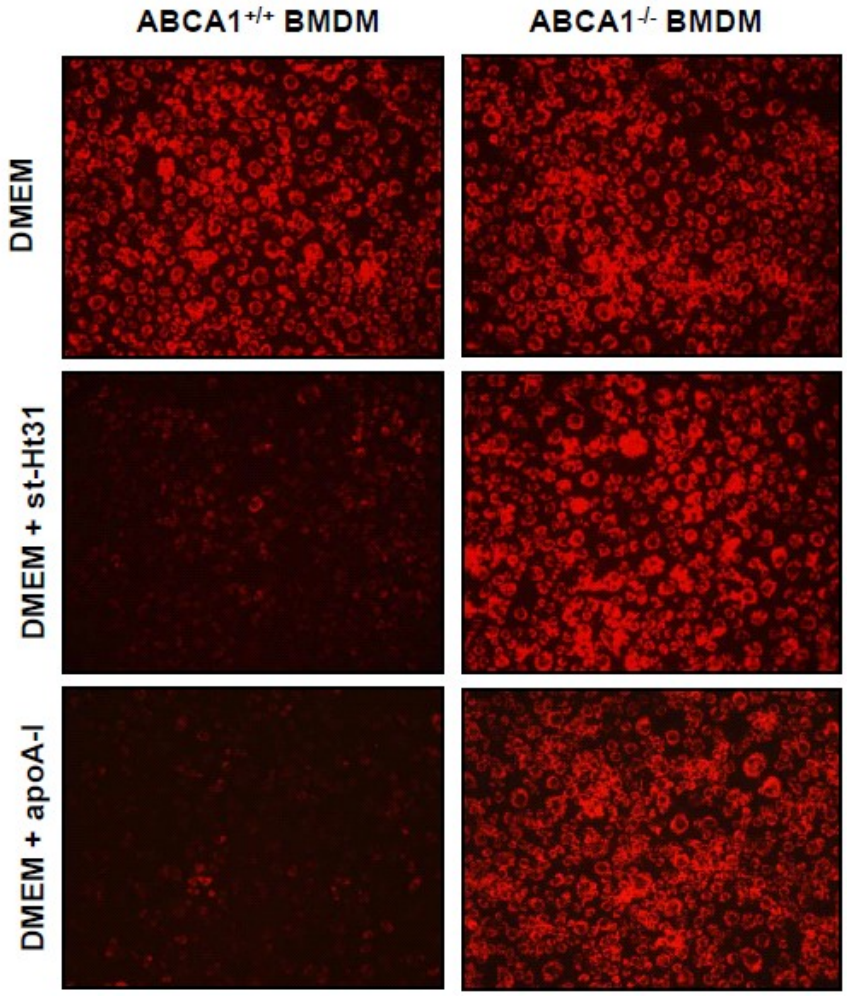
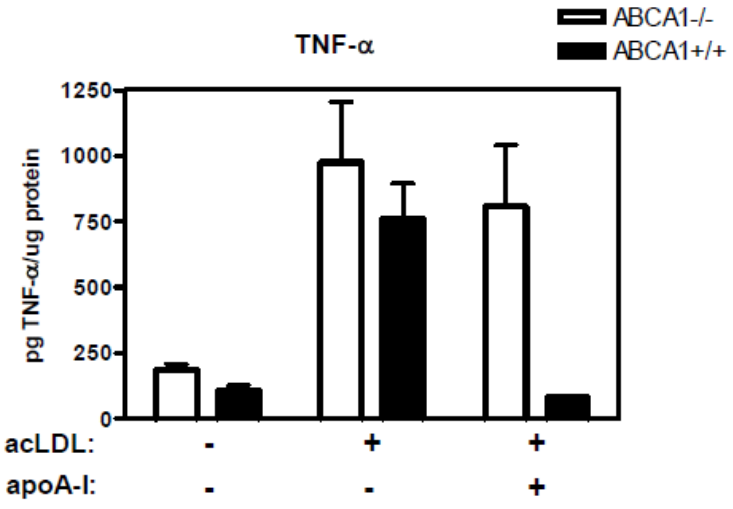

ABCA1+/+ BMDM
ABCA1-/- BMDM
- st-Ht31
+ st-Ht31
A
B
C
D
E
ABCA1-/-
ABCA1+/+
Oil Red O (FI) / Cell
0.15
0.10
0.05
0.00
***
st-Ht31
-
+
F
TNF-α
ABCA1-/-
ABCA1+/+
pg TNF-α/ug protein
1250
1000
750
500
250
0
**
acLDL:
st-Ht31:
*** p<0.0001
** p<0.001

Figure 2.10 **Effect of st-Ht31 and apoA-I on foam cell formation. A.** BMDM from ABCA1$^{+/+}$ and ABCA1$^{-/-}$ mice were incubated with acetyl-LDL (100 μg/ml) for 2 days, then washed and changed into the fresh medium with or without either st-Ht31 (10 μg/ml) or apoA-I (10 μg/ml) for another day (day 3). Cells were then stained with Oil red O. Fluorescent images of Oil red O were taken and displayed under identical settings. **B.** Similarly treated BMDM as in *A*. were challenged after day 3 with LPS (100 ng/ml). The medium was collected and analyzed by ELISA for secreted TNF-α.

A


ABCA1+/+ BMDM
ABCA1-/- BMDM
DMEM
DMEM + st-Ht31
DMEM + apoA-I

B


TNF-α
ABCA1-/-
ABCA1+/+
pg TNF-α/ug protein
1250
1000
750
500
250
0
acLDL: - + +
apoA-I: - - +

macrophages such that they can now mount a well modulated inflammatory response to LPS challenge.

2.7 Discussion

In this study, we identify a novel mechanism that promotes robust cholesterol export in an ABCA1-dependent fashion. We provide evidence that, by inhibiting PKA anchoring on AKAPs, st-Ht31 enhances cytosolic PKA activity, and this PKA activity is essential for cholesterol export. ABCA1 facilitates st-Ht31 uptake and thereby increases the effective activity of PKA in the cytoplasm. This ultimately leads to efficient cholesterol export. Significantly, at 10 μm, st-Ht31 is able to exclusively remove lipids from ABCA1+/+ foam cells and restore these cells back to healthy macrophages. st-Ht31 at 10 μm has little effect on ABCA1−/− BMDM foam cells and on BHK cells or RAW macrophages that do not express ABCA1.

In many ways, the effect of st-Ht31 is similar to that of apoA-I. It is well established that apoA-I can relieve lipid burden from macrophage foam cells through ABCA1. We indeed observed a similar reversing effect on ABCA1+/+ foam cells by apoA-I. Through this process, apoA-I initiates the so-called "reverse cholesterol transport" that shuttles excess cholesterol to the liver for excretion. Perhaps more importantly, by removing excess lipids, apoA-I restores the healthy homeostasis in macrophages. This ensures that the macrophages can continue with their anti-inflammatory and anti-atherogenic functions. These include eliminating oxidized LDL and apoptotic bodies, modulating inflammatory processes, and repairing tissue damage. As we show here, st-Ht31 is equally as effective in reversing foam cell formation as apoA-I. In this way, st-Ht31 could play an important role in promoting healthy macrophages. Indeed, st-Ht31-treated macrophages display well modulated inflammatory responses, similar to the ones treated with apoA-I.

It is noteworthy that st-Ht31 is less than 1/10 of apoA-I in size (24 amino acids versus 244 amino acids). This will likely provide a significant advantage if st-Ht31 were to be used in therapeutic interventions. st-Ht31 is similar in some ways to apoA-I mimic peptides, particularly 5A, one of the 37-pA derivatives (21). However, there is a fundamental difference between st-Ht31 and apoA-I mimics. apoA-I mimics, as the name implies, have apoA-I-like structure motifs that are capable of loading lipids onto themselves. Ht31 does not sequester lipids onto itself. Instead, it targets to a particular domain of a known molecule,

PKA RII subunit, to release cellular cholesterol. This is a novel and unique advantage. The crystal structure of the PKA anchoring domain is known (22), and small nonpeptide molecules can be readily developed to target this domain. These small molecules can be further tailored to target ABCA1-expressing cells, which would allow cholesterol removal specifically from foam cells.

Suppression of inflammatory cytokine secretion by st-Ht31 is not entirely unexpected, because depleting cellular cholesterol by cyclodextrin is known to have similar effects (25). However, MCD removes cholesterol indiscriminately and suppresses inflammatory responses independent of ABCA1 expression (25). This could potentially suppress constructive inflammatory responses aimed to restore tissue homeostasis. st-Ht31, conversely, specifically modulates the pool of macrophages that are highly lipid-loaded and at risk of causing damage to blood vessels. Therefore, this has to be beneficial in atherogenic environments.

Currently, we do not understand the fate of the cholesterol released by st-Ht31. However, compared with the whole plasma cholesterol pool, the amount of cholesterol released from lipid-laden foam cells must be very small and will not likely influence plasma lipid profile. This is supported by the observation that macrophage-specific deletion of ABCA1 in Ldlr−/− or apoE−/− mouse models does not alter plasma lipid profile including HDL levels. Macrophage-specific deletion nevertheless significantly exaggerates atherosclerotic lesion formation (26–28). This again strongly supports the critical importance of healthy macrophages at potential lesion sites. If these macrophages remain healthy, there is a much better chance for injured vessels to be repaired even under highly unfavorable lipid environments such as those in Ldlr−/− or apoE−/− animals. It is therefore tempting to speculate that st-Ht31 or its nonpeptide analogues could exert significant anti-atherogenic functions in vivo by specifically relieving cholesterol burden from lesion foam macrophages.

This is also the first report to identify a spatial pool of PKA for cholesterol export. Interestingly, when we increased PKA activities by artificially raising cAMP levels using forskolin/isobutylmethylxanthine presumably at all cellular locations (**Figure 2.4**), cholesterol efflux was not altered. This may reflect a necessity for finely orchestrated spatial and temporal PKA activation in biological events. The mechanism by which cytosolic PKA activation promotes cholesterol efflux remains to be determined and is currently under

investigation. Another interesting aspect from the current study is that disrupting PKA anchoring on AKAP by st-Ht31 has been frequently used to inhibit local PKA activity in cells. Our study here demonstrates a previously unforeseen event: by removing PKA from anchoring sites (thus diminishing local PKA activity), st-Ht31 redistributes PKA and PKA activity. This, as shown here, has significant consequences in at least cholesterol homeostasis. At 50 μm, a frequently used concentration (29), st-Ht31 activates cytosolic PKA and depletes 10% cellular cholesterol in 2 h in cells without ABCA1. Moreover, if cells happened to express ABCA1, they could lose up to 40% cellular cholesterol in 2 h. This, undoubtedly, will significantly influence many physiological events, particularly the ones sensitive to the lipid rafts.

ABCA1/st-Ht31 releases cholesterol mainly in the form of microparticles. We presently do not understand the detailed cholesterol trafficking pathway that generates microparticles. These microparticles are uniform in size (<200 nm) and between 1.063 and 1.21 in density (6). We provide evidence here that these microparticles contain flotillin-2, an exosome marker and also a potential molecular module for exosome release (16). This suggests that these microparticles may be related to exosomes. ABCA1 and st-Ht31 may influence multivesicular body biogenesis and eventually exosome release, which is a topic currently under investigation.

In summary, we report here that de-anchoring PKA by st-Ht31 triggers robust cholesterol export preferentially from ABCA1-expressing cells. This effectively reverses macrophage foam cell formation and restores healthy inflammatory response. We speculate that PKA anchoring could be used as a therapeutic target to relieve lipid burden from lesion macrophages and promote lesion regression.

2.8 Acknowledgements

We thank Dr. Jin Zhang (Johns Hopkins University School of Medicine) for sharing AKAR constructs and also acknowledge Dr. Yves Marcel (University of Ottawa Heart Institute) for providing bone marrow-derived macrophages. This work is supported in part by a grant from Canadian Institutes of Health Research. LM was a recipient of an Ontario Graduate Scholarship in Science and Technology and a Canada Graduate Scholarship.

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Chapter 3: ABCA1 enhances TLR4-stimulated IL-10 secretion through PKA activation

Authors: Loretta Ma, Fumin Dong, Maryam Zaid, Ashok Kumar and Xiaohui Zha

Journal of Biological Chemistry

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3.1 Significance of this Manuscript

The research presented in this manuscript uncovers the anti-inflammatory functions of macrophage ABCA1, which is through its activation of PKA activity. Our results emphasize the importance of macrophage ABCA1 in modulating immune response by suppressing pro-inflammatory cytokine secretion and promoting anti-inflammatory IL-10 secretion. Furthermore, these immunomodulatory functions are contributed by ABCA1 promoting PKA activity, and we indicate that this increase in PKA activity is most likely by regulating cellular cholesterol. Our findings highlight the novel aspect of ABCA1 that can be beneficial in targeting against the chronic inflammation in atherosclerosis.

3.2 Author Contributions

Experiments were planned by both Dr. Zha and I, and the majority of the experiments were performed by me. Dr. Fumin Dong provided his assistance with the P-PKA substrate western blotting. Maryam Zaid contributed significantly in the initial cytokine ELISA experiments to optimize the protocols. Also, Dr. Ashok Kumar provided human monocyte derived macrophages and helped with optimizing the cytokine ELISA protocol. I wrote the first draft of the manuscript, and the final version of the paper was established after several rounds of editing between Dr. Zha and I, with the advice of others.

3.3 Abstract

Nonresolving inflammatory response from macrophages is a major characteristic of atherosclerosis. Macrophage ABCA1 has been previously shown to suppress the secretion of proinflammatory cytokine. In the present study, we demonstrate that ABCA1 also promotes the secretion of IL-10, an anti-inflammatory cytokine critical for inflammation resolution. $ABCA1^{+/+}$ bone marrow-derived macrophages secrete more IL-10 but less proinflammatory cytokines than $ABCA1^{-/-}$ bone marrow-derived macrophages, similar to alternatively activated (M2) macrophages. We present evidence that ABCA1 activates PKA and that this elevated PKA activity contributes to M2-like inflammatory response from $ABCA1^{+/+}$ bone marrow-derived macrophages. Furthermore, cholesterol lowering by statins, methyl-β-cyclodextrin, or filipin also activates PKA and, consequently, transforms macrophages toward M2-like phenotype. Conversely, cholesterol enrichment suppresses PKA activity and promotes M1-like inflammatory response. As the primary function of ABCA1 is cholesterol removal, our results suggest that ABCA1 activates PKA by regulating cholesterol. Indeed, forced cholesterol enrichment in ABCA1-expressing macrophages suppresses PKA activation and elicits M1-like response. Collectively, these findings reveal a novel protective process by ABCA1-activated PKA in macrophages. They also suggest cholesterol lowering in extra-hepatic tissues by statins as an anti-inflammation strategy.

3.4 Introduction

The onset of atherosclerosis is characterized by two fundamental hallmarks: cholesterol accumulation and inflammation, particularly of macrophages. Cholesterol accumulation is due to elevated plasma cholesterol and, consequently, building up of cholesterol-rich lipoproteins within the artery, resulting in recruitment and retention of macrophages. Macrophages are subsequently converted into cholesterol-loaded foam cells by engulfing lipoproteins (1). Foam cells further fuel inflammation by secreting proinflammatory cytokines, thereby delaying and impairing inflammation resolution. Although the detailed mechanisms for this action remain largely elusive, excess cholesterol in macrophages is thought to hyperactivate inflammatory response such as those triggered by Toll-like receptors (TLRs) (2). Indeed, cholesterol accumulation was shown to exacerbate LPS-stimulated secretion of TNF-α and other proinflammatory cytokines (3).

Excess cholesterol in macrophages is normally countered by cholesterol efflux mediated by ABC transporters. ABCA1 primarily facilitates cholesterol and phospholipid efflux to lipid-poor apolipoprotein A-I (apoA-I), which generates nascent HDL (4, 5). Individuals defective in ABCA1 have almost no HDL and elevated atherosclerosis. Another ABC transporter, ABCG1, also removes cholesterol but has been suggested to function downstream of ABCA1 as it needs nascent HDL as acceptor (6). Interestingly, both ABCA1 and ABCG1 have significant impact on inflammation. Macrophages without ABCA1 or ABCG1 secrete more proinflammatory cytokines even in the absence of excessive cholesterol loading (3, 7). ABCA1/ABCG1 double deletion further exacerbates this response (7). Currently, this is thought to be primarily due to increased recruitment of TLRs to the cholesterol-rich membrane microdomains such as lipid rafts, which is more abundant in the absence of ABCA1 or ABCG1 (8). However, the precise mechanisms by which these ABC transporters or cholesterol modulate TLR-mediated immune response are largely unknown.

It is widely accepted now that atherosclerosis is a chronic inflammatory disease, marked by dysregulation of the inflammatory process and, particularly, the failure to effectively resolve the inflammation. An effective inflammatory response requires rapid and appropriate activation of inflammatory mechanisms but equally timely and effective resolution of the inflammatory state. This balance is orchestrated, at least in part, by the balance of pro- and anti-inflammatory cytokines secreted. In this context, it is interesting to note that apoA-I can activate STAT3 through JAK2 (9). STAT3 is known to modulate inflammation by promoting the secretion of anti-inflammatory cytokines such as IL-10 (10, 11). In leukocytes, IL-10 expression is positively regulated by PKA through CREB (10). Consistent with this notion, cAMP, an agonist of PKA and exchange protein activated by cAMP (Epac), has been known to influence TLR-mediated cytokine release from macrophages (12–14). Most remarkably, cAMP does not simply suppress or enhance TLR signaling. Rather, it suppresses proinflammatory cytokine, but enhances anti-inflammatory cytokine secretion at the same time, similar to phenotypic transformation from classically activated (M1) to alternatively activated macrophages (M2) (15). More recently, PKA, not Epac, has been identified as the direct downstream target of cAMP for this immune modulation effect (16). Because PKA activity is also essential for ABCA1 function, we wondered whether ABCA1 actually promotes the expression of anti-inflammatory cytokines,

in addition to suppression of proinflammatory cytokines. Perhaps through PKA activation, ABCA1 is able to poise macrophages for M2-like immune response, thus limiting inflammation.

3.5 Experimental Procedures

Materials & Reagents: Cell culture growth medium, antibiotics (penicillin and streptomycin), and fetal calf serum (FCS) were purchased from Invitrogen. Baby hamster kidney (BHK) cells that are stably transfected with a mifepristone-inducible vector with or without ABCA1 gene insert were from Drs. Oram and Vaughan (University of Washington, Seattle, WA). The RAW 264.7 cell line was purchased from the ATCC. Mouse bone marrow-derived macrophages and human acLDL were kindly provided by Dr. Marcel (Ottawa University Heart Institute). Mifepristone was from Invitrogen, and T0901317 was from Sigma. The following antibodies were acquired from the following vendors: mouse monoclonal anti-ABCA1 (Upstate Millipore), rabbit polyclonal anti-phosphorylated-PKA substrate (Cell Signaling), rabbit polyclonal anti-phosphorylated-CREB (Cell Signaling), mouse monoclonal anti-CREB (Cell Signaling), and mouse monoclonal anti-Hsp70 (BD Transduction Laboratories). Protease inhibitor mixture and phosphatase inhibitor (PhosSTOP) were purchased from Roche Applied Science. Methyl-β-cyclodextrin, filipin, compactin, simvastatin, and mevalonate were from Sigma-Aldrich, and PKA inhibitor (PKI) was from Enzo Bioscience.

Cell Cultures: Both BHK cells and RAW 267.4 macrophage cells were maintained in DMEM supplemented with 10% FCS at 37 °C in a 5% CO2 incubator. ABCA1 expression was induced during 16–18 h of incubation in DMEM with 1 mg/ml BSA containing either 5 nm mifepristone or 10 μm T0901317 for BHK or RAW264.7 and BMDM, respectively. Mock-transfected cells were used as negative controls in experiments with BHK cells, whereas T0901317 was withheld for negative controls in experiments with RAW cells. T0901317, a liver X receptor agonist, increases ABCA1 gene expression by binding liver X receptor response element within the ABCA1 gene promoter (17). Mouse BMDM were obtained by flushing the femurs of ABCA1+/+ and ABCA1−/− C57 mice and allowed to differentiate into macrophages by incubation with DMEM containing 10% FBS and 10% L929 conditioned medium for 7 days.

Cytokine ELISA Assay: RAW264.7 and BMDM were first induced to express ABCA1 with 10 μm T0901317 for 18–20 h. Some of the cells were pretreated with either 50 μm PKI or other compounds, such as methyl-β-cyclodextrin (MCD) or filipin, and then stimulated with 100 ng/ml LPS in DMEM supplemented with 1 mg/ml BSA for 6 h. The medium was collected, and cell debris was removed by centrifuging at 12,000 × g for 5 min. Cells were lysed with 1× SDS lysis buffer (50 mm Tris-Cl, pH 6.8, 100 mm dithiothreitol, 2% SDS, 10% glycerol, protease inhibitor mixture, and one tablet of PhosSTOP per 10 ml of buffer). IL-10 and TNF-α in the medium were determined using a kit from R&D Systems Inc. Protein levels in cell lysates were used to normalize cytokine levels in the medium.

Cytokine Array Assay: BMDM cells were induced to express ABCA1 with 10 μm T0901317 for 18–20 h. Cells were incubated with 100 ng/ml LPS in DMEM supplemented with 1 mg/ml BSA for 6 h. Medium and cells were collected as described above. Cytokine in the medium was determined using RayBio® Cytokine Antibody Array 1 from Raybiotech Inc.

Immunofluorescent Staining: BHK cells were seeded in glass bottom coverslip microscopy dishes and grown to 50–70% confluency. Cells were fixed with 4% paraformaldehyde in PBS for 10 min and subsequently permeabilized with 0.1 mg/ml saponin in PBS for 30 min. Nonspecific binding was blocked with 5% calf serum and 50 mm NH4Cl in PBS for 20 min. The primary antibodies (P-PKA substrate or ABCA1) were then added at 1:200 in 5% calf serum/PBS for 30 min followed by incubation with secondary antibodies (Alexa Fluor-488 goat anti-rabbit IgG and Alexa Fluor-547 goat anti-mouse IgG, 1:200) for 30 min. Fluorescent images were taken using a Nikon TE2000-E inverted fluorescent microscope with a 60× objective. Identical settings were used to take images of ABCA1 and mock cells. Fluorescent intensities of individual cells were analyzed with MetaMorph software.

Statistics: Statistical analyses between data groups were performed with PRISM software (GraphPad). Data for Western blot analyses and ELISA experiments are presented as the mean ± S.E. or S.D. as indicated. For quantification of immunoblots, relative unit values were measured using the Image Lab software. The statistical significance of differences between groups was analyzed by Student's t test. Differences were considered significant at a p value < 0.05.

3.6 Results

ABCA1 expression enhances IL-10 secretion

It has been widely reported that ABCA1 suppresses TLR4-mediated TNF-α secretion in various tissue culture and animal models. To test whether ABCA1 enhances the release of anti-inflammatory cytokines at the same time, BMDM from WT and $ABCA1^{-/-}$ mice were stimulated with LPS, a TLR4 ligand. The medium was first analyzed using a cytokine array (**Figure 3.1**). Consistent with previous findings including ours (3, 18), $ABCA1^{+/+}$ BMDM (WT) secreted fewer proinflammatory cytokines, such as TNF-α and IL-12p40 (**Figure 3.2A**), in comparison with $ABCA1^{-/-}$ BMDM. However, ABCA1 expression in BMDM also significantly enhanced the secretion of IL-10. This enhanced IL-10 secretion was further verified by ELISA in both primary BMDM and RAW macrophages; ABCA1-expressing macrophages produced significantly more IL-10 but less TNF-α (**Figure 3.2, B and C**). Collectively, these results reveal an important and novel immune regulatory function of ABCA1, not simply immune suppression as previously reported (3).

ABCA1 activates PKA and this activation requires functional ATP binding domain of ABCA1

ABCA1 has been reported to decrease TLR4 surface presentation and recruitment to lipid rafts (7, 19), which could explain less TNF-α release by LPS. However, as we showed above, ABCA1 also robustly enhances IL-10 secretion. As LPS/TLR4 is required for the release of both IL-10 and TNF-α (neither was detectable without LPS), the initial TLR4 signaling, *i.e.* TLR4 surface presentation or recruitment to lipid raft, is not likely compromised significantly by ABCA1. Rather, some factors, which are induced by ABCA1 and act downstream of the initial TLR4 signaling, have poised macrophages toward an M2-like response. One candidate of such factors is PKA. PKA activation is known to switch macrophages to M2-like responses, *i.e.* high IL-10 and low TNF-α secretion (16). We thus wondered whether ABCA1 could activate PKA. To test this, we first analyzed the phosphorylation of CREB, a PKA substrate, in BHK cells. These cells are stable transfectants that inducibly express ABCA1, its mutants, and mock cells (generated with identical plasmids but without ABCA1 insert), respectively (20). We found that p-CREB level is clearly elevated in BHK cells expressing WT ABCA1, relative to that of mock cells (**Figure 3.3A**). A nonfunctional ABCA1 mutant, A937V, failed to increase p-CREB despite

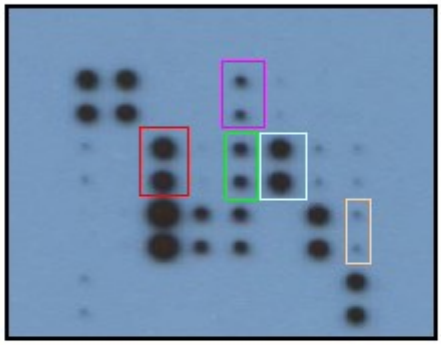
Figure 3.1 **Map of Cytokine array Ray Bio ® Cytokine Antibody Array 1 (A)** indicating the cytokines detected. Array blots used to determine the levels of cytokine secretion from WT **(B)** and ABCA1-/- **(C)** BMDMs.

A

	A	B	C	D	E	F	G	H
1	Pos	Pos	Neg	Neg	GCSF	GM-CSF	IL-2	IL-3
2	Pos	Pos	Neg	Neg	GCSF	GM-CSF	IL-2	IL-3
3	IL-4	IL-5	IL-6	IL-9	IL-10	IL-12 p40p70	IL-12p70	IL-13
4	IL-4	IL-5	IL-6	IL-9	IL-10	IL-12 p40p70	IL-12p70	IL-13
5	IL-17	IFN-γ	MCP-1	MCP-5	RANTES	SCF	sTNFRI	TNF-α
6	IL-17	IFN-γ	MCP-1	MCP-5	RANTES	SCF	sTNFRI	TNF-α
7	Thrombopoietin	VEGF	Blank	Blank	Blank	Blank	Blank	Pos
8	Thrombopoietin	VEGF	Blank	Blank	Blank	Blank	Blank	Pos

B

ABCA1$^{+/+}$



C

ABCA1$^{-/-}$

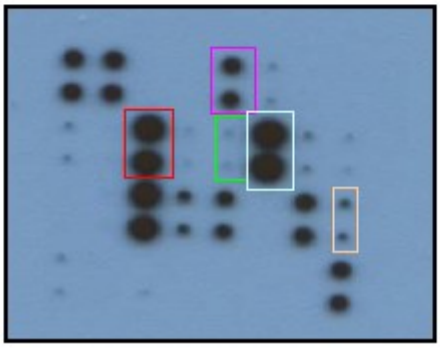


Figure 3.2 **ABCA1 expression in primary mouse BMDM increases IL-10 secretion and decreases pro-inflammatory cytokine secretion.** WT and ABCA1-/- primary mouse BMDMs were induced with 10 μM T0901317 overnight, followed by 100 ng/ml LPS treatment for 6 hours. TOA mouse cytokine array was used to determine the levels of cytokine secretion from WT and ABCA1-/- BMDMs **(A)**. IL-10 and TNFα levels in the medium from primary mouse BMDM from WT and ABCA1-/- **(B)** and RAW 264.7 macrophages **(C)** were measured by mouse cytokine ELISAs. Data is presented as average of duplicated samples with standard deviation.

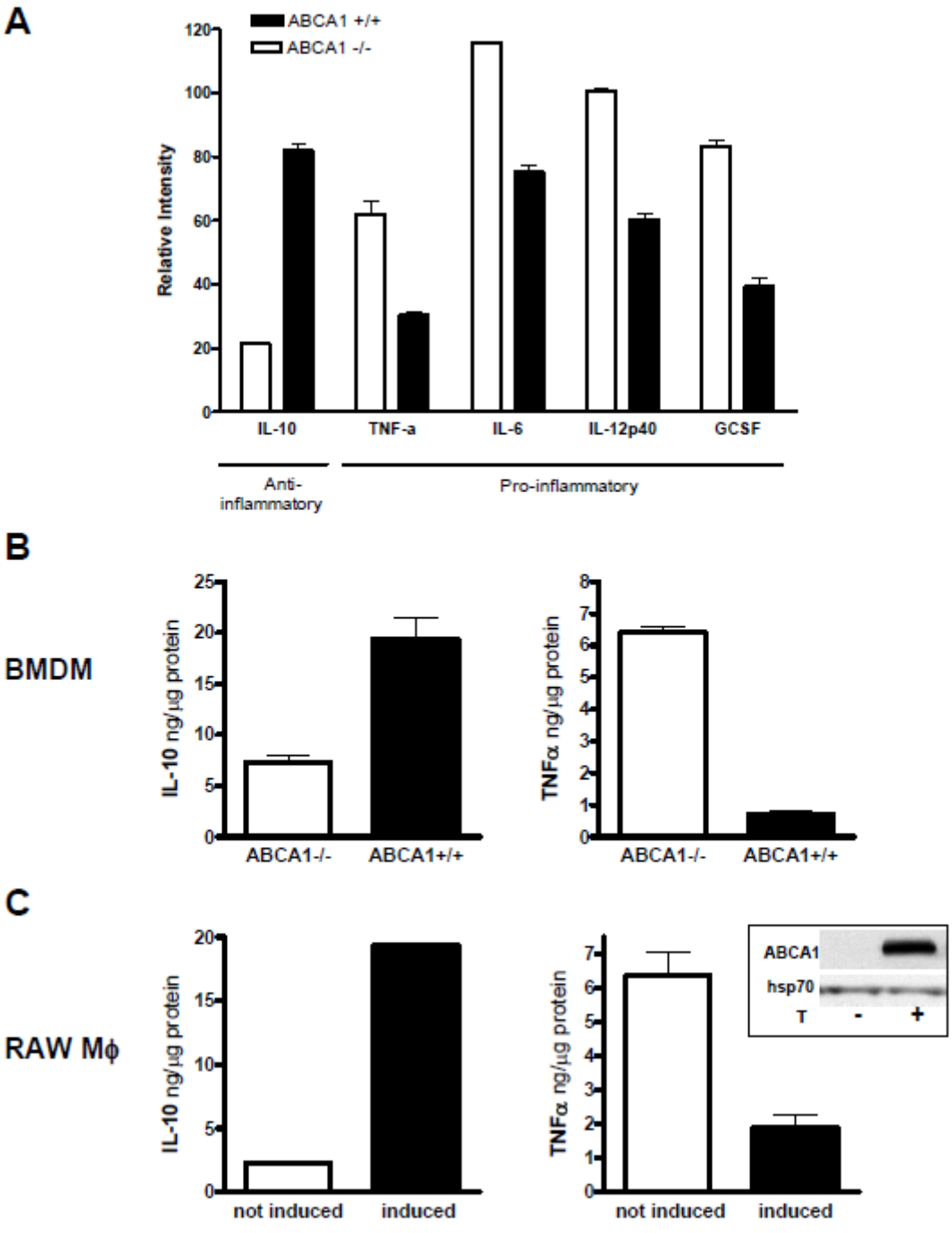
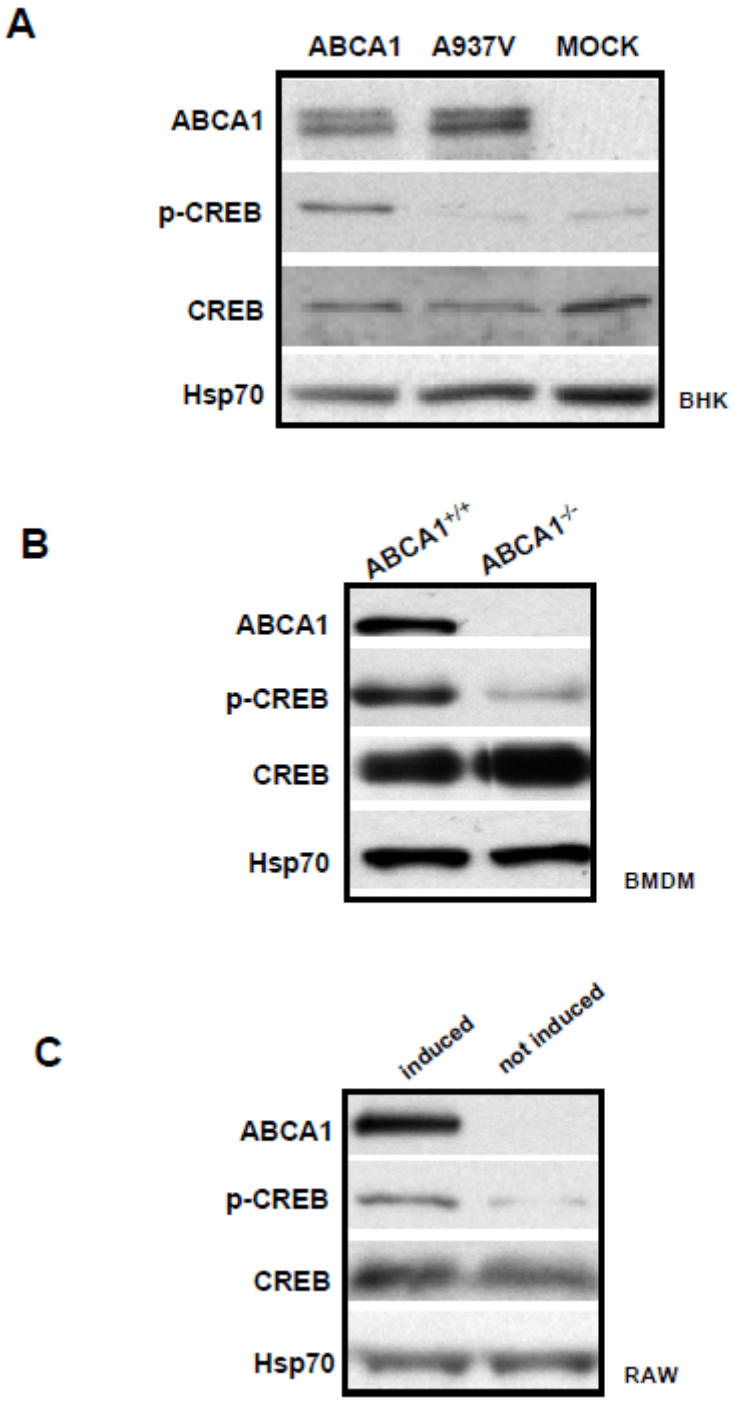

A
ABCA1 +/+
ABCA1 -/-
Relative Intensity
120
100
80
60
40
20
0
IL-10
TNF-a
IL-6
IL-12p40
GCSF
Anti-inflammatory
Pro-inflammatory
B
BMDM
IL-10 ng/µg protein
25
20
15
10
5
0
ABCA1-/-
ABCA1+/+
TNFα ng/µg protein
8
7
6
5
4
3
2
1
0
ABCA1-/-
ABCA1+/+
C
RAW Mϕ
IL-10 ng/µg protein
20
15
10
5
0
not induced
induced
TNFα ng/µg protein
7
6
5
4
3
2
1
0
not induced
induced
ABCA1
hsp70
T
-
+

Figure 3.3 **CREB phosphorylation is increased with the expression of functional ABCA1.** BHK cells were treated overnight with 5 nM mifepristone. BMDMs and RAW macrophages were treated overnight with T0901317. Cell lysates were immunobloted for ABCA1, p-CREB and total CREB in BHK cells expressing ABCA1 wt, A937V mutant, and MOCK control **(A)**, BMDMs from WT ABCA1+/+ and ABCA1-/- **(B)**, and RAW macrophages **(C)**. Hsp70 was used as loading control. Results are representative of at least three independent experiments.


A
ABCA1
A937V
MOCK
ABCA1
p-CREB
CREB
Hsp70
BHK
B
ABCA1+/+
ABCA1-/-
ABCA1
p-CREB
CREB
Hsp70
BMDM
C
induced
not induced
ABCA1
p-CREB
CREB
Hsp70
RAW

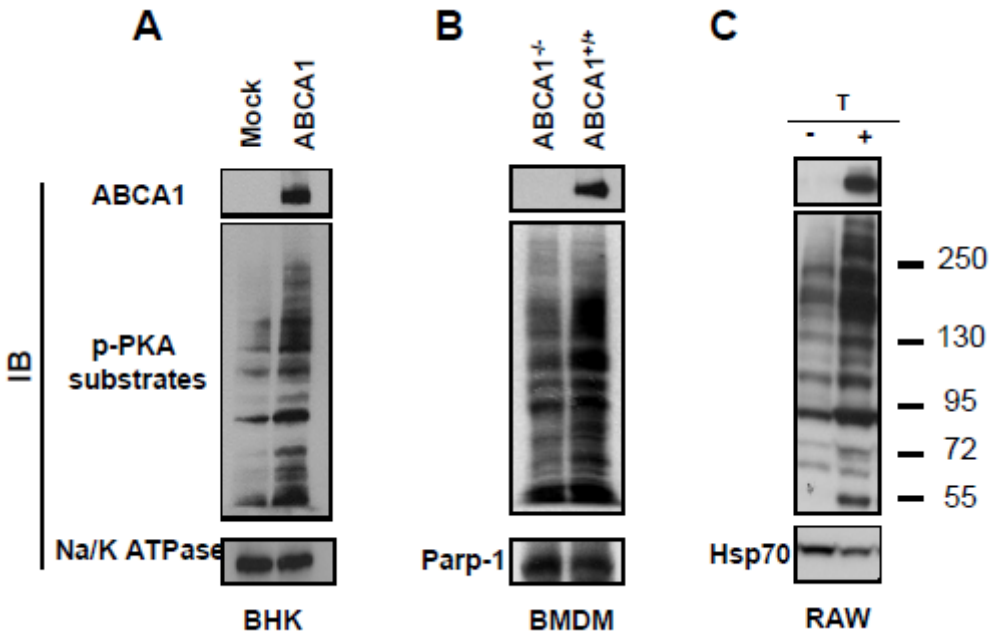
being expressed at a similar level as WT ABCA1 (**Figure 3.3A**) with correct targeting to the plasma membrane (21). $ABCA1^{A937V}$ is defective in ATP binding and consequently unable to efflux cholesterol to apoA-I or perturb lipid rafts in the plasma membrane (20, 21). This suggests that PKA activation is a functional consequence of ABCA1. Indeed, such PKA activation by ABCA1 was further confirmed in macrophages; WT BMDM exhibited higher levels of p-CREB when compared with $ABCA1^{-/-}$ BMDM (**Figure 3.3B**), which was also observed with ABCA1-expressing RAW macrophages (**Figure 3.3C**).

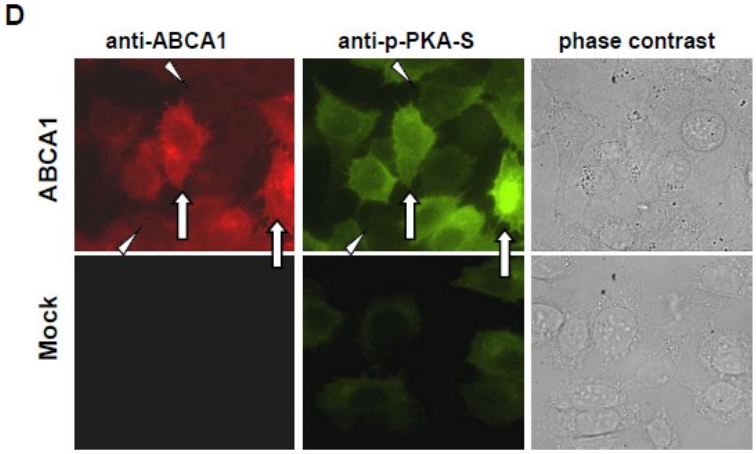
To further substantiate these observations, we next assessed PKA activity with an antibody raised against PKA-phosphorylated proteins. Consistent with p-CREB results above, there were more PKA-phosphorylated proteins in ABCA1-expressing BHK cells relative to those in mock cells (**Figure 3.4A**). PKA-phosphorylated proteins were also significantly elevated in $ABCA1^{+/+}$ BMDM in comparison with $ABCA1^{-/-}$ BMDM (**Figure 3.4B**). Similarly, RAW macrophages had more PKA-phosphorylated proteins when induced to express ABCA1 (**Figure 3.4C**). Furthermore, elevation of PKA-phosphorylated proteins by ABCA1 was clearly observed at the single BHK cell level by microscopy. Higher ABCA1 expression is correlated with more PKA-phosphorylated proteins (**Figure 3.4D, arrows**). Conversely, adjacent cells with low or little ABCA1 expression have fewer PKA-phosphorylated proteins (**Figure 3.4D, arrowheads**), similar to that of the mock cells. A positive correlation between ABCA1 expression level and the level of PKA-phosphorylated proteins was also observed from a large number of individual cells (**Figure 3.4E**). Together with p-CREB results, we conclude that there is most likely a causal relationship between ABCA1 function and PKA activity.

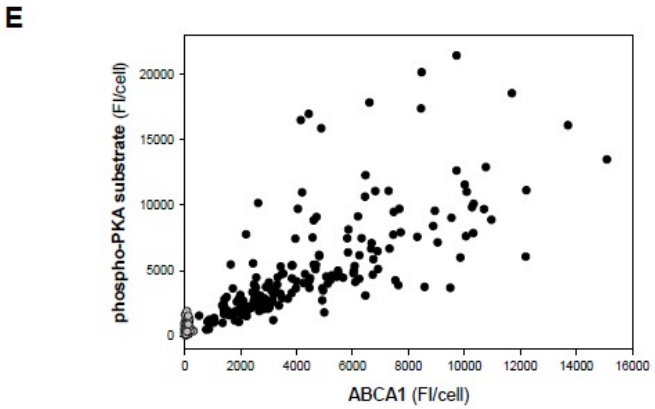
PKA Activity Is Required for ABCA1 to Exert Its Immune Regulatory Function

We next investigated whether elevated PKA activity by ABCA1 was at least partially responsible for the more favorable IL-10/TNF-α release profile. For this, we used a cell-permeable short peptide (6 amino acids), PKI, to acutely block PKA function. One technical limitation is that $ABCA1^{+/+}$ BMDM had been expressing ABCA1 for more than 24 h since T0901317 induction. Accordingly, PKA activity should be continuously elevated during this period. To avoid a broad effect, we only pretreated BMDM with PKI for 30 min before LPS addition. Such short treatment may not be able to completely reverse all the proteins

Figure 3.4 **PKA substrate phosphorylation is higher with expressing ABCA1.** Cells were prepared as in fig.2. Cell lysates were immunoblotted for phospho-PKA substrates in BHK cells **(A)**, BMDMs **(B)**, and RAW macrophages **(C)**. **(D)** Immunofluorescence staining of BHK cells with anti-ABCA1 and anti-phosphorylated PKA substrate antibodies. Na/K ATPase, parp-1 and hsp70 were used as loading control, respectively. **(E)** Correlation between the level of phospho-PKA substrates and level of ABCA1 expression in individual cells.


A
Mock
ABCA1
ABCA1
IB
p-PKA substrates
Na/K ATPase
BHK
B
ABCA1-/-
ABCA1+/+
Parp-1
BMDM
C
T
-
+
250
130
95
72
55
Hsp70
RAW


D
anti-ABCA1
anti-p-PKA-S
phase contrast
ABCA1
Mock


E
phospho-PKA substrate (FI/cell)
20000
15000
10000
5000
0
0
2000
4000
6000
8000
10000
12000
14000
16000
ABCA1 (FI/cell)

phosphorylated by ABCA1-activated PKA, particularly for these with a slow dephosphorylation rate. Nevertheless, as shown in **Figure 3.5A**, PKI at a concentration with negligible effect on ABCA1$^{-/-}$ BMDM, was able to significantly suppress IL-10 secretion from ABCA1$^{+/+}$ BMDM. Similarly, ABCA1$^{+/+}$ BMDM released more TNF-α in the presence of PKI when ABCA1$^{-/-}$ BMDM was not significantly altered (**Figure 3.5B**). These results demonstrate that PKA activation is significantly contributing to the regulation of inflammatory response by ABCA1.

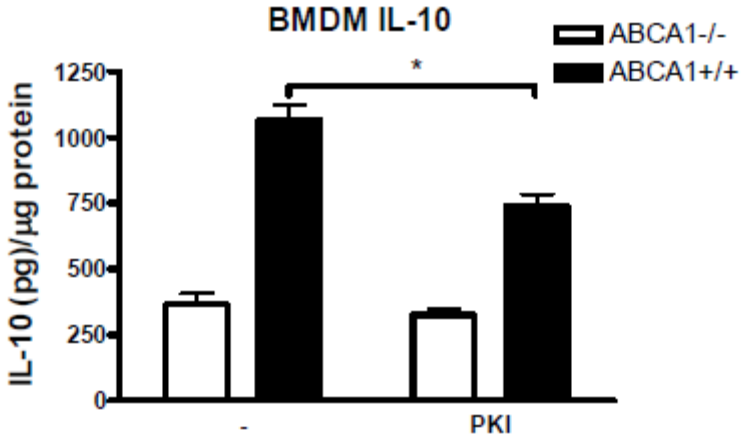
Cholesterol Influences Steady State PKA Activity

To understand how ABCA1 activates PKA, we reviewed some well established ABCA1 functions. The first and foremost, ABCA1 interacts with apoA-I and mediates cholesterol efflux to apoA-I. ApoA-I is known to increase PKA activity (22) and suppress TNF-α secretion (9, 18). However, all the cell models used here including macrophages were not at all exposed to apoA-I; they were induced to express ABCA1 in BSA-only medium. ApoA-I therefore cannot be a significant contributor to PKA activation observed here. Another widely reported observation is that ABCA1 decreases lipid raft content in the plasma membrane (18, 19, 21), independent of apoA-I. Thus, we tested whether modulating lipid rafts influences PKA activity in macrophages. This was first achieved by incubating RAW macrophages with increasing concentrations of cyclodextin for 30 min. Cyclodextrin is a cholesterol-sequestering agent, thereby removing cholesterol from the plasma membrane (23). This in general depletes lipid rafts (24). We found that p-CREB levels were dose-dependently elevated by cyclodextrin (**Figure 3.6A**). Also, macrophages were treated with filipin for 1 h. Filipin is known to bind free cholesterol on the plasma membrane. This sequesters cholesterol away from the general area of the plasma membrane. We observed a similar increase of p-CREB in filipin-treated cells (**Figure 3.6B**). Furthermore, macrophages were treated with statins in lipoprotein-deficient serum, which again exhibited a dose-dependent elevation of p-CREB (**Figure 3.6C**).

The levels of PKA-phosphorylated proteins were also similarly elevated. Both MCD and filipin led to more PKA-phosphorylated proteins (**Figure 3.7A, lanes 2 and 4, relative to lane 1**). If PKI is present during the treatment, neither MCD nor filipin can raise PKA-phosphorylated protein levels (**Figure 3.7A, lanes 3 and 5**). PKI did not interfere with

Figure 3.5 **PKI suppresses IL-10 secretion but increases TNF-α release in ABCA1+/+ BMDM, without significant effect on ABCA1-/- BMDM.** Primary mouse ABCA1+/+ and ABCA1-/- BMDMs were treated with 10 μM TO-901317 overnight. BMDM were then pre-treated with 50 μM PKI for 30 min before LPS challenge (100 ng/ml) for 6 hours. IL-10 **(A)** and TNFα **(B)** levels in the media were measured by mouse cytokine ELISA, and presented as average of duplicates + standard deviation.

A


BMDM IL-10
ABCA1-/-
ABCA1+/+
*
IL-10 (pg)/µg protein
1250
1000
750
500
250
0
-
PKI

B

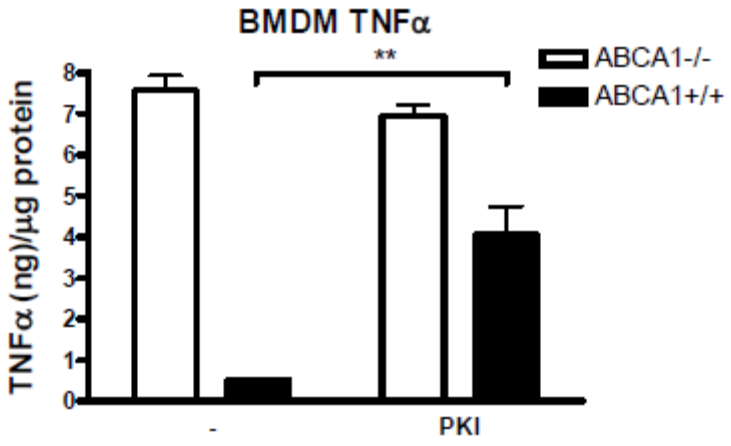
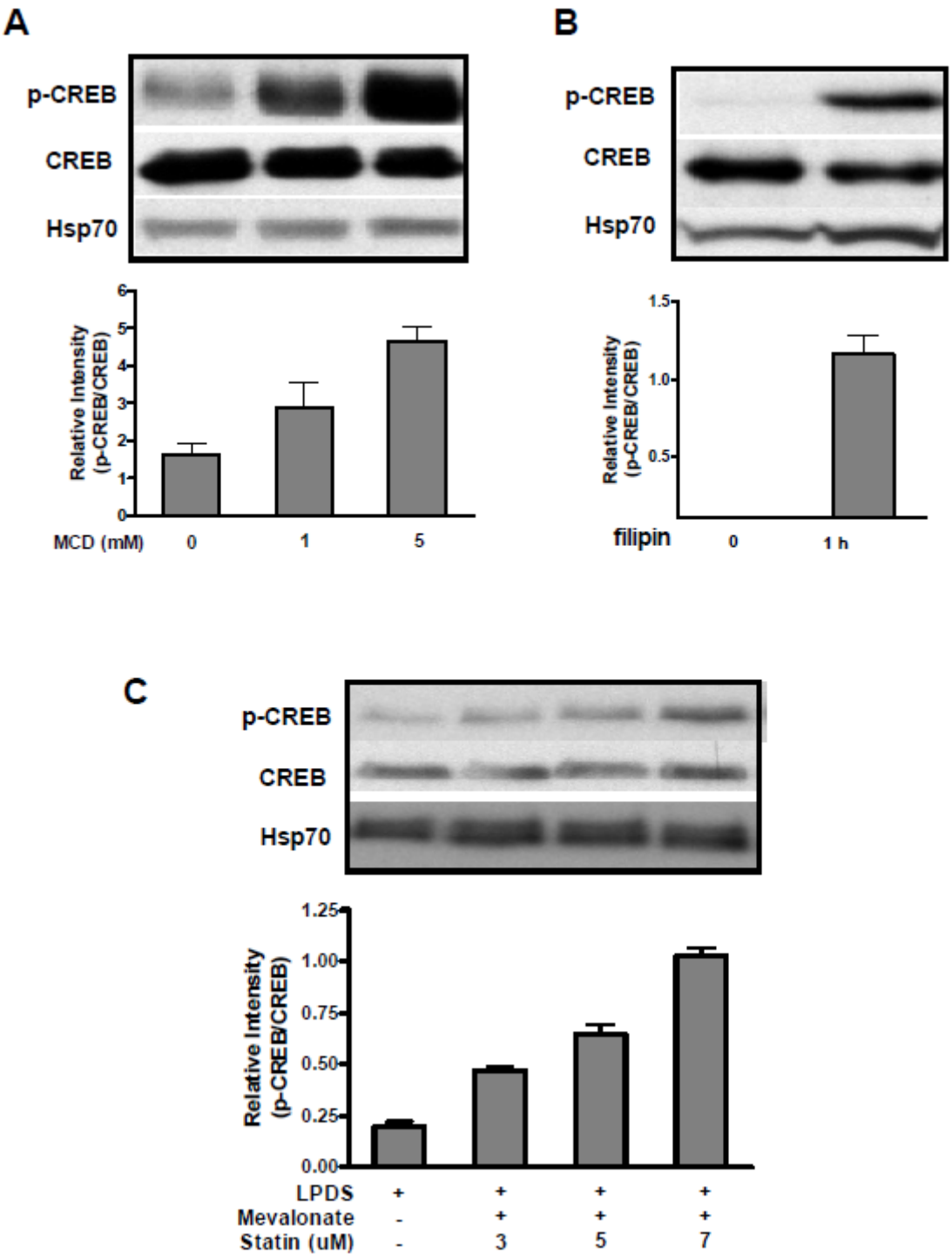

BMDM TNFα
**
ABCA1-/-
ABCA1+/+
TNFα (ng)/µg protein
8
7
6
5
4
3
2
1
0
-
PKI

Figure 3.6 **Cholesterol depletion increases p-CREB.** RAW macrophages were treated with 0, 1, or 5 mM MCD for 30 minutes **(A)**, 3 μg/mL filipin for 1 h **(B)** or 3, 5, or 7 μM statins (compactin and simvastain) for 48 h **(C)**. Cell lysates were immunoblotted for p-CREB and total CREB with hsp70 as loading control. Bar graphs represent averages from at least two independent experiments with standard deviations.


A
p-CREB
CREB
Hsp70
Relative Intensity (p-CREB/CREB)
0
1
2
3
4
5
6
MCD (mM)
0
1
5
B
p-CREB
CREB
Hsp70
Relative Intensity (p-CREB/CREB)
0.5
1.0
1.5
filipin
0
1 h
C
p-CREB
CREB
Hsp70
Relative Intensity (p-CREB/CREB)
0.00
0.25
0.50
0.75
1.00
1.25
LPDS + + + +
Mevalonate - + + +
Statin (uM) - 3 5 7

cholesterol modulation. In line with MCD and filipin, statin also raised the level of PKA-phosphorylated proteins (**Figure 3.7B**). Together, our results support the notion that reduction in membrane cholesterol, presumably lipid rafts, activates PKA.

Cholesterol Regulates TLR4-mediated Cytokine Secretion through PKA

To determine the role of cellular cholesterol on cytokine secretion, RAW macrophages were stimulated with LPS under cholesterol-depleted conditions. We once again chose IL-10, an anti-inflammatory cytokine, and TNF-α, a proinflammatory cytokine, as bench markers for potential inflammation regulation. We found that in accordance with their capacity to activate PKA, both MCD and filipin significantly increased LPS-stimulated IL-10 release, whereas TNF-α secretion was suppressed (**Figure 3.8A**). Noticeably, MCD at 5 mm is more potent in enhancing IL-10 or reducing TNF-α release than filipin, apparently correlated with its capacity of being more potent in activating PKA (**Figure 3.7A**). We then tested whether PKA activity is necessary for cholesterol to regulate cytokine secretion. Indeed, the effect of cholesterol depletion on cytokine secretion is mostly abolished if PKA activation is prevented by PKI. MCD or filipin can no longer boost IL-10 secretion or suppress TNF-α release without PKA activation (**Figure 3.8B**). Thus, PKA activity is necessary for cholesterol depletion to regulate immune response to LPS. Collectively, we conclude that cholesterol exerts significant influence on TLR4-mediated immune response through its role in PKA activation.

Cholesterol Loading Can Override the Influence of ABCA1 on PKA Activity and on Cytokine Secretion

We have so far established that disrupting lipid rafts increases PKA activity. This poises macrophages for M2-like anti-inflammatory response to LPS. As demonstrated earlier, ABCA1 also shares these capacities on lipid rafts and on PKA. We thus wondered whether cholesterol loading, which increases lipid rafts, in ABCA1-expressing cells can counter this ABCA1 effect. RAW macrophages were incubated with or without acetylated LDL and induced to express ABCA1. ABCA1 expression in loaded or nonloaded cells was identical. However, cholesterol loading substantially decreased PKA activity, indicated by diminished levels of p-CREB and PKA-phosphorylated proteins (**Figure 3.9A**). Cytokine secretion was also significantly affected; cholesterol-loaded macrophages secreted less IL-10 (**Figure 3.9B**)

Figure 3.7 **Cholesterol depletion increases PKA phosphorylated proteins.** RAW macrophages were pretreated with or without 50 μM PKI for 1 hour and subsequently treated 30 minutes with 5mM MCD or 10 μg/mL filipin **(A)**, or incubated with statins in 10% LPDS medium for 48 hours **(B)**. Whole cell lysates were analyzed by immunoblotting for phospho-PKA substrates with hsp70 as loading control. Bar graphs represent averages from multiple independent experiments with standard deviations.

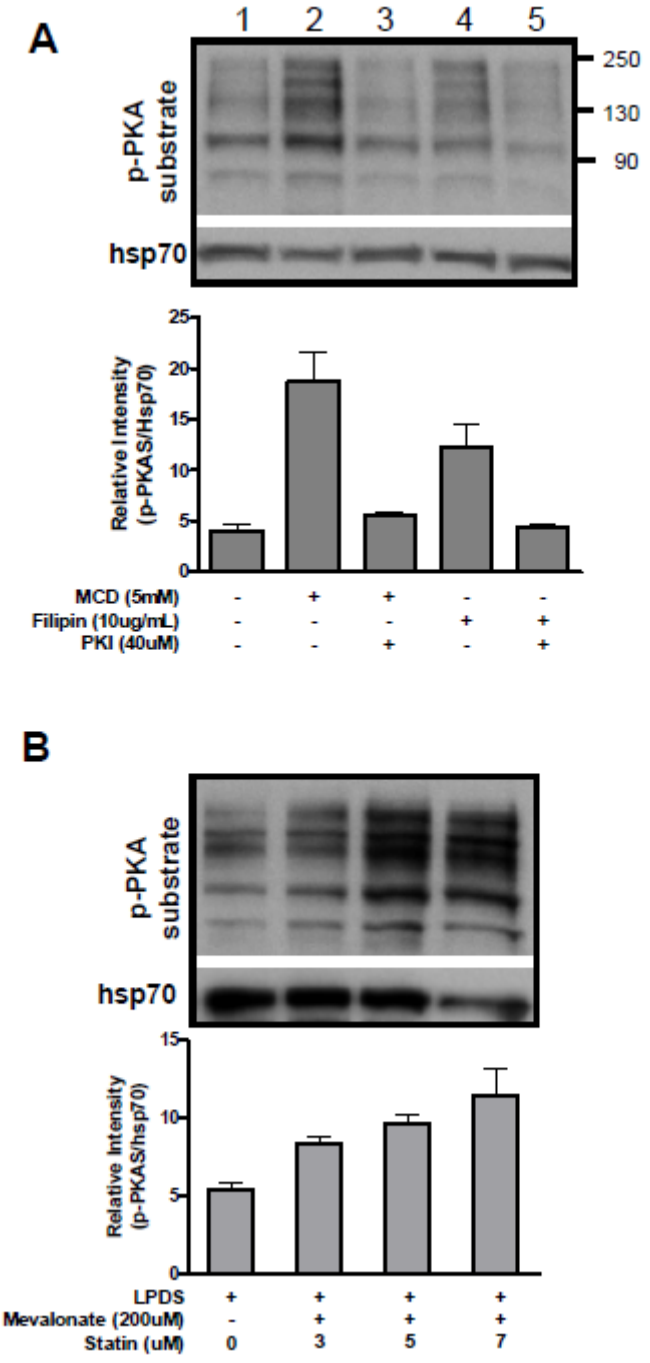

A
1
2
3
4
5
250
130
90
p-PKA substrate
hsp70
Relative Intensity (p-PKAS/Hsp70)
25
20
15
10
5
0
MCD (5mM) - + + - -
Filipin (10ug/mL) - - - + +
PKI (40uM) - - + - +
B
p-PKA substrate
hsp70
Relative Intensity (p-PKAS/hsp70)
15
10
5
0
LPDS + + + +
Mevalonate (200uM) - + + +
Statin (uM) 0 3 5 7

Figure 3.8 **Cholesterol depletion promotes IL-10 secretion but suppresses TNF-α release.** RAW macrophages were treated with 10 μM TO-901317 overnight and followed by 100 ng/ml LPS treatment in combination with MCD or filipin for 6 hours **(A)**. Some of cells were pre-incubated with 50 μM PKI and then treated as above **(B)**. Medium IL-10 and TNF-α levels were measured by ELISA. Data is presented as average of duplicated samples with standard deviation.

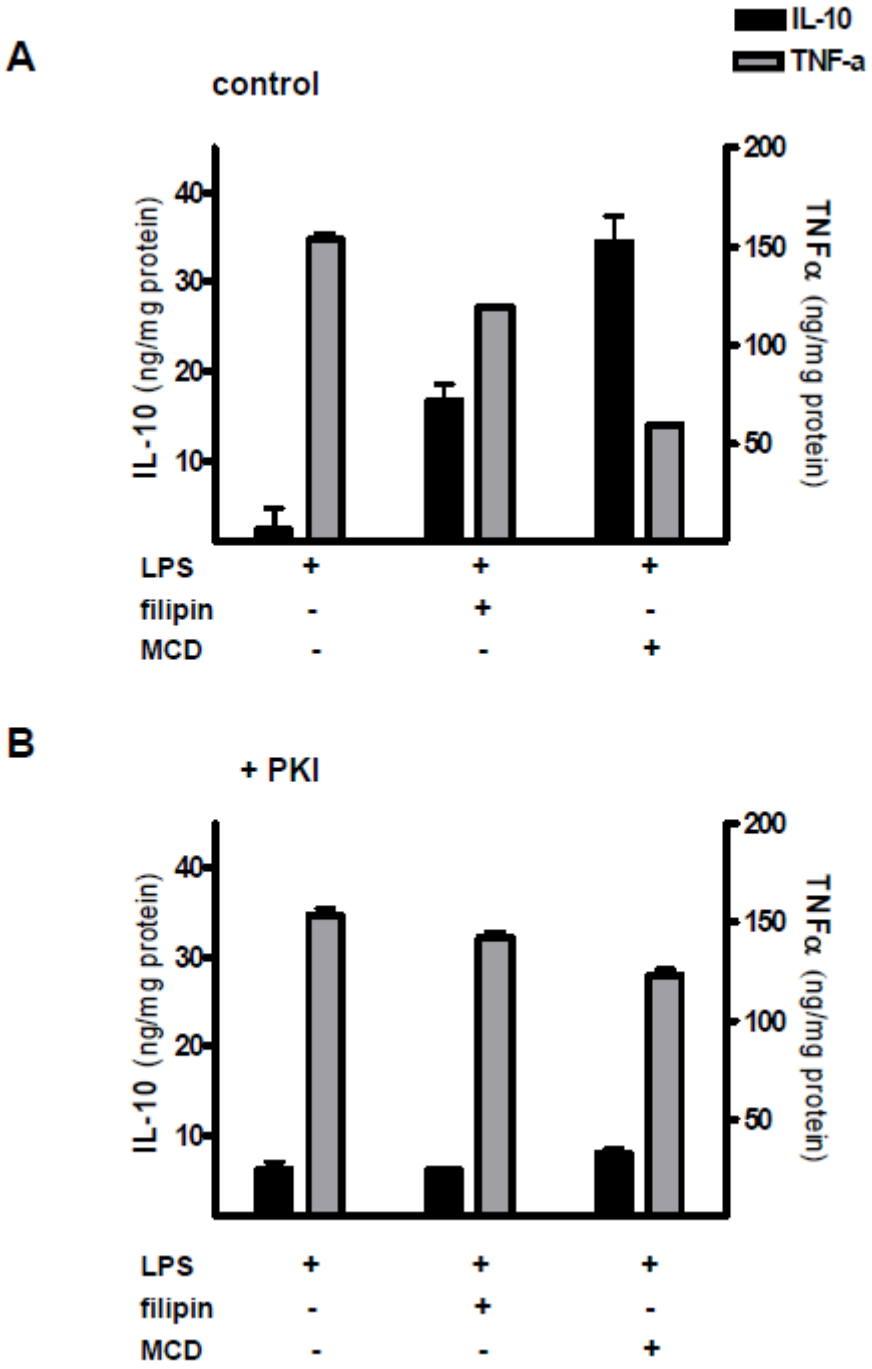
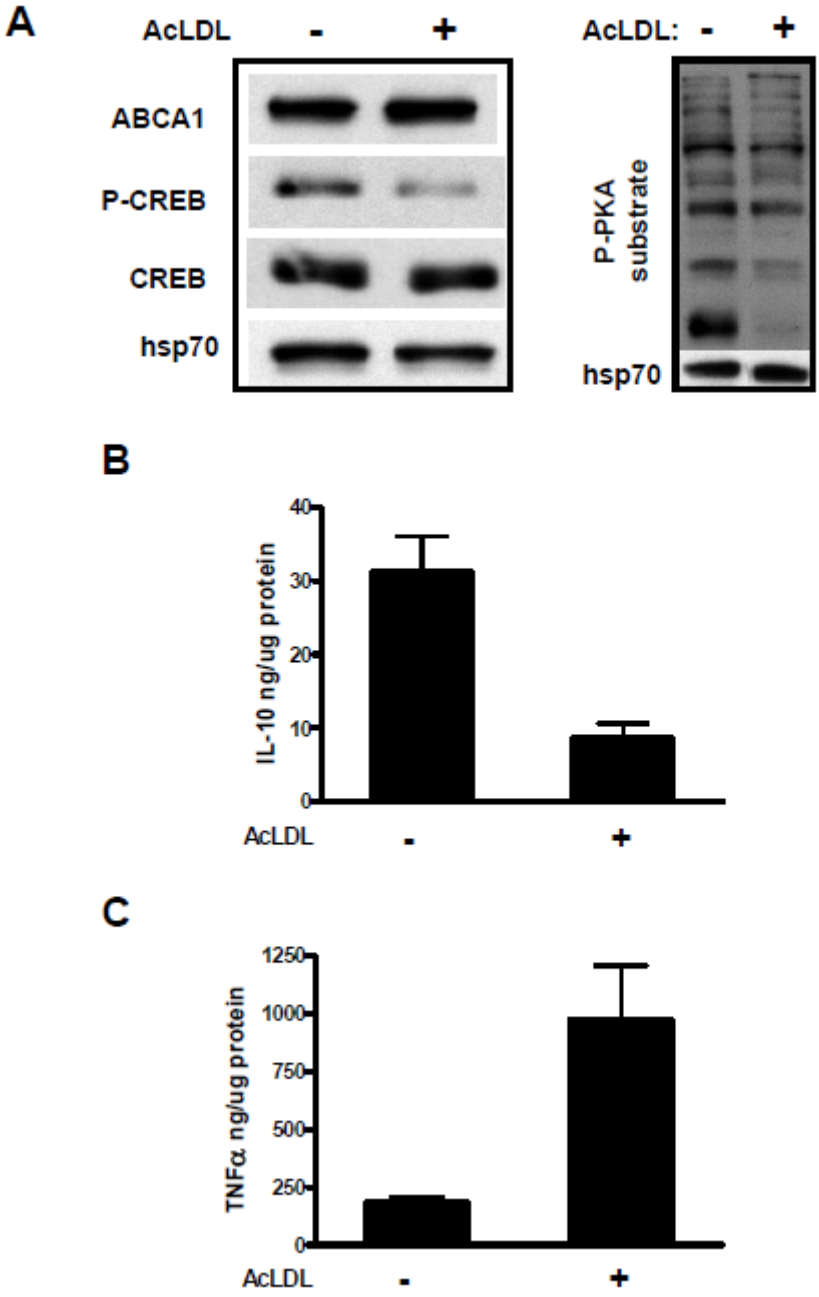

A
control
IL-10
TNF-a
IL-10 (ng/mg protein)
TNFα (ng/mg protein)
40
30
20
10
200
150
100
50
LPS + + +
filipin - + -
MCD - - +
B
+ PKI
IL-10 (ng/mg protein)
TNFα (ng/mg protein)
40
30
20
10
200
150
100
50
LPS + + +
filipin - + -
MCD - - +

Figure 3.9 **Cholesterol loading decreases p-CREB and promote pro-inflammatory response.** RAW macrophages were incubated with 50 μg/mL acLDL or without for 24 hours and then 10 μM TO-901317 overnight. Some of the cells were then treated with 100 ng/ml LPS for 6 h. Cell lysates were immunoblotted for p-CREB, total CREB, PKA phosphorylated proteins with hsp70 as loading control **(A)**. IL-10 **(B)** and TNFα **(C)** levels in the medium were measured by ELISA. Data is presented as average of duplicated samples with standard deviation.


A
AcLDL - +
ABCA1
P-CREB
CREB
hsp70
AcLDL: - +
P-PKA substrate
hsp70
B
IL-10 ng/ug protein
40
30
20
10
0
AcLDL - +
C
TNFα ng/ug protein
1250
1000
750
500
250
0
AcLDL - +

but more TNF-α (**Figure 3.9C**), consistent with less PKA activity in these cells. Thus, the plasma membrane likely plays a more proximal role in immunomodulation than ABCA1.

3.7 Discussion

The major conclusion from this study is that ABCA1 activates PKA. Elevated PKA activity significantly assists ABCA1 to poise macrophages to an M2-like response when exposed to LPS, such as releasing more IL-10 but less TNF-α. IL-10 is a major anti-inflammatory cytokine. To our knowledge, this is the first study demonstrating the role of ABCA1 in enhancing the anti-inflammatory arm of the immune response.

It is generally accepted that atherosclerosis is initiated and propagated by the response of the innate immune system. The innate immune system has evolved to mount robust response to infection and injury and, at the same time, be self-limiting to avoid excessive damage and to promote recovery. As such, a well orchestrated balance of pro- and anti-inflammatory programs is essential for the eventual inflammation resolution. Atherosclerosis is a chronic inflammatory disease and, in a sense, the consequence of excessive proinflammatory activities and failure to proceed to inflammation resolution. Indeed, in this context, strengthening the anti-inflammatory arm has proven to be beneficial. For example, IL-10-deficient mice develop more fatty streaks when compared with WT animals when fed high fat diet (25). By contrast, IL-10 transgenic mice do not develop fatty streaks under the same condition (26). Also, IL-10 deficiency in apoE−/− mice increases atherosclerosis (27). ABCA1 is significantly antiatherogenic both in human and in all animal models tested to date. As we reported here, ABCA1 enhances IL-10 secretion but limits TNF-α release, in addition to its role in HDL biogenesis. It is thus tempting to speculate that ABCA1 may prevent atherosclerosis partially by promoting M2-like immune responses.

It is known that macrophages can undergo classical (M1) or alternative (M2) activation, which represent extremes of a continuum in a universe of activation states (28). Among other things, the M1 phenotype is characterized by the expression of high levels of proinflammatory cytokines/chemokines. In contrast, M2 macrophages have immune regulatory functions, characterized by efficient phagocytic activity, high expression of scavenging molecules, and an IL-12lowIL-10hi phenotype. Although we only analyzed IL-10 in the current study, Chimini and colleagues (29) have performed a comprehensive screen

of ABCA1-expressing or -nonexpressing macrophages from various mouse models and concluded that ABCA1 is a positive factor to promote the appearance of M2 markers, including CD163 and ARG1. In fact, ABCA1 itself behaves as an exquisite M2 marker as its expression is positively regulated by IL-4 and parallels the expression of most established M2 markers (29), This is in good agreement with our observations here. Also, M2 macrophages exhibit enhanced IL-10 but suppressed TNF-α secretion when challenged by LPS. Interestingly, IL-10 itself can also significantly promote M1 to M2 transition (30). In this context, by promoting IL-10 secretion, ABCA1 should further strengthen and enrich M2 phenotypes, thereby facilitating inflammation resolution.

We provide evidence that this immune regulatory function of ABCA1 is at least partially due to its ability to activate PKA. It has long been established that cAMP elicits an anti-inflammatory effect on the innate immune system (14). cAMP can activate both PKA and Epac. However, its immune regulatory function was recently reported to be primarily through PKA activation in macrophages. For example, PKA knockdown completely abolishes the immune regulatory effects of cAMP, whereas Epac antagonist has little effect (16). At the molecular level, cAMP activates PKA to phosphorylate several immune regulatory molecules including NF-κB p65 and CREB. This leads to the suppression of NF-κB-mediated proinflammatory cytokine expression but enhancement of IL-10 generation, respectively (16). Interestingly, ABCA1 can also activate STAT3 through apoA-I, which is known to up-regulate IL-10 expression. However, without apoA-I as in the present study, ABCA1 is unable to influence STAT3 activity (9). Therefore, STAT3 is not likely the direct mechanism by which ABCA1 promotes IL-10 secretion.

The mechanisms by which ABCA1 increases PKA activity remain to be elucidated. Elevated cellular cAMP can certainly increase PKA activity. Alternatively, PKA activity could be regulated by PKA cellular localizations. In recent years, it has become increasingly recognized that intracellular cAMP is distributed in a highly non-uniform fashion. For example, many PKA-anchoring proteins, i.e. AKAPs, also anchor adenyl cyclases to produce cAMP and diesterases to degrade cAMP locally. This provides a localized and also temporal pool of cAMP for PKA activation. ABCA1 can increase PKA activity potentially by influencing any of these molecules.

However, the most well established function of ABCA1 is its regulation of cholesterol. ABCA1 regulates cellular cholesterol at two levels. First, ABCA1 facilitates cholesterol efflux to apoA-I. This decreases overall cellular cholesterol contents. Secondly, ABCA1 weakens cholesterol interaction with phospholipids in the membrane, perhaps similar to flippases, and disrupts the formation of microdomains, such as lipid rafts. These cholesterol regulation functions are likely essential for PKA activation by ABCA1. Indeed, once cholesterol-loaded, ABCA1 fails to elevate PKA and also fails to poise macrophage for the M2-like inflammatory responses. These cholesterol-enriched macrophages secrete more TNF-α but less IL-10 than non-loaded cells, although ABCA1 expression remains unchanged. Thus, it is likely that cholesterol acts more proximally than ABCA1 to PKA activation. Consistent with this notion, we found that cholesterol depletion by various reagents, a common approach for lipid raft disruption, activates PKA and modulates cytokine secretion accordingly, without change in ABCA1 expression.

Interestingly, similar cholesterol manipulations were found to increase adenylyl cyclase activity. For example, in cells treated with MCD (and therefore with fewer lipid rafts), β2 adrenergic receptor can more efficiently form a complex with adenylyl cyclase and G protein (Gs). This leads to activation of adenylyl cyclase and increased cAMP production both under basal condition (i.e. without β2 adrenergic receptor stimuli) and with stimulation (32). PKA could be activated in ABCA1-expressing cells by similar adenylyl cyclase-mediated mechanism. This higher steady state PKA activity then poises macrophages to M2-like inflammatory responses.

It has been suggested that lowering cellular cholesterol (thus resulting in fewer lipid rafts) disrupts initial TLR4 signaling, resulting in an overall suppression of inflammatory response. Indeed, high concentration of MCD is known to inhibit MyD88-dependent TLR recruitment to lipid rafts and thus prevent TLR from forming complexes with accessory proteins (33). Drawing an analogy to this, ABCA1 is speculated to suppress TLRs, particularly TLR4, by removing cholesterol and disrupting lipid rafts. ABCA1 is widely reported to suppress LPS-stimulated TNF-α release. However, the secretion of IL-10, another direct downstream event of LPS-TLR4 signaling, is increased by ABCA1 as we reported here. This argues against a simple immune suppression. Rather, by activating PKA, ABCA1 poises macrophages to a different state. Such a state allows macrophages to mount a distinct

and more favorable response when challenged by LPS. Intriguingly, a recent comprehensive genome-wide analysis in macrophages suggests that LPS-stimulated gene expression primarily results from LPS-independent transcription factor positioning (therefore poised). LPS stimulation merely amplifies the transcription from this predetermined positioning pattern (34). It would be interesting to see whether ABCA1 or cholesterol depletion influences this prepositioning of transcription factors. Perhaps PKA could influence this event. On this note, PKA was shown to modulate TLR4 inflammatory responses through a nuclear PKA-anchoring protein, AKAP-95 (16). AKAP-95 was recently reported to regulate the activity of S6 kinase (S6K), a key mediator of mTORC1 to regulate mRNA transcription (31). Also consistent with this global prepositioning (or a poise state), the work from Chimini and colleagues (29) concluded that ABCA1+/+ macrophages are M2-like, versus an M1-like phonotype in ABCA1−/− macrophages without inflammatory stimuli.

Another relevant and perhaps equally important finding here is how cholesterol loading with modified LDL tips the balance toward M1-like immune response in macrophages. Conversely, cholesterol lowering by statins switches macrophages toward M2-like inflammatory response. Given that the primary cause of atherosclerosis is the elevated LDL in the circulation, it is plausible that high cholesterol weakens the defense capacity of the immune system by suppressing macrophage M2 polarization. In support of this, statins are known to offer anti-inflammatory functions, independent of their LDL-lowering capacity. Perhaps statins could also decrease cellular cholesterol in peripheral tissues including macrophages, in addition to its well established up-regulation of LDL receptor in the liver. This could make immune cells more resilient to environmental challenges, thereby resulting in less chronic inflammation and less atherosclerosis.

In summary, the present study demonstrates for the first time that ABCA1 directly promotes the anti-inflammatory arm of the immune response. This is most likely through PKA activation. Perhaps equally importantly, we provide evidence that cholesterol has a direct role in immune regulation.

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Chapter 4: Discussion

This thesis presents new insight towards mechanisms underlying ABCA1 mediated functions. While ABCA1 encompasses a range of anti-atherogenic roles, signaling pathways, such as cAMP/PKA, are crucial integral factors that support ABCA1 functions. A major finding in this thesis is that ABCA1 functionally activates PKA. This PKA signal transduction is necessary for various aspects of ABCA1 function, including cholesterol efflux and anti-inflammatory functions.

First of all, cholesterol efflux, the most extensively studied function of ABCA1, requires PKA activity, as a highly specific PKA inhibitor, PKI, can completely block this process (1). Cholesterol efflux from ABCA1-expressing cells consists of two independent components: cholesterol efflux to apoA-I that generates HDL and cholesterol efflux to form microparticles. We show here that a specific cellular pool of PKA is utilized to promote microparticle secretion. Inhibition of PKA anchoring with st-Ht31 enhances PKA activity in the cytoplasm, likely at the expense of PKA in other cellular pools that normally anchor through AKAPs. Such cytoplasmic PKA activation robustly enhances microparticle formation, reverse cholesterol accumulation in macrophage foam cells, and promote M2-like inflammatory response in an ABCA1-dependent manner. This demonstrates that PKA signaling is a critical factor which is extensively integrated into the intrinsic activities of ABCA1. Indeed, we further demonstrate that ABCA1 activates PKA, likely through its capacity of modulating cholesterol. It is through this PKA activation that ABCA1 influences inflammatory responses and transform macrophages towards the M2 phenotype. The implications of these results in the perspective of current knowledge of ABCA1 activities, including cellular lipid export and macrophage inflammatory signaling, as well as future prospects of ABCA1-associated PKA signaling in relation to progression of atherosclerosis are discussed below.

4.1 ABCA1-mediated Cholesterol Efflux

4.1.1 ApoA-I Lipidation and Microparticle Formation

The role of ABCA1 to promote efflux of cholesterol to apoA-I, i.e.HDL formation, is well established. Only very recently has ABCA1 been shown to also export a significant amount of cholesterol independent of apoA-I in the form of microparticles (1, 2). Inhibition of PKA

with PKI drastically inhibited both apoA-I-dependent and microparticle formation pathways. ABCA1 is known to be phosphorylated by PKA and its proper phosphorylation is necessary for apoA-I lipidation, which implies that microparticle formation may also depend on properly phosporylated ABCA1. However, we found that increasing the level of microparticle release by st-Ht31 is not correlated with increase in the level of ABCA1 phosphorylation by PKA (**Figure 2.3B**). While further studies are needed to resolve the precise mechanism by which ABCA1 releases microparticles, we can conclude that apoA-I lipidation and the release of microparticles are regulated by two distinct branches of PKA signaling.

This conclusion is also consistent with previous studies from our laboratory. For example, a report by Karwatsky *et al.* shows that antagonizing Ca^{2+}/calcineurin signaling completely blocks cholesterol efflux to apoA-I but has no effect on apoA-I independent cholesterol efflux (microparticle formation) (3). Similar distinction was also observed with eATP signaling pathway; cholesterol efflux to apoA-I requires eATP, but not basal or st-Ht31 induced cholesterol efflux (4). Thus, experimental evidence collectively supports the concept that ABCA1 functions in a dualistic manner; one encompasses microparticle release, perhaps by exerting more general effects on membrane packing and fluidity (1, 5), and the other facilitates the interaction between apoA-I and the plasma membrane to allow apoA-I lipidation. Also, this general effect of ABCA1 on the plasma membrane is likely necessary to prime cells for cholesterol and phospholipid efflux to apoA-I.

4.1.2 Characteristics of Microparticles

Unlike cholesterol efflux to apoA-I that has a well-established role in RCT, the physiological role of non-HDL microparticles remains to be elucitated. Microparticles have been extensively characterized as cholesterol/phospholipid-rich particles with size much larger than HDL (9-12 nm) but less than 200 nm (1). The density of the microparticles is similar to HDL (between 1.063 and 1.21), suggesting that it contains more proteins than LDL (density <1.063). Microparticles are previously reported to contain lipid-raft-associated markers, CD14 and ganglioside, and thus thought to originate from plasma membrane raft microdomains. However, we found flotillin-2, an exosome marker (6), associated with these microparticles, suggesting that an exosome pathway may be a plausible origin. Although

flotillin-2 is also expressed on plasma membrane, ABCA1, a plasma membrane protein itself, was not associated with these microparticles, which is more consistent with an exosomal origin (**Figure 2.8**). Additional studies are required to determine whether other components in the exosomal pathway are associated with the microparticles, such as ESCRT proteins and cytoskeletal proteins (7). Similarly, the protein composition of these microparticles may also provide clues about their physiological functions, as the anti-oxidative functions of HDL partially come from associated enzymes such as PON1 and PAF-AH (8).

The amount of cholesterol released as microparticles is approximately 30% of total ABCA1-mediated cholesterol efflux, while 70% cholesterol efflux goes to apoA-I to form HDL. Thus, compared to HDL, the amount of cholesterol in the microparticles may not be as significant in contributing to the plasma lipid profile. However, as one of the major pathological features of atherosclerosis is the accumulation of cellular cholesterol in foam cells, even a small degree of alleviating the lipid burden could still be beneficial and atheroprotective, particularly if it could be greatly enhanced by Ht31 as we showed in Chapter 2. However, several key questions remain. First, the removal of cellular cholesterol from peripheral tissues, such as from foam cells in the atherosclerotic lesion, is only the very first step in RCT. We do not know presently whether cholesterol carried by microparticles can be effectively shuttled to the liver through the circulation. Secondly, the effectiveness of microparticles in RCT likely also depends on its protein component, as microparticles have to interact with hepatic cells for potential uptake and for eventual excretion. Future studies are needed to determine whether microparticles can interact with known lipoprotein receptors in the liver, including SRBI, CD36, SRA, and LDL receptor related protein (LRP). The fate of these microparticles is as important as the mechanism of their initial release, if this efflux pathway were to have therapeutic potential in suppressing atherosclerosis.

4.1.3 Ht31-Stimulated Microparticle Formation

We have demonstrated that disruption of PKA anchoring to AKAPs with st-Ht31 significantly enhances ABCA1-mediated cholesterol efflux as microparticles. Interestingly, st-Ht31 consists of an amphipathic helical structure, which is the essential component for apoA-I to interact with the membrane and promote cholesterol efflux. Our observations that membrane impermeable Ht31 does not produce microparticles (**Figure 2.6**) may indicate that

st-Ht31 does not act like apoA-I to associate with lipids. Only when Ht31 enters cells through stearic acid modification, disrupts PKA anchoring, and raises PKA activity specifically in the cytoplasm (**Figure 2.5**) could microparticles be generated. We also show that st-Ht31 is much more effective in stimulating microparticle release from ABCA1-expressing cells. This is likely due to higher permeability of st-Ht31 to ABCA1-expressing cells than to Mock cells (**Figure 2.7A**). Previous studies have reported that ABCA1 can alter the membrane lipid packing, such as disruption of lipid raft microdomains on the plasma membrane and increase in surface membrane fluidity, which could be the underlying mechanism for increased permeability of st-Ht31 (1,5). This preferential uptake of st-Ht31 to ABCA1-expressing cells may present an advantage as atherosclerotic lesions are rich in cholesterol-loaded macrophages that express high levels of ABCA1. Indeed, we observed that st-Ht31 was most effective in eliminating the accumulated lipid droplets within macrophage foam cells expressing ABCA1, in comparison with ABCA1 knockout foam cells (**Figure 2.9, B and D**).

Since st-Ht31 augments events downstream of ABCA1, treating ABCA1-null cells with a sufficiently high concentration of st-Ht31 was also capable of releasing microparticles (**Figure 2.7, B and C**). If this is so, how then could ABCA1-expressing cells also release microparticles in the absence of st-Ht31, i.e. basal level? One possibility is that the increase in cellular PKA activity by ABCA1 expression itself is sufficient to release microparticles, as shown in Chapter 3. Presently, we do not know how each individual pool of PKA at steady state, i.e. without st-Ht31, is affected by ABCA1 due to technical limitations. FRET experiment could only analyze changes that occurred in the same cells, such as before and after adding st-Ht31 or apoA-I, and within a relatively short period. However, we speculate that an overall increase in PKA activity by ABCA1 may encompass an increase in cytoplasmic PKA activity. This would promote microparticle release from ABCA1-expressing cells, although to a much lesser extent than from cells treated with st-Ht31.

4.1.4 Microparticles and its Atheroprotective Properties

The most interesting aspect of st-Ht31 is that it can stimulate microparticle release from lipid-laden macrophage foam cells. This reverses foam cell formation and restores macrophages back to a healthy metabolic state, in terms of both cholesterol homeostasis and

immune response (**Figure 2.9**). Thus, enhancing microparticle formation may have potential as a treatment to alleviate the lipid burden associated with atherosclerosis. Currently, an attractive concept for a potential therapy is the use of various apoA-I mimetic peptides, which takes advantage of the amphipathic helical structure of apoA-I (8). The amphipathic helical motif of apoA-I presents both a hydrophilic and hydrophobic face that mediates several cardioprotective functions attributed to apoA-I, including cellular cholesterol removal and inhibition of inflammatory processes. For example, the bihelical apoA-I mimetic peptide 5A complexed with phospholipids enhances ABCA1-mediated cholesterol efflux and increases whole-body RCT in atherosclerotic prone apoE knockout mice (9). ApoA-I mimetic peptides can also have broad anti-inflammatory functions, including suppressed expression of endothelial cell adhesion molecules and reduce macrophage TLR inflammatory signaling to produce pro-inflammatory cytokines (10). Further studies are required to investigate whether microparticles released by st-Ht31 treatment can contribute to RCT *in vivo* and have similar RCT-promoting functions as apoA-I mimetic peptides.

It has been shown that apoA-I mimetic peptides also have beneficial effects towards cardiovascular outcomes in various animal models. For instance, intravenous administration of mimetic peptide 5A complexed with phospholipids in apoE knockout mice reduced aortic plaque surface area (9), while another mimetic peptide, ETC-642, prevented increase in aortic plaque burden in hyperlipidemic rabbits (11). Similar *in vivo* experiments could be performed with st-Ht31 to induce atherosclerotic lesion regression. Although the study of apoA-I mimetic peptides have advanced significantly, the efficacy of apoA-I mimetic peptides still remains to be elucidated. For instance, subcutaneous and intravenous administration of L4F apoA-I mimetic peptide did not improve HDL function significantly in phase 1 clinical trials (12). This failure to improve HDL function has been speculated to be due to saturation of the apoA-I mimetic peptide with plasma lipids in vivo, since its mechanism is through their ability to bind lipids. In contrast, the mechanism of st-Ht31 is specifically targeting the anchoring domain of PKA-R subunits. Thus, there are unique opportunities to explore the use of st-Ht31 in anti-atherogenic therapy. For example, as the crystal structure of the PKA anchoring domain is well known (13), it is possible to develop small non-peptide molecules to target the same domain. If these non-peptide analogs can be modified to specifically target ABCA1-expressing cells, for instance, by differential

membrane permeability, they could relieve lipid burden from foam cells effectively and promote lesion regression.

4.2 Insights into PKA Activity and ABCA1

4.2.1 Elevated PKA Activity by ABCA1

We provided evidence that ABCA1 increases overall PKA activity; however, the exact mechanism is still unclear. ABCA1 is known to influence lipid-lipid interactions within the plasma membrane, giving rise to a reduction in lipid raft microdomains. We speculate that this disruption in raft microdomains induced by ABCA1 is the cause for the activation of PKA. Indeed, PKA can be activated with pharmacological agents that disrupt lipid rafts (**Figure 6b**). Components that regulate local cAMP concentrations, such as ACs (AC1, AC3, AC5, AC6, and AC8), AKAPs (AKAP79), G proteins, and PDEs, can localize to lipid rafts (14), which may facilitate protein complex formation and activation of specific cAMP signaling pathways. For instance, reducing lipid rafts impairs the sensitivity of AC6 and AC8 to Ca^{2+}-stimulated regulation (14). Thus, lipid raft microdomains appear to be crucial in preserving ACs responsiveness to specific receptor-mediated activation.

Interestingly, disruption of lipid rafts by MCD significantly increases basal, unstimulated activity of AC8 (15). This may reflect a lipid raft associated factor exerting inhibitory influences on basal AC activity or AC8 may come into contact with non-lipid raft factors to enhance its activity. Aligned with this concept, ABCA1 may provoke an increase in PKA activity through intrinsic changes in raft microdomains to influence factors within the PKA signaling cascade, such as enhancing the activation of AC to generate cAMP or prevent cAMP degradation by diesterases. Additional studies are necessary to determine the existence of ABCA1-induced changes in localized cAMP concentrations and identify factors that may associate or dissociate with PKA signaling complexes to influence their basal activities.

4.2.2 Compartmentalized PKA Activity and ABCA1

In recent years, the concept of compartmentalized PKA signaling has come to light with the discovery of AKAPs. Each type of AKAP dictates local PKA dynamics through its anchoring of PKA, AC, PDEs, and phosphatases. Thus, these AKAP-anchored complexes

form localized pockets of cAMP, providing a higher degree of specificity towards PKA signal transduction. Unexpectedly, apoA-I binding and lipidation requires anchored PKA signaling, as de-anchoring PKA with st-Ht31 impaired these ABCA1 activities (**Appendix II**). Furthermore, ABCA1 is itself a substrate of PKA and plasma membrane protein (16). Our recent observations found that apoA-I increases plasma membrane localized PKA activity in ABCA1-expressing cells (**Appendix I**) and enhances ABCA1 phosphorylation by PKA (**Appendix III**), which suggests a role for plasma membrane-anchored PKA activity in apoA-I-related ABCA1 functions. Consequently, impairing PKA phosphorylation of ABCA1 impairs its function to lipidate apoA-I. Conversely, cytoplasmic-localized PKA, as mentioned above, is another subcellular pool of PKA that we observed to regulate the microparticle generation aspect of ABCA1 function.

We show that st-Ht31 robustly enhances microparticle release, and cytoplasmic-localized PKA activity is a critical factor in this process. However, we do not understand which isoform of PKA (type I or II) and the specific AKAP involved. The PKA regulatory binding domain of AKAPs can vary, resulting in preferential interactions to PKA-RI or PKA-RII. Type I PKA is predominantly cytoplasmically localized while the majority of type II PKA associates at structural components within the cell. Since st-Ht31 peptide does not discriminate between RI and RII, it would be informative to investigate specific disruption of AKAP-RI and -RII anchoring interactions with the use of more recently optimized PKA-anchoring disruptor peptides. Another important reason for using specifically tailored disruptors is that plasma membrane-associated PKA activity is necessary for cholesterol efflux to apoA-I (**Appendix II**). In the presence of st-Ht31, plasma membrane-associated PKA activity was diminished, likely due to de-anchoring (**Figure 2.5**). Cholesterol efflux to apoA-I was also abolished due probably to the lack of proper phosphorylation of ABCA1 by plasma membrane anchored PKA (**Appendix III**). This would be a potential disadvantage of st-Ht31, even though it can stimulate cholesterol efflux through microparticles more than that normally brought out by apoA-I. Thus, our next focus should progress towards targeting more specific AKAP-PKA interactions, perhaps type I PKA anchoring. Such de-anchoring may offer more localized elevation of PKA activity without significantly affect plasma membrane bound PKA. This would allow more microparticle release but preserve apoA-I lipidation.

Additional studies are necessary to elucidate the downstream effectors of PKA and how the cellular pathways are influenced by ABCA1-induced PKA. While ABCA1 is a direct substrate of PKA, it is not the only factor that PKA targets. st-Ht31 potentially activates an exosomal secretory pathway, and there may be a causal relationship between PKA activity and exosome secretion. Interestingly, brefeldin A-inhibited guanine nucleotide exchange protein 2 (BIG2), an AKAP that is shuttled between the cytoplasm and intracellular secretory vesicles, is known to promote exosome-like vesicle release from endothelial cells (17). BIG2 can also be phosphorylated by PKA to modulate its activities to facilitate the activation of ADP-ribosylation factors (ARFs) that have crucial roles in intracellular vesicular trafficking (18). ABCA1-mediated microparticle release may also exploit similar machinery. Cytosolic proteins that are involved in the mechanism of exosome formation may be associated with released exosomes (i.e. Rab and ARF proteins), thus identifying exosome-associated proteins may provide more insight on the mechanism of microparticle formation. On the other hand, both ABCA1 and st-Ht31 increases CREB phosphorylation (**Figure 2.5D and 3.3**). CREB is a transcription factor known to modulate inflammatory signaling, which may link PKA activity to the anti-inflammatory aspects of ABCA1 function.

4.3 Inflammatory Response and ABCA1

4.3.1 Pro-inflammatory Suppression by ABCA1

Atherosclerosis is well established as an inflammatory disease; inflammation at the site of lesions is part of its pathology. Macrophages are immune cells that play a central role in the progression of atherosclerosis. They are recruited to atherosclerotic lesions to engulf modified LDL particles and expect to egress as inflammation resolves. However, under sustained lipid overloading, macrophages convert into lipid-laden foam cells. These foam cells continuously elicit pro-inflammatory response and hence hinder inflammation resolution. Indeed, ABCA1 deficiency in macrophage leads to increased levels of free cellular cholesterol and enhanced pro-inflammatory response. It is speculated that, by reducing free cholesterol content in lipid rafts, ABCA1 dampens pro-inflammatory response by suppressing TLR4 signaling.

Cholesterol-rich microdomains are proposed to be platforms to concentrate signaling factors involved in the initial events triggering protein signaling cascades. The macrophage TLR4 signaling cascade is one such pathway that implicates lipid raft microdomains in inflammatory response. Reduction of raft cholesterol by MCD through extracting membrane FC is directly coupled to diminished pro-inflammatory cytokine secretion in response to TLR4 agonists (19). This was thought to be due to a decrease in the initial TLR4 activation, since TLR4 activation requires the trafficking of TLRs into lipid rafts to form the TLR4/MD2/CD14 protein complex. We also observed less pro-inflammatory cytokine secretion by ABCA1 expression and cholesterol depletion. However, we provide evidence that this reduction in pro-inflammatory response is primarily due to activation of PKA (**Figure 3.8**).

As the primary function of ABCA1 is regulating cellular cholesterol through cholesterol export and disrupting raft microdomains, it is likely that ABCA1 activates PKA (as shown in **Figure 3.4**) through these cholesterol regulating activities. PKA is one of the two cAMP downstream effectors, the other being Epac, implicated to exert broad immunosuppressive effects (20). However, primarily PKA modulates pro-inflammatory cytokine secretion in macrophages (21). Currently, the underlying mechanism in which ABCA1-activated PKA reduces pro-inflammatory response remains to be elucidated. A recent study demonstrated PKA is targeted to NF-κB p105 subunit by AKAP95 to phosphorylate p105, which is suggested to repress NF-κB transcription activity and suppress LPS-induced expression of proinflammatory cytokine genes (21). Perhaps the elevation of PKA by cholesterol depletion and ABCA1 employs a similar mechanism to suppress LPS-induced pro-inflammatory response.

4.3.2 Anti-inflammatory IL-10 Induction by ABCA1

In addition to observing a suppression of pro-inflammatory response, we demonstrated an enhancement of anti-inflammatory IL-10 secretion by ABCA1, which resembles an M2-like response (**Figure 3.2**). Such increase in IL-10 secretion was also seen with reduction of cholesterol (**Figure 3.8**). If the initial event of TLR4 signaling is suppressed by cholesterol reduction, we would expect all downstream responses to be suppressed. Instead, the secretion of IL-10, which is a major downstream event of LPS-induced TLR4 signaling, is increased,

which goes against the notion of suppressed initial TLR4 signaling by reducing lipid raft cholesterol. Indeed, MCD treatment also enhanced LPS-mediated IL-10 production from differentiated THP-1 cells, although the signaling pathways were not addressed in this study (22).

PKA-dependent enhancement of LPS-induced IL-10 secretion has been proposed to occur through CREB transcription activity, since *IL-10* is a target gene of CREB (21). On the other hand, it has also been suggested that AKAP10-anchored PKA signaling contributes to IL-10 secretion (20). Further studies are required to determine the precise mechanism that governs this PKA-induced IL-10 production. The IL-10 signaling cascade is well established as an immunosuppressive mediator to down-regulate and limit pro-inflammatory cytokine expression in order to resolve inflammation (23). This allows macrophages and other immune cells to maintain a controlled immune response during injuries or infections and ultimately prevent chronic inflammation, as in atherosclerosis. ABCA1, by enhancing anti-inflammatory IL-10 production, may impede the progression of atherosclerosis.

4.4 Cytokines and Atherosclerosis

As immune responses are involved in atherosclerosis, it is evident that cytokines play a major role. Several cytokines, including IL-12 and TNF-α, are expressed within atherosclerotic lesions, resulting in disease progression. Cytokines are suggested to be key players in regulating macrophage foam cell formation, a central process in atherogenesis. A number of studies have reported pro-inflammatory cytokines promote lipid retention in macrophages and accelerated murine atherosclerosis *in vivo*. For instance, Xanthoulea *et al.* showed that TNF-α target receptor deficiency reduced foam cell formation in $LDL^{-/-}$ mice (24). Conversely, anti-atherogenic cytokines have also emerged. Anti-inflammatory IL-10 has been demonstrated to reduce cholesterol accumulation in human macrophages by promoting ABCA1 and prevent foam cell formation and atherosclerosis *in vivo* (25). It is becoming quite evident that the presence of pro- and anti-inflammatory cytokines modulates the rate of disease progression (26). Since pro-inflammatory cytokines predominantly promote atherogenesis, this emphasizes the importance of a balance between pro-inflammatory and anti-inflammatory responses in atherosclerosis. Indeed, this has led investigators to explore the possibility of therapeutic intervention of pro-inflammatory

cytokines as treatment for CVD. Blocking TNF-α has reported to lower incidences of cardiovascular events in rheumatoid arthritis patients (27). Alternatively, administering anti-inflammatory IL-10 has also been proposed as a potential treatment against atherosclerosis (28). However, these potential therapies to suppress pro-inflammatory response may pose a risk of impairing normal immune functions to fight against viral and microbial infections.

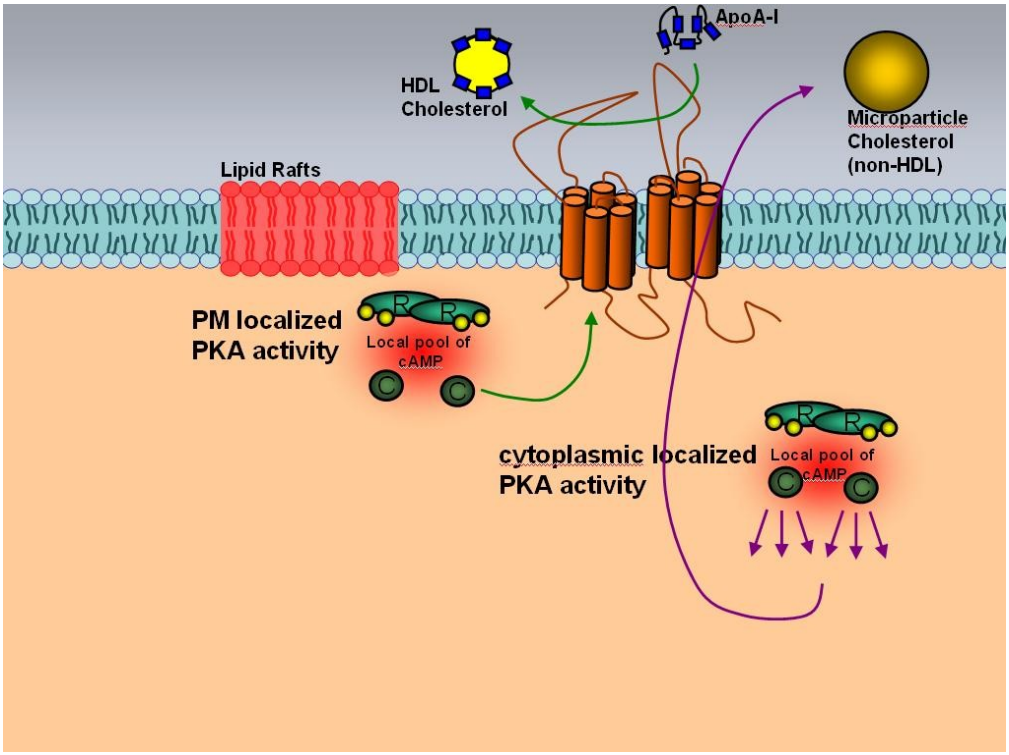
Our observations on ABCA1 and its regulatory functions on cytokines may provide significant insight into developing promising therapies to target atherosclerosis. Nuclear receptors, such as LXR, have been shown to have immunomodulatory functions to favour an anti-inflammatory M2 phenotype (29). Also, LXR agonists (eg. T0901317) have anti-atherogenic effects (30). Our findings that ABCA1 itself drives macrophages towards an anti-inflammatory M2-like phenotype provides an additional pathway in which LXRs agonists can contribute to atheroprotection. Since ABCA1 transcription is induced by LXR activation, at least part of the anti-inflammatory and atheroprotective effects of LXR agonists may perhaps be due to its upregulation of ABCA1. Furthermore, cholesterol-lowering agents, such as statins, are currently the most successful treatment for CVD. Statins are also known to modulate serum levels of inflammatory cytokines and exhibit anti-inflammatory activities, such as upregulating IL-10 and suppressing TNF-α (26). Thus, some of these cytokine-modulating actions of statins may be contributed by enhancing PKA signaling due to its cholesterol-lowering effect in lesional macrophages. Our findings here provide a possible mechanism in which current therapies, such as cholesterol-lower agents, may lead to their anti-inflammatory actions.

4.5 Conclusion

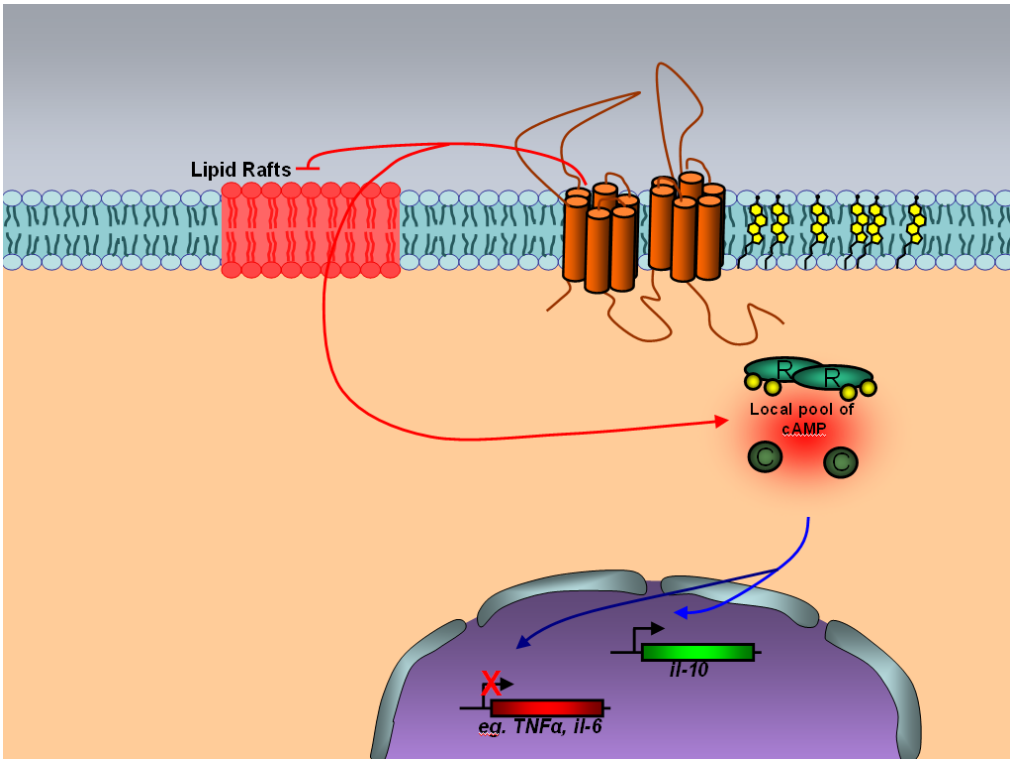
Work presented in this thesis demonstrates that PKA is a critical component of ABCA1 function (**Figure 4.1**). First, compartmentalized PKA signaling plays a key role in ABCA1-mediated cholesterol efflux to specifically promote the release of microparticles and lipidate apoA-I. Future studies to understand these pathways in more detail will undoubtedly provide new insights into cellular cholesterol modulation and atheroprotective functions of ABCA1. Targeting compartmentalized PKA activation has potential as a future therapeutic strategy against atherosclerosis by reversing foam cell formation and suppressing inflammation. Secondly, we provide evidence that the activation of PKA by ABCA1 or by cellular

Figure 4.1 **Model diagrams summarizing the role of PKA in ABCA1 function.** Compartmentalized PKA activity is important in ABCA1-mediated cholesterol efflux. While lipidation of apoA-I is dependent upon PM localized PKA activity, microparticle release requires cytoplasmical localized PKA activity **(A)**. Cholesterol depletion by ABCA1 promotes M2-like inflammatory response in macrophages through increasing PKA activity **(B)**.

A


ApoA-I
HDL
Cholesterol
Microparticle
Cholesterol
(non-HDL)
Lipid Rafts
PM localized
PKA activity
Local pool of
cAMP
cytoplasmic localized
PKA activity
Local pool of
cAMP

B


Lipid Rafts
Local pool of
cAMP
il-10
eg. TNFα, il-6

cholesterol reduction is a critical underlying mechanism to promote a M2-like anti-inflammatory response in macrophages. This suggests that ABCA1 and/or statins can provide a beneficial role by promoting inflammation resolution in atherosclerotic plaques. Thus, upregulating macrophage ABCA1 expression and function is emerging to be a potential approach to treat and prevent atherosclerosis.

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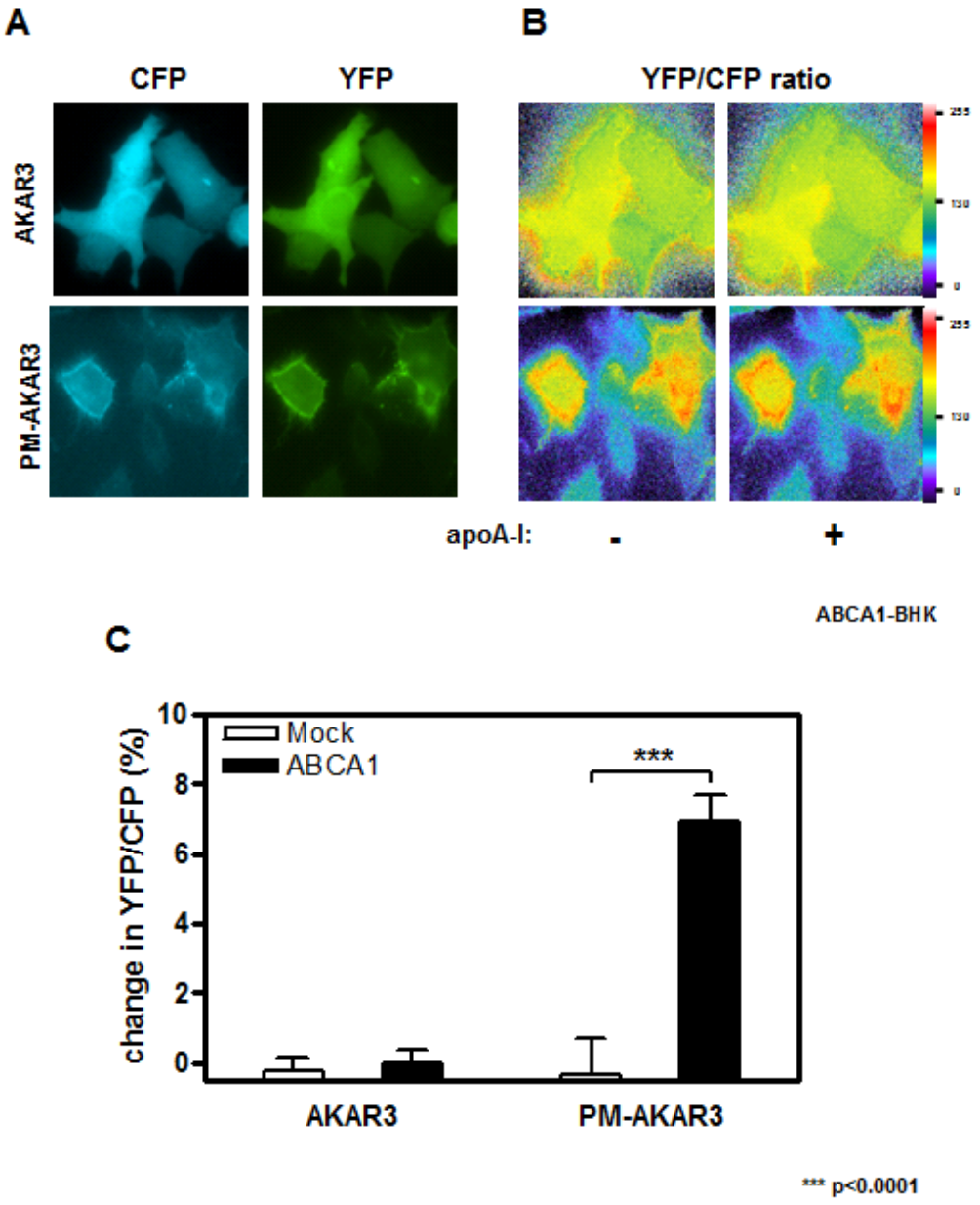
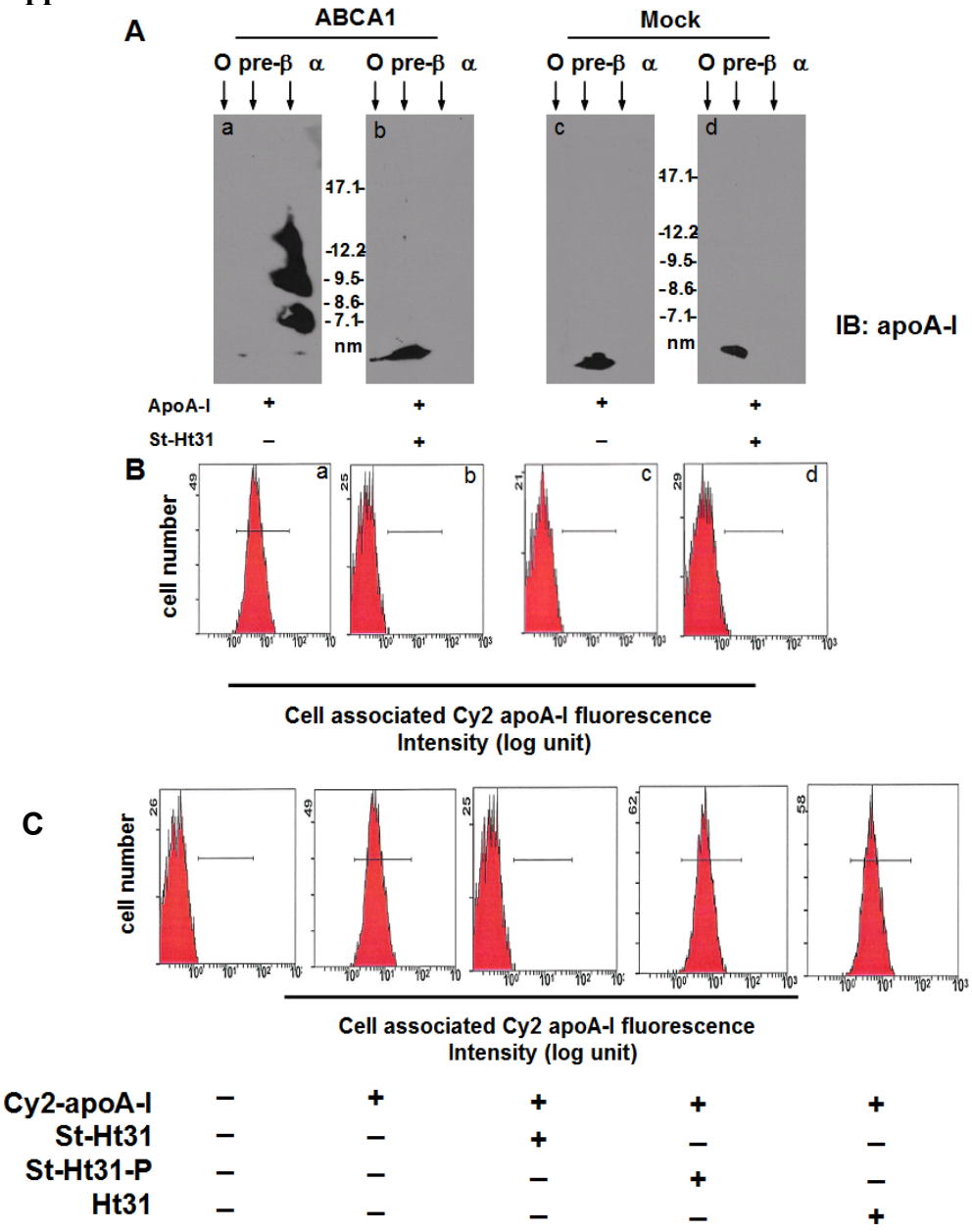
Appendix I:



ApoA-I increases plasma membrane localized PKA activity in ABCA1-expressing cells. A. ABCA1-BHK cells were transfected with either AKAR3 or pm-AKAR3 and induced overnight with mifepristone. Representative images from CFP and sensitized YFP channels were shown. **B.** CFP and sensitized YFP images were taken before and after addition of apoA-I (10 min). After background and cross-talk correction, a pair of ratio images (YFP/CFP) was produced and displayed under identical setting. **C.** FRET intensities (YFP/CFP) were calculated for each individual cell and presented as percentages of change before and after apoA-I addition. The data represent the averages from 50–100 cells and standard errors of the mean.

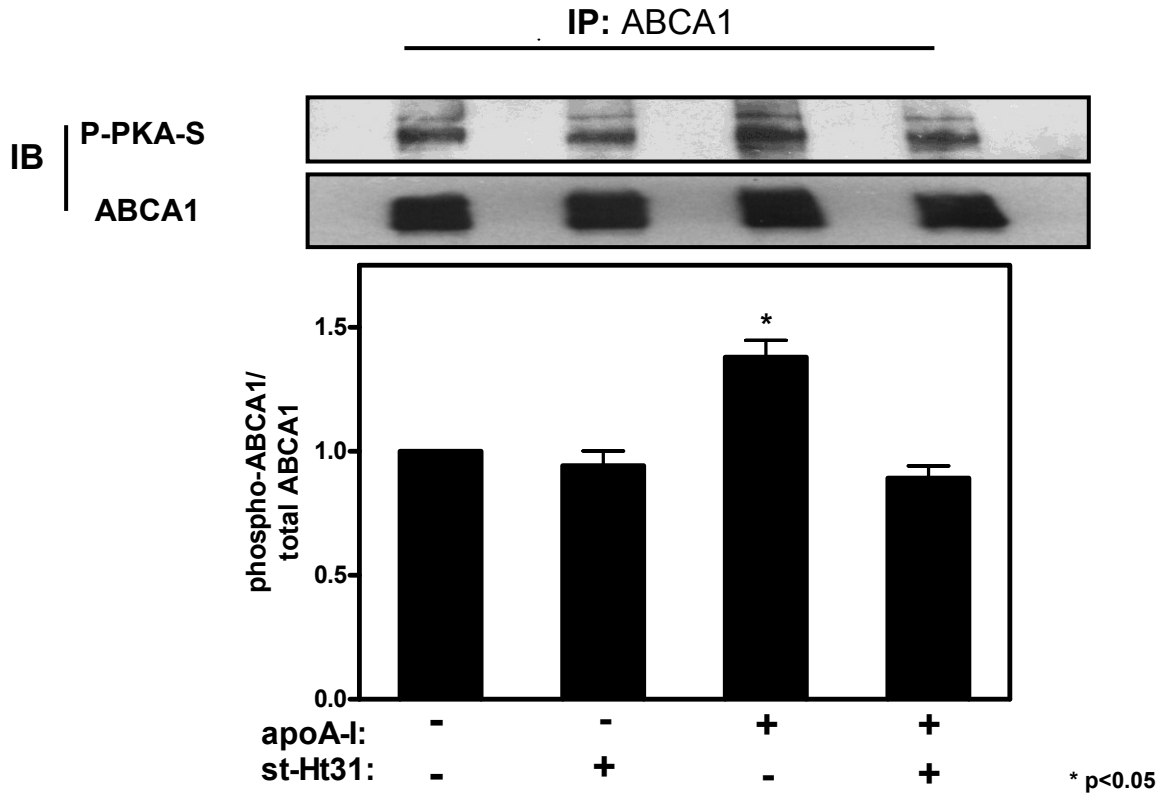
Appendix II:



Cy2-apoA-I – fluorescently labeled apoA-I
st-Ht31 – cell permeable peptide that inhibits anchoring of PKA
st-Ht31-P – null mutant control peptide
Ht31 – cell impermeable peptide

ApoA-I binding and lipidation requires anchored PKA signaling. A. Lipidated and unlipidated apoA-I was analyzed by nondenaturing 2D gel electrophoresis. Experiment performed by Dr. Maxime Denis **B & C** Cells were preincubated with Cy2 labeled apoA-I for 30 mins before apoA-I binding analysis with flow cytometry.

Appendix III:



ApoA-I enhances ABCA1 phosphorylation by anchored PKA. Induced ABCA1-BHK cells were treated with apoA-I (5 μg/mL) or st-Ht31 (5 μM) for 2 h. ABCA1 was immunoprecipitated and blotted using phospho-PKA substrate antibody and ABCA1 antibody, respectively. Representative blots from three independent experiments were shown. The *bars* represent the means ± standard deviations. Experiment performed by Dr. Fumin Dong.

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