THE HIGH DENSITY LIPOPROTEIN RECEPTOR, SR-BI

INSIGHTS INTO THE DEVELOPMENT OF ATHEROSCLEROSIS AND CORONARY ARTERY DISEASE: STUDIES FROM GENE TARGETED MICE LACKING THE HIGH DENSITY LIPOPROTEIN RECEPTOR, SR-BI

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ABSTRACT

High density lipoprotein (HDL) is an independent risk factor for the development of coronary heart disease. HDL mediated reverse cholesterol transport is a key element responsible for the cardioprotective effects of HDL. In addition HDL exerts other atheroprotective effects in vascular cells. The HDL receptor, scavenger receptor class I type B (SR-BI) derives the process of reverse cholesterol transport, mediates HDL signaling in the vasculature and protects against atherosclerosis. However, the exact atheroprotective mechanisms of HDL and SR-BI are not clearly understood.

This thesis starts by characterizing a model of occlusive coronary artery atherosclerosis, the SR-BI/apolipoprotein E double knockout mice and tests the effects of phenolic rich pomegranate extract on disease progression. Coronary artery disease in these mice starts at three weeks of age and progresses rapidly leading to sudden death within three to five weeks. The administration of pomegranate extract reduced the extent of coronary artery atherosclerosis possibly via mechanisms that involved alterations in lipid metabolism and reduced inflammation and oxidative stress.

The next two chapters aimed to gain better understanding of the atheroprotective actions of HDL and SR-BI. Increased macrophage apoptosis is a key event in the development of atherosclerotic plaques. HDL signaling via SR-BI reduced macrophage apoptosis while the lack of macrophage SR-BI was associated with increased macrophage apoptosis and necrotic core areas, features of plaque instability. Next HDL and SR-BI effects on macrophage migration, a key event in atherosclerotic plaque

regression, are described. HDL stimulated the migration of macrophages in a manner that was dependent on SR-BI, its adaptor protein, PDZK1, and the G-protein coupled receptor, sphingosine-1-phosphate receptor 1. SR-BI mediated macrophage migration may suggest a potential role of SR-BI in atherosclerotic plaque regression.

To expand our view of HDL effects on macrophages we have used proteomics as an approach. HDL treatment of macrophages altered the expression of multiple proteins. Validation experiment confirmed changes in interesting and particularly relevant protein targets in HDL mediated protection against macrophage apoptosis and inflammation and in HDL induced macrophage migration. Follow up experiments will determine their involvement in HDL and SR-BI mediated signaling. Overall this work represents a milestone in understanding the atheroprotective effects of HDL and SR-BI in macrophages.

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LIST OF ABBREVIATIONS

2D-DIGE	Two Dimensional-Difference In Gel Electrophoresis
ABC	ATP Binding Cassette Transporter
ACAT	Acyl-Coenzyme A: Cholesterol Acyltransferase
AcLDL	Acetyl-Low Density Lipoprotein
ANOVA	Analysis of Variance
Apo	Apolipoprotein
ApoE KO	Apolipoprotein E Knockout
Bad	Bcl-2 Associated Death Promoter
Bax	Bcl-2–associated X protein
Bcl-2	B- Cell Lymphoma-2
Bcl-xL	B- Cell Lymphoma Extra Large
BH3	Bcl-2 Homolog Domain 3
Bid	BH3 Interacting Domain Death Agonist
Bim	BCL-2-Interacting Mediator of Cell Death
BM	Bone Marrow
BMT	Bone Marrow Transplantation
C5a	Complement Protein 5 A
CA	Coronary Arteries
CapG	Gelsolin Like Capping Protien
CCR2	Chemokine Receptor 2
CD11b	Cluster Differentiation Molecule 11b
CD36	Cluster Differentiation Molecule 36
CE	Cholesterol Esters
CETP	Cholesterol Ester Transfer Protein
CHO	Chinese Hamster Ovary Cells
CHOP/GADD153	C/EBP Homologous Protein / Growth Arrest and DNA-Damage-Inducible Protein
COS-M6	African Green Monkey SV40-transfected Kidney Fibroblasts
dKO	Double Knockouts
DIGE	Difference In Gel Electrophoresis
DMEM	Dulbecco's Modified Eagle's Medium
dNT-1	Deoxyribonucleotide Transferase-1
E2-25K/HIP2	Ubiquitin Conjugating Enzyme-2/Huntington Interacting Protein 2
ECs	Endothelial Cells
eNOS	Endothelial Nitric Oxide Synthase
ER	Endoplasmic Reticulum
ERK	Extracellular Signal Regulated Kinase
$E_x\lambda$	Excitation Wavelength

FBS	Fetal Bovine Serum
FC	Free Cholesterol
Foxo3a	Forkhead Box O3
FPLC	Fast Pressure Liquid Chromatography
GAC	Galic Acid Equivalents
GPCR's	G-Protein Coupled Receptors
Grp78	Glucose Regulated Protein 78
HDL	High Density Lipoprotein
HMG-CoA	Hydroxy-Methley Glutaryl Coenzyme A
HRP	Horse Raddish Peroxidase
Hypo	Hypomorphic
ICAM-1	Inter-Cellular Adhesion Molecule-1
IEF	Isoelectric Focusing
IFN-γ	Interferon-γ
IgG	Immunoglobulin-G
IL-1	Interluikin-1
IL-6	Interluikin-6
IκB	Inhibitor of Nuclear Factor- κB
JNK	c-Jun N-Terminal Kinases
KO	Knockout
LCAT	Licithin Cholesteryl Acayl Transferase
LC-MS/MS	Liquid Chromatography - Mass Spectrometry/Mass Spectrometry
LDL	Low Density Lipoprotein
LDLR	Low Density Lipoprotein Receptor
LPS	Lipopolysaccharide
MAPKs	Mitogen Activated Protein Kinases
MCP-1	Monocyte Chemotactic Protein-1
MDM	Monocyte Derived Macrophages
mmLDL	Minimally Modified Low Density Lipoprotein
MPM	Mouse Peritoneal Macrophages
NCLPDS	Newborn Calf Lipoprotein Deficient Serum
NF-κB	Nuclear Factor- κB
NO	Nitric Oxide
oxLDL	Oxidized Low Density Lipoprotein
p38 MAPK	P38 Mitogen Activated Protein Kinase
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PDGF	Platelets Derived Growth Factor
PDGFR	Platelets Derived Growth Factor Receptor
PDZK1	Postsynaptic Density Protein (PSD-95)/Drosophila Discs-Large (Dlg)/Tight-Junction Protein (ZO1)

p-eIF2α	Phopshorylated- Eukaryotic Translation initiation Factor 2 α
PH	Plekstrin Homology Domain
PI(3,4)P2	Phosphatidylinositol (3,4)-bisphosphate
PI3Ks	Phosphoinositide 3-Kinases
PIP3	Phosphatidylinositol(3,4,5)-trisphosphate
PKC	Protein Kinase C
POMlx	Pomegranate Liquid Extract
PTX	Pertussis Toxin
QRT-PCR	Quantitative Real Time-Polymerase Chain Reaction
RCT	Reverse Cholesterol Transport
rHDL	Reconstituted HDL
ROCK	RhoA-Activated Kinase
ROS	Reactive Oxygen Species
RT	Room Temperature
S1P	Sphingosine-1-Phosphate
S1PR	Sphingosine-1-Phosphate Receptor
SAA	Serum Amyloid A
Scap	SREBP Cleavage Activating Protein
SDS-PAGE	Sodium Dodecyle Sulfate Poly Acrylamide Gel Electrophoresis
SK	Sphingosine Kinase
SR-A	Scavenger Receptor Class A
SR-BI	Scavenger Receptor Class B Type-I
SR-BI/apoE dKO	Scavenger Receptor Class B Type I/Apolipoprotein E Double Knockout
TC	Total Cholesterol
TLR	Toll Like Receptor
TNF-α	Tumor Necrosis Factor-α
TUNEL	Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling
UC	Unesterified Cholesterol
VCAM-1	Vascular Cell Adhesion Molecule-1
VLDL	Very Low Density Lipoprotein
VSMCs	Vascular Smooth Muscle Cells
WT	Wild Type

CHAPTER ONE

INTRODUCTION

PREFACE

The majority of this work has previously been published in:

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This work has been modified to set context for the upcoming chapters of the thesis.

1. Abstract

High density lipoprotein (HDL) levels are inversely proportional to the risk of coronary heart disease. HDL mediates various anti-atherogenic pathways including reverse cholesterol transport from cells of the arterial wall to the liver and steroidogenic tissues. In addition HDL activates various intracellular signaling events that confer atheroprotection. The HDL receptor, scavenger receptor class B type I (SR-BI) has been implicated directly and indirectly in HDL mediated atheroprotection. In this chapter the involvement of SR-BI in atherosclerosis and its role HDL induced signaling in the vasculature will be discussed.

2. HDL structure and function

High density lipoprotein (HDL) constitutes a heterogeneous population of lipoproteins with differences in shape, size, density, apolipoprotein and lipid composition and surface charge. The structure and complexity of HDL has been covered extensively in a number of recent reviews [1-7]**.** In vivo, HDL particles undergo continuous remodeling which has a significant impact on their properties and adds further to their heterogeneity (reviewed in [1, 7, 8]). Human HDL can be separated by ultracentrifugation into two major subfractions: HDL_2 ($1.063 < d < 1.125$ g/ml) and HDL_3 ($1.125 < d < 1.215$ g/ml) and can be classified based on size into five distinct subpopulations ranging from 10.6 to 7.6 nm in diameter [9]. HDLs consist of a hydrophobic core (mainly cholesteryl esters and small amounts of

triacylglyceride) surrounded by a monolayer of phospholipids, unesterified cholesterol and apolipoproteins (apo) [1-7]. HDL particles can be separated by agarose gel electrophoresis into alpha, pre-beta or gamma migrating particles relative to the migration of plasma proteins [10, 11]. Spherical HDL particles, including HDL_2 and HDL_3, migrate predominantly at an α- position during agarose gel electrophoresis however some large HDL particles migrate at a pre-β position and are known as pre-β-2 HDL. On the other hand the majority of lipid free apoA-I and discoidal HDLs migrate at a pre-β position and are designated as pre-β-1 HDL ([12], reviewed in [13]). The two major apoliporoteins of HDL are apoA-I and apoA-II and HDL particles can be classified based on their apoA-I composition into apoA-I HDL or apoA-I/apoA-II HDL (reviewed in [7]). ApoA-I HDL contain apoA-I but not apoA-II and are larger particles that appear in the HDL_2 subfraction, while the apoA-I/apoA-II HDL are smaller and denser particles that contain both apoA-I and apoA-II and are enriched in the HDL_3 subfraction [14]. Additional apolipoproteins that are associated with HDL include apoA-IV, apoA-V, apoC-I, apoC-II, apoC-III, apoD, apoE, apoJ, apoM, apoF and apoL [15, 16]. Several enzymes involved in plasma lipid metabolism are transported by HDL such as cholesteryl ester transfer protein (CETP), lecithin:cholesterol acyl transferase (LACT) and phospholipid transfer protein (reviewed in [1]). Moreover recent proteomics studies revealed that proteins and enzymes other than those involved in lipid metabolism are also carried by HDL including proteins involved in complement regulation, acute phase responses and protease inhibition [17] (reviewed in [3-6, 18, 19]). HDL also carries a variety of

biologically active lipids including lysosphingolids such as sphingosine-1-phosphate (S1P), lysophosophatidic acid and sphingophosphorylcholine (reviewed in [18]) and diverse lipid soluble vitamins and hormones [20-25].

Clinical and epidemiological studies have established a strong inverse relationship between HDL cholesterol levels and the risk of coronary heart disease [26-31]. More recent studies suggest a stronger relationship between the levels of HDL's major apolipoprotein, apoA-I and CHD risk, suggesting that HDL mediated atheroprotection is more likely dependent on HDL particle number and/or activity rather than HDL cholesterol [32]. Additionally, increased HDL levels have been correlated with a more stable plaque morphology [33] and reduced plaque lipid core size [34, 35] and appear to promote plaque regression [36-38]. The most well studied mechanism by which HDL protects against atherosclerosis, initially proposed by Glomset, is reverse cholesterol transport (RCT) [39]. RCT involves the removal of membrane cholesterol from cells, including macrophages in the artery wall, and its delivery to the liver for bile acid biosynthesis, biliary excretion or recycling in new lipoproteins or to steroidogenic tissues for steroid hormone production (reviewed in [2, 6, 40-44]). The first step in the RCT pathway involves the lipidation of apoA-I by the ATP-binding cassette transporter A1 (ABCA1) leading ultimately to the formation of spherical particles that accept additional cholesterol and phospholipids by other ABC transporters including ABCG1 and ABCG4 ([45-47] and references therein). This sequential lipidation process results in the net removal of cholesterol

from peripheral cells and leads to the formation of cholesterol ester enriched large spherical HDL particles, the preferred ligand of the hepatic scavenger receptor class B type I (SR-BI) [48, 49]. Studies in mice suggest that cholesteryl esters from these particles can be selectively taken up by hepatic SR-BI [50-53]; in humans, they can also be transferred from HDL to LDL, via CETP (recently reviewed in [54]). LDL particles are then cleared from the circulation by the hepatic LDL receptor (LDL-R) [55].

In addition to its role in RCT, HDL has anti-inflammatory, anti-thrombotic and anti-oxidant properties and promotes endothelial repair (reviewed in [7, 18, 56]). Several of these properties are mediated by the activation of signaling pathways in endothelial cells [57-66]. Moreover, many of the atheroprotective actions of HDL appear to be mediated by interactions with SR-BI. This receptor can mediate selective HDL lipid uptake and cholesterol efflux from cells and studies in mouse models suggest that hepatic SR-BI drives HDL RCT (reviewed in [67-69]). In addition SR-BI appears to mediate HDL dependent activation of signaling pathways in endothelial cells (Fig.1.1).

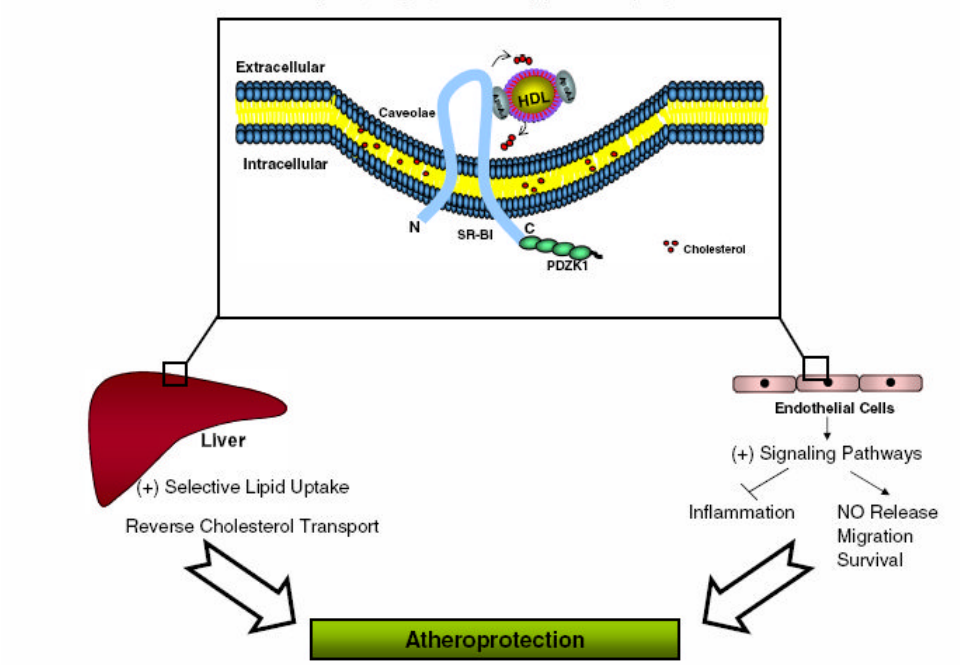

Fig. 1.1: Atheroprotective pathways mediated by SR-BI. SR-BI binds to HDL, via apoA-I, and mediates selective uptake of HDL cholesterol (top; arrows indicate cholesterol movement). In this way, SR-BI in liver hepatocytes drives reverse cholesterol transport by mediating hepatic clearance of HDL cholesterol (left). In vascular endothelial cells (right) HDL binding to SR-BI triggers the activation of PI3K/Akt and ERK1/2 signaling pathways leading to the suppression of inflammation and the stimulation of NO production, cell migration and prevention apoptosis.

This chapter will introduce SR-BI structure, function and role in atherosclerosis. It will also review the current state of knowledge of the role of HDL and SR-BI in signaling, focusing on endothelial cells, where most of the research has been done, to set the stage for the analysis of SR-BI mediated HDL signaling in macrophages which will be the focus of chapters 3-5.

3. The HDL receptor SR-BI

3.1. SR-BI structure and function

SR-BI, originally identified as a receptor able to bind to acetyl-LDL [70], was the first molecularly characterized HDL receptor [71]. The SR-BI protein is a 509 amino acid polypeptide with two hydrophobic, membrane spanning domains flanking a large, heavily N-glycosylated extracellular domain. It has two relatively short cytoplasmic tails of 10 and 40 amino acids at the N and C termini, respectively. The C-terminal cytoplasmic tail of SR-BI is also fatty acylated. SR-BII is generated through alternative splicing of the transcript and has a distinct C-terminal cytoplasmic tail. The structure of SR-BI has been comprehensively reviewed previously [69, 72]. The terminal 4 residues (EAKL) of SR-BI's C-terminal cytoplasmic tail interact with the multi-subunit adaptor protein, PDZK1 [PDZ-(postsynaptic density protein (PSD-95)/*Drosophila* discs-large (dlg)/tight-junction protein ZO1) containing 1] [73], a 70 kDa cytosolic protein that was shown to be important for the stability of SR-BI in liver hepatocytes and for SR-BI-dependent signaling in endothelial cells [63, 74]. SR-BI binds

to a variety of ligands including native and modified lipoproteins, modified serum proteins such as advanced glycation end product-modified serum albumin and maleylated serum albumin, lipid vesicles containing anionic phospholipids, apoptotic cells and bacterial cell surface components such as lipopolysaccharide (LPS) (reviewed in [69, 72, 75]). Despite its broad ligand binding specificity, studies of knockout and transgenic mice reveal that SR-BI plays a critical role in HDL metabolism [69, 71, 72]. Larger, cholesterol ester rich α-HDL particles bind to SR-BI with greater affinity than smaller, lipid poor pre-β HDL or lipid free apo-AI [48] (reviewed in [76]). The relative content of apo-AI vs. apo-AII in α-HDL affects the interaction of the HDL particle with SR-BI, such that SR-BI binds with greater affinity to apoA-II enriched particles yet with reduced lipid uptake activity [77]. In contrast de Beer et al. reported that SR-BI binds to apoA-I reconstituted HDL with greater affinity than apoA-I/apoAII reconstituted HDL, however the latter is more efficient in mediating SR-BI selective lipid uptake [78].

SR-BI mediates selective lipid uptake from lipoprotein ligands [69, 72]. Selective lipid uptake refers to the transfer of HDL lipids into cells without the net internalization and degradation of the entire HDL particle, itself [69, 72, 79]. SR-BI mediates the uptake of diverse lipids from HDL particles, including cholesteryl esters (CE), unesterified cholesterol, phospholipids, triglycerides and lipid soluble vitamins including α-tocopherol (reviewed in [69]). SR-BI also mediates the efflux of unesterified (free) cholesterol (FC) from cells to lipoproteins via a passive, energy

independent process [80-85]. Thus SR-BI mediates the bidirectional movement of lipids between cells and bound lipoproteins, the net direction of which depends on the concentration gradient [80-85] (reviewed in [86]).

SR-BI is a key player in RCT. It has been proposed that SR-BI participates in cholesterol efflux from cells to HDL, although this has been controversial; while overexpression of SR-BI in cultured cells generally increases efflux of cholesterol tracer to HDL, elimination of SR-BI expression from macrophages does not consistently result in altered HDL dependent cholesterol efflux [84, 87-91] (reviewed in [42, 92, 93]). The underlying reasons for this discrepancy are not well understood. On the other hand, studies involving overexpression or inhibition of SR-BI in cells and in mouse models have consistently demonstrated a key role for hepatic SR-BI in the selective uptake of HDL lipids [51-53, 71, 94-97]. HDL cholesterol taken up by liver hepatocytes via SR-BI appears to be secreted into bile supporting a role for hepatic SR-BI in RCT [51, 52, 96, 98-101].

3.2. SR-BI in atherosclerosis

Several studies established a role of SR-BI expressed in hepatocytes and in bone marrow derived cells in the protection against atherosclerosis [91, 101-107] Hepatic overexpression of SR-BI reduced atherosclerosis in homozygous and heterozygous LDL-receptor knockout (LDL-R KO) mice [104, 108] and in apoB transgenic mice [103, 104, 108]. Similarly, reduced expression of SR-BI in livers, due

to induced hypomorphic mutations [97, 109], Cre-LoxP mediated liver specific targeting [109] or due to knockout of PDZK1 [110, 111] result in increased atherosclerosis in chow fed apoE KO or high fat diet fed mice on wild type or LDLR KO backgrounds.

The role of SR-BI in bone marrow derived cells in the protection against atherosclerosis was demonstrated by transplanting bone marrow from SR-BI deficient mice into SR-BI$^{+/+}$ mice that were susceptible to atherosclerosis due to deficiency in apoE, or LDLR or due to high fat diet feeding [91, 102, 105]. Reconstitution of LDLR KO recipients with SR-BI KO bone marrow stem cells resulted in increased levels of high fat diet induced aortic atherosclerosis when compared with control LDLR KO mice reconstituted with bone marrow from SR-BI$^{+/+}$ donors [102, 105]. Similar results were obtained when wild type mice were used as recipients [102]. Similarly, the transplantation of bone marrow from SR-BI/apoE dKO mice into apoE single KO recipients resulted in increased levels of spontaneous aortic atherosclerosis compared to control apoE KO mice transplanted with bone marrow from apoE KO recipients [91]. Finally, liver specific targeted deletion of SR-BI in hypomorphic mice with reduced SR-BI expression in steroidogenic tissues but normal expression in macrophages, resulted in lower levels of diet induced atherosclerosis induction and reduced lesional macrophage content than did whole body SR-BI deletion in SR-BI KO mice [109]. This led to the suggestion that SR-BI in macrophages may protect against atherosclerosis, accounting for the differences in lesion size and composition

seen in the "liver specific" and whole body SR-BI KO mice. Together, these studies demonstrate that SR-BI plays an important atheroprotective role in bone marrow derived cells, possibly macrophages, in atherosclerotic plaques.

In addition to developing increased atherosclerosis in the aortic sinus, mice lacking SR-BI and apoE develop spontaneous occlusive atherosclerotic lesions in their coronary arteries, resulting in myocardial infarctions, reduced heart function, cardiac conductance abnormalities and ultimately death by 5-8 weeks of age [107]. These phenotypes can be delayed by treatment with the anti-oxidant drug probucol [112], by prevention of intestinal absorption of cholesterol with ezetimibe or of bile acids with an inhibitor of the apical sodium-dependent bile acid transporter [113], or by knockout of hepatic lipase [114]. On the other hand, elimination of lymphocytes by knockout of the recombination activating gene 2 did not reduce the onset or degree of occlusive coronary artery atherosclerosis, myocardial infarction, cardiac functional or conductance abnormalities or the survival of SR-BI/apoE dKO mice [115].

Similar to SR-BI/apoE dKO mice, SR-BI KO mice that also contain an induced hypomorphic mutation in the apoE gene, (resulting in a 95% reduction in apoE in normal chow fed mice) develop high fat, high cholesterol diet-induced occlusive coronary artery atherosclerosis, accompanied by similar outcomes of myocardial infarction, reduced heart function, cardiac conductance abnormalities and premature death (after 3-4 weeks of diet induction) [116]. Recently, Krieger and co-workers have demonstrated a similar, though less severe phenotype of diet induced

coronary artery atherosclerosis in PDZK1/apoE dKO mice [117]. These findings suggest that SR-BI may play a particularly important role in the protection of coronary arteries against development of occlusive atherosclerosis. However the mechanisms of plaque development and the role of SR-BI in coronary arteries of mice lacking SR-BI on an atherogenic background are not clearly understood.

In an attempt to understand the mechanisms of disease development in coronary arteries of mice lacking SR-BI we have tested the role of inflammation and oxidative stress in coronary artery atherosclerosis in the SR-BI/apoE dKO mice. Several clinical and experimental studies have tested the effects of anti-oxidant rich natural products on the development of coronary heart disease [118-120]. However the effects of anti-oxidant rich natural products on coronary artery atherosclerosis have not been directly tested. Pomegranate is an anti-oxidant rich fruit that has frequently been described as cardioprotective [121]. Therefore we have tested if coronary artery atherosclerosis in the SR-IB/apoE dKO mice can be reduced by the administration pomegranate extract. In chapter two the involvement of inflammation and oxidative stress in the development of coronary artery atherosclerosis in the SR-BI/apoE dKO mice and the effects of pomegranate extract on disease development are described.

4. HDL and SR-BI Signaling

In addition to mediating RCT, HDL can also activate cellular signaling pathways. This has been most well studied in endothelial cells and a review of this literature sets the stage for my investigation of SR-BI's role in HDL signaling in macrophages. SR-BI may participate in HDL induced signaling via functional and/or direct interactions with signaling receptors and/or down-stream signaling molecules to activate pathways that confer atheroprotection in vascular cells. In the following sections HDL effects on the endothelium, the most well studied target of HDL/SR-BI signaling, will be described followed by HDL and role in macrophages and potential mechanisms of HDL/SR-BI-dependent signaling.

4.1. HDL effects on endothelial cells

4.1.1. HDL modulation of eNOS activity

Among the most well-demonstrated atheroprotective actions of HDL on endothelium is the modulation of endothelial nitric oxide synthase (eNOS) activity (reviewed in [64, 122, 123]). A direct association between HDL levels and endothelium dependent flow-mediated dilation was demonstrated in hypercholesterolemic individuals by acute administration of reconstituted HDL [124]. Increased bioavailability of nitric oxide (NO) was the mechanism responsible for improved endothelial function by reconstituted HDL [124]. Both basal and the stimulated eNOS activity in HDL deficient patients were shown to be reduced [125].

Surprisingly, the administration of a single rapid infusion of reconstituted HDL resulted in the recovery of endothelial vasomotor responses [125]. Moreover, infusions of human HDL into mice resulted in NO-dependent increase in myocardial perfusion [126]. Thus NO appears to be an essential cellular messenger in the regulation of vascular tone by HDL. NO is a potent vasodilator with additional effects on the vasculature [127]. In endothelial cells, NO is generated by the conversion of L-arginine into L-citrulline by eNOS, the activity of which is modulated by several cell surface receptors and physical stimuli [128]. Several studies demonstrated that eNOS is atheroprotective and is critically involved in the protection against hypercholesterolemia induced vascular disease [129-132].

SR-BI plays an important role in HDL induced activation of eNOS [25, 63, 133, 134]. HDL activates eNOS in endothelial cell caveolae and antibodies against SR-BI or apo-AI completely abolish HDL effects [25, 133-135]. Moreover, HDL induced the NO dependent vasorelaxation in aortas from wild type mice but not SR-BI KO mice [133]. This data suggests that HDL induced activation of eNOS involves HDL binding to SR-BI [133]. Similar results were observed upon treatment with cyclodextrin. This has been interpreted as suggesting the involvement of cholesterol efflux in SR-BI activation of eNOS by HDL (see below) rather than the delivery of bioactive lipids by HDL [63, 134]. The role of SR-BI in the regulation of plasma membrane cholesterol will be discussed in more detail later. On the other hand,

several lines of evidence suggest the involvement of bioactive lipid cargo of HDL in HDL mediated signaling in cells.

4.1.2. HDL protection of endothelial cells from apoptosis

HDL also appears to protect endothelial cells against apoptosis [136-138]. Many risk factors that induce endothelial dysfunction and atherosclerosis trigger endothelial cell apoptosis [139]. These include oxidized lipoproteins [140, 141], inflammatory mediators such as tumor necrosis factor-α (TNF-α) [141, 142], homocysteine [143] and angiotensin II [144]. HDL was shown to protect against endothelial cell apoptosis via multiple mechanisms [136-138]. For instance HDL protected against TNF-α induced apoptosis via a pathway involving attenuated caspase-3 activity. [137]. HDL also suppressed endothelial cell apoptosis induced by growth factor deprivation [136]. This involved blockade of the mitochondrial pathway of apoptosis by inhibition of caspase-3 and 9 activation, reduced generation of reactive oxygen species and prevention of the dissipation of the mitochondrial transmembrane potential and cytochrome c release [136]. Additionally, HDL induces Akt phosphorylation and subsequent phosphorylation and dissociation of BAD from BCL-XL, which becomes available to inhibit the mitochondrial apoptotic pathway [136, 145]. OxLDL induced apoptosis of endothelial cells via a sustained increase in intracellular Ca^{2+} levels and these effects were reversed by HDL and were mimicked by purified apo-AI but not apo-AII [138]. This process involved HDL binding to the cells and induction of protein synthesis [138], however the nature of the HDL binding

molecule(s) was not addressed. Moreover these effects were mimicked by HDL associated lysosphingolipids including S1P, lysophosphatidic acid and sphingophosphorylcholine [136, 146, 147], and involved eNOS as well as extracellular signal-regulated kinase (ERK)1/2 and phospholipase C [146, 147]**.** In addition, S1P induced endothelial cell survival with levels similar to HDL and the knockdown of S1PR1 but not S1PR3 abolished these effects [62]. Furthermore, SR-BI has been shown to mediate the activation of phosphatidyl inositol 3 kinase (PI3K) leading to activation of Akt in MCF-7 human breast cancer cells leading to the suppression of apoptosis [145]. As discussed below, and in light of the ability of SR-BI to mediate HDL-dependent induction of eNOS, and PI3K/Akt signaling, and the participation of S1P and S1PR's in at least some of the HDL induced signaling pathways, SR-BI and S1PR's appear to participate in signaling leading to anti-apoptotic pathways in endothelial cells.

On the other hand, one report provided evidence supporting an alternative role for SR-BI in apoptosis [148]. Overexpression of SR-BI in cultured Chinese hamster ovary cells induced, and knockout of SR-BI in cultured mouse embryonic fibroblasts and aortic endothelial cells, prevented induction of apoptosis in the absence of HDL and eNOS activity [148]. This SR-BI dependent apoptosis pathway involved activation of caspase 8 and was prevented by the activation of eNOS or by the addition of HDL [148]. These findings suggested that in the absence of its HDL ligand, and when eNOS is suppressed, SR-BI may activate apoptosis, and that the

effect of HDL is to turn off this pathway [148]. On the other hand, in vivo data from knockout mice lacking SR-BI are more consistent with increased rather than decreased endothelial cell apoptosis, suggesting, at least in the presence of HDL and eNOS, that SR-BI normally plays a role in preventing endothelial cell apoptosis [149] (for review, see [64]). Whether SR-BI may stimulate apoptosis of endothelial cells in vivo in the absence of either HDL or eNOS expression remains to be determined.

4.1.3. HDL-stimulated endothelial cell migration

Endothelial cell migration plays a key role in neovascularization and maintenance of the integrity of the arterial intima [150] (reviewed in [151]). HDL treatment triggered actin cytoskeleton rearrangements and lamellipodia formation in endothelial cells and induced their migration [149] (reviewed in [64]). Knockdown of SR-BI impaired HDL's ability to induce Rac activation, lamellipodia formation and endothelial cell migration [149]. The in vivo significance of these findings was demonstrated when it was shown that reendothelialization of carotid arteries after injury was impaired in SR-BI null mice compared to SR-BI-expressing controls [149]. Effects of the altered size, composition and levels of HDL from SR-BI-deficient mice were ruled out as causative factors [149]. S1PR's also appear to participate because knockdown of either S1PR1 or 3 was shown to dramatically impair the ability of HDL to induce endothelial cell migration. [62].

The participation of both SR-BI and S1PR's in HDL signaling may occur by a number of different mechanisms. Firstly, SR-BI, by virtue of mediating HDL lipid uptake into cells, may mediate the uptake of HDL associated S1P or its precursor, sphingosine. Sphingosine can be converted to S1P by sphingosine kinases within the cell and S1P can activate S1P receptors. It was suggested that SR-BI may even anchor HDL to the membrane in the vicinity of S1PR's to facilitate the transfer of HDL associated S1P [152]. As yet, however, physical interaction between SR-BI and S1PR's has not been reported. Furthermore, the observation that reconstituted HDL, lacking S1P or its precursor sphingosine, exhibits many of the signaling activities of HDL prepared from human plasma, suggests that SR-BI may have a more direct role in signaling than merely facilitating S1P uptake [153]. S1PR's such as S1PR1, have been shown to transactivate growth factor receptor tyrosine kinases such as the platelet derived growth factor receptor, through direct interactions between the two receptors or through indirect interactions via scaffolding proteins [154]**.** Alternatively, signaling downstream of growth factor receptor tyrosine kinases, leading to ERK1/2 activation, has been shown to result in the phosphorylation of sphingosine kinase 1 leading to its translocation and activation [155], as well as increased expression of S1PR genes [156]. It is tempting to speculate that similar pathways may at least in part underlie the participation of S1PR's in SR-BI-dependent HDL mediated signaling.

4.2. HDL effects on macrophages

4.2.1 HDL and macrophage RCT

Macrophages and macrophage foam cells are the predominant cell type in atherosclerotic lesions (recently reviewed in [157]). Cholesterol efflux from these cells to mature/premature HDL particles is the first step in the process of RCT. Several efflux pathways contribute to the net cholesterol efflux from macrophage foam cells and these include energy dependent pathways mediated by the ABC transporters ABCA1 and ABCG1 and facilitated passive transport via SR-BI or simple aqueous diffusion (recently reviewed in [158]). The contribution of receptor mediated efflux pathways in lesional foam cell depends on receptor activity, interaction with other receptors and ligand availability [158]. ABCA1 facilitate phospholipids and cholesterol efflux to lipid free or lipid poor apoA-I, while ABCG1 mediates the transfer of free cholesterol to α-HDL particles [158]. Knockout of both ABCA1 and ABCG1 resulted in a dramatic reduction in macrophage RCT in vivo and a large decrease in net cholesterol efflux to HDL in vitro [89, 159, 160]. However the single deletion of ABCA1 or ABCG1 did not result in drastic effects, rather it led to the accumulation of a smaller number of foam cells suggesting the importance of ABCA1 and ABCG1 in macrophage cholesterol efflux and the possible interactions between these transporters [159, 160]. The role of SR-BI in cholesterol efflux from macrophages has been rather controversial. Some reports suggest that knockout of SR-BI in macrophages exerts only a minor effect on cholesterol efflux to HDL in

vitro or no effect in vivo [89, 102, 161, 162]. Alternatively other studies suggest that SR-BI does play an important role in cholesterol efflux from macrophages [163-166]. Although the reasons for the controversial results are not clear, effects of cholesterol loading and or cell culture conditions on the levels of expression of SR-BI may contribute to the reported contraversy. For example, cholesterol loading of macrophages in culture reduces SR-BI expression [167].

4.2.2 HDL effects on macrophage inflammation and oxidative stress

An inverse relationship exists between HDL-cholesterol levels and proinflammatory cytokines such as tumor necrosis factor-α (TNF- α), interlukin-1β (IL-1β), IL-2, IL-6 and monocyte chemotactic protein-1(MCP-1) (reviewed in [168]). HDL and its major apolipoprotein, apo-AI, were reported to exhibit anti-inflammatory properties in various cell types including monocytes / macrophages [169-171]. HDL treatment of monocytes reduced nuclear factor-ƙB (NF-ƙB) nuclear translocation, inhibited the activation and the kinase activity of NF-ƙB inhibitor (I ƙB), reduced MCP-1, chemokine (C-C motif 5) and chemokine (C-X3-C motif) ligand 1 expression levels [172]. Additionally reduced levels of cluster differentiation molecule 11b (CD11b), a monocyte adhesion molecule, were reported in HDL treated human monocytes derived macrophages with apoA-I being responsible for these effects [173]. The anti-inflammatory effects of HDL were suggested to be due to the

activation of cellular signaling pathways stimulated by cholesterol efflux and changes in plasma membrane lipid composition [173-175]. Recently however ; the group of Dr. Jay Heinecke elegantly demonstrated that HDL treatment of mouse peritoneal macrophages inhibited the transcription of a group of lipopolysaccharide-stimulated genes in a manner that is independent of cholesterol metabolism [176]. Thus the involvement of cholesterol efflux in the anti-inflammatory effects of HDL is not clearly understood. This reported discrepancy could be due to differences in the experimental conditions such as the type of macrophages or proinflammatory stimuli used or the length of treatment.

Moreover HDL posses potent anti-oxidant properties [177, 178]. There are several mechanisms by which HDL protects against oxidative stress. HDL binding and transport of oxidants is a major pathway by which HDL reduces oxidative stress [179, 180]. HDL also carries anti-oxidant vitamins such as vitamin E and enzymes and proteins with anti-oxidant properties including glutathione peroxidase, LCAT, CETP, platelets activating factor acetylhydrolase , paraoxonases 1 and 3, apo-AI, apo-AII , apo-AIV, apo-E and apo-J (recently review in [181]). Importantly SR-BI was reported to determine HDL level's of paraoxonase 1, a major antioxidant enzyme carried by HDL [182]. Finally HDL mediated efflux of cytoxic oxysterols and reduced inflammation can indirectly contribute to its anti-oxidant properties [183].

As reviewed above, the majority of our understanding of the molecular mechanisms of HDL signaling has been demonstrated in endothelial cells. Yet not as much is known in macrophages. In an attempt to gain a better understanding of the molecular mechanisms of the atheroprotective effects of HDL we have tested the role of HDL and SR-BI in signaling in macrophages (chapters 3 and 4).

4.3. Potential mechanisms of SR-BI-dependent signaling

4.3.1. SR-BI as a mediator of bioactive lipid uptake

HDL carries a variety of bioactive lipids including phospholipid derivatives, steroid hormones (e.g. estrogen) and lipid soluble vitamins (e.g. vitamin E). SR-BI is able to mediate the cellular uptake of a variety of different lipids from HDL carriers, including both sterols (cholesterol, cholesteryl ester), phospholipids and derivatives, and lipid soluble vitamins (such as vitamin E) [71, 184, 185]. This raises the possibility that SR-BI mediated uptake of one or more bioactive lipids from HDL into cells may contribute to HDL dependent activation of cell signaling pathways (Fig. 1.2). Of the various bioactive lipids carried by HDL, S1P and its precursor sphingosine have received considerable attention lately. This is because HDL serves as the major plasma carrier of S1P and, as described above, many atheroprotective actions of HDL have been attributed to S1P (recently reviewed in [186, 187]). On the other hand, there are clear differences in the actions of HDL, S1P and S1P associated with HDL (see reference [187] for a recent review in this topic).

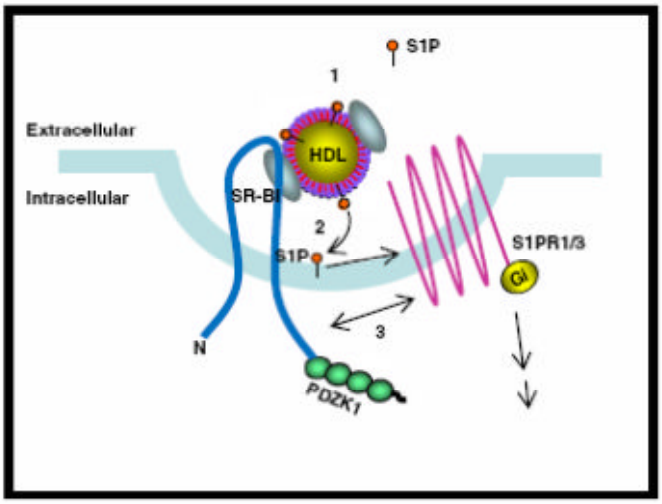

Fig. 1.2: SR-BI as a mediator of lipid uptake. HDL sequesters S1P reducing the effective concentration of free S1P influencing S1P interaction with S1P receptors (1). SR-BI may mediate the transfer of HDL associated S1P into the cell membrane (2), allowing S1P to access G_i-coupled S1P receptors and initiate signaling. S1P receptors have been shown to physically and functionally associate with growth factor receptors raising the possibility that SR-BI may also associate directly (or indirectly via adaptor proteins) with one or more S1P receptor to engage in signaling (3). This remains to be tested.

Several lines of evidence suggest that S1P is involved in HDL induced eNOS activation, including the fact that S1P is carried by HDL, the finding that delipidation of HDL impaired its ability to induce vasodilatation, and the finding that S1P added directly to cells can activate eNOS [147, 152, 188, 189]. Studies in Chinese hamster ovary cells overexpressing SR-BI showed that HDL increases the intracellular levels

of ceramide, a precursor of sphingosine and S1P, and that this results in eNOS activation [25]. Furthermore, deficiency of S1P receptor 3 (S1PR3) in mice resulted in ~50% reduced HDL-mediated vasodilation, accompanied by defects in HDL-triggered eNOS signaling in endothelial cells [152]. However the nature of the remaining half of HDL-mediated vasodilatation that is S1PR3 independent remains unclear. HDL was shown to protect against ischemia/reperfusion injury via S1PR3 in an NO-dependent manner [83]. In contrast intravenous administration of HDL was shown to stimulate myocardial perfusion in vivo equally well in wild type and S1PR3 deficient mice but not in eNOS-deficient mice [126]. HDL has also been shown to inhibit the activation of NF-ƙB and subsequent expression of the adhesion molecules VCAM- 1 and ICAM in endothelial cells treated with an inflammatory stimulus [63]. This has been shown to involve eNOS activation and these effects were partially inhibited by siRNA-mediated knockdown of either SR-BI or S1P receptors 1 and 3, and were completely inhibited by knockdown of both S1PR1 and 3 together with SR-BI [63]. Moreover, reconstituted HDL containing only apoA-I, phosphatidyl choline and unesterified cholesterol exerted effects that were similar to native HDL and which were completely abolished by inhibition of SR-BI. On the other hand, S1P added alone also induced eNOS activation, although for a shorter duration, and, unlike HDL, stimulated rather than repressed VCAM1 and ICAM expression [63]. These effects were also reduced by knockdown of S1PR1 and S1PR3 [63]. This raises the possibility that HDL may modulate the effects of S1P on S1P receptors, for example, by altering the local concentration of S1P (reviewed in [187]). Furthermore,

SR-BI appears to participate in HDL dependent signaling but may not participate directly in S1P dependent signaling through S1P receptors. On the other hand, these findings may suggest the involvement either of other S1P receptors or of S1P independent pathways in HDL dependent effects. For example, HDL was shown to augment statin induced NO production in endothelial cells in a manner that involved S1PR1 but neither S1PR3 nor SR-BI [190]. Thus the involvement of different S1P receptors, i.e. S1PR3 vs. S1PR1, in HDL's actions on the endothelium appears to be complex and may depend on the vascular sources of endothelial cells used, or potential compensatory regulation of the expression of different S1P receptors.

HDL also carries other bioactive lipids that may participate in HDL signaling. One such lipid is estradiol. One study reported that HDL isolated from females was more potent in stimulating eNOS than HDL from males [24]. HDL from male mice, that had been either enriched with estradiol ex vivo, or isolated from male mice treated with estradiol in vivo, resulted in the activation of eNOS nearly as well as HDL isolated from female mice [24]. Thus it has been suggested that HDL associated estradiol participates in eNOS activation [24], [25, 133, 191-193]. SR-BI, the estrogen receptor and eNOS all localize in plasma membranes to caveolae (reviewed in [128]). This close physical proximity may facilitate functional interactions between them. In vivo, HDL associated estradiol stimulated the relaxation of precontracted arteries from wild type mice, and this was abolished in arteries from SR-BI-deficient mice [24]. Thus it has been proposed that HDL binding to SR-BI facilitates estradiol

uptake and targets its delivery to eNOS and/or to the estrogen receptor in caveolae [24]. In contrast, other studies have reported that eNOS activation by HDL isolated from males and females was similar [152, 194]. Thus the role of estradiol in HDL induced activation of eNOS appears controversial.

4.3.2. SR-BI as a regulator of plasma membrane cholesterol

Several reports indicated that SR-BI influences the local structural organization of the plasma membrane [195-199]. SR-BI overexpression in cultured cells increased the size of the cholesterol oxidase accessible pool of cholesterol in the plasma membrane [195] and the availability of cholesterol for extraction from the outer leaflet of the plasma membrane [196]. This suggests that SR-BI increases either the amount of cholesterol or its accessibility in the plasma membrane's extracellular leaflet. This does not appear to be solely the consequence of HDL binding in close proximity to the plasma membrane because CD36, a closely related scavenger receptor that also binds HDL with high affinity, does not mediate HDL selective lipid uptake by cells [200] or increases in the cholesterol oxidase accessible pool of cholesterol in the plasma membrane [195]. The alterations in plasma membrane cholesterol content and distribution resulting from SR-BI overexpression may affect the structure/morphology and function of the plasma membrane. For example SR-BI overexpression induces the formation of microvillar channels which may be sites of SR-BI mediated HDL lipid uptake in adrenocortical cells [197, 198].

SR-BI may interact directly with cholesterol in the plasma membrane based on the ability of photoactivatible derivatives of cholesterol to crosslink SR-BI [134]. The binding site for cholesterol resides in the C-terminal transmembrane domain of SR-BI [134]. This domain was shown to be necessary for SR-BI mediated activation of eNOS in response to HDL [134]. It is not clear if this acts as a sensor for cholesterol in the membrane in a manner analogous to sterol sensing domains, as found in Scap, HMG-CoAR and other sterol sensing membrane proteins (reviewed in [201]), or whether this participates in the modulation of plasma membrane cholesterol levels/distribution by SR-BI [134]. Furthermore, SR-BI mediated cholesterol efflux to cyclodextrin increased eNOS activity in COS-M6 cells overexpressing SR-BI [134]. Thus it has been suggested that SR-BI dependent cholesterol efflux may participate in signaling in response to HDL (Fig. 1.3) [134].

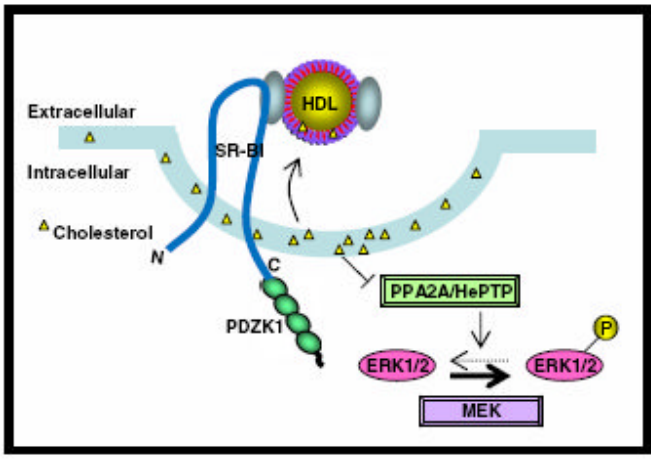

Fig. 1.3: SR-BI as a regulator of plasma membrane cholesterol. SR-BI expression and activity modifies the amount and/or distribution of cholesterol in the plasma membrane. The localization of SR-BI in caveolae in the plasma membrane and the finding that altered plasma membrane cholesterol affects the activity of a protein phosphatase complex that dephosphorylates ERK1/2 raises the possibility that SR-BI dependent HDL stimulated ERK1/2 phosphorylation may be the consequence of SR-BI dependent alterations in the amount or distribution of cholesterol in plasma membrane caveolae upon HDL binding to SR-BI—possibly due to HDL dependent cholesterol efflux.

SR-BI is localized to caveolae in a number of cell types and Anderson and co-workers have described a caveolae-cholesterol dependent pathway by which the activity of a high molecular weight phosphatase complex is regulated [202]. This 440kDa PPA2A/HePTP phosphatase complex has a dual serine/thereonine and tyrosine phosphatase activities [202]. The assembly and activity of this complex is

dependent on caveolae cholesterol and the extraction of cholesterol from caveolae inhibits this complex [202]. This in turn reduces the dephosphorylation of protein kinase targets such as ERK1/2, prolonging the activation of these kinase signaling pathways [202]. ERK1/2 is a known downstream target of SR-BI mediated signaling [65, 203-205]. Thus, HDL dependent and SR-BI mediated alterations in the distribution/content of cholesterol in plasma membrane may, at least in part, explain the apparent activation of ERK1/2 signaling pathways by SR-BI, although this has not been demonstrated directly.

4.3.3. SR-BI and downstream signaling events

The interaction of HDL with SR-BI and the subsequent activation of downstream signaling pathways were demonstrated in many of the atheroprotective actions of HDL such as the activation of eNOS [25, 149], the regulation of endothelial cell migration [149] and inhibition of adhesion molecules [63] (recently reviewed in [64, 206]). The activity of eNOS is regulated by several signaling pathways [207-213]. The engagement of receptor or non receptor tyrosine kinases (TK's) and subsequent activation of the PI3K/Akt signaling pathway results in the phosphorylation of eNOS at ser 1179 and its activation [212, 214-216]. In contrast, phosphorylation of eNOS at Thr497 inhibits its enzyme activity [213, 217]. The mitogen activated protein kinases ERK1/2 could have either positive or negative effects on the enzyme activity [207, 218-220]. HDL induced the phosphorylation of eNOS at Ser1179 in bovine aortic endothelial cells and in COS-M6 cells

overexpressing either SR-BI or eNOS [65]. In contrast however, phosphorylation at Thr497 of eNOS was not affected by HDL [65]. Inhibition of Akt1 inhibited HDL induced phosphorylation of eNOS at Ser1179 and reduced eNOS activity [65]. Additionally, HDL was shown to activate ERK1/2 via PI3K and the inhibition of ERK1/2 completely abolished HDL-dependent eNOS activation without affecting Akt1 or eNOS phosphorylation at Ser1179 [65]. On the other hand, dominant negative Akt1 did not influence HDL induced activation of ERK1/2 suggesting that HDL induced eNOS activation involves two independent pathways, Akt and ERK1/2, which lie downstream of PI3K [65].

HDL binding to SR-BI also leads to the activation of the non-receptor tyrosine kinase, Src, which lies upstream of PI3K [65]. Src appears to directly interact with SR-BI as shown by co-immunoprecipitation [153]. This interaction may be independent of the adaptor protein, PDZK1, however PDZK1 was essential for HDL mediated Src phosphorylation and for SR-BI mediated activation of many downstream pathways of HDL stimulation [153]. The precise role of PDZK1 in SR-BI dependent signaling remains unclear, however (Fig.1.4).

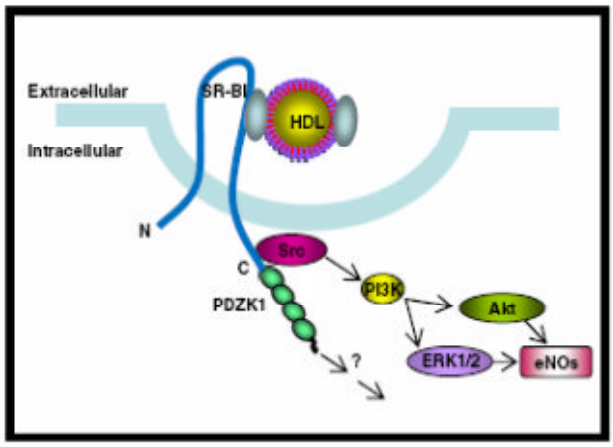

Fig. 1.4: SR-BI interaction with down-stream signaling molecules. SR-BI has been shown to interact with down-stream signaling molecules, such as the non-receptor Tyrosine kinase, Src, which activates PI3K and results in parallel activation of Akt and ERK1/2 signaling pathways leading to phosphorylation of eNOS. Alternatively, it is possible that signaling events might be initiated down-stream of PDZK1, although this remains to be established.

The activity of eNOS is also regulated by binding to calcium (Ca^{2+})-dependent calmodulin [221]. HDL has been shown to increase intracellular Ca^{2+} levels via the activation of phospholipase C in endothelial cells [222]. Ca^{2+} responses to HDL were shown to be pertussis toxin sensitive and involve lipid moieties of HDL [222], implicating the possible involvement of S1PR's or one or more other G-protein coupled receptors. Together, these studies demonstrate that HDL, via SR-BI activates multiple signaling events to promote NO production by eNOS, leading to the

activation of a variety of atheroprotective pathways such as inhibition of the induction of VCAM-1 and ICAM [63]. At the same time, SR-BI activates apparently eNOS-independent atheroprotective pathways such as endothelial cell migration via similar down-stream signaling events like PI3K/Akt and MAPK signaling [149].

5. Overall objectives, major findings and flow of the thesis

The overall objective of this thesis is to investigate the atheroprotective roles of SR-BI in vivo and in vitro. The thesis starts with in vivo studies describing mechanisms of plaque development in coronary arteries and the effects of anti-oxidant rich pomegranate extract on coronary artery atherosclerosis in the SR-BI/apoE dKO mouse model. The specific aims of this project include (1) testing the role of inflammation and oxidative stress in the development of coronary artery atherosclerosis, (2) directly testing the effects of pomegranate extract on CA atherosclerosis in the SR-BI/apoE dKO mice. Pomegranate extract administration to the SR-BI/apoE dKO mice changed the levels and compositions of serum lipoproteins and significantly reduced the extent of aortic sinus and coronary artery atherosclerosis, accompanied by reduced inflammation and oxidative stress. Moreover pomegranate extract reduced lipid accumulation, macrophage infiltration, myocardial inflammation and fibrosis and prevented heart enlargements in these mice. These studies demonstrate the involvement of inflammation and oxidative

stress in the development of coronary artery atherosclerosis in the SR-BI/apoE dKO mice and that coronary artery atherosclerosis can be reduced by the short term administration of pomegranate extract.

Due to the significant role of SR-BI in HDL metabolism and function (as discussed above), its role in bone marrow derived cells, including macrophages, in the protection against atherosclerosis [91, 102, 105], and its emerging role in human's lipoprotein metabolism and atherosclerosis [223-228], the next three chapters (3-5) of this thesis were dedicated to gain a better understanding of the role of HDL and SR-BI signaling in macrophages and to examine HDL effects on macrophage protein expression.

Plaque cellularity is determined by the fine balance between monocyte migration into the vessel wall, macrophage apoptosis and clearance of apoptotic cells. Macrophage apoptosis has been suggested to play a dual role in plaque development [229]. In early lesions macrophage apoptosis reduces plaque cellularity, inflammation and limits plaque development [229], however in more advanced lesions increased macrophage apoptosis accompanied by reduced phagocytic clearance of apoptotic cells further contributes to inflammation, necrotic core formation and acute lesional thrombosis [229]. In more advanced lesions macrophage apoptosis occurs via mechanisms that are unique to these lesions, e.g. free cholesterol loading, and its proatherogenic [230, 231]. In chapter 3 the effects of HDL on macrophage apoptosis induced by free cholesterol loading and the role of SR-BI in the protection against

macrophage apoptosis will be described. HDL treatment of macrophages protected against free cholesterol loading induced macrophage apoptosis. These effects were SR-BI dependent and involved PI3K/Akt-1, ERK1/2, JNK-1 and Foxo3a signaling cascades accompanied by changes in the balance of pro and anti-apoptotic members of the Bcl family, Bim and Mcl-1. SR-BI protection against macrophage apoptosis was further demonstrated in vivo using bone marrow transplantation experiments. The lack of SR-BI in bone marrow derived cells was associated with an increase in the number of apoptotic nuclei and cell free areas in atherosclerotic lesions compared to lesions of similar sizes in mice expressing SR-BI in bone marrow stem cells. Collectively, this data suggests that HDL protects against macrophage apoptosis in an SR-BI dependent manner. Importantly, reduced apoptotic nuclei and necrotic cores in mice transplanted with bone marrow cells expressing SR-BI suggest a potential role of SR-BI in plaque stability.

In addition to monocyte recruitment, macrophage apoptosis and phagocytic clearance of apoptotic cells, plaque cellularity is determined by macrophage egress out of the plaque [232, 233]. Macrophages can migrate into the lymphatics or into the circulation [232, 233]. Due to the presence of higher concentration of lipoproteins in circulation compared to interstitial spaces [234], we propose that HDL induces macrophage migration. HDL induced macrophage migration maybe a mechanism by which HDL protects against atherosclerosis and meditate plaque regression. The effects of HDL on macrophage migration are described in (chapter 4). HDL

stimulated macrophage migration in an SR-BI, PDZK1 and S1P receptor 1 dependent manner. These effects involved the activation of downstream kinases including PI3K/Akt1, ERK1/2, p38MAPK, PKC and ROCK.

Finally we have used proteomics to test HDL effects on macrophage protein expression (chapter 5). 2D electrophoretic analysis of HDL treated macrophages identified multiple proteins and confirmed changes in the expression levels of some that are involved in cell migration, apoptosis and inflammation. Subsequent analysis of these proteins will provide further insights into the atheroprotective actions of HDL.

6. References

[1] P. Barter, J. Kastelein, A. Nunn and R. Hobbs, High density lipoproteins (HDLs) and atherosclerosis; the unanswered questions, *Atherosclerosis.* **168** (2003), pp. 195-211.

[2] K.H. Cho, Biomedicinal implications of high-density lipoprotein: its composition, structure, functions, and clinical applications, *BMB Rep.* **42** (2009), pp. 393-400.

[3] W.S. Davidson and T.B. Thompson, The structure of apolipoprotein A-I in high density lipoproteins, *J Biol Chem.* **282** (2007), pp. 22249-53.

[4] P. Davidsson, J. Hulthe, B. Fagerberg and G. Camejo, Proteomics of Apolipoproteins and Associated Proteins From Plasma High-Density Lipoproteins, *Arterioscler Thromb Vasc Biol.* (2009), pp.

[5] J.W. Heinecke, The HDL proteome: a marker--and perhaps mediator--of coronary artery disease, *J Lipid Res.* **50 Suppl** (2009), pp. S167-71.

[6] R. Movva and D.J. Rader, Laboratory assessment of HDL heterogeneity and function, *Clin Chem.* **54** (2008), pp. 788-800.

[7] F. Tabet and K.A. Rye, High-density lipoproteins, inflammation and oxidative stress, *Clin Sci (Lond).* **116** (2009), pp. 87-98.

[8] G.F. Lewis and D.J. Rader, New insights into the regulation of HDL metabolism and reverse cholesterol transport, *Circ Res.* **96** (2005), pp. 1221-32.

[9] P.J. Blanche, E.L. Gong, T.M. Forte and A.V. Nichols, Characterization of human high-density lipoproteins by gradient gel electrophoresis, *Biochim Biophys Acta.* **665** (1981), pp. 408-19.

[10] G.R. Castro and C.J. Fielding, Early incorporation of cell-derived cholesterol into pre-beta-migrating high-density lipoprotein, *Biochemistry.* **27** (1988), pp. 25-9.

[11] B.F. Asztalos, C.H. Sloop, L. Wong and P.S. Roheim, Two-dimensional electrophoresis of plasma lipoproteins: recognition of new apo A-I-containing subpopulations, *Biochim Biophys Acta.* **1169** (1993), pp. 291-300.

[12] B.F. Asztalos, L.A. Cupples, S. Demissie, K.V. Horvath, C.E. Cox, M.C. Batista and E.J. Schaefer, High-density lipoprotein subpopulation profile and coronary heart disease prevalence in male participants of the Framingham Offspring Study, *Arterioscler Thromb Vasc Biol.* **24** (2004), pp. 2181-7.

[13] R.S. Rosenson, H.B. Brewer, Jr., M.J. Chapman, S. Fazio, M.M. Hussain, A. Kontush, R.M. Krauss, J.D. Otvos, A.T. Remaley and E.J. Schaefer, HDL measures, particle heterogeneity, proposed nomenclature, and relation to atherosclerotic cardiovascular events, *Clin Chem.* **57** (2011), pp. 392-410.

[14] M.C. Cheung and J.J. Albers, Characterization of lipoprotein particles isolated by immunoaffinity chromatography. Particles containing A-I and A-II and particles containing A-I but no A-II, *J Biol Chem.* **259** (1984), pp. 12201-9.

[15] H. Karlsson, P. Leanderson, C. Tagesson and M. Lindahl, Lipoproteomics II: mapping of proteins in high-density lipoprotein using two-dimensional gel electrophoresis and mass spectrometry, *Proteomics.* **5** (2005), pp. 1431-45.

[16] P.N. Duchateau, C.R. Pullinger, R.E. Orellana, S.T. Kunitake, J. Naya-Vigne, P.M. O'Connor, M.J. Malloy and J.P. Kane, Apolipoprotein L, a new human high density lipoprotein apolipoprotein expressed by the pancreas. Identification, cloning, characterization, and plasma distribution of apolipoprotein L, *J Biol Chem.* **272** (1997), pp. 25576-82.

[17] T. Vaisar, S. Pennathur, P.S. Green, S.A. Gharib, A.N. Hoofnagle, M.C. Cheung, J. Byun, S. Vuletic, S. Kassim, P. Singh, H. Chea, R.H. Knopp, J. Brunzell, R. Geary, A. Chait, X.Q. Zhao, K. Elkon, S. Marcovina, P. Ridker, J.F. Oram and J.W. Heinecke, Shotgun proteomics implicates protease inhibition and complement activation in the antiinflammatory properties of HDL, *J Clin Invest.* **117** (2007), pp. 746-56.

[18] A.M. Scanu and C. Edelstein, HDL: bridging past and present with a look at the future, *Faseb J.* **22** (2008), pp. 4044-54.

[19] M.P. Reilly and A.R. Tall, HDL proteomics: pot of gold or Pandora's box?, *J Clin Invest.* **117** (2007), pp. 595-8.

[20] D. Goti, A. Hammer, H.J. Galla, E. Malle and W. Sattler, Uptake of lipoprotein-associated alpha-tocopherol by primary porcine brain capillary endothelial cells, *J Neurochem.* **74** (2000), pp. 1374-83.

[21] H.J. Kayden and M.G. Traber, Absorption, lipoprotein transport, and regulation of plasma concentrations of vitamin E in humans, *J Lipid Res.* **34** (1993), pp. 343-58.

[22] F. Okajima, Plasma lipoproteins behave as carriers of extracellular sphingosine 1-phosphate: is this an atherogenic mediator or an anti-atherogenic mediator?, *Biochim Biophys Acta.* **1582** (2002), pp. 132-7.

[23] J.T. Clarke, The glycosphingolipids of human plasma lipoproteins, *Can J Biochem.* **59** (1981), pp. 412-7.

[24] M. Gong, M. Wilson, T. Kelly, W. Su, J. Dressman, J. Kincer, S.V. Matveev, L. Guo, T. Guerin, X.A. Li, W. Zhu, A. Uittenbogaard and E.J. Smart, HDL-associated estradiol stimulates endothelial NO synthase and vasodilation in an SR-BI-dependent manner, *J Clin Invest.* **111** (2003), pp. 1579-87.

[25] X.A. Li, W.B. Titlow, B.A. Jackson, N. Giltiay, M. Nikolova-Karakashian, A. Uittenbogaard and E.J. Smart, High density lipoprotein binding to scavenger receptor, Class B, type I activates endothelial nitric-oxide synthase in a ceramide-dependent manner, *J Biol Chem.* **277** (2002), pp. 11058-63.

[26] D.P. Barr, E.M. Russ and H.A. Eder, Protein-lipid relationships in human plasma. II. In atherosclerosis and related conditions, *Am J Med.* **11** (1951), pp. 480-93.

[27] G.S. Boyd and M.F. Oliver, The circulating lipids and lipoproteins in coronary artery disease, *Postgrad Med J.* **32** (1957), pp. 2-6; passim.

[28] D.R. Jacobs, Jr., I.L. Mebane, S.I. Bangdiwala, M.H. Criqui and H.A. Tyroler, High density lipoprotein cholesterol as a predictor of cardiovascular disease mortality in men and women: the follow-up study of the Lipid Research Clinics Prevalence Study, *Am J Epidemiol.* **131** (1990), pp. 32-47.

[29] G. Assmann, H. Schulte, A. von Eckardstein and Y. Huang, High-density lipoprotein cholesterol as a predictor of coronary heart disease risk. The PROCAM experience and pathophysiological implications for reverse cholesterol transport, *Atherosclerosis.* **124 Suppl** (1996), pp. S11-20.

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

[31] M.S. Brown and J.L. Goldstein, Lipoprotein metabolism in the macrophage: implications for cholesterol deposition in atherosclerosis, *Annu Rev Biochem.* **52** (1983), pp. 223-61.

[32] M.J. McQueen, S. Hawken, X. Wang, S. Ounpuu, A. Sniderman, J. Probstfield, K. Steyn, J.E. Sanderson, M. Hasani, E. Volkova, K. Kazmi and S. Yusuf, Lipids, lipoproteins, and apolipoproteins as risk markers of myocardial infarction in 52 countries (the INTERHEART study): a case-control study, *Lancet.* **372** (2008), pp. 224-33.

[33] J.C. Tardif, J. Gregoire, P.L. L'Allier, R. Ibrahim, J. Lesperance, T.M. Heinonen, S. Kouz, C. Berry, R. Basser, M.A. Lavoie, M.C. Guertin and J. Rodes-Cabau, Effects of reconstituted high-density lipoprotein infusions on coronary atherosclerosis: a randomized controlled trial, *Jama.* **297** (2007), pp. 1675-82.

[34] J.A. Shaw, A. Bobik, A. Murphy, P. Kanellakis, P. Blombery, N. Mukhamedova, K. Woollard, S. Lyon, D. Sviridov and A.M. Dart, Infusion of reconstituted high-density lipoprotein leads to acute changes in human atherosclerotic plaque, *Circ Res.* **103** (2008), pp. 1084-91.

[35] B.A. Phan, B. Chu, N. Polissar, T.S. Hatsukami, C. Yuan and X.Q. Zhao, Association of high-density lipoprotein levels and carotid atherosclerotic plaque characteristics by magnetic resonance imaging, *Int J Cardiovasc Imaging.* **23** (2007), pp. 337-42.

[36] R.P. Choudhury, J.X. Rong, E. Trogan, V.I. Elmalem, H.M. Dansky, J.L. Breslow, J.L. Witztum, J.T. Fallon and E.A. Fisher, High-density lipoproteins retard the progression of atherosclerosis and favorably remodel lesions without suppressing indices of inflammation or oxidation, *Arterioscler Thromb Vasc Biol.* **24** (2004), pp. 1904-9.

[37] C. von Birgelen, M. Hartmann, G.S. Mintz, D. Baumgart, A. Schmermund and R. Erbel, Relation between progression and regression of atherosclerotic left main coronary artery disease and serum cholesterol levels as assessed with serial long-term (> or =12 months) follow-up intravascular ultrasound, *Circulation.* **108** (2003), pp. 2757-62.

[38] S.J. Nicholls, E.M. Tuzcu, I. Sipahi, A.W. Grasso, P. Schoenhagen, T. Hu, K. Wolski, T. Crowe, M.Y. Desai, S.L. Hazen, S.R. Kapadia and S.E. Nissen, Statins, high-density lipoprotein cholesterol, and regression of coronary atherosclerosis, *Jama.* **297** (2007), pp. 499-508.

[39] J.A. Glomset, The plasma lecithins:cholesterol acyltransferase reaction, *J Lipid Res.* **9** (1968), pp. 155-67.

[40] M. Krieger, Charting the fate of the "good cholesterol": identification and characterization of the high-density lipoprotein receptor SR-BI, *Annu Rev Biochem.* **68** (1999), pp. 523-58.

[41] D.L. Williams, M.A. Connelly, R.E. Temel, S. Swarnakar, M.C. Phillips, M. de la Llera-Moya and G.H. Rothblat, Scavenger receptor BI and cholesterol trafficking, *Curr Opin Lipidol.* **10** (1999), pp. 329-39.

[42] J.C. Escola-Gil, N. Rotllan, J. Julve and F. Blanco-Vaca, In vivo macrophage-specific RCT and antioxidant and antiinflammatory HDL activity measurements: New tools for predicting HDL atheroprotection, *Atherosclerosis.* **206** (2009), pp. 321-7.

[43] R. Ohashi, H. Mu, X. Wang, Q. Yao and C. Chen, Reverse cholesterol transport and cholesterol efflux in atherosclerosis, *Qjm.* **98** (2005), pp. 845-56.

[44] G. Schmitz and M. Grandl, The molecular mechanisms of HDL and associated vesicular trafficking mechanisms to mediate cellular lipid homeostasis, *Arterioscler Thromb Vasc Biol.* **29** (2009), pp. 1718-22.

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

[46] A.M. Vaughan and J.F. Oram, ABCA1 and ABCG1 or ABCG4 act sequentially to remove cellular cholesterol and generate cholesterol-rich HDL, *J Lipid Res.* **47** (2006), pp. 2433-43.

[47] I.C. Gelissen, M. Harris, K.A. Rye, C. Quinn, A.J. Brown, M. Kockx, S. Cartland, M. Packianathan, L. Kritharides and W. Jessup, ABCA1 and ABCG1 synergize to mediate cholesterol export to apoA-I, *Arterioscler Thromb Vasc Biol.* **26** (2006), pp. 534-40.

[48] K.N. Liadaki, T. Liu, S. Xu, B.Y. Ishida, P.N. Duchateaux, J.P. Krieger, J. Kane, M. Krieger and V.I. Zannis, Binding of high density lipoprotein (HDL) and discoidal reconstituted HDL to the HDL receptor scavenger receptor class B type I. Effect of lipid association and APOA-I mutations on receptor binding, *J Biol Chem.* **275** (2000), pp. 21262-71.

[49] M.C. de Beer, D.M. Durbin, L. Cai, A. Jonas, F.C. de Beer and D.R. van der Westhuyzen, Apolipoprotein A-I conformation markedly influences HDL interaction with scavenger receptor BI, *J Lipid Res.* **42** (2001), pp. 309-13.

[50] R. Out, M. Hoekstra, S.C. de Jager, P. de Vos, D.R. van der Westhuyzen, N.R. Webb, M. Van Eck, E.A. Biessen and T.J. Van Berkel, Adenovirus-mediated hepatic overexpression of scavenger receptor class B type I accelerates chylomicron metabolism in C57BL/6J mice, *J Lipid Res.* **46** (2005), pp. 1172-81.

[51] N. Wang, T. Arai, Y. Ji, F. Rinninger and A.R. Tall, Liver-specific overexpression of scavenger receptor BI decreases levels of very low density

lipoprotein ApoB, low density lipoprotein ApoB, and high density lipoprotein in transgenic mice, *J Biol Chem.* **273** (1998), pp. 32920-6.

[52] K.F. Kozarsky, M.H. Donahee, A. Rigotti, S.N. Iqbal, E.R. Edelman and M. Krieger, Overexpression of the HDL receptor SR-BI alters plasma HDL and bile cholesterol levels, *Nature.* **387** (1997), pp. 414-7.

[53] M. Brundert, Ewert A, Heeren J, Behrendt B, Ramakrishnan R, Greten H, Merkel M, Rinninger F., Scavenger receptor class B type I mediates the selective uptake of high-density lipoprotein-associated cholesteryl ester by the liver in mice., *Arterioscler Thromb Vasc Biol.* **25** (2005), pp. 143-8.

[54] E.C. Quintao and P.M. Cazita, Lipid transfer proteins: past, present and perspectives, *Atherosclerosis.* **209** (2010), pp. 1-9.

[55] D.K. Spady, Hepatic clearance of plasma low density lipoproteins, *Semin Liver Dis.* **12** (1992), pp. 373-85.

[56] M. Florentin, E.N. Liberopoulos, A.S. Wierzbicki and D.P. Mikhailidis, Multiple actions of high-density lipoprotein, *Curr Opin Cardiol.* **23** (2008), pp. 370-8.

[57] G.W. Cockerill, J. Saklatvala, S.H. Ridley, H. Yarwood, N.E. Miller, B. Oral, S. Nithyanathan, G. Taylor and D.O. Haskard, High-density lipoproteins differentially modulate cytokine-induced expression of E-selectin and cyclooxygenase-2, *Arterioscler Thromb Vasc Biol.* **19** (1999), pp. 910-7.

[58] B.G. Drew, N.H. Fidge, G. Gallon-Beaumier, B.E. Kemp and B.A. Kingwell, High-density lipoprotein and apolipoprotein AI increase endothelial NO

synthase activity by protein association and multisite phosphorylation, *Proc Natl Acad Sci U S A.* **101** (2004), pp. 6999-7004.

[59] T. Grewal, de Diego, I., Kirchhoff, MF., Tebar, F., Heeren, J., Rinninger, F., et al., High density lipoprotein-induced signaling of the MAPK pathway involves scavenger receptor type BI-mediated activation of Ras., *J. Biol. Chem* . **278** (2003), pp. 16478-81.

[60] A.J. Murphy, J.P. Chin-Dusting, D. Sviridov and K.J. Woollard, The anti inflammatory effects of high density lipoproteins, *Curr Med Chem.* **16** (2009), pp. 667-75.

[61] F. Robbesyn, N. Auge, C. Vindis, A.V. Cantero, R. Barbaras, A. Negre-Salvayre and R. Salvayre, High-density lipoproteins prevent the oxidized low-density lipoprotein-induced epidermal [corrected] growth factor receptor activation and subsequent matrix metalloproteinase-2 upregulation, *Arterioscler Thromb Vasc Biol.* **25** (2005), pp. 1206-12.

[62] T. Kimura, K. Sato, E. Malchinkhuu, H. Tomura, K. Tamama, A. Kuwabara, M. Murakami and F. Okajima, High-density lipoprotein stimulates endothelial cell migration and survival through sphingosine 1-phosphate and its receptors, *Arterioscler Thromb Vasc Biol.* **23** (2003), pp. 1283-8.

[63] T. Kimura, H. Tomura, C. Mogi, A. Kuwabara, A. Damirin, T. Ishizuka, A. Sekiguchi, M. Ishiwara, D.S. Im, K. Sato, M. Murakami and F. Okajima, Role of scavenger receptor class B type I and sphingosine 1-phosphate receptors in

high density lipoprotein-induced inhibition of adhesion molecule expression in endothelial cells, *J Biol Chem.* **281** (2006), pp. 37457-67.

[64] C. Mineo and P.W. Shaul, Role of high-density lipoprotein and scavenger receptor B type I in the promotion of endothelial repair, *Trends Cardiovasc Med.* **17** (2007), pp. 156-61.

[65] C. Mineo, I.S. Yuhanna, M.J. Quon and P.W. Shaul, High density lipoprotein-induced endothelial nitric-oxide synthase activation is mediated by Akt and MAP kinases, *J Biol Chem.* **278** (2003), pp. 9142-9.

[66] M. Valiyaveettil, N. Kar, M.Z. Ashraf, T.V. Byzova, M. Febbraio and E.A. Podrez, Oxidized high-density lipoprotein inhibits platelet activation and aggregation via scavenger receptor BI, *Blood.* **111** (2008), pp. 1962-71.

[67] V.I. Zannis, A. Chroni and M. Krieger, Role of apoA-I, ABCA1, LCAT, and SR-BI in the biogenesis of HDL, *J Mol Med.* **84** (2006), pp. 276-94.

[68] M. Krieger and K. Kozarsky, Influence of the HDL receptor SR-BI on atherosclerosis, *Curr Opin Lipidol.* **10** (1999), pp. 491-7.

[69] A. Rigotti, H.E. Miettinen and M. Krieger, The role of the high-density lipoprotein receptor SR-BI in the lipid metabolism of endocrine and other tissues, *Endocr Rev.* **24** (2003), pp. 357-87.

[70] S.L. Acton, P.E. Scherer, H.F. Lodish and M. Krieger, Expression cloning of SR-BI, a CD36-related class B scavenger receptor, *J Biol Chem.* **269** (1994), pp. 21003-9.

[71] S. Acton, A. Rigotti, K.T. Landschulz, S. Xu, H.H. Hobbs and M. Krieger, Identification of scavenger receptor SR-BI as a high density lipoprotein receptor, *Science.* **271** (1996), pp. 518-20.

[72] D. Rhainds and L. Brissette, The role of scavenger receptor class B type I (SR-BI) in lipid trafficking. defining the rules for lipid traders, *Int J Biochem Cell Biol.* **36** (2004), pp. 39-77.

[73] M. Ikemoto, H. Arai, D. Feng, K. Tanaka, J. Aoki, N. Dohmae, K. Takio, H. Adachi, M. Tsujimoto and K. Inoue, Identification of a PDZ-domain-containing protein that interacts with the scavenger receptor class B type I, *Proc Natl Acad Sci U S A.* **97** (2000), pp. 6538-43.

[74] A. Yesilaltay, O. Kocher, R. Pal, A. Leiva, V. Quinones, A. Rigotti and M. Krieger, PDZK1 is required for maintaining hepatic scavenger receptor, class B, type I (SR-BI) steady state levels but not its surface localization or function, *J Biol Chem.* **281** (2006), pp. 28975-80.

[75] N. Ohgami, R. Nagai, A. Miyazaki, M. Ikemoto, H. Arai, S. Horiuchi and H. Nakayama, Scavenger receptor class B type I-mediated reverse cholesterol transport is inhibited by advanced glycation end products, *J Biol Chem.* **276** (2001), pp. 13348-55.

[76] S. Lund-Katz, L. Liu, S.T. Thuahnai and M.C. Phillips, High density lipoprotein structure, *Front Biosci.* **8** (2003), pp. d1044-54.

[77] A. Pilon, O. Briand, S. Lestavel, C. Copin, Z. Majd, J.C. Fruchart, G. Castro and V. Clavey, Apolipoprotein AII enrichment of HDL enhances their affinity for

class B type I scavenger receptor but inhibits specific cholesteryl ester uptake, *Arterioscler Thromb Vasc Biol.* **20** (2000), pp. 1074-81.

[78] M.C. de Beer, D.M. Durbin, L. Cai, N. Mirocha, A. Jonas, N.R. Webb, F.C. de Beer and D.R. van Der Westhuyzen, Apolipoprotein A-II modulates the binding and selective lipid uptake of reconstituted high density lipoprotein by scavenger receptor BI, *J Biol Chem.* **276** (2001), pp. 15832-9.

[79] M.A. Connelly and D.L. Williams, Scavenger receptor BI: a scavenger receptor with a mission to transport high density lipoprotein lipids, *Curr Opin Lipidol.* **15** (2004), pp. 287-95.

[80] M. de La Llera-Moya, M.A. Connelly, D. Drazul, S.M. Klein, E. Favari, P.G. Yancey, D.L. Williams and G.H. Rothblat, Scavenger receptor class B type I affects cholesterol homeostasis by magnifying cholesterol flux between cells and HDL, *J Lipid Res.* **42** (2001), pp. 1969-78.

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

[82] H.J. Pownall, Detergent-mediated phospholipidation of plasma lipoproteins increases HDL cholesterophilicity and cholesterol efflux via SR-BI, *Biochemistry.* **45** (2006), pp. 11514-22.

[83] S.T. Thuahnai, S. Lund-Katz, P. Dhanasekaran, M. de la Llera-Moya, M.A. Connelly, D.L. Williams, G.H. Rothblat and M.C. Phillips, Scavenger receptor class B type I-mediated cholesteryl ester-selective uptake and efflux of unesterified cholesterol. Influence of high density lipoprotein size and structure, *J Biol Chem.* **279** (2004), pp. 12448-55.

[84] Y. Ji, B. Jian, N. Wang, Y. Sun, M.L. Moya, M.C. Phillips, G.H. Rothblat, J.B. Swaney and A.R. Tall, Scavenger receptor BI promotes high density lipoprotein-mediated cellular cholesterol efflux, *J Biol Chem.* **272** (1997), pp. 20982-5.

[85] B. Jian, M. de la Llera-Moya, Y. Ji, N. Wang, M.C. Phillips, J.B. Swaney, A.R. Tall and G.H. Rothblat, Scavenger receptor class B type I as a mediator of cellular cholesterol efflux to lipoproteins and phospholipid acceptors, *J Biol Chem.* **273** (1998), pp. 5599-606.

[86] A. von Eckardstein, J.R. Nofer and G. Assmann, High density lipoproteins and arteriosclerosis. Role of cholesterol efflux and reverse cholesterol transport, *Arterioscler Thromb Vasc Biol.* **21** (2001), pp. 13-27.

[87] M. Brundert, J. Heeren, M. Bahar-Bayansar, A. Ewert, K.J. Moore and F. Rinninger, Selective uptake of HDL cholesteryl esters and cholesterol efflux from mouse peritoneal macrophages independent of SR-BI, *J Lipid Res.* **47** (2006), pp. 2408-21.

[88] X. Gu, K. Kozarsky and M. Krieger, Scavenger receptor class B, type I-mediated [3H]cholesterol efflux to high and low density lipoproteins is dependent on

lipoprotein binding to the receptor, *J Biol Chem.* **275** (2000), pp. 29993-30001.

[89] X. Wang, H.L. Collins, M. Ranalletta, I.V. Fuki, J.T. Billheimer, G.H. Rothblat, A.R. Tall and D.J. Rader, Macrophage ABCA1 and ABCG1, but not SR-BI, promote macrophage reverse cholesterol transport in vivo, *J Clin Invest.* **117** (2007), pp. 2216-24.

[90] Y. Zhang, J.R. Da Silva, M. Reilly, J.T. Billheimer, G.H. Rothblat and D.J. Rader, Hepatic expression of scavenger receptor class B type I (SR-BI) is a positive regulator of macrophage reverse cholesterol transport in vivo, *J Clin Invest.* **115** (2005), pp. 2870-4.

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

[92] X. Wang and D.J. Rader, Molecular regulation of macrophage reverse cholesterol transport, *Curr Opin Cardiol.* **22** (2007), pp. 368-72.

[93] M. Pennings, I. Meurs, D. Ye, R. Out, M. Hoekstra, T.J. Van Berkel and M. Van Eck, Regulation of cholesterol homeostasis in macrophages and consequences for atherosclerotic lesion development, *FEBS Lett.* **580** (2006), pp. 5588-96.

[94] R. Out, M. Hoekstra, J.A. Spijkers, J.K. Kruijt, M. van Eck, I.S. Bos, J. Twisk and T.J. Van Berkel, Scavenger receptor class B type I is solely responsible

for the selective uptake of cholesteryl esters from HDL by the liver and the adrenals in mice, *J Lipid Res.* **45** (2004), pp. 2088-95.

[95] T.J. Nieland, M. Penman, L. Dori, M. Krieger and T. Kirchhausen, Discovery of chemical inhibitors of the selective transfer of lipids mediated by the HDL receptor SR-BI, *Proc Natl Acad Sci U S A.* **99** (2002), pp. 15422-7.

[96] Y. Ji, N. Wang, R. Ramakrishnan, E. Sehayek, D. Huszar, J.L. Breslow and A.R. Tall, Hepatic scavenger receptor BI promotes rapid clearance of high density lipoprotein free cholesterol and its transport into bile, *J Biol Chem.* **274** (1999), pp. 33398-402.

[97] M.L. Varban, F. Rinninger, N. Wang, V. Fairchild-Huntress, J.H. Dunmore, Q. Fang, M.L. Gosselin, K.L. Dixon, J.D. Deeds, S.L. Acton, A.R. Tall and D. Huszar, Targeted mutation reveals a central role for SR-BI in hepatic selective uptake of high density lipoprotein cholesterol, *Proc Natl Acad Sci U S A.* **95** (1998), pp. 4619-24.

[98] P. Mardones, V. Quinones, L. Amigo, M. Moreno, J.F. Miquel, M. Schwarz, H.E. Miettinen, B. Trigatti, M. Krieger, S. VanPatten, D.E. Cohen and A. Rigotti, Hepatic cholesterol and bile acid metabolism and intestinal cholesterol absorption in scavenger receptor class B type I-deficient mice, *J Lipid Res.* **42** (2001), pp. 170-80.

[99] E. Sehayek, J.G. Ono, S. Shefer, L.B. Nguyen, N. Wang, A.K. Batta, G. Salen, J.D. Smith, A.R. Tall and J.L. Breslow, Biliary cholesterol excretion: a novel

mechanism that regulates dietary cholesterol absorption, *Proc Natl Acad Sci U S A.* **95** (1998), pp. 10194-9.

[100] Y. Ueda, L. Royer, E. Gong, J. Zhang, P.N. Cooper, O. Francone and E.M. Rubin, Lower plasma levels and accelerated clearance of high density lipoprotein (HDL) and non-HDL cholesterol in scavenger receptor class B type I transgenic mice, *J Biol Chem.* **274** (1999), pp. 7165-71.

[101] B. Trigatti, H. Rayburn, M. Vinals, A. Braun, H. Miettinen, M. Penman, M. Hertz, M. Schrenzel, L. Amigo, A. Rigotti and M. Krieger, Influence of the high density lipoprotein receptor SR-BI on reproductive and cardiovascular pathophysiology, *Proc Natl Acad Sci U S A.* **96** (1999), pp. 9322-7.

[102] M. Van Eck, I.S. Bos, R.B. Hildebrand, B.T. Van Rij and T.J. Van Berkel, Dual role for scavenger receptor class B, type I on bone marrow-derived cells in atherosclerotic lesion development, *Am J Pathol.* **165** (2004), pp. 785-94.

[103] Y. Ueda, E. Gong, L. Royer, P.N. Cooper, O.L. Francone and E.M. Rubin, Relationship between expression levels and atherogenesis in scavenger receptor class B, type I transgenics, *J Biol Chem.* **275** (2000), pp. 20368-73.

[104] K.F. Kozarsky, M.H. Donahee, J.M. Glick, M. Krieger and D.J. Rader, Gene transfer and hepatic overexpression of the HDL receptor SR-BI reduces atherosclerosis in the cholesterol-fed LDL receptor-deficient mouse, *Arterioscler Thromb Vasc Biol.* **20** (2000), pp. 721-7.

[105] S.D. Covey, M. Krieger, W. Wang, M. Penman and B.L. Trigatti, Scavenger receptor class B type I-mediated protection against atherosclerosis in LDL

receptor-negative mice involves its expression in bone marrow-derived cells, *Arterioscler Thromb Vasc Biol.* **23** (2003), pp. 1589-94.

[106] T. Arai, N. Wang, M. Bezouevski, C. Welch and A.R. Tall, Decreased atherosclerosis in heterozygous low density lipoprotein receptor-deficient mice expressing the scavenger receptor BI transgene, *J Biol Chem.* **274** (1999), pp. 2366-71.

[107] A. Braun, B.L. Trigatti, M.J. Post, K. Sato, M. Simons, J.M. Edelberg, R.D. Rosenberg, M. Schrenzel and M. Krieger, Loss of SR-BI expression leads to the early onset of occlusive atherosclerotic coronary artery disease, spontaneous myocardial infarctions, severe cardiac dysfunction, and premature death in apolipoprotein E-deficient mice, *Circ Res.* **90** (2002), pp. 270-6.

[108] T. Arai, F. Rinninger, L. Varban, V. Fairchild-Huntress, C.P. Liang, W. Chen, T. Seo, R. Deckelbaum, D. Huszar and A.R. Tall, Decreased selective uptake of high density lipoprotein cholesteryl esters in apolipoprotein E knock-out mice, *Proc Natl Acad Sci U S A.* **96** (1999), pp. 12050-5.

[109] T. Huby, C. Doucet, C. Dachet, B. Ouzilleau, Y. Ueda, V. Afzal, E. Rubin, M.J. Chapman and P. Lesnik, Knockdown expression and hepatic deficiency reveal an atheroprotective role for SR-BI in liver and peripheral tissues, *J Clin Invest.* **116** (2006), pp. 2767-76.

[110] O. Kocher, A. Yesilaltay, C.H. Shen, S. Zhang, K. Daniels, R. Pal, J. Chen and M. Krieger, Influence of PDZK1 on lipoprotein metabolism and atherosclerosis, *Biochim Biophys Acta.* **1782** (2008), pp. 310-6.

[111] O. Kocher, A. Yesilaltay, C. Cirovic, R. Pal, A. Rigotti and M. Krieger, Targeted disruption of the PDZK1 gene in mice causes tissue-specific depletion of the high density lipoprotein receptor scavenger receptor class B type I and altered lipoprotein metabolism, *J Biol Chem.* **278** (2003), pp. 52820-5.

[112] A. Braun, S. Zhang, H.E. Miettinen, S. Ebrahim, T.M. Holm, E. Vasile, M.J. Post, D.M. Yoerger, M.H. Picard, J.L. Krieger, N.C. Andrews, M. Simons and M. Krieger, Probucol prevents early coronary heart disease and death in the high-density lipoprotein receptor SR-BI/apolipoprotein E double knockout mouse, *Proc Natl Acad Sci U S A.* **100** (2003), pp. 7283-8.

[113] A. Braun, A. Yesilaltay, S. Acton, K.O. Broschat, E.S. Krul, N. Napawan, N. Stagliano and M. Krieger, Inhibition of intestinal absorption of cholesterol by ezetimibe or bile acids by SC-435 alters lipoprotein metabolism and extends the lifespan of SR-BI/apoE double knockout mice, *Atherosclerosis.* **198** (2008), pp. 77-84.

[114] S.L. Karackattu, B. Trigatti and M. Krieger, Hepatic lipase deficiency delays atherosclerosis, myocardial infarction, and cardiac dysfunction and extends lifespan in SR-BI/apolipoprotein E double knockout mice, *Arterioscler Thromb Vasc Biol.* **26** (2006), pp. 548-54.

[115] S.L. Karackattu, M.H. Picard and M. Krieger, Lymphocytes are not required for the rapid onset of coronary heart disease in scavenger receptor class B type I/apolipoprotein E double knockout mice, *Arterioscler Thromb Vasc Biol.* **25** (2005), pp. 803-8.

[116] S. Zhang, M.H. Picard, E. Vasile, Y. Zhu, R.L. Raffai, K.H. Weisgraber and M. Krieger, Diet-induced occlusive coronary atherosclerosis, myocardial infarction, cardiac dysfunction, and premature death in scavenger receptor class B type I-deficient, hypomorphic apolipoprotein ER61 mice, *Circulation.* **111** (2005), pp. 3457-64.

[117] A. Yesilaltay, K. Daniels, R. Pal, M. Krieger and O. Kocher, Loss of PDZK1 causes coronary artery occlusion and myocardial infarction in Paigen diet-fed apolipoprotein E deficient mice, *PLoS One.* **4** (2009), pp. e8103.

[118] M. Fito, M. Cladellas, R. de la Torre, J. Marti, D. Munoz, H. Schroder, M. Alcantara, M. Pujadas-Bastardes, J. Marrugat, M.C. Lopez-Sabater, J. Bruguera and M.I. Covas, Anti-inflammatory effect of virgin olive oil in stable coronary disease patients: a randomized, crossover, controlled trial, *Eur J Clin Nutr.* **62** (2008), pp. 570-4.

[119] P. Bogani, C. Galli, M. Villa and F. Visioli, Postprandial anti-inflammatory and antioxidant effects of extra virgin olive oil, *Atherosclerosis.* **190** (2007), pp. 181-6.

[120] Y.M. Yu, W.C. Chang, C.H. Wu and S.Y. Chiang, Reduction of oxidative stress and apoptosis in hyperlipidemic rabbits by ellagic acid, *J Nutr Biochem.* **16** (2005), pp. 675-81.

[121] A. Basu and K. Penugonda, Pomegranate juice: a heart-healthy fruit juice, *Nutr Rev.* **67** (2009), pp. 49-56.

[122] C. Mineo and P.W. Shaul, Circulating cardiovascular disease risk factors and signaling in endothelial cell caveolae, *Cardiovasc Res.* **70** (2006), pp. 31-41.

[123] C. Mineo, H. Deguchi, J.H. Griffin and P.W. Shaul, Endothelial and antithrombotic actions of HDL, *Circ Res.* **98** (2006), pp. 1352-64.

[124] L.E. Spieker, I. Sudano, D. Hurlimann, P.G. Lerch, M.G. Lang, C. Binggeli, R. Corti, F. Ruschitzka, T.F. Luscher and G. Noll, High-density lipoprotein restores endothelial function in hypercholesterolemic men, *Circulation.* **105** (2002), pp. 1399-402.

[125] R.J. Bisoendial, G.K. Hovingh, J.H. Levels, P.G. Lerch, I. Andresen, M.R. Hayden, J.J. Kastelein and E.S. Stroes, Restoration of endothelial function by increasing high-density lipoprotein in subjects with isolated low high-density lipoprotein, *Circulation.* **107** (2003), pp. 2944-8.

[126] B. Levkau, S. Hermann, G. Theilmeier, M. van der Giet, J. Chun, O. Schober and M. Schafers, High-density lipoprotein stimulates myocardial perfusion in vivo, *Circulation.* **110** (2004), pp. 3355-9.

[127] R.A. Cohen, The role of nitric oxide and other endothelium-derived vasoactive substances in vascular disease, *Prog Cardiovasc Dis.* **38** (1995), pp. 105-28.

[128] P.W. Shaul, Regulation of endothelial nitric oxide synthase: location, location, location, *Annu Rev Physiol.* **64** (2002), pp. 749-74.

[129] S. Moncada and A. Higgs, The L-arginine-nitric oxide pathway, *N Engl J Med.* **329** (1993), pp. 2002-12.

[130] P.J. Kuhlencordt, R. Gyurko, F. Han, M. Scherrer-Crosbie, T.H. Aretz, R. Hajjar, M.H. Picard and P.L. Huang, Accelerated atherosclerosis, aortic aneurysm formation, and ischemic heart disease in apolipoprotein E/endothelial nitric oxide synthase double-knockout mice, *Circulation.* **104** (2001), pp. 448-54.

[131] K. Kauser, V. da Cunha, R. Fitch, C. Mallari and G.M. Rubanyi, Role of endogenous nitric oxide in progression of atherosclerosis in apolipoprotein E-deficient mice, *Am J Physiol Heart Circ Physiol.* **278** (2000), pp. H1679-85.

[132] R. Yang, L. Powell-Braxton, A.K. Ogaoawara, N. Dybdal, S. Bunting, O. Ohneda and H. Jin, Hypertension and endothelial dysfunction in apolipoprotein E knockout mice, *Arterioscler Thromb Vasc Biol.* **19** (1999), pp. 2762-8.

[133] I.S. Yuhanna, Y. Zhu, B.E. Cox, L.D. Hahner, S. Osborne-Lawrence, P. Lu, Y.L. Marcel, R.G. Anderson, M.E. Mendelsohn, H.H. Hobbs and P.W. Shaul,

High-density lipoprotein binding to scavenger receptor-BI activates endothelial nitric oxide synthase, *Nat Med.* **7** (2001), pp. 853-7.

[134] C. Assanasen, C. Mineo, D. Seetharam, I.S. Yuhanna, Y.L. Marcel, M.A. Connelly, D.L. Williams, M. de la Llera-Moya, P.W. Shaul and D.L. Silver, Cholesterol binding, efflux, and a PDZ-interacting domain of scavenger receptor-BI mediate HDL-initiated signaling, *J Clin Invest.* **115** (2005), pp. 969-77.

[135] A. Uittenbogaard, P.W. Shaul, I.S. Yuhanna, A. Blair and E.J. Smart, High density lipoprotein prevents oxidized low density lipoprotein-induced inhibition of endothelial nitric-oxide synthase localization and activation in caveolae, *J Biol Chem.* **275** (2000), pp. 11278-83.

[136] J.R. Nofer, B. Levkau, I. Wolinska, R. Junker, M. Fobker, A. von Eckardstein, U. Seedorf and G. Assmann, Suppression of endothelial cell apoptosis by high density lipoproteins (HDL) and HDL-associated lysosphingolipids, *J Biol Chem.* **276** (2001), pp. 34480-5.

[137] M. Sugano, K. Tsuchida and N. Makino, High-density lipoproteins protect endothelial cells from tumor necrosis factor-alpha-induced apoptosis, *Biochem Biophys Res Commun.* **272** (2000), pp. 872-6.

[138] I. Suc, I. Escargueil-Blanc, M. Troly, R. Salvayre and A. Negre-Salvayre, HDL and ApoA prevent cell death of endothelial cells induced by oxidized LDL, *Arterioscler Thromb Vasc Biol.* **17** (1997), pp. 2158-66.

[139] S. Dimmeler, J. Haendeler and A.M. Zeiher, Regulation of endothelial cell apoptosis in atherothrombosis, *Curr Opin Lipidol.* **13** (2002), pp. 531-6.

[140] D. Li, B. Yang and J.L. Mehta, Ox-LDL induces apoptosis in human coronary artery endothelial cells: role of PKC, PTK, bcl-2, and Fas, *Am J Physiol.* **275** (1998), pp. H568-76.

[141] J.C. Choy, D.J. Granville, D.W. Hunt and B.M. McManus, Endothelial cell apoptosis: biochemical characteristics and potential implications for atherosclerosis, *J Mol Cell Cardiol.* **33** (2001), pp. 1673-90.

[142] S. Dimmeler, K. Breitschopf, J. Haendeler and A.M. Zeiher, Dephosphorylation targets Bcl-2 for ubiquitin-dependent degradation: a link between the apoptosome and the proteasome pathway, *J Exp Med.* **189** (1999), pp. 1815-22.

[143] G.N. Welch and J. Loscalzo, Homocysteine and atherothrombosis, *N Engl J Med.* **338** (1998), pp. 1042-50.

[144] W.B. Strawn and C.M. Ferrario, Mechanisms linking angiotensin II and atherogenesis, *Curr Opin Lipidol.* **13** (2002), pp. 505-12.

[145] W. Cao, Murao, K., Imachi, H., Yu, X., Abe, H., Yamauchi, A., et al., A mutant high-density lipoprotein receptor inhibits proliferation of human breast cancer cells., *Cancer Res.* **64** (2004), pp. 1515-21.

[146] Y.G. Kwon, J.K. Min, K.M. Kim, D.J. Lee, T.R. Billiar and Y.M. Kim, Sphingosine 1-phosphate protects human umbilical vein endothelial cells from

serum-deprived apoptosis by nitric oxide production, *J Biol Chem.* **276** (2001), pp. 10627-33.

[147] T. Kimura, K. Sato, A. Kuwabara, H. Tomura, M. Ishiwara, I. Kobayashi, M. Ui and F. Okajima, Sphingosine 1-phosphate may be a major component of plasma lipoproteins responsible for the cytoprotective actions in human umbilical vein endothelial cells, *J Biol Chem.* **276** (2001), pp. 31780-5.

[148] X. Li, Guo, L., Dressman, JL., Asmis, R., Smart, EJ., A novel ligand-independent apoptotic pathway induced by scavenger receptor class B, type I and suppressed by endothelial nitric-oxide synthase and high density lipoprotein., *J. Biol. Chem.* **280** (2005), pp. 19087-96.

[149] D. Seetharam, C. Mineo, A.K. Gormley, L.L. Gibson, W. Vongpatanasin, K.L. Chambliss, L.D. Hahner, M.L. Cummings, R.L. Kitchens, Y.L. Marcel, D.J. Rader and P.W. Shaul, High-density lipoprotein promotes endothelial cell migration and reendothelialization via scavenger receptor-B type I, *Circ Res.* **98** (2006), pp. 63-72.

[150] K. Kawasaki, R.S. Smith, Jr., C.M. Hsieh, J. Sun, J. Chao and J.K. Liao, Activation of the phosphatidylinositol 3-kinase/protein kinase Akt pathway mediates nitric oxide-induced endothelial cell migration and angiogenesis, *Mol Cell Biol.* **23** (2003), pp. 5726-37.

[151] L. Rossig, S. Dimmeler and A.M. Zeiher, Apoptosis in the vascular wall and atherosclerosis, *Basic Res Cardiol.* **96** (2001), pp. 11-22.

[152] J.R. Nofer, M. van der Giet, M. Tolle, I. Wolinska, K. von Wnuck Lipinski, H.A. Baba, U.J. Tietge, A. Godecke, I. Ishii, B. Kleuser, M. Schafers, M. Fobker, W. Zidek, G. Assmann, J. Chun and B. Levkau, HDL induces NO-dependent vasorelaxation via the lysophospholipid receptor S1P3, *J Clin Invest.* **113** (2004), pp. 569-81.

[153] W. Zhu, S. Saddar, D. Seetharam, K.L. Chambliss, C. Longoria, D.L. Silver, I.S. Yuhanna, P.W. Shaul and C. Mineo, The scavenger receptor class B type I adaptor protein PDZK1 maintains endothelial monolayer integrity, *Circ Res.* **102** (2008), pp. 480-7.

[154] T. Tanimoto, A.O. Lungu and B.C. Berk, Sphingosine 1-phosphate transactivates the platelet-derived growth factor beta receptor and epidermal growth factor receptor in vascular smooth muscle cells, *Circ Res.* **94** (2004), pp. 1050-8.

[155] S.M. Pitson, P.A. Moretti, J.R. Zebol, H.E. Lynn, P. Xia, M.A. Vadas and B.W. Wattenberg, Activation of sphingosine kinase 1 by ERK1/2-mediated phosphorylation, *Embo J.* **22** (2003), pp. 5491-500.

[156] L.K. Landeen, N. Aroonsakool, J.H. Haga, B.S. Hu and W.R. Giles, Sphingosine-1-phosphate receptor expression in cardiac fibroblasts is modulated by in vitro culture conditions, *Am J Physiol Heart Circ Physiol.* **292** (2007), pp. H2698-711.

[157] H.M. Wilson, Macrophages heterogeneity in atherosclerosis - implications for therapy, *J Cell Mol Med.* **14** (2010), pp. 2055-65.

[158] Y. Zhao, T.J. Van Berkel and M. Van Eck, Relative roles of various efflux pathways in net cholesterol efflux from macrophage foam cells in atherosclerotic lesions, *Curr Opin Lipidol.* **21** (2010), pp. 441-53.

[159] R. Out, M. Hoekstra, K. Habets, I. Meurs, V. de Waard, R.B. Hildebrand, Y. Wang, G. Chimini, J. Kuiper, T.J. Van Berkel and M. Van Eck, Combined deletion of macrophage ABCA1 and ABCG1 leads to massive lipid accumulation in tissue macrophages and distinct atherosclerosis at relatively low plasma cholesterol levels, *Arterioscler Thromb Vasc Biol.* **28** (2008), pp. 258-64.

[160] L. Yvan-Charvet, M. Ranalletta, N. Wang, S. Han, N. Terasaka, R. Li, C. Welch and A.R. Tall, Combined deficiency of ABCA1 and ABCG1 promotes foam cell accumulation and accelerates atherosclerosis in mice, *J Clin Invest.* **117** (2007), pp. 3900-8.

[161] M. Duong, H.L. Collins, W. Jin, I. Zanotti, E. Favari and G.H. Rothblat, Relative contributions of ABCA1 and SR-BI to cholesterol efflux to serum from fibroblasts and macrophages, *Arterioscler Thromb Vasc Biol.* **26** (2006), pp. 541-7.

[162] M.P. Adorni, F. Zimetti, J.T. Billheimer, N. Wang, D.J. Rader, M.C. Phillips and G.H. Rothblat, The roles of different pathways in the release of cholesterol from macrophages, *J Lipid Res.* (2007), pp.

[163] M. Cuchel, S. Lund-Katz, M. de la Llera-Moya, J.S. Millar, D. Chang, I. Fuki, G.H. Rothblat, M.C. Phillips and D.J. Rader, Pathways by which reconstituted

high-density lipoprotein mobilizes free cholesterol from whole body and from macrophages, *Arterioscler Thromb Vasc Biol.* **30** (2010), pp. 526-32.

[164] S. Larrede, C.M. Quinn, W. Jessup, E. Frisdal, M. Olivier, V. Hsieh, M.J. Kim, M. Van Eck, P. Couvert, A. Carrie, P. Giral, M.J. Chapman, M. Guerin and W. Le Goff, Stimulation of cholesterol efflux by LXR agonists in cholesterol-loaded human macrophages is ABCA1-dependent but ABCG1-independent, *Arterioscler Thromb Vasc Biol.* **29** (2009), pp. 1930-6.

[165] Y. Zhao, M. Pennings, R.B. Hildebrand, D. Ye, L. Calpe-Berdiel, R. Out, M. Kjerrulf, E. Hurt-Camejo, A.K. Groen, M. Hoekstra, W. Jessup, G. Chimini, T.J. Van Berkel and M. Van Eck, Enhanced foam cell formation, atherosclerotic lesion development, and inflammation by combined deletion of ABCA1 and SR-BI in Bone marrow-derived cells in LDL receptor knockout mice on western-type diet, *Circ Res.* **107** (2010), pp. e20-31.

[166] P.G. Yancey, W.G. Jerome, H. Yu, E.E. Griffin, B.E. Cox, V.R. Babaev, S. Fazio and M.F. Linton, Severely altered cholesterol homeostasis in macrophages lacking apoE and SR-BI, *J Lipid Res.* **48** (2007), pp. 1140-9.

[167] A. Ji, J.M. Meyer, L. Cai, A. Akinmusire, M.C. de Beer, N.R. Webb and D.R. van der Westhuyzen, Scavenger receptor SR-BI in macrophage lipid metabolism, *Atherosclerosis.* **217** (2011), pp. 106-12.

[168] M.J. Haas and A.D. Mooradian, Inflammation, high-density lipoprotein and cardiovascular dysfunction, *Curr Opin Infect Dis.* **24** (2011), pp. 265-72.

[169] D.T. Ashby, K.A. Rye, M.A. Clay, M.A. Vadas, J.R. Gamble and P.J. Barter, Factors influencing the ability of HDL to inhibit expression of vascular cell adhesion molecule-1 in endothelial cells, *Arterioscler Thromb Vasc Biol.* **18** (1998), pp. 1450-5.

[170] N. Hyka, J.M. Dayer, C. Modoux, T. Kohno, C.K. Edwards, 3rd, P. Roux-Lombard and D. Burger, Apolipoprotein A-I inhibits the production of interleukin-1beta and tumor necrosis factor-alpha by blocking contact-mediated activation of monocytes by T lymphocytes, *Blood.* **97** (2001), pp. 2381-9.

[171] M.D. Saemann, M. Poglitsch, C. Kopecky, M. Haidinger, W.H. Horl and T. Weichhart, The versatility of HDL: a crucial anti-inflammatory regulator, *Eur J Clin Invest.* **40** (2011), pp. 1131-43.

[172] C.A. Bursill, M.L. Castro, D.T. Beattie, S. Nakhla, E. van der Vorst, A.K. Heather, P.J. Barter and K.A. Rye, High-density lipoproteins suppress chemokines and chemokine receptors in vitro and in vivo, *Arterioscler Thromb Vasc Biol.* **30** (2010), pp. 1773-8.

[173] A.J. Murphy, K.J. Woollard, A. Hoang, N. Mukhamedova, R.A. Stirzaker, S.P. McCormick, A.T. Remaley, D. Sviridov and J. Chin-Dusting, High-density lipoprotein reduces the human monocyte inflammatory response, *Arterioscler Thromb Vasc Biol.* **28** (2008), pp. 2071-7.

[174] X. Zhu, J.Y. Lee, J.M. Timmins, J.M. Brown, E. Boudyguina, A. Mulya, A.K. Gebre, M.C. Willingham, E.M. Hiltbold, N. Mishra, N. Maeda and J.S. Parks,

Increased cellular free cholesterol in macrophage-specific Abca1 knock-out mice enhances pro-inflammatory response of macrophages, *J Biol Chem.* **283** (2008), pp. 22930-41.

[175] L. Yvan-Charvet, C. Welch, T.A. Pagler, M. Ranalletta, M. Lamkanfi, S. Han, M. Ishibashi, R. Li, N. Wang and A.R. Tall, Increased inflammatory gene expression in ABC transporter-deficient macrophages: free cholesterol accumulation, increased signaling via toll-like receptors, and neutrophil infiltration of atherosclerotic lesions, *Circulation.* **118** (2008), pp. 1837-47.

[176] M. Suzuki, D.K. Pritchard, L. Becker, A.N. Hoofnagle, N. Tanimura, T.K. Bammler, R.P. Beyer, R. Bumgarner, T. Vaisar, M.C. de Beer, F.C. de Beer, K. Miyake, J.F. Oram and J.W. Heinecke, High-density lipoprotein suppresses the type I interferon response, a family of potent antiviral immunoregulators, in macrophages challenged with lipopolysaccharide, *Circulation.* **122** (2010), pp. 1919-27.

[177] G. Theilmeier, B. De Geest, P.P. Van Veldhoven, D. Stengel, C. Michiels, M. Lox, M. Landeloos, M.J. Chapman, E. Ninio, D. Collen, B. Himpens and P. Holvoet, HDL-associated PAF-AH reduces endothelial adhesiveness in apoE-/- mice, *Faseb J.* **14** (2000), pp. 2032-9.

[178] A. Kontush, S. Chantepie and M.J. Chapman, Small, dense HDL particles exert potent protection of atherogenic LDL against oxidative stress, *Arterioscler Thromb Vasc Biol.* **23** (2003), pp. 1881-8.

[179] J. Christison, A. Karjalainen, J. Brauman, F. Bygrave and R. Stocker, Rapid reduction and removal of HDL- but not LDL-associated cholesteryl ester hydroperoxides by rat liver perfused in situ, *Biochem J.* **314 (Pt 3)** (1996), pp. 739-42.

[180] W. Sattler and R. Stocker, Greater selective uptake by Hep G2 cells of high-density lipoprotein cholesteryl ester hydroperoxides than of unoxidized cholesteryl esters, *Biochem J.* **294 (Pt 3)** (1993), pp. 771-8.

[181] E.A. Podrez, Anti-oxidant properties of high-density lipoprotein and atherosclerosis, *Clin Exp Pharmacol Physiol.* **37** (2010), pp. 719-25.

[182] R.W. James, M.C. Brulhart-Meynet, A.K. Singh, B. Riederer, U. Seidler, R. Out, T.J. Van Berkel and S. Deakin, The scavenger receptor class B, type I is a primary determinant of paraoxonase-1 association with high-density lipoproteins, *Arterioscler Thromb Vasc Biol.* **30** (2010), pp. 2121-7.

[183] N. Terasaka, N. Wang, L. Yvan-Charvet and A.R. Tall, High-density lipoprotein protects macrophages from oxidized low-density lipoprotein-induced apoptosis by promoting efflux of 7-ketocholesterol via ABCG1, *Proc Natl Acad Sci U S A.* **104** (2007), pp. 15093-8.

[184] P. Mardones, P. Strobel, S. Miranda, F. Leighton, V. Quinones, L. Amigo, J. Rozowski, M. Krieger and A. Rigotti, Alpha-tocopherol metabolism is abnormal in scavenger receptor class B type I (SR-BI)-deficient mice, *J Nutr.* **132** (2002), pp. 443-9.

[185] B. Engelmann and M.K. Wiedmann, Cellular phospholipid uptake: Flexible paths to coregulate the functions of intracellular lipids, *Biochim Biophys Acta.* (2010), pp.

[186] C. Rodriguez, M. Gonzalez-Diez, L. Badimon and J. Martinez-Gonzalez, Sphingosine-1-phosphate: A bioactive lipid that confers high-density lipoprotein with vasculoprotection mediated by nitric oxide and prostacyclin, *Thromb Haemost.* **101** (2009), pp. 665-73.

[187] K. Sattler and B. Levkau, Sphingosine-1-phosphate as a mediator of high-density lipoprotein effects in cardiovascular protection, *Cardiovasc Res.* **82** (2009), pp. 201-11.

[188] N. Murata, K. Sato, J. Kon, H. Tomura, M. Yanagita, A. Kuwabara, M. Ui and F. Okajima, Interaction of sphingosine 1-phosphate with plasma components, including lipoproteins, regulates the lipid receptor-mediated actions, *Biochem J.* **352 Pt 3** (2000), pp. 809-15.

[189] J. Igarashi and T. Michel, Sphingosine 1-phosphate and isoform-specific activation of phosphoinositide 3-kinase beta. Evidence for divergence and convergence of receptor-regulated endothelial nitric-oxide synthase signaling pathways, *J Biol Chem.* **276** (2001), pp. 36281-8.

[190] J. Igarashi, M. Miyoshi, T. Hashimoto, Y. Kubota and H. Kosaka, Statins induce S1P1 receptors and enhance endothelial nitric oxide production in response to high-density lipoproteins, *Br J Pharmacol.* **150** (2007), pp. 470-9.

[191] K. Hisamoto, M. Ohmichi, H. Kurachi, J. Hayakawa, Y. Kanda, Y. Nishio, K. Adachi, K. Tasaka, E. Miyoshi, N. Fujiwara, N. Taniguchi and Y. Murata, Estrogen induces the Akt-dependent activation of endothelial nitric-oxide synthase in vascular endothelial cells, *J Biol Chem.* **276** (2001), pp. 3459-67.

[192] K.L. Chambliss, I.S. Yuhanna, C. Mineo, P. Liu, Z. German, T.S. Sherman, M.E. Mendelsohn, R.G. Anderson and P.W. Shaul, Estrogen receptor alpha and endothelial nitric oxide synthase are organized into a functional signaling module in caveolae, *Circ Res.* **87** (2000), pp. E44-52.

[193] K.L. Chambliss, I.S. Yuhanna, R.G. Anderson, M.E. Mendelsohn and P.W. Shaul, ERbeta has nongenomic action in caveolae, *Mol Endocrinol.* **16** (2002), pp. 938-46.

[194] J.T. Kuvin, M.E. Ramet, A.R. Patel, N.G. Pandian, M.E. Mendelsohn and R.H. Karas, A novel mechanism for the beneficial vascular effects of high-density lipoprotein cholesterol: enhanced vasorelaxation and increased endothelial nitric oxide synthase expression, *Am Heart J.* **144** (2002), pp. 165-72.

[195] M. de la Llera-Moya, G.H. Rothblat, M.A. Connelly, G. Kellner-Weibel, S.W. Sakr, M.C. Phillips and D.L. Williams, Scavenger receptor BI (SR-BI) mediates free cholesterol flux independently of HDL tethering to the cell surface, *J Lipid Res.* **40** (1999), pp. 575-80.

[196] G. Kellner-Weibel, M. de La Llera-Moya, M.A. Connelly, G. Stoudt, A.E. Christian, M.P. Haynes, D.L. Williams and G.H. Rothblat, Expression of

scavenger receptor BI in COS-7 cells alters cholesterol content and distribution, *Biochemistry.* **39** (2000), pp. 221-9.

[197] D.L. Williams, J.S. Wong and R.L. Hamilton, SR-BI is required for microvillar channel formation and the localization of HDL particles to the surface of adrenocortical cells in vivo, *J Lipid Res.* **43** (2002), pp. 544-9.

[198] E. Reaven, S. Leers-Sucheta, A. Nomoto and S. Azhar, Expression of scavenger receptor class B type 1 (SR-BI) promotes microvillar channel formation and selective cholesteryl ester transport in a heterologous reconstituted system, *Proc Natl Acad Sci U S A.* **98** (2001), pp. 1613-8.

[199] S. Parathath, M.A. Connelly, R.A. Rieger, S.M. Klein, N.A. Abumrad, M. De La Llera-Moya, C.R. Iden, G.H. Rothblat and D.L. Williams, Changes in plasma membrane properties and phosphatidylcholine subspecies of insect Sf9 cells due to expression of scavenger receptor class B, type I, and CD36, *J Biol Chem.* **279** (2004), pp. 41310-8.

[200] X. Gu, B. Trigatti, S. Xu, S. Acton, J. Babitt and M. Krieger, The efficient cellular uptake of high density lipoprotein lipids via scavenger receptor class B type I requires not only receptor-mediated surface binding but also receptor-specific lipid transfer mediated by its extracellular domain, *J Biol Chem.* **273** (1998), pp. 26338-48.

[201] T.Y. Chang, C.C. Chang, N. Ohgami and Y. Yamauchi, Cholesterol sensing, trafficking, and esterification, *Annu Rev Cell Dev Biol.* **22** (2006), pp. 129-57.

[202] P.Y. Wang, P. Liu, J. Weng, E. Sontag and R.G. Anderson, A cholesterol-regulated PP2A/HePTP complex with dual specificity ERK1/2 phosphatase activity, *Embo J.* **22** (2003), pp. 2658-67.

[203] Y. Zhang, A.M. Ahmed, N. McFarlane, C. Capone, D.R. Boreham, R. Truant, S.A. Igdoura and B.L. Trigatti, Regulation of SR-BI-mediated selective lipid uptake in Chinese hamster ovary-derived cells by protein kinase signaling pathways, *J Lipid Res.* **48** (2007), pp. 405-16.

[204] Y. Osada, T. Sunatani, I.S. Kim, Y. Nakanishi and A. Shiratsuchi, Signalling pathway involving GULP, MAPK and Rac1 for SR-BI-induced phagocytosis of apoptotic cells, *J Biochem.* **145** (2009), pp. 387-94.

[205] I.N. Baranova, T.G. Vishnyakova, A.V. Bocharov, R. Kurlander, Z. Chen, M.L. Kimelman, A.T. Remaley, G. Csako, F. Thomas, T.L. Eggerman and A.P. Patterson, Serum amyloid A binding to CLA-1 (CD36 and LIMPII analogous-1) mediates serum amyloid A protein-induced activation of ERK1/2 and p38 mitogen-activated protein kinases, *J Biol Chem.* **280** (2005), pp. 8031-40.

[206] F. Okajima, K. Sato and T. Kimura, Anti-atherogenic actions of high-density lipoprotein through sphingosine 1-phosphate receptors and scavenger receptor class B type I, *Endocr J.* **56** (2009), pp. 317-34.

[207] M.A. Corson, N.L. James, S.E. Latta, R.M. Nerem, B.C. Berk and D.G. Harrison, Phosphorylation of endothelial nitric oxide synthase in response to fluid shear stress, *Circ Res.* **79** (1996), pp. 984-91.

[208] T. Michel, G.K. Li and L. Busconi, Phosphorylation and subcellular translocation of endothelial nitric oxide synthase, *Proc Natl Acad Sci U S A.* **90** (1993), pp. 6252-6.

[209] G. Garcia-Cardena, R. Fan, D.F. Stern, J. Liu and W.C. Sessa, Endothelial nitric oxide synthase is regulated by tyrosine phosphorylation and interacts with caveolin-1, *J Biol Chem.* **271** (1996), pp. 27237-40.

[210] Y.C. Boo, G. Sorescu, N. Boyd, I. Shiojima, K. Walsh, J. Du and H. Jo, Shear stress stimulates phosphorylation of endothelial nitric-oxide synthase at Ser1179 by Akt-independent mechanisms: role of protein kinase A, *J Biol Chem.* **277** (2002), pp. 3388-96.

[211] E. Butt, M. Bernhardt, A. Smolenski, P. Kotsonis, L.G. Frohlich, A. Sickmann, H.E. Meyer, S.M. Lohmann and H.H. Schmidt, Endothelial nitric-oxide synthase (type III) is activated and becomes calcium independent upon phosphorylation by cyclic nucleotide-dependent protein kinases, *J Biol Chem.* **275** (2000), pp. 5179-87.

[212] M.E. Davis, H. Cai, G.R. Drummond and D.G. Harrison, Shear stress regulates endothelial nitric oxide synthase expression through c-Src by divergent signaling pathways, *Circ Res.* **89** (2001), pp. 1073-80.

[213] M.B. Harris, H. Ju, V.J. Venema, H. Liang, R. Zou, B.J. Michell, Z.P. Chen, B.E. Kemp and R.C. Venema, Reciprocal phosphorylation and regulation of endothelial nitric-oxide synthase in response to bradykinin stimulation, *J Biol Chem.* **276** (2001), pp. 16587-91.

[214] B. Gallis, G.L. Corthals, D.R. Goodlett, H. Ueba, F. Kim, S.R. Presnell, D. Figeys, D.G. Harrison, B.C. Berk, R. Aebersold and M.A. Corson, Identification of flow-dependent endothelial nitric-oxide synthase phosphorylation sites by mass spectrometry and regulation of phosphorylation and nitric oxide production by the phosphatidylinositol 3-kinase inhibitor LY294002, *J Biol Chem.* **274** (1999), pp. 30101-8.

[215] M. Montagnani, H. Chen, V.A. Barr and M.J. Quon, Insulin-stimulated activation of eNOS is independent of Ca2+ but requires phosphorylation by Akt at Ser(1179), *J Biol Chem.* **276** (2001), pp. 30392-8.

[216] S.R. Thomas, K. Chen and J.F. Keaney, Jr., Hydrogen peroxide activates endothelial nitric-oxide synthase through coordinated phosphorylation and dephosphorylation via a phosphoinositide 3-kinase-dependent signaling pathway, *J Biol Chem.* **277** (2002), pp. 6017-24.

[217] B.J. Michell, Z. Chen, T. Tiganis, D. Stapleton, F. Katsis, D.A. Power, A.T. Sim and B.E. Kemp, Coordinated control of endothelial nitric-oxide synthase phosphorylation by protein kinase C and the cAMP-dependent protein kinase, *J Biol Chem.* **276** (2001), pp. 17625-8.

[218] I. Fleming, J. Bauersachs, A. Schafer, D. Scholz, J. Aldershvile and R. Busse, Isometric contraction induces the Ca2+-independent activation of the endothelial nitric oxide synthase, *Proc Natl Acad Sci U S A.* **96** (1999), pp. 1123-8.

[219] Z. Chen, I.S. Yuhanna, Z. Galcheva-Gargova, R.H. Karas, M.E. Mendelsohn and P.W. Shaul, Estrogen receptor alpha mediates the nongenomic activation of endothelial nitric oxide synthase by estrogen, *J Clin Invest.* **103** (1999), pp. 401-6.

[220] S.G. Bernier, S. Haldar and T. Michel, Bradykinin-regulated interactions of the mitogen-activated protein kinase pathway with the endothelial nitric-oxide synthase, *J Biol Chem.* **275** (2000), pp. 30707-15.

[221] P.W. Shaul, E.J. Smart, L.J. Robinson, Z. German, I.S. Yuhanna, Y. Ying, R.G. Anderson and T. Michel, Acylation targets emdothelial nitric-oxide synthase to plasmalemmal caveolae, *J Biol Chem.* **271** (1996), pp. 6518-22.

[222] H.M. Honda, B.K. Wakamatsu, J.I. Goldhaber, J.A. Berliner, M. Navab and J.N. Weiss, High-density lipoprotein increases intracellular calcium levels by releasing calcium from internal stores in human endothelial cells, *Atherosclerosis.* **143** (1999), pp. 299-306.

[223] J.J. McCarthy, T. Lehner, C. Reeves, D.J. Moliterno, L.K. Newby, W.J. Rogers and E.J. Topol, Association of genetic variants in the HDL receptor, SR-B1, with abnormal lipids in women with coronary artery disease, *J Med Genet.* **40** (2003), pp. 453-8.

[224] A.C. Naj, M. West, S.S. Rich, W. Post, W.H. Kao, B.A. Wasserman, D.M. Herrington and A. Rodriguez, Association of scavenger receptor class B type I polymorphisms with subclinical atherosclerosis: the Multi-Ethnic Study of Atherosclerosis, *Circ Cardiovasc Genet.* **3** (2010), pp. 47-52.

[225] L. Brunham, I. Tietjen, A. Bochem, R. Singaraja, P. Franchini, C. Radomski, M. Mattice, A. Legendre, G. Hovingh, J. Kastelein and M. Hayden, Novel mutations in scavenger receptor BI associated with high HDL cholesterol in humans, *Clin Genet.* **79** (2011), pp. 575-81.

[226] J. Aron-Wisnewsky, Z. Julia, C. Poitou, J.L. Bouillot, A. Basdevant, M.J. Chapman, K. Clement and M. Guerin, Effect of bariatric surgery-induced weight loss on SR-BI-, ABCG1-, and ABCA1-mediated cellular cholesterol efflux in obese women, *J Clin Endocrinol Metab.* **96** pp. 1151-9.

[227] A. Ritsch, G. Sonderegger, A. Sandhofer, U. Stanzl, I. Tancevski, P. Eller, W. Schgoer, A. Wehinger, T. Mueller, M. Haltmayer and J.R. Patsch, Scavenger receptor class B type I polymorphisms and peripheral arterial disease, *Metabolism.* **56** (2007), pp. 1135-41.

[228] M. Vergeer, S.J. Korporaal, R. Franssen, I. Meurs, R. Out, G.K. Hovingh, M. Hoekstra, J.A. Sierts, G.M. Dallinga-Thie, M.M. Motazacker, A.G. Holleboom, T.J. Van Berkel, J.J. Kastelein, M. Van Eck and J.A. Kuivenhoven, Genetic variant of the scavenger receptor BI in humans, *N Engl J Med.* **364** (2011), pp. 136-45.

[229] I. Tabas, Macrophage death and defective inflammation resolution in atherosclerosis, *Nat Rev Immunol.* **10** (2009), pp. 36-46.

[230] I. Tabas, Free cholesterol-induced cytotoxicity a possible contributing factor to macrophage foam cell necrosis in advanced atherosclerotic lesions, *Trends Cardiovasc Med.* **7** (1997), pp. 256-63.

[231] S.S. Katz, G.G. Shipley and D.M. Small, Physical chemistry of the lipids of human atherosclerotic lesions. Demonstration of a lesion intermediate between fatty streaks and advanced plaques, *J Clin Invest.* **58** (1976), pp. 200-11.

[232] E. Trogan, J.E. Feig, S. Dogan, G.H. Rothblat, V. Angeli, F. Tacke, G.J. Randolph and E.A. Fisher, Gene expression changes in foam cells and the role of chemokine receptor CCR7 during atherosclerosis regression in ApoE-deficient mice, *Proc Natl Acad Sci U S A.* **103** (2006), pp. 3781-6.

[233] J. Llodra, V. Angeli, J. Liu, E. Trogan, E.A. Fisher and G.J. Randolph, Emigration of monocyte-derived cells from atherosclerotic lesions characterizes regressive, but not progressive, plaques, *Proc Natl Acad Sci U S A.* **101** (2004), pp. 11779-84.

[234] G. Luc, Z. Majd, P. Poulain, L. Elkhalil and J.C. Fruchart, Interstitial fluid apolipoprotein A-II: an association with the occurrence of myocardial infarction, *Atherosclerosis.* **127** (1996), pp. 131-7.

CHAPTER TWO

THE EFFECT OF POMEGRANATE EXTRACT ON CORONARY ARTERY ATHEROSCLEROSIS IN SR-BI/APOE DOUBLE KNOCKOUT MICE

CHAPTER TWO PREFACE

This manuscript is in the process of being submitted to *Atherosclerosis*.

I have conducted all the experiments in this chapter except for the representative images of fibrosis staining (Figure 2.6 A) which were done by Christine Tenedero and Fatima Igdoura. Supplementary (Figure 2.6) was generated by Christine Tenedero. MCP-1 ELISA of cell culture media (Supplementary Figure 2.1 B) and serum samples (Supplementary Figure 2.3 B) were done by Elizabeth White.

THE EFFECT OF POMEGRANATE EXTRACT ON CORONARY ARTERY ATHEROSCLEROSIS IN SR-BI/APOE DOUBLE KNOCKOUT MICE

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1. Abstract

Objective: To test the effect of pomegranate extract on inflammation and oxidative stress and the development of spontaneous occlusive coronary artery atherosclerosis in the SR-BI/apoE double knockout mouse model of coronary heart disease.

Methods and results: SR-BI/apoE dKO mice were treated for two weeks with pomegranate extract via drinking water. Treatment with pomegranate extract increased cholesterol ester content, reducing the abnormally high unesterified:esterified cholesterol ratio of VLDL sized lipoproteins. Pomegranate extract treatment also reduced atherosclerosis in the aortic sinus and reduced the proportion of coronary arteries with occlusive atherosclerotic plaques. MCP-1 and SAA levels in serum were substantially elevated in SR-BI/apoE dKO mice compared to age matched apoE single KO control mice but were not reduced by treatment with pomegranate extract. Instead, treatment with pomegranate extract resulted in substantial reductions in levels of oxidative stress and MCP-1 immunoreactivity in atherosclerotic aortic sinus and coronary arteries. Treatment with pomegranate extract also reduced lipid accumulation, macrophage infiltration, levels of MCP-1 and fibrosis in the myocardium, and prevented cardiac enlargement in SR-BI/apoE dKO mice.

Conclusion: Pomegranate extract reduced aortic sinus and occlusive coronary artery atherosclerosis in the SR-BI/apoE dKO mice via mechanisms that involve alterations in lipoprotein metabolism and reduced inflammation and oxidative stress.

Key words:

Pomegranate extract, coronary arteries, atherosclerosis, inflammation, oxidative stress.

2. Introduction

Increased inflammation, dysregulated lipid metabolism and increased oxidative stress are key elements in the development of atherosclerosis [1]. Complex networks with complex interactions exist between these factors [1]. Oxidative stress may act as an initiator of the process resulting in lipid peroxidation and increased inflammation [2] and as an important player in plaque rupture leading to atherothrombosis and myocardial infarction [3]. Multiple experimental studies and clinical trials have used anti-oxidants as therapeutic agents [4]. However, anti-oxidant therapies in coronary heart disease patients were not effective [5-10]. Many of these studies involved treatment with single anti-oxidants (reviewed in [4]). However patients with coronary heart disease may be deficient in multiple antioxidants or micronutrients affecting different pathways [11]. Interestingly, the properties of polyphenolic anti-oxidants have been shown to be enhanced when present in combination as in natural fruit extracts [12].

Pomegranate (*Punica granatum*) fruit is rich in anti-oxidant polyphenolics was and has been shown to have multiple cardioprotective effects [13]. Several studies focused on its antioxidant actions in vitro, ex vivo and in vivo while others have elaborated on the ability of different components of the fruit, including the juice, seed oil, peel, flower extract or their derivatives to kill bacteria and viruses, fight vascular disease, diabetes and cancer (reviewed in [13]). Studies in patients with carotid artery stenosis showed that the consumption of pomegranate juice for a period of three years increased serum paraoxonase-1 activity, reduced serum oxidative stress and reduced intima-media

thickness in these patients [14]. Other studies demonstrated that pomegranate juice or the liquid extract of pomegranate fruit (POMlx) reduce development of aortic atherosclerosis in apolipoprotein (apo)E deficient mice [15-18]. Recently, pomegranate juice administration to mice was shown to reduce macrophage total cholesterol and triglycerides' contents by inhibiting their synthesis and increasing cholesterol efflux to HDL [19] as well as reducing low density lipoprotein (LDL) and oxidized (ox) LDL uptake [18].

Despite developing spontaneous and diet accelerated atherosclerosis in a number of arteries including the aorta and branching arteries, apoE knockout (apoE KO) mice do not reproducibly develop coronary artery atherosclerosis with high frequency [20]. In contrast, apoE KO mice that also lack the scavenger receptor, class B type I (SR-BI) spontaneously develop occlusive coronary artery (CA) atherosclerosis, myocardial infarction cardiac enlargement and severe cardiac dysfunction [21]. The coronary artery disease and accompanying sequelae in SR-BI/apoE double KO (dKO) mice exhibit features reminiscent of human coronary heart disease suggesting that they may be a useful mouse model of human coronary heart disease [21-23]. These mice exhibit premature death between 5 and 8 weeks of age [19]. Inhibition of intestinal cholesterol absorption by ezetimibe, or reduction of bile acid recirculation by the small molecule, SC-435, have both been shown to reduce plasma lipoprotein cholesterol levels and delay disease development in these mice [23].

The role of vascular inflammation and/or oxidative stress in these mice has not been explored. In this study, we set demonstrate the presence of markers of oxidative stress and inflammation in atherosclerotic vessels and in myocardial tissue in SR-BI/apoE dKO mice and we demonstrate that supplementation of their drinking water with pomegranate extract reduces aortic and coronary artery atherosclerosis, oxidative stress and inflammatory markers in atherosclerotic vessels and heart tissue, cardiac enlargement and cardiac fibrosis.

3. Materials and methods

Materials

Pomegranate extract (POMlx) or juice (POM Wonderful, CA, USA) were purchased from local grocery stores. All other materials were purchased from Sigma Aldrich (St. Louis, MO, USA) unless stated otherwise.

Mice

All procedures involving mice were approved by McMaster University Animal Research Ethics Board and were in accordance with the guidelines of the Canadian

Council on Animal Care. C57BL/6J (Jackson Labs, Bar Harbor ME, USA) or SR-BI$^{+/-}$ apoE KO mice (mixed C57BL/6J and 129Sv genetic background, originally provided by Monty Krieger, Massachusetts Institute of Technology, Cambridge, USA) were bred and housed at McMaster University or TaARI animal facilities and had free access to food and water. SR-BI/apoE dKO and apoE single KO littermates received either water alone (control group) or water containing pomegranate extract (307.5 µl/L) beginning at three weeks of age. Control and pomegranate extract-treated mice were euthanized at 5 weeks of age. Additional control SR-BI/apoE dKO and apoE single KO mice were euthanized at 3 weeks of age. All pomegranate extract-treated and untreated mice were fasted from 4-16 hrs prior to euthanasia. Blood was collected by cardiac puncture and serum was prepared using serum collection tubes (Sarstedt Inc., Montreal, PQ, Canada) and frozen at -80 ºC for later analysis. Mice were perfused with phosphate buffered saline containing 10 U of heparin and the harvested tissues frozen in Shandon Cyromatrix (Thermo Fisher Scientific, Ottawa, ON, Canada) and stored at -80^{o}C for further analysis.

Histology

Cryosections (10µm) of hearts, or aortic sinus were stained for lipids with oil red-O [21], for oxidative stress markers with dihydroethidium (DHE) [24] or for myocardial fibrosis with Trichrome (Thermo Fisher Scientific, Ottawa, ON, Canada), according to manufacturer's protocols. Macrophage infiltration and MCP-1 levels were detected using

rat anti-CD68 (Gene Tex Inc, Irvine, CA, USA) or rabbit anti-MCP-1 (Abcam Inc, Boston, MA, USA) with the corresponding rabbit anti-rat or goat anti-rabbit alexa-594 conjugated secondary antibodies (Invitrogen Life Technologies, Burlington, ON, Canada). Sections subjected to immunofluorescence staining were counterstained for nuclei with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI). Images were taken using either a Zeiss Axiovert 200 M inverted microscope or Olympus BX41 Clinical Microscope.

Lipoprotein analysis

Serum was fractionated by gel filtration fast-protein liquid chromatography (FPLC) as previously described [25]. Enzymatic assay kits were used to measure total cholesterol (TC, Thermo Fisher Scientific, Ottawa, ON, Canada) and unesterified cholesterol (UC, Wako Diagnostics, Richmond, VA, USA) in each fraction [22],[25-27]. Cholesterol ester (CE) levels were calculated by subtracting UC from TC.

Statistical analysis

Data was analyzed using the Student's T-test (Microsoft Excel) or one-way ANOVA (GraphPad Prism) and was considered statistically significant when $P < 0.05$.

4. Experimental results

4.1. Pomegranate extract reduces macrophage inflammation and foam cell formation

The effect of pomegranate extract on macrophage inflammation was tested in macrophages treated with lipopolysaccahride (LPS) or acetylated LDL (AcLDL) . LPS treatment of macrophages increased the production of interleukin (IL)-6 which was significantly reduced in a dose dependent manner by pomegranate extract or pomegranate juice treatment (supplementary Fig. 2.1 A). Similarly, pomegranate extract reduced both basal and AcLDL stimulated MCP-1 production (supplementary Fig. 2.1 B). Similar results were observed in interferon (IFN-) γ stimulated macrophages (data not shown). Pomegranate extract was almost 100 times more potent in suppressing LPS-induced IL-6 production than pomegranate juice suggesting that the extract exhibits a greater anti-inflammatory activity (supplementary Fig. 2.1 A). We therefore chose pomegranate extract over juice for our subsequent in vivo studies.

4.2. Pomegranate extract increases serum lipoprotein cholesterol ester levels

To test the effects of pomegranate extract on coronary artery atherosclerosis and myocardial infarction directly, we treated SR-BI/apoE dKO mice for 2 weeks beginning at 3 weeks of age with pomegranate extract via their drinking water. We administered 307.5 µl pomegranate extract per L of drinking water, which was calculated to be approximately equivalent to 200 µg of galic acid equivalents (GAE) per mouse per day [18]. As reported previously total cholesterol levels in VLDL- and intermediate and low density lipoprotein (IDL/LDL)-sized particles were substantially higher in serum from SR-BI/apoE dKO than from apoE single KO mice (Fig. 2.1 A, B) , and this was primarily due to increased unesterified cholesterol (UC) (Fig. 2.1 C, D) [22, 27]. No differences were observed between males and females (data not shown) and pooled data are shown. Pomegranate extract treatment resulted in substantial increases in total cholesterol contents of VLDL- and IDL/LDL-sized particles for both SR-BI/apoE dKO and apoE single KO mice (Fig. 2.1 A, B). The increase in total cholesterol levels was due to increased lipoprotein cholesterol ester (CE) contents without any significant changes in UC contents compared to untreated SR-BI/apoE dKO or apoE single KO mice (Fig. 2.1 C-F). These changes in lipoprotein CE levels were not accompanied by changes in levels of apolipoprotein (apo) B 100, apoB 48 or apoA-I in pomegranate extract treated mice (supplementary Fig. 2.2), suggesting that pomegranate extract treatment does not alter steady state lipoprotein concentrations, but rather affects the cholesterol composition of these lipoproteins.

4.3. The effects of pomegranate extract treatment on atherosclerosis in the SR-BI/apoE dKO mice

SR-BI/apoE dKO mice do not exhibit aortic sinus atherosclerosis at 3 weeks of age (not shown), but develop substantial atherosclerosis in the aortic sinus by 5 weeks of age (Fig. 2.2 B). In contrast, apoE single KO mice aged 5 weeks exhibit no aortic sinus atherosclerosis. This is consistent with the previously described accelerated aortic sinus atherosclerosis in SR-BI/apoE dKO mice [21]. Treatment of SR-BI/apoE dKO mice for two weeks with pomegranate extract significantly reduced the size of aortic sinus plaques (Fig. 2.2 D, E). SR-BI/apoE dKO mice also develop spontaneous CA atherosclerosis and die prematurely by 6-8 weeks of age whereas control apoE single KO mice do not [22] (Fig. 2.2 F-K and data not shown). Atherosclerosis in CA's was examined in pomegranate extract treated and untreated SR-BI/apoE dKO mice (Fig. 2.2 F-K). No coronary artery atherosclerosis was detected in 3 week old SR-BI/apoE dKO mice although approximately 15 % of coronary arteries examined exhibited fatty streaks (Supplementary figure 6). Approximately 35 % of coronary arteries from untreated SR-BI/apoE dKO mice contained atherosclerotic plaques, with approximately 7 % being completely occluded, 14 % being > 50 % occluded, and 14 % being < 50 % occluded. 31% of coronary arteries analyzed contained fatty streaks and the remaining 34 % of coronary arteries examined showed no evidence of lipid accumulation ("no atherosclerosis" in Fig. 2.2 F). Hearts from pomegranate extract treated SR-BI/apoE dKO mice had significantly higher proportions (60 % vs. 34 % in untreated mice) of coronary

arteries that showed no evidence of lipid accumulation, similar proportions (25 %) of coronary arteries with fatty streaks, and lower proportions (23 % vs. 35 % in untreated mice) of coronary arteries with atherosclerotic plaques, with 8% being < 50 % occluded, 6 % > 50 % occluded and 2 % that were completely occluded (Fig. 2.2 K). Therefore, treatment with pomegranate extract reduced both aortic sinus and coronary artery atherosclerosis in SR-BI/apoE dKO mice.

4.4. The effects of pomegranate extract treatment on oxidative stress and inflammation in the SR-BI/apoE dKO mice

Oxidative stress plays an important role in the development of atherosclerosis [2] and pomegranate extract is reported to contain multiple anti-oxidant compounds [16-18, 28, 29]. Treatment of SR-BI/apoE dKO mice with pomegranate extract for 2 weeks resulted in a marked reduction in cellular reactive oxygen species (ROS) within cells in atherosclerotic plaques in the aortic sinus, as detected by reduced DHE staining of nuclei in plaques of similar sizes (Fig. 2.3 A-E). Similarly reduced oxidative stress was observed in the walls of coronary arteries in SR-BI/apoE dKO mice treated with pomegranate extract (Fig. 2.3 F-J). This suggests that reduced oxidative stress may contribute to the observed reduced atherosclerosis in the aortic sinus and coronary arteries in pomegranate extract treated SR-BI/apoE dKO mice.

Inflammation is a key factor in plaque development, that often accompanies oxidative stress [30]. In clinical practice, inflammatory biomarkers are often used in the diagnosis and risk assessment of patients with coronary heart disease [30]. Increased circulating SAA levels were reported in patients with acute myocardial infarction [31] and were related to the severity of acute CA disease [32]. SAA is the predominant acute phase reactant in mice [33] and increased plasma levels were associated with increased arthrosclerosis in mice [34]. MCP-1 is a proinflammatory cytokine that plays an important role in the recruitment of monocytes to atherosclerotic lesions and has been implicated in both atherosclerotic plaque development and stability, myocardial infarction and acute coronary syndromes[35-38]. Levels of SAA and MCP-1 in serum were significantly higher in SR-BI/apoE dKO mice than in apoE single KO controls (supplementary Fig. 2.3), consistent with increased atherosclerosis in these mice (Fig. 2.2). Treatment with pomegranate extract however, did not significantly reduce levels of SAA or MCP-1 in serum (supplementary Fig. 2.3 A, B). In contrast, treatment with pomegranate extract did reduce levels of immunodetectable MCP-1 in atherosclerotic plaques in the aortic sinus (Fig. 2.4 A-C) and in distal coronary arteries (Fig. 2.4 D-L) in SR-BI/apoE dKO mice. Moreover the reduction of MCP-1 levels appeared to be independent of the extent of atherosclerosis in coronary arteries (Fig.2.4 D-K) suggesting that Pomegranate extract not only reduced lesional inflammation, but also reduced the inflammatory burden of CA, thus possibly reducing their susceptibility to develop atherosclerosis. This is consistent with increased number of CA with “no atherosclerosis”

in pomegranate extract treated SR-BI/apoE dKO mice compared to untreated mice (Fig. 2.2 K).

4.5. The effects of pomegranate extract on the myocardium

Previous studies have demonstrated that occlusive coronary artery atherosclerosis in SR-BI/apoE dKO mice is accompanied by macrophage infiltration and lipid accumulation in the myocardium, myocardial fibrosis an cardiac enlargement [21, 22]. Similarly, we observed extensive oil red O staining, indicative of lipid accumulation (Fig. 2.5 A, B) and CD68 staining indicative of macrophage infiltration (Fig. 2.5 C, D) within the myocardium of SR-BI/apoE dKO mice. Consistent with previous reports [21], the extent of lipid accumulation was greater towards the base and reduced towards the apex of hearts (Fig. 2.5 A, B). We also detected substantial MCP-1 immunostaining within myocardial tissue from SR-BI/apoE dKO mice (Fig. 2.5 E-F). Treatment of SR-BI/apoE dKO mice with pomegranate extract reduced levels of lipid accumulation in regions closer to the base and in regions towards the apex of the heart (Fig. 2.5 A, B). Pomegranate extract treated mice also exhibited reduced macrophage CD68 staining in regions towards the apex of the hearts but not in regions closer to the base (Fig. 2.5 C, D). This data suggests that pomegranate extract treatment reduced macrophage infiltration and to a greater extent, reduced the accumulation of lipids within the infiltrated macrophages. These findings are consistent with our observation that treatment of

macrophages in culture with pomegranate extract reduced oxLDL induced foam cell formation (Supplementary Fig. 2.4 A, B) and altered expression of genes involved in cholesterol homeostasis (supplementary Fig. 2.5). Furthermore, treatment with pomegranate extract resulted in substantial reductions in levels of MCP-1 immunostaining within the myocardium, both towards the apex and towards the base of hearts. (Fig. 2.5 E, F).

Hearts from 5 week old SR-BI/apoE dKO mice also exhibited extensive fibrosis as indicated by trichrome staining (Fig. 2.6 A). As reported previously, fibrosis was not detected in apoE single KO mice (not shown) [21, 22]. Treatment with pomegranate extract resulted in substantially reduced levels of fibrosis as detected by reduced collagen deposition (Fig. 2.6 A, B). We began our treatment of SR-BI/apoE dKO mice at 3 weeks of age. Mice at this age do not exhibit atherosclerosis in coronary arteries (supplementary Fig. 2.6), or the aortic sinus (not shown), nor do they exhibit myocardial fibrosis or cardiac enlargement (Fig. 2.6). SR-BI/apoE dKO mice develop a substantial increase in heart:body weight ratios between three and five weeks of age, corresponding to the development of cardiac pathology (Fig. 2.6 C). Heart:body weight ratio's of apoE single KO mice, by contrast, decreased over this time (Fig. 2.6C). Treatment of mice with pomegranate extract reduced the cardiac enlargement that occurred between 3 and 5 weeks of age in SR-BI/apoE dKO mice (Fig. 2.6 C). In contrast, it did not substantially affect the heart:body weight ratios of control apoE single KO mice. Importantly, reduced heart: body weight ratios of treated SR-BI/apoE dKO mice were due to reduced heart size

and corresponded to reduced fibrosis (Fig. 2.6 B) since there were no differences in body weights of untreated or pomegranate extract treated SR-BI/apoE dKO mice (data not shown).

5. Discussion

In the present study we tested the involvement of inflammation and oxidative stress in the development of spontaneous aortic sinus and occlusive coronary artery atherosclerosis and myocardial infarction in SR-BI/apoE dKO mice and examined the ability of treatment with the anti-oxidant rich pomegranate extract to reduce disease development. Treatment of SR-BI/apoE dKO with pomegranate extract affected various parameters that contribute to development of occlusive coronary artery atherosclerosis and myocardial infarction. Pomegranate extract treatment reduced the UC:CE ratios in VLDL and IDL/LDL sized lipoproteins, reduced levels of oxidative stress and MCP-1 in coronary arteries and atherosclerotic plaques in the aortic sinus, and reduced levels of macrophage infiltration, lipid accumulation and MCP-1 in myocardial tissue. These were accompanied by reduced atherosclerosis in the aortic sinus and coronary arteries, reduced myocardial infarction and reduced cardiac enlargement in treated SR-BI/apoE dKO mice.

Previous studies have demonstrated that SR-BI/apoE dKO mice and related high fat/high cholesterol fed SR-BI KO apoE-R61 hypomorphic mutant mice accumulate large sized lipoproteins with abnormally increased UC:TC ratios [22, 39]. This altered composition of surface to core lipids is accompanied by drastically altered lipoprotein structures [22, 39]. The altered lipoprotein structure and composition may, at least in part, contribute to the development of accelerated occlusive coronary artery and aortic sinus atherosclerosis in these mice [22, 39]. This may be the consequence of reduced selective uptake of UC from lipoproteins [40] or to reduced LCAT activity in SR-BI-deficient mice [41]. Reduced UC:TC ratios were reported in hypercholesterolemic rats fed green tea extract, in manner that was dependent on the concentration of phenolic compounds [42]. Moreover, treatment with the anti-oxidant, lipid lowering drug probucol reduced lipoprotein UC:TC ratios, coronary artery atherosclerosis and myocardial infarction and prolonged survival of SR-BI/apoE dKO mice [22]. Similarly, inhibition of cholesterol absorption by treatment with ezetimibe, an inhibitor of NPC1L1 mediated cholesterol absorption, or SC-435, an inhibitor of bile acid recirculation, reduced lipoprotein TC levels and reduced coronary artery and aortic atherosclerosis, and myocardial infarction and prolonged survival of SR-BI/apoE dKO mice [23].

Our data demonstrates that treatment with pomegranate extract resulted in a reduction in the UC:TC ratios of VLDL and IDL/LDL sized lipoproteins from both SR-BI/apoE dKO and apoE single KO mice. This appears to be the result of increased levels of CE rather than reduced levels of UC associated with lipoproteins. Whether this is the

result of altered LCAT activities remains to be determined. It also remains to be determined if the effects of pomegranate extract treatment on lipoprotein CE composition are the consequence, cause or independent of effects on oxidative stress and inflammation.

We demonstrate that SR-BI/apoE dKO mice exhibited increased levels of serum MCP-1 and SAA, markers of systemic inflammation, as well as evidence of substantial local inflammation (MCP-1 staining) and oxidative stress (DHE staining) in aortic sinus atherosclerosis, coronary vessels and/or in myocardial tissue. Pomegranate extract treatment substantially reduced oxidative stress in atherosclerotic vessels from SR-BI/apoE dKO mice. Consistent with the reported decrease in malondialdehyde positive cells in LDL receptor KO mice treated with pomegranate juice or flower extract [28]. The reduction in oxidative stress could either be due to the free radical scavenging properties of pomegranate polyphenolics or alternatively due to the activation of cardiac anti-oxidative defenses [29]. MCP-1 gene expression is regulated by redox-sensitive transcription factors and is sensitive to oxidative stress [43]. On the other hand treatment of monocytes with recombinant MCP-1 can increase oxidative stress [44]. Consistent with the relationship between oxidative stress and MCP-1 mediated inflammation, pomegranate extract treatment also reduced MCP-1 in atherosclerotic plaques in the aortic sinus and in coronary arteries as well as in the myocardium. This may have been related to the reduced macrophage infiltration and lipid accumulation in the myocardium. Indeed, we found that treatment of macrophages in vitro with pomegranate extract

reduced LPS or AcLDL induced production of IL-6 or MCP-1, and oxLDL induced foam cell formation. Target gene expression analysis suggests that pomegranate extract treatment of cells resulted in alterations in levels of expression of genes involved in cellular cholesterol homeostasis, perhaps underlying the reduced foam cell formation.

Increased MCP-1 levels have been reported in patients with acute coronary syndrome and have been closely associated with the development of atherosclerosis [38]. Perhaps surprisingly, we did not observe any statistically significant reductions in levels of serum MCP-1 or SAA. On the other hand, our data suggest that pomegranate extract-mediated suppression of tissue inflammation may contribute to the reduced atherosclerosis and myocardial infarction in these mice.

Preliminary analysis, however, suggested that treatment with pomegranate extract at the dose used did not prolong the survival of SR-BI/apoE dKO mice. This may be because coronary artery atherosclerosis and myocardial infarction still occurred all be it to a lesser extent, in the treated mice. Nevertheless, these data demonstrate that pomegranate extract is effective at reducing aortic sinus, and coronary artery atherosclerosis, myocardial infarction and cardiac enlargement through its effects on lipid metabolism, oxidative stress and/or inflammation.

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7. References

[1] V. Mallika, B. Goswami and M. Rajappa, Atherosclerosis pathophysiology and the role of novel risk factors: a clinicobiochemical perspective, Angiology. 58 (2007) 513-22.

[2] R. Stocker and J.F. Keaney, Jr., Role of oxidative modifications in atherosclerosis, Physiol Rev. 84 (2004) 1381-478.

[3] S. De Rosa, P. Cirillo, A. Paglia, L. Sasso, V. Di Palma and M. Chiariello, Reactive oxygen species and antioxidants in the pathophysiology of cardiovascular disease: does the actual knowledge justify a clinical approach?, Curr Vasc Pharmacol. 8 (2010) 259-75.

[4] A. Mente, L. de Koning, H.S. Shannon and S.S. Anand, A systematic review of the evidence supporting a causal link between dietary factors and coronary heart disease, Arch Intern Med. 169 (2009) 659-69.

[5] C.H. Hennekens, J.E. Buring, J.E. Manson, M. Stampfer, B. Rosner, N.R. Cook, C. Belanger, F. LaMotte, J.M. Gaziano, P.M. Ridker, W. Willett and R. Peto, Lack of effect of long-term supplementation with beta carotene on the incidence of malignant neoplasms and cardiovascular disease, N Engl J Med. 334 (1996) 1145-9.

[6] G.S. Omenn, G.E. Goodman, M.D. Thornquist, J. Balmes, M.R. Cullen, A. Glass, J.P. Keogh, F.L. Meyskens, B. Valanis, J.H. Williams, S. Barnhart and S. Hammar, Effects of a combination of beta carotene and vitamin A on lung cancer and cardiovascular disease, N Engl J Med. 334 (1996) 1150-5.

[7] J.M. Rapola, J. Virtamo, S. Ripatti, J.K. Huttunen, D. Albanes, P.R. Taylor and O.P. Heinonen, Randomised trial of alpha-tocopherol and beta-carotene supplements on incidence of major coronary events in men with previous myocardial infarction, Lancet. 349 (1997) 1715-20.

[8] D.A. Street, G.W. Comstock, R.M. Salkeld, W. Schuep and M.J. Klag, Serum antioxidants and myocardial infarction. Are low levels of carotenoids and alpha-tocopherol risk factors for myocardial infarction?, Circulation. 90 (1994) 1154-61.

[9] N.R. Cook, C.M. Albert, J.M. Gaziano, E. Zaharris, J. MacFadyen, E. Danielson, J.E. Buring and J.E. Manson, A randomized factorial trial of vitamins C and E and beta carotene in the secondary prevention of cardiovascular events in women: results from the Women's Antioxidant Cardiovascular Study, Arch Intern Med. 167 (2007) 1610-8.

[10] A. Shihabi, W.G. Li, F.J. Miller, Jr. and N.L. Weintraub, Antioxidant therapy for atherosclerotic vascular disease: the promise and the pitfalls, Am J Physiol Heart Circ Physiol. 282 (2002) H797-802.

[11] K.K. Witte and A.L. Clark, Micronutrients and their supplementation in chronic cardiac failure. An update beyond theoretical perspectives, Heart Fail Rev. 11 (2006) 65-74.

[12] N.P. Seeram, L.S. Adams, S.M. Henning, Y. Niu, Y. Zhang, M.G. Nair and D. Heber, In vitro antiproliferative, apoptotic and antioxidant activities of punicalagin, ellagic acid and a total pomegranate tannin extract are enhanced in combination with other polyphenols as found in pomegranate juice, J Nutr Biochem. 16 (2005) 360-7.

[13] A. Basu and K. Penugonda, Pomegranate juice: a heart-healthy fruit juice, Nutr Rev. 67 (2009) 49-56.

[14] M. Aviram, M. Rosenblat, D. Gaitini, S. Nitecki, A. Hoffman, L. Dornfeld, N. Volkova, D. Presser, J. Attias, H. Liker and T. Hayek, Pomegranate juice consumption for 3 years by patients with carotid artery stenosis reduces common carotid intima-media thickness, blood pressure and LDL oxidation, Clin Nutr. 23 (2004) 423-33.

[15] M. Kaplan, T. Hayek, A. Raz, R. Coleman, L. Dornfeld, J. Vaya and M. Aviram, Pomegranate juice supplementation to atherosclerotic mice reduces macrophage lipid peroxidation, cellular cholesterol accumulation and development of atherosclerosis, J Nutr. 131 (2001) 2082-9.

[16] M. Aviram, L. Dornfeld, M. Rosenblat, N. Volkova, M. Kaplan, R. Coleman, T. Hayek, D. Presser and B. Fuhrman, Pomegranate juice consumption reduces oxidative stress, atherogenic modifications to LDL, and platelet aggregation: studies in humans and in atherosclerotic apolipoprotein E-deficient mice, Am J Clin Nutr. 71 (2000) 1062-76.

[17] M. Aviram, L. Dornfeld, M. Kaplan, R. Coleman, D. Gaitini, S. Nitecki, A. Hofman, M. Rosenblat, N. Volkova, D. Presser, J. Attias, T. Hayek and B. Fuhrman, Pomegranate juice flavonoids inhibit low-density lipoprotein oxidation and cardiovascular diseases: studies in atherosclerotic mice and in humans, Drugs Exp Clin Res. 28 (2002) 49-62.

[18] M. Aviram, N. Volkova, R. Coleman, M. Dreher, M.K. Reddy, D. Ferreira and M. Rosenblat, Pomegranate phenolics from the peels, arils, and flowers are antiatherogenic: studies in vivo in atherosclerotic apolipoprotein e-deficient (E 0) mice and in vitro in cultured macrophages and lipoproteins, J Agric Food Chem. 56 (2008) 1148-57.

[19] M. Rosenblat, N. Volkova and M. Aviram, Pomegranate juice (PJ) consumption antioxidative properties on mouse macrophages, but not PJ beneficial effects on macrophage cholesterol and triglyceride metabolism, are mediated via PJ-induced stimulation of macrophage PON2, Atherosclerosis. 212 (2010) 86-92.

[20] A. Braun, A. Rigotti and B.L. Trigatti, Myocardial infarction following atherosclerosis in murine models, Curr Drug Targets. 9 (2008) 217-23.

[21] A. Braun, B.L. Trigatti, M.J. Post, K. Sato, M. Simons, J.M. Edelberg, R.D. Rosenberg, M. Schrenzel and M. Krieger, Loss of SR-BI expression leads to the early onset of occlusive atherosclerotic coronary artery disease, spontaneous myocardial infarctions, severe cardiac dysfunction, and premature death in apolipoprotein E-deficient mice, Circ Res. 90 (2002) 270-6.

[22] A. Braun, S. Zhang, H.E. Miettinen, S. Ebrahim, T.M. Holm, E. Vasile, M.J. Post, D.M. Yoerger, M.H. Picard, J.L. Krieger, N.C. Andrews, M. Simons and M. Krieger, Probucol prevents early coronary heart disease and death in the high-density lipoprotein receptor SR-BI/apolipoprotein E double knockout mouse, Proc Natl Acad Sci U S A. 100 (2003) 7283-8.

[23] A. Braun, A. Yesilaltay, S. Acton, K.O. Broschat, E.S. Krul, N. Napawan, N. Stagliano and M. Krieger, Inhibition of intestinal absorption of cholesterol by ezetimibe or bile acids by SC-435 alters lipoprotein metabolism and extends the lifespan of SR-BI/apoE double knockout mice, Atherosclerosis. 198 (2008) 77-84.

[24] J. Fennel, Baker, AH (2005) *Hypertension Methods and Protocols* (Humana Press, New Jersey).

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

[26] A. Rigotti, B.L. Trigatti, M. Penman, H. Rayburn, J. Herz and M. Krieger, A targeted mutation in the murine gene encoding the high density lipoprotein (HDL) receptor scavenger receptor class B type I reveals its key role in HDL metabolism, Proc Natl Acad Sci U S A. 94 (1997) 12610-5.

[27] B. Trigatti, H. Rayburn, M. Vinals, A. Braun, H. Miettinen, M. Penman, M. Hertz, M. Schrenzel, L. Amigo, A. Rigotti and M. Krieger, Influence of the high density lipoprotein receptor SR-BI on reproductive and cardiovascular pathophysiology, Proc Natl Acad Sci U S A. 96 (1999) 9322-7.

[28] F. de Nigris, S. Williams-Ignarro, L.O. Lerman, E. Crimi, C. Botti, G. Mansueto, F.P. D'Armiento, G. De Rosa, V. Sica, L.J. Ignarro and C. Napoli, Beneficial effects of pomegranate juice on oxidation-sensitive genes and endothelial nitric oxide synthase activity at sites of perturbed shear stress, Proc Natl Acad Sci U S A. 102 (2005) 4896-901.

[29] R.N. Jadeja, M.C. Thounaojam, D.K. Patel, R.V. Devkar and A.V. Ramachandran, Pomegranate (Punica granatum L.) juice supplementation

attenuates isoproterenol-induced cardiac necrosis in rats, Cardiovasc Toxicol. 10 (2010) 174-80.

[30] G. Biasillo, M. Leo, R. Della Bona and L.M. Biasucci, Inflammatory biomarkers and coronary heart disease: from bench to bedside and back, Intern Emerg Med. 5 (2010) 225-33.

[31] G. Liuzzo, L.M. Biasucci, J.R. Gallimore, R.L. Grillo, A.G. Rebuzzi, M.B. Pepys and A. Maseri, The prognostic value of C-reactive protein and serum amyloid a protein in severe unstable angina, N Engl J Med. 331 (1994) 417-24.

[32] B.D. Johnson, K.E. Kip, O.C. Marroquin, P.M. Ridker, S.F. Kelsey, L.J. Shaw, C.J. Pepine, B. Sharaf, C.N. Bairey Merz, G. Sopko, M.B. Olson and S.E. Reis, Serum amyloid A as a predictor of coronary artery disease and cardiovascular outcome in women: the National Heart, Lung, and Blood Institute-Sponsored Women's Ischemia Syndrome Evaluation (WISE), Circulation. 109 (2004) 726-32.

[33] C. Cray, J. Zaias and N.H. Altman, Acute phase response in animals: a review, Comp Med. 59 (2009) 517-26.

[34] K.E. Lewis, E.A. Kirk, T.O. McDonald, S. Wang, T.N. Wight, K.D. O'Brien and A. Chait, Increase in serum amyloid a evoked by dietary cholesterol is associated with increased atherosclerosis in mice, Circulation. 110 (2004) 540-5.

[35] J. Reckless, E.M. Rubin, J.B. Verstuyft, J.C. Metcalfe and D.J. Grainger, Monocyte chemoattractant protein-1 but not tumor necrosis factor-alpha is correlated with monocyte infiltration in mouse lipid lesions, Circulation. 99 (1999) 2310-6.

[36] R.J. Aiello, P.A. Bourassa, S. Lindsey, W. Weng, E. Natoli, B.J. Rollins and P.M. Milos, Monocyte chemoattractant protein-1 accelerates atherosclerosis in apolipoprotein E-deficient mice, Arterioscler Thromb Vasc Biol. 19 (1999) 1518-25.

[37] P. Libby, Inflammation in atherosclerosis, Nature. 420 (2002) 868-74.

[38] U. Ikeda, K. Matsui, Y. Murakami and K. Shimada, Monocyte chemoattractant protein-1 and coronary artery disease, Clin Cardiol. 25 (2002) 143-7.

[39] S. Zhang, M.H. Picard, E. Vasile, Y. Zhu, R.L. Raffai, K.H. Weisgraber and M. Krieger, Diet-induced occlusive coronary atherosclerosis, myocardial infarction, cardiac dysfunction, and premature death in scavenger receptor class B type I-deficient, hypomorphic apolipoprotein ER61 mice, Circulation. 111 (2005) 3457-64.

[40] P.G. Yancey, M. de la Llera-Moya, S. Swarnakar, P. Monzo, S.M. Klein, M.A. Connelly, W.J. Johnson, D.L. Williams and G.H. Rothblat, High density lipoprotein phospholipid composition is a major determinant of the bi-directional

flux and net movement of cellular free cholesterol mediated by scavenger receptor BI, J Biol Chem. 275 (2000) 36596-604.

[41] J.Y. Lee, R.M. Badeau, A. Mulya, E. Boudyguina, A.K. Gebre, T.L. Smith and J.S. Parks, Functional LCAT deficiency in human apolipoprotein A-I transgenic, SR-BI knockout mice, J Lipid Res. 48 (2007) 1052-61.

[42] T. Yokozawa, T. Nakagawa and K. Kitani, Antioxidative activity of green tea polyphenol in cholesterol-fed rats, J Agric Food Chem. 50 (2002) 3549-52.

[43] D. Ping, G.H. Boekhoudt, E.M. Rogers and J.M. Boss, Nuclear factor-kappa B p65 mediates the assembly and activation of the TNF-responsive element of the murine monocyte chemoattractant-1 gene, J Immunol. 162 (1999) 727-34.

[44] P. Aukrust, R.K. Berge, T. Ueland, E. Aaser, J.K. Damas, L. Wikeby, A. Brunsvig, F. Muller, K. Forfang, S.S. Froland and L. Gullestad, Interaction between chemokines and oxidative stress: possible pathogenic role in acute coronary syndromes, J Am Coll Cardiol. 37 (2001) 485-91.

8. Figure legends

Fig. 2.1: The effects of pomegranate extract treatment on serum lipoprotein profiles. Serum lipoproteins were fractionated by FPLC and TC (A and B) UC (C and D) and CE levels (E and F) were determined as indicated in Experimental Procedures. Representative profiles from untreated or pomegranate extract treated apoE KO mice (n=7) (A) and SR-BI/apoE dKO mice (n=10) (B) are shown. Positions of human lipoprotein standards are indicated for reference.

Fig. 2.2: The effect of pomegranate extract treatment on atherosclerosis in the aortic sinus and coronary arteries of SR-BI/apoE dKO mice. Representative images of plaques at the aortic sinus of treated or untreated apoE KO (A, C) or SR-BI/apoE dKO mice stained with oil red O (B, D) are shown. Scale bars= 300 μm. Plaque areas at the aortic root were quantified from untreated (n = 10) and pomegranate extract treated (n = 8) SR-BI/apoE dKO mice (E). Oil red O stained and hematoxylin counter stained CA's were classified according to the extent of atherosclerosis (F-J). Scale bars= 60 μm.Quantification of CA occlusion in pomegranate extract treated (n = 9) and untreated (n = 11) SR-BI/apoE dKO mice (K). Data are means ± standard deviations. $^{\S}P < 0.05$ and $*P < 0.01$ vs. untreated dKO controls.

Fig. 2.3: The effects of pomegranate extract treatment on oxidative stress in atherosclerotic vessels in the SR-BI/apoE dKO mice. Oil red O (A, C, F, H) and DHE stained (B, D, G, I) sections of atherosclerotic plaques in the aortic roots (A-D) or coronary arteries (F-I) from untreated or pomegranate treated SR-BI/apoE dKO mice. Representative sections are shown. Scale bars= 30 μm. (E) Quantification of DHE staining in atherosclerotic plaques in aortic sinus sections from untreated (n = 4) and pomegranate extract-treated (n = 6) mice. (J) Quantification of DHE staining in coronary arteries of untreated control (n = 3) or pomegranate extract treated mice (n = 4). Data is expressed as mean ± standard deviation, * $P < 0.05$, vs. untreated dKO controls.

Fig. 2.4: The effect of pomegranate extract treatment on inflammation atherosclerotic vessels in SR-BI/apoE dKO mice. MCP-1 immunofluorescence of aortic root lesions from untreated (A) and pomegranate extract-treated (B) SR-BI/apoE dKO mice with staining intensity normalized to lesion area (C). Oil red O (D, F, H, J) and MCP-1 immunoflurescence staining (E, G, I, K) in CA's of SR-BI/apoE dKO mice that were either untreated (D-J) or treated with pomegranate extract (H-K). Representative sections of arteries either with atherosclerosis (D, E, H, I) or without atherosclerosis (F, G, J, K) are shown. Scale bars= 60 μm. Staining intensity was normalized to atherosclerotic plaque area (C) or artery circumference (L). n= 6 untreated and n=5

pomegranate extract treated mice were analyzed. Data is expressed as mean ± standard deviation, * $P < 0.05$, vs. untreated dKO controls.

Fig. 2.5: Effects of pomegranate extract on lipid accumulation, macrophage infiltration and inflammation in the hearts of SR-BI/apoE dKO mice. Representative images of oil red O stained sections taken towards the upper ("base") or lower ("apex") portions of hearts from untreated or pomegranate extract treated SR-BI/apoE dKO mice (A). Quantification of lipid accumulation in the myocardium from untreated or pomegranate extract treated SR-BI/apoE dKO (n = 5-7) (B). Immunofluorescent staining of CD68 (C) or MCP-1 (E) (red) in treated or untreated SR-BI/apoE dKO mice. Nuclei are counter stained with DAPI (C, E) (blue). Scale bars= 60 μm. Quantification of CD68 (D) or MCP-1 (F) staining (n=3-6). Values are means ± standard deviations, * $P < 0.05$ vs. untreated controls.

Fig. 2.6: The effects of pomegranate extract on myocardial fibrosis and cardiac enlargement in the SR-BI/apoE dKO mice. Representative images of trichrome stained cross-sections from the upper portions of hearts (towards the base in Fig. 2.5A) from untreated 3 week old and untreated or treated 5 week old SR-BI/apoE dKO mice (A). Quantification of myocardial fibrosis (blue staining in A) (n = 6-8 mice per group). Data

are means± standard deviations. § $P < 0.01$, * $P < 0.05$ vs. untreated 5 week old mice (B). Heart to body weight ratios of untreated three week old apoE KO (n = 4) or SR-BI/apoE dKO (n = 8) and five week old treated apoE KO (n = 11) or SR-BI/apoE dKO (n=15) and untreated apoE KO (n = 17) or SR-BI/apoE dKO (n = 10) mice (C). Data are means ± standard deviations. § $P < 0.0001$ vs. 3 weeks old mice, * $P < 0.01$ vs. apoE KO or SR-BI/apoE dKO at 5 weeks of age.

9. Supplementary materials and methods

Cells and cell culture, foam cell formation and cytokine analysis

RAW264.7 cells were cultured at 37 ºC in and the presence of 5 % CO_2 in air, in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine and 50 μg/ml penicillin/streptomycin (Invitrogen Life Technologies, Burlington, ON, Canada) and were passaged by scraping.

Thioglycolate elicited mouse peritoneal macrophages (MPMs) were isolated from C57BL6/J mice four days after injection with 1 ml of 10 % thioglycolate. MPMs were allowed to adhere for 2 hrs, non-adherent cells were removed and MPMs were cultured in DMEM containing 10% FBS. Cells were pretreated (2 hrs) with media containing pomegranate extract or pomegranate juice at the indicated concentrations. Cells were then treated with or without LPS, AcLDL or oxLDL (Biomedical Technologies, Inc., Stoughton, MA, USA) for 24 hrs in the presence of pomegranate juice or extract as indicated. For analysis of foam cell formation, cells were fixed and stained for 1hr with oil-red O [45]. Serum amyloid alpha (SAA) and MCP-1 levels were measured in serum samples from treated or untreated mice. MCP-1 or IL-6 levels in cell culture media or mouse serum were measured by ELISA using kits from R&D Systems (Minneapolis, MN, USA). Serum amyloid alpha (SAA) in mouse serum was measured by ELISA using a kit from Invitrogen Life Technologies (Burlington, ON, Canada).

Quantitative real time PCR and immunoblotting

Total RNA was extracted from cells using Qiagen RNeasy Mini kit (Qiagen, ON, Canada). cDNA was prepared using Quanti Tect reverse transcription kit (Qiagen, ON, Canada). Quantitative eal time PCR analysis was performed using Platinum SYBR Green Supermix-UDG (Invitrogen Life Technologies, Burlington, ON, Canada) [46] with primers (Invitrogen Life Technologies, Burlington, ON, Canada) specific for murine hydroxymethylglutaryl coenzyme A (HMG-CoA) reductase (forward, 5'-GGGAGCATAGGCGGCT-3'; reverse, 5'- TGCGATGTAGATAGCAGTGACA-3'), ATP-binding cassette transporter (ABC) A1 (forward, 5'-ATTGCCAGACGGAGCCG-3'; reverse5'-TGCCAAAGGGTGGCACA-3'), ABCG1 (forward, 5'-CAAGACCCTTTTGAAAGGGATCTC-3'; reverse, 5'-GCCAGAATATTCATGAGTGTGGAC-3') and cyclophilin A (forward, 5'-GGCCGATG-3'; reverse, 5-TGTCTTTGGAACTTTGTCTGCAA-3').

For immunoblotting, cells were lysed in an ice cold solution containing 0.2 x PBS, 0.1 % Triton-X 100, and protease inhibitors (20 µg/ml aprotinin, 10 µg/ml leupeptin, 1 mM APMSF and 10 µg/ml pepstatinA). Lysates were centrifuged for 10 min at maximum speed in a bench top microcentrifuge and protein concentrations in the supernatants were measured using the BCA assay kit (Thermoscientific, Ottawa, CA). Cleared lysates (50 µg protein) or 1 µl of serum from mice were subjected to SDS-PAGE [3] electrophoretic transfer to PVDF membranes [4] and immunoblotting with rabbit-anti-

LDL receptor (R&D Systems, Minnesota, USA) or rabbit anti-p38 MAPK (Cell Signaling Technology Inc., Danvers MA, USA) or goat anti-human apoA-I or goat anti-human apoB100/48 (Midland Bioproducts Corp. Boone, IA, USA) and detected with HRP-conjugated anti-rabbit or anti-goat antibodies (Jackson Immuno Research, West Grove, PA, USA).

References

[1] N.G. Barra, S. Reid, R. MacKenzie, G. Werstuck, B.L. Trigatti, C. Richards, A.C. Holloway and A.A. Ashkar, Interleukin-15 contributes to the regulation of murine adipose tissue and human adipocytes, Obesity (Silver Spring). 18 (2010) 1601-7.

[2] C.J. Delvecchio, P. Bilan, K. Radford, J. Stephen, B.L. Trigatti, G. Cox, K. Parameswaran and J.P. Capone, Liver X receptor stimulates cholesterol efflux and inhibits expression of proinflammatory mediators in human airway smooth muscle cells, Mol Endocrinol. 21 (2007) 1324-34.

[3] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature. 227 (1970) 680-5.

[4] H. Towbin, T. Staehelin and J. Gordon, Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications, Proc Natl Acad Sci U S A. 76 (1979) 4350-4.

10. Supplementary figure legends

Supplementary Fig. 2.1: The effects of pomegranate extract on macrophage inflammation. RAW264.7 macrophages were pretreated (2 hrs) with pomegranate extract (POMlx) or pomegranate juice (PJ) at the concentrations indicated and then incubated in the continued presence of pomegranate extract or juice with either no addition or with LPS (10 ng/ml) **(A)**. MPMS were treated with or without AcLDL (100 μg/ml) in the presence or absence of pomegranate extract (0.769 μl/ml) (B). After 24 hrs, cell culture media was assayed for IL-6 (A) or MCP-1 (B) by ELISA. Values are means ± standard deviations of 3-4 independent samples, $^{\S}P \leq 0.01$,*$P \leq 0.01$ vs. untreated controls or LPS/AcLDL treated macrophages respectively.

Supplementary Fig. 2.2: The effects of pomegranate extract on serum apolipoprotein levels. Serum samples (1 μl) from 4 untreated or pomegranate extract treated SR-BI/apoE dKO mice were subjected to SDS-PAGE and immunoblotting for apoA-I or apoB 100/48.

Supplementary Fig. 2.3: Levels of SAA and MCP-1 in apoE or SR-BI/apoE dKO mice treated with pomegranate extract. SAA was measured by ELISA in serum

samples from untreated (n = 5), or pomegranate extract treated (n = 5) apoE KO mice and untreated (n = 5) or pomegranate extract treated (n = 7) SR-BI/apoE dKO mice (A). MCP-1 levels were measured by ELISA from untreated (n=4) or pomegranate extract treated (n=5) apoE KO and untreated (n = 10) or pomegranate extract treated (n = 8) SR-BI/apoE dKO mice (B). Data are means ± standard deviations, NS= not statistically significantly different.

Supplementary Fig. 2.4: Effect of pomegranate extract on foam cell formation. MPMs were treated with or without oxLDL (100 μg/ml) in the presence or absence of pomegranate extract (0.769 μl/ml) as described in the methods section. Cells were stained with oil red O (A) and quantified (B). Representative images from three independent mice are shown in (A) and data in (B) are means ± standard deviations of 10-14 images from each mouse (B), $^{\S}P \leq 0.01$,*$P \leq 0.01$ vs. untreated control or AcLDL treated macrophages respectively.

Supplementary Fig. 2.5: The effect of pomegranate extract on transcript and protein levels of genes involved in cholesterol homeostasis. RAW264.7 macrophages were treated with or without pomegranate extract (0.769 μl/ml). RNA was isolated and cDNA was prepared from 4 independent samples in each group subjected to real time

PCR amplification using specific primers to the indicated genes (A). The values represent fold induction relative to untreated cells normalized to cyclophilin A as an internal standard. Data are expressed as means ± standard deviations, § $P < 0.05$, *$P < 0.001$ vs. untreated controls. Total cell lysates (50 µg protein) from cells treated without or with the indicated concentrations of pomegranate extract were analyzed by immunoblotting for the LDLR and p38 MAK as the loading control (B).

Supplementary Fig. 2.6: Coronary artery atherosclerosis in three weeks old SR-BI/apoE dKO mice. Quantification of coronary artery atherosclerosis in three week old SR-BI/apoE dKO mice (n=8). Data are means ± standard deviations, * $P < 0.001$ vs. plaque containing arteries (one way ANOVA).

CHAPTER TWO
FIGURES

Fig. 2.1: The effect of pomegranate extract on serum lipoprotein profiles and composition

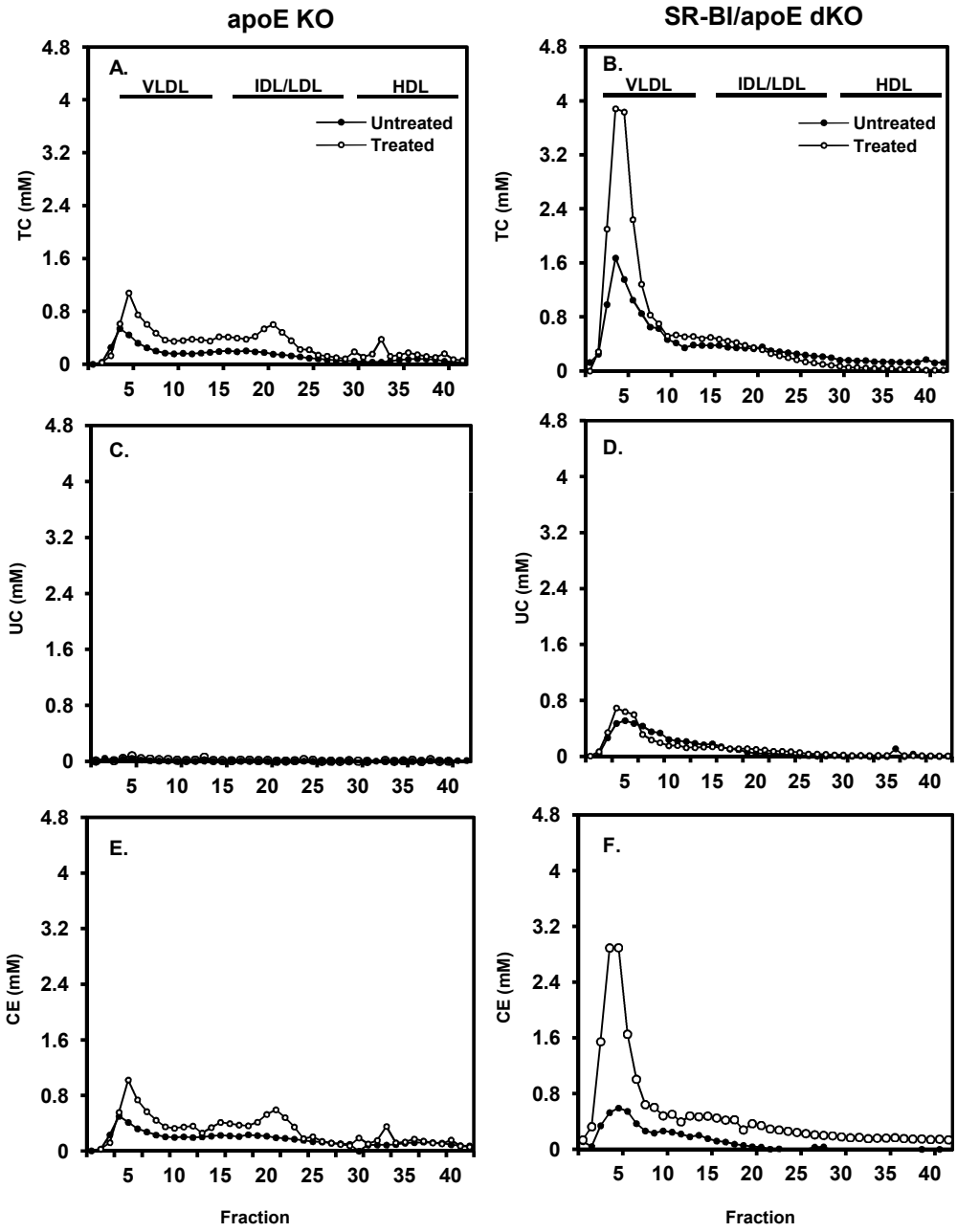


Fig. 2.2: The effect of pomegranate extract on atherosclerosis in SR-BI/apoE dKO mice

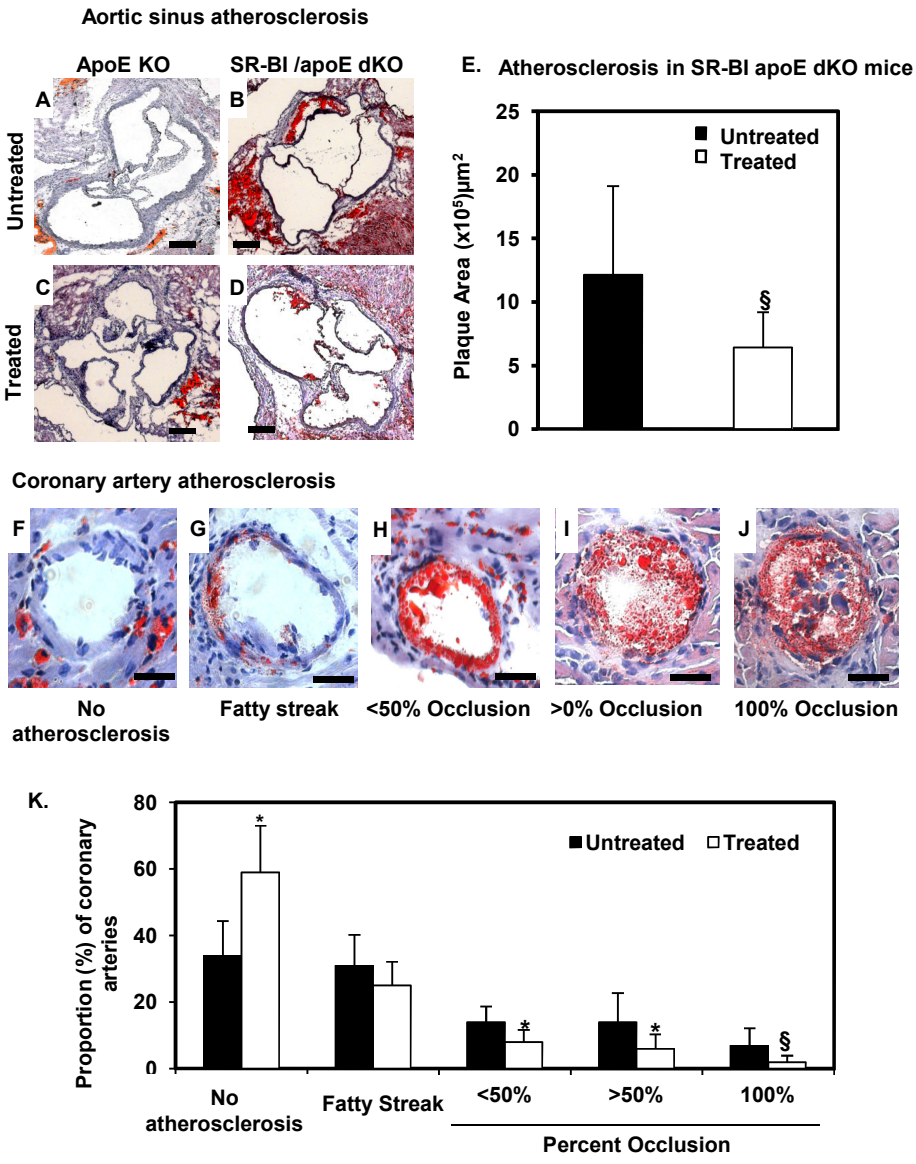


Fig. 2.3: The effect of pomegranate extract on oxidative stress in the SR-BI/apoE dKO mice

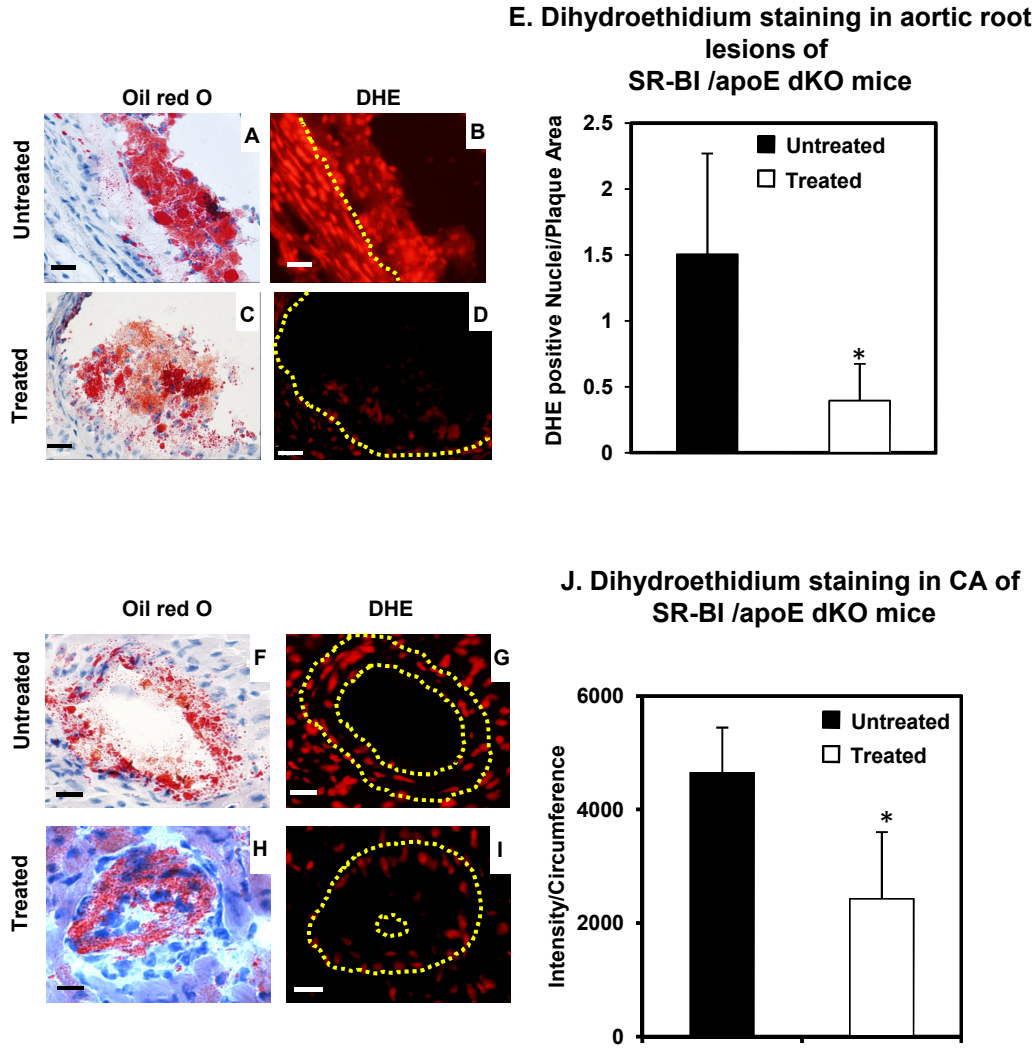


Fig. 2.4: The effect of pomegranate extract on inflammation in the SR-BI/apoE dKO mice.

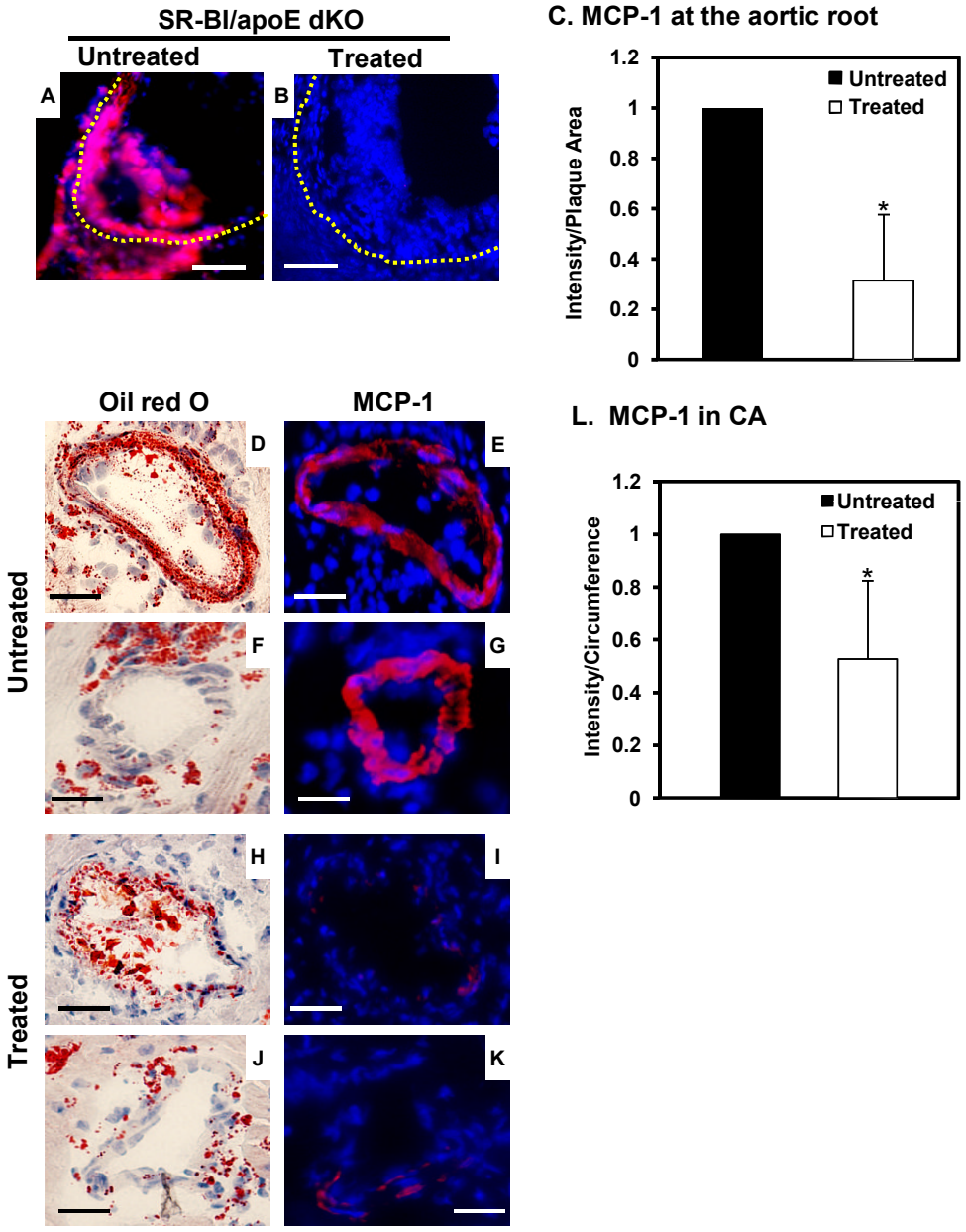


Fig. 2.5: The effects of POMlx on lipid accumulation, macrophage infiltration and inflammation in the heart.

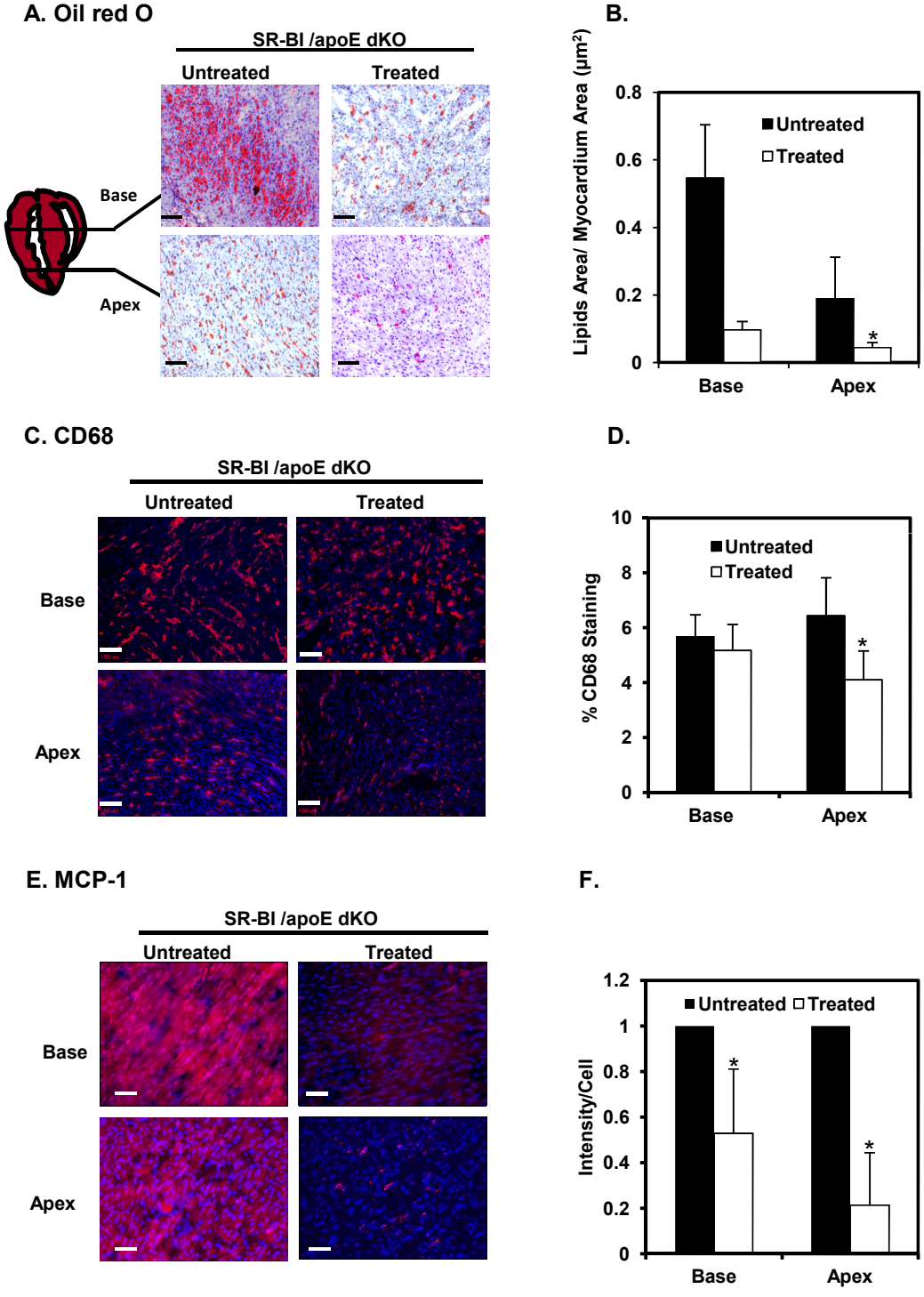
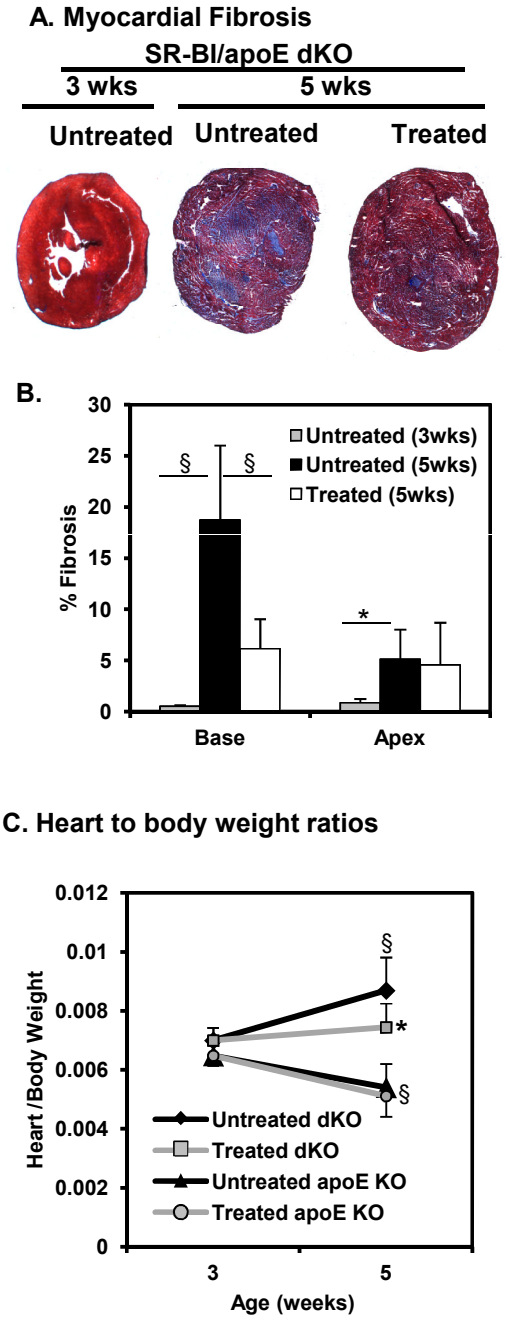
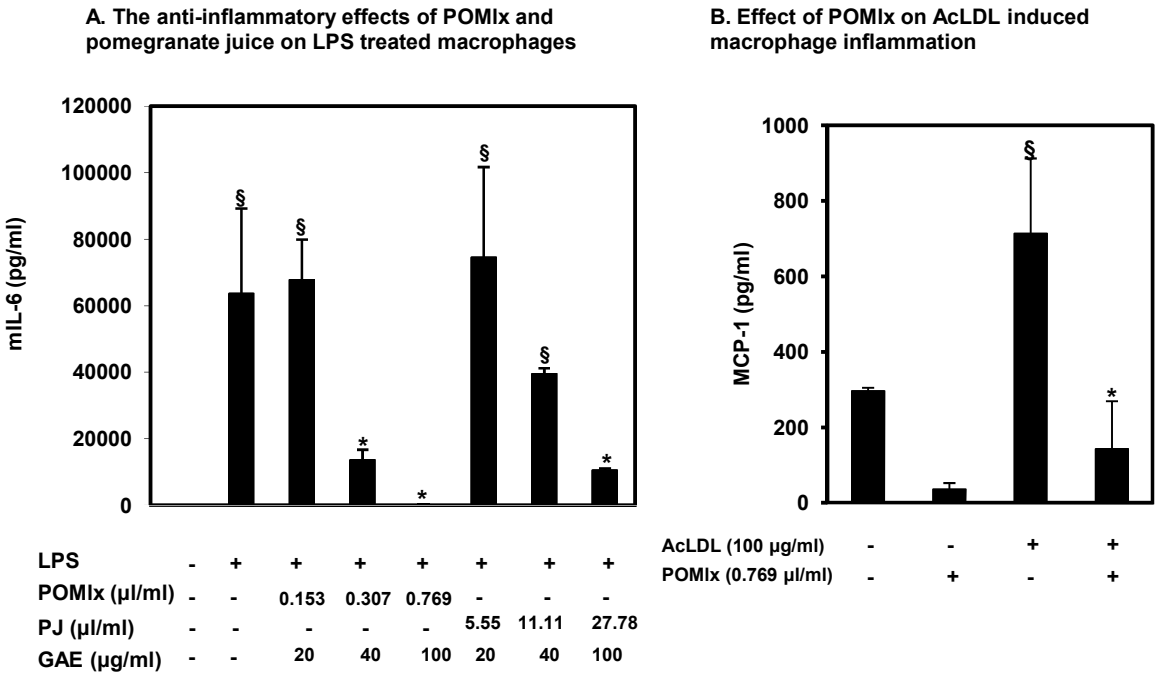


Fig. 2.6: The effects of pomegranate extract on myocardial fibrosis and cardiac enlargements in the SR-BI/ apoE dKO mice.

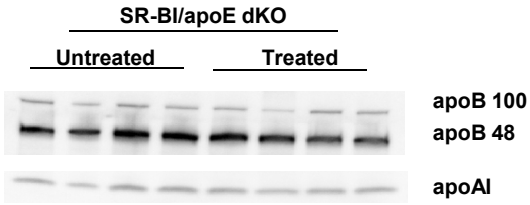


CHAPTER TWO
SUPPLEMENTARY FIGURES

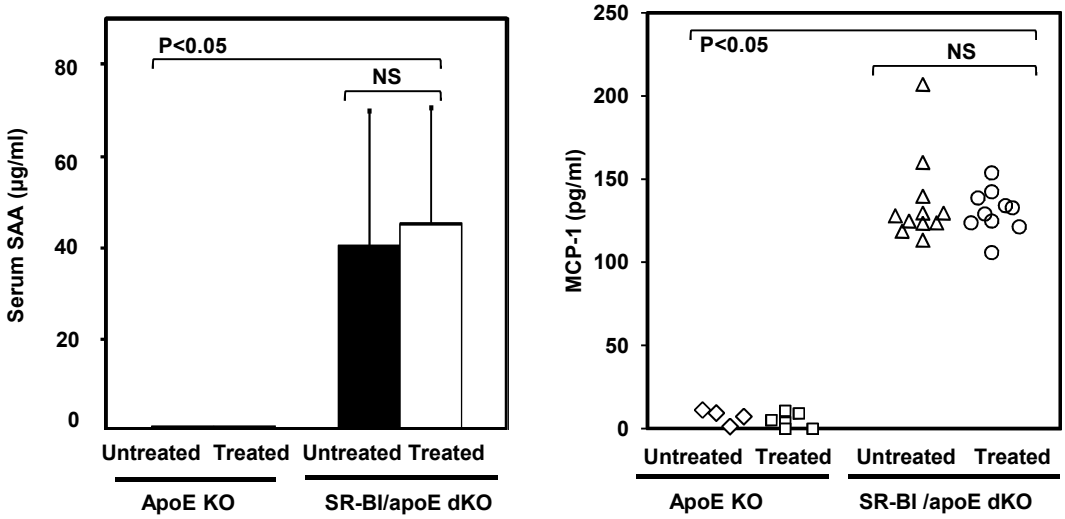
Supplementary Fig. 2.1: Effect of pomegranate extract on macrophage inflammation.



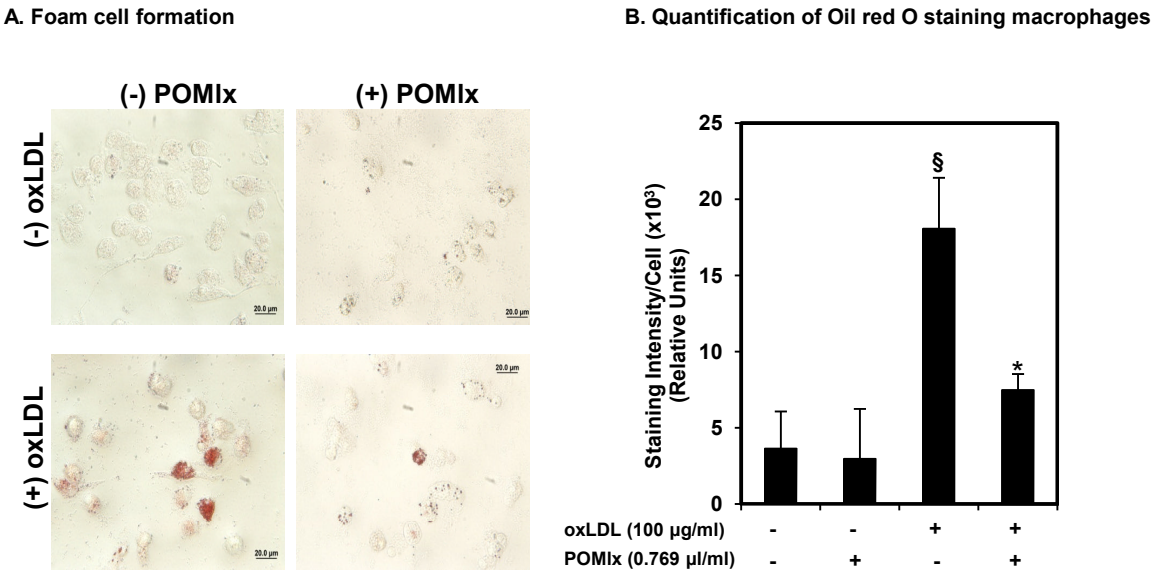
Supplementary Fig. 2.2: The effects of pomegranate extract on serum apolipoproteins levels.



Supplementary Fig. 2.3: The effect of pomegranate extract on systemic inflammation in the SR-BI/apoE dKO mice

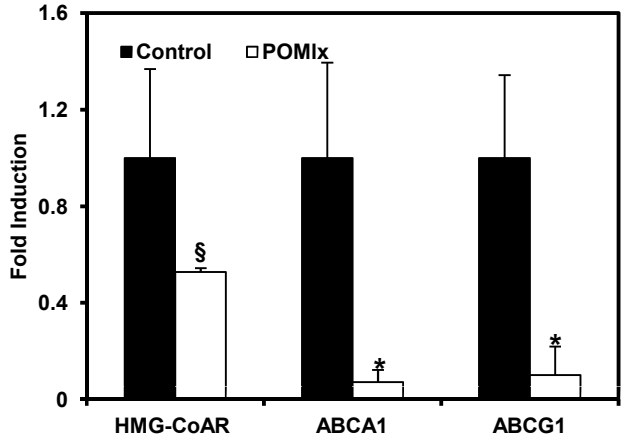


Supplementary Fig. 2.4: The effect of pomegranate extract on foam cell formation.

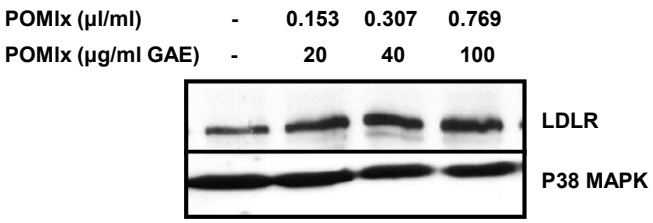


Supplementary Fig. 2.5: The effect of pomegranate extract on transcript and protein levels of genes involved in cholesterol homeostasis

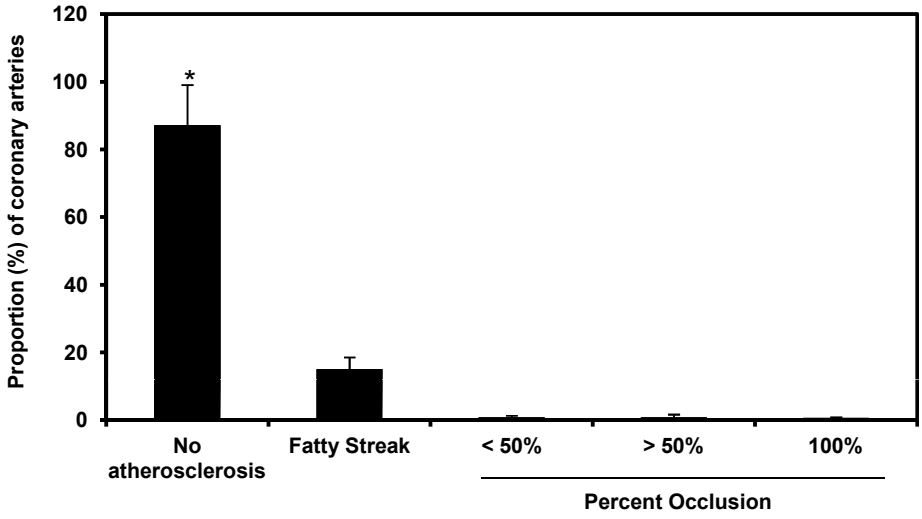
A.



B.



Supplementary Fig. 2.6: Coronary artery atherosclerosis in three weeks old SR-BI/apoE dKO mice



CHAPTER THREE

SR-BI PROTECTS MACROPHAGES AGAINST FREE CHOLESTEROL-INDUCED APOPTOSIS IN AN HDL DEPENDENT MANNER

CHAPTER THREE PREFACE

This work has been submitted to *Atherosclerosis Thrombosis Vascular Biology*. The reviewer's comments are being addressed and the manuscript will be resubmitted.

My contribution to this work include intellectual discussions with Dr.Trigatti about possible molecular mechanisms that may explain SR-BI mediated protection against atherosclerosis in bone marrow derived cells, writing the initial draft of the manuscript and editing subsequent versions.

My contribution to the manuscript figures includes designing and conducting the experiments in the following figures: (Fig. 3.1 C and D), (Fig. 3.5 D-G), (Fig. 3.6 A-D), (Supplementary Fig. 3.3 E-G). Cells' isolation, treatments and data analysis of (Fig. 3.2 A and B).

SR-BI PROTECTS MACROPHAGES AGAINST FREE CHOLESTEROL-INDUCED APOPTOSIS IN AN HDL DEPENDENT MANNER

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Abbreviated Title: HDL protects against macrophage apoptosis

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1. Abstract

Objectives:

The objective was to determine if the HDL receptor SR-BI protected macrophages against apoptosis, and if this contributed to its protection against plaque development in vivo.

Methods and Results:

Mouse peritoneal macrophages were loaded with free cholesterol (FC) by incubation with acetylated LDL and an acyl-CoA-cholesterol acyltransferase inhibitor. HDL did not affect the extent of FC loading or induction of ER stress markers, but protected against FC-induced apoptosis. This was reduced in SR-BI KO macrophages or in wild type macrophages treated with an anti-SR-BI blocking antibody. HDL triggered the phosphorylation of Akt1, Erk1/2, JNK-1 and Foxo3a, reduced pro-apoptotic Bim and increased anti-apoptotic Mcl-1 levels. HDL mediated reduction in Bim and suppression of apoptosis were prevented by disruption of PI3K, Akt1, JNK-1 and Erk1/2 signaling. ApoE KO mice transplanted with bone marrow from SR-BI knockout donors exhibited increased accumulation of apoptotic, TUNEL-positive cells and increased necrotic core sizes in advanced atherosclerotic plaques in the aortic sinus.

Conclusion:

SR-BI mediates protection against FC-induced macrophage apoptosis through a signaling pathway involving PI3K/Akt1 signaling and activation of the MAPK's Erk1/2 and JNK-1 and leading to alterations in pro-and anti-apoptotic factors Bim and Mcl-1.

This may contribute to protection against atherosclerotic plaque macrophage apoptosis and necrotic core formation, features associated with plaque instability.

Supplementary Key Words: Apoptosis; atherosclerosis; HDL; SR-BI; macrophages

2. Condensed abstract

HDL protected against free cholesterol induced macrophage apoptosis in an SR-BI, PI3K/Akt1 and MAPK-dependent manner. The elimination of SR-BI in bone marrow derived cells in apoE KO mice resulted in increased apoptosis and necrotic core formation in advanced atherosclerotic plaques in vivo.

3. Introduction

Macrophage apoptosis is thought to promote the development of advanced atherosclerotic lesions, where apoptotic cell clearance is impaired[1]. This results in the accumulation of apoptotic macrophages, which contribute to necrotic core development [1]. Dead macrophages that persist in advanced plaques release proteases, which contribute to degradation of extracellular matrix, as well as tissue factor, which increases the thrombotic potential of the plaque contents [1, 2]. Plaques with large necrotic cores and thin fibrous caps are vulnerable to rupture, leading to vascular thrombosis [1]. In advanced lesions macrophage apoptosis may be triggered by diverse factors including free cholesterol (FC) loading or exposure to oxysterols [3]. Tabas and co-workers have demonstrated that FC loading of macrophages induces endoplasmic reticulum (ER) stress leading to the upregulation of the pro-apoptotic transcription factor CHOP/GADD153[4, 5]. This appears to prime cells for apoptosis, which is triggered by the engagement of pattern recognition receptors including the scavenger receptor class A (SR-AI/II), toll-like receptors and cluster differentiation molecule 36 (CD36)[6, 7]. The inactivation of CHOP reduces apoptosis and necrotic core sizes in atherosclerotic plaques in mice [5, 8]. Conversely, the activation of apoptotic pathways or the disruption of pro-survival pathways that normally antagonize them result in increased apoptosis in atherosclerotic plaques in animal models, leading to increased necrotic core formation [7, 9-11]. FC loading of macrophages has been reported to activate the mitochondrial apoptosis pathway [12]. B-cell lymphoma-2 (Bcl-2) family proteins play a central role in

this pathway [13]. "BH3-only" Bcl-2 family members, such as Bim (Bcl-2 interacting mediator of cell death), bind to pro-survival family members, such as Bcl-2, Bcl-xL and Mcl-1, allowing the release of pro-apoptotic Bcl-2 family members Bax and Bad which form pores in the mitochondrial membrane in response to caspase 8 mediated cleavage of Bid to t-Bid [13]. This allows for release of cytochrome C and formation of the apoptosome, triggering cell death. A key mechanism of control is the balance of pro-apoptotic BH3 only family members, such as Bim, and pro-survival Bcl-2 family members such as Mcl-1 [13-15].

High density lipoproteins (HDL) protect against atherosclerosis by several mechanisms. The most well-studied is HDL mediated cholesterol efflux from macrophages, the first step in reverse cholesterol transport (RCT). The ATP-binding cassette transporters ABCA1 and ABCG1, mediate phospholipid and cholesterol efflux to apolipoprotein (apo) A-I or mature HDL respectively. The HDL receptor SR-BI has also been implicated in RCT since hepatic SR-BI mediates HDL cholesterol clearance[16-21] . Furthermore, SR-BI overexpression in cultured cells increases FC efflux to bound HDL (reviewed in [22]). On the other hand, elimination of SR-BI from macrophages does not consistently result in impaired cholesterol efflux to HDL [23-26]. Despite this, the elimination of SR-BI in bone marrow (BM) derived cells has been shown in a number of studies to increase atherosclerosis in the aortic sinus and/or descending aortas of mice subjected to BM transplantation [23, 24, 27].

In addition to its role in cholesterol transport, SR-BI can also mediate HDL dependent activation of cell signaling pathways in different cell types [28-35]. This has been

most studied in endothelial cells where SR-BI mediates eNOS activation, cell proliferation and migration, and suppresses inflammatory gene expression via a pathway that is dependent on the SR-BI adaptor protein PDZK1 and engagement of sphingosine 1 phosphate (S1P) receptors [35-38]. HDL has also been shown to suppress apoptosis in different cell types including macrophages, endothelial and smooth muscle cells [28, 39-43]. A recent study demonstrated that HDL protects against oxidized LDL (oxLDL)-induced macrophage apoptosis by mediating 7-ketocholesterol efflux via ABCG1 [44].

The mechanisms by which FC loading induces apoptosis appear to be distinct from those in which oxLDL and 7-ketocholesterol induce apoptosis, although both appear to involve induction of ER stress [12]. In this study, we demonstrate that HDL, via SR-BI, protects against macrophage apoptosis induced by FC-loading in vitro and in atherosclerotic plaques in vivo raising the possibility that activation of cell signaling pathways by SR-BI in macrophages contributes to protection against atherosclerosis.

4. Methods

Mice: Procedures were in accordance with Canadian Council on Animal Care guidelines and approved by the McMaster University Animal Research Ethics Board. Mice were bred and housed in the McMaster University Central Animal Facility and had free access to food and water. Mice were originally from Jackson Labs except for Akt1 KO (originally provided by Morris Birnbaum, University of Pennsylvania), and SR-BI KO (C57BL/6 background) and SR-BI$^{+/-}$apoE$^{-/-}$ (mixed 129 x C57BL6/J background) (originally provided by Monty Krieger, Massachusetts Institute of Technology).

Cell preparation, culture and treatment: Mouse peritoneal macrophages (MPMs) were collected by peritoneal lavage 4 days after mice were injected i.p. with 1 ml of 10 % thioglycollate. Cells were cultured overnight in DMEM + 10 % FBS, 2 mM L-glutamine, and 50 U/ml penicillin/50 μg/ml streptomycin. The next day, 10 % FBS was replaced with 3 % newborn calf lipoprotein deficient serum (NCLPDS)[33] and cells were cultured for 1 day. For analyses of protein phosphorylation, cells were serum starved for 2 hrs prior to the start of the experiment. Cells were treated for 24 hrs (unless indicated otherwise) with either no additions, or with 100 μg/ml AcLDL and 10 μg/ml of the ACAT inhibitor Sandoz 58-035, with or without HDL (100 μg/ml unless otherwise indicated). All lipoproteins were from Biomedical Technologies Inc, Boston MA; other reagents from Sigma St Louis MO. In some experiments cells were pretreated for 30 min with inhibitors of signaling pathways (LY294002 to inhibit PI3K; U0126 to inhibit

MEK and block Erk1/2 activation; CalBiochem, San Diego CA) at the concentrations specified prior to FC-loading and incubation with or without HDL in the continued presence of the inhibitors.

Analysis of apoptosis and cell signaling: Cells or frozen sections were fixed with 2.5 % paraformaldehyde (30 min) and stained using the Apoptag Fluorescein In Situ TUNEL staining kit (Chemicon, Temecula, CA) and DAPI or propidium iodide (PI) counter staining. Alternatively, cells were lysed at different times in the presence of protease and phosphatase inhibitors (Sigma, St. Louis, MO) and SDS-PAGE and immunoblotting were carried out as previously described [33, 45] using anti-GRP78 (anti-KDEL antibody), CHOP/GADD153, phospho-eIF2α, calreticulin, phospho-Akt, phospho-Foxo3a, phospho-Erk1/2 (Cell Signaling Technology, Danvers, MA), Bim, Mcl-1 and actin (MP Biomedicals LLC, Solon, OH) antibodies and horseradish peroxidase conjugated anti-rabbit or mouse secondary antibodies (Jackson Immunoresearch Laboratories Inc, West Grove, PA). Phospho-JNK-1 was detected by immunoprecipitation with rabbit anti-JNK-1 followed by immunoblotting with chicken anti-phospho-JNK and horseradish peroxidase conjugated anti-chicken antibody (Abcam Inc, Boston USA).

Measurement of cellular cholesterol: Cholesterol loaded MPMs were lysed in ice-cold PBS by sonication and centrifuged at 1500 rpm for 5 min to remove cell debris. The supernatant was recovered, protein concentrations were determined by the Bradford

assay (BioRad Laboratories, Hercules CA) and cell homogenates were processed for lipid analyses, as previously described[46, 47].

Bone marrow transplantation (BMT): BM donor SR-BI$^{-/-}$apoE$^{-/-}$ and control donor SR-BI$^{+/+}$apoE$^{-/-}$ mice were littermates generated by breeding SR-BI$^{+/-}$apoE$^{-/-}$ mice. BM recipient mice were 10 week old female C57BL/6 background apoE$^{-/-}$ mice. BMT was as previously described [27, 48] except that recipient mice were lethally irradiated with 11 Gy using a Gammacel 3000 small animal irradiator and received 6 x10^6 donor BM cells i.v. Four weeks after BMT, mice were fed a 20% fat, 0.15 % cholesterol, Western-type diet (Dyets Inc, Bethlehem, PA) for 4 or 12 weeks. Genotyping of BM derived cells was performed 4 weeks after BMT (blood cells) and 4 weeks after Western diet feeding (blood cells and/or BM-derived macrophages) as previously described [16, 27]. Mice were then fasted, euthanized by avertin anesthetic overdose, and blood and tissues were collected for analysis of atherosclerosis as described previously [27, 49]. Frozen sections (10 μm) through the aortic sinus were stained with oil red O (lipid) and hematoxylin (nuclei), or trichrome and analyzed by bright field microscopy. Alternatively sections were stained with picrosirius red (collagen), with DAPI (nuclei), Fluorescein -TUNEL (apoptotic nuclei) or immunostained for macrophages using a rabbit anti-mouse macrophage antibody[50] (CLA31240 Cedarlane Laboratories, Hornby, Ontario, Canada) and an alexa-594 labeled goat anti-rabbit secondary antibody (Invitrogen, Burlington, Ontario, Canada).

Statistical Analysis: Data was analyzed using the Student's *T*-Test (Microsoft Excel) and was considered statistically significant when $P < 0.05$.

5. Results

HDL protects macrophages against FC-induced apoptosis

FC loading coupled with engagement of pattern recognition receptors including SR-AI/II triggers a cascade of events leading to macrophage apoptosis [4, 6](Figure 3.1A). FC loading and SR-AI/II engagement was achieved by incubating MPMs with AcLDL (which is endocytosed by SR-AI/II) and an ACAT inhibitor, 58-035, to prevent re-esterification of AcLDL derived FC [4]. MPMs that were FC-loaded in this way exhibited time dependent increases in TUNEL staining for fragmented DNA but negligible PI staining (Figure 3.1A), confirming that FC loading increases cellular apoptosis but not necrosis as reported previously[4]. HDL suppressed the induction of apopotosis (Figure 3.1A) in a saturable manner, with a half-maximal concentration of 0.8 µg/ml (Figure 3.1B). This suggested the involvement of a high affinity HDL receptor. FC-loading induced but HDL did not suppress the induction of apoptosis in MPMs from mice lacking the HDL receptor, SR-BI (Figure 3.1C,D). Furthermore, an anti-SR-BI antibody that blocks HDL binding [51] also prevented HDL-mediated suppression of apoptosis by FC loading in wild type MPMs (Figure 3.1E).

HDL did not prevent the ability of AcLDL and ACAT inhibition to trigger FC accumulation in either wild type or SR-BI KO MPMs (Figure 3.2A). Furthermore, HDL

did not prevent the FC-loading-mediated upregulation of the ER stress markers GRP-78, phospho-eIF-2α or calreticulin, or the transcription factor CHOP in wild type MPMs (Figure 3.2B). These data demonstrate that HDL suppresses the ability of FC loading to induce apoptosis in MPM via an SR-BI dependent pathway that acts downstream of the induction of ER stress.

HDL triggers the activation of Akt1 and Erk1/2 leading to alterations in BH3 family apoptotic effectors

HDL triggers the activation of diverse cell signaling pathways including PI3K/Akt in different cells such as endothelial, Chinese hamster ovary and MCF-7 mammary tumor cells [28, 32, 33, 52]. We examined the time-dependent effects of HDL on the activation of signaling pathways in MPMs. We examined early events up to 4 hrs after treatment with HDL to avoid potential confounding effects of active cell death. Treatment of macrophages from wild type mice with HDL from 10 min to 4 hrs resulted in a time dependent increase in Akt phosphorylation at Ser473 which peaked at 2 hrs and a corresponding increase in phosphorylation of the transcription factor Foxo3a (Figure 3.3A). Foxo3a upregulates expression of the pro-apoptotic factor Bim, and Akt mediated phosphorylation of Foxo3a prevents its accumulation in the nucleus [53, 54]. Consistent with this, HDL treatment resulted in a time dependent decrease in Bim levels, up to 4 hrs. One of the critical signals triggering apoptosis is the balance of pro- and anti-apoptosis Bcl family members [13, 15]. Bim and Mcl-1 have been shown to be critical for controlling macrophage apoptosis [55]. As Bim levels decreased, Mcl-1 levels

increased slightly upon HDL treatment. Thus HDL treatment altered the balance of Bim to Mcl-1, favoring cell survival.

In addition to transcriptional regulation by Foxo3a, Bim can be phosphorylated by Erk1/2, targeting Bim for degradation [13, 15]. HDL treated MPMs exhibited a transient increase in phospho-Erk1/2 which peaked by 10 min and returned to baseline by 30 min (Figure 3.3A). HDL did not trigger increased phospho-Akt, phospho-Foxo3a or phospho-Erk1/2, or reduced Bim or increased Mcl-1 in MPMs lacking Akt-1 one of 3 isoforms of Akt in mammals (Figure 3.3B). This suggests that HDL signaling via SR-BI in macrophages requires Akt1 and is consistent with the ability of the PI3K/Akt1 pathway to activate the Erk1/2 pathway in certain cell types [56]. We therefore examined the inhibition of PI3K, KO of Akt1 or inhibition of Erk1/2 on HDL's ability to suppress FC-induced apoptosis. Inhibition of PI3K with 10 μM LY294002 or knockout of Akt1 appeared to reduce the extent of FC-induced apoptosis (Figure 3.3D and E compared to C). Nevertheless, HDL treatment did not suppress FC-induced apoptosis in the presence of LY294002 or the absence of Akt1 KO macrophages (Figure 3.3D, E). Treatment of macrophages from WT mice with the Erk1/2 pathway inhibitor U0126, itself, triggered substantial apoptosis, which was not reduced by HDL (Figure 3.3F), consistent with the important protective role exerted by the Erk1/2 pathway. Together these results demonstrate that HDL suppression of FC-loading induced apoptosis involves Akt1 signaling, which mediates suppression of Bim levels through phosphorylation of Foxo3a and Erk1/2 activation.

JNK-1 participates in HDL mediated suppression of apoptosis induced by FC loading:

FC loading also activates the MAP kinase c-jun N-terminal kinase (JNK)2 [4]. JNK1 and 2 play anti- and pro-apoptotic roles in different cells depending on the circumstances of cell activation and the time course and duration of JNK phosphorylation: Short term (1-2 hr) increases in phospho-JNK1 have been associated with activation of anti-apoptotic pathways, whereas long term, sustained activation of phospho-JNK has been associated with activation of pro-apoptotic pathways (reviewed in [57]). We therefore examined the phosphorylation status of JNK-1 in cells treated with AcLDL and 58-035 in the absence or presence of HDL. FC loading did not significantly alter JNK-1 phosphorylation, whereas HDL increased phospho-JNK-1 levels in a time dependent manner, with the greatest increases seen at 60 and 120 min of incubation (Figure 3.4A). JNK's have also been reported to modulate Bim, [57]. We therefore tested the effects of JNK-1 deficiency on levels of Bim, Mcl-1 and FC-loading induced apoptosis in HDL treated macrophages. JNK1 KO macrophages treated with HDL exhibited time-dependent increases in Bim levels, although Mcl-1 levels also appeared to increase (Figure 3.4A,B). Importantly, however, HDL did not reduce the ratio of Bim to Mcl-1 in JNK-1 KO macrophages (Figure 3.4B). HDL treatment was not able to suppress apoptosis induced by FC-loading in JNK-1 KO macrophages (Figure 3.4C,D). This suggested that JNK1 activation in response to HDL also contributed to the ability of HDL to reduce Bim and suppress apoptosis induced by FC loading. A model of HDL mediated protection against FC-loading induced macrophage apoptosis is summarized in (Fig. 3.7).

ApoE KO mice lacking SR-BI in macrophages exhibit increased apoptosis in atherosclerotic plaques after prolonged HF-diet feeding

To test the role of SR-BI in protection against apoptosis in atherosclerotic plaques, we transplanted 10 week old female ApoE KO mice (which lacked significant aortic sinus atherosclerosis (supplementary figure 3.3A)) with bone marrow (BM) from either SR-BI$^{-/-}$apoE$^{-/-}$ or control SR-BI$^{+/+}$apoE$^{-/-}$ donors. Four weeks after BMT, mice were fed an atherogenic Western type diet for 4 or 12 weeks. Genotyping of blood cell and BM derived macrophage DNA verified donor BM engraftment (supplementary figure 3.1). BM specific deletion of SR-BI did not affect fasting lipoprotein total cholesterol profiles for mice fed either normal or Western type diets for 4 or 12 weeks (supplementary figure 3.2). BM specific deletion of SR-BI did not affect the sizes or the collagen content/distribution of atherosclerotic plaques after 12 weeks of Western diet feeding (Figure 3.5). Similar results for plaque sizes were observed after 4 weeks of Western diet feeding (supplementary figure 3.3).

The similarity in the sizes allowed for a direct comparison of the extent of apoptosis and necrotic core sizes in advanced plaques in aortic sinuses of the BM transplanted mice. Apoptotic TUNEL^{+} nuclei were detected in regions that also stained positively for macrophages (Figure 3.6A,B). Greater numbers of apoptotic nuclei were detected in plaques from apoE KO mice transplanted with SR-BI-deficient BM (Figure 3.6A-C). The regions of the plaque that were devoid of nuclei stained strongly for macrophages, suggesting that they may represent necrotic cores containing macrophage debris (Figure 3.6A,B). Similar patterns of macrophage staining have been reported by

others [58] and we have seen similar patterns in unrelated experiments, when staining for other macrophage markers including CD11b (data not shown). The average sizes of the anuclear areas were greater in plaques from mice transplanted with SR-BI$^{-/-}$ApoE$^{-/-}$ BM than those from mice transplanted with SR-BI$^{+/+}$ApoE$^{-/-}$ BM (Figure 3.6D). These data suggest that deficiency of SR-BI in BM-derived cells results in increased plaque apoptosis and increased necrotic core sizes in high fat diet fed apoE KO mice. Similar results were obtained when we analyzed plaques from mice that had been fed the western type diet for only 4 weeks (supplementary figure 3.3E-G).

6. Discussion

FC-induced apoptosis in macrophages involves the mitochondrial apoptosis pathway which is regulated by the balance of pro- and anti-apoptotic Bcl-2 family members [12, 15]. We demonstrate that SR-BI can mediate HDL signaling in macrophages involving activation of Akt1 and JNK-1, reducing levels of Bim, and increasing Mcl-1-to-Bim ratios, tipping the balance of these anti-and pro-apoptotic factors towards protection against FC-induced apoptosis. Consistent with a role for SR-BI in suppression of apoptosis, a deficiency of SR-BI in BM-derived cells results in increased apoptosis and increased necrotic core sizes in atherosclerotic plaques in vivo in experimental mice. HDL has previously been shown to protect macrophages against apoptosis triggered by oxLDL by mediating the ABCG1 dependent efflux of 7-ketocholesterol[44]. However, FC-loading induced apoptosis appears to involve a mechanism distinct from that involved in

oxysterol mediated apoptosis [12]. Despite the well documented ability of HDL to mediate FC efflux, our data demonstrates that HDL did not suppress the FC-loading of macrophages by incubation with AcLDL and inhibition of ACAT. This is most likely because the capacity of AcLDL to deliver cholesterol to macrophages via SRA-dependent endocytosis exceeds the capacity of HDL to efflux cholesterol from cells, resulting in the net accumulation of FC when cells are incubated with both AcLDL and HDL. In contrast, when we pre-incubated macrophages with AcLDL for 24 hrs, and then removed the AcLDL, HDL did accelerate the reduction in cellular cholesterol (data not shown). Consistent with the finding that HDL did not suppress FC loading of macrophages, HDL also did not suppress the induction of ER-stress markers GRP-78, phospho-eIF-2α, calreticulin or CHOP. This suggests that HDL triggered anti-apoptotic responses in MPMs that were downstream of the induction of ER-stress by FC loading. Recently, Aviram and co-workers demonstrated that paraoxanase 1 deficiency was associated with reduced macrophage SR-BI expression in mice, reduced HDL binding to macrophages and a reduced ability of HDL to protect macrophages against apoptosis induced by tunicamycin, which inhibits protein glycosylation and induces ER stress [59]. The mechanisms, however, were not explored. We have also seen a reduced capacity of HDL to protect against tunicamycin-induced apoptosis in SR-BI KO macrophages (data not shown). Our findings demonstrate that HDL treatment induced the phosphorylation of Akt-1, Foxo3a and the MAPK's Erk1/2 and JNK-1 and decreased the levels of the pro-apoptotic factor Bim while increasing anti-apoptotic Mcl-1. Bim is regulated at the transcriptional level by Foxo3a, which becomes sequestered in the cytosol and

inactivated upon phosphorylation by Akt1. The PI3K/Akt pathway has also been reported to activate the Erk1/2 pathway in certain cell types such as endothelial cells [56]. This likely explains our observation that the transient HDL-stimulated Erk1/2 phosphorylation was dependent on Akt1. Erk1/2 can directly phosphorylate Bim targeting it for ubiquitination and proteasomal degradation [14]. HDL treatment also led to increased phospho-JNK-1 and elimination of JNK-1 led to the accumulation of Bim and the inability of HDL to suppress FC-induced apoptosis. Therefore our data suggests that HDL triggers the activation of PI3K/Akt1 signaling which leads to phosphorylation and inactivation of Foxo3a, activation of Erk1/2 and JNK-1, all of which result in reduced levels of Bim and increased Mcl-1 (Fig. 3.7).

Recently, the lack of Akt1 was shown to induce severe atherosclerosis and occlusive coronary artery disease in high fat diet fed apoE KO mice[60], a phenotype reminiscent of the spontaneous occlusive coronary artery atherosclerosis that develops in SR-BI$^{-/-}$apoE $^{-/-}$ mice[61, 62]. The lack of Akt1 was associated with increased apoptosis of macrophages after free cholesterol loading and in lesions from high fat diet fed Akt1/ApoE double KO mice [60]. Conversely, constitutively active Akt1 protects FC-loaded macrophages from apoptosis [3]. Impaired PI3K/Akt signaling was also implicated in increased susceptibility of insulin receptor deficient macrophages to apoptosis as a result of increased Foxo1 activity and expression of IκBε, suppressing the p65/NF-κB mediated induction of a variety of anti-apoptotic factors [9,63].Whether similar effects of PI3K/Akt1 signaling occur downstream of HDL is not known. Minimally oxidized

forms of LDL (mmLDL) are also able to protect macrophages from apoptosis triggered by FC loading or oxLDL, via a pathway that involves PI3K/Akt signaling [64].SR-BI is a multi-ligand receptor. Whether SR-BI, in response to ligands other than HDL, can mediate protection against apoptosis remains to be determined.

Our findings that SR-BI mediated HDL dependent protection against apoptosis in macrophages by a pathway that involved phosphorylation of Akt1 are consistent with reports that SR-BI mediates HDL dependent protection of human MCF7 breast tumor cells against apoptosis [28], but appear to differ from reports that SR-BI promotes apoptosis in the absence of HDL in endothelial cells or Chinese hamster ovary derived cells overexpressing SR-BI [41]. Our results in macrophages, however, did not demonstrate consistently decreased apoptosis in SR-BI KO cells (data not shown) suggesting that SR-BI may influence apoptosis in macrophages and endothelial cells via distinct pathways.

We and others have shown previously that bone marrow specific deletion of SR-BI results in increased atherosclerosis in high fat diet fed LDL receptor KO or normal chow diet fed apoE KO mice [23, 24, 27]. To determine if a lack of SR-BI in macrophages affected apoptosis in atherosclerotic plaques *in vivo* we used BMT to generate apoE KO mice lacking SR-BI only in BM-derived cells, and fed these mice a high fat, Western diet to trigger the development of advanced, complex atherosclerotic plaques. Surprisingly, we saw no differences in the cross sectional areas, or fibrous cap thickness

in atherosclerotic plaques in mice with or without SR-BI in BM-derived cells, after either 4 or 12 weeks of high fat diet feeding. However plaques from apoE KO mice that lacked SR-BI in BM-derived cells exhibited increased abundance of apoptotic nuclei adjacent to larger necrotic cores compared to plaques from mice with SR-BI$^{+/+}$ bone marrow after either 4 or 12 weeks of Western diet feeding. This data is consistent with our *in vitro* findings that SR-BI mediates HDL dependent protection against apoptosis and with reports that Akt1 KO also results in increased apoptosis in atherosclerotic plaques. We cannot rule out the possibility, however, that the increased numbers of apoptotic nuclei in plaques from mice lacking SR-BI in BM-derived cells result at least in part from impaired SR-BI-dependent clearance of apoptotic corpses[10, 65]. Nevertheless, these findings suggest that SR-BI plays a significant role in macrophage apoptosis in plaques and contributes to prevention of necrotic core formation and plaque destabilization at least in part by mediating HDL dependent protection against apoptosis.

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8. References

1. Seimon T, Tabas I. Mechanisms and consequences of macrophage apoptosis in atherosclerosis. *J Lipid Res.* 2009;50 Suppl:S382-387.
2. Libby P, Geng YJ, Aikawa M, Schoenbeck U, Mach F, Clinton SK, Sukhova GK, Lee RT. Macrophages and atherosclerotic plaque stability. *Curr Opin Lipidol.* 1996;7:330-335.
3. Seimon TA, Wang Y, Han S, Senokuchi T, Schrijvers DM, Kuriakose G, Tall AR, Tabas IA. Macrophage deficiency of p38alpha MAPK promotes apoptosis and plaque necrosis in advanced atherosclerotic lesions in mice. *J Clin Invest.* 2009;119:886-898.
4. Devries-Seimon T, Li Y, Yao PM, Stone E, Wang Y, Davis RJ, Flavell R, Tabas I. Cholesterol-induced macrophage apoptosis requires ER stress pathways and engagement of the type A scavenger receptor. *J Cell Biol.* 2005;171:61-73.
5. Thorp E, Li G, Seimon TA, Kuriakose G, Ron D, Tabas I. Reduced apoptosis and plaque necrosis in advanced atherosclerotic lesions of Apoe-/- and Ldlr-/- mice lacking CHOP. *Cell Metab.* 2009;9:474-481.
6. Seimon TA, Obstfeld A, Moore KJ, Golenbock DT, Tabas I. Combinatorial pattern recognition receptor signaling alters the balance of life and death in macrophages. *Proc Natl Acad Sci U S A.* 2006;103:19794-19799.
7. Manning-Tobin JJ, Moore KJ, Seimon TA, Bell SA, Sharuk M, Alvarez-Leite JI, de Winther MP, Tabas I, Freeman MW. Loss of SR-A and CD36 activity reduces

atherosclerotic lesion complexity without abrogating foam cell formation in hyperlipidemic mice. *Arterioscler Thromb Vasc Biol.* 2009;29:19-26.

8. Tsukano H, Gotoh T, Endo M, Miyata K, Tazume H, Kadomatsu T, Yano M, Iwawaki T, Kohno K, Araki K, Mizuta H, Oike Y. The Endoplasmic Reticulum Stress-C/EBP Homologous Protein Pathway-Mediated Apoptosis in Macrophages Contributes to the Instability of Atherosclerotic Plaques. *Arterioscler Thromb Vasc Biol*;30:1925-1932.
9. Han S, Liang CP, DeVries-Seimon T, Ranalletta M, Welch CL, Collins-Fletcher K, Accili D, Tabas I, Tall AR. Macrophage insulin receptor deficiency increases ER stress-induced apoptosis and necrotic core formation in advanced atherosclerotic lesions. *Cell Metab.* 2006;3:257-266.
10. Thorp E, Cui D, Schrijvers DM, Kuriakose G, Tabas I. Mertk receptor mutation reduces efferocytosis efficiency and promotes apoptotic cell accumulation and plaque necrosis in atherosclerotic lesions of apoe-/- mice. *Arterioscler Thromb Vasc Biol.* 2008;28:1421-1428.
11. Thorp E, Li Y, Bao L, Yao PM, Kuriakose G, Rong J, Fisher EA, Tabas I. Brief report: increased apoptosis in advanced atherosclerotic lesions of Apoe-/- mice lacking macrophage Bcl-2. *Arterioscler Thromb Vasc Biol.* 2009;29:169-172.
12. Yao PM, Tabas I. Free cholesterol loading of macrophages is associated with widespread mitochondrial dysfunction and activation of the mitochondrial apoptosis pathway. *J Biol Chem.* 2001;276:42468-42476.

13. Olson M, Kornbluth S. Mitochondria in apoptosis and human disease. *Curr Mol Med.* 2001;1:91-122.

14. Ewings KE, Wiggins CM, Cook SJ. Bim and the pro-survival Bcl-2 proteins: opposites attract, ERK repels. *Cell Cycle.* 2007;6:2236-2240.

15. Huntington ND, Puthalakath H, Gunn P, Naik E, Michalak EM, Smyth MJ, Tabarias H, Degli-Esposti MA, Dewson G, Willis SN, Motoyama N, Huang DC, Nutt SL, Tarlinton DM, Strasser A. Interleukin 15-mediated survival of natural killer cells is determined by interactions among Bim, Noxa and Mcl-1. *Nat Immunol.* 2007;8:856-863.

16. Rigotti A, Trigatti BL, Penman M, Rayburn H, Herz J, Krieger M. A targeted mutation in the murine gene encoding the high density lipoprotein (HDL) receptor scavenger receptor class B type I reveals its key role in HDL metabolism. *Proc Natl Acad Sci U S A.* 1997;94:12610-12615.

17. Temel RE, Trigatti B, DeMattos RB, Azhar S, Krieger M, Williams DL. Scavenger receptor class B, type I (SR-BI) is the major route for the delivery of high density lipoprotein cholesterol to the steroidogenic pathway in cultured mouse adrenocortical cells. *Proc Natl Acad Sci U S A.* 1997;94:13600-13605.

18. Out R, Hoekstra M, Spijkers JA, Kruijt JK, van Eck M, Bos IS, Twisk J, Van Berkel TJ. Scavenger receptor class B type I is solely responsible for the selective uptake of cholesteryl esters from HDL by the liver and the adrenals in mice. *J Lipid Res.* 2004;45:2088-2095.

19. Brundert M, Ewert A, Heeren J, Behrendt B, Ramakrishnan R, Greten H, Merkel M, Rinninger F. Scavenger receptor class B type I mediates the selective uptake of high-density lipoprotein-associated cholesteryl ester by the liver in mice. *Arterioscler Thromb Vasc Biol.* 2005;25:143-148.

20. Hoekstra M, Ye D, Hildebrand RB, Zhao Y, Lammers B, Stitzinger M, Kuiper J, Van Berkel TJ, Van Eck M. Scavenger receptor class B type I-mediated uptake of serum cholesterol is essential for optimal adrenal glucocorticoid production. *J Lipid Res.* 2009;50:1039-1046.

21. Zhang Y, Da Silva JR, Reilly M, Billheimer JT, Rothblat GH, Rader DJ. Hepatic expression of scavenger receptor class B type I (SR-BI) is a positive regulator of macrophage reverse cholesterol transport in vivo. *J Clin Invest.* 2005;115:2870-2874.

22. Yancey PG, Bortnick AE, Kellner-Weibel G, de la Llera-Moya M, Phillips MC, Rothblat GH. Importance of different pathways of cellular cholesterol efflux. *Arterioscler Thromb Vasc Biol.* 2003;23:712-719.

23. Zhang W, Yancey PG, Su YR, Babaev VR, Zhang Y, Fazio S, Linton MF. Inactivation of macrophage scavenger receptor class B type I promotes atherosclerotic lesion development in apolipoprotein E-deficient mice. *Circulation.* 2003;108:2258-2263.

24. Van Eck M, Bos IS, Hildebrand RB, Van Rij BT, Van Berkel TJ. Dual role for scavenger receptor class B, type I on bone marrow-derived cells in atherosclerotic lesion development. *Am J Pathol.* 2004;165:785-794.

25. Yancey PG, Jerome WG, Yu H, Griffin EE, Cox BE, Babaev VR, Fazio S, Linton MF. Severely altered cholesterol homeostasis in macrophages lacking apoE and SR-BI. *J Lipid Res.* 2007;48:1140-1149.

26. Wang X, Collins HL, Ranalletta M, Fuki IV, Billheimer JT, Rothblat GH, Tall AR, Rader DJ. Macrophage ABCA1 and ABCG1, but not SR-BI, promote macrophage reverse cholesterol transport in vivo. *J Clin Invest.* 2007;117:2216-2224.

27. Covey SD, Krieger M, Wang W, Penman M, Trigatti BL. Scavenger receptor class B type I-mediated protection against atherosclerosis in LDL receptor-negative mice involves its expression in bone marrow-derived cells. *Arterioscler Thromb Vasc Biol.* 2003;23:1589-1594.

28. Cao WM, Murao K, Imachi H, Yu X, Abe H, Yamauchi A, Niimi M, Miyauchi A, Wong NC, Ishida T. A mutant high-density lipoprotein receptor inhibits proliferation of human breast cancer cells. *Cancer Res.* 2004;64:1515-1521.

29. Grewal T, de Diego I, Kirchhoff MF, Tebar F, Heeren J, Rinninger F, Enrich C. High density lipoprotein-induced signaling of the MAPK pathway involves scavenger receptor type BI-mediated activation of Ras. *J Biol Chem.* 2003;278:16478-16481.

30. Li XA, Titlow WB, Jackson BA, Giltiay N, Nikolova-Karakashian M, Uittenbogaard A, Smart EJ. High density lipoprotein binding to scavenger receptor, Class B, type I activates endothelial nitric-oxide synthase in a ceramide-dependent manner. *J Biol Chem.* 2002;277:11058-11063.

31. Gong M, Wilson M, Kelly T, Su W, Dressman J, Kincer J, Matveev SV, Guo L, Guerin T, Li XA, Zhu W, Uittenbogaard A, Smart EJ. HDL-associated estradiol stimulates endothelial NO synthase and vasodilation in an SR-BI-dependent manner. *J Clin Invest.* 2003;111:1579-1587.

32. Mineo C, Yuhanna IS, Quon MJ, Shaul PW. High density lipoprotein-induced endothelial nitric-oxide synthase activation is mediated by Akt and MAP kinases. *J Biol Chem.* 2003;278:9142-9149.

33. Zhang Y, Ahmed AM, McFarlane N, Capone C, Boreham DR, Truant R, Igdoura SA, Trigatti BL. Regulation of SR-BI-mediated selective lipid uptake in Chinese hamster ovary-derived cells by protein kinase signaling pathways. *J Lipid Res.* 2007;48:405-416.

34. Zhu P, Liu X, Treml LS, Cancro MP, Freedman BD. Mechanism and Regulatory Function of CpG Signaling via Scavenger Receptor B1 in Primary B Cells. *J Biol Chem.* 2009;284:22878-22887.

35. Kimura T, Tomura H, Mogi C, Kuwabara A, Damirin A, Ishizuka T, Sekiguchi A, Ishiwara M, Im DS, Sato K, Murakami M, Okajima F. Role of scavenger receptor class B type I and sphingosine 1-phosphate receptors in high density lipoprotein-induced inhibition of adhesion molecule expression in endothelial cells. *J Biol Chem.* 2006;281:37457-37467.

36. Assanasen C, Mineo C, Seetharam D, Yuhanna IS, Marcel YL, Connelly MA, Williams DL, de la Llera-Moya M, Shaul PW, Silver DL. Cholesterol binding,

efflux, and a PDZ-interacting domain of scavenger receptor-BI mediate HDL-initiated signaling. *Journal of Clinical Investigation.* 2005;115:969-977.

37. Seetharam D, Mineo C, Gormley AK, Gibson LL, Vongpatanasin W, Chambliss KL, Hahner LD, Cummings ML, Kitchens RL, Marcel YL, Rader DJ, Shaul PW. High-density lipoprotein promotes endothelial cell migration and reendothelialization via scavenger receptor-B type I. *Circ Res.* 2006;98:63-72.
38. Kimura T, Mogi C, Tomura H, Kuwabara A, Im DS, Sato K, Kurose H, Murakami M, Okajima F. Induction of scavenger receptor class B type I is critical for simvastatin enhancement of high-density lipoprotein-induced anti-inflammatory actions in endothelial cells. *J Immunol.* 2008;181:7332-7340.
39. Nofer JR, Junker R, Pulawski E, Fobker M, Levkau B, von Eckardstein A, Seedorf U, Assmann G, Walter M. High density lipoproteins induce cell cycle entry in vascular smooth muscle cells via mitogen activated protein kinase-dependent pathway. *Thromb Haemost.* 2001;85:730-735.
40. Nofer JR, Levkau B, Wolinska I, Junker R, Fobker M, von Eckardstein A, Seedorf U, Assmann G. Suppression of endothelial cell apoptosis by high density lipoproteins (HDL) and HDL-associated lysosphingolipids. *J Biol Chem.* 2001;276:34480-34485.
41. Li XA, Guo L, Dressman JL, Asmis R, Smart EJ. A novel ligand-independent apoptotic pathway induced by scavenger receptor class B, type I and suppressed by endothelial nitric-oxide synthase and high density lipoprotein. *J Biol Chem.* 2005;280:19087-19096.

42. Petoumenos V, Nickenig G, Werner N. High density lipoprotein exerts vasculoprotection via endothelial progenitor cells. *J Cell Mol Med.* 2008.

43. de Souza JA, Vindis C, Negre-Salvayre A, Rye KA, Couturier M, Therond P, Chantepie S, Salvayre R, Chapman MJ, Kontush A. Small, dense HDL3 particles attenuates apoptosis in endothelial cells: Pivotal role of apolipoprotein A-I. *J Cell Mol Med.* 2009.

44. Terasaka N, Wang N, Yvan-Charvet L, Tall AR. High-density lipoprotein protects macrophages from oxidized low-density lipoprotein-induced apoptosis by promoting efflux of 7-ketocholesterol via ABCG1. *Proc Natl Acad Sci U S A.* 2007;104:15093-15098.

45. Zhang Y, Ahmed AM, Tran TL, Lin J, McFarlane N, Boreham DR, Igdoura SA, Truant R, Trigatti BL. The inhibition of endocytosis affects HDL-lipid uptake mediated by the human scavenger receptor class B type I. *Mol Membr Biol.* 2007;24:442-454.

46. Wei E, Alam M, Sun F, Agellon LB, Vance DE, Lehner R. Apolipoprotein B and triacylglycerol secretion in human triacylglycerol hydrolase transgenic mice. *J Lipid Res.* 2007;48:2597-2606.

47. Sahoo D, Trischuk TC, Chan T, Drover VA, Ho S, Chimini G, Agellon LB, Agnihotri R, Francis GA, Lehner R. ABCA1-dependent lipid efflux to apolipoprotein A-I mediates HDL particle formation and decreases VLDL secretion from murine hepatocytes. *J Lipid Res.* 2004;45:1122-1131.

48. Holm TM, Braun A, Trigatti BL, Brugnara C, Sakamoto M, Krieger M, Andrews NC. Failure of red blood cell maturation in mice with defects in the high-density lipoprotein receptor SR-BI. *Blood.* 2002;99:1817-1824.

49. Trigatti B, Rayburn H, Vinals M, Braun A, Miettinen H, Penman M, Hertz M, Schrenzel M, Amigo L, Rigotti A, Krieger M. Influence of the high density lipoprotein receptor SR-BI on reproductive and cardiovascular pathophysiology. *Proc Natl Acad Sci U S A.* 1999;96:9322-9327.

50. Whitman SC, Rateri DL, Szilvassy SJ, Cornicelli JA, Daugherty A. Macrophage-specific expression of class A scavenger receptors in LDL receptor(-/-) mice decreases atherosclerosis and changes spleen morphology. *J Lipid Res.* 2002;43:1201-1208.

51. Gu X, Kozarsky K, Krieger M. Scavenger receptor class B, type I-mediated [3H]cholesterol efflux to high and low density lipoproteins is dependent on lipoprotein binding to the receptor. *J Biol Chem.* 2000;275:29993-30001.

52. Yuhanna IS, Zhu Y, Cox BE, Hahner LD, Osborne-Lawrence S, Lu P, Marcel YL, Anderson RG, Mendelsohn ME, Hobbs HH, Shaul PW. High-density lipoprotein binding to scavenger receptor-BI activates endothelial nitric oxide synthase. *Nat Med.* 2001;7:853-857.

53. Brunet A, Bonni A, Zigmond MJ, Lin MZ, Juo P, Hu LS, Anderson MJ, Arden KC, Blenis J, Greenberg ME. Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. *Cell.* 1999;96:857-868.

54. Dijkers PF, Medema RH, Lammers JW, Koenderman L, Coffer PJ. Expression of the pro-apoptotic Bcl-2 family member Bim is regulated by the forkhead transcription factor FKHR-L1. *Curr Biol.* 2000;10:1201-1204.

55. Steimer DA, Boyd K, Takeuchi O, Fisher JK, Zambetti GP, Opferman JT. Selective roles for antiapoptotic MCL-1 during granulocyte development and macrophage effector function. *Blood.* 2009;113:2805-2815.

56. Harfouche R, Gratton JP, Yancopoulos GD, Noseda M, Karsan A, Hussain SN. Angiopoietin-1 activates both anti- and proapoptotic mitogen-activated protein kinases. *Faseb J.* 2003;17:1523-1525.

57. Dhanasekaran DN, Reddy EP. JNK signaling in apoptosis. *Oncogene.* 2008;27:6245-6251.

58. Tacke F, Alvarez D, Kaplan TJ, Jakubzick C, Spanbroek R, Llodra J, Garin A, Liu J, Mack M, van Rooijen N, Lira SA, Habenicht AJ, Randolph GJ. Monocyte subsets differentially employ CCR2, CCR5, and CX3CR1 to accumulate within atherosclerotic plaques. *J Clin Invest.* 2007;117:185-194.

59. Fuhrman B, Gantman A, Aviram M. Paraoxonase 1 (PON1) deficiency in mice is associated with reduced expression of macrophage SR-BI and consequently the loss of HDL cytoprotection against apoptosis. *Atherosclerosis*;211:61-68.

60. Fernandez-Hernando C, Ackah E, Yu J, Suarez Y, Murata T, Iwakiri Y, Prendergast J, Miao RQ, Birnbaum MJ, Sessa WC. Loss of Akt1 leads to severe atherosclerosis and occlusive coronary artery disease. *Cell Metab.* 2007;6:446-457.

61. Braun A, Trigatti BL, Post MJ, Sato K, Simons M, Edelberg JM, Rosenberg RD, Schrenzel M, Krieger M. Loss of SR-BI expression leads to the early onset of occlusive atherosclerotic coronary artery disease, spontaneous myocardial infarctions, severe cardiac dysfunction, and premature death in apolipoprotein E-deficient mice. *Circ Res.* 2002;90:270-276.

62. Karackattu SL, Trigatti B, Krieger M. Hepatic lipase deficiency delays atherosclerosis, myocardial infarction, and cardiac dysfunction and extends lifespan in SR-BI/apolipoprotein E double knockout mice. *Arterioscler Thromb Vasc Biol.* 2006;26:548-554.

63. Senokuchi T, Liang CP, Seimon TA, Han S, Matsumoto M, Banks AS, Paik JH, DePinho RA, Accili D, Tabas I, Tall AR. Forkhead transcription factors (FoxOs) promote apoptosis of insulin-resistant macrophages during cholesterol-induced endoplasmic reticulum stress. *Diabetes.* 2008;57:2967-2976.

64. Boullier A, Li Y, Quehenberger O, Palinski W, Tabas I, Witztum JL, Miller YI. Minimally oxidized LDL offsets the apoptotic effects of extensively oxidized LDL and free cholesterol in macrophages. *Arterioscler Thromb Vasc Biol.* 2006;26:1169-1176.

65. Murao K, Terpstra V, Green SR, Kondratenko N, Steinberg D, Quehenberger O. Characterization of CLA-1, a human homologue of rodent scavenger receptor BI, as a receptor for high density lipoprotein and apoptotic thymocytes. *J Biol Chem.* 1997;272:17551-17557.

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9. Figure Legends

Figure 3.1: HDL protects against FC-induced apoptosis in an SR-BI dependent manner. Thioglycollate-elicited peritoneal macrophages were loaded with FC by incubation with 100 μg/ml AcLDL plus 10 μg/ml of the ACAT inhibitor 58-035 with or without 100 μg/ml HDL, as indicated. Cells were stained with DAPI for nuclei, TUNEL for apoptotic nuclei or propidium iodide for necrosis, as indicated. **(A)** Time course of induction of apoptosis by FC loading. Apoptosis is expressed as the numbers of TUNEL-positive nuclei relative to the total number of DAPI stained nuclei. **(B)** HDL concentration dependent suppression of apoptosis. **(C)** Wild type and **(D)** SR-BI KO macrophages loaded with FC in the absence or presence of 100 μg/ml HDL for 24 h. Control cells were incubated in parallel without AcLDL, 58-035 or HDL. **(E)** Wild type macrophages were treated with either a non-immune control IgG or with an anti-SR-BI IgG for 30 min prior to, and during FC-loading either in the absence or the presence of 100 μg/ml HDL. Control cells were incubated in parallel. Apoptosis was determined after 24 h. The data represent averages ± standard deviations (SD) of at least three separate determinations. * $P<0.05$; NS, $P>0.05$ between cells treated with or without HDL.

Figure 3.2: HDL does not suppress FC-loading or induction of ER-stress markers. Macrophages from wild type **(A and B)** or SR-BI KO mice **(A)** were FC-loaded in the absence or the presence of 100 μg/ml HDL. Control cells were incubated in parallel

without AcLDL, 58-035 or HDL. **(A)** Cellular free cholesterol (FC) levels in wild type (white bars) or SR-BI KO macrophages (black bars) were measured after 24 h. Data represent the averages ± SD of 6 determinations over two experiments. * $P<0.05$; NS, $P>0.05$. **(B)** The ER stress markers, Grp78, p-eIF2α, calreticulin, and CHOP were examined by immunoblotting in lysates of wild type macrophages that were either not loaded (lane 1) or FC-loaded in the absence (lane 2) or presence of HDL (lane 3) for 24 h as above. Actin was examined as a control for equal loading. Representative images from triplicates are shown.

Figure 3.3: Role of Akt1 and Erk1/2 in HDL mediated suppression of apoptosis.

Macrophages from (**A)** wild type or **(B)** Akt1 KO mice were incubated with 100 μg/ml HDL for the indicated times from 10-240 min. Control cells (time 0) were harvested without HDL addition. Levels of phospho-Akt, phospho-Foxo3a, phospho-Erk1/2, Bim, Mcl-1 and actin (loading control) were determined by immunoblotting. Data are representative of three experiments. **(C-F)** Apoptosis was examined by TUNEL staining in untreated (control) and in FC-loaded cells incubated for 24 h in the absence (white bars) or presence of 100 μg/ml HDL (black bars). **(C)** Wild type MPMs. (D) Wild type MPMs treated with 10 μM LY294002 beginning 30 min prior to FC-loading. **(E)** MPMs from Akt1 KO mice. **(F)** Wild type MPMs were treated with the MEK inhibitor U0126 (10 μM) in the absence (white bars) or presence of 100 μg/ml HDL (black bars) for 24 hrs. Data in **(C-F)** represent averages ± SD of 3 determinations and are representative of at least 2 separate experiments. * $P<0.05$; NS, $P>0.05$.

Figure 3.4: Involvement of JNK-1 in HDL mediated suppression of apoptosis. Peritoneal macrophages from wild type mice **(A and C)** or JNK-1 KO mice **(B and D)** were FC-loaded in the absence or presence of 100 μg/ml HDL. **(A,B)** Time course from 10-120 min of FC-loading in the absence or presence of HDL. Control cells (time 0) were harvested without addition of AcLDL, 58-035 or HDL. **(A)** Phospho-JNK-1 was analyzed by immunoprecipitation with chicken anti-JNK-1 followed by immunoblotting with rabbit anti-phospho-JNK. **(A, B)** Bim, Mcl-1 and actin (loading control) in cell lysates were analyzed by immunoblotting. Data are representative of 2-3 separate experiments. **(C, D)** Apoptosis, measured by TUNEL staining in wild type **(C)** or JNK-1 KO macrophages **(D)** that were FC loaded in the absence (white bars) or presence of 100 μg/ml HDL (black bars) for 24 h. Control cells were untreated. Data represent averages ± SD of 3 determinations and are representative of at least 2 separate experiments. * $P<0.05$; NS, $P>0.05$.

Figure 3.5: Atherosclerosis in apoE KO mice transplanted with either SR-BI$^{-/-}$ apoE$^{-/-}$ or control SR-BI$^{+/+}$apoE$^{-/-}$ bone marrow and fed a Western type diet for 12 weeks. Atherosclerotic plaque sizes in the aortic sinus were determined in frozen sections stained by oil-red O for lipids and hematoxylin for nuclei. **(A, B)** Representative sections are shown. Scale bars=250 μm. C. Average ± SD for plaque cross sectional areas for 8 mice. Plaque cross-sectional areas were not significantly different between groups. Frozen sections from mice transplanted with either SR-

$BI^{+/+}apoE^{-/-}$ **(D, F)** or $SR\text{-}BI^{-/-}apoE^{-/-}$ **(E, G)** BM were stained with Trichrome **(D, E)** for fibrous tissue or pico-sirius red **(F, G)** for collagen. Representative sections are shown.

Figure 3.6: Apoptosis in atherosclerotic plaques from apoE KO mice lacking SR-BI in bone marrow. **(A,B)** Frozen sections were stained by FITC-TUNEL (green) for apoptotic nuclei (also visible is autofluorescence of elastin), DAPI (blue) for all nuclei, or macrophages (red). Representative sections from a mouse transplanted with $SR\text{-}BI^{+/+}apoE^{-/-}$ BM **(A)** or $SR\text{-}BI^{-/-}apoE^{-/-}$ BM **(B)** are shown. Filled arrows point to TUNEL-positive (apoptotic) nuclei. Open arrows indicate anuclear areas (necrotic cores). **(C)** Apoptotic nuclei were counted and divided by the total area of the atherosclerotic plaque for each section. Necrotic cores were determined as the area within the plaque that was free of DAPI staining nuclei. **(D)** Necrotic core areas were divided by the corresponding total plaque areas. Data in C and D are the averages ± SD of 7-8 individuals. * $P<0.05$.

Figure 3.7: HDL mediated suppression of FC-loading induced macrophage apoptosis. HDL protects against FC loading induced macrophage apoptosis by orchestrating proteins levels of pro-apoptotic and anti-apoptotic members of Bcl-2 family. HDL via SR-BI and PDZK1 activates multiple downstream signaling pathways (1-4) that regulate transcriptional and post transcriptional levels of the pro-apoptotic protein Bim. HDL induced activation of PI3K/Akt-1 (1) leads to phosphorylation inactivation of Bim transcription factor, Foxo3a, (2) and phosphorylation activation of

ERK1/2 MAPK, a negative regulator of Bim (3). HDL activates JNK-1 MAPK, independent of PI3K/Akt-1, further reducing Bim protein levels (4). The net effect of HDL stimulated signaling is reduced Bim transcript and protein levels and subsequently reduced Bim : Mcl-1 ratio thus protecting against FC-loading induced macrophage apoptosis.

10. Supplementary Figures' Legends

Supplementary Figure 3.1: SR-BI genotyping of blood cells and BM-derived macrophages harvested from BM-transplanted mice after 4 weeks of HF diet feeding. Lanes 1-3 are control tail-DNA from SR-BI+/+, +/- and -/- mice. Lanes 4 and 5 correspond to blood cell DNA and lanes 6 and 7 to macrophages derived from BM harvested from representative apoE KO mice transplanted with BM from either control SR-BI$^{+/+}$apoE-/-("+/+") or from SR-BI-/-apoE-/- ("-/-") donors. Sizes in base pairs of the wild type and mutant PCR products are indicated on the right.

Supplementary Figure 3.2: Elimination of SR-BI from BM-derived cells does not alter steady state plasma lipoprotein total cholesterol levels in apo E KO mice. Plasma lipoproteins were size-fractionated and total cholesterol in each fraction was assayed as previously described [27]. Representative plasma lipoprotein total cholesterol profiles are shown for mice transplanted with BM from SR-BI$^{-/-}$apoE$^{-/-}$ (closed symbols) or SR-BI$^{+/+}$apoE$^{-/-}$ donors (open symbols). Mice were fed normal chow for 4 weeks post BMT and then either maintained on normal chow for 12 weeks **(A)** or fed a high fat diet for either 4 **(B)** or 12 weeks **(C)**. Human lipoprotein standards are shown for reference.

Supplementary Figure 3.3: Atherosclerosis levels and apoptosis in atherosclerotic plaques in apoE KO mice transplanted with SR-BI$^{-/-}$apoE$^{-/-}$ bone marrow and fed Western type diet for 4 weeks. 10 μm frozen sections through the aortic sinus were stained with oil red O and hematoxylin **(A-C)** or FITC-TUNEL (green-apoptotic nuclei, arrows) and DAPI (blue-all nuclei) **(E,F)**. **(A)** Representative sections from 10 week old mice (age of BM transplantation) showing baseline atherosclerosis levels at the age of transplantation. **(B,E)** ApoE$^{-/-}$ mice that had been transplanted with BM from SR-BI$^{+/+}$ApoE$^{-/-}$ donors or **(C, F)** SR-BI$^{-/-}$ApoE$^{-/-}$ donors were fed the high fat Western type diet for 4 weeks, beginning 4 weeks after BM transplantation. **(D)** Atherosclerotic plaque sizes were determined from oil red O/hematoxylin stained sections using Axiovision software (Carl Zeiss Canada Inc). **(G)** Numbers of apoptotic (TUNEL+) nuclei per μm^2 in plaques. *=P<0.05 by Student's T test. NS=No statistically significant differences comparing SR-BI$^{+/+}$ApoE$^{-/-}$ (n=12) and SR-BI$^{-/-}$ApoE$^{-/-}$ donors (n=9).

CHAPTER THREE

FIGURES

Figure 3.1: HDL protects against FC-induced apoptosis in an SR-BI dependent manner.

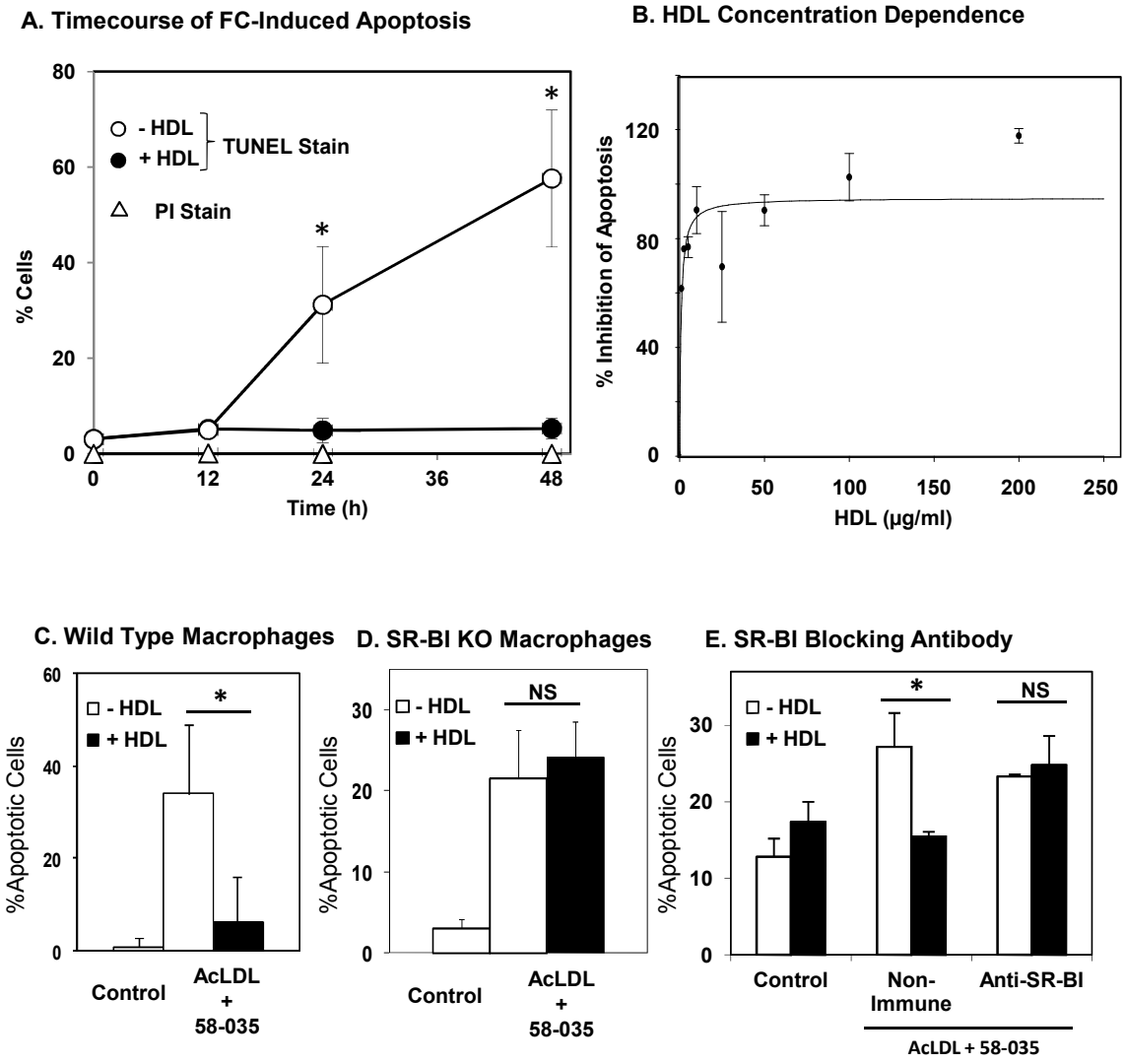


Figure 3.2: HDL does not suppress FC-loading or induction of ER-stress markers.

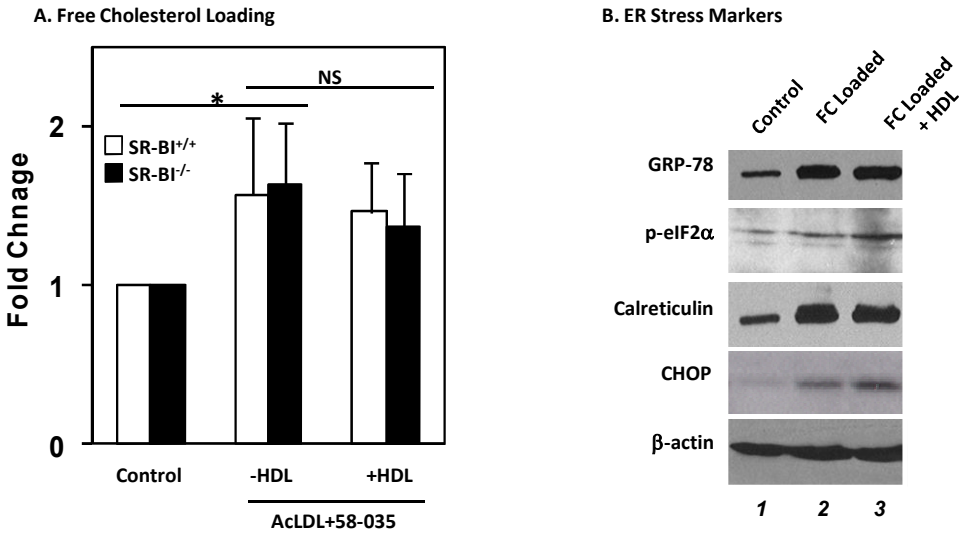


Figure 3.3: Role of Akt1 and Erk1/2 in HDL mediated suppression of apoptosis.

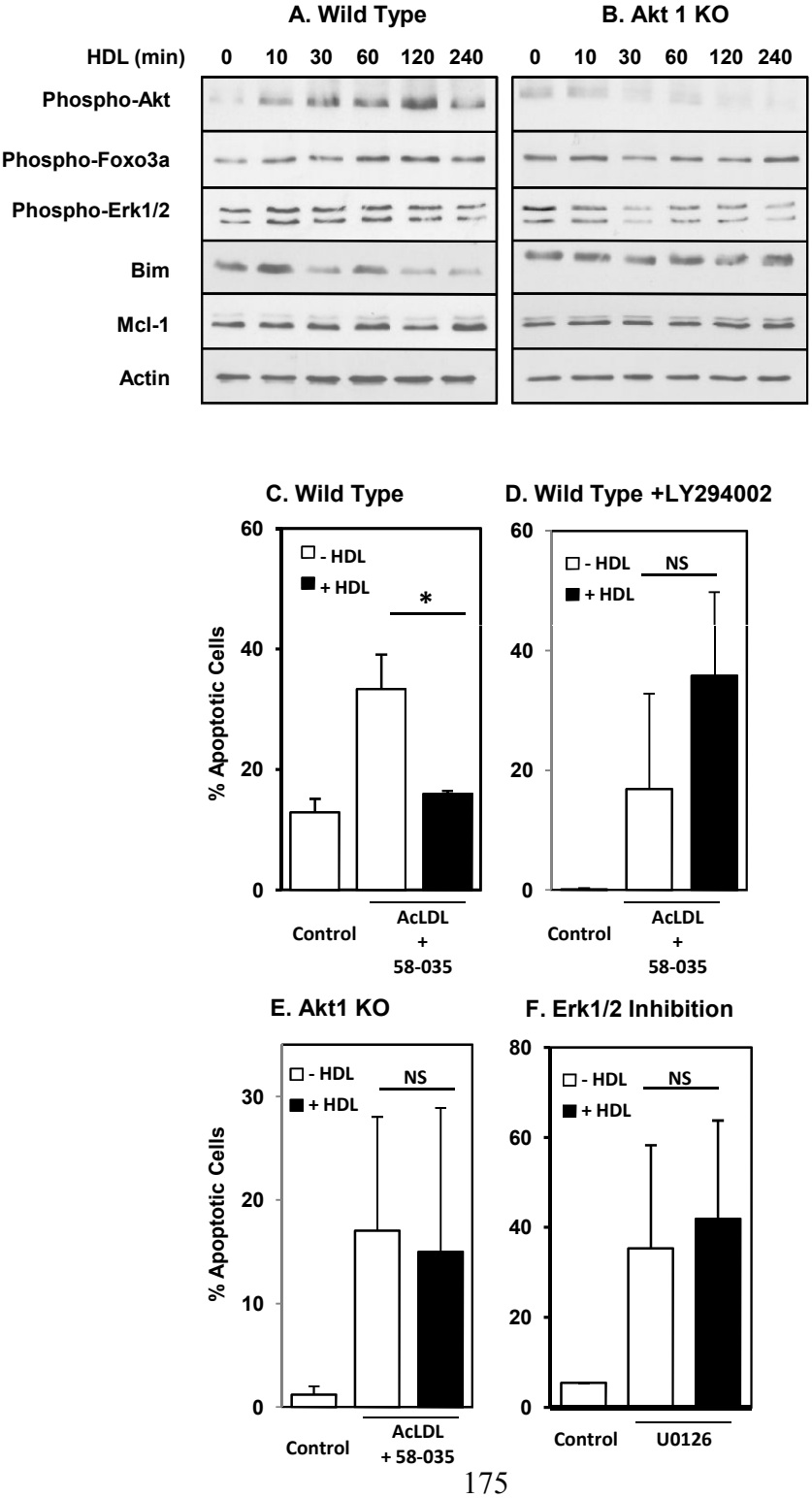


Figure 3.4: of JNK-1 in HDL mediated suppression of apoptosis.

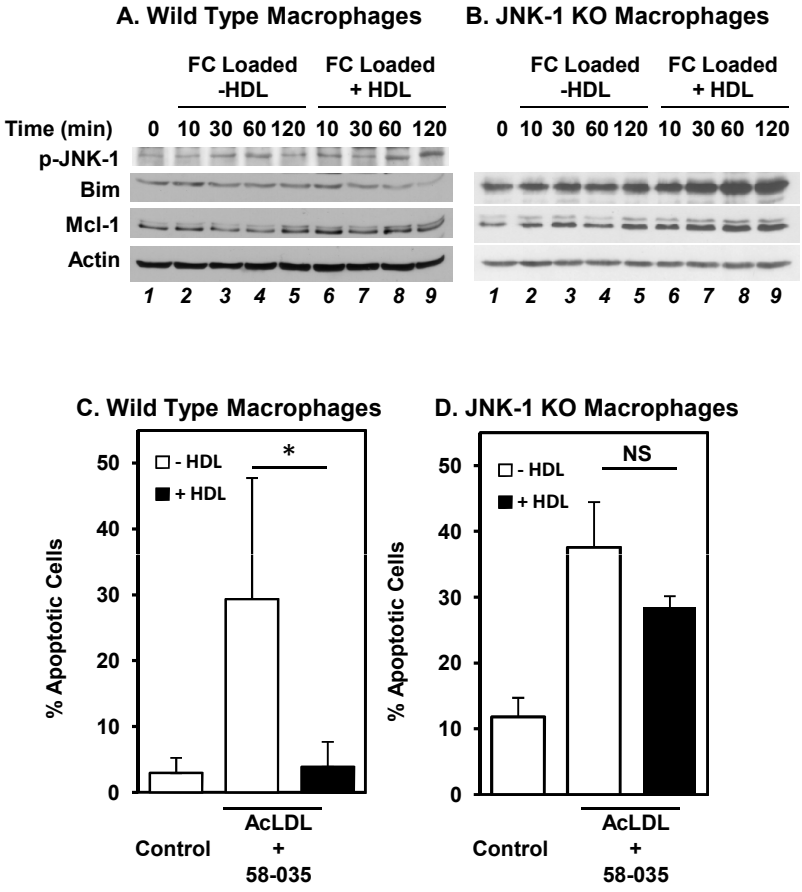


Figure 3.5: Atherosclerosis in apoE KO mice transplanted with either SR-BI$^{-/-}$ apoE$^{-/-}$ or control SR-BI$^{+/+}$apoE$^{-/-}$ bone marrow and fed a Western type diet for 12 weeks.

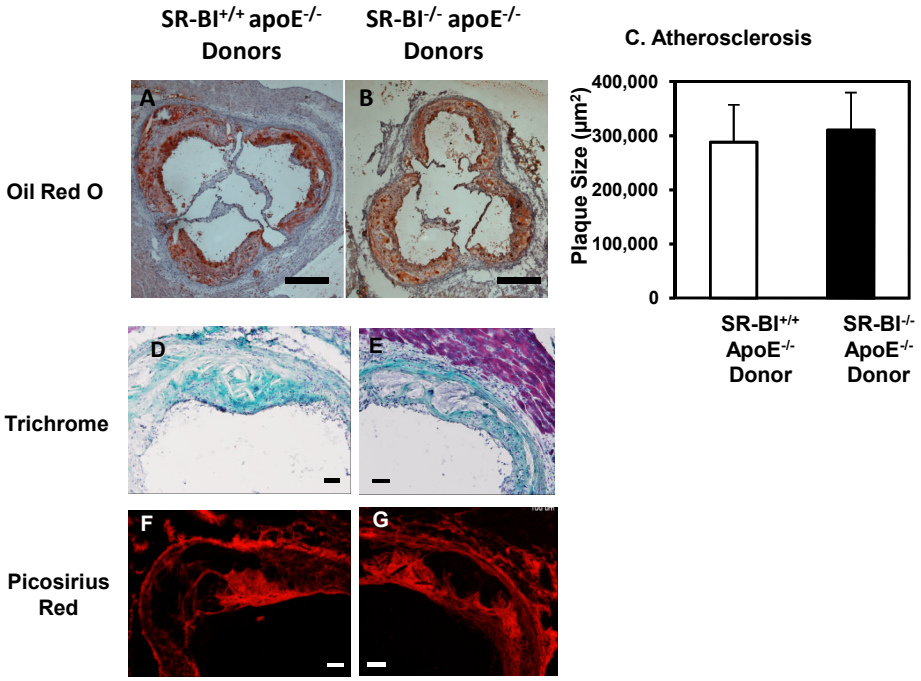


Figure 3.6: Apoptosis in atherosclerotic plaques from apoE KO mice lacking SR-BI in bone marrow.

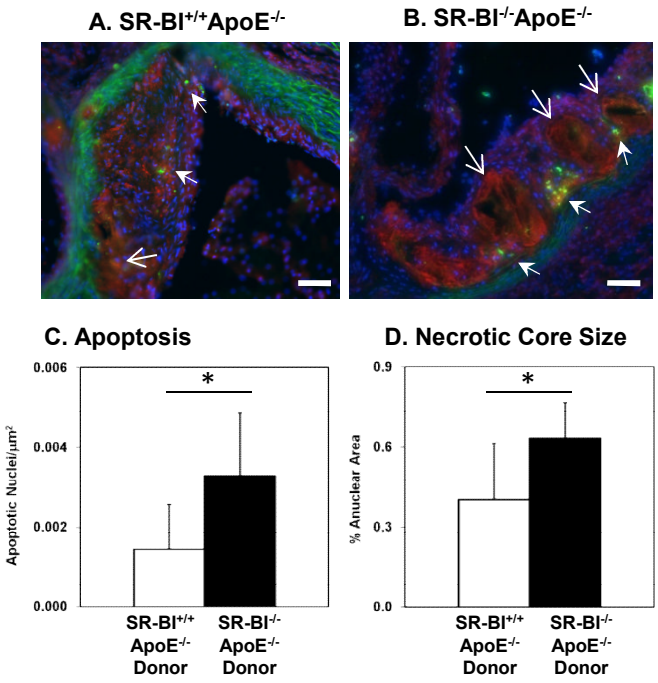
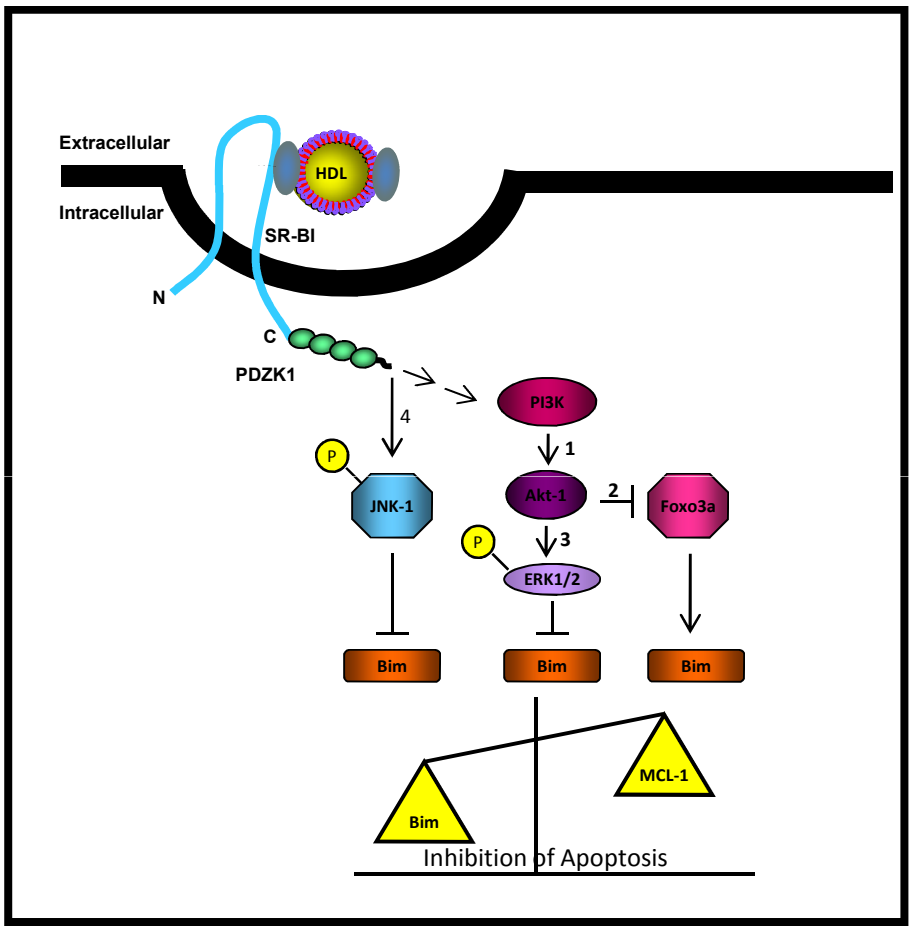


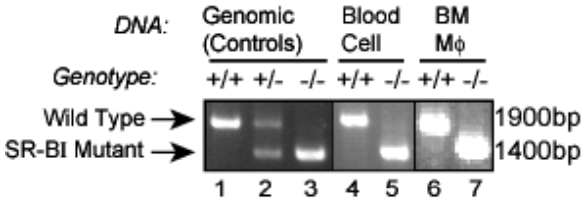
Figure 3.7: HDL mediated suppression of FC-loading induced macrophage apoptosis.



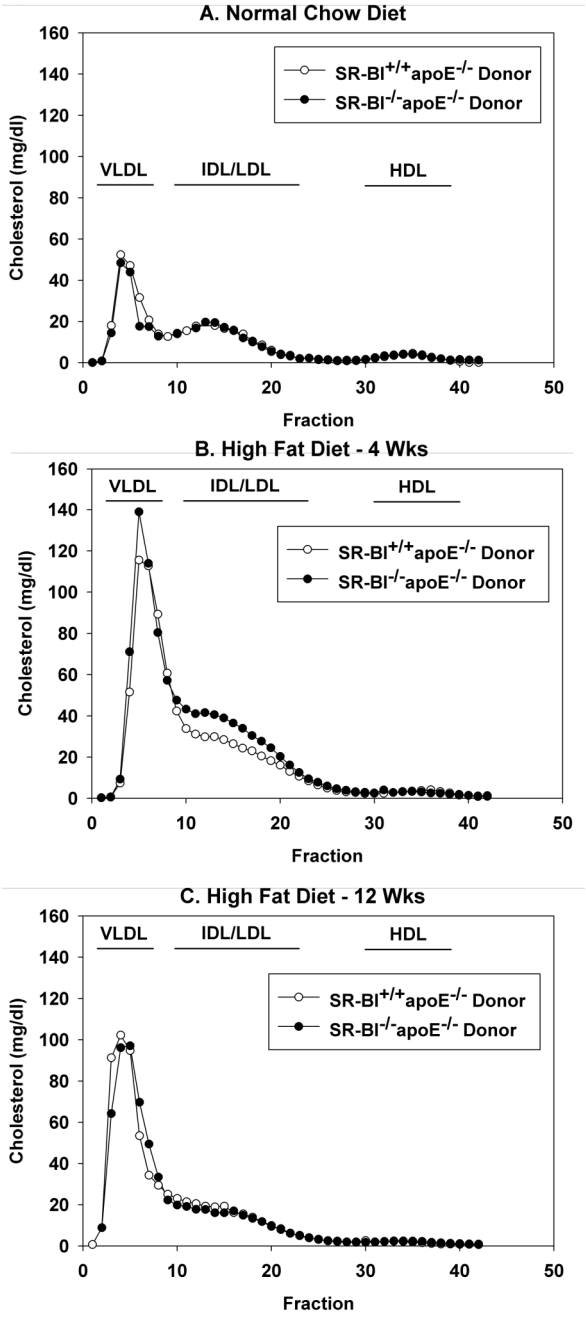
CHAPTER THREE

SUPPLEMENTARY FIGURES

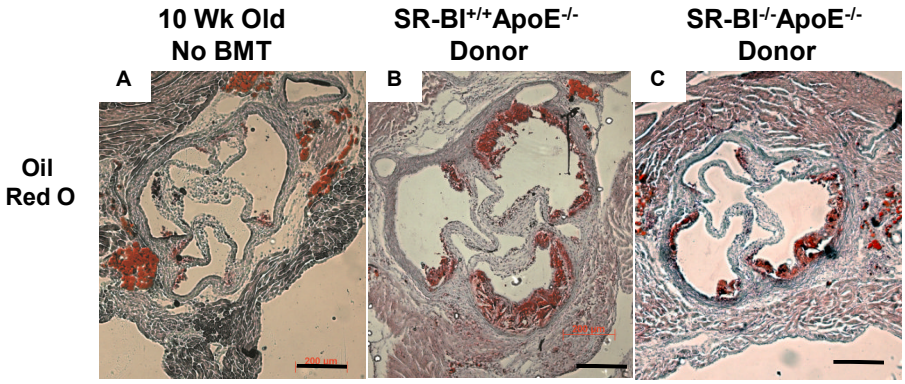
Supplementary Figure 3.1: SR-BI genotyping of blood cells and BM-derived macrophages harvested from BM-transplanted mice after 4 weeks of HF diet feeding.

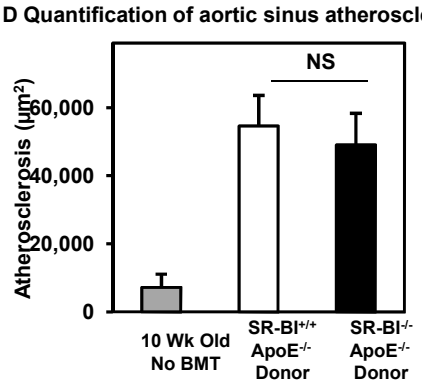


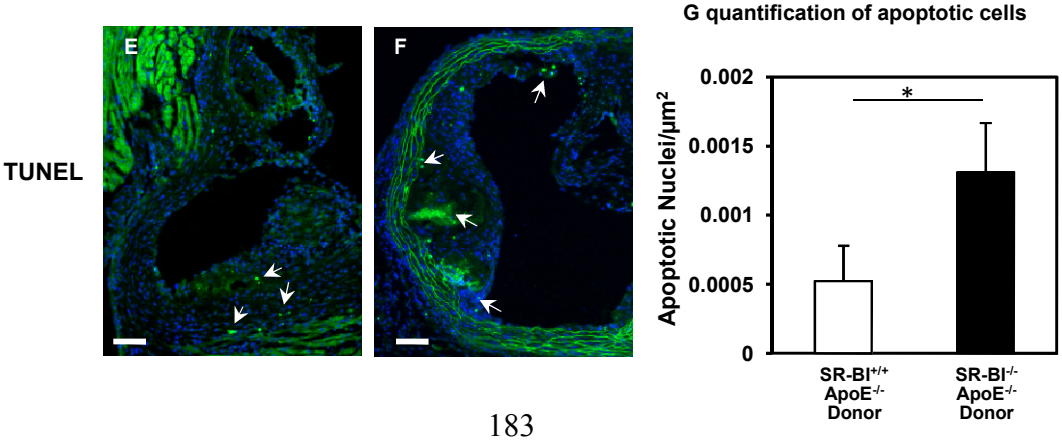
Supplementary Figure 3.2: Elimination of SR-BI from BM-derived cells does not alter steady state plasma lipoprotein total cholesterol levels in apo E KO mice.



Supplementary Figure 3.3: Atherosclerosis levels and apoptosis in atherosclerotic plaques in apoE KO mice transplanted with SR-BI$^{-/-}$apoE$^{-/-}$ bone marrow and fed Western type diet for 4 weeks.







CHAPTER FOUR

HDL INDUCES MACROPHAGE MIGRATION IN SR-BI AND S1P RECEPTOR 1 DEPENDENT MANNER

CHAPTER FOUR PREFACE

This work will be submitted to *Journal of Cell Science*.

I have conducted all the experiments in this chapter with the exception of (Figure 4.1.A) which was generated by Dr.Xing Chen.

HDL INDUCES MACROPHAGE MIGRATION IN SR-BI AND S1P RECEPTOR 1 DEPENDENT MANNER

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1. Summary

We and others have previously demonstrated that HDL stimulates a variety of cell signaling pathways in diverse cell types. HDL carries biologically active lipids such as sphingosine-1-phosphate (S1P) which may contribute to its atheroprotective actions. We set out to determine if HDL and the sphingosine analogue, FTY720 triggered macrophage migration. Treatment of mouse peritoneal, RAW264.7 or human THP-1 derived macrophages with HDL stimulated their migration. HDL stimulated migration appears to be dependent on HDL binding to SR-BI and on SR-BI lipid transfer activity however the response to FTY720 was not. Both HDL and FTY720 stimulated migration were inhibited by treatment of macrophages with a sphingosine 1 phosphate receptor 1 antagonist, or in macrophages from SR-BI KO mice or mice lacking the SR-BI adaptor protein, PDZK1. The reduced response to FTY720 in macrophages lacking SR-BI or PDZK1 appears to be due to reduced S1P receptor 1 protein levels in these cells. S1P receptors are G-protein coupled receptors sensitive to inhibition by pertussis toxin and activate downstream pathways including PI3K-Akt, PKC, p38 MAPK, ERK1/2 and Rho kinases. Using selective inhibitors or macrophages from gene targeted mice, we have demonstrated the involvement of each of these downstream signaling factors in HDL and FTY720 dependent macrophage migration. HDL stimulated migration of macrophages out of the artery wall, may be one mechanism by which HDL may contribute to protection against atherosclerosis.

Key words: Migration, macrophage, HDL, S1P, SR-BI, PDZK1, S1PR1, signaling,

Short title: HDL induced Macrophage Migration

2. Introduction

An inverse relationship between circulating levels of high density lipoprotein (HDL) and coronary heart disease has been reported in numerous clinical and epidemiological studies (Assmann and Schulte, 1992; Assmann et al., 1996; Gordon and Rifkind, 1989). HDL particles as well as HDL associated proteins and lipids were shown to exert a broad scope of potentially anti-atherogenic effects (Florentin et al., 2008; Mineo et al., 2006; Nofer, 2002). These include the ability to mediate reverse cholesterol transport from atherosclerotic plaque resident foam cells to the liver (Assmann and Nofer, 2003; Cuchel and Rader, 2006; Hersberger and von Eckardstein, 2005). HDL also exhibits various anti-inflammatory and anti-oxidative properties (Florentin et al., 2008; Mineo et al., 2006; Nofer, 2002). Short term weekly infusions of reconstituted HDL (rHDL) particles resulted in rapid and significant regression of coronary atherosclerosis in patients with acute coronary syndrome (Nissen et al., 2003). Similar, though more striking, results have been obtained in apolipoprotein E knockout (apoE KO) mice injected with rHDL (Cho, 2009; Cho and Kim, 2009). A recent study of atherosclerotic plaque regression in mice has reported significant alterations in the expression of a variety of genes in inflammatory cells in the regressing plaques, including significantly increased expression of the scavenger receptor class B type I (SR-BI) (Trogan et al., 2006).

SR-BI is a high affinity HDL receptor that mediates selective HDL lipid uptake (Acton et al., 1996). Data from genetically altered mice demonstrates that overexpression of SR-BI in livers protects against atherosclerosis while knockout of SR-BI either in all tissues or in bone marrow derived cells promotes atherosclerosis (Covey et al., 2003; Kozarsky et al., 1997; Trigatti et al., 1999). The interaction of HDL with SR-BI leads to both bi-directional lipid exchange between the bound particle and cells (Acton et al., 1996; Ji et al., 1997) as well as the activation of various signaling pathways (Florentin et al., 2008; Mineo et al., 2006; Norata and Catapano, 2005). HDL induced activation of protein kinase C (PKC) was reported in Chinese hamster ovary (CHO)-derived cells overexpressing SR-BI cells and PKC activity, in turn, appears to increase the selective lipid uptake activity of SR-BI (Brunet et al., 2011; Rentero et al., 2006; Zhang et al., 2007). HDL dependent signaling mediated by SR-BI is also well demonstrated in endothelial cells (ECs), where the interaction of HDL with SR-BI activated various signaling pathways such as the phosphoinositide 3- kinases (PI3K)-Akt, p38 mitogen activated protein kinase (MAPK) and extracellular signal regulated kinases 1/2 (ERK1/2) resulting in induction of endothelial nitric oxide synthase (eNOS) and cell migration but suppression of adhesion molecules expression and apoptosis (Kimura et al., 2006; Mineo et al., 2006). Studies in endothelial cells demonstrated that cholesterol efflux, cholesterol binding to the C-terminal transmembrane domain of SR-BI and the adaptor protein, PDZK1, were all required for HDL-induced signaling (Assanasen, 2005; Mineo et al.,

2006) (recently reviewed in (Al-Jarallah and Trigatti, 2010). In contrast little is known about HDL-SR-BI induced signaling in macrophages.

Postsynaptic Density Protein (PSD-95)/Drosophila Discs-Large (Dlg)/Tight-Junction Protein (ZO1) (PDZK1) is a 519 amino acid, 63 kDa adapter protein that contains four PDZ protein-protein interaction domains (Kocher et al., 1998; Silver, 2002; Yesilaltay et al., 2006). The first and third PDZ domain of PDZK1 interact with the terminal three to four amino acids, EAKL, of SR-BI's C-terminal cytoplasmic tail (Ikemoto et al., 2000; Kocher et al., 2011; Silver, 2002). In liver hepatocytes, PDZK1 is required to stabilize SR-BI protein from degradation, but does not appear to affect its lipid transport function or cell surface localization (Yesilaltay et al., 2006). PDZK1 is also expressed in ECs and macrophages (Kocher et al., 2008; Zhu et al., 2008). In ECs and macrophages, deletion or knockdown of PDZK1 does not affect SR-BI protein levels suggesting that it is not required to stabilize SR-BI protein in these cell types (Kimura et al., 2006; Kocher et al., 2008; Zhu et al., 2008). Instead, knockdown of PDZK1 or deletion of the last three amino acids of SR-BI's C-terminal tail impaired HDL dependent signaling in endothelial cells (Assanasen et al., 2005; Kimura et al., 2006).

Some of the atheroprotective actions of HDL may involve the delivery of bioactive lipids to cells which appears to involve uptake pathways including SR-BI mediated selective uptake (Gong et al., 2003; Li et al., 2002; Okajima et al., 2009). For example, sphingosine 1 phosphate (S1P) and related lysosphingolipids are carried by HDL and exhibit atheroprotective effects on the endothelium (Argraves and Argraves,

2007; Murata et al., 2000; Okajima et al., 2009; Sattler and Levkau, 2009). S1P signaling is mediated in a variety of cell types by a family of five G-protein coupled S1P receptors, S1PR1-5, encoded by distinct genes (Rivera et al., 2008; Takabe et al., 2008). Bone marrow derived and mouse peritoneal macrophages were shown to express S1PR1 and S1PR2 (Hughes et al., 2008) and to a lesser extent S1PR3 (Keul et al., 2011). In macrophages, S1P suppresses proinflammatory cytokine production in response to LPS, apparently through S1PR1 (Hughes et al., 2008). In ECs, S1P increases eNOS activity and abundance and induces cell survival via S1PR1 and in part via S1PR3 (Kimura, 2003; Kimura et al., 2006). S1P also induced ECs migration in an S1PR1 and S1PR3 dependent manner involving Rho kinase (Kimura, 2003; Liu et al., 2001), p38 MAPK (Kimura et al., 2000; Liu et al., 2001) and PI3-Akt-Rac (Morales-Ruiz et al., 2001; Rikitake et al., 2002; Ryu et al., 2002) pathways. ECs migration in response to S1P was accompanied by stimulation of EC proliferation and tube formation (Matsuo et al., 2007). S1P can be supplied exogenously to cells or generated intracellularly (Rivera et al., 2008; Takabe et al., 2008). S1P in the circulation is carried primarily by HDL (Argraves and Argraves, 2007; Murata et al., 2000; Okajima et al., 2009; Sattler and Levkau, 2009). rHDL containing apolipoprotein A-I (apoA-I), phosphatidyl choline and S1P can efficiently delivers S1P to S1P receptors on cells (Frias et al., 2009; Matsuo et al., 2007). For example S1P containing rHDL particles efficiently induced endothelial tube formation in an S1PR2 and S1PR3 dependent manner (Matsuo et al., 2007). This, coupled with SR-BI's ability to mediate selective lipid uptake from HDL into cells

suggests that SR-BI might mediate HDL signaling in part by mediating the transfer of S1P from HDL into cells.

Given the ability of HDL to stimulate the migration of ECs and the role of SR-BI and S1P receptors in that process, and given the importance of macrophage migration for atherosclerotic plaque regression, we have investigated if HDL can stimulate migration of macrophages. We demonstrate that HDL induced macrophage migration in an SR-BI, PDZK1 and S1P receptor dependent manner and it was inhibited by pertussis toxin and inactivation of PI3K-Akt1, p38MAPK, ERK1/2, PKC and Rho kinase pathways. Collectively this data suggests the possibility that SR-BI may mediate HDL dependent macrophage migration by mediating the uptake of S1P from HDL.

3. Results

HDL induces macrophage migration in SR-BI and PDZK1 dependent manner

Cell migration is a highly coordinated process that involves dynamic rearrangement of the actin cytoskeleton and extensive cross talk between signaling molecules at the leading edge of a contracting cell followed by down regulation of adhesion molecules at the trailing end of the cell (Jones, 2000). We aimed to test the effect of HDL on macrophage migration. Lamellar extension is one of the early morphological changes in response to chemotactic stimuli (Jones, 2000). Exposure of

mouse peritoneal macrophages (MPMs) to HDL resulted in rearrangement in the actin cytoskeleton and increased the formation of cellular lamellipodia as revealed by fluorescence microscopic analysis of fixed cells stained with alexa-488 labeled phalloidin (Fig. 4.1A). Similar results were obtained using murine RAW264.7 macrophages (not shown). These alterations in the actin cytoskeleton in response to HDL were not observed in cells lacking the HDL receptor, SR-BI (Fig. 4.1B), suggesting the involvement of SR-BI in HDL stimulated lamellipodia formation. This observation prompted us to test if HDL can stimulate macrophage migration using a transwell migration assay in which cells were added to the top chamber. HDL (100μg/ml, added to the lower chamber) significantly stimulated the migration of wild type (WT) MPMs from the upper to the lower chamber through the collagen I coated filters (Fig. 4.2A). Similar findings were observed with human THP-1 and mouse RAW264.7 macrophage cell lines (data not shown). Cell migration was not observed however, if HDL was added to the upper chamber of the apparatus (data not shown). This indicated that HDL was acting as a chemotactic factor rather than generally increasing cell motility. In contrast HDL did not stimulate the migration of foam cells that were derived from WT MPM's by culturing them for 48 hours in the presence of acetylated LDL (Supplementary Fig. 4.1). This is consistent with previously reported findings that cholesterol loading of macrophages reduced migration in response to complement protein C5a (Nagao et al., 2007). Immunological blockade of SR-BI substantially reduced HDL stimulated migration (Fig. 4.2A). Furthermore the requirement of SR-BI activity was tested using, BLT-1, a small

molecule that does not inhibit HDL binding to SR-BI but blocks SR-BI lipid transfer activity (Nieland et al., 2002) . Treatment of macrophages with BLT-1 also reduced HDL stimulated migration (Fig. 4.2B) suggesting that SR-BI lipid transfer activity is required for HDL stimulated migration. Consistent with this, HDL did not stimulate the migration of macrophages from mice lacking either SR-BI or PDZK1, an adaptor protein that binds to SR-BI C-terminus (Supplementary Fig. 4.2). The inability of HDL to stimulate migration in PDZK1 KO cells was not due to reduced levels of SR-BI in these cells since PDZK1 KO macrophages express similar levels of SR-BI at the cell surface as wild type macrophages (Supplementary Fig. 4.3A). In contrast, HDL did not induce and the lack of SR-BI did not affect macrophage adhesion to collagen I (Supplementary Fig. 4.4 and data not shown). Moreover, immunological blockage, inhibition of SR-BI activity and the lack of SR-BI or PDZK1 did not affect the ability of macrophages to migrate in response to monocyte chemotactic protein-1 (MCP-1) (Figs 4.2A, 2B, S2), suggesting that SR-BI and PDZK1 are specifically required for macrophages to respond to HDL.

HDL stimulated migration is dependent on sphingosine-1-phosphate receptor

Unlike HDL, lipid free apoA-I did not induce macrophage migration (Supplementary Fig. 4.5) suggesting that apoA-I, the major protein constituent of HDL is not sufficient for HDL mediated migration. This together with the finding that SR-BI's lipid transfer activity is required for HDL stimulated migration suggests the possibility

that intact HDL particles and/or lipid constituents of HDL may be required to stimulate macrophage migration. HDL serves as the major carrier of plasma sphingosine 1 phosphate (S1P) (Murata et al., 2000). S1P is known to be a modulator of immune cell migration and appears to mediate the migration of endothelial cells treated with HDL (Argraves and Argraves, 2007; Rivera et al., 2008; Takabe et al., 2008). Therefore we tested the involvement of S1P receptor (s) (S1PRs) activity in HDL dependent macrophage migration. The sphingosine analog FTY720, which is phosphorylated in vivo into FTY720-phosphate (Billich et al., 2003), a broad spectrum S1PR agonist, induced macrophage migration in a manner that was not diminished by anti-SR-BI blocking antibody or by BLT-1 (Fig. 4.2 A,B). S1PRs are pertussis toxin (PTX) sensitive G-protein coupled receptors (GPCR's). We therefore tested the effects of PTX treatment on macrophage migration stimulated by HDL, FTY720, and as a control, MCP-1, which signals via chemokine receptor 2 (CCR2), another PTX sensitive GPCR (Charo et al., 1994). HDL, FTY720 and MCP-1 induced macrophage migration (Fig. 4.3A) without affecting macrophage adhesion to collagen I (Fig. S4.4). Furthermore, treatment of cells with PTX blocked HDL, FTY720 and MCP-1 stimulated macrophage migration (Fig. 4.3A), demonstrating the involvement of $G_{i/o}$-coupled receptors. Macrophages were reported to express S1PR1 and S1PR2 and lower levels of S1PR3 (Keul et al., 2011). S1PR2 was recently shown to serve as a negative modulator of macrophage migration in vivo and in vitro (Michaud et al., 2011). As a result we tested the role of S1PR1 and S1PR3 in HDL induced migration. Treatment of macrophages with VPC23019, an

S1PR1 and S1PR3 selective antagonist (Davis et al., 2005), blocked migration induced by FTY720 and HDL but not by MCP-1 (Fig. 4.3B) suggesting the possible involvement of S1PR1 and/or S1PR3 in HDL and FTY720 stimulated migration. Because S1PR1 exhibits higher affinity to S1P (Anliker and Chun, 2004) and is expressed at higher levels than S1PR3 in macrophages (Keul et al., 2011) we tested its role in HDL induced migration using specific pharmacological reagents. The S1PR1specific agonist, SEW28719 stimulated macrophage migration (Fig. 4.3C). On the other hand migration in response to HDL, FTY720 and SEW28719 was blocked by treatment with the S1PR1 specific antagonist W146 (Fig. 4.3C). However migration stimulated by MCP-1 was not affected (Fig. 4.3C). This data suggests that S1PR1 is required for HDL stimulated migration of macrophages, acting downstream of SR-BI and that SR-BI might participate by mediating sphingosine or S1P uptake from HDL particles and their delivery to S1PR1. This is consistent with the inability of SR-BI blocking antibody or BLT-1, the inhibitor of SR-BI activity, to alter migration in response to FTY720, a direct activator of S1PR1. To our surprise, knockout of either SR-BI or PDZK1 reduced macrophage migration in response to FTY720 (Supplementary Fig. 4.2). This appears to be due to substantially reduced S1PR1 protein levels in macrophages lacking SR-BI or PDZK1 (Supplementary Fig. 4.6). This suggested that the presence of SR-BI and/or PDZK1 is required for normal steady state levels of S1PR1 protein in macrophages. The underlying mechanisms remain to be tested.

HDL stimulated macrophage migration is mediated by multiple downstream kinases

PI3K and protein kinase B/Akt have been implicated in driving initial steps in cell polarization and migration in response to various stimuli (Burgering and Coffer, 1995; Kimura et al., 2003; Manning and Cantley, 2007). To explore the molecular mechanism of HDL-induced macrophage migration, the effects of HDL on the PI3K-Akt pathway were examined. Akt phosphorylation in response to HDL was examined by immunoblotting in RAW264.7 macrophages. HDL (100μg/ml) induced Akt phosphorylation in a time dependent manner over 60 minutes with a peak at 30 minutes (Fig. 4.4A). Moreover HDL, FTY720 and MCP-1 induced migration was inhibited in RAW264.7 cells treated with the PI3K inhibitor LY294002 (Fig. 4.4B). Wortmannin, another inhibitor of PI3K had similar effects (data not shown). Together, this data suggests the involvement of PI3K in HDL, FTY720 and MCP-1-induced macrophage migration.

The Akt family consists of three isoforms: Akt-1, 2 and 3, encoded by different genes, all of which are reportedly expressed in macrophages (Shiratsuchi and Basson, 2007). The involvement of each Akt isoform was tested using MPMs from Akt1, 2, or 3 KO mice. Isoform specific KO macrophages revealed distinct roles of Ak1 and Akt2 in macrophage migration. HDL was unable to induce the migration of Akt1 KO MPMs (Fig. 4.5A). Akt2 KO MPMs exhibited a five-fold increase in migration in the absence of stimulation compared to wild type MPMs (Fig. 4.5A). This was reduced by greater than

50 % by HDL suggesting that Akt2 KO MPMs were still able to respond to HDL (Fig. 4.5A). On the other hand, neither the basal migration nor the migration in response to HDL were affected in Akt3 KO MPMs (Fig. 4.5A) suggesting that Akt3 was not required for either basal or HDL stimulated macrophage migration. The inability of Akt1 KO MPMs to respond to HDL was not due to differences in SR-BI expression levels at the cell surface (Fig. S4.3B). Similarly, Akt2 and Akt3 KO MPMs had normal cell surface levels of SR-BI (data not shown). The knockout of Akt1 also prevented MPM migration in response to FTY720 (Fig. 4.5B), and MCP-1 (Fig. 4.5C). On the other hand FTY720, like HDL, reduced the enhanced basal migration of Akt2 KO macrophages (Fig. 4.5B) whereas MCP-1 had no effect (Fig. 4.5C). Both FTY720 and MCP-1 stimulated migration of Akt3 KO macrophages.

HDL induced activation of ERK1/2 and p38 MAPK has been reported in vascular smooth muscle cells, endothelial cells and in CHO cells overexpressing SR-BI (Baranova et al., 2005; Mineo et al., 2003; Zhang et al., 2007). Therefore we tested the effects of inhibiting these kinase pathways on the ability of macrophages to migrate in response to HDL. Inhibition of the Erk1/2 pathway with PD98059 did not affect basal macrophage migration but prevented macrophage migration in response to HDL, FTY720, and MCP-1 (Supplementary Fig. 4.7A). Similarly, inhibition of p38 MAPK with SB230580 also had no effect on basal macrophage migration but prevented migration in response to HDL, FTY720 and MCP-1 (Supplementary Fig. 4.7B). This is consistent reports that

MAPK pathways are involved in HDL and S1P induced migration of endothelial cells (Kimura et al., 2003).

HDL also induces the activation of PKC in different cell types (Mendez et al., 1991; Rentero et al., 2006; Zhang et al., 2007), and S1P induced cell migration has been shown to require PKC activity (Gorshkova et al., 2008). We therefore tested the involvement of PKC activity using a general PKC inhibitor Ro31-8220 and an inhibitor of Ca^{2+}-dependent PKC isoforms, Go6976 (Martiny-Baron et al., 1993; Nixon et al., 1992). Both of these inhibitors reduced basal migration and blocked the induction of migration of macrophages in response to HDL or FTY720 (Fig. S4.7C), suggesting the involvement of a Ca^{2+}-dependent PKC.

Rho signaling plays an important role in cell migration in response to various stimuli (Jones, 2000; Jones et al., 2000; Ridley, 2004,2001c; Ridley et al., 1999). Cholesterol loading of macrophages has been reported to decrease their migration in response to C5a in part due to inactivation of RhoA (Murao, 2006; Nagao et al., 2007). We therefore examined the role of Rho-kinase signaling in HDL and FTY720 mediated macrophage migration using the Rho-kinase inhibitor, Y-27632 (Supplementary Fig. 4.7D). Inhibition of Rho-kinase did not affect basal migration of macrophages but prevented the induction of migration by HDL, FTY720 or MCP-1, suggesting that migration in response to these stimuli requires active Rho-kinase signaling. A proposed model of HDL stimulated migration is summarized in (Fig. 4.6).

4. Discussion

Macrophages are motile cells and macrophage migration was demonstrated in atherosclerotic plaques undergoing regression (Llodra et al., 2004; Trogan et al., 2006). The reduction of $CD68^+$ cells in regressing plaques was dependent on HDL cholesterol levels despite elevated non-HDL cholesterol levels (Feig et al., 2011). The migration of MPMs to native and modified lipoproteins was previously demonstrated, however the underlying mechanisms were not investigated (Trach et al., 1996). In the present study we show that HDL, but not lipid free apoA-I induced macrophage migration. This finding is consistent with studies that investigated the role of apoA-I in eNOS activation where it was shown that lipid free apoA-I is necessary but not sufficient for eNOS stimulation (Yuhanna et al., 2001). The role of apoA-I in cell migration was investigated in endothelial cells using lipoprotein particles reconstituted with phosphatidylcholine, cholesterol and apoA-I where endothelial cells migration was comparable to HDL induced migration (Seetharam et al., 2006). Moreover, HDL induced macrophage migration in a manner that is dependent on the HDL receptor, SR-BI and its adaptor protein, PDZK1, and sensitive to antagonists of the G-protein coupled receptor, S1PR1. Functional studies on SR-BI using SR-BI blocking antibody and an inhibitor of SR-BI meditated lipid transfer, BLT-1, showed that HDL binding to SR-BI and SR-BI mediated lipid transfer activities are both required for HDL induced migration. SR-BI could be involved in the uptake of HDL associated lipids such as sphingosine and/or S1P and

mediating their delivery to S1PR(s), however the involvement of SR-BI mediated cholesterol efflux cannot be excluded (Fig. 4.6).

Our data suggests that HDL induced macrophage migration involves an S1PR, likely S1PR1. The involvement of S1PR1 in HDL mediated eNOS activation, cell survival, migration and inhibition of adhesion molecules was previously demonstrated in ECs (Kimura et al., 2003; Kimura et al., 2006). SR-BI binding and lipid transfer activities were required for HDL but not FTY720 mediated migration. Surprisingly however the migration in response to FTY720 was blunted in macrophages lacking SR-BI or PDZK1. This appears to be due to reduced protein levels of S1PR1 in cells lacking SR-BI or PDZK1. Mechanisms by which SR-BI and PDZK1 affect S1PR1 protein levels are not yet clear. In addition to effects on S1PR1 protein levels there are several ways in which SR-BI and S1PR might interact functionally. These include: (1) direct physical interaction, (2) mutual transactivation or (3) regulatory interactions. Both SR-BI and S1PR1 were shown to localize in membrane caveolae in different cell types including ECs (Igarashi et al., 2007; Uittenbogaard et al., 2000), thus a direct physical interaction at the receptor level itself or via scaffolding proteins is plausible spatially since SR-BI interacts with the scaffolding protein PDZK1 (Ikemoto et al., 2000) and was shown to mutlimerize and/or dimerize (Sahoo et al., 2007a; Sahoo et al., 2007b). On the other hand, S1PR1 was shown to interact with growth factor receptors including platelets derived growth factor receptor (PDGFR) (Tanimoto et al., 2004) and vascular endothelial growth factor receptor (Bergelin et al., 2010). Alternatively mutual transactivation

between the two receptors could occur when the binding of one receptor to its ligand activates the other. Transactivation of S1PR1 via an Akt dependent pathway was reported in oxidized phospholipid treated ECs which in turn resulted in cortical actin rearrangement and enhancement of ECs barrier function (Singleton et al., 2009). Akt is a signaling molecule downstream of SR-BI (Mineo et al., 2003), thus HDL SR-BI interaction and subsequent activation of Akt may lead to S1PR1 transactivation. Moreover, Erk1/2 is also downstream of SR-BI (Grewal, 2003; Mineo et al., 2003). The phosphorylation of sphingosine kinase1 (SK1) at Ser225 by ERK1/2 induces SK1 activation and plasma membrane translocation which places SK1 in the vicinity of its substrate, sphingosine (Pitson et al., 2003). Thus HDL's interaction with SR-BI could possibly activate S1PR1 via the generation of intracellular S1P. To summarize, the interactions between SR-BI and S1PR1 could possibly occur at multiple levels and may involve one or more of the mechanisms mentioned above. These possibilities however remain to be tested.

Our data suggests that HDL and FTY720 share common downstream signaling pathways leading to macrophage migration which may suggest the involvement of common upstream signal transducers such as S1PR1. We demonstrate that HDL induced migration is dependent on the activation of PI3K-Akt pathway consistent with the requirement of PI3K-Akt in HDL stimulated ECs migration (Seetharam et al., 2006). Moreover we report distinct roles of Akt isoforms in HDL stimulated macrophage migration. Interestingly, basal migration was enhanced ~5 fold in Akt2 KO MPMs. HDL

did not further increase migration, but rather reduced the enhanced basal migration by half, suggesting that Akt2 KO MPMs could still respond to HDL. Akt3 did not play a significant role in migration in response to HDL. On the other hand HDL induced migration was dependent on Akt1. Similar data was observed in FTY720 stimulated migration. The difference in HDL mediated migration in Akt1, 2, and 3 KO macrophage was not due to differences in expression levels of SR-BI as these cells express similar levels of SR-BI at the cell surface compared to wild type macrophages. Akt isoforms seems to play non redundant and sometimes opposing functions in mediating cell migration (Zhou et al., 2006), their role appears to be cell type specific and depends on the chemotactic agent tested (Arboleda et al., 2003; Irie et al., 2005; Yoeli-Lerner et al., 2005). Our data suggests that Akt1 is downstream of HDL and is the key Akt isoform in mediating HDL induced migration. Several studies reported the involvement of Akt1 in the development of atherosclerosis (Fernandez-Hernando et al., 2007; Fernandez-Hernando et al., 2009), macrophage inflammation (Androulidaki et al., 2009) (Fernandez-Hernando et al., 2007), ECs migration (Ackah, 2005) and VSMCs proliferation and protection against apoptosis (Fernandez-Hernando et al., 2009). Overall these studies suggest an atheroprotective function of Akt1. The requirement of Akt1 in HDL induced macrophage migration may suggest that at least some of the atheroprotective actions of HDL are mediated by Akt1, yet this possibility remains to be tested.

In addition to the PI3K-Akt pathway we demonstrated the involvement of p38MAPK and ERK1/2 in HDL mediated macrophage migration. Kimura et al. (2003) showed that p38MAPK was involved in HDL induced ECs migration and lies downstream of PI3K-Rac however; ERK1/2 was activated independently of PI3K and was not involved in ECs migration (Kimura et al., 2003). We also demonstrated the involvement of Ca^{2+}-dependent PKCs in response to HDL, FTY720 and MCP-1. HDL has been shown to increase intracellular calcium concentration in CHO cells (Grewal et al., 2005), human skin fibroblasts (Nofer et al., 2000; Porn et al., 1991), smooth muscle cells (Bochkov et al., 1992) and endothelial cells (Honda et al., 1999; Nofer et al., 2004). Elevation of intracellular calcium upon HDL stimulation would result in inducible targeting of PKC to the plasma membrane which may partially explain the PKC-dependent activation of Raf-1-MEK-ERK1/2 upon HDL treatment of SR-BI expressing CHO cells (Rentero et al., 2006). Pilon et al. reported that SR-BI expression is increased in human adrenocortical cells in response to PKC activators, resulting in higher lipoprotein binding and specific cholesteryl ester uptake utilized for steroidogenesis (Pilon et al., 2003). HDL-dependent activation of PKC, mediated by HDL binding to SR-BI, in transfected CHO-derived cells, may also directly modulate the activity of SR-BI (Zhang et al., 2007). Thus, SR-BI may be a mediator and/or a target of HDL-dependent PKC activation. Small GTPases of the Rho family play a key role in actin cytoskeleton organization and cell migration in response to chemokines and cytokines (Jones, 2000; Jones et al., 2000; Ridley, 2004,2001a,2001b,2001c; Ridley et al., 1999). Blockage of

Rho-kinase reduced HDL stimulated migration suggesting the requirement of this pathway in response to HDL. Similar results were obtained for FTY720 and MCP-1 mediated migration suggesting the importance of the Rho-kinase pathway in macrophage migration in response to these stimuli. Although our data suggests the involvement of different kinases in HDL stimulated macrophage migration the hierarchy and the interaction between multiple downstream signaling pathways remain to be established.

In conclusion we have demonstrated that HDL induces macrophage migration in an SR-BI, PDZK1 and S1PR1 dependent manner. HDL induced macrophage migration involves the PI3K-Akt1, p38MAPK, ERK1/2, PKC and Rho kinase pathways. HDL mediated macrophage migration may be a mechanism by which it protects against atherosclerosis.

5. Materials and Methods

Mice

All mice were on a C57BL6 background, except PDZK1 KO mice, which were on a mixed C57BL/6J X 129S6 background. C57BL6 mice were bred from founders purchased from the Jackson Laboratories. SR-BI KO mice were bred from founders provided by Monty Krieger (Massachusetts Institute of Technology). Akt1, 2 and 3 KO mice were bred from founders provided by Morris Birnbaum (University of Pennsylvania). PDZK1 KO mice were from Jackson Laboratories (Bar Harbor, Maine). All procedures involving mice were approved by McMaster University Animal Research Ethics Board and were in accordance with the guidelines of the Canadian Council on Animal Care. All mice were bred and housed in the Central Animal Facility at McMaster University and provided with free access to food and water.

Cells and cell culture

All reagents used in cell culture were from Invitrogen unless otherwise specified. All cells were cultured at 37 ºC in an atmosphere of 5 % CO_2 in air. RAW264.7 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10 % heat-inactivated fetal bovine serum (FBS), 2mM L-glutamine and 50µg/ml

penicillin/streptomycin and were passaged by scraping. THP-1 cells were cultured in RPMI 1640 (containing 10% FBS, 2mM L-glutamine and 50μg/ml penicillin/streptomycin, and were stimulated to differentiate into macrophages using 8.1 nM phorbol 12-myristate 13-acetate (PMA) (Sigma) for 48 hours (Park et al., 2007). Primary macrophages were isolated from mice injected (day 0) with 1 ml of 10% thioglycolate (Sigma). On day 4 the mice were euthanized and peritoneal cells were collected with 10 ml PBS containing 5mM EDTA. The cells were then washed once in DMEM containing 20 % FBS and plated in DMEM containing 10 % FBS ($8x10^6$ cells/10 cm dish). Foam cells were prepared by incubating MPMs with acetyl-LDL (AcLDL) (100 μg/ml, Biomedical Technologies, Inc.) for 48 hours. Prior to experiments, cells were washed twice with serum free media and cultured for 16 hours either in the absence of serum or in the presence of 3% newborn calf lipoprotein deficient serum (NCLPDS) (Krieger et al., 1981) as indicated.

Macrophage migration and adhesion assays

Macrophage migration was tested in a chemotaxis assay (Nagao et al., 2007; Qin et al., 2006) using Transwell Inserts (Costar). The Transwell migration inserts with pore size of 5μm were pre-coated with rat tail collagen I (110μg/ml) (BD Biosciences) and $4x10^5$ cells were added and incubated for 2 hours at 37 ^{0}C in media containing 5% NCLPDS. The media was then removed and replaced with media containing 0.5%

NCLPDS. These filter inserts were placed in wells containing the same media with either no further additives or one of the following: HDL or apoA-I (100 μg/ml, Biomedical Technologies, Inc.), FTY720 (2ng/ml, Cayman Chemical) or MCP-1 (100 ng/ml, Fitzgerald Industries International Inc.). In migration assays testing the effects of immunological or pharmacological inhibitors, the cells were pre-incubated with the inhibitor for 30 min and the migration assays were performed in the presence of the inhibitors. The following compounds were used: rabbit anti-SR-BI blocking antibody (0.5 μg/ml, generously provided by Karen Kozarsky, ReGenX Biosciences), BTL-1 (0.3 μM, ID 5234221, ChemBridge Corp.), VPC32019, (10 μM, Avanti Polar Lipids, Inc.), PTX (100 ng/ml, Sigma), wortmannin or LY294002 (10 μM, CalBioChem) , SB203580 (1 μM, CalBioChem), PD98059 (10μM, CalBioChem), Go6976 and Ro31-8220 (5 μM, CalBioChem) and Y-27632 (10 μM, Tocris). After 4 hours, cells on the filters were fixed and stained with Quick Stain II (Camco) or with 300 nM DAPI (Sigma) for 10 min and rinsed twice with water. The non-migrated cells on the upper surface of the filters were removed and the cells that had migrated to the lower face of the filter were visualized by bright field or fluorescent microscopy (using a Carl Zeiss Axiovert 200M inverted microscope with a 10X objective) and counted. Cell adhesion to collagen I was tested by plating the same number of cells (as for migration assays) in each well of a 96 well dish pre-coated with rat tail collagen I under the same conditions as those used to coat the Transwell filters. After 4 hrs non-adhered cells were washed away and adherent cells

were fixed, stained as above with DAPI, and cell adhesion was measured by counting DAPI stained nuclei.

Phalloidin staining

To visualize F-actin, RAW264.7 macrophages and MPMs were cultured on sterile, untreated glass coverslips, as above, in the presence or absence of HDL (100 μg/ml) for different times. The cells were then washed twice with PBS, fixed with 3 % paraformaldehyde pH 7.4 for 30 min. and permeabilized with 0.1 % Triton-X100 for 5 min. at room temprature. F-actin was stained using 25 units/ml AlexaFlour 488 phalliodin (Molecular Probes, Invitrogen) in PBS for 30 minutes at RT. The coverslips were then mounted (Vectasheild, Vector Laboratories) and imaged using a Zeiss Axiovert 200M fluorescence microscope with standard FITC filters using a 40x objective.

SDS-PAGE and immunoblotting

RAW264.7 cells were serum starved overnight and incubated with HDL (100 μg/ml) for different times, washed and lysed in ice cold lysis buffer containing: 0.2x PBS, 0.1 % Triton-X100, 1x phosphatase inhibitor mixture and protease inhibitors (20 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM APMSF and 10 μg/ml pepstatinA) (Sigma). To prepare total membranes, MPMs were homogenized on ice for 1 minute in 20 mM

Tris-HCl, pH 7.5 containing 2 mM $MgCl_2$, 0.25 M sucrose, and protease inhibitors with the concentrations indicated above. Homogenates were centrifuged at 3000xg for 10 minutes at 4 ^{o}C and the supernatant was subjected to another centrifugation step at 100,000 x g for 1 hour at 4 ^{o}C. The pellet was then suspended in 10 mM sodium phosphate, pH 7.0 containing the protease inhibitors listed above. After boiling, the samples were subjected to SDS-PAGE followed by immunoblotting with rabbit anti-Akt, rabbit anti-phospho-Akt (Ser473) (Cell signaling Technology, Inc), rabbit anti-S1PR1 (Cayman Chemical), rabbit anti-SR-BI (Novus Biologicals) or mouse anti-actin (MP Biomedicals). HRP-conjugated donkey-anti-rabbit and donkey-anti-mouse antibodies (Jackson ImmunoResearch) were used as secondary antibodies and were detected using Western lightning ECL reagent kit (Perkin Elmer).

Flow cytomery

SR-BI surface expression was measured in unfixed non-permeabilized MPMs. Macrophages were treated with rat anti-CD16/32 (eBiosciences) to block FC receptors then incubated with rabbit anti-SR-BI blocking antibody (KK-1B, 0.5µg/ml) followed by anti-rabbit FITC conjugated antibody. Cell sorting was performed using BD FACSCalibur instrument (BD Biosciences) and data was analyzed using CellQuest Pro software (Nieland et al., 2002).

Statistical analysis

Data was analyzed using the Student's *T*-Test (Microsoft Excel) and was considered statistically significant when $P<0.05$.

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7. References

Ackah, E., Yu J, Zoellner S, Iwakiri Y, Skurk C, Shibata R, Ouchi N, Easton RM, Galasso G, Birnbaum MJ, Walsh K, Sessa WC. (2005). Akt1/protein kinase Balpha is critical for ischemic and VEGF-mediated angiogenesis. *J Clin Invest.* **115**, 2119-27.

Acton, S., Rigotti, A., Landschulz, K.T., Xu, S., Hobbs, H.H. and Krieger, M. (1996).Identification of scavenger receptor SR-BI as a high density lipoprotein receptor. *Science***. 271**, 518-20.

Al-Jarallah, A. and Trigatti, B.L. (2010).A role for the scavenger receptor, class B type I in high density lipoprotein dependent activation of cellular signaling pathways. *Biochim Biophys Acta***. 1801**, 1239-48.

Androulidaki, A., Iliopoulos, D., Arranz, A., Doxaki, C., Schworer, S., Zacharioudaki, V., Margioris, A.N., Tsichlis, P.N. and Tsatsanis, C. (2009).The kinase Akt1 controls macrophage response to lipopolysaccharide by regulating microRNAs. *Immunity***. 31**, 220-31.

Anliker, B. and Chun, J. (2004).Lysophospholipid G protein-coupled receptors. *J Biol Chem***. 279**, 20555-8.

Arboleda, M.J., Lyons, J.F., Kabbinavar, F.F., Bray, M.R., Snow, B.E., Ayala, R., Danino, M., Karlan, B.Y. and Slamon, D.J. (2003).Overexpression of AKT2/protein kinase Bbeta leads to up-regulation of beta1 integrins, increased

invasion, and metastasis of human breast and ovarian cancer cells. *Cancer Res*. **63**, 196-206.

Argraves, K.M. and Argraves, W.S. (2007).HDL serves as an S1P signaling platform mediating a multitude of cardiovascular effects. *J Lipid Res*. **48**, 2325-2333.

Assanasen, C., Mineo, C., Seetharam, D., Yuhanna, I.S., Marcel, Y.L., Connelly, M.A., Williams, D.L., de la Llera-Moya, M., Shaul, P.W. and Silver, D.L. (2005).Cholesterol binding, efflux, and a PDZ-interacting domain of scavenger receptor-BI mediate HDL-initiated signaling. *J Clin Invest*. **115**, 969-77.

Assanasen, C., Mineo, C., Seetharam, D., Yuhanna, IS., Marcel, YL., Connelly, MA., et al. (2005).Cholesterol binding, efflux, and a PDZ-interacting domain of scavenger receptor-BI mediate HDL-initiated signaling. . *J. Clin. Invest.* **115**, 969-77.

Assmann, G. and Nofer, J.R. (2003).Atheroprotective effects of high-density lipoproteins. *Annu Rev Med*. **54**, 321-41.

Assmann, G. and Schulte, H. (1992).Relation of high-density lipoprotein cholesterol and triglycerides to incidence of atherosclerotic coronary artery disease (the PROCAM experience). Prospective Cardiovascular Munster study. *Am J Cardiol*. **70**, 733-7.

Assmann, G., Schulte, H., von Eckardstein, A. and Huang, Y. (1996).High-density lipoprotein cholesterol as a predictor of coronary heart disease risk. The

PROCAM experience and pathophysiological implications for reverse cholesterol transport. *Atherosclerosis*. **124 Suppl**, S11-20.

Baranova, I.N., Vishnyakova, T.G., Bocharov, A.V., Kurlander, R., Chen, Z., Kimelman, M.L., Remaley, A.T., Csako, G., Thomas, F., Eggerman, T.L., et al. (2005).Serum amyloid A binding to CLA-1 (CD36 and LIMPII analogous-1) mediates serum amyloid A protein-induced activation of ERK1/2 and p38 mitogen-activated protein kinases. *J Biol Chem*. **280**, 8031-40.

Bergelin, N., Lof, C., Balthasar, S., Kalhori, V. and Tornquist, K. (2010).S1P1 and VEGFR-2 form a signaling complex with extracellularly regulated kinase 1/2 and protein kinase C-alpha regulating ML-1 thyroid carcinoma cell migration. *Endocrinology*. **151**, 2994-3005.

Billich, A., Bornancin, F., Devay, P., Mechtcheriakova, D., Urtz, N. and Baumruker, T. (2003).Phosphorylation of the immunomodulatory drug FTY720 by sphingosine kinases. *J Biol Chem*. **278**, 47408-15.

Bochkov, V., Tkachuk, V., Buhler, F. and Resink, T. (1992).Phosphoinositide and calcium signalling responses in smooth muscle cells: comparison between lipoproteins, Ang II, and PDGF. *Biochem Biophys Res Commun*. **188**, 1295-304.

Brunet, R., How, M. and Trigatti, B.L. (2011).Modulators of Protein Kinase C Affect SR-BI-Dependent HDL Lipid Uptake in Transfected HepG2 Cells. *Cholesterol*. **2011**, 687939.

Burgering, B.M. and Coffer, P.J. (1995).Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction. *Nature***. 376**, 599-602.

Charo, I.F., Myers, S.J., Herman, A., Franci, C., Connolly, A.J. and Coughlin, S.R. (1994).Molecular cloning and functional expression of two monocyte chemoattractant protein 1 receptors reveals alternative splicing of the carboxyl-terminal tails. *Proc Natl Acad Sci U S A***. 91**, 2752-6.

Cho, K.H. (2009).A reconstituted high density lipoprotein containing the V156E mutant of apolipoprotein A-I exhibits anti-atherosclerotic activity in Apo-E deficient mice. *J Atheroscler Thromb***. 16**, 217-29.

Cho, K.H. and Kim, J.R. (2009).A reconstituted HDL containing V156K or R173C apoA-I exhibited anti-inflammatory activity in apo-E deficient mice and showed resistance to myeloperoxidase-mediated oxidation. *Exp Mol Med***. 41**, 417-28.

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

Cuchel, M. and Rader, D.J. (2006).Macrophage reverse cholesterol transport: key to the regression of atherosclerosis? *Circulation***. 113**, 2548-55.

Davis, M.D., Clemens, J.J., Macdonald, T.L. and Lynch, K.R. (2005).Sphingosine 1-phosphate analogs as receptor antagonists. *J Biol Chem***. 280**, 9833-41.

Feig, J.E., Rong, J.X., Shamir, R., Sanson, M., Vengrenyuk, Y., Liu, J., Rayner, K., Moore, K., Garabedian, M. and Fisher, E.A. (2011).HDL promotes rapid atherosclerosis regression in mice and alters inflammatory properties of plaque monocyte-derived cells. *Proc Natl Acad Sci U S A***. 108**, 7166-71.

Fernandez-Hernando, C., Ackah, E., Yu, J., Suarez, Y., Murata, T., Iwakiri, Y., Prendergast, J., Miao, R.Q., Birnbaum, M.J. and Sessa, W.C. (2007).Loss of Akt1 leads to severe atherosclerosis and occlusive coronary artery disease. *Cell Metab***. 6**, 446-57.

Fernandez-Hernando, C., Jozsef, L., Jenkins, D., Di Lorenzo, A. and Sessa, W.C. (2009).Absence of Akt1 reduces vascular smooth muscle cell migration and survival and induces features of plaque vulnerability and cardiac dysfunction during atherosclerosis. *Arterioscler Thromb Vasc Biol***. 29**, 2033-40.

Florentin, M., Liberopoulos, E.N., Wierzbicki, A.S. and Mikhailidis, D.P. (2008).Multiple actions of high-density lipoprotein. *Curr Opin Cardiol***. 23**, 370-8.

Frias, M.A., James, R.W., Gerber-Wicht, C. and Lang, U. (2009).Native and reconstituted HDL activate Stat3 in ventricular cardiomyocytes via ERK1/2: role of sphingosine-1-phosphate. *Cardiovasc Res***. 82**, 313-23.

Gong, M., Wilson, M., Kelly, T., Su, W., Dressman, J., Kincer, J., Matveev, S.V., Guo, L., Guerin, T., Li, X.A., et al. (2003).HDL-associated estradiol stimulates

endothelial NO synthase and vasodilation in an SR-BI-dependent manner. *J Clin Invest***. 111**, 1579-87.

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

Gorshkova, I., He, D., Berdyshev, E., Usatuyk, P., Burns, M., Kalari, S., Zhao, Y., Pendyala, S., Garcia, J.G., Pyne, N.J., et al. (2008).Protein kinase C-epsilon regulates sphingosine 1-phosphate-mediated migration of human lung endothelial cells through activation of phospholipase D2, protein kinase C-zeta, and Rac1. *J Biol Chem***. 283**, 11794-806.

Grewal, T., de Diego, I., Kirchhoff, MF., Tebar, F., Heeren, J., Rinninger, F., et al. (2003).High density lipoprotein-induced signaling of the MAPK pathway involves scavenger receptor type BI-mediated activation of Ras. *J. Biol. Chem* . **278**, 16478-81.

Grewal, T., Evans, R., Rentero, C., Tebar, F., Cubells, L., de Diego, I., Kirchhoff, M.F., Hughes, W.E., Heeren, J., Rye, K.A., et al. (2005).Annexin A6 stimulates the membrane recruitment of p120GAP to modulate Ras and Raf-1 activity. *Oncogene***. 24**, 5809-20.

Hersberger, M. and von Eckardstein, A. (2005).Modulation of high-density lipoprotein cholesterol metabolism and reverse cholesterol transport. *Handb Exp Pharmacol***.** 537-61.

Honda, H.M., Wakamatsu, B.K., Goldhaber, J.I., Berliner, J.A., Navab, M. and Weiss, J.N. (1999).High-density lipoprotein increases intracellular calcium levels by releasing calcium from internal stores in human endothelial cells. *Atherosclerosis***. 143**, 299-306.

Hughes, J.E., Srinivasan, S., Lynch, K.R., Proia, R.L., Ferdek, P. and Hedrick, C.C. (2008).Sphingosine-1-phosphate induces an antiinflammatory phenotype in macrophages. *Circ Res***. 102**, 950-8.

Igarashi, J., Miyoshi, M., Hashimoto, T., Kubota, Y. and Kosaka, H. (2007).Hydrogen peroxide induces S1P1 receptors and sensitizes vascular endothelial cells to sphingosine 1-phosphate, a platelet-derived lipid mediator. *Am J Physiol Cell Physiol***. 292**, C740-8.

Ikemoto, M., Arai, H., Feng, D., Tanaka, K., Aoki, J., Dohmae, N., Takio, K., Adachi, H., Tsujimoto, M. and Inoue, K. (2000).Identification of a PDZ-domain-containing protein that interacts with the scavenger receptor class B type I. *Proc Natl Acad Sci U S A***. 97**, 6538-43.

Irie, H.Y., Pearline, R.V., Grueneberg, D., Hsia, M., Ravichandran, P., Kothari, N., Natesan, S. and Brugge, J.S. (2005).Distinct roles of Akt1 and Akt2 in regulating cell migration and epithelial-mesenchymal transition. *J Cell Biol***. 171**, 1023-34.

Ji, Y., Jian, B., Wang, N., Sun, Y., Moya, M.L., Phillips, M.C., Rothblat, G.H., Swaney, J.B. and Tall, A.R. (1997).Scavenger receptor BI promotes high density lipoprotein-mediated cellular cholesterol efflux. *J Biol Chem***. 272**, 20982-5.

Jones, G.E. (2000).Cellular signaling in macrophage migration and chemotaxis. *J Leukoc Biol***. 68**, 593-602.

Jones, G.E., Ridley, A.J. and Zicha, D. (2000).Rho GTPases and cell migration: measurement of macrophage chemotaxis. *Methods Enzymol***. 325**, 449-62.

Keul, P., Lucke, S., von Wnuck Lipinski, K., Bode, C., Graler, M., Heusch, G. and Levkau, B. (2011).Sphingosine-1-phosphate receptor 3 promotes recruitment of monocyte/macrophages in inflammation and atherosclerosis. *Circ Res***. 108**, 314-23.

Kimura, T., Sato, K., Malchinkhuu, E., Tomura, H., Tamama, K., Kuwabara, A., Murakami, M. and Okajima, F. (2003).High-density lipoprotein stimulates endothelial cell migration and survival through sphingosine 1-phosphate and its receptors. *Arterioscler Thromb Vasc Biol***. 23**, 1283-8.

Kimura, T., Sato K, Malchinkhuu E, Tomura H, Tamama K, Kuwabara A, Murakami M, Okajima F. (2003).High-density lipoprotein stimulates endothelial cell migration and survival through sphingosine 1-phosphate and its receptors. *Arterioscler Thromb Vasc Biol.,***. 23**, 1283-8.

Kimura, T., Tomura, H., Mogi, C., Kuwabara, A., Damirin, A., Ishizuka, T., Sekiguchi, A., Ishiwara, M., Im, D.S., Sato, K., et al. (2006).Role of scavenger

receptor class B type I and sphingosine 1-phosphate receptors in high density lipoprotein-induced inhibition of adhesion molecule expression in endothelial cells. *J Biol Chem*. **281**, 37457-67.

Kimura, T., Watanabe, T., Sato, K., Kon, J., Tomura, H., Tamama, K., Kuwabara, A., Kanda, T., Kobayashi, I., Ohta, H., et al. (2000).Sphingosine 1-phosphate stimulates proliferation and migration of human endothelial cells possibly through the lipid receptors, Edg-1 and Edg-3. *Biochem J*. **348 Pt 1**, 71-6.

Kocher, O., Birrane, G., Yesilaltay, A., Shechter, S., Pal, R., Daniels, K. and Krieger, M. (2011).Identification of the PDZ3 Domain of the Adaptor Protein PDZK1 as a Second, Physiologically Functional Binding Site for the C Terminus of the High Density Lipoprotein Receptor Scavenger Receptor Class B Type I. *J Biol Chem*. **286**, 25171-86.

Kocher, O., Comella, N., Tognazzi, K. and Brown, L.F. (1998).Identification and partial characterization of PDZK1: a novel protein containing PDZ interaction domains. *Lab Invest*. **78**, 117-25.

Kocher, O., Yesilaltay, A., Shen, C.H., Zhang, S., Daniels, K., Pal, R., Chen, J. and Krieger, M. (2008).Influence of PDZK1 on lipoprotein metabolism and atherosclerosis. *Biochim Biophys Acta*. **1782**, 310-6.

Kozarsky, K.F., Donahee, M.H., Rigotti, A., Iqbal, S.N., Edelman, E.R. and Krieger, M. (1997).Overexpression of the HDL receptor SR-BI alters plasma HDL and bile cholesterol levels. *Nature*. **387**, 414-7.

Krieger, M., Brown, M.S. and Goldstein, J.L. (1981).Isolation of Chinese hamster cell mutants defective in the receptor-mediated endocytosis of low density lipoprotein. *J Mol Biol***. 150**, 167-84.

Li, X.A., Titlow, W.B., Jackson, B.A., Giltiay, N., Nikolova-Karakashian, M., Uittenbogaard, A. and Smart, E.J. (2002).High density lipoprotein binding to scavenger receptor, Class B, type I activates endothelial nitric-oxide synthase in a ceramide-dependent manner. *J Biol Chem***. 277**, 11058-63.

Liu, F., Verin, A.D., Wang, P., Day, R., Wersto, R.P., Chrest, F.J., English, D.K. and Garcia, J.G. (2001).Differential regulation of sphingosine-1-phosphate- and VEGF-induced endothelial cell chemotaxis. Involvement of G(ialpha2)-linked Rho kinase activity. *Am J Respir Cell Mol Biol***. 24**, 711-9.

Llodra, J., Angeli, V., Liu, J., Trogan, E., Fisher, E.A. and Randolph, G.J. (2004).Emigration of monocyte-derived cells from atherosclerotic lesions characterizes regressive, but not progressive, plaques. *Proc Natl Acad Sci U S A***. 101**, 11779-84.

Manning, B.D. and Cantley, L.C. (2007).AKT/PKB signaling: navigating downstream. *Cell***. 129**, 1261-74.

Martiny-Baron, G., Kazanietz, M.G., Mischak, H., Blumberg, P.M., Kochs, G., Hug, H., Marme, D. and Schachtele, C. (1993).Selective inhibition of protein kinase C isozymes by the indolocarbazole Go 6976. *J Biol Chem***. 268**, 9194-7.

Matsuo, Y., Miura, S., Kawamura, A., Uehara, Y., Rye, K.A. and Saku, K. (2007).Newly developed reconstituted high-density lipoprotein containing sphingosine-1-phosphate induces endothelial tube formation. *Atherosclerosis***.** **194**, 159-68.

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

Michaud, J., Im, D.S. and Hla, T. (2011).Inhibitory role of sphingosine 1-phosphate receptor 2 in macrophage recruitment during inflammation. *J Immunol***. 184**, 1475-83.

Mineo, C., Deguchi, H., Griffin, J.H. and Shaul, P.W. (2006).Endothelial and antithrombotic actions of HDL. *Circ Res***. 98**, 1352-64.

Mineo, C., Yuhanna, I.S., Quon, M.J. and Shaul, P.W. (2003).High density lipoprotein-induced endothelial nitric-oxide synthase activation is mediated by Akt and MAP kinases. *J Biol Chem***. 278**, 9142-9.

Morales-Ruiz, M., Lee, M.J., Zollner, S., Gratton, J.P., Scotland, R., Shiojima, I., Walsh, K., Hla, T. and Sessa, W.C. (2001).Sphingosine 1-phosphate activates Akt, nitric oxide production, and chemotaxis through a Gi protein/phosphoinositide 3-kinase pathway in endothelial cells. *J Biol Chem***. 276**, 19672-7.

Murao, K., Imachi H, Cao W, Yu X, Li J, Yoshida K, Ahmed RA, Matsumoto K, Nishiuchi T, Wong NC, Ishida T. (2006).High-density lipoprotein is a potential growth factor for adrenocortical cells. *Biochem Biophys Res Commun.* **344**, 226-32.

Murata, N., Sato, K., Kon, J., Tomura, H., Yanagita, M., Kuwabara, A., Ui, M. and Okajima, F. (2000).Interaction of sphingosine 1-phosphate with plasma components, including lipoproteins, regulates the lipid receptor-mediated actions. *Biochem J*. **352 Pt 3**, 809-15.

Nagao, T., Qin, C., Grosheva, I., Maxfield, F.R. and Pierini, L.M. (2007).Elevated cholesterol levels in the plasma membranes of macrophages inhibit migration by disrupting RhoA regulation. *Arterioscler Thromb Vasc Biol*. **27**, 1596-602.

Nieland, T.J., Penman, M., Dori, L., Krieger, M. and Kirchhausen, T. (2002).Discovery of chemical inhibitors of the selective transfer of lipids mediated by the HDL receptor SR-BI. *Proc Natl Acad Sci U S A*. **99**, 15422-7.

Nissen, S.E., Tsunoda, T., Tuzcu, E.M., Schoenhagen, P., Cooper, C.J., Yasin, M., Eaton, G.M., Lauer, M.A., Sheldon, W.S., Grines, C.L., et al. (2003).Effect of recombinant ApoA-I Milano on coronary atherosclerosis in patients with acute coronary syndromes: a randomized controlled trial. *Jama*. **290**, 2292-300.

Nixon, J.S., Bishop, J., Bradshaw, D., Davis, P.D., Hill, C.H., Elliott, L.H., Kumar, H., Lawton, G., Lewis, E.J., Mulqueen, M., et al. (1992).The design and

biological properties of potent and selective inhibitors of protein kinase C. *Biochem Soc Trans*. **20**, 419-25.

Nofer, J., Kehrel, B., Fobker, M., Levkau, B., Assmann, G., von Eckardstein, A. (2002).HDL and arteriosclerosis: beyond reverse cholesterol transport. *Atherosclerosis*. **161**, 1-16.

Nofer, J.R., Fobker, M., Hobbel, G., Voss, R., Wolinska, I., Tepel, M., Zidek, W., Junker, R., Seedorf, U., von Eckardstein, A., et al. (2000).Activation of phosphatidylinositol-specific phospholipase C by HDL-associated lysosphingolipid. Involvement in mitogenesis but not in cholesterol efflux. *Biochemistry*. **39**, 15199-207.

Nofer, J.R., van der Giet, M., Tolle, M., Wolinska, I., von Wnuck Lipinski, K., Baba, H.A., Tietge, U.J., Godecke, A., Ishii, I., Kleuser, B., et al. (2004).HDL induces NO-dependent vasorelaxation via the lysophospholipid receptor S1P3. *J Clin Invest*. **113**, 569-81.

Norata, G.D. and Catapano, A.L. (2005).Molecular mechanisms responsible for the antiinflammatory and protective effect of HDL on the endothelium. *Vasc Health Risk Manag*. **1**, 119-29.

Okajima, F., Sato, K. and Kimura, T. (2009).Anti-atherogenic actions of high-density lipoprotein through sphingosine 1-phosphate receptors and scavenger receptor class B type I. *Endocr J*. **56**, 317-34.

Pilon, A., Martin, G., Bultel-Brienne, S., Junquero, D., Delhon, A., Fruchart, J.C., Staels, B. and Clavey, V. (2003).Regulation of the scavenger receptor BI and the LDL receptor by activators of aldosterone production, angiotensin II and PMA, in the human NCI-H295R adrenocortical cell line. *Biochim Biophys Acta***. 1631**, 218-28.

Pitson, S.M., Moretti, P.A., Zebol, J.R., Lynn, H.E., Xia, P., Vadas, M.A. and Wattenberg, B.W. (2003).Activation of sphingosine kinase 1 by ERK1/2-mediated phosphorylation. *Embo J***. 22**, 5491-500.

Porn, M.I., Akerman, K.E. and Slotte, J.P. (1991).High-density lipoproteins induce a rapid and transient release of Ca2+ in cultured fibroblasts. *Biochem J***. 279 (Pt 1)**, 29-33.

Qin, C., Nagao, T., Grosheva, I., Maxfield, F.R. and Pierini, L.M. (2006).Elevated plasma membrane cholesterol content alters macrophage signaling and function. *Arterioscler Thromb Vasc Biol***. 26**, 372-8.

Rentero, C., Evans, R., Wood, P., Tebar, F., Vila de Muga, S., Cubells, L., de Diego, I., Hayes, T.E., Hughes, W.E., Pol, A., et al. (2006).Inhibition of H-Ras and MAPK is compensated by PKC-dependent pathways in annexin A6 expressing cells. *Cell Signal***. 18**, 1006-16.

Ridley, A.J. (2004).Rho proteins and cancer. *Breast Cancer Res Treat***. 84**, 13-9.

Ridley, A.J. (2001a).Rho family proteins: coordinating cell responses. *Trends Cell Biol***. 11**, 471-7.

Ridley, A.J. (2001b).Rho proteins: linking signaling with membrane trafficking. *Traffic***.** **2**, 303-10.

Ridley, A.J. (2001c).Rho GTPases and cell migration. *J Cell Sci***. 114**, 2713-22.

Ridley, A.J., Allen, W.E., Peppelenbosch, M. and Jones, G.E. (1999).Rho family proteins and cell migration. *Biochem Soc Symp***. 65**, 111-23.

Rikitake, Y., Hirata, K., Kawashima, S., Ozaki, M., Takahashi, T., Ogawa, W., Inoue, N. and Yokoyama, M. (2002).Involvement of endothelial nitric oxide in sphingosine-1-phosphate-induced angiogenesis. *Arterioscler Thromb Vasc Biol***. 22**, 108-14.

Rivera, J., Proia, R.L. and Olivera, A. (2008).The alliance of sphingosine-1-phosphate and its receptors in immunity. *Nat Rev Immunol***. 8**, 753-63.

Ryu, Y., Takuwa, N., Sugimoto, N., Sakurada, S., Usui, S., Okamoto, H., Matsui, O. and Takuwa, Y. (2002).Sphingosine-1-phosphate, a platelet-derived lysophospholipid mediator, negatively regulates cellular Rac activity and cell migration in vascular smooth muscle cells. *Circ Res***. 90**, 325-32.

Sahoo, D., Darlington, Y.F., Pop, D., Williams, D.L. and Connelly, M.A. (2007a).Scavenger receptor class B Type I (SR-BI) assembles into detergent-sensitive dimers and tetramers. *Biochim Biophys Acta***. 1771**, 807-17.

Sahoo, D., Peng, Y., Smith, J.R., Darlington, Y.F. and Connelly, M.A. (2007b).Scavenger receptor class B, type I (SR-BI) homo-dimerizes via its C-

terminal region: fluorescence resonance energy transfer analysis. *Biochim Biophys Acta***. 1771**, 818-29.

Sattler, K. and Levkau, B. (2009).Sphingosine-1-phosphate as a mediator of high-density lipoprotein effects in cardiovascular protection. *Cardiovasc Res***. 82**, 201-11.

Seetharam, D., Mineo, C., Gormley, A.K., Gibson, L.L., Vongpatanasin, W., Chambliss, K.L., Hahner, L.D., Cummings, M.L., Kitchens, R.L., Marcel, Y.L., et al. (2006).High-density lipoprotein promotes endothelial cell migration and reendothelialization via scavenger receptor-B type I. *Circ Res***. 98**, 63-72.

Shiratsuchi, H. and Basson, M.D. (2007).Akt2, but not Akt1 or Akt3 mediates pressure-stimulated serum-opsonized latex bead phagocytosis through activating mTOR and p70 S6 kinase. *J Cell Biochem***. 102**, 353-67.

Silver, D.L. (2002).A carboxyl-terminal PDZ-interacting domain of scavenger receptor B, type I is essential for cell surface expression in liver. *J Biol Chem***. 277**, 34042-7.

Singleton, P.A., Chatchavalvanich, S., Fu, P., Xing, J., Birukova, A.A., Fortune, J.A., Klibanov, A.M., Garcia, J.G. and Birukov, K.G. (2009).Akt-mediated transactivation of the S1P1 receptor in caveolin-enriched microdomains regulates endothelial barrier enhancement by oxidized phospholipids. *Circ Res***. 104**, 978-86.

Takabe, K., Paugh, S.W., Milstien, S. and Spiegel, S. (2008)."Inside-out" signaling of sphingosine-1-phosphate: therapeutic targets. *Pharmacol Rev***. 60**, 181-95.

Tanimoto, T., Lungu, A.O. and Berk, B.C. (2004).Sphingosine 1-phosphate transactivates the platelet-derived growth factor beta receptor and epidermal growth factor receptor in vascular smooth muscle cells. *Circ Res***. 94**, 1050-8.

Trach, C.C., Wulfroth, P.M., Severs, N.J. and Robenek, H. (1996).Influence of native and modified lipoproteins on migration of mouse peritoneal macrophages and the effect of the antioxidants vitamin E and Probucol. *Eur J Cell Biol***. 71**, 199-205.

Trigatti, B., Rayburn, H., Vinals, M., Braun, A., Miettinen, H., Penman, M., Hertz, M., Schrenzel, M., Amigo, L., Rigotti, A., et al. (1999).Influence of the high density lipoprotein receptor SR-BI on reproductive and cardiovascular pathophysiology. *Proc Natl Acad Sci U S A***. 96**, 9322-7.

Trogan, E., Feig, J.E., Dogan, S., Rothblat, G.H., Angeli, V., Tacke, F., Randolph, G.J. and Fisher, E.A. (2006).Gene expression changes in foam cells and the role of chemokine receptor CCR7 during atherosclerosis regression in ApoE-deficient mice. *Proc Natl Acad Sci U S A***. 103**, 3781-6.

Uittenbogaard, A., Shaul, P.W., Yuhanna, I.S., Blair, A. and Smart, E.J. (2000).High density lipoprotein prevents oxidized low density lipoprotein-induced inhibition of endothelial nitric-oxide synthase localization and activation in caveolae. *J Biol Chem***. 275**, 11278-83.

Yesilaltay, A., Kocher, O., Pal, R., Leiva, A., Quinones, V., Rigotti, A. and Krieger, M. (2006).PDZK1 is required for maintaining hepatic scavenger receptor, class B, type I (SR-BI) steady state levels but not its surface localization or function. *J Biol Chem***. 281**, 28975-80.

Yoeli-Lerner, M., Yiu, G.K., Rabinovitz, I., Erhardt, P., Jauliac, S. and Toker, A. (2005).Akt blocks breast cancer cell motility and invasion through the transcription factor NFAT. *Mol Cell***. 20**, 539-50.

Yuhanna, I.S., Zhu, Y., Cox, B.E., Hahner, L.D., Osborne-Lawrence, S., Lu, P., Marcel, Y.L., Anderson, R.G., Mendelsohn, M.E., Hobbs, H.H., et al. (2001).High-density lipoprotein binding to scavenger receptor-BI activates endothelial nitric oxide synthase. *Nat Med***. 7**, 853-7.

Zhang, Y., Ahmed, A.M., McFarlane, N., Capone, C., Boreham, D.R., Truant, R., Igdoura, S.A. and Trigatti, B.L. (2007).Regulation of SR-BI-mediated selective lipid uptake in Chinese hamster ovary-derived cells by protein kinase signaling pathways. *J Lipid Res***. 48**, 405-16.

Zhou, G.L., Tucker, D.F., Bae, S.S., Bhatheja, K., Birnbaum, M.J. and Field, J. (2006).Opposing roles for Akt1 and Akt2 in Rac/Pak signaling and cell migration. *J Biol Chem***. 281**, 36443-53.

Zhu, W., Saddar, S., Seetharam, D., Chambliss, K.L., Longoria, C., Silver, D.L., Yuhanna, I.S., Shaul, P.W. and Mineo, C. (2008).The scavenger receptor class

B type I adaptor protein PDZK1 maintains endothelial monolayer integrity. *Circ Res*. **102**, 480-7.

8. Figure legends

Fig. 4.1: HDL induces lamellipodia formation in an SR-BI dependent manner. (**A)** MPMs prepared from either SR-BI$^{+/+}$ or SR-BI$^{-/-}$ mice were cultured in the presence of 3 % NCLPDS for 16 hours before analysis. Cells were incubated with or without HDL (100 μg/ml) for the times indicated. Actin filaments were visualized by fluorescence microscopy after alexa488-phalloidin staining. (**B)** Numbers of cells with lamellipodia (arrows in **A**) were counted (~100 cells over 4-5 fields) for cells isolated from three mice from each genotype. *P<0.05 compared to time 0, NS indicates a non-significant difference.

Fig. 4.2: HDL stimulated migration requires binding to SR-BI and SR-BI lipid transfer activity. Wild type MPMs were pretreated with anti-SR-BI blocking antibody (0.5 μg/ml) (**A**) or BLT-1, an inhibitor of SR-BI mediated lipid transfer (0.3 μM) (**B**) for 30 min and cell migration in response to HDL (100 μg/ml), FTY720 (2 ng/ml) or MCP-1 (100 ng/ml) was measured. Values are means ± standard deviations of cells from 6 different mice,*P<0.05 vs. unstimulated control, #P<0.05 vs. pre-immune serum (**A**) or DMSO control (**B**).

Fig. 4.3: HDL stimulated migration involves S1PR1. RAW264.7 macrophages (**A, B**) or MPMs (**C**) were pretreated for 30 min with PTX (2 ng/ml) (**A**), VPC23019 (10 μM) (**B**) or W146 (10 μM) (**C**) and cell migration in response to HDL (100 μg/ml), FTY720 (2 ng/ml), SEW28719 (5 nM) or MCP-1 (100 ng/ml) was measured in the continued presence of the inhibitors as described in the legend to Fig. 2. Data is mean ± standard deviations of 3-6 wells (**A** and **B**) or of single wells from three independent mice (**C**). *P<0.05 vs. unstimulated control, #P<0.05 compared to vehicle control (white bars).

Fig. 4.4: HDL stimulated macrophage migration involves PI3K-Akt signaling. RAW264.7 macrophages were serum starved for ~18 hours, washed and treated with or without HDL (100 μg/ml) for 10, 30 or 60 minutes. Equal amounts of proteins were analyzed by SDS-PAGE and immunoblotting for either phospho-Ser473 or total Akt (**A**). Cells were pretreated with 10 μM LY29004 for 30 min and basal migration and migration in response to HDL (100 μg/ml), FTY720 (2 ng/ml) or MCP-1 (100 ng/ml) was measured (**B**). Data is mean ± standard deviations of 6 replicates done over two independent experiments. *P<0.05 vs. unstimulated control, #P<0.05 compared to vehicle control.

Fig. 4.5: Inactivation of Akt1 or 2 have distinct effects on macrophage migration. MPMs were isolated from WT, Akt1, Akt2 or Akt3 KO mice, incubated overnight in 3 % NCLPDS and migration in response to HDL (100 μg/ml) (**A**), FTY720 (2 ng/ml) (**B**) and

MCP-1 (100 ng/ml) (**C**) was measured. Data is mean ± standard deviations of cells from 6 mice done over two independent experiments. *P<0.05 vs. unstimulated control, #P<0.05 vs. wild type macrophages.

Fig. 4.6: HDL stimulated macrophage migration. HDL induced macrophage migration requires the HDL receptor, SR-BI and its adaptor protein, PDZK1, and the G-protein coupled receptor, S1PR1. SR-BI binds to HDL (1) and mediates cholesterol efflux and uptake of bioactive lipids such as S1P from HDL particle (2) and its delivery to S1P receptor, S1PR1(3). S1PR1 and/or SR-BI activate down stream kinases including : Ca^{2+}-dependent protein kinase C, PKC, PI3K/Akt-1, ERK1/2 and p38 MAPK as well as small monomeric GTP-binding proteins, RhoA and Rac resulting in alterations in the actin cytoskeleton and cell migration.

9. Supplementary figure legends

Supplementary Fig. 4.1: HDL does not induce foam cell migration. Foam cells from wild type MPMs were generated by culture for 48 hours in the presence of AcLDL (100μg/ml). Cells were washed and migration in response to HDL was measured using a Transwell migration assay as described in the Methods section. NS: indicates no statistically significant difference was detected.

Supplementary Fig. 4.2: The lack of SR-BI and PDZK1 reduce HDL and FTY720 stimulated macrophage migration. The migration of MPMs from WT (white bars) and SR-BI KO (**A**) or PDZK1 KO (**B**) mice (black bars) in response to HDL (100 μg/ml), FTY720 (2 ng/ml) or MCP-1 (100 ng/ml) was performed as described in the Methods section. Data is mean ± standard deviations of cells from three independent mice. . *P<0.05.

Supplementary Fig. 4.3: SR-BI surface expression in WT, SR-BI KO and PDZK1 KO MPMs. Histogram representation of SR-BI surface expression in thiogycolate elicited MPMs from PDZK1 KO (A) and Akt1 KO (B) mice compared to control WT and SR-BI KO mice. Cells were preincubated overnight in 3% NCLPDS and assayed for SR-BI surface expression using anti-SR-BI blocking antibody. Shown are representative histograms of an experiment done at least twice.

Supplementary Fig. 4.4: HDL, FTY720 and MCP-1 do not affect macrophage adhesion. RAW264.7 cell were incubated with either HDL (100μg/ml), FTY720 (2ng/ml) or MCP-1 (100mg/ml) in conditions paralleling the migration assay. Cell adhesion was measured by counting DAPI stained nuclei. NS: indicates no significance.

Supplementary Fig. 4.5: ApoA-I does not increase macrophage migration. Wild type MPMs were incubated in lipoprotein deficient serum overnight and the transwell migration assay in response to apoA-I (100 μg/ml), HDL (100 μg/ml) and MCP-1 (100 ng/ml) was performed as described in the Methods section. Data from three independent samples from each group is represented as mean ± standard deviations. *P<0.05.

Supplementary Fig. 4.6: S1PR1 expression is reduced in macrophages lacking SR-BI, PDZK1 or Akt1. Total membranes were prepared from WT, SR-BI KO, PDZK1 KO or Akt1 KO MPMs and equal amounts of protein were analyzed by SDS-PAGE and immunoblotting for SR-BI, S1PR1 and actin.

Supplementary Fig. 4.7: HDL stimulated macrophage migration is mediated by multiple protein kinases. RAW264.7 cells were pre-incubated in media containing 3% NCLPDS for 18 hours. Cells were preincubated with the indicated inhibitors for 30 min then the migration in response to HDL (100 μg/ml), FTY720 (2 ng/ml) or MCP-1 (100

ng/ml) was carried in the presence or absence of 10 μM PD98059 (**A**), 1 μM SB230580 (**B**), 5 μM of either Ro31-8220 or Go6976 (**C**), or 10 μM Y-27632, (**D**). Values are means ± standard deviations of 6 replicates done over two experiments. *P<0.05.

CHAPTER FOUR

FIGURES

Fig. 4.1: HDL induces lamellipodia formation in an SR-BI dependent manner.

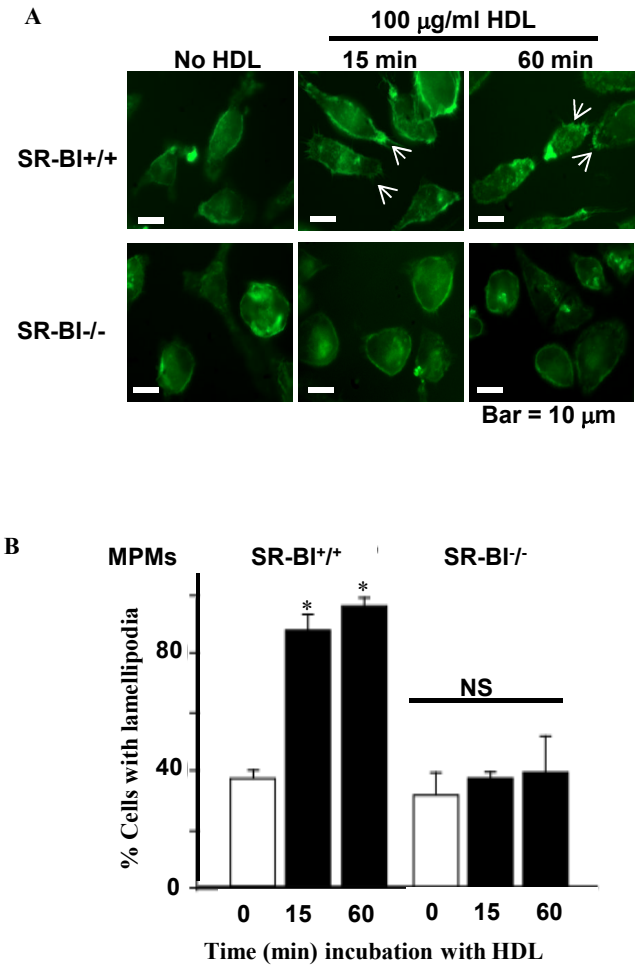


Fig. 4.2: HDL stimulated migration requires binding to SR-BI and SR-BI lipid transfer activity.

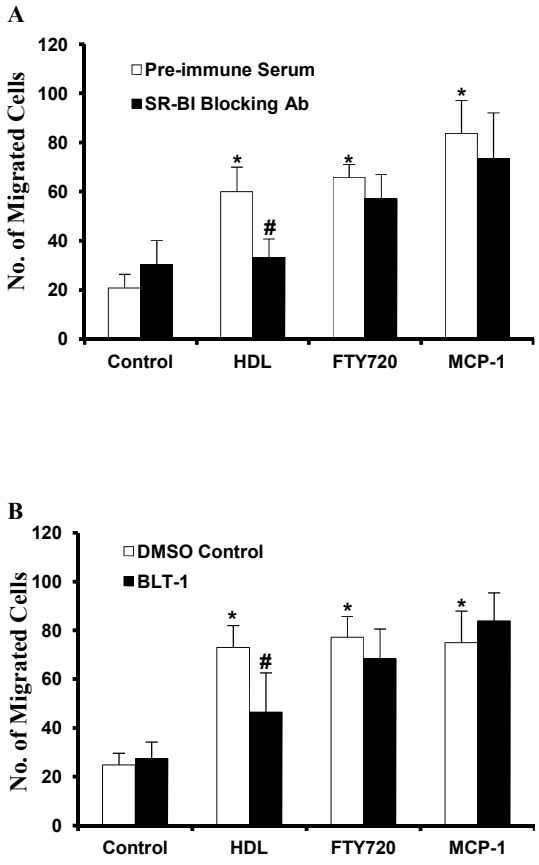


Fig. 4.3: HDL stimulated migration involves S1PR1.

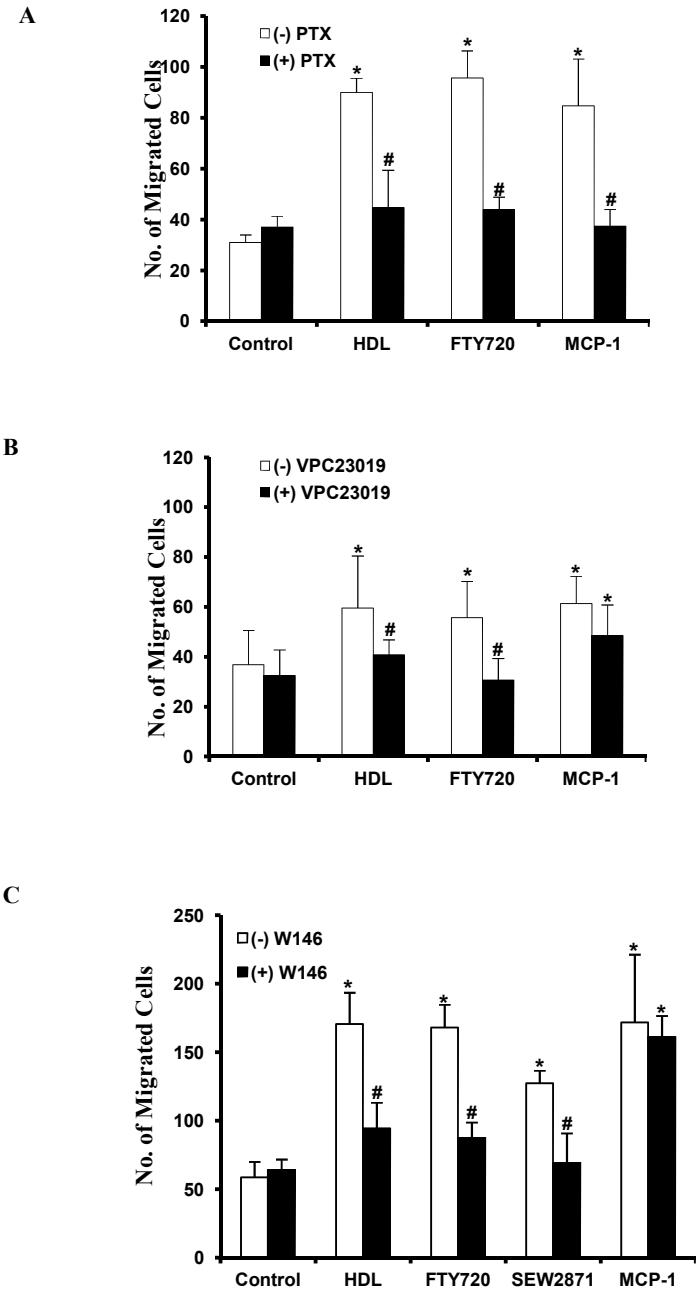


Fig. 4.4: HDL stimulated macrophage migration involves PI3K-Akt signaling.

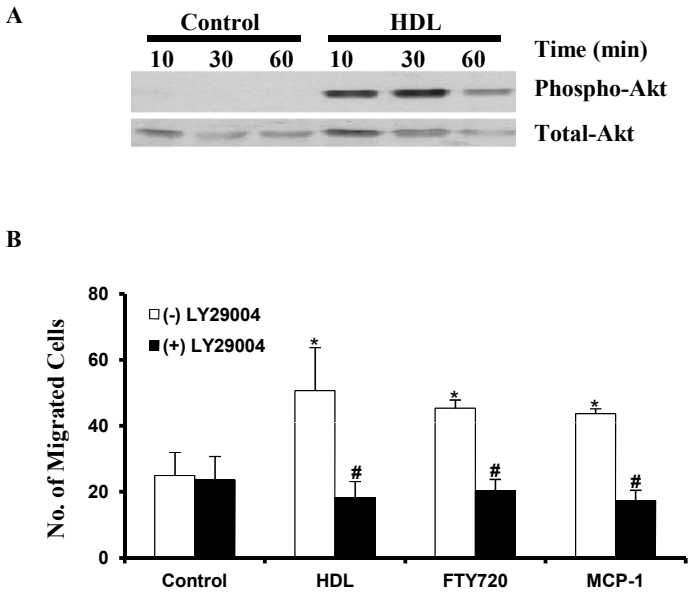


Fig. 4.5: Inactivation of Akt 1 or 2 have distinct effects on macrophage migration.

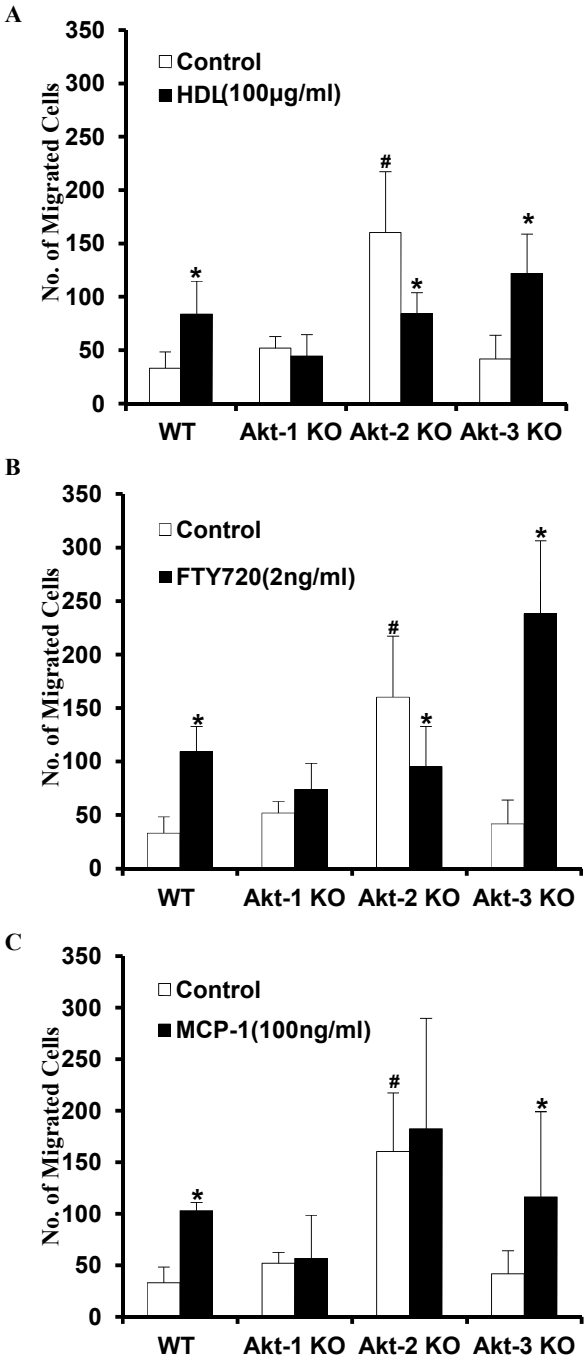
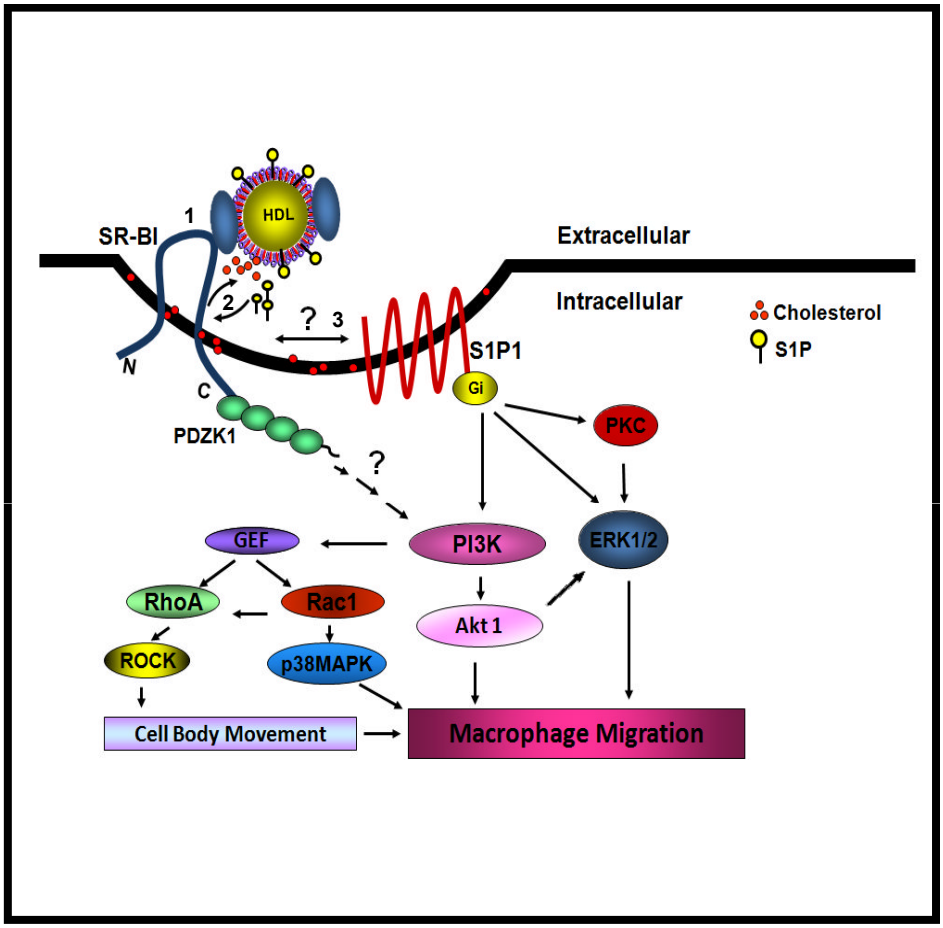


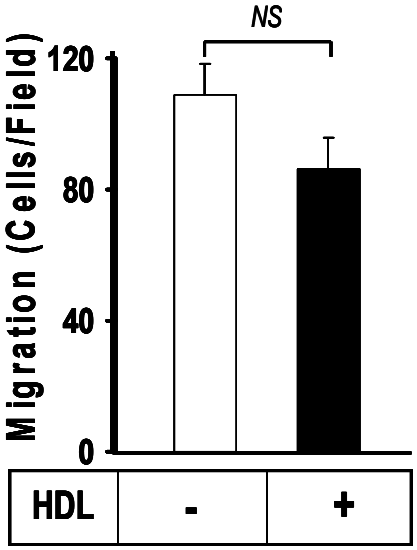
Fig. 4.6: Fig. 4.6: Proposed model of HDL stimulated macrophage migration.



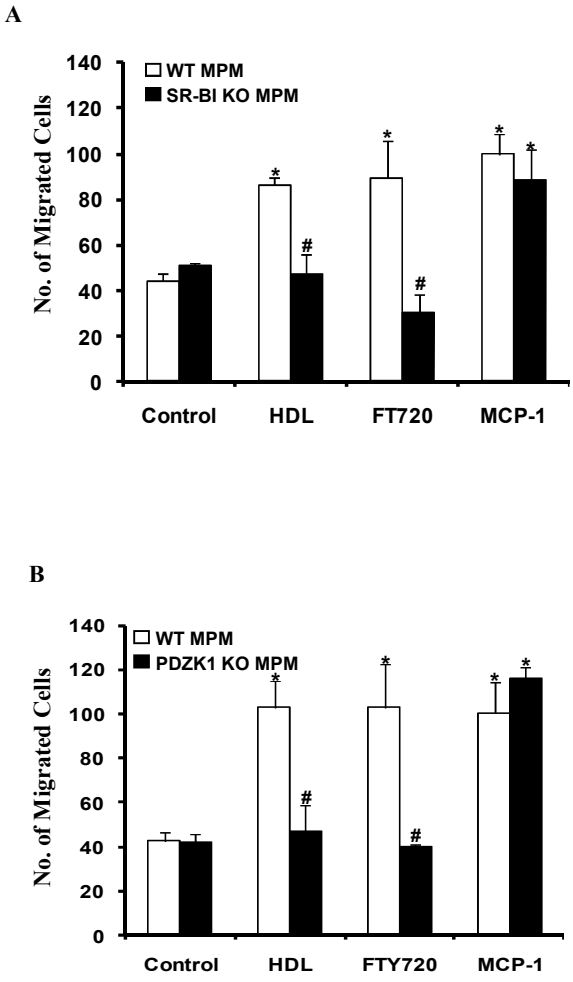
CHAPTER FOUR

SUPPLEMENTARY FIGURES

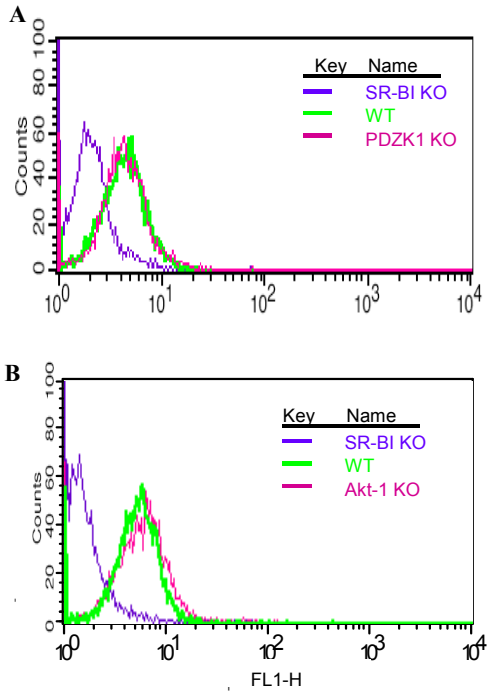
Supplementary Fig. 4.1: HDL does not induce foam cell migration.



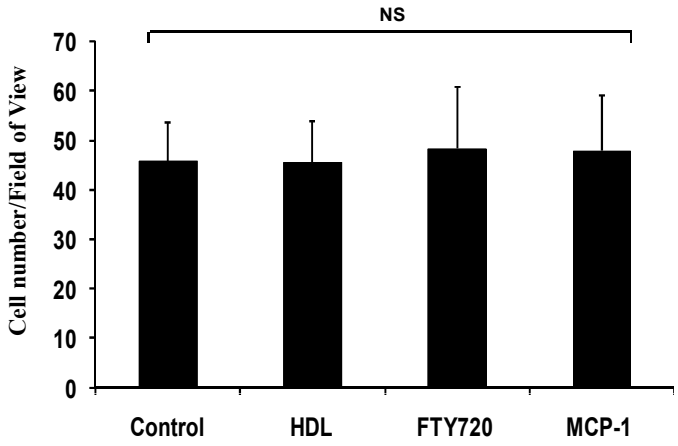
Supplementary Fig. 4.2: The lack of SR-BI and PDZK1 reduce HDL and FTY720 stimulated macrophage migration.



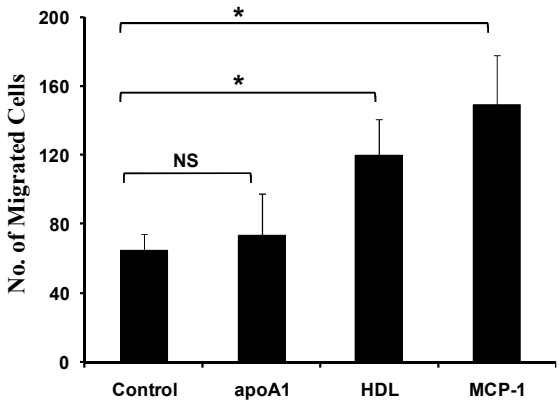
Supplementary Fig. 4.3: SR-BI Surface Expression in WT, SR-BI KO and PDZK1 KO MPMs.



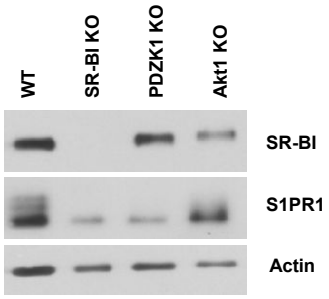
Supplementary Fig. 4.4: HDL, FTY720 and MCP-1 do not affect macrophage adhesion.



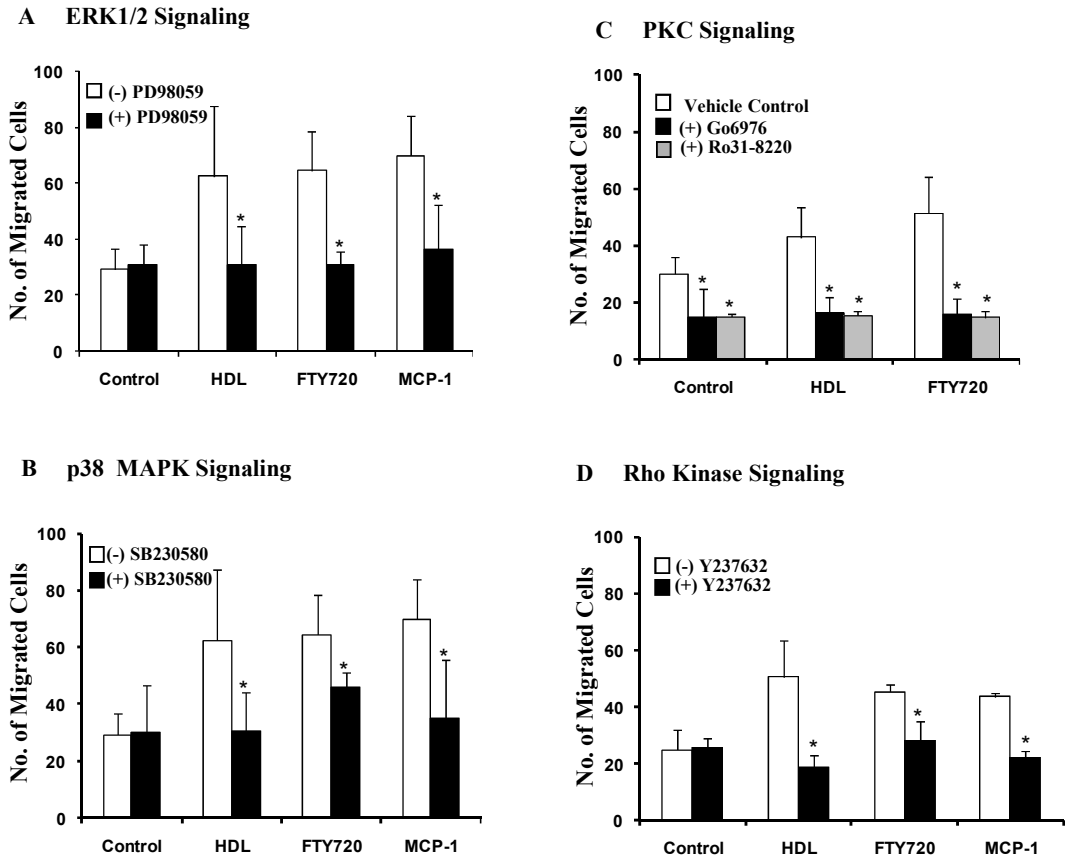
Supplementary Fig. 4.5: ApoA-I does not increase macrophage migration.



Supplementary Fig. 4.6: S1PR1 expression is reduced in macrophages lacking SR-BI, PDZK1 or Akt1.



Supplementary Fig. 4.7: HDL stimulated macrophage migration is mediated by multiple protein kinases.



CHAPTER FIVE

PROTEOMICS ANALYSIS OF HDL EFFECTS ON MACROPHAGES

CHAPTER FIVE PREFACE

The work described in this chapter is in the process of being submitted.

PROTEOMICS ANALYSIS OF HDL EFFECTS ON MACROPHAGES

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1. Abstract

Objective: High density lipoprotein (HDL) mediated reverse cholesterol transport and activation of cellular signaling pathways in the vasculature have been implicated in the atheroprotective actions of HDL. HDL mediated cholesterol efflux from macrophages is the first step in reverse cholesterol transport. We and others have shown that macrophages are targets for HDL mediated signaling. To gain insights into the effects of HDL on macrophages, we used two dimensional electrophoretic analysis to examine alterations in macrophage proteins triggered by HDL.

Methods and results: Two dimensional-difference in gel electrophoresis (2D-DIGE) was used to examine alterations in proteins in RAW264.7 macrophages after incubation (24 hrs) with HDL (100 μg/ml). One protein spot was reproducibly and statistically significantly increased more than 1.5-fold, and one region was reproducibly and statistically significantly decreased by at least 33 % in extracts from HDL treated compared to control cells. To identify altered spots, 1 mg of pooled extracts from either HDL treated or control cells were separated by two dimensional electrophoresis and proteins were stained with Deep Purple. Differentially stained spots were picked using 2D-DIGE images as guides, subjected to limited tryptic digestion and analyzed by liquid chromatography, tandem mass spectroscopy. This led to the identification of a number of proteins in total cell extracts that appeared to be altered by HDL treatment. These included alpha fetoprotein and deoxyribonucleotidase-1, actins and ubiquitin conjugating

enzyme/huntington interacting protein-2 (E2-25K/HIP2), a protein involved in ubiquitnylation, cell apoptosis and inflammation. Immunoblotting confirmed that HDL increased β-actin and decreased E2-25K/HIP2 in macrophages.

Conclusion: Proteomics analysis of HDL treated macrophages identified novel targets of HDL. Further analysis of these proteins will determine their role in HDL mediated macrophage migration and/or protection against apoptosis and inflammation.

2. Introduction

Clinical and epidemiological studies demonstrated an inverse relationship between plasma high density lipoprotein (HDL) levels and the risk of coronary heart disease [1-6]. HDL mediated cholesterol efflux from macrophages is a well appreciated mechanism by which HDL protects against atherosclerosis. More recently HDL induced activation of signaling pathways in vascular cells including macrophages has been suggested as an additional mechanism by which HDL protects against atherosclerosis (chapters 1, 3, 4 and references therein). We have shown that HDL treatment protectsagainst free cholesterol loading induced apoptosis of macrophages (Al-Jarallah A. et al. submitted, chapter 3). HDL treatment of monocytes reduced nuclear factor- ƙB (NF-ƙB) nuclear translocation, inhibited the activation and the kinase activity of NF-ƙB inhibitor(IƙB), reduced monocyte chemotactic protein-1, chemokine (C-C motif 5) and chemokine (C-X3-C motif) ligand 1 expression levels [7]. Additionally levels of cluster differentiation molecule 11b (CD11b), a monocyte adhesion molecule, were reduced in HDL treated human monocyte-derived macrophages (MDM) with apoA-I being responsible for these effects [8]. Recently microarray analysis of lipopolyscaccharide (LPS) challenged mouse peritoneal macrophages identified novel anti-inflammatory properties of HDL [9]. HDL selectively inhibited type-I interferon response genes in LPS treated macrophages in a manner that was independent of sterol metabolism [9]. HDL promoted the translocation of TRAM, a component of TRAM/TRIF complex and a

mediator of Toll-like receptor-4 (TLR-4) signaling, into an intracellular compartment which abrogated subsequent signaling by TLR-4 [9]. Although a large number of studies reported signaling properties of HDL in the vasculature, the effects of long term exposure of HDL on vascular cells' proteome are not clearly understood. Several "omics" studies examined the effects of low density lipoprotein (LDL), oxidized low density lipoprotein (oxLDL) or very low density lipoprotein (VLDL) on macrophages. Moreover increased inflammation was reported under acute and chronic exposure to oxLDL [10]. Proteomic analysis of oxLDL treated human THP-1 macrophages lead to the identification of 104 proteins that were altered in response to oxLDL treatment; these were involved in cell growth and maintenance, metabolism, apoptosis, signal transduction and immune responses [11]. In addition treatment of human MDM with oxLDL upregulated expression of 9 intracellular and 3 secreted proteins and downregulated 11 intracellular proteins and 1 secreted protein [12]. Several upregulated proteins were members of the heat shock protein family and actin cytoskeletal proteins, which are implicated in oxLDL induced endoplasmic reticulum (ER) stress and changes in the actin cytoskeleton respectively [12]. Mass spectrometric analysis of human MDM isolated from autosomal dominant hypercholesterolemic patients treated with oxLDL identified glutathione-S-transferase omega 1 and gelsolin like capping protein (CapG) as two proteins with large variability among subjects [13]. CapG is a protein that represents 1% of total cytosolic proteins in macrophages and is involved in actin dynamics and cell motility [14]. Interestingly CapG gene polymorphism in hypercholesterolemic patients was

significantly associated with carotid artery atherosclerosis [13]. In addition proteomics studies on VLDL-laden, triglyceride rich , THP-1 macrophages identified 8 upregulated proteins and 6 downregulated proteins that were involved in energy metabolism, oxidative stress, differentiation and apoptosis [15]. Moreover oxLDL treatment of THP-1 macrophages altered the expression of proteins involved in cell growth, adhesion, signal transduction, immune response and apoptosis [11]. In contrast, the long term effects of HDL on macrophage proteome are not clear. Understanding HDL effects on macrophages may uncover novel targets that could be responsible for the atheroprotective actions of HDL. The objective of this study is to identify HDL dependent alterations in macrophage proteins. Identification of HDL protein targets in macrophages will help in gaining better understanding of the antiatherogenic actions of HDL.

3. Methods

Mice

All procedures involving mice were approved by the McMaster University Animal Research Ethics Board and were in accordance with the guidelines of the Canadian Council on Animal Care. C57BL/6J mice (Jackson Laboratories) were injected intraperitoneally with 1 ml of 10% thioglycollate (Sigma Chemical Co, St Louis MO), and euthanized 4 days later. Mouse peritoneal macrophages (MPMs) were collected in 10 ml of sterile PBS, pelleted at 300 x g for 10 min and plated in cell culture dishes in

Dulbecco's modified eagles medium (DMEM) containing 2 mM L-glutamine 50 µg/ml penicillin and 50 units/ml streptomycin DMEM supplemented with 10 % fetal bovine serum (FBS).

Cells and cell culture

All reagents for cell culture were from Invitrogen Corporation (Burlington, ON, Canada) unless stated otherwise. Cells were cultured at 37 ºC in 5 % CO_2 / 95 % air. MPMs plated in DMEM + 10 % FBS were allowed to adhere to cell culture dishes for 2 hrs, after which media containing non-adhered cells was removed and replaced with DMEM containing 1 % newborn calf lipoprotein deficient serum (NCLPDS), prepared as described previously [16]. MPMs were used the following day. RAW264.7 cells were cultured in DMEM + 10 % heat inactivated FBS until nearly confluent. Cells were removed from dishes by scraping, diluted into fresh medium and seeded at 1:5 onto new dishes. One day prior to each experiment, cell culture media was replaced with DMEM + 1 % NCLPDS. RAW264.7 cells or MPMs were cultured for further 24 hrs in DMEM + 1 % NCLPDS without further additions (control) or containing 100 µg/ml human HDL (Biomedical Technologies Inc, MA USA).

2D electrophoresis

Cells were lysed (9M urea, 2M thiourea, 30mM Tris, pH8.5, 4% CHAPS) in the presence of protease inhibitors and nuclease mix (GE Healthcare, Quebec, CA) by

passage 8 times through 18G needles, followed by incubation at room temperature for 40 minutes. Cell debris was removed by centrifugation at 14,000 rpm for 10 min using a Spectrafuge 16M microcentrifuge (Labnet International Inc., Ontario, CA). Protein concentrations were then measured using Coomassie Plus Assay (ThermoScientific, Ontario, CA) and samples were stored at -80^{o} C prior to analysis.

2D-Differential in gel electrophoresis (2D-DIGE)

Proteins (50 µg) from untreated controls or HDL treated macrophages were each labeled with Cy3 or Cy5, respectively (or vice versa to control for differential labeling) and pooled. A separate pool of 50µg protein (25 µg from all of control and 25 µg from all of HDL treated macrophages) were labeled with Cy2 according to the manufacturer's instructions (GE Healthcare, Quebec, CA). Cy2 labeled pooled samples served as an internal standard. Cy3, Cy5 and Cy2 labeled samples were mixed (50 µg protein each) together with rehydration buffer (9 M urea, 2 M thiourea, 4% CHAPS, 60 mM DTT) and IPG buffer (GE Healthcare, Quebec, CA). Proteins were separated by isoelectric focusing on non linear 24-cm pH 4-7 IPG strips using an IPGphore (GE Healthcare, Quebec, CA). Samples were run at 150 volts (V), 500 V, 1000 V, and 2500 V for 1 hr each. The voltage was then increased to 8000 V over 1 hr, and held at 8000 V for a total of 80 kV·hrs. The strips were then equilibrated in 6 M urea, 30% w/v glycerol and 2% w/v SDS in 0.05 M Tris-HCl pH 8.8 containing a trace of bromophenol blue dye, and applied to large format polyacrylamide gels (10%) and run as described [17, 18]. Gels were

scanned using a Typhoon 9400 Variable Mode Imager (GE Healthcare, Quebec, CA). Excitation/emission wavelengths used for Cy 2, Cy3 and Cy 5 were 280/530 nm, 540/620 nm and 590/680 nm, respectively [19]. Images were analyzed using DeCyder 7.0 software. Internal standard images from the four gels were compared and the gel with highest number of spots was designated as a master gel. Spots were compared to the internal standard of each gel and to the master gel image. Spots were selected based on an abundance ratio of greater than or equal to 1.5 fold and a $P < 0.05$ between control and HDL-treated samples.

Preparative 2D electrophoresis

Pooled samples totaling 1 mg protein from either control or HDL-treated RAW264.7 cells were analyzed by isoelectric focusing and denaturing SDS-PAGE as described above. Gels were stained with Deep Purple (GE Healthcare, Quebec, CA) and scanned using a Typhoon 9400 Variable Mode Imager (GE Healthcare, Quebec, CA). Images were analyzed using DeCyder 7.0 to match spots with those of the corresponding DIGE gels. Spots of interest were picked using an Ettan Spot Picker (GE Healthcare, USA).

LC-MS/MS analysis

Gel fragments were washed twice with 50 mM ammonium bicarbonate / 50% methanol and once with 100 % acetonitrile and dried at room temperature for 20 minutes.

Proteins were digested with trypsin (20 ng/µl) in 25 µl of 10 mM ammonium bicarbonate (Promega, Wisconsin, USA). Samples were incubated at 37 °C for 2 hrs and 8 µl of digested sample was analyzed by LC-MS/MS using the Agilent LC/MSD Trap Ultra 6330 Mass Spectrometer (Agilent Technologies, California, USA) [17, 18]. The obtained peptide spectra were analyzed using Spectrum Mill MS Proteomics Workbench Rev. A.03.03.084 SR4 (Agilent Technologies, California, USA). Spectra were compared to the mouse subset of the NCBI nr protein database with the following search parameters: (1) specificity of trypsin as the proteolytic enzyme with two missed cleavages were allowed, (2) cystine modification by carbamidomethylation, (3) precursor and fragment ion tolerance of ± 2.5 & 0.7 Da respectively. Proteins with at least two peptides and an MS/MS score of ≥ 25 were chosen as true IDs.

Immunoblotting

For one dimensional SDS-PAGE analysis, total cell lysates (50 µg protein) from control or HDL treated RAW264.7 macrophages or MPMs were analyzed by 15 % acrylamide SDS-PAGE as described previously [16, 20]. For analytical scale 2D electrophoresis, total cell lysates (250 µg protein) from HDL treated or untreated samples were subjected to isoelectric (IEF) using 7 cm non linear 4-7 IPG strips. Samples were prepared as described above and IEF strips were run at 150 V, 500 V, 1000 V and 2500 V for 1hr each, from 2500 to 8000 for 1hr, then at 800 V until 15 kV·hrs was reached. The strips were then equilibrated as described above and analyzed by denaturing SDS-PAGE

on 4-20% acrylamide gradient gels (BioRad, California, USA). The gels were run at 100V for 10 min and then at 200V until the dye front reached the bottom of the gel. Proteins were electrophoretically transferred to PVDF membranes [21]. Membranes were blocked in 5% fat free milk in Tris-Buffered saline, pH7.4, containing 0.1% Tween-20 for 1hr at room temprature and immunoblotting was performed as described previously [16, 20] using rabbit anti human E2-25K/HIP2 (Cell Signaling Technology, Massachusetts, USA), rabbit anti-human β actin (Cell Signaling Technology, Massachusetts, USA), mouse anti-human γ actin (Sigma Aldrich, Missori, USA), goat anti human apoA-I (Midland BioProducts Corporation, Iowa, USA) or rabbit polyclonal anti-GAPDH (Cell Signaling Technology, Massachusetts, USA). Corresponding secondary HRP-conjugated donkey anti-rabbit, rabbit anti-goat or donkey anti-mouse antibodies (Jackson ImmunoResearch, Pennsylvania, USA) were used. Band intensities were quantified using ImageLab software (BioRad, California, USA). Data are represented as mean ± standard deviations. Differences between means were considered significant when $P < 0.05$ according to Student's t test (Microsoft Excel).

4. Results

2D-DIGE identification of differentially expressed proteins

RAW264.7 macrophages were cultured in the presence or absence of HDL (100 μg/ml) for 24 hrs in media containing 1% NCLPDS. Lysates were prepared from treated

and untreated cells, differentially labeled with fluorescent protein stains, mixed and analyzed by 2D-DIGE. Four gels, each representing a different pool of one control and one HDL treated sample, were processed simultaneously and analyzed with DeCyder 7.0 Software. More than 3000 spots were detected. 1500 spots were matched between all four gels and analysis revealed the presence of two regions containing differentially expressed spots between control and HDL treated cells that were reproducibly different between the four gels (Fig. 5.1). One region contained a train of spots (spot train a) that was downregulated (-2.23 folds, P = 0.0012) and the other region contained a prominent spot of lower molecular weight (spot b) that was upregulated (+1.5 folds, P = 0.017) with HDL treatment (Fig. 5.1). This experiment demonstrates at an analytical scale that there are differences between control and HDL treated samples. However protein identities could not be determined directly from the CyDye gels, presumably due to the low amounts of protein.

Analysis of preparative 2D gels

In order to identify proteins altered by HDL treatment, preparative gels containing 1mg of pooled protein from each of the 4 control or HDL treated samples were run and stained with Deep Purple protein stain (Fig. 5.2). This was repeated with a different set of 4 control and HDL treated samples. Using the spot patterns from the DIGE gels as a reference, matching spots were picked from the preparative Deep Purple stained gels (Fig. 5.2). Other spots that appeared different between the preparative gels of lysates from control and HDL treated cells were also picked (Fig.5.2). Proteins in picked spots

were subjected to trypsin digestion and peptide sequences were identified by LC-MS/MS. This lead to the identification of a number of candidate proteins that appeared to be different between control and HDL treated macrophages (Table).

A number of the protein candidates identified as potentially altered by HDL treatment appear to have roles in signal transduction, apoptosis and/or inflammation. We and others have shown that HDL induces signaling in different cell types (chapters 1, 3, 4 and references therein). Therefore we attempted to validate these candidates by immunoblotting. For some (deoxyribonucleotide transferase-1 (dNT-1), alpha-fetoprotein) antibodies either were not specific, yielding multiple bands, or did not yield bands on immunoblotting analysis of either RAW264.7 or MPM cell lysates (data not shown). Therefore we did not attempt to analyze these candidates further.

We have previously found that HDL induces macrophage migration and suppresses apoptosis induced by free cholesterol (chapters 3 and 4). Others have also reported that HDL suppresses the induction of inflammatory gene expression in macrophages [9]. Therefore we focused our attention on some protein candidates that were linked to these pathways, namely the ubiquitin conjugating enzyme-2 (E2-25K)/huntington interacting protein 2 (HIP2) and β- and γ-actins.

Cell lysates from control and HDL treated macrophages were first probed for apoA-I, the major apolipoprotein component of HDL (Fig. 5.3 A, B). ApoA-I was detected in lysates of HDL treated cells. This is most likely HDL derived apoA-I

associated with cells. No apoA-I was detected in lysates from control cells confirming the absence of HDL in those samples.

β- and/or γ-actins were identified as spot 26 on the preparative 2D gels (Figure 5.2 and Table). We have previously shown that shorter term incubation with HDL (1-4 hrs) resulted in the rearrangement of actin and induced migration of macrophages (chapter 4). The role of β-actin in cell migration is well understood however that of γ-actin is not clear (reviewed in [22]). Immunoblotting using antibodies specific for either β- or γ-actin showed that HDL treatment for 24 hrs statistically significantly increased β-actin levels (Fig. 5.3 A,C) but had no effect on γ-actin levels (Fig 5.3 A,D). These findings are consistent with our previous demonstration that HDL stimulates macrophage migration.

E2-25K/HIP2 is a ubiquitin conjugating enzyme that has been implicated in inflammation and apoptosis [23-26]. HDL has been shown to suppress LPS induced inflammation in macrophages [9] and we have demonstrated that HDL suppresses the activation of apoptosis by free cholesterol loading in macrophages (chapter 3). To verify that HDL treatment altered E2-25K/HIP2 levels in macrophages, cell lysates separated by 1D SDS-PAGE were immunoblotted with an antibody against E2-25K/HIP2. This revealed that E2-25K/HIP2 protein levels were reduced in HDL treated macrophages relative to controls (Fig. 5.3 A, D). Three splice variants encoding distinct isoforms have been reported for human's E2-25K/HIP2 (NP_001104582.1, NP_005330.1, NP_001104583.1), however differences in tissue distribution, expression levels, or

regulation of these isoforms have not been characterized. Two dimensional immunoblotting indicates the presence of two immunoreactive E2-25K/HIP2 spots in murine macrophages, both of which are reduced by HDL treatment (Fig. 5.4). To confirm these results in primary macrophages, we have treated thioglycolate elicited MPMs with HDL under the same experimental conditions. HDL reduced E2-25K/HIP2 levels in MPMs (Fig. 5.5) to a similar extent as that seen in RAW264.7 macrophages.

5. Discussion

The objective of this study was to identify new proteins altered by HDL in macrophages, that my play roles in HDL's anti-atherogenic effects. 2D-DIGE analysis identified two regions that were altered in HDL treated macrophages. Because the amounts of proteins in the analytical 2D-DIGE gels were too low, we were not able to detect proteins by LC-MS/MS analysis of picked spots. Analysis of preparative Deep Purple stained gels suggests that a number of other proteins may be altered by HDL treatment. Matching the spot patterns from the preparative Deep Purple stained gels with those of the analytical 2D-DIGE gels suggests that the protein that was seen to be strongly upregulated by HDL on the 2D-DIGE analysis (spot b in Figure 5.1) is likely the deoxyribonucleotide transferase-1, dNT-1 (spot 24 in Fig 5.2 and Table). On the other hand protein spots that were found by analytical 2D-DIGE to be strongly downregulated by HDL treatment (spot train a in Fig 5.1) likely correspond to alpha fetoprotein (spots

28-30 in Fig 5.2 and Table). Although these have been associated with signal transduction and/or inflammation and apoptosis, pathways relevant to HDL mediated signaling and anti-atherogenic effects in macrophages, we have not yet been able to validate them by immunoblotting due to the absence of adequate reagents.

Although we only detected two spots/regions by analytical 2D-DIGE that met our stringent criteria for differences (Fig 3.1) we noticed a number of other spots that appeared to be different in preparative total protein stained gels (Fig 5.2). We therefore picked and identified some these spots as well (Table). Of these, we chose to focus on β– and γ–actin and E2-25K/HIP2 due to the availability of antibodies for immunoblotting and their reported involvement in cell migration or apoptosis, which we have found to be altered in macrophages treated with HDL (chapters 3 and 4).

β- and γ-actin, are ubiquitous components of the microfilament system and differ in only 4 amino acids at the amino terminus [27]. Despite the great degree of homology exhibited between β- and γ-actin, these actin isoforms exhibit differences in their function, cellular localization and post-translational modifications. β- and γ-actin associate with different tropomyosin isoforms, resulting in the formation stress fibers in different regions of the cell. β-actin forms stress fibers that are localized to the cell periphery at ruffling membranes, whereas γ–actin forms stress fibers throughout the cytosol of mouse fibroblasts ([28], reviewed in [29]) . Post-translational modification of β-actin but not γ-actin by arginylation was reported to control actin polymerization and lamella formation in motile cells [30]. The actin cytoskeleton was built normally in γ-

actin knockout cells suggesting that it is not an essential building block of the actin cytoskeleton, rather it is required for the long term stability of F-actin [31]. Our data suggests that HDL treatment of macrophages increases β-actin protein levels but has no effect on γ-actin levels. This is consistent with our observation that HDL stimulates macrophage migration (chapter 4) and suggests that HDL treatment may also increase the capacity of cells to migrate.

OxLDL has also been reported to induce the expression of actin and actin related proteins in human macrophages [11, 13]. Although oxLDL increased actin expression, it increased cell spreading and inhibited macrophage migration in a manner that is dependent on CD36 signaling [32]. Thus despite the increased actin expression in response to HDL and oxLDL these lipoproteins appear to affect macrophage migration in opposite ways. Reduced macrophage migration triggered by oxLDL has been suggested to contribute to atherosclerotic plaque growth [33, 34], whereas increased macrophage migration triggered by HDL could potentially contribute to macrophage egress from plaques and atherosclerotic plaque regression. Alterations in β-actin may contribute to these effects.

Amyloid β (Aβ) treatment of neurons was reported to increase levels of E2-25K/HIP2, caspase 12, ER stress and apoptosis [25]. Disruption of E2-25K/HIP2 expression triggered proteasomal degradation of caspase 12 and reduced the extent of ER stress and apoptosis in Aβ treated neurons. Conversely overexpression of E2-25K/HIP2 resulted in increased caspase 12 protein levels, increased ER stress and increased

apoptosis in neurons [25]. These data suggest that E2-25K/HIP2 may play a role in promoting ER stress induced apoptosis. We have demonstrated that HDL protects macrophages against apoptosis induced by loading with free cholesterol (chapter 3), and we and others have found that HDL suppresses apoptosis induced by other ER stress inducing agents including tunicamycin and thapsigargin (data not shown and [35]). We have demonstrated that HDL induces multiple signaling pathways that may contribute to suppression of pro-apoptotic factors such as the BH3 only pro-apoptotic protein Bim. Here we demonstrate that HDL also suppresses expression of E2-25K/HIP2, suggesting another possible pathway by which it may suppress apoptosis. Interestingly 2D immunoblotting revealed the presence of two E2-25K/HIP2 spots, both of which were reduced in HDL treated macrophages. Three isoforms have been described for human E2-25K/HIP2. It is not clear which of these isoforms the two spots detected in macrophage lysates correspond to, or how these isoforms may differ in activity or function. Our finding that E2-25K/HIP2 is suppressed by HDL treatment suggests that this may contribute to HDL mediated suppression of apoptosis.

E2-25K/HIP2 has also been implicated in cellular inflammation [26]. Likewise, HDL has been demonstrated to suppress inflammatory gene expression in both endothelial cells and macrophages [9, 36]. Nuclear factor-κB (NF-κB) is a central regulator of cellular inflammatory gene expression. It is a heterodimeric protein composed of the p50 (NF-κB1) and p65 (RelA) subunits (reviewed in [37]). The p50 subunit is produced by proteolytic processing subsequent to ubiquitinylation of a p105

precursor [37]. In unstimulated cells the p50/p65 complex is maintained in an inactive state via the interaction with inhibitor of nuclear factor-κB (IκBα) which prevents its nuclear translocation (recently reviewed in [38]). The activity of IκBα is also regulated by proteasome mediated proteolytic degradation [38]. E2-25K/HIP2 has been isolated as a ubiquitin conjugating enzyme that participates in the ubiqutinylation and subsequent processing of p105 suggesting a potential role in regulating cellular inflammation [26]. Therefore the identification of E2-25K/HIP2 as a protein downregulated by HDL suggests that this could also participate in HDL mediated suppression of inflammatory gene expression.

Further studies are necessary to determine if in fact, downregulation of E2-25K/HIP2 by HDL participates in HDL dependent suppression of apoptosis and/or inflammatory gene expression. These include testing whether reducing E2-25K/HIP2 expression, itself, will protect macrophages from ER stress induced apoptosis and will suppress activation of NF-κB and LPS induced expression of inflammatory genes. In addition, validation of HDL's effects on some of the other proteins identified may provide further insight into mechanisms by which HDL exerts anti-atherogenic effects in macrophages.

6. Figure legends

Fig. 5.1: 2D-DIGE analysis of control and HDL treated macrophages. Total cell lysates of RAW264.7 cells either untreated or treated with HDL (100μg/ml) and proteins were differentially labeled with Cy3 (green, control lysate) or Cy5 (red, HDL treated). 50 μg of each were mixed together with 50 μg of pooled Cy2 labeled lysates (not shown). Samples were subjected to IEF on non linear 4-7 IPG strips followed by SDS-PAGE on 10% polyacrylamide gels. A representative gel scan of 5 independently run sample pairs is shown. Differentially expressed spots are indicated with arrows. Spot train a (red) was reduced and spot b (green), was increased in samples from HDL treated cells. The acidic and basic sides of the gel are indicated.

Fig. 5.2: Deep Purple stained gels of samples from control and HDL treated macrophages. Protein (1 mg) pooled from 4 lysates of control (A) or 4 lysates of HDL treated cells (B) were separated by 2D electrophoresis and stained with Deep Purple total protein stain. Gels were matched to the CyDye labeled gels using DeCyder software and some of the spots that appeared to be different were picked and identified by LC-MS/MS. The spots that were picked and identified by LC-MS/MS were arbitrarily numbered as marked on the gel. Protein identities are summarized in (Table). All spots with the exception of 27-30 were picked from the HDL gel. Another two sets of gels from a separate pool of 4 control vs. 4 HDL treated samples and individual samples were ran.

Fig. 5.3: Validation of selected proteins by immunoblotting. RAW264.7 cells were incubated without or with HDL as in (Fig 5.1).Total cell lysates (50μg protein) were separated SDS-PAGE on 15 % acrylamide gels, transferred to PVDF membrane and immunoblotted for the indicated proteins (A). Immunoblots were analyzed using a Gel Doc system (BioRad Laboratories). Band intensities of apoA-I (B), β-actin (C), γ-actin (D) and E2-25K/HIP2 (E) were quantified and normalized to those of GAPDH. Data represents means ± standard deviations of the four replicates, * $P < 0.05$. Data was reproducible in another experiment using the four control and HDL treated samples from Fig 5.1.

Fig. 5.4: Immunoblting of E2-25K/HIP2 separated by 2D-electrophoresis. Lysates from control (A) or HDL-treated RAW264.7 cells (B) were separated by analytical 2D electrophoresis, transferred to PVDF membranes and probed for E2-25K/HIP2 as described in the methods section. A similar pattern was observed when an independent pool of 4 lysates of control or HDL treated macrophages were analyzed.

Fig. 5.4: HDL reduces E2-25K/HIP2 protein levels in mouse peritoneal macrophages. Thioglycollate elicited MPM were prepared from three different mice, incubated without or with HDL, lysed and proteins were analyzed by SDS-PAGE and immunoblotting as described above (A). Band intensities were quantified relative to GAPDH levels (B). Values are means ± standard deviations, * $P < 0.05$.

7. Tables

Table: List of proteins picked from Deep Purple stained gels.

8. References

[1] D.P. Barr, E.M. Russ and H.A. Eder, Protein-lipid relationships in human plasma. II. In atherosclerosis and related conditions, *Am J Med.* **11** (1951), pp. 480-93.

[2] G.S. Boyd and M.F. Oliver, The circulating lipids and lipoproteins in coronary artery disease, *Postgrad Med J.* **32** (1957), pp. 2-6; passim.

[3] G. Assmann, H. Schulte, A. von Eckardstein and Y. Huang, High-density lipoprotein cholesterol as a predictor of coronary heart disease risk. The PROCAM experience and pathophysiological implications for reverse cholesterol transport, *Atherosclerosis.* **124 Suppl** (1996), pp. S11-20.

[4] M.S. Brown and J.L. Goldstein, Lipoprotein metabolism in the macrophage: implications for cholesterol deposition in atherosclerosis, *Annu Rev Biochem.* **52** (1983), pp. 223-61.

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

[6] D.R. Jacobs, Jr., I.L. Mebane, S.I. Bangdiwala, M.H. Criqui and H.A. Tyroler, High density lipoprotein cholesterol as a predictor of cardiovascular disease mortality in men and women: the follow-up study of the Lipid Research Clinics Prevalence Study, *Am J Epidemiol.* **131** (1990), pp. 32-47.

[7] C.A. Bursill, M.L. Castro, D.T. Beattie, S. Nakhla, E. van der Vorst, A.K. Heather, P.J. Barter and K.A. Rye, High-density lipoproteins suppress

chemokines and chemokine receptors in vitro and in vivo, *Arterioscler Thromb Vasc Biol.* **30** (2010), pp. 1773-8.

[8] A.J. Murphy, K.J. Woollard, A. Hoang, N. Mukhamedova, R.A. Stirzaker, S.P. McCormick, A.T. Remaley, D. Sviridov and J. Chin-Dusting, High-density lipoprotein reduces the human monocyte inflammatory response, *Arterioscler Thromb Vasc Biol.* **28** (2008), pp. 2071-7.

[9] M. Suzuki, D.K. Pritchard, L. Becker, A.N. Hoofnagle, N. Tanimura, T.K. Bammler, R.P. Beyer, R. Bumgarner, T. Vaisar, M.C. de Beer, F.C. de Beer, K. Miyake, J.F. Oram and J.W. Heinecke, High-density lipoprotein suppresses the type I interferon response, a family of potent antiviral immunoregulators, in macrophages challenged with lipopolysaccharide, *Circulation.* **122** (2010), pp. 1919-27.

[10] J.P. Conway and M. Kinter, Proteomic and transcriptomic analyses of macrophages with an increased resistance to oxidized low density lipoprotein (oxLDL)-induced cytotoxicity generated by chronic exposure to oxLDL, *Mol Cell Proteomics.* **4** (2005), pp. 1522-40.

[11] J.H. Kang, H.S. Ryu, H.T. Kim, S.J. Lee, U.K. Choi, Y.B. Park, T.L. Huh, M.S. Choi, T.C. Kang, S.Y. Choi and O.S. Kwon, Proteomic analysis of human macrophages exposed to hypochlorite-oxidized low-density lipoprotein, *Biochim Biophys Acta.* **1794** (2009), pp. 446-58.

[12] A. Dupont, M. Chwastyniak, O. Beseme, A.L. Guihot, H. Drobecq, P. Amouyel and F. Pinet, Application of saturation dye 2D-DIGE proteomics to characterize proteins modulated by oxidized low density lipoprotein treatment of human macrophages, *J Proteome Res.* **7** (2008), pp. 3572-82.

[13] E. Burillo, D. Recalde, E. Jarauta, S. Fiddyment, A.L. Garcia-Otin, R. Mateo-Gallego, A. Cenarro and F. Civeira, Proteomic study of macrophages exposed to oxLDL identifies a CAPG polymorphism associated with carotid atherosclerosis, *Atherosclerosis.* **207** (2009), pp. 32-7.

[14] G.A. Dabiri, C.L. Young, J. Rosenbloom and F.S. Southwick, Molecular cloning of human macrophage capping protein cDNA. A unique member of the gelsolin/villin family expressed primarily in macrophages, *J Biol Chem.* **267** (1992), pp. 16545-52.

[15] Y. Lu, J. Guo, Y. Di, Y. Zong, S. Qu and J. Tian, Proteomic analysis of the triglyceride-rich lipoprotein-laden foam cells, *Mol Cells.* **28** (2009), pp. 175-81.

[16] Y. Zhang, A.M. Ahmed, N. McFarlane, C. Capone, D.R. Boreham, R. Truant, S.A. Igdoura and B.L. Trigatti, Regulation of SR-BI-mediated selective lipid uptake in Chinese hamster ovary-derived cells by protein kinase signaling pathways, *J Lipid Res.* **48** (2007), pp. 405-16.

[17] D.M. Wong, J.P. Webb, P.M. Malinowski, J. Macri and K. Adeli, Proteomic profiling of the prechylomicron transport vesicle involved in the assembly and

secretion of apoB-48-containing chylomicrons in the intestinal enterocytes, *Proteomics.* **9** (2009), pp. 3698-711.

[18] D.M. Wong, J.P. Webb, P.M. Malinowski, E. Xu, J. Macri and K. Adeli, Proteomic profiling of intestinal prechylomicron transport vesicle (PCTV)-associated proteins in an animal model of insulin resistance (94 char), *J Proteomics.* **73** (2010), pp. 1291-305.

[19] J.F. Timms and R. Cramer, Difference gel electrophoresis, *Proteomics.* **8** (2008), pp. 4886-97.

[20] Y. Zhang, Ahmed, A. M. , Tran, T. , Lin, J. ,McFarlane, N. , Boreham, D. R. , Idgdoura, S. A., Truant, R., Trigatti, B. L., The inhibition of endocytosis affects HDL-lipid uptake mediated by the human scavenger receptor class B type I., *Mol Membr Biol.* **24** (2007), pp. 442-454.

[21] H. Towbin, T. Staehelin and J. Gordon, Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications, *Proc Natl Acad Sci U S A.* **76** (1979), pp. 4350-4.

[22] D. Tondeleir, D. Vandamme, J. Vandekerckhove, C. Ampe and A. Lambrechts, Actin isoform expression patterns during mammalian development and in pathology: insights from mouse models, *Cell Motil Cytoskeleton.* **66** (2009), pp. 798-815.

[23] S. Song and Y.K. Jung, Alzheimer's disease meets the ubiquitin-proteasome system, *Trends Mol Med.* **10** (2004), pp. 565-70.

[24] S. Song, S.Y. Kim, Y.M. Hong, D.G. Jo, J.Y. Lee, S.M. Shim, C.W. Chung, S.J. Seo, Y.J. Yoo, J.Y. Koh, M.C. Lee, A.J. Yates, H. Ichijo and Y.K. Jung, Essential role of E2-25K/Hip-2 in mediating amyloid-beta neurotoxicity, *Mol Cell.* **12** (2003), pp. 553-63.

[25] S. Song, H. Lee, T.I. Kam, M.L. Tai, J.Y. Lee, J.Y. Noh, S.M. Shim, S.J. Seo, Y.Y. Kong, T. Nakagawa, C.W. Chung, D.Y. Choi, H. Oubrahim and Y.K. Jung, E2-25K/Hip-2 regulates caspase-12 in ER stress-mediated Abeta neurotoxicity, *J Cell Biol.* **182** (2008), pp. 675-84.

[26] O. Coux and A.L. Goldberg, Enzymes catalyzing ubiquitination and proteolytic processing of the p105 precursor of nuclear factor kappaB1, *J Biol Chem.* **273** (1998), pp. 8820-8.

[27] J. Vandekerckhove and K. Weber, The complete amino acid sequence of actins from bovine aorta, bovine heart, bovine fast skeletal muscle, and rabbit slow skeletal muscle. A protein-chemical analysis of muscle actin differentiation, *Differentiation.* **14** (1979), pp. 123-33.

[28] G. Schevzov, B. Vrhovski, N.S. Bryce, S. Elmir, M.R. Qiu, M. O'Neill G, N. Yang, N.M. Verrills, M. Kavallaris and P.W. Gunning, Tissue-specific tropomyosin isoform composition, *J Histochem Cytochem.* **53** (2005), pp. 557-70.

[29] I.R. Nabi, The polarization of the motile cell, *J Cell Sci.* **112 (Pt 12)** (1999), pp. 1803-11.

[30] M. Karakozova, M. Kozak, C.C. Wong, A.O. Bailey, J.R. Yates, 3rd, A. Mogilner, H. Zebroski and A. Kashina, Arginylation of beta-actin regulates actin cytoskeleton and cell motility, *Science.* **313** (2006), pp. 192-6.

[31] I.A. Belyantseva, B.J. Perrin, K.J. Sonnemann, M. Zhu, R. Stepanyan, J. McGee, G.I. Frolenkov, E.J. Walsh, K.H. Friderici, T.B. Friedman and J.M. Ervasti, Gamma-actin is required for cytoskeletal maintenance but not development, *Proc Natl Acad Sci U S A.* **106** (2009), pp. 9703-8.

[32] Y.M. Park, M. Febbraio and R.L. Silverstein, CD36 modulates migration of mouse and human macrophages in response to oxidized LDL and may contribute to macrophage trapping in the arterial intima, *J Clin Invest.* **119** (2009), pp. 136-45.

[33] T. Nagao, C. Qin, I. Grosheva, F.R. Maxfield and L.M. Pierini, Elevated cholesterol levels in the plasma membranes of macrophages inhibit migration by disrupting RhoA regulation, *Arterioscler Thromb Vasc Biol.* **27** (2007), pp. 1596-602.

[34] C. Qin, T. Nagao, I. Grosheva, F.R. Maxfield and L.M. Pierini, Elevated plasma membrane cholesterol content alters macrophage signaling and function, *Arterioscler Thromb Vasc Biol.* **26** (2006), pp. 372-8.

[35] B. Fuhrman, A. Gantman and M. Aviram, Paraoxonase 1 (PON1) deficiency in mice is associated with reduced expression of macrophage SR-BI and

consequently the loss of HDL cytoprotection against apoptosis, *Atherosclerosis.* **211** (2011), pp. 61-8.

[36] T. Kimura, H. Tomura, C. Mogi, A. Kuwabara, A. Damirin, T. Ishizuka, A. Sekiguchi, M. Ishiwara, D.S. Im, K. Sato, M. Murakami and F. Okajima, Role of scavenger receptor class B type I and sphingosine 1-phosphate receptors in high density lipoprotein-induced inhibition of adhesion molecule expression in endothelial cells, *J Biol Chem.* **281** (2006), pp. 37457-67.

[37] F. Wan and M.J. Lenardo, The nuclear signaling of NF-kappaB: current knowledge, new insights, and future perspectives, *Cell Res.* **20** (2010), pp. 24-33.

[38] A. Israel, The IKK complex, a central regulator of NF-kappaB activation, *Cold Spring Harb Perspect Biol.* **2** (2010), pp. a000158.

CHAPTER FIVE

FIGURES

Fig. 5.1: 2D-DIGE analysis of control and HDL treated macrophages

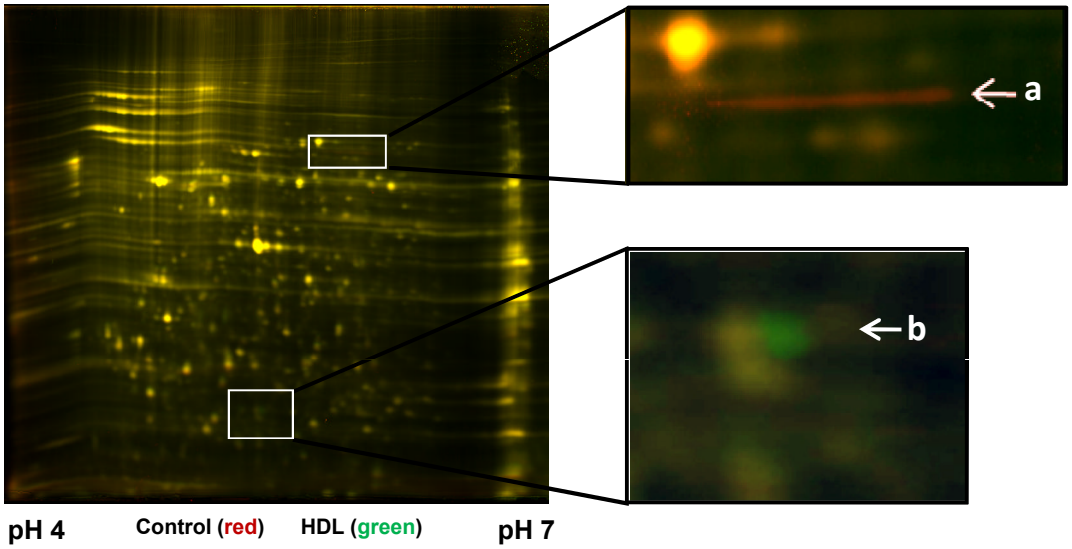
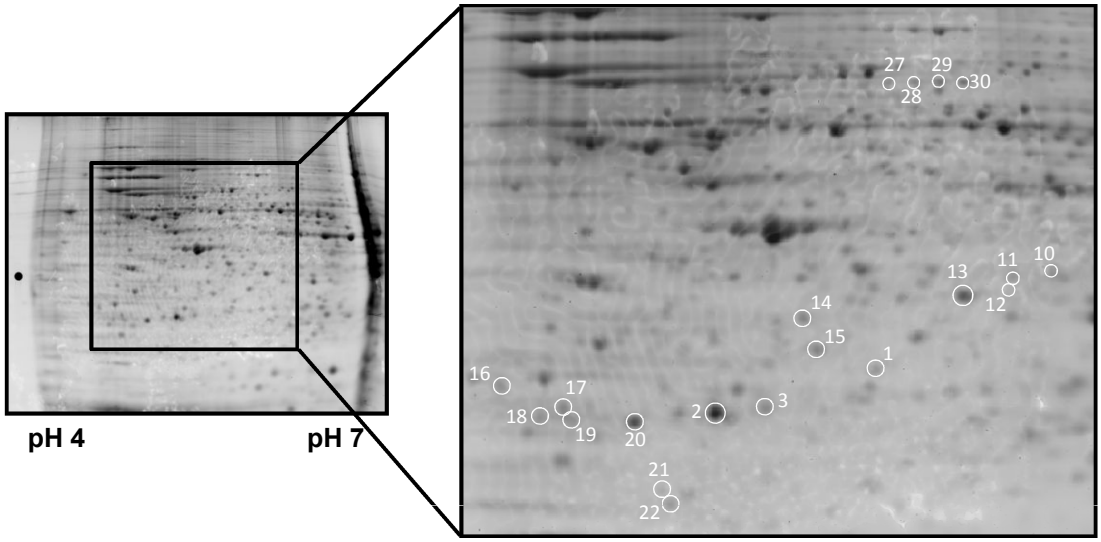


Fig. 5.2 : Deep Purple stained gels of untreated and HDL treated macrophages

A. Control



B. HDL

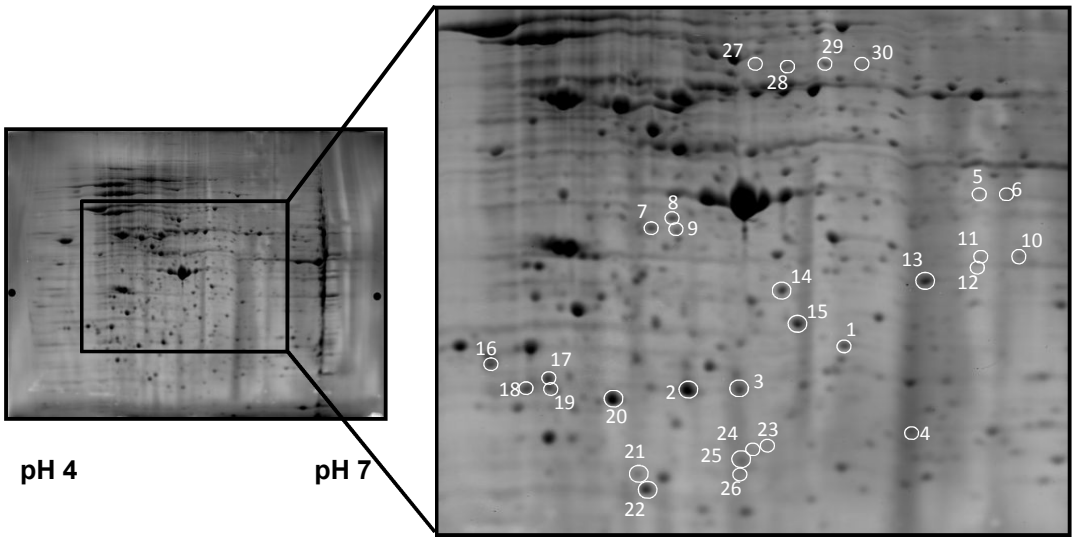


Fig. 5.3 : Validation of selected proteins by immunoblotting.

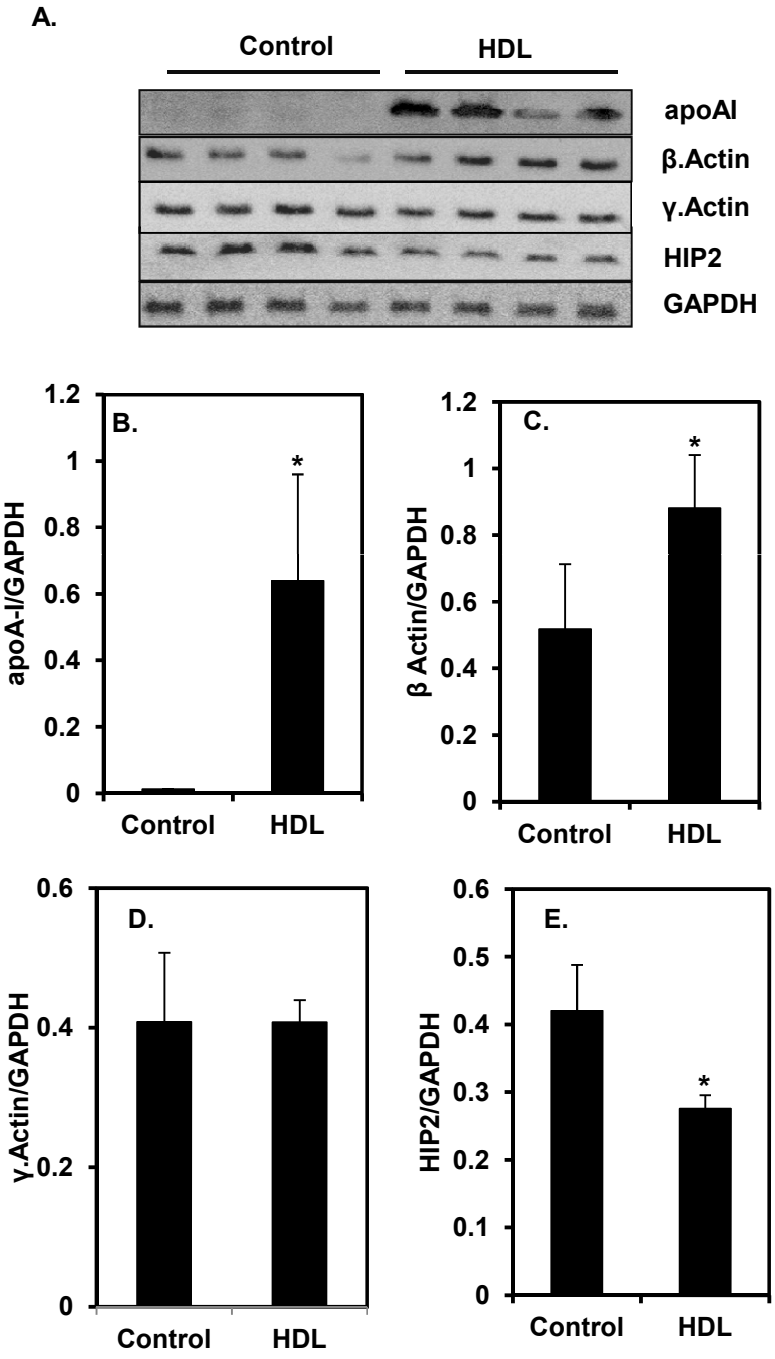


Fig. 5.4 : Immunobltting of E2-25K/HIP2 separated by 2D-lectrophoresis.

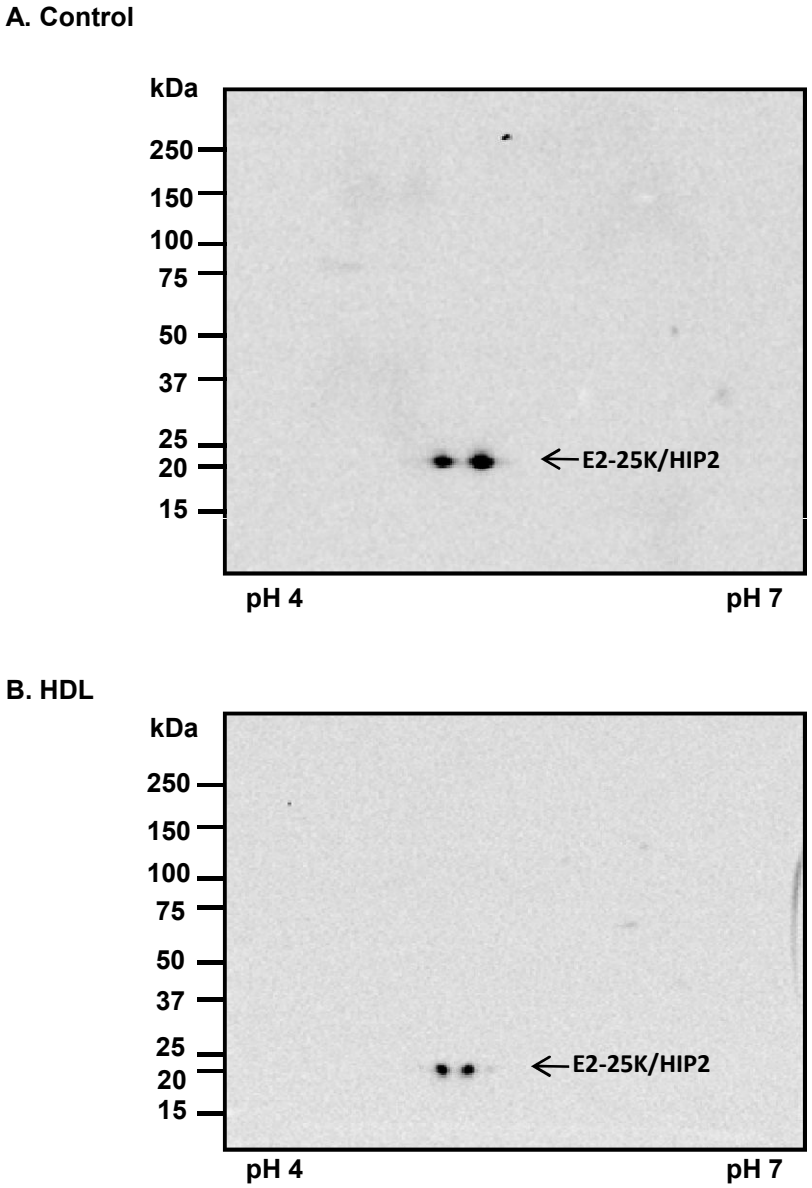
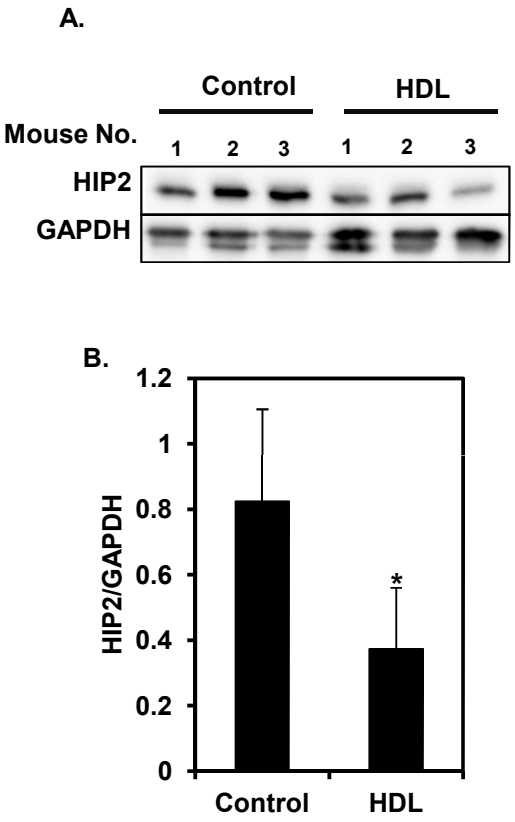


Fig. 5.5: HDL reduces E2-25K/HIP2 protein levels in mouse peritoneal macrophages



CHAPTER FIVE

TABLE

Table 5.1: List of proteins identified from Deep Purple stained gels

Spot number	Accession number	Protein name	Function	MW(kDa) [1]	pI [2]	#Peptides	Score [3]	% Coverage [4] [5]
1	46360168	Prohibitin	Regulation of gene transcription and cell proliferation	30	5.57	9	161	49
	34364645	Immunoglobulin heavy chain constant α	Second most abundant immunoglobulin in human serum	56	6.52	4	65	9
	189053741	C20orf114	Innate immune response to bacteria	53	7.1	2	35	6
2	4757768	Rho GDP dissociation inhibitor 1 isoform a	Prevents dissociation of GDP from Rho-G proteins and prevents their recruitment to the plasma membrane	23	5.02	6	98	19
3	148704620	Proteasome subunit α type 3	A core subunit of 20S proteasomecore structure	30	5.54	4	77	16
4	74212099	Proteasome subunit β type 4	A core subunit of 20S proteasomecore structure	29	5.6	7	111	39
5	74191897	Protein phosphatase methyl transferase I	Methylation of Protein phosphatase 2A	48	6.42	5	68	13
6	26346452	Putative N acetylglucose amine-6-phosohatase deacetylase	Amino acids and carbohydrates metabolism	44	5.78	6	111	20
	117606366	UPM-CMP kinase 2 mitochonderial precursor	Component of the salvage pathway of nucleotide synthesis	50	6.89	7	101	17
	14250208	mevalonatediphosphate decarboxylase	Decarboxylates and dehydrates mevalonate in an early step of cholesterol biosynthesis	44	5.96	4	66	18
7	74218228	Heterogeneous nuclear ribonucleoprotein C1/C2 isoform 1	Splisosome assembly and mRNA splicing	37	6.88	3	53	11
8	74218228	Heterogeneous nuclear ribonucleoprotein C1/C2 isoform 1	Splisosome assembly and mRNA splicing	37	6.88	2	32	6
9	74218228	Heterogeneous nuclear ribonucleoprotein C1/C2 isoform 1	Splisosome assembly and mRNA splicing	37	6.88	6	87	17
	192050	mitochondrial aspartate aminotransferase	L-aspartate:2-oxoglutarate aminotransferase activity	48	9.05	2	22	5

Spot number	Accession number	Protein name	Function	MW(kDa)	pI	#Peptides	Score	% Coverage
10	12805125	Nmi protein	Interacts with 6 out of7 STAT proteins and Augment STAT transcription in response to IL-2 and IFN-γ	35	5.08	4	59	14
11	4063383	Serine/Threonine kinase receptor associated protein	Inhibits apoptosis signal-regulating kinase 1 and suppression of JNK-1 mediated apoptosis	39	4.99	4	59	16
12	74218228	Heterogeneous nuclear ribonucleoprotein C1/C2 isiform 1	Splisosome assembly and mRNA splicing	37	6.88	6	99	19
13	13994195	Serine/Threonine protein phosphatase (PP1)-α catalytic subunit	Protein dephosphorylation and regulation of cellular activity	38	5.94	8	144	27
14	26354210	Serine/Threonine protein phosphatase (PP1)-α catalytic subunit	Protein dephosphorylation and regulation of cellular activity	37	5.84	8	133	23
	26348803	Pyridoxal kinase	Nitamin B6 metabolism	35	5.88	2	33	7
15	148681747	aldose reductase	Development of diabetic retinopathy	38	6.51	2	32	6
17	33416530	Annexin 4A	Binds Annexin V and mutations have been linked to α thalassemia	36	5.43	7	108	28
18	74215924	14-3-3 gamma	Mediates signal transduction by binding to phosphoserine-containing proteins, regulates cell proliferation	35	5.36	8	123	27
19	148676868	14-3-3 zeta / Protein kinase C inhibitor protein 1	Regulation of apoptotic and mitogenic signaling pathways by binding to phosphoserine/phosphotheroniene motifs	29	4.71	5	81	19
20	74191394	14-3-3 beta	Raf activation and augmentation of MAPK signaling pathway	28	4.82	6	106	28
	74215924	14-3-3 gamma	Mediates signal transduction by binding to phosphoserine-containing proteins, regulates cell proliferation	35	5.36	3	42	10
	6756037	14-3-3 eta	Regulation of cellular signaling pathways	28	4.81	2	27	7

Spot number	Accession number	Protein name	Function	MW(kDa)	pI	#Peptides	Score	% Coverage
21	238231384	Proteasome subunit β type 6 precursor	Protein catabolism	25	4.97	2	36	8
22	309264127	Peroxiredoxine type II	Redox homeostasis	22	5.85	2	31	10
23	13385320	vacuolar protein sorting-associated protein 28 homolog	Protien sorting nad traficking	25452	5.36	6	108.99	33
24	7657031	Deoxyribonucleotide transferase	Nucleotide metabolism	23077	5.31	7	125.74	45
25	148705798	E2-25/ Huntington interacting protein2	Apoptosis and inflammation	20873	6.94	9	173.69	62
26	74204414	β-actin and γ-actin	Cellular cytoskeleton components	41886	5.37	4	62.71	12
	21311849	γ-glutamyl cyclotransferase	Amino acids metabolism	21166	5.52	2	36.86	12
27	74184909	ATPase H^+ transporting lysosomal V1	Maintinance of lysosome's acidic pH	68369	5.42	2	34.36	5
28	74137565	Alpha fetoprotein, albumin precursor	Cellular inflammation and apoptosis	68733	5.79	2	25.42	4
29	74137565	Alpha fetoprotein, albumin precursor	Cellular inflammation and apoptosis	68733	5.79	2	32.49	4
30	74137565	Alpha fetoprotein, albumin precursor	Cellular inflammation and apoptosis	68733	5.79	1	16.41	2

[1] MW: Predicted molecular weight based on primary amino acids sequence.
[2] pI: Predicted isoelectric point based on primary amino acids sequence.
[3] # Peptides: The number of peptides detected for each protein.
[4] Score: Refers to protein score in Spectrum Mill software.
[5] % Coverage: Percentage of the published protein sequence covered by the MS/MS peptides sequences.

CHAPTER SIX

FINAL CONCLUSIONS AND EXTENSIONS OF THE WORK

1. Summary

Reverse cholesterol transport is the most well recognized mechanism by which high density lipoprotein (HDL) and its receptor, scavenge receptor class B type I (SR-BI) protect against atherosclerosis (chapter 1 and references therein). HDL also exhibits anti-inflammatory, anti-oxidant and anti-thrombotic properties (reviewed in [1-3]). HDL and SR-BI interaction and the activation of downstream signaling pathways in vascular cells have been suggested as additional mechanisms by which HDL and SR-BI protect against atherosclerosis. Endothelial cells have so far been the center of studying HDL and SR-BI signaling (reviewed in [4]). In contrast HDL and SR-BI signaling in the predominant cell type in atherosclerotic lesions, macrophages, has not been carefully looked at. The aim of this dissertation was to examine the atheroprotective roles of macrophage SR-BI in vivo and in vitro. In this chapter thesis contributions to the field and its implications in future research areas is described.

2. Contributions of the thesis in understanding the atheroprotective actions of high density lipoprotein and its receptor, SR-BI

The lack of SR-BI is associated with the development of spontaneous occlusive coronary artery atherosclerosis in mice lacking apolipoprotein E (apoE), the SR-BI/apoE double knockout (dKO) mice [5], however the exact mechanisms and factors contributing

to disease development are not clearly understood. The findings described in chapter 2 clearly demonstrate the onset of disease development, the role of inflammation and oxidative stress, and suggest a possible model of disease progression in SR-BI/apoE dKO mice (discussed further below). Moreover we demonstrate, for the first time, that occlusive coronary artery atherosclerosis can be reduced by the administration of polyphenolic rich pomegranate extract; possibly by its anti-inflammatory and anti-oxidant properties and effects on lipid metabolism.

Furthermore, discoveries in this work add to our growing knowledge of SR-BI's role in atherosclerosis and focus on SR-BI as a signaling molecule. This work is among the first to demonstrate a role of HDL and SR-BI signaling in macrophages (chapters 3 and 4). The involvement of HDL and SR-BI in macrophage apoptosis, a key event in atherosclerotic plaque development, was examined in chapter 3. HDL and SR-BI protected against macrophage apoptosis. SR-BI protection against macrophage apoptosis in vivo may explain some of the atheroprotective effects of SR-BI in bone marrow derived cells and may implicate its involvement in plaque stability.

Moreover migration of macrophages out of atherosclerotic plaques has been linked to plaque regression [6, 7]. SR-BI transcripts levels were upregulated in plaques undergoing regression [6]. We demonstrate in chapter 4 that HDL stimulated SR-BI mediated signaling results in alterations of the actin cytoskeleton and induces

macrophage migration. These findings provide the framework for future experiments testing the role of SR-BI in plaque regression in vivo.

In addition to testing the short term effects of HDL including post translational modifications of signaling proteins, we were interested in identifying longer term effects of HDL on protein expression in macrophages. Work accomplished in this thesis (chapter 5) identified changes in expression of multiple signaling proteins. Validation experiments of selected targets confirmed some of these changes in both a macrophage cell line and primary macrophages from mice. This sets the stage for further biochemical analyses that will determine the roles of some of these proteins in HDL mediated atheroprotective pathways and provides a valuable contribution to our understanding of the atheroprotective actions of HDL.

3. Characterization of coronary artery atherosclerosis in the SR-BI/apoE dKO mouse model

As discussed earlier, the SR-BI/apoE dKO mouse model has been described as a model of spontaneous occlusive coronary artery atherosclerosis; however features contributing to plaque development in the aortic sinus and in coronary arteries are not fully characterized. The findings in chapter 2 suggest, to the first time, that occlusive coronary artery disease in the SR-BI/apoE dKO mice starts after 3 weeks of age and

imply the involvement of oxidative stress and inflammation in the development of coronary artery atherosclerosis in these mice. Based on these findings the following sequence of events for disease development in these mice is likely to occur: Occlusive coronary artery atherosclerosis, induced presumably by dislipidemia and increased systemic inflammation, results in myocardial ischemia, macrophage infiltration followed by myocardial fibrosis, most likely as a consequence of cell apoptosis. We have shown that the lack of SR-BI sensitizes macrophages to apoptosis. Yet it remains to be established if SR-BI exhibits similar effects in cardiomyocytes. All together these findings significantly add to our understanding of the SR-BI/apoE dKO model of coronary artery disease.

Clinical studies demonstrated that pomegranate juice has cardioprotective effects [8, 9]. One year consumption of pomegranate juice by patients with carotid artery stenosis lead to significant reductions in mean intima-media thickness, serum lipids peroxidation and systolic blood pressure compared to baseline values [8]. These patients were treated for hypercholesterolemia and hypertension throughout the study suggesting an additive or synergistic effects of pomegranate juice [8]. In another study pomegranate juice consumption for three months improved myocardial perfusion and reduced stress induced myocardial ischemia in patient with coronary heart disease [9]. Remarkably coronary artery atherosclerosis, cardiac inflammation, oxidative stress and myocardial fibrosis were reduced by the short term administration of pomegranate extract to the SR-

BI/apoE dKO mice (chapter 2) suggesting that the cardioprotective effects of pomegranate extract could partially be attributed to its anti-inflammatory and anti-oxidant properties. We have demonstrated that pomegranate extract exhibits more potent anti-inflammatory properties when compared to pomegranate juice (chapter 2). The polyphenolic contents in pomegranate extract was reported to be about 36 times higher than in pomegranate juice [10]. Based on this and the encouraging effects of pomegranate juice in patients with coronary artery disease, we anticipate that pomegranate extract may exert more beneficial effects on the cardiovascular system. Once established in humans, this discovery is likely to have potential implications in the treatment of coronary artery atherosclerosis. Indeed continuation of treatments and changes in the lifestyle such as the diet, exercise, smoking habits, etc. will be necessary and complementary to pomegranate extract supplementation. Lastly the findings that polyphenolic rich pomegranate extract had pleiotropic effects including effects on lipoprotein metabolism and inflammation would imply a broader view of polyphenolic rich natural products. Nevertheless it needs to be determined whether these effects are dependent or independent on the anti-oxidant properties of pomegranate extract.

4. HDL and macrophage SR-BI signaling in atherosclerosis

Bone marrow transplantation experiments suggested an atheroprotective role of SR-BI [11-13]. Reduced atherosclerotic lesions and lesional macrophage contents were reported in hypomorphic mice that lacked hepatic SR-BI but expressed normal levels of macrophage SR-BI when compared to whole body deletion of SR-BI [14]. Increased macrophage apoptosis and impaired phagocytic clearance of apoptotic cells are important factors in the development of advanced atherosclerotic lesions (reviewed in [15, 16]). We demonstrate that HDL and SR-BI protected against free cholesterol loading induced macrophage apoptosis (chapter 3). This involved the activation of multiple signaling pathways downstream of SR-BI. The lack of SR-BI in bone marrow derived cells, was associated with increased cellular apoptosis and necrotic areas in plaques in vivo, features of plaque instability (chapter 3). These findings provide one possible mechanism by which macrophage SR-BI protects against atherosclerosis and may contribute to plaque stability. Although this study answered important questions it generated many others. For instance would SR-BI protect macrophages from other apoptosis inducers such as oxidized low density lipoproteins, endoplasmic reticulum stress inducers, tunicamycin or thapsigargin, and UV irradiation? Is lipid transfer activity of SR-BI involved in this process? Is HDL mediated protection against apoptosis confined to certain HDL subpopulations? What is/are the active component(s) in HDL responsible for the observed effects? What is the role of SR-BI mediated phagocytosis and clearance of

apoptotic cell debris? Answers to these questions will indeed add to our understanding of the atheroprotective actions of SR-BI.

As discussed earlier, induced SR-BI expression and increased macrophage migration were reported in atherosclerotic lesions undergoing regression [6]. HDL regulation of monocyte-derived cell's migration in vivo was reported as a mechanism by which HDL promotes atherosclerotic plaque regression [17]. However the molecular mechanisms are not clearly understood. Studies described in chapter 4 demonstrate the involvement of multiple pathways in HDL stimulated macrophage migration and indicate that HDL induces macrophage migration in a manner that is dependent on the HDL receptor, SR-BI, its adaptor protein, PDZK1 and sphingosine-1-phosphare receptor 1(S1PR1). Importantly the lack of SR-BI reduced protein levels of S1PR1 suggesting for the first time that SR-BI expression may influence steady state levels of other plasma membrane proteins. While this work provides valuable contributions in understanding the role of HDL and SR-BI signaling in macrophages and identifies novel functions of SR-BI, it uncovered many other interesting research areas. Specifically; determination of cross talk between HDL and SR-BI stimulated signaling pathways. The nature of interactions between SR-BI and S1PR1, whether it is a direct physical or regulatory interaction, needs further investigations. It also remains to be determined if SR-BI interacts with other plasma membrane proteins in its vicinity and/or affect their functions.

5. HDL and SR-BI signaling in other pathophysiological conditions.

In addition to atherosclerosis the involvement of SR-BI in was reported in other metabolic and inflammatory diseases [18-23]. Whole body deletion of SR-BI was associated with increased susceptibility to cecal ligation and puncture induced septic death in mice [21]. The role of macrophage SR-BI in protecting against inflammation was suggested as a possible mechanism [21]. In addition to mediating systemic clearance of lipopolysccaharides (LPS) [22, 23], SR-BI reduced LPS-induced macrophage inflammation via the suppression of Toll-like receptor 4 signaling pathway [21]. Moreover SR-BI role in adaptive immunity has recently been characterized [24]. Mice lacking SR-BI had impaired lymphocytes homeostasis characterized by increased inflammation and active T and B cells subpopulations [24]. These mice also developed autoimmune disorder as indicated by increased levels of circulating autoantibodies, renal immune complex deposition and leukocyte infiltration [24]. All together these studies suggest a role of SR-BI in cellular and systemic inflammation and suggest the possible involvement of SR-BI in autoimmune disorders.

Interestingly an association between SR-BI gene variants and lipid profiles of patients with type2 diabetes was reported in the Framingham study [25]. Serum capacity to induce SR-BI mediated cholesterol efflux was significantly reduced in diabetic patients with renal complications which could potentially accelerate the development of cardiovascular disease in these patients [18]. Single nucleotide polymorphism in SR-BI

gene, Scarb1, was associated with insulin resistance in sex dependent manner [19]. Further studies will reveal the exact role of SR-BI in diabetes and its associated complications including nephropathy. Additionally HDL cholesterol levels and SR-BI function were associated with obesity and adipocytes physiology [26]. Interestingly adipocytes were shown to contribute to cholesterol efflux in vivo and ex vivo studies demonstrated the involvement of SR-BI and ATP-binding cassettes transporter A1 (ABCA1) in the process [20]. Insulin and angiotensin II increased HDL cholesterol ester uptake by adipocytes in a manner that was dependent on SR-BI [27]. Importantly, HDL via SR-BI increased triglycerides contents in adipocytes implicating their involvement in lipogenesis [27]. However signaling pathways controlled by HDL and SR-BI in adipocytes remain unclear. An important feature of obesity is increased inflammation and macrophage infiltration in the adipose tissue (reviewed in [28]). Given importance of HDL and SR-BI in adipose tissue homeostasis and their role in macrophage migration it is tempting to speculate that HDL induced macrophage migration may contribute to its effects on tissue inflammation. Certainly this possibility and the direct effects of HDL on adipocytes inflammation remain to be tested. In conclusion studying HDL and SR-BI signaling in macrophages has significant implications in inflammatory diseases that could lead to atherosclerosis. Mapping these signaling pathways in normal physiology or dysfunctional pathophysiology will set framework for future experiments and will provide insights into the molecular mechanisms of disease development.

6. HDL and macrophage proteome: The road a head

Based on the work described here and by others we realized the importance of having a larger scale image of HDL effects on macrophages. Therefore we examined HDL effects on protein expression by macrophages. Studies in (chapter 5) identified several HDL protein targets that could potentially be implicated in its anti-inflammatory and anti-apoptotic properties. Confirmation of changes in proteins levels of actin and ubiquitin conjugating enzyme 2-25K (E2-25K) / Huntington interacting protein2 (HIP2) is an encouraging step towards pursuing further biochemical studies to test their possible involvement in HDL induced migration (chapter 4) or protection against apoptosis (chapter 3) and inflammation, respectively. Evaluation of other protein targets may reveal the involvement of HDL in previously uncharacterized signaling pathways, such as amino acid and nucleotide metabolism. Identifying the role of SR-BI in these functions of HDL will represent a new area for research.

In conclusion, with the recently reported involvement of SR-BI/CLA-1 in patients with coronary heart disease and the involvement of HDL and SR-BI in the progression and/or regression of atherosclerosis, a better understanding of the role of SR-BI and HDL in disease development is required. The body of this work identified novel functions of HDL and SR-BI in macrophages and opened doors for many other future research areas. These discoveries will certainly add to the growing knowledge of the atheroprotective roles of SR-BI in mice which could possibly be implicated in HDL and

SR-BI functions in humans. Finally, the involvement of HDL and SR-BI signaling in macrophages could be implicated in other pathophysiological conditions in addition to atherosclerosis.

7. References

[1] A.M. Scanu and C. Edelstein, HDL: bridging past and present with a look at the future, *Faseb J.* **22** (2008), pp. 4044-54.

[2] F. Tabet and K.A. Rye, High-density lipoproteins, inflammation and oxidative stress, *Clin Sci (Lond).* **116** (2009), pp. 87-98.

[3] M. Florentin, E.N. Liberopoulos, A.S. Wierzbicki and D.P. Mikhailidis, Multiple actions of high-density lipoprotein, *Curr Opin Cardiol.* **23** (2008), pp. 370-8.

[4] A. Al-Jarallah and B.L. Trigatti, A role for the scavenger receptor, class B type I in high density lipoprotein dependent activation of cellular signaling pathways, *Biochim Biophys Acta.* **1801** (2010), pp. 1239-48.

[5] A. Braun, B.L. Trigatti, M.J. Post, K. Sato, M. Simons, J.M. Edelberg, R.D. Rosenberg, M. Schrenzel and M. Krieger, Loss of SR-BI expression leads to the early onset of occlusive atherosclerotic coronary artery disease, spontaneous myocardial infarctions, severe cardiac dysfunction, and premature death in apolipoprotein E-deficient mice, *Circ Res.* **90** (2002), pp. 270-6.

[6] E. Trogan, J.E. Feig, S. Dogan, G.H. Rothblat, V. Angeli, F. Tacke, G.J. Randolph and E.A. Fisher, Gene expression changes in foam cells and the role of chemokine receptor CCR7 during atherosclerosis regression in ApoE-deficient mice, *Proc Natl Acad Sci U S A.* **103** (2006), pp. 3781-6.

[7] J. Llodra, V. Angeli, J. Liu, E. Trogan, E.A. Fisher and G.J. Randolph, Emigration of monocyte-derived cells from atherosclerotic lesions characterizes regressive, but not progressive, plaques, *Proc Natl Acad Sci U S A.* **101** (2004), pp. 11779-84.

[8] M. Aviram, M. Rosenblat, D. Gaitini, S. Nitecki, A. Hoffman, L. Dornfeld, N. Volkova, D. Presser, J. Attias, H. Liker and T. Hayek, Pomegranate juice consumption for 3 years by patients with carotid artery stenosis reduces common carotid intima-media thickness, blood pressure and LDL oxidation, *Clin Nutr.* **23** (2004), pp. 423-33.

[9] M.D. Sumner, M. Elliott-Eller, G. Weidner, J.J. Daubenmier, M.H. Chew, R. Marlin, C.J. Raisin and D. Ornish, Effects of pomegranate juice consumption on myocardial perfusion in patients with coronary heart disease, *Am J Cardiol.* **96** (2005), pp. 810-4.

[10] M. Aviram, N. Volkova, R. Coleman, M. Dreher, M.K. Reddy, D. Ferreira and M. Rosenblat, Pomegranate phenolics from the peels, arils, and flowers are antiatherogenic: studies in vivo in atherosclerotic apolipoprotein e-deficient (E 0) mice and in vitro in cultured macrophages and lipoproteins, *J Agric Food Chem.* **56** (2008), pp. 1148-57.

[11] S.D. Covey, M. Krieger, W. Wang, M. Penman and B.L. Trigatti, Scavenger receptor class B type I-mediated protection against atherosclerosis in LDL

receptor-negative mice involves its expression in bone marrow-derived cells, *Arterioscler Thromb Vasc Biol.* **23** (2003), pp. 1589-94.

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

[13] M. Van Eck, I.S. Bos, R.B. Hildebrand, B.T. Van Rij and T.J. Van Berkel, Dual role for scavenger receptor class B, type I on bone marrow-derived cells in atherosclerotic lesion development, *Am J Pathol.* **165** (2004), pp. 785-94.

[14] T. Huby, C. Doucet, C. Dachet, B. Ouzilleau, Y. Ueda, V. Afzal, E. Rubin, M.J. Chapman and P. Lesnik, Knockdown expression and hepatic deficiency reveal an atheroprotective role for SR-BI in liver and peripheral tissues, *J Clin Invest.* **116** (2006), pp. 2767-76.

[15] I. Tabas, Consequences and therapeutic implications of macrophage apoptosis in atherosclerosis: the importance of lesion stage and phagocytic efficiency., *Arterioscler Thromb Vasc Biol.* **25.** (2005), pp. 2255-64.

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

[17] J.E. Feig, J.X. Rong, R. Shamir, M. Sanson, Y. Vengrenyuk, J. Liu, K. Rayner, K. Moore, M. Garabedian and E.A. Fisher, HDL promotes rapid atherosclerosis

regression in mice and alters inflammatory properties of plaque monocyte-derived cells, *Proc Natl Acad Sci U S A.* **108** (2011), pp. 7166-71.

[18] H. Zhou, K.C. Tan, S.W. Shiu and Y. Wong, Cellular cholesterol efflux to serum is impaired in diabetic nephropathy, *Diabetes Metab Res Rev.* **24** (2008), pp. 617-23.

[19] J.J. McCarthy, A. Somji, L.A. Weiss, B. Steffy, R. Vega, E. Barrett-Connor, G. Talavera and R. Glynne, Polymorphisms of the scavenger receptor class B member 1 are associated with insulin resistance with evidence of gene by sex interaction, *J Clin Endocrinol Metab.* **94** (2009), pp. 1789-96.

[20] Y. Zhang, F.C. McGillicuddy, C.C. Hinkle, S. O'Neill, J.M. Glick, G.H. Rothblat and M.P. Reilly, Adipocyte modulation of high-density lipoprotein cholesterol, *Circulation.* **121** (2010), pp. 1347-55.

[21] L. Guo, Z. Song, M. Li, Q. Wu, D. Wang, H. Feng, P. Bernard, A. Daugherty, B. Huang and X.A. Li, Scavenger Receptor BI Protects against Septic Death through Its Role in Modulating Inflammatory Response, *J Biol Chem.* **284** (2009), pp. 19826-34.

[22] K.S. Michelsen, M.H. Wong, P.K. Shah, W. Zhang, J. Yano, T.M. Doherty, S. Akira, T.B. Rajavashisth and M. Arditi, Lack of Toll-like receptor 4 or myeloid differentiation factor 88 reduces atherosclerosis and alters plaque phenotype in

mice deficient in apolipoprotein E, *Proc Natl Acad Sci U S A.* **101** (2004), pp. 10679-84.

[23] L. Cai, A. Ji, F.C. de Beer, L.R. Tannock and D.R. van der Westhuyzen, SR-BI protects against endotoxemia in mice through its roles in glucocorticoid production and hepatic clearance, *J Clin Invest.* **118** (2008), pp. 364-75.

[24] H. Feng, L. Guo, D. Wang, H. Gao, G. Hou, Z. Zheng, J. Ai, O. Foreman, A. Daugherty and X.A. Li, Deficiency of Scavenger Receptor BI Leads to Impaired Lymphocyte Homeostasis and Autoimmune Disorders in Mice, *Arterioscler Thromb Vasc Biol.* **(Ahead of print)** (2011), pp.

[25] D. Osgood, D. Corella, S. Demissie, L.A. Cupples, P.W. Wilson, J.B. Meigs, E.J. Schaefer, O. Coltell and J.M. Ordovas, Genetic variation at the scavenger receptor class B type I gene locus determines plasma lipoprotein concentrations and particle size and interacts with type 2 diabetes: the framingham study, *J Clin Endocrinol Metab.* **88** (2003), pp. 2869-79.

[26] J. Aron-Wisnewsky, Z. Julia, C. Poitou, J.L. Bouillot, A. Basdevant, M.J. Chapman, K. Clement and M. Guerin, Effect of bariatric surgery-induced weight loss on SR-BI-, ABCG1-, and ABCA1-mediated cellular cholesterol efflux in obese women, *J Clin Endocrinol Metab.* **96** (2011), pp. 1151-9.

[27] L. Yvan-Charvet, A. Bobard, P. Bossard, F. Massiera, X. Rousset, G. Ailhaud, M. Teboul, P. Ferre, G. Dagher and A. Quignard-Boulange, In vivo evidence for a

role of adipose tissue SR-BI in the nutritional and hormonal regulation of adiposity and cholesterol homeostasis, *Arterioscler Thromb Vasc Biol.* **27** (2007), pp. 1340-5.

[28] K.E. Wellen and G.S. Hotamisligil, Obesity-induced inflammatory changes in adipose tissue, *J Clin Invest.* **112** (2003), pp. 1785-8.