MACROPHAGE SR-BI AND ATHEROSCLEROSIS

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A Thesis

Submitted to the School of Graduate Studies

in Partial Fulfillment of the Requirements

for the Degree

Master of Science

McMaster University

MASTER OF SCIENCE (2006) McMaster University
(Biochemistry and Biomedical Sciences) Hamilton, Ontario

TITLE: Macrophage SR-BI and Atherosclerosis

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NUMBER OF PAGES: xi, 97

Abstract

The Scavenger Receptor, Class B, Type I (SR-BI) is an integral membrane protein whose expression in the liver is critical to reverse cholesterol transport by mediating the selective uptake of HDL-derived cholesterol. SR-BI is expressed in a variety of tissues including bone marrow derived macrophages and foam cells in atherosclerotic lesions. We have explored the effect of eliminating SR-BI in leukocytes on advanced stages of atherosclerotic plaque development in apoE KO mice. We observed statistically significant cardiomegaly as a result of the elimination of SR-BI in bone marrow derived cells compared to controls (P=0.02). We report that the elimination of SR-BI in bone marrow derived cells in apoE KO mice induced to undergo atherosclerosis by feeding a high fat diet for four weeks leads to no significant difference in cross-sectional atherosclerotic plaque area at the aortic root ($4.9 \pm 0.9\times10^4 \mu m^2$ when SR-BI-/- apoE-/- → apoE-/- [n=9] and $5.5 \pm 0.9\times10^4 \mu m^2$ when SR-BI +/+ apoE-/- → apoE -/- [n=12], P=0.68) or plaque volume through the aortic sinus ($1.8 \pm 0.3\times10^7 \mu m^3$ when SR-BI-/- apoE-/- → apoE-/- [n=9] and $1.9 \pm 0.3\times10^7 \mu m^3$ when SR-BI +/+ apoE-/- → apoE -/- [n=12], P=0.69). We demonstrate that macrophage SR-BI protein expression can be decreased by cholesterol associated with lipoproteins. Furthermore, we report that in Raw 264.7 macrophage-like cells the expression of SR-BI can also decrease in response to glucosamine treatment. The expression of SR-BI is decreased significantly in cells overexpressing SR-BI (ldlA[mSR-BI] cells [P=0.003]) due to treatment with glucosamine with increased protein mobility. We support this finding by demonstrating that this difference may be the result of altered glycosylation.

Acknowledgments

Foremost, I would like to extend my sincere gratitude to my supervisor, Dr. Bernardo L. Trigatti. His guidance, enthusiasm, and motivation have influenced me professionally and personally. I have learned volumes about the scientific process from him and he has constantly challenged me to push my limits by continually "raising the bar." I am grateful to my committee members Dr. Geoff Werstuck and Dr. Jonathon Bramson for their support and valuable suggestions throughout my graduate work. I am also grateful for the opportunity to collaborate with Dr. Werstuck and with Dr. Suleiman Igdoura. The potential of side-projects like these are my driving force.

My Fulbright Fellowship would not have been possible without Dr. Peter Whyte and Dr. Nancy Krucher, and I am grateful for the inspiration they have given me as a scientist and for their unyielding support and encouragement.

I am grateful to have known Dr. Trigatti's lab members, particularly Scott Covey in appreciation for his guidance and Rachelle Brunet for the camaraderie, support, and atmosphere. I would also like to thank lab members Dan Kim, Ali Rizvi, Ayesha Ahmed, Yi Zhang, Xing Chen, Rebecca Pierce, and Heather Grey who have all helped make this experience enjoyable and vibrant.

I extend my sincere gratitude and appreciation to my parents, Raffaele Tedesco and Susan Tedesco who taught me the value of hard work by their own example and for their sacrifice in support of me. Their strength, motivation, and support have been instrumental in my achieving this goal. I would also like to thank my grandmother, Edna Tedesco for her friendship, support, and for always making me feel loved. I would like to thank my brother and best friend, Richard Tedesco, for always making me laugh. I am also grateful to my mother, Janet Tedesco, for providing me with the tenacity to complete this task.

I am also grateful to Barbra and Jan DuBois and the Ciavarra Family for providing me a warm and loving home away from home. This would not be complete without me offering my heart-felt thanks to Gianni Ciavarra for his partnership and for the inspiration and moral support he provided throughout my research and graduate experience. His ability to keep science in perspective and kind manner kept me grounded.

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List of Abbreviations

ABCA1	ATP-binding cassette Transporter I
apoA-I	Apolipoprotein A-I
apoA-II	Apolipoprotein A-II
apoC-III	Apolipoprotein C-II
apoE	Apolipoprotein E
ATF6	Activating Transcription Factor 6
BM	Bone Marrow
CETP	Cholesteryl Ester Transfer Protein
CHO	Chinese Hamster Ovary
CLA-1	CD36- and LIMPII analogous 1
CM	Chylomicrons
ER	Endoplasmic Reticulum
ERAD	Endoplasmic Reticulum-Associated Degradation
GFAT	Glutamine:Fructose-6-phosphate Amidotransferase
GLUTs	Glucose Transport Proteins
GRP78	Glucose regulated protein 78
GRP94	Glucose regulated protein 94
HDL	High Density Lipoproteins
HMG-CoA reductase	3-hydroxy-3-methylglutaryl coenzyme A reductase
IDL	Intermediate Density Lipoproteins
IRE1	Inositol-Requiring Transmembrane Kinase/Endoribonuclease

List of Abbreviations (continued)

KO	Knockout
LDL	Low Density Lipoprotein
ldlA7	Mutant Chinese Hamster Ovary (CHO) cell line lacking a functional LDL receptor, clone 7
ldlA[mSR-BI]	ldla7 cells over-expressing murine SR-BI
LDLR	Low Density Lipoprotein Receptor
LCAT	Lecithin-Cholesterol Acyltransferase
LXR	Liver X Receptor
NCLPDS	Lipoprotein Deficient Newborn Calf Serum
PERK	PKR-like ER Kinase
SCAP	SREBP Cleavage-Activating Protein
S1P	Site-1 Protease
S2P	Site-2 Protease
SR-A	Scavenger Receptor, Class A
SR-BI	Scavenger Receptor Class B type I
SREBPs	Sterol Regulatory Binding Proteins
UPR	Unfolded Protein Response
VLDL	Very Low Density Lipoproteins

1. Introduction

1.1 Plasma Lipoproteins and Atherosclerosis

Atherosclerosis is a complex multifactorial disease [1], a major reason for heart disease and stroke, and a leading cause of death in the developed world. In 2002 the World Health Organization estimated that 16.7 million people worldwide die each year of cardiovascular disease, accounting for one third of all deaths globally [2]. Elevated cholesterol levels in the form of low density lipoproteins, diabetes, hypertension, and smoking are the major risk factors correlated with atherosclerosis [3]. Plasma lipoproteins play critical roles in the formation and progression of atherosclerotic lesions. High blood glucose causes metabolic abnormalities that induce vascular dysfunction and predisposes diabetics to atherosclerotic plaque development [4].

Lipoproteins are soluble complexes that transport cholesterol in the circulation. Although the composition of plasma lipoproteins is highly dynamic, the general structure is similar. They consist of a core of neutral lipids (predominantly cholesteryl esters and triacylglycerol) surrounded by a monolayer of amphipathic lipids (phospholipids and unesterified cholesterol) and apolipoproteins [5].

Lipoproteins are separated into classes based on their buoyancy by density gradient ultracentrifugation [6]. They are classified as chylomicrons (CM), very low density lipoproteins (VLDL), low density lipoproteins (LDL), and high density lipoproteins (HDL). Chylomicrons have the lowest protein to lipid ratio and are the least

dense while HDL have the highest protein to lipid ratio and are therefore the most dense [5].

Chylomicrons are lipoproteins that transport dietary lipids (primarily triacylglycerol) and are synthesized in the intestine [5]. Chylomicrons are metabolized by lipoprotein lipase into chylomicron remnants which are cleared by the liver [7]. VLDL are assembled in the liver for the transport of endogenous triacylglycerols [5]. Lipoprotein lipase converts VLDL into intermediate density lipoproteins (IDL) which are subsequently converted into LDL by hepatic lipase [7]. LDL is the major lipoprotein which transports cholesteryl esters to peripheral tissues. HDL is formed in plasma either by the assembly of cholesterol/phospholipids effluxed by cells with protein components such as apoA-I [5]. HDL mediates the delivery of cholesterol back to the liver for metabolism or excretion and to steroidogenic tissues for steroid biosynthesis, a process called reverse cholesterol transport [8]. HDL cholesterol can also be reassembled into larger lipoproteins (i.e. VLDL and LDL) by the cholesteryl transfer protein (CETP) in the liver [5].

Lipids associated with HDL originate in the liver and peripheral tissues while HDL protein components are synthesized in the liver and intestine [5]. The principle apolipoprotein associated with HDL is apoA-I [9]. Minor protein components include apoA-II, apoC-II, and apoE [7]. apoA-I is an exchangeable apolipoprotein that spontaneously interacts with phospholipids to form discoidal HDL complexes [5]. The subsequent addition of cholesterol to discoidal phospholipid-rich HDL leads to its maturation [9]. Partially lipidated apoA-I matures into spherical HDL via cholesterol

esterification by lecithin-cholesterol acyltransferase (LCAT) in the plasma, and HDL particles are processed and remodeled by CETP, phospholipid transfer protein, and hepatic lipase [8]. Discoidal HDL and mature spherical HDL are the major fraction found in plasma [10,11].

Epidemiological evidence from the Framingham Heart Study indicated that low levels of plasma HDL is an independent risk factor for coronary heart disease, and implied that elevated levels of plasma HDL was inversely related to this risk [12]. Potential mechanisms by which HDL could protect against atherosclerosis include reverse cholesterol transport, protection against endothelial dysfunction, and inhibition of oxidative stress [13].

High levels of low density lipoproteins (LDL) are directly correlated with risk for heart disease [14,15]. Atherogenic lipoproteins such as LDL become trapped in the sub-endothelial space of artery walls, called the intima [16], and can be modified by means of oxidation, glycation, aggregation, or association with proteoglycans leading to smooth muscle and endothelial injury [17,18]. In response to retained modified lipoproteins circulating bone marrow derived monocytes are recruited to the intima [19]. Additionally, modified LDL is chemotactic for other monocytes and can stimulate the inflammatory response recruiting more macrophages to the artery wall [20]. Inside the sub-endothelial space, monocytes differentiate into macrophages [21]. Modified lipoproteins are internalized by macrophages [22,23]. These phagocytic cells internalize large quantities of oxidized LDL and therefore large quantities of cholesterol without negative feedback regulation [24]. The accumulation of cholesteryl esters from

atherogenic lipoproteins results in the formation of macrophage foam cells, the hallmark of an atherosclerotic plaque [25]. Macrophages are the major cellular component of both early and advanced atherosclerotic lesions [26].

1.2 Regulation of Cholesterol Homeostasis

Cholesterol levels are controlled by numerous processes including reverse cholesterol transport [27,28], *de novo* synthesis [29], lipoprotein uptake [30], and cholesterol esterification [31]. Mammalian cells control cholesterol homeostasis by regulating transcription factors called sterol regulatory element binding proteins (SREBPs) [32]. SREBPs are synthesized as transmembrane precursors in the endoplasmic reticulum (ER) membrane [32,33], and they form complexes with SREBP cleavage-activating protein (SCAP). In the absence of sterols, SCAP escorts SREBP to the Golgi apparatus where SREBP is cleaved by Site-1 protease (S1P) and Site-2 protease (S2P) [32,33]. This generates a fragment of SREBP which travels to the nucleus and activates more than 35 genes, including those whose products are involved in cholesterol synthesis and lipid uptake [34]. Among the genes transcribed are the low density lipoprotein receptor (LDLR) [34] and 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase) [35]. The increased expression of the LDLR gene in the absence of sterols results in increased LDLR expression on the cell surface and increases the internalization of LDL from plasma, increasing cellular cholesterol levels and lowering LDL cholesterol in the plasma [36]. Additionally, HMG-CoA reductase (the rate limiting

enzyme in cholesterol biosynthesis) leads to increased production of endogenous cholesterol [35]. As cellular sterol levels increase, the SREBP/SCAP complex is retained in the ER membrane through the binding of SCAP to ER retention proteins called Insigs [37,38]. This prevents cleavage of SREBP by S1P and S2P. Thus, the active fragment of SREBP is not released and cannot enter the nucleus to stimulate the transcription of genes including LDLR and HMG-CoA reductase in the presence of sterols. This leads to attenuated cholesterol synthesis and uptake in the presence of sterols [38].

1.3 Scavenger Receptors

Scavenger receptors are cell surface transmembrane proteins that bind a broad variety of ligands including chemically modified lipoproteins, modified proteins, and sulfated polysaccharides [27], and several of them have been shown to play critical roles in lipoprotein metabolism and atherosclerosis [39]. These receptors were first characterized in cultured macrophages as mediators of cholesterol uptake from modified lipoproteins, leading to the formation of lipid-loaded macrophages that resemble foam cells in atherosclerotic lesions [40].

Scavenger receptors are characterized based on structural features and are divided into classes. Among the many classes of scavenger receptors are class A and class B. Class A scavenger receptors (SR-A) were first cloned from cDNA in macrophages and are homotrimeric integral membrane proteins that are structurally characterized by an elongated extracellular domain composed of an alpha-helical coiled coil region and a collagenous domain [41-43]. The SR-A gene encodes two scavenger receptor proteins

generated by translation of alternatively spliced transcripts [41,44]. SR-AI and SR-AII bind acetylated LDL and oxidized LDL [24]. Class B scavenger receptors have two transmembrane domains [27]. This family includes CD36 which binds oxidized LDL [45,46]. Additional members of this family are the scavenger receptor class B type I (SR-BI) [47] and its splice variant SR-BII [48]. The human homologue of SR-BI is CD36- and LIMPII analogous 1 (CLA-1) [49].

1.4 Scavenger Receptor class B type I

1.4.1 SR-BI expression and binding partners

SR-BI has 30% sequence homology to CD36 [50], and it is an integral membrane protein that is heavily glycosylated and is approximately 82kDa in size [51]. SR-BI has a horseshoe-like membrane topology composed of a short intracellular *N*-terminus, a large extracellular portion, and a relatively short *C*-terminus that is intracellular [27,50]. SR-BI is highly expressed in steroidogenic tissues, adipocytes, and in hepatocytes [47,52-54]. Expression of SR-BI is also found in endothelial cells, macrophages, and in foam cells of atherosclerotic lesions [55-59].

SR-BI binds a wide array of ligands including HDL [52], and several apolipoproteins associated with HDL, namely apoA-I [60,61], apoA-II, apoC-III [61], and apoE [62]. SR-BI also binds VLDL [63], and native or modified LDL [47,64], yet its binding to modified lipoproteins does not lead to foam cell formation [65]. SR-BI also binds protein-free lipid vesicles containing anionic phospholipids [66], maleylated bovine

serum albumin [47], and advanced glycation endproduct modified proteins [67]. Additionally, SR-BI has been reported to bind apoptotic thymocytes [64] and phosphatidylserine in apoptotic testicular Sertoli cells [68]. The diversity of binding partners associated with SR-BI suggests multiple functions of the receptor.

1.4.2 SR-BI and Selective Uptake

SR-BI expression in the liver is crucial for normal murine lipoprotein metabolism [69,70]. One pathway by which hepatocytes can take up cholesterol from HDL and other lipoproteins is mediated by SR-BI [52,71]. SR-BI mediates the selective uptake of cholesteryl esters from HDL in hepatocytes without net internalization of the lipoprotein [39,50]. HDL-derived cholesterol in the liver is secreted into the bile, used for bile acid synthesis, or packaged and secreted in newly synthesized lipoproteins. This leads to the clearance of plasma HDL cholesterol and ultimately to whole-body elimination of excess cholesterol in bile [28,72]. Selective uptake therefore appears to be important for the clearance of HDL derived cholesterol [70].

A definitive role for SR-BI in HDL metabolism and reverse cholesterol transport *in vivo* has been demonstrated using different transgenic and knockout mouse models. Overexpression of SR-BI in liver reduced HDL cholesterol levels, increased reverse cholesterol transport [73,74], and decreased susceptibility to atherosclerosis [69,75,76]. Atherosclerosis reduction associated with hepatic SR-BI overexpression may be the result of an increase in HDL-derived cholesterol clearance [69,74-76]. As SR-BI can also bind lipoproteins such as LDL and VLDL [47], it is possible that SR-BI might influence atherosclerosis by influencing the levels of these atherogenic lipoproteins [69]. In

contrast, the genetic suppression of SR-BI activity in mice leads to increased susceptibility to diet-induced atherosclerosis [77]. Additionally, increased diet-induced atherosclerosis has been reported in SR-BI/LDLR double knock out mice [78], and in LDLR knockout mice with an attenuated SR-BI expression [79]. Genetic suppression of SR-BI on an apoE knockout background leads to hypercholesterolemia, dramatically accelerates the onset of atherosclerosis, and is lethal [80,81]. The mice die between six to eight weeks of age with characteristics much like human coronary heart disease; this includes occlusive atherosclerosis, myocardial infarction and cardiac dysfunction [80,81]. SR-BI apoE double knockout mice have an abnormally high heart to body weight ratio which is 1.6 -1.8 fold greater than apoE knockout mice, and this cardiomegaly was the result of heart damage and compensatory hypertrophy [80,81].

1.4.3 SR-BI in Hematopoietic-Derived Cells

In addition to an atheroprotective role in hepatocytes, SR-BI expression in macrophages appears to play a role in protection against atherosclerosis [78,82,83]. This was shown initially by our laboratory using tissue specific elimination of SR-BI expression [78]. Bone marrow transplantations (using either SR-BI knockout or wild-type mice as donors) were performed on lethally irradiated LDL receptor knockout mice to generate mice with selective elimination of SR-BI in bone marrow derived cells. Atherosclerosis was induced by feeding the mice a high fat diet for four months. The aortas of LDLR KO mice have an increased amount of atherosclerotic plaque coverage when fed a high fat diet [84]. This study demonstrated that the selective elimination of SR-BI in bone marrow derived cells resulted in increased atherosclerosis [78], and

implied that the expression of SR-BI in bone marrow derived cells leads to atheroprotection. Others confirmed this finding using bone marrow specific elimination of SR-BI in apoE knockout mice fed a chow diet. The mice that received SR-BI knockout bone marrow had a 1.4-fold increase in cross-sectional area of plaque in the aortic sinus compared to wild type controls [83]. Interestingly, another study in which SR-BI was eliminated in bone marrow derived cells in LDLR knockout animals indicated a possible dual role for SR-BI. At four weeks on a high fat diet, there was an increase in cross-sectional area of plaque in the aortic sinus due to the presence of bone marrow derived SR-BI while at 9-12 weeks, there was a decrease in cross-sectional area of atherosclerotic plaque due to the presence of bone marrow derived SR-BI [82]. When SR-BI was eliminated from bone marrow derived cells in wild type mice fed a high cholesterol diet containing cholate for eight weeks, there was also an increase in cross-sectional area of plaque in the aortic sinus due to the presence of bone marrow derived SR-BI [82]. The goal of this study is to determine the effect of a lack of macrophage SR-BI at various stages of atherosclerotic development in apoE KO mice.

1.4.4 SR-BI and Cholesterol Homeostasis

SR-BI is a major determinant of murine plasma HDL concentrations [52,70,73,85], and therefore plays an important role in cholesterol homeostasis. Sequence analysis of the human SR-BI promoter revealed a 9-bp sequence containing an E box [86], an element which was previously shown to bind SREBP-1a [87,88]. SR-BI expression in macrophages appears to be regulated by sterols, however this point is

somewhat controversial [56,89,90]. In culture, macrophage SR-BI expression is decreased in response to cholesterol loading from lipoproteins and 25-hydroxy-cholesterol [90]. A recent study demonstrated SR-BI expression was elevated in human macrophage foam cells in response to modified lipoproteins [91]. Furthermore, there are conflicting reports regarding the binding of the SREBP transcription factor to the SR-BI promoter [88,90]. The effect of native lipoprotein derived cholesterol on SR-BI expression is not clear.

1.5 Cholesterol Efflux from Macrophages and Atheroprotection

The efflux of cholesterol from macrophages is an important first step in reverse cholesterol transport that plays a critical role in HDL-mediated atheroprotection. HDL has been shown to remove cholesterol from cells [9,92,93], and cholesterol efflux from macrophages can protect the artery wall from cholesterol overload. *In vitro* studies indicated an increase in cholesterol efflux to discoidal HDL (an HDL cholesterol acceptor) which appeared to be related to over-expression of SR-BI [55,94]. Murine SR-BI (mSR-BI) was over-expressed in Chinese Hamster Ovary (CHO) cells with a mutant LDL receptor (ldlA clone 7), and there was a statistically significant increase in efflux of [^{3}H] cholesterol tracer to cholesterol acceptors in ldlA[mSR-BI] cells compared to ldlA7 controls [55]. It has also been reported that efflux to HDL was dependent on lipoprotein binding to SR-BI [95]. This suggests that SR-BI expression promotes the efflux of cholesterol to HDL. It has been hypothesized that SR-BI expression in macrophages also

promotes the efflux of cholesterol to HDL, thereby proposing a mechanism where SR-BI protects against atherosclerosis by protecting artery walls from cholesterol accumulation.

The theory that SR-BI plays a role in macrophage cholesterol efflux is intriguing, yet this point is controversial. Two reports cite no significant decreases in efflux when SR-BI expression was eliminated in primary macrophages [78,83], and another report cited no significant macrophage cholesterol efflux due to SR-BI or to the ATP-binding cassette transporter A1 (ABCA1) in J774 cells [96]. Meanwhile, two other studies show minor decreases in cholesterol efflux to HDL when SR-BI was eliminated from primary macrophages [82,97]. Definitive biological data supporting SR-BI expression and macrophage cholesterol efflux is lacking.

Interestingly, the overexpression of murine SR-BI in these cells has also been linked to the accumulation of HDL-derived cholesterol without net internalization of the protein component [52]. ldlA[mSR-BI] cells were incubated with HDL and this led to an overall increase in HDL derived cholesteryl esters within the cells [52]. This implied that SR-BI led to an overall influx of cholesterol in ldlA[mSR-BI] cells, and it demonstrates that net flux is dependent on the cholesterol concentration gradient.

SR-BI is among a group of proteins including those of the ATP-binding cassette (ABC) super-family of proteins implicated in macrophage cholesterol efflux. ABC proteins are membrane transporters that use ATP hydrolysis to transport solute molecules against a concentration gradient [98]. Among the ATP-binding cassette proteins, ABCA1 is the most well-understood efflux protein [72,99]. It has been demonstrated that macrophage-expressed ABCA1 mediates the efflux of cholesterol to lipid-free apoA-I

[99], an exchangeable protein component predominantly associated with HDL. ABCA1 has been shown to preferentially bind free apoA-I and apoA-I associated with discoidal HDL rather than binding to mature, spherical, lipid-rich HDL particles [10,11]. ABCA1 expression also increases in response to macrophage cholesterol loading [100-102]. Conversely, SR-BI preferentially binds spherical, lipid-rich HDL particles [103].

Proteins within the ABCG family have also been implicated in cholesterol efflux. Recently, ABCG1 which is highly expressed in macrophages has been shown to efflux cholesterol to partially lipidated HDL among other lipoprotein and non-lipoprotein acceptors [104]. ABCG1 expression is regulated by an oxysterol activated transcription factor called liver X receptor (LXR) [105]. Additionally, ABCG4 is expressed in the bone marrow and in macrophages [106] and has been be implicated in cholesterol efflux in many cell types [104]. It has been hypothesized that ABCG4 may play a role in macrophage efflux [104]. Other macrophage efflux proteins that have been identified include the moesin-like HDL binding protein [107].

1.6 Glucose Transport

Glucose is hydrophilic and cannot penetrate the lipid bilayer. It is transported into cells by Glucose Transport Proteins (GLUTs) whose genes are regulated by hyperglycemia, insulin, and cellular translocation. GLUT1, 3, and 5 are expressed in macrophages and foam cells [108,109]. GLUT3 is expressed in a variety of cells including platelets and macrophages and is the predominant transporter for glucose [110,111]. One major response of tissues to insulin is the recruitment of GLUTs,

including GLUT3, to the cell surface [112,113]. Reduced rates of insulin-mediated glucose uptake characterize insulin resistance [114,115]. Glucose-induced insulin resistance involves the impaired recruitment of (GLUTs) to the cell surface without a change in total cell number of transporters [116,117]. Hyperglycemia negatively affects insulin secretion and insulin action [115,118].

The metabolism of glucose can lead to the hexosamine biosynthesis pathway [119]. Upon entry into non-hepatic cells, glucose is phosphorylated by hexokinase. Glucose-6-phosphate can then be converted into fructose-6-phosphate. Through the hexosamine pathway, fructose-6-phosphate and glutamine are ultimately converted into glucosamine-6-phosphate by the enzymatic actions of glutamine:fructose-6-phosphate amidotransferase (GFAT). This is the rate-limiting step of the hexosamine pathway [120]. Through this pathway, glucose is converted to glucosamine [121]. Acetylation leads to the production of *N*-acetylglucosamine-6-phosphate which is a component of many molecules including glycoproteins [121]. High levels of glucose [122], and free fatty acids [123] can accelerate the hexosamine pathway.

It has been postulated that glucosamine and the hexosamine pathway play an important role in the development of insulin resistance [121,123,124]. High levels of blood glucose lead to insulin resistance by accelerating this pathway [121,125,126]. Glucosamine is more potent than glucose for the impairment of glucose transporters reaching the cell surface [117]. Glucosamine is widely used to accelerate the hexosamine pathway flux, independently of glucose [114,122,127-132].

1.7 Interference with Glycosylation and ER stress

Many proteins require *N*-linked glycans for folding and transport out of the ER but not necessarily for biological function [133]. The oligosaccharides in glycoproteins have several functions including facilitation of protein folding, protection against proteolysis, direction of intracellular trafficking and secretion, participation in intermolecular interactions, and the control of cell surface expression and activity [133-135]. Glucosamine is a necessary component for glycosylation as *N*-linked glycans are attached to proteins through *N*-acetylglucosamine [120]. Although the observed mass of SR-BI based on immunoblot and immunoprecipitation experiments is ~82 kDa, the mass predicted from the receptor's deduced primary amino acid sequence is ~57 kDa [52]. This is due to extensive *N*-glycosylation of SR-BI which occurs both cotranslationally and posttranslationally [51]. SR-BI is initially synthesized with multiple high mannose *N*-linked oligosaccharide chains, with the mature protein containing both complex as well as hybrid and/or high mannose chains [51]. There are 11 potential sites for *N*-linked glycosylation on mSR-BI; two of which affect SR-BI expression and appear to have an important role in ER folding and/or intracellular transport [136].

A variety of conditions or agents (including altered protein glycosylation, calcium depletion, reductive stress, and others) have been shown to interfere with the proper folding of proteins in the ER leading to disruption of the ER processing system [137-139], resulting in a condition termed ER stress [138] which leads to the accumulation of unfolded or misfolded proteins in the ER. Excess glucosamine can inhibit protein glycosylation [140]. When *N*-linked glycosylation is prevented, proteins typically

misfold, aggregate, and are retained in the ER [141]. Improperly folded proteins accumulated in the ER can induce an adaptive mechanism called the unfolded protein response [142].

1.8 The Unfolded Protein Response

In response to ER stress, the synthesis of chaperones (i.e. GRP78) is induced at the transcriptional level [143] through an intracellular signaling pathway from the ER to the nucleus, called the unfolded protein response (UPR) [144,145]. GRP78 is induced when cells are glucose-starved [146,147], treated with tunicamycin [148] or glycoprotein-containing viruses [149,150]. Unsalvageable proteins are targeted for translocation back to the cytosol for proteosomal degradation [151-153].

GRP78 negatively regulates the three signaling mechanisms that activate the UPR. GRP78 binds the luminal domain of the ER stress sensors inositol-requiring transmembrane kinase/endoribonuclease (IRE1) and PKR-like ER kinase (PERK), inhibits their dimerization, and maintains them in an inactive state [154,155]. GRP78 also binds the activating transcription factor 6 (ATF6) and prevents its translocation to the Golgi apparatus for processing and activation [156,157]. In the presence of misfolded proteins, GRP78 dissociates from the sensors and binds the misfolded proteins thereby releasing the sensors and leading to their activation [154].

The oligomerization of PERK leads to autophosphorylation and subsequent phosphorylation of the alpha subunit of the eukaryotic translation initiation factor 2 (eIF2α) [158,159]. This leads to transient inhibition of the translation initiating factor

eIF2α and a general decrease in translation thereby limiting the new protein load in the ER [160]. IRE1 is activated by dimerization and autophosphorylation. When IRE1 is activated, its endoribonuclease activity snips a pre-existing substrate mRNA which subsequently leads to the activation of a transcription factor X-box-binding protein (XBP-1) [155,161]. XBP-1 upregulates genes involved in ER-associated degradation (ERAD) and the retrotranslocation of proteins [162,163] thereby limiting the protein load of the ER. The UPR also leads to the trafficking of ATF6 to the Golgi from the ER where it is cleaved by S1P and S2P, the same proteases that cleave SREBP [32,33,164]. This leads to the release of a cytosolic domain that subsequently enters the nucleus and transactivates the genes encoding ER chaperone proteins (GRP78) to alleviate the protein-folding load on the ER [144,156,164].

1.9 Mammalian Response Downstream of ER Stress/UPR

Mammalian cells also respond to the UPR by the activation of programmed cell death or apoptosis [165-167]. This appears to be a result of an inability to maintain ER homeostasis as the result of severe ER stress. Another consequence of ER stress is the activation of SREBP [139]. ER stress has been shown to lead SREBP activation and subsequent promotion of lipid accumulation in human aortic smooth muscle cells and hepatocytes [139]. This dysregulation of SREBP may lead to dysregulation of multiple pathways involved in lipid metabolism [139,168], and indicates a link between ER stress and cholesterol homeostasis.

Interestingly, there appears to be a correlation between ER stress and atherosclerotic lesion development in apoE KO mice [169], and it has most recently been shown that hyperglycemia is associated with tissue-specific ER stress and accelerated atherosclerosis in streptozotocin-induced apoE KO mice [170].

The presence of SR-BI in bone marrow derived macrophages may protect against atherosclerotic plaque formation in apoE KO mice, yet the effects of eliminating SR-BI in later stages of plaque development in apoE KO mice is unknown. Additionally, the effects of ER stress and increased cellular glucosamine levels on SR-BI expression in macrophages are not known. This thesis attempts to address some of these questions using bone marrow transplantation in mouse models of atherosclerosis and cell culture models.

2. Materials and Methods

2.1 Materials

Reagents and chemicals are detailed in Table 1.

Table 1. Reagents and Suppliers

Chemical or Reagent (Supplier)	
2,2,2-tribromoethanol (Sigma)	2-methylbutane (Fisher Scientific)
2-methylbutanol (Sigma)	Agarose (Bioshop)
APMSF (Sigma)	ApoA-1 antibody (Sigma)
Aprotinin (Sigma)	NH_4Cl (BDH, Inc.)
β-actin antibody (MP Biomedicals, Inc.)	Bicinchoninic Acid Assay (BCA)
Bovine serum albumin (Sigma)	$CaCl_2$ (BDH, Inc.)
CD36 antibody (Cascade Biosciences)	Cell strainer, 100m (Flacon)
Crystalmount (Biomeda)	Cycloheximide (Sigma)
DMSO (Sigma)	DTT (Roche)
EDTA (EMD)	Ethidium Bromide (Bioshop)
Fetal Bovine Serum (Hyclone)	Filter Flask, 0.22m (Nalgene)
37% formaldehyde (Caledon)	10% formalin (Fisher Scientific)
Glucosamine (Sigma)	Glucose (Sigma)
Glycine (Bioshop)	Heparin (Sigma)
HRP conjugated Donkey∝Goat IgG (Jackson Immunoresearch)	High Fat Diet (Dyet's, Inc.)
HRP conjugated Donkey∝Mouse IgG (Jackson Immunoresearch)	Hepes (Bioshop)
HRP conjugated Donkey∝Rabbit IgG (Jackson Immunoresearch)	Iscove's Medium (Gibco)
Infinity Cholesterol Liquid Stable Reagent (Thermo Electron Corp.)	Jell-O (Kraft Foods, Inc.)
$KHCO_3$ (EMD)	KCl (JT Baker)
KH_2PO_4 (BDH, Inc.)	KBr (Bioshop)
KDEL antibody (Stressgen Biotechnologies)	$KHCO_3$ (EM Science)
Leupeptin (Sigma)	L-glutamine (Gibco)
$MgSO_4$ (BDH, Inc.)	$MgCl_2$ (EM Science)
Mannitol (Sigma)	Mayer's Hematoxylin (Fluka)
NaCl (Bioshop)	$NaHCO_3$ (EMD)
$NaPO_4$ (BDH, Inc.)	Newborn Calf Serum (Gibco)
N-glycosidase F (Roche)	Nutrical (Evsco)
Nucleospin Blood Quick Pure Isolation Kit (BD Biosciences)	Oil Red O (Sigma)
Penicillin/Streptomycin (Gibco)	Pep A (Sigma)
PVDF (Perkin Elmer)	Septra (Novopharm)
Shandon Cryomatrix (Thermo Electron Corp.)	Sucrose (Bioshop)
SR-BI antibody, 400-101 (Novus Biologicals)	Tris (Bioshop)
Triton-X 100 (Sigma)	Tween 20 (Sigma)
Western Lightning Chemiluminescent Reagent (Perkin Elmer)	

2.2 Methods

2.2.1 Mice

Experimental protocols involving mice were in approved by McMaster University's Animal Research Ethics Board. SR-BI+/- apoE knockout (KO) mice with a mixed C57BL/6J:129 agouti background were originally obtained from Dr. Monty Krieger (Massachusetts Institute of Technology). These mice were mated to generate SR-BI-/- apoE-/- (SR-BI/apoE double KO) and control SR-BI+/+ apoE-/- mice. ApoE KO mice on a C57BL/6J background were originally obtained from The Jackson Laboratories. Mice were bred and housed in micro-isolator cages in the Barrier facility of the Central Animal Facility at McMaster University (Hamilton, Ontario). The mice were provided food and water *ad libitum*. Food was either low fat chow diet or an atherogenic high fat diet (Dyets, Inc: 21% butterfat, 0.15% cholesterol), as indicated.

2.2.2 Preparation of Bone Marrow Recipient Mice

Seven days prior to scheduled bone marrow transplant, 9 week old female apoE KO recipient mice were separated into individual sterile micro-isolator cages and acclimatized to the antibiotic Septra (40mg/mL sulfamethoxazole and 8mg/mL trimethoprim), which is widely used to block internal folic acid production in the gut (final concentrations: 1.25mg/mL trimethoprim and 0.25mg/mL sulfamethoxazole in sterile water) [171,172]. Each day the mice were provided with sterile pelleted food and sterile powdered food soaked with Septra-water and mixed with Nutrical, a calcium-rich

nutritional supplement paste. The mice were also given approximately 6mL (½ cube) strawberry Jell-O prepared with Septra and sterile water. The mice remained on this Septra regimen for one month post transplant. The mice were monitored twice daily and were euthanized in accordance with the endpoint policies of McMaster University.

2.2.3 Harvest of Bone Marrow from Donor Mice

Mice were euthanized by carbon dioxide asphyxiation, and using sterile instruments, a superficial incision was made into the abdomen leaving the peritoneal membrane intact. Femurs and tibias were dissected free of muscle and extraneous tissue and were placed in ice cold Medium A (Iscove's medium containing 2% Heat-Inactivated FBS supplemented with 2mM L-glutamine and 50U/mL penicillin- 50 µg/mL streptomycin). Under aseptic conditions, the ends of the bones were snipped and the bone marrow was flushed out with Medium A (as described above) using a one milliliter syringe with a 23G1 needle for femurs and a 25G5/8 needle for tibias. The bone marrow cells were dispersed by passage and expulsion through an 18G1½ needle five times followed by subsequent dispersions using a 20G1½, 23G1, 25G5/8, and 26G½ needles five times each. Cells were then filtered using a 100µm sterile cell strainer. Bone marrow cells were pelleted by centrifugation at 500×g for 10 minutes and either resuspended in Medium A for subsequent counting and immediate transplant or resuspended in Heat-Inactivated FBS with 10% DMSO and frozen at -20°C for one hour, -80°C overnight, then in liquid nitrogen for later use. At the time of transplant, bone marrow was thawed and a small sample of bone marrow derived cells was diluted 1:10 in sterile ACK

(150mM NH_4Cl, 10mM $KHCO_3$, 0.1M EDTA. pH 7.2-7.4) to lyse erythrocytes and cells were counted using a hemacytometer. Concentrations were adjusted with Medium A as indicated.

2.2.4 Irradiation and Bone Marrow Transplantation

To determine the appropriate lethal irradiation dose for 10 week old female apoE KO mice, groups of mice were exposed to 9, 10, or 11Gy of $^{137}Cs\gamma$ irradiation (Gammacell 3000). Two thirds of the dose was administered during a first session and the remaining third was administered three hours later [78]. Bone marrow recipient mice were lethally irradiated with 11Gy in the same manner using the same source. Immediately following irradiation, 6×10^6 donor bone marrow cells were injected via the tail vein.

2.2.5 Induction of Atherosclerosis

Four weeks after transplantation the mice were fed an atherogenic, high fat western-type diet to accelerate the development of atherosclerosis [173,174]. Mice were fasted overnight, and plasma and tissue samples were subsequently prepared as described previously [81].

2.2.6 Blood Collection/Plasma and Serum Preparation

Mice were anaesthetized by intraperitoneal (IP) injection using 2.5% avertin [81]. Four weeks after transplant and at harvest, mice were fasted overnight and blood was collected via tail vein under anesthesia into heparinized microtubes. At harvest blood was

collected by cardiac puncture into microtubes containing 1μL of the anticoagulant heparin ($10\times^4$U heparin/mL PBS). The blood was centrifuged at 14,000rpm at 4°C in a Spectrafuge microcentrifuge (Labnet) for five minutes to separate blood cells from the plasma. Plasma was stored at 4°C or used immediately for lipoprotein separation and cholesterol analysis. Cells were either stored at -20°C or used immediately for DNA isolation.

2.2.7 Genotyping

Genomic DNA was isolated as described previously from tail biopsies of pups from SR-BI+/- apoE-/- breeding pairs [175]. A minimum of 50μL (packed volume) of blood cells were used for DNA isolation using a NucleoSpin Blood Quick Pure kit (BD Biosciences). Resultant DNA was diluted 1:100 and 1:20 in ultra-pure H_2O and genotyping was determined by multiplex PCR (see Table 2 for primers) [70]. The PCR reaction was initiated by denaturation at 94°C for 2 minutes, annealing at 57°C for two minutes, and extension at 65°C for 5 minutes. This was followed by forty cycles of denaturation at 94°C for 30 seconds, annealing at 57°C for 30 seconds and extension at 65°C for 90 seconds. Final extension was carried out at 65°C for 10 minutes. The ramping speed was 1°/second. PCR products were separated on a 1% agarose gel containing 0.5μg/mL ethidium bromide. Wild-type and mutant alleles of SR-BI were visible as 1.9kb and 1.4kb bands, respectively and heterozygotes exhibited both the 1.9kb and 1.4kb amplicons (Table 2) [70,78].

Table 2. Primer sequences and amplicon size information for SR-BI genotyping

Allele	Primer Name	Primer Pair (5'-3')	Amplicon Size (bp)
SR-BI Wild-Type	oDT44	TGA AGG TGG TCT TCA AGA GCA GTC CT	1900
	oDT66	TAT CCT CGG CAG ACC TGA GTC GTG T	
SR-BI Mutant	oDT44	TGA AGG TGG TCT TCA AGA GCA GTC CT	1400
	oSi75	GAT TGG GAA GAC AAT AGC AGG CAT GC	

2.2.8 Analysis of Plasma and Lipoprotein Cholesterol

The volume of plasma prepared from each mouse was determined. Plasma or purified HDL (see below) was separated by gel filtration chromatography using an AKTA FPLC with a Superose 6 HR 10/30 column and eluted (154mM NaCl, 1mM EDTA, pH 8.0) into 250μL fractions [78]. Total cholesterol analyses were performed using a coupled spectrophotometric enzymatic assay comprised of cholesterol esterase, cholesterol oxidase, and peroxidase (Infinity Cholesterol Liquid Stable Reagent Kit). Each fraction was mixed and 100μL from each fraction was added to a 96 well plate. 200μL cholesterol reagent was added to each sample. After incubation at 37°C for 30 minutes, absorbance values were determined at 500nm using a 96 well plate reader. A standard curve of absorbance vs. cholesterol concentration was used to determine the total cholesterol in each sample.

2.2.9 Tissue Collection

An incision was made into the abdominal cavity and up through the thorax to open the ribs which were then pinned back to allow access to the thoracic cavity. The circulatory system of each mouse was gravity perfused with 10mL ice cold PBS (0.14M NaCl, 2.7mM KCl, 15mM Na_2PO_4, 1.5mM KH_2PO_4, pH 7.5) containing 1mM EDTA pH 8.0 (elevated one meter above the heart) using a winged butterfly needle (23G1x0.75" needle, 0.4mL tubing) inserted into the left ventricle of the heart (outflow from the right atrium). The heart and intact aorta were dissected from the mouse and extraneous tissue was removed under a dissecting scope. The aorta was dissected from the heart proximal to the emergence of the brachiocephalic and carotid artery branches and then fixed in 10% formalin and stored at room temperature. Excised hearts were rinsed with PBS and incubated for 30 min at room temperature in Kreb-Henseleit buffer (118mM NaCl, 4.7mM KCl, 1.2mM KH_2PO_4, 1.2mM $MgSO_4$, 25mM $NaHCO_3$, and 11mM glucose), then fixed in 10% formalin for 24hrs at 4°C. The hearts were then sliced in half horizontally and rinsed in PBS for 24hrs at 4°C. The samples were incubated in 30% sucrose in PBS for 24hrs at 4°C and then frozen in Cryomatrix in a dry ice/2-methylbutane bath and stored at -80°C for subsequent sectioning. The lobes of the liver were divided into four equal quantities, placed in cryovials, snap-frozen in liquid nitrogen, and stored at -80°C.

2.2.10 Histology

Cross sections (10μm) of the hearts were generated using a cryotome (ThermoShandon). Sections were collected onto microscope slides (Aptex treated slides from Hamilton

Health Sciences Histology Lab) starting at the aortic root and moving distally. Sections were fixed with 37% formaldehyde, stained with Oil Red O and counterstained with Mayer's Hematoxylin as previously described [176] and mounted with Crystalmount.

2.2.11 Analysis of Atherosclerotic Plaque

Digital images of stained slides were obtained in brightfield at 5× magnification using a Zeiss Axiovert 200M inverted microscope (Carl Zeiss, Inc) fitted with an Axiocam digital color camera. The section at the aortic root showing three complete aortic valves attached was assigned as the first section (0μm) (see Figure 6A). That section and serial sections at 80μm intervals distal to that section were analyzed. Cross-sectional areas of atherosclerotic lesions in each section were quantified using the Axiovision 3.1 software. The total atherosclerotic lesion cross-sectional area in a given section was calculated as the sum of the cross-sectional areas of each of the individual atherosclerotic lesions in that section [81]. The average atherosclerotic plaque volume in a 320μm long segment of the aortic sinus beginning at the aortic root and extending distally was calculated as follows: The cross-sectional area of atherosclerotic plaque measured in a given section was taken as the average area for a segment extending 40μm in either direction. The volume was calculated as the average area × linear distance (80μm). This was done for sections taken at 80μm intervals (see above) and summed to obtain the average atherosclerotic plaque volume over 320μm.

2.2.12 DiIAcLDL Uptake Assay

Cells to be assayed were washed in Dulbecco's Modified Eagle's Medium (DMEM) containing 50ug/mL penicillin/streptomycin, 2mM L-glutamine, and 0.5% bovine serum albumin (Media B). Media B was supplemented with 5μg/mL 1'1'-dioctadecyl-3,3,3',3'-tetramethylindo iodide (DiI) labeled AcLDL in the dark. Cells were incubated in the dark at 37°C for 2 hrs. The media was removed and cells were washed twice in ice-cold complete PBS (PBS containing 0.5mM $MgCl_2$ and 0.68mM $CaCl_2$) containing 0.5%BSA and then washed with warm complete PBS +0.5%BSA [52]. DiI fluorescence was detected using a Zeiss Axiovert 200 fluorescent microscope (Carl Zeiss, Inc.).

2.2.13 Preparation of Lipoprotein Deficient Serum

Potassium bromide was dissolved in ice-cold Newborn Calf Serum to adjust its density to 1.215g/mL (50.5g KBr/150ml serum). The serum was then subjected to ultracentrifugation in Quickseal ultracentrifuge tubes (Beckman 342414 1×3 ½ polyallomer tube) at 55,000rpm for 52 hours at 4°C (Ti70 rotor) [177,178], to float lipoproteins up to the top of the tube. Lipoprotein-deficient serum was collected from the bottom half of the tube and was dialyzed (12,000-14,000Da cut off) eight times at 4°C against 0.9% sodium chloride for a minimum of 4 hours. The protein concentration of the serum was determined and adjusted to 70mg/ml with 0.9% sodium chloride and sterilized by filtration through a 0.22μm pore size filter. Aliquots were stored at -20°C.

2.2.14 Preparation of Lipoproteins from Human Plasma

Citrated human plasma was obtained from the laboratory of Dr. F. Ofosu (McMaster University, Hamilton, Ontario) and EDTA was added to 3mM. All procedures were conducted at 4°C. Potassium bromide was added to adjust its density to 1.019g/mL (2.86g KBr/150mL plasma). The plasma was subjected to ultracentrifugation in Quickseal ultracentrifuge tubes (Beckman 342414 1×3 ½ polyallomer tube) at 55,000rpm for 15.2 hours (Ti70 rotor) to isolate and subsequently remove VLDL and chylomicrons. The plasma containing LDL and HDL was recovered from the bottom (approximately 50%) of the tubes. The density of the recovered plasma was adjusted to 1.063g/mL with KBr and it was subjected to ultracentrifugation as described above. LDL was recovered from the top (approximately 25%) of the tubes. HDL remained within the plasma in the bottom (approximately 50%) of the tubes, and was collected separately. This ultracentrifugation step was repeated to remove residual LDL. Fresh butylated hydroxytoluene (20mM) was added to the remaining plasma, the density was adjusted to 1.215g/mL with KBr, and samples were subjected to ultracentrifugation as described above. HDL was recovered from the top of the tubes. Lipoproteins were dialyzed as described above against 0.9%NaCl containing 3mM EDTA pH 7.4, saturated with N_2 (g). Protein concentrations were determined as described above.

2.2.15 Cell Culture

2.2.15.1 Maintenance

Raw 264.7 (murine monocyte/macrophage cells) were cultured in DMEM supplemented with 5% heat-inactivated FBS, 2mM L-glutamine, and 50μg/mL penicillin/streptomycin (Media C) and were passaged by scraping. The ldlA7 mutant Chinese Hamster Ovary (CHO) cell line lacks a functional LDL receptor (LDLR) due to a mutation in the LDLR gene [179]. ldlA[mSR-BI] cells are ldlA7 cells that overexpress murine SR-BI [47]. ldlA7 and ldlA[mSR-BI] cells were cultured in HAMS F12 media supplemented with 5% heat-inactivated FBS, 2mM L-glutamine, and 50μg/mL penicillin/streptomycin (Media D). All cells were maintained in a humidified incubator at 37°C with 5% CO_2.

2.2.15.2 Experimental Treatment Conditions

Prior to each experiment cell culture media was replaced with media containing either 10% FBS or 3% NCLPDS. Raw 264.7 cells and ldlA[mSR-BI] cells were maintained in either 10% heat-inactivated FBS or 3% NCLPDS (see Preparation of Lipoprotein Deficient Serum), in DMEM and HAMS F12, respectively. Where indicated, media was supplemented with the following: glucosamine, glucose, mannitol, DTT, and/or cycloheximide (concentrations and time-points as indicated).

2.2.15.3 Preparation & Differentiation of Bone Marrow Derived Macrophages in Culture

Bone marrow, isolated as described above, was plated in DMEM supplemented with 10% heat-inactivated FBS, 50ug/mL penicillin/streptomycin, 2mM L-glutamine (Media E) for 2hrs to allow the adherence of differentiated macrophages. Suspended cells were then re-plated in Media E for 12hrs to allow for the adherence of fibroblasts. The remaining cells in suspension were removed and cultured for seven days in the presence of 20% L-cell conditioned media which contains macrophage colony stimulating factor [180-182]. To prepare L-cell conditioned medium, murine L929 cells were propagated in Media C for one week without reaching confluence [183]. The media was removed, filter sterilized, and stored at -20°C.

2.2.16 Preparation of Cell Lysates

Cells were washed twice with ice cold PBS and then scraped on ice in lysis buffer (0.2× PBS containing 0.1% Triton-X 100) and containing protease inhibitors (20μg/mL aprotinin, 10μg/mL leupeptin, 1mM APMSF, and 10μg/mL pepstatinA). Lysates were then centrifuged at 14,000rpm in a Spectrafuge microcentrifuge at 4°C for 15 minutes to remove nuclei and cellular debris. Protein concentrations in the supernatants were determined using the bicinchoninic acid (BCA) assay using BSA as a standard. Where indicated, lysates were treated with N-glycosidase F (1U/50μg lysate) for 16hr at 37°C.

2.2.17 Western Blotting

Equal amounts of protein were solubilized by boiling 5 minutes in sample buffer containing 2%SDS [w/v], 10%glycerol [v/v], 100mM dithiothreitol, 0.1% bromophenol blue [w/v], and 50mM Tris-HCl pH 6.8. The samples were subjected to SDS-PAGE (Separating gel: 12% acrylamide [30:1 bis-acrylamide], 0.4M Tris-HCl pH 8.8, 0.1%SDS. Stacking gel: 4% acrylamide [30:1 bis-acrylamide], 0.13M Tris-HCl pH 6.8, 0.1% SDS) in electrophoresis tank containing running buffer (50mM Tris, 196mM glycine, and 0.1% SDS) [184]. Samples were electrophoretically transferred to activated PVDF membrane using a transfer buffer consisting of 14.4mM Tris pH 8.3, 122mM Glycine, 20% methanol and an Idea Scientific transfer apparatus at 24V for 45 minutes [185]. Non-specific protein binding sites on membranes were blocked by incubation for one hour in 5% non-fat dry milk in PBST (PBS containing 0.01% Tween-20). Membranes were then incubated for one hour at room temperature with primary antibodies at the following concentrations (β-actin, 1:50,000. CD36, 1:1000. ε-COP (a generous gift from Dr. M. Krieger [MIT]), 1:5000. KDEL, 1:1000. SR-BI 400-101, 1:1000 all in PBST). Membranes were washed three times for 10 min each in PBST and then incubated with secondary antibodies conjugated to horseradish peroxidase (HRP) anti-IgG (donkey α rabbit, donkey α mouse, or rabbit α goat, where appropriate) for one hour in PBST at room temperature. The membranes were then washed twice for 10 min with PBST followed by one wash with PBS. HRP activity was detected using Western Lightning Chemiluminescence Reagent. Images were captured on autoradiography film

and/or using a Kodak Imagestation 440CF. Net intensity of bands was quantified on digital images using Kodak 1D 3.5 image analysis software.

2.2.18 Preparation of Thioglycollate Elicited Murine Peritoneal Macrophages

Murine peritoneal macrophages (MPM) were elicited, collected, and cultured as described previously [186]. Mice were injected intraperitoneally with 1mL of sterile 10% thioglycollate. Four days later mice were euthanized by carbon dioxide asphyxiation and cervical dislocation. PBS (10mL) containing EDTA (5mM) (37°C) was injected IP using a 25G⅝ needle. Mice were rolled vigorously for 5 minutes on bench-coat to dislodge peritoneal macrophages. The outer dermal layer of abdomen was opened leaving the peritoneum intact. The peritoneal membrane was tented and the peritoneal fluid was slowly aspirated using a 21G needle and collected into sterile tubes. Cells were pelleted by centrifugation for 10 minutes at 500×g. The cell pellet was washed in Media C and pelleted as described above. Cells were resuspended in Media C, counted, and plated at 5×10^5cells/cm^2 in 35mm dishes and cultured as previously described. The media and any non-adhering cells were removed after 2hrs, and attached cells were washed with unsupplemented DMEM at least three times to remove red blood cells. Cells were cultured in Media C as described above.

2.2.19 Cholesterol Efflux Assay

MPM were elicited from C57 BL6/J SR-BI KO and wild-type mice as described above. For each mouse, 50μCi of [1, 2-^{3}H] cholesterol was prepared by incubation overnight at

37°C in 50μL heat-inactivated fetal bovine serum. Four days after thioglycollate-injection, cells within the peritoneal cavity were loaded with radioactive cholesterol *in situ* by injection with 50μCi [1, 2-^{3}H] cholesterol in 500μL PBS. Three hours later, mice were euthanized and MPM were harvested as described above. MPM were plated at 5×10^5cells/cm^2 in 35mm dishes and any non-adhering cells were removed after 2hrs, and attached cells were washed with unsupplemented DMEM at least three times to remove red blood cells. MPM were then cultured overnight in DMEM containing 3% NCLPDS (lipoprotein deficient serum). The following day, the media was changed to 2mL of DMEM containing 0.2% BSA without or with 25μg/mL HDL as a cholesterol acceptor (time 0). At time 0, and at each time point, 100μL aliquots of the efflux media were collected and centrifuged at 500×g for 10 minutes to remove cells. A 90μL sample of each aliquot was added to 5mL of aqueous counting scintillant (ACS) and radioactivity was determined by scintillation counting. After the final time point, cells were washed with PBS and cellular lysates were collected as described in 2.2.16. The amount of cellular [^{3}H] cholesterol was determined by scintillation counting of an aliquot representing 17% of the total lysate. Efflux was expressed as the proportion of radioactive cholesterol in the media at each time point relative to the total amount of radioactive cholesterol associated with cells.

2.2.20 Statistical Analysis

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

3. Results

3.1 Diet-induced atherosclerosis in apoE KO mice transplanted with bone marrow from either SR-BI/apoE double KO or apoE single KO donors

3.1.1 Establishing conditions for bone marrow transplantation

The presence of SR-BI in bone marrow derived cells including macrophages appears to be important for protection against atherosclerosis at early stages of lesion development [78,83,184] (however, for an alternative see [82]). Little is known about the role of SR-BI in macrophages in later events in the maturation of plaques. The goal of this study was to evaluate the effect of eliminating SR-BI in bone marrow derived cells on development of advanced stages of atherosclerosis in apoE knockout mice. The following experimental approach was taken: Bone marrow from either SR-BI/apoE double KO or control apoE single KO mice from the same colony was transplanted into lethally irradiated apoE knockout recipients that contain a wild type SR-BI gene. The purpose of these experiments was to generate experimental groups of mice which lacked a normal SR-BI gene in all hematopoietic cells including monocyte derived macrophages. One month later, atherosclerosis was induced in the mice by feeding them a high fat diet for either four or twelve weeks (Figure 2).

C57BL6/J apoE KO mice were chosen for this study as this is a standard model for atherosclerosis. These mice spontaneously develop foam cell-rich depositions in their proximal aortas by three months of age, yet most of these mice survive without heart

disease to eight months of age [173,187]. Atherosclerotic plaque development is accelerated by feeding the mice a high fat diet [173].

First, the dosage required for lethal irradiation was tested in a pilot study. Ten week old female apoE KO mice were exposed to 9, 10, or 11Gy of whole body irradiation and monitored twice daily (see Methods 2.2.2 and 2.2.4). The mice that received 9 or 10Gy survived up to 40 days while none of the mice that received 11Gy survived past 16 days post irradiation (Figure 1). We therefore used 11Gy for subsequent bone marrow transplant studies.

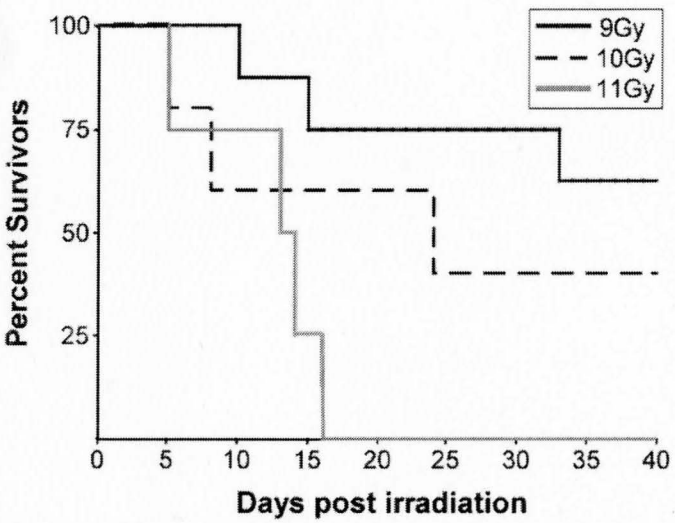


Figure 1. Effect of radiation dose on survival of ten week old female apoE KO mice. Ten week old female apoE KO mice were irradiated with 9Gy (n=8), 10Gy (n=5), 11Gy (n=4) of ^{137}Csγ irradiation using a Gammacell 3000. Two thirds of the dose was administered during a first session and the remaining third was administered three hours later (see Methods 2.2.4). Mice were monitored twice daily and received post-irradiation treatment as described (see Methods 2.2.2).

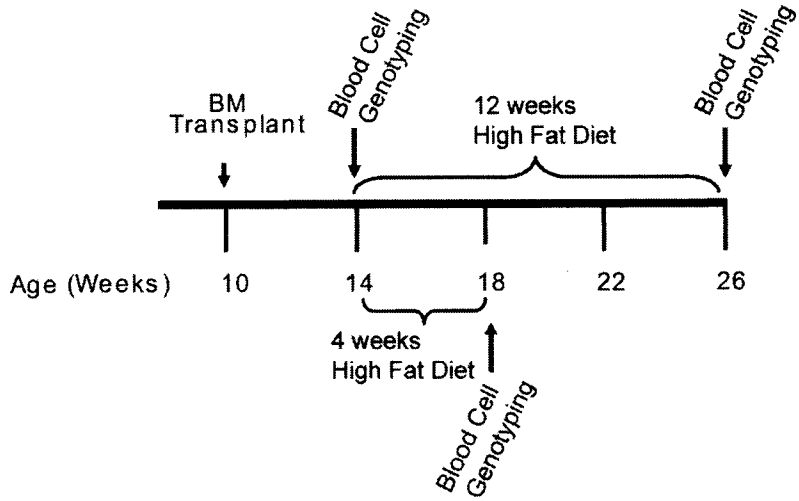


Figure 2. Timeline for the generation of bone marrow transplant mice. Ten week old female apoE KO mice were lethally irradiated and transplanted with SR-BI-/- apoE -/- or SR-BI+/+ apoE-/- bone marrow. After a four week recovery period, the genotype of circulating blood cells was determined to assess the extent of donor bone marrow engraftment. The mice were then induced to develop atherosclerosis by feeding a high fat diet for 4 weeks/12 weeks as indicated. Donor bone marrow repopulation was also assessed at harvest.

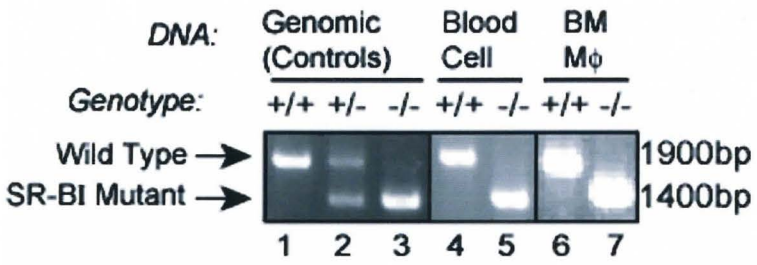
3.1.2 Bone marrow transplantation and PCR to test repopulation

Ten week old female apoE knockout recipient mice were irradiated with 11Gy, and underwent bone marrow transplantation using donors that were SR-BI positive or SR-BI KO on an apoE KO background (SR-BI +/+ apoE-/- → apoE-/-, control or SR-BI-/- apoE-/- → apoE-/-, experimental). We assessed the success of donor bone marrow engraftment in the transplanted mice one month later (data not shown) and after harvesting the mice (Figure 3A). Blood cell DNA was isolated and multiplex PCR was performed for the mutant and wild-type SR-BI alleles (Table 2 and Figure 3A). The majority of mice that received SR-BI KO donor bone marrow had circulating blood cells

that carried only the SR-BI KO allele indicating the absence of circulating blood cells derived from endogenous bone marrow. When a band corresponding to the wild-type allele was detected in blood cell derived DNA, the mouse was removed from the study (2 cases).

Macrophages are derived from circulating monocytes which originate from hematopoietic stem cells in the bone marrow. Monocytes constitute a very small percentage (1-6%) of the circulating blood cell population [188]. It was therefore important to verify that stem cell-derived macrophage precursors from the BM transplant mice were donor derived. We cultured macrophage-depleted bone marrow cells collected from select transplant mice (n=3 experimental, n=2 controls) at the time of harvest under conditions in which they differentiate into macrophages [183]. Macrophages express class A scavenger receptors (SR-AI and SR-AII) and can internalize acetylated LDL [45,189-191]. We used DiI labeled acetylated LDL to monitor acetylated LDL uptake by fluorescence microscopy. All of the bone marrow cells exhibited DiI uptake, suggesting that all of the cells differentiated into macrophages under the conditions employed (Figure 3B). We isolated DNA from these *in vitro* bone marrow derived macrophage cells and analyzed the SR-BI genotype. The mice that received SR-BI KO donor bone marrow had bone marrow derived macrophages that carried the SR-BI KO allele. PCR confirmed that the macrophage-like cells were donor derived in the transplant mice (Figure 3A).

A.



B. DiI-AcLDL uptake by bone marrow derived macrophages

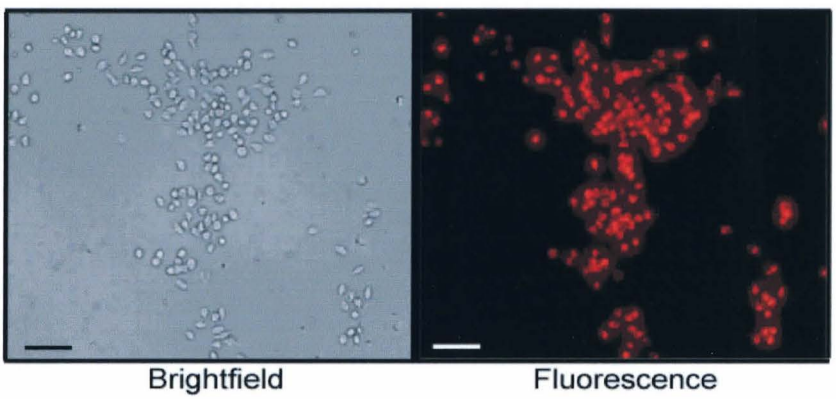


Figure 3. Genotype analysis of circulating blood cells and bone marrow precursor-derived macrophages. The positions of PCR products corresponding to the wild type (1.9kb) and mutant alleles (1.4kb) are shown on the right. The panels on the left correspond to control DNA prepared from tails of mice of known genotypes (A, Lanes 1-3). Blood cell DNA was prepared at harvest and qualitative analysis of SR-BI genotype was determined by PCR (A, Lanes 4-5) (see Methods 2.2.6 and 2.2.7). This data corresponds to DNA from representative bone marrow transplanted mice and demonstrates complete repopulation of hematopoietic stem cells. Bone marrow was prepared from mice when they were harvested and was stimulated to differentiate into macrophages by culturing in the presence of MCSF (L-cell conditioned medium) for seven days (A, Lanes 6-7 and B) (see Methods 2.2.15.3). The cells were assessed to be macrophages indirectly by testing DiI-AcLDL uptake (B, scale=50μm) (see Methods 2.2.12). Cells were incubated with 5 mg/ml DiI-acetyl LDL and DiI fluorescence was visualized using a Zeiss Axiovert 200 fluorescent microscope. DNA was prepared, and qualitative analysis of SR-BI genotype in representative samples was determined by PCR (A, Lanes 6-7). These data show that bone marrow derived macrophages differentiated in culture taken from recipient mice post-transplantation have the mutant allele.

3.1.3 Analysis of plasma cholesterol levels and lipoprotein profiles

SR-BI plays an important role in HDL metabolism [39,69,70,73,76,78-83,145,192,193]. The elimination of SR-BI on an apoE knockout background leads to altered lipoprotein sizes and distribution and doubled total cholesterol content, attributable to hepatic SR-BI [81]. Previous reports indicated that a lack of SR-BI in BM derived cells did not affect plasma total cholesterol levels in high fat diet fed LDLR KO mice, chow fed apoE KO mice, or in high cholesterol/cholate fed wild-type mice [78,82,83]. We monitored lipoprotein total cholesterol to determine if eliminating SR-BI in BM derived cells altered lipoprotein total cholesterol levels in high fat diet fed apoE KO mice. Analysis of plasma lipoproteins indicated no statistically significant differences in plasma total cholesterol or cholesterol content of any of the lipoprotein fractions when SR-BI was eliminated from bone marrow compared to mice that received bone marrow from SR-BI+/+ apoE-/- donors either after four (n=9, SR-BI-/- apoE-/- → apoE-/- and n=14, SR-BI+/+ apoE-/- → apoE-/-, Table 3 and Figure 4A) or twelve weeks (n=9 per group, Table 4 and Figure 4B) of high fat diet feeding. Thus the elimination of SR-BI in bone marrow derived cells did not influence the levels of total cholesterol levels in plasma or in individual lipoproteins in apoE KO mice fed a high fat, western-type diet. This is consistent with findings in SR-BI KO transplanted high fat diet fed LDLR KO mice [78,82], chow fed apoE KO mice [83], or in high cholesterol/cholate fed wild-type mice [82].

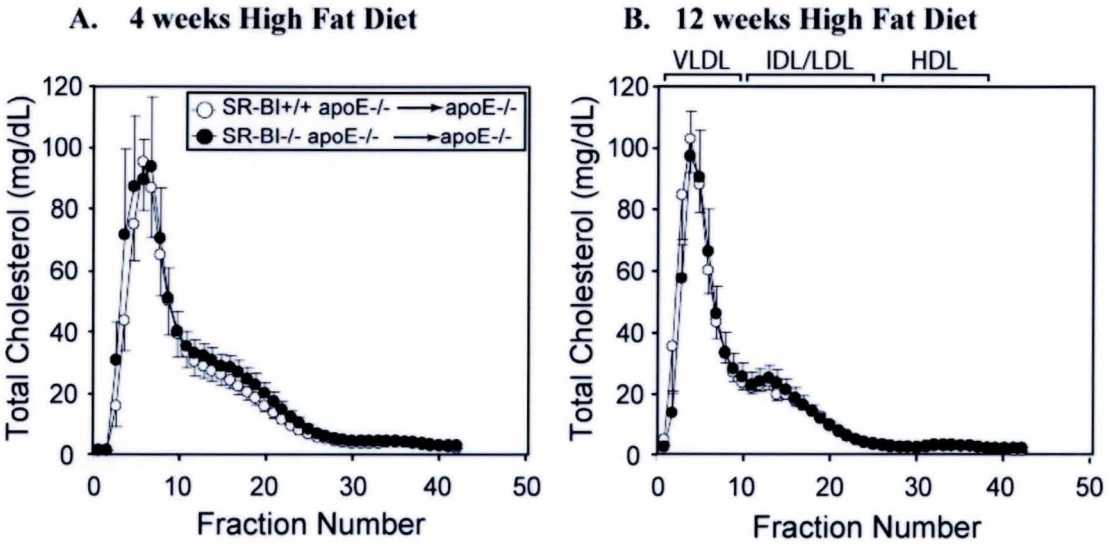


Figure 4. Plasma lipoprotein total cholesterol profiles of high fat diet fed apoE KO mice transplanted with either SR-BI/apoE double KO or apoE single KO BM. ApoE KO mice were transplanted and fed a high fat diet as described (see Methods 2.2.4 and 2.2.5) for either 4 weeks (A) or 12 weeks (B). ApoE KO mice received either SR-BI/apoE double KO (filled symbols [●], n=9 for 4 weeks [A] n=9 for 12 weeks [B]) or control apoE single KO BM (open symbols [○], n=14 for 4 weeks [A] or n=9 for 12 weeks [B]). Plasma lipoproteins from the transplanted mice were separated by size exclusion chromatography (see Methods 2.2.6 and 2.2.8). Total cholesterol in each fraction was determined (see Methods 2.2.8). The fractions in which purified human VLDL, IDL/LDL, or HDL elute are indicated [70,194]. Profiles are the averages of those of individual mice ± SEM, $P > 0.05$ indicating no statistically significant differences between mice receiving SR-BI/apoE double KO or control apoE single KO BM after 4 or 12 weeks of high fat diet feeding.

Table 3. Plasma total cholesterol in SR-BI/ apoE double KO or apoE single KO BM transplanted apoE KO mice after 4 weeks of high fat diet feeding. Plasma lipoproteins from individual fasted mice were separated by size by gel filtration-FPLC, and the concentration of total cholesterol in each fraction was measured. VLDL cholesterol is the sum of cholesterol in fractions 1 through 9; IDL/LDL cholesterol is the sum of cholesterol in fractions 10 through 25; HDL cholesterol is the sum of cholesterol levels in fractions 26 through 38. Values are mean ± SEM (standard error of the mean). Group numbers are indicated in parenthesis. *P* values were determined by Student's *t*-test for comparison between apoE KO mice receiving apoE single KO or SR-BI/apoE double KO BM.

Genotype	Total Cholesterol (mg/dL)	VLDL cholesterol (mg/dL)	IDL/LDL cholesterol (mg/dL)	HDL cholesterol (mg/dL)
SR-BI+/+ apoE-/- → apoE-/- (n=14)	777 ± 97	421 ± 54	317 ± 44	39 ± 3.9
SR-BI-/- apoE-/- → apoE-/- (n=9)	898 ± 113	483 ± 72	367 ± 54	48 ± 7.3
***P* value**	0.43	0.49	0.48	0.25

Table 4. Plasma total cholesterol in SR-BI/ apoE double KO or apoE single KO BM transplanted apoE KO mice after 12 weeks of high fat diet feeding. Plasma lipoproteins from individual fasted mice were separated by size by gel filtration-FPLC, and the concentration of total cholesterol in each fraction was measured. VLDL cholesterol is the sum of cholesterol in fractions 1 through 9; IDL/LDL cholesterol is the sum of cholesterol in fractions 10 through 25; HDL cholesterol is the sum of cholesterol levels in fractions 26 through 38. Values are mean ± SEM. Group numbers are indicated in parenthesis. *P* values were determined by Student's *t*-test for comparison between apoE KO mice receiving apoE single KO or SR-BI/apoE double KO BM.

Genotype	Total Cholesterol (mg/dL)	VLDL cholesterol (mg/dL)	IDL/LDL cholesterol (mg/dL)	HDL cholesterol (mg/dL)
SR-BI+/+ apoE-/- → apoE-/- (n=9)	717 ± 57	469 ± 46	217 ± 19	28 ± 1.8
SR-BI-/- apoE-/- → apoE-/- (n=9)	682 ± 107	424 ± 71	225 ± 38	26 ± 3.8
***P* value**	0.77	0.61	0.85	0.74

3.1.4 Histological assessment of atherosclerotic plaque development

At harvest, it was apparent that the hearts of the transplanted apoE KO mice that received SR-BI/apoE double KO bone marrow were enlarged compared to those that received apoE KO bone marrow. We measured heart to body weight ratios in the bone marrow transplant mice fed a high fat diet for four weeks, and we observed that the hearts of the mice that received SR-BI apoE double KO bone marrow were enlarged 17% relative to apoE KO controls (Figure 5B, Student's *t*-test, P=0.02) with no statistically significant differences in total weight of the mice (Figure 5A, Student's *t*-test, P=0.66). Histological analysis showed healthy myocardium in transplant mice induced to develop atherosclerosis by feeding a high fat diet for four or twelve weeks (not shown). It is not yet clear whether this is accompanied by a biologically significant alteration in heart function. Further studies such as magnetic resonance imaging (MRI) or echocardiography are required [195-199].

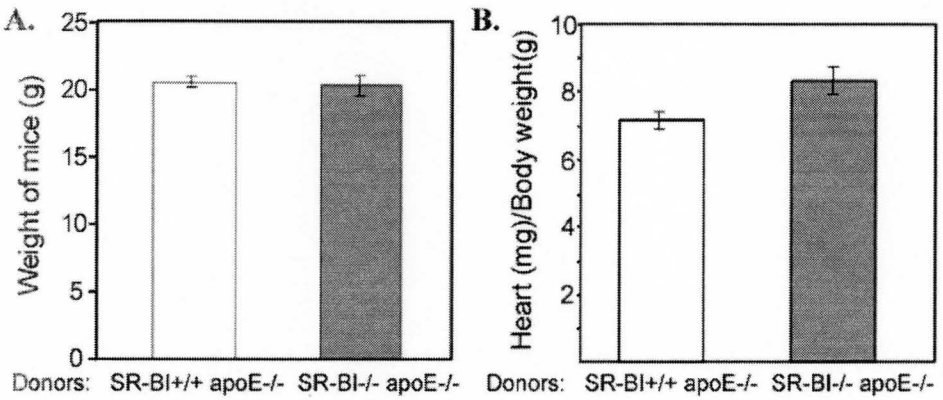


Figure 5. Gravimetric analysis of hearts from BM transplanted apoE KO mice fed a high fat diet for 4 weeks. ApoE KO mice were transplanted with BM from either SR-BI/apoE double KO (white bars) or apoE single KO (grey bars) and were fed a high fat diet for 4 weeks. Prior to euthanasia, the mice were fasted overnight, weighed, and tissues were harvested as described (see Methods 2.2.6 and 2.2.9). Hearts were weighed after harvest. Data are mean values ± SEM for apoE KO mice transplanted with BM from SR-BI/apoE double KO donors (n=7) and apoE single KO donors (n=12). (A) Body weights were 20.3g ± 0.66 (SR-BI/apoE double KO donors) and 20.5g ± 0.41 (apoE single KO donors), Using Student's *t*-test, $P = 0.66$ when transplant groups were compared. (B) Ratio of heart weight to body weight were 8.3 ± 0.41 (SR-BI/apoE double KO donors) and 7.00 ± 0.26 (apoE single KO donors). Using Student's *t*-test, $P = 0.02$ when transplant groups were compared.

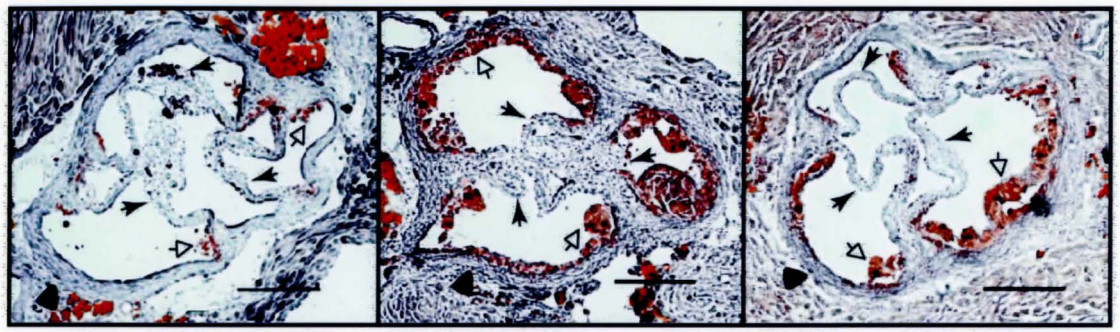
Atherosclerosis initially develops in the aortic sinus of atherosclerotic mouse models, including apoE KO mice [173]. As atherosclerosis advances, plaque development is observed in the aortic arch and descending aorta [173,187]. We first measured the amount of atherosclerosis in 10 week old female apoE knockout mice (n=8) corresponding in age and sex to the recipient mice used in bone marrow transplants. The amount of atherosclerosis was determined by measuring the cross-sectional area of Oil Red O stained plaques in sections through the aortic sinus (Figure 6A). The atherosclerotic lesions detected exhibited small, isolated groups of cells with lipid

deposits. These likely represented macrophage foam cells and appeared to be fatty streaks or Type I lesions based on Stary's histological classification of atherosclerosis [200]. Atherosclerosis was measured from the aortic valve extending 560μm distally (Figure 6B). The average plaque area at the aortic root was 7.2± 0.1x10^3μm^2 (n=8). The total plaque volume in this region was 2.1 ± 0.3x10^6μm^3 (n=7) (Figure 6C). This data is consistent with studies that demonstrated that apoE KO mice develop only small atherosclerotic plaques by this age [173,187].

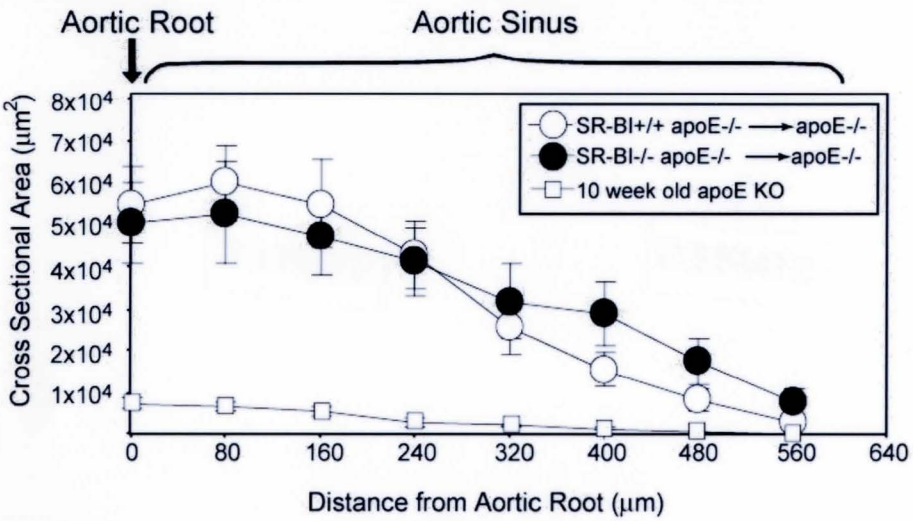
We measured atherosclerotic plaque sizes in apoE KO mice reconstituted with bone marrow from either SR-BI/apoE double KO or apoE single KO donors and fed a high fat diet for four weeks. Large complex atherosclerotic plaques were detected that exhibited extracellular lipid accumulation and what appeared to be layers of cells that may represent smooth muscle cells. There were fissures within some of the atherosclerotic plaques and cholesterol clefts were visible, indicating Type II atherosclerotic lesions based on Stary's histological classification of atherosclerosis (Figure 6A) [200]. Atherosclerosis was measured as the cross-sectional areas of plaques at the aortic roots and in sections spaced at 80μm intervals in a segment (560μm) distal to the aortic root (Figure 6B). Mean cross-sectional areas through the aortic root were 4.9 ± 0.9×10^4μm^2 when SR-BI/apoE double KO → apoE KO (n=9) and 5.5×10^4 ± 0.9×10^4μm^2 when apoE single KO → apoE KO (n=12) (Student's *t*-test *P*=0.68). Atherosclerotic plaque sizes decreased with distance from the aortic root, and the differences between transplant groups did not reach statistical significance (Figure 6B). Total plaque volume was calculated, and the averages were consistent with areas (1.9± 0.3×10^7μm^3 for SR-

BI+/+ apoE-/- donors and $1.7\pm 0.3\times10^{7}\mu m^{3}$ for SR-BI-/- apoE-/- donors [P=0.69]) demonstrating no difference in plaque volume in the aortic sinus due to the elimination of SR-BI in apoE KO mice after 4 weeks of feeding a high fat diet (Figure 6C). Interestingly, however, there was a trend towards increased atherosclerosis in more distal regions of the aortic root when apoE KO mice were reconstituted with SR-BI/ apoE double KO bone marrow. Atherosclerotic plaque sizes decrease with distance from the aortic root, yet the decrease appears to be less when apoE KO are transplanted with SR-BI/apoE double KO bone marrow. At positions 320, 400, 480, and 560μm from the aortic root, there is less of a decrease in area of atherosclerotic plaque when apoE KO mice are reconstituted with SR-BI/apoE double KO-/- bone marrow compared to apoE single KO controls (Figure 6B). Although this difference does not reach statistical significance, the differences in area of this region may suggest that presence of SR-BI influences less advanced, smaller-sized plaques.

A.


10 week old apoE-/-
SR-BI+/+ apoE-/- → apoE -/-
SR-BI-/- apoE-/- → apoE-/-

B.


Aortic Root
Aortic Sinus
Cross Sectional Area (μm²)
8x10⁴
7x10⁴
6x10⁴
5x10⁴
4x10⁴
3x10⁴
2x10⁴
1x10⁴
0
80
160
240
320
400
480
560
640
Distance from Aortic Root (μm)
SR-BI+/+ apoE-/- → apoE-/-
SR-BI-/- apoE-/- → apoE-/-
10 week old apoE KO

C.

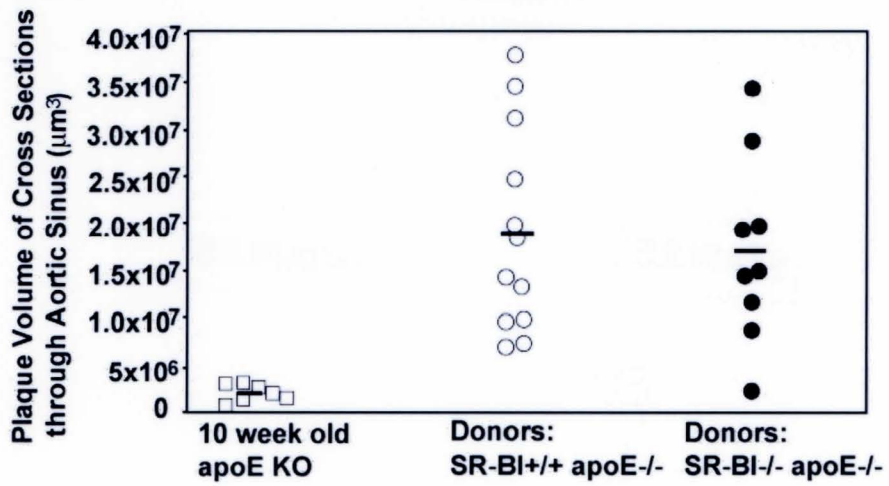

Plaque Volume of Cross Sections through Aortic Sinus (μm³)
4.0x10⁷
3.5x10⁷
3.0x10⁷
2.5x10⁷
2.0x10⁷
1.5x10⁷
1.0x10⁷
5x10⁶
0
10 week old apoE KO
Donors: SR-BI+/+ apoE-/-
Donors: SR-BI-/- apoE-/-

Figure 6. Aortic sinus atherosclerosis in apoE KO mice at 10 weeks of age, and apoE KO mice transplanted with either SR-BI/apoE double KO or apoE single KO BM and fed a high fat diet for four weeks. Atherosclerosis was measured in Oil Red O and hematoxylin stained cross sections through the aortic sinus. Atherosclerotic plaque development was assessed in apoE KO mice transplanted with either SR-BI/apoE double KO (●) or apoE single KO BM (○) and in 10 week old apoE KO mice corresponding to the age of transplant (□). (A) Representative histological images with plaque sizes corresponding to the average cross-sectional areas determined for each group. Arrow heads point to the artery wall, filled arrows point to valve leaflets, open arrows point to atherosclerotic plaque. Scale= 200μm. (B) Plaque cross-sectional area was analyzed in the sections separated by 80μm covering 560μm the vessel. Values are averages ±SEM, and at the aortic root were $4.9 \pm 0.9 \times 10^4 \mu m^2$ when SR-BI/apoE double KO → apoE KO (●, n=9) and $5.5 \pm 0.9 \times 10^4 \mu m^2$ when apoE single KO → apoE KO (○, n=12). *P* value =0.68 when comparing transplant groups. *P* values >0.05 at each interval (n=8/group) when comparing transplant groups. In 10 week old apoE KO mice corresponding to the age of transplant (□), the average plaque area at the aortic root was $7.2 \pm 0.1 \times 10^3 \mu m^2$ (n=8). (C) Plaque volume was analyzed starting at the aortic root and spanned a 320μm segment of the aorta distal to the root. Each point represents an individual animal, horizontal bars represent mean values which were as follows: ApoE KO mice transplanted with BM from SR-BI/apoE double KO donors $1.8 \pm 0.3 \times 10^7 \mu m^3$ (n=9) or apoE single KO donors $1.9 \pm 0.3 \times 10^7 \mu m^3$ (n=13). *P* value = 0.69. Plaque volume of apoE KO mice at transplant age (□) was $2.1 \pm 0.3 \times 10^6 \mu m^3$ (n=7).

Previously, atherosclerotic plaque was measured in the aorta of a similar set of transplanted apoE KO mice fed a high fat diet for 12 weeks. The absence of SR-BI in bone marrow derived cells resulted in a statistically significant increases in the amount of atherosclerotic plaque in the descending aorta (ratio of area of lipid-rich Sudan IV stained plaque to the total area of the aorta and ratio of area of plaque in abdominal aorta to total area of the abdominal aorta for each mouse (unpublished results [201]). There were no statistically significant differences in cross-sectional area of plaque in the aortic sinuses of mice fed a high fat diet for 12 weeks (unpublished results [201]) which is consistent with the results described in this study in transplanted apoE KO mice fed a high fat diet for four weeks. These observations, together with the findings reported by others [83], suggest that the elimination of SR-BI from bone marrow derived cells increases early atherosclerotic plaque development in apoE KO mice but does not affect the size or morphology of more advanced plaques such as those closer to the aortic root in high fat diet fed apoE KO mice. This appears to be different from results in high fat diet fed LDLR KO mice suggesting that the elimination of SR-BI in bone marrow derived cells increased the development of more advanced atherosclerotic plaque [78,82], but decreased the development of very early stage atherosclerotic plaque [82]. The reasons for these differences are not presently clear.

3.2 Effect of lipoproteins on SR-BI protein levels in macrophages

SR-BI is expressed in macrophage cell lines and atherosclerotic plaques [55-57]. Figure 7A shows the results of an immunoblot for SR-BI in lysates from mouse peritoneal macrophages (MPM) isolated from SR-BI+/+ or control SR-BI-/- mice. Cells were cultured for 16h in the presence of lipoprotein deficient serum. SR-BI was detected as an 82kDa band in the lysate from SR-BI+/+ cells, and no SR-BI immunoreactive band was detected in the lysate of SR-BI -/- cells. To control for equal loading ε-COP was included. Therefore, SR-BI is also expressed in elicited peritoneal macrophages (Figure 7A).

There has been conflicting data regarding the regulation of SR-BI expression by modified lipoproteins [56,89]. Oxidized and acetylated LDL have been shown to increase SR-BI transcript and protein levels in human macrophages [56]. A recent study demonstrated SR-Bl expression is elevated in human macrophage foam cells in response to modified lipoproteins [91]. Conversely, it has been demonstrated that SR-BI expression in Raw 264.7 cells (murine macrophages) is decreased upon treatment with oxidized LDL [89]. It was also determined that SR-BI expression is down-regulated in the presence of sterols such 25-hydroxy-cholesterol and acetylated LDL [90]. SR-BI appears to be a target gene of the SREBP transcription factor family [88,202], although there are conflicting reports [90]. To test whether native lipoproteins affected SR-BI protein levels in macrophages, we cultured elicited MPM and Raw 264.7 cells in the presence of lipoprotein deficient newborn calf serum (NCLPDS) without or with increasing amounts of purified human LDL or HDL for 16 hours. Murine peritoneal

macrophages and macrophage-like Raw 264.7 cells are normally cultured in media containing 10% FBS. SR-BI protein levels were compared in both MPM and Raw 264.7 cells cultured in media containing either 10% FBS (containing lipoproteins) or 3% NCLPDS (lacking lipoproteins) (Figure 7B). Cells cultured in 3% NCLPDS (Lanes 2 and 4) exhibited higher SR-BI protein levels than cells cultured in 10% FBS (Lanes 1 and 3). In contrast, equal protein levels were detected for CD36 (middle panels) and for ε-COP (bottom panels). The expression of SR-BI increased when MPM and Raw 264.7 cells were cultured without lipoproteins (Figure 7B). Raw 264.7 cells were cultured in media lacking serum (Figure 7C, Lane 1) or containing lipoprotein deficient serum in the absence (Lane 2) or presence of increasing concentrations of either LDL (Lanes 3-6) or HDL (Lanes 7-10). The levels of SR-BI protein were highest when Raw 264.7 cells were cultured in the absence of serum (Lane 1). SR-BI protein levels decreased in a dose-dependent manner in response to increasing concentrations of LDL (Lanes 3-6) and HDL (Lanes 7-10). This is consistent with the notion that lipoprotein-delivered cholesterol can down-regulate SR-BI expression. SR-BI expression levels appeared to decrease more in the presence of LDL compared to HDL at the same concentration.

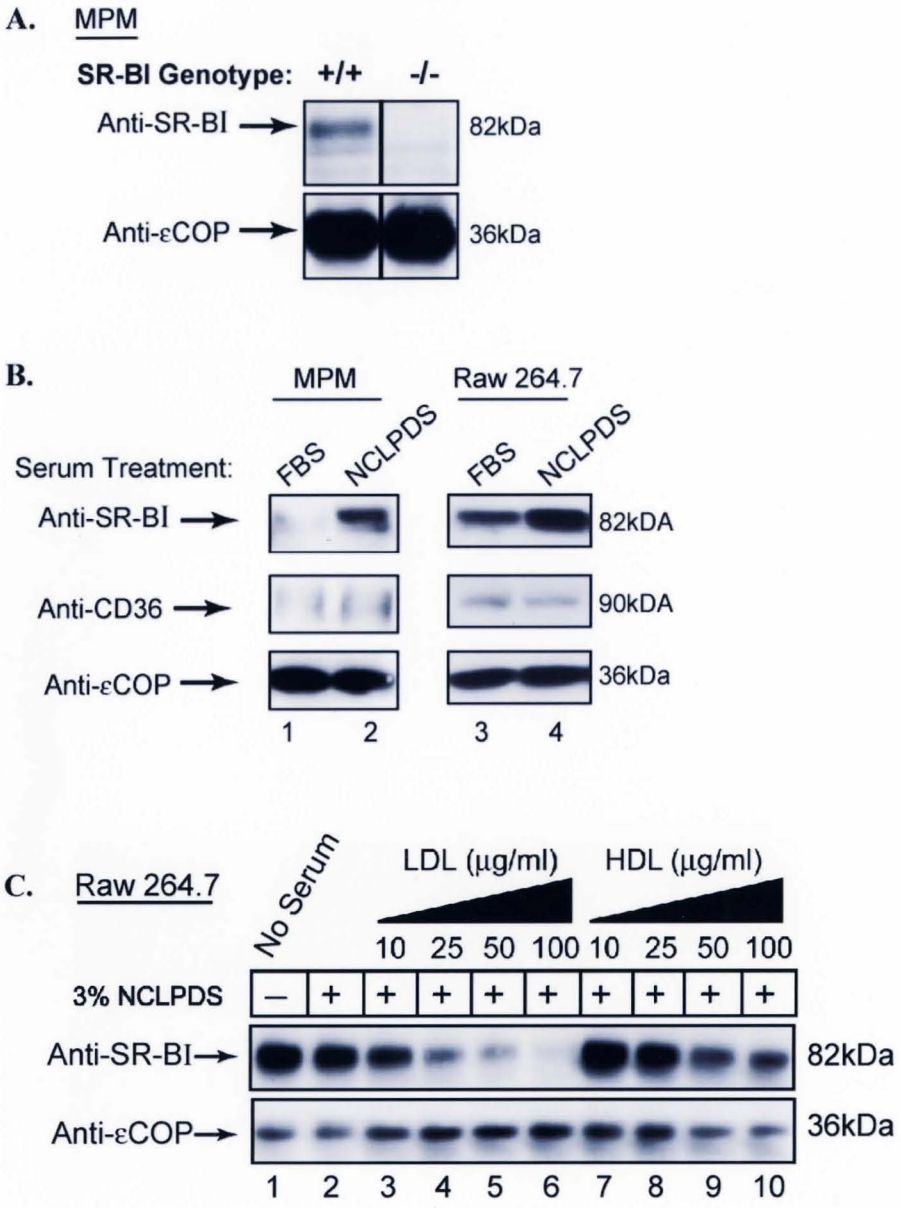

A.
MPM
SR-BI Genotype: +/+ -/-
Anti-SR-BI
82kDa
Anti-εCOP
36kDa
B.
MPM
Raw 264.7
Serum Treatment:
FBS
NCLPDS
FBS
NCLPDS
Anti-SR-BI
82kDA
Anti-CD36
90kDA
Anti-εCOP
36kDa
1 2 3 4
C.
Raw 264.7
No Serum
LDL (μg/ml)
HDL (μg/ml)
10 25 50 100 10 25 50 100
3% NCLPDS
– + + + + + + + + +
Anti-SR-BI
82kDa
Anti-εCOP
36kDa
1 2 3 4 5 6 7 8 9 10

Figure 7. SR-BI expression in macrophages is regulated by lipoproteins. (A) Elicited MPM were collected from wild-type and SR-BI KO mice and cultured for 16h in DMEM with 3% NCLPDS (lipoprotein deficient serum). This immunoblot shows SR-BI protein expression in murine wild-type MPM, and the lack of SR-BI protein expression in SR-BI KO MPM. Experiments were run in duplicate. (B) Elicited MPM were collected from wild-type mice (left panel) and Raw 264.7 cells (right panel) were cultured for 16h in 10%FBS (containing lipoproteins) or 3% NCLPDS (lacking lipoproteins). This immunoblot demonstrates that SR-BI expression is up regulated in the absence of lipoproteins in MPM (comparing Lanes 1 and 2) and Raw 264.7 cells (comparing Lanes 3-4). Images are representative of either MPM were run in duplicate or Raw 264.7 cells run in triplicate. (C) Raw 264.7 cells were incubated for 16h without serum, in 3%NCLPDS, or in the presence of 3% NCLPDS with 10, 25, 50, 100 μg/ml LDL or HDL. This immunoblot indicates decreased SR-BI expression in response to LDL (Lanes 3-6) and HDL (Lanes 7-10). The levels of SR-BI protein were highest when Raw 264.7 cells were cultured in the absence of serum (Lane 1). Experiment was run in triplicate. Equal amounts of protein were run on SDS-PAGE, blotted, probed with antibodies for SR-BI, CD36 and ε-COP (loading control), and detected by chemiluminescence as described (see Methods 2.2.16 and 2.2.17).

3.3 Cholesterol efflux from MPM from wild-type and SR-BI KO mice and SR-BI expression during the time-course of efflux

It has been hypothesized that SR-BI expression in peripheral tissues, such as macrophages in the artery wall, may play a role in cholesterol efflux [55,82,83,94,203,204]. However, the evidence regarding macrophage SR-BI and cholesterol efflux is conflicting. As described earlier, overexpression of SR-BI in a variety of cell lines leads to increased levels of efflux of ^{3}H cholesterol tracer to HDL and phospholipid vesicles [55,94]. In contrast, both our lab and others have reported that efflux of ^{3}H cholesterol tracer to HDL acceptors is either unaffected [78,83,194] or only very slightly [82,89] reduced in macrophages from SR-BI KO mice compared to those from wild-type mice with intact SR-BI expression. Similar results have been reported for endothelial cells treated with the SR-BI inhibitor BLT-1 [205]. In contrast, BLT-1 appears to partially reduce cholesterol efflux from hepatocytes to HDL [205]. It is interesting that SR-BI expression in endothelial cells has been reported to be reduced by increased cellular cholesterol/oxysterols, whereas hepatocyte SR-BI appears to be insensitive to cellular sterol levels [205]. This, together with our results and those of others [90] raises the possibility that the finding that HDL-dependent cholesterol efflux is not substantially reduced in SR-BI KO macrophages may be because SR-BI expression is reduced in the wild-type control cells in the presence of HDL over the course of the efflux assay. The following experiment was designed to test this possibility.

Figure 8A shows preliminary data reproducing the experiment carried out by Scott Covey [194] and reported previously by our lab [78]. Elicited MPM from SR-BI KO and wild-type mice were loaded *in situ* with [1,2-^{3}H] cholesterol as described in

Methods 2.2.19. Three hours later MPM were harvested and cultured overnight in lipoprotein deficient media. Cholesterol efflux was initiated by the addition of media with 25μg/mL HDL (closed symbols) or without HDL (open symbols) and the level of efflux was determined as the amount of radioactivity in the media at each time point relative to the total amount associated with cells. In each case, cholesterol efflux was higher in the presence of HDL (compare filled with open symbols) as expected. Although there was substantial sample-to-sample variation (compare SR-BI+/+ #1 with SR-BI+/+ #2, which refer to cells from two independent mice), this preliminary data indicate no apparent decrease in cholesterol efflux from SR-BI-/- cells (circles representing cells from one mouse). This is consistent with previous findings reported by our lab [78,194] and others [83].

To directly test whether the level of SR-BI expression decreased in cells from wild-type mice in the presence of HDL over the course of the efflux assay, we carried out a "mock" efflux experiment. Cells were treated as above (without ^{3}H cholesterol loading) and harvested at different points in the efflux assay (see Figure 8B, left panel). Lysates were prepared and SR-BI levels were analyzed by SDS-PAGE and immunoblotting. This analysis revealed that SR-BI levels were similar at the start of the efflux period and 4 and 8 hours after the incubation with HDL during the efflux period (Figure 8B, right panel). Note that for this assay, 100μg/mL of HDL was used rather than the 25μg/mL used in the efflux assay presented in Figure 8A. Furthermore, SR-BI levels in the cell lysates collected in the experiment shown in Figure 8A were also analyzed and compared to those from a parallel dish of SR-BI+/+ cells collected at time

0. Consistent with the data in Figure 8B, that analysis showed no changes in the levels of SR-BI from SR-BI+/+ cells over the course of the efflux assay, and no SR-BI was detected in the lysate from SR-BI-/- cells (data not shown).

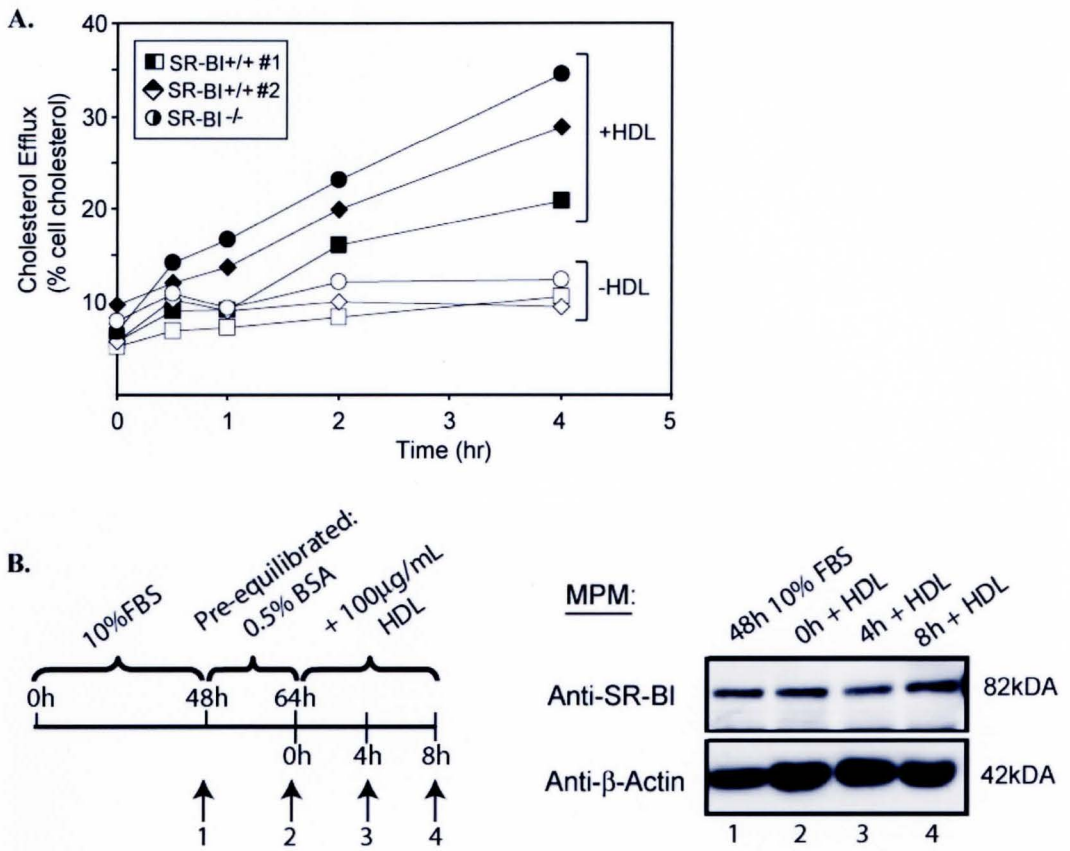


Figure 8. Effect of a lack of SR-BI on HDL-dependent cholesterol efflux. (A) MPM were elicited with thioglycollate in one SR-BI-/- (circles) and two control wild-type mice (squares and triangles) (see Methods 2.2.18). Cells were labeled *in situ* with [^{3}H]-cholesterol by direct injection into the peritoneal cavity (see Methods 2.2.19). Cells were then harvested and cultured overnight in 35mm dishes (5×10^5 cells/cm^2)in DMEM containing 3% NCLPDS. Efflux assays were initiated by replacement of the media with DMEM containing 0.2% BSA with (closed symbols) or without (open symbols) 25µg/mL HDL as a cholesterol acceptor. The amount of [^{3}H]-cholesterol in the media at different time points was detected by scintillation counting and is plotted as relative to the total amount of cellular [^{3}H]-cholesterol. Shown are efflux time courses with or without HDL from parallel dishes of cells from each of three individual mice (n=1 with and 1 without HDL, per mouse). (B) In a separate experiment, MPM were isolated from wild-type mice and processed as for a cholesterol efflux experiment. Cells were lysed either before or at t=0, 4, or 8h after the addition of 100µg/mL HDL (left panel). Equal amounts of protein (100µg) were analyzed by SDS-PAGE and immunoblotted (right panel) using antibodies for either SR-BI (top) or β-actin (bottom, loading control) and detected by chemiluminescence as described (see Methods 2.2.16 and 2.2.17).

One potential explanation for our finding that cholesterol efflux was not decreased in SR-BI KO compared to wild-type MPM may be the presence of substantial amounts of free apoA-I in the preparation of HDL used as a cholesterol acceptor. ApoA-I is the major apolipoprotein associated with HDL [9], and free apoA-I is also an efficient acceptor of cholesterol efflux mediated by ABCA1 [99]. To test this possibility, we analyzed our HDL preparation for the presence of free apoA-I. This was done by separating HDL using size exclusion chromatography. HDL is between 175-360kDa [206] and typically elutes between fractions 26-38 [78]. Lipid free apoA-I is 30kDa and is expected to elute between fractions 42-46 (see protein standards profile, Figure 9B). As expected, the majority of apoA-I was associated with cholesterol and eluted in fractions 27-39 consistent with it being associated with spherical HDL particles. Only minor amounts of apoA-I were detected in FPLC fractions corresponding to smaller sized particles including lipid-free apoA-I (Figure 9A). Therefore it is not likely that efflux of cholesterol measured in Figure 8A and in previous data generated by our lab [194] was the result of efflux to lipid-free apoA-I. Instead, it appears that SR-BI is not required for HDL dependent cholesterol efflux.

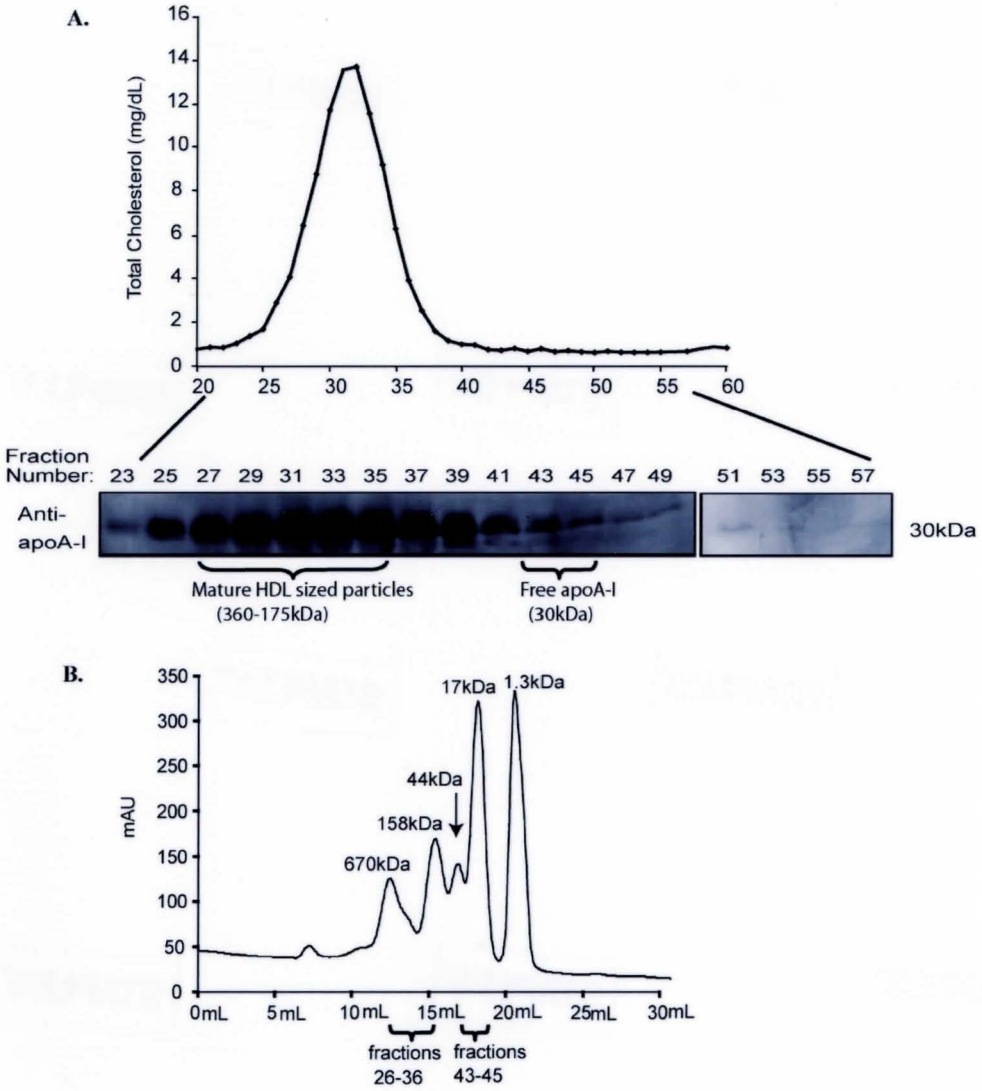


Figure 9. Assessment of HDL preparation for free apoA-I. (A) HDL prepared from human plasma (800µg) was passed through a gel filtration column to analyze HDL for the presence of free apoA-I. Aliquots of fractions associated with HDL sized particles and subsequent fractions that elute smaller particles (i.e. free apoA-I) were separated via SDS-PAGE, blotted, probed with an antibody for apoA-I, and detected by chemiluminescence. Total cholesterol from each fraction was assayed. (B) Protein standards (BioRad Gel Filtration Standards; the proteins are thyroglobulin [670kDa], gamma globulin [158kDa], chicken ovalbumin [44kDa], equine myoglobin [17kDa], and vitamin B_{12} [1.3kDa]) were run through an FPLC column. Based on the volume of eluent associated with each standard, we deduced where HDL sized particles (175-360kDa) and free apoA-I (30kDa) would be expected to elute.

3.4 Affect of glucosamine treatment on macrophage SR-BI expression

Atherosclerosis is a frequent complication of diabetes [4]. One of the effects of elevated levels of glucose in diabetes is a concomitant increase in intracellular glucosamine levels resulting from increased flux of glucose through the hexosamine pathway [122]. Glucosamine exerts a number of effects on cells including interference with normal protein *N*-linked glycosylation, induction of ER stress (possibly as a consequence of interference with normal protein *N*-linked glycosylation), and increased protein *O*-linked glycosylation.

Many proteins require *N*-linked glycans for folding and transport out of the ER, although glycosylation may not be necessary for biological function [133]. There are eleven sites for *N*-glycosylation on SR-BI; two of which affect SR-BI expression and function and also appear to have an important role in protein folding within the ER and/or intracellular transport [136].

There appears to be a link between ER stress and the development of atherosclerosis. Elevated plasma concentrations of total homocysteine, a condition that leads to ER stress, has been linked with atherosclerotic plaque development in apoE KO (diet and/or genetically induced hyperhomocysteinemia) [169,207-210]. Additionally, a report has indicated that glucosamine may be linked to increased atherosclerosis associated with diabetes mellitus through the induction of ER stress [170].

We tested the consequences of glucosamine or DTT on SR-BI expression levels in macrophage-like Raw 264.7 cells. Both in the presence (Figure 10A) and absence of lipoproteins (Figure 10B), treatment with 5mM glucosamine resulted in apparent

decreases in SR-BI expression after 8 hours (Figure 10, panels A and B, Lane 2) and 16 hours (Figure 10, panels A and B, Lane 6). Mannitol (5mM) was included as a control for hyperosmotic stress [211] and resulted in an apparent slight increase in SR-BI expression (Figure 10A, Lanes 3 and 7). In the presence of DTT (2.5mM), a reducing agent that is known to induce ER stress [212], SR-BI levels also tended to increase, although the differences did not reach statistical significance (due in part to large sample to sample variation) (Figure 10B, Lane 8).

To determine if this might be due to altered glycosylation of SR-BI Raw 264.7 cells were treated without or with 5mM glucosamine, 5mM mannitol, or 2.5mM DTT (as described above), lysed, and then treated without or with *N*-glycanase for 16hours (Figure 10C). Control lysates (Figure 10C, Lanes 5-8) exhibited similar changes in the level of the 82kDa band corresponding to the mature glycosylated SR-BI [51] (as in Figure 10, panels A and B). Additionally, (as shown in Lanes 5-8), a significant amount of SR-BI protein migrates with an apparent molecular weight of 55kDa, consistent with a poorly glycosylated form of the protein. Upon treatment with *N*-glycanase, the 82kDa band disappeared. There were no apparent differences in the amount of the non-glycosylated 55kDa SR-BI protein (Figure 10C, Lanes 1-4) from cells treated with glucosamine, mannitol, or DTT. This suggests that changes in the levels of the 82kDa band most likely reflected altered glycosylation of SR-BI rather than altered protein levels.

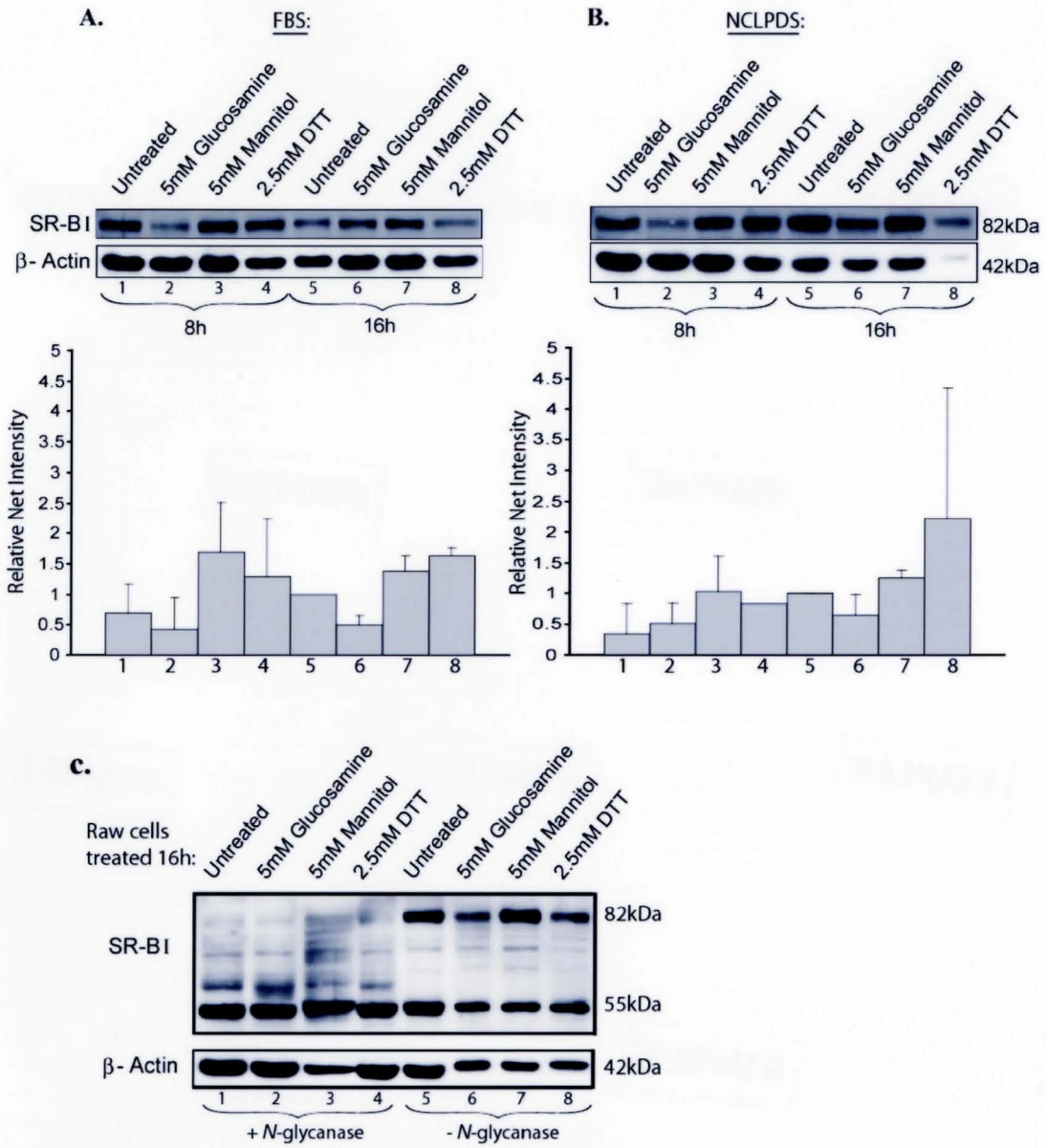


Figure 10. Glucosamine treatment in Raw 264.7 cells. Raw 264.7 cells cultured for 8 or 16h in the presence of FBS (A) or NCLPDS (B) were treated with 5mM glucosamine, mannitol, or 2.5mM DTT, as indicated. Cell lysates (100μg) were incubated without or with *N*-glycosidase F for 16h at 37°C to remove N-linked glycans (C). Cell lysates (100μg) were run on SDS-PAGE, blotted, probed with antibodies for SR-BI and β-actin (loading control), and detected by chemiluminescence (see Methods 2.2.16 and 2.2.17). The net intensity of each band was quantified using Kodak ID software. The ratio of SR-BI to β-actin was determined, and values were normalized to 16h NCLPDS treatment. The error bars represent standard deviation (n=2, differences did not reach statistical significance when comparing glucosamine treatment to untreated and mannitol treated cells, $P>0.05$).

To determine if ER stress was induced by these treatments, we probed expression of GRP78 using an antibody against the KDEL peptide. GRP78 contains the KDEL ER retention sequence, is up-regulated in response to ER stress, and is the major 78kDa protein detected using the anti-KDEL antibody. GRP78 expression increased in Raw 264.7 cells treated with glucosamine (Figure 11, Lane 2 compared to 1), mannitol (Lane 2 compared to 3) or DTT (Lane 4 compared to 2 and 1). This suggests that ER stress was induced in each case.

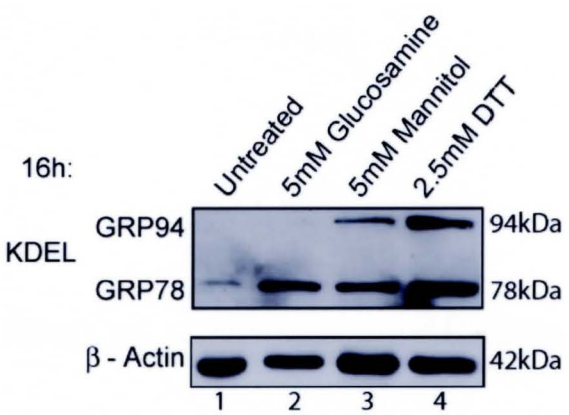
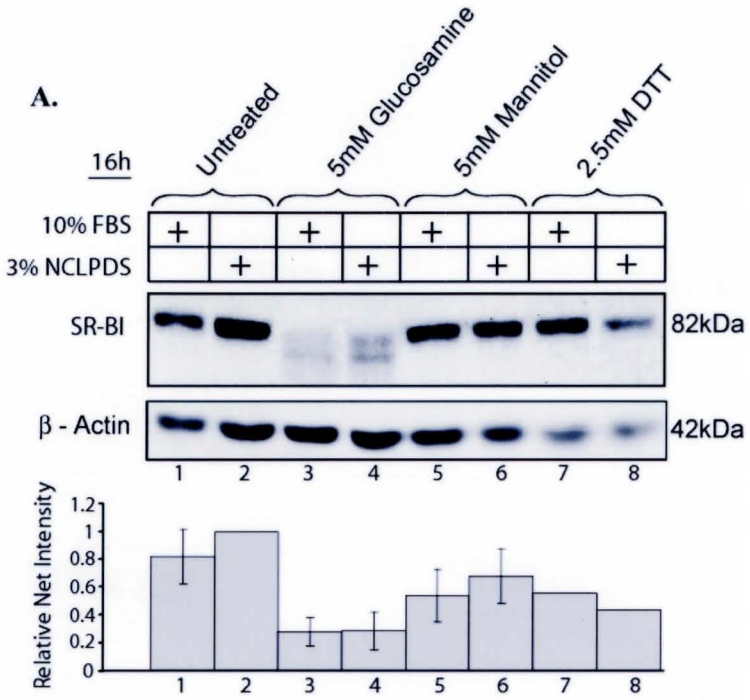


Figure 11. Immunoblot for KDEL in glucosamine treated Raw 264.7 cells. Raw 264.7 cells were incubated with NCLPDS and glucosamine, mannitol, or DTT for 16hours. Cell lysates were prepared and equal amounts of protein (100μg) were run on SDS-PAGE, blotted, probed with antibodies for KDEL and β-actin (loading control), and detected by chemiluminescence (see Methods 2.2.16 and 2.2.17). A representative experiment is shown (n=3).

3.5 Affect of glucosamine treatment on SR-BI expression in ldlA[mSR-BI] cells

We also tested the effects of glucosamine on levels of SR-BI in CHO-derived ldlA7 that stably overexpress murine SR-BI (mSR-BI) (Figure 12). When cells were cultured in either lipoprotein deficient or lipoprotein containing serum, glucosamine treatment for 16 hours resulted in decreased levels and altered migration of SR-BI (Figure 12A, Lanes 3-4 compared to Lanes 1-2 and 5-8, and panel B Lane 5 compared to 4 and 6). SR-BI migrated as a doublet with increased mobility in lysates from glucosamine-treated ldlA[mSR-BI] cells. Quantitation of bands from a number of experiments (n=3) suggested that mannitol or DTT treatment resulted in less substantial decreases in SR-BI with no changes in protein mobility (Figure 12A, Lanes 5-8, and 12B, Lane 6).

As described for Raw 264.7 cells, we compared the levels of the mature 82kDa and 55kDa precursor proteins in lysates treated without or with *N*-glycanase. Unlike Raw 264.7 cells, ldlA[mSR-BI] cells contained no 55kDa band. Upon treatment with *N*-glycanase, lower mobility SR-BI bands disappeared and were replaced by the 55kDa non-glycosylated form of SR-BI. Consistent with Raw 264.7 cells, the levels of the 55kDa band in ldlA[mSR-BI] cells were the same in control, glucosamine and mannitol treated cells. Thus it appears that glucosamine treatment results in altered glycosylation of SR-BI.


A.
16h
Untreated
5mM Glucosamine
5mM Mannitol
2.5mM DTT
10% FBS
3% NCLPDS
SR-BI
82kDa
β - Actin
42kDa
1 2 3 4 5 6 7 8
Relative Net Intensity
1.2
1
0.8
0.6
0.4
0.2
0
1 2 3 4 5 6 7 8

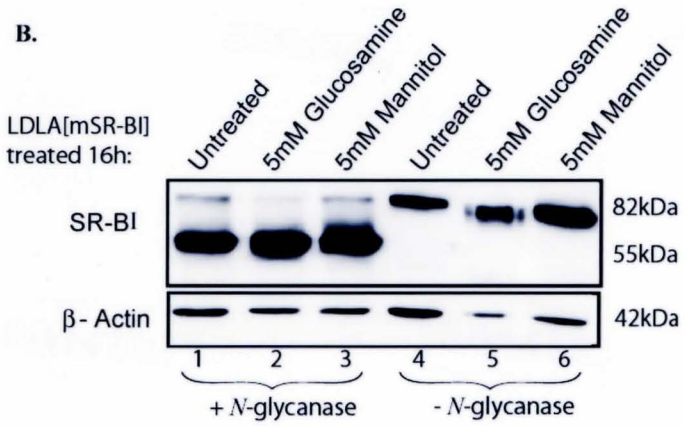

B.
LDLA[mSR-BI]
treated 16h:
Untreated
5mM Glucosamine
5mM Mannitol
Untreated
5mM Glucosamine
5mM Mannitol
SR-BI
82kDa
55kDa
β - Actin
42kDa
1 2 3 4 5 6
+ N-glycanase
- N-glycanase

Figure 12. Glucosamine treatment in ldlA[mSR-BI] cells. (A) ldlA[mSR-BI] cells were cultured for16h in the presence of 10% FBS or 3% NCLPDS and were treated with 5mM glucosamine, mannitol, or 2.5mM DTT, as indicated. Cell lysates were prepared and equal amounts of protein (25μg) were run on SDS-PAGE, blotted, probed with antibodies for SR-BI and β-actin (loading control), and detected by chemiluminescence. The net intensity of each band was quantified using Kodak ID software. The ratio of SR-BI to β-actin was determined, and values were normalized to 16h NCLPDS treatment. The error bars represent SEM (n=3, comparing Lanes 2 and 4, P=0.003). A representative experiment is shown. (B) ldlA[mSR-BI] cells were cultured for16h in the presence of 5mM glucosamine, mannitol, or 2.5mM DTT and cell lysates (50μg) were incubated without or with *N*-glycosidase F for 16h at 37°C to remove N-linked glycans. Cell lysates (50μg) were run on SDS-PAGE, blotted, probed as described in (A).

We tested the involvement of newly synthesized proteins in the altered expression of SR-BI associated with glucosamine treatment. We incubated ldlA[mSR-BI] cells with cycloheximide (70μm), a general inhibitor of protein synthesis, for one hour, at which time glucosamine (5mM) or mannitol (5mM) was added to the cycloheximide treated cells as indicated. SR-BI levels were tested at different times for up to 16 hours after the addition of glucosamine or mannitol (Figure 13B-D). Treatment of cells with glucosamine did not affect the migration of SR-BI after 4h but did after 8 and 16h (Figure 13, panel A). Treatment with cycloheximide alone resulted in a very gradual decline in the levels of SR-BI over 16h (Figure13, panel B and E) consistent with the reported half-life of the protein [51]. No effect on the time course was observed when either glucosamine or mannitol were included (Figure 13, panels C, D, and E). This suggests that the altered glycosylation induced by glucosamine requires new protein synthesis. This may reflect co-translational glycosylation of newly synthesized SR-BI. It is surprising, however, that when protein synthesis was not blocked and cells were treated with glucosamine for as short as 8h there was little or no normally glycosylated 82kDa SR-BI detected, even though substantial amounts were present after 16h in the presence of cycloheximide. This suggests the possibility that in addition to altering the glycosylation of newly synthesized SR-BI, glucosamine may alter either the glycosylation or stability of mature previously synthesized SR-BI. Further experiments would be required to test this.

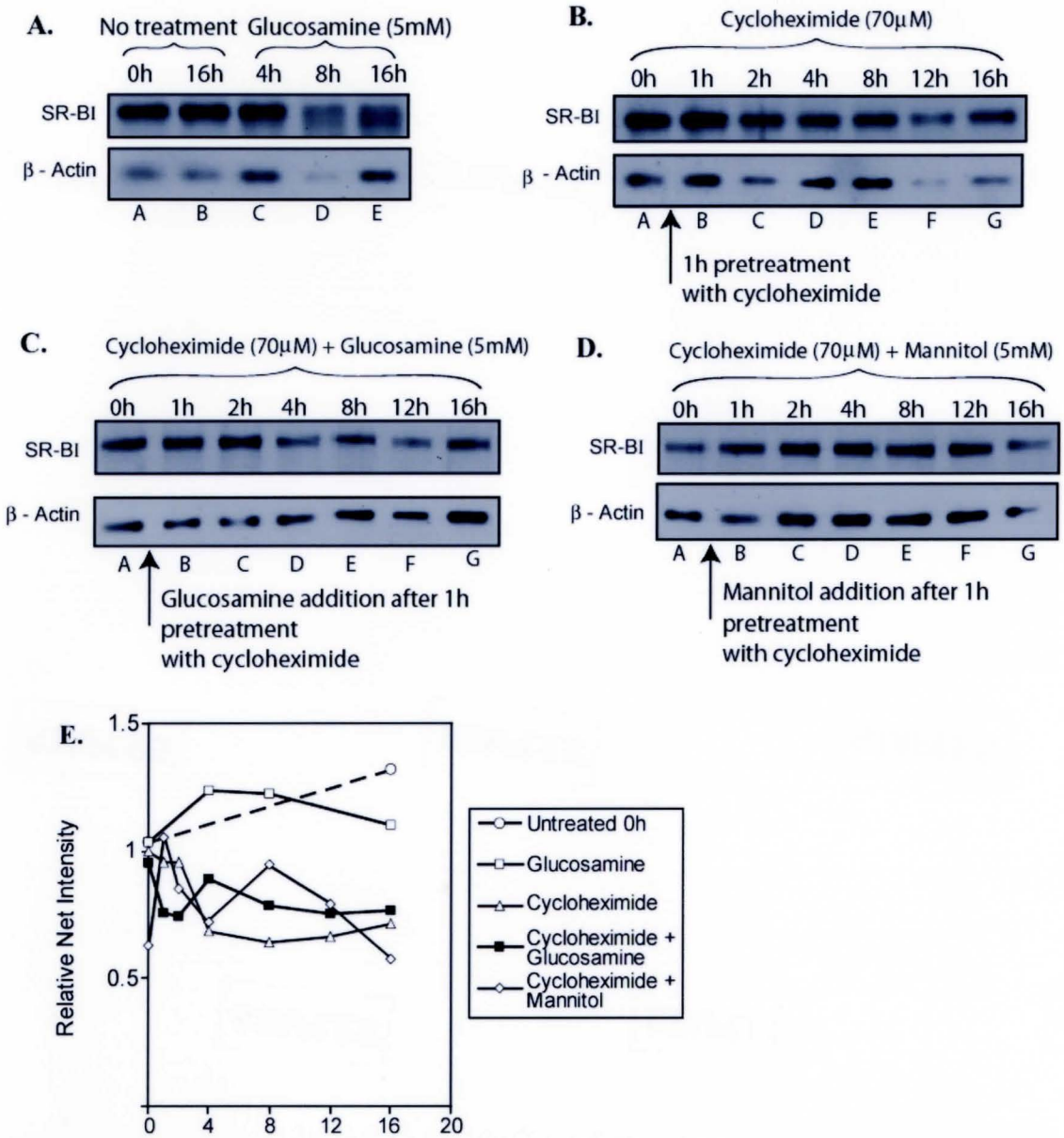


Figure 13. Test for the involvement of new protein synthesis in decreased SR-BI expression associated with glucosamine treatment in ldlA[mSR-BI] cells. ldlA[mSR-BI] cells were pretreated with cycloheximide, as indicated for one hour followed by treatment with (A) glucosamine (B) cycloheximide only (C) cycloheximide and glucosamine or (D) cycloheximide and mannitol. Cells lysates were prepared at each time point (0-16h) and equal amounts of protein (25μg) were run on SDS-PAGE, blotted, probed with antibodies for SR-BI and β-actin (loading control). Representative experiment shown (n=3). (E) The net intensity of each band was quantified using Kodak ID software. The ratio of SR-BI to β-actin was determined, and values were normalized to 0h cycloheximide (after 1h pretreatment). Averages are shown (n=3, $P>0.05$).

4. Discussion

4.1 Consequences of the elimination of SR-BI in bone marrow derived cells in diet-induced apoE KO mice

Hepatic SR-BI expression has a profound effect on plasma HDL levels, influences plasma HDL subpopulations, and protects against atherosclerosis in mouse models by promoting hepatic clearance of HDL derived cholesterol [14,69,70,73-76]. SR-BI binds a wide variety of ligands [47,52,60-64,66-68] and is expressed in cell types relevant to atherosclerotic plaque development, such as macrophages, endothelial cells, and smooth muscle cells [55-59]. As macrophages are the predominant cell type associated with atherosclerotic plaque, we and others have asked whether eliminating SR-BI in bone marrow derived cells such as macrophages also influenced atherosclerotic plaque development. In this study we have examined the effect of the bone marrow specific elimination of SR-BI at advanced stages of atherosclerotic development on an apoE KO background. In doing so, we have expanded unpublished data previously generated in our lab by Ali Rizvi [201] and our published data [78].

In this study we demonstrated no significant differences in plasma lipoprotein profiles nor total cholesterol levels due to the elimination of SR-BI in bone marrow derived cells when transplanted apoE KO mice were fed a high fat diet for four or twelve weeks. These findings agree with our lab's previous work and the findings of others where SR-BI was eliminated from bone marrow derived cells in LDLR KO mice fed a high fat diet [78,82], chow fed apoE KO mice [83], and wild-type mice fed a high cholesterol diet containing cholate [82]. This suggests that SR-BI in bone marrow

derived cells does not appear to play a major role in regulating plasma lipoprotein cholesterol levels. In contrast, hepatic SR-BI does play an important role in lipoprotein and cholesterol metabolism [14,69,70,73,76].

Previously our lab reported that the selective elimination of SR-BI in bone marrow derived cells in LDL receptor KO mice fed a high fat diet for four months leads to increased atherosclerotic lesion development in the aortic arch [78]. It has also been shown that bone marrow specific elimination of SR-BI leads to increased atherosclerosis in the aortic sinus in 18 week old apoE KO mice when the mice were fed a normal chow diet [83]. Taken together, this data indicated that the presence of SR-BI in bone marrow derived cells protected against atherosclerosis. Others have reported that the selective elimination of SR-BI in bone marrow derived cells in LDLR KO mice led to reduced atherosclerosis when mice were fed a high fat diet for four weeks [82], but increased atherosclerosis when mice were fed a high fat diet for up to 12 weeks [78,82]. Furthermore, the elimination of SR-BI from bone marrow derived cells in wild-type mice fed a high cholesterol diet containing cholate for eight weeks led to reduced atherosclerosis [82]. This suggests that bone marrow derived SR-BI may have a complex effect on atherosclerosis.

In contrast, we have shown in this study that the elimination of SR-BI in bone marrow derived cells leads to no statistically significant difference in cross-sectional area of atherosclerotic plaques in the aortic sinus in apoE KO mice when the mice were fed a high fat diet for four weeks. Ali Rizvi from our lab has obtained similar results in apoE KO mice fed a high fat diet for 12 weeks [201]. However, he did find a statistically

significant increase in the distribution of atherosclerosis descending aorta (unpublished data) [201]. Notably, in this study we found a trend towards increased lesion size in apoE KO mice transplanted with SR-BI/apoE double KO bone marrow, as lesion size decreases. Although the differences did not reach statistical significance, this trend may be consistent with the increased plaque distribution in more distal portions of the aorta. This suggests the possibility that presence of SR-BI might influence less advanced, smaller-sized plaque.

It remains to be determined if plaque distribution is effected due to a lack of SR-BI in bone marrow derived cells in apoE KO mice fed a high fat diet for four weeks. We predict that the presence of SR-BI in bone marrow derived cells would lead to atheroprotection based on the trend observed in this study and previously published reports. We expect that plaque development in the aortic arch will be minimal based on the age of the mice and length of atherosclerotic plaque induction by feeding a high fat diet. Taken together this data clearly illustrates that the stage of plaque formation, the manner and time-course of atherosclerotic induction, and the experimental model used are important variables in assessing the development of atherosclerosis in transplanted mice.

Interestingly, apoE KO mice transplanted with SR-BI/apoE double KO bone marrow and fed a high fat diet for four weeks showed statistically significant macroscopic changes of intact animal hearts based on heart:body weight ratios indicating cardiomegaly. Although this correlates with the phenotype of SR-BI/apoE double KO mice which includes cardiomegaly, and multiple myocardial infarct resulting in

premature death, the cardiomegaly reported in the mice transplanted with SR-BI/apoE double KO bone marrow mice was less pronounced. Biological significance in the transplanted mice has not been determined, and this observation would have to be supported by evaluation of cardiac function. Magnetic resonance imaging (MRI) would accurately determine murine myocardial mass, assess heart damage and measure blood flow [195-197] and echocardiograms would measure pumping abilities and to measure the size of the chambers of the heart, including the dimension or volume of the cavity and the thickness of the walls [199]. SR-BI/apoE double KO mice also develop extensive cardiac fibrosis [80]. We therefore examined heart sections for fibrosis in transplanted mice induced to develop atherosclerosis by feeding a high fat diet for four or twelve weeks. There was no observable fibrosis in any sections (data not shown). It is possible that the cardiomegaly we observed may indicate early stages of heart disease but the disease has not progressed to myocardial infarction and fibrosis. More research is required to determine if bone marrow derived SR-BI influences coronary heart disease in apoE KO mice.

The bone marrow transplantation technique allows for a quick, straightforward means to genetically eliminate SR-BI from bone marrow derived cells, yet it has limitations. Recipient mice were transplanted with crude bone marrow which consists of pluripotent hematopoietic stem cells surrounded by stromal cells and multipotent mesenchymal stem cells [213]. SR-BI is expressed in cell types that arise from the bone marrow including macrophages [55-57], yet circulating endothelial cell precursors [214] and cardiomyocytes [47] also originate in the bone marrow. It is not clear whether any

influence of SR-BI *in vivo* are the direct result of macrophages, macrophage foam cells, or another hematopoietic derivative such as endothelial cells.

4.2 Implications of dysregulated SR-BI expression by lipoprotein-derived cholesterol

SR-BI expression in macrophages is down regulated by oxidized LDL [89], acetylated LDL and 25-hydroxy-cholesterol [90]. In this study we report that murine SR-BI expression in macrophages (elicited peritoneal macrophages and Raw 264.7 cells) is down-regulated in response to cholesterol associated with native lipoproteins. This may affect SR-BI mediated cholesterol exchange between cells and lipoproteins.

Macrophages are the principle cell type in both early and advanced atherosclerotic plaque [26], and these phagocytic cells take up large quantities of lipoproteins without negative feedback control [24]. It is conceivable that in advanced plaques, which accumulate increasing quantities of lipoprotein derived cholesterol and accumulate cholesterol in lipid droplets, that macrophage SR-BI *in vivo* is down-regulated in a similar manner to what we report in this study in culture. This supports the atherosclerotic analysis which indicates no statistically significant changes in the sizes of advanced plaques in the aortic sinus when SR-BI is eliminated from bone marrow derived cells when apoE KO mice are fed a high fat diet. It remains to be determined if the levels of SR-BI expression in macrophages differ in early versus advanced plaques and immunohistochemistry experiments would be required to assess this.

The overexpression of murine SR-BI in cell culture resulted in increased HDL cholesterol uptake and increased cellular cholesterol content [52]. Nonetheless, studies using cells overexpressing SR-BI demonstrate that SR-BI can mediate the efflux of unesterified cholesterol tracer to HDL and phospholipid acceptors [55,96], and the net flux of HDL cholesterol/lipids is dependent on the cholesterol concentration gradient [52,96]. Cholesterol efflux is a popular hypothesis explaining atheroprotection associated with SR-BI, yet in macrophages definitive biological data supporting this is lacking. In primary macrophages two reports cite no significant decreases in efflux due to the genetic elimination of SR-BI [78,83], and another report cited no significant cholesterol efflux due to SR-BI or ABCA1 in J774 macrophage-like cells [96]. On the other hand, two other studies show minor decreases in cholesterol efflux to HDL when SR-BI was eliminated from primary macrophages [82,97]. Work reported by our lab and this study demonstrate no decrease in cholesterol efflux due to the inactivation of the SR-BI gene [78]. We have shown that macrophage SR-BI is expressed throughout the conditions of the efflux experiment, and we have demonstrated that the quantity of free apoA-I, an established cholesterol acceptor for ABCA1 mediated efflux, is minor in our HDL preparation. Our laboratory's data and the supporting evidence presented in this study indicate no cholesterol efflux due to the presence of SR-BI in macrophages.

4.3 Implications of dysregulated SR-BI expression by glucosamine

High blood glucose causes metabolic abnormalities that predispose diabetics to atherosclerosis [4], and elevated levels of glucose promotes increased glucose uptake and leads to increased levels of intracellular glucosamine [122]. Increased glucosamine levels have been shown to interfere with protein glycosylation [148] and can lead to ER stress [215]. It has recently been shown that ER stress correlates with atherogenic lesion development in hyperhomocyteinemic apoE knockout mice [169] and that ER stress may be linked to accelerated atherosclerosis associated with diabetes mellitus [170].

It has also been reported that the induction of ER stress in cells leads to the alteration of SREBP independent of cellular sterol levels [168]. Our finding that SR-BI levels are negatively regulated by lipoproteins and those of others demonstrating negative regulation by sterols [90] and/or transcription activation of the SR-BI promoter by SREBP [87,88], suggest the possibility that ER stress may affect SR-BI levels in cells. We tested this by culturing cells in the presence of DTT or glucosamine (or mannitol) to induce ER stress. While treatment with DTT tended to result in either unchanged or slightly increased levels of SR-BI (as did mannitol), exposure to glucosamine tended to result in reduced levels of the mature 82kDa protein in both Raw 264.7 cells and ldlA[mSR-BI] cells. This appeared to be due to altered glycosylation of SR-BI rather than decreased polypeptide levels. Interestingly, in the absence of protein synthesis SR-BI appeared to be rather stable with substantial amounts of mature 82kDa SR-BI remaining 17 hours after the addition of cycloheximide. In contrast, in the presence of

protein synthesis, glucosamine appeared to result in the replacement of an 82kDa band with one of altered mobility probably due to altered glycosylation.

It remains to be determined if SR-BI expression *in vivo* is effected by agents that induce ER stress. Hyperhomocysteinemia has been linked to ER stress in apoE KO mice [169]. One approach would be to assess the expression of SR-BI in atherosclerotic lesions of apoE KO mice made hyperhomocysteinemic by feeding a high methionine diet [207,209]. Furthermore, SR-BI expression could be assessed in atherosclerotic lesions of streptozoticin-induced hyperglycemic apoE KO mice. Effects on SR-BI function by glucosamine and other ER stress agents have yet to be explored.

5. Conclusion

In this study we tested the effect of bone marrow specific elimination of SR-BI in apoE KO mice induced to develop advanced atherosclerosis by feeding a high fat diet for four weeks. We report statistically significant cardiomegaly when SR-BI/apoE double KO bone marrow was transplanted into apoE KO mice, yet the influence of the elimination of bone marrow specific SR-BI in apoE KO mice on coronary heart disease has not been explored. We demonstrate that the elimination of bone marrow specific SR-BI in apoE KO mice leads to no statistically significant changes in cross-sectional atherosclerotic plaque area or plaque volume in the aortic sinus when the transplanted mice were fed a high fat diet for four weeks. We observed a trend towards increased lesion size in apoE KO mice transplanted with SR-BI/apoE double KO bone marrow, as lesion size decreases yet this did not reach statistical significance.

We demonstrate that SR-BI expression in macrophages is decreased in response to native lipoproteins (LDL and HDL). It is unclear whether macrophage SR-BI expression is dysregulated *in vivo*. Based on these findings, it is conceivable that the expression of macrophage SR-BI in atherosclerotic plaques may not be static.

Treatment with glucosamine leads to a slight decrease in SR-BI expression in Raw 264.7 cells and in ldlA[mSR-BI] cells. In Raw cells it is potentially a result of ER stress. Glucosamine treatment appears to result in altered glycosylation of SR-BI in Raw 264.7 cells and in ldlA[mSR-BI] cells, and may require new protein synthesis. It is possible that decreased SR-BI expression is associated with diminished function of SR-BI, although this has not been explored.

Taken together, these *in vitro* studies highlight conditions that are relevant to an *in vivo* model of atherosclerosis, such as accumulated cholesterol in the artery wall or elevated plasma glucose levels that lead to increased intracellular glucosamine and ER stress. We speculate that conditions that lead to decreased SR-BI expression in macrophages may influence the progression of the advanced disease state in mice.

This study focuses on SR-BI in mice and murine cell lines. The expression of CLA-1, the human homologue of SR-BI, is also decreased by acetylated LDL and 25-hydroxycholesterol [90]. It is unknown how glucosamine affects CLA-1. It appears that the expression of SR-BI may be down-regulated with glucosamine or in response lipoproteins.

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