The Role of Surfactant Protein D In Atherosclerosis

by

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Abstract

Elevated concentrations of surfactant protein D (SP-D) in the serum have been associated with cardiovascular disease (CVD) mortality. It is not known, however, whether this relationship is causal or an epiphenomenon of lung disease or inflammation. The primary purpose of this thesis was to investigate the effects of SP-D on atherosclerosis. The overarching hypothesis driving this study is that SP-D is pro-atherogenic by modulating plasma lipids and systemic inflammation. These studies will enable development of SP-D as a biomarker of atherosclerosis, ischemic heart disease, and stroke and determine whether SP-D can be a therapeutic target to reduce CVD.

To address the hypothesis, SP-D-knockout (KO) mice were crossed with apolipoprotein E (ApoE)-KO mice to produce mice deficient in both SP-D and ApoE. These mice were fed a high fat diet (HFD) for twelve weeks. We then measured the size of atherosclerotic lesions in aorta, determined the lipid profile in the serum, and measured circulating inflammatory cytokines and white blood cell counts in mice. We also challenged SP-D-deficient mice with lipopolysaccharide (LPS), which was microsprayed directly into the trachea. We then determined endothelial function of the descending aorta, 24 hours after the LPS challenge.

We found that in the atherosclerotic plaque was significantly smaller in ApoE-KO mice lacking SP-D compared with apoE mice with intact SP-D. Absence of SP-D in the apo-E KO mice resulted in reduced levels of low density lipoprotein (LDL) cholesterol and total cholesterols, and a marked attenuation of certain parameters of systemic inflammation including neutrophil to lymphocyte ratios (NLR) and interleukin (IL)-6 in serum. Deficiency

of SP-D was also associated with improved endothelial function following intratracheal LPS challenge.

Together, these findings indicate that SP-D plays an active role in modulating lipid profile and systemic inflammation and most importantly may enhance atherosclerosis and thus contribute directly to excess cardiovascular morbidity and mortality.

Preface

This dissertation is original, unpublished, independent work by the author, C, Go Eun. Ethics approval was given by the Animal Care Committee of the UBC Research Ethics Board (A10-0257: SP-D and Atherosclerosis: Linking Lung Injury with Cardiovascular Diseases).

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List of Abbreviations

ApoE Apolipoprotein E

BALF Broncho alveolar lavage fluid

CRD Carbohydrate recognition domain

CVD Cardiovascular disease

HDL High density lipoprotein

HFD High fat diet

ICAM Intercellular adhesion molecule

IL Interleukin

KC Keratinocyte chemoattractant

LPS Lipopolysaccharide

LDL Low density lipoprotein

MMP Metalloproteinase

MCP Monocyte chemoattractant protein

NLR Neutrophil to lymphocyte ratio

NO Nitric Oxide

Ox Oxidized

PBS Phosphate buffered saline

SMC Smooth muscle cell

SNP Sodium nitroprusside

SP-D Surfactant protein D

TNF Tumor necrotic factor

VLDL Very low-density lipoprotein

VCAM Vascular cell adhesion molecule

WHO World health organization

WT Wild type

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I've read many others' thesis and this is the part I love to read most. I was imagining how nice it would be if I'm the one who get to thank everyone and I'm finally writing the acknowledgements.

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Dedication

This work is dedicated to my parents (Y. Chung and S. Choi) and all of my cheerful friends (W1, W2, D, M, D, S, Z), in appreciation of the many insights into life that they have shared with me, and in the hope that they will continue to enjoy their lives to the fullest for many years to come.

Chapter 1: Introduction

1.1 Atherosclerosis

1.1.1 Atherosclerosis and Coronary Artery Disease

Cardiovascular disease (CVD) is the leading cause of morbidity and mortality in the Western world. The global burden of these diseases has sharply increased in last two decades and continues to increase due to the growth in aging population and health risk behaviors (Jemal *et al.,* 2011; Sanderson *et al.,* 2007). The World Health Organization predicts that by 2030, CVD will kill about 23.6 million people, making it the leading cause of global death (WHO, 2012).

'Athere' in Greek means porridge and refers to the soft lipid-filled core of plaques and 'sclerosis' implies hardening and refers to the hard fibrotic cap separating blood from the thrombogenic material within atherosclerotic lesions. Atherosclerosis is thought to be caused by multiple risk factors including tobacco smoking, aging, elevated cholesterol levels, unhealthy diet, elevated blood pressure and heredity in the civilized society, especially in the Western world. (Li *et al.,* 2004; Ruffer *et al.,* 1911).

Atherosclerosis is a complex, chronic process that results in the formation of stratified lesions in arterial wall (Star *et al.,* 1994; Virmani *et al.,* 2000). In arteries of healthy people, oxygenated blood can freely travel to the organs but in arteries of atherosclerotic patients the flow of blood is limited owing to the thickened intima. At the initial stage of atherosclerosis, the blood vessels expands outwards to compensate against intimal thickening and to maintain luminar area. As atherosclerosis progresses, intimal thickening outpaces the vessels' ability to expand outwards, causing the lumen to narrow and eventually leading to the formation of a fibrous cap. If the fibrous cap ruptures, thrombosis

ensues, causing acute occlusion of lumen and myocardial infarction (Mansaray *et al.,* 1999; Davies *et al.,* 1995).

1.1.2 Initiation and Development of Atherosclerosis

Atherosclerosis is initiated when the cholesterol-rich very low-density lipoprotein (VLDL) levels and LDL levels in plasma are elevated. These lipoproteins can infiltrate into endothelial cells on arterial wall, and when the infiltration rate exceeds the ability of the vessels to remove them, accumulation occurs in the vessel wall (Camejo *et al.,* 1998; Skålén *et al.,* 2002). Once retained in the vessel wall, LDL undergoes oxidative modification, which then becomes a substrate for the release bioactive phospholipids that can activate endothelial cells and coax the expression of leukocyte adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) (Kume *et al.,* 1994; Leitinger *et al.,* 2003). These molecules in turn promote adhesion of monocytes and T cells to the endothelium. Once inside the artery wall, monocytes transmigrate across the endothelial monolayer into the intima where they can proliferate, and differentiate into macrophages under the guidance of cytokines such as monocyte/macrophage chemoattractant MCP-1 (Rice *et al.,* 2003; Cybulsky *et al.,* 1991). Macrophages accumulate large amounts of lipid by ingesting modified lipoproteins, leading to the formation of foam cells. Foam cells are the hallmark of atherosclerosis and become the substrate for fatty streaks. Macrophage-derived foam cells can stimulate smooth muscle cell (SMC) to migrate from the media into the intima forming a fibrous cap that is rich in collagen (Herity *et al.,* 1999). SMCs, foam cells and extracellular matrix together become the necrotic core (Figure 1.1.2). As atherosclerosis progresses, macrophages and macrophage derived foam cells

release matrix metalloproteinases (MMPs), which then degrade extracellular matrix resulting in an unstable plaque. Unstable plaque is characterized by a thin, collagen-poor fibrous cap with an enlarged necrotic core that is filled with dead cells, accumulated lipid and cellular debris, and cholesterol crystals (Kocks *et al.,* 2000). When this unstable plaque ruptures, the lipid core becomes exposed, which is extremely thrombogenic, leading to recruitment of platelets and active coagulation on the plaque surface. Together, this leads to acute thrombus formation, which can cause acute coronary syndromes (Herity *et al.,* 1999).

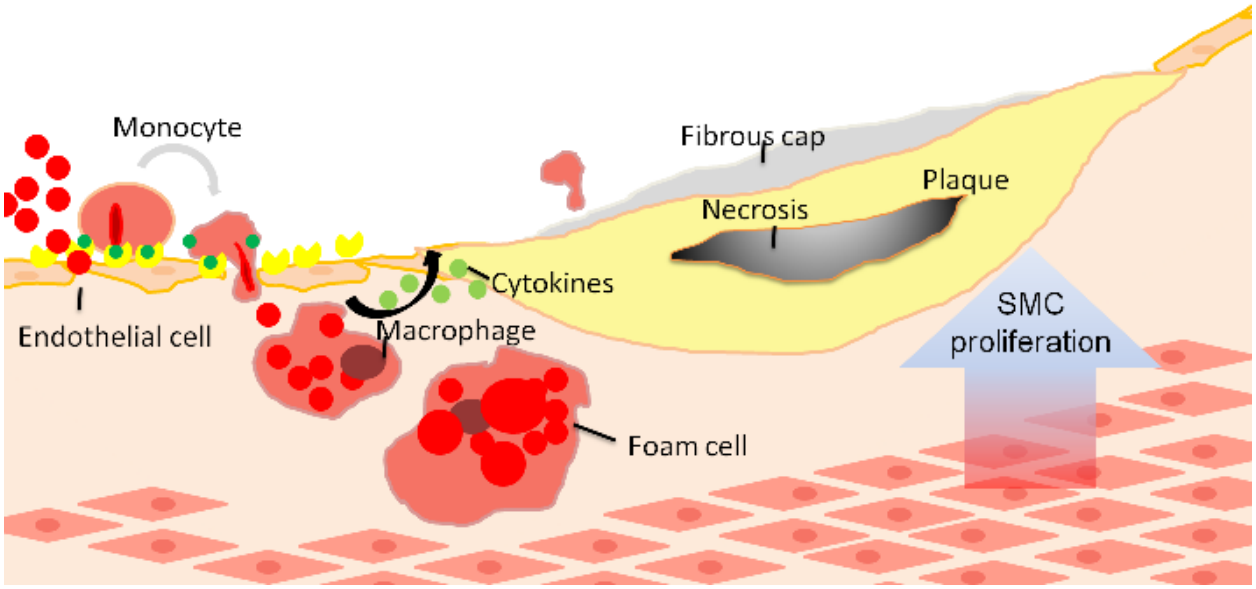


Figure 1. The Development of Atherosclerotic Lesions. Dyslipidemia leading to retention of LDLs in the intima causes expression of vascular cell adhesion molecules that recruit leukocytes onto endothelial surfaces. Once inside, monocytes migrate and differentiate into macrophages. Macrophages engulf lipoproteins and become lipid-laden foam cells. As atherosclerosis advances, smooth muscle cells (SMCs) are recruited from the tunica media and proliferate in response to platelet-derived growth factor. In the intima, SMCs produce extracellular matrix molecules such as collagen and elastin. This results in a fibrous cap that covers the plaque. Foam cells undergo apoptosis and release lipids. The inefficient clearance of apoptotic cells results in accumulation of cellular debris and formation of a necrotic core. Unstable plaque has a collagen-poor fibrous cap with SMCs and abundant macrophages.

1.1.3 Endothelial Dysfunction in Atherosclerosis

Endothelial dysfunction has been well known to be the first step in progression towards atherosclerosis. This dysfunction is caused by oxidized LDL, infection, free radical generation, hypertension and diabetes. In healthy endothelium, endothelial cells prevent the attachment of circulating leukocytes and produces nitric oxide (NO), a major vascular dilator that possesses anti-inflammatory, anti-thrombotic properties. Dysfunctional endothelium is characterized by reduced vasodilatation, over-expression of adhesion molecules and chemokines and increased permeability (Endermann *et al.,* 2004; Kaperonis *et al.,* 2006).

1.1.4 Systemic Inflammation and Atherosclerosis

Inflammation plays a pivotal role in causing plaque instability. Inflammatory cell recruitment is mediated by various chemotactic cytokines which cause proliferation, differentiation and cellular dysfunction (Girn *et al.,* 2007). Inflammatory cytokines that play an active role in atherosclerosis have been identified.

The interleukin (IL)-6 and its signaling molecules have been shown to contribute to atherosclerotic plaque genesis, progression and instability. It functions as a pro-atherogenic cytokine by coaxing the synthesis and recruitment of other pro-inflammatory cytokines, by inducing phospolipases and oxidizing lipoproteins, stimulating acute phase protein and pro-thrombotic mediator synthesis and secretion and activating matrix metalloproteinase (MMPs) (Yudkin *et al.,* 2000; Ross *et al.,* 1999; Harbarth *al.,* 2001; McCarty *al.,* 1999). IL-6 also promotes endothelial dysfunction, SMC proliferation and migration, recruitment and activation of leukocytes, thereby amplifying vascular

inflammation (Von der Thusen *et al.,* 2003; Klouche *et al.,* 2000). IL-6 is synthesized by many tissues, including the lungs (Libby *et al.,* 2002; Zhou *et al.,* 1999; Sukovich *et al.,* 1998).

Monocyte chemoattractant protein-1 (MCP-1) promotes atherosclerosis by recruiting monocytes to the vessel wall and is detectable in atherosclerotic lesions (Schwartz *et al.,* 1991; Taub *et al.,* 1996). It is responsible for regulating local expression of adhesion molecules, IL-1, IL-6 and tissue factor (Jiang *et al.,* 1992; Schecter *et al.,* 1997). Our lab previously found that MCP-1 level in serum was elevated in atherosclerosis model (unpublished data).

In humans, IL-8 demarginates neturophils from bone marrow and promotes the movement of immature neutrophil precursors into the circulation (Tarashima *et al.,* 1998). As the homolog to human IL-8, mice keratinocyte chemoattractant (KC) is the initial chemokine responsible for monocyte arrest on native atherosclerotic endothelium. It also recruits neutrophils by increasing their rolling, adhesion and extravascular accumulation and mediates TNF-alpha induced neutrophil activation (Huo *et al.,* 2001; Zhang *et al.,* 2001; Rollins *et al.,* 1997).

The involvement of TNF-alpha in the pathogenesis of atherosclerosis is supported by its presence in human atherosclerotic plaques. Furthermore, circulating TNF-alpha levels are associated with age-related atherosclerosis and with increased risk of recurrent myocardial infarction, atherosclerotic thickening of carotid intima-media *(Kleemann et al.,* 2008). TNF-alpha is primarily produced by monocytes and macrophages and is an early mediator of the acute-phase response (Gauldie, 1995). TNF-alpha has been shown to influence lipid metabolism by decreasing activity of lipoprotein lipase which results in liver

production of triglycerides (Grunfeld *et al.,* 1990; Feingold *et al.,* 1996; Feingold *et al.,* 1992; Fried *et al.,* 1989; Feingold *et al.,* 1989). It also induces smooth muscle proliferation, activates vascular endothelial, and upregulates ICAM-1 and VCAM-1 promoting the recruitment of monocytes into the endothelium (Thorne *et al.,* 1996; Krishnaswamy *et al.,* 1999).

White blood cells (WBC) are an independent predictor of CVD and mortality and it can be used to identify high-risk individuals for CVD (Margolis *et al.,* 2005). There are considerable data that leukocytosis directly enhance atherosclerosis (Coller *et al.,* 2005; Barron *et al.,* 2000; Cannon *et al.,* 2001; Friedman *et al.,* 1974; Sabatine *et al.,* 2002; Rothe *et al.,* 1996). A variety of risk factors of CVD including obesity, smoking, sedentary lifestyles and metabolic syndrome, have been associated with leukocytosis (Ortlepp *et al.,* 2004; Bovill *et al.,* 1996). Monocytosis by itself has been associated with CVD and with atherosclerotic plaque burden (Tani *et al.,* 2009; Chapman *et al.,* 2004; Afiune *et al.,* 2006; Stewart *et al.,* 2005; Swirski *et al.,* 2007; Horne *et al* 2005; Wildmer *et al.,* 2003; Drechsler *et al.,* 2010; Zernecke *et al.,* 2008). Neutrophil plays an important role in atherogenesis and atherothrombosis; while lymphocytosis has been inversely associated with atherosclerosis. Thus, neutrophil lymphocyte ratio (NLR) is considered reliable biomarker of systemic inflammation relevant for atherosclerosis (Gibson *et al.,* 2007; Walsh *et al.,* 2005; Sarraf *et al.,* 2009; Jilma *et al.,* 1999).

1.1.5 Lipid Homeostasis and Atherosclerosis

Hyperlipidemia due to elevated LDL-cholesterol is an important marker for atherosclerosis. Disruption in lipid homeostasis with elevated plasma cholesterol level is a

risk factor for the development of CVD (Miller *et al.,* 2005; Rader *et al.,* 2007). Low density lipoproteins (LDL) are recognized as the major atherogenic lipoprotein. Triglycerides (TG) are mainly present in triglyceride-rich lipoproteins such as chylomicrons and very low density lipoproteins, and fasting TG level has been associated with atherosclerosis especially when high density lipoprotein (HDL) concentration is low (Barcat *et al.,* 2006). HDL is a well-known atheroprotective molecule. The major mechanism by which HDL confers protection against atherosclerosis is by acting as a reverse cholesterol transporter, and thus transfers cholesterol from peripheral tissues to the liver, where it is excreted from the body directly or following conversion into bile acids. This process involves the unloading of cholesterol from foam cells in atherosclerotic plaque by ATP-binding cassette transporters (Bancells *et al*., 2010).

1.2 Apolippoprotein Knockout Mice

Apolipoprotein E (ApoE) is a 34-kDa arginine-rich glycoprotein found in chylomicrons, chymolomicron remants, VLDL, and some isotypes of HDL. It is synthesized in various tissue including brain, spleen, lung, ovaries, adrenal gland, kidney and muscles, but it is in the liver where the greatest amount of production occurs. A small amount of ApoE is also synthesized by macrophages/monocytes. The synthesis and secretion of ApoE by macrophages is influenced by the cellular cholesterol content. ApoE plays a central role in lipoprotein metabolism. It clears remnant lipoproteins in plasma by promoting reverse cholesterol transporter system and inducing assembly of nascent VLDL particles. It also regulates triglyceride flux in adipocytes and cholesterol efflux from cholesterol-loaded macrophages (Mensencamp *et al*., 1999; Yue *et al*., 2004; Curtis *et al*., 2000). In addition to

its role in lipoprotein metabolism, ApoE can also directly inhibit LDL oxidation, SMC proliferation, and endothelial cell proliferation, protecting cells and tissues from atherosclerosis (Zhang *et al*., 1992; Plump *et al*., 1992).

Plasma cholesterols in mice are carried mostly by HDL and mice have very little LDL or VLDL remnants. This is why mice are not prone to atherosclerosis even when fed a high fat diet in contrast to humans, who use LDL as the major lipoprotein for carrying cholesterol (Mahley *et al*., 1998; Miller *et al*., 1982). In 1980s, ApoE-KO mice were generated and since then, these are well used to study atherosclerosis due to the fact they have five times more cholesterol than wild type mice when fed with chow diet (Zhang *et al*., 1992; Plump *et al*., 1992). These mice manifest significant hypercholesterolemia and accelerated atherosclerosis on chow diet, and develop lesions in a similar manner as in humans (Huang *et al*., 2012). Atherosclerotic lesions in mice are located mainly in aortic root, brachiocephalic artery, aortic arch, and the branch points of the left common carotid and left subclavian arteries (Libby *et al*., 2012).

1.3 Surfactant Protein D

Pulmonary surfactant is a surface-active lipoprotein complex synthesized by type II alveolar cells and is composed of surfactant proteins (SP) and lipids (Griese *et al*., 1999). There are two groups of SPs. SP-B and SP-C are hydrophobic and they alter lipid packing and spreading, stabilize lipid layers during the respiratory cycle, and reduce surface tension in lungs. SP-A and SP-D are hydrophilic and are members of the collagen-like lectin family. SP-A and SP-D mediate host-defenses. They are potent immune molecules involved in viral neutralization, clearance of bacteria, fungi, apoptotic and necrotic cells and

resolution of inflammation (Crouch *et al*., 1998; Kegami *et al*., 1998; Hoppe *et al*., 1994; Kishore *et al*., 2006).

SP-D is a 43kDa protein containing an N-terminal, triple-helical collagen region and a C-terminal homotrimeric C-type lectin or carbohydrate recognition domain (CRD). It can be oligomerized into multimers, particularly trimers and dodecamers. The basic unit of SP-D is a homotrimer. The CRD mediates interactions with the pathogens, thereby mediating cellular phagocytosis (Crouch *et al*., 1998). Trimerization occurs by triple helix formation in a collagen-like domain and bundled alpha-helical coiled-coil formation in the neck region. Further oligomerization into dodecamers occurs when N-termini of trimers form disfulfide bonds, resulting in structures with the CRDs pointing outward (Crouch *et al*., 1994). SP-D dodecamers can modulate the function of various inflammatory cells such as macrophages, neutrophils, eosinophils and lymphocytes (Wright *et al*., 2005).

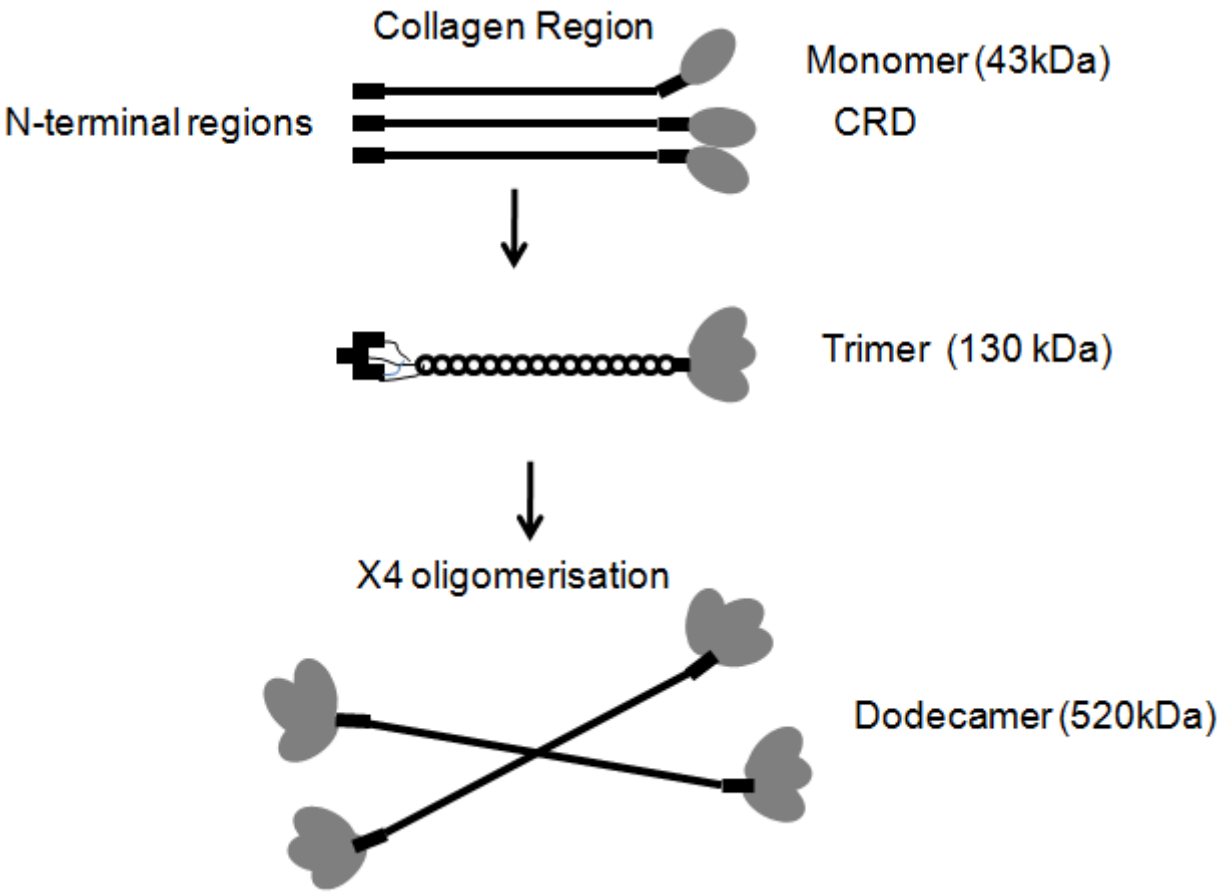


Figure 2. Surfactant Protein D Structure. SP-D is composed of polypeptide chains of 43 kDa that assemble into homotrimeric subunits by assembly of the neck. The structure of the collectin is characterized by four domains consisting of: 1) an N terminus involved in interchain disulfide bonding, 2) a collagen-like domain, 3) a coiled-coil neck domain, and 4) a carbohydrate recognition domain (CRD). The homotrimeric subunits of SP-D may further associate at their NH_2 termini to form cruciform molecules with four collagenous arms and peripheral, globular sugar-binding domains (Crouch *et al*., 2001).

SP-D is synthesized in alveolar type II cells and nonciliated airway epithelial cells (club cells) of lungs. SP-D's production is constitutive within the lung, but the synthesis and secretion of SP-D increase in response to lung injury from cigarette smoking and disease states such as cystic fibrosis, asthma, pneumonia, and idiopathic pulmonary fibrosis (Jobe *et al.,* 1998; Nordenbaek *et al.,* 2003). Besides type II pneumocytes, SP-D gene expression has been found in epithelial cells in the gastrointestinal, genitourinary tract and exocrine glands (Kleim *et al.,* 2000; Voorhout *et al.,* 1992).

SP-D acts as a pattern recognition receptor which binds to a variety of bacteria, viruses and fungi. SP-D binds to most gram-negative bacteria and some gram-positive organisms via binding of CRD to the bacterial cell wall components lipoteichoic acid and peptidoglycan and enhancing the phagocytosis and promoting mucocilliary clearance. SP-D also plays a role in fungi infection. The interaction of SP-D with fungi enhances phagocytosis and killing by neutrophils and macrophages (Griese *et al.,* 2005; Ofek *et al.,* 2001; Hartshom *et al.*, 1998; Van de Wetering *et al.*, 2001; Madan *et al.*, 1997).

SP-D-KO mice have been generated to study the function of this protein. These knockout mice show no noticeable physiological abnormalities at birth. However, during the post-natal developmental period, they demonstrate some lung abnormalities including alveolar lipidosis with type II cell hypertrophy, accumulation of enlarged and foamy macrophages, and expansion of lymphoid tissue (Korfhagen *et al,* 1998). The mice eventually develop dista-acinar emphysema and fibrosis and excess inflammatory reaction related to abnormal oxidant metabolism and metalloproteinase activity (Botas *et al.,* 1998; Wert *et al.,* 2000). These mice also show decreased viral clearance and enhanced inflammation following challenge with respiratory syncytial virus. In addition, they show

increased inflammation, increased oxidant production and decreased macrophage phagocytosis in response to infection (Le Vine *et al.,* 2000; Fisher *et al.,* 2000).

1.4 Rationale

A low-grade systemic inflammation is important to initiate plaque formation and progression to atherosclerosis (Ross *et al.,* 1999). Emerging data suggest that there is an important relationship between systemic inflammation induced by lung inflammation and atherosclerosis (Suwa *et al.,* 2002). Also, with the airway inflammation, the endothelium is activated and intracellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) are upregulated on the endothelia (Van Eden *et al.,* 2005).

SP-D has been extensively studied regard to pulmonary disease yet it is only recent been shown to be associated with CVD. A recent study by our laboratory showed a significant relationship between circulating SP-D and cardiovascular disease mortality in two independent cohorts. The first cohort of patients had coronary angiography for suspected coronary artery disease and the second cohort of patients was younger smokers with mild airflow limitation without a known CVD history. Data showed the patients with the highest quintile of SP-D had a 4.4 fold higher risk of CVD mortality compared with those in the lowest quintile independent of age, sex and plasma lipid levels (Hill *et al.,* 2011).

SP-D is mostly found in the lung and likely spill over into systemic circulation during acute or chronic inflammatory states (Suda *et al.,* 2011). The serum concentration of SP-D has been proposed as a noninvasive biomarker to assess the permeability or integrity of the alveolar-capillary barrier in pulmonary disease. Also, recent data showed that SP-D is increased in serum of patients with COPD while broncho alveolar lavage fluids (BALF) are

known to be reduced in the same patient (Lomas *et al.,* 2009). This makes SP-D an interesting molecule that could potentially link lung inflammation to systemic inflammation and atherosclerosis. The first reports of SP-D in atherosclerosis were published by Dr. Sorensen's laboratory. Their study showed that SP-D-KO mice had a 5.6 fold smaller atherosclerotic lesions, 18% higher plasma HDL-cholesterol level, and 45% reduced TNF-alpha concentrations compared to WT mice. Recombinant SP-D treatment reversed these findings (decrease in 21% of HDL-cholesterol, 26% total cholesterol and 27% LDL-cholesterol) (Sorensen *et al.,* 2006). This suggests the role of SP-D in regulating lipid homeostasis and affecting atherosclerosis. However, because mice are generally not prone to develop atherosclerotic lesion even when fed a high fat diet, SP-D-KO mice are suboptimal in elucidating the role of SP-D in atherosclerosis. To overcome this limitation, we generated double knock out (DKO) mice by cross-breeding with ApoE-KO mice with SP-D-KO mice, all on a C57/BL genetic background. The purpose of this study was to determine whether or not SP-D deficiency would lead to reduced atherosclerotic burden in these mice. To address this objective, we carefully looked at the effect of SP-D on three most prominent atherosclerotic features; systemic inflammation, lipid profile change and endothelial function, which can independently and/or synergistically induce atherosclerosis.

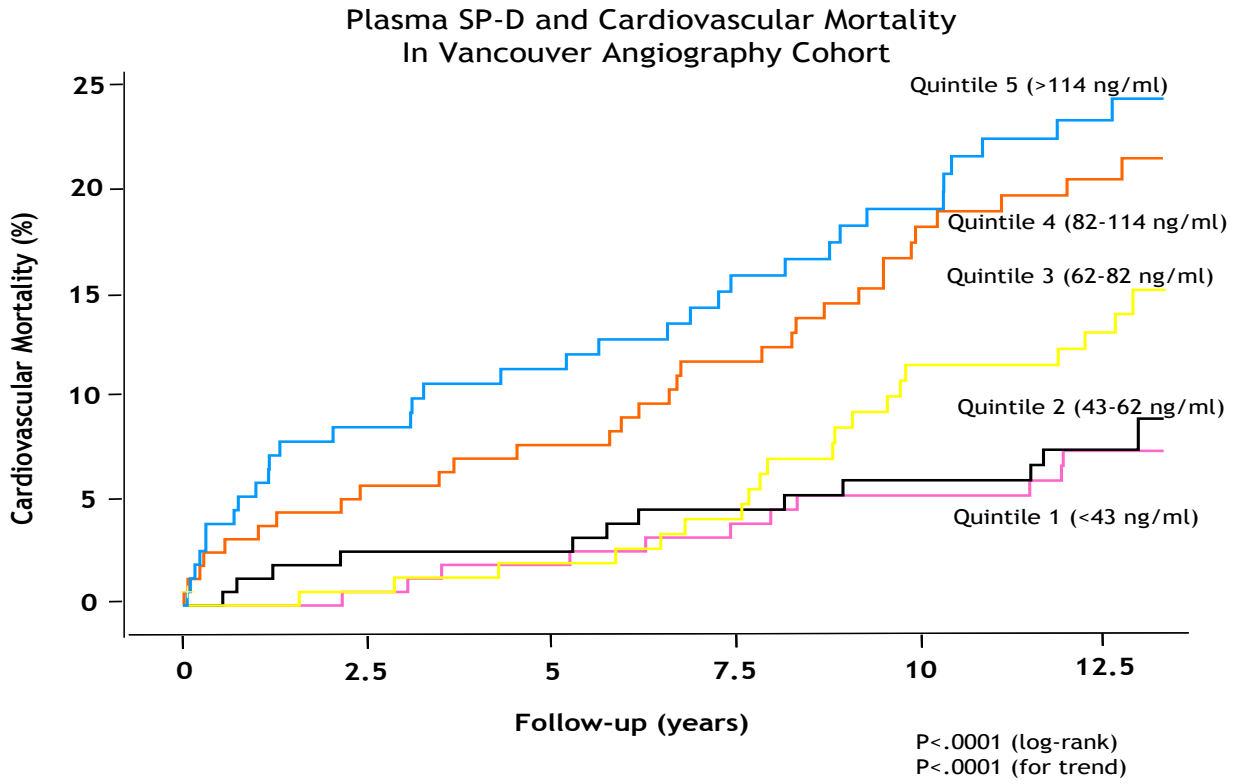


Figure 3. The Relationship of SP-D in Plasma and Cardiovascular Mortality. From quintile 1 to quintile 5, the level of SP-D increases and the higher the serum SP-D is found in patients, the higher mortality chance are due to cardiovascular diseases. Those in the highest quintile of plasma SP-D showed a 4.4 fold higher risk of CVD mortality than those in the lower quintile independent of age, sex and plasma lipid levels (Hill *et al.,* 2011 used with permission from EHJ).

1.5 Objective and Hypothesis

The main objective of this study was to elucidate the role of SP-D in the development of atherosclerosis, and we hypothesized that the deficiency of SP-D will lead to less atherosclerosis in ApoE deficient mice. Our specific aims were to determine the effects of SP-D deficiency on:

1. atherosclerotic plaque formation.
2. serum lipid profile.
3. systemic inflammation.
4. endothelial function of aorta.

Chapter 2: Methods

2.1 Generation of DKO Mice

We took homozygous ApoE-KO mice (from Jackson Laboratory) and crossbred them with SP-D-KO mice obtained from Dr. Hawgood's laboratory (UCSF). Both were on a C57BL/6J background. Breeding for 10 generations was done to ensure these mice had these same C57BL/6J genetic backgrounds as the wildtype (WT). Genotyping of the SP-D locus was performed by polymerase chain reaction (PCR) using mouse specific SP-D specific primers (mouse sense, 5′-CGTCTAGAGGTTGCCTTCTCC-3′, and antisense, 3′-GGCTCAGACCTGTATGTTGCCA-5′). Polymerase chain reaction (PCR) protocols were designed from Jackson Laboratories. All animal experiments were conducted according to the Guidelines for Animal Experiments of UBC.

ApoE-KO mice and DKO mice were housed for 20 weeks. For first 8 weeks, they were on a regular rodent chow diet, and for the remaining 12 weeks, they were on high fat diet (HFD). Neither ApoE-KO mice nor DKO mice showed any significant skin lesions while on a high fat diet and there were no specific healthy issues related to the genetic modification.

Table 1. Number of Mice Used in Atherosclerosis Experiment

Mice	Hematology	Lesion Area	Serum Lipid	Serum Cytokines
ApoE-KO	9	8	8	8
DKO	11	8	8	8
SP-D-KO	8	8	8	8
C57BL/6J	8	8	8	8

To study endothelial function, we used SP-D-KO mice and C57 mice. They were on normal chow diet and the ages of mice were from 8 to 10 weeks old.

Table 2. Number of Mice Used in Endothelial Function Experiment

Mice	PBS treatment	LPS treatment
C57BL/6J	8	4
SP-D-KO	6	7

2.2 Plaque Measurement

We measured morphologic and biochemical features of plaque including lipid content and distribution using quantitative histological methods that have been extensively used for atherosclerosis studies. Using ice cold PBS solution, the whole aortas were liberated from the surrounding fat tissues. The root of aorta were isolated and embedded in Tissue-Tek® O.C.T.™. The fixed tissues were then divided into 10 μm slices. Lipids in atherosclerotic lesions were quantified using oil-red-O stained sections prepared from formalin-fixed frozen O.C.T.™ tissue. The images of all sections were captured by a spot digital camera (Microspot, Nikon, Tokyo, Japan), and examined. The oil-red-O stain was used to identify the plaque, which is colored in red. The whole stained areas for aortic root were measured using imagescope Aperio software. Secondly, the atherosclerotic process in the whole aorta was sampled from aortic arch to iliac bifurcation. After surrounding excessive fat was removed, the whole aorta was dissected and pinned and stained with Sudan IV staining. The staining detected the plaque by staining lesion in red. The whole aortas were examined and pictures were taken at the light microscopic level using standardized morphometric techniques. The percentage of plaque detected in the whole

aorta was calculated by red stained area and measuring the red stained whole aorta area using the Image Pro Plus software.

2.3 WBC Counting

Blood was drawn in tubes containing 4mmol/L EDTA. Whole blood samples were obtained and cell counting was performed using hemocytometer, Cell-Dyn* 3700 (Abbott Diagnostics) (n= 9 ApoE-KO, 11 DKO, 8 SP-D-KO mice and 8 C57 mice). Neutrophil to lymphocyte ratio (NLR) was calculated based on the obtained hematology data.

2.4 Cytokine Assay

Following a 3 hour fast, blood samples were collected and serum obtained by centrifugation of blood samples at 8000 rpm for 5 minutes at 4 °C, separated and divided into aliquots, then stored frozen (-80 °C) until analyzed. We measured mouse cytokines by the Milliplex® Mouse cytokine kit from Milliopore (Billerica, MA, U.S.A) using mouse standards according to the manufacturer's guidelines. Serum was plated in 96-well plates 25ul/well and the concentration was determined by the fluorescent reporter signals that detects a biotinylated individual cytokine antibody. The cytokines used included IL-6, MCP-1, KC and TNF-alpha. The assay was performed in duplicate and the lower limit of detection limitation was 6.4pg/mL.

2.5 Lipid Profile Analysis

Serum total cholesterol, triglycerides, LDL-cholesterol and HDL-cholesterol were measured individually by colorimetric enzymatic methods (Wako Diagnostics, Richmond,

VA, USA) according to the manufacture's protocols. Spectrophotometer was used to measure the absorbance and the concentration was calculated as manufacture's protocols.

2.6 Endothelial Function

We evaluated the impact of SP-D on endothelial function via acute lung injury. Wild-type mice C57BL/6J and SP-D-KO on normal diet were divided into two groups: (1) exposed to LPS (n=6, n=8) and (2) exposed to PBS as a control treatment (n=7, n=4). The LPS used in mice was 2ug of LPS per each gram of mice body weight and it was instilled intratracheally, using a microsprayer. 24 hours later, arterial blood and abdominal aortas were collected. To determine the impact of lung inflammation on extrapulmonary blood vessels, we examined the endothelial function of the abdominal aorta, using wire myography which is a well established technique for evaluation of endothelial function. It assesses the change in the response to graded concentrations of a vasodilator. Vasodilatory response to acetylcholine (Ach) was determined to evaluate the endothelium-dependent vasorelaxation, while vasodilatory responses to sodium nitroprusside (SNP) were determined to evaluate endothelium-independent vasorelaxation.

2.7 Statistic Analysis

Data were analyzed using GraphPad Prism 5.00 (GraphPad Software, San Deigo, CA). Mean values for plaque quantification data, biochemical data and myography data (logEC50) were determined by unpaired two-tailed t-test for comparing two groups. One way ANOVA parameter (Kruskal-Wallis test with Dunn's post test) applied to compare the four groups of mice. For Kruskal-Wallis test, the pair showed significant difference with $p <$

0.05, was indicated with following numbers at the summary table (page 56). 1: C57 mice and SP-D-KO mice, 2: C57 mice and ApoE-KO mice, 3: C57 mice and DKO mice, 4: SP-D-KO mice and ApoE-KO mice, 5: SP-D-KO mice and DKO mice and 6: ApoE-KO mice and DKO mice. Experimental results are shown as means±SEM. All statistical tests with $p < 0.05$ were considered significant.

Chapter 3: Results

3.1 The Generation of DKO Mice and Their Body Weight

ApoE-KO mice and SP-D-KO mice were crossbred and genotyping using PCR protocol from Jackson Laboratories was performed to ensure both SP-D gene and ApoE gene were knocked out.

Table 3. The Weight of Mice at Age of 20 Weeks Old

	C57 Mice	SP-D-KO Mice	ApoE-KO Mice	DKO Mice	P value
Mice weight (g)	40.93±2.16	48.37±1.80	32.48±1.36	43.74±2.74	<0.0001 (1,2,4,5,6)

P value for Kruskal-Wallis test is shown with the number of each pair showing significance with $p < 0.05$. 1: C57 mice and SP-D-KO mice, 2: C57 mice and ApoE-KO mice, 3: C57 mice and DKO mice, 4: SP-D-KO mice and ApoE-KO mice, 5: SP-D-KO mice and DKO mice and 6: ApoE-KO mice and DKO mice.

3.2 The Effect of SP-D on Atherosclerotic Lesion Formation

We first examined the effects of SP-D deficiency on the development of atherosclerotic lesion. It was done by measuring the size of the aortic root lesion and the overall atherosclerotic plaque area in the whole aorta.

3.2. 1 The Atherosclerotic Plaque Size in Aortic Root.

Quantitative analysis of aortic roots revealed that C57 mice had plaque size of 13990 ± 6095 μm^2 and ApoE mice had 218689 ± 10756 μm^2 ($P<0.0001$). SP-D-KO mice had a plaque size of 3818 ± 706.8 μm^2. The difference between SP-D-KO mice and C57 mice

were also significant (p=0.0158). Compared to ApoE-KO mice, DKO mice had 15.5% less lesion size (184790 ± 11385 μm^2) in aortic roots (P=0.0482).

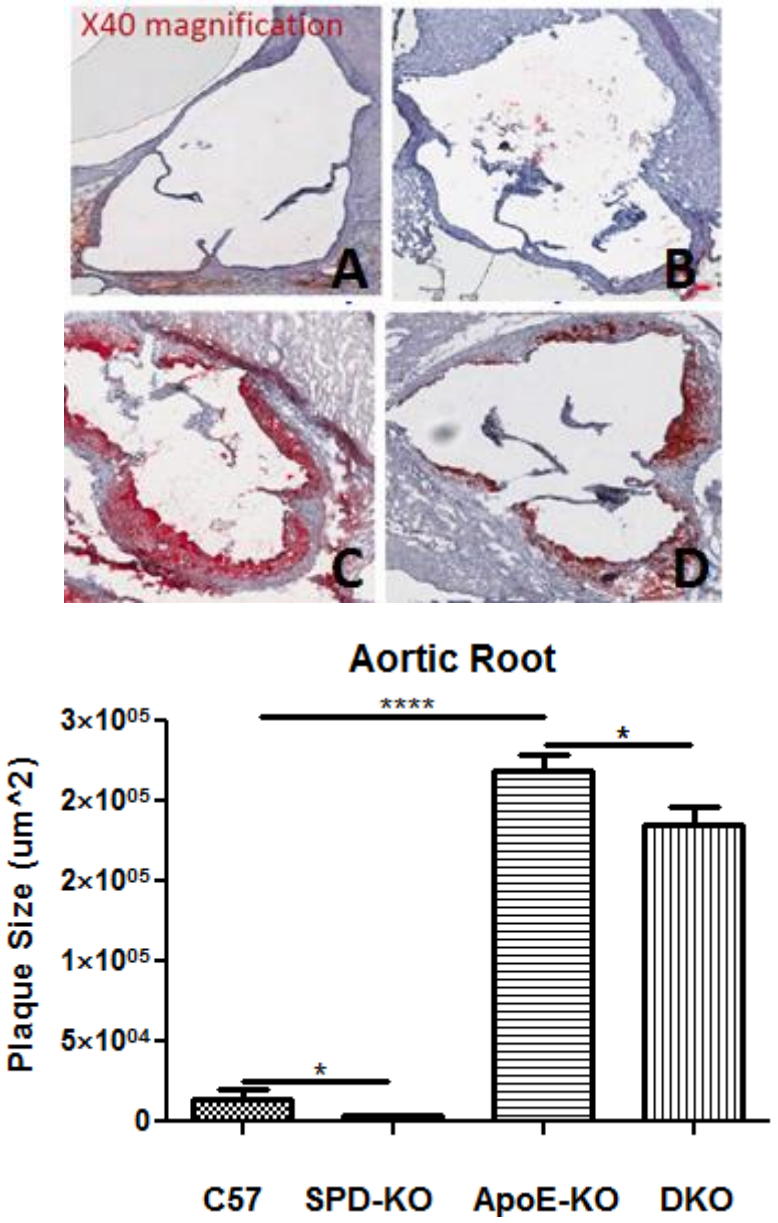


Figure 4. The Effect of SP-D Deficiency on Formation of Atherosclerotic Plaque in Aortic Root Area. The O.C.T. embedded frozen aortic root areas were sectioned at 10um in thickness and stained with oil-red-O, making lipid-laden areas appear red. Red stained areas were measured using Imagescope Aperio. A: C57 mice, B: SP-D-KO mice, C: ApoE-KO

mice and D: DKO mice. Unpaired two-tailed t-test was used. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001; mean±SEM of n=8 animals per group.

3.2.2 The Atherosclerotic Plaque Area in Whole Aorta Intima

Secondly we looked at the distribution of the plaque formation in the intima of the entire aorta. Quantitative analysis revealed that plaque coverage in C57 mice was 1.305 ± 0.3765 % of the entire aorta area and that in ApoE-KO mice was 14.72 ± 1.060% (P<0.0001). Plaque coverage in SP-D-KO mice was 1.197 ± 0.2662 %. The difference between SP-D-KO mice and C57 mice were not significant. Compared to ApoE-KO mice, double knock out (DKO) mice had 23% less atherosclerotic plaque area coverage (11.32 ± 1.138 ;P=0.0463).

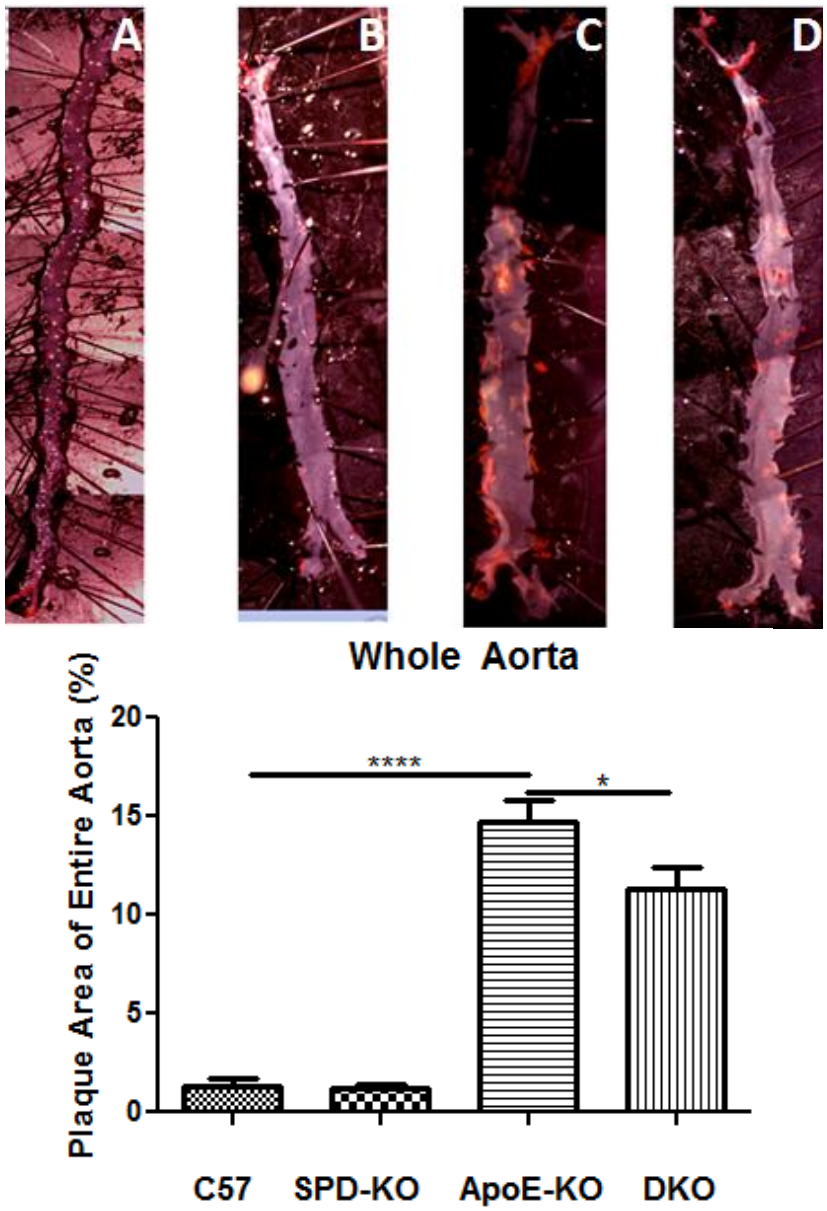


Figure 5. The Effect of SP-D Deficiency on Coverage of Atherosclerotic Plaque in Whole Aortic Intima. Aortas were prepared for en face analysis and stained with Sudan IV staining, making lipid-laden areas appear red. The coverage (%) of whole aorta was analyzed using Image-Pro software. A: C57 mice, B: SP-D-KO mice, C: ApoE-KO mice and D: DKO mice. Unpaired two-tailed t-test was used. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001; mean±SEM of n=8 animals per group.

3.3 The Effect of SP-D on Systemic Inflammation

Several studies have explored the relationship between systemic inflammation and cardiovascular mortality. Elevated levels of systemic inflammatory markers have been associated with increased risk of cardiovascular diseases. We thus examined the effects of SP-D on systemic inflammation by measuring circulating inflammatory cytokines and white blood cells (WBC).

3.3.1 The Effect of SP-D on Serum Inflammatory Cytokines

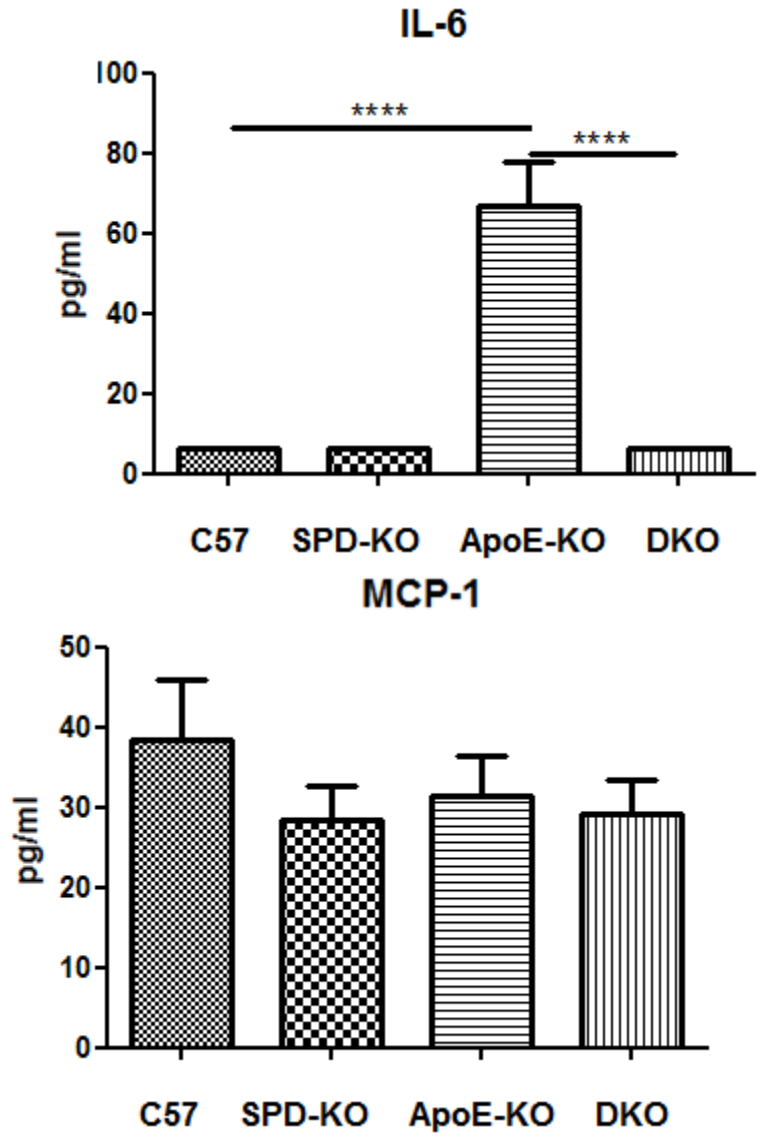
IL-6 promotes endothelial dysfunction, SMC proliferation and migration, and recruitment and activation of inflammatory cells thereby perpetuating vascular inflammation. As our experiments showed, WT mice C57 have serum IL-6 levels below the detection limit of the assay. On the other hand, IL-6 levels in ApoE-KO mice were significantly increased to 66.77 ± 11.27 pg/ml ($P<0.0001$). SP-D-KO mice did not show significant difference in IL-6 level compared to the C57 mice. IL-6 levels in DKO were similar to SP-D-KO mice and significantly lower than that in ApoE-KO mice ($P<0.0001$) (Fig 3.3.1).

MCP-1 promotes atherosclerosis by recruiting monocytes to the vessel wall and they are detected in atherosclerotic lesions. In these experiments, we did not observe any significant difference in MCP-1 level among the four groups of mice (Fig 3.3.1).

KC is able to specifically recruit neutrophils to local endothelium and mediate the TNF-alpha induced neutrophil activation and endothelial damage. In our study, ApoE-KO mice had dramatically increased KC level compared to WT C57 mice (8.2 folds and $P=0.0026$). SP-D-KO mice had significantly lower KC level compared to WT C57 mice, while

DKO mice also had decreased level of KC compared with ApoE-KO mice though the difference did not reach the significance (Fig 3.3.1).

TNF-alpha induces smooth muscle proliferation, activates vascular endothelia, and recruits monocytes into the endothelium. In these experiments, we did not observe significant difference in TNF-alpha level due to very low level detection compared to other cytokines.


IL-6
100
80
60
40
20
0
pg/ml
****
****
C57
SPD-KO
ApoE-KO
DKO
MCP-1
50
40
30
20
10
0
pg/ml
C57
SPD-KO
ApoE-KO
DKO

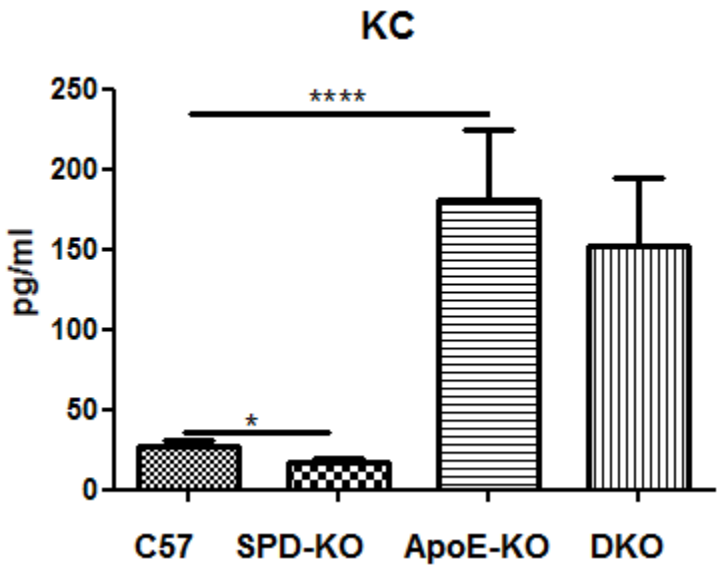


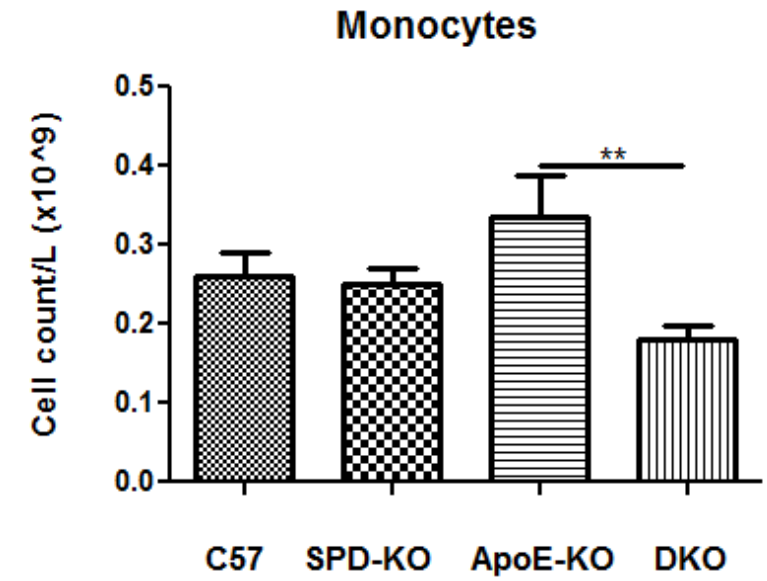
Figure 6. The Effect of SP-D Deficiency on Inflammatory Cytokines; IL-6, MCP-1 and KC in Serum. Serum of each mice group were collected and obtained by centrifugation at 8000 rpm for 5 minutes at °C, separated and divided into aliquots and then stored frozen at -80°C. Individual cytokine was measured by the Milliplex Mouse cytokine kit from Milliopore using mouse standards. The limitation of detection was the concentration below 6.4 pg/ml. Unpaired two-tailed t-test was used. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001; mean±SEM of n=8 animals per group.

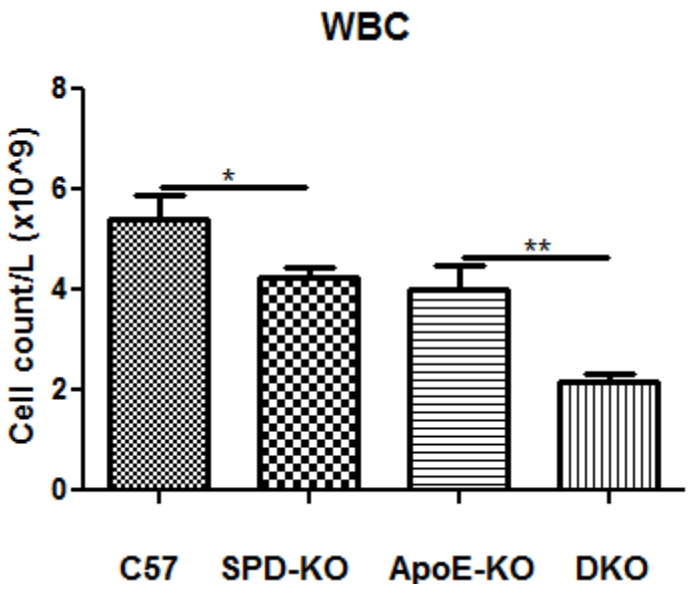
3.3.2 WBC Counts

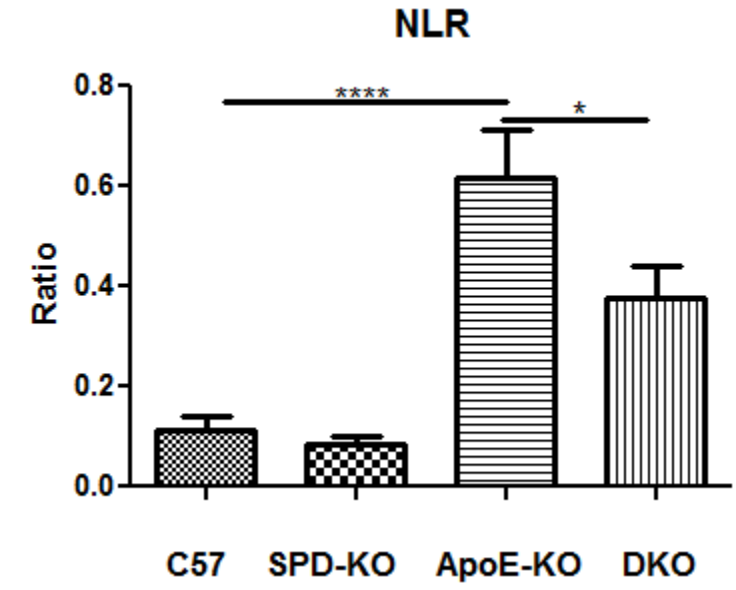
Many studies have shown that there is a correlation between leukocytosis and CVD. As shown in Fig 3.3.2, ApoE-KO mice did not show significant difference in whole blood WBC counts compared with WT C57 mice. Although SP-D-KO mice also had no significant effect on WBC counts compared to WT, DKO mice showed significantly lower WBC counts compared to ApoE-KO mice (P=0.0010).

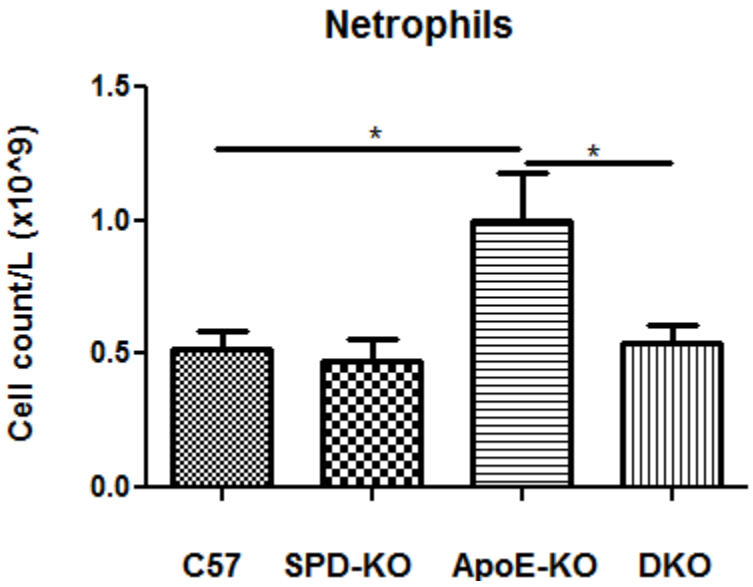
Furthermore, ApoE-KO mice showed higher monocyte counts compared to background C57 mice though the difference was not significant. Although SP-D-KO mice had no significant effect on monocyte count, the DKO mice showed significantly lower monocyte counts compared to ApoE-KO mice (P=0.0070) (Fig 3.3.2).

Additionally, ApoE-KO mice also showed higher neutrophil counts compared to WT C57 mice (P=0.0358). In DKO mice, neutrophil counts were completely normal (P=0.0331). Interestingly, ApoE-KO mice also showed significantly lower numbers of circulating lymphocytes compared to WT mice (from 5.161 ± 0.4295 x 10^9 cells/L to 1.968 ± 0.2358 x 10^9 cells/L, P< 0.0001). The lymphocyte count was lowest in DKO mice (1.438 ± 0.1622 10^9 cells/L). In ApoE-KO mice, the NLR was more than five fold higher compared to WT C57 mice (P=0.0064). This ratio was significantly reduced in DKO (P=0.0471) (Fig 3.3.2).


Monocytes
Cell count/L (x10^9)
0.5
0.4
0.3
0.2
0.1
0.0
**
C57
SPD-KO
ApoE-KO
DKO


WBC
Cell count/L (x10^9)
8
6
4
2
0
*
**
C57
SPD-KO
ApoE-KO
DKO


NLR
Ratio
0.8
0.6
0.4
0.2
0.0
****
*
C57
SPD-KO
ApoE-KO
DKO


Netrophils
Cell count/L (x10^9)
1.5
1.0
0.5
0.0
*
*
C57
SPD-KO
ApoE-KO
DKO

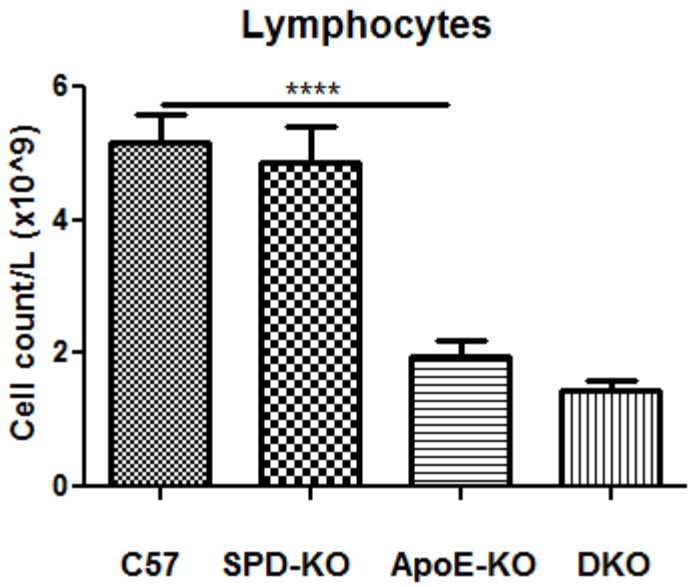
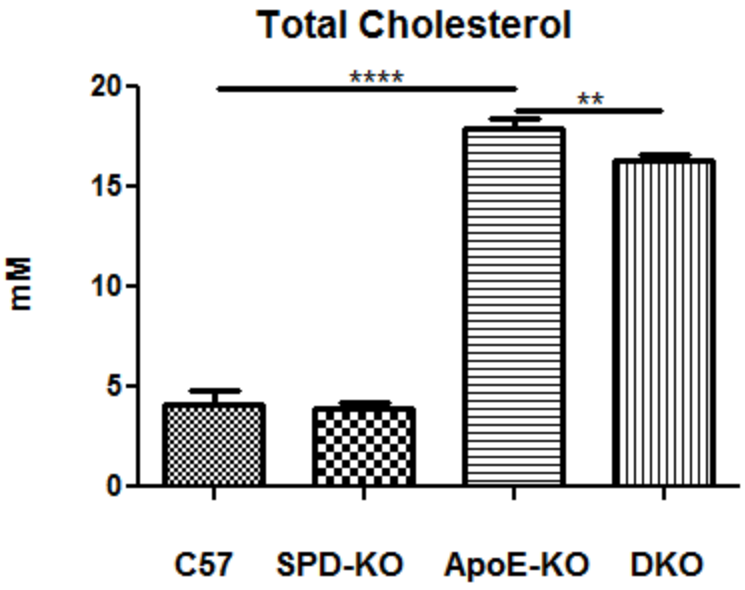
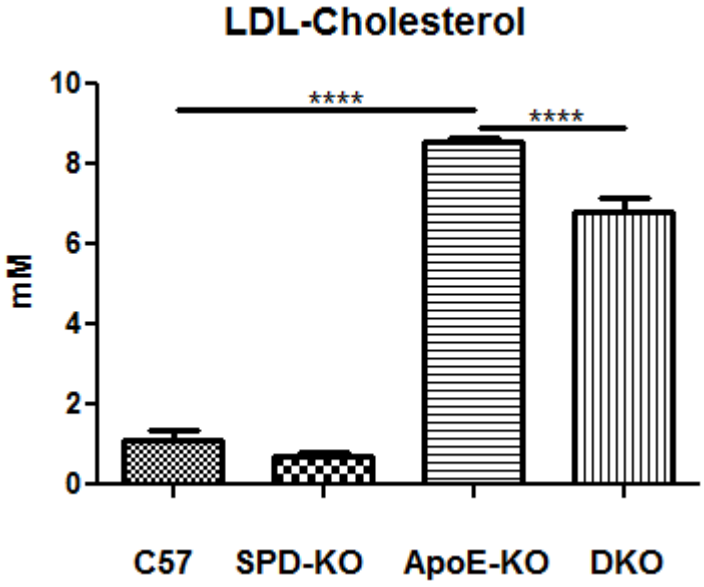


Figure 7. The Effects of SP-D on Monocyte Count, Leukocyte Count, Neutrophil to Lymphocyte Ratio (NLR), Neutrophil Counts and Lymphocyte Counts in Serum. Blood was drawn in tube containing 4mmol/L EDTA. Whole blood samples were obtained and WBC cell counting was performed using hemocytometer, Cell-Dyn* 3700 (Abbott Diagnostics). NLR was calculated based on the obtained WBC counting. Unpaired two-tailed t-test was used. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001; mean±SEM of n=9 for ApoE-KO, 11 for DKO and 8 for SP-D-KO and 8 for C57 animals.

3.4 The Effects of SP-D on Lipid Profile

Hyperlipidemia due to elevated LDL-cholesterol is an important risk factor for atherosclerosis while HDL-cholesterol is atheroprotective. Compared to WT C57 mice, ApoE-KO mice had significantly higher total cholesterol ($P < 0.0001$), LDL-cholesterol ($P < 0.0001$) and triglyceride levels ($P=0.0107$). SP-D-KO mice did not show significant changes in above measurements. However, DKO mice showed significantly lower total cholesterol ($P=0.0089$) and LDL-cholesterol ($P < 0.0001$) levels compared to ApoE-KO mice (Fig 3.4). ApoE-KO mice and DKO mice did not show significant difference in the level of HDL compared to WT mice, but SP-D-KO mice showed significantly higher levels of HDL compared to all the other groups ($P < 0.0001$) (Fig 3.4).


Total Cholesterol
****
**
20
15
10
5
0
mM
C57
SPD-KO
ApoE-KO
DKO


LDL-Cholesterol
****
****
10
8
6
4
2
0
mM
C57
SPD-KO
ApoE-KO
DKO

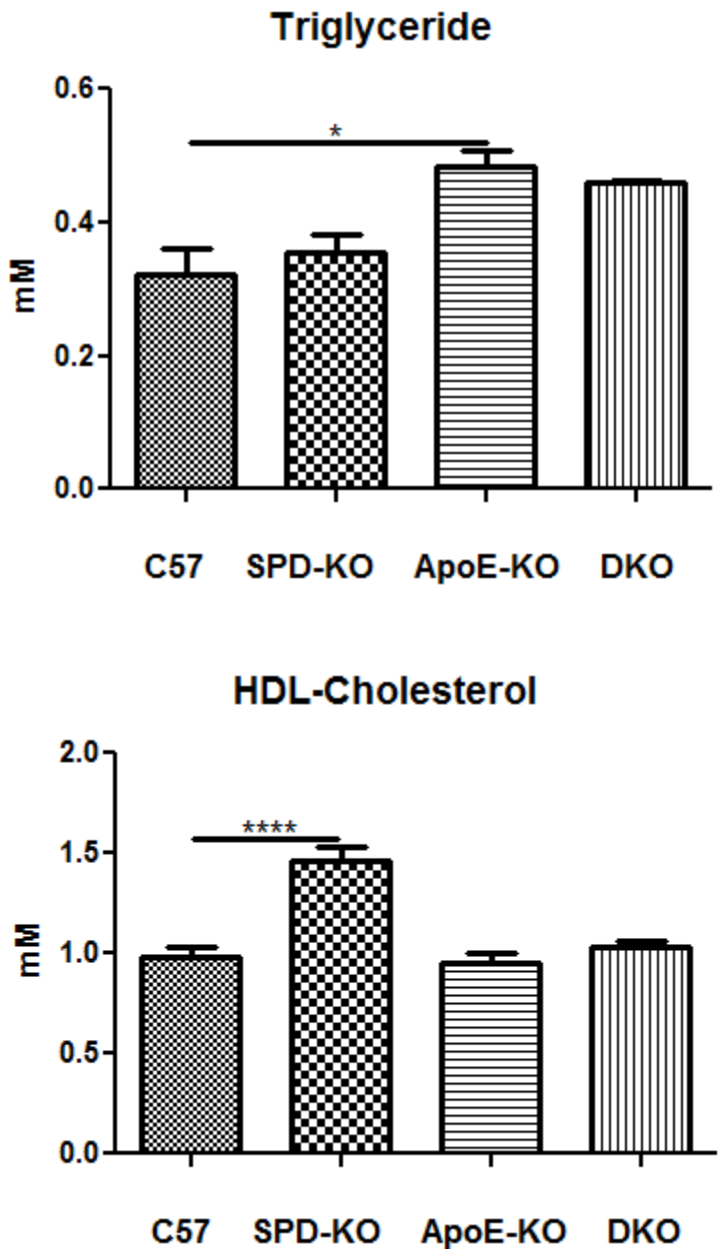


Figure 8. The Effect of SP-D on Endothelial Function, 24 Hours After Exposure to PBS. Prior to euthanization, all mice were fasted for three hours. Serum of each mice group were collected and obtained by centrifugation at 8000 rpm for 5 minutes at °C, separated and divided into aliquots and then stored frozen at -80°C. Serum total cholesterol, triglycerides, LDL-cholesterol and HDL-cholesterol were measured individually by colorimetric

enzymatic methods (Wako Diagnostics). Unpaired two-tailed t-test was used. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001; mean±SEM of n=8 animals per group.

3.5 The Effect of SP-D on Endothelial Function

Acute lung injury (ALI) disturbs the alveolar-capillary barrier and results in increased lung permeability with disturbed surfactant pools and the infiltration of activated neutrophils into the lungs and systemic inflammation (Abraham *et al.,* 2003; Reutershan *et al.,* 2005). Systemic inflammation is associated with endothelial dysfunction of the vessels, reduced cardiac output, and increased risks of cardiovascular events (Charlmers *et al.*, 2008; Spodick *et al.,* 1984). LPS is a principal component of the outer membrane of Gram-negative bacteria that activates macrophages and induces a variety of inflammatory mediators, including TNF-α and interferon-1(Tominaga *et al.*, 1999). Instillation of LPS induces acute lung inflammation mimicking acute lung injury (Mei *et al.*, 2007). Previous study from our lab found that intratracheal LPS instillation causes egress of SP-D from lungs into systemic circulation, which in turn is accompanied by systemic inflammation and endothelial dysfunction, a process that is in part mediated by IL-6. Based on this observation, we wanted to examine whether SP-D affects endothelial function.

Endothelial function as indicated by vasodilatation related to Ach showed no significant difference with PBS instillation (control treatment). The LPS instillation induced a right shift of the response curve, indicating impairment of endothelial function. SP-D-KO mice exposed to LPS demonstrated normal vasodilatory response to Ach, indicating normal endothelial function and resistance to LPS (P=0.0034) (Fig 3.5.2).

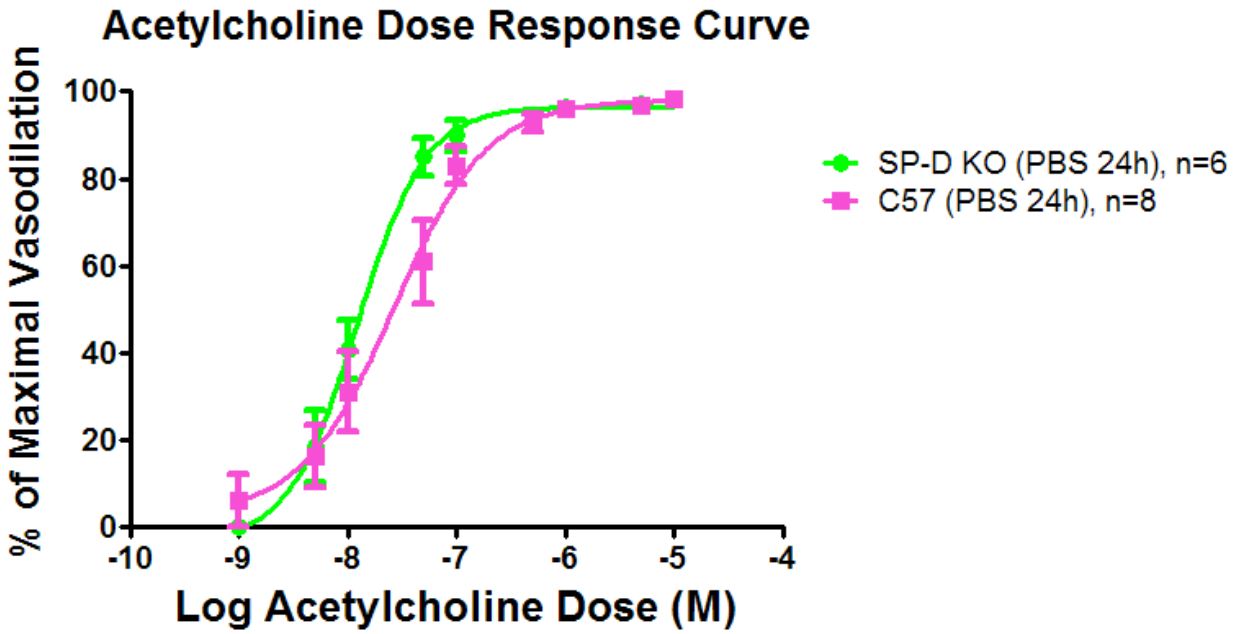


Figure 9. The Effect of SP-D on Endothelial Function, 24 Hours After Exposure to PBS. Each mouse was exposed to PBS (control) intratracheally using microsprayer. 24 after the PBS instillation, the mice were euthanized and abdominal aorta size of 2mm was taken from the whole aorta of each mice. These aortas were treated with endothelial dependent vasodilator, Ach to measure maximal vasodilatation % to gradient acetylcholine, to show the endothelial function. Unpaired two-tailed t-test was used. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001; mean±SEM of n=8 for C57 mice and 6 for SP-D-KO mice.

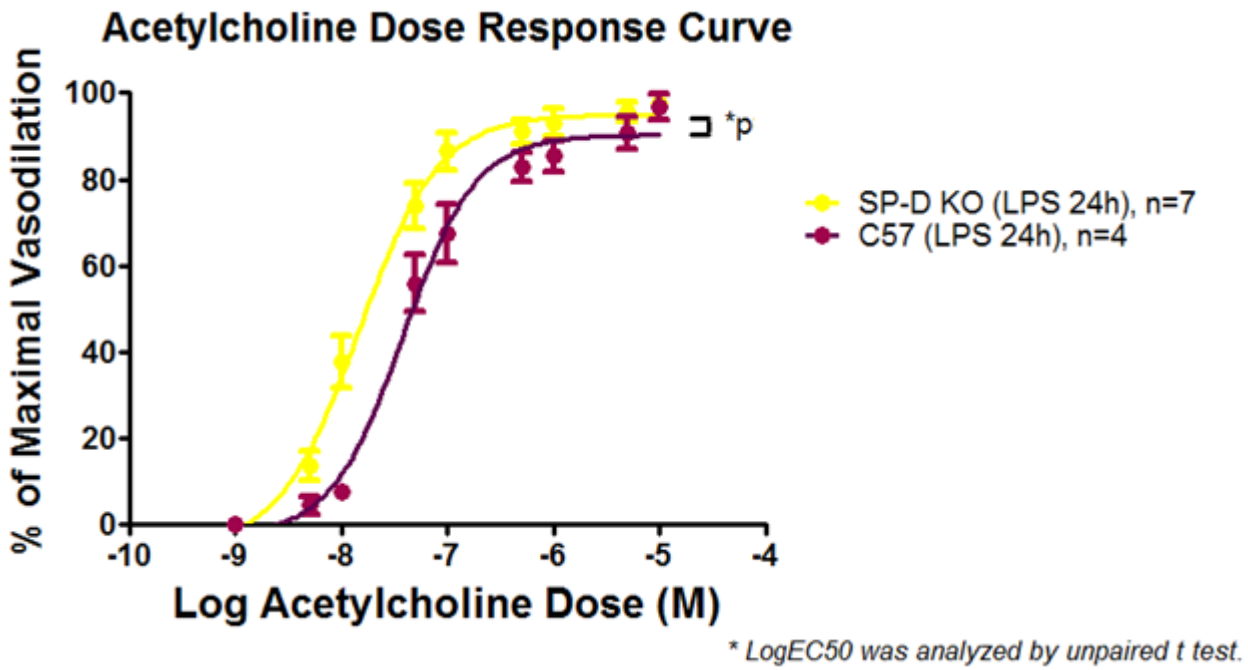


Figure 10. The Effect of SP-D on Endothelial Function, 24 Hours After Exposure to LPS. Each mouse was exposed to LPS intratracheally (2ug /gram of weight) using microsprayer. 24 after the LPS instillation, the mice were euthanized and abdominal aorta size of 2mm was taken from the whole aorta of each mice. These aortas were treated with endothelial dependent vasodilator, Ach to induce maximal vasodillation % to gradient acetylcholine, to show the endothelial function. Unpaired two-tailed t-test was used. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001; mean±SEM of n=4 for C57 mice and 7 for SP-D-KO mice.

Chapter 4: Summary and Discussion

This study was to characterize the atherogenic effects of SP-D. The major results from the experiments on our mice model were that: compared with ApoE gene knockout alone, additional SP-D gene knockout decreased the size of atherosclerotic lesions in both the aortic root (16%) and entire aorta (24%), reduced the elevated circulating IL-6, total WBC count and NLR, and reduced the cholesterol burden induced by ApoE gene knockout (Table 4.). Additionally, SP-D deficiency made aortas resistant to endothelial dysfunction caused by LPS exposure.

To evaluate the role of SP-D in plaque pathogenesis, generation of DKO mice by cross breeding SP-D-KO mice with ApoE-KO mice was critical because WT mice are resistant to atherosclerosis. We chose ApoE-KO mice as an atherosclerosis model for the reasons below: 1) it can develop the aortic lesion mimicking human atherosclerosis, 2) the mice are grossly healthy and have comparable life span as WT mice for us to study the initiation and progression of atherosclerosis over a manageable time scale, 3) The plaques are developed in the aorta and are easy to quantify, and 4) ApoE-KO mice demonstrate abnormal cholesterol physiology and endothelial function. It is important, however, to recognize that there are some differences in lipoprotein metabolism between human and mice. In particular, mice lack the cholesteryl ester transfer protein, an enzyme that transfers cholesterol ester from HDL to VLDL and LDL. This may explain the finding that there is no significant change in HDL in ApoE-KO mice compared with WT mice (Kako *et al.,* 2002).

There are several published methods for plaque quantification. Among these, we chose 2 different methods to ensure robustness of our data. In both aortic root and whole aorta intimal analysis, DKO mice had significantly less plaque burden compared with ApoE-KO mice. Serial sectioning of the entire aorta to calculate the plaque volume may have improved the accuracy of the measurement. Measuring atherosclerosis biochemically by determining cholesterol ester content of aorta may have provided additional information.

To examine the effects of SP-D on lipid homeostasis, we measured individual lipid composition using colorimetric enzymatic method. We found significantly reduced LDL-cholesterol and total cholesterol in the absence of SP-D in ApoE-KO mice. HDL-cholesterol and triglyceride levels showed no significant differences. The kit we used contained phosphotungstate and magnesium salt which can precipitate and remove nonspecific lipid fractions and enable us to measure specific lipid profile fractions. Remaining supernatant was tested for certain cholesterol particles by enzymatic colorimetric procedure. This method does not require centrifugation of blood samples which can result in various values based on techniques and machines. Alternative method such as high-performance liquid chromatography (HPLC) may have provided more accurate and comprehensive lipid profiling.

To explore the effect of SP-D on systemic inflammation, we measured serum levels of atherosclerosis related cytokines and WBC among different groups of mice. We found that ApoE-KO mice had significantly elevated IL-6 and KC levels. DKO blocked the elevation of IL-6 and partially inhibited elevation of KC (although differences were not significant). For MCP-1, ApoE-KO mice had elevated MCP-1 and it was partially blocked by DKO (although it was not significant). For TNF-alpha, there were no significant changes among

different groups. However, the overall level of TNF-alpha was extremely low compared to other chemokines. It might be due to the short half-life of this molecule (Wouters *et al.,* 2009). Other than the effect on serum chemokines, SP-D also influenced circulating WBC. In our study, we first confirmed what other studies demonstrated in that ApoE-KO mice had elevated WBC, monocytes, neutrophils and NLR. We also showed these effects were attenuated by SP-D deficiency. However, the total leukocyte counts did not show significant change in ApoE-KO mice compared to WT C57 mice. It could be explained by the changes in lymphocytes, the major population of the WBC in the blood. Interestingly, ApoE-KO mice also showed significant lower circulating lymphocyte counts compared to background mice (from from $5.161 \pm 0.4295 \times 10^9$ cells/L to $1.968 \pm 0.2358 \times 10^9$ cells/L, $P < 0.0001$), and DKO mice additional decreased the lymphocyte number. ApoE has been shown to modulate immune system including the proliferation and function of lymphocytes (Laskowitz, 2000). But the detailed mechanisms including the roles of SP-D in these processes need to be further elucidated.

Finally, to test the effect of SP-D on endothelial function, we induced acute lung inflammation by instilling LPS. The dosage we applied (2ug /gram of weight) was determined from previous study and it was confirmed that SP-D level in blood increases at this level of LPS. We showed that LPS significantly induced a right shift in the Ach response curve which indicates endothelial dysfunction. The SP-D-KO mice restored the function curve to the baseline. This result suggested that SP-D mediates the endothelial dysfunction induced by LPS instillation in systemic inflammation.

Table 4. The Summary Data on Atherosclerotic Size, Inflammatory Markers, WBCs and Lipids among Mice Groups.

	C57 mice	SP-D-KO mice	ApoE-KO mice	DKO mice	P value
Aortic Root Size (μm^2)	13,990±6,095	3,818±706.8	218,689±10,756	184,790±11,385	<0.0001 (2,4,5)
Whole Aorta Coverage (%)	1.305±0.377	1.197±0.266	14.72±1.060	11.32±1.138	<0.0001 (2,3,4,5,6)
IL- 6 (pg/ml)	0.0±0.0	0.0±0.0	66.77±11.27	0.0±0.0	<0.0001 (2,4,6)
MCP-1 (pg/ml)	38.43±7.546	28.51 ± 4.222	31.35±5.063	29.06±4.390	0.5619
KC (pg/ml)	15.65±2.953	21.62 ± 4.569	180.8±43.23	151.5±42.60	<0.0001 (2,3,4,5)
WBC ($x10^9$ cells/L)	5.422±0.464	4.227±0.234	3.661±0.3742	1.946±0.2480	<0.0001 (1,2,3,5,6)
Monocytes ($x10^9$ cells/L)	0.2599±0.03077	0.1795±0.01959	0.3362±0.05221	0.1795±0.01959	0.0225 (6)
NLR	0.1111±0.02895	0.08569±0.01532	0.6149±0.09681	0.3748±0.06438	0.0003 (2,4,5)
Neutrophils ($x10^9$ cells/L)	0.5200±0.06897	0.4740 ± 0.08341	1.000±0.1813	0.5380±0.07333	0.0748
Lymphocytes ($x10^9$ cells/L)	5.161±0.4295	4.854 ± 0.5372	1.968±0.2358	1.438±0.1622	<0.0001 (2,4,5)
Total cholesterol (mM)	4.138±0.664	3.896 ± 0.319	17.91±0.465	16.31±0.328	<0.0001 (2,3,4,5)
LDL-cholesterol (mM)	1.091±0.2434	0.7205±0.0751	8.566±0.0972	6.810±0.342	<0.0001 (2,3,4,5)

	C57 mice	SP-D-KO mice	ApoE-KO mice	DKO mice	P value
Triglyceride (mM)	0.3534±0.02684	0.3223±0.03865	0.4840±0.02255	0.4588±0.004517	<0.0001 (2,3,4,5)
HDL-cholesterol (mM)	0.9813±0.04751	1.465±0.06462	0.9528±0.04615	1.029±0.03731	< 0.0001 (1,4,5)

P value for Kruskal-Wallis test is shown with the number of each pair showing significance with $p < 0.05$. 1: C57 mice and SP-D-KO mice, 2: C57 mice and ApoE-KO mice, 3: C57 mice and DKO mice, 4: SP-D-KO mice and ApoE-KO mice, 5: SP-D-KO mice and DKO mice and 6: ApoE-KO mice and DKO mice.

Chapter 5: Conclusions

The data in the present study provides novel evidence that SP-D contributes causally to the formation of atherosclerosis. The exact mechanism by which this occurs remains not fully understood. One intriguing possibility is that it does so by amplifying systemic inflammation; another is that it perturbs lipid homeostasis. To fully understand how SP-D regulates systemic inflammation and lipid metabolism, further studies with more detailed analyses of lipid and cytokines will be needed. Notwithstanding this limitation, our study provides a plausible pathway to link lung disease with atherosclerosis, which has been well described epidemiologically but its mechanisms are unknown. Because SP-D is largely expressed in the lungs and spills into the systemic circulation, we speculate that persistent systemic over-expression of SP-D in diseases such as chronic obstructive pulmonary disease (COPD) contributes to the increased incidence of ischemic heart disease in these patients. Thus, SP-D presents an interesting and novel target for biomarker and therapeutic discovery in patients with chronic lung disease.

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