NOVEL ROLES FOR NEU1 SIALIDASE IN ATHEROSCLEROSIS

NOVEL ROLES FOR NEU1 SIALIDASE IN LIPOPROTEIN METABOLISM AND INFLAMMATION: BRIDGING MOLECULAR MECHANISMS IN ATHEROSCLEROSIS

By GABRIEL GYULAY (B.Sc.)

A Thesis Submitted to the School of Graduate Studies in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy

Doctor of Philosophy (Biology, 2014)

McMaster University, Hamilton, Ontario, Canada

TITLE: Novel Roles for Neu1 Sialidase in Lipoprotein Metabolism and Inflammation: Bridging Molecular Mechanisms in Atherosclerosis

AUTHOR: Gabriel Gyulay

SUPERVISOR: Dr. S. Igdoura

NUMBER OF PAGES: 248

ABSTRACT

Atherosclerosis is a complex multi-factorial disease that involves the interaction of many cell types and a plethora of molecular events. Initiation of the disease occurs when circulating LDL gets trapped in the sub-endothelial space of arteries, where LDL is oxidized causing inflammatory responses by endothelial cells. This results in recruitment and differentiation of monocytes into macrophages; macrophages in turn continue to take up cholesterol and propagate inflammation. Such a gloomy milieu of immune cells, lipids, and smooth muscle cells can give rise to atherosclerotic plaques, which then cause stenosis, vascular stiffening and eventually thrombosis. Common risk factors such as cholesterol levels, lifestyle and genetic predisposition can accelerate this potentially life threatening series of events. The downstream long-term effects of atherosclerosis, including heart disease and strokes, are now the number one cause of death in the world. While a large amount of knowledge and evidence is available in understanding this disease, prevention and treatment strategies remain somewhat ineffective. Sialylation of immune cells, lipoproteins and cellular receptors has been previously implicated in metabolic and molecular pathways relevant to atherosclerosis; however, little is known about the functional role of sialidase in these processes. Sialidase cleaves sialic acid, and is a ubiquitously expressed and evolutionarily conserved protein with essential functions in many life forms. In this study, we sought to investigate the impact of sialidase activity on atherosclerosis, emphasizing the interaction of lipid metabolism and inflammation. We have demonstrated a significant role for sialidase in cholesterol

and lipoprotein metabolism *in vivo.* Specifically, hypomorphic sialidase mice have increased hepatic storage of lipids and triglycerides, decreased VLDL production, lower circulating LDL levels and alterations in regulation of LDLR. Mice over-expressing hepatic human sialidase have increased atherosclerotic lesion formation, higher serum cholesterol esters and lower levels of hepatic LDLR and SRB-1 protein. *In vitro*, we have shown that VLDL can induce differentiation and cytokine production in monocytes coupled with an up-regulation of Neu1. Inhibition of sialidase using DANA attenuated VLDL-induced monocyte differentiation and lipid uptake, as well as activation of macrophages, implicating Neu1 in inflammatory processes associated with initiation of atherosclerosis. Furthermore, we have shown that hypomorphic sialidase activity increases LDLR-dependent LDL uptake and cholesterol efflux to HDL in macrophages. We conclude that reduction of sialidase activity can lead to an atheroprotective phenotype with multiple effects on mechanisms involved in disease progression. This work represents novel contributions into delineating both metabolic and inflammatory processes of atherosclerosis and enables the advancement of future treatment strategies.

ACKNOWLEDGEMENTS

I would like to profoundly thank Dr. Suleiman Igdoura for his excellent guidance and supervision. I will forever be grateful for all the opportunities he has provided me and everything he has taught me. He has shaped my mind as a scientist and as an individual and has instilled in me a passion for thinking and innovation. I would also like to thank Dr. Trigatti and Dr. McClelland for their exceptional instruction, technical advice and support. To Dr. Elizabeth White, a very special and well-deserved thank you for all the help over the years. I would also like to thank all of the current and past members of the Igdoura lab that I've worked with, the memories and laughs will last a lifetime.

Last but not least, I would like to thank my wonderful family, friends, girlfriend and the McMaster swim team for all of their never-ending support. I am lucky to have such people in my life and you've managed to keep me motivated and happy through thick and thin. In closing, I will strive to ensure that this thesis is only the beginning of a successful and fulfilling career.

TABLE OF CONTENTS

LIST OF FIGURES

ABBREVIATIONS

^{3}H: Tritium (radioactive label)
4-Mu-NANA: 4-Methylumbelliferyl-N-acetyl-α-D-neuraminic acid
ABCA1: ATP-binding cassette transporter A1
ABCG1: ATP-binding cassette transporter G1
ACAT: Acyl-CoA cholesterol acyltransferase
acLDL: acetylated LDL
AKT: Thymoma causing oncogene in Ak mouse strain, also known as Protein kinase B
AP: Activator protein
Apo-A: Apolipoprotein A
Apo-B: Apolipoprotein B
Apo-C: Apolipoprotein C
Apo-E: Apolipoprotein E
ApoER2: Apolipoprotein E Receptor 2
ATP: Adenosine-5'-triphosphate
BMDM: Bone marrow derived macrophages
BSA: Bovine serum albumin
CathA: Cathepsin A
CCL5: Chemokine (C-C motif) ligand 5
CD: Cluster of differentiation
cDNA: Complementary DNA
CEH: Cholesterol ester hydrolase
CETP: Cholesterol ester transfer protein
CX3CL1: Chemokine (C-X3-C motif) ligand 1
DANA: 2-deoxy-2,3-dehydro-N-acetylneuraminic acid
DGAT: Diglyceride acyltranferase
DMEM: Dulbecco's modified eagle medium
DNA: Deoxyribonucleic acid
EDTA: Ethylenediaminetetraacetic acid
EL: Endothelial lipase
E-LDL: Enzymatically modified LDL
ELISA: Enzyme-linked immunosorbent assay
ER: Endoplasmic reticulum
ERK: Extracellular signal-regulated kinase

FA: Fatty acid
FAS: Fatty acid synthase
FBS: Fetal bovine serum
FPLC: Fast protein liquid chromatography
GAPDH: Glyceraldehyde 3-phosphate dehydrogenase
GM-CSF: Granulocyte macrophage-colony stimulating factor
GRP-78: 78 kDa glucose-regulated protein
HA: Hyaluronic acid
HDL: High density lipoprotein
HEPES: 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
HL: Hepatic lipase
HMG-CoA Reductase: 3 hydroxy-3-methyl-glutaryl-CoA reductase
HRP: Horseradish peroxidise
hSial: Human Sialidase (hNEU1)
ICAM-1: Intercellular adhesion molecule-1
IDL: Intermediate density lipoprotein
Idol: Inducible degrader of LDLR
IFN-γ: Interferon-gamma
IL-1β: Interleukin-1 beta
IL-4: Interleukin-4
IL-6: Interleukin-6
Insig: Insulin-induced gene protein
kDa: Kilodalton
LacZ: β-galactosidase
LCAT: Lecithin-cholesterol acyltransferase
LDL: Low density lipoprotein
LDLR: Low density lipoprotein receptor
LOX-1: lectin-like oxLDL receptor
LPDS: Lipoprotein deficient serum
LPL: Lipoprotein lipase
LPS: Lipopolysaccharide
LRP-1: Low density lipoprotein receptor-related protein 1
LXR: Liver X receptor
M: Molar
MAL: Maackia amurensis leukoagglutinin
MAP: 2-amino-2-methyl-1-propanol

MAPK: Mitogen-activated protein kinase
MARCO: Macrophage receptor with collagenous structure
MCP-1: Monocyte chemotactic protein 1
M-CSF: Macrophage-colony stimulating factor
MEK: MAPK/ERK kinase
MPG-1: Macrophage expressed gene 1
mLDL: Modified LDL
mRNA: Messenger RNA
MTP: Microsomal triglyceride transfer protein
MUP: Major urinary protein
MyD88: Myeloid differentiation primary response gene 88
Neu1: Neuraminidase 1
Neu2: Neuraminidase 2
Neu3: Neuraminidase 3
Neu4: Neuraminidase 4
NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells
NPC1: Niemann-Pick disease type C1
oxLDL: Oxidized LDL
PAGE: Polyacrylamide gel electrophoresis
PCSK9: Proprotein convertase subtilisin/kexin type 9
PCR: Polymerase chain reaction
PBS: Phosphate-buffered saline
PI3K: Phosphoinositide 3-kinase
PKC: Protein kinase C
PMA*:* Phorbol-12-myristate-13-acetate
PPAR: Peroxisome proliferator-activated receptors
PPCA: Protective protein cathepsin A
qPCR: Quantitative real-time PCR
qRT-PCR: Quantitative reverse transcription PCR
RCT: Reverse cholesterol transport
RIPA: Radio immunoprecipitation assay
RNA: Ribonucleic acid
RMPI: Roswell Park Memorial Institute medium
RT-PCR: Reverse transcription PCR
RXR: Retinoid X receptor
SCAP: SREBP cleavage-activating protein

SDS: Sodium dodecyl sulphate
SMC: Smooth muscle cell
SNA: Sambucus nigra agglutinin
SR-A: Scavenger receptor A
SRB-1: Scavenger receptor class B member 1
SR-C: Scavenger receptor C
SREBP: Sterol regulatory element binding protein
SREC: Scavenger receptor expressed by endothelial cells
STAT: Signal transducer and activator of transcription
TG: Triglyceride (triacylglycerol)
Th1: T helper 1
Th2: T helper 2
THP-1: Tamm-Horsfall protein 1 (Human acute monocytic leukemia cell line)
TLR: Toll-like receptor
TNF: Tumor necrosis factor
tTA: Tetracycline regulated transcriptional activator (tetracycline transactivator)
VCAM-1: Vascular cell adhesion molecule 1
VLDL: Very low density lipoprotein
VLDLR: Very low density lipoprotein receptor

DECLARATION

All work was performed by Gabriel Gyulay except the following:

Chapter 2: Figure 2.1B, Table 2.1/2.3, Figure 2.2, Figure 2.3, Figure 2.4A-B, Figure 2.5A and C, and Figure 2.6B. Elizabeth White performed sialidase activity assay (Figure 2.1B). Abraham Yang performed FPLC analysis (Figure 2.2), serum lipid measurements (Table 2.1), VLDL production assay and MTP western blot (Figure 2.3, Table 2.3), SREBP2 and ACAT2 westerns (Figure 2.5A, C) and LRP-1 western blot (Figure 2.6B). Mark Mitchell generated the Neu1 vector and adenovirus (Figure 2.4A-B).

Chapter 3: Figures 3.1A, 3.3C, 3.3D, 3.3E, 3.3F, 3.6 and 3.7. Elizabeth White performed flow cytometry and analysis (Figure 3.6A-C, 3.7A-E). Abraham Yang performed atherosclerotic lesion staining and analysis (Figure 3.3C, D, E, F), and assisted with lipid measurements and FPLC profiling (Figure 3.4). J.P. King generated the plasmid pBIG-N2.1hSial (Figure 3.1A).

CHAPTER 1: Introduction

1.1 PREFACE

As life and humans have evolved, so has science, yet it has always been fuelled by instinctive curiosity and a resolve to explain that which is not known. Whether trying to create fire, questioning the shape of the earth, or dissecting intricate molecular pathways, a pervasive theme of human resilience and intrigue is evident. This consistent pursuit of knowledge has changed life extensively, and will continue to do so at an exponential rate due to technological advancements and the constant rise of scientific complexity. Perhaps the root of current scientific motivation is the hope that discoveries will eventually contribute to not only an ascent in knowledge, but also to long-term improvements in quality of life, no matter how minute they may appear at the time. An obvious and direct application of biological research is its effects on health care and treatment of disease, with direct ramifications on humanity. Scientific research is an absolute necessity in understanding and treating diseases, and in progressing as humans. While the ultimate naïve goal of ubiquitous quality of life and equality might never be reached, scientific advancements will continue to guide humanity onto avenues with brightly lit futures.

1.2 ATHEROSCLEROSIS

Atherosclerosis is the leading preventable cause of death worldwide, and is the underlying cause for the majority of all circulatory problems including coronary heart disease, myocardial infarction and stroke (Hegele, 2009). The main risk factors for atherosclerosis include sedentary lifestyle, obesity, hypertension, dyslipidemia, diabetes, smoking, high fat or 'westernized' diet as well as genetic susceptibility (Barton, 2013). The initial stage of the disease involves the accumulation of subendothelial low density lipoprotein (LDL) in the arteries, especially at branch points and regions with disrupted laminar flow, and also at 'leaky junctions' or gaps between endothelial cells (Dabagh et al., 2009). Proteoglycans in the extracellular matrix of the subendothelium are also responsible for binding and aggregating LDL (Skalen et al., 2002). This accumulation is heightened when the body's LDL levels are elevated (due to diet or genetics), or when there has been remodelling of the extracellular matrix of the arterial wall (Glass and Witztum, 2001).

The next crucial step in the initiation of atherosclerosis is the oxidation, or enzymatic modification, of LDL and its protein moiety apolipoprotein-B (Apo-B), so that it cannot be bound or internalized by the LDL receptor (LDLR) and thus remains in circulation (Levitan et al., 2010). Minimal oxidation will still allow LDL to be taken up by LDLR; however, once extensive oxidation has occurred the Apo-B component can become covalently altered and even fragmented rendering it unidentifiable to LDLR (Levitan et

al., 2010). Normally, LDL is protected from oxidation in serum; however, its susceptibility to enzymatic, nitric oxide and free-radical oxidation is greatly heightened when it is trapped in the subendothelial layer (Levitan et al., 2010). This aggregation of oxidized LDL (oxLDL) triggers early inflammatory responses and expression of endothelial cell adhesion molecules such as vascular cell adhesion molecule 1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), P- and E-selectins, and CD44 (Glass and Witztum, 2001). Expression of these molecules initiates binding of monocytes and T-lymphocytes to the endothelium, allowing these immune cells to be recruited into the intima by monocyte chemotactic protein 1 (MCP-1), chemokine C-C motif ligand 5 (CCL5), chemokine C-X3-C motif ligand 1 (CX3CL1), and macrophage-colony stimulating factor (M-CSF) (Hansson, 2009). Subsequently, monocytes differentiate into macrophages and begin the task of clearing accumulated modified lipoproteins; mainly oxLDL, but also enzymatically modified (E-) LDL, and this occurs via a process known as scavenger receptor endocytosis (Yamada et al., 1998). There are several macrophage scavenger receptors for modified LDL that have been identified in humans including, but not limited to, scavenger receptor AI/II (SR-AI/II), macrophage receptor with collagenous structure (MARCO), lectin-like oxLDL receptor (LOX-1), cluster of differentiation 68 (CD68), cluster of differentiation 36 (CD36) and scavenger receptor class B member 1 (SRB-1) (Daugherty et al., 2005;Steinbrecher, 1999). The expression of most scavenger receptors is not tightly regulated by macrophage cholesterol content at the transcriptional level, thus enabling uninhibited uptake of oxLDL and causing the

formation of lipid filled foam cells, named so due to their white and soapy appearance (Steinbrecher, 1999). Foam cells can have a 10-fold greater diameter compared to normal macrophages due to engorgement with lipids and this also contributes to their trapping in the subendothelial space (Daugherty et al., 2005). The macrophage can modify excess amassed oxLDL to yield more soluble forms of storage in the cell such as lipid droplets which are characteristic of foam cells (Moore and Tabas, 2011). Once macrophages have entered the intima and accumulate intracellular lipids, they can continue to potentiate atherosclerosis by oxidizing lipoproteins, remodelling the extracellular environment and promoting local coagulation (Aviram and Rosenblat, 1994;Daugherty et al., 2005).

Foam cells and their gross subendothelial accumulation on the artery wall (also termed 'fatty streaks'), are early events in the progression of atherosclerosis but they are the direct cause of atherosclerotic lesions (Moore et al., 2013;Ross, 1999). Maturation of the fatty streak and progression of the disease occurs through the expression of a diverse variety of cytokines and inflammatory molecules, as well as the upregulation of cytokine receptors and toll-like receptors (TLR) by the macrophages and T-cells in the early lesion (Daugherty et al., 2005;Hansson, 2009). T-Cell activation occurs and cells in the lesion can express both Th1 and Th2 cytokines, including interferon gamma (IFN-γ) and interleukin-4 (IL-4), respectively (Hansson, 1997). Additionally, macrophages and T cells undergo significant crosstalk involving many inflammatory molecules, which propagate the disease (Glass and Witztum, 2001).

Activated Th1 T-cells produce effector molecules like cluster of differentiation 40 (CD40) ligand and IFN-γ to induce the expression of multiple pro-inflammatory genes in the macrophage (Hansson, 2009). These stimuli, along with TLR signalling, can unleash the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway resulting in activated macrophages in the developing lesion which drive atherogenesis (Libby et al., 2013;Moore et al., 2013). It is important to emphasize that immune cell content is very heterogeneous and diverse at most stages of atherosclerosis, with T-cells, B lymphocytes, monocytes, macrophages, neutrophils, dendritic cells, and mast cells all playing important roles and contributing to the dynamics of disease progression (Libby et al., 2013). Macrophages appear to have the most prominent and pervasive role primarily due to foam cell formation, presence during early and late atherosclerosis, and propagation of inflammation (Gui et al., 2012;Libby et al., 2011;Williams et al., 2012). Macrophages also secrete a realm of pro-inflammatory cytokines, such as interleukin-1β (IL-1β), interleukin-6 (IL-6), MCP-1 and tumour necrosis factor (TNF) which recruit more macrophages and other cell types as the disease progresses (Daugherty et al., 2005;Moore et al., 2013). These cytokines can influence lipid accumulation as well, primarily by inducing lipid retention in macrophages (Persson et al., 2008).

Endoplasmic reticulum (ER) stress, autophagy, apoptosis, as well as defective efferocytosis contribute to atherosclerotic disease advancement (Moore et al., 2013;Moore and Tabas, 2011). Atherosclerotic lesions, triggered by foam cell formation and the recruitment of inflammatory cells, occur when smooth muscle cells (SMCs)

migrate from the arterial media into the subendothelial space or intima, resulting in their proliferation and internalization of more modified LDL (Ross, 1999). Scavenger receptors on smooth muscle cells include scavenger receptor expressed by endothelial cells (SREC), CD36 and LOX-1 (Steinbrecher, 1999). Additionally, β-very low density lipoprotein (VLDL), which is a heterogeneous cholesterol ester-rich and atherogenic variant of native VLDL and chylomicron remnants, can also attract more smooth muscle cells to the developing lesion directly, thus further propagating the matter (Zhao et al., 2001). These smooth muscle cells add complexity to the plaque and continue to generate an inflammatory response. Mature lesions can also be referred to as plaques due to their high content of smooth muscle cells, necrotic foam cells, calcification and pro-coagulant activity (Businaro et al., 2012;Moore and Tabas, 2011). In addition to narrowing the artery, plaques can also cause platelet accumulation, which further drives disease progression (Glass and Witztum, 2001;Nofer et al., 2010). Large, unstable plaques are especially dangerous, as they are susceptible to rupture and release into the bloodstream due to the presence of a thin fibrous cap of smooth muscle cells covering the plaque (Fishbein, 2008). Thrombus attachment can also occur in macrophage rich areas of lesions, causing catastrophic consequences of late atherosclerosis (Daugherty et al., 2005). Thrombosis, stenosis and disrupted plaques may lead to myocardial infarctions and stroke which can be lethal (Gui et al., 2012;Moore and Tabas, 2011).

Individuals with genetic predispositions, those who lead an unhealthy lifestyle, and those with metabolic syndrome are especially susceptible to atherosclerosis (Glass and

Witztum, 2001;Goldstein et al., 1985). In spite of recent discoveries of disease mechanisms and advancements in prevention strategies, occurrences of life-threatening events due to atherosclerosis continue to rise (Barton, 2013;Libby et al., 2011). In fact, prominent challenges remain in order to fully understand the interactions of all contributing factors of the disease and to design appropriate treatment strategies (Barton, 2013;Libby et al., 2011).

1.3 LIPOPROTEIN METABOLISM

Cholesterol is an important steroid and plasma membrane constituent which is produced by many cells, can be obtained from external sources such as food, and is excreted minimally via bile (Maxfield and Wustner, 2002). Due to their hydrophobicity, cholesterol, fatty acids (FAs) and triglycerides (TGs) are transported in the blood by several lipoproteins with the majority of serum cholesterol being transported by LDL (Steinberg and Witztum, 1990). Lipoproteins are stable moieties of lipids and proteins that are arranged in spherical structures consisting of an outside surface containing a single layer of phospholipids that surrounds a neutral lipid core consisting of varying amounts of triglycerides and cholesterol esters (Hoofnagle and Heinecke, 2009). Lipoproteins serve to deliver cholesterol and lipids to several cell types and tissues in the body. Lipoproteins contain a diverse array of amphipathic protein constituents on their surface which are essential for mediating the movement, interaction, and uptake of these particles *in vivo* (Hoofnagle and Heinecke, 2009). Apolipoprotein-B is synthesized in hepatocytes and enterocytes, where it serves to initiate VLDL or chylomicron

assembly respectively (Davis, 1997;Shelness et al., 1999). However, intestinal Apo-B is a smaller variant of hepatic Apo-B (created by mRNA editing) and is solely found in chylomicrons and chylomicron remnants in humans, which transport dietary fats to the liver or peripheral tissues after absorption (Mehta et al., 1996). Apolipoprotein E (Apo-E) and apolipoprotein A1 (Apo-A1) are also secreted from the liver; Apo-A1 is the major protein constituent of high density lipoprotein (HDL) (Sundaram and Yao, 2012). Cholesterol-containing VLDL is originally secreted by the liver through the addition of triglycerides to Apo-B by the enzyme microsomal triglyceride transfer protein (MTP) (Gordon et al., 1995). Triglyceride precursors of VLDL are also synthesized in the liver and begin to associate with Apo-B in the endoplasmic reticulum (Davis, 1997). Other protein components including apolipoproteins-Cs and –E are then added during the secretion process (Davis, 1997;Hegele, 2009;Shelness et al., 1999). Through the actions of enzymes such as lipoprotein lipase (LPL) and its interaction with Apo-CII, the VLDL particle undergoes a change into intermediate-LDL (IDL) and subsequently LDL through the loss of triglyceride components, structural remodelling and the loss of all protein constituents other than Apo-B (Hegele, 2009). Interestingly, Apo-CIII can inhibit lipolysis by LPL and it is involved in VLDL production as well (Yao and Wang, 2012). It should also be noted that lipolysis of VLDL by LPL occurs at the vascular endothelial surface and LPL can be synthesized and secreted by adipocytes, muscle cells and macrophages (Babaev et al., 1999;Mead et al., 2002). Other enzymes involved in intravascular lipolysis of TG-rich lipoproteins include hepatic lipase (HL) and endothelial lipase (EL) (Young and

Zechner, 2013). Cholesterol content of lipoproteins can also be modified extracellularly via the actions of lecithin-cholesterol acyltransferase (LCAT), which can esterify cholesterol on lipoproteins and is involved in maturation of HDL (Kunnen and Van, 2012). Furthermore, cholesterol ester transfer protein (CETP) can transfer cholesterol esters between lipoproteins in humans, but is absent in mice (Barter et al., 2003).

Absorption of all lipoproteins containing Apo-B and/or Apo-E occurs through their binding and internalization by the LDLR both in peripheral cells as well as the liver (Goldstein et al., 1985). Specific receptors for Apo-E containing lipoproteins include very low density lipoprotein receptor (VLDLR), LDLR-related protein 1 (LRP-1) and Apo-E receptor 2 (apoER2), although their roles have not been as thoroughly studied as LDLR (Lillis et al., 2008;May et al., 2005). LDLR protein levels are intricately regulated in the liver through several pathways, and this protein is crucial to cholesterol homeostasis (Faiz et al., 2012;Goldstein et al., 1985). The transcriptional regulation of LDLR has been delineated below; however, microRNAs, proprotein convertase subtilisin/kexin type 9 (PCSK9) and inducible degrader of the LDLR (IDOL) all play important roles in regulating levels of LDLR and add complexity to the hepatic expression of this important receptor (Goedeke and Fernandez-Hernando, 2012;Lopez, 2008;Zelcer et al., 2009). PCSK9 is a secreted protein that binds to LDLR protein at the cell surface and targets it for degradation (Grefhorst et al., 2008).

In addition, reverse cholesterol transport (RCT) from periphery cells back to the liver can also occur via ATP-binding cassette transporter's (ABCA1/G1) lipidation of Apo-A1

and high density lipoprotein (HDL) and subsequent uptake by the liver via the SRB-1 receptor (Hegele, 2009). Overall, cholesterol and lipid homeostasis of the organism is maintained via the balance of secretion and uptake of lipoproteins by the liver, in addition to intracellular synthesis and trafficking of these compounds (Goedeke and Fernandez-Hernando, 2012).

1.4 INTRACELLULAR CHOLESTEROL AND TRIGLYCERIDE METABOLISM

Inside the cell, cholesterol can reside in cytosolic lipid droplets as stored cholesterol esters, and in endocytic compartments following uptake (Soccio and Breslow, 2004). Additionally, cholesterol is synthesized endogenously in the ER where esterification also occurs (Feng et al., 2003). The main players in control of cholesterol synthesis are the Sterol Regulatory Element Binding Protein (SREBP) family of transcription factors with SREBP-2 primarily involved in activating genes of cholesterol synthesis and SREBP-1a and -1c connected to fatty acid synthesis (Soccio and Breslow, 2004). SREBP regulation is extremely complex, but it functions using a homeostatic mechanism where cholesterol levels in the cell can exert a negative feedback on cholesterol synthesis; in times of cholesterol abundance, SREBPs remain in the ER associated with SREBP cleavage activating protein (SCAP) and the ER retention insulin-induced gene protein (Insig), halting the transcription of genes involved in cholesterol synthesis and uptake (Radhakrishnan et al., 2008;Yang et al., 2002). On the other hand, low cholesterol causes a structural change in SCAP, releasing Insig and allowing SREBP-SCAP to reach the Golgi where SREBP is cleaved by proteases, releasing its active form to the nucleus for

transcriptional activation of many cholesterol synthesis and uptake genes such as LDLR, PSCK9 and 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMG-CoA reductase) (Brown et al., 2002;Horton et al., 2002;Horton et al., 2003;Yang et al., 2002). In addition to suppressing its own synthesis, high cholesterol levels can activate acyl-coenzyme A:cholesterol acyltranferase (ACAT) which leads to esterification of cholesterol and subsequently its storage along with triglycerides inside cytosolic lipid droplets (Soccio and Breslow, 2004). Conversely, cholesterol esters are degraded by the enzyme cholesterol ester hydrolase (CEH) to generate free cholesterol, which can make it available for bile secretion and efflux (Bie et al., 2013;Sekiya et al., 2011). Triglycerides are formed from the addition of glycerol to fatty acid chains by the cell using the enzymes diglyceride acyltransferase 1 and 2 (DGAT1/2) (Yen et al., 2008). FAs are originally synthesized by fatty acid synthase (FAS) and this enzyme's transcription is regulated by SREBP-1c (Latasa et al., 2003).

Internalized cholesterol and endogenously synthesised cholesterol can both produce oxysterols (including 24(S),25-epoxycholesterol and 25-hydrocycholesterol) that act as agonists to the liver X receptor alpha and beta (LXRα/β) ligand-activated transcription factors (Lund et al., 1998;Spencer et al., 2001). LXRα is predominantly expressed in metabolically active tissues (such as liver, intestine, adipocytes and macrophages), while LXRβ displays a more ubiquitous expression pattern, and they are both extremely important regulators of intracellular cholesterol metabolism as well as cholesterol homeostasis in the body (Zhao and Dahlman-Wright, 2010). LXRs can activate

cholesterol efflux by inducing expression of ABCA1/G1 and Apo-E in peripheral tissues, and they can regulate expression of IDOL, FAS and genes involved in bile secretion and cholesterol synthesis in the liver; this is accomplished by detecting cellular levels of oxysterols and forming heterodimers with retinoid X receptor (RXR) and inducing gene transcription (Lund et al., 1998;Spencer et al., 2001;Zelcer et al., 2009;Zhao and Dahlman-Wright, 2010). Another key player in intracellular cholesterol transport is Niemann-Pick C1 (NPC1) which is a protein involved in mediating the trafficking of free cholesterol from endosomal compartments to the ER and plasma membrane (Zhang et al., 2008).

1.5 MACROPHAGE CHOLESTEROL/LIPID HOMEOSTASIS AND INFLAMMATION

Macrophages can take up LDL to meet their lipid and cholesterol needs, but also readily participate in scavenger receptor internalization of lipoproteins (Glass and Witztum, 2001;Moore et al., 2013). This includes primarily uptake of oxLDL, and is the direct cause of foam cells and atherosclerosis, as mentioned previously. Other lipoproteins, such as β-VLDL and modified Apo-B and Apo-E containing particles can contribute to foam cell formation as well (Tabas et al., 1990;Veniant et al., 2008). Macrophages express LDLR, like any other cell type, and its expression is regulated by intracellular cholesterol levels (as outlined previously) and the cell's state of differentiation and activation (Boisvert et al., 1997). Macrophages exhibit a plasticity of functions in atherosclerosis, and the contribution of macrophage native LDL uptake to atherosclerosis is under debate; however, human peripheral blood monocytes and

macrophages, and murine and human macrophage cell lines can readily take up LDL (Boisvert et al., 1997;Daugherty et al., 2005;Wang et al., 2007). Several bone marrow transplant experiments have been performed to assess the importance of the macrophage LDLR on lesion formation. LDLR-/- mice receiving bone marrow from LDLR+/+ donors show no difference compared to those that received bone marrow from LDLR-/- donors (Fazio et al., 1997). However, when the recipient is C57Bl/6, donation of LDLR+/+ bone marrow induces more atherosclerosis than donation of LDLR-/- bone marrow (Herijgers et al., 2000;Linton et al., 1999). Despite these findings, the macrophage LDLR still remains somewhat enigmatic with regards to its mechanism of lipoprotein uptake and molecular role in foam cell formation and inflammation. Nevertheless, it is an important contributor to cholesterol homeostasis in the macrophage and potentially atherogenesis as well. Minimal amounts of LDL can also be taken up by the macrophage via LDLR-independent means such as macropinocytosis as well (Wang et al., 2007). LDL can also induce CD11b expression and increase MCP-1 induced cell migration in THP-1 monocytes in an LDLR-dependent manner (Han et al., 2003). Interestingly, even chylomicron remnant uptake by macrophage LDLR can influence the cell's inflammatory status (Graham et al., 2011). Other important lipoprotein receptors in macrophages include LRP-1 and VLDLR; tissue-specific deletion and bone marrow studies indicate that macrophage LRP-1 expression is atheroprotective without changing lipoprotein levels, while macrophage VLDLR induces foam cell formation and lesion development by accumulation of atherogenic

lipoproteins, yet their overall contribution to cholesterol homeostasis is poorly defined (Eck et al., 2005;Lillis et al., 2008).

Furthermore, there is also abundant evidence that macrophage cholesterol metabolism is directly linked to inflammation as part of a complex biological system contributing to disease progression in unison (Daugherty et al., 2005;Guo et al., 2009;Hansson, 2007;Hong and Tontonoz, 2008;Lemaire-Ewing et al., 2012;Libby, 2007). Peroxisome proliferator-activated receptors (PPAR- α, δ and γ) are involved in inflammatory response as well as cholesterol efflux in the macrophage, along with the LXR family of transcription factors (Chawla et al., 2001;Chawla, 2010;Hong and Tontonoz, 2008). Furthermore, the LXR and PPAR family of transcription factors are involved in a myriad of inflammatory and immune responses in the plaque including cytokine production, and macrophage and T-cell activation (Hong and Tontonoz, 2008). PPARs can be activated by oxidized LDL in the macrophage and can activate the CD36 scavenger receptor gene as well as activate the LXR gene itself (Chawla et al., 2001). Additionally, oxidation products of cholesterol and lipids can have a realm of inflammatory effects in the macrophage (Leonarduzzi et al., 2012), and even TGs have been invoked as a pro-inflammatory stimulus in immune cells (Libby, 2007;Libby et al., 2011;Persson et al., 2008).

LRP-1 is also involved in a plethora of cellular signalling pathways and can have multiple ligands; its importance is highlighted by the fact that knocking out the gene is

embryonic lethal in mice (Herz and Strickland, 2001). In addition to its roles in lipid homeostasis, LRP-1 has been found to suppress NF-κB and it is also a regulator of proteolytic activity (Gaultier et al., 2008;May et al., 2005). Other roles of LRP-1 include binding of extracellular membrane proteins, regulation of vascular permeability, and remodelling of the protein composition of the plasma membrane (Boucher and Herz, 2011;Gonias et al., 2004). In SMCs, LRP-1 is involved in reducing proliferation stimulated by growth factors, as well as in modulating cell migration (Lillis et al., 2005). In adipocytes, LRP-1 can serve to regulate cholesterol metabolism and cell surface lipolysis (Terrand et al., 2009). *In vivo* studies using macrophage knockouts of LRP-1 have shown increased atherosclerosis independent of lipid alterations, thus indicating that LRP-1 is involved in inflammatory mechanisms of atherosclerosis (Lillis et al., 2008;Overton et al., 2007). This receptor also has important roles in neuronal signal transduction, embryonic development, and human immunodeficiency virus infection (Hennen et al., 2013;May et al., 2005;Strickland et al., 2002). LRP-1 has several anti-inflammatory effects and can affect multiple signalling pathways in the macrophage, thus its diverse role in atherosclerotic mechanisms is not to be underestimated. LRP-1, PPARs and LXRs along with SRB-1, Apo-Cs, TLRs and NF-κB provide evidence that cholesterol/lipid metabolism and inflammation are one complex biological system with significant crosstalk (Guo et al., 2009;Hansson, 2007;Hong and Tontonoz, 2008;Kawakami et al., 2007;Kawakami and Yoshida, 2009;Lemaire-Ewing et al., 2012;Leonarduzzi et al., 2012;Yasuda et al., 2007).

Recent reports have attributed triglyceride and fatty acid metabolism in macrophages directly to their inflammatory response (Libby, 2007). Specifically, VLDL appears to have unique roles in the macrophage, although its exact interaction with cell surface receptors and its uptake/signalling mechanisms are still being explored (Bojic et al., 2012;Lu et al., 2009;Milosavljevic et al., 2003;Palmer et al., 2005). Human monocytes isolated from blood donors and differentiated with Granulocyte macrophage-colony stimulating factor (GM-CSF) were treated with VLDL *in vitro* and resulted in foam cell formation and secretion of Il-1β (Persson et al., 2006). In addition, it was shown that caspase-1 activation after VLDL-treatment was required for secretion of mature Il-1β protein (Stollenwerk et al., 2005a). VLDL is also able to induce TNF production in human monocyte-derived macrophages and to phosphorylate mitogen-activated protein kinase-extracellular signal-regulated kinase (MEK1/2) via activator protein-1 (AP-1) but not NFκB (Stollenwerk et al., 2005b). Incubation of macrophages with Il-1β and TNF increases their VLDL-derived lipid content, indicative of a pro-atherogenic cycle and a direct correlation between VLDL uptake and inflammation (Persson et al., 2008). Huang et al have shown that saturated FA treatment activates TLR pathways in mouse macrophages leading to NFκB activation (Huang et al., 2012). VLDL also induces Il-1β and TNF production in THP-1 macrophages along with phosphorylation of Extracellular Signal-related Kinase (ERK1/2) and p38 MAPK and upregulation of ICAM-1 (Bojic et al., 2012;Jinno et al., 2011). This was found to be decreased by PPAR-δ activation, and involved AKT signalling and was dependent on LPL (Bojic et al., 2012). In fact, several

studies indicate that LPL is necessary for VLDL-induced foam cell formation and inflammation in human and mouse macrophages (Milosavljevic et al., 2003;Saraswathi and Hasty, 2006) and that VLDL can induce LPL expression (Pou et al., 2011). Reduction of LPL activity by deletion of Apo-CII on VLDL or by incubation with the drug Orlistat resulted in reduction of lipid accumulation and amelioration of cellular signalling activation of ERK and p38 MAPK cascades (Milosavljevic et al., 2003;Saraswathi and Hasty, 2006).

Interestingly, VLDL can activate p38 MAPK, Protein Kinase C (PKC) and NFκB in endothelial cells leading to increased arterial inflammation and VCAM-1 expression (Kawakami et al., 2006a;Libby, 2007), and it can induce cytokine secretion in T-cells as well (Sampedro et al., 2001). Kawakami et al have demonstrated that the Apo-CIII component is responsible for the inflammatory actions of VLDL and that Apo-CIII can induce adhesion of monocytes to endothelial cells (Kawakami et al., 2006a;Kawakami et al., 2006b;Kawakami et al., 2007). They show activation of PKC and NFκB in THP-1 monocytes leading to activation of Ras homolog gene family member A (RhoA) and β1-integrin, which are involved in cytoskeleton remodelling and cell adhesion (Kawakami et al., 2006b;Kawakami et al., 2007). However, the authors acknowledge that Apo-CIII activates both monocytic and endothelial components and they used an Il1-β pre-treatment on endothelial cells to prime them for adhesion, making it difficult to assess the direct role of VLDL/Apo-CIII on monocytes alone (Kawakami et al., 2006b;Kawakami et al., 2007). Nevertheless, their data does show that Apo-CIII can be a potent pro-

inflammatory moiety in monocytes, macrophages and endothelial cells and as such plays a role in vascular adhesion processes. It is interesting to note that serum Apo-CIII is a risk factor for atherosclerosis and obesity, and could be contributing to disease progression via the pathways outlined above (Harvey et al., 2009;Ooi et al., 2008). However, the exact interactions of Apo-CIII with each cell type and the cell surface receptors that are involved remain unclear. Apo-E from VLDL-lipolysis products can also modulate LPS induced monocyte inflammation, further adding to the notion that apolipoproteins can influence immune mechanisms of atherosclerosis (den Hartigh et al., 2012). Furthermore, even addition of LPL alone to endothelial cells was found to be sufficient in inducing human monocyte adhesion (Mamputu et al., 1997). Overall, VLDL/Apo-CIII metabolism and lipolysis have very unique roles in macrophage inflammatory and adhesion mechanisms, in addition to their established functions in lipid homeostasis. It is thus crucial to further study their involvement and molecular actions, especially in early atherosclerotic events such as monocyte adhesion and differentiation.

As described previously, the macrophage can efflux excess cholesterol via the RCT pathway, in addition to conversion of excess cholesterol into nontoxic cholesterol esters for storage (Rosenson et al., 2012). Reverse cholesterol transport is mediated by HDL delivery of cholesterol to the liver using the receptor SRB-1, and this is the reason why low HDL levels are proatherogenic (Glass and Witztum, 2001;Hegele, 2009). The export of cholesterol from macrophages occurs by mechanisms that involve translocation of cholesterol to nascent HDL via ABCG1 or to lipid poor apoA1 via the ABCA1 transporter

pathway (Lorkowski, 2008). These transporters are crucial to RCT, as a double knockout of both ABC members drastically exacerbated atherosclerosis on an LDLR-/- background (Yvan-Charvet et al., 2007). Macrophage SRB-1 and apo-E play roles in efflux as well, and lipoproteins other than HDL can act as cholesterol acceptors, although these processes are still under investigation (Getz and Reardon, 2009;Gu et al., 2000;Ji et al., 2011;Lammers et al., 2011). Recent studies have identified roles for autophagy and lysosomal acid lipase in cholesterol efflux, as they can increase the availability of cholesterol to ABC transporters by breaking down lipid droplets in the macrophage (Ouimet et al., 2011;Ouimet and Marcel, 2012). Therefore, macrophage cholesterol and lipid homeostasis are directly related to vascular inflammation and atherosclerosis, thus warranting further investigation into these complex processes.

1.6 SIALIC ACID AND SIALIDASE

Sialic acid, also known as neuraminic acid, is a negatively charged 9 carbon hexose terminal sugar residue found on a variety of oligosaccharides (Chen and Varki, 2010). It is most commonly bound by an α-2,3 or α-2,6 glycosidic linkage to other sugars, with the most widespread being an α-2,3 linkage to galactose (Millar, 2001). Sialic acids are found as glycoconjucates on proteins and lipids, and are in abundance on the cell surfaces of bacteria and animals (Pshezhetsky and Hinek, 2011). Sialic acids have diverse and important functional roles in a vast array of biological processes; these include conformational stabilization of molecules, cell surface charge, cell recognition, and immune response (Chen and Varki, 2010;Schauer, 2009;Varki and Gagneux, 2012).

Sialidase, also referred to as neuraminidase, is the enzyme that cleaves sialic acid, while sialyltransferases, catalyze the addition of sialic acid residues (Chen and Varki, 2010). In mammals there are 4 genes encoding sialidases which differ based on their cellular localization and enzymatic characteristics (Miyagi and Yamaguchi, 2012). Sialidases can exist in the lysosome (NEU1), the cytosol (NEU2), the cell membrane (NEU3, NEU1, NEU4), and the mitochondria/ER (NEU4) (Cross et al., 2003;Liang et al., 2006;Miyagi and Yamaguchi, 2012;Rottier et al., 1998). NEU1 is ubiquitous and highly expressed in many human tissues, having 10-20 fold greater abundance of mRNA compared to NEU3/4 and 5000 fold greater expression compared to NEU2 in most tissues (Hata et al., 2008;Pshezhetsky and Ashmarina, 2013). NEU1 exhibits an extremely high functional and structural homology between mouse and human (Igdoura et al., 1998). The naturally occurring inbred mouse strain SM/J was identified to have a point mutation in the Neu1 locus and exhibits reduced sialidase activity (20-30 % of normal), hypersialylation of glycoproteins and altered immune response (Champigny et al., 2009;Rottier et al., 1998). The mouse knockout of Neu1 results in a severe phenotype similar to the human disease sialidosis (de Geest et al, 2002). Sialidosis is a rare and fatal autosomal recessive disorder characterized by the accumulation of sialylglycoconjucates resulting in muscular and mental deterioration: several mutations of NEU1 with negligible sialidase activity have been identified and characterized in patients of this disease by our lab (Pattison et al., 2004). General cellular functions of NEU1 sialidase include glycoconjugate catabolism in lysosomes, exocytosis, phagocytosis

and elastic fiber assembly (Miyagi and Yamaguchi, 2012;Seyrantepe et al., 2008;Seyrantepe et al., 2010).

NEU1 requires association with lysosomal protective protein Cathepsin A (CathA) to trigger its enzymatic activity; however, the exact mechanisms of this activation and sialidase's overall intracellular transport remain unclear (Pshezhetsky and Hinek, 2011). NEU1 has a C-terminal lysosomal targeting motif and forms a heterodimer with CathA and this association is necessary for the trafficking of NEU1 to the lysosome and its subsequent activation (Bonten et al., 2009). The N-terminal glycans of NEU1 are important for its stability and activity, and CathA can counteract against defects in N-glycosylation (Wang et al., 2009). CathA is extremely important as knockout mice exhibit severe disease resembling the human lysosomal storage disorder sialidosis (Miyagi and Yamaguchi, 2012). While the presence of NEU1 in the cell membrane is not disputed, its exact trafficking mechanisms and interactions with the membrane are under debate. One study proposed that NEU1 is an integral membrane protein with a C-terminal trans-membrane domain that can then be internalized to the lysosome and subsequently cleaved yielding a soluble form (Lukong et al., 2001). Homology models of NEU1 do not predict any trans-membrane domains, and whether the enzyme's presence at the cell surface can be attributed to fusion of lysosomal and plasma membranes during exocytosis or other anchors/mechanisms remains to be seen (Pshezhetsky and Hinek, 2011).

Our lab has several tools which enable us to study NEU1 and its effects. Measurement of live sialidase activity can be performed using 4-Methylumbelliferyl-N-acetyl-α-D-neuraminic acid (4-Mu-NANA) by measuring the fluorescence of the 4-Mu liberated from the NANA substrate by the enzyme. Detection of linked sialic acids is accomplished by using SNA (Sambucus nigra agglutinin) and MALII (Maackia amurensis leucoagglutinin) lectins which bind specific α-2,6 or α-2,3 linked sialic acids, respectively (Knibbs et al., 1991). Lectins can be used to detect cell surface sialylation by flow cytometry or they can be utilized to pull down glycoproteins for immunoblot analysis. SM/J mice were backcrossed to C57Bl/6 mice 6 times (B6.SM) or 10 times (B10.SM) in order to isolate the point mutation and to allow for *in vitro* studies of hypomorphic sialidase expression with comparison to a wild-type mouse. Both of these Neu1 deficient strains have hypomorphic expression of Neu1 and are demarcated as $Neu1^{hypo}$. The chemical inhibitor 2-deoxy-2,3-dehydro-N-acetylneuraminic acid (DANA) is a potent inhibitor of all sialidases and can be used to downregulate sialidase activity (Zhang et al., 2013). Furthermore, fibroblasts isolated from sialidosis patients will serve as another tool to study the effects of null NEU1 activity *in vitro* (Pattison et al., 2004). Sialidase over-expression can be accomplished by using helper-dependent adenovirus generated by our lab for both mouse and human sialidase.

1.7 SIALYLATION OF LIPOPROTEINS AND RECEPTORS

Glycosylation plays an important role in LDL, as all LDL particles contain sialylated oligosaccharides (Millar, 2001;Orekhov et al., 1991). Apo-B contains many mono- and di-

sialylated sugars, with every Apo-B molecule containing approximately 12-14 sialic acid residues in human plasma (Millar, 2001). It has been shown that patients with atherosclerosis contain LDL in their blood with a 2.5-5 fold lower sialic acid content compared to that of healthy subjects, with no difference in protein and lipid content between the two (Orekhov et al., 1991). Also, the sialylation of Apo-B has been directly implicated with risk of atherosclerosis (Mel'nichenko et al., 2005). Similarly, Sobenin et al demonstrated that LDL desialylated with neuraminidase causes a doubling of intracellular cholesterol, thus directly correlating the sialylation of LDL with its metabolism (Sobenin et al., 1991). In addition, approximately 20% of all Apo-E molecules are sialylated in human plasma and a study has linked sialylation to their clearance rate (Millar, 2001). Apolipoproteins CII and CIII, which are found on VLDL and are responsible for VLDL's interaction with lipoprotein lipase, are also heavily sialylated with functional consequences (Mann et al., 1997;Mauger et al., 2006;Stoline et al., 1985).

Sialylation of LDLR is predominately on O-linked glycosylation near the extracellular domain near the membrane spanning region and these sugars are believed to act as physically supportive struts, as their deletion does not hamper LDLR function (Cummings et al., 1983;Davis et al., 1986;Goldstein et al., 1985;Kingsley et al., 1986). However, there is evidence for dispersed O-linked oligosaccharides near the N-terminal (ligand binding) region of the receptor, which are believed to be essential for LDLR function along with the 2 N-linked oligosaccharides (Filipovic, 1989;Kingsley et al., 1986;Kozarsky et al., 1988). Treatments with sialidase have shown a reduction of 7-11 kDa of the LDL

receptor in human fibroblast cells, demonstrating that sialic acid is a large component of the receptor's mature structure (Cummings et al., 1983). Additionally, sialic acid molecules on the LDLR cause a net negative charge (Goldstein et al., 1985). Sialylation of LDLR also plays important roles in its interaction with LDL, evidenced by the fact that fibroblasts and endothelial cells that were treated with bacterial sialidase had significantly altered LDL binding, internalization and degradation (Sprague et al., 1988). PCSK9 also binds to the highly glycosylated ligand-binding region of LDLR, thus adding further potential implications for sialylation in LDLR regulation and degradation (Yamamoto et al., 2011).

1.8 SIALIDASE AND IMMUNE RESPONSE

Unlike lipoprotein metabolism, the role of sialylation in immunity has been well studied (Varki and Gagneux, 2012). Sialidase activity is higher in macrophages compared to monocytes, and differentiation of monocytes into macrophages induces upregulation of Neu1 and Neu3 (Lambre et al., 1990;Stamatos et al., 2005). Sialidase activation is also required for CD44-HA adhesion of monocytes and T-cells (Gee et al., 2002;Katoh et al., 1999;Katoh et al., 2010). Furthermore, THP-1 monocyte differentiation involves upregulation of Neu1 and its targeting to the cell membrane (Liang et al., 2006). Upon activation of T-cells, sialidase expression is induced and is involved in the secretion of many inflammatory factors, namely IL-4 (Chen et al., 1997;Wang et al., 2004). Recruitment and subsequent differentiation of monocytes and leukocytes play a pivotal role in the inflammatory response that is prominent in atherosclerosis. There is also

abundant evidence for activation and movement of sialidase to the cell surface during adhesion and diapedisis of neutrophils (Cross et al., 2003;Cross and Wright, 1991). Sialidase treatment or sialidase up-regulation can also affect many cell surface glycoproteins on endothelial cells leading to increased adherence of neutrophils (Feng et al., 2011;Sakarya et al., 2004). Inhibition of sialidase in human dendritic cells reduces LPS-induced cytokine production (Stamatos et al., 2010). CD15s or sialyl-Lewis X, a glycan moiety abundant on myeloid lineage cell surface glycolipids and glypoproteins, has been shown to be desialylated by Neu1 during differentiation and is linked to selectin binding (Gadhoum and Sackstein, 2008). Additionally, consequences of sialic acid biology are prominent in a plethora of cell surface clusters of differentiation (CDs) that are involved in immune response and adhesion (Varki and Gagneux, 2012). Historically, sialidase has been heavily implicated in adherence of bacteria and in influenza infection (Galen et al., 1992;McCullers and Bartmess, 2003;Varki, 2008), and recent evidence involves this enzyme in cancer as well (Miyagi and Yamaguchi, 2012).

1.9 OTHER ROLES FOR SIALIDASE IN ATHEROSCLEROSIS

Elevated serum levels of sialic acid are markers of atherosclerosis, and one hypothesis suggests that these sialic acids originate from the endothelium and circulating cells that have been desialylated (Lindberg, 2007;Rastam et al., 1996). In fact, even global sialylation status of the blood and endothelium has been linked to hypertension and risk of atherosclerosis (Sathiyapriya et al., 2008). Moreover, a recent finding concluded that upon binding to a ligand, toll like receptors (TLRs) require Neu1

sialidase for activation and subsequent cytokine secretion in macrophages, another crucial step in atherogenesis (Abdulkhalek et al., 2011;Amith et al., 2009). Interestingly, TLR4 activation by LPS has also been linked to EL upregulation and increased native LDL uptake in macrophages, providing further evidence for crosstalk between inflammation and cholesterol metabolism (Yasuda et al., 2007;Ye et al., 2009). Another study found that treatment with gangliosides (which contain sialic acids) can stimulate smooth muscle cell proliferation, and this is not surprising as lactosylceramide, the precursor for these gangliosides, is abundant in many cell types in the plaque (Chatterjee, 1998;Gouni-Berthold et al., 2001). Recently, Neu1 has been linked to insulin receptor activation and glucose metabolism in mice (Dridi et al., 2013) and Neu1 activity is altered in epididymal fat and livers of obese and diabetic mice (Natori et al., 2013). There is an array of evidence that implicates sialidase and sialic acid in diverse areas of atherosclerosis, yet there are still unknown targets and functions of sialylation in both lipid and inflammatory mechanisms.

1.10 RATIONALE AND OBJECTIVES

While it is clear that Neu1 is involved in several molecular functions relevant to lipoproteins, cholesterol metabolism and inflammation, the details and breadth of its involvement are still under investigation. In fact, no *in vivo* or functional studies have directly delineated sialidase's role in atherosclerosis disease progression, and many of the *in vitro* experiments lack molecular mechanisms and direct relevance to processes elicited in atherogenesis. We thus aim to further study the effects of sialidase on both

inflammation and lipoprotein metabolism, using a combination of *in vivo* and *in vitro* approaches. This will allow us to further elucidate the mechanisms of Neu1 on its molecular targets, highlighting the interaction of lipids and inflammatory events in atherosclerosis. We hypothesize that modulation of Neu1 sialidase expression and/or activity *in vivo* and *in vitro* will directly affect lipoprotein metabolism, cholesterol metabolism and inflammatory mechanisms. Overall, reducing sialidase activity will have anti-atherogenic effects.

Objectives:

1. To investigate the role of sialidase as a modulator of lipoprotein metabolism *in vivo*: We will utilize mice with hypomorphic expression of Neu1 as well as Neu1 helper dependent adenovirus to genetically modify the activity of Neu1 *in vivo*. These mice will be characterized and compared to wild-type controls with regards to their hepatic and serum lipid/cholesterol content, lipoprotein metabolism, and molecular mechanisms that are involved in these processes. We aim to establish a direct role for Neu1 in cholesterol and TG metabolism *in vivo.*

2. To examine the effects of hepatic sialidase over-expression on lipoprotein metabolism and atherosclerosis *in vivo*: We will create and utilize novel transgenic mice that express hepatic human NEU1 to analyze atherosclerosis after high fat diet feeding. We will analyze cholesterol and lipoprotein

metabolism as well as atherosclerotic lesion formation, in order to assess the effects of over-expression of hepatic NEU1 on atherogenesis *in vivo.* This will allow us to explore a causal role for human NEU1 in atherosclerosis progression.

3. To investigate the effects of sialidase on VLDL-induced monocyte/macrophage inflammation and lipid metabolism: We will utilize the THP-1 cell line to assess the effects of modulating sialidase activity on VLDL-induced monocyte differentiation and activation, as well as foam cell formation *in vitro.* The sialidase inhibitor DANA will be used to assess the effects of reducing sialidase activity on lipid and inflammatory mechanisms in response to VLDL. We will investigate relevant molecular pathways that are invoked by VLDL and influenced by sialidase in order to further delineate atherosclerosis disease mechanisms involved during macrophage differentiation and activation.

4. To investigate the role of sialidase on macrophage cholesterol metabolism: We will utilize macrophages from hypomorphic Neu1 animals and the THP-1 cell line (with DANA) to assess the effects of modulating sialidase activity on macrophage cholesterol homeostasis *in vitro.* Macrophages with reduced sialidase activity will be analyzed for LDL uptake, protein expression, and cholesterol efflux in comparison to wild type controls. We aim to deduce novel and relevant

pathways in macrophage cholesterol metabolism that are influenced by Neu1 with potential implications in progression of atherosclerosis.

CHAPTER 2 (Published Paper): Hypomorphic sialidase expression decreases serum cholesterol by downregulation of VLDL production in mice

This article has been published in the Journal of Lipid Research. First authorship is shared between Gabriel Gyulay and Abraham Yang. The article citation is as follows: Yang A, Gyulay G, Mitchell M, White E, Trigatti BL, Igdoura SA. Hypomorphic sialidase expression decreases serum cholesterol by downregulation of VLDL production in mice. *J Lipid Res* 2012 December;53(12):2573-85.

All work was performed by Gabriel Gyulay except for Figure 1B, Table 1/3, Figure 2, Figure 3, Figure 4A-B, Figure 5A and C, and Figure 6B. Elizabeth White performed sialidase activity assay (Figure 1B). Abraham Yang performed FPLC analysis (Figure 2), serum lipid measurements (Table 1), VLDL production assay and MTP western blot (Figure 3, Table 3), SREBP2 and ACAT2 westerns (Figure 5A,C) and LRP-1 western blot (Figure 6B). Mark Mitchell generated the Neu1 vector and adenovirus (Figure 4A-B). A reprint of the original is enclosed below.

Hypomorphic sialidase expression decreases serum cholesterol by downregulation of VLDL production in mice

Abraham Yang,[1,*] Gabriel Gyulay,[1,*] Mark Mitchell,[*] Elizabeth White,[*] Bernardo L. Trigatti,[†,] and Suleiman A. Igdoura[2,*,§]**

Departments of Biology,[*] Biochemistry and Biomedical Sciences,[†] and Pathology and Molecular Medicine,[§] and Thrombosis and Atherosclerosis Research Institute,[**] McMaster University, Hamilton, Ontario, Canada

Abstract **Lipoprotein metabolism is an important contributing factor in the development and progression of atherosclerosis. Plasma lipoproteins and their receptors are heavily glycosylated and sialylated, and levels of sialic acids modulate their biological functions. Sialylation is controlled by the activities of sialyltranferases and sialidases. 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 the rate of hepatic lipoprotein secretion and lipoprotein uptake. Our results indicate that hepatic levels of cholesterol and triglycerides are significantly higher in B6.SM mice compared with C57Bl/6 mice; however, VLDL-triglyceride production rate is lower. In addition, B6.SM mice show significantly lower levels of hepatic microsomal triglyceride transfer protein (MTP) and active sterol-regulatory element binding protein (SREBP)-2 but higher levels of diglyceride acyltransferase (DGAT)2; these are all indicative of increased hepatic lipid storage. Rescue of sialidase activity in hypomorphic sialidase mice using helper-dependent adenovirus resulted in increased VLDL production and an increase in MTP levels. Furthermore, hypomorphic sialidase expression results in stabilization of hepatic LDL receptor (LDLR) protein expression, which enhances LDL uptake. These findings provide novel evidence for a central role of sialidase in the cross talk between the uptake and production of lipoproteins.**—Yang, A., G. Gyulay, M. Mitchell, E. White, B. L. Trigatti, and S. A. Igdoura. **Hypomorphic sialidase expression decreases serum cholesterol by downregulation of VLDL production in mice.** ***J. Lipid Res.*** **2012.** 53: **2573–2585.**

Supplementary key words neu1 • very low density lipoprotein • cholesterol metabolism • adenovirus

Atherosclerosis is a chronic inflammation of the arteries caused by subendothelial accumulation of modified lipoproteins and their interactions with components of the vasculature and immune cells (1). This complex and multifactorial disease is the leading preventable cause of death in the modern world due to complications involved with plaque formation and rupture, which lead to coronary heart disease, myocardial infarctions, and strokes (2). Neu1 sialidase belongs to a family of hydrolytic enzymes that cleave terminal sialyl linkages of glycoproteins, glycolipids, and oligosaccharides (3). 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 (4–8). Some of its documented roles include regulation of the function of cell-surface molecules, such as TLR4, CD15, CD22, CD43, CD44, and CD45 (9–17). In inflammatory response, sialidase is involved in modulating the function of macrophages (18–20), T cells (21–23), and neutrophils (24–26), indicating its potential effect in chronic inflammation, such as atherosclerosis, rheumatoid arthritis, and inflammatory bowel disease. To date, genes encoding lysosomal/membrane (neu1) (27–29), cytosolic (neu2) (30, 31), plasma membrane-bound (neu3) (32, 33) and mitochondrial (neu4) (34–36) sialidase 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 peripheral tissues (37). Furthermore, early reports have indicated

This work was funded by Heart & Stroke Foundation (HSF) of Canada and CIHR grants to S.A.I. E.J.W. is the recipient of a Canadian Institutes of Health Research (CIHR) postdoctoral fellowship.

Manuscript received 10 April 2012 and in revised form 14 September 2012.

Published, JLR Papers in Press, September 14, 2012
DOI 10.1194/jlr.M027300

Abbreviations: DGAT, diglyceride acyltransferase; ER, endoplasmic reticulum; FPLC, fast-protein liquid chromatography; ITR, inverted terminal repeat; LDLR, LDL receptor; MTP, microsomal triglyceride transfer protein; PCSK9, proprotein convertase subtilisin/kexin type 9; SREBP, sterol-regulatory element binding protein; TG, triglyceride.

[1] A. Yang and G. Gyulay contributed equally to this work.
[2] To whom correspondence should be addressed.
e-mail: igdoura@mcmaster.ca

that sialidase can affect LDL metabolism in vitro and that serum sialylation levels can be used as an indicator of cardiovascular disease risk (38–42). Additionally, low-density lipoprotein receptor (LDLR) and apolipoprotein (apo)B, CII, CIII, and E are all heavily sialylated, and several studies postulate that sialic acids can have functional significance on these proteins (43–52). Despite their apparent roles in lipid metabolism and atherosclerotic disease progression, the functional effects of sialic acids on these glycoconjugates have not been significantly addressed. To assess the effects of sialidase on lipoprotein and cholesterol metabolism in vivo, we sought to generate and analyze mice with hypomorphic sialidase expression. A partial deficiency of sialidase was identified in the SM/J mouse in the 1970s (53, 54), and these animals have abnormal sialylation of glycoproteins and an impaired immune response (55). Campbell and colleagues have demonstrated that the SM/J liver expresses low sialidase mRNA (28), and 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 (56). SM/J mice, however, harbor mutations in a number of other genes (57, 58), complicating analysis of the physiological consequences of low sialidase. We have therefore isolated this mutation from the SM/J mice by backcrossing onto a C57Bl/6 genetic background, generating a hypomorphic sialidase mouse, named B6.SM, which has reduced sialidase protein levels and activity. 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 hepatic lipid metabolism. This study points to sialidase as an important player in lipoprotein metabolism and as a potential therapeutic target for metabolic syndrome diseases, such as hypercholesterolemia and atherosclerosis.

METHODS

Mice

B6.SM mice were obtained by crossing SM/J mice with C57Bl/6 mice six times. The presence of 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′ and 5′ CTT AAG GGC ATT GGG GTC AT 3′, synthesized by Mobix facility at McMaster University. PCR (40 cycles) was performed with denaturing temperature at 94°C for 2 min, annealing temperature at 60°C for 30 s, and elongation temperature at 72°C for 30 s. PCR products were digested with MspA1I (New England BioLab), which serves as a genetic diagnostic as it only cleaves the PCR product carrying the B6.SM mutation. Mice were housed in microisolator cages in a room with a 12 h light and dark cycle and given unlimited access to food and water. All experimental protocols were approved by our Institutional Animal Research Ethics Committee.

Cell lines

Three human fibroblast cell lines were used: normal (MCH64) and sialidosis (WG544 and W) lines. The cell line W was isolated from a sialidosis patient; it has a premature stop codon (69G>A) in the neu1 gene and null sialidase activity (59). MCH64 and WG544 were obtained from the Montreal Children's Hospital Research Institute. Cells were cultured in DMEM with 10% FBS and maintained in a 37°C 5% CO_2 incubator. Optimem Low Serum (Invitrogen) media was used prior to receptor and lipoprotein uptake studies to starve cells and upregulate LDLR. The human embryonic kidney cell lines 293 and 293Cre4 were generous gifts from Dr. Frank Graham (McMaster University, Hamilton, ON) and were grown in F-11 medium with 10% FBS and maintained in a 37°C 5% CO2 incubator. 293Cre4 cells were grown similarly but with 0.4 mg/ml G418 antibiotic.

Adenoviral infections

All adenovirus was propagated in F-11 medium supplemented with 5% horse serum with antibiotics and fungizone. Adenoviral infections were carried out by adding the adenovirus in PBS++ on 90% confluent dishes immediately after removal of cell medium. Adenovirus was allowed to adsorb to the cell monolayer for 1 h.

Cloning of mouse sialidase gene into helper-dependent vector

A genomic fragment containing the mouse lysosomal sialidase gene and promoter was isolated from a BAC (NCBI accession number AF109906) containing 180 kb spanning the sialidase locus (Genome Systems) and was subcloned into a pBSK plasmid (60). pBSKS+msialR plasmid containing the mouse neu1 gene was digested with NotI and RsrII, yielding a 10.6 kb fragment. The helper-dependent plasmid pC4HSU was similarly digested with NotI and RsrII, producing a 19.3 kb fragment containing the adenoviral components necessary for viral encapsulation, the left and right inverted terminal repeats (ITR) and the packaging signal (Ψ). The 10.6 kb mouse sialidase fragment and 19.3 kb adenoviral vector fragment were both purified with the Geneclean II with Spin kit (Q-BIOgene). The 10.6 kb mouse sialidase fragment was then ligated by compatible cohesive ends to the 19.3 kb adenoviral vector fragment, producing a 29.9 kb helper-dependent vector containing the mouse sialidase gene, pC4HSUmsial, verified by restriction digestion (Fig. 4A).

Propagation of HD-Ad containing mouse sialidase gene

The helper-dependent plasmid pC4HSUmsial was digested with *PmeI* to release the bacterial amplification elements, ampicillin resistance, and the origin of replication, producing a 27.2 kb fragment and exposing the two ITRs on the ends of the fragment. Following heat inactivation, the digested plasmid was transfected into 293Cre4 cells by calcium phosphate transfection. After adsorption and addition of maintenance medium, complete cytopathic effect (CPE) was seen 48 h postinfection. The cells were then scraped and stored. This lysate was used to coinfect a 90% confluent dish of 293Cre4 cells along with the helper virus at an MOI of 1 PFU/cell. After complete CPE was seen, the cell lysate was harvested and stored. Coinfections continued serially up until eight lysates had been harvested. After each lysate had been collected, pronase/SDS was added to the dishes to digest the remaining adenovirus to confirm correct adenoviral propagation. Phenol extraction and ethanol precipitation purified the adenoviral DNA, and restriction digestion confirmed proper adenoviral sequence. Sequencing of the purified adenovirus DNA for the mouse sialidase promoter also confirmed the presence of the mouse sialidase gene within the viral genome. The helper-dependent vector AdC4HSULacZ was provided by

Chang-Xin Shi (McMaster University, Hamilton, ON) and was serially passaged as above.

Purification and concentration determination of adenovirus

After CPE was reached postinfection, cells were scraped into 10 mM Tris-HCl, pH 8.0. Sodium deoxycholate (5%) was added to lyse the cells, followed by the addition of 2M $MgCl_2$ and DNAase I to digest any unpackaged viral DNA as well as cellular DNA. The lysate was then spun, and the supernatant was collected and ultracentrifugated twice through CsCl density gradient (61). The lower viral bands were collected with an 18 gauge needle and syringe through the side of the tube. The collected adenovirus was then injected into a Slide-A-Lyzer dialysis cassette (Pierce) where the virus was dialyzed against three changes of 500 ml 10 mM Tris-HCl (pH 8.0) over 24 h. The adenovirus was collected from the dialysis cassette, and sterile glycerol was added to a final concentration of 10%. The concentration of helper-dependent adenovirus was determined through fluorometric analysis using Hoechst dye (Boehringer Mannheim). CsCl-banded adenovirus (20 μl) was treated with 20 μl of pronase/SDS overnight at 37°C to degrade the viral capsid. The following day, 20 μl of the pronase/SDS-treated virus was exposed to the Hoechst dye, and fluorometric analysis was measured using the Hoefer Fluorometer (Hoefer). Adenoviral particle count was based on the fluorometric result (μg/ml) of inserting this value into the following: Viral DNA Concentration (μg/ml) × 9.48 × 10^{11} / Length of Viral DNA (Kb).

Adenoviral administration in vivo

Helper-dependent adenoviruses containing mouse sialidase gene or lacZ cDNA (100 μl; 10^9 particles/mouse in sterile PBS) were injected into the tail vein of 5-month-old male B6.SM mice under isofluorane anesthetic. Mice were monitored for the incubation period of 14 days until VLDL-production experiments.

Collection of blood and tissues

Mice were anesthetized with ketamine/xylazine and euthanized by exposure of their thoracic cavity. Blood was obtained by cardiac puncture. Serum was obtained by centrifugation of blood for 5 min at 15,000 rpm using serum collection tubes (Sarstedt). Mice were perfused with PBS through the left ventricle of the heart. The liver was harvested, frozen in liquid nitrogen, and stored at −80°C for further use in protein and RNA studies.

Sialidase activity assay

Approximately 0.15 g of tissue was minced on ice and homogenized in 1.5 ml water. Tissue homogenate (50 μl) was then incubated for 1 h at 37°C with 60 μl of 0.4 mM 4-Mu-NANA in acetate buffer (pH 4.2) with 10% BSA. Assay was performed similarly for WG544 cell lysates infected with the HD-Ad sialidase. Activity is measured as the amount of fluorescence generated from the liberation of umbelliferone (4-Mu) from the NANA substrate. The reaction was stopped by the addition of 2 ml of basic 0.1 M MAP buffer. Fluorscence was then measured using a plate fluoremeter (PerkinElmer) and normalized to protein concentration.

RNA isolation and quantitative real-time PCR

Livers were homogenized in RNA lysis buffer, and then RNA was isolated using Norgen Total RNA Isolation Kit. Total RNA (1–5 μg) was 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 a 20 μl reaction per well and included appropriate blanks and standard. (PCR cycle was as follows: 10 min 95°C, 40× 15 sec 95°C, 60 sec 60°C). Primers (synthesized by MOBIX facility, McMaster University) for LDLR qRT-PCR were: 5′ TGACTCAGACGAACAAGGCTG 3′ and 5′ ATCTAGGCAATCTCGGTCTCC 3′ and for SREBP2 qRT-PCR were: 5′ GCAGCAACGGGACCATTCT 3′ and 5′ CCCCATGACTAAGTCCTTCAACT 3′.

Immunoblot analysis

Livers or cells were homogenized in RIPA buffer containing protease inhibitors (Roche), and the protein concentration was determined using the Bradford assay (Bio-Rad). Samples were separated on SDS/polyacrylamide gel and transferred to nitrocellulose membranes using Tris-Glycine buffers. The following antibodies were used: anti-MTP (mouse anti-MTP, 1:2500, BD Biosciences); anti-ACAT2 (mouse anti-ACAT2, 1:200, Santa Cruz Biotechnology); anti-SREBP-2 (mouse anti-SREBP-2, 1:500, Pharminogen); anti-SREBP-1a/c (rabbit anti-SREBP-1, 1:1000, Novus); anti-LDLR (mouse anti-LDLR, 1:1000, Calbiochem, and goat anti-LDLR 1:1000, R and D); anti-LRP1 (rabbit anti-LRP1, 1:10000, Epitomics); anti-neu1 sialidase (rabbit anti-neu1 1:500, Rockland); anti-DGAT2 (rabbit anti-DGAT2 1:1000, Novus); anti-GAPDH (goat anti-GAPDH 1:2000, R and D); anti-β-tubulin (mouse anti-β-tubulin, 1:1000, Chemicon International); and anti-β-actin (mouse anti-β-actin, 1:1000, Cell Signaling Technology). Secondary HRP-conjugated antibodies (1:10000, Santa Cruz Biotechnology) were used and visualized with chemiluminescence (ECL, Amersham). Intensity was measured by ImageJ densitometry software.

Immunoprecipitation and lectin pull-downs

Liver membrane-enriched lysates with equal amounts of protein were immunoprecipitated using LDLR antibody and Protein A prior to immunoblot analysis, as described before (62). We utilized SNA (Sambucus nigra agglutinin), which binds α 2,6 linkages of sialic acid and MALII (Maackia amurensis leucoagglutinin), which binds α 2,3 linkages of sialic acid (63). The biotin-labeled lectins (Vector Laboratories) were incubated with membrane-enriched lysates and pulled down with streptavidin beads. The enriched samples, containing glycoproteins pulled down by their sialic acids, were used for immunoblot analysis.

TrueBlot immunoprecipitation of PCSK9

Serum was incubated with anti mouse PCSK9 antibody (courtesy of Dr. Nabil Seidah, University of Montreal, Montreal, Canada) and TrueBlot agarose anti-rabbit beads (eBioscience) overnight at 4°C. Samples were spun and washed with RIPA buffer with protease inhibitors (EDTA-free). The resulting pellet was boiled in Laemlli Sample Buffer, spun down, and then 30 μl of the supernatant was subjected to SDS-PAGE and blotted with the same antibody. A special secondary antibody (TrueBlot anti-Rabbit IgG HRP, eBioscience), which only detects full-length immunoglobulin, was used on the Western blot to avoid nonspecific bands.

Lipid analyses

For hepatic lipid analyses, 150 mg of liver was homogenized in 1 ml 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 300 μl of liver homogenates, and the tubes were mixed for 1 min. After that, 0.6 ml of distilled water was added to the tubes, and the tubes were mixed for 1 min. The extraction mixture was left at 4°C for 2 h. After 2 h, the tubes were centrifuged at low speed to facilitate phase separation. The lower phase (chloroform phase) was dried completely by sitting in a water bath at 37°C. The dry chloroform phase was resuspended in 60 μl of isopropanol. Hepatic total cholesterol was analyzed with enzymatic

assay (Infinity Cholesterol Liquid Stable Reagent, Thermo Scientific). The enzymatic colorimetric assay product was measured at 500 nm. Free cholesterol was analyzed with Free Cholesterol E Reagent (Wako Diagnostics). The absorbance of the reaction product was measured at 600 nm. Cholesteryl ester concentration was calculated by subtracting free cholesterol measurements from total cholesterol concentration. Triglyceride was analyzed with enzymatic colorimetric assay (L-Type Triglyceride H, Wako Diagnostics). The absorbance of the reaction product was measured at 600 nm. Serum samples were measured directly as above. For lipoprotein cholesterol analyses, 300 µl of serum was fractionated by gel filtration-FPLC using a Superose 6 column (64), and lipid levels were measured with enzymatic assay as above.

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) (15 g/dl in 0.9% NaCl). Mice were fasted overnight prior to the experiments, and 500 mg/kg mg of Triton WR 1339 was injected. Blood samples were taken from the cheek under light anesthesia before and at 1, 2, 3, and 4 h after Triton injection for triglyceride, cholesterol, free cholesterol, and cholesteryl ester measurements. VLDL-triglyceride, cholesterol, free cholesterol, and cholesteryl ester production rates were obtained by calculating the slope of the regression line of the graph with VLDL-triglyceride, cholesterol, free cholesterol, and cholesteryl ester concentration, respectively, versus time in hours.

Fibroblast Oil Red O staining

For staining of neutral lipids in cells, Oil Red O powder from Sigma-Aldrich (O0625) was prepared by dissolving 2.5 g in 500 ml of isopropanol. Prior to experimental use, this mixture was diluted 3:2 with isopropanol and filtered to remove any particulate matter. Cells were grown on uncoated glass coverslips in a 24-well plate and incubated with 50 µg/ml LDL (BTI Inc.) for 24 h after growing in Optimem for 72 h. Cells were washed, fixed with 3.7% formaldehyde, and then washed with 60% isopropanol prior to Oil Red O staining for 1 h. This was followed by another 60% isopropanol rinse and four PBS washes. Hematoxylin staining was then performed, and the coverslips were mounted on microscope slides using Aqua Mount from Fisher. Pictures were taken using brightfield and phase microscopy with Zeiss Axiovision software. Quantification was performed by measuring the red stained area and dividing by nuclear area per cell and averaging each group.

Immunofluorescence

Cells were grown in Optimem for 72 h then fixed with 3.7% formaldehyde and permeabilized with 0.5% Triton-X 100 in PBS. Cells were blocked in 20% goat serum in PBS, incubated overnight with anti-LDLR antibody (R and D) at 1:400, washed, incubated with Texas Red anti-goat secondary at 1:400, stained with DAPI, and then mounted using ProLong Gold Antifade reagent from Invitrogen. Images were taken and analyzed with AxioVision software from Zeiss.

Fibroblast cholesterol assay

Fibroblasts were grown in Optimem for 72 h then incubated with 50 µg/ml LDL for 24 h. Briefly, cells were washed and lipids were extracted using a hexane/isopropanol mixture overlaid on the cells for 1 h. The mixture was allowed to evaporate and lipids were resuspended in 100 µl isopropanol for enzymatic measurements of cholesterol as described above. After lipid extraction, cells were scraped in 0.1% SDS, and protein content was measured using a Lowry assay to normalize cholesterol readings.

Statistical analysis

Statistical analyses between multiple groups of data were analyzed by one-way ANOVA followed by Tukey comparison test and multiple comparison test using Prism 5 (version 5.04). Statistical analyses between two groups were performed using an unpaired Student's *t*-test. Error bars represent SEM unless otherwise noted. Data were considered statistically different if $P < 0.05$.

RESULTS

Sialidase protein expression and activity in B6.SM mice

Western blot analysis of hepatic neu1 sialidase reveals a significant reduction (approximately 85%) in sialidase expression in B6.SM compared with C57Bl/6 mice (**Fig. 1A**). Sialidase activity is significantly reduced in the brain, liver, spleen, and kidney of B6.SM males compared with C57Bl/6 controls (Fig. 1B). The sialidase enzymatic assay of organ

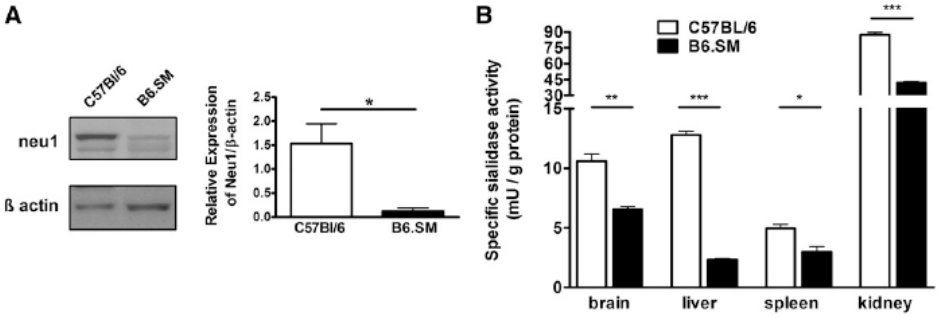


Fig. 1. Sialidase protein expression and activity in C57Bl/6 and B6.SM mice. (A) B6.SM males show a significant reduction in hepatic neu1 sialidase expression compared with C57Bl/6 controls ($P = 0.03$). Representative blots of n = 3 for each group. Liver lysates were subjected to SDS-PAGE (8%). Membranes were probed with anti-neu1 Sialidase antibody and anti-β-actin as a control. Intensities of bands were measured by ImageJ densitometry software. (B) B6.SM tissues have significantly lower levels of sialidase activity, and this is especially prominent in the liver where levels are reduced to approximately 20% of C57Bl/6. Brain, liver, spleen, and kidney lysates were assessed for sialidase activity using fluorescent 4-Mu-NANA. $*P = 0.01$, $**P = 0.001$, $***P < 0.0001$. mU = µMol/hr.

extracts from B6.SM mice indicated that this strain recapitulates a tissue-specific hypomorphic sialidase mouse model.

Effect on serum cholesterol levels and hepatic lipid levels

Measurement of fasted serum levels of total cholesterol and triglycerides revealed no significant difference between C57Bl/6 and B6.SM mice (**Table 1**). However, when serum lipoproteins from unfasted male mice were fractionated by size exclusion using fast-protein liquid chromatography (FPLC) with a superose 6 gel filtration column (**Fig. 2**), the cholesterol profiles of B6.SM mice show significantly lower cholesterol levels in LDL fractions (fractions 20–30) than those of the corresponding C57Bl/6 controls. Also, the HDL peak shows a slight shift indicative of smaller-sized HDL particles in B6.SM mice (64–66) (Fig. 2). Additionally, the VLDL peak appears to be slightly smaller in B6.SM mice (Fig. 2) and to shift to the right, indicative of smaller-sized VLDL particles (Fig. 2, inset). These results indicate that hypomorphic sialidase expression appears to lower cholesterol levels of LDL-sized particles. To determine whether the altered cholesterol profile is associated with altered hepatic lipid metabolism, we measured the hepatic total cholesterol, triglyceride, free cholesterol, and cholesteryl ester content in both B6.SM and C57Bl/6 mice. We observed a significant increase in hepatic total cholesterol, cholesteryl esters, and triglyceride in B6.SM mice compared with C57Bl/6 mice (**Table 2**). A trend of increase in hepatic free cholesterol of B6.SM mice compared with C57Bl/6 mice is also noted. These results point to sialidase activity as having a role in modulation of lipid metabolism and homeostasis in the liver.

In vivo effect on hepatic VLDL-lipid production and MTP expression

To determine whether the changes in serum and hepatic cholesterol levels are caused by a decrease in 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 hepatic production rates of VLDL. Our results indicate that B6.SM mice had significantly lower production rates of VLDL-triglyceride (VLDL-TG) over a 4 h period (**Fig. 3A** and **Table 3**). These results show that hypomorphic sialidase expression decreases VLDL-TG production, which coincided with lower serum cholesterol levels in B6.SM mice. This decrease in VLDL-TG production is accompanied by lower microsomal triglyceride transfer protein (MTP) protein expression in the liver

TABLE 1. Serum lipid levels in C57Bl/6 and B6SM mice

	Serum Total Cholesterol	Serum Triglycerides
	mM	*mM*
C57Bl/6 Male	3.33 ± 0.94	0.63 ± 0.25
B6.SM Male	2.97 ± 0.29	0.52 ± 0.10

Fasted serum levels of cholesterol and triglycerides from six-week-old C57Bl/6 and B6SM males on a standard chow diet (n = 3). Mean ± SD are shown.

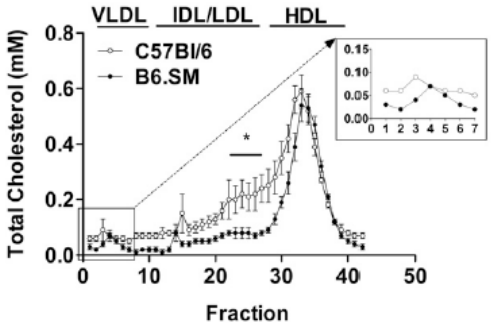


Fig. 2. FPLC cholesterol profiles of C57Bl/6 and B6.SM mice. Serum lipoproteins were fractionated by size using FPLC using a superose 6 gel filtration column. The FPLC cholesterol profiles of the unfasted male B6.SM animals (n = 3) have significantly less total cholesterol in LDL-sized particles than those of the corresponding C57Bl/6 controls (n = 3). In addition, there is a tendency of the HDL and VLDL (inset) peaks to be shifted slightly to the right. Each circle represents mean ± SE (*$P < 0.05$).

(Fig. 3E), which is required for triglyceride loading onto apoB and subsequently VLDL synthesis (67–69). As a result, the decrease in VLDL-triglyceride appears to be a direct result of the decreased protein expression of MTP. These data show that B6.SM mice exhibit a drastically altered lipoprotein metabolism initiated by reductions in VLDL assembly and production.

Hepatic VLDL production and MTP expression after sialidase gene therapy in B6.SM mice

The mouse lysosomal sialidase gene (10.6 kb) was ligated via compatible cohesive ends into the 19.3 kb adenoviral vector pC4HSU that contained the two ITRs and the packaging signal (Ψ). The resultant plasmid, pC4HSU-msial, was confirmed by digesting with EcoRI, yielding the expected fragment sizes (**Fig. 4A**). After the helper-dependent mouse sialidase adenovirus was properly characterized and CsCl purified (see Methods), we analyzed its effects on sialidase activity. Sialidosis fibroblast cells were infected with increasing doses of viral particles, which caused a steady, almost linear increase in sialidase activity with increasing virus concentration (Fig. 4B). These results demonstrate that this helper-dependent mouse sialidase (HD-AdSial) adenovirus is a functional vector capable of producing active mouse neu1 sialidase in vitro. Because B6.SM male mice have lower VLDL-TG production compared with C57Bl/6 controls (Fig. 3), we sought to determine whether adenoviral sialidase gene therapy would rescue the phenotype in B6.SM animals. Thus, we infected B6.SM sialidase-deficient male mice with helper-dependent mouse sialidase (HD-AdSial) or LacZ (HD-AdlacZ) adenovirus, and then measured VLDL-TG production. The HD-AdSial group had significantly higher expression of neu1 sialidase protein in the liver compared with the HD-AdlacZ group, confirming expression of the virus (Fig. 4C). B6.SM mice infected with sialidase virus had significantly higher VLDL-TG production compared with LacZ controls (Fig. 4D). These data directly demonstrate that

TABLE 2. Hepatic lipid levels in C57Bl/6 and B6.SM mice

	Hepatic Total Cholesterol	Hepatic Free Cholesterol	Hepatic Cholesteryl Esters	Hepatic Triglycerides
	mM	*mM*	*mM*	*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[a]	0.76 ± 0.04	0.61 ± 0.004[a]	8.56 ± 0.16[a]

Lipids from livers of C57Bl/6 (n = 3) and B6.SM mice (n = 3) were isolated using Folch extraction. There is a significant increase in hepatic total cholesterol, cholesteryl esters, and triglyceride levels in B6.SM male mice compared with C57Bl/6 ($P = 0.01$, $P = 0.02$, and $P = .048$, respectively). In addition, there is a trend of increase in hepatic free cholesterol levels in B6.SM mice compared with C57Bl/6 ($P = 0.19$). Mean ± SE are shown.
[a]$P < 0.05$.

rescuing sialidase deficiency via adenovirus increases VLDL-TG production, similar to what was observed in C57Bl/6 mice. The HD-AdSial group also had an increase in hepatic MTP protein (Fig. 4E) compared with the LacZ controls, indicating that sialidase can affect MTP levels, although the mechanism is yet to be determined. These findings enable us to conclude that low levels of neu1 sialidase in B6.SM mice are directly driving reduced VLDL-TG production.

Hypomorphic sialidase expression decreases hepatic SREBP-2 and increases hepatic DGAT2 expression

To investigate the mechanisms behind the changes in hepatic lipid levels and lipoprotein metabolism, we analyzed protein expression of several important enzymes and transcription factors. We found a significant decrease in cleaved hepatic sterol-regulatory element binding protein (SREBP)-2 expression in B6.SM mice (**Fig. 5A**). It has been shown that the MTP promoter contains SREBP-2 response elements (70, 71), suggesting that reduced SREBP-2 may contribute to the reduced MTP levels. Nevertheless, higher levels of cholesterol in the livers of B6.SM mice reduce active SREBP-2 and affect downstream gene expression. Additionally, protein levels of cleaved SREBP-1a/c remain unchanged between the two strains (Fig. 5B). It appears that the phenotype observed in B6.SM livers is primarily due to SREBP-2 and independent of SREBP-1. Furthermore, we assessed the expression of hepatic acylCoA:cholesterol acyltransferase (ACAT-2), which mediates esterification of hepatic cholesterol (72), and we found a trend of higher ACAT-2 expression, implying that there is sufficient excess of cholesterol to be esterified (Fig. 5C). Due to the increases in triglyceride levels observed in the livers of B6.SM mice, it was important to analyze the diglyceride acyltransferase

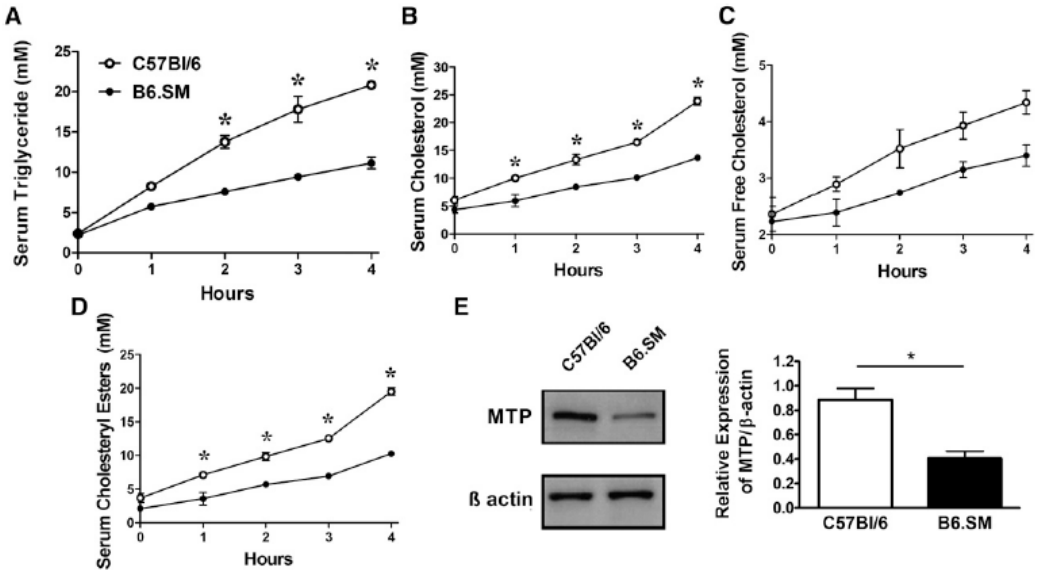


Fig. 3. In vivo hepatic VLDL-TG production in C57Bl/6 and B6.SM mice. C57Bl/6 (n = 3) and B6.SM mice (n = 3) were fasted overnight and injected with the lipoprotein lipase inhibitor Triton WR1339 (500 mg/kg). Serum samples were drawn just before the injection (time 0 h) and at 1, 2, 3, and 4 h postinjection. There is a decrease in serum (A) VLDL-triglyceride, (B) VLDL-cholesterol, (C) VLDL-free cholesterol, and (D) VLDL-cholesteryl esters concentrations at different indicated time points after 0 h (*$P < 0.05$). Also note that there is a decrease in the steepness of the slope of the B6.SM mice compared with that of the C57Bl/6 mice. This indicates that hypomorphic sialidase expression causes decreased hepatic VLDL-TG production rates. Values represent means ± SE. (E) B6.SM mice have a significant decrease in the protein expression of MTP compared with C57Bl/6 ($P = 0.002$), which further supports that hypomorphic sialidase expression results in decreased VLDL-TG production. Mouse liver lysates were subjected to SDS-PAGE (8%), and membranes were probed with anti-MTP and anti-β-actin antibodies. Intensities of bands were measured by ImageJ densitometry software.

TABLE 3. In vivo hepatic VLDL-lipid production rates (PRs) in C57Bl/6 and B6.SM mice

	Triglyceride PR	Total Cholesterol PR	Free Cholesterol PR	Cholesteryl Esters PR
	mM/hr	*mM/hr*	*mM/hr*	*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[a]	2.28 ± 0.16[a]	0.31 ± 0.06[a]	1.97 ± 0.17[a]

Male C57Bl/6 (n = 3) and male B6.SM mice (n = 3) were fasted overnight. Serum was collected just before the injection of the lipoprotein lipase inhibitor Triton WR1339 (time 0 h) and at 1, 2, 3, and 4 h post administration. 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 with C57Bl/6 mice. Mean ± SE are shown.
[a]$P < 0.05$.

(DGAT) enzyme, which facilitates the final step in endogenous triglyceride synthesis (73). We observed an increase in DGAT2 (the major enzyme) in B6.SM mice (Fig. 5D), which is indicative of increased triglyceride synthesis and changes in hepatic lipid homeostasis (73). Thus, increased hepatic retention of lipids, along with downregulated SREBP-2 and upregulated DGAT2 protein levels, appears to alter lipid homeostasis in B6.SM mice.

Modulation of hepatic expression of LDLR

To determine whether the decrease in serum cholesterol levels and the increase in hepatic cholesterol levels in B6.SM mice are caused by altered expression of lipoprotein receptors, we have evaluated the expression of LDLR and LRP-1. Although there is no significant difference in hepatic protein levels of LDLR or LRP-1 in B6.SM male mice compared with C57Bl/6 controls (**Fig. 6A**, B), there is a significant reduction in LDLR mRNA levels as measured by qRT-PCR (Fig. 6C). The decrease in SREBP-2 protein expression observed in B6.SM livers is consistent with a decrease in LDLR transcript, as the LDLR promoter contains SREBP-2 response elements. Thus, the maintenance at the protein level caused by hypomorphic sialidase expression could be due to a posttranslational mechanism, such as slower receptor trafficking/recycling or decreased degradation. 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 (74–77). Our results indicate that B6.SM mice show a decrease in the serum levels of PCSK9 compared with C57Bl/6 mice (Fig. 6D). PCSK9 gets secreted and can bind to LDL receptors at the cell surface and target them for degradation instead of recycling (78). Therefore, the lower level of PCSK9 expression may result in an increase in the rate of receptor recycling and retention of LDLR protein, despite lower mRNA levels. To determine whether hypomorphic sialidase expression affects the sialylation of LDLR directly, we performed lectin pull-downs followed by Western blotting. Membrane-enriched liver lysates were pulled down with streptavidin beads using biotin-labeled SNA or MAL II, which bind specific α-2,6 and α-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 included control samples immunoprecipitated with LDLR and blotted for total LDLR to ensure equal starting amounts. We observed higher levels of LDLR-associated sialic acids in livers of B6.SM mice compared with C57Bl/6 (Fig. 6E). This indicates that sialic acid molecules on LDLR are directly affected by reduced sialidase expression, with potential functional consequences. Thus, hypomorphic sialidase expression leads to hypersialylation of LDLR, which in turn could affect the trafficking, degradation, or turnover of the receptor.

Sialidase-null mutation increases LDL uptake in human fibroblasts

To address the direct functional effect of sialidase on LDLR, we utilized human fibroblasts that have null neu1 sialidase activity. The aim of this experiment was to complement the in vivo data with human cells in vitro. We observed no differences in LDLR protein expression between normal and sialidase-null cells, but sialidase-null cells had slightly higher molecular weight LDLR protein, potentially due to hypersialylation (**Fig. 7A**). Furthermore, LDLR immunofluorescence has shown that the receptors appear to cluster next to the nucleus to a greater extent in the sialidosis cell line (Fig. 7B, arrows), although there were no gross changes in expression. To measure the function of the LDLR, we analyzed LDL uptake and lipid droplet formation via Oil Red O staining. Both cell types showed minimal staining during serum starvation but exhibited lipid droplet formation after 24 h of LDL treatment, indicating significant LDL uptake. Neutral lipid accumulation appeared to be slightly higher (although not significant) in sialidase-null versus normal control cells (Fig. 7C), as measured by Oil Red O quantification. To assess this uptake more quantitatively, we utilized lipid extraction followed by enzymatic cholesterol level measurements in these cells. Both cell types had significant increases in cholesterol levels when treated with LDL, indicating internalization of the lipoprotein. However, sialidase-null fibroblasts treated with LDL had significantly higher total cholesterol levels than wild-type cells treated with LDL (Fig. 7D). These data indicate that the absence of neu1 sialidase activity results in higher cholesterol levels due to increased LDL uptake in sialidase-null fibroblasts, consistent with our findings in livers of B6.SM mice.

DISCUSSION

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

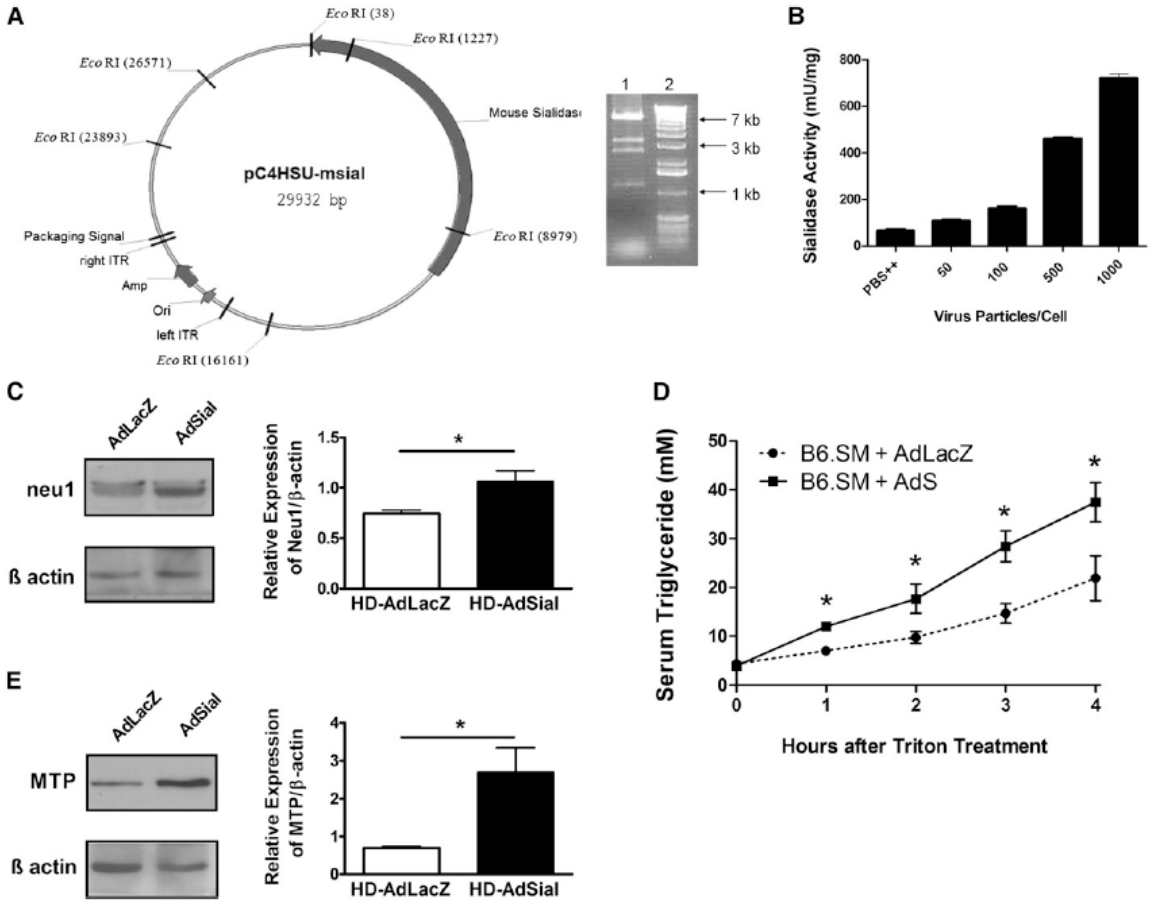


Fig. 4. In vivo hepatic VLDL-TG production in B6.SM mice rescued with sialidase gene therapy. (A) Plasmid map of pC4HSUmsial helper-dependent adenovirus displaying fragment sizes corresponding to an *Eco*RI digestion. Restriction analysis of pC4HSUmsial using *Eco*RI reveals the appropriate fragments following digestion (lane 1, pC4HSUmsial; lane 2, 1 kb ladder). This virus was combined into a helper-dependent vector, yielding HD-AdSial (see Methods). (B) Sialidase activity of sialidosis fibroblast cells (WG544) that were infected with HD-AdSial at various particles/cell. Viral infection increases sialidase activity at a particle-dependent rate (n = 3, error bars represent SE). B6.SM male mice (n = 4 for each group) were infected with helper-dependent adenovirus containing mouse sialidase (HD-AdSial) or LacZ (HD-AdlacZ) and monitored for 14 days. (C) Neu1 sialidase protein is upregulated in liver lysates of the HD-AdSial group as measured by Western blotting, indicating expression of the viral vector. VLDL-TG production was analyzed by hourly serum triglyceride measurement following injection of LPL inhibitor Triton WR1339, as described above. (D) B6.SM mice infected with mouse sialidase helper-dependent adenovirus have significantly higher VLDL-TG production compared with B6.SM mice infected with HD-AdlacZ control adenovirus. (E) MTP protein expression is increased in B6.SM mice that were infected with HD-AdSial compared with HD-AdlacZ as measured by Western blotting. $^*P < 0.05$.

The B6.SM mouse shows a drastic reduction in sialidase protein and activity, and our study revealed a significant decrease in cholesterol levels in IDL/LDL-sized particles in B6.SM mice with a shift in the size of HDL and VLDL particles as revealed by FPLC cholesterol profiles. We demonstrated that hypomorphic sialidase mice have lower serum cholesterol levels as a result of lower hepatic VLDL production and potentially higher hepatic LDL uptake. This is coupled with an increase in hepatic lipid storage and a decrease in hepatic MTP protein, all of which are suggestive of an atheroprotective phenotype, which appears to be driven by reduced VLDL secretion. To confirm that the lower VLDL-TG production phenotype is a direct result of the hypomorphic neu1 gene, we transduced neu1 sialidase expression using helper-dependent adenovirus in B6.SM livers and were able to show a significant increase in both VLDL-TG production and hepatic MTP protein levels compared with HD-AdlacZ controls.

To dissect the mechanisms of increased hepatic lipid storage and decreased VLDL-production in B6.SM mice, hepatic cholesterol and triglyceride metabolism in the liver were investigated. The SREBP transcription factors are master regulators of hepatic lipid homeostasis (79). Our observations of higher hepatic cholesterol levels resulting in lower active SREBP-2 and lower VLDL production

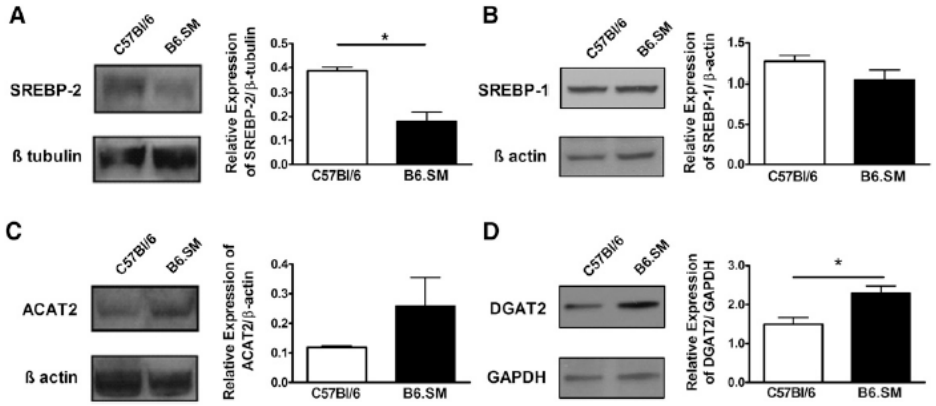


Fig. 5. Hepatic SREBP-2, SREBP-1, ACAT-2, and DGAT2 expression in C57Bl/6 and B6.SM mice. Liver lysates were subjected to SDS-PAGE (8%). Membranes were probed with (A) anti-SREBP-2, (B) anti-SREBP-1a/c, (C) anti-ACAT-2, and (D) anti-DGAT2 and anti-β-actin or anti-β-tubulin or anti-GAPDH antibodies as a control. Intensities of bands were measured by ImageJ densitometry software. B6.SM mice have a significant decrease in the protein expression of cleaved SREBP-2 ($P = 0.004$), a significant increase in DGAT2 protein expression ($P = 0.01$), and a trend of increase in the protein expression of ACAT-2 ($P = 0.11$) in liver compared with C57Bl/6 mice. Representative blots of n = 3 for each group.

is consistent with previous studies (80). SREBP-2 is typically endoplasmic reticulum (ER)-resident and bound to SREBP cleavage activating protein (SCAP), and it can be escorted to the Golgi when sterol levels are low, where site-1 and site-2 proteases can convert SREBP to its active form, which induces the transcription of genes involved in cholesterol synthesis (81). As intracellular cholesterol levels increase in hepatocytes of hypomorphic sialidase mice, the insulin-induced gene 1 (Insig) can bind to SCAP, and the Insig and SCAP complex will retain SREBP in the ER (82), limiting the transcriptional activation of cholesterol synthesis genes as well as MTP and LDLR. Thus, the decreased hepatic expression of MTP and lower VLDL production in B6.SM mice could be due to a downstream effect of decreased levels of active SREBP-2. Interestingly, B6.SM mice have no significant difference in hepatic SREBP-2 mRNA compared with 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. In fact, Horton and colleagues have reported that transgenic mice overexpressing SREBP-2, but not SREBP-1, exhibit increased MTP gene transcription (71, 83). This implies that hepatic cholesterol levels could be the driving force for our observed phenotype, primarily through a decrease in active SREBP-2. In contrast, Sato and colleagues have shown that SREBP negatively regulates MTP gene transcription, although their work was performed in human HepG2 cells (84). Further work is required to consolidate these results with our observations. Furthermore, we have shown that protein levels of cleaved SREBP-1a/c, which is a master transcriptional regulator of fatty acid synthesis (80), are unchanged between C57Bl/6 and B6.SM male mice, indicating that SREBP-driven regulation of hepatic fatty acid synthesis is unaffected by sialidase levels. It appears that the phenotype observed in B6.SM livers is primarily due to SREBP-2 and is independent of SREBP-1a/c. In addition, higher levels of hepatic esterified cholesterol as a result of high levels of ACAT-2 expression were observed in hypomorphic sialidase mice. ACAT-2 is an ER-bound enzyme that forms cholesterol esters from cholesterol (85). ACAT-2 activity decreases the solubility of cholesterol and prevents its incorporation into lipid membranes. As ACAT-2 expression is limited to hepatocytes and enterocytes, ACAT2-derived cholesteryl esters can be packaged directly into VLDL via MTP or stored as neutral lipid droplets in the cytosol. The latter option is more likely in our model as B6.SM mice show lower production levels of VLDL and higher hepatic cholesterol content. We also observed an increase in DGAT2 protein in the livers of B6.SM mice compared with C57Bl/6, which is indicative of increased triglyceride synthesis (73). These results, along with higher levels of hepatic triglyceride, favor the idea that these animals have increased lipid droplet formation and triglyceride storage, as DGAT does not affect the VLDL production rate (86). Interestingly, DGAT2 is insensitive to SREBP regulation (79). Clearly there are other factors that influence levels of DGAT2; nevertheless, changes in DGAT2 and SREBP-2 protein levels contribute to alterations in hepatic lipid homeostasis in hypomorphic sialidase mice. Overall, the decrease in VLDL-lipid production rate observed in B6.SM mice is caused by decreased MTP expression, which appears to be caused by increased hepatic retention of lipids and down-regulated SREBP-2 levels.

In addition to VLDL production, hepatic cholesterol content is primarily dependent on LDL endocytosis via LDLR or on chylomicron uptake via LRP-1. The stabilized levels of hepatic LDLR protein in the hypomorphic sialidase mice appear to be due to posttranslational events, as the LDLR mRNA level is in fact lower in B6.SM mouse livers.

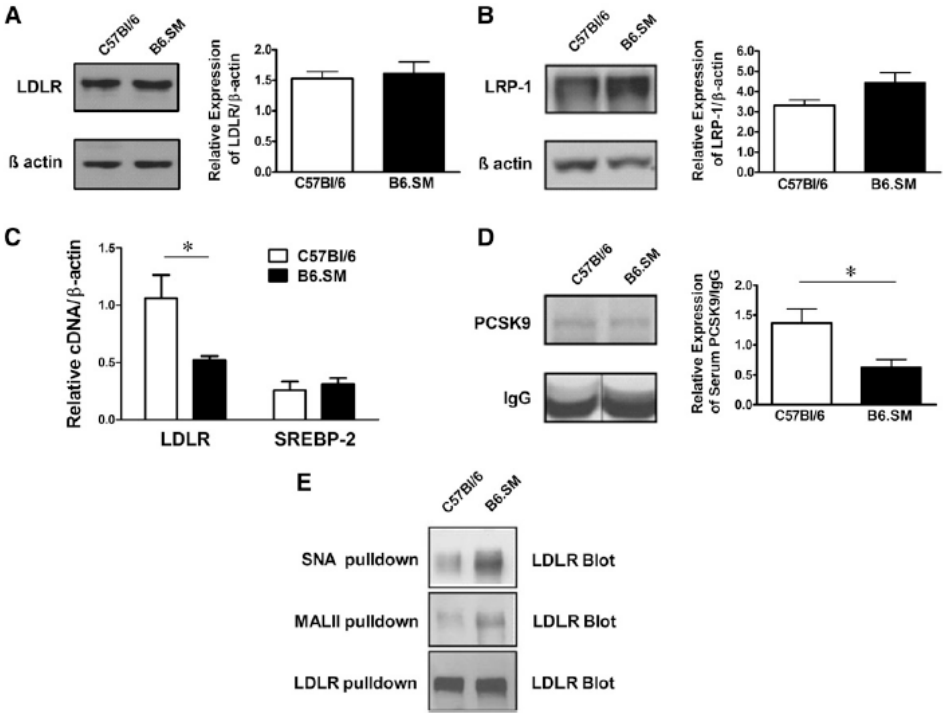


Fig. 6. Hypomorphic sialidase expression influences hepatic expression and glycosylation of LDLR. (A) B6.SM male mice have equal hepatic protein levels of LDLR compared with C57Bl/6 controls. Representative blots of n = 3 for each group. Liver lysates were subjected to SDS-PAGE followed by Western blotting for LDLR and β-actin (as a control). Intensities of bands were measured by ImageJ densitometry software. (B) B6.SM male mice have a trend of increase in hepatic protein levels of LRP-1 compared with C57Bl/6 mice, although this is not significant ($P = 0.06$). (C) B6.SM mice have lower levels of hepatic LDLR mRNA compared with C57Bl/6 controls while showing no difference in SREBP-2 mRNA ($P = 0.03$ and $P = 0.3$, n = 3 in each group). (D) PCSK9 TrueBlot immunoprecipitation of B6.SM and C57Bl/6 mouse serum. B6.SM mice have lower levels of secreted PCSK9 compared with C57Bl/6 controls ($P = 0.026$, n = 3 in each group). (E) LDLR from B6.SM mice has higher levels of terminal α-2,6 linked and α-2,3 linked sialic acids compared with C57Bl/6 mice, due to lower sialidase levels. 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 equal quantities of LDLR in starting lysates.

The lower LDLR mRNA levels are as expected due to lower SREBP-2 protein levels, but the maintenance of the protein is a unique finding in the B6.SM mice. The increased sialic acid content of the LDLR protein appears to result in its retention, potentially by increasing its half-life or recycling or decreasing its degradation. We postulate that significant alterations in the sialylation of LDLR (as observed in B6.SM mice) could have functional implications by altering its interaction with LDL, PCSK9, or the recycling machinery. Interestingly, the lower levels of PCSK9 in hypomorphic sialidase mice are likely responsible for maintaining steady-state levels of LDL receptors in spite of lower LDLR transcripts. As a result, the reduced level of LDLR degradation enables its recycling and its higher efficiency for LDL internalization. The dependency on PCSK9 for LDLR stabilization does not seem to be a requirement for LDL uptake in human fibroblasts, where significantly higher levels of LDL were internalized by sialidase-null compared with normal cells. The latter points more toward differences in ligand-receptor interactions between sialidase-null and normal fibroblasts, as earlier reports had suggested dependency on sialylation for LDL uptake in vitro (38). This also provides verification that sialidase can affect LDLR function directly and complements the mechanism delineated in vivo. These human fibroblast data provide strong evidence for the role of sialidase in LDLR function and suggest a role for sialidase in human lipoprotein metabolism.

Overall, we demonstrate that the changes in lipoprotein metabolism observed in hypomorphic sialidase mice are mediated by a decrease in the production of VLDL-TG in the liver, which is driven by reduced MTP expression and also by hepatic retention of cholesterol and triglycerides. Despite having similar levels of LDLR protein in the liver, hypersialylated LDL receptors in the B6.SM mice are more effective in internalizing LDL, and as a result, hypomorphic sialidase mice show lower serum LDL cholesterol. Although the exact mechanism is unknown, sialylation of LDLR appears to affect

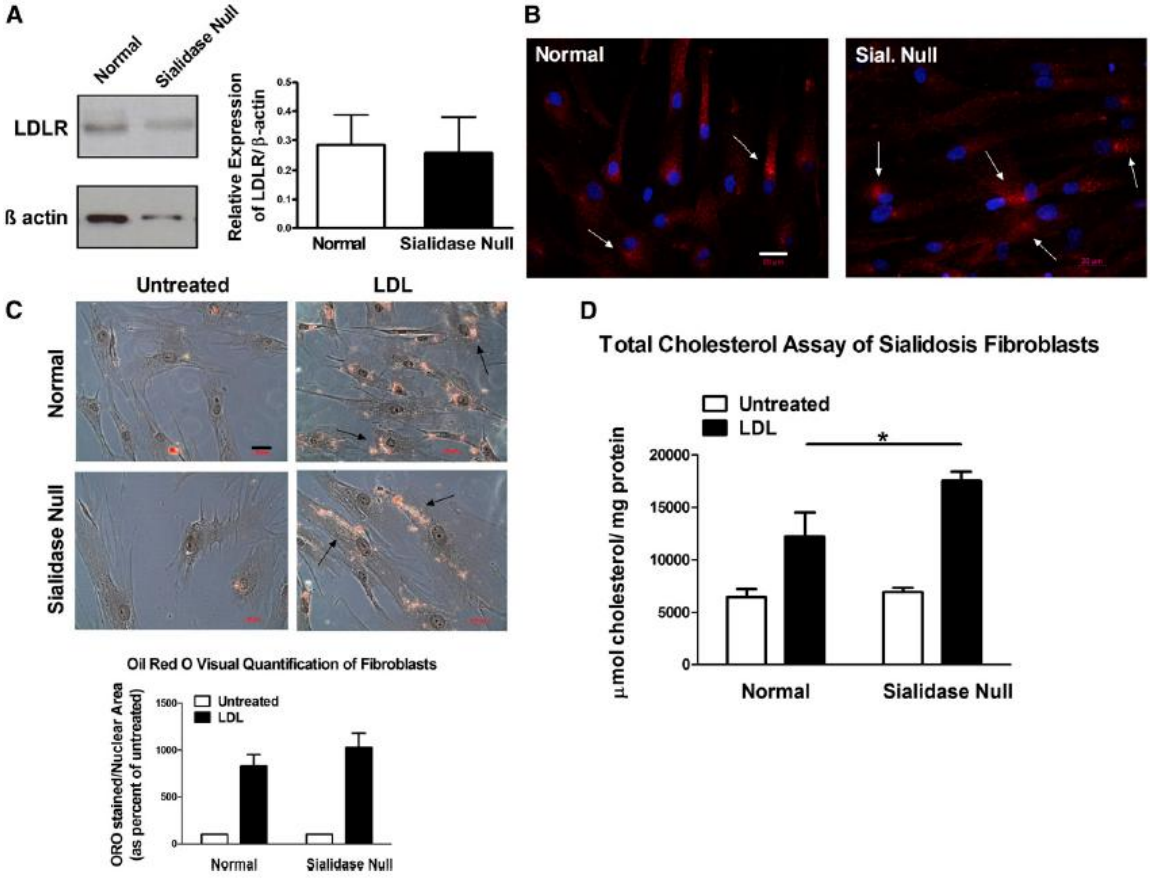


Fig. 7. LDLR expression and function in human fibroblasts with a Neu1 sialidase-null mutation. Cells were serum starved in Optimem media to upregulate LDLR and analyzed by Western blotting, immunofluorescence, Oil Red O staining, and enzymatic cholesterol assay. (A) Sialidase-null fibroblasts do not show significantly different LDLR protein expression, although their receptors appear to be of a higher molecular weight (representative of four blots). (B) LDLR immunofluorescence shows localization and clustering of receptors in fibroblasts (arrows), but no gross expression changes between the two cell types (bar = 20 µm). (C) Oil Red O staining after LDL incubation shows lipoprotein uptake and lipid droplet formation (arrows) in the fibroblasts, although there was no significant difference in neutral lipid accumulation (bar = 20 µm). (D) Sialidase-null fibroblasts show significantly higher total cholesterol levels as measured by enzymatic cholesterol assay after LDL incubation compared with wild-type cells, indicating more LDL uptake ($P = 0.048$, n = 3 in each group).

its recycling or internalization, potentially through PCSK9. Taken together, these events are expected to lead to an atheroprotective effect through the lowering of LDL cholesterol in the serum. These findings provide evidence of a central role for sialidase in cholesterol metabolism and set the stage for examining polymorphisms in the human neu1 gene and potential links to cardiovascular disease.

REFERENCES

1. Hegele, R. A. 2009. Plasma lipoproteins: genetic influences and clinical implications. *Nat. Rev. Genet.* **10:** 109–121.
2. Glass, C. K., and J. L. Witztum. 2001. Atherosclerosis: the road ahead. *Cell.* **104:** 503–516.
3. Reuter, G., and H. J. Gabius. 1996. Sialic acids structure-analysis-metabolism-occurrence-recognition. *Biol. Chem. Hoppe Seyler.* **377:** 325–342.
4. Collard, J. G., J. F. Schijven, A. Bikker, R. G. La, J. G. Bolscher, and E. Roos. 1986. Cell surface sialic acid and the invasive and metastatic potential of T-cell hybridomas. *Cancer Res.* **46:** 3521–3527.
5. Pilatte, Y., J. Bignon, and C. R. Lambre. 1993. Sialic acids as important molecules in the regulation of the immune system: pathophysiological implications of sialidases in immunity. *Glycobiology.* **3:** 201–218.
6. Varki, A. 1997. Sialic acids as ligands in recognition phenomena. *FASEB J.* **11:** 248–255.
7. Bonten, E., A. van der Spoel, M. Fornerod, G. Grosveld, and A. d'Azzo. 1996. Characterization of human lysosomal neuraminidase defines the molecular basis of the metabolic storage disorder sialidosis. *Genes Dev.* **10:** 3156–3169.
8. Varki, A. 2008. Sialic acids in human health and disease. *Trends Mol. Med.* **14:** 351–360.
9. Abdulkhalek, S., S. R. Amith, S. L. Franchuk, P. Jayanth, M. Guo, T. Finlay, A. Gilmour, C. Guzzo, K. Gee, R. Beyaert, et al. 2011. Neu1 sialidase and matrix metalloproteinase-9 cross-talk is essential for Toll-like receptor activation and cellular signaling. *J. Biol. Chem.* **286:** 36532–36549.
10. Katoh, S., T. Miyagi, H. Taniguchi, Y. Matsubara, J. Kadota, A. Tominaga, P. W. Kincade, S. Matsukura, and S. Kohno. 1999. Cutting edge: an inducible sialidase regulates the hyaluronic acid binding ability of CD44-bearing human monocytes. *J. Immunol.* **162:** 5058–5061.
11. Cuff, C. A., D. Kothapalli, I. Azonobi, S. Chun, Y. Zhang, R. Belkin, C. Yeh, A. Secreto, R. K. Assoian, D. J. Rader, et al. 2001. The adhesion

receptor CD44 promotes atherosclerosis by mediating inflammatory cell recruitment and vascular cell activation. *J. Clin. Invest.* **108:** 1031–1040.
12. Gee, K., M. Kozlowski, and A. Kumar. 2003. Tumor necrosis factor-alpha induces functionally active hyaluronan-adhesive CD44 by activating sialidase through p38 mitogen-activated protein kinase in lipopolysaccharide-stimulated human monocytic cells. *J. Biol. Chem.* **278:** 37275–37287.
13. Khan, A. I., S. M. Kerfoot, B. Heit, L. Liu, G. Andonegui, B. Ruffell, P. Johnson, and P. Kubes. 2004. Role of CD44 and hyaluronan in neutrophil recruitment. *J. Immunol.* **173:** 7594–7601.
14. Gadhoum, S. Z., and R. Sackstein. 2008. CD15 expression in human myeloid cell differentiation is regulated by sialidase activity. *Nat. Chem. Biol.* **4:** 751–757.
15. Katoh, S., S. Maeda, H. Fukuoka, T. Wada, S. Moriya, A. Mori, K. Yamaguchi, S. Senda, and T. Miyagi. 2010. A crucial role of sialidase Neu1 in hyaluronan receptor function of CD44 in T helper type 2-mediated airway inflammation of murine acute asthmatic model. *Clin. Exp. Immunol.* **161:** 233–241.
16. Katoh, S., O. Kaminuma, T. Hiroi, A. Mori, T. Ohtomo, S. Maeda, H. Shimizu, Y. Obase, and M. Oka. 2011. CD44 is critical for airway accumulation of antigen-specific Th2, but not Th1, cells induced by antigen challenge in mice. *Eur. J. Immunol.* **41:** 3198–3207.
17. Feng, C., L. Zhang, L. Almulki, S. Faez, M. Whitford, A. Hafezi-Moghadam, and A. S. Cross. 2011. Endogenous PMN sialidase activity exposes activation epitope on CD11b/CD18 which enhances its binding interaction with ICAM-1. *J. Leukoc. Biol.* **90:** 313–321.
18. Lambré, C. R., A. Greffard, L. Gattegno, and L. Saffar. 1990. Modifications of sialidase activity during the monocyte-macrophage differentiation in vitro. *Immunol. Lett.* **23:** 179–182.
19. Liang, F., V. Seyrantepe, K. Landry, R. Ahmad, A. Ahmad, N. M. Stamatos, and A. V. Pshezhetsky. 2006. Monocyte differentiation up-regulates the expression of the lysosomal sialidase, Neu1, and triggers its targeting to the plasma membrane via major histocompatibility complex class II-positive compartments. *J. Biol. Chem.* **281:** 27526–27538.
20. Stamatos, N. M., F. Liang, X. Nan, K. Landry, A. S. Cross, L. X. Wang, and A. V. Pshezhetsky. 2005. Differential expression of endogenous sialidases of human monocytes during cellular differentiation into macrophages. *FEBS J.* **272:** 2545–2556.
21. Pappu, B. P., and P. A. Shrikant. 2004. Alteration of cell surface sialylation regulates antigen-induced naive CD8+ T cell responses. *J. Immunol.* **173:** 275–284.
22. Xu, Z., and A. Weiss. 2002. Negative regulation of CD45 by differential homodimerization of the alternatively spliced isoforms. *Nat. Immunol.* **3:** 764–771.
23. Chen, X. P., E. Y. Enioutina, and R. A. Daynes. 1997. The control of IL-4 gene expression in activated murine T lymphocytes: a novel role for neu-1 sialidase. *J. Immunol.* **158:** 3070–3080.
24. Cross, A. S., and D. G. Wright. 1991. Mobilization of sialidase from intracellular stores to the surface of human neutrophils and its role in stimulated adhesion responses of these cells. *J. Clin. Invest.* **88:** 2067–2076.
25. Cross, A. S., S. Sakarya, S. Rifat, T. K. Held, B. E. Drysdale, P. A. Grange, F. J. Cassels, L. X. Wang, N. Stamatos, A. Farese, et al. 2003. Recruitment of murine neutrophils in vivo through endogenous sialidase activity. *J. Biol. Chem.* **278:** 4112–4120.
26. Sakarya, S., S. Rifat, J. Zhou, D. D. Bannerman, N. M. Stamatos, A. S. Cross, and S. E. Goldblum. 2004. Mobilization of neutrophil sialidase activity desialylates the pulmonary vascular endothelial surface and increases resting neutrophil adhesion to and migration across the endothelium. *Glycobiology.* **14:** 481–494.
27. Igdoura, S. A., C. Gafuik, C. Mertineit, F. Saberi, A. V. Pshezhetsky, M. Potier, J. M. Trasler, and R. A. Gravel. 1998. Cloning of the cDNA and gene encoding mouse lysosomal sialidase and correction of sialidase deficiency in human sialidosis and mouse SM/J fibroblasts. *Hum. Mol. Genet.* **7:** 115–121.
28. Carrillo, M. B., C. M. Milner, S. T. Ball, M. Snoek, and R. D. Campbell. 1997. Cloning and characterization of a sialidase from the murine histocompatibility-2 complex: low levels of mRNA and a single amino acid mutation are responsible for reduced sialidase activity in mice carrying the Neu1a allele. *Glycobiology.* **7:** 975–986.
29. Pshezhetsky, A. V., C. Richard, L. Michaud, S. Igdoura, S. Wang, M. A. Elsliger, J. Qu, D. Leclerc, R. Gravel, L. Dallaire, et al. 1997. Cloning, expression and chromosomal mapping of human lysosomal sialidase and characterization of mutations in sialidosis. *Nat. Genet.* **15:** 316–320.
30. Monti, E., A. Preti, E. Rossi, A. Ballabio, and G. Borsani. 1999. Cloning and characterization of NEU2, a human gene homologous to rodent soluble sialidases. *Genomics.* **57:** 137–143.
31. Ferrari, J., R. Harris, and T. G. Warner. 1994. Cloning and expression of a soluble sialidase from Chinese hamster ovary cells: sequence alignment similarities to bacterial sialidases. *Glycobiology.* **4:** 367–373.
32. Miyagi, T., T. Wada, A. Iwamatsu, K. Hata, Y. Yoshikawa, S. Tokuyama, and M. Sawada. 1999. Molecular cloning and characterization of a plasma membrane-associated sialidase specific for gangliosides. *J. Biol. Chem.* **274:** 5004–5011.
33. Wada, T., Y. Yoshikawa, S. Tokuyama, M. Kuwabara, H. Akita, and T. Miyagi. 1999. Cloning, expression, and chromosomal mapping of a human ganglioside sialidase. *Biochem. Biophys. Res. Commun.* **261:** 21–27.
34. Comelli, E. M., M. Amado, S. R. Lustig, and J. C. Paulson. 2003. Identification and expression of Neu4, a novel murine sialidase. *Gene.* **321:** 155–161.
35. Monti, E., M. T. Bassi, R. Bresciani, S. Civini, G. L. Croci, N. Papini, M. Riboni, G. Zanchetti, A. Ballabio, A. Preti, et al. 2004. Molecular cloning and characterization of NEU4, the fourth member of the human sialidase gene family. *Genomics.* **83:** 445–453.
36. Seyrantepe, V., A. Hinek, J. Peng, M. Fedjaev, S. Ernest, Y. Kadota, M. Canuel, K. Itoh, C. R. Morales, J. Lavoie, et al. 2008. Enzymatic activity of lysosomal carboxypeptidase (cathepsin) A is required for proper elastic fiber formation and inactivation of endothelin-1. *Circulation.* **117:** 1973–1981.
37. O'Brien, J. S., and T. G. Warner. 1980. Sialidosis: delineation of subtypes by neuraminidase assay. *Clin. Genet.* **17:** 35–38.
38. Sprague, E. A., M. Moser, E. H. Edwards, and C. J. Schwartz. 1988. Stimulation of receptor-mediated low density lipoprotein endocytosis in neuraminidase-treated cultured bovine aortic endothelial cells. *J. Cell. Physiol.* **137:** 251–262.
39. Afzali, B., R. S. Bakri, P. Bharma-Ariza, P. J. Lumb, N. Dalton, N. C. Turner, A. S. Wierzbicki, M. A. Crook, and D. J. Goldsmith. 2003. Raised plasma total sialic acid levels are markers of cardiovascular disease in renal dialysis patients. *J. Nephrol.* **16:** 540–545.
40. Gokmen, S. S., G. Kilicli, F. Ozcelik, M. Ture, and S. Gulen. 2002. Association between serum total and lipid-bound sialic acid concentration and the severity of coronary atherosclerosis. *J. Lab. Clin. Med.* **140:** 110–118.
41. Sathiyapriya, V., N. Selvaraj, H. Nandeesha, Z. Bobby, A. Aparna, and P. Pavithran. 2008. Association between protein bound sialic acid and high sensitivity C-reactive protein in prehypertension: a possible indication of underlying cardiovascular risk. *Clin. Exp. Hypertens.* **30:** 367–374.
42. Orekhov, A. N., V. V. Tertov, I. A. Sobenin, V. N. Smirnov, D. P. Via, J. Guevara, Jr., A. M. Gotto, Jr., and J. D. Morrisett. 1992. Sialic acid content of human low density lipoproteins affects their interaction with cell receptors and intracellular lipid accumulation. *J. Lipid Res.* **33:** 805–817.
43. Goldstein, J. L., M. S. Brown, R. G. Anderson, D. W. Russell, and W. J. Schneider. 1985. Receptor-mediated endocytosis: concepts emerging from the LDL receptor system. *Annu. Rev. Cell Biol.* **1:** 1–39.
44. Davis, C. G., A. Elhammer, D. W. Russell, W. J. Schneider, S. Kornfeld, M. S. Brown, and J. L. Goldstein. 1986. Deletion of clustered O-linked carbohydrates does not impair function of low density lipoprotein receptor in transfected fibroblasts. *J. Biol. Chem.* **261:** 2828–2838.
45. Kozarsky, K., D. Kingsley, and M. Krieger. 1988. Use of a mutant cell line to study the kinetics and function of O-linked glycosylation of low density lipoprotein receptors. *Proc. Natl. Acad. Sci. USA.* **85:** 4335–4339.
46. Kingsley, D. M., K. F. Kozarsky, L. Hobbie, and M. Krieger. 1986. Reversible defects in O-linked glycosylation and LDL receptor expression in a UDP-Gal/UDP-GalNAc 4-epimerase deficient mutant. *Cell.* **44:** 749–759.
47. Cummings, R. D., S. Kornfeld, W. J. Schneider, K. K. Hobgood, H. Tolleshaug, M. S. Brown, and J. L. Goldstein. 1983. Biosynthesis of N- and O-linked oligosaccharides of the low density lipoprotein receptor. *J. Biol. Chem.* **258:** 15261–15273.
48. Filipovic, I. 1989. Effect of inhibiting N-glycosylation on the stability and binding activity of the low density lipoprotein receptor. *J. Biol. Chem.* **264:** 8815–8820.
49. Millar, J. S. 2001. The sialylation of plasma lipoproteins. *Atherosclerosis.* **154:** 1–13.

50. Stoline, A. M., K. Saku, B. A. Hynd, and M. L. Kashyap. 1985. Effect of desialylation of very low-density lipoproteins on their catabolism by lipoprotein lipase. *Metabolism.* **34:** 30–35.
51. Lee, Y., M. Kockx, M. J. Raftery, W. Jessup, R. Griffith, and L. Kritharides. 2010. Glycosylation and sialylation of macrophage-derived human apolipoprotein E analyzed by SDS-PAGE and mass spectrometry: evidence for a novel site of glycosylation on Ser290. *Mol. Cell. Proteomics.* **9:** 1968–1981.
52. Kawakami, A., M. Aikawa, P. Libby, P. Alcaide, F. W. Luscinskas, and F. M. Sacks. 2006. Apolipoprotein CIII in apolipoprotein B lipoproteins enhances the adhesion of human monocytic cells to endothelial cells. *Circulation.* **113:** 691–700.
53. Potier, M., Y. D. Lu Shun, and J. E. Womack. 1979. Neuraminidase deficiency in the mouse. *FEBS Lett.* **108:** 345–348.
54. Womack, J. E., D. L. Yan, and M. Potier. 1981. Gene for neuraminidase activity on mouse chromosome 17 near h-2: pleiotropic effects on multiple hydrolases. *Science.* **212:** 63–65.
55. Clark, E. A., D. Engel, and N. T. Windsor. 1981. Immune responsiveness of SM/J mice: hyper NK cell activity mediated by NK 1+ Qa 5- cells. *J. Immunol.* **127:** 2391–2395.
56. Champigny, M. J., M. Mitchell, A. Fox-Robichaud, B. L. Trigatti, and S. A. Igdoura. 2009. A point mutation in the neu1 promoter recruits an ectopic repressor, Nkx3.2 and results in a mouse model of sialidase deficiency. *Mol. Genet. Metab.* **97:** 43–52.
57. Hara, T., M. Ichihara, M. Takagi, and A. Miyajima. 1995. Interleukin-3 (IL-3) poor-responsive inbred mouse strains carry the identical deletion of a branch point in the IL-3 receptor alpha subunit gene. *Blood.* **85:** 2331–2336.
58. Blankenhorn, E. P., G. Bryan, A. V. Kossenkov, L. D. Clark, X. M. Zhang, C. Chang, W. Horng, L. S. Pletscher, J. M. Cheverud, L. C. Showe, et al. 2009. Genetic loci that regulate healing and regeneration in LG/J and SM/J mice. *Mamm. Genome.* **20:** 720–733.
59. Pattison, S., M. Pankarican, C. A. Rupar, F. L. Graham, and S. A. Igdoura. 2004. Five novel mutations in the lysosomal sialidase gene (NEU1) in type II sialidosis patients and assessment of their impact on enzyme activity and intracellular targeting using adenovirus-mediated expression. *Hum. Mutat.* **23:** 32–39.
60. Champigny, M. J., M. Johnson, and S. A. Igdoura. 2003. Characterization of the mouse lysosomal sialidase promoter. *Gene.* **319:** 177–187.
61. Hitt, M. M., and F. L. Graham. 2000. Adenovirus vectors for human gene therapy. *Adv. Virus Res.* **55:** 479–505.
62. Kaboord, B., and M. Perr. 2008. Isolation of proteins and protein complexes by immunoprecipitation. *Methods Mol. Biol.* **424:** 349–364.
63. Knibbs, R. N., I. J. Goldstein, R. M. Ratcliffe, and N. Shibuya. 1991. Characterization of the carbohydrate binding specificity of the leukoagglutinating lectin from Maackia amurensis. Comparison with other sialic acid-specific lectins. *J. Biol. Chem.* **266:** 83–88.
64. Rigotti, A., B. L. Trigatti, M. Penman, H. Rayburn, J. Herz, and M. Krieger. 1997. A targeted mutation in the murine gene encoding the high density lipoprotein (HDL) receptor scavenger receptor class B type I reveals its key role in HDL metabolism. *Proc. Natl. Acad. Sci. USA.* **94:** 12610–12615.
65. Vaisman, B. L., H. G. Klein, M. Rouis, A. M. Berard, M. R. Kindt, G. D. Talley, S. M. Meyn, R. F. Hoyt, Jr., S. M. Marcovina, and J. J. Albers. 1995. Overexpression of human lecithin cholesterol acyltransferase leads to hyperalphalipoproteinemia in transgenic mice. *J. Biol. Chem.* **270:** 12269–12275.
66. de Silva, H. V., J. Mas-Oliva, J. M. Taylor, and R. W. Mahley. 1994. Identification of apolipoprotein B-100 low density lipoproteins, apolipoprotein B-48 remnants, and apolipoprotein E-rich high density lipoproteins in the mouse. *J. Lipid Res.* **35:** 1297–1310.
67. Gordon, D. A., J. R. Wetterau, and R. E. Gregg. 1995. Microsomal triglyceride transfer protein: a protein complex required for the assembly of lipoprotein particles. *Trends Cell Biol.* **5:** 317–321.
68. Wetterau, J. R., R. E. Gregg, T. W. Harrity, C. Arbeeny, M. Cap, F. Connolly, C. H. Chu, R. J. George, D. A. Gordon, H. Jamil, et al. 1998. An MTP inhibitor that normalizes atherogenic lipoprotein levels in WHHL rabbits. *Science.* **282:** 751–754.
69. Gordon, D. A., H. Jamil, R. E. Gregg, S. O. Olofsson, and J. Boren. 1996. Inhibition of the microsomal triglyceride transfer protein blocks the first step of apolipoprotein B lipoprotein assembly but not the addition of bulk core lipids in the second step. *J. Biol. Chem.* **271:** 33047–33053.
70. Rubin, D., A. Schneider-Muntau, M. Klapper, I. Nitz, U. Helwig, U. R. Folsch, J. Schrezenmeir, and F. Doring. 2008. Functional analysis of promoter variants in the microsomal triglyceride transfer protein (MTTP) gene. *Hum. Mutat.* **29:** 123–129.
71. Horton, J. D., I. Shimomura, M. S. Brown, R. E. Hammer, J. L. Goldstein, and H. Shimano. 1998. Activation of cholesterol synthesis in preference to fatty acid synthesis in liver and adipose tissue of transgenic mice overproducing sterol regulatory element-binding protein-2. *J. Clin. Invest.* **101:** 2331–2339.
72. Cases, S., S. Novak, Y. W. Zheng, H. M. Myers, S. R. Lear, E. Sande, C. B. Welch, A. J. Lusis, T. A. Spencer, B. R. Krause, et al. 1998. ACAT-2, a second mammalian acyl-CoA:cholesterol acyltransferase. Its cloning, expression, and characterization. *J. Biol. Chem.* **273:** 26755–26764.
73. Yen, C. L., S. J. Stone, S. Koliwad, C. Harris, and R. V. Farese, Jr. 2008. Thematic review series: glycerolipids. DGAT enzymes and triacylglycerol biosynthesis. *J. Lipid Res.* **49:** 2283–2301.
74. Abifadel, M., M. Varret, J. P. Rabes, D. Allard, K. Ouguerram, M. Devillers, C. Cruaud, S. Benjannet, L. Wickham, D. Erlich, et al. 2003. Mutations in PCSK9 cause autosomal dominant hypercholesterolemia. *Nat. Genet.* **34:** 154–156.
75. Cohen, J., A. Pertsemlidis, I. K. Kotowski, R. Graham, C. K. Garcia, and H. H. Hobbs. 2005. Low LDL cholesterol in individuals of African descent resulting from frequent nonsense mutations in PCSK9. *Nat. Genet.* **37:** 161–165.
76. Maxwell, K. N., R. E. Soccio, E. M. Duncan, E. Sehayek, and J. L. Breslow. 2003. Novel putative SREBP and LXR target genes identified by microarray analysis in liver of cholesterol-fed mice. *J. Lipid Res.* **44:** 2109–2119.
77. Park, S. W., Y. A. Moon, and J. D. Horton. 2004. Post-transcriptional regulation of low density lipoprotein receptor protein by proprotein convertase subtilisin/kexin type 9a in mouse liver. *J. Biol. Chem.* **279:** 50630–50638.
78. Horton, J. D., J. C. Cohen, and H. H. Hobbs. 2007. Molecular biology of PCSK9: its role in LDL metabolism. *Trends Biochem. Sci.* **32:** 71–77.
79. Horton, J. D., N. A. Shah, J. A. Warrington, N. N. Anderson, S. W. Park, M. S. Brown, and J. L. Goldstein. 2003. Combined analysis of oligonucleotide microarray data from transgenic and knockout mice identifies direct SREBP target genes. *Proc. Natl. Acad. Sci. USA.* **100:** 12027–12032.
80. Horton, J. D., J. L. Goldstein, and M. S. Brown. 2002. SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver. *J. Clin. Invest.* **109:** 1125–1131.
81. Brown, M. S., and J. L. Goldstein. 1997. The SREBP pathway: regulation of cholesterol metabolism by proteolysis of a membrane-bound transcription factor. *Cell.* **89:** 331–340.
82. Sun, L. P., L. Li, J. L. Goldstein, and M. S. Brown. 2005. Insig required for sterol-mediated inhibition of Scap/SREBP binding to COPII proteins in vitro. *J. Biol. Chem.* **280:** 26483–26490.
83. Horton, J. D., H. Shimano, R. L. Hamilton, M. S. Brown, and J. L. Goldstein. 1999. Disruption of LDL receptor gene in transgenic SREBP-1a mice unmasks hyperlipidemia resulting from production of lipid-rich VLDL. *J. Clin. Invest.* **103:** 1067–1076.
84. Sato, R., W. Miyamoto, J. Inoue, T. Terada, T. Imanaka, and M. Maeda. 1999. Sterol regulatory element-binding protein negatively regulates microsomal triglyceride transfer protein gene transcription. *J. Biol. Chem.* **274:** 24714–24720.
85. Chang, T. Y., B. L. Li, C. C. Chang, and Y. Urano. 2009. Acyl-coenzyme A:cholesterol acyltransferases. *Am. J. Physiol. Endocrinol. Metab.* **297:** E1–E9.
86. Millar, J. S., S. J. Stone, U. J. Tietge, B. Tow, J. T. Billheimer, J. S. Wong, R. L. Hamilton, R. V. Farese, Jr., and D. J. Rader. 2006. Short-term overexpression of DGAT1 or DGAT2 increases hepatic triglyceride but not VLDL triglyceride or apoB production. *J. Lipid Res.* **47:** 2297–2305.

CHAPTER 3: High fat diet induces atherosclerosis in transgenic mice over-expressing hepatic NEU1

Gabriel Gyulay[1], Elizabeth White[1], Abraham E. Yang[1], J.P. King[1], Bernardo L. Trigatti[2,4], and Suleiman A. Igdoura[1,3]

Departments of [1]Biology, [2]Biochemistry and Biomedical Sciences, [3]Pathology and Molecular Medicine, [4]Thrombosis and Atherosclerosis Research Institute, McMaster University, Hamilton, Ontario, Canada

Correspondence:
Suleiman Igdoura, Ph.D.
McMaster University, 1280 Main St. W. LSB 335
Hamilton, Ontario L8S 4K1 Canada
Tel. (905) 525-9140ex27729
Fax: (905) 522-6066
E-Mail: igdoura@mcmaster.ca

PREFACE

Gabriel Gyulay performed all experiments except Figures 3.1A, 3.3C, 3.3D, 3.3E, 3.3F, 3.6 and 3.7. Elizabeth White performed flow cytometry and analysis (Figure 3.6A-C, 3.7A-E). Abraham Yang performed atherosclerotic lesion staining and analysis (Figure 3.3C, D, E, F), and assisted with lipid measurements and FPLC profiling (Figure 3.4). J.P. King generated the plasmid pBIG-N2.1hSial (Figure 3.1A).

3.1 ABSTRACT

Atherosclerosis is a complex and prevalent disease propagated by immune cells and initiated by the interaction of lipoproteins with the vasculature. Sialidase is an enzyme that cleaves sialic acid from glycoproteins and has important functional roles in several metabolic and immunological pathways. We have established that hypomorphic expression of Neu1 sialidase causes significant changes in murine lipoprotein metabolism *in vivo* and results in an atheroprotective phenotype. Neu1 hypomorphic mice have increased hepatic lipid and cholesterol storage, lower VLDL production, decreased serum LDL and enhanced LDL uptake. Next, we wanted to investigate if human sialidase can affect molecular pathways involved in atherogenesis as well. In order to test whether hepatic over-expression of NEU1 impacts lipoprotein metabolism, sialylation of immune cells, and subsequently atherosclerosis, we have generated a transgenic mouse line expressing human sialidase (hNEU1) and β-galactosidase (LacZ) under the control of a bidirectional promoter responsive to the tetracycline transactivator (tTA) regulatory protein. When crossed with animals expressing a hepatocyte-specific tTA under the control of the mouse urinary protein promoter (mTA), these $mTA^{+}hNEU1^{+}$ mice have significantly higher hepatic levels of human sialidase mRNA and sialidase activity compared to controls, as well as β-galactosidase expression in their liver. In order to assess the impact of NEU1 over-expression on atherosclerosis, the $mTA^{+}hNEU1^{+}$ mice (and controls) were fed a high fat western diet for 6 months and our results indicate that sialidase over-expression caused atherosclerotic lesion

formation in the aortic root of mice compared to controls, but showed no change in hepatic and serum triglycerides. However, these transgenic mice show a shift in the size of HDL cholesterol particles, a reduction in hepatic cholesterol ester levels, and a rise in serum cholesterol ester levels. This cholesterol ester re-distribution to the serum, along with reduced hepatic protein expression of LDLR and SRB-1, may contribute to an increased susceptibility to atherosclerosis. Additionally, we observed that overall cell-surface sialylation of blood and spleen leukocytes is also decreased in $mTA^{+}hNEU1^{+}$ mice, potentially implicating effects of secreted hepatic NEU1 on immune cells in these animals. Decreased sialylation of adhesion molecules on leukocytes may impact homing and adhesion mechanisms leading to increased atherogenesis. Our novel transgenic mouse allows for direct examination of hepatic NEU1 *in vivo* and suggests a role for human sialidase as a direct player in progression of atherosclerosis.

3.2 INTRODUCTION

Atherosclerosis is a chronic state of inflammation of the arteries initiated by high levels of LDL cholesterol (Libby et al., 2011). There are several genetic and environmental factors that contribute to elevated levels of LDL and risk of atherosclerosis, such as hypertension, obesity, sedentary lifestyle, genetic dyslipidemia, smoking, diabetes and aging (Barton, 2013). LDL is susceptible to oxidation as it accumulates in the subendothelial space of the artery and this modification causes endothelial cells to initiate the recruitment and adhesion of monocytes (Gui et al., 2012). These monocytes then differentiate into macrophages where they amass cholesterol and lipids and become pro-atherogenic foam cells (Moore et al., 2013). Foam cells secrete cytokines and recruit other immune cells resulting in a pro-inflammatory milieu which triggers the migration of smooth muscle cells of the artery into the developing lesion (Businaro et al., 2012). Lesions then gain complexity due to the propagation of inflammatory signals and continued aggregation of modified lipoproteins and immune cells (Businaro et al., 2012). Eventually this results in the formation of plaques which can cause hypertension, rupture and thrombosis, triggering severe circulatory problems; including coronary heart disease, myocardial infarctions and strokes (Barton, 2013;Hegele, 2009;Libby et al., 2013).

Sialic acid, also known as neuraminic acid, is a terminal monosaccharide found on an abundance of glycoproteins and glycolipids and its negative charge is crucial for conformational stabilization as well as cell recognition and adhesion (Cross and Wright,

1991;Pshezhetsky and Hinek, 2011;Rottier et al., 1998). Sialidases, also referred to as neuraminidases, are ubiquitously expressed enzymes that cleave sialic acid and NEU1 sialidase is located in the lysosomes and plasma membranes of cells (Liang et al., 2006;Rottier et al., 1998). NEU1 sialidase activity has been implicated in biological mechanisms, including immune cell differentiation, activation and adhesion (Amith et al., 2009;Chen et al., 1997;Gadhoum and Sackstein, 2008;Gee et al., 2002;Lambre et al., 1990;Liang et al., 2006;Ross, 1999;Stamatos et al., 2005;Wang et al., 2004). Furthermore, low density lipoprotein receptor (LDLR), apolipoprotein-B, C and E are all heavily sialylated and several studies postulate that sialic acids can have functional significance on these proteins (Cummings et al., 1983;Davis et al., 1986;Filipovic, 1989;Goldstein et al., 1985;Kawakami et al., 2008;Kingsley et al., 1986;Kozarsky et al., 1988;Millar, 2001;Stoline et al., 1985). The SM/J mouse strain has a partial deficiency of Neu1 sialidase and exhibits aberrant sialylation of glycoproteins and an impaired immune response (Clark et al., 1981;Potier et al., 1979;Rottier et al., 1998). Upon crossing these mice onto a C57Bl/6 background, we have shown that hypomorphic sialidase ($Neu1^{hypo}$) expression modulates VLDL production, hepatic lipid metabolism and LDLR function in mice (Yang et al., 2012). The Neu1 knockout mouse exhibits a severe phenotype similar to the human disease sialidosis, limiting its use for examination of other diseases and mechanisms (de Geest et al., 2002).

However, despite its vital role in lipoprotein metabolism and inflammation, the functional effect of NEU1 on atherosclerotic disease progression *in vivo* has not been

significantly addressed. Given that the liver is the hub of cholesterol metabolism and lipoprotein synthesis, we aimed to test the impact of hepatic NEU1 over-expression on atherosclerosis *in vivo*. Thus, we generated a model of hepatic over-expression of human sialidase in FVB mice to examine the consequences of altered sialylation on lipid metabolism and atherogenesis. Importantly, human NEU1 and mouse Neu1 share high structural and functional homology at the DNA and protein level, including their glycosylation and dependence on protective protein Cathepsin A for trafficking and activity (Igdoura et al., 1998;Wang et al., 2009). Additionally, Neu1 deficient mouse cells were successfully transduced with a retrovirus containing human NEU1 cDNA to yield active and functional protein (Wang et al., 2009). Based on these experiments and the homology of this enzyme, we are able to study the effects of this human gene *in vivo* in the mouse. To accomplish this, we bred mice harbouring the human sialidase (hNEU1) gene controlled by a TRE promoter with mice expressing the tTA gene under the control of the liver-specific major urinary protein (MUPtTA), abbreviated as mTA for this paper (Manickan et al., 2001). Thus, $mTA^{+}hNEU1^{+}$ animals are a model of hepatic over-expression of human sialidase and allow for direct examination of the effects of sialidase over-expression on lipid metabolism and atherosclerosis. After characterizing the function of our transgenic construct, we found that mice over-expressing NEU1 have significant aortic sinus lipid deposition and changes in hepatic and serum cholesterol ester content compared to littermate controls after 6 months of high fat diet feeding. Additionally, $mTA^{+}hNEU1^{+}$ mice also exhibit lower protein levels of hepatic LDLR and

SRB-1, while having reduced blood and splenic leukocyte sialylation. These phenomena are indicative of reduced hepatic lipoprotein uptake, and increased leukocyte recruitment potential, both of which are pro-atherogenic. In this study, we have established a novel transgenic line which revealed a role for hepatic NEU1 sialidase in atherosclerosis.

3.3 MATERIALS AND METHODS

3.3.1 Animals

FVB, mTA, hNEU1, $mTA^{+}hNEU1^{+}$, $mTA^{+}hNEU1^{-}$, $mTA^{-}hNEU1^{+}$, and $mTA^{-}hNEU1^{-}$ mice were are all housed in the Central Animal Facility at McMaster University, in accordance with all animal ethics requirements, and had unlimited access to food and water. For atherosclerosis experiments, animals were fed a high fat diet (HFD) for 6 months (Modified Stanford University diet from Dyets Inc., 21% Butterfat and 0.15% cholesterol with 1% safflower oil), which started at 3 months of age. Mice were anaesthetized by injection with 12.5 µl/gram body weight of 5% tribromoethanol in sterile PBS prior to perfusion, euthanasia and tissue harvesting. Age matched littermate males were utilized for all experiments.

3.3.2 Targeting Construct

The plasmid pBIG-N2.1 was modified by the Mobix Facility at McMaster University to contain an artificial multiple cloning site for incorporation of additional restriction sites into the vector. Human NEU1 was cloned from the plasmid pzeohSial using XhoI and HindIII and ligated to pBIG-N2.1 (Mansoor Husain, University of Toronto) cut with HindII and SalI. Vectors were sent to the Mobix Facility to confirm the sequences. Cloning of the plasmids was performed by transforming E.Coli with the plasmid followed by growth and selection in LB media. Plasmid DNA was extracted using the Norgen MiniPrep Kit.

3.3.3 Generation of Transgenic Animals

Female FVB mice were injected with pregnant mare serum gonadotrophin intraperitoneally prior to being paired with males. Post insemination, embryos were extracted and placed in HEPES-buffered KSOM Embryo Culture Media (Millipore). Pronuclei of the embryos were injected with the linearized plasmid using a microinjector apparatus. Embryos were then re-implanted into the infundibulum of the uterine tube of pseudopregnant FVB females. Females were made pseudopregnant by mating with a vasectomised male. Offspring were tested for incorporation of transgene using PCR genotyping and selected for further breeding. FVB mice harbouring the knock-in of the NEU1 gene (under the control of a TRE promoter) were crossed with MUPtTA (abbreviated mTA) mice, also on an FVB background, yielding $mTA^{+}hNEU1^{+}$ mice and controls (see details in figure 3.1 for generation). We wish to acknowledge Jake Liang from the National Institutes of Health, Bethesda, Maryland for the MUPtTA mice.

3.3.4 Genotyping and PCR

Tail clippings were lysed in tail lysis buffer (2 mM Tris pH 8.0, 5 mM NaCL, 0.5 mM EDTA, 100 µg/mL proteinase K) at 55°C overnight. Phenol extraction and ethanol precipitation of DNA was then performed to isolate genomic DNA. This was followed either by qPCR of genomic DNA (see below) or by PCR and agarose gel electrophoresis and ethidium bromide visualization to select and breed mice. To genotype the mice, the thermocycler program consisted of an initial step of 4 min at 94°C, followed by 40 cycles

of (30 sec at 94°C, 30 sec at 60°C, 45 sec at 72°C), and 1 cycle of 7 min at 72°C). Primers were designed by our lab and synthesized by MOBIX (5' to 3'):

hNEU1: Forward- CTGTAGGGTTTGGGTGTTTG, Reverse- ACATCGCTCACTACTGCCCCAA

mTA: Forward- CGCTGTGGGGCATTTTACTTTAG, Reverse- CATGTCCAGATCGAAATCGTC

The following cycles and primers were used for characterization of plasmid integration:

Region 1, spanning the end of the hNEU1 cDNA and part of the plasmid backbone:

Thermocycler program: 5 min at 95°C; 40 cycles of (20 sec at 94°C, 30 sec at 57.2°C, 20 sec at 72°C); 7 min at 68°C

Primers: Forward- CAGTTCCCTGAGTTCACATT, Reverse- AGAGAGAGTCCAGCTATGGC

Region 2, spanning part of TRE and part of hNEU1 cDNA:

Thermocycler program: 5 min at 95°C; 40 cycles of (20 sec at 94°C, 30 sec at 51.6°C, 20 sec at 72°C); 7 min at 68°C

Primers: Forward- GACATCCCCATCATTGACAA, Reverse- GTAAACTCGACCTATATAAG

Region 3, spanning part of TRE and part of LacZ:

Thermocycler program: 5 min at 95°C; 40 cycles of (20 sec at 94°C, 55 sec at 58.8°C, 20 sec at 72°C); 7 min at 68°C

Primers: Forward- GTCGAGTAGGGGTGTACGGT, Reverse- CACATCTGAACTTCAGCCTC

3.3.5 RNA Isolation and Quantitative Real-Time PCR

Liver samples were homogenized and RNA was isolated using the Trizol (Invitrogen) method after which it was purified using Norgen Total RNA II Isolation Kit and genomic DNA was removed using an extra DNAse step. 1-5 µg of total RNA were

then reverse transcribed using oligoDT primers following the protocol of Invitrogen's SSIII RT reverse transcriptase (18080044) and 5 µl of resulting cDNA was used for qRT-PCR. Alternatively, genomic DNA for qPCR was isolated as in 3.2.2 and diluted to 0.1 ng/µl of which 10 µl was used in the qPCR reaction. These PCRs were performed using Applied Biosystems Power Sybr Green (4367659). Plates were loaded with a 20 µl reaction per well and included appropriate blanks and standard. (Thermocycler program for cDNA: 10 min at 95°C, 40 cycles of 15 sec at 95°C, 60 sec at 60°C. Thermocycler program for genomic DNA: 10 min at 95°C, 40 cycles of 15 sec at 95°C, 30 sec at 60°C, 35 sec at 72°C). Primers were designed by our lab and synthesized by MOBIX: (5' to 3')

hNEU1: Forward-AACCTTGGGGCAGTAGTGAG, Reverse-GGCAAACACTTCAGTGCCAATA

tTA: Forward- GCCATTGAGATGTTAGATAGGCACC,

Reverse- TCGCGATGACTTAGTAAAGCACATC

Actin: Forward- GGCTGTATTCCCCTCCATCG, Reverse- CCAGTTGGTAACAATGCCATGT

3.3.6 Protein Assay

Lowry (LOWRY et al., 1951) or Bradford (Bradford, 1976) protein assays (Bio-Rad) were performed on all lysates to determine protein concentrations so that normalized, appropriate comparisons can be made between them. Bovine Serum Albumin (BSA) was used as a standard.

3.3.7 Western Blotting

Tissue was homogenized in RIPA buffer containing protease inhibitors (Complete Mini, Roche 11873580001, 1 tablet per 10 mL) with 1 mM EDTA, then boiled in Laemmli Sample Buffer prior to loading. Proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes then blocked using TBST with 5% milk. Antibodies were used at 1:1000 in TBST with 5% milk. HRP-bound appropriate secondary antibodies were then detected by ECL Reagent (GE) after which membranes were exposed onto film and developed (Freeman, 2013). Antibodies: rabbit anti - *E. coli* β-Galactosidase (Genway 18-783-78938), goat anti-LDLR (R&D AF2255), mouse anti-MTP (BD Biosciences 612022), rabbit anti-SRB-1 (Novus NB400-101), mouse anti-ACAT2 (Santa Cruz sc-30279), mouse anti β-actin (Cell Signalling 3700). Band Intensity was measured by densitometry analysis using ImageJ software and normalized to loading controls.

3.3.8 Sialidase activity assay

After dissection of liver, approximately 0.15 g of liver tissue was minced on ice followed by homogenization in water. Tissue homogenate was then incubated for 1 h at 37°C with 0.4 mM 4-Methylumbelliferyl-*N*-acetyl-α-D-neuraminic acid (4-Mu-NANA, Toronto Research Chemicals) in acetate buffer (pH 4.2) with 10% BSA. Activity was measured as the amount of fluorescence generated from the liberation of umbelliferone (4-Mu) from the NANA substrate. The reaction was stopped by the addition of 2 ml of basic 0.1 M MAP (2-amino-2-methyl-1-propanol) buffer. Fluorescence was then

measured using a plate fluorometer (PerkinElmer) and normalized to protein concentration.

3.3.9 Immunohistochemistry

While under anaesthesia, animals were perfused with PBS and then 3.7% formaldehyde in PBS through the left ventricle of the heart and livers were excised and fixed in 3.7% formaldehyde in PBS followed by embedding in paraffin wax and sectioning by the histology laboratory at McMaster University Medical Center. De-paraffinization and dehydration steps were performed using sequential xylene and ethanol baths. Staining was performed using our human sialidase primary antibody made against the carboxyl terminus of human NEU1 synthetic peptide (anti-75). Primary antibody signal was amplified using HRP secondary antibody and DAB (3,3'-Diaminobenzidine). Slides were counterstained with methylene blue and coverslips were mounted on the slides using Permount (Fisher). Images were captured using a Zeiss Axiovert 200 microscope using an MRc AxioCam camera in combination with a Kodak Wratten 47 blue gelatin filter (Sigma). Images were processed and scale bars were added using Zeiss Axiovision Release 4.8.2 software. Hematoxylin and Eosin staining was performed by the Pathology lab in the McMaster University Medical Center, and pictures were taken as above but without the filter.

3.3.10 Oil Red O Preparation

For staining of neutral lipids in cells, Oil Red O powder from Sigma-Aldrich (O0625) was prepared by dissolving 2.5 grams in 500mL of isopropanol. Prior to experimental use, this mixture was diluted 3:2 with double distilled H_20 and filtered to remove any particulate matter.

3.3.11 Aortic Sinus Lesion Analysis

While under anaesthesia, mice were perfused with PBS and then hearts were perfused with 3.7% formaldehyde in PBS. Hearts were dissected and placed in 30% sucrose in PBS overnight and then embedded in Shandon cryomatrix solution (VWR). The processed hearts were cut into serial 10 μm cross sections using a Shandon Cryostat (VWR) which were then collected onto coverslides. Sections were stained with Oil Red O for neutral lipids and stained with hematoxylin for cell nuclei. Pictures were taken using a Zeiss Axiovert 200 microscope. Atherosclerosis was quantified as the total cross sectional area of atherosclerotic lesion in each given section using Zeiss Axiovision Release 4.8.2 microscope software.

3.3.12 Cholesterol and Triglyceride Assay

For hepatic lipid analyses, 150 mg of liver was homogenized in 1 ml 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 300 μl of liver homogenates, and the tubes were mixed for 1 min. After that, 0.6 ml of distilled water was added to the tubes,

and the tubes were mixed for 1 min. The extraction mixture was left at 4°C for 2 h, after which the tubes were centrifuged at low speed to facilitate phase separation. The lower phase (chloroform phase) was dried completely by sitting in a water bath at 37°C. The dry chloroform phase was resuspended in 60 µl of isopropanol, yielding a measurable sample. Serum lipids were analyzed directly from samples obtained after blood was centrifuged using serum collection tubes (Sarstedt 41.1378.005). Total Cholesterol was measured using a colorimetric enzymatic assay (Infinity™ Cholesterol Liquid Stable Reagent, Thermo Scientific TR13421) with appropriate standards and blanks, with reading of the final reaction product at 500 nm using a spectrophotometer plate reader. To obtain cholesterol ester measurements, free cholesterol levels were subtracted from total, after measurement with Free Cholesterol E assay kit (Wako Diagnostics 435-35801) and reading at 600 nm. Triglycerides were measured using L-type TG H enzymatic kit (Wako Diagnostics, discontinued) with readings at 600 and 700 nm.

3.3.13 Fast Protein Liquid Chromatography (FPLC)

Gel Filtration Fast Protein Liquid Chromatography using a Superose 6 gel filtration column was used to analyze serum lipoprotein compositions of mice. 200 µl of serum was loaded and fractionated into respective VLDL, IDL/LDL and HDL fractions. These fractions were then analyzed for total cholesterol using an enzymatic assay (Infinity™ Cholesterol Liquid Stable Reagent, Thermo Scientific), with reading of the final reaction product at 500 nm using a spectrophotometer.

3.3.14 Flow Cytometry

Blood was collected via cheek bleeding of mice for peripheral leukocyte analysis. For splenic studies, spleens were excised after perfusion with PBS and pressed through a 40 µm nylon cell strainer into 1 ml PBS supplemented with 2% FBS. Erythrocytes in samples were lysed using BD Pharm Lyse (BD Biosciences) and leukocytes were pelleted (500x G) and re-suspended in PBS with 2% FBS. Samples were then stained with SNA (Sambucus nigra agglutinin)-FITC, CD115-PE, CD3ε-APC-Cy7, Ter199 (erythroid cell marker)-Alexa Fluor 700 and Ly6C (Lymphocyte Antigen 6C)-V450 in PBS supplemented with 0.2% FBS. All antibodies were obtained from BD Biosciences, and SNA lectin was obtained from Vector Labs. Samples were washed with PBS 2% FBS, fixed with BD Cytofix^{TM} Fixation Buffer (BD Biosciences) and washed again before they were run on a LSR II flow cytometer (Beckman Coulter). For intracellular analysis of Neu1, cells were permeabilized with 0.5% Triton-X in PBS and stained with rabbit anti-Neu1 (Rockland 100-401-396) and Alexa Fluor 488 anti-rabbit secondary antibody. Data was analyzed using FlowJo v9.2. SNA binds α-2,6 linkages of sialic acid (Knibbs et al., 1991). Total leukocytes were identified by Ter119^{neg} and forward scatter (FSC)/side scatter(SSC) characteristics. CD115^{+} and FSC^{hi}SSC^{int} were used to identify monocytes. CD3ε^{+} was used to identify T-cells. SSC^{hi}Ly6C^{int} were used to identify neutrophils.

3.3.15 Statistical Analysis

For all experiments, data were as analyzed by Student's *t* test or one way ANOVA with Dunnett's post hoc test. Analysis was performed using GraphPad Prism 5 software and error bars represent SEM, unless otherwise stated.

3.4 RESULTS

3.4.1 Generation of transgenic FVB mice

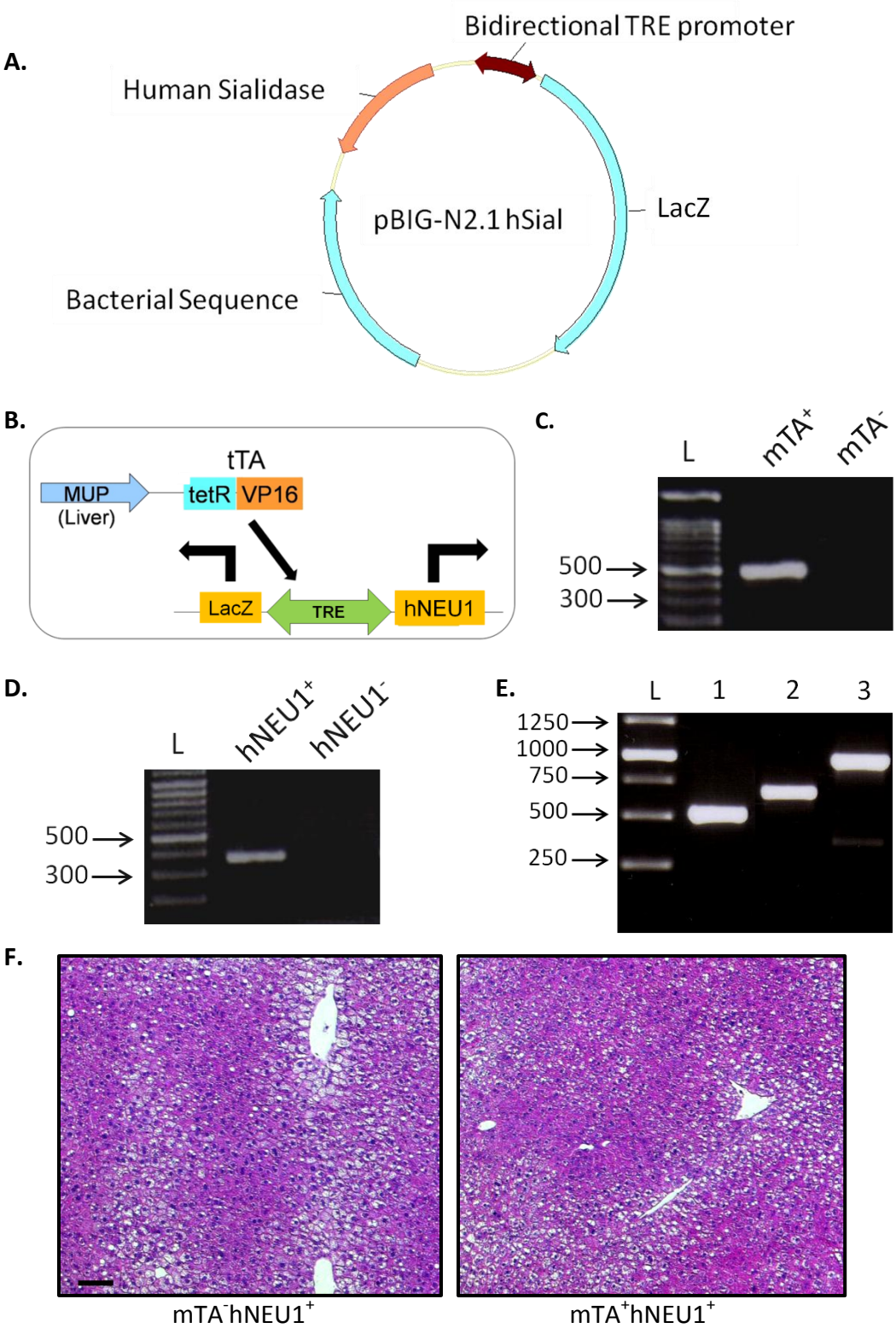
Plasmid pBIG-N2.1 was constructed containing human NEU1 (hNEU1) and *E. coli* Beta-Galactosidase (LacZ) cDNA under the control of a bi-directional tetracycline responsive element (TRE) promoter (Figure 3.1A). The linearized plasmid was injected into the pronuclei of FVB mouse embryos that were extracted. The embryos were re-implanted into pseudopregnant female mice and genotyped for integration. These transgenic mice contained human NEU1 and *E. coli* Beta-Galactosidase (LacZ) cDNA and were labelled as $hNEU1^{+}$, short for human Neuraminidase. This strain was then crossed with FVB mice that express the tetracyline transactivator (tTA) under the control of the liver specific major urinary protein (MUP) promoter (Manickan et al., 2001). This yielded animals that constitutively express human sialidase in their livers ($mTA^{+}hNEU1^{+}$) (Figure 3.1B). The MUP-induced expression of tTA (abbreviated mTA) in the liver drives expression of human NEU1 as well as the reporter gene LacZ via its tetracycline response element. These mice exhibit average litter sizes of 6 when the mating pairs are $mTA^{+}hNEU1^{+}$ with $mTA^{+}hNEU1^{+}$, and average litter sizes of 6.5 when the mating pairs are $mTA^{-}hNEU1^{+}$ with $mTA^{+}hNEU1^{-}$. PCR genotyping of tail genomic DNA shows the presence of tTA in mTA^{+} mice (Figure 3.1C, 469 bp), and the presence of hNEU1 in $NEU1^{+}$ mice (Figure 3.1D, 363 bp), along with no band in negative controls. Animals were genotyped

for both of these amplicons for selecting mice for mating and experiments. Additionally, we also wanted to characterize integration of our plasmid into the genomic DNA of $hNEU1^{+}$ animals prior to assessing protein expression and activity. Figure 3.1E shows amplification of the region spanning the end of the human sialidase cDNA and extending into the plasmid backbone (Lane 1, 518 bp), the region spanning the TRE and the start of the LacZ cDNA (Lane 2, 676 bp) and the region spanning the TRE and the start of the human sialidase cDNA (Lane 3, 974 bp) in DNA from a $mTA^{+}hNEU1^{+}$ mouse. The livers of $mTA^{+}hNEU1^{+}$ animals appear to have no gross morphological differences compared to controls as analyzed by Hematoxylin and Eosin staining (Figure 3.1F).

Figure 3.1 Plasmid construct and generation of transgenic mice

(A) Recombinant cDNA of human Sialidase (hNEU1) and Beta-Galactosidase (LacZ) were constructed in plasmid pBIG-N2.1. hNEU1 and LacZ cDNA are controlled by a bi-directional promoter responsive to the tetracylcine transactivator (tTA), as it contains tet-responsive elements (TRE). (B) FVB mice containing the hNEU1 insert were crossed with a mouse strain expressing the tetracyline transactivator (under the control of the liver specific murine urinary promoter (mTA). This yields a tet-off system in which $mTA^{+}hNEU1^{+}$ FVB mice express human sialidase in their livers until shut off if tetracycline is introduced. (C) Genotyping PCR and agarose gel electrophoresis of mTA in mTA^{+} and mTA^{-} mouse genomic DNA samples. (D) Genotyping PCR and agarose gel electrophoresis of hNEU1 in $hNEU1^{+}$ and $hNEU1^{-}$ mouse genomic DNA samples. (E) PCR and agarose gel electrophoresis of different parts of the pBIG-N2.1 hSial plasmid in tail genomic DNA of $mTA^{+}hNEU1^{+}$ mice. Lane 1: region spanning human sialidase cDNA and extending into the plasmid backbone, 518 bp. Lane 2: region spanning the TRE and the start of the LacZ cDNA, 676 bp. Lane 3: region spanning the TRE and the start of the human sialidase cDNA, 974 bp. L= ladder, with size indicated in base bairs. (F) Hematoxylin and Eosin staining of $mTA^{-}hNEU1^{+}$ and $mTA^{+}hNEU1^{+}$ liver sections show no gross morphological differences. Scale bar = 100 μm.

Figure 3.1


A.
Bidirectional TRE promoter
Human Sialidase
pBIG-N2.1 hSial
LacZ
Bacterial Sequence
B.
tTA
MUP
(Liver)
tetR
VP16
LacZ
TRE
hNEU1
C.
L
mTA+
mTA-
500
300
D.
L
hNEU1+
hNEU1-
500
300
E.
L
1
2
3
1250
1000
750
500
250
F.
mTA-hNEU1+
mTA+hNEU1+

3.4.2 mTA$^+$hNEU1$^+$ mice express hepatic human NEU1 mRNA, LacZ protein, and have increased Sialidase Activity

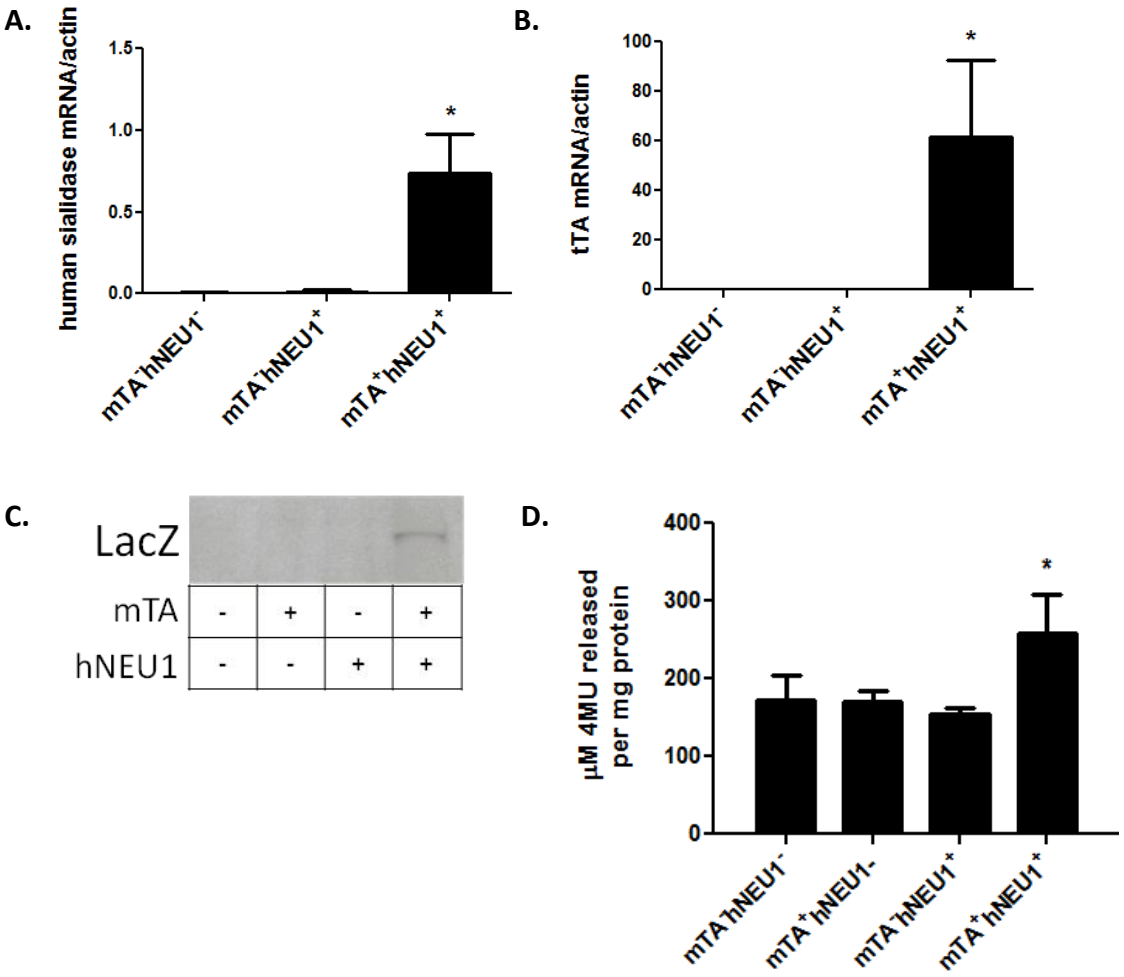
After generation and genotyping of mTA$^+$hNEU1$^+$ mice and controls, we assessed the expression and function of the transgene. Quantitative Real-Time PCR analysis revealed that mTA$^+$hNEU1$^+$ mice have significantly higher expression levels of human sialidase mRNA in their livers compared to mTA$^-$hNEU1$^-$ (wild type) and mTA$^-$hNEU1$^+$ controls, which almost completely lack human NEU1 expression (Figure 3.2A). Furthermore, tTA cDNA was highly expressed in mTA$^+$hNEU1$^+$ animals, but entirely lacking in controls, thus linking tTA expression with the expression of the human sialidase (Figure 3.2B). It is important to note that mTA$^+$hNEU1$^+$ exhibited incomplete penetrance (86%) with regards to human NEU1 mRNA expression in the liver. However, all of the mTA$^+$hNEU1$^+$ mice utilized for the high fat diet atherosclerosis studies, lipid analysis and western blots exhibited significant hepatic human NEU1 mRNA expression. We also utilized western blotting to test for protein expression of the reporter β-Galactosidase. mTA$^+$hNEU1$^+$ mice expressed hepatic β-galactosidase protein, while control animals did not (Figure 3.2C). Furthermore, we performed a sialidase activity assay using 4MU-NANA on the livers of mTA$^+$hNEU1$^+$ mice and controls. Mice expressing human NEU1 had significantly higher sialidase activity than controls (Figure 3.2D), indicating that the expression of the transgene resulted in active protein. Immunohistochemistry was also utilized to analyze human sialidase protein expression using an antibody generated by our laboratory against the C-terminal portion of the

human NEU1 protein. $mTA^{+}hNEU1^{+}$ mice exhibit specific punctuate staining, while control $mTA^{-}hNEU1^{+}$ animals have almost no staining (Figure 3.2E). These data indicated human NEU1 hepatic protein expression and further validated this transgenic system.

Figure 3.2 Characterization of $mTA^{+}hNEU1^{+}$ transgenic Mice

(A) $mTA^{+}hNEU1^{+}$ mice have significantly higher levels of human sialidase mRNA compared to controls as measured by qRT-PCR and this expression is driven by the expression of the tetracycline transactivator (tTA) mRNA (B) which the control mice lack (n=5). (C) Western blotting illustrates that $mTA^{+}hNEU1^{+}$ mice express LacZ in liver lysates while the control mice do not. (D) $mTA^{+}hNEU1^{+}$ animals display higher hepatic sialidase activity levels compared to controls (n=2-4). Livers were harvested, freshly homogenized and activity was measured using the fluorescent substrate 4MU-NANA. (E) $mTA^{+}hNEU1^{+}$ hepatocytes exhibit specific staining for human sialidase in their livers as measured by immunohistochemistry (scale bar=20µm). (* denotes $p<0.05$)

Figure 3.2


A.
human sialidase mRNA/actin
1.5
1.0
0.5
0.0
*
mTA-hNEU1-
mTA-hNEU1+
mTA+hNEU1+
B.
tTA mRNA/actin
100
80
60
40
20
0
*
mTA-hNEU1-
mTA-hNEU1+
mTA+hNEU1+
C.
LacZ
mTA - + - +
hNEU1 - - + +
D.
µM 4MU released per mg protein
400
300
200
100
0
*
mTA-hNEU1-
mTA+hNEU1-
mTA-hNEU1+
mTA+hNEU1+
E.
20 µm
mTA-hNEU1+
mTA+hNEU1+

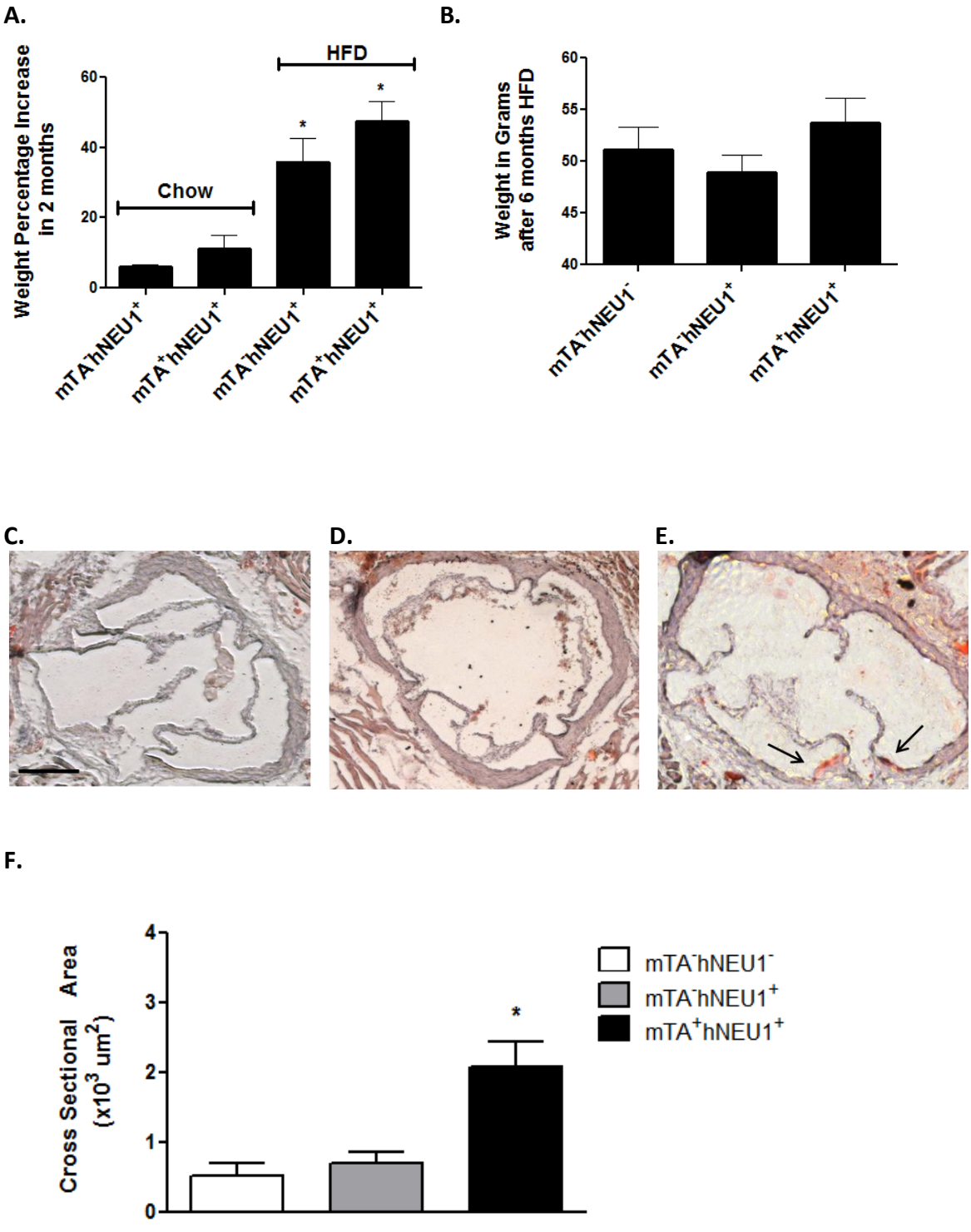
3.4.3 Impact of high-fat diet on $mTA^{+}hNEU1^{+}$ mice: assessment of aortic lesions

In order to investigate whether hepatic over-expression of sialidase impacts the initiation of atherosclerosis, we fed $mTA^{+}hNEU1^{+}$ mice and age matched controls a high fat diet for 6 months and analyzed aortic lesion formation. The high fat diet significantly increased the weight gain of all animals (Figure 3.3A) even after 2 months, but there was no significant change in weight between $mTA^{+}hNEU1^{+}$ mice and controls at the time of harvest (Figure 3.3B). Atherosclerotic lesion formation was analyzed by Oil Red O staining and wild type $mTA^{-}hNEU1^{-}$ mice (Figure 3.3C) and $mTA^{-}hNEU1^{+}$ mice (Figure 3.3D) almost completely lacked lipid deposition on the aortic sinus. However, $mTA^{+}hNEU1^{+}$ aortic sinuses displayed positive Oil Red O staining and signs of developing lesions (Figure 3.3D). Therefore, human hepatic NEU1 expression significantly induced fatty streak formation in the aortic root as quantified by the total lesion area in the aortic sinus (Figure 3.3E). It is noteworthy that $mTA^{+}hNEU1^{+}$ mice exhibit signs of lesions, despite the strong resistance to atherosclerosis of the FVB background of these mice (Stein et al., 2006).

Figure 3.3 High fat diet induced weight gain and atherosclerosis lesion analysis of transgenic mice

Hepatic human sialidase over-expression in FVB mice did not significantly change body weight after (A) 2-month and (B) 6-month high fat diet feeding compared to control mice. However, high fat diet did increase weight compared to chow-fed mice, as expected (n=4-6). (C, D) Control and (E) $mTA^{+}hNEU1^{+}$ mouse aortic sinus atherosclerosis was analyzed by Oil Red O staining and $mTA^{+}hNEU1^{+}$ mice displayed aortic sinus fatty streak formation. (F) Hepatic human sialidase over-expression in FVB mice caused lesion formation as quantified by total Oil Red O positive cross-sectional area in the aortic sinus (N= 3,4,6 $mTA^{-}hNEU1^{-}$, $mTA^{-}hNEU1^{+}$, $mTA^{+}hNEU1^{+}$ respectively). Mice were fed a high fat diet for 6 months prior to analysis. Arrows indicate lipid deposition on the endothelium (scale bar=200 μm). (* denotes $p<0.05$)

Figure 3.3


A.
Weight Percentage Increase in 2 months
60
40
20
0
Chow
HFD
*
*
mTA-hNEU1+
mTA+hNEU1+
mTA-hNEU1+
mTA+hNEU1+
B.
Weight in Grams after 6 months HFD
60
55
50
45
40
mTA-hNEU1-
mTA-hNEU1+
mTA+hNEU1+
C.
D.
E.
F.
Cross Sectional Area (x10^3 um^2)
4
3
2
1
0
*
mTA-hNEU1-
mTA-hNEU1+
mTA+hNEU1+

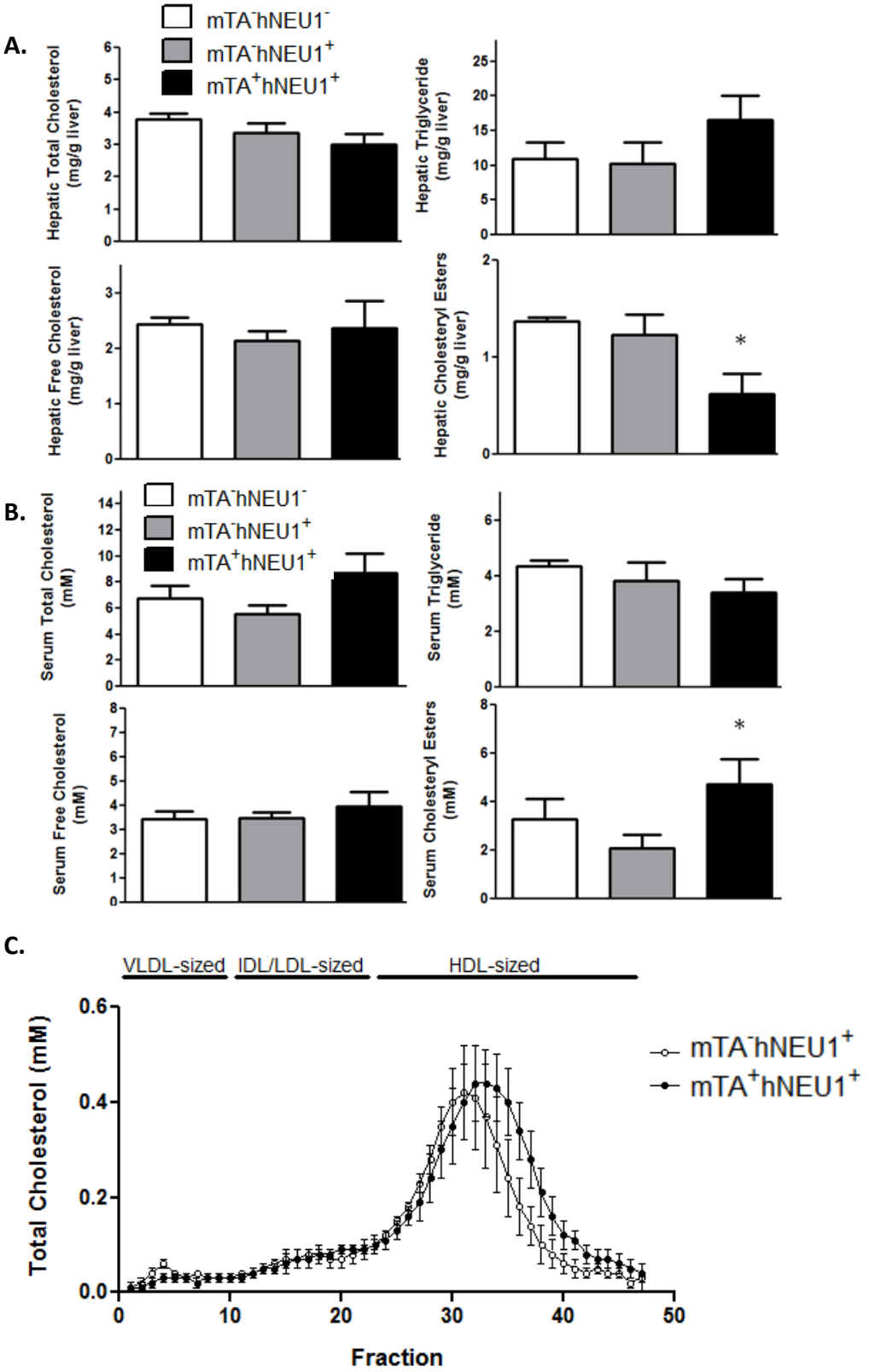
3.4.4 Cholesterol ester re-distribution in high fat diet fed mTA$^+$hNEU1$^+$ mice

We analyzed serum and hepatic lipid levels and FPLC cholesterol profiles of the HFD-fed mice in order to gain insight into the pro-atherogenic effects observed in mTA$^+$hNEU1$^+$ animals. Interestingly, while total cholesterol concentrations showed no difference, the mTA$^+$hNEU1$^+$ mice exhibited higher cholesterol ester levels in their serum and lower cholesterol ester levels in the liver (Figure 3.4 A&B). Sialidase over-expression redistributed the cholesterol ester content from the liver to the serum, where it potentially propagated atherosclerosis, since heightened blood cholesterol is a risk factor. Triglyceride levels remained unchanged both in the liver and serum. Fast Protein Liquid Chromatography (FPLC) lipoprotein cholesterol profiles showed no significant changes in lipoprotein content, although mTA$^+$hNEU1$^+$ animals had a slight shift in the HDL peak, indicating a trend towards smaller HDL particles (Figure 3.4C).

Figure 3.4 Lipid profiling of transgenic mice after high fat diet feeding

(A) $mTA^{+}hNEU1^{+}$ mice had significantly lower hepatic cholesterol ester levels compared to both controls. Total cholesterol and free cholesterol levels remained unchanged, while triglycerides showed a slight trend towards increase when human sialidase is over-expressed. (B) $mTA^{+}hNEU1^{+}$ animals also had significantly higher levels of serum cholesterol esters, while other parameters remained unchanged (n=3,6,6 for $mTA^{-}hNEU1^{-}$, $mTA^{-}hNEU1^{+}$, and $mTA^{+}hNEU1^{+}$ respectively). (C) Human hepatic sialidase over-expression resulted in slightly smaller and more cholesterol rich HDL particles as measured by FPLC cholesterol profiling (N=3 and 5). (* denotes $p<0.05$)

Figure 3.4


A.
mTA-hNEU1-
mTA-hNEU1+
mTA+hNEU1+
Hepatic Total Cholesterol (mg/g liver)
Hepatic Triglyceride (mg/g liver)
Hepatic Free Cholesterol (mg/g liver)
Hepatic Cholesteryl Esters (mg/g liver)
*
B.
Serum Total Cholesterol (mM)
Serum Triglyceride (mM)
Serum Free Cholesterol (mM)
Serum Cholesteryl Esters (mM)
*
C.
VLDL-sized
IDL/LDL-sized
HDL-sized
Total Cholesterol (mM)
Fraction
mTA-hNEU1+
mTA+hNEU1+

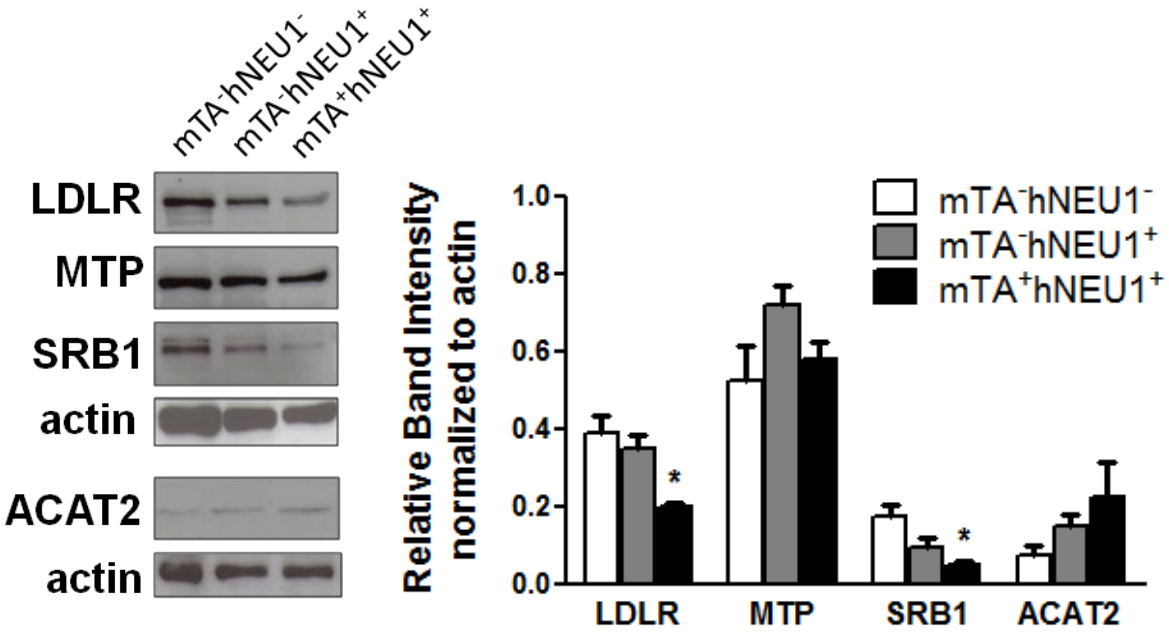
3.4.5 Hepatic sialidase over-expression decreases LDLR and SRB-1 protein levels in HFD-fed mice

In order to investigate lipoprotein metabolism further in these mice, we analyzed expression of several important hepatic proteins by western blotting in the mice which were used in the atherosclerosis analysis. Our data show that LDLR and SRB-1 protein levels were significantly decreased in $mTA^{+}hNEU1^{+}$ livers, and this suggests alterations in lipid metabolism and susceptibility to atherosclerosis due to over-expression of hepatic NEU1 (Figure 3.5). MTP and ACAT expression did not differ significantly between $mTA^{+}hNEU1^{+}$ and the controls. The hepatic enzyme ACAT2 (Acyl-CoA cholesteryl acyl transferase 2) is directly involved in esterifcation of cholesterol in the liver (Buhman et al., 2000). Since hepatic protein expression of ACAT2 was not significantly different between groups, this indicates that the changes observed in hepatic cholesterol esters (Figure 3.4) were due to a mechanism independent of ACAT2 protein levels.

Figure 3.5 Hepatic western blot analyses of HFD-fed mTA$^+$hNEU1$^+$ and control mice

The hepatic protein expression of several proteins involved in lipid metabolism was measured by SDS-PAGE and western blot analysis of liver lysates from mTA$^+$hNEU1$^+$, mTA$^-$hNEU1$^+$ and mTA$^-$hNEU1$^-$ mice fed a high fat diet for 6 months. LDLR and SRB-1 protein levels were significantly decreased in mTA$^+$hNEU1$^+$ mice, compared to controls, while MTP and ACAT2 levels were unchanged. Graphs represent average band intensity normalized to actin (representative blots are shown, N=3). (* denotes $P<0.05$ compared to both control groups)

Figure 3.5


mTA-hNEU1-
mTA-hNEU1+
mTA+hNEU1+
LDLR
MTP
SRB1
actin
ACAT2
actin
Relative Band Intensity normalized to actin
1.0
0.8
0.6
0.4
0.2
0.0
*
*
LDLR
MTP
SRB1
ACAT2
mTA-hNEU1-
mTA-hNEU1+
mTA+hNEU1+

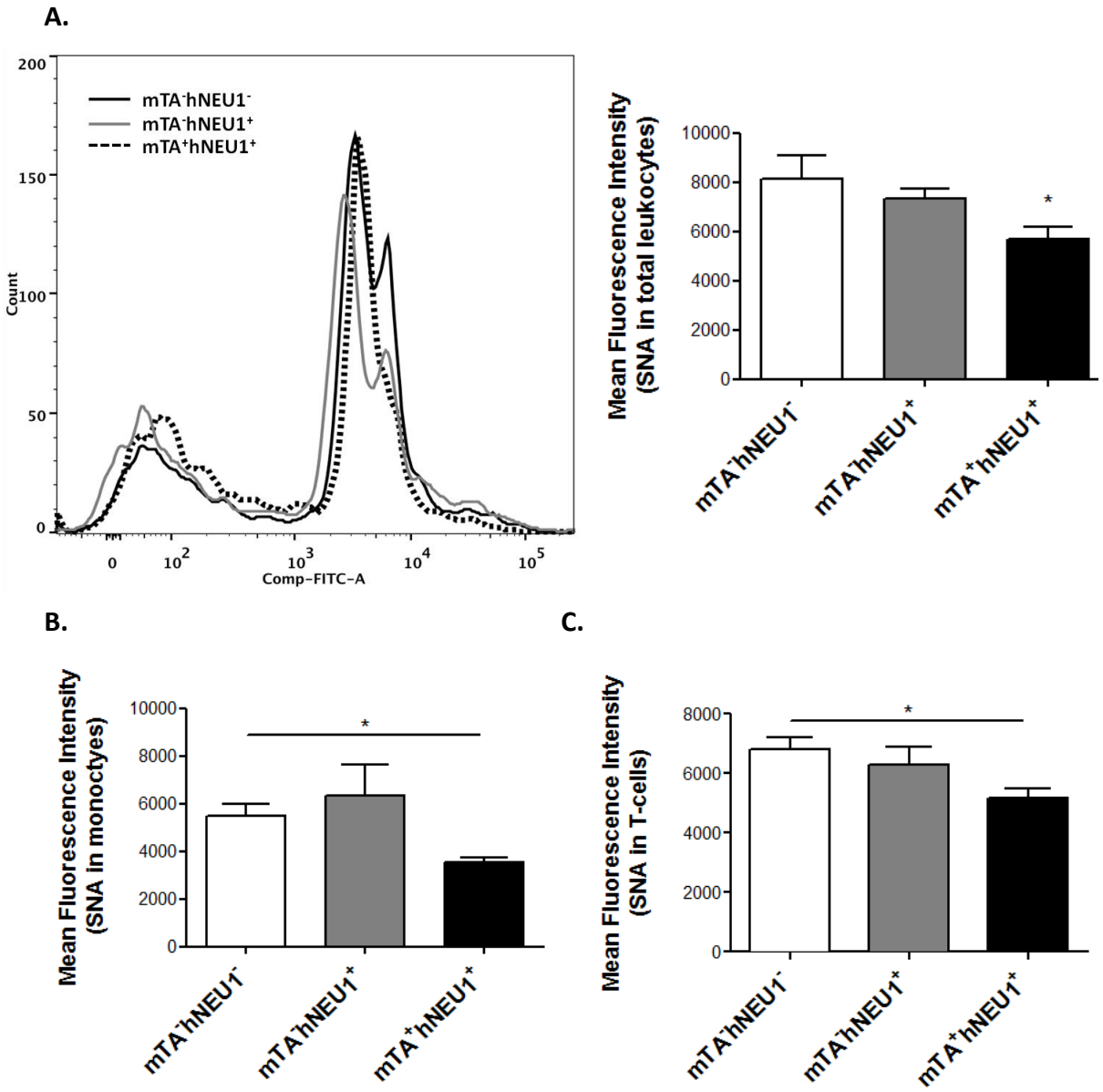
3.4.6 Hepatic sialidase over-expression decreases α-2,6-linked sialic acid on the cell surface of blood leukocytes

In this experiment, we wanted to assess inflammatory mechanisms in $mTA^{+}hNEU1^{+}$ mice. Since sialidase is heavily involved in immune response, we hypothesize that immune cells could be affected and could be contributing to atherogenesis in mice. Interestingly, we found that blood leukocytes of $mTA^{+}hNEU1^{+}$ mice had less α-2,6-linked sialic acids than controls (Figure 3.6A). This was examined by flow cytometry using SNA (Sambucus nigra agglutinin) lectin, and showed that leukocytes had less sialylation in mice over-expressing hepatic human sialidase. The same trend was also observed when analyzing monocyte (Figure 3.6B) and T-cell (Figure 3.6C) subsets using SNA flow cytometry. This was quite remarkable, as we showed evidence for desialylation of blood cells in mice expressing hepatic human sialidase, yet the percentage of total leukocytes and subsets did not differ significantly.

Figure 3.6 Flow cytometry analysis of sialylation of blood leukocytes

(A) Flow cytometry using SNA lectin showed reduced sialylation of blood leukocytes from $mTA^{+}hNEU1^{+}$ mice as analyzed by mean fluorescence intensity (representative graph of n=3). Control mice consistently exhibited two sharp SNA peaks, indicative of populations with high and low content of α-2,6 sialic acid, while $mTA^{+}hNEU1^{+}$ only had the lower peak. (B) monocyte and (C) T-Cell leukocyte populations exhibited the same trend of reduced α-2,6 sialylation in $mTA^{+}hNEU1^{+}$ mice. Total blood leukocytes, monoctyes and T-cells from mice over-expressing hepatic human sialidase had less α-2,6-linked sialic acid compared to controls (n=4). (* denotes $P<0.05$)

Figure 3.6


A.
mTA-hNEU1-
mTA-hNEU1+
mTA+hNEU1+
Count
Comp-FITC-A
Mean Fluorescence Intensity (SNA in total leukocytes)
B.
Mean Fluorescence Intensity (SNA in monoctyes)
C.
Mean Fluorescence Intensity (SNA in T-cells)
mTA-hNEU1-
mTA-hNEU1+
mTA+hNEU1+

3.4.7 $mTA^{+}hNEU1^{+}$ mice have decreased α-2,6-linked sialic acid on the cell surface of splenic leukocytes

In order to further investigate the sialylation of immune cells in our transgenic mice, we analyzed the level of cell surface α-2,6-linked sialic acids on splenic leukocytes by flow cytometry, using SNA lectin. We observed that total splenic leukocytes of $mTA^{+}hNEU1^{+}$ mice had less α-2,6-linked sialic acids compared to $mTA^{-}hNEU1^{-}$ but not $mTA^{-}hNEU1^{+}$ controls (Figure 3.7A). This shows that leukocytes from the spleen had less sialylation in mice over-expressing hepatic human sialidase. The same pattern was also observed when analyzing monocyte (Figure 3.7B) and T-cell (Figure 3.7C) subsets, while neutrophil (Figure 3.7D) α-2,6-sialylation remained unchanged between groups. We consistently observed an intermediate amount of SNA binding in monocytes and T-cells from $mTA^{-}hNEU1^{+}$ spleens, although this was not significantly different from $mTA^{+}hNEU1^{+}$ or $mTA^{-}hNEU1^{-}$ spleen cells. This data was similar to results observed in blood leukocytes and implicates that hepatic sialidase expression might affect splenic leukocytes. In order to test whether NEU1 was over-expressed on the splenic leukocytes themselves, we performed flow cytometry using an antibody against human NEU1. Total spleen leukocytes exhibited a trend of increased cell surface sialidase expression in $mTA^{+}hNEU1^{+}$ animals, but did not differ significantly from controls (Figure 3.7E).

Figure 3.7 Flow cytometry analysis of sialylation of spleen leukocytes

(A) Flow cytometry using SNA lectin showed reduced sialylation of leukocytes from spleen of $mTA^{+}hNEU1^{+}$ mice as analyzed by mean fluorescence intensity (representative graph of N=3). Control mice exhibited distinct SNA peaks which were consistently shifted to the left in $mTA^{+}hNEU1^{+}$ samples, indicating lower SNA binding. (B) monocyte and (C) T-Cell leukocyte populations exhibited the same trend of reduced α-2,6 sialylation in mice over expressing hepatic human sialidase compared to $mTA^{-}hNEU1^{-}$ spleens. (D) SNA binding in splenic neutrophils did not change. (E) Flow cytometry using NEU1 antibody showed a trend for increased staining in $mTA^{+}hNEU1^{+}$ samples as evidenced by a shift to the right in the NEU1 peak, although mean fluorescence intensity analysis did not show a statistically significant difference. This data shows that monoctyes, T-cells and leukocytes as a whole from spleens of $mTA^{+}hNEU1^{+}$ mice had less α-2,6-linked sialic acid compared to $mTA^{-}hNEU1^{-}$ controls (N=4). (* denotes $P<0.05$)

Figure 3.7

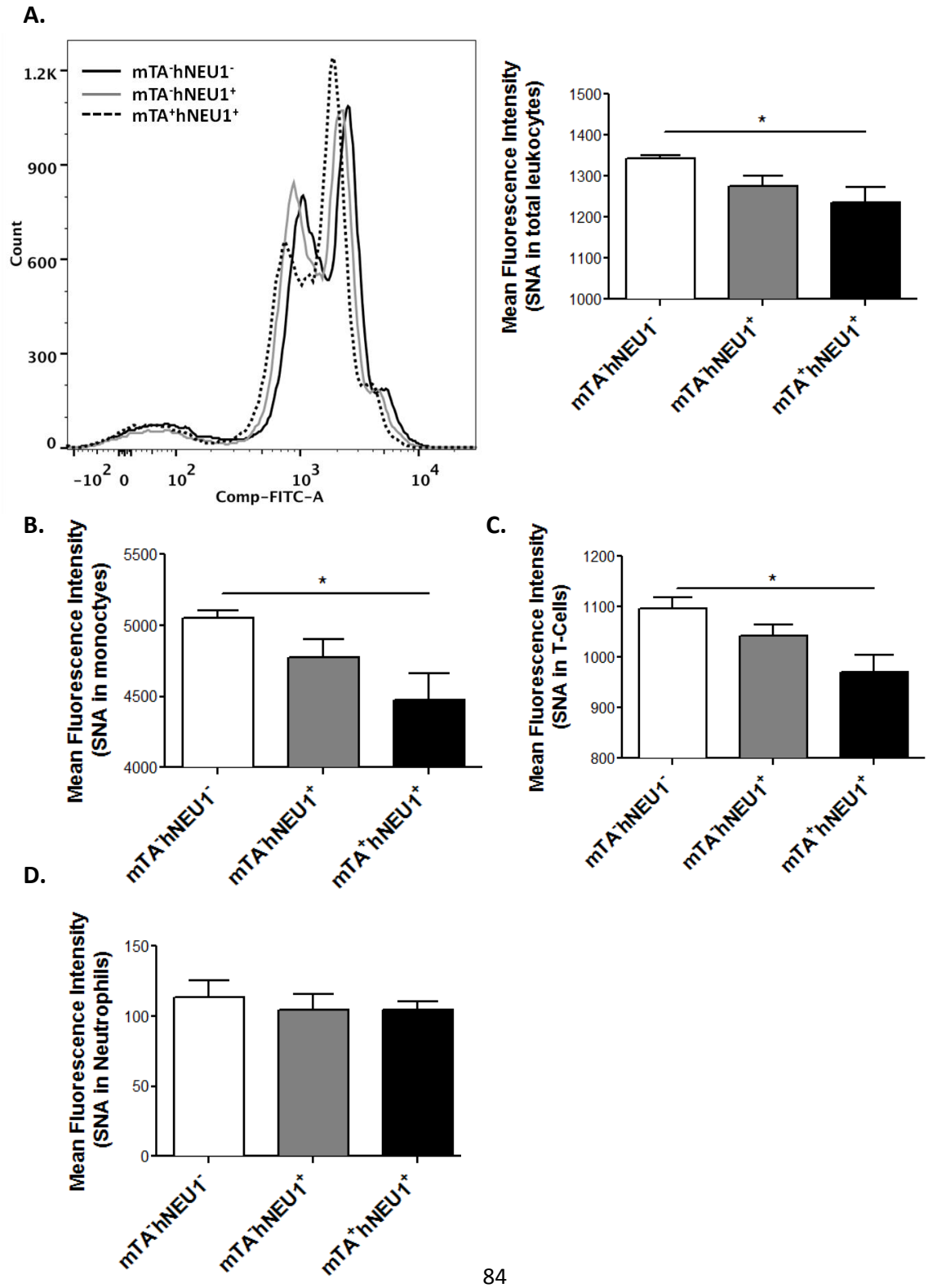
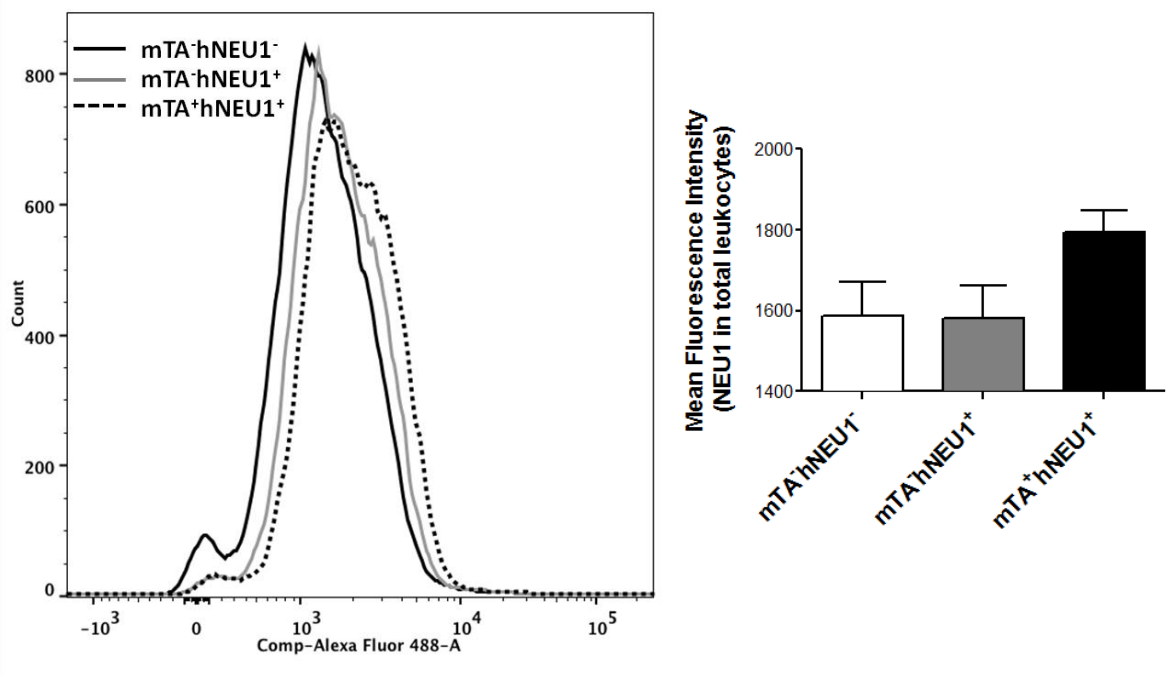

A.
mTA-hNEU1-
mTA-hNEU1+
mTA+hNEU1+
Count
Comp-FITC-A
Mean Fluorescence Intensity (SNA in total leukocytes)
B.
Mean Fluorescence Intensity (SNA in monoctyes)
C.
Mean Fluorescence Intensity (SNA in T-Cells)
D.
Mean Fluorescence Intensity (SNA in Neutrophils)
mTA-hNEU1-
mTA-hNEU1+
mTA+hNEU1+

Figure 3.7 continued

E.



3.5 DISCUSSION

Due to its multifaceted involvement in pathways relevant to cardiovascular disease, we investigated the effects of NEU1 sialidase on lipid metabolism and atherosclerosis. We generated transgenic mice that constitutively over-express human sialidase in the liver and observed an increase in aortic sinus lesion formation after high fat diet feeding. Additionally, these animals had a reduction in hepatic cholesterol ester levels while exhibiting a rise in serum cholesterol ester levels, and also had lower hepatic protein expression of LDLR and SRB-1. Furthermore, NEU1 sialidase over-expression also resulted in reduced α2,6-linked sialylation of blood leukocytes. All of these effects are potentially contributing to the increased susceptibility to atherosclerosis observed in $mTA^{+}hNEU1^{+}$ mice. These findings suggest a novel role for hepatic NEU1 sialidase as a direct player in cholesterol metabolism and progression of atherosclerosis *in vivo*.

In the current study, we generated transgenic mice to assess whether hepatic NEU1 sialidase can have an effect on atherosclerosis and cholesterol metabolism *in vivo*. Human sialidase quantitative real time PCR, beta galactosidase western blots, and hepatic sialidase activity assays provide conclusive evidence for function of the hNEU1 transgene in mTA positive mice. It was important to characterize the proper insertion of the plasmid and function of the transgene since this is a novel mouse model. Presumably due to the high homology of mouse and human sialidase, we observed over-expression of active sialidase in $mTA^{+}hNEU1^{+}$ livers despite the fact that we did not up-regulate Cathepsin A, which is crucial for the transport and function of NEU1 (Bonten et al.,

2009;Igdoura et al., 1998;Wang et al., 2009). It appears that the human protein from the transgene is utilizing the endogenous mouse Cathepsin A, which appears to be sufficient for proper function of the protein, as determined by sialidase activity assays. Thus, $mTA^{+}hNEU1^{+}$ mice over-express active human sialidase in their liver and enable investigation of the direct effects of hepatic NEU1 on atherosclerosis. It is important to note that $mTA^{+}hNEU1^{+}$ mice exhibit incomplete penetrance, as determined by percentage of $mTA^{+}hNEU1^{+}$ mice that express hNEU1 mRNA; however, all of the $mTA^{+}hNEU1^{+}$ animals utilized for the atherosclerosis experiments had hNEU1 mRNA expression. This enabled us to study the function and potential molecular targets of human sialidase in order to further elucidate the role of NEU1 in atherosclerosis progression *in vivo*.

We have found that human sialidase over-expression in FVB mice induces lipid deposition and lesion formation in the aortic sinus after six months of high fat diet feeding. A long duration of high fat diet feeding was required to induce any lesion formation, as this mouse strain is not an atherosclerosis susceptible strain. We sought to investigate lipid levels and lipoprotein metabolism in this model of sialidase over-expression in order to delineate the mechanisms behind the increase in atherosclerosis susceptibility. Furthermore, we have shown that mice with hypomorphic Neu1 expression exhibit reduced serum cholesterol levels, increased hepatic lipid storage, decreased VLDL-TG production and alterations in LDLR function, implicating sialidase in lipoprotein metabolism (Yang et al., 2012). $mTA^{+}hNEU1^{+}$ mice show lesion formation in

the aortic root and this was accompanied by increased serum cholesterol esters, but lower hepatic cholesterol esters. Additionally, $mTA^{+}hNEU1^{+}$ mice exhibited HDL particles that are slightly smaller in size, also indicative of a pro-atherogenic phenotype, as lower amounts of HDL cholesterol are a risk factor for atherosclerosis (Rosenson et al., 2012). Total cholesterol and triglyceride levels were not significantly altered, but nevertheless the cholesterol ester phenotype warranted further investigation into mechanisms controlling lipoprotein and cholesterol metabolism.

We then analyzed proteins involved in hepatic lipoprotein metabolism by western blotting of liver lysates from animals used in the atherosclerosis and lipid studies. LDLR protein levels are significantly decreased when hepatic sialidase is over-expressed, suggesting an increased susceptibility to atherosclerosis, as LDLR removes LDL cholesterol from circulation (Goldstein et al., 1985). The decreased expression of hepatic LDLR protein in $mTA^{+}hNEU1^{+}$ mice did not result in significantly increased LDL cholesterol in the FPLC profile. We hypothesize that this difference is not detectable since the VLDL and LDL peaks are extremely low. Hepatic SRB-1 protein levels were also significantly reduced in $mTA^{+}hNEU1^{+}$ mice, again indicative of increased susceptibility to atherosclerosis since SRB-1 is crucial for reverse cholesterol transport and uptake of HDL (Trigatti et al., 1999). The down-regulated protein expression of these important lipoprotein receptors in $mTA^{+}hNEU1^{+}$ animals implicates over-expression of hepatic human NEU1 in modification of lipoprotein uptake and metabolism resulting in a pro-atherogenic phenotype. Interestingly, protein expression of MTP remained unchanged,

indicative of no gross changes in VLDL production and synthesis (Bartels et al., 2002). This was unexpected, since mice with hypomorphic expression of Neu1 had lower MTP levels and decreased VLDL-TG production; however, $Neu1^{hypo}$ mice were on a C57Bl/6 background and were not fed a high fat diet. We also analyzed protein expression of Acyl-CoA cholesteryl acyl transferase (ACAT2), which converts free cholesterol to cholesterol esters in the liver (Chang et al., 2009). Hepatic protein expression of ACAT2 was not changed in $mTA^{+}hNEU1^{+}$ mice, despite the fact that human sialidase over-expression changes the distribution of cholesterol esters from the liver to the serum. Potential causes for the cholesterol ester redistribution could be due to differential uptake and processing of lipoproteins, changes in ACAT2 activity or cholesterol ester hydrolysis, as well as remodelling of lipoproteins by LCAT (Lecithin-cholesterol acyltransferase), but this remains to be determined (Lee et al., 2005). Regardless of the mechanism, the cholesterol ester redistribution and western blot data point to significant changes in cholesterol metabolism and susceptibility to atherosclerosis in high fat diet fed $mTA^{+}hNEU1^{+}$ animals.

Hepatic over-expression of human sialidase results in a pro-atherogenic phenotype by modulating expression of LDLR and SRB-1, as well as affecting the cholesterol ester distribution and HDL particle size. While these modifications to lipid metabolism can explain our pro-atherogenic phenotype, we wanted to assess inflammatory mechanisms as well, to obtain a full picture of NEU1's diverse effects on atherogenesis. Since sialidase is heavily involved in immune response and trafficking of

leukocytes, we hypothesized that immune cells could be affected by sialidase over-expression, and that these inflammatory modifications are another contributing factor to atherogenesis (Varki and Gagneux, 2012). Interestingly, we found that blood and spleen leukocytes of $mTA^{+}hNEU1^{+}$ mice have less α2,6-linked sialic acids than controls. When examined by flow cytometry using SNA lectin, leukocytes have less cell surface sialylation in mice over-expressing hepatic human sialidase. The same trend is also observed when analyzing monocyte and T cell subsets. We show evidence for desialylation of blood and spleen cells by over-expression of hepatic human sialidase, yet the percentage of total leukocytes and subsets do not differ significantly. This indicates that sialidase is potentially secreted into the blood where it can act on these leukocytes or that it is expressed in tissues involved in hematopoiesis and immune cell maturation such as the spleen or bone marrow. While our data examined global sialylation, we expect that desialylation of cell surface molecules, such as CD44, will increase leukocyte binding and differentiation potential (Gee et al., 2003;Lambre et al., 1990;Stamatos et al., 2005). This is potentially another contributing mechanism to the atherosclerosis observed in $mTA^{+}hNEU1^{+}$ animals, and offers further insight into the role of hepatic sialidase in cardiovascular disease.

Based on the SNA flow cytometry data, we sought to examine expression of sialidase protein in splenic leukocytes. There appears to be a trend for increased NEU1 expression on the surface of cells in $mTA^{+}hNEU1^{+}$ mice compared to controls, although the difference is not statistically significant. The decrease in sialylation of splenic and

blood leukocytes in $mTA^{+}hNEU1^{+}$ animals could be partially attributed to the splenic leukocyte expression of NEU1. It is also important to note that due to the high homology of mouse and human sialidase, we were unable to detect solely human NEU1 protein using our antibody. Using more specific means of measuring exclusively human NEU1 protein on these cells might provide a clearer picture of its expression in leukocytes. Another interesting observation is that $mTA^{-}hNEU1^{+}$ animals tend to exhibit an intermediary phenotype (between $mTA^{+}hNEU1^{+}$ and $mTA^{-}hNEU1^{-}$), especially in our SNA flow cytometry data. These mice could be undergoing tTA-independent expression of hNEU1 in their liver or other tissues and this points to non-specific expression in some $mTA^{-}hNEU1^{+}$ animals. Further investigation of NEU1 expression of all the tissues utilized in the above experiments would answer these questions and aid in elucidating any issues with leakiness in expression of the hNEU1 transgene. Importantly, however, all of the $mTA^{-}hNEU1^{+}$ mice used for the atherosclerosis analysis lacked human NEU1 cDNA in their liver. Although non-specific human NEU1 expression might be possible in other tissue types in our mice, the atherogenic phenotype can still be directly attributed to hepatic NEU1 expression in $mTA^{+}hNEU1^{+}$ animals.

Overall, our findings represent a novel involvement of hepatic NEU1 in both lipid and inflammatory atherosclerotic disease processes. It is interesting to note that even on an Apo-E knockout background, FVB mice have 8-fold lower lesion areas than C57Bl/6 animals (Smith et al., 2001). Yet, despite this fact, we observed a pro-atherogenic phenotype and aortic sinus lipid deposition when sialidase is over-expressed

in the livers of FVB mice. We utilized the FVB strain because it is desirable for transgenic work since these mice exhibit reliable breeding, large litter sizes, and prominent pronuclei for injection (Taketo et al., 1991). Nevertheless, future studies will involve placing mTAhNEU1 animals on more atherosclerosis prone backgrounds (such as Apo-E or LDLR knockout), to enable the study of more severe atherogenesis. Furthermore, the hNEU1 transgene can be coupled with tTA expression from other tissues. For example, $hNEU1^{+}$ mice could be bred with FVB.Cg-Tg(Tal1-tTA)19Dgt/J mice which express tTA in their hematopoietic lineage and are available from Jackson Labs. This would enable us to study the direct effects of leukocyte Neu1 on atherosclerosis. Nevertheless, this unique mouse model provides enormous potential for studying the effects of human NEU1 *in vivo* and adds insight to the many roles exhibited by sialidase in atherosclerosis disease progression. Overall, we have generated a new avenue for investigating the contributions of NEU1 to *in vivo* cholesterol metabolism, inflammation and atherosclerosis and our data solidifies sialidase as a potential therapeutic target for atherosclerosis, which is an inflammatory and dyslipidemic disease.

3.6 REFERENCES

Amith SR, Jayanth P, Franchuk S, Siddiqui S, Seyrantepe V, Gee K, Basta S, Beyaert R, Pshezhetsky AV, Szewczuk MR (2009) Dependence of pathogen molecule-induced Toll-like receptor activation and cell function on Neu1 sialidase. Glycoconj J 26:1197-212

Bartels ED, Lauritsen M, Nielsen LB (2002) Hepatic expression of microsomal triglyceride transfer protein and in vivo secretion of triglyceride-rich lipoproteins are increased in obese diabetic mice. Diabetes 51:1233-1239

Barton M (2013) Mechanisms and therapy of atherosclerosis and its clinical complications. Curr Opin Pharmacol 13:149-153

Bonten EJ, Campos Y, Zaitsev V, Nourse A, Waddell B, Lewis W, Taylor G, d'Azzo A (2009) Heterodimerization of the sialidase NEU1 with the chaperone protective protein/cathepsin A prevents its premature oligomerization. J Biol Chem 284:28430-28441

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248-254

Buhman KK, Accad M, Novak S, Choi RS, Wong JS, Hamilton RL, Turley S, Farese RV, Jr. (2000) Resistance to diet-induced hypercholesterolemia and gallstone formation in ACAT2-deficient mice. Nat Med 6:1341-1347

Businaro R, Tagliani A, Buttari B, Profumo E, Ippoliti F, Di CC, Capoano R, Salvati B, Rigano R (2012) Cellular and molecular players in the atherosclerotic plaque progression. Ann N Y Acad Sci 1262:134-141

Chang TY, Li BL, Chang CC, Urano Y (2009) Acyl-coenzyme A:cholesterol acyltransferases. Am J Physiol Endocrinol Metab 297:E1-E9

Chen XP, Enioutina EY, Daynes RA (1997) The control of IL-4 gene expression in activated murine T lymphocytes: a novel role for neu-1 sialidase. J Immunol 158:3070-3080

Clark EA, Engel D, Windsor NT (1981) Immune responsiveness of SM/J mice: hyper NK cell activity mediated by NK 1+ Qa 5- cells. J Immunol 127:2391-2395

Cross AS, Wright DG (1991) Mobilization of sialidase from intracellular stores to the surface of human neutrophils and its role in stimulated adhesion responses of these cells. J Clin Invest 88:2067-2076

Cummings RD, Kornfeld S, Schneider WJ, Hobgood KK, Tolleshaug H, Brown MS, Goldstein JL (1983) Biosynthesis of N- and O-linked oligosaccharides of the low density lipoprotein receptor. J Biol Chem 258:15261-15273

Davis CG, Elhammer A, Russell DW, Schneider WJ, Kornfeld S, Brown MS, Goldstein JL (1986) Deletion of clustered O-linked carbohydrates does not impair function of low density lipoprotein receptor in transfected fibroblasts. J Biol Chem 261:2828-2838

de GN, Bonten E, Mann L, de Sousa-Hitzler J, Hahn C, d'Azzo A (2002) Systemic and neurologic abnormalities distinguish the lysosomal disorders sialidosis and galactosialidosis in mice. Hum Mol Genet 11:1455-1464

Filipovic I (1989) Effect of inhibiting N-glycosylation on the stability and binding activity of the low density lipoprotein receptor. J Biol Chem 264:8815-8820

Freeman LA (2013) Western blots. Methods Mol Biol 1027:369-385

Gadhoum SZ, Sackstein R (2008) CD15 expression in human myeloid cell differentiation is regulated by sialidase activity. Nat Chem Biol 4:751-757

Gee K, Kozlowski M, Kumar A (2003) Tumor necrosis factor-alpha induces functionally active hyaluronan-adhesive CD44 by activating sialidase through p38 mitogen-activated protein kinase in lipopolysaccharide-stimulated human monocytic cells. J Biol Chem 278:37275-37287

Gee K, Lim W, Ma W, Nandan D, az-Mitoma F, Kozlowski M, Kumar A (2002) Differential regulation of CD44 expression by lipopolysaccharide (LPS) and TNF-alpha in human monocytic cells: distinct involvement of c-Jun N-terminal kinase in LPS-induced CD44 expression. J Immunol 169:5660-5672

Goldstein JL, Brown MS, Anderson RG, Russell DW, Schneider WJ (1985) Receptor-mediated endocytosis: concepts emerging from the LDL receptor system. Annu Rev Cell Biol 1:1-39

Gui T, Shimokado A, Sun Y, Akasaka T, Muragaki Y (2012) Diverse roles of macrophages in atherosclerosis: from inflammatory biology to biomarker discovery. Mediators Inflamm 2012:693083

Hegele RA (2009) Plasma lipoproteins: genetic influences and clinical implications. Nat Rev Genet 10:109-121

Igdoura SA, Gafuik C, Mertineit C, Saberi F, Pshezhetsky AV, Potier M, Trasler JM, Gravel RA (1998) Cloning of the cDNA and gene encoding mouse lysosomal sialidase and

correction of sialidase deficiency in human sialidosis and mouse SM/J fibroblasts. Hum Mol Genet 7:115-121

Kawakami A, Osaka M, Aikawa M, Uematsu S, Akira S, Libby P, Shimokado K, Sacks FM, Yoshida M (2008) Toll-like receptor 2 mediates apolipoprotein CIII-induced monocyte activation. Circ Res 103:1402-1409

Kingsley DM, Kozarsky KF, Hobbie L, Krieger M (1986) Reversible defects in O-linked glycosylation and LDL receptor expression in a UDP-Gal/UDP-GalNAc 4-epimerase deficient mutant. Cell 44:749-759

Knibbs RN, Goldstein IJ, Ratcliffe RM, Shibuya N (1991) Characterization of the carbohydrate binding specificity of the leukoagglutinating lectin from Maackia amurensis. Comparison with other sialic acid-specific lectins. J Biol Chem 266:83-88

Kozarsky K, Kingsley D, Krieger M (1988) Use of a mutant cell line to study the kinetics and function of O-linked glycosylation of low density lipoprotein receptors. Proc Natl Acad Sci U S A 85:4335-4339

Lambre CR, Greffard A, Gattegno L, Saffar L (1990) Modifications of sialidase activity during the monocyte-macrophage differentiation in vitro. Immunol Lett 23:179-182

Lee RG, Shah R, Sawyer JK, Hamilton RL, Parks JS, Rudel LL (2005) ACAT2 contributes cholesteryl esters to newly secreted VLDL, whereas LCAT adds cholesteryl ester to LDL in mice. J Lipid Res 46:1205-1212

Liang F, Seyrantepe V, Landry K, Ahmad R, Ahmad A, Stamatos NM, Pshezhetsky AV (2006) Monocyte differentiation up-regulates the expression of the lysosomal sialidase, Neu1, and triggers its targeting to the plasma membrane via major histocompatibility complex class II-positive compartments. J Biol Chem 281:27526-27538

Libby P, Lichtman AH, Hansson GK (2013) Immune effector mechanisms implicated in atherosclerosis: from mice to humans. Immunity 38:1092-1104

Libby P, Ridker PM, Hansson GK (2011) Progress and challenges in translating the biology of atherosclerosis. Nature 473:317-325

LOWRY OH, ROSEBROUGH NJ, FARR AL, RANDALL RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193:265-275

Manickan E, Satoi J, Wang TC, Liang TJ (2001) Conditional liver-specific expression of simian virus 40 T antigen leads to regulatable development of hepatic neoplasm in transgenic mice. J Biol Chem 276:13989-94

Millar JS (2001) The sialylation of plasma lipoproteins. Atherosclerosis 154:1-13

Moore KJ, Sheedy FJ, Fisher EA (2013) Macrophages in atherosclerosis: a dynamic balance. Nat Rev Immunol 10:709-21

Potier M, Lu Shun YD, Womack JE (1979) Neuraminidase deficiency in the mouse. FEBS Lett 108:345-348

Pshezhetsky AV, Hinek A (2011) Where catabolism meets signalling: neuraminidase 1 as a modulator of cell receptors. Glycoconj J 28:441-452

Rosenson RS, Brewer HB, Jr., Davidson WS, Fayad ZA, Fuster V, Goldstein J, Hellerstein M, Jiang XC, Phillips MC, Rader DJ, Remaley AT, Rothblat GH, Tall AR, Yvan-Charvet L (2012) Cholesterol efflux and atheroprotection: advancing the concept of reverse cholesterol transport. Circulation 125:1905-1919

Ross R (1999) Atherosclerosis--an inflammatory disease. N Engl J Med 340:115-126

Rottier RJ, Bonten E, d'Azzo A (1998) A point mutation in the neu-1 locus causes the neuraminidase defect in the SM/J mouse. Hum Mol Genet 7:313-321

Smith JD, Dansky HM, Breslow JL (2001) Genetic modifiers of atherosclerosis in mice. Ann N Y Acad Sci 947:247-252

Stamatos NM, Liang F, Nan X, Landry K, Cross AS, Wang LX, Pshezhetsky AV (2005) Differential expression of endogenous sialidases of human monocytes during cellular differentiation into macrophages. FEBS J 272:2545-2556

Stein O, Dabach Y, Ben-Naim M, Halperin G, Stein Y (2006) Lower macrophage recruitment and atherosclerosis resistance in FVB mice. Atherosclerosis 189:336-341

Stoline AM, Saku K, Hynd BA, Kashyap ML (1985) Effect of desialylation of very low-density lipoproteins on their catabolism by lipoprotein lipase. Metabolism 34:30-35

Taketo M, Schroeder AC, Mobraaten LE, Gunning KB, Hanten G, Fox RR, Roderick TH, Stewart CL, Lilly F, Hansen CT, . (1991) FVB/N: an inbred mouse strain preferable for transgenic analyses. Proc Natl Acad Sci U S A 88:2065-2069

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

Varki A, Gagneux P (2012) Multifarious roles of sialic acids in immunity. Ann N Y Acad Sci 1253:16-36

Wang D, Zaitsev S, Taylor G, d'Azzo A, Bonten E (2009) Protective protein/cathepsin A rescues N-glycosylation defects in neuraminidase-1. Biochim Biophys Acta 1790:275-282

Wang P, Zhang J, Bian H, Wu P, Kuvelkar R, Kung TT, Crawley Y, Egan RW, Billah MM (2004) Induction of lysosomal and plasma membrane-bound sialidases in human T-cells via T-cell receptor. Biochem J 380:425-433

Yang A, Gyulay G, Mitchell M, White E, Trigatti BL, Igdoura SA (2012) Hypomorphic sialidase expression decreases serum cholesterol by downregulation of VLDL production in mice. J Lipid Res 53:2573-2585

CHAPTER 4: VLDL-dependent monocyte activation is mediated by sialidase (NEU1) activity

Gabriel Gyulay[1], Bernardo L. Trigatti[2,4], and Suleiman A. Igdoura[1,3]

Departments of [1]Biology, [2]Biochemistry and Biomedical Sciences, [3]Pathology and Molecular Medicine, [4]Thrombosis and Atherosclerosis Research Institute, McMaster University, Hamilton, Ontario, Canada

Correspondence:
Suleiman Igdoura, Ph.D.
McMaster University, 1280 Main St. W. LSB 335
Hamilton, Ontario L8S 4K1 Canada
Tel. (905) 525-9140ex27729
Fax: (905) 522-6066
E-Mail: igdoura@mcmaster.ca

4.1 ABSTRACT

Atherosclerosis is an inflammatory disease that is initiated and propagated by dyslipidemia. The differentiation of monocytes into macrophages and their subsequent lipid accumulation and foam cell formation are central to atherosclerosis. Very low density lipoprotein can enhance both lipid and immune related mechanisms in atherosclerotic disease progression; however, the exact pro-atherogenic effects of VLDL on leukocytes are still not fully known. The enzyme sialidase (NEU1), which cleaves sialic acid, has been invoked in inflammatory responses as well as lipoprotein function, yet its complex role in propagation of atherosclerosis by monocytes and macrophages is not fully understood. Many functionally important leukocyte cell surface molecules are sialylated, and sialic acid is abundant on lipoprotein receptors as well as VLDL. Thus, we analyzed the effect of VLDL on monocyte differentiation and activation. We have found that VLDL stimulation of THP-1 monocytes increases their differentiation to macrophages and cytokine production. The differentiated cells also accumulate triglycerides, express macrophage markers, and exhibit a foam-cell phenotype, pointing to a novel and important role for VLDL in the initiation of atherosclerosis. VLDL also affects mature THP-1 macrophages by causing lipid accumulation, down-regulation of ABCG1, and triggering secretion of cytokines. We also investigated whether sialidase can affect VLDL-induced inflammation and foam cell formation. We found that VLDL up-regulates NEU1 protein as THP-1 monocytes differentiate, and that the sialidase inhibitor DANA drastically reduced this differentiation, and accompanying lipid uptake.

DANA also dampened VLDL-induced activation of mature macrophages, thus showing that sialidase plays an important role in the diverse pro-atherogenic actions of VLDL. We propose novel mechanisms for VLDL-induced monocyte to foam cell transition and lay the groundwork for the use of sialidase inhibition as a potent therapeutic target in atherosclerosis.

4.2 INTRODUCTION

Atherosclerosis is a complex disease involving several metabolic pathways and inflammatory cascades (Glass and Witztum, 2001). The oxidation and subendothelial retention of low density lipoprotein (LDL) are the best characterized triggers of atherosclerosis, but there are a plethora of atherogenic lipoproteins and inflammatory mechanisms that contribute to atherosclerotic lesion formation (Businaro et al., 2012;Skalen et al., 2002;Tabas et al., 1990;Ye et al., 2009). While hypercholesterolemia is the primary risk factor, the driving force behind plaque formation and subsequent ischemic events is the propagation of an immune response by the macrophage (Hansson, 2009;Ross, 1999). Furthermore, lipoprotein metabolism and leukocyte inflammation are not separate pathways in atherosclerosis, but are part of one complex biological system contributing to disease progression in unison (Daugherty et al., 2005;Guo et al., 2009;Hansson, 2007;Hong and Tontonoz, 2008;Libby, 2007). Monocytes and macrophages are at the heart of this cross-talk and are prevalent at many critical steps of atherogenesis; they can interact with several other cell types (T-cells, smooth muscle cells, etc.) and internalize a vast array of lipoproteins (Chawla, 2010;Gui et al., 2012;Palmer et al., 2005a;Persson et al., 2008a). Not only is the differentiation of monocytes into macrophages a necessary requirement in the initiation of atherosclerosis, but the activation and ongoing immune response of mature lipid-rich macrophage foam cells potentiates disease progression (Gui et al., 2012;Libby et al., 2011;Williams et al., 2012). The transition of monocytes to foam cells is a complex

continuum that can be triggered by endothelial dysfunction or direct activation of monocytes themselves, and monocyte-macrophages exhibit heterogeneous morphology and behaviour, especially in the plaque microenvironment (Correa et al., 2011;Shalhoub et al., 2011). Macrophages differ from monocytes in that they are larger, non-migratory, have catabolic abilities and large changes in gene and protein expression; yet monocyte differentiation into macrophages is a complex and variable process (Satpathy et al., 2012).

High levels of very low density lipoprotein (VLDL) due to hypertriglyceremia contribute to lipid accumulation as well as the inflammatory response of macrophages (Sampedro et al., 2001;Stollenwerk et al., 2005a). Cholesterol and triglyceride containing VLDL is assembled by the liver and secreted into the bloodstream (Shelness et al., 1999). Its apolipoprotein-E (Apo-E) and apolipoprotein-B (Apo-B) components facilitate its binding not only to VLDL receptor (VLDLR), but also to LDL receptor related protein 1 (LRP-1) and LDL receptor (LDLR) (May et al., 2005), and other VLDL apolipoproteins such as apolipoprotein-CII (Apo-CII) and apolipoprotein-CIII (Apo-CIII) mediate its interaction with lipoprotein lipase (LPL), leading to its lipolysis and maturation into intermediate density lipoprotein (IDL) and then LDL (Hegele, 2009). VLDL is composed primarily of triglycerides and contains several apolipoproteins, while LDL is cholesterol rich and contains only Apo-B (Glass and Witztum, 2001). VLDL can induce TG accumulation and inflammatory response of macrophages *in vitro* (Bojic et al., 2012;Milosavljevic et al., 2003). Specifically, VLDL causes macrophages to secrete IL-1β and TNF, and induces

foam cell formation and the activation of ERK/MEK and p38 MAPK cell signalling pathways (Jinno et al., 2011;Persson et al., 2008b;Stollenwerk et al., 2005a). The Apo-CIII component of VLDL has been suggested to mediate endothelial inflammation and adhesion of monocytes to endothelial cells, via activation of PKC and NFκB and subsequent activation of β1-integrin (Kawakami et al., 2006a;Kawakami et al., 2006b;Kawakami et al., 2007). Nevertheless, the molecular mechanisms which mediate VLDL-monocyte interactions and subsequent downstream effects are still unclear and require more investigation.

NEU1 sialidase also plays a prevalent role in monocyte differentiation and macrophage inflammatory response (Varki and Gagneux, 2012). Sialidase belongs to a family of hydrolytic enzymes that cleave terminal sialic acid residues of glycoproteins, glycolipids and oligosaccharides, and NEU1 sialidase is localized to the lysosome and the cell surface of cells (Pshezhetsky and Ashmarina, 2013;Reuter and Gabius, 1996). Sialic acid, also known as neuraminic acid, is a negatively charged 9 carbon hexose terminal sugar residue found on a variety of cell surface moieties (Millar, 2001;Pappu and Shrikant, 2004;Varki, 2008). Sialic acids have important biological roles in conformational stabilization, cell surface charge, cell recognition, and immune response (Chen and Varki, 2010;Schauer, 2009;Varki and Gagneux, 2012). NEU1 sialidase is up-regulated and targeted to the cell surface as monocytes differentiate into macrophages, and it is required for Cluster of Differentiation-Hyaluronic Acid (CD44-HA) adhesion, selectin binding and toll like receptor (TLR) activation (Amith et al., 2009;Gadhoum and

Sackstein, 2008;Gee et al., 2002;Gee et al., 2003;Lambre et al., 1990;Liang et al., 2006;Stamatos et al., 2005). In addition to their roles in macrophage inflammatory response, sialic acid and sialidase have also been implicated in lipoprotein metabolism (Lindberg, 2007;Rastam et al., 1996;Sprague et al., 1988;Stoline et al., 1985;Yang et al., 2012). However, the exact involvement of NEU1 in macrophage lipid metabolism and VLDL-induced inflammatory response is unknown.

In this study, we show that VLDL induced adhesion and differentiation of THP-1 monocytes onto glass, in a PI3K and p38 MAPK dependent manner. VLDL treatment of monocytes also resulted in lipid accumulation, cytokine production and upregulation of NEU1 protein as well as markers of differentiation and ER stress in adhered cells (Dickhout et al., 2011;Du et al., 2011;Isa et al., 2011;Spilsbury et al., 1995). Additionally, this monocyte to foam cell transition was reduced when sialidase was inhibited using 2-deoxy-2,3-dehydro-N-acetyl neuraminic acid (DANA). DANA had similar effects when studying mature PMA differentiated macrophages, as it damped VLDL induced cytokine secretion and lipid uptake. We also show that VLDL treatment can induce changes in protein expression in differentiated macrophages, implicating pathways involved in cholesterol efflux and immune response (Rosenson et al., 2012;Zhang et al., 2013a). Our data adds to the notion that VLDL has diverse effects on leukocytes by affecting both lipid metabolism and inflammation (den Hartigh et al., 2010;Libby, 2007;Lu et al., 2009;Stollenwerk et al., 2005b). Additionally, we show that inhibition of sialidase not only decreases VLDL-induced THP-1 differentiation, but also has anti-inflammatory

effects in VLDL-treated mature macrophages. It is evident that sialidase is involved in several facets of atherosclerosis and we seek to investigate further mechanisms that pave way for therapeutic options that address both inflammation and dyslipidemia.

4.3 MATERIALS AND METHODS

4.3.1 Macrophages

THP-1 cells are a human monocytic cell line derived from a 1-year old male with acute leukemia and were grown in RPMI1640 medium wtih 0.1 mM L-glutamine supplemented with 10% FBS, 1% antibacterial solution and 0.1% fungizone. For macrophage experiments, differentiation of monocytes to macrophages was performed with 0.2 µM PMA (phorbol 12-myristate 13-acetate) for 72 hours. Cells were cultured at 37ºC at 5% CO2 and 90% humidity in a standard water jacketed incubator. Macrophages were lipid starved prior to lipoprotein studies by replacing 10% FBS with 5% lipoprotein deficient serum (LPDS) in RPMI. THP-1 cells readily internalize LDL and VLDL (Lu et al., 2009;Palmer et al., 2005b;Ye et al., 2009).

4.3.2 Lipoproteins

LDL, acLDL and VLDL were purchased from Biomedical Technologies Inc. and were used in LPDS (lipoprotein deficient serum) in RPMI to avoid any FBS-derived lipoprotein interference. Concentrations of lipoproteins were 50 µg/mL and incubation periods were 48 hours unless otherwise stated.

4.3.3 THP-1 Adhesion Studies

THP-1 monocytes were cultured in lipoprotein deficient media throughout the experiment, with supplements listed below. THP-1 monocytes were cultured on 24-well plates containing glass coverslips and incubated with lipoproteins or PMA for 48 hours

with and without 200 μM DANA or galactose. For inhibitor experiments, THP-1 monocytes were incubated with PMA or VLDL for 48 hours in combination with several cell signalling pathway inhibitors (see below). Equal amounts of cells were loaded in each well. Cells that adhered to coverslips were washed and fixed with formaldehyde and then stained with Oil Red O (Sigma-Aldrich O0625). After extensive washing, Haematoxylin staining was performed and the cover slips were mounted on microscope slides using Aqua Mount (Fisher). Pictures were taken using bright field and phase microscopy with Zeiss Axiovision Release 4.8.2 software using an MRc Axiocam camera at 40X magnification on a Zeiss Axiovert 200 microscope. Adhered cells were counted in random fields of view and normalized per millimeter squared. Alternatively, flattened cells that had a diameter greater than 20 μm were scored as differentiated (Krombach et al., 1997). At least 50 random fields of view were counted for each group in counting assays. To assess cell area, bright field pictures at 10X were analyzed using ImageJ software, by quantifying the area of all cells greater than 100 μm^2 using the analyze particles function after conversion of the picture to a threshold binary image. Mean cell area was determined from at least 500 cells for each condition. Oil Red O quantification was performed by measuring the area of the red stained lipid droplets of a cell normalized to its nuclear size and averaged. Inhibitor concentrations were as follows: Protein kinase C: Calphostin C - 2 μM (Calbiochem), Phosphoinositide 3-kinase: LY294004 - 50 μM (Calbiochem), p38 Mitogen-activated protein kinase: Inhibitor VIII- 1 μM (Santa Cruz), Extracellular signal-regulated kinase/Mitogen-activated protein kinase

kinase: PD98059 - 20 μM (Calbiochem). Adhesion/Differentiation assays were also reproduced using 8-well Nunc® Lab-Tek® Glass Chamber Slides (Sigma).

4.3.4 Protein Assay

DC protein assays (Bio-Rad 500-0113/4) using a modified method of Lowry (Lowry et al., 1951) were performed on all lysates to determine protein concentrations to allow for normalized comparisons and equal loading. Bovine Serum Albumin (BSA) was used as a standard.

4.3.5 Western Blot Analysis

After washing with cold PBS, adhered cells were scraped and lysed in RIPA buffer containing protease inhibitors (Complete Mini, Roche 11873580001, 1 tablet per 10 mL) with 1 mM EDTA. Cells in suspension were centrifuged out from media and resuspended in RIPA with protease inhibitors. Samples were then sonicated and boiled in Laemmli Sample Buffer prior to loading. Proteins were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and then blocked using 5% milk in TBST. Antibodies were used at 1:1000 in 5% milk in TBST. HRP-bound appropriate secondary antibodies were then detected by ECL Reagent (GE) after which membranes were exposed onto film and developed (Freeman, 2013). Antibodies: rabbit anti-ABCG1 (Novus Biologicals NB400-132), mouse anti-ABCA1 (GenScript A00121), goat anti-VLDLR (R & D Systems AF2258), rabbit anti-LRP1 (Epitomics 2703-1, detects cleaved 85kDa fragment), rabbit anti-SRB-1 (Novus NB400-101), rabbit anti-STAT3 (Cell Signalling

Technology 4904), rabbit anti-phospho-STAT3 (Cell Signalling Technology 9145), mouse anti-BiP/GRP78 (BD Biosciences 610979), rabbit anti-MPG-1 (Cell Sciences CPM700) goat anti-GAPDH (R & D Systems AF5718), mouse anti-β-actin (Cell Signalling 3700), rabbit anti-sialidase (synthesized by our lab against the carboxyl terminus of human NEU1). Band intensity was measured by densitometry analysis using ImageJ software and normalized to loading controls.

4.3.6 Sialidase Inhibition

Inhibition of sialidase activity was performed using 2-deoxy-2,3-dehydro-N-acetyl neuraminic acid (DANA). DANA is an established potent competitive inhibitor for sialidase, that functions by mimicking the sialic acid substrate and it has been routinely used in our laboratory and elsewhere (Bonnet and Bryce, 2004;Luo et al., 1995;Meindl et al., 1974;Zhang et al., 2013b). DANA was dissolved in PBS as a stock and final concentrations in media were 200 μM unless otherwise stated. Galactose, which is also a monosaccharide, was used as a control at 200 μM, since DANA has a similar structure but galactose lacks inhibitory activity.

4.3.7 Enzyme-Linked Immunosorbent Assay

ELISA kits from BD Biosciences were used to assess cytokine concentrations in media using the sandwich method. Macrophages were differentiated with PMA for 72 hours, lipid starved, and then treated with VLDL or VLDL and DANA for 48 hours. Media was collected and cells were washed and lysed for protein estimation. Alternatively, for

cell adhesion experiments, media was collected after 48 hour treatment of THP-1 monocytes with VLDL or PMA (equal amounts of cells were plated). After centrifugation to remove cells and debris, media was plated and the ELISA was performed as per the manufacturer's protocol (BD OptEIA™). Cytokine kits used (catalogue #): TNF (550610), IL-1β (557966), IL-6 (550719), MCP-1 (559017). A standard curve of purified cytokine was used to determine exact levels in samples after measuring the optical density in a plate reader and then normalizing to cellular protein content.

4.3.8 Triglyceride Assay

PMA-differentiated macrophages were incubated with lipoproteins for 48 hours in LPDS. Cells were washed and lipids were extracted using a hexane/isopropanol mixture overlaid on the cells for 1 hour. The mixture was allowed to evaporate and lipids were resuspended in 100 µl isopropanol for enzymatic measurements of Triglycerides using a colorometric assay kit with appropriate blanks and standards (Wako Diagnostics L-type TG H, discontinued). After lipid extraction, cells were scraped in 0.1% SDS and protein content was measured to normalize readings. Technical triplicates were used and data is representative of multiple experiments.

4.3.9 Statistical Significance

For all experiments, data were as analyzed by Student's *t* test or one way ANOVA followed by a Bonferroni multiple comparison test. Analysis was performed using GraphPad Prism 5 software and error bars represent SEM, unless otherwise stated.

4.4 RESULTS

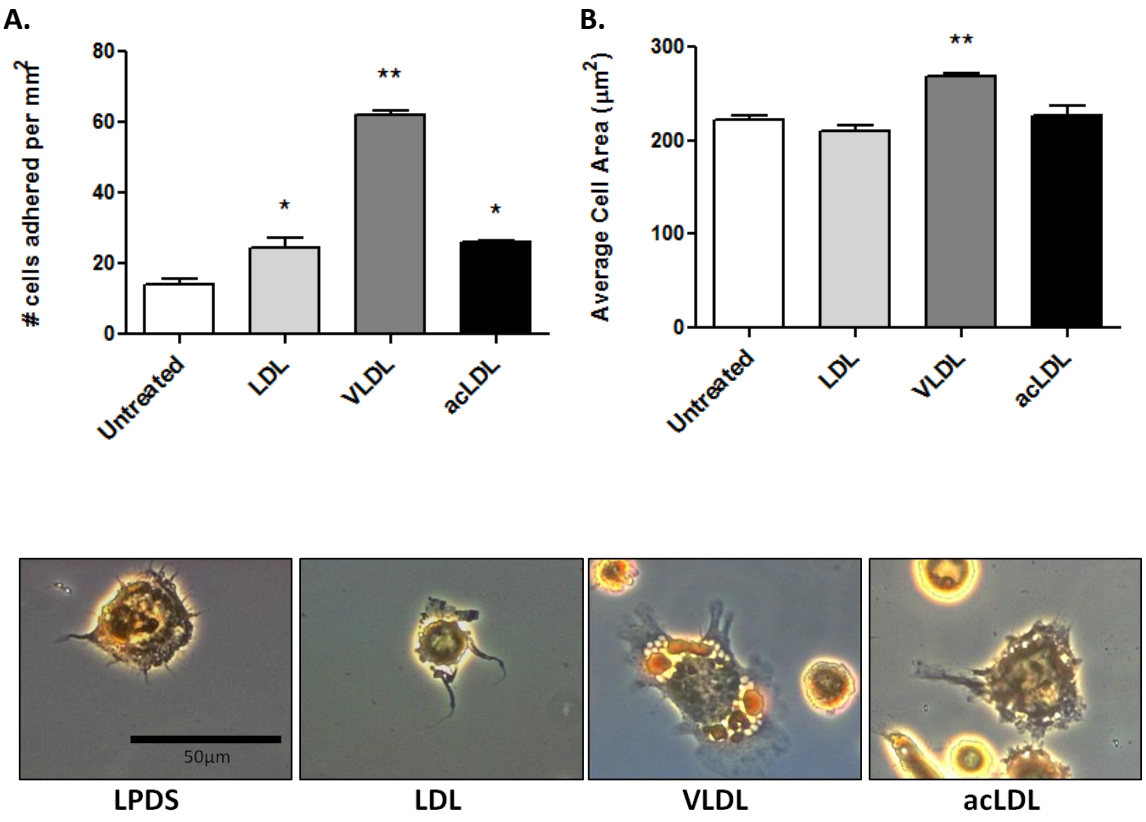
4.4.1 VLDL treatment induces THP-1 monocyte adhesion

In order to determine how VLDL affects monocytes, we sought to analyze whether it can induce adhesion of THP-1 monocytes onto a glass surface without confounding effects from other cell types. VLDL caused a large amount of cells to adhere compared to other lipoproteins and controls using a cell counting assay (Figure 4.1A). Untreated cells in lipoprotein deficient serum (LPDS) adhered to a small extent, but lacked lipid accumulation. Other lipoproteins also caused an increase in adhesion, but VLDL stimulation caused the most drastic increase, and this was correlated with increased cell area (Figure 4.1B). The adhered cells exhibited various phenotypes and sizes, but many cells in the VLDL group had large lipid droplets and macrophage-like characteristics.

Figure 4.1 THP-1 monocyte adhesion and cell area quantification after incubation with lipoproteins

Adhered cells were counted and quantified for cell area after THP-1 monocytes were stimulated with lipoproteins for 48 hours. Cells were lipid starved in LPDS for 24 hours prior to and during the incubation. (A) LDL and acLDL caused a doubling in adhesion onto glass compared to untreated controls, while VLDL treatment induced a large amount of cells to adhere (n=50 fields of view). (B) VLDL treatment resulted in significantly larger adhered cells compared to controls. N=500 cells per group. (*=$p<0.05$ compared to untreated, **=$p<0.05$ compared to all controls)

Figure 4.1


A.
cells adhered per mm2
80
60
40
20
0
**
*
*
Untreated
LDL
VLDL
acLDL
B.
Average Cell Area (µm2)
300
200
100
0
**
Untreated
LDL
VLDL
acLDL
50µm
LPDS
LDL
VLDL
acLDL

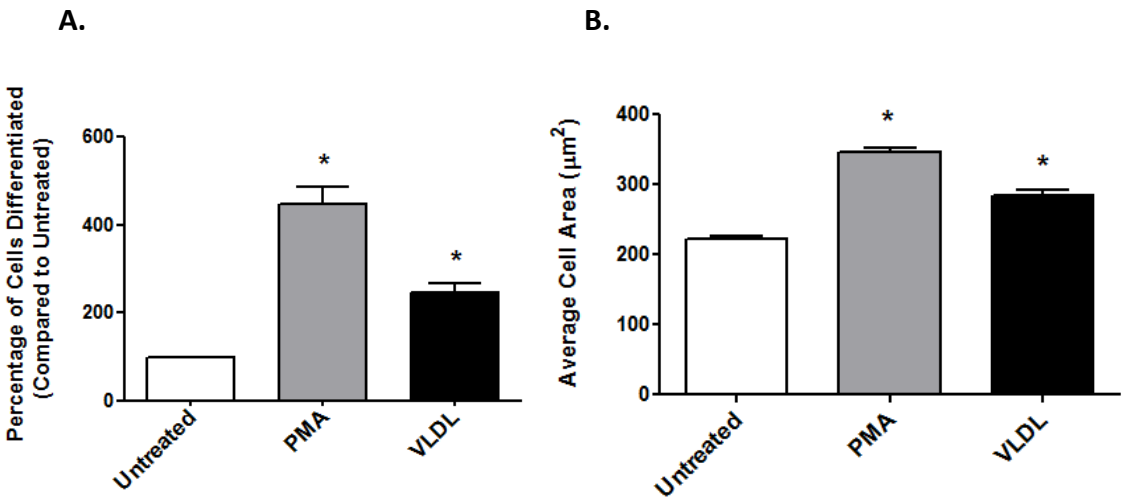
4.4.2 VLDL treatment induces THP-1 monocyte differentiation

Additionally, another experiment was performed, in which large (> 20 µm diameter) macrophage-like cells were scored, as to avoid inaccuracy due to small cells that have adhered non-specifically. We observed the same potent induction of differentiation by VLDL, this time with PMA as a positive control (Figure 4.2A). PMA treatment resulted in many large and differentiated cells, as expected. VLDL-treated cells also had an increase in cell area compared to untreated, but not quite as large as PMA (Figure 4.2B). This is evidence for VLDL inducing THP-1 monocytes not only to adhere, but also to exhibit macrophage-like characteristics and size.

Figure 4.2 THP-1 monocyte differentiation and cell area quantification after VLDL or PMA treatment

Large differentiated cells were counted and all adhered cells were quantified for cell area after THP-1 monocytes were stimulated with PMA or VLDL for 48 hours. Cells were lipid starved in LPDS media for 24 hours prior to and during the incubation. (A) Both PMA and VLDL induced cells to differentiate significantly compared to untreated, while the PMA group had twice as many cells differentiate than VLDL. (B) These treatments also resulted in larger cell areas of adhered cells compared to control, with the PMA group having the largest. (*=$p<0.05$ compared to the other 2 bars)

Figure 4.2


A.
B.
Percentage of Cells Differentiated (Compared to Untreated)
600
400
200
0
*
*
Untreated
PMA
VLDL
Average Cell Area (μm²)
400
300
200
100
0
*
*
Untreated
PMA
VLDL

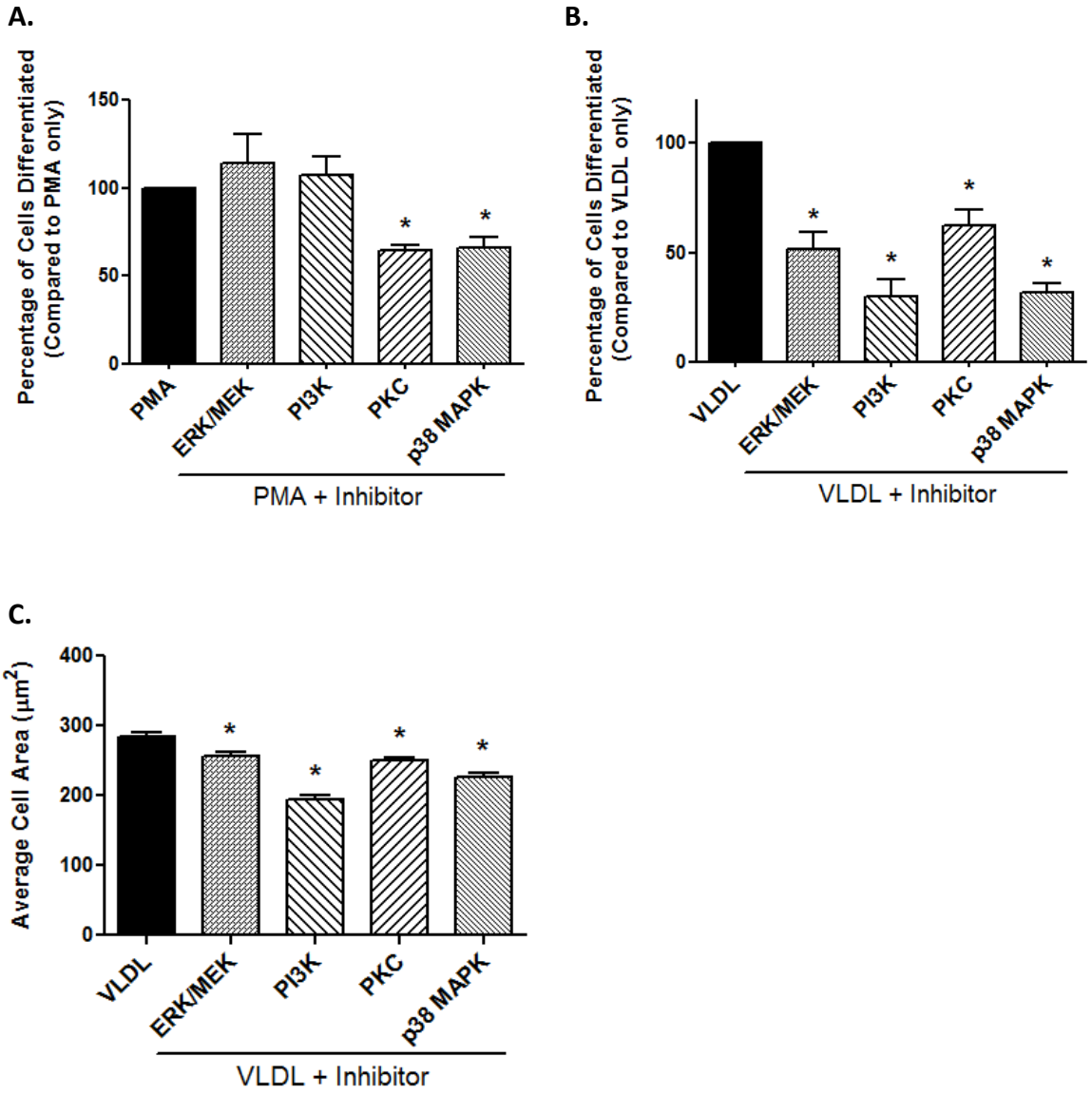
4.4.3 Inhibition of PI3 Kinase and p38 MAPK reduce VLDL-induced THP-1 monocyte differentiation

We aimed to gain insight into the cellular pathways elicited by VLDL during monocyte adhesion and differentiation. We performed experiments where PMA or VLDL was used to stimulate THP-1 monocytes along with several major cell signalling inhibitors. PI3K, p38 MAPK, PKC and ERK/MEK pathways have been cited in leukocyte growth, signalling, and inflammatory response (Foster et al., 2012;Langlois et al., 2010;O'Sullivan et al., 2009). As a control, we show that PMA differentiated THP-1 monocytes into macrophages, and this was reduced by blocking Protein Kinase C and p38 MAPK pathways (Figure 4.3A), which has been published (O'Sullivan et al., 2009). All of the inhibitors used reduced VLDL-induced THP-1 differentiation; however PI3 Kinase and p38 MAPK inhibitors had the greatest effect, inhibiting THP-1 monocyte differentiation by 75% (Figure 4.3B). Additionally, these 2 inhibitors had the greatest effect in reducing average cell area of VLDL-induced cells (Figure 4.3C). This indicates that VLDL-induced differentiation of THP-1 cells is heavily dependent on PI3K and p38 MAPK cell signalling pathways.

Figure 4.3 THP-1 monocyte VLDL- and PMA-induced differentiation in the presence of cell signaling inhibitors

Differentiated cells were counted and quantified for cell area after THP-1 monocytes were stimulated with VLDL or PMA for 48 hours with various cell signaling inhibitors. (A) PMA-induced differentiation was significantly reduced by PKC and p38 MAPK inhibitors but not by ERK/MEK and PI3K inhibitors. (B and C) While all inhibitors caused a reduction in VLDL-induced differentiated cells and total cell areas, PI3K and p38 MAPK inhibitors had the greatest effects. (*=$p<0.05$ compared to control)

Figure 4.3


A.
Percentage of Cells Differentiated (Compared to PMA only)
150
100
50
0
PMA
ERK/MEK
PI3K
PKC
p38 MAPK
PMA + Inhibitor
B.
Percentage of Cells Differentiated (Compared to VLDL only)
100
50
0
VLDL
ERK/MEK
PI3K
PKC
p38 MAPK
VLDL + Inhibitor
C.
Average Cell Area (μm²)
400
300
200
100
0
VLDL
ERK/MEK
PI3K
PKC
p38 MAPK
VLDL + Inhibitor

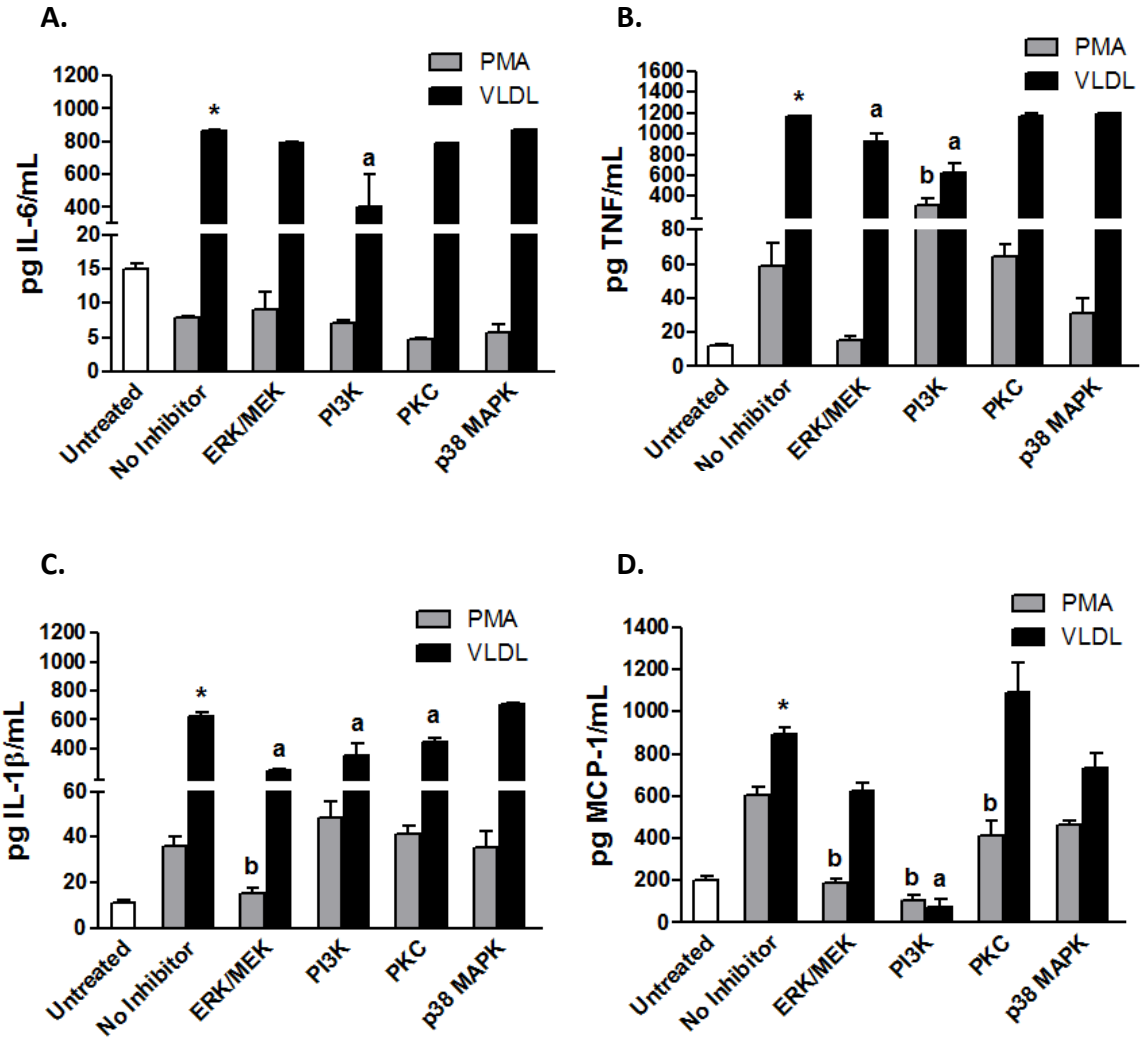
4.4.4 VLDL treatment of THP-1 monocytes induces cytokine secretion

In order to assess inflammatory effects of VLDL on THP-1 monocytes, we measured cytokine levels in the media after 48 hours of incubation with PMA, VLDL and cell signaling inhibitors. VLDL treatment caused a drastic increase in the secretion of IL-6 (Figure 4.4A), TNF (4.4B), IL-1β (4.4C) and MCP-1 (4.4D). PMA did not have the same effect, although it did increase MCP-1 to comparable levels. This data indicates that VLDL potentiated pro-inflammatory effects in THP-1 monocytes that culminated in cytokine secretion. Interestingly, the PI3K inhibitor reduced VLDL-induced production of all 4 cytokines (Figure 4.4), with the most potent effect occurring on MCP-1. Whether these cytokines are being secreted by the cells that have adhered and differentiated or by the cells left in suspension is unknown, however we hypothesize it is the former. Evidence for this is the fact that PI3K inhibition reduced cytokine production and this correlates with our previous data of PI3K inhibition reducing differentiation by VLDL. However, p38 MAPK inhibition did not reduce cytokine production, while it did reduce differentiation, providing some evidence against this. Interestingly, the ERK/MEK, PI3K and PKC inhibitors reduced PMA-induced levels of MCP-1 (Figure 4.4D), while the PI3K inhibitor actually increased PMA-induced TNF in the media (4.4B). The ERK/MEK inhibitor reduced VLDL-induced secretion of TNF (4.4B) and IL-1β (4.4C) as well. While the exact molecular cascades and cytokine feedback loops are not fully known, VLDL is a potent inducer of cytokine production in THP-1 monocytes and this is dependent on PI3K signaling.

Figure 4.4 ELISA of media from THP-1 monocytes treated with PMA or VLDL

THP-1 monocytes were stimulated with VLDL or PMA for 48 hours with various cell signaling inhibitors. The media was analyzed by ELISA for production of (A) IL-6, (B) TNF, (C) IL-1β and (D) MCP-1. VLDL treatment induced all 4 cytokines compared to untreated or PMA treated cells, which exhibited very low levels except for MCP-1. Furthermore, the PI3K inhibitor significantly reduced levels of all 4 cytokines when used in combination with VLDL. The ERK/MEK inhibitor also reduced levels of TNF and IL-1β when added to VLDL, while the PKC inhibitor lowered VLDL-induced IL-1β production as well. The PI3K inhibitor potentiated PMA-induced TNF production while significantly lowering PMA-induced MCP-1 levels. N=3 (*=$p<0.05$ compared to untreated or PMA only, a=$p<0.05$ compared to VLDL only, b=$p<0.05$ compared to PMA only)

Figure 4.4


A.
pg IL-6/mL
PMA
VLDL
Untreated
No Inhibitor
ERK/MEK
PI3K
PKC
p38 MAPK
B.
pg TNF/mL
C.
pg IL-1β/mL
D.
pg MCP-1/mL

4.4.5 PI3 Kinase inhibition abolishes VLDL-induced THP-1 differentiation

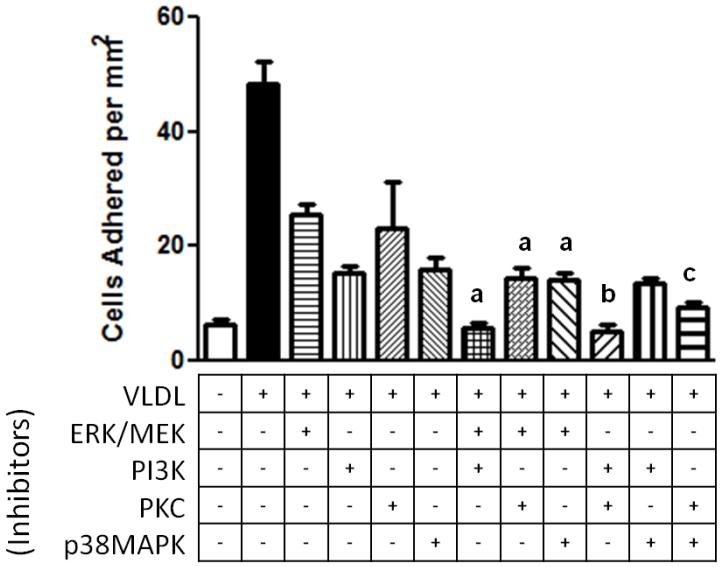
VLDL-induced differentiation of THP-1 monocytes differs from PMA-induced differentiation in that the PI3 kinase pathway appears to be playing a unique role. This data gives insight into what cellular pathways were elicited by VLDL during THP-1 differentiation, and we used these inhibitors in combination in order to observe any unique or additive effects. Inhibition of PI3 kinase in combination with ERK/MEK or PKC inhibitors completely eliminated VLDL-induced differentiation (Figure 4.5A) and significantly reduced cell areas (Figure 4.5B). This implies that ERK/MEK and PKC signalling acted separately from PI3K signalling during VLDL-induced differentiation. Interestingly, PI3K and p38MAPK inhibitors did not have an additive effect when combined. These results, in addition to previous data using PMA and cell signalling inhibitors, implicates PI3 kinase signalling as a unique and necessary axis for VLDL-induced THP-1 monocyte differentiation. How VLDL invokes the PI3K cascade remains to be seen, but we aim to see what proteins are induced by VLDL in addition to its effects on cytokines.

Figure 4.5 VLDL-induced THP-1 differentiation with combinations of cell signaling inhibitors

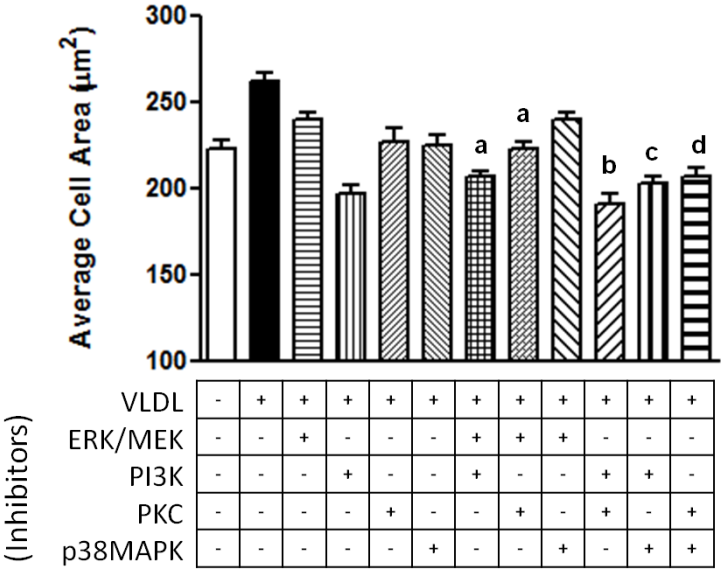
Differentiated cells were counted and quantified after THP-1 monocytes were stimulated with VLDL and various cell signaling inhibitors in combination. VLDL caused significant differentiation and this was reduced by PI3K and p38 MAPK inhibitors. (A) Treatment using the PI3K inhibitor combined with the ERK/MEK or PKC inhibitor completely eliminated VLDL-induced differentiation. (B) The PI3K inhibitor was also potent in reducing cell area when used alone or in combination. (All inhibitor groups are significantly different ($p<0.05$) compared to VLDL only, a=$p<0.05$ compared to VLDL+ERK/MEK, b=$p<0.05$ compared to VLDL+PKC, c=$p<0.05$ compared to VLDL+p38MAPK, d=$p<0.05$ compared to VLDL+PKC and VLDL+p38MAPK)

Figure 4.5

A.


Cells Adhered per mm2
60
40
20
0
a
a
a
b
c
(Inhibitors)
VLDL - + + + + + + + + + + +
ERK/MEK - - + - - - + + + - - -
PI3K - - - + - - + - - + + -
PKC - - - - + - - + - + - +
p38MAPK - - - - - + - - + - + +

B.


Average Cell Area (μm2)
300
250
200
150
100
a
a
b
c
d
(Inhibitors)
VLDL - + + + + + + + + + + +
ERK/MEK - - + - - - + + + - - -
PI3K - - - + - - + - - + + -
PKC - - - - + - - + - + - +
p38MAPK - - - - - + - - + - + +

4.4.6 VLDL treatment up-regulates LRP-1, NEU1, GRP-78 and MPG-1 protein levels in adhered THP-1 cells

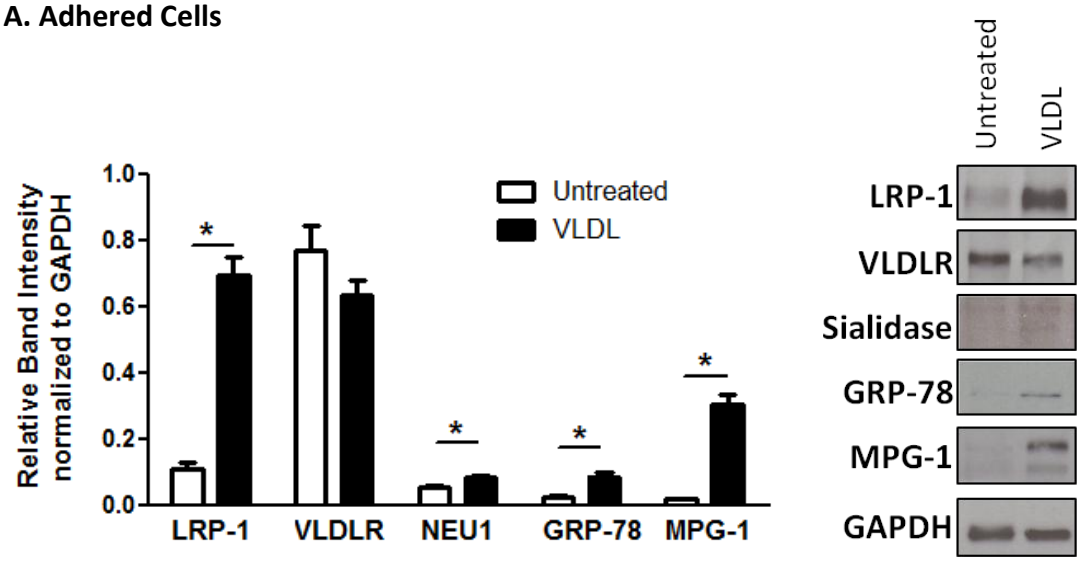
We analyzed protein expression by western blotting of THP-1 cells (that have adhered from VLDL-treatment or that remained in suspension) to gain more insight into VLDL's effects. LRP-1 protein levels were significantly up-regulated by VLDL treatment in adhered THP-1 monocytes, while VLDLR protein levels remained unchanged (Figure 4.6A). This could aid in explaining the potent pro-atherogenic effects of VLDL, as one of its key receptors actually gets over-expressed after treatment with the ligand. LRP-1 has important functions in immune response and growth factor recognition in macrophages and other cell types, and it is quite plausible that VLDL is stimulating and propagating these responses via this receptor. MPG-1 and GRP-78 protein levels were also up-regulated by VLDL treatment in adhered cells, indicating that these cells are differentiating and are exhibiting ER stress, respectively (4.6A). NEU1 sialidase protein levels also increased upon VLDL stimulation and this further implicates this enzyme in monocyte differentiation (4.6A). These trends were not evident in suspension cells, and NEU1 and MPG-1 levels were not detectable (Figure 4.6B).

Figure 4.6 Western blotting of VLDL-treated adhered and suspension THP-1 cells

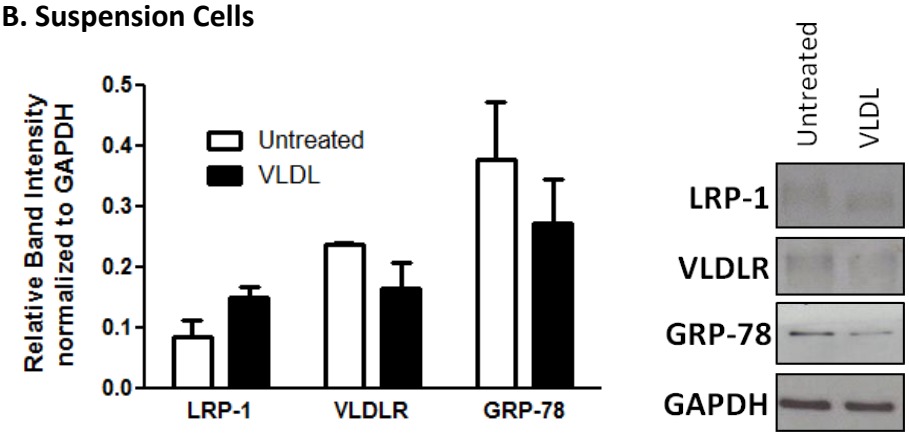
THP-1 monocytes were incubated with VLDL for 48 hours. Adhered cells were harvested and analyzed by western blotting and densitometry relative to GAPDH. (A) Adhered cells that were treated with VLDL had significantly higher protein levels of LRP-1 (85 kDa fragment), NEU1, GRP-78 and MPG-1 protein compared to untreated adhered control cells, while having no difference in VLDLR expression. (B) Blotting of the same samples from the cells that remained in suspension showed no difference in expression of LRP-1, VLDLR and GRP-78, while having undetectable levels of MPG-1 and NEU1 altogether. Blots are representative of n=3. (*=$p<0.05$)

Figure 4.6

A. Adhered Cells



B. Suspension Cells



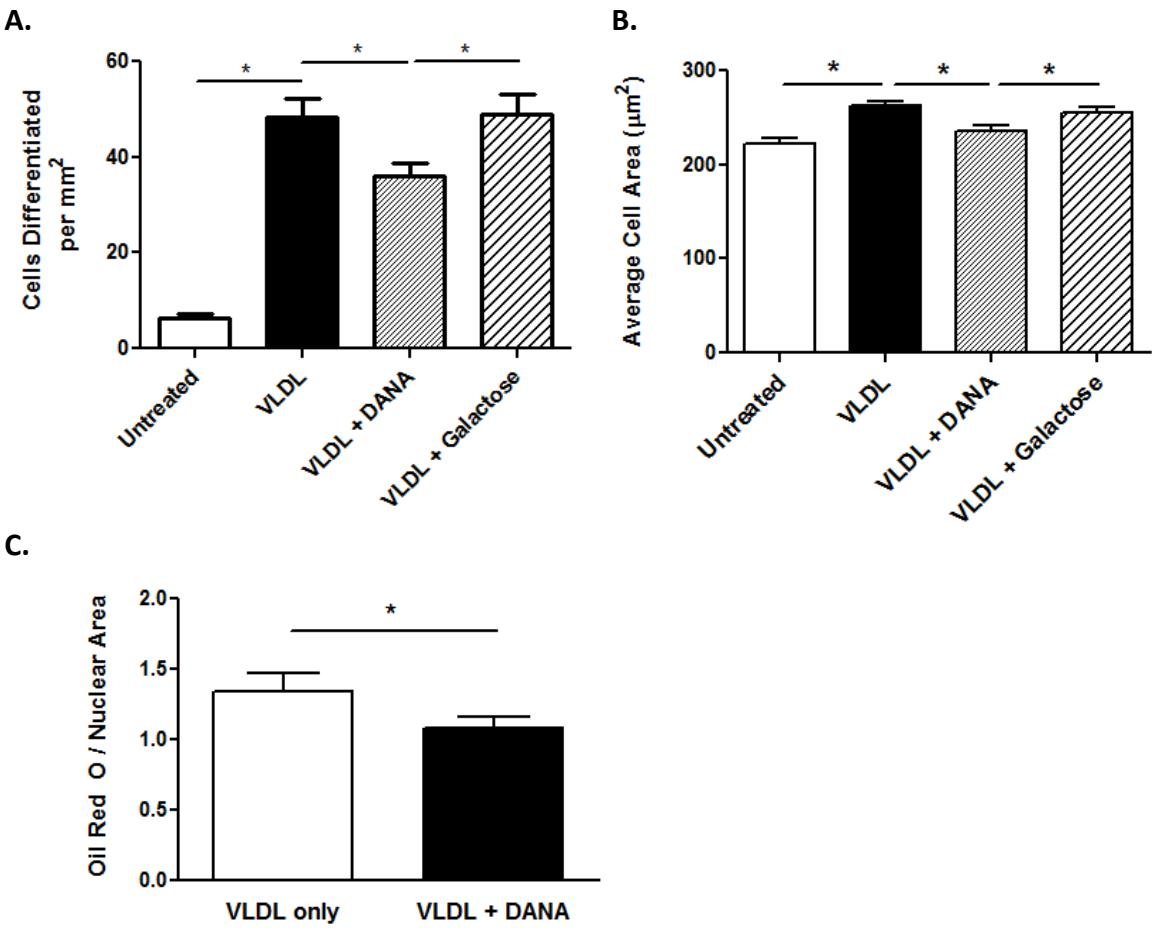
4.4.7 Sialidase inhibition decreases VLDL-induced THP1 monocyte differentiation and lipid content

Due the fact that NEU1 gets up-regulated as monocytes differentiate, we analyzed whether sialidase inhibition using DANA affects VLDL-induced THP-1 differentiation. DANA decreased VLDL-induced differentiation of THP-1 monocytes (Figure 4.7A), as well as their cell areas (Figure 4.7B), while the monosaccharide galactose did not cause significant changes and was used as a control to rule out any non-sialidase specific effects on the cells. This effect of sialidase inhibition was accompanied by a reduction in lipid accumulation of adhered cells treated with DANA and VLDL vs VLDL alone (Figure 4.7C). This data not only confirms that VLDL is a potent inducer of THP-1 monocyte differentiation, but that sialidase inhibition can reduce this process, further involving this enzyme as a player in these cellular events.

Figure 4.7 VLDL-induced differentiation and Oil Red O staining of THP-1 monocytes treated with the sialidase inhibitor DANA

Differentiated cells were counted and quantified for cell area after THP-1 monocytes were stimulated with VLDL. (A) Co-treatment with DANA significantly reduced the number of differentiated cells while the monosaccharide galactose had no effect. (B) Results were similar when cell area was assessed, as DANA caused a significant reduction in cell size compared to VLDL alone, while galactose had no effect. (C) DANA also significantly reduced lipid accumulation of VLDL-treated adhered cells as measured by ORO staining, indicative of reduced foam cell formation. Approximately 400 cells were quantified for each treatment. (*=$p<0.05$)

Figure 4.7


A.
Cells Differentiated per mm2
60
40
20
0
*
*
*
Untreated
VLDL
VLDL + DANA
VLDL + Galactose
B.
Average Cell Area (µm2)
300
200
100
0
*
*
*
Untreated
VLDL
VLDL + DANA
VLDL + Galactose
C.
Oil Red O / Nuclear Area
2.0
1.5
1.0
0.5
0.0
*
VLDL only
VLDL + DANA

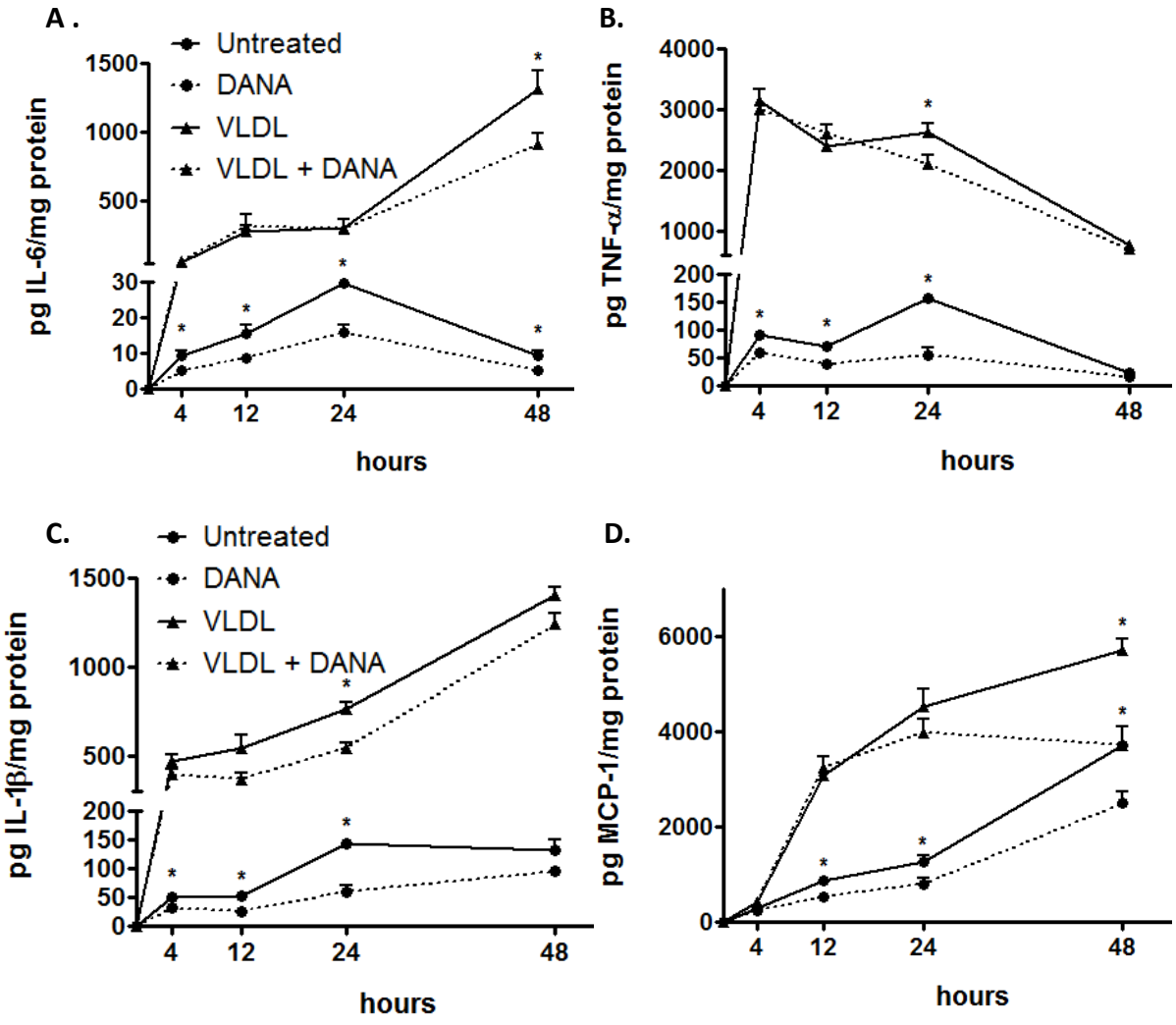
4.4.8 Sialidase inhibition decreases VLDL-induced cytokine production in PMA-differentiated macrophages

After analyzing the effects of VLDL on monocyte adhesion, differentiation and inflammation, we sought to further probe into the effects of VLDL on mature macrophages. THP-1 cells were differentiated with PMA and incubated with VLDL for 48 hours, causing a massive secretion of IL-6 (Figure 4.8A), TNF (4.8B), IL-1β (4.8C) and MCP-1 (4.8D) into the media. DANA treatment decreased VLDL-induced secretion of all four cytokines by THP-1 macrophages (Figure 4.8). The most drastic reductions occurred in MCP-1 and IL-6 after 48 hours of VLDL and DANA co-incubation. MCP-1 baseline levels were relatively high, and this is most likely attributed to residual activation from when the monocytes were differentiated into macrophages using PMA (Figure 4.8D). DANA actually reduced VLDL-induced levels of MCP-1 down to baseline by 48 hours, indicating a potent anti-inflammatory effect (Figure 4.8D). DANA even reduced the already low baseline levels of all four cytokines (4.8). Overall, we observed that VLDL treatment can lead to activation of THP-1 macrophages, and that sialidase inhibition has an ameliorative effect.

Figure 4.8 ELISA of VLDL-treated mature macrophages using the sialidase inhibitor DANA

PMA-differentiated THP-1 macrophages were treated with VLDL for 48 hours resulting in a drastic increase in secretion of (A) IL-6, (B) TNF, (C) IL1-β and (D) MCP-1 as measured by ELISA of cell media. Sialidase inhibition using DANA dampened these pro-inflammatory effects of VLDL and reduced cytokine accumulation in the media by 24 and 48 hours. MCP-1 levels were reduced to untreated levels when DANA was added to VLDL. DANA also significantly reduced baseline levels of cytokines (n=3). (*=$p<0.05$)

Figure 4.8


A .
Untreated
DANA
VLDL
VLDL + DANA
pg IL-6/mg protein
1500
1000
500
30
20
10
0
4
12
24
48
hours
B.
pg TNF-α/mg protein
4000
3000
2000
1000
200
150
100
50
0
4
12
24
48
hours
C.
Untreated
DANA
VLDL
VLDL + DANA
pg IL-1β/mg protein
1500
1000
500
200
150
100
50
0
4
12
24
48
hours
D.
pg MCP-1/mg protein
6000
4000
2000
0
4
12
24
48
hours

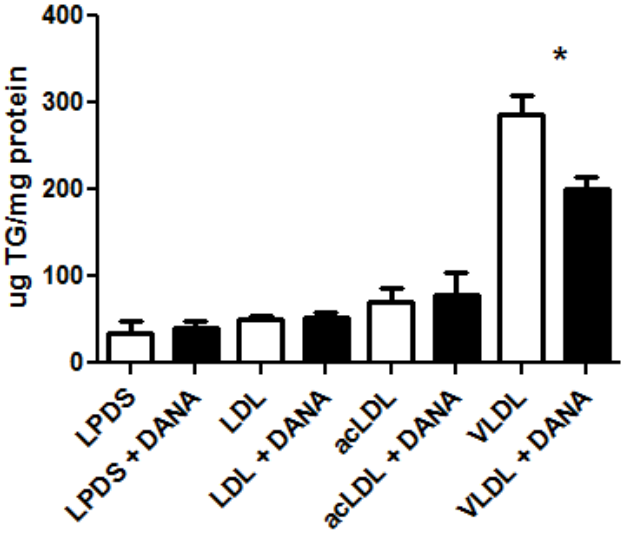
4.4.9 Sialidase inhibition reduces triglyceride accumulation from VLDL in mature THP-1 macrophages

In addition to its inflammatory effects, VLDL treatment of mature macrophages induced significant triglyceride (TG) accumulation in THP-1 macrophages, as expected, with only minor accumulations from other cholesterol-rich lipoproteins (Figure 4.9). Interestingly, DANA decreased TG levels significantly in the VLDL-treated group, indicating that VLDL uptake or accumulation from VLDL lipolysis was reduced. This could explain the reductions in inflammatory response observed above (Figure 4.8), as the reductions in TG accumulations could correlate with lower activation of macrophages and amelioration of foam cell formation. Inhibition of sialidase has potent atheroprotective effects by decreasing VLDL-stimulated cytokine secretion and accumulation of VLDL derived lipids in THP-1 macrophages, in addition to its effects on THP-1 monocyte differentiation.

Figure 4.9 Triglyceride assay of lipoprotein THP-1 macrophages

PMA-differentiated THP-1 macrophages were incubated with lipoproteins (with and without DANA) for 48 hours. Lipid extraction from cells was performed and TG levels were assayed using an enzymatic kit. VLDL caused a massive increase in TG levels and DANA significantly reduced this triglyceride accumulation in macrophages (n=3). *=$p<0.05$

Figure 4.9


400
300
200
100
0
ug TG/mg protein
*
LPDS
LPDS + DANA
LDL
LDL + DANA
acLDL
acLDL + DANA
VLDL
VLDL + DANA

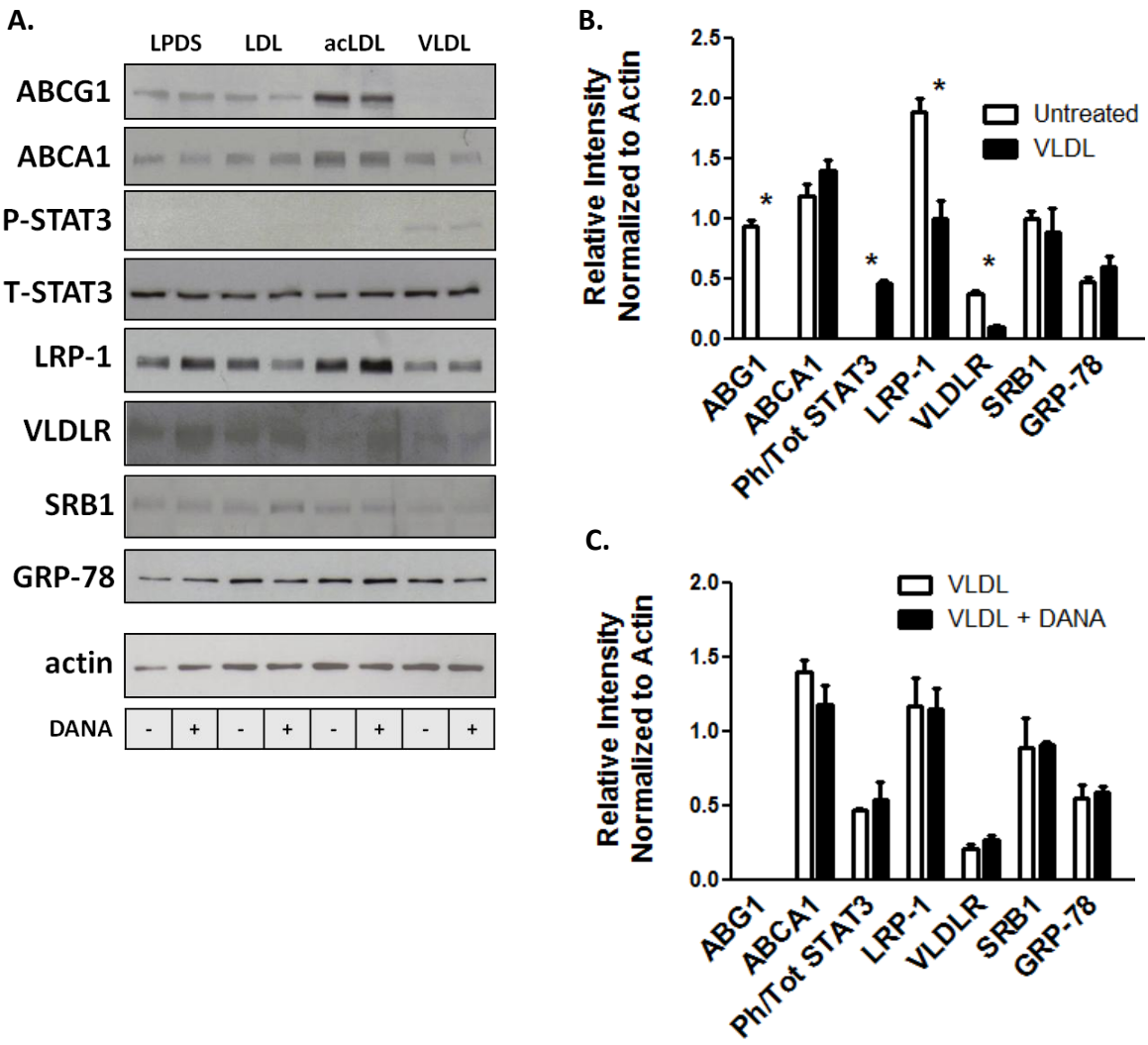
4.4.10 VLDL treatment abolishes ABCG1 expression and phosphorylates STAT3 in THP-1 Macrophages

We sought to analyze how VLDL causes lipid accumulation and propagation of a foam cell phenotype in THP-1 macrophages by analyzing expression of several proteins involved in lipoprotein metabolism and macrophage activation. While control acLDL treatment caused increased expression of ABCG1 and ABCA1, VLDL did not induce expression of ABCA1 and actually abolished protein expression of ABCG1 (Figure 4.10A and B). This is striking, and lack of ABCG1 would certainly contribute to lipid accumulation and foam cell formation from VLDL-induced lipid accumulation. Additionally, VLDL also increased phosphorylation of STAT3, which could be contributing to inflammation and proliferation (Figure 4.10A and B). STAT3 is most likely activated downstream of IL6 (Tacke et al., 2011;Zhang et al., 2013a) and STAT3 has also been linked to PI3K activation (Hart et al., 2011). Eliminating ABCG1 protein would reduce cholesterol efflux and activation of STAT3 would promote proliferation and inflammation, all of which would contribute to foam cell formation and atherosclerosis. Furthermore, VLDL treatment lowered protein expression of LRP-1 and VLDLR, both of which are receptors for VLDL, while SRB-1 and GRP-78 levels remained unchanged (Figure 4.10B). Additionally, DANA had no significant effect on expression of these proteins when comparing VLDL and VLDL plus DANA (Figure 4.10C).

Figure 4.10 Western blotting of lipoprotein treated PMA-differentiated THP-1 macrophages

PMA-differentiated THP-1 macrophages were treated with lipoproteins for 48 hours (with and without DANA) and analyzed by western blotting and densitometry analysis. (A) While acLDL caused upregulation of ABC efflux transporters, VLDL abolished protein expression of ABCG1. (B) VLDL treatment also increased protein levels of phosphorylated STAT3, while decreasing LRP-1 and VLDLR compared to untreated. (C) DANA had no significant effect on the expression of these proteins when added to the VLDL treatment. Representative blots of multiple experiments are shown, densitometry analysis was performed on separate gels containing n=3 for untreated, VLDL and VLDL + DANA). *=$p<0.05$

Figure 4.10


A.
LPDS
LDL
acLDL
VLDL
ABCG1
ABCA1
P-STAT3
T-STAT3
LRP-1
VLDLR
SRB1
GRP-78
actin
DANA
- + - + - + - +
B.
Relative Intensity Normalized to Actin
2.5
2.0
1.5
1.0
0.5
0.0
Untreated
VLDL
*
ABG1
ABCA1
Ph/Tot STAT3
LRP-1
VLDLR
SRB1
GRP-78
C.
Relative Intensity Normalized to Actin
2.0
1.5
1.0
0.5
0.0
VLDL
VLDL + DANA
ABG1
ABCA1
Ph/Tot STAT3
LRP-1
VLDLR
SRB1
GRP-78

4.5 DISCUSSION

We have shown that VLDL induces a monocyte to foam cell transition and has multifaceted effects on lipoprotein metabolism and inflammation in the monocyte/macrophage. It can induce lipid accumulation and evoke cell signalling cascades, which increase cytokine production and progression of atherosclerosis. Importantly, VLDL is involved in monocyte differentiation associated with early atherosclerosis, and also with pro-inflammatory and dyslipidemic mechanisms of later atherosclerosis. We have found that sialidase appears to be pivotal to these events, with its inhibition having profound anti-inflammatory effects, including a reduction of differentiation, lowered TG accumulation and decreased cytokine secretion.

The exact pathway by which VLDL causes THP-1 monocyte adhesion and differentiation is unclear and we aimed to delineate this further. In their endothelial cell experiments, Kawakami et al postulate that the pro-adhesive effects of VLDL are caused solely by Apo-CIII, and that this activates the Protein Kinase C (PKC) pathway and Nuclear factor kappa B (NFκB), although this has yet to be confirmed (Kawakami et al., 2006b;Kawakami et al., 2007). LRP-1, which is expressed in macrophages and can bind VLDL, amongst other ligands, has also been linked to growth factor response, proliferation, and cellular signalling cascades (Lillis et al., 2005;Lillis et al., 2008;May et al., 2005). Furthermore, other groups have shown that lipoprotein lipase is necessary for VLDL stimulated lipid accumulation and subsequent inflammatory response in THP-1 macrophages (Jinno et al., 2011;Palmer et al., 2005a). Lastly, the VLDL receptor (which

is abundant in THP1 cells) is not downregulated by sterol loading, and its expression is atherogenic (Eck et al., 2005;Kosaka et al., 2001;Sakthivel et al., 2001). Therefore, these proteins and interactions represent potential contributions to VLDL-induced THP-1 differentiation and subsequent cellular effects.

VLDL-induced THP-1 adhesion and differentiation is a complex process and seems to involve several major cellular pathways. Kawakami et al state that Apo-CIII activates both monocytic and endothelial components when an IL-1β pre-treatment of endothelial cells is used to prime them for adhesion, thus making it difficult to assess the direct role of VLDL/Apo-CIII on monocytes alone (Kawakami et al., 2006b;Kawakami et al., 2007). IL-1β up-regulates many endothelial cell genes and promotes a pro-inflammatory environment which stimulates cells to adhere (Puhlmann et al., 2005;Williams et al., 2008). Thus, we sought to study leukocytes in isolation, and perform differentiation assays onto glass, and not endothelial cells or cells of the vasculature. This enabled us to dissect monocyte/macrophage specific mechanisms without interference from other factors and secretions or interactions from other cell types. Specifically, this allows for testing of whether VLDL has direct effects on monocyte adhesion and differentiation, and we conclude that it does by increasing the number of cells adhered, and also their size and complexity, which are indicative of differentiation. While our inhibitor data invokes mainly the PI3 Kinase intracellular pathway in VLDL-induced monocyte differentiation, we also see reductions in differentiation from p38MAPK, MEK/ERK and PKC inhibitors. This indicates that VLDL or

its components are stimulating adhesion, differentiation and cell signalling involved in proliferation, inflammation and growth factor response (O'Sullivan et al., 2009;Wegiel et al., 2008). More evidence for these effects is that VLDL treatment caused THP-1 monocytes to secrete large amounts of cytokines into the media. It induced production of IL-6, TNF, IL-1β and MCP-1. Interestingly, incubation of macrophages with IL-1β and TNF has been shown to increase their VLDL-derived lipid content, indicative of a pro-atherogenic cycle and a direct correlation between VLDL uptake and inflammation (Persson et al., 2008b). Cytokine production was also sensitive to PI3K inhibition, further indicating the importance of this signalling cascade axis in VLDL-induced monocyte to foam cell transition. PI3K and its related pathways protein kinase B (AKT), mammalian target of rapamycin (mTOR) and NFκB, all have been linked to leukocyte migration, growth, survival and inflammation (Foster et al., 2012;Hoesel and Schmid, 2013). Clearly, VLDL is inducing drastic changes in the monocyte including adhesion, differentiation and inflammation using these signalling pathways. While the mechanism by which VLDL initially triggers these effects is still not known, we show that the end result is a potent and multi-faceted pro-atherogenic effect.

Moreover, western blotting of VLDL-treated cells that adhered shows an up-regulation of LRP-1, GRP-78, MPG-1 and NEU1, while cells that remained in suspension had no changes in levels of these proteins. This data further supports that the adhered cells are differentiating and are eliciting an immune response. MPG-1 is a marker of differentiated macrophages, and GRP-78 is up-regulated during the unfolded protein

response and ER stress (Dickhout et al., 2011;Du et al., 2011;Isa et al., 2011;Spilsbury et al., 1995). LRP-1 has been linked to activation of the PI3K pathway in other cell types (Fuentealba et al., 2009;Zhou et al., 2009), and we hypothesize that VLDL binding to LRP-1 is having similar effects in THP-1 cells. Furthermore, NEU1 sialidase is important in macrophage inflammatory response and it translocates to the cell surface upon differentiation of THP-1 monocytes (Liang et al., 2006;Stamatos et al., 2005). We thus wanted to study whether inhibiting sialidase activity would dampen the pro-atherogenic effects of VLDL. Inhibition of sialidase with DANA reduced THP-1 cell differentiation and size, while also lowering the amount of lipid accumulation in differentiated cells. This data directly implicates sialidase a key player in the VLDL-induced monocyte to foam cell transition.

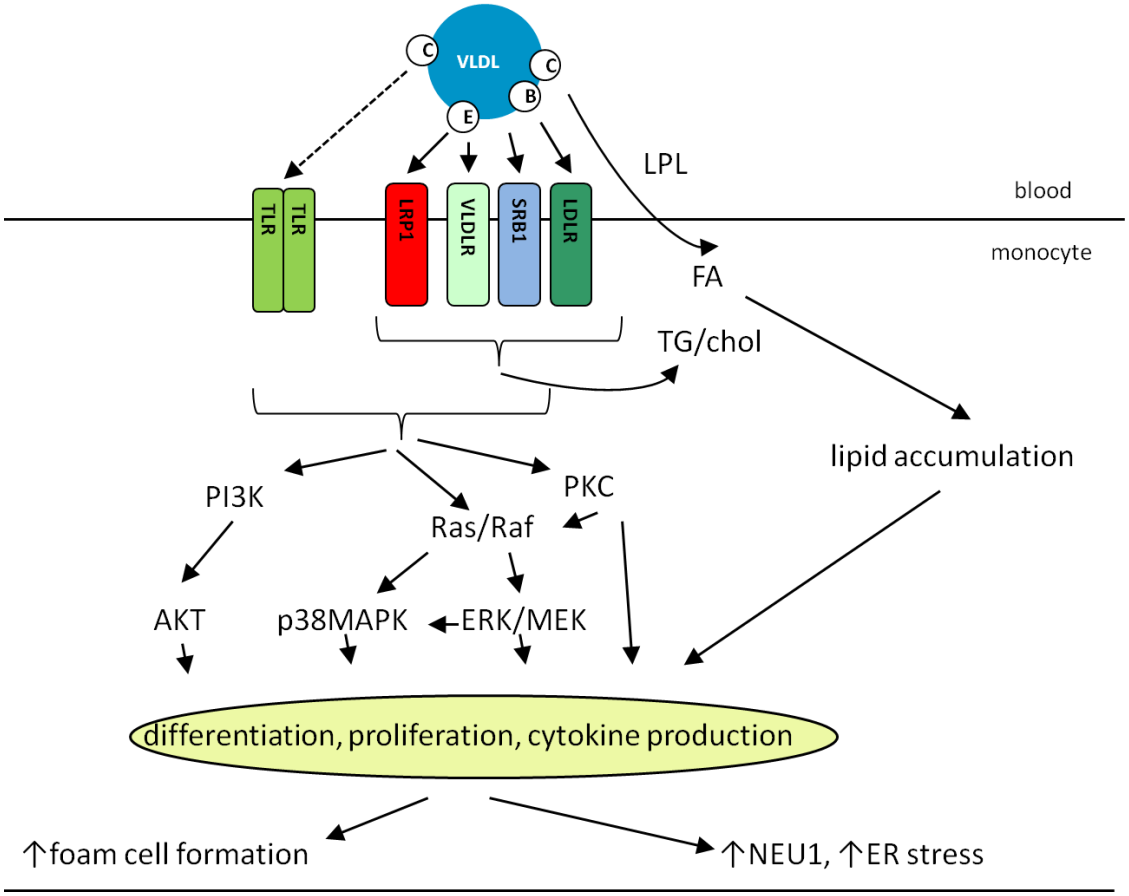
Sialidase could be playing a role in VLDL-induced differentiation and inflammation by altering the interaction of lipoproteins with lipoprotein receptors at the cell surface. Modifying this interaction could result in direct effects on cell signalling through several plausible pathways as discussed above, or as an artefact of altered lipid accumulation downstream of reduced VLDL uptake. Not only could these pathways be involved in the reduced differentiation observed with sialidase inhibition, but they are also playing a role in reducing VLDL-induced triglyceride accumulation and cytokine production. It is also extremely interesting that Apo-CIII and CII are sialylated and that the number of sialic acid molecules have striking impacts on its function and atherogenicity (Jong et al., 1999;Mann et al., 1997;Melajarvi et al., 1996;Millar, 2001). We postulate that

potentially altering sialidase activity with DANA could alter the sialylation of Apo-C on VLDL, with functional consequences, resulting in decreased lipolysis (Stoline et al., 1985). In fact, several studies indicate that LPL is necessary for VLDL-induced foam cell formation and inflammation in human and mouse macrophages (Milosavljevic et al., 2003;Saraswathi and Hasty, 2006) and that VLDL can induce LPL expression (Pou et al., 2011). Reduction of LPL activity by deletion of Apo-CII on VLDL or the drug Orlistat resulted in reduction of lipid accumulation and amelioration of cellular signalling activation of ERK and p38 MAPK cascades (Milosavljevic et al., 2003;Saraswathi and Hasty, 2006). We hypothesize that similar pathways are being elicited if the sialylation of Apo-CII was altered and thus slows VLDL-induced foam cell formation with DANA. Furthermore, Apo-B (also found on VLDL), which is used for receptor-mediated endocytosis by LRP-1, is also sialylated (Millar, 2001). It appears that sialidase inhibition could be affecting these interactions of VLDL with LRP-1, which could lead to reduced lipid uptake and/or signalling by LRP-1.

In addition to LRP1, VLDL can also be internalized by LDLR, apoER2/LRP8 and VLDLR (Herijgers et al., 2000;Lillis et al., 2008;May et al., 2005;Nofer et al., 2010;Takahashi et al., 2004), but these receptors do not have nearly as many roles in cellular signalling. Interestingly, the LDLR family of receptors contain homologous regions that are glycosylated and sialylated, and we hypothesize that DANA could be resulting in hypersialylation of these receptors (Go and Mani, 2012;Lillis et al., 2008;Millar, 2001;Yang et al., 2012). PI3 kinase inhibition stopped VLDL-induced

differentiation but not PMA-induced differentiation, linking this cellular signalling pathway to a unique role in activation by VLDL. VLDL also caused a drastic increase in LRP-1 protein levels as the cells are differentiating, and this could lead to continued uptake and propagation of inflammation. As mentioned previously, LRP-1 has important functions in immune response and growth factor recognition in macrophages and other cell types, and it is quite plausible that VLDL is stimulating and propagating these responses (Langlois et al., 2010;Lillis et al., 2008;Mantuano et al., 2010;Perrot et al., 2012). The contributions of receptor mediated endocytosis, LPL-mediated lipolysis and cell signalling stimulation remain to be fully elucidated in VLDL-induced differentiation. Nevertheless, VLDL treatment upregulates sialidase in differentiated cells and inhibition using DANA reduces this differentiation and the accompanying TG accumulation. A summary of the effects of VLDL on monocytes as discussed above is shown in Figure 4.11.

Figure 4.11 Overview of VLDL-induced effects on monocytes

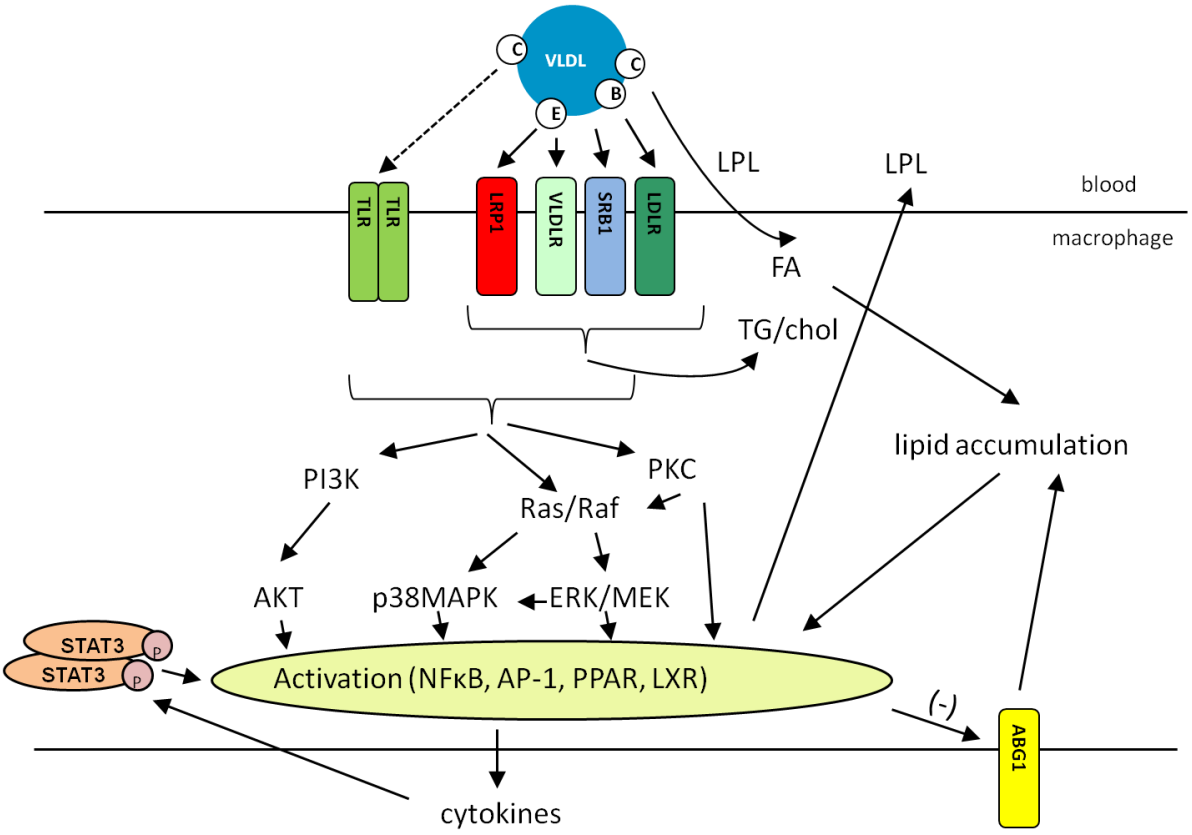


VLDL appears to be interacting with the cells in a similar manner even in PMA-differentiated macrophages and is propagating foam cell formation. Since VLDL has been shown to elicit cytokine secretion in mature THP-1 macrophages as well, we wanted to test whether sialidase inhibition can affect PMA-differentiated macrophage activation (Jinno et al., 2011;O'Sullivan et al., 2009;Pou et al., 2011). Sialidase inhibition had anti-inflammatory effects in mature macrophages, as DANA treatment reduced macrophage production of IL6, MCP-1, IL1-β and TNF upon VLDL stimulation. The most prominent reductions were observed in MCP-1, where sialidase inhibition brings VLDL-treated levels down to those of untreated cells. This cytokine reduction was coupled with lower TG content of macrophages. This could be quite relevant in an atherosclerotic plaque and would result in reduced inflammation, macrophage infiltration and foam cell formation (Daugherty et al., 2005;Moore et al., 2013). Cytokines can influence lipid accumulation in macrophages as well, further indicating that DANA can reduce overall activation in the macrophage (Persson et al., 2008b).

In order to examine the mechanisms behind the unique pro-atherogenic effects of VLDL in mature macrophages, we analyzed protein expression of ABC cholesterol efflux transporters and STAT3 (signal transducer and activator of transcription 3), as these proteins are involved in lipid homeostasis and macrophage proliferation, respectively (Yvan-Charvet et al., 2007;Zhang et al., 2013a). In addition to its published roles in lipid accumulation and cytokine stimulation (Bojic et al., 2012), we have found that VLDL abolishes ABCG1 protein expression and induces phosphorylation of STAT3 in

macrophages. STAT3 can be activated indirectly by IL-6 (Tacke et al., 2011;Zhang et al., 2013a) and PI3K activation can play a role in STAT3 phosphorylation as well (Hart et al., 2011). The reduction of ABCG1 protein would reduce cholesterol efflux and activation of STAT3 would promote proliferation and inflammation and potentially lipid uptake, all of which would contribute to foam cell formation and atherosclerosis. Furthermore, we show that VLDL treatment causes downregulation of LRP-1 and VLDLR, indicating that uptake of VLDL is potentially occurring through these receptors. Interestingly, the DANA treatment had no impact on VLDL-induced expression of these proteins, showing that sialidase inhibition is having anti-atherogenic effects independent of gross changes in expression of the proteins analyzed. We hypothesize that DANA is having similar effects as it did in monocyte differentiation, by modulating the sialic acid dependent functional interactions of VLDL and its receptors or LPL. A summary of the effects of VLDL on macrophages is shown in Figure 4.12.

Figure 4.11 Overview of VLDL-induced effects on macrophages



As we have shown, VLDL is a potent inflammatory moiety in events associated with early atherosclerosis; however, it also promotes dyslipidemia and inflammation in mature PMA-differentiated THP-1 macrophages. It is intriguing, that while not traditionally classified as a modified lipoprotein, we have uncovered direct and diverse atherogenic effects of VLDL. We also have evidence that sialidase plays a pivotal role in this VLDL-induced monocyte to foam cell transition. VLDL appears to be unique from acLDL, oxLDL and other 'classic' pro-atherogenic lipoproteins, by having multiple effects on monoctye differentiation and foam cell formation. VLDL has potent effects on monoctye differentiation involving PI3K signalling, LRP-1 and NEU1 sialidase, and it can also induce subsequent lipid uptake and inflammatory response in macrophages through effects on ABCG1 and STAT3. These observations suggest a significant role for VLDL in atherosclerosis, through diverse actions that bridge immune response and lipid metabolism. Our work suggests that sialidase is a modulator of VLDL function during foam cell formation. We thus outline a novel mechanism of VLDL-induced atherogenesis with NEU1 sialidase playing a prominent role. This data could pave the avenue for using DANA or a similar compound as a potent and multi-pronged therapeutic agent for atherosclerosis.

4.6 REFERENCES

Amith SR, Jayanth P, Franchuk S, Siddiqui S, Seyrantepe V, Gee K, Basta S, Beyaert R, Pshezhetsky AV, Szewczuk MR (2009) Dependence of pathogen molecule-induced Toll-like receptor activation and cell function on Neu1 sialidase. Glycoconj J 26:1197-212

Bojic LA, Sawyez CG, Telford DE, Edwards JY, Hegele RA, Huff MW (2012) Activation of peroxisome proliferator-activated receptor delta inhibits human macrophage foam cell formation and the inflammatory response induced by very low-density lipoprotein. Arterioscler Thromb Vasc Biol 32:2919-2928

Bonnet P, Bryce RA (2004) Molecular dynamics and free energy analysis of neuraminidase-ligand interactions. Protein Sci 13:946-957

Businaro R, Tagliani A, Buttari B, Profumo E, Ippoliti F, Di CC, Capoano R, Salvati B, Rigano R (2012) Cellular and molecular players in the atherosclerotic plaque progression. Ann N Y Acad Sci 1262:134-141

Chawla A (2010) Control of macrophage activation and function by PPARs. Circ Res 106:1559-1569

Chen X, Varki A (2010) Advances in the biology and chemistry of sialic acids. ACS Chem Biol 5:163-176

Correa CR, Dias-Melicio LA, Calvi SA, Lastoria S, Soares AM (2011) Activation of monocytes and cytokine production in patients with peripheral atherosclerosis obliterans. J Inflamm (Lond) 8:23

Daugherty A, Webb NR, Rateri DL, King VL (2005) Thematic review series: The immune system and atherogenesis. Cytokine regulation of macrophage functions in atherogenesis. J Lipid Res 46:1812-1822

den Hartigh LJ, Connolly-Rohrbach JE, Fore S, Huser TR, Rutledge JC (2010) Fatty acids from very low-density lipoprotein lipolysis products induce lipid droplet accumulation in human monocytes. J Immunol 184:3927-3936

Dickhout JG, Lhotak S, Hilditch BA, Basseri S, Colgan SM, Lynn EG, Carlisle RE, Zhou J, Sood SK, Ingram AJ, Austin RC (2011) Induction of the unfolded protein response after monocyte to macrophage differentiation augments cell survival in early atherosclerotic lesions. FASEB J 25:576-589

Du ZY, Ma T, Lock EJ, Hao Q, Kristiansen K, Froyland L, Madsen L (2011) Depot-dependent effects of adipose tissue explants on co-cultured hepatocytes. PLoS One 6:e20917

Eck MV, Oost J, Goudriaan JR, Hoekstra M, Hildebrand RB, Bos IS, van Dijk KW, Van Berkel TJ (2005) Role of the macrophage very-low-density lipoprotein receptor in atherosclerotic lesion development. Atherosclerosis 183:230-237

Foster JG, Blunt MD, Carter E, Ward SG (2012) Inhibition of PI3K signaling spurs new therapeutic opportunities in inflammatory/autoimmune diseases and hematological malignancies. Pharmacol Rev 64:1027-1054

Freeman LA (2013) Western blots. Methods Mol Biol 1027:369-385

Fuentealba RA, Liu Q, Kanekiyo T, Zhang J, Bu G (2009) Low density lipoprotein receptor-related protein 1 promotes anti-apoptotic signaling in neurons by activating Akt survival pathway. J Biol Chem 284:34045-34053

Gadhoum SZ, Sackstein R (2008) CD15 expression in human myeloid cell differentiation is regulated by sialidase activity. Nat Chem Biol 4:751-757

Gee K, Kozlowski M, Kumar A (2003) Tumor necrosis factor-alpha induces functionally active hyaluronan-adhesive CD44 by activating sialidase through p38 mitogen-activated protein kinase in lipopolysaccharide-stimulated human monocytic cells. J Biol Chem 278:37275-37287

Gee K, Lim W, Ma W, Nandan D, az-Mitoma F, Kozlowski M, Kumar A (2002) Differential regulation of CD44 expression by lipopolysaccharide (LPS) and TNF-alpha in human monocytic cells: distinct involvement of c-Jun N-terminal kinase in LPS-induced CD44 expression. J Immunol 169:5660-5672

Glass CK, Witztum JL (2001) Atherosclerosis. the road ahead. Cell 104:503-516

Go GW, Mani A (2012) Low-density lipoprotein receptor (LDLR) family orchestrates cholesterol homeostasis. Yale J Biol Med 85:19-28

Gui T, Shimokado A, Sun Y, Akasaka T, Muragaki Y (2012) Diverse roles of macrophages in atherosclerosis: from inflammatory biology to biomarker discovery. Mediators Inflamm 2012:693083

Guo L, Song Z, Li M, Wu Q, Wang D, Feng H, Bernard P, Daugherty A, Huang B, Li XA (2009) Scavenger Receptor BI Protects against Septic Death through Its Role in Modulating Inflammatory Response. J Biol Chem 284:19826-19834

Hansson GK (2007) Medicine. LIGHT hits the liver. Science 316:206-207

Hansson GK (2009) Inflammatory mechanisms in atherosclerosis. J Thromb Haemost 7 Suppl 1:328-331

Hart JR, Liao L, Yates JR, III, Vogt PK (2011) Essential role of Stat3 in PI3K-induced oncogenic transformation. Proc Natl Acad Sci U S A 108:13247-13252

Hegele RA (2009) Plasma lipoproteins: genetic influences and clinical implications. Nat Rev Genet 10:109-121

Herijgers N, Van EM, Groot PH, Hoogerbrugge PM, Van Berkel TJ (2000) Low density lipoprotein receptor of macrophages facilitates atherosclerotic lesion formation in C57Bl/6 mice. Arterioscler Thromb Vasc Biol 20:1961-1967

Hoesel B, Schmid JA (2013) The complexity of NF-kappaB signaling in inflammation and cancer. Mol Cancer 12:86

Hong C, Tontonoz P (2008) Coordination of inflammation and metabolism by PPAR and LXR nuclear receptors. Curr Opin Genet Dev 18:461-467

Isa SA, Ruffino JS, Ahluwalia M, Thomas AW, Morris K, Webb R (2011) M2 macrophages exhibit higher sensitivity to oxLDL-induced lipotoxicity than other monocyte/macrophage subtypes. Lipids Health Dis 10:229

Jinno Y, Nakakuki M, Kawano H, Notsu T, Mizuguchi K, Imada K (2011) Eicosapentaenoic acid administration attenuates the pro-inflammatory properties of VLDL by decreasing its susceptibility to lipoprotein lipase in macrophages. Atherosclerosis 219:566-572

Jong MC, Hofker MH, Havekes LM (1999) Role of ApoCs in lipoprotein metabolism: functional differences between ApoC1, ApoC2, and ApoC3. Arterioscler Thromb Vasc Biol 19:472-484

Kawakami A, Aikawa M, Alcaide P, Luscinskas FW, Libby P, Sacks FM (2006a) Apolipoprotein CIII induces expression of vascular cell adhesion molecule-1 in vascular endothelial cells and increases adhesion of monocytic cells. Circulation 114:681-687

Kawakami A, Aikawa M, Libby P, Alcaide P, Luscinskas FW, Sacks FM (2006b) Apolipoprotein CIII in apolipoprotein B lipoproteins enhances the adhesion of human monocytic cells to endothelial cells. Circulation 113:691-700

Kawakami A, Aikawa M, Nitta N, Yoshida M, Libby P, Sacks FM (2007) Apolipoprotein CIII-induced THP-1 cell adhesion to endothelial cells involves pertussis toxin-sensitive G

protein- and protein kinase C alpha-mediated nuclear factor-kappaB activation. Arterioscler Thromb Vasc Biol 27:219-225

Kosaka S, Takahashi S, Masamura K, Kanehara H, Sakai J, Tohda G, Okada E, Oida K, Iwasaki T, Hattori H, Kodama T, Yamamoto T, Miyamori I (2001) Evidence of macrophage foam cell formation by very low-density lipoprotein receptor: interferon-gamma inhibition of very low-density lipoprotein receptor expression and foam cell formation in macrophages. Circulation 103:1142-1147

Krombach F, Munzing S, Allmeling AM, Gerlach JT, Behr J, Dorger M (1997) Cell size of alveolar macrophages: an interspecies comparison. Environ Health Perspect 105 Suppl 5:1261-1263

Lambre CR, Greffard A, Gattegno L, Saffar L (1990) Modifications of sialidase activity during the monocyte-macrophage differentiation in vitro. Immunol Lett 23:179-182

Langlois B, Perrot G, Schneider C, Henriet P, Emonard H, Martiny L, Dedieu S (2010) LRP-1 promotes cancer cell invasion by supporting ERK and inhibiting JNK signaling pathways. PLoS One 5:e11584

Liang F, Seyrantepe V, Landry K, Ahmad R, Ahmad A, Stamatos NM, Pshezhetsky AV (2006) Monocyte differentiation up-regulates the expression of the lysosomal sialidase, Neu1, and triggers its targeting to the plasma membrane via major histocompatibility complex class II-positive compartments. J Biol Chem 281:27526-27538

Libby P (2007) Fat fuels the flame: triglyceride-rich lipoproteins and arterial inflammation. Circ Res 100:299-301

Libby P, Ridker PM, Hansson GK (2011) Progress and challenges in translating the biology of atherosclerosis. Nature 473:317-325

Lillis AP, Mikhailenko I, Strickland DK (2005) Beyond endocytosis: LRP function in cell migration, proliferation and vascular permeability. J Thromb Haemost 3:1884-1893

Lillis AP, Van Duyn LB, Murphy-Ullrich JE, Strickland DK (2008) LDL receptor-related protein 1: unique tissue-specific functions revealed by selective gene knockout studies. Physiol Rev 88:887-918

Lindberg G (2007) Resialylation of sialic acid deficit vascular endothelium, circulating cells and macromolecules may counteract the development of atherosclerosis: a hypothesis. Atherosclerosis 192:243-245

LOWRY OH, ROSEBROUGH NJ, FARR AL, RANDALL RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193:265-275

Lu Y, Guo J, Di Y, Zong Y, Qu S, Tian J (2009) Proteomic analysis of the triglyceride-rich lipoprotein-laden foam cells. Mol Cells 28:175-181

Luo M, Jedrzejas MJ, Singh S, White CL, Brouillette WJ, Air GM, Laver WG (1995) Benzoic acid inhibitors of influenza virus neuraminidase. Acta Crystallogr D Biol Crystallogr 51:504-510

Mann CJ, Troussard AA, Yen FT, Hannouche N, Najib J, Fruchart JC, Lotteau V, Andre P, Bihain BE (1997) Inhibitory effects of specific apolipoprotein C-III isoforms on the binding of triglyceride-rich lipoproteins to the lipolysis-stimulated receptor. J Biol Chem 272:31348-31354

Mantuano E, Jo M, Gonias SL, Campana WM (2010) Low density lipoprotein receptor-related protein (LRP1) regulates Rac1 and RhoA reciprocally to control Schwann cell adhesion and migration. J Biol Chem 285:14259-14266

May P, Herz J, Bock HH (2005) Molecular mechanisms of lipoprotein receptor signalling. Cell Mol Life Sci 62:2325-2338

Meindl P, Bodo G, Palese P, Schulman J, Tuppy H (1974) Inhibition of neuraminidase activity by derivatives of 2-deoxy-2,3-dehydro-N-acetylneuraminic acid. Virology 58:457-463

Melajarvi N, Gylling H, Miettinen TA (1996) Sialic acids and the metabolism of low density lipoprotein. J Lipid Res 37:1625-1631

Millar JS (2001) The sialylation of plasma lipoproteins. Atherosclerosis 154:1-13

Milosavljevic D, Kontush A, Griglio S, Le NG, Thillet J, Chapman MJ (2003) VLDL-induced triglyceride accumulation in human macrophages is mediated by modulation of LPL lipolytic activity in the absence of change in LPL mass. Biochim Biophys Acta 1631:51-60

Moore KJ, Sheedy FJ, Fisher EA (2013) Macrophages in atherosclerosis: a dynamic balance. Nat Rev Immunol 10:709-21

Nofer JR, Brodde MF, Kehrel BE (2010) High-density lipoproteins, platelets and the pathogenesis of atherosclerosis. Clin Exp Pharmacol Physiol 37:726-735

O'Sullivan AW, Wang JH, Redmond HP (2009) The role of P38 MAPK and PKC in BLP induced TNF-alpha release, apoptosis, and NFkappaB activation in THP-1 monocyte cells. J Surg Res 151:138-144

Palmer AM, Nova E, Anil E, Jackson K, Bateman P, Wolstencroft E, Williams CM, Yaqoob P (2005a) Differential uptake of subfractions of triglyceride-rich lipoproteins by THP-1 macrophages. Atherosclerosis 180:233-244

Palmer AM, Nova E, Anil E, Jackson K, Bateman P, Wolstencroft E, Williams CM, Yaqoob P (2005b) Differential uptake of subfractions of triglyceride-rich lipoproteins by THP-1 macrophages. Atherosclerosis 180:233-244

Pappu BP, Shrikant PA (2004) Alteration of cell surface sialylation regulates antigen-induced naive CD8+ T cell responses. J Immunol 173:275-284

Perrot G, Langlois B, Devy J, Jeanne A, Verzeaux L, Almagro S, Sartelet H, Hachet C, Schneider C, Sick E, David M, Khrestchatisky M, Emonard H, Martiny L, Dedieu S (2012) LRP-1--CD44, a new cell surface complex regulating tumor cell adhesion. Mol Cell Biol 32:3293-3307

Persson J, Nilsson J, Lindholm MW (2008a) Interleukin-1beta and tumour necrosis factor-alpha impede neutral lipid turnover in macrophage-derived foam cells. BMC Immunol 9:70

Persson J, Nilsson J, Lindholm MW (2008b) Interleukin-1beta and tumour necrosis factor-alpha impede neutral lipid turnover in macrophage-derived foam cells. BMC Immunol 9:70

Pou J, Martinez-Gonzalez J, Rebollo A, Rodriguez C, Rodriguez-Calvo R, Martin-Fuentes P, Cenarro A, Civeira F, Laguna JC, Alegret M (2011) Type II interleukin-1 receptor expression is reduced in monocytes/macrophages and atherosclerotic lesions. Biochim Biophys Acta 1811:556-563

Pshezhetsky AV, Ashmarina LI (2013) Desialylation of surface receptors as a new dimension in cell signaling. Biochemistry (Mosc) 78:736-745

Puhlmann M, Weinreich DM, Farma JM, Carroll NM, Turner EM, Alexander HR, Jr. (2005) Interleukin-1beta induced vascular permeability is dependent on induction of endothelial tissue factor (TF) activity. J Transl Med 3:37

Rastam L, Lindberg G, Folsom AR, Burke GL, Nilsson-Ehle P, Lundblad A (1996) Association between serum sialic acid concentration and carotid atherosclerosis measured by B-mode ultrasound. The ARIC Investigators. Atherosclerosis Risk in Communities Study. Int J Epidemiol 25:953-958

Reuter G, Gabius HJ (1996) Sialic acids structure-analysis-metabolism-occurrence-recognition. Biol Chem Hoppe Seyler 377:325-342

Rosenson RS, Brewer HB, Jr., Davidson WS, Fayad ZA, Fuster V, Goldstein J, Hellerstein M, Jiang XC, Phillips MC, Rader DJ, Remaley AT, Rothblat GH, Tall AR, Yvan-Charvet L (2012) Cholesterol efflux and atheroprotection: advancing the concept of reverse cholesterol transport. Circulation 125:1905-1919

Ross R (1999) Atherosclerosis--an inflammatory disease. N Engl J Med 340:115-126

Sakthivel R, Zhang JC, Strickland DK, Gafvels M, McCrae KR (2001) Regulation of the ligand binding activity of the human very low density lipoprotein receptor by protein kinase C-dependent phosphorylation. J Biol Chem 276:555-562

Sampedro MC, Motran C, Gruppi A, Kivatinitz SC (2001) VLDL modulates the cytokine secretion profile to a proinflammatory pattern. Biochem Biophys Res Commun 285:393-399

Saraswathi V, Hasty AH (2006) The role of lipolysis in mediating the proinflammatory effects of very low density lipoproteins in mouse peritoneal macrophages. J Lipid Res 47:1406-1415

Satpathy AT, Wu X, Albring JC, Murphy KM (2012) Re(de)fining the dendritic cell lineage. Nat Immunol 13:1145-1154

Schauer R (2009) Sialic acids as regulators of molecular and cellular interactions. Curr Opin Struct Biol 19:507-514

Shalhoub J, Falck-Hansen MA, Davies AH, Monaco C (2011) Innate immunity and monocyte-macrophage activation in atherosclerosis. J Inflamm (Lond) 8:9

Shelness GS, Ingram MF, Huang XF, DeLozier JA (1999) Apolipoprotein B in the rough endoplasmic reticulum: translation, translocation and the initiation of lipoprotein assembly. J Nutr 129:456S-462S

Skalen K, Gustafsson M, Rydberg EK, Hulten LM, Wiklund O, Innerarity TL, Boren J (2002) Subendothelial retention of atherogenic lipoproteins in early atherosclerosis. Nature 417:750-754

Spilsbury K, O'Mara MA, Wu WM, Rowe PB, Symonds G, Takayama Y (1995) Isolation of a novel macrophage-specific gene by differential cDNA analysis. Blood 85:1620-1629

Sprague EA, Moser M, Edwards EH, Schwartz CJ (1988) Stimulation of receptor-mediated low density lipoprotein endocytosis in neuraminidase-treated cultured bovine aortic endothelial cells. J Cell Physiol 137:251-262

Stamatos NM, Liang F, Nan X, Landry K, Cross AS, Wang LX, Pshezhetsky AV (2005) Differential expression of endogenous sialidases of human monocytes during cellular differentiation into macrophages. FEBS J 272:2545-2556

Stoline AM, Saku K, Hynd BA, Kashyap ML (1985) Effect of desialylation of very low-density lipoproteins on their catabolism by lipoprotein lipase. Metabolism 34:30-35

Stollenwerk MM, Lindholm MW, Porn-Ares MI, Larsson A, Nilsson J, Ares MP (2005a) Very low-density lipoprotein induces interleukin-1beta expression in macrophages. Biochem Biophys Res Commun 335:603-608

Stollenwerk MM, Schiopu A, Fredrikson GN, Dichtl W, Nilsson J, Ares MP (2005b) Very low density lipoprotein potentiates tumor necrosis factor-alpha expression in macrophages. Atherosclerosis 179:247-254

Tabas I, Lim S, Xu XX, Maxfield FR (1990) Endocytosed beta-VLDL and LDL are delivered to different intracellular vesicles in mouse peritoneal macrophages. J Cell Biol 111:929-940

Tacke RS, Tosello-Trampont A, Nguyen V, Mullins DW, Hahn YS (2011) Extracellular hepatitis C virus core protein activates STAT3 in human monocytes/macrophages/dendritic cells via an IL-6 autocrine pathway. J Biol Chem 286:10847-10855

Takahashi S, Sakai J, Fujino T, Hattori H, Zenimaru Y, Suzuki J, Miyamori I, Yamamoto TT (2004) The very low-density lipoprotein (VLDL) receptor: characterization and functions as a peripheral lipoprotein receptor. J Atheroscler Thromb 11:200-208

Varki A (2008) Sialic acids in human health and disease. Trends Mol Med 14:351-360

Varki A, Gagneux P (2012) Multifarious roles of sialic acids in immunity. Ann N Y Acad Sci 1253:16-36

Wegiel B, Bjartell A, Culig Z, Persson JL (2008) Interleukin-6 activates PI3K/Akt pathway and regulates cyclin A1 to promote prostate cancer cell survival. Int J Cancer 122:1521-1529

Williams HJ, Fisher EA, Greaves DR (2012) Macrophage differentiation and function in atherosclerosis: opportunities for therapeutic intervention? J Innate Immun 4:498-508

Williams MR, Kataoka N, Sakurai Y, Powers CM, Eskin SG, McIntire LV (2008) Gene expression of endothelial cells due to interleukin-1 beta stimulation and neutrophil transmigration. Endothelium 15:73-165

Yang A, Gyulay G, Mitchell M, White E, Trigatti BL, Igdoura SA (2012) Hypomorphic sialidase expression decreases serum cholesterol by downregulation of VLDL production in mice. J Lipid Res 53:2573-2585

Ye Q, Chen Y, Lei H, Liu Q, Moorhead JF, Varghese Z, Ruan XZ (2009) Inflammatory stress increases unmodified LDL uptake via LDL receptor: an alternative pathway for macrophage foam-cell formation. Inflamm Res 11:809-18

Yvan-Charvet L, Ranalletta M, Wang N, Han S, Terasaka N, Li R, Welch C, Tall AR (2007) Combined deficiency of ABCA1 and ABCG1 promotes foam cell accumulation and accelerates atherosclerosis in mice. J Clin Invest 117:3900-3908

Zhang C, Li Y, Wu Y, Wang L, Wang X, Du J (2013a) Interleukin-6/signal transducer and activator of transcription 3 (STAT3) pathway is essential for macrophage infiltration and myoblast proliferation during muscle regeneration. J Biol Chem 288:1489-1499

Zhang Y, Albohy A, Zou Y, Smutova V, Pshezhetsky AV, Cairo CW (2013b) Identification of selective inhibitors for human neuraminidase isoenzymes using C4,C7-modified 2-deoxy-2,3-didehydro-N-acetylneuraminic acid (DANA) analogues. J Med Chem 56:2948-2958

Zhou L, Takayama Y, Boucher P, Tallquist MD, Herz J (2009) LRP1 regulates architecture of the vascular wall by controlling PDGFRbeta-dependent phosphatidylinositol 3-kinase activation. PLoS One 4:e6922

CHAPTER 5: Hypomorphic sialidase expression increases LDL uptake and cholesterol efflux in macrophages

Gabriel Gyulay[1], Bernado L. Trigatti[2,3], and Suleiman A. Igdoura[1,4]

Departments of [1]Biology, 2Biochemistry and Biomedical Sciences, [3]Thrombosis and Atherosclerosis Research Institute, and [4]Pathology and Molecular Medicine, McMaster University, Hamilton, Ontario, Canada

Correspondence:
Suleiman Igdoura, Ph.D.
McMaster University, 1280 Main St. W. LSB 335
Hamilton, Ontario L8S 4K1 Canada
Tel. (905) 525-9140ex27729
Fax: (905) 522-6066
E-Mail: igdoura@mcmaster.ca

5.1 ABSTRACT

Atherosclerosis and its associated health risks represent a significant medical challenge in modern society. Atherosclerosis is initiated by high circulating levels of LDL cholesterol and its subsequent oxidation which causes macrophages to form foam cells and atherosclerotic lesions. Disease progression is ameliorated by LDL clearance from circulation via LDL receptors or through reverse cholesterol transport by macrophages via lipidation of HDL by ABC cholesterol transporters. The LDLR protein is crucial in cholesterol homeostasis since patients with familial hypercholesterolemia lack LDLR and develop severe atherosclerosis. Previous studies, including ours, have shown that LDLR is heavily glycosylated and sialylated and that these glycans can play important functional roles. Sialidase (Neu1) is a ubiquitously expressed enzyme found in the lysosome and on the cell surface that catalyzes the cleavage of sialic acids from glycoconjucates. We have shown that hypomorphic sialidase ($Neu1^{hypo}$) mice harbour a point mutation in the Neu1 promoter causing a drastic reduction in sialidase expression which is pronounced in immune cells. Immune cells, and especially macrophages, are of direct relevance to atherosclerosis and therefore we aimed to characterize the impact of hypomorphic sialidase expression on macrophage cholesterol metabolism. Both THP-1 macrophages treated with the sialidase inhibitor DANA and macrophages from $Neu1^{hypo}$ mice display increased LDL uptake and aberrant regulation of LDLR compared to controls. Furthermore, mouse bone marrow derived macrophages from $Neu1^{hypo}$ mice display higher levels of ABCG1 and increased cholesterol efflux to HDL, in an LDLR

dependent manner. Hypomorphic sialidase expression results in increased LDL uptake and increased cholesterol efflux. This study establishes sialidase as a potent modulator of LDLR function and macrophage cholesterol homeostasis, and as a result an important enzyme in lipoprotein metabolism and mechanisms of atherosclerosis.

5.2 INTRODUCTION

Atherosclerosis is a dynamic disease and its progression is highly dependent on circulating cholesterol and lipid levels (Glass and Witztum, 2001). Lipoproteins are the main carriers of cholesterol, triglycerides and fatty acids in circulation, with the majority of serum cholesterol being transported by low density lipoprotein (LDL) (Steinberg and Witztum, 1990). LDL serves to deliver cholesterol and lipids to cells in different tissues of the body, and high levels of this lipoprotein are atherogenic (Libby et al., 2011). Cholesterol-containing very low density lipoprotein (VLDL) is originally produced by the liver through the addition of triglycerides to apolipoprotein-B (Apo-B) and subsequent secretion into the blood (Shelness et al., 1999). The VLDL particle undergoes conformational and compositional changes in circulation and matures into LDL, allowing for its LDL-receptor (LDLR) mediated endocytosis by cells (Hegele, 2009).

Cells can regulate their cholesterol content via endogenous synthesis, receptor mediated endocytosis, as well as reverse cholesterol transport (Goedeke and Fernandez-Hernando, 2012). Cholesterol uptake occurs primarily via uptake of LDL by LDLR, while cholesterol efflux to high density lipoprotein (HDL) and apolipoprotein-A1 (Apo-A1) occurs via the ATP-binding cassette transporters G1 and A1 (ABCA1/ABCG1) and subsequent cholesterol delivery to the liver using Scavenger receptor class B type 1 (SRB-1) (Go and Mani, 2012;Rosenson et al., 2012). Intracellular cholesterol resides primarily in lipid droplets and the cell membrane, although sterol regulatory element binding protein 2 (SREBP-2)-mediated sensing and regulation of cholesterol

synthesis/uptake is initiated in the endoplasmic reticulum (ER) (Radhakrishnan et al., 2008;Soccio and Breslow, 2004). While cholesterol homeostasis is important in all cell types, the balance of cholesterol uptake and efflux in macrophages is critical to atherosclerosis (Moore et al., 2013;Ouimet and Marcel, 2012). Macrophages are pervasive throughout all stages of atherogenesis, and their cholesterol content and subsequent inflammatory reaction drives disease progression (Gui et al., 2012). Macrophages are unique from other periphery cell types, in that they can take up modified lipoproteins via scavenger receptor internalization to further contribute to their cholesterol pool as well (Glass and Witztum, 2001;Moore et al., 2013). This primarily includes uptake of oxidized LDL, and is a direct cause of foam cell formation and immune response in the plaque, as macrophage reverse cholesterol transport cannot keep up with incoming cholesterol (Levitan et al., 2010;Moore and Tabas, 2011). Additionally, macrophages readily internalize LDL, but the exact role of macrophage LDLR and LDL metabolism in disease progression is unknown (Boisvert et al., 1997;Daugherty et al., 2005;Fazio et al., 1997;Herijgers et al., 2000;Linton et al., 1999;Wang et al., 2007). Levels of extracellular cholesterol-containing lipoproteins and macrophage cholesterol homeostasis are both critical factors in the initiation and propagation of atherosclerosis (Barton, 2013).

Neu1 sialidase is a ubiquitously expressed enzyme that cleaves sialic acids from glycoconjucates and it is localized to the lyosome and the cell surface of cells (Pattison et al., 2004;Pshezhetsky and Hinek, 2011). Sialic acid is a terminal monosaccharide

abundant on many proteins and cell surface moieties, and its functions include conformational stabilization and cell surface recognition (Varki, 2008). We have previously demonstrated that hypomorphic expression of Neu1 sialidase in mice causes a reduction in serum LDL and alterations in hepatic LDLR function (Yang et al., 2012). Furthermore, human Neu1 null fibroblasts exhibit increased LDL uptake, and the importance of sialylation on LDLR function has been previously noted (Goldstein et al., 1985;Shite et al., 1990;Sprague et al., 1988;Yang et al., 2012). While the role of macrophage Neu1 sialidase in immunity has been extensively studied, the function of Neu1 in cholesterol and lipid metabolism of macrophages remains unclear (Miyagi and Yamaguchi, 2012). Neu1 sialidase is highly expressed on the cell surface of differentiated macrophages and we aim to study its effect on LDLR function and macrophage cholesterol homeostasis as a whole (Liang et al., 2006). We have found that sialidase inhibition using 2-deoxy-2,3-dehydro-N-acetylneuraminic acid (DANA) resulted in increased LDL uptake and aberrant regulation of LDLR mRNA in human THP-1 macrophages. Furthermore, macrophages from hypomorphic Neu1 mice had increased LDL uptake and increased expression of ABCG1 protein compared to C57Bl/6 and LDLR-/- control cells. This was coupled with increased cholesterol efflux to HDL in $Neu1^{hypo}$ macrophages and was dependent on LDLR. We show strong evidence for modulation of macrophage cholesterol metabolism by Neu1 sialidase and hypothesize that hypersialylation of macrophage LDLR will lead to an atheroprotective phenotype in vivo. Increased LDL uptake would lower serum cholesterol levels and also reduce the LDL

available for oxidation. These mechanisms, when coupled with increased cholesterol efflux, would result in amelioration of the large cholesterol load encountered by macrophages in the developing plaque. Therefore, Neu1 sialidase plays important roles in macrophage cholesterol homeostasis, and its inhibition or reduction represent potential mechanisms and therapies for atheroprotection which warrant further investigation.

5.3 MATERIALS AND METHODS

5.3.1 THP-1 Macrophages

THP-1 monocytes are a leukemia cell line obtained from a 1-year old male and were grown in RPMI1640 medium wtih 0.1 mM L-glutamine supplemented with 10% FBS, 1% antibiotic solution and 0.1% fungizone. Differentiation into macrophages was performed with 0.2 µM PMA (phorbol 12-myristate 13-acetate) for 72 hours. Cells were cultured at 37ºC at 5% CO2 and 90% humidity in a standard incubator. Macrophages were lipid starved for 24 hours in 5% lipoprotein deficient serum (LPDS) prior to addition of lipoproteins.

5.3.2 Intraperitoneal Macrophage Isolation

For intraperitoneal (IP) macrophage isolation, mice were injected intraperitoneally with 1 mL of 10% thioglycollate in PBS and sacrificed 4 days later by cervical dislocation. The peritoneal cavity was injected with 10 mL PBS to loosen elicited macrophages for removal and plating. Cells were removed with a 20-gauge needle and syringe from the peritoneal cavity in a sterile biosafety cabinet, and spun down at 1400RPM. The cells from the pellet were suspended and grown in RPMI1640 medium supplemented with 10% FBS, 1% antibiotic solution and 0.1% fungizone. Cells were cultured at 37ºC at 5% CO_2 and 90% humidity in a standard Incubator.

5.3.3 Bone Marrow Macrophage Isolation

Mouse femurs and tibias were removed from animals and placed in sterile media in a biosafety cabinet. The ends of the bones were cut, and the inside of the bones was flushed with warm RPMI media using a 26-gauge needle. The cell suspension was then passed through a 100 µm nylon cell strainer, placed on ice, then spun at 3300 RPM for 10 minutes. The supernanant was removed, and the cell pellet was reconstituted and plated in RPMI media supplemented with 10% FBS, 1% antibiotic solution, 0.1% fungizone, and 20 mM HEPES. Monocyte colony stimulating factor (M-CSF) at 5 ng/mL was added to the media for the duration of the experiment, for differentiation into macrophages. Cultures were grown for 9 days, with washing and replenishing of media every third day. FBS was then switched to LPDS for 24 hours to lipid starve cells prior to incubation with lipoproteins. 2 genotypes were examined and compared; C57Bl/6 (wild-type) and $Neu1^{hypo}$ (hypomorphic sialidase). $Neu1^{hypo}$ mice are derived from backcrossing SM/J mice with C57Bl6 mice 6 times. C57Bl/6 is a standard inbred strain that is commonly used while SM/J is a strain that contains a point mutation in the Neu1 gene which codes for lysosomal sialidase (Champigny et al., 2009;Rottier et al., 1998). This allows for adequate comparisons between genotypes as the only genetic difference between them is the hypomorphic expression of Neu1. BMDMs are routinely assessed for sialidase activity by our laboratory and $Neu1^{hypo}$ cells have approximately 40-50% activity compared to C57Bl/6. LDLR-/- animals are on a C57Bl/6 background and lack the LDLR gene (Ishibashi et al., 1993). LDLR-/- mice were crossed with $Neu1^{hypo}$ mice to

generate LDLR-/- Neu1^{hypo} animals. Mice were housed at the Central Animal Facility at McMaster University and were fed a regular chow diet, and are in accordance with all animal ethics regulations. Animals were euthanized by cervical dislocation prior to bone marrow macrophage isolation.

5.3.3 Macrophage Experimental Design

Plated/differentiated macrophages were lipid starved by incubation for 24 hours in LPDS-containing media followed by a 48 hour incubation with LDL, acLDL, VLDL or no lipoproteins. Cells were then subjected to lipid analysis or harvested for RNA or protein work.

5.3.4 Lipoproteins

LDL, acLDL, HDL and VLDL were purchased from Biomedical Technologies Inc. and were used in LPDS (lipoprotein deficient serum) in RPMI to avoid any medium-resident lipoprotein interference. Concentrations of lipoproteins were 50 µg/mL and incubation periods were 48 hours unless otherwise stated.

5.3.5 Immunoblot Analysis

After washing with cold PBS, adhered cells were scraped and lysed in RIPA buffer containing protease inhibitors (Complete Mini, Roche 11873580001, 1 tablet per 10 mL) with 1 mM EDTA. Cells in suspension were centrifuged out from media, then resuspended in the same buffer. Samples were then sonicated and boiled in Laemmli Sample Buffer prior to loading. Proteins were resolved by SDS-PAGE and transferred to

nitrocellulose membranes, then blocked using TBST w/ 5% milk. Antibodies were used at 1:1000 in TBST w/ 5% milk. HRP-bound species appropriate secondary antibodies were then detected by ECL Reagent (GE RPN2106) after which membranes were exposed onto film and developed (Freeman, 2013). Antibodies: rabbit anti-human/mouse ABCG1 (Novus Biologicals NB400-132), mouse anti-human/mouse ABCA1 (GenScript A00121), goat anti-mouse LDLR (R & D Systems AF2255) and goat anti-human/mouse GAPDH (R & D Systems AF5718). Band intensity was measured by densitometry analysis using ImageJ software and normalized to loading controls.

5.3.6 Sialidase Inhibition

Inhibition of cell surface sialidase activity was performed using 2-deoxy-2,3-dehydro-N-acetyl neuraminic acid (DANA). It is an established competitive inhibitor for sialidase, that functions by mimicking the sialic acid substrate and it has been routinely used in our laboratory and elsewhere (Bonnet and Bryce, 2004;Luo et al., 1995;Meindl et al., 1974). DANA, dissolved in PBS was used at concentrations of 200 µM in media unless otherwise stated. For lipoprotein experiments, DANA was added 24 hours prior to the starvation phase and was also present in the media for 48 hours during the treatment stage.

5.3.7 Cholesterol Efflux Assays

BMDMs were differentiated and maintained with RPMI supplemented growth medium (+ M-CSF) for 10 days. Cells were washed and then incubated with 2 µCi/mL

[1,2-^{3}H(N)] cholesterol (American Radiolabeled Chemicals Inc. ART 0255) in heat-inactivated FBS (Final FBS concentration in media was 10 %) for 2 days. The labelling medium was removed, cells were washed twice and incubated overnight in equilibration medium (supplemented RPMI containing 0.2 % fatty acid free BSA (Sigma) and no FBS). Equilibration medium was replaced with efflux medium (containing 50 µg/ml HDL or 0.2% BSA) and sampling of media was performed at various timepoints to measure cholesterol efflux. Wells containing no cells or no radioactivity were used as controls. Radioactivity in harvested cells and in the media at 0, 2, 4, 6, 8 and 24 hours was measured using a liquid scintillation counter. Final data was expressed as percent cholesterol efflux of total radioactive cholesterol and was obtained by dividing the radioactivity of each timepoint by the sum of all the timepoints and the final lysate (multiplied by 100). Media was spun down and 90 µl of the supernanant was used per timepoint, cell lysates were obtained by overlaying 0.1N NaOH after washing cells at the end of the assay. 90 µl of sample was mixed in glass vials containing 4 mL of scintillation fluid from Perkin Elmer (1200-436) prior to reading. The 90 µl readings were extrapolated to the total volume of the sample.

5.3.8 Sialidase Activity Assay

PMA-differentiated THP-1 macrophages were cultured in a 96-well, black, clear bottomed plate and treated with LPS alone or LPS and DANA for 1 hour (6 wells for each treatment). After washing, a neutral pH mixture containing 0.2 mM 4-Mu-NANA (4-

methylumbelliferyl-N-acetylneuraminic acid) and 10% BSA in acetate buffer was added and incubated for 1 hour at 37°C. The reaction was stopped by the addition of MAP (0.1 M 2-amino-2-methyl-1-propanol) buffer. Fluorescence was measured using a plate fluorometer (Perkin Elmer) and normalized to protein concentration. Activity was measured as the amount of fluorescence generated from the liberation of umbelliferone (4-Mu) from the NANA substrate by THP-1 cell surface sialidase (Amith et al., 2009).

5.3.9 ORO Extraction Quantification

THP-1 cells were differentiated with 2 µm PMA in lipoprotein deficient media for 48 hours in a 24-well plate. DANA (at the indicated concentrations) was added for 24 hours followed by 48 hours of LDL treatment (with and without DANA). Cells were washed, fixed in 2% formaldehyde in PBS and incubated in Oil Red O for 1 hour, followed by PBS washes. Reconstitution of lipid bound Oil Red O was performed in 0.5 mL isopropanol for each well after shaking. Intensity was then measured at 510 nm in a plate reader with appropriate blanks. After extraction, cells were stained with haematoxylin and counted to ensure equal cell counts. Technical triplicates were used.

5.3.10 Macrophage Oil Red O Staining and Visual Quantification

In a 24-well plate, cells were incubated with LDL for 48 hours at a concentration of 50 ug/mL after growing in LPDS for 24 hours. Glass cover slips were placed on the bottom of the wells prior to fibroblast plating. Cells were washed once with PBS, fixed in formaldehyde for 30 minutes then washed with 60% isopropanol prior to Oil Red O

staining for 60 minutes. This is followed by another 60% isopropanol rinse and 4 PBS washes. Hematoxylin staining was then performed and glass cover slips were then lifted and mounted on microscope slides using aqua mount (Fisher), avoiding any air bubbles. Hematoxylin staining was then performed followed by mounting onto microscope slides. Quantification was then performed by measuring the red stained area, normalized to the nuclear stained area on an individual cell basis. AxioVision Release 4.8.2 software was used for image acquisition and processing. These methods were adapted from the literature (Kozaki et al., 1997;Qiu and Hill, 2007;Scholz et al., 2004).

5.3.11 Triglyceride Assay

Macrophages were incubated with lipoproteins for 48 hours in LPDS. Cells were washed and lipids were extracted using a hexane/isopropanol mixture overlaid on the cells for 1 hour. The mixture was allowed to evaporate and lipids were resuspended in 100 µl isopropanol for enzymatic measurements of triglycerides using a colorometric assay kit from Wako (L-type TG H, discontinued) with appropriate blanks and standards. After lipid extraction, cells were scraped in 0.1% SDS and protein content was measured to normalize readings. Technical triplicates were used and data is representative of multiple experiments.

5.3.12 Protein Assay

DC protein assays (Bio-Rad 500-0113/4) using a modified method of Lowry (Lowry et al., 1951) were performed on all lysates to determine protein concentrations

to allow for normalized comparisons and equal loading. Bovine serum albumin (BSA, Sigma) was used as a standard.

5.3.13 Quantitative Real Time PCR

THP-1 derived macrophages were differentiated in lipoprotein deficient media for 48 hours and treated with 200 μM DANA for 24 hours. LDL (with or without DANA) was then added for 48 hours and RNA was isolated using Norgen Total RNA Isolation kit. Reverse transcriptase was performed using oligoDT primers and SSIII RT from Invitrogen. cDNA was then subjected to qRT-PCR analysis using Power SYBR green from ABI with primers for human LDLR (Forward: GACGTGGCGTGAACATCTG, Reverse: CTGGCAGGCAATGCTTTGG) and GAPDH (Forward: AGGTCGGTGTGAACGGATTTG, Reverse: TGTAGACCATGTAGTTGAGGTCA) and appropriate controls and standards. PCR cycle: 10min 95°C, 40x – 15sec 95°C, 60sec 60°C – followed by a dissociation stage. Technical replicates of 4 were used.

5.3.14 Statistical Significance

Statistical analyses between multiple groups of data were analyzed by one-way analysis of variance (one-way ANOVA) followed by a Bonferroni multiple comparison test using GraphPad Prism 5. Statistical analyses between 2 groups were performed using an unpaired student's T test. Error bars represent standard error of the mean. Data were considered statistically different only if p value is < 0.05, and demarcated by a *.

5.4 RESULTS

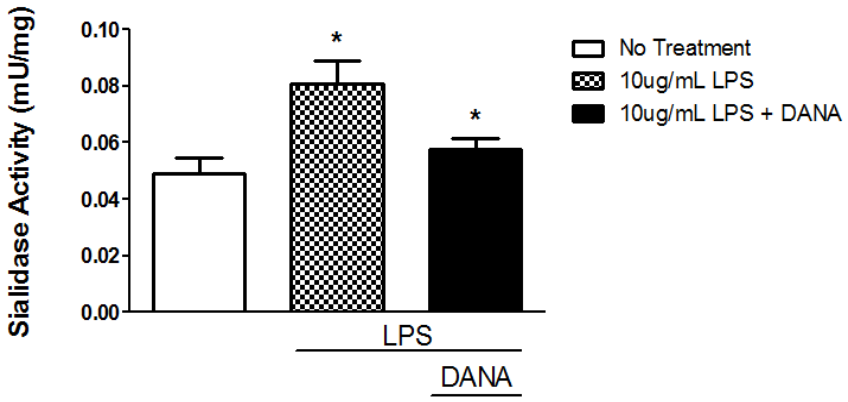
5.4.1 DANA is potent cell surface sialidase inhibitor

In order to validate the effect of the sialidase inhibitor 2,3-dehydro-2-deoxy-N-acetylneuraminic acid (DANA) on cell surface sialidase, a sialidase activity assay was performed on live cells. DANA is a modified form of sialic acid and is a potent and non-toxic mammalian sialidase inhibitor (Meindl et al., 1974;Zhang et al., 2013). Sialidase activity was measured using 4MU-NANA substrate at neutral pH, as to allow for detection of cell surface activity. THP-1 Cells were differentiated with PMA and incubated with 4MU-NANA for quantitative measurement of cell surface sialidase activity. LPS treatment caused a significant increase in sialidase activity and this increase vanished when DANA was present (Figure 5.1). This experiment provides proof that cell surface sialidase in THP-1 macrophages is being inhibited by DANA and gives merit to using this compound in our study.

Figure 5.1 Sialidase activity assay of live THP-1 macrophages

Live PMA-differentiated THP-1 macrophages were assayed for sialidase activity using 4-muNANA at a neutral pH. LPS increased activity, as expected, and DANA reduced this increase significantly (n=6). This validates the use of the sialidase inhibitor DANA on THP-1 cells. mU=nmol/hr, *=$p<0.05$

Figure 5.1


Sialidase Activity (mU/mg)
0.10
0.08
0.06
0.04
0.02
0.00
*
*
No Treatment
10ug/mL LPS
10ug/mL LPS + DANA
LPS
DANA

5.4.2 DANA increases LDL uptake and LDLR transcript in THP-1 macrophages

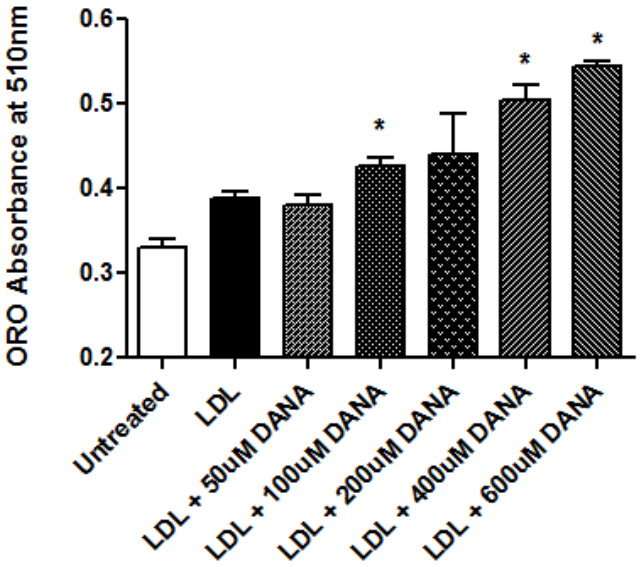
We have previously shown that LDLR is sialylated, and its function is altered in the liver of hypomorphic Neu1 mice and NEU1 null human fibroblasts (Yang et al., 2012). We aimed to test whether modifying sialidase activity can affect LDLR expression and function in vitro. LDL uptake by THP-1 macrophages was significantly increased with DANA treatment, in a dose dependent manner, as measured by Oil Red O staining and extraction (Figure 5.2A). Furthermore, qRT-PCR analysis shows maintenance of LDLR transcript when DANA is present along with LDL (Figure 5.2B). LDLR mRNA was reduced by LDL, as expected, but was reduced to a lesser degree when DANA was added as well. This correlates with the findings in Figure 5.2A since LDL uptake is a direct result of LDLR expression and function. This data provides a direct link between sialidase and LDLR-mediated macrophage lipid homeostasis.

Figure 5.2 Oil Red O staining and LDLR mRNA levels of LDL-treated THP-1 macrophages

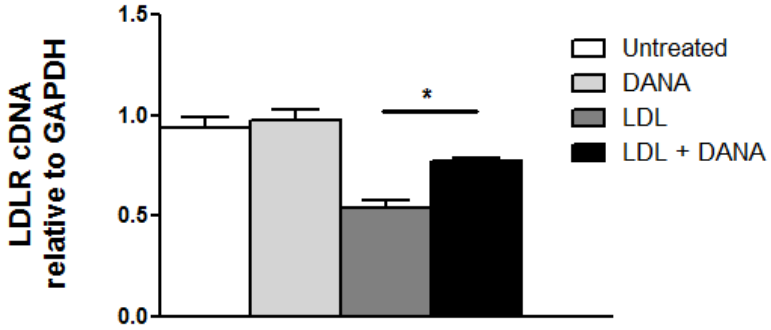
PMA differentiated THP-1 cells were lipid starved and treated with 50 μg/mL LDL for 48 hours, then anaylzed by Oil Red O staining or qRT-PCR. DANA was present in the media during the starvation and incubation period. (A) THP-1 macrophages show increased LDL uptake with increasing DANA concentrations as measured by Oil Red O extraction quantification (n=3). (B) DANA treatment also hampers reduction of LDLR mRNA expression by LDL, resulting in maintenance of LDLR expression despite higher lipid levels (n=4). *=$p<0.05$

Figure 5.2

A.


ORO Absorbance at 510nm
0.6
0.5
0.4
0.3
0.2
*
*
*
Untreated
LDL
LDL + 50uM DANA
LDL + 100uM DANA
LDL + 200uM DANA
LDL + 400uM DANA
LDL + 600uM DANA

B.


LDLR cDNA relative to GAPDH
1.5
1.0
0.5
0.0
*
Untreated
DANA
LDL
LDL + DANA

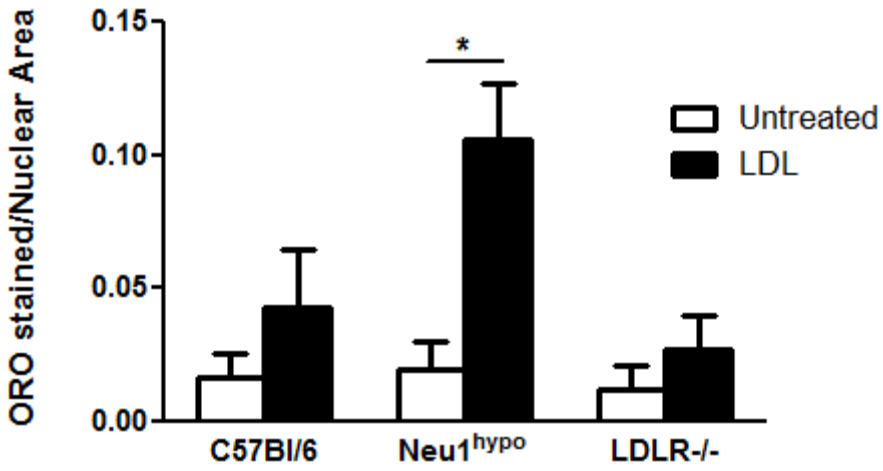
5.4.3 Sialidase deficient macrophages accumulate more lipids after LDL treatment compared to C57Bl/6 and LDLR-/- controls as measured by ORO staining

We also analyzed LDL uptake using Oil Red O staining of mouse intraperitoneal thioglycollate-elicited macrophages. $Neu1^{hypo}$ macrophages have been previously characterized and display approximately 40% lower sialidase activity than C57Bl/6. $Neu1^{hypo}$ macrophages have significantly higher ORO staining after LDL treatment, whereas C57Bl/6 and LDLR-/- macrophages do not. Cells from C57Bl/6 and LDLR-/- mice show just slight increases in lipid staining after LDL treatment, as expected, but nevertheless, sialidase deficiency resulted in more lipid accumulation in IP macrophages. This increase could be explained by modulation of LDLR function due to hypersialylation and/or expression as caused by sialidase deficiency.

Figure 5.3 Oil Red O staining of LDL treated intraperitoneal macrophages

Sialidase deficiency increases LDL uptake in thioglycollate-elicited macrophages compared to LDLR-/- and C57Bl/6, after 48 hours. Sialidase deficient 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 $Neu1^{hypo}$ cells that received LDL with no such effect in wild-type and LDLR-/- controls (n=3 mice). *=p<.0.05

Figure 5.3


ORO stained/Nuclear Area
0.15
0.10
0.05
0.00
*
Untreated
LDL
C57Bl/6
Neu1hypo
LDLR-/-

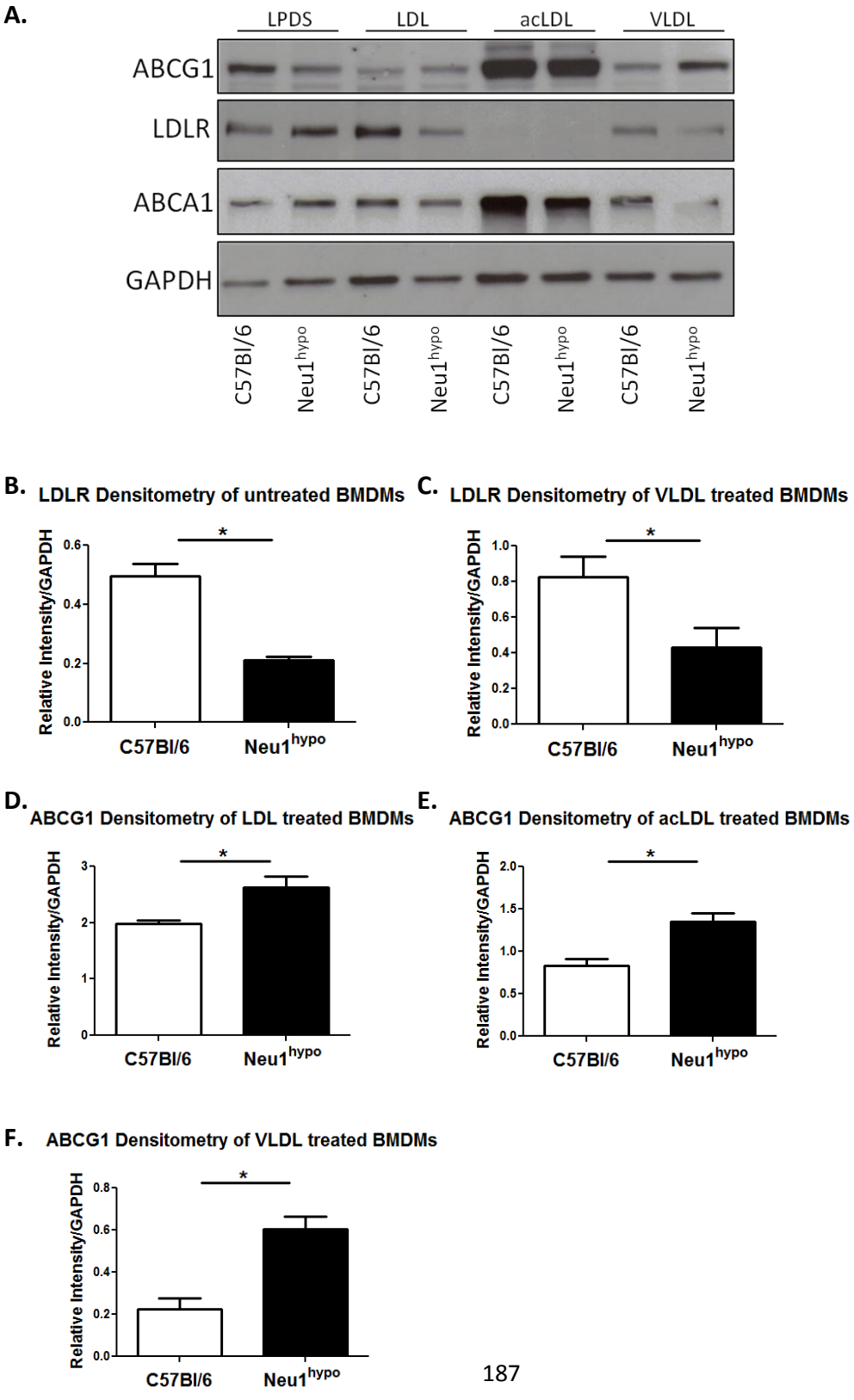
5.4.4 Neu1^{hypo} macrophages have increased expression of ABCG1 protein and decreased expression of LDLR protein

Neu1^{hypo} mouse bone marrow derived macrophages (BMDMs) have lower levels of LDLR protein when untreated or treated with VLDL, while showing no difference when treated with LDL or acLDL (Figure 5.4 A-C). Sialidase deficiency 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, although LDLR levels were not significantly lower between genotypes in the LDL treated group. ABCG1 protein levels are higher in Neu1^{hypo} BMDMs treated with LDL, acLDL or VLDL, compared to C57Bl/6 controls, while showing no changes in ABCA1 protein (Figure 5.4 A, D-F). Increased ABCG1 could lead to increased cholesterol efflux to HDL and atheroprotection, as more cholesterol would be removed from the lipid-engorged macrophages. This data implicates sialidase in regulation of macrophage cholesterol levels and potentially in regulation of uptake and efflux, although the exact mechanism is still unknown.

Figure 5.4 Western blot analysis of lipoprotein treated bone marrow derived macrophages

BMDMs were lipid starved and treated with lipoproteins for 48 hours after which protein levels were assessed by western blotting of lysates and band intensity was normalized to GAPDH. (A) acLDL treatment drastically increased ABC transporter (A1/G1) protein expression, while down-regulating LDLR protein expression, while LDL and VLDL treatments only had slight effects compared to untreated on the expression of these proteins. $Neu1^{hypo}$ macrophages in (B) untreated and (C) VLDL-treated groups have significantly lower expression of LDLR compared to C57Bl/6. $Neu1^{hypo}$ macrophages in (D) LDL, (E) acLDL and (F) VLDL-treated groups have increased levels of ABCG1 protein levels. ABCA1 levels remained unchanged between genotypes. Densitometry of protein levels that were not statistically significantly different were not shown in graphs. Representative blot shown, densitometry was performed on separate gels of n=3. *=p<.0.05

Figure 5.4


A.
LPDS
LDL
acLDL
VLDL
ABCG1
LDLR
ABCA1
GAPDH
C57Bl/6
Neu1hypo
B. LDLR Densitometry of untreated BMDMs
C. LDLR Densitometry of VLDL treated BMDMs
D. ABCG1 Densitometry of LDL treated BMDMs
E. ABCG1 Densitometry of acLDL treated BMDMs
F. ABCG1 Densitometry of VLDL treated BMDMs
Relative Intensity/GAPDH
*

5.4.5 Bone marrow derived macrophages from hypomorphic sialidase mice have increased efflux to HDL compared to C57Bl/6 controls.

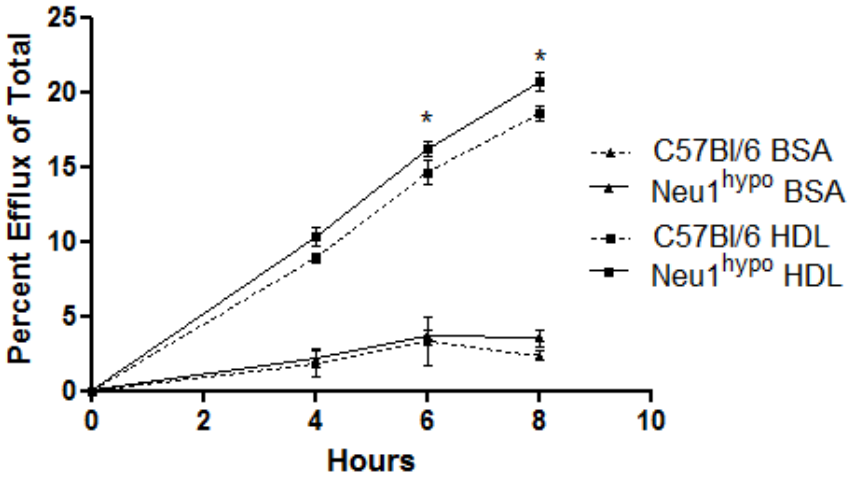
We assayed cholesterol efflux of bone marrow derived macrophages to HDL. $Neu1^{hypo}$ macrophages have significantly higher efflux compared to C57Bl/6 controls, enabling them to clear intracellular cholesterol more efficiently (Figure 5.5 A, C). Increased efflux is atheroprotective and appears to be LDLR dependent, as this difference disappears on macrophages from LDLR-/- background mice (Figure 5.5 B, D). Efflux to BSA control acceptor is minimal (Figure 5). Increased cholesterol efflux to HDL correlates with the higher levels of ABCG1 protein observed in $Neu1^{hypo}$ macrophages. This data can aid in explaining how increased lipoprotein uptake by the macrophage can be beneficial, if cholesterol can leave the cell via reverse cholesterol transport. Sialidase deficiency appears to accelerate cholesterol uptake followed by increased efflux, which could result in a net clearance of cholesterol and lipids from the plaque *in vivo* and cause atheroprotection.

Figure 5.5 Cholesterol efflux to HDL by bone marrow derived macrophages

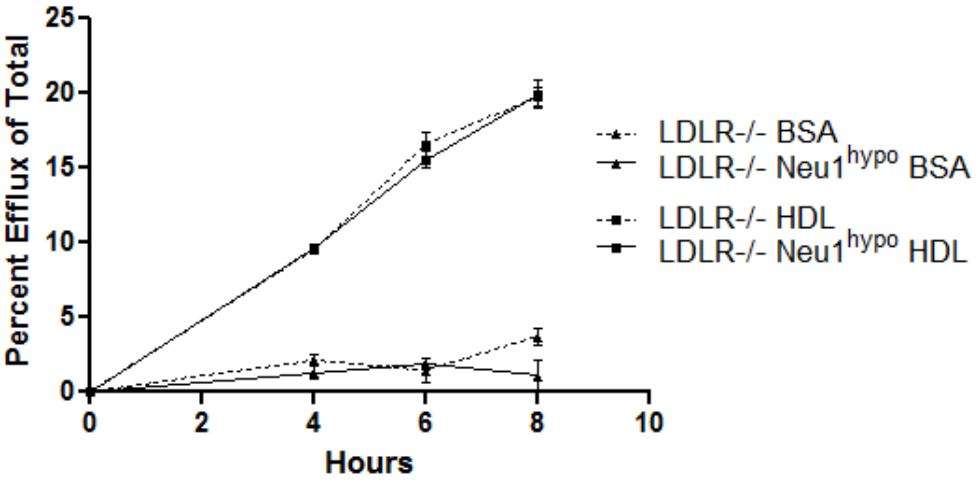
Hypomorphic sialidase expression increases cholesterol efflux in mouse macrophages, and this difference disappears on an LDLR-/- background. BMDMs were loaded with [^{3}H] cholesterol in FBS and assayed for efflux to HDL in the media. Neu1^{hypo} macrophages show a significant increase at (A) 6, 8, and (C) 24 hours compared to C57Bl/6, while this difference disappears between LDLR-/- and LDLR-/-Neu1^{hypo} cells at (C) 6, 8, and (D) 24 hours. Efflux to BSA was used as a negative control and showed negligible levels and no difference between groups. Efflux is expressed as a percent of total radioactive cholesterol originally taken up by the cell (n=3). *=p<.0.05

Figure 5.5

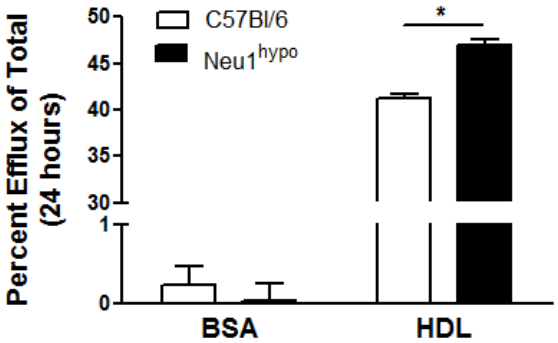
A.



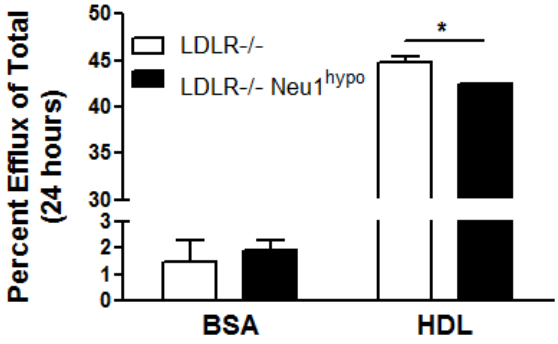
B.



C.



D.



5.5 DISCUSSION

We have previously shown that sialidase (Neu1) deficiency results in hypersialylation of LDLR, alterations in function of hepatic LDLR, and reductions of serum LDL in mice. We also have previous data showing increased LDL uptake in Neu1 null fibroblasts. Furthermore, we have observed that sialidase inhibition using DANA can modulate VLDL uptake and inflammatory response in THP-1 macrophages. Due to its apparent atheroprotective effect, we wanted to investigate the role of sialidase deficiency and inhibition on macrophage cholesterol homeostasis. Specifically, we sought to analyze lipid uptake and cholesterol efflux using murine hypomorphic Neu1 macrophages and human THP-1 macrophages treated with the sialidase inhibitor DANA. Macrophages are critical to progression of atherosclerosis and in this study we show that reduction in sialidase activity can increase LDL uptake and cholesterol efflux in an LDLR-dependent manner, presenting a novel role for Neu1 sialidase in macrophage lipoprotein metabolism and atherosclerotic mechanisms.

To test the effects of sialidase inhibition on LDL metabolism, we analyzed THP-1 human macrophages using Oil Red O (ORO) staining after LDL treatment. The concentration of DANA (and thus the amount of sialidase inhibition) showed a direct correlation with increased LDL uptake as measured by ORO, and this indicates that conditions with low sialidase activity are causing the macrophage to take up more LDL, presumably due to effects on LDLR. To investigate this phenomenon, we utilized quantitative real time PCR on cDNA from THP-1 macrophages that were treated with

LDL, DANA or both. We show that LDLR gene expression is reduced by LDL, as expected, but is reduced much less by LDL in combination with DANA. Sialidase inhibition reduces the macrophage's response to LDL and down-regulates receptor levels to a lesser degree. This correlates with the previous findings, as LDL uptake is a direct result of LDLR expression and function, and DANA treated cells would continue to internalize more LDL if the mRNA expression remains high. These high levels persist independently of the associated high cholesterol content, and this is unexpected, since SREBP-2 would presumably down-regulate LDLR transcript if the macrophage is engorged with cholesterol (Brown and Goldstein, 1997). This implies that sialidase inhibition is causing drastic changes in macrophage cholesterol regulation or intracellular distribution, partly due to hypersialyaltion of LDLR, although the exact mechanism remains unknown.

We also analyzed LDL uptake in macrophages from Neu1hypo mice that have hypomorphic Neu1 expression and reduced sialidase activity in hematopoeitic immune cell lineages (Champigny et al., 2009). We treated thioglycollate elicited intraperitoneal macrophages with LDL and analyzed lipid droplet formation by ORO staining. Macrophages show significantly increased staining in $Neu1^{hypo}$ cells that received LDL versus no LDL and no significant effect was seen in wild-type C57Bl/6 controls, although there was a slight increase. Negligible increases in staining were present in the LDLR-/- controls most likely due to non-selective macropinocytosis (Kruth et al., 2005). These data reveal that LDL uptake is increased under sialidase deficient conditions in mouse

macrophages, most likely due to effects on LDLR. We hypothesize that increased LDL uptake could facilitate removal of lipoproteins from circulation.

Bone marrow derived macrophages (BMDMs) were isolated from Neu1^{hypo} and wild-type C57Bl/6 mice, after which they were cultured and treated with lipoproteins. We utilized BMDMs to further study lipoprotein uptake and protein expression of macrophages under conditions of reduced sialidase activity. Untreated and VLDL-treated Neu1^{hypo} macrophages had significantly lower LDLR protein levels compared to C57Bl/6, while showing no significant difference in LDL and acLDL treated group. This indicates that sialidase deficiency is reducing the quantity of LDLR protein, either by reducing the cell's cholesterol need or through other post transcriptional mechanisms. We have shown in a previous study that hepatic Neu1^{hypo} LDLR is hypersialylated, and hypothesize that this could have effects on receptor-ligand interactions or recycling of LDLR (Yang et al., 2012). Based on the THP-1 and BMDM VLDL data, it is curious that LDLR protein levels remained unchanged in LDL-treated macrophages; however, this can be explained if Neu1^{hypo} macrophages maintain their levels of LDLR despite higher intracellular cholesterol content, which would result in continued uptake of LDL. This further implicates Neu1 in regulation of LDLR and macrophage cholesterol homeostasis. Hypomorphic sialidase expression also causes increases in ABCG1 protein levels after lipid loading of macrophages compared to C57Bl/6, while ABCA1 levels remain unchanged between genotypes. These ATP binding cassette molecules play important roles in cholesterol efflux and macrophage lipid homeostasis and could be responsible

for altering cholesterol content in Neu1^{hypo} mouse macrophages (Yvan-Charvet et al., 2007). Increased ABCG1 protein indicates that Neu1^{hypo} macrophages could have increased cholesterol efflux to HDL, as well as alterations in intracellular cholesterol trafficking (Tarling, 2013). ABCG1 has been shown to redistribute cholesterol away from the ER and result in processing and activation of SREBP2 (Tarling and Edwards, 2011). This could aid in explaining the phenotype observed in Neu1^{hypo} macrophages, as heightened levels of ABCG1 would result in re-distribution of cholesterol away from the ER, and thus processing of SREBP2 and maintenance of LDLR transcript. In addition to effects on cholesterol levels and SREBP2 due to hypomorphic sialidase expression, we have observed effects on proprotein convertase subtilisin/kexin type 9 (PCSK9) levels in Neu1^{hypo} hepatocytes in a previous study (Yang et al., 2012). We can rule out any effects of PCSK9 on degradation of LDLR in this study, as macrophages do not express this protein, and it is quite plausible that sialidase's effects on LDLR regulation exhibit different mechanisms in different cell types (Ferri et al., 2012;Horton et al., 2007).

Nevertheless, functional analysis of increased macrophage ABCG1 levels was necessary in order to elucidate any effects on lipoprotein metabolism and atherosclerosis. Therefore we performed cholesterol efflux assays to HDL using radioactively labelled cholesterol. Expectedly, Neu1^{hypo} BMDMs had significantly higher cholesterol efflux to HDL compared to C57Bl/6 and this trend continued to increase up to 24 hours. Hypomorphic Neu1 expression had no such effect on cholesterol efflux when the mice were on an LDLR-/- background, indicating that LDLR is necessary for this

atheroprotective phenotype. Macrophages from hypomorphic sialidase mice appear to be taking up more LDL cholesterol via LDLR and then unloading it to HDL. Not only would increased LDL uptake by macrophages reduce circulating LDL concentrations and lower chance of oxLDL formation, but increasing cholesterol efflux would result in less cholesterol accumulation by macrophages and decreased foam cell formation (Moore and Tabas, 2011;Soccio and Breslow, 2004). LDLR could be involved more directly in efflux or cholesterol trafficking mechanisms, but this is not known. While the heightened cholesterol efflux to HDL in Neu1^{hypo} macrophages is primarily due to up-regulation of ABCG1, alterations in intracellular cholesterol distribution, or cholesterol efflux via SRB-1 cannot be ruled out (Gu et al., 2000;Ouimet and Marcel, 2012). Furthermore, the exact contributions of ABCG1 to lipoprotein levels and atherogenesis remain under debate, as the results of knockout studies are clouded by compensation of ABCA1 and mixed effects on lesion formation (Tarling, 2013;Yvan-Charvet et al., 2007). Ubiquitous over-expression of ABCG1 has been shown to increase atherosclerosis on an LDLR-/- background; however, over-expression of macrophage ABCG1 and adenoviral mediated up-regulation of ABCG1 in the vasculature of rabbits have been shown to promote HDL-mediated cholesterol efflux and atheroprotection (Basso et al., 2006;Munch et al., 2012;Tarling, 2013). Clearly, the role of ABCG1 in atherosclerosis progression and cholesterol metabolism is quite complex; however, we show that increased protein levels of ACBG1 could be the cause behind the increased cholesterol efflux to HDL in Neu1^{hypo} macrophages. Up-regulating macrophage-specific ABCG1 in vivo, would

presumably increase reverse cholesterol transport via HDL and confer atheroprotection. We are currently investigating the role of hematopoietic hypomorphic expression of Neu1 on atherosclerosis progression in vivo in LDLR-/- and Apo-E-/- mouse models.

In this study, we have shown that Neu1 plays an intricate role in macrophage cholesterol homeostasis and that its reduction or inhibition would result in an atheroprotective phenotype due to increased LDL uptake and heightened ABCG1-mediated cholesterol efflux to HDL. While the mechanism is still under investigation, this data indicates that sialidase deficiency can directly influence macrophage LDLR regulation and function with direct effects on macrophage cholesterol homeostasis. We provide strong evidence for roles of Neu1 in LDL cholesterol metabolism and atherosclerosis, and lay a foundation for further investigation of the exact molecular mechanisms involved in order to clarify implications for future novel therapies.

5.6 REFERENCES

Barton M (2013) Mechanisms and therapy of atherosclerosis and its clinical complications. Curr Opin Pharmacol 13:149-153

Basso F, Amar MJ, Wagner EM, Vaisman B, Paigen B, Santamarina-Fojo S, Remaley AT (2006) Enhanced ABCG1 expression increases atherosclerosis in LDLr-KO mice on a western diet. Biochem Biophys Res Commun 351:398-404

Boisvert WA, Spangenberg J, Curtiss LK (1997) Role of leukocyte-specific LDL receptors on plasma lipoprotein cholesterol and atherosclerosis in mice. Arterioscler Thromb Vasc Biol 17:340-347

Bonnet P, Bryce RA (2004) Molecular dynamics and free energy analysis of neuraminidase-ligand interactions. Protein Sci 13:946-957

Brown MS, Goldstein JL (1997) The SREBP pathway: regulation of cholesterol metabolism by proteolysis of a membrane-bound transcription factor. Cell 89:331-340

Champigny MJ, Mitchell M, Fox-Robichaud A, Trigatti BL, Igdoura SA (2009) A point mutation in the neu1 promoter recruits an ectopic repressor, Nkx3.2 and results in a mouse model of sialidase deficiency. Mol Genet Metab 97:43-52

Daugherty A, Webb NR, Rateri DL, King VL (2005) Thematic review series: The immune system and atherogenesis. Cytokine regulation of macrophage functions in atherogenesis. J Lipid Res 46:1812-1822

Fazio S, Hasty AH, Carter KJ, Murray AB, Price JO, Linton MF (1997) Leukocyte low density lipoprotein receptor (LDL-R) does not contribute to LDL clearance in vivo: bone marrow transplantation studies in the mouse. J Lipid Res 38:391-400

Ferri N, Tibolla G, Pirillo A, Cipollone F, Mezzetti A, Pacia S, Corsini A, Catapano AL (2012) Proprotein convertase subtilisin kexin type 9 (PCSK9) secreted by cultured smooth muscle cells reduces macrophages LDLR levels. Atherosclerosis 220:381-386

Freeman LA (2013) Western blots. Methods Mol Biol 1027:369-385

Getz GS, Reardon CA (2009) Apoprotein E as a lipid transport and signaling protein in the blood, liver, and artery wall. J Lipid Res 50 Suppl:S156-S161

Glass CK, Witztum JL (2001) Atherosclerosis. the road ahead. Cell 104:503-516

Go GW, Mani A (2012) Low-density lipoprotein receptor (LDLR) family orchestrates cholesterol homeostasis. Yale J Biol Med 85:19-28

Goedeke L, Fernandez-Hernando C (2012) Regulation of cholesterol homeostasis. Cell Mol Life Sci 69:915-930

Goldstein JL, Brown MS, Anderson RG, Russell DW, Schneider WJ (1985) Receptor-mediated endocytosis: concepts emerging from the LDL receptor system. Annu Rev Cell Biol 1:1-39

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

Gui T, Shimokado A, Sun Y, Akasaka T, Muragaki Y (2012) Diverse roles of macrophages in atherosclerosis: from inflammatory biology to biomarker discovery. Mediators Inflamm 2012:693083

Hegele RA (2009) Plasma lipoproteins: genetic influences and clinical implications. Nat Rev Genet 10:109-121

Herijgers N, Van EM, Groot PH, Hoogerbrugge PM, Van Berkel TJ (2000) Low density lipoprotein receptor of macrophages facilitates atherosclerotic lesion formation in C57Bl/6 mice. Arterioscler Thromb Vasc Biol 20:1961-1967

Horton JD, Cohen JC, Hobbs HH (2007) Molecular biology of PCSK9: its role in LDL metabolism. Trends Biochem Sci 32:71-77

Ishibashi S, Brown MS, Goldstein JL, Gerard RD, Hammer RE, Herz J (1993) Hypercholesterolemia in low density lipoprotein receptor knockout mice and its reversal by adenovirus-mediated gene delivery. J Clin Invest 92:883-893

Kockx M, Jessup W, Kritharides L (2008) Regulation of endogenous apolipoprotein E secretion by macrophages. Arterioscler Thromb Vasc Biol 28:1060-1067

Kozaki K, Akishita M, Eto M, Yoshizumi M, Toba K, Inoue S, Ishikawa M, Hashimoto M, Kodama T, Yamada N, Orimo H, Ouchi Y (1997) Role of activin-A and follistatin in foam cell formation of THP-1 macrophages. Arterioscler Thromb Vasc Biol 17:2389-2394

Kruth HS, Jones NL, Huang W, Zhao B, Ishii I, Chang J, Combs CA, Malide D, Zhang WY (2005) Macropinocytosis is the endocytic pathway that mediates macrophage foam cell formation with native low density lipoprotein. J Biol Chem 280:2352-2360

Levitan I, Volkov S, Subbaiah PV (2010) Oxidized LDL: diversity, patterns of recognition, and pathophysiology. Antioxid Redox Signal 13:39-75

Liang F, Seyrantepe V, Landry K, Ahmad R, Ahmad A, Stamatos NM, Pshezhetsky AV (2006) Monocyte differentiation up-regulates the expression of the lysosomal sialidase, Neu1, and triggers its targeting to the plasma membrane via major histocompatibility complex class II-positive compartments. J Biol Chem 281:27526-27538

Libby P, Ridker PM, Hansson GK (2011) Progress and challenges in translating the biology of atherosclerosis. Nature 473:317-325

Linton MF, Babaev VR, Gleaves LA, Fazio S (1999) A direct role for the macrophage low density lipoprotein receptor in atherosclerotic lesion formation. J Biol Chem 274:19204-19210

LOWRY OH, ROSEBROUGH NJ, FARR AL, RANDALL RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193:265-275

Luo M, Jedrzejas MJ, Singh S, White CL, Brouillette WJ, Air GM, Laver WG (1995) Benzoic acid inhibitors of influenza virus neuraminidase. Acta Crystallogr D Biol Crystallogr 51:504-510

Meindl P, Bodo G, Palese P, Schulman J, Tuppy H (1974) Inhibition of neuraminidase activity by derivatives of 2-deoxy-2,3-dehydro-N-acetylneuraminic acid. Virology 58:457-463

Miyagi T, Yamaguchi K (2012) Mammalian sialidases: physiological and pathological roles in cellular functions. Glycobiology 22:880-896

Moore KJ, Sheedy FJ, Fisher EA (2013) Macrophages in atherosclerosis: a dynamic balance. Nat Rev Immunol 10:709-21

Moore KJ, Tabas I (2011) Macrophages in the pathogenesis of atherosclerosis. Cell 145:341-355

Munch G, Bultmann A, Li Z, Holthoff HP, Ullrich J, Wagner S, Ungerer M (2012) Overexpression of ABCG1 protein attenuates arteriosclerosis and endothelial dysfunction in atherosclerotic rabbits. Heart Int 7:e12

Ouimet M, Marcel YL (2012) Regulation of lipid droplet cholesterol efflux from macrophage foam cells. Arterioscler Thromb Vasc Biol 32:575-581

Pattison S, Pankarican M, Rupar CA, Graham FL, Igdoura SA (2004) Five novel mutations in the lysosomal sialidase gene (NEU1) in type II sialidosis patients and assessment of

their impact on enzyme activity and intracellular targeting using adenovirus-mediated expression. Hum Mutat 23:32-39

Pshezhetsky AV, Hinek A (2011) Where catabolism meets signalling: neuraminidase 1 as a modulator of cell receptors. Glycoconj J 28:441-452

Qiu G, Hill JS (2007) Atorvastatin decreases lipoprotein lipase and endothelial lipase expression in human THP-1 macrophages. J Lipid Res 48:2112-2122

Radhakrishnan A, Goldstein JL, McDonald JG, Brown MS (2008) Switch-like control of SREBP-2 transport triggered by small changes in ER cholesterol: a delicate balance. Cell Metab 8:512-521

Rosenson RS, Brewer HB, Jr., Davidson WS, Fayad ZA, Fuster V, Goldstein J, Hellerstein M, Jiang XC, Phillips MC, Rader DJ, Remaley AT, Rothblat GH, Tall AR, Yvan-Charvet L (2012) Cholesterol efflux and atheroprotection: advancing the concept of reverse cholesterol transport. Circulation 125:1905-1919

Rottier RJ, Bonten E, d'Azzo A (1998) A point mutation in the neu-1 locus causes the neuraminidase defect in the SM/J mouse. Hum Mol Genet 7:313-321

Scholz H, Aukrust P, Damas JK, Tonstad S, Sagen EL, Kolset SO, Hall C, Yndestad A, Halvorsen B (2004) 8-isoprostane increases scavenger receptor A and matrix metalloproteinase activity in THP-1 macrophages, resulting in long-lived foam cells. Eur J Clin Invest 34:451-458

Shelness GS, Ingram MF, Huang XF, DeLozier JA (1999) Apolipoprotein B in the rough endoplasmic reticulum: translation, translocation and the initiation of lipoprotein assembly. J Nutr 129:456S-462S

Shite S, Seguchi T, Mizoguchi H, Ono M, Kuwano M (1990) Differential effects of brefeldin A on sialylation of N- and O-linked oligosaccharides in low density lipoprotein receptor and epidermal growth factor receptor. J Biol Chem 265:17385-17388

Soccio RE, Breslow JL (2004) Intracellular cholesterol transport. Arterioscler Thromb Vasc Biol 24:1150-1160

Sprague EA, Moser M, Edwards EH, Schwartz CJ (1988) Stimulation of receptor-mediated low density lipoprotein endocytosis in neuraminidase-treated cultured bovine aortic endothelial cells. J Cell Physiol 137:251-262

Steinberg D, Witztum JL (1990) Lipoproteins and atherogenesis. Current concepts. JAMA 264:3047-3052

Tarling EJ (2013) Expanding roles of ABCG1 and sterol transport. Curr Opin Lipidol 24:138-146

Tarling EJ, Edwards PA (2011) ATP binding cassette transporter G1 (ABCG1) is an intracellular sterol transporter. Proc Natl Acad Sci U S A 108:19719-19724

Varki A (2008) Sialic acids in human health and disease. Trends Mol Med 14:351-360

Wang MD, Kiss RS, Franklin V, McBride HM, Whitman SC, Marcel YL (2007) Different cellular traffic of LDL-cholesterol and acetylated LDL-cholesterol leads to distinct reverse cholesterol transport pathways. J Lipid Res 48:633-645

Yang A, Gyulay G, Mitchell M, White E, Trigatti BL, Igdoura SA (2012) Hypomorphic sialidase expression decreases serum cholesterol by downregulation of VLDL production in mice. J Lipid Res 53:2573-2585

Yvan-Charvet L, Ranalletta M, Wang N, Han S, Terasaka N, Li R, Welch C, Tall AR (2007) Combined deficiency of ABCA1 and ABCG1 promotes foam cell accumulation and accelerates atherosclerosis in mice. J Clin Invest 117:3900-3908

Zhang Y, Albohy A, Zou Y, Smutova V, Pshezhetsky AV, Cairo CW (2013) Identification of selective inhibitors for human neuraminidase isoenzymes using C4,C7-modified 2-deoxy-2,3-didehydro-N-acetylneuraminic acid (DANA) analogues. J Med Chem 56:2948-2958

CHAPTER 6: Discussion

6.1 GENERAL REVIEW

Atherosclerosis is a multifaceted disease involving a plethora of risk factors and mechanisms of disease progression (Barton, 2013). The interplay of lipoprotein metabolism and inflammation emerges as a recurring and central theme in atherogenesis, with macrophages appearing at the crux of this intricate relationship (Moore et al., 2013). Continuing to delineate the molecular dynamics of lipoprotein metabolism and inflammation are crucial in advancing knowledge of atherosclerosis and in discovering treatment strategies. The importance of Neu1 sialidase has been well characterized in diverse molecular, cellular and physiological processes, yet its direct function in atherosclerotic mechanisms remains enigmatic. We demonstrate a significant role for sialidase in several major pathways relevant to atherosclerosis, including regulation of hepatic cholesterol and triglyceride (TG) metabolism, lipoprotein metabolism, LDLR function, aortic sinus lipid deposition, monocyte differentiation, foam cell formation, inflammation, and macrophage cholesterol homeostasis. We have shown that modification of sialidase activity can affect a diverse array of molecular targets and glycogonjucates that play pivotal functional roles in atherosclerosis. This work presents novel implications for sialidase as a player in metabolic and inflammatory processes of atherosclerosis and sets the foundation for further investigation and future treatment strategies.

Specifically, this body of evidence shows that hypomorphic expression of Neu1 (using $Neu1^{hypo}$ mice) increases hepatic cholesterol and lipid storage *in vivo* compared to C57Bl/6 controls. These mice also had a reduction in VLDL-TG production, lower MTP and SREBP-2 protein levels, and higher levels of DGAT-2. Rescue of sialidase activity using helper dependent adenovirus caused increased VLDL production and an increase in MTP levels. We also showed that hypomorphic sialidase expression results in stabilization of hepatic LDLR protein levels, and all of these findings point to hypomorphic sialidase deficiency resulting in an atheroprotective phenotype. Additionally, we have generated a novel transgenic mouse that expresses human sialidase (hNEU1) in the liver and these mice showed an increased susceptibility to atherosclerosis after high fat diet feeding compared to controls. Mice expressing hNEU1 had a reduction in hepatic cholesterol ester levels and a rise in serum cholesterol ester levels and this was coupled with reduced hepatic protein expression of LDLR and SRB-1 compared to controls. We also observed that overall cell-surface sialylation of blood and spleen leukocytes was decreased in mice expressing hepatic hNEU1 compared to controls, and these findings indicate that hepatic human sialidase can induce atherogenesis and alterations in lipoprotein metabolism and immune cell sialylation in mice. Furthermore, *in vitro* studies have demonstrated that sialidase inhibition using DANA can attenuate VLDL induced differentiation, lipid uptake, and activation of THP-1 monocytes. Not only did we uncover novel roles for VLDL in early atherosclerotic mechanisms, but we showed that sialidase activity can modulate these potent and

diverse atherogenic effects of VLDL. Lastly, we have shown that sialidase activity is directly linked to macrophage cholesterol metabolism, and that hypomorphic expression of Neu1 or sialidase inhibition using DANA results in increased LDL uptake and aberrant regulation of LDLR in macrophages. Neu1^{hypo} macrophages also have increased ABCG1 levels and heightened cholesterol efflux to HDL, indicative of an atheroprotective phenotype.

We have utilized a diverse battery of techniques and experimental models in order to gain a more complete understanding of the effects sialidase activity has on inflammation and lipid/cholesterol metabolism. Our *in vivo* and *in vitro* approaches have allowed us to address both murine and human Neu1 in several different tissues and cell types. This work has relevance in unveiling potential treatment strategies for atherosclerosis that target Neu1 and it also sets the bar for further investigation of the molecular mechanisms affected by sialidase.

6.2 ANALYSIS, FUTURE DIRECTIONS, AND LIMITATIONS

In the first study, we examined the lipoprotein and cholesterol metabolism of a mouse model expressing hypomorphic levels of Neu1. We determined that Neu1^{hypo} livers have higher cholesterol content, resulting in lower levels of active SREBP-2, and reduced MTP protein and VLDL-TG production. We hypothesize that the higher cholesterol levels are driving the reduction in MTP activity through SREBP-2, and that this is contributing to the phenotype of the mouse. Several questions remain, including what causes the initial rise in increased hepatic cholesterol, and also how SREBP-2

affects MTP and VLDL-TG production. The heightened cholesterol content could be explained due to redistribution of cholesterol from the serum (which has lower LDL compared to C57Bl/6) into the liver, and due to alterations in intestinal cholesterol absorption/trafficking in Neu1^{hypo} animals. These hypotheses, along with the phenotype of adipocytes, could be explored further in order to gain a better understanding of what is occurring systemically in hypomorphic Neu1 mice. SREBP-2 can directly regulate MTP gene expression, but there is conflicting evidence as to how they interact (Horton et al., 1998;Horton et al., 1999;Sato et al., 1999). Further elucidation of the relationship between MTP levels and SREBP-2 activity would aid in confirming our results that lower SREBP-2 is the driving force behind the lower VLDL-TG production and subsequent decrease in LDL. Additionally, while we show that LDLR is hypersialylated, we cannot quantitatively state to what extent, and in what part of the molecule. LDLR is heavily glycoyslated and sialylated, and it would be interesting to test which regions of LDLR are affected by reduced sialidase expression, and with what functional consequences. The amount of sialylation on LDLR could be measured more quantitatively using mass spectrometry or 2-dimensional gel electrophoresis in Neu1^{hypo} mice and controls. Functional assays of LDL internalization and binding to PCSK9 could be performed using LDL receptors that have different glycosylation sites removed using mutation or glycanase enzymes. This would enable dissection of the exact glycans and sialic acid residues that are involved in LDLR function and are affected by hypomorphic Neu1

expression. This information would aid in explaining the mechanism behind sialidase's effect on LDLR function and regulation.

Secondly, we examined the effects of hepatic expression of NEU1 sialidase on lipoprotein metabolism and atherosclerosis in FVB mice. The creation of this novel transgenic mouse enabled us to study human sialidase *in vivo* and to determine its effects in a tissue-specific manner. We attribute the increase in aortic sinus lipid deposition in $mTA^{+}hNEU1^{+}$ mice to redistribution of cholesterol esters from the liver into the bloodstream, and due to lower hepatic protein levels of LDLR and SRB-1 compared to controls. While these observations point to alterations in cholesterol metabolism in $mTA^{+}hNEU1^{+}$ animals, we did not determine the exact molecular interactions of hNEU1 and these targets. As mentioned above, determination of the exact sialylation and functional effects on LDLR and SRB-1 would be quite beneficial in determining the molecular mechanisms involved. Investigation of other hepatic and serum proteins that play roles in cholesterol metabolism, and specifically esterification and its regulation, would also aid in understanding the phenotype observed. While all the mice used for our experimental analyses displayed hNEU1 expression in their livers, we are still currently characterizing the expression of hNEU1 in other tissues of these mice and investigating the incomplete penetrance, as some $mTA^{+}hNEU1^{+}$ do not express hNEU1 cDNA or protein. A large number of mice from all genotype groups and thorough qRT-PCR and protein analysis of several tissues would serve this purpose, and aid in fully characterizing this novel transgenic model. Additionally, sequencing of these animals

would yield information into where the transgene has inserted into the genome, and could provide key information with regards to transcriptional regulation. Furthermore, we found that both serum and spleen leukocyte sialylation is reduced in $mTA^{+}hNEU1^{+}$ animals compared to controls, and this indicated that the human sialidase is having an extrahepatic effect. It would be interesting to elaborate on these findings and to investigate which cell surface glycomoeties are being desialylated, and if there are any potential functional consequences on their adhesion, rolling and differentiation (Gee et al., 2003;Lambre et al., 1990;Stamatos et al., 2005). Conclusive characterization of human sialidase expression on blood and splenic leukocytes would also be informative in understanding where this desialylation is occurring; there could be leakiness of the transgene which would lead to increased NEU1 on leukocytes, or perhaps sialidase is being secreted from the liver. Utilizing the tet-off system and treating mice with tetracycline will also serve as an important control. Future projects that would make use of this novel strain include using other tissue specific tTA mice, and to create a full transgenic where human NEU1 is over-expressed ubiquitously. Furthermore, these animals could be crossed to Apo-E-/- and LDLR-/- backgrounds in order to study more advanced atherosclerosis.

Our THP-1 *in vitro* study has shown that VLDL can induce monocyte differentiation, foam cell formation, and inflammatory response. Sialidase plays a key role in these early atherosclerotic events induced by VLDL, as its inhibition reduced monocyte differentiation, lipid uptake and cytokine production. In order to delineate

the mechanism of how VLDL can initially stimulate the differentiation and activation of monocytes, knockout and inhibition experiments using antibodies/siRNA would be necessary. Based on our data and the literature, potential targets such as LRP-1, VLDLR, LPL and TLRs can be down-regulated to see if VLDL is still having similar influences (Eck et al., 2005;Jinno et al., 2011;Kosaka et al., 2001;Lillis et al., 2005;Lillis et al., 2008;May et al., 2005;Palmer et al., 2005;Sakthivel et al., 2001). We have clarified the downstream effects in terms of cellular phenotype, signalling pathways elicited, and proteins involved, but it is important to know how VLDL is initiating these events at the cell surface. Investigation of other cytokines and transcription factors elicited could also prove worthwhile in sorting out the molecular events of VLDL induction in monocytes and macrophages. The THP-1 cell line is useful for studying monocyte and macrophage dynamics and metabolism, but showing similar effects of VLDL on other cell types, such as monocytes isolated from human blood, would add more validity and direct clinical relevance to our work. Teasing out exactly how sialidase inhibition dampens the effects of VLDL could be accomplished using knockout and down-regulation studies as above. It is important to determine whether DANA is affecting the potential interaction of Apo-CIII and TLR2, as this pathway has been postulated previously to induce monocyte adherence and TLR's require sialidase activity (Amith et al., 2009;Kawakami et al., 2006b). It is also important to determine whether DANA is having its effect by directly altering the lipoprotein's interaction with receptors at the cell surface and influencing cell signalling, or whether the reduction in differentiation and inflammation observed is

due to reductions in lipid accumulation, which can then affect cell signalling pathways. Again, we show the end result and phenotype of sialidase inhibition on VLDL treatment of macrophages, but its potential molecular actions remain complex. Flow cytometry analysis of cell surface adhesion molecules and their sialylation would also be beneficial in determining the mechanisms of how DANA is influencing VLDL-induced differentiation of monocytes.

Lastly, we have shown that decreasing sialidase activity can increase LDL uptake and cholesterol efflux in an LDLR-dependent manner in macrophages. Similar to our other work, we show that the function and regulation of LDLR is altered in hypomorphic NEU1 macrophages or THP-1 macrophages treated with DANA, leading to higher cholesterol content. It would be useful to investigate the protein levels of SREBP-2, in addition to our LDLR protein and q-RT analyses, in order to delineate how LDLR is regulated in these cell types. Furthermore, levels of PPAR and LXR groups of transcription factors could be investigated as these proteins can regulate cholesterol efflux and homeostasis in macrophages (Chawla et al., 2001;Hong and Tontonoz, 2008). The measurement of cholesterol efflux to Apo-A1 might also prove worthwhile in order to delineate whether sialidase is affecting solely HDL loading of cholesterol or not. The *in vivo* implications of these findings are currently being investigated in other projects in our lab using bone marrow transplantation of hypomorphic sialidase bone marrow in Apo-E-/- and LDLR-/- knockout donors. Abraham Yang has shown that transplantation of $Neu1^{hypo}$ bone marrow results in reduced aortic sinus lesions compared to bone marrow

with WT sialidase on both these atherogenic backgrounds. Increased LDL clearance and efflux to HDL by macrophages (derived from the bone marrow) are plausible hypotheses that can explain this atheroprotection.

Overall, we have proven that sialidase has a prevalent function in several important mechanisms involved in cholesterol metabolism and immune response. Neu1 is a contributing factor in the complex and multifarious disease process of atherosclerosis, and it would be interesting to perform genome wide association studies to potentially link Neu1 with other loci in cardiovascular disease. Furthermore, our studies and animal models can be extended to investigate other metabolic syndrome and inflammatory diseases. Analysing glucose metabolism, adipocyte metabolism and diabetes are all fruitful avenues for investigation of Neu1. In fact, we have preliminary data implicating hypomorphic Neu1 expression in AKT signalling, glucose clearance, and diabetes. Our current mouse models can be used to investigate these pathways more thoroughly and to expand the breadth of metabolic pathways that are influenced by sialidase.

6.3 CLINICAL IMPLICATIONS AND PERSPECTIVES

Atherosclerosis is an extremely prominent and deadly disease, especially in the western world; its associated clinical complications represent an enormous financial and social burden on society (Barton, 2013). Several treatment strategies, including statins, fibrates, MTP inhibitors, Apo-B inhibitors, PCSK9 inhibitors, and intestinal cholesterol

absorption inhibitors are currently being developed and utilized with mixed success (Faiz et al., 2012;Katsiki et al., 2013;Kramer et al., 2005;Lopez, 2008). The goal of these therapies is to lower LDL and to increase HDL, yet some patients often cannot tolerate the side effects of these medications, nor are the drugs even effective in the first place (Faiz et al., 2012). Cholesterol ester transfer protein inhibitors were initially deemed to be a promising family of atherosclerosis drugs, yet their safety and efficacy is still under debate (Bochem et al., 2013). Drugs that address hypertension are useful in preventing acute complications of atherosclerosis, but do little to reduce cholesterol levels and plaque progression (Barton, 2013). Simple preventative strategies such as exercise and a healthy diet are theoretically sufficient for non-genetically predisposed individuals to avoid atherosclerosis, yet environmental, socioeconomic and lifestyle factors appear to cloud this seemingly simple solution (Barton, 2013;Glass and Witztum, 2001). More research into personalized medicine and pharmacogenomics has been proposed in cardiovascular disease and atherosclerosis as well (Yip and Pirmohamed, 2013). Thus, novel therapies are currently under development, and a theme for comprehensive and multi-mechanistic treatment plans is emerging in hopes of better management of risk factors and disease progression (Kones, 2013;Yip and Pirmohamed, 2013).

Our work represents not only insight into molecular and cellular pathways in atherosclerosis, but it also paves the way for new treatment strategies that address lipoprotein metabolism and inflammation in unison. The importance of inflammation in atherosclerosis is only recently being emphasized, and treatment plans are still primarily

targeting only dyslipidemia or hypertension (Gui et al., 2012;Hansson, 2009). NEU1 sialidase represents a unique target that could potentially address multiple mechanisms of atherosclerosis; including LDLR, hepatic cholesterol/TG metabolism, VLDL production, immune cell sialylation, RCT, monocyte differentiation and foam cell formation. Although significantly more work and elucidation of molecular mechanisms need to be performed, inhibition or reduction of sialidase activity represents a modern and comprehensive therapeutic strategy. Our mouse and *in vitro* models set the stage for further work and potentially future clinical trials that would utilize NEU1 as an attractive pharmaceutical or genetic target. Determining the appropriate amount of inhibition, the tissue specificity as well as the cellular localization of the NEU1 being inhibited are extremely important challenges for the future that must be addressed. We hypothesize that reduction of NEU1 activity in humans would reduce serum LDL, increase cholesterol clearance from macrophages, and reduce arterial inflammation. Potential adverse effects of *in vivo* sialidase inhibition include accumulation of cellular glycoconjucates, damped systemic immune response, and defects in lysosomal trafficking/function, and these caveats warrant stringent consideration. Nevertheless, while the end result appears distant, our discoveries in this field will lay the groundwork for future molecular and clinical studies, and have solidified the importance of sialidase in atherosclerosis.

6.4 CONCLUSION

We have utilized a diverse and multi-faceted approach in order to study Neu1 sialidase and its involvement in multiple mechanisms of atherosclerosis. We have teased apart and analyzed several pathways and opened the doors for future questions and studies. We are obtaining a reliable overall picture of how sialidase can alter cholesterol homeostasis, lipoprotein metabolism, monocyte differentiation and inflammation. Not only have we shown that the effects of this enzyme permeate many pathways, cells, and tissue types involved in atherosclerosis, but we have identified a potential novel target for future treatment strategies. Neu1 has unique and prevalent functions in atherosclerosis and its associated pathways, and the work and ideas presented here could lead to fascinating future studies and eventual development of a multi-pronged therapeutic agent.

REFERENCES

Abdulkhalek S, Amith SR, Franchuk SL, Jayanth P, Guo M, Finlay T, Gilmour A, Guzzo C, Gee K, Beyaert R, Szewczuk MR (2011) Neu1 sialidase and matrix metalloproteinase-9 cross-talk is essential for Toll-like receptor activation and cellular signaling. J Biol Chem 286:36532-36549

Amith SR, Jayanth P, Franchuk S, Siddiqui S, Seyrantepe V, Gee K, Basta S, Beyaert R, Pshezhetsky AV, Szewczuk MR (2009) Dependence of pathogen molecule-induced Toll-like receptor activation and cell function on Neu1 sialidase. Glycoconj J 26:1197-212

Aviram M, Rosenblat M (1994) Macrophage-mediated oxidation of extracellular low density lipoprotein requires an initial binding of the lipoprotein to its receptor. J Lipid Res 35:385-398

Babaev VR, Fazio S, Gleaves LA, Carter KJ, Semenkovich CF, Linton MF (1999) Macrophage lipoprotein lipase promotes foam cell formation and atherosclerosis in vivo. J Clin Invest 103:1697-1705

Barter PJ, Brewer HB, Jr., Chapman MJ, Hennekens CH, Rader DJ, Tall AR (2003) Cholesteryl ester transfer protein: a novel target for raising HDL and inhibiting atherosclerosis. Arterioscler Thromb Vasc Biol 23:160-167

Barton M (2013) Mechanisms and therapy of atherosclerosis and its clinical complications. Curr Opin Pharmacol 13:149-153

Bie J, Wang J, Marqueen KE, Osborne R, Kakiyama G, Korzun W, Ghosh SS, Ghosh S (2013) Liver-specific cholesteryl ester hydrolase deficiency attenuates sterol elimination in the feces and increases atherosclerosis in ldlr-/- mice. Arterioscler Thromb Vasc Biol 33:1795-1802

Bochem AE, Kuivenhoven JA, Stroes ES (2013) The promise of cholesteryl ester transfer protein (CETP) inhibition in the treatment of cardiovascular disease. Curr Pharm Des 19:3143-3149

Boisvert WA, Spangenberg J, Curtiss LK (1997) Role of leukocyte-specific LDL receptors on plasma lipoprotein cholesterol and atherosclerosis in mice. Arterioscler Thromb Vasc Biol 17:340-347

Bojic LA, Sawyez CG, Telford DE, Edwards JY, Hegele RA, Huff MW (2012) Activation of peroxisome proliferator-activated receptor delta inhibits human macrophage foam cell

formation and the inflammatory response induced by very low-density lipoprotein. Arterioscler Thromb Vasc Biol 32:2919-2928

Bonten EJ, Campos Y, Zaitsev V, Nourse A, Waddell B, Lewis W, Taylor G, d'Azzo A (2009) Heterodimerization of the sialidase NEU1 with the chaperone protective protein/cathepsin A prevents its premature oligomerization. J Biol Chem 284:28430-28441

Boucher P, Herz J (2011) Signaling through LRP1: Protection from atherosclerosis and beyond. Biochem Pharmacol 81:1-5

Brown AJ, Sun L, Feramisco JD, Brown MS, Goldstein JL (2002) Cholesterol addition to ER membranes alters conformation of SCAP, the SREBP escort protein that regulates cholesterol metabolism. Mol Cell 10:237-245

Businaro R, Tagliani A, Buttari B, Profumo E, Ippoliti F, Di CC, Capoano R, Salvati B, Rigano R (2012) Cellular and molecular players in the atherosclerotic plaque progression. Ann N Y Acad Sci 1262:134-141

Champigny MJ, Mitchell M, Fox-Robichaud A, Trigatti BL, Igdoura SA (2009) A point mutation in the neu1 promoter recruits an ectopic repressor, Nkx3.2 and results in a mouse model of sialidase deficiency. Mol Genet Metab 97:43-52

Chatterjee S (1998) Sphingolipids in atherosclerosis and vascular biology. Arterioscler Thromb Vasc Biol 18:1523-1533

Chawla A (2010) Control of macrophage activation and function by PPARs. Circ Res 106:1559-1569

Chawla A, Boisvert WA, Lee CH, Laffitte BA, Barak Y, Joseph SB, Liao D, Nagy L, Edwards PA, Curtiss LK, Evans RM, Tontonoz P (2001) A PPAR gamma-LXR-ABCA1 pathway in macrophages is involved in cholesterol efflux and atherogenesis. Mol Cell 7:161-171

Chen X, Varki A (2010) Advances in the biology and chemistry of sialic acids. ACS Chem Biol 5:163-176

Chen XP, Enioutina EY, Daynes RA (1997) The control of IL-4 gene expression in activated murine T lymphocytes: a novel role for neu-1 sialidase. J Immunol 158:3070-3080

Cross AS, Sakarya S, Rifat S, Held TK, Drysdale BE, Grange PA, Cassels FJ, Wang LX, Stamatos N, Farese A, Casey D, Powell J, Bhattacharjee AK, Kleinberg M, Goldblum SE (2003) Recruitment of murine neutrophils in vivo through endogenous sialidase activity. J Biol Chem 278:4112-4120

Cross AS, Wright DG (1991) Mobilization of sialidase from intracellular stores to the surface of human neutrophils and its role in stimulated adhesion responses of these cells. J Clin Invest 88:2067-2076

Cummings RD, Kornfeld S, Schneider WJ, Hobgood KK, Tolleshaug H, Brown MS, Goldstein JL (1983) Biosynthesis of N- and O-linked oligosaccharides of the low density lipoprotein receptor. J Biol Chem 258:15261-15273

Dabagh M, Jalali PP, Tarbell JM (2009) The transport of LDL across the deformable arterial wall: the effect of endothelial cell turnover and intimal deformation under hypertension. Am J Physiol Heart Circ Physiol 297:H983-96

Daugherty A, Webb NR, Rateri DL, King VL (2005) Thematic review series: The immune system and atherogenesis. Cytokine regulation of macrophage functions in atherogenesis. J Lipid Res 46:1812-1822

Davis CG, Elhammer A, Russell DW, Schneider WJ, Kornfeld S, Brown MS, Goldstein JL (1986) Deletion of clustered O-linked carbohydrates does not impair function of low density lipoprotein receptor in transfected fibroblasts. J Biol Chem 261:2828-2838

Davis RA (1997) Evolution of processes and regulators of lipoprotein synthesis: from birds to mammals. J Nutr 127:795S-800S

de GN, Bonten E, Mann L, de Sousa-Hitzler J, Hahn C, d'Azzo A (2002) Systemic and neurologic abnormalities distinguish the lysosomal disorders sialidosis and galactosialidosis in mice. Hum Mol Genet 11:1455-1464

den Hartigh LJ, Altman R, Hutchinson R, Petrlova J, Budamagunta MS, Tetali SD, Lagerstedt JO, Voss JC, Rutledge JC (2012) Postprandial apoE isoform and conformational changes associated with VLDL lipolysis products modulate monocyte inflammation. PLoS One 7:e50513

Dridi L, Seyrantepe V, Fougerat A, Pan X, Bonneil E, Thibault P, Moreau A, Mitchell GA, Heveker N, Cairo CW, Issad T, Hinek A, Pshezhetsky AV (2013) Positive Regulation of Insulin Signaling by Neuraminidase 1. Diabetes 62:2338-46

Eck MV, Oost J, Goudriaan JR, Hoekstra M, Hildebrand RB, Bos IS, van Dijk KW, Van Berkel TJ (2005) Role of the macrophage very-low-density lipoprotein receptor in atherosclerotic lesion development. Atherosclerosis 183:230-237

Faiz F, Hooper AJ, van Bockxmeer FM (2012) Molecular pathology of familial hypercholesterolemia, related dyslipidemias and therapies beyond the statins. Crit Rev Clin Lab Sci 49:1-17

Fazio S, Hasty AH, Carter KJ, Murray AB, Price JO, Linton MF (1997) Leukocyte low density lipoprotein receptor (LDL-R) does not contribute to LDL clearance in vivo: bone marrow transplantation studies in the mouse. J Lipid Res 38:391-400

Feng B, Yao PM, Li Y, Devlin CM, Zhang D, Harding HP, Sweeney M, Rong JX, Kuriakose G, Fisher EA, Marks AR, Ron D, Tabas I (2003) The endoplasmic reticulum is the site of cholesterol-induced cytotoxicity in macrophages. Nat Cell Biol 5:781-792

Feng C, Zhang L, Almulki L, Faez S, Whitford M, Hafezi-Moghadam A, Cross AS (2011) Endogenous PMN sialidase activity exposes activation epitope on CD11b/CD18 which enhances its binding interaction with ICAM-1. J Leukoc Biol 90:313-321

Filipovic I (1989) Effect of inhibiting N-glycosylation on the stability and binding activity of the low density lipoprotein receptor. J Biol Chem 264:8815-8820

Fishbein MC (2008) The vulnerable and unstable atherosclerotic plaque. Cardiovasc Pathol 19:6-11

Gadhoum SZ, Sackstein R (2008) CD15 expression in human myeloid cell differentiation is regulated by sialidase activity. Nat Chem Biol 4:751-757

Galen JE, Ketley JM, Fasano A, Richardson SH, Wasserman SS, Kaper JB (1992) Role of Vibrio cholerae neuraminidase in the function of cholera toxin. Infect Immun 60:406-415

Gaultier A, Arandjelovic S, Niessen S, Overton CD, Linton MF, Fazio S, Campana WM, Cravatt BF, III, Gonias SL (2008) Regulation of tumor necrosis factor receptor-1 and the IKK-NF-kappaB pathway by LDL receptor-related protein explains the antiinflammatory activity of this receptor. Blood 111:5316-5325

Gee K, Kozlowski M, Kumar A (2003) Tumor necrosis factor-alpha induces functionally active hyaluronan-adhesive CD44 by activating sialidase through p38 mitogen-activated protein kinase in lipopolysaccharide-stimulated human monocytic cells. J Biol Chem 278:37275-37287

Gee K, Lim W, Ma W, Nandan D, az-Mitoma F, Kozlowski M, Kumar A (2002) Differential regulation of CD44 expression by lipopolysaccharide (LPS) and TNF-alpha in human monocytic cells: distinct involvement of c-Jun N-terminal kinase in LPS-induced CD44 expression. J Immunol 169:5660-5672

Getz GS, Reardon CA (2009) Apoprotein E as a lipid transport and signaling protein in the blood, liver, and artery wall. J Lipid Res 50 Suppl:S156-S161

Glass CK, Witztum JL (2001) Atherosclerosis. the road ahead. Cell 104:503-516

Goedeke L, Fernandez-Hernando C (2012) Regulation of cholesterol homeostasis. Cell Mol Life Sci 69:915-930

Goldstein JL, Brown MS, Anderson RG, Russell DW, Schneider WJ (1985) Receptor-mediated endocytosis: concepts emerging from the LDL receptor system. Annu Rev Cell Biol 1:1-39

Gonias SL, Wu L, Salicioni AM (2004) Low density lipoprotein receptor-related protein: regulation of the plasma membrane proteome. Thromb Haemost 91:1056-1064

Gordon DA, Wetterau JR, Gregg RE (1995) Microsomal triglyceride transfer protein: a protein complex required for the assembly of lipoprotein particles. Trends Cell Biol 5:317-321

Gouni-Berthold I, Seul C, Ko Y, Hescheler J, Sachinidis A (2001) Gangliosides GM1 and GM2 induce vascular smooth muscle cell proliferation via extracellular signal-regulated kinase 1/2 pathway. Hypertension 38:1030-1037

Graham VS, Di MP, Armengol S, Lawson C, Wheeler-Jones CP, Botham KM (2011) Inhibition of macrophage inflammatory cytokine secretion by chylomicron remnants is dependent on their uptake by the low density lipoprotein receptor. Biochim Biophys Acta 1811:209-220

Grefhorst A, McNutt MC, Lagace TA, Horton JD (2008) Plasma PCSK9 preferentially reduces liver LDL receptors in mice. J Lipid Res 49:1303-1311

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

Gui T, Shimokado A, Sun Y, Akasaka T, Muragaki Y (2012) Diverse roles of macrophages in atherosclerosis: from inflammatory biology to biomarker discovery. Mediators Inflamm 2012:693083

Guo L, Song Z, Li M, Wu Q, Wang D, Feng H, Bernard P, Daugherty A, Huang B, Li XA (2009) Scavenger Receptor BI Protects against Septic Death through Its Role in Modulating Inflammatory Response. J Biol Chem 284:19826-19834

Han KH, Chen Y, Chang MK, Han YC, Park JH, Green SR, Boullier A, Quehenberger O (2003) LDL activates signaling pathways leading to an increase in cytosolic free calcium and stimulation of CD11b expression in monocytes. J Lipid Res 44:1332-1340

Hansson GK (1997) Cell-mediated immunity in atherosclerosis. Curr Opin Lipidol 8:301-311

Hansson GK (2007) Medicine. LIGHT hits the liver. Science 316:206-207

Hansson GK (2009) Inflammatory mechanisms in atherosclerosis. J Thromb Haemost 7 Suppl 1:328-331

Harvey SB, Zhang Y, Wilson-Grady J, Monkkonen T, Nelsestuen GL, Kasthuri RS, Verneris MR, Lund TC, Ely EW, Bernard GR, Zeisler H, Homoncik M, Jilma B, Swan T, Kellogg TA (2009) O-glycoside biomarker of apolipoprotein C3: responsiveness to obesity, bariatric surgery, and therapy with metformin, to chronic or severe liver disease and to mortality in severe sepsis and graft vs host disease. J Proteome Res 8:603-612

Hata K, Koseki K, Yamaguchi K, Moriya S, Suzuki Y, Yingsakmongkon S, Hirai G, Sodeoka M, von IM, Miyagi T (2008) Limited inhibitory effects of oseltamivir and zanamivir on human sialidases. Antimicrob Agents Chemother 52:3484-3491

Hegele RA (2009) Plasma lipoproteins: genetic influences and clinical implications. Nat Rev Genet 10:109-121

Hennen E, Safina D, Haussmann U, Worsdorfer P, Edenhofer F, Poetsch A, Faissner A (2013) A LewisX glycoprotein screen identifies the low density lipoprotein receptor-related protein 1 (LRP1) as a modulator of oligodendrogenesis in mice. J Biol Chem 288:16538-16545

Herijgers N, Van EM, Groot PH, Hoogerbrugge PM, Van Berkel TJ (2000) Low density lipoprotein receptor of macrophages facilitates atherosclerotic lesion formation in C57Bl/6 mice. Arterioscler Thromb Vasc Biol 20:1961-1967

Herz J, Strickland DK (2001) LRP: a multifunctional scavenger and signaling receptor. J Clin Invest 108:779-784

Hong C, Tontonoz P (2008) Coordination of inflammation and metabolism by PPAR and LXR nuclear receptors. Curr Opin Genet Dev 18:461-467

Hoofnagle AN, Heinecke JW (2009) Lipoproteomics: using mass spectrometry-based proteomics to explore the assembly, structure, and function of lipoproteins. J Lipid Res 50:1967-1975

Horton JD, Goldstein JL, Brown MS (2002) SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver. J Clin Invest 109:1125-1131

Horton JD, Shah NA, Warrington JA, Anderson NN, Park SW, Brown MS, Goldstein JL (2003) Combined analysis of oligonucleotide microarray data from transgenic and knockout mice identifies direct SREBP target genes. Proc Natl Acad Sci U S A 100:12027-12032

Horton JD, Shimano H, Hamilton RL, Brown MS, Goldstein JL (1999) Disruption of LDL receptor gene in transgenic SREBP-1a mice unmasks hyperlipidemia resulting from production of lipid-rich VLDL. J Clin Invest 103:1067-1076

Horton JD, Shimomura I, Brown MS, Hammer RE, Goldstein JL, Shimano H (1998) Activation of cholesterol synthesis in preference to fatty acid synthesis in liver and adipose tissue of transgenic mice overproducing sterol regulatory element-binding protein-2. J Clin Invest 101:2331-2339

Huang S, Rutkowsky JM, Snodgrass RG, Ono-Moore KD, Schneider DA, Newman JW, Adams SH, Hwang DH (2012) Saturated fatty acids activate TLR-mediated proinflammatory signaling pathways. J Lipid Res 53:2002-2013

Igdoura SA, Gafuik C, Mertineit C, Saberi F, Pshezhetsky AV, Potier M, Trasler JM, Gravel RA (1998) Cloning of the cDNA and gene encoding mouse lysosomal sialidase and correction of sialidase deficiency in human sialidosis and mouse SM/J fibroblasts. Hum Mol Genet 7:115-121

Ji A, Meyer JM, Cai L, Akinmusire A, de Beer MC, Webb NR, van der Westhuyzen DR (2011) Scavenger receptor SR-BI in macrophage lipid metabolism. Atherosclerosis 217:106-112

Jinno Y, Nakakuki M, Kawano H, Notsu T, Mizuguchi K, Imada K (2011) Eicosapentaenoic acid administration attenuates the pro-inflammatory properties of VLDL by decreasing its susceptibility to lipoprotein lipase in macrophages. Atherosclerosis 219:566-572

Katoh S, Maeda S, Fukuoka H, Wada T, Moriya S, Mori A, Yamaguchi K, Senda S, Miyagi T (2010) A crucial role of sialidase Neu1 in hyaluronan receptor function of CD44 in T helper type 2-mediated airway inflammation of murine acute asthmatic model. Clin Exp Immunol 161:233-241

Katoh S, Miyagi T, Taniguchi H, Matsubara Y, Kadota J, Tominaga A, Kincade PW, Matsukura S, Kohno S (1999) Cutting edge: an inducible sialidase regulates the hyaluronic acid binding ability of CD44-bearing human monocytes. J Immunol 162:5058-5061

Katsiki N, Nikolic D, Montalto G, Banach M, Mikhailidis DP, Rizzo M (2013) The role of fibrate treatment in dyslipidemia: an overview. Curr Pharm Des 19:3124-3131

Kawakami A, Aikawa M, Alcaide P, Luscinskas FW, Libby P, Sacks FM (2006a) Apolipoprotein CIII induces expression of vascular cell adhesion molecule-1 in vascular endothelial cells and increases adhesion of monocytic cells. Circulation 114:681-687

Kawakami A, Aikawa M, Libby P, Alcaide P, Luscinskas FW, Sacks FM (2006b) Apolipoprotein CIII in apolipoprotein B lipoproteins enhances the adhesion of human monocytic cells to endothelial cells. Circulation 113:691-700

Kawakami A, Aikawa M, Nitta N, Yoshida M, Libby P, Sacks FM (2007) Apolipoprotein CIII-induced THP-1 cell adhesion to endothelial cells involves pertussis toxin-sensitive G protein- and protein kinase C alpha-mediated nuclear factor-kappaB activation. Arterioscler Thromb Vasc Biol 27:219-225

Kawakami A, Yoshida M (2009) Apolipoprotein CIII links dyslipidemia with atherosclerosis. J Atheroscler Thromb 16:6-11

Kingsley DM, Kozarsky KF, Hobbie L, Krieger M (1986) Reversible defects in O-linked glycosylation and LDL receptor expression in a UDP-Gal/UDP-GalNAc 4-epimerase deficient mutant. Cell 44:749-759

Knibbs RN, Goldstein IJ, Ratcliffe RM, Shibuya N (1991) Characterization of the carbohydrate binding specificity of the leukoagglutinating lectin from Maackia amurensis. Comparison with other sialic acid-specific lectins. J Biol Chem 266:83-88

Kones R (2013) Molecular sources of residual cardiovascular risk, clinical signals, and innovative solutions: relationship with subclinical disease, undertreatment, and poor adherence: implications of new evidence upon optimizing cardiovascular patient outcomes. Vasc Health Risk Manag 9:617-670

Kosaka S, Takahashi S, Masamura K, Kanehara H, Sakai J, Tohda G, Okada E, Oida K, Iwasaki T, Hattori H, Kodama T, Yamamoto T, Miyamori I (2001) Evidence of macrophage foam cell formation by very low-density lipoprotein receptor: interferon-gamma inhibition of very low-density lipoprotein receptor expression and foam cell formation in macrophages. Circulation 103:1142-1147

Kozarsky K, Kingsley D, Krieger M (1988) Use of a mutant cell line to study the kinetics and function of O-linked glycosylation of low density lipoprotein receptors. Proc Natl Acad Sci U S A 85:4335-4339

Kramer W, Girbig F, Corsiero D, Pfenninger A, Frick W, Jahne G, Rhein M, Wendler W, Lottspeich F, Hochleitner EO, Orso E, Schmitz G (2005) Aminopeptidase N (CD13) is a molecular target of the cholesterol absorption inhibitor ezetimibe in the enterocyte brush border membrane. J Biol Chem 280:1306-1320

Kunnen S, Van EM (2012) Lecithin:cholesterol acyltransferase: old friend or foe in atherosclerosis? J Lipid Res 53:1783-1799

Lambre CR, Greffard A, Gattegno L, Saffar L (1990) Modifications of sialidase activity during the monocyte-macrophage differentiation in vitro. Immunol Lett 23:179-182

Lammers B, Zhao Y, Hoekstra M, Hildebrand RB, Ye D, Meurs I, Van Berkel TJ, Van EM (2011) Augmented atherogenesis in LDL receptor deficient mice lacking both macrophage ABCA1 and ApoE. PLoS One 6:e26095

Latasa MJ, Griffin MJ, Moon YS, Kang C, Sul HS (2003) Occupancy and function of the -150 sterol regulatory element and -65 E-box in nutritional regulation of the fatty acid synthase gene in living animals. Mol Cell Biol 23:5896-5907

Lemaire-Ewing S, Lagrost L, Neel D (2012) Lipid rafts: a signalling platform linking lipoprotein metabolism to atherogenesis. Atherosclerosis 221:303-310

Leonarduzzi G, Gamba P, Gargiulo S, Biasi F, Poli G (2012) Inflammation-related gene expression by lipid oxidation-derived products in the progression of atherosclerosis. Free Radic Biol Med 52:19-34

Levitan I, Volkov S, Subbaiah PV (2010) Oxidized LDL: diversity, patterns of recognition, and pathophysiology. Antioxid Redox Signal 13:39-75

Liang F, Seyrantepe V, Landry K, Ahmad R, Ahmad A, Stamatos NM, Pshezhetsky AV (2006) Monocyte differentiation up-regulates the expression of the lysosomal sialidase, Neu1, and triggers its targeting to the plasma membrane via major histocompatibility complex class II-positive compartments. J Biol Chem 281:27526-27538

Libby P (2007) Fat fuels the flame: triglyceride-rich lipoproteins and arterial inflammation. Circ Res 100:299-301

Libby P, Lichtman AH, Hansson GK (2013) Immune effector mechanisms implicated in atherosclerosis: from mice to humans. Immunity 38:1092-1104

Libby P, Ridker PM, Hansson GK (2011) Progress and challenges in translating the biology of atherosclerosis. Nature 473:317-325

Lillis AP, Mikhailenko I, Strickland DK (2005) Beyond endocytosis: LRP function in cell migration, proliferation and vascular permeability. J Thromb Haemost 3:1884-1893

Lillis AP, Van Duyn LB, Murphy-Ullrich JE, Strickland DK (2008) LDL receptor-related protein 1: unique tissue-specific functions revealed by selective gene knockout studies. Physiol Rev 88:887-918

Lindberg G (2007) Resialylation of sialic acid deficit vascular endothelium, circulating cells and macromolecules may counteract the development of atherosclerosis: a hypothesis. Atherosclerosis 192:243-245

Linton MF, Babaev VR, Gleaves LA, Fazio S (1999) A direct role for the macrophage low density lipoprotein receptor in atherosclerotic lesion formation. J Biol Chem 274:19204-19210

Lopez D (2008) Inhibition of PCSK9 as a novel strategy for the treatment of hypercholesterolemia. Drug News Perspect 21:323-330

Lorkowski S (2008) The ins and outs of lipid efflux. J Mol Med (Berl) 86:129-134

Lu Y, Guo J, Di Y, Zong Y, Qu S, Tian J (2009) Proteomic analysis of the triglyceride-rich lipoprotein-laden foam cells. Mol Cells 28:175-181

Lukong KE, Seyrantepe V, Landry K, Trudel S, Ahmad A, Gahl WA, Lefrancois S, Morales CR, Pshezhetsky AV (2001) Intracellular distribution of lysosomal sialidase is controlled by the internalization signal in its cytoplasmic tail. J Biol Chem 276:46172-46181

Lund EG, Kerr TA, Sakai J, Li WP, Russell DW (1998) cDNA cloning of mouse and human cholesterol 25-hydroxylases, polytopic membrane proteins that synthesize a potent oxysterol regulator of lipid metabolism. J Biol Chem 273:34316-34327

Mamputu JC, Desfaits AC, Renier G (1997) Lipoprotein lipase enhances human monocyte adhesion to aortic endothelial cells. J Lipid Res 38:1722-1729

Mann CJ, Troussard AA, Yen FT, Hannouche N, Najib J, Fruchart JC, Lotteau V, Andre P, Bihain BE (1997) Inhibitory effects of specific apolipoprotein C-III isoforms on the binding of triglyceride-rich lipoproteins to the lipolysis-stimulated receptor. J Biol Chem 272:31348-31354

Mauger JF, Couture P, Bergeron N, Lamarche B (2006) Apolipoprotein C-III isoforms: kinetics and relative implication in lipid metabolism. J Lipid Res 47:1212-1218

Maxfield FR, Wustner D (2002) Intracellular cholesterol transport. J Clin Invest 110:891-898

May P, Herz J, Bock HH (2005) Molecular mechanisms of lipoprotein receptor signalling. Cell Mol Life Sci 62:2325-2338

McCullers JA, Bartmess KC (2003) Role of neuraminidase in lethal synergism between influenza virus and Streptococcus pneumoniae. J Infect Dis 187:1000-1009

Mead JR, Irvine SA, Ramji DP (2002) Lipoprotein lipase: structure, function, regulation, and role in disease. J Mol Med 80:753-769

Mehta A, Banerjee S, Driscoll DM (1996) Apobec-1 interacts with a 65-kDa complementing protein to edit apolipoprotein-B mRNA in vitro. J Biol Chem 271:28294-28299

Mel'nichenko AA, Tertov VV, Ivanova OA, Aksenov DV, Sobenin IA, Popov EV, Kaplun VV, Suprun IV, Panasenko OM, Orekhov AN (2005) Desialylation decreases the resistance of apo B-containing lipoproteins to aggregation and increases their atherogenic potential. Bull Exp Biol Med 140:51-54

Millar JS (2001) The sialylation of plasma lipoproteins. Atherosclerosis 154:1-13

Milosavljevic D, Kontush A, Griglio S, Le NG, Thillet J, Chapman MJ (2003) VLDL-induced triglyceride accumulation in human macrophages is mediated by modulation of LPL lipolytic activity in the absence of change in LPL mass. Biochim Biophys Acta 1631:51-60

Miyagi T, Yamaguchi K (2012) Mammalian sialidases: physiological and pathological roles in cellular functions. Glycobiology 22:880-896

Moore KJ, Sheedy FJ, Fisher EA (2013) Macrophages in atherosclerosis: a dynamic balance. Nat Rev Immunol 10:709-21

Moore KJ, Tabas I (2011) Macrophages in the pathogenesis of atherosclerosis. Cell 145:341-355

Natori Y, Ohkura N, Nasui M, Atsumi G, Kihara-Negishi F (2013) Acidic sialidase activity is aberrant in obese and diabetic mice. Biol Pharm Bull 36:1027-1031

Nofer JR, Brodde MF, Kehrel BE (2010) High-density lipoproteins, platelets and the pathogenesis of atherosclerosis. Clin Exp Pharmacol Physiol 37:726-735

Ooi EM, Barrett PH, Chan DC, Watts GF (2008) Apolipoprotein C-III: understanding an emerging cardiovascular risk factor. Clin Sci (Lond) 114:611-624

Orekhov AN, Tertov VV, Mukhin DN (1991) Desialylated low density lipoprotein--naturally occurring modified lipoprotein with atherogenic potency. Atherosclerosis 86:153-161

Ouimet M, Franklin V, Mak E, Liao X, Tabas I, Marcel YL (2011) Autophagy regulates cholesterol efflux from macrophage foam cells via lysosomal acid lipase. Cell Metab 13:655-667

Ouimet M, Marcel YL (2012) Regulation of lipid droplet cholesterol efflux from macrophage foam cells. Arterioscler Thromb Vasc Biol 32:575-581

Overton CD, Yancey PG, Major AS, Linton MF, Fazio S (2007) Deletion of macrophage LDL receptor-related protein increases atherogenesis in the mouse. Circ Res 100:670-677

Palmer AM, Nova E, Anil E, Jackson K, Bateman P, Wolstencroft E, Williams CM, Yaqoob P (2005) Differential uptake of subfractions of triglyceride-rich lipoproteins by THP-1 macrophages. Atherosclerosis 180:233-244

Pattison S, Pankarican M, Rupar CA, Graham FL, Igdoura SA (2004) Five novel mutations in the lysosomal sialidase gene (NEU1) in type II sialidosis patients and assessment of their impact on enzyme activity and intracellular targeting using adenovirus-mediated expression. Hum Mutat 23:32-39

Persson J, Nilsson J, Lindholm MW (2006) Cytokine response to lipoprotein lipid loading in human monocyte-derived macrophages. Lipids Health Dis 5:17

Persson J, Nilsson J, Lindholm MW (2008) Interleukin-1beta and tumour necrosis factor-alpha impede neutral lipid turnover in macrophage-derived foam cells. BMC Immunol 9:70

Pou J, Martinez-Gonzalez J, Rebollo A, Rodriguez C, Rodriguez-Calvo R, Martin-Fuentes P, Cenarro A, Civeira F, Laguna JC, Alegret M (2011) Type II interleukin-1 receptor expression is reduced in monocytes/macrophages and atherosclerotic lesions. Biochim Biophys Acta 1811:556-563

Pshezhetsky AV, Ashmarina LI (2013) Desialylation of surface receptors as a new dimension in cell signaling. Biochemistry (Mosc) 78:736-745

Pshezhetsky AV, Hinek A (2011) Where catabolism meets signalling: neuraminidase 1 as a modulator of cell receptors. Glycoconj J 28:441-452

Radhakrishnan A, Goldstein JL, McDonald JG, Brown MS (2008) Switch-like control of SREBP-2 transport triggered by small changes in ER cholesterol: a delicate balance. Cell Metab 8:512-521

Rastam L, Lindberg G, Folsom AR, Burke GL, Nilsson-Ehle P, Lundblad A (1996) Association between serum sialic acid concentration and carotid atherosclerosis measured by B-mode ultrasound. The ARIC Investigators. Atherosclerosis Risk in Communities Study. Int J Epidemiol 25:953-958

Rosenson RS, Brewer HB, Jr., Davidson WS, Fayad ZA, Fuster V, Goldstein J, Hellerstein M, Jiang XC, Phillips MC, Rader DJ, Remaley AT, Rothblat GH, Tall AR, Yvan-Charvet L (2012) Cholesterol efflux and atheroprotection: advancing the concept of reverse cholesterol transport. Circulation 125:1905-1919

Ross R (1999) Atherosclerosis--an inflammatory disease. N Engl J Med 340:115-126

Rottier RJ, Bonten E, d'Azzo A (1998) A point mutation in the neu-1 locus causes the neuraminidase defect in the SM/J mouse. Hum Mol Genet 7:313-321

Sakarya S, Rifat S, Zhou J, Bannerman DD, Stamatos NM, Cross AS, Goldblum SE (2004) Mobilization of neutrophil sialidase activity desialylates the pulmonary vascular endothelial surface and increases resting neutrophil adhesion to and migration across the endothelium. Glycobiology 14:481-494

Sakthivel R, Zhang JC, Strickland DK, Gafvels M, McCrae KR (2001) Regulation of the ligand binding activity of the human very low density lipoprotein receptor by protein kinase C-dependent phosphorylation. J Biol Chem 276:555-562

Sampedro MC, Motran C, Gruppi A, Kivatinitz SC (2001) VLDL modulates the cytokine secretion profile to a proinflammatory pattern. Biochem Biophys Res Commun 285:393-399

Saraswathi V, Hasty AH (2006) The role of lipolysis in mediating the proinflammatory effects of very low density lipoproteins in mouse peritoneal macrophages. J Lipid Res 47:1406-1415

Sathiyapriya V, Selvaraj N, Nandeesha H, Bobby Z, Aparna A, Pavithran P (2008) Association between protein bound sialic acid and high sensitivity C-reactive protein in prehypertension: a possible indication of underlying cardiovascular risk. Clin Exp Hypertens 30:367-374

Sato R, Miyamoto W, Inoue J, Terada T, Imanaka T, Maeda M (1999) Sterol regulatory element-binding protein negatively regulates microsomal triglyceride transfer protein gene transcription. J Biol Chem 274:24714-24720

Schauer R (2009) Sialic acids as regulators of molecular and cellular interactions. Curr Opin Struct Biol 19:507-514

Sekiya M, Osuga J, Igarashi M, Okazaki H, Ishibashi S (2011) The role of neutral cholesterol ester hydrolysis in macrophage foam cells. J Atheroscler Thromb 18:359-364

Seyrantepe V, Hinek A, Peng J, Fedjaev M, Ernest S, Kadota Y, Canuel M, Itoh K, Morales CR, Lavoie J, Tremblay J, Pshezhetsky AV (2008) Enzymatic activity of lysosomal carboxypeptidase (cathepsin) A is required for proper elastic fiber formation and inactivation of endothelin-1. Circulation 117:1973-1981

Seyrantepe V, Iannello A, Liang F, Kanshin E, Jayanth P, Samarani S, Szewczuk MR, Ahmad A, Pshezhetsky AV (2010) Regulation of phagocytosis in macrophages by neuraminidase 1. J Biol Chem 285:206-215

Shelness GS, Ingram MF, Huang XF, DeLozier JA (1999) Apolipoprotein B in the rough endoplasmic reticulum: translation, translocation and the initiation of lipoprotein assembly. J Nutr 129:456S-462S

Skalen K, Gustafsson M, Rydberg EK, Hulten LM, Wiklund O, Innerarity TL, Boren J (2002) Subendothelial retention of atherogenic lipoproteins in early atherosclerosis. Nature 417:750-754

Sobenin IA, Tertov VV, Orekhov AN, Smirnov VN (1991) Synergetic effect of desialylated and glycated low density lipoproteins on cholesterol accumulation in cultured smooth muscle intimal cells. Atherosclerosis 89:151-154

Soccio RE, Breslow JL (2004) Intracellular cholesterol transport. Arterioscler Thromb Vasc Biol 24:1150-1160

Spencer TA, Li D, Russel JS, Collins JL, Bledsoe RK, Consler TG, Moore LB, Galardi CM, McKee DD, Moore JT, Watson MA, Parks DJ, Lambert MH, Willson TM (2001) Pharmacophore analysis of the nuclear oxysterol receptor LXRalpha. J Med Chem 44:886-897

Sprague EA, Moser M, Edwards EH, Schwartz CJ (1988) Stimulation of receptor-mediated low density lipoprotein endocytosis in neuraminidase-treated cultured bovine aortic endothelial cells. J Cell Physiol 137:251-262

Stamatos NM, Carubelli I, van d, V, Bonten EJ, Papini N, Feng C, Venerando B, d'Azzo A, Cross AS, Wang LX, Gomatos PJ (2010) LPS-induced cytokine production in human dendritic cells is regulated by sialidase activity. J Leukoc Biol 88:1227-1239

Stamatos NM, Liang F, Nan X, Landry K, Cross AS, Wang LX, Pshezhetsky AV (2005) Differential expression of endogenous sialidases of human monocytes during cellular differentiation into macrophages. FEBS J 272:2545-2556

Steinberg D, Witztum JL (1990) Lipoproteins and atherogenesis. Current concepts. JAMA 264:3047-3052

Steinbrecher UP (1999) Receptors for oxidized low density lipoprotein. Biochim Biophys Acta 1436:279-298

Stoline AM, Saku K, Hynd BA, Kashyap ML (1985) Effect of desialylation of very low-density lipoproteins on their catabolism by lipoprotein lipase. Metabolism 34:30-35

Stollenwerk MM, Lindholm MW, Porn-Ares MI, Larsson A, Nilsson J, Ares MP (2005a) Very low-density lipoprotein induces interleukin-1beta expression in macrophages. Biochem Biophys Res Commun 335:603-608

Stollenwerk MM, Schiopu A, Fredrikson GN, Dichtl W, Nilsson J, Ares MP (2005b) Very low density lipoprotein potentiates tumor necrosis factor-alpha expression in macrophages. Atherosclerosis 179:247-254

Strickland DK, Gonias SL, Argraves WS (2002) Diverse roles for the LDL receptor family. Trends Endocrinol Metab 13:66-74

Sundaram M, Yao Z (2012) Intrahepatic role of exchangeable apolipoproteins in lipoprotein assembly and secretion. Arterioscler Thromb Vasc Biol 32:1073-1078

Tabas I, Lim S, Xu XX, Maxfield FR (1990) Endocytosed beta-VLDL and LDL are delivered to different intracellular vesicles in mouse peritoneal macrophages. J Cell Biol 111:929-940

Terrand J, Bruban V, Zhou L, Gong W, El AZ, May P, Zurhove K, Haffner P, Philippe C, Woldt E, Matz RL, Gracia C, Metzger D, Auwerx J, Herz J, Boucher P (2009) LRP1 controls intracellular cholesterol storage and fatty acid synthesis through modulation of Wnt signaling. J Biol Chem 284:381-388

Varki A (2008) Sialic acids in human health and disease. Trends Mol Med 14:351-360

Varki A, Gagneux P (2012) Multifarious roles of sialic acids in immunity. Ann N Y Acad Sci 1253:16-36

Veniant MM, Beigneux AP, Bensadoun A, Fong LG, Young SG (2008) Lipoprotein size and susceptibility to atherosclerosis--insights from genetically modified mouse models. Curr Drug Targets 9:174-189

Wang D, Zaitsev S, Taylor G, d'Azzo A, Bonten E (2009) Protective protein/cathepsin A rescues N-glycosylation defects in neuraminidase-1. Biochim Biophys Acta 1790:275-282

Wang MD, Kiss RS, Franklin V, McBride HM, Whitman SC, Marcel YL (2007) Different cellular traffic of LDL-cholesterol and acetylated LDL-cholesterol leads to distinct reverse cholesterol transport pathways. J Lipid Res 48:633-645

Wang P, Zhang J, Bian H, Wu P, Kuvelkar R, Kung TT, Crawley Y, Egan RW, Billah MM (2004) Induction of lysosomal and plasma membrane-bound sialidases in human T-cells via T-cell receptor. Biochem J 380:425-433

Williams HJ, Fisher EA, Greaves DR (2012) Macrophage differentiation and function in atherosclerosis: opportunities for therapeutic intervention? J Innate Immun 4:498-508

Yamada Y, Doi T, Hamakubo T, Kodama T (1998) Scavenger receptor family proteins: roles for atherosclerosis, host defence and disorders of the central nervous system. Cell Mol Life Sci 54:628-640

Yamamoto T, Lu C, Ryan RO (2011) A two-step binding model of PCSK9 interaction with the low density lipoprotein receptor. J Biol Chem 286:5464-5470

Yang T, Espenshade PJ, Wright ME, Yabe D, Gong Y, Aebersold R, Goldstein JL, Brown MS (2002) Crucial step in cholesterol homeostasis: sterols promote binding of SCAP to INSIG-1, a membrane protein that facilitates retention of SREBPs in ER. Cell 110:489-500

Yao Z, Wang Y (2012) Apolipoprotein C-III and hepatic triglyceride-rich lipoprotein production. Curr Opin Lipidol 23:206-212

Yasuda T, Hirata K, Ishida T, Kojima Y, Tanaka H, Okada T, Quertermous T, Yokoyama M (2007) Endothelial lipase is increased by inflammation and promotes LDL uptake in macrophages. J Atheroscler Thromb 14:192-201

Ye Q, Chen Y, Lei H, Liu Q, Moorhead JF, Varghese Z, Ruan XZ (2009) Inflammatory stress increases unmodified LDL uptake via LDL receptor: an alternative pathway for macrophage foam-cell formation. Inflamm Res

Yen CL, Stone SJ, Koliwad S, Harris C, Farese RV, Jr. (2008) Thematic review series: glycerolipids. DGAT enzymes and triacylglycerol biosynthesis. J Lipid Res 49:2283-2301

Yip VL, Pirmohamed M (2013) Expanding role of pharmacogenomics in the management of cardiovascular disorders. Am J Cardiovasc Drugs 13:151-162

Young SG, Zechner R (2013) Biochemistry and pathophysiology of intravascular and intracellular lipolysis. Genes Dev 27:459-484

Yvan-Charvet L, Ranalletta M, Wang N, Han S, Terasaka N, Li R, Welch C, Tall AR (2007) Combined deficiency of ABCA1 and ABCG1 promotes foam cell accumulation and accelerates atherosclerosis in mice. J Clin Invest 117:3900-3908

Zelcer N, Hong C, Boyadjian R, Tontonoz P (2009) LXR regulates cholesterol uptake through Idol-dependent ubiquitination of the LDL receptor. Science 325:100-104

Zhang JR, Coleman T, Langmade SJ, Scherrer DE, Lane L, Lanier MH, Feng C, Sands MS, Schaffer JE, Semenkovich CF, Ory DS (2008) Niemann-Pick C1 protects against atherosclerosis in mice via regulation of macrophage intracellular cholesterol trafficking. J Clin Invest 118:2281-2290

Zhang Y, Albohy A, Zou Y, Smutova V, Pshezhetsky AV, Cairo CW (2013) Identification of selective inhibitors for human neuraminidase isoenzymes using C4,C7-modified 2-deoxy-2,3-didehydro-N-acetylneuraminic acid (DANA) analogues. J Med Chem 56:2948-2958

Zhao C, Dahlman-Wright K (2010) Liver X receptor in cholesterol metabolism. J Endocrinol 204:233-240

Zhao D, Letterman J, Schreiber BM (2001) beta-Migrating very low density lipoprotein (beta VLDL) activates smooth muscle cell mitogen-activated protein (MAP) kinase via G protein-coupled receptor-mediated transactivation of the epidermal growth factor (EGF) receptor: effect of MAP kinase activation on beta VLDL plus EGF-induced cell proliferation. J Biol Chem 276:30579-30588