THE EFFECTS OF HYPOMORPHIC SIALIDASE EXPRESSION ON ATHEROSCLEROSIS

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By

ABRAHAM ERNEST YANG, HON. B. SC.

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AUTHOR: Abraham Ernest Yang, Hon. B. Sc. (University of Toronto)

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Abstract

Atherosclerosis is a complex multifactorial disease which is an important risk factor for morbidity and mortality related to coronary heart disease and stroke in humans. Both lipoprotein metabolism and inflammation are related to atherosclerosis. Sialidase, a hydrolytic enzyme that is present in viruses, bacteria and vertebrates and catalyzes the removal of terminal sialic acid residues from glycoproteins, glycolipids and oligosaccharides, has impacts on the activities of many blood cells and lipoproteins. Therefore, we aim to determine the effects of hypomorphic sialidase expression on atherosclerosis. Our first objective has shown that hypomorphic sialidase expression alters lipoprotein metabolism in C57Bl/6 mice. We have dissected the mechanism showing that sialidase-deficient B6.SM mice have decreased hepatic cholesterol production via decreased microsomal triglyceride transfer protein (MTP) expression. We have further shown that a decrease in sterol regulatory element binding protein-2 (SREBP-2) expression is responsible for the decreased expression of MTP. Our second objective has shown that hypomorphic sialidase expression confers atheroprotection in *apoe-/-* mice as seen by the rescue of hypercholesterolemia cholesterol profiles and a decreased migration and infiltration of leuckocytes into atherosclerotic lesions. We have further shown that hypomorphic sialidase expression specifically in blood cells is sufficient to affect atherogenesis. Most importantly, we have discovered that treating *apoe-/-* mice with 2-deoxy-2,3-dehydro-N-acetylneuraminic acid (DANA), a sialidase inhibitor, is a potent treatment for atherosclerosis. Our third objective has shown that hypomorphic sialidase expression confers atheroprotection via an increase in monocytic cholesterol uptake and

macrophage cholesterol efflux to HDL. Taken together, hypomorphic sialidase expression is atheroprotective in C57Bl/6, ApoE-deficient and LDLR-deficient mouse models. Overall, these studies suggest that sialidase is a novel risk factor for atherosclerosis in humans and may set the stage to investigate the contribution of genetic variation within the sialidase gene to atherosclerotic cardiovascular disease in humans.

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Preface

All the work has been performed by Abraham Yang with the following exceptions: Gabriel Gyulay performed LDLR and PCSK9 immunblotting (Fig. 2.3.5 A and C), LDLR quantitative real-time PCR (Fig. 2.3.5 D), lectins and pull downs (Fig. 2.3.5 E), macrophage Oil Red O staining (Fig. 5.3.10) and cholesterol efflux assay (Fig. 5.3.8 and 5.3.9). Gabriel Gyulay also contributed to folch lipid extraction in Ch. 2, Ch. 3 and Ch. 5. David Egier assisted in castration in Ch.3. Elizabeth White performed peripheral blood immunophenotyping, selectin and hyaluronic acid binding assay (Fig. 4.3.1, 4.3.6, 4.3.7 and 4.3.8), serum cytokine measurement (Fig. 4.3.5) and flow cytometry (Fig. 5.3.3, 5.3.4, 5.3.5 and 5.3.6). Šárka Lhoták performed paraffin sectioning and immunostaining in Ch. 3. Vivienne Tedesco and Mohamed Alsaied contributed to bone marrow transplantation of LDLR-/- mice in Ch. 5.

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List of Abbreviations

µm=Micrometer

4AAP=4-Aminoantipyrine

ABCA1= ATP-binding cassette transporter A1

ABCG1= ATP-binding cassette transporter G1

ACAT=Acyl-CoA cholesterol acyltransferase

ACAT1=Acyl-CoA cholesterol acyltransferase 1

ACAT2=Acyl-CoA cholesterol acyltransferase 2

ApoA-I=Apolipoprotein A-I

ApoB=Apolipoprotein B

ApoB-100=Apolipoprotein B-100

ApoB-48=Apolipoprotein B-48

ApoE=Apolipoprotein E

ARH=Autosomoal recessive hypercholesterolemia

ATP= Adenosine-5'-tryphosphate

Bp=Base pair

BSA=Bovine serum albumin

CD11b=Cluster of differentiation11b

CD15=Cluster of differentiation15

CD22=Cluster of differentiation22

CD3ε =Cluster of differentiation3 epsilon

CD43=Cluster of differentiation 43

CD44=Cluster of differentiation 44

CD45=Cluster of differentiation 45

CE= Cholesterol esterase

CO= Cholesterol oxidase

Cre=Cre recombinase

DANA=2-deoxy-2,3-dehydro-N-acetylneuraminic acid (Neu5Ac2en)

DBP= Vitamin D3-binding Protein

DNA=Deoxyribonucleic acid

ER=Endoplasmic reticulum

FPLC=Fast protein liquid chromatography

GCS complex=β-galactosidase, protective protein cathepsin A and sialidase complex

GK= Glycerol kinase

GM=Monosialotetrahexosylganglioside

Gy=Gray

HA=Hyaluronic acid

HBA= Hydroxybenzoic acid

HDL=High density lipoprotein

HMG-CoA Reductase=3 hydroxy-3-methyl-glutaryl-CoA reductase

HRP=Horseradish peroxidase

ICAM-1=Intercellular cell adhesion molecule-1

IDL=Intermediate density lipoprotein

Idol= Inducible degrader of LDLR

IFN-γ=Interferon-gamma

IL-1β=Interleukin-1 beta

IL-2=Interleukin-2

IL-4=Interleukin-4

IL-6=Interleukin-6

IL-10=Interleukin-10

IMDM= Iscove minimum Dulbecco medium

IκB-α=Inhibitor of kappa B-alpha

kDa=Kilo dalton

LAMP1= Lysosomal-associated membrane protein1

LDL=Low density lipoprotein

LDLR=Low density lipoprotein receptor

LPL=Lipoprotein lipase

LPS=Lipopolysaccharide

LRP=Low density lipoprotein receptor-related protein

LXR= Liver X Receptor

MAC-3=Macrophage-3 antigen

Maf= Macrophage-activating factor

MCP-1= Monocyte chemotactic protein 1

M-CSF= Macrophage-colony stimulating factor

MHC= Major histocompatibility complex

Min=Minute

ml=Milliliter

MTP=Microsomal triglyceride transfer protein

Mup= Mouse urinary promoter

MyD88=Myeloid differentiation primary response gene 88

Neu1=Neuraminidase 1

Neu2=Neuraminidase 2

Neu3=Neuraminidase 3

Neu4=Neuraminidase 4

NFκB=Nuclear factor kappa-light-chain-enhancer of activated B cells

Nm=Nanometer

PBS= Phosphate-buffered saline

PCSK9=

POD= Peroxidase

PPCA= Protective protein cathepsin A

RAG=Recombination activating gene

RCT=Reverse cholesterol transport

RNA=Ribonucleic acid

RT-PCR=Reverse transcription polymerase chain reaction

S=Second

SCID= Severe combined immunodeficiency

SR-BI= Scavenger receptor class B type 1

SREBP-1=Sterol regulatory element binding protein-1

SREBP-2= Sterol regulatory element binding protein-2

Th1=T helper 1

Th2=T helper 2

THP-1=Human acute monocytic leukemia cell line

TLR=Toll-like receptor

TLR4=Toll-like receptor 4

TNF-α= Tumor necrosis factor-α

TRAF6=TNF receptor-associated factor 6

VCAM-1 =Vascular cell adhesion molecule 1

VLDL=Very low density lipoprotein

VLDLR=Very low density lipoprotein receptor

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Chapter 1

Introduction and Relevant Literature Review

1.1 Atherosclerosis

Atherosclerosis is a leading preventable cause of death worldwide and a complex multifactorial disease which accounts for most of the morbidity and mortality seen in humans with coronary heart disease, myocardial infarction and stroke (Hegele RA, 2009). Cardiovascular disease remains the number one source of mortality in North America, Europe and Asia although many approaches have been implemented to decrease the occurrence and/or severity of atherosclerosis (Braunwald E., 1997). There are numerous risk factors for developing atherosclerosis (Steinberg and Witztum, 1990) such as obesity, high blood pressure, dyslipidemia, diabetes, smoking and genetic susceptibility (Ross R., 1995). Numerous studies have suggested that genetic and environmental factors can affect atherosclerosis (Hegele R., 1992). It has also been suggested that the composition and plasma levels of lipoproteins are strongly associated with atherosclerosis (Kashyap M., 1997). Genetic variation in the genes for several apolipoproteins and lipoprotein receptors affects lipoprotein metabolism additively and as such affects atherogenesis (Breslow J., 1988; Chen et al., 2003; Luc et al., 1994, Ross R., 1993). The extent of the contribution of each gene to the disease is under extensive investigation (Braunwald E., 1997), as many recognize that dissection of the relative contribution of the various genetic and environmental factors is necessary for developing effective therapies.

1.2 Atherosclerotic Progression

The progression and the development of atherosclerotic lesions are highly dependent on the presence of disrupted laminar flow as well as gaps between endothelial cells and proteoglycans in the extracellular matrix of the subendothelium (Dabagh et al., 2009; Skålén et al., 2002). The accumulation of subendothelial low density lipoprotein (LDL) at these sites is amplified if there is remodeling of the extracellular matrix and/or if there is an elevation of the LDL level in blood (Glass and Witztum, 2001).

LDL is protected from oxidation in blood. However, enzymatic modification, and specifically oxidation increases significantly when LDL particles accumulate in the subendothelium (Parthasarathy et al., 1992; Schwenke et al., 1989a; Schwenke et al., 1989b). Minimal oxidation will still allow LDLs to be taken up by the LDL receptors (LDLR). However, if there is extensive oxidation or enzymatic modification of LDL and its protein moiety ApoB, the ApoB component will become unidentifiable to the LDLR. As a result, the LDLs cannot be bound or internalized by LDLR (Navab et al., 1996; Steinberg and Gotto, 1999). The accumulation of oxidized LDL induces early inflammatory responses and expression of cell adhesion molecules on endothelial cells such as vascular cell adhesion molecule 1 (VCAM-1), inter cellular adhesion molecule 1 (ICAM-1), P-selections, E-selectins, and hyaluronic acid (HA) enabling the binding of leukocytes to the endothelium (Glass and Witztum, 2001). These leukocytes will then migrate into the intima in response to monocyte chemotactic protein 1 (MCP-1), and macrophage-colony stimulating factor (M-CSF) (Glass and Witztum, 2001; Hansson G., 2005). Scavenger receptors allow scavenger receptor-mediated endocytosis of the modified LDL (Daugherty et al., 2005; Steinbrecher U., 1999;

Trigatti et al., 2003). Furthermore, since scavenger receptor expression is not regulated by macrophage cholesterol content, this will lead to unregulated uptake of oxidized LDL and modified LDL (Steinbrecher U., 1999; Trigatti et al., 2003). Eventually, the unregulated uptake of oxidized LDL and modified LDL by macrophages will induce macrophages to release more MCP-1 resulting in additional monocyte recruitment. These monocytes then differentiate into macrophages, releasing more MCP-1 and recruiting more monocytes, further propagating the recruitment pathway (Lucas A and Greaves D, 2001). These macrophages continue to take up oxidized LDL and modified LDL. Eventually, these subendothelial macrophages become engorged with lipid droplets and a triglyceride core surrounded by a lipid monolayer (Brown and Goldstein, 1983; Daugherty et al., 2005; Parthasarathy et al., 1992; Steinbrecher U., 1999). These lipid filled cells are called foam cells which can continue to enhance atherosclerotic progression by oxidizing lipoproteins and remodeling the extracellular matrix (Aviram and Rosenblat, 1994; Daugherty et al., 2005). In addition, when these foam cells die, they can propagate the inflammatory process (Lucas A and Greaves D, 2001).

Another pathway exists that enables macrophage cholesterol efflux. This pathway is called reverse cholesterol transport (RCT) (Hegele R., 2009). Macrophage cholesterol efflux utilizes ATP-binding cassette transporters, ABCA1 and ABCG1, which allows transport of intracellular cholesterol to ApoA-I and high density lipoprotein (HDL) acceptor molecules respectively (Goldstein and Brown, 1975; Simons and Ikonen, 2000). Subsequently, HDL will deliver cholesterol to hepatocytes via SR-BI receptor (Hegele R, 2009; Suzuki et al.1997).

The development of fatty streaks resulting from accumulation of sub-endothelial LDL and macrophages mentioned above is followed by excessive inflammatory response (Ross R., 1999). The maturation of fatty streaks and the progression of atherosclerotic lesions occur through the expression of a variety of cytokines and inflammatory molecules as well as through the upregulation of cytokine receptors and toll-like receptors (TLR) on the macrophages and T-cells (Daugherty et al., 2005; Hansson G., 2005). T-cell activation occurs by inducing Th1 and Th2 responses which entails the release of cytokines, including but not limited to IFN-γ and IL-4, respectively (Hansson G., 1997). At the same time, Th1 cells can produce IFN-γ to induce the expression of various proinflammatory genes in the macrophage (Hansson G., 2005). IFN-γ can as well upregulate LDLR and downregulate VLDLR in the macrophage (Daugherty et al., 2005). The formation of atherosclerotic lesions expands when smooth muscle cells migrate into the intima from the tunica media resulting in smooth muscle cells proliferation, binding and uptake of more modified LDL by scavenger receptors (Lucas and Greaves, 2001; Ross R., 1999; Steinbrecher U., 1999). Eventually, these smooth muscle cells can dedifferentiate to become foam cells (Ross R., 1993).

VLDL can attract additional smooth muscle cells to the atherosclerotic lesion (Endemann et al., 1987; Zhao et al., 2001). At this stage, smooth muscle cells add complexity to the atherosclerotic lesion by forming fibrous layers between the fatty deposits and the intima. Eventually, calcification of the vascular smooth muscle cells will lead to calcium deposits (Ross R., 1993). Due to the appearance of necrotic foam cells, smooth muscle cells and calcification content, these mature atherosclerotic

lesions are also referred to as plaques (Ross R., 1995). In addition to narrowing the artery, these plaques can cause the accumulation of blood platelets, which may eventually lead to stenosis and thrombosis (Glass and Witztum, 2001). Nevertheless, plaque rupturing can also occur in areas where a thin fibrous cap of smooth muscle cells covering the plaque is present (Fishbein M., 2010). Thrombus usually develops in macrophage-rich regions of the artery (Daugherty et al., 2005). Thrombosis and stenosis can potentially lead to myocardial ischemia and ultimately infarction, which are often lethal (Glass and Witztum, 2001).

1.3 Lipoprotein Metabolism

Cholesterol is a steroid that is essential for a variety of cellular functions including phospholipid membrane integrity and steroid synthesis (Maxfield and Wüstner, 2002; Simons and Ikonen, 2000). Cholesterol is produced by many cells and can be obtained both exogenously and endogenously. Cholesterol and other lipids such as fatty acids and triglycerides, are highly hydrophobic and as a result are transported in the blood by lipoproteins (Steinberg and Witztum, 1990). The major carrier of blood cholesterol is LDL; however, other lipoproteins also play roles in its transport (Simons and Ikonen, 2000). Lipoprotein metabolism is important to understanding atherosclerosis and cholesterol homeostasis. Liver is the major organ for cholesterol metabolism (Goldstein and Brown, 1975; Simons and Ikonen, 2000).

Typically, chylomicrons are assembled in the intestinal mucosa as a means to transport dietary cholesterol and triglycerides to the rest of the body. The predominant lipids of chylomicrons are triacylglycerides (Hegele R., 2009). The major

apolipoproteins in chylomicrons are ApoB-48 (Hegele R., 2009; Karpe et al., 1996). Chylomicrons are then secreted into the lymphatic system and enter the circulation where they acquire ApoE and ApoC from high-density lipoproteins (HDL) (Redgrave, 1983). In the capillaries of adipose tissue and muscle, triacylglycerides are removed from chylomicrons by the action of lipoprotein lipase (LPL), which is found on the surface of the endothelial cells of the capillaries (Hegele R., 2009). Chylomicron remnants, containing primarily cholesterol, ApoE and ApoB-48, are delivered to, and taken up by the LDLR (ApoE/ApoB receptors) both in peripheral cells as well as the liver (Goldstein et al., 1985).

Hepatically synthesized triglycerides and cholesterol are packaged into very-low-density lipoproteins (VLDL) and released into the blood circulation for delivery to extrahepatic tissues for storage or production of energy through oxidation (Mahley et al., 1984). In addition to triglycerides and cholesterol, VLDL contain ApoB-100, ApoC and ApoE. Like chylomicrons, newly released VLDL acquire ApoC and ApoE from circulating HDL. The action of lipoprotein lipase converts VLDL to intermediate density lipoproteins (IDL), also termed VLDL remnants (Berneis and Krauss, 2002; Packard et al., 2000; Packard et al., 1984) that are transient intermediates in the delipidation cascade from VLDL to LDL. IDL are lipolysed by hepatic lipase, converting into LDL, or taken up by the liver through the interaction with hepatic ApoE/ApoB receptors (the LDL receptor is also called the ApoE/ApoB receptor) and/or LDL-related protein (LRP) (Chappell and Medh, 1998). LDL are taken up by cells via receptor-mediated endocytosis through the interaction of ApoB-100 component of LDL with LDL receptors located on plasma membranes (Twisk et al.,

2000). Thus, ApoB-100 can be used as a marker for tracing the metabolism of VLDL, IDL and LDL with the use of labelled amino acid precursors.

Receptors for ApoE containing lipoproteins include VLDLR, LDLR-related protein 1 (LRP1) and ApoE receptor 2, although their roles have not been as well studied as LDLR (Lillis et al., 2008; May et al., 2005). After the binding of LDL, LDLR is internalized into primary and early endosomes (Linton et al., 1999). Depending upon the intracellular cholesterol level, the LDLR is recycled back from late endosomes to the cell surface to be reused for further LDL endocytosis (Linton et al., 1999). Subsequent lysosomal degradation yields cholesterol and amino acids to the cell (Linton et al., 1999).

Excess cellular cholesterol is processed via reverse cholesterol transport (Hegele R, 2009; Suzuki et al.1997). It can occur from periphery cells back to the liver via ATP-binding cassette transporter's (ABCA1/G1) lipidation of ApoA-I and High Density Lipoprotein (HDL), respectively (Goldstein and Brown. 1975; Simons and Ikonen, 2000). Subsequently, these molecules will be taken up by the liver via the SR-BI receptor (Hegele R, 2009; Suzuki et al.1997).

Intracellular cholesterol level is controlled by sterol regulatory element binding protein (SREBP). SREBP is membrane-bound transcription factor that is responsible for the activation of genes involved in cholesterol synthesis (Brown and Goldstein, 1997; Horton et al., 2002). There are three mammalian isoforms of SREBP, namely, SREBP-1a, SREBP-1c and SREBP-2 (Yokoyama et al., 1993; Hua et al., 1993;

Brown and Goldstein, 1997). SREBP-1a and -1c are transcribed from the same gene with alternative mRNA splicing. SREBP-1a and -1c are mainly involved in activating genes of fatty acid synthesis (Soccio and Breslow, 2004). SREBP-2 is responsible for activating genes of cholesterol synthesis (Soccio and Breslow, 2004).

SREBPs consist of four domains with two membrane-spanning regions. Their N- and C-terminuses project into the cytoplasm (Yokoyama et al., 1993). The N-terminus is constituted of a transcription factor, being followed by a glycine, serine, proline, and glutamine rich region. The glycine, serine, proline and glutamine rich region is followed by a basic-helix-loop-helix leucine zipper (bHLHZip) protein. The C-terminus is a regulatory domain which allows interaction with cleavage activating protein (SCAP) (Hua et al., 1996; Sakai et al., 1997). Scap is embedded in ER membranes via its N-terminal domain. The Scap N-terminus is composed of eight transmembrane (TM) helices separated by hydrophilic loops with TM helices 2-6 being involved in cholesterol-sensing (Nohturftt et al., 1998a; Nohturfft et al., 1998b; Hua et al., 1996).

When the cholesterol level is low, the Scap/SREBP complex exits the ER in COPII-coated vesicles that bud from ER membranes (Nohturfft et al., 2000; Espenshade et al., 2002; Sun et al., 2005). The Scap/SREBP complex enters the Golgi apparatus where site-1 protease, a serine protease of the subtilisin family, cleaves SREBP at the loop region (Espenshade et al., 1999), followed by the second cleavage in the first membrane spanning region by site-2 protease, a Zn2+ metalloprotease (Zelenski et al., 1999), to release the N-terminal of SREBP (bHLH-Zip-containing domain) (Sakai et

al., 1998; Duncan et al., 1997; Espenshade et al., 1999; Duncan et al., 1998; Rawson et al., 1997). The cleaved form of SREBP (bHLH-Zip-containing domain) is transported into the nucleus by binding directly to importin β with Ran-GTP causing dissociation of the SREBP-importin β complex (Nagoshi et al., 1999). Scap travels back to the ER to interact with another SREBP molecule. In the nucleus, SREBP activates transcription of target genes by binding to the sterol regulatory element (SRE) in the promoter region of LDLR and 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCoA reductase) genes (Briggs et al., 1993; Wang et al., 1993). When the cholesterol level is high, choesterol binds to Scap causing a structural change in SCAP. This structural change in SCAP allows Insig to bind to Scap. There are two isoforms of Insig. Insig-1 is a SREBP target gene (Janowski B., 2002; Horton et al., 2002). Insig-2 is expressed at a low but constitutive level and is not regulated by SREBP (Yabe et al., 2002). When Insig binds to Scap, the Scap/SREBP complex remains in the ER (Sun et al., 2005). Thus, transcription of genes involved in cholesterol synthesis decreases.

In order to monitor the level of intracellular sterols, cells have, in addition to SREBP, an ER resident transmembrane protein, HMG CoA reductase,. At the transcriptional level, HMG CoA reductase is controlled by SREBPs. At the posttranscriptional level, it is regulated by sterol-accelerated degradation (Gil et al., 1985; Skalnik et al., 1988; Sever et al., 2003b). HMG CoA reductase is embedded in ER membranes through its N-terminal domain that contains eight TM helices (Roitelman et al., 1992). TM helices 2-6 are involved in the sterol-sensing (Hua et al., 1996; Nohturfft et al., 1998a; Sever et al., 2003a). The C-terminal domain of the reductase is projected into the

cytoplasm and is attached to the ER membrane by a proline-rich region (Liscum et al., 1985). The mechanism for sterol-regulated HMG CoA reductase occurs when the cholesterol precursor, lanosterol, triggers the binding of the reductase to Insig (Sever et al., 2003a; Song et al., 2005a). Insig exists in a complex with E3 ubiquitin ligase, gp78, which binds to the E2 ubiquitin-conjugating enzyme Ubc7 (Song et al., 2005b; Ponting, 2000). Gp78 also binds to Valosin-containing protein (VCP), an ATPase implicated in the postubiquitination steps of ER-associated protein degradation (ERAD) (Zhong et al., 2004; Ye et al., 2005). ERAD is the process of retrotranslocating denatured ER proteins across the ER membrane for degradation by the cytosolic proteasome (Meusser et al., 2005). Gp78 ubiquitinates the reductase and then the ubiquitinated reductase is extracted from the membrane by VCP and delivered to the proteasome for degradation (Sever et al., 2003a; Song et al., 2005b).

1.4 LDLR and LDL

Cholesterol, a complex four-ring structure, was first isolated from gallstones in 1784. Cholesterol transport in blood requires esterification and packaging of these esters within the hydrophobic cores of lipoproteins. In the 1950s, four major classes of lipoproteins were identified, i.e., VLDL, IDL, LDL and HDL (Gofman et al., 1954). LDL, being the most abundant cholesterol-carrying lipoprotein in blood, plays a significant role in cholesterol homeostasis by a receptor-mediated pathway. LDLR is a cell surface glycoprotein that contains two asparagine-linked (N-linked) oligosaccharides (Cummings et al., 1983; Kozarsky et al., 1988). About two-thirds of the O-linked glycans are clustered in the extracellular region of the protein near the membrane spanning region (Davis et al., 1986; Kingsley et al., 1986; Kozarsky et al.,

1988). The binding domain of LDLR contains 202 amino acids and consists of regions that bind ApoB-100 and ApoE respectively. It was shown that lipoproteins that contain multiple copies of ApoE bind to LDLR with higher efficiency compared to lipoproteins that contain only one copy of ApoB, e.g., LDL (Innerarity and Mahley, 1978).

The expression of LDLR is tightly regulated at both the transcriptional and posttranscritpional levels. Transcription of the LDLR gene is regulated by intracellular cholesterol levels via sterol regulatory element binding protein (SREBP) (Yokoyama et al., 1993; Hua et al., 1993). When intracellular cholesterol decreases, the more active form of SREBP is processed and can induce the transcription of the LDLR gene (Goldstein et al., 2006).

Post-transcriptional regulation of LDLR expression is regulated by a LDLR adaptor protein that is involved in autosomal recessive hypercholesterolemia (ARH) (Garcia et al., 2001; Cohen et al., 2003) and proprotein convertase subtilisin/kexin 9 (PCSK9) (Abifadel et al., 2003; Cohen et al., 2005; Seidah et al., 2003; Maxwell and Breslow, 2004; Park et al., 2004), which itself is a SREBP target gene. Recently, Zelcer and colleagues (2009) have demonstrated that Liver X Receptor (LXR), an important regulator of metabolic and inflammatory signalling, has the ability to regulate LDLR expression via inducible degradation of LDLR (idol). Idol is a protein that interacts with myosin regulatory light chain (Olsson et al., 1999). The authors believe that the altered expression of LDLR is caused by idol-mediated ubiquitination of LDLR.

In addition to the cholesterol-mediated expression of LDLR, the degree of sialylation and glycosylation has also been shown to affect LDLR activity (Zelcer et al., 2009). When human fibroblast cells are treated with sialidase, which cleaves sialic acid from oligosaccharides, there is a 7 to 11 kDa reduction of LDLR size. In addition, sialic acids on the LDLR contribute a net negative charge (Shite et al., 1990). Most importantly, treatment of human fibroblasts with sialidase results in reduced LDL binding and internalization compared to LDLR with normal sialylation (Sprague et al., 1988).

The sialic acid content of lipoproteins varies between different lipoprotein fractions (Millar et al, 1999; Sobenin et al., 1998; Anber et al., 1997). Sialic acid content of lipoproteins is determined by the action of sialyltransferases that sialylate apolipoproteins prior to their secretion into blood. The site of sialylation on apolipoproteins is influenced by the conditions under which the apolippoprotein is synthesized and is highly dependent on the level and type of sialyltransferases since sialyltransferases are differentially expressed in different tissues (Dwek et al., 1993; Ravindranaths et al., 1988). It has been shown that sialic acid level of ApoE produced by monocytes is much higher than those in blood (Ravindranaths et al., 1988). Tissue-specific sialylation of apolipoproteins can be affected by hypercholesterolemia and inflammation (Tsunemitsu et al., 1990; Ghosh et al., 1991; Ghosh et al., 1999).

Sialidase activity has been detected in blood. Interestingly, its pH optimum is between 4 and 5 (Schauer et al., 1976). While sialidase levels in blood increase following myocardial infarction, little is known about the source of the enzyme (Hanson et al.,

1987). However, many believe that the presence of sialidase activity in the blood is likely due to tissue injury (Gross and Brossmer, 1991). Desialylated ApoB-100 has normal clearance from blood compared to the sialylated controls (Malmendier et al., 1980; Attie et al., 1979; Hatton et al., 1978; Morell et al., 1971). It has been shown that LDL with low level of sialylation has increased propensity to aggregate (Tertov and Orekhov, 1994). However, desialylation of LDL has no effect on LDL binding and uptake by fibroblasts or hepatocytes (Attie et al., 1979; Shireman and Fisher, 1979). LDL and LDL with a low level of sialylation both show an increase in uptake by aortic smooth muscle cells (Orekhov et al., 1989; Filipovic et al., 1979). Camejo and colleagues have demonstrated that desialylation of LDL increases its interaction with chondroitin-6-sulfate-rich proteoglycans isolated from arterial wall matrix (Camejo et al., 1985). Furthermore, sialylated ApoE promotes cholesteryl ester uptake from HDL to HepG2 cells more efficiently than sialidase treated HDL or desialylated ApoE (Ghiselli et al., 1986).

1.5 Factors affecting VLDL assembly/secretion

VLDL assembly/secretion pathway is dependent upon: (1) the production of apolipoprotein B (ApoB); (2) the availability of sufficient lipids (triglycerides, free cholesterol and cholesterol esters); (3) the normal activity of microsomal triglyceride transfer protein (MTP). In the absence of any one or more of these factors, ApoB is diverted from the VLDL assembly/secretion pathway and is degraded (Davis R, 1999; Gibbons G, 1990; Kang and Davis, 2000; Thompson et al., 1996).

Triglyceride enters the cisternal space of the endoplasmic reticulum (ER) when ApoB is present in the membrane but into the cytoplasmic space in the absence of ApoB (Olofsson et al., 1987). Thus, the presence of ApoB is critical to the VLDL assembly/secretion pathway. It has been suggested that MTP, a specific triglyceride transfer protein, play a role in this process (Wetterau and Zilversmit, 1984; Wetterau and Zilversmit , 1985). MTP facilitates the transfer of lipids between membranes and is important in the assembly of lipoproteins in the lumen of the ER (Wetterau and Zilversmit, 1986). This is supported by the discovery that MTP is the defective "abetalipoproteinemia" gene responsible for the inability to secrete ApoB-containing lipoproteins (Wetterau et al., 1992; Sharp et al., 1993). In addition, heterozygous mice expressing a mutated MTP gene have decreased levels of ApoB-containing lipoproteins (Raabe et al., 1998). Furthermore, conditional cre/lox liver MTP gene knockout mice showed a marked impairment in the production and secretion of ApoB-containing lipoproteins (Chang et al., 1999). Therefore, MTP plays an essential and rate-limiting role in the secretion of ApoB-containing lipoproteins in mice.

1.6 Sialic Acid

Sialic acid, also known as neuraminic acid, is a nine carbon hexose terminal sugar residue found on a variety of oligosaccharides. It is commonly bound by an α2-3, α2-6 or α2-8 glycosidic linkage to other sugars (Cross and Wright, 1991; Millar J., 2001). The most common linkage is α2-3 linkage to galactose. Sialic acid is found in animal tissue and in bacteria bound to glycoproteins and glycolipids and is a significant contributor to the overall net negative charge of the cell surface. It compresses 50% of the overall charge (Carrillo et al., 1997; Millar J., 2001; Sprague E. et al., 1988). In

addition, the degree of sialylation regulates cell identification, cell adhesion, cell-cell interactions, cell proliferation and conformational stabilization (Millar J., 2001; Monti et al., 2000).

1.7 Sialidase

Lysosomal storage diseases are metabolic disorders caused by defects in the synthesis, targeting, or functioning of lysosomal enzymes. They are characterized by a massive accumulation of undegraded substrates of deficient enzymes in the lysosomes. A major fraction of lysosomal storage diseases is caused by deficiency in glycosidases involved in the catabolism of the sugar chains of oligosaccharides, glycoproteins and glycolipids (Kornfeld and Sly, 2001). It includes Tay-Sachs disease caused by a deficiency of beta-hexosaminidase A; Fabry disease which is caused by a deficiency of acid alpha-galactosidase A; Gaucher disease which is caused by a deficiency of acid beta-glucosidase (Kornfeld and Sly, 2001).

Sialidosis and galactosialidosis are autosomal recessive disorders. They arise due to a deficiency of lysosoaml sialidase and are caused by two different primary genetic defects. Sialidosis is an autosomal recessive disease caused by mutations in the sialidase gene (Thomas G., 2001). Galactosialidosis is an autosomal recessive disease caused by mutations in the protective protein cathepsin A (PPCA) gene, encoding a lysosomal carboxypeptidase (d'Azzo et al., 2001; Galjart et al., 1988). PPCA was cloned in 1988 and characterized for its mutation associated with galactosialidosis. It is responsible for the stabilization of the active conformation of sialidase and protects

it against proteolytic degradation in the lysosome (Vinogradova et al., 1998; van der Horst et al., 1989; d'Azzo et al., 1982).

There are two major types of sialidosis. They are the late-onset sialidosis type I and early-onset sialidosis type II. Type I and type II dffer in both the age of onset and severity. Late-onset, Type I sialidosis manifests in the second or third decade of life. Patients of Type I sialidosis usually develop bilateral cherry-red spots, impaired vision, myoclonus of the extremities and gait abnormalities (O'Brien and Warner, 1980; Sasagasako et al., 1993; Bonten et al., 2000). Early-onset, Type II sialidosis may be diagnosed in utero or during infancy. Patients of Type II sialidosis exhibit developmental delay, short stature, mental retardation, hepatosplenomegaly and corneal clouding (Lowden and O'Brian, 1979; Matsuo et al., 1983; Young et al., 1987).

Sialidases constitute a family of hydrolytic enzymes that are found in viruses, bacteria and vertebrates and catalyze the removal of terminal sialic acid residues from glycoproteins, glycolipids and oligosaccharides whereas sialyltransferases catalyze the addition of sialic acid to oligosaccharides (Achyuthan and Achyuthan, 2001; Pilatte et al., 1993; Pshezhetsky and Ashmarina, 2001; Vimr and Lichtensteiger, 2002). Neu1, the lysosomal form of the sialidase enzyme, cleaves terminal α2-3 and α2-6 sialic acid residues with an optimal activity pH of 4.5 (Igdoura et al., 1998; Pattison et al., 2004). These enzymes influence a number of important biological processes including cell-cell interactions, T-cell activation, catabolism and antigenicity (Varki, 1997; Pilatte et al., 1993; Corfield, 1992; Reuter and Gabius, 1996). In mammals, four

distinct sialidases have been identified. Genes encoding lysosomal (*neu1*) (Igdoura et al., 1998; Carrillo et al., 1997; Pshezhesky et al., 1997), cytosolic (*neu2*) (Monti et al., 1999; Ferrari et al., 1994), ganglioside specific (*neu3*) (Miyagi et al., 1999; Wada et al., 1999), and mitochondrial sialidases (*neu4*) (Comelli et al., 2003; Monti et al., 2004; Yamaguchi et al., 2005) have been cloned. It has been reported that neu1 sialidase is present in the cell membrane in addition to the lysosome (Liang et al., 2006; Millar J., 2001; Rottier et al., 1998). In mice, there are three *neu* alleles (a, b and c) that have different rates of activity (Carrillo et al., 1997).

The human *neu1* gene is located in the HLA histocompatibility locus on chromosome 6p21.3. The murine *neu1* gene maps to the H-2 locus on chromosome 17 (Bonten et al., 1996; Pshezhetsky et al., 1997; Rottier et al., 1998; Igdoura et al., 1998). The human *neu1* mRNA shares 90% sequence identity with the murine *neu1*. Both the human and the murine neu1 have a signal sequence which initiates the translocation of the polypeptide into the ER. There are three Asn-linked glycosylation sites in the human neu1 and four Asn-linked glycosylation sites in the murine neu1 (Bonten et al., 1996; Rottier et al., 1998). It has been shown that glycosylation sites can affect the stability and folding of the proteins (Wang et al., 2009). Neu1 has a sequence motif of eight amino acids, designated as “Asp-box” which is highly conserved among sialidases (Roggentin et al., 1993). The precise role of this Asp-box is still uncertain. Neu1 also has a (F/Y) RIP motif. This motif is located near the N-terminus of neu1 and is a highly conserved domain in non-viral sialidases (Bonten et al., 1996).

Sialidase (neu1) plays major roles in homeostasis. Neu1 is a negative regulator of lysosomal exocytosis (Yogalingam et al., 2008). It controls the extent of sialylation and the turnover of lysosomal-associated membrane protein (LAMP). Loss of neu1 is associated with hypersialylation of LAMP1 at the lysosomal membrane. Hypersialylated LAMP1 are more prone to dock at the plasma membrane causing excessive exocytosis of lysosomal content. This results in premature degradation of VCAM-1 in the bone niche by excessive serine proteases (Yogalingam et al., 2008). Loss of neu1 has also been shown to be associated with hearing loss and muscle atrophy which is believed to be due to hypersialylation of LAMPs, particularly LAMP1 (Wu et al., 2010; Zanoteli et al., 2010). Loss of neu1 in skeletal muscle is associated with excessive lysosomal exocytosis of fibroblasts which can lead to abnormal remodeling of the extracellular matrix and excessive proliferation of connective tissue (Zanoteli et al., 2010).

Lysosomal sialidase, a 46kDa enzyme, exists in a multienzyme complex consisting of β-galactosidase, protective protein cathepsin A (PPCA) and sialidase (GCS complex) (Igdoura et al., 1998; van der Spoel et al., 1998). All sialidase enzymes have a similar conformation consisting of six, four-stranded antiparallel beta-sheets arranged in a propeller-like fashion (Igdoura et al., 1998). The active site of sialidase consists of an arginine triad, a hydrophobic pocket, a tyrosine residue and glutamic acid residue. The length and conformation of the loops connecting the beta-sheets may play a role in determining sialidase substrate specificity (Magesh et al., 2006).

For the lysosomal routing, sialidase is inefficiently transported to the lysosome via interaction with mannose 6-phosphate receptor (MPR), the canonical mode of lysosomal transport for soluble lysosomal enzymes (Hille-Rehfeld et al., 1995), and is believed to be due to low mannose 6-phosphorylation (van der Spoel et al., 1998). Two different mechanisms of lysosomal targeting of sialidase have been described. For the first mechanism, sialidase is proposed to be an integral membrane protein that is compartmentalized in lysosomes via the interaction of its C-terminal tetrapeptide (YGTL) with the adaptor protein complex-3 (AP-3). Sialidase is transported to the lysosomes, linked to membrane vesicles, and is released as soluble enzyme into the lumen by proteolytic cleavage (Lukong et al., 2001). It remains to be determined whether lysosomal proteolytic cleavage exists. For the second mechanism, PPCA is required for optimal lysosomal targeting as illustrated by changes in biochemical and structural properties of sialidase when in complex with PPCA (Bonten and d'Azzo, 2000). PPCA has a functional mannose 6-phosphate receptor (MPR). Therefore, by interacting with PPCA, sialidase is compartmentalized in lysosomes via the MPR pathway. When PPCA is absent, sialidase remains in the ER and is eventually degraded (van der Spoel et al., 1998).

PPCA, sialidase and β-galactosidase forms a high molecular weight multienzyme complex that enables subsequent activation of the sialidase enzyme (van der Spoel et al., 1998; Bonten et al., 1995; Rudenko et al., 1995; Igdoura et al., 1998; Bonten and d'Azzo, 2000). The carboxypeptidase activity of PPCA does not appear to play a role in sialidase activation (Galjart et al., 1991). The exact mechanism of the catalytic activation of sialidase is still uncertain. Nevertheless, PPCA has been shown to be the

rate limiting factor for sialidase activation as demonstrated by a positive correlation between the proportional increase of sialidase activity and PPCA levels in the lysosomes (Bonten et al., 2009). Mutations in the neuraminidase gene can lead to sialidosis, whereas defects in PPCA can cause galactosialidosis (van der Spoel et al., 1998). In addition to PPCA, another glycosidase, GALNS (N-acetylgalactosamine-6-sulfate sulfatase), is found to be associated with the GCS complex and may play roles in additional hydrolases (Pshezhetsky and Potier, 1996; van der Spoel et al., 1998; Yamamoto and Nishimura, 1987; Zhou et al., 1995).

PPCA mutations have been shown to associate with the loss of neu1 activity and reduced β-galactosidase activity (Itoh et al., 2000). Galactosialidosis patients differ in both the age of onset and severity of the disease. The early-onset form has symptoms of cardiac and renal failure, visceromegaly, skeletal dysplasia and early death. They have similar phenotypic manifestations of sialidosis, caused by neu1 deficiency. PPCA-/- mice have been generated and display symptoms similar to that of galactosialidosis patients including early death, renal failure and hepatosplenomegaly (Zhou et al., 1995). Loss of Purkinje cells has been detected in the PPCA-/- mice although researchers are still uncertain of the cause. In addition, PPCA has been shown to play a role in regulating blood pressure and circulation (d'Azzo et al., 2001). Recently, the cardiovascular role of PPCA has been demonstrated by a PPCA-deficient mouse model showing that PPCA acts as an endothelin-1-inactivating enzyme. Endothelin-1 is a vasoconstrictive peptide. PPCA-deficient mice have a reduced degradation rate of endothelin-1 and increased arterial blood pressure (Seyrantepe et al., 2008).

1.8 Sialic acid and sialidase involvement in immune, inflammatory response and atherosclerosis

In the immune system, sialidase and sialic acid are mainly involved in the activation of macrophages (Lambré et al., 1990; Liang et al., 2006; Stamatos et al., 2005), T cells (Pappu and Shrikant, 2004; Xu and Weiss, 2002) and neutrophils (Cross et al., 1991; Cross et al., 2003; Sakarya et al., 2004). It has been demonstrated that THP-1 monocyte differentiation involves upregulation of neu1 sialidase and its targeting to the cell membrane (Liang et al., 2006). The CD45 antigen, required for T-cell activation, is negatively regulated by homodimer formation (Xu and Weiss, 2002). The rate of homodimerization is controlled, at least in part, by the degree of sialylation and O-glycosylation of CD45 (Pappu and Shrikant, 2004). T-cell activation is increased as a result of desialylation, sialidase expression and secretion of many inflammatory factors, such as, IL-4 (Chen et al., 1997; Pappu and Shrikant, 2004; Wang et al., 2004). In addition, sialidase activation plays a significant role in CD44-HA adhesion in LPS and TNF stimulated THP-1 monocytes (Gee et al., 2002; Gee et al., 2003). Sialidase movement to the plasma membrane plays a role in activation, adhesion, and migration of neutrophils (Cross et al., 1991; Cross et al., 2003; Sakarya et al., 2004). The activation and adhesion can be blocked by sialidase inhibitor, 2-deoxy-2,3-dehydro-N-acetyl-neuraminic acid (DANA) (Cross et al., 1991). Furthermore, Vitamin D3-binding Protein (DBP) is converted to DBP-*maf* (macrophage-activating factor) via post-translational modification with β-galactosidase from B-cells and sialidase from T-cells (Swamy et al., 2001). DBP-*maf* converts monocytes into macrophages, which play role in immune response (Yamamoto et al., 1991).

Recently, CD15s (sialyl Lewis X), a glycan moiety which is commonly found on myeloid lineage cell surface glycolipids and glypoproteins, was shown to be desialylated during differentiation which enable selectin binding (Gadhoum and Sackstein, 2008). Activation, recruitment and differentiation of monocytes and leukocytes play an important role in the inflammatory response which is prominent in the atherosclerosis process (Ross R., 1999). Vascular cell adhesion molecule 1 (VCAM-1), enables leukocytes to bind to the endothelial cells (Glass and Witztum, 2001). Recently, it was demonstrated that neu1 deficiency results in hypersialylation of LAMP1 (lysosomal-associated membrane protein) at the lysosomal membrane and excessive exocytosis of lysosomal content. These lead to premature degradation of VCAM-1 in the bone niche by excessive serine proteases (Yogalingam et al., 2008). VCAM-1-deficient mice are less susceptible to the initiation of atherosclerosis compared to control in LDLR-/- background (Cybulsky et al., 2001). Therefore, neu1 sialidase deficiency has atheroprotective potential on atherosclerosis.

The investigation of atherosclerosis needs to consider inflammation. This is because atherosclerosis is not due solely to lipid accumulation. Many inflammatory molecules were affect atherosclerosis. This view is supported by the finding of decreased atherosclerosis in IL-4/ApoE double knockout mice compared to ApoE knockout mice (Davenport and Tipping, 2003). Simiarly, inhibition of tumor necrosis factor-α (TNF-α) in ApoE knockout mice reduces atherosclerosis (Branen et al., 2004). RAG-1 knockout mice, which lack functional T and B cells, were crossed with ApoE knockout mice to produce RAG-1/ApoE double knockout mice. RAG-1/ApoE double knockout mice mice exhibited a reduction of atherosclerosis compared to ApoE

knockout mice (Dansky et al., 1997). Severe combined immunodeficient mice (SCID) carrying ApoE knockout mutation, showed a decrease in the level of atherosclerotic lesion (Zhou et al., 2000).

Atherosclerotic lesions are mostly made up of macrophages, smooth muscle cells and T lymphocytes (Jonasson et al., 1986; Emeson et al., 1988; Hansson et al., 1988; Katsuda et al., 1992). Recently, several studies have described a key role for sialylation in the regulation of CD44 and CD45 important in the activation and/or recruitment of monocytes/macrophages (Gee et al., 2003; Katoh et al., 1999; Xu and Weiss, 2002). In addition, CD44 promotes atherosclerosis by inducing activation of both inflammatory and vascular cells (DeGrendele et al., 1996; Hodge-Dufour et al., 1997; McKee et al., 1996). Most importantly, CD44-null mice had reduced atherosclerosis compared with heterozygote and wild-type mice (Cuff et al., 2001).

1.9 Sialidase Inhibitors

In 1942, Hirst discovered an enzyme activity on the influenza virus surface that removed virus receptors from erythrocytes. Burnet used this concept and deduced that inhibition of an enzyme involved in this receptor-destroying mechanism could lead to the treatment of the influenza virus. After that, Gottschalk characterized the chemical structure of neuraminic acid (sialic acid) (Neu5Ac), its linkage to glycoconjugates, and the specificity of the enzyme for terminal sialic acid residues. It was not until 1969 when the first inhibitor of influenza virus sialidase, 2-deoxy-2,3-dehydro-N-acetylneuraminic acid (Neu5Ac2en) (DANA) was developed (Meindl et al., 1969; Meindl et al., 1971; Meindl et al., 1974). DANA functions as a competitor with sialic

acid for binding to the active site of the sialidase. It resembles the transition state configuration of sialic acid during the enzymatic reaction. However, DANA had low potency and specificity.

In the 1980s, Colman and colleagues reported the crystal structure of influenza virus sialidase and of its complex with sialic acid (Colman et al., 1989). This set the stage for the development of sialic acid derivatives that better suited to function as competitors of sialic acid in the binding to the active side of the sialidase. In 1993, von Itzstein and his colleagues attempted to increase the interaction between Neu5An2en and the amino acid residues forming the enzyme active site by substituting a guanidinyl group for a hydroxyl on carbon atom 4. This led to the development of 4-guanidino-Neu5Ac2en (Zanamir). Von Itzstein and his colleagues also demonstrated that Zanamir had high poteny and specificity (von Itzstein et al., 1993). However, Zanamir needed to be inhaled. The first orally active sialidase inhibitor, Oseltamivir, was developed by Kim and colleagues in 1997 by using a cyclohexene ring and by replacing a polar glycerol with lipophilic side chains (Kim et al., 1997). Thus, Oseltamivir was not only the first orally active sialidase inhibitor, but also it was the first intracellular sialidase inhibitor as the lipophilic side allowed entry through the cell membrane. In 2000, Babu and colleagues combined the guanidinyl group and lipophilic side chains concepts from Zanamir and Oseltamivir and developed Peramivir (Babu et al., 2000). Peramivir is a cyclopentane derivative with a guanidinyl group and lipophilic side chains. The inhibition caused by these inhibitors is reversible. Inhibition of non-influenza virus sialidases requires concentrations at

least a million times higher that of influenza virus sialidases and cellular cytotoxicity has not been determined (Gubareva et al., 2000).

1.10 Mouse Model

The mouse (*mus musculus*) has evolved an invaluable tool for identifying genetic factors that affect atherosclerosis and lipid metabolism. Extensive genetic characterization is available (Lyons and Searle, 1989) as seen by the relative ease of mapping relative genes for atherogenesis (Paigen et al., 1987).

Inbred mouse strains differ in plasma lipoprotein concentrations, responsiveness to high-fat, high-cholesterol diets, and susceptibility to aortic lesion development. Interestingly, comparison of nine inbred strains of mice fed high-fat diet identified a sialidase-deficient inbred mouse strain, SM/J, as susceptible to aortic lesion formation but maintained the same high-density lipoprotein cholesterol (HDL-C) level (Nishina et al., 1993; Paigen, 1995).

A related line of mice, B10.S/SgSlc (B10.S;H-2^{s}), which also lacks sialidase shows increased atherosclerosis susceptibility relative to a control line, SJL. This atherosclerosis susceptibility has been associated with bone marrow derived cells. However, this study is not conclusive since B10.S/SgSlc or SJL bone marrow were mixed with wild type ApoE-/- bone marrow (Ishimori et al., 2001) producing a heterozygote genotype for sialidase deficiency.

1.10.1 SM/J mice

In the early 1940s, the SM/J mouse was characterized by its small body size following cross matings with seven different inbred strains. A partial deficiency of sialidase was first identified in the SM/J strain by Potier and colleagues (Potier et al., 1979). In 1981, Womack and colleagues demonstrated that there were abnormal sialylation of at least four lysosomal glycoproteins (Womack et al., 1981). Womack and colleagues deduced that this hypersialylation was attributed to a reduction in sialidase activity. In the same year, Clark and colleagues demonstrated that SM/J mice had an impaired immune response, which is thought to result from the altered processing of sialic acids present on cell surface molecules of subpopolations of T cells (Clark et al., 1981). The SM/J mice can only stimulate the IFN-γ-producing (Th1)-mediated immune response, but not the IL-4-producing (Th2)-mediated immune response (Chen et al., 1997; Chen et al., 2000). This altered response has been attributed to reduced sialidase activity (*neu1* gene), which is thought to prevent desialylation of surface molecules on naïve T cells. D'Azzo and colleagues suggested that the SM/J strain sialidase deficiency resulted from a missense mutation (Leu209Ile) (Rottier et al., 1998). Carrillo et al. showed that SM/J liver expressed low sialidase mRNA and suggested a possible promoter defect (Carrillo et al., 1997). We examined the sialidase promoter region and identified a point mutation (-517G→A) in the SM/J strain, one of which results in reduced sialidase gene transcription (Champigny et al., 2009).

Nkx3.2 is highly expressed in liver and leukocytes (Schneider et al., 1999). Nkx3.2 can repress transcription by recruiting HDAc/SinsA complex in a smad-dependent manner (Kim and Lassar, 2003). Previous studies from our laboratory have shown that

Nkx3.2-mediated repression does not depend on the Nkx3.2 DNA binding domain. This implies that Nkx3.2 suppression of the *neu1* gene works in an HDAc-independent manner. In addition to Nkx3.2, Nkx3.1, which is an estrogen-dependent transcription factor, can bind to the sialidase promoter and may compete with Nkx3.2 resulting in increased sialidase gene transcription (Champigny et al., 2009).

We have isolated the *neu1* mutation from the SM/J background by backcrossing onto the C57Bl/6 inbred genetic background, producing B6.SM. This new strain provides us with the opportunity to test whether this mutation in isolation can affect atherosclerosis susceptibility. B6.SM mice are optimal for our study because they do not show the sialidosis phenotype unlike the Neu1-/- mice which have a complete knockout of the *neu1* gene (de Geest et al., 2002). Jacob and colleagues showed that peritoneal macrophages of SM/J mice had decreased levels of TNF-α production relative to that of C57Bl/10 (Jacob et al., 1993). Branen and colleagues reported that atherosclerosis was decreased by inhibiting tumor necrosis factor-α (TNF-α) in ApoE-/- mice (Branen et al., 2004). Recently, we demonstrated that there is a decrease in the level of TNF-α stimulated sialidase-deficient leukocyte adhesion to the walls of hepatic sinusoids although TNF-α stimulated leukocyte adhesion to the walls of post-sinusoidal venules is not affected significantly (Champigny et al., 2009). In addition, an increase in the TNF-α stimulated sialidase-deficient leukocyte rolling in post-sinusoidal venules was observed (Champigny et al., 2009). Therefore, these results infer that sialidase deficiency may cause reduced atherosclerosis susceptibility.

1.10.2 ApoE-deficient mouse

Inbred mouse strain, C57Bl/6, fed high fat diets develops very limited atherosclerotic lesions in the arterial intima of the proximal aorta (Nishina et al., 1990; Paigen et al., 1985; Paigen et al., 1987). Thus, a more atherosclerosis-susceptible model is needed. ApoE-deficient mice generated by gene targeting show elevated plasma cholesterol levels, even when fed a regular chow diet and the mice develop severe atherosclerotic lesions (Piedrahita et al., 1992; Plump et al., 1992). Lesions are found dispersed throughout the arterial tree forming at the base of the aorta, and along the entire length of the aorta. The development of atherosclerotic lesions in this model more closely resembles those of humans than did the previous atherosclerotic model (Zhang et al., 1992; Plump et al., 1992; Zhang et al., 1994). Many different approaches have been performed to study atherosclerosis using the *apoe-/-* mouse model, such as, nutritional intervention, pharmacological and genetic studies. However, when using this mouse strain, we need to consider the site specificity of atherosclerosis, age of lesion, and gender-specific effects. For example, estrogen has been shown to be atheroprotective in several mouse models (Hodgin and Maeda, 2002). In contrast, both ApoE-deficient and LDL receptor-deficient female mice generally develop larger lesions than males at the aortic sinus (Pastzy et al., 1994; Reardon et al., unpublished data, 2003 mentioned in Vanderlaan et al., 2004).

1.10.3 LDLR-deficient mice

After the generation of ApoE-deficient mice in 1992, LDLR-deficient mice were generated as another widely used model to study atherosclerosis (Ishibashi et al, 1993). ApoE-deficient mice and LDLR-deficient mice differ in both the distribution of

cholesterol in lipoprotein particles and the susceptibility to atherogenesis. In ApoE-deficient mice, cholesterol is mostly found in the VLDL particles (Reddick et al., 1994; Nakashima et al., 1994). In LDLR-deficient mice, cholesterol accumulates in the LDL particles (Ishibashi et al., 1993). ApoE-deficient mice develop atherosclerotic lesions spontaneously and these are found dispersed throughout the arterial tree forming at the base of the aorta, and along the entire length of the aorta (Zhang et al., 1992; Plump et al., 1992; Zhang et al., 1994). However, the development of atherosclerotic lesions in LDLR-deficient mice is limited on normal chow diet (Ishibashi et al., 1993). Nevertheless, LDLR-deficient mice develop extensive atherosclerotic lesions feeding on a western diet.

LDLR-/- mouse models have been used to study the role of very low density lipoprotein receptor (VLDLR) on lipoprotein metabolism. Adenovirus-mediated gene transfer of VLDLR reverses hypercholesterolemia in LDLR-/- mice (Kobayashi et al., 1996; Kozarsky et al., 1996). In addition to the work with the first generation adenovirus, helper-dependent adenovirus-mediated gene transfer of VLDLR has been shown to correct the hypercholesterolemia phenotype in LDLR-/- mice (Oka et al., 2001). Moreover, it has been shown that VLDLR-/-/LDLR-/- mice show a significant increase in serum lipid levels, whereas overexpression of VLDLR in LDLR-/- mice causes a significant decrease in the serum lipid levels (Tacken et al., 2000).

Bone marrow transplantation experiments have been carried out to determine the effects of the LDLR in macrophage on atherosclerosis (Boisvert et al., 1997; Herijgers et al., 1997; Herijgers et al., 2000; Linton et al., 1999). Bone marrow of LDLR+/+

donors transplanted into LDLR-/- recipients show no significant difference in atherosclerotic lesion areas compared to LDLR-/- donors (Herijgers et al., 1997; Herijgers et al., 2000; Linton et al., 1999). However, when the recipient is C57Bl/6, the LDLR +/+ donors showed more atherosclerosis compared to the LDLR-/- donors (Herijgers et al., 2000; Linton et al., 1999). The researchers believed that this outcome was caused by the reduced expression of LDLR in a hyperlipidemia environment.

1.11 Objectives

In this study, we aim to define the role of sialidase (neu1) in atherosclerosis. Development and progression of atherosclerosis is dependent on lipoprotein metabolism and inflammation which, in turn, are interconnected. Our first objective was to study the effects of hypomorphic sialidase expression on lipoprotein metabolism in C57Bl/6 mice using both static and kinetic parameters. Our second objective was to study the effects of hypomorphic sialidase expression on lipoprotein metabolism and atherosclerosis in *apoe-/-* mice. In this objective, we determined the effects of hypomorphic sialidase expression on atherosclerosis using genetic and chemical approaches. The genetic approach involved comparing atheroscelerotic lesion areas of *apoe-/-* to that of B6.SM/*apoe-/-* mice. The chemical approach involved the use of sialidase inhibitors such as DANA, and comparing atherosclerotic lesion areas found in *apoe-/-* to that of DANA-treated *apoe-/-* mice. As mentioned above, inflammation is an important factor for atherogenesis and is related to lipoprotein metabolism. Blood cells, including monocytes/macrophages and T cells, play roles in the inflammatory process that leads to atherosclerosis. Thus, we determined the blood cell specific sialidase effects on atherosclerosis using bone

marrow transplanted B6.SM/*apoe-/-* mice. Our third objective was to study the effects of hypomorphic sialidase expression on lipoprotein metabolism and atherosclerosis in LDLR-deficient mice. To that end, we conducted studies to determine the role of hypomorphic sialidase blood cells in the uptake of serum cholesterol and how it impacts the atherosclerotic lesion progression in LDLR-deficient mice transplanted with hypomorphic sialidase hematopoietic cells. This study defined a novel role for hypomorphic sialidase leukocytes in the regulation of lipoprotein metabolism. Overall, these studies showed that sialidase is a novel risk factor for atherosclerosis and that sialidase inhibition is a potent treatment for atherosclerosis.

Chapter 2

Hypomorphic Sialidase Expression Decreases Serum Cholesterol by Down- Regulation of VLDL Production in Mice

Abraham E. Yang[1], Gabriel Gyulay[1], Bernardo L. Trigatti[2] and Suleiman A. Igdoura[1,3]

Department of [1]Biology, [2]Biochemistry and Biomedical Sciences, [3]Pathology and Molecular Medicine, McMaster University, Hamilton, Ontario, Canada

Preface

Abraham Yang performed FPLC cholesterol profiles (Fig. 2.3.1), hepatic lipid analyses (Table 2.3.1), hepatic VLDL-lipid production (Fig. 2.3.2 and Table 2.3.2), hepatic VLDL-ApoB-100 and –ApoB-48 secretion (Fig. 2.3.3), hepatic MTP (Fig. 2.3.4 A), SREBP-2 (Fig. 2.3.4 B), ACAT-2 (Fig. 2.3.4 C), LRP-1 (Fig. 2.3.5 B), intestinal MTP (Fig. 2.3.6 A), ApoB-48 (Fig. 2.3.6 B) and SREBP-2 immunoblotting (Fig. 2.3.6 C). Gabriel Gyulay performed folch extraction for hepatic lipid analyses, LDLR (Fig. 2.3.5 A) and PCSK9 immunblotting (Fig. 2.3.5 C), LDLR quantitative real-time PCR (Fig. 2.3.5 D), lectins and pull downs (Fig. 2.3.5 E).

Chapter 2

Hypomorphic Sialidase Expression Decreases Serum Cholesterol by Down- Regulation of VLDL Production in Mice

Summary

Lipoprotein metabolism is an important contributing factor in the development and progression of atherosclerosis. Plasma lipoproteins and their receptors are heavily glycosylated and sialylated. Sialylation of lipoproteins has been associated with lipoprotein assembly, secretion, uptake and lipid-binding. Sialylation is modulated by the activities of sialyltranferases and sialidases. In order to address the impact of sialidase (neu1) activity on lipoprotein metabolism, we have generated a mouse model with a hypomorphic neu1 allele (B6.SM) that displays reduced sialidase expression and sialidase activity. The objectives of this study are to determine the impact of sialidase on serum cholesterol and to determine if hypomorphic sialidase expression affects the rate of hepatic lipoprotein secretion, and/or rate of lipoprotein uptake. Our results indicate that hepatic levels of cholesterol and triglyceride are significantly higher in B6.SM mice compared to C57Bl/6 mice; however, VLDL-cholesterol and VLDL-triglyceride production rates in B6.SM mice are significantly lower than those of C57Bl/6 mice. In addition, significantly lower levels of SREBP-2 as well as hepatic microsomal triglyceride transfer protein (MTP) are observed in B6.SM mice indicating that loading of triglyceride and cholesterol esters onto Apo-B during the formation of VLDL particles is reduced. Furthermore, hypomorphic sialidase expression results in stabilization in hepatic protein expression of LDLR and decreased PCSK9 secretion. This allows sialidase to promote lipid clearance via

LDLR. These findings provide evidence for a central role for sialidase in the cross talk between the uptake and production of lipoproteins.

2.1 Introduction

Previous studies have suggested that different level of sialylation of apolipoproteins may impact lipoprotein metabolism and atherosclerosis (Orekhov et al., 1989; Sobenin et al., 1991; Orekhov et al., 1991; Tertov et al., 1992a; Tertov et al., 1992b; Orekhov et al., 1992). Sialylation is modulated by the activities of sialyltranferases and sialidases. Neu1 sialidase belongs to a family of hydrolytic enzymes that cleave terminal α2-3 and α2-6 sialyl linkages of glycoproteins, glycolipids and oligosaccharides (Reuter et al., 1996). The enzyme is found in a multienzyme complex which includes β-galactosidase (β-gal) and protective protein/cathepsin A (ppca) (van der Horst et al., 1989; van der Spoel et al., 1998; Bonten et al., 1995; Galjart et al., 1988, Galjart et al., 1991). While the role of the enzyme in the lysosome is well defined, its functional consequences on the cell surface, including its role in cell-surface receptor recognition, catabolism, tumorigenicity and antigen masking, are still being explored (Collard et al., 1986; Pilatte et al., 1993; Varki et al., 1997; Bonten et al., 1996). Sialidase activity can also regulate the function of cell surface molecules, such as, TLR4, CD15, CD22, CD43, CD44 and CD45 (Braesch-Andersen and Stamenkovic, 1994; Katoh et al., 1999; Cuff et al., 2001; Gee et al., 2003; Khan et al., 2004; Gadhoum and Sackstein, 2008; Amith et al., 2009; Amith et al., 2010; Katoh et al., 2010; Katoh et al., 2011; Feng et al., 2011). In the inflammatory response, sialidase is involved in modulating the function of macrophages (Lambré et al., 1990; Liang et al., 2006; Stamatos et al., 2005), T cells (Pappu and Shrikant, 2004; Xu and Weiss, 2002) and neutrophils (Cross et al., 1991; Cross et al., 2003; Sakarya et al., 2004) indicating their potential effect in chronic inflammation, such as, atherosclerosis,

rheumatoid arthritis and inflammatory bowel disease. To date, genes encoding lysosomal/membrane, neu1 (Igdoura et al., 1998; Carrillo et al., 1997; Pshezhetsky et al., 1997), cytosolic, neu2 (Monti et al., 1999; Ferrari et al., 1994), plasma membrane-bound, neu3 (Miyagi et al., 1999; Wada et al., 1999) and mitochondrial forms, neu4 (Comelli et al., 2003; Monti et al., 2004; Seyrantepe et al., 2008) have been cloned. A deficiency in lysosomal/membrane sialidase (neu1) is associated with sialidosis, an autosomal recessive disease, which is characterized by accumulation of sialylconjugates within the lysosomes in the central nervous system as well as in the peripheral tissues (O'Brien and Warner, 1980).

A partial deficiency of sialidase was identified in the SM/J mice in the 1970s (Potier et al., 1979; Womack et al., 1981). In addition to the abnormal sialylation of glycoproteins, SM/J mice show an impaired immune response resulting from the altered processing of sialic acids present on cell surface molecules of T cells (Clark et al., 1981). D'Azzo and colleagues suggested that the SM/J sialidase deficiency resulted from a missense mutation (Rottier et al., 1998). Campbell and colleagues demonstrated that the SM/J liver expresses low level of sialidase mRNA (Carrillo et al., 1997). We have recently identified a point mutation (-519G→A) in the mouse lysosomal sialidase promoter (neu1), which results in reduced sialidase gene transcription in the SM/J mice. This promoter mutation creates a binding site for a transcriptional repressor, Nkx3.2, resulting in reduced gene expression (Champigny et al., 2009). We have isolated this mutation from the SM/J mice by backcrossing onto a C57Bl/6 genetic background, generating a hypomorphic sialidase mouse, namely B6.SM.

In this study, we focus on the effect of hypomorphic sialidase (neu1) expression on the regulation of lipoprotein metabolism in the liver. We demonstrate that hypomorphic sialidase expression lowers serum cholesterol levels by modulating hepatic VLDL production as well as LDL uptake. The results described in this chapter show that sialidase is a novel contributor to impact lipoprotein metabolism.

2.2 Methods

2.2.1 Mice

B6.SM mice were obtained by crossing SM/J mice with C57Bl/6 mice six times. The presence of the the regulatory mutation, (-519G→A) within the *neu1* promoter was confirmed by PCR using DNA extracted from tail biopsies. The following primers were used for the PCR: 5' ATC CCT GTC CAG GAA CTG GT 3' (Primer ML-07-06, Mobix, McMaster University) and 5' CTT AAG GGC ATT GGG GTC AT 3' (Primer ML-07-07, Mobix, McMaster University). PCR (40 cycles) were performed with denaturing temperature of 94°C for 2 minutes, annealing temperature of 60°C for 30 seconds and elongation temperature of 72°C for 30 seconds. PCR products were digested with MspA1I (New England BioLab) which serves as a genetic diagnostic since it only cleaves the PCR product carrying the B6.SM mutation. Mice were housed in filter-top cages and given unlimited access to food and water. Mice in cages were housed in a room with 12-hour light and dark cycle. All experimental protocols were approved by institutional animal research ethics committees.

2.2.2 Collection of blood and tissues

Under anesthesia with ketamine/xylazine, blood was obtained by cardiac puncture. Serum was obtained by centrifugation of blood for 5 min at 15,000 rpm using serum collection tubes. Mice were perfused with phosphate-buffered saline (PBS) through the left ventricle of the heart. The liver was harvested and frozen in liquid nitrogen, then stored at -80°C for further use in protein and RNA studies.

2.2.3 Serum lipid analyses

Serum was fractionated by gel filtration-FPLC using a Superose 6 column, and serum total cholesterol was analyzed with enzymatic assay (Infinity™ Cholesterol Liquid Stable Reagent, Thermo Scientific). The enzymatic colorimetric assay product was measured at 500nm. Serum free cholesterol was analyzed with Free Cholesterol E Reagent (Wako Diagnostics). The absorbance of the reaction product was measured at 600 nm. Serum cholesteryl esters concentration was calculated by subtracting serum free cholesterol concentration from serum total cholesterol concentration. Serum triglyceride was analyzed with enzymatic colorimetric assay (L-Type Triglyceride H Reagents 1 and 2, Wako Diagnostics). The absorbance of the reaction product was measured at 600 nm.

2.2.4 Hepatic lipid analyses

Part of the liver (150 mg) was homogenized in 1ml of TNES (10 mM Tris, pH 7.5, 400 mM NaCl, 100 mM EDTA, 0.6% SDS). Folch mixture (chloroform/methanol, 2:1; 3 ml) was added to 300ul of liver homogenates and the tubes were vortexed for 1 minute. After that, 0.6 ml of distilled water was added to the tubes and the tubes were vortexed for 1 minute. Then, the extraction mixture was left at 4°C for 2 hours. After 2 hours, the tubes were centrifuged at low speed to facilitate phase separation. The lower phase (chloroform phase) was dried completely by sitting in water bath at 37°C. The dry chloroform phase was resuspended in 60ul of isopropanol. Hepatic total cholesterol, free cholesterol, cholesteryl esters and triglyceride concentrations were measured as described in serum lipid analyses using enzymatic colorimetric assays.

2.2.5 *In vivo* hepatic VLDL-lipid secretion

Hepatic production of VLDL-triglyceride, cholesterol, free cholesterol, and cholesteryl esters were measured in 3-month-old male C57Bl/6 and B6.SM after intravenous injection of Triton WR 1339 (Tyloxapol T0307-10G, Sigma BioXtra, Sigma-Aldrich) [15g/dl in 0.9% NaCl]. Mice were fasted overnight prior to the experiments, and 500mb/kg mg of Triton WR1339 was injected. Blood samples were taken from the cheek under light anesthesia before and at 1, 2, 3 and 4 hours after Triton injection for triglyceride, cholesterol, free cholesterol, and cholesteryl esters measurements. VLDL-triglyceride, cholesterol, free cholesterol and cholesteryl esters production rates were obtained by calculating the slope of the regression line of the graph with VLDL-triglyceride, cholesterol, free cholesterol, and cholesteryl esters concentration respectively versus time in hours.

2.2.6 Immunoblot analysis

Liver lysates were prepared in RIPA buffer containing protease inhibitors (Roche) and the protein concentration was determined using the Bradford assay (Biorad). Samples were separated on SDS/polyacrylamid gel and transferred to nitrocellulose membranes. The following antibodies were used: anti-ApoB (goat anti-ApoB, 1:6000, Midland Bioproducts), anti-MTP (mouse anti-MTP, 1:2500, BD Biosciences), anti-ACAT2 (mouse anti-ACAT2, 1:200, Santa Cruz), anti-SREBP-2 (mouse 1:500, Pharmingen), anti-LDLR (mouse anti-LDLR, 1:1000, Calbiochem), anti-LRP1 (rabbit anti-LRP1, 1:10000, Epitomics), anti-β-actin (mouse anti-β-actin, 1:1000, Cell Signalling Technology) and anti-beta-tubulin (mouse anti-beta-tubulin, 1:1000). Secondary HRP-conjugated antibodies (1:10000, Santa Crutz Biotechnology) were used and visualized

with chemiluminescence (ECL, Amersham). Intensity was measured by ImageJ densitometry software.

2.2.7 RNA Isolation and Quantitative Real-Time PCR

Livers were homogenized and RNA was isolated using Norgen Total RNA Isolation Kit. Total RNA (1-5 ug) were then reverse transcribed using oligoDT primers following the protocol of Invitrogen's SSIII RT reverse transcriptase. cDNA was then used for qRT PCR using Applied Biosystems Power Sybr Green. Plates were loaded with 20ul reaction per well including appropriate blanks and standard. (PCR cycle was as follows: 10min 95°C, 40x – 15sec 95°C, 60sec 60°C). Primers were designed by our laboratory and synthesized by MOBIX facility, McMaster University.

2.2.8 Mouse Liver Membrane Enrichment

Livers were perfused with saline followed by removal and chopping of the liver at 4°C. They were then homogenized in a 0.25M sucrose solution. After that, samples were centrifuged at 3000xG, and the supernatant was centrifuged at 170000xG yielding a pellet. The pellet was resuspended in RIPA buffer with protease inhibitors.

2.2.9 Immunoprecipitation

Liver lysates with equal amount of protein were immunoprecipitated using LDLR antibody and Protein A, prior to immunoblot analysis.

2.2.10 Lectins and Pull-Downs

We utilized SNA (Sambucus nigra agglutinin) which binds alpha 2,6 linkages of sialic acid and MAL (Maackia amurensis leucoagglutinin) which binds alpha 2,3 linkages of sialic acid. The biotin labelled lectins were incubated with lysates and pulled down with streptavidin. The enriched samples, containing glycoproteins pulled down by their sialic acids, were used for immunoblot analysis

2.2.11Trueblot Immunoprecipitation of PCSK9

Serum (200 ul) was incubated with anti-mouse PCSK9 antibody (courtesy of Dr. Nabil Seidah) and TrueBlot (30ul, eBioscience) agarose anti-rabbit beads overnight at 4°C. Samples were spun and washed with RIPA 5X. Resulting pellet was boiled in 60ul Laemlli Sample Buffer, spun down, and 30ul of the supernatant was subjected to SDS-PAGE and blotting with the same antibody. A special secondary antibody which only detects full length Immunoglobulins and not reduced ones was used on the western blot to avoid non-specific bands.

2.2.12 Statistical analysis

Statistical analyses between groups of data were analyzed by one-way analysis of variance (one-way ANOVA) followed by Tukey Comparison Test and *t* test using Prism 5 (version 5.04). Error bars represent standard error of the mean. Data were considered statistically different only if p value is less than 0.05.

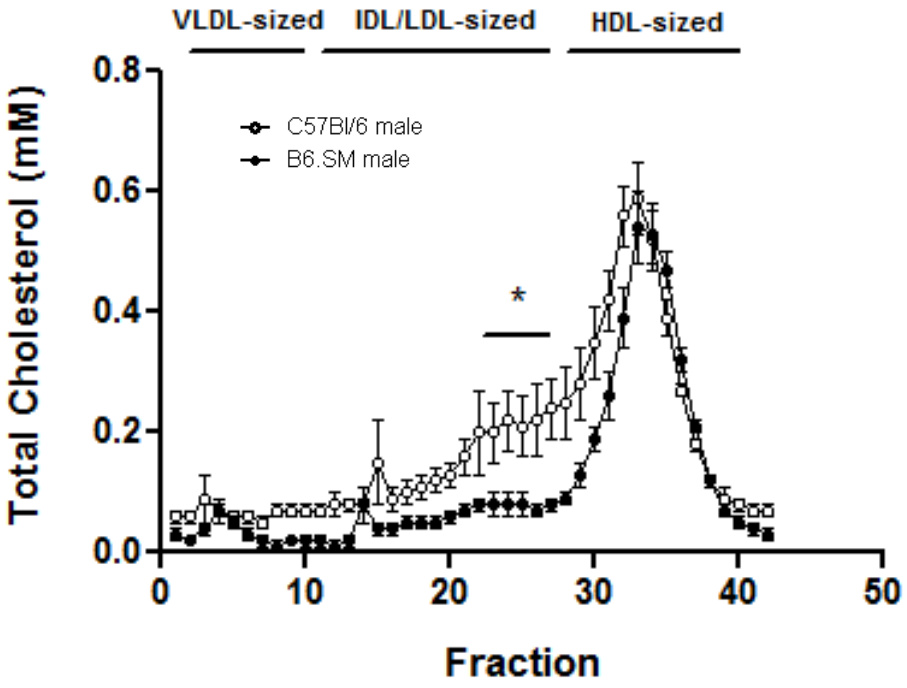
2.3 Results

2.3.1 Hypomorphic sialidase expression decreases serum cholesterol levels.

In order to determine the effects of hypomorphic sialidase expression on cholesterol levels of serum lipoproteins, we separated serum lipoproteins by size exclusion using Fast Protein Liquid Chromatography (FPLC) with a superose 6 gel filtration column. The cholesterol profiles of B6.SM mice show significantly lower cholesterol levels in low density lipoproteins (fractions 20 -30) than those of the corresponding C57Bl/6 controls as illustrated by the left shoulder difference (Fig. 2.3.1). Also, the HDL peak appears to shift slightly toward a higher fraction number (smaller apparent size), which may represent a smaller HDL particle in B6.SM mice (Vaisman et al., 1995; de Silva et al., 1994; Rigotti et al., 1997) (Fig.2.3.1). This result suggests that hypomorphic sialidase expression appears to lower cholesterol levels in LDL-sized particles because of the decreases observed in the IDL/LDL-sized fractions (fractions 20-30).

Fig. 2.3.1 FPLC cholesterol profiles of C57Bl/6 and B6.SM mice. Serum lipoproteins from unfasted mice were fractionated by size using Fast Protein Liquid Chromatography (FPLC) using a superose 6 gel filtration column. The FPLC cholesterol profiles of the male B6.SM animals (n=3) have significantly less total cholesterol in LDL-sized particles than those of the corresponding C57Bl/6 controls (n=3). In addition, there is a tendency of the HDL peak to be shifted slightly to the right. Each circle represents mean±SE. (VLDL-sized: Very low density lipoprotein sized particle, IDL-sized: Intermediate density lipoprotein sized particle, LDL-sized: Low density lipoprotein sized particle, HDL-sized: High density lipoprotein sized particle).

Fig. 2.3.1


VLDL-sized
IDL/LDL-sized
HDL-sized
C57Bl/6 male
B6.SM male
*
Total Cholesterol (mM)
0.0
0.2
0.4
0.6
0.8
0
10
20
30
40
50
Fraction

2.3.2 Hypomorphic sialidase expression increases hepatic lipid levels.

In order to determine whether the altered cholesterol profile is caused by altered hepatic lipid metabolism, we have measured the hepatic total cholesterol, triglyceride, free cholesterol and cholesteryl ester levels in both the B6.SM and C57Bl/6 mice. We observed a significant increase in hepatic total cholesterol, cholesteryl esters and triglyceride in B6.SM mice compared to C57Bl/6 mice (Table 2.3.1). A trend of increase in hepatic free cholesterol of B6.SM mice compared to C57Bl/6 mice is also observed. These results point to sialidase activity as playing a role in modulation of lipid metabolism in the liver.

2.3.3 Hypomorphic sialidase expression decreases *in vivo* hepatic VLDL-lipid production.

In order to determine whether the decrease in serum cholesterol levels is caused by a decrease in hepatic VLDL-lipid production, we have measured the lipid concentrations at several intervals post lipoprotein lipase inhibition. Administration of Triton WR1339 prevents the hydrolysis of triglyceride and the uptake of VLDL, and as a result, allows for the assessment of the production rates of VLDL-cholesterol and VLDL-triglyceride. Our results indicate that B6.SM mice have significantly lower production rates in VLDL-triglyceride and VLDL cholesterol over a 4-hour period (Fig. 2.3.2 A-D) (Table 2.3.2). These results show that hypomorphic sialidase expression decreases VLDL-cholesterol production which results in lower serum cholesterol levels in B6.SM mice.

In addition to the lipids, VLDL is composed of Apolipoprotein-B. Apolipoprotein B exists in VLDL, IDL and LDL. Therefore, it is useful as an indicator of quantities of these atherogenic lipoproteins. By Western blot analysis, we examined serum levels of Apo-B100 and Apo-B48 at 0, 1, 2, 3 and 4 hours after Triton WR1339 injection in B6.SM and C57Bl/6 mice. No significant difference was observed in ApoB-100 or in Apo-B48 between C57Bl/6 and B6.SM mice (Fig. 2.3.3 A and B). Therefore, these results indicate changes in the composition of VLDL and not a change in particle number, as each lipoprotein only bears one Apo-B molecule. This further leads us to consider levels of MTP which mediates loading of triglyceride to Apo-B during the formation of VLDL.

Table 2.3.1 Hepatic lipids levels in C57Bl/6 and B6.SM mice. Lipids from livers of C57Bl/6 (n=3) and B6.SM mice (n=3) were extracted using folch extraction. There is a significant increase in hepatic total cholesterol, cholesteryl esters and triglyceride levels in B6.SM mice compared to C57Bl/6 (P=0.048, P=0.02 and P=0.01 respectively). In addition, there is a trend of increase in hepatic free cholesterol levels in B6.SM mice compared to C57Bl/6 (P=0.19 respectively). Mean±SE are shown.

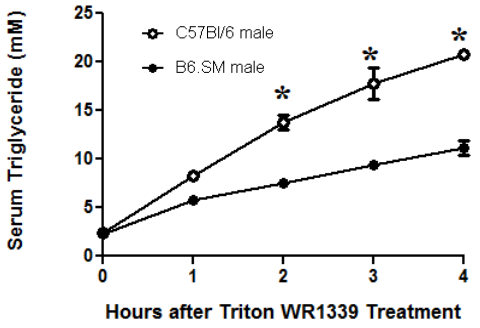
Table 2.3.1

	Hepatic total cholesterol (mM)	Hepatic free cholesterol (mM)	Hepatic cholesteryl esters (mM)	Hepatic triglyceride (mM)
C57Bl/6 Male	1.13±0.11	0.67±0.08	0.46±0.04	7.78±0.13
B6.SM Male	1.37±0.01*	0.76±0.04	0.61±0.004*	8.56±0.16*

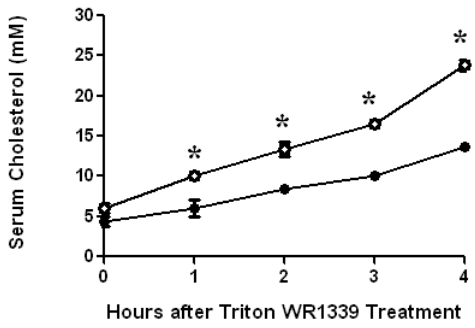
Fig. 2.3.2 *In vivo* hepatic VLDL-lipid production in C57Bl/6 and B6.SM mice. Male C57Bl/6 (n=3) and male B6.SM mice (n=3) were fasted overnight and injected with Triton WR1339 (500mg/kg). Serum samples were drawn at just before the injection of Triton WR1339 (time 0 hour) and at 1, 2, 3, and 4 hours after the injection of Triton WR1339. There is a decrease in serum (A) VLDL-triglyceride, (B) VLDL-cholesterol, (C) VLDL-free cholesterol and (D) VLDL-cholesteryl esters concentrations in different indicated times after 0 hour (* denotes $P<0.05$). Note that there is a decrease in the steepness of the slope of the B6.SM mice compared to that of the C57Bl/6 mice. These imply that hypomorphic sialidase expression causes decreased hepatic VLDL-lipid production rates. Values represent the means±S.E. with n=3 in all groups.

Fig. 2.3.2

A

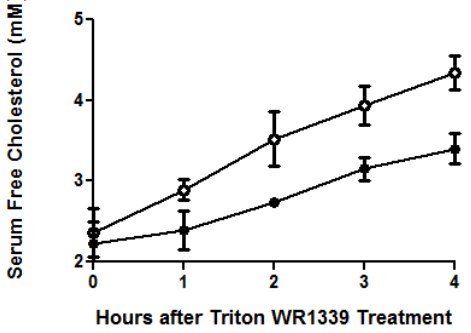

C57Bl/6 male
B6.SM male
Serum Triglyceride (mM)
Hours after Triton WR1339 Treatment

B


Serum Cholesterol (mM)
Hours after Triton WR1339 Treatment

C

D


Serum Free Cholesterol (mM)
Hours after Triton WR1339 Treatment

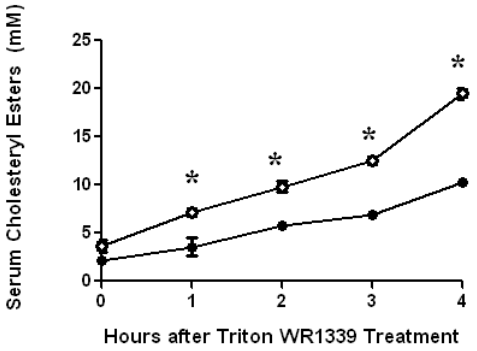

Serum Cholesteryl Esters (mM)
Hours after Triton WR1339 Treatment

Table 2.3.2 *In vivo* hepatic VLDL-lipid production rates in C57Bl/6 and B6.SM mice. Male C57Bl/6 (n=3) and male B6.SM mice (n=3) were fasted overnight. Serum were collected at just before the administration of Triton WR1339 (time 0 hour) and at 1, 2, 3, and 4 hours after the administration of Triton WR1339. There is a significant decrease in hepatic VLDL-triglyceride, VLDL-cholesterol, VLDL-free cholesterol and VLDL-cholesteryl esters production rate in B6.SM mice compared to C57Bl/6 mice. Values represent the means±S.E. with n=3 in all groups.

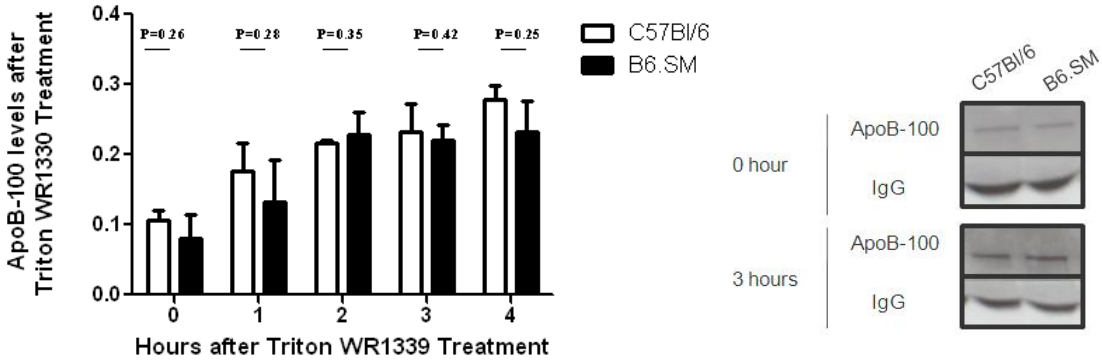
Table 2.3.2

	Triglyceride PR (mM/hr	Total Cholesterol PR (mM/hr	Free Cholesterol PR (mM/hr	Cholesteryl Esters PR (mM/hr)
C57Bl/6 Male	4.64 + 0.26	4.21 + 0.18	0.50 + 0.02	3.71 + 0.21
B6.SM Male	* 2.15 + 0.11	* 2.28 + 0.16	* 0.31 + 0.06	* 1.97 + 0.17

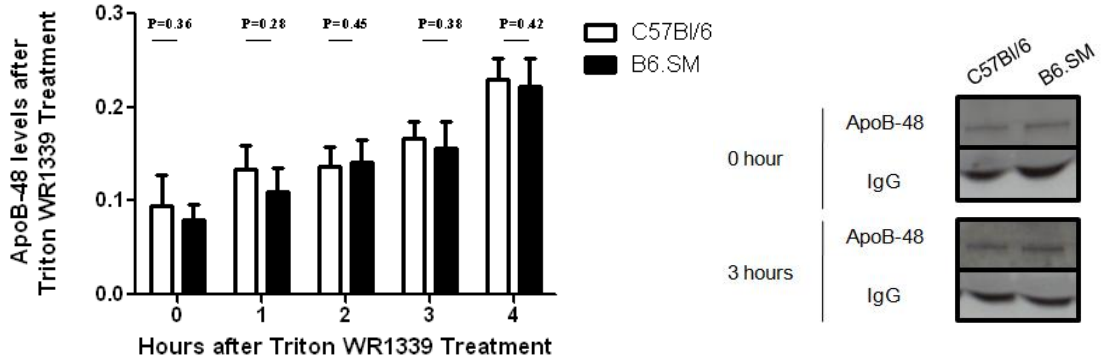
Fig. 2.3.3 Hepatic VLDL-ApoB-100 and –ApoB-48 secretion in C57Bl/6 and B6.SM mice. Serum samples were obtained at just before the injection of Triton WR1339 (time 0 hour) and at 1, 2, 3, and 4 hours after the injection of Triton WR1339. Serum was subjected to SDS-PAGE (4-20%). Membranes were probed with anti-ApoB (goat anti-ApoB, 1:6000) and HRP-conjugated antibodies (donkey anti-goat, 1:10000). No significant difference was observed in serum (A) ApoB-100 and (B) ApoB-48 levels between C57Bl/6 (n=3) and B6.SM mice (n=3). Apo-B levels were determined by the intensities of ApoB bands normalized to IgG bands. Intensities of bands were measured by ImageJ densitometry software. This implied that decreased VLDL lipid secretion is not caused by decreased VLDL particle secretion.

Fig. 2.3.3

A


ApoB-100 levels after Triton WR1330 Treatment
0.4
0.3
0.2
0.1
0.0
P=0.26
P=0.28
P=0.35
P=0.42
P=0.25
0
1
2
3
4
Hours after Triton WR1339 Treatment
C57Bl/6
B6.SM
C57Bl/6
B6.SM
0 hour
ApoB-100
IgG
3 hours
ApoB-100
IgG

B


ApoB-48 levels after Triton WR1339 Treatment
0.3
0.2
0.1
0.0
P=0.36
P=0.28
P=0.45
P=0.38
P=0.42
0
1
2
3
4
Hours after Triton WR1339 Treatment
C57Bl/6
B6.SM
C57Bl/6
B6.SM
0 hour
ApoB-48
IgG
3 hours
ApoB-48
IgG

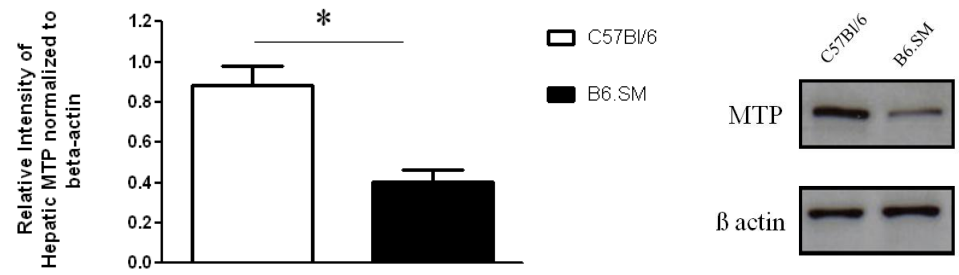
2.3.4 Hypomorphic sialidase expression decreases hepatic MTP expression.

In order to explain the decreased loading of lipids to Apo-B, we evaluated the expression level of hepatic microsomal triglyceride transfer protein (MTP). MTP is required for the secretion of Apo-B-containing lipoproteins from hepatocytes and from the enterocytes of the intestine (Gordon D., 1996; Wetterau et al., 1998). Our data show a significant decrease in hepatic MTP protein expression in B6.SM mice compared to C57Bl/6 mice (Fig. 2.3.4 A). As a result, the decrease in VLDL-triglyceride appears to be due to decreased protein expression of MTP. We also examined hepatic sterol regulatory element binding protein-2 (SREBP-2) protein expression and found a significant decrease in its expression in B6.SM mice (Fig. 2.3.4 B). It has been shown that MTP promoter contains sterol regulatory element binding protein response elements (Rubin et al., 2008; Sato et al., 1999). Furtheremore, we assessed the expression of hepatic acylCoA: cholesterol acyltransferase (ACAT2), which mediates esterification of hepatic cholesterol and found an increase in ACAT2 expression implying that there is sufficient excess of cholesterol esters (Fig. 2.3.4 C). Overall, the decrease in VLDL-lipid production rate observed in B6SM mice appears to be caused by decreased MTP expression which is due to down-regulated SREBP-2 levels.

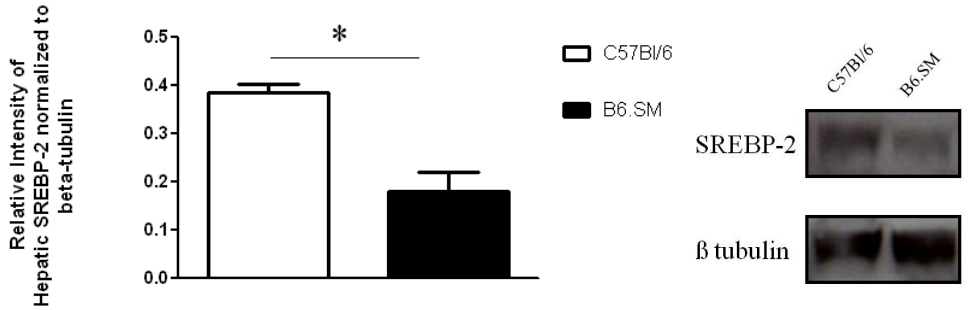
Fig. 2.3.4 Hepatic MTP, SREBP2, ACAT2 expression in C57Bl/6 and B6.SM mice. Liver lysates were subjected to SDS-PAGE (8%). Membranes were probed with (A) anti-MTP (mouse anti-MTP, 1:2500), (B) anti-SREBP-2 (mouse anti-SREBP-2, 1:500), (C) anti-ACAT2 (mouse anti-ACAT2, 1:200), anti-β-actin (mouse anti-β-actin, 1:1000), or anti-beta-tubulin (moue anti-beta-tubulin, 1:1000) and HRP-conjugated antibodies (goat anti-mouse, 1:10000). Intensities of bands were measured by ImageJ densitometry software. B6.SM mice have shown a significant decrease in the protein expression of MTP (* denotes $P<0.05$) and SREBP2 (* denotes $P<0.05$) and a trend of increase in the protein expression of ACAT2 ($P=0.11$) in liver compared to C57Bl/6 mice.

Fig. 2.3.4

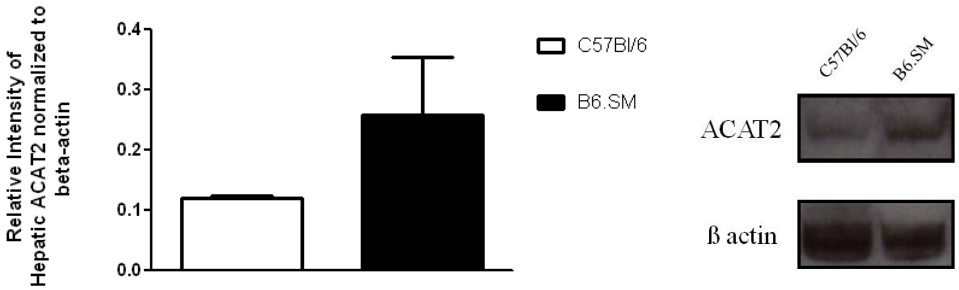
A


Relative Intensity of Hepatic MTP normalized to beta-actin
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
C57Bl/6
B6.SM
C57Bl/6
B6.SM
MTP
ß actin

B


Relative Intensity of Hepatic SREBP-2 normalized to beta-tubulin
0.5
0.4
0.3
0.2
0.1
0.0
*
C57Bl/6
B6.SM
C57Bl/6
B6.SM
SREBP-2
ß tubulin

C


Relative Intensity of Hepatic ACAT2 normalized to beta-actin
0.4
0.3
0.2
0.1
0.0
C57Bl/6
B6.SM
C57Bl/6
B6.SM
ACAT2
ß actin

2.3.5 Hypomorphic sialidase expression attenuates hepatic expression of LDLR.

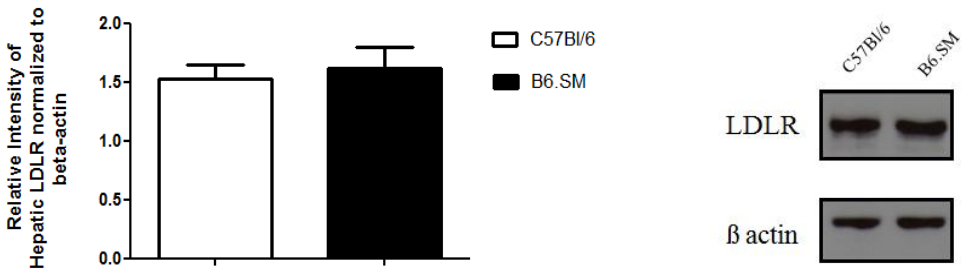
In order to determine whether the decrease in serum cholesterol levels in B6.SM mice is caused by an increase in uptake via increased expression of lipoprotein receptors, we have evaluated the hepatic expression of LDLR and LRP-1. Although there is no significant difference in hepatic protein levels of LRP-1 or LDLR in B6.SM male mice compared to C57Bl/6 controls (Fig. 2.3.5 A and B), there is a significant reduction in LDLR mRNA levels compared to C57Bl6 mice with no differences in hepatic SREBP-2 mRNA levels (Fig. 2.3.5 D). This is reasonable because an increase in hepatic cholesterol levels plays a role in reducing the cleaved form of SREBP-2 protein without affecting the SREBP-2 mRNA levels directly. The decrease in SREBP-2 protein expression observed in B6SM livers is consistent with a decrease in LDLR transcription since LDLR promoter contains SREBP-2 response elements. Thus, the maintanence at the protein level caused by hypomorphic sialidase expression can be attributed to a post translational mechanism such as slower receptor trafficking/recycling. In view of these results, we have measured the serum protein expression of proprotein convertase subtilisin/kexin 9 (PCSK9), a SREBP-2 target gene that affects the turnover of the LDLR (Abifadel et al., 2003; Cohen et al., 2005; Seidah et al., 2003; Maxell and Breslow, 2004; Park et al., 2004). Our results indicate that B6.SM mice show a decrease in the serum protein expression of PCSK9 compared to C57Bl/6 mice (Fig. 2.3.5 C). It has been demonstrated that PCSK9 can bind to LDLR at the cell surface and target them for degradation instead of recycling (Horton et al., 2007). Therefore, the lower level of PCSK9 expression may result in an increase in the rate of receptor recycling and could be the reason behind the higher level of hepatic cholesterol in B6.SM mice.

In order to determine whether the hypomorphic sialidase expression is affecting the sialic acids on LDLR in C57Bl/6 and B6.SM mice, we performed lectin pull downs followed by western blotting. Membrane enriched liver lysates were pulled down with streptavidin using biotin labelled SNA or MAL II which bind specific alpha 2,6 and alpha 2,3 linkages of sialic acid respectively. This was followed by LDLR blotting to assess how much LDLR was pulled down via the sialic acids. We have included control samples blotted for total LDLR to ensure equal starting amounts. We have observed higher levels of LDLR-associated sialic acid in livers of B6.SM mice compared to C57Bl/6 (Fig 2.3.5 E). Thus, hypomorphic sialidase expression leads to hypersialylation of LDLR which may affect the binding of the LDLR.

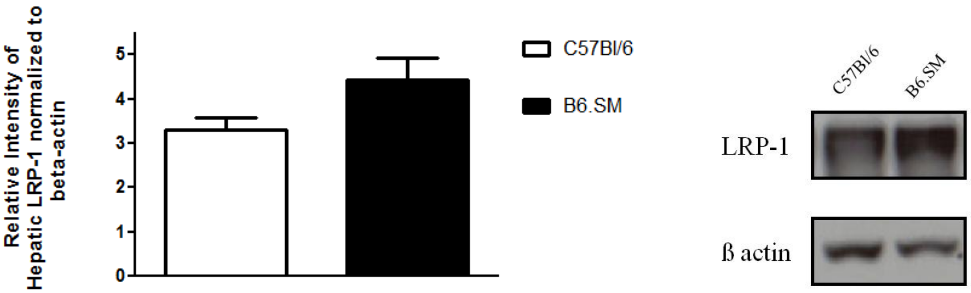
Fig. 2.3.5 mRNA-independent preservation of LDLR protein expression via decreased PCSK9 expression in B6.SM mice. (A) B6.SM male mice have equal hepatic protein levels of LDLR compared to C57Bl/6 controls. Liver lysates were subjected to SDS-PAGE followed by western blotting for LDLR and SR-BI (as a control). Intensities of bands were measured by ImageJ densitometry software (* denotes $P<0.05$; n=3 in each group). (B) B6.SM male mice have a trend of increase in hepatic protein levels of LRP-1 compared to C57Bl/6 mice. (C) PCSK9 trueblot immunoprecipitation of B6.SM and C57Bl/6 mouse serum. B6.SM mice have lower levels of secreted PCSK9 compared to C57Bl/6 controls. (D) B6.SM mice have lower levels of hepatic LDLR mRNA compared to C57Bl/6 controls while showing no difference in SREBP-2 mRNA (* denotes $P<0.05$; n=3 in each group). (E) LDLR from B6.SM mice have higher levels of terminal α-2,6 linked and α-2,3 linked sialic acids when compared to C57Bl/6 mice. Sialylated conjugates from membrane-enriched liver lysates (equal protein) were pulled down using biotinylated SNA and MALII lectins followed by streptavidin sepharose. Pulled down glycoproteins were immunoblotted for LDLR. Control LDLR blotting of LDLR IP samples show no starting difference between C57Bl/6 and B6.SM mice.

Fig. 2.3.5

A


Relative Intensity of Hepatic LDLR normalized to beta-actin
2.0
1.5
1.0
0.5
0.0
C57Bl/6
B6.SM
C57Bl/6
B6.SM
LDLR
ß actin

B


Relative Intensity of Hepatic LRP-1 normalized to beta-actin
5
4
3
2
1
0
C57Bl/6
B6.SM
C57Bl/6
B6.SM
LRP-1
ß actin

C

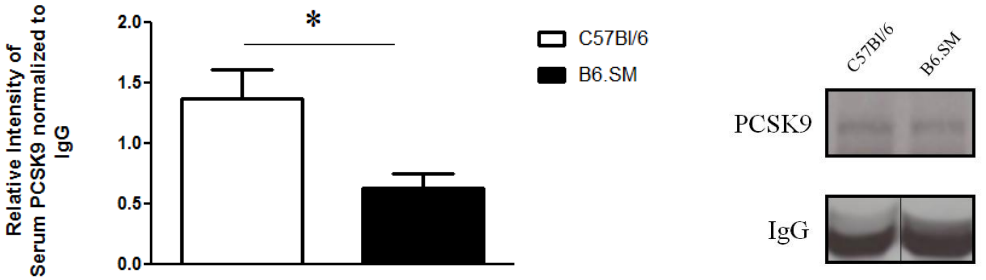
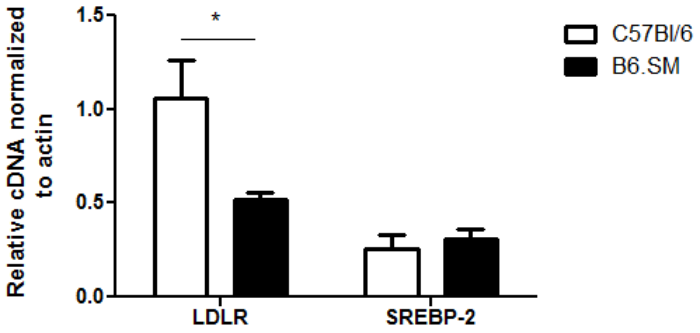
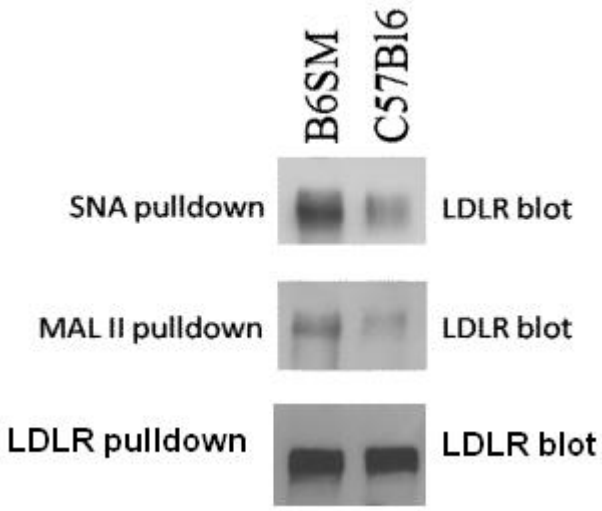

Relative Intensity of Serum PCSK9 normalized to IgG
*
2.0
1.5
1.0
0.5
0.0
C57Bl/6
B6.SM
C57Bl/6
B6.SM
PCSK9
IgG

Fig. 2.3.5

D


Relative cDNA normalized to actin
1.5
1.0
0.5
0.0
*
LDLR
SREBP-2
C57Bl/6
B6.SM

E


B6SM
C57Bl6
SNA pulldown
LDLR blot
MAL II pulldown
LDLR blot
LDLR pulldown
LDLR blot

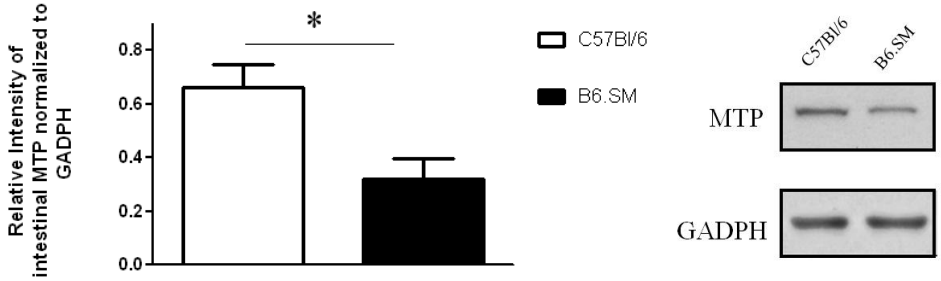
2.3.6 Hypomorphic sialidase expression decreases intestinal MTP and SREBP2 expression.

The intestine contributes to the production of ApoB48-containing lipoproteins and is another important compartment for manipulating lipoprotein metabolism. Most importantly, MTP is highly expressed in the intestine in addition to the liver. Therefore, we evaluated the intestinal expression of MTP protein by western blotting. There is a significant decrease in intestinal MTP of sialidase-deficient mice compared to controls (Fig. 2.3.6 A). ApoB-48 is the only ApoB secreted from the enterocytes and its production implicates intestinal chylomicron production. Thus, we evaluated the intestinal expression of ApoB-48 protein and observed that intestinal ApoB-48 is significantly decreased in sialidase-deficient mice compared to controls (Fig. 2.3.6 B). In addition, there is a significant decrease in SREBP-2 in sialidase-deficient mice compared to controls (Fig. 2.3.6 C). Taken together, these imply that hypomorphic sialidase expression reduces the intestinal MTP level via decreased intestinal SREBP-2 which eventually leads to decreased chylomicron production. These results implicate a common molecular switch between the liver and intestine which maintains higher levels of hepatic cholesterol and triglycerides in the livers.

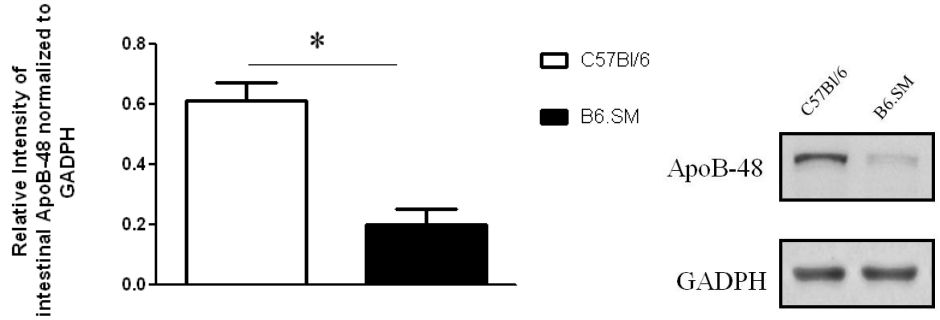
Fig. 2.3.6 Intestinal MTP, ApoB-48 and SREBP-2 expression in C57Bl/6 and B6.SM mice. Lamina propria of proximal jejunum lysates were subjected to SDS-PAGE (8%). Membranes were probed with anti-MTP (mouse anti-MTP, 1:2500), anti-ApoB-48 (goat anti-ApoB, 1:6000), anti-SREBP-2 (mouse anti-SREBP-2, 1:500), or anti-GADPH (goat anti-GADPH, 1:2000) and HRP-conjugated antibodies (donkey anti-goat or goat anti-mouse, 1:10000). Intensities of bands were measured by ImageJ densitometry software. B6.SM mice have shown a significant decrease in the protein expression of intestinal (A) MTP (* denotes $P<0.05$), (B) ApoB-48 (* denotes $P<0.05$) and (C) SREBP-2 (* denotes $P<0.05$) compared to C57Bl/6 mice.

Fig. 2.3.6

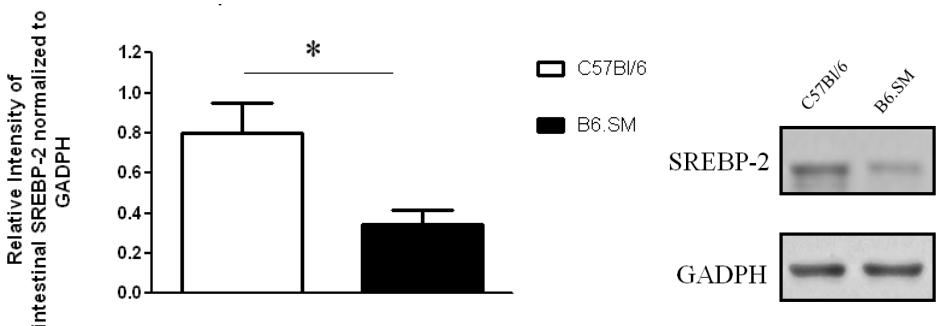
A


Relative Intensity of intestinal MTP normalized to GADPH
0.0
0.2
0.4
0.6
0.8
*
C57Bl/6
B6.SM
C57Bl/6
B6.SM
MTP
GADPH

B


Relative Intensity of intestinal ApoB-48 normalized to GADPH
0.0
0.2
0.4
0.6
0.8
*
C57Bl/6
B6.SM
C57Bl/6
B6.SM
ApoB-48
GADPH

C


Relative Intensity of intestinal SREBP-2 normalized to GADPH
0.0
0.2
0.4
0.6
0.8
1.0
1.2
*
C57Bl/6
B6.SM
C57Bl/6
B6.SM
SREBP-2
GADPH

2.4 Discussion

In this study, we examined lipoprotein metabolism in a unique mouse model expressing hypomorphic levels of sialidase (neu1). While sialylation of lipoproteins and lipoprotein receptors has been invoked frequently as an important determinant in lipoprotein metabolism, little has been reported toward dissecting the impact of sialidase (neu1) on lipoprotein production or clearance.

Our study has revealed a significant decrease in cholesterol levels in the IDL/LDL-sized particles in the B6.SM mice with a shift in the HDL-sized particles' peak in our FPLC cholesterol profiles. We demonstrated that hypomorphic sialidase mice have lower serum cholesterol levels as a result of lower hepatic VLDL production and higher hepatic LDL uptake. In addition, higher levels of hepatic esterified cholesterol as a result of high level of Acyl-CoA: cholesterol acyltransferase (ACAT2) expression were observed in hypomorphic sialidase mice. ACAT2 is an ER-bound intracellular enzyme that forms cholesterol esters from cholesterol. ACAT2 activity decreases the solubility of cholesterol and prevents its incorporation into lipid membranes. As ACAT2 is mostly expressed in hepatocytes and enterocytes, ACAT2-derived CE can be packaged directly into VLDL via MTP or stored as neutral lipid droplets in the cytosol. The latter option is more likely since B6.SM mice show reduced lipid incorporation.

Our observations of higher hepatic cholesterol levels resulting in lower hepatic SREBP-2 and lower MTP is consistent with previous studies. The antibody shows

band for the cleaved form of the SREBP-2 instead of the uncleaved form. SREBP-2 is typically bound to SCAP which can escort SREBP to the Golgi where Site-1 and Site-2 protease can convert SREBP to its cleaved form which in turn induces the transcription of genes involved in cholesterol synthesis (Brown and Goldstein, 1997). As intracellular cholesterol levels increase in heptocytes of hypomorphic sialidase mice, Insig can bind to SCAP. The insig and SCAP complex will lock SREBP in the ER (Sun et al., 2005) limiting the transcriptional activation of cholesterol synthesis genes as well as the MTP and LDLR genes. We conclude that the decreased hepatic expression of MTP in B6SM mice is likely due to reduction in hepatic SREBP-2. Interestingly, B6.SM mice have no significant difference in hepatic SREBP-2 mRNA compared to C57Bl/6 mice as determined by quantitative RT-PCR. Therefore, our model indicates that the decreased SREBP-2 protein expression is a direct result of the altered cholesterol levels in the liver and not a downstream negative feedback on SREBP-2 gene transcription. For the SREBP-mediated effect of MTP, Sato and colleagues have shown that SREBP negatively regulates MTP gene transcription (Sato et al., 1999). However, Horton and colleagues have reported that transgenic mice overexpressing SREBP-2, but not SREBP-1, can positively regulate MTP gene transcription (Horton et al., 1998; Horton et al., 1999). Nevertheless, Bartels and co-workers have demonstrated that SREBP-1 positively regulates MTP gene transcription (Bartels et al., 2002). Our results appear to be consistent with the work reported by Horton and colleagues by showing that a decreased hepatic expression of MTP is regulated by a decreased expression of SREBP-2. The contradictory results between the reports can be due to the lipid level in the liver as SREBP is affected by intracellular cholesterol level as discussed above. In addition, this altered SREBP

level can cause changes in the level of transcription facuters such as hepatic nuclear factor 4 (HNF-4) and/or activator protein 1 (AP-1) (Hagan et al., 1994). These transcription factors are capable to modulate MTP gene transcription (Hagan et al., 1994).

Hepatic cholesterol is typically dependent on LDL endocytosis via LDLR or chyomicron uptake via LRP-1. The stabilized levels of hepatic LDLR in the hypomorphic sialidase mice appear to be due to post-translational events since LDLR mRNA level is in fact lower in B6SM mice livers. The hypersialylation of LDLR appears to increase the effeciency of LDL endocytosis indicating that sialidase typically targets LDLR for processing, most likely in a late endosomal/recycling compartment. Interestingly, the levels of PCSK9 which are dependent on SREBP-2 are lower in hypomorphic sialidase mice enabling LDLR protein to recycle more and potentially to endocytose more LDL from serum. This sialidase-dependent increase in LDLR recycling is an interesting aspect of this work which requires further investigation.

In addition to the liver, the intestine contributes significantly to lipoprotein metabolism. Chylomicron production by entrocytes requires MTP to load lipids to ApoB-48 (Hussain M., 2000; Xiao et al., 2011). We have observed a significant decrease in SREBP-2, MTP and ApoB-48 in entrocytes from hypomorphic sialidase mice compared to C57Bl6 mice. Intestinal absorption involves several transporter proteins including adenosine-triphosphate binding cassette A1 (ABCA1), ABCG5, ABCG8, Niemann-Pick C1 like 1 (NPC1L1) and scavenger receptor class B type 1

(SR-BI). NPC1L1 and SR-BI are responsible for intestinal uptake of cholesterol (Sane et al., 2006). NPC1L1-deficient mice have 69% reduction in cholesterol absorption (Kramer et al., 2005). NPC1L1 is heavily glycoslylated (Iyer et al., 2005) and is shown to be regulated by peroxisome proliferator-activated receptor (PPAR) by decreasing its transcription, and thereby decreasing intestinal cholesterol absorption (van der Veen et al., 2005). ABCG5 and ABCG8 deficient mice have increased cholesterol absorption efficiency (Yu et al., 2002b). Overexpression of ABCG5 and ABCG8 in mice reduces cholesterol absorption (Plösch et al., 2004; Yu et al., 2002a; Yu et al., 2003). Transcription of ABCG5 and ABCG8 is regulated by liver X receptor (LXR) (Zelcer and Tontonoz, 2006). SR-BI deficient mice show minor reduction of cholesterol absorption (Mardones et al., 2001). Overexpression of SR-BI specific in intestine leads to increased cholesterol absorption (Bietrix et al., 2006). Mice deficient in ABCA1 show minor effects in cholesterol absorption (Drobnik et al., 2001; McNeish et al., 2000). Therefore, ABCA1, ABCG5, ABCG8, NPC1L1 and SR-BI are potential targets for further studies of the impact of sialidase deficieny on intestinal cholesterol absorption. We deduce that the reduction in cholesterol levels is due to a decreased production of the chylomicron from the enterocytes in addition to a reduced production of hepatic VLDL. Thus, hypomorphic sialidase expression modulates lipoprotein metabolism in both the liver and intestine. While it was expected to see lower levels of serum cholesterol levels as a result of lower chylmomicron production, the significantly higher cholesterol levels in the liver was not expected. It points to a dysregulation of hepatic cholesterol uptake as a result of the sialidase expression in the liver.

Overexpression of PCSK9 typically causes an increase in LDL cholesterol levels (Maxwell and Breslow, 2004). While overexpression of PCSK9 has no effect on LDLR mRNA levels, LDLR protein was nearly not detected (Maxwell and Breslow, 2004). PCSK9 gene transcription is regulated by SREBP-2 (Horton et al., 2003; Maxwell et al., 2003). PCSK9 has a SRE in the promoter region which enables SREBP-2 binding (Dubuc et al., 2004; Jeong et al., 2008). As a result, PCSK9 is highly responsive to changes in intracellular cholesterol levels and as seen in B6.SM mice, increased hepatic cholesterol levels lower hepatic SREBP-2, which in turn result in reduction in the transcription of the PCSK9 gene. Therefore, the observed decrease in hepatic PCSK9 in B6.SM mice would be expected to increase the half life of LDL receptors. In fact, we have observed a maintenance in hepatic LDLR protein in B6.SM mice. Various studies have implicated PCSK9 in the degradation and turnover of LDLR (Park et al., 2004; Benjannet et al., 2004; Maxwell et al., 2005; Sun et al., 2005). Currently, three major lines of evidence pointing to the role of PCSK9 in LDLR degradation have been suggested. First, cells overexpressing PCSK9 with clathrin heavy chain deficiency show a reduction in LDLR degradation (Nassoury et al. 2007). Second, PCSK9 co-localizes with markers of late endocytic compartment (Legace et al., 2006; Benjannet al., 2006). Specifically, PCSK9-mediated degradation of LDLR occurs in the lysosomal pathway as demonstrated by the inhibitory effects of acidotropic agents (Lagace et al., 2006; Benjann et al., 2004; Maxwell et al., 2005; Nassoury et al., 2007; Holla et al., 2007). Third, PCSK9 requires the epidermal growth factor (EGF)-like repeat A of the LDLR for it to bind (Kwon et al., 2008). It has been reported that the structure of PCSK9 rather than its catalytic activity is responsible for inducing LDLR degradation (Grefhorst et al., 2008; Li et al., 2007;

McNutt et al., 2007; Schmidt et al., 2008). However, it remains to be determined if sialylation has an impact on the formation of PCSK9-LDLR complex which leads to LDLR degradation. The binding site of PCSK9 has been localized to the EGF-like repeat A domain of the LDLR. Whether the EGF-A region contains hypersialylated glycosylation sites which are targets of sialidase is still to be determined.

In conclusion, we demonstrated that the changes in lipoprotein metabolism observed in hypomorphic sialidase mice are mediated by both a decrease in the production of VLDL-lipid in the liver which is mediated by a reduced MTP expression and an increase in uptake of LDL regulated by an increased stabilization of LDLR. Taken together, these events are predictive to lead to an overall atheroprotective effect through lowering LDL cholesterol in serum. These findings provided evidence for a central role for sialidase in the cross talk between the uptake and production of lipoproteins.

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Chapter 3

Inhibition of Sialidase as a Potent Treatment for Atherosclerosis in ApoE Knockout Mice

Abraham E. Yang[1], Gabriel Gyulay[1], David A. Egier[1], Richard C. Austin[4], Bernardo L. Trigatti[2], Suleiman A. Igdoura[1,3]

Department of [1]Biology, [2]Biochemistry and Biomedical Sciences, [3]Pathology and Molecular Medicine, McMaster University, Hamilton, Ontario, Canada, [4]Department of Medicine, Division of Nephrology, McMaster University, St. Joseph's Healthcare Hamilton and Hamilton Centre for Kidney Research, Hamilton, Ontario, Canada

Preface

Abraham Yang performed the followings: FPLC cholesterol profiles of *apoe-/-* and B6.SM/*apoe-/-* mice (Fig. 3.3.1), serum and hepatic lipids levels in *apoe-/-* and B6.SM/*apoe-/-* mice (Table 3.3.1), *in vivo* hepatic VLDL-lipids production in *apoe-/-* and B6.SM/*apoe-/-* mice (Fig. 3.3.2), *in vivo* hepatic VLDL-lipids production rates in *apoe-/-* and B6.SM/*apoe-/-*mice (Table 3.3.2), hepatic VLDL-ApoB-100 and –ApoB-48 secretion in *apoe-/-* and B6.SM/*apoe-/-* mice (Fig. 3.3.3), hepatic MTP, SREBP2 and ACAT2 expression in *apoe-/-* and B6.SM/*apoe-/-* mice (Fig. 3.3.4), atherosclerotic lesion area in *apoe-/-* and B6.SM/*apoe-/-* mice (Fig. 3.3.5), quantification of macrophages and T cells in atherosclerotic lesions in *apoe-/-* and B6.SM/*apoe-/-* mice (Fig. 3.3.6), FPLC cholesterol profiles in western diet-fed male *apoe-/-* and B6.SM/*apoe-/-* mice (Fig. 3.3.7), serum total cholesterol levels in western diet-fed male *apoe-/-* and B6.SM/*apoe-/-* mice (Table 3.3.3), atherosclerotic lesion area in western diet-fed male *apoe-/-* and B6.SM/*apoe-/-* mice (Fig. 3.3.8), FPLC cholesterol profiles in *apoe-/-* treated with DANA and control *apoe-/-* mice (Fig. 3.3.9), serum and hepatic lipids levels in *apoe-/-* treated with DANA and control *apoe-/-* mice (Table 3.3.4), atherosclerotic lesion area in *apoe-/-* treated with DANA and control *apoe-/-* mice (Fig. 3.3.10), the effects of sialidase inhibition using Oseltamivir on spontaneous atherosclerosis in male *apoe-/-* mice (Fig. 3.3.11) and the effects of hypomorphic sialidase expression on castrated and sham male B6.SM/*apoe-/-* mice (Fig. 3.3.12).

David Egier contributed to castration. Šárka Lhoták performed paraffin sectioning and immunostaining. Jordon Wronzberg and Darren de Sa contributed to FPLC (Fig. 3.3.9

and Fig. 3.3.11 A respectively), Sheila Brown contributed to cryosectioning, Aline Fiebig contributed to technical assistance for mini osmotic pumps implantation.

Chapter 3

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Summary

Atherosclerosis is an inflammatory process, which is highly dependent on lipoprotein metabolism. The level of sialic acid modification of several cell surface molecules as well as serum apolipoproteins have been implicated in the modulation of inflammation and lipoprotein metabolism respectively. In order to examine the role of sialidase during the development of atherosclerosis, we generated ApoE knockout mice expressing hypomorphic levels of sialidase (B6.SM/*apoe*-/-). These hypomorphic sialidase *apoe*-/- mice show decreased VLDL production rate and decreased recruitment and infiltration of monocytes and T cells in the atherosclerotic lesions as well as reduced aortic sinus atherosclerosis. In addition, the atheroprotective effects of hypomorphic sialidase expression are maintained in *apoe*-/- mice fed western diet and are not altered by androgen withdrawal. When male *apoe*-/- mice are treated with a mammalian sialidase inhibitor, 2-deoxy-2,3-dehydro-N-acetylneuraminic acid (DANA), the size of aortic atherosclerotic lesions are significantly reduced by 40% in comparison to saline-treated male *apoe*-/- controls. On the other hand, viral sialidase inhibitor, Oseltamivir has no effect. Thus, hypomorphic sialidase expression confers protection against atherosclerosis in male ApoE-deficient mice and, as a result, neu1 sialidase represent a novel target for the treatment of atherosclerosis.

3.1 Introduction

Atherosclerosis causes thickening of the innermost layer of the artery, the intima, and is responsible for causing myocardial infarction. Inflammation and lipoprotein metabolism play important roles in the development of atherosclerosis. Atherosclerotic lesions, the asymmetric focal thickenings of the intima, involve the transmigration of macrophages and T cells. Despite keen interest in atherosclerosis, the molecular events that lead to atherosclerotic progression have not been fully elucidated. Several studies have suggested that different levels of sialylation of apolipoproteins may affect atherogenic potential (Orekhov et al., 1989; Sobenin et al., 1991; Orekhov et al., 1991; Tertov et al., 1992a; Tertov et al., 1992b; Orekhov et al., 1992) and that different level of sialylation of certain cell surface molecules modulate inflammation which in turn drives atherosclerosis (Gee et al., 2003; Katoh et al., 1999).

Sialic acids are a family of N- and O-substituted derivatives of sialic acid. Over 40 different derivatives of sialic acid have been identified. They are generally found as terminal sugar residues, frequently found in α-2,3- or α-2,6-linkages with galactose, on oligosaccharides of both glycoproteins and glycolipids. The function of sialic acid on apolipoproteins is not completely understood but has been associated with secretion, lipid-binding, and plasma clearance of apolipoproteins (Paulson J., 1989). Sialylation is modulated through the action of sialyltransferases and sialidases.

Sialidases constitute a family of hydrolytic enzymes that are present in viruses, bacteria and vertebrates and catalyze the removal of terminal sialic acid residues from glycoproteins, glycolipids and oligosaccharides (Achyuthan K. and Achyuthan A., 2001; Pilate et al., 1993; Pshezhetsky and Ashmarina, 2001; Vimr and Lichtensteinger, 2002). Desialylation influences a number of important biological processes including cell-cell interactions, T-cell activation, catabolism and antigenicity (Varki A., 1997; Corfield T., 1992; Reuter and Gabius, 1996). In mammals, four distinct sialidases have been identified. Genes encoding lysosomal (*neu1*) (Igdoura et al., 1998; Carrillo et al., 1997; Pshezhetsky et al., 1997), cytosolic (*neu2*) (Monti et al., 1999; Ferrari et al., 1994), ganglioside specific (*neu3*) (Miyagi et al., 1999; Wada et al., 1999), and mitochondrial sialidases (*neu4*) (Comelli et al., 2003; Monti et al., 2004; Yamaguchi et al., 2005) have been cloned. Among them, neu1 is expressed and targeted to the cell surface and lysosomes during the activation of T cells, B cells, macrophages, and neutrophils (Landolfi et al., 1985; Landolfi and Cook, 1986; Cross and Wright, 1991; Stamatos et al., 2005). We have previously demonstrated that *neu1* overexpression results in an overall reduction in cell surface sialylation in C2C12 cells (Champigny et al., 2005). More recent evidence has implicated a sialidase in the conversion of Sialyl-CD15 to CD15 on myeloid cells. This desialylation occurs on glycoproteins such as PSGL-1 and CD43, which are essential for homing of leukocytes during inflammation (Gadhoum and Sackstein, 2008).

There is mounting evidence that sialylation of apolipoproteins is an important determinant of plasma lipoproteins levels. For example, desialylated ApoB LDL has a more rapid rate of clearance than sialylated ApoB LDL (Malmendier et al., 1980), and

desialylated LDL is taken up more readily by aortic smooth muscle cells than sialylated LDL (Orekhov et al., 1989; Filipovic et al., 1979). A strong negative correlation between LDL sialic acid content and the degree of cholesterol accumulation has been shown in vitro (Orekhov et al., 1989; Sobenin et al., 1991; Orekhov et al., 1991; Tertov et al., 1992a; Tertov et al., 1992b; Orekhov et al., 1992; Filipovic et al., 1979). Furthermore, the LDL of patients with coronary artery disease (CAD) has low sialic acid content as compared to healthy controls (Orekhov et al., 1989; Orekhov et al., 1991; Tertov et al., 1992a; Tertov et al., 1992b; Ruelland et al., 1993).

An inbred strain, SM/J mice show a partial deficiency of sialidase leading to hypersialyated hepatic enzymes and cell surface molecules on leukocytes (Potier et al., 1979; Clark et al., 1981). Hypomorphic sialidase mice (B6.SM) are generated by backcrossing SM/J mice carrying the mutant *neu1* allele to C57Bl/6 mice. B6.SM mice become a valuable model since they do not show the fatal Sialidosis phenotype seen in *neu1*$^{-/-}$ mice (de Geest et al., 2002). Therefore, we utilized these mice to examine if hypomorphic neu1 sialidase expression attenuates serum lipoprotein levels and/or inflammation and as a result may have an impact on atherosclerosis. In this study, we present evidence that hypomorphic sialidase expression results in reduced VLDL production in ApoE knockout mice. We also show that low sialidase reduced leukocytes frequency within atherosclerotic lesions and significantly reduced aortic sinus atherosclerosis in ApoE knockout mice. In addition, we demonstrate that pharmacological inhibition of sialidase significantly reduced (>50%) atherosclerotic lesion formation in ApoE-deficient mice. Therefore, our study not only reveals a novel

role for neu1 sialidase but also points to a new avenue for treatment of cardiovascular disease.

3.2 Material and Methods

3.2.1 Generation of mice

B6.SM mice were crossed with *apoe-/-* mice to generate compound B6.SM/*apoe-/-* mice on a C57Bl/6 background. *apoe-/-* and B6.SM/*apoe-/-* were used for the spontaneous atherosclerosis studies. Mice were housed in microisolator cages, maintained on a 12-hr light/dark cycle, and given free access to food and water. All procedures involving animals were in accordance with guidelines established by the Canadian Council on Animal Care and were subjected to prior approval by the institutional Animal Research Ethics Board.

3.2.2 Treatment of mice

Spontaneous atherosclerosis studies were performed in 7- to 9-month-old, male and female, *apoe*-/- mice and B6.SM/*apoe*-/- mice. For the sialidase inhibition studies, at 8 months of age, one group of male *apoe-/-* mice was sacrificed for baseline quantification and examination of atherosclerotic lesions. The remaining male mice were administered with the sialidase inhibitor N-acetyl-2,3-dehydro-2-deoxyneuraminic acid (DANA) or saline using mini-osmotic pumps (Alzet osmotic pump, Model 2004, DURECT Corporation). DANA was administered at a rate of 0.06µg per hour for six weeks. Mice were sacrificed at 6 weeks after injection (9.5 months of age) for quantification and morphological characterization of atherosclerotic lesions. Serum was obtained on weeks 0, 4, and 6 after DANA injection for analysis of lipids. For the Oseltamivir-treated *apoe-/-* mice, Oseltamivir was dissolved in drinking and was administered daily to male *apoe-/-* mice for 6

weeks. For the western diet-fed male *apoe*-/- and B6.SM/*apoe*-/- mice, western diet feeding was started when the mice were 1 month of age. Western diet used contains 21% butterfat and 0.15% cholesterol diet with 1% safflower oil (Modified Stanford University, Dyets Inc, catalogue #112286). The mice were fed with western diet for 1 month. The mice were harvested when they were 2 months of age.

3.2.3 Surgical castration

B6.SM/*apoe*-/- male mice (4 months old) were anesthetized and two separate incisions were made through the skin on the ventral side of the scrotum. The vas deferens and the testicular blood vessels at the junction between the testis and epididymis were ligated using synthetic absorbable 4.0 gauge sutures. The testicle was then removed. The remaining pieces of the vas deferens, the fat, and the epididymis were replaced back into the scrotal sac. The wound was closed with surgical staples. This procedure was repeated for the other testis. Sham-castration followed the above procedure without ligation of the testicular vessels or removal of the testes was used as control. The sham and castrated B6.SM/*apoe*-/- male mice were fed western diet starting at 24 hours after the surgery for 4 weeks.

3.2.4 Collection of blood and tissues

Mice were anesthetized with ketamine/xylazine. The abdominal and thoracic cavities were opened and the heart was perfused with phosphate-buffered saline (PBS) through the left ventricle of the heart (drainage via the right atrium). At the termination of the experiment, blood was obtained by cardiac puncture. Serum was prepared by centrifugation of whole blood for 5 min at 15,000 rpm using serum collection tubes.

The heart was removed and placed in Krebs Henseleit Solution. After 30 min, the heart was placed in 4% formaldehyde. The liver was removed, frozen in liquid nitrogen and stored in -80ºC.

3.2.5 Serum lipid and lipoprotein analysis

Serum was fractionated by gel filtration-FPLC, and serum total cholesterol was analyzed by enzymatic assay (Infinity™ Cholesterol Liquid Stable Reagent, Thermo Scientific) (Covey et al., 2003; Rigotti et al., 1997). Briefly, cholesterol esters in the serum were enzymatically hydrolysed by cholesterol esterase (CE) to cholesterol and free fatty acids. Free cholesterol was then oxidized by cholesterol oxidase (CO) to cholest-4-en-3-one and hydrogen peroxide. The hydrogen peroxide combined with hydroxybenzoic acid (HBA) and 4-aminoantipyrine (4AAP) in the presence of peroxidase (POD) to form a chromophore (quinoeimine dye) which may be quantified at 500nm. The absorbance of the reaction product was measured at 500 nm using spectrophotometer. Serum free cholesterol was analyzed by enzymatic assay (Free Cholesterol E Reagent, Wako Diagnostics). Briefly, free cholesterol in the serum was oxidized by cholesterol oxidase (CO) to Δ^4-cholestenone and hydrogen peroxide. The hydrogen peroxide caused DAOS and 4-aminoantipyrine (4AAP) to undergo an oxidative condensation in the presence of peroxidase (POD), to produce a blue color which may be quantified at 600nm. The absorbance of the reaction product was measured at 600 nm using spectrophotometer. Serum esterified cholesterol concentration was calculated by subtracting serum free cholesterol concentration from serum total cholesterol concentration. Serum triglyceride was analyzed with enzymatic assay (L-Type Triglyceride H Reagents 1 and 2, Wako Diagnostics).

Briefly, free glycerol in the serum was decomposed by glycerol kinase (GK), GPO and catalase in reagent 1. When reagent 2 was added after decomposition of free glycerol, triglycerides in the serum were hydrolyzed to glycerol and fatty acids in a reaction catalyzed by lipoprotein lipase (LPL). Glycerol is converted to glycerol-3 phosphate by GK in the presence of adenosine-5'-tryphosphate (ATP). Glyerol-3-phosphate formed is then oxidized by GPO in a reaction that produced hydrogen peroxide. The hydrogen peroxide produced caused HDAOS and 4-aminoantipyrine to undergo a quantitative oxidative condensation catalyzed by peroxidase (POD), producing a blue color which may be quantified at 600nm. The absorbance of the reaction product was measured at 600 nm using spectrophotometer.

3.2.6 Evaluation of atherosclerotic lesions

Hearts were embedded in Cryomatrix (Shandon Corp) and serial 10 μm sections were collected using a Shandon Cryostat. Sections were stained with Oil Red O for neutral lipid and hematoxylin for nuclei, and atherosclerosis was quantified as the total cross sectional area of atherosclerotic lesion in each given section. Lesion volume was determined by measuring the cross sectional area of lesions in sections spaced 100 μm apart as the average lesion area for the 100 μm stretch of the aortic sinus, multiplying by 100 μm, and then adding these values together.

3.2.7 Histology and Immunocytochemistry

Paraffin sections of the aortic root were blocked with rabbit serum for MAC-3 or goat serum for CD-3, antigen heat retrieval in citrate buffer pH 6.0 and stained with a monoclonal rat antibody MAC-3 (Pharmingen; 1:500 dilution) or CD-3 (Dako; 1:200

dilution), followed by detection with biotinylated secondary antibody (Vector; 1:500 dilution) (Zhou et al., 2005). For smooth muscle cells detection, paraffin sections were blocked with goat serum, and stained with a monoclonal mouse antibody SMA clone 1A4 (Neomakers; 1:200 dilution), followed by detection with anti-mouse Envision-HRP secondary antibody (Dako) (Zhou et al., 2005). Immunoreactivity was visualized by using NovaRED (Vector). Sections were counterstained with hematoxylin (Sigma).

3.2.8 Hepatic and blood cell lipid analyses

Contents of total cholesterol, free cholesterol, cholesterol esters and triglyceride in liver or blood cells (obtained from the low phrase in the serum collection tubes after centrifugation as mentioned in collection of blood and tissues) were determined after lipid extraction (Folch et al., 1957) using an enzymatic method. Briefly, liver or blood cell homogenates were prepared by homogenizing 150 mg of liver in 1ml of TNES (10 mM Tris, pH 7.5, 400 mM NaCl, 100 mM EDTA, 0.6% SDS). Folch mixture (6ml; chloroform/methanol, 2:1) was added to 300ul of liver homogenates and the tubes were vortexed for 1 minute. 1.2ml of distilled water was added and the tubes were vortexed for 1 minute vigorously. The extraction mixture was left at 4ºC for 2 hours. The tubes were centrifuged at low speed to facilitate phase separation. The upper phase (methanol/water phase) was discarded and the lower phase was saved. The lower phase (chloroform phase) was dried completely. The dry chloroform phase was resuspended in 60 μl of isopropanol. Hepatic total cholesterol, free cholesterol, cholesterol esters and triglyceride concentrations were measured as described in serum lipid and lipoprotein analysis. Blood cell lipid analysis was performed the same as the

hepatic lipid analysis except that lipids concentrations were normalized to protein concentrations after performing protein assays.

3.2.9 *In vivo* measurement of hepatic VLDL-triglyceride secretion

Male *apoe-/-* and B6.SM/*apoe-/-* mice (3 months old) were used for hepatic VLDL production studies. Mice were fasted overnight before the initiation of synthesis studies. To determine the rate of lipid synthesis, after injection of 500mg/kg of Triton WR1339 mixture ((Tyloxapol T0307-10G, Sigma BioXtra) (Sigma-Aldrich)) [15g/dl in 0.9% NaCl] to inhibit plasma lipoprotein lipase, blood was collected hourly at 0 hour (before Triton WR1339 injection) and 1, 2, 3 and 4 hours after Triton WR1339 injection (Kuipers et al., 1997; S. Otway and Robinson, 1957; Li et al., 1996; J. Millar et al., 2005). Blood was collected by serum collection tubes. Serum was obtained from the supernatant by centrifuging the blood in serum collection tubes at 15000rpm for 5 min. Serum was used for triglyceride, cholesterol and free cholesterol assays. At the termination of the experiment, mice were sacrificed. VLDL-triglyceride, cholesterol, free cholesterol and cholesteryl esters production rates were obtained by the regression lines of the graph with VLDL-triglyceride, cholesterol, free cholesterol, and cholesteryl esters concentration respectively versus time in hours.

3.2.10 Immunoblot analysis

Livers of male *apoe-/-* or B6.SM/*apoe-/-* mice were removed and immediately frozen with liquid nitrogen. Liver lysates were prepared in RIPA buffer supplemented with protease inhibitors. Protein concentration was determined using the Bradford assay (Biorad) with BSA as standard. Samples (50 μg) were separated on 6%

SDS/polyacrylamide gel and transferred to nitrocellulose membranes. Serum (1 µl) was subjected to SDS/4-15% or 4-20% PAGE and transferred to nitrocellulose membranes. Membranes were probed with the following antibodies: anti-ApoB (goat anti-ApoB, 1:6000 from Midland Bioproducts), anti-MTP (mouse anti-MTP, 1:2500 from BD Biosciences), anti-ACAT2 (mouse anti-ACAT2, 1:200 from Santa Cruz), anti-SREBP-2 (mouse 1:500 from Pharmingen), anti-β-actin (mouse anti-β-actin, 1:1000 from Cell Signalling Technology) and anti-GADPH (goat anti-GADPH, 1:1000 from R&D). Anti-goat or anti-mouse secondary HRP-conjugated antibodies (1:10000, Santa Crutz Biotechnology) were used and visualized with chemiluminescence (ECL, Amersham). Intensities of bands were measured by ImageJ densitometry software.

3.2.11 Statistical analysis

One-way analysis of variance (one-way ANOVA) followed by Tukey's Post Hoc Multiple Comparison Test, and additional statistical analyses were conducted using Prism 5 (version 5.04, GraphPad). Mean±SE were shown with error bars. Data were considered statistically significant different only if $P < 0.05$.

3.3 Results

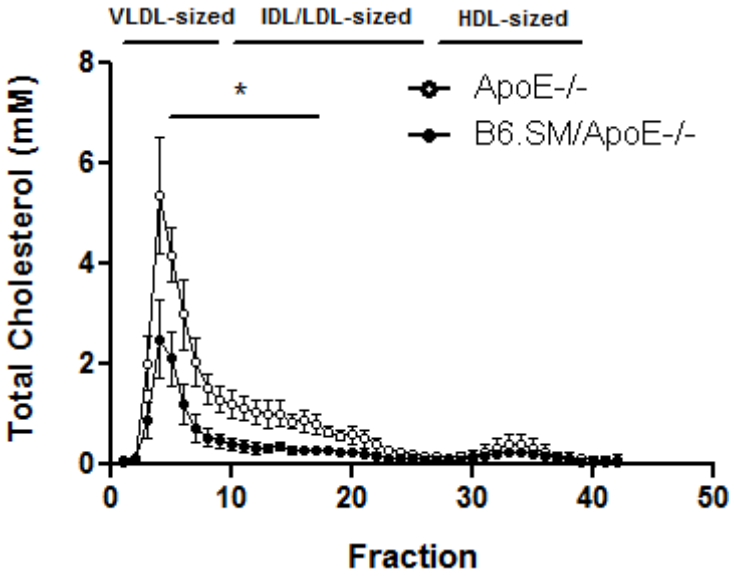
3.3.1 Hypomorphic sialidase expression decreases cholesterol levels in VLDL and LDL-sized lipoproteins in *apoe-/-* mice.

In order to determine the effects of hypomorphic sialidase expression on blood lipid profile, serum lipoproteins arre separated using Fast Protein Liquid Chromatography (FPLC) utilizing a superose 6 gel filtration column. Serum cholesterol associated with IDL/LDL-sized particles is evaluated and found to be significantly lower in male B6.SM/*apoe-/-* mice compared to *apoe-/-* mice (Fig. 3.3.1 A). However, there is no difference in fasted serum cholesterol FPLC profile of male B6.SM/*apoe-/-* mice compared to *apoe-/-* mice (Fig. 3.3.1 C). In addition, there is no difference in serum cholesterol FPLC profile of fasted or unfasted female B6.SM/*apoe-/-* mice compared to *apoe-/-* mice (Fig. 3.3.1 B and D). The majority of serum total cholesterol is found in fractions corresponding to VLDL and larger sized particles (fractions 3-10) and to IDL/LDL sized particles (fractions 11-25). Our data indicate that hypomorphic sialidase expression affects lipoprotein cholesterol level in a gender-specific manner, such that male mice show lower levels of serum lipoprotein cholesterol than female mice.

Fig. 3.3.1 FPLC cholesterol profiles of *apoe-/-* and B6.SM/*apoe-/-* mice. (A) Serum cholesterol FPLC profile show a significant decrease in VLDL- and LDL-sized particles between unfasted male B6.SM/*apoe-/-* mice (n=3) and *apoe-/-* controls (n=3) at 7 months of age. (B) Serum cholesterol FPLC profile show no differences between unfasted female B6.SM/*apoe-/-* mice (n=3) and *apoe-/-* controls (n=3) at 7 months of age. (C) There is no significant difference in cholesterol FPLC profile of fasted male B6.SM/*apoe-/-* mice (n=3) compared to *apoe-/-* controls (n=3), and (D) fasted female B6.SM/*apoe-/-* mice (n=3) compared to *apoe-/-* controls (n=3). Each circle represents mean ± SE. * denotes $P<0.05$ (VLDL-sized: Very low density lipoprotein sized particle, IDL-sized: Intermediate density lipoprotein sized particle, LDL-sized: Low density lipoprotein sized particle, HDL-sized: High density lipoprotein sized particle).

Fig. 3.3.1

A


VLDL-sized
IDL/LDL-sized
HDL-sized
*
ApoE-/-
B6.SM/ApoE-/-
Total Cholesterol (mM)
Fraction
0
2
4
6
8
0
10
20
30
40
50

B

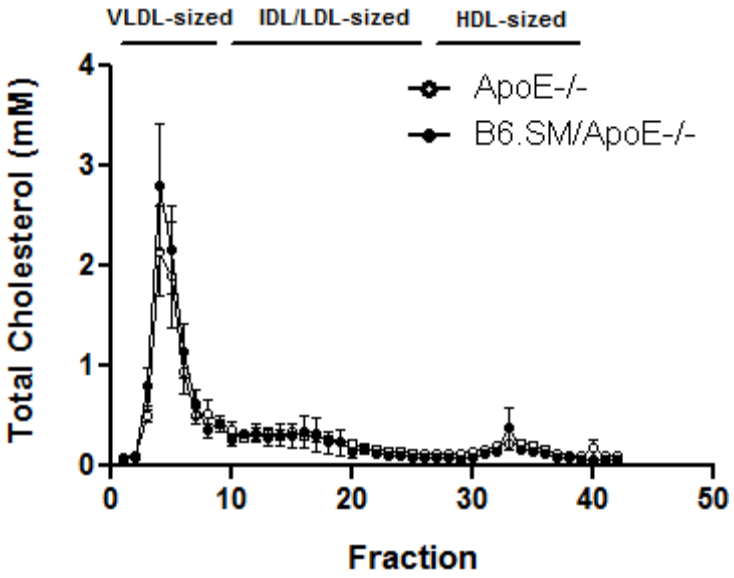
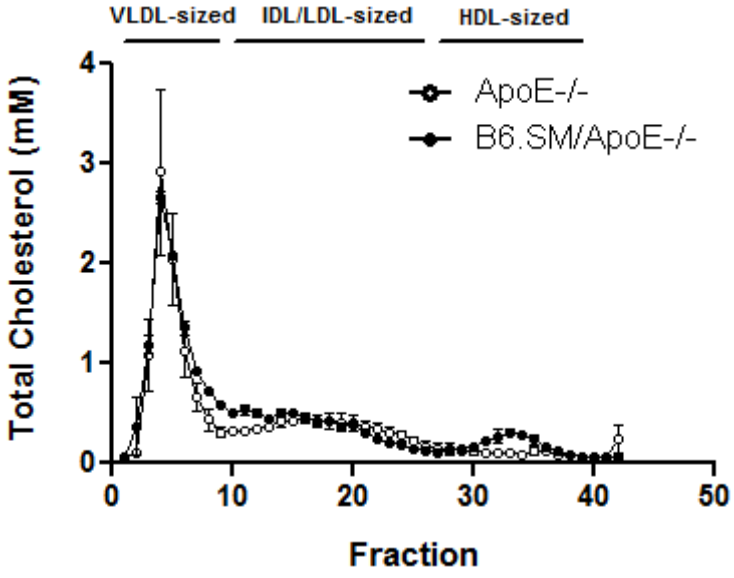
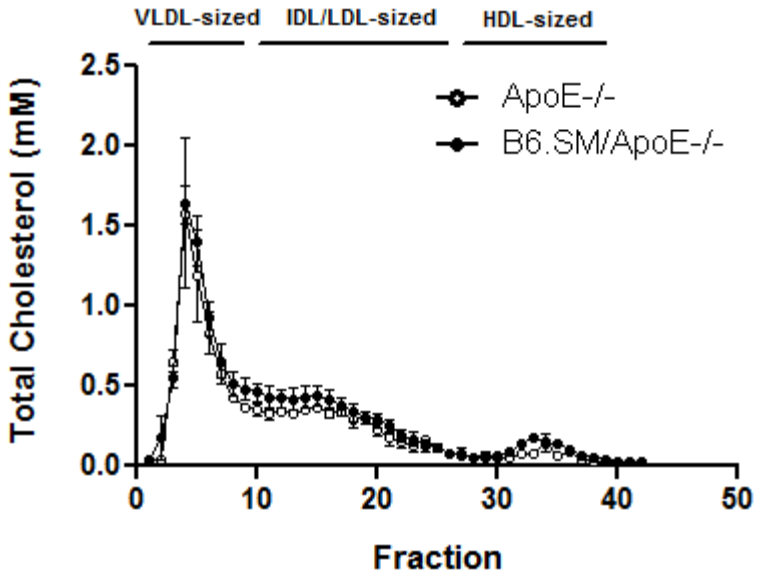

VLDL-sized
IDL/LDL-sized
HDL-sized
ApoE-/-
B6.SM/ApoE-/-
Total Cholesterol (mM)
Fraction
0
1
2
3
4
0
10
20
30
40
50

Fig. 3.3.1

C


VLDL-sized
IDL/LDL-sized
HDL-sized
ApoE-/-
B6.SM/ApoE-/-
Total Cholesterol (mM)
Fraction
0
1
2
3
4
0
10
20
30
40
50

D


VLDL-sized
IDL/LDL-sized
HDL-sized
ApoE-/-
B6.SM/ApoE-/-
Total Cholesterol (mM)
Fraction
0.0
0.5
1.0
1.5
2.0
2.5
0
10
20
30
40
50

3.3.2 The effects of hypomorphic sialidase expression on serum and hepatic lipids levels in *apoe-/-* mice.

After observing changes in cholesterol level in VLDL and IDL-LDL-sized particles in B6.SM/*apoe-/-* mice compared to *apoe-/-* controls, we investigated the levels of other lipid parameters, such as, triglyceride (a major component of chylomicrons and VLDL), cholesterol esters and free cholesterol (components of all lipoproteins). We demonstrate that hepatic cholesteryl esters concentration is increased and hepatic free cholesterol concentration is decreased in B6.SM/*apoe-/-* mice when compared to *apoe-/-* mice although hepatic triglyceride concentration is unchanged (Table 3.3.1). Serum free cholesterol and cholesteryl esters concentrations are decreased in B6.SM/*apoe-/-* mice when compared to *apoe-/-* mice with no change in serum triglyceride levels. In fact, the decrease in serum total cholesterol concentration is consistent and refected in the FPLC profile illustrated earlier (Table 3.3.1).

Table 3.3.1 Serum and hepatic lipids levels in *apoe*-/- and B6.SM/*apoe*-/- mice. We used enzymatic assay to measure lipids in serum of mice. There is a significant decrease in serum total cholesterol, free cholesterol and cholesteryl esters in B6.SM/*apoe*-/- mice (n=8) compared to *apoe*-/- mice (n=3). We used folch extraction to extract lipids from liver samples that we obtained from the mice and then used enzymatic assays to analyze lipid levels. Hepatic cholesteryl esters concentration is increased in B6.SM/*apoe*-/- mice (n=8) when compared to *apoe*-/- mice (n=3).* denotes $P<0.05$; ** denotes $P<0.001$.

Table 3.3.1

	Hepatic total cholesterol (mg/g liver)	Serum total cholesterol (mM)	Hepatic free cholesterol (mg/g liver)	Serum free cholesterol (mM)	Hepatic cholesteryl esters (mg/g liver)	Serum cholesteryl esters (mM)	Hepatic triglyceride (mg/g liver)	Serum triglyceride (mM)
ApoE-/-	3.62±0.29	32.24±2.12	3.16±0.30	14.43±2.38	0.46±0.13	17.81±0.69	16.98±1.52	3.32±0.19
B6.SM/ApoE-/-	2.22±0.11 **	14.97±1.42**	1.52±0.18 *	7.45±0.75 **	0.70±0.18	7.52±1.14**	14.69±1.74	4.38±0.49

3.3.3 Hypomorphic sialidase expression decreases *in vivo* hepatic VLDL-lipids production rates in *apoe-/-* mice.

In order to assess the potential mechanism behind the lower levels of serum VLDL-sized particles observed in B6.SM/*apoe-/-* mice compared to those of the *apoe-/-* mice, we measured hepatic VLDL production *in vivo* over 4 hours. Our results show that hypomorphic sialidase *apoe-/-* mice produces significantly lower levels of VLDL-triglyceride and VLDL cholesterol (free cholesterol and cholesteryl esters) over 4 hours in comparison to *apoe-/-* mice (Fig. 3.3.2 A to D). The production rates of VLDL-lipids as illustrated by the slope are also significantly lower (Table 3.3.2). The difference was greatest in VLDL-triglyceride production rate with 60% less VLDL-TG in hypomorphic sialidase mice. The levels of ApoB100 and ApoB48 which are components of VLDL show little difference between hypomorphic sialidase *apoe-/-* and *apoe-/-* mice at the end of 4 hours (Fig. 3.3.3 A and B). Thus, sialidase expression is a significant determinant of hepatic VLDL-lipid secretion but not of ApoB expression. Therefore, we performed further experiments to assess the expression of MTP, which mediates loading of lipids to ApoB.

Fig. 3.3.2 *In vivo* hepatic VLDL-lipids production in *apoe-/-* and B6.SM/*apoe-/-* mice. Male *apoe-/-* (n=4) and male B6.SM/*apoe-/-* mice (n=4) were fasted overnight and injected with Triton WR1339 (500mg/kg). Serum samples were drawn at just before the injection of Triton WR1339 (time 0 hour) and at 1, 2, 3, and 4 hours after the injection of Triton WR1339. There is a significant decrease in hepatic (A) VLDL-triglyceride, (B) VLDL-cholesterol, (C) VLDL-free cholesterol and (D) VLDL-cholesteryl esters concentrations in different indicated times after 0 hour (* denotes $P<0.05$). In addition, there is a decrease in the steepness of the slope of the B6.SM/*apoe-/-* mice compared to that of the *apoe-/-* mice. These imply that hypomorphic sialidase expression causes decreased hepatic VLDL-lipids production. Values represent the means ± S.E.

Fig. 3.3.2

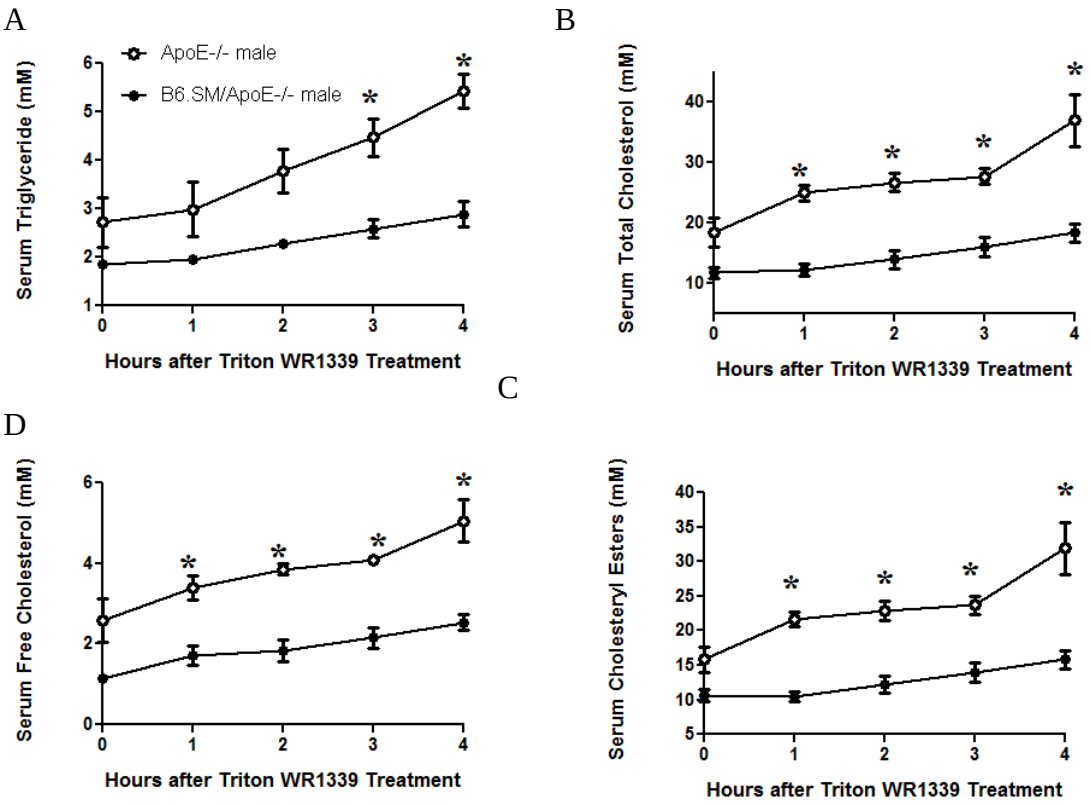

A
ApoE-/- male
B6.SM/ApoE-/- male
Serum Triglyceride (mM)
Hours after Triton WR1339 Treatment
B
Serum Total Cholesterol (mM)
Hours after Triton WR1339 Treatment
C
D
Serum Free Cholesterol (mM)
Hours after Triton WR1339 Treatment
Serum Cholesteryl Esters (mM)
Hours after Triton WR1339 Treatment

Table 3.3.2 *In vivo* hepatic VLDL-lipids production rates in *apoe-/-* and B6.SM/*apoe-/-*mice. Male *apoe-/-* (n=4) and male B6.SM/*apoe-/-* mice (n=4) were fasted overnight and administered Triton WR1339 (500mg/kg). Serum samples were collected at 0, 1, 2, 3, and 4 hours after the administration of Triton WR1339. Hepatic VLDL-triglyceride, total cholesterol, free cholesterol and cholesteryl esters production rates are calculated from the slope of the VLDL-triglyceride, and VLDL-cholesterol levels versus time curves, which were fitted by linear regression over the 4-hours period. There is a significant decrease in hepatic VLDL-triglyceride, VLDL-cholesterol, VLDL-free cholesterol and VLDL-cholesteryl esters production rate in B6.SM/*apoe-/-* mice compared to *apoe-/-* mice. Values represent the means±S.E.

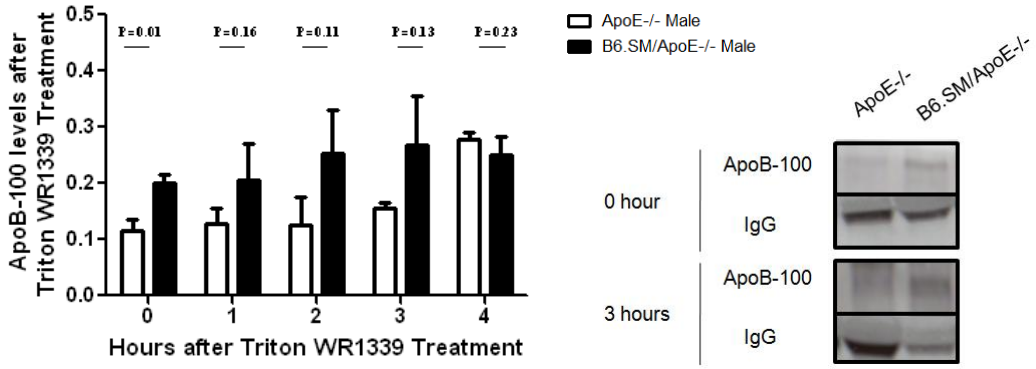
Table 3.3.2

	Triglyceride PR (mM/hr	Total Cholesterol PR (mM/hr	Free Cholesterol PR (mM/hr	Cholesteryl Esters PR (mM/hr)
ApoE-/- Male	0.69 + 0.14	3.98 + 1.11	0.56 + 0.21	3.42 + 0.90
B6.SM/ApoE-/- Male	0.27 + 0.08	1.70 + 0.23	0.32 + 0.04	1.38 + 0.19
P value	0.02	0.045	0.15	0.03

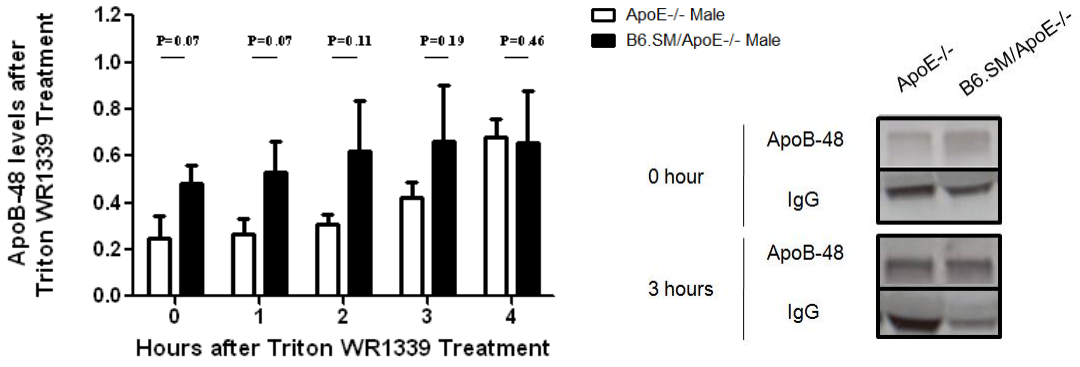
Fig. 3.3.3 Hepatic VLDL-ApoB-100 and –ApoB-48 secretion in *apoe-/-* and B6.SM/*apoe-/-* mice. For the determination of VLDL contents of (A) ApoB-100 and (B) ApoB-48, serum samples obtained at 0, 1, 2, 3, and 4 hours, were probed for ApoB. There is no significant difference in the levels of either ApoB-100 or ApoB-48 between *apoe-/-* (n=3) and B6.SM/*apoe-/-* mice (n=3). ApoB levels were determined by the intensities of ApoB bands normalized to IgG bands. Intensities of bands were measured by ImageJ densitometry software. This implies that decreased VLDL lipids secretion is not caused by decreased VLDL particle secretion.

Fig. 3.3.3

A


ApoB-100 levels after Triton WR1339 Treatment
0.5
0.4
0.3
0.2
0.1
0.0
P=0.01
P=0.16
P=0.11
P=0.13
P=0.23
0
1
2
3
4
Hours after Triton WR1339 Treatment
ApoE-/- Male
B6.SM/ApoE-/- Male
ApoE-/-
B6.SM/ApoE-/-
0 hour
ApoB-100
IgG
3 hours
ApoB-100
IgG

B


ApoB-48 levels after Triton WR1339 Treatment
1.2
1.0
0.8
0.6
0.4
0.2
0.0
P=0.07
P=0.07
P=0.11
P=0.19
P=0.46
0
1
2
3
4
Hours after Triton WR1339 Treatment
ApoE-/- Male
B6.SM/ApoE-/- Male
ApoE-/-
B6.SM/ApoE-/-
0 hour
ApoB-48
IgG
3 hours
ApoB-48
IgG

3.3.4 Hypomorphic sialidase expression decreases hepatic MTP and SREBP2 expression in *apoe-/-* mice.

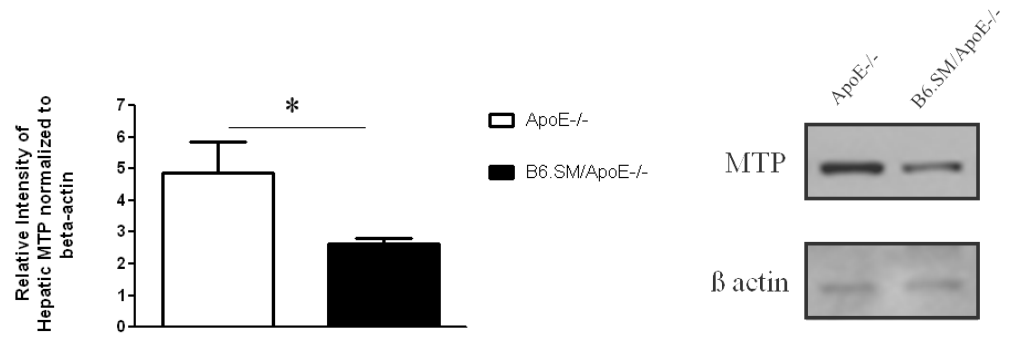
We have shown that sialidase-deficient mice have decreased VLDL-lipids production rate compared to controls in *apoe-/-* background. In addition, we have demonstrated that there is no significant difference in hepatic ApoB-100 and ApoB-48 production implying that the decrease in VLDL-lipids production rate is not caused by decreased hepatic VLDL particle production. In order to explain the decrease in the hepatic VLDL-lipids production rate, we examined hepatic expression of microsomal transfer protein (MTP). MTP is required for the secretion of ApoB-containing lipoproteins from hepatocytes and from enterocytes (Gordon D., 1996; Wetterau et al., 1998). Our results indicate a significant decrease in hepatic protein expression of MTP in B6.SM/*apoe-/-* mice compared to *apoe-/-* mice (Fig. 3.3.4 A). Thus, the decrease in VLDL-lipids appears to be the result of lower expression levels of hepatic MTP. Since the MTP promoter contains a SREBP binding site (Rubin et al., 2008; Sato et al., 1999) and since transgenic mice overexpressing SREBP-2 show increased MTP mRNA level in the liver (Horton et al., 1998), we examined the levels of hepatic SREBP-2 in B6.SM/*apoe-/-* and *apoe-/-* mice. Our results demonstrate that the hepatic expression of SREBP-2 is significantly reduced in hypomorphic sialidase *apoe-/-* mice compared to *apoe-/-* mice (Fig. 3.3.4 B). On the other hand, the hepatic expression of ACAT2, which performs intracellular cholesterol esterification (Chang et al., 1993) is significantly increased in hypomorphic sialidase *apoe-/-* mice compared to *apoe-/-* controls (Fig. 3.3.4 C). ACAT2 is instrumental in providing cholesteryl esters for VLDL assembly in the liver (Chang et al., 2009). Thus, an

increase in hepatic ACAT2 implies that the decreased expression of hepatic MTP is not a downstream effect of insufficient supply of cholesteryl esters.

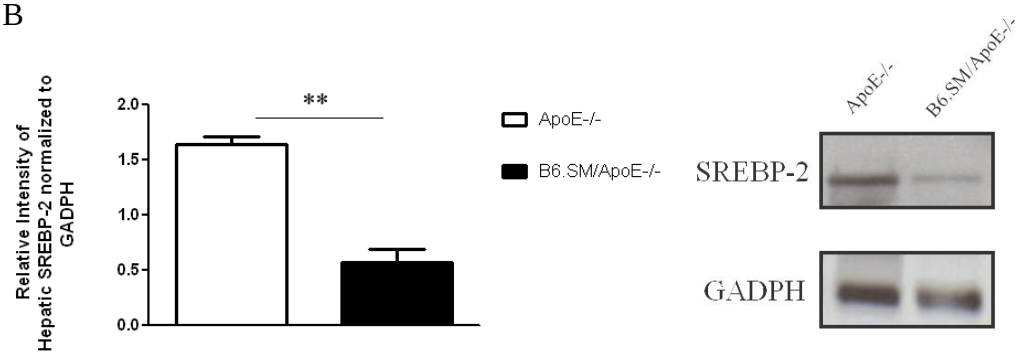
Fig. 3.3.4 Hepatic MTP, SREBP2 and ACAT2 expression in *apoe*-/- and B6.SM/*apoe*-/- mice. Liver lysates were subjected to SDS-PAGE (8%). Membranes were probed for MTP (A), SREBP-2 (B), ACAT2 (C), and GADPH anti-β-actin. B6.SM/*apoe*-/- mice show a significant decrease in the expression of MTP and SREBP2, and a significant increase in the expression of ACAT2 in the liver compared to *apoe*-/- mice. Representative blots and graphs representing quantification of normalized band intensities are shown. Intensities of bands are measured by ImageJ densitometry software. Numbers of animals used are as follows: MTP (n=3 for *apoe*-/- ; n=3 for B6.SM/*apoe*-/-), SREBP-2 (n=4 for *apoe*-/-; n=5 for B6.SM/*apoe*-/-), ACAT2 (n=4 for *apoe*-/-; n=5 for B6.SM/*apoe*-/-). * denotes $P<0.05$; ** denotes $P<0.001$. Mean ± SE are shown.

Fig. 3.3.4

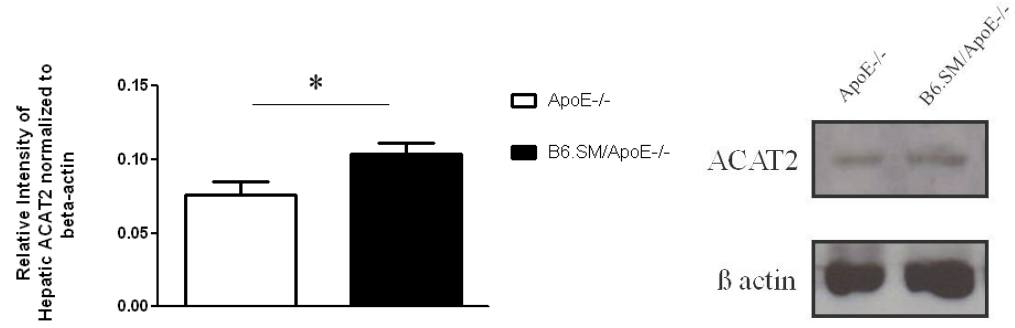
A


Relative Intensity of Hepatic MTP normalized to beta-actin
7
6
5
4
3
2
1
0
*
ApoE-/-
B6.SM/ApoE-/-
ApoE-/-
B6.SM/ApoE-/-
MTP
ß actin

B


Relative Intensity of Hepatic SREBP-2 normalized to GADPH
2.0
1.5
1.0
0.5
0.0
**
ApoE-/-
B6.SM/ApoE-/-
ApoE-/-
B6.SM/ApoE-/-
SREBP-2
GADPH

C

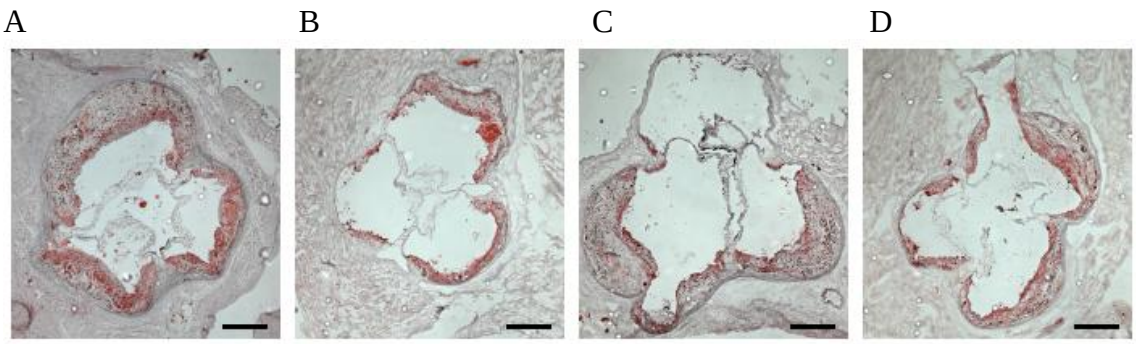

Relative Intensity of Hepatic ACAT2 normalized to beta-actin
0.15
0.10
0.05
0.00
*
ApoE-/-
B6.SM/ApoE-/-
ApoE-/-
B6.SM/ApoE-/-
ACAT2
ß actin

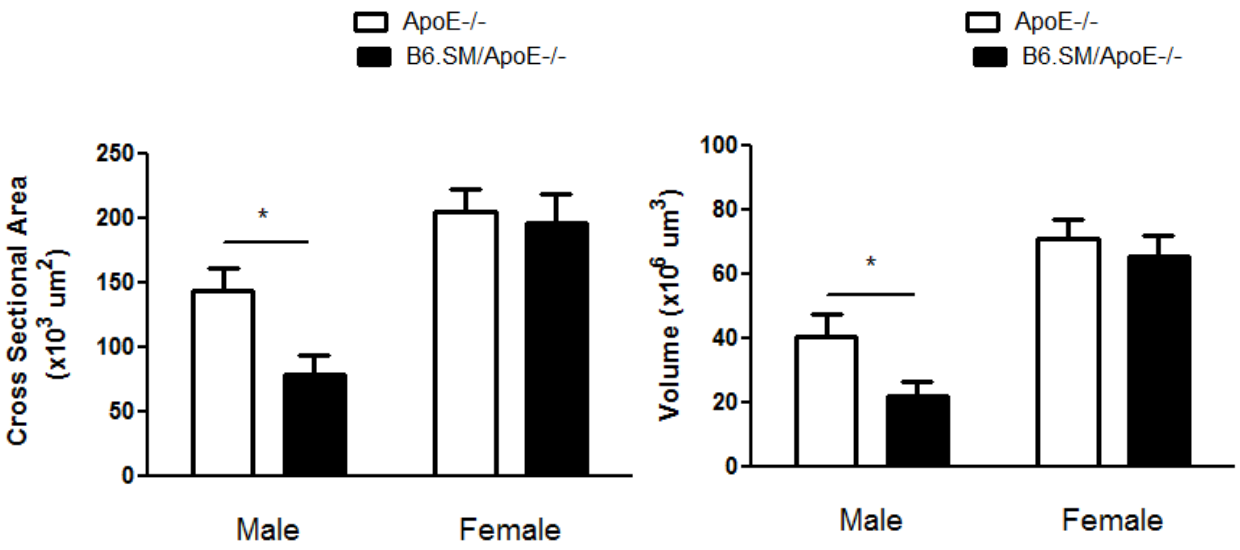
3.3. 5 Hypomorphic sialidase expression decreases atherogenesis in *apoe-/-* mice.

To assess the effects of hypomorphic sialidase expression on atherosclerosis, aortic root sections from mice were stained with Oil Red O. Histologically, atherosclerotic lesions were smaller in male B6.SM/*apoe-/-* mice than corresponding *apoe-/-* controls (Fig. 3.3.5 A and B). This significant reduction in lesion area was only observed in male mice (Fig. 3.3.5 C and D). Quantitatively, we evaluated atherosclerosis by measuring the sizes of lesions in the aortic sinuses of male and female B6.SM/*apoe-/-* and *apoe-/-* mice. The average size of the atherosclerotic deposits in the male B6.SM/ *apoe-/-* mice was one half of the average size measured in the male *apoe-/-* mice (P=0.0097) (Fig. 3.3.5 E). The average size of the atherosclerotic deposits in the female B6.SM/*apoe-/-* mice was not significantly different from the average size measured in the female *apoe-/-* mice. Similar results were obtained when the volumes of atherosclerotic lesions were determined in male and female B6.SM/*apoe-/-* and *apoe-/-* mice (Fig. 3.3.5 F).

Fig. 3.3.5 Atherosclerotic lesion area in *apoe-/-* and B6.SM/*apoe-/-* mice. Cross-sections of the aortic sinus from (A) male *apoe-/-*, (B) male B6.SM/*apoe-/-*, (C) female *apoe-/-* and (D) female B6.SM/*apoe-/-* mice. Sections were stained with Oil Red O for neutral lipid and hematoxylin for nuclei. Scale bar = 200μm. Atherosclerotic lesion is significantly reduced in male B6.SM/*apoe-/-* mice (n=15) compared to male *apoe-/-* mice (n=17) (P=0.0097 and P=0.032 for (E) lesion area and (F) volume respectively). Female B6.SM/*apoe-/-* mice (n=15) reveals a similar amount of atherosclerosis as in female *apoe-/-* mice (n=15) (P=0.79 and P=0.57 for lesion area and volume respectively). * denotes $P<0.05$. Each bar represents mean±SE.

Fig. 3.3.5


A
B
C
D


E
F
ApoE-/-
B6.SM/ApoE-/-
Cross Sectional Area (x10^3 um^2)
250
200
150
100
50
0
*
Male
Female
ApoE-/-
B6.SM/ApoE-/-
Volume (x10^6 um^3)
100
80
60
40
20
0
*
Male
Female

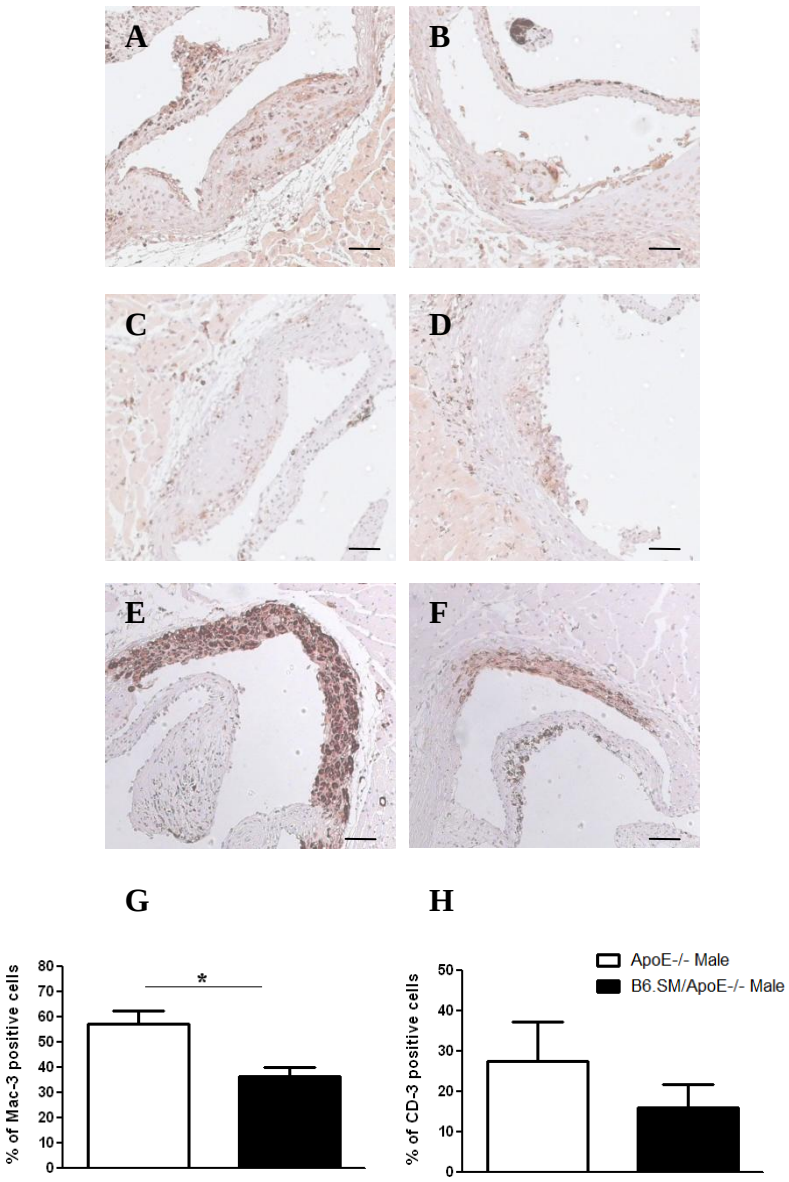
3.3. 6 Hypomorphic sialidase *apoe-/-* mice have less macrophages and T cells in atherosclerotic lesions.

Atherosclerotic lesions are mostly made up of macrophages and T cells (Jonasson et al., 1986; Hansson et al., 1988; Katsuda et al., 1992). To assess whether the decrease in atherosclerotic lesion is associated with the quantity of macrophage in the lesion, aortic root sections from male mice were immunostained with anti-MAC-3, a macrophage-specific antibody. Macrophages were abundant throughout the atherosclerotic lesion of *apoe-/-* mice, and constituted most of the cells in the lesion (Fig. 3.3.6 A). In contrast, markedly fewer macrophages were present in the aortic sinuses of B6.SM/ *apoe-/-* mice (Fig. 3.3.6 B). There was a significant reduction in 43% of MAC-3 positive cells in atherosclerotic lesions in B6.SM/ apoe-/- male compared to apoe-/- male (P=0.029) (Fig. 3.3.6 G). These data suggest that the decrease in the size of the atherosclerotic lesions is associated with a reduction in the number of macrophage in the lesion, implying that sialidase was important in homing of monocytes as well as recruitment and maturation of macrophages into atherosclerotic lesions. To assess whether the decrease in atherosclerotic lesion is associated with the quantity of T cells in the lesion, aortic root sections from male mice were immunostained with anti-CD-3, a T-cell-specific antibody (Fig. 3.3.6 C and D). No significant difference in % of CD-3 positive cells in atherosclerotic lesions in B6.SM/*apoe-/-* male compared to *apoe-/-* male (P=0.37) (Fig. 3.3.6 H). To assess whether the decrease in atherosclerotic lesion is associated with the quantity of smooth muscle cells in the lesion, aortic root sections from male mice were immunostained with anti-SMA, a smooth muscle cell-specific antibody. Markedly more smooth muscle cells were present in media layer of *apoe-/-* male compared to

B6.SM/*apoe-/-* male (Fig. 3.3.6 E and F). These data suggest that the decrease in atherosclerotic lesion is associated with a reduction in number of smooth muscle cells in the media layer. Overall, B6.SM/*apoe-/-* male mice have less macrophages, T cells and smooth muscle cells in the atherosclerotic lesions compared to *apoe-/-* male mice. These results point to a reduced degree of inflammation within atherosclertoic lesions.

Fig. 3.3.6 Macrophages and T cells quantities in atherosclerotic lesions in *apoe*-/- and B6.SM/*apoe*-/- mice. (A) Macrophage, (C) T cells, and (E) smooth muscle cells infiltration of the aortic sinus in *apoe*-/- male mice are shown. (B) Macrophage, (D) T cells, and (F) smooth muscle cells infiltration of the aortic sinus in B6.SM/*apoe*-/- male mice are shown. Macrophages were detected with MAC-3 (red). T cells were detected with CD-3. Smooth muscle cells were detected with SMA. Hematoxylin (blue) was used for nuclei staining. Shown are representative sections from mice of male *apoe*-/-, male B6.SM/*apoe*-/-. Scale bar = 50μm for MAC-3, CD-3, SMA. (G) Quantification of the frequency of MAC-3 positive cells and frequency of CD-3 positive cells in atherosclerotic lesions. B6.SM/*apoe*-/- male show a 43% reduction of MAC-3 positive cells in atherosclerotic lesions in comparison to controls (P=0.029). (H) No significant difference in % of CD-3 positive cells is found (P=0.37).

Fig. 3.3.6


A
B
C
D
E
F
G
H
% of Mac-3 positive cells
80
70
60
50
40
30
20
10
0
*
% of CD-3 positive cells
50
40
30
20
10
0
ApoE-/- Male
B6.SM/ApoE-/- Male

3.3. 7 Hypomorphic sialidase expression decreases serum cholesterol levels and atherosclerosis in western diet-fed male *apoe-/-* mice.

In order to assess the role of hypomorphic sialidase expression in an accelerated atherosclerotic model, we fed B6.SM/*apoe-/-* and *apoe-/-* mice with western diet for 4 weeks. To determine the effects of hypomorphic sialidase expression on the serum lipoprotein profile after feeding the western diet, the serum was fractionated and lipoprotein cholesterol levels were measured. Our result shows reduced serum cholesterol concentrations in the IDL/LDL-sized particles in western diet-fed male B6.SM/*apoe-/-* mice compared to controls (Fig. 3.3.7). In addition, cholesterol concentrations in the VLDL-sized particles appear to decrease in the B6.SM/*apoe-/-* mice compared to *apoe-/-* mice although the decrease is not statistically significant. No significant difference in cholesterol profiles was observed in female *apoe-/-* and B6.SM/*apoe-/-* mice. Although the reduction in cholesterol level is not as dramatic as that seen in *apoe-/-* mice fed normal diet, these results indicate that hypomorphic sialidase expression results in reduced cholesterol concentrations in IDL/LDL-sized particles in *apoe-/-* mice fed western diet.

The changes in cholesterol levels in FPLC profiles of *apoe-/-* and B6.SM/*apoe-/-* mice fed western diet led us to assess whether there are differences in the ratio of free cholesterol to cholesteryl esters. We demonstrate a decrease in serum levels of cholesteryl ester in male B6.SM/*apoe-/-* mice compared to male *apoe-/-* mice fed western diet. Serum triglyceride and free cholesterol concentrations are not significantly different (Table 3.3.3). Therefore, the decrease in total serum cholesterol is reflective of a decrease in cholesterol concentration in IDL/LDL-sized particles

seen on the FPLC profile. Since cholesteryl ester is a major component of LDL, this decrease in cholesterol ester concentrations actually reflects the decreased cholesterol concentration in IDL/LDL-sized particles. Furthermore, atherosclerotic lesion areas and volumes in western diet-fed male B6.SM/*apoe-/-* mice are significantly reduced compared to controls (P=0.0085 and P=0.016 for lesion area and volume respectively) (Fig. 3.3.8 A and B). These results show that hypomorphic sialidase expression protects from accelerated atherosclerosis.

Fig. 3.3.7 FPLC cholesterol profiles in western diet-fed male *apoe*-/- and B6.SM/*apoe*-/- mice. There is a decrease in serum cholesterol levels in LDL-sized particles in unfasted male B6.SM/*apoe*-/- mice (n=3) compared to *apoe*-/- controls (n=3) at 1 month of age after feeding with western diet for 1 month. Mean±SE are shown. * denotes $P<0.05$. (VLDL-sized: Very low density lipoprotein sized particle, IDL-sized: Intermediate density lipoprotein sized particle, LDL-sized: Low density lipoprotein sized particle, HDL-sized: High density lipoprotein sized particle).

Fig. 3.3.7

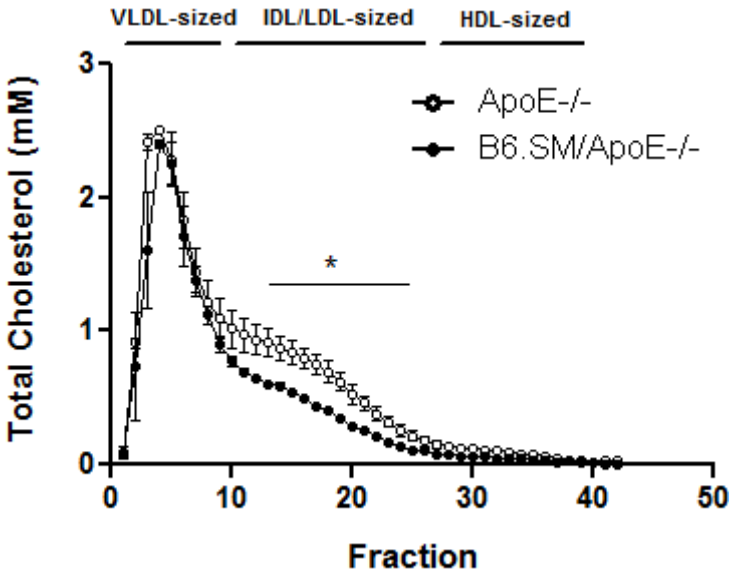

VLDL-sized
IDL/LDL-sized
HDL-sized
ApoE-/-
B6.SM/ApoE-/-
*
Total Cholesterol (mM)
Fraction
0
1
2
3
0
10
20
30
40
50

Table 3.3.3 Serum total cholesterol levels in western diet-fed male *apoe-/-* and B6.SM/*apoe-/-* mice. Serum total cholesterol, free cholesterol, cholesteryl esters, and triglyceride concentrations in western diet-fed male *apoe-/-* (n=3) and B6.SM/*apoe-/-* mice (n=3). Western diet-fed male B6.SM/ *apoe-/-* mice have a decreased level of total cholesterol (* denotes $P<0.05$) and decreased level of cholesteryl esters concentration and no significant difference in free cholesterol and triglyceride concentration compared to western diet-fed male *apoe-/-* controls.

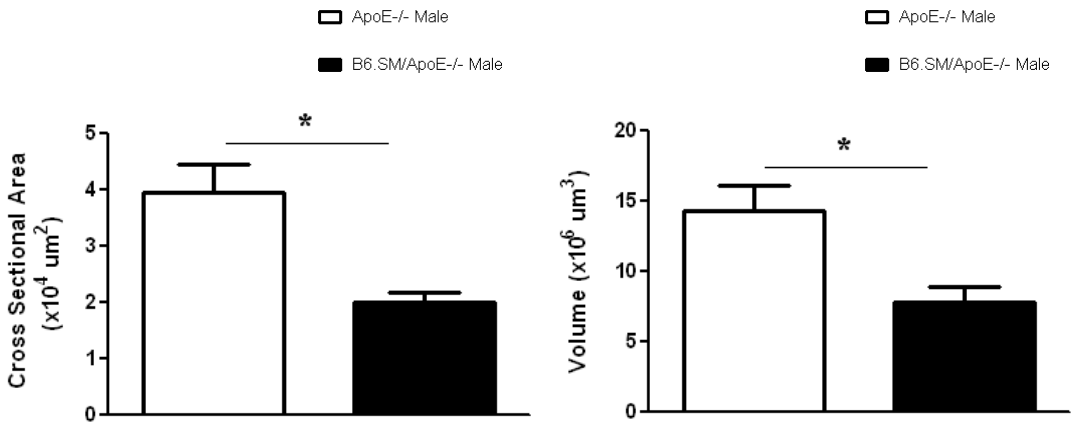
Table 3.3.3

	Serum total cholesterol (mM)	Serum free cholesterol (mM)	Serum cholesteryl esters (mM)	Serum triglyceride (mM)
ApoE-/- Male	25.64±0.81	11.37±0.37	14.27±0.75	21.18±1.62
B6.SM/ApoE-/- Male	20.78±1.09*	9.78±0.64	11.00±0.53*	28.16±4.25

Fig. 3.3.8 Atherosclerotic lesion area in western diet-fed male *apoe-/-* and B6.SM/*apoe-/-* mice. There is decreased atherogenesis in male hypomorphic sialidase *apoe-/-* mice at 2 month of age after feeding with western diet for 1 month. (A) Atherosclerotic lesion area and (B) volume are significantly reduced in male B6.SM/*apoe-/-* mice compared to male *apoe-/-* mice (P=0.0085 and P=0.016 for lesion area and volume respectively). Each bar represents mean±SE. * denotes P<0.05.

Fig 3.3.8

A B


ApoE-/- Male
B6.SM/ApoE-/- Male
Cross Sectional Area (x10^4 um^2)
0
1
2
3
4
5
*
ApoE-/- Male
B6.SM/ApoE-/- Male
Volume (x10^6 um^3)
0
5
10
15
20
*

3.3.8 The impact of pharmacological inhibition of sialidase on levels of serum cholesterol and atherosclerosis in male *apoe-/-* mice.

Male double homozygous mutant mice had decreased IDL and LDL-cholesterol levels compared to ApoE-/- mice (Fig. 3.3.1). In addition, aortic atherosclerotic lesions in double homozygous mutant mice were significantly reduced ~50% in males when compared to ApoE-/- male mice (Fig. 3.3.5). The atheroprotective impact of sialidase deficiency on ApoE-/- mice makes it a potential target for inhibition. In our study, we utilized one common mammalian sialidase inhibitor, 2-deoxy-2,3-dehydro-N-acetylneuraminic acid (DANA) (Meindl and Tuppy, 1969; Meindl et al., 1971; Meindl et al., 1974). DANA is used to determine the effects of sialidase inhibition on atherosclerosis in male *apoe-/-* mice. The non-permeability of DANA allows it to inhibit the sialidase on the surface of the cell membrane without affecting the intracellular sialidase. We therefore administered DANA to male *apoe-/-* mice using mini-osmotic pumps (0.06µg per hour) and analyzed serum lipoprotein levels at various intervals for 6 weeks. Our results show no differences in serum cholesterol FPLC profile between DANA-treated and saline-treated male *apoe-/-* mice (Fig. 3.3.9). In addition, levels of serum triglyceride, free cholesterol and cholesteryl esters show no significant differences. However, we observe a significant decrease in hepatic free cholesterol, a significant increase in hepatic cholesteryl esters and no change in hepatic triglycerides when comparing DANA-treated to saline-treated male *apoe-/-* mice (Table 3.3.4).

After administering DANA or saline for 6 weeks, mice were sacrificed, their aortic root sinus was dissected, sectioned and stained with Oil Red O. Histologically, lesions

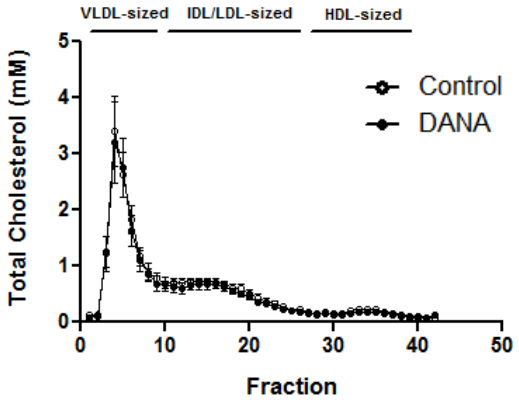
from DANA-treated male *apoe-/-* mice were significantly smaller than lesions from saline-treated control male *apoe-/-* mice (Fig. 3.3.10 A, B and C). Quantitatively, attenuation of atherosclerosis progressionin the aortic root in the DANA-treated male *apoe-/-* mice are noted, compared with those treated with saline and baseline ($P<0.001$) (Fig. 3.3.10 D). Similar results were obtained when we determined the volumes of atherosclerotic lesions (Fig. 3.3.10 E). These results suggested that sialidase inhibition by DANA is effective in halting atherosclerotic lesion progression at the aortic sinus.

In order to assess the specificity of DANA on atherogenesis, we have treated male *apoe-/-* mice with a viral sialidase inhibitor, Oseltamivir. Oseltamivir is the drug used to treat influenza virus with common name called Tamiflu. Male *apoe-/-* male mice (7-month old) are administered the sialidase inhibitor Oseltamivir for 6 weeks. Our results show no difference in cholesterol FPLC profile of Oseltamivir-treated compared to saline-treated male *apoe-/-* mice (Fig. 3.3.11 A). Quantitatively, no significant difference of atherosclerotic lesion area in the aortic sinus in the Oseltamivir-treated male *apoe-/-* mice is noted in comparison to controls ($P=0.12$) (Fig. 3.3.11 B). In addition, no differences are observed in the volumes of atherosclerotic lesions ($P=0.34$) (Fig. 3.3.11 C). Therefore, the atheroprotective effects of sialidase inhibition in male *apoe-/-* mice are specifically caused by mammalian sialidase inhibition.

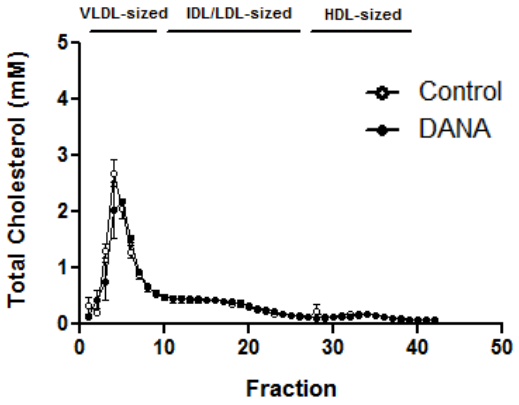
Fig. 3.3.9 FPLC cholesterol profiles in male *apoe-/-* treated with DANA and control male *apoe-/-* mice. Comparison of serum cholesterol profiles of saline-treated male *apoe-/-* (open circle) and DANA-treated male *apoe-/-* mice (black circle). All male *apoe-/-* mice were fasted before collection of serum. There is no significant difference in serum cholesterol levels in VLDL-sized, IDL/LDL-sized and HDL-sized particles in male *apoe-/-* mice treated with DANA for (A) 0 week, (B) 4 weeks and (C) 6 weeks compared to saline-treated male *apoe-/-* controls. Cholesterol levels in different lipoproteins were determined by FPLC (VLDL-sized: Very low density lipoprotein sized particle, IDL-sized: Intermediate density lipoprotein sized particle, LDL-sized: Low density lipoprotein sized particle, HDL-sized: High density lipoprotein sized particle).

Fig. 3.3.9

A


VLDL-sized
IDL/LDL-sized
HDL-sized
Control
DANA
Total Cholesterol (mM)
Fraction
0
1
2
3
4
5
0
10
20
30
40
50

B


VLDL-sized
IDL/LDL-sized
HDL-sized
Control
DANA
Total Cholesterol (mM)
Fraction
0
1
2
3
4
5
0
10
20
30
40
50

C

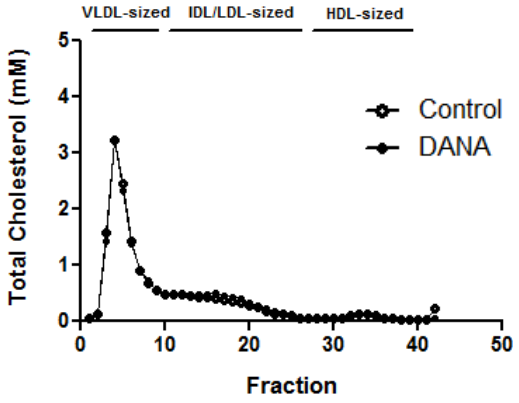

VLDL-sized
IDL/LDL-sized
HDL-sized
Control
DANA
Total Cholesterol (mM)
Fraction
0
1
2
3
4
5
0
10
20
30
40
50

Table 3.3.4 Serum and hepatic lipids levels in male *apoe-/-* treated with DANA and control male *apoe-/-* mice. Total serum cholesterol, triglyceride, free cholesterol and cholesteryl esters show no significant differences which explain the FPLC profiles. However, there are significant decreases in total cholesterol and free cholesterol in the liver when comparing DANA-treated mice (n=8) to saline-treated mice (n=8). There is a trend of decrease in triglyceride although it is not significant. In addition, there is a significant increase in cholesteryl esters. * denotes $P<0.05$.

Table 3.3.4

	Hepatic total cholesterol (mg/g liver)	Serum total cholesterol (mM)	Hepatic free cholesterol (mg/g liver)	Serum free cholesterol (mM)	Hepatic cholesteryl esters (mg/g liver)	Serum cholesteryl esters (mM)	Hepatic triglyceride (mg/g liver)	Serum triglyceride (mM)
Baseline	3.61±0.02	26.04±2.11	3.36±0.09	9.01±1.01	0.25±0.08	17.03±1.38	13.60±3.21	2.61±0.30
Saline	4.48±0.07	19.16±3.29	3.82±0.21	8.62±1.35	0.66±0.25	10.54±2.57	10.07±1.77	2.26±0.25
DANA	3.44±0.20*	24.77±1.84	1.82±0.32*	9.14±0.45	1.62±0.35*	15.63±1.61	7.02±0.62	2.56±0.31

Fig. 3.3.10 Atherosclerotic lesion area in male *apoe-/-* treated with DANA and control male *apoe-/-* mice. Cross-sections of the aortic sinus from (A) baseline (n=8), (B) male *apoe-/-* (n=8) and (C) male *apoe-/-* mice treated with DANA (n=8). Sections were stained with Oil Red O for neutral lipid and hematoxylin for nuclei. Scale bar = 200μm. Atherosclerotic lesion area (D) and volume (E) are significantly reduced in DANA-treated male *apoe-/-* mice compared to untreated male *apoe-/-* mice. Each bar represents mean ± SE. * denotes $P<0.05$ and ** denotes $P<0.001$.

Fig. 3.3.10

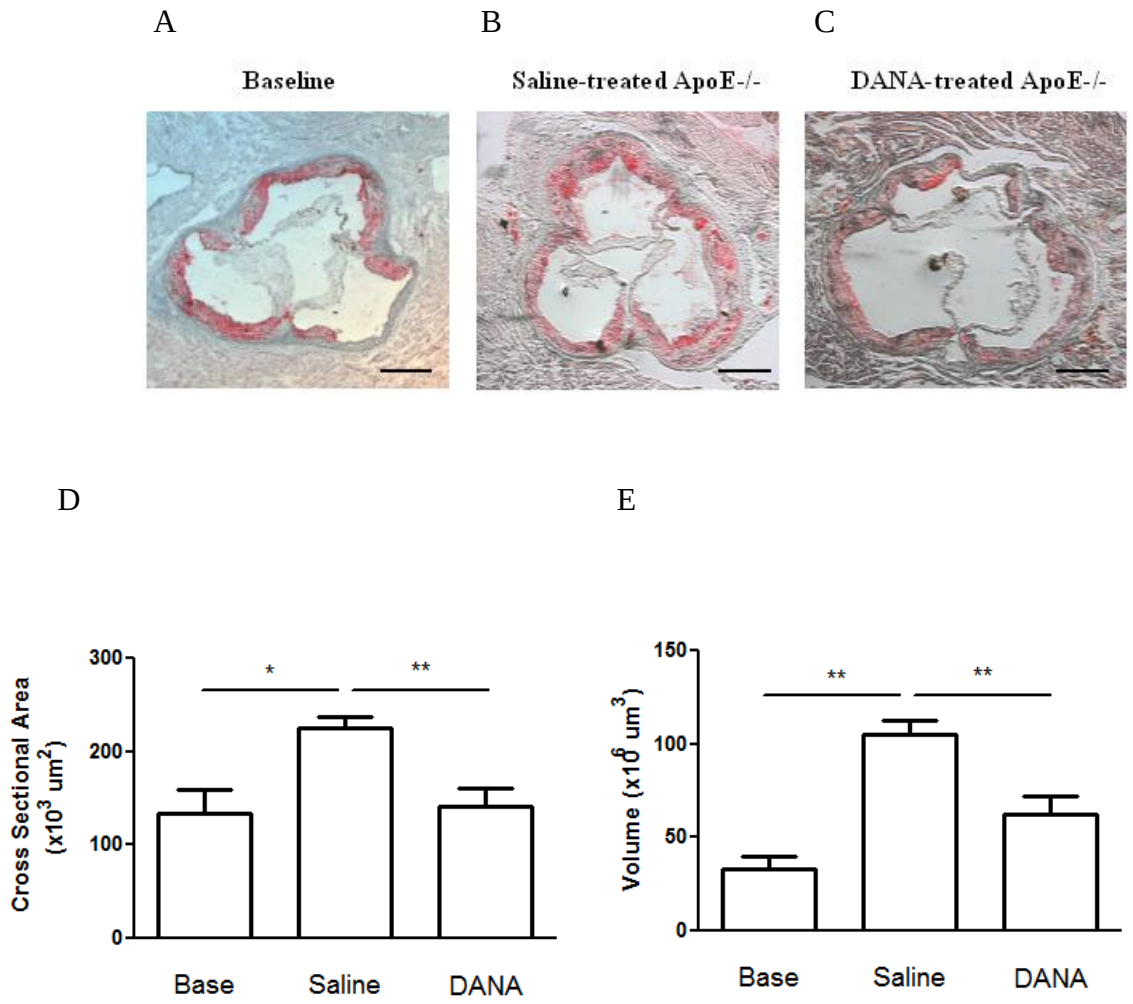
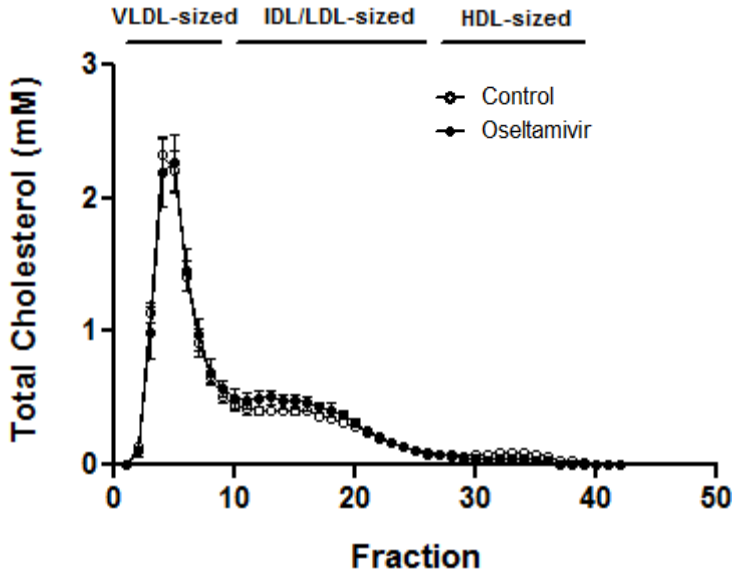

A
B
C
Baseline
Saline-treated ApoE-/-
DANA-treated ApoE-/-
D
E
Cross Sectional Area (x10^3 um^2)
300
200
100
0
*
**
Base
Saline
DANA
Volume (x10^6 um^3)
150
100
50
0
**
**
Base
Saline
DANA

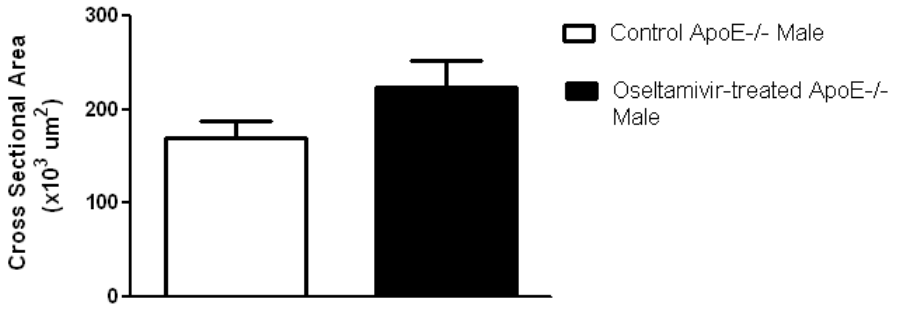
Fig. 3.3.11. The effects of sialidase inhibition using Oseltamivir on spontaneous atherosclerosis in male *apoe-/-* mice. (A) There is no significant difference in serum cholesterol FPLC profile in Oseltamivir-treated male *apoe-/-* mice (n=7) compared to *apoe-/-* controls (n=7). There is no significant difference in (B) atherosclerotic lesion area and (C) volume in Oseltamivir-treated male *apoe-/-* mice (n=7) compared to untreated male *apoe-/-* mice (n=7) (P=0.12 and P=0.34 for lesion area and volume respectively). The mean values ± standard error are shown.

Fig. 3.3.11

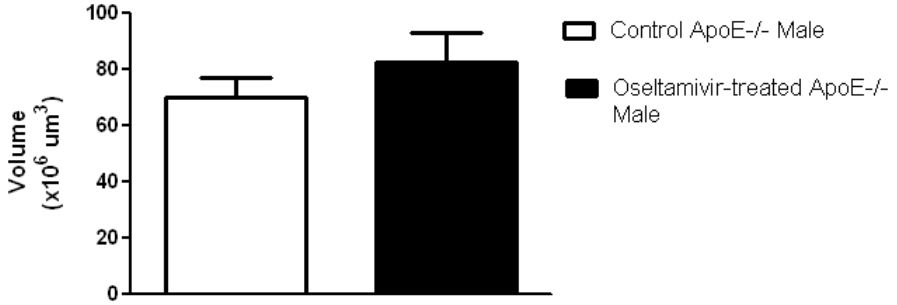
A


VLDL-sized
IDL/LDL-sized
HDL-sized
Control
Oseltamivir
Total Cholesterol (mM)
Fraction
0
1
2
3
0
10
20
30
40
50

B


Cross Sectional Area (x10^3 um^2)
Control ApoE-/- Male
Oseltamivir-treated ApoE-/- Male
0
100
200
300

C


Volume (x10^6 um^3)
Control ApoE-/- Male
Oseltamivir-treated ApoE-/- Male
0
20
40
60
80
100

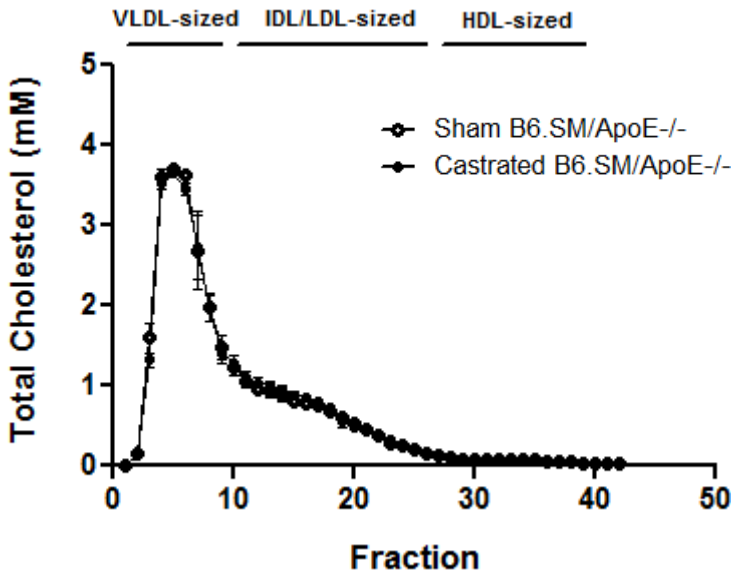
3.3. 9 Impact of androgen withdrawal on serum cholesterol levels and atherosclerosis in hypomorphic sialidase *apoe-/-* mice .

Our previous results indicated that both the areas and volumes of aortic atherosclerotic lesions in male B6.SM/*apoe-/-* mice were significantly reduced by ~50% in males when compared to *apoe-/-* mice. However, there was no significant difference in both lesion areas and volumes of atherosclerotic lesions in female *apoe-/-* compared to female B6.SM/*apoe-/-* mice. These data suggest that hypomorphic sialidase expression affected atherosclerosis in a gender-specific manner, such that male mice are more protected than females. In view of these results, we set to examine whether the effects of hypomorphic sialidase expression on atherosclerosis are dependent on male sex hormones. In order to determine whether the effects of hypomorphic sialidase expression on atherosclerosis are dependent on male sex hormones, we compared the cholesterol concentrations in different lipoprotein particles and atherosclerotic lesion areas and volumes between castrated male B6.SM/*apoe-/-* and sham male B6.SM/*apoe-/-* mice. The mice were castrated at 4 months of age. Sham or castrated B6.SM/*apoe-/-* males fed western diet for 4 weeks show no significant difference in cholesterol concentrations of VLDL, IDL/LDL, or HDL-sized particles (Fig. 3.3.12 A). In addition, there are no significant difference in both the areas and volumes of atherosclerotic lesions between sham and castrated B6.SM/*apoe-/-* mice (P=0.38 and P=0.47 for lesion area and volume respectively) (Fig. 3.3.12 B and C). Therefore, androgens may not be the only source of this gender-specific effects of hypomorphic sialidase expression on atherosclerosis in *apoe-/-*.

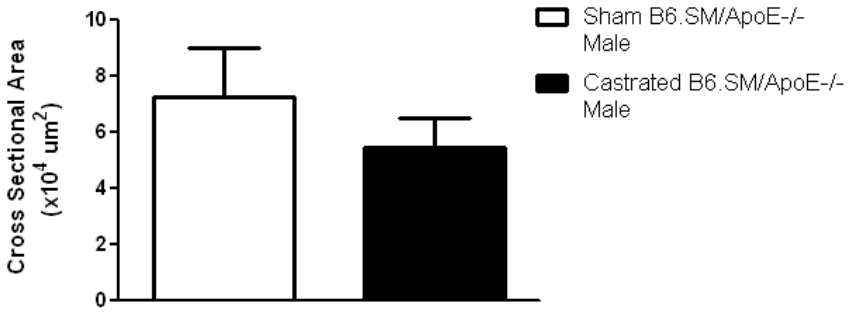
Fig. 3.3.12 The effects of hypomorphic sialidase expression on castrated and sham male B6.SM/*apoe*-/- mice. (A) No significant difference in serum cholesterol concentrations was observed between sham (n=4) and castrated B6.SM/*apoe*-/- (n=5)) after feeding with western diet for 1 month. Each circle represents mean±SE. (VLDL-sized: Very low density lipoprotein sized particle, IDL-sized: Intermediate density lipoprotein sized particle, LDL-sized: Low density lipoprotein sized particle, HDL-sized: High density lipoprotein sized particle). There is no significant difference in the size of (B) the atherosclerotic lesion area and (C) volume in sham (n=4) and castrated B6.SM/*apoe*-/- mice (n=5) (P=0.38 and P=0.47 for lesion area and volume respectively). Each bar represents mean±SE.

Fig. 3.3.12

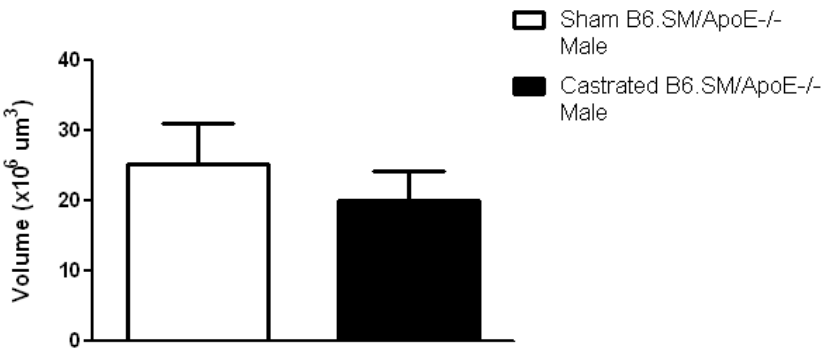
A


VLDL-sized
IDL/LDL-sized
HDL-sized
Total Cholesterol (mM)
Fraction
Sham B6.SM/ApoE-/-
Castrated B6.SM/ApoE-/-

B


Cross Sectional Area (x10^4 um^2)
Sham B6.SM/ApoE-/- Male
Castrated B6.SM/ApoE-/- Male

C


Volume (x10^6 um^3)
Sham B6.SM/ApoE-/- Male
Castrated B6.SM/ApoE-/- Male

3.4 Discussion

Neu1 is implicated in the activation of blood cells, including macrophages, T cells, B cells, and neutrophils (Landolfi et al., 1985; Landolfi and Cook, 1986; Cross and Wright, 1991; Stamatos et al., 2005). In addition, neu1 is implicated in the desialylation of blood cell surface adhesion molecules including CD44 and sialyl Lewis X motifs (Katoh et al., 1999; Gee et al., 2003; Gadhoum et al., 2008; Katoh et al., 2010). Furthermore, neu1 plays roles in blood cell Toll-like receptor 4 (TLR4) signalling which activates the sescretion of inflammatory cytokines (Amith et al., 2009; Amith et al., 2010; Stamatos et al., 2010). In addition to inflammatory processes, modification of sialylation on apolipoproteins has impacts on lipoprotein metabolism (Orekhov et al., 1989; Sobenin et al., 1991; Orekhov et al., 1991; Tertov et al., 1992a; Tertov et al., 1992b; Orekhov et al., 1992; Filipovic et al., 1979). In addition, patients of coronary artery disease (CAD) have lower LDL sialylation compared to healthy individuals (Orekhov et al., 1989; Orekhov et al., 1991; Tertov et al., 1992a; Tertov et al., 1992b; Ruelland et al., 1993). However, no *in vivo* studies of the impact of sialidase on atherogenesis and lipoprotein metabolism have been performed. Therefore, we aim to determine the effects of hypomorphic sialidase expression on atherosclerosis. The data we have presented here show that in response to hypomorphic sialidase expression, cholesterol level decreased in the male B6.SM/*apoe-/-* mice especially in the VLDL and LDL-sized particles. The size of atherosclerotic lesions was also decreased in the male B6.SM/*apoe-/-* mice. However, no difference was found in female B6.SM/*apoe-/-* mice compared to the *apoe-/-* controls. In fact, we have also analyzed 1-month-old male *apoe-/-* and B6.SM/*apoe-/-*

mice treated with western diet for 1 month. Similar results were observed for the male mice. Thus, these results showed that western diet does not have significant impact on the effects of hypomorphic sialidase expression on atherosclerosis, hypomorphic sialidase expression decreases atherosclerosis under both normal diet and western diet-treated mice and there appears to be a gender-specific effect. We have already eliminated that androgen is the only source of causing this gender-specific effects because no significant difference in cholesterol concentrations in different lipoprotein particles and atherosclerotic lesion area and volume between sham and castrated B6.SM/*apoe-/-* mice was observed. SM/J has Th1 cells, but not Th2 cells (Clark et al., 1981). Th1 cells produce IFN-γ and Th2 cells produce IL4 (Constant et al., 1997). Whitman et al. (2002) demonstrated that the decrease in atherosclerotic lesion areas in *apoe-/-* mice with a deficiency of IFN-γ is more pronounced in male compared to female (Whitman et al., 2002). This is consistent with our results as we showed that there was a significant decrease in atherosclerotic lesion area and volume in male hypomorphic sialidase *apoe-/-* but not in the female. Thus, this may be an IFN-γ -mediated effect.

We have observed that B6.SM/*apoe-/-* mice have more cholesteryl esters and less free cholesterol in liver, and less cholesteryl esters in serum compared to *apoe-/-* mice. In the hepatocyte, cholesteryl esters are hydrolyzed to free cholesterol by lysosomal cholesterol esterase (Rudel and Shelness, 2000). The conversion of cholesterol esters into free cholesterol by lysosomal cholesterol esterase in B6.SM/*apoe-/-* mice may not be as efficient as the conversion in *apoe-/-* mice. We have demonstrated that the decrease in VLDL-triglyceride, total cholesterol, free cholesterol and cholesteryl

esters production rates in hypomorphic sialidase mice is not caused by decreased VLDL particle production as illustrated by a non-significant difference in the ApoB production between the mice.

Microsomal triglyceride transfer protein (MTP) regulates the loading of lipids to ApoB during VLDL assembly in the ER (Wetterau and Zilversmit, 1985; Gregg and Wetterau, 1994; Gordon et al., 1995). Nevertheless, MTP requires supply of cholesterol esters in the ER. We have shown that MTP has sufficient supply of cholesterol esters by studying the expression of ACAT2, the major cholesterol esterifying enzyme in liver (Anderson et al., 1998; Cases et al., 1998; Oelkers et al., 1998). Nevertheless, the question of how neu1 affects the protein expression of the ER-resident MTP is still unknown. However, the MTP gene is a SREBP responsive gene and is directly affected by lower levels of hepatic cholesterol. Typically, the regulatory domain in the C-terminal of SREBP-2 interacts with cleavage activating protein (SCAP) in the ER which can escort SREBP to Golgi where Site-1 and Site-2 protease can cleave SREBP to its active form. Cleaved SREBP-2 activates genes involved in cholesterol synthesis during cholesterol deprivation (Nohturfft et al., 2000; Espenshade et al., 2002; Sun et al., 2005). When there is high intracellular cholesterol as in hypomorphic sialidase mice, cholesterol binds to Scap, which causes a structural change in SCAP that enables Insig to bind to Scap. When Insig binds to Scap, the Scap/SREBP complex remains in the ER (Sun et al., 2005). As a result, the transcription activation of the target gene, i.e., MTP, does not occur and the expression of MTP as a result is reduced.

We have previously shown that hepatic microcirculation in hypomorphic sialidase mice show increased rolling and decreased adhesion of leukocytes (Champigny et al., 2009). Sialidase (neu1) plays roles in the desialylation of cell surface cell adhesion molecules including CD44 (Katoh et al., 1999; Katoh et al., 2000; Gee et al., 2003; Katoh et al., 2010) and sialyl Lewis X motifs contained in selectin ligands such as P-selectin glycoprotein ligand 1 (PSGL-1) and E-selectin ligand 1 (ESL-1) (Gadhoum et al., 2008). These imply that sialidase may play roles in intercellular adhesion and may impact the recruitment and infiltration of blood cells into atherosclerostic lesions. We have observed that there are fewer macrophages and T cells in the atherosclerotic lesions in the aortic sinuses of B6.SM/*apoe-/-* mice compared to *apoe-/-* mice. Therefore, sialylation may play an important role in macrophage recruitment and homing. In fact, our results demonstrate that low sialidase reduced the numbers of macrophages and T cells in the atherosclerotic lesions. Hypomorphic sialidase expression is likely to cause a reduction in macrophage recruitment and homing to the endothelium by negatively affecting the binding of cell adhesion molecules such as P-selectin, E-selectin and hyaluronic acid. Further experiments to determine whether the decreased atherosclerosis is caused by a decreased number of leukocytes infiltration and/or the secretion of pro-inflammatory cytokines by the leukocytes such as TNFα, IL-1β, IL-6 and MCP-1 will be worthwhile.

Our discovery of the novel role for sialidase in atherosclerosis makes it a potential target for pharmacological inhibition. There are different kinds of sialidase inhibitors, such as, 2-deoxy-2,3-dehydro-N-acetylneuraminic acid (DANA). In 1969, DANA was developed as a synthetic sialic acid derivative which differs from naturally occurring

sialic acid by one double bond between carbon atom 2 and carbon atom 3. DANA can inhibit mammalian, viral and bacterial sialidases (Meindl and Tuppy, 1969; Meindl et al., 1971; Meindl et al., 1974). Other viral sialidase inhibitors include Zanamivir (von Itzstein et al., 1993), Oseltamivir (Kim et al., 1997), and Peramivir (Babu et al., 2000). We used DANA, the modified form of sialic acid, to determine the effects of sialidase inhibition on spontaneous atherosclerosis in *apoe-/-* mice because of its non-permeability of cell membrane and non-toxicity. The non-permeability of DANA allows it to inhibit the sialidase on the surface of the cell membrane without affecting the intracellular sialidase. Therefore, the DANA-injected mice will not develop sialidosis, a lysosomal disease caused by hypomorphic sialidase expression. Normally, sialidase has an active site that allows the sialic acid to bind. During the binding, the sialic acid is in the transition-state conformation. DANA resembles this transition-state conformation and binds to the active site of sialidase. Thus, DANA competes with sialic acid to bind to the active site of sialidase. Treating *apoe-/-* mice with DANA for 6 weeks is prominent to reduce atherogenesis as shown by the atherosclerotic lesion areas. The reduced atherosclerotic lesion areas can be due to attenuation in the progression of atherogenesis which is illustrated by the DANA treatment slowing down atherogenesis to comparable baseline levels. Nevertheless, regression of atherosclerosis may occur concurrently which reduces the atherosclerotic area directly. Several studies were done showing the atherosclerotic plaque regression by emigration of monocyte-derived cells from atherosclerotic lesions in response to normalization of serum cholesterol in a CCR7-dependent manner (Feig et al., 2009; Llodra et al., 2004; Trogan et al., 2006; Trogan et al., 2004).

Sialidase plays roles in the activation of leukocytes. During the activation, sialidase migrates to the cell surface to interact with cell surface molecules such as TLR4, CD44 and CD15 (Amith et al., 2009; Amith et al., 2010; Katoh et al., 1999; Katoh et al., 2010; Gadhoum et al., 2008; Gee et al., 2003). Sialidase inhibition was shown to be able to decrease the pro-inflammatory cytokine secretion and binding of leukocytes to endothelial cells (Katoh et al., 1999; Katoh et al., 2010; Nan et al., 2007). Although leukocytes are important contributors to confer atheroprotection, lipoprotein metabolism may be able to have synergistic impact with leukocytes. LIGHT and lymphotoxin, are members of the tumor necrosis factor super family (TNFSF) and ligands that bind to lymphotoxin β receptor (LTβR), and can promote inflammation, immune host defense, cell death and survival, and atherosclerosis. LIGHT mainly expresses on the surface of T cells and dendrite cells (Schnelder et al., 2004). Lo and colleagues have shown that overexpressing LIGHT on T cells causes the transgenic mice to have an increase in triglyceride and cholesterol concentrations in blood (Lo et al., 2007). Therefore, the changes in lipoprotein cholesterol levels can be affected by the activation of leukocytes.

When *apoe-/-* mice were treated with Oseltamivir, no significant difference in atherosclerotic lesion areas was observed. In fact, Oseltamivir is the drug used to treat influenza virus with brand name called Tamiflu. Thus, it is only specific to viral sialidase and does not inhibit mammalian sialidase. Therefore, *apoe-/-* mice treated with Oseltamvir do not show a reduction in atherosclerosis as observed in DANA-treated *apoe-/-* mice. Taken together, it appeared that non-cell membrane permeable sialidase inhibitor, DANA, can serve as an effective agent to reduce atherosclerosis.

In this study, we provide *in vivo* evidence that hypomorphic sialidase expression reduces serum cholesterol levels and reduces atherosclerosis in *apoe-/-* mice. In addition to genetic approach, we demonstrate that sialidase inhibition on the cell membrane is effective to reduce atherosclerosis. In conclusion, our studies so far present evidence that hypomorphic sialidase expression can protect against the atherogenic effects of ApoE deficiency in mice and inhibiting sialidase is a potent treatment for atherosclerosis.

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Chapter 4

Hypomorphic Sialidase Expression in Bone Marrow Derived Leukocytes Reduces Atherogenesis in ApoE Knockout Mice

Abraham E. Yang[1ǂ], Elizabeth J. White[1ǂ], Gabriel Gyulay[1], Bernardo L. Trigatti[2], Suleiman A. Igdoura[1,3]

Department of [1]Biology, [2]Biochemistry and Biomedical Sciences, [3]Pathology and Molecular Medicine, McMaster University, Hamilton, Ontario, Canada,

[ǂ]Abraham E. Yang and Elizabeth J. White contributed equally to this work and are co-first authors.

Preface

Abraham Yang performed the followings: weights of male and female B6.SM/*apoe-/-* mice transplanted with B6.SM/*apoe-/-* or *apoe-/-* bone marrow after feeding with western diet (Fig. 4.3.2), serum and hepatic lipids levels in B6.SM/*apoe-/-* mice transplanted with B6.SM/*apoe-/-* or *apoe-/-* bone marrow (Table 4.3.1), blood cells lipids levels in B6.SM/*apoe-/-* mice transplanted with apoe-/- or B6.SM/*apoe-/-* bone marrow (Fig. 4.3.3), atherosclerotic lesion area of B6.SM/*apoe-/-* mice transplanted with B6.SM/*apoe-/-* or *apoe-/-* bone marrow (Fig. 4.3.4). Elizabeth White performed peripheral blood immunophenotyping, selectin and hyaluronic acid binding assay (Fig. 4.3.1, 4.3.6-4.3.8), serum cytokine measurement (Fig. 4.3.5). Gabriel Gyulay contributed to folch lipid extraction. Aline Fiebig contributed to technical assistance for measuring weights and bone marrow transplantation (Fig. 4.3.2).

Chapter 4

Hypomorphic Sialidase Expression in Bone Marrow Derived Leukocytes Reduces Atherogenesis in ApoE Knockout Mice

Summary

Atherosclerosis is dependent on both lipoprotein metabolism and inflammatory cascades, involving immune cells and cytokine signaling in the peripheral blood and within developing lesion areas. We have shown previously that hypomorphic sialidase expression in macrophages results in reduced secretion of inflammatory cytokines, including TNFα, IL-1β, IL-6 and MCP-1 when stimulated with LPS. We have generated a congenic mouse strain with hypomorphic sialidase expression which shows significantly reduced atherosclerotic lesion development on an ApoE knockout background. In this study, we examined whether hematopoietic cell derived sialidase contributes significantly to atherogenesis. We first assessed the effect of hypomorphic sialidase expression on immunophenotypes of peripheral blood cells in *apoe-/-* mice, and its consequences on *in vivo* cytokine secretion and *in vitro* function of cell surface cell adhesion molecules. In addition, we transplanted male B6.SM/*apoe-/-* or *apoe-/-* bone marrow to female and male B6.SM/*apoe-/-* mice to test if normal sialidase expression in hematopoietic cells affects atherosclerosis in B6.SM/*apoe-/-*mice. Our results show increased populations of $CD4^+$ T and $CD11b^{hi}$ cells, but significantly reduced proportions of $CD11b^{lo}$ cells in B6.SM/*apoe-/-* mice compared to *apoe-/-* controls. When challenged with LPS, B6.SM/*apoe-/-* mice show delayed secretion of IFNγ and secrete less IL-10 than *apoe-/-*mice, indicating decreased inflammatory potential as a result of hypomorphic sialidase expression. B6.SM/*apoe-/-* peripheral

blood shows decreased adhesion function of P-selectin ligands on CD3ε+ cells, as well as a trend for decreased hyaluronic acid binding on CD11b+ cells, which suggests compromised extravasation. Furthermore, female or male B6.SM/*apoe-/-* mice transplanted with male B6.SM/*apoe-/-* bone marrow show a significant decrease in both the atherosclerotic lesion area and volume compared to female or male B6.SM/*apoe-/-* mice treated with male *apoe-/-* bone marrow. No significant differences in serum and hepatic levels of cholesterol and triglyceride are observed between bone marrow transplanted mice with B6.SM/*apoe-/-* or *apoe-/-* bone marrow. Therefore, we conclude that sialidase expression in hematopoietic cells is a major contributor to atherogenesis via modulating leukocytes homing and their overall inflammatory function without any effect on serum lipoprotein levels.

4.1 Introduction

Sialidases belong to a family of hydrolytic enzymes that cleave sialic acid, an acidic sugar, from glycoproteins, glycolipids and oligosaccharides (Reuter and Gabius, 1996). The enzyme exists in a multi-enzyme complex consisting of β-galactosidase, cathepsin A and sialidase (GCS complex) (van der Horst et al., 1989; van der Spoel et al., 1998; Bonten et al., 1995; Galjart et al., 1988, Galjart et al., 1991). The functional consequences of sialidases on the cell surface include cellular differentiation, tumorigenicity and metastatic behaviour of tumor cells (Collard et al., 1986; Pilatte et al., 1993; Varki A., 1997; Bonten et al., 1996). Sialylaton plays a role in the regulation of receptor-ligand interaction, dimerization, activation, as well as the activity of cell surface molecules, such as, CD44 and CD45 (Cuff et al., 2001; Katoh et al., 1995; Khan et al., 2004). Sialidase and sialic acid play key roles in inflammatory responses involving the activation of macrophages (Lambré et al., 1990; Liang et al., 2006; Stamatos et al., 2005), T cells (Pappu and Shrikant, 2004; Xu and Weiss, 2002) and neutrophils (Cross et al., 1991; Cross et al., 2003; Sakarya et al., 2004) indicating their effects in chronic inflammation such as atherosclerosis.

The mammalian sialidases differ in subcellular localization and substrate specificity. To date, genes encoding four sialidase isozymes have been cloned. These are lysosomal, *neu1* (Igdoura et al., 1998; Carrillo et al., 1997; Pshezhetsky et al., 1997), cytosolic, *neu2* (Monti et al., 1999; Ferrari et al., 1994), plasma membrane-bound, *neu3* (Miyagi et al., 1999; Wada et al., 1999) and mitochondrial forms, *neu4* (Comelli et al., 2003; Monti et al., 2004; Seyranptepe et al., 2004). Deficiency of the lysosomal

sialidase (Neu1) causes the autosomal recessive disease sialidosis, which is characterized by the accumulation of sialylated glycoconjugates such as ganglioside GM3 within the lysosomes in the central nervous system as well as in peripheral tissues (O'Brien and Warner, 1980). Sialidosis has two distinct phenotypes differentiated by the severity of symptoms and age of onset.Type I sialidosis has a late onset and is associated with less severe symptoms, while Type II sialidosis has an early onset and is accompanied by severe symptoms (O'Brien and Warner, 1980; Sasagasako et al., 1993; Bonten et al., 2000).

In 1979, it was first demonstrated that SM/J mouse has a partial deficiency of sialidase (Potier et al., 1979; Womack et al., 1981). SM/J mice have an impaired immune response resulting from the altered processing of sialic acids present on cell surface molecules of T cells. Also, SM/J mice can stimulate a Th1-mediated immune response (IFN-γ-producing T cells), but not a Th2-mediated immune response (IL-4-producing T cells) (Clark et al., 1981; Constant et al., 1997; Chen et al., 1997; Chen et al., 2000). It was reported that SM/J sialidase deficiency resulted from a missense mutation (Rottier et al., 1998). In addition, we identified a point mutation (-519G→A) in the mouse lysosomal sialidase promoter (*neu1*). This mutation creates a binding site for a transcriptional repressor, Nkx3.2, resulting in reduced gene expression (Champigny et al., 2009). We have isolated this mutation from the SM/J mice by backcrossing onto the C57Bl/6 strain, generating a hypomorphic sialidase congenic mouse strain namely, B6.SM, providing us with the opportunity to test whether this mutation in isolation can affect atherosclerosis susceptibility. We have shown recently that LPS-stimulated hypomorphic sialidase bone marrow derived macrophages show reduced secretion of

inflammatory cytokines, such as, TNFα, IL-1β, IL-6 and MCP-1. In addition, there have been many reports demonstrating the protective role of reduced cytokines during atherosclerosis in *apoe-/-* mice. For example, mice with a double knockout for ApoE and IL4 show decreased atherosclerosis compared to ApoE knockout mice (Davenport and Tipping, 2003). In addition, atherogenesis is decreased by inhibiting tumor necrosis factor-alpha (TNFα) in *apoe-/-* mice (Branen et al., 2004).

Leukocytes include monocytes, granulocytes, T cells and natural killer cells. Common markers that have been used for these cell typtes include CD11b for monocytes, Gr-1 for granulocytes, CD3ε for T cells, NK1.1 for natural killer cells. Leukocyte recruitment starts with capturing of leukocytes to the endothelium. Capture of leukocytes is followed by rolling of leukocytes along the endothelium. The capture and rolling depend on cell surface molecules such as P-selectin glycoprotein ligand-1 (PSGL-1) and CD44. This process involves interaction between PSGL-1 and CD44 with P-selectin and E-selectin on endothelial cells (McEver and Cummings, 1997). CD44 can also interact with hyaluronic acid (HA) (Ponta et al., 2003). Leukocyte capture and rolling is followed by leukocyte activation and adhesion which involves chemokine receptors and integrins such as CD11b. This is followed by transmigration which allows leukocytes to migrate through the endothelium into the intima layer of the artery (Springer T., 1994).

Sialylation plays a significant role in the regulation of the cell adhesion molecules, CD44 and CD45, which both play roles in the activation and/or recruitment of monocytes/macrophages after LPS stimulation (Gee et al., 2003; Katoh et al., 1999;

Xu and Weiss, 2002). Furthermore, CD44 promotes atherosclerosis by inducing activation of inflammatory cells (DeGrendele et al., 1996; Hodge-Dufour et al., 1997; McKee et al., 1996). Cuff and colleagues have also demonstrated that CD44-null mice had reduced atherosclerosis compared with wild-type mice (Cuff et al., 2001). Therefore, there is much interest in defining major determinants for the pro-inflammatory cascade leading to atherogenesis. One may hypothesize that the modulation of sialylation on leukocytes by neu1 sialidase may be one of these determinants.

Our recent work has shown that hypomorphic sialidase expression significantly reduces atherosclerosis in ApoE knockout mice. Furthermore, administration of a sialidase inhibitor, 2,3-dehydro-2-deoxy-N-acetylneuraminic acid (DANA), significantly reduced atherosclerotic lesion area and volume in *apoe-/-* mice compared to controls. Thus, it became imperative to determine whether hematopoietic cells with reduced or inhibited sialidase underlie this atheroprotective effect. In this report, we demonstrate that hypomorphic sialidase expression in hematopoietic cells may reduce extravasation leading to reduced atherosclerosis with no effect on serum and hepatic levels of total cholesterol and triglyceride. These results demonstrate that reduced neu1 sialidase activity in blood cells can be protective from atherosclerosis.

4.2 Methods

4.2.1 Generation of the donor and recipient mice

B6.SM mice were generated by crossing SM/J mice with C57Bl/6 mouse strain six times. B6.SM/*apoe-/-* mice were generated by crossing the B6.SM mice with *apoe-/-* mice on a C57Bl/6 background. B6.SM/*apoe-/-* and *apoe-/-* mice were used for the bone marrow transplantation studies in which B6.SM/*apoe-/-* mice were used as the recipients and *apoe-/-* or B6.SM/*apoe-/-* mice were used as the donors. Mice were housed in microisolator cages, and had unlimited access to food and water. All experimental procedures involving animals were approved by the McMaster Animal Research Ethics Board.

4.2.2 Peripheral blood immunophenotyping

Peripheral blood was collected from male *apoe-/-* and B6.SM/*apoe-/-* mice by terminal cardiac puncture with a heparinized needle. Erythrocytes were lysed using ACK lysis buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, 100 mM Na_2EDTA), leukocytes were counted and cells were pre-incubated with rat anti-mouse CD16/CD32 (10 μg/ml, BD Pharmingen, Mississauga, Canada) and immunostained for cell surface markers using 1 μg of each antibody for 10^6 cells in FACS buffer (PBS, 0.2% BSA): hamster anti-mouse CD3ε-APC-Cy7 (clone 145-2C11), rat anti-mouse CD11b-APC (clone M1/70), rat anti-mouse CD19-V450 (clone 1D3), rat anti-mouse Gr-1-FITC (clone RB6-8C5), and mouse anti-mouse NK1.1-PE-Cy7 (clone PK136). Separate reactions were used to assess hamster anti-mouse CD3ε-APC-Cy7 (clone 145-2C11), rat anti-mouse CD4-PE (clone GK1.5) and rat anti-mouse CD8a-Pacific Blue™ (clone 53-6.7).

Samples were washed with FACS buffer, fixed with BD Cytofix^{TM} Fixation Buffer (BD Biosciences, Mississauga, Canada) and washed again before they were run on a LSR II flow cytometer (Beckman Coulter).

4.2.3 Selectin and hyaluronic acid binding assay

Peripheral blood was isolated by cardiac puncture and red blood cells were lysed using ACK lysis buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, 100 mM Na_2EDTA). Cells were incubated in Hank's balanced salt solution containing calcium and magnesium at 37^{o}C for 1h with 4 μg P-selectin-human IgG chimera, 4 μg E-selectin-human IgG chimera or 200 μg hyaluronic acid conjugated to fluorescein. To detect bound selectin chimera protein, cells were incubated on ice with anti-human-IgG conjugated to Alexa Fluor 488. Cells were incubated on ice with hamster anti-mouse CD3ε-Pacific Blue (clone 145-2C11) and rat anti-mouse CD11b-APC (clone M1/70) to detect T cells and monocyte/granulocyte populations, respectively. Samples were fixed with BD Cytofix^{TM} Fixation Buffer (BD Biosciences, Mississauga, Canada) and run on a LSR II flow cytometer.

4.2.4 Serum cytokine measurement

Male *apoe-/-* and B6.SM/*apoe-/-* mice were injected with 200 μg lipopolysaccharide (*E. coli* 026:B6 LPS, Sigma-Aldrich) or 25 μg anti-CD3ε antibody (clone 145-2C11, BD Pharmingen, Mississauga, Canada) in sterile saline. Blood was harvested at 2h, 4h and 8h, in serum collection tubes, spun at 10 000 x g for 5 min. and stored at -80^{o}C until analysis. For time 0h, blood was collected from untreated animals and used to determine baseline levels. Serum IL-2, IL-4, IL-10 and IFNγ cytokine levels were

analyzed according to the mouse Th1/Th2 Ready-SET-Go! ELISA Set (eBioscience, California, USA).

4.2.5 Treatment of mice

The transplanted mice were given septra antibiotic water and jello for 4 weeks after transplantation. Mice wee fed with western diet 4 weeks post transplantation for 2 months. After 3 months post transplantation, the mice were sacrificed for atherosclerosis and lipoprotein analyses.

4.2.6 Bone marrow transplantation

One week before irradiation, the recipient mice were given septra antibiotic water and jello. On the day of transplantation, the donor mice were euthanized by cervical dislocation. Femurs and humurus were harvested from the donor mice. Femurs and humurus were then placed in a 35mm tissue culture dish containing 1ml of the following filtered medium: Iscove minimum Dulbecco medium (IMDM) with penicillin and streptomycin and fungizone. The ends of the femurs and humurus were cut. The femurs and humurus were flushed with filtered IMDM with penicillin and streptomycin and fungizone three times using 26G 1/2 and 26G 3/4 needles. The solution was put through a sterile 40-um nylon Cell Strainer (Falcon 352340) and collected in a 1.5ml eppendorf tube. The solution was centrifuged at 3300 rpm for 10 minutes at 4C. The upper phase was disposed and the cells were resuspended in 300ul of filtered IMDM with penicillin and streptomycin and fungizone. Cell suspension (5ul) was added to ACK buffer (45ul). Number of cells was determined by mixing the cell suspension (5ul) and ACK buffer (45ul) and the mixture (15ul) was then counted

using a hemocytometer. The recipient mice were irradiated with 11Gy.The recipient mice were then retro orbital-injected with 295 ul of the cell suspension, equivalent to $1x10^6$ cells. Transplantation efficacy was assessed by genotyping blood cells using PCR.

4.2.7 Blood and tissue collection

Under anesthesia with ketamine/xylazine, mouse was flushed with phosphate-buffered saline (PBS) through the left ventricle of the heart. Heart was then harvested and was placed in Krebs Henseleit Solution for 30 min. After 30 min, the heart was fixed in 4% formaldehyde. Under anesthesia and before the perfusion, blood was obtained by cardiac puncture. Serum was prepared by centrifugation of blood for 5 min at 15,000 rpm using serum collection tubes. The liver was removed and was immediately frozen in liquid nitrogen.

4.2.8 Serum lipids analysis

Serum total cholesterol levels were measured by Infinity reagent as described by manufacturer (Thermo) and the absorbance of the reaction product was read at 500nm. Serum free cholesterol levels were measured using free cholesterol E regent (Wako Diagnostics) and was read at 600nm. Serum triglyceride levels were measured with L-Type Triglyceride H Reagents 1 and 2 (Wako Diagnostics) and were read at 600nm.

4.2.9 Hepatic and blood cell lipid analyses

Hepatic total cholesterol, free cholesterol, cholesterol esters and triglyceride levels were determined after lipid extraction using an enzymatic assay. Briefly, liver was

homogenized in 1ml of TNES (10 mM Tris, pH 7.5, 400mM NaCl, 100mM EDTA, 0.6% SDS). Folch mixture (6ml of chloroform/methanol, 2:1) was added to 300ul of liver homogenates. After vortexing, 1.2ml of distilled water was added and the tube was vortexed. The extraction mixture was left at 4ºC for 2 hours. The tubes were centrifuged at low speed to facilitate phase separation. The upper phase (methanol/water phase) was discarded and the lower phase was saved for the hepatic lipid enzymatic assay. The lower phase (chloroform phase) was dried completely. The dry chloroform phase was resuspended in 60ul of isopropanol. Hepatic total cholesterol, free cholesterol, cholesterol esters and triglyceride concentrations were measured using an enzymatic method as described in serum lipids analysis. Blood cell lipid analysis was performed the same as the hepatic lipid analysis except that the lipids concentrations were normalized to protein concentrations after performing protein assays.

4.2.10 Analysis of lesions in the aortic sinus

Hearts were processed as described in blood and tissues collection. After 4% formaldehyde fixation, hearts were placed in 30% sucrose in PBS overnight at 4˚C. Hearts were embedded in cryomatrix (Shandon Thermo) and were frozen on dry ice. Serial 10 um sections were collected using a Shandon Cryostat. Sections were stained with Oil Red O for neutral lipids and stained with hematoxylin for cell nuclei. Atherosclerosis was quantified as the total cross sectional area of atherosclerotic plaque in each given section using a Zeiss Axiovision light microscope software. Lesion volume was determined by measuring the cross sectional area of lesions in

sections spaced 100 μm apart as the average lesion area for the 100μm stretch of the aortic sinus, multiplying by 100 μm, and then adding these values together.

4.2.11 Statistical analysis

One-way analysis of variance (one-way ANOVA) followed by Tukey's Post Hoc Multiple Comparison Test, and additional statistical analyses were conducted using Prism 5 (version 5.04, GraphPad). Mean±SE were shown with error bars. Comparison of data was considered statistically significant different only if $P < 0.05$, determined by Student's *t* test.

4.3 Results

4.3.1 B6.SM/*apoe*-/- mice have increased CD3ε$^+$ and decreased CD11b^{lo}populations.

Peripheral blood leukocytes from *apoe*-/- and B6.SM/*apoe*-/- mice were analyzed by flow cytometry (Fig. 4.3.1). The peripheral blood CD3ε$^+$ cell population was significantly increased in B6.SM/*apoe*-/- mice (39±5%) compared to *apoe*-/- mice (15±2%) (p=0.0006, Fig. 4.3.1 A). Within the CD3ε$^+$ subset, the CD3ε$^+$CD4$^+$ population was significantly increased in the B6.SM/*apoe*-/- mice (39±6%) compared to *apoe*-/- mice (22±1%) (p=0.014, Fig. 4.3.1 B), but the CD3ε$^+$CD8$^+$ subset was not different between B6.SM/*apoe*-/- (38±5%) and mice (35±6%).

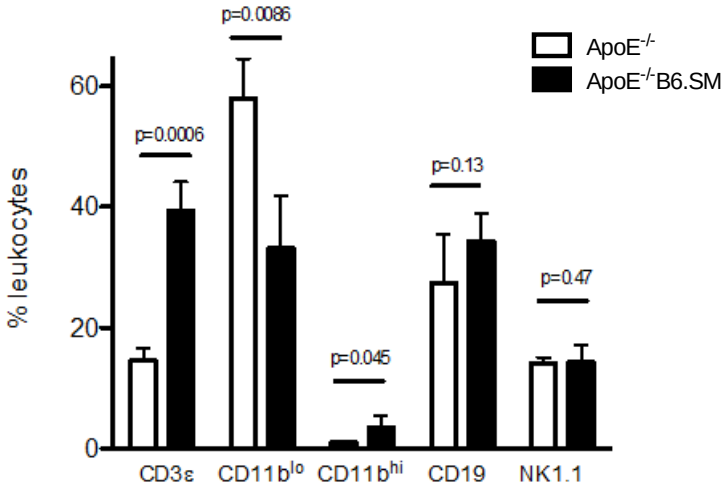
Two CD11b$^+$ subsets were apparent in both *apoe*-/-and B6.SM/*apoe*-/-mice (Fig. 4.3.1 A), including CD11b^{lo} and CD11b^{hi} subsets. CD11b^{lo} was significantly decreased in B6.SM/*apoe*-/- (33±9%) compared to *apoe*-/- mice (58±7%) (p=0.0086), while the CD11b^{hi} subset was significantly increased in B6.SM/*apoe*-/- mice (3.5±2%) compared to *apoe*-/- controls (1±0.1%) (p=0.045). Populations of CD11b^{lo}Gr-1^{lo} and CD11b^{lo}Gr-1^{hi} were not significantly different between B6.SM/*apoe*-/- and *apoe*-/- mice (Fig. 4.3.1 C). In CD11b^{hi} cells, no difference was observed in CD11b^{hi}Gr-1^{lo} populations in B6.SM/*apoe*-/- mice compared to *apoe*-/- mice, but a significant decrease in the CD11b^{hi}Gr-1^{hi} population was observed in B6.SM/*apoe*-/- mice (1.0±0.6%) compared to *apoe*-/- mice (7±3%) (p=0.014, Fig. 4.3.1 D). The proportion of CD19$^+$ cells was not significantly different in B6.SM/*apoe*-/- (34±4%) peripheral blood compared to *apoe*-/- (27±8%) (Fig. 4.3.1 A). As well, there is no difference in

NK1.1^{+} cell populations in B6.SM/*apoe-/-* (14±3%) peripheral blood compared to *apoe-/-* (14±1%) (Fig. 4.3.1 A).

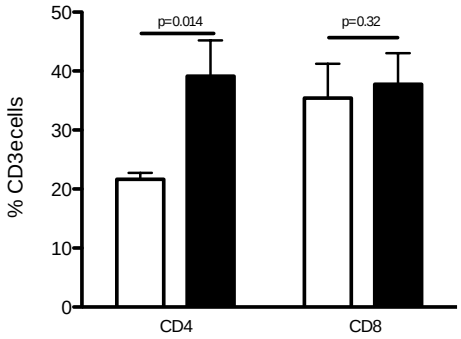
Fig. 4.3.1 Peripheral blood immmunophenotype comparison between *apoe-/-* and hypomorphic sialidase B6.SM/*apoe-/-*. Samples were treated with ACK lysis buffer, blocked with CD16/CD32 antibody, stained with directly conjugated antibodies for cell surface markers, fixed and run on a LSR II flow cytometer. (A) Proportions of $CD3\varepsilon^{+}$ (T cell marker, epsilon chain of the T cell receptor associated complex), $CD11b^{lo}$ and $CD11b^{hi}$ (monocyte/granulocyte marker αM integrin), $CD19^{+}$ (B cell differentiation antigen) and $NK1.1^{+}$ (NK and NK T cell antigen) cells in peripheral blood. (B) CD4+ (helper T cells) and $CD8^{+}$ (cytotoxic T cells) subsets in the $CD3\varepsilon^{+}$ gate are presented. (C) Proportions of $Gr\text{-}1^{lo}$ and $Gr\text{-}1^{hi}$ cells in the $CD11b^{lo}$ gate. (D) Proportions of $Gr\text{-}1^{lo}$ and $Gr\text{-}1^{hi}$ cells in the $CD11b^{hi}$ gate. Mean proportion ± standard deviation is presented in each graph.

Fig. 4.3.1

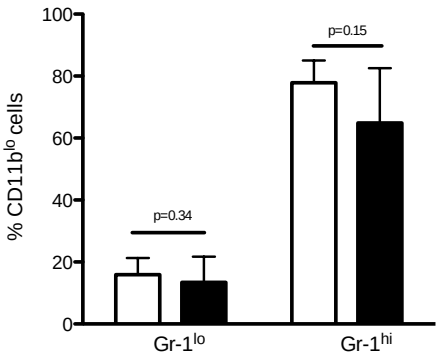
A


p=0.0086
ApoE-/-
ApoE-/-B6.SM
60
p=0.0006
p=0.13
40
% leukocytes
20
p=0.47
p=0.045
0
CD3ε
CD11blo
CD11bhi
CD19
NK1.1

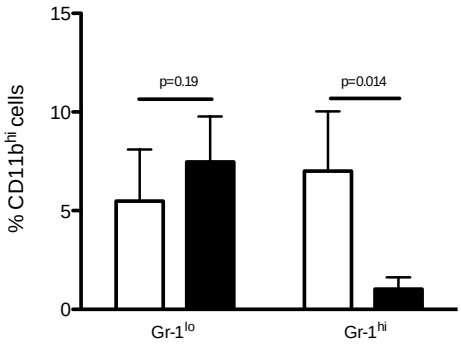
B


50
p=0.014
p=0.32
40
30
% CD3ecells
20
10
0
CD4
CD8

C


100
p=0.15
80
60
% CD11blo cells
40
p=0.34
20
0
Gr-1lo
Gr-1hi

D


15
p=0.19
p=0.014
10
% CD11bhi cells
5
0
Gr-1lo
Gr-1hi

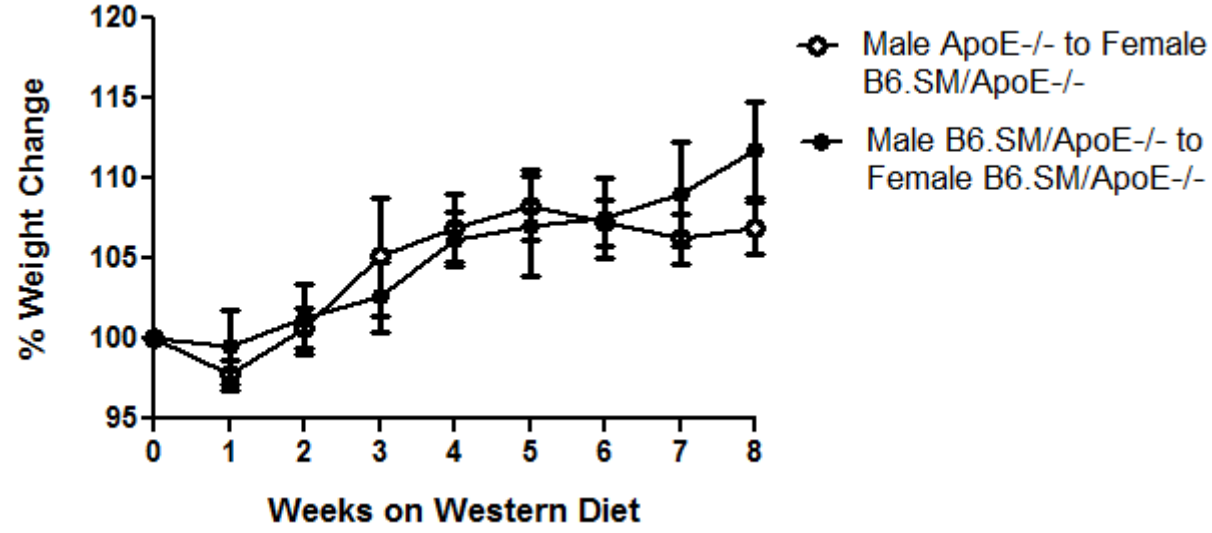
4.3.2 The effects of hypomorphic sialidase expression in hematopoietic cells on serum and hepatic lipids levels in B6.SM/*apoe*-/- mice.

In order to test the effects of sialidase expression in blood cells on atherosclerosis *in vivo*, we performed bone marrow transplantation experiments. Bone marrow (BM) from the femurs of *apoe*-/- or B6.SM/*apoe*-/- mice was harvested and then injected intravenously into 2-month-old, lethally irradiated B6.SM/*apoe*-/- mice. Mice were fed western diet 30 days post transplantation for 60 days. Weights of the mice were measured weekly (Fig. 4.3.2). Weights of the male B6.SM/*apoe*-/- mice transplanted with B6.SM/*apoe*-/- bone marrow are significantly higher than those of B6.SM/*apoe*-/-mice transplanted with *apoe*-/- bone marrow, after 5 weeks of western diet (Fig. 4.3.2 B). However, there is no difference in weights of the female B6.SM/*apoe*-/- mice transplanted with B6.SM/*apoe*-/-bone marrow and the female B6.SM/*apoe*-/- mice transplanted with *apoe*-/- bone marrow (Fig. 4.3.2 A). After 3 months post transplantation, the mice were sacrificed for atherosclerosis and lipid analyses. No significant difference in hepatic or serum total cholesterol, triglyceride, cholesteryl esters and free cholesterol concentrations were found in B6.SM/*apoe*-/- female transplanted with B6.SM/*apoe*-/- or *apoe*-/- bone marrow (Table 4.3.1). Similar results were observed for the male recipients. In addition, mice transplanted with either bone marrow from normal sialidase *apoe*-/- or from hypomorphic sialidase *apoe*-/- mice show no significant differences in the levels of cholesterol and triglyceride within blood cells (Fig. 4.3.3 A-D).

Fig. 4.3.2 Weights of male and female B6.SM/*apoe-/-* mice transplanted with B6.SM/*apoe-/-* or *apoe-/-* bone marrow after feeding with western diet. There is no significant difference in weights (%) between (A) female B6.SM/*apoe-/-* mice transplanted with B6.SM/*apoe-/-* (n=10) and *apoe-/-* (n=10) bone marrow. There is an increase in weights (%) for the (B) male B6.SM/*apoe-/-* mice transplanted with B6.SM/*apoe-/-* (n=9) bone marrow compared to those transplanted with *apoe-/-(*n=7) bone marrow. Each dot represents mean ±SE. * denotes $P<0.05$.

Fig. 4.3.2

A


% Weight Change
120
115
110
105
100
95
0
1
2
3
4
5
6
7
8
Weeks on Western Diet
Male ApoE-/- to Female B6.SM/ApoE-/-
Male B6.SM/ApoE-/- to Female B6.SM/ApoE-/-

B

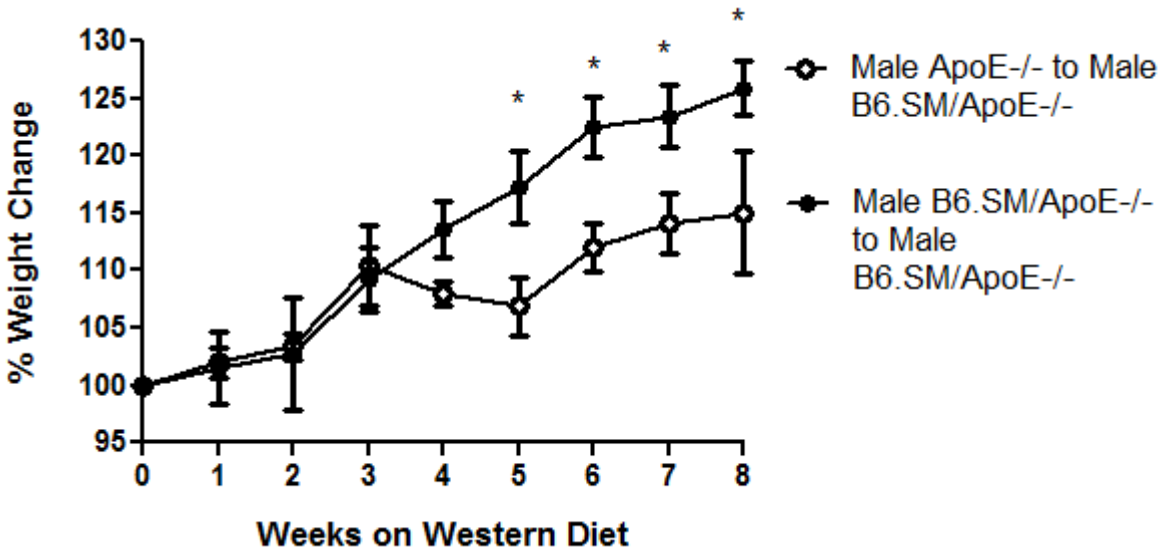

% Weight Change
130
125
120
115
110
105
100
95
0
1
2
3
4
5
6
7
8
*
*
*
*
Weeks on Western Diet
Male ApoE-/- to Male B6.SM/ApoE-/-
Male B6.SM/ApoE-/- to Male B6.SM/ApoE-/-

Table 4.3.1 Serum and hepatic lipids levels in B6.SM/*apoe-/-* mice transplanted with B6.SM/*apoe-/-* or *apoe-/-* bone marrow. There is no significant difference in hepatic total cholesterol, free cholesterol, cholesteryl esters, and triglyceride concentrations in bone marrow transplanted mice with (A) female B6.SM/*apoe-/-* mice transplanted with B6.SM/*apoe-/-* (n=5) or *apoe-/-* bone marrow and (B) male B6.SM/*apoe-/-* mice transplanted with B6.SM/*apoe-/-* or *apoe-/-* bone marrow (n=5) . In addition, there is no significant difference in serum total cholesterol, free cholesterol, cholesteryl esters, and triglyceride concentrations in bone marrow transplanted mice with female B6.SM/*apoe-/-* mice transplanted with B6.SM/*apoe-/-* (n=7) or *apoe-/-* bone marrow and male B6.SM/*apoe-/-* mice transplanted with B6.SM/*apoe-/-* or *apoe-/-*bone marrow (n=7).

Table 4.3.1

A

	Hepatic total cholesterol (mg/g liver)	Serum total cholesterol (mM)	Hepatic free cholesterol (mg/g liver)	Serum free cholesterol (mM)	Hepatic cholesteryl esters (mg/g liver)	Serum cholesteryl esters (mM)	Hepatic triglyceride (mg/g liver)	Serum triglyceride (mM)
B6.SM/ApoE-/- -> Female B6.SM.ApoE-/-	4.30±0.40	32.85±1.41	2.29±0.53	13.89±0.72	2.02±0.41	18.95±1.24	15.06±1.55	2.21±0.06
ApoE-/- -> Female B6.SM.ApoE-/-	3.38±0.48	30.18±2.15	2.15±0.40	11.53±0.90	1.23±0.42	18.65±1.66	16.00±1.37	2.89±0.60

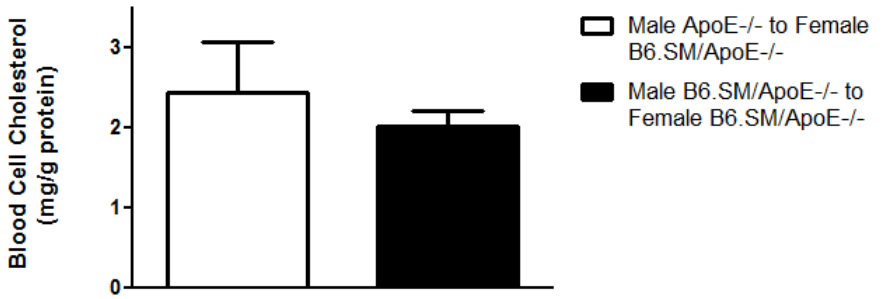
B

	Hepatic total cholesterol (mg/g liver)	Serum total cholesterol (mM)	Hepatic free cholesterol (mg/g liver)	Serum free cholesterol (mM)	Hepatic cholesteryl esters (mg/g liver)	Serum cholesteryl esters (mM)	Hepatic triglyceride (mg/g liver)	Serum triglyceride (mM)
B6.SM/ApoE-/- -> Male B6.SM.ApoE-/-	3.13±0.18	31.59±0.84	1.93±0.23	10.78±0.63	1.20±0.35	20.81±0.81	13.72±1.22	2.82±0.34
ApoE-/- -> Male B6.SM.ApoE-/-	3.43±0.59	31.31±0.64	1.54±0.24	12.66±1.48	1.89±0.45	18.65±0.98	12.94±2.46	2.87±0.33

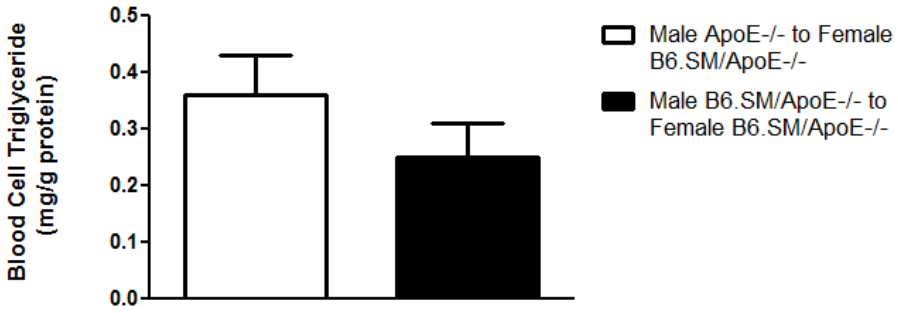
Fig. 4.3.3 Blood cells lipids levels in B6.SM/*apoe-/-* mice transplanted with apoe-/- or B6.SM/*apoe-/-* bone marrow. Blood cell cholesterol and triglyceride levels in bone marrow transplanted mice with (A and B) female B6.SM/ApoE mice transplanted with B6.SM/ apoe-/-(n=6) or *apoe-/-* bone marrow (n=3) and with (C and D) male B6.SM/*apoe-/-* mice transplanted with B6.SM/*apoe-/-* (n=6) or *apoe-/-* bone marrow (n=8). There is no significant difference in blood cell cholesterol and triglyceride levels in B6.SM/*apoe-/-* mice transplanted with B6.SM/*apoe-/-* bone marrow and those transplanted with *apoe-/-* bone marrow. Peripheral blood cell cholesterol and triglyceride were determined using an enzymatic method after extracting blood cell lipids by folch extraction. The mean values ± standard error are shown.

Fig. 4.3.3

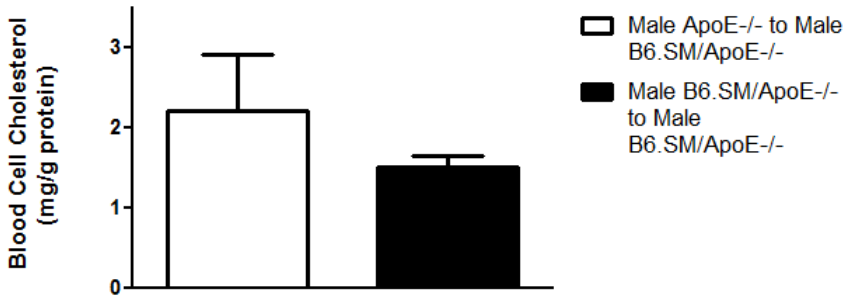
A


Blood Cell Cholesterol (mg/g protein)
0
1
2
3
Male ApoE-/- to Female B6.SM/ApoE-/-
Male B6.SM/ApoE-/- to Female B6.SM/ApoE-/-

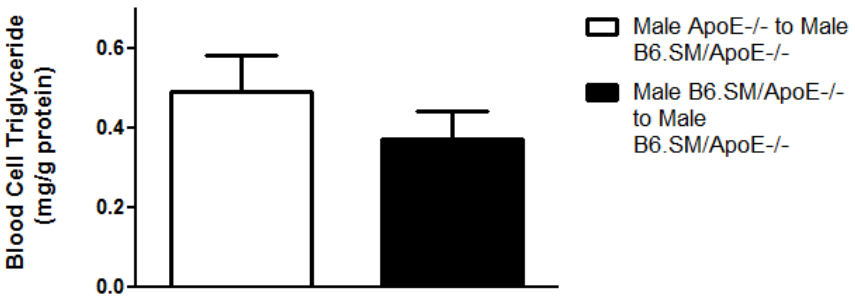
B


Blood Cell Triglyceride (mg/g protein)
0.0
0.1
0.2
0.3
0.4
0.5
Male ApoE-/- to Female B6.SM/ApoE-/-
Male B6.SM/ApoE-/- to Female B6.SM/ApoE-/-

C


Blood Cell Cholesterol (mg/g protein)
0
1
2
3
Male ApoE-/- to Male B6.SM/ApoE-/-
Male B6.SM/ApoE-/- to Male B6.SM/ApoE-/-

D

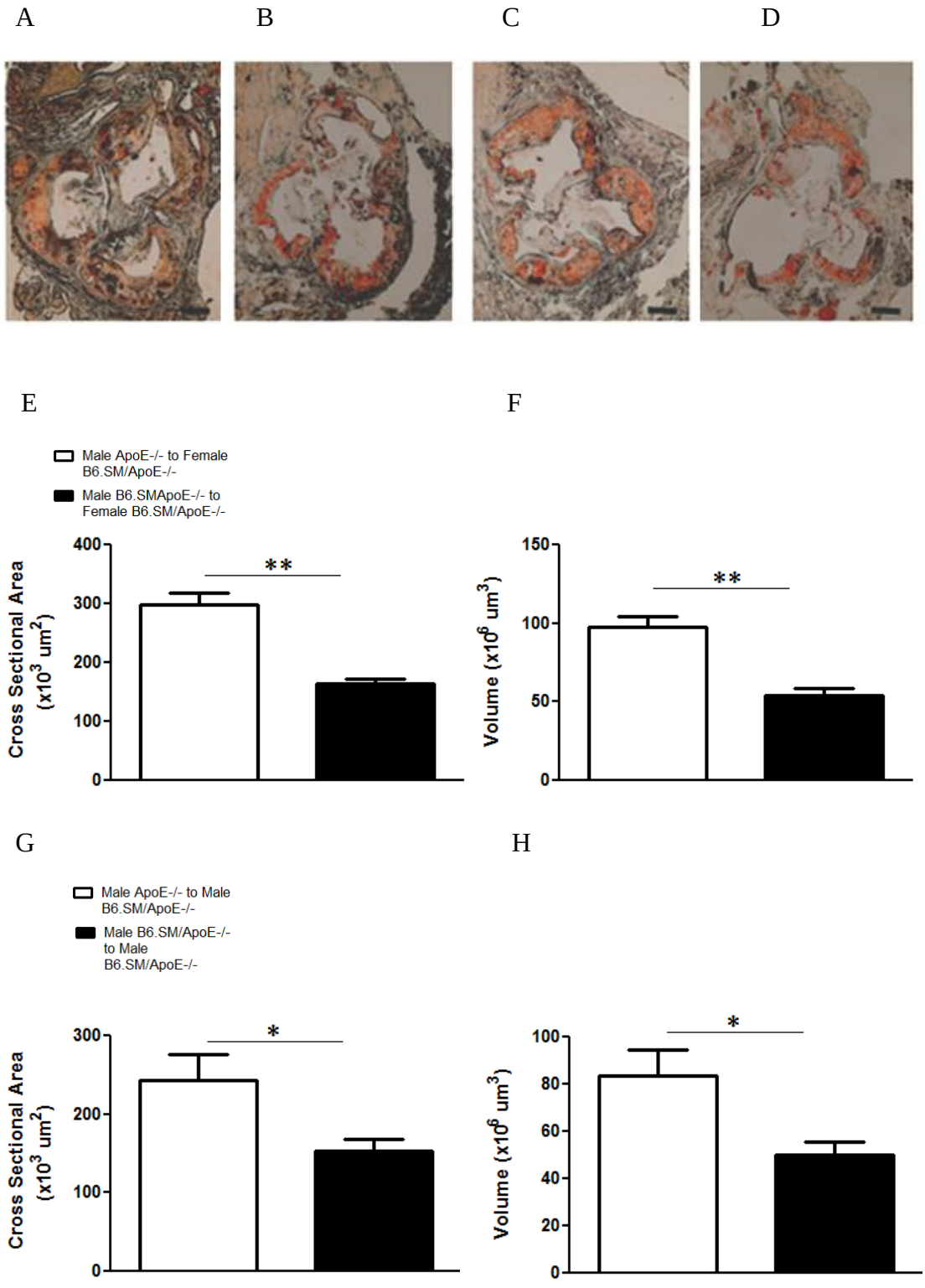

Blood Cell Triglyceride (mg/g protein)
0.0
0.2
0.4
0.6
Male ApoE-/- to Male B6.SM/ApoE-/-
Male B6.SM/ApoE-/- to Male B6.SM/ApoE-/-

4.3.3 Hypomorphic sialidase expression in hematopoietic cells reduces atherosclerosis.

Transplantation of female B6.SM/*apoe-/-* mice with bone marrow from male B6.SM/*apoe-/-* mice resulted in significantly reduced area and volume of atherosclerotic plaques when compared to similar mice transplanted with bone marrow from male *apoe-/-* mice (Fig. 4.3.4 A and B). Transplantation of male B6.SM/*apoe-/-* bone marrow into male B6.SM/*apoe-/-*mice also showed decreased atherosclerosis compared to controls (Fig. 4.3.4 C and D). Thus, hypomorphic sialidase expression in blood cells reduces atherogenesis.

Fig. 4.3.4 Atherosclerotic lesion area of B6.SM/*apoe-/-* mice transplanted with B6.SM/*apoe-/-* or *apoe-/-* bone marrow. Oil Red O stained sections (Quantification data are shown in E to H) of aortic sinuses of female B6.SM/*apoe-/-* mice transplanted with (A) *apoe-/-* bone marrow and (B) B6.SM/*apoe-/-* bone marrow, and male B6.SM/*apoe-/-* mice transplanted with (C) *apoe-/-* bone marrow and (D) B6.SM/*apoe-/-* bone marrow. (E and F) Transplantation of male B6.SM/*apoe-/-* bone marrow into female B6.SM/*apoe-/-*mice (n=10) shows decreased atherosclerosis compared to female B6.SM/*apoe-/-* mice transplanted with male *apoe-/-* bone marrow (n=10) (P=0.016 and P=0.015 for lesion area and volume respectively). (G and H) Transplantation of male B6.SM/*apoe-/-* bone marrow into male B6.SM/*apoe-/-* mice (n=7) shows decreased atherosclerosis compared to male B6.SM/*apoe-/-* mice transplanted with male *apoe-/-* bone marrow (n=9) (P=0.000010 and P=0.000055 for lesion area and volume respectively). Each bar represents mean±SE. * denotes $P<0.05$; ** denotes $P<0.001$. Scale bar = 200μm.

Fig. 4.3.4


A
B
C
D
E
F
Male ApoE-/- to Female B6.SM/ApoE-/-
Male B6.SMApoE-/- to Female B6.SM/ApoE-/-
Cross Sectional Area (x10^3 um^2)
400
300
200
100
0
**
Volume (x10^6 um^3)
150
100
50
0
**
G
H
Male ApoE-/- to Male B6.SM/ApoE-/-
Male B6.SM/ApoE-/- to Male B6.SM/ApoE-/-
Cross Sectional Area (x10^3 um^2)
300
200
100
0
*
Volume (x10^6 um^3)
100
80
60
40
20
0
*

4.3.4 LPS-injected B6.SM/*apoe-/-* mice secrete less IL-10 and show delayed IFNγ secretion.

Cytokines were measured in blood serum in untreated animals and at 2h, 4h, and 8h after intraperitoneal LPS injection (Fig. 4.3.5 A). IL-2 and IL-4 were not induced by LPS within 8h of treatment (Fig. 4.3.5 A). IL-10 was produced within 2h of LPS injection, but B6.SM/*apoe-/-* mice produced significantly less IL-10 (3778±824 pg/ml IL-10) compared to *apoe-/-* mice (10126±2015 pg/ml IL-10) at 2h ($p<0.05$). Within 2-4h post LPS injection, IL-10 secretion was reduced but B6.SM/*apoe-/-* mice continued to secrete significantly less IL-10 (1515±500 pg/ml IL-10) compared to *apoe-/-*mice (5340±3268 pg/ml IL-10) ($p<0.05$). Within 4-8h post LPS injection, IL-10 serum levels dropped even further but B6.SM/*apoe-/-* mice produced significantly less than *apoe-/-* mice (358±192 pg/ml and 1000±177 pg/ml IL-10, respectively, $p<0.05$). IFNγ secretion was significantly higher in blood serum at 4h in B6.SM/*apoe-/-* (1241±680 pg/ml IFNγ) compared to *apoe-/-* (not detectable), although similar levels of IFNγ were observed at 8h in B6.SM/*apoe-/-* mice (1028±657 pg/ml IFNγ) and *apoe-/-* mice (1300±471 pg/ml IFNγ).

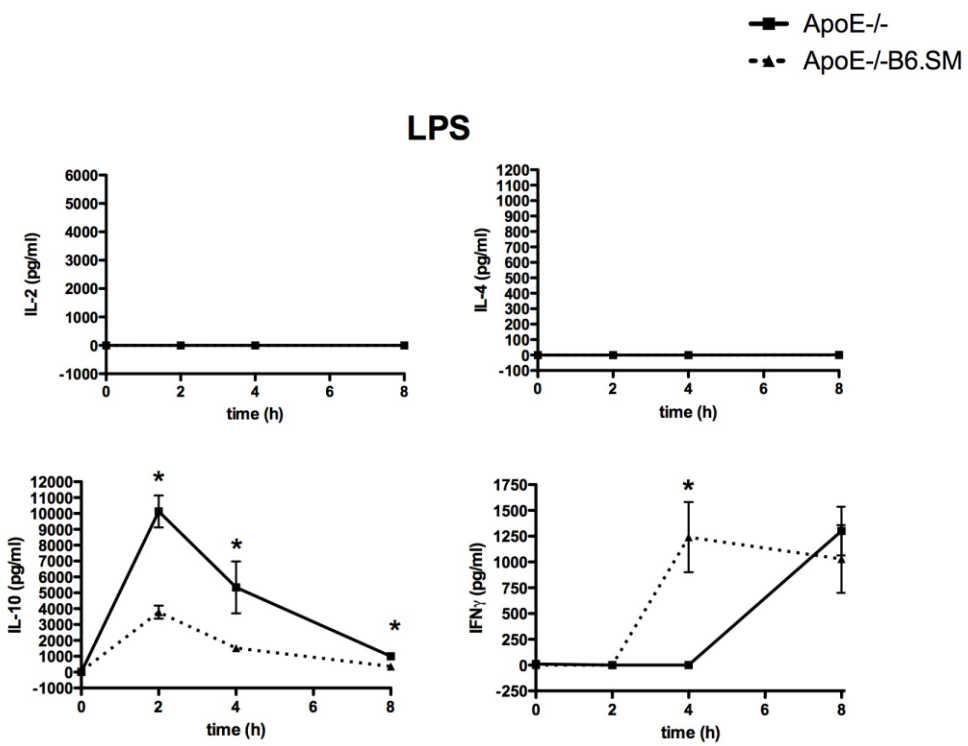
4.3.5 Anti-CD3ε injected B6.SM/*apoe*-/- mice secrete less IL-4 and IFNγ.

Soluble anti-CD3ε antibody (145-2C11) was injected IP to induce signal transduction and cytokine secretion from T cell receptor expressing cells (Fig. 4.3.5 B). IL-2 secretion was induced within 2h of anti-CD3ε injection, but was not different between B6.SM/*apoe*-/- (4727±1606 pg/ml) and *apoe*-/-mice (4159±953 pg/ml). At 4h and 8h, IL-2 secretion was reduced but similar quantities were observed in both B6.SM/*apoe*-/- (1066±205 pg/ml at 4h, 128±76 pg/ml at 8h) and *apoe*-/-mice (1459±405 pg/ml at 4h, 94±26 pg/ml at 8h). IL-4 secretion was induced within 2h of anti-CD3ε injection, but B6.SM/*apoe*-/- mice produced significantly less IL-4 (493±184 pg/ml) than *apoe*-/-mice (839±386 pg/ml). By 4h, IL-4 production was not detectable. Within 2h post anti-CD3ε injection, IL-10 was secreted into the serum of B6.SM/*apoe*-/-(4517±1028 pg/ml) and *apoe*-/-mice (3375±2667 pg/ml), but hypomorphic sialidase expression did not significantly affect secretion. At 4h, IL-10 secretion was reduced, and B6.SM/*apoe*-/- (1020±722 pg/ml) and *apoe*-/-mice (1168±461 pg/ml) produced similar amounts. By 8h, IL-10 secretion dropped further in both B6.SM/*apoe*-/- (470±196 pg/ml) and *apoe*-/- mice (885±276 pg/ml), but was not different with hypomorphic sialidase expression. IFNγ was not detectable until 4h post anti-CD3ε injection, and was only found in *apoe*-/- serum at this time point (801±764 pg/ml, $p<0.05$). By 8h post anti-CD3ε injection, IFNγ secretion was detectable but significantly less in B6.SM/*apoe*-/- (280±167 pg/ml) than *apoe*-/- mice (1066±484 pg/ml).

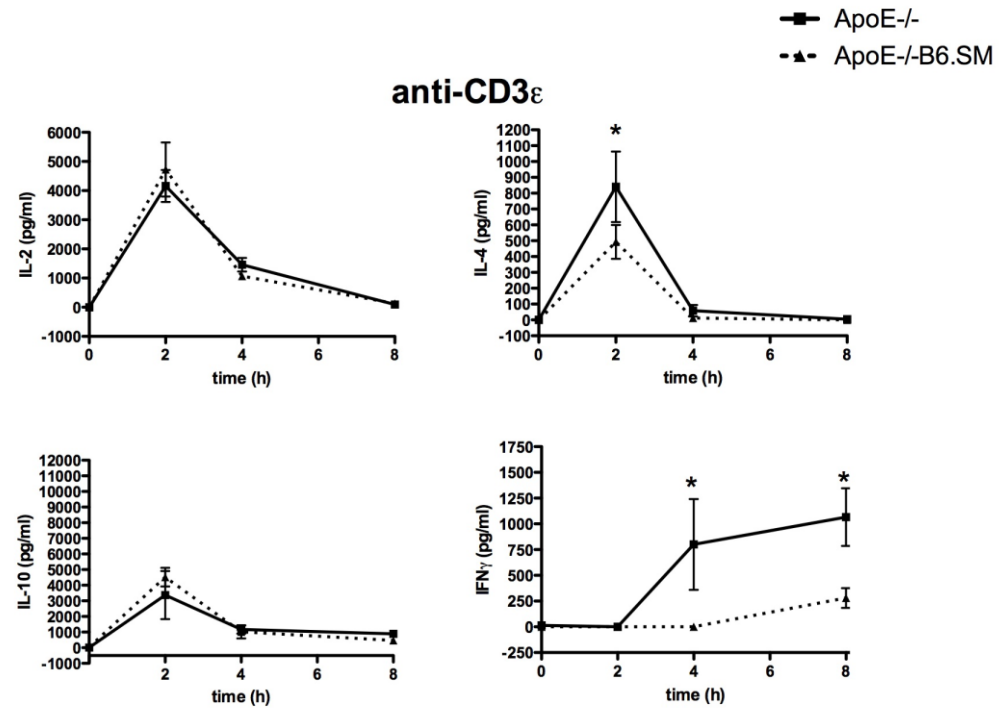
Fig. 4.3.5 Blood serum cytokine levels in male *apoe*-/- and B6.SM/*apoe*-/- in response to stimulation by (A) LPS (n=4 each time point) or (B) anti-CD3ε antibody (n=3 each time point). Untreated animals were used to determine baseline cytokine levels at time 0. The Wilcoxon Rank Sum test was used to determine differences in cytokine concentration at each time point between *apoe*-/- and B6.SM/*apoe*-/-. Asterisks (*) indicate $p<0.05$.

Fig. 4.3.5

A


ApoE-/-
ApoE-/-B6.SM
LPS
IL-2 (pg/ml)
IL-4 (pg/ml)
IL-10 (pg/ml)
IFNγ (pg/ml)
time (h)

B


ApoE-/-
ApoE-/-B6.SM
anti-CD3ε
IL-2 (pg/ml)
IL-4 (pg/ml)
IL-10 (pg/ml)
IFNγ (pg/ml)
time (h)

4.3.6 B6.SM/*apoe-/-* hematopoietic cells show impaired cell adhesion function *in vitro*.

To assess the function of cell adhesion molecules on the cell surface of $CD11b^+$ and $CD3\varepsilon^+$ peripheral blood cells, we incubated peripheral blood with P-selectin-human IgG, E-selectin-human-IgG or fluoroscein-labeled hyaluronic acid. There was no significant difference (p=0.70) in the geometric mean fluorescence intensity of P-selectin on $CD11b^+$ cells isolated from *apoe-/-* (100.0±10.2% of average *apoe-/-* geometric MFI) and B6.SM/*apoe-/-* mice (101.7±7.6% of average *apoe-/-* geometric MFI), (Fig. 4.3.6). On the other hand, $CD3\varepsilon^+$ cells from B6.SM/*apoe-/-* mice bound significantly less P-selectin fusion protein (80.7±22.8% of average *apoe-/-* geometric MFI) compared to *apoe-/-* $CD3\varepsilon^+$ cells (100.0±9.8% of average *apoe-/-* geometric MFI)(p=0.043). No difference was observed in E-selectin fusion protein binding to the cell surface of *apoe-/-* and B6.SM/*apoe-/-* $CD11b^+$ cells (100.0±19.9% versus 81.1±6.1% of average *apoe-/-* geometric MFI, respectively, p=0.19) or $CD3\varepsilon^+$ (100.0±13.4% versus 88.1±4.1% of average *apoe-/-* geometric MFI, respectively, p=0.21) (Fig. 4.3.7). Finally, there was a significant decrease (p=0.015) in hyaluronic acid binding to the cell surface of $CD11b^+$ cells from hypomorphic sialidase B6.SM/*apoe-/-* mice (60.6±8.1% of average *apoe-/-* geometric MFI) compared to *apoe-/-* (100.0±35.9% of average *apoe-/-* geometric MFI) (Fig. 4.3.8). $CD3\varepsilon^+$ cells bound hyaluronic acid at two distinct levels in both *apoe-/-* and B6.SM/*apoe-/-*, HA^{lo} and HA^{hi}, and showed a significant reduction of hyaluronic acid binding in B6.SM/*apoe-/-* mice (81.8±12.6% of average *apoe-/-* geometric MFI) versus *apoe-/-* *mice* (100.0±9.7% of average *apoe-/-* geometric MFI) at the HA^{lo}level (P=0.011) and 85.9±7.8% of average *apoe-/-* geometric MFI in B6.SM/*apoe-/-* versus 100.0±14.4%

of average *apoe-/-* geometric MFI in *apoe-/-* mice at the HA^{hi} level (P=0.042) (Fig. 4.3.8).

Fig. 4.3.6 P-selectin binding assay in CD11b$^+$ and CD3ε$^+$ peripheral blood subsets in *apoe-/-* and B6.SM/*apoe-/-* mice. Hypomorphic sialidase *apoe-/-* peripheral blood showed no difference in P-selectin binding on CD11b$^+$ subsets (P=0.70), but a significant reduction of P-selectin binding on CD3ε$^+$ subsets was observed (P=0.043). Peripheral blood was incubated with 4 μg P-selectin-human-IgG chimera for 1h, then incubated with anti-human-IgG-AF488, anti-CD11b-APC and anti-CD3ε-Pacific Blue. Samples were analyzed on an LSR II, n=3 each group, data analyzed using two-tailed student's *t* test.

Fig. 4.3.6

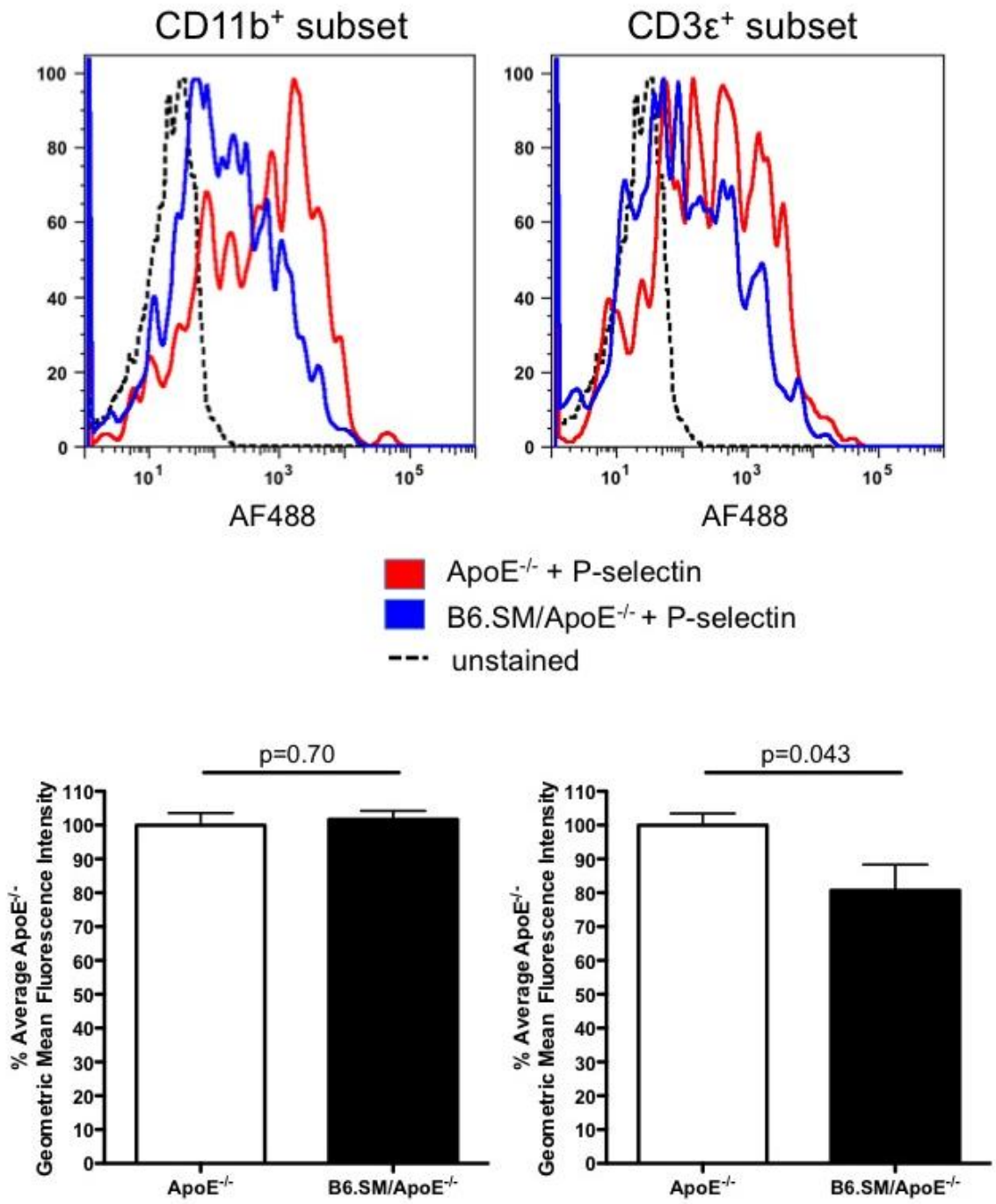

CD11b+ subset
CD3ε+ subset
AF488
AF488
ApoE-/- + P-selectin
B6.SM/ApoE-/- + P-selectin
unstained
p=0.70
p=0.043
% Average ApoE-/- Geometric Mean Fluorescence Intensity
ApoE-/-
B6.SM/ApoE-/-

Fig. 4.3.7 E-selectin binding assay in CD11b$^+$ and CD3ε$^+$ peripheral blood subsets in *apoe-/-* and B6.SM/*apoe-/-* mice. Hypomorphic sialidase *apoe-/-* peripheral blood showed no difference in E-selectin binding on CD11b$^+$ subsets (P=0.19) and CD3ε$^+$ subsets (P=0.21). Peripheral blood was incubated with 4 μg E-selectin-human-IgG chimera for 1h, then incubated with anti-human-IgG-AF488, anti-CD11b-APC and anti-CD3ε-Pacific Blue. Samples were analyzed on an LSR II, n=8 *apoe-/-*, n=9 B6.SM/*apoe-/-* each group, data analyzed using two-tailed student's *t* test.

Fig. 4.3.7

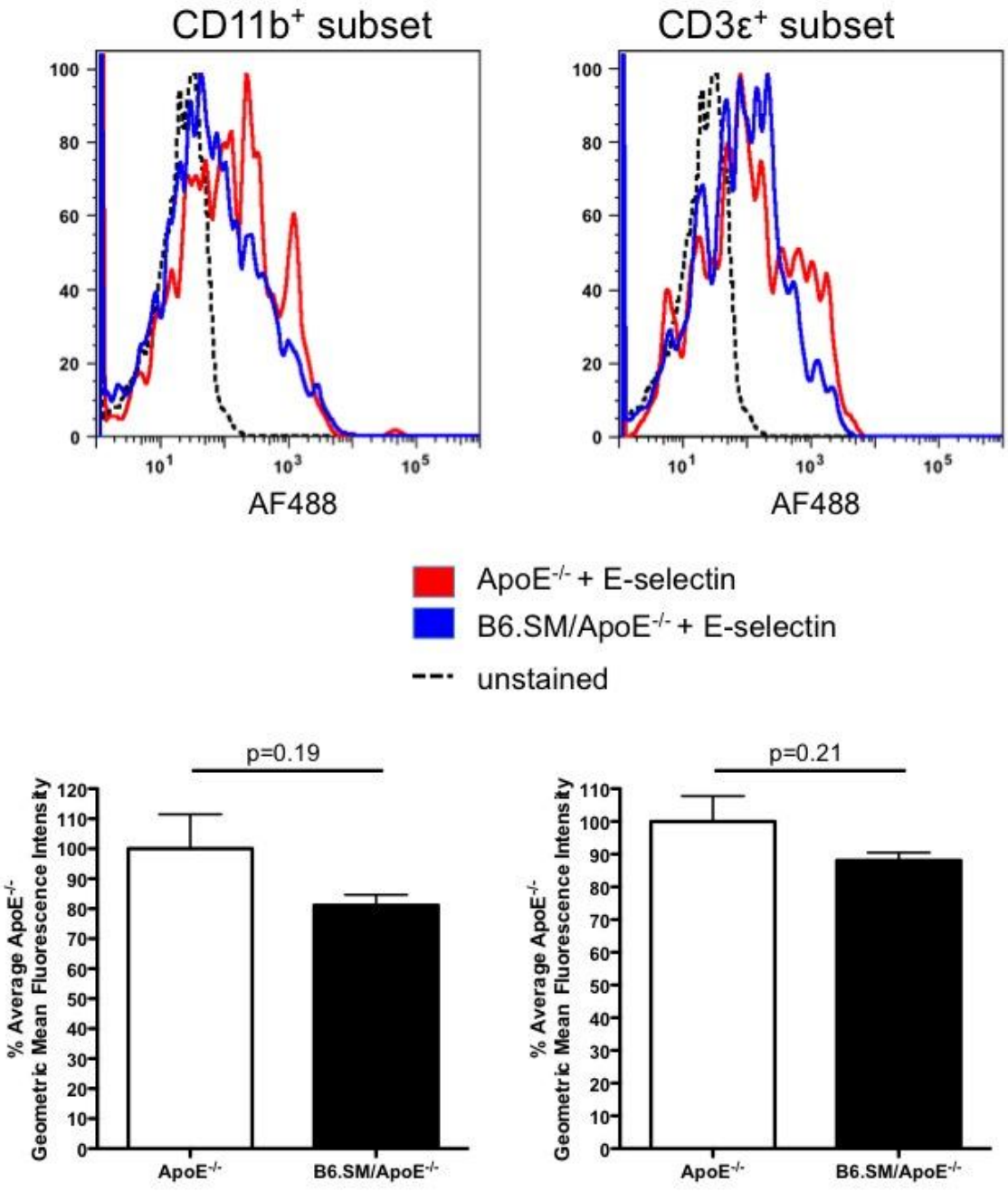
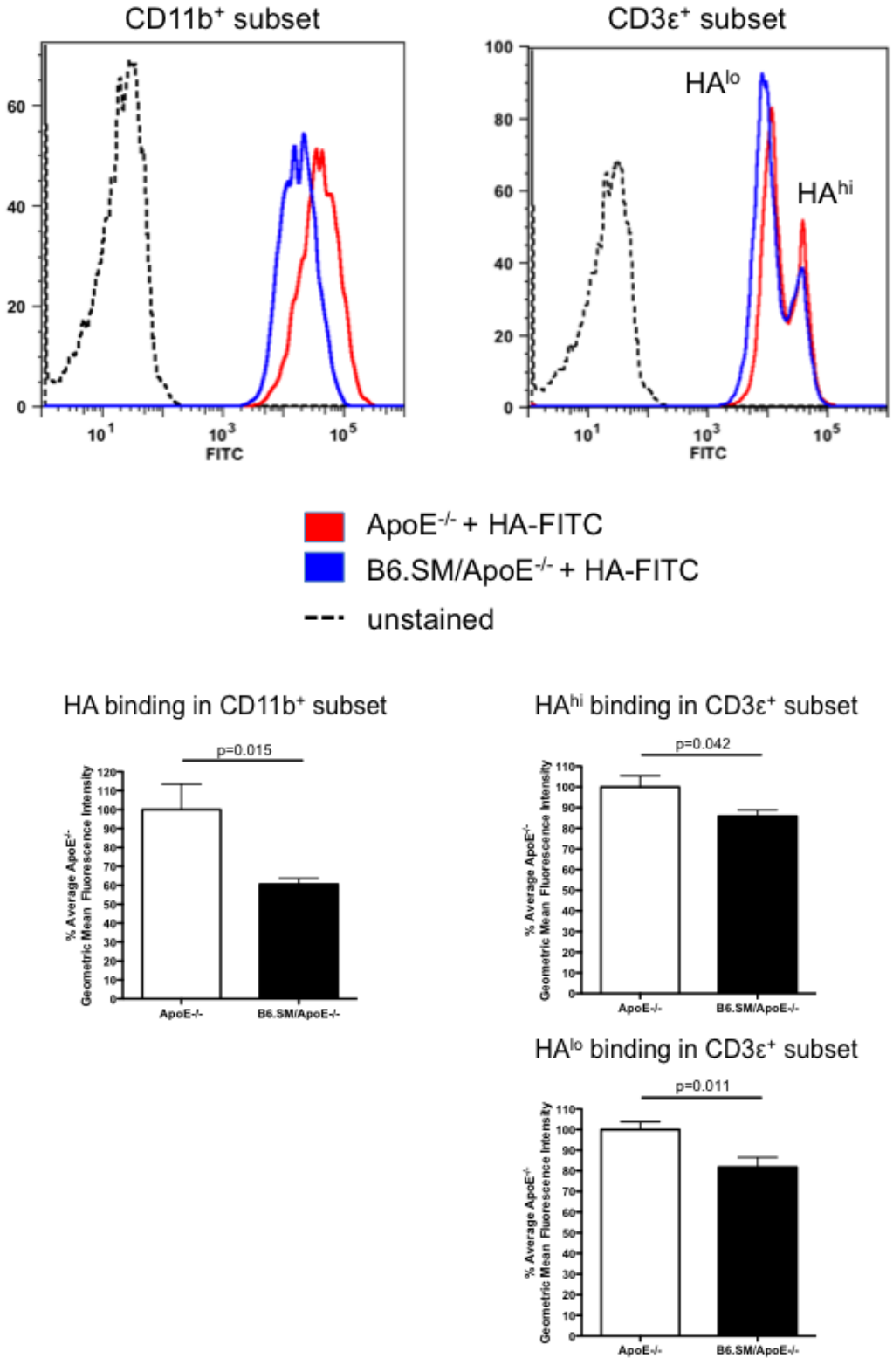

CD11b+ subset
CD3ε+ subset
AF488
AF488
ApoE-/- + E-selectin
B6.SM/ApoE-/- + E-selectin
unstained
p=0.19
p=0.21
% Average ApoE-/- Geometric Mean Fluorescence Intensity
ApoE-/-
B6.SM/ApoE-/-

Fig. 4.3.8 Hyaluronic acid binding assay in CD11b$^+$ and CD3ε$^+$ peripheral blood subsets in *apoe-/-* and B6.SM/*apoe-/-* mice. Hypomorphic sialidase *apoe-/-* peripheral blood CD11b$^+$ cells show significantly reduced hyaluronic acid binding (P=0.015), while CD3ε$^+$ cells show two distinct populations that bind hyaluronic acid at different amounts, HA^{lo} and HA^{hi}, and show significantly reduced hyaluronic acid binding in B6.SM/*apoe-/-* cells (P=0.042 for the HA^{hi} group, P=0.011 for the HA^{lo} group, two-tailed student's *t* test).

Fig. 4.3.8


CD11b+ subset
CD3ε+ subset
HAlo
HAhi
FITC
ApoE-/- + HA-FITC
B6.SM/ApoE-/- + HA-FITC
unstained
HA binding in CD11b+ subset
HAhi binding in CD3ε+ subset
HAlo binding in CD3ε+ subset
p=0.015
p=0.042
p=0.011
% Average ApoE-/- Geometric Mean Fluorescence Intensity
ApoE-/-
B6.SM/ApoE-/-

4.4 Discussion

In this study, we demonstrate that hypomorphic sialidase expression in *apoe-/-* mice alters immune cell proportions in the peripheral blood, and also that these hematopoietic derived cells have lower pro-inflammatory cytokine production and extravasation potential. These data provide support for a significant role of sialidase in peripheral blood cells in the inflammatory component of atherogenesis. Our results indicate that hypomorphic sialidase expression in hematopoietic cells decreases atherosclerotic lesion area and volume in *apoe-/-* mice with hypomorphic sialidase expression. This effect is demonstrated in both the male and female recipients with male bone marrow donors. In addition, sialidase expression in *apoe* deficient hematopoietic cells does not affect serum and hepatic cholesterol and triglyceride levels. These data imply that sialidase activity in hematopoietic cells contributes significantly to atherogenesis and is enough to affect atherogenesis without a synergic effect of lipoprotein metabolism.

Immunophenotyping data shows increased proportions of $CD3\varepsilon^{+}CD4^{+}$ T cells and $CD11b^{hi}$ monocytes, but reduced numbers of $CD11b^{lo}$ cells, in the hypomorphic sialidase B6.SM/*apoe-/-* mice compared to *apoe-/-* mice.This indicates that hypomorphic sialidase expression alters the development of hematopoietic cells in *apoe*-/- mice, or the expression of these molecular markers is abnormal with reduced sialidase activity. The increased proportion of $CD4^{+}$ T cells could reflect a bias to the development of lymphoid lineages, since this is accompanied by a decrease in the $CD11b^{lo}$ myeloid lineage. $Gr\text{-}1^{hi}$ populations are mobilized from the bone marrow and

spleen in response to inflammatory stimuli, and include myeloid progenitors, neutrophils and inflammatory monocyte subpopulations (Swirski et al., 2009). The reduced proportion of Gr-1^{hi} cells observed in B6.SM/*apoe*-/- mice may reflect decreased pro-inflammatory activity in these mice. In contrast to the B6.SM/*apoe*-/- mice, $Neu1^{-/-}$ mice show increased production of granulocyte/erythroid/macrophage/megakaryocyte colony forming units due to extramedullary hematopoiesis (De Geest et al., 2002).

Hypomorphic sialidase expression in hematopoietic cells decreases atherosclerosis in a hepatic and serum cholesterol independent manner. Lipopolysaccharide is first detected by cells expressing TLR4 including monocytes, dendritic cells, mast cells and NK cells (Chow et al., 1999; Palsson-McDermott and O'Neill, 2004; Lauzon et al., 2006). The resultant signaling in these cells leads to the expression and secretion of cytokines, predominantly TNFα from antigen presenting cells and IFNγ from NK cells (Beutler and Rietschel, 2003). LPS-free, minimally modified LDL (mmLDL) has also been shown to stimulate the TLR4 pathway, and presents as an endogenous ligand in the context of atherogenesis (Bae et al., 2009). Our LPS-stimulation experiments suggest B6.SM/*apoe-/-* mice would be less sensitive to minimally modified LDL (mmLDL) and secrete less pro-inflammatory cytokines. Early IL-10 release after LPS treatment is considered an anti-inflammatory response to reduce the toxicity of LPS, and has been demonstrated to suppress inflammatory cytokine release including IFNγ (Marchant et al., 1994). The early and transient peak in IL-10 in *apoe-/-* blood serum could explain the delay in IFNγ induction. Decreased IL-10 production by early responding cells in B6.SM/*apoe-/-* is likely responsible for the relatively early

increase in IFNγ in these animals at 4h compared to levels produced in *apoe-/-*, although this difference between IFNγ production in *apoe-/-* and B6.SM/*apoe-/-* disappears sometime between 4h and 8h. The cell type that rapidly releases IL-10 after LPS may be NK or NK T cells, and dysfunction of these cell types could contribute to the effect of hypomorphic sialidase expression on altered immune response and reduced atherosclerosis in male B6.SM/*apoe-/-* mice. Anti-CD3ε *in vivo* immune stimulations reveal reduced production of Th2-type cytokine IL-4 in male B6.SM/*apoe-/-* mice, which is expected given that SM/J and B10.SM mice show defective IL-4 production and IL-4 signaling as a result of hypomorphic sialidase expression (Chen et al., 1997; Wang et al., 2004). Although IL-4 and IFNγ characteristically suppress each other's production, IL-4 has also been shown to induce IFNγ production by NK and NK T cells (Morris et al., 2006), both of which are found in atherosclerotic plaques. IFNγ is one essential component to the development of atherosclerosis in *apoe-/-*mice as demonstrated in male ApoE$^{-/-}$IFNγ$^{-/-}$ and ApoE$^{-/-}$ IFNγR$^{-/-}$ mice that have attenuated plaque formation (Gupta et al., 1997; Whitman et al., 2002). Inhibition of sialidase using DANA also directly interferes with IFNγ production (Nan et al., 2007). This could be a major underlying reason for the reduced development of atherosclerosis in B6.SM/*apoe-/-* transplanted with B6.SM/*apoe-/-* bone marrow compared to those transplanted with *apoe-/-* bone marrow.

The extravasation of blood cells into the vessel wall is a critical pathological component of atherogenesis, and allows the accumulation of peripheral blood cells into sites of inflammation. Leukocyte rolling on endothelial cells requires the coordinated expression of selectins and selectin ligands. Inflamed endothelial cells

express P-selectin and E-selectin, which recognize the sialyl Lewis X motif contained in ligands such as P-selectin glycoprotein ligand 1 (PSGL-1), CD44 and CD34 expressed on leukocytes (reviewed in Sperandio et al., 2009). These interactions trigger intracellular signaling and up regulate the expression of integrins (I-CAM or V-CAM) on the leukocyte cell surface, permitting firm adhesion and recruitment of the leukocytes into the tissue (Sperandio et al., 2009). Hyaluronic acid, a component of the extracellular matrix, can act as a ligand for CD44, and has been shown to have greater binding when CD44 is desialylated (Gee et al., 2003; Katoh et al. 2010; Katoh et al., 1999; Katoh et al., 1995). We have previously observed reduced infiltration of CD11b+ cells and a trend for reduced infiltration of CD3+ cells into atherosclerotic lesions in B6.SM/*apoe-/-* mice. By incubating leukocytes with P- and E-selectin fusion proteins or fluorescent labeled hyaluronic acid, we get a functional assessment of the selectin ligands and functional CD44 expressed by the leukocytes. In B6.SM/*apoe-/-* $CD11b^+$ cells, we observe a trend for reduced E-selectin and hyaluronic acid binding, while in $CD3\varepsilon^+$cells, we observed a reduction of functional P-selectin binding. Since hypomorphic sialidase expression should be associated with hypersialylation of cell surface molecules, and selectin ligands require a sialylated motif, we suggest the motifs are obscured by hyper-sialylation, or the presentation of selectin ligands is reduced. Reduced hyaluronic acid binding on $CD11b^+$ cells provides evidence that CD44 is hyper-sialylated in the B6.SM/*apoe-/-* mice and thus reduces the migratory and extravasation potential of peripheral blood monocytes, and thereby causes a reduction in atherosclerotic lesion areas in B6.SM/*apoe-/-* mice transplanted with B6.SM/*apoe-/-* bone marrow compared to those transplanted with *apoe-/-* bone marrow.

In conclusion, we have demonstrated that hypomorphic sialidase expression in blood cells is sufficient to reduce atherosclerosis in hypomorphic sialidase *apoe-/-* mice. The analysis of the effects of hypomorphic sialidase expression in B6.SM/*apoe-/-* peripheral blood cells shows altered hematopoietic development of myeloid and lymphoid lineages as well as decreased pro-inflammatory cytokine production and extravasation potential. This atheroprotective effect is independent of the hepatic and serum lipids levels.

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Chapter 5

Hypomorphic Sialidase Expression Modulates Serum Cholesterol and Atherosclerosis by Enhancing Monocytic Uptake of Serum Lipoproteins in LDLR Knockout Mice

Abraham E. Yang[1], Gabriel Gyulay[1], Elizabeth J. White[1], Vivienne C. Tedesco[2], Mohamed Alsaied[1], Bernardo L. Trigatti[2], Suleiman A. Igdoura[1,3]

Department of [1]Biology, [2]Biochemistry and Biomedical Sciences, [3]Pathology and Molecular Medicine, McMaster University, Hamilton, Ontario, Canada

Preface

Abraham Yang performed the following: FPLC cholesterol profiles of LDLR-/- mice transplanted with LDLR+/+, B6.SM/LDLR+/+, LDLR-/- or B6.SM/LDLR-/- bone marrow (Fig. 5.3.1), Serum and hepatic lipids levels in LDLR-/- mice transplanted with LDLR+/+, B6.SM/LDLR+/+, LDLR-/- or B6.SM/LDLR-/- bone marrow (Table 5.3.1), blood cells lipids levels in LDLR-/- mice transplanted with LDLR+/+, B6.SM/LDLR+/+, LDLR-/- or B6.SM/LDLR-/- bone marrow (Fig. 5.3.2), atherosclerotic lesion area in LDLR-/- mice transplanted with LDLR+/+, B6.SM/LDLR+/+, LDLR-/- or B6.SM/LDLR-/- bone marrow (Fig. 5.3.7), FPLC cholesterol profiles of LDLR-/- and B6.SM/LDLR-/- mice (Fig. S5.3.1), serum and hepatic lipids levels in LDLR-/- and B6.SM/LDLR-/- mice (Table S5.3.1), hepatic VLDL production in LDLR-/- and B6.SM/LDLR-/- mice (Fig. S5.3.2), hepatic VLDL production rates in LDLR-/- and B6.SM/LDLR-/- mice (Table S5.3.2), hepatic VLDL-ApoB-100 and –ApoB-48 secretion in LDLR-/- and B6.SM/LDLR-/- mice (Fig. S5.3.3), atherosclerotic lesion area in LDLR-/- and B6.SM/LDLR-/- mice after feeding western diet for 6 weeks (Fig. S5.3.4).

Gabriel Gyulay performed the followings: bone marrow derived macrophages from hypomorphic sialidase mice have increased efflux to HDL compared to C57Bl/6 controls (Fig. 5.3.8), B6.SM BMDMs have higher levels of ABCG1 protein and lower levels of LDLR protein (Fig. 5.3.9) and intraperitoneal hypomorphic sialidase macrophages accumulate more lipids after LDL treatment compared to C57Bl/6 and LDLR -/- controls as measured using ORO staining (Fig. 5.3.10). Gabriel Gyulay also contributed to folch lipid extraction.

Elizabeth White performed the followings: $CD11b^+$ and $CD3\varepsilon^+$ subsets in peripheral blood of LDLR-/- and B6.SM/LDLR-/- mice (Fig. 5.3.3 and Fig. 5.3.5), free cholesterol content of $CD11b^+$ and s $CD3\varepsilon^+$ subsets in LDLR-/- and B6.SM/LDLR-/- peripheral blood (Fig. 5.3.4 and Fig. 5.3.6).

Vivienne Tedesco and Mohamed Alsaied contributed to bone marrow transplantation for LDLR-/- mice transplanted with LDLR+/+ and B6.SM/LDLR+/+ bone marrow. Aline Fiebig and Hatem Abo-ouf contributed to technical assistance for bone marrow transplantation.

Chapter 5

Hypomorphic Sialidase Expression Modulates Serum Cholesterol and Atherosclerosis by Enhancing Monocytic Uptake of Serum Lipoproteins in LDLR Knockout Mice

Summary

Bone marrow-derived blood cells, including monocyte-derived macrophages, express lipoprotein receptors which endocytose atherogenic lipoproteins, i.e., VLDL, IDL and LDL. The dynamic of lipoprotein uptake by monocytes is tightly controlled by the expression and the down regulation of lipoproteins receptors. Sialidase (neu1) desialylates lipoproteins and their receptors and thereby,has an impact on receptor efficacy and specificity and consequently lipoprotein metabolism. In this study, we examined whether sialidase plays a role in blood cellmediated lipoprotein cholesterol uptake and reverse cholesterol transport. First, we generated LDLR knockout mice expressing hypomorphic levels of sialidase (B6.SM/LDLR-/-). Second, bone marrow from LDLR+/+, B6.SM/LDLR+/+, LDLR-/- or B6.SM/LDLR-/-donors were transplanted into lethally irradiated LDLR-/- recipients. LDLR-/- mice receiving B6.SM/LDLR+/+ bone marrow showed a significant decrease in IDL/LDL cholesterol compared with LDLR-/- mice transplanted with LDLR+/+ bone marrow. In addition, LDLR-/- mice receiving B6.SM/LDLR+/+ bone marrow showed significantly smaller lesion area than LDLR-/- mice receiving LDLR+/+ bone. The decrease in cholesterol levels and lesion size in mice with hypomorphic sialidase hematopoietic cells appears to be a result of an increase in lipoprotein uptake by leukocytes. Our results indicate

that peripheral blood monocytes ($CD11b^+$) of hypomorphic sialidase B6.SM/LDLR-/- mice contain significantly higher free cholesterol levels than LDLR-/- mice. In addition, hypomorphic sialidase macrophages appear to upregulate ABCG1 expression and to increase cholesterol efflux to HDL. Taken together, we conclude that hypomorphic sialidase hematopoietic cells confer atheroprotection in LDLR-/- mice primarily due to reduced serum lipoprotein cholesterol levels and increased cholesterol efflux. In addition, the absence of LDLR in leukocytes, including monocytes, amplifies the extent of lipoprotein cholesterol uptake. Therefore, we demonstrate a novel role for hematopoietic cells in the regulation of lipoprotein cholesterol levels.

5.1 Introduction

Sialidases are hydrolytic enzymes that cleave sialic acid from glycoproteins, glycolipids and oligosaccharides (Reuter and Gabius, 1996). Neu1 sialidase is expressed ubiquitously in the cell membrane in addition to the lysosome, and cleaves terminal α2-3 and α2-6 sialic acid residues (Igdoura et al., 1998; Pattison et al., 2004; Liang et al., 2006; Millar J., 2001; Rottier et al., 1998). These processes influence a number of important biological processes including cell-cell interactions, macrophage and T-cell activation, catabolism and antigenicity (Varki, 1997; Pilatte et al., 1993; Corfield, 1992; Reuter and Gabius, 1996). Since inflammation and immune response are directly related to the progression of atherosclerosis, sialidases which are essential for the activation of macrophages (Lambré et al., 1990; Liang et al., 2006; Stamatos et al., 2005), T cells (Pappu and Shrikant, 2004; Xu and Weiss, 2002) and neutrophils (Cross et al., 1991; Cross et al., 2003; Sakarya et al., 2004) would have to play a role in atherosclerosis. THP-1 monocyte differentiation involves upregulation of neu1 sialidase and its targeting to the cell membrane (Liang et al., 2006). In addition, desialylation of T-cell enhances its activation and secretion of inflammatory factors, such as, IL-4 (Chen et al., 1997; Pappu and Shrikant, 2004; Wang et al., 2004). Furthermore, sialidase activation contributes to CD44-HA adhesion in LPS and TNF stimulated THP-1 monocytes (Gee et al., 2002; Gee et al., 2003). For neutrophils, sialidase movement to the plasma membrane plays role in its activation, adhesion and migration (Cross et al., 1991; Cross et al., 2003; Sakarya et al., 2004). The activation of these leukocytes is important because recruitment and differentiation of monocytes and leukocytes play an important role in the inflammatory response that is prominent

in atherosclerosis (Ross R., 1999). Therefore, sialidase has atheroprotective potential on atherosclerosis.

In addition to inflammation, lipoprotein uptake also plays a significant role in the progression of atherosclerosis. The majority of cholesterol in the blood is transported by low density lipoprotein (LDL) (Steinberg and Witztum, 1990). Enzymatic modification and specifically oxidation increase significantly when LDLs accumulate in the subendothelium (Parthasarathy et al., 1992; Schwenke et al., 1989). Uptake of LDL occurs through its binding and internalization by the LDL receptor (LDLR). LDLR is a transmembrane protein that is expressed ubiquitously. The unglycosylated precursor and glycosylated mature form are about 120kDa and 164kDa in size respectively (Filipovic I., 1989; Gent and Braakman, 2004). The LDLR is heavily glycosylated with 80% of the O-linked glycosylation occurring in the extracellular region of the protein near the membrane spanning region (Davis et al., 1986; Kozarsky et al., 1988; Kingsley et al., 1986). These glycans are not responsible for normal function of the LDLR, as their deletion does not hamper LDLR function. However, it was demonstrated that 20% of the O-linked glycosylation near the N-terminal (ligand binding) region of the receptor, is essential for LDLR function along with the 2 N-linked oligosaccharides (Kozarsky et al., 1988; Kingsley et al., 1986). It was shown that sialidase treatment of LDLR utilizing *Clostridium perfringens* neuraminidase with preference in the order of α2-3>α2-6>α2-8 resulted in a 7-11 kDa reduction of the receptor in human fibroblast cells, indicating the presence of terminal sialic acids (Schneider et al., 1982). In addition, sialic acids on the LDLR not only contribute a net negative charge, but also play a significant role for regulating

LDL-LDLR interaction. Furthermore, treatment of human fibroblasts with sialidase results in reduced LDL binding and internalization compared to LDLR with normal sialylation (Sprague et al., 1988). LDLR-deficient mice were generated as an animal model to study western diet induced atherosclerosis with the majority of its cholesterol being accumulated in the VLDL and LDL-sized particles (Ishibashi et al, 1993).

There have been many studies published utilizing bone marrow transplantation as a method to assess the impact of bone marrow-derived blood cells during atherosclerosis (Boisvert et al., 1997; Fazio et al., 1997; Herijgers et al., 1997; Herijgers et al., 2000; Linton et al., 1999). Lipoprotein receptors such as LDLR, VLDLR and LRP, on leukocytes have been shown to modulate VLDL- and/or IDL/LDL-cholesterol levels as demonstrated by the FPLC profiles (Boisvert et al., 1997; Herijgers et al., 1997; Herijgers et al., 2000; Linton et al., 1999; Van Eck et al, 2005; Overton et al., 2007). However, monocytic uptake of lipoprotein cholesterol has yet to be investigated (Nguyen et al., 1988; Pietsch et al., 1996). In addition to the uptake of lipoprotein cholesterol, macrophages can shed excess intracellular cholesterol by effluxing cholesterol via ATP-binding cassette transporter AI (ABCAI) and ATP-binding cassette transporter GI (ABCG1) (Wang et al., 2000; Wang et al., 2004). This initial efflux of cellular cholesterol is critical for the reverse cholesterol transport pathway which transfers excess cholesterol from peripheral tissues to the liver for catabolism and excretion (Fielding and Fielding, 2001). The efflux of cholesterol involves acceptors such as high density lipoprotein (HDL) and/or lipid-poor apoproteins. HDL levels in blood are inversely related to the susceptibility of atherosclerosis (Hayden et al., 2000). Transplantation of bone marrow from mice

lacking ABCG1 affects atherogenesis (Baldan et al., 2006; Out et al., 2006; Ranalletta et al., 2006). Inactivation of ABCA1 in macrophages increases atherosclerosis (Aiello et al., 2002; van Eck et al., 2002). However, the effects of sialidase on the function of these lipoprotein receptors and transporters, and the consequences of these effects on atherogenesis are largely unknown.

To test the effects of hypomorphic sialidase expression in leukocytes on atherosclerosis in LDLR-/- mice, we have compared atherogenesis in LDLR-/- mice transplanted with C57Bl/6 (LDLR+/+), B6.SM/LDLR+/+, LDLR-/-, or B6.SM/LDLR-/- bone marrow.In this report, we have demonstrated that hypomorphic sialidase hematopoetic cells significantly reduce serum VLDL and LDL cholesterol levels in LDLR-/- mice fed western diet for 6 weeks. The decrease in serum cholesterol levels appears to be a result of an increase in cholesterol uptake and an increase in reverse cholesterol transport. In addition, the decrease in serum lipoprotein levels resulted in reduced atherogenesis. This study illustrates a potential role for leukocytes in the modulation of serum lipoprotein and as a consequence atherosclerosis.

5.2 Methods

5.2.1 Mice

B6.SM/LDLR-/- mice were generated by crossing LDLR-/- and B6.SM mice. All mice were maintained in microisolator cages and had free access to food and water. Atherosclerosis studies were performed in 3-month-old male LDLR-/- and B6.SM/LDLR-/- mice fed western diet for 6 weeks. For flow cytometry, male LDLR-/- and B6.SM/LDLR-/- were fed western diet for 3 weeks before peripheral blood was collected by cardiac puncture. Western diet used contains 21% butterfat and 0.15% cholesterol diet with 1% safflower oil (Modified Stanford University, Dyets Inc, catalogue #112286). Animal care and experimental procedures were performed in accordance with institutional guidelines and were approved by Animal Research Ethics Board at McMaster University.

5.2.2 Bone marrow transplantation

Bone marrow was collected from donor mice by flushing femurs and humurus with Iscove minimum Dulbecco medium (IMDM) with penicillin, streptomycin and fungizone. Recipient mice were irradiated 11Gy. Bone marrow cells (10^7 cells in 295ul) were administered retro orbitally under anesthesia. One week pre-transplantation and 4 weeks post-bone marrow transplantation, the recipient mice were given septra antibiotic water and jello.

5.2.3 Polymerase chain reaction (PCR) of genomic DNA

Genomic DNA was prepared from the tail of LDLR-/-, B6.SM/LDLR-/- and peripheral blood cells of BMT mice. For the identification of B6.SM, the following primer sequences were used: 5’ ATC CCT GTC CAG GAA CTG GT 3’ (Primer ML-07-06, Mobix, McMaster University) and 5’ CTT AAG GGC ATT GGG GTC AT 3’ (Primer ML-07-07, Mobix, McMaster University). PCR (40 cycles) was performed with denaturation temperature at 94°C for 2 minutes, annealing temperature at 60°C for 30 seconds and elongation temperature at 72°C for 30 seconds. PCR products were digested with MspA1I (New England BioLab R0577) in order to genotype for *neu1* mutation specific for B6.SM mice. The digested products were separated on a 1.5% agarose gel, 1X TBE buffer. This reaction yields a 450-bp band which is specific for the wild type and both the 275-bp and 225-bp which are specific for the mutant (B6.SM). Two different PCR amplifications were performed for the LDLR allele. The first reaction was performed for the normal LDLR allele and the second reaction was performed for the targetted allele. Primer sequences for the normal LDLR allele are: 5’ ACC CCA AGA CGT GCT CCC AGG ATG A 3’ (Primer ML-07-4550, Mobix, McMaster University) and 5’ CGC AGT GCT CCT CAT CTG ACT TCT 3’ (Primer ML-07-1200, Mobix, McMaster University); primer sequences for the disrupted allele which hybridize to a region in the Neo-R gene close to the 5' end of the coding region are: 5’ GGC AAG ATG GCT CAG CAA GCA AAG GC 3’ (Primer ML-07-4243, Morbix, McMaster University) and 5’ GAT TGG GAA GAC AAT AGC AGG CAT GC 3’ (Primer ML-07-4244, Morbix, McMaster University). PCR (40 cycles) was performed with denaturation temperature at 94°C for 2 minutes, annealing temperature at 57°C for 2 minutes and elongation temperature at 65°C for 5 minutes.

PCR products were separated on a 1% agarose gel, 1X TBE buffer. The reaction for the normal LDLR allele yields a 383-bp band which is specific for the normal allele. The reaction for the targetted allele yields a 260-bp band from the disrupted LDLR allele, and no amplification from the normal allele.

5.2.4 Lipoprotein and serum lipids analysis

In order to examine serum lipoprotein cholesterol profile, mice were anesthesized with ketamine/xylazine i.p. and blood samples were collected by cardiac puncture. Serum was prepared by centrifugation of blood for 5 min at 15000 rpm using serum collection tubes. Serum was fractionated by Gel Filtration Fast Protein Liquid Chromatography AKTA P-950 (GE Healthcare). Serum (100 ul) was injected into the Superose 6 column (GE Heathcare) and was run at a rate of 0.5ml/min. At the termination of the fractionation, 100 ul fractions in FPLC buffer were collected in as 96-well microplate. Serum cholesterol levels in VLDL-sized, IDL/LDL-sized and HDL-sized particles were measured by Infinity reagent (Thermo) and the absorbance of the reaction product was read at 500nm. Serum total cholesterol levels were also measured using Infinity reagent (Thermo) and was read at 500nm. Serum free cholesterol levels were measured using free cholesterol E reagent (Wako Diagnostics) and was read at 600nm. Serum triglyceride levels were measured with L-Type Triglyceride H Reagents 1 and 2 (Wako Diagnostics) and were read at 600nm.

5.2.5 Hepatic and blood cell lipid analysis

Hepatic or blood cell total cholesterol, free cholesterol, cholesteryl esters and triglyceride were determined using an enzymatic method after lipid extraction from

the liver or blood cell. Briefly, liver or blood cell homogenates were suspended in 900 ul of TNES (10 mM Tris, pH 7.5, 400 mM NaCl, 100 mM EDTA, 0.6% SDS). Chloroform/methanol mixture (6 ml) in a ratio of 2:1 was added to 300 ul of liver homogenates. Distilled water (1.2 ml) was added, and the extraction mixture was allowed to stay at 4°C for 2 hours for phase separation. The lower phase was dried completely and was resuspended in 60 ul of isopropanol. Hepatic or blood cell total cholesterol, free cholesterol, cholesteryl esters and triglyceride concentrations were measured as described in lipoprotein and serum lipids analysis.

5.2.6 *In vivo* measurement of hepatic VLDL-triglyceride secretion

Three-month-old male LDLR-/- and B6.SM/LDLR-/- mice were fasted overnight. Twenty four hours later, the mice were injected with Triton WR1339 mixture (Tyloxapol T0307-10G, Sigma BioXtra) at 500mg/kg body weight. Blood samples were drawn into serum collection tubes at 0, 1, 2, 3 and 4 hours after Triton WR1339 administration. Serum was obtained by centrifugation and was assayed for total cholesterol, triglyceride, free cholesterol and cholesteryl esters using enzymatic assays as described in lipoprotein and serum lipids analysis. Hepatic VLDL-triglyceride, cholesterol, free cholesterol and cholesteryl esters production rates were calculated from the slope of the linear regression over a time interval of 4 hours and were expressed in mM/hour.

5.2.7 Immunoblot analysis

Serum was separated on SDS/polyacrylamid gel and transferred to nitrocellulose membranes. Bone marrow-derived macrophages were lysed in RIPA buffer with

protease inhibitors and homogenized then boiled in Laemmli Sample Buffer prior to loading. Blots were probed using anti-apoB antibodies (1:6000, Midland Bioproducts, Boone, USA), anti-LDLR (1:1000, R&D systems), anti-GADPH (1:1000, R&D systems), anti-ABCG1 (1:1000, Novus Biologicals), anti-ABCA1 (1:1000, GenScript), secondary HRP-conjugated antibodies (1:10000, Santa Crutz Biotechnology) and finally visualized with chemiluminescence (ECL, Amersham). Intensities of bands were measured by ImageJ densitometry software.

5.2.8 Flow cytometry

In order to perform flow cytometry, peripheral blood was obtained and erythrocytes were lysed using 1X BD Pharm Lyse. Leukocytes were re-suspended in phosphate buffered saline (PBS) with 1% fetal bovine serum (FBS). For blocking, cells were treated with rat anti-mouse CD16/CD32 (Fc Block, 10 μg/ml, BD Pharmingen). Cells were incubated with primary antibodies for 40 min. on ice including 1 μg each of hamster anti-mouse-CD3ε-APC-Cy7 (clone 145-2C11) and rat anti-mouse CD11b-APC (clone M1/70) (BD Biosciences). After primary antibodies incubation, cells were washed with PBS 1% FBS and fixed with BD Cytofix for 20 min on ice. Cells were washed with PBS 1% FBS again and then were incubated with 50 μg/ml filipin III (diluted from fresh 10 μg/ml filipin in DMSO stock, stored under nitrogen gas) for 50 min. After 50 min. of filipin III incubation, cells were washed with PBS 1% FBS, were run through a cell strainer, and then were read on a BD FacsAria III cell sorter. Filipin III was excited using a 375 nm near-UV laser and emission wavelengths were detected using a 450/20 band pass filter. Data was analyzed using FlowJo (Treestar).

5.2.9 Quantification of atherosclerotic lesion at aortic sinus

Mice were perfused with phosphate-buffered saline (PBS) through the left ventricle with drainage via the right atrium. Hearts were removed and placed in Krebs Henseleit Solution for 30 min. After treatment with Krebs Henseleit Solution, hearts were fixed in 4% formaldehyde. After fixation, hearts were placed in 30% sucrose in PBS overnight at 4˚C. Hearts were embedded in cryomatrix (Shandon Thermo) and were frozen on dry ice. Serial 10 um cryosections were obtained using a Cryostat (Shandon Thermo). Cryosections were stained with Oil Red O for neutral lipids for 10 minutes and stained with hematoxylin for nuclei for 10 minutes. Atherosclerosis was quantified as the total cross sectional area of atherosclerotic lesion in each section using a Zeiss Axiovision Microscopic Software.

5.2.10 Macrophages

Sources of macrophages included mouse intraperitoneal isolation or those from mouse bone marrow isolation and differentiation. These cells were grown in RPMI medium supplemented with 10% FBS, 1% antibacterial solution and 0.1% fungizone. For BMDM culture, bone marrow cells isolated from mouse femurs were differentiated with M-CSF (monocyte colony stimulating factor) (Invitrogen) for 9 days. For IP macrophage isolation, mice were injected intraperitoneally with thioglycollate and sacrificed 4 days after. The peritoneal cavity was injected with PBS to loosen elicited macrophages for removal and plating. Cells were cultured at 37ºC at 5% CO_2 and 90% humidity in a standard Incubator. Macrophages were cholesterol starved prior to lipoprotein uptake studies in order to upregulate receptors by using lipoprotein deficient serum (LPDS) for 48 hours.

5.2.11 Lipoproteins

Lipoproteins for in vitro work were purchased from Biomedical Technologies Inc. These were used with their Lipoprotein deficient serum (LPDS) RPMI media to avoid any medium-resident lipoprotein interference. Concentrations are 50ug/mL with 48 hours incubation unless otherwise stated.

5.2.12 Macrophage Oil Red O Staining and Quantification

For visual quantification, IP macrophages were plated on glass cover slips placed in 24-well plates. After LPDS starvation and 50ug/mL LDL incubation for 48 hours, cells were rinsed, fixed in formaldehyde, ORO stained and washed. Hematoxylin staining was then performed followed by water washes and mounting of glass cover slips onto microscope slides using aqueous mounting media. Quantification was then performed by measuring the red stained area normalized to the nuclear stained area on an individual cell basis using AxioVision software. 200-300 cells were counted and quantified for each treatment.

5.2.13 Cholesterol Efflux Assay

Differentiated BMDMs were loaded with [^{3}H] cholesterol (American Radiolabeled Chemicals Inc.) in FBS for 2 days. Cells were washed and incubated overnight in equilibration medium then provided with efflux medium containing 50ug/mL HDL or LDL. Media was sampled at various timepoints and radioactivity was measured using a liquid scintillation counter. Efflux at each time point is expressed as a percentage of total radioactive cholesterol in the cells at time 0. BSA was used as a control acceptor, instead of HDL and LDL, and accumulated minimal radioactivity.

5.2.14 Statistical analysis

Statistical analyses of data were performed by one-way analysis of variance (one-way ANOVA) followed by Tukey's Post Hoc Multiple Comparison Test, and additional statistical tests for comparison from Prism 5 (version 5.04, GraphPad). Mean±SE were shown with error bars. Data were considered statistically significant different only if $P < 0.05$.

5.3 Results

5.3.1 Hypomorphic sialidase hematopoietic cells decreases cholesterol levels in VLDL and LDL-sized lipoproteins in LDLR-/- mice.

In order to assess the role of hematopoietic cells in lipoprotein metabolism in LDLR-/- versus hypomorphic sialidase LDLR-/- mice, we transplanted bone marrow from LDLR+/+, B6.SM/LDLR+/+, LDLR-/- or B6.SM/LDLR-/-donors into lethally irradiated LDLR-/- recipients. Bone marrow of LDLR+/+, B6.SM/LDLR+/+, LDLR-/- and B6.SM/LDLR-/- mice was harvested and then injected intravenously into recipient mice. Mice were fed western diet 1 month post transplantation for 6 weeks. After 6 weeks of western diet, transplanted mice were sacrificed for atherosclerosis and lipoprotein analyses.

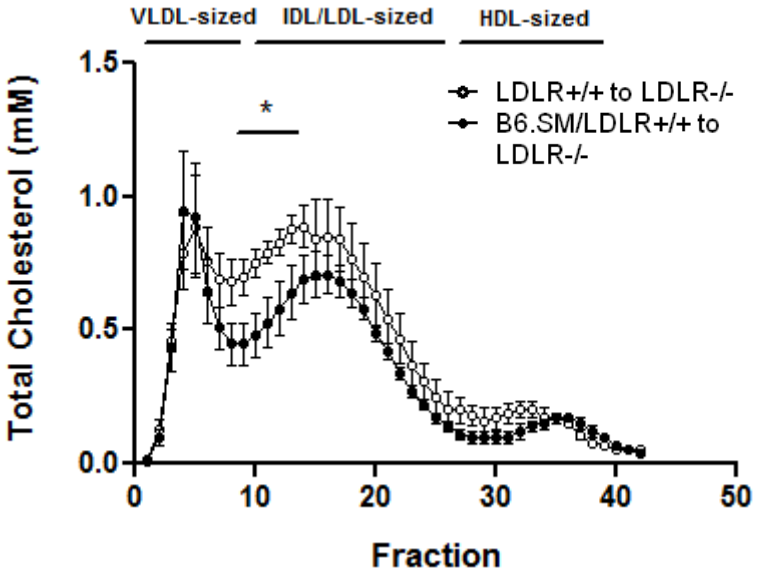
LDLR-/- mice with B6.SM/LDLR+/+ bone marrow appears to have lower cholesterol concentrations in IDL/LDL-sized particles compared to LDLR-/- mice with LDLR+/+ bone marrow (Fig. 5.3.1 A). In addition, hypomorphic sialidase blood cells with intact LDLR show lower cholesterol concentrations in HDL-sized particles compared to the group with LDLR+/+ bone marrow. Furthermore, there was a shift in the FPLC profile only in the HDL-sized particles range. This shift implied that LDLR-/- mice with hypomorphic sialidase bone marrow with the presence of LDLR had a smaller average size of the HDL-sized particles. LDLR-/- mice with LDLR-/- bone marrow have higher cholesterol concentrations in VLDL-sized particles and IDL/LDL-sized particles compared to LDLR-/- mice with B6.SM/LDLR-/- bone marrow (Fig. 5.3.1 B). These implied that hypomorphic sialidase expression specific in blood cells with

the presence of LDLR causes lower cholesterol concentrations in IDL/LDL-sized particles. And, hypomorphic sialidase expression specific in blood cells with the absence of LDLR causes lower cholesterol concentrations in VLDL-sized and IDL/LDL-sized particles. Thus, mice with hypomorphic sialidase expression in blood cells may show higher atherogenic lipoprotein clearance rate compared to control mice.

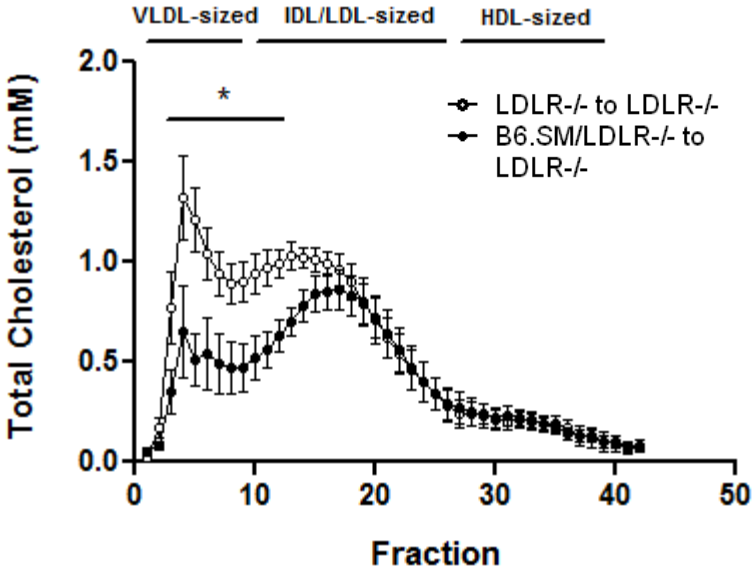
Fig. 5.3.1 FPLC cholesterol profiles of LDLR-/- mice transplanted with LDLR+/+, B6.SM/LDLR+/+, LDLR-/- or B6.SM/LDLR-/- bone marrow. LDLR-/- mice were transplanted with bone marrow and after 4 weeks were fed western for 6 weeks. (A) LDLR-/- mice with B6.SM/LDLR+/+ bone marrow (n=4) has lower cholesterol concentrations in IDL/LDL-sized particles compared to LDLR-/- mice receiving LDLR+/+ bone marrow (n=4). In addition, (B) LDLR-/- mice with B6.SM/LDLR-/- bone marrow (n=5) has lower cholesterol concentrations in VLDL-sized and IDL/LDL-sized particles compared to LDLR-/- mice receiving LDLR-/- bone marrow (n=5). Each circle represents mean ±SE. (VLDL-sized: Very low density lipoprotein sized particle, IDL-sized: Intermediate density lipoprotein sized particle, LDL-sized: Low density lipoprotein sized particle, HDL-sized: High density lipoprotein sized particle). * denotes $P<0.05$.

Fig. 5.3.1

A


VLDL-sized
IDL/LDL-sized
HDL-sized
Total Cholesterol (mM)
1.5
1.0
0.5
0.0
*
LDLR+/+ to LDLR-/-
B6.SM/LDLR+/+ to LDLR-/-
0
10
20
30
40
50
Fraction

B


VLDL-sized
IDL/LDL-sized
HDL-sized
Total Cholesterol (mM)
2.0
1.5
1.0
0.5
0.0
*
LDLR-/- to LDLR-/-
B6.SM/LDLR-/- to LDLR-/-
0
10
20
30
40
50
Fraction

5.3.2 The effects of hypomorphic sialidase hematopoietic cells on serum and hepatic lipids levels in LDLR-/- mice.

The total serum cholesterol concentration in LDLR-/- mice transplanted with B6.SM/LDLR-/- bone marrow compared to LDLR-/- mice transplanted with LDLR-/- bone marrow was significantly decreased (Table 5.3.1). There was a trend toward a reduction in total serum cholesterol concentration in LDLR-/- mice transplanted with B6.SM/LDLR+/+ bone marrow compared to LDLR-/- mice transplanted with LDLR+/+ bone marrow (Table 5.3.1). The total serum cholesterol concentrations in LDLR-/- mice transplanted with LDLR+/+, B6.SM/LDLR+/+, LDLR-/- or B6.SM/LDLR-/- bone marrow appeared to be consistent with the FPLC profiles (Fig. 5.3.1). There was a reduction in total serum cholesteryl esters concentration in LDLR-/- mice transplanted with B6.SM/LDLR+/+ bone marrow compared to LDLR-/- mice transplanted with LDLR+/+ bone marrow and LDLR-/- mice transplanted with B6.SM/LDLR-/- bone marrow compared to LDLR-/- mice transplanted with LDLR-/- bone marrow (Table 5.3.1). For the hepatic cholesterol concentration, there was a reduction in cholesterol concentration when there is a hypomorphic sialidase expression in blood cells (Table 5.3.1). In addition, there was a reduction in free cholesterol concentration when there is a hypomorphic sialidase expression in blood cells. On the other hand, a trend of increase in cholesteryl esters concentration was observed with hypomorphic sialidase expression in bone marrow transplantation with intact LDLR (Table 5.3.1).

Table 5.3.1 Serum and hepatic lipids levels in LDLR-/- mice transplanted with LDLR+/+, B6.SM/LDLR+/+, LDLR-/- or B6.SM/LDLR-/- bone marrow. Serum total cholesterol, free cholesterol, cholesteryl esters, and triglyceride concentrations in bone marrow transplanted mice with female B6.SM/LDLR-/- mice transplanted with LDLR+/+ (n=4) or B6.SM/LDLR+/+ bone marrow (n=4) or LDLR-/- (n=6) or B6.SM/LDLR-/- bone marrow (n=6). Total cholesterol, free cholesterol, cholesterol esters and triglyceride in serum were determined using an enzymatic method. Hepatic total cholesterol, free cholesterol, cholesteryl esters, and triglyceride concentrations in bone marrow transplanted mice with female B6.SM/LDLR-/- mice transplanted with LDLR+/+ (n=4) or B6.SM/LDLR+/+ bone marrow (n=4) or LDLR-/- (n=4) or B6.SM/LDLR-/- bone marrow (n=3). Hepatic total cholesterol, free cholesterol, cholesterol esters and triglyceride were determined using an enzymatic method after extracting hepatic lipids by folch extraction. The mean values ± standard error are shown.

Table 5.3.1

	Hepatic total cholesterol (mg/g liver)	Serum total cholesterol (mM)	Hepatic free cholesterol (mg/g liver)	Serum free cholesterol (mM)	Hepatic cholesteryl esters (mg/g liver)	Serum cholesteryl esters (mM)	Hepatic triglyceride (mg/g liver)	Serum triglyceride (mM)
LDLR+/+ -> LDLR-/-	3.48±0.19	22.00±2.45	2.54±0.14	9.87±0.54	0.94±0.18	12.13±2.08	9.29±1.93	12.48±3.61
B6.SM/LDLR+/+ -> LDLR-/-	2.93±0.67	18.81±0.31	1.52±0.60*	9.08±0.12	1.41±0.19	9.73±0.40	8.57±2.99	6.05±1.08
LDLR-/- -> LDLR-/-	3.27±0.74	26.49±1.91	2.28±0.50	10.19±1.27	0.99±0.26	16.30±2.33	11.92±3.58	14.10±1.72
B6.SM/LDLR-/- -> LDLR-/-	2.11±0.18	18.12±2.27*	1.25±0.23	8.36±0.90	0.86±0.15	9.75±1.45*	4.35±1.56	10.69±2.87

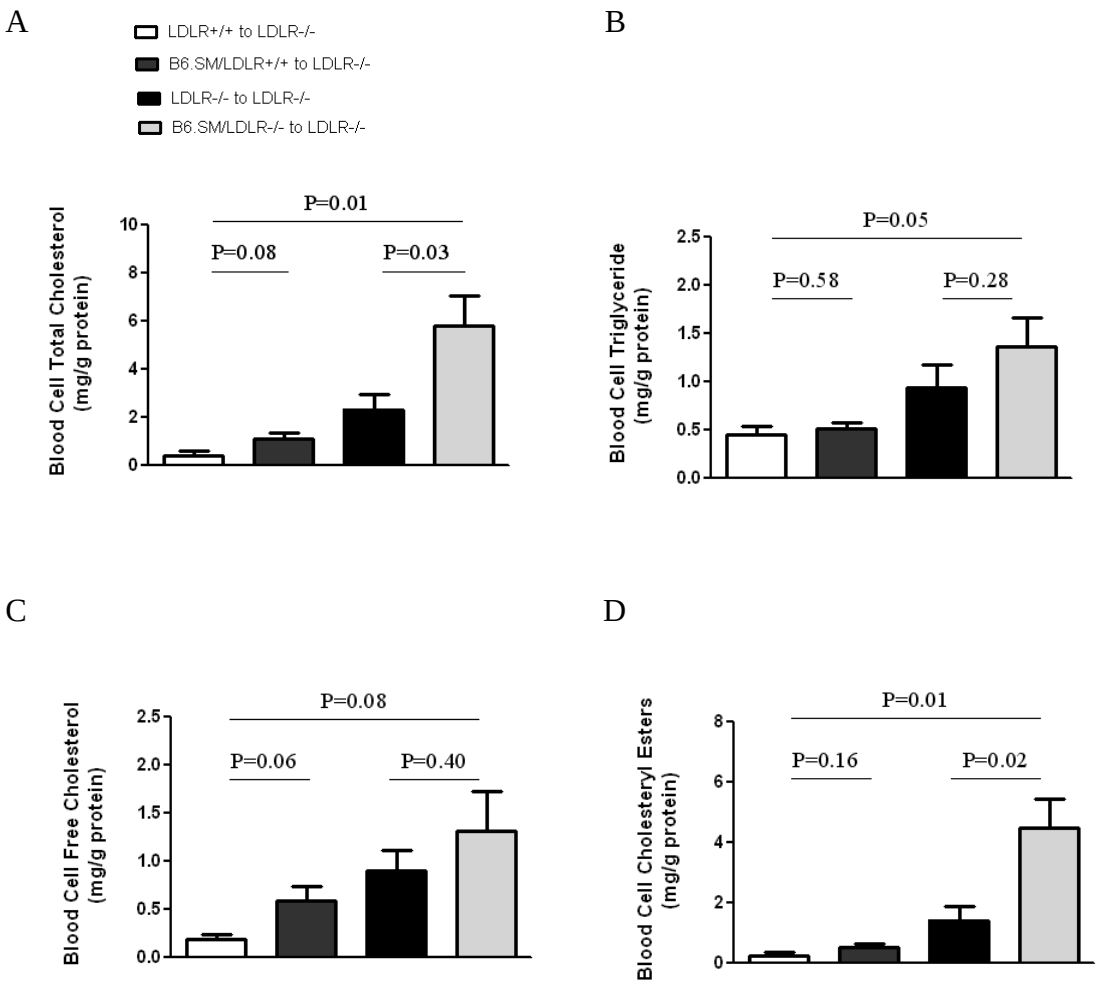
5.3.3 Hypomorphic sialidase hematopoietic cells have higher blood cells lipids levels in LDLR-/- mice.

After we observed that there was a significant decrease in the cholesterol levels in the IDL/LDL sized particles in mice with hypomorphic sialidase blood cells, we wanted to determine whether the decrease in the cholesterol levels is caused by an increased uptake by the peripheral blood cells. Most importantly, this would explain whether the significant decrease in cholesterol levels in the VLDL-sized particles in mice with hypomorphic sialidase blood cells when LDLR is absent is caused by the different extent of uptake of VLDL compared to mice with hypomorphic sialidase blood cells when LDLR is present. Thus, we performed enzymatic lipid assays after extracting lipids from blood cell pellets obtained from the lower phrase of the serum collection tubes. There was an increase in the cholesterol, triglyceride, free cholesterol and cholesteryl esters concentrations in hypomorphic sialidase blood cells compared to controls (Fig. 5.3.2 A-D). Specifically, this increase is significantly greater when LDLR is absent in the hypomorphic sialidase blood cells compared to LDLR-/- mice transplanted with bone marrow with intact sialidase. Critically, the increase is greatest in the cholesteryl esters concentrations when LDLR is absent in the hypomorphic sialidase blood cells compared to blood cells with intact sialidase. These implied that hypomorphic sialidase blood cells can uptake cholesterol and cholesteryl esters more efficiently, and, specifically, the uptake is more pronounced when LDLR is absent. Blood cell cholesterol in the LDLR-/- mice with LDLR-/- bone marrow appears to be higher than that for LDLR-/- mice with LDLR+/+ bone marrow (Fig. 5.3.2 A). This is supported by studies showing that VLDLR and LRP play a significant role in uptaking ApoB48-containing lipoproteins when LDLR is absent (Veniant et al., 1998; Martins

et al., 2000; Tacken et al., 2000). Nevertheless, ACAT, which plays a role in esteriflying free cholesterol to become esterified cholesterol, may be modulated by hypomorphic sialidase expression. In fact, hypomorphic sialidase expression alters hepatic ACAT levels as shown in Chapters 2 and 3.

Fig. 5.3.2 Blood cells lipids levels in LDLR-/- mice transplanted with LDLR+/+, B6.SM/LDLR+/+, LDLR-/- or B6.SM/LDLR-/- bone marrow. Blood cell (A) total cholesterol, (B) triglyceride (C) free cholesterol, and (D) cholesteryl esters concentrations in LDLR-/- mice transplanted with LDLR+/+ (n=4) or B6.SM/LDLR+/+ bone marrow (n=5) or LDLR-/- (n=7) or B6.SM/LDLR-/- bone marrow (n=7). There is a significant increase in cholesterol and cholesteryl esters concentrations in mice transplanted with B6.SM/LDLR+/+ or B6.SM/LDLR-/- blood cells. This increase is most notable in mice with B6.SM/LDLR-/- blood cells, i.e., when LDLR is absent indicating an up-regulation in LDL-independent VLDL/LDL lipoprotein uptake mechanism, e.g., VLDLR. Peripheral blood cell total cholesterol, free cholesterol, cholesterol esters and triglyceride were determined using an enzymatic method after extracting blood cell lipids by folch extraction. The mean values ± standard error are shown.

Fig. 5.3.2


A
LDLR+/+ to LDLR-/-
B6.SM/LDLR+/+ to LDLR-/-
LDLR-/- to LDLR-/-
B6.SM/LDLR-/- to LDLR-/-
Blood Cell Total Cholesterol (mg/g protein)
P=0.01
P=0.08
P=0.03
B
Blood Cell Triglyceride (mg/g protein)
P=0.05
P=0.58
P=0.28
C
Blood Cell Free Cholesterol (mg/g protein)
P=0.08
P=0.06
P=0.40
D
Blood Cell Cholesteryl Esters (mg/g protein)
P=0.01
P=0.16
P=0.02

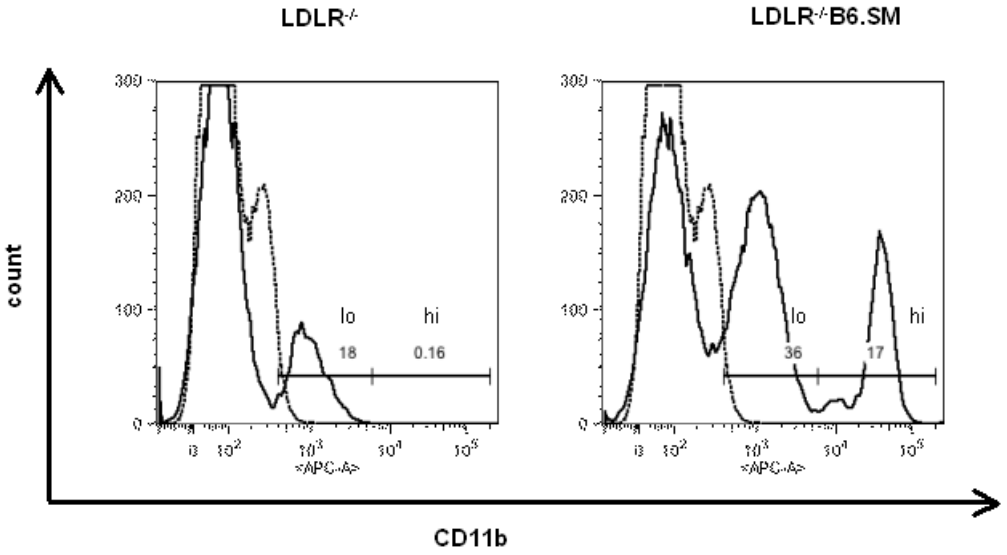
5.3.4 Peripheral blood CD11b$^+$ subsets of hypomorphic sialidase B6.SM/LDLR-/- mice contain higher free cholesterol than LDLR-/- mice after 3 weeks of western diet feeding.

In combination with CD11b, a monocyte/granulocyte marker, filipin III was used to detect intracellular free cholesterol by flow cytometry within leukocytes. In both LDLR-/- and B6.SM/LDLR-/- CD11b$^+$ subsets, two populations were observed, i.e., are CD11b^{lo} and CD11b^{hi} (Fig. 5.3.3 A). Peripheral blood of hypomorphic sialidase B6.SM/LDLR-/- mice had a trend for an increased proportion of each of the CD11b$^+$ subsets in the leukocyte gate with 32.8±10% CD11b^{lo} and 15±11% CD11b^{hi}, compared to 24.3±5.1% CD11b^{lo}, and 2.7±4.5% CD11b^{hi} in LDLR-/- mice (Fig. 5.3.3 B and C). Most importantly, free cholesterol levels were significantly higher in each CD11b$^+$ subset in peripheral blood of hypomorphic sialidase B6.SM/LDLR-/- mice compared to peripheral blood of LDLR-/- mice (Fig.5.3.4 A to C). Mean fluorescence intensity (MFI) of filipin III (using arbitrary units) in CD11b^{lo} cells was 11742±2675 in peripheral blood of LDLR-/- mice and 15492±420 in peripheral blood of hypomorphic sialidase B6.SM/LDLR-/- mice (P=0.037, Fig.5.3.4 B); in CD11b^{hi} cells filipinIII MFI was 37076±4471 in peripheral blood of LDLR-/- mice and 57548±3042 in peripheral blood of B6.SM/LDLR-/- mice (P=0.0014, Fig.5.3.4 C).

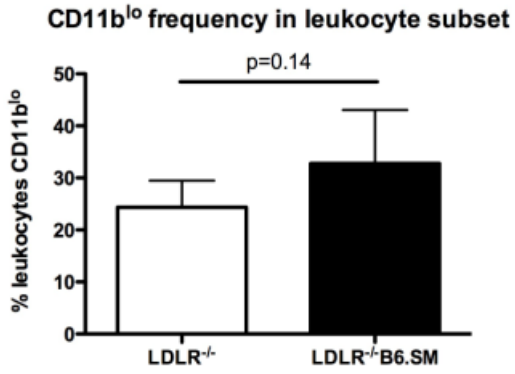
Fig. 5.3.3 $CD11b^+$ subsets in peripheral blood of LDLR-/- and B6.SM/LDLR-/- mice fed western diet for 3 weeks. (A) Two populations of $CD11b^+$ cells ($CD11b^{lo}$, $CD11b^{hi}$) show a trend for increased abundance in B6.SM/LDLR-/- peripheral blood (representative graph of n=3 for each genotype is shown). The abundance of each subset (B) $CD11b^{lo}$, and (C) $CD11b^{hi}$ is compared between LDLR-/- (n=3) and B6.SM/LDLR-/- (n=3) peripheral blood gated on leukocytes.

Fig. 5.3.3

A


LDLR-/-
LDLR-/-B6.SM
count
lo
hi
18
0.16
36
17
CD11b

B


CD11blo frequency in leukocyte subset
p=0.14
% leukocytes CD11blo
LDLR-/-
LDLR-/-B6.SM

C

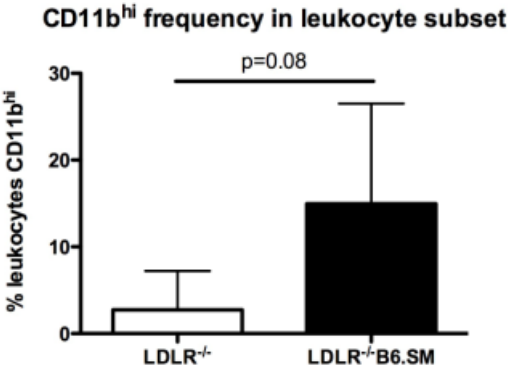
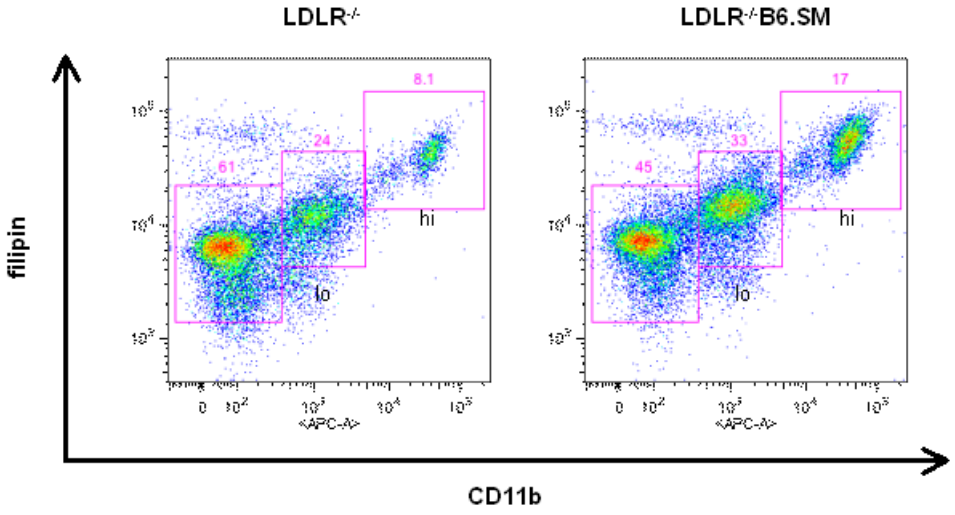

CD11bhi frequency in leukocyte subset
p=0.08
% leukocytes CD11bhi
LDLR-/-
LDLR-/-B6.SM

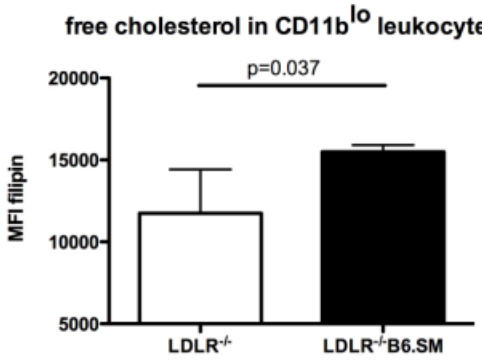
Fig. 5.3.4 Free cholesterol content of CD11b^{+} subsets in LDLR-/- and B6.SM/LDLR-/- peripheral blood. (A) An increase in free cholesterol content is demonstrated by filipin III staining which correlates with the level of CD11b^{+} expression. CD11b^{lo} subsets show the lowest free cholesterol and CD11b^{hi} subsets show the highest levels of free cholesterol. Mean fluorescence intensity (MFI) of filipin staining in each (B) CD11b^{lo} and (C) CD11b^{hi} subsets in LDLR-/- and B6.SM/LDLR-/- reveals that each B6.SM/LDLR-/- CD11b^{+} subset has significantly higher free cholesterol content than each LDLR-/- CD11b^{+} subset.

Fig. 5.3.4

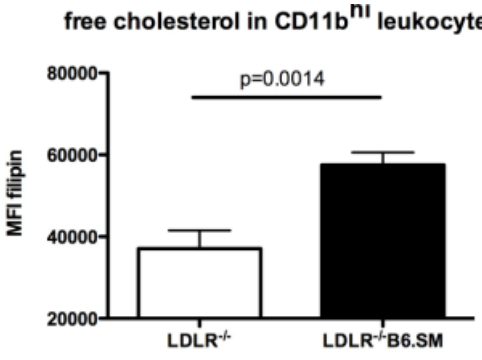
A


LDLR-/-
LDLR-/-B6.SM
filipin
CD11b
8.1
24
61
hi
lo
17
33
45
hi
lo
<APC-A>

B


free cholesterol in CD11blo leukocytes
p=0.037
MFI filipin
20000
15000
10000
5000
LDLR-/-
LDLR-/-B6.SM

C


free cholesterol in CD11bhi leukocytes
p=0.0014
MFI filipin
80000
60000
40000
20000
LDLR-/-
LDLR-/-B6.SM

5.3.5 Peripheral blood CD3ε$^+$ subset of hypomorphic sialidase B6.SM/LDLR-/- shows a trend for increased free cholesterol compared to LDLR-/- after 3 weeks of western diet feeding.

Peripheral blood of hypomorphic sialidase B6.SM/LDLR-/- mice had a trend for a decreased abundance of CD3ε$^+$ cells at 20.6±3.7% of the leukocyte gate versus 25.2±1.8% of the leukocyte gate in peripheral blood of LDLR-/- mice (P=0.06, Fig. 5.3.5 A and B). Most importantly, a trend for increased free cholesterol levels was also observed in peripheral blood CD3ε$^+$ cells of hypomorphic sialidase B6.SM/LDLR-/- mice with MFI 4850±121 compared to 3698±971 in the peripheral blood CD3ε$^+$ subset of LDLR-/- mice (P=0.055, Fig. 5.3.6 A and B).

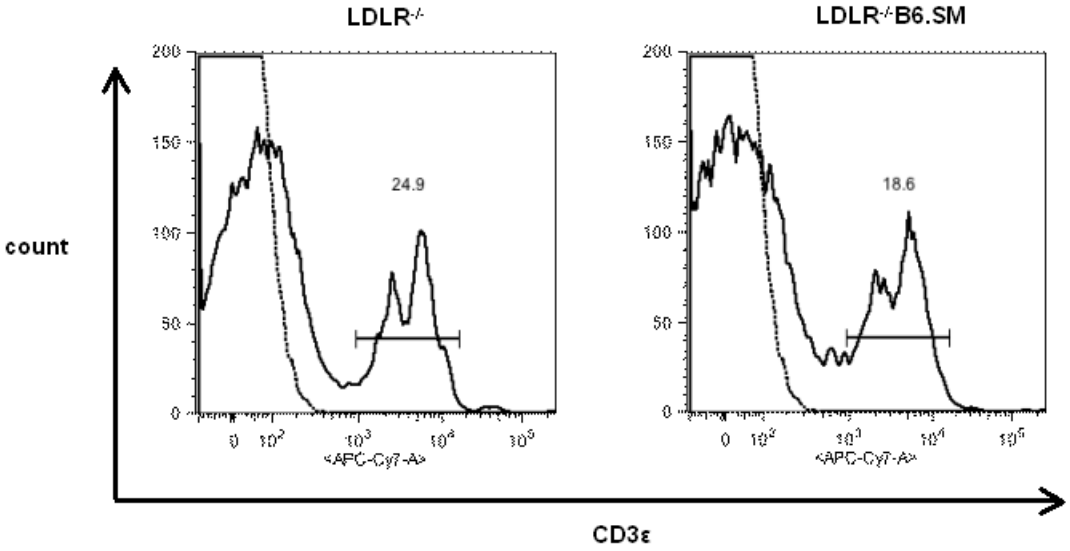
5.3.6 Hypomorphic sialidase hematopoietic cells confer atheroprotection in LDLR-/- mice.

LDLR-/- mice transplanted with B6.SM/LDLR+/+ bone marrow developed smaller atherosclerotic plaques compared to LDLR-/- mice transplanted with LDLR+/+ bone marrow (Fig. 5.3.7). In addition, LDLR-/- mice transplanted with B6.SM/LDLR-/- bone marrow developed smaller atherosclerotic plaques compared to LDLR-/- mice transplanted with LDLR-/- bone marrow. Thus, the groups with higher blood cell lipid levels correlate with lower atherosclerotic lesion areas. This implies that cholesterol efflux may occur which transports cholesterol from blood cells.

Fig. 5.3.5 CD3ε$^+$ subsets in peripheral blood of LDLR-/- and B6.SM/LDLR-/- mice fed western diet for 3 weeks. Representative of n=3 are presented in (A). There was a trend for decreased abundance of CD3ε$^+$ cells in the leukocyte gate of B6.SM/LDLR-/- peripheral blood compared to LDLR-/- peripheral blood (B). Leukocytes from peripheral blood were incubated with hamster anti-mouse-CD3ε-APC-Cy7 (clone 145-2C11). After primary antibodies incubation, leukocytes were incubated with 50 μg/ml filipin III. After filipin III incubation, cells were run through a cell strainer, and then were read on a BD FacsAria III cell sorter. Filipin III was excited using a 375 nm near-UV laser and emission wavelengths were detected using a 450/20 band pass filter. Data was analyzed using FlowJo.

Fig. 5.3.5

A


LDLR-/-
LDLR-/-B6.SM
count
24.9
18.6
CD3ε

B

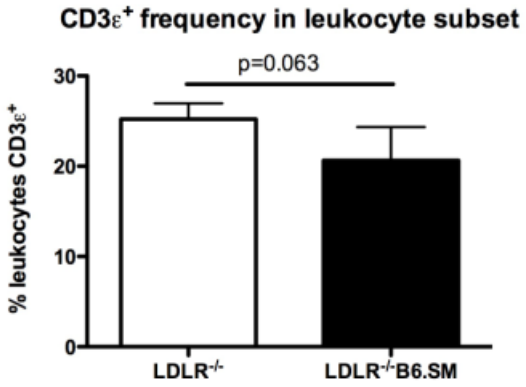
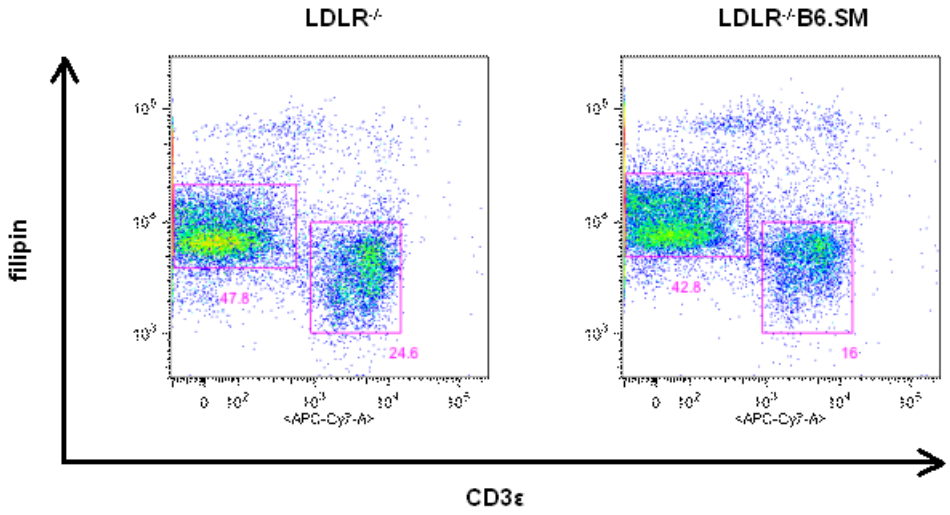

CD3ε+ frequency in leukocyte subset
p=0.063
% leukocytes CD3ε+
LDLR-/-
LDLR-/-B6.SM

Fig. 5.3.6 Free cholesterol content of CD3ε^{+} subsets in LDLR-/- and B6.SM/LDLR-/-peripheral blood. Representative histograms of n=3 are presented in (A). A trend for increased free cholesterol content in hypomorphic sialidase LDLR$^{-/-}$ B6.SM peripheral blood CD3ε^{+} cells can be observed compared to LDLR$^{-/-}$. Leukocytes from peripheral blood were incubated with hamster anti-mouse-CD3ε-APC-Cy7 (clone 145-2C11). After anti-mouse-CD3ε-APC-Cy7 incubation, leukocytes were incubated with filipin III and were read on a BD FacsAria III cell sorter with 375 nm emission wavelengths. Data was analyzed using FlowJo.

Fig. 5.3.6

A


LDLR-/-
LDLR-/-B6.SM
filipin
CD3ε
47.8
24.6
42.8
16
<APC-Cy7-A>

B

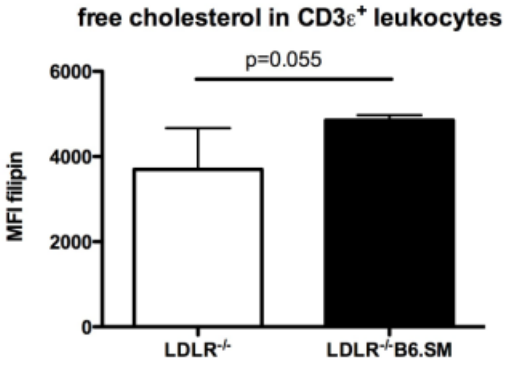
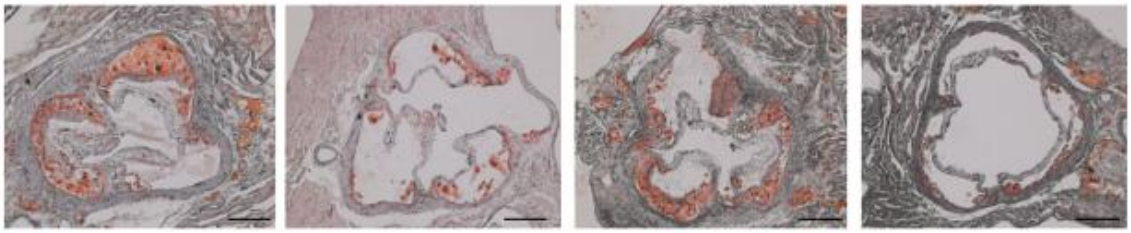

free cholesterol in CD3ε+ leukocytes
p=0.055
MFI filipin
6000
4000
2000
0
LDLR-/-
LDLR-/-B6.SM

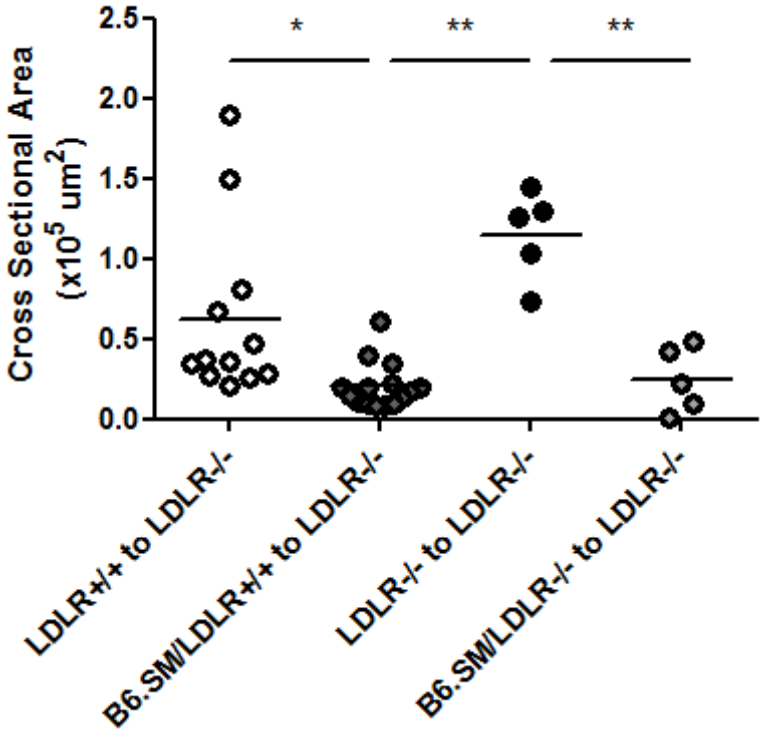
Fig. 5.3.7 Atherosclerotic lesion area in LDLR-/- mice transplanted with LDLR+/+, B6.SM/LDLR+/+, LDLR-/- or B6.SM/LDLR-/- bone marrow after feeding western diet for 6 weeks. LDLR-/- mice with B6.SM/LDLR+/+ bone marrow (n=15; dark grey circle) has lower lesion area compared to LDLR-/- mice with LDLR+/+ bone marrow (n=12; white circle); LDLR-/- mice with B6.SM/LDLR-/- bone marrow (n=5; light grey circle) has lower lesion area compared to LDLR-/- mice with LDLR-/- bone marrow (n=5; black circle). Scale bar = 200μm. Atherosclerosis was quantified as the total cross sectional area of atherosclerotic plaque in each section. * denotes $P<0.05$; ** denotes $P<0.001$.

Fig. 5.3.7

A B C D



E



5.3.7 Hypomorphic sialidase bone marrow derived macrophages have increased cholesterol efflux to HDL.

We assayed cholesterol efflux of bone marrow derived macrophages to HDL. B6.SM macrophages have significantly higher efflux to HDL compared to C57Bl/6 controls (Fig. 5.3.8 A), enabling them to clear intracellular cholesterol more efficiently. In addition, B6.SM macrophages have significantly lower efflux to LDL compared to C57Bl/6 controls (Fig. 5.3.8 B). Efflux to BSA control acceptor is minimal. This data can aid in explaining how increased lipid uptake by the macrophage can be beneficial, if these lipids can leave the cell via reverse cholesterol transport. Hypomorphic sialidase expression appears to accelerate cholesterol uptake followed by increased efflux, which could result in a net clearance of lipids from the atherosclerotic liesion and cause atheroprotection.

5.3.8 Hypomorphic sialidase bone marrow derived macrophages have an increased expression of ABCG1.

B6.SM mouse BMDMs have lower levels of LDLR protein when untreated or with VLDL, while showing no difference when treated with LDL or acLDL (Fig. 5.3.9). ABCG1 protein levels are higher in B6.SM BMDMs treated with LDL acLDL or VLDL, compared to C57Bl/6 controls, while showing no changes in ABCA1 protein (Fig. 5.3.9). Increased ABCG1 would lead to increased cholesterol efflux to HDL and atheroprotection, as cholesterol would be removed from foam cells in atherosclerotic lesions. This data implicates sialidase in regulation of macrophage cholesterol levels and regulation of uptake and efflux, although the exact mechanism is still unknown. Hypomorphic sialidase expression is reducing the quantity of LDLR protein, either by

reducing the cell's cholesterol need or through other post transcriptional mechanisms. Nevertheless, this is indicative of increased lipid levels in the cell. The combined uptake of lipid and increase efflux can be atheroprotective by having a higher rate of efflux compared to uptake. Higher ratio of efflux to uptake is a causative factor in plaque regression (Ibanez et al., 2007). Hypomorphic sialidase expression also causes increases in ABCG1 protein levels, but not ABCA1, after lipid loading of macrophages, which will lead to increased efflux to HDL.

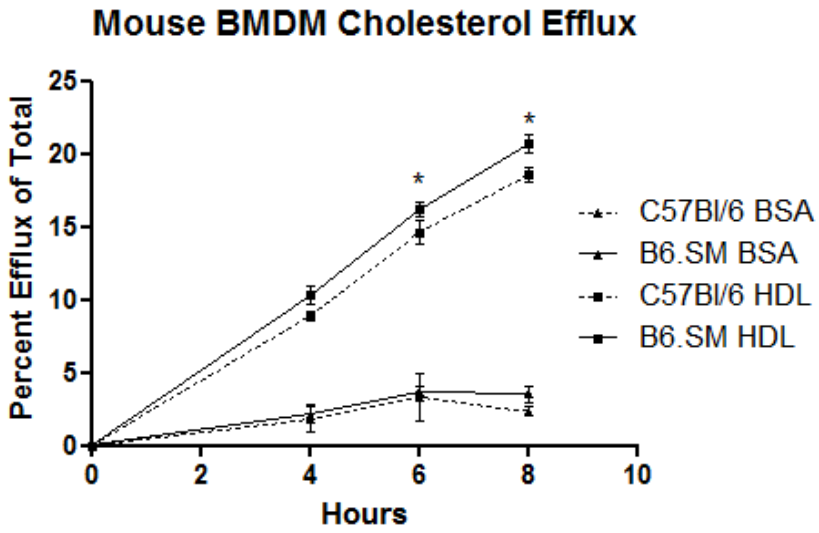
5.3.9 Hypomorphic sialidase intraperitoneal macrophages accumulate more lipids after LDL treatment.

We analyzed LDL uptake *in vitro* using Oil Red O staining of mouse intraperitoneal thiglycollate-elicited macrophages. B6.SM macrophages have significantly higher Oil Red O staining after LDL treatment, whereas C57Bl/6 and LDLR-/- macrophages do not (Fig. 5.3.10). These macrophages show just slight increases in lipid staining after LDL treatment, as expected, but nevertheless, hypomorphic sialidase expression resulted in more lipid accumulation in IP macrophages. This increase could be explained by modulation of LDLR function and/or expression due to hypomorphic sialidase expression.

Fig. 5.3.8 Bone marrow derived macrophages from hypomorphic sialidase mice have increased efflux to HDL compared to C57Bl/6 controls. Hypomorphic sialidase expression increases cholesterol efflux in C57Bl/6 macrophages. BMDMs were loaded with [^{3}H] cholesterol and assayed for efflux to (A) HDL and (B) LDL in the media. B6.SM macrophages show a significant increase in efflux to HDL at 6 and 8 hours, and a significant decrease in efflux to LDL at 8 hours. These imply that increased cholesterol uptake by the hypomorphic sialidase macrophage can be atheroprotective, providing that the cholesterol can leave the macrophage via reverse cholesterol transport. n=3, * denotes $P<0.05$.

Fig. 5.3.8

A


Mouse BMDM Cholesterol Efflux
Percent Efflux of Total
25
20
15
10
5
0
0
2
4
6
8
10
Hours
*
*
C57Bl/6 BSA
B6.SM BSA
C57Bl/6 HDL
B6.SM HDL

B

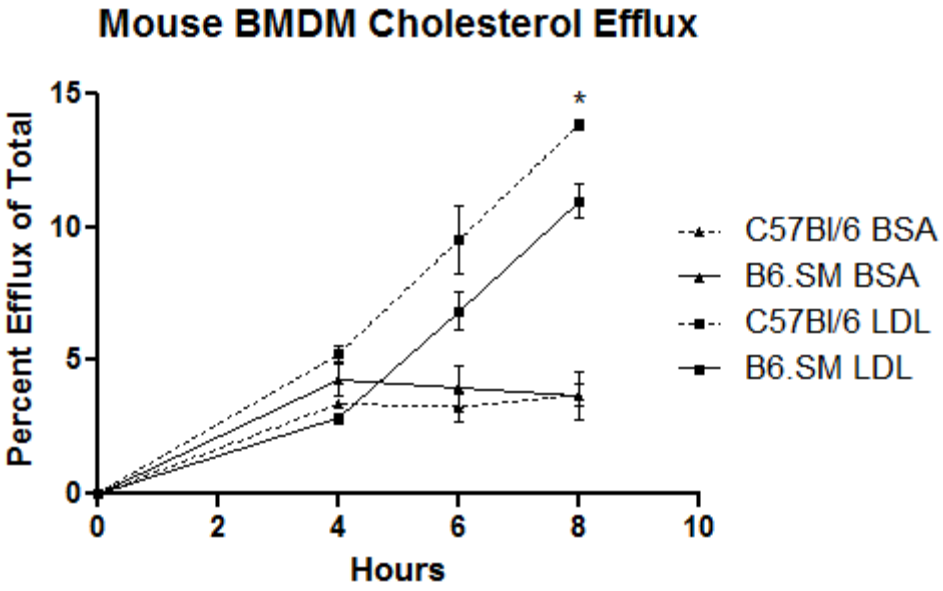
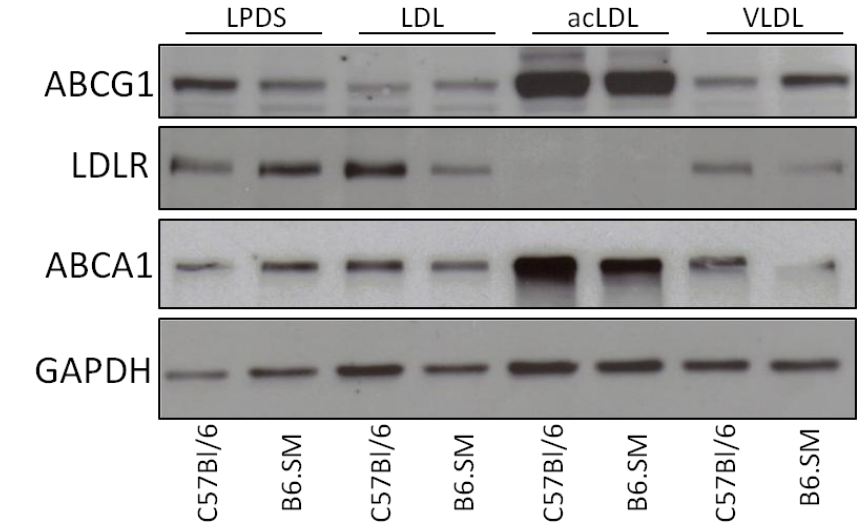
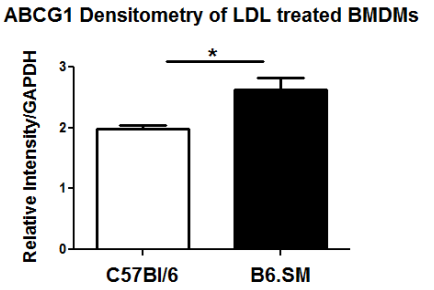
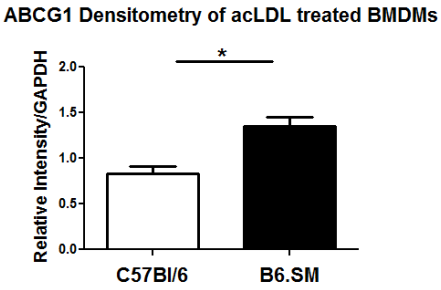

Mouse BMDM Cholesterol Efflux
Percent Efflux of Total
15
10
5
0
0
2
4
6
8
10
Hours
*
C57Bl/6 BSA
B6.SM BSA
C57Bl/6 LDL
B6.SM LDL

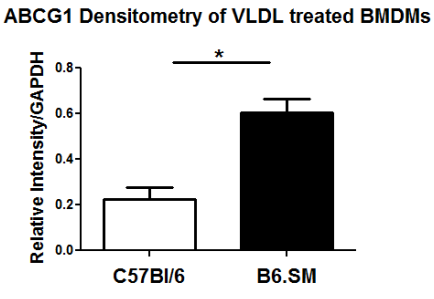
Fig. 5.3.9 B6.SM BMDMs have higher levels of ABCG1 protein and lower levels of LDLR protein. ABCG1 levels are higher in B6.SM cells when treated with lipoproteins, while LDLR levels are lower. ABCA1 shows no significant difference in any group. Lysates of bone marrow derived macrophages were analyzed by immunoblotting and band intensity was normalized to GAPDH. Hypomorphic sialidase expression results in protein expression changes that indicate increased cholesterol uptake and efflux. n=3, * denotes $P<0.05$.

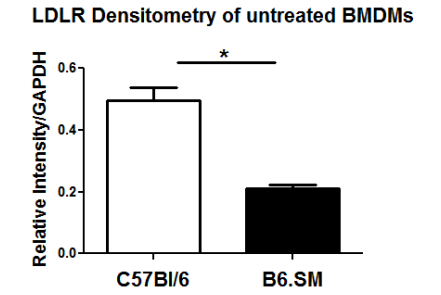
Fig. 5.3.9


LPDS
LDL
acLDL
VLDL
ABCG1
LDLR
ABCA1
GAPDH
C57Bl/6
B6.SM
C57Bl/6
B6.SM
C57Bl/6
B6.SM
C57Bl/6
B6.SM


ABCG1 Densitometry of LDL treated BMDMs
Relative Intensity/GAPDH
*
C57Bl/6
B6.SM


ABCG1 Densitometry of acLDL treated BMDMs
Relative Intensity/GAPDH
*
C57Bl/6
B6.SM


ABCG1 Densitometry of VLDL treated BMDMs
Relative Intensity/GAPDH
*
C57Bl/6
B6.SM


LDLR Densitometry of untreated BMDMs
Relative Intensity/GAPDH
*
C57Bl/6
B6.SM

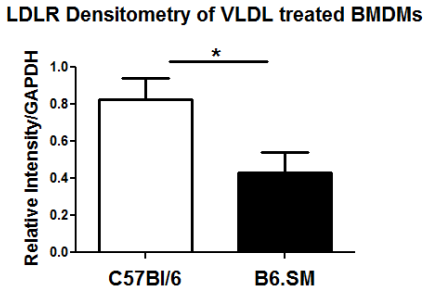
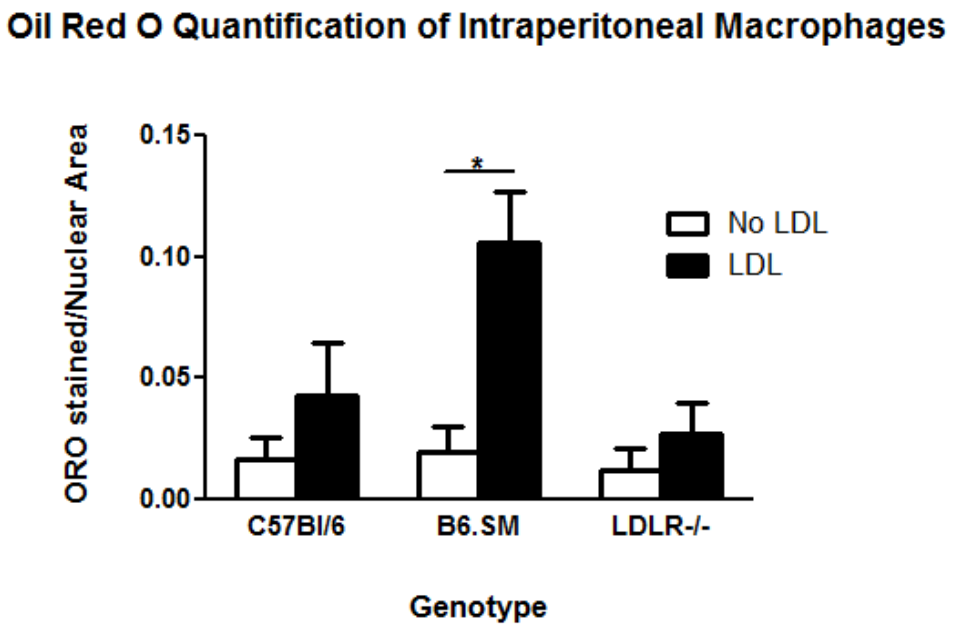

LDLR Densitometry of VLDL treated BMDMs
Relative Intensity/GAPDH
*
C57Bl/6
B6.SM

Fig. 5.3.10 Intraperitoneal hypomorphic sialidase macrophages accumulate more lipids after LDL treatment compared to C57Bl/6 and LDLR -/- controls as measured using ORO staining. Hypomorphic sialidase expression increases LDL uptake in thioglycollate-elicited macrophages compared to LDLR-/- and C57BL/6, after 48hour. Hypomorphic sialidase intraperitoneal macrophages accumulate more lipids after 48 hour LDL incubation than controls. Visual quantification of Oil Red O stained cells shows a significant increase in B6.SM cells that received LDL vs. no LDL with no such effect in wild-type and LDLR-/- controls. n=3, * denotes $P<0.05$.

Fig. 5.3.10


Oil Red O Quantification of Intraperitoneal Macrophages
ORO stained/Nuclear Area
0.15
0.10
0.05
0.00
*
No LDL
LDL
C57Bl/6
B6.SM
LDLR-/-
Genotype

5.4 Discussion

In the 1980s, Brown and Goldstein have identified LDLR as the receptor that can affect lipoprotein cholesterol levels via binding and uptaking LDL (Tolleshaug et al., 1983; Ishibashi et al., 1993). After the discovery of LDLR, other family members of LDLR have been identified, including VLDL (Sakai et al., 1994) and LRP (Kristensen et al., 1990). From the 1990s to the 2000s, researchers have started to focus at the effects of bone marrow-derived blood cells, including monocytes-derived macrophages, in affecting lipoprotein cholesterol uptake and atherosclerosis (Boisvert et al., 1997; Fazio et al., 1997; Herijgers et al., 1997; Herijgers et al., 2000; Linton et al., 1999). Blood cell LDLR has been shown to play roles in affecting VLDL- and/or IDL/LDL-cholesterol levels as demonstrated by the FPLC profiles (Boisvert et al., 1997; Herijgers et al., 1997; Herijgers et al., 2000; Linton et al., 1999). In addition to blood cell LDLR, blood cell VLDLR and LRP have been shown to play roles in affecting cholesterol levels (Van Eck et al, 2005; Overton et al., 2007). Blood cell LDLR and VLDLR deficiency appears to play a prominent role in in increasing VLDL-cholesterol after feeding western diet (Linton et al., 1999; Herijgers et al., 2000; Van Eck et al., 2005). These studies imply that blood cell lipoprotein receptors, including monocytic LDLR, VLDL and LRP may be able to take up lipoprotein cholesterol in blood. Nevertheless, this implication is not addressed in these studies. Instead of the monocytic uptake of lipoprotein cholesterol, they have shown that macrophage lipoprotein receptors are capable of binding and taking up VLDL-cholesterol (Herijgers et al, 2000; Overton et al., 2007). Up to the year of 2012, only a few studies have been published showing monocytic uptake of lipoprotein cholesterol

(Nguyen et al., 1988; Pietsch et al., 1996). Monocytic uptake of lipoprotein cholesterol via VLDLR and/or LRP is still unknown. Most importantly, the effects of sialidase on blood cell lipoprotein receptor function and subsequently lipoprotein metabolism and atherogenesis as a whole, are largely unknown. Therefore, we have addressed this question by determining the effects of blood cell hypomorphic sialidase expression on lipoprotein cholesterol levels and atherosclerosis Our studies have shown that LDLR-/- mice with bone marrow transplanted from hypomorphic sialidase mice had less atherosclerotic lesion area than LDLR-/- mice transplanted with intact sialidase bone marrow. Interestingly, our results have shown that this decrease in atherosclerotic lesion area corresponds to a reduction in serum lipoprotein cholesterol levels in mice transplanted with hypomorphic sialidase hematopoietic cells which is caused by an increase in the cholesterol levels in hypomorphic sialidase peripheral leukocytes, possibly via an increase in VLDL uptake.

We have observed that the decrease in the cholesterol concentrations in VLDL is much greater when LDLR is absent in hypomorphic sialidase blood cells. These results implied that the absence of LDLR in blood cells can enhance the extent of the reduction of cholesterol concentrations in VLDL-sized particles. In addition, the presence or absence of LDLR in blood cells seemed to play a role for the extent of decrease caused by hypomorphic sialidase expression. In other words, the effects of hypomorphic sialidase expression appeared to be dependent on the presence or absence of LDLR in blood cells. We observed that there was still a difference in the VLDL-lipids production rates between mice with and without hypomorphic sialidase expression in the absence of LDLR (Fig. S5.3.2). Therefore, LDLR could not be the

only contributing factor for the decreased cholesterol concentration in the VLDL-sized particles. Nonetheless, the decrease in the VLDL-sized particles was greater when LDLR was absent in the blood cells. Therefore, other receptors (LRP and VLDLR) that can take up VLDL must be playing a role when the LDLR is absent. VLDLR and LRP play roles in the removal of ApoB48-containing lipoproteins from the blood and are present in macrophages (Willnow et al., 1994; Kobayashi et al., 1996; Kozarsky et al., 1996; Rohlmann et al., 1998; van Dijk et al., 1998; Oka et al., 2001). Also, it has been suggested that VLDLR and LRP play a significant role in ApoB48-containing lipoproteins uptake when LDLR is absent (Veniant et al., 1998; Martins et al., 2000; Tacken et al., 2000). Overall, hypomorphic sialidase monocytes may be playing a role in affecting the uptake of cholesterol via VLDLR and/or LRP contributing to the decrease in cholesterol levels in the serum. Nevertheless, we are still uncertain whether monocyte is the only cell type responsible for this cholesterol uptake in the peripheral blood.

In order to answer the question from our previous discussion emphasizing which cell type(s) is/are responsible for governing the uptake of cholesterol in the peripheral blood, we have measured the abundance of monocytes and granulocytes with CD11b primary antibody through flow cytometry. There is a trend of increased abundance of monocytes in B6.SM/LDLR-/- mice compared to LDLR-/- mice. Most importantly, this increased abundance of monocytes is accompanied by a significant increased cellular quantity of free cholesterol as illustrated by the filipin marker which detects non-esterified cholesterol. In addition to monocytes and granulocytes, B6.SM/LDLR-/- mice also have slight changes in abundance of T cells as illustrated by the CD3ε

marker. Specifically, the B6.SM/LDLR-/- T cells also have higher free cholesterol levels compared to LDLR-/- as demonstrated by filipin marker although the increase is not as great as that of the $CD11b^+$ cells. Taken together, these results suggest that $CD11b^+$ cells, such as, monocytes, are more important in regulating the uptake of cholesterol compared to T cells. In addition, these results tell us that monocyte is a cell type that is responsible for the observed increase in blood cell lipid contents in our previous section showing that LDLR-/- mice transplanted with hypomorphic sialidase bone marrow has higher blood cell total cholesterol levels compared to LDLR-/- mice transplanted with bone marrow with the presence of sialidase. Our results have indicated that a decrease in atherosclerotic lesion area in LDLR-/- mice transplanted with hypomorphic sialidase bone marrow corresponds to an increase in cholesterol levels in monocytes. Reverse cholesterol transport may be playing a role such that hypomorphic sialidase macrophages in intima have a higher rate of cholesterol efflux although they are higher in cholesterol levels before they migrate into the intima. The downstream effect of higher cholesterol efflux causes a reduction in the atherosclerotic lesion area in LDLR-/- mice transplanted with hypomorphic sialidase bone marrow. In fact, results have shown that the expression of ABCG1 in the bone marrow-derived macrophages of hypomorphic sialidase mice is significantly higher compared to controls. In addition, bone marrow-derived macrophages isolated from B6.SM mice have a significantly higher rate of cholesterol efflux to HDL compared with bone marrow macrophages isolated from C57Bl/6 mice. Furthermore, peritoneal macrophages of B6.SM mice have higher cholesterol contents compared to C57Bl/6 mice as shown by Oil Red O staining. Higher cholesterol contents in macrophages can cause the activation of liver X recepters (LXRs) (Zelcer and Tontonoz, 2006). LXR,

being an important transcriptional regulator of lipoprotein metabolism, can enhance transcription of target genes, including ABCG1 (Repa et al., 2000; Kennedy et al., 2005). Taken together, higher cholesterol uptake exists in serum hypomorphic sialidase monocytes. These monocytes with higher cholesterol levels are differentiated to macrophages in the intima. Concurrently, higher cholesterol levels cause the activation of LXR which increases the expression of ABCG1 in hypomorphic sialidase macrophages. Increased ABCG1 expression in hypomorphic sialidase macrophages causes an increase in cholesterol efflux. The downstream effect of these causes a reduction in atherosclerotic lesions in mice with hypomorphic sialidase bone marrow-derived blood cells.

Overall, these studies lead us to a novel concept of blood cells manipulating the lipoprotein metabolism in serum. In conclusion, our results demonstrated that hypomorphic sialidase hematopoietic cells confer atheroprotection in LDLR-/- mice by having lower serum lipoprotein cholesterol levels. Specifically, the absence of LDLR in leukocytes, e.g., monocytes, increases the extent of lipoprotein cholesterol uptake. Furthermore, this study shows a novel role for hypomorphic sialidase leukocytes in the regulation of lipoprotein metabolism. Most importantly, these sialidase mediated effects of cholesterol uptake in leukocytes are prominent in reducing atherosclerosis, and thus, will shed light on anti-atherosclerotic therapy.

Supplementary Section

S5.3.1 Hypomorphic sialidase expression decreases cholesterol levels in VLDL and LDL-sized lipoproteins in male LDLR-/- mice.

In order to test the impact of combined deficiencies in LDLR and sialidase on serum cholesterol levels of VLDL and LDL, serum collected from male LDLR-/- and B6.SM/LDLR-/- mice, fed western diet for 6 weeks, were fractionated by Fast Protein Liquid Chromatography (FPLC). Male hypomorphic sialidase B6.SM/LDLR-/- mice show significantly lower cholesterol levels of VLDL and LDL when compared with male LDLR-/- mice (Fig. S5.3.1). Thus, hypomorphic sialidase expression has impacts on cholesterol levels in the VLDL and LDL.

S5.3.2 Hypomorphic sialidase expression decreases serum and increases hepatic lipids levels in male LDLR-/- mice.

Enzymatic lipid analysis revealed a significant decrease in both serum cholesterol and cholesteryl esters concentrations in hypomorphic sialidase LDLR-/- male mice (Table S5.3.1). These results were consistent with our FPLC cholesterol profiles which also showed a decrease in the cholesterol concentrations (Table S5.3.1). The decrease in the cholesterol concentrations originates mainly from reduced cholesterol levels of VLDL and IDL/LDL-sized particles. The VLDL, IDL and LDL particles contain a significant amount of cholesteryl esters. Therefore, the decrease in the total serum cholesteryl esters in hypomorphic sialidase LDLR-/- male mice was reflected in the FPLC profile. Hepatic lipid concentrations were determined and an increase in the hepatic cholesteryl esters and triglyceride concentrations were observed in

hypomorphic sialidase LDLR-/- male mice compared to LDLR-/- male mice (Table S5.3.1). The increase in the cholesteryl esters in the liver and the decrease in the cholesteryl esters in the serum implied that hypomorphic sialidase expression may affect the cholesteryl esters to be secreted from the liver. Cholesteryl esters are mainly secreted from the liver in VLDL. Therefore, we performed VLDL–lipids production rate analysis in LDLR-/- and B6.SM/LDLR-/- male mice.

Fig. S5.3.1 FPLC cholesterol profiles of male LDLR-/- and male B6.SM/LDLR-/- mice. B6.SM/LDLR-/- male mice (n=3) fed western diet for 6 weeks starting at age of 3 months have a significant decrease in cholesterol level in VLDL and LDL-sized particles compared to LDLR-/- mice (n=3). (VLDL-sized: Very low density lipoprotein sized particle, IDL-sized: Intermediate density lipoprotein sized particle, LDL-sized: Low density lipoprotein sized particle, HDL-sized: High density lipoprotein sized particle). Each circle represents mean±SE. * denotes $P<0.05$.

Fig. S5.3.1

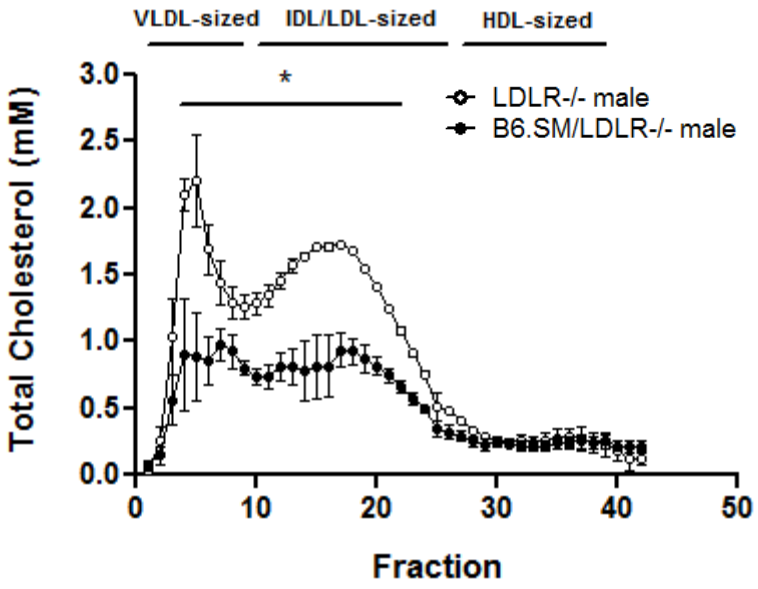

VLDL-sized
IDL/LDL-sized
HDL-sized
*
LDLR-/- male
B6.SM/LDLR-/- male
Total Cholesterol (mM)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
10
20
30
40
50
Fraction

Table S5.3.1 Serum and hepatic lipids levels in male LDLR-/- and B6.SM/LDLR-/- mice. An increase in the hepatic triglyceride (P=0.02) and a trend of increase in cholesteryl esters levels (P=0.12) in male B6.SM/LDLR-/- mice (n=5) fed western diet for 6 weeks was observed compared to male LDLR-/- mice (n=4). Consistent with the FPLC, a significant decrease in total serum cholesterol and cholesteryl esters were observed in B6.SM/LDLR-/- mice (n=3) compared to LDLR-/- mice (n=4) fed western diet for 6 weeks (* denotes $P<0.05$). Mean±SE are shown.

Table S5.3.1

	Hepatic total cholesterol (mg/g liver)	Serum total cholesterol (mM)	Hepatic free cholesterol (mg/g liver)	Serum free cholesterol (mM)	Hepatic cholesteryl esters (mg/g liver)	Serum cholesteryl esters (mM)	Hepatic triglyceride (mg/g liver)	Serum triglyceride (mM)
LDLR-/- Male	3.66±0.21	29.23±0.20	2.88±0.17	13.50±1.51	0.78±0.04	15.76±1.52	8.65±1.71	15.12±0.70
B6.SM/LDLR-/- Male	3.54±0.36	22.19±0.83*	2.21±0.50	10.75±0.20	1.33±0.38	11.37±0.69*	14.33±1.54*	17.79±3.76

S5.3.3 Hypomorphic sialidase expression decreases hepatic VLDL production in male LDLR-/- mice.

Majority of the VLDL is produced by the liver. In order to assess the kinetics of the lipoprotein secretion in the presence of hypomorphic sialidase expression, cholesterol and triglyceride concentrations were measured after lipoprotein lipase inhibition with Triton WR1339. Our results indicate a significant increase in VLDL-triglyceride concentration over the time period of 4 hours in the male B6.SM/LDLR-/- and LDLR-/- mice (Fig. S5.3.2 A). There were still differences in VLDL-triglyceride concentrations in 4-hour period after inhibiting lipoprotein lipase. Similar results were obtained for the VLDL-cholesterol, VLDL-free cholesterol and VLDL-cholesteryl esters (Fig. S5.3.2 B, C and D). In order to assess any difference in VLDL-lipids production rate between hypomorphic sialidase mice and controls with the absence of LDLR., the slope of the regression line over a 4-hour period of time was determined and found to show a significant decrease in VLDL-triglyceride, VLDL-cholesterol and VLDL-cholesteryl esters production rates over the 4-hour period (Table S5.3.2).

Fig. S5.3.2 Hepatic VLDL production in male LDLR-/- and B6.SM/LDLR-/- mice. LDLR-/- (n=3) and B6.SM/LDLR-/- mice (n=3) were fasted overnight and injected with Triton WR1339 (500mg/kg). Serum samples were drawn at 0, 1, 2, 3, and 4 hours after the injection of Triton WR1339. There is a decrease in hepatic (A) VLDL-triglyceride, (B) VLDL-cholesterol, (C) VLDL-free cholesterol and (D) VLDL-cholesteryl esters concentrations in different indicated times after 0 hour in LDLR-/- mice and B6.SM/LDLR-/- mice. Each circle represents mean±SE. * denotes $P<0.05$.

Fig. S5.3.2

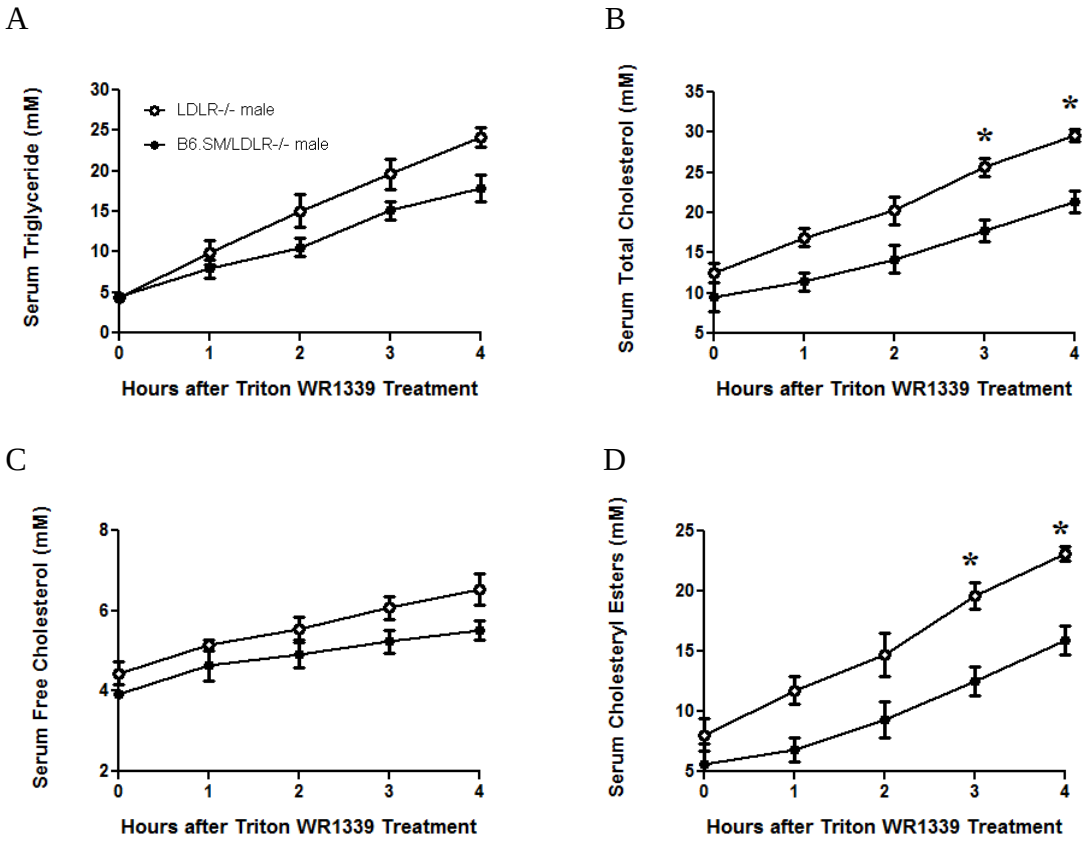

A
B
C
D
LDLR-/- male
B6.SM/LDLR-/- male
Serum Triglyceride (mM)
Serum Total Cholesterol (mM)
Serum Free Cholesterol (mM)
Serum Cholesteryl Esters (mM)
Hours after Triton WR1339 Treatment
*

Table S5.3.2 Hepatic VLDL production rates in male LDLR-/- and B6.SM/LDLR-/- mice. Male LDLR-/- (n=3) and B6.SM/LDLR-/- mice (n=3) were fasted overnight and Triton WR1339 was administered (500mg/kg). Serum was collected at 0, 1, 2, 3, and 4 hours after the administration of Triton WR1339. There is a decrease in hepatic VLDL-triglyceride, VLDL-cholesterol, and VLDL-cholesteryl esters esters production rates in male B6.SM/LDLR-/- mice compared to LDLR-/- mice. Mean±SE are shown.

Table S5.3.2

	Triglyceride PR (mM/hr	Total Cholesterol PR (mM/hr	Free Cholesterol PR (mM/hr	Cholesteryl Esters PR (mM/hr)
LDLR-/- Male	4.94 + 0.27	4.31 + 0.12	0.51 + 0.06	3.80 + 0.15
B6.SM/LDLR-/- Mal	3.38 + 0.33	3.01 + 0.17	0.38 + 0.02	2.63 + 0.18
P value	0.02	0.003	0.1	0.004

S5.3.4 The effects of hypomorphic sialidase expression on hepatic VLDL-ApoB-100 and –ApoB-48 secretion in male LDLR-/- mice.

After observing changes in the VLDL-lipid production rare between male B6.SM/LDLR-/- and LDLR-/- mice, we want to determine whether the observed difference is caused by decreased VLDL particle production. Thus, we measured the protein expression of ApoB-100 and ApoB-48. We did not observe any significant changes in rate of ApoB-100 secretion in the 4-hour period after Triton WR1339 injection (Fig. 5.3.3 A). However, a trend toward a decrease in ApoB-48 levels were observed in male B6.SM/LDLR-/- mice compared to male LDLR-/- mice (Fig. 5.3.3 B). This implies that receptors that can bind ApoB-48 may be involved in the uptake of VLDL particle that contains ApoB-48.

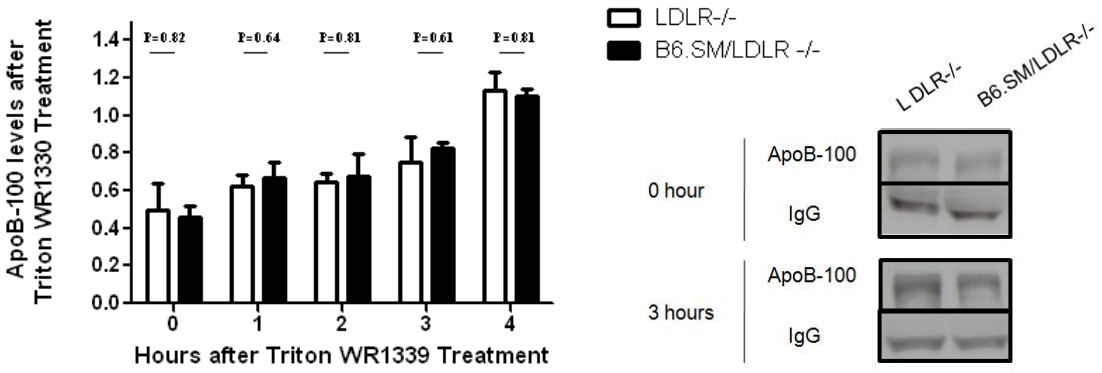
S5.3.5 Hypomorphic sialidase expression decreases atherogenesis in male LDLR-/- mice.

In order to see whether the decrease in cholesterol level is reflective of atherosclerosis in hypomorphic sialidase LDLR-/- male mice, atherosclerotic lesion areas were evaluated at the aortic sinus of male LDLR-/- and B6.SM/LDLR-/- mice. There was a trend of decrease in atherosclerotic lesion area at the aortic sinus of male B6.SM/LDLR-/- mice compared to male LDLR-/- mice (P=0.06) (Fig. 5.3.4). Thus, hypomorphic sialidase expression is atheroprotective in male LDLR-/- mice.

Fig. S5.3.3 Hepatic VLDL-ApoB-100 and –ApoB-48 secretion in male LDLR-/- and B6.SM/LDLR-/- mice. (A) Serum ApoB-100 levels between male LDLR-/- (n=3) and B6.SM/LDLR-/- mice (n=3). No differences in ApoB-100 levels were found between the two mice strain at each time interval. (B) Serum ApoB-48 levels between male LDLR-/- (n=3) and male B6.SM/LDLR-/- mice (n=3). Differences in ApoB-48 levels were found between male LDLR-/- and B6.SM/LDLR-/- mice indicating differences in the uptake of VLDL via ApoB-48 receptors. For the determination of ApoB-100 and ApoB-48 secretion, serum samples were obtained at 0, 1, 2, 3, and 4 hours were probed with ApoB (goat anti-ApoB, 1:6000) and HRP-conjugated antibodies (donkey anti-goat, 1:10000). ApoB levels were determined by the intensities of ApoB bands normalized to IgG bands.

Fig. S5.3.3

A


ApoB-100 levels after Triton WR1330 Treatment
P=0.82
P=0.64
P=0.81
P=0.61
P=0.81
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
1
2
3
4
Hours after Triton WR1339 Treatment
LDLR-/-
B6.SM/LDLR -/-
LDLR-/-
B6.SM/LDLR-/-
0 hour
ApoB-100
IgG
3 hours
ApoB-100
IgG

B

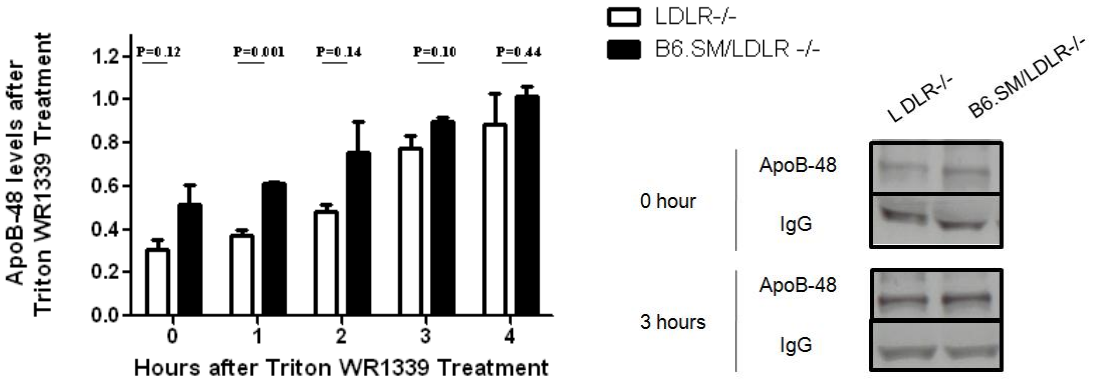
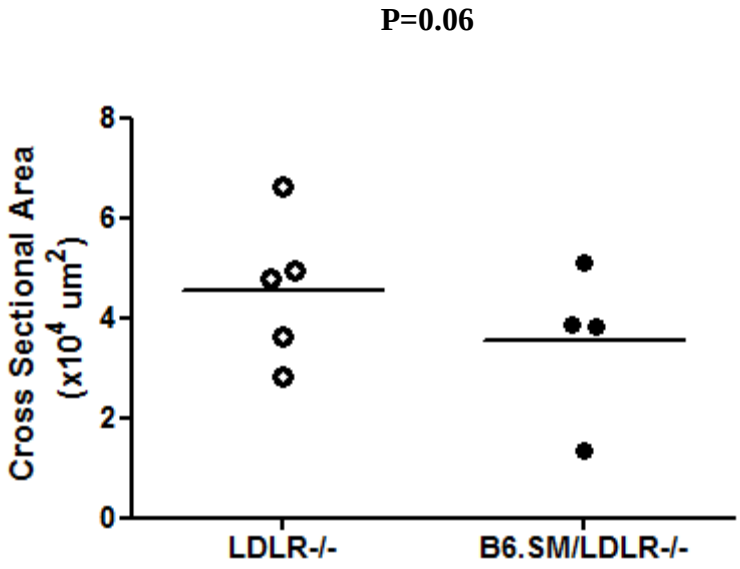

ApoB-48 levels after Triton WR1339 Treatment
P=0.12
P=0.001
P=0.14
P=0.10
P=0.44
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
1
2
3
4
Hours after Triton WR1339 Treatment
LDLR-/-
B6.SM/LDLR -/-
LDLR-/-
B6.SM/LDLR-/-
0 hour
ApoB-48
IgG
3 hours
ApoB-48
IgG

Fig. S5.3.4 Atherosclerotic lesion area in male LDLR-/- and B6.SM/LDLR-/- mice after feeding western diet for 6 weeks. A decrease in atherosclerotic lesion area at the aortic sinus of B6.SM/LDLR-/- male mice (n=4) compared to LDLR-/- male mice (n=5) was observed (P=0.06). Atherosclerosis was quantified as the total cross sectional area of atherosclerotic plaque in each section.

Fig. S5.3.4



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Chapter 6
Significance, Conclusions and Looking Forward

Atherosclerosis is an inflammatiory event which is dependent on lipoprotein metabolism. Sialylation of certain cell surface molecules on leukocytes, including macrophages, neutrophils and T-cells, may modulate inflammation. Modulation of the level of sialylation on apolipoproteins in lipoproteins, including VLDL, LDL and HDL, may affect lipoprotein metabolism. We have generated hypomorphic sialidase (*neu1*) mice, B6.SM, by backcrossing a mutation in the inbred mouse strain, SM/J, on C57Bl/6 background. B6.SM mice have decreased serum cholesterol levels compared to C57Bl/6 mice. In addition, the hepatic VLDL-triglyceride, total cholesterol, free cholesterol and cholesteryl esters production rates of the B6.SM mice are decreased compared to C57Bl/6 mice. Furthermore, B6.SM mice have decreased hepatic expression of microsomal triglyceride transfer protein (MTP), which loads triglyceride and cholestesteryl esters onto ApoB during the formation of VLDL particle, compared to C57Bl/6 mice. B6.SM mice also have decreased hepatic expression of sterol responsive element binding protein-2 (SREBP-2), a transcription factor that activates transcription of genes including MTP. Taken together, reduced SREBP-2 expression causes a decrease in MTP expression which causes a reduction in hepatic VLDL-lipid production. In addition to VLDL production, hypomorphic sialidase expression also plays a role in VLDL/LDL cholesterol clearance by maintaining the hepatic protein expression of LDLR without any changes in the LDLR mRNA levels. B6.SM mice have reduced expression of proprotein convertase subtilisin/kexin 9 (PCSK9), a SREBP-2 target gene that affects recycling of the LDLR. Taken together, reduced SREBP-2 expression causes a decrease in PCSK9 expression which causes an

increase in LDLR recycling. The increase in LDLR recycling causes an increase in VLDL/LDL clearance in B6.SM mice compared to C57Bl/6 mice. In conclusion, hypomorphic sialidase expression alters lipoprotein metabolism by both decreasing hepatic cholesterol production and increasing cholesrerol clearance.

We have generated B6.SM/*apoe-/-* mice harboring a mutated neu1 sialidase gene by crossing B6.SM mice with *apoe-/-* mice in order to determine the effects of hypomorphic sialidase expression on atherogenesis. B6.SM/*apoe-/-* mice have decreased atherosclerotic lesion areas at the aortic sinus compared to *apoe-/-* mice. When *apoe-/-* mice were treated with 2-deoxy-2,3-dehydro-N-acetylneuraminic acid (DANA), a sialidase inhibitor, atherosclerotic lesion areas were decreased compared to saline-treated *apoe-/-* mice. In addition, B6.SM/*apoe-/-* mice transplanted with B6.SM/*apoe-/-* bone marrow have decreased atherosclerotic lesion areas compared to B6.SM/*apoe-/-* mice receiving *apoe-/-* bone marrow. We have dissected the mechanism for the atheroprotective effect of B6.SM/*apoe-/-* mice by showing that B6.SM/*apoe-/-* mice have decreased atherogenic VLDL-lipid production rate and decreased recruitment and infiltration of monocytes in the atherosclerotic lesions compared to *apoe-/-* mice. In conclusion, hypomorphic sialidase expression is atheroprotective in *apoe-/-* mice and inhibiting sialidase is a potential treatment for atherosclerosis.

Leukocytes, including monocyte-derived macrophages, express lipoprotein receptors which enable the binding and uptaking of atherogenic lipoproteins, i.e., VLDL, IDL and LDL. Many lipoproteins are sialylated and their receptors have different

efficiency of their binding and uptake with modulation of sialylation. We have transplanted bone marrow from LDLR+/+, B6.SM/LDLR+/+, LDLR-/- or B6.SM/LDLR-/- mice into LDLR-/- mice in order to determine whether sialidase affects leukocyte mediated lipoprotein cholesterol uptake, and therby affects atherogenesis. LDLR-/- mice receiving B6.SM/LDLR+/+ bone marrow have decreased cholesterol levels in IDL/LDL-sized particles compared to LDLR-/- mice receiving LDLR+/+ bone marrow. Concurrently, LDLR-/- mice receiving B6.SM/LDLR+/+ bone marrow have decreased atherosclerotic lesion areas at the aortic sinus compared to LDLR-/- mice receiving LDLR+/+ bone. In addition, peripheral blood monocytes ($CD11b^{+}$) of B6.SM/LDLR-/- mice have increased free cholesterol levels compared to LDLR-/- mice. Furthermore, sialidase-deficient macrophages have increased cholesterol efflux to HDL with increased expression of adenosine triphosphate binding cassette G1 (ABCG1), a transporter which plays roles in transporting intracellular cholesterol to HDL. Taken together, hypomorphic sialidase bone marrow-derived cells, including monocytes/macrophages, confer atheroprotection in LDLR-/- mice with reduced serum lipoprotein cholesterol levels and increased cholesterol efflux from macrophages. Specifically, the absence of LDLR in leukocytes, including monocytes, amplifies the extent of lipoprotein cholesterol uptake. This uptake is most prominent for VLDL-cholesterol when LDLR is absent. In conclusion, we have discovered a novel role for monocytes in the regulation of lipoprotein cholesterol levels via hypomorphic sialidase expression.

In order to target sialidase as therapy for treating atherosclerosis, we need to confirm that using DANA as a sialidase inhibitior is not toxic. In fact, this is supported by previous studies showing that DANA is not toxic (Hunt and Brown, 2007). In addition,

we need to confirm whether DANA has any effect on atherosclerotic progression in female mice at the same age compared to male mice. Furthermore, performing experiments on the effects of sialidase inhibition using DANA can be done by extracting the intracellular organelles and measuring the sialidase activity. Last but not least, delivering sialidase inhibitor to specific cell types in the body can be done by liposomes. Studies on administrating a monosaccharide mimetic by targeted liposome delivery have been done with success (Pollock et al., 2008). These will shed light on the potential therapy for treating atherosclerosis with minimization of off-target effects.

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