THE ROLE OF INFLAMMATION IN ATHEROSCLEROSIS

By

Fatma Saaoud

Bachelor of Medicine and Surgery
Zawia University, 2005

Master of Science
University of South Carolina, 2012

Submitted in Partial Fulfillment of the Requirements
For the Degree of Doctor of Philosophy in
Biomedical Science
School of Medicine
University of South Carolina

2017

Accepted by:
Daping Fan, Major Professor
Susan Lessner, Committee Member
Holly LaVoie, Committee Member
Angela Murphy, Committee Member
Saurabh Chatterjee, Committee Member
Cheryl L. Addy, Vice Provost and Dean of the Graduate School
Dedication

To the best people I have ever had in my life, my parents, Elmokhtar Saaoud and Salma Saaoud, for their endless love, support, and encouragements throughout my life pushing me always toward success.

To my dear husband, Ismail Ben Issa, I give my deepest expression of love and appreciation for your support, encouragement, and the sacrifice you made during this graduate program. Thank you for your practical and emotional support.

To my precious kids, Mohammed, Abdalmuizz, Danah, and Maryam who have been affected in every possible way by this quest. My love for you all can never be quantified.
Acknowledgements

First and for most, I would like to thank my advisor, Dr. Daping Fan, for his admirable guidance, excellent advice, and patience over the past several years. I am very grateful to him for providing me such opportunity to work in his laboratory and great training that is valuable to my future development. I would like to thank all current and past members of Dr. Fan’s lab, in particularly Xuemei Jia and Junfeng Wang for their invaluable training and assistance since the first day I joined the group. I would like to thank Yuzhen Wang for her help with the histological analysis. I would like to thank my committee members Dr. Susan Lessner, Dr. Angela Murphy, Dr. Holly LaVoie, and Dr. Saurabh Chatterjee for serving on my dissertation committee and for their comments, help and advice.

Finally, my family is the most important thing in my life. My amazing husband, Ismail, for his understanding, patience, and encouragement, especially during times when nothing seemed to work right. This work would have been impossible without his love and support. My greatest thanks go to my lovely kids, for those warm and emotional smiles and hugs on days when I felt so disappointed. To my friend Marwa Belhaj. I'm so grateful to have you as a friend. There are many people through time and space that have been important to me, and I have not forgotten you. Thank you all.
Abstract

Atherosclerosis is both a chronic inflammatory disease and lipid deposition disorder characterized by accumulation of lipids, fibrous tissue, and inflammatory cells in the arterial wall. Thus, investigating the role of inflammation and the immune system in the progression of atherosclerosis may help in the development of novel therapeutics for atherosclerotic disease. Current atherosclerosis therapy is aimed at lipid targets and focused primarily on reducing plasma cholesterol levels. Clinical and experimental data support the critical role of inflammation in atherosclerosis and suggest that reducing inflammation even without affecting lipid levels may reduce the event rate of cardiovascular disease. Yet, no pure anti-inflammatory drugs are used to treat patients with atherosclerotic diseases. Recently, the Canakinumab Anti-inflammatory Thrombosis Outcomes Study (CANTOS) provided evidence that targeting inflammation by inhibiting IL1-β reduced the rate of cardiovascular events, providing support for the inflammation hypothesis of atherosclerosis. In this study, we aim to test the inflammation hypothesis using various immunomodulatory approaches. First, we measured the expression of inflammation markers in human atherosclerotic carotid arteries. We demonstrated that markers of inflammation were significantly increased in plaques compared to normal media and those markers positively correlate with miR155, a pro-inflammatory microRNA. We observed that miR155 was up-regulated in the
calcified media occurred concomitant with osteogenic genes; and cell culture and mouse studies further suggested a causal role of miR155 in vascular calcification. In the second study we observed that bone marrow tristetraprolin (TTP) deficiency in LDLR<sup>−/−</sup> mice significantly increased systemic inflammation. Unexpectedly, serum lipid levels and hepatic steatosis were dramatically reduced, likely due to reduced lipid production in the liver by downregulating SREBF1. Increased inflammation and reduced serum lipid offset each other and result in unchanged atherosclerosis. This model may provide an opportunity to examine the relationship between inflammation and lipoprotein metabolism. We also investigated the effect of macrophage-specific GP96 deficiency on atherosclerosis to determine the role of toll like receptors (TLRs) during atherogenesis. Our results show that macrophage-specific GP96 deficiency did not change inflammatory status, serum lipid, and atherosclerosis in LDLR<sup>−/−</sup> mice. Sparstolonin (SsnB), a selective TLR2/TLR4 inhibitor, is known to have anti-inflammatory effects. In this study, we injected mice with super-low dose LPS or LPS plus SsnB. Super-low dose LPS increased serum chemo-attractants, while SsnB reduced these chemokines. However, super-low dose LPS and SsnB administration did not affect atherosclerotic lesions. Atherosclerosis is a complex, multifactorial process, and the interaction between inflammation and atherogenesis is complicated as indicated by our results. Since CANTOS shows that targeting inflammation reduced the rate of cardiovascular events, it opens the field to further examine the inflammation hypothesis of atherosclerosis and to explore novel therapeutic avenues by targeting inflammation.
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Chapter I

Introduction

1.1 Atherosclerotic cardiovascular diseases

Cardiovascular diseases (CVDs), including myocardial infarction (heart attack) and angina pectoris (chest pain), represent the leading causes of mortality and long-term morbidity worldwide\(^1\). The incidence of CVDs continues to rise and is projected to remain as the number one cause of death globally by 2020\(^2\). Atherosclerosis is by far the main underlying pathological process of coronary artery disease, carotid artery disease, and peripheral arterial disease — which are among the most prevalent CVDs\(^3\).

The term 'atherosclerosis' comes from Greek word "athero" meaning *gruel*, and *sclerosis* means *hard* — which is hardening with loss of elasticity leading to narrowing of the arteries. Atherosclerosis is both a lipid deposition disorder and a chronic inflammatory disease, characterized by formation of atherosclerotic plaque in the arterial wall\(^4,5\). It mainly affects medium-sized and large arteries such as: the aorta, coronary arteries, carotid arteries, and renal arteries, in focal areas — particularly regions in which laminar flow is disturbed (e.g. arterial branch points and bifurcations)\(^6,7\). The disease begins early in life and goes undetected until thrombotic complications, such as acute coronary syndromes and stroke occur\(^8\). There are numerous genetic and environmental risk factors associated with atherosclerotic diseases. Most of the environmental risk factors are modifiable and
reversible, whereas the genetic factors are not. Clinically significant lesions are usually associated with hypercholesterolemia, smoking, diabetes mellitus, hypertension, obesity, physical inactivity, family history, and high-fat diet (HFD)\textsuperscript{8-9}. It has been shown that hypercholesterolemia alone is sufficient to drive the development of atherosclerosis. However, the presence of other risk factors appears to accelerate disease progression, driven by the atherogenic lipoproteins (i.e. low-density lipoprotein; LDL)\textsuperscript{10}.

1.2 Structure of blood vessel wall

The normal wall of blood vessels consists of endothelial cells, vascular smooth muscle cells (VSMCs), and extracellular matrix (ECM) arranged in three different layers (from innermost to the outermost): the intima, media, and adventitia\textsuperscript{11}. The tunica intima is composed of a single monolayer of simple squamous cells known as endothelial cells. It lies on the internal elastic lamina, a layer of elastic fibers, which defines the outermost boundary of the intimal space and separates the intima from the media. The endothelial layer represents the interface between circulating blood flow and the vascular wall — providing a smooth lining for blood vessels\textsuperscript{12}. The endothelial cells have a significant role in regulation of vascular permeability, regulation of immune responses, modulation of blood flow and vascular resistance, platelet aggregation, and thrombosis prevention. These are achieved by releasing anti-platelet, anti-coagulant, anti-thrombogenic, and fibrinolytic factors in addition to the production of the potent vasodilator nitric oxide (NO)\textsuperscript{13}. Besides the vasodilators, endothelial cells also produce some vasoconstrictor molecules, such as endothelin and angiotensin-II,
which can also promote proliferation of smooth muscle cells and thereby contribute to plaque formation\textsuperscript{14,15}. Therefore, the ability of the endothelial cell layer to repair itself and to maintain functional and structural integrity, is key to the prevention of atherosclerosis\textsuperscript{16,17}.

The middle layer of the arterial wall is the tunica media, which is composed primarily of VSMCs surrounded by a network of ECM proteins including elastic fibers, collagen, and proteoglycans. This layer provides strength and elasticity to the blood vessels so they can stretch or expand without tearing\textsuperscript{18}. The outermost layer is the tunica adventitia, which is primarily composed of irregularly arranged collagen bundles, scattered fibroblasts, a few elastic fibers, and small blood vessels called vasa vasorum. Vasa vasorum provides oxygen and nutrients to outer layers of the vascular wall. The elastic collagen fibers allow arteries to stretch to prevent over-expansion due to higher pressures in these blood vessels\textsuperscript{19-20}. Understanding the structure and function relationship of each layer in the arterial wall provides insight into the pathophysiology of arterial wall diseases, such as atherosclerosis, and allows for better predictions for prevention and treatment strategies.

1.3 Pathogenesis of atherosclerosis

Atherosclerosis is a complex and multifactorial disease characterized by systemic and vascular wall inflammation and activation of immune pathways, dyslipidemia, endothelial dysfunction, foam cell formation, VSMC activation, platelet activation, and thrombosis\textsuperscript{21,22}. A number of hypotheses have been proposed to describe the pathogenesis of atherosclerosis including the: response
to injury hypothesis\textsuperscript{23}, response to retention hypothesis\textsuperscript{24}, LDL-oxidation hypothesis\textsuperscript{25}, lipid hypothesis\textsuperscript{26}, and inflammation hypothesis\textsuperscript{27}. A better understanding of the pathogenesis of atherosclerosis will aid in formulating preventive and therapeutic strategies to reduce the mortality rate of cardiovascular events.

1.3.1 Response to injury hypothesis

The response to injury hypothesis suggests that atherosclerosis is initiated with injury-induced damage and dysfunction of the endothelial cells lining the intima. This damage caused by local disturbances of blood flow at branch points, along with systemic risk factors, perpetuates a series of events that culminate in the development of atherosclerotic plaque. Endothelial dysfunction is the initial step that allows entry and retention of cholesterol-containing LDL particles and inflammatory cells (i.e. monocytes, T lymphocytes) into the sub-endothelial spaces, altering normal homeostasis\textsuperscript{28,29,30}. Under normal homeostatic conditions, the white blood cells do not adhere to the endothelial cells lining the intima. However, under atherogenic conditions, the damaged endothelial cells express surface adhesion molecules such as vascular adhesion molecule-1 (VCAM-1), leading to adhesion of circulating blood monocytes to the endothelial surfaces. Those monocytes ultimately migrate to the sub-endothelial space. When acting together with imbalance between vasoconstriction and vasodilation, these processes promote increased vascular permeability and platelet aggregation\textsuperscript{31}. Furthermore, endothelial dysfunction is associated with decreased production of NO and increased cellular permeability to lipoproteins, each thought to be a key event in the development and progression of atherosclerosis\textsuperscript{32}. 
1.3.2 Response to retention hypothesis

The response to retention hypothesis of atherosclerosis was initially proposed in 1995\textsuperscript{24}. Since then there has been several line of experimental evidence supporting this hypothesis. The response to retention hypothesis suggests that retention of cholesterol-rich, atherogenic lipoproteins within the sub-endothelial space is the key initiating event in early atherosclerosis. Once retained, these lipoproteins increased susceptibility for modification/oxidation, and subsequently provoke the inflammatory response with increasing smooth muscle cell migration and phenotype switching as well as infiltration of inflammatory cells including macrophages\textsuperscript{24,30}.

1.3.3 LDL oxidation hypothesis

The LDL oxidation hypothesis suggests that oxidative modification of lipoproteins, in particular LDL, within the arterial wall by reactive oxygen species (ROS) plays an important role in the pathogenesis of atherosclerosis\textsuperscript{25}. Monocytes/macrophages express scavenger receptors that engulf oxidized oxLDL in an uncontrolled manner. In turn, uptake of oxLDL leads to accumulation of lipids and lipid-laden foam cells to form a fatty streaks — an early feature of atherosclerotic plaque formation\textsuperscript{25,33,34}. Experimental data indicate that oxLDL within the arterial wall promotes the development of atherogenesis\textsuperscript{25}. Additionally, inhibiting the oxidation of LDL could decrease or prevent atherosclerosis development. Previous studies have shown the efficacy of anti-oxidant treatment, such as probucol and vitamin E, which significantly reduced the severity of atherosclerosis in different atherogenic animal models. These studies supported
the oxidative modification hypothesis\textsuperscript{35}. Furthermore, targeting some proteins involved in LDL oxidation and uptake of oxLDL (e.g. 12/15 lipoxygenase and scavenger receptors (SRA and SRB)) in various atherogenic murine models significantly attenuated the severity of atherosclerosis\textsuperscript{36–38}.

1.3.4 Lipid hypothesis

The lipid hypothesis of atherosclerosis states that both lesion initiation and progression of atherosclerosis is primarily associated with hyperlipidemia\textsuperscript{26}. In the last decade, considerable advances in the understanding of cardiovascular risk factors have been established, and dyslipidemia has been shown as one of the most powerful risk factors. Controlling blood cholesterol is efficient at reducing cardiovascular risk and an important therapeutic decision, as the rate of CVDs were significantly reduced with decreased blood cholesterol\textsuperscript{39}. The link between hyperlipidemia and atherosclerosis predominate, until the 1970s, based on strong experimental and clinical relationships between hypercholesterolemia and severity of atherosclerotic lesions. This was further supported by the fact that statins, lipid lowering agents, were able to significantly reduce atherosclerotic diseases\textsuperscript{40}.

The first description of an atherosclerotic lesion was recorded in 1908 when Alexander I. Ignatowski, an experimental pathologist, fed rabbits cholesterol for 17 weeks followed by chow diet for 14 weeks. These rabbits developed pronounced aortic atherosclerosis\textsuperscript{41}. In 1913, Anitschkow and colleagues proposed the fundamental role of cholesterol in the pathogenesis of atherosclerosis. They fed rabbits with a high-cholesterol diet and found that the blood cholesterol levels were
significantly increased, and indeed, within weeks those rabbits displayed vascular atherosclerotic lesions similar to those of human atherosclerosis.\textsuperscript{42} 

In order to examine the lipid hypothesis of atherosclerosis induced by cholesterol, Clarkson and Newburgh (1926) fed rabbits a normal diet with various cholesterol doses for 47-87 days. They discovered atherosclerosis in rabbits fed high cholesterol doses\textsuperscript{43}. These observations strongly supported the theory that cholesterol was the main driving force in the development of atherosclerotic CVDs\textsuperscript{44}.

In the early stages of atherosclerosis, plasma LDL crosses the endothelial barrier and enters the intima. Once accumulated in the sub-endothelial arterial intima, LDL undergoes oxidative modification via the reactive oxygen species produced by macrophages and damaged endothelial cells, forming oxLDL\textsuperscript{34,22,45}. oxLDL is a pivotal molecule representing the initial event in atherosclerotic lesion formation and progression by promoting inflammation, foam cell formation, and smooth muscle cell migration/proliferation\textsuperscript{46}. oxLDL is also considered a potent chemo-attractant and strong pro-inflammatory stimulus, which recruits the circulating blood monocytes and T-cells to the sub-endothelial space\textsuperscript{47}. Once retained in the intima, oxLDL activates endothelial cells and up-regulates the expression of adhesion molecules, such as VCAM-1 and intercellular adhesion molecule-1 (ICAM-1), and the secretion of chemokine/cytokines that contribute to the recruitment of circulating leukocytes\textsuperscript{48,49}. oxLDL taken up by activated macrophages via scavenger receptors (SR-A, CD36, and Lox1) form lipid-laden foam cells — a hallmark of atherosclerosis. Activated macrophages and foam cells
secrete different pro-inflammatory cytokines and chemokines, which in turn attract more monocytes to the intima and potentiate the inflammatory response\textsuperscript{22,31,50}.

The role of plasma lipoproteins in atherosclerosis development has gained significant attention and has been demonstrated by randomized controlled trials. These studies revealed the rate of cardiovascular events were significantly reduced via inhibition of cholesterol biosynthesis using lipid lowering agents, mainly statins. Statins act by inhibiting 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMG-CoA Reductase); the rate limiting step in the cholesterol biosynthesis pathway\textsuperscript{40}. The lipid hypothesis of atherosclerosis is now considered fact, supported by: 1) epidemiological studies that have shown a positive relationship between total cholesterol concentrations and the mortality rate of CVDs, and 2) the success of statin drug therapy in the past 40 years; statins significantly reduced atherosclerotic disease mortality through reducing plasma LDL levels\textsuperscript{39}.

1.3.5 Inflammation hypothesis

In the last century, it was believed that atherosclerosis was merely a lipid storage disease, associated with hyperlipidemia and accumulation of cholesterol and fatty streaks in the arterial wall. Over the past two decades, studies demonstrated the complexity of atherosclerosis and the involvement of both the innate and adaptive immune systems by detecting different immune cells (e.g. monocytes, macrophages, dendritic cells (DCs) and T cells) associated with production of immune mediators within the arterial wall\textsuperscript{21}. Presently, the role of inflammation in atherosclerosis is well recognized and accumulating evidence
indicates that inflammation plays a key role in the pathophysiology of all stages of atherosclerosis from lesion initiation, through progression, and ultimately the thrombotic complications of atherosclerosis\textsuperscript{51}.

Inflammation in the arterial wall proceeds as a cascade, which begins with endothelial cell activation and expression of surface adhesion molecules such as VCAM-1, ICAM-1, selectins, and integrins that increase the adhesion of circulating blood monocytes to the endothelial cell layer lining the blood vessel wall\textsuperscript{52,53}. Furthermore, the activated endothelial cells will secrete chemoattractants, such as monocyte chemoattractant protein-1 (MCP-1) also known as CCL2, that interact with chemokine receptors on blood monocytes and promote their recruitment and entry into sub-endothelial intimal space in a phenomenon known as diapedesis\textsuperscript{54,55,56}. Once the blood monocytes migrate into the sub-endothelial space, they activate and differentiate into macrophages, stimulated by macrophage colony stimulating factors (M-CSF)\textsuperscript{57–59}. These activated macrophages exhibit high expression levels of surface recognition receptors called scavenger receptors (SR-A and CD36), which have the ability to engulf oxLDL and form lipid-laden foam cells within the sub-endothelial space. Further, these activated macrophages secrete inflammatory cytokines/chemokines including MCP-1, interleukin-1 (IL-1), and tumor necrosis factor-α (TNF-α) that increase adhesion and recruitment of monocytes into the lesion, potentiating the inflammatory response of atherosclerosis\textsuperscript{60,60,61}. Over time, the lipid-laden foam cells coalesce and form a fatty streaks — characteristic of atherosclerosis and presenting at a very young age. Excessive lipid accumulation followed by
macrophage death results in enlargement of the atherosclerotic core, deposition of collagen fibers, and migration of smooth muscle cells from media into the intima. This ultimately leads to the irreversible formation of fibrous atherosclerotic plaques. Fibrous lesions usually have a fibrous cap composed of smooth muscle cells and ECM, which encloses a lipid-rich, necrotic core. In addition to macrophages, atherosclerotic lesions contain other immune cells including dendritic cells, B lymphocytes, and T lymphocytes, notably CD4+ T cells and regulatory T cells, which regulate many innate immune pathways.

Vascular smooth muscle cells (VSMCs) play a fundamental role in atherosclerosis development. The inflammatory mediators such as cytokines/chemokines and growth factors released by injured endothelial cells and inflammatory cells stimulate migration of VSMCs from the underlying media to the sub-endothelial space resulting in intimal area expansion. Additionally, VSMCs will switch from quiescent contractile phenotype to the proliferative, synthetic phenotype, characterized by excess production of ECM. This results in deposition of ECM proteins and formation of a fibrotic cap that covers the necrotic core and stabilizes/prevents plaque rupture. Over time, these lesions become calcified and grow toward the adventitia. At a certain point, they begin to encroach to the lumen leading to narrowing of the blood vessels and the complications associated with atherosclerosis.

In the atherosclerotic lesion, monocytes can differentiate into two major types of macrophages: classically activated (M1) macrophages which promote inflammation and alternatively activated (M2) macrophages which stimulate
resolution of inflammation. An imbalance in the ratio of classically versus alternatively activated macrophages in advanced atherosclerosis leads to impaired resolution\textsuperscript{71,72}. One important function of M2 macrophages is the clearance of apoptotic cells via a process called efferocytosis. Efferocytosis is a term refer to the engulfment or phagocytosis of antigen presenting cells such as macrophages and dendritic cells\textsuperscript{73}. As macrophages engulf oxLDL in the arterial wall during early stages of atherosclerosis, these macrophages undergo apoptosis\textsuperscript{74}. Apoptosis is a physiological, programmed, and energy-dependent cell death cascade. In early atherosclerosis, macrophage apoptosis is associated with reduced atherosclerosis progression. This is most likely due to effective efferocytosis by M2 macrophages\textsuperscript{75,76}. Efficient efferocytosis has been shown to induce anti-inflammatory mediators, such as interleukin-10 (IL-10) and transforming growth factor-\(\beta\) (TGF-\(\beta\))\textsuperscript{77}. As atherosclerosis progresses, efferocytosis is thought to become impaired. A lack of efferocytosis leads to secondary necrosis, where macrophages die and release their cellular contents including oxidized lipids and pro-inflammatory mediators. Secondary necrosis amplifies the inflammatory response and leads to the development of a necrotic core in the plaque\textsuperscript{78}. Additionally, inflammatory cells and VSMCs in the atherosclerotic lesion release matrix metalloproteinases (MMPs), a family of proteolytic enzymes that degrade ECM, resulting in the thinning and degradation of the fibrous cap. This promotes plaque instability, leading to rupture of the atherosclerotic plaque mainly at the plaque shoulder region and the resultant clinical consequences, such as myocardial infarction and other thrombotic complications\textsuperscript{79–81}. 
Taken together, it is clear that inflammation indeed drives all phases of atherosclerosis. Therefore, several anti-inflammatory compounds have been examined as potential therapeutic strategies for the prevention of atherosclerotic complications using various atherosclerotic animal models\textsuperscript{82}. Despite strong evidence arising from animal studies that lowering inflammation may be a promising strategy for decreasing atherosclerosis and its complications, results have yet to be recapitulated in humans. Various protein therapeutic strategies, such as anti-cytokine therapies, have received noticeable appreciation for clinical application because of their potential direct anti-inflammatory effects. However, these strategies will require extensive clinical evaluation and testing in randomized trials before implementation into practice.

There are two large randomized controlled clinical trials testing the inflammation hypothesis of human atherosclerosis. These studies aim to evaluate whether inhibiting inflammation will decrease event rates and improve prognosis among patients with heart disease using anti-inflammatory agents. The Canakinumab Anti-inflammatory Thrombosis Outcomes Study (CANTOS) is evaluating whether interleukin-1β (IL-1β) inhibition by anti-human IL-1β monoclonal antibody (Canakinumab) can reduce the rates of myocardial infarction, stroke, and cardiovascular death among patients with a history of previous myocardial infarction and elevated levels of high-sensitivity C-reactive protein (hs-CRP) — a clinical marker for chronic inflammatory conditions\textsuperscript{83}. This study showed that the hs-CRP was reduced by 39% and reduced cardiovascular event rates by 17%. This study validated the concept that targeting inflammation and
manipulating the immune system can reduce atherosclerosis without affecting lipid levels and provide a novel cytokine-based therapy for the secondary prevention of CVDs\textsuperscript{84}. The second clinical trial that is still on-going is Cardiovascular Inflammation Reduction Trial (CIRT) which aims to elucidate whether treatment with low-dose methotrexate (a common drug used in the treatment of autoimmune disorders such as rheumatoid arthritis and psoriasis arthritis) will reduce major vascular events among patients with a history of myocardial infarction\textsuperscript{85}.

1.4 Animal models of atherosclerosis

Atherosclerosis animal models have been developed to understand the cellular and molecular mechanisms of atherosclerosis pathogenesis, and to drive research that evaluates the efficacy of newly developed preventive and therapeutic atherosclerotic drugs\textsuperscript{86}. Animal models used to study atherosclerosis and thrombosis include: mouse, rat, rabbit, and pig\textsuperscript{87}. The majority of animal models are based on the presence or induction of hyperlipidemia, primarily hypercholesterolemia, which is the most important risk factor influencing atherosclerosis development. In 1908, Ignatowski demonstrated that the experimental atherosclerosis could be induced in animals, when he fed rabbits a diet enriched in animal proteins including milk, eggs, and meat. At the experimental endpoint, these rabbits presented with atherosclerotic lesions in the aortic wall\textsuperscript{41,44}. After that, several animal models were employed to understand the mechanisms involved in both induction and regression of atherosclerotic lesions\textsuperscript{87,88}.

Recently, the mouse models have become the most commonly used animal model for atherosclerosis experimentation. These animal models are easy to
handle, inexpensive to maintain, reproduce quickly, and are easily to genetically manipulate. The development of atherosclerosis in mice is typically based on genetic modifications of lipoprotein metabolism, including deletion of LDL-receptor (LDLR) or apolipoprotein-E (apoE) gene, followed by administration of a high fat diet to induce hyperlipidemia. High-fat diet (HFD) in experimental research has been found to elevate serum LDL and atherosclerosis in different atherosclerotic animal models, including mice. The apoE-deficient (apoE−/−) mice and LDLR-deficient (LDLR−/−) mice are the most widely used murine models. Apolipoprotein-E is a plasma lipoprotein secreted from liver and promotes hepatic binding, uptake, catabolism, and clearance of triglyceride-rich lipoproteins such as very low density lipoprotein (VLDL) and low density lipoprotein (LDL). In apoE−/− mice, the total plasma cholesterol levels are dramatically increased and extensive atherosclerotic lesions form (widely distributed throughout the aorta) even in the absence of cholesterol– enriched diet, where lesions progress and advance with age. LDLR is a cell-surface receptor expressed in different cell types, where they pick up low-density lipoproteins circulating in the bloodstream and transport them into the cell. They are particularly abundant in the liver that mediates the endocytosis of LDL particles, thereby removing excess LDL-cholesterol from the circulation. LDLR expression is tightly controlled by feedback mechanisms at the transcriptional and post-transcriptional levels. When intracellular cholesterol levels are highly elevated, the transcription factor, sterol regulatory element-binding proteins (SREBPs) will down-regulate the rate-limiting enzyme of cholesterol biosynthesis (HMG-COR), as well as the LDLR, and scavenger receptor class B
member 1 (SCARB1)\textsuperscript{96}. Therefore, LDLR\textsuperscript{−/−} mice exhibit a delayed clearance and consequently, massive accumulation of plasma LDL\textsuperscript{93}. Thus, the LDLR\textsuperscript{−/−} mouse model is characterized by elevated plasma cholesterol levels and develop atherosclerotic lesions slowly when fed a normal chow diet. When fed HFD, LDLR\textsuperscript{−/−} mice develop hypercholesterolemia and extensive lesions throughout the entire aorta and aortic root\textsuperscript{97}.

1.5 Bone marrow transplantation and total body irradiation

Lethal total body irradiation of atherosclerotic mice followed by bone marrow transplantation from donor mice with transgenic alterations in the innate and adaptive immune systems is a common method utilized to reconstitute the immune system and experimentally used to identify the role of bone marrow-derived cells in atherosclerosis. The experimental mice are compared to control mice that have undergone comparable irradiation and bone marrow transplantation with syngeneic wild-type bone marrow cells\textsuperscript{98}. This technique is commonly used in atherosclerosis research to determine the contribution of different hematopoietic cells of interested genotype to the pathogenesis of atherosclerosis\textsuperscript{99}. The high dose total body irradiation is to completely deplete the hematopoietic system of the recipients and to make sure the engraftment of the bone marrow cells from the donors. Because the total body irradiation causes damage to the small intestine, the recipient mice should be provided with antibiotics one week before and two weeks after bone marrow transplantation to avoid septic shock caused by the intestinal bacteria crossing into the blood stream due to their suppressed immune system\textsuperscript{100}. LDLR\textsuperscript{−/−} mice are the most common mouse model used as recipient
mice. ApoE\textsuperscript{−/−} mice are not commonly used as the recipients, because ApoE produced by the transplanted bone marrow cells are sufficient to reverse the atherogenic phenotype\textsuperscript{101}. After bone marrow transplantation, mice were allowed 4 – 5 weeks to reconstitute the hematopoietic and immune system. After this period, majority of the myeloid cells in peripheral blood and bone marrow will be a donor derived cells\textsuperscript{98}.

1.6 microRNA-155 in atherosclerosis

MicroRNAs (miRNAs) are small, noncoding RNA molecules that regulate gene expression at the post-transcriptional level through base pairing with mRNAs, resulting in either translational repression or mRNA degradation. The expression and targets of miRNAs are cell type dependent, which determines the biological function of miRNAs\textsuperscript{102,103}. microRNA-155 (miR155), a typical multi-functional miRNA, has recently emerged to play a significant role in atherosclerosis development\textsuperscript{104}. It has been shown that a hematopoietic miR-155 deficiency reduced atherosclerosis in partial carotid artery ligation Apoe\textsuperscript{−/−} mouse model by increasing the expression of B-cell leukemia/lymphoma 6 (Bcl6) in macrophages thus reducing vascular inflammation\textsuperscript{105}. Additionally, miR155 inhibition by antagonimrs in Apoe\textsuperscript{−/−} mice significantly reduced lesion formation after high-fat diet feeding\textsuperscript{106}. However, hematopoietic miR155 deficiency increased atherosclerosis in LDLR\textsuperscript{−/−} mice fed a high-fat diet by generating a more pro-atherogenic immune cell profile and a more pro-inflammatory monocyte/macrophage phenotype\textsuperscript{107}. Previously we showed that miR155 deficiency in apoE\textsuperscript{−/−} mice attenuated atherogenesis by reducing macrophage inflammation\textsuperscript{108}. 
My studies aim to further investigate the inflammation hypothesis of atherosclerosis in human atherosclerotic carotid lesions as well as in atherosclerotic mouse models. We measured the expression levels of inflammation markers in human carotid atherosclerotic lesions and studied the role of microRNA-155 in vascular calcification. We also studied the role of tristetraprolin (TTP) in inflammation and atherosclerosis. Additionally, we examined the effect of macrophage-specific GP96 deficiency on inflammation and atherosclerosis development. Finally, we examined the therapeutic value of an anti-inflammatory — Chinese herb compound, SsnB, for atherosclerosis.
Chapter II

Inflammation markers in human atherosclerotic lesions and the role of microRNA-155 in vascular calcification

2.1 Background

Inflammation plays a critical role in all stages of atherosclerosis: endothelial activation resulting in a chemokine-mediated recruitment of different immune cells; modification and uptake of oxLDL by macrophages and defective cholesterol efflux leading to formation of foam cells and fatty streaks in the early stage of atherosclerosis. Over time, the disease progresses and complex fibrotic plaques are produced as a result of foam cell death, migration and proliferation of VSMCs and a continued inflammatory response. The production of ECM by VSMCs leads to stabilization of plaques, whereas the production of matrix metalloproteinases (MMPs) from macrophages resulted in destabilization and rupture of unstable plaques and subsequently thrombosis and clinical complications (e.g. myocardial infarction)\(^{31,51,109}\). The inflammatory response in atherosclerosis is regulated by both the innate and adaptive immune system coordinated by different cytokines\(^ {110}\). Knowledge of the roles of cytokines in all stages of atherosclerosis has recently advanced considerably mainly by studies using mouse model systems\(^ {51,110,111}\). Therefore, investigating the role of the immune system in the progression of atherosclerosis and reducing inflammation represent a potential novel therapeutic
approach for prevention and treatment of atherosclerotic vascular diseases. The inflammatory cytokine IL1β is the central cytokine in the inflammatory response in atherogenesis which drives IL6 signaling pathway. The CANTOS study using Canakinumab, IL-1β neutralizing antibody validated the role of inflammation in CVDs and revealed a significant 15% reduction of major adverse cardiovascular events in patient with a prior heart attack and inflammatory atherosclerosis.

The expression of inflammatory markers in human atherosclerotic plaques has been studied since 1985 using different techniques including RT-PCR, immunohistochemistry, and in situ hybridization. Interleukin 1-β (IL-1β) and interleukin 6 (IL-6) expression have been observed in atherosclerotic lesions in humans, hypercholesterolemic rabbits, and apoE−/− mice and their roles in nearly all phases of atherosclerosis have been widely documented, indicating the pro-inflammatory role of these cytokines. We recently demonstrated a pro-atherogenic role of microRNA-155 (miR155) in mouse models. However, if the pro-atherogenic role of miR155 also holds true in humans has not been examined.

In the current study, we performed qRT-PCR to examine the expression levels of inflammation markers including — IL-6, IL-1β, miR155, MMP-9, Adiporedoxin (Adrx) in human atherosclerotic carotid arteries in different locations — normal media, diseased media, atheroma, and fibrous cap from patients undergoing surgical carotid endarterectomy. Furthermore, the expression of miR155 and its correlation to osteogenic genes in calcified media have been examined. In our study, we demonstrated that most of the inflammatory markers listed above were significantly up-regulated in atheroma, fibrous cap, and diseased
media of the carotid plaque and that miR155 positively correlated with IL-6, IL-1β, and MMP-9. We showed that miR155 expression was significantly increased in the fibrous caps of lesions in symptomatic patients compared to those in asymptomatic patients. We also showed that miR155 expression is increased in calcified media and its expression levels positively correlates with osteogenic genes, which are confirmed in cell culture and in vivo animal studies. We found that miR155 deficiency significantly attenuated VSMCs and aortic calcification induced by calcification media and reduced osteogenic gene expression. Compared to wildtype mice, miR155 deficient mice showed a significant decrease in vascular calcification induced by vitamin D3 (vitD3).

2.2 Material and Methods

Human carotid atherosclerotic specimens

Human carotid artery specimens were collected from symptomatic or asymptomatic carotid atherosclerotic patients undergoing carotid endarterectomy (n = 81) with patients’ informed consent at Greenville Memorial Hospital (Greenville health system, Greenville, SC, USA) and Palmetto Hospital (Palmetto Health System, Columbia, SC, USA). Specimens were sliced transversely into 7 mm segments. One segment from each specimen was dissected into separate samples representing normal media, atheroma, underlying diseased media, and fibrous cap.

Vascular smooth muscle cell isolation and culture

Vascular smooth muscle cells were isolated from wild-type or miR155−/− mice using collagenase digestion as previously described116. The isolated cells
were cultured in DMEM supplemented with 10% fetal bovine serum, penicillin-streptomycin, and 2 mM L-glutamine. Cells passage five were used in all of the experiments. To induce calcification, cells were cultured in calcification media (2 mM CaCl$_2$, and 2 mM inorganic phosphate) for 7 days. Calcification media was changed every other day.

**RNA extraction, cDNA synthesis, and quantitative real-time PCR**

Quantitative real-time PCR (qRT-PCR) was utilized to measure the expression levels of human interleukins (IL-6 and IL-1β), matrix metalloproteinase-9 (MMP-9), and miR-155, Adiporedoxin (Adrx), and osteogenic genes (OPN, BMP-2, RUNX-2, and COL-1) in carotid artery specimens. Total RNA, including miRNAs, were isolated from cultured VSMCs or human specimens after homogenization in Qiazol lysis reagent (Qiagen, Maryland, USA) using miRNeasy Mini kit (Qiagen, Maryland, USA) and reverse transcribed to cDNA using miScrip Reverse Transcription Kit (Qiagen, Maryland, USA). qRT-PCR was performed using miScript SYBR Green PCR Kits (Qiagen, Maryland, USA). Human inflammatory gene expression was normalized with 18S as endogenous control. miR155 expression was detected according to the manufacturer’s instructions using the miScript PCR System (Qiagen, Valencia, CA, USA) and miScript Primer Assays (Qiagen, Maryland, USA). Human miR155 gene expression was normalized to U6 as endogenous control. Gene expression levels are reported as ΔCt values relative to expression of 18S or U6. ΔCt is the difference in threshold cycle between the gene of interest and the housekeeping gene (18S or U6) as a control. ΔCt levels are inversely proportional to the gene expression in the sample (the lower the ΔCt
level the greater the higher expression of target gene in the sample). Primers used are listed in table 2.1.

**Aortic ring culture**

Aortas were dissected from wild-type or miR155\(^{-/-}\) mice, cut into small rings, then cultured in calcification media which was changed every other day. After 7 days, aortic rings were harvested for calcium content quantification and embedded in optimal cutting temperature (OCT) compound (Sakura Finetek USA, Inc., Torrance, CA, USA) for frozen section and Alizarin Red staining for calcium deposition.

**Induction of vascular calcification in mice**

Twenty week-old male C57BL/6J mice and miR155\(^{-/-}\) mice (n=5 for each group) were used to induce vascular calcification by administration of Vitamin D3 (VitD3) — Cholecalciferol (Sigma Aldrich, St. Louis, MO, USA). VitD3 was dissolved in absolute ethanol (5 mg per 40 μL) then diluted to a concentration of 3.75 mg/mL with highly refined olive oil (Sigma Aldrich, St. Louis, MO, USA). Stock solutions of VitD3 were prepared fresh for each 3-day injection cycle and then placed in foil wrapped containers and stored at 4°C. The mice were intraperitoneally injected with a dose of VitD3 (100 μL/30 g body weight, 500000 IU/kg, per day) or vehicle (100 μL olive oil, per day) for 10 days. On the 14th day after injection, blood and aorta tissues were harvested for further analysis.

**Calcium content quantification**

After decalcification with 0.6 N HCl at 4 °C for 24 hour, the calcium content from cells and tissues were determined in the HCl supernatants using colorimetric
assay with calcium assay kit (QuantiChromTM Calcium Assay Kit, BioAssay Systems, Hayward, CA, USA) according to the manufacturer's instructions. Briefly, 5 μL of the samples was added to a 96-well plate. 200 μL of working reagent was added and absorbance was measured at 570 nm using a microplate ELISA reader (BioTek Instruments, Winooski, VT, USA). After decalcification, cells were washed three times with PBS and solubilized with 0.1 N NaOH/0.1% SDS. The protein content was measured with a DCTM Protein Assay kit (Bio-Rad). The calcium content of VSMCs was then normalized to the protein content, whereas that of the tissues was normalized to tissue dry weight.

For serum calcium measurement, blood was collected from mice by retro-orbital venous plexus puncture, serum obtained via centrifugation at 4000 x g for 20 minutes. Serum calcium concentration was measured using the QuantiChrom calcium assay kit (BioAssay Systems, Hayward, CA, USA) according to the manufacturer's instructions.

**Alizarin Red S staining**

Slides were hydrated with Xylene/Alcohol and washed three times with tap water. Slides were stained in 1% Alizarin Red S solution (Sigma Aldrich, St. Louis, MO, USA) at pH 9.0 for 30 minutes. Next, slides were dehydrated in Acetone for 15 seconds, then in Acetone/Xylene for 15 seconds. Then slides were cleaned in 3 Xylene changes, coverslipped and visualized under microscope to detect vascular calcium deposition.
Western blot analysis

To obtain the total cell lysates, cells were incubated and lysed for 30 minutes on ice in RIPA buffer (Pierce™, Rockford, IL, USA) supplemented with protease inhibitor cocktail and phosphatase inhibitors (Sigma, Sigma Aldrich, St. Louis, MO, USA). Equal amount of total cell proteins (30 µg) were separated by 4-20% SDS-polyacrylamide gel electrophoresis (Bio-Rad) and transferred onto nitrocellulose membranes (Millipore Corp., Bedford, MA, USA). After blocking with 5% fat-free milk solution, primary antibodies and HRP-conjugated secondary antibodies were used to detect the protein of interest. The primary antibodies used were as follows: anti-RUNX2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-OPN (Bioworld Technology, MN, USA), and anti-GAPDH (GeneTex, Irvine, CA, USA). Signal was detected using Pierce ECL Western Blotting Substrate (Pierce™, Rockford, IL, USA) and exposed to X-ray film to obtain optimal results in the dark room. The density of blots was quantified by Image-Pro Plus 6.0 software. For stripping, membranes were submerged for 30 minutes at 55 – 60°C in a buffer containing 100 mM 2-mercaptoethanol, 2% SDS and 62.5 mM Tris-HCl, pH 6.7, and washed three times with PBST.

Statistical analyses

Statistical analysis was performed with Graph-Pad Prism 6.0 software (Graph-Pad Software Inc, San Diego, CA, USA). Statistical significance was determined using one-way analysis of variance (ANOVA). The correlations between expression of inflammatory genes and miR-155 expression were analyzed by linear regression. For mouse experiments, All measurement data
were expressed as the mean ± standard error of the mean (SEM). Statistical differences were determined using a Student’s t-test for two group comparisons and one-way analysis of variance (ANOVA) for multiple group comparison. Data were considered statistically significant at *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

2.3 Results

**Increased expression of inflammatory cytokines in human carotid atherosclerotic lesions**

First, we examined the expression levels of IL-6 and IL1-β in human atherosclerotic carotid artery using qRT-PCR. Atheroma, underlying diseased media, fibrous cap covering the atheroma, and the surrounding normal media from human atherosclerotic carotid artery were dissected and total RNA was extracted for measurement of human IL-6 and IL1-β expression. In agreement with the growing body of literature that demonstrates the higher expression of inflammatory cytokines in atherosclerotic plaques\textsuperscript{114,117,118}, our results showed that IL-6 and IL-1β mRNA expression levels were significantly increased in human carotid plaques. The expression of IL-6 (Figure 2.1A and 2.1B) and IL-1β (Figure 2.1C and 2.1D) in different locations of the lesion — atheroma, underlying diseased media and fibrous cap were significantly higher compared to surrounding normal carotid media in atherosclerotic carotid artery for the same patient ($p < 0.05$).

**Increased expression of matrix metalloproteinase-9 (MMP-9) in human carotid atherosclerotic lesions**

In addition to the uptake of lipoproteins, macrophages secrete inflammatory mediators and proteinases such as matrix metalloproteinases (MMPs), which play
a critical role in atherosclerotic plaque progression\textsuperscript{22}. Collagen, a key component in the fibrous cap of atherosclerotic plaques, helps maintain the plaque stability. MMPs degrade collagen, leading to increased plaque instability\textsuperscript{119}. Therefore, inflammation and MMPs contribute to all phases of atherosclerosis, from initiation through progression and eventually to the thrombotic complications. MMP-9 represents a novel inflammation marker in coronary artery disease patients and its expression is found to be increased in advanced lesions in atherosclerotic mouse models and in progressive carotid atherosclerotic plaques obtained from humans undergoing carotid endarterectomy, especially in the fibrous cap regions where macrophages accumulate, implicating MMP-9 and macrophages in plaque rupture\textsuperscript{120,121}. Our data showed that MMP-9 expression was significantly increased in atheromatous tissue, diseased media and fibrous caps of human atherosclerotic carotid plaques compared to the adjacent normal media of the same patient (Figure 2.1E and 2.1F) (p<0.001).

**Increased expression of Adiporedoxin in atheroma tissue of human carotid atherosclerotic plaques**

Adiporedoxin (Adrx) is a redox regulatory protein that is expressed in adipose tissue and plays a critical role in the adipocyte function and regulates metabolism by modulating secretion of adiponectin that protect from the development of diabetes and heart disease\textsuperscript{122}. Adrx has been shown to have anti-inflammatory effects by negatively regulating macrophage inflammation\textsuperscript{123}. Recently, it has been shown that Adrx was expressed in human vascular endothelial cells, and its expression significantly induced by oxLDL, IL-1\beta, and
TNF-α stimulation\textsuperscript{124}. In this study, we examined expression of Adrx in atheroma tissue from human carotid atherosclerotic plaques. We found that Adrx expression was significantly increased in the atheroma compared to normal tissue from the same carotid arteries (Figure 2.1G) (\(p<0.05\)). These results suggest induction of Adrx expression may serve as an intrinsic anti-atherogenic mechanism which can be exploited for atherosclerosis treatment.

**miR155 expression is up-regulated in human carotid atherosclerotic lesions**

miR155 has been shown to enhance macrophage inflammation and proved to be pro-atherogenic\textsuperscript{115}. Recently, we and others showed that the macrophage miR155 may be pro-atherogenic\textsuperscript{115,125}. We therefore examined the miR155 expression in different locations of human carotid atherosclerotic plaque and compared to the normal media in the same arteries. In agreement with these previous studies, we demonstrated that miR155 expression levels were significantly up-regulated in the atheroma, underlying diseased media, and fibrous cap compared to the adjacent normal media (Figure 2.2A and 2.2B)(\(p<0.05\)). Moreover, the expression levels of miR155 were compared in the fibrous caps from a-symptomatic (\(n=69\)) and symptomatic (\(n=12\)) patients. The results showed that fibrous caps obtained from symptomatic patients were characterized by significantly higher expression levels of miR155 compared to those from a-symptomatic patients (Figure 2.2C) (\(p=0.0331\)).
Expression levels of miR155 positively correlate with the expression of inflammatory cytokines and MMP-9 in human carotid plaques

To test whether the expression levels of miR155 are correlated with pro-inflammatory cytokine expression, we analyzed the correlation between miR155 expression and the expression of inflammation markers; IL-1β, IL-6, and MMP-9 using linear regression. Gene expression levels of inflammation markers, IL-6, IL-1β as well as MMP-9 in human carotid plaques showed highly significant positive correlations with miR155 expression levels (r=0.377, r=0.238, r=0.432 respectively, p<0.0001) (Figure 2.3A, 2.3B, and 2.3C) suggesting that increased miR155 expression is associated with more inflammation and plaque instability in human carotid atherosclerotic diseases.

miR155 expression is up-regulated in calcified media and positively correlated with osteogenic genes

Cardiovascular calcification is a major feature of chronic inflammatory disorders — such as atherosclerosis and calcific aortic valve disease (CAVD) — that associate with increased mortality and morbidity. There has been evidence that specific miRNAs such as miR-125b, miR-133a, miR-29a/b, miR-204, and miR-30b/c — play critical roles in the regulation of vascular calcification, in vitro and in vivo by regulating the trans-differentiation of VSMCs to osteoblast-like cells. The role of miR155 in vascular calcification has never been studied. In the current study, we examined the expression levels of miR155 in calcified media and correlated that with the expression levels of calcification markers. The results showed that miR155 was highly expressed in calcified media compared to
adjacent normal non-calcified media, $p=0.0356$ (Figure 2.4A). Concurrently, we examined the expression levels of some calcification (osteogenic) genes, such as Osteopontin (OPN), Bone morphogenetic protein-2 (BMP-2), Runt-related transcription factor-2 (RUNX-2), and Collagen Type I Alpha 1 (COL-1). The expression levels of these genes were significantly up-regulated in calcified media compared to adjacent non-calcific media, $p<0.05$ and $p<0.01$ (Figures 2.4B, 2.4C 2.4D and 2.4E). Next, we analyzed the correlation between expression of miR155 and osteogenic genes. We plotted the correlation of miR155 levels and OPN, BMP-2, or RUNX-2 levels, showing a clear positive correlation between expression levels of miR155 with OPN and RUNX-2 expression levels ($r=0.446$, $r=0.322$ respectively) (Figure 2.4F). Thus, miR155 seems to influence plaque stability by influencing the expression levels of osteogenic genes, and thus the grade of calcification.

**miR155 deficiency attenuates VSMCs calcification in vitro**

To further study the role of miR155 in vascular calcification and to confirm the data obtained using human samples, mouse VSMCs were isolated from aorta of wild-type or miR155$^{-/-}$ mice, cultured in DMEM media supplemented with 5% FBS, penicillin/streptomycin, and treated with calcification media containing 2 mM CaCl$_2$ and 2 mM of inorganic phosphate (Pi) to induce calcification. Calcification media was changed every other day. After seven days, cells were collected, cellular calcium content was measured. miR155$^{-/-}$ VSMCs showed a significant decrease in calcium deposition compared to wild-type VSMCs (Figure 2.5A). Then cells were lysed in Trizol for mRNA extraction. The mRNA expression levels of
osteogenic genes including BMP-2, RUNX-2, OPN and OCN were detected. The results showed that the expression of these genes was significantly increased after calcification media treatment and that the miR155−/− VSMCs had a lower expression than the wild-type VSMCs, p-value<0.05 (Figure 2.5B). This result indicated that miR155 deficiency significantly reduced expression of osteogenic genes in calcified VSMCs. Furthermore, we measured the protein levels of RUNX-2 and OPN. The results showed that miR155 deficiency significantly reduced protein expression levels of RUNX-2 and OPN (Figure 2.5C). These data indicated that miR155 deficiency attenuated VSMCs calcification by reducing expression of osteogenic genes.

**miR155 deficiency attenuates aortic calcification ex vivo and in vivo**

The effect of miR155 on vascular calcification was further assessed ex vivo and in vivo. The aortas were dissected from miR155−/− or wild-type mice, cut into rings, and cultured in calcification media. Also, aortas from mice injected with vitD3 were dissected and cut into small rings. The degree of calcification was determined using alizarin-red staining and tissue calcium content was detected using colorimetric assays with calcium assay kit. The results demonstrated that miR155 deficiency reduced calcium tissue content and calcium deposition in the calcified cultured aortic ring (Figures 2.6A and 2.6B) and aortic ring from vitD3 injected mice (Figures 2.6C and 2.6D). We also measured serum calcium concentration after vitD3 injection. Interestingly, miR155−/− mice displayed higher serum calcium than wild-type mice p-value<0.05 (Figure 2.6E). Collectively, these data indicated that
miR155 deficiency reduced aortic calcification *ex vivo* and in the aorta of vitD3 injected mice.

**2.4 Discussion**

Inflammation plays a significant role in all stages of atherosclerosis. The cytokines secreted by innate and adaptive immune cells regulate the inflammatory response in atherosclerosis. The expression of inflammatory cytokines in mouse models of atherosclerosis has been extensively studied. A previous study found that the expression of the pro-inflammatory cytokines in apoE*−/−* mice after 4 weeks of high fat diet was significantly increased. The expression levels of several pro-inflammatory cytokines in the atherosclerotic plaques were studied and reported from the mid-1980s mainly in human carotid endarterectomy specimens. Better understanding of cytokine gene expression is important for successful development of therapeutic approaches that decrease the inflammatory response. Inhibiting cytokine-induced inflammation and promoting the anti-inflammatory cytokine response represent potential therapeutic approaches for the prevention of disease development and progression. Currently available therapies against atherosclerosis are mainly lipid modulators. While lipid lowering drugs are successful at reducing the risk of death result from cardiovascular diseases, there is a need for new therapies that directly target the inflammation to further attenuate atherosclerosis and improve cardiovascular outcome.

The expression of inflammation markers in different locations in the human carotid plaque has not been addressed. In our study we examined the mRNA expression levels of some inflammation markers in human carotid atherosclerotic
arteries obtained from patients undergoing carotid endarterectomy. The specimens were dissected into atheroma, underlying diseased media, fibrous cap, and the surrounding non-diseased media. The expression levels of inflammation markers (such as IL-6, IL-1β, MMP-9, miR155, and Adiporedoxin) and calcification markers (such as OPN, RUNX-2, BMP-2, and COL-1) were measured. IL-6, a pro-inflammatory cytokine mainly expressed by activated macrophages and VSMCs, was significantly higher in atheroma, diseased media, and in the fibrous cap compared to the normal non-diseased media. Similarly, expression of pro-inflammatory cytokine IL-1β was significantly higher in atheroma, diseased media, and in the fibrous cap compared to the normal non-diseased media. The expression level of MMP-9 was higher in atheroma, diseased media, and in the fibrous cap compared to the normal non-diseased media. Furthermore, the expression level of miR155, a recently reported pro-inflammatory and pro-atherogenic microRNA, was significantly up-regulated in the atheroma, diseased media, and fibrous cap compared to the normal media. Increased expression of these mRNAs in different locations in human carotid atherosclerotic plaque suggests they may play a role in atherosclerotic plaque progression. Furthermore, when we analyzed the expression levels of miR155 in the fibrous caps in symptomatic patients compared to asymptomatic patients, we found that the expression levels of miR155 in fibrous cap were significantly higher in symptomatic compared to those in asymptomatic patients. This might suggest the important role of miR155 in modulating plaque stability and clinical manifestation of plaque rupture. The expression of miR155 and calcification markers — such as OPN,
RUNX-2, Col-1, and BMP-2 in calcified media were also analyzed. The results showed that miR155 was up-regulated in calcified media occurred concomitant with increased expression levels of calcification markers, suggesting miR155 might be involved in vascular calcification. Moreover, we showed a positive correlation between miR155 expression level and osteogenic gene expression levels. We also used in vitro, ex vivo, and in vivo experiments to determine the effect of miR155 deficiency on vascular calcification. The results showed that miR155 deficiency attenuated vascular calcification in VSMCs after calcification media treatment as well as decreased aortic calcification in vitD3 injected mice. Furthermore, the expression levels of osteogenic genes in miR155−/− VSMCs after treatment with calcification media were significantly reduced compared to wild-type VSMCs. These results were in agreement with the data obtained from human samples as the increased miR155 expression levels in calcified media occurred concomitant with increased expression levels of osteogenic genes. Collectively, these data indicated that miR155 plays a critical role in VSMCs calcification by increasing expression of osteogenic genes, and inhibiting miR155 may represent a novel approach to reduce vascular calcification. Inhibition of miR-155 by antagomiR-155 may provide new therapeutic approach for the prevention and treatment of vascular calcification caused by a variety of diseases. Investigating the role of miRNAs in VSMCs calcification will lead to a better understanding of the mechanisms and to developing drugs directed against specific gene targets for the treatment of cardiovascular diseases associated with vascular calcification.
Table 2. Primers that were used for qRT-PCR in this study

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<th>Reverse (5’ → 3’)</th>
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<td>AGTCGGCAGCGTTTATGTC</td>
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<td>TTTTCTGCGAGTCCTCCTTT</td>
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<td>hIL-1β</td>
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<td>hOPN</td>
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<td>CAGGTCGCAAACCTTTCTTAG</td>
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<td>hRUNX2</td>
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<td>ACCCGCTGTCTTCTTAGC</td>
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Figure 2. Increased expression of inflammation markers in human carotid atherosclerotic atheroma

Carotid artery plaque samples of patients undergoing carotid endarterectomy were collected. mRNA was isolated from tissue extracts and mRNA transcripts of genes of interest were measured by qRT-PCR. Results were represented as ΔCt values after being normalized to the expression of 18s rRNA. ΔCt is the difference in threshold cycle between the gene of interest and the housekeeping gene (18s rRNA). ΔCt levels are inversely proportional to the gene expression levels (the lower the ΔCt level, the higher expression of target gene). (A, B) IL-6 mRNA, (C, D) IL-1β mRNA, (E, F) MMP-9, and (G) Adrx mRNA in different locations for the same patients were detected. Each line connects data from a same patient (*p<0.05, **p<0.01, ***p<0.001)
Figure 2. 2 Increased expression of miR155 in human carotid atherosclerotic plaques

Carotid artery plaque samples of patients undergoing a carotid endarterectomy were collected. mRNA was isolated from tissue. miR155 mRNA levels were measured using qRT-PCR. Results were represented as ΔCt values after being normalized to the expression of U6. ΔCt is the difference in threshold cycle between miR155 and U6. (A, B) Expression levels of miR155 were significantly increased in atheroma, diseased media, and fibrous cap from human carotid lesions, compared to those in the normal media. Each line connects data from a same patient. (*p<0.05, **p<0.01, ***p<0.001). (C) Expression of miR155 in fibrous cap of carotid atherosclerotic plaques is higher in symptomatic than asymptomatic patients. (*p=0.033).
Figure 2. 3 Expression levels of miR155 correlate with the levels of inflammatory cytokines and MMP-9 in human carotid artery atheroma
Correlation between miR155 expression and pro-inflammatory cytokines IL6 (A), IL1β (B), and miR155 expression and MMP-9 expression (C) using linear regression.
Figure 2. 4 The expression of miR155 and calcification gene in human atherosclerotic carotid artery

(A) The expression level of miR155 in human atherosclerotic carotid artery was assessed by qRT-PCR (n=6 for each group). Results were represented as ΔCt values after being normalized to the expression of U6. (B, C, D, and E) the expression levels of osteogenic genes including OPN, COL-1, BMP-2, and RUNX-2 in human atherosclerotic carotid artery were assessed by qRT-PCR. Results were presented as ΔCt values after being normalized to the expression of 18s rRNA. Each line connects data from a same patient. (n=5 for each group). (F, G) Correlation between miR155 expression and osteogenic markers (OPN and RUNX2) using linear regression. *P<0.05, **P<0.01. NM: Normal media; CM: Calcification media
Figure 2. 5 miR155 deficiency attenuates calcification in vascular smooth muscle cells

(A) Calcium content of VSMCs was measured using colorimetric assay with a calcium assay kit. The expression levels of calcification genes; BMP-2, RUNX-2, OPN and OCN in VSMCs after treatment with calcification media were detected by qRT-PCR. (C) Protein levels of RUNX2 and OPN were assessed by western blotting. *p<0.05
Figure 2. 6 miR155 deficiency attenuates aortic calcification in vivo and ex vivo

(A) Calcium deposition in cultured aortic rings from wild-type or miR155−/− mice was detected by alizarin Red staining. (B) Quantification of calcium content in the aortic rings using colorimetric assays with calcium assay kit. (C) Calcium deposition in the aortas of wild-type or miR155−/− mice injected with VitD3 was determined by Alizarin Red staining. (n=5 for each group). (D) Quantification of aortic calcium content. (E) Serum calcium concentration was measured by colorimetric assay using a calcium assay kit. Scale bar, 500 μm. *P<0.05, **P<0.01.
Chapter III

The role of Tristetraprolin (TTP) in inflammation and atherosclerosis

3.1 Background

Tristetraprolin (TTP) — also known as ZFP36 (zinc finger protein of 36 kDa)\textsuperscript{134}, Nup 475\textsuperscript{135}, TIS11\textsuperscript{136}, or G0S24\textsuperscript{137} — is a member of a small family of mRNA-binding proteins containing a tandem Cys-Cys-Cys-His (CCCH) zinc finger (TZF) domain. The initial description of TTP was in 1990 in mouse 3T3-L1 fibroblasts stimulated with insulin and serum\textsuperscript{138}. This protein is encoded by the immediate – early response gene called Zinc Finger Protein 36 (ZFP36)\textsuperscript{139}. It is highly expressed in immune organs in a variety of cell types including monocytes and macrophages\textsuperscript{138}. Normally, TTP protein levels are low and the protein is primarily localized in the nucleus of quiescent cells. However, mitogenic stimulation of cells induces TTP expression and promotes rapid translocation from the nucleus into the cytoplasm\textsuperscript{140}. Through the TZF domains, TTP can bind directly to the adenosine-uridine (AU)-rich elements (AREs) in the 3' untranslated region (3'-UTRs) of their target mRNAs removing its poly(A) tail; in a process known as ‘deadenylation’— the rate-limiting step in mRNA decay resulting in suppression of translation, destabilization and subsequently degradation of the mRNA transcript through the recruitment of deadenylases or exonucleases\textsuperscript{141,142}. 
TTP acts at the post-transcriptional level to inhibit the expression of a number of pro-inflammatory cytokine/chemokines and growth factors. The most apparent physiological targets of TTP are the mRNAs encoding TNF-α\textsuperscript{143–145}, granulocyte-macrophage colony-stimulating factor (GM-CSF)\textsuperscript{146}, Interleukin-2 (IL-2)\textsuperscript{147}, interleukin-3 (IL-3)\textsuperscript{148}, Interleukin-10 (IL-10)\textsuperscript{149}, Interleukin-12 (IL-12)\textsuperscript{150}, and Interferon-γ (IFN-γ)\textsuperscript{151}. The role of TTP as an mRNA destabilizing protein initially comes from the characterization of TTP knockout mice, which appeared normal and healthy at birth, but after few weeks displayed a complex inflammatory syndrome manifested by growth retardation, erosive poly-articular arthritis, conjunctivitis, dermatitis, myeloid hyperplasia, splenomegaly, kidney lesions and autoimmunity\textsuperscript{152}. This inflammatory phenotype was due to excess production of the potent pro-inflammatory cytokine, TNF-α\textsuperscript{145}. Furthermore, macrophages isolated from the TTP knockout mice and stimulated with lipopolysaccharide (LPS) showed a significant increase in TNF-α mRNA and protein levels due to stabilization of TNF-α mRNA\textsuperscript{145,153}. The inflammatory phenotype of TTP knockout mice was significantly attenuated by injecting the mice, soon after birth, with anti-TNF-α antibodies or by breeding with TNF-α receptor deficient mice\textsuperscript{143,154}.

To determine whether the excess TNF-α which causes the TTP deficiency inflammatory phenotype is produced by myeloid cells, myeloid-specific TTP deficient mice have been generated. Results revealed that mice with myeloid-specific TTP deficiency appeared normal under laboratory conditions. However, when these mice were challenged with low-dose LPS, they developed severe endotoxic shock associated with increasing serum TNF-α levels compared to the
control mice. These studies demonstrated that myeloid cell-specific TTP deficiency did not completely phenocopy whole-body TTP deficiency in mice under normal laboratory conditions suggesting that other cell types were involved in the pathogenesis of the TTP deficiency syndrome\textsuperscript{155,156}. Bone marrow transplantation has been used to determine if it could transfer the inflammatory phenotype of TTP knockout mice to recipient mice. Recombination activating gene-2 knockout (RAG-2\textsuperscript{−/−}) mice transplanted with TTP deficient bone marrow cells reproduce the TTP deficiency inflammatory syndrome, which may indicate that hematopoietic cells play a significant role in excess production of TNF-α that leads to the pathology reported in TTP knockout mice\textsuperscript{153}. Recently, it has been shown that TTP is a potent modulator of TNF-α secretion from dendritic cells after LPS stimulation\textsuperscript{157,158}.

TTP has been shown to be highly expressed in the vascular endothelium of atherosclerotic mice but not in the vascular endothelium of healthy mice. The anti-inflammatory effects of endothelial cell TTP might post it as a potential therapeutic target for the prevention and treatment of atherosclerosis\textsuperscript{159}.

Hematopoietic cells are crucial players in the inflammatory reactions driving atherosclerosis. In this study, we transplant TTP deficient bone marrow cells into LDLR\textsuperscript{−/−} mice to examine the effect of hematopoietic TTP deficiency on inflammation and atherosclerotic lesion development. We investigated whether bone marrow transplantation transfers the inflammatory phenotype of TTP knockout and determine its role in atherosclerosis development in LDLR\textsuperscript{−/−} mice. We first transplanted bone marrow cells from either TTP deficient or wild type mice into LDLR\textsuperscript{−/−} mice after lethal irradiation, and fed a western diet for 12 weeks to
induce atherosclerosis. We demonstrate that TTP deficiency in bone marrow cells transferred the phenotype of TTP deficiency including systemic and multi-organ inflammation. Surprisingly, we found that TTP deficiency in bone marrow cells did not affect atherosclerosis development. Unexpectedly, TTP deficiency in bone marrow cells dramatically reduced plasma lipid and hepatic lipid accumulation (hepatic steatosis). Increased inflammation and attenuated hyperlipidemia offset each other leading to unchanged atherosclerosis in LDLR−/− mice.

To define the role of macrophage-specific TTP deficiency on atherosclerosis, LDLR−/− mice were lethally irradiated and reconstituted with either macrophage-specific TTP deficient bone marrow cells or WT bone marrow cells. Four weeks after bone marrow transplantation, mice were fed a western diet for 9 weeks. This study reveals that macrophage-specific TTP deficiency did not mimic the inflammatory phenotype of whole myeloid cell TTP deficiency in mice. In addition, these mice had comparable lesion size in the aorta and aortic root. Interestingly, macrophage infiltration and lipid deposition in the aortic root were reduced in macrophage-specific TTP deficient mice, which might be due to efficient removal by healthy macrophages in early atherosclerosis.

3.2 Material and Methods

Human carotid atherosclerotic specimens

Human carotid artery samples were collected as described in chapter 2.

Mice

Six to eight week-old female LDLR−/− mice were obtained from Jackson Laboratories (Bar Harbor, MA, USA). Mice were maintained in pathogen-free
conditions at the University of South Carolina according to National Institutes of Health (NIH) guidelines. Animal care and experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of South Carolina.

**Bone Marrow Transplantation (BMT) and atherosclerosis induction in mice**

Bone marrow transplantation was performed as previously described \(^{160}\). Briefly, bone marrow cells from donor WT or TTP deficient mice were harvested from cleaned femurs and tibias by flushing with sterile RPMI-1640 media (Invitrogen Life Technologies, Grand Island, NY, USA). The cell suspension was centrifuged at 300 x g for 5 minutes and re-suspended in cold phosphate buffer saline (PBS). One week before and two weeks after BMT, recipient mice (LDLR\(^{-/-}\) mice) were given autoclaved antibiotic water supplemented with 100 mg/L neomycin and 500,000 U/L polymyxin B sulfate. Early morning, all recipient mice were lethally irradiated (900 rad) using a cesium gamma source. Six hours after irradiation, mice were injected in the retro-orbital venous plexus with nucleated bone marrow cell (5 X 10\(^6\) in 200 µL PBS). After BMT, mice were observed for any signs of bone marrow transplant rejection. To confirm the success of total body irradiation, one mouse did not transplanted with bone marrow cells. This mouse died exactly after 2 weeks of total body irradiation. After four weeks recovery, the resulting chimeric mice were fed a western diet for 12 weeks to induce atherosclerosis (Figure 3.1A). In order to assess the effect of bone marrow TTP deficiency in macrophages on atherosclerosis development, lethally irradiated LDLR\(^{-/-}\) mice were reconstituted with macrophage-specific TTP deficient bone
marrow cells or control (WT) bone marrow cells. Four weeks later, mice were put on western diet for 9 weeks (Figure 3.1B). During the western diet period, body weight and food intake were measured weekly. At the experimental endpoint, mice were euthanized by isoflurane and venous blood from the eyes was collected. Tissues including: heart, aorta, liver, lung, and spleen were collected and analyzed.

**Peritoneal macrophage isolation and culture**

Standard techniques were used to isolate thioglycollate-elicited peritoneal macrophages. Briefly, mice were injected intraperitoneally (i.p.) with 3 mL of 3% (w/v) sterile thioglycollate (BD Biosciences Clontech; Palo Alto CA, USA). After 3 days, peritoneal macrophages were collected by washing the peritoneal cavity with 10 mL of cold PBS twice. The cells were centrifuged at 300 x g for 5 minutes and re-suspended in high-glucose Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS), a combination of penicillin/streptomycin (Sigma-Aldrich, St. Louis, MO, USA), and 50 μM of β-mercaptoethanol. Two million cells/well were cultured in a 12-well plate. Two hours later, cells were washed with PBS to remove the non-adherent cells, and the adherent macrophages cultured in serum free media (SFM) overnight at 37°C and 5% CO₂. Macrophages were stimulated with LPS (50 ng/mL, Sigma-Aldrich) for 6 hours under the same conditions. Treated cells were lysed with Trizol reagent (Invitrogen, Grand Island, NY, USA) for mRNA extraction. Expressions of pro-inflammatory cytokines; TNF-α and IL-6 expression were detected by qRT-PCR.
**VetScan hematology analysis**

Blood was drawn into heparinized tubes by retro-orbital puncture. Blood samples were run on the VetScan HMT hematology analyzer (Abaxis, Union City, CA, USA). The following parameters were measured: total red blood cells (RBC), hemoglobin, mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), hematocrit (Hct), platelet number, mean platelet volume (MPV), platelet distribution width (PDW), total white blood cells (WBC), lymphocytes, mean cell volume (MCV), and red cell distribution width (RDW).

**Blood Glucose Measurement**

Blood glucose concentration was measured using OneTouch Ultra Blood Glucose Monitoring System (LifeScan, Inc., Milpitas, CA, USA) following the manufacturer’s instructions.

**Serum Lipid and Lipoprotein Analysis**

Total serum cholesterol and triglycerides were determined using enzymatic colorimetric assays with Cholesterol Reagent and Triglycerides GPO reagent kits (Raichem; San Diego, CA, USA) at the indicated time points, and analyzed by Soft-Max Pro5 software (Molecular Devices, Sunnyvale CA, USA). In brief, mice were anesthetized with isoflurane, blood was collected by retro-orbital venous plexus puncture, plasma obtained via centrifugation at 4000 x g for 20 minutes. Serum was diluted 1:100 in sterile water, 100 µL was loaded on the microplate well and 100 µL of freshly prepared cholesterol and triglyceride reagent were added to the wells. The plates were incubated 10 minutes at 37°C and the absorbance was measured at 540 nm. Serum lipoproteins were measured using a fast performance
liquid chromatography (FPLC) system (AKTA purifier, GE Healthcare Biosciences, Pittsburgh, PA, USA) equipped with a Superose 6 10/300 GL column (GE Healthcare). Pooled mouse serum (100 µL) was loaded onto the column, and eluted at a constant flow rate of 0.5 mL/min with 1 mM sodium EDTA and 0.15 M NaCl. Fractions of 0.5 mL were collected and cholesterol concentration from each fraction was measured.

**Total RNA extraction, cDNA synthesis, and quantitative Real-Time PCR**

Total RNAs were extracted from liver tissues and cultured peritoneal macrophages incubated in SFM with or without LPS stimulation using Trizol reagent (Invitrogen, Grand Island, NY, USA) following the manufacturer’s instructions. One microgram of isolated RNA of each sample was reverse transcribed into complementary DNA (cDNA) in 20 µL reactions using iScript™ cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). qRT-PCR was performed on a Bio-Rad CFX96 system using iQ™ SYBR® Green Supermix (Bio-Rad) according to the manufacturer’s instructions. The relative amount of target mRNA was calculated using the $2^{(\Delta\Delta\text{Ct})}$ method by normalizing target mRNA Ct values to those of housekeeping gene, 18S rRNA expression. PCR thermal cycling conditions were 3 minutes at 95°C, and 40 cycles of 15 s at 95°C and 58 s at 60°C. Samples were run in triplicate. Primers used are listed in table 3.1

**Flow cytometry analysis**

The spleen was removed from freshly euthanized mice and smashed in 10 mL RPMI-1640 medium supplemented with 10% FBS using Stomacher® 80 Biomaster (Seward Laboratory Systems Inc., Port St. Lucie, FL, USA). To obtain
single cell suspensions, tissues were passed through a 70 µM cell strainer (Life Sciences, Tewksbury, MA, USA). After red blood cell lysis, cells were stained for 30 minutes on ice in dark with anti-CD19 mAb, anti-CD3 PE mAb, anti-CD4 FITC mAb, anti-CD8a FITC mAb, anti-FOXP3 mAb, anti-ly6C FITC mAb, anti-CD11b PE mAb, anti-ly6G FITC mAb, and anti F4/80 FITC mAb (eBioscienceTM, Invitrogen, Grand Island, NY, USA) in staining buffer. Stained cells were acquired on a Cytomics FC 500 flow cytometer and CXP software version 2.2 (Beckman Coulter, Brea, CA, USA). Data were collected for 10,000 live events per sample.

**Enzyme-linked immunosorbent assay (ELISA)**

ELISA assays were performed per the manufacturer’s protocol. Briefly, blood was collected and centrifuged at 4,000 x g for 20 minute. Serum layer was removed and diluted 1:5. 96-well ELISA plates were coated with 100 µL of TNF-α capture antibody (2 µg/mL) and incubated overnight at 4°C. The cells were incubated with 300 µL of blocking solution (1% BSA, 5% sucrose, and 0.05% NaN3) for 1 hour at room temperature. Next, 100 µL of each sample were loaded to each well and incubated for 2 hour at room temperature. After washing three times, the plates were incubated with biotinylated anti-mouse TNF-α (250 ng/mL), 1 µg/mL horseradish peroxidase streptavidin and substrate solution. The reaction was stopped by adding 50 µL of 1M H2SO4 solution. Recombinant mouse TNF-α was used to generate a linear standard curve. Optical density was determined with a SpectraMax M5 microplate reader at 450 nm (Molecular Devices). All samples were tested in triplicate.
Bioplex analysis of serum cytokine/chemokines

Serum levels of 23 cytokine/chemokines from mice were measured using a Bio-Plex Pro™ Mouse Cytokine 23-plex Assay (Bio-Rad, Hercules, CA, USA) on a Bio-Plex system following the manufacturer's instructions.

Quantification of atherosclerosis

At the experimental endpoint, all mice were euthanized and the vasculature was perfused with cold phosphate buffer solution (PBS). The aorta from arch to bifurcation was fixed in 10% neutral buffered formalin. Then, it was opened longitudinally, pinned onto black wax plates, and stained with Sudan-IV (Sigma-Aldrich, St. Louis, MO, USA) for aortic enface analysis. Aortic lesion areas were quantified and analyzed by ImageJ software. The aortic root was isolated and embedded in Optimal Cutting Temperature (OCT; Tissue-tek) compound (Sakura Finetek USA, Inc., Torrance, CA, USA) in a plastic mold, stored at -20 °C. Ten-micrometer-thick cryosections were cut from the proximal 1 mm of the aortic root. Aortic root sections were assessed for atherosclerotic plaque size with Hematoxylin and Eosin (H&E) staining method. To analyze the accumulation of macrophages in the atherosclerotic plaque, Moma-2 (Abcam, Cambridge, MA, USA) staining was performed. Sections were fixed in acetone for 20 minutes at room temperature, rinsed with PBS, followed by block in immunostain blocker solution, and then rinsed with PBS again. The endogenous peroxidase activity was blocked with 3% hydrogen peroxide. Sections were blocked for non-specific staining with 5% normal goat serum for 30 minutes at room temperature followed by incubation with the primary antibody (Moma-2, 1:400 dilution) at 4°C overnight.
After washing with PBS three times, the biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA) was used as the secondary antibody. Immunoreactivity was amplified using the Vectastain ABC kit (Vector Laboratories), and signal was enhanced by peroxidase enhancer (GeneTex, Irvine, CA; USA) and reacted with the substrate from AEC Chromogen/FRP substrate kit (GeneTex Irvine, CA; USA). Nuclei and cytoplasm were counterstained with haematoxylin for 1 min. Finally, sections washed with deionized water and air-dry before getting covered with coverslips. Macrophage infiltration was analyzed via microscope. Inflammatory cell infiltration was expressed as Moma-2 positive area. Oil red O staining was performed to detect lipid content in atherosclerotic plaques. Slides placed in oil red O (ORO; Sigma-Aldrich) working solution (0.5% in 100% propylene glycol) for 4 hours at room temperature. Differentiate slides in 85% aqueous propylene glycol three times for 1 minute. Then slides rinsed with deionized water for 2 minutes. Sections were stained with aqueous hematoxylin (GeneTex, Irvine, CA; USA) for 20 seconds. Slides washed with deionized water and air-dry. Sections were covered with cover glasses and analyzed under a microscope. Plaque collagen content was analyzed using Masson’s trichrome staining. Slides were fixed in Bouin’s fluid for 1 hour at 60 °C, washed with water, and then placed in Working Weigert’s Iron Hematoxylin Stain for 10 minutes. Section were stained with Biebrich Scarlet-acid Fuchs solution for 15 minutes and rinsed in distilled water. Next, sections were placed in Phosphotungstic-Phosphomolybdic Acid solution for 10 minutes, then placed in Aniline blue for 5 minutes followed by 1% Acetic Acid solution for 3 minutes.
slides were dehydrated and cleared through 95% ethanol, 100% ethanol, and xylenes, 2 changes each, mounted with permount, and coversliped. All images were recorded with a Nikon E600 Wide field Epifluorescence microscope and Micropublisher digital camera with Q-imaging software. Plaque size, lipid content, collagen percentage, and macrophage cell content were quantified by computerized image Pro-Plus.

**Histological analysis**

Tissue samples (Liver, lung, kidney and spleen) were fixed in 10% neutral buffered formalin. H&E staining and microscopy were implemented to detect infiltrating inflammatory cells. All tissue sections were visualized with a Nikon E 600 microscope.

**Microarray analysis**

For microarray analysis, liver tissue was lysed with Qiazol, and total RNA was extracted using RNeasy Mini kit (Qiagen, Germantown, MD, USA). RNA quality and quantity analysis were determined using Agilent 2100 Bioanalyzer. All RNA samples had an RNA integrity number (RIN) of 9.2 or higher. The RNA was amplified and labeled with the Agilent Low Input Quick Amp labeling kit according to the instructions of the manufacturer. Labeled RNA was then purified using the Qiagen RNeasy mini kit. mRNA contained in 200 ng of total RNA was converted into cDNA using a poly-dT primer that also contained the T7 RNA polymerase promoter sequence. Subsequently, T7 RNA polymerase was added to cDNA samples to amplify the original mRNA molecules and to simultaneously incorporate cyanine-3 labeled CTP into the amplification product (cRNA). Labeled cRNA
molecules were purified using Qiagen’s RNeasy Mini Kit (Valencia, CA). After spectrophotometric assessment of dye incorporation and cRNA yield, samples were hybridized to Agilent whole mouse genome microarrays 8 × 60,000 using a gene expression hybridization kit (Agilent) according to the manufacturer’s recommendation. Microarray analysis was performed using an Agilent DNA microarray scanner system. After washes, arrays were scanned using a High Resolution Agilent DNA Microarray Scanner and images saved in TIFF format. A heat map of genes from relevant pathways identified by Ingenuity pathway analysis was generated using R function heatmap.2.

**Statistical analysis**

Data were presented as mean ± standard error of the mean (SEM). Statistical analysis was performed with Graph-Pad Prism 6.0 software (Graph-Pad Software Inc, San Diego, CA, USA). Statistical significance was determined by one-way analysis of variance (ANOVA) for multi-group comparison and Student’s t test for two-group comparison. Data were considered statistically significant at *P < 0.05, **P<0.01, and ***P<0.001.

3.3 Results

**TTP is up-regulated in human carotid atherosclerotic lesions**

We examined the expression levels of TTP in human carotid atherosclerotic artery using qRT-PCR. Atherosclerotic lesion and the surrounding normal media from human carotid artery were dissected from patients undergoing carotid endarterectomy. Total RNA extraction and qRT-PCR analysis were performed to measure the expression of human TTP mRNA. In agreement with a previous
study\textsuperscript{159}, we demonstrated that TTP is highly expressed in atheromatous tissue compared to the adjacent normal media in the carotid atherosclerotic plaques $p$-value $<$0.05 (Figure 3.2).

**TTP deficiency in bone marrow-derived cells causes growth retardation, but did not affect food intake**

Five million bone marrow cells from either TTP deficient mice or WT mice were transplanted into lethally irradiated LDLR$^{-/-}$ mice. After 4 weeks recovery, the recipient mice were challenged with a western diet for 12 weeks, after which mice were sacrificed. During western diet period, mouse food intake and body weight were measured weekly. None of these mice showed any signs or symptoms of bone marrow transplant rejection. We did not observe significant differences in food intake between bone marrow TTP deficient mice and control mice (Figure 3.3A). There was, however, a reduction in the mouse body weight with bone marrow TTP deficiency (Figure 3.3B). This reduction in body weight and cachexia was the most important characteristic phenotype of TTP knockout mice\textsuperscript{161}.

**TTP deficiency in bone marrow-derived cells causes hematological and Immunological disturbance**

Previous studies showed that there is a hematological and immunological disturbance associated with TTP deficiency\textsuperscript{161–163}. To determine the effect of bone marrow TTP deficiency on hematological and immunological status of TTP deficient bone marrow transplanted LDLR$^{-/-}$ mice, we performed Vetscan HMT analysis of peripheral blood at the experimental endpoint. The results showed an increase in the number of white blood cells, number and percentage of blood
monocytes, but these changes did not reach statistical significance, perhaps because of the relatively small sample number (n=5-6). There was, however, a significant increase in the number and percentage of neutrophils (P=0.0037, P=0.0336 respectively) of bone marrow TTP deficient mice compared to the control mice (Figure 3.4A). Total platelet number, platelet hematocrit were significantly increased (P=0.0107, P=0.0053 respectively) in bone marrow TTP deficient mice compared to the control mice (Figure 3.4B). On the other hand, we did not observe any differences in the red blood cell count, hemoglobin concentration, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, MCHC, and the RDWC between the two groups (Figure 3.4C). Flow cytometric analysis of the leukocytes population in the spleen showed a significant decrease in the number and percentage of CD3+/CD4+ T lymphocytes, and a significant increase in CD11b+/GR1+ myeloid cells, CD11b+/Ly6G+ (neutrophils), CD11b+/Ly6C<sup>high</sup> (inflammatory monocytes). Whereas, the number and percentage of CD19+ B lymphocytes, CD3+/CD8a+ T lymphocytes, CD4+/FoxP3+, CD4+/IL17+, CD11C+, F4/80+, and CD11b+/Ly6C<sup>low</sup> were comparable between the two groups (Figures 3.5A & 3.5B). Even though the spleens of bone marrow TTP deficient mice were slightly enlarged (Figure 3.5C), the absolute cell numbers were comparable to those of control mice (Figure 3.5D). These results indicated that the bone marrow TTP deficiency causes hematological and immunological disturbance as previously reported in TTP knockout mice<sup>164</sup>. 
TTP deficiency in bone marrow-derived cells increases expression of pro-inflammatory cytokines

A number of pro-inflammatory cytokines produced by macrophages including, TNF-α and IL-6, were used to assess the ability of macrophages to produce cytokines\textsuperscript{163}. These cytokines play a crucial role in the macrophage response to LPS stimulation. Peritoneal macrophages of bone marrow TTP deficient mice and WT mice were isolated by washing the peritoneal cavity, cultured and treated with LPS (50 ng/mL) for 6 hours. The expression of pro-inflammatory cytokines TNF-α and IL-6 mRNA were measured by qRT-PCR. The results showed a significantly higher expression of TNF-α mRNA, \( p \)-value 0.0071 (Figure 3.6A) and IL-6 mRNA (Figure 3.6B) in TTP deficient macrophages after stimulation with LPS compared to WT ones. These data indicated that TTP deficient macrophages had a higher ability to produce TNF-α after LPS stimulation and confirmed that TNF-α is one of the physiological-targets of TTP\textsuperscript{165}.

TTP deficiency in bone marrow-derived cells causes systemic and multi-organ inflammation

As the TTP knockout mice developed a severe inflammatory phenotype\textsuperscript{161}, we next evaluated the inflammatory phenotype of bone marrow TTP deficient mice. Mouse tissues were isolated, fixed, and stained with H&E. The histological analysis of these mice showed a wide degree of variation, where some of them had severe abnormal findings, while others had a mild abnormal finding typical of whole body TTP knockout. The bone marrow TTP deficient mice had no difference in liver weight (Figure 3.6C), however, H&E staining showed a significant inflammatory
cell infiltration and foci of liver necrosis around the central vein (Figure 3.6D). In
the lung of bone marrow TTP deficient mice, there was a great deal of inflammatory
cell infiltration surrounding the vasculature, indicating the movement and migration
of the inflammatory cells. In addition, these mice exhibit more inflammatory
exudate in the alveolar space compared to the control group (Figure 3.6E). The
spleen showed a loss of the normal organization with no clear demarcation
between the red and the white pulp due to inflammatory infiltration (Figure 3.6F).

To further define the effect of bone marrow TTP deficiency on systemic
inflammation, we performed ELISA assays to detect serum TNF-α levels. The
serum TNF-α concentration was increased in the bone marrow TTP deficient mice
compared to control mice $p$-value 0.0004, further indicating that these mice have
systemic inflammation (Figure 3.6G). Taken together, these data indicate that bone
marrow TTP deficiency in LDLR$^{-/-}$ mice significantly increased systemic and multi-
organ inflammation.

**TTP deficiency in bone marrow-derived cells does not affect atherosclerosis
in LDLR$^{-/-}$ mice**

In order to assess the effect of bone marrow TTP deficiency on
atherosclerosis, we fed the chimeric mice a western diet for 12 weeks and
quantified atherosclerotic lesion formation and parameters of plaque complexity.
Even though the bone marrow TTP deficient mice have a systemic inflammation,
the bone marrow TTP deficiency did not significantly modify the atherosclerotic
lesion size (Figure 3.7A). Similarly, the lipid contents in the aortic root were found
to be the same (Figure 3.7B); there was no significant difference in the
macrophage content (Figure 3.7C) and collagen percentage (Figure 3.7D) in bone marrow TTP deficient compared with the control mice. These results indicated that the bone marrow TTP deficiency did not result in alteration of atherosclerosis in LDLR−/− mice.

**TTP deficiency in bone marrow-derived cells reduces plasma lipid and attenuated hepatic steatosis**

Atherosclerosis is both a lipid deposition disorder and a chronic inflammatory disease\(^{166}\). Previous studies have shown that pro-inflammatory signaling in pre-clinical models does affect lipid metabolism\(^{167}\). Hyperlipidemia is usually associated with systemic inflammation\(^ {168}\). Next, we investigated the effect of bone marrow TTP deficiency on serum lipid. Serum total cholesterol and triglyceride levels were measured at the indicated time points. Interestingly, after 8 weeks of western diet the bone marrow TTP deficient mice had dramatically reduced serum total cholesterol and triglyceride levels compared to the control mice \(p\)-value 0.0001, 0.0006 respectively (Figures 3.8A and 3.8B). In agreement with 8 weeks western diet, at the experimental endpoint, bone marrow TTP deficient mice showed a significant reduction in serum cholesterol and triglyceride \(p\)-value 0.0009, 0.0022 respectively (Figures 3.8C and 3.8D). Fast Protein Liquid Chromatography (FPLC) analyses of serum lipoproteins showed the difference in very low-density lipoprotein (VLDL), LDL, and high-density lipoprotein (HDL) cholesterol peaks between bone marrow TTP deficient mice and control mice (Figures 2.8E and 2.8F). Interestingly, bone marrow TTP deficient mice exhibited significantly lower VLDL cholesterol levels, whereas no difference was observed
in LDL and HDL cholesterol. The liver is the most important organ involved in lipid metabolism\textsuperscript{169}; we measured the liver lipid content of the frozen liver sections using the Oil-Red-O (ORO) staining and showed that hepatocytes of bone marrow TTP deficient mice had a dramatically reduced lipid accumulation compared to the levels in control mice (Figure 3.8G). Additionally, cholesterol excretion in the feces were significantly decreased in bone marrow TTP deficient mice p-value<0.0001 (Figure 3.8H). We also measured the blood glucose levels and the body temperature at the end point. No significant differences in these parameters were detected between the two groups (Figures 3.8I and 3.8J). Collectively, these data suggest that the bone marrow TTP deficiency in atherogenic LDLR\textsuperscript{−/−} mice dramatically reduced plasma total cholesterol levels particularly in VLDL fraction, and hepatic steatosis.

**TTP deficiency in bone marrow-derived cells alters expression of liver genes involved in inflammation and lipid metabolism**

To gain further insight into the genes related to hepatic lipid metabolism and inflammation in bone marrow TTP deficient mice and to further explore the underlying molecular mechanisms for the above observed phenotype, a microarray gene analysis was performed on mRNA isolated from liver of bone marrow TTP deficient mice and control mice. We found that bone marrow TTP deficiency significantly modulated 236 genes with corrected \( p \)-value cut-off:0.05 and an absolute fold change >2.00 (Figure 3.9A). Of the 236 genes, there were 27 genes involved in lipid metabolism, and inflammation that were significantly changed in bone marrow TTP deficient mice compared to mice with WT bone
marrow cells (Figure 3.9B). Furthermore, the lipid related genes such as sterol regulatory element binding transcription factor-1 (SREBF-1), which controls the expression of enzymes involved in fatty acid and cholesterol biosynthesis were significantly downregulated whereas the genes involved in modulating immune response and inflammation such as Serum Amyloid A1 (SAA1), CC chemokine receptor-2 (CCR2) were highly expressed in the livers of bone marrow TTP deficient mice.

To confirm the gene expression patterns identified by the microarray analysis, qRT-PCR experiments for the mRNA expression of selected genes (SAA1, CCR2, and SREBF1) were performed. Indeed the expression of several genes from microarray data were validated by qRT-PCR. SREBF1 was significantly down-regulated in the liver of bone marrow TTP deficient mice; however, the mRNA expression of the inflammatory CCR2 and SAA1 genes were significantly upregulated p-value<0.05 (Figure 3.9C).

Taken together, although we did not analyze in detail all of the differentially expressed genes related to lipid metabolism, our study indicated that many key genes related to lipid metabolism were influenced by bone marrow TTP deficiency, suggesting that the bone marrow TTP deficiency could change the expression of lipid metabolism genes particularly SREBP1 resulting in decreased lipid production by the liver and subsequently reduced serum lipid, hepatic steatosis, and lipid excretion.
Effect of macrophage-specific TTP deficiency on inflammation and atherosclerosis development

Macrophages play a central role in the initial step of atherosclerotic lesion formation\textsuperscript{171}. To evaluate the contribution of macrophage TTP to atherosclerosis development in LDLR\textsuperscript{−/−} mice, we utilized bone marrow transplantation studies to generate a macrophage-specific TTP deficient mouse model. We transplanted atherosclerosis susceptible six to eight week-old female LDLR\textsuperscript{−/−} mice after lethal irradiation with bone marrow from either macrophage-specific TTP knockout (LysM-cre/TTP\textsuperscript{fl/fl}, mac-TTP\textsuperscript{−/−}) or control (TTP\textsuperscript{fl/fl}, WT) mice. After a recovery period of 4 weeks on regular murine chow diet, the transplanted mice were challenged with a western diet for 9 weeks to induce atherosclerosis (Figure 3.1B). Western blot analysis and PCR were utilized to confirm that the reconstitution of the macrophage-specific TTP deficient bone marrow was efficient indicated by the lack of expression of TTP in the resident peritoneal macrophages (Figures 3.10A and 3.10B). During western diet period, mice body weight and food intake were monitored weekly. Throughout the experiment, macrophage-specific TTP deficient mice and control mice showed a similar food intake (Figure 3.11A) while the weight gain slightly reduced in macrophage-specific TTP deficient mice (Figure 3.11B). In addition, there were no differences in the spleen weight between the two groups (Figure 3.12A). Flow cytometric analysis of splenocytes showed no difference in leukocyte population (Figure 3.12B). To further assess the influence of macrophage-specific TTP deficiency on systemic inflammation, we utilized the Bio-Plex Assays to measure the levels of cytokine/chemokines in the serum. We found
that macrophage-specific TTP deficiency significantly increased the plasma levels interleukin-12 (IL12-P40), one of the known TTP targets (Figure 3.12C). At the experimental endpoint, we measured serum total cholesterol (Figure 3.13A), triglyceride (Figure 3.13B) and lipoprotein fractions (Figure 3.13C). Interestingly, macrophage-specific TTP deficiency had no significant effects on serum cholesterol and triglyceride as well as lipoprotein fractions. After overnight fasting, these mice also showed similar blood glucose levels (Figure 3.13D).

To investigate the effect of macrophage-specific TTP deficiency on atherosclerotic lesion development, en face aortic atherosclerotic lesion and aortic root lesion sizes were quantified after 9 weeks of western diet. Macrophage-specific TTP deficiency did not significantly affect atherosclerotic lesion size as compared to the control mice (Figures 3.14A and 3.14B). Interestingly, ORO staining of the aortic root showed a significant reduction in the lipid accumulation in macrophage-specific TTP deficient mice p-value 0.009 (Figure 3.14C). Moreover, the macrophage infiltration in the atherosclerotic lesion was significantly reduced p-value 0.022 (Figure 3.14D). The plaque stability was also assessed by using trichrome staining of the aortic root section, and we found that there was no difference in the collagen percentage with macrophage-specific TTP deficiency (Figure 3.14E).

3.4 Discussion

TTP is a member of CCCH tandem zinc finger proteins family. It binds via its tandem zinc finger domain to AU rich elements (AREs) found in 3’ UTR of the target mRNAs such as that of TNF-α, resulting in deadenylatation and destabilization
of the target mRNAs\textsuperscript{164,172}. TTP has been demonstrated to play a significant role in controlling inflammation in the vascular endothelial cells\textsuperscript{159}.

Previous studies demonstrated that TTP deficiency resulted in development of a complex syndrome of myeloid hyperplasia with cachexia, skin lesions, arthritis, conjunctivitis, kidney lesions, and that treatment with TNF-\(\alpha\) antibodies\textsuperscript{161} or interbreeding the mice with TNF-R deficient mice\textsuperscript{173} could attenuate development of above inflammatory phenotype in the TTP deficient mice. Transplantation of TTP deficient hematopoietic cells reproduces the whole body TTP knockout inflammatory syndrome in RAG-2 deficient mice, indicating that hematopoietic cells contribute dominantly to the phenotype\textsuperscript{153}. However, the effects of bone marrow TTP deficiency on atherosclerosis have not been studied. In our study, we transplanted bone marrow cells from either TTP knockout mice or WT mice into LDLR\textsuperscript{−/−} mice, and we wanted to test if the bone marrow transplantation of TTP deficient cells could transfer the inflammatory phenotype of TTP knockout mice into recipient atherogenic LDLR\textsuperscript{−/−} mice and to examine the impact of bone marrow TTP deficiency on atherosclerosis development. TTP has anti-inflammatory properties, and inflammation plays a significant role in the development of atherosclerosis\textsuperscript{174}; therefore, we expected that the bone marrow TTP deficiency would increase inflammation and lead to increased atherosclerosis in hyperlipidemic LDLR\textsuperscript{−/−} mice. First we studied the inflammatory phenotype in bone marrow TTP deficient mice and found that TTP deficient bone marrow transplantation into LDLR\textsuperscript{−/−} mice resulted in all aspects of TTP knockout syndrome including: growth retardation, systemic and multi-organ inflammation, suggesting
that hematopoietic progenitors are responsible for the development of the inflammatory syndrome. We then aimed to determine whether bone marrow TTP deficiency affects atherosclerosis development. A comparable lesion size in the aortic root was observed between bone marrow TTP deficient mice and control mice. Plaque composition analysis showed no effects of bone marrow TTP deficiency on the lipid content or macrophage content either, as well as amount of collagen. Then we evaluated the effect of bone marrow TTP deficiency on lipid profile and found that these mice had a significant reduction in plasma lipid and hepatic steatosis. Increased inflammation and reduced plasma lipid might thus counteract each other leaving atherosclerosis development unchanged in bone marrow TTP deficient mice compared to bone marrow WT mice. Previous studies demonstrated the inflammatory phenotype of TTP knockout mice, and that transplantation of TTP deficient bone marrow cells into RAG2 deficient mice could transfer all aspects of TTP deficiency syndrome. We, for the first time, show that bone marrow TTP deficiency significantly increased inflammation, reduced plasma lipid, and attenuated hepatic steatosis, but did not affect western diet-induced atherosclerotic lesion development in LDLR−/− mice. Bone marrow TTP deficient mice had lower body weight than control mice; these mice also displayed hematopoietic abnormalities including slightly elevated white blood cell count, increased absolute number and percentage of neutrophils, increased absolute number and percentage of blood monocytes. The peripheral red blood cell count, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin were not different between the two groups. However, the platelet count, and
platelet hematocrit were significantly increased \((P=0.0107, P=0.0054\) respectively) in bone marrow TTP deficient mice. Flow cytometric analysis of the leukocyte population in the spleen revealed that CD3+/CD4+ T cell numbers and percentage decreased significantly with bone marrow TTP deficiency, whereas the number and percentage of neutrophils and inflammatory monocytes were increased significantly in bone marrow TTP deficient mice compared to the control mice. No difference was observed in the B lymphocytes between the two groups. In addition, the isolated peritoneal macrophages were cultured and treated with LPS (TLR4 ligand), and shown to have a significantly higher expression of pro-inflammatory cytokines, TNF-α and IL-6, indicating that TTP deficiency increased responsiveness of macrophages to LPS stimulation. The most striking observed feature of TTP deficiency is the diffuse multi-organ inflammation\(^{161,162}\). Our data demonstrated that the bone marrow TTP deficiency results in inflammatory cell infiltration in multiple organs including liver and lung. The spleen weight of bone marrow TTP deficient mice was slightly enlarged compared to the control mice. In addition, microscopically there is a disturbed morphology of the spleen due to a large inflammatory cell infiltration in bone marrow TTP deficient mice. Those mice also had increased plasma TNF-α levels. We expected that, with the increase in inflammation caused by bone marrow TTP deficiency, more severe atherosclerosis would develop in these mice. However, bone marrow TTP deficiency did not increase atherosclerosis in LDLR\(^{-/-}\) mice after 12 weeks of western diet feeding. Interestingly, the plasma total cholesterol and triglyceride levels, especially those in the VLDL fractions were significantly reduced in bone
marrow TTP deficient mice. Additionally, these mice showed significantly decreased hepatic lipid accumulation (hepatic steatosis) compared to bone marrow WT mice.

To gain further insight into the genes related to lipid metabolism and inflammation in the bone marrow TTP deficient mice, and to understand the underlying molecular mechanism by which bone marrow TTP deficiency reduces liver lipid production, we performed a microarray analysis to compare the liver gene expression patterns of these mice. We found that there were 236 genes significantly changed with bone marrow TTP deficiency; among these there were 27 genes involved in lipid metabolism and inflammation that were significantly up or down regulated with bone marrow TTP deficiency. Those down-regulated genes tend to regulate cholesterol and fatty acid metabolism such as SREBF1 and ABCG8, whereas up-regulated genes were involved in inflammation such as CCR2, SAA1, and Calgranulin A (S100A8) a biomarker for chronic inflammatory diseases\textsuperscript{175}. SREBF-1, a key transcription factor regulating lipogenic enzymes\textsuperscript{170}.

To further dissect the role of macrophage TTP in atherosclerosis development, we transplanted the bone marrow cells from macrophage-specific TTP knockout mice or WT mice to LDLR\textsuperscript{−/−} mice. After 4 weeks of hematopoietic reconstitution followed by 9 weeks of western diet, the serum concentration of IL-12P(40) was the only cytokine significantly increased in macrophage-specific TTP deficient mice than the control. Furthermore, no significant change in serum cholesterol and triglyceride levels were observed with macrophage-specific TTP deficiency. Finally, the atherosclerotic lesion size in LDLR\textsuperscript{−/−} mice was unchanged.
However, macrophage-specific TTP deficiency resulted in significant changes in lesion composition during the initial stages of plaque development. This occurred through reduction in macrophage infiltration, and lipid content. Overall, our data demonstrated that loss of macrophage-specific TTP expression in LDLR\(^{-/-}\) mice results in unchanged atherosclerosis indicating that TTP expression in macrophages does not play an important role in atherogenesis.

In conclusion, our study provides evidence that bone marrow TTP deficiency in LDLR\(^{-/-}\) mice results in significantly increased inflammation and attenuated hyperlipidemia. The increased systemic inflammation and reduced serum lipid levels in bone marrow TTP deficient mice offset each other and resulted in no significant change in the atherosclerosis development in mice. Although it is unknown if TTP directly acts on lipoprotein metabolism related genes, it is intriguing to hypothesize that targeting certain components of immune system and inflammation may have unexpected effects on hepatic lipoprotein metabolism. This aspect is a subject for further investigation. In addition, our results demonstrate that macrophage-specific TTP deficiency had less robust effects than whole bone marrow cell TTP deficiency on inflammation and serum lipid, suggesting TTP expression in other bone marrow-derived cells contributes more to inflammation and lipoprotein metabolism.
Table 3. 1 Primers that were used for qRT-PCR in this study

<table>
<thead>
<tr>
<th>Primers</th>
<th>Forward (5’ → 3’)</th>
<th>Reverse (5’ → 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>m18s</td>
<td>CGCGGTTCTATTTTGTGTT</td>
<td>AGTCGGCATCGTTTTATGGTC</td>
</tr>
<tr>
<td>hTTP</td>
<td>TACGAGAGCCTCCAGTGCAT</td>
<td>GCGAAGTGGTGAGGGTGAC</td>
</tr>
<tr>
<td>mTNF-α</td>
<td>CGT CAG CCG ATT TGC TAT CT</td>
<td>CGGACTCCGAAAGTCAAAG</td>
</tr>
<tr>
<td>mIL6</td>
<td>AGTTGCCTTCTTGGGACTGA</td>
<td>TCCACGATTTCCAGAGAAC</td>
</tr>
<tr>
<td>mSREBF 1</td>
<td>GCCCACAATGCCATGGA</td>
<td>GCAAGAAGCGGATGTCGAT</td>
</tr>
<tr>
<td>mCCR2</td>
<td>ATTCTCACCACCTGTGTTGC</td>
<td>GATTCCTGGAAGGTGGTC</td>
</tr>
</tbody>
</table>
Figure 3. 1 In vivo experimental design, mouse model and bone marrow transplantation used for assessment of atherosclerosis

(A) six to eight week-old female LDLR−/− mice were provided with autoclaved antibiotics water containing neomycin (100 mg/L) and polymyxin B sulphate (60,000 U/L) one week before and two weeks after bone marrow transplantation. Mice received total body irradiation. Bone marrow cells were isolated from TTP knockout mice or control mice and 5 x 10⁶ cells injected into LDLR−/− mouse in the retro-orbital plexus. Four weeks after the BMT, mice were fed a western diet for a period of 12 weeks, then the mice were sacrificed for atherosclerosis analysis. (B) six to eight week-old female LDLR−/− mice were provided with autoclaved antibiotics water one week before and two weeks after BMT. Mice received total body irradiation. Bone marrow cells were isolated from macrophage-specific TTP deficient mice or control mice and 5 x 10⁶ cells injected into LDLR−/− mouse. Four weeks after BMT, mice were fed a western diet for 9 weeks, then the mice were sacrificed for atherosclerosis analysis.
Figure 3. 2 TTP expression is increased in human carotid atherosclerotic lesions

TTP expression in human carotid artery was analyzed by qRT-PCR. Expression levels of TTP were significantly increased in carotid atherosclerotic lesions compared to those in the non-lesion carotid medial tissue. Results were represented as ΔCt values after being normalized to the expression of 18s rRNA. ΔCt is the difference in threshold cycle between the gene of interest and the housekeeping gene (18s). Each line connects data from a same patient. (*p<0.05).
Figure 3. Bone marrow TTP deficiency causes growth retardation, but did not affect food intake

Six to eight week-old female LDLR<sup>−/−</sup> mice were transplanted with TTP knockout or WT bone marrow cells. Four weeks after BMT, mice were fed a western diet for 12 weeks. (A) Food intake and (B) body weight were monitored weekly. The average daily intake of western diet per mouse was calculated. Data are presented as mean ± SEM.
Figure 3. 4 Bone marrow TTP deficiency causes hematological disturbance
VetScan hematology analyzer was utilized to measure the following parameters: (A) white blood cell and white blood cells differential counts in peripheral blood at the experimental endpoint. (B) Total platelet number, mean platelet volume, platelet distribution width (PDWc), and the platelet hematocrit. (C) Red blood cell (RBC) number, hemoglobin (HGB) concentration and hematocrit (HCT), mean corpuscular volume, mean corpuscular hemoglobin concentration (MCHC), red cell distribution width (RDWc). All data were presented as the mean ± SEM.
Figure 3. 5 Bone marrow TTP deficiency causes immunological disturbance
Spleens from bone marrow TTP deficient mice and bone marrow WT mice were collected at experimental end point. A single cell suspension was made, and the cells were stained with different antibodies to detect different mouse leukocyte populations by flow cytometry. (A) Absolute number and (B) percentage of leukocytes in the spleen. (C) Spleen weight of LDLR−/− mice that received WT or TTP deficient bone marrow cells. (D) Total spleen cell numbers of LDLR−/− mice that received WT or TTP deficient bone marrow cells. (*P<0.05; **P<0.01).
Figure 3. 6 TTP deficiency increased macrophage responses to LPS stimulation, and bone marrow TTP deficiency resulted in extensive inflammatory tissue damage and systemic inflammation

Residential peritoneal macrophages were obtained from mice at the experimental endpoint. The cells were treated with LPS (50 ng/mL) for 6 h. Total mRNA was extracted for qRT-PCR measurement of the inflammatory cytokine expression (A) TNF-α, (B) IL-6 (N=3 for each group). (C) Liver weight of LDLR<sup>−/−</sup> mice received WT or TTP deficient bone marrow cells. Representative photomicrographs showing H&E staining in the (D) liver (20x), (E) lung (20x), and (F) spleen (10x) in wild-type recipient mice (right panels) and TTP deficiency recipient mice (left panels). (G) Serum TNF-α concentrations of bone marrow TTP deficient mice and control mice were determined by ELISA. Data were analyzed by Student’s t test. *<i>p < 0.05</i>; **<i>p < 0.01</i>; ***<i>p < 0.001</i>. 
Figure 3. 7 Bone marrow TTP deficiency had no impact on atherosclerosis after 12 weeks of western diet in LDLR$^{-/-}$ mice

To induce atherosclerosis, bone marrow transplanted LDLR$^{-/-}$ mice were fed a western diet for 12 weeks. The aortic roots were embedded in OCT and frozen at -20 °C. For analysis of atherosclerosis, 10 µm thick sections were collected. (A) The aortic root lesion area of the frozen sections was detected by H&E staining and quantified by Image-ProPlus 6.0. The representative images and quantitative analysis are shown. (B) Lipid contents in the aortic root lesions were determined by ORO staining. The representative images and quantitative analysis are shown. (C) The areas of macrophages in the aortic root lesions were determined by immunostaining for MOMA-2 (a macrophage marker). The representative images and quantitative analysis are shown. (D) Collagen content in the aortic root lesions was determined by masson’s trichrome staining. The representative images and quantitative analysis are shown.
Figure 3. 8 Bone marrow TTP deficiency markedly reduced serum lipid levels and attenuated hepatic steatosis

Mouse serum cholesterol (A) and triglyceride (B) levels were measured after 8 weeks of western diet. Mouse serum cholesterol (C) and triglyceride (D) levels
were measured at the experimental end point. (E, F) Pooled serum samples from mice at the experimental endpoint were analyzed for the lipoprotein profile using FPLC analysis. Cholesterol or triglyceride content in each fraction was determined. Data represent the mean ±SEM. (G) Representative images and quantification of ORO stained liver sections. (H) Fecal cholesterol excretion was detected at the experimental end point. (I) Fasting blood glucose levels of mice at the experimental end point were measured. (J) Body temperatures at the experimental end point were measured. *p < 0.05; **p < 0.01; ***p < 0.001.
Figure 3. 9 Bone marrow TTP deficiency alters expression of liver genes involved in inflammation and lipid metabolism
(A) Heat map showing the expression of 236 genes significantly changed in the liver of bone marrow TTP deficient mice compared to control mice (2.00 fold
change cutoff). (B) Heat map of 27 genes involved in inflammation and lipid metabolism significantly changed with bone marrow TTP deficiency. Color coding indicates increased gene expression in red, and decreased expression in green. The columns and rows in the heat maps represent samples and genes, respectively. (C) Quantitative real-time RT-PCR analysis of liver mRNA was used to verify the results of selected genes from microarray data.
Figure 3. 10 Efficiency of macrophage-specific TTP deficiency in the bone marrow transplantation model
Thioglycollate-elicited peritoneal macrophages were lysed in Trizol reagent for mRNA isolation and PCR analysis or RIPA buffer for protein isolation and western blot analysis. Western blot and PCR were utilized to detect TTP protein (A) and TTP mRNA (B) expression.
Figure 3. 11 macrophage-specific TTP deficiency did not affect mouse food intake, but reduced body weight
LDLR\(^{-/-}\) mice were transplanted with WT or macrophage-specific TTP deficient bone marrow cells. Four weeks after bone marrow transplantation, mice were fed a western diet for 9 weeks. (A) Food intake and (B) body weight were monitored weekly. The average daily intake of western diet per mouse was calculated. Data are presented as mean ± SEM.
Figure 3. 12 Effect of macrophage-specific TTP deficiency on inflammation
(A) Spleen weight of LDLR−/− mice that received WT or macrophage-specific TTP deficient bone marrow cells at the experimental endpoint. (B) Flow cytometry analysis of leukocyte population in the spleen. Percentage of each subset of cells were shown. Data are represented as the mean ± SEM. (C) Serum levels of 23 chemokine/cytokines were measured by Bio-Plex Pro™ Mouse Cytokine 23-plex Assay. Data are presented as the mean ± SEM. *P<0.05.
Figure 3. 13 Effect of macrophage-specific TTP deficiency on plasma lipid levels and lipoprotein profile

LDLR−/− mice reconstituted with WT or macrophage-specific TTP deficient bone marrow cells were fed a western diet for 9 weeks. Blood samples were drawn after overnight fasting. The concentrations of cholesterol (A) and triglycerides (B) in serum were determined using enzymatic colorimetric assays. (C) Plasma lipoproteins profile was determined by FPLC analysis. Fractions represent VLDL, LDL, and HDL, respectively. (D) Fast blood glucose levels after were determined.
Figure 3. 14 Effect of macrophage-specific TTP deficiency on atherosclerosis
(A) Representative image of the aorta stained with Sudan IV and quantification of the surface area occupied by the lesions. (B) Representative images and quantitative analysis of atherosclerotic lesion size in the aortic root of WT and macrophage-specific TTP deficient mice. (C) Representative images and quantitative analysis of ORO stained aortic root. **p<0.001. (D) Representative images and quantitative analysis of MOMA2 stained area in the aortic root. *P<0.05 (E) Representative images and quantitative analysis of collagen content in the aortic root.
Chapter IV
The effect of macrophage-specific GP-96 deficiency on inflammation and atherosclerosis development

4.1 Background

Glycoprotein-96 (GP-96), also known as glucose regulated protein-94 (GRP-94), endoplasmic, or 99-kDa endoplasmic reticulum protein (ERP99), is an endoplasmic reticulum (ER)-resident chaperone/stress protein\textsuperscript{176}. GP-96 is expressed in various cell types, and its expression can be upregulated by accumulation of misfolded proteins and other stress conditions that leads to disturbance in the ER functions\textsuperscript{177,178}. Initially, GP-96 was discovered as a protein strongly induced by glucose starvation in hamster fibroblasts as a major endoplasmic reticulum calcium-binding protein\textsuperscript{179}. Recently, the role of GP-96 in innate immunity, inflammation and oncogenesis started to be appreciated\textsuperscript{180,181}.

GP-96 plays a major role in maintaining protein homeostasis by catalyzes the folding of the newly synthesized proteins such as Toll like receptors (TLRs), Wnt co-receptor LRP6, insulin-like growth factors, immunoglobulins (Ig), and some integrins\textsuperscript{180,182,183}. Global deletion of GP-96 is embryonically lethal and not compatible with life because it is responsible for chaperoning of multiple proteins induction and muscle development\textsuperscript{183}.

To study the effect of GP-96 on TLR expression and the immune function, myeloid-specific GP96 deficient mice were generated and extensively studied.
LysM-Cre/ GP-96^{fl/fl} is highly macrophage selective, without affecting other cell populations\textsuperscript{180}. Macrophage-specific GP-96 deficient mice have normal hematopoiesis and normal immune cell numbers (e.g. macrophages, dendritic cells, and leukocytes). \textit{In-vivo} studies revealed that macrophage-specific GP-96 deficient mice had a significant decrease in the production of pro-inflammatory cytokines and were relatively resistant to endotoxic shock induced by LPS injection (TLR4 ligand). GP-96 is capable of activating the TLR signaling pathways determined by the ability to induce pro-inflammatory cytokine/chemokines. \textit{In vitro} studies demonstrated that macrophages isolated from macrophage-specific GP-96 deficient mice significantly reduced production of pro-inflammatory cytokines such as TNF-\(\alpha\) upon stimulation with TLRs agonist, indicating the role of GP-96 in TLR expression\textsuperscript{180,182}.

Toll like receptors (TLRs) are the best characterized pattern recognition receptors (PRRs). They are prominently involved in the first line defense against infection by recognizing conserved motifs expressed by pathogens called pathogen-associated molecular patterns (PAMPs) and thereby initiate the innate immune response. TLRs are highly expressed by a number of immune cells such as macrophages and dendritic cells (DCs) as well as non-immune cells such as epithelial cells and fibroblasts\textsuperscript{184,185}. To date, thirteen functional TLRs have been identified in mouse that are either expressed on the cell surface and recognize microbial membrane components (e.g. TLR1, TLR2, TLR4, TLR5, TLR6, TLR11, and TLR12) or are associated with intracellular vesicles and recognizes nucleic acids (e.g. TLR3, TLR7, TLR8, TLR9 and TLR13)\textsuperscript{186}. The role of TLRs in
atherosclerosis development is still controversial. Recent evidence suggests that TLRs have both protective and detrimental roles in atherosclerosis. Previous study suggests a detrimental role for TLR signaling in atherosclerosis, demonstrating that the whole-body deficiency of MyD88 reduced both atherosclerotic lesion development and macrophage accumulation in apoE<sup>−/−</sup> mice. This study was confirmed and followed by another study demonstrating that whole body TLR2/TLR4 deficiency in murine models of atherosclerosis results in a significant reduction in atherosclerotic lesion formation via inhibition of lipid deposition, inflammatory cell recruitment and activation<sup>187,188</sup>. Other studies showed the athero-protective functions of TLRs in atherosclerosis<sup>189,190</sup>.

Blocking TLRs signaling in the macrophage is considered to be a promising strategy to halt the inflammation and attenuate atherosclerosis. While GP-96 has been shown to modulate both the innate and adaptive immune systems by controlling most TLRs, the role of GP-96 in atherosclerosis has not been investigated. To address the role of GP-96 in immune responses during atherogenesis, we transplanted macrophage-specific GP-96 deficient or WT bone marrow cells into LDLR<sup>−/−</sup> mice and fed them a western diet for 9 weeks. Recipient mice reconstituted with macrophage-specific GP-96 deficient bone marrow cells had no significant difference in inflammation compared with control mice transplanted with WT bone marrow cells. In addition, macrophage-specific GP-96 deficient mice displayed a comparable serum lipid, resulting in unchanged atherosclerosis in LDLR<sup>−/−</sup> mice.
4.2 Material and Methods

Mice

Six to eight week-old female LDLR<sup>−/−</sup> mice (C57BL/6J background) were purchased from the Jackson Laboratory (Bar Harbor, MA, USA). Mice were maintained in pathogen-free conditions at the University of South Carolina Animal Research Facility according to National Institutes of Health (NIH) guidelines. All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of South Carolina.

Bone Marrow Transplantation (BMT) and atherosclerosis induction

BMT was performed as previously described in chapter 2. Briefly, six to eight week-old female LDLR<sup>−/−</sup> mice were maintained on antibiotic water containing 100 mg/L neomycin and 10 mg/L polymyxin B, one week before and 2 weeks after BMT. Six hours before BMT, recipient mice were lethally irradiated (900 rad) using a cesium gamma source. Bone marrow cells were harvested by flushing both femurs and tibias from WT or macrophage-specific GP-96 deficient mice (kindly provided by Dr. Zihai Li at the Medical University of South Carolina) with sterile RPMI-1640 medium (Invitrogen Life Technologies, Grand Island, NY, USA) containing 2% fetal bovine serum (Invitrogen, Grand Island, NY, USA) and heparin (5 units/mL, Sigma, St. Louis, MO, USA). The cell suspension was centrifuged for 5 minutes at 300 x g and re-suspended in ice cold phosphate buffer saline (PBS). Five million bone marrow cells from donor mice were injected into the retro-orbital venous plexus of the recipient irradiated LDLR<sup>−/−</sup> mice. After 4 weeks recovery, mice were fed a western diet containing 21% (w/v) anhydrous
milkfat, 34% (w/v) sucrose, and a total of 0.2% (w/v) cholesterol (Harlan laboratories; Indianapolis, IN, USA) for a duration of 9 weeks, after which mice were sacrificed (Figure 4.1). During a western diet period, mouse food intake and body weight were measured weekly throughout the study.

**Peritoneal macrophage isolation and culture**

Mice were injected i.p. with 3 mL of 3% (w/v) thioglycolate solution (BD Biosciences Clontech; Palo Alto CA, USA). Three days later, mice were anesthetized with isoflurane, and macrophages were obtained by washing peritoneal cavity with 10 mL of cold PBS twice. The cells were then re-suspended in high-glucose Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS), a combination of penicillin/streptomycin (Sigma-Aldrich, St. Louis, MO, USA), and 50 μM of β-mercaptoethanol. After incubation for 2 hours at 37°C, non-adherent cells were washed away with PBS, and adherent cells were cultured overnight in serum free DMEM. Then macrophages were treated with DMEM containing LPS (50 ng/mL, Sigma-Aldrich, St. Louis, MO, USA) for 6 hours. Next, cells were washed with Dulbecco’s phosphate-buffered saline (DPBS) twice before being lysed for total RNA or protein extraction.

**Assessment of successful bone marrow reconstitution**

The success of bone marrow reconstitution of LDLR−/− mice was determined at the experiment endpoint. Isolated peritoneal macrophages were lysed in Trizol reagent for PCR analysis or in RIPA buffer for western blot analysis. GP-96 mRNA and protein levels were detected.
RNA extraction, cDNA synthesis, and quantitative real-time PCR

Total RNA was extracted using TRIzol reagent (Life Technologies, Grand Island, NY, USA) according to the standard protocol. One microgram of RNA of each sample was reverse transcribed into cDNA using iScript™ cDNA Synthesis Kit (Bio-Rad Life Science, Hercules, CA). qRT-PCR was performed using iQ™ SYBR® Green Supermix (Bio-Rad, Hercules, CA, USA) according to the manufacturers’ instructions. The relative amount of target mRNA was determined using the comparative threshold (Ct) method by normalizing target mRNA Ct values to those of 18S RNA. PCR thermal cycling conditions were 3 min at 95°C, and 40 cycles of 15 s at 95°C and 58 s at 60°C. Samples were run in triplicate. The primers used for qRT-PCR are shown in table 4.1.

Western blot analysis

After 9 weeks of western diet, peritoneal macrophages were isolated, cultured, and incubated in the absence or presence of LPS (50 ng/mL) for 24 hours. To obtain the total cell lysates, the attached cells were incubated and lysed for 30 min on ice in RIPA buffer (Pierce™, Rockford, IL, USA) supplemented with protease inhibitor cocktail and phosphatase inhibitors (Sigma-Aldrich, St. Louis, MO, USA). Equal amounts of total cell proteins (30 µg) were separated in 4-20 % SDS-PAGE pre-cast gels (Bio-Rad, Hercules, CA, USA) for electrophoresis and transferred onto Nitrocellulose membranes (Millipore Corp., Bedford, MA, USA). After blocking with 5% non-fat dry milk, primary antibodies and HRP-conjugated secondary antibodies were used to detect target proteins. Signal was detected using Pierce ECL Western Blotting Substrate (Pierce™, Rockford, IL, USA). For
stripping, membranes were submerged for 30 minutes at 55 – 60°C in a buffer containing 100 mM 2-mercaptoethanol, 2% SDS and 62.5 mM Tris-HCl, pH 6.7, and washed three times with PBST.

**Serum lipid and lipoprotein distribution analysis**

After an overnight fast, ~ 500 µL blood was collected from each mouse by retro-orbital venous plexus puncture and centrifuged at 4,000 x g for 20 min. Serum total cholesterol and triglyceride levels were determined by enzymatic colorimetric assays with Cholesterol Reagent and Triglycerides GPO reagent kits (Raichem, San Diego, CA, USA), and analyzed by SoftMax Pro5 software (Molecular Devices, Sunnyvale CA, USA). Serum lipoproteins were determined using a fast-performance liquid chromatography (FPLC) system (AKTA purifier, GE Healthcare Biosciences, Pittsburgh, PA, USA) equipped with a Superose 6 10/300 GL column (GE Healthcare). Pooled mouse plasma (100 µL) was loaded into the column, and eluted at a constant flow rate of 0.5 mL/min with 1 mM sodium EDTA and 0.15 M NaCl. Fractions of 0.5 mL were collected and cholesterol concentration from each fraction was measured.

**Blood glucose measurement**

OneTouch Ultra Blood Glucose Monitoring System (LifeScan, Inc., Milpitas, CA, USA) was used to measure the blood glucose concentration following the manufacturer’s instructions.

**Flow cytometry analysis**

After 9 weeks of western diet feeding, the mice (not thioglycollate stimulated) were sacrificed. Spleens were obtained and smashed in 10 mL RPMI
1640 medium supplemented with 10% FBS using Stomacher® 80 Biomaster (Seward Laboratory Systems Inc., Port St. Lucie, FL, USA). Red blood cells in spleen samples were lysed using red blood cell lysing buffer (Sigma-Aldrich, St. Louis, MO, USA). To obtain single cell suspensions, tissue were passed through a 70 µM cell strainer (Life Sciences, Tewksbury, MA, USA). Cells were fixed, permeabilized, and stained for 30 min on ice in dark using the following antibodies: PE-anti-CD3, FITC-anti-CD4, FITC-anti-CD8a, FITC-anti-CD19, FITC-anti-CD11c, PE-anti-CD11b, FITC-anti-Ly6C, FITC-anti-Ly6G, and FITC- anti -F4/80 mAb (eBioscience™, Invitrogen) in staining buffer. Stained cells were acquired on a Cytomics FC 500 flow cytometer and further analyzed with CXP software version 2.2 (Beckman Coulter, Brea, CA, USA). Data were collected for 10,000 live events per sample.

Bioplex analysis of serum cytokine/chemokines

Serum levels of 23 cytokine/chemokines from mice were measured using a Bio-Plex Pro™ Mouse Cytokine 23-plex Assay (Bio-Rad, Hercules, CA, USA) on a Bio-Plex system following the manufacturer’s instructions.

Atherosclerosis analysis

To induce atherosclerosis, mice were fed a western diet for 9 weeks. At the experimental endpoint, mice were sacrificed and their vasculature perfused with 10 mL of cold PBS via cardiac puncture to remove blood contamination from vascular tissue. Hearts and aortas were dissected, and the aortic root was isolated and embedded in OCT compound in a plastic mold, frozen at -20 °C, and cut into 10 µm sections. For the aortic en face assay, aortas were fixed in 10% neutral
buffered formalin, cleaned, opened longitudinally, pinned onto black wax plates, and stained with Sudan-IV (Sigma-Aldrich, St. Louis, MO, USA). The lesion areas were quantified and analyzed by ImageJ software. Atherosclerotic lesion areas in the aortic root were visualized with H&E staining, and lipid-deposition with ORO staining and quantified by Image-Pro Plus 6.0 as previously described in chapter 3. For macrophage analysis, rat anti-mouse MOMA2 (Abcam, Cambridge, MA, USA) was used for immunostaining on serial sections of the aortic root, and biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA, USA) was used as the secondary antibody. Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA) was used to amplify the immunoreactivity, and signal was enhanced by Peroxidase Enhancer (GeneTex, Irvine, CA, USA), and finally reacted with the substrate from AEC Chromogen/FRP substrate kit (GeneTex, Irvine, CA, USA). Masson’s trichrome staining was used as previously described in chapter 3 to detect collagen content in the aortic root sections. All images were recorded with a Nikon E600 Wide field Epifluorescence microscope and Micropublisher digital camera with Q-imaging software. Data were quantified by computerized image Pro-Plus.

Statistical analyses

Data were presented as mean ± SEM. Statistical significance was calculated by Student’s $t$ test for two-group comparison or one-way analysis of variance (ANOVA) for multi-group comparison using the Graph-Pad Prism statistical program (Graph-Pad Software, Inc., San Diego, CA). $p<0.05$ was considered statistically significant.
4.3 Results

**Efficiency of macrophage-specific GP-96 knockout**

After bone marrow transplantation and 9 weeks of western diet, PCR and western blot analysis were used to confirm the efficiency of macrophage-specific GP-96 knockout. Macrophage-specific GP-96 deficient mice exhibit a significant loss (but not complete absence) of GP-96 in peritoneal and splenic macrophages, while the GP-96 expression was unaffected in splenic B-cells and T-cells (Figures 4.2A, 4.2B and 4.2C).

As GP-96 is the master chaperone for multiple TLRs, we next detected the expression of TLR4 in peritoneal macrophages using western blot analysis. Macrophage-specific GP-96 deficient macrophages displayed a significant decrease in TLR4 expression (Figure 4.2D).

**Macrophage-specific GP-96 deficiency does not affect mouse food intake and body weight**

We transplanted five million bone marrow cells from either WT (control) or macrophage-specific GP-96 deficient mice to lethally irradiated LDLR\(^{-/-}\) mice. Four weeks following the bone marrow transplantation, the recipients LDLR\(^{-/-}\) mice were fed a western diet for 9 weeks to induce atherosclerosis. During the western diet feeding period, we measured the food intake and body weight weekly. Throughout the experiment, macrophage-specific GP-96 deficient mice and control mice showed no difference in food intake (Figure 4.3A) and body weight (Figure 4.3B).
Macrophage GP96 is not required for the inflammatory response during atherosclerosis progression

The effect of macrophage-specific GP96 deficiency on inflammation has been examined. Initially, the spleen weight was measured at the experimental endpoint. No significant differences were observed between the two groups (Figure 4.4A). Next, flow cytometric analysis of the splenocytes was performed to determine the leukocyte population in the spleen. Several different leukocyte populations were investigated and the results showed that there were no significant differences in the percentage of leukocyte populations in the spleen between macrophage-specific GP96 deficient mice and control mice (Figure 4.4B).

To obtain further insight into the role of macrophage-specific GP96 deficiency in systemic inflammation during atherosclerosis, we measured serum levels of cytokine/chemokines using Bio-plex assays. We found that there was no difference in these cytokine/chemokines between the two groups (Figure 4.4C). This indicates that macrophage-specific GP96 deficiency did not significantly affect inflammation in LDLR−/− mice.

Macrophage-specific GP96 deficiency does not affect serum lipid levels

After 9 weeks of western diet feeding, serum cholesterol and triglyceride levels were measured and there were no significant differences between the macrophage-specific GP-96 deficient group and the control group (Figures 4.5A and 4.5B). Analysis of the plasma lipoprotein profile by FPLC showed no significant difference in VLDL, LDL and HDL cholesterol fractions in macrophage-specific GP-96 deficient mice compared to the control mice (Figure 4.5C). In addition, blood
glucose levels were comparable between the two groups (Figure 4.5D). These findings indicated that macrophage-specific GP-96 deficiency did not affect serum lipid levels.

**Macrophage-specific GP-96 deficiency does not influence progression of atherosclerosis**

Bone marrow recipient mice were sacrificed and the extent of early stages of atherosclerosis in the aorta and aortic root were analyzed. A comparable lesion size in the aorta and aortic root was observed between macrophage-specific GP-96 deficient mice and control mice (Figure 4.6A and 4.6B). Moreover, all parameters of plaque complexity including lipid accumulation (Figure 4.6C), macrophage infiltration (Figure 4.6D) as well as collagen content (Figure 4.6E) in the atherosclerotic lesions were similar between the two groups of mice. This indicates that macrophage GP-96 is dispensable for both inflammation and atherosclerosis development in LDLR−/− mice.

**4.4 Discussion**

GP-96 has been implicated in antigen presentation and activation of the innate and adaptive immunity191. GP-96 participates in the folding and assembly of many secretory and membrane proteins, and is essential for the cell-surface expression of TLRs including TLR2 and TLR4180,192. Given the fact that GP-96 deficiency is associated with loss of multiple TLRs, macrophage-specific GP-96 deficient mice are relatively resistant to shock induced by systemic administration of LPS (TLR4 ligand), and macrophages isolated from these mice showed a significant decrease in cytokine production upon stimulation with multiple TLR
agonists, we aimed to investigate if the reduced TLR dependent responsiveness of GP-96 deficient macrophages would result in reduced inflammation and subsequently attenuated atherosclerosis in hyperlipidemic mice. The major finding in this study is that macrophage-specific GP-96 deficiency did not result in alteration in atherosclerosis in high fat diet-fed LDLR−/− mice, suggesting that GP-96 does not play a significant role in macrophage inflammatory response in atherogenesis.

GP-96 contributes to the correct functioning of TLR mediated responses. Myeloid GP-96 deficiency leads to loss of multiple TLRs including TLR1-9 except TLR3180. Increasing evidence suggests that TLRs are key orchestrators in atherosclerosis development. Activation of all TLRs except TLR3 triggers an intracellular signaling pathway mediated by myeloid differentiation primary-response protein 88 (MyD88) or TIR domain-containing adaptor inducing IFN-β (TRIF), leading to the production of pro- and anti-inflammatory cytokines193. Previous evidence suggests that MyD88 plays a significant role in atherosclerosis. Deletion of the MyD88 gene in apoE−/− mice results in a significant reduction in aortic atherosclerosis and reduced macrophage accumulation194. However, bone marrow transplantation of MyD88 deficient DCs to LDLR−/− mice had atheroprotective effect195. The role of TLRs in atherosclerosis is still controversial; deficiency of TLRs in atherosclerosis mouse models showed conflicting effects on atherogenesis, e.g. TLR2/LDLR deficient mice developed less atherosclerosis compared to LDLR−/− mice. This result was confirmed in apoE−/− mice. However, no difference was observed in bone marrow transplantation studies, this finding
suggests that selective TLR2 cellular-type expression contributes differentially to lesion development\textsuperscript{196,197}. TLR1 and TLR6 did not affect atherosclerosis development in high fat diet-fed LDLR\textsuperscript{−/−} mice\textsuperscript{198}. Genetic deletion of TLR4 dramatically reduced macrophage infiltration and atherosclerotic lesion development in apoE\textsuperscript{−/−} mice\textsuperscript{187}; whereas transplantation of TLR2/TLR4 deficient bone marrow cells to LDLR\textsuperscript{−/−} mice fed a high fat diet did not result in changes in atherosclerosis\textsuperscript{198}. Furthermore, TLR3 has been shown to be atheroprotective as the TLR3\textsuperscript{−/−}/apoE\textsuperscript{−/−} mice had more atherosclerosis than ApoE\textsuperscript{−/−} mice\textsuperscript{189}. Taken together, these studies showed conflicting outcomes of TLR manipulation depending on experimental conditions and models. Our study added another example of the complex role of TLRs in atherogenesis.
Table 4. 1 Primers that were used for qRT-PCR in this study

<table>
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<td>mIL6</td>
<td>AGTTGCCTTCTTGGGACTGA</td>
<td>TCCACGATT TCCCAGAAGAC</td>
</tr>
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</table>
Figure 4. 1 In vivo experimental design, mouse model and bone marrow transplantation used for assessment of atherosclerosis

Six to eight week-old female LDLR<sup>−/−</sup> mice were provided with antibiotics water containing neomycin (100 mg/L) and polymyxin B sulphate (60.000 U/L) one week before and two weeks after BMT. Mice received total body irradiation. Bone marrow cells were isolated from macrophage-specific GP-96 deficient mice or WT mice and 5 x 10<sup>6</sup> cells were injected into LDLR<sup>−/−</sup> mice in the retro-orbital plexus. Four weeks after the BMT, mice were fed a western diet for 9 weeks.
Figure 4.2 Characterization of macrophage-specific GP-96 deficient mice
Expression of the GP-96 isolated from peritoneal macrophages of macrophage-specific GP-96 deficient mice and control (WT mice) (n = 3 per group) was examined by PCR (A) and western blot (B). Expression of GP-96 protein in macrophage, B cells and T cells isolated from spleen of macrophage-specific GP-96 deficient mice and WT mice (C). Expression of TLR4 in peritoneal macrophages was assessed by western blot (n=3 per group).
Figure 4. 3 Effect of macrophage-specific GP-96 deficiency on mouse body weight, and food intake
LDLR−/− mice were transplanted with bone marrow cells from macrophage-specific GP-96 deficient mice or control mice, allowed to recover for 4 weeks and subsequently fed a western diet for 9 weeks. Food intake (A) and body weight (B) were monitored weekly during the whole experiment.
Figure 4. Effect of macrophage-specific GP-96 deficiency on inflammation
(A) Spleen weight, (B) Flow cytometric analysis of splenocytes of LDLR^{−/−} mice transplanted with bone marrow cells from macrophage-specific GP-96 deficient or WT mice and fed a western diet for 9 weeks. (C) Serum cytokine/chemokines were measured by Bio-Plex assays.
Figure 4. 5 Effect of macrophage-specific GP-96 deficiency on serum lipids
After 9 weeks of western diet feeding, blood samples were drawn from retro-orbital puncture and the concentrations of cholesterol (A) and triglycerides (B) in the serum were determined. The distribution of cholesterol over the different lipoproteins was determined by FPLC. Cholesterol content in VLDL, LDL, and HDL fractions were measured (C). Fast blood glucose levels were determined (D).
Figure 4. 6 Effect of macrophage-specific GP-96 deficiency on atherosclerotic lesion development
After 9 weeks of western diet, mice were sacrificed and their aortas and hearts were dissected. Aortas were cleaned, fixed in 10 % PFA, and stained with Sudan IV en face (A). Cross-sections throughout the aortic root area starting from the appearance of open aortic valve leaflets were used for atherosclerosis measurements. (B) Representative images and quantification of lesion area stained with H&E. (C) Representative images and quantification of lipid content in the aortic root as determined by ORO staining. (D) Representative images and quantification of aortic root macrophage infiltration as detected by immunostaining using MOMA-2 antibody. (E) Representative images and quantification of aortic root collagen content stained with trichrome.
Chapter V

The therapeutic value of an anti-inflammatory Chinese herb compound, SsnB, for atherosclerosis

5.1 Background

Amounting clinical studies have shown that persistent low-grade inflammation is associated with the pathogenesis of chronic diseases such as atherosclerosis. Although high dose endotoxemia causes a significant increase in the inflammatory cytokines and acute tissue damage, low levels of circulating LPS have been implicated in initiating low-grade inflammation by mild and persistent increase in the expression of pro-inflammatory cytokines. Low-grade endotoxemia may change the host immune environment into a mild non-resolving pro-inflammatory state, which eventually leads to slow and progressive tissue damage, leading to the pathogenesis and progression of chronic inflammatory diseases. Recently, it has been shown that super-low dose LPS administration significantly promoted inflammation and exacerbated steatohepatitis in high fat diet-fed apoE−/− mice.

Sparstolonin B (SsnB) is a newly described, bioactive natural compound isolated and characterized from the Chinese herb Sparganium stoloniferum tubers which are used in Traditional Chinese Medicine (TCM) as a safe, non-toxic pharmaceutical agent for the treatment of several inflammatory diseases and as an anti-tumor agent. Our laboratory first isolated the compound and determined its
structure using NMR spectroscopy and X-ray crystallography. We showed that SsnB is a polyphenol with structural features of xanthone and isocoumarin, which has anti-oxidation and anti-inflammatory property. We have previously shown: 1) SsnB is a selective inhibitor of TLR2/TLR4 mediated inflammatory signaling via disruption of TIRAP/MyD88 interaction; 2) SsnB attenuates inflammation and reduces production of inflammatory cytokines in macrophages and endothelial cells by selectively blocking TLR2/TLR4 signaling; 3) SsnB significantly inhibited VSMCs proliferation, migration, inflammatory responses, and lipid accumulation in vitro; 4) SsnB strongly attenuated cardiomyocyte inflammation in an ischemia/reperfusion injury model in vitro; 5) SsnB inhibits pro-angiogenic functions and blocks cell cycle progression in human umbilical vein endothelial cells (HUVECs); and 6) SsnB is able to protect mice against endotoxin shock via inhibiting production of multiple cytokines and to attenuate hypoxia-induced apoptosis, necrosis, and inflammation in cultured rat left ventricular tissue slices.

All of these studies suggested that SsnB may act as a potential therapeutic agent for inflammatory diseases, such as atherosclerosis. Given the facts that macrophages and VSMCs play a significant role in atherosclerosis, and the anti-inflammatory properties of SsnB on endothelial cells, VSMCs, and macrophages, we expected that SsnB could be a promising new agent for the prevention and treatment of inflammatory cardiovascular atherosclerotic diseases. In this study we tested the effect of SsnB on vascular inflammation and atherosclerosis development in an atherogenic mouse model after super-low dose LPS administration. We hypothesized that super-low dose LPS injection would promote
low grade chronic inflammation, and that SsnB may reduce the LPS induced inflammation and thereby attenuate atherosclerosis development.

5.2 Material and Methods

Mouse models

Six to eight week-old female LDLR<sup>−/−</sup> mice on C57BL/6J background were purchased from the Jackson Laboratories (Bar Harbor, MA, USA) and maintained in pathogen-free conditions at the University of South Carolina Animal Research Facility according to National Institutes of Health (NIH) guidelines. All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of South Carolina. Mice were placed on a western diet containing 21% (w/v) anhydrous milk-fat, 34% (w/v) sucrose, and a total of 0.2% (w/v) cholesterol (Harlan laboratories, Indianapolis, IN, USA) for 10 weeks. After two weeks of western diet, mice were randomly divided into 3 groups as follows: (1) LPS group, injected intraperitoneally (i.p.) with super-low dose LPS (4 ng/kg body weight, ~100 pg/mouse) dissolved in endotoxin free PBS; (2) SsnB group, injected i.p. with SsnB (4 mg/kg body weight) plus LPS (4 ng/kg body weight), and (3) PBS group, injected i.p. with the same volume of PBS (No LPS). Initially, mice were injected every other day for the first 4 weeks, then twice a week for another 4 weeks. Body weight and food intake were monitored weekly. At the experimental endpoints, all mice were sacrificed; blood and serum were collected for analysis, aortas and aortic roots were harvested for atherosclerosis analysis. Liver samples were lysed in Triazol for qPCR.
**VetScan hematology analysis**

Blood was collected from retro-orbital puncture into heparinized tubes. Blood samples were run on the VetScan HMT hematology analyzer (Abaxis, Union City, CA, USA). Different blood parameters were detected: total red blood cells (RBC), hemoglobin, mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), hematocrit (Hct), platelet number, mean platelet volume (MPV), platelet distribution width (PDW), total white blood cells (WBC), lymphocytes, mean cell volume (MCV), and red cell distribution width (RDW).

**Serum lipid analysis**

Total serum cholesterol and triglyceride levels were measured using enzymatic colorimetric assays with Cholesterol Reagent and Triglycerides GPO reagent kits (Raichem; San Diego, CA, USA) and analyzed by SoftMax Pro5 software (Molecular Devices, Sunnyvale CA, USA). Briefly, retro-orbital venous blood was collected. Serum was obtained via centrifugation at 4000 x g for 20 minutes. Then, serum was diluted 1:100 in sterile water, 100 µL was loaded on the microplate well and 100 µL of freshly prepared cholesterol and triglyceride reagent were added to wells. The plates were incubated 10 minutes at 37°C and the absorbance was measured at 540 nm.

**Blood glucose measurement**

Blood glucose concentration was measured using OneTouch Ultra Blood Glucose Monitoring System (LifeScan, Inc., Milpitas, CA, USA) following the manufacturer’s instructions.
Bio-plex analysis of serum cytokine/chemokines

Mouse serum levels of 23 cytokine/chemokines were measured using a Bio-Plex Pro™ Mouse Cytokine 23-plex Assay (Bio-Rad, Hercules, CA, USA) on a Bio-Plex system following the manufacturer’s instructions.

Alanine Aminotransferase (ALT) measurement

Liver function was assessed by measuring plasma alanine amino transferase (ALT) levels. At the experimental endpoint, blood was collected and serum isolated by centrifugation at 4000 x g for 20 minutes. ALT levels were measured using a Liquid ALT (SGPT) Reagent Set (Pointe Scientific, Inc. Canton, MI, USA) according to the manufacturer’s instructions.

RNA extraction, cDNA synthesis, and quantitative real-time PCR

Total mRNAs were extracted from liver tissue using Trizol reagent (Invitrogen) following the manufacturer’s instructions. One microgram of isolated mRNA was reverse transcribed into cDNA in 20 μL reactions using iScript™ cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). qRT-PCR was performed on a Bio-Rad CFX96 system (Bio-Rad, Hercules, CA, USA) using iQ™ SYBR® Green Supermix (Bio-Rad, Hercules, CA, USA) according to the manufacturer’s instructions. The relative amount of target mRNA was calculated using the $2^{(\Delta\Delta Ct)}$ method by normalizing target mRNA Ct values to those of housekeeping gene, 18S mRNA expression. PCR thermal cycling conditions were 3 minutes at 95°C, and 40 cycles of 15 s at 95°C and 58 s at 60°C. Samples were run in triplicate. Primers used are listed in table 5.1
Quantification of atherosclerosis

At the experimental end point, mice were sacrificed. The aortas and aortic root were collected and processed as previously described in chapter 3 for atherosclerosis analysis. Aortas were stained with Sudan IV for aortic *en face* assay. Aortic root sections were stained with H&E, ORO, immunostaining using MOMA-2 antibody, and Masson’s trichrome to measure the atherosclerotic plaque area, lipid deposition, macrophage infiltration, and collagen percentage respectively. All images were recorded with a Nikon DS-Ri2 microscope and Micropublisher digital camera with Q-imaging software. Plaque size, lipid content, collagen percentage and macrophage cell content were quantified by computerized image Pro-Plus.

**Statistical analyses**

Data were presented as mean ± standard error of the mean (SEM). Statistical analysis was performed with GraphPad Prism 6.0 software (GraphPad Software Inc, San Diego, CA, USA). Statistical significance was determined by one-way analysis of variance (ANOVA) for multi-group comparison and Student’s *t* test for two-group comparison. Data were considered statistically significant at *P* < 0.05, **P**<0.01, and ***P***<0.001.

5.3 Results

**SsnB treatment and super-low dose LPS administration did not affect mouse food intake and body weight**

To induce atherosclerosis, LDLR−/− mice were fed a western diet for 10 weeks to induce hyperlipidemia. After two weeks of western diet, mice were
divided into three groups. In the first group, mice were injected i.p. with super-low dose LPS (4 ng/kg body weight). While in the second group, mice were injected i.p. with SsnB (4 mg/kg body weight) along with LPS (4 ng/kg body weight), in the third group, mice were injected i.p. with 1 mL PBS. Food intake and body weight of the mice were monitored weekly. We did not observe significant differences in body weight and food intake between the groups over the course of the experiment (Figure 5.1A and 5.1B).

**SsnB treatment and super-low dose LPS administration did not affect blood cell profile**

To examine the effect of SsnB treatment after super-low dose LPS administration on blood cell profile, we performed a detailed hematological analysis. Vetscan HMT analysis indicated that there were no significant differences in all blood parameters including total white blood cells and white blood cell differential count, red blood cell number, hemoglobin concentration and hematocrit, Total blood platelet numbers and platelet hematocrit between the groups (Figures 5.2A, 5.2B, and 5.2C).

**SsnB treatment and super-low dose LPS administration did not affect serum lipid levels and blood glucose levels**

After 10 weeks of western diet and 8 weeks of super-low dose LPS and SsnB treatment, mice were fasted overnight, sacrificed, blood were collected and serum isolated as previously described. Serum total cholesterol and triglyceride levels were measured as well as fasting blood glucose levels. We did not observe any significant difference in the serum total cholesterol (Figure 5.3A), triglyceride
(Figure 5.3B), and fast blood glucose levels (Figure 5.3C) between PBS, LPS and SsnB groups. Indicating that superlow dose LPS administration and SsnB treatment did not affect lipid profile.

**SsnB treatment reduced serum chemokines induced by super-low dose LPS administration**

Chronic injection of subclinical super-low dose LPS has been shown to sustain a low grade inflammation *in vitro* and *in vivo*\(^{201,208}\). Furthermore, SsnB treatment dramatically reduced macrophage and endothelial cell inflammation *in vitro*\(^{202,203}\). We measured the spleen weight for these mice and found no difference between the groups (Figure 5.4A). We next detected the effects of super-low dose LPS and SsnB treatment on the inflammation in LDLR\(^{-/-}\) mice fed a western diet by measuring several cytokine/chemokines levels in the serum using Bio-Plex assay. In agreement with a previous study\(^{201}\) several inflammatory biomarkers including keratinocyte-derived chemokine (KC), Macrophage inflammatory protein (MIP1-\(\alpha\) and MIP1-\(\beta\)), and RANTES were increased by super-low dose LPS injection. Furthermore, SsnB treatment was able to significantly reduce the inflammatory chemokines *p*-value 0.0121, 0.0240, 0.0452 respectively (Figure 5.4B). These chemokines are potent macrophage and lymphocyte chemoattractant and play a critical role in chronic inflammation. Macrophage inflammatory proteins (MIPs) are markedly up-regulated in macrophages upon exposure to bacterial LPS and increase their ability to recruit more inflammatory cells\(^{209}\). It has been shown that KC plays a central role in macrophage infiltration and accumulation in atherosclerotic lesions in mice\(^{210}\). In addition to that, some
other inflammatory cytokines such as IL3 and IL12(P40) were slightly increased after super-low dose LPS injection and SsnB slightly reduced these cytokines but without statistical significance.

**SsnB treatment has no impact on atherogenesis in LDLR^{−/−} mice administered with super-low dose LPS and fed an atherogenic diet**

For atherosclerotic lesion analysis, the aorta and aortic root were isolated for atherosclerosis staining. In brief, the aortic roots were embedded in OCT for cryo-sectioning. Aortas were fixed in 10% neutral buffered formalin and stained with Sudan IV for the aortic enface analysis. The results showed that most of the lesions were localized specifically in the aortic sinus and aortic arch. The super-low dose LPS injection as well as SsnB treatment did not significantly affect atherosclerotic lesion area of the aorta as and aortic root after 10 weeks of western diet (Figure 5.5A and 5.5B). Furthermore, the super-low dose LPS injection and SsnB treatment did not significantly affect lipid deposition, lesional MOMA-2 positive macrophage infiltration, and collagen deposition in the aortic root lesions (Figure 5.5C, 5.5D, and 5.5E).

**Effects of SsnB treatment on liver function after super-low dose LPS administration**

Subclinical super-low dose LPS administration has been shown to initiate low grade-chronic inflammation and exacerbates liver inflammation as well as lipid accumulation\textsuperscript{201,208}. We have shown previously that SsnB administration in experimental animal models attenuates early liver inflammation and injury in toxin-induced steatohepatitis\textsuperscript{211}. We therefore examined the effects of super-low dose
LPS injection and SsnB treatment on liver function in our experimental mouse model. At the experimental endpoint, we measured liver weight. Neither super-low dose LPS nor SsnB treatment changed the liver weight at the experimental endpoint (Figure 5.6A). Further, we collected serum from mice and examined the liver function by detecting serum ALT levels. As previously reported\textsuperscript{201}, our results showed that super-low dose LPS administration slightly increased plasma ALT levels compared to the PBS group but without statistical significance. However, SsnB treatment did not affect ALT levels (Figure 5.6B). qRT-PCR was used to examine the expression levels of pro-inflammatory cytokines in the liver tissue. LDLR\textsuperscript{-/-} mice injected with super-low dose LPS had a higher expression of inflammatory markers such as TNF-α and IL-6 compared to PBS group. Whereas, SsnB treatment slightly reduced the LPS induced IL6 expression and made no difference in TNF-α expression levels (Figure 5.6C and 5.6D).

5.4 Discussion

Atherosclerosis is a complex process involving many steps. Several lines of evidence ranging from animal experimental models to epidemiological studies support the notion that inflammation is one of the driving forces of atherogenesis\textsuperscript{22,62}. However, the current treatments of atherosclerotic diseases are focused exclusively on reducing plasma lipid levels rather than reducing the harmful effects of acute and chronic inflammation. Recently, there has been intense interest in exploring specific anti-inflammation therapies to reduce the risk of cardiovascular diseases and offer an opportunity to directly test the inflammation hypothesis of atherosclerosis. Therefore, modulating systemic and local
inflammatory responses and targeting different inflammatory pathways might be effective in the treatment/prevention of atherosclerosis and may thereby reduce the risk of CVDs\textsuperscript{22,31,109}.

High dose LPS injection has been shown to significantly aggravate atherosclerosis via the activation of the nuclear factor kappa-B (NF-κB) pathway to secrete pro-inflammatory cytokines, including IL-6, MCP-1, and TNF-α\textsuperscript{212}; but high dose LPS is not commonly observed in humans and thus not particularly clinically relevant. Subclinical super-low dose LPS is associated with persistent non-resolving chronic low-grade inflammation during the pathogenesis and progression of chronic diseases such as atherosclerosis and diabetes; and chronic exposure to low or very low dose of LPS is common and clinically relevant. Unlike high dose LPS, the super-low dose LPS does not activate NFκB pathway or induce the anti-inflammatory mediators\textsuperscript{213,214}. Therefore, strategies targeting this low grade inflammation may demonstrate significant promise in not only the treatment, but also the reversal and prevention of chronic inflammatory diseases.

A Chinese herb-derived compound, sparstolonin B (SsnB), selectively blocks TLR2/TLR4 mediated inflammatory signaling pathways such as MAPK and NF-κB pathways in macrophages\textsuperscript{202,215}. SsnB has been shown to significantly reduce the expression of pro-inflammatory cytokines in high dose LPS-challenged mice\textsuperscript{202}. However, the effect of SsnB on inflammation and atherosclerosis in atherogenic mice after super-low dose LPS administration has not been elucidated. In the present study, we found that super-low dose LPS and SsnB administration for 8 weeks did not affect mouse food intake and body weight after
10 weeks of western diet. In addition, blood cell profile and serum lipid levels did not change with SsnB treatment after super-low dose LPS administration. On the other hand, super-low dose LPS administration increased serum chemoattractant chemokines (RANTES, MIP1-α, and KC) and SsnB treatment attenuated the increase of these chemokines. Our study also showed that subclinical super-low dose LPS and SsnB treatment did not significantly alter lesion size in the aortic enface and aortic root lesions. Regarding plaque composition, no significant differences were observed in lipid deposition, macrophage infiltration and collagen content between groups.

In conclusion, we show here that subclinical super-low dose LPS injection in LDLR$^{-/-}$ mice after 10 weeks of western diet did not significantly increased systemic inflammation and atherosclerotic lesion size at early stages as we expected. Furthermore, SsnB administration did not significantly affect plaque size or composition after super-low dose LPS injection. The effect of SsnB on atherosclerosis through suppressing TLR2/4 signaling warrants further investigations.
### Table 5.1 Primers that were used for qRT-PCR in this study

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Figure 5. 1 SsnB treatment and super-low dose LPS administration did not affect mouse food intake and body weight
Six to eight week-old female LDLR−/− mice were fed a western diet for 10 weeks. During the last eight weeks, the mice were injected intraperitoneally with PBS, super-low dose LPS and SsnB. At the end of each indicated week mouse body weights (A) and food intake (B) were measured. The average daily intake of western diet per mouse during the week was calculated.
Figure 5. 2 SsnB treatment and super-low dose LPS administration did not affect blood cell profile

Vetscan HMT blood cell analysis showed: (A) white blood leukocyte profile: total white blood cell number and white blood cell differential count (lymphocytes, monocytes, and neutrophils numbers and percentage). (B) Red blood cell (RBC) number, hemoglobin (HGB) concentration and hematocrit (HCT), as well as mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and red cell distribution width (RDWc) which is calculated as Standard deviation ÷ mean cell volume x 100. (C) Platelet related parameters of mice obtained by Vetscan HMT analysis. Parameters include total blood platelet count, mean platelet volume (MVP), platelet hematocrit (PCT), and platelet distribution width (PDWc) which is calculated using equation \( RDWc(\%) = \frac{\text{Standard deviation}}{\text{mean cell volume}} \times 100 \). N=7 mice each group.
Figure 5. 3 SsnB treatment and super-low dose LPS administration did not alter serum lipid and blood glucose levels
Female LDLR−/− mice were fed a western diet for 10 weeks. During the last eight weeks, the mice were injected with LPS with or without SsnB every other day for the first four weeks then twice a week for another four weeks. At the experimental endpoint all mice were sacrificed and serum collected. Serum total cholesterol (A) and triglyceride (B). Fast blood glucose levels were determined (C).
Figure 5. 4 SsnB treatment reduced serum chemokines induced by super-low dose LPS administration

(A) Mouse spleen weight. (B) Serum levels of 23 cytokine/chemokines were measured by Bio-Plex Pro™ Mouse Cytokine 23-plex Assay. Data are presented as the mean ± SEM (n = 10 per group). *P < 0.05, **P < 0.01
Figure 5. SsnB treatment has no impact on atherogenesis in LDLR−/− mice administered with super-low dose LPS and fed an atherogenic diet

LDLR−/− mice were fed a western diet for 10 weeks to induce atherosclerosis. Mice were injected i.p. with super-low dose LPS (4 ng/kg body weight, ~100 pg/mouse) or LPS plus SsnB (4 mg/kg body weight) for 8 weeks. (A) Representative images and quantification of aortic enface stained with Sudan IV. (B) Representative images and quantification of lesion area in the aortic root frozen section of the control (PBS), LPS, and SsnB treatment mice stained with H&E. (C) Representative images and quantification of lipid content in the aortic root frozen section stained with ORO. (D) Representative images and quantification of macrophage infiltration in the aortic root frozen section stained with immunostaining using MOMA2 antibody. (E) Representative images and quantification of collagen content in the aortic root frozen section stained with trichrome staining. Data are presented as mean ± SEM.
**Figure 5.6** Effects of SsnB treatment on liver function after super-low dose LPS administration

After sacrificing, mouse liver was thoroughly perfused to remove blood and then weighed. (A) Liver weight. (B) Serum ALT levels were determined at the end point. (d) Liver IL6, TNF-α gene expression were examined by qRT-PCR (*P < 0.05)
Chapter VI
Summary and conclusion

Atherosclerosis is a multi-stage and multifaceted disease. Both innate and adaptive immunity are involved in all phases of atherogenesis, from lesion initiation through progression and ultimately to the clinical complications of this disease\textsuperscript{50}. All types of immune cells have been found in atherosclerotic lesions indicating that all immune components may participate in atherogenesis. Inflammation is a basic pathogenic element in the development and progression of atherosclerotic disease and in its manifestation. Inflammation is caused by complex interactions involving different cell types, different mediators, and different cellular processes. All of these factors form the basis of the “inflammation hypothesis of atherosclerosis”\textsuperscript{45}. The relationship between inflammation and atherogenesis is complex. The link between inflammation and atherosclerosis provides a new avenue for future pharmacologic intervention that may slow the progression of atherosclerosis by targeting inflammation. Directly testing the inflammation hypothesis of atherosclerosis requires drugs that have systemic anti-inflammatory effect without affecting lipid levels. Experimental and clinical studies suggest that reducing inflammation without affecting lipid levels may reduce the rate of CVDs\textsuperscript{4,22,27,31}. Recently, the phase III CANTOS study demonstrated that Canakinumab (IL1-\(\beta\) antibody) is the first and only drug that selectively targets inflammation and significantly reduces cardiovascular risk in patients with a prior heart attack and
inflammatory atherosclerosis. IL1-β has been identified in early human atherosclerotic plaques and mouse models of atherosclerosis suggesting a causal role for IL-1β in atherogenesis. oxLDL, cholesterol crystals, and apoptotic cells have been identified as endogenous triggers of the NLRP3 inflammasome and secretion of IL-1β by plaque macrophages in atherosclerosis. The expression of inflammation markers in atherosclerosis has been shown to be predictive of future cardiovascular risk. The detection of these inflammatory markers and immune profile may lead to a better stratification of asymptomatic patients. In particular, master cytokines such as IL-1β and IL-6 have been shown to independently predict the presence and the characteristics of the atherosclerotic plaque, whereas novel markers such as MMPs family including MMP-9 have been shown to be implicated in its destabilization. Inflammation markers may also provide a tool to follow atherosclerotic patients to assess their response to different therapies, and then allow for a better selection of appropriate strategy.

TTP is an anti-inflammatory protein that functions to promote mRNA decay. The anti-inflammatory role of TTP has been extensively studied in mouse models and cell lines, such as blood monocytes and bone marrow–derived macrophages. The anti-inflammatory functions of TTP have been primarily studied in rheumatoid arthritis. However, the role of TTP in atherosclerosis has not been examined. Our study shows that transplantation of TTP deficient bone marrow into atherogenic LDLR−/− mice resulted in dramatically increased systemic and multi-organ inflammation. However, atherosclerotic lesion size and plaque composition were not significantly affected. Surprisingly, bone marrow TTP deficient mice
displayed a significant reduction in their plasma lipid and hepatic lipid accumulation, with reduced expression of SREBF-1 in the liver. It remains to be further determined how serum lipid and hepatic steatosis were severely reduced. Increased inflammation is counterbalanced by the reduction in serum lipid levels, resulting in no alteration in atherosclerosis development. This result reveals that targeting hematopoietic TTP activity may not be a suitable therapeutic approach.

GP-96 is an essential chaperone for folding and function of multiple TLRs except TLR3. TLRs recognize pathogens and thereby initiate innate immune response. Deletion of GP-96 results in the post-translational loss of multiple TLRs, suggesting that this chaperone protein occupies a central position in the innate immunity. TLRs are implicated in the pathogenesis of atherosclerosis\textsuperscript{180,182,193}. Targeting TLRs may provide alternative approaches and new therapy to halt atherosclerosis. However, evidence for the role of TLRs in atherosclerosis in mouse models is controversial. Some studies showed atheroprotective effects of TLRs, whereas others showed pro-atherogenic effects. This discrepancy can be partially explained by the use of different background strains of the mice\textsuperscript{221}. Whether targeting TLRs is a successful approach for the treatment of atherosclerotic diseases remains to be determined. In this study we show that bone marrow transplantation of macrophage-specific GP-96 deficient cells into LDLR$^{-/-}$ mice did not result in any difference in inflammatory status, even though we confirmed the significant reduction in GP-96 and TLR4 expression in the peritoneal macrophages. Serum lipid levels were comparable between groups. Finally, these mice showed no difference in atherosclerotic lesion size and plaque
composition. Although more details regarding the TLRs function have recently been studied, the extent to which the different TLRs affect inflammation and immunity in atherosclerosis remains unclear.

Traditional Chinese medicine (TCM), especially herbal medicine, has been used for the treatment of cardiovascular diseases for a very long time. Many compounds from these Chinese herbes are often capable of targeting multiple pathways. SsnB is a novel bioactive compound isolated and characterized in our lab; SsnB has anti-inflammatory properties by inhibiting TLR mediated signaling\textsuperscript{203,211,215}. Inhibiting TLR2/TLR4 signaling pathway may be a fascinating therapeutic approach for atherosclerosis, however, no TLR2 or TLR4 antagonists have been approved for clinical use. SsnB has robust anti-inflammatory properties, but its effect on atherosclerosis is unknown. After super-low dose LPS injection, LDLR\textsuperscript{−/−} mice showed a slight increase in inflammation and no change in serum lipid levels. Atherosclerotic lesions were comparable with the control mice. Furthermore, SsnB treatment had no effects on inflammation and atherosclerosis after super-low dose LPS injection. These results indicate that SsnB administration to western diet fed LDLR\textsuperscript{−/−} mice is not effective to change inflammation and atherosclerosis development when the mice were injected with super-low dose LPS. The effect of SsnB on inflammation and atherosclerosis in atherogenic mice warrants further investigation using other animal models.

In conclusion, our studies aimed to test the inflammation hypothesis of atherosclerosis using various models suggest that the role of inflammation in atherogenesis is complex. Some novel findings may guide us in future
investigations. First, we discovered a new role of miR155, a pro-inflammatory molecule, in promoting vascular calcification, suggesting that targeting vascular miR155 may be an effective preventive and therapeutic approach for cardiovascular diseases with vascular calcification as a pathological feature. Second, we discovered that systemic inflammation resulted from myeloid cell TTP deficiency surprisingly reduced plasma lipid levels and liver lipid accumulation, pointing to a possibility that a certain component of inflammation may actually benefit hepatic lipoprotein metabolism. Third, although TLR signaling has been implicated in the pathogenesis of atherosclerosis, direct targeting TLRs may not be an optimal approach for chronic diseases with low-level systemic inflammation, such as atherosclerosis.
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