A NOVEL ROLE FOR HDL AND D-HDL IN PULMONARY IMMUNITY

by

Michael Yaeger

July 2018

Director of Thesis: Dr. Kymberly Gowdy

Major Department: Engineering

Cardiovascular and pulmonary diseases are leading causes of morbidity and mortality worldwide. Studies report an inverse correlation between levels of serum high-density lipoprotein (HDL) and the severity of cardiovascular and lung diseases. HDL has also been shown to be anti-inflammatory, anti-atherosclerotic, and anti-oxidative. HDL's cardioprotective functions are well understood through the reverse cholesterol transport process. However, how HDL effects the immune system in the lungs is still unknown. We hypothesize that HDL is critical in preventing pulmonary injury from lipopolysaccharide (LPS) through inhibiting neutrophil (PMN) chemotaxis. While HDL is known to be biologically protective, it has also been reported that HDL can become dysfunctional (D-HDL) in chronic inflammatory diseases. HDL is characterized as dysfunctional when it does not perform its protective mechanisms. It has been challenging to study D-HDL, in part, because D-HDL is found in specific patient populations commonly burdened with comorbidities and subsequent medications. Apolipoprotein A-I (apoA-I), the major protein component of HDL, is primarily responsible for HDL's beneficial properties. There are apoA-I mimetic peptides, such as L-4F, available to study the biological properties of HDL in both *in vitro* and *in vivo* models. However, there is no such research tool available to study D-HDL. Therefore, to better understand how D-HDL differs from HDL, we also sought to design a D-HDL mimetic peptide that can be used to examine the biological mechanisms of how HDL and D-HDL differ.

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A Thesis

Presented to the Faculty of the Department of Engineering

East Carolina University

In Partial Fulfillment of the Requirements for the Degree

Master of Science in Biomedical Engineering

by

Michael Yaeger

July 2018

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Michael Yaeger

APPROVED BY:

DIRECTOR OF
THESIS: ____

Kymberly Gowdy, PhD

COMMITTEE MEMBER: ______________________________________

Barbara Muller-Borer, PhD

COMMITTEE MEMBER: ______________________________________

Loren Limberis, PhD

COMMITTEE MEMBER: ______________________________________

Kim Kew, PhD

COMMITTEE MEMBER: ______________________________________

Tonya Zeczycki, PhD

CHAIR OF THE DEPARTMENT
OF ENGINEERING: ____

Barbara Muller-Borer, PhD

DEAN OF THE
GRADUATE SCHOOL: _______________________________________

Paul J. Gemperline, PhD

ACKNOWLEDGEMENTS

First, I would like to express my deepest gratitude to my mentor, Dr. Kymberly Gowdy. Without her guidance, I never would have been able to accomplish a fraction of what I have achieved during my time at ECU. Dr. Gowdy's patience, wisdom, and passion for her work have always been an inspiration for me. She has helped me learn how to grow from being a student in the classroom to becoming a critically thinking scientist in the lab. Her guidance has helped me develop the skills necessary to work in this field and has helped me to prepare for, and find, work after school. I would also like to thank the remainder of my committee members Dr. Barbara Muller-Borer, Dr. Loren Limberis, Dr. Kim Kew, and Dr. Tonya Zeczycki who have all been instrumental in helping me complete my graduate degree and to grow as a scientist.

I also would like to thank everyone in Dr. Gowdy's lab for all the time they have dedicated to helping me with my research, and teaching me about the science and techniques that I have used throughout my education. I am deeply grateful to Dr. Sky Reece, Dr. Brita Kilburg-Basnyat, Myles Hodge, Christine Psaltis, and Bin Lou for all the help they have given me.

Additionally, I'd like to thank all of the collaborators who have contributed to my research. I have been truly impressed by the professors at ECU's willingness to work together and support me through my research. The list of people to thank is far too long to write out, but in particular I have to thank Dr. Toby Allen for all of his help with the peptide synthesis component of my project.

Lastly, I would like to thank my family for all of their support and guidance throughout these challenging past few years. I would never have been able to get this far without their endless support and advice. I am so thankful to have such a loving and supportive family.

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LIST OF ABBREVIATIONS

AAT	α-1 antitrypsin	3
ABCA1	ATP binding cassette transporter A-1	4
ABCG1	ATP binding cassette transporter G-1	4
Apo	Apolipoprotein	3
BALF	Bronchoalveolar lavage fluid	14
CD11b	Cluster of differentiation molecule 11b	5
CD14	Cluster of differentiation molecule 14	2
Ct	Cycle threshold	15
CVD	Cardiovascular disease	4
CXCR2	C-X-C motif chemokine receptor 2	12
DAMP	Damage associated molecular pattern	2
D-HDL	Dysfunctional high-density lipoprotein	8
G-CSF	Granulocyte colony-stimulating factor	6
HDL	High-density lipoprotein	1
HDL-C	High-density lipoprotein-cholesterol	4
HOCl	Hypochlorite	40
HUVEC	Human umbilical vein endothelial cell	5
ICAM-1	Intracellular adhesion molecule-1	5
IL-1β	Interleukin-1β	2
IL-6	Interleukin-6	2
KC	Keratinocyte chemoattractant	2
LBP	Lipopolysaccharide binding protein	2

LCAT	Lecithin: cholesterol acetyltransferase	3
LDL	Low-density lipoprotein	5
LDLr	Low-density lipoprotein receptor	15
LPS	Lipopolysaccharide	2
LXR	Liver-X-receptor	52
MALDI-TOF	Matrix assisted laser desorption/ionization time of flight mass spectrometry	53
MCP-1	Monocyte chemoattractant protein-1	6
MIP-2	Macrophage inflammatory protein-2	2
MPO	Myeloperoxidase	43
MyD88	Myeloid differentiation primary response protein 88	2
MΦ	Macrophage	25
NF-κB	Nuclear factor-κB	2
PAMP	Pathogen associated molecular pattern	2
PLTP	Phospholipid transfer protein	3
PMN	Neutrophil	2
PON1	Paraoxonase-1	3
RCT	Reverse cholesterol transport	4
ROS	Reactive oxygen species	2
SR-BI	Scavenger receptor class B-I	4
TLR4	Toll-like receptor 4	2
TNF-α	Tumor necrosis factor-α	2
VCAM	Vascular adhesion molecule	6
WT	C57BL/6J	13

CHAPTER I: INTRODUCTION

Introduction

Cardiovascular and lung diseases were reported to be two of the top three leading causes of death in the United States as of 2015 (1). These diseases are commonly driven by chronic inflammation which can lead to severe tissue injury. As research has been conducted, an emerging role for cholesterol in the pathogenesis of cardiovascular and pulmonary diseases has developed (2, 3). High-density lipoprotein (HDL), a cholesterol transporter, has been shown to be protective of the cardiovascular and respiratory systems (4-7). Cholesterol is essential in the body for integrity of cellular membranes, cellular signaling, steroid production, and as a form of energy (8, 9). However, excess levels of cholesterol termed 'hypercholesteremia' can be pathological in the pulmonary and circulatory system (10, 11). Therefore, balanced cholesterol homeostasis is necessary and, when thrown out of balance, can exacerbate chronic inflammation. This has driven a need for pharmaceuticals that balance cholesterol levels in the body, such as the use of statins. However, these treatments have not been effective in treating cardiovascular diseases (12). As a result, there has been a call for new therapeutic targets for balancing cholesterol in the body. HDL plays a critical role in the balance of cholesterol by transporting cholesterol out of the circulatory system (13). However, the connection between HDL's ability to transport cholesterol and its protective of role is unknown. It is only understood that HDL carries cholesterol and has an anti-inflammatory role in the immune system. Therefore, developing a better understanding of the role of HDL in the immune system is important for the development of better therapeutics for these diseases.

The innate immune response during chronic inflammation

Cardiovascular and pulmonary diseases are considered to be the result of chronic inflammation from an acute immune response to a stimulus (14-16). The innate immune system generally recognizes two types of stimuli, a damaged-associated molecular pattern (DAMP) or a pathogen-associated molecular pattern (PAMP). Lipopolysaccharide (LPS) is an PAMP commonly used to induce acute and chronic inflammatory responses. LPS is a lipid found in the outer membrane of gram-negative bacteria (17). When LPS enters the body, it binds to LPS-binding protein (LBP). This catalyzes LPS binding to cluster of differentiation molecule 14 (CD14), a receptor commonly found on myeloid cells, facilitating the activation of toll-like receptor 4 (TLR4) (18). Once TLR4 is activated, an extensive cascade is triggered beginning with myeloid differentiation primary response protein 88 (MyD88) and ending with nuclear factor-κB (NF-κB). NF-κB is the responsible for the transcription and release of chemokines such as keratinocyte chemoattractant (KC) and macrophage inflammatory protein-2 (MIP-2) in rodents, or interleukin-8 (IL-8) in humans. NF-κB also contributes to transcription of pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and interleukin-1β (IL-1β) (19, 20). Because LPS so easily binds to LBP and triggers the TLR4 cascade, an overly amplified immunological response can occur at certain exposure concentrations (21). Hyperinflammation can then damage the local tissue due to increased neutrophilia, reactive oxygen species (ROS) production, and protease release. Increased blood flow to the site of inflammation also perpetuates the inflammatory response as more macrophages, neutrophils (PMNs), and pro-inflammatory cytokines accumulate in the site of injury (22).

High-Density Lipoprotein

HDL is an 8-10 nm particle composed of a core with cholesterol, cholesteryl ester, fatty acids, triglycerides, proteins, enzymes, and micro RNAs and a phospholipid monolayer membrane interwoven with several apolipoprotein(apo)s (23-25). HDL's primary function is cholesterol transport and is understood to be protective in the cardiovascular and respiratory systems. There are two HDL subpopulations based on density, HDL2, and HDL3 with HDL2 being less dense and larger than HDL3 (26). ApoA-I is the primary structural protein of HDL which makes up 70% of its protein content (27, 28). The second major structural protein is apoA-II, making up 15-20% of total HDL protein (29). HDL's remaining protein content is composed of several other apolipoproteins, enzymes, lipid transfer proteins, acute phase proteins, proteinase inhibitors and various other proteins (27). Notable proteins are lecithin: cholesterol acetyltransferase (LCAT), phospholipid transfer protein (PLTP), paraoxonase 1 (PON1), and α-1-antitrypsin (AAT). LCAT is necessary for the esterification of cholesterol as it is transferred from tissue to nascent HDL to form mature HDL (30). PLTP transfers phospholipids from triglyceride-rich lipoproteins to HDL and has also been shown to bind other lipids such as LPS (31). PON1 is an enzyme found in HDL that has been shown to be anti-atherosclerotic due to hydrolyzing specific oxidized phospholipids in lipoproteins (32, 33), and increasing HDL binding to foamy macrophages (34). AAT is an enzyme that neutralizes elastase, the proteinase enzyme released by PMNs during inflammation (35). These proteins and enzymes all contribute to the overall function of HDL in the body, whether that be cholesterol transportation, or inhibition of inflammation, atherosclerosis, oxidation, or thrombosis.

HDL in reverse cholesterol transport

The primary function of HDL is to perform reverse cholesterol transport (RCT) (30). RCT is the process in which HDL takes up cholesterol from the cells of peripheral tissues or lipid-laden macrophages and transports the cholesterol to the liver to produce sterols, steroids, glucocorticoids, or excrete the cholesterol. Cholesterol is effluxed from peripheral tissues by ATP binding cassette transporter A-1 (ABCA1) and is transported to pre-β HDL (nascent/immature HDL) which is synthesized in the liver. The cholesterol is then esterified by LCAT, making the cholesterol more hydrophobic and stable within the HDL particle. As pre-β HDL fills with cholesteryl ester, it grows to HDL3, where ATP binding cassette transporter G-1 (ABCG1), in addition to ABCA1, effluxes cholesterol from macrophages and transports it to HDL. HDL then grows to HDL2, the largest and least dense HDL molecule. The filled HDL particle then passively passes through circulation until reaching the liver. Once HDL reaches the liver, scavenger receptor B class I (SR-BI) binds to the HDL particle and transfers the cholesteryl ester into the liver (36, 37).

The cardioprotective role of HDL

HDL is most widely known for being protective in the cardiovascular system. There is an inverse relationship between plasma HDL and the severity of cardiovascular disease (CVD) (4, 5). The leading pathway in HDL's protective function has generally been thought to be the result of HDL performing RCT (38). Through RCT, HDL takes up cholesterol from foamy macrophages that are attempting to destroy plaque lesions (39). As a result, HDL inhibits the development of atherosclerosis or other forms of CVD. It is for this reason that clinicians measure the concentration of systemic HDL-cholesterol (HDL-C) as a diagnostic measure of early CVD development. Additionally, HDL has been shown to play a protective role in many other ways. For example,

HDL has been shown to lower the susceptibility of low-density lipoprotein (LDL) oxidation which is pro-atherosclerotic (40, 41). Also, HDL carries another protein, sphingosine-1-phosphate, which facilitates nitric oxide production and facilitates vascular tone modulation and circulatory homeostasis (42). Additionally, HDL has been shown to inhibit neutrophil adhesion to and migration through endothelial cells by suppressing adhesion molecule surface display (43). The ability of HDL to suppress intracellular adhesion molecule-1 (ICAM-1) is thought to be the result of HDL carrying micro RNA miR-223, which blunts the expression of ICAM-1 (44, 45). HDL is also beneficial to endothelial cell proliferation and migration, increasing the wound healing of human umbilical vein endothelial cells (HUVECs) (46). It has also been described that HDL can inhibit the activation of monocytes due to the decreased display of cluster of differentiation molecule 11b (CD11b) (47, 48). Along with these anti-atherosclerotic and anti-inflammatory properties, HDL has also demonstrated anti-oxidative (49-51), and some anti-thrombotic (52, 53) functions.

The pulmonary protective role of HDL

While HDL is primarily understood as cardioprotective, recent literature has indicated a protective role for HDL in the lungs. It has been recently reported that low HDL-C levels are associated with the development of asthma in adolescents (6). Also, apoA-I deficiency, which essentially prevents HDL formation, induces increased oxidative stress, inflammation, collagen deposition, and airway hyperresponsiveness (7). These findings indicate HDL is necessary for fundamental lung function. Additional research has demonstrated that HDL also plays a significant role in the pulmonary inflammatory response. It was found that HDL will accumulate in the lungs in a mouse model of pulmonary emphysema (54). Likewise, apoA-I deficient mice were found to have increased airway

PMNs after ovalbumin-induced airway inflammation (55). In that study, pulmonary neutrophilia was then suppressed by administration of an anti-granulocyte colony-stimulating factor (G-CSF) antibody, indicating the necessity of apoA-I to suppress PMN production after pulmonary injury. Furthermore, excess apoA-I administration decreases pulmonary neutrophilia and collagen deposition after bleomycin challenge (55), pulmonary inflammation after LPS challenge (56), and pulmonary inflammation from house dust mite-driven asthma (57). Collectively, this research demonstrates the necessity of HDL, and its primary structural protein apoA-I, in the pulmonary immune response and its ability to inhibit that response when given in excess.

Apolipoprotein A-I

ApoA-I is the major structural protein of HDL. It is a 243 amino acid protein with an amphipathic alpha-helix structure (23). The amphipathic alpha-helix structure of apoA-I and other apolipoproteins allows for the formation of lipoproteins by providing a hydrophobic side to face lipids and a hydrophilic side to allow lipoproteins to be soluble. There are many apolipoproteins within HDL and other lipoproteins, but apoA-I is unique to HDL and thought to be responsible for HDL’s protective function (58). For example, apoA-I is primarily responsible for the activity of HDL in RCT. ApoA-I selectively interacts with lipoprotein receptors to efflux cholesterol from lipid-laden macrophages or peripheral tissue and take up cholesterol within HDL (59-63). ApoA-I is also a cofactor for LCAT to esterify cholesterol and increase its hydrophobicity, stabilizing cholesterol within HDL (64). While this role of apoA-I in RCT makes apoA-I anti-atherosclerotic, there have also been many studies demonstrating delipidated apoA-I to have anti-inflammatory properties similar to HDL. ApoA-I was shown to inhibit nonocclusive carotid periarterial collar-induced ROS, ICAM-1, vascular cell adhesion molecule (VCAM), and monocyte chemoattractant

protein-1 (MCP-1) in rabbits (65). Additionally, in an LPS-induced inflammatory model, genetically transferred human apoA-I by adenoviral vector to mice increased mouse survival and suppressed TLR4 expression (66). PMN density and myeloperoxidase concentrations in the lungs, markers of TLR4 induced inflammation, were also suppressed by apoA-I. Continued research has implicated apoA-I to play a role in the immune response through TLR4. Completely delipidated apoA-I can initiate TLR4 activation and through MyD88-dependent and –independent pathways, can activate NF-κB, and induce cytokine production in macrophages (67). Taken together, this information suggests that apoA-I has the ability to directly influence components of the immune system including modulation of the TLR4 pathway.

ApoA-I mimetic peptides

Because of the anti-inflammatory properties of apoA-I, apoA-I mimetic peptides have been designed to replicate endogenous apoA-I. These peptides are unique sequences designed to replicate the class A amphipathic alpha-helix of apoA-I (68). The original apoA-I mimetic, 18A, was shown to replace 40% of apoA-I within HDL. The modified HDL was then shown to be equally effectual as unmodified HDL in acting as an LCAT substrate, indicating 18A sufficiently mimics the structural motif of apoA-I (69). 18A has since been altered to increase lipid affinity, and thus increase the solubilization of lipids and prevent atherosclerosis (70, 71). While these peptides have been shown to increase lipid affinity and more effectively induce cholesterol efflux, the high lipid affinity is associated with cytotoxicity because of adverse effects on the integrity of the plasma membrane (72). Consequently, more peptides have been designed to reduce general lipid affinity, but induced cholesterol efflux specifically through a lipoprotein receptor (73-75). Despite these advances, the most commonly studied peptide is L-4F (76) due to its simple 18

amino-acid sequence with effective cholesterol efflux and anti-inflammatory properties (77-79). While these peptides have been primarily studied in the context of CVD, L-4F has also been shown to be protective in the lungs (80, 81). These peptides have been designed as tools to further study the role of HDL, as well as to be studied as potential therapeutics for CVD and pulmonary diseases.

Dysfunctional HDL

While HDL is generally necessary for lung/cardiovascular homeostasis and protection, it can become dysfunctional in individuals with chronic inflammatory diseases. Dysfunctional HDL (D-HDL) is identified as being larger in size or as having decreased protective function. As a result, D-HDL is less anti-inflammatory (82), anti-oxidative (83), anti-apoptotic, and anti-atherosclerotic (84). D-HDL develops in chronic inflammatory diseases such as coronary artery disease (85), type II diabetes mellitus (86), or obesity (87) due to the high levels of oxidative stress throughout the body in these diseases. D-HDL may also contribute to the development of lung diseases such as chronic obstructive pulmonary disease (88) or viral pneumonia (89). D-HDL has been shown to be unable to prevent LDL oxidation in woman with systemic lupus erythematosus (90). Also, D-HDL from diabetics is unable to increase endothelial cell wound healing (84). The definition of D-HDL is unclear, with the notion of it not behaving as functional HDL being the primary stipulation. As a result, there are many forms of D-HDL. One can be found to have D-HDL in a circumstance as simple as having a greater density of the larger sized subpopulation of HDL (HDL2) in circulation (91). The smaller sized subpopulation of HDL (HDL3) has been reported to be more protective than HDL2 (92), however, this is somewhat controversial as there have been conflicting reports on this finding (93, 94). Another form of D-HDL occurs during the acute phase inflammatory response as serum amyloid A replaces apoA-I in HDL (95), significantly reducing

the cholesterol efflux, anti-inflammatory (96), and the anti-oxidative ability of HDL (97, 98). HDL can also become dysfunctional when apoA-I is glycated, which usually is found in type II diabetics (99). D-HDL by glycation has an impaired ability to induce monocyte cholesterol efflux (100) and suppress the inflammatory response of monocytes/macrophages to LPS (101). The most commonly studied (102), and ubiquitously identified, type of D-HDL is induced by oxidation of apoA-I. Oxidized HDL has been found in type II diabetes (86), coronary artery disease (103), obesity (87), chronic kidney disease (104), atherosclerosis (105), and after acute inflammation (89). Most research has discovered where D-HDL can be found, however, there is very little literature on the ramifications of developing D-HDL, particularly in pulmonary disease.

Goal of research and statement of hypothesis

The role of HDL in CVD has been studied for decades, however the role of HDL in pulmonary immunity is severely understudied. As described earlier, HDL is necessary for fundamental lung function, and is involved in the innate immune response of the lungs. Yet, how HDL is incorporated into lung function is unknown. With the lack of clarity for the role of HDL in pulmonary immunity, the consequences of D-HDL in the pulmonary immune response is even less understood. ApoA-1 has been shown to be critical in the protective role of HDL in the lung and, as a result, apoA-1 mimetic peptides have been designed to study HDL. However, there are currently no D-HDL mimetic peptides that could be used to study the consequences of D-HDL in cardiovascular or pulmonary disease pathogenesis. The goal of this project is to further elucidate the role of HDL in the pulmonary immune system, and to design a D-HDL mimetic peptide to be used as a tool to study D-HDL in the context of pulmonary inflammation that can drive the development of chronic lung diseases.

Specific aims

Aim 1: Determine if HDL protects the lungs from inflammation by decreasing PMN chemotaxis. *Hypothesis: HDL decreases pulmonary inflammation by decreasing the display of CXCR2 on PMNs, thus inhibiting PMN chemotaxis and chemokine potency.*

A. Observe if HDL suppresses the LPS-induced increase in pulmonary neutrophilia and inflammation.
B. Evaluate if HDL suppresses the LPS-induced increase in surface display of CXCR2 on blood PMNs.
C. Determine the impact of LPS and HDL on cholesterol transportation in the lungs.

Aim 2: Develop a model for evaluating the functionality of a novel D-HDL mimetic peptide. *Hypothesis: The apoA-I mimetic peptide, L-4F, will replicate the protective functions of HDL during endothelial cell inflammation.*

A. Reveal if HDL suppresses the LPS-induced increases in VCAM and ICAM-1 expression on endothelial cells *in vitro*.
B. Ascertain if L-4F mimics the protective properties of HDL on endothelial cells
C. Engineer a novel D-HDL mimetic peptide (L-2W) for future study of D-HDL

CHAPTER II: A NOVEL ROLE FOR HDL IN PULMONARY IMMUNITY

INTRODUCTION

Pulmonary diseases are the third leading cause of death in the United States, accounting for more than 30% of deaths in 2015 (106). Additionally, metabolic and cardiovascular diseases can contribute to pulmonary disease onset and progression (107, 108). It has long been appreciated that there is an inverse relationship between plasma high-density lipoprotein (HDL) and cardiovascular disease (CVD) (4, 5). Recently, it has been reported that there is a similar relationship between serum HDL-cholesterol and the severity of pulmonary diseases (109-111). However, the mechanistic interaction of HDL influencing the severity of lung disease is understudied. Therefore, it is critical to further understand how HDL interacts with the pulmonary immune system.

Several pulmonary diseases, such as chronic obstructive pulmonary disease, are the result of continuous acute phase inflammation (16). The innate immune response is generally stimulated by damage-associated molecular patterns (DAMPs) or pathogen-associated molecular patterns (PAMPs). A commonly used PAMP for inducing acute phase inflammation is a lipid known as lipopolysaccharide (LPS), which activates the immune system through the toll-like receptor 4 (TLR4) pathway (17, 18). This pathway leads to the production of pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and interleukin-1β (IL-1β), neutrophil (PMN) chemokines such as keratinocyte chemoattractant (KC) and macrophage inflammatory protein-2 (MIP-2), and adhesion molecules such as intracellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule (VCAM) (19, 20, 112).

During host defense, activated PMNs display a CD11b/CD18 complex (113-115). Additionally, PMN trafficking is facilitated by chemotaxis, which involves the secretion of

chemokine molecules to create a chemotactic gradient to communicate to immune cells in circulation (116). MIP-2 and KC are the ligands for the C-X-C Motif Chemokine Receptor 2 (CXCR2) on PMNs (117), which facilitates PMN chemotaxis (118). Production of these molecules contribute to trafficking leukocytes to the cite of inflammation for the body to resolve the inflammatory stimulus. However, hyperinflammation can damage the local tissue due to increased neutrophilia, reactive oxygen species production (ROS), and protease release (119). Due to the severity of pulmonary diseases across the world, it is critical to further understand the pulmonary immune response. Through further understanding, better treatments can be developed to reduce the burden of pulmonary diseases on society.

It has recently become understood that HDL plays a significant role in the pulmonary immune system (120). HDL is an 8-10 nm particle composed of a core with cholesterol, cholesteryl ester, fatty acids, triglycerides, proteins, enzymes, and micro RNAs and a phospholipid monolayer membrane interwoven with several apolipoproteins (23-25). The protective properties of HDL have primarily been attributed to its major structural protein, apolipoprotein A-I (apoA-I) (58). ApoA-I deficient mice have increased pulmonary inflammation, oxidative stress, collagen deposition, and airway hyperresponsiveness (7). HDL is also beneficial to the lungs by increasing type II alveolar cell proliferation (121). Additionally, isolated apoA-I protects mouse lungs from LPS-induced inflammation (56). Also, HDL has been shown to accumulate in the lungs of mice with induced pulmonary emphysema (54). Congruently, HDL has been shown to decrease ICAM-1 and VCAM expression after tumor necrosis factor α (TNF-α) incubation *in vitro* (43).

Many of HDL's cardioprotective properties have been attributed to its role in reverse cholesterol transport (RCT) (36, 122). During RCT, lipoprotein receptors ATP binding cassette transporter A1 (ABCA1), ATP binding cassette transporter G1 (ABCG1), and scavenger receptor

B-I (SR-BI) transport cholesterol from tissues/cells to HDL (30, 36, 123, 124). These lipoprotein receptors used in RCT are expressed in the lung on type I and type II alveolar epithelial cells and alveolar macrophages (61, 125, 126). These receptors allow HDL to interact with the lungs directly, promoting the RCT of lipids from type I and type II alveolar epithelial cells and surfactant secretion (127, 128). In addition to RCT, lipoprotein receptors have been reported to be protective against lung injury and development of chronic lung diseases (129-132).

The hypothesis of this study was that increasing circulating levels of HDL will mitigate pulmonary injury and inflammation through modulation of lipoprotein receptors. This study evaluated the mechanism of how circulating HDL can mitigate pulmonary inflammation utilizing a well characterized model of pulmonary inflammation (LPS-induced injury). These data indicate that HDL inhibits the pulmonary inflammatory response by suppressing the chemotactic and transmigratory potential of blood PMNs. Additionally, these findings show that HDL abrogates pulmonary lipoprotein receptor expression during lung injury. To our knowledge, these findings are the first to implicate the ability of HDL to inhibit PMN chemotactic potential through the modulation of lipoprotein receptors in the lungs.

METHODS

Murine HDL pretreatment and whole body LPS exposure. C57BL/6J (WT) male mice, 8-12 weeks were purchased from Jackson Laboratories (Bar Harbor, ME). HDL was purchased from Kalen biomedical (Germantown, MD). *Escherichia coli* 0111:B4 LPS was from Sigma (St. Louis, MO). All experiments were performed in accordance with the Animal Welfare Act and the U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals after review by East Carolina University's Animal Care and Use Committee. Mice were pretreated with HDL

(40mg/kg), or phosphate buffered saline (PBS) through retro-orbital injection (200 μL) and then exposed to aerosolized LPS (300 μg/mL) or were unexposed. Mice were exposed to aerosolized LPS for 30 min and necropsied 2 or 24 hours after exposure.

Murine sample collection. Mice were first anesthetized with 990 mg/kg ketamine and 10 mg/kg xylazine, and then euthanized by a bilateral thoracotomy. During necropsy, blood, bronchoalveolar lavage fluid (BALF), and lung tissue were collected. Blood was collected by puncturing the right atrium with a 25-gauge needle and collecting from venous flow. Three quarter BALF was collected by actively instilling and withdrawing 26.25 $ml/kg_{(body\ wt)}$ PBS three times from the right lung with the left lung clamped off. The left lung was then excised, flash frozen, and stored at -80°C.

Assessment of airspace inflammation and cholesterol content. Cell differentials were performed as previously described (129). Total cholesterol content in BALF was measured using an Amplex Red cholesterol assay kit from ThermoFisher (Wilmington, DE). BALF protein was measured using a Pierce BCA Protein Assay Kit from ThermoFisher (Wilmington, DE) and microplate photometer set to 562 nm .

Pro-inflammatory cytokines, chemokines, and lipoprotein receptors expression in the lung. The left lungs of mice were flash frozen and total RNA was isolated using a Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA). Concentrations of RNA for each sample were determined using the NanoDrop 2000 (ThermoFisher Scientific, Wilmington, DE). RNA was reverse transcribed, using the High Capacity RNA-to-cDNA Kit or High Capacity cDNA Reverse Transcription Kit, and real-time polymerase chain reaction (RT-PCR) was performed in a one-step reaction using iScript

One-Step RT-PCR kit (ThermoFisher Scientific, Waltham, MA). RT-PCR was completed with the Taqman Universal PCR Master Mix from Thermofisher (Waltham, MA). Taqman primers were obtained from Invitrogen (Waltham, MA). Primers used for RT-PCR were 18s (Mm03928990), Abcg1 (Mm00437390), Abca1 (Mm00442646), Cd36 (Mm00432403), Scarb1 (Mm00450234), Ldlr (Mm01177349), Cxcl2 (Mm00436450), Cxcl1 (Mm04207460), IL-1β (Mm00434228), TNF (Mm00443258), IL-6 (Mm00446190), ICAM-1 (Mm00516023), and VCAM (Mm01320970) from Thermofisher (Waltham, MA). Genes were amplified and detected using a ViiA 7 RT-PCR System (ThermoFisher, Waltham, MA) to obtain cycle threshold (Ct) values for target and internal reference cDNA levels. Fold changes in expression for mRNA quantities were calculated using the $2^{-\Delta\Delta}$Ct method and Ct values. Samples were normalized to 18S as previously described (133). Taqman primers identified pro-inflammatory cytokines (TNF-α, IL-6, IL-1β) PMN chemokines (MIP-2, and KC) and lipoprotein receptors (ABCA1, ABCG1, SR-BI, Cluster of Differentiation 36 (CD36), and LDL receptor (LDLr)) gene expression.

Blood flow cytometry. During necropsy, blood was collected into EDTA tubes. Cells were blocked using 5% normal mouse serum and 5% normal rat serum (Jackson ImmunoResearch Laboratories Inc, West Grove, PA), and 1% Fc-receptor-block (anti-mouse CD16/32; eBioscience, San Diego, CA), and then stained with anti-mouse antibodies for anti-Ly6G-APC, anti-C-X-C Motif Chemokine Receptor 2 (CXCR2)-PerCp, anti-CD45-Pacific Blue, and anti-cluster of differentiation molecule 11B (CD11b)-BUV. Antibodies used were from Biolegend (San Diego, CA). Laser refraction was measured (BD LSRII flow cytometer) and analyzed using FlowJo software (Tree Star Inc, Ashland, OR). PMNs were identified as CD45 and Ly6G positive.

Statistical analysis. Data were analyzed using parametric or nonparametric one-way ANOVA (Kruskal-Wallis test) followed by comparison using a Dunn's multiple comparisons test to correct for multiple comparisons using statistical hypothesis testing in GraphPad Prism 7.0 (San Diego, CA). Where deemed appropriate, unpaired nonparametric t-test (Mann-Whitney tests) were utilized to analyze data. A value of $p<0.05$ was deemed significant.

RESULTS

HDL suppresses LPS induced pulmonary neutrophils and macrophages

LPS exposure has been shown to induce pulmonary inflammation (18), which is inhibited by apoA-I and apoA-I mimetic peptides (56, 81, 121). However, the anti-inflammatory effects of HDL in the context of aerosolized LPS-induced acute lung injury has not been studied. Mice were pretreated with HDL retro-orbitally, then challenged with aerosolized LPS to induce an acute pulmonary inflammatory response. 24 hours after pretreatment and challenge, cell differentials from BALF were analyzed (Figure 2.1). HDL pretreatment alone did not alter pulmonary inflammation in unexposed mice. LPS exposure increased the number of airspace PMNs and decreased airspace macrophages. HDL pretreatment before LPS exposure caused a decrease in airway PMNs and macrophages compared to LPS exposed mice.

LPS-increased airway protein concentration was not suppressed by HDL

LPS is known to increase markers of injury such as lung protein (134-137). Given HDL pretreatment prevented leukocyte influx into the lungs, it was of interest to determine if HDL influced lung injury. 24 hours after pretreating mice with HDL and exposing them to LPS, BALF was collected to measure total protein (Figure 2.2). HDL pretreatment had no effect on BALF

protein when mice were unexposed. LPS exposure significantly increased lung protein. HDL pretreatment did not suppress the LPS-induced increase of protein in the lungs.

HDL inhibits select LPS induced pro-inflammatory cytokines

HDL has been reported to suppress circulating levels of pro-inflammatory cytokines IL-6 and TNF-α in various animal models and models of acute lung injury (138-140). Given that pretreatment with HDL suppressed LPS induced pulmonary neutrophilia, the influence of HDL on pro-inflammatory cytokine expression was determined. Mice pretreated with HDL and exposed to LPS were necropsied 2 h after exposure to assess the inflammatory response of pulmonary resident cells, or 24 h after exposure to observe peak lung inflammation (141). RNA was isolated from whole lung tissue and expression of pro-inflammatory cytokines IL-6, TNF-α, IL-1β, and PMN recruiting chemokines MIP-2, and KC were measured by RT-PCR 2 and 24hrs post exposure (Figure 2.3). Pro-inflammatory cytokines and chemokines were unaffected by HDL in unexposed mice. After LPS exposure, all measured genes were upregulated both 2 and 24 hours after exposure compared to respective controls. Pro-inflammatory cytokine TNF-α was unaffected by HDL pretreatment 2 and 24 hours after LPS exposure. IL-6 was inhibited in HDL pretreated mice 2 hours after LPS exposure and IL-1β 24 hours after LPS exposure. However, MIP-2 expression was suppressed by HDL 2 hours after LPS exposure, and KC was suppressed 2 and 24 hours after exposure.

HDL decreases PMN migration adhesion molecules

Downregulation of pulmonary PMNs and suppression of PMN chemoattractants suggested that HDL inhibits general PMN trafficking. Another component of PMN trafficking involves the use

of adhesion molecules ICAM-1 and VCAM, which are crucial for endothelial adhesion and transmigration (142), and have previously been shown to be suppressed by HDL (43). However, the effect of HDL on aerosolized LPS-induced expression of pulmonary ICAM-1 and VCAM has not been studied. mRNA gene expression of ICAM-1 and VCAM was measured by RT-PCR from the lungs of WT mice pretreated with HDL and exposed to LPS (Figure 2.4). HDL did not affect either adhesion molecule in unexposed mice. LPS exposure increased the expression of ICAM-1 and VCAM 2 hours after injury. ICAM-1 and VCAM were not suppressed by HDL 2 hours after LPS exposure. 24 hours after LPS exposure, pulmonary ICAM-1 expression was increased. HDL pretreatment suppressed ICAM-1 and VCAM gene expression in the lungs 24 hours after LPS exposure.

HDL blunts LPS-induced upregulation of PMN chemotaxis receptor, CXCR2

Decreased pulmonary PMNs, PMN chemokines expression, and adhesion molecule expression suggests HDL would suppress PMN chemotaxis receptor, CXCR2; however, no report to which we are aware has demonstrated the effect of HDL on CXCR2 surface display on PMNs. Blood collected from HDL pretreated, and LPS exposed, mice was labeled with CD45, Ly6G, CD11b and CXCR2 antibodies and surface display on PMNs was observed by flow cytometry (Figure 2.5). The percent of PMNs present in the blood (out of all hemopoietic cells) was suppressed in unexposed mice pretreated with HDL. LPS exposure did not cause a change in percent neutrophils. HDL pretreatment did not change percent neutrophils after LPS exposure. CD11b, a marker of PMN activation (113-115), was not altered by HDL pretreatment. Display of CXCR2 was decreased in HDL pretreated mice both unexposed and exposed to LPS.

LPS exposure increases pulmonary cholesterol content

After finding HDL inhibits PMN chemotaxis during LPS exposure, we sought to begin to uncover the mechanism associated with this anti-inflammatory role in the lung. HDL is a cholesterol transporter, thus many of its protective functions are dependent on removing cholesterol from specific tissues. Since HDL modulates pulmonary cholesterol homeostasis and pulmonary inflammation, we evaluated the impact of HDL on the concentration of cholesterol in the BALF of LPS exposed mice. Surprisingly, we found that HDL pretreatment alone did not affect airspace cholesterol. LPS exposure significantly increased the concentration of total cholesterol in the lungs, which was then suppressed in HDL pretreated mice (Figure 2.6).

HDL suppresses lipoprotein receptors after exposure to LPS

HDL is actively involved in the transport of cholesterol via lipoprotein receptors such as ABCG1, ABCA1, and SR-BI. These receptors have also been tied to the pulmonary immune response in several studies (129, 131, 143). Because of these connections, the impact of HDL pretreatment on the gene expression of these receptors in the lungs of mice exposed to LPS was evaluated. The gene expression of CD36 was also observed because of its many overlapping roles with SR-BI (144), and LDLr expression was assessed because of its close relationship with HDL (145) (Figure 2.7). HDL pretreatment alone had no effect on these lipoprotein receptors. LPS exposure caused a marked increase in gene expression of ABCG1, ABCA1, SR-BI, and LDLr 24 hours after, which was strongly suppressed by HDL pretreatment. CD36 was suppressed 24 hours after LPS exposure and suppressed further in HDL pretreated mice. There was also found to be an increase in gene expression of SR-BI 2 hours after LPS exposure, which increased further with HDL pretreatment.

DISCUSSION

Pulmonary diseases are serious conditions with considerable impact on current and future society. In 2010 lung diseases were reported to be the third leading cause of death worldwide (146). Medical costs associated with pulmonary diseases were reported to be $32.1 billion in 2010 and are expected to rise to $49 billion by 2020 (147). As there is an inverse relationship between HDL-cholesterol and pulmonary diseases, advances in the understanding of HDL's interaction with the pulmonary immune system may be necessary to alleviate some of the burden of pulmonary diseases across the world.

In this study it was demonstrated that HDL inhibits PMN chemotaxis in the lung after acute lung injury. This was concluded from finding that HDL suppressed pulmonary neutrophilia, PMN recruiting chemokines, and PMN chemokine receptor CXCR2. It was later demonstrated that this inhibition may be the result of a decreased cholesterol burden in the lungs due to the decreased pulmonary cholesterol content and lipoprotein receptor gene expression in the lung after HDL pretreatment. Overall, this report demonstrates a potential mechanistic outline of HDL's role in pulmonary protection from acute lung injury.

To uncover the role of HDL in the pulmonary immune system, it was first demonstrated that HDL decreases pulmonary neutrophilia after LPS exposure, indicating an abrogation in the inflammatory response. However, it was found that HDL does not suppress airway protein concentration after LPS exposure. This indicates that alveolar damage was not prevented by HDL pretreatment. The inflammatory signaling response was then evaluated to determine the cause for decreased airway neutrophilia, but not decreased damage. The gene expression of pro-inflammatory cytokines and PMN chemokines was assessed, and select suppression of pro-

inflammatory cytokines and PMN chemokines at 2 and 24 hours after injury was observed. By observing the response 2 hours after exposure, the responses of local resident cells were evaluated because there is minimal systemic migration to the site of injury after 2 hours. Consequently, 24 hours after exposure peak leukocyte infiltration occurs (141), giving an indication of the complete inflammatory response. The only pro-inflammatory cytokine that was not affected by HDL was TNF-α. This is contradictory to previous studies; however, previous studies have used different routes of HDL delivery, LPS exposure, and animal models (56, 148-152). The early inhibition of IL-6 observed could suggest inhibition of resident alveolar macrophage activation and pro-inflammatory response. IL-1β was only suppressed 24 hours after exposure in HDL pretreated mice, suggesting the pro-inflammatory state was becoming resolved after 24 hours in HDL pretreated mice. IL-6 can also play an anti-inflammatory role later after acute injury (153, 154), correlating with HDL pretreated mice having a resolving inflammatory state after 24 hours. Interestingly, PMN chemokines MIP-2 and KC were both suppressed 2 hours after LPS exposure. MIP-2 and KC are primarily secreted by macrophages, further supporting HDL effects alveolar macrophages early during injury (155, 156). KC also continued to be suppressed 24 hours after LPS exposure. The suppression of these PMN chemokines suggests a possible explanation to the decrease in pulmonary neutrophilia previously observed. Taken together, it appears HDL partially suppressed early secretion of pro-inflammatory cytokines, which lead to the suppression of alveolar macrophage mediated PMN recruitment.

Additionally, the effect of HDL on PMN transmigratory potential was evaluated by measuring the expression of adhesion molecules ICAM-1 and VCAM in the lungs. It was found that HDL pretreatment suppresses the gene expression of ICAM-1 and VCAM in LPS exposed mice. The suppression of ICAM-1 has been thought to be the result of HDL carrying micro RNA

miR-223, which blunts the expression of ICAM-1 (44, 45). By inhibiting adhesion molecule expression, HDL effectively mitigated the ability of PMN's to migrate into the lungs where chemokines would be secreted. The additional suppression of PMN chemokines with adhesion molecules leads to decreased chemotactic potential of PMNs during inflammation. Previously, HDL has indirectly been shown to influence leukocyte chemotaxis in pulmonary inflammation. Addition of an apoA-I mimetic peptide decreases PMN chemotaxis as well as CXCR2 surface display in apoA-I deficient murine PMNs (77). Also, isolated apoA-I has been shown to decrease monocyte chemotaxis in rabbits (157), and the number of BALF PMNs is increased in ovalbumin-challenged apoA-I knockout mice when compared to ovalbumin-challenged WT mice (55). Additionally, HDL has been shown to decrease PMN migration to a sight of cardiac infarction in mice (158), indicating the potential to decrease PMN migration to the sight of injury in the lungs. This information was supported when it was observed that HDL pretreatment suppresses the surface display of CXCR2, but not percent PMNs after LPS exposure. This data suggests signaling for PMN production and activation was not inhibited by HDL pretreatment. However, PMN chemotactic potential through the endo/epithelium and into the lung was suppressed, possibly preventing pulmonary neutrophilia. If HDL prevents PMN transmigration through the endo/epithelium, then secretion of oxidative enzymes, ROS, and PMN phagocytosis would be prevented in the lungs which would reduce pulmonary inflammation.

Knowing HDL can take up cholesterol from macrophages, we evaluated the cholesterol content in the lungs and found HDL slightly inhibits the LPS-induced increase of cholesterol in the lung BALF. The increase in cholesterol after LPS exposure may have been the result of systemic leak into the lung because of damaged alveoli from increased pulmonary neutrophilia. As more PMNs migrated to the lungs, they caused temporary pores in the endo/epithelium, which

could then have been expanded by secreted ROS, and oxidative enzymes (159, 160). The decrease in observed cholesterol in the BALF might then be the result of an attenuated inflammatory response, and less damage to the alveolar membrane. Several studies have reported that the increased lipid raft formation that occurs in ABCG1 and ABCA1 deficient macrophages could account for enhanced inflammatory responses, especially after exposure to LPS (161-165). It is possible that the increased cholesterol deposition observed here during LPS injury exacerbated the inflammatory response. Additionally, ABCG1, ABCA1, and SR-BI have been shown to play a protective role in the lungs. ABCG1 deficient mice have been shown to have increased airway PMNs after ovalbumin sensitization and challenge (35). SR-BI deficient mice were also shown to have increased PMN recruitment and cytokine production in the airspace of lungs injured by bacterial pneumonia (33). ABCA1 has been shown to be activated by LPS exposure in THP-1 monocytes and WT mice (54). Therefore, HDL pretreatment may have attenuated the inflammatory response by reducing the cholesterol burden on alveolar macrophages and consequently decreased PMN chemotaxis. To answer this question, the gene expression of cholesterol transporters and lipoprotein receptors ABCG1, ABCA1, SR-BI, CD36 and LDLr was measured. It was found that LPS significantly increased the expression of ABCG1, ABCA1, SR-BI, and LDLr 24 hours after exposure. This expression was then suppressed by HDL pretreatment. We believe the increases in expression of the lipoprotein receptors was the result of cholesterol loading of alveolar macrophages after lung injury. When mice were pretreated with HDL, SR-BI was then responsible for transporting cholesterol from the alveolar macrophages to HDL 2 hours after exposure, thus reducing the cholesterol burden in the lungs. This reduced burden resulted in a decreased demand for the lipoprotein receptors 24 hours after exposure, and thus decreased gene expression.

Overall, it has been demonstrated that HDL suppresses PMN chemotaxis by inhibiting MIP-2/KC production, suppressing adhesion molecules ICAM-1/VCAM production, and decreasing the surface display of CXCR2 on PMNs. It has also been shown that LPS exposure increases cholesterol content in the lungs and gene expression of several lipoprotein receptors. The increases in cholesterol and lipoprotein receptors was then suppressed by HDL. We have proposed a connection between these two phenotypes, stating increased cholesterol load on alveolar macrophages increases the inflammatory response, and HDL prevents that increased inflammation. With this information, treatments could be developed to target this anti-inflammatory role of HDL in the pulmonary immune system to help alleviate the severity of pulmonary diseases.

FIGURES

Figure 2.1 HDL suppresses lung macrophage and neutrophil populations after lung injury. Mice were pretreated with PBS or HDL retro-orbitally and exposed to aerosolized LPS or were unexposed and then necropsied 24 hours after exposure to measure BALF cell differentials. Macrophage (MΦ) and neutrophil (PMN) populations were recorded (n=5 per group; * $p<0.05$ from PBS; # $p<0.05$).

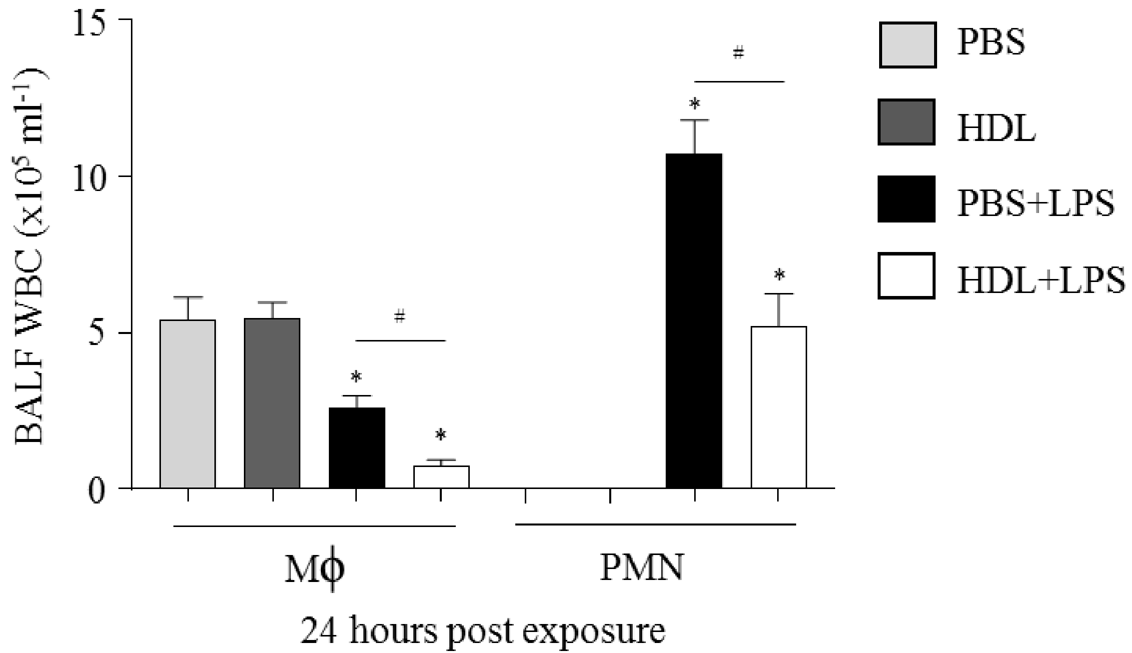


Figure 2.2 HDL does not suppress LPS-increased airway protein. Mice were pretreated with HDL, exposed to LPS, and BALF was collected 24 hours after LPS exposure to measure BALF protein. (n=5 per group; * p<0.05 from PBS).

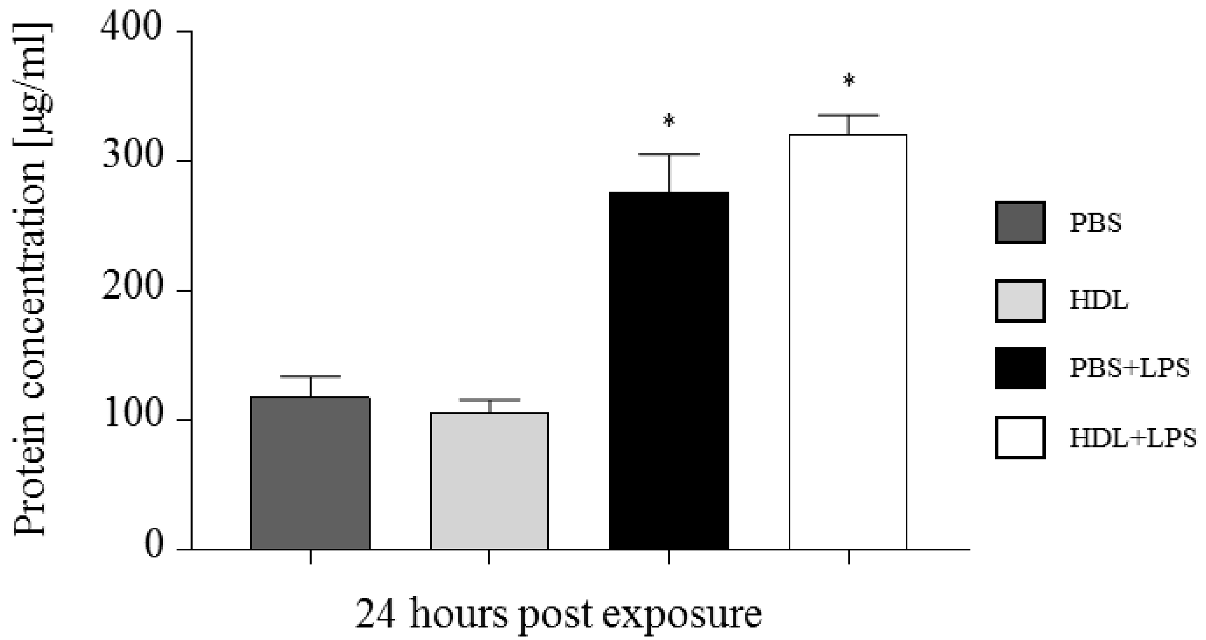


Figure 2.3 HDL suppresses select pro-inflammatory cytokines and PMN chemokines after lung injury by LPS. WT mice were pretreated with PBS or HDL and were then left unexposed or exposed to 300 µg/mL of LPS. 2h and 24h after LPS exposure, mice were necropsied. RNA was isolated from whole lung tissue and proinflammatory cytokines **(A)** TNF-a, **(B)** IL-6, **(C)** IL-1β and neutrophil chemoattractants **(D)** MIP-2 and **(E)** KC were measured by real time PCR. Data is presented as ddCt compared to 18S (n=5 per group; * p<0.05 from PBS of respective time point; # p<0.05).

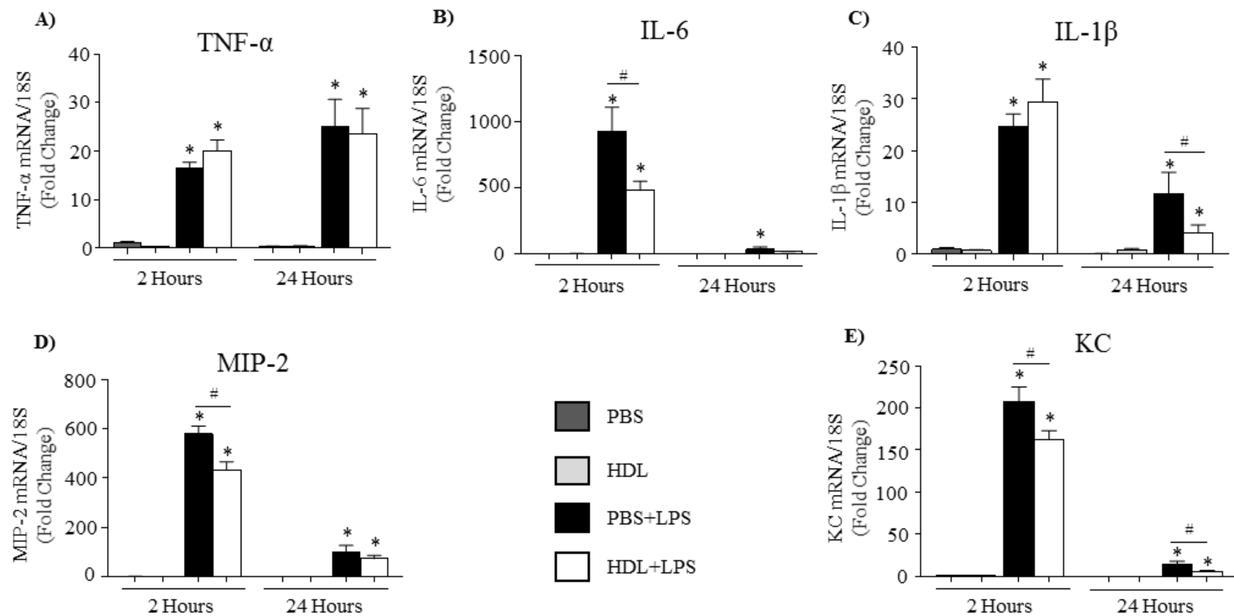


Figure 2.4 HDL suppresses adhesion molecule expression after LPS exposure. WT mice were pretreated with PBS or HDL and were then left unexposed or exposed to 300 μg/mL of LPS. 2h and 24h later, mice were necropsied. RNA was isolated from the lungs and adhesion molecules **(A)** ICAM-1 and **(B)** VCAM expression were measured by real time PCR. Data is presented as ddCt compared to 18S (n=5 per group; * $p<0.05$ from PBS of respective time point; # $p<0.05$).

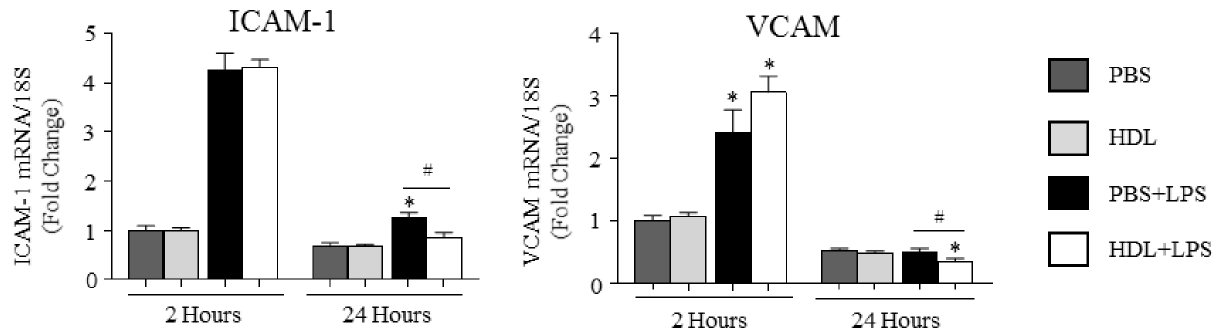


Figure 2.5 CXCR2 surface display, but not percent PMNs, is suppressed by HDL. WT mice were pretreated with PBS or HDL and were then left unexposed or exposed to 300 µg/mL of LPS. 24h later, mice were necropsied. Blood was collected into EDTA tubes and labeled with Ly6g, CD45, CD11b, and CXCR2 antibodies and analyzed by flow cytometry. HDL pretreatment suppressed **(A)** scatter plot of percent PMN **B)** histogram of CXCR2 surface display **(C)** quantified percent PMN, **(D)** quantified CXCR2 surface display were analyzed (n=5 per group; * $p<0.05$ from PBS; # $p<0.05$).

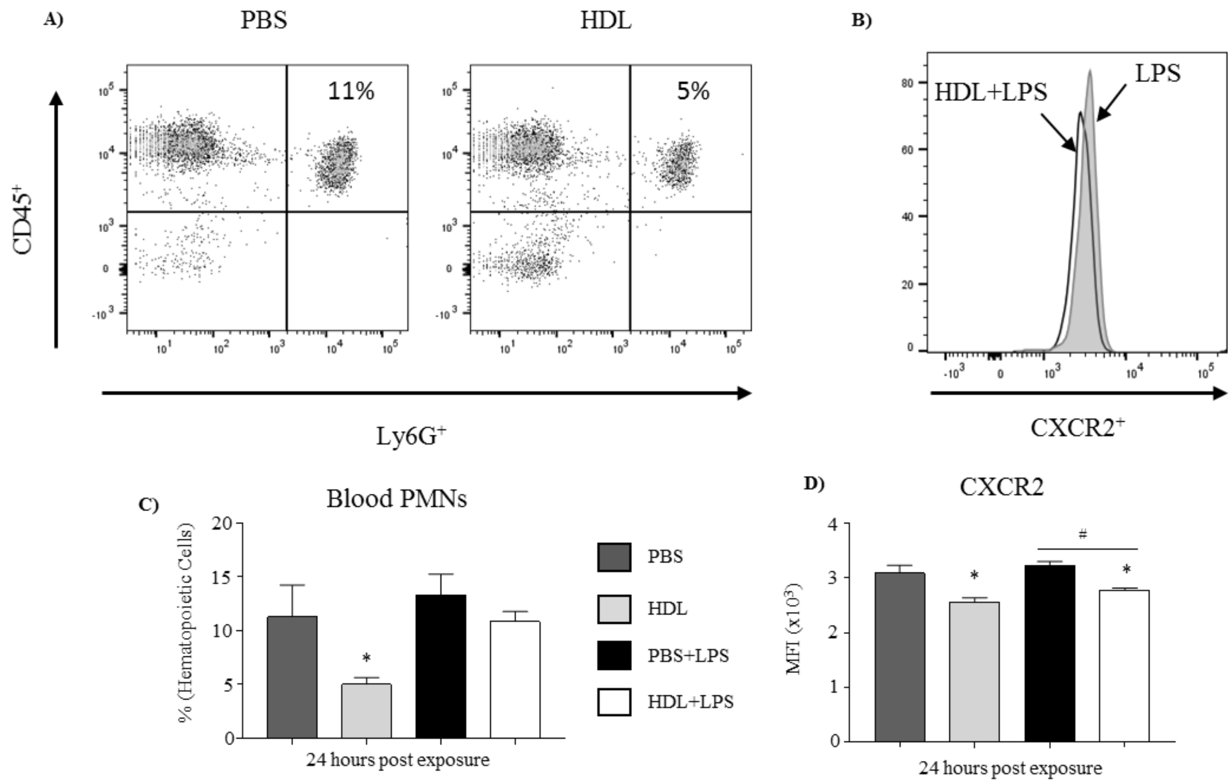


Figure 2.6 LPS exposure increases airway cholesterol concentration. WT mice were pretreated with PBS or HDL and were then left unexposed or exposed to 300 µg/mL of LPS. 2 and 24h later, BALF was analyzed for cholesterol content (measured by the Amplex Red Cholesterol Assay; n=5 per group; * p<0.05 from PBS; # p<0.05).

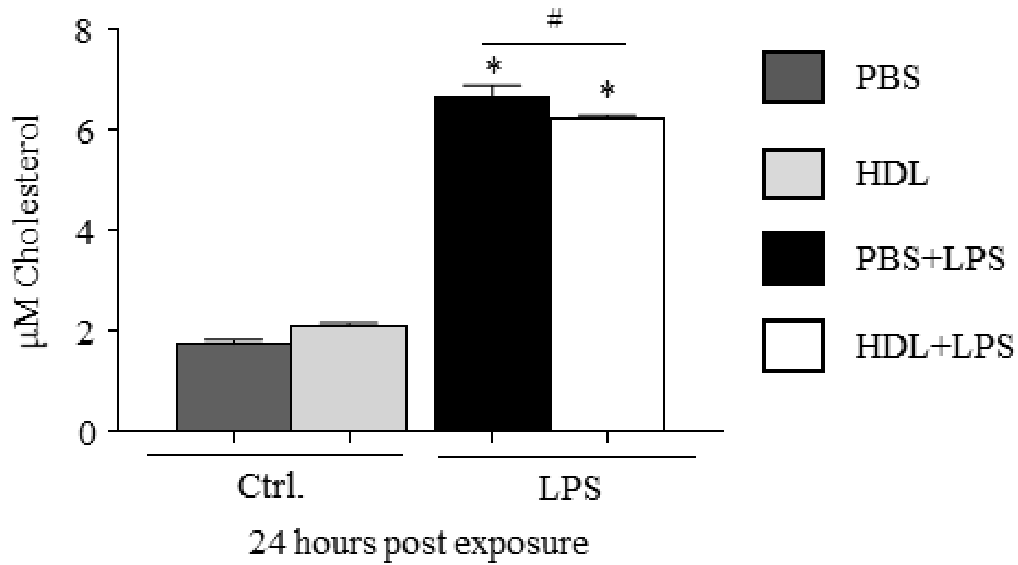
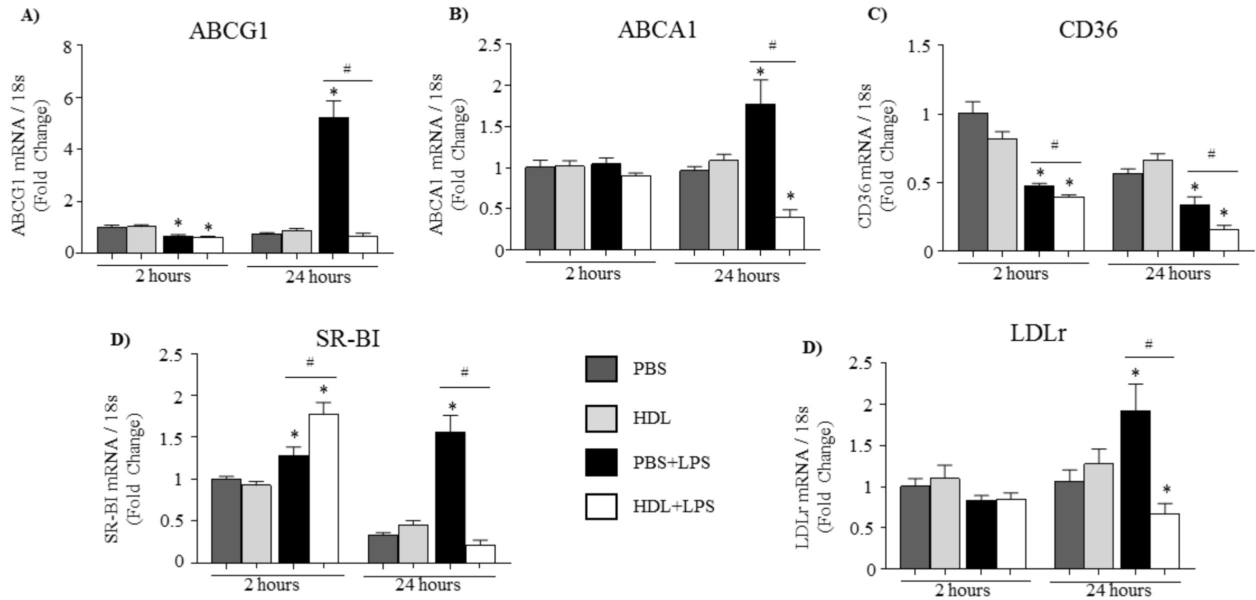


Figure 2.7 HDL suppresses LPS-increased lipoprotein receptor gene expression. WT mice were pretreated with PBS or HDL and were then unexposed or exposed to 300 μg/mL of LPS. 2h and 24h later, mice were necropsied. RNA was isolated from whole lung tissue and lipoprotein receptors **(A)** ABCG1, **(B)** ABCA1, **(C)** CD36 **(D)** SR-BI, and **(E)** LDLr expression was measured by real time PCR. Data is presented as ddCt compared to 18S (n=5 per group; * p<0.05 from PBS of respective time point; # p<0.05).



CHAPTER III: ENGINEERING A NOVEL DYSFUNCTIONAL HDL MIMETIC PEPTIDE

INTRODUCTION

In 2015 pulmonary diseases contributed to almost 8% of reported deaths in the United States, ranking third behind cardiovascular disease and cancer (106). Pulmonary diseases are a continuously growing epidemic that have a severe impact on our current society. One avenue that has recently been pursued to develop therapies for these diseases is with high-density lipoprotein (HDL). There is an inverse relationship between HDL-cholesterol levels and the severity of pulmonary disease (109-111). Additionally, a potentially pathogenic post-translational modification to HDL, known as dysfunctional HDL (D-HDL), has been found in subjects with pulmonary disease (88). The repercussions of developing D-HDL could be severe, however, there are currently few effective methods to study the consequences of D-HDL in the lung.

HDL is an 8-10 nm cholesterol transporter composed of a core with cholesterol, cholesteryl ester, fatty acids, triglycerides, proteins, enzymes, and micro RNAs and a phospholipid monolayer membrane interwoven with several apolipoproteins (23-25). Many of HDL's protective properties have been attributed to its role in reverse cholesterol transport (RCT) (36, 122). During RCT, HDL takes up cholesterol from peripheral tissue and lipid-laden macrophages and transports that cholesterol to the liver for production of glucocorticoids, steroids, or excretion of cholesterol (36, 37). This action gives HDL its anti-atherosclerotic properties. By removing cholesterol from lipid-laden macrophages on atherosclerotic plaques (166), and altering the monocyte/macrophage lipid rafts (47), HDL inhibits vascular plaque buildup and inflammation. Along with this anti-atherosclerotic role, HDL is also anti-inflammatory (167) anti-oxidative (49-51), and anti-thrombotic (52, 53).

Many of HDL's protective functions are thought to be attributed to HDL's major structural protein, apolipoprotein A-I (apoA-I). ApoA-I is a 243 amino acid protein with an amphipathic alpha-helix structure (23). It makes up 70% of its protein content (27, 28), and selectively interacts with lipoprotein receptors to efflux cholesterol from lipid-laden macrophages or peripheral tissue and take up cholesterol within HDL (59-63, 168). Delipidated apoA-I has also been shown to be anti-inflammatory, suppressing tumor necrosis factor α (TNF-α), interleukin-1β, (IL-1β), interleukin-6 (IL-6), reactive oxygen species (ROS), intracellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule (VCAM), monocyte chemoattractant protein-1 (MCP-1), and toll-like receptor 4 (TLR4) in various inflammatory models (56, 65, 66). ApoA-I mimetic peptides have been created to replicate the cholesterol efflux and anti-inflammatory abilities of endogenous apoA-I (69). These peptides are unique sequences designed to replicate the class A amphipathic alpha-helix of apoA-I (68). The most commonly studied apoA-I mimetic peptide is L-4F (76), which has been shown to effectively replicate the cholesterol efflux and anti-inflammatory properties of HDL (77-79).

HDL is normally protective in the body, however, in chronic inflammatory diseases HDL can become dysfunctional. D-HDL is identified as being larger in size or as having decreased protective function. As a result, D-HDL is less anti-inflammatory (82), anti-oxidative (83), and anti-atherosclerotic (84). The definition of D-HDL is unclear, with the notion of it not behaving as functional HDL being the primary stipulation. As a result, there are many forms of D-HDL. There can be an imbalance in HDL subpopulations (91), replacement of apoA-I with other proteins (95), glycation of apoA-I (99), or oxidation of apoA-I. The most commonly studied (102), and most ubiquitously identified, type of D-HDL is induced by oxidation of apoA-I. Oxidized D-HDL has been found in type II diabetes (86), coronary artery disease (103), obesity (87), chronic kidney

disease (104), atherosclerosis (105), and after acute inflammation (89). This oxidation inhibits apoA-I's endothelial repair (169), cholesterol transport (170), and anti-inflammatory properties (171), indicating oxidized HDL is dysfunctional. While the development of D-HDL has begun to become uncovered, the consequences of D-HDL in pulmonary diseases has yet to be elucidated.

Here in it was hypothesized that the apoA-I mimetic peptide, L-4F, would replicate the protective functions of HDL during endothelial cell inflammation. In this chapter, a model was established for reproducibly testing the mimicking ability of an apoA-I mimetic peptide and a D-HDL mimetic peptide. Additionally, a D-HDL mimetic peptide (L-2W) was carefully designed to best reproduce the dysfunction of endogenous D-HDL. To our knowledge, this is the first D-HDL mimetic peptide that has been designed with the purpose of assessing the consequences of D-HDL in pulmonary disease.

METHODS

Peptide synthesis. L-4F (Ac-DWFKAFYDKVAEKFKEAF-NH_2) was synthesized using an automated solid phase synthesizer (PS3 from Protein Technologies Inc., Tucson, AZ). Fluorinylmethyloxycarbonyl (FMOC)-amino acids were coupled to a rink amide resin from Millipore Sigma (Burlington, MA) in the presence of 2-(H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), and acetylated with acetic anhydride at the N-terminus. The peptide was cleaved from the solid support using 90% TFA in the presence of anisole (5%) and triethylsilane (5%) during a 3-hour incubation, purified by high performance liquid chromatography (HPLC), lyophilized, and stored in a sealed container at room temperature. Peptides were diluted in phosphate buffered saline (PBS) when used.

Murine HDL pretreatment, whole body LPS exposure, and sample collection. All experiments were performed in accordance with the Animal Welfare Act and the U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals after review by East Carolina University's Animal Care and Use Committee. C57BL/6J (WT) male mice, 8-12 weeks were purchased from Jackson Laboratories (Bar Harbor, ME). HDL was purchased from Kalen biomedical (Germantown, MD). *Escherichia coli* 0111:B4 lipopolysaccharide (LPS) was from Sigma (St. Louis, MO). RNeasey minikit was from Qiagen (Germantown, MD). Mice were pretreated with HDL (40mg/kg), or PBS through retro-orbital injection (200 µL) and then exposed to aerosolized LPS (300 µg/mL) or room air (RA). Mice were exposed to aerosolized LPS for 30 min and necropsied 24 hours after exposure. Mice were anesthetized with 990 mg/kg ketamine and 10 mg/kg xylazine, and then euthanized by a bilateral thoracotomy. During necropsy the left lung was excised, flash frozen, and stored at -80°C.

Endothelial cell culture and exposures. Human umbilical vein endothelial cells (HUVECs) were purchased from Lonza (Morristown, NJ) and cultured in Endothelial Cell Medium from ScienCell Research Laboratories (Carlsbad, CA). Cells were cultured in T-75 culture flasks from ThermoFisher (Waltham, MA), or Falcon Tissue Culture Dish plates (100 x 20 mm) from Corning (Corning, NY). Cells were passaged until P5, P6, or P7 and stored in liquid nitrogen until use. After thawing, cells were passaged once and seeded in 12-well culture plates for about one day or until wells reached ~80% confluency, or if a monolayer was formed when desired. Cells were then incubated with PBS, HDL (200 µg/ml), or L-4F (50 µg/ml). Either at the same time, or 16-20 hours after incubation with PBS, HDL, or L-4F, cells were exposed to LPS (1 µg/ml or 0.1 µg/ml). Cells were then lysed lysed by 1% β-Mercaptoethanol in RLT buffer and collected.

Wound healing assay. Cell migration ability can be determined by performing a wound healing migration assay (46). HUVECs were plated in 12-well plates with 1 ml of endothelial cell medium containing 1% fetal bovine serum and cultured until ~80% confluency or until monolayers formed. PBS, HDL (200 μg/ml), or L-4F (50 μg/ml) were incubated with the cells at ~80% confluency for 16-20 hours before wounding, or treatments were incubated with the cells forming a monolayer immediately before wounding. Cells were then exposed to LPS (1 μg/ml or 0.1 μg/ml), and a wound was formed by scratching HUVECs with a 200 μl micropipette tip. Wound healing was observed by photographing the scratch with the EVOS FL Cell Imaging System 0, 4, 8, and 12 hours after wounding. The width of the wound was measured at 6 locations per well using ImageJ software and a single averaged width per well was reported.

Pro-inflammatory cytokine, and adhesion molecule expression in the lung. The left lungs of mice were flash frozen and total RNA was isolated using a Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA). RNA was also collected from HUVECs. Concentrations of RNA for each sample were determined using the NanoDrop 2000 (ThermoFisher Scientific, Wilmington, DE). RNA was reverse transcribed, using the High Capacity RNA-to-cDNA Kit or High Capacity cDNA Reverse Transcription Kit, and real-time polymerase chain reaction (RT-PCR) was performed in a one-step reaction using iScript One-Step RT-PCR kit (ThermoFisher Scientific, Waltham, MA). RT-PCR was completed with the Taqman Universal PCR Master Mix from Thermofisher (Waltham, MA). Taqman primers were obtained from Invitrogen (Waltham, MA). Primers used for RT-PCR were 18s (Mm03928990 or Hs03003631), Abcg1 (Mm00437390), Abca1 (Mm00442646), Cd36 (Mm00432403), Scarb1 (Mm00450234), Ldlr (Mm01177349), Cxcl2 (Mm00436450), Cxcl1

(Mm04207460), IL-1β (Mm00434228 or Hs01555410), TNF (Mm00443258 or Hs), IL-6 (Mm00446190 or Hs00985639), ICAM-1 (Mm00516023 or Hs00164932), VCAM (Mm01320970 or Hs01003372), and IL-8 (Hs00174360) from Thermofisher (Waltham, MA). Genes were amplified and detected using a ViiA 7 RT-PCR System (ThermoFisher, Waltham, MA) to obtain cycle threshold (Ct) values for target and internal reference cDNA levels. Fold changes in expression for mRNA quantities were calculated using the $2^{-\Delta\Delta}$Ct method and Ct values. Samples were normalized to 18S as previously described (133). Taqman primers used were pro-inflammatory cytokine TNF-α, ICAM-1, and VCAM.

RESULTS

HDL *in vivo* suppresses LPS-increased expression of adhesion molecules

There is currently no universal model *in vitro* used to test the functional ability of an HDL or D-HDL mimetic peptide in relation to pulmonary disease. To determine which cell line to use *in vitro*, HDL's ability to influence the lungs *in vivo* was evaluated. WT mice were pretreated with HDL retro-orbitally, then exposed to aerosolized LPS, and necropsied 24 hours after exposure. Whole lung tissue was excised, and RNA was collected to measure adhesion molecules ICAM-1, and VCAM gene expression by RT-PCR (Figure 3.1). HDL pretreatment did not affect ICAM-1 and VCAM levels before exposure. LPS exposure caused an increase in ICAM-1 gene expression. HDL pretreated and then LPS exposed mice had suppressed gene expression of ICAM-1 and VCAM compared to LPS alone.

HDL suppresses LPS-induced inflammation of endothelial cells

HDL has been shown to suppress ICAM-1 and VCAM secretion from endothelial cells exposed to TNF-α (45, 172). Given that the data indicates that HDL can alter the expression of endothelial cell expressed adhesion molecules after pulmonary inflammation, endothelial cells were specifically assessed. HUVECs were incubated with HDL for 16 hours, exposed to LPS, and RNA was collected to measure ICAM-1, VCAM, and TNF-α by RT-PCR (Figure 3.2). HDL incubation alone had no effect on ICAM-1, VCAM, and TNF-α expression 16 hours after treatment. LPS exposure increased gene expression of ICAM-1, VCAM, and TNF-α. HDL incubation along with simultaneous LPS exposure suppressed gene expression of ICAM-1, VCAM, and TNF-α compared to LPS alone.

L-4F mimics HDL's ability to suppress LPS-induced inflammation of endothelial cells

After demonstrating endothelial cells are suitable for the mimetic peptide testing model, L-4F's ability to mimic HDL was assessed. L-4F has been shown to inhibit the production of ICAM-1, VCAM, and TNF-α from endothelial cells co-cultured with monocytes that had been pretreated with LPS (78). However, there is limited literature on the optimal LPS exposure concentration for assessing L-4F function on endothelial cells. Because of this, HUVECs were exposed to L-4F and LPS at 0.1 μg/ml or 1 μg/ml simultaneously (L-4F+LPS) for 12 hours and were collected to measure gene expression of ICAM-1, VCAM, and TNF-α by RT-PCR (Figure 3.3). L-4F alone did not affect expression. 0.1 μg/ml LPS increased gene expression for all markers, and 1 μg/ml of LPS increased gene expression more than 0.1 μg/ml of LPS. L-4F+LPS(1 μg/ml) cells had suppressed expression of ICAM-1, VCAM, and TNF-α and L-4F+LPS(0.1 μg/ml) cells had further suppressed expression of all markers.

LPS suppresses wound healing of endothelial cells

HDL has been shown to increase endothelial cell wound healing (84), however, the effect of L-4F on HUVEC wound healing has not been assessed. Likewise, the impact of LPS on wound healing has been understudied. To uncover these effects, HUVECs were exposed to L-4F and LPS at 0.1 μg/ml or 1 μg/ml, scratched, and photographed 0, 4, 8, and 12 hours after exposure. Percent wound closure after 12 hours was recorded (Figure 3.4). Cells exposed to PBS as a control averaged 89.8% wound closure 12 hours after scratching. L-4F did not alter wound healing. LPS exposure at 0.1 μg/ml did not alter wound healing. LPS exposure at 1 μg/ml significantly suppressed wound healing 12 hours after exposure. Cells exposed to LPS+L-4F also had significantly suppressed wound healing 12 hours after exposure, indicating L-4F did not rescue wound healing.

L-4F rescues endothelial cell wound healing from LPS-induced suppression

Despite initially finding L-4F did not rescue wound healing, it seemed unlikely the apoA-I mimetic peptide would be unable to replicate the wound healing ability of HDL. L-4F has been shown to bind to LPS and suppress LPS induced inflammation (78), suggesting L-4F should inhibit the deleterious effects of LPS on wound healing. In one report, HDL has been shown to rescue wound healing after being incubated with cells 16 hours before TNF-α exposure (43). Therefore, HUVECs were incubated with L-4F 16 hours before LPS exposure, scratched, and wound healing was observed 12 hours later (Figure 3.5). L-4F alone did not influence endothelial wound healing. LPS exposure decreased wound healing. Cells incubated with L-4F for 16 hours and then exposed to LPS saw partially rescued wound healing, although not back to baseline.

A novel D-HDL mimetic peptide retains the alpha-helix of L-4F

Here in a model has been developed to assess the functionality of an HDL or D-HDL mimetic peptide. Subsequently, a D-HDL mimetic peptide has been designed to be used for studying the impact of developing D-HDL in the context of pulmonary disease. Due to the high propensity of oxidized D-HDL, oxidation of an apoA-I mimetic peptide is the foundation behind the novel peptide's design. It was reported that hypochlorite (HOCl) oxidation of L-4F does not inhibit its ability to efflux cholesterol (173). Therefore, the novel D-HDL mimetic peptide was designed to increase L-4F's susceptibility to oxidation. The novel mimetic, L-2W, was designed by substituting the carboxyl-terminal phenylalanine of L-4F for a tryptophan and oxidizing the peptide with HOCl (Figure 3.6A). This design was constructed to maintain the amphipathic alpha helix of L-4F before oxidation occurs. Using 3D molecular dynamics modeling, L-2W was shown to retain an alpha-helix structure (Figure 3.6B) and the chosen amino acid substitution maintains amphipathicity.

DISCUSSION

Research on HDL has mostly been focused on its protective role in cardiovascular disease, however, there is a growing need to evaluate the role of HDL in the context of pulmonary disease (120). Along with the protective role of HDL in the lungs, the consequences of D-HDL in the lungs are critically important to understand. However, due to the heterogeneous nature of HDL (174, 175), and the variety of D-HDL that develops (91, 95, 99, 102), this pathologic condition is difficult to study. Because of these difficulties, the goal of this research is to develop a model for reproducibly testing the functionality of a HDL and D-HDL mimetic peptide. Furthermore, a D-HDL mimetic peptide is being designed to be used as a tool for evaluating the consequences of D-

HDL in pulmonary diseases. With this goal in mind, it is hypothesized that the apoA-I mimetic peptide, L-4F, will replicate the protective functions of HDL on endothelial cells exposed to LPS.

A model was to be developed by identifying the parameters required for the HDL mimetic peptide, L-4F, to accurately mimic the protective functions of HDL. The first challenge was to determine what cell type in the lungs is consistently protected by HDL. It is known that HDL can interact with macrophages, endothelial cells, and epithelial cells in the lungs through lipoprotein receptors to transport cholesterol (61, 125, 126). Macrophages may be a suitable cell type to use for assessing HDL mimetic peptide function because of HDL's ability to suppress their inflammatory response (176) and efflux cholesterol (177). However, delipidated apoA-I can cause a pro-inflammatory response in macrophages (67). This is contrary to the normal anti-inflammatory role of HDL and requires more research to increase the understanding of HDL, thus making macrophages an unsuitable cell type for developing a model to assess HDL mimetic peptide function. Because of this, endothelial or epithelial cells were decided to be the more likely candidates to be used for developing an HDL mimetic peptide testing model. This was verified when WT mice were pretreated with HDL, exposed to LPS, and observed to have decreased adhesion molecule expression (ICAM-1, and VCAM) in isolated lung tissue. Endothelial cells were then selected over epithelial cells because HDL more commonly interacts with endothelial cells since it passes through circulation, there is no concern of air-liquid interface culturing, and HDL can increase wound healing of endothelial cells (84, 178). Endothelial cells were confirmed to be a suitable cell type when HUVECs had suppressed expression of adhesion molecules ICAM-1, and VCAM when treated with HDL and LPS compared to LPS alone.

After establishing that HDL protects endothelial cells *in vitro* from LPS-induced inflammation, the anti-inflammatory abilities of L-4F needed to be evaluated. Also, the optimal

concentration of LPS for assessing the anti-inflammatory ability of L-4F was evaluated because there is limited literature on the use of LPS in combination with L-4F with endothelial cells. It was reported that L-4F suppressed the gene expression of adhesion molecules, ICAM-1 and VCAM, as well as pro-inflammatory cytokine TNF-α. It was also shown that suppression was concentration dependent for LPS exposure, with the lower concentration of LPS (0.1 μg/ml) being suppressed more than the higher concentration of LPS (1 μg/ml). Consequently, there was not complete immunosuppression in the L-4F treated cells. This is likely the result of concentration dependent binding of L-4F to LPS. It was reported that L-4F likely binds LPS as L-4F incubated with LPS had a single size exclusion chromatographic peak distinct from individual LPS or L-4F peaks (78). It is likely that L-4F binds the lipid A moiety of LPS because of its anionic and amphipathic structure making an ideal target for L-4F binding (179). This binding would inhibit the LPS-induced inflammatory response by competitively inhibiting the lipid binding protein (LBP)/cluster of differentiation 14 (CD14) complex necessary for activating the TLR4 inflammatory pathway (112, 180). Thus, by increasing the concentration of LPS from 0.1 μg/ml to 1 μg/ml without changing the concentration of L-4F, there was less competitive inhibition of LBP/CD14, and less suppression of inflammation.

Another method for determining HDL mimetic peptide functionality in this model is to assess the wound healing ability of the mimetic peptides. It has been well established that HDL increases endothelial repair, proliferation, and migration (46, 84, 178, 181-184). However, to the best of our knowledge, only one group has evaluated the endothelial repair abilities of L-4F, using human aorta endothelial cells (178). It was reported that L-4F did not increase HUVEC wound healing when incubated with endothelial cells for 12 hours. There has also been little research on the impact of LPS on endothelial cell migration, and proliferation. Furthermore, current research

shows controversial data of LPS either increasing or decreasing migration (185, 186). Therefore, the impact of LPS at 0.1 μg/ml or 1 μg/ml on wound healing of endothelial cells was assessed in the presence or absence of L-4F after 12 hours. LPS decreased wound healing at 1 μg/ml, which was not prevented by co-incubation with L-4F. After further investigation, it was found that incubation of L-4F with endothelial cells for 16 hours before LPS exposure and scratching rescued the decreased wound healing caused by LPS. This is a novel finding that requires further investigation because of the potential impact to cardiovascular and pulmonary diseases.

Once a model had been established for evaluating the functionality of HDL mimetic peptides, a D-HDL mimetic peptide could be designed. The idea behind the D-HDL mimetic peptide design was to create an oxidized HDL mimetic peptide since oxidized HDL is the most commonly studied type of D-HDL (102). HDL oxidation usually occurs, but not exclusively, through a reaction involving an enzyme secreted by PMNs and macrophages called myeloperoxidase (MPO) (105). MPO catalyzes a reaction utilizing hydrogen peroxide and a chlorine containing compound, such as sodium chloride, to produce HOCl (187). MPO has been shown to bind selectively to apoA-I on HDL, likely facilitating the oxidation of apoA-I from produced HOCl (188), which has been shown to occur on varying amino acids in apoA-I's sequence (170, 189-191). Oxidizing the currently studied apoA-I mimetic peptide, L-4F, is not enough to induce peptide dysfunction, as the oxidized peptide's ability to induce cholesterol efflux on macrophages is preserved (173). However, it is still true that oxidation of apoA-I causes HDL dysfunction (169, 178, 189, 191, 192), therefore it is likely that increasing the susceptibility of the L-4F would induce peptide dysfunction. By increasing susceptibility of oxidation, the amphipathicity, and hydrophobicity of the peptide would be disrupted once oxidized, possibly preventing lipid binding and inhibiting the protective functions of the peptide. Susceptibility of

oxidation was increased by substituting the carboxyl-terminal phenylalanine for tryptophan because tryptophan was the one amino acid oxidized when incubated with HOCl (173). The phenylalanine was chosen because tryptophan and phenylalanine have similar non-polar tendencies and have similar structural shapes. These similarities allow the substitution to occur without denaturing the amphipathic alpha-helix of all other apoA-I mimetic peptides. The terminal phenylalanine was also chosen to avoid denaturing, as a more central phenylalanine might have caused a kink in the peptide since tryptophan is slightly bulkier than phenylalanine. The new peptide, L-2W, was confirmed to retain the necessary alpha-helix structure by 3D molecular dynamic modeling. L-2W, a hypothetically functional peptide, could then be oxidized by HOCl and then become dysfunctional because of the increased susceptibility to oxidation.

In this study, a model has been developed to assess the functional capabilities of HDL or D-HDL mimetic peptides. It has been reported that HDL suppresses the LPS-induced expression of adhesion molecules in the lungs. HDL was confirmed to influence the endothelial cell inflammatory response to LPS *in vitro*, which was shown to be effectively mimicked by L-4F. The anti-inflammatory effect of L-4F was shown to be influenced by the concentration of LPS exposure. The concentration of LPS was also shown to influence wound healing of endothelial cells. Also, incubation period of L-4F before LPS exposure was shown to influence wound healing. By developing this model, the designed D-HDL mimetic peptide, or future HDL or D-HDL mimetic peptides, could be evaluated to test their functional capabilities. With these tools, the consequences of D-HDL in the context of pulmonary disease can be further understood. Furthermore, by understanding the consequences of D-HDL development, therapies for pulmonary disease can be more accurately optimized for effective treatment of pulmonary disease.

FIGURES

Figure 3.1 HDL suppresses LPS-induced inflammation in the lungs. WT mice were pretreated with PBS or HDL and were then exposed to RA or 300 µg/mL of LPS. 24h later, mice were necropsied. Whole lung RNA was isolated and adhesion molecules (A) ICAM-1 and (B) VCAM expression were measured by RT-PCR. Data is presented as ddCt compared to 18S (n=5 per group; * $p<0.05$ from PBS; # $p<0.05$).

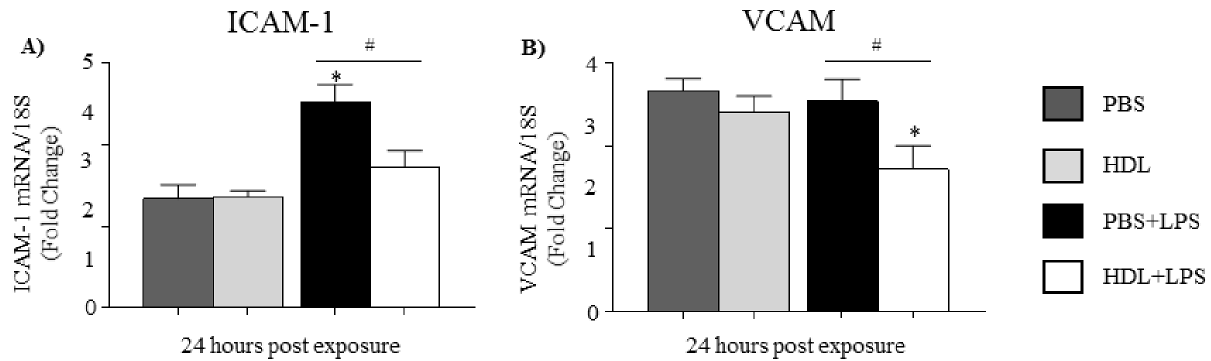


Figure 3.2 HDL suppresses LPS-induced inflammation in endothelial cells. HUVECs were incubated with HDL (200 μg/ml) for 16 hours and exposed to LPS (1 μg/ml) for 12 hours. Cells were lysed and collected to measure RNA. Gene expression of **A)** ICAM-1, **B)** VCAM, and **C)** TNF-α was measured by RT-PCR. Data is presented as ddCt compared to 18S (n=3 per group; * $p<0.05$ from PBS; # $p<0.05$).

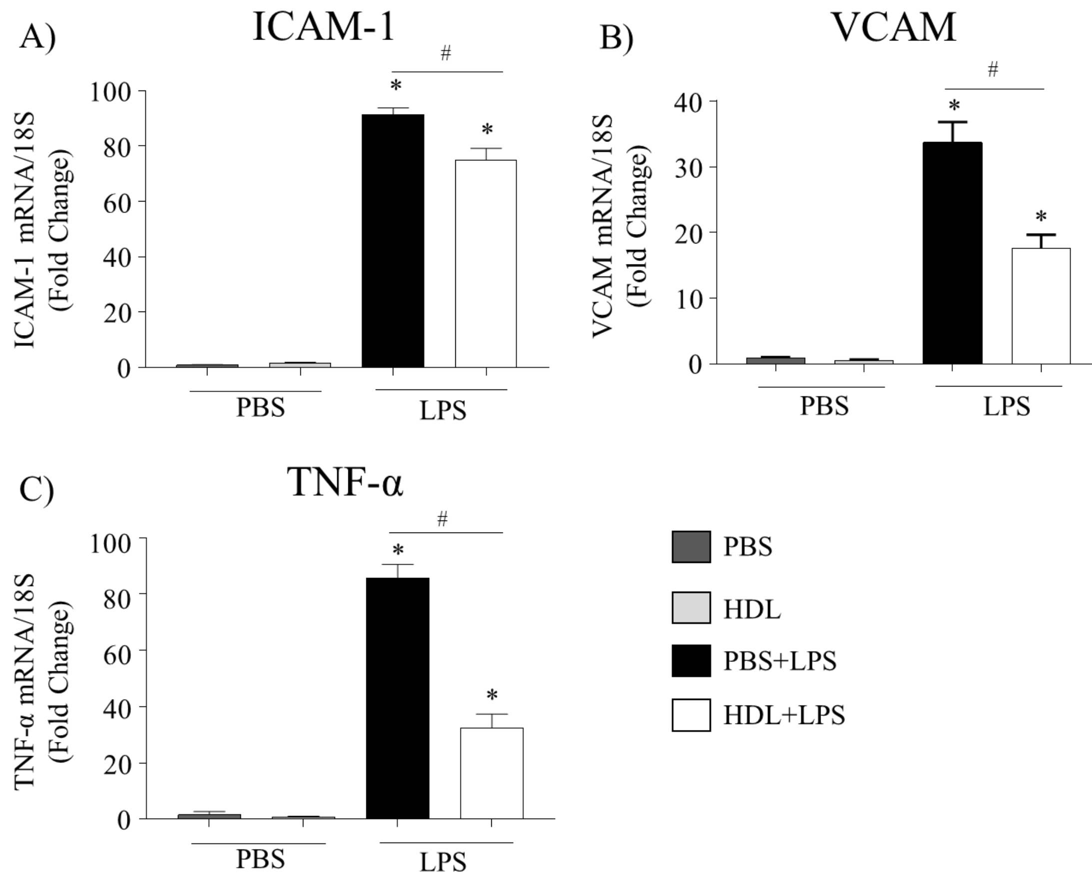


Figure 3.3 L-4F suppresses LPS-induced inflammation in endothelial cells. HUVECs were exposed to L-4F (50 μg/ml) and LPS (0.1 μg/ml or 1 μg/ml) for 12 hours. Cells were then lysed and collected to measure gene expression of ICAM-1, VCAM, and TNF-α by RT-PCR. Data is presented as ddCt compared to 18S (n=3 per group; * $p<0.05$ from PBS; # $p<0.05$).

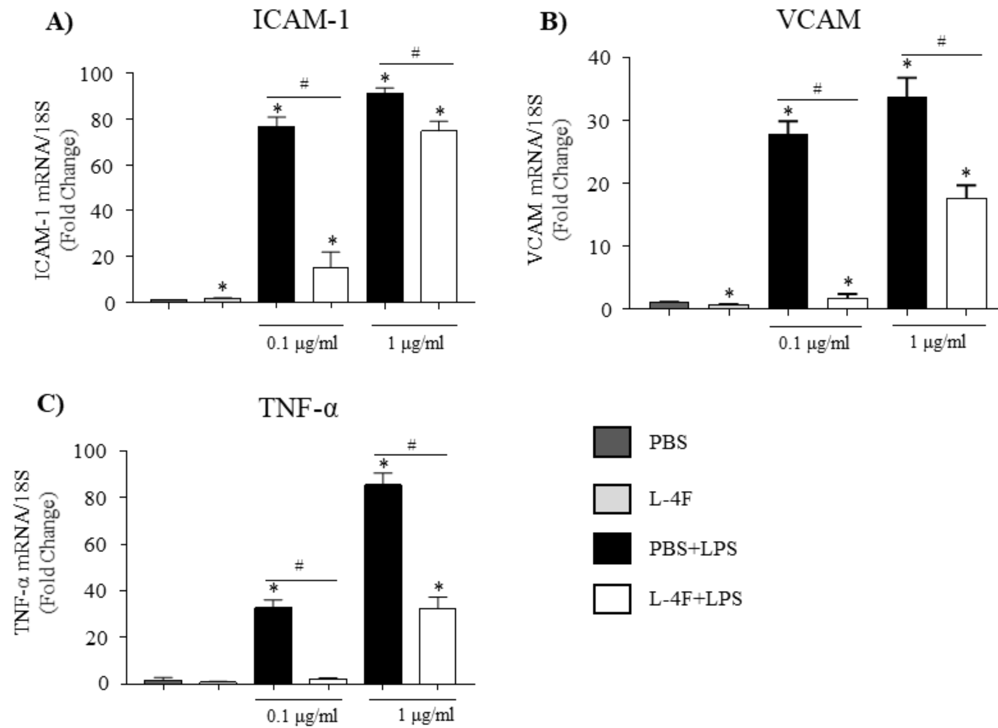


Figure 3.4 LPS suppressed endothelial cell wound healing at 1 µg/ml. HUVECs were exposed to L-4F (50 µg/ml) and LPS (0.1 µg/ml or 1 µg/ml), scratched with a 200 µl pipette tip, and imaged 0, 4, 8, and 12 hours after exposure. Width of wound was measured by ImageJ, and percent wound closure from 0 to 12 hours was recorded. (n=3 per group; * $p<0.05$ from PBS).

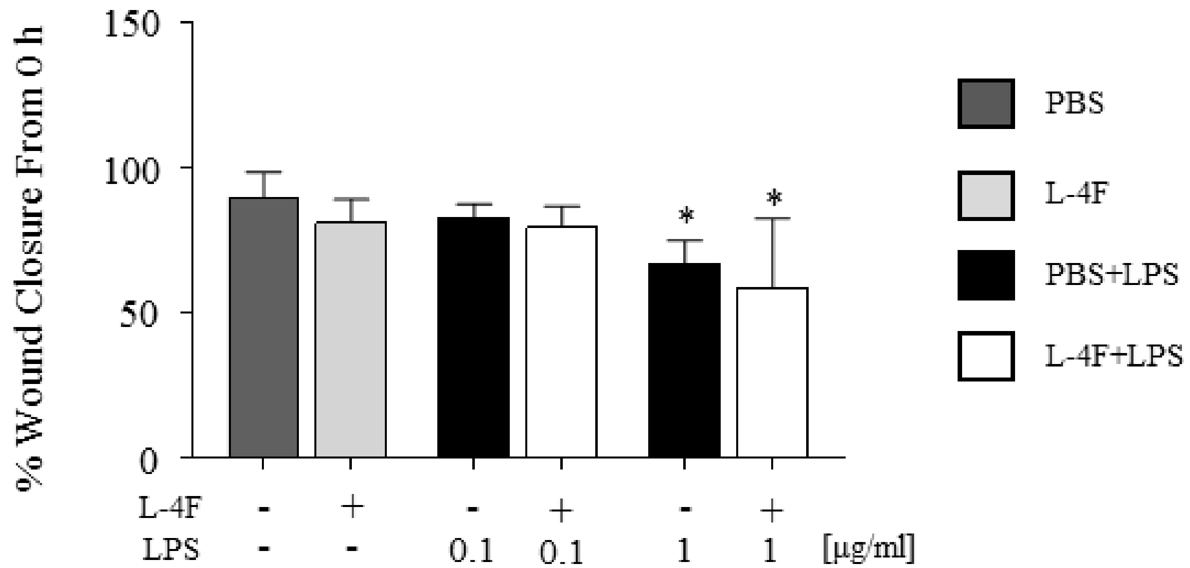


Figure 3.5 L-4F abrogates LPS-suppressed endothelial cell wound healing. HUVECs were seeded in a 12 well plate, and incubated with L-4F (50 µg/ml) after reaching ~80% confluency. 16 hours after incubation, cells were exposed to LPS (1 µg/ml) and scratched with a 200 µl pipette tip. Cells were then imaged 0, and 12 hours after exposure and percent wound closure was assessed using ImageJ. (n=3 per group; * $p<0.05$ from PBS; # $p<0.05$).

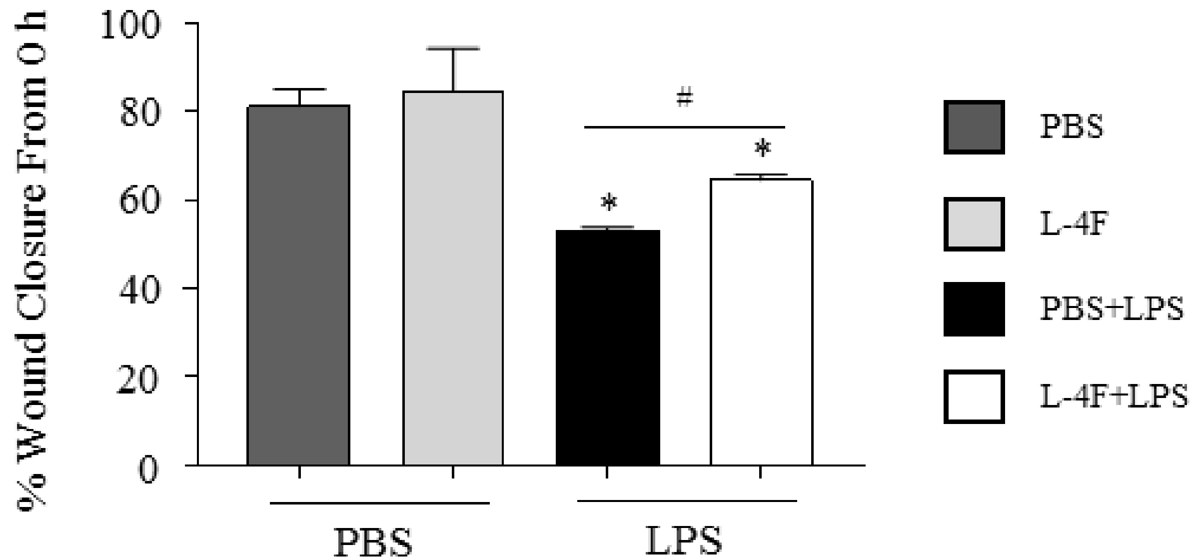
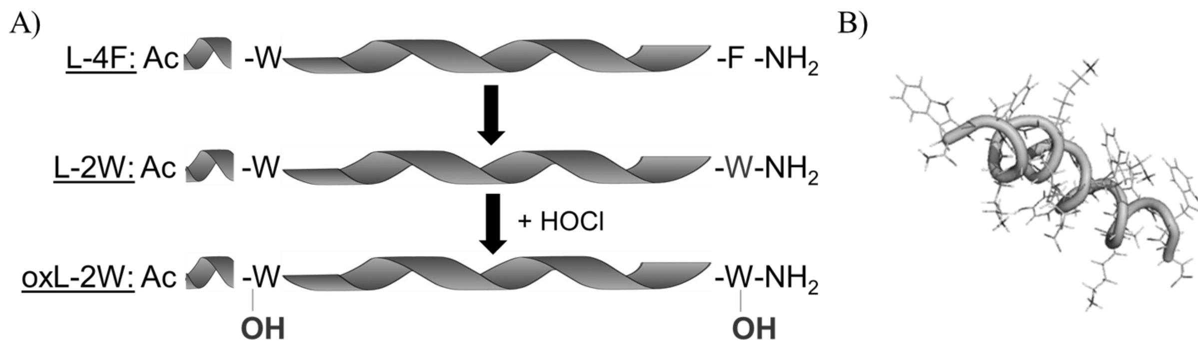


Figure 3.6 A designed novel D-HDL mimetic peptide retains an alpha-helix. **(A)**L-2W was designed by substituting the carboxyl-terminus phenylalanine for a tryptophan and the D-HDL mimetic peptide was then designed by oxidizing L-2W with HOCl to make oxL-2W. **(B)** L-2W molecular dynamics was performed on the AMBER 14 computer software.



CHAPTER IV: CONCLUSIONS AND FUTURE DIRECTIONS

CONCLUSIONS/DISSCUSION

This thesis contributes to developing a higher knowledge of the role of HDL in the pulmonary immune system and developing tools to study HDL and D-HDL in the lungs. In chapter II, HDL was reported to inhibit the chemotactic potential of PMNs while modulation of several lipoprotein receptors in the lungs was observed. This significant finding implicates the crucial role of HDL in the pulmonary immune response. In chapter III, a model was developed for testing the functionality of HDL or D-HDL mimetic peptides and a novel D-HDL mimetic peptide was designed. These tools were created to further study the roles of HDL and D-HDL in the respiratory and circulatory systems. With these new technologies, the consequences of developing D-HDL can be understood, and therapies can be produced to protect the body from this pathological condition.

Chapter II investigates the role of HDL in the pulmonary inflammatory response to LPS. It was suggested that the inhibition of chemotactic potential, and the observed suppression of the inflammatory response, is the result of HDL taking up cholesterol from alveolar macrophages to reduce their inflammatory response. It has already been reported that HDL reducing the lipid load of monocytes/macrophages, and altering their lipid rafts, reduces their inflammatory response (47). It is likely this occurred in the lungs when mice were pretreated with HDL. Once mice received LPS exposure their alveolar macrophages may have already had a reduced lipid load, or HDL was still passing through circulation and taking up cholesterol after exposure. SR-BI was the only lipoprotein receptor shown to be upregulated by HDL at the early time point, 2 hours after exposure. Increased expression of ABCG1/ABCA1 by HDL was not observed in this report, but it is possible increased expression occurred at an earlier or later time point. Increased

ABCG1/ABCA1 transcription will have allowed for increased cholesterol uptake from macrophages to occur, thus attenuating the inflammatory response of alveolar macrophages.

Transcription of ABCG1 and ABCA1 can be activated by liver X receptor (LXR) which is known to be activated by physiologic sterol ligands (e.g. oxysterols, cholesterol intermediates (193, 194)). Also, LXR agonist supplementation has been reported to decrease human PMN chemotaxis to IL-8 (the human analog to MIP-2/KC) (195), as well as knocking in LXR has been shown to protect mice from acute lung injury from intranasally instilled LPS (196). There is a clear effect of LXR on PMN chemotaxis (197), however the mechanism is unclear. It is possible increased LXR leads to decreased CXCR2, preventing PMN migration towards MIP-2/KC. Because LXR can be activated by sterol ligands, increased levels of alveolar macrophage cholesterol may correlate with an increased expression of the ABCG1, and ABCA1 as part of the inflammatory response to LPS.

HDL may serve one of two purposes in the observed inflammatory response to LPS. First HDL may decrease inflammation by decreasing cholesterol concentrations in lung tissue, and thus decreasing activation of LXR and lipoprotein receptor transcription after 24 hours. Second, HDL can serve as an LXR agonist, possibly by acting directly on circulating PMNs. This would decrease PMN chemotaxis towards any MIP-2/KC secreted in the lungs, possibly by inhibiting CXCR2 surface display. Also, increased LXR would increase the transcription of ABCG1 and ABCA1 which we've proposed would consequently lead to decreased MIP-2/KC secretion. This two-fold impact of HDL on CXCR2 and MIP-2/KC would then considerably inhibit PMN chemotaxis, which would explain the decreased pulmonary neutrophilia observed. This is a novel application to the already understood ability of HDL to interact with lipoprotein receptors found in the lung.

In chapter III, a model was developed to assess the functional capabilities of HDL and D-HDL mimetic peptides. Then a D-HDL mimetic peptide was designed by increasing the susceptibility of L-4F to oxidation. HOCl was chosen as the oxidizing agent to maintain physiological relevance. HDL is most commonly oxidized by MPO in the body because of the tendency for HDL and MPO to accumulate in atherosclerotic plaques (103). MPO produces HOCl and has been found to oxidize several different sites of apoA-I to make it dysfunctional (190, 198-200). Originally, it was intended to use the enzymatic reaction of MPO with H_2O_2 and NaCl to produce HOCl and oxidize L-2W. However, it quickly became understood that analyzing the oxidation of the mimetic peptide would be complicated because of the size of MPO compared to L-2W. Oxidation was intended to be verified by recording ultraviolet light visual spectrophotometry, circular dichroism, and matrix assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF) measurements of the peptides. These measurements would require the filtration of MPO and the other substrates out of solution after peptide oxidation, otherwise observation of the peptide would be obscured by the large enzyme. This additional step simply added an unnecessary complication to the procedure since the oxidative interaction is solely between HOCl and apoA-I, with MPO only producing HOCl. The intention of developing a D-HDL mimetic peptide was to create a simple tool to be used to study the more complicated consequences of D-HDL in pulmonary diseases. Therefore, simply incubating L-2W with HOCl was decided to be the optimal method of oxidation. By creating a simple and reproducible model for evaluating peptide function, and engineering a novel D-HDL mimetic peptide, valuable research can be conducted to understand the complications of D-HDL.

In conclusion, HDL plays a critical role in the pulmonary inflammatory response, and a D-HDL mimetic peptide has been designed to asses the impact of developing D-HDL on the lungs.

Understanding the ability of HDL to suppress the inflammatory response of alveolar macrophages highlights the possible ramifications of D-HDL. It is possible D-HDL does not have the ability to suppress the inflammatory response. This could lead to more severe pulmonary injury in subjects suffering from obesity or diabetes because of a compromised immune system. More research needs to be conducted to understand how exactly D-HDL fails to match up to HDL. The D-HDL mimetic peptide designed here can be used to conduct this research. Also, the model for evaluating peptide function can also be used to assess other D-HDL mimetic peptides that replicate the other types of endogenous D-HDL. Overall, this research contributes to the scientific fields of pulmonary immunology, cardiovascular health, and lipid research to assist in the medical fields of pulmonary and cardiovascular diseases.

FUTURE DIRECTIONS

Future work may be aimed at further understanding the mechanisms of HDL's anti-inflammatory role, and how that is perturbed in D-HDL. This research was primarily conducted to elucidate what the role is of HDL in the lungs, and to create a tool for studying D-HDL. HDL has been shown to suppress production of inflammatory cytokines, chemokines, and to reduce injury. However, there has been little work demonstrating the mechanism of how HDL influences those responses. Likewise, there has been even less work on how that influence is altered when HDL becomes dysfunctional. The goal would be to identify therapeutic targets encouraging the protective effects of HDL and inhibiting the deleterious effects of D-HDL.

Dependency of HDL's anti-inflammatory response on lipoprotein receptors could be further studied. Here we observed the influence of HDL on lipoprotein receptor expression, so it would be enlightening to evaluate the anti-inflammatory properties of HDL when specific

lipoprotein receptors were genetically removed or pharmacologically inhibited. Alveolar macrophage specific inhibition of SR-BI could be used to determine the direct interaction point of HDL with the pulmonary immune system. It has been reported here that HDL increases SR-BI expression 2 hours after LPS exposure, and suppresses its expression 24 hours after LPS exposure. It has been proposed that HDL inhibits alveolar macrophage inflammation by reducing intracellular cholesterol concentration by transporting cholesterol through SR-BI. Therefore, if SR-BI was abolished, alveolar macrophage inflammation would hypothetically increase. Investigating the intracellular cholesterol of alveolar macrophages in SR-BI deficient models could elucidate the mechanism of HDL's anti-inflammatory role in the lung.

The ability of L-4F, and oxL-2W to suppress the pulmonary immune response is another area that could be further studied. L-4F has been shown to be suppress the inflammatory response to LPS in some models (80, 81). However, there has been no report on the influence of a D-HDL mimetic peptide on the pulmonary immune response. An interesting study would be to genetically knock out apoA-I, and then administer either L-4F or oxL-2W, or a ratio of the two, and observe the inflammatory response of the lungs to LPS. This would first clarify the therapeutic potential of L-4F in the face of developed D-HDL, and second could highlight how exactly the pulmonary immune system is compromised when D-HDL is present. Presently, there is no good model for evaluating the presence of D-HDL via genetic knock out. SR-BI or apoE deficiencies are potential options, but because of their widespread necessity throughout the body, expanding beyond the role of HDL, it would be impossible to discern between phenotypes of obesity (201), Alzheimer's (202), dyslipidemia (203, 204), or immune suppression (129) and D-HDL.

Other possibilities exist for further research around the role of HDL and D-HDL in the lungs. There is a lot that is not understood about HDL and D-HDL, while there is significant

clinical relevance revolving around these lipoproteins. Continued research on the influence of HDL on migrating PMNs, endothelial cell inflammation, epithelial cell inflammation and the connection to surfactant secretion are all areas that are understudied. Consequently, the impact of D-HDL on each of these areas needs to be evaluated if we are to attempt to reduce the drastic burden of pulmonary diseases on the global population.

REFERENCES

1. Statistics NCfH. Health, united states, 2016: With chartbook on long-term trends in health. *Center for Disease Control* 2017.
2. Kuklina EV, Yoon PW, Keenan NL. Trends in high levels of low-density lipoprotein cholesterol in the united states, 1999-2006. *JAMA* 2009;302(19):2104-2110.
3. Grundy SM. Inflammation, metabolic syndrome, and diet responsiveness. *Circulation* 2003;108(2):126-128.
4. Kingwell BA, Chapman MJ, Kontush A, Miller NE. Hdl-targeted therapies: Progress, failures and future. *Nat Rev Drug Discov* 2014;13(6):445-464.
5. Rader DJ, Hovingh GK. Hdl and cardiovascular disease. *Lancet* 2014;384(9943):618-625.
6. Yiallouros PK, Savva SC, Kolokotroni O, Behbod B, Zeniou M, Economou M, Chadjigeorgiou C, Kourides YA, Tornaritis MJ, Lamnisos D, et al. Low serum high-density lipoprotein cholesterol in childhood is associated with adolescent asthma. *Clin Exp Allergy* 2012;42(3):423-432.
7. Wang W, Xu H, Shi Y, Nandedkar S, Zhang H, Gao H, Feroah T, Weihrauch D, Schulte ML, Jones DW, et al. Genetic deletion of apolipoprotein a-i increases airway hyperresponsiveness, inflammation, and collagen deposition in the lung. *J Lipid Res* 2010;51(9):2560-2570.
8. True R. The role of cholesterol in human body metabolism. 2013 5/25/2018]. Available from: https://www.truemd.com/general-health/the-role-of-cholesterol-in-human-body-metabolism/.
9. Gowdy KM, Fessler MB. Emerging roles for cholesterol and lipoproteins in lung disease. *Pulm Pharmacol Ther* 2013;26(4):430-437.
10. Hegele RA. Plasma lipoproteins: Genetic influences and clinical implications. *Nat Rev Genet* 2009;10(2):109-121.
11. Vockeroth D, Gunasekara L, Amrein M, Possmayer F, Lewis JF, Veldhuizen RA. Role of cholesterol in the biophysical dysfunction of surfactant in ventilator-induced lung injury. *Am J Physiol Lung Cell Mol Physiol* 2010;298(1):L117-125.
12. Diamond DM, Ravnskov U. How statistical deception created the appearance that statins are safe and effective in primary and secondary prevention of cardiovascular disease. *Expert Rev Clin Pharmacol* 2015;8(2):201-210.
13. Marques LR, Diniz TA, Antunes BM, Rossi FE, Caperuto EC, Lira FS, Goncalves DC. Reverse cholesterol transport: Molecular mechanisms and the non-medical approach to enhance hdl cholesterol. *Front Physiol* 2018;9:526.
14. Gan WQ, Man SF, Senthilselvan A, Sin DD. Association between chronic obstructive pulmonary disease and systemic inflammation: A systematic review and a meta-analysis. *Thorax* 2004;59(7):574-580.
15. Koenig W, Sund M, Frohlich M, Fischer HG, Lowel H, Doring A, Hutchinson WL, Pepys MB. C-reactive protein, a sensitive marker of inflammation, predicts future risk of coronary heart disease in initially healthy middle-aged men: Results from the monica (monitoring trends and determinants in cardiovascular disease) augsburg cohort study, 1984 to 1992. *Circulation* 1999;99(2):237-242.
16. Vestbo J, Hurd SS, Agusti AG, Jones PW, Vogelmeier C, Anzueto A, Barnes PJ, Fabbri LM, Martinez FJ, Nishimura M, et al. Global strategy for the diagnosis, management, and

prevention of chronic obstructive pulmonary disease: Gold executive summary. *Am J Respir Crit Care Med* 2013;187(4):347-365.
17. Whitfield C, Trent MS. Biosynthesis and export of bacterial lipopolysaccharides. *Annu Rev Biochem* 2014;83:99-128.
18. Kitz R, Rose MA, Placzek K, Schulze J, Zielen S, Schubert R. Lps inhalation challenge: A new tool to characterize the inflammatory response in humans. *Med Microbiol Immunol* 2008;197(1):13-19.
19. Svanborg C, Godaly G, Hedlund M. Cytokine responses during mucosal infections: Role in disease pathogenesis and host defence. *Curr Opin Microbiol* 1999;2(1):99-105.
20. Wu Y, Jin F, Wang Y, Li F, Wang L, Wang Q, Ren Z, Wang Y. In vitro and in vivo anti-inflammatory effects of theaflavin-3,3'-digallate on lipopolysaccharide-induced inflammation. *Eur J Pharmacol* 2017;794:52-60.
21. Cohen J. The immunopathogenesis of sepsis. *Nature* 2002;420(6917):885-891.
22. Janeway CAT, P. Walport, M. Shlomchik, M. Immunobiology: The immune system in health and disease. Garland; 2001.
23. Lund-Katz S, Phillips MC. High density lipoprotein structure-function and role in reverse cholesterol transport. *Subcell Biochem* 2010;51:183-227.
24. Schiller J, Zschornig O, Petkovic M, Muller M, Arnhold J, Arnold K. Lipid analysis of human hdl and ldl by maldi-tof mass spectrometry and (31)p-nmr. *J Lipid Res* 2001;42(9):1501-1508.
25. Gordon SM, Deng J, Lu LJ, Davidson WS. Proteomic characterization of human plasma high density lipoprotein fractionated by gel filtration chromatography. *J Proteome Res* 2010;9(10):5239-5249.
26. Franceschini G, Tosi C, Moreno Y, Sirtori CR. Effects of storage on the distribution of high density lipoprotein subfractions in human sera. *J Lipid Res* 1985;26(11):1368-1373.
27. Kontush A, Lindahl M, Lhomme M, Calabresi L, Chapman MJ, Davidson WS. Structure of hdl: Particle subclasses and molecular components. *Handb Exp Pharmacol* 2015;224:3-51.
28. Asztalos BF, Schaefer EJ. High-density lipoprotein subpopulations in pathologic conditions. *Am J Cardiol* 2003;91(7A):12E-17E.
29. Duriez P, Fruchart JC. High-density lipoprotein subclasses and apolipoprotein a-i. *Clin Chim Acta* 1999;286(1-2):97-114.
30. Azzam KM, Fessler MB. Crosstalk between reverse cholesterol transport and innate immunity. *Trends Endocrinol Metab* 2012;23(4):169-178.
31. Massey JB, Hickson D, She HS, Sparrow JT, Via DP, Gotto AM, Jr., Pownall HJ. Measurement and prediction of the rates of spontaneous transfer of phospholipids between plasma lipoproteins. *Biochim Biophys Acta* 1984;794(2):274-280.
32. Ahmed Z, Ravandi A, Maguire GF, Emili A, Draganov D, La Du BN, Kuksis A, Connelly PW. Apolipoprotein a-i promotes the formation of phosphatidylcholine core aldehydes that are hydrolyzed by paraoxonase (pon-1) during high density lipoprotein oxidation with a peroxynitrite donor. *J Biol Chem* 2001;276(27):24473-24481.
33. Aviram M, Rosenblat M, Bisgaier CL, Newton RS, Primo-Parmo SL, La Du BN. Paraoxonase inhibits high-density lipoprotein oxidation and preserves its functions. A possible peroxidative role for paraoxonase. *J Clin Invest* 1998;101(8):1581-1590.
34. Rosenblat M, Vaya J, Shih D, Aviram M. Paraoxonase 1 (pon1) enhances hdl-mediated macrophage cholesterol efflux via the abca1 transporter in association with increased hdl binding to the cells: A possible role for lysophosphatidylcholine. *Atherosclerosis* 2005;179(1):69-77.

35. Hubbard RC, Crystal RG. Alpha-1-antitrypsin augmentation therapy for alpha-1-antitrypsin deficiency. *Am J Med* 1988;84(6A):52-62.
36. Kellner-Weibel G, de la Llera-Moya M. Update on hdl receptors and cellular cholesterol transport. *Curr Atheroscler Rep* 2011;13(3):233-241.
37. Acton S, Rigotti A, Landschulz KT, Xu S, Hobbs HH, Krieger M. Identification of scavenger receptor sr-bi as a high density lipoprotein receptor. *Science* 1996;271(5248):518-520.
38. Murphy AJ, Chin-Dusting JP, Sviridov D, Woollard KJ. The anti inflammatory effects of high density lipoproteins. *Curr Med Chem* 2009;16(6):667-675.
39. Bobryshev YV, Ivanova EA, Chistiakov DA, Nikiforov NG, Orekhov AN. Macrophages and their role in atherosclerosis: Pathophysiology and transcriptome analysis. *Biomed Res Int* 2016;2016:9582430.
40. Penn MS, Chisolm GM. Oxidized lipoproteins, altered cell function and atherosclerosis. *Atherosclerosis* 1994;108 Suppl:S21-29.
41. Steinberg D, Parthasarathy S, Carew TE, Khoo JC, Witztum JL. Beyond cholesterol. Modifications of low-density lipoprotein that increase its atherogenicity. *N Engl J Med* 1989;320(14):915-924.
42. Sattler K, Graler M, Keul P, Weske S, Reimann CM, Jindrova H, Kleinbongard P, Sabbadini R, Brocker-Preuss M, Erbel R, et al. Defects of high-density lipoproteins in coronary artery disease caused by low sphingosine-1-phosphate content: Correction by sphingosine-1-phosphate-loading. *J Am Coll Cardiol* 2015;66(13):1470-1485.
43. Cockerill GW, Rye KA, Gamble JR, Vadas MA, Barter PJ. High-density lipoproteins inhibit cytokine-induced expression of endothelial cell adhesion molecules. *Arterioscler Thromb Vasc Biol* 1995;15(11):1987-1994.
44. Tabet F, Vickers KC, Cuesta Torres LF, Wiese CB, Shoucri BM, Lambert G, Catherinet C, Prado-Lourenco L, Levin MG, Thacker S, et al. Hdl-transferred microrna-223 regulates icam-1 expression in endothelial cells. *Nat Commun* 2014;5:3292.
45. Carvalho LS, Panzoldo N, Santos SN, Modolo R, Almeida B, Quinaglia ESJC, Nadruz W, Jr., de Faria EC, Sposito AC, Brasilia Heart Study G. Hdl levels and oxidizability during myocardial infarction are associated with reduced endothelial-mediated vasodilation and nitric oxide bioavailability. *Atherosclerosis* 2014;237(2):840-846.
46. Pan B, Ren H, Ma Y, Liu D, Yu B, Ji L, Pan L, Li J, Yang L, Lv X, et al. High-density lipoprotein of patients with type 2 diabetes mellitus elevates the capability of promoting migration and invasion of breast cancer cells. *Int J Cancer* 2012;131(1):70-82.
47. Murphy AJ, Woollard KJ, Hoang A, Mukhamedova N, Stirzaker RA, McCormick SP, Remaley AT, Sviridov D, Chin-Dusting J. High-density lipoprotein reduces the human monocyte inflammatory response. *Arterioscler Thromb Vasc Biol* 2008;28(11):2071-2077.
48. Kekulawala JR, Murphy A, D'Souza W, Wai C, Chin-Dusting J, Kingwell B, Sviridov D, Mukhamedova N. Impact of freezing on high-density lipoprotein functionality. *Anal Biochem* 2008;379(2):213-215.
49. Luscher TF, Landmesser U, von Eckardstein A, Fogelman AM. High-density lipoprotein: Vascular protective effects, dysfunction, and potential as therapeutic target. *Circ Res* 2014;114(1):171-182.
50. Rozenberg O, Rosenblat M, Coleman R, Shih DM, Aviram M. Paraoxonase (pon1) deficiency is associated with increased macrophage oxidative stress: Studies in pon1-knockout mice. *Free Radic Biol Med* 2003;34(6):774-784.

51. Shih DM, Xia YR, Wang XP, Miller E, Castellani LW, Subbanagounder G, Cheroutre H, Faull KF, Berliner JA, Witztum JL, et al. Combined serum paraoxonase knockout/apolipoprotein e knockout mice exhibit increased lipoprotein oxidation and atherosclerosis. *J Biol Chem* 2000;275(23):17527-17535.
52. Li D, Weng S, Yang B, Zander DS, Saldeen T, Nichols WW, Khan S, Mehta JL. Inhibition of arterial thrombus formation by apoa1 milano. *Arterioscler Thromb Vasc Biol* 1999;19(2):378-383.
53. Nofer JR, Walter M, Kehrel B, Wierwille S, Tepel M, Seedorf U, Assmann G. Hdl3-mediated inhibition of thrombin-induced platelet aggregation and fibrinogen binding occurs via decreased production of phosphoinositide-derived second messengers 1,2-diacylglycerol and inositol 1,4,5-tris-phosphate. *Arterioscler Thromb Vasc Biol* 1998;18(6):861-869.
54. Moreno JA, Ortega-Gomez A, Rubio-Navarro A, Louedec L, Ho-Tin-Noe B, Caligiuri G, Nicoletti A, Levoye A, Plantier L, Meilhac O. High-density lipoproteins potentiate alpha1-antitrypsin therapy in elastase-induced pulmonary emphysema. *Am J Respir Cell Mol Biol* 2014;51(4):536-549.
55. Dai C, Yao X, Keeran KJ, Zywicke GJ, Qu X, Yu ZX, Dagur PK, McCoy JP, Remaley AT, Levine SJ. Apolipoprotein a-i attenuates ovalbumin-induced neutrophilic airway inflammation via a granulocyte colony-stimulating factor-dependent mechanism. *Am J Respir Cell Mol Biol* 2012;47(2):186-195.
56. Yan YJ, Li Y, Lou B, Wu MP. Beneficial effects of apoa-i on lps-induced acute lung injury and endotoxemia in mice. *Life Sci* 2006;79(2):210-215.
57. Park SW, Lee EH, Lee EJ, Kim HJ, Bae DJ, Han S, Kim D, Jang AS, Uh ST, Kim YH, et al. Apolipoprotein a1 potentiates lipoxin a4 synthesis and recovery of allergen-induced disrupted tight junctions in the airway epithelium. *Clin Exp Allergy* 2013;43(8):914-927.
58. Mangaraj M, Nanda R, Panda S. Apolipoprotein a-i: A molecule of diverse function. *Indian J Clin Biochem* 2016;31(3):253-259.
59. Thuahnai ST, Lund-Katz S, Dhanasekaran P, de la Llera-Moya M, Connelly MA, Williams DL, Rothblat GH, Phillips MC. Scavenger receptor class b type i-mediated cholesteryl ester-selective uptake and efflux of unesterified cholesterol. Influence of high density lipoprotein size and structure. *J Biol Chem* 2004;279(13):12448-12455.
60. Gelissen IC, Harris M, Rye KA, Quinn C, Brown AJ, Kockx M, Cartland S, Packianathan M, Kritharides L, Jessup W. Abca1 and abcg1 synergize to mediate cholesterol export to apoa-i. *Arterioscler Thromb Vasc Biol* 2006;26(3):534-540.
61. Kennedy MA, Barrera GC, Nakamura K, Baldan A, Tarr P, Fishbein MC, Frank J, Francone OL, Edwards PA. Abcg1 has a critical role in mediating cholesterol efflux to hdl and preventing cellular lipid accumulation. *Cell Metab* 2005;1(2):121-131.
62. Lawn RM, Wade DP, Garvin MR, Wang X, Schwartz K, Porter JG, Seilhamer JJ, Vaughan AM, Oram JF. The tangier disease gene product abc1 controls the cellular apolipoprotein-mediated lipid removal pathway. *J Clin Invest* 1999;104(8):R25-31.
63. Oram JF. Hdl apolipoproteins and abca1: Partners in the removal of excess cellular cholesterol. *Arterioscler Thromb Vasc Biol* 2003;23(5):720-727.
64. Phillips JC, Wriggers W, Li Z, Jonas A, Schulten K. Predicting the structure of apolipoprotein a-i in reconstituted high-density lipoprotein disks. *Biophys J* 1997;73(5):2337-2346.
65. Nicholls SJ, Dusting GJ, Cutri B, Bao S, Drummond GR, Rye KA, Barter PJ. Reconstituted high-density lipoproteins inhibit the acute pro-oxidant and proinflammatory

vascular changes induced by a periarterial collar in normocholesterolemic rabbits. *Circulation* 2005;111(12):1543-1550.
66. Van Linthout S, Spillmann F, Graiani G, Miteva K, Peng J, Van Craeyveld E, Meloni M, Tolle M, Escher F, Subasiguller A, et al. Down-regulation of endothelial tlr4 signalling after apo a-i gene transfer contributes to improved survival in an experimental model of lipopolysaccharide-induced inflammation. *J Mol Med (Berl)* 2011;89(2):151-160.
67. Smoak KA, Aloor JJ, Madenspacher J, Merrick BA, Collins JB, Zhu X, Cavigiolio G, Oda MN, Parks JS, Fessler MB. Myeloid differentiation primary response protein 88 couples reverse cholesterol transport to inflammation. *Cell Metab* 2010;11(6):493-502.
68. Anantharamaiah GM, Mishra VK, Garber DW, Datta G, Handattu SP, Palgunachari MN, Chaddha M, Navab M, Reddy ST, Segrest JP, et al. Structural requirements for antioxidative and anti-inflammatory properties of apolipoprotein a-i mimetic peptides. *J Lipid Res* 2007;48(9):1915-1923.
69. Chung BH, Anatharamaiah GM, Brouillette CG, Nishida T, Segrest JP. Studies of synthetic peptide analogs of the amphipathic helix. Correlation of structure with function. *J Biol Chem* 1985;260(18):10256-10262.
70. Mendez AJ, Anantharamaiah GM, Segrest JP, Oram JF. Synthetic amphipathic helical peptides that mimic apolipoprotein a-i in clearing cellular cholesterol. *J Clin Invest* 1994;94(4):1698-1705.
71. Datta G, Chaddha M, Hama S, Navab M, Fogelman AM, Garber DW, Mishra VK, Epand RM, Epand RF, Lund-Katz S, et al. Effects of increasing hydrophobicity on the physical-chemical and biological properties of a class a amphipathic helical peptide. *J Lipid Res* 2001;42(7):1096-1104.
72. Remaley AT, Thomas F, Stonik JA, Demosky SJ, Bark SE, Neufeld EB, Bocharov AV, Vishnyakova TG, Patterson AP, Eggerman TL, et al. Synthetic amphipathic helical peptides promote lipid efflux from cells by an abca1-dependent and an abca1-independent pathway. *J Lipid Res* 2003;44(4):828-836.
73. Sethi AA, Stonik JA, Thomas F, Demosky SJ, Amar M, Neufeld E, Brewer HB, Davidson WS, D'Souza W, Sviridov D, et al. Asymmetry in the lipid affinity of bihelical amphipathic peptides. A structural determinant for the specificity of abca1-dependent cholesterol efflux by peptides. *J Biol Chem* 2008;283(47):32273-32282.
74. Bielicki JK, Zhang H, Cortez Y, Zheng Y, Narayanaswami V, Patel A, Johansson J, Azhar S. A new hdl mimetic peptide that stimulates cellular cholesterol efflux with high efficiency greatly reduces atherosclerosis in mice. *J Lipid Res* 2010;51(6):1496-1503.
75. Di Bartolo BA, Vanags LZ, Tan JT, Bao S, Rye KA, Barter PJ, Bursill CA. The apolipoprotein a-i mimetic peptide, etc-642, reduces chronic vascular inflammation in the rabbit. *Lipids Health Dis* 2011;10:224.
76. Navab M, Anantharamaiah GM, Hama S, Garber DW, Chaddha M, Hough G, Lallone R, Fogelman AM. Oral administration of an apo a-i mimetic peptide synthesized from d-amino acids dramatically reduces atherosclerosis in mice independent of plasma cholesterol. *Circulation* 2002;105(3):290-292.
77. Madenspacher JH, Azzam KM, Gong W, Gowdy KM, Vitek MP, Laskowitz DT, Remaley AT, Wang JM, Fessler MB. Apolipoproteins and apolipoprotein mimetic peptides modulate phagocyte trafficking through chemotactic activity. *J Biol Chem* 2012;287(52):43730-43740.

78. Gupta H, Dai L, Datta G, Garber DW, Grenett H, Li Y, Mishra V, Palgunachari MN, Handattu S, Gianturco SH, et al. Inhibition of lipopolysaccharide-induced inflammatory responses by an apolipoprotein ai mimetic peptide. *Circ Res* 2005;97(3):236-243.
79. Smythies LE, White CR, Maheshwari A, Palgunachari MN, Anantharamaiah GM, Chaddha M, Kurundkar AR, Datta G. Apolipoprotein a-i mimetic 4f alters the function of human monocyte-derived macrophages. *Am J Physiol Cell Physiol* 2010;298(6):C1538-1548.
80. Kwon WY, Suh GJ, Kim KS, Kwak YH, Kim K. 4f, apolipoprotein ai mimetic peptide, attenuates acute lung injury and improves survival in endotoxemic rats. *J Trauma Acute Care Surg* 2012;72(6):1576-1583.
81. Sharifov OF, Xu X, Gaggar A, Grizzle WE, Mishra VK, Honavar J, Litovsky SH, Palgunachari MN, White CR, Anantharamaiah GM, et al. Anti-inflammatory mechanisms of apolipoprotein a-i mimetic peptide in acute respiratory distress syndrome secondary to sepsis. *PLoS One* 2013;8(5):e64486.
82. Bergt C, Pennathur S, Fu X, Byun J, O'Brien K, McDonald TO, Singh P, Anantharamaiah GM, Chait A, Brunzell J, et al. The myeloperoxidase product hypochlorous acid oxidizes hdl in the human artery wall and impairs abca1-dependent cholesterol transport. *Proc Natl Acad Sci U S A* 2004;101(35):13032-13037.
83. Hansel B, Giral P, Nobecourt E, Chantepie S, Bruckert E, Chapman MJ, Kontush A. Metabolic syndrome is associated with elevated oxidative stress and dysfunctional dense high-density lipoprotein particles displaying impaired antioxidative activity. *J Clin Endocrinol Metab* 2004;89(10):4963-4971.
84. Li Y, Zhao M, He D, Zhao X, Zhang W, Wei L, Huang E, Ji L, Zhang M, Willard B, et al. Hdl in diabetic nephropathy has less effect in endothelial repairing than diabetes without complications. *Lipids Health Dis* 2016;15:76.
85. Riwanto M, Rohrer L, Roschitzki B, Besler C, Mocharla P, Mueller M, Perisa D, Heinrich K, Altwegg L, von Eckardstein A, et al. Altered activation of endothelial anti- and proapoptotic pathways by high-density lipoprotein from patients with coronary artery disease: Role of high-density lipoprotein-proteome remodeling. *Circulation* 2013;127(8):891-904.
86. Marin MT, Dasari PS, Tryggestad JB, Aston CE, Teague AM, Short KR. Oxidized hdl and ldl in adolescents with type 2 diabetes compared to normal weight and obese peers. *J Diabetes Complications* 2015;29(5):679-685.
87. Roberts CK, Katiraie M, Croymans DM, Yang OO, Kelesidis T. Untrained young men have dysfunctional hdl compared with strength-trained men irrespective of body weight status. *J Appl Physiol (1985)* 2013;115(7):1043-1049.
88. Burkart KM, Manichaikul A, Wilk JB, Ahmed FS, Burke GL, Enright P, Hansel NN, Haynes D, Heckbert SR, Hoffman EA, et al. Apom and high-density lipoprotein cholesterol are associated with lung function and per cent emphysema. *Eur Respir J* 2014;43(4):1003-1017.
89. Van Lenten BJ, Wagner AC, Nayak DP, Hama S, Navab M, Fogelman AM. High-density lipoprotein loses its anti-inflammatory properties during acute influenza a infection. *Circulation* 2001;103(18):2283-2288.
90. McMahon M, Grossman J, FitzGerald J, Dahlin-Lee E, Wallace DJ, Thong BY, Badsha H, Kalunian K, Charles C, Navab M, et al. Proinflammatory high-density lipoprotein as a biomarker for atherosclerosis in patients with systemic lupus erythematosus and rheumatoid arthritis. *Arthritis Rheum* 2006;54(8):2541-2549.
91. Ortiz-Munoz G, Couret D, Lapergue B, Bruckert E, Meseguer E, Amarenco P, Meilhac O. Dysfunctional hdl in acute stroke. *Atherosclerosis* 2016;253:75-80.

92. Rizzo M, Otvos J, Nikolic D, Montalto G, Toth PP, Banach M. Subfractions and subpopulations of hdl: An update. *Curr Med Chem* 2014;21(25):2881-2891.
93. Mora S, Glynn RJ, Ridker PM. High-density lipoprotein cholesterol, size, particle number, and residual vascular risk after potent statin therapy. *Circulation* 2013;128(11):1189-1197.
94. Zeljkovic A, Vekic J, Spasojevic-Kalimanovska V, Jelic-Ivanovic Z, Bogavac-Stanojevic N, Gulan B, Spasic S. Ldl and hdl subclasses in acute ischemic stroke: Prediction of risk and short-term mortality. *Atherosclerosis* 2010;210(2):548-554.
95. Whitehead AS, de Beer MC, Steel DM, Rits M, Lelias JM, Lane WS, de Beer FC. Identification of novel members of the serum amyloid a protein superfamily as constitutive apolipoproteins of high density lipoprotein. *J Biol Chem* 1992;267(6):3862-3867.
96. Han CY, Tang C, Guevara ME, Wei H, Wietecha T, Shao B, Subramanian S, Omer M, Wang S, O'Brien KD, et al. Serum amyloid a impairs the antiinflammatory properties of hdl. *J Clin Invest* 2016;126(1):266-281.
97. Wroblewski JM, Jahangiri A, Ji A, de Beer FC, van der Westhuyzen DR, Webb NR. Nascent hdl formation by hepatocytes is reduced by the concerted action of serum amyloid a and endothelial lipase. *J Lipid Res* 2011;52(12):2255-2261.
98. Kotani K, Yamada T, Gugliucci A. Paired measurements of paraoxonase 1 and serum amyloid a as useful disease markers. *Biomed Res Int* 2013;2013:481437.
99. Curtiss LK, Witztum JL. Plasma apolipoproteins ai, aii, b, ci, and e are glucosylated in hyperglycemic diabetic subjects. *Diabetes* 1985;34(5):452-461.
100. Hoang A, Murphy AJ, Coughlan MT, Thomas MC, Forbes JM, O'Brien R, Cooper ME, Chin-Dusting JP, Sviridov D. Advanced glycation of apolipoprotein a-i impairs its anti-atherogenic properties. *Diabetologia* 2007;50(8):1770-1779.
101. Liu D, Ji L, Zhang D, Tong X, Pan B, Liu P, Zhang Y, Huang Y, Su J, Willard B, et al. Nonenzymatic glycation of high-density lipoprotein impairs its anti-inflammatory effects in innate immunity. *Diabetes Metab Res Rev* 2012;28(2):186-195.
102. Fisher EA, Feig JE, Hewing B, Hazen SL, Smith JD. High-density lipoprotein function, dysfunction, and reverse cholesterol transport. *Arterioscler Thromb Vasc Biol* 2012;32(12):2813-2820.
103. Annema W, von Eckardstein A. Dysfunctional high-density lipoproteins in coronary heart disease: Implications for diagnostics and therapy. *Transl Res* 2016;173:30-57.
104. Rysz-Gorzynska M, Banach M. Subfractions of high-density lipoprotein (hdl) and dysfunctional hdl in chronic kidney disease patients. *Arch Med Sci* 2016;12(4):844-849.
105. Daugherty A, Dunn JL, Rateri DL, Heinecke JW. Myeloperoxidase, a catalyst for lipoprotein oxidation, is expressed in human atherosclerotic lesions. *J Clin Invest* 1994;94(1):437-444.
106. CDC. Leading causes of death and numbers of deaths, by sex, race, and hispanic origin: United states, 1980 and 2015. 2015 3/17/2017 [cited 2018 2/1]. Available from: https://www.cdc.gov/nchs/data/hus/hus16.pdf#019.
107. Naveed B, Weiden MD, Kwon S, Gracely EJ, Comfort AL, Ferrier N, Kasturiarachchi KJ, Cohen HW, Aldrich TK, Rom WN, et al. Metabolic syndrome biomarkers predict lung function impairment: A nested case-control study. *Am J Respir Crit Care Med* 2012;185(4):392-399.

108. Borrell LN, Nguyen EA, Roth LA, Oh SS, Tcheurekdjian H, Sen S, Davis A, Farber HJ, Avila PC, Brigino-Buenaventura E, et al. Childhood obesity and asthma control in the gala ii and sage ii studies. *Am J Respir Crit Care Med* 2013;187(7):697-702.
109. Kim TH, Lee YH, Kim KH, Lee SH, Cha JY, Shin EK, Jung S, Jang AS, Park SW, Uh ST, et al. Role of lung apolipoprotein a-i in idiopathic pulmonary fibrosis: Antiinflammatory and antifibrotic effect on experimental lung injury and fibrosis. *Am J Respir Crit Care Med* 2010;182(5):633-642.
110. Cirillo DJ, Agrawal Y, Cassano PA. Lipids and pulmonary function in the third national health and nutrition examination survey. *Am J Epidemiol* 2002;155(9):842-848.
111. Rastogi D, Fraser S, Oh J, Huber AM, Schulman Y, Bhagtani RH, Khan ZS, Tesfa L, Hall CB, Macian F. Inflammation, metabolic dysregulation, and pulmonary function among obese urban adolescents with asthma. *Am J Respir Crit Care Med* 2015;191(2):149-160.
112. Tobias PS, Tapping RI, Gegner JA. Endotoxin interactions with lipopolysaccharide-responsive cells. *Clin Infect Dis* 1999;28(3):476-481.
113. Murphy AJ, Woollard KJ, Suhartoyo A, Stirzaker RA, Shaw J, Sviridov D, Chin-Dusting JP. Neutrophil activation is attenuated by high-density lipoprotein and apolipoprotein a-i in in vitro and in vivo models of inflammation. *Arterioscler Thromb Vasc Biol* 2011;31(6):1333-1341.
114. Nupponen I, Andersson S, Jarvenpaa AL, Kautiainen H, Repo H. Neutrophil cd11b expression and circulating interleukin-8 as diagnostic markers for early-onset neonatal sepsis. *Pediatrics* 2001;108(1):E12.
115. Fortunati E, Kazemier KM, Grutters JC, Koenderman L, Van den Bosch v J. Human neutrophils switch to an activated phenotype after homing to the lung irrespective of inflammatory disease. *Clin Exp Immunol* 2009;155(3):559-566.
116. Weathington NM, van Houwelingen AH, Noerager BD, Jackson PL, Kraneveld AD, Galin FS, Folkerts G, Nijkamp FP, Blalock JE. A novel peptide cxcr ligand derived from extracellular matrix degradation during airway inflammation. *Nat Med* 2006;12(3):317-323.
117. Eggesbo JB, Hjermann I, Hostmark AT, Kierulf P. Lps induced release of il-1 beta, il-6, il-8 and tnf-alpha in edta or heparin anticoagulated whole blood from persons with high or low levels of serum hdl. *Cytokine* 1996;8(2):152-160.
118. Chintakuntlawar AV, Chodosh J. Chemokine cxcl1/kc and its receptor cxcr2 are responsible for neutrophil chemotaxis in adenoviral keratitis. *J Interferon Cytokine Res* 2009;29(10):657-666.
119. Deutschman CS, Tracey KJ. Sepsis: Current dogma and new perspectives. *Immunity* 2014;40(4):463-475.
120. Fessler MB. Next stop for hdl: The lung. *Clin Exp Allergy* 2012;42(3):340-342.
121. Yu Z, Jin J, Wang Y, Sun J. High density lipoprotein promoting proliferation and migration of type ii alveolar epithelial cells during inflammation state. *Lipids Health Dis* 2017;16(1):91.
122. Navab M, Hama SY, Anantharamaiah GM, Hassan K, Hough GP, Watson AD, Reddy ST, Sevanian A, Fonarow GC, Fogelman AM. Normal high density lipoprotein inhibits three steps in the formation of mildly oxidized low density lipoprotein: Steps 2 and 3. *J Lipid Res* 2000;41(9):1495-1508.
123. Oram JF, Vaughan AM. Atp-binding cassette cholesterol transporters and cardiovascular disease. *Circ Res* 2006;99(10):1031-1043.

124. Tall AR, Yvan-Charvet L, Terasaka N, Pagler T, Wang N. Hdl, abc transporters, and cholesterol efflux: Implications for the treatment of atherosclerosis. *Cell Metab* 2008;7(5):365-375.
125. Kielar D, Dietmaier W, Langmann T, Aslanidis C, Probst M, Naruszewicz M, Schmitz G. Rapid quantification of human abca1 mrna in various cell types and tissues by real-time reverse transcription-pcr. *Clin Chem* 2001;47(12):2089-2097.
126. Kolleck I, Sinha P, Rustow B. Vitamin e as an antioxidant of the lung: Mechanisms of vitamin e delivery to alveolar type ii cells. *Am J Respir Crit Care Med* 2002;166(12 Pt 2):S62-66.
127. Bates SR, Tao JQ, Yu KJ, Borok Z, Crandall ED, Collins HL, Rothblat GH. Expression and biological activity of abca1 in alveolar epithelial cells. *Am J Respir Cell Mol Biol* 2008;38(3):283-292.
128. Bortnick AE, Favari E, Tao JQ, Francone OL, Reilly M, Zhang Y, Rothblat GH, Bates SR. Identification and characterization of rodent abca1 in isolated type ii pneumocytes. *Am J Physiol Lung Cell Mol Physiol* 2003;285(4):L869-878.
129. Gowdy KM, Madenspacher JH, Azzam KM, Gabor KA, Janardhan KS, Aloor JJ, Fessler MB. Key role for scavenger receptor b-i in the integrative physiology of host defense during bacterial pneumonia. *Mucosal Immunol* 2015;8(3):559-571.
130. Aiello RJ, Brees D, Francone OL. Abca1-deficient mice: Insights into the role of monocyte lipid efflux in hdl formation and inflammation. *Arterioscler Thromb Vasc Biol* 2003;23(6):972-980.
131. Draper DW, Gowdy KM, Madenspacher JH, Wilson RH, Whitehead GS, Nakano H, Pandiri AR, Foley JF, Remaley AT, Cook DN, et al. Atp binding cassette transporter g1 deletion induces il-17-dependent dysregulation of pulmonary adaptive immunity. *J Immunol* 2012;188(11):5327-5336.
132. Chai AB, Ammit AJ, Gelissen IC. Examining the role of abc lipid transporters in pulmonary lipid homeostasis and inflammation. *Respir Res* 2017;18(1):41.
133. Sullivan EM, Fix A, Crouch MJ, Sparagna GC, Zeczycki TN, Brown DA, Shaikh SR. Murine diet-induced obesity remodels cardiac and liver mitochondrial phospholipid acyl chains with differential effects on respiratory enzyme activity. *J Nutr Biochem* 2017;45:94-103.
134. O'Kane CM, McKeown SW, Perkins GD, Bassford CR, Gao F, Thickett DR, McAuley DF. Salbutamol up-regulates matrix metalloproteinase-9 in the alveolar space in the acute respiratory distress syndrome. *Crit Care Med* 2009;37(7):2242-2249.
135. Nathani N, Perkins GD, Tunnicliffe W, Murphy N, Manji M, Thickett DR. Kerbs von lungren 6 antigen is a marker of alveolar inflammation but not of infection in patients with acute respiratory distress syndrome. *Crit Care* 2008;12(1):R12.
136. Ware LB, Matthay MA. The acute respiratory distress syndrome. *N Engl J Med* 2000;342(18):1334-1349.
137. Parekh D, Dancer RC, Thickett DR. Acute lung injury. *Clin Med (Lond)* 2011;11(6):615-618.
138. Pajkrt D, Doran JE, Koster F, Lerch PG, Arnet B, van der Poll T, ten Cate JW, van Deventer SJ. Antiinflammatory effects of reconstituted high-density lipoprotein during human endotoxemia. *J Exp Med* 1996;184(5):1601-1608.
139. Petropoulou PI, Berbee JF, Theodoropoulos V, Hatziri A, Stamou P, Karavia EA, Spyridonidis A, Karagiannides I, Kypreos KE. Lack of lcat reduces the lps-neutralizing capacity

of hdl and enhances lps-induced inflammation in mice. *Biochim Biophys Acta* 2015;1852(10 Pt A):2106-2115.
140. Yu Y, Cui Y, Zhao Y, Liu S, Song G, Jiao P, Li B, Luo T, Guo S, Zhang X, et al. The binding capability of plasma phospholipid transfer protein, but not hdl pool size, is critical to repress lps induced inflammation. *Sci Rep* 2016;6:20845.
141. Blackwell TS, Lancaster LH, Blackwell TR, Venkatakrishnan A, Christman JW. Chemotactic gradients predict neutrophilic alveolitis in endotoxin-treated rats. *Am J Respir Crit Care Med* 1999;159(5 Pt 1):1644-1652.
142. Ley K, Laudanna C, Cybulsky MI, Nourshargh S. Getting to the site of inflammation: The leukocyte adhesion cascade updated. *Nat Rev Immunol* 2007;7(9):678-689.
143. Kaplan R, Gan X, Menke JG, Wright SD, Cai TQ. Bacterial lipopolysaccharide induces expression of abca1 but not abcg1 via an lxr-independent pathway. *J Lipid Res* 2002;43(6):952-959.
144. Silverstein RL, Febbraio M. Cd36, a scavenger receptor involved in immunity, metabolism, angiogenesis, and behavior. *Sci Signal* 2009;2(72):re3.
145. Topchiy E, Cirstea M, Kong HJ, Boyd JH, Wang Y, Russell JA, Walley KR. Lipopolysaccharide is cleared from the circulation by hepatocytes via the low density lipoprotein receptor. *PLoS One* 2016;11(5):e0155030.
146. Lozano R, Naghavi M, Foreman K, Lim S, Shibuya K, Aboyans V, Abraham J, Adair T, Aggarwal R, Ahn SY, et al. Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010: A systematic analysis for the global burden of disease study 2010. *Lancet* 2012;380(9859):2095-2128.
147. Murray CJ, Lopez AD. Alternative projections of mortality and disability by cause 1990-2020: Global burden of disease study. *Lancet* 1997;349(9064):1498-1504.
148. Levine DM, Parker TS, Donnelly TM, Walsh A, Rubin AL. In vivo protection against endotoxin by plasma high density lipoprotein. *Proc Natl Acad Sci U S A* 1993;90(24):12040-12044.
149. Casas AT, Hubsch AP, Rogers BC, Doran JE. Reconstituted high-density lipoprotein reduces lps-stimulated tnf alpha. *J Surg Res* 1995;59(5):544-552.
150. Hubsch AP, Casas AT, Doran JE. Protective effects of reconstituted high-density lipoprotein in rabbit gram-negative bacteremia models. *J Lab Clin Med* 1995;126(6):548-558.
151. Quezado ZM, Natanson C, Banks SM, Alling DW, Koev CA, Danner RL, Elin RJ, Hosseini JM, Parker TS, Levine DM, et al. Therapeutic trial of reconstituted human high-density lipoprotein in a canine model of gram-negative septic shock. *J Pharmacol Exp Ther* 1995;272(2):604-611.
152. Lee RP, Lin NT, Chao YF, Lin CC, Harn HJ, Chen HI. High-density lipoprotein prevents organ damage in endotoxemia. *Res Nurs Health* 2007;30(3):250-260.
153. Xing Z, Gauldie J, Cox G, Baumann H, Jordana M, Lei XF, Achong MK. Il-6 is an antiinflammatory cytokine required for controlling local or systemic acute inflammatory responses. *J Clin Invest* 1998;101(2):311-320.
154. Scheller J, Chalaris A, Schmidt-Arras D, Rose-John S. The pro- and anti-inflammatory properties of the cytokine interleukin-6. *Biochim Biophys Acta* 2011;1813(5):878-888.
155. Wolpe SD, Sherry B, Juers D, Davatelis G, Yurt RW, Cerami A. Identification and characterization of macrophage inflammatory protein 2. *Proc Natl Acad Sci U S A* 1989;86(2):612-616.

156. Iida N, Grotendorst GR. Cloning and sequencing of a new gro transcript from activated human monocytes: Expression in leukocytes and wound tissue. *Mol Cell Biol* 1990;10(10):5596-5599.
157. Wang L, Chen WZ, Wu MP. Apolipoprotein a-i inhibits chemotaxis, adhesion, activation of thp-1 cells and improves the plasma hdl inflammatory index. *Cytokine* 2010;49(2):194-200.
158. Theilmeier G, Schmidt C, Herrmann J, Keul P, Schafers M, Herrgott I, Mersmann J, Larmann J, Hermann S, Stypmann J, et al. High-density lipoproteins and their constituent, sphingosine-1-phosphate, directly protect the heart against ischemia/reperfusion injury in vivo via the s1p3 lysophospholipid receptor. *Circulation* 2006;114(13):1403-1409.
159. Ferguson ND, Fan E, Camporota L, Antonelli M, Anzueto A, Beale R, Brochard L, Brower R, Esteban A, Gattinoni L, et al. The berlin definition of ards: An expanded rationale, justification, and supplementary material. *Intensive Care Med* 2012;38(10):1573-1582.
160. Zemans RL, Matthay MA. What drives neutrophils to the alveoli in ards? *Thorax* 2017;72(1):1-3.
161. Francone OL, Royer L, Boucher G, Haghpassand M, Freeman A, Brees D, Aiello RJ. Increased cholesterol deposition, expression of scavenger receptors, and response to chemotactic factors in abca1-deficient macrophages. *Arterioscler Thromb Vasc Biol* 2005;25(6):1198-1205.
162. Koseki M, Hirano K, Masuda D, Ikegami C, Tanaka M, Ota A, Sandoval JC, Nakagawa-Toyama Y, Sato SB, Kobayashi T, et al. Increased lipid rafts and accelerated lipopolysaccharide-induced tumor necrosis factor-alpha secretion in abca1-deficient macrophages. *J Lipid Res* 2007;48(2):299-306.
163. Zhu X, Lee JY, Timmins JM, Brown JM, Boudyguina E, Mulya A, Gebre AK, Willingham MC, Hiltbold EM, Mishra N, et al. Increased cellular free cholesterol in macrophage-specific abca1 knock-out mice enhances pro-inflammatory response of macrophages. *J Biol Chem* 2008;283(34):22930-22941.
164. Yvan-Charvet L, Welch C, Pagler TA, Ranalletta M, Lamkanfi M, Han S, Ishibashi M, Li R, Wang N, Tall AR. Increased inflammatory gene expression in abc transporter-deficient macrophages: Free cholesterol accumulation, increased signaling via toll-like receptors, and neutrophil infiltration of atherosclerotic lesions. *Circulation* 2008;118(18):1837-1847.
165. Baldan A, Gomes AV, Ping P, Edwards PA. Loss of abcg1 results in chronic pulmonary inflammation. *J Immunol* 2008;180(5):3560-3568.
166. Bandeali S, Farmer J. High-density lipoprotein and atherosclerosis: The role of antioxidant activity. *Curr Atheroscler Rep* 2012;14(2):101-107.
167. Tang C, Liu Y, Kessler PS, Vaughan AM, Oram JF. The macrophage cholesterol exporter abca1 functions as an anti-inflammatory receptor. *J Biol Chem* 2009;284(47):32336-32343.
168. Zhao Y, Van Berkel TJ, Van Eck M. Relative roles of various efflux pathways in net cholesterol efflux from macrophage foam cells in atherosclerotic lesions. *Curr Opin Lipidol* 2010;21(5):441-453.
169. Pan B, Yu B, Ren H, Willard B, Pan L, Zu L, Shen X, Ma Y, Li X, Niu C, et al. High-density lipoprotein nitration and chlorination catalyzed by myeloperoxidase impair its effect of promoting endothelial repair. *Free Radic Biol Med* 2013;60:272-281.
170. Shao B, Cavigiolio G, Brot N, Oda MN, Heinecke JW. Methionine oxidation impairs reverse cholesterol transport by apolipoprotein a-i. *Proc Natl Acad Sci U S A* 2008;105(34):12224-12229.

171. Undurti A, Huang Y, Lupica JA, Smith JD, DiDonato JA, Hazen SL. Modification of high density lipoprotein by myeloperoxidase generates a pro-inflammatory particle. *J Biol Chem* 2009;284(45):30825-30835.
172. Kimura T, Tomura H, Mogi C, Kuwabara A, Damirin A, Ishizuka T, Sekiguchi A, Ishiwara M, Im DS, Sato K, et al. Role of scavenger receptor class b type i and sphingosine 1-phosphate receptors in high density lipoprotein-induced inhibition of adhesion molecule expression in endothelial cells. *J Biol Chem* 2006;281(49):37457-37467.
173. White CR, Datta G, Buck AK, Chaddha M, Reddy G, Wilson L, Palgunachari MN, Abbasi M, Anantharamaiah GM. Preservation of biological function despite oxidative modification of the apolipoprotein a-i mimetic peptide 4f. *J Lipid Res* 2012;53(8):1576-1587.
174. Cai L, Ji A, de Beer FC, Tannock LR, van der Westhuyzen DR. Sr-bi protects against endotoxemia in mice through its roles in glucocorticoid production and hepatic clearance. *J Clin Invest* 2008;118(1):364-375.
175. Gordon S, Durairaj A, Lu JL, Davidson WS. High-density lipoprotein proteomics: Identifying new drug targets and biomarkers by understanding functionality. *Curr Cardiovasc Risk Rep* 2010;4(1):1-8.
176. Inoue M, Niki M, Ozeki Y, Nagi S, Chadeka EA, Yamaguchi T, Osada-Oka M, Ono K, Oda T, Mwende F, et al. High-density lipoprotein suppresses tumor necrosis factor alpha production by mycobacteria-infected human macrophages. *Sci Rep* 2018;8(1):6736.
177. Tall AR, Costet P, Wang N. Regulation and mechanisms of macrophage cholesterol efflux. *J Clin Invest* 2002;110(7):899-904.
178. He D, Zhao M, Wu C, Zhang W, Niu C, Yu B, Jin J, Ji L, Willard B, Mathew AV, et al. Apolipoprotein a-1 mimetic peptide 4f promotes endothelial repairing and compromises reendothelialization impaired by oxidized hdl through sr-b1. *Redox Biol* 2018;15:228-242.
179. David SA. Towards a rational development of anti-endotoxin agents: Novel approaches to sequestration of bacterial endotoxins with small molecules. *J Mol Recognit* 2001;14(6):370-387.
180. Parker TS, Levine DM, Chang JC, Laxer J, Coffin CC, Rubin AL. Reconstituted high-density lipoprotein neutralizes gram-negative bacterial lipopolysaccharides in human whole blood. *Infect Immun* 1995;63(1):253-258.
181. Zhu W, Saddar S, Seetharam D, Chambliss KL, Longoria C, Silver DL, Yuhanna IS, Shaul PW, Mineo C. The scavenger receptor class b type i adaptor protein pdzk1 maintains endothelial monolayer integrity. *Circ Res* 2008;102(4):480-487.
182. Giannotti G, Doerries C, Mocharla PS, Mueller MF, Bahlmann FH, Horvath T, Jiang H, Sorrentino SA, Steenken N, Manes C, et al. Impaired endothelial repair capacity of early endothelial progenitor cells in prehypertension: Relation to endothelial dysfunction. *Hypertension* 2010;55(6):1389-1397.
183. He D, Pan B, Ren H, Zheng L. Effects of diabetic hdl on endothelial cell function. *Cardiovasc Hematol Disord Drug Targets* 2014;14(2):137-141.
184. Spieker LE, Sudano I, Hurlimann D, Lerch PG, Lang MG, Binggeli C, Corti R, Ruschitzka F, Luscher TF, Noll G. High-density lipoprotein restores endothelial function in hypercholesterolemic men. *Circulation* 2002;105(12):1399-1402.
185. Wu X, Liu Z, Hu L, Gu W, Zhu L. Exosomes derived from endothelial progenitor cells ameliorate acute lung injury by transferring mir-126. *Exp Cell Res* 2018.
186. Li Y, Zhu H, Wei X, Li H, Yu Z, Zhang H, Liu W. Lps induces huvec angiogenesis in vitro through mir-146a-mediated tgf-beta1 inhibition. *Am J Transl Res* 2017;9(2):591-600.

187. Furtmuller PG, Obinger C, Hsuanyu Y, Dunford HB. Mechanism of reaction of myeloperoxidase with hydrogen peroxide and chloride ion. *Eur J Biochem* 2000;267(19):5858-5864.
188. Zheng L, Nukuna B, Brennan ML, Sun M, Goormastic M, Settle M, Schmitt D, Fu X, Thomson L, Fox PL, et al. Apolipoprotein a-i is a selective target for myeloperoxidase-catalyzed oxidation and functional impairment in subjects with cardiovascular disease. *J Clin Invest* 2004;114(4):529-541.
189. Chan GK, Witkowski A, Gantz DL, Zhang TO, Zanni MT, Jayaraman S, Cavigiolio G. Myeloperoxidase-mediated methionine oxidation promotes an amyloidogenic outcome for apolipoprotein a-i. *J Biol Chem* 2015;290(17):10958-10971.
190. Zheng L, Settle M, Brubaker G, Schmitt D, Hazen SL, Smith JD, Kinter M. Localization of nitration and chlorination sites on apolipoprotein a-i catalyzed by myeloperoxidase in human atheroma and associated oxidative impairment in abca1-dependent cholesterol efflux from macrophages. *J Biol Chem* 2005;280(1):38-47.
191. Peng DQ, Wu Z, Brubaker G, Zheng L, Settle M, Gross E, Kinter M, Hazen SL, Smith JD. Tyrosine modification is not required for myeloperoxidase-induced loss of apolipoprotein a-i functional activities. *J Biol Chem* 2005;280(40):33775-33784.
192. Kameda T, Ohkawa R, Yano K, Usami Y, Miyazaki A, Matsuda K, Kawasaki K, Sugano M, Kubota T, Tozuka M. Effects of myeloperoxidase-induced oxidation on antiatherogenic functions of high-density lipoprotein. *J Lipids* 2015;2015:592594.
193. Janowski BA, Grogan MJ, Jones SA, Wisely GB, Kliewer SA, Corey EJ, Mangelsdorf DJ. Structural requirements of ligands for the oxysterol liver x receptors lxralpha and lxrbeta. *Proc Natl Acad Sci U S A* 1999;96(1):266-271.
194. Janowski BA, Willy PJ, Devi TR, Falck JR, Mangelsdorf DJ. An oxysterol signalling pathway mediated by the nuclear receptor lxr alpha. *Nature* 1996;383(6602):728-731.
195. Smoak K, Madenspacher J, Jeyaseelan S, Williams B, Dixon D, Poch KR, Nick JA, Worthen GS, Fessler MB. Effects of liver x receptor agonist treatment on pulmonary inflammation and host defense. *J Immunol* 2008;180(5):3305-3312.
196. Gong H, He J, Lee JH, Mallick E, Gao X, Li S, Homanics GE, Xie W. Activation of the liver x receptor prevents lipopolysaccharide-induced lung injury. *J Biol Chem* 2009;284(44):30113-30121.
197. Beceiro S, Pap A, Czimmerer Z, Sallam T, Guillen JA, Gallardo G, Hong C, N AG, Tabraue C, Diaz M, et al. Lxr nuclear receptors are transcriptional regulators of dendritic cell chemotaxis. *Mol Cell Biol* 2018.
198. Shao B, Pennathur S, Heinecke JW. Myeloperoxidase targets apolipoprotein a-i, the major high density lipoprotein protein, for site-specific oxidation in human atherosclerotic lesions. *J Biol Chem* 2012;287(9):6375-6386.
199. DiDonato JA, Aulak K, Huang Y, Wagner M, Gerstenecker G, Topbas C, Gogonea V, DiDonato AJ, Tang WH, Mehl RA, et al. Site-specific nitration of apolipoprotein a-i at tyrosine 166 is both abundant within human atherosclerotic plaque and dysfunctional. *J Biol Chem* 2014;289(15):10276-10292.
200. Huang Y, DiDonato JA, Levison BS, Schmitt D, Li L, Wu Y, Buffa J, Kim T, Gerstenecker GS, Gu X, et al. An abundant dysfunctional apolipoprotein a1 in human atheroma. *Nat Med* 2014;20(2):193-203.

201. Plump AS, Smith JD, Hayek T, Aalto-Setala K, Walsh A, Verstuyft JG, Rubin EM, Breslow JL. Severe hypercholesterolemia and atherosclerosis in apolipoprotein e-deficient mice created by homologous recombination in es cells. *Cell* 1992;71(2):343-353.
202. Choi J, Forster MJ, McDonald SR, Weintraub ST, Carroll CA, Gracy RW. Proteomic identification of specific oxidized proteins in apoe-knockout mice: Relevance to alzheimer's disease. *Free Radic Biol Med* 2004;36(9):1155-1162.
203. Liao J, Liu X, Gao M, Wang M, Wang Y, Wang F, Huang W, Liu G. Dyslipidemia, steatohepatitis and atherogenesis in lipodystrophic apoe deficient mice with seipin deletion. *Gene* 2018;648:82-88.
204. Pal R, Ke Q, Pihan GA, Yesilaltay A, Penman ML, Wang L, Chitraju C, Kang PM, Krieger M, Kocher O. Carboxy-terminal deletion of the hdl receptor reduces receptor levels in liver and steroidogenic tissues, induces hypercholesterolemia, and causes fatal heart disease. *Am J Physiol Heart Circ Physiol* 2016;311(6):H1392-H1408.

APPENDIX: IACUC Approval Letters

Animal Care and Use Committee
212 Ed Warren Life Sciences Building
East Carolina University
Greenville, NC 27834-4354

252-744-2436 office
252-744-2355 fax

October 17, 2017

Kymberly Gowdy, Ph.D.
Department of Pharmacology
Brody 6S-10
East Carolina University

Dear Dr. Gowdy:

Your Animal Use Protocol entitled, "Breeding Protocol for Class B Scavenger Receptor Deficient Mice" (AUP #W242a) was reviewed by this institution's Animal Care and Use Committee on October 17, 2017. The following action was taken by the Committee:

"Approved as submitted"

Commendation for justification of animal numbers and very well written AUP overall!

Please contact Aaron Hinkle at 744-2997 prior to hazard use

A copy is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies. **Please ensure that all personnel associated with this protocol have access to this approved copy of the AUP and are familiar with its contents.**

Sincerely yours,

Susan McRae, Ph.D.
Chair, Animal Care and Use Committee

SM/jd

Enclosure

Animal Care and
Use Committee
212 Ed Warren Life
Sciences Building
East Carolina University
Greenville, NC 27834-4354

252-744-2436 office
252-744-2355 fax

February 13, 2018

Kym Gowdy, Ph.D.
Department of Pharmacology
EW Life Sciences Building
East Carolina University

Dear Dr. Gowdy:

Your Animal Use Protocol entitled, "The Role of Scavenger Receptor B11 in Pulmonary Bacterial and Viral Infections in Mice" (AUP #W243a) was reviewed by this institution's Animal Care and Use Committee on February 13, 2018. The following action was taken by the Committee:

"Approved as submitted"

Please contact Aaron Hinkle at 744-2997 prior to hazard use

A copy is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies. **Please ensure that all personnel associated with this protocol have access to this approved copy of the AUP and are familiar with its contents.**

Sincerely yours,

Susan McRae, Ph.D.
Chair, Animal Care and Use Committee

SM/jd

Enclosure

East Carolina University is a constituent institution of the
University of North Carolina. An equal opportunity university.

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