The Expression and Function of CCL5 in Early Atherosclerosis

by

Kelly Tai

A thesis submitted in conformity with the requirements for the degree of Master of Science

Department of Immunology
University of Toronto

© Copyright by Kelly Tai 2017
The Expression and Function of CCL5 in Early Atherosclerosis

Kelly Tai
Master of Science
Department of Immunology
University of Toronto
2017

Abstract

Atherosclerosis is a chronic cardiovascular disease initiated by the accumulation of lipid-loaded macrophages within the artery wall. Chemokines can have several potential functions in atherosclerosis. CCL5, an inflammatory chemokine, is considered proatherogenic. Since CCL5 expression is dramatically increased in early mouse atherosclerotic lesions, the goals were to identify which cells express CCL5 and the functions of CCL5 in early lesion development. CCL5 mRNA and protein were up-regulated both by myeloid and endothelial cells in lesions. Bone marrow transplantation studies demonstrated that CCL5 deficiency in bone marrow-derived cells transiently affected lesion myeloid cell content but did not significantly affect macrophage proliferation or monocyte recruitment. Additionally, CCL5 deficiency in non-hematopoietic cells had no effect on lesion parameters. These studies suggest that although CCL5 is expressed abundantly within lesions, the effects of CCL5 deficiency in early lesion development is minimal and this may be due to compensatory functions of related chemokines.
Acknowledgments

I would like to thank my supervisors, Dr. Myron Cybulsky and Dr. Jenny Jongstra-Bilen, for their mentorship, supervision, guidance, support, and continuous encouragement throughout my graduate school experience. Thank you so much for the effort and time you have invested into this project and my growth as a student. In addition, thank you to my supervisory committee members: Dr. Eleanor Fish and Dr. Clinton Robbins. Thank you for your patience, wisdom, guidance, and collaboration.

Thank you to the past and present lab members for their much-appreciated support. Thanks to Allan Siu who’s work provided much support to start this project and the radiation project in Appendix I. Your work has been very influential in helping me shape my work. Thank you to Dr. Mark Roufaiel for teaching me all the techniques and helping me with the first bone marrow chimeras generated. Thank you so much for your time and patience with me. Dr. Sharon Hyduk, thank you for all that you continuously do for all of us in the lab. Thank you especially for your time in revising my thesis. You are an invaluable member of the lab. Thank you, Marwan Althagafi, for always lending a helping hand with experiments and preparing many of the reagents for everyone to use. Hisham Ibrahim, you have been a voice of wisdom and comfort for me in the lab. Your friendship, encouragement, and support is so greatly appreciated, thank you. Xiaotang Gao, you are a very intelligent student, and it was my pleasure to have had the opportunity to work with you in the lab. Thank you for your assistance and analysis of the bone marrow transplantation experiments in Appendix I. Chanele Polenz, thank you for the much-needed yoga sessions. Andrew Rajkumar, thank you for your company and support. Good luck to you all in your future endeavours in the Cybulsky lab.

I am thankful for the support from my family all my life. I’m not certain whether they truly understood what I’ve been doing the past few years in graduate school, but I am grateful that they’ve allowed me to “search” for my path. It has not been perfect, but I thank them for their understanding and unrelenting support.

Finally, I am forever grateful for our God whose provision and grace always surpasses our expectations when we remember His promises. I thank God for placing me where I am today and for my church at Harvest Bible Chapel York Region. I always feel at home when I’m at Harvest.
Table of Contents

Acknowledgments ........................................................................................................ iii

Table of Contents ......................................................................................................... iv

List of Tables ................................................................................................................ vii

List of Figures .............................................................................................................. viii

List of Abbreviations ................................................................................................. ix

List of Appendices ....................................................................................................... xii

1 Introduction ............................................................................................................... 1

1.1 Cardiovascular Disease and Atherosclerosis ....................................................... 1

1.2 Atherosclerotic Mouse Models ........................................................................... 3

1.2.1 ApoE−/− mice .................................................................................................. 3

1.2.2 Ldlr−/− mice .................................................................................................. 4

1.3 Stages of Plaque Development ......................................................................... 6

1.3.1 Atherosclerosis susceptibility ....................................................................... 6

1.3.2 Intima ............................................................................................................ 9

1.3.3 Lipid loading and nascent foam cell formation ............................................ 9

1.3.4 Advanced lesion progression ....................................................................... 10

1.4 Mechanisms of Early Lesion Formation ............................................................ 12

1.4.1 Monocyte recruitment .................................................................................. 12

1.4.2 Proliferation .................................................................................................. 12

1.4.3 Survival ........................................................................................................ 13

1.4.4 Egress .......................................................................................................... 14

1.4.5 Pro-inflammatory gene expression ............................................................... 15

1.5 Mechanisms of Monocyte Recruitment .............................................................. 17

1.5.1 Monocyte subsets ......................................................................................... 17

1.5.2 Leukocyte adhesion cascade ....................................................................... 17

1.6 Chemokines in Atherosclerosis ......................................................................... 20

1.6.1 Chemokine receptors ................................................................................... 21

1.6.2 CCR1, CCR3, and CCR5 in atherosclerosis .................................................. 23

1.6.3 CCL3 and CCL4 in atherosclerosis ............................................................. 24

1.7 CCL5 in Atherosclerosis ..................................................................................... 27

1.8 Rationale .............................................................................................................. 29
1.9 Hypothesis and aims .................................................................................................................. 30

2 Methods ...................................................................................................................................... 35
  2.1 Mice ........................................................................................................................................ 35
  2.2 Isolation of Aortic Arch ........................................................................................................... 35
  2.3 Staining Ascending Aortic Arch ............................................................................................. 36
  2.4 Confocal Microscopy ............................................................................................................... 36
  2.5 Isolating Intimal cell mRNA from Ascending Aortic Arch ....................................................... 37
  2.6 RT Real-time qPCR ................................................................................................................ 37
  2.7 Bone Marrow Cell Isolation ................................................................................................... 38
  2.8 Bone Marrow Reconstitution ................................................................................................. 38
  2.9 BrdU Pulse Labelling ............................................................................................................. 39
  2.10 Immunostaining Frozen Aorta Sections ................................................................................. 39
  2.11 Statistics .............................................................................................................................. 39

3 Results ......................................................................................................................................... 42
  3.1 Ccl5 is expressed by hematopoietic and non-hematopoietic cells within early lesions ....... 43
  3.2 Hematopoietic CCL5 deficiency did not affect lipid area but reduced CD45^+ area in 3-week lesions .................................................................................................................................. 47
  3.3 Hematopoietic CCL5 deficiency did not affect leukocyte proliferation in 3-week lesions 49
  3.4 Hematopoietic CCL5 deficiency did not affect lipid area and myeloid cell area in 6-week lesions .................................................................................................................................. 51
  3.5 Hematopoietic CCL5 deficiency did not affect leukocyte proliferation or recruitment in 6-week lesions ........................................................................................................... 54
  3.6 Hematopoietic CCL5 deficiency reduced Ccl3 and myeloid cell marker mRNA expression in 3-week lesions ........................................................................................................... 55
  3.7 Hematopoietic CCL5 deficiency did not reduce of myeloid cell marker mRNA expression in 6-week lesions ........................................................................................................... 57
  3.8 Non-hematopoietic CCL5 deficiency did not affect lipid area or myeloid cell accumulation in 3-week lesions ........................................................................................................... 59
  3.9 Non-hematopoietic CCL5 deficiency did not significantly affect expression of chemokines and myeloid cell markers in 3-week lesions ........................................................................... 61
  3.10 Complete CCL5 deficiency did not affect lipid or myeloid cell accumulation in 3-week lesions .................................................................................................................................. 63
  3.11 Ccl4 expression is elevated in 3-week lesions with global CCL5 deficiency ..................... 65

4 Discussion ..................................................................................................................................... 67
5 Future Directions .................................................................................................................. 71

References ........................................................................................................................................... 74

6 Appendix ........................................................................................................................................... 90
6.1 Rationale .......................................................................................................................................... 90
6.2 Methods .......................................................................................................................................... 91
   6.2.1 Mice ........................................................................................................................................ 91
   6.2.2 Bone marrow transplantation (BMT) ...................................................................................... 91
   6.2.3 Isolation of Aortic Arch ......................................................................................................... 91
   6.2.4 Analysis of atherosclerosis ..................................................................................................... 91
   6.2.5 Confocal Microscopy ............................................................................................................. 91
   6.2.6 Isolating Intimal mRNA from Ascending Aortic Arch ............................................................. 92
   6.2.7 RT Real-time qPCR ................................................................................................................ 92
   6.2.8 Statistics .................................................................................................................................. 92
6.3 Results .............................................................................................................................................. 93
   6.3.1 Bone marrow transplantation reduced lipid area in 3-week but not 6-week lesions .......... 93
   6.3.2 Bone marrow transplantation reduced myeloid cell area in 3-week lesions ...................... 96
   6.3.3 Bone marrow transplantation significantly reduced lipid area in 12-week lesions as shown
        by Oil Red O staining .................................................................................................................. 98
   6.3.4 Bone marrow transplantation reduced lesion thickness in progressing lesions .............. 100
   6.3.5 Bone marrow transplantation suppresses CRD-induced gene expression in early lesions .. 102
6.4 Discussion ...................................................................................................................................... 104
List of Tables

**Table 1:** Stages of atherosclerotic lesion formation in $Ldlr^{−/−}$ mice

**Table 2:** Real-time PCR primers
List of Figures

**Figure 1:** Lesser curvature and mapping of the ascending aortic arch.

**Figure 2:** *Ccl3, Ccl4, and Ccl5* are located on chromosome 11 in mice and chromosome 17 in humans.

**Figure 3:** Blocking CCR5 with monoclonal antibody MC-68 inhibits monocyte recruitment into the lesser curvature of the ascending aortic arch in 3-week lesions.

**Figure 4:** CRD induces expression of chemokines and adhesion molecules.

**Figure 5:** Deletion of CD11c$^+$ cells reduced chemokine mRNA level in the ascending aortic arch of *CD11c-DTR Ldlr$^{-/-}$* BM chimeras injected with Diptheria Toxin (DT).

**Figure 6:** Potential roles of CCL5 in early atherosclerotic lesion formation.

**Figure 7:** *Ccl5* is expressed by hematopoietic and non-hematopoietic cells in early lesions.

**Figure 8:** CCL5 is colocalized with hematopoietic and non-hematopoietic cells within 12-week lesions.

**Figure 9:** Hematopoietic CCL5 deficiency significantly reduced lesion CD45$^+$ leukocyte area in 3-week lesions.

**Figure 10:** Lesion Nile Red$^+$ lipid and CD45$^+$ leukocyte area is significantly increased between 3-week and 6-week lesions.

**Figure 11:** Hematopoietic CCL5 deficiency has no effect on Nile Red$^+$ lipid area, CD45$^+$ leukocyte area, or monocyte proliferation and recruitment in 6-week early lesions.

**Figure 12:** Hematopoietic CCL5 deficiency reduced mRNA expression of chemokines and myeloid cell markers in 3-week lesions.

**Figure 13:** Hematopoietic CCL5 deficiency did not significantly affect mRNA expression of myeloid cell markers in 6-week lesions.
Figure 14: Non-hematopoietic CCL5 deficiency does not affect Nile Red$^+$ lipid area or CD45$^+$ leukocyte area in 3-week lesions.

Figure 15: Non-hematopoietic CCL5 deficiency did not significantly affect mRNA expression of chemokines Ccl3, Ccl4, Ccl5 and myeloid cell markers in 3-week lesions.

Figure 16: Complete CCL5 deficiency does not affect Nile Red$^+$ lipid area or CD45$^+$ leukocyte area in 3-week lesions.

Figure 17: Ccl4 expression is elevated in complete CCL5 deficient 3-week lesions.

Figure 18: Apoptosis in myeloid foam cell lesions.
List of Abbreviations

ABCA1 – ATP-binding cassette transporter 1
Akt – aka Protein Kinase B (PKB)
ApoE – apolipoprotein E
ApoE<sup>−/−</sup> – apolipoprotein E deficient mice
BrdU – 5-bromo-2'-deoxyuridine
BM – bone marrow
BMT – bone marrow transplantation
CCL2 – Chemokine (C-C) motif ligand 2 aka monocyte chemoattractant protein 1 (abbrev MCP1)
CCL3 – Chemokine (C-C) motif ligand 2 aka macrophage inflammatory protein 1α (abbrev MIP1α)
CCL4 – Chemokine (C-C) motif ligand 4 aka macrophage inflammatory protein 1β (abbrev MIP1β)
CCL5 – Chemokine (C-C) motif ligand 5 aka RANTES
CCL19 – Chemokine (C-C) motif ligand 19
CCR5 – CC chemokine receptor 5
CCR7 – CC chemokine receptor 7
CD11c – cluster of differentiation 11c, integrin alpha<sub>x</sub>
CD31 – cluster of differentiation 31, platelet EC adhesion molecules-1 (PECAM-1)
CD36 – cluster of differentiation 36
CD45 – cluster of differentiation 45
CD68 – cluster of differentiation 68 aka macrosialin
CRD – cholesterol rich diet
CX3CL1 – chemokine (C-X3-C) motif ligand 1 aka fractalkine
DAG – diacylglycerol
DC – dendritic cells
EC – endothelial cell
eNOS – endothelial nitric oxide synthase
EDTA – ethylenediaminetetraacetic acid
FBS – fetal bovine serum
FITC – fluorescein isothiocyanate
GAG – glycosaminoglycan
GC – greater curvature of the ascending aortic arch
GDP – guanosine diphosphate
GTP – guanosine triphosphate
GM-CSF – granulocyte monocyte colony-stimulating factor
HDL – high density lipoprotein
hrs – hours
ICAM1- Intercellular adhesion molecule 1
IDL – intermediate density lipoprotein particles
IL-1 – interleukin 1
IL-6 – interleukin 6
IP<sub>3</sub> – inositol triphosphate
IRF-3 – interferon regulatory factor 3
JAM-1 – junctional adhesion molecule 1
LC – lesser curvature of the ascending aortic arch
Ldlr – low density lipoprotein receptor
LFA-1 – lymphocyte function-associated antigen 1
LPS – lipopolysaccharide (endotoxin)
LRP – lipoprotein receptor-related protein
LXRα – Liver X receptor alpha
mRNA – messenger ribonucleic acid
MARCO – macrophage receptor with collagenous structure
Mer – Tyrosine-protein kinase Mer
MMP – matrix metalloproteinase
NF-κB – nuclear factor κB
PCR – polymerase chain reaction
PFA – paraformaldehyde
PI3K – Phosphoinositide 3-kinase
PIP2 – Phosphatidylinositol 4,5-bisphosphate
PKC – Protein kinase C
PLC – Phospholipase C
PPAR-γ – Peroxisome proliferator-activated receptor gamma
PSGL1 – P-selectin glycoprotein ligand 1
RANTES – Regulated on activation, normal T cell expressed and secreted
RTM – Reverse transmigration
SCD – standard chow diet
SMC – smooth muscle cell
SRA-I/II – scavenger receptor type I/II
STAT-1 – Signal transducer and activator of transcription 1
THP-1 – Human leukemic monocyte cell line
TLR – Toll-like receptor
TM – thrombomodulin
TNF-α – Tumor necrosis factor alpha
TUNEL – Terminal deoxynucleotidyl transferase dUTP Nick-End Labeling
VCAM-1 – Vascular cell adhesion molecule 1
WT – wildtype
µm – micrometer
°C – degrees Celsius
List of Appendices

Appendix 1: Effects of irradiation and bone marrow reconstitution on early stages of atherosclerotic lesion development in the mouse aortic arch
1 Introduction

1.1 Cardiovascular Disease and Atherosclerosis

Cardiovascular disease (CVD) is a family of heart and blood vessel disorders including myocardial infarction and ischemic stroke. Atherosclerosis is a disease of arteries and is the most common underlying cause of CVD. In 2010, atherosclerosis was responsible for one in four deaths globally.\(^1\) Many major modifiable risk factors of atherosclerosis have been identified including high blood cholesterol, smoking, obesity, high blood pressure, and diabetes mellitus.\(^2\) Epidemiological studies have shown the association of several genetic and environmental factors with the occurrence of atherosclerosis, revealing the complexity of this disease.

The study of atherosclerosis was prompted by a discovery in 1913 by German scientist, Nikolai Anitschkow, who observed that feeding cholesterol to rabbits elicits atherosclerosis and concluded that “without cholesterol there is no atherosclerosis”.\(^3\) His classic experiments lead to today’s understanding of cholesterol in cardiovascular disease. It is now known that cholesterol trafficking in the circulation is facilitated via hydrophobic lipoprotein particles: chylomicrons, very low density lipoprotein particles (VLDL), intermediate density lipoprotein particles (IDL), low density lipoprotein particles (LDL), and high density lipoprotein particles (HDL). Dietary lipids and cholesterol are packaged into chylomicrons for transport to the liver. VLDL is generated by the liver and functions to distribute cholesterol and triglycerides to other parts of the body. IDL is generated by lipases reducing triglyceride content from VLDL. HDL mediates the return of excess cholesterol and lipids from peripheral tissues to the liver for excretion or for biosynthesis of vitamin D and steroid hormones.\(^4\) VLDL and LDL are atherogenic lipoproteins
while HDL has been identified to have atheroprotective properties. Abnormal lipoprotein levels are associated with several human diseases, most commonly atherosclerosis.

Atherosclerosis initiates when oxidatively modified lipids accumulate in the inner arterial wall, also known as the intima. Within the intima, resident myeloid cells engulf the modified lipids and enlarge, becoming what are known as foam cells. The earliest lesions are composed of mainly foam cells, known as ‘fatty streaks’, and are the precursors of more advanced lesions. Advanced lesions are characterized by the buildup of lipid-rich necrotic debris, covered by smooth muscle cells (SMCs) that form a fibrous cap. Ultimately, plaque progression can lead to the severe complications that include acute occlusion of the artery lumen by the formation of a thrombus. This is often the underlying cause of myocardial infarction or stroke.

Human atherosclerosis is a complex disease often with multifactorial risk factors. This makes atherosclerosis a difficult disease to model in laboratory animals. Although limitations and challenges exist in modeling the human disease, the mouse remains as an effective tool to study the mechanisms involved in atherosclerotic plaque development. The mouse has been used not only to reveal atherosclerosis as a disorder in cholesterol metabolism, but also a chronic inflammatory condition that can result in acute cardiovascular complications.
1.2 Atherosclerotic Mouse Models

In the late 1970s and early 1980s, scientists began research on the mouse model for atherosclerosis studies. Due to the low level of atherogenic lipoproteins in mice, lesion development does not occur in wildtype mice when fed a high cholesterol diet. In the early 1990s, through genetic engineering, mice with elevated circulating levels of cholesterol were generated. These mice are now two of the most commonly studied atherosclerosis susceptible mouse models: the apolipoprotein E deficient mouse (ApoE\(^{-/-}\))\(^6\) and the low-density lipoprotein receptor knockout mouse (Ldlr\(^{-/-}\)).\(^7\)

1.2.1 ApoE\(^{-/-}\) mice

The ApoE deficient transgenic mouse is among the most widely used mouse models for atherosclerosis. ApoE is a glycoprotein synthesized primarily in the liver but also in other cell types, including macrophages.\(^8\) It is a main constituent on the surface of lipoprotein particles and a ligand for multiple cholesterol receptors responsible for lipoprotein uptake. In addition to the LDL receptor, ApoE is a ligand for other lipoprotein clearing receptors including the lipoprotein receptor-related protein (LRP) and putative remnant receptor.\(^9\) Due to the important role of ApoE in VLDL and chylomicron remnant clearance from the plasma, the targeted deletion of the apoE gene results in severe hypercholesterolemia and spontaneous atherosclerosis. The plasma cholesterol level of an apoE-deficient mice fed a standard chow diet ranges from 360 to 885mg/dL which is substantially elevated compared to levels found in normal control mice at 101-119mg/dL.\(^10\) Feeding apoE deficient mice a Western-type diet results in substantially higher
cholesterol levels ranging from 1085 to 4402mg/dL. In comparison, normal control mice fed a Western-type diet resulted in minimally elevated cholesterol levels (154 to 301mg/dL). Fatty streak formation and intimal foam cell deposits can be found at valve attachment sites in the aorta of ApoE deficient mice even when fed a standard chow diet. The presence of these preatherosclerotic lesions in apoE-deficient mice demonstrates that the lack of apoE is sufficient to initiate atherogenesis. A disadvantage to the ApoE deficient mouse model is that apoE sufficient hematopoietic cells transplanted into an apoE-deficient mouse is capable of clearing lipoproteins and normalizing serum cholesterol levels. Hence, the transfer of bone marrow (BM) from a mouse expressing apoE into an apoE-deficient recipient not only significantly reduces plasma lipid levels but also rescues atherosclerosis.

1.2.2 Ldlr−/− mice

Ldlr knockout mice, unlike apoE-deficient mice, do not spontaneously develop hypercholesterolemia and are widely used in diet-induced atherosclerosis studies. These mice encode a non-functional receptor that is truncated within the ligand-binding domain and lacks the cell membrane spanning segment. This receptor fragment does not bind LDL. Since mouse LDL particles contain ApoE, they can still be taken up from plasma by hepatocytes via LRP receptors, and this results in a mild elevation of circulating cholesterol levels (175-225 mg/dL) while on a standard chow diet (SCD). Because hypercholesterolemia and lesion formation in Ldlr−/− mice are readily enhanced by a diet supplemented with cholesterol, these mice can develop lesions depending on the duration of this diet. Ldlr−/− mice fed a cholesterol-rich diet (CRD) can develop nascent lesions (0-1 week diet), early lesions (≤8 week diet), and complex lesions (≥12 week diet), (Table 1).
Although lesion development in these mouse models exhibits a similar pathology observed in human disease, there are some distinct differences. Like humans, mice develop lesions at regions of disturbed blood flow, however, unlike humans, lesions in mice tend to develop in the aorta and carotids but not the coronary arteries. In addition, mouse models seldom exhibit lesion rupture, which is a key feature precipitating thrombotic complications and heart attacks.
1.3 Stages of Plaque Development

1.3.1 Atherosclerosis susceptibility

Plaque formation occurs at specific regions of the vasculature. This is due to the heterogeneity in hemodynamics that act along the vessel wall, altering endothelial cell gene expression at differing regions. At relatively straight regions of the vasculature, one observes uniform laminar flow of blood and high wall shear stress. In these regions, endothelial cells are protected from the expression of atherogenic genes and upregulate the expression of anti-inflammatory and anti-coagulant genes (e.g., endothelial nitric oxide synthase and thrombomodulin). The overall effect is atheroprotection, reduced endothelial permeability, reduced migration of leukocytes, and reduced SMC proliferation. In contrast, in regions of branch points, bifurcations and curvatures, one finds disturbed blood flow with oscillatory shear stress, chronic low-grade inflammation, and the expression of atherogenic genes (e.g. monocyte chemoattractant protein-1 or MCP-1), leading to a greater susceptibility for atherosclerotic lesion. An example of a lesion pre-disposed site is lesser curvature (LC) (Figure 1a) of the ascending aortic arch (Figure 1b). An example of a low-potential site for atherosclerosis susceptibility is the greater curvature (GC) (Figure 1c).

Endothelial cells (ECs) are equipped with receptors that allow for the detection and response to mechanical forces created by shear stress. These mechanosensing and mechanotransducing mechanisms involve mediators including integrins, platelet EC adhesion molecules-1 (PECAM-1), tyrosine kinase receptors (e.g. VEGFR2), VE-cadherin, G-proteins and ion channels that have been demonstrated to function in transducing mechanical signals across the EC membrane into the interior of the cell. Initially, mechanical forces stimulate conformational activation of
integrins on the EC surface. Then the clustering of integrins, also known as focal adhesion formation, activates intracellular signal transduction pathways including focal adhesion kinase, MAP kinase cascade, and NF-κB.\textsuperscript{23} Low or oscillating shear stress is shown to activate NF-κB, promoting chronic low-grade inflammation in these areas of the endothelium.\textsuperscript{24} The balance between nitric oxide (NO) synthesis, reactive oxygen species (ROS) production, as well as NF-κB induction determines the probability for atherosclerotic lesion formation.\textsuperscript{23} Hence, the endothelial response to hemodynamics establishes susceptibility for atherosclerotic lesion formation in specific regions.
Figure 1. Lesser curvature and mapping of the ascending aortic arch.

Figure of branch point and lesser and greater curvature (a) adapted from Won et al.\textsuperscript{16} Dissection of the aorta and proximal arch (b). Dashed lines indicate the cuts for \textit{en face} imaging and gene analysis. Schematic of \textit{en face} view of dissected proximal aorta (c). High probability area (HP) or lesser curvature (LC) and low probability area (LP) or greater curvature (GC) areas are indicated in grey. Small notches at the commissures of the aortic valve (A and B) and midpoint of the lesser curvature (C) are indicated. Figure adapted from Iiyama \textit{et al} and used with permission granted.$^{102}$
1.3.2 Intima

The normal artery consists of three layers: tunica intima, tunica media, and tunica adventitia. The intima is the innermost layer of the arterial wall located closest to the lumen. It consists of a monolayer of endothelial cells and subendothelial space overlying the internal elastic lamina. Vascular endothelial cells make up a significant part of the intima and serves as a physical barrier between the vessel wall and the lumen. Because of its location, the intima is most directly influenced by hemodynamic forces. Atherogenesis is accompanied by alterations in the endothelium which normally secretes compounds that regulate vascular tone, inhibit platelet aggregation, promote fibrinolysis and prevent the adhesion of circulating inflammatory cells; however endothelial dysfunction results in the loss of these atheroprotective properties.

1.3.3 Lipid loading and nascent foam cell formation

The initial event propelling the development of atherosclerosis is the increased accumulation of lipoproteins in the subendothelial space. Foam cell formation is a key event in the initiation of atherosclerosis. Upon induction of hypercholesterolemia, the overabundance of circulating cholesterol results in the accumulation of atherogenic lipoproteins (e.g., LDL, VLDL) in the arterial intimal space. Accumulated LDL can become modified oxidatively or non-oxidatively. Lipoproteins can be modified by various enzymes such as myeloperoxidases, lipoxygenases, and reactive oxygen species expressed in macrophages and can generate a modified form of atherogenic LDL known as oxidized LDL (oxLDL). Resident intimal dendritic cells that reside in the arterial intima at sites predisposed to atherosclerosis are the first cells to take up lipids and form the initial foam cells. This occurs prior to increased
macrophage proliferation or monocyte recruitment. The increased production of cytokines, chemokines, and adhesion molecules induces the recruitment of monocytes to the area.

Infiltrating monocytes differentiate into macrophages expressing scavenger receptors and begin to engulf modified LDL, becoming macrophage foam cells. Intimal macrophages engulf lipoproteins by endocytosis of aggregated and native LDL through the LDL receptor and uptake modified lipoproteins, such as oxLDL, are ingested by macrophages through scavenger receptors including class A scavenger receptors type I and II (SRA-I/II), MARCO, CD36, and CD68. This increased cholesterol lipid accumulation in foam cells promotes inflammatory responses including the induction of TLR signaling and inflammasome activation. Macrophage specific NF-κB signaling was shown to be important in lipid loading of macrophages. Interestingly, inhibiting NF-κB in macrophages resulted in an upregulation of scavenger receptor CD36 through PPARγ induction; however, PPARγ simultaneously enhanced expression LXRα resulting in subsequent induction of cholesterol efflux ABCA1 transporter protein. Taken together, the balance between cholesterol uptake and efflux is controlled by the PPARγ pathway, and constitutes a principal determinant for the transformation of macrophages into foam cells. Initially, the lipid-scavenging function of macrophages is favorable, but as lipid uptake leads to excessive accumulation of cholesterol esters in macrophages, overwhelmed intimal myeloid cells become intimal foam cells. Overtime, as the cycle of leukocyte recruitment, proliferation, and foam cell formation perpetuates, early lesions become more complex lesions.

1.3.4 Advanced lesion progression

The advanced lesion is characterized by the presence of a necrotic core with extracellular cholesterol clefts, increased macrophage proliferation, SMC extracellular matrix production, and
dystrophic calcification. The necrotic core is formed over time as foam cells die. This forms acellular lipid-rich regions surrounded by foam cells. SMCs migrate from the media into the intima over the necrotic core, proliferate, and secrete extracellular matrix molecules including interstitial collagen and elastin to form a fibrous cap that covers the necrotic core of the plaque.
1.4 Mechanisms of Early Lesion Formation

1.4.1 Monocyte recruitment

Atherosclerosis-predisposed endothelium is activated and upregulates the expression of adhesion molecules and chemokines leading to the recruitment of monocytes to susceptible sites of lesion formation. Circulating leukocytes are recruited by first adhering to endothelium. The initial step is leukocyte capture and rolling is mediated by selectin molecules binding to their ligands. L-selectin is expressed by most leukocytes, whereas E-selectin and P-selectin are expressed by inflamed endothelial cells. Upon binding to its appropriately glycosylated ligand, P-selectin glycoprotein ligand 1 (PSGL1), this enables leukocytes to adhere to inflamed endothelium. Following leukocyte rolling, leukocyte arrest is triggered by chemokines and is mediated by the binding of activated leukocyte integrins to endothelial expressed adhesion molecules, VCAM1 and ICAM1. Following arrest, leukocytes may emigrate through endothelial walls and into inflamed tissue by transendothelial cell migration (TEM). Infiltrating monocytes differentiate into macrophages in response to M-CSF secreted by endothelial cells. Overtime, mature macrophages engulf modified lipids and become foam cells. In LDL receptor deficient mice, monocyte recruitment to the artery wall is increased overtime as foam cells of the fatty streak appear.

1.4.2 Proliferation

The local proliferation of macrophages is an important driving force in the accumulation of immune cells in lesions. Proliferation in atherosclerotic lesions was first observed when McMillan et al. identified mitotic activity in foam cell lesions in cholesterol fed rabbits.
Additionally, Gordon et al., utilizing antibodies specific for cell cycle proteins, identified the proliferation of macrophages and smooth muscle cells in lesions of human coronary arteries. A technique to detect proliferation is through the administration of 5-bromo-2’deoxyuridine (BrdU) in mice. BrdU is a thymidine analog that is stably incorporated into the DNA of cells during S phase of the cell cycle. Using this method, our lab identified that the proliferation of intimal CD11c+ myeloid cells initiates in nascent lesions and is regulated by GM-CSF. More recently, Robbins et al. demonstrated that monocyte recruitment was driving the progression of early stage lesions; however, in established atherosclerotic lesions, macrophage proliferation dominated over recruitment in maintenance of lesion macrophages. Hence, local macrophage proliferation is a significant cause of atherosclerotic plaque progression but especially in late stages of lesion development.

### 1.4.3 Survival

Macrophages in atherosclerotic plaques have an exceptional capacity to survive different types of cellular stress. In early plaques, the activation of autophagy protects macrophages from oxidative stress by removing damaged proteins and organelles within the cytoplasm. Cholesterol esters in lipid droplets are efficiently hydrolyzed in autolysosomes to generate free cholesterol for effective efflux via the transporter ABCA1. Additionally, the unfolded protein response (UPR) protects macrophages from apoptosis by ER stress-inducing agents due to the accumulation of cholesterol. Apoptotic cells are rapidly cleared via phagocytosis in this early stage and PI3K/Akt and NF-κB are several survival pathways that are activated. Overall, in early plaques, macrophages can effectively metabolize cholesterol and efficiently remove waste and apoptotic bodies to maintain cell survival.
In advanced plaques, autophagy and efferocytosis (phagocytosis of apoptotic cells) are defective.\textsuperscript{52} Ineffective clearing of apoptotic cells contributes to secondary necrosis with release of inflammatory mediators.\textsuperscript{53} The accumulation of non-phagocyted apoptotic bodies undergoing necrosis contributes to the enlargement of the necrotic core and promotes plaque progression. One way in which macrophages remove debris is to prevent secondary necrosis and inflammation is through the process of efferocytosis.\textsuperscript{54} In advanced plaques, defective kinase activity by Mertk resulted in deficient macrophage efferocytosis, resulting in furthering necrosis in plaques in \textit{ApoE}^{-/-} mice.\textsuperscript{55} Hence, a primary mediator of efferocytosis in macrophages is Mertk.

1.4.4 Egress

Defective cellular egress from lesions promotes the progression of atherosclerotic plaques. Van Gils and colleagues established that a neuronal guidance molecule with immunomodulatory functions, Netrin-1, and its receptor UNC5b, are expressed by macrophage foam cells in human and mouse atherosclerotic plaques.\textsuperscript{56} They demonstrated that recombinant netrin-1 and foam cell secreted netrin-1 can potently block the migration of macrophages towards CCL19 \textit{in vitro}. Netrin-1 also blocked the migration of macrophages towards CCL2, through its receptor CCR2. This data indicated that the expression of netrin-1 produced by macrophage foam cells was pro-atherosclerotic due to its inhibitory effect on macrophage egress.\textsuperscript{56}

A recent paper from our laboratory identified that intimal dendritic cells undergo protective reverse transendothelial migration (RTM) after systemic stimulation of pattern-recognition receptors.\textsuperscript{57} In a CCR7 and CCL19 dependent manner, intimal myeloid cells are able to return into the arterial circulation while removing intracellular pathogens such as \textit{Chlamydia}
*muridarum* after TLR stimulation; however this process was inhibited by hypercholesterolemia. Hence, atherogenesis is accompanied by compromised RTM of intimal myeloid cells; however, the precise mechanisms involved in this inhibition of RTM are the subject of ongoing studies in our laboratory.

### 1.4.5 Pro-inflammatory gene expression

Inflammation is an important governing factor at all stages of atherosclerosis, from lesion formation to plaque build-up and final end-stage rupture and thrombosis. Atherosclerosis is a chronic inflammatory disease where pro-inflammatory gene expression promotes the production of effectors that enhance lesion progression. These effectors include various enzymes, stimulatory cytokines, chemokines, growth factors, survival factors, and receptors. Several genes are known to be associated with CAD: *Il-6, Tnfa*, and *Il-1*.

A key component of inflammatory gene expression in atherosclerosis is interleukin 1 (IL-1). In early stages of atherosclerosis, IL-1 enhances leukocyte adhesion and transmigration as well as induces foam cell and lesion formation. In humans, the expression of IL-1β correlates with disease severity in ischemic heart disease patients. IL-1β knockout or antagonism in *apoE*−/− mice attenuated atherosclerosis disease development. IL-1 signaling through interleukin 1 receptor 1 (IL-1R1), activates transcription factor NF-κB and results in the upregulation of a large number of inflammatory genes including chemokines.

Chemokines also contribute to pro-inflammatory gene expression in atherosclerosis and enhance lesion progression. These chemotactic cytokines play a role not only in recruiting leukocytes and adhesion to activated endothelium, but also during plaque progression. The topic of
chemokines in atherosclerosis is the focus of this thesis and will be elaborated in the following sections.

In addition to the secretion of inflammatory mediators, macrophages in advanced lesions promote the growth of the necrotic core and thinning and weakening of the fibrous cap through the secretion of proteases that degrade the extracellular matrix, such as matrix metalloproteases (MMPs), serine proteases and cysteine proteases, making the plaque vulnerable for rupture.
1.5 Mechanisms of Monocyte Recruitment

1.5.1 Monocyte subsets

Monocytes represent a heterogeneous population of cells that circulate in the blood. Circulating monocytes in mice consist of two major subsets, Ly-6C^{hi}CCR2^{hi}CX3CR1^{lo} monocytes, which express high levels of CCR2 but low levels of CX3CR1, and Ly-6C^{lo}CCR2^{lo}CX3CR1^{hi} monocytes, which express low levels of CCR2 but high levels of CX3CR1. Ly-6C^{hi}CCR2^{hi}CX3CR1^{lo} monocytes are “classical” monocytes. They are sometimes referred to as “inflammatory monocytes” due to their potent phagocytosis capacity, their preferential recruitment into inflamed tissues, and they are more likely to mature into inflammatory M1 macrophages, which are characterized by their secretion of pro-inflammatory cytokines TNFα and IL-6. Ly-6C^{lo}CCR2^{lo}CX3CR1^{hi} monocytes are “non-classical” monocytes that patrol the luminal side of endothelium via CX3CR1 and secrete anti-inflammatory cytokine, IL-10. In vascular inflammation, Ly6C^{lo} monocytes are recruited to tissue and more likely to differentiate into M2 macrophages that contribute towards tissue repair. Hypercholesterolemia is associated with elevated levels of circulating monocytes. In Apoe^{−/−} and Ldlr^{−/−} mice, Ly6C^{hi} circulating monocyte levels are significantly increased compared to wildtype mice and are of the subset preferentially recruited to plaques.

1.5.2 Leukocyte adhesion cascade

The leukocyte adhesion cascade is a central paradigm in inflammation and immunity. It describes the sequential process of a leukocyte’s journey towards the site of inflammation: integrin-mediated leukocyte rolling, leukocyte activation and arrest, and transendothelial cell
migration. In atherosclerosis, endothelial cells are activated at the site of inflammation, upregulating the expression of adhesion molecules and chemoattractants and present these on their luminal surface. The first stage of leukocyte rolling on endothelium is mediated by L-selectin, P-selectin, and E-selectin which interact with P-selectin glycoprotein ligand 1 (PSGL1) on leukocytes. The interaction of these selectins with their ligands promote the capture of leukocytes onto inflamed endothelium despite conditions of blood flow. Thereafter, chemokines presented on the endothelium by glycosaminoglycans (GAGs) bind to high affinity G-protein coupled receptors (GPCRs) on leukocytes. Following GPCR-triggered signaling, integrins are rapidly activated and bind to immunoglobulin superfamily adhesion molecules, such as VCAM1 and ICAM1 on endothelial cells. This combination of inside-out and outside-in signaling contributes to adhesion strengthening and arrest.

Transmigration through the endothelial barrier is the final step in the process of leukocyte emigration into the inflamed tissue. Transendothelial cell migration can occur paracellularly or transcellularly and can occur with minimal disruption to the structure of vessel walls. The paracellular method of leukocyte trafficking occurs as the expression of interendothelial cell contacts reduce and cell junction proteins are redistributed, permitting leukocyte migration between endothelial cells. Some endothelial junctional molecules actively mediate leukocyte transendothelial migration including the immunoglobulin superfamily proteins platelet endothelial adhesion molecule (PECAM)-1 and junctional adhesion molecule-1 (JAM-1). These molecules are concentrated at endothelial cell-cell contacts and may serve as “gatekeepers” that regulate the transmigration of leukocytes. For example, blocking PECAM-1 homophilic interactions at the endothelial cell junctions was shown to block transendothelial migration of leukocytes. In contrast, JAM-1 is a ligand for the β2 integrin lymphocyte function
associated antigen (LFA)-1 on leukocytes, and this heterophilic interaction is important for permitting leukocyte transmigration.\textsuperscript{73} Hence, the binding of LFA-1 on leukocytes to JAM-1 at the interendothelial junctions guide leukocytes during transendothelial migration.
1.6 Chemokines in Atherosclerosis

Chemokines are a family of small soluble signaling proteins that have roles in health and disease by orchestrating the infiltration of leukocytes. These chemotactic cytokines are classified into four subfamilies based on the position of the amino-terminal cysteine residues (CC, CXC, C, CX3C) and bind to specific chemokine receptors.\textsuperscript{63} Chemokines function by establishing a chemokine gradient to direct receptor expressing immune cells towards the site of inflammation. They bind to their cognate seven trans-membrane GPCRs to mediate cellular migration.\textsuperscript{74}

In atherosclerosis, chemokines are involved in regulating the processes of integrin-dependent leukocyte arrest, transendothelial migration, and anti-apoptotic survival signaling.\textsuperscript{75} The localization of the chemokine often dictates the function or role of the chemokine. For example, when immobilized on the surface of the endothelium, chemokines presented by glycosaminoglycans (GAGs) to establish a gradient for recruiting leukocytes to inflamed tissues. Upon binding to GPCRs on the surface of leukocytes, a signal transduction cascade is initiated to activate surface integrins for mediating leukocyte arrest.\textsuperscript{76}

Investigation on the role of chemokines in atherosclerosis have been generated from hypercholesterolemic Apoe\textsuperscript{-/-} and Ldlr\textsuperscript{-/-} mice.\textsuperscript{77} The first chemokine to be shown to have a role in the pathogenesis of atherosclerosis was CCL2 (MCP-1). Absence of CCL2 in Ldlr\textsuperscript{-/-} mice fed a high cholesterol diet reduced atherosclerosis significantly.\textsuperscript{78} Further studies showed that a deficiency in CCR2, the receptor for CCL2, abolished egress of monocytes from the bone marrow and significantly reduced CCL2-induced recruitment of monocytes into inflammatory sites.\textsuperscript{79,80,81} Later studies showed that three major receptor-chemokine pairs are critical in facilitating monocyte transmigration: CCR2-CCL2, CX3C-chemokine receptor 1 (CX3CR1)-
CX3C-chemokine ligand 1 (CX3CL1 or fractalkine) and CCR5-CCL5, \(^{82}\) of which CCR5-CCL5 is the subject of this thesis.

Beyond facilitating recruitment of monocytes, atherogenic chemokines can participate in atherosclerosis development in other ways. For example, dendritic cell derived CCL17 was shown to drive atherosclerosis development through inhibiting the expansion of T regulatory cells. \(^{83}\) The general understanding of chemokines in atherosclerosis has revealed the functional versatility, redundancy and robustness of the chemokine system.

**1.6.1 Chemokine receptors**

Upon ligand binding, chemokine receptors activate a series of coordinated intracellular signaling events that eventually lead to cellular responses. All chemokine receptors identified thus far are membrane bound molecules composed of seven transmembrane domains and coupled to G-proteins. \(^{84}\) Upon chemokine binding, these receptors associate with and activate G-proteins by exchanging guanosine diphosphate (GDP) for guanosine triphosphate (GTP). Subsequent activation of the enzyme phospholipase C (PLC) results in cleavage of phosphatidylinositol 4,5-biphosphate (PIP\(_2\)) forming secondary messengers, phosphatidylinositol 1,4,5-triphosphate (IP\(_3\)) and diacyl-glycerol (DAG). IP\(_3\) mobilizes calcium from intracellular stores and DAG acts to activate protein kinase C (PKC). \(^{84}\) This activates various calcium-sensitive protein kinases which in turn activates a series of signaling events leading to cellular responses.

In atherosclerosis, atherogenic chemokine receptors can have different modes of action. Most chemokine receptors have a specialized role in regulating the recruitment and function for specific cell populations. Monocyte recruitment is facilitated largely by chemokines and chemokine receptor signaling. Classical monocytes known as Ly6C\(^{hi}\) CCR2\(^+\) expressing cells
depend on CCR2 for entry into atherosclerotic plaques.\textsuperscript{82} This subset of monocytes also depend on CX3CR1 to migrate into lesions. Non-classical monocytes, Ly6C\textsuperscript{lo}CCR2\textsuperscript{−} monocytes, do not employ CX3CR1 to enter plaques but instead required CCR5 for trafficking.\textsuperscript{82}

Other chemokine receptors and their ligands have been identified to have different roles in atherosclerotic mouse models. CXCL1-CXCR2 mediates macrophage accumulation in established lesions,\textsuperscript{85} and its ligand, CXCL1, triggers monocyte arrest on early atherosclerotic endothelium.\textsuperscript{86} In some cases, the role of certain receptors can be ambivalent and controversial. For instance, genetic deletion of CCR7 in \textit{Ldlr}\textsuperscript{−/−} mice reduced the size and macrophage accumulation in atherosclerotic plaques;\textsuperscript{87} however, in \textit{apoE}\textsuperscript{−/−} mice, CCR7 deficiency did not affect size or macrophage content.\textsuperscript{88}

Chemokine receptors can also serve an atheroprotective role in atherosclerosis development. The blockade of CCR7 ligands, CCL19 and CCL21, inhibited plaque regression and foam cell reduction, suggesting that CCR7 may drive the egress of macrophages from lesions and have an atheroprotective role.\textsuperscript{89} CCR1 deficient bone marrow reconstitution of \textit{Ldlr}\textsuperscript{−/−} mice resulted in markedly increased atherosclerotic lesion size.\textsuperscript{90} This was similarly replicated in \textit{Ccr1}\textsuperscript{−/−} \textit{apoE}\textsuperscript{−/−} mice.\textsuperscript{91} Reconstituting \textit{Ldlr}\textsuperscript{−/−} mice with \textit{Cxcr4}\textsuperscript{−/−} bone marrow was shown to increase atherosclerotic lesions in the whole aorta and aortic root. Thus, demonstrating that CXCR4, which binds CXCL12, plays an atheroprotective role in atherosclerosis development.\textsuperscript{92} Overall, chemokine receptors in atherosclerosis have been demonstrated to have atherogenic functions (eg. CCR2, CCR5, CXCR2, CX3CR1), atheroprotective functions (eg. CCR7, CCR1, CXCR4)\textsuperscript{77} and have influential roles in the pathophysiology of atherosclerosis development.
1.6.2 CCR1, CCR3, and CCR5 in atherosclerosis

CCR1, CCR3, and CCR5 are the receptors for the chemokines CCL3, CCL4, and CCL5. CCL3 binds to CCR1 and CCR5, CCL4 binds selectively to CCR5, and CCL5 binds to all 3 receptors.\(^9^3\) Like most chemokine receptors, CCR1, CCR3, and CCR5 are G protein-coupled seven transmembrane receptors that activate downstream intracellular signaling upon ligand binding.\(^7^4\)

CCR1 is expressed on monocytes, memory T cells, basophils, and dendritic cells.\(^9^4\) Its ligands include CCL3 and CCL5. As mentioned previously, CCR1 deficiency in bone marrow did not protect but rather enhanced the progression of atherosclerosis in *Ldlr\(^{-/-}\)* mice, suggesting that CCR1 plays a protective role in atherogenesis.\(^9^0\) Similarly in *Ccr1\(^{-/-}\)* *apoE\(^{-/-}\)* mice, mice showed increased atherosclerotic lesion formation compared to control mice and this may have been due to the significant increase of T cells and IFN\(\gamma\) production in plaques since macrophage content was not affected.\(^9^1\) These studies reveal that although CCL5 has been shown to be an atherogenic chemokine, its engagement with distinct receptors can produce atheroprotective functions.

CCR3 is expressed on eosinophils basophils and T cells.\(^8^4\) In atherosclerosis, CCR3 is relatively understudied and a role of this CCL5 receptor in atherosclerosis has not been identified. Much of literature on CCR3 revolves around its participation in lung inflammation, particularly in asthma pathology.\(^9^5\) However, CCR3 and one of its ligands, eotaxin, was shown to be upregulated in human atheroma, suggesting that they may participate in the pathology of the disease.\(^9^6\)

CCR5 binds to all 3 ligands: CCL3, CCL4, and CCL5. This receptor is most known for its role as a co-receptor in HIV infection.\(^9^7\) The role of CCR5 in atherosclerosis has been investigated by both receptor antagonism and genetic deletion however the results from these studies are not clear.\(^9^3\),\(^9^8\) Kuziel *et al* identified that complete CCR5 deficiency in *apoE\(^{-/-}\)* mice had no effect in
early atherosclerosis after 16 weeks of normal chow. Quinones et al confirmed that CCR5 deficiency in apoE−/− mice had no influence on lesions at the early-atherosclerotic stage but the CCR5 deficiency protected against advanced atherosclerosis. In contrast, complete CCR5 deficiency protected apoE−/− mice from atherosclerosis after a 10-12 week high-fat diet. Bone marrow CCR5 deficiency in Ldlr−/− mice after 8 weeks of fat diet accounted for a 30% reduction in macrophage accumulation in lesions within the aortic sinus but there were no significant differences in plaque size. In addition, lesion size and composition of advanced lesions in these mice were unaffected. Hematopoietic CCR5 deficiency was shown to upregulate interleukin-10 in the spleen and down regulation of TNFα in T cells but this effect was lost in advanced lesions. The understanding of the role of CCR5 in atherosclerosis is generally atherogenic however the mechanism at which CCR5 and its ligands, CCL3, CCL4, and CCL5, modulates atherosclerosis has not been clearly defined.

### 1.6.3 CCL3 and CCL4 in atherosclerosis

CCL3 (MIP-1α) and CCL4 (MIP-1β) are macrophage inflammatory proteins and part of the CC chemokine family. They were first isolated from culture medium of LPS-activated macrophages. Both human and murine CCL3 and CCL4 genes are inducible in most mature hematopoietic cells, although low levels of CCL3 are expressed constitutively. They are both ligands to CCR5 and have been relatively understudied in atherosclerosis.

CCL3 is a chemokine shown to attract neutrophils, monocytes, eosinophils, basophils and lymphocytes by binding to CCR1 and CCR5. It is encoded in a single-copy gene, located on chromosome 11 in mice and chromosome 17 in humans (Figure 2). The expression of CCL3 is mainly found in macrophages, although evidence also points to the release of CCL3 from
activated platelets, neutrophils and mast cells.\textsuperscript{106} During atherosclerotic lesion formation in ApoE\textsuperscript{-/-} mice, CCL3 expression is significantly increased and is shown to play a role in neutrophil accumulation.\textsuperscript{107} CCL3 has been reported to augment neutrophil chemotaxis induced by the pro-inflammatory cytokine TNF\textgreek{a} in a CCR5-dependent manner.\textsuperscript{108} Deficiency of bone marrow-derived CCL3 led to a reduction of plasma lipids and atherosclerotic lesion area; however, no differences in lipid metabolism or atherosclerosis were observed in global CCL3 deficiency in LDLR\textsuperscript{-/-} mice.\textsuperscript{104} Taken together, CCL3 has a role in atherosclerosis, more specifically in neutrophil migration; however direct mechanistic understanding of CCL3 functions in this disease has yet to be elucidated.

CCL4 is encoded by a single gene on chromosome 11, adjacent to Ccl3.\textsuperscript{105} CCL4 binds selectively to CCR5.\textsuperscript{109} This chemokine was first demonstrated to play a role in enhancing T cell adhesion to VCAM-1 by binding to its receptor when immobilized on endothelial cells through GAG binding sites.\textsuperscript{110} Its most prominent activity described is the induction of monocyte recruitment at inflammatory sites. In the field of atherosclerosis, CCL4 is relatively understudied. The expression of CCL4 is upregulated in advanced plaques.\textsuperscript{111,75} In vitro, human CCL4 stimulation of human monocytic cells (THP-1) increased reactive oxygen species (ROS) production and increased the adhesion of monocytes to inflamed human umbilical vein endothelial cells through oxidative stress via PI3K-Rac1 cascades.\textsuperscript{112} Serum CCL4 levels may also be a potential predictor for cardiovascular events in hypertensive patients as individuals with the highest quartile of serum CCL4 showed a higher risk of stroke and cardiovascular events.\textsuperscript{112} Although there is increasing evidence supporting the potential role of CCL4 in atherosclerosis, the mechanistic understanding of CCL4-related atherosclerosis disease is unknown.
Figure 2. *Ccl3*, *Ccl4*, and *Ccl5* are located on chromosome 11 in mice and chromosome 17 in humans.

Genomic organization of the CC chemokine major cluster. *Ccl5*, *Ccl3*, and *Ccl4* are located closely on mouse chromosome 11. Rectangles represent functional genes. Black arrows represent transcriptional orientation. Black dots represent cDNA that has been reported. Ovals and ps indicate non-functional pseudogenes. Maps shown are based on the Ensembl Genome Browser and analyses from Shibata *et al.*

105
1.7 CCL5 in Atherosclerosis

CCL5 (RANTES, Regulated on Activation, Normal T cell Expressed and Secreted) is a small protein of 68 amino acids and is a pro-inflammatory chemokine that is detected in atherosclerotic plaques. Regulation of CCL5 expression in immune cells is well studied. It has been reported that transcription factors such as interferon regulatory factor-3 (IRF-3), STAT1, and NF-κB mediate CCL5 transcription and production. In macrophages, CCL5 is predominantly regulated by the prototypic p50/p65 heterodimers binding to its promoter NF-κB sites. Kruppel-like factor 13 has been identified as a transcription factor regulating the expression of CCL5 in T cells. In smooth muscle cells, the expression of CCL5 has been found to be controlled by Y-box binding protein 1 and contributes to neointimal and medial hyperplasia in an experimental model for accelerated atherosclerosis.

CCL5 is a relatively indiscriminate chemokine in that it binds to multiple receptors (CCR1, CCR3, and CCR5) and is capable of forming many different heterodimers. CCL5 is expressed in different cell types including macrophages, CD8+ T cells, platelets and smooth muscle cells. Elevated CCL5 expression is associated with a wide range of inflammatory disorders and diseases, including asthma, glomerulonephritis, arthritis, and atherosclerosis.

In atherosclerotic lesions, CCL5 has been shown to play a role in the recruitment of monocytes to regions prone for lesion formation. Most notably, Christian Weber et al. demonstrated the role of platelet-derived CCL5. CCL5 can be stored and released from α-granules by platelets and CCL5-CXCL4 heterodimers deposited onto activated endothelium constitutes an important mechanism exacerbating lesion formation. Disruption of the heterodimerization between CCL5 and CXCL4 attenuated monocyte recruitment and reduced atherosclerosis.
Antagonism of CCL5 signaling attenuates lesion formation in mice. Met-RANTES, a version of CCL5 synthesized recombinantly in prokaryotic cells that retains an initial methionine residue, is utilized as a CCL5 receptor antagonist to effectively block calcium mobilization and chemotaxis. Administration of Met-RANTES showed reduced lesion formation in Ldlr\textsuperscript{-/-} mice fed with high-cholesterol diet for 14 weeks. Treatment with another dominant negative CCL5 mutant \textsuperscript{44}AANA\textsuperscript{47}, a version of CCL5 that interferes with GAG binding and oligomerization by forming heterodimers with wildtype CCL5 in Ldlr\textsuperscript{-/-}, limited atherosclerotic lesion formation by reducing infiltration of T cells and macrophages and reduced production of matrix metalloproteinase (MMP)-9.

Overall, CCL5 has been implicated to function in the pathology of atherosclerosis; however, the exact source and role of this chemokine within early stage atherosclerosis has not been investigated.
1.8 Rationale

CCL5 and its receptor CCR5, have been shown to play a role in recruiting monocytes to atherosclerotic lesions\textsuperscript{56}. Allan Siu, a previous graduate student in our lab, showed that blocking CCR5 with a monoclonal antibody (MC-68) reduced monocyte recruitment in early lesions (Figure 3). Allan also found that CCL5 mRNA expression was substantially elevated in early lesions (Figure 4), suggesting that CCL5 is produced locally. In addition, deletion of CD11c positive myeloid cells in $Ldlr^{-/-}$ mice transplanted with CD11c-diphtheria toxin receptor bone marrow significantly reduced CCL5 expression in early lesions, suggesting that CD11c$^+$ intimal foam cells produce the bulk of intimal CCL5 (Figure 5). However, endothelial cells, which are not derived from bone marrow precursors, may also produce CCL5 because intimal CCL5 levels were not completely abolished in these experiments. We were intrigued by these data and decided to investigate the source and functions of CCL5 in early atherosclerotic lesion development (Figure 6).

The local production and functions of CCL5 produced by cells within atherosclerotic lesions has not been published. Other groups showed that platelets produce and deposit CCL5 onto the endothelium over atherosclerotic lesions\textsuperscript{7,8,9}. In one of these studies, $ApoE^{-/-}$ mice were reconstituted with CCL5-deficient but $ApoE$ sufficient bone marrow, and the control group consisted of both CCL5- and $ApoE$-sufficient bone marrow. The fact that $ApoE^{+/+}$ bone marrow was used complicated the interpretation of the results because the lesions were dramatically reduced in the control group.
1.9 Hypothesis and aims

We hypothesize that both myeloid and endothelial cells produce CCL5 in early atherosclerotic lesions, and that these sources of CCL5 are functionally distinct.

The aims of my project are:

1) To identify the source(s) of CCL5 in early atherosclerotic lesions

2) To elucidate the role of myeloid cell and endothelial cell-derived CCL5 in early lesion development.
Figure 3: Blocking CCR5 with monoclonal antibody MC-68 inhibits monocyte recruitment into the lesser curvature of the ascending aortic arch in 3-week lesions.

Schematic showing the experimental protocol (A). Experiment: MC-68 (50 ug/mouse) or IgG2b isotype control was injected intravenously 3 hours after BrdU administration and analysis was performed at 24h. Control: Baseline intimal cell proliferation level was determined by 3 hour BrdU pulse. Quantification of CD45<sup>+</sup> BrdU<sup>+</sup> nuclei in the lesser curvature (LC) of the ascending aortic arch in Ldlr<sup>-/-</sup> mice fed cholesterol rich diet (CRD) for 3 weeks after BrdU pulse labeling (B). Values expressed as mean ± SEM; n=6-8 mice per group. Significant differences in cells in the lesser curvature are indicated * P < 0.05, ** P < 0.01, *** P < 0.001; One-way ANOVA and Tukey’s post-hoc test were performed (Allan Siu, unpublished data).
Figure 4: CRD induces intimal expression of the chemokines and adhesion molecules.

Ccl3, Ccl4, Ccl5, Cx3cl1, Vcam-1, and Icam-1 mRNA expression was measured by RT real-time q-PCR in the lesser curvature (LC) of the ascending aortic arch in Ldlr⁻/⁻ mice fed a standard chow diet (SCD) or cholesterol rich diet (CRD) for 3 weeks. Values represent mean ± SEM normalized to VE-Cadherin and relative to lesser curvature of SCD fed Ldlr⁻/⁻ mice, which was assigned a value of 1. Significant differences in mRNA expression in the lesser curvature between SCD (n=6) and CRD (n=7) fed Ldlr⁻/⁻ mice are indicated; ** P < 0.01; Student’s t-test was performed. (Allan Siu, unpublished data).
Figure 5: Deletion of CD11c+ cells reduced chemokine mRNA level in the ascending aortic arch of CD11c-DTR Ldlr−/− BM chimeras injected with Diptheria Toxin (DT).

Intimal mRNA expression of chemokines (Ccl3, Ccl4, Ccl5, Cx3cl1, Cxcl1) and adhesion molecules (Vcam1 and Icam1) was quantified by real-time PCR in the lesser curvatures (LC) of the ascending aortic arches in Ldlr−/− BM chimeras reconstituted with WT (n=11) or CD11c-DTR (n=9) BM treated with DT and fed CRD for 3 weeks. Values represent mean ± SEM normalized to endothelial VE-Cadherin expression and relative to Ldlr−/− mice reconstituted with WT BM fed a standard chow diet (SCD). Significant differences in mRNA expression in the lesser curvature between WT and CD11c-deleted Ldlr−/− mice are indicated * P < 0.05, ** P < 0.01; Student’s t-test was performed. (Allan Siu, unpublished data).
Figure 6: Potential roles of CCL5 in early atherosclerotic lesion formation.

Production of CCL5 by macrophage foam cell (A). Potential signaling/communication and activation of endothelial cells (B) (upregulation of chemokine and adhesion molecule expression). Potential transport of chemokines to the endothelial surface and presentation on proteoglycans (C). Potential autocrine loop or survival cue for myeloid cells (D). Potential source of endothelial-derived CCL5 presented on endothelial surface for monocyte recruitment (E). Platelet-derived CCL5 deposited onto activated endothelium during the recruitment of leukocytes (F) as described by Weber et al.96
2 Methods

2.1 Mice

Male and female C57BL/6J, \( Ldlr^{+/-} \), and \( Ccl5^{-/-} \) strains of mice from Jackson laboratories were used between 6 weeks and 12 weeks of age. \( Ccl5^{-/-} \) mice from Jackson Laboratories were bred into the \( Ldlr^{-/-} \) background and then further bred via a heterozygous intercross to generate heterozygote littermates. \( Ccl5 Ldlr \) double knockout mice were kept in the hemizygous state for breeding, and litters were genotyped by PCR and gel electrophoresis using the following primer set to \( Ccl5 \): wildtype 5'- ATG CAT CTC CCA CAG CCT CT-3'; mutant reverse 5'- TGG ATG TGG AAT GTG TGC GAG-3'; common 5'-TTGGAA AGA AGG GGA GGT CT-3'. \( Ccl5^{-/-} \) \( Ldlr^{-/-} \) and \( Ccl5^{-/-} Ldlr^{-/-} \) littermates were used for experiments. Mouse colonies were maintained in a pathogen-free environment at the University Health Network animal facility with 12h light-dark cycles. Mice were fed a standard rodent chow. A cholesterol rich diet (CRD) consisting of 1.25% cholesterol and 40% kcal fat (Lichtman et al., 1999) (Research Diets, diet D12108) was used to initiate atherosclerotic lesion formation in \( Ldlr^{-/-} \) mice. All protocols were performed in adherence to the guidelines of the Canadian Council of Animal Care.

2.2 Isolation of Aortic Arch

Mice were sacrificed with isofluorane overdose and were perfused through the left ventricle with 1X PBS followed by 4% paraformaldehyde (PFA) at 1.5 psi. Aortas were removed and fixed in 4% PFA for 1 hour prior to removing surrounding fat tissue.
2.3 Staining Ascending Aortic Arch

The ascending aortic arch was dissected and fixed in 4% PFA for 45 minutes after isolation. Aortic arches were permeabilized in 0.5% Triton X-100 in PBS for 15 minutes. Endogenous catalases and peroxidases were quenched with 3% hydrogen peroxide in PBS. Aortas were treated with DNase I for 1.5 hours at 37°C to cleave DNA for BrdU staining. Primary antibodies used included Alexa647 conjugated anti-mouse CD45 (2 µg/mL clone 30-F11; BioLegend) and biotin conjugated anti-BrdU (4 µg/mL clone Bu20a; BioLegend) and were incubated overnight at 4°C. TSA™ Fluorescein System –Tyramide Signal Amplification kit (Perkin Elmer) was used for immunostaining with biotin conjugated antibodies. Aortas were incubated with streptavidin-conjugated horseradish peroxidase for 20 minutes and FITC-conjugated tyramide reagent for 7 minutes (Perkin Elmer). Lipid was stained with neutral lipid stain Nile Red 5 µg/mL (Sigma) for 30 minutes. Nuclei were counterstained with 10 µg/mL HOECHST 33342 (Invitrogen) for 30 minutes. Aortas were opened as shown in Figure 1. Aortas were flattened on slides and mounted with DAKO fluorescence mounting medium to allow for en face visualization.

2.4 Confocal Microscopy

Aortas were visualized en face and tiled images spanning the entire ascending aortic arch were obtained using a 40x oil objective (NA1.3) on an Olympus FluoView 1000 Laser Scanning Confocal microscope with 405, 488, 543 and 633 nm lasers and Olympus FV10-ASW acquisition software. Acquired images were compiled in Adobe Photoshop software, version CS3. Later experiments in the project utilized Nikon A1R Resonance Scanning Confocal using a 40x oil objective where tiling images was automated and compiled using Nikon NIS Elements Version 4.51 acquisition and analysis software. BrdU+ nuclei of CD45+ leukocytes were counted.
Nile Red-positive area and CD45-positive areas were determined as percentage of total aorta using Image J software version 1.51f.

2.5 Isolating Intimal cell mRNA from Ascending Aortic Arch

Mice were perfused with 1X PBS in a 10mL syringe. The heart and aorta were dissected and immediately placed on a dish containing 1mM Aurintricarboxylic acid (ATA) RNase inhibitor in PBS to isolate the ascending aortic arch. The aortic arch was dissected, opened, and pinned down for cell isolation. 100 μL of 25 µg/mL liberase blenzyme (Roche) was added to luminal surface of the lesser curvature and incubated for 10 minutes at 37°C. The digestion of extracellular matrix and loosening of endothelial cells was terminated by rinsing with 1mM ATA. 2μL of 0.1M FITC beads (Polysciences) was added to the luminal surface to assist in visualizing the endothelial cell surface during intimal cell harvesting and collection. Intimal cells were collected by gentle scraping with a 30 gauge needle and were directly transferred into RLT lysis buffer from the RNeasy micro kit (Qiagen). Total RNA was isolated following the manufacturer’s protocol. A single aorta was used for each isolation.

2.6 RT Real-time qPCR

Reverse transcription of mRNA was completed using random primers and the High Capacity cDNA reverse transcription kit (Invitrogen). mRNA levels were quantified by real-time PCR using Lightcycler 480 SYBR Green I Master Mix and a Roche Lightcycler 480. Quantitative PCR cycle settings used were: 95°C for 5 minutes, followed by 45 cycles of 95°C for 10 seconds, 60°C for 20 seconds and 72°C for 30 seconds. Melt curve analysis was carried out at the end of 45 cycles to determine product purity. Standard curves were generated for each primer
pair to determine primer efficiency (Table 2) with 10 fold serial dilution of reference mRNA prepared from heart, lung and liver or lymph node of C57BL/6 mouse injected with 10 μg of LPS. Quantification of relative amounts of mRNA was performed by comparative standard curve method and normalized to endothelial-specific gene, \(VE-Cadherin\).

2.7 Bone Marrow Cell Isolation

Both femurs and tibias were isolated from donor mice, C57BL/6J (WT) or \(Ccl5^{-/-}\), and flushed with 10mL RPMI with 10% FBS and 1% Penicillin/Streptomycin. The bone marrow (BM) cell suspension was filtered through a 40μm pore nylon cell strainer to remove debris and bone fragments and pelleted by centrifugation at 1000 rpm for 10 minutes. The cell pellet was resuspended in RPMI at 5 x 10^6 cells for each 300 uL injection.

2.8 Bone Marrow Reconstitution

6-week-old recipient \(Ldlr^{-/-}\) mice were irradiated with 10 Grays of gamma irradiation using a cesium 137 radioactive source. 5 x 10^6 BM cells from either C57BL/6J (WT) or \(Ccl5^{-/-}\) donors were injected intravenously through the tail vein of the recipient mice the same day as their irradiation to reconstitute hematopoietic cells for studies of hematopoietic \(Ccl5\). Extent of reconstitution was determined via qPCR of blood. Reconstituted mice were left to recover for 6 weeks before the initiation of cholesterol rich diet. In order to study non-hematopoietic \(Ccl5\), WT BM donors were injected to irradiated \(Ccl5^{-/-}Ldlr^{-/-}\) or \(Ccl5^{+/+}Ldlr^{-/-}\) recipients.
2.9 BrdU Pulse Labelling

As described previously\textsuperscript{32}, 2mg of BrdU in 200uL PBS was injected intravenously via mouse tail vein, 3 hours or 24 hours prior to sacrifice to pulse label proliferating and proliferating plus recruited cells respectively.

2.10 Immunostaining Frozen Aorta Sections

Mice were perfused with 1X PBS followed by 4% PFA at 1.5 psi for 2 minutes. The aortic arch was carefully isolated, immediately embedded into OCT, and frozen over dry ice for 30 minutes before storing in -80°C. 10µm cross sections of ascending aortic arch were cut using a Leica CM3050 S cryostat. Serial aortic sections from each mouse were stained with rat anti-mouse CD31 (BD Pharmigen, clone MEC 13.3), Alexa647-conjugated anti-mouse CD68 (BioLegend, clone FA-11), and polyclonal goat anti-mouse CCL5 (R&D systems, Cat. AF478). Cy3-conjugated donkey anti-rat IgG (Jackson Immunoresearch,) was used as secondary staining for CD31. Biotin-SP-conjugated donkey anti-goat IgG followed by streptavidin-conjugated horseradish peroxidase and FITC-conjugated tyramide reagent (Perkin Elmer) were used for amplifying CCL5 signal.

2.11 Statistics

Statistical analysis was conducted using Prism Software version 6.0. Statistical tests included one-way analysis of variance (ANOVA) with Tukey’s post-hoc test, two-way ANOVA with Tukey’s multiple comparisons tests, and unpaired two-tailed Student’s t test.
Table 1: Stage of atherosclerotic lesion formation in $Ldlr^{-/-}$ mice

<table>
<thead>
<tr>
<th>Stage</th>
<th>Description</th>
<th>Duration of CRD (weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Nascent lesions</td>
<td>Uptake of lipid by resident intimal myeloid cells</td>
<td>&lt;1</td>
</tr>
<tr>
<td>2. Early lesions</td>
<td>Composed predominantly of myeloid foam cells, with minimal intimal smooth muscle cells (SMCs) and no fibrous cap or necrotic regions. Lesions grow laterally (increased lesion surface area) and vertically</td>
<td>≤12</td>
</tr>
<tr>
<td>3. Complex lesions</td>
<td>Atheromatous core composed of macrophage foam cells extracellular lipid, cholesterol clefts, necrotic cell debris, foci of calcification, and a fibrous cap composed of intimal SMCs and matrix. Lesions grow vertically.</td>
<td>&gt;12</td>
</tr>
<tr>
<td>4. Advanced hemorrhagic lesions</td>
<td>Complex lesions with hemorrhage (in the innominate artery of old $ApoE^{-/-}$ mice)</td>
<td>&gt;40</td>
</tr>
</tbody>
</table>
Table 2: Real-time PCR primers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
<th>Efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>VE-Cadherin</td>
<td>Forward</td>
<td>GAAAACCAGAAGAAACCGCTGAT</td>
<td>94.20%</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CACTGGTCTTTGCGGATGGA</td>
<td></td>
</tr>
<tr>
<td>E-selectin</td>
<td>Forward</td>
<td>GAAACAAAGACTCGGGCATGT</td>
<td>95.50%</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>ATGACCACTGCAGGATGCATT</td>
<td></td>
</tr>
<tr>
<td>Icam1</td>
<td>Forward</td>
<td>CTGCCCTTGGTAGAGGTGACTGA</td>
<td>97.89%</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>AGGACAGGAGCTGAAAAAGTTGTAGA</td>
<td></td>
</tr>
<tr>
<td>Vcam1</td>
<td>Forward</td>
<td>GCACACAAAGAAGGCTTTGAAGCA</td>
<td>98.84%</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GATTGGAGCAATCGTTTTGTATTC AG</td>
<td></td>
</tr>
<tr>
<td>Cd45</td>
<td>Forward</td>
<td>TCCACGCGTATTTCAGCAAGTT</td>
<td>97.00%</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>ACACGGTTTATAATCATAGGGAAGAATG</td>
<td></td>
</tr>
<tr>
<td>Cd11c</td>
<td>Forward</td>
<td>CAGTGGGCTTTTGCTCATCGCCTGA</td>
<td>90.40%</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>AGCCCTGGGATCCAGATGCTTC</td>
<td></td>
</tr>
<tr>
<td>Cd68</td>
<td>Forward</td>
<td>AGCTGCGCTGACAAGGGACACT</td>
<td>100.3%</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>AGGAGGACCAGGCCAATGAT</td>
<td></td>
</tr>
<tr>
<td>Cx3cl1</td>
<td>Forward</td>
<td>CAGTGGGCTTTTGCTCATCCGGCTGA</td>
<td>96.69%</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>AGCCTGGGTGATCCAGATGCTTC</td>
<td></td>
</tr>
<tr>
<td>Ccl2</td>
<td>Forward</td>
<td>GTCCCTGTCATGCTTTCTGG</td>
<td>92.40%</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>ATTGGGATCATCTTTGCTGG</td>
<td></td>
</tr>
<tr>
<td>Ccl3</td>
<td>Forward</td>
<td>ACTGACCCTGCTCGCTCTCTCTACAA</td>
<td>100.90%</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>ATGACACCTGGCCTGGAGCAAA</td>
<td></td>
</tr>
<tr>
<td>Ccl4</td>
<td>Forward</td>
<td>ACCCTCCCACCTCTTGCTGTGTT</td>
<td>98.00%</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CTGTCTGCCTCTTTGTTGTCAGG</td>
<td></td>
</tr>
<tr>
<td>Ccl5</td>
<td>Forward</td>
<td>TGCAGAGGACTCTGAGACAGC</td>
<td>92.05%</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>AGAGGTAGGCAAGCAGCAG</td>
<td></td>
</tr>
</tbody>
</table>

Table 2: Primer pairs were designed to target two separate exons spanned by an intron for the indicated genes according to mRNA sequences. Efficiencies were determined by serial dilution of mRNA isolated from heart, lung and lymph node of C57BL6 mice stimulated with 10 µg of LPS.
3 Results

Previous data from our laboratory indicated that CCL5 mRNA expression is significantly upregulated locally within early lesions (Figure 4) and that antibody blockade of CCR5 reduces monocyte recruitment in early lesions (Figure 3). This suggests that CCL5 signaling through CCR5 may be a critical component in facilitating monocyte recruitment in early lesion development.

We undertook to identify the importance of CCL5 (one of the CCR5 ligands) in early atherosclerotic lesion formation. Our first goal was to identify the local production of CCL5 during atherogenesis in the Ldlr\(^{-}\) mouse model in hematopoietic and non-hematopoietic cells. Ldlr\(^{-}\) mice were irradiated and reconstituted with CCL5 deficient BM. Following recovery, mice were fed a cholesterol rich diet (CRD) for either 3-weeks or 6-weeks to induce atherosclerosis lesion development. mRNA expression and protein immunostaining was used to identify the sources of CCL5 in early atherosclerosis. In addition, lesion parameters were quantified and analyzed to identify the role of CCL5 in early atherosclerosis.
3.1 Ccl5 is expressed by hematopoietic and non-hematopoietic cells within early lesions

The mRNA expression of endothelial cell molecules (VCAM-1, ICAM-1, and E-Selectin) and chemokines (CCL5, CX3CL1, and CCL2) was quantified in the lesser curvature of the ascending aortic arch of Ldlr<sup>−/−</sup> BM chimeras reconstituted with either WT or CCL5-deficient BM and fed a CRD for 3 weeks. There were no significant differences in the mRNA expression of endothelial cell adhesion molecules Vcam1, Icam1, E-selectin as well as chemokines Cx3cl1 and Ccl2 between WT and CCL5-deficient chimeric mice (Figure 7) indicating that hematopoietic CCL5 deficiency has no effect on the expression of these genes. However, Ccl5 mRNA expression was significantly reduced in mice that were reconstituted with CCL5 deficient BM, but not completely absent. After ensuring that incomplete BM chimerism did not account for the remaining CCL5 expression via RT-PCR of whole blood, our data suggest that the majority of CCL5 expression is hematopoietic-derived and that the residual Ccl5 mRNA expression is derived from a non-hematopoietic source.

The expression of CCL5 protein was investigated in atherosclerotic lesions in Ldlr<sup>−/−</sup> and Ccl5<sup>−/−</sup> Ldlr<sup>−/−</sup> double knock out (DKO) mice fed a CRD for 12 weeks. This time point was selected in order to reproducibly obtain lesions of adequate size for immunofluorescent staining of frozen cross-sections of the ascending aortic arch. Sections were stained with a polyclonal goat anti-mouse CCL5 antibody. Fluorescently-labelled anti-CD31, anti-CD68 were also used to visualize the co-localization of CCL5 protein with endothelial and myeloid cell type-specific markers, respectively, within the plaque. CCL5 protein was found in 12-week lesions of Ldlr<sup>−/−</sup> but not Ccl5<sup>−/−</sup> Ldlr<sup>−/−</sup> mice, confirming the specificity of the antibody. CCL5 co-localized not only with CD68 within the intima of the arterial wall, but was also found on the surface of the endothelium.
(Figure 8). Immunostaining of chimeric \( Ldlr^{-/-} \) and \( Ccl5^{-/-} Ldlr^{-/-} \) mice will be completed to confirm endothelial source of CCL5.
Figure 7. *Ccl5* is expressed by hematopoietic and non-hematopoietic cells in early lesions.

Expression of chemokine (*Cx3cl1, Ccl2, and Ccl5, n=14 per group*) and endothelial adhesion molecule (*Vcam1, Icam-1, and E-selectin, n=7 per group*) mRNA in micro-dissected intimal lesions located in the lesser curvature of the ascending aortic arch. *Ldlr*<sup>−/−</sup> chimeric mice reconstituted with either WT (black) or CCL5 knockout (grey) bone marrow and fed a CRD for 3 weeks. All mRNA expression values (mean ± SEM) were normalized to *VE-Cadherin* and are shown relative to the expression in the corresponding WT BM chimeras assigned a value of 1. Significant differences in mRNA expression are indicated; * P < 0.05; Student’s t test was performed.
Figure 8. CCL5 is co-localized with hematopoietic and non-hematopoietic cells within 12-week lesions.

Confocal microscopy images (40X) of a representative ascending aortic arch cross section after immunostaining with antibodies against CCL5 (green), endothelial cell marker CD31(red), and myeloid cell marker CD68 (magenta). White arrows show co-localization of CCL5 and endothelial CD31 (yellow) and co-localization of CCL5 and myeloid CD68 (white). Nuclei were stained with Hoechst 33342 (blue). Asterisks indicate location of lumen. Lack of CCL5 staining in Ccl5−/−Ldlr−/− mice shows the specificity of anti-CCL5 antibody. Representative image of 3 experiments.
3.2 Hematopoietic CCL5 deficiency did not affect lipid area but reduced CD45⁺ area in 3-week lesions

The effect of CCL5 deficiency on hematopoietic cell content and on lipid accumulation in lesions of the ascending aortic arch was assessed after 3-weeks of CRD in Ldlr⁻/⁻ chimeras reconstituted with either WT or CCL5 deficient BM. Ascending aortic arches were stained with Nile Red, a neutral lipid stain, imaged en face by confocal microscopy, and the area of Nile Red positive pixels was quantified with Image J. This experiment revealed comparable Nile Red-positive areas in intimal lesions of WT and CCL5 deficient BM chimeras (Figure 9A). Similar analysis of CD45-positive pixels revealed a significant reduction in CCL5 deficient BM chimeras (Figure 9B), suggesting that hematopoietic CCL5 deficiency may influence either CD45 expression or leukocyte accumulation at this early stage. This observation was unexpected, because the majority of lipid in early lesions accumulates within intimal myeloid cells; therefore, one would expect a direct correlation between the accumulation of intimal leukocytes and lipid.
Figure 9. Hematopoietic CCL5 deficiency significantly reduced lesion CD45⁺ leukocyte area in 3-week lesions.

Lesions in chimeric Ldlr⁻/⁻ mice reconstituted with WT or CCL5 deficient BM fed 3-week CRD were analyzed for Nile red lipid area (A) (n=11-13 per genotype), CD45⁺ leukocyte area (B) (n=10-11 per genotype), and BrdU⁺ CD45⁺ nuclei at 3h and 24h post BrdU injection (C) (n=6-8 per group). Number of pixels positive for Nile Red or CD45 were normalized to the total number of pixels for each aorta then presented relative to WT chimera. Values represented as mean ± SEM. Student’s t-test was performed; * P < 0.05; ns=no significance.
3.3 Hematopoietic CCL5 deficiency did not affect leukocyte proliferation in 3-week lesions

The abundance of myeloid cells in lesions is dependent on local cell proliferation and the recruitment of blood monocytes. The rate of leukocyte proliferation in lesions was quantified 3 hours after intravenous injection of BrdU. Injected BrdU has a very short half-life and is cleared from the blood rapidly; thus, a single injection of BrdU can be considered as pulse labeling of cells in S phase of the cell cycle. At the 3-hour time point labeled leukocytes are not found in the circulation. This means that BrdU-labeled cells in the aorta at 3 hours represent cells that were labeled in situ and underwent mitotic cell division. BrdU also labels classical (Ly-6C$^{\text{high}}$) monocyte precursors in the bone marrow. Classical monocytes mature rapidly, are released into the blood after 4 to 6 hours and peak of BrdU$^+$ Ly-6C$^{\text{hi}}$ monocytes in the blood (40 to 50% of circulating classical monocytes) is detected between 12 and 24 hours. We assessed BrdU$^+$ cells in lesions at 24 hours after pulse labeling. At this time point, BrdU$^+$ cells consist of locally labeled cells as well as recruited classical monocytes. We estimated the number of recruited monocytes by subtracting the 3 hour value from the 24 hour.

BrdU$^+$ CD45$^+$ leukocytes were assessed in 3-week intimal lesions at 3 and 24 hours after BrdU injection. There was a comparable number of BrdU$^+$CD45$^+$ cells at 3h post BrdU injection in WT and CCL5 KO chimeras, suggesting no difference in the rate of local intimal leukocyte proliferation (Figure 9C). We concluded that hematopoietic cell-derived CCL5 does not influence leukocyte proliferation in 3-week lesions. At 24 hours post-BrdU injection, the number of BrdU$^+$ cells was increased modestly in WT chimeras, but this increase was not significant. These data suggest that the recruitment of classical monocytes is very low in 3-week lesions in radiation chimeras. This finding is distinct from previous data from our laboratory showing
robust monocyte recruitment to 2- and 3-week lesions in non-irradiated Ldlr<sup>-/-</sup> mice.<sup>24</sup> At 24 hours after BrdU pulse labeling, the number of BrdU<sup>+</sup> CD45<sup>+</sup> leukocytes was not different in CCL5 deficient and control WT BM chimeras. These data are consistent with comparable leukocyte proliferation in lesions (3 hour post-BrdU data) and very low monocyte recruitment at this early stage of lesion formation. In light of these findings, we decided to determine if monocyte recruitment is increased in 6-week lesions of chimeric mice and if so, determine the role of hematopoietic CCL5.
3.4 Hematopoietic CCL5 deficiency did not affect lipid area and myeloid cell area in 6-week lesions

The effect of hematopoietic CCL5 deficiency on lipid accumulation and myeloid cell accumulation in early lesions was assessed after 6-weeks of CRD in the lesser curvature of the ascending aortic arch of Ldlr⁻/⁻ chimeras reconstituted with either WT or CCL5 deficient BM. Nile Red positive lipid area was increased 3-fold, and CD45 positive area was approximately increased 2-fold in 6-week lesions compared to 3-week lesions in both WT and CCL5 deficient BM chimeras (Figure 10 A,B). This confirms that with increased duration of the CRD, lesion surface area increases. However, significant differences in areas of Nile Red-stained lipid or CD45⁺ cells were not observed when comparing WT or CCL5-deficient BM chimeras (Figure 11 A, B). These data suggest that in 6-week lesions, myeloid cell abundance is comparable in WT and CCL5-deficient BM chimeras. Since CD45⁺ area was reduced in CCL5-deficient BM chimeras at 3-weeks post CRD, our 6-week lesion data suggest that between 3 and 6 weeks of CRD the abundance of myeloid cells in lesions increases at a comparable or slightly higher rate in mice with hematopoietic CCL5 deficiency. Overall, our data indicate that hematopoietic CCL5 has no significant effect on the accumulation of lipid and myeloid cells in 6-week atherosclerotic lesions.
Figure 10. Lesion Nile Red$^+$ lipid and CD45$^+$ leukocyte area is significantly increased between 3-week and 6-week lesions.

Quantification of Nile Red$^+$ lipid area (A) (n=9-13) and CD45$^+$ leukocyte area (B) (n=7-10) of lesions in Ldlr$^{-/-}$ mice reconstituted with WT or Ccl5$^{-/-}$ BM and fed 3 and 6 weeks of CRD.

Number of pixels positive for Nile Red or CD45 were normalized to the total number of pixels for each ascending aorta. Values presented as mean ± SEM. Two-way ANOVA and Tukey’s multiple comparisons tests were performed; * $P < 0.05$, **** $P < 0.0001$. 
Figure 11. Hematopoietic CCL5 deficiency has no effect on Nile Red$^+$ lipid area, CD45$^+$ leukocyte area, or monocyte proliferation and recruitment in 6-week early lesions.

Quantification of Nile red$^+$ lipid area (A) (n=7-9 per genotype), CD45$^+$ leukocyte area (B) (n=7-9 per genotype), and BrdU$^+$/CD45$^+$ nuclei at 3h (n=2 per group) and 24h (n=3-4 per group) post BrdU injection (C). Number of pixels positive for Nile Red or CD45 were normalized to the total number of pixels for each aorta then presented relative to WT chimera. Values represent mean ± SEM Student’s t-test was performed * $P < 0.05$, ** $P < 0.01$; ns=no significance.
3.5 Hematopoietic CCL5 deficiency did not affect leukocyte proliferation or recruitment in 6-week lesions

The rate of leukocyte proliferation was quantified and analyzed by counting the BrdU$^+$ CD45$^+$ nuclei within the lesion in the lesser curvature of the ascending aortic arch 3 hours after BrdU pulse labeling. Similar to our data from 3-week lesions, the number of BrdU$^+$CD45$^+$ nuclei was comparable in CCL5-deficient and WT chimeras, suggesting that hematopoietic deficiency of CCL5 did not affect the rate of leukocyte proliferation in 6-week lesions (Figure 11C).

At 24 hours post-BrdU pulse labeling, the number of BrdU$^+$ cells was increased dramatically in WT chimeras (Figure 11C). These data suggest that the recruitment of classical monocytes is robust in 6-week lesions of radiation chimeras, unlike the 3-week time point. This finding suggests that enhanced monocyte recruitment is delayed in radiation chimeras as compared to non-irradiated Ldlr$^{-/-}$ mice. The number of CD45$^+$ BrdU$^+$ nuclei at 24h, was comparable between WT and CCL5-deficient BM chimeras, suggesting that hematopoietic CCL5 deficiency did not affect classical monocyte recruitment in 6-week lesions, possibly implicating a redundant or compensatory mechanism.
3.6 Hematopoietic CCL5 deficiency reduced Ccl3 and myeloid cell marker mRNA expression in 3-week lesions

RNA was isolated from microdissected lesions in the lesser curvature of the ascending aortic arch and reverse transcribed into cDNA for analysis by quantitative PCR. mRNA expression of chemokines (CCL3, CCL4, and CCL5) and leukocyte/myeloid cell markers (CD45, CD68, CD11c) was analyzed by qPCR with the primers listed in Table 2. As expected, the mRNA expression of Ccl5 was significantly reduced but not completely ablated, confirming our previous results indicating that CCL5 is produced in lesions mostly by hematopoietic cells. Moreover, hematopoietic Ccl5 deficiency also significantly reduced the mRNA expression of Ccl3 but not Ccl4 (Figure 12A). Whether hematopoietic CCL5 deficiency is directly influencing the expression of Ccl3 expression is unclear because mRNA expression of Cd45, Cd11c, and Cd68 in 3-week lesions was also significantly reduced (Figure 12B). A reduction of lesion leukocyte and myeloid cell mRNA markers is consistent with reduced CD45+ surface area in 3-week lesions (Figure 9B), and supports the conclusion that hematopoietic CCL5 deficiency reduced myeloid cell abundance in 3-weeks lesions.
Figure 12. Hematopoietic CCL5 deficiency reduced mRNA expression of chemokines and myeloid cell markers in 3-week lesions.

Relative mRNA expression of chemokines and myeloid cell markers in the LC intima of chimeric Ldlr−/− mice reconstituted with WT or CCL5 deficient BM fed 3-weeks CRD: mRNA expression of chemokines (A) and myeloid cell markers (B) (n=16-17 per genotype) normalized to endothelial VE-Cadherin expression levels and presented relative to WT chimera fed 3 weeks CRD. Values presented as mean ± SEM. Student’s t-test was performed; * P < 0.05, ** P < 0.01; ns=no significance.
3.7 Hematopoietic CCL5 deficiency did not reduce myeloid cell marker mRNA expression in 6-week lesions

The expression of chemokines (CCL3, CCL4, and CCL5) and myeloid cell markers (CD45, CD68, CD11c) was analyzed in 6-week lesions. The expression of Ccl5 mRNA was substantially reduced in CCL5 deficient BM chimeras, but ~30% residual CCL5 expression remained (Figure 13A), consistent with data from 3-week lesions (Figure 12A). This supports our earlier observations that non-hematopoietic cells in the lesion also produce CCL5. The expression of Ccl3 mRNA remained significantly reduced in CCL5 deficient chimeras (Figure 13A), while Ccl4 mRNA expression in CCL5 deficient chimeras remained comparable to WT. The expression of leukocyte/myeloid cell marker mRNAs was comparable between CCL5 deficient and WT chimeras in 6-week lesions (Figure 13B). These data are consistent with comparable CD45+ surface area in 3-week lesions (Figure 11B) and supports comparable abundance of myeloid cells. The fact that Ccl3 mRNA was significantly reduced in CCL5 deficient chimeras in spite of comparable myeloid cells suggests that deficiency of Ccl5 in hematopoietic cells influences Ccl3 expression in atherosclerotic lesions. Yet in spite of this, intimal leukocyte proliferation and monocyte recruitment were comparable (Figure 11C).
Figure 13. Hematopoietic CCL5 deficiency did not significantly affect mRNA expression of myeloid cell markers in 6-week lesions.

Relative mRNA expression of chemokines and myeloid cell markers in the LC intima of chimeric Ldlr<sup>−/−</sup> mice reconstituted with WT or CCL5 deficient BM fed 6-weeks CRD. mRNA expression of chemokines (A) or myeloid cell markers (B) (n=9-11 per genotype) normalized to endothelial VE-Cadherin expression are relative to WT chimera fed 6-weeks CRD. Mean ± SEM. Student’s t-test was performed; * P < 0.05 **** P < 0.0001.
3.8 Non-hematopoietic CCL5 deficiency did not affect lipid area or myeloid cell accumulation in 3-week lesions

*Ldlr<sup>−/−</sup> or Ccl5<sup>−/−</sup> Ldlr<sup>−/−</sup> mice that were reconstituted with WT BM were used to investigate the role of non-hematopoietic CCL5 in lesion development. After 3 weeks of CRD, lesion lipid area and myeloid cell abundance was analyzed within the ascending aortic arch. Nile Red lipid area was comparable between both control WT→Ldlr<sup>−/−</sup> and WT→Ccl5<sup>−/−</sup>Ldlr<sup>−/−</sup> groups, indicating that non-hematopoietic CCL5 deficiency does not affect lesion lipid area (Figure 14A). Moreover, CD45 positive area was also comparable between both control WT→Ldlr<sup>−/−</sup> and WT→Ccl5<sup>−/−</sup>Ldlr<sup>−/−</sup> groups after 3-weeks of CRD (Figure 14B). These data indicate that deficiency of CCL5 in non-hematopoietic cells does not affect lipid or myeloid cell accumulation in early atherosclerosis.
Figure 14. Non-hematopoietic CCL5 deficiency does not affect Nile Red$^+$ lipid area or CD45$^+$ leukocyte area in 3-week lesions.

Lesion analysis of chimeric $Ldlr^{-/-}$ mice deficient in non-hematopoietic CCL5 fed for 3 weeks CRD. Quantification of Nile red$^+$ lipid area (A) (n=10) and CD45$^+$ leukocyte area (B) (n=5) normalized to total ascending aortic arch area and presented relative to WT chimera. Student’s t-test was performed; ns=no significance between groups.
3.9 Non-hematopoietic CCL5 deficiency did not significantly affect expression of chemokines and myeloid cell markers in 3-week lesions

The expression of CCL3, CCL4, and CCL5 as well as CD45, CD68, CD11c mRNAs were analyzed in Ldlr<sup>−/−</sup> or Cell5<sup>−/−</sup> Ldlr<sup>−/−</sup> mice reconstituted with WT BM by qPCR. Cell5 expression was not significantly reduced in chimeric lesions with CCL5 deficiency in non-hematopoietic cells (Figure 15A). This result agrees with our prior observation that hematopoietic CCL5 is the dominant source of Cell5 production in early lesions. Moreover, deficiency in non-hematopoietic CCL5 did not significantly affect expression of Cell3 or Cell4. The expression of CD45, CD68, CD11c mRNAs was not significantly different between non-hematopoietic CCL5 deficient and WT BM chimeric lesions (Figure 15B). Overall, non-hematopoietic CCL5 deficiency did not affect the expression of chemokines and myeloid cell markers in 3-week lesions.
Figure 15. Non-hematopoietic CCL5 deficiency did not significantly affect mRNA expression of chemokines Ccl3, Ccl4, Ccl5 and myeloid cell markers in 3-week lesions.

Relative mRNA expression of chemokines and myeloid cell markers in the lesser curvature (LC) of either Ldlr−/− or Ccl5−/−Ldlr−/− recipients reconstituted with WT BM and fed 3-weeks CRD. mRNA expression of chemokines (A) or myeloid cell markers (B) (n=5 per genotype) normalized to endothelial VE-Cadherin expression and are presented relative to WT chimera fed 3 weeks CRD. Values represented as mean ± SEM. Student’s t-test was performed; ns=no significance.
3.10 Complete CCL5 deficiency did not affect lipid or myeloid cell accumulation in 3-week lesions

*Ccl5<sup>-/-</sup> Ldlr<sup>-/-</sup>* double knockout mice were generated to assess the effects of global CCL5 deficiency. Lesions in the lesser curvature of the ascending aortic arch were compared and analyzed in *Ccl5<sup>+/+</sup>Ldlr<sup>-/-</sup>, Ccl5<sup>+-</sup>Ldlr<sup>-/-</sup>,* and *Ccl5<sup>-/-</sup>Ldlr<sup>-/-</sup>* mice fed a CRD for 3 weeks. There were no significant differences in lipid area regardless of *Ccl5* deficiency (Figure 16A). CD45<sup>+</sup> area was also comparable between all three groups (Figure 16B). These results suggest that a combined deficiency of CCL5 from hematopoietic and non-hematopoietic cells has no effect on lipid or myeloid cell accumulation in 3-week lesions.
Figure 16. Complete CCL5 deficiency does not affect Nile Red$^+$ lipid area or CD45$^+$ leukocyte area in 3-week lesions.

Lesion analysis in Ccl5$^{+/+}$/Ldlr$^{-/-}$, Ccl5$^{+/+}$/Ldlr$^{-/-}$, and Ccl5$^{-/-}$/Ldlr$^{-/-}$ mice fed 3-weeks CRD. Quantification of Nile Red$^+$ lipid area (A, n=6-14) and CD45$^+$ leukocyte area (B, n=8-9) normalized to total ascending aortic arch area of Ccl5$^{+/+}$/Ldlr$^{-/-}$ mice. One-way ANOVA performed showing no significance among groups.
3.11 Ccl4 expression is elevated in 3-week lesions with global CCL5 deficiency

The expression of CCL3, CCL4, and CCL5 as well as CD45, CD68, CD11c mRNAs was analyzed in Ccl5+/+ Ldlr−/+ and Ccl5−/+ Ldlr−/+ mice after being fed a CRD for 3 weeks. As expected, Ccl5 expression was not detectable in Ccl5−/+ Ldlr−/+ mice. This result confirmed the absence of functional CCL5 mRNA in Ccl5−/− mice. Ccl3 mRNA expression in lesions was comparable; however, a trend toward an increased Ccl4 mRNA expression was observed in Ccl5−/− Ldlr−/− lesions relative to Ccl5+/+ Ldlr−/+ lesions (Figure 17A), but this increase was not statistically significant (P=0.09). Nevertheless, elevated Ccl4 expression may compensate for the lack of CCL5 in 3-week lesions. The expression of CD45, CD68, CD11c mRNAs was comparable in Ccl5+/+ Ldlr−/+ and Ccl5−/− Ldlr−/− mice (Figure 17B), consistent with comparable CD45+ area (Figure 15B).
Figure 17. *Ccl4* expression is elevated in complete CCL5 deficient 3-week lesions.

Relative mRNA expression of chemokines and myeloid cell markers in *Ccl5*+/+ Ldlr−/- and *Ccl5*−/− Ldlr−/- mice fed for 3 weeks CRD. mRNA levels of chemokines (A) and myeloid cell markers (B) (n=8-11 per genotype) were normalized to endothelial *VE-Cadherin* expression and presented relative to *Ccl5*+/+ Ldlr−/- mice. Values represented as mean ± SEM; Student’s t-test was performed, ns=no significance.
4 Discussion

Chemokines and their receptors are critical effectors within the inflammatory environment of atherosclerotic lesions. Our laboratory has previously confirmed the role of CCR5 in the recruitment of leukocytes to early lesions (Figure 3). Among CCR5 ligands (CCL3, CCL4, and CCL5), CCL5 is the chemokine that has been most studied in the context of atherosclerosis. CCL5 has been previously associated with lesion progression in multiple studies	extsuperscript{96,97,99}; however, the mechanisms involved and the sources of CCL5 expression within the atherosclerotic lesion have not been investigated thoroughly. This study examines which cell type(s) within lesions produce CCL5 and their potential roles in early atherogenesis. Residual expression of CCL5 after deletion of CD11c\(^+\) cells or in chimeric mice reconstituted with CCL5-deficient BM indicated that there are two major sources of intimal CCL5 expression in early atherosclerotic lesions: hematopoietic-derived and non-hematopoietic endothelial cells, with the most abundant expression originating from the hematopoietic compartment, i.e., intimal myeloid cells (Figures 5 and 7). I have generated chimeric \textit{Ldlr}\(^{-/-}\) mice that are deficient in either hematopoietic-derived CCL5 or non-hematopoietic derived CCL5. Several parameters of lesion development were assessed at 3- or 6-weeks of CRD: lesion neutral lipid area, myeloid cell area, local proliferation, monocyte recruitment and gene expression.

Hematopoietic CCL5 was critical for lesion myeloid cell abundance after 3-weeks of CRD (Figures 9B and 12B), while differences in lesion lipid area were not observed at this time point. The intimal leukocyte proliferation rate was comparable in WT and CCL5-deficient chimeric mice and monocyte recruitment was minimal. Thus it is possible that other parameters, such as intimal myeloid cell apoptosis or reverse transendothelial migration, are increased in CCL5-deficient chimeric mice and account for reduced myeloid cell content. This remains to be
investigated. After 6 weeks of CRD, myeloid cell content was comparable in lesions of WT and hematopoietic CCL5 deficient chimeric mice (Figures 11B and 13B). Monocyte recruitment to lesions, which was detected only in 6-week lesions was not affected by CCL5 deficiency (Figure 9C and 11C). These data suggest that hematopoietic-derived CCL5 has a transient role at the very early stages of lesion development. However, the data presented in Appendix 1 indicate that lesions grow vertically at and beyond 6 weeks CRD. Thus, our data with mice fed 6 week CRD, which rely on en face assessment of lesion area may need to be verified by other approaches such as histological assessment of lesion cross-sections that provide more precise information related to lesion height and will be a subject for future studies.

The non-hematopoietic compartment of CCL5 expression in early lesions was identified as endothelial cells, based on immunostaining (Figure 8). Analysis of chimeric mice lacking CCL5 in endothelial cells showed that, unlike myeloid CCL5, endothelial CCL5 does not regulate the myeloid cell content in 3-week lesions (Figures 14B and 15B). These differences in the functions of the two compartments of CCL5 may derive from differential heterodimerization with other chemokines, which was previously described to yield distinct functions.\textsuperscript{118}

The function of hematopoietic CCL5 in maintaining myeloid cell abundance in 3-weeks lesions was not reproduced when there was a complete deficiency of CCL5 i.e., in both hematopoietic and non-hematopoietic compartments (Figure 16B and 17B). Chemokines are commonly redundant in their actions on target cells and often promiscuous in their receptor interactions. These characteristics confer a robustness to the chemokine/chemokine receptor system and allow for the proper performance by individual chemokines regardless of any functional perturbation. As mentioned previously, in addition to CCL5, CCL3 and CCL4 are also ligands for CCR5.\textsuperscript{6,7} Our laboratory showed that the expression of all three chemokines is elevated in early lesions
The expression of the *Ccl4* gene appeared to be upregulated in mice with complete CCL5 deficiency as compared to *Ldlr*−/− mice (Figure 17A) suggesting that a compensatory mechanism may be in place. Compensation for CCL5 deficiency by CCL4 has been observed previously in the context of bacterial infection in the lungs of CCL5 knockout mice. Vesosky *et al.* identified that CCL4 protein levels in mycobacterium tuberculosis infected lungs peaked earlier and achieved significantly higher levels in CCL5 knockout mice than in WT controls. This group concluded that the up-regulation of CCL4 may have partially compensated for the lack of CCL5 and recruited IFN-γ producing cells into the lungs of CCL5 knockout mice. Whether CCL4 compensation contributed to the lack of effect in complete CCL5 deficiency in myeloid cell content will be investigated in future experiments.

Parallel to our findings above, Kennedy *et al.* detected a moderate reduction in lesion area when *Ldlr*−/− mice were reconstituted with CCL3-deficient BM after being fed 6-weeks of Western Diet. Moreover, atherosclerotic lesions were not affected when CCL3 was globally deficient in *Ccl3*−/− *Ldlr*−/− double knockout mice. This group concluded that CCL3 potentially plays a role in lipid metabolism in bone marrow reconstituted hyperlipidemic *Ldlr*−/− mice; however, the mechanism for this is still unclear. Although the primary role of CCL5 is reported to be chemotaxis, it is possible that CCL5 plays a role in cell metabolism in atherosclerosis. CCL5 has been demonstrated to have a role in promoting glucose uptake and ATP production in T cells. In addition, CCL5 has been associated with lipid metabolism in breast cancer cells through activation of CCR5. These findings raise the question whether the deficiency of hematopoietic CCL5 could affect cellular metabolism of intimal myeloid cells in chimeric mice that have undergone BMT.
Since Ccl3, Ccl4 and Ccl5 expression are similarly increased in early atherosclerotic plaques (Figure 4) the transcription of these genes may be regulated by common mechanisms. Interestingly, Ccl3, Ccl4 and Ccl5 loci are closely located on Chromosome 11 in mice and Chromosome 17 in humans. The proximity of the loci of these three chemokines raises the possibility that they share common enhancers, promoters or other regulatory elements (Figure 2).

Indeed, it was previously shown by Chandrasekar et al. that Ccl3 and Ccl4 expression may be co-regulated in macrophages. Our data showed that Ccl3 expression in early lesions was significantly repressed in chimeric hematopoietic-derived CCL5-deficient mice (Figure 12A and 13A), suggesting that the expression of Ccl5 and Ccl3 in lesion myeloid cells is under the control of common regulatory mechanisms.

Our results describing a mild effect for CCL5 deficiency in early atherosclerosis was unexpected considering the bulk of literature emphasizing the role of CCL5 and CCR5 in atherosclerosis. It is important to note that other studies investigating CCL5 in atherosclerosis mostly utilized the ApoE−/− mouse model and/or studied advanced lesions. Although it is possible that CCL5 may not have a prominent function in early atherosclerosis as compared to later stages, different experimental conditions may also contribute to the discrepancy. For instance, the importance of platelet-derived CCL5 at 12-week high-fat diet was shown when BMT was performed with Ccl5−/− ApoE+/+ BM cells to reconstitute Ccl5+/+ ApoE−/− recipient mice, which would rescue the ApoE−/− phenotype. The hematopoietic CCL5-deficiency in Ldlr−/− mice that was studied here may also implicate platelet CCL5. Whether platelet CCL5 contributed to myeloid cell abundance at 3-week CRD remains to be determined.
5 Future Directions

This study showed a mild impact of CCL5-deficiency on myeloid cell content in early (3-week) atherogenesis when comparing chimeric \( Ldlr^{-/-} \) mice reconstituted with \( Ccl5^{-/-} \) BM to WT BM. In the appendix, we showed that BMT of WT BM into \( Ldlr^{-/-} \) mice resulted in lower levels \( Ccl5, Ccl4 \) and \( Ccl3 \) mRNA in 3-week lesions when compared to \( Ldlr^{-/-} \) mice without BMT (Figure F). Thus, it appears that in experiments interrogating hematopoietic CCL5, chimeric \( Ldlr^{-/-} \) mice with CCL5 KO BM were compared to the corresponding mice with WT BM, where the myeloid cell content would be reduced as a consequence of BMT. This may have resulted in smaller differences between the experimental groups. An alternative to BMT is to generate transgenic mice on \( Ldlr^{-/-} \) background that are deficient in CCR5 ligands in a cell type-specific manner. For example, mice can be generated where \( Ccl3, Ccl4, \) and \( Ccl5 \) genes will be flanked by LoxP sites and crossed with mice that express Cre recombinase under the control of a cell type-specific promoter. Due to the proximity of \( Ccl3, Ccl4, \) and \( Ccl5 \) genes it is possible to flank these three genes simultaneously with minimal disruption of other genes. Thereafter, crossing the floxed mouse with an endothelial expressing Cre, such as Tie2-Cre or myeloid-specific LysM Cre mice, would selectively delete \( Ccl3, Ccl4, \) and \( Ccl5 \) genes from these cells. Endothelial, myeloid, and platelet specific Cre/lox system will enable elucidation of function of these chemokines derived from specific cell types. While the same parameters that we assessed for lesion development will be analyzed, we expect a faster development of atherosclerotic lesions in the non-BMT mice; thus, 1 to 3-week lesions will be analyzed.

Function blocking antibodies against CCL3 and CCL4 administered together or independently into \( Ccl5^{-/-} Ldlr^{-/-} \) mice during atherosclerosis lesion development will evaluate the potential compensatory effects of CCL3 or CCL4. Continuous administration of the blocking antibodies
will be achieved by implantation of subcutaneous osmotic pumps. We will assess the same parameters of lesion development as we performed in this study after 1 to 3 weeks of CRD.

The chemokine CX3CL1 was shown to promote the survival of monocytes in addition to its role in chemotaxis. Since in 3-week lesions, macrophage content was affected by hematopoietic CCL5-deficiency without any effects on proliferation and with minimal recruitment at this time point, it is possible that CCL5 regulates intimal resident myeloid cell survival. The role CCL5 and CCR5 in macrophage survival has been shown in other contexts. CCL5 has a distinct role in the survival of macrophages in white adipose tissue (WAT). Furthermore, CCL5-CCR5 signaling was shown to activate PI3K-AKT and MEK-ERK signaling pathways and inhibit apoptosis of virus infected macrophages. The terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling (TUNEL) assay will be used to detect apoptotic cells that undergo extensive DNA degradation during the late stages of apoptosis. Previous data from the laboratory identified that the levels of apoptotic cells in early lesions of Ldlr<sup>−/−</sup> mice remains low (less than 10 TUNEL<sup>+</sup> cells per ascending aorta) until after a feeding CRD for 8 weeks (Figure 18). Hence, the role of CCL5 will be studied in Ccl5<sup>−/−</sup>Ldlr<sup>−/−</sup> mice or the LoxP/Cre mice described above.

Efforts are underway in the laboratory to physically isolate aortic endothelial cells and intimal myeloid cells. Once the methodology is in place, changes in the expression of Ccl3, Ccl4 and Ccl5 will be assessed in the two cell types isolated during early lesion development in Ldlr<sup>−/−</sup> mice. Moreover, upon deletion of these genes or function blocking with approaches described above, the impact on the expression of other pro-inflammatory genes will be assessed in order to gain insights on the functions of CCR5 ligands in the two different intimal cell types during early development of atherosclerotic lesions.
Figure 18. Apoptosis in myeloid foam cell lesions.

Apoptosis was assessed in myeloid foam cell lesions of Ldlr\textsuperscript{-/-} mice at different time points of CRD feeding. The number of TUNEL\textsuperscript{+} cells did not increase from baseline levels until after 8-weeks of CRD. Values represented as mean ± SD, n=4-8 mice, * P < 0.05, (Suning Zhu, unpublished data).
References


60. Galea, J. et al. Interleukin-1 beta in coronary arteries of patients with ischemic heart disease. 


113. RANTES/CCL5 and risk for coronary events: results from the MONICA/KORA Augsburg case-cohort, Athero-Express and CARDIoGRAM studies. - PubMed - NCBI. Available at: https://www.ncbi.nlm.nih.gov/myaccess.library.utoronto.ca/pubmed/?term=RANTES%2FCCL5+and+Risk+for+Coronary+Events%3A+Results+from+the+MONICA%2FKORA+Augsburg+Case-Cohort%2C+Athero-Express+and+CARDIoGRAM+Studies. (Accessed: 2nd August 2017)


6 Appendix

The Effects of Irradiation and Bone Marrow Reconstitution on Early Stages of Atherosclerotic Lesion Development in the Mouse Aortic Arch

6.1 Rationale

Bone marrow transplantation (BMT) in the Ldlr\(^{-/-}\) mouse model is a common tool used in identifying the role of hematopoietic and non-hematopoietic derived cells in atherosclerosis research. BMT, however, has been reported to affect lesion development in these mice. Linda Curtiss’ group studied the effects of BMT on atherosclerosis development. They identified that BMT worsened atherosclerosis within the aortic root yet suppressed lesion progression across the surface of the thoracic aorta in Ldlr\(^{-/-}\) mice fed 16 weeks of high fat diet. They suggested that the discrepancy between the BM transplanted and non-BM transplanted mice in atherosclerotic lesion development at the two different anatomic sites may be a result of differences in hemodynamics and endothelial responses; however, the underlying mechanism for these results remain uncertain. Our laboratory utilizes BMT to study the function of hematopoietic and non-hematopoietic cells in the early stages of atherosclerosis. From our experiments, we have observed that Ldlr\(^{-/-}\) mice transplanted with WT (C57BL/6) BM cells develop smaller lesions than non-BM transplanted Ldlr\(^{-/-}\) mice and ongoing studies in the laboratory are investigating the mechanisms that are involved.
6.2 Methods

6.2.1 Mice

Mice usage and protocols were described in Methods section 2.1.

6.2.2 Bone marrow transplantation (BMT)

BMT was performed as described in the Methods section 2.8.

6.2.3 Isolation of Aortic Arch

Isolation of aortic arch for staining was performed as described in the Methods section 2.2.

6.2.4 Analysis of atherosclerosis

Staining of aortic arches for atherosclerosis analysis was performed as described in Methods section 2.3. Additional primary antibodies used included Alexa647 conjugated hamster anti-mouse CD11c (2 µg/mL clone N418; BioLegend) were incubated overnight at 4°C. In addition to Nile Red staining, ascending aortic arches with 12-week lesions were stained with Oil Red O (ORO) and photographed at a fixed magnification. Aortas were opened as described in the Methods section and Figure 1 to allow for en face visualization.

6.2.5 Confocal Microscopy

Aortas were visualized en face via Nikon A1R Resonance Scanning Confocal Microscope using a 40x oil objective where tiling images was automated and compiled using Nikon NIS Elements Version 4.51 acquisition and analysis software. Nile Red positive area and CD11c positive area was determined as a percentage of total aorta using Image J software version 1.51f. Lesion
thickness was measured by confocal scanning in the z axis at 5 individual points within each lesion (3 points across length of lesion and 1 point above and below the midline).

6.2.6 Isolating Intimal mRNA from Ascending Aortic Arch
Isolation of intimal mRNA from the ascending aortic arch was performed as described in Methods section 2.5.

6.2.7 RT Real-time qPCR
Reverse transcription of mRNA and analysis of gene expression by real-time qPCR was performed as described in Methods section 2.6.

6.2.8 Statistics
Statistical analysis was conducted as described in Methods section 2.11.
6.3 Results

6.3.1 Bone marrow transplantation reduced lipid area in 3-week but not 6-week lesions

To determine whether BM transplantation affects early lesion development, Ldlr−/− mice were irradiated and reconstituted with WT BM. Lesion areas in the lesser curvature of the ascending arch were compared between BM transplanted and non-transplanted Ldlr−/− mice after increasing durations of CRD (5 days, 3 weeks, and 6 weeks). In BM transplanted and non-BM transplanted Ldlr−/− mice, Nile Red positive lipid areas increased significantly with prolonged durations of CRD confirming that the onset and progression of lesion formation in these mice depend on feeding a high cholesterol diet (Figure A). At 3-weeks of CRD, lesion lipid area is significantly reduced in transplanted mice (Figure A and B2), suggesting that BM transplantation affected lesion development at 3-weeks CRD. This difference between transplanted and non-transplanted mice was not seen in 5 day lesions (Figure B1) or 6-week lesions (Figure B3). Furthermore, the increase in lesion lipid area in transplanted mice was significantly increased between 3 and 6 weeks of CRD, however this was not significantly different in non-transplanted mice (Figure A). This indicated that between 3 and 6 weeks of CRD, lesion lipid area increased at a higher rate in the transplanted mice compared to the non-transplanted ones.
Lesion lipid area is reduced in BM transplanted mice at 3-weeks CRD but catches up by 6-weeks.

Lesional lipid area was analyzed by Nile Red staining in the lesser curvature of the ascending aortic arch of Ldlr\(^{-/-}\) mice with or without BM transplantation. Lipid areas were quantified after 5 days (n=4), 3 weeks (n=9), and 6 weeks (n=5) CRD. Pixels positive for Nile Red were presented as a percentage of the total number of pixels for each ascending aortic arch (Mean ± SEM). Two-way ANOVA and Tukey’s multiple comparison tests were performed. ** P < 0.01, *** P < 0.001.
Figure B. Nile Red⁺ lipid area is significantly reduced in 3-week lesions.

Lesional lipid area was analyzed by Nile Red staining in the lesser curvature of the ascending aortic arch of non-BM transplanted and BM-transplanted Ldlr⁻/⁻ mice. Quantification of lipid area was measured in mice fed CRD for 5 days (B1, n=4), 3 weeks (B2, n=7-9), and 6 weeks (B3, n=5-7). Pixels positive for Nile Red were presented as percentage of total number of pixels for each ascending aorta (Mean ± SEM). Student’s t-test was performed, * P < 0.05.
6.3.2 Bone marrow transplantation reduced myeloid cell area in 3-week lesions

In order to investigate any effects of bone marrow transplantation may have on the abundance of myeloid cells in early lesions, the area of CD11c+ myeloid cells was compared between Ldlr<sup>-/-</sup> mice reconstituted with WT BM with non-BM transplanted Ldlr<sup>-/-</sup> mice after feeding CRD for 5 days (Figure C1), 3 weeks (Figure C2), and 6 weeks (Figure C3). The lesser curvature of the ascending aortic arch was imaged by en face confocal microscopy and CD11c<sup>+</sup> pixels were normalized to total ascending aortic arch area and quantified using ImageJ. CD11c<sup>+</sup> cell areas were comparable after 5 days of CRD. In contrast, after 3 weeks of CRD, CD11c<sup>+</sup> area was reduced in BM transplanted mice. Subsequently, no differences were observed between non-transplanted and transplanted mice at 6-weeks CRD. Overall these data indicate that CD11c myeloid cell content is transiently reduced in 3-week lesions.
Figure C. CD11c positive myeloid cell area is significantly reduced in 3-week lesions.

Quantification of myeloid cell area was performed on lesions in the lesser curvature of the ascending aortic arch from non-BM-transplanted and BM-transplanted Ldlr−/− mice fed CRD for 5 days (C1, n=6), 3 weeks (C2, n=7-9), and 6 weeks (C3, n=5-7). Lesional myeloid cell area was analyzed by staining with anti-CD11c antibodies and imaged using confocal microscopy. CD11c positive area was calculated by normalizing CD11c positive pixels to the total ascending aortic arch area and presented relative to non-BM-transplanted Ldlr−/− control. All values represented as mean ± SEM Student’s t-test was performed, * P < 0.05.
6.3.3 Bone marrow transplantation significantly reduced lipid area in 12-week lesions as shown by Oil Red O staining

To assess lesion lipid area after 12-week CRD, ascending aortic arches of BM transplanted and non-BM transplanted Ldlr⁻/⁻ mice were stained with Oil Red O (ORO) (Figure D1, D2). ORO, unlike Nile Red, can be visualized in both bright field and fluorescent microscopy and is a less sensitive lipid dye compared to Nile Red.¹²⁸ We found that the high sensitivity of Nile Red in detecting lipid droplets obscured the assessment of lesion area of mature lesions. Therefore, due to the large abundance of lipid found in 12-week lesions, ORO was used in replacement of Nile Red. In Figure D, ORO positive area was significantly reduced in BM transplanted mice compared to non-BM transplanted mice (Figure D3). This data suggests that BM transplantation significantly reduced lipid area in 3-week and 12-week lesions.
Figure D. Oil Red O positive lipid area is significantly reduced in 12-week lesions.

Representative en face images of the dissected and flattened ascending aortic arch of the non-BM transplanted (D1) and BM transplanted (D2) Ldlr⁻/⁻ mice fed 12-weeks CRD. Lesional lipid area was analyzed by Oil Red O staining in the lesser curvature of the ascending aortic arch. Oil Red O positive pixels were presented as a percentage of total number of pixels for each ascending aortic arch and (D3). * P < 0.05 (n=4 per group).
6.3.4 Bone marrow transplantation reduced lesion thickness in progressing lesions

After 6 weeks of CRD, non-transplanted Ldlr<sup>−/−</sup> mice displayed similar lesion area as compared to 3 week lesions (Figures A and B). This was puzzling given that increased monocyte recruitment and local myeloid cell proliferation, the prototypic parameters of lesion progression, are observed beyond 3-week CRD in Ldlr<sup>−/−</sup> mice.<sup>46</sup> We predicted that the reason for this paradox may be due to the method of measuring lesion progression by en face imaging, since beyond covering the lesser curvature area, lesions may grow vertically. To verify this possibility, lesion thickness was measured by confocal scanning at 5 individual points within the lesion. We averaged the measurements gathered from each lesion and compared the lesion thickness between BM transplanted and non-transplanted Ldlr<sup>−/−</sup> mice fed CRD for various durations. As expected, lesion thickness increased progressively with longer durations of CRD in non-transplanted Ldlr<sup>−/−</sup> mice (Figure E1) beyond 3 weeks, suggesting that lesions grow in height. When comparing lesion thickness between BM transplanted and non-BM transplanted Ldlr<sup>−/−</sup> mice, lesion thickness was significantly reduced in BM transplanted mice in 6-week (Figure E2) and 12-week lesions (Figure E3), suggesting that BMT diminished lesion abundance at these stages.
Figure E. Lesion thickness increases with prolonged CRD and is significantly reduced in 6-week and 12-week lesions in BM-transplanted Ldlr<sup>-/-</sup> mice.

Lesion thickness was measured by confocal microscopy imaging of Nile Red<sup>+</sup> after 3 time points of CRD (3, 6, and 12 weeks) (E1, n=3-4). Lesion thickness was averaged over 5 separate points within each lesion located in the lesser curvature (3 points across the length of the lesion and 2 points above and below the midline). Measurements of lesion thickness at 6 week (E2, n=3-5 per group) and 12-week (E3, n=4 per group) CRD was compared between non-BM transplanted and BM-transplanted Ldlr<sup>-/-</sup> mice. All values are represented as mean ± SEM. Student’s t-test was performed, ** P < 0.01, *** P < 0.001.
6.3.5 Bone marrow transplantation suppresses CRD-induced gene expression in early lesions

To assess the effects of BM transplantation on gene expression in lesions, mRNA was isolated from the lesser curvature of the ascending aortic arch in non-BM transplanted mice and BM-transplanted Ldlr\(^{-/-}\) mice fed a CRD for 3 weeks. mRNA levels were quantified by qPCR analysis. CRD-induced gene expression of chemokines (Ccl3, Ccl4, Ccl5, and Cx3c11), endothelial adhesion markers (Vcam1, Icam1, E-Selectin) and myeloid cell markers (Cd45 and Cd11c) was analyzed relative to mice fed a standard chow diet. As was already shown in Figure 4, section 1.8, Ccl3, Ccl4, and Ccl5 mRNA expression was significantly induced in non-BM transplanted Ldlr\(^{-/-}\) mice by CRD; however, this induction by CRD was substantially reduced in BM-transplanted Ldlr\(^{-/-}\) mice (Figure F1, F2). Furthermore, the induction of Cd45 expression by CRD was also reduced in BM transplanted Ldlr\(^{-/-}\) mice, consistent with the fact that leukocyte abundance is reduced at 3-weeks CRD. E-Selectin mRNA induction by CRD was also significantly reduced in BM-transplanted Ldlr\(^{-/-}\) mice (Figure F3, F4). Overall, these data indicate that BM transplantation significantly suppresses the CRD induced expression of chemokine genes (i.e. Ccl3, Ccl4, and Ccl5), the expression of endothelial cell adhesion molecule E-Selectin, and myeloid cell marker CD45.
Figure F. Bone marrow transplantation suppresses CRD induced gene expression in early lesions.

mRNA expression of chemokines (F1, F2) and endothelial adhesion markers and myeloid cell markers (F3, F4) in the lesser curvature (LC) of the ascending aortic arch was analyzed in non-BM transplanted $Ldlr^{-/-}$ mice (F1, F3) and BM-transplanted $Ldlr^{-/-}$ mice (F2, F4) fed 3 weeks of CRD. mRNA expression is calculated relative to the expression in corresponding mice fed SCD that is given a value of 1. Gene expression is represented as mean ± SEM normalized to $VE$-$Cadherin$. Significant differences between SCD and CRD are indicated: n=6-16 per group. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$ paired student’s t-test. ns = no significance.
6.4 Discussion

BMT is a commonly used tool to study the role of BM-derived cells in the field of atherosclerosis research. Previous observations in Ldlr<sup>−/−</sup> mice fed a 16-week CRD suggested that BMT exacerbated atherosclerosis at the aortic root and reduced atherosclerosis in the thoracic aorta. Our objective is to identify the effects of BMT on early lesion development which will provide insights to the critical mechanisms that govern this stage of atherogenesis. Previously, our laboratory discovered that resident intimal CD11c-positive cells ingest lipid and become foam cells in Ldlr<sup>−/−</sup> mice fed for 5 days CRD<sup>15</sup>, a time point prior to the induction of monocyte recruitment or local proliferation.<sup>32,129</sup> Our data indicate that lipid and myeloid cell areas are comparable in 5-day lesions between BM-transplanted and non-BM transplanted Ldlr<sup>−/−</sup> mice (Figures A and B1), suggesting that BM transplantation did not significantly affect the process of lipid loading 5 days after initiating CRD. At 3-weeks CRD, lipid and CD11c-positive myeloid cell areas found in the LC of the ascending aortic arch were significantly reduced in BM-transplanted Ldlr<sup>−/−</sup> mice (Figure A, B2, C2). This is in line with the reduction in mRNA levels of chemokines and E-selectin observed after BMT, implicating impairment in monocyte recruitment. Although the reduction in lipid and myeloid cell areas due to BMT appeared to be lost in 6-week lesions (Figure A, B3, C3), experiments measuring the thickness of lesions (Figure E) suggested that these results may be due to a technical problem as to how these lesions are analyzed. Lesion size measured by ORO at 12-week lesions (Figures D3), showed that lesions continue to grow beyond 6-week lesions and the reduction in lesion progression by BMT is exacerbated when CRD is prolonged to 12 weeks. The measurements of lesion thickness showed that lipid areas between 6-week and 12-week lesions are affected by BMT (Figure E2, E3). These data show that evaluating lesion load by analysis of en face lipid and myeloid cell
areas are inadequate for assessing total lesion development once lesions reach a certain size. A more accurate analysis of lesion abundance would require assessing lesion volume. Future experiments in our laboratory will utilize imaging tools that gather 3-dimensional information of lesions for this purpose. Based on the fact that chemokine gene expression was dampened in BM transplanted mice (Figure F1, F2) future experiments will investigate whether reduction in monocyte recruitment is the underlying cause for reduced lesion size in BMT mice by quantifying monocyte recruitment rates using in vivo pulse labelling with BrdU at 24h.

These and future investigations into the mechanisms of lesion formation that are affected by BMT are expected to shed light on the critical mechanisms that underlie early lesion formation during atherogenesis.