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Role of Cannabinoid Receptor Type 2 (CB2) in Late Stage Atherosclerosis

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A dissertation
presented to
the faculty of the Department of Biomedical Sciences
East Tennessee State University

In partial fulfillment
of the requirements for the degree
Doctor of Philosophy in Biomedical Sciences
Biochemistry & Molecular Biology Concentration

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by
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December 2017

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ABSTRACT

Role of Cannabinoid Receptor Type 2 (CB2) in Late Stage Atherosclerosis

by

Makenzie L. Fulmer

Atherosclerosis is a chronic inflammatory disorder of medium and large vessels. Immune signaling and dyslipidemia are two of several processes which influence lesion development in atherosclerosis. Cannabinoids, such as those found in marijuana, exert their effects through two cannabinoid receptors, CB1 and CB2. Recent studies using CB2 knockout mice and CB2-selective ligands have shed light on a protective role of CB2 in early stages of atherosclerosis. However, the role of CB2 in advanced stages of atherosclerosis remains unclear. To determine if CB2 plays a role in advanced atherosclerotic lesion composition and progression, we investigated the effects of systemic CB2 gene deletion on advanced atherogenesis in Ldlr-null mice fed an atherogenic high fat diet (HFD) for 20-24 weeks. CB2 deficiency did not significantly affect aortic root lesion area, however, $CB2^{-/-}$ mice had a significant increase (~1.9 fold) in the percentage of abdominal aorta surface occupied by lesion. $CB2^{-/-}$ mice also displayed increased lesional macrophage content (~2.3 fold) and an unstable phenotype characterized by significantly reduced smooth muscle cell/macrophage ratio and increased matrix metalloproteinase-9 activity and mineralization. These results suggest that although CB2 does not affect the size of atherosclerotic lesions, it does modulate the cellular and extracellular matrix composition and promotes a stable phenotype. $CB2^{+/+}$ and $CB2^{-/-}$ mice were also subjected to treatments with either CB2-selective agonist, JWH-015, or antagonist, SR144528, over the last four weeks of a 24 week atherogenic diet to identify the effects of CB2 stimulation on

calcification of advanced lesions. No change was observed in body weight or cholesterol in response to either treatment. SR144528 reduced triglycerides and mineralization of aortic root lesions in $CB2^{+/+}$ mice only. Aortic Runx2 and osteopontin were increased in response to JWH-015 by a CB2-dependent mechanism. Administration of synthetic cannabinoids in an *ex vivo* organ culture of $CB2^{+/+}$ aortas revealed increased vascular calcification in response to CB2 blockade and decreased vascular calcification in response to CB2 activation. All together, these results support a protective role for CB2 in late stages of atherosclerosis and suggests that drugs targeting CB2 may be beneficial in the treatment of advanced atherosclerosis by affecting osteogenic mechanisms implicated in the mineralization of lesions.

DEDICATION

This manuscript is being dedicated to the following:

My mother, Kimberly Fulmer, who has never stopped believing in me. I would not be where I am today without your love, guidance, and support, and I hope I can one day be half the person you are. Thank you for selflessly putting my wants and desires before your own as you raised me all by yourself.

My grandfather, Donald Beltz, who taught me how to fish and create things with my hands; who taught me to love people well and with purpose; who showed me the importance of putting hot sauce on every food item; and the person on this Earth whom I cherish the most.

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ABBREVIATIONS

2-AG	2-arachidonoyl glycerol
AA	arachidonic acid
AEA	anandamide
ApoE	apolipoprotein E
ALP	alkaline phosphatase
CB1	cannabinoid receptor 1
CB2	cannabinoid receptor 2
CE	cholesteryl ester
CVD	cardiovascular disease
DMSO	dimethyl sulfoxide
ECM	extracellular matrix
ECS	endocannabinoid system
FAAH	fatty acid amide hydrolase
FBS	fetal bovine serum
HDL	high density lipoprotein
HFD	high fat diet
ICAM-1	intracellular adhesion molecule-1
IFN	interferon
IL	interleukin
LDL	low density lipoprotein
Ldlr	low density lipoprotein receptor
MCP-1	monocyte chemoattractant protein-1
MMP	matrix metalloproteinase
OBT	osteoblastic transdifferentiation
OCN	osteocalcin
OxLDL	oxidized low density lipoprotein
OPG	osteoprotegerin
OPN	osteopontin
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PRR	pattern recognition receptor
RANKL	receptor activator of nuclear k-B ligand
ROS	reactive oxygen species
Runx2	runt-related transcription factor 2
SMC	smooth muscle cell
THC	(-)delta-9-tetrahydrocannabinol
TLR	toll-like receptor
TNF	tumor necrosis factor
VCAM-1	vascular cell adhesion molecule-1
VSMC	vascular smooth muscle cell

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CHAPTER 1

INTRODUCTION

Cardiovascular disease (CVD) is the leading cause of death in the U.S., and has accounted for more deaths than any other cause of death in the U.S. every year since 1919 (Benjamin et al. 2017). With advancements in medicine, deaths caused by CVD declined 28.8% from 2003 to 2013, but despite this, CVD still accounts for 30.8% of all reported deaths in America; over 2200 Americans die from CVD daily, averaging one death every 40 seconds (Mozaffarian et al. 2016). The most prevalent form of CVD is coronary heart disease (CHD), which alone causes 1 out of 7 deaths in the US. Approximately every 34 seconds, an American suffers from a coronary event, with a death occurring every 1 minute and 24 seconds (Mozaffarian et al. 2016). The underlying cause of CHD is atherosclerosis, a chronic inflammatory disease characterized by the formation of cholesterol-rich plaques within the vessel wall of large- and medium-sized arteries. Atherosclerosis is a multifactorial disease and several genetic and environmental risk factors of atherosclerosis have been identified, such as dyslipidemia, obesity, high-fat diet, diabetes, smoking, and hypertension (C.K. Glass and Witztum 2001).

The wall of arteries is comprised of three distinct layers, shown in Figure 1.1. The intima is the innermost layer and consists mostly of endothelial and smooth muscle cells (SMCs) with macrophages present in irregular intervals. At the most luminal portion of the intima is a single layer of endothelial cells, and the outermost portion consists of an elastic lamina (EL) that helps to provide a connection between the intima and the middle layer. This middle layer, called the media, consists of concentric sheets of SMCs that function in constriction and dilation of the vessel and collagen, which also serves as a means to connect with the intima. A second EL is

found at the outermost portion of the media and connects the media with the adventitia, the final outer layer of the vessel that connects the vessel to surrounding tissue. The adventitia is mostly made of connective tissue with unsystematically arranged collagen and elastin fibers.

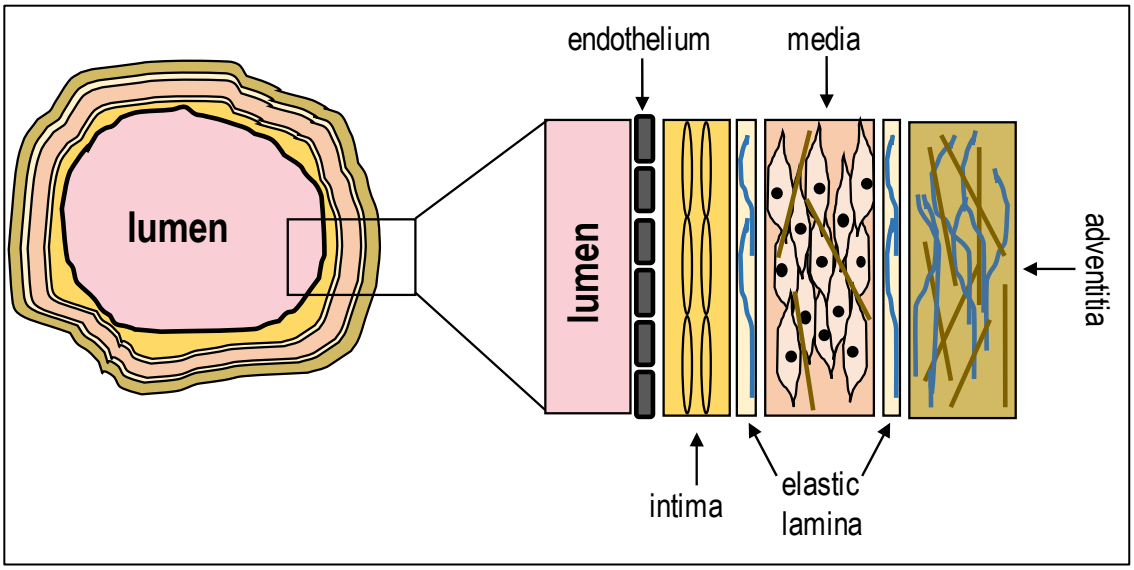

Figure 1.1 Cross-sectional diagram of artery layers. The vessel wall is composed of three distinct layers known as the intima, media, and adventitia. Two elastic laminas serve to separate the media from the inner and outer layers. A layer of endothelial cells separates the intima from the lumen, which is where oxygenated blood flows.

Atherosclerotic plaques form in the intimal layer of arteries over several decades. These plaques are generally divided in to three successive stages based on their morphological and stabilizing characteristics: fatty streak, intermediate fibrous lesion, and advanced lesion (Figure 1.2). Fatty streaks (Figure 1.2b) are the first sign of atherogenesis that can be visualized macroscopically on the intimal surface with the unaided eye and are present in the aorta of 99% of children aged 2 to 15 years (Stary et al. 1994). This early, asymptomatic stage is characterized by an influx of monocytes and low-density lipoprotein (LDL) particles that eventually give rise to adjacent, stratified layers of macrophage-derived foam cells, creating a yellow streaking

appearance on the intimal surface. Foam cells, considered the hallmark of atherosclerosis, are retained in plaques and eventually contribute to disease progression by promoting insufficient lipid metabolism and further compromising fundamental immune functions (Hansson and Hermansson 2011). Lipids, which are mainly in the form of cholesteryl esters (CE) (Katz et al. 1976), are also contained within intimal SMCs at this stage but to a lesser degree than in macrophages. Thinly dispersed lipid droplets are also found in the extracellular space in small quantities, however most of the lipid seen during the fatty streak stage is within cells (Stary et al. 1994).

Fatty streaks can progress into intermediate fibrous lesions (Figure 1.2c) over several years, a stage that serves as a morphological and chemical bridge between fatty streaks and advanced lesions. On gross appearance, intermediate lesions resemble fatty streaks but are widely different microscopically. These fibrous lesions contain increased amounts of extracellular lipid droplets that bear more free cholesterol, fatty acid, and triglyceride components than in fatty streaks (Stary et al. 1994). At this stage, SMCs deposit collagen and other extracellular matrix (ECM) components in to the lesion to create a stabilizing fibrous cap. A large lipid core is not well defined yet but the accumulation of several separate extracellular lipid pools layered beneath the macrophages and foam cells disrupt coherent layers of intimal SMCs and intercellular matrix proteoglycans and fibers (Stary et al. 1994). Like in the case of fatty streaks, this second stage of atherosclerotic plaque development is not clinically significant. However, culmination of this process over decades leads to the formation of large, structurally unstable advanced lesions that give rise to incidences of myocardial infarction and stroke. Advanced lesions, also known as atheromas (Figure 1.2d), are characterized by the presence of a large necrotic core that is absent in preceding stages (Stary et al. 1995). Lesions contain an

abundance of extracellular lipids forming lipid cores, pro-inflammatory cytokines, increased amounts of cell death via apoptosis and necrosis, and enhanced collagen deposition (Ball et al. 1995; Kolodgie 2004). In more advanced lesions, mineralization and increased secretion of proteolytic enzymes that diminish and damage collagen and elastin fibers in the internal EL, eventually degrading the ECM, leading to lesional instability. Resulting consequences are plaque fissuring and thrombolytic rupture, a predecessor to a series of detrimental coronary events.

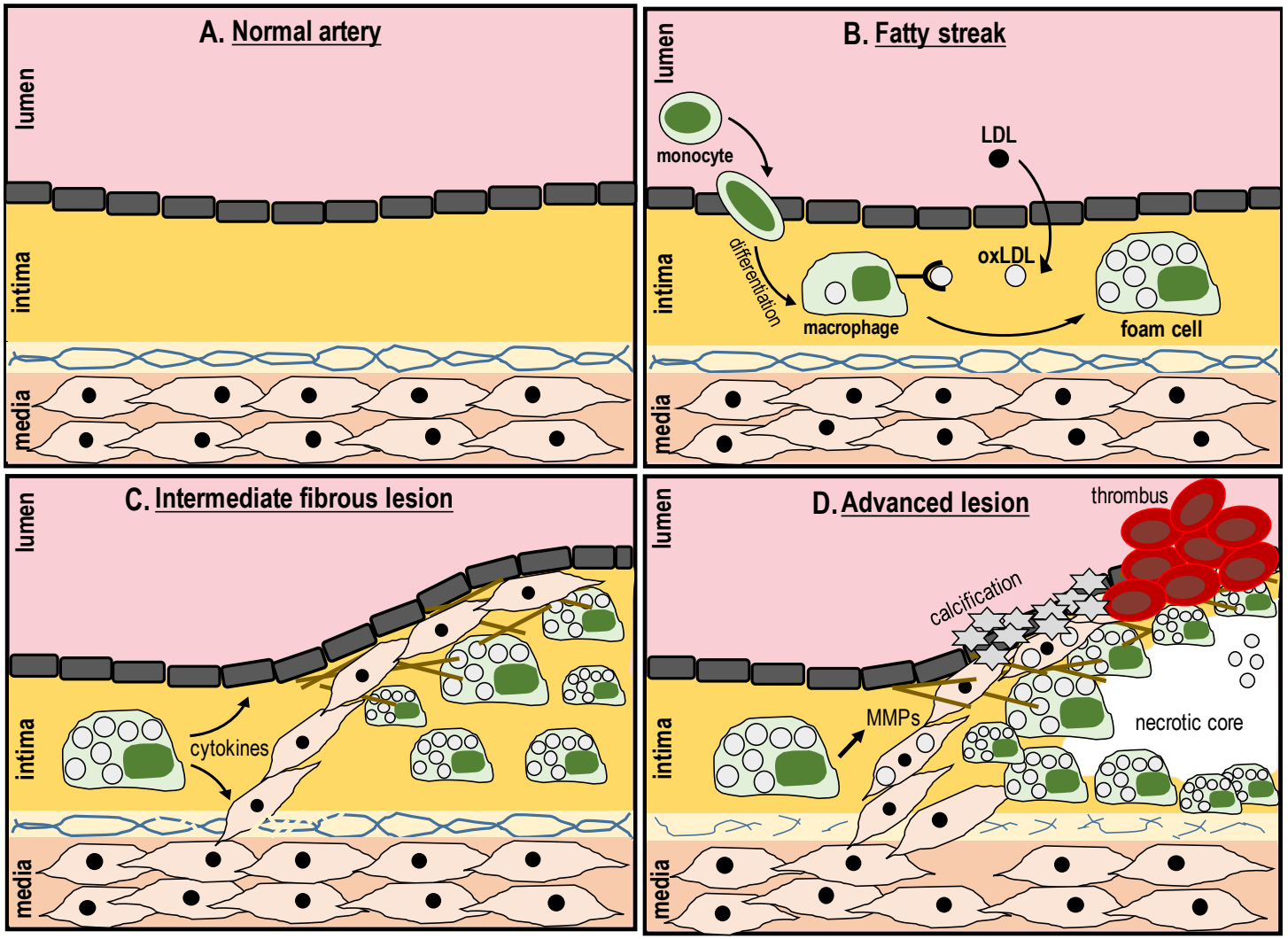

Figure 1.2 Stages of atherosclerotic progression. A) Non-diseased artery under normal conditions. The initial retention of modified LDL particles and circulating monocytes in the subendothelial space marks the initiation of a fatty streak (B). Culmination of this process over several years results in an abundance of immune cells and heightened secretion of proinflammatory cytokines. Smooth muscle cells begin to migrate toward the lumen in response to increased inflammatory signaling in an attempt to stabilize the intermediate fibrous lesion (C). Increased amounts of cell death results in a large, unstable necrotic core underneath the stabilizing fibrous cap. Proteolytic degradation of ECM components and mineralization of the fibrous cap occur in advanced lesions (D) and can lead to an unstable phenotype that is prone to rupture and thrombolytic occlusion.

While there is no cure for atherosclerosis, a common therapeutic strategy is to manipulate serum lipid levels by the use of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (Buhaescu and Izzedine 2007). These drugs, commonly referred to as statins, reduce cholesterol synthesis by inhibiting the rate limiting enzyme of the cholesterol synthesis pathway. Reduction in cholesterol synthesis results in the upregulation of expression of LDL receptor by hepatic cells, allowing for enhanced clearance of cholesterol-packed LDL from the circulation. Statins also play a beneficial immunomodulatory role and have the ability to affect several processes involved in the progression of atherosclerotic plaque formation. These include enhancing endothelial cell differentiation, suppressing macrophage activation and endothelial cell adhesion molecule expression, and inhibiting the migration and proliferation of vascular smooth muscle cells (VSMCs) (Jones et al. 1998; Pitt et al. 1999; Vaughan et al. 2000; Bonetti et al. 2003; Davignon and Ganz 2004; Ray and Cannon 2005). Despite the ability of statins to reduce cardiovascular events in human and animal studies, this therapy has not been successful in drastically decreasing mortality rates as 40% of patients with normal plasma lipid levels still develop atherosclerotic plaques (Genest et al. 1991; Rubins et al. 1995; Yusuf et al. 2016). Also, some patients are intolerant to statins. New therapies for managing atherosclerosis are needed, independent of managing hypercholesterolemia.

Pathogenesis of Atherosclerosis

Under normal conditions, the monolayer of endothelial cells lining the intima comes in to contact with flowing blood with high ability to resist adhesion of leukocytes and various other immune cells. Unprovoked, spontaneous atherosclerosis is thought to occur through “response to

injury," a hypothesis proposed by Rudolph Virchow in 1856 that is still widely accepted today. The basic premise of this hypothesis is that the formation of atherosclerotic lesions is a response to an initial injury to arterial endothelial cells, which can be initiated by hyperglycemia, increased levels of oxidized LDL (oxLDL) in circulation, sheer stress, and infectious pathogens. After initial injury to the endothelial layer, an inflammatory response is initiated by lipids, cellular debris, and inflammatory cells that become retained in the intima of the arterial wall.

Atherogenesis begins when various stimuli induce inflammatory cytokine release and vascular endothelial expression of adhesion molecules. Elevated levels of LDL in plasma, which can be attributed to high-fat diets (HFD) and genetic disorders, leads to retention of LDL in the intima of the arterial wall where it becomes modified by several processes. One modification in particular is the oxidation of LDL particles to become oxLDL, which stimulates the production and secretion of pro-inflammatory cytokines, such as TNFα, IL-6, and IL-10 (Hamilton et al. 1990; Fong et al. 1991). This inflammatory response in turn initiates endothelial cell expression of luminal adhesion molecules, such as intracellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1, and p-selectin (Dustin et al. 1986; Cybulsky and Gimbrone 1991; Takahashi et al. 1996). Expression of these adhesion molecules allows for transmigration of leukocytes, mainly blood monocytes, into the subendothelial space, where they differentiate into macrophages and express scavenger receptors that bind and facilitate internalization of oxLDL (Dhaliwal and Steinbrecher 1999). Receptor-mediated endocytosis of oxLDL by macrophages is a continual process, and the cholesterol derived from the oxLDL particles is converted into cholesteryl esters (CEs) by acyl coenzyme A: cholesterol acyltransferase (ACAT). CEs are stored in lipid droplets in the cytoplasm of macrophages which give these cells a "foamy" or "frothy" appearance, giving rise to the term "foam cell" which is the first hallmark of

atherosclerosis (Moore et al. 2013).

Fatty streaks progress to intermediate plaques and the inflammatory process continues with the recruitment of T cells (Jonasson et al. 1986) and B cells (Zhou and Hansson 1999). T lymphocytes activate and perpetuate inflammation by secreting pro-inflammatory cytokines and chemokines and recruiting additional immune cells. VSMCs are normally present in the medial layer of the artery where they function as quiescent, contractile cells; however, they possess the ability to transition to a proliferative phenotype when provoked following vascular injury, playing in important role in the pathophysiology of atherosclerosis. Recruitment of immune cells secreting pro-inflammatory cytokines and chemokines signals VSMCs to begin proliferating and migrating from the medial layer of the artery towards the intima (Schönbeck et al. 2000). This process marks the "fibrous plaque" stage of atherosclerotic lesion development, and the migrating VSMCs attempt to stabilize the pro-inflammatory lipid core by depositing collagen and other ECM components to create a fibrous cap. This mechanism can later be altered by the pro-inflammatory cytokines and chemokines secreted by macrophages and T lymphocytes, which can destabilize the ECM by impairing the synthesis of collagen by VSMCs and increasing expression of MMPs (Hansson and Libby 2006).

As atherosclerotic lesions advance, they begin to undergo calcification by an active process resembling embryonic osteogenic mechanisms and normal bone remodeling (Steitz et al. 2001; Trion and van der Laarse 2004; Bab et al. 2008). VSMCs transdifferentiate to an osteoprogenitor cell type, and the presence of several transcription factors and other regulatory proteins promote expression of osteogenic genes, allowing these cells to acquire a functional osteoblast phenotype. Vascular calcium deposition reduces vessel elasticity and vessel wall compliance (Trion and van der Laarse 2004; Sun et al. 2012) and is therefore a factor

contributing to plaque instability. In addition to this, excess buildup of oxLDL and free cholesterol in foam cells eventually initiates apoptotic cell death (Marchant et al. 1995). As the number of apoptotic cells increases, inefficient clearance of these cells (efferocytosis) can result in secondary necrosis. During secondary necrosis, lipids and other cellular debris are deposited in the extracellular space (Tabas 2007), giving rise to a necrotic core. Stabilization of this lipid-rich core by the fibrous cap is lost when inflammatory cells secrete MMPs which serve to degrade ECM components, resulting in the weakening/thinning of the fibrous cap. When the stability of atherosclerotic plaques in compromised, the risk of plaque rupture is very high. The formation of a thrombus is initiated after a plaque ruptures; a result of pro-thrombotic materials from the lesion being exposed to blood. Life threatening complications can arise from this process, as thrombi can induce hypoxia by occlusion of the artery, leading to myocardial infarction and stroke.

The Immune System in Atherosclerosis

The progression of atherosclerotic plaque development is influenced by both innate and adaptive immune responses. The innate immune system is a subset of the overall immune system that responds to pathogens in a non-specific but immediate fashion, and adaptive immunity is an antigen-specific immune response that gives rise to immunological memory. Activation of the innate immune system is important for eliciting an inflammatory response. Pattern recognition receptors (PRRs) are necessary for detecting and clearing harmful materials. Cells of the innate immune system are involved in several aspects of atherogenesis and express a diverse set of PRRs that recognize both exogenous pathogenic components and endogenous products (Akira et al. 2006). These PRRs can functionally be classified into two groups: endocytic receptors, which facilitate the uptake of extracellular molecules and macromolecular complexes; and signaling

receptors, which function to activate pro-inflammatory signaling pathways through upregulation of pro-inflammatory genes. Examples of endocytic receptors are scavenger receptors, such as SR-A (Lundberg and Hansson 2010) which is expressed on the surface of retained macrophages and facilitates the clearance of oxLDL and formation of foam cells.

The major signaling PRRs are toll-like receptors (TLRs), which provide a link between activation of innate immune responses through triggering acute inflammation and induction of adaptive immune responses by activating co-stimulatory molecules necessary for cell-mediated immunity. TLRs are type I transmembrane receptors and are ubiquitously expressed throughout the body, with upregulated expression in atherosclerotic lesions on VSMCs, endothelial cells, and macrophages (Edfeldt et al. 2002; Otsui et al. 2007). Stimulation of TLRs on macrophages can result in release of proinflammatory cytokines TNFα, IL-1, and IL-6 as well as induction of MMP expression. Recruitment and activation of T cells also occurs in response to TLR stimulation, which potentiates the inflammatory process through production of more pro-inflammatory mediators, such as IFNγ and TNFα, further stimulating SMCs, endothelial cells, and macrophages within the lesion (Lundberg and Hansson 2010). Several TLRs exist and collectively they recognize a diverse set of pathogen-associated molecular patterns (PAMPs) that share commonality among many microorganisms (Akira et al. 2006). TLR2 and TLR4 have been implicated in atherosclerosis. TLR4 is the receptor for lipopolysaccharide (LPS), a major component in the cell wall of Gram negative bacteria, and systemic LPS administration in hypercholesterolemic rabbits and atherogenic mice demonstrated a pro-atherosclerotic role (Lehr et al. 2001; Ostos et al. 2002). TLR2 recognizes the largest variety of PAMPs on bacteria, fungal, and viral products (Schwandner et al. 1999; Vabulas et al. 2001; Takeuchi et al. 2002; Compton et al. 2003), and injection of a TLR2 ligand into hypercholesterolemic and atherogenic

mice resulted in increased lesion severity (Mullick et al. 2005). In addition to these studies showing that pathogenic products have the ability to promote atherosclerosis through TLR signaling, several reports from epidemiological studies show an increased risk of atherosclerosis associated with the following bacterial and viral pathogens: *Chlamydia pneumoniae, Helicobacter pylori, Porphyromonas gingivalis*, Cytomegalovirus, Epstein-Barr virus, Human Immunodeficiency virus, and certain strains of Herpes simplex virus, Influenza virus, and hepatitis viruses (Michelsen et al. 2004; Desvarieux et al. 2005; Guan et al. 2008; Harskamp and van Ginkel 2008).

Macrophages are key players in the initiation and progression of atherosclerotic plaques, through their role in both cholesterol metabolism and innate immune response regulation. Macrophages are phagocytic janitors that function to remove cellular debris and pathogenic agents and secrete cytokines in order to regulate other immune processes. Most macrophages present within atherosclerotic plaques originate from bone marrow-derived blood monocytes (Mallat 2017), although recent evidence from lineage tracing studies has shown that many macrophages transdifferentiate from SMCs within the plaque (Shankman et al. 2015). In the beginning stages of atherogenesis, macrophages play a beneficial role through their ability to ingest and clear accumulated lipoproteins from the subendothelial space. However, uptake of modified lipoproteins is not well regulated and eventually macrophages become grossly engorged with lipids, resulting in the formation of foam cells and dysregulation of lipid metabolism (Moore et al. 2013). The LDL receptor is negatively regulated at the transcriptional level by increases in cellular cholesterol. However, CD36, a type B scavenger receptor, is positively regulated by oxLDL, and is one of the main receptors responsible for macrophage uptake of modified lipoproteins (Van Berkel et al. 1998; de Winther et al. 2000; Nicholson and

Hajjar 2004). These lipid-laden foam cells have diminished capacity to migrate out and therefore accumulate, giving rise to a frothy appearance in the arterial wall characteristic of a fatty streak. Foam cells impart a negative impact on disease progression two-fold: in beginning stages, with compromised immune functions resulting in diminished capacity to migrate and resolve inflammation; and in advanced stages, where they contribute to the inflammatory response by secretion of pro-inflammatory cytokines and promote plaque instability by secretion of matrix-degrading proteases (Randolph 2008; Moore et al. 2013).

Macrophages exhibit incredible plasticity and are able to change their physiological function in response to their surrounding environment. The two most commonly seen and studied macrophage phenotypes are classified as "pro-inflammatory" M1 and "anti-inflammatory" M2, and switching to either phenotype occurs under the appropriate stimuli. The phenotype of a macrophage has a large effect on its overall contribution to atherosclerotic plaque development. M1 macrophage phenotype is induced by stimulation with Th1-secreted IFNγ and LPS from Gram-negative bacteria, and promotes inflammation by producing pro-inflammatory cytokines, such as IL-1β, IL-12, and TNFα; and by attracting more monocytes through production of monocyte chemoattractant protein (MCP)-1 (Tabas and Bornfeldt 2016). TLR4 signaling plays a large role in the M1 response, as it can activate NFκB, ERK, p38 MAPK, and JNK signaling to promote transcription of pro-inflammatory genes and enhance foam cell formation (Muslin 2008).

The M2 phenotype can be acquired in the presence of IL-4 and IL-13, two cytokines that are largely produced by Th2 T cells. Ligand binding of IL-4 to the IL-4 receptor causes STAT6 activation, which can function to suppress TLR4 signaling. M2 macrophages produce anti-inflammatory molecules, such as IL-10 and TGFβ (Tabas and Bornfeldt 2016), that lessen the

pro-inflammatory response of Th1 cells and control cellular functions involved in tissue remodeling and repair. M2 macrophages could be extremely beneficial in the regression of atherosclerotic plaques, as they secrete factors that can promote collagen formation and can function in the clearance of dying cells and debris within the lesion (Moore et al. 2013). However, human atherosclerotic lesions show a Th1-dominant plaque environment, bearing more IFN than IL-4 (Frostegård et al. 1999), resulting in more macrophages with the M1 phenotype. As these cells are not considered to have a pre-determined fate, it is likely they can switch between phenotypes in the presence of an always changing microenvironment. Further, the microenvironment of atherosclerotic plaques is highly variable depending on the location and stage of development, which can affect the M1/M2 macrophage balance at any given moment.

TLR signaling also plays a role in antigen presentation by professional antigen presenting cells (APCs), such as macrophages, contributing to activation and polarization of adaptive immune processes. APCs present antigenic peptides to T cells to activate several different immune functions depending on the class of T cell and type of antigen. The majority of T cells are classified as either CD4+ or CD8+ T cells. CD4+ T cells are considered "helper" cells that can illicit either a pro- or anti-inflammatory response depending on the cytokine environment. CD8+ T cells are cytotoxic T cells that function to kill cells that are infected with intracellular pathogens, such as viruses. Activated T cells are found in human and murine atherosclerotic plaques (Hansson et al. 1989) and the magnitude of the pro-inflammatory response during advanced atherosclerotic lesion development is largely regulated by T cells (Andersson et al. 2010). Although all subsets of T cells have been found in atherosclerotic plaques (Andersson et al. 2010; Hansson and Hermansson 2011; Lahoute et al. 2011), several studies have shown more CD4+ than CD8+ T cells (Stemme et al. 1995; de Boer et al. 1999; Andersson et al. 2010). Like

macrophages, CD4+ T cells can acquire different phenotypes depending on the signals present in the surrounding microenvironment. The most common subtypes present in atherosclerotic lesions are Th1 and Th2, with Th1 cells being the most dominant T helper subtype (Laurat et al. 2001; Mallat et al. 2009).

T cells become activated within the atherosclerotic lesion when APCs present them antigenic fragments, such as peptide fragments from modified LDL particles that mimic patterns associated with pathogens, causing them to differentiate. Differentiation into a Th1 phenotype occurs in the presence of macrophage-secreted IL-12 and IL-18 (Elhage et al. 2003), and Th1 cells are considered to be pro-atherogenic largely due to their secretion of IFNγ. A few studies have shown that IFNγ exacerbates plaque development, where injection of recombinant IFNγ into ApoE$^{-/-}$ mice resulted in increased lesion size, and knockdown of IFNγ in ApoE$^{-/-}$ mice resulted in decreased lesion size (Whitman et al. 2000). Blockade of IL-12 and IL-18 signaling in ApoE$^{-/-}$ mice also resulted in decreased plaque size (Mallat et al. 2001; Davenport and Tipping 2003) and increased collagen content (Mallat et al. 2001; Hauer et al. 2005), likely due to the lost ability for Th1 differentiation and a subsequent decrease in IFNγ secretion. The Th2 subset, like the M2 macrophage phenotype, is characterized by anti-inflammatory actions; however, the role of Th2 cells in the development of atherosclerotic lesions is controversial. Th2 differentiation is stimulated in the presence of IL-4, a cytokine which opposes pro-atherogenic Th1 differentiation and IFNγ secretion. Whether or not Th2 cells are anti-atherogenic or not remains to be elucidated, as IL-4 expression also stimulates some pro-atherogenic events, such as VCAM-1 and MMP expression and increased MCP-1 secretion (Barks et al. 1997; Y.W. Lee et al. 2001).

The role of CD8+ T cells in the progression of atherosclerosis is less clear than that of CD4+ T cells. Knockdown of CD8+ in ApoE$^{-/-}$ mice resulted in no appreciable change in plaque

burden (Elhage et al. 2004), however, CD8+ activation in ApoE$^{-/-}$ mice showed an increase in plaque size (Olofsson et al. 2008). This data could indicate that the role of CD8+ T cells is minor compared to the role of CD4+ cells under normal conditions, possibly bearing a more significant role in the presence of a stimulus, such as an intracellular infection.

Atherosclerotic Plaque Instability

Atherosclerotic plaques, for the most part, remain "silent" throughout their progression, bearing no complications. However, certain factors can provoke plaque rupture and endothelial erosion, leading to ischemia and other life-threatening complications. Stable plaques contain a fibrous cap made of a collagenous matrix, which serves to stabilize and protect the contents within the lipid-rich core. It is when the surface continuity of the endothelium and fibrous cap becomes compromised that complications can arise. Plaque rupture, also called fissuring, is seen in about 75% of patients that present with acute coronary syndrome (Davies and Thomas 1984), while over 25% of coronary thrombosis cases present with plaque surface erosion (Farb et al. 1996). Research on plaque disruption has been hindered due to lack of appropriate animal models, as rodents rarely yield thrombolytic activity within plaques (Johnson and Jackson 2001; Schwartz et al. 2007). Studies have shown, so far, that the actions of MMPs within the lesions, as well as deposition of calcium, are among the processes contributing to unstable plaques and plaque rupture.

Matrix Metalloproteinases (MMPs)

MMPs are endopeptidases that play an important role in controlling the composition of the ECM. There are several classifications of these zinc-dependent proteases based on their substrate specificity and function within the ECM: gelatinases, collagenases, matrilysins,

stromelysins, and other membrane type (MT) and unclassified MMPs (Snoek-van Beurden and den Hoff 2005; Roy et al. 2006). At least twenty-three different MMPs have been identified in humans (Raffetto and Khalil 2008), playing important roles in processes such as wound healing and tissue repair (Johnson 2017 Sep 9). Some classes of MMPs are implicated in a range of different pathologies, where they have increased expression and activity. Therapeutic targeting of MMPs has been explored in the treatment of several maladies, including arthritis, cancer, neurodegenerative diseases, and arthritis (Morgan et al. 2004; Fingleton 2007).

MMP activity is regulated at the level of gene transcription as well as by post-translational mechanisms. These proteolytic enzymes are secreted in an inactive zymogen form that requires subsequent stepwise activation, through a process of cleavage and conformational change (Nagase et al. 2006). Multiple pathways for MMP activation exist within the atherosclerotic plaque environment. Activation of several different MMPs occurs in the presence of mast cell-secreted chymase and tryptase (Lees et al. 1994; Johnson et al. 1998), thrombin (Galis et al. 1997), cathepsin G and K (Sukhova et al. 1998; Legedz et al. 2004), and members of the plasmin system (Lijnen 2001), all of which are present in atherosclerotic plaques. Tight regulation of MMPs is further seen with endogenous inhibitors, proteolysis, and internalization and recycling (Nagase et al. 2006). The endogenous inhibitors, termed tissue inhibitors of metalloproteinases (TIMPs), irreversibly bind to and inactive MMPs and are considered to be the key regulators of MMPs under both normal and abnormal physiological conditions. Four TIMPs have been identified, and all function to inhibit different MMP classes/molecules. A finely tuned equilibrium of MMPs and TIMPs is needed in order to maintain a balance in ECM homeostasis.

It is known that plaque vulnerability is associated with increased inflammation, proteolysis of structural ECM proteins, and reduced collagen content. High expression and

disconcerted proteolytic activity of MMPs has been implicated in early and late stages of atherogenesis. It is thought that in the early stages of plaque development, MMPs promote migration and proliferation of immune cells and SMCs; in advanced plaques, excess inflammation drives increased proteolytic activity of MMPs, leading to a weakened fibrous cap and increased vulnerability to rupture (Wågsäter et al. 2011). In addition to inflammatory cytokines functioning to provoke certain immune responses, molecules such as IL-1β, TNFα, and IFNγ also induce MMP expression by macrophages (Amento et al. 1991; Sarén et al. 1996).

The gelatinase class of MMPs, which includes MMP-2 and MMP-9, has been studied extensively in the setting of cardiovascular diseases due to their regulatory function in VSMC migration and proliferation (Johnson 2007). Clinical studies have shown that MMP-2 levels are increased in patients suffering from acute myocardial infarction compared to healthy subjects, and MMP-9 levels are increased in patients with more severe vessel disease compared to healthy subject and subjects with less developed disease (Kai et al. 1998). Other studies have shown increased MMP-9 expression in SMCs and macrophages within advanced plaques, as well as increased MMP-9 proteolytic activity, compared to non-diseased vessels (Galis, Muszynski, et al. 1994; Galis, Sukhova, et al. 1994). Although increased circulating MMP-9 levels are not a clear indication for the presence of unstable plaques, several groups have shown the association between acute coronary syndrome and heightened MMP-9 levels in circulation (Kai et al. 1998; Inokubo et al. 2001). Most studies aimed at determining MMP relation to severity of atherosclerotic disease has been conducted in later stages of atherogenesis from tissues collected from human biopsies or mice genetically altered to overexpress or lack MMPs. One study used a mouse model of atherosclerosis to determine expression and activity patterns of several MMPs throughout the course of atherogenesis, and found that increases in MMP-2 and MMP-9

expression and proteolytic activity specifically in atherosclerotic plaques are associated with the progression of disease (Wågsäter et al. 2011).

Calcification

Mineralization of hard tissue, such as teeth and bones, occurs as a regulated process under normal physiological conditions and is required in order for these tissues to properly serve their purpose. It is only under pathological conditions that soft tissues become mineralized, and these situations often give rise to consequences that are detrimental. Advanced atherosclerotic plaques are among these scenarios of soft tissue mineralization that can result in adverse clinical outcomes. Once thought to be a passive process, research has shed light on this ectopic mineralization process, recognizing it as an active process resembling embryonic osteogenic mechanisms and normal bone remodeling (Steitz et al. 2001; Trion and van der Laarse 2004; Bab et al. 2008; Moore et al. 2013). Vascular calcium deposition reduces vessel elasticity and augments plaque brittleness (Trion and van der Laarse 2004; Sun et al. 2012). Therefore, mineralization of plaques affects overall vascular compliance and is a risk factor for myocardial infarction and known to contribute substantially to the morbidity and mortality of cardiovascular diseases (Detrano et al. 2008; Polonsky et al. 2010).

Current anti-atherosclerosis therapies, such as statins and beta-blockers, are aimed at modulating the disease risk factors hypercholesterolemia and hypertension, respectively. However, these therapies have not been successful in drastically decreasing mortality rates. Therefore, development of new therapeutic approaches that are aimed at controlling or decreasing calcification of atherosclerotic lesions could be beneficial in stabilizing late stage atherosclerotic lesions and further decrease mortality rates.

Lesion development involves many different cell types, such as monocytes/macrophages and VSMC. Specifically, VSMCs are key contributors to lesion development through osteogenic differentiation (Byon et al. 2008). Studies have provided much evidence to support the hypothesis that vascular calcification mirrors regulatory processes seen in osteogenesis, involving a balance between inhibitory and stimulatory molecules. Proliferation, extracellular matrix maturation, and mineralization are the three major sequential phases of osteogenesis, involving many key components that help facilitate the phase progressions (Vimalraj et al. 2015). Osteoblasts originate from mesenchymal stem cells and are matrix-producing cells that are responsible for bone formation. Runt-related transcription factor 2 (Runx2) is a major modulator in the process of osteogenesis and serves as a key transcription factor in the regulation of osteoblast differentiation genes (Komori 2009; Vimalraj et al. 2015). Runx2 has been shown to be essential in the early stages of osteogenic phenotype transition and phosphate-induced matrix calcification of SMCs, which serve as a primary mediator in vascular calcification (Lin et al. 2015). Downstream from Runx2 is a zinc-finger containing transcription factor, Osterix (Osx), which is necessary for osteoblast differentiation and further bone formation (K. Nakashima et al. 2002; K. Nakashima and de Crombrugghe 2003; C. Zhang 2012). Osx is needed for the commitment of pre-osteoblasts to differentiate into mature, functioning osteoblasts (C. Zhang 2012) and the specificity of Osx expression to osteoblasts is higher than that of Runx2 expression (K. Nakashima and de Crombrugghe 2003). Several noncollagenous bone proteins that are present during hard tissue calcification are also involved in the calcification process of soft tissues, such as alkaline phosphatase (ALP) (Giachelli 1999), osteocalcin (OCN) (Levy et al. 1983), and bone morphogenetic protein type 2 (BMP-2) (Boström et al. 1993). Osteopontin (OPN) is another protein that plays a role in ectopic

calcification of soft tissues. OPN is a ubiquitously expressed protein serving many different functions depending on the environment, and specifically serves as a potent inhibitor of vascular calcification (Lund et al. 2009).

While majority of cell types undergo terminal differentiation, the SMC phenotype bears much plasticity. Characteristically, VSMCs exist as a mature contractile phenotype but have the ability to differentiate into a synthetic fibroblastic phenotype in response to injury or disease (Steitz et al. 2001). Osteoblastic transdifferentiation (OBT) of plaque VSMCs is a key step in the atherosclerotic calcification process, sharing many features to the synthetic VSMC phenotype. During this transition, VSMCs dedifferentiate into a mesenchymal stem cell that serves as a precursor to osteochondrogenic cell lineages (Figure 1.3).

Under appropriate stimuli, VSMC begin acquiring an osteoblast-like phenotype, characterized by the loss of smooth muscle (SM)22α and SM α-actin markers and the upregulation of Runx2 (Steitz et al. 2001). Increased expression of Runx2 can directly stimulate the transcription of type I collagen (ColI) (Franceschi et al. 2003), which is necessary before osteoblasts can differentiate and express other osteogenic genes, like OCN and ALP (Franceschi 1999). While increased Runx2 is necessary during the beginning stages of OBT, its presence can serve to inhibit maturation of pre-osteoblasts in later stages (McGee-Lawrence et al. 2014). Once these cells have committed to the osteoblast lineage and adopt a pre-osteoblast phenotype, only Osx is required for maturation into a calcifying cell. Presence of Runx2 or OPN at this stage can halt the calcification process (Figure 1.3).

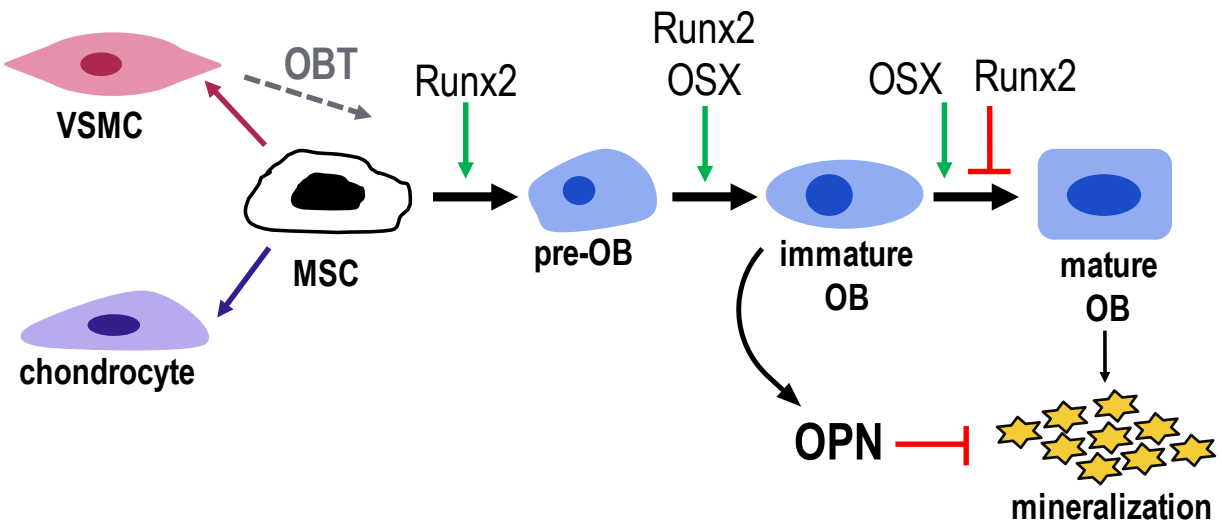

Figure 1.3 Vascular smooth muscle cell (VSMC) osteoblastic transdifferentiation (OBT). VSMCs dedifferentiate into a mesenchymal stem cell (MSC), a precursor to osteoblasts. Runx2 is necessary for the commitment of MSCs to the osteoblast lineage, but negatively regulates the maturation of immature osteoblasts into mineralizing cells. Osterix (OSX) is necessary for final stages of osteoblast maturation. Osteopontin (OPN) transcription is regulated by Runx2 and functions as a negative regulator of ectopic calcification.

A homeostatic balance between osteoblasts and osteoclasts is essential for normal bone remodeling. Opposite from osteoblasts, osteoclasts are large, multinucleated cells that function to absorb bone tissue during repair and remodeling. Osteoclasts can differentiate from monocytes/macrophages and share many signaling molecules and regulatory mechanisms with immune cells (Takayanagi 2007). The most important cytokine involved in osteoclast differentiation and activation is receptor activator of nuclear factor κ-B ligand (RANKL). RANKL is expressed by mesenchymally-derived cells, such as osteoblasts, and proteolytic cleavage of this membrane-anchored molecule allows for soluble RANKL to bind to RANK to initiate osteoclastogenesis (T. Nakashima et al. 2000). Differentiation and activation of osteoclasts can be inhibited by osteoprotegerin (OPG), a soluble decoy receptor for RANKL expressed by mesenchymal cells. The presence of OPG inhibits RANKL binding to RANK on

osteoclasts, resulting in diminished NFkB activation. Evidence from several studies has demonstrated an essential role for the RANKL/RANK/OPG system in maintaining normal bone homeostasis, and mutations in these genes can result in bone disorders, such as autosomal recessive osteopetrosis in the case of mutated RANKL (T. Nakashima and Takayanagi 2009). Exact mechanisms of plaque calcification remain to be elucidated, but evidence is accumulating that shows an association between increased inflammation and uncontrolled biomineralization of plaques.

Mouse Models of Atherosclerosis

The most common way to study atherosclerosis *in vivo* is by use of mice that have genetic modifications resulting in hypercholesterolemia. Wild-type mice are resistant to atherosclerosis and have high levels of high-density lipoproteins (HDL) in plasma. Mice are beneficial due to their short breeding times and production of large litters, ease of achieving genetic modifications, and low cost of maintenance compared to other animal models. While almost ten murine models of atherosclerosis exist, the most widely used strains are knockouts that are deficient in either the low-density lipoprotein receptor ($Ldlr^{-/-}$) or apolipoprotein-E ($ApoE^{-/-}$), the first gene-knockout mice to develop advanced atherosclerosis coupled with severe hypercholesterolemia.

ApoE, a major component of very low-density lipoproteins (VLDL), is synthesized by the liver and macrophages and functions to package cholesterol for lipoprotein transport, playing an atheroprotective role. Ldlr expression is mostly on hepatocytes (Traber and Kayden 1980; Jaakkola et al. 1989) and functions to facilitate lipoprotein clearance by binding apoB100 and ApoE. Familial hypercholesterolemia is caused by mutations in the LDLR gene resulting in inefficient clearance of lipoproteins (Goldstein et al. 1983; Tolleshaug et al. 1983). The major

differences between these two mice strains are the induction of atherosclerotic disease and the pace of progression (Maganto Garcia et al. 2012). ApoE$^{-/-}$ mice are severely hypercholesterolemic from birth while Ldlr$^{-/-}$ mice only have mildly elevated circulating cholesterol levels. Differences are also seen in the lipoprotein profiles of these mice, where ApoE-null mice have more cholesterol contained in VLDL, and Ldlr-null mice have more cholesterol contained in LDL particles which more closely resembles the lipoprotein profile of humans. On a standard chow diet, ApoE$^{-/-}$ mice can develop quantifiable lesions within six weeks, and lesion formation and progression can be exacerbated with the addition of a cholesterol-rich atherogenic diet, which is a common practice. On the other hand, Ldlr$^{-/-}$ mice will not develop quantifiable lesions for at least six months on a standard diet, as the extent of disease is mostly correlated with the degree of hypercholesterolemia. Most studies utilizing Ldlr$^{-/-}$ mice thereby are conducted by feeding an atherogenic diet to induce hypercholesterolemia required for the formation of atherosclerotic lesions. A typical atherogenic diet used with both of these mice models is the "Western" diet. Also referred to as the "meat-sweet diet" or "standard American diet", the Western diet mimics a typical diet seen in modern Western society consisting of high amounts of saturated fat and trans-fatty acids (Odermatt 2011). A positive correlation exists between higher incidence of cardiovascular complications and obesity with a western-style dietary pattern (Kant 2004; Zarraga and Schwarz 2006), making this diet appropriate for diet-induced hypercholesterolemia and atherogenesis with murine models of atherosclerosis. The typical Western diet fed to these mice usually contains 21% butterfat and 0.2% cholesterol, although cholesterol levels may vary (Merat et al. 1999).

While these mice are cost effective and beneficial for studying atherosclerosis, there are a few drawbacks seen with use of these mouse models. For instance, atherogenic diets can cause

potential inflammatory effects that are not related to the actual progression of atherosclerosis, and mice lack late stage plaque rupture, a complication commonly seen in humans. Further, similar treatments can result in varying effects between the different mouse models used, and this must be taken into consideration when selecting a model, as further genetic manipulation or treatments can be affected by the preceding genetic and physiological background.

The Endocannabinoid System

The medicinal usage of *Cannabis* for the treatment of a wide variety of human ailments, from headaches to sexually transmitted diseases to cancers, have been documented as far back as the 15th century (Grotenhermen and Müller-Vahl 2012; Borgelt et al. 2013). The medicinal properties of *Cannabis* are attributable to the unique aryl-substituted meroterpenoid compounds, called cannabinoids, produced in the *Cannabis* genera of plants (Ashton 2001). Over 50 years ago researchers at the Hebrew University of Jerusalem isolated and characterized the first cannabinoid, (-)delta-9-tetrahydrocannabinol (THC), the main psychoactive component of marijuana (Gaoni and Mechoulam 1964; Gaoni and Mechoulam 2002) . Since then, more than 100 different cannabinoid compounds have been identified in *Cannabis*, however, only a few of these have been extensively studied (Aizpurua-Olaizola et al. 2016). For the next nearly thirty years, how THC (and other cannabinoids) produced specific biological effects was unknown, with most speculating that the hydrophobic nature of cannabinoids resulted in nonspecific interactions with cell membranes. However, in the late 1980s evidence accumulated supporting a specific signal transduction mechanism for cannabinoids and in the early 1990s two membrane-bound cannabinoid receptors were identified, cloned and characterized (Matsuda et al. 1990; Munro et al. 1993). These cannabinoid receptors, and their later discovered endogenous ligands (endocannabinoids) and metabolic enzymes, are collectively termed the endocannabinoid system

(ECS) (Fig. 1.4). Over the past 30 years, preclinical studies have shown that the ECS functions in a wide variety of physiological and pathological processes; with alterations in both the levels of CB1 and CB2 receptor expression and levels of their endogenous ligands being associated with several human diseases including neurological conditions, metabolic disorders, cardiovascular diseases, as well as some cancers (Pacher et al. 2006; Pacher and Kunos 2013).

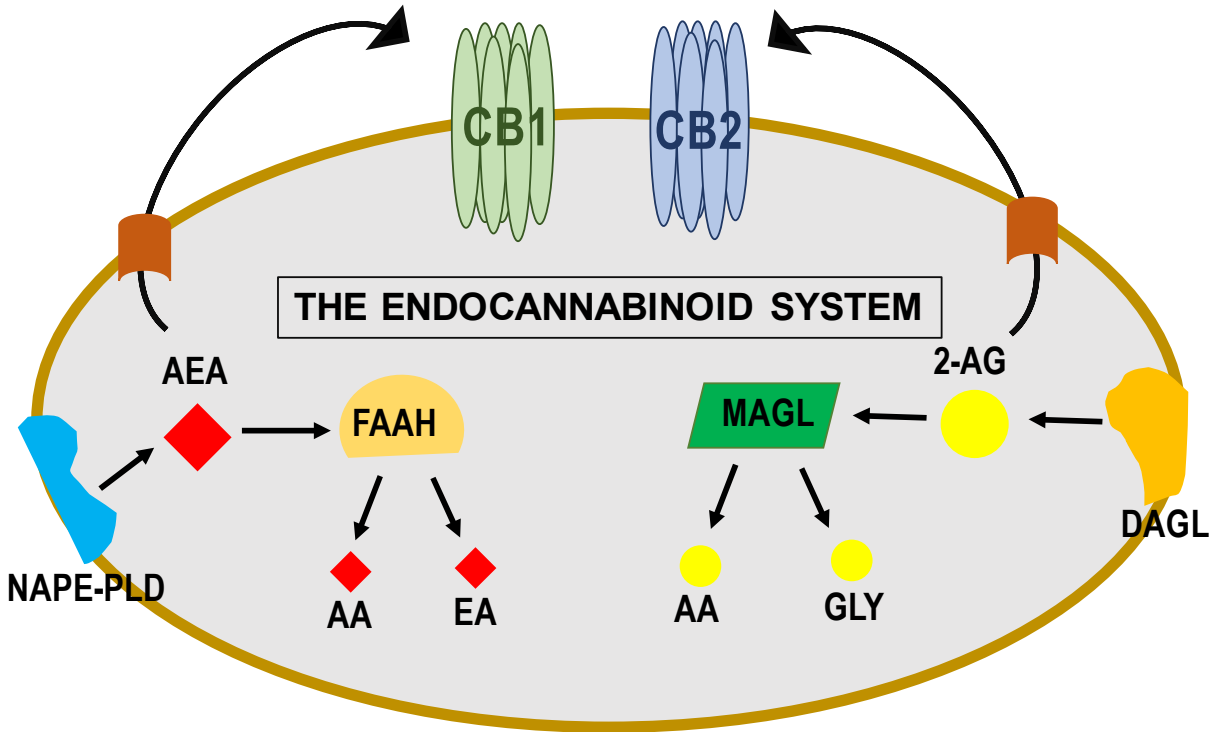

Figure 1.4 The Endocannabinoid System (ECS). The ECS consists of two known receptors, CB1 and CB2, the endogenous ligands for these receptors (endocannabinoids), and the metabolic enzymes involved in the synthesis and degradation of endocannabinoids. Endocannabinoids are synthesized intracellularly and transported to the extracellular space where they bind to and mediate signaling of the cannabinoid receptors.

Cannabinoid receptors

The first cannabinoid receptor identified, and later termed cannabinoid receptor type 1 (CB1), was independently cloned from rat and human brain cDNA libraries in 1990 (Gérard et

al. 1990; Matsuda et al. 1990). Shortly thereafter, another cannabinoid receptor, termed cannabinoid receptor type 2 (CB2), was cloned from HL60 cells, a human promyelocytic leukemia cell line (Munro et al. 1993). CB1 and CB2 are products of distinct intronless genes (*CNR1* and *CNR2*, respectively) with *CNR1* located on chromosome 6 in humans and chromosome 4 in mice, and *CNR2* located on chromosome 1 in humans and chromosome 4 in mice. Both are members of the G-protein coupled receptors (GPCR) superfamily of cell membrane receptors, containing seven transmembrane spanning domains separated by three intracellular and three extracellular loops, a glycosylated extracellular N-terminus and an intracellular C-terminus. Overall, human CB1 and CB2 share 44% amino acid similarity, with 68% homology within their transmembrane domains (Munro et al. 1993). Both receptors are relatively highly conserved among mammals, for example CB2 sequence identity between humans and rodents is ≥80%.

CB1 is abundantly expressed by neurons in the brain, most notably in the cerebral cortex, basal ganglia, cerebellum, and hippocampus (Matsuda et al. 1990) where it is responsible for the psychotropic effects of THC. CB1 is also expressed at lower levels in parts of the peripheral and autonomic nervous system, as well as several tissues, including heart, lung, thymus, spleen, and reproductive organs (Howlett et al. 2002; Bonz et al. 2003; Quarta et al. 2010). Expression of CB1 has also been noted in immune cells, but at levels up to 100 fold lower than CB2 (Galiègue, Mary, Marchand, Dussossoy, Carrière, Carayon, Bouaboula, SHIRE, et al. 1995). Although CB1 has been shown to play a role in modulating nociception, anxiety, energy metabolism and lipogenesis, the potential for undesirable psychotropic effects has greatly hindered the development of CB1-targeted pharmacologic therapies. For example, Rimonabant, a CB1-selective antagonist developed by Sanofi-Aventis was demonstrated to reduce body weight and

improve cardiovascular risk factors in humans and was given approval as an anti-obesity drug in Europe in 2006 (Ravinet Trillou et al. 2003) (Mølhøj et al. 2010) (Després et al. 2005) (Pi-Sunyer et al. 2006) (Van Gaal et al. 2005; Scheen and Van Gaal 2007). However, in 2008, despite producing beneficial metabolic and cardiovascular effects, Rimonabant was withdrawn from use in Europe due to adverse psychiatric side effects resulting from its activity in the CNS. Rimonabant never gained approval from the FDA for use in the United States.

CB2 is mainly expressed by cells of the peripheral immune system where it mediates the immunosuppressive effects of THC (Buckley et al. 2000; Eisenstein et al. 2007). Expression of CB2 by immune cells varies (B lymphocytes> natural killer cells >macrophages, ≥monocytes ≥ neutrophils>T-cells) (Bouaboula et al. 1993; Munro et al. 1993; Galiègue, Mary, Marchand, Dussossoy, Carrière, Carayon, Bouaboula, Shire, et al. 1995) and can be greatly affected by the differentiation and activation state of the cell. For example, macrophages activated by thioglycollate or IFN-γ express significantly more CB2 than unstimulated macrophages (Carlisle et al. 2002). Generally, the expression of CB2 by non-immune cells is very low, but it has been observed in several other cell types including osteogenic cells (Ofek et al. 2006), cardiomyocytes (Bouchard et al. 2003), fibroblasts (Defer et al. 2009), endothelial cells (Ronco et al. 2007), and VSMCs (Rajesh et al. 2008). Expression of CB2 in the CNS is controversial, as some studies failed to detect CB2 mRNA in neurons by Northern blotting and *in situ* hybridization analysis (Munro et al. 1993), while others found very low levels using quantitative PCR analysis (Maresz et al. 2005). However, expression by hematopoietic-derived microglial cells, rather than neurons, could not be ruled out (Maresz et al. 2005). A number of immunostaining studies have reported low levels of CB2 expression in some areas of the brain (Van Sickle et al. 2005; Gong et al. 2006; Brusco et al. 2008; Callén et al. 2012; Kim and Li 2015), however, inconsistent results

obtained with CB2 antibodies from different sources, as well as similar staining patterns produced in mice genetically altered to lack CB2, makes interpretation of these immunostaining experiments tenuous (Buckley et al. 2000; Baek et al. 2013). Validation of CB2 expression in the CNS awaits more specific CB2 antibodies or implementation of other methods specific for CB2 detection.

CB1 and CB2 were initially characterized as GPCR that couple primarily to pertussis toxin sensitive $G_{i/o}$ signal transduction proteins and, when activated, inhibit adenylate cyclase to reduce intracellular cyclic AMP levels and modulate downstream cascades under the control of protein kinase A (PKA). In addition to affecting cAMP/PKA and Ca^{2+} signaling, emerging evidence indicates that cannabinoid receptors modulate multiple diverse signaling networks in a variety of cell types, including the mitogen-activated protein kinases/extracellular signal–regulated kinases (MAPK/ERK1/2), phosphoinositide-3-kinase–protein kinase B/Akt (PI3K-PKB/Akt), phospholipase C, Janus kinase and Signal Transducer and Activator of Transcription (JAK/STAT) and nuclear factor kappa-light-chain-enhancer of activated B cells (NFkB) pathways (Bouaboula et al. 1995; Bouaboula et al. 1996; Howlett 2005; Demuth and Molleman 2006).

Endocannabinoids

The cloning of CB1 and CB2 led to the discovery of endogenous cannabinoids, or endocannabinoids (ECs), that act as ligands of these receptors, including N-arachidonoylethanolamide (anandamide, AEA) (Devane et al. 1992), 2-arachidonoylglycerol (2-AG) (Mechoulam et al. 1995; Sugiura et al. 1995), 2-arachidonyl glyceryl ether (2-AGE, noladin ether) (Hanus et al. 2001), O-arachidonoyl-ethanolamine (OAE, virodhamine) (Porter et al.

2002), and N-arachidonoyl dopamine (NADA) (Bisogno et al. 2000). These ECs are bioactive lipid mediators that share a common backbone structure resulting from their synthesis from membrane phospholipid precursors containing arachidonic acid. Of these, AEA and 2-AG are considered to be the primary endogenous activators of CB1 and CB2 and are the best studied, while the functions and physiological roles of the others are unclear and require further investigation. AEA is a partial agonist of CB1 (Mackie et al. 1993; Atmaram D Khanolkar et al. 1996; Felder et al. 1999; M. Glass and Northup 1999) and a weak partial agonist of CB2 (Gonsiorek et al. 2000). 2-AG is a full agonist of both CB1 and CB2 (Mechoulam et al. 1995; Gonsiorek et al. 2000). Compared to most tissues, AEA and 2-AG levels are significantly higher in brain, however, detectable levels of both have been found in numerous other mammalian organ systems, including bone marrow, kidney, liver, spleen, plasma, adipose, and gut, with 2-AG being more abundant than AEA in most tissues (Mechoulam et al. 1998).

Metabolism of Endocannabinoids

Generation of AEA and 2-AG has been shown for only a limited number of cell types, most notably in stimulated neurons, platelets and macrophages (Sugiura et al. 2002). Both AEA and 2-AG are synthesized and released only on demand after an appropriate stimulation and, in most cases, uptake and intracellular degradation rapidly terminates their activity. Synthesis of AEA occurs as a two-step process, beginning with the formation of N-arachidonoyl phosphatidylethanolamine (NAPE) from phosphatidylethanolamine (Sugiura et al. 1996) followed by the cleavage of NAPE by NAPE phospholipase D to generate AEA (Basavarajappa 2007). Like AEA, 2-AG is also synthesized in a two-step process, where diacylglycerol (DAG)

is generated by phospholipid C-regulated hydrolysis of membrane phospholipids and converted to 2-AG by DAG lipase (Basavarajappa 2007).

Two enzymes are primarily responsible for the rapid turnover of ECs; fatty acid amide hydrolase (FAAH) which metabolizes AEA to free arachidonic acid and ethanolamine, and monoacylglycerol lipase (MAGL) which metabolizes 2-AG to free arachidonic acid and glycerol (Deutsch and Chin 1993; Cravatt et al. 1996; Maccarrone et al. 1998; Dinh et al. 2002; Saario et al. 2004). FAAH has also been reported to metabolize 2-AG, at least under some conditions (Goparaju et al. 1999). AEA and 2-AG metabolism is summarized in Figure 1.5. Transport mechanisms for uptake and reuptake of AEA and 2-AG are not completely understood, but in addition to their lipophilic nature that allows them to passively diffuse across cell membranes, facilitated diffusion by protein transporter mechanisms is likely to also occur (Basavarajappa 2007).

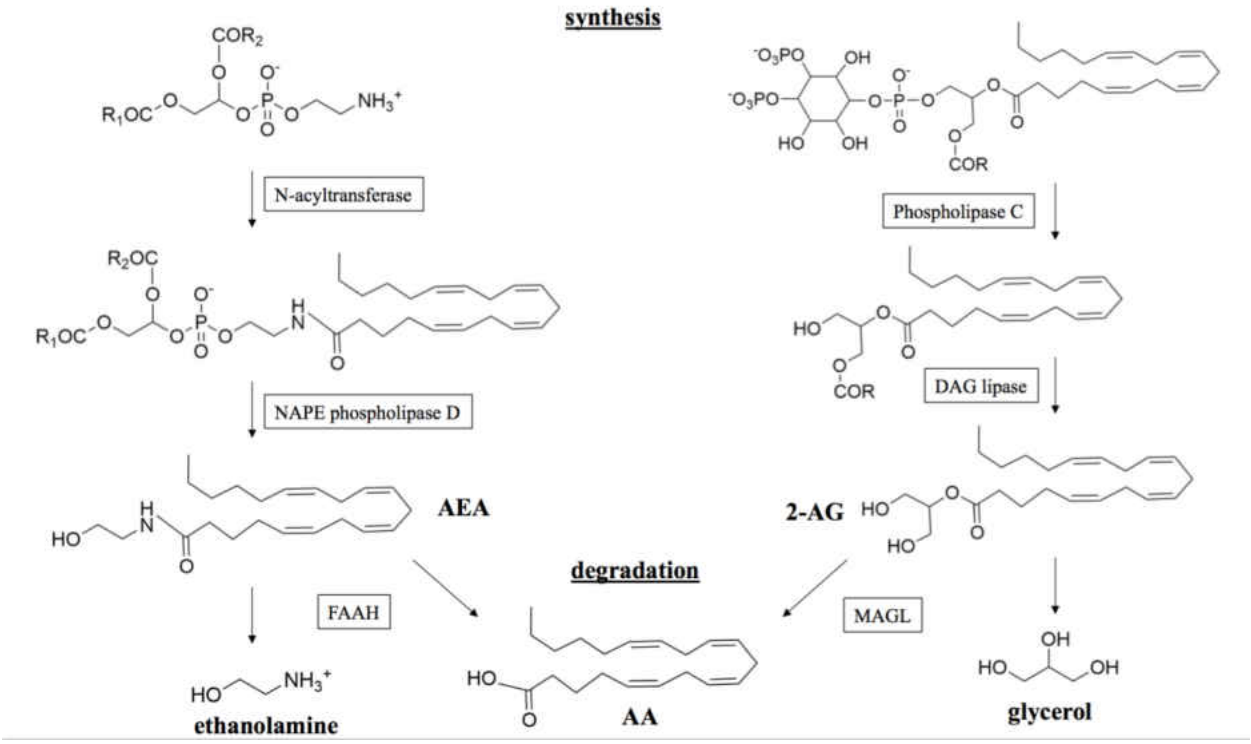

Figure 1.5 Metabolism of 2-AG and AEA. Synthesis of both 2-AG and AEA occurs as a two-step process, and degradation of both yields arachidonic acid (AA).

CB2-deficient mouse models

The use of mice genetically altered to lack CB2 expression has enabled investigators to examine how CB2 specifically functions in various disease states, giving rise to potential novel CB2-directed therapies aimed at reducing the burden of disease. The first CB2 knockout mouse ($Cnr2^{tmZim}$) was generated in 2000 in chimeras of 129 (H-2b) and C57BL/6 (H2-b) mice by inactivation of the *CNR2* gene using homologous recombination to eliminate part of the intracellular loop 3, transmembrane domains 6 and 7, and the carboxyl extremity of the receptor (Buckley et al. 2000). In this model of CB2 inactivation, CB1 expression and function is unaltered and there are no morphological differences between these mice and their wild type counterparts. At a cellular level, one study found that CB2-null mice present with abnormal development of certain subsets of T and B cells (Ziring et al. 2006), however, spontaneous

development of any observable immune disorder has never been noted in these mice. It should be noted that strain-specific genetic effects can still be observed even after extensive backcrossing. WT mice should be obtained from the same breeder in order to identify whether an experimental outcome is CB2-dependent or a genetic effect specific to the strain.

A second CB2 knockout mouse was generated by Deltagen ($Cnr2^{tm1Dgen}$) and is available from Jackson Labs (Bar Harbor, ME). Inactivation of CNR2 was achieved by homologous recombination in E14 stem cells from129P2/OlaHsd mice followed by at least 5 generations of backcrossing with a mixed C57BL/6J;C57BL/6N background. In a study comparing $Cnr2^{tmZim}$ and $Cnr2^{tm1Dgen}$, it was found that GRP55 signaling was impaired only in $Cnr2^{tmZim}$ mice (Sisay et al. 2013). $Cnr2^{tmZim}$ mice still have the ability to generate a truncated CB2 message due to the presence of an intact CNR2 promotor and N terminus, and it is possible that GRP55 signaling is affected by a dysfunctional CB2 message or signal (Buckley et al. 2000; Sisay et al. 2013). Other differences seen between the two CB2 knockout models may reflect differences in their genetic backgrounds. Despite this, both CB2-deficient mice models are widely accepted and used; the use of CB2-null mice has proven to be an instrumental tool in cannabinoid research and defining a role for CB2 in various inflammatory disease models.

ECS and Atherosclerosis

Clinical and experimental data has emerged indicating that local and systemic fluctuations in expression of ECS components is strongly associated with atherosclerosis, suggesting that alterations in EC levels or CB receptor expression may represent biomarkers of ongoing vascular disease. Increased systemic ECs are present in patients with coronary artery disease (Sugamura et al. 2009), and CB2 expression is upregulated in activated macrophages

(Carlisle et al. 2002; Rajesh et al. 2008) and has been detected within atherosclerotic plaques of both human coronary arteries and aortas of mice, but is not found in non-diseased vessels (Steffens, Veillard, Arnaud, Pelli, Burger, Staub, Zimmer, Frossard, and Mach 2005a; Montecucco et al. 2012).

The first experimental evidence indicating a role for CB2 in atherosclerosis came from a study by Steffens et al. in 2005, demonstrating that $ApoE^{-/-}$ mice given a low oral dose of THC developed significantly smaller plaques with fewer macrophages than untreated control mice (Steffens, Veillard, Arnaud, Pelli, Burger, Staub, Zimmer, Frossard, and Mach 2005b). Co-administration of SR144528, a CB2-selective antagonist, reversed the effects of THC on lesion progression, providing strong evidence that the effects of THC on atherosclerosis were mediated by CB2. It should be noted that the dose of THC used in this study was below that at which psychotropic effects are produced, a major concern for the development of cannabinoid-based therapies. Similar effects were seen with WIN55,212-2, a potent synthetic CB receptor agonist that produces similar effects as those seen with THC. In one study by our laboratory, two weeks of daily intraperitoneal injections of WIN55,212-2 in early atherogenic $Ldlr^{-/-}CB2^{-/-}$ mice resulted in reduced plasma triglycerides and decreased macrophage accumulation in aortic root plaques (Netherland-Van Dyke et al. 2015). These results were consistent with our previous findings that systemic CB2-deletion resulted in increased macrophage infiltration in hyperlipidemic Ldlr-null mice (Netherland et al. 2010). Administration of WIN55,212-2 in the $ApoE^{-/-}$ mouse model not only reduced atherosclerotic plaque size and macrophage content, but also decreased expression of VCAM-1 and reduced expression of pro-inflammatory mediators, including blocking NFκB activation by oxLDL (Zhao, Y. Liu, et al. 2010; Zhao, Yuan, Y. Liu, Xue, Tian, W. Liu, W. Zhang, Shen, Xu, Liang, and Chen 2010a). These effects were blunted in

the presence of a CB2-selective antagonist AM630, providing evidence of these results also being CB2-dependent. Since VCAM-1 is a necessary component of transendothelial migration of blood monocytes and other components of the immune system, a reduction in its expression on the endothelium could hinder the progression of plaque size by limiting the rate at which monocytes migrate into the subendothelial space. Several *in vitro* studies have also confirmed the role of CB2 in macrophage infiltration and expression of cell adhesion molecules. Administration of both HU-308 and JWH-133 blunted TNFα-induced VCAM-1 and ICAM-1 expression on human coronary artery endothelial cells (HCAECs) (Rajesh et al. 2007), and this effect was also seen with WIN55,212-2 administration in human umbilical vein endothelial cells (HUVECs) (Zhao, Y. Liu, et al. 2010). Both studies also showed a reduction in adhesion of monocytes, and the effects on both endothelial cells and monocytes were all blunted in the presence of a CB2-selective antagonist. Steffens et. al. also provided evidence for low doses of THC inhibiting macrophage migration in response to MCP-1, which was blocked by SR144528 (Steffens, Veillard, Arnaud, Pelli, Burger, Staub, Zimmer, Frossard, and Mach 2005b). Further, the effects of THC were not seen in macrophages isolated from CB2-null mice.

In addition to affecting migration of macrophages, CB2 activation may also affect migration of other cell types in atherosclerosis. Our laboratory observed that CB2 deficiency resulted in increased SMC content within atherosclerotic plaques of $Ldlr^{-/-}$ mice (Netherland et al. 2010). In agreement with this, activation of CB2 with JWH-133 or HU-308 blunted the TNFα-induced proliferation and migration of SMCs isolated from human coronary arteries (HCASMCs) *in vitro*, by diminishing the activation of the MAPK pathway involved in VSMC migration and proliferation in humans (Rajesh et al. 2008). This effect was blocked by co-

administration of CB2 antagonists AM630 and SR144528 but was unchanged by the presence of a CB1-specific antagonist.

CB2 also modulates protease-mediated vulnerability of atherosclerotic plaques. Components of the ECM, particularly collagen, play a very important role in maintaining the structural integrity of vessels and plaques. Degradation of the ECM by MMPs leads to breakdown of collagen fibers, leaving atherosclerotic plaques weakened and more prone to rupture (Pasterkamp et al. 2000). The most common MMPs implicated in rupture-prone regions of atherosclerotic plaques are MMP-1, MMP-3, and MMP-9 (Newby 2005; Dollery and Libby 2006) and an inverse correlation exists between the levels of CB2 and MMP9 content in atherogenic vessels from humans (Montecucco et al. 2012) and mice (Netherland et al. 2010). Further, our laboratory found that systemic CB2 deficiency resulted in decreased collagen content and increased elastin fragmentation in aortic plaques of $Ldlr^{-/-}$ mice after 12 weeks of HFD, in addition to a 57% increase in MMP9 activity (Netherland et al. 2010). Other studies have discovered that MMP9 activity in immune cells is suppressed by pharmacological activation of CB2 and induced by CB2 antagonism (Ghosh et al. 2006; Tauber et al. 2009; Netherland et al. 2010). *In vitro* studies have shown that pre-incubation of human primary neutrophils with JWH-133 results in a reduction in MMP9 release stimulated by TNF-α, which is blunted with co-administration with AM630 (Montecucco et al. 2012). It is possible that CB2-mediated increases in MMP9 are, at least in part, responsible for subsequent increases in infiltration of immune cells into the plaque area due to the breakdown of structural components within the luminal endothelium. Interestingly, CB2 deficiency had no effect on collagen content, elastin fragmentation, or MMP9 activity in plaques of $Ldlr^{-/-}$ mice after 8 weeks of HFD feeding (Netherland et al. 2010). This suggests that CB2-dependent mechanisms involved in regulation

of ECM degradation may not come into play in initial stages of plaque development but play a protective role in more advanced lesions.

Due to their rapid catabolism, the administration of ECs in murine models to evaluate the effects of chronically elevated EC levels on atherosclerosis is not feasible. To overcome this limitation, mice lacking either FAAH or MAGL, the enzymes primarily responsible for catabolism of AEA and 2-AG, respectively, were developed. Mice lacking FAAH cannot efficiently degrade AEA and therefore accumulate substantially higher AEA levels in brain and peripheral tissues (Cravatt et al. 2001). FAAH-deficient mice have exaggerated CB1-dependent behavioral responses to AEA, including hypomotility, analgesia, catalepsy, and hypothermia, and also display an anti-inflammatory phenotype (Schlosburg et al. 2009; Zimmer 2015). Lenglet et al. created $FAAH^{-/-}ApoE^{-/-}$ mice to evaluate atherosclerosis in the setting of enhanced AEA levels due to FAAH deficiency (Lenglet et al. 2013). Compared to $ApoE^{-/-}$ mice, $FAAH^{-/-}ApoE^{-/-}$ mice have significantly elevated plasma AEA levels and develop smaller plaques with characteristics strongly associated with increased vulnerability to rupture, including elevated neutrophils, increased MMP-9 expression and lower SMC content. Pharmacological inhibition of FAAH activity in $ApoE^{-/-}$ mice produced very similar results (Hoyer et al. 2014). The role of cannabinoid receptors in the observed plaque destabilizing effects of FAAH deficiency was not evaluated in these studies. The fact that AEA is a weak partial CB1 agonist and is nearly inactive as a CB2 agonist (Sugiura et al. 1995; Gonsiorek et al. 2000; Di Marzo and De Petrocellis 2012), makes it likely that elevated AEA levels promote formation of less stable plaque via CB1-mediated mechanisms. Conclusive evidence of the role of CB1/CB2 in promoting the vulnerable plaque phenotype resulting from impaired FAAH activity will require additional studies of the effects of either genetic ablation, or pharmacological antagonism, of

CB1 and CB2 on the plaque phenotype in $FAAH^{-/-}ApoE^{-/-}$ mice and/or $ApoE^{-/-}$ mice treated with a FAAH inhibitor.

In 2011, Taschler et al. generated MAGL knockout mice to investigate the pathophysiological consequences of systemic elevation of 2-AG. These mice exhibit substantially less MAGL activity with concomitant increases in 2-AG levels in all tissues examined (white adipose, liver and brain) (Taschler et al. 2011). Vujic et. al. determined the effects of impaired 2-AG metabolism on atherosclerosis by creating $MAGL^{-/-}ApoE^{-/-}$ mice (Vujic et al. 2016). When fed an atherogenic diet, $MAGL^{-/-}ApoE^{-/-}$ mice develop significantly elevated 2-AG levels in plasma and in aortic tissue compared to $ApoE^{-/-}$ mice. *In vitro*, macrophages isolated from $MAGL^{-/-}ApoE^{-/-}$ mice displayed impaired capacity to form foam cells as evidenced by significantly reduced lipid loading and expression of CD36. Somewhat surprisingly, the plaques in $MAGL^{-/-}ApoE^{-/-}$ mice were larger than in $ApoE^{-/-}$ mice, even though macrophage and lipid content were slightly reduced. This apparent discrepancy was explained by the observation that collagen and SMC content were both substantially increased in $MAGL^{-/-}ApoE^{-/-}$ plaques, resulting in thicker fibrous caps and a more stable plaque phenotype. Notably, when $MAGL^{-/-}ApoE^{-/-}$ mice were given SR144528, plaques increased in size and became less stable resembling those in $ApoE^{-/-}$ mice, indicating that plaque stabilization in the setting of MAGL deficiency likely results from 2-AG activation of CB2-dependent mechanisms.

In contrast to the studies performed in the setting of reduced systemic MAGL-mediated degradation of 2-AG (Hoyer et al. 2014; Vujic et al. 2016), Jehle et. al. employed adoptive transfer of bone marrow stem cells to evaluated atherosclerosis under conditions of myeloid-specific deficiency in DAG lipase α (DAGLα), a key enzyme in the biosynthesis of 2-AG (Jehle et al. 2016). Macrophages isolated from $ApoE^{-/-}$ mice reconstituted with bone marrow from

mice lacking DAGLα had greatly reduced DAGLα activity and 2-AG levels compared to wild type macrophages; however, plasma levels of 2-AG were notably unchanged and only slightly reduced in aortic tissue, indicating that macrophage synthesis of 2-AG does not significantly contribute to circulating 2-AG levels in plasma or in aortic tissue. Consistent with the correlation established between decreased 2-AG degradation and enhanced plaque size in studies of MAGL deficiency, myeloid-specific deficiency in 2-AG synthesis correlated with decreased plaque size. Interestingly, systemic MAGL deficiency and myeloid-specific DAGLα deficiency, despite having opposite effects on 2-AG levels and plaque size, were both associated with substantially reduced immune cell infiltration (neutrophil granulocytes and macrophages). However, only 2-AG elevation resulting from MAGL-deficiency altered the SMC and collagen content of plaques. Together, these results indicate that macrophage 2-AG exerts a proinflammatory effect on early lesion formation by accelerating the infiltration of immune cells into the vessel wall, and suggests that as plaques progress, non-myeloid-derived cells become active to exert plaque stabilizing mechanisms in response to chronic elevation of 2-AG.

Although FAAH and MAGL have substrates in addition to AEA and 2-AG which may contribute to the observed effects on atherosclerosis, these studies provide compelling evidence that alteration of AEA and 2-AG homeostasis affects plaque phenotype. The opposite effects on plaque stability imparted by systemic MAGL deficiency and systemic FAAH deficiency strongly suggests that the primary endocannabinoid substrates of these enzymes (2-AG and AEA) are modulators of plaque phenotype, with AEA activation of CB1-dependent mechanisms decreasing plaque stability and 2-AG activation of CB2-dependent mechanisms increasing stability. Further, although there were some contradictory conclusions among the early studies of the effects of CB2 gene deletion on atherogenesis (Netherland et al. 2010; Delsing et al. 2011;

Hoyer et al. 2011; Willecke et al. 2011), most likely arising from differences in the genetic backgrounds of the CB2 knockout mice strains ($Cnr2^{tmZim}$ vs $Cnr2^{tm1Dgen}$), the atherosclerosis-prone murine models ($ApoE^{-/-}$ vs $Ldlr^{-/-}$), and the methods of analysis used in the studies, the accumulating evidence from *in vivo* and *in vitro* studies has coalesced into a consensus interpretation that CB2 signaling modulates the formation and progression of atherosclerotic plaque. Taken together, these results support an anti-atherosclerotic role for CB2 activation, at least in part, through modulation of inflammatory processes and biochemical signaling pathways involved in atherosclerotic plaque formation and stability. Further studies could potentially result in a CB2-selective therapy that aims to reduce inflammation and related plaque vulnerability in atherosclerosis.

Our laboratory previously found that systemic CB2 gene deletion resulted in changes in plaque cellularity of Ldlr-null mice fed a HFD for up to 12 weeks, a time frame known to induce beginning stages of atherosclerotic plaque development. We hypothesized that CB2 modulates key pathways in later stages of atherogenesis, some of which affect plaque stability and predisposition to rupture. The clinical relevance of our research lies in the time of analysis, as most patients do not begin treatment until plaques have already progressed over several decades. Our lab is also the first, to my knowledge, to assess CB2-dependent effects on plaque calcification in advanced lesions. Results from this basic research provides a promising strategy to reduce the burden of atherosclerosis, especially among populations in Western countries where atherosclerosis and associated complications account for the highest cause of death.

Specific Aims

Aim 1: Determine the effect of systemic CB2 deletion on plaque cellularity in advanced atherosclerotic lesions in Ldlr-null mice fed an atherogenic diet.

Aim 2: Determine the effect of systemic CB2 deletion on calcification of advanced plaques in Ldlr-null mice.

Aim 3: Identify effects of CB2 pharmacological targeting on atherosclerotic and vascular calcification.

CHAPTER 2

CANNABINOID RECEPTOR TYPE 2 (CB2) DEFICIENCY ALTERS LESION CELLULARITY OF ADVANCED ATHEROSCLEROTIC PLAQUES IN HYPERLIPIDEMIC LDLR-NULL MICE

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Abstract

Objective: Cannabinoid receptor 2 (CB2) has been shown to play a role in the early stages of atherogenesis, however, the involvement of CB2 in advanced atherosclerotic lesion progression has not yet been examined. To determine if CB2 plays a role in advanced atherosclerotic lesion composition and progression, we investigated the effects of systemic CB2 gene deletion on advanced atherogenesis in Ldlr-null mice fed an atherogenic high fat diet (HFD).

Methods and results: $Ldlr^{-/-}CB2^{+/+}$ ($CB2^{+/+}$) and $Ldlr^{-/-}CB2^{-/-}$ ($CB2^{-/-}$) were fed an atherogenic diet for at least 20 weeks to induce the formation of advanced lesions. Morphometric analysis revealed no significant difference between the atherosclerotic lesion area in the aortic root of $CB2^{+/+}$ and $CB2^{-/-}$ mice after 20 or 24 weeks of HFD, however, there was an increase in abdominal lesions present in aortas of $CB2^{-/-}$ mice after 20 weeks of atherogenic diet (17.0±1.3 versus 9.0±1.3%, $p<0.05$). Immunohistochemical staining of the aortic root revealed no difference in smooth muscle cell (SMC) content between the two genotypes, but a significant increase in the presence of macrophages in $CB2^{-/-}$ mice compared the $CB2^{+/+}$ mice (18.8±3.5 versus 8.7±1.7%; $p<0.05$) after 20 weeks of HFD feeding. The ratio of atherosclerotic lesion SMCs/macrophages is used as a determinant of lesion stability and was significantly reduced in $CB2^{-/-}$ mice (0.94±0.09 versus 1.96±0.24; $p=0.001$). Histological staining revealed no discernible difference in collagen content in $CB2^{+/+}$ and $CB2^{-/-}$ mice after 20 weeks. Initial zymography studies show increased matrix metalloproteinase (MMP)-9 activity in aortic lysates at several time points in $CB2^{-/-}$ mice compared to $CB2^{+/+}$ mice, with a 5-fold increase observed after 22 weeks of atherogenic diet.

Conclusions: CB2 receptor deficiency affects late stages of atherogenesis by increasing lesional macrophage content, decreasing lesion stability through decreasing SMC/macrophage ratio and

upregulating MMP activity. This data supports a protective role for CB2 in late stages of atherosclerosis. Information obtained from this study could be used to produce a CB2-specific therapy aimed at reducing the morbidity and mortality from atherosclerosis by reducing macrophage accumulation and increasing lesional stability.

Introduction

Heart disease, specifically coronary heart disease (CHD), is the leading cause of death in the U.S. (Hansson and Hermansson, 2011; Benjamin et al., 2017). The underlying cause of CHD is atherosclerosis, a chronic inflammatory disease of the vascular system that is characterized by the buildup of plaques, composed of lipids and other cellular debris, within arterial walls (Libby, 2012). Atherosclerosis begins when apolipoprotein B (apoB)-containing low-density lipoproteins (LDL) become sequestered in the intima of arterial walls, rendering them vulnerable to a series of chemical changes, such as oxidation to form oxLDL. These modifications allow the LDL particles to mimic molecular patterns associated with pathogens that trigger a pro-inflammatory immune response. An initial response seen to oxLDL accumulation is the activation of endothelial cells, characterized by upregulation of integrins that interact with adhesion molecules that facilitate the entry of immune cells such as monocytes and T lymphocytes (Amberger et al., 1997). Monocytes are recruited to the subendothelial regions where modified LDL is accumulated, differentiate into macrophages, and act as phagocytes to engulf the modified LDL. Expression of scavenger receptors leads to increased influx of LDL particles, leading to the conversion of macrophages to cholesterol-laden foam cells, a hallmark of atherosclerosis. Foam cells are retained in plaques and eventually contribute to disease progression by promoting insufficient lipid metabolism, further compromising fundamental immune functions. Foam cells that are accumulated in plaques become stationary and frequently die via necrosis or apoptosis, releasing their internal lipid content and immune components, which contributes to plaque instability and rupture (Hansson and Hermansson, 2011).

Retention of more lipids and secretion of pro-inflammatory cytokines, such as IFNγ, IL-1β, and TNFα, by immune cells aids in the multifactorial progression of atherosclerotic plaques.

Early atherosclerotic lesions are typically found in younger individuals with no clinical consequences. These lesions are classified as either “fatty streaks” or “intimal thickening”. Fatty streaks are characterized by foam cell accumulation that lacks a necrotic core, while intimal thickening is SMC accumulation without the presence of foam cells or lipids (Rosenfeld et al., 2000). During intermediate stages of lesion development, partial resolution is obtained by the formation of a fibrous cap from smooth muscle cells (SMCs) and collagen. The fibrous cap acts to stabilize the contents of the plaque and serves as a barrier to separate platelets from the blood and clotting factors contained within the lesion (Tabas et al., 2015). Lesions in this stage are considered to be stable and therefore typically do not result in adverse clinical events (Virmani et al., 2002). Over time lesions can develop a vulnerable phenotype, rendering them prone to rupture and initiating acute thrombotic complications. Features of this advanced lesion include large areas of necrosis, fibrous cap thinning, and increase inflammation. Thinning of the fibrous cap is caused by increased degradation of collagen and other ECM proteins by proteolytic enzymes called matrix metalloproteinases (MMPs). Several classes of MMPs exist, and the gelatinase MMP-9 has gained much interest in the role it plays in atherosclerosis. MMP-9 expression by macrophages within the plaque is positively correlated with oxLDL concentration (Suzue et al., 2007) and is associated with fibrous cap thinning and plaque rupture (Newby, 2007). Heightened inflammatory responses seen in advanced plaques leads to excess MMP secretion coupled with diminished capacity of SMCs to synthesize collagen. Weakening of the fibrous cap can allow fissures to form, exposing macrophage-derived clotting factors to the blood flowing through the lumen of the artery, leading to detrimental thrombolytic activity that can result in acute heart attack or stroke.

The endocannabinoid system consists of endocannabinoid ligands, their catabolic enzymes, and the receptors at which they function (Smith et al., 2015). Two G-protein coupled receptors, cannabinoid (CB) type 1 (CB1) and type 2 (CB2), have been characterized (Matsuda et al., 1990; Munro, 2015). The CB1 receptor is primarily located in the CNS and is responsible for the psychotropic effects of cannabinoids (Schlicker and Kathmann, 2001). CB2 is primarily expressed on peripheral immune cells, exerting an immunomodulatory role in response to cannabinoids (Galiègue et al., 1995; Buckley et al., 2000; Netherland et al., 2010) and further gaining interest as a drug target for inflammatory diseases (Mach et al., 2008; Pacher and Ungvari, 2008). Patients with coronary artery disease have elevated levels of circulating endocannabinoids (Sugamura et al., 2009), and both humans and mice express the CB2 receptor in atherosclerotic lesions but not in non-diseased vessels (Steffens et al., 2005), suggesting a functional signaling role for CB2 in atherosclerosis. Further studies have also supported this finding by showing that cannabinoid therapy reduces atherosclerotic lesion progression, an incidence that is blunted by co-administration of a CB2-selective antagonist (Steffens et al., 2005; Zhao et al., 2010b). Several *in vitro* studies have shown CB2-dependent effects on monocyte adhesion and infiltration (Rajesh et al., 2007; Zhao et al., 2010a), and our laboratory was the first to confirm a role for CB2 in early plaque macrophage content by CB2 gene deletion in a murine model of atherosclerosis (Netherland et al., 2010).

To our knowledge, no other studies have explored the effects of genetic knockdown of CB2 on advanced atherosclerotic plaque formation in Ldlr-null mice fed an extensive high fat diet. In this study, we examined the effects of CB2 deletion on the cellularity and stability of advanced atherosclerotic lesions. Due to the fact that patients typically do not begin intervention until they are in the advanced stages of atherosclerosis, it is important to uncover treatments that

can be beneficial in later disease stages. The results from this study may hold important clinical implications for the development and of CB2-directed drug therapies that could potentially reduce the burden of unstable atherosclerosis and cardiovascular disease.

Materials and Methods

Animals and diet-induced atherosclerosis protocol

All mice were housed in a pathogen-free, temperature- and humidity-controlled room in the Animal Research Facility at East Tennessee State University. Mice were maintained on a standard chow diet (Ralston Purina, St. Louis, MO) with water provided *ad libitum*. All animal procedures were approved by and conducted in accordance with the guidelines administered by the Institutional Animal Care and Usage Committee of East Tennessee State University and in conformity with the Public Health Service Policy on Humane Care and Use of Laboratory Animals published by the National Institutes of Health. CB2 receptor knockout (Buckley et al., 2000) in the C57BL/J6 background (a generous gift from Dr. Nancy Buckley, California State Polytechnic University, Panoma, CA) were crossed with Ldlr-null mice backcrossed to the C57BL/J6 background for ten generations (The Jackson Laboratories, Bar Harbor, ME) to generate $CB2^{-/-}Ldlr^{-/-}$ ($CB2^{-/-}$) and $CB2^{+/+}Ldlr^{-/-}$ ($CB2^{+/+}$) mice as described (Netherland et al., 2010). Genotyping was performed using PCR analysis of DNA isolated from tail clips using Ldlr- and CB2-specific primers. Age-matched male and female $CB2^{+/+}$ and $CB2^{-/-}$ mice were used for all experiments. At eight weeks of age, groups of male and female $CB2^{+/+}$ and $CB2^{-/-}$ mice were placed on an atherogenic, high-fat diet (HFD) consisting of 21% fat and 0.15% cholesterol (Harlan Teklad, Madison, WI) for at least 20 weeks.

Analysis of atherosclerosis

Animals were euthanized as previously described (Netherland et al., 2010). Briefly, mice were anesthetized and perfused via left ventricle with phosphate-buffered saline (PBS). The heart and aorta were dissected and the aortas were placed in 10% formalin at 4ºC and the heart tissue

was embedded in OCT medium and snap frozen. OCT-embedded hearts were mounted in a Leica CM1850 cryostat and 8μm sections of the aortic root were collected. Tissue cryosections were stained for neutral lipids using oil-red O and counterstained with hematoxylin as described (Netherland et al., 2010; Netherland-Van Dyke et al., 2015). Digital images were captured using an EVOS FL digital imaging system (Electron Microscopy Sciences, Hartfield, PA). Fixed aortas were prepared using *en face* method and lipids were stained using Sudan IV reagent. Atherosclerotic lesions present in the aortic root and throughout the aorta were measured morphometrically using the ImageJ software (NIH, Bethesda, MD). Measurements were independently performed by two observers in a blind fashion.

Physiological parameters

Blood samples were collected and analyzed as previously described (Netherland et al., 2010). Briefly, overnight-fasted blood was collected from anesthetized mice via cardiac puncture. Plasma was separated by centrifugation and analyzed for total plasma cholesterol and triglycerides levels using commercially available kits (Pointe Scientific, Inc., Canton, MI) according to the manufacturer's protocol.

Histological and immunohistochemical staining

Prior to histological staining, fresh frozen aortic root sections were thawed and air dried for 1 h and rehydrated. The presence of collagen was determined using Mason's Trichrome staining. For immunohistochemical staining, cryosections were thawed and air dried at room temperature for 1h and fixed in ice-cold acetone for 10min. Sections were dried and pretreated with 1% H_2O_2 in PBS for 0.5h before being rinsed and blocked in normal serum for 0.5h at room

temperature. Detection of VSMCs and macrophages were accomplished by using anti-smooth muscle α-actin (ThermoFisher, Waltham, MA; RB-9010PO) (diluted 1:200) and MOMA2 (Chemicon International, Temecula, CA; MAB1852) (diluted 1:1000), respectively. Sections were developed using a Vector Labs Elite ABC kit (Burlingame, CA) and counterstained with methyl green at 55ºC for 5 min. Quantification of staining was performed by use of computer-assisted image analysis using ImageJ, and a standard threshold for staining that discriminated between positive and negative lesion areas was applied to all specimens analyzed. All evaluations were performed by a trained observer unaware of genotype or sample type.

Zymography

Aortic lysates were resolved on 10% SDS-PAGE gels containing gelatin (1mg/ml) and electrophoresed under nonreducing conditions. Gel proteins were renatured, washed, and activated as previously described (Netherland et al., 2010). Proteolytic activity was represented as zones of lysis that could be visualized in the background of Coomassie blue staining. Quantification was performed using ImageJ software.

LC-MS/MS measurement of aortic endocannabinoid levels

Quantification of aortic 2-arachydonoyl glycerol (2-AG) and anandamide (AEA) levels was performed using a Shimadzu liquid chromatography system coupled to a Shimadzu IT-TOF mass spectrometer with an electrospray (Columbia, MD) as previously described (Garst et al., 2016). Briefly, analytes were extracted using a liquid-liquid extraction method using toluene, evaporated to dryness under nitrogen and reconstituted in acetonitrile. Spiked standards were prepared using octadeuterated internal standards. Injections were performed with 10µL of reconstituted sample at a flow rate of 0.200mL/min and detection was achieved utilizing a direct MS/MS method in positive electrospray. Amount of 2-AG and AEA was normalized to the

weight of the tissue sample.

Statistical Analysis

Data is presented as mean± SEM. Data were analyzed by Student's t-test or one-way ANOVA using SigmaPlot Software. $p<0.05$ was considered to be statistically significant.

Results

CB2 deficiency affects the extent of lesion formation in the distal aorta

Throughout the study, no significant effect on plasma lipid levels was observed between the two genotypes (Figure 2.1c-d). Increases in mean body mass were observed in $CB2^{-/-}$ mice compared to $CB2^{+/+}$ mice after HFD feeding for 20 weeks (males: 42.1±1.9 versus 34.1±2.2 grams, respectively; p=0.01, females: 27.4±1.7 versus 22.8±0.6 grams, respectively; p=0.003) and 24 weeks (males: 42.1±2.1 versus 33.2±2.1 grams, respectively; p=0.01, females: 32.6±1.7 versus 22.3±1.0 grams, respectively; p=0.0001) (Figure 2.1a-b). Atherosclerotic lesion development was quantified in the aortic root and throughout the thoracic and abdominal aorta (Figure 2.2). No difference in mean percent of aortic root occupied by lesion was observed between $CB2^{+/+}$ or $CB2^{-/-}$ mice after 20 weeks (55.7±3.8 versus 52.2±3.7%, respectively; p=0.527) or 24 weeks (53.2±1.3 versus 52.3±2.6%, respectively; p=0.734) of HFD feeding. Examination of the extent of atherosclerosis in *en face* prepared aortas was performed in designated aortic segments: aortic arch, thoracic aorta, abdominal aorta. Quantification revealed a significant increase (~1.9 fold) in the percentage of abdominal aorta surface occupied by lesions in $CB2^{-/-}$ mice compared to $CB2^{+/+}$ mice after 24 weeks of HFD (17.0±1.3 versus 9.0±1.3%, respectively; p=0.002). However, no difference in percentage of area occupied by lesion was observed between $CB2^{+/+}$ and $CB2^{-/-}$ mice in the aortic arch (57.1±1.8 versus 54.9±2.0%, respectively; p=0.4) or thoracic aorta (17.4±2.6 versus 12.9±2.1%, respectively; p=0.2).

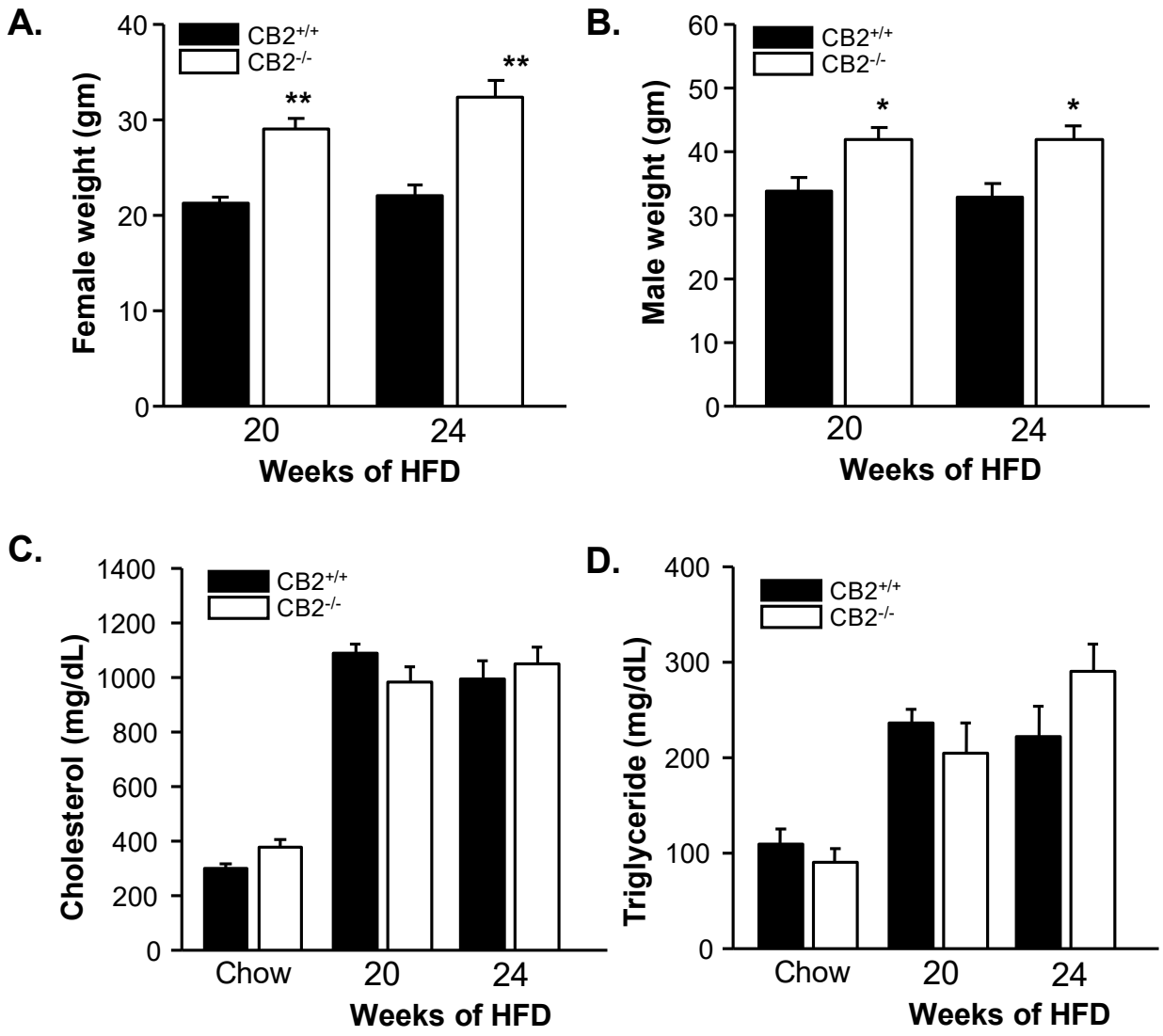

Figure 2.1 CB2 deficiency increases weight in Ldlr-null mice after 20 weeks of atherogenic diet. Weights of female (A) and male (B) $CB2^{+/+}$ and $CB2^{-/-}$ mice after 20 and 24 weeks of atherogenic diet. Serum levels of cholesterol (C) and triglycerides (D) from $CB2^{+/+}$ and $CB2^{-/-}$ mice after 20 and 24 weeks of atherogenic diet. Data represents means ($n \geq 8$)±SEM. *$p<0.05$; **$p<0.001$.

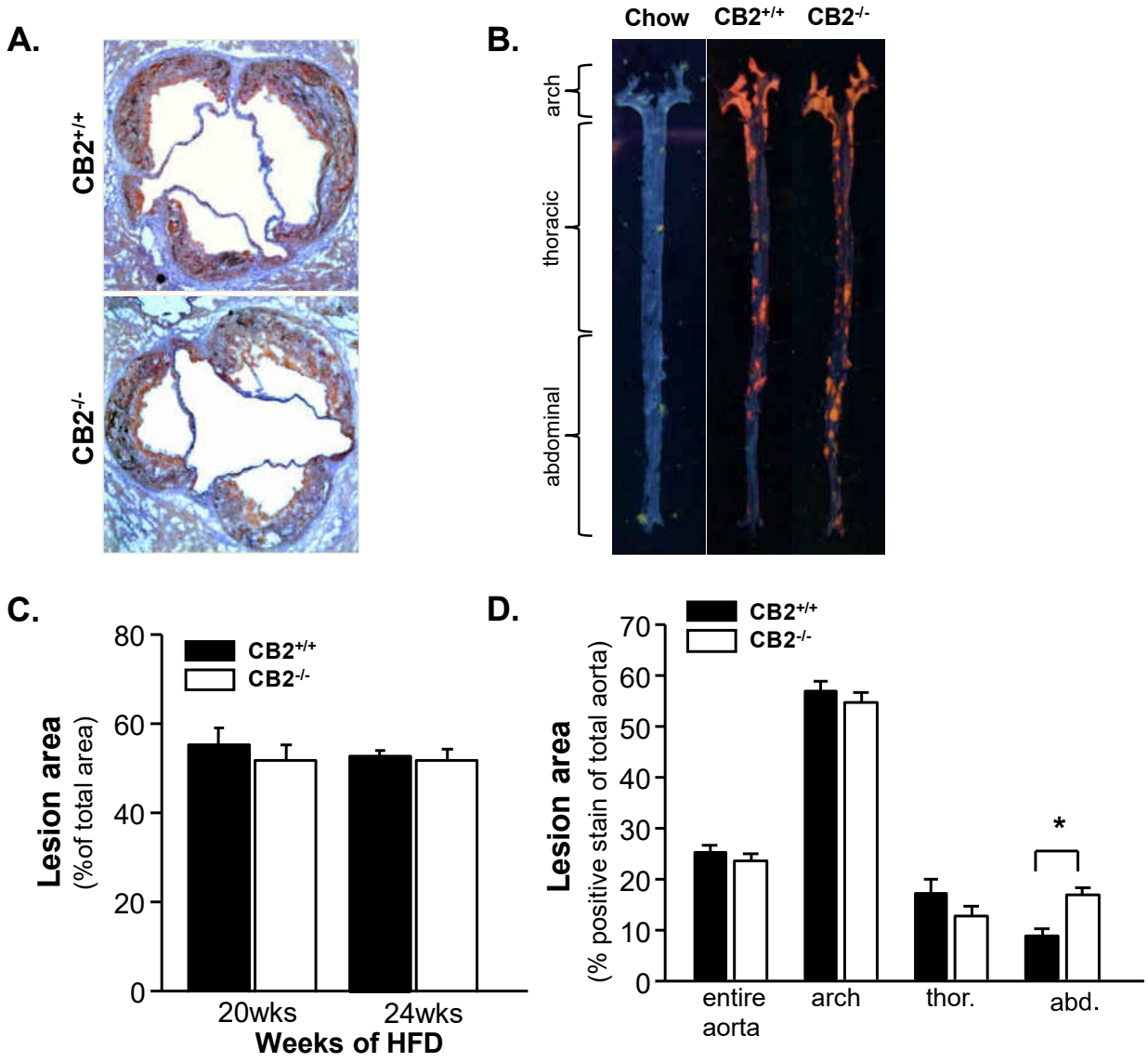

Figure 2.2 CB2 deficiency does not affect extent of atherosclerosis development in the aortic root but does affect plaque development in the abdominal aorta of Ldlr-null mice after 20 weeks of HFD. (A) Representative photomicrographs of oil red O-stained aortic root cross sections from $CB2^{+/+}$ and $CB2^{-/-}$ mice after 20 weeks of HFD. B) Representative photographs of *en faced* aortas from $CB2^{+/+}$ and $CB2^{-/-}$ mice stained with Sudan IV after 24 weeks of HFD. Quantification of the percent lesion area in the aortic root (C) and aortic lumen (D) of $CB2^{+/+}$ and $CB2^{-/-}$ mice. Data represents means (n=6-8)±SEM. *$p<0.05$ compared to $CB2^{+/+}$

<u>CB2 deficiency slightly elevates aortic 2-AG levels with no effect on AEA</u>

Aortas were analyzed to determine levels of two endocannabinoids, 2-arachydonoyl glycerol (2-AG) and anandamide (AEA) (Figure 2.3). LC-MS/MS analysis of aortic tissue

revealed no difference in either 2-AG or AEA between either genotype or chow-fed controls after 8 weeks of HFD. After 12 weeks of HFD, aortic levels of 2-AG and AEA were increased significantly in both $CB2^{+/+}$ and $CB2^{-/-}$ mice compared to chow-fed controls ($CB2^{+/+}$ versus chow 2-AG, p=0.004; $CB2^{+/+}$ versus chow AEA, p=0.003; $CB2^{-/-}$ versus chow 2-AG, p=0.0001; $CB2^{-/-}$ versus chow AEA, p=0.0006). 2-AG levels were significantly higher in $CB2^{-/-}$ mice compared to $CB2^{+/+}$ mice (0.6±0.05 versus 0.4±0.04 nM/mg, respectively; p=0.03). Although aortas from $CB2^{-/-}$ mice had slightly more AEA than $CB2^{+/+}$ mice, the levels were not statistically significant between genotypes (155.2±11.3 versus 146.3±11.8 nM/mg, respectively; p=0.6).

After 24 weeks of HFD, the aortic levels of 2-AG were further increased significantly compared to the levels after 12 weeks of HFD for both genotypes, with aortas of $CB2^{-/-}$ mice having significantly more 2-AG than $CB2^{+/+}$ mice (1.7±0.30 versus 0.7±0.05 nM/mg, respectively; p=0.0004). Interestingly, after 24 weeks the aortic levels of AEA decreased dramatically to levels below that of chow controls for both $CB2^{-/-}$ mice (0.6±0.06 versus 80.4±13.6 nM/mg; $p<0.001$) and $CB2^{+/+}$ mice (0.7±0.1 versus 80.4±13.6 nM/mg; $p<0.001$). The AEA levels between both genotypes at this time point were comparable (p=0.2). Elevations of endocannabinoids in relation to obesity have been accompanied with subsequent decreases in fatty acid amide hydrolase (FAAH) in humans (Engeli et al., 2005). To parallel the increased body mass and 2-AG levels we observed in $CB2^{-/-}$ mice, we also observed a reduction in protein expression of FAAH in CB2-deficient aortas; however, these levels never reached significance (Supplemental Figure 2.6).

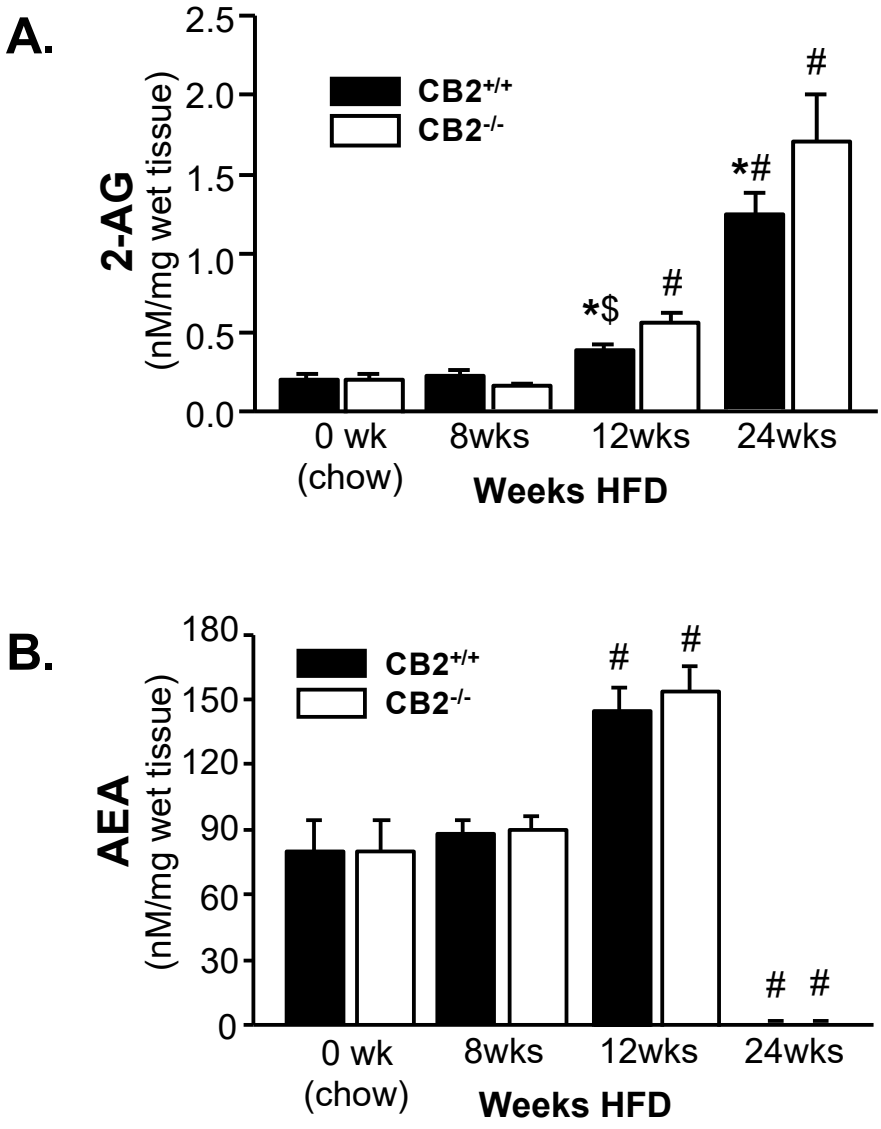

Figure 2.3 CB2 deficiency increases aortic 2-AG levels in Ldlr-null mice. Concentrations of 2-AG (panel A) and AEA (panel B) in aortas from $CB2^{+/+}$ and $CB2^{-/-}$ mice after HFD feeding for 0, 8, 12, and 24 weeks. Data represents means ($n \geq 8$)±SEM. #$p<0.001$ compared to chow; \$$p<0.05$ compared to chow; *$p<0.05$ between age-matched genotypes.

CB2 deficiency increases macrophage accumulation in atherosclerotic lesions

Macrophage accumulation in the intima of atherosclerotic lesions was readily detected after 20 weeks of atherogenic diet in both $CB2^{+/+}$ and $CB2^{-/-}$ mice as determined by immunostaining with a MOMA2 antibody (Figure 2.4b). Quantification of the areas staining positive for MOMA2 revealed a significant (~2.2 fold) increase in the macrophage content of aortic root lesions in $CB2^{-/-}$ mice compared to $CB2^{+/+}$ mice (18.8±3.5 versus 8.7±1.7%, respectively; p=0.03). Immunostaining with a smooth muscle α-actin antibody revealed migration of SMC from the intima towards the lumen in lesions of wildtype and CB2-deficient mice (Figure 2.4a). Quantification showed equivalent SMC immunoreactivity in both $CB2^{+/+}$ and $CB2^{-/-}$ lesions (14.0±1.1 versus 15.2±0.6%, respectively; p=0.4). Unstable atherosclerotic lesions contain a reduced ratio of lesional VSMCs relative to macrophages (Tabas et al., 2015). We observed a significant decrease in the ratio of VSMCs relative to macrophages in lesions of $CB2^{-/-}$ mice compared to $CB2^{+/+}$ mice (0.94±0.09 versus 1.96±0.24, respectively; p=0.001) (Figure 2.4e). Fibrous caps were also significantly thinner in $CB2^{-/-}$ mice compared to $CB2^{+/+}$ mice (52.8±3.8 versus 75.7±5.9 μm, respectively; p=0.002) (Figure 2.4f). These results indicate that although $CB2^{+/+}$ mice exhibit equally large lesions, their cellular composition is characterized by a more stable phenotype.

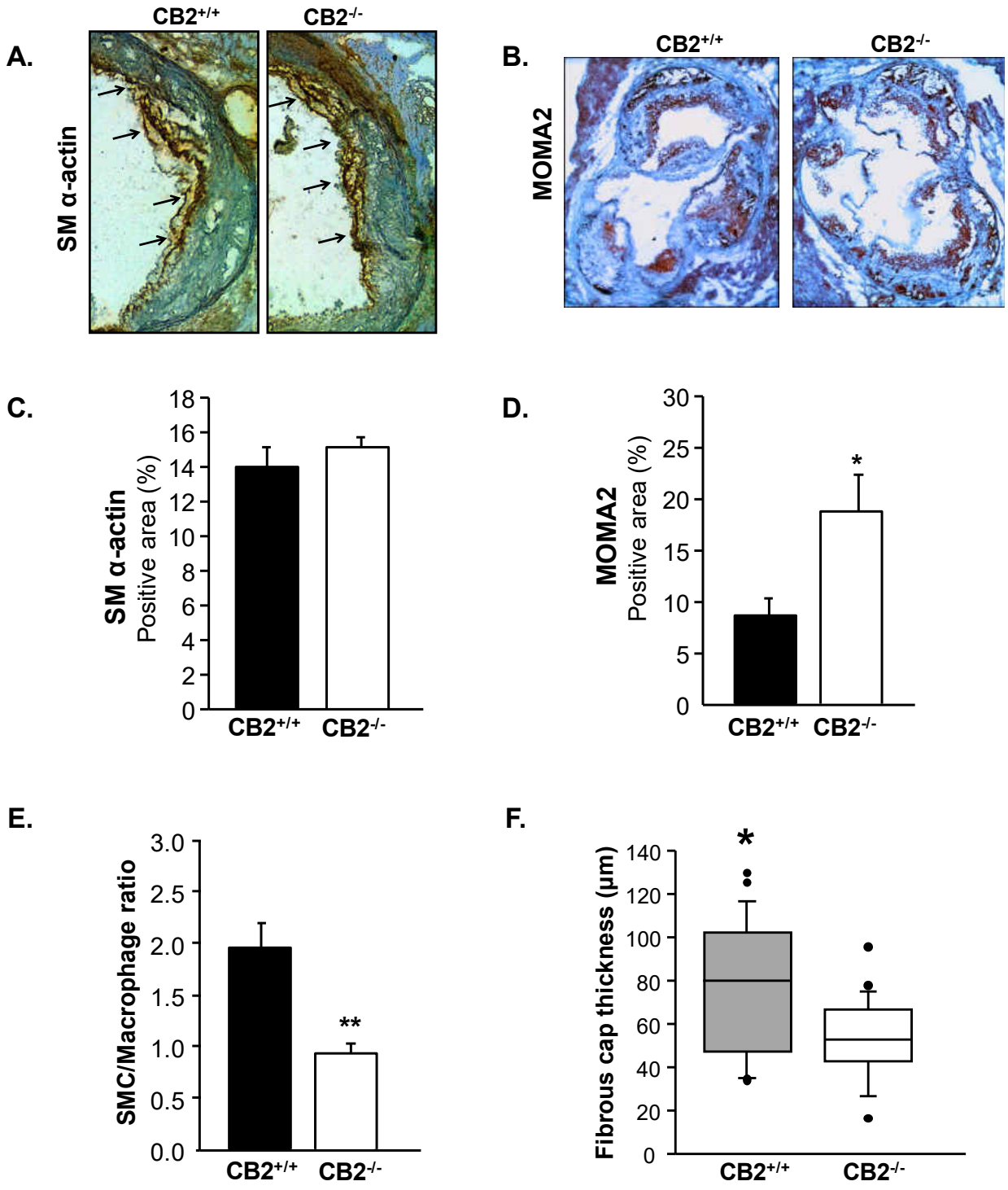

Figure 2.4 Cellular composition of atherosclerotic lesions in $CB2^{+/+}$ and $CB2^{-/-}$ mice fed an atherogenic diet for 20 weeks. Representative photomicrographs of aortic root cross sections from $CB2^{+/+}$ and $CB2^{-/-}$ mice fed an atherogenic diet for 20 weeks and immunohistochemically stained for SMCs (A) and macrophages (B) using SM α-actin and MOMA2 antibodies, respectively. Quantification of SMC (C) and macrophage (D) content of aortic root lesions from

$CB2^{+/+}$ and $CB2^{-/-}$ mice. Positive immunostaining was quantified and represented as the percentage of the total lesional area. E) Ratio of VSMCs relative to macrophages within atherosclerotic lesions in $CB2^{+/+}$ and $CB2^{-/-}$ mice. Ratios were calculated from percentage of positive SM α-actin signal relative to positive MOMA2 signal for all samples. F) Quantification of fibrous cap thickness. Each data point represents an average of 8-10 measurements from each mouse. Data represents means (n=6-8)±SEM.*$p<0.05$, **$p<0.001$

CB2 deficiency alters the ECM of atherosclerotic lesions

Collagen content of aortic root lesions was assessed by use of Mason's trichrome staining (Figure 2.5a), which revealed no statistical difference between $CB2^{+/+}$ and $CB2^{-/-}$ mice after 22 weeks of atherogenic diet (45.2±3.7 versus 50.8±4.6%, respectively; $p=0.4$). Our laboratory previously detected increased MMP9 activity in atherosclerotic lesions in the aortic root, as well as isolated peritoneal macrophages, of $CB2^{-/-}$ mice in early lesions (Netherland et al., 2010). We analyzed lysates prepared from the aortic arches of $CB2^{+/+}$ and $CB2^{-/-}$ mice to determine MMP9 activity, as this portion of the aorta displays extensive atherosclerosis. Zymography of aortic arch lysates from $CB2^{+/+}$ and $CB2^{-/-}$ mice showed a band of gelatinase activity at 92kDa, corresponding with the approximate weight of MMP9 (Figure 2.5b). Quantification of the zymograms confirmed larger zones of lysis corresponding to MMP9 activity in lysates from $CB2^{-/-}$ mice compared to those from $CB2^{+/+}$ after 22 weeks of atherogenic diet (4728.9±1306.1 versus 900.6±276.4, respectively; $p=0.04$).

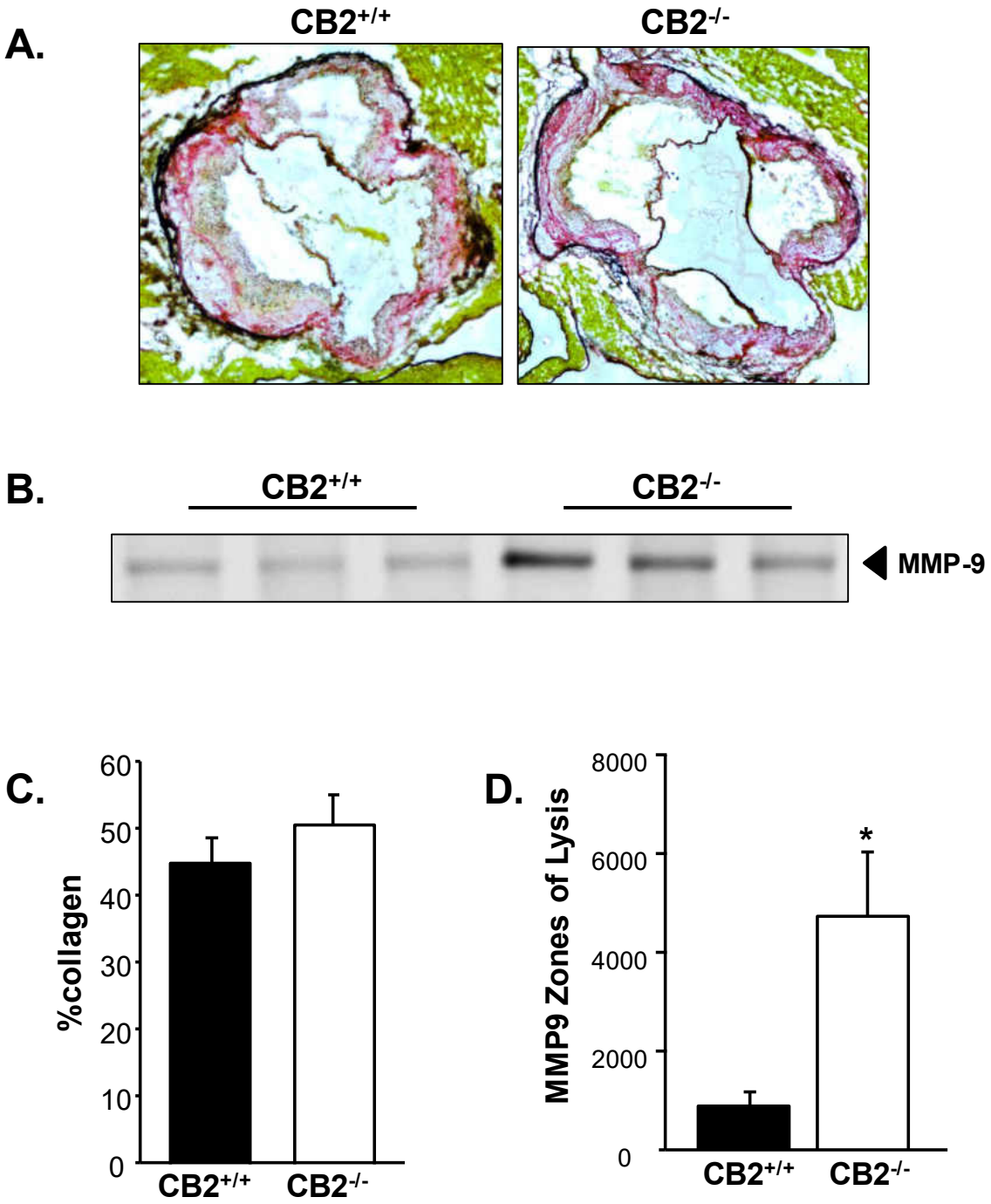

Figure 2.5 Collagen content and MMP9 activity in advanced atherosclerotic lesions in $CB2^{+/+}$ and $CB2^{-/-}$ mice. A) Representative photomicrographs of atherosclerotic lesions in aortic root cross sections stained for collagen after 22 weeks of atherogenic diet (pink). B) Zymogram of MMP9 activity from aortas of $CB2^{+/+}$ and $CB2^{-/-}$ mice after 22 weeks of atherogenic diet. C) Quantification of the collagen content of aortic root lesions from $CB2^{+/+}$ and $CB2^{-/-}$ mice (n=6). The amount of collagen is represented as a percentage of total lesional area. D) Digital quantification of the MMP9 zones of lysis in the zymogram (n=3). Graphical representation is shown as the band density for 15 µm of protein. Data represented as mean±SEM. *$p<0.05$.

Discussion

Several *in vivo* and *in vitro* studies suggest a role for CB2 in atherosclerosis (Steffens et al., 2005; Mach et al., 2008; Rajesh et al., 2008; Netherland et al., 2010; Zhao et al., 2010c; Netherland-Van Dyke et al., 2015), however, these studies have not been carried out to mimic advanced lesion progression. In the current study, we directly examined the role of CB2 in more advanced atherosclerosis by investigating the effects of systemic CB2 gene deletion on atherosclerosis in Ldlr-null mice fed an atherogenic diet for an extended period of up to 24 weeks. Our study showed that deletion of CB2 did not affect the lesion area in the aortic root of Ldlr-null mice despite the fact that CB2 activation has been shown to decrease lesion size previously. Pharmacological studies have shown that administration of exogenous CB ligands reduced aortic root atherosclerotic lesion size in ApoE-null mice in a CB2-dependent manner (Steffens et al., 2005; Zhao et al., 2010c). Alternatively, our laboratory failed to detect a difference in aortic root lesion size after administration of WIN55,212-2, a CB1/CB2 mixed agonist, in Ldlr-null mice (Netherland-Van Dyke et al., 2015). Contradiction in these results could be attributed to differences between the two murine models of atherosclerosis, as it is well documented that $Ldlr^{-/-}$ and $ApoE^{-/-}$ mice show variation in lipoprotein profiles and onset of atherogenesis (Daugherty and Rateri). Our study did, however, show an increase in the amount of lesion that occupied the abdominal aorta in CB2-deficient mice. No study until now has shown how CB2 deletion in Ldlr-null mice differentially affects lesion development in specific regions of the aorta.

Our laboratory and others have previously shown a CB2-dependent role in macrophage accumulation in early aortic root lesions (Steffens et al., 2005; Netherland et al., 2010; Zhao et al., 2010c). The current study confirms that CB2 deficiency also increases macrophage

accumulation in more advanced atherosclerotic lesions, suggesting that CB2 signaling plays a role in monocyte/macrophage accumulation with effects lasting into later stages of atherogenesis. The result is consistent with studies showing that blocking CB2 signaling results in increased transendothelial migration of monocytes into the vessel wall due to upregulation of adhesion molecules (VCAM-1, ICAM-1, p-selectin) on the surface of endothelial cells (Rajesh et al., 2007; Zhao et al., 2010b). However, our study did not measure expression of endothelial cell adhesion molecules, and to our knowledge, tissue macrophage proliferation has never been assessed in a CB2-dependent way. Therefore, it is possible that in advanced lesions the increased macrophage content of $CB2^{-/-}$ lesions is due to increased proliferation of lesional macrophages and not to increased transendothelial migration of blood monocytes. We plan to address this issue in future studies.

Proliferation and migration of VSMCs is an important mechanism for stabilizing atherogenic plaques. Our laboratory previously showed that CB2 deficiency increases SMC migration to the fibrous cap regions in early lesions (Netherland et al., 2010) and *in vitro* studies have shown attenuation of TNFα-induced SMC proliferation and migration in a CB2-dependent manner (Rajesh et al., 2008). However, our current study failed to show CB2-dependent loss of SMC staining in late atherosclerotic lesions. There are a few possible explanations for these somewhat contradictory results. First, there are many biochemical cell signaling pathways that can affect SMC migration and proliferation, and it is possible that CB2 modulation of these processes is overshadowed by compensatory pathways that become more involved as the disease progresses. Secondly, recent studies involving lineage tracing of cells in atherosclerotic plaques has provided evidence of phenotype-switching of certain cell types throughout atherogenesis (Lineage tracing of cells involved in atherosclerosis., 2016). Notably, this study found that

almost 20% of CD68-positive macrophage-like cells did not originate from the myeloid lineage, but instead, were derived from mature SM cells. It is possible that CB2 signaling affects SMC phenotype switching. CB2 activation results in an anti-inflammatory response, and its plausible that CB2 deletion increases the transition of SMCs to a macrophage-like phenotype, resulting in increased expression of inflammatory genes. Experiments utilizing a combination of genetic fate mapping and single cell expression analysis in the setting of CB2 gene deletion would have to be employed in a murine model of atherosclerosis to provide insight into the role of CB2, if any, in this process.

Although we previously showed that CB2 deletion resulted in decreased collagen content in early atherosclerotic lesions, the current study did not find an appreciable difference in collagen content between the two genotypes in late lesions. As our results were calculated as a percentage of total lesion area, an increase in acellular regions of lesions from $CB2^{-/-}$ mice affecting the total lesion area could be responsible for this result. Another explanation for this could be the fact that SMC content was similar between both groups, and SMCs are the primary producer of collagen. Our data suggests that CB2 modulation of SMC proliferation is only observed early in disease development, and becomes less prevalent in advanced stages.

Expression of MMP9 by immune cells is suppressed in the presence of cannabinoid agonists (Ghosh et al., 2006; Tauber et al., 2009), while a dose-dependent increase in MMP9 expression has been observed in macrophages exposed to a CB2-specific antagonist (Netherland et al., 2010). Consistent with this, we found an increase in MMP9 activity in aortic arch lysates of CB2-deficient mice. Increased serum MMP9 levels have been associated with increased risk of acute coronary events in patients with atherosclerosis (Kai et al., 1998; Inokubo et al., 2001) and increased MMP9 expression in atherosclerotic plaques is associated with disease progression

(Wågsäter et al., 2011). Another factor contributing to plaque instability beyond ECM degradation is a reduced ratio of VSMCs to macrophages within the lesion (Tabas et al., 2015), and our data showed a significant reduction in this ratio associated with CB2 gene deletion. However, as mentioned before, lineage tracing has provided evidence for switching of VSMCs to a macrophage-like phenotype, which potentially could affect this result if the phenotype switching is affected by CB2 signaling. Due to the lack of a definitive lineage tracing study in the setting of CB2 gene deletion, a considerable ambiguity exists regarding which cells are in fact derived from SMC mesenchymal lineage versus macrophage myeloid lineage.

The role of the ECS and CB1 specifically in the control of food intake, energy balance and body mass has been well-documented (Kirkham et al., 2002; Cota et al., 2003; Matias et al., 2006; Dodd et al., 2009). CB1 activation results in increased adipocyte differentiation and lipogenesis (Cota et al., 2003; Gasperi et al., 2007), and, accordingly, CB1 inhibition has been shown to protect against HFD-induced weight gain and also reduces body weight in obese animals and humans (Després et al., 2005; Pi-Sunyer et al., 2006; Scheen and Van Gaal, 2007; Dol-Gleizes et al., 2009; Engeli, 2012). The observed increases in body weight and aortic 2-AG levels in CB2-deficient mice are likely associated with each other, and could be explained by increased CB1 expression/signaling, as it is possible that CB1 is upregulated as a compensatory mechanism due to the absence of CB2. It has also been observed that CB1 expression in adipose tissue is increased in the presence of HFD-induced obesity in mice (Osei-Hyiaman et al., 2005), and 2-AG levels are increased in epididymal fat pads of obese animals in comparison to lean mice (Matias et al., 2006). Further, 2-AG administration in the limbic forebrain of animals increases food intake in a CB1-mediated, dose-dependent manner (Kirkham et al., 2002). It appears that CB1 and 2-AG both exhibit a “cause-and-effect” phenomenon, where both are

upregulated as a result of obesity, but also promote obesity through their biological functions. It is also likely that upregulation of CB1 affects 2-AG production, as biosynthesis of 2-AG in pathological conditions appears to occur selectively, requiring retrograde regulation mediated by CB1 (Di Marzo and De Petrocellis, 2012).

Alterations in the concentrations of 2-AG and AEA occur in many pathological conditions, and it is not uncommon to see opposite regulation of the two (Ligresti et al., 2009). The observed decrease in aortic AEA levels in both genotypes after 24 weeks of HFD is likely due to increased leptin production occurring in response to enhanced fat storage. Leptin is a hormone secreted by adipocytes that modulates food intake and energy balance by upregulating anorexigenic factors (Di Marzo et al., 2001; Brennan and Mantzoros, 2006). Several studies have shown an inverse correlation between AEA and leptin levels, and leptin inhibits endogenous AEA levels in the brain and uterus of rodents as well as in human blood (Maccarrone et al., 2003; Di Marzo et al., 2004; Maccarrone et al., 2005).

Collectively, these results provide additional evidence that supports a protective role of CB2 signaling in the progression of atherosclerosis. Previous studies reported an anti-atherogenic role for CB2 in early atherosclerotic lesion formation and progression, and our present study provides the first direct evidence of CB2 gene expression playing a role in modulating the cellular composition and stability of advanced lesions. These results support a potential use for pharmacological compounds with selective activity at CB2 in the treatment of atherosclerotic lesions which have progressed to an advanced stage. Uncovering a treatment regimen which could provide beneficial modifications of lesion compositions in later stages of disease development. This would be extremely clinically relevant, as most affected patients do not begin

receiving pharmacological intervention until after suffering from an initial acute episode resulting from rupture of an advanced lesion.

Conflict of interest

The authors have no conflict of interest to disclose.

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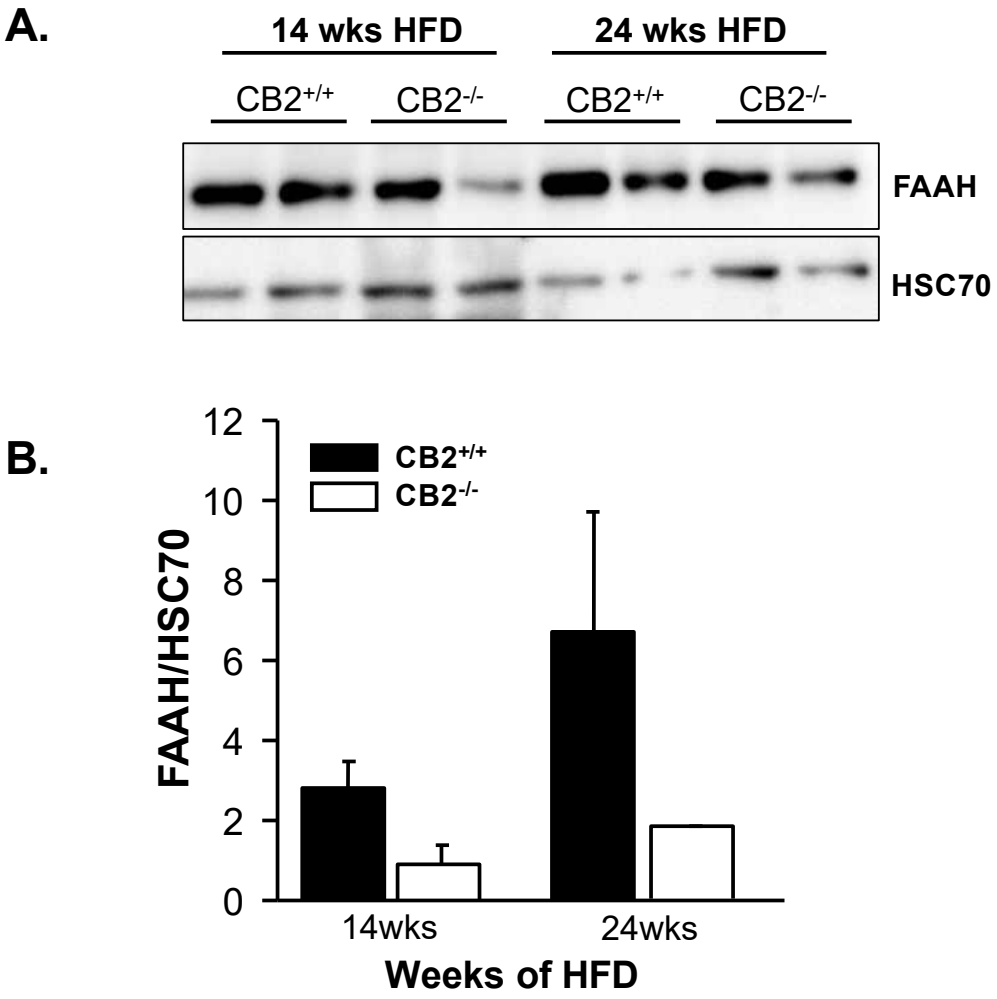
Supplemental Figures



Supplemental Figure 2.6 Effects of CB2 gene deletion of aortic FAAH levels in Ldlr-null mice. A) Immunoblot showing levels of FAAH in aortic arch lysates from $CB2^{+/+}$ and $CB2^{-/-}$ mice after 14 and 24 weeks of HFD. B) Quantification of FAAH levels relative to the housekeeping protein, HSC70. Data are means±S.D. (n=2) for each genotype.

CHAPTER 3

CANNABINOID RECEPTOR TYPE 2 (CB2) DEFICIENCY INCREASES CALCIFICATION OF ATHEROSCLEROTIC LESIONS IN HYPERLIPIDEMIC LDLR-NULL MICE

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Abstract

Objective: Calcification of atherosclerotic lesions is a risk factor for future adverse cardiovascular events. Cannabinoid receptor type 2 (CB2) signaling modulates bone remodeling processes and affects development of atherosclerotic lesions. To determine if CB2 signaling impacts lesion calcification, we investigated the effects of systemic CB2 gene deletion on the development of calcified lesions in Ldlr-null mice fed an atherogenic diet.

Methods and results: At 8 weeks of age, $Ldlr^{-/-}CB2^{+/+}$ ($CB2^{+/+}$) and $Ldlr^{-/-}CB2^{-/-}$ ($CB2^{-/-}$) mice were placed on an atherogenic diet for 20-24 weeks. Morphometric analysis of von Kossa-stained aortic root cross sections revealed a significant increase in calcium deposition in $CB2^{-/-}$ mice compared to $CB2^{+/+}$ mice (13.0±1.1 versus 5.6±1.2%; $p<0.05$) after 20 weeks of atherogenic diet. Biochemical analysis of the activity of the osteoclast marker enzyme, tartrate-resistant acid phosphatase (TRAP), revealed significantly less osteoclast activity in aortic root lesions of $CB2^{-/-}$ mice compared to $CB2^{+/+}$ mice (9.7±0.05 versus 4.9±0.16%; $p<0.05$). Immunofluorescent staining of aortic root cross sections with an antibody against soluble receptor activator of nuclear factor kappa-B ligand (RANKL) similarly showed reduced RANKL expression in lesions from $CB2^{-/-}$ mice compared to $CB2^{+/+}$ mice (0.2±0.0004 versus 0.04±0.0040; $p<0.001$). Immunoblotting detected significantly decreased expression of osteogenic regulatory proteins runt-related transcription factor 2 (Runx2) ($p<0.05$) and osteopontin (OPN) ($p<0.05$). Semiquantitative RT-PCR analysis of RNA extracted from aortic arch tissue revealed elevated mRNA levels of osteogenic genes, alkaline phosphatase (ALP), type I collagen (ColI), and osteoprotegerin (OPG) in $CB2^{-/-}$ mice ($P<0.05$ for all genes).

Conclusions: CB2 receptor deficiency increases calcification of atherosclerotic lesions in Ldlr-null mice, at least in part, by enhancing osteoblast activity and decreasing osteoclast activity

within lesions. Our data indicates a novel therapeutic potential for CB2 agonists to reduce lesion calcification, and therefore the risk for myocardial infarction and stroke.

Introduction

Cardiovascular disease (CVD) claims more lives every single year than all forms of cancer combined. The most prevalent form of CVD is coronary artery disease (CAD), a disease that is responsible for over 360,000 deaths every year in the U.S. (Benjamin et al., 2017). Atherosclerosis is the underlying cause of CAD and other CVDs, resulting in myocardial infarction, stroke, and peripheral vascular disease. Atherosclerosis is a chronic inflammatory disorder that progresses over the course of decades and is characterized by the buildup of atherosclerotic lesions (or plaque) containing lipids and immune cells within the walls of arteries. The disease is multifaceted, initially elicited by the subendothelial retention of lipoproteins and gradually evolving with the infiltration of inflammatory cells, the proliferation and migration of smooth muscle cells (SMCs), cellular apoptosis and necrosis, arterial remodeling and calcification, all of which may lead to plaque rupture and thrombosis (Bui et al., 2009; Bentzon et al., 2014). The majority of plaques are subclinical; however, a percentage of individuals develop unstable lesions which puts them at risk for adverse clinical outcomes.

Several factors are attributed to the formation of unstable plaques, such as increased proteolytic activity of extracellular matrix (ECM) components, necrotic core formation, and calcification. In particular, calcification of atherosclerotic plaques has gained increasing recognition over the past 15 years due to consistent findings of a correlation with increased risk of cardiovascular mortality (Sage et al., 2010). Vascular SMCs (VSMC) are key contributors to plaque calcification as a result of their phenotypic plasticity (Byon et al., 2008). VSMCs typically exhibit a contractile phenotype and are present in the medial layer of arteries to function in maintaining vascular tone. In response to injury or disease, VSMCs can be induced to switch to a synthetic phenotype in order to proliferate and migrate to sites in need of repair

(Shanahan et al., 1993; Shanahan and Weissberg, 1998). This is seen in the context of atherosclerosis, when VSMCs migrate into the intima and create a protective fibrous cap at site of injury. In normal instances, VSMCs are highly regulated and return to their contractile, non-proliferating phenotype. However, regulatory signals become compromised in the setting of atherogenesis, causing dysregulation in VSMC differentiation (Iyemere et al., 2006). Under these conditions, VSMCs transdifferentiate to a synthetic phenotype characterized by features of other mesenchymal lineages, such as osteoblasts (Tintut et al., 2003).

Osteoblasts are cells that synthesize a matrix that then becomes mineralized, and are important in the formation and remodeling of bones. Runt-related transcription factor 2 (Runx2) is a complex transcription factor necessary for the differentiation of osteoblasts (Ducy and Karsenty, 1995; Komori et al., 1997; Otto et al., 1997), and is considered to be the master regulator of osteoblastogenesis. Regulation of osteoblast differentiation by Runx2 is dynamic, and a shift from positive to negative regulation is seen from initial stages to final stages of osteoblast maturation. Runx2 also regulates the expression of several osteoblast marker genes, such as osteocalcin (Ducy and Karsenty, 1995), collagen I (ColI), osteopontin (OPN) (Ducy et al., 1997; Sato et al., 1998), osteoprotegerin (OPG) (Thirunavukkarasu et al., 2000), and alkaline phosphatase (ALP) (Bruderer et al., 2014). These proteins provide a variety of functions in the regulation of calcification, both negative and positive. For example, increased ALP is needed for progression of osteoblast maturation (Takeuchi et al., 1996) while OPN is a potent negative regulator of calcification and binds to apatite crystals to inhibit their growth (Wada et al., 1999).

The endocannabinoid system consists of two known G-protein coupled receptors, termed cannabinoid receptor 1 (CB1) and 2 (CB2), endogenous ligands (called endocannabinoids) for these receptors, and their metabolic enzymes. CB1 is largely expressed in the central nervous

system where it mediates the psychotropic effects associated with cannabinoids. CB2 is primarily expressed in peripheral immune cells, such as macrophages and T cells (Munro et al., 1993; Galiègue et al., 1995; Buckley et al., 2000), subsequently producing immunosuppressive effects. Emerging evidence indicates a role for the endocannabinoid system and CB2 specifically in atherosclerosis. Circulating endocannabinoid levels are increased in patients with coronary artery disease (Sugamura et al., 2009) and CB2 expression is present in atherosclerotic plaques but not in healthy vessels (Steffens et al., 2005a). Specifically, CB2 expression is upregulated in plaque macrophages (Carlisle et al., 2002) and has been implicated in pathways involved with ongoing atherogenesis (Steffens et al., 2005a; Montecucco et al., 2008; Netherland et al., 2010).

Interestingly, CB2 deficient mice display an age-related decrease in bone mass resembling osteoporosis, and polymorphisms in *CNR2*, the gene encoding CB2, are strongly associated with osteoporosis in postmenopausal women (Bab et al., 2009). Further, a negative correlation exists between osteoporosis and atherosclerotic calcification in humans (Hamerman, 2005; Farhat and Cauley, 2008; Stojanovic et al., 2011). Therefore, we hypothesized that CB2 plays a protective role in calcification of atherosclerotic plaques. In this study, we have provided the first evidence to our knowledge that CB2 inhibits lesional osteogenic processes and calcification in a mouse model of atherosclerosis. Furthermore, we identify key osteogenic regulatory mechanisms altered by CB2 deficiency within lesions and aorta. These finding provide molecular insights into the regulation of atherosclerotic calcification by CB2, and uncover potentially novel therapeutic uses for CB2 agonists to reduce adverse complications of atherosclerotic calcification.

Materials and Methods

Animals and diet-induced atherosclerosis protocol

All mice were housed in a pathogen-free, temperature- and humidity-controlled room in the Animal Research Facility at East Tennessee State University. Mice were maintained on a standard chow diet (Ralston Purina, St. Louis, MO) with water provided *ad libitum*. All animal procedures were approved by and conducted in accordance with the guidelines administered by the Institutional Animal Care and Usage Committee of East Tennessee State University and in conformity with the Public Health Service Policy on Humane Care and Use of Laboratory Animals published by the National Institutes of Health. CB2 receptor knockout mice in the C57BL/J6 background (a generous gift from Dr. Nancy Buckley, California State Polytechnic University, Panoma, CA) were crossed with Ldlr-null mice as previously described (Netherland et al., 2010). Age-matched $CB2^{+/+}$ and $CB2^{-/-}$ mice were used for all experiments. At eight weeks of age, groups of $CB2^{+/+}$ and $CB2^{-/-}$ mice were placed on an atherogenic, high-fat diet (HFD) consisting of 21% fat and 0.15% cholesterol (Harlan Teklad, Madison, WI) for 20-24 weeks to induce the formation of advanced atherosclerotic lesions.

Histological and immunofluorescent staining

Animals were euthanized as previously described (Netherland et al., 2010). Briefly, mice were anesthetized and euthanized by cardiac perfusion with 10 ml ice-cold phosphate buffered saline. The heart and aorta were dissected and snap frozen at -80°C, with heart tissue embedded in OCT medium. OCT-embedded hearts were mounted in a Leica CM1850 cryostat and 8µm sections of the aortic root were collected. Tissue cryosections were stained for calcium deposition using von Kossa method. *In situ* osteoclast activity was measured biochemically using

a tartrate-resistant acid phosphatase (TRAP) leukocyte kit (Sigma, St. Louis, MO) according to the manufacturer's protocol. For immunofluorescent staining, tissue cryosections were thoroughly rinsed with tris-buffered saline containing 0.1% tween 20 (TBS-T) and blocked in 5% serum in PBS-T for 1h at RT. Sections were then probed with an antibody against sRANKL (ProSci Inc, Poway, CA; #3963) (1:200 in blocking buffer) overnight at 4°C, rinsed with PBS-T, and incubated with a FITC-conjugated secondary antibody (Santa Cruz Biotechnologies, Dallas, TX) (1:1000 in blocking buffer) for 1h at RT in the dark. Sections were rinsed three times with PBS-T, once with PBS, and mounted with Pro-Long mounting medium with DAPI (ThermoFisher, Waltham, MA). Digital images were captured using an EVOS FL digital imaging system (Electron Microscopy Sciences, Hartfield, PA). Computer-assisted morphometric analysis was performed using ImageJ software (NIH, Bethesda, MD). A threshold for positive staining was measured and applied uniformly to all images. For each sample, a minimum of four slides containing four cross sections were evaluated and averaged. All measurements were performed by a trained observer in blinded fashion.

Immunoblotting

Aortic arch tissue was lysed in buffer containing 20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Triton-X100, 1% DOC, 1 mM EDTA, 0.05% SDS and freshly supplemented with protease inhibitor cocktail (Sigma, St. Louis, MO) by use of a handheld homogenizer. Lysates were centrifuged at 12,000 x g for 15 min at 4°C and the supernatant was collected and the protein content determined using a microBCA kit (ThermoFisher, Waltham, MA). Samples containing 20μg of protein were denatured at 95°C in the presence of DTT for 5 min and subjected to electrophoresis using 4-12% Bis-Tris gels (Invitrogen, Carlsbad, CA). Proteins were

transferred to a polyvinylidene fluoride membrane, blocked in TBS-T containing 5% non-fat milk and probed with specific antibodies to Runx2 (Santa Cruz, Dallas, TX; sc-10758) (1:2000), OCN (MyBioSource, San Diego, CA; MBS2003553) (1:1000), and OPN (R&D Systems, Minneapolis, MN; AF808) (1:3000) overnight at 4℃. After incubation with proper horse radish peroxidase (HRP) conjugated secondary antibody (Santa Cruz, Dallas, TX) (1:2000), membranes were developed using Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare Bio-Sciences, Pittsburgh, PA) and a Fujifilm Fla-4000 imager. Membranes were stripped and probed with an HSC70 antibody (Abcam, Cambridge, United Kingdom; ab19136) (1:10,000) to serve as a loading control. Quantitation of band densities was performed using ImageJ software (NIH, Bethesda, MD).

Semiquantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from aortas using Direct-zol RNA MiniPrep (Zymo Research, Irvine, CA). The concentration and purity ($A_{260/280}$) of RNA samples was determined using a Nanodrop ND-1000 Spectrophotometer (Nanodrop Technologies, Wilmington, DE). RT-PCR was carried out using a Verso HotStart 1-Step kit (Life Technologies, Carlsbad, CA) according the manufacturer's instructions in an Eppendorf Mastercycler (Hamburg, Germany). Typical cycling reactions were a first cycle of 15 minutes at 50°C, 2 minutes at 95°C followed by 30-35 cycles of 95°C for 20 seconds, 50-60°C for 30 seconds and 1 minute at 72°C. Conditions were optimized so that none of the PCR products analyzed reached a plateau by the end of the amplification protocol, i.e. they were in the exponential phase of amplification. The reactions were run along with a no-RNA sample negative control and the absence of genomic DNA contamination was verified by performing control reactions in which the reverse transcriptase

was inactivated by heating at 95°C for 5 minutes prior to performing the standard RT-PCR protocol.

All primers were synthesized by Integrated DNA Technologies (Coralville, IA). The primer sequences were determined using Primer 3 software (developed by Steve Rozen, Helen J. Skaletsky, 1996, 1997) available at http://www-genome.wi.mit.edu. All primers were selected with the following parameters: length 20-22 bases; Tm 57 and 65°C, optimal Tm 60-62°C; amplification product length 200-500 bp. When possible, the primers were selected to span different exons, so that the amplification product obtained from cDNA would be of different length from that obtained from any contaminant genomic DNA. Sequence specificity was confirmed using Genbank Blast at the National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov). The following primers were used: Runx2 forward 5′-CCAAATTTGCCTAACCAGAATG-3′; Runx2 reverse 5′-GAGGCTGTGGTTTCAAAGCA-3′; CollagenI forward 5′-GAGCTGGTGTAATGGGTCCT-3’; Collagen 1 reverse 5′-GAGACCCAGGAAGACCTCTG-3; ALP forward 5’-GCCCTCTCCAAGACATATA-3’; ALP reverse 5’-CCATGATCACGTCGATATCC-3’; OPG forward 5’-ATCAGAGCCTCATCACCTT-3’; OPG reverse 5’-CTTAGGTCCAACTACAGAGGAAC-3’. Results were expressed as the ratio of the target gene transcript to the reference gene transcript (GAPDH; forward 5’-CTCACTGGCATGGCCTTCCG-3’; reverse 5’-ACCACCCTGTTGCTGTAGCC-3’).

PCR products were resolved on 1.5 % agarose gels in 1X TBE impregnated with ethidium bromide. A 1 kb DNA ladder (ThermoFisher, Waltham, MA) was run on each gel to confirm the expected length of amplification products. Digital images of the ethidium bromide-stained agarose gels were acquired using a Fujifilm Fla-4000 imager and the band intensities

were determined using ImageJ software (NIH, Bethesda, MD and expressed as relative absorbance units. Each primer product was normalized to the intensity of bands produced with GAPDH primers for each RNA sample.

Results

CB2 gene deletion increases atherosclerotic plaque calcification

The presence of calcium deposits in atherosclerotic lesions is a well-known risk factor for adverse cardiovascular events. We assessed the degree of calcium deposition in lesions of $CB2^{+/+}$ and $CB2^{-/-}$ mice after 20 weeks of atherogenic diet by staining aortic root cross sections using the von Kossa method (Figure 3.1). We observed that calcium deposits were mainly present on the lumenal side of the intima, as expected, coincident with increased presence of VSMCs in this area (data not shown). Digital quantification of the area staining positive for calcium revealed ~2.3 fold more calcification present in lesions of $CB2^{-/-}$ mice compared to $CB2^{+/+}$ mice (13.0±1.1 versus 5.6±1.2%, respectively; p=0.002). Similar results were obtained when we stained aortic root cross sections for calcium deposition using another calcium stain, Alizarin red S (data not shown).

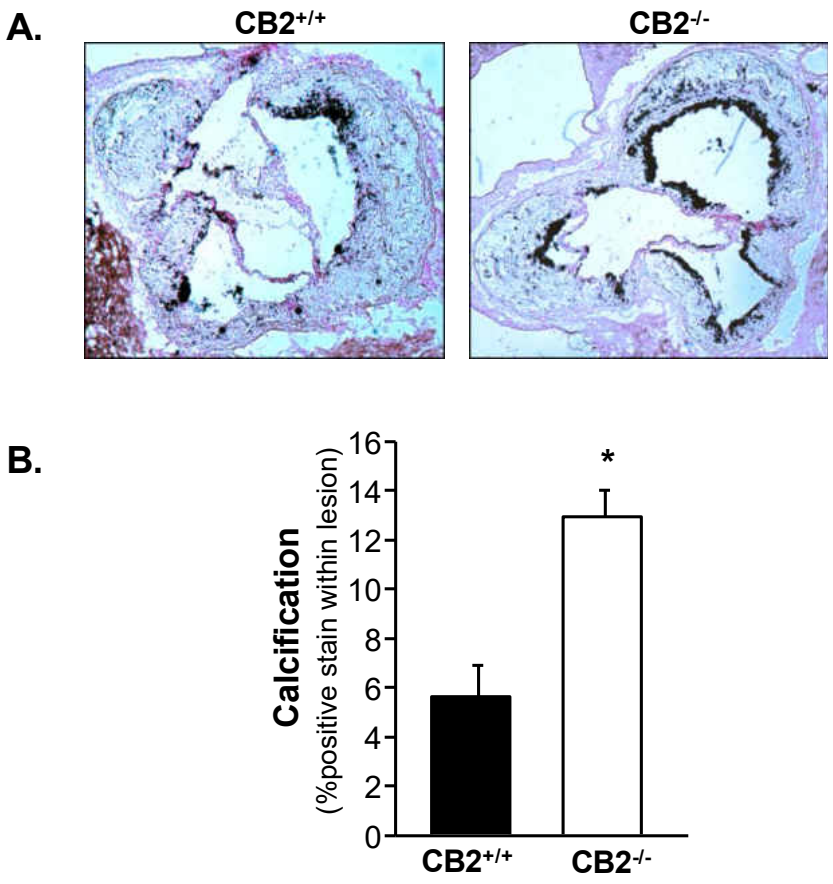

Figure 3.1 CB2 gene deletion increases calcium deposition in atherosclerotic lesions of Ldlr-null mice. A) Representative photomicrographs of von Kossa stained aortic root cross sections from $CB2^{+/+}$ and $CB2^{-/-}$ mice fed an atherogenic diet for 20 weeks. B) Quantification of calcium deposition within aortic root lesions of $CB2^{+/+}$ and $CB2^{-/-}$ mice. Data is represented as the percent of positive stain area per total lesion area. *$p<0.05$. Data is the mean (n=8) ±S.D.

CB2 deletion reduces osteoclast activity in advanced atherosclerotic lesions

Mineralization can occur as a result of an imbalance between osteoblasts, bone-forming cells, and osteoclasts, bone-resorbing cells. Active osteoblasts demonstrate alkaline phosphatase (ALP) activity. We stained aortic root cross sections for ALP activity using BCIP/NBT substrate and found no significant difference in ALP activity in lesions of $CB2^{+/+}$ and $CB2^{-/-}$ mice fed an atherogenic diet for 20 weeks (Supplemental Figure 3.5). We next evaluated osteoclast activity in the aortic root lesions by staining for tartrate-resistant acid phosphatase (TRAP) activity

(Figure 3.2a). TRAP-positive staining was mostly present in the shoulder regions of the aortic root lesions and was significantly increased (~2.2 fold) in $CB2^{+/+}$ mice compared to $CB2^{-/-}$ mice (9.7±0.05 versus 4.9±0.16%, respectively; p=0.02). Since osteoclast formation is centrally regulated by RANKL, and osteoblast production of RANKL induces osteoclastic differentiation and maturation of osteoclast precursors (Walsh and Choi, 2003), the levels of RANKL present in aortic root lesions was evaluated by immunofluorescence using an antibody to soluble RANKL. The majority of RANKL immunofluorescence was observed in the shoulder regions of the lesions (Figure 3.2c), in the same regions showing TRAP activity. Digital quantitation of the fluorescent intensity found significantly more RANKL staining in aortic root lesions of $CB2^{+/+}$ mice compared to $CB2^{-/-}$ mice (0.2±0.0004 versus 0.04±0.0040, respectively; $p<0.001$).

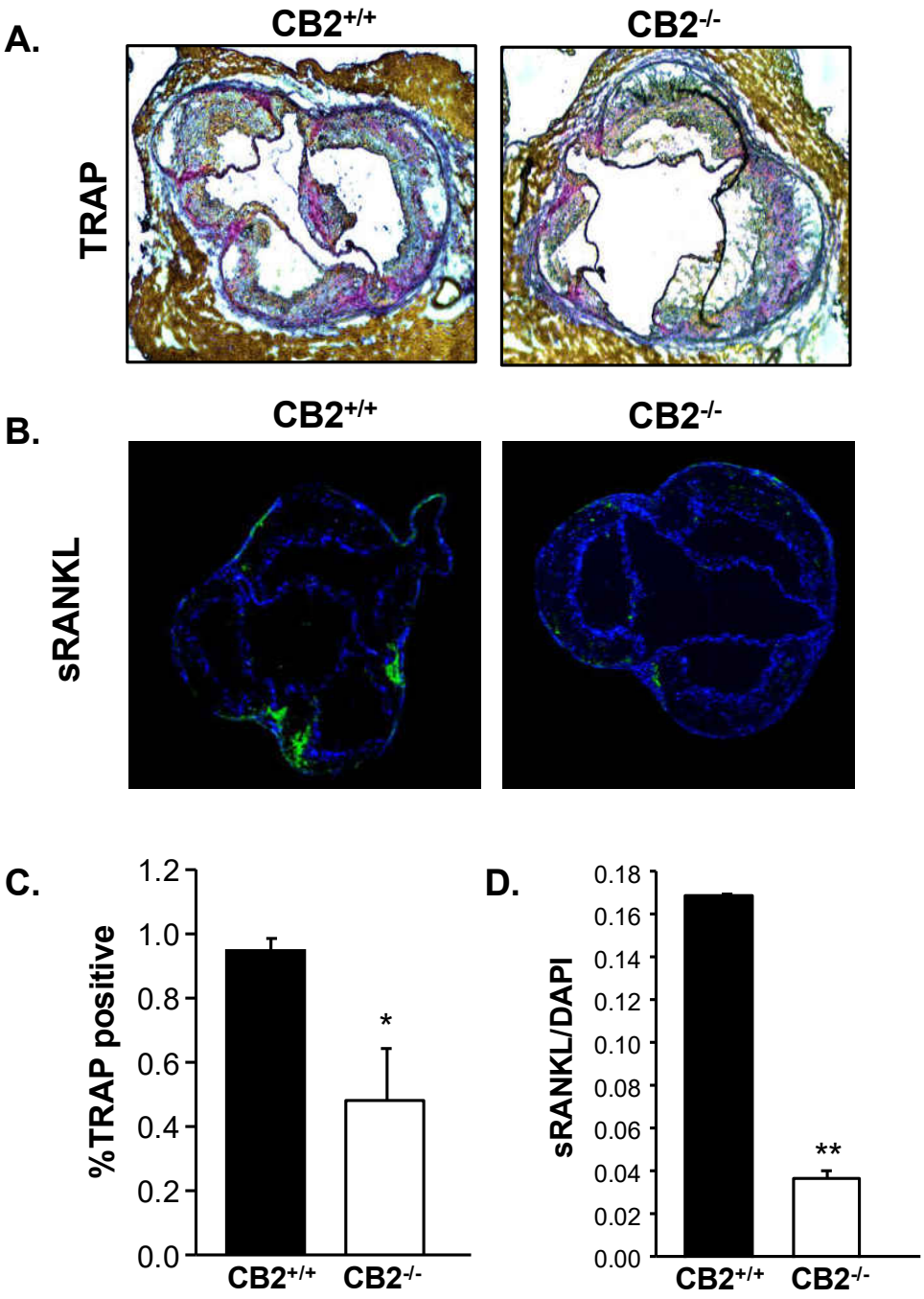

Figure 3.2 CB2 deficiency reduces TRAP activity within atherosclerotic lesions of Ldlr-null mice. A) Representative photomicrographs showing TRAP activity (pink) in aortic root lesions of $CB2^{+/+}$ and $CB2^{-/-}$ mice fed an atherogenic diet for 20 weeks. B) Representative fluorescent micrographs of sRANKL immunoreactivity in aortic root lesions in $CB2^{+/+}$ and $CB2^{-/-}$ mice. C) Quantification of TRAP activity within aortic root lesions. Data is represented as percentage of positive stain per total lesion area. D) Quantification of sRANKL immunoreactivity within the aortic root lesions. Data is represented as the mean ±S.D. of the ratio of FITC signal intensity relative to DAPI signal intensity. n=6. *$p<0.05$; **$p<0.001$.

CB2 gene deletion alters aortic levels of key osteogenic proteins, Runx2, OPN and OCN

We next used immunoblotting to examine aortic levels of three factors (Runx2, OPN, and OCN) known to be important for vascular calcification (Figure 3.3). Runx2 is a master transcriptional regulator of osteoblastogenesis, and its expression functions in both a positive and negative fashion during the maturation of osteoblast precursors into calcifying cells (Bruderer et al., 2014). SMC-specific deletion of Runx2 greatly reduces medial and HFD-induced vascular calcification in mice (Sun et al., 2012; Lin et al., 2015). OPN is biologically diverse but in the setting of biomineralization it specifically functions as a potent inhibitor of ectopic vascular calcification (Lund et al., 2009). OCN, on the other hand, is secreted by mature osteoblasts and is a positive regulator in calcification (Sila-Asna et al., 2007). Aortas of $CB2^{+/+}$ mice contained dramatically greater amounts of Runx2 (~2.5 fold, p=0.03) and OPN (~3.7 fold, p=0.02) compared to aortas of $CB2^{-/-}$ after 20 weeks of HFD. In contrast, the level of OCN in aortas of $CB2^{+/+}$ mice was reduced compared that of aortas from $CB2^{-/-}$ mice, although the difference did not reach statistical significance.

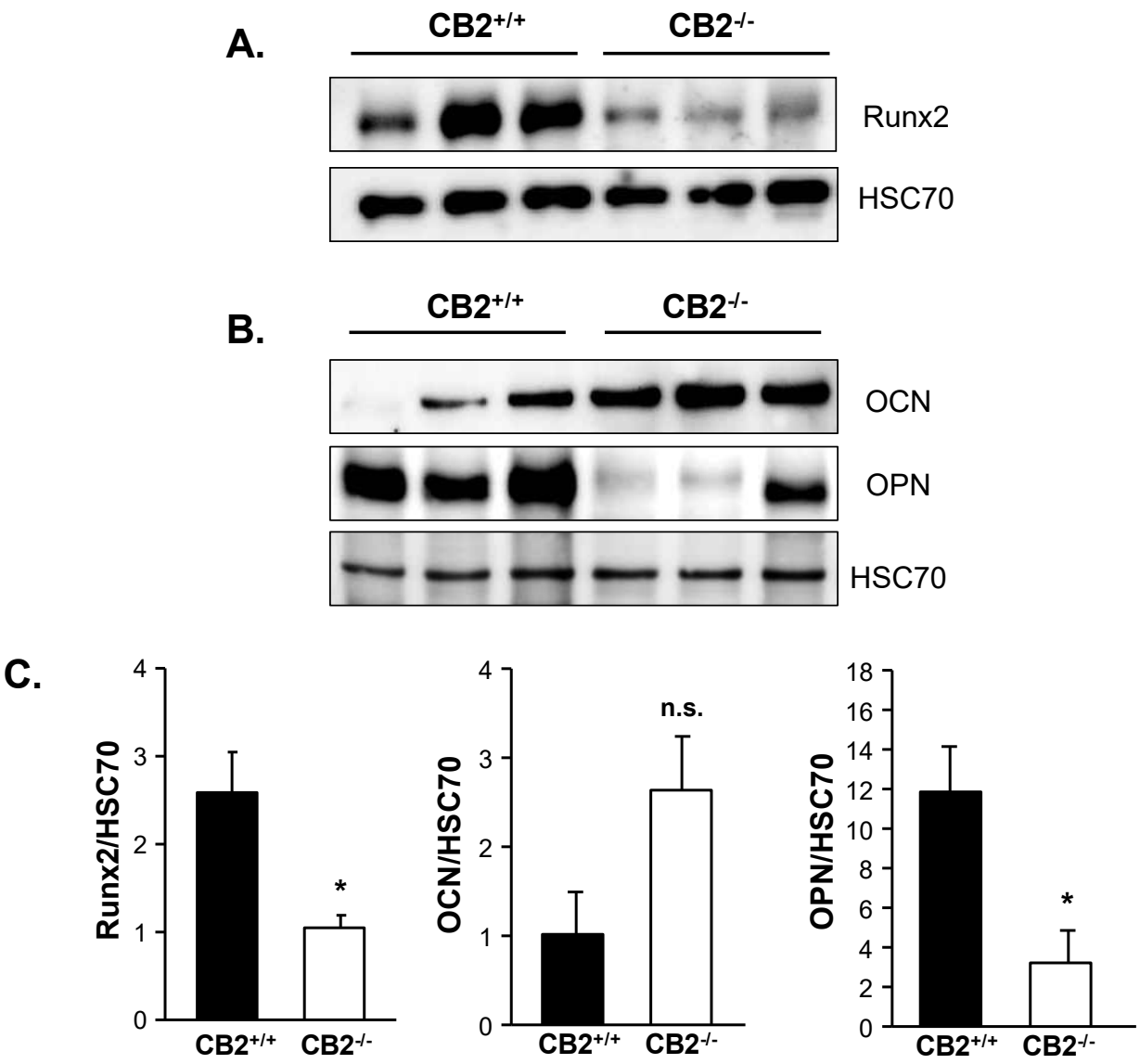

Figure 3.3 CB2 deficiency alters protein expression of osteogenic regulatory proteins in Ldlr-null mice. Immunoblot showing levels of Runx2 (A), OCN and OPN (B) in aorta from three $CB2^{+/+}$ and three $CB2^{-/-}$ mice after 20 weeks of HFD. C) Quantification of Runx2, OCN, and OPN levels relative to the housekeeping protein, HSC70. Data are means±S.D. (n=3) for each genotype. *$p<0.05$. n.s. = not significant

CB2 gene deletion alters osteoblast gene expression

In addition to Runx2, OPN, and OCN, which have been demonstrated to play a role in vascular calcification in animal models, other osteoblast-specific genes implicated in soft tissue mineralization, as well as normal bone formation, may also be modulated by CB2 in vascular

tissue (Sage et al., 2010). Total aortic mRNA for Runx2, Collagen 1 (Col1), ALP, and osteoprotegerin (OPG) was analyzed by semiquantitative RT-PCR to further assess differences in relative mRNA expression of osteogenic genes between $CB2^{+/+}$ mice and $CB2^{-/-}$ mice (Figure 3.4). After normalizing to GAPDH expression levels, we found that after 20 weeks of atherogenic diet, $CB2^{-/-}$ mice had significantly ($p<0.05$) greater relative mRNA expression of Runx2 (~1.6 fold), ALP (~4.8 fold), ColI (~1.7 fold) and OPG (~1.3 fold) compared to $CB2^{+/+}$ mice.

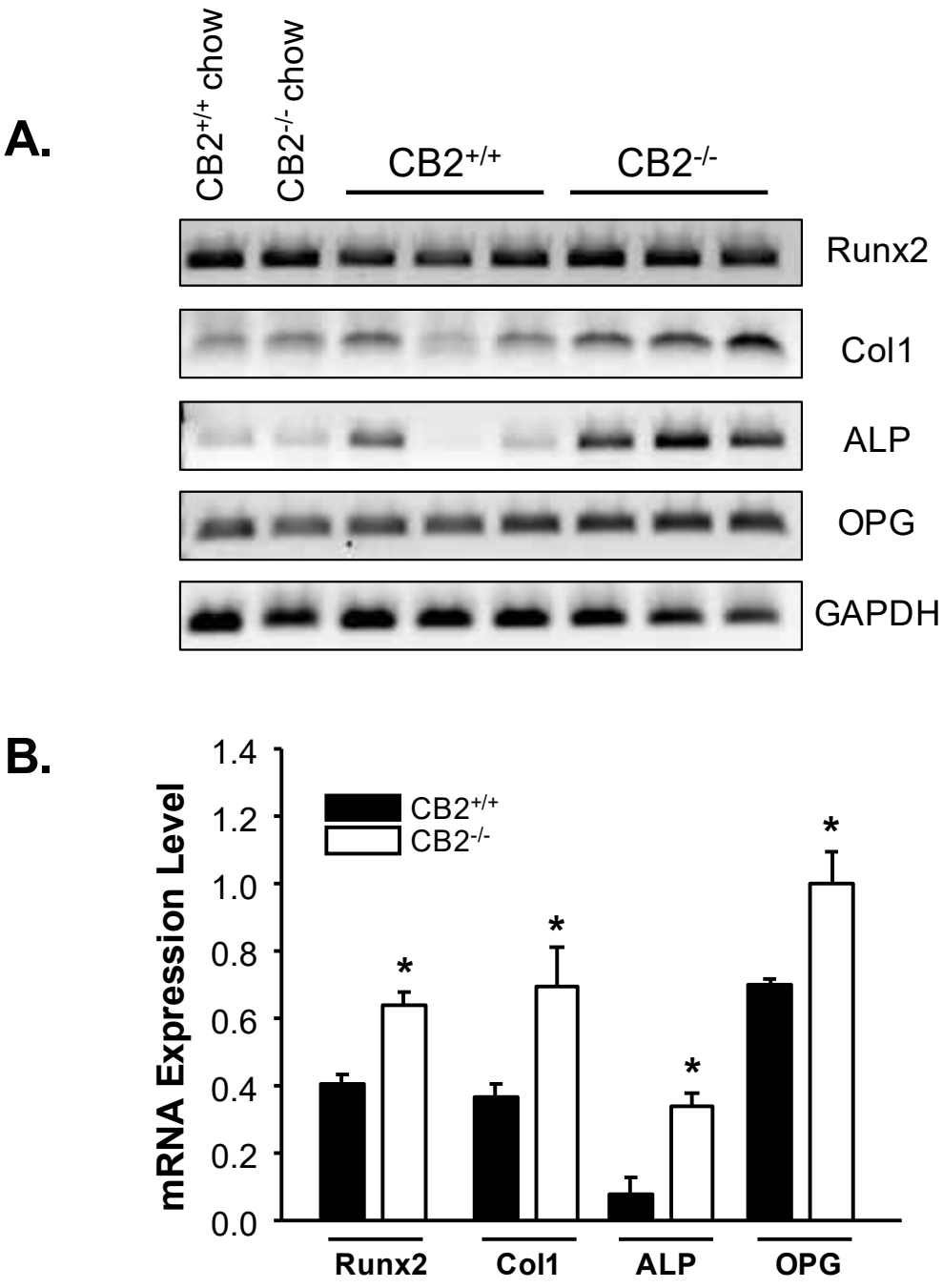

Figure 3.4 CB2 deficiency alters the relative levels of mRNA for osteogenic genes in Ldlr-null mice. A) Semiquantitative RT-PCR of Runx2, ColI, ALP, and OPG expression in aortas from $CB2^{+/+}$ and $CB2^{-/-}$ mice (n=3 for each genotype) fed an atherogenic diet for 20 weeks. RT-PCR of mRNA levels in aortas of chow fed $CB2^{+/+}$ and $CB2^{-/-}$ mice is shown for comparison. B) Quantification of Runx2, ColI, ALP, OPG gene expression in $CB2^{+/+}$ and $CB2^{-/-}$ mice. Bar graphs represent the mean ± S.D. relative levels for each target mRNA:GAPDH mRNA ratio for the data shown in panel A. Results shown are from one analysis performed in triplicate and representative of two independent experiments. *$p<0.05$.

Discussion

Many *in vivo* and *in vitro* studies have provided evidence of a role for CB2 signaling in regulating immune processes involved in atherogenesis (Steffens et al., 2005b; Rajesh et al., 2008; Netherland et al., 2010; Zhao et al., 2010). However, no studies have yet explored the role of CB2 in atherosclerotic lesion calcification. Mineralization of arterial plaques is a pathological condition that results in reduced vessel elasticity and increased susceptibility to plaque rupture, and is associated with increased risk of heart attack (Beadenkopf et al., 1964). In the current study, we explored the role of CB2 in atherosclerotic mineralization by determining the effects of systemic CB2 gene deficiency on calcification of advanced lesions in Ldlr-null mice fed an atherogenic diet. The results of our study show that the absence of CB2 exerts a pro-calcific effect that enhances mineralization of atherosclerotic lesions.

Calcification of vessels occurs in two distinct locations: the media and the intima. Ectopic calcification of atherosclerotic plaques in the intimal layer of the vessel is a cell-mediated process that is modulated by a delicate balance of positive and negative regulators, much like osteogenic pathways controlling normal bone formation. A regulated balance between osteoblast and osteoclast is crucial for maintaining bone homeostasis. We found that CB2 deletion resulted in decreased osteoclast activity, as determined by analysis of TRAP activity, in aortic root lesions of Ldlr-null mice. RANK/RANKL signaling is necessary for osteoclast maturation and function. Consistent with decreased osteoclast activity in lesions of CB2-null mice, they also displayed decreased expression of soluble RANKL. In addition, osteoclastogenesis is restrained in tissues expressing OPG, a soluble decoy receptor for RANKL, and mRNA levels of OPG were found to be significantly elevated in atherosclerotic aortas of CB2-null mice. Together,

these observations suggest that CB2 deficiency enhances lesion calcification, in part, by reducing osteoclast-like activity in lesions.

VSMCs play an important role in plaque calcification, as they adopt an osteoblast-like phenotype through a process of osteoblastic transdifferentiation that is regulated by a network of transcription factors and bone matrix proteins (Shanahan et al., 1993; 1999; Tyson et al., 2003). Runx2 is a master transcriptional regulator of osteoblast gene expression and osteoblastogenesis, exhibiting a stage-dependent positive and negative role in osteoblast maturation. Inactivation of the Runx2 gene results in unmineralized skeletons in mice (Ducy et al., 1997) and SMC-specific deletion of Runx2 in ApoE-null mice reduces formation of osteoblast-like cells and calcium deposition in atherosclerotic lesions (Sun et al., 2012; Lin et al., 2015). Although the commitment of precursor cells to the osteoblast lineage is dependent upon Runx2 expression, expression of Runx2 beyond early commitment is detrimental and diminishes the capability of pre-osteoblasts to mature into functional osteoblasts (Bruderer et al., 2014). Here we observed that enhanced lesion calcification in the setting of CB2 deficiency was associated with decreased Runx2 protein levels and slightly increased Runx2 mRNA levels in atherosclerotic aortas. Several studies have reported a similar discordance between Runx2 mRNA and protein levels, concluding that Runx2 expression is regulated at the post-translational level (Prince et al., 2001; Sudhakar et al., 2001). Osteoblast differentiation is also negatively regulated by ubiquitin-mediated degradation of Runx2 (Shen et al., 2006; Jonason et al., 2009). Together, our results are consistent with CB2 exerting anti-calcifying effects in lesions, in part, by inhibiting Runx2 degradation and restraining the maturation of pre-osteoblasts. Recent studies indicate that phosphorylation, acetylation, SUMOylation and ubiquitination regulate Runx2 functions differently (Wee et al., 2002; Qiao et al., 2006; Shen et al., 2006; Huang et al., 2012) and the

effects of CB2 signaling on post-translational regulation of Runx2 functions are currently unknown and merit further investigation.

An ECM containing an abundance of type I collagen is necessary for osteoblast differentiation and expression of osteoblast-related genes (Franceschi, 1999). These genes, which encode for proteins such as OCN and ALP, are necessary for mineralization (Murshed et al., 2005). We found a significant increase of ALP and ColI mRNA in atherosclerotic aortas of CB2-null mice, accompanied with an increase in OCN protein expression. While ALP mRNA levels are upregulated in CB2-deficient mice, biochemical staining failed to detect enhanced ALP activity in CB2-deficient lesions. Future studies utilizing immunoblotting and immunohistochemical analysis are needed to clarify the effect of CB2 deficiency on ALP expression in lesions.

In addition to regulating the expression of several osteoblast marker genes, Runx2 also regulates the expression of OPN in non-osteoblastic cells (Ducy et al., 1997), such as macrophages. OPN regulates mineralization by acting as a potent inhibitor of vascular calcification (Wada et al., 1999; Lund et al., 2009) and is also crucial for normal bone resorption through its role in osteoclast migration and differentiation (Chellaiah et al., 2003). Here we found a significant decrease in OPN protein expression in lysates of atherosclerotic aortas from CB2-deficient mice. This observation suggests that CB2 may exert anti-calcifying effects by elevating OPN levels in lesions.

In summary, the current study provides the first evidence for a role of CB2 in calcification of atherosclerotic lesions in a murine model of atherosclerosis. While more studies need to be done in order to further delineate the mechanisms by which CB2 signaling alters plaque calcification, our data indicates that CB2 reduces calcification, at least partially, through

down-regulation of osteoblastic gene expression and upregulation of osteoclast activity. These results provide a foundation for potential CB2-directed drug development that can help to reduce or slow calcification of plaques, thereby reducing the morbidity and mortality associated with advanced disease progression.

Conflict of interest

The authors have no conflict of interest to disclose.

Acknowledgements

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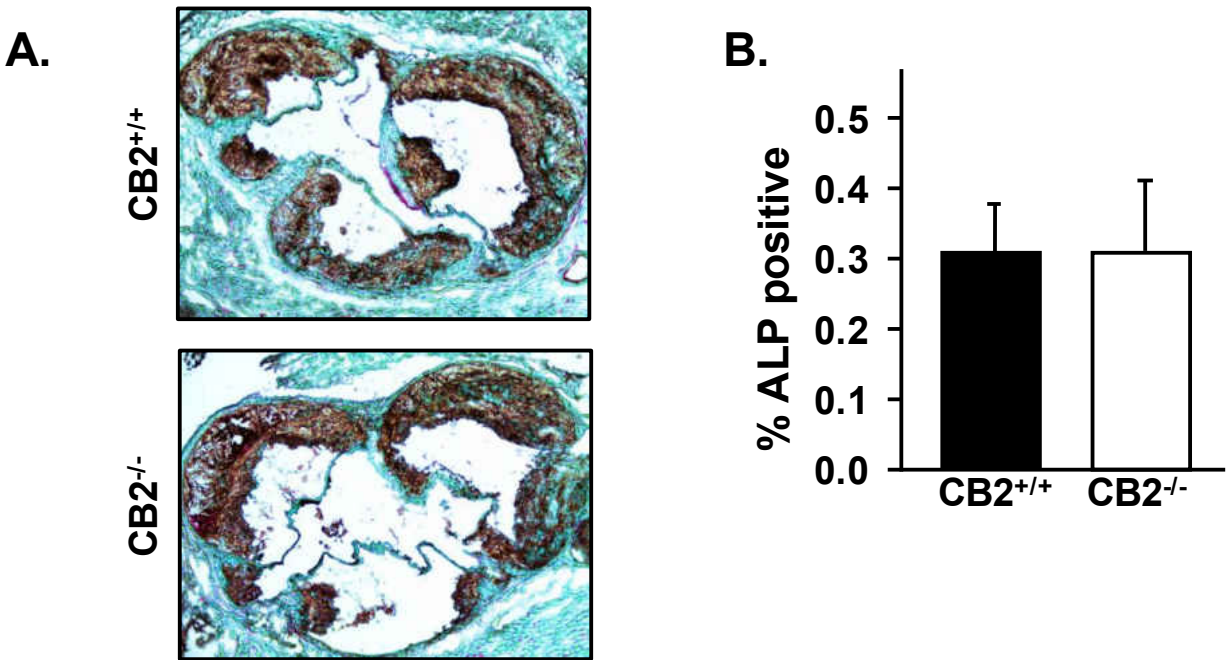
Supplemental Figures



Supplemental Figure 3.5 CB2 deficiency does not affect ALP activity within atherosclerotic lesions of Ldlr-null mice. A) Representative photomicrographs showing ALP activity (pink) in aortic root lesions of $CB2^{+/+}$ and $CB2^{-/-}$ mice fed an atherogenic diet for 20 weeks. B) Quantification of ALP activity within aortic root lesions. Data is represented as percentage of positive stain per total lesion area and mean±S.D. n=6 for each group.

CHAPTER 4

EFFECTS OF EXOGENOUS CB2 LIGANDS ON CALCIFICATION OF ADVANCED ATHEROSCLEROTIC PLAQUES IN LDLR-NULL MICE

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Keywords: Atherosclerosis, Cannabinoid receptor 2, Calcification, Smooth Muscle Cell, JWH015, SR144528

Abstract

Objective: Atherosclerotic calcification is a risk factor for future adverse cardiovascular events. Cannabinoid receptor type 2 (CB2) activation reduces plaque burden in murine models of atherosclerosis. CB2 is well known to modulate bone remodeling processes and we recently observed that CB2 deficiency in Ldlr-null mice enhances lesion calcification. However, the ability to affect atherosclerotic calcification by pharmacologically targeting CB2 is unknown. Therefore, we investigated the effects of JWH-015 (CB2-selective agonist) and SR144528 (CB2-seelctive inverse agonist/antagonist) on calcification of atherosclerotic lesions in hyperlipidemic low-density lipoprotein receptor (Ldlr) null mice.

Methods and results: At 8 weeks of age, Ldlr-null mice were placed on a high fat diet (HFD) for 24 weeks with administration of either JWH-015 (1mg/kg) or SR144528 (1mg/kg) three times a week for the final four weeks. No change was observed in mean body mass or total plasma cholesterol in response to either treatment; however, total plasma triglycerides were significantly decreased in $CB2^{+/+}$ mice in response to SR144528 administration (116.7±13.3 versus 292.1±37.1 mg/dL; p=0.0009). Aortic root lesion area was not discernably affected by treatment with either JWH-015 or SR144528. Von kossa staining of aortic root lesions revealed Ldlr-null mice treated with SR144528 had significantly less calcium deposition compared to controls (12.7±0.9 versus 15.8±0.7%; p=0.03). Treatment with JWH-015 also reduced calcium deposition in Ldlr-null mice, but the decrease was not significant. Immunoblot analysis revealed increased Runx2 and osteopontin (OPN) expression in aortic arch lysates of Ldlr-null mice treated with JWH-015. Analysis of aortas cultured *ex vivo* in an osteogenic media revealed that supplementation of the media with a CB2 agonist decreased aortic calcification while supplementation with a CB2 antagonist increased aortic calcium deposition.

Conclusion: Pharmacological targeting of CB2 does not alter lesion size in more advanced atherosclerotic lesions of Ldlr-null mice, but does affect circulating triglycerides levels and osteogenic processes in vascular and lesional calcification. This data provides the proof of principle that CB2-specific therapies might be developed to exert beneficial effects on vascular and atherosclerotic calcification.

Introduction

Myocardial infarction and stroke, acute clinical consequences of atherosclerosis, remains to be the leading causes of morbidity and mortality in the developed world (Benjamin et al., 2017). Decades of research has provided insight into the mechanisms involved in atherogenesis, a disease that was once thought to be simply caused by a dysregulation of lipid metabolism. Atherosclerosis is now recognized as a progressive inflammatory condition of medium- and large-sized arteries (Libby et al., 2002) involving a complex network of immune cells, lipids, and other cellular debris over the course of several decades. Currently, the most wildly used therapies for patients with atherosclerosis are drugs that target hypercholesterolemia (statins) and hypertension (beta-blockers), two cardiovascular disease risk factors. While these therapies have been successful in reducing the severity of risk factors in affected patients, they have only slightly decreased mortality rates associated with heart disease. It is therefore extremely important for new treatment regimens to be uncovered, and much interest has been gained in targeting the immune system as a therapeutic approach in an attempt to further decrease mortality rates associated with atherosclerosis.

Endocannabinoids and other cannabinoids exert the majority of their effects through binding two G-protein coupled receptors, cannabinoid receptor type 1 (CB1) and cannabinoid receptor type 2 (CB2). Exogenous cannabinoids such as delta9-tetrahydrocannabinol (THC) are well-known for their psychotropic effects, and increasing knowledge is being obtained regarding their anti-inflammatory and immunosuppressive effects. Expression of CB1 is primarily seen in the central nervous system where its activation results in psychotropic effects, but is also found in the heart (Bátkai et al., 2004), liver (Osei-Hyiaman et al., 2005), adipocytes (Cota et al., 2003), and vasculature (Liu et al., 2000). Immunosuppressive effects of cannabinoids are

mediated through CB2, which is prominently expressed by cells of the peripheral immune system, but also found on adipocytes (Roche et al., 2006), endothelial cells (Blázquez et al., 2003; Rajesh et al., 2007), and in the pancreas (Juan-Picó et al., 2006).

Research over the past 15 years has provided evidence for a beneficial role of cannabinoids in the treatment of inflammatory disorders (Di Marzo et al., 2004; Klein, 2005; Steffens et al., 2005a; Pacher et al., 2006; Mach and Steffens, 2008; Netherland et al., 2010; Zhao et al., 2010a; 2010b; Hoyer et al., 2011). Use of cannabinoid agonists have shown beneficial CB2-dependent effects in animal models of gastrointestinal diseases (Wright et al., 2008; Storr et al., 2009), cardiac and hepatic ischemia-reperfusion (Bátkai et al., 2007; Defer et al., 2009), and various neurological disorders (Malan et al., 2001; Klegeris et al., 2003). In the setting of atherosclerosis, the role of CB2 is not completely understood, although there is strong evidence for an atheroprotective function. Reduction of atherosclerosis has been shown in ApoE-null mice after administration of plant-derived (THC) (Steffens et al., 2005a) and synthetic (Win55,212-2) (Zhao et al., 2010b) cannabinoids. These effects were attributed to decreased adhesion molecule activation and monocyte recruitment, and were abolished in the presence of CB2, but not CB1, antagonists. A study from our laboratory using WIN55,212-2 in Ldlr-null mice did not find CB2-dependent effects on lesional macrophage infiltration, but did find reductions in lesional smooth muscle cell (SMC) content and apoptosis (Netherland-Van Dyke et al., 2015a). In another study, we observed increased lesional macrophage infiltration in $Ldlr^{-/-}$ mice lacking CB2 (Netherland et al., 2010). Other independent investigators have also reported systemic and hematopoietic-specific CB2 gene deletion increases macrophage content in atherosclerotic lesions from Ldlr-null (Delsing et al., 2011; Willecke et al., 2011) and ApoE-null mice (Hoyer et al., 2011). Yet, other investigators have reported seemingly conflicting results of

the effects of the synthetic CB2 agonist JWH-133, with lesion progression being either unaffected in Ldlr-null mice (Willecke et al., 2011) or reduced in ApoE-null mice (Hoyer et al., 2011). The variability in these results, possibly due to strain-specific differences in the atherosclerosis mouse models ($Ldlr^{-/-}$ vs $ApoE^{-/-}$) and differences in the chemical composition of synthetic drugs, has made it difficult to uncover a consensus view of the therapeutic potential for CB2 in the progression of atherosclerosis.

Calcification of advanced atherosclerotic plaques is a risk factor for adverse cardiovascular events, independent of traditional risk factors such as smoking, diabetes, obesity, and physical inactivity. Recently, we found that CB2 gene deficiency increases plaque calcification in Ldlr-null mice fed an atherogenic diet for 20 weeks (manuscript in preparation). In this study, we investigated the effects of pharmacologically targeting CB2 with an agonist and an antagonist on atherosclerotic calcification in Ldlr-null mice *in vivo* and in aortas cultured in an *ex vivo* model of vascular calcification.

Materials and Methods

Animal atherosclerosis and pharmacological administration protocol

Ldlr-null mice ($CB2^{+/+}$) and Ldlr-null mice lacking CB2 ($CB2^{-/-}$) were as previously described (Netherland et al., 2010). At 8 weeks of age, groups (n=8) of male mice were placed on an high fat diet (HFD) consisting of 21% fat and 0.15% cholesterol (Harlan Teklad, Madison, WI) for a total of 24 weeks. During the final 4 weeks of HFD feeding, groups of mice received intraperitoneal (i.p.) injections of either 1mg/kg JWH-015 (Tocris, Bristol, United Kingdom) or 1mg/kg SR144528 (Tocris, Bristol, United Kingdom) diluted in 50% DMSO/50% saline three times per week (Monday, Wednesday, Friday). Control mice received i.p. injections of an equivalent volume of vehicle (100 µL).

All mice were housed in a pathogen-free, temperature- and humidity-controlled room in the Animal Research Facility at East Tennessee State University. Mice were maintained on a standard chow diet (Ralston Purina, St. Louis, MO) with water provided *ad libitum*. All animal procedures were approved by and conducted in accordance with the guidelines administered by the Institutional Animal Care and Usage Committee of East Tennessee State University and in conformity with the Public Health Service Policy on Humane Care and Use of Laboratory Animals published by the National Institutes of Health.

Physiological Parameters

Following an overnight fast, blood samples were collected under anesthesia by cardiac puncture. Blood was separated by centrifugation and plasma was assayed for total cholesterol and triglyceride levels using commercially available kits (Pointe Scientific, Inc., Canton, MI) according to manufacturer's protocol.

Analysis of Atherosclerosis

Anesthetized mice were euthanized as previously described (Netherland et al., 2010) and hearts containing proximal aortic arch and brachiocephalic arteries were dissected and snap frozen in OCT embedding medium. Serial 8µm cross-sections of the aortic root were collected as previously described (Liu et al., 2005; Netherland et al., 2010). The extent of atherosclerosis was determined by computer-assisted morphometric analysis using ImageJ (NIH, Bethesda, MD) following oil-red O staining for neutral lipid content as previously described.

Histological Staining

Calcium deposition was determined using the von Kossa method. Positive staining areas were quantified using NIH ImageJ software and represented as the percentage of total atherosclerotic plaque area relative to control. For each sample, a minimum of four slides containing four cross sections were evaluated and averaged.

Immunoblotting

Atherosclerotic aortas (aortic arch and thoracic aorta) were homogenized in 1X cell lysis buffer (Cell Signaling Technology, Danvers, MA) supplemented with 1X protease inhibitor mix (Sigma-Aldrich, St. Louis, MO). Protein content was determined using a microBCA kit (Thermo Scientific, Waltham, MA). Lysates containing 20µg of protein were separated on 4-12% Bis/Tris gels (Invitrogen, Carlsbad, CA) and transferred to a polyvinylidene fluoride membrane. Membranes were blocked for 1h at room temperature in tris-buffered saline 0.1% tween 20 (TBS-T) containing 5% non-fat dry milk and probed with antibodies to Runx2 (Santa Cruz, Dallas, TX; sc-10758) (1:2000 dilution) and OPN (R&D Systems, Minneapolis, MN; AF808) (1:3000) overnight at 4ºC. Membranes were washed with TBS-T and incubated with either goat

anti-rabbit (1:2000 dilution, for Runx2) or rabbit anti-goat (1:2000, for OPN) horseradish peroxidase-conjugated secondary antibodies (Santa Cruz, Dallas, TX) and developed using Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare Bio-Sciences, Pittsburgh, PA) and a Fujifilm Fla-4000 imager. Membranes were stripped and probed with a β-actin antibody (Neomarkers, Portsmouth, NH; RB-9421-PO) (1:3000) to serve as a loading control.

Ex vivo organ culture

Thoracic aortas were dissected from $CB2^{+/+}$ mice maintained on a standard chow diet and cultured in DMEM supplemented with 15% fetal bovine serum, 100 U/mL penicillin, and 100 mg/mL streptomycin at 37ºC in a humidified atmosphere containing 5% CO_2. Induction of calcification was achieved by addition of 2.6 mmol/L Pi (Na_2HPO_4/NaH_2PO_4, pH 7.4) to the culture medium (osteogenic media). Aortas were treated with either CB2 agonist HU-308 (Cayman Chemical, Ann Arbor, MI) or CB2 antagonist SR144528 (Tocris, Bristol, United Kingdom) in osteogenic media at a final concentration of 2 μM. Segments of the same aorta were placed in separate culture wells, with one segment incubated with osteogenic media alone (control) and the other segments cultured in osteogenic media supplemented with either HU-308 or SR144528. At the end of 4 days, the segments were washed with phosphate buffered saline and snap frozen in OCT medium and cryosectioned at 8 μm. Calcium deposition was visualized by staining of thawed cryosections with 2% Alizarin red (pH 4.2) for 5 min followed by rinsing with dH_2O three times. Digital images were acquired and the extent of calcium deposition was determined by quantifying the Alizarin red stained area by computer assisted morphometric analysis with ImageJ software (NIH, Bethesda, MD).

Statistical Analysis

Data are presented as means±S.D. Student's t-test or ANOVA methods were employed using SigmaPlot (StyStat Software, Chicago, IL) to assess differences between groups, with probability values of less than 0.05 considered statistically significant.

Properties of all synthetic cannabinoids used in this study are listed in the supplemental (SF 4.6).

Results

SR144528 decrease plasma triglyceride levels in Ldlr-null mice

Ldlr-null mice with and without CB2 ($CB2^{+/+}$ and $CB2^{-/-}$, respectively) were fed an atherogenic diet for 24 weeks to induce the formation advanced atherosclerosis. During the last 4 weeks, groups of mice we given i.p. injections of either JWH-015 or SR144528 at a final concentration of 1mg/kg three times per week. Control mice were given the same volume of vehicle. After treatment with exogenous CB2 ligands, no significant differences in mean body mass (Figure 4.1a) or plasma cholesterol (Figure 4.1b) were observed for either genotype. Although plasma triglyceride levels tended to be lower in $CB2^{-/-}$ mice compared to $CB2^{+/+}$ mice, the difference did not reach statistical significance (Figure 4.1c). Treatment of $CB2^{+/+}$ mice with SR144528 resulted in a ~60% decrease in plasma triglyceride levels compared to controls (116.7±13.3 versus 292.1±37.1 mg/dL, respectively; p=0.0009), an effect which was absent in $CB2^{-/-}$ mice (264.4±31.8 versus 191.7±33.1 mg/dL, respectively; p=0.1). Treatment with either JWH-015 or SR144528 did not affect the size of established aortic root lesions in either genotype (Figure 4.1d).

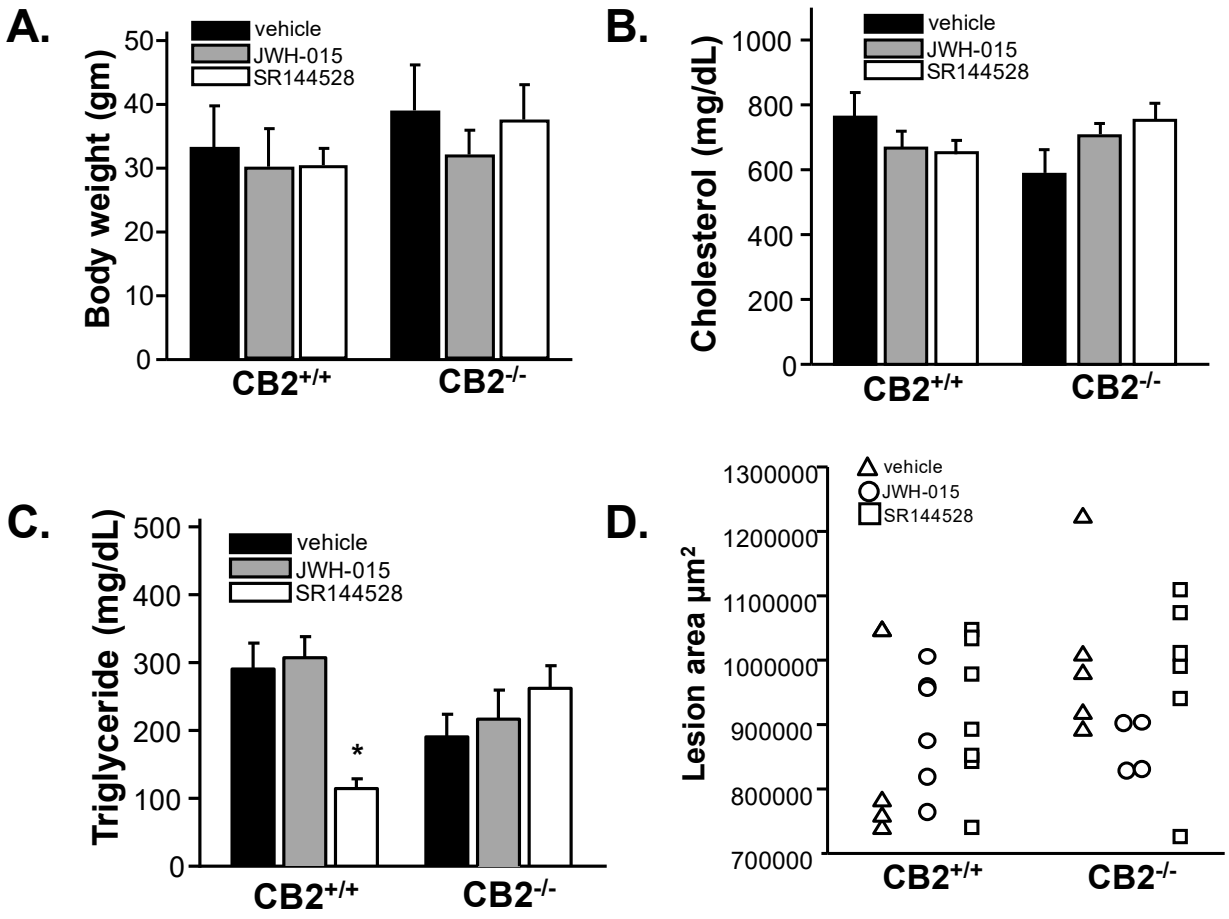

Figure 4.1 SR144528 reduces triglyceride levels in Ldlr-null mice by a CB2-dependent mechanism. Body weights (A) serum cholesterol (B) triglyceride (C) and aortic root lesion size (D) of $CB2^{+/+}$ and $CB2^{-/-}$ mice after 24 weeks of HFD in which the mice were given i.p. injections of JWH-015 or SR144528 (1mg/kg, 3x per week) for the final 4 weeks. The results are the mean±S.D. (n=6-8). *$p<0.001$

SR144528 treatment reduces calcium deposition in atherosclerotic plaques

We next compared the extent of atherosclerotic calcification in Ldlr-null ($CB2^{+/+}$) mice treated with JWH-015 or SR144528 by von Kossa staining aortic root cross sections (Figure 4.2). Quantification of the lesion area staining positive for calcium revealed that treatment with the CB2 agonist, JWH-015, reduced the calcium deposition compared to controls, but the decrease did not reach statistical significance. Surprisingly, treatment with the CB2 antagonist,

SR1144528, also resulted in less calcium deposition (12.7±0.9 versus 15.8±0.7% for controls; p=0.03). Treatment with either compound did not significantly affect the extent of calcium deposition in aortic root lesions of CB2$^{-/-}$ Ldlr-null mice (see supplemental Table 4.1).

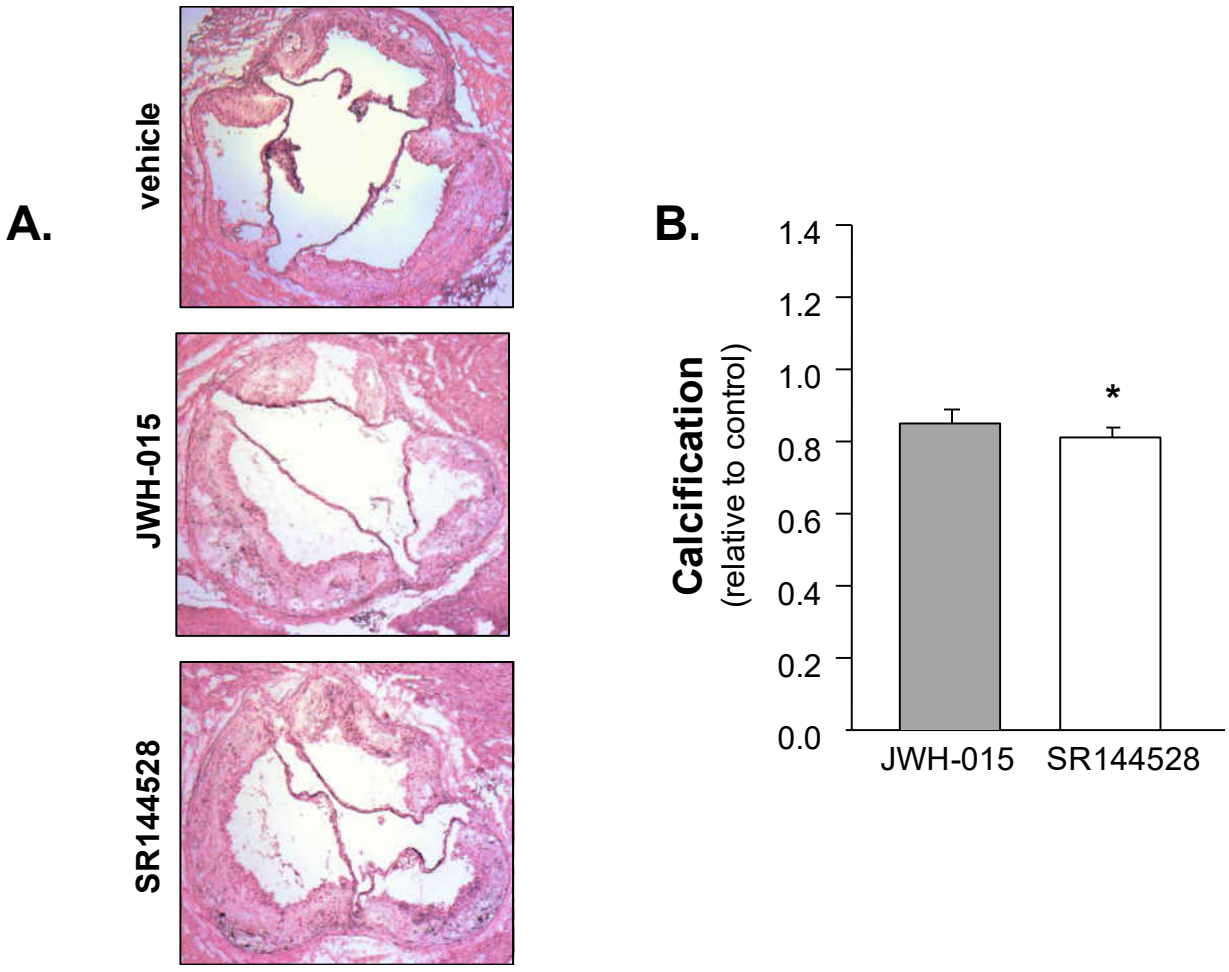

Figure 4.2 CB2 antagonism reduces atherosclerotic calcification in Ldlr-null mice. A) Representative photomicrographs of von Kossa stained aortic root cross sections from Ldlr-null mice following treatment with JWH-015 or SR144528. B) Quantification of total aortic root lesion area occupied by calcium as indicated by positive von Kossa-staining. Data represented as relative staining compared to control (vehicle). The results are the mean±S.D. (n=8) *p<0.05.

We previously observed CB2-dependent effects on lesional osteoclast activity (Figure 3.2). Biochemical staining of tartrate-resistant phosphatase (TRAP) activity in aortic root lesions of

JWH-015 or SR144528 treated Ldlr-null mice revealed no differences in osteoclast activity from that in vehicle treated controls (Figure 4.3).

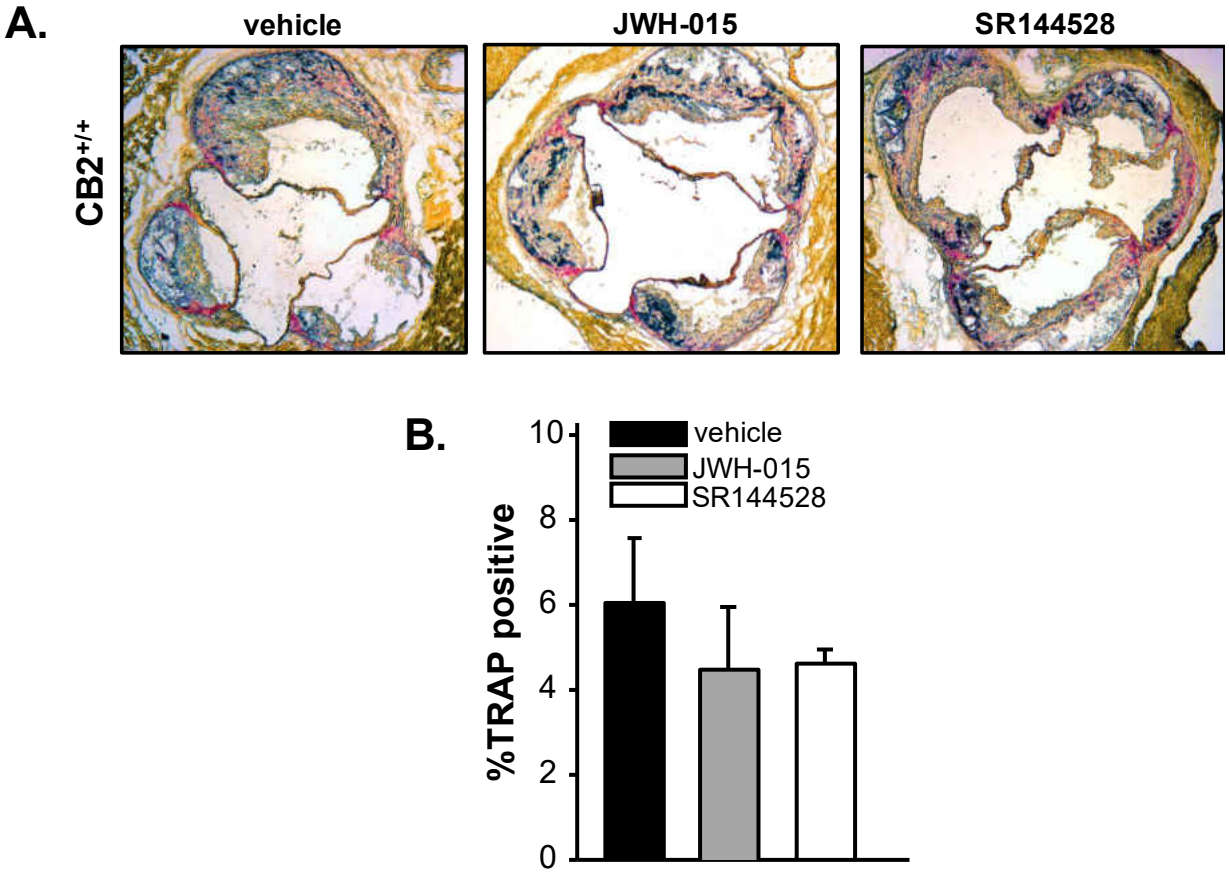

Figure 4.3 Administration of a CB2 agonist and antagonist does not affect TRAP activity in Ldlr-null mice. A) Representative photomicrographs of aortic root cross sections stained for TRAP activity from $CB2^{+/+}$ mice after 24 weeks of HFD with i.p. injections of either JWH-015 or SR144528 over the final 4 weeks. B) Quantification of percent of total lesion area staining positive for TRAP activity. Data is presented as the mean±S.D. (n=8)

Administration of exogenous ligands alters aortic Runx2 and OPN protein levels in Ldlr-null mice

Runx2 is a master transcription factor controlling osteoblastogenesis in a stage-dependent manner (Bruderer et al., 2014). At early stages of development, Runx2 functions in the commitment of precursor cells to the osteoblast lineage; however, Runx2 inhibits the maturation of pre-osteoblasts in later stages. We previously found that Runx2 was decreased in calcified aortic lesions of $CB2^{-/-}$ mice compared to $CB2^{+/+}$ mice (Figure 3.3). Further, in early atherosclerotic lesions of Ldlr-null mice fed HFD for 12 weeks, treatment with WIN55,212-2, a mixed CB1/CB2 agonist, increased aortic expression of Runx2 in a dose-dependent manner (Supplemental Fig. 4.7). Therefore, we determined if the treatment of Ldlr-null mice with JWH-015 and SR144528 also affected the level of Runx2 detected in calcified aortic tissues (Figure 4.4a,b). Immunoblot analysis using showed no discernable differences between the amount of Runx2 in aortas from SR144528-treated mice and vehicle control mice. However, Runx2 levels were significantly elevated in calcified aortas from JWH-015-treated mice compared to the vehicle control.

Osteopontin (OPN) is an important regulator of osteogenesis, as it serves as a potent negative regulator of ectopic mineralization in vascular tissue (Scatena et al., 2007). In early atherosclerotic lesions of Ldlr-null mice fed HFD for 12 weeks, administration of WIN55,212-2 dose-dependently increases in aortic OPN expression (Supplemental Fig. 4.7). Immunoblot analysis showed greater OPN levels in aortas of JWH-015-treated Ldlr-null mice and decreased levels in aortas of SR144528-treated mice compared to control mice (Supplemental Figure 4.8).

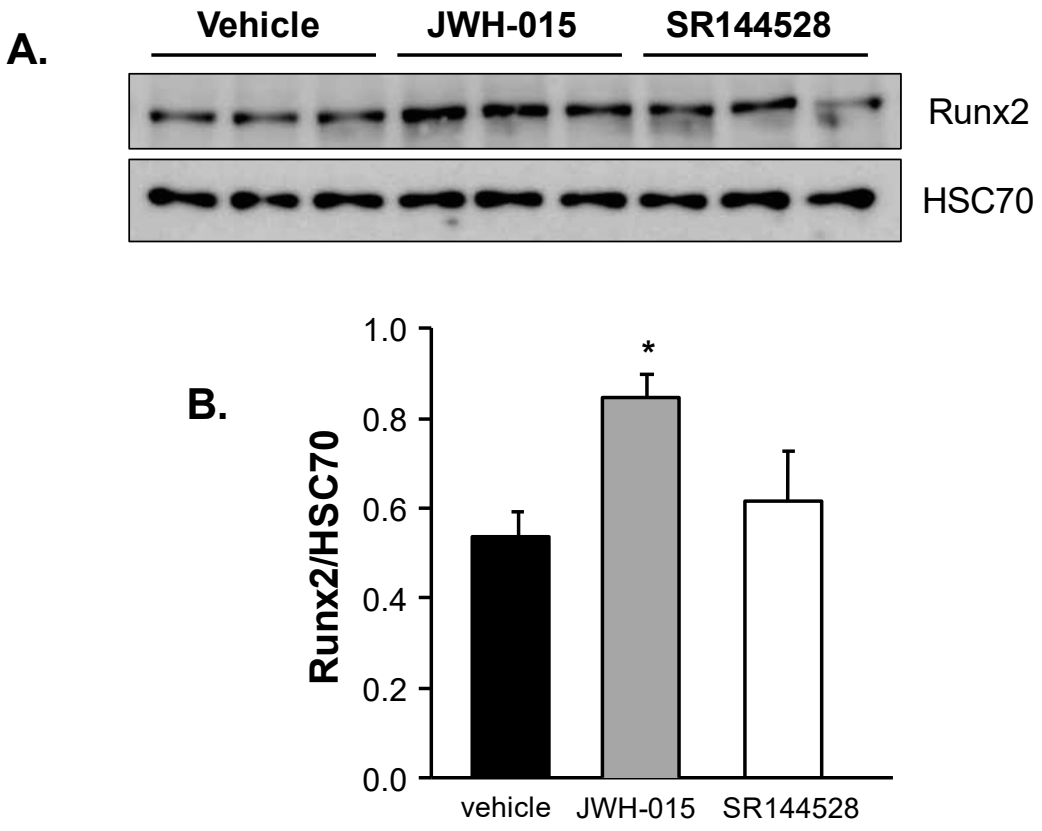

Figure 4.4 Upregulation of Runx2 levels in calcified aortas of Ldlr-null mice treated with JWH-015. Immunoblot analysis of aortic arch lysates from Ldlr-null mice after 24 weeks of HFD and 4 weeks of i.p. administration of JWH-015 or SR144528. A) Representative immunoblot of aortic tissue probed with Runx2 antibody. B) Quantification of Runx2 protein levels detected by immunoblotting of aortic arch lysate. Data is presented as the mean±SD ratio of the intensity of the target protein relative to a loading control protein (HSC70). (n=3) *$p<0.05$.

Administration of exogenous ligands alters vascular calcification in *ex vivo* cultured aortas

To more rapidly screen exogenous CB2 ligands for effects on vascular calcification we utilized a recently developed culture method of inducing calcification of aortas *ex vivo* (Akiyoshi et al., 2016). This method uses an osteogenic media containing 2.6 mM Pi to induce aortic calcification in 3-5 days of culture (Akiyoshi et al., 2016). Each aorta dissected from an Ldlr-

null mouse fed a normal chow diet was cut in half, with one half cultured in osteogenic media (OG) and the other half cultured in OG media supplemented with a CB2 agonist (HU-308) or OG media supplemented with a CB2 antagonist (SR144528) (Figure 4.5a). After 4 days in culture, the aortas were removed and adjacent 8 μm cryosections were prepared for analysis of calcium deposition by staining with alizarin red (Figure 4.5b). Calcium deposition in aorta segments cultured in the presence of HU-308 was reduced compared to that seen in the corresponding segments cultured in OG media alone, while aorta segments cultured in the presence of SR144528 had increased calcium deposition relative to their corresponding control aorta segments (Figure 4.5c). Aorta segments cultured in standard media (0.9 mM Pi) did not show positive staining with alizarin red (data not shown).

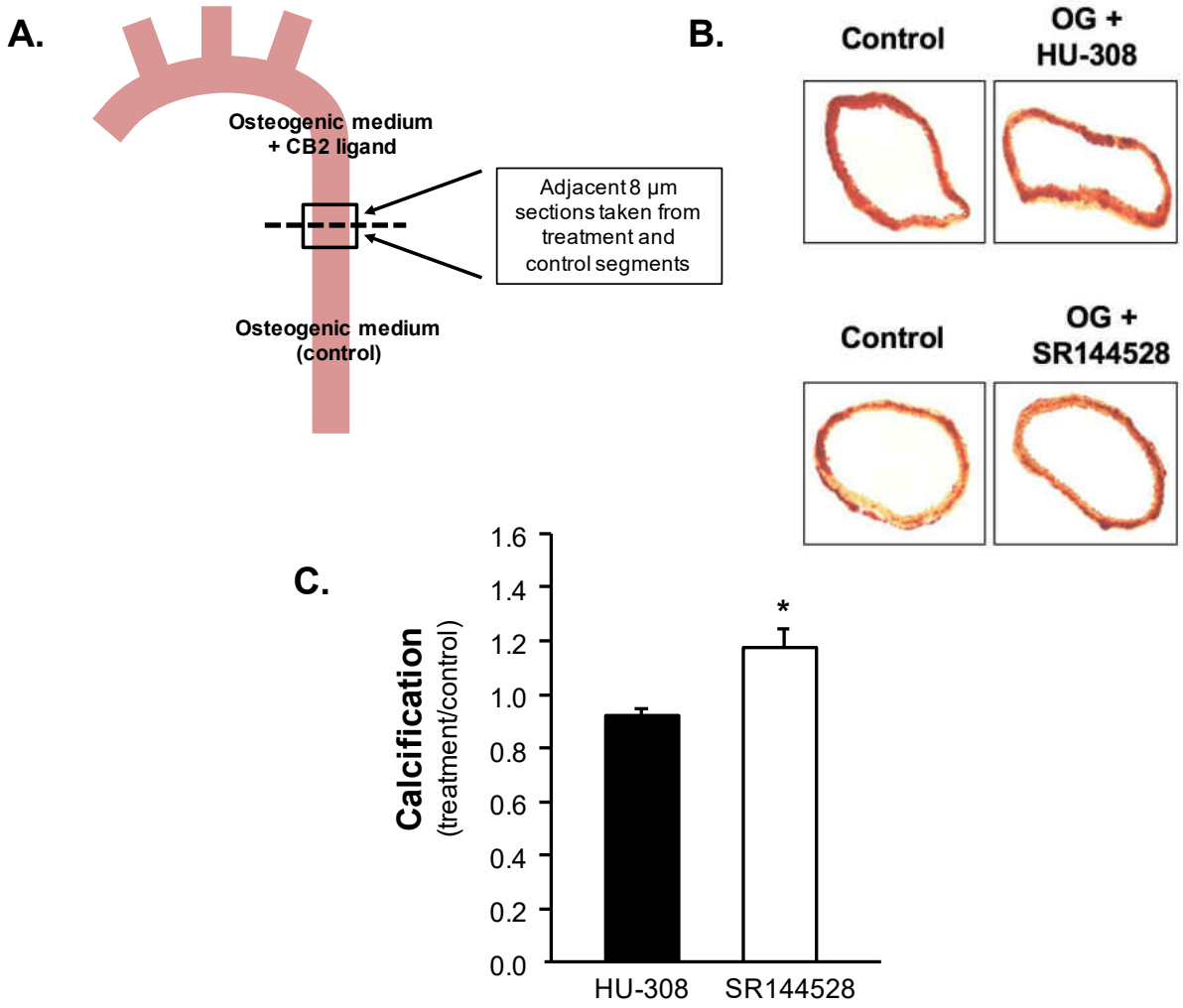

Figure 4.5 CB2 agonist and antagonist differentially affect calcification of aortas *ex vivo*. A) Schematic of culture method. B) Representative photomicrographs of alizarin red stained cross sections of aorta segments after culture in osteogenic medium (control), and osteogenic medium containing either 2µM HU-308 or 2µM SR144528 for 4 days. B) Quantification of the effect of HU308 and SR144528 on calcium deposition in aorta segments from 6 different Ldlr-null mice. Data is presented and the mean±S.D. of the ratio of the % area staining positive for the treatment and the % area staining positive in the corresponding control aorta segment from the same mouse. A total of four sections were stained for each segment and averaged. *$p<0.05$

Discussion

Previous studies have provided evidence supporting a CB2-dependent reduction in early atherosclerosis progression in atherogenic ApoE-null mice treated with synthetic cannabinoids WIN55,212-2 (Zhao et al., 2010a; 2010b) and JWH-133 (Hoyer et al., 2011). Further, CB2-dependent and –independent roles were observed in Ldlr-null mice treated with WIN55,212-2 after initiation of early atherosclerotic lesions (Netherland-Van Dyke et al., 2015a). These studies assessed CB2-dependent characteristics such as lesion size and monocyte entry/macrophage content, but with some contradicting results. For instance, administration of natural or synthetic CB1/CB2 agonists in ApoE-null mice demonstrates atherosclerotic lesion size regression in a CB2-dependent manner (Steffens et al., 2005b; Zhao et al., 2010a; 2010b; Hoyer et al., 2011); however, this drug-induced effect on lesion burden has not been observed in Ldlr-null mice (Delsing et al., 2011; Willecke et al., 2011; Netherland-Van Dyke et al., 2015b). These apparently contradictory results could be explained, at least in part, by the strain-specific differences between ApoE- and Ldlr-null mice. In support of this, it is well-known that these two murine models of atherosclerosis differ in a number of parameters which can affect the pathophysiological characteristics of atherosclerosis (Daugherty and Rateri).

While increasing evidence has accumulated supporting a role for CB2 in modulation of early atherogenesis, to date, the effects of synthetic cannabinoids on processes in advanced atherosclerosis has not yet been explored. Most atherosclerotic plaques remain clinically insignificant, remaining stable enough to never initiate adverse cardiovascular events. However, among a significant population of people, unstable plaques become prone to rupture and subsequent thrombolytic activity creates the potential for myocardial infarction or stroke to occur. Beyond traditional risk factors, calcification of atherosclerotic plaques is a major risk

factor for lesion instability and is highly correlated with mortality associated with atherosclerosis. In the present study, we provide the first evidence of the effects of synthetic cannabinoid administration on calcification of advanced atherosclerotic plaques in a murine model of atherosclerosis.

Consistent with our previous studies of CB2 deficiency in Ldlr-null mice, we found no effect on plaque burden or mean body mass of targeting CB2 in Ldlr-null mice with JWH-015 or SR144528. We did however, note SR144528 significantly reduced plasma triglyceride levels. Previously, our laboratory showed a reduction of plasma triglyceride levels associated with WIN55,212-2 administration in early atherogenesis (Netherland-Van Dyke et al., 2015a), but this effect was not CB2-dependent and was likely mediated through CB1 which is known to modulate plasma triglyceride levels in humans (Després et al., 2005; Van Gaal et al., 2005; Pi-Sunyer et al., 2006; Scheen and Van Gaal, 2007) and in mice (Dol-Gleizes et al., 2009). In another study from our laboratory, we found SR144528 blocked acyl-coenzyme A: cholesterol acyltransferase (ACAT) activity and lipid accumulation in macrophages (Thewke et al., 2009), providing evidence for a role of CB2 blockade in lipoprotein metabolism while another group demonstrated AM1241 resulted in CB2-dependent intracellular lipid accumulation in hepatocytes *in vitro* (De Gottardi et al., 2010). These studies provide evidence that CB2-dependent mechanisms also affecting lipid metabolism independently from CB1. However, it has been shown that SR144528 shares structural similarity to CB1 antagonist SR141716A, and it could be that in the absence of CB2 signaling, CB1 expression in the liver becomes down-regulated in the presence of SR144528.

Our observation that blockade of CB2 by SR144528 reduced atherosclerotic lesion calcification in Ldlr-null mice, was somewhat surprising, as we observed lesion calcification to

increase in the setting of systemic CB2 deficiency in Ldlr-null mice. We speculate that this seemingly CB2-dependent effect might be related to the drastic decrease in plasma triglycerides elicited by SR144528, and not directly involve modulation of CB2 signaling in osteogenic processes. Evidence for this is two-fold: first, there is an invariable association between lipids and deposition of calcium crystals in atherosclerotic plaques (Hirsch et al., 1993; Parhami et al., 2002; Iyemere et al., 2006; Laird et al., 2006), and second, the expression of Runx2 and OPN in aorta was not consistent with that expected in an environment of reduced calcium deposition. We found that activation of CB2 with both WIN55,212-2 and JWH-015 increased Runx2 and OPN levels in early and late atherogenesis, respectively, which we and others have found to inhibit maturation of osteoblasts and inhibit calcification (Wada et al., 1999; Prince et al., 2001; Bruderer et al., 2014; McGee-Lawrence et al., 2014) (unpublished observation, Fulmer ML and Thewke DP).

To address the effects of pharmacological manipulation of CB2 on vascular calcification independently of extravascular parameters, such as hepatic triglyceride synthesis, we used an *ex vivo* aorta organ culture model. Our preliminary data shows that *ex vivo* aortas cultured in a high Pi osteogenic media rapidly induce calcium deposition by mechanism(s) which are inhibited by CB2 activation and provoked by CB2 antagonism. These results provide additional evidence of a protective role for CB2 signaling in atherosclerotic calcification and suggest that reduced atherosclerotic calcification seen in mice treated with SR144528 may be related to off-target or extravascular effects of SR144528. Further studies are required to determine the mechanism by which SR144528 reduces lesion calcification.

In conclusion, although administration of CB2-specific ligands does not induce lesion regression, SR144528 does reduce plasma triglycerides and plaque calcification. CB2 activation

with JWH-015 alters expression of Runx2 and OPN, key osteogenic regulatory proteins, in Ldlr-null mice. These results demonstrate that CB2 signaling might be a novel target to affect calcifying mechanisms in later stages of atherosclerotic lesion development, which may influence lesion stability. Understanding the exact mechanisms by which CB2 signaling directly and indirectly affects all aspects of lesion development need to be further explored. Many variable parameters are present in the current study, such as type of synthetic cannabinoid used as well as initiation, frequency, and duration of dosing. Further studies will need to be done that addresses these variables in order to provide better insight on how to best target CB2 to achieve beneficial results.

Conflict of interest

The authors have no conflict of interest to disclose.

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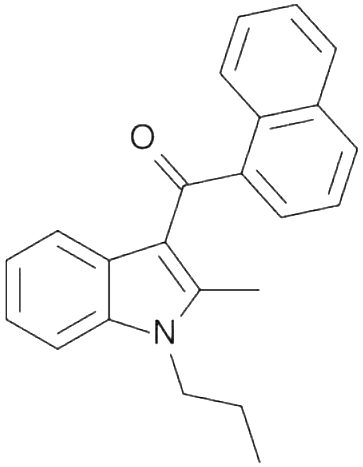
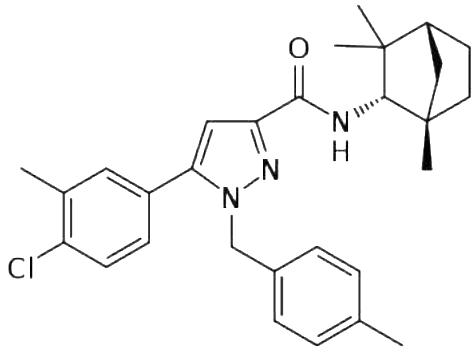
Supplemental Figures and Tables

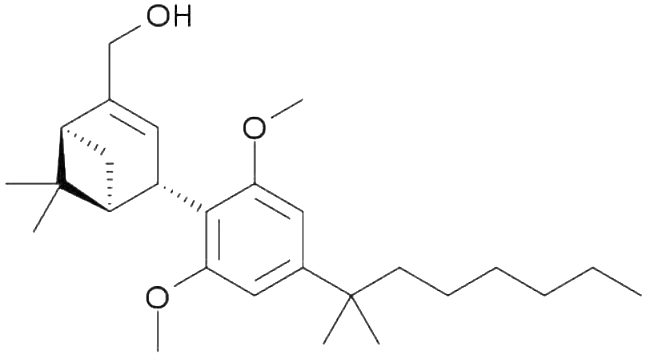
JWH-015
CB2 agonist
Ki= 13.8nM (CB2),
383nM (CB1)



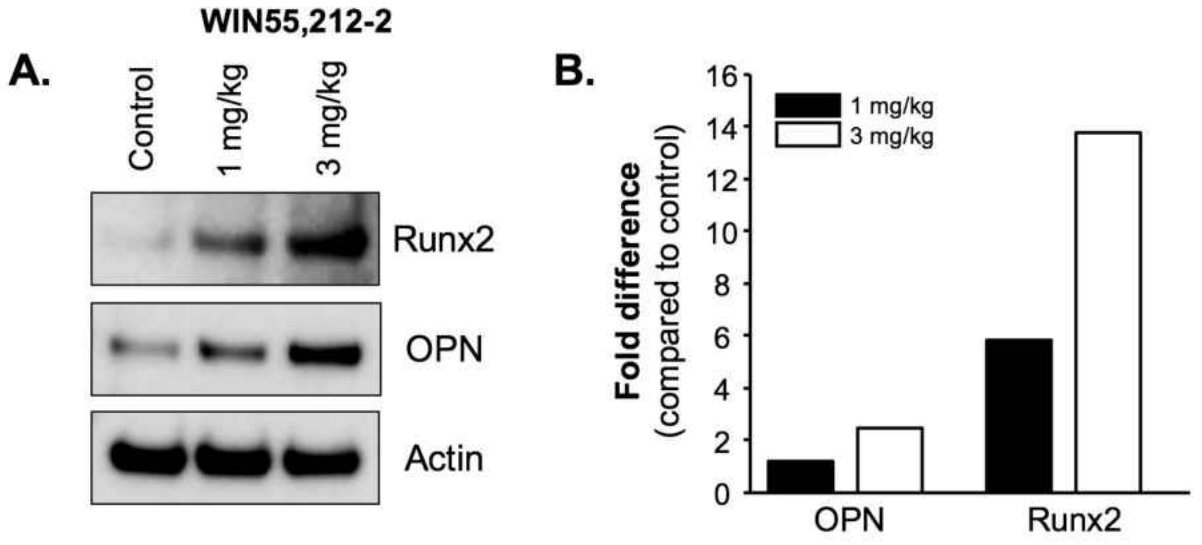
SR144528
CB2 antagonist/inverse agonist
Ki= 0.6nM (CB2),
400nM (CB1)



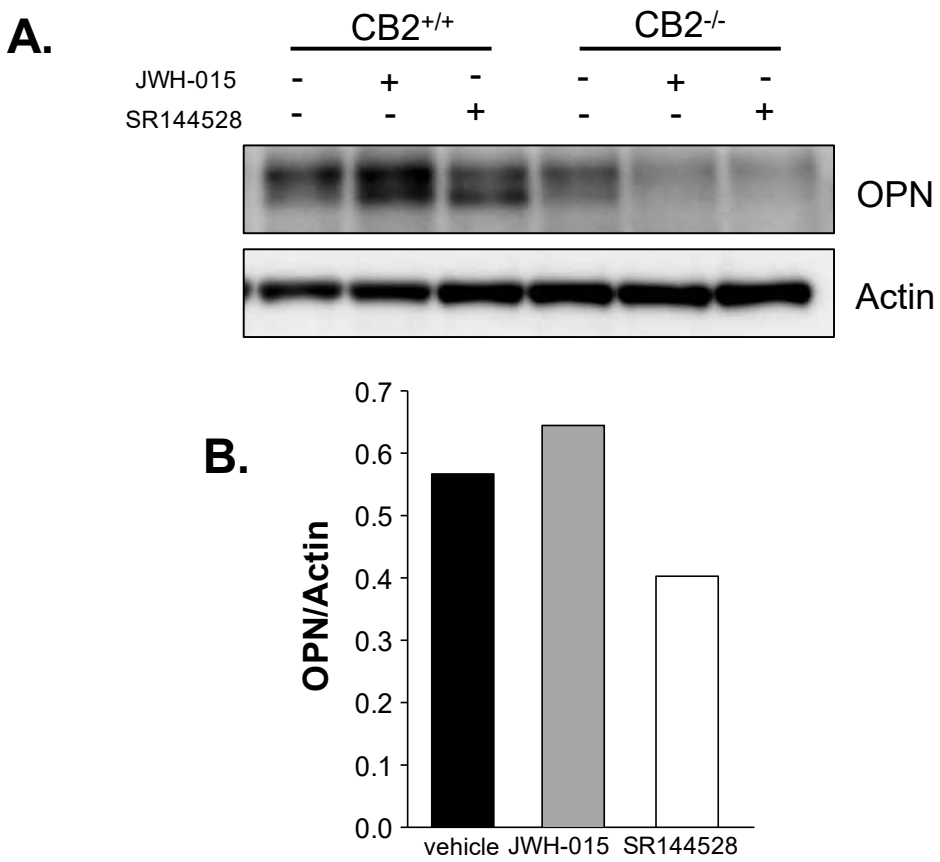
HU-308
CB2 agonist
Ki= 20nM (CB2),
>10μM (CB1)



Supplemental Figure 4.6 Chemical structures of CB2 agonists and antagonist used in this study.



Supplemental Figure 4.7 Dose-dependent response of WIN55,212-2 on OPN and Runx2 in aortic arch lysates. $CB2^{+/+}$ mice were fed a HFD for 8 weeks, receiving daily i.p. injections of CB1/CB2 mixed agonist WIN55,212-2 at either 1mg/kg or 3mg/kg in 50%DMSO/50%PBS. A) Western blot analysis of aortic arch lysates from $CB2^{+/+}$ mice after 8 weeks of HFD and 2 weeks of daily i.p. administration of WIN55,212-2. B) Quantification of OPN and Runx2 protein, expressed as a fold difference over control (50%DMSO/50%PBS).



Supplemental Figure 4.8 Upregulation of OPN levels in calcified aortas of Ldlr-null mice treated with JWH-015. Immunoblot analysis of aortic arch lysates $CB2^{+/+}$ and $CB2^{-/-}$ mice after 24 weeks of HFD and 4 weeks of i.p. administration of JWH-015 or SR144528. A) Representative immunoblot of aortic tissue probed with OPN antibody. B) Quantification of OPN protein levels detected by immunoblotting of aortic arch lysate. Data is presented as the ratio of the intensity of the target protein relative to a loading control protein (Beta actin). n=1.

Analysis of von Kossa staining in aortic root of $CB2^{-/-}$ mice after JWH-015 or SR144528 administration

Treatment	**mean±SEM**	**Probability value versus control**
Control (vehicle)	15.8±0.9%	-
JWH-015	13.4±1.1%	p=0.119
SR144528	17.4±0.9%	p=0.247

Supplemental Table 4.1 Quantification of von Kossa stained aortic root cross sections from $CB2^{-/-}$ mice after 24 weeks of HFD and 4 weeks of i.p. JWH-015 or SR144528 injections. Data represented as percent of positive stain per total lesion area.

CHAPTER 5

CONCLUSIONS

Over the last 15 years, studies involving cannabinoid receptor manipulation has greatly improved our knowledge surrounding the impact of cannabinoid signaling on atherosclerotic lesion formation and progression to an advanced disease state. The initial focus of research in regards to cannabinoid-based therapies for atherosclerosis were centered on actions at CB1, when the CB1-inverse agonist Rimonabant (SR141716A) was used clinically in Europe as an anti-obesity therapy and further showed improvements of cardiovascular risk factors which could not be fully attributed to CB1-mediated weight loss (Després et al. 2005; Poirier et al. 2005; Pi-Sunyer et al. 2006; Scheen and Van Gaal 2007). Beyond its therapeutic potential as a treatment in the setting of obesity and atherosclerosis, Rimonanbant also produces detrimental psychological effects through its actions in the CNS. Consequently, Rimonabant was withdrawn from the European market and never gained approval for use in the U.S. Interest in CB2-mediated effects on atherosclerosis came to light in 2005 when Steffens et. al. discovered that CB2 was expressed in atherogenic vessels, but not in non-diseased areas of vasculature, and that low-doses of THC could induce plaque regression in a manner that was sensitive to CB2 blockade by SR144528 (Steffens, Veillard, Arnaud, Pelli, Burger, Staub, Zimmer, Frossard, and Mach 2005b). These initial studies involving CB1 and CB2 provided insight into the opposing effects of cannabinoid receptor signaling in the face of atherogenesis, and much interest has been gained in the realm of CB2 due to the lack of psychotropic effects involved in CB2-specific signaling.

Information gained from the studies described in this dissertation aid in our comprehension of the role of CB2 in the pathophysiology of atherosclerosis. Specifically, a role

for CB2 in the modulation of the cellular and extracellular composition of advanced lesions, a novel avenue explored in the setting of CB2 and atherogenesis. Data uncovered from these studies indicates a need for future research focused on the identification and development of CB2-specific ligands and CB2-directed therapies as an interventional approach for atherosclerosis.

It is well-recognized that cardiovascular disease claims more lives in the developed world than any other disease, and majority of those affected experience adverse cardiovascular events that result from complications arising from advanced atherosclerosis (Benjamin et al. 2017). Atherosclerosis is a decades-long progression of plaque buildup in vessel walls, fueled by disconcerted inflammatory processes and dysfunctions in lipid homeostasis. Atherosclerotic lesions can remain virtually asymptomatic for several decades, and in fact, only an estimated 3% of lesions actually result in acute clinical events (Virmani et al. 2002). Despite this, the need for intervention of the progression of this disease is not overshadowed by this relatively low statistic, as complications from this disease still kills thousands of people each year.

In the last 10 years, most initial research on the role of CB2 in atherosclerosis was focused on the potential for regression of lesion size in the response to CB2 activation. However, increasing knowledge has emerged indicating a weak correlation between lesion size and progression to a rupture-prone phenotype. It is now more accepted that alteration in lesion composition accompanied with disease progression is more prognostic of the transition from a stable to unstable phenotype. Majority of these alterations in lesion composition are a result of increased pro-inflammatory signaling, resulting in increased influx of immune cells, defects in cellular clearance, alterations in extracellular matrix (ECM) composition, and phenotypic

switching of cell types that bare a high degree of plasticity such as smooth muscle cells (SMCs) (Finn et al. 2010; Moore and Tabas 2011).

Atherogenesis begins decades before intervention is initiated, often times as early as childhood. The first histological change in this process that both chemically and microscopically detectable is the presence of small, isolated groups of macrophages and lipid-laden macrophages, called "foam cells", within the human intima. Also termed an "initial lesion" or "fatty streak," this initial cellular change is mostly present in children and been found in the coronary arteries of 45% of infants within their first 8 months of life (Stary 1987; Stary 1989). Macrophages develop from circulating monocytes which migrate to tissues in response to chemoattractant signals and mature into tissue macrophages where they perform their functions. These cells are versatile immune cells that serve to phagocytose antigens and other cellular debris and initiate immune responses by secretion of effector cytokines and presentation of antigens to T lymphocytes, and are also the predominant cell type found in all stages of atherosclerotic lesion development (Stary 1992).

Macrophages contribute to the progression of atherosclerosis through propagation of inflammation and further recruitment of immune cells, making them a potential target for an anti-inflammatory approach in treatment of atherosclerosis. Administration of natural and synthetic cannabinoids, THC and WIN55,212-2, has been shown to decrease lesional macrophage content in ApoE-null mice and inhibit migration and proliferation of macrophages *in vivo* and *in vitro*, by mechanisms sensitive to CB2 blockade (Steffens, Veillard, Arnaud, Pelli, Burger, Staub, Zimmer, Frossard, and Mach 2005b; Zhao, Y. Liu, et al. 2010; Zhao, Yuan, Y. Liu, Xue, Tian, W. Liu, W. Zhang, Shen, Xu, Liang, and Chen 2010b). The effects of WIN55,212-2 administration on macrophage content were two-fold: by limiting monocyte entry

into the intima through downregulating endothelial adhesion molecule expression (ICAM-1, VCAM-1, and p-selectin), and by reducing the inflammatory response through downregulation of proinflammatory cytokines (Zhao, Y. Liu, et al. 2010; Zhao, Yuan, Y. Liu, Xue, Tian, W. Liu, W. Zhang, Shen, Xu, Liang, and Chen 2010a). However, definitive conclusions were difficult to come to in these early pharmacological studies due to the effects of these compounds acting independently of cannabinoid receptors. THC and WIN55,212-2 both activate PPAR receptors (O'Sullivan and Kendall 2010) and WIN55,212-2 has been shown to negate IL-1-induced expression of adhesion molecules independent of cannabinoid receptor signaling (Curran et al. 2005). A role for CB2 in lesional macrophage accumulation was supported in studies utilizing systemic and bone marrow-derived leukocyte CB2 deficiency in ApoE- and Ldlr-null mice (Netherland et al. 2010; Delsing et al. 2011; Willecke et al. 2011). However, CB2-dependent effects of pharmacological manipulation could not be fully appreciated when assessed in a model containing a functional CB2 receptor. It wasn't until 2015 that our lab provided the first evidence of WIN55,212-2 administration reducing lesional macrophage accumulation by a true CB2-dependent mechanism (Netherland-Van Dyke et al. 2015). This study also provided evidence of CB2-independent effects of WIN55,212-2 administration, which is something that should be considered in pharmacological studies. All of these studies have been performed in models of atherosclerosis which mimic the initial stages, when pharmacological intervention would not yet be initiated in human subjects. Here we provide the first evidence of increased macrophage content in advanced atherosclerotic lesions of Ldlr-null mice lacking CB2 function, suggesting a role for CB2 throughout many stages of atherogenesis.

As atherosclerotic plaques advance, vascular SMCs (VSMCs) play an important role in attempting to stabilize the lesion. In response to increased inflammation and a growing necrotic

core, VSMCs migrate from the media to the luminal side of the intima where they secrete collagen and other ECM components to form a stabilizing fibrous cap to protect the necrotic core and prevent prothrombotic components from interacting with the bloodstream (Rudijanto 2007). The collagen, which makes up over half of the protein content present in atherosclerotic plaques (Smith 1965), provides strength and stability and is another feature which protects the necrotic core in lesions which have become moderately advanced (Burleigh et al. 1992; R.T. Lee and Libby 1997). Thinning of the fibrous cap and proteolytic degradation of collagen and other ECM components by macrophage-secreted matrix metalloproteinases (MMPs) are processes which can initiate instability of advanced atherosclerotic lesions (Galis, Muszynski, et al. 1994). Several MMPs exist, but MMP-9 has been implicated in atherosclerosis and expression of constitutively active MMP-9 was found to increase plaque fissuring and rupture of lesions in ApoE-null mice (Gough et al. 2006). Here we provide evidence for a positive role for CB2 in modulating MMP9 expression in advanced plaques, consistent with our previous study performed in early lesions (Netherland et al. 2010). We also found that fibrous cap thickness was significantly reduced in the setting of CB2 gene deletion. This suggests that CB2-mediated therapies may be beneficial in advanced stages for reducing the expression or activity of MMP-9, thereby reducing degradation of stabilizing ECM components.

Beyond the formation of a fibrous cap, the plasticity of VSMCs contributes to other processes in advanced lesions which promote an unstable phenotype. VSMCs transdifferentiate into a synthetic osteoprogenitor cell type where they commit to an osteoblast lineage in the presence of certain transcription factors and osteogenic regulatory proteins (Steitz et al. 2001). This process results in ectopic calcification of lesions which augments plaque brittleness and reduces vessel elasticity (Trion and van der Laarse 2004; Sun et al. 2012) and is a well-known

major risk factor for adverse cardiovascular events (Detrano et al. 2008; Polonsky et al. 2010). Here we provide the first evidence for a role of CB2 in calcification of advanced plaques and the osteogenic processes involved.

Once thought of as a passive process, plaque calcification is now accepted as an active cellular process similar to normal bone remodeling. Runx2 is a master transcription factor controlling osteoblastogenesis, bearing both positive and negative regulatory functions. Runx2 is necessary for commitment of osteochondrocytic precursors to the osteoblast lineage, however, its expression in subsequent steps of osteoblast maturation are detrimental and blocks the ability of pre-osteoblasts to become functional, bone-forming cells (Prince et al. 2001; Komori 2009; Bruderer et al. 2014; Vimalraj et al. 2015). We found that CB2 deficiency resulted in increased calcification of plaques characterized by decreased expression of Runx2 as well as decreased expression of OPN, a potent inhibitor of calcification. Regulation of these proteins was also achieved through pharmacological activation of CB2 at both early and late stages of atherogenesis. However, pharmacological blockade of CB2 resulted in a slight reduction in advanced plaque calcification. The inconsistency in this data could be attributed to the marked reduction of triglycerides also observed in our antagonist-treated mice. It has been well observed that a uniform association between lipids and calcium crystals exist, and that lipids promote calcium crystal deposition by stimulating osteogenic differentiation of VSMCs and also by serving as an independent site of origin for calcification (Hirsch et al. 1993; Parhami et al. 2002; Laird et al. 2006). Other factors that could affect the results in this study are the concentration and type of synthetic ligands used as well as the onset, frequency, and duration of treatment. We utilized an innovative osteogenic *ex vivo* organ culture method to address the effects of pharmacological manipulation of CB2 on aortic calcification. Use of this system provides an

environment more representative of the natural milieu than what is seen in a single cell culture model and allowed us to assess CB2-dependent effects on vascular calcification that were independent of extravascular parameters, such as hepatic triglyceride synthesis. We found that calcium deposition induced by osteogenic media was reduced in the presence of a CB2 agonist and exacerbated in the presence of a CB2 antagonist. Our data here provides the first evidence of CB2-mediated effects on osteogenic processes involved in atherosclerotic and vascular calcification. Further studies need to be employed to address potential off-target or extravascular effects of CB2 pharmacological manipulation in order to determine if a CB2-mediated therapy could be beneficial in reducing or slowing down calcification of plaques in an attempt to stabilize lesions.

Currently, the only approved therapy for atherosclerosis is use of drugs which aim to reduce serum lipid levels and blood pressure. Reduction of LDL cholesterol with statin therapy has been the standard treatment since the 1980s (Mozaffarian et al. 2016). However, these drugs are only shown to reduce the risk of adverse cardiovascular outcomes by 30% (MS et al. 2017). Further, some patients are intolerant to statins and almost half of patients with normal plasma lipid levels still develop atherosclerotic plaques (Genest et al. 1991; Rubins et al. 1995; Yusuf et al. 2016), highlighting the need for new therapies that can be used in the treatment of atherosclerosis.

Summarized in Figure 5.1, the results presented in this dissertation provide evidence supporting the potential of CB2 as a novel therapeutic target that could promote lesion stability by impacting several processes involved in the progression of atherosclerotic lesions to an unstable phenotype. Future directions include cell-specific CB2 knockdown in both SMCs and macrophages using adoptive transfer. This will provide further insight into how CB2 signaling

specifically affects the function of these cells in atherogenesis and lesion stability. Research efforts aimed at analyzing components of atherosclerotic plaque stability are hindered due to the inability of mouse models to exhibit true plaque rupture as it occurs in humans. Therefore, it is hard to make a full comparison of how CB2 signaling regulating these processes in mice will compare in the setting of human disease. Lesional overexpression of MMP-9 in ApoE-null mice results in increased intraplaque hemorrhaging and outward remodeling, both signs of plaque vulnerability (de Nooijer et al. 2006). However, true plaque fissure was not consistently observed in this model and these findings did not correlate with adverse events seen in humans. Development of a mouse model which better replicates plaque rupture is needed in order to gain further insight on the implications of CB2 signaling in these processes.

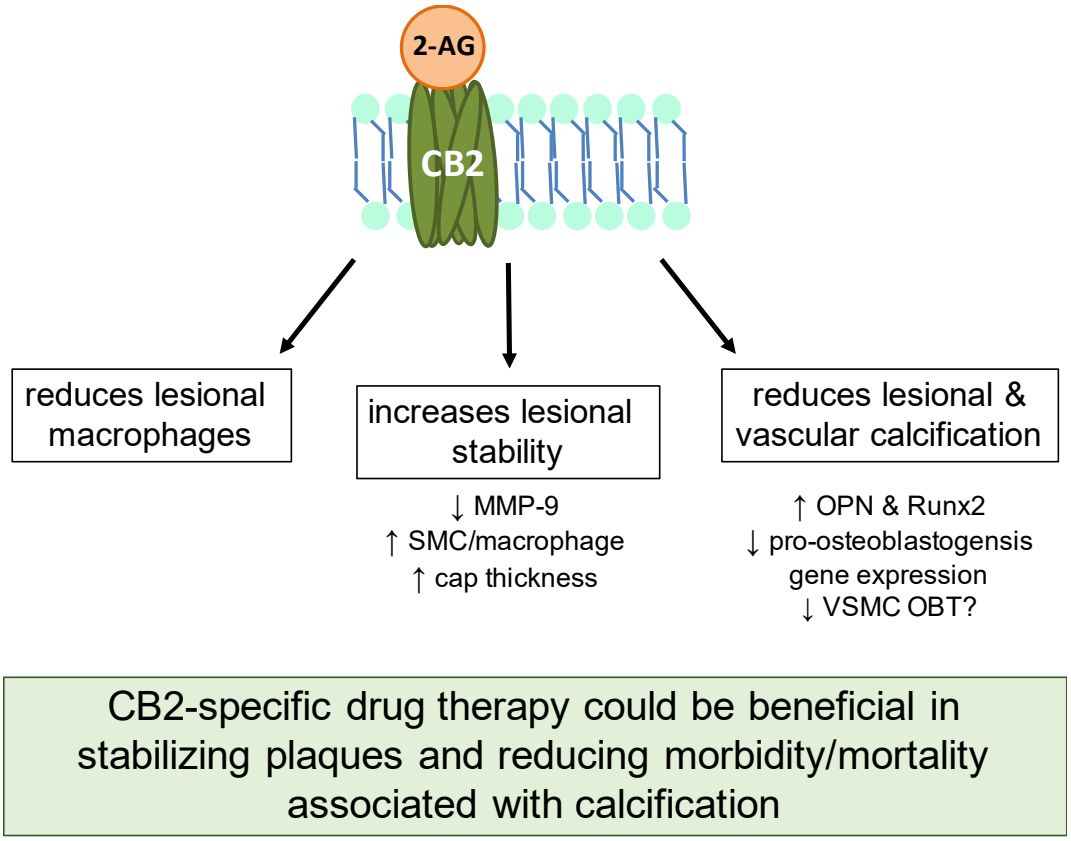


Figure 5.1 Summary of dissertation research findings.

Careful consideration must also be taken when employing cannabinoid-based therapies, as other pathological conditions have been found to be affected by both CB1 and CB2 signaling. For instance, we observed a decrease in serum triglycerides in response to SR144528 administration, and previously found that SR144528 blocked ACAT activity and lipid accumulation in macrophages (Thewke et al. 2009). Increases in intracellular lipid accumulation in hepatocytes has also been observed in response to CB2-selective agonists (De Gottardi et al. 2010). Therefore, although CB2 activation shows to be atheroprotective in terms of stabilizing lesions, it may be detrimental in modulating lipid metabolism. Further, cannabinoid receptor manipulation has also been shown to affect the pathophysiology of other diseases. CB2 activation has been shown to produce anti-inflammatory and immunosuppressive effects in rheumatoid arthritis, liver cirrhosis, gastrointestinal disorders, and neurodegenerative diseases (Sumariwalla et al. 2004; Buckley 2008; George et al. 2008; Parker et al. 2008), offering varying levels of protection in each condition. Studies will need to be done to determine how CB2-specific compounds used in the treatment of atherosclerosis may affect the pathology of other diseases that could result in beneficial and/or adverse effects. The use of tissue-specific drug intervention may be necessary to avoid any potential complications arising from systemic CB2 activation.

Several CB1/CB2 agonists are used clinically to treat maladies such as side effects produced by chemotherapy treatment or neuropathic pain. As of 2017, there are 29 states in the U.S. that have approved the use of cannabis for medical purposes, with each state governing its regulation independent of another (National Academies of Sciences, Engineering, and Medicine et al. 2017 Jan 12). The marijuana plant, *Cannabis sativa*, contains over 700 chemical compounds, 104 of which are considered to be unique cannabinoids (Mechoulam and Hanuš

2000; Izzo et al. 2009). The U.S. Food and Drug Administration has not approved marijuana as a "safe or effective drug for any indication," but states their awareness regarding research interests in testing marijuana in the treatment of several medical condition and supports the medical research community studying the effects of this drug. While marijuana appears to be beneficial in some disease states, increasing knowledge is being obtained about the adverse cardiovascular effects of the drug, such as myocardial infarction, cardiomyopathy, stroke, arrhythmias, and sudden cardiac arrest, all which are mediated through CB1 (Pacher et al. 2017). THC is a partial agonist at CB1 and likely would not produce harmful cardiovascular effects at lower concentrations, but over the past 10 years the potency of THC in marijuana has increased 10-fold, which has paralleled a substantial rise in severe, and sometimes fatal, adverse cardiovascular effects. Further, THC is also a partial agonist at CB2 and has been shown to produce beneficial effects on atherosclerosis when administered at a dose known to be too low to produce effects at CB1 (Steffens, Veillard, Arnaud, Pelli, Burger, Staub, Zimmer, Frossard, and Mach 2005a). Therefore, it appears that the composition of the plant has a large effect on the likelihood of adverse events to occur. Further, poisoning outbreaks and deaths have been on the rise with the increased use of synthetic cannabinoids, consisting of several CB1 agonists that have an overall potency of up to 200-times greater than that of THC. These drugs, referred to as "designer drugs," have been manufactured to exhibit increased potency over the last 2 years, coinciding with an increase in fatalities (Banister et al. 2015; Kasper et al. 2015; Law et al. 2015; Adams et al. 2017).

The rise in adverse effects associated with recreational cannabinoid use has made it difficult to expand the clinical indication for marijuana or marijuana-based products in the U.S. The results in this dissertation provide a novel understanding regarding the potential therapeutic

nature of CB2 signaling in advance atherosclerosis. It further provides a framework for synthetic cannabinoid administration in advanced disease that serves to stabilize lesions and is devoid of adverse cardiovascular effects associated with CB1 signaling.

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